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Petition for an Extension of the Determination of Nonregulated Status for Male Sterile, Glufosinate-Ammonium Tolerant *Brassica napus* Transformation Event MS11

OECD Unique Identifier BCS-BNØ12-7

Submitted By
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RELEASE OF 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 MS11 *Brassica napus* 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.

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SUMMARY

Bayer CropScience LP requests a determination from USDA APHIS that male sterile, glufosinate-ammonium tolerant *Brassica napus* event MS11 and any progeny derived from crosses of this event with traditional or transgenic *Brassica spp.* varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340, and that APHIS consider this document as an extension to petition 98-278-01p. The subject of petition 98-278-01p, MS8 *B. napus* x RF3 *B. napus*, received a determination of non-regulated status on March 22, 1999.

MS11 *B. napus* (male sterile line) was produced by means of *Agrobacterium*-mediated transformation using the vector pTCO113. MS11 *B. napus* contains the *barnase* gene (origin *Bacillus amyloliquefaciens*) coding for a ribonuclease, Barnase. The *barnase* gene is driven by the Pta29 promoter that restricts gene expression to the tapetum cells during anther development. Expression of Barnase in the tapetum cells of MS11 *B. napus* results in lack of viable pollen and male sterility. MS11 *B. napus* contains the *barstar* gene (origin *B. amyloliquefaciens*) coding for the Barstar protein, which is an inhibitor of the Barnase protein. This prophylactic *barstar* gene, driven by the Pnos promoter, is included to enhance transformation frequency. MS11 *B. napus* also contains the *bar* gene (origin *Streptomyces hygroscopicus*) coding for phosphinothricin acetyl transferase (PAT/*bar*) conferring tolerance to glufosinate-ammonium. The *bar* gene is driven by the PssuAt plant promoter that is active in all green tissues of the plant. The OECD identifier of MS11 *B. napus* is BCS-BNØ12-7.

The incorporation and expression of the MS11 transgenic locus in the *B. napus* genome has been characterized according to international standards for the safety assessment of biotechnology products. This information is included with this application to support the plant pest risk assessment of MS11 *B. napus*. Hybrid *B. napus* varieties containing the MS11 event will be grown commercially in the *B. napus* producing areas of Canada, USA and Australia.

The *bar*, *barnase* and *barstar* genes were introduced into the *B. napus* genome in a single gene construct via direct-gene transfer. 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.

In the molecular characterization of the MS11 transgenic locus, bioinformatics analysis of the full DNA sequence revealed no evidence supporting cryptic gene expression or unintended effects resulting from the genetic modification. The transgenic locus also shows structural stability over different generations and growing environments, and in different genetic backgrounds.

The agronomic performance of MS11 was observed in ten field trials conducted in the Canola growing regions of Western Canada and Northwestern USA. Based on the agronomic assessment, the MS11 *B. napus* demonstrated no biologically relevant differences compared to the non-GM conventional counterpart and showed equivalent agronomic performance in the field to *B. napus* reference varieties.

ACRONYMS AND SCIENTIFIC TERMS

ai	Active Ingredient
AOSA	Association of Official Seed Analysts
Bayer CS	Crop Science, A Division of Bayer
BBCH Scale	Scale used to identify the phenlogical development stages of a plant
BC1, BCn	First backcross into the non-transformed parental line, and subsequent backcrosses
bp(s)	Base Pair(s)
BLAST	Basic Local Alignment Search Tool
CFR	Code of Federal Regulations
DNA	Deoxyribonucleic Acid
DW	Dry Weight
EC	Embryonic Callus
ELISA	Enzyme-Linked Immunosorbent Assay
EMBOSS	European Molecular Biology Open Software Suite
EST	Expressed Sequence Tag
F1, Fn	First filial generation, or cross to dissimilar genetic background, and subsequent crosses to this genetic background.
FASTA	A text-based format for representing either nucleotide or peptide sequences
FARRP	Food Allergy Research and Resource Program
FW	Fresh Weight
g	Gram
gDNA	Genomic DNA
GM	Genetically Modified
ha	Hectare
HT	Herbicide Tolerant or Tolerance
ILSI	International Life Sciences Institute
kb	Kilobase, 1,000 Base Pairs
kDa	Kilodalton
kg	Kilogram
L	Liter
LB	Left Border
LLOQ	Lower Limit of Quantification
L-PPT	L-isomer of Phosphinothricin
m	Meter
µg	Microgram
MW	Molecular Weight
NCBI	National Center for Biotechnology Information
ND	Not Detected
ng	Nanogram
OECD	Organisation for Economic Cooperation and Development
ORF	Open Reading Frame
PAT	Phosphinothricin Acetyl Transferase
PCR	Polymerase Chain Reaction
pg	Picogram
RB	Right Border
RNA	Ribonucleic Acid
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SL	Soluble Liquid
T-DNA	Transfer DNA
Ti	Tumor Inducing
T0, T1, Tn	Initial transformation event and subsequent generations
TSD	Target Site Deletion

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I. RATIONALE FOR NONREGULATED STATUS

The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Services (APHIS) is responsible for protection of the US 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 an Extension of the *Petition for Determination of Nonregulated Status: InVigor® Hybrid Canola Transformation Events MS8/RF3*. The petition for MS8/RF3 (98-278-01p) received a determination of non-regulated status on March 22, 1999. Event MS11 demonstrates the same phenotype as event MS8: male sterility conferred by the expression of Barnase proteins in the tapetum and glufosinate-ammonium tolerance conferred by the expression of Pat/*bar* protein in all green tissue. Therefore, there are no changes in the rationale from petition 98-278-01p.

I.A. Description of the Trait and Intended Use of the Product

Bayer's Crop Science division (Bayer CS) has developed a highly successful breeding tool that is used to produce *B. napus* glufosinate-ammonium tolerant hybrids that are sold in Canada and the USA. The hybrid technology comprises three components: a dominant gene for male sterility – the *barnase* gene (event MS11), a dominant gene for fertility restoration – the *barstar* gene (event RF3) and the *bar* gene (found in both MS11 and RF3) conferring tolerance to glufosinate-ammonium. Currently, Bayer CS *B. napus* canola hybrids are based on events MS8 *B. napus* and RF3 *B. napus*. MS8 *B. napus* will be phased out of use by the mid-2020's and MS11 *B. napus* will be the replacement event.

MS11 *B. napus* was produced by means of *Agrobacterium*-mediated transformation using the vector pTCO113. MS11 *B. napus* contains the *barnase* gene (origin *Bacillus amyloliquefaciens*) coding for a ribonuclease, Barnase. The *barnase* gene is driven by the Pta29 promoter that restricts gene expression to the tapetum cells during anther development. Expression of Barnase in the tapetum cells of MS11 *B. napus* results in lack of viable pollen and male sterility. MS11 *B. napus* contains the *barstar* gene (origin *B.amyloliquefaciens*) coding for the Barstar protein, which is an inhibitor of the Barnase protein. This prophylactic *barstar* gene, driven by the Pnos promoter, is included to enhance transformation frequency. MS11 *B. napus* also contains the *bar* gene (origin *Streptomyces hygroscopicus*) coding for phosphinothricin acetyltransferase (PAT/*bar*) conferring tolerance to glufosinate-ammonium. The *bar* gene is driven by the PssuAt plant promoter that is active in all green tissues of the plant.

The hybrid technology comprises three components: a dominant gene for male sterility – the *barnase* gene (event MS11), a dominant gene for fertility restoration – the *barstar* gene (event RF3) and the *bar* gene (found in both MS11 and RF3) conferring tolerance to glufosinate-ammonium. MS11 *B. napus* is a male sterile line that segregates 1:1 for sterility and fertility and is only used for the production of the MS11xRF3 *B. napus* hybrid seed. It will never be commercialized as a standalone product.

B. napus oilseed rape has been developed for human consumption to have low erucic acid in its oil as well as low glucosinolate content in its meal by-product, which is used as a high protein animal feed (OGTR, 2002). These so-called 'double low' commercial varieties of *B. napus* dominate the oilseed *Brassica* production area in developed countries. In North America, these species are considered to be of canola quality (OECD, 2012).

Hybrids based on MS11 will be commercialized in the canola growing regions of Canada, USA, and Australia.

I.B. Description of the Benefits and Anticipated Adoption of the Product

F1 hybrids of canola varieties yield 20-25% more than the best open-pollinated canola varieties. The uniformity of the hybrid plants is an advantage in commercial fields, facilitating harvesting and marketing. The incorporation of the *bar* gene provides a weed management tool to canola growers. The *bar* gene confers tolerance to glufosinate-ammonium, a broad spectrum herbicide. Herbicide-tolerant canola varieties were quickly adopted in Canada and the USA since their introduction in the mid-1990's. Approximately 95% of Canada's canola quality *B. napus* has been genetically modified for herbicide tolerance (CCC, 2016a). The benefits of high yielding herbicide-tolerant canola varieties are many:

- Increased yields – the hybrid system allows production of higher yielding canola varieties. This results in a more consistent supply for oilseed crushers, exporters and consumers.
- Better weed control – Glufosinate-ammonium provides broad spectrum weed control in the crop and also provides growers with an additional tool for their weed resistance management strategy.
- The use of herbicide-tolerant canola has reduced fuel use and tillage practices, resulting in soil conservation and related environmental benefits such as carbon sequestering. It is estimated that in Canada in 2013 the use of genetically modified canola resulted in a fuel saving of 69 million liters and reduced carbon dioxide emissions by 185 million kg (Table I-1) (Brookes and Barfoot, 2015).
- The harvested crop has less dockage (such as weed seed and chaff in the harvested seed) so farmers get higher prices for their canola.
- Farmers use less tillage and more direct-seeding. The reduction of tillage reduces soil erosion, contributes to less air pollution from dust, improves soil moisture retention, and reduces soil compaction.
- Less herbicide is used. In 2013, the use of genetically modified herbicide-tolerant canola resulted in a 2.1 million kg reduction in the amount of herbicide active ingredient use (-17.1%) (Brookes and Barfoot, 2015).

Because of these advantages, herbicide-tolerant varieties have quickly grown in popularity since their introduction in 1995.

Table I-1: Carbon storage/sequestration from reduced fuel use with GM crops 2013

Crop/Trait/Country	Fuel Saving (million liters)	Permanent carbon dioxide savings arising from reduced fuel use (million kg of carbon dioxide)	Permanent fuel savings: as average family car equivalents removed from the road for a year ('000s)[a]
Canada: GM HT canola	69	185	82

Source Brookes and Barfoot, 2015

[a]Assumes an average family car produces 150 g of carbon dioxide per km. A car travels an average of 15,000 km/year and therefore produces 2,250 kg of carbon dioxide/year.

Canola Export Data

Approximately 95% of Canada's canola has been genetically modified for herbicide tolerance. Canada exports 90% of its canola as seed, oil or meal to 55 markets around the world. The biggest buyer of canola oil and meal is the United States, accounting for about 65% of oil exports and 96% of meal exports in 2014. For raw seed, the most important destinations are China, Japan, Mexico, and the United States (CCC, 2016b). 2013 canola/oilseed rape production, import and export data are presented in Table I-2.

Table I-2: 2013 World production, imports and exports of Oilseed Rape (selected countries)

	Rapeseed Production 2013 [hectares]	Exports Rapeseed 2013 [tonnes]	Imports Rapeseed 2013 [tonnes]	Exports Rapeseed Oil 2013 [tonnes]	Imports Rapeseed Oil 2013 [tonnes]	Exports Rapeseed Cake (Meal) [tonnes]	Import Rapeseed Cake (Meal) [tonnes]
Australia	4,141,731	3,795,677	738	142,901	18,071	54,554	0
Canada	17,954,800	9,680,373	75,302	2,287,283	81,440	3,295,662	11,566
China	14,458,029	162	3,662,688	17,021	1,567,894	72,992	125,879
France	4,370,075	1,316,923	1,105,211	298,970	268,830	477,879	585,442
Germany	5,784,300	119,003	4,594,632	1,157,979	185,945	1,729,341	527,574
Poland	2,677,665	751,326	225,803	348,805	83,284	603,626	60,816
Ukraine	2,351,730	2,346,699	2,623	48,271	937	65,703	0
United Kingdom	2,128,000	431,000	177,380	220,016	125,030	139,060	272,178
Russian Federation	1,393,263	124,182	2,003	304,788	1,391	289,593	1,520
Romania	666,097	471,928	26,841	21,440	22,704	156,051	3,865
South Korea	2,000	1	51,141	30	66,292	16,316	439,557
Mexico	3,000	0	1,386,125	1,900	47,738	0	65,372
Japan	1,770	48	2,461,041	502	20,370	10	68,451
Thailand	0	0	0	171	553	0	377,179
Vietnam	0	0	0	0	2,799	0	0
Philippines	0	0	8	0	409	0	3,498
Malaysia	0	0	13,258	35,889	51,734	6,136	29,009
Indonesia	0	0	686	24	4,529	100	152,446
Taiwan	14	0	84	356	21,337	0	63,717
USA	512,420	126,351	589,351	138,493	1,271,531	59,709	3,135,230

Source FAOSTAT, 2016

I.C. Comparison of MS11 to the Antecedent Organism MS8

As described in section I.A. of this petition, event MS11 *B. napus* is intended as the replacement event for the antecedent organism event MS8 *B. napus* as the male sterile component in the Bayer CS hybrid canola breeding system. In both MS11 and MS8, the male sterility phenotype is achieved via *Agrobacterium*-mediated transformation using a vector containing the *barnase* and *bar* gene. The *barnase* gene, driven by the Pta29 promoter, confers male sterility by driving expression of the Barnase protein in the tapetum cells during anther development resulting in a lack of viable pollen. To maintain the male sterile line, MS11 and MS8 must be backcrossed to a maintainer line, producing a 1:1 heterozygous population of male sterile and fully fertile lines. Therefore, in both events MS11 and MS8, the *bar* gene is included in the genetic modification for expression of PAT/*bar* protein, conferring tolerance to glufosinate-ammonium. This allows glufosinate-ammonium to be sprayed over the heterozygous population, selecting for the male sterile phenotype which segregates in the population along with the herbicide-tolerant phenotype. Hybrid commercial lines are produced by planting heterozygous blocks of the event MS11 inbred line (or the antecedent organism MS8), spraying the blocks with glufosinate-ammonium to select for plants only with the intended trait, and crossing with an inbred line with a fertility restorer trait, event RF3 *B. napus.* Event RF3 *B. napus* expresses Barstar protein, which counteracts the effects of the Barnase protein. Event RF3 *B. napus* was deregulated along with MS8 *B. napus* under petition 98-278-01p.

Event MS11 and the antecedent organism event MS8 have the same mechanism of action for achieving male sterility and glufosinate-ammonium tolerance. The only difference between MS11 and the antecedent organism MS8 as it pertains to the genetic modification is that the MS11 transformation includes the *barstar* gene cassette, driven by a weak Pnos promoter, for low level prophylactic expression of the Barstar protein. As described above, this protein is expressed by the restorer event RF3 as well. This low level expression of the Barstar protein was included to increase transformation efficiency. It has no effect on the male sterile or herbicide tolerant phenotype of event MS11. Therefore, the phenotype of event MS11 and the antecedent organism MS8 are fundamentally the same in the context of a plant pest risk assessment. Table I-3 summarizes the comparison of event MS11 *B. napus* and the antecedent organism event MS8 *B. napus*. Based on these similarities, event MS11 *B. napus* is no more likely to act as a plant pest or noxious weed than deregulated antecedent organism MS8 *B. napus.* Therefore the determination of non-regulated status for event MS8 *B. napus* can be extended to event MS11 *B. napus.*

Table I-3: Comparison of event MS11 and the antecedent organism event MS8

	Characteristic	MS11	MS8
Recipient organism and phenotype	Recipient Organism	*Brassica napus*	*Brassica napus*
	Parent Line	N90-740	Drakkar
	Phenotype	Glufosinate-ammonium tolerant; male-sterile	Glufosinate-ammonium tolerant; male-sterile
	Mechanism of action	Male sterility by expression of Barnase in the tapetum cells; Glufosinate-ammonium tolerance by expression of PAT/*bar*	Male sterility by expression of Barnase in the tapetum cells; Glufosinate-ammonium tolerance by expression of PAT/*bar*
	Gene Products	PAT/*bar*, Barnase, Barstar	Pat/*bar*, Barnase
Transformation method	Transformation Method	*Agrobacterium*-mediated	*Agrobacterium*-mediated
	Vector	pTCO113	pTHW107
bar cassette	Gene/Donor	*bar / Streptomyces hygroscopicus*	*bar / Streptomyces hygroscopicus*
	Promoter/Donor	PssuAt / *Arabidopsis thaliana*	PssuAt / *Arabidopsis thaliana*
	Terminator/Donor	3'g7 / *Agrobacterium tumefaciens*	3'g7 / *Agrobacterium tumefaciens*
barnase cassette	Gene/Donor	*barnase / Bacillus amyloliquefaciens*	*barnase / Bacillus amyloliquefaciens*
	Promoter/Donor	Pta29 / *Nicotiana tabacum*	Pta29 / *Nicotiana tabacum*
	Terminator/Donor	3'barnase / *Bacillus amyloliquefaciens*; and 3'nos / *Agrobacterium tumefaciens*	3'barnase / *Bacillus amyloliquefaciens*; and 3'nos / *Agrobacterium tumefaciens*
barstar cassette	Gene/Donor	*barstar / Bacillus amyloliquefaciens*	Not present
	Promoter/Donor	Pnos / *Agrobacterium tumefaciens*	Not present
	Terminator/Donor	3'g7 / *Agrobacterium tumefaciens*	Not present

II. BIOLOGY OF *BRASSICA NAPUS*

The genus *Brassica* is classified under family *Cruciferae*. *B. napus* is commonly referred to as oilseed rape. The term “canola” refers to oilseed rape plants of the species *B. napus, B. rapa, or B. juncea* from which the oil contains less than 2% erucic acid in its fatty acid profile and the solid component contains less than 20 micromoles of glucosinolates.

The OECD consensus document on oilseed rape biology (OECD, 2012) provides information pertaining to the following aspects of oilseed rape 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

III. DEVELOPMENT OF EVENT MS11

III.A. Transformation Methods

Seeds of *B. napus* variety N90-740 were germinated on solid germination medium. Hypocotyl segments were dissected from the *B. napus* seedlings and incubated on solid modified Murashige & Skoog (MS) medium for callus induction. Callus was isolated from the wounded sites of the hypocotyls and transferred to the same medium for embryogenic callus (EC) development. Small clumps of EC were transformed with the transformation vector pTCO113 using an *A. tumefaciens* transformation method.

III.B. Breeding History

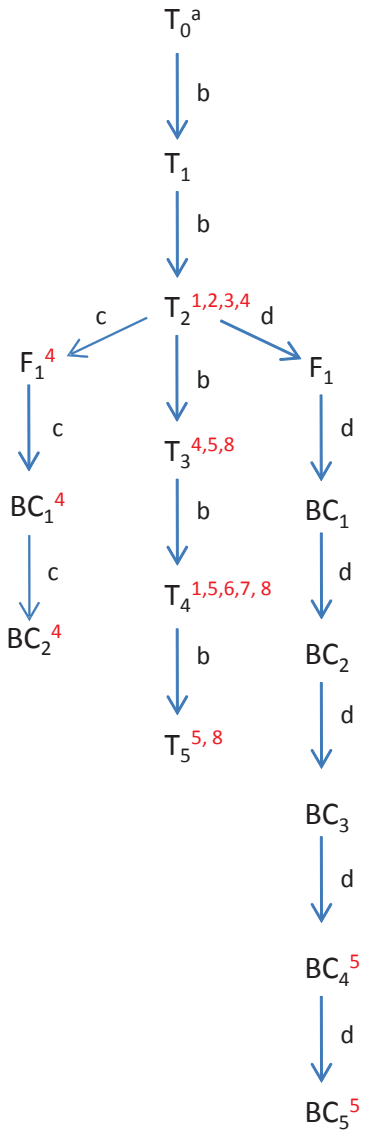
Following *Agrobacterium*-mediated transformation of the conventional breeding line N90-740 resulting in event MS11, T_0 plants were treated with glufosinate-ammonium to select for the expression of the *bar* gene. T_0 hemizygous MS11 plants were cross-pollinated with non-genetically modified (non-GM) plants (N90-740 variety) to produce the T_1 generation. MS11 hemizygous plants from the T_1 generation were cross-pollinated with non-GM plants (N90-740 variety) to produce the T_2 generation. The process of crossing MS11 hemizygous plants with non-GM plants (N90-740 variety) was repeated to produce the T_3, T_4, and T_5 generations.

MS11 hemizygous plants from the T_2 generation were also cross-pollinated with non-GM plants (B144 variety) creating a F_1 generation. MS11 hemizygous plants from the F_1 generation were backcrossed to non-GM plants (B144 variety) to produce a BC_1 generation. The process of backcrossing MS11 hemizygous plants with non-GM B144 plants was repeated to produce the BC_2, BC_3, BC_4, and BC_5 generations.

MS11 hemizygous plants from the T_2 generation were also cross-pollinated with non-GM plants (Ebony variety) creating a F_1 generation. MS11 hemizygous plants from the F_1 generation were backcrossed to non-GM plants (Ebony variety) to produce a BC_1 generation. The process of backcrossing MS11 hemizygous plants with non-GM plants (Ebony variety) was repeated to produce a BC_2 generation.

The breeding program for the development of event MS11 and its introgression into *B. napus* germplasm is demonstrated in Figure III-1 below. Table III-1 describes the MS11 generations used for analysis and the associated studies.

Figure III-1: Pedigree of MS11



a: N90-740 variety was used for transformation
b: crossing with N90-740 variety
c: crossing with Ebony variety
d: crossing with B144 variety

Table III-1: Generations used for analysis

No. in Tree	Experiment	Generation(s)	Comparator
1	DNA sequencing of insert and flanking region	T_2 T_4	N90-740 None
2	Insert Characterization by Southern Analysis	T_2	N90-740
3	Absence of Vector Backbone by Southern Analysis	T_2	N90-740
4	Structural Stability by Southern Analysis	T_2 T_3 F_1 (Ebony) BC_1 (Ebony) BC_2 (Ebony)	N90-740
5	Inheritance of the Insert	T_3 T_4 T_5 BC_4 (B144) BC_5 (B144)	None
6	Agronomic and phenotypic Analysis	T_4	N90-740
7	Protein Expression Analysis	T_4	N90-740
8	Protein Expression over generations	T_3 T_4 T_5	N90-740

IV. GENETIC MATERIAL USED FOR TRANSFORMATION OF EVENT MS11

IV.A. Description of the Transformation Vector and Gene Construct

The vector pTCO113 is derived from pGSC1700. The map of the vector pTCO113 is presented in Figure IV-1 and the genetic elements are described in Table IV-1.

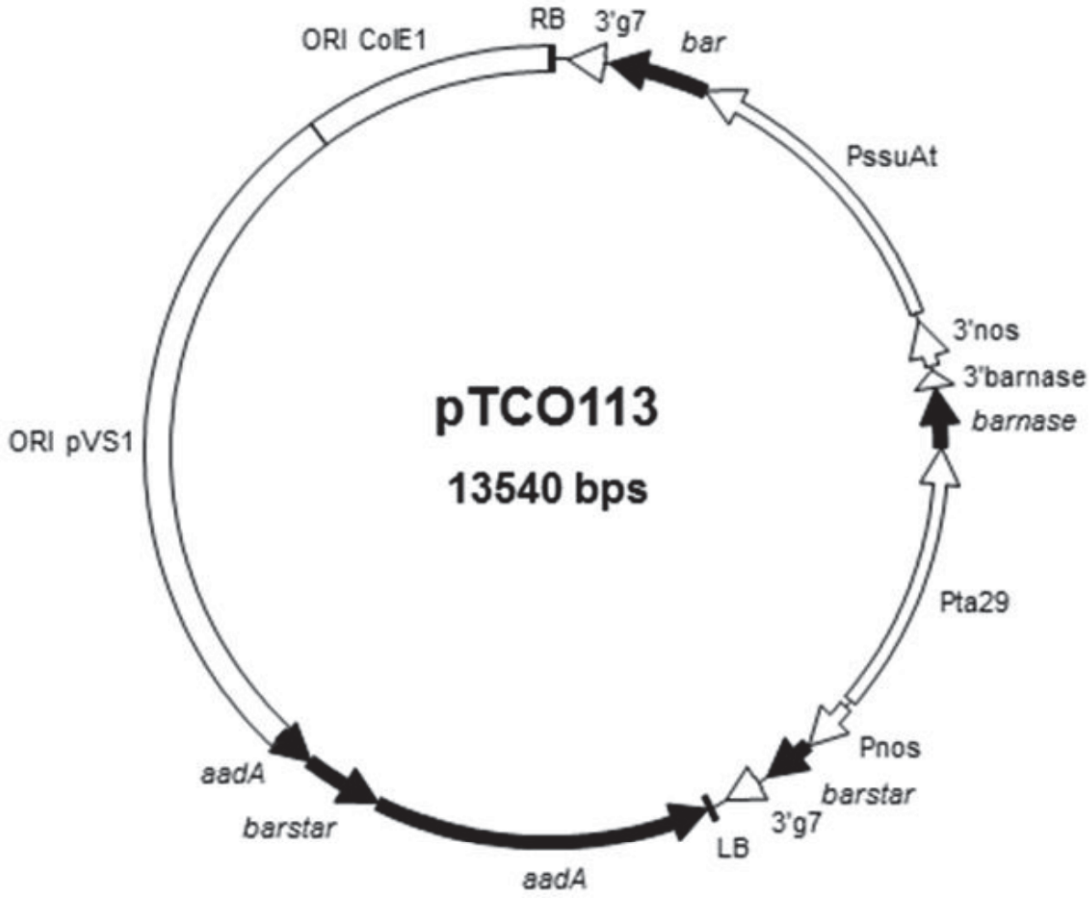


Figure IV-1: Map of vector pTCO113

Table IV-1: Description of the genetic elements of pTCO113

Nt Positions	Orientation	Origin
1 - 25		**RB:** right border region of the T-DNA of *Agrobacterium tumefaciens* (Zambryski,1988)
26 - 97		Polylinker sequences: sequence used in cloning
98 - 309	Counter clockwise	**3'g7:** 3´ untranslated region of the TL-DNA gene 7 of the *Agrobacterium tumefaciens* octopine Ti plasmid. (Dhaese et al., 1983)
310 - 331		Polylinker sequences: sequence used in cloning
332 - 883	Counter clockwise	***bar:*** coding sequence of the phosphinothricin acetyltransferase gene of *Streptomyces hygroscopicus* (Thompson et al., 1987).
884 - 2613	Counter clockwise	**PssuAt:** promoter region of the ribulose-1,5-biphosphate carboxylase small subunit gene of *Arabidopsis thaliana* (Krebbers et al., 1988)
2614 - 2658		Polylinker sequences: sequence used in cloning
2659 - 2919	Counter clockwise	**3'nos:** 3' untranslated region of the nopaline synthase gene from the T-DNA of pTiT37 (Depicker et al., 1982)
2920 - 2935		Polylinker sequences: sequence used in cloning
2936 - 3033	Counter clockwise	**3'barnase:** 3´ untranslated region of the *barnase* gene from *Bacillus amyloliquefaciens* (Hartley, 1988)
3034 - 3369	Counter clockwise	***barnase:*** coding sequence of the *barnase* gene of *Bacillus amyloliquefaciens* (Hartley, 1988)
3370 - 3371		Polylinker sequences: sequence used in cloning
3372 - 4879	Counter clockwise	**Pta29:** promoter of the anther-specific gene TA29 of *Nicotiana tabacum* (tobacco). (Seurinck et al., 1990)
4880 - 4920		Polylinker sequences: sequence used in cloning
4921 - 5214	Clockwise	**Pnos:** promoter region of the nopaline synthase gene of *Agrobacterium tumefaciens* (Depicker et al., 1982)
5215 - 5216		Polylinker sequences: sequence used in cloning
5217 - 5489	Clockwise	***barstar:*** coding sequence of the *barstar* gene of *Bacillus amyloliquefaciens* (Hartley, 1988)
5490 - 5554		Polylinker sequences: sequence used in cloning
5555 - 5766	Clockwise	**3'g7:** 3´ untranslated region of the TL-DNA gene 7 of the *Agrobacterium tumefaciens* octopine Ti plasmid. (Dhaese et al., 1983)
5767 - 5840		Polylinker sequences: sequence used in cloning
5841 - 5865		**LB:** left border region of the T-DNA of *Agrobacterium tumefaciens* (Zambryski, 1988)
5866 - 7745	Counter clockwise	***aadA*:** fragment including the aminoglycoside adenyltransferase gene of *Escherichia coli* (Fling et al., 1985)
7746 - 8181	Counter clockwise	***barstar:*** fragment including the *barstar* gene of *Bacillus amyloliquefaciens* (Hartley, 1988)
8182 - 8405	Counter clockwise	***aadA*:** fragment including the residual upstream sequences of the aminoglycoside adenyltransferase gene of *Escherichia coli* (Fling et al., 1985)
8406 - 12177		**ORI pVS1:** fragment including the origin of replication of the plasmid pVS1 of *Pseudomonas aeruginosa* (Heeb et al., 2000)
12178 - 13540		**ORI ColE1:** fragment including the origin of replication from the plasmid pBR322 for replication in *Escherichia coli* (Bolivar et al., 1977).

IV.B. Description of the Transferred Genes and Gene Products

barnase

The *barnase* gene, encoding for an extracellular ribonuclease, was isolated from *B. amyloliquefaciens* (Hartley,1988). The gene was engineered with an Asp718 site at the start of the coding sequence, substituting alanine and glutamine for valine and proline respectively. The Asp718 site was digested to give a blunt end and fused to the ATG initiation codon.

The *barnase* gene in MS11 *B. napus* is driven by the Pta29 promoter that restricts gene expression to the tapetum cells during anther development. Expression of Barnase in the tapetum cells of MS11 *B. napus* results in lack of viable pollen and male sterility.

```
  1  mvpvintfdg vadylqtyhk lpdnyitkse aqalgwvask gnladvapgk siggdifsnr

 61  egklpgksgr twreadinyt sgfrnsdril yssdwliykt tdhyqtftki r
```

Figure IV-2: Amino acid sequence of the Barnase protein (111 amino acids)

barstar

The *barstar* gene, an intracellular inhibitor of the Barnase ribonuclease, was isolated from *B. amyloliquefaciens* (Hartley, 1988). It encodes for the Barstar protein.

