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01-206-01p
Revised

Aventis CropScience

Aventis

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October 8, 2001

Robert I. Rose, Ph.D
Arthropod Biotechnologist
Permits and Risk Assessment, Unit 147
Plant Protection and Quarantine
United States Department of Agriculture
Animal and Plant Health Inspection Service
4700 River Road
Riverdale, MD 20737

Re: Application for and Extension of the Determination of Nonregulated Status for Glufosinate-Tolerant Canola Transformation (98-278-01p): MS1/RF1/RF2 (01-206-01p)

Dear Dr. Rose:

Enclosed please find 2 originals and 4 photocopies of the above referenced petition extension. This revised extension addresses the deficiencies listed in your September 18, 2001, letter. Specifically, where available, better quality gels have been provided. Also included throughout the applicable sections, are better comparisons of the MS1/RF1/RF2 events with the antecedent organism MS8/RF3 (petition 98-278-01p).

The basic presentation issues, e.g., page numbering, have also been addressed.

If you have any questions, please feel free to contact me at 919-549-2748 or Susan MacIntosh at 919-549-2780, 919-549-3929 (fax) or susan.macintosh@aventis.com.

Respectfully,

Luann Powell

Luann Powell
Registration Manager – Biotechnology

Enclosures (Contains Confidential Business Information)

Cc: Susan MacIntosh, Manager, Regulatory Affairs – Biotechnology
Rob MacDonald, Global Product Safety Management

10/9/01

01-206-01p
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Application for an Extension of the Determination of Nonregulated Status for Glufosinate-Tolerant Canola Transformation (98-278-01p):

MS1/RF1/RF2

The undersigned submits this petition under 7 CFR 340.6 to request that the Director, Scientific Services, makes a determination that the article should not be regulated under 7 CFR 340.

Submitted by:

Luann Powell
Registration Manager
Regulatory Affairs – Biotechnology

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Contributors:

Rob MacDonald

October 8, 2001

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10/9/01

Summary

Male sterile canola with glufosinate herbicide tolerance transformation event MS1 with fertility restorers RF1 and RF2, received an opinion of "No Concern" from the Federal Drug Administration April 4, 1996, for food and feed uses (Attachment I). On March 22, 1996, Plant Genetics Systems, (now Aventis CropScience) received an opinion letter from the United States Department of Agriculture indicating that APHIS will not consider transformation event MS1 with fertility restorer, RF1 and RF2, and any hybrids or varieties developed from these events moved from Canada to the United States for processing as regulated articles (Attachment II). MS1/RF1 and RF2 approvals from the Canadian Food Inspection Agency for unconfined release into the environment and use as livestock feed were received in April 1995 and December 1995, respectively (Attachment III). Food safety approvals for MS1/RF1 and RF2 in September 1994, and August 1995, respectively, from Health Canada (Attachment IV).

Prior to commercialization in Canada, MS1/RF1 and RF2 were extensively field tested by Aventis Crop Science Canada Inc., formerly Plant Genetic Systems Canada and Plant Genetic Systems NV. Extensive multi-location field trials have been conducted in Canada, USA and Europe. Data collected from these trials, laboratory analyses, reports and literature references presented herein demonstrate that male sterile canola with glufosinate herbicide tolerance event MS1 with fertility restorers RF1 and RF2: 1) exhibits no plant pathogenic properties; 2) is no more likely to become a weed than non-modified canola; 3) is unlikely to increase the weediness potential of any other cultivated plant or native wild species; 4) does not cause damage to processed agricultural commodities; and 5) is unlikely to harm other organisms that are beneficial to agriculture.

Glufosinate tolerant hybrid lines developed from these events have been marketed commercially in Canada since 1996. Since 1996 greater that 3 million acres of canola derived from these events has been cultivated in Western Canada. Aventis continues to conduct field trials as part of Aventis' monitoring and stewardship program. The results of post commercialization monitoring of volunteers confirm the conclusions of the risk assessment that essentially the presence of these traits has not lead to any increase in the weediness of volunteers.

Aventis CropScience requests a determination from APHIS that male sterile canola with glufosinate herbicide tolerance transformation event MS1 with fertility restorers RF1 and RF2 and any progeny derived from crosses of this event with tradition canola varieties, and any progeny derived from crosses of this event with transgenic canola 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 the 98-278-01p petition entitled, "Petition for the Determination of Nonregulated Status: InVigor® Hybrid Canola Transformation Events MS8/RF3." The events MS1/RF1 and RF2 were produced using the same transformation technology and contain the same genetic elements as the events MS8/RF3 with the notable exception that the former contain the antibiotic resistance marker gene *nptII* which was utilized solely for selection of transformants in tissue culture. The events MS1/RF1 and RF2 as well as MS8/RF3 originated from the same parent line, Drakkar, a common variety in the canola growing regions of western Canada and Europe. Also, as was seen for the previous deregulated events MS8/RF3, there were no

morphological, beneficial organism, disease or pest differences between the events MS1/RF1/RF2 and the previously considered event MS8/RF3.

Certification

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.

Luann Powell
Registration Manager
Regulatory Affairs – Biotechnology

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ACRONYMS, SYNONMYMS AND SCIENTIFIC TERMS

APHIS - Animal and Plant Health Inspection Service

B0154 – S3 generation of RF2 (B94-2)

B93-101 – RF1

B93-n – refers to different fertility restorer (RF1) transformation events

B91-4 – MS1

B91-n – refers to different male sterile (MS1) transformation events

B94-n – refers to different fertility restorer (RF2) transformation events

B94-2 – RF2

bar - *phosphinothricin acetyltransferase* gene (origin *Streptomyces hygroscopicus*)

barnase – *barnase* gene (origin *Bacillus amyloliquefaciens*)

barstar – *barstar* gene (origin *Bacillus amyloliquefaciens*)

BC - Backcross

CFIA – Canadian Food Inspection Agency

ELISA – enzyme linked immunosorbent assay

FDA – Federal Drug Administration

GA – glufosinate-ammonium

GTC – glufosinate tolerant canola

LibertyLink – Aventis trade name given to those events that are glufosinate-ammonium (Liberty) tolerant

neo - *Neomycin phosphotransferease* II (gene) (also called the *nptII* gene)

MS – Male Sterile (also referred to as Nuclear Male Sterile)

nptII - *Neomycin phosphotransferase II* (gene)

NPTII - Neomycin phosphotransferease II (protein)

NMS – Nuclear Male Sterile

P1132 – S1 generation of RF2 (B94-2)

PAT - phosphinothricin acetyltransferase (protein)

RF – Fertility Restorer

SeedLink – Aventis trade name given to those events that have the male sterility/fertility restorer traits

T-DNA - transferred DNA

Ti-DNA - tumor inducing DNA

Ti-plasmid - tumor inducing plasmid

USDA - United States Department of Agriculture

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Statement of Grounds for Nonregulated Status

I. Rationale for Submission of Request for Extension

There are no changes in the rationale from the previously approved petition number 98-278-01p, entitled "Petition for Determination of Nonregulated Status: InVigor® Hybrid Canola Transformation Events MS8/RF3." The specific differences between MS1/RF1/RF2 and its progeny and the event in the previous petition are discussed in the appropriate sections. The events MS1/RF1 and RF2 were produced using the same transformation technology and contain the same genetic elements as the events MS8/RF3 with the notable exception that the former contain the antibiotic resistance marker gene *nptII* which was utilized solely for selection of transformants in tissue culture. The events MS1/RF1 and RF2 as well as MS8/RF3 originated from the same parent line, Drakkar, a common variety in the canola growing regions of western Canada and Europe. Also, as was seen for the previous deregulated events MS8/RF3, there were no morphological, beneficial organism, disease or pest differences between the events MS1/RF1/RF2 and the previously considered event MS8/RF3. There is no reason to think cultivation of events MS1/RF1 and RF2 and it's progeny will have environmental effects different that the cultivation of events MS8/RF3 which have already been considered by APHIS. The new events to be considered under this extension are MS1/RF1/RF2.

Table 1. Identity and Function of DNA Elements for Canola Events MS8/RF3 and MS1/RF1/RF2

Characteristic	**Events MS8/RF3**	**Events MS1/RF1 and RF2**
Crop	Canola	Canola
Cultivar Species name	*Brassica napus* L.	*Brassica napus* L.
Parent Line	Drakkar	Drakkar
Transformation Method	*Agrobaterium tumefaciens* mediated transformation	*Agrobaterium tumefaciens* mediated transformation
Vectors	PTHW107 PTHW118	PTTM8RE PTVE74RE
Trait	Male Sterility; Fertility Restorer; Tolerance to glufosinate ammonium	Male Sterility; Fertility Restorer; Tolerance to glufosinate ammonium
	Vector pTHW107	**Vector pTTM8RE**
Gene 1/Donor	*Phosphinothricin acetyltransferase* (*bar*) gene/*Streptomyces hygroscopicus*	*Phosphinothricin acetyltransferase* (*bar*) gene/*Streptomyces hygroscopicus*
Gene 1 Promoter/Donor	PSsuAra/*Arabidopsis thaliana*	PSsuAra/*Arabidopsis thaliana*
Gene 1 Terminator/Donor	3' untranslated end of the TL-DNA gene/ *Agrobacterium tumefaciens*	3' untranslated end of the TL-DNA gene/ *Agrobacterium tumefaciens*
Gene 2/Donor	*Barnase* gene/*Bacillus*	*Barnase* gene/*Bacillus*

	amyloliquefaciens	*amyloliquefaciens*
Gene 2 Promoter/Donor	Anther-specific region of *TA29* gene/*Nicotiana tabacum*	Anther-specific region of *TA29* gene/*Nicotiana tabacum*
Gene 2 Terminator/Donor	*Nopaline synthase* gene/*Agrobacterium tumefaciens*	*Nopaline synthase* gene/*Agrobacterium tumefaciens*
Gene 3/Donor	N/A	*Neomycin phosphotransferase II* (*nptII*) gene/*Escherichia coli*
Gene 3 Promoter/Donor	N/A	*Nopaline synthase* gene/ *Agrobacterium tumefaciens*
Gene 3 Terminator/Donor	N/A	*Octopine synthase* gene/ *Escherichia coli*
	Vector pTHW118	**VectorpTVE74RE**
Gene 1/Donor	*Phosphinothricin acetyltransferase* (*bar*) gene/*Streptomyces hygroscopicus*	*Phosphinothricin acetyltransferase* (*bar*) gene/*Streptomyces hygroscopicus*
Gene 1 Promoter/Donor	PSsuAra/*Arabidopsis thaliana*	PSsuAra/*Arabidopsis thaliana*
Gene 1Terminator/Donor	*3' untranslated end of the TL-DNA* gene/*Agrobacterium tumefaciens*	*3' untranslated end of the TL-DNA* gene/*Agrobacterium tumefaciens*
Gene 2/Donor	*Barstar* gene/*Bacillus amyloliquefaciens*	*Barstar* gene/*Bacillus amyloliquefaciens*
Gene 2 Promoter/Donor	Anther-specific region of *TA29* gene/*Nicotiana tabacum*	Anther-specific region of *TA29* gene/*Nicotiana tabacum*
Gene 2 Terminator/Donor	*Nopaline synthase* gene/*Agrobacterium tumefaciens*	*Nopaline synthase* gene/*Agrobacterium tumefaciens*
Gene 3/Donor	N/A	*Neomycin phosphotransferase II* (*nptII*) gene *Escherichia coli*
Gene 3 Promoter/Donor	N/A	*Nopaline synthase* gene/ *Agrobacterium tumefaciens*
Gene 3 Terminator/Donor	N/A	*Octopine synthase* gene/ *Escherichia coli*

II. The Canola Family

There are no changes from the previously approved petition submission.