The Barstar protein is an inhibitor of the Barnase protein. The prophylactic *barstar* gene in MS11 *B. napus*, driven by the Pnos promoter, was included to enhance transformation frequency.

```
 1  mkkavingeq irsisdlhqt lkkelalpey ygenldalwd cltgwveypl vlewrqfeqs

61  kqltengaes vlqvfreaka egcditiils
```

Figure IV-3: Amino acid sequence of the Barstar protein (90 amino acids)

bar

The *bar* gene was isolated from *S. hygroscopicus* (Murakami et al., 1986; Thompson et al., 1987). The *bar* gene encodes the enzyme phosphinothricin acetyltransferase (PAT/*bar*), which confers resistance to the phytotoxic activity of glufosinate-ammonium. An *Nco*I site was created at the initiation codon. Accordingly, the second codon of the *bar* gene - AGC (Serine) has been modified to a GAC (Aspartic acid) codon (Botterman et al., 1991).

```
  1  mdperrpadi rrateadmpa vctivnhyie tstvnfrtep qepqewtddl vrlrerypwl

 61  vaevdgevag iayagpwkar naydwtaest vyvsprhqrt glgstlythl lksleaqgfk

121  svvaviglpn dpsvrmheal gyaprgmlra agfkhgnwhd vgfwqldfsl pvpprpvlpv

181  tei
```

Figure IV-4: Amino acid sequence of the PAT/*bar* protein (183 amino acids)

V. GENETIC CHARACTERIZATION OF EVENT MS11

V.A. Structural Stability

Southern blot analysis

The structural stability of the MS11 transgenic locus in *B. napus* was demonstrated by assessing individual MS11 *B. napus* plants from five generations (T_2, T_3, F_1, BC_1, and BC_2) by means of Southern blot analysis.

Seeds from five different seed lots were used to produce MS11 *B. napus* leaf material. The identity and zygosity of the individual plants were confirmed. Non-genetically modified (non-GM) *B. napus* variety N90-740 (non-GM counterpart) was used as a negative control. The positive control was the transforming plasmid of MS11 *B. napus* (pTCO113).

The MS11 *B. napus* and non-GM counterpart genomic DNA (gDNA) samples were digested with the *Eco*RV restriction enzyme. Plasmid DNA of pTCO113 was digested with the *Eco*RI restriction enzyme. The resulting DNA fragments were separated by agarose gel-electrophoresis. Transfer of the separated DNA fragments from the agarose gel to a positively charged nylon membrane was performed by a neutral Southern blotting procedure. The resulting membranes were hybridized with a DIG-labeled T-DNA probe P028 (Table V-1). A schematic overview of the MS11 transgenic locus, with indication of the restriction sites, the T-DNA probe used, and the expected fragments is presented in Figure V-1.

Each membrane used for the analysis contained one negative control. For all hybridizations, this negative control showed no hybridization with the T-DNA probe, confirming the absence of any background hybridization with the probe used. Similarly, each membrane contained one positive control. For all hybridizations, the expected fragments were detected for the positive control indicating that the conditions of Southern blot experiments allowed specific hybridization of the T-DNA probe with the target sequences. A number of small, weak additional fragments were obtained for the positive control on the membrane containing the T_3 generation (Figure V-3). These additional fragments are artifacts, which are most likely due to star activity since these fragments were no longer observed after hybridization of a freshly digested transforming plasmid pTCO113 with the same T-DNA probe (Figure V-2; Figure V-4; Figure V-5; and Figure V-6).

Genomic DNA from individual MS11 *B. napus* plants was digested with *Eco*RV. For all individual plants confirmed as positive for the presence of MS11 *B. napus* from the T_2, T_3, F_1, BC_1 and BC_2 generations, both expected fragments (4400 bp and 4900 bp) were obtained (Figure V-2 to Figure V-6 and Table V-2).

These results demonstrate the structural stability of MS11 *B. napus* in the T_2, T_3, F_1, BC_1 and BC_2 generations.

Table V-1: Information on the probe used for structural stability analysis

Probe ID	Description	Primer pair	Primer sequence (5' → 3')	Primer position in pTCO113 (bp)	Probe size (bp)
P028	T-DNA	GLPA174	AATTACAACGGTATATATCCTGCCA	1 → 25	5865
		GLPA359	CGGCAGGATATATTCAATTGTAAAT	5865 → 5841	

Table V-2: Stability of MS11 *B. napus* in the individual plants - Expected and obtained hybridization fragments

Sample	Reference to figure[$]	Fragment size (bp)	Fragment description	Probe P028 T-DNA	
				Exp.	Obt.
10 samples scoring positive for MS11 - T2 generation – EcoRV	Figure 2	approx. 4900 *	5' integration fragment	Yes	Yes
		approx. 4400 *	3' integration fragment	Yes	Yes
10 samples scoring positive for MS11 – T3 generation – *Eco*RV	Figure 3	approx. 4900 *	5' integration fragment	Yes	Yes
		approx. 4400 *	3' integration fragment	Yes	Yes
9 samples scoring positive for MS11 – F1 generation – *Eco*RV	Figure 4	approx. 4900 *	5' integration fragment	Yes	Yes
		approx. 4400 *	3' integration fragment	Yes	Yes
10 samples scoring positive for MS11 – BC1 generation – *Eco*RV	Figure 5	approx. 4900 *	5' integration fragment	Yes	Yes
		approx. 4400 *	3' integration fragment	Yes	Yes
10 samples scoring positive for MS11 – BC2 generation – *Eco*RV	Figure 6	approx. 4900 *	5' integration fragment	Yes	Yes
		approx. 4400 *	3' integration fragment	Yes	Yes
non-GM counterpart – *Eco*RV	Figure 2 to Figure 6	/	Negative control	/	/
non-GM counterpart – *Eco*RI digested + an equimolar amount of pTCO113 – *Eco*RI digested	Figure 2 to Figure 6	2260	Positive control°	Yes	Yes
		11280		Yes	Yes

[$] lane numbers see legend of figures
* Fragment sizes as determined in the "Detailed insert characterization and confirmation of the absence of vector backbone sequences in MS11 *B. napus"* study
°For the membrane containing the T3 generation samples, additional weak fragments were obtained in the positive control, see Figure 3

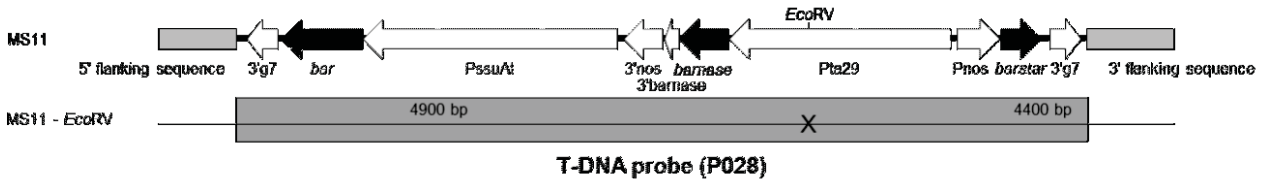


Figure V-1: Schematic overview of the MS11 *B. napus* transgenic locus with indication of the restriction sites, the probe used and expected fragment sizes in bp

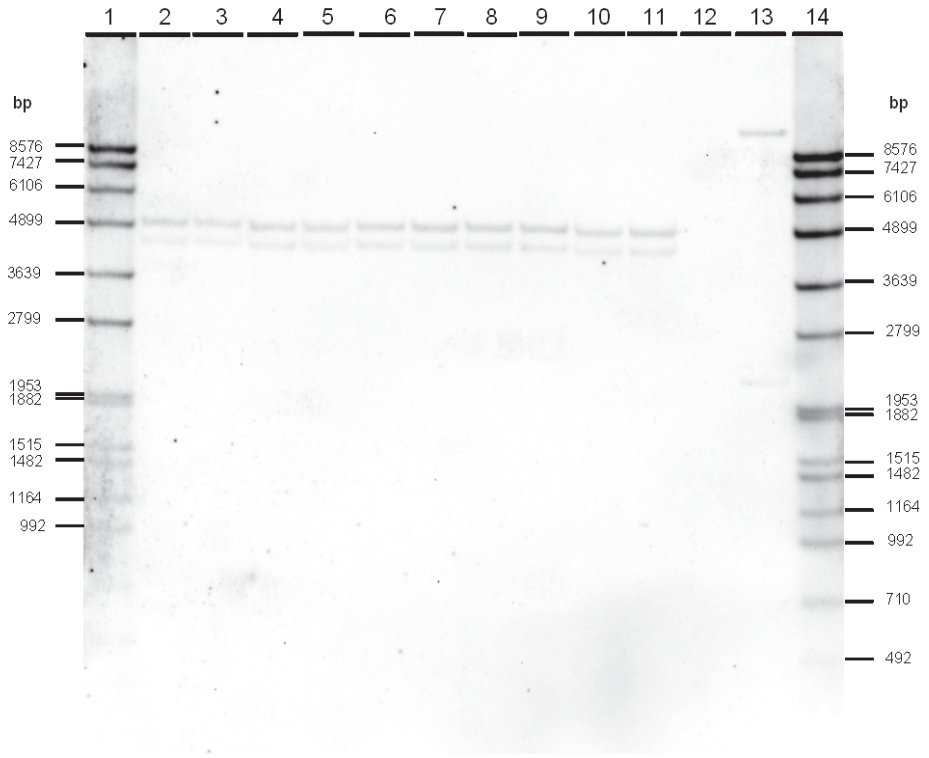


Figure V-2: Southern blot analysis of MS11 *B. napus* – Hybridization performed with a MS11 *B. napus* T-DNA probe to assess structural stability of the individual plants of the T2 generation

Digital image ID: H1/LJS017/08-F2

Genomic DNA was isolated from individual MS11 *B. napus* plants of the T2 generation confirmed as positive for the presence of MS11 *B. napus* and from the non-GM counterpart. The gDNA samples were digested with the restriction enzyme *Eco*RV and hybridized with a probe corresponding to the MS11 *B. napus* T-DNA region (P028-13).

Lane 1: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 3 µg of gDNA of the non-GM counterpart – *Eco*RI digested
Lane 2 to 11: 3 µg gDNA of individual samples of MS11 *B. napus* of the T2 generation scoring positive for the presence of MS11 *B. napus* – *Eco*RV digested
Lane 12: 3 µg gDNA of the non-GM counterpart – *Eco*RV digested (negative control)
Lane 13: 3 µg gDNA of the non-GM counterpart – *Eco*RI digested + an equimolar amount of plasmid pTCO113 – *Eco*RI digested (positive control)
Lane 14: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 3 µg of gDNA of the non-GM counterpart – *Eco*RI digested

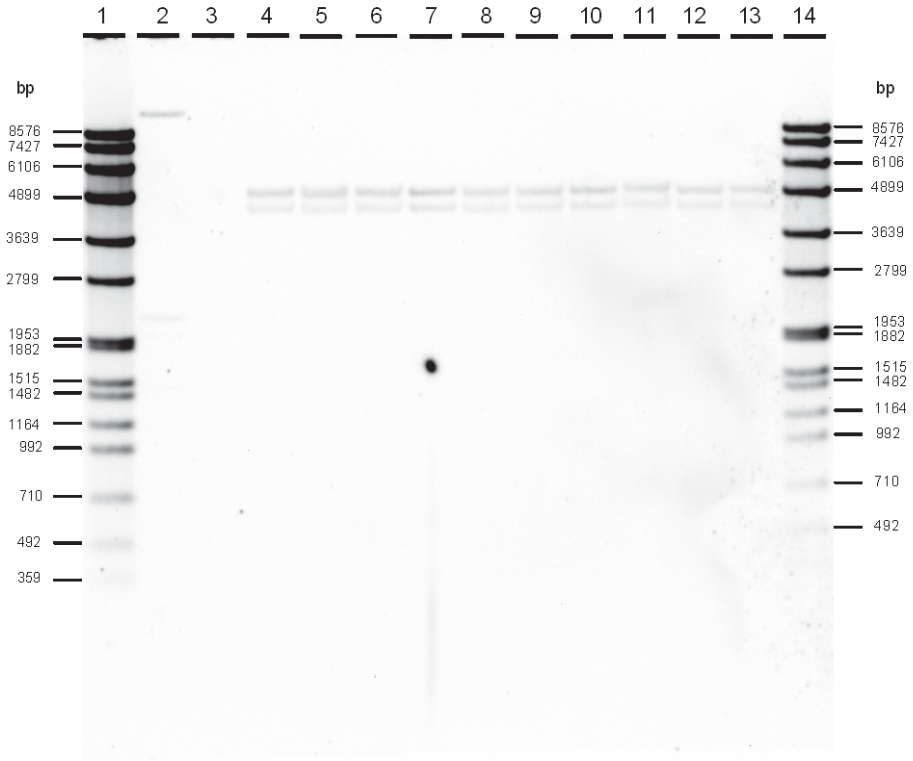


Figure V-3: Southern blot analysis of MS11 *B. napus* – Hybridization performed with a MS11 *B. napus* T-DNA probe to assess structural stability of the individual plants of the T3 generation

Digital image ID: H1/LJS017/01-F3

Genomic DNA was isolated from individual MS11 *B. napus* plants of the T3 generation confirmed as positive for the presence of MS11 *B. napus* and from the non-GM counterpart. The gDNA samples were digested with the restriction enzyme *Eco*RV and hybridized with a probe corresponding to the MS11 *B. napus* T-DNA region (P028-02).

Lane 1: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 3 µg of gDNA of the non-GM counterpart – *Eco*RI digested
Lane 2: 3 µg gDNA of the non-GM counterpart – *Eco*RI digested + an equimolar amount of plasmid pTCO113 – *Eco*RI digested (positive control)
Lane 3: 3 µg gDNA of the non-GM counterpart – *Eco*RV digested (negative control)
Lane 4 to 13: 3 µg gDNA of individual samples of MS11 *B. napus* of the T3 generation scoring positive for the presence of MS11 *B. napus* – *Eco*RV digested
Lane 14: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 3 µg of gDNA of the non-GM counterpart – *Eco*RI digested

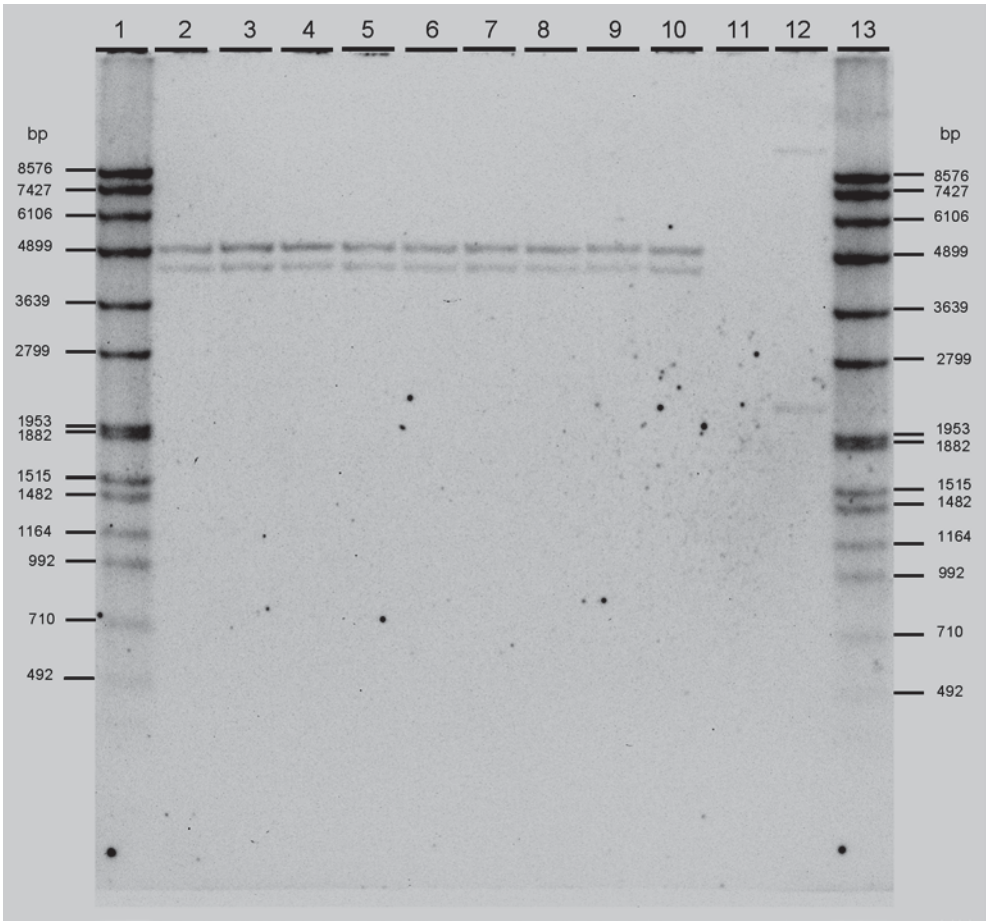


Figure V-4: Southern blot analysis of MS11 *B. napus* – Hybridization performed with a MS11 *B. napus* T-DNA probe to assess structural stability of the individual plants of the F1 generation

Digital image ID: H1/LJS017/02-F4

Genomic DNA was isolated from individual MS11 *B. napus* plants of the F1 generation confirmed as positive for the presence of MS11 *B. napus* and from the non-GM counterpart. The gDNA samples were digested with the restriction enzyme *Eco*RV and hybridized with a probe corresponding to the MS11 *B. napus* T-DNA region (P028-05).

Lane 1: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 3 µg of gDNA of the non-GM counterpart – *Eco*RI digested
Lane 2 to 10: 3 µg gDNA of individual samples of MS11 *B. napus* of the F1 generation scoring positive for the presence of MS11 *B. napus* – *Eco*RV digested
Lane 11: 3 µg gDNA of the non-GM counterpart – *Eco*RV digested (negative control)
Lane 12: 3 µg gDNA of the non-GM counterpart – *Eco*RI digested + an equimolar amount of plasmid pTCO113 – *Eco*RI digested (positive control)
Lane 13: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 3 µg of gDNA of the non-GM counterpart – *Eco*RI digested

Note: Image enhanced (brightness, -40%, contrast +40%) from original submission to increase visibility of positive control, lane 12.

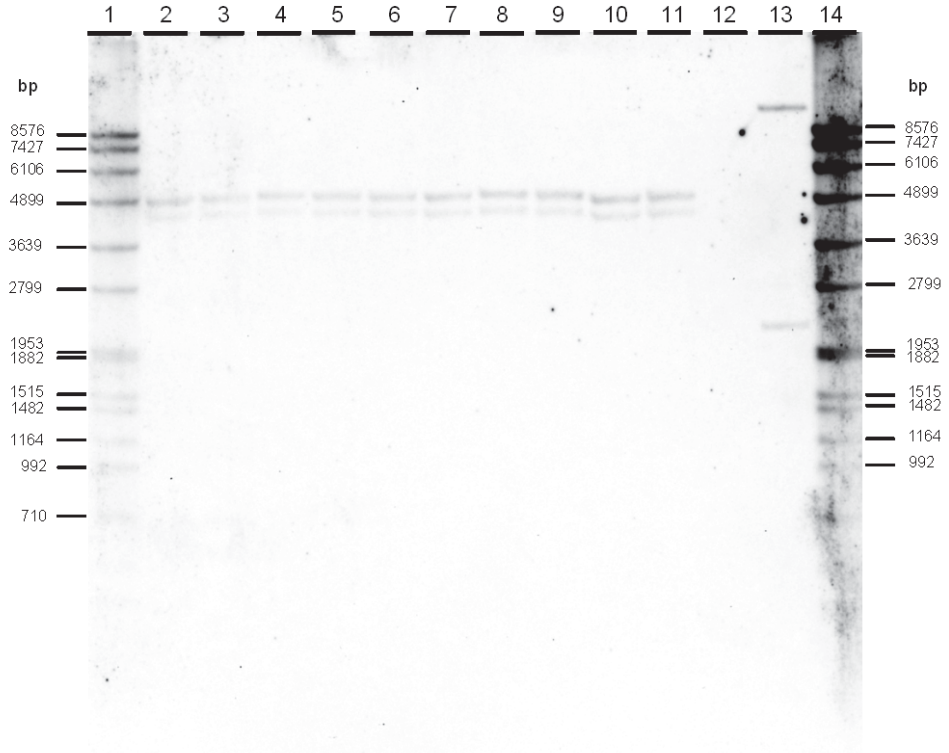


Figure V-5: Southern blot analysis of MS11 *B. napus* – Hybridization performed with a MS11 *B. napus* T-DNA probe to assess structural stability of the individual plants of the BC1 generation

Digital image ID: H1/LJS017/03-F2

Genomic DNA was isolated from individual MS11 *B. napus* plants of the BC1 generation confirmed as positive for the presence of MS11 *B. napus* and from the non-GM counterpart. The gDNA samples were digested with the restriction enzyme *Eco*RV and hybridized with a probe corresponding to the MS11 *B. napus* T-DNA region (P028-05).

Lane 1: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 3 µg of gDNA of the non-GM counterpart – *Eco*RI digested
Lane 2 to 11: 3 µg gDNA of individual samples of MS11 *B. napus* of the BC1 generation scoring positive for the presence of MS11 *B. napus* – *Eco*RV digested
Lane 12: 3 µg gDNA of the non-GM counterpart – *Eco*RV digested (negative control)
Lane 13: 3 µg gDNA of the non-GM counterpart – *Eco*RI digested + an equimolar amount of plasmid pTCO113 – *Eco*RI digested (positive control)
Lane 14: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 3 µg of gDNA of the non-GM counterpart – *Eco*RI digested

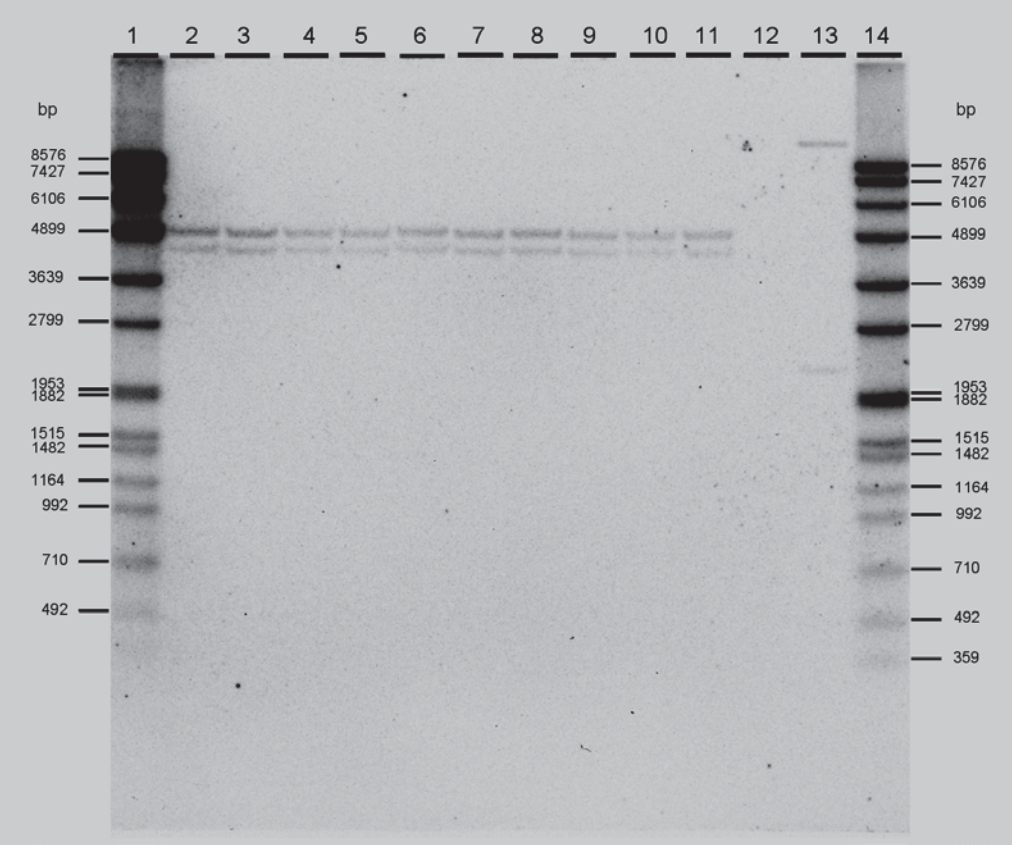


Figure V-6: Southern blot analysis of MS11 *B. napus* – Hybridization performed with a MS11 *B. napus* T-DNA probe to assess structural stability of the individual plants of the BC2 generation

Digital image ID: H1/LJS017/04-F1

Genomic DNA was isolated from individual MS11 *B. napus* plants of the BC2 generation confirmed as positive for the presence of MS11 *B. napus* and from the non-GM counterpart. The gDNA samples were digested with the restriction enzyme *Eco*RV and hybridized with a probe corresponding to the MS11 *B. napus* T-DNA region (P028-05).

Lane 1: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 3 µg of gDNA of the non-GM counterpart – *Eco*RI digested
Lane 2 to 11: 3 µg gDNA of individual samples of MS11 *B. napus* of the BC2 generation scoring positive for the presence of MS11 *B. napus* – *Eco*RV digested
Lane 12: 3 µg gDNA of the non-GM counterpart – *Eco*RV digested (negative control)
Lane 13: 3 µg gDNA of the non-GM counterpart – *Eco*RI digested + an equimolar amount of plasmid pTCO113 – *Eco*RI digested (positive control)
Lane 14: 10 ng DNA Molecular Weight marker VII, DIG-labeled + 3 µg of gDNA of the non-GM counterpart – *Eco*RI digested

V.B. Number of Insertion Sites, Arrangement and Copy Number of Transferred DNA

Insert characterization by Southern blot analysis

The transgenic locus of MS11 *B. napus* was characterized by means of Southern blot analysis.

Seeds from the T_2 generation were used to produce MS11 *B. napus* leaf material. The identity of the leaf material was confirmed. Non-GM *B. napus* variety N90-740 (non-GM counterpart) was used as a negative control. The positive control was the transforming plasmid of MS11 *B. napus* (pTCO113).

To characterize the transgenic locus of MS11, pooled gDNA from MS11 *B. napus* samples were digested with the restriction enzymes *Afl*III, *Bcl*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Hpa*I, *Kpn*I, *Mfe*I, *Nco*I, *Nde*I and *Sty*I. Pooled gDNA from plants of the non-GM counterpart was digested with the restriction enzyme *Eco*RI. Plasmid DNA of pTCO113 was digested with the *Eco*RI restriction enzyme.

The resulting DNA fragments were separated by agarose gel-electrophoresis. Transfer of the separated DNA fragments from the agarose gel to a positively charged nylon membrane was performed by a neutral Southern blotting procedure. The resulting membranes were hybridized with DIG-labeled probes covering the different components of the transgenic cassettes as well as the full T-DNA (P014, P016 to P023 and P028) (Figure V-7). Table V-3 provides details of the probes used in the Southern blot analysis. A schematic overview of the MS11 transgenic locus, with indication of the restriction enzymes, the probes used and the expected fragments is presented in Figure V-8.

Each membrane contained one negative control, in which the template DNA was digested gDNA prepared from the non-GM counterpart. This negative control showed no hybridization with any of the probes used, confirming the absence of any background hybridization. Similarly, each membrane contained a positive control of digested gDNA prepared from non-GM plant material, supplemented with an equimolar amount of digested transforming plasmid pTCO113. For each of the probes used, the expected fragments were detected for the positive control, confirming that the applied experimental conditions allowed specific hybridization of the probes used with the target sequences. Hybridization of the positive control with the Pta29 probe showed a second band of >10 kb which is the result of incomplete digestion of the plasmid (Figure V-15, lane 15).

The banding pattern expected for a single insertion was observed for MS11 *B. napus* samples with all restriction digests and probe combinations tested (Table V-4, Figure V-9 to Figure V-21).

Membranes containing gDNA digested with *Hpa*I and hybridized with the *barstar*, 3'barnase-*barnase*, Pta29, Pnos and the T-DNA probes (Figure V-10, Figure V-14, Figure V-15, Figure V-16 and Figure V-19, lane 8) showed an additional weak fragment of approximately 10 kb. The probes with which this fragment is visualized and the fact that the size of this fragment (approximately 10 kb) is an approximate summation of the 2296 bp internal fragment and a 8200 bp 3' integration fragment demonstrates that this fragment is the result of an incomplete digestion of the *Hpa*I restriction site within the Pta29 promoter. Hybridization of a freshly-prepared *Hpa*I digested gDNA sample with the T-DNA probe confirmed the presence of this incomplete digested fragment (Figure V-20, lane 3).

Additionally, membranes containing gDNA digested with *Sty*I and hybridized with any of the probes (Figure V-10, Figure V-11 and Figure V-16, lane 12; Figure V-9, Figure V-12 to Figure V-15, Figure V-18 and Figure V-19, lane 13) resulted in a smear of fragments as a consequence of partially digested gDNA. To confirm the single copy model as present in the pTCO113 plasmid for MS11, the *Sty*I restriction digestion and Southern blot analysis was repeated. Hybridization of this freshly-prepared *Sty*I restriction digestion with the T-DNA probe (Figure V-21, lane 3) resulted in all expected fragments and confirmed the single copy model as present in the pTCO113 plasmid for MS11.

Finally, the membrane containing gDNA digested with *Nco*I and hybridized with the Pnos probe (Figure V-16, lane 11) showed two bands corresponding to both the integration fragments, whereas only hybridization with the 2500 bp fragment was expected. Since the hybridization signal with the 5300 bp fragment was assumed to be the result of a not fully stripped membrane, the experiment was repeated. This hybridization (Figure V-17, lane 3) confirmed the absence of a signal of the Pnos probe with the 5300 bp fragment.

In conclusion, the Southern blot results demonstrated the presence of one complete T-DNA insert containing the *bar*, the *barnase* and the *barstar* gene cassettes in MS11 *B. napus*.

Table V-3: Information on the probes used for insert characterization

Probe ID	Probe template ID	Description	Primer pair/ Restr. digest	Primer sequence (5' □ 3')	Primer position on pTCO113 (bp)	Size probe template (bp)
P014	PT023	*bar*	GLPA343	GAAACCCACGTCATGCCAGTTCC	395 → 417	425
			GLPA344	GCACCATCGTCAACCACTACATCG	819 → 796	
P016	PT035	*barstar*	GLPA345°	GCAGTCATTAACGGGGAACAAATC	5226 → 5249 and 8049 → 8026	262°
			GLPA346°	AAGAAAGTATGATGGTGATGTCG	5487 → 5465 and 7788 → 7810	
P017	PT073	RB - 3'g7	GLPA174	AATTACAACGGTATATATCCTGCCA	1 → 25	317 8311**
			GLPA048	GGATCCCCCGATGAGCTAAGCTAGC	317 → 293 and 5547 → 5571	
P018	PT092	3'nos	GLPA348	GTAACATAGATGACACCGCGC	2666 → 2686	217
			GLPA349	TTAAGATTGAATCCTGTTGCCG	2882 → 2861	
P019	PT108	PssuAt	GLPA001	ATGTCGGCCGGGCGTCGTTCTG	855 → 876	1870
			GLPA005	AAACAAAATATAGCGCGCAAACT	2724 → 2702	
P020	PT109	3'barnase - *barnase*	GLPA006	CCGGCAACAGGATTCAATCT	2860 → 2879	573
			GLPA009	GCAAGTGTAACAGTACAACATCATCACT	3432 → 3405	
P021	PT110	Pta29	GLPA012	GTAATGTAATTATCAGGTAGCTTATGATATGTCTG	3290 → 3324	1660
			GLPA013	CTCCCTTAATTCTCCGCTCATG	4949 → 4928	
P022	PT111	Pnos	GLPA015	TAATCGACGGATCCCCGGG	4873 → 4891	374 10758***
			GLPA017	TTGTTCCCCGTTAATGACTGCT	5246 → 5225 and 8029 → 8050	
P023	PT116	3'g7 - LB	GLPA020	GGAAACACAAACCCGCAAGC	5516 → 5535 and 7759 → 7740	350 2244****
			GLPA359	CGGCAGGATATATTCAATTGTAAAT	5865 → 5841	
P028	PT108	T-DNA	GLPA174	AATTACAACGGTATATATCCTGCCA	1 → 25	5865
			GLPA359	CGGCAGGATATATTCAATTGTAAAT	5865 → 5841	

** An additional PCR product of 8311 bp can be produced
*** An additional PCR product of 10758 bp can be produced
**** An additional PCR product of 2244 bp can be produced
° These primers amplify two identical regions

Table V-4: Expected and obtained hybridization fragments determined for the insert characterization of MS11 *B. napus*

Part 1: Probes P014-2, P016-1, P017-3, P018-4, P019-2, and P020-2.

Enzyme	Expected fragment size (bp)	Fragment description	Obtained fragment size (bp)	H4/LJS018/08-F5		H1/LJS018/06-F9		H4/LJS018/06-F4		H8/LJS018/09-F2		H5/LJS018/09-F3		H1/LJS018/10-F4	
				P014-2		P016-1		P017-3		P018-4		P019-2		P020-2	
				bar		*barstar*		RB-3'g7		3'nos		PssuAt		3'barnase-*barnase*	
				Figure 9		Figure 10		Figure 11		Figure 12		Figure 13		Figure 14	
				Expected	Obtained	Expected	Obtained	Expected	Obtained	Expected	Obtained	Expected	Obtained	Expected	Obtained
***Afl*III**	>305	5' integration fr.	4300	No	No	No	No	Yes	Yes	No	No	No	No	No	No
	2476	internal fr.	2476	Yes	Yes	No	No	No	No	Yes ** (132)	Yes	Yes	Yes	No	No
	550	internal fr.	550	No	No	No	No	No	No	Yes ** (84)	No	No	No	Yes	Yes
	>2467	3' integration fr	2500	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes ** (84)	No
***Bcl*I**	>1637	5' integration fr.	1850	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes	No	No
	2761	internal fr.	2761	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
	509	internal fr.	509	No	No	No	No	No	No	No	No	No	No	No	No
	>891	3' integration fr.	2650	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No
***Eco*RI**	>2614	5' integration fr.	> 10 kb	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes	No	No
	2260	internal fr.	2260	No	No	No	No	No	No	Yes	Yes	Yes ** (93)	No	Yes	Yes
	>924	3' integration fr.	8400	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No
***Eco*RV**	>3895	5' integration fr.	4900	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	>1903	3' integration fr.	4400	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No
***Hin*dIII**	>948	5' integration fr.	2100	Yes	Yes	No	No	Yes	Yes	No	No	Yes ** (110)	No	No	No
	3938	internal fr.	3938	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
***Hin*dIII**	629	internal fr.	629	No	No	Yes	Yes	No	No	No	No	No	No	No	No
	>283	3' integration fr.	1450	No	No	No	No	Yes	Yes	No	No	No	No	No	No
***Hpa*I**	>1867	5' integration fr.	3200	Yes	Yes	No	No	Yes	Yes	No	No	Yes	Yes	No	No
	2296	internal fr.	2296	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
	>1635	3' integration fr.	8200	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No

Enzyme	Expected fragment size (bp)	Fragment description	Obtained fragment size (bp)	H4/LJS018/08-F5		H1/LJS018/06-F9		H4/LJS018/06-F4		H8/LJS018/09-F2		H5/LJS018/09-F3		H1/LJS018/10-F4	
				P014-2		P016-1		P017-3		P018-4		P019-2		P020-2	
				bar		*barstar*		RB-3'g7		3'nos		PssuAt		3'barnase-*barnase*	
				Figure 9		Figure 10		Figure 11		Figure 12		Figure 13		Figure 14	
				Expected	Obtained	Expected	Obtained	Expected	Obtained	Expected	Obtained	Expected	Obtained	Expected	Obtained
		Additional fr.	>10 kb	No	No	No	Yes	No	No	No	No	No	No	No	Yes
***Kpn*I**	>349	5' integration fr.	7000	No	No	No	No	Yes	Yes	No	No	No	No	No	No
	2256	internal fr.	2256	Yes	Yes	No	No	No	No	No	No	Yes	Yes	No	No
	21*	internal fr.	NA	No	No	No	No	No	No	No	No	Yes ** (21)	No	No	No
	719	internal fr.	719	No	No	No	No	No	No	Yes	Yes	Yes ** (81)	No	Yes	Yes
	>2453	3' integration fr.	>10 kb	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes ** (70)	No
***Mfe*I**	>3827	5' integration fr.	4100	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	>1971	3' integration fr.	5000	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No
***Nco*I**	>3349	5' integration fr.	5300	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	>2449	3' integration fr.	2500	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes ** (66)	No
		Additional fr.	5300	No	No	No	No	No	No	No	No	No	No	No	No
***Nde*I**	>4557	5' integration fr.	6900	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	64*	internal fr.	NA	No	No	No	No	No	No	No	No	No	No	No	No
	>1177	3' integration fr.	1600	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No
***Sty*I**	>1279	5' integration fr.	3000	Yes	Unknown	No	Unknown	Yes	Unknown	No	Unknown	Yes	Unknown	No	No
	2070	internal fr.	2070	No		No		No		Yes		Yes		Yes	No
	982	internal fr.	982	No		No		No		No		No		Yes ** (66)	No
	>1467	3' integration fr.	1500	No		Yes		Yes		No		No		No	No
		Additional fr.		No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	No
Non-GM counterpart genomic DNA *Eco*RI digested	/	negative control	NA	No	No	No	No	No	No	No	No	No	No	No	No

Enzyme	Expected fragment size (bp)	Fragment description	Obtained fragment size (bp)	H4/LJS018/08-F5		H1/LJS018/06-F9		H4/LJS018/06-F4		H8/LJS018/09-F2		H5/LJS018/09-F3		H1/LJS018/10-F4	
				P014-2		P016-1		P017-3		P018-4		P019-2		P020-2	
				bar		*barstar*		RB-3'g7		3'nos		PssuAt		3'barnase-*barnase*	
				Figure 9		Figure 10		Figure 11		Figure 12		Figure 13		Figure 14	
				Expected	Obtained	Expected	Obtained	Expected	Obtained	Expected	Obtained	Expected	Obtained	Expected	Obtained
Non-GM counterpart genomic DNA *Eco*RI digested + 1 equimolar amount pTCO113 *Eco*RI digested	2260	positive control	2260	No	No	No	No	No	No	Yes	Yes	No	No	Yes	Yes
	11280		11280	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	No	No

Table V-4: Expected and obtained hybridization fragments determined for the insert characterization of MS11 *B. napus*

Part 2: Probes P021-2, P022-2, P022-3, P023-2, P028-12, P028-8, and P028-12.

Enzyme	Expected fragment size (bp)	Fragment description	Obtained fragment size (bp)	H3/LJS018/09-F2		H6/LJS018/06-F2		H1/LJS018/18-F2	H5/LJS018/08-F4		H6/LJS018/08-F4		H1/LJS018/16-F5	H1/LJS018/15-F3
				P021-2		P022-2		P022-3	P023-2		P028-12		P028-8	P028-12
				Pta29		Pnos			3'g7-LB		T-DNA probe			
				Figure 15			Figure 16	Figure 17	Figure 18			Figure 19	Figure 20	Figure 21
				Expected	Obtained	Expected	Obtained		Expected	Obtained	Expected	Obtained		
***Afl*III**	>305	5' integration fr.	4300	No	No	No	No	N.A.	Yes	Yes	Yes ** (119)	No	N.A.	N.A.
	2476	internal fr.	2476	No	No	No	No	N.A.	No	No	Yes	Yes$	N.A.	N.A.
	550	internal fr.	550	Yes ** (58)	No	No	No	N.A.	No	No	Yes	No	N.A.	N.A.
	>2467	3' integration fr.	2500	Yes	Yes	Yes	Yes	N.A.	Yes	Yes	Yes	Yes$	N.A.	N.A.
***Bcl*I**	>1637	5' integration fr.	1850	No	No	No	No	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
	2761	internal fr.	2761	Yes	Yes	No	No	N.A.	No	No	Yes	Yes$	N.A.	N.A.
	509	internal fr.	509	Yes	Yes	Yes ** (52)	No	N.A.	No	No	Yes	No °	N.A.	N.A.
	>891	3' integration fr.	2650	Yes ** (25)	No	Yes	Yes	N.A.	Yes	Yes	Yes	Yes$	N.A.	N.A.
***Eco*RI**	>2614	5' integration fr.	> 10 kb	No	No	No	No	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
	2260	internal fr.	2260	Yes	Yes	Yes ** (19)	No	N.A.	No	No	Yes	Yes	N.A.	N.A.
	>924	3' integration fr.	8400	Yes ** (58)	Yes	Yes	Yes	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
***Eco*RV**	>3895	5' integration fr.	4900	Yes	Yes	No	No	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
	>1903	3' integration fr.	4400	Yes	Yes	Yes	Yes	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
***Hin*dIII**	>948	5' integration fr.	2100	No	No	No	No	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
	3938	internal fr.	3938	Yes	Yes	Yes ** (31)	No	N.A.	No	No	Yes	Yes	N.A.	N.A.
	629	internal fr.	629	Yes ** (46)	Yes	Yes	Yes	N.A.	Yes ** (16)	No	Yes	No	N.A.	N.A.
***Hin*dIII**	>283	3' integration fr.	1450	No	No	No	No	N.A.	Yes	Yes	Yes ** (128)	No	N.A.	N.A.
***Hpa*I**	>1867	5' integration fr.	3200	No	No	No	No	N.A.	Yes	Yes	Yes	Yes	Yes	N.A.
	2296	internal fr.	2296	Yes	Yes	No	No	N.A.	No	No	Yes	Yes	Yes	N.A.
	>1635	3' integration fr.	8200	Yes	Yes	Yes	Yes	N.A.	Yes	Yes	Yes	Yes	Yes	N.A.

Enzyme	Expected fragment size (bp)	Fragment description	Obtained fragment size (bp)	H3/LJS018/09-F2		H6/LJS018/06-F2		H1/LJS018/18-F2	H5/LJS018/08-F4		H6/LJS018/08-F4		H1/LJS018/16-F5	H1/LJS018/15-F3
				P021-2		P022-2		P022-3	P023-2		P028-12		P028-8	P028-12
				Pta29		Pnos			3'g7-LB		T-DNA probe			
				Figure 15			Figure 16	Figure 17	Figure 18			Figure 19	Figure 20	Figure 21
				Expected	Obtained	Expected	Obtained		Expected	Obtained	Expected	Obtained		
		Additional fr.	>10 kb	No	Yes	No	Yes	N.A.	No	No	No	Yes	Yes	N.A.
***Kpn*I**	>349	5' integration fr.	7000	No	No	No	No	N.A.	Yes	Yes	Yes ** (163)	No	N.A.	N.A.
	2256	internal fr.	2256	No	No	No	No	N.A.	No	No	Yes	Yes	N.A.	N.A.
	21*	internal fr.	NA	No	No	No	No	N.A.	No	No	Yes ** (21)	No	N.A.	N.A.
	719	internal fr.	719	Yes ** (72)	Yes	No	No	N.A.	No	No	Yes	No	N.A.	N.A.
	>2453	3' integration fr.	>10 kb	Yes	Yes	Yes	Yes	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
***Mfe*I**	>3827	5' integration fr.	4100	Yes	Yes	No	No	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
	>1971	3' integration fr.	5000	Yes	Yes	Yes	Yes	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
***Nco*I**	>3349	5' integration fr.	5300	Yes ** (76)	Yes	No	No	No	Yes	Yes	Yes	Yes	N.A.	N.A.
	>2449	3' integration fr.	2500	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	N.A.	N.A.
		Additional fr.	5300	No	No	No	Yes	No	No	No	No	No	N.A.	N.A.
***Nde*I**	>4557	5' integration fr.	6900	Yes	Yes	No	No	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
	64*	internal fr.	NA	Yes ** (64)	No	No	No	N.A.	No	No	Yes ** (64)	No	N.A.	N.A.
	>1177	3' integration fr.	1600	Yes	Yes	Yes	Yes	N.A.	Yes	Yes	Yes	Yes	N.A.	N.A.
***Sty*I**	>1279	5' integration fr.	3000 [b]	No	Unknown	No	Unknown	N.A.	Yes	Unknown	Yes	Unknown	N.A.	Yes [b]
	2070	internal fr.	2070	Yes ** (76)		No		N.A.	No		Yes		N.A.	Yes
	982	internal fr.	982	Yes		No		N.A.	No		Yes		N.A.	Yes
	>1467	3' integration fr.	1500 [b]	Yes		Yes		N.A.	Yes		Yes		N.A.	Yes [b]
		Additional fr.		No	Yes	No	Yes	N.A.	No	Yes	No	Yes	N.A.	No
Non-GM counterpart genomic DNA *Eco*RI digested	/	negative control	NA	No	No	No	No	No	No	No	No	No	No	No

Enzyme	Expected fragment size (bp)	Fragment description	Obtained fragment size (bp)	H3/LJS018/09-F2		H6/LJS018/06-F2		H1/LJS018/18-F2	H5/LJS018/08-F4		H6/LJS018/08-F4		H1/LJS018/16-F5	H1/LJS018/15-F3
				P021-2		P022-2		P022-3	P023-2		P028-12		P028-8	P028-12
				Pta29		Pnos			3'g7-LB		T-DNA probe			
				Figure 15			Figure 16	Figure 17	Figure 18			Figure 19	Figure 20	Figure 21
				Expected	Obtained	Expected	Obtained		Expected	Obtained	Expected	Obtained		
Non-GM counterpart genomic DNA *Eco*RI digested + 1 equimolar amount pTCO113 *Eco*RI digested	2260	positive control	2260	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes	Yes
	11280		11280	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

* Based on the technical limits of Southern Blotting, this fragment might be too small to be visualized.

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

$: These bands have a comparable size and cannot be distinguished using this experimental setup

° This band is not observed because of the small size of the fragment in comparison with the large probe

[a] this band overlaps with the 2761 bp internal fragment

[b] With this experimental setup, it is not possible to determine if this fragment represents the 5' or 3' integration fragment

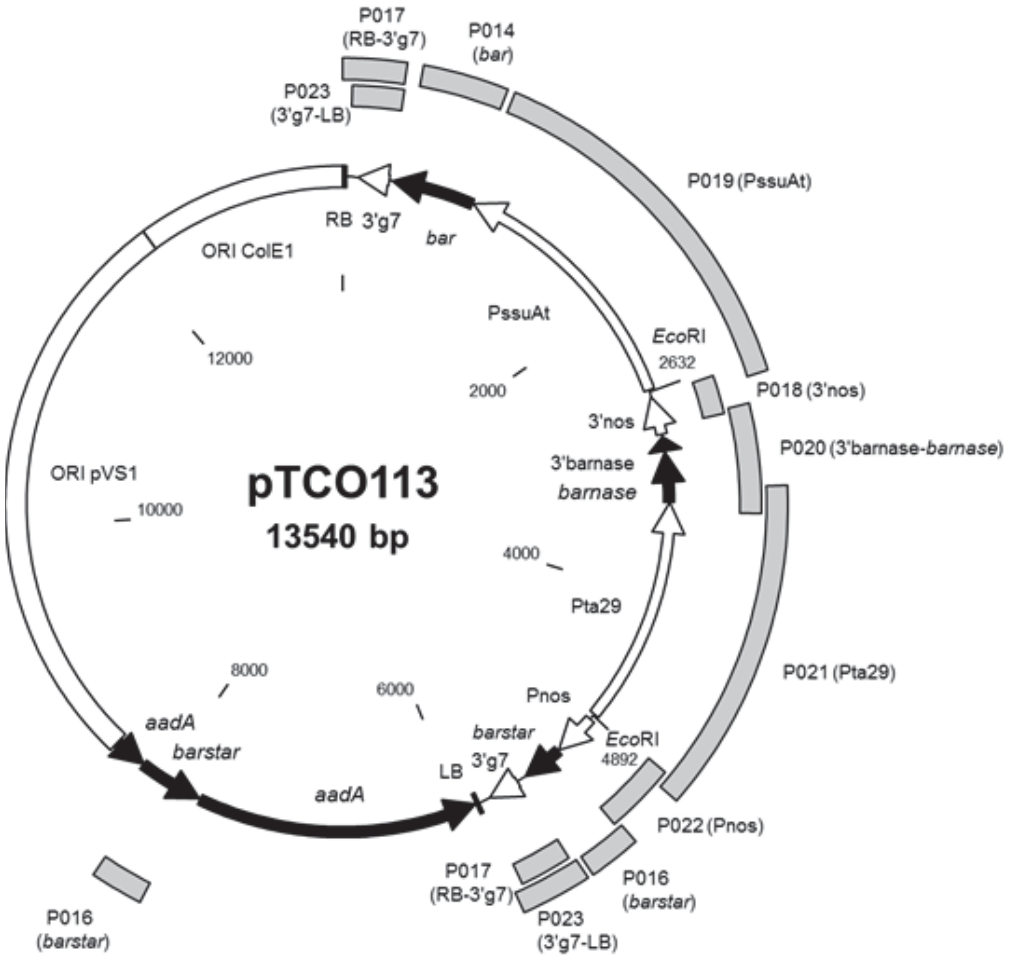


Figure V-7: Map of transformation vector pTCO113 with indication of the position of enzymes used for plasmid digestion in this study and the probes covering the different individual features of the T-DNA region

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

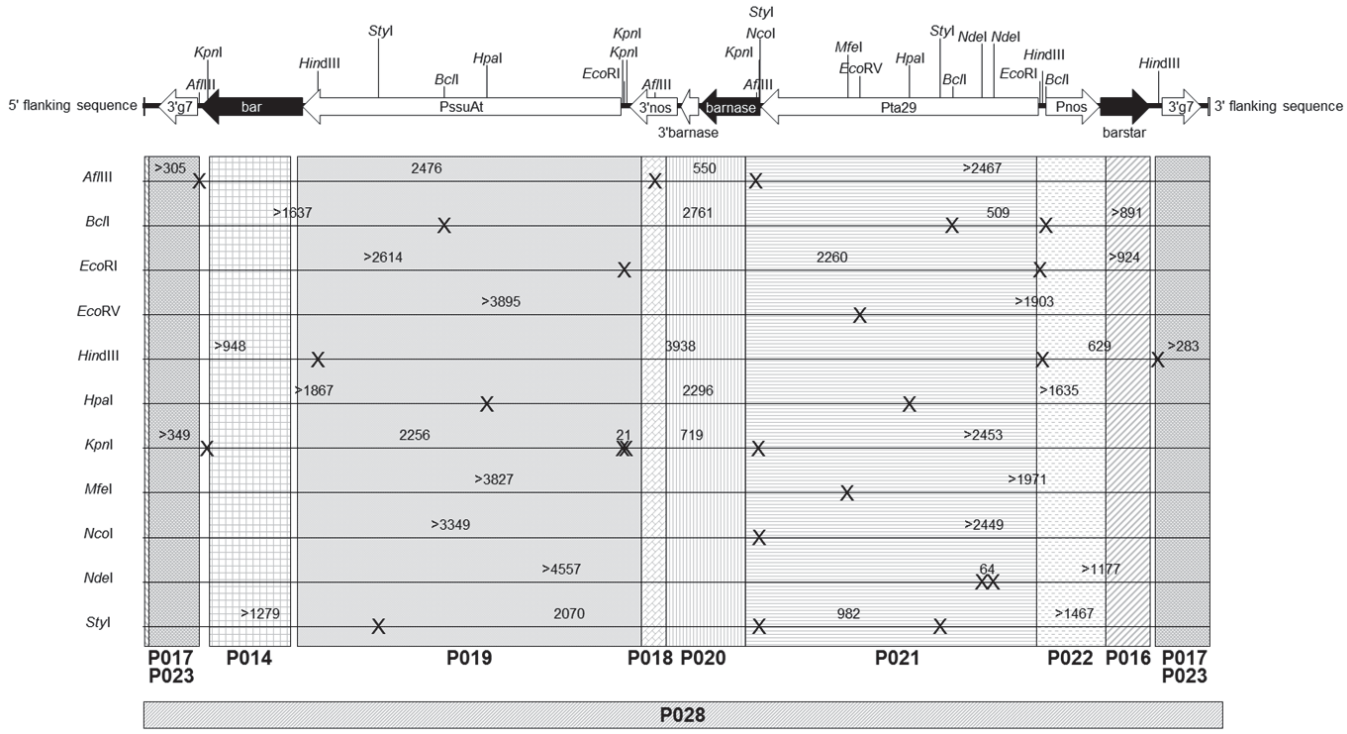


Figure V-8: Schematic overview of the MS11 transgenic locus with indication of the different restriction enzymes and probes used in this study to assess the insert organization, and expected fragment sizes in bp

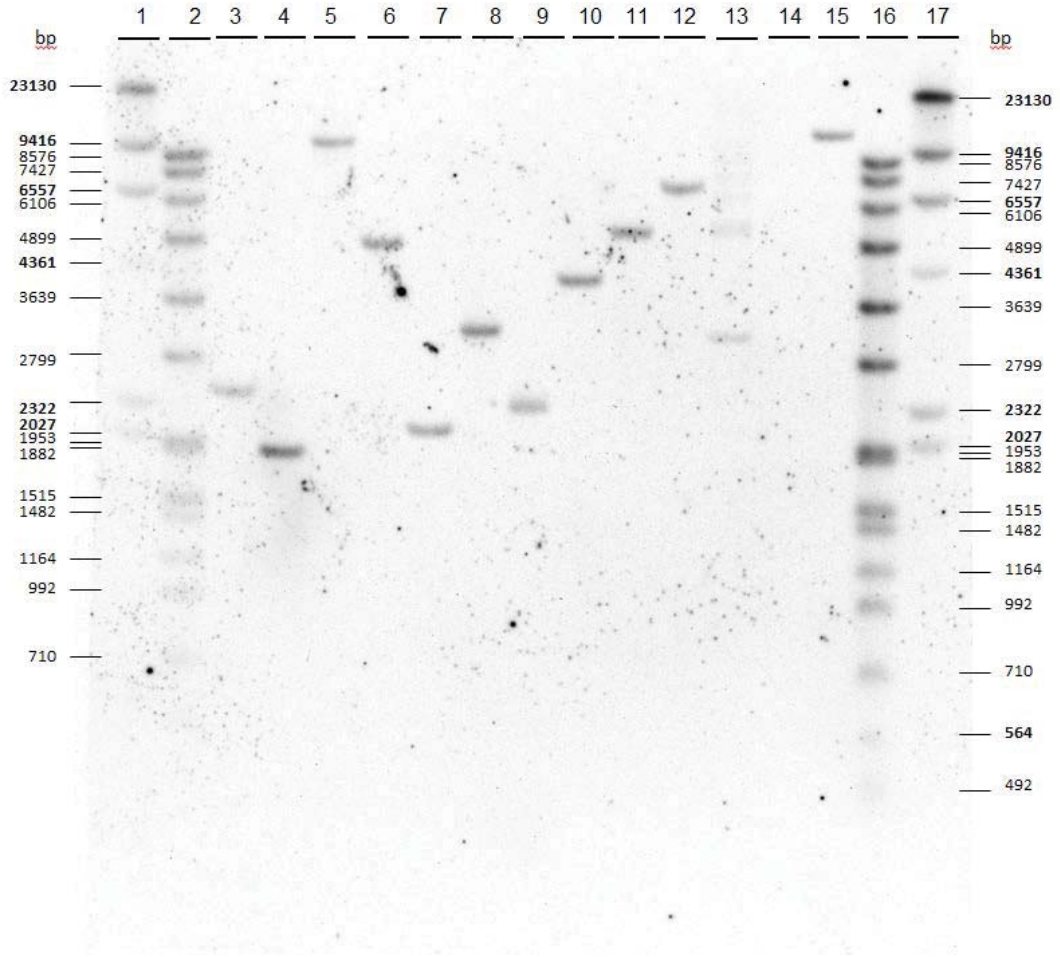


Figure V-9: Hybridization performed with a *bar* probe (P014) to determine the insert organization of MS11

Digital image: H4/LJS018/08-F5

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 *bar* sequence (P014-2, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 5 µg gDNA from MS11 *B. napus* - *Bcl*I digested
Lane 5: 5 µg gDNA from MS11 *B. napus* - *Eco*RI digested
Lane 6: 5 µg gDNA from MS11 *B. napus* - *Eco*RV digested
Lane 7: 5 µg gDNA from MS11 *B. napus* - *Hin*dIII digested
Lane 8: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 9: 5 µg gDNA from MS11 *B. napus* - *Kpn*I digested
Lane 10: 5 µg gDNA from MS11 *B. napus* - *Mfe*I digested
Lane 11: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 12: 5 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 13: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 14: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 15: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 16: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 17: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

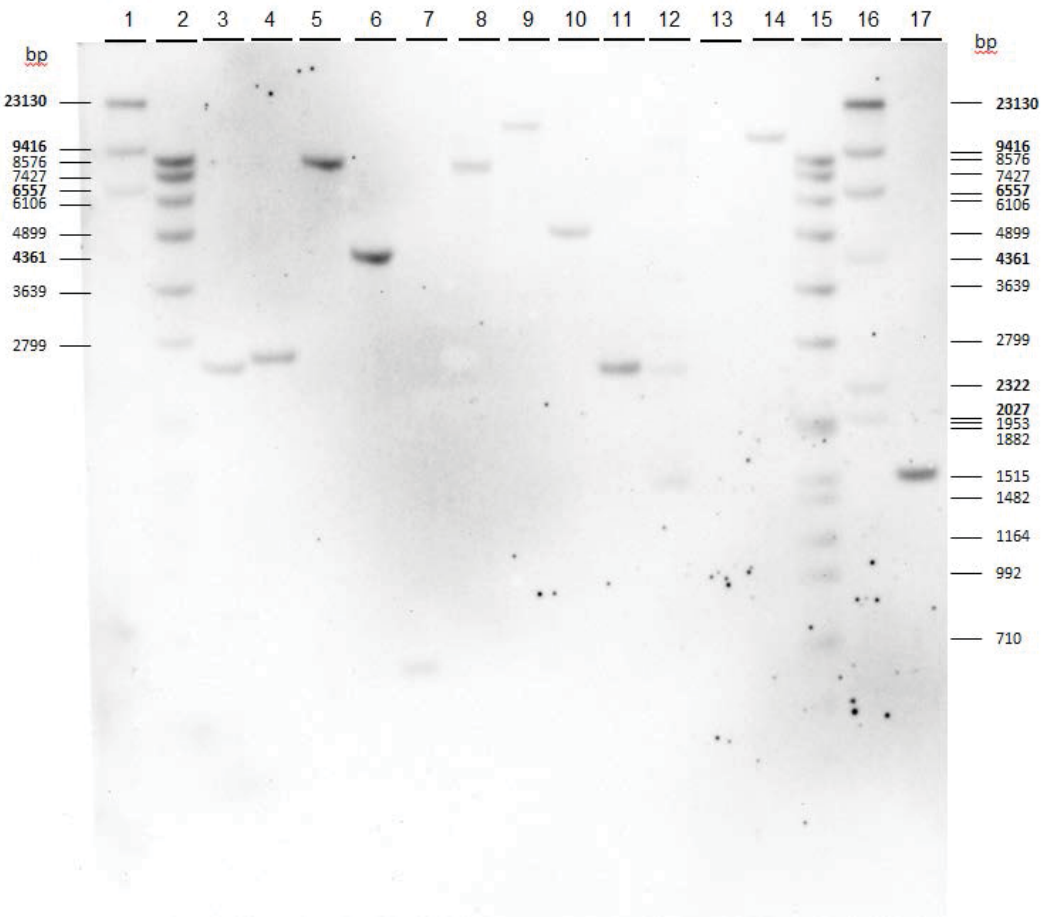


Figure V-10: Hybridization performed with a *barstar* probe (P016) to determine the insert organization of MS11

Digital image: H1/LJS018/06-F9

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 *barstar* sequence (P016-1, random primed labeling).The size of the low molecular weight band in lane 7 is determined using another exposure of this membrane (data not shown).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 5 µg gDNA from MS11 *B. napus* - *Bcl*I digested
Lane 5: 5 µg gDNA from MS11 *B. napus* - *Eco*RI digested
Lane 6: 5 µg gDNA from MS11 *B. napus* - *Eco*RV digested
Lane 7: 5 µg gDNA from MS11 *B. napus* - *Hin*dIII digested
Lane 8: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 9: 5 µg gDNA from MS11 *B. napus* - *Kpn*I digested
Lane 10: 5 µg gDNA from MS11 *B. napus* - *Mfe*I digested
Lane 11: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 12: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 13: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 14: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 15: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 17: 5 µg gDNA from MS11 *B. napus* - *Nde*I digested