III. The Transformation System, Plasmid and Parent Line Used

Brassica napus was transformed using *Agrobaterium tumefaciens* mediated transformation to create the events MS1/RF1 and RF2. This is the same transformation technology utilized for the deregulated events MS8/RF3, (98-278-01p). Cointegrated disarmed plasmids were constructed to transfer the DNA sequences of interest to *B. napus*. [

] After transfer into the appropriate *Agrobaterium* strain, the intermediate vector will cointegrate into the acceptor Ti-plasmid, giving rise to a simple T-DNA configuration without duplications between border sequences (Deblaere *et al.*, 1985). Upon cointegration, the resulting T-DNA is large and contains duplications between border sequences. Based on the procedure described by Deblaere *et al* (1985), cointegrative diarmed plasmids pTTM8RE and pTVE74RE were used to engineer male sterility and restoration of fertility, respectfully. These plasmids contain the same genetic elements at the deregulated event MS8/RF3 (98-278-01p) with the exception of the selection marker gene, *nptII*, present in MS1/RF1 and RF2.

The *Brassica napus* line used for transformation was the winter oilseed rape line Drakkar, the same parent line used for the transformation event MS8/R3, which is no longer regulated by USDA APHIS (petition 98-278-01p).

Regulatory Sequences for Plasmids pTTM8RE and pTVE74RE

PNos-*neo*-3'ocs

This sequence contains the weak constitutive *nopaline synthase* promoter (nos) from *Agrobacterium tumefaciens*. The nos promoter regulates the expression of the *neo* gene isolated from the bacterium *Escherichia coli*. The *neo* gene (also referred to as the *nptII* gene) codes for the neomycin phosphotransferase II (NPTII) enzyme and was used as a selectable marker to allow for the selection of transgenic cells in the early in vitro stage carrying the inserted DNA segments. It confers resistance to kanamycin. The terminator at the end of the sequence is the 3'end
of the *octopine synthase* gene of *Agrobacterium tumefaciens* (Beck *et al.*, 1982; De Greve *et al.*, 1983; Harpster *et al.*, 1988; Mitra *et al.*, *1989*).

PSsu-Ara-tp-*bar*-3'g7

This sequence contains the promoter SsuAra and a transit peptide signal (tp) for the translocation of the expressed protein to the chloroplast. The SsuAra promoter was isolated from *Arabidopsis thaliana*. The SsuAra promoter regulates the expression of the *bar* gene, isolated from *Streptomyces hygroscopicus*. The *bar* gene codes for the enzyme phosphinothricin acetyltransferase (PAT). It confers resistance to the herbicide glufosinate-ammonium. Polyadenylation signals are provided by the 3'end of the T-DNA gene 7 of *Agrobacterium tumefaciens* (Van den Broeck *et al.*, 1985; Thompson *et al.*, 1987; Krebbers *et al.*, 1988; De Almeida *et al.*, 1989)

PTA29-*barnase*-3'nos

The promoter TA29 of *Nicotiana tabacum* regulates the expression of the *barnase* gene isolated from the bacterium *Bacillus amyloliquefaciens*. The TA29 promoter allows to limit the activity of the *barnase* gene in tissues (the tapetum cells of the pollen sac) as well as in time (only when flowering during anther development). The sequence also contains the 3'end of the *nopaline synthase* gene of *Agrobacterium tumefaciens* (Depicker *et al.*, 1982; Hartley 1988; Kaul 1988; Koltunow *et al.*, 1990; Seurinck *et al.*, 1990; Be Block *et al.*, 1992; Goldberg *et al.*, 1993).

PTA29-*barstar*-3'nos

The promoter TA29 of *Nicotiana tabacum* regulates the expression of the *barstar* gene isolated from the bacterium *Bacillus amyloliquefaciens*. This sequence also contains the 3'end of the *nopaline-synthase* gene of *Agrobacterium tumefaciens* (Hartley 1988; Koltunow *et al.*, 1990; Seurinck *et al.*, 1990).

The maps of vectors pTTM8RE and pTVE74RE are shown in Figures 1 and 2, respectfully. Descriptions of the genetic elements for plasmids pTTM8RE and pTVE74RE are shown in Tables 2 and 3, respectively.

Figure 1. Vector map of plasmid pTTM8RE

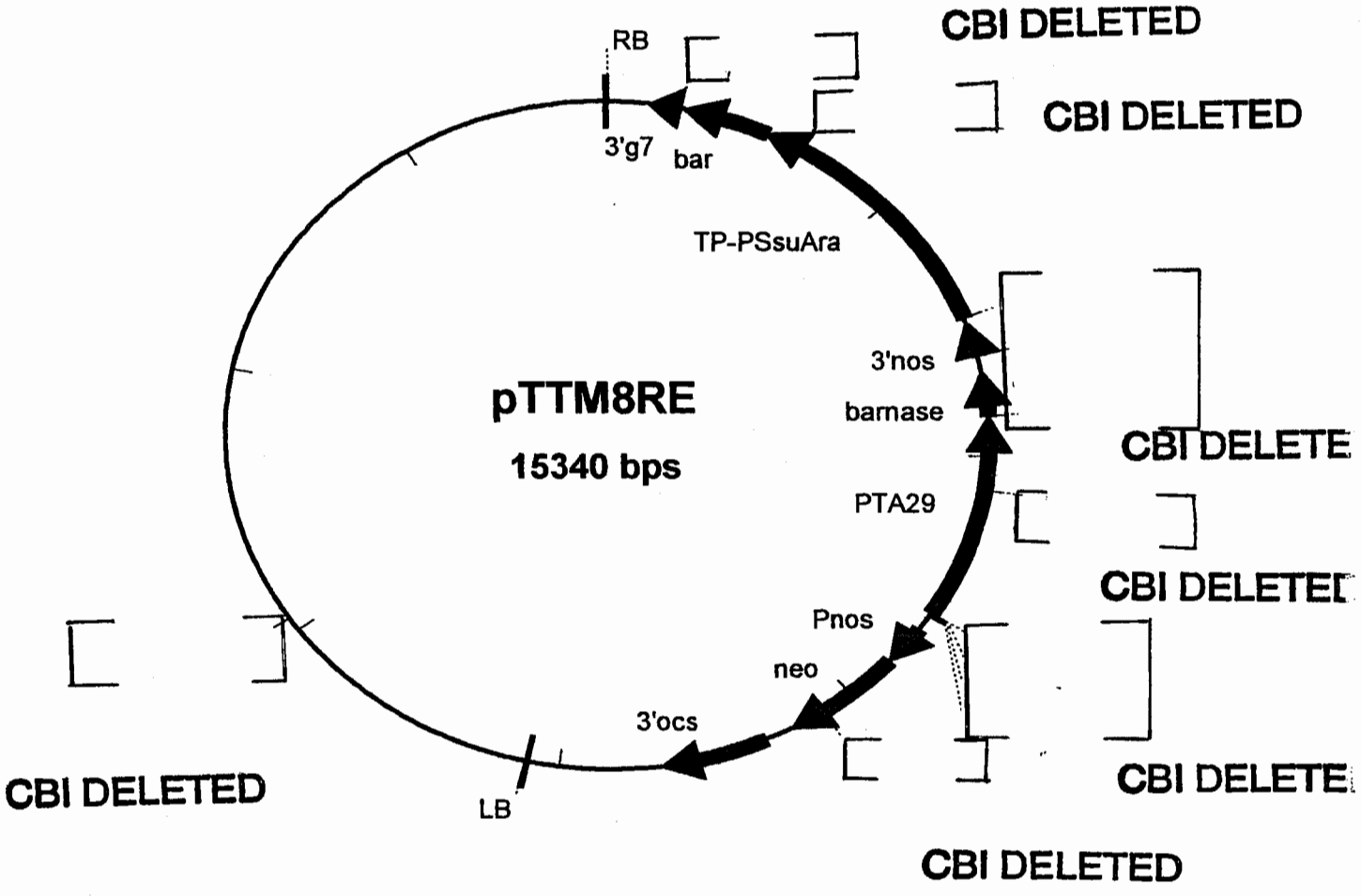

Table 2. Genetic Elements of the plasmid pTTM8RE

Genetic Element	Size (Kb)	Function and Source
RB	0.28	Right border repeat from the TL-DNA from pTiB6S3 and residual sequence.
g7 3'	0.2	Plant polyadenylation signals and obtained from the 3'untranslated end from the TL-DNA gene 7 (3'g7) of pTiB6S3 (Velten and Schell., 1985; Dhaese *et al.*, 1983).
bar	0.5	The coding sequence of the bialaphos resistance gene (*bar*) of *Streptomyces hygroscopicus* (Thompson *et al.*, 1987).
PssuAra	2.0	The promoter from the atS1A ribulose-1,5-biphosphate carboxylase small subunit gene from *Arabidopsis thaliana* (PssuAra). The promoter fragment comprises the 1.7 kb fragment upstream from the atS1A ATG codon (Krebbers *et al.*, 1988) and the transit peptide (tp) sequence (1240-1076) for targeting to the chloroplast.
NOS 3'	0.25	Nopaline synthase gene (3'nos) from the T-DNA of pTiT37 and containing plant polyadenylation signals (Depicker *et al.*, 1982).
Barnase	0.34	Region encoding mature *barnase* from *Bacillus amyloliquefaciens*.
TA29	1.5	The promoter region of the anther-specific gene TA29 from *Nicotiana tabacum*. The PTA29 promoter comprises the 1.5 kb of the sequence upstream from the ATG initiation codon (Seurinck *et al.*, 1990).
NOS	0.4	The promoter from the *nopaline synthase* gene from the T-DNA of pTiT37 of *Agrobacterium tumefaciens* (PNos); the nucleotide sequence of the PNos promoter is described by Depicker *et al.*(1982).
neo	1.0	The coding sequence from the *neo* gene encoding neomycin phosphotransferase II. This sequence corresponds with the sequence from *Tn*5 of *Escherichia coli* as described by Beck *et al.* (1982). The ATG initiation codon of the *neo* coding region has been substituted for a linker sequence as described by Reiss *et al.* (1984). There are 171 bp of the *Tn*5 sequence downstream from the *neo* coding region present (Beck *et al.*, 1982).
OCS 3'	0.9	The 3' untranslated end from the *octopine synthase* gene (3'ocs). This corresponds with a 706 bp PvuII fragment from the *octopine synthase* gene (Gielen *et al.*, 1984). The PvuII fragment has been cloned in the SmaI site of the *Tn*5 sequence.
LB	0.7	Left border repeat from the TL-DNA from pTiB6S3 and residual sequence.

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Figure 2. Vector map of the plasmid pTVE74RE

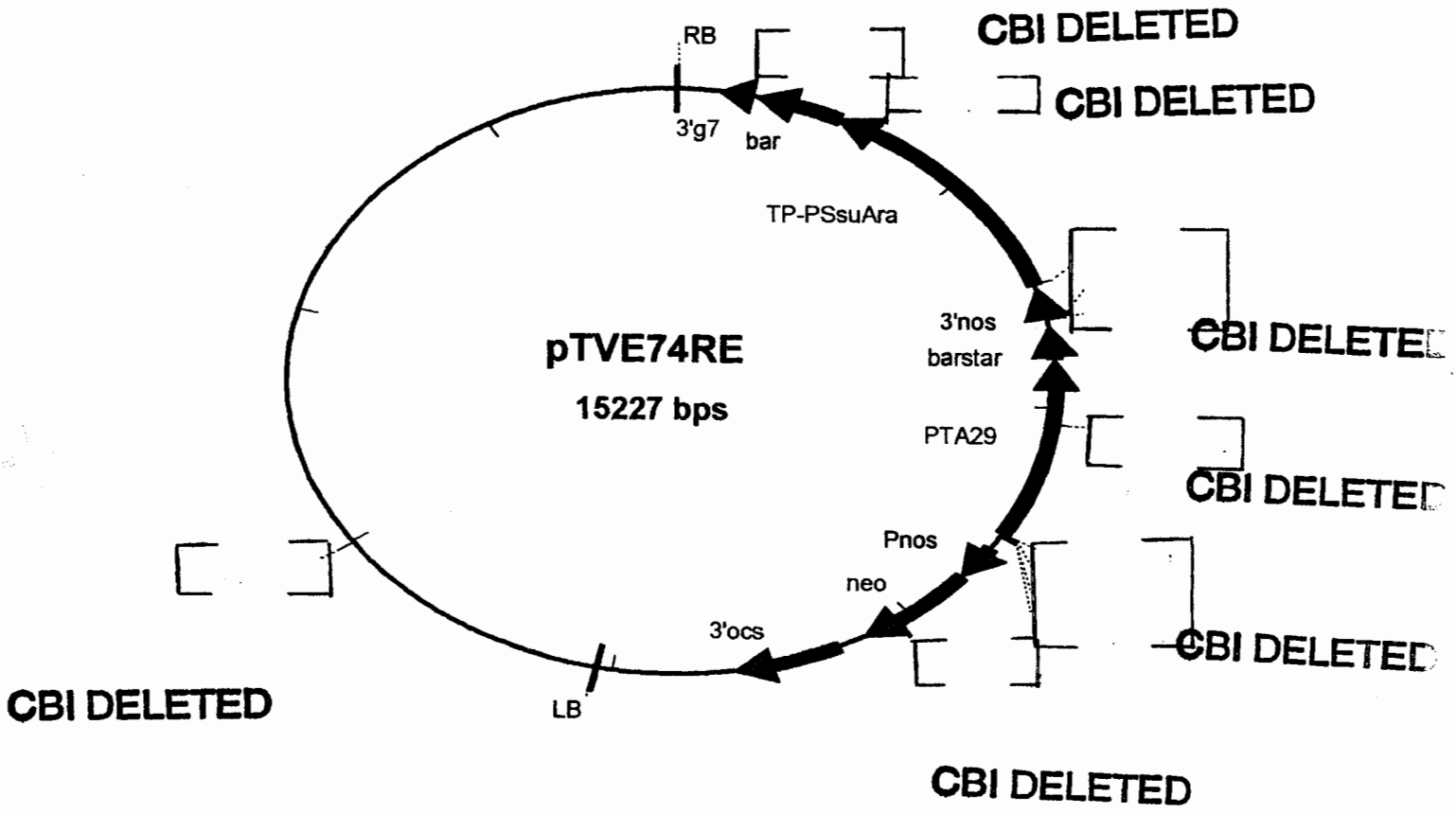

Table 3. Genetic Elements of the plasmid pTVE74RE

Genetic Element	Size (Kb)	Function and Source
RB	0.28	Right border repeat from the TL-DNA from pTiB6S3 and residual sequence.
g7 3'	0.2	Plant polyadenylation signals and obtained from the 3' untranslated end from the TL-DNA gene 7 (3'g7) from pTiB6S3 (Velten and Schell., 1985; Dhaese *et al.*, 1983).
bar	0.5	The coding sequence of the bialaphos resistance gene (*bar*) of *Streptomyces hygroscopicus* (Thompson *et al.*, 1987).
PssuAra	2.0	The promoter from atS1A ribulose-1,5-biphosphate carboxylase small subunit gene from *Arabidopsis thaliana* (PssuAra). The promoter fragment comprises the 1.7 kb fragment upstream from the atS1A ATG codon (Krebbers *et al.*, 1988) (PSsuAra) and the transit peptide (tp) sequence for targeting to the chloroplast.
NOS 3'	0.25	Nopaline synthase gene (3'nos) from the T-DNA of pTiT37 (Depicker *et al.* 1982).
Barstar	0.34	The region of the *barstar* gene of *Bacillus amyloliquefaciens*
PTA29	1.5	The promoter region of the anther specific gene TA29 from *Nicotiana tabacum* (PTA29). The promoter comprises the 1.5 kb of the sequence upstream from the ATG initiation codon (Seurinck *et al.*, 1990).
NOS	0.4	The promoter from the *nopaline synthase* gene of the T-DNA of pTiT37 from *Agrobacterium tumefaciens* (PNos); the nucleotide sequence of the PNos promoter is described by Depicker *et al.*(1982).
neo	1.0	The coding sequence from the *neo* gene encoding neomycin phosphotransferase II. This sequence corresponds with the sequence from *Tn*5 from *Escherichia coli* as described by Beck *et al.* (1982). The ATG initiation codon of the *neo* coding region has been substituted for a linker sequence as described by Reiss *et al.* (1984). There are 171 bp of the *Tn*5 sequence downstream from the *neo* coding region present (Beck *et al.*; 1982.
OCS 3'	0.9	The 3' untranslated end from the *octopine synthase* gene (3'ocs). This corresponds with a 706 bp PvuII fragment from *the octopine synthase* gene (Gielen *et al.*, 1984). The PvuII fragment has been cloned in the SmaI site of the *Tn*5 sequence (Beck et al., 1982) The construction of the chimeric PNos-*neo*-3'ocs gene has been described by Hain *et al.* (1985).

LB	0.7	Left border repeat from the TL-DNA from pTiB6S3 and residual sequence.

In summary, the events MS1/RF1 and RF2 contain the same genetic elements as the nonregulated events MS8/RF3 (98-278-01p) with the exception of the antibiotic resistance marker gene, *nptII*, present in the MS1/RF1 and RF2 events that was used solely as a transformant selection tool.

IV. Genetic Characterization of Event MS1/RF1 and RF2

A. Description, History and Mendelian Inheritance

There have been extensive field tests conducted with MS1, RF1, and RF2 prior to the commercial release of the first hybrid lines. Field studies date back to 1991 and have been conducted in Canada, USA, Europe and South America. Table 4 presented a summary of the field trials and associated authorization permits for field trials conducted in 1991, 1992 and 1993. The objective of these trials was in part to evaluate the material in highly diverse a range of environments over multiple years to fully assess the stability and performance of the introduced traits. The results of the field trial experience confirm the stability and performance of the introduced traits. This field trial experience has further been confirmed by the commercial experience of cultivating hybrid lines derived from these events in Canada since 1996.

Table 4. Summary of Field Trials and Authorizations

Company Code	Country	Authorization Code	Goal
3. Field			
3.1. 1991			
FBN9102-1	Canada	91-UOG-CAN-01	Agronomic evaluation of a hybrid system introduced via genetic engineering in spring oilseed rape.
FBN9102-2	Canada	91-UOG-CAN-02	Evaluation of the backcross program of male sterile and restorer lines.
FBN9105	Sweden	33 54/91	Field evaluation of the NMS lines of spring oilseed rape.
FBN9107	France	91/01/03	Evaluation au champ d'un hybride et d'une lignée restauratrice de fertilité de colza obtenus par génie génétique.
FBN9109	Belgium	BIOT/91/M04	Agronomic evaluation of an unrestored hybrid and its parents (preregistration trial).
FBN9110	Belgium	BIOT/91/M04	Evaluation of the backcross program of male sterile and restorer lines.
FBN9111	Belgium	BIOT/91/M04	Evaluation of the effect of the hybridization level on yield and competitive performance and determination of the different characteristics between the hybrid and its parents in a mixed stand.
FBN9112	Belgium	BIOT/91/M04	Comparison of NMS lines in spring oilseed rape.
FBN9113	Belgium	BIOT/91/M04	Seed production of oilseed rape hybrids based on the NMS system.
FBN9114	Belgium	BIOT/91/M13	Seed production of oilseed rape hybrids based on the NMS system.

Continuing Table 4.

Company code	Country	Authorization code	Goal
3.2. 1992			
FBN9201/3201	Belgium	BIOT/92/M03	A hybrid system in spring oilseed rape introduced via genetic engineering.
FBN9201/3301	France	91/12/01	Etude de la faisabilité du système hybride en colza de printemps.
FBN9201/3302	France	91/12/01	Etude de la faisabilité du système hybride en colza de printemps.
FBN9201/4401	U.Kingdom	PR 18/4	Feasibility study of a hybrid system in spring oilseed rape.
FBN9201/4601	Sweden	22 2268/91	Feasibility study of a hybrid system in spring oilseed rape.
FBN9201/0001	USA	92-017-01	Feasibility study of a hybrid system in spring oilseed rape.
FBN9201/01xx	Canada	T92-PGS-CAN-01..06	Feasibility study of a hybrid system in Canola.
FBN9202/3201	Belgium	BIOT/92/M04	Evaluation of a Canola production scheme based on a hybrid system.
FBN9202/0105	Canada	T92-PGS-CAN-10	Evaluation of a Canola production scheme based on a hybrid system.
FBN9203/3201	Belgium	BIOT/92/M05	Evaluation of backcross program of male sterile and restorer lines.
FBN9203/0101	Canada	T92-PGS-CAN-12	Evaluation of the backcross program for the hybrid system in Canola.

Continuing Table 4.

Company code	Country	Authorization code	Goal
FBN9213/3201	Belgium	BIOT/92/M07	Evaluation of new combinations in male sterile and restorer lines in Canola
FBN9213/0101	Canada	T92-PGS-CAN-16	Evaluation of new combinations in male sterile and restorer lines in Canola
FBN9216/3201	Belgium	BIOT/92/M08	Evaluation of the herbicide tolerance level on plant growth, development and fertility.
FBN9221/3201	Belgium	BIOT/92/M10	Evaluation of Canola hybrids based on a hybrid system obtained via genetic engineering.
FBN9221/0101	Canada	T92-PGS-CAN-18	Evaluation of Canola hybrids based on a hybrid system obtained via genetic engineering
FBN9241/3205	Belgium	BIOT/92/M21	Hybrid system safety assessment.
FBN9251/3206	Belgium	BIOT/92/M12	Seed production of a male sterile and restorer line.
FBN92W/Chile	Chile	Notified to SAG	Production of experimental hybrids
FBN92W41/3202	Belgium	BIOT/92/M27	Safety assessment for the introduction of male sterile winter oilseed rape.

Continuing Table 4.

3.3. 1993			
FBN9301/3202	Belgium	BIOT/93/M03	Evaluation of the stability of nuclear male sterile and restorer spring oilseed rape lines
FBN9301/4401	U.Kingdom	PR18/5	Evaluation of the stability of the nuclear male sterile and restorer spring oilseed rape lines
FBN9301/4601	Sweden	22 4390/92	Evaluation of the stability of the nuclear male sterile and restorer spring oilseed rape lines
FBN9301/3501	Spain	B/E/93/1	Evaluation of the stability of the nuclear male sterile and restorer spring oilseed rape lines
FBN9301/3302	France	92/12/02	Etude de la stabilité d'un système hybride transgénique en colza de printemps
FBN9301/0001	USA	93-049-02	Evaluation of the stability of the nuclear male sterile and restorer spring oilseed rape lines
FBN9301/0101-7	Canada	93-PGS-CAN-01..04	Evaluation of the stability of the nuclear male sterile and restorer spring oilseed rape lines
FBN9316/3302	Belgium	BIOT/93/M03	Evaluation of Basta tolerance of nuclear male sterile and restorer spring oilseed rape lines
FBN9316/0101	Canada	93-PGS-CAN-01..04	Evaluation of Basta tolerance of nuclear male sterile and restorer spring oilseed rape lines
FBN9361/3202	Belgium	BIOT/93/M03	Ecological evaluation of competitiveness of transgenic oilseed rape modified to obtain a hybrid system
FBN9361/4501	Denmark	D 2823-0003	Ecological evaluation of competitiveness of transgenic oilseed rape modified to obtain a hybrid system
FBN9341/3202	Belgium	BIOT/93/M08	BRIDGE safety assessment program : pollen dispersal from a herbicide resistant spring oilseed rape
FBN9302/3202	Belgium	BIOT/93/M03	Basta tolerance used as a selectable marker in production schemes
FBN9302/0101	Canada	93-PGS-CAN-01..04	Basta tolerance used as a selectable marker in production schemes
FBN9305/3202	Belgium	BIOT/93/M03	Phosphinothricin in different formulations in production schemes

MS1

The first greenhouse evaluation of the T_1 progeny of the original male sterile transformant was conducted to observe the inheritance of the transgenic DNA and to verify the *bar* and *barnase* gene expression. Two weeks after sowing, the seedlings were sprayed with Basta® and tolerant plants were transplanted and grown to maturity. A 1:1 segregation of PAT sensitive and tolerant plants was obtained. The *bar* and *barnase* locus has been inherited in the Mendelian fashion, fitting the prediction of a single locus. Seeds from surviving plants from the event were advanced to accumulate additional information. T_2 seeds were sown and geminated seedlings were sprayed with different concentrations of Basta®. Again T_2 plants segregated 1:1 according to the Mendelian fashion.