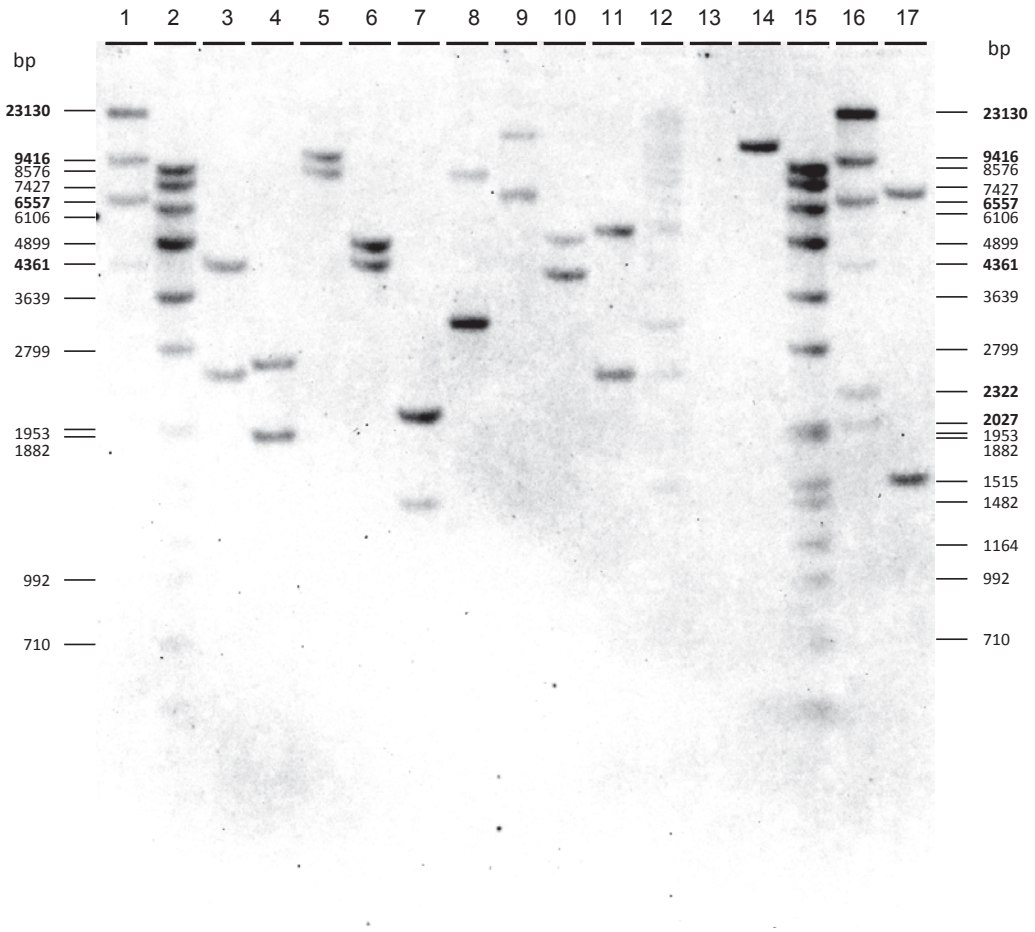


Figure V-11: Hybridization performed with a RB-3'g7 probe (P017) to determine the insert organization of MS11

Digital image: H4/LJS018/06-F4

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 RB-3'g7 sequence (P017-3, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 5 µg gDNA from MS11 *B. napus* - *Bcl*I digested
Lane 5: 5 µg gDNA from MS11 *B. napus* - *Eco*RI digested
Lane 6: 5 µg gDNA from MS11 *B. napus* - *Eco*RV digested
Lane 7: 5 µg gDNA from MS11 *B. napus* - *Hin*dIII digested
Lane 8: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 9: 5 µg gDNA from MS11 *B. napus* - *Kpn*I digested
Lane 10: 5 µg gDNA from MS11 *B. napus* - *Mfe*I digested
Lane 11: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 12: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 13: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 14: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 15: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 17: 5 µg gDNA from MS11 *B. napus* - *Nde*I digested

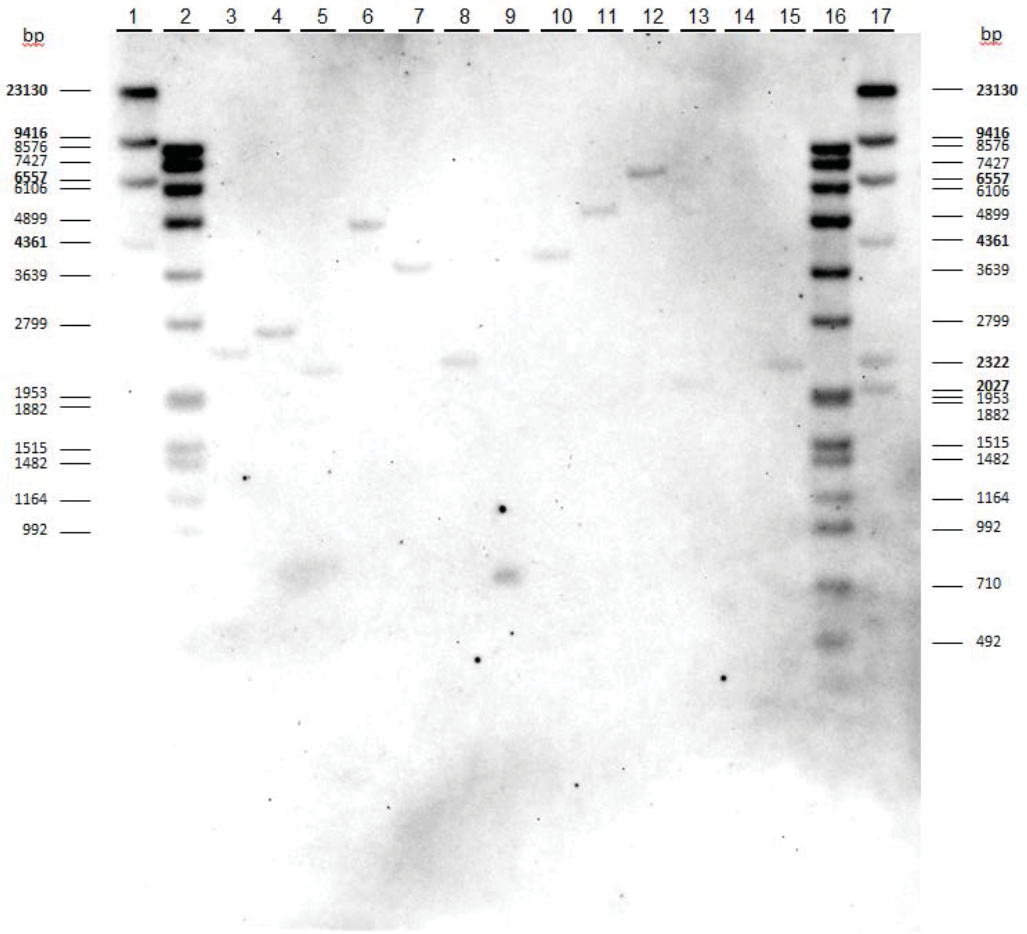


Figure V-12: Hybridization performed with a 3'nos probe (P018) to determine the insert organization of MS11

Digital image: H8/LJS018/09-F2

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 3'nos sequence (P018-4, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 5 µg gDNA from MS11 *B. napus* - *Bcl*I digested
Lane 5: 5 µg gDNA from MS11 *B. napus* - *Eco*RI digested
Lane 6: 5 µg gDNA from MS11 *B. napus* - *Eco*RV digested
Lane 7: 5 µg gDNA from MS11 *B. napus* - *Hin*dIII digested
Lane 8: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 9: 5 µg gDNA from MS11 *B. napus* - *Kpn*I digested
Lane 10: 5 µg gDNA from MS11 *B. napus* - *Mfe*I digested
Lane 11: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 12: 5 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 13: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 14: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 15: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 16: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 17: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

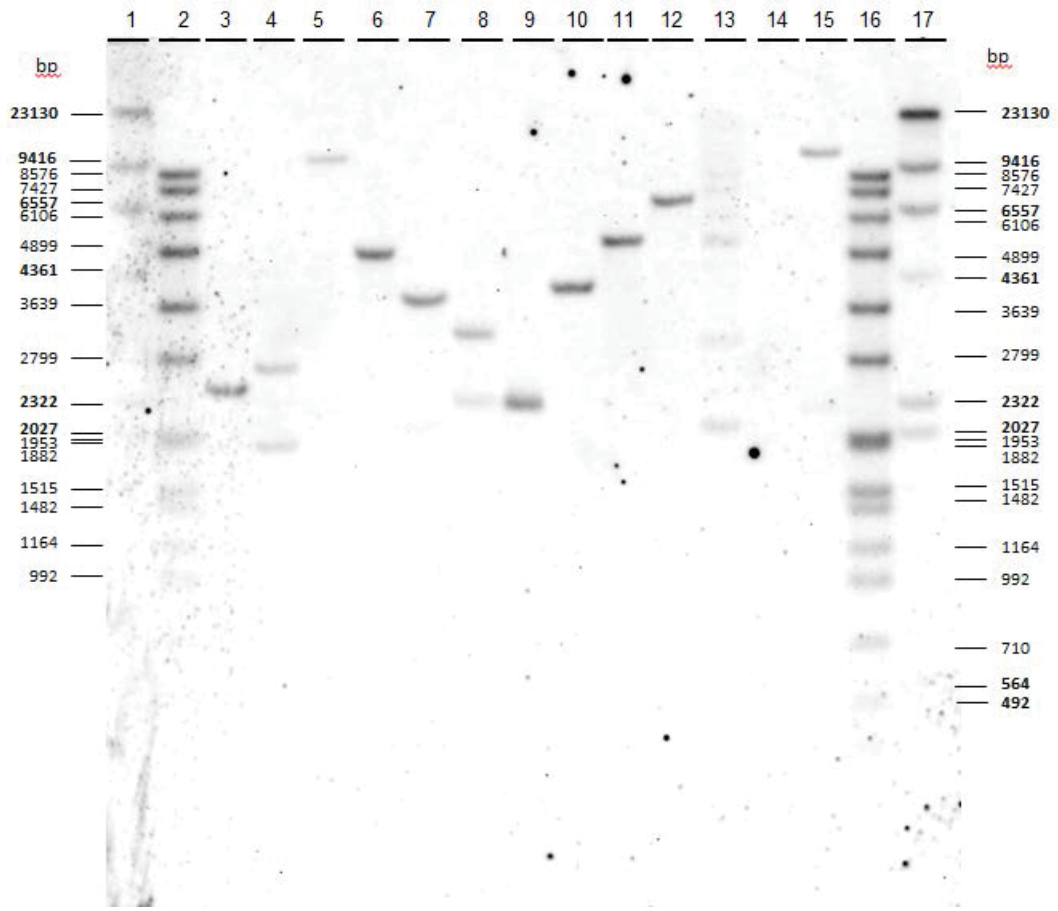


Figure V-13: Hybridization performed with a PssuAt probe (P019) to determine the insert organization of MS11

Digital image: H5/LJS018/09-F3

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 PssuAt sequence (P019-2, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 5 µg gDNA from MS11 *B. napus* - *Bcl*I digested
Lane 5: 5 µg gDNA from MS11 *B. napus* - *Eco*RI digested
Lane 6: 5 µg gDNA from MS11 *B. napus* - *Eco*RV digested
Lane 7: 5 µg gDNA from MS11 *B. napus* - *Hin*dIII digested
Lane 8: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 9: 5 µg gDNA from MS11 *B. napus* - *Kpn*I digested
Lane 10: 5 µg gDNA from MS11 *B. napus* - *Mfe*I digested
Lane 11: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 12: 5 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 13: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 14: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 15: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 16: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 17: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

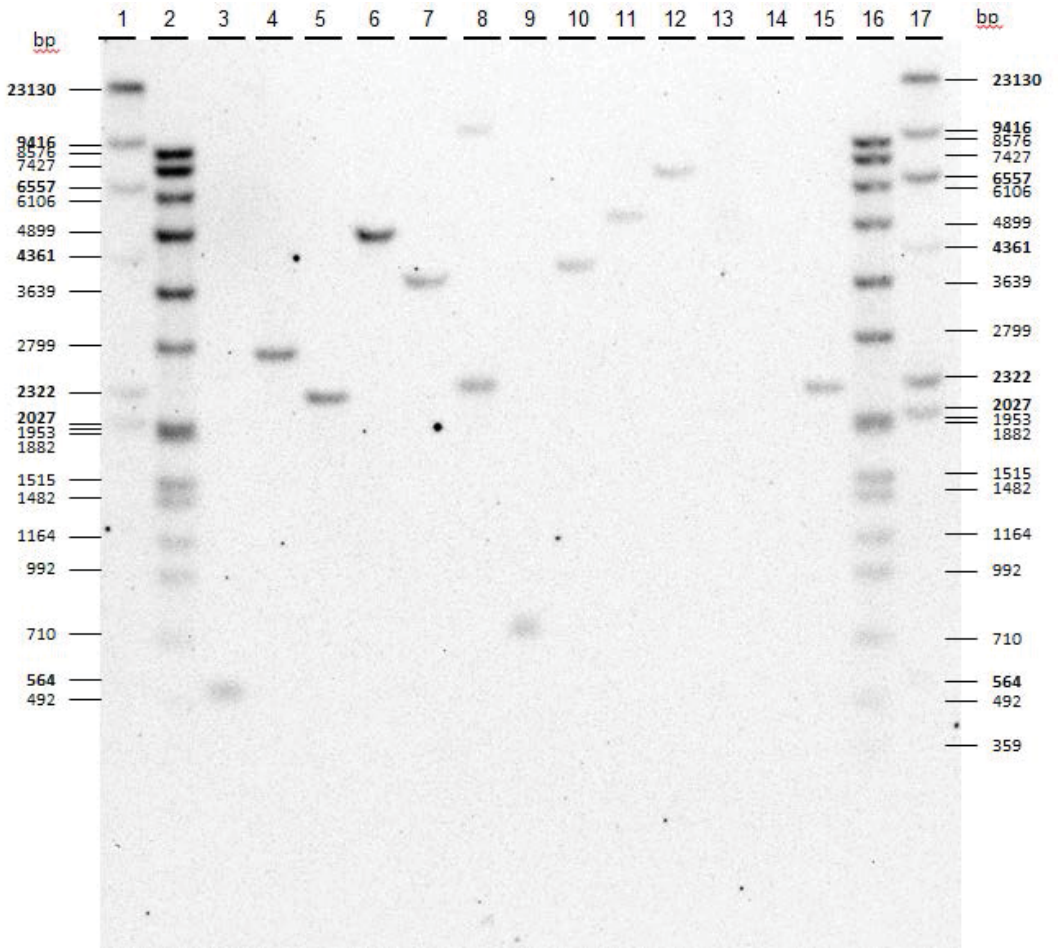


Figure V-14: Hybridization performed with a 3'barnase-*barnase* probe (P020) to determine the insert organization of MS11

Digital image: H1/LJS018/10-F4

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 3'barnase-*barnase* sequence (P020-2, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 5 µg gDNA from MS11 *B. napus* - *Bcl*I digested
Lane 5: 5 µg gDNA from MS11 *B. napus* - *Eco*RI digested
Lane 6: 5 µg gDNA from MS11 *B. napus* - *Eco*RV digested
Lane 7: 5 µg gDNA from MS11 *B. napus* - *Hin*dIII digested
Lane 8: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 9: 5 µg gDNA from MS11 *B. napus* - *Kpn*I digested
Lane 10: 5 µg gDNA from MS11 *B. napus* - *Mfe*I digested
Lane 11: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 12: 5 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 13: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 14: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 15: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 16: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 17: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

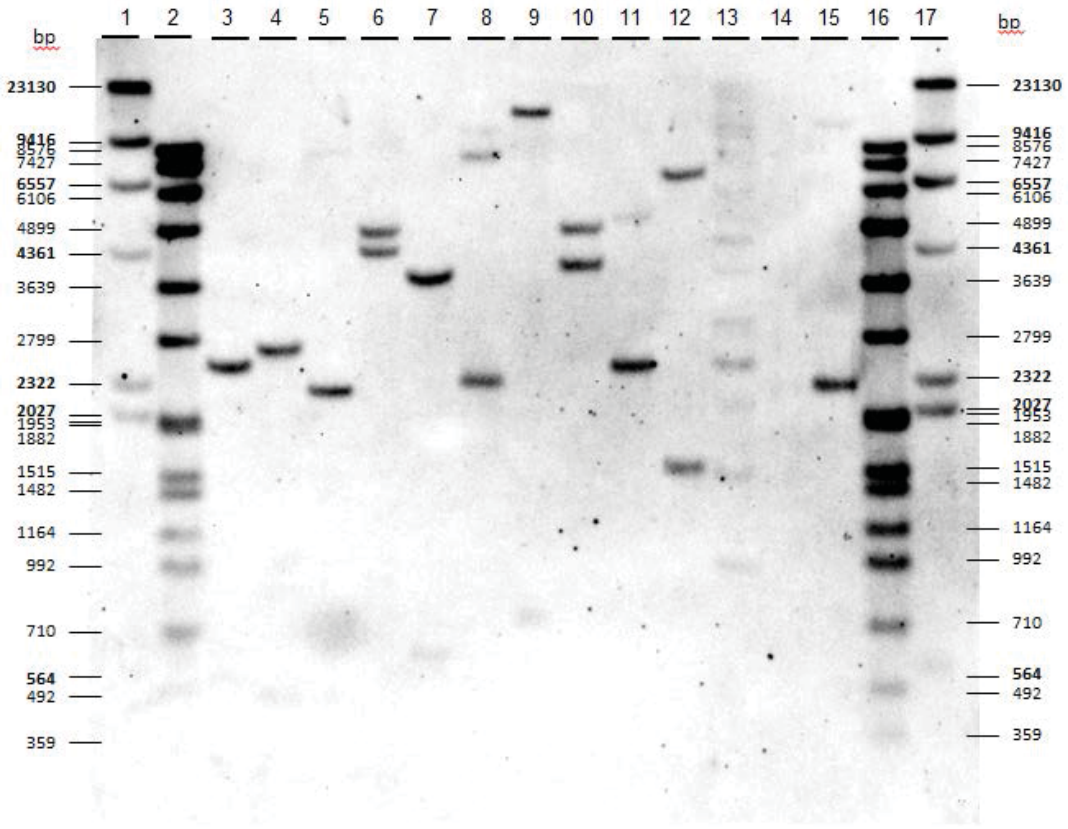


Figure V-15: Hybridization performed with a Pta29 probe (P021) to determine the insert organization of MS11

Digital image: H3/LJS018/09-F2

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 Pta29 sequence (P021-2, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 5 µg gDNA from MS11 *B. napus* - *Bcl*I digested
Lane 5: 5 µg gDNA from MS11 *B. napus* - *Eco*RI digested
Lane 6: 5 µg gDNA from MS11 *B. napus* - *Eco*RV digested
Lane 7: 5 µg gDNA from MS11 *B. napus* - *Hin*dIII digested
Lane 8: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 9: 5 µg gDNA from MS11 *B. napus* - *Kpn*I digested
Lane 10: 5 µg gDNA from MS11 *B. napus* - *Mfe*I digested
Lane 11: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 12: 5 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 13: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 14: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 15: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 16: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 17: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

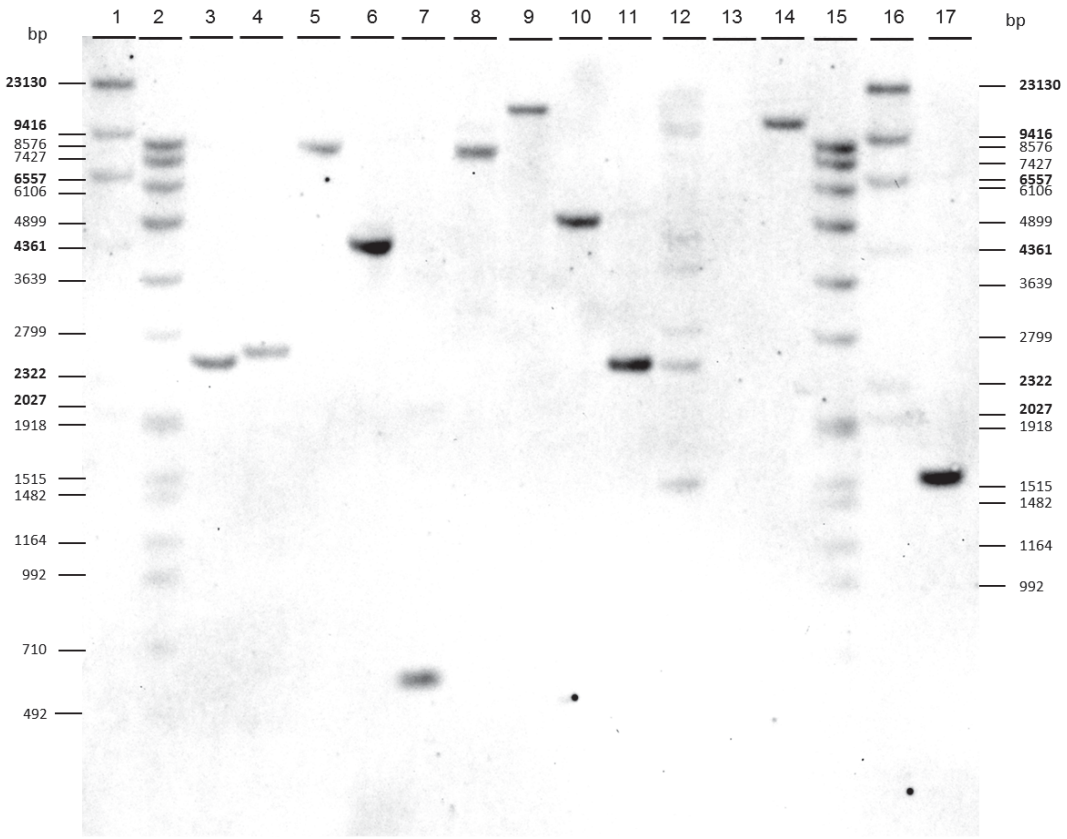


Figure V-16: Hybridization performed with a Pnos probe (P022) to determine the insert organization of MS11 (multiple digests)

Digital image: H6/LJS018/06-F2

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 Pnos sequence (P022-2, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 5 µg gDNA from MS11 *B. napus* - *Bcl*I digested
Lane 5: 5 µg gDNA from MS11 *B. napus* - *Eco*RI digested
Lane 6: 5 µg gDNA from MS11 *B. napus* - *Eco*RV digested
Lane 7: 5 µg gDNA from MS11 *B. napus* - *Hin*dIII digested
Lane 8: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 9: 5 µg gDNA from MS11 *B. napus* - *Kpn*I digested
Lane 10: 5 µg gDNA from MS11 *B. napus* - *Mfe*I digested
Lane 11: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 12: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 13: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 14: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 15: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 16: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 17: 5 µg gDNA from MS11 *B. napus* - *Nde*I digested

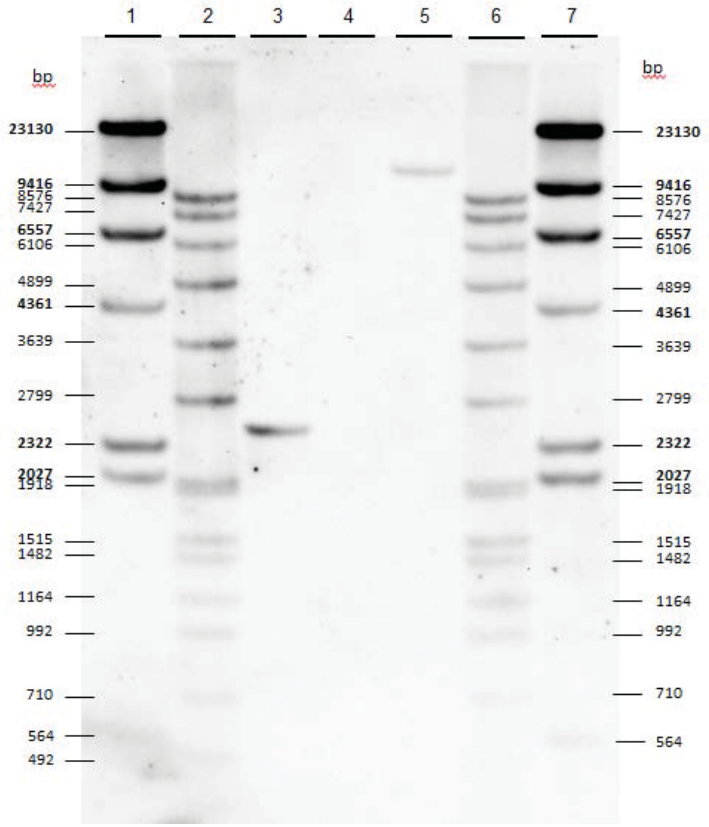


Figure V-17: Hybridization performed with a Pnos probe (P022) to determine the insert organization of MS11 (*Nco*I digest)

Digital image: H1/LJS018/18-F2

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 Pnos sequence (P022-3, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 4: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 5: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 6: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 7: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

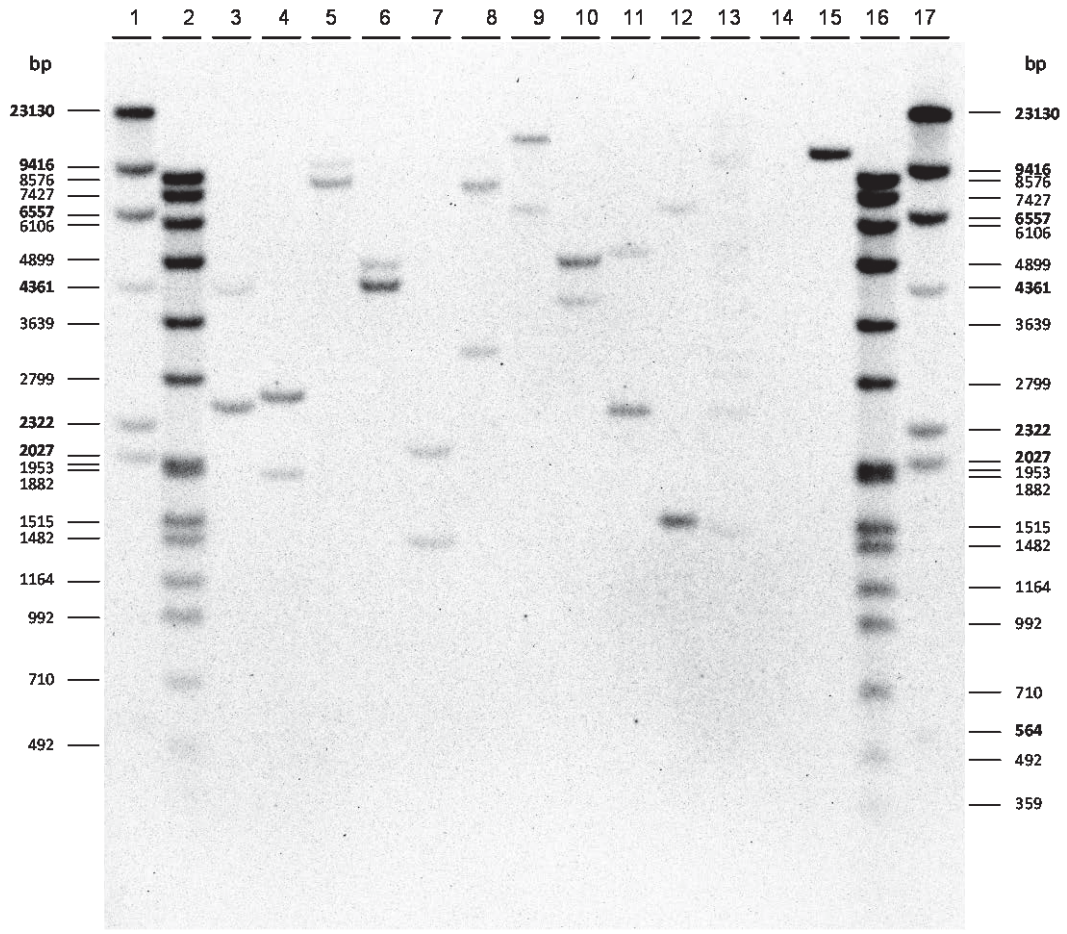


Figure V-18: Hybridization performed with a 3'g7-LB probe (P023) to determine the insert organization of MS11

Digital image: H5/LJS018/08-F4

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 3'g7-LB sequence (P023-2, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 5 µg gDNA from MS11 *B. napus* - *Bcl*I digested
Lane 5: 5 µg gDNA from MS11 *B. napus* - *Eco*RI digested
Lane 6: 5 µg gDNA from MS11 *B. napus* - *Eco*RV digested
Lane 7: 5 µg gDNA from MS11 *B. napus* - *Hind*III digested
Lane 8: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 9: 5 µg gDNA from MS11 *B. napus* - *Kpn*I digested
Lane 10: 5 µg gDNA from MS11 *B. napus* - *Mfe*I digested
Lane 11: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 12: 5 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 13: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 14: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 15: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 16: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 17: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