Evaluation of the Mendelian inheritance of the *barnase* gene in T_2 MS1 progeny was evaluated. Seeds were grown in the greenhouse and three-week-old seedlings were sprayed with Basta at 7.5 L/ha. Tolerant plants were identified, transplanted and grown to maturity. The MS1 plants were pollinated with pollen from a nontransgenic canola line. For that purpose, special oilseed rape cages were constructed in which small bumblebee colonies were placed. Table 5 shows the segregation of the T_2 progeny.

Table 5. Basta Segregation of MS1 T_2 Progeny

Plant material	Total number of seedlings	Number of seedlings that survived Basta	Chi² (1/2 sensitive)
T_2 MS1	99	61	Marginally significant

All Basta tolerant oilseed rape plants appeared to be male sterile, confirming the linkage between the *bar* and *barnase* gene in the plant genome.

Sixty T_3 generation seeds along with a nontransformed control were sown in the greenhouse to further evaluate segregation. Three-week-old seedlings were sprayed with Basta (7.5 L/ha). Tolerant plants were identified, transplanted and grown to maturity. Bumblebees were used to pollinate the male sterile plants along with the nontransformed control plants. Table 6 shows the segregation of the T_3 progeny.

Table 6. Basta Segregation of the MS1 T_3 Progeny

Plant material	Total number of seedlings	Number of seedlings that survived Basta	Chi² (1/2 sensitive)
T_3 MS1	24	12	NS
	24	11	NS
	12	8	NS
Total	60	31	NS

NS – Not significant

All Basta tolerant oilseed rape plants appear to be male sterile indicating the linkage between the *bar* and *barnase* gene in the plant genome. Bumblebees intensively foraged on the transgenic (MS1) and nontransformed control oilseed rape plants. No preference in bumblebee foraging was observed and this was confirmed by good seed production for both the MS1 and nontransformed oilseed rape plants.

RF1

A number of S_1 seeds were used as starting plant material for the search for a homozygous fertility restorer line. S_1 plants were sown and sprayed with Basta (Table 7).

Table 7. Basta (8 L/ha) Segregation Data (first selfings of RF1)

Plant Material	Total number of seedlings	Number of seedlings that survived Basta	Chi^2 (1/4 sensitive)
S_1 (RF1)	292	213	Not significant

A number of Basta tolerant plants were isolated and allowed to self-pollinate. S_2 seed of RF1 was sown and sprayed. Homozygous S_1 plants were then identified (Table 8).

Table 8. Basta (8 L/ha) Segregation Data of the Progeny of Selected RF1 Plants

S_1 Plant material	Total Number of S_1 seedlings	Number of S_2 seedlings that survived Basta	Comments
Seedlot 1			
Plant 1	19	11	
Plant 30	10	8	
Plant 24	10	7	
Plant 26	14	11	
Plant 21	16	10	
Plant 22	7	6	
Plant 19	20	20	Homozygous
Plant 31	17	10	
Plant 18	18	15	
Plant 20	18	18	Homozygous
Plant 23	14	10	
Plant 8	16	16	Homozygous
Plant 11	15	11	
Plant 2	17	17	Homozygous
Plant 4	18	18	Homozygous

Seedlot 2			
Plant 37	19	15	
Plant 36	18	15	
Plant 40	19	19	Homozygous
Plant 39	20	20	Homozygous
Plant 38	20	20	Homozygous
Plant 45	20	17	
Plant 43	18	14	
Plant 41	19	16	
Plant 46	20	14	
Plant 35	15	10	
Plant 34	16	10	
Plant 44	14	10	
Plant 20	11	6	
Plant 17	15	11	
Plant 13	17	13	
Plant 15	18	18	Homozygous
Plant 9	18	12	
Plant 18	7	6	
Plant 10	16	15	
Plant 11	19	12	
Plant 14	16	13	
Plant 3	17	11	
Plant 6	20	19	
Plant 5	13	10	
Control	18	0	

Selected seedlots of the homozygous restorer plants were further used to for the restoration of MS1 plants.

MS1/RF1

A primary evaluation of the F_1 progeny of the original male transformant (MS1) and the fertility restorer (RF1) was carried out in the greenhouse to evaluate the efficacy of the TA29-*barstar* constructs for fertility restoration of plants containing the TA29-*barnase* gene. MS1 flowers of T_0 transformants containing the *barnase* gene were crossed with T_0 RF1 transformants. The F_1 progenies of these crosses were grown in the greenhouse. Table 9 shows the segregation data for Basta tolerance.

Table 9. Basta (8 L/ha) Segregation Data for T_0 MS1 x T_0 RF1 Plants

Plant material	**Total number of seedlings**	**Number of seedlings that survived Basta (8 L/ha)**	**Chi^2**
T_0 MS1 x T_0 RF1	76	61	NS

NS – Not significant

The segregation ratio suggests that TA29-*barstar* plants can induce restoration of fertility of TA29-*barnase* plants.

MS1/ RF2

T_0 male sterile transformant (MS1) plants were crossed with T_0 fertility restorer transformant (RF2) plants in the greenhouse evaluate the efficacy of the TA29-*barstar* construct for fertility restoration on plants containing the TA29-*barnase* gene. The F_1seeds were collected, sown the following fall, and sprayed with Basta (8 L/ha). Segregation data for Basta tolerance is shown in Table 10 and phenotypic evaluation is shown in Table 11.

Table 10. Segregation Data for Basta Tolerance of the F_1 Progeny of MS1 Crossed with RF2

Plant material	Total number of seedlings	Number of seedlings that survived Basta
MS1 x RF2	3	2

Table 11. Phenotypic Evaluation of the TA29-*barstar* Restoration of the *barnase* Containing Male Sterile Plants

Plant material	Total number of Basta tolerant plants tested	Number of male fertile plants	Number of male sterile plants
MS1 x RF2	2	1	1

Segregation ratios (fertile/sterile) suggest that the TA29-*barstar* containing plants can induce restoration of the fertility TA29-*barnase* plants.

To confirm the Mendelian segregation of RF2, 100 S_1 RF2 seeds were sown in the greenhouse in the spring of 1991. Seedlings were sprayed with Basta (8 L/ha). A number of the survivor plants were isolated and allowed to self. These plants were individually harvested. Twenty S_2 RF2 seeds per harvested plant were resown and grown to the 2-4-leaf stage. Basta (8 L/ha) was applied on the seedlings. Based on the segregation results, candidate homozygous RF2 S_1 plants were identified. Table 12 shows the Basta segregation data for the progeny of selected S_2 plants.

Table 12. Basta Segregation Data of the Progeny of Selected RF2 S_2 Plants

S_1 plant material	Total number of S_2 seedlings	Number of S_2 seedlings that survived Basta	Chi^2 (1/6 sensitive)
Plant 55	19	16	
Plant 49	19	17	
Plant 11	8	3	
Plant 56	19	19	
Plant 13	17	10	
Plant 14	18	14	
Plant 17	20	15	
Plant 16	12	12	
Plant 12	19	11	
Plant 8	14	11	
Plant 3	14	10	
Plant 5	13	13	
Plant 2	14	11	
Plant 7	20	17	
Plant 47	20	19	
Plant 57	20	20	
Plant 59	19	19	
Plant 58	20	18	
Plant 19	16	15	
Total	311	260	NS

NS – Not significant

S_1 and S_2 RF2 plants germinated uniformly and showed the expected Basta segregation ratio.

An additional study was conducted to further evaluate the Mendelian segregation of RF2. Twenty RF2 S_2 seeds of individually harvested RF2 S_1 plants were sown in the greenhouse during the fall of 1991. Seedlings were sprayed with Basta (8 L/ha). S_3 seeds were produced via bumblebee pollination. Table 13 shows the Basta segregation rates.

Table 13. Basta Segregation Data of the S_2 Progeny of RF2 Plants

Plant material	Total number of S_2 seedlings	Number of seedlings that survived Basta	Chi^2 (1/6 sensitive)
Nontransgenic control	20	0	
Plant 1	20	20	
Plant 2	18	14	
Plant 3	19	13	
Plant 4	20	16	
Total	77	63	NS

NS – Not significant

Basta data were as expected. Based on Basta segregation data of the progeny of individual plants, a homozygous S_2 RF2 plant could be identified and S_3 seeds were successfully produced. No effects on the pollinating bumblebees could be observed.

B. DNA Analysis and Gene Expression of Events MS1/RF1/RF2

Characterization of MS1 and RF1

Laboratory Studies conducted and presented in detail in Appendix IX.

- L01 Primary analysis of male sterile transformants on laboratory scale
- L02 Primary analysis of original fertility restorer transformants on laboratory scale
- L03a Molecular analysis of the integration of the chimeric gene in the transgenic fertility restorer B93-101 (RF1) line
- L03b Molecular analysis of the integration of the chimeric gene in the transgenic male sterile B91-4 (MS1) line
- L04 The transformants are free of *Agrobacterium tumefaciens*
- L05 Stability of integration of the T-DNA over a number of generations via Southern blot analysis
- L06 Confirmation that the inserted DNA corresponds with the plasmid T-DNA
- L07 Characterization of the T-DNA integration via molecular analysis of the regions flanking the T-DNA
- L08 Molecular analysis of the target site deletion present on the homologous chromosome
- L09 Homology search between plant DNA sequences and B91-4 (MS1) and B93-101 target sequences
- L10 Expression of the introduced genes via Northern analysis and evaluation of occurrence of cryptic gene expression
- L11 NPTII activity assay over a number of generations
- L12 Spectrophotometer PAT assay over a number of generations
- L13 Identification of the genome on which the genes are inserted

Conclusions

- The primary transformants MS1 and RF1 contain a single locus of the chimeric *barnase*, respectively *barstar* gene.
- The transformants are unambiguously characterized by RFLP pattern.
- The transfer of the inserted DNA is limited to the sequences comprised between the border repeats.
- The descendants of the MS1 and Rf1 transformants are free of *Agrobacterium tumefaciens*.
- The inserted T-DNA remains genetically stable in distinct generations.
- The insertion site has been in detail characterized.
- There is no indication of the insertion of the T-DNA in a functional gene.
- The expression of the inserted T-DNA is limited to the introduced coding sequences. There is no indication of expression of other newly inserted sequences (cryptic genes).
- PAT and NPTII activity of the transgenic lines has been assessed and is consistent between tested generations

Characterization of RF2

- L01 Primary analysis of the original fertility restorer transformant on laboratory scale
- L02 Molecular analysis of the integration of the chimeric gene in the transgenic fertility restorer B94-2 (RF2) line
- L03 Determination of Agrobateria is present in the transgenic oilseed rape line
- L04 Stability of integration of the T-DNA over a number of generations via Southern blot analysis
- L05 Confirmation that the inserted DNA corresponds with the plasmid T-DNA
- L06 Characterization of the T-DNA integration via molecular analysis of the regions flanking the T-DNA
- L07 Molecular analysis of the target site deletion present on the homologous chromosome
- L08 Homology search between plant DNA sequences and B94-2 target sequences
- L09 Expression of the introduced genes via Northern analysis and evaluation of occurrence of cryptic gene expression
- L10 Assay of NPTII activity over a number of generations
- L12 Assay of PAT activity over a number of generations

Conclusions

- The primary transformant RF2 contains a single locus of the chimeric *barnase*, respectively *barstar* gene.
- The transformants are unambiguously characterized by RFLP pattern.
- The transfer of the inserted DNA is limited to the sequences comprised between the border repeats.
- The descendants of the RF2 transformant are free of *Agrobacterium tumefaciens*.

- The inserted T-DNA remains genetically stable in distinct generations.
- The insertion site has been in detail characterized.
- There is no indication of the insertion of the T-DNA in a functional gene.
- The expression of the inserted T-DNA is limited to the introduced coding sequences. There is no indication of expression of other newly inserted sequences (cryptic genes).
- PAT and NPTII activity of the transgenic lines has been assessed and is consistent between tested generations

V. Agronomic Performance of Events MS1/RF1/RF2

As was seen for the previous deregulated events MS8/RF3, there were no differences in morphology and in disease or insect resistance between the events MS1/RF1/RF2 and its nontransgenic counterpart. There were no morphological, beneficial organism, disease and pest differences between MS1/RF1 and RF2 compared to the previously considered event MS8/RF3. There is no obvious increase in volunteers, difference in seed germination, vigor, plant establishment, days to flower, days to maturity, yield and lodging, or any other variation indicative of increased weediness. There is no reason to think cultivation of events MS1/RF1 and RF2 and it's progeny will have environmental effects different that the cultivation of events MS8/RF3 which have already been considered by APHIS.

In addition, the expected segregation ratios were observed for a single locus. In the field trials, when sprayed with Basta, all plants exhibited a high level of glufosinate resistance, indicating that the respective genes are stably integrated and expressed.

A. Field Tests of Events MS1/RF1/RF2

MS1/RF1/RF2 events were extensively field tested (globally) prior to receiving the necessary Canadian approvals for commercialization under various global authorization numbers as detailed in Section IV A. above.

B. Agronomic, Disease and Pest Characteristics

MS1

Seed characteristics of MS1 seed were compared with a nontransgenic control variety. Hectoliter weight and 1000-kernel weight (measurement of 2x4x100 seeds) were determined. The results of several germination tests (two different methods) were compared. In addition, an accelerated aging test was done, where the seeds were kept for a number of days at 40-50°C and then germinated at 20°C. The germination of 4 times 100 seeds of each entry was evaluated. Seed characteristic results are presented in Tables 14, 15, 16, 17 and 18.

Table 14. 1000 Kernel Weight and Hectoliter Weight of MS1 Canola Seeds and Nontransgenic Control Canola Seeds

Plant Material	1000 Kernel weight (g)[1]	Hectoliter weight of seeds (g)[1]
MS1	3.3022	63.0
Nontransgenic control	3.3593	62.5

[1] Mean over two determinations

Table 15. Germination Percentage of 4 Times 100 T_2 MS1 Seeds Per Entry and Per Method

Germination test method	**Nontransgenic control**	**T_2 MS1**
Copenhagen table	87	85
	86	84
	88	88
	89	87
	Mean 88	Mean 86
Sand method	85	80
	87	74
	80	72
	78	71
	Mean 83	Mean 74
Top of paper method	89	88
	84	84
	85	85
	77	89
	Mean 84	Mean 87
Between paper method	79	82
	86	86
	87	85
	89	88
	Mean 82	Mean 85

Table 16. Germination Percentage of MS1 and Nontransformed Control Plant Material With and Without 7 Days Pretreatment in a 10°C Room

Plant material	Without pretreatment	With pretreatment
Nontransformed control	82	84
	83	85
	84	74
	82	78
	Mean 82	Mean 80
MS1	88	79
	84	77
	85	76
	89	79
	Mean 87	Mean 78

Table 17. Germination Percentage of MS1 and Nontransformed Control Plant Material after a 7 Day Germination Period at 10°C or 20°C

	Number of normal seedlings		Number of abnormal seedlings	
Plant material	**7 days at 10°C**	**7 days at 20°C**	**7 days at 10°C**	**7 days at 20°C**
Nontransformed control	81	82	14	10
	77	83	16	9
	79	84	11	9
	81	82	11	9
	Mean 80	Mean 83	Mean 13	Mean 9
MS1	79	88	10	7
	78	84	15	9
	79	85	12	8
	69	89	19	6
	Mean 76	Mean 87	mean 14	Mean 8

Table 18. Germination Percentage Under Accelerated Aging Conditions

	Number of days the seeds were kept at 40-45°C									
Plant Material	0	3	8	10	12	15	17	19	22	24
MS1	88	81	76	82	79	77	79	80	81	73
	84	84	81	86	85	77	80	77	80	73
	85	82	84	78	77	78	74	76	78	71
	89	89	89	76	75	81	80	80	77	73
Nontransformed control	82	78	82	82	83	81	80	79	76	75
	84	83	80	81	76	80	79	76	77	77
	83	85	80	83	80	81	83	83	72	77
	82	87	82	82	84	80	84	83	74	76

The introduction of the chimeric *barnase* gene in MS1 has not provoked any specific effects on the ability to survive or germinate.

MS1/RF1/RF2

Evaluation of the stability and reliability of restoration of RF2 and RF2 in different climactic conditions as well as various agronomic evaluations were obtained. Tables 19 through 24 provide these data.

Table 19. Germination and Plant Establishment (two locations, mean of 4 replications) for Transformation Events MS1, RF1 and RF2

Genotype	Belguim[1]	UK[2] (Great Dunmow)
MS1	4.00	7.25
RF2	4.75	7.50
RF1	3.75	7.25
MS1 x RF2	4.00	7.00
MS1 x RF1	4.25	7.00
Nontransgenic Control	4.00	7.00

[1] Scale: 0=poor, 5=good
[2] Scale: 1=poor, 9=good

Table 20. Vigor of Transformation Events MS1, RF1 and RF2

Genotype	Belgium[1]	France[1]	UK[2]
MS1	3.75	2.00	7
RF2	4.00	2.50	6
RF1	3.75	2.75	8
MS1 x RF2	4.00	2.63	8
MS1 x RF1	3.75	2.75	8
Nontransgenic Control	4.00	2.63	--

[1] Scale is 1 to 5
[2] Scale is 1 to 9

Table 21. Number of Days to Start Flowering for Transformation Event MS1, RF1 and RF2

Genotype	**Belgium**	**Sweden**	**UK (Great Dunmow)**	**UK Aberdeen**	**Canada**
MS1	54	66.5	65.8	61	95
RF2	53	68.7	65.5	59	99
RF1	54	68.3	66.8	60	103
MS1 x RF2	53	69.0	66.5	60	99
MS1 x RF1	53	68.0	66.3	60	108
Nontransgenic Control	53	68.3	65.8	--	96

Table 22. Number of days to maturity for Transformation Events MS1, RF1 and RF2

Genotype	Sweden	UK (Aberdeen)
MS1	115	82.67
RF2	116	85.91
RF1	116	84.86
MS1 x RF2	117	84.19
MS1 x RF1	116	83.37
Nontransgenic Control	115	85.32

Table 23. Lodging resistance for Transformation Events MS1, RF1 and RF2

Genotype	Canada[1]	UK (Great Dunmow)[2]
MS1	2	4.24
RF2	1	2.25
RF1	2	2.25
MS1 x RF2	2	2.00
MS1 x RF1	2	2.25
Nontransgenic Control	2	2.67

[1] Scale: 1=no lodging, 3=severe lodging
[2] Scale: 1=no lodging, 5=severe lodging

Table 24. Seed yield (kg/ha) for Transformation Events MS1, RF1 and RF2

Genotype	Belgium	Sweden	UK (Great Dunmow)	UK (Aberdeen)	France
MS1	2641	1074	1230	2710	2145
RF2	2848	976	1494	2694	2760
RF1	2953	866	1652	2499	2580
MS1 x RF2	2891	1031	1411	2976	2602
MS1 x RF1	2980	933	1561	3022	2703
Nontransgenic Control	3117	976	1505	2709	2842

No differences in vigor, number of days to start flowering, number of days to maturity and lodging resistance were observed between the transgenic entries and their nontransgenic counterpart. Statistical analysis of seed yield was carried out via Agrobase Software using ANOVA tools. No statistically significant differences between the restorer fertility entries, the restored combinations and the nontransgenic counterpart were observed.

As seen for events MS8/RF3, there were no differences in morphology and in disease and insect resistance between the events MS1/RF1/RF2 compared with the non-transgenic counterpart. In addition, the expected segregation ratios were observed for a single insert.

It can be concluded that there are no substantial differences in the agronomic characteristics between MS1/RF1/RF2 and commercially available nontransgenic counterpart, Drakkar.

The FDA issued a finding of "No Concern" on April 4, 1996 for these canola transformation events and the use of these canola events for food and feed purposes has also been granted in Canada. Eruric acid and glucosinolates are the only two toxicants known in rapeseed. MS1/RF1 and RF2 canola has been developed from low erucic acid and low glucosinolate canola varieties, and these transformation events were chosen, in part, for normal seed and oil quality. Agriculture and Agri-Food Canada (1995) concluded that AgrEvo (Aventis) provided data that demonstrated that the nutritional composition of whole seed, processed meal or oil derived from events MS1/RF1 and RF2 and their hybrids are substantially equivalent to conventional canola varieties. Therefore, MS1/RF1 and RF2 and their hybrids should have no indirect or direct plant pest effects on any processed commodities.

APHIS has previously issued determinations of nonregulated status to other genetically engineered glufosinate-tolerant canola (98-278-01p and 97-205-01p) with similar genetic constructs as those used in MS1/RF1 and RF2 canola. No adverse impacts on agricultural practices associated with the cultivation of these events have been observed.

C. Effects on Nontarget Organisms

Several laboratory studies were undertaken to examine the potential adverse effects of MS1/RF1/RF2 on nontarget organisms. A study looking at the effect of various intake levels of transgenic oilseed rape leaves on the zootechnical performance of rabbits was conducted. No signs of toxicity of the transgenic leaves were found. Rabbits partially or exclusively fed with the transgenic leaves showed no clinical symptoms, no was mortality observed.

In addition to the rabbit study, a 28-day avian dietary study was conducted. There were no differences in food consumption, behavior and body weight gain between the birds fed transgenic diet and nontransgenic diet.

A caged experiment with honeybees was conducted to evaluate the effects of foraging on transgenic verse nontransgenic oilseed rape. The observed foraging activity and mortality rate was not statistically significant between the transgenic and nontransgenic oilseed rape. It was therefore concluded that there is no influence on the honeybees that are foraging on flowers from modified oilseed rape plants. The results from this laboratory experiment are supported by data collected during greenhouse trials where bumblebees were used to pollinate the modified plants.

An additional study was conducted to evaluate the rhizobacterial flora on transformed and nontransformed oilseed rape plants. The purpose of the study was to investigate possible effects of transformed rapeseed plants on the bacterial population on the root surface. The study showed that transformation of the plants for male sterility (MS1) and restoration (RF1 and RF2) had no detectable effect on the rhizobacterial flora that was screened, and

that no specific bacterial flora was found to be associated with either transformed or nontransformed plants.

1. Summary

As is the case with the previously deregulated MS8/RF3, the new male sterility trait (MS1) effects only anther and pollen development. The nectar, which provides a source of nutrients for pollinators, as well as the flowers, develops normally. The RF1 and RF2 plants and the hybrids have normal flower morphology, fertility and attractiveness to insect pollinators. Normal insect activity and behavior was observed in insects foraging on these plants. Common insects that feed on canola are not on the threatened or endangered species list. In addition, the proteins expressed in MS1/RF1/RF2 hybrids are derived from a common soil bacterium and are expected to be a normal part of the diet of animals, humans and insects.

D. Monitoring

Monitoring residual effects in crops grown at Canadian locations previously used for culturing transgenic oilseed rape

To determine if any residual effect of growing transgenic oilseed rape could be observed on the following crop, a sampling study was conducted in more detail in 1993 in Canada. At the Laird location of 1992, wheat was sown in 1993. These wheat plants (0.25 m^2) were sampled in July 1993. Plant cuttings were taken when plants were at the early flag stage. All samples were completely dried prior to being weighed. Results of the studies are indicated in Table 25. An analysis of variance indicated that no significant differences were observed between the biomasses of wheat plants growing at a previous transgenic oilseed rape location versus a location with no history of growing transgenic oilseed rape plants.

In addition, visual observations of plant growth and yield results of different crops grown on previous transgenic oilseed rape locations did not give indications of a residual effect on these crops due to the cultivation of the male sterile, fertility restorer and restored hybrids.

Table 25. Dried Wheat biomass (g) (4 replicates) of plant cuttings taken in July 1993 at a 1992 transgenic canola test site

1992 Treatment	1993 Wheat biomass (g) (4 replicates)
Non-transgenic variety	81.0 61.3 68.0 45.6
Drakkar	81.8 64.7 61.6 72.4
B91-4 progeny	58.4 59.9 54.2 57.2
B93-101 progeny	53.3 58.0 51.2 81.8
Restored B91-4 x B93-101 hybrid	99.8 64.5 56.9 62.4

Post Commercialization Monitoring of SeedLink Canola Volunteers in Rotational Crops

In the 2000-growing season, a monitoring study was conducted to evaluate the persistence and fate of SeedLink hybrid canola volunteers in rotational crops,

Based on the first year of the survey, no evidence was found to suggest that SeedLink canola behaves any differently in managed and unmanaged environments than does standard, non-transgenic canola. The results from this survey are consistent with those reported earlier by (Deschamps *et al.*, 1998) which reported on the persistence and control of glufosinate tolerant canola volunteers (LibertyLink).