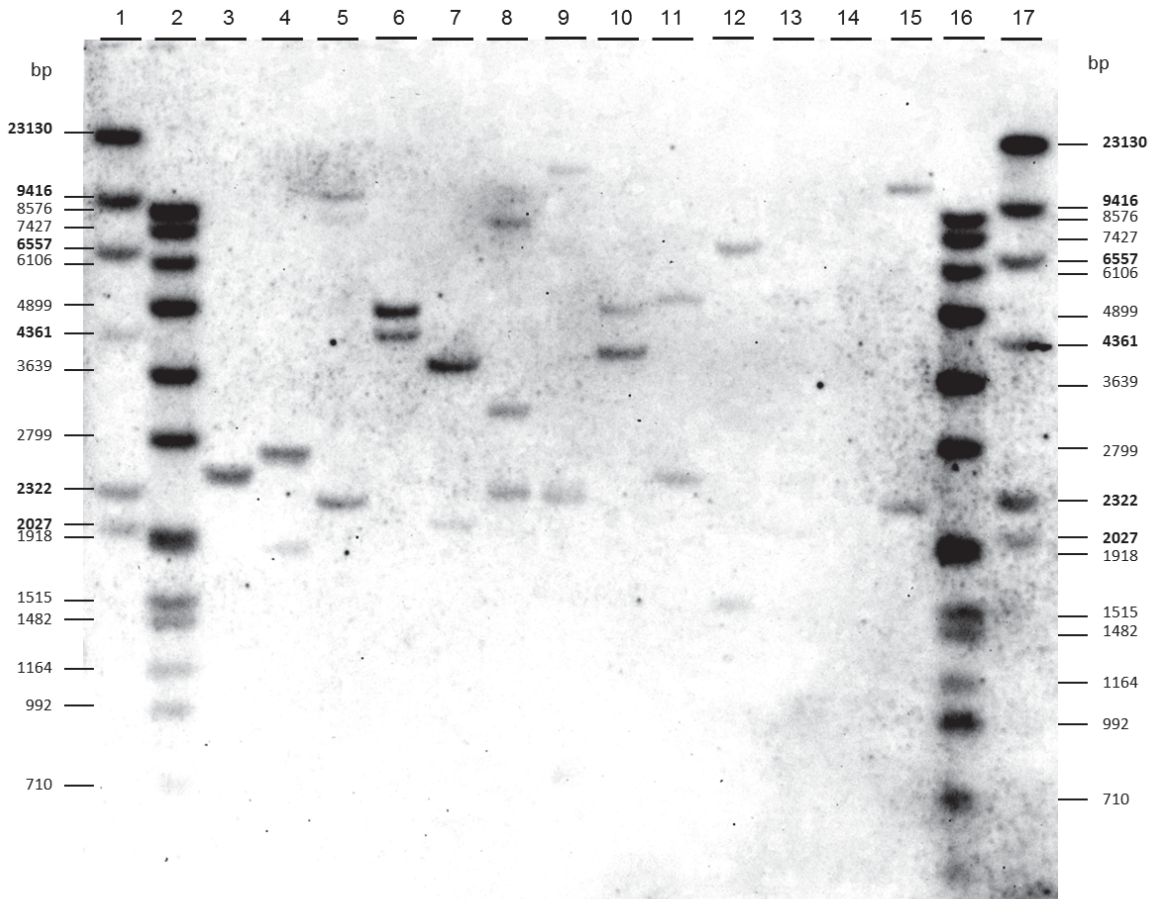


Figure V-19: Hybridization performed with a T-DNA probe (P028) to determine the insert organization of MS11 (multiple digests)

Digital image: H6/LJS018/08-F4

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 T-DNA sequence (P028-12, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 5 µg gDNA from MS11 *B. napus* - *Bcl*I digested
Lane 5: 5 µg gDNA from MS11 *B. napus* - *Eco*RI digested
Lane 6: 5 µg gDNA from MS11 *B. napus* - *Eco*RV digested
Lane 7: 5 µg gDNA from MS11 *B. napus* - *Hind*III digested
Lane 8: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 9: 5 µg gDNA from MS11 *B. napus* - *Kpn*I digested
Lane 10: 5 µg gDNA from MS11 *B. napus* - *Mfe*I digested
Lane 11: 5 µg gDNA from MS11 *B. napus* - *Nco*I digested
Lane 12: 5 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 13: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 14: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 15: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 16: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 17: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

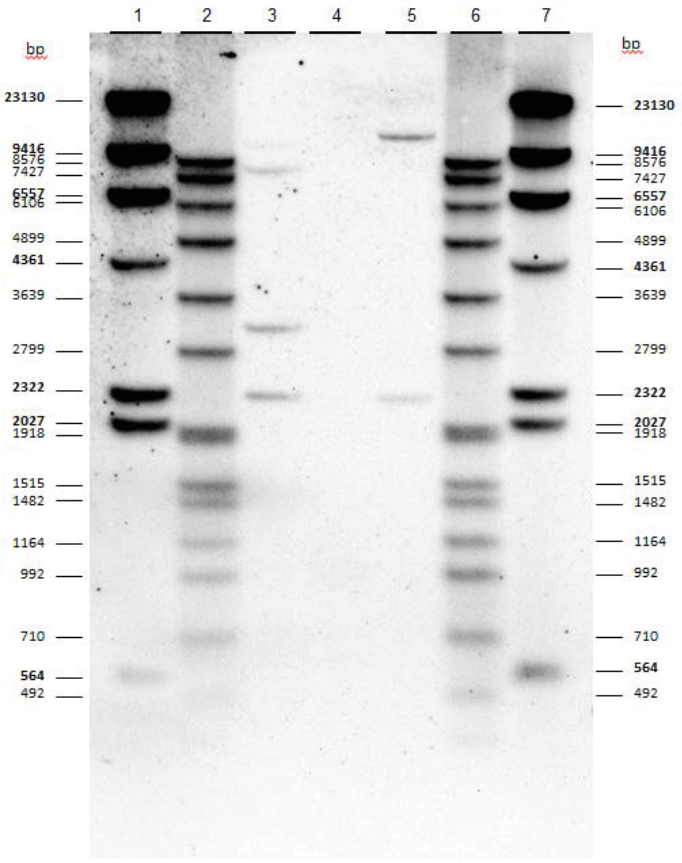


Figure V-20: Hybridization performed with a T-DNA probe (P028) to determine the insert organization of MS11 (*Hpa*I digest)

Digital image: H1/LJS018/16-F5

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 T-DNA sequence (P028-8, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Hpa*I digested
Lane 4: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 5: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 6: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 7: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

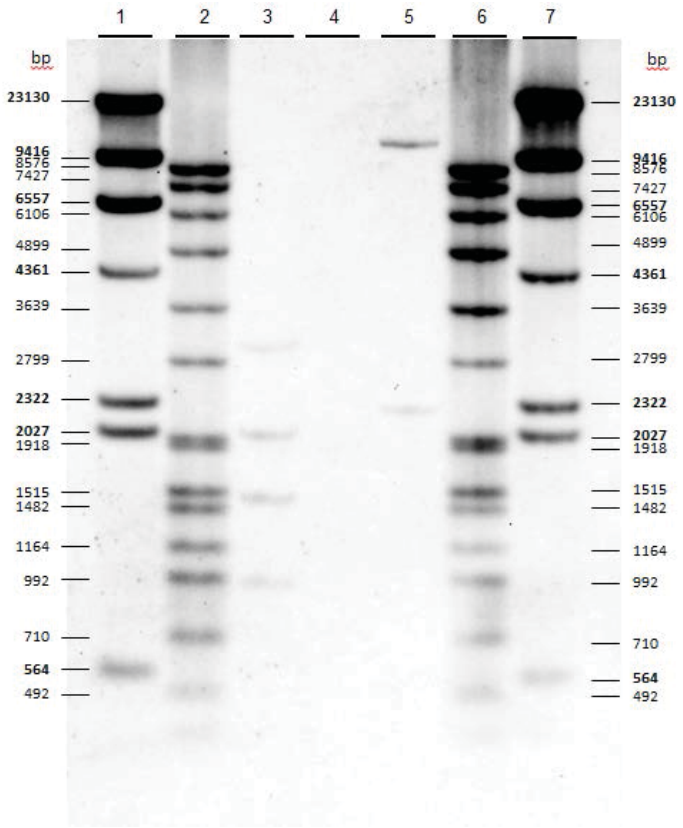


Figure V-21: Hybridization performed with a T-DNA probe (P028) to determine the insert organization of MS11

Digital image: H1/LJS018/15-F3

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with different restriction enzymes and hybridized with a probe corresponding to the MS11 T-DNA sequence (P028-12, PCR labeling).

Lane 1: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 5 µg gDNA from MS11 *B. napus* - *Sty*I digested
Lane 4: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested
Lane 5: 5 µg gDNA from the non-GM counterpart - *Eco*RI digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 6: 5 µg gDNA from the non-GM counterpart- *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 7: 5 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

Inheritance of the Insert

Genomic DNA from individual plants of five MS11 *B. napus* generations (T_3, T_4, T_5, BC_4, and BC_5) was tested for the absence or presence of MS11 by polymerase chain reaction (PCR) analysis. The results from event-specific PCR analysis were used to calculate the segregation ratios of the MS11 insert.

Chi-square analysis of the segregation data for each of the five generations was performed to test the hypothesis that the MS11 *B. napus* 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 *B. napus* nuclear genome.

Plant samples were analyzed using event-specific PCR to determine the presence or absence of the MS11 insert. PCR analysis included the amplification of the MS11 event-specific sequence and the amplification of an endogenous gene sequence. Samples with signal corresponding to the MS11 event-specific sequence and the endogenous sequence were recorded as positive for the MS11 insert. Samples with signal corresponding to the endogenous sequence only were recorded as negative.

The Chi-square analysis is based on testing the observed segregation ratio relative to the segregation ratio expected from Mendelian inheritance principles. For the T_3, T_4, T_5, BC_4, and BC_5 generations of MS11 *B. napus*, the expected segregation ratio of positive and negative was 1:1. The $\chi 2$ values were calculated using the following equation.

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

The results for MS11 event-specific PCR are summarized in Table V-5. In addition, the absence or presence of the *bar*, *barstar*, and *barnase* genes was determined using gene-specific PCR analysis. The results from the gene-specific PCR analysis confirmed that the *bar*, *barstar*, and *barnase* genes are present for samples positive for MS11 and are absent for samples negative for MS11.

Segregation ratios determined for five generations of MS11 *B. napus* confirmed that the MS11 insert is inherited in a predictable manner and as expected for a single insertion. These data are consistent with Mendelian principles and support the conclusion that MS11 *B. napus* consists of a single insert integrated at a single chromosomal locus within the *B. napus* nuclear genome.

Table V-5: Observed versus expected identity for MS11 in T_3, T_4, T_5, BC_4, and BC_5 as determined by PCR analysis

MS11 Insert	T_3		T_4		T_5		BC_4		BC_5	
	Observed	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Positive	42	42	48	46	39	47.5	43	44.5	51	49
Negative	42	42	44	46	56	47.5	46	44.5	47	49
χ^2 Value *	0		0.174		3.042		0.101		0.163	

* The critical value to reject the null hypothesis at the 5% confidence level is < 3.84 with one degree of freedom.

V.C. Presence of Vector and/or Other Non-Host Origin Sequences

The potential presence of vector backbone sequences in MS11 *B. napus* was assessed by means of Southern blot and PCR analysis.

Seeds from the T_2 generation were used to produce MS11 *B. napus* leaf material. The identity of the leaf material was confirmed. Non-genetically modified (non-GM) *B. napus* variety N90-740 (non-GM counterpart) was used as a negative control. The positive control was the transforming plasmid of MS11 *B. napus* (pTCO113).

To assess the presence of vector backbone sequences in MS11, the gDNA from individual MS11 plants were digested with the restriction enzymes *Afl*III and *Nde*I. Equal amounts of digested gDNA of five different MS11 plants were pooled for each restriction digestion and further analyzed. Pooled gDNA from plants of the non-GM counterpart was digested with the restriction enzyme *Nde*I. Plasmid DNA of pTCO113 was digested with the *Eco*RI restriction enzyme.

The resulting DNA fragments were separated by agarose gel-electrophoresis. Transfer of the separated DNA fragments from the agarose gel to a positively charged nylon membrane was performed by a neutral Southern blotting procedure. The resulting membranes were hybridized with four overlapping, DIG-labeled vector backbone probes (P024 to P027) that cover every bp of the vector backbone except the *barstar* sequences. The absence of the *barstar* gene contained within the vector backbone could not be confirmed by Southern blot analysis since the gene is also part of the MS11 insert sequence. Therefore, the absence of the *barstar* gene as part of the vector backbone was confirmed by means of PCR analysis.

Table V-6 provides details of the probes used in the Southern blot analysis. A schematic overview of the plasmid pTCO113 with indication of the restriction enzymes and probes used to assess the presence of vector backbone sequences in MS11 is presented in Figure V-22.

Each membrane contained one negative control, in which the template DNA was digested gDNA prepared from the non-GM counterpart. This negative control showed no hybridization with any of the probes used, confirming the absence of any background hybridization with all the probes used. Similarly, each membrane contained two positive controls, one consisting of digested gDNA prepared from non-GM plant material and supplemented with an equimolar amount of digested transforming plasmid pTCO113, and a second positive control consisting of digested gDNA prepared from non-GM plant material that was supplemented with 0.1 equimolar amount of pTCO113 digested plasmid DNA. Both positive controls showed the expected hybridization fragments after hybridization with the vector backbone probes (Figure V-23 to Figure V-26, lanes 6 and 7). This demonstrated that the hybridizations were performed under conditions allowing detection of the possible presence of vector backbone sequences in one of the five pooled MS11 plants tested.

Hybridization of the digested MS11 gDNA samples with the vector backbone probes resulted in no hybridization fragments, as expected (Table V-8, Figure V-23 to Figure V-26, lanes 3 and 4). This demonstrated the absence of vector backbone sequences in MS11 gDNA samples. When hybridizing the same membranes with the T-DNA probe, all expected fragments were obtained. This demonstrated that an adequate amount of a sufficient quality of digested MS11 gDNA was loaded on the gels to be able to detect vector backbone sequences in MS11, if present.

The absence of *barstar* sequence originating from the vector backbone was verified by PCR analysis. Five primer combinations were used to perform the PCR analysis. Primers targeting T-DNA sequences at the RB were included to serve as an internal control. Primer sequences and the positions of the primers in plasmid pTCO113 are presented in Table V-7 and Figure V-22. No amplicons were obtained using MS11 gDNA as template in PCR analysis to test for the presence of *barstar* sequence originating from the vector backbone (Table V-9, Figure V-27, panel A: lane 2, 7 and 12; panel B: lane 2 and 7). As a result, the absence of *barstar* originating from the vector backbone sequence was demonstrated.

In conclusion, the Southern blot and PCR results demonstrated the absence of vector backbone sequences in MS11 *B. napus*.

Table V-6: Information on the probes used for presence of vector backbone

Probe ID	Probe template ID	Description	Primer pair/ Restr. digest	Primer sequence (5' → 3')	Primer position on pTCO113 (bp)	Size probe template (bp)	Overlap between probe
P024	PT112	Vector backbone - *aadA*	GLPA019	GCTTGCATGTTGGTTTCTACGC	5891 → 5912	1840	No overlap with PT113 → *barstar* gene
			GLPA361	GAGACTTCATCCGGGGTCAG	7730 → 7711		
P025	PT113	Vector backbone - 5'ORI pVS1, version 1	GLPA380	TGGAAGGCGAGCATCGTTTG	8214 → 8233	2382	
			GLPA396	AgACAACCCAgCCgCTTACg	10595 → 10576		611 bp (version1) or 282 bp (version 2)
		Vector backbone - 5'ORI pVS1, version 2	GLPA378	AAGGCGAGCATCGTTTGTTC	8217 → 8236	2050	
			GLPA151	GCCGGCACTTAGCGTGTTTG	10266 → 10247		
P026	PT114	Vector backbone - 3' ORI pVS1	GLPA148	AAGCGGCCTTTGTCGTGTCG	9984 → 10003	2246	
			GLPA156	GAGCTGCATGTGTCAGAGGT	12229 → 12210		39 bp
P027	PT115	Vector backbone - ORI ColE1	GLPA160	TTCGGTGATGACGGTGAAAACC	12191 → 12212	1389	
			GLPA162	CGACGGCCGAGTACTGGCAG	39 → 20		
P028	PT108	T-DNA	GLPA174	AATTACAACGGTATATATCCTGCCA	1 → 25	5865	NA[a]
			GLPA359	CGGCAGGATATATTCAATTGTAAAT	5865 → 5841		

[a]NA means not applicable

Table V-7: Information on the primers used in the PCR analysis to test for presence of the *barstar* sequence originating from the vector backbone

Description amplicon	Primer pair	Primer sequence (5' → 3') *	Primer position in pTCO113 (bp)	PCR amplicon position in pTCO113 (bp)	Amplicon size (bp)
T-DNA (part of 3'g7)	GLPA047	gTCAggTATTATAgTCCAAgC	143 →163 5721→5701**	143 →317 5547 →5721 143 →5521*** 5547 →317***	175 5579*** 8311***
	GLPA048	ggATCCCCCgATgAgCTAAgCTAgC	317 →293 5547→5571**		
complete *barstar* in vector backbone	GLPA049	CTTCAggAgATCggAAgACC	7663 →7682	7663 →8218	556
	GLPA050	TTCCAgAAAACCgAggATgCg	8218 →8198		
barstar + downstream sequences in vector backbone	GLPA181	ACTgggTTCgTgCCTTCATC	7478 →7497	7478 →8027 5248 → 8027 ***	550 2780***
	GLPA045	TCAgAAgTATCAgCgACCTCCACC	8027 →8004 5248→5271**		
	GLPA181	ACTgggTTCgTgCCTTCATC	7478 →7497	7478 →8049 5226 → 8049***	572 2824***
	GLPA345	gCAgTCATTAACggggAACAAATC	8049 →8026 5226→5249**		
	GLPA180	gAACCgAACAggCTTATgTC	7457 →7476	7457 →8049 5226 → 8049***	593 2824***
	GLPA345	gCAgTCATTAACggggAACAAATC	8049 →8026 5226→5249**		
barstar + upstream sequences in vector backbone	GLPA046	AAgTATgATggTgATgTCgCAgCC	7792 → 7815 5483→5460**	7792 →8218 7792 → 5483 ***	427 11232 ***
	GLPA050	TTCCAgAAAACCgAggATgCg	8218 →8198		

* A lowercase 'g' is used to avoid confusion between 'G' and 'C'
** An additional binding site is present for this primer
*** Additional PCR products can be produced

Table V-8: Expected and obtained hybridization fragments determined for the vector backbone assessment of MS11
Part 1: Probes P024-2, P025-2, P028-1, and P028-2.

Sample	T-DNA or plasmid fragment sizes (bp)	Fragment description	Obtained fragment size (bp)	H1/LJS018/11-F4		H2/LJS018/11-F2		H1/LJS018/12-F6		H3/LJS018/12-F3	
				P024-2		P028-1		P025-2		P028-2	
				Vector backbone probe (*aad*A)		T-DNA probe		Vector backbone probe (5' ORI pVS1)		T-DNA probe	
				Figure 23				Figure 24			
				Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
Ms11 - *Afl*III	>305	5' integration fr.	NA	No	No	Yes ** (119)	No	No	No	Yes ** (119)	No
	2476	internal fr.	2476	No	No	Yes	Yes$	No	No	Yes	Yes$
	550	internal fr.	550	No	No	Yes	No °	No	No	Yes	No °
	>2467	3' integration fr.	2500	No	No	Yes	Yes$	No	No	Yes	Yes$
Ms11 - *Nde*I	>4557	5' integration fr.	6900	No	No	Yes	Yes	No	No	Yes	Yes
	64*	internal fr.	NA	No	No	Yes	No	No	No	Yes	No
	>1177	3' integration fr.	1600	No	No	Yes	Yes	No	No	Yes	Yes
Non-GM counterpart - *Nde*I	/	Negative control	NA	No	No	No	No	No	No	No	No
Non-GM counterpart - *Nde*I + 0.1 equimolar amount pTCO113 - *Eco*RI	2260	Positive control	2260	No	No	Yes	No	No	No	Yes	No
	11280	Positive control	11280	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Non-GM counterpart - *Nde*I + 1 equimolar amount pTCO113 - *Eco*RI	2260	Positive control	2260	No	No	Yes	Yes	No	No	Yes	Yes
	11280	Positive control	11280	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Table V-8: Expected and obtained hybridization fragments determined for the vector backbone assessment of MS11
Part 2: Probes P026-2, P027-2, P028-3, and P028-10.

Sample	T-DNA or plasmid fragment sizes (bp)	Fragment description	Obtained fragment size (bp)	H1/LJS018/13-F2		H3/LJS018/13-F1		H1/LJS018/14-F5		H3/LJS018/14-F1	
				P026-2		P028-3		P027-2		P028-10	
				Vector backbone probe (3' ORI pVS1)		T-DNA probe		Vector backbone probe (ORI Col E1)		T-DNA probe	
				Figure 25				Figure 26			
				Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
Ms11 - *Afl*III	>305	5' integration fr.	NA	No	No	Yes ** (119)	No	No	No	Yes ** (119)	No
	2476	internal fr.	2476	No	No	Yes	Yes$	No	No	Yes	Yes$
	550	internal fr.	550	No	No	Yes	No °	No	No	Yes	No °
	>2467	3' integration fr.	2500	No	No	Yes	Yes$	No	No	Yes	Yes$
Ms11 - *Nde*I	>4557	5' integration fr.	6900	No	No	Yes	Yes	No	No	Yes	Yes
	64*	internal fr.	NA	No	No	Yes	No	No	No	Yes	No
	>1177	3' integration fr.	1600	No	No	Yes	Yes	No	No	Yes	Yes
Non-GM counterpart - *Nde*I	/	Negative control	NA	No	No	No	No	No	No	No	No
Non-GM counterpart - *Nde*I + 0.1 equimolar amount pTCO113 - *Eco*RI	2260	Positive control	2260	No	No	Yes	No	No	No	Yes	No
	11280	Positive control	11280	Yes	Yes	Yes	No	Yes	Yes	Yes	No
Non-GM counterpart - *Nde*I + 1 equimolar amount pTCO113 - *Eco*RI	2260	Positive control	2260	No	No	Yes	Yes	No	No	Yes	Yes
	11280	Positive control	11280	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

$ These bands have a comparable size and cannot be distinguished using this experimental setup
* Based on the technical limits of Southern Blotting, this fragment might be too small to be visualized
** Due to a small overlap with the probe, these fragments may not be visible. The size of the overlap is indicated between brackets
° This band is probably not observed because of the small size of the fragment in comparison with the large probe

Table V-9: Expected and obtained PCR results to investigate the potential presence of *barstar* sequences as part of the vector backbone

Primer combinations	Target	MS11	WT (negative control)	WT + equimolar amount of pTCO113 (positive control)
GLPA049 - GLPA050 GLPA047 - GLPA048*	complete *barstar* in vector backbone	/ 175 bp°	/ /	556 bp 175 bp°
GLPA181 - GLPA045 GLPA047 - GLPA048*	*barstar* + downstream sequences in vector backbone	/ 175 bp° 474 bp[a]	/ /	550 bp 2780 bp** 175 bp° 474 bp[a]
GLPA181 - GLPA345 GLPA047 - GLPA048*		/ 175 bp° 496 bp[b]	/ /	572 bp 2824 bp** 175 bp° 496 bp[b]
GLPA180 - GLPA345 GLPA047 - GLPA048*		/ 175 bp° 496 bp[b]	/ /	593 bp 2824 bp** 175 bp° 496 bp[b]
GLPA046 - GLPA050 GLPA047 - GLPA048*	*barstar* + upstream sequences in vector backbone	/ 175 bp°	/ /	427 bp 11232 bp** 175 bp°

* Primers targeting T-DNA sequences at the RB are included to serve as an internal control
** An additional PCR product can be produced, see also Table 5
° Additional PCR products might be obtained also for the positive control, see Table 5 for the expected amplicon sizes of these additional fragments
[a] Additional PCR product of 474 bp may be expected as a result of the combination of primers GLPA045 and GLPA047.
[b] Additional PCR product of 496 bp may be expected as a result of the combination of primers GLPA047 and GLPA345

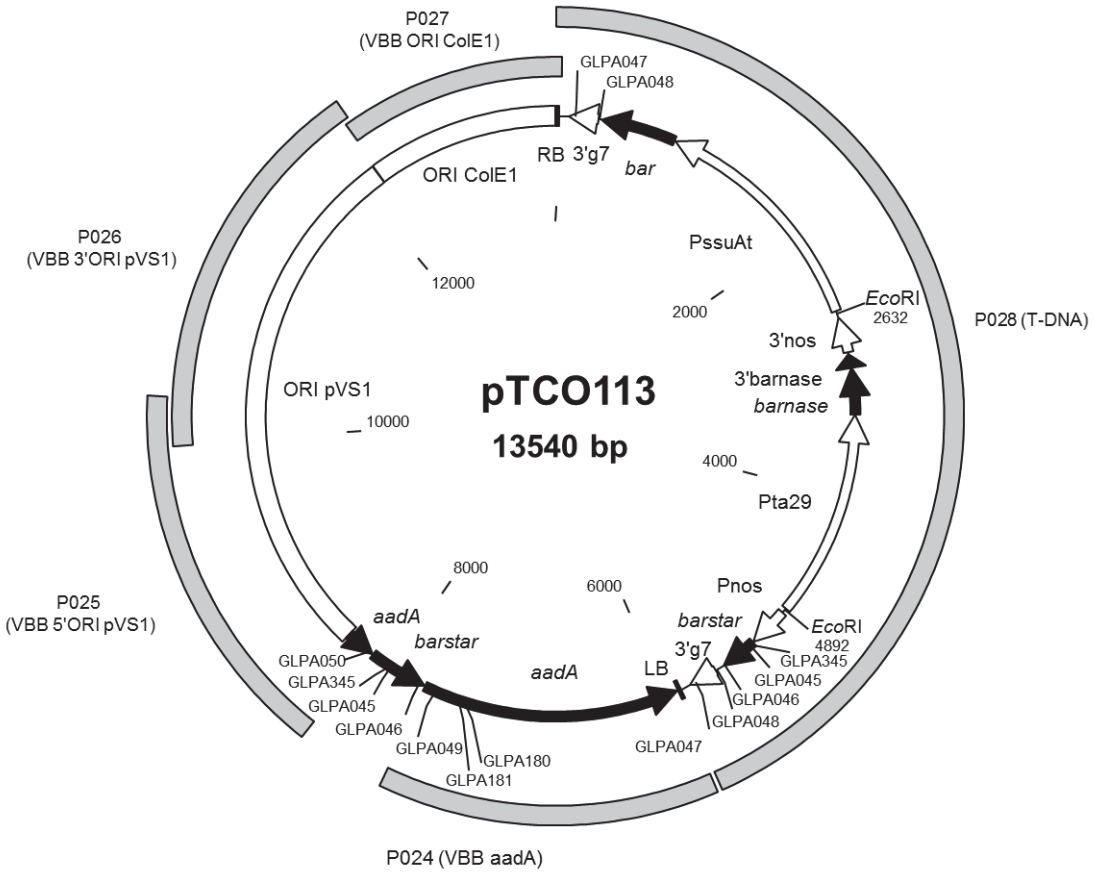


Figure V-22: Map of transformation vector pTCO113 with indication of the primers used for the investigation of the presence of *barstar* sequences as part of the vector backbone, the position of enzymes used for plasmid digestion in this study and the vector backbone probes and T-DNA probe (P028) indicated

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

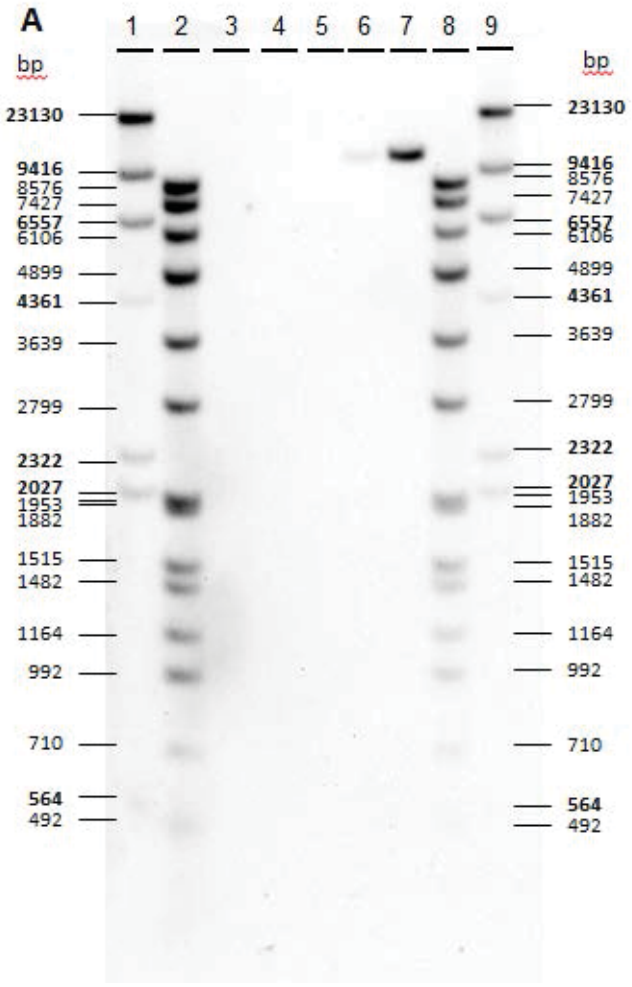


Figure V-23: Hybridization performed with a vector backbone probe covering the *aadA* sequence (P024) to assess the vector backbone presence in the T_2 generation of MS11

Digital image: H1/LJS018/11-F4

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with restriction enzymes *Afl*III and *Nde*I and hybridized with a vector backbone probe (P024-2, PCR labeling) and with the T-DNA probe (P028-01, PCR labeling) (data not shown).

Lane 1: 3 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from the non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 3 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 5: 3 µg gDNA from the non-GM counterpart - *Nde*I digested
Lane 6: 3 µg gDNA from the non-GM counterpart - *Nde*I digested + 1/10th of an equimolar amount of pTCO113 - *Eco*RI digested
Lane 7: 3 µg gDNA from the non-GM counterpart - *Nde*I digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 8: 3 µg gDNA from the non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 9: 3 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

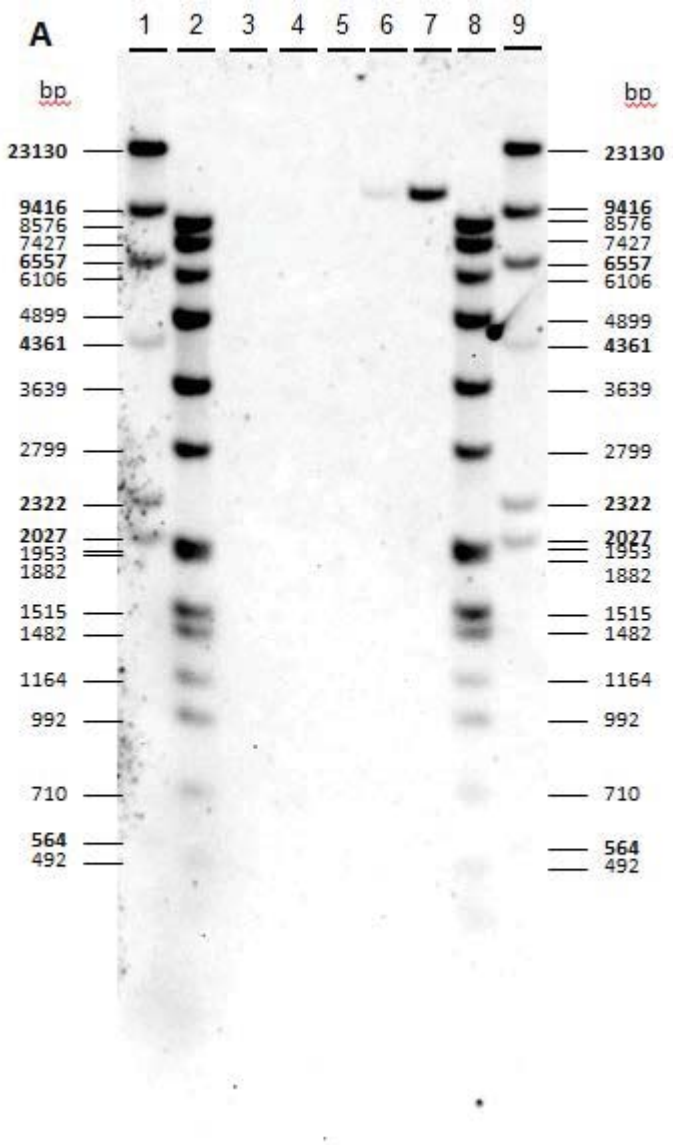


Figure V-24: Hybridization performed with a vector backbone probe covering the 5'ORI pVS1 sequence (P025) to assess the vector backbone presence in the T_2 generation of MS11

Digital image: H1/LJS018/12-F6

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with restriction enzymes *Afl*III and *Nde*I and hybridized with a vector backbone probe (P025-2, PCR labeling) and with the T-DNA probe (P028-02, PCR labeling) (data not shown).