VI. Potential for Environmental Impact from Non-contained Use of Events MS1/RF1/RF2

There were no significant differences, apart from the intended changes, demonstrated in field tests of events MS1/RF1/RF2 compared with a non-transgenic variety. No morphological, beneficial organisms, disease or pest differences between event MS1/RF1/RF2 and the previously considered events, MS8/RF3, were noted. There is no reason to think cultivation of events MS1/RF1/RF2 will have environmental effects different from cultivation of MS8/RF3, the other male sterile, fertility restorer event previously considered by APHIS. No adverse consequences from the introduction of events MS1/RF1/RF2 are expected.

VII. Statement of Grounds Unfavorable

No unfavorable information and data have been demonstrated for the male sterile, fertility restorer transformation events MS1/RF1/RF2.

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Appendix I

Health Canada Food Approval Letter for Transformation Events MS1/RF1

Health Canada / Santé Canada
Health Protection Branch / Direction générale de la protection de la santé

Tunney's Pasture
Ottawa, Ontario
K1A 0L2

September 8, 1994

Dr. Patrick Rüdelsheim
Registration Manager
Plant Genetic Systems N.V.
Jozef Plateaustraat 22 - B 9000
Gent, Belgium

Dear Dr. Rüdelsheim:

This will refer to your Novel Food Submission dated May 20, 1994 concerning a transgenic variety of canola developed through the use of a new hybridization system.

The hybridization system of note consists of the following elements:

(i) Introduction of a "nuclear male sterility" gene construct containing the t-DNA derived from pTTM8RE (disarmed *Agrobacterium tumefaciens*) into one line of canola (*Brassica napus*) to cause direct expression of RNase (*barnase*) and hence induction of male sterility;

(ii) Introduction of a "fertility restorer" gene construct containing t-DNA derived from pTVE74RE (disarmed *Agrobacterium tumefaciens*) into another line of canola to cause direct expression of the protein, *barstar*, which is a specific inhibitor of barnase; and

(iii) Crossing of the two lines in (i) and (ii) to produce a fertile hybrid which contains both the *barnase* and *barstar* genes. As well, the hybrid contains two marker genes, the kanamycin resistant gene *NPTII* (derived from *Escherichia coli*) and the *bar* gene (derived from *Streptomyces hygroscopicus*) which confers tolerance to phosphinothricin.

Canada

Officers of the Health Protection Branch have reviewed the information you provided to characterize the transgenic (hybrid) canola varieties. They have also noted that neither the canola wholeseed nor the meal is expected to be used for human consumption. On the basis of the data submitted, we have no objection to food uses of the refined oil from a canola variety developed through the use of the above-noted hybridization system.

Concerning the acceptability of the meal for use in animal feed, we would suggest that you continue to keep in direct contact with the Department of Agriculture and Agri-Food Canada as the evaluation of animal feed falls under the purview of that Department.

By copy of this letter, we are informing our colleagues in the Department of Agriculture and Agri-Food Canada.

Yours truly,

S.W. Gunner, Ph.D.
Director General
Food Directorate

cc: S.W. Ormrod
Director General
Plant Industry Directorate
Food Production and Inspection Branch
Agriculture and Agri-Food Canad

Appendix II

Health Canada Food Approval Letter for Transformation Event RF2

Health Canada
Santé Canada

Health Protection Branch
Direction générale de la protection de la santé

17 AUG. 1995

Tunney's Pasture
Ottawa, Ontario
K1A 0L2

August 3, 1995

Dr. Patrick Rüdelsheim
Registration Manager
Plant Genetic Systems N.V.
Jozef Plateaustraat 22 - B 9000
Gent, Belgium

Dear Dr. Rüdelsheim:

This will refer to your Novel Food Submission dated April 13, 1995 concerning a transgenic variety of canola developed through the use of a new hybridization system.

Officers of the Health Protection Branch have reviewed the information Plant Genetic Systems N.V. provided for the safety assessment of the novel hybridized canola variety. According to the submitted data, the procedure used in developing the new hybridization system of note consisted of the following:

(i) Introduction of a "nuclear male sterility" gene construct containing the t-DNA derived from pTTM8RE (disarmed *Agrobacterium tumefaciens*) into one line of canola (*Brassica napus*) to cause direct expression of RNase (*barnase*) and hence induction of male sterility. This line, designated MS1(B91-4), was previously assessed by the Branch and in our letter of opinion dated September 8, 1994, no objection was taken to its use;

(ii) Introduction of a "fertility restorer" gene construct containing t-DNA derived from pTVE74RE (disarmed *Agrobacterium tumefaciens*) into another line of canola to cause direct expression of the protein, *barstar*, which is a specific inhibitor of barnase. This line, designated RF2(B94-2), is the novel component of the submission; and

Canada

(iii) Crossing of the two lines in (i) and (ii) to produce a fertile hybrid which contains both the *barnase* and *barstar* genes.

The barnase and barstar genes are both linked to the kanamycin resistant gene *NPTII* (derived from *Escherichia coli*), as well as the *bar* gene (derived from *Streptomyces hygroscopicus*) which confers tolerance to phosphinothricin. Hence, the canola hybrid of note contains 4 new genes and the proteins expressed by them.

We also note that neither the canola wholeseed nor the meal is expected to be used for human consumption. On the basis of the data submitted, we have no objection to food uses of the refined oil from the canola variety developed through the use of the above-noted hybridization system.

Concerning the acceptability of the meal for use in animal feed, we would suggest that you continue to keep in direct contact with the Department of Agriculture and Agri-food as the evaluation of animal feed falls under the purview of that Department.

Please note that we are providing our colleagues in the Department of Agriculture and Agri-food with a copy of this letter.

Yours truly,

S.W. Gunner, Ph.D.
Director General
Food Directorate

cc: Dr. A. MacKenzie
Director General
Food Inspection Directorate

Appendix III

Agriculture and Agri-Food Canada Environmental and Feed Approval Letter for Transformation Events MS1/RF1

Agriculture and Agri-Food Canada
Agriculture et Agro-alimentaire Canada

Food Production and Inspection Branch
Direction générale de la production et de l'inspection des aliments

Post-it™ FAX TRANSMITTAL MEMO 7671 | 12 NO. OF PAGES

TO: Barb Fowler	FROM: Anne-Christine
CO.:	CO.:
DEPT.:	PHONE #:
FAX #: 306 975-0120	FAX #: 613 9925219

Plant Products Division
59 Camelot Drive
Nepean, Ontario K1A 0Y9

April 28, 1995

Dr. Patrick Rüdelsheim
Plant Genetic Systems Inc.
Regulatory Department
Jozef Plateaustraat
22 - B 9000 Gent
Belgium

3625-6-10P2

Dear Dr. Rüdelsheim:

We have reviewed your application for unconfined field release and for livestock feed use of Plant Genetic Systems novel hybridization system for canola, that involves the transformed MS1 and RF1 canola lines (*Brassica napus*), and their hybrid MS1xRF1. These plants have been transformed with genes that confer nuclear male sterility, male fertility restoration, tolerance to the herbicide glufosinate ammonium, and a selectable marker.

On the basis of the information provided to us, the unconfined release of MS1, RF1 and MS1xRF1 should not pose any concern to environmental safety, and is therefore authorized in Canada. The hybridization system does not in itself raise any concerns regarding the safety or nutritional composition of lines derived from it. These hybrid lines are therefore approved for use as livestock feed ingredients in Canada, provided that the feed ingredients produced therefrom conform to the definitions for canola seed, canola meal or canola oil as listed in Schedule IV of the Feeds Regulations. The enclosed Decision Document, dated April 28, 1995, which explains the rationale behind our decision, will be made publicly available.

The present authorization relates to MS1, RF1 and MS1xRF1, all other *Brassica napus* varieties resulting from the same transformation event, and all their descendants, provided no inter-specific crosses are performed, provided the intended use is similar, and provided these plants do not display any additional novel traits. The present authorization letter also relates to other *B. napus* varieties transformed with the same genetic construct, provided that the resulting lines can be shown to be substantially equivalent to MS1 or RF1 in terms of their potential environmental impact and livestock safety.

If at any time, your company becomes aware of any new information regarding risk to the environment, including risk to animal or human health, that could result from this release, you must immediately provide such information to this office.

Please note that, while determining the environmental safety of plants with novel traits is a critical step in the commercialization of these plant types, other requirements may still need to be addressed, including a food safety assessment by Health Canada, and Variety Registration by Agriculture and Agri-Food Canada. We will inform provincial agencies of this decision.

Yours sincerely,

Glenn Hansen
Director
cc. Provincial Contacts, EC, HC, Seed Program Officers, Variety Section, Feed Section, Director, Plant Protection. Enclosure (DD95-04)

Canada

Appendix IV

Agriculture and Agri-Food Canada Environmental and Feed Approval Letter for Transformation Event RF2

FK → IM / DT

Official copy will follow soon!
Another step on the road.

Food Inspection Directorate
Plant Products Division
Nepean, Ontario
K1A 0Y9

20 December, 1995

Dr. Patrick Rüdelsheim
Registration Manager
Plant Genetic Systems
Jozef Plateaustraat 22
B 9000 Gent
Belgium

Dear Dr. Rüdelsheim,

We have reviewed the submission respecting the feed safety and nutritional utility of the **Modified Hybridization System for Oilseed Rape, RF2**, as livestock feed.

Canola and several byproducts are currently described in Schedule IV of the Feeds Regulations which specifies labelling requirements for these feed ingredients. RF2 and its byproducts have been assessed and found to be substantially equivalent to traditional canola varieties and RF1 (previously approved) in terms of feed safety and nutritional composition. RF2, and its subsequent sister lines are approved for use as livestock feed ingredients in Canada provided that the feed ingredients produced therefrom conform to the definitions for canola seed, canola meal or canola oil as listed in Schedule IV of the Feeds Regulations.

If you have any questions regarding this approval, please do not hesitate to contact me at (613) 952-8000, ext. 4374.

Sincerely,

Catherine Italiano
Toxicologist

cc: Simon Barber

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Appendix V

United States Department of Agriculture Import to Process Approval Letter for Transformation Events MS1/RF1/RF2

FILE: FIST, RF1, RF2
USDA petition
Rec. file.

United States Department of Agriculture

Animal and Plant Health Inspection Service

Biotechnology, Biologics, and Environmental Protection

MAR 22 1996

Dr. Keith Newhouse
Director
Plant Genetic Systems (America) Inc.
7200 Hickman Road
Des Moines, IA 50322

Dr. Patrick Rudelsheim
Registration Manager
Plant Genetic Systems, N.V.
J. Plateaustratt 22
B-9000 Ghent, Belgium

Dear Drs. Newhouse and Rudelsheim:

We are writing in response to your letter of February 7, 1996, in which you requested an opinion on the regulatory authority of the Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture (USDA), with respect to your company's male sterile canola (*Brassica napus*) with glufosinate herbicide tolerance gene as a marker gene (transformation event MS1), the fertility restorer canola with the same marker gene (transformation events RF1 and RF2), and any hybrids or varieties developed from these events. Based upon the information in your letter and the intended use of this plant material exclusively for processing, we believe that these lines should pose no plant pest risk. Therefore, APHIS will not consider MS1, RF1, RF2, and progeny canola seed, imported into the United States for processing, as regulated articles under our regulations (7 CFR Part 340).

We have based our decision on the factors summarized below:

1. The intended use of the canola seeds is processing the seeds to extract the oil. After processing, the remaining plant material is not viable.

2. For years, canola seeds have been shipped and imported for processing at facilities located in the United States. APHIS is unaware of any plant pest problems that have been associated with such seed shipments or the handling of the remaining plant material after processing of the seeds.

3. APHIS takes note of the environmental analysis on canola line conducted by USDA and Agri-Food Canada in which they concluded that the canola is safe for cultivation in Canada and that the canola is no more competitive than other canola varieties.