Lane 1: 3 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from the non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 3 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 5: 3 µg gDNA from the non-GM counterpart - *Nde*I digested
Lane 6: 3 µg gDNA from the non-GM counterpart - *Nde*I digested + 1/10th of an equimolar amount of pTCO113 - *Eco*RI digested
Lane 7: 3 µg gDNA from the non-GM counterpart - *Nde*I digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 8: 3 µg gDNA from the non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 9: 3 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

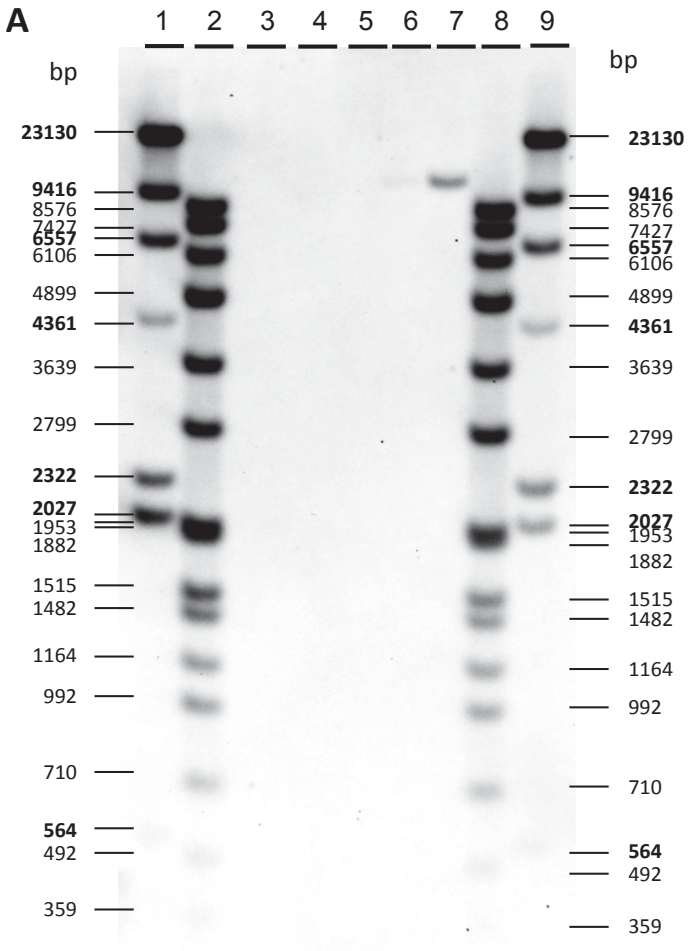


Figure V-25: Hybridization performed with a vector backbone probe covering the 3'ORI pVS1 sequence (P026) to assess the vector backbone presence in the T_2 generation of MS11

Digital image: H1/LJS018/13-F2

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with restriction enzymes *Afl*III and *Nde*I and hybridized with a vector backbone probe (P026-2, PCR labeling) and with the T-DNA probe (P028-03, PCR labeling) (data not shown).

Lane 1: 3 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from the non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 3 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 5: 3 µg gDNA from the non-GM counterpart - *Nde*I digested
Lane 6: 3 µg gDNA from the non-GM counterpart - *Nde*I digested + 1/10th of an equimolar amount of pTCO113 - *Eco*RI digested
Lane 7: 3 µg gDNA from the non-GM counterpart - *Nde*I digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 8: 3 µg gDNA from the non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 9: 3 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

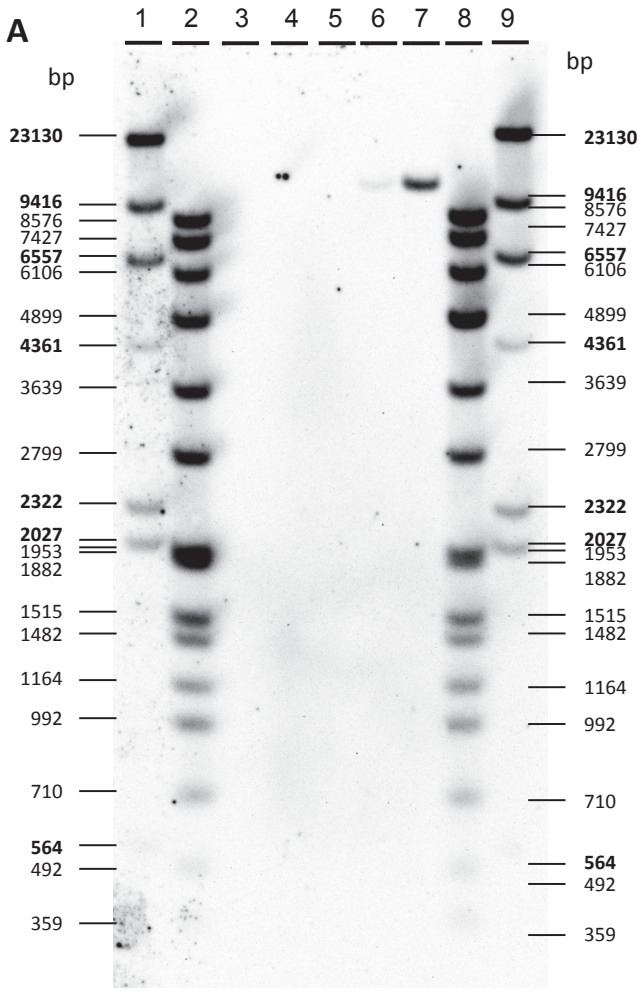


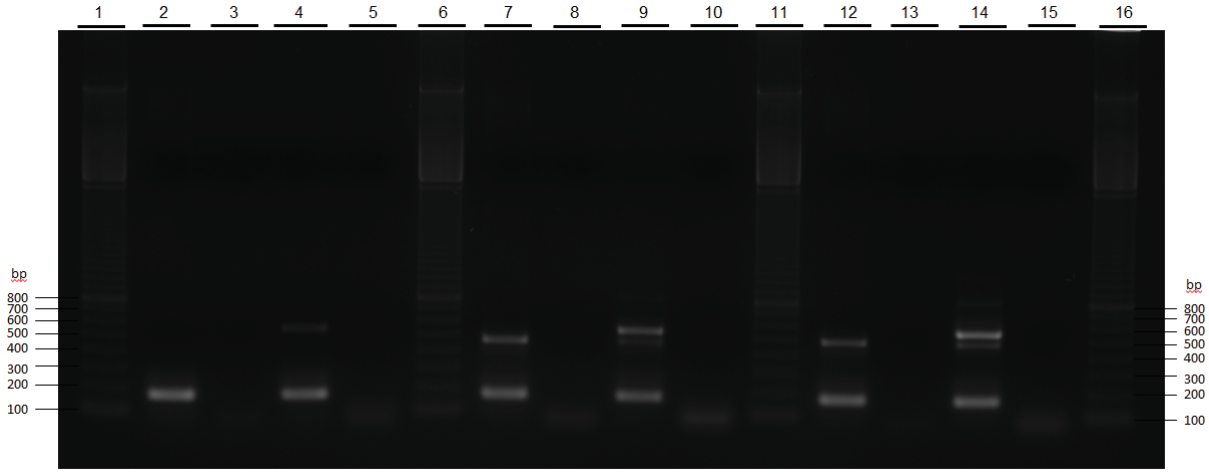
Figure V-26: Hybridization performed with a vector backbone probe covering the 3'ORI colE1 sequence (P027) to assess the vector backbone presence in the T_2 generation of MS11

Digital image: H1/LJS018/14-F5

gDNA was isolated from MS11 *B. napus* plants and from the non-GM counterpart. The gDNA samples were digested with restriction enzymes *Afl*III and *Nde*I and hybridized with a vector backbone probe (P027-2, PCR labeling) and with the T-DNA probe (P028-10, PCR labeling) (data not shown).

Lane 1: 3 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)
Lane 2: 3 µg gDNA from the non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 3: 3 µg gDNA from MS11 *B. napus* - *Afl*III digested
Lane 4: 3 µg gDNA from MS11 *B. napus* - *Nde*I digested
Lane 5: 3 µg gDNA from the non-GM counterpart - *Nde*I digested
Lane 6: 3 µg gDNA from the non-GM counterpart - *Nde*I digested + 1/10th of an equimolar amount of pTCO113 - *Eco*RI digested
Lane 7: 3 µg gDNA from the non-GM counterpart - *Nde*I digested + an equimolar amount of pTCO113 - *Eco*RI digested
Lane 8: 3 µg gDNA from the non-GM counterpart - *Eco*RI digested + 10 ng DNA Molecular Weight Marker VII, DIG-labeled (Roche)
Lane 9: 3 µg gDNA from the non-GM counterpart - *Hin*dIII digested + 10 ng DNA Molecular Weight Marker II, DIG-labeled (Roche)

Panel A:



Panel B:

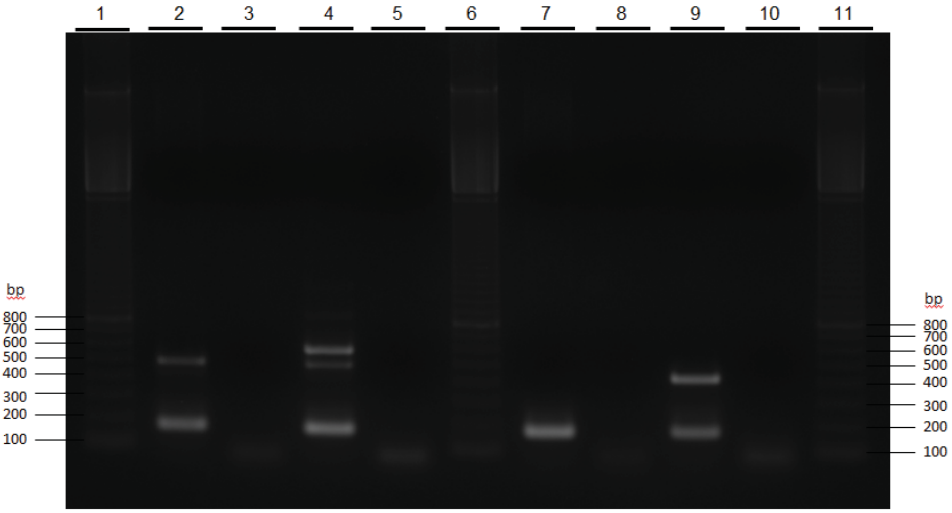


Figure V-27: PCR analysis to assess absence of *barstar* originating from vector backbone sequence in MS11

Panel A:

Lane 1, 6, 11 and 16: 100 bp molecular weight marker

PCR template:
Lane 2, 7 and 12: gDNA from *B. napus* MS11
Lane 3, 8 and 13: gDNA from *B. napus* N90-740 (negative control)
Lane 4, 9 and 14: gDNA from *B. napus* N90-740 + equimolar amount of pTCO113 (positive control)
Lane 5, 10 and 15: water sample (no template control)

Primer combinations used:
Lane 2 to 5: GLPA049-GLPA050 (complete *barstar* in vector backbone; 556 bp); GLPA047-GLPA048 (part of 3'g7 in T-DNA; 175 bp)
Lane 7 to 10: GLPA181-GLPA045 (*barstar* + downstream sequences in vector backbone; 550 bp); GLPA047-GLPA048 (part of 3'g7 in T-DNA; 175 bp)
Lane 12 to 15: GLPA345-GLPA181 (*barstar* + downstream sequences in vector backbone; 572 bp); GLPA047-GLPA048 (part of 3'g7 in T-DNA; 175 bp)

Panel B:

Lane 1, 6 and 11: 100 bp molecular weight marker

PCR template:
Lane 2 and 7: gDNA from *B. napus* MS11
Lane 3 and 8: gDNA from *B. napus* N90-740 (negative control)
Lane 4 and 9: gDNA from *B. napus* N90-740 + equimolar amount of pTCO113 (positive control)
Lane 5 and 10: water sample (no template control)

Primer combinations used:
Lane 2 to 5: GLPA345-GLPA180 (*barstar* + downstream sequences in vector backbone; 593 bp); GLPA047-GLPA048 (part of 3'g7 in T-DNA; 175 bp)
Lane 7 to 10: GLPA050-GLPA046 (*barstar* + upstream sequences in vector backbone; 427 bp); GLPA047-GLPA048 (part of 3'g7 in T-DNA; 175 bp)

V.D. Description of the Insertion Locus

Refer to V.E. for experimental design.

The MS11 *B. napus* transgenic sequence was compared with the MS11 insertion locus sequence, and no differences were observed in the overlapping flanking sequences (Table V-10). The corresponding MS11 insertion locus consisted of 2471 bp, which included 1129 bp of sequence 100% identical to the 5' flanking sequence of MS11 *B. napus*, 1302 bp of sequence 100% identical to the 3' flanking sequence MS11 *B. napus*, and a target site deletion (TSD) of 40 bp.

Table V-10: Alignment between the final MS11 transgenic sequence and the final MS11 insertion locus sequence

Region of identity	% identity	Length (bp)	MS11 *B. napus* transgenic locus		MS11 *B. napus* insertion locus	
			start	end	start	end
5' flanking sequence	100	1129	bp 1	bp 1129	bp 1	bp 1129
3' flanking sequence	100	1302	bp 6908	bp 8209	bp 1170	bp 2471

V.E. DNA Sequence of the Transgenic and Insertion Locus

The DNA sequence of the MS11 *B. napus* transgenic locus and the corresponding insertion locus was determined.

In initial experiments, six overlapping fragments were prepared to determine the sequence of the MS11 *B. napus* transgenic locus. The insertion locus was amplified in one fragment. As the MS11 plants used in these experiments were hemizygous, containing one copy of the MS11 transgenic locus and one copy of the insertion locus, gDNA extracted from leaf material of MS11 *B. napus* plants was used as template for all amplifications. For each PCR fragment, multiple identical PCR reactions were performed. After amplification all identical PCR reactions were pooled for sequencing. Sanger sequencing was performed.

The obtained consensus sequences of the transgenic and insertion loci were annotated by pairwise alignments using the Clone Manager software. The consensus sequence of the MS11 transgenic locus was compared with the pTCO113 plasmid sequence to identify the T-DNA region. The consensus sequence of the MS11 transgenic locus was also compared to the MS11 insertion locus sequence to identify sequence regions of *B. napus* origin within the MS11 transgenic locus as well as the TSD within the MS11 insertion locus.

To determine additional 5' and 3' flanking sequences of MS11 *B. napus* to obtain at least 1 kb of the flanking regions, and the corresponding insertion locus sequence of MS11 *B. napus*, three additional fragments were prepared to generate additional sequence. To determine additional MS11 flanking sequences, gDNA extracted from leaf material of MS11 *B. napus* plants was used as a template. To extend the MS11 insertion locus sequence, gDNA extracted from leaf material of non-GM *B. napus* variety N90-740 was used as a template. For each PCR fragment, multiple identical PCR reactions were performed. After amplification all identical PCR reactions were pooled for sequencing. Sanger sequencing was performed using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). A consensus sequence of all sequencing reads was generated. Each bp of the consensus sequence had 4-fold coverage, 4 independent sequencing reads.

The extended MS11 transgenic locus sequence was validated by comparing with the sequences of the MS11 transgenic locus obtained from previous experiments. A pairwise alignment demonstrated 100% identity in the overlapping regions of the sequenced fragments of the MS11 transgenic locus with the previously determined MS11 transgenic locus sequence.

The extended MS11 insertion locus sequence was validated by comparing with the sequences of the MS11 insertion locus obtained from previous experiments. A pairwise alignment demonstrated 100% identity in the overlapping regions of the sequences determined for the amplified MS11 insertion locus fragment with the previously determined MS11 insertion locus sequence.

The final MS11 transgenic locus with extended flanking sequences consisted of 8209 bp, which included 1129 bp of 5' flanking sequence and 1302 bp of 3' flanking sequence. The corresponding MS11 insertion locus consisted of 2471 bp, which included 1129 bp of sequence 100% identical to the 5' flanking sequence, 1302 bp of sequence 100% identical to the 3' flanking sequence, and a TSD of 40 bp. The results demonstrated that upon transformation, 40 bp from the MS11 insertion locus were replaced by 5778 bp of T-DNA from plasmid pTCO113. The flanking sequences obtained at the MS11 *B.* napus transgenic locus were identical to the

corresponding sequences obtained from the insertion locus. This demonstrates that the MS11 *B. napus* flanking sequences are of *B. napus* origin within its original genomic organization.

V.F. Bioinformatics Analyses of the Transgenic and Insertion Locus

Bioinformatic analysis of the MS11 insertion locus

Bioinformatics analysis on the MS11 insertion locus sequence was performed to identify the insertion locus and to determine whether regulatory sequences or endogenous *B. napus* genes were interrupted upon the insertion of T-DNA sequences.

BLAST (Basic Local Alignment Search Tool) searches were performed in order to search for identity with known genes and proteins. The BLAST tool available on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov) was used with default parameters. Database definitions are provided in Table V-11.

BLASTn, which compares a nucleotide query sequence against a nucleic sequence database, was used to identify similarities between the MS11 insertion locus and sequences within the nucleotide collection and the Expressed Sequence Tag (EST) databases available on the NCBI website.

BLASTx, which compares the six-frame theoretical translation products of the nucleotide query sequence (both strands) against a protein sequence database, was used to compare the MS11 insertion locus sequence to the NCBI non-redundant protein database.

Similarities between the MS11 insertion locus and *B. napus* genome were identified using the BLAT tool (BLAST-like alignment tool) and a *B. napus* reference genome (Chalhoub et al., 2014) available on the Genoscope website (http://www.genoscope.cns.fr/brassicanapus/). Default parameters were used.

Based on the bioinformatics analysis performed, the MS11 insertion locus originates from *B. napus* chromosome A03. Similarity searches indicated the presence of an endogenous gene in the 3' flanking sequence region of the MS11 insertion locus. The coding sequence of this gene is not interrupted upon insertion of T-DNA sequences. Therefore, the insertion of T-DNA sequences in the MS11 insertion locus is unlikely to interrupt or alter transcriptional or translational activity of known endogenous *B. napus* genes.

Table V-11: Database definitions used for BLAST analysis

Database	Algorithm	Posted Date	Analysis date	Number of sequences	Number of letters
NCBI Nucleotide collection (nr/nt)	BLASTN 2.3.1+	Feb 29, 2016 8:13 AM	Mar 02, 2016	34,921,546	113,216,291,083
NCBI Expressed Sequence Tags (EST)	BLASTN 2.3.1+	Feb 28, 2016 6:19 AM	Mar 02, 2016	76,144,851	42,428,242,625
NCBI Non-redundant protein sequences (nr)	BLASTX 2.3.1+	Feb 29, 2016 8 :12 AM	Mar 02, 2016	82,777,350	30,298,809,097
Genoscope Brassica napus L Reference Genome	BLAT	NA[a]	Mar 02, 2016	NA	NA

[a]NA stands for non-applicable.

Bioinformatic analysis of the MS11 *B. napus* transgenic locus

A bioinformatics analysis was performed on the transgenic locus sequence of MS11 *Brassica napus* to identify potential open reading frames (ORF).

The ORF search was performed using the GetORF search program from the European Molecular Biology Open Software Suite (EMBOSS) tools (version 6.3.1, July 2010). An ORF was defined as the region between two translation stop codons (TAA, TAG, or TGA) with a minimum size coding for 3 amino acids. All ORF crossing a junction or overlapping the inserted DNA were reported.

Translated amino acid sequences from all identified ORF with a minimum size of 30 amino acids were used as query sequences for homology search with known allergens and known toxins.

The 8-mer homology search was carried out to identify any short sequences of 8 amino acids or longer that share 100% identity to an allergenic protein. Additionally, each complete query sequence was compared with all the sequences available in the allergen database (FARRP; www.allergenonline.org) (Table V-12). The overall homology search used the FASTA program (version 35.04, Jan. 15, 2009). Only the matches of ≥35% identity over at least 80 amino acids were considered potentially relevant. For all ORF shorter than 80 amino acids, the percentage of identity was calculated over a hypothetical 80 amino acid window, with gaps treated as mismatches:

$$\text{Calculated \% identity} = \frac{\text{\% identity} \times \text{length of ORF coverage}}{80}$$

Each complete query sequence was also compared with all the sequences available in the NCBI non-redundant protein database (Table V-12) using the FASTA program. For each ORF, only the best scoring 1000 matches were reported when more than 1000 matches were found.

The biological relevance of the matches was further assessed by examining the alignments (e.g., identity, length of alignment, presence of gaps, E-value), as well as the published information on toxicity of the matching proteins. The biologically relevant matches provided insight on the familiarity and potential toxic properties of the potential polypeptide.

In the MS11 transgenic locus, GetORF identified 554 ORF (corresponding to 526 unique sequences) defined between two stop codons and with a minimum size of 3 amino acids. After elimination of duplicates, translated amino acid sequences of at least 30 amino acids length represented 107 unique sequences.

No 100% identities were found between the 8 or longer linearly contiguous amino acid blocks that compose the query sequences and known allergens. Additionally, no biologically relevant identities were found between the query sequences and known allergens. For all ORFs of 80 amino acids or more, there was no match of ≥ 35% identity over at least 80 amino acids. For all ORFs shorter than 80 amino acids, there was no match of ≥ 35% identity recalculated over 80 amino acids.

None of the matches obtained from the NCBI non-redundant database were toxicologically relevant (i.e., indicative of a potential identity with a toxin), for one of the following reasons:

The match was not biologically relevant (e.g., short alignment, low % identity, presence of gaps, high E-value) or the matching protein was not a known toxin.

Results indicate that these ORF sequences of ≥30 amino acids length showed no biologically relevant sequence identities with known allergens and known toxins. Therefore, there are neither allergenic nor toxicological *in silico* findings associated with the presence of the potential ORF polypeptides.

Table V-12: Summary of the database releases and date of search

Name	Database type	Number of sequences	Version	Date of release (Year-Month-Day)
AOL	Allergen database	1,956	16	2016-01-26
NCBI non-redundant protein database	General database	81,622,391	2016.0206	2016-02-19

V.G. Gene Product Expression Analysis

Protein expression in field grown plants

Protein expression analysis was conducted on tissue samples harvested from *B. napus* plants grown in the USA and Canada in 2014 using methods typical of commercial *B. napus* production. Plants for all sample analysis except grain were produced in Manitoba, CN (two sites) and Washington, USA. Plants for grain sample analysis were produced in Manitoba, CN, Quebec, CN, and Washington, USA. There were two plots of MS11 included at each site. One plot was treated with trait-specific herbicide (Liberty® 280 SL) at a nominal rate of 500 g ai/Ha while the other plot was not treated. Protein expression levels of PAT/*bar,* Barstar, and Barnase were determined on a fresh weight (FW) and dry weight (DW) basis by sandwich enzyme-linked immunosorbent assay (ELISA) in the following sample matrices: whole plant during leaf development (BBCH 13-16), stem elongation (BBCH 30-39), and inflorescence (BBCH 57-65); root during stem elongation and inflorescence; raceme during inflorescence; and grain at maturity. The BBCH-scale is a system for a uniform coding of phenologically similar growth stages of mono- and dicotyledonous plant species.

The quantitation of PAT/*bar* protein was conducted with a validated PAT/*bar* -specific ELISA method using the Envirologix QualiPlate™ Kit for LibertyLink® PAT/*bar* (Catalog number: AP 013). The quantitation of Barstar protein was conducted with a validated Barstar-specific ELISA method using the EnviroLogix Barstar Plate Kit (Catalog Number: AP 125). The quantitation of Barnase protein was conducted with a validated Barnase-specific ELISA method using the EnviroLogix Barnase Plate Kit (Catalog Number: AP 127).

Mean, standard deviation, and range for each entry matrix was based on the total matrix sample population (n=15) (three trial sites x five independent matrix samples analyzed per trial site), except as otherwise noted. Where n<15, the sample values excluded from calculations were below the lower limit of quantification (LLOQ) or not available for analysis.

The level of PAT/*bar* expression in untreated and treated MS11 *B. napus* matrices ranged from

<LLOQ to 74.44 µg/g DW (Table V-13). Root (BBCH 30-39 and BBCH 57-65 growth stages) and grain matrices all exhibited lower mean PAT/*bar* DW expression levels relative to mean DW values for other matrices of MS11 *B. napus*. Mean PAT/*bar* DW expression levels were highest in whole plant samples of untreated and treated MS11 *B. napus* at BBCH 30-39 and BBCH 57-65 growth stages, respectively.

The level of Barstar expression in untreated and treated MS11 *B. napus* matrices ranged from <LLOQ to 1.0 µg/g DW (Table V-14). Low protein expression levels of Barstar were consistently observed in root samples (BBCH 30-39 and BBCH 57-65 growth stages). Only two samples of treated MS11 raceme and three samples of treated MS11 whole plant were above LLOQ. The level of Barstar expression was <LLOQ in all other matrices.

The level of Barnase expression was <LLOQ in all untreated and treated MS11 matrices. The LLOQ for Barnase is 0.500 ng/mL, 1.000 ng/mL, 0.750 ng/ml, 1.000 ng/ml, and 2.500 ng/mL in leaf, forage, raceme, grain, and root, respectively.

In conclusion, the expression levels of PAT/*bar*, Barstar, and Barnase in all matrices were similar between MS11 *B. napus* treated with trait-specific herbicide and untreated MS11 *B. napus*.

Table V-13: Mean concentrations and expression ranges of PAT/*bar* in plant matrices harvested from untreated (U) and treated (T) MS11 *B. napus* entries grown at three sites

Matrix	BBCH Growth Stage	MS11 Entry[a]	n (Number of samples analyzed)	Number of samples above LLOQ[b]	PAT/*bar* (µg/g FW)				PAT/*bar* (µg/g DW)			
					Mean	SD[c]	Min	Max	Mean	SD	Min	Max
Whole Plant	13-15	U	15	4	2.34	0.13	2.20	2.52	22.02	7.09	14.93	30.88
		T	15	15	2.95	0.64	2.25	4.01	35.40	16.22	7.32	74.44
Whole Plant	30-39	U	15	6	3.29	1.75	0.70	4.83	24.68	12.02	9.53	40.73
		T	15	14	2.72	1.82	0.54	5.14	21.89	9.59	7.35	40.66
Root	30-39	U	15	3	0.04	0.01	0.03	0.05	0.17	0.03	0.15	0.20
		T	15	6	0.09	0.07	0.03	0.22	0.39	0.19	0.18	0.64
Whole Plant	57-65	U	14	3	2.12	1.11	0.84	2.85	18.93	9.55	7.91	24.54
		T	15	14	1.85	0.83	0.97	3.47	14.82	5.01	6.13	27.52
Root	57-65	U	15	1	0.03	ND[d]	<LLOQ	0.03	0.17	ND	<LLOQ	0.17
		T	15	6	0.09	0.06	0.03	0.16	0.37	0.25	0.15	0.76
Raceme	57-65	U	15	4	2.32	0.31	2.03	2.68	13.95	1.50	12.54	16.06
		T	15	14	2.99	0.78	1.91	4.78	23.89	10.73	9.37	55.29
Grain	89-99	U	15	9	0.30	0.17	0.06	0.56	0.34	0.18	0.06	0.59
		T	15	15	0.44	0.18	0.27	0.77	0.49	0.18	0.31	0.84

[a](T)reated or (U)ntreated with glufosinate-ammonium at 500 g ai./ha at 2-4 leaf stage.
[b]The Lower Limit of Quantification (LLOQ) for PAT/*bar* in all tissues is 0.469 ng/mL.
[c]Standard Deviation
[d]Not Determined

The untreated MS11 *B. napus* entry contained positive plants as well as negative segregants, as expected. All samples with PAT/*bar* expression levels <LLOQ except for root samples were excluded from mean and standard deviation calculations and ranges as they were determined to be negative segregants. Protein expression levels in positive roots samples were close to the LLOQ, and it was not possible to distinguish between root samples with low expression levels and root samples obtained from negative segregants. As a result, root samples with PAT/*bar* protein expression levels <LLOQ were excluded from mean and standard deviation calculations but included in the ranges.

Table V-14: Mean concentrations and expression ranges of Barstar in plant matrices harvested from untreated (U) and treated (T) MS11 *B. napus* entries grown at three sites

Matrix	BBCH Growth Stage	MS11 Entry[a]	n (Number of samples analyzed)	Number of samples above LLOQ[b]	Barstar (µg/g FW)				Barstar (µg/g DW)			
					Mean	SD[c]	Min	Max	Mean	SD	Min	Max
Whole Plant	13-15	U	15	0	ND[d]		<LLOQ		ND		<LLOQ	
		T	15	0	ND		<LLOQ		ND		<LLOQ	
Whole Plant	30-39	U	15	0	ND		<LLOQ		ND		<LLOQ	
		T	15	0	ND		<LLOQ		ND		<LLOQ	
Root	30-39	U	15	4	0.08	0.04	0.05	0.13	0.43	0.38	0.20	1.00
		T	15	12	0.09	0.02	0.06	0.14	0.50	0.24	0.27	1.04
Whole Plant	57-65	U	14	0	ND		<LLOQ		ND		<LLOQ	
		T	15	3	0.03	0.00	0.03	0.03	0.21	0.08	0.13	0.28
Root	57-65	U	15	3	0.08	0.01	0.07	0.09	0.40	0.09	0.31	0.49
		T	15	10	0.09	0.02	0.06	0.13	0.39	0.10	0.22	0.56
Raceme	57-65	U	15	0	ND		<LLOQ		ND		<LLOQ	
		T	15	2	0.09	0.07	0.04	0.13	0.68	0.31	0.46	0.90
Grain	89-99	U	15	0	ND		<LLOQ		ND		<LLOQ	
		T	15	0	ND		<LLOQ		ND		<LLOQ	

[a](T)reated or (U)ntreated with glufosinate-ammonium at 500 g ai./ha at 2-4 leaf stage.
[b]The Lower Limit of Quantification (LLOQ) for Barstar in all tissues is 0.500 ng/mL.
[c]Standard Deviation
[d]Not Determined

The untreated MS11 *B. napus* entry contained positive plants as well as negative segregants, as expected. All samples with Barstar expression levels <LLOQ except for whole plant samples were excluded from mean and standard deviation calculations and ranges as they were determined to be negative segregants. Protein expression levels in positive whole plant samples were close to the LLOQ, and it was not possible to distinguish between whole plant samples with low expression levels and whole plant samples obtained from negative segregants. As a result, whole plant samples with Barstar protein expression levels <LLOQ were excluded from mean and standard deviation calculations but included in the ranges.

Protein expression over different generations

Protein expression levels of PAT/*bar,* Barstar, and Barnase were determined by protein-specific sandwich enzyme-linked immunosorbent assay (ELISA) in whole plant and raceme matrices collected from three generations (T_3, T_4, T_5) of MS11 *B. napus*. Protein expression analysis was conducted on tissue samples harvested from *B. napus* plants cultivated in growth chambers. For each generation, four whole plant samples were collected at BBCH 30-39 and again at BBCH 57-65. Four raceme samples were also collected at BBCH 57-65.

The quantitative analysis of PAT/*bar* protein was conducted using a validated PAT/*bar*-specific ELISA method using the Envirologix QualiPlate™ Kit for LibertyLink® PAT/*bar* (Catalog number: AP 013). The quantitative analysis of Barstar protein was conducted with a validated Barstar-specific ELISA method using the EnviroLogix Barstar Plate Kit (Catalog Number: AP 125). The quantitative analysis of Barnase protein was conducted using a validated Barnase specific ELISA method using the EnviroLogix Barnase Plate Kit (Catalog Number: AP 127).

Mean expression levels of PAT/*bar* in whole plant and raceme matrices are presented in Table V-15. Measured mean expression levels of PAT/*bar* in whole plant samples (stem elongation or BBCH 30-39) across the three generations (T_3, T_4, T_5) were 15.73, 14.96, and 17.03 µg/g DW, respectively. Measured mean expression levels of PAT/*bar* in raceme samples (inflorescence or BBCH 57-65) across the three generations were 30.72, 20.23, and 20.38 µg/g DW, respectively. Measured mean expression levels of PAT/*bar* in whole plant samples (BBCH 57-65) across the three generations were 11.94, 11.93, and 13.98 µg/g DW, respectively.

Mean expression levels of Barstar and Barnase in all three generations of *B. napus* whole plant and raceme samples were below the lower limit of quantitation (LLOQ) for the ELISA method.

The measured expression levels across each generation of the matching tissue type exhibited similar overlapping expression ranges when comparing three generations of MS11 *B. napus*.

Table V-15: Expression levels of PAT/*bar* across three generations of MS11 *B. napus*

Matrix	BBCH Growth Stage	MS11 Generation	PAT/*bar* (µg/g FW)				PAT/*bar* (µg/g DW)			
			Mean	SD	Min	Max	Mean	SD	Min	Max
Whole Plant	30-39	T_3	1.27	0.18	1.03	1.46	15.73	3.17	12.12	19.78
		T_4	1.22	0.10	1.15	1.36	14.96	0.36	14.43	15.25
		T_5	1.25	0.09	1.19	1.37	17.03	4.58	12.16	23.09
Raceme	57-65	T_3	3.71	2.40	2.25	7.30	30.72	23.49	15.72	65.71
		T_4	2.67	0.17	2.53	2.93	20.23	2.04	17.66	22.51
		T_5	2.51	0.22	2.25	2.79	20.38	3.62	17.50	25.45
Whole Plant	57-65	T_3	1.15	0.04	1.11	1.19	11.94	0.66	11.25	12.76
		T_4	1.15	0.03	1.12	1.19	11.93	1.02	10.56	12.99
		T_5	1.29	0.16	1.12	1.46	13.98	0.99	13.13	15.34

Mean and standard deviation (SD) for each entry tissue type was based on the total sample population (N = 4).

VI. CHARACTERIZATION OF THE INTRODUCED PROTEINS

VI.A. Identity and Function of the Barnase Protein

Barnase protein is a ribonuclease which, when expressed in the tapetum cells of the anthers during pollen development, leads to cell death and consequently, to male sterility. The Barnase protein encoded by the *barnase* gene has 111 amino acids with a theoretical molecular weight of 12.5 kDa.

Background information and history of use

Barnase protein is derived from a well-known source organism, *B. amyloliquefaciens*, which is ubiquitous in nature and has an excellent safety profile. Because of its relatively small and simple structure, Barnase has been extensively studied in terms of structure, function, enzymatic activity, and molecular interactions for several years.

Barnase occurs frequently in nature for four reasons:

- There are many similar species of *B. amyloliquefaciens* in nature.
- *B. amyloliquefaciens* is used in detergent and food industries.
- Barnase is also present in other bacteria such as other *Bacilli* species, *Clostridium acetobutylicum* and the Gram-negative *Yersinia pestis*.
- The RNase family and RNase inhibitors play a central role in every aspect of cellular RNA metabolism, not only in prokaryotes but also in eukaryotes

Therefore, exposure to Barnase protein is not new.

Barnase (and its inhibitor Barstar) have been utilized to develop plant phenotypes with direct agronomic application. Male sterility and restoration of fertility was one of the first to be reported, and hybrid canola varieties engineered with this technology have been commercialized since 1996. Bayer CS has utilized the antecedent organism, MS8 *B. napus*, for hybrid canola variety production for over a decade.

Mode of action

The mode of action of Barnase is well known. The Barnase protein is an endoribonuclease; it cleaves RNA at internal sites. Barnase catalyzes the cleavage of phosphodiester linkages in RNA oligo- and polynucleotides. This reaction leads to the formation of intermediate nucleoside-2'3'-cyclophosphates and mono- and small oligonucleotides as final products. Barnase shows preference towards phosphodiester bonds with guanosine at their 3' end when cleaving RNA (Yakovlev et al., 1993).

Protein characterization of *in planta* Barnase

In MS11 *Brassica napus*, the *barnase* gene expression is under the control of a tapetum-specific promoter**,** Pta29 (Mariani et al., 1990). Therefore, the Barnase protein is expected to be specifically expressed in flower buds during anther development. Barnase exhibits RNase activity; hence, presence of Barnase protein leads to RNA degradation, cell disruption, and cell death (Mariani et al., 1992; Hartley, 1989). Since cells expressing Barnase protein are quickly disrupted, the levels of Barnase protein in MS11 *B. napus* tissues would be expected to be low.

This was substantiated in protein expression studies where expression levels of Barnase protein determined in different matrices of MS11 *B. napus,* including flower buds, were below the LLOQ for the ELISA method in all matrices analyzed. Furthermore, Barnase was not detected by western blot analysis in crude extracts or upon immuno-affinity purification attempts.

Due to the low levels of Barnase in MS11 *B. napus* tissues, protein of sufficient quantity and quality could not be extracted from the MS11 *B. napus* plant to experimentally confirm the equivalence the MS11 *B. napus* plant-produced Barnase protein with that of the antecedent organism, MS8 or other microbially-produced sources. As such, the Barnase protein in MS11 *B. napus* would be classified as an intractable protein as described in Bushey et al., 2014. Similarly, Barnase as expressed by the antecedent organism MS8, is also considered intractable. Therefore, a weight of evidence approach was used to assess the equivalence of the intractable protein expressed by MS11 and MS8.

Sequence analysis of the MS11 *B. napus* insert confirmed the sequence of the *barnase* gene was as expected. MS11 *B. napus* plants exhibited the male sterile phenotype of the antecedent organism MS8, demonstrating that an active Barnase was expressed and was efficacious.

This information cumulatively provides evidence that the Barnase in MS11 *B. napus* was produced as intended and that MS8 is an appropriate antecedent organism, as the male sterile phenotype is achieved by the same mode of action as MS8, the apparent expression of Barnase in the tapetum cells of flowers of transformed plants.

VI.B. Identity and Function of the Barstar Protein

The *barstar* gene, an intracellular inhibitor of the Barnase ribonuclease, was isolated from *Bacillus amyloliquefaciens* (Hartley, 1988). The Barstar protein encoded by the *barstar* gene has 90 amino acids, and a theoretical molecular weight of 10.3 kDa.

The Barstar protein is an inhibitor of the Barnase protein. The prophylactic *barstar* gene in MS11 *B. napus*, driven by the Pnos promoter, was included to enhance transformation frequency.

Background information and history of use

Barstar protein is derived from a well-known source organism, *B. amyloliquefaciens*, which is ubiquitous in nature and has an excellent safety profile. Because of its relatively small and simple structure, Barstar has been extensively studied in terms of structure, function, enzymatic activity, and molecular interactions for several years.

Barstar occurs frequently in nature for four reasons:

- There are many similar species of *B. amyloliquefaciens* in nature.
- *B. amyloliquefaciens* is used in detergent and food industries.
- Barstar is also present in other bacteria such as other *Bacilli* species, *C. acetobutylicum* and the Gram-negative *Y. pestis*.
- The RNase family and RNase inhibitors play a central role in every aspect of cellular RNA metabolism, not only in prokaryotes but also in eukaryotes

Therefore, exposure to Barstar protein is not new.

While the Barstar protein is not prophylactically expressed by the antecedent organism MS8, it is expressed by event RF3, which along with MS8 was also subject of petition 98-278-01p. Barstar as the inhibitor of Barnase has been utilized to develop plant phenotypes with direct agronomic application. Male sterility and restoration of fertility was one of the first to be reported, and hybrid canola varieties engineered with this technology have been commercialized since 1996. Bayer CS has utilized the antecedent organism, MS8 *B. napus*, for hybrid canola variety production for over a decade.

Mode of action of Barstar

The only known function of the Barstar protein is to protect the bacteria from the lethal effect of the Barnase activity (Hartley, 1988; Smeaton et al., 1965). The inhibition of Barnase by Barstar is highly specific and non-covalent. The Barstar protein sterically blocks the active site of the Barnase protein with an alpha-helix and adjacent loop (Hartley, 1989).

Protein characterization of *in planta* Barstar

MS11 *Brassica napus* contains the *barstar* gene (origin *B. amyloliquefaciens*) coding for the Barstar protein, which is an inhibitor of the Barnase protein. This prophylactic *barstar* gene, driven by the constitutive Pnos promoter, was included to enhance transformation frequency.

Barstar protein was only consistently expressed in roots from field grown samples treated with glufosinate-ammonium. The protein expression levels of Barstar were consistently below LLOQ in all grain samples and most raceme and whole plant samples. Western blot analysis of crude root extracts only very faintly detected Barstar protein, but a band of the anticipated size was detected in concentrated preparations. However, repeated attempts to purify or further enrich the Barstar protein using immuno-affinity chromatography were unsuccessful, as contaminants were also co-purified during this process.

As such, the Barstar protein in MS11 *B. napus* would be classified as an intractable protein as described in Bushey et al., 2014. Therefore, a weight of evidence approach was used to assess the equivalence of the intractable protein with that expressed by event RF3.

Sequence analysis of the MS11 *B. napus* insert confirmed that the sequence of the *barstar* gene was as expected. Since no additional start codon is present in the MS11 *B. napus* insert sequence that could result in a slightly different protein, it was concluded that the amino acid sequence of the Barstar protein expressed in MS11 *B. napus* is identical to the amino acid sequence for that of event RF3.

Additionally, concentrated crude protein extract was used to confirm the molecular weight and immuno-reactivity of the Barstar protein expressed in MS11 *B. napus*. Enough protein of sufficient quantity and quality could not be extracted from the MS11 *B. napus* plants to perform additional experiments typically conducted to demonstrate equivalency.

This information cumulatively provides evidence that the Barstar in MS11 *B. napus* was produced as intended and can be considered as equivalent to that expressed by RF3 *B. napus* which was previously reviewed in petition 98-278-01p. Further, Barstar expression has no effect on the actual male sterile phenotype of MS11. It is included only to enhance transformation efficiency. Therefore MS8 is the appropriate antecedent organism for MS11.

VI.C. Identity and Function of the PAT/*bar* Protein

The phosphinothricin acetyltransferase (PAT) protein is encoded by the *bar* gene that was isolated from *S. hygroscopicus* in the mid-1980s (Murakami et al., 1986). The PAT/*bar* protein encoded by the *bar* gene has 183 amino acids (Herouet et al., 2005) with a theoretical molecular weight of 20.7 kDa.

Background information and history of use

PAT/*bar* protein is derived from a well-known source organism, *S. hygroscopicus*, which is a common saprophytic bacterial species that is found worldwide (Kutzner, 1981) and has an excellent safety profile. *S. hygroscopicus* is not known to be a pathogen of plants, humans or other animals (OECD, 1999), and the PAT protein, like other acetyltransferases, is not known to have any allergenic or toxic properties, and has a well-characterized activity and substrate specificity. A battery of tests performed according to internationally accepted methods and standards have established that the PAT protein does not possess structural or functional similarity with known toxic proteins or allergens; it shares no sequence homology with known allergens and toxins, no N-glycosylation sites, and rapidly degrades in simulated digestive environments (Herouet et al., 2005).

Transgenic crops expressing the PAT protein have been grown for more than a decade in the USA and Canada. In the 2011 review by ILSI, it was estimated that regulatory authorities in seven countries had issued approvals for the environmental release of six transgenic crop species that express PAT proteins (encoded by the *bar* gene), either alone or in combination with genes for other traits (e.g. insect resistance) (ILSI-CERA, 2011).

Mode of action

The mode of action of the PAT protein has been well characterized (ILSI-CERA, 2011; OECD, 1999; Herouet et al., 2005). The L-isomer of phosphinothricin (L-phosphinothricin or L-PPT) is the active ingredient of glufosinate-ammonium. The herbicidal effect of L-PPT is achieved through the inhibition of glutamine synthetase, the only enzyme in plants that can detoxify ammonia released by photorespiration, nitrate reduction, and amino acid degradation. L-PPT is a structural analogue of glutamate, the usual substrate of glutamine synthetase. When L-PPT competitively binds to glutamine synthetase, the result is accumulation of phytotoxic levels of ammonia in plant tissues and the inhibition of photosynthesis. The PAT protein acetylates L-PPT at the N-terminus, causing it to become an inactive derivative and thereby conferring tolerance to glufosinate-ammonium (Thompson et al., 1987).

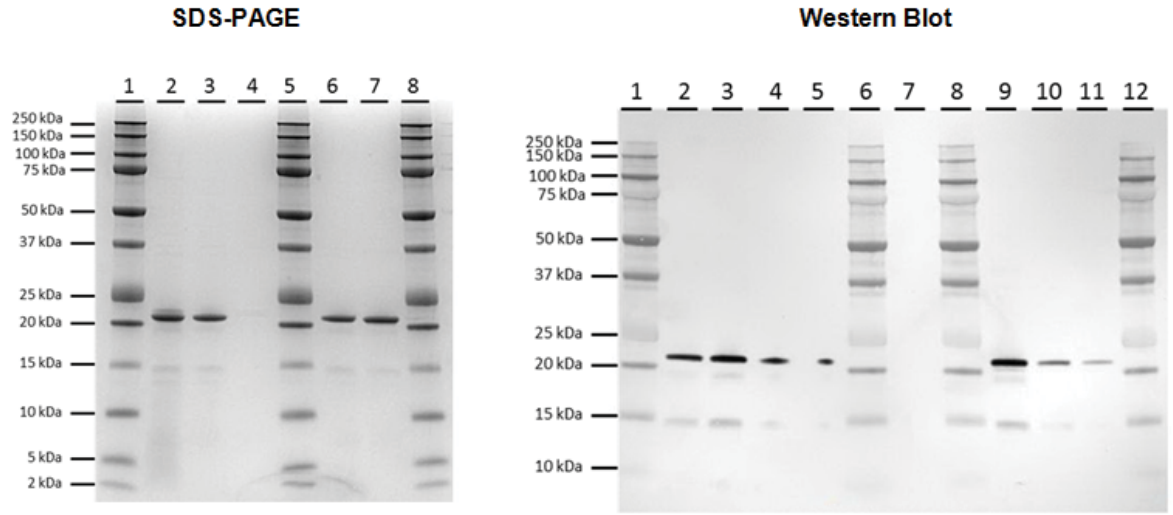
Protein characterization of *in planta* produced PAT/*bar*

PAT/*bar* protein was extracted and purified from MS11 *Brassica napus* leaves to determine the structural and functional characteristics of the plant-purified protein. The structural and functional comparability with the microbially-produced PAT/*bar* protein batch 1215_PATbar was assessed by several complementary methods: molecular weight and purity determination by SDS-PAGE or UPLC-UV-MS, immuno-reactivity by western blot towards an anti- Pat/*bar* antibody, peptide mapping by mass spectrometry, N-terminal sequencing by Edman Degradation, glycosylation by glycostaining, and enzymatic activity assay using a qualitative and quantitative spectrophotometric method.

For the plant-purified PAT/*bar* protein, SDS-PAGE analysis demonstrated a purity of 74%. Peptide mapping against the theoretical amino acid sequence of the PAT/*bar* protein resulted in coverage of 100%. The N-terminal sequence (MDPER) was consistent with the expected theoretical sequence although acetylation of the N-terminus was observed, and the intact molecular mass confirmed the theoretical molecular mass of the acetylated PAT/*bar* protein (21 kDa).

Comparisons of the plant-purified and the microbially-produced PAT/*bar* proteins by SDS-PAGE demonstrated comparable molecular masses, and the immuno-reactivity of the plant-purified PAT/*bar* protein was confirmed (Figure VI-1). Neither the plant-purified nor the microbially-produced PAT/*bar* proteins were glycosylated. The activity of the plant-purified and microbially-produced PAT/*bar* proteins were functionally equivalent.

The comparison of the structural and functional characteristics of the plant-purified PAT/*bar* protein with the microbially-produced PAT/*bar* protein batch 1215_PATbar demonstrated that both PAT/*bar* proteins have similar protein-specific characteristics. The identity of the plant-purified PAT/*bar* protein was confirmed and both plant-purified and the microbially-produced PAT/*bar* proteins are structurally and functionally equivalent.



Lane 1: MW Marker
Lane 2: 2 µg plant purified protein
Lane 3: 1 µg plant purified protein
Lane 4: negative control
Lane 5: MW Marker
Lane 6: 1 µg plant purified protein
Lane 7: 1 µg 1215_PATbar
Lane 8: MW Marker

Lane 1: MW Marker
Lane 2: 40 ng plant purified protein
Lane 3: 40 ng plant purified protein
Lane 4: 10 ng plant purified protein
Lane 5: 4 ng plant purified protein
Lane 6: MW Marker
Lane 7: negative control
Lane 8: MW Marker
Lane 9: negative control + 40 ng 1215_PATbar
Lane 10: negative control + 10 ng 1215_PATbar
Lane 11: negative control + 4 ng 1215_PATbar
Lane 12: MW Marker

Figure VI-1: SDS-PAGE and western blot comparisons of plant-purified and microbially-produced (Batch 1215_PATbar) PAT/*bar* protein

VII. AGRONOMIC AND PHENOTYPIC EVALUATION

VII.A. History of Field Activities

MS11 *B. napus* has been field tested since 1999. The majority of field trials have been carried out in canola growing regions of Canada. Field trials were conducted in the United States only in 2014 under USDA notification 14-087-103n. The field trial data report for this trial has been submitted to USDA, and is provided again in Appendix 1 to this petition.

VII.B. Agronomic Assessment

MS11 *B. napus* is an essential element in the production of Bayer canola hybrids. The hybrid technology comprises three components: a dominant gene for male sterility – the *barnase* gene (event MS11), a dominant gene for fertility restoration – the *barstar* gene (event RF3) and a selectable marker gene to make the system more convenient for breeding and seed production – the *bar* gene (found in both MS11 and RF3) conferring tolerance to glufosinate-ammonium.

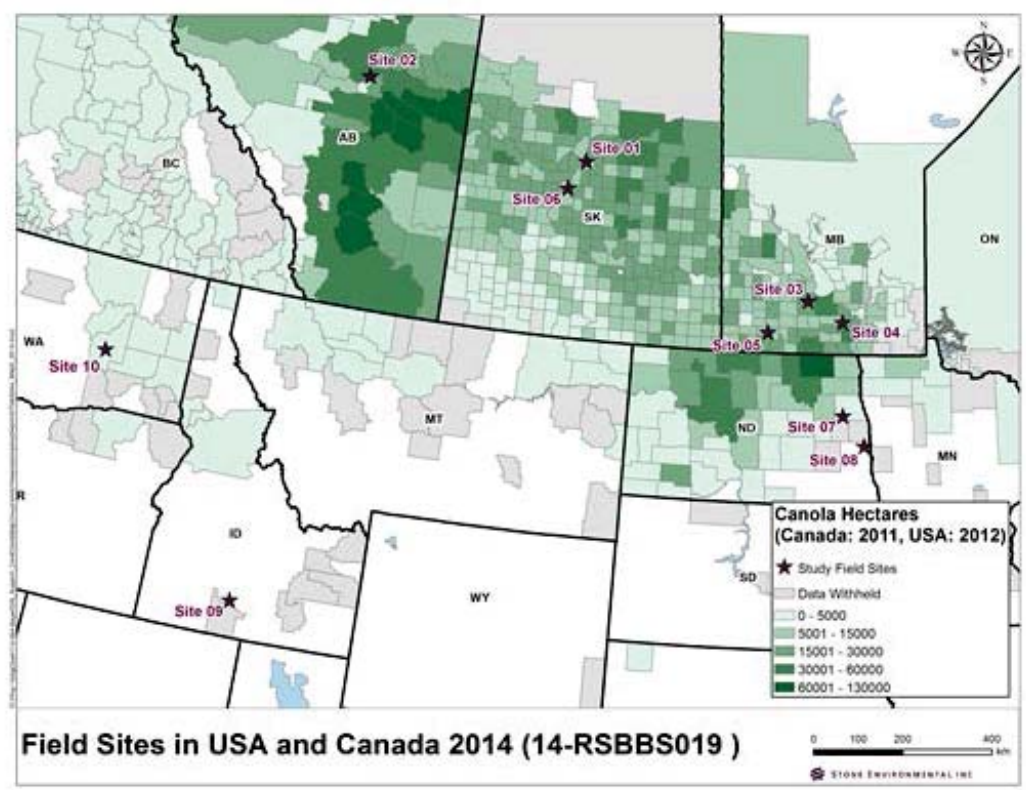
MS11 *B*. *napus* provides the male sterile parent line of the hybridization system and will not be marketed as a single event. Due to nuclear male sterility, when the MS11 seed is planted, half of the plants produce pollen and are not glufosinate-ammonium tolerant. The other half contains the *barnase* and *bar* genes and are male sterile as well as glufosinate-ammonium tolerant. To produce F1 certified seed for commercial sale, the pollen producing segregants in MS11 *B. napus* are removed by spraying with glufosinate-ammonium. The remaining sterile plants are fertilized by adjacent strips of RF3 *B. napus.* RF3 *B. napus* contains the *barstar* gene, expressing Barstar, that inhibits Barnase and therefore restores fertility. The resulting F1 seed is both fully fertile and tolerant to glufosinate-ammonium.

The agronomic performance of MS11 was observed in ten field trials conducted in the canola growing regions of Western Canada and Northwestern USA (Figure VII-1 and Table VII-1) during 2014.

Table VII-1: Trial site locations for the 2014 field tests

Trial No.	County (USA) or Rural Municipality (CA)	Nearest Town	State (USA) or Province (CA), Country
01	Hoodoo	Wakaw	SK, Canada
02	Sturgeon	Gibbons	AB, Canada
03	North Norfolk	MacGregor	MB, Canada
04	MacDonald	Starbuck	MB, Canada
05	Whitewater	Minto	MB, Canada
06	Corman Park	Saskatoon	SK, Canada
07	Grand Forks	Northwood	ND, USA
08*	Case	Gardner	ND, USA
09	Jerome	Jerome	ID, USA
10	Grant	Ephrata	WA, USA

*Site excluded from statistical analyses due to flooding



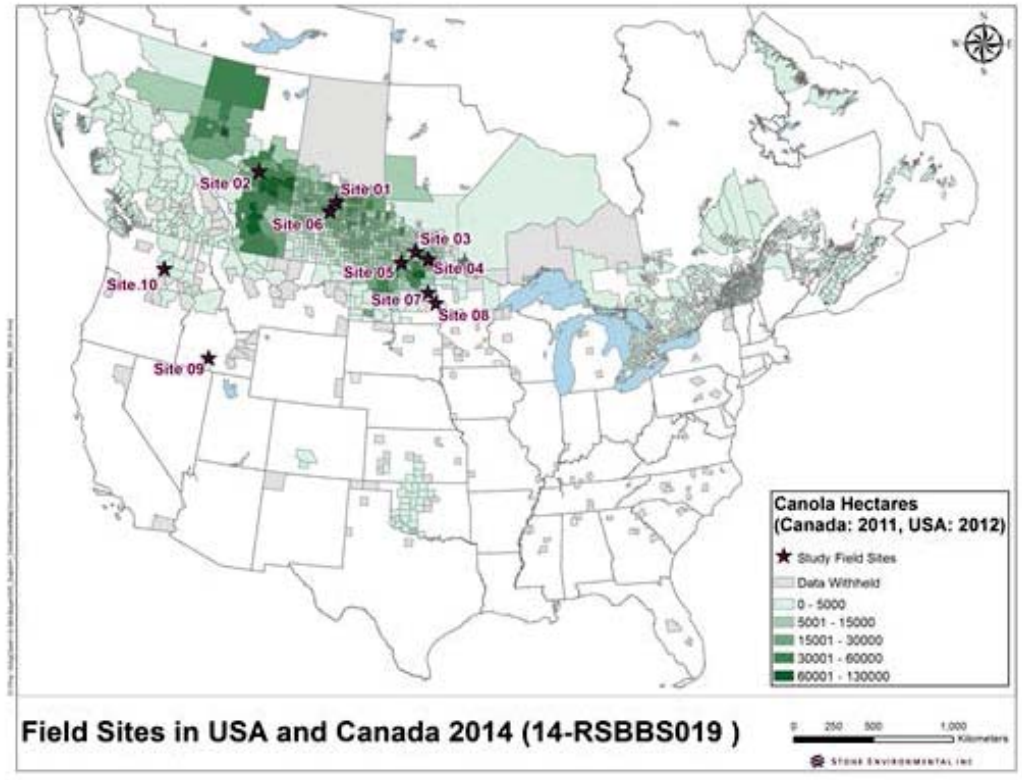


Figure VII-1: 2014 Trial site locations and the main canola production areas in Canada and USA

Entries relevant for the agronomic assessment of MS11 included at each field trial site are presented in Table VII-2. Each entry was replicated four times in a randomized complete block design. Three of six possible reference varieties that represent the variability existing in commercial *B. napus* lines were grown at each site to generate reference ranges for agronomic parameters for comparison.

Table VII-2: Description of entries in 2014 field trials

Entry ID	Description	Background	Trait-Specific Herbicide Treatment	Trial No. Locations
A	Non-GM Conventional Counterpart (N90-740)	N90-740	Not Treated	All
B	MS11	N90-740	Not Treated	All
C	MS11	N90-740	Treated	All
F	46A65 (Reference Variety)	Non-GM	Not Treated	1, 2, 3, 7 and 9
G	AC Elect (Reference Variety)	Non-GM	Not Treated	1, 2, 3, 7 and 9
H	AC Excel (Reference Variety)	Non-GM	Not Treated	1, 2, 3, 7 and 9
I	Peace (Reference Variety)	Non-GM	Not Treated	4, 5, 6, 8 and 10
J	Spectrum (Reference Variety)	Non-GM	Not Treated	4, 5, 6, 8 and 10
K	Westar (Reference Variety)	Non-GM	Not Treated	4, 5, 6, 8 and 10

The plots at each field trial site consisted of a minimum of 6 rows that were at least 5 m in length. Plots were separated by a minimum of 1.5 m fallow alleyway. The seeding density (sowing rate) was 1600 seeds per plot for all entries except Entry C. The seeding density for Entry C, the MS11 plots treated with glufosinate-ammonium, was doubled to 3200 seeds per plot because approximately 50 % of seedlings would not be tolerant to the glufosinate-ammonium application. Therefore, all plots resulted in the same plant density after glufosinate-ammonium treatment.

The plots were large enough to allow treatment with commercial type or small plot application equipment. MS11 *B. napus* plots treated with trait-specific herbicide received one spray application of glufosinate-ammonium (Liberty 280 SL; 2.34 g ai/L) at a target rate of 500 g ai/ha at BBCH Growth Stage 12 to 14. Since the trait-specific herbicide treatment removed the non-tolerant segregants from the treated plots, the remaining plants in these plots were sterile and depended on pollen from neighboring plots for fertilization.

Table VII-3 summarizes the agronomic parameters evaluated throughout the growing season at each field trial site. Growth stages and the dates of collection were also recorded with the agronomic observations.

Table VII-3: Agronomic parameters evaluated in 2014 field trials

Parameters	Parameters Type
Early Stand Count	Continuous
Seedling Vigor	Continuous
Days to Flowering (50% Plants Flower)	Continuous
Days to 10% Plants Remain Flowering	Continuous
Days to Maturity	Continuous
Seed Yield	Continuous
Plant Height	Continuous
Final Stand Count	Continuous
Seedling Vigor	Categorical
Sterile Plants per Plot	Categorical
Lodging Resistance	Categorical
Pod Shattering	Categorical
Environmental Interactions	Categorical

MS11 male sterility (having flowers but lacking anthers) was confirmed to be as expected when evaluated appropriately at several sites, confirming expected functioning of the introduced trait. Male sterility data was not further evaluated.