4. APHIS also believes that standard industry practices for the shipment of the canola to a processing plant are adequate and should

not present any plant pest risk. There is no indication that the shipment of other canola varieties has ever resulted in a plant pest risk.

5. The four new traits introduced into these three canola lines: *barnase* (a ribonuclease), *barstar* (a highly specific inhibitor of *barnase*), phosphinothricin acetyltransferase, and neomycin phosphotransferase (both marker genes) have not altered the canola's plant pest risk potential. The use of phosphinotricin acetyltransferase, which encodes glufosinate resistance as a marker gene, would confer a selective advantage only if these plants or their offspring were treated with glufosinate. APHIS believes that it is very unlikely that canola will escape, germinate, grow to reproductive maturity, and pollinate wild or cultivated relatives whose offspring will be treated with glufosinate and thereby exhibit a selective advantage. Even if such an unlikely sequence of events were to occur and result in a plant population that could not be controlled with glufosinate, alternative chemical and mechanical control practices are currently available that should be effective.

We must emphasize that our opinion regarding these canola lines is expressly limited to the conditions that you have described, namely, shipment of seed to processing plants in the United States. Under the circumstance of this intended use (i.e., shipment to a processing facility), APHIS would not regulate these canola lines found under our 7 CFR Part 340. This opinion includes canola lines MS1, RF 1, RF2, crosses between these lines, and progeny of crosses with other canola lines that are not regulated articles under 7 CFR Part 340. However, if this plant material is imported for other uses or purposes, it may be subject to regulation under 7 CFR Part 340. The canola seed is subject to all other applicable phytosanitary standards and regulations.

If you have any further questions about this matter, please feel free to contact Dr. James L. White at Area Code (301) 734-5940.

Sincerely,

John H. Payne, Ph.D.
Acting Director

Appendix VI

United States Food and Drug Administration Letter of Opinion of No Concern for Transformation Events MS1/RF1/RF2

APR 4 1996

Dr. Patrick Rudelsheim
Registration Manager
Plant Genetic Systems, N.V. (PGS)
Jozef Plateaustraat 22
B-9000 Gent
Belgium

Dear Dr. Rudelsheim:

This is in regard to PGS's consultation with the Food and Drug Administration (FDA) (Center for Veterinary Medicine and Center for Food Safety and Applied Nutrition) on genetically modified oilseed rape, specifically transformation events B91-4, B93-101, and B94-2. According to PGS, transformation event B91-4 oilseed rape is modified to express the *neo* (*kanR*) gene from a *Tn5* carrying plasmid from *Escherichia coli*, the *bar* gene from *Streptomyces hygroscopicus*, and the *barnase* gene from *Bacillus amyloliquefaciens*. These genes encode the respective proteins for aminoglycoside resistance (NPTII), glufosinate herbicide tolerance (PAT), and male sterility inducing RNase (barnase; a specific RNase). Lines containing transformation events B93-101 and B94-2 are modified to express *neo* and *bar*, as above, as well as the *barstar* gene from *B. amyloliquefaciens*. The latter gene encodes a specific RNase inhibitor protein (barstar), thereby making these oilseed rape lines fertility restorers to the male sterile rape line (B91-4) when cross-pollinated (i.e., pollination control system), leading to the development of 100% hybrid seed for commercial purposes.

In November of 1992, PGS met with FDA to discuss their proposed safety and nutritional assessment of oilseed rape containing the aforementioned transformation events. As part of bringing PGS's consultation regarding these products to closure, PGS submitted a summary of the assessments of oilseed rape containing transformation events B91-4 and B93-101 on July 6, 1995. A second submission for transformation event B94-2 was submitted by PGS on October 23, 1995.

These communications informed FDA of the steps taken by PGS to ensure that these products comply with the legal and regulatory requirements that fall within FDA's jurisdiction. Based on the safety and nutritional assessment that you have conducted, it is our understanding that PGS has concluded that oilseed rape oil, honey (nectar) and cake (meal), derived from the hybrid seed, are not materially different in composition, safety, and other relevant parameters from oilseed rape oil, honey (nectar) and cake (meal) presently on the market, and that genetically modified oilseed rape does not raise issues that would require

premarket review or approval by FDA. All materials relevant to this notification have been placed in a file designated BNF0032. This file will be maintained in the Office of Premarket Approval.

Based on the information PGS has presented, we have no further questions concerning oil, honey (nectar) and cake (meal) from hybrid seed derived from transformation events B91-4, B93-101, and B94-2 at this time. However, as you are aware, it is PGS's continued responsibility to ensure that foods marketed by the firm are safe, wholesome and in compliance with all applicable legal and regulatory requirements.

Sincerely yours,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety
and Applied Nutrition

Appendix VII

Field Trial Reports for Transformation Events MS1/RF1/RF2

EXPERIMENT FBN9201

Authorization numbers : BIOT/92/M03 (Deinze, Belgium)
91/12/01 (Paris Sud, France)
91/12/01 (Cambrai, France)
PR 18/4 (King's Lynn, United Kingdom)
(Landskrona, Sweden)
92-017-01 (Idaho Falls, USA)
T92-PGS-CAN-01 (Laird, Canada)
T92-PGS-CAN-02 (Saskatoon, Canada)
T92-PGS-CAN-03 (Saskatoon, Canada)
T92-PGS-CAN-05 (Guelph, Canada)
T92-PGS-CAN-06 (Olds, Canada)

Field trial responsible : ir G. De Both, Field trials agronomist

FBN9201 Goal of the experiment

Agronomical evaluation of the male sterile B91-4 progeny, the B93-101 restorer line progeny, their restored product and some control varieties adapted to the different local conditions. Evaluation of the stability of the male sterility and reliability of the restoration under specific environmental conditions.

FBN9201 Plant material

Table FBN9201 Origin of the plant material

Plant material	Origin of the plant material
Drakkar	Cultivar
B91-4 out FBN9114	T_3 generation of B91-4
B93-101 (homozygous)	S_3(B93-101)
B91-4 x B93-101 (homozygous)	T_1(B91-4) x S_2(B93-101)
Pactol (in Belgium and France), Puma (in Sweden and UK), Westar (in USA), Delta (in Guelph (Canada)), Legend (in Saskatoon (Canada)), Excel (in Laird (Canada)), (in Olds (Canada))	Local cultivars

* : see Table 3 in annex VI for a detailed description of the exact generation of the used plant material

FBN9201 Methods

The field trials were designed as complete randomized blocks with four replicates. The size of the experimental plots and their pathways were chosen in function of each cooperator. Non-transgenic oilseed rape was planted on a border zone (varying from 2m to 10m) around the trial sites (see Table 1 in annex VI).

The trials were sown with a precision drill at a density normally practiced at the different locations, with exception of the B91-4 plots which were sown at double density. The latter entry was sprayed with Basta (5l/ha) at the 5-6 leaves stage. To prevent bird damage at the end of the season, plots were sometimes netted before maturity. Additional herbicides and insecticides were applied conforming to normal agricultural practices.

The following observations were made : germination and establishment, number of plants before and after Basta treatment in the NMS B91-4 plots, flowering dates and flower morphology (male sterile or male fertile) of 100 plants were scored. Ten inflorescences in the male sterile plots were isolated. Yield was determined. A seed sample of each entry was analyzed to determine the following parameters : humidity %, oil %, fatty acid composition, protein % and glucosinolate content and composition.

FBN9201 Results and conclusions

FBN9201 Results

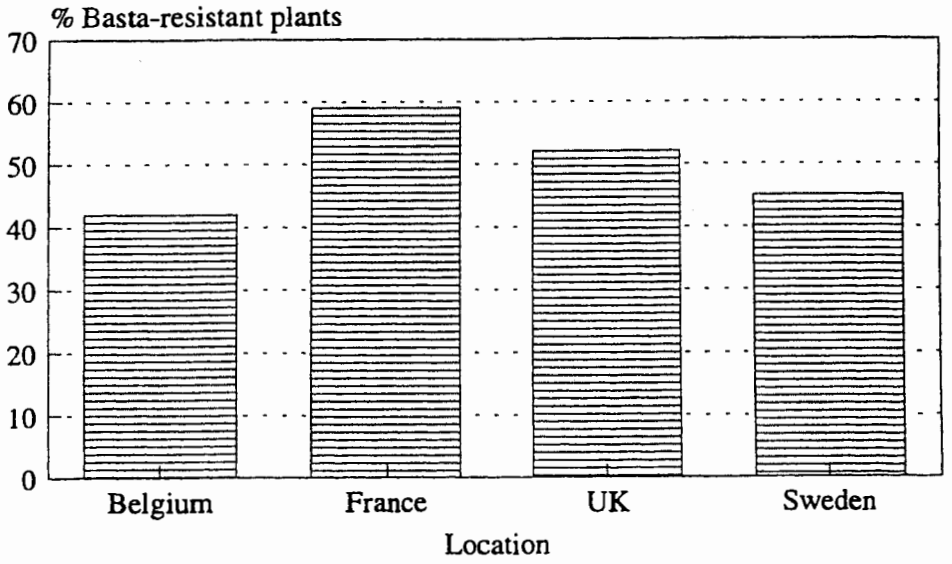

Figure 1. Basta segregation data of the male sterile B91-4 progeny as obtained over different locations

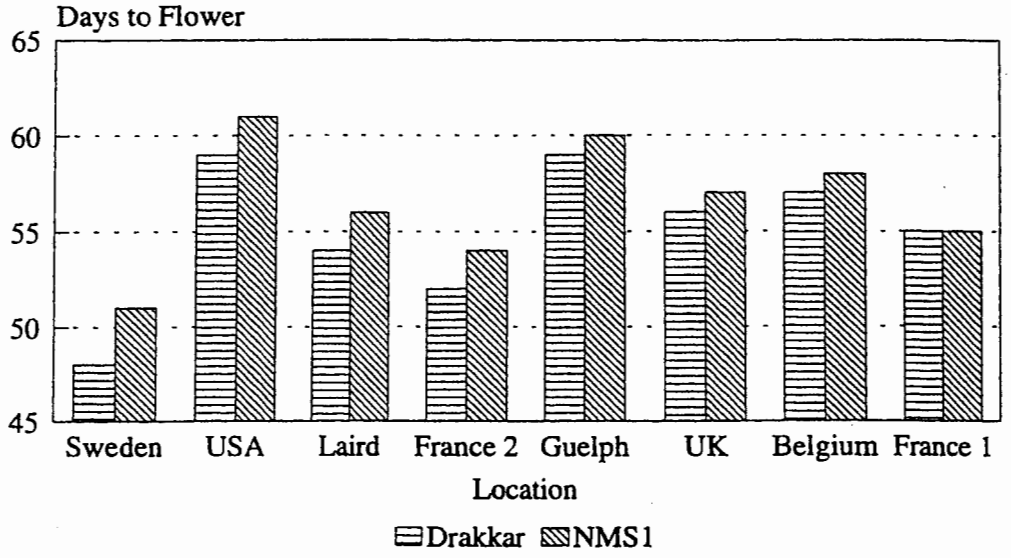

Figure 2. **Number of days to flowering of the male sterile B91-4 plants as obtained in different locations**

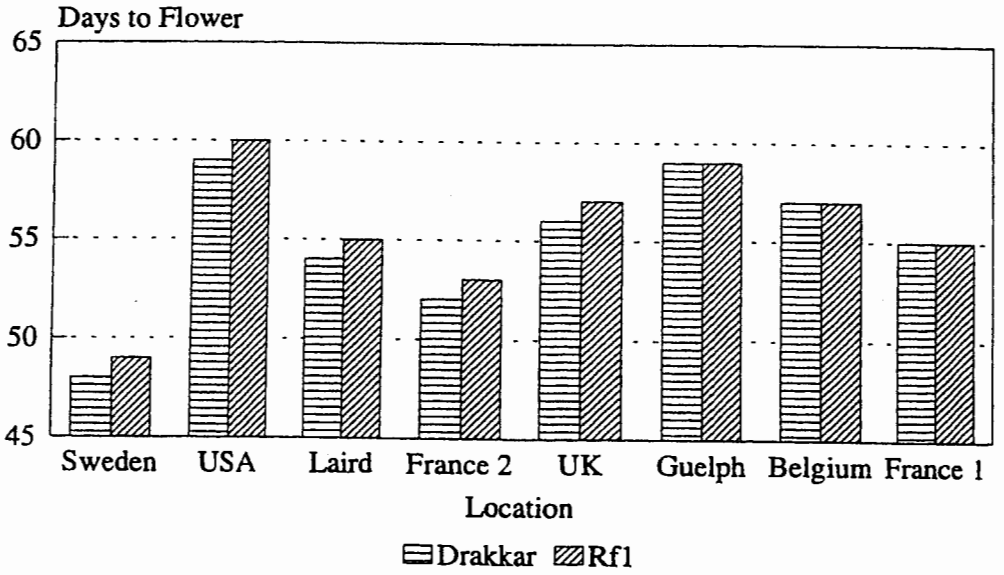

Figure 3. **Number of days to flowering of the restorer B93-101 plants as obtained in different locations**