Agronomic data from nine sites were selected for statistical analysis as site 08 was excluded due to flooding which resulted in missing data from multiple plots, but no loss of containment or confinement.

Descriptive statistics for the continuous agronomic parameters are summarized in Table VII-4 for the combined site analysis. Comparative assessments between the different entries (A vs B and A vs C) were performed using a mixed model analysis of variance. No statistical differences ($p < 0.05$) were observed between the non-GM conventional counterpart (Entry A) and MS11 *B. napus* not treated with trait-specific herbicide (Entry B) for any of the continuous agronomic parameters. No statistical differences were observed between the non-GM conventional counterpart (Entry A) and MS11 *B. napus* treated with trait-specific herbicide (Entry C) for three continuous agronomic parameters; final stand count, days to flowering, and average plant height. However, statistically significant differences were observed for other continuous agronomic parameters; early stand count, days to 10% flowers remaining, days to maturity, and yield. As the Entry C plots were seeded at twice the seeding rate as Entry A, the statistically high early stand count was expected.

Descriptive statistics for the categorical parameters are summarized in Table VII-5. No statistical differences were observed in any of the categorical agronomic parameters and environmental interaction ratings, except for seedling vigor and abiotic stress at BBCH 30-39, which showed statistically significant differences between Entry A and C because of the higher seeding rate of Entry C.

Agronomic parameters for the six reference varieties, Entries F through K, were evaluated as a single entry for the descriptive statistics. In addition, tolerance intervals, specified to contain 99% of the population with 95% confidence, were calculated for the continuous parameters for the reference varieties across all sites. All continuous and categorical mean values were within the range of the reference varieties and the tolerance intervals except for early stand count which was expected to be higher in Entry C.

Based on the agronomic assessment, the MS11 *B. napus* demonstrated no biologically relevant differences compared to the non-GM conventional counterpart and showed equivalent agronomic performance in the field to *B. napus* reference varieties.

Biotic (disease and insect) stressors were evaluated four times (BBCH 12-14, 30-39, 60-69, and 79-87) during the 2014 growing season (Table VII-5). No statistical differences were observed in any of the biotic environmental interaction ratings in comparisons between the non-GM conventional counterpart and the MS11 *B. napus*.

Table VII-4: Comparison of continuous agronomic parameters of MS11 *B. napus* with its non-GM conventional counterpart

Parameter	Non-GM Conventional Counterpart (Entry A) Mean ± SD	MS11 Not Treated (Entry B) Mean ± SD	MS11 Treated (Entry C) Mean ± SD	Non-GM Reference Varieties Range (Entries F-K) [a] (Min-Max)	Tolerance Interval Non-GM Reference Varieties (Entries F-K) [b] (Lower-Upper)	Comparison t-test A vs B [c] p-value	Comparison t-test A vs C [c] p-value
Early Stand Count	158.3 ± 49.2	168.8 ± 57.8	267.3 ± 121.7	65 - 312	20.0 - 265.6	0.858	<.001
Final Stand Count	95.9 ± 32.4	113.5 ± 36.1	90.3 ± 48.0	15 - 174	0 - 199.3	0.319	0.792
Days to Flowering	43.5 ± 4.07	43.9 ± 3.79	44.0 ± 4.10	37 - 55	29.7 - 56.8	0.845	0.370
Days to Flowering - 10% remains	61.8 ± 8.67	65.0 ± 6.80	62.9 ± 10.37	46 - 76	37.8 - 88.1	0.243	0.004
Days to Maturity	100.5 ± 10.11	100.3 ± 10.85	105.9 ± 11.71	80 - 125	68.2 - 134.2	0.745	0.010
Average Plant Height (cm)	112.7 ± 22.7	110.5 ± 22.8	112.8 ± 19.9	76.8 - 154.5	55.9 - 163.0	0.584	0.890
Yield (Kg/Ha@8%)	1638.0 ± 959.5	1535.9 ± 981.8	1333.5 ± 793.0	241.0 - 3760.4	0 - 4125.0	0.797	0.017

[a] Range of results from six reference lines (Entries F-K).
[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 conventional counterpart (Entry A). Statistical significance was evaluated at $p < 0.05$.

Table VII-5: Comparison of categorical agronomic parameters of MS11 *B. napus* with its non-GM conventional counterpart

Parameter	Non-GM Conventional Counterpart (Entry A) ---------- Mean ± SD	MS11 Not Treated (Entry B) ---------- Mean ± SD	MS11 Treated (Entry C) ---------- Mean ± SD	Non-GM Reference Varieties Range (Entries F-K) [a] ---------- (Min-Max)	A vs B CMH-Test ---------- p-value [b]	A vs C CMH-Test ---------- p-value [b]
Seeding Vigor (1-9)	6.83 ± 1.65	7.13 ± 1.56	7.53 ± 1.71	1 – 9	0.410	0.007
Lodged Plants (1-9)	5.53 ± 2.21	5.47 ± 2.42	5.70 ± 2.36	1 – 9	0.777	0.647
Pod Shattering (1-9)	7.97 ± 1.08	8.16 ± 1.08	8.13 ± 1.04	4 – 9	0.317	0.133
Abiotic Stress Rating (1-9) BBCH 12-14	2.17 ± 1.93	2.44 ± 1.98	2.20 ± 1.96	1 – 9	0.533	0.485
Abiotic Stress Rating (1-9) BBCH 30-39	1.67 ± 0.96	1.72 ± 1.02	2.30 ± 1.73	1 – 4	0.655	0.002
Abiotic Stress Rating (1-9) BBCH 60-69	1.42 ± 0.87	1.47 ± 1.05	1.43 ± 1.41	1 – 7	0.728	0.886
Abiotic Stress Rating (1-9) BBCH 79-87	3.06 ± 2.24	3.25 ± 2.18	3.08 ± 2.36	1 – 7	0.317	0.286
Disease Stress Rating (1-9) BBCH 12-14	1.14 ± 0.49	1.00 ± 0.00	1.05 ± 0.22	1 – 3	0.111	0.298
Disease Stress Rating (1-9) BBCH 30-39	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1 – 1	NA	NA
Disease Stress Rating (1-9) BBCH 60-69	1.25 ± 0.65	1.25 ± 0.67	1.20 ± 0.61	1 – 3	1.000	0.727
Disease Stress Rating (1-9) BBCH 79-87	1.86 ± 1.05	1.88 ± 1.16	1.85 ± 1.19	1 – 5	0.802	0.782
Insect Stress Rating (1-9) BBCH 12-14	1.17 ± 0.51	1.13 ± 0.49	1.13 ± 0.40	1 – 3	1.000	0.471
Insect Stress Rating (1-9) BBCH 30-39	1.56 ± 1.00	1.50 ± 0.98	1.45 ± 0.85	1 – 4	0.243	0.502
Insect Stress Rating (1-9) BBCH 60-69	1.69 ± 1.35	1.81 ± 1.42	1.70 ± 1.30	1 – 5	0.635	0.654
Insect Stress Rating (1-9) BBCH 79-87	1.39 ± 0.80	1.38 ± 0.79	1.50 ± 0.99	1 – 3	0.495	0.180

[a] Range of results from six reference lines (Entries F-K).
[b] Cochran-Mantel-Haenszel Test p-value: comparison to the non-GM conventional counterpart (Entry A). Statistical significance was evaluated at $p < 0.05$.
NA: Analysis could not be run due to lack of variability

VII.C. Seed Germination and Dormancy Evaluation

Germination Study

To compare the germination potential of the MS11 *B. napus* to that of the non-GM conventional counterpart, a warm-cold germination test following AOSA seed evaluation guidelines was conducted (AOSA, 2013a; AOSA, 2013b).

Analysis of the warm germination data (Table VII-6) showed that MS11 and N90-740 *B. napus* had germination percentages of 97.75% and 98.00%, respectively. MS11 was not significantly different from that of the non-GM conventional counterpart.

Table VII-6: Comparison of germination categories between MS11 and N90-740 in the warm germination test

Genotype	Category	Count	Percent	p-value[a]
MS11	Abnormal	9	2.25	
MS11	Normal	391	97.75	
MS11	Un-germinated	0	0.00	
N90-740	Abnormal	8	2.00	
N90-740	Normal	392	98.00	
N90-740	Un-germinated	0	0.00	
				1.000

[a] Fisher's Exact Test p-value for comparison of germination categories between MS11 and N90-740 in the Warm Germination Test

In the cold germination test, both MS11 and N90-740 *B. napus* had germination percentages of 98.50% (Table VII-7). The cold germination potential of MS11 was not significantly different from that of the non-GM conventional counterpart.

Table VII-7: Comparison of germination categories between MS11 and N90-740 in the cold germination test

Genotype	Category	Count	Percent	p-value[a]
MS11	Abnormal	3	1.50	
MS11	Normal	197	98.50	
MS11	Un-germinated	0	0.00	
N90-740	Abnormal	3	1.50	
N90-740	Normal	197	98.50	
N90-740	Un-germinated	0	0.00	
				1.000

[a] Fisher's Exact Test p-value for comparison of germination categories between MS11 and N90-740 in the Cold Germination Test

Cold Tolerance Study

The cold tolerance of MS11 *B.* napus was compared to that of the non-GM conventional counterpart N90-740 using AOSA guidelines as a reference (AOSA, 2013a; AOSA, 2013b).

Cold tolerance test results for MS11 and N90-740 are summarized in Table VII-8. MS11 *B. napus* had an overall germination percentage of 2.00% while N90-740 had an overall

germination percentage of 3.50%. There was no significant difference ($p < 0.05$) in the cold tolerance of MS11 and N90-740 ($p = 0.221$).

Table VII-8: Comparison of germination categories between MS11 and N90-740 in the cold tolerance test

Genotype	Category	Count	Percent	p-value[a]
MS11	Abnormal/un-germinated Normal	392 8	98.00 2.00	
N90-740	Abnormal/un-germinated Normal	386 14	96.5 3.5	
				0.221

[a] Fisher's Exact Test p-value for comparison of germination categories between MS11 and N90-740 in the Cold Tolerance Test

VIII. ENVIRONMENTAL SAFETY AND IMPACT ON AGRONOMIC PRACTICES

VIII.A. Environmental Safety

Persistence, weediness and invasiveness

Since *B. napus* is the result of an interspecific cross between a plant or plants of *B. rapa* and the *B. oleracea* complex, it could only have arisen in the Mediterranean or the European west coastal regions, where the two species were growing in close proximity (OECD, 2011). Commercial *B. napus* varieties in the United States and Canada were introduced by Europeans around 17th -18th centuries. *B. napus* is not considered a weed in managed ecosystems.

The potential of *B. napus* to become a problematic weed in succeeding crops depends on the management practices used in the production of the crop, the setup of the harvesting equipment and the speed of the harvesting operation. These parameters will determine the seed lost from the harvester. The comparative assessment has confirmed the substantial equivalence of MS11 *B. napus* to its conventional counterpart confirming that it is very unlikely that MS11 *B. napus* plants will be more persistent or will present different weed-related characteristics than the conventional counterpart. MS11 *B. napus* plants that remain in the field after cultivation can be controlled using the same methods as for conventional *B. napus*, except glufosinate-ammonium.

In addition, the comparative assessment (Section VII) has confirmed the substantial equivalence of MS11 *B. napus* to its conventional counterpart for all phenotypic and agronomic parameters except pollen formation and glufosinate-ammonium tolerance, the predicted traits, confirming that it is very unlikely that MS11 *B. napus* plants would be more persistent or would present different weed-related characteristics than the conventional counterpart.

In conclusion there are no differences between MS11 *B. napus* and its conventional counterpart that could contribute to increased weediness potential of MS 11 *B. napus*.

Gene flow and its consequences

Brassica pollen, although heavy and sticky can still become air-borne and float on the wind due to its minute size. In addition to wind, pollen can be transferred by insects, primarily honey bees (OECD, 2012). Wind pollination is more common in areas were big fields are cultivated (> 60 Ha) as in Canada, as bees cannot services all the flowers (OECD, 2016). Gene flow can occur into an adjacent oilseed rape crop but the frequency of gene flow decreases with the distance to the pollen source. Most of the pollen (90%) is expected to travel less than 20 m distance (OECD, 2016).

B. napus could hybridize with some members of the Brassicaceae, and this species can act as a bridge to other species, but the likelihood and success of spontaneous hybridization is low compared to normal intraspecific crosses. Studies in both Canada and Europe have demonstrated that the incorporation of genes for resistance to specific herbicides imparts no altered weediness or invasive potential (OECD, 2016). Some factors important for hybridization to occur include the presence of vectors for pollination and the mechanism for pollination. Many hybrids fail due to lack of development of the endosperm. The chromosome numbers of *B. napus* and the related species are also important. The ratio of maternal and parental chromosomes must be of 2:1 or higher (Nishiyama and Inomata, 1966).

The male sterile MS11 line does not produce pollen. Since this line will only be grown in the presence of a pollen source (a control line for maintenance of the trait or a restorer-of-fertility line for hybrid seed production), pollination by wild relatives is highly unlikely. Such pollination would lead to impurity in pure seed production and is therefore tightly controlled. The fertility restorer RF3 line and the fertile restored hybrid combination (MS11xRF3) do not differ in their pollination capacity and pollen acceptor capacity from control plants.

It is highly unlikely that outcrossing via pollen flow to *B. napus* relatives might occur under natural conditions. An interspecific cross pollination between *B. napus* plants and their (wild) relatives can only take place if plants are established in close proximity and if both species flower during the same period of time. Additionally, interspecific cross pollination between two species has to take place. This is a rare event compared to intraspecific pollination where no crossing barriers have to be broken. Furthermore, if F_1 progeny develop, these plants need to be as competitive as the parental plants in order to be able to permanently establish in the environment. For a trait to become incorporated into a species genome, recurrent backcrossing of plants of that species by the hybrid intermediaries, and survival and fertility of the resulting offspring, will be necessary (OECD, 2016). None of the physical, genetic, and ecological barriers of MS11 *B. napus* are influenced by the expression of the transgenes, with the exception of the reduction of pollen emission in the male sterile line. Therefore, it is concluded that the scope and the extent of exchange of genes between the genetically modified oilseed rape and wild relatives is identical to what is occurring today for traditionally derived varieties.

Potential selective advantage to wild relatives

It is possible that MS11 *B. napus* crosses with related weed species as previously discussed, but the success of the interspecific F_1 hybrids depends on their growth, vigor, fertility, ability to propagate vegetatively, ability to give viable F_2 and backcross progeny, and the ability to survive over subsequent generations. These hybrids would also need to compete for space with the progeny of its parental plants (OECD, 2016). In the event that MS11 *B. napus* was able to successfully hybridize with related species, the offspring would not be expected to behave differently than other *B. napus* hybrids and could be effectively controlled with herbicides approved for oilseed rape (except glufosinate-ammonium).

In regions with low exposure potential to MS11 *B. napus*, such as countries that only import canola seed, it is improbable that MS11 *B. napus* plants would hybridize with related species. The occurrence of such an event would most likely take place close to the importing and handling areas or around transportation routes. Again, if by some chance these hybrids germinated successfully, they could be controlled using authorized herbicides for controlling oilseed rape (except glufosinate-ammonium).

Potential for horizontal gene transfer

Current scientific knowledge indicates that horizontal gene transfer of non-mobile DNA fragments between unrelated organisms (such as plants to microorganisms) is extremely unlikely to occur under natural conditions (EFSA, 2015).

Altered disease or pest susceptibility

Biotic (disease and insect) stressors evaluations were performed four times during the growing season at all trial sites in Canada in 2014 (Section VII.A.). No significant difference was found between MS11 *B. napus* (treated with the intended herbicide or not treated) and the conventional counterpart in disease or insect stress at any of the four evaluation time points.

Potential interactions with abiotic environment

Abiotic stressors evaluations were performed four times during the growing season at 10 trial sites in Canada in 2014 (Section VII.A.). Only one comparison at BBCH 30-39 showed statistically significant differences between MS11 *B. napus* treated with the intended herbicide and its conventional counterpart. However the comparison between MS11 not treated and the conventional counterpart was not significant and the values of MS11 *B. napus* were well within the range of the commercial reference varieties used. Therefore it was conclude that MS11 *B. napus* was equivalent to its conventional counterpart.

Survival and dormancy

Two studies were performed to test the germination potential of MS11 *B. napus* seeds (Section VII.C).

The first study was a standard warm-cold germination test. Analysis of warm germination data demonstrated that MS11 and N90-740 (its non-GM conventional counterpart) *B. napus* had germination percentages of 97.75% and 98.00%, respectively. Analysis of cold germination data demonstrated that both MS11 and N90-740 *B. napus* had germination percentages of 98.50%. Both the warm and cold germination potential of MS11 *B. napus* were not significantly different from that of the non-GM conventional counterpart.

In the second study, a cold tolerance test was conducted where the seeds of MS11 and N90-740 *B. napus* were incubated at -10 ± 5°C for 10 days prior to performing the standard warm germination protocol. Evaluation of the seeds for MS11 and N90-740 *B. napus* indicated only 2.00% and 3.50%, respectively, survived to germinate normally. Statistical analysis of the cold tolerance data concluded that there was no significant difference between the two genotypes.

VIII.B. Impact on Agronomic Practices

Current agronomic practices for canola

Canada was the first country to commercially use Genetically Modified Herbicide Tolerant (GM HT) canola in 1996. Since then the area planted to varieties containing GM HT traits has increased significantly, and in 2012 was 98% of the total crop (8.37 million ha) (Brookes and Barfoot, 2015). Reasons for the adoption of GM HT canola varieties include improved weed control, earlier spring planting (resulting in increased yield due to better soil moisture utilization), less dockage (weed seeds), increased seed quality and reduced herbicide and tillage costs. Hybrid HT canola varieties are increasingly popular with growers due to their yield advantage and in 2010 hybrid canola varieties constituted most of the canola market (Beckie et al., 2011).

The use of these new hybrid HT canola varieties has altered weed management practices in the canola growing areas of western Canada. Much of the tillage associated with HT canola production has been eliminated as 64% of producers are now using zero or minimum tillage as their preferred form of crop and soil management. Producers are getting very high levels of

weed control in fields seeded with HT canola, to the level that there is no longer a need to prework fields before seeding in the following crop year. Traditionally, producers have tilled their fields prior to seeding as a form of early weed control. The use of HT canola would appear to have eliminated this practice as producers now apply herbicides to 'burnoff' weeds prior to seeding (Smyth et al., 2011).

In western Canada, canola is typically grown in the same field once in every two to four years, primarily to reduce disease problems. Typical crop rotation sequences in the Canadian prairies would include canola followed by a cereal crop, followed by a pulse crop or flax followed by a cereal crop.

Anticipated change in practice with new crop/trait

The use of hybrid canola varieties produced with MS11 *B. napus* are not anticipated to change current agronomic practices. Glufosinate-ammonium tolerant hybrid canola varieties have been produced in Canada and the USA for approximately 20 years.

Impact on agricultural practices for canola

The use of hybrid canola varieties produced with MS11 *B. napus* are not anticipated to impact current agricultural practices for canola. Glufosinate-ammonium tolerant hybrid canola varieties have been produced in Canada and the USA for approximately 20 years.

Value to agriculture and country

F1 hybrid canola varieties yield 20-25% more than the best open-pollinated canola varieties. The uniformity of the hybrid plants is an advantage in commercial fields, facilitating harvesting and marketing. The incorporation of the *bar* gene provides a weed management tool to canola growers. The *bar* gene confers tolerance to glufosinate-ammonium, a broad spectrum herbicide. Herbicide-tolerant canola varieties were quickly adopted in Canada and the USA since their introduction in mid-1990. Approximately 95% of Canada's canola has been genetically modified for herbicide tolerance (CCC, 2016a). The benefits of high yielding herbicide-tolerant canola varieties are many.

The economic benefits to growers include:

- Increased yields – the hybrid system allows production of higher yielding canola varieties. This results in a more consistent supply for oilseed crushers, exporters and consumers.
- Better weed control – Glufosinate-ammonium provides broad spectrum weed control in the crop and also provides growers with an additional tool for their weed resistance management strategy.
- The harvested crop has less dockage (such as weed seed and chaff in the harvested seed) so farmers get higher prices for their canola.

Environmental benefits

The use of herbicide tolerant canola has reduced fuel use and tillage practices, resulting in soil conservation and related environmental benefits such as carbon sequestering. It is estimated that in Canada in 2013 the use of genetically modified canola resulted in a fuel saving of 69

million liters and reduced carbon dioxide emissions by 185 million kg (Brookes and Barfoot, 2015).

Farmers use less tillage and more direct-seeding in herbicide tolerant canola cropping systems. The reduction of tillage reduces soil erosion, contributes to less air pollution from dust, improves soil moisture retention, and reduces soil compaction.

Less herbicide is used in herbicide tolerant canola cropping systems. In 2013, the use of genetically modified herbicide tolerant canola resulted in a 2.1 million kg reduction in the amount of herbicide active ingredient use (-17.1%) (Brookes and Barfoot, 2015).

IX. STATEMENT OF GROUNDS UNFAVORABLE

Bayer CropScience LP knows of no study data and/or observations associated with male sterile, glufosinate-ammonium tolerant event MS11 *B. napus* that will result in adverse environmental consequences for its introduction. The Invigor Canola hybrid system, which contains the antecedent event MS8 *B. napus*, received nonregulated status in March 1999. Since the phenotype and mechanism of achieving the phenotype in MS11 *B. napus* are the same as those of the antecedent event, the assessment for MS8 informs the assessment of MS11.

The evidence and data provided in this petition supports the conclusion that event MS11 *B. napus* does not pose a plant pest risk.

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Appendix 1. Field Trial Data Reports

Field Test Report: Notification 14-087-103n: Location ID NB0088IDJeromeRS01

Field Trial Information for Site Planted

Study (Protocol) ID	Experiment Code	Experiment County	Experiment Location
14-087-103n	B0088IDJeromeRS01	Jerome	ID

Harvest/Termination

Unique planting ID for harvest or termination record -	Planting 01
How was this trial concluded -	Harvest-Saved Seed
Date of trial conclusion (dd-Mon-yyyy)	26-Sep-2014
What was the method of harvest or termination	Harvest-Mechanical
If other for method of harvest or termination, describe	Disking
How was Saved Seed or Grain handled after harvest -	Transported/shipped off site for analysis and destruction
If other for how Saved Seed or Grain was handled after harvest, describe	Seed was stored in [ ] GMO seed storage locker until it could be shipped.
Date (dd-Mon-yyyy) when Saved Seed or Grain was stored, shipped, or destroyed	29-Sep-2014
Amount of Saved Seed or Grain material stored, shipped, or destroyed	18.000
Amount of Saved Seed or Grain material stored, shipped, or destroyed units -	lb
Destroyed - method of Saved Seed or Grain destruction -	Unshipped seeds was Burned / incinerated
Destroyed - If other for method of Saved Seed or Grain destruction, describe	NA
Shipment - Saved Seed or Grain material sent to (Name and Company)	[ ]

Observation Methods for Unusual or Unexpected Effects

The field trial was inspected at approximately 10 to 15, 30, 60, 90, and 120 Days After Planting. Comparisons were made between plots with plants expressing constructs/events authorized under this Notification and plots null for the construct/event. Observations were specifically made for unusual or unexpected differences in insect pests, beneficial insects, plant disease, phenotype/morphology of the plants particularly as it relates to potential weediness of plants expressing the construct/event.

Resulting Data and Analysis for Unusual or Unexpected Effects

No unexpected or unusual differences in insect pests, beneficial insects, plant disease, or phenotype/morphology were observed in comparisons between plots with plants expressing the constructs/events authorized under this notification and plots null for the constructs/events.

Potential Compliance Incident (PCI)

1.	Submitted report for an accidental and/or unauthorized release	(Y/N)	N
2.	Submitted report for substantially different characteristics	(Y/N)	N
3.	Submitted report for any unusual occurrence	(Y/N)	N

Field Test Report: Notification 14-087-103n; Location ID B0088NDCassRS02

Field Trial Information for Site Planted

Study (Protocol) ID	Experiment Code	Experiment County	Experiment Location
14-087-103n	B0088NDCassRS02	Cass	ND

Harvest/Termination

Unique planting ID for harvest or termination record -	Planting 01
How was this trial concluded -	Harvest-Saved Grain
Date of trial conclusion (dd-Mon-yyyy)	26-Sep-2014
What was the method of harvest or termination	Harvest-Mechanical
If other for method of harvest or termination, describe	NA
How was Saved Seed or Grain handled after harvest -	Transported/shipped off site for analysis and destruction
If other for how Saved Seed or Grain was handled after harvest, describe	NA
Date (dd-Mon-yyyy) when Saved Seed or Grain was stored, shipped, or destroyed	17-Nov-2014
Amount of Saved Seed or Grain material stored, shipped, or destroyed	20.000
Amount of Saved Seed or Grain material stored, shipped, or destroyed units -	lb.
Destroyed - method of Saved Seed or Grain destruction -	Burned / incinerated
Destroyed - If other for method of Saved Seed or Grain destruction, describe	Remaining after shipping was burned
Shipment - Saved Seed or Grain material sent to (Name and Company)	[ ]

Observation Methods for Unusual or Unexpected Effects

The field trial was inspected at approximately 10 to 15, 30, 60, 90, and 120 Days After Planting. Comparisons were made between plots with plants expressing constructs/events authorized under this Notification and plots null for the construct/event. Observations were specifically made for unusual or unexpected differences in insect pests, beneficial insects, plant disease, phenotype/morphology of the plants particularly as it relates to potential weediness of plants expressing the construct/event.

Resulting Data and Analysis for Unusual or Unexpected Effects

No unexpected or unusual differences in insect pests, beneficial insects, plant disease, or phenotype/morphology were observed in comparisons between plots with plants expressing the constructs/events authorized under this notification and plots null for the constructs/events.

Potential Compliance Incident (PCI)

4.	Submitted report for an accidental and/or unauthorized release	(Y/N)	N
5.	Submitted report for substantially different characteristics	(Y/N)	N
6.	Submitted report for any unusual occurrence	(Y/N)	N

Field Test Report: Notification 14-087-103n; Location ID B0088NDGrandForksRS03

Field Trial Information for Site Planted

Study (Protocol) ID	Experiment Code	Experiment County	Experiment Location
14-087-103n	B0088NDGrandForksRS03	Grand Forks	ND

Harvest/Termination

Unique planting ID for harvest or termination	Planting 01
How was this trial concluded -	Harvest-Saved Grain
Date of trial conclusion (dd-Mon-yyyy)	20-Sep-2014
What was the method of harvest or termination -	Harvest-Mechanical
If other for method of harvest or termination,	NA
How was Saved Seed or Grain handled after harvest -	Transported/shipped off site for analysis and destruction
If other for how Saved Seed or Grain was handled after harvest, describe	NA
Date (dd-Mon-yyyy) when Saved Seed or Grain was stored, shipped, or destroyed	24-Sep-2014
Amount of Saved Seed or Grain material stored, shipped, or destroyed	10.000
Amount of Saved Seed or Grain material stored, shipped, or destroyed units -	Kg
Destroyed - method of Saved Seed or Grain	NA
Destroyed - If other for method of Saved Seed or Grain destruction, describe	NA
Shipment - Saved Seed or Grain material sent to (Name and Company)	BCS, 407 Davis Dr., Durham, NC 27560

Observation Methods for Unusual or Unexpected Effects

The field trial was inspected at approximately 10 to 15, 30, 60, 90, and 120 Days After Planting. Comparisons were made between plots with plants expressing constructs/events authorized under this Notification and plots null for the construct/event. Observations were specifically made for unusual or unexpected differences in insect pests, beneficial insects, plant disease, phenotype/morphology of the plants particularly as it relates to potential weediness of plants expressing the construct/event.

Resulting Data and Analysis for Unusual or Unexpected Effects

No unexpected or unusual differences in insect pests, beneficial insects, plant disease, or phenotype/morphology were observed in comparisons between plots with plants expressing

the constructs/events authorized under this notification and plots null for the constructs/events.

Potential Compliance Incident (PCI)

7. Submitted report for an accidental and/or unauthorized release	(Y/N)	N
8. Submitted report for substantially different characteristics	(Y/N)	N
9. Submitted report for any unusual occurrence	(Y/N)	N

Field Test Report: Notification 14-087-103n; Location ID B0088WAGrantRS04

Field Trial Information for Site Planted

Study (Protocol) ID	Experiment Code	Experiment County	Experiment Location
14-087-103n	B0088WAGrantRS04	Grant	WA

Harvest/Termination

Unique planting ID for harvest or termination record -	Planting 01
How was this trial concluded -	Harvest-Grain Seed
Date of trial conclusion (dd-Mon-yyyy)	19-Sep-2014
What was the method of harvest or termination -	Harvest-Mechanical
If other for method of harvest or termination, describe	NA
How was Saved Seed or Grain handled after harvest -	Transported/shipped off site for analysis and destruction
If other for how Saved Seed or Grain was handled after harvest, describe	NA
Date (dd-Mon-yyyy) when Saved Seed or Grain was stored, shipped, or destroyed	19-Sep-2014
Amount of Saved Seed or Grain material stored, shipped, or destroyed	12.000
Amount of Saved Seed or Grain material stored, shipped, or destroyed units -	kg
Destroyed - method of Saved Seed or Grain destruction -	Seed not shipped was destroyed via deep burial
Destroyed - If other for method of Saved Seed or Grain destruction, describe	NA
Shipment - Saved Seed or Grain material sent to (Name and Company)	[ ]

Observation Methods for Unusual or Unexpected Effects

The field trial was inspected at approximately 10 to 15, 30, 60, 90, and 120 Days After Planting. Comparisons were made between plots with plants expressing constructs/events authorized under this Notification and plots null for the construct/event. Observations were specifically made for unusual or unexpected differences in insect pests, beneficial insects, plant disease, phenotype/morphology of the plants particularly as it relates to potential weediness of plants expressing the construct/event.

Resulting Data and Analysis for Unusual or Unexpected Effects

No unexpected or unusual differences in insect pests, beneficial insects, plant disease, or phenotype/morphology were observed in comparisons between plots with plants expressing the constructs/events authorized under this notification and plots null for the constructs/events.

Potential Compliance Incident (PCI)

10. Submitted report for an accidental and/or unauthorized release	(Y/N)	N
11. Submitted report for substantially different characteristics	(Y/N)	N
12. Submitted report for any unusual occurrence	(Y/N)	N