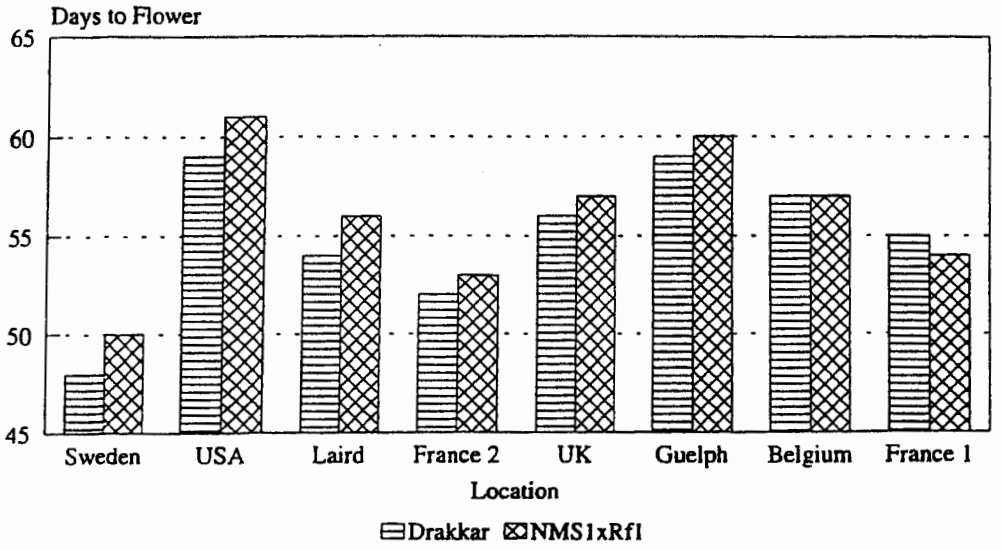

Figure 4. **Number of days to flowering of the restored hybrids as obtained in different locations**

Table 1. Seed yield (kg/ha) of the transformants and their control varieties

Entry	Belgium	France Paris Sud	France Cambrai	United Kingdom	Sweden	Canada Guelph	Canada Saskatoon
B91-4	3728	2120	1446	930	1346	2647	2536
B93-101	3728	2376	2077	890	1849	3022	2443
Hybrid	3744	2363	1925	980	1914	2644	2781
Drakkar	4504	2447	1770	1090	1989	2590	2723
Local variety	3888	2052	1886	1570	2174	2648	2743

Table 2. Seed yield (% of Drakkar) of the transformants and their control varieties

Entry	Belgium	France Paris Sud	France Cambrai	United Kingdom	Sweden	Canada Guelph	Canada Saskatoon
Drakkar	100	100	100	100	100	100	100
B93-101	83	87	82	85	68	102	93
Hybrid	83	97	117	82	93	117	90
B91-4	83	97	109	90	96	102	102

Table 3. Quality analysis of the transgenic plants and their control varieties

Location	Entry	Oil (%)	Protein (%)	Glucosinolates (µmol/g)	Erucic acid (%)
Belgium	Drakkar	37.8	30.9	27.8	< 0.05
	B91-4	37.7	30.8	31.8	< 0.05
	B93-101	36.3	51.5	26.7	< 0.05
	Hybrid	35.6	31.6	24.8	0.3
	Local var.	42.8	26.2	14.8	< 0.05
France Paris Sud	Drakkar	44.3	25.6	15.0	Not determ.
	B91-4	43.0	26.2	15.0	
	B93-101	43.7	25.9	15.0	
	Hybrid	43.0	26.3	17.0	
	Local var.	43.8	24.3	10.0	
France Cambrai	Drakkar	43.0	25.3	18.0	< 0.05
	B91-4	42.7	25.8	23.8	< 0.05
	B93-101	43.3	25.3	22.5	< 0.05
	Hybrid	42.9	25.3	23.4	< 0.05
	Local var.	44.4	24.0	18.9	< 0.05
Sweden	Drakkar	38.3	28.2	27.0	< 0.05
	B91-4	35.2	29.7	27.7	< 0.05
	B93-101	37.1	28.6	25.0	< 0.05
	Hybrid	37.1	28.6	24.1	< 0.05
	Local var.	38.8	29.4	20.4	< 0.05

FBN9201 Conclusions

Over all locations, the different entries *germinated* and established well. No differences were noticed between the transformed and non-transformed lines. Although a uniform plant stand was observed at the seedling stage, the double density B91-4 plots appeared to contain somewhat thinner plants.

In most of the different locations, the double density made it very difficult to apply the herbicide in a proper way (some plants were covered by others). At four sites (Belgium, France (Cambrai), United Kingdom and Sweden) counts were done before and after *Basta treatment*. The segregation ratio ranged between 42 and 59 % phosphinothricin tolerant plants, the average being 49.5 % (Figure 1).

In Figure 2, an overview of the *number of days to flowering* is given for the different entries. The flowering of the male sterile line showed a maximum delay of three days compared to Drakkar in Sweden. At the other locations, the flowering of the B91-4 progeny was delayed by two days (USA, Canada (Laird)), one day (Canada (Guelph), United Kingdom, Belgium) or was flowering as early as Drakkar (France (Paris Sud)).

Flowering of the restorer line showed a maximum delay of one day in comparison with Drakkar (Sweden, USA, Canada (Laird), France (Cambrai), United Kingdom). At the other locations, flowering started as early as Drakkar (Figure 3).
The restored hybrid showed a maximum retardation of two days compared to Drakkar (Sweden, USA, Canada (Laird)). In Cambrai (France), United Kingdom and Guelph (Canada), it started flowering one day later than Drakkar. In Belgium, the hybrid was as early as Drakkar and in France (Paris Sud) flowering of the hybrid started even one day earlier than the non-transformed Drakkar cultivar (Figure 4).

At all locations, the restorer B93-101 plants showed only fertile flowers.

Seed yield was only determined at a limited number of the locations since the Western Canadian field trials (Laird and Olds) were hit by an early frost and snow in late August. These trials were finished before maturity. In Belgium, Guelph and Saskatoon, seed yield was not significantly different for the 5 entries at these 3 locations. At the other sites, the

male sterile line showed a significant yield penalty in comparison to the other entries. Yield of the restorer line and the hybrid was comparable to the non-transformed Drakkar variety (Table 1 and Table 2).

Quality analysis of the different entries at four different locations is outlined in Table 3.

EXPERIMENT FBN9301 **Evaluation of the stability of the nuclear male sterility trait and reliability of restoration in different climatic conditions : agronomical evaluation of the B91-4 male sterile line, the B93-101 restorer line, their restored product and two control varieties**

Authorization numbers : BIOT/93/M03 (Gent, Belgium)
91/12/02 (Cambrai, France)
22 4390/92 (Malmö, Sweden)
PR18/5 (Terrington, United Kingdom)
93-049-02 (Moscow, USA)
93-PGS-CAN-01..04 (Saskatoon, Canada 1)
93-PGS-CAN-01..04 (Guelph, Canada 7)

Responsible : ir G. De Both, PGS Product Development Manager Oilseeds

FBN9301 Goal of the experiment

Evaluation of the stability of NMS and reliability of restoration in different climatic conditions : agronomical evaluation of the B91-4 male sterile line, the B93-101 restorer line, their restored product and two control varieties.

FBN9301 Plant material

Table FBN9301 Origin of the plant material

Plant material	Origin of the plant material
Drakkar (normal density)	Cultivar
Drakkar (1/2 density)	Cultivar
B91-4	T_3 generation of B91-4
B93-101 (homozygous)	S_3(B93-101)
B91-4 x B93-101 (homozygous)	T_3(B91-4) x S_3(B93-101)
Local check 1	Cultivars,B:Starlight,S:Paroll,F:Mars, UK:Puma,Can7:Springfield,USA:Helios,Can1:Excel
Local check 2	Cultivars B:Mars,S:Kristina, F:Starlight,UK:Global,Can7:Global, USA:HC120,Can1:Delta
Local check 3 (in Canada)	Cultivar Legend

FBN9301 Methods

The field trial was designed as a completely randomized block with four replicates (size of the plots, pathways, etc. in function of the cooperator). Non-transgenic oilseed rape was planted on border zone around the trial site.

The trials were sown with a precision drill at normal plant density. Emergence and establishment were noted (subjective rating). Number of plants before and after Basta treatment (5l/ha) in the NMS B91-4, RF B93-101 and their hybrid plots were counted.

Flowering dates (beginning and end) and the number of male sterile and male fertile plants of 100 plants per plot were scored. Ten inflorescences in the male sterile plots were isolated. The absence of seed set in these individual isolations is a strong indication for male sterility, stably expressed during the season.

Any differences in plant growth, vigor or morphology were noted. Plots were harvested. The obtained seeds were cleaned and weighed and yield was determined.

FBN9301 Results and conclusions

FBN9301 Results

FBN9301$_1$ Germination of the plants : number of plants per square meter before Basta treatment (mean of four replicates)

Genotype	B	UK	USA	Can01	Can07	Sweden
B91-4	88	85	127	63	104	197
B93-101	57	90	77	51	64	229
B91-4 x B93-101	82		119			
Drakkar	77	97	115			
Drakkar 1/2	38	61	63			
Local check 1	93	49	154			
Local check 2	90	56	230			

FBN9301$_2$ Basta segregation results : percentage of phosphinothricin tolerant plants (mean of 4 reps)

Genotype	B	F	UK[1]	USA	Can01	Can07	Average[2]
B91-4	50	51	67	41	52	50	**52**
B93-101	100	100	100	100	100	100	**100**

Remark 1 : these aberrant segregation figures can be result of the very low establishment percentages (varying between 19 and 23 % of the planted seeds)

Table FBN9301$_3$ Vigor

Genotype	B[1]	USA[3]	Can01[2]
B91-4	2.8--	6.5	4.75
B93-101	3.0-	6.8	8.5
B91-4 x B93-101	4.3	7.5	9.00
Drakkar	4.0	7.3	8.50
Drakkar 1/2	3.0-	6.3	7.75
Local check 1	5.0*	7.8	10.00
Local check 2	5.0*	8.3	9.75
Local check 3			9.50

Remarks : [1] : 1 to 5 scale (1 bad - 5 good)
- means significantly lower than general mean at the 0.05 level (-- at the 0.01 level)
* means significantly higher than general mean at the 0.05 level (** at the 0.01 level)

[2] : 1 to 10 scale (1 bad - 10 good)

[3] : 1 to 9 scale (1 bad - 9 excellent)

Table FBN9301$_4$ Number of days to flower

Genotype	B	F	USA	Can01	Can07	Sweden
B91-4	51.0	55	59.5	63	57	59
B93-101	51.0	53.5	59.5	63.5	55	58
B91-4 x B93-101	50.0	54	59	64	55	58
Drakkar	50.0	53	59	62.5	54	57
Drakkar 1/2	49.3	54	59	63	55	57
Local check 1	44.0	52	58	57	48	53
Local check 2	48.0	49	51	58	53	52
Local check 3				56		

UK : All modified lines uniform but some 3 days later than Drakkar

Table FBN9301$_5$ Number of days to end of flowering

Genotype	B	USA	Can01	Can07	Sweden
B91-4	76.3	80	90	77	80
B93-101	76.0	81	86	75	82
B91-4 x B93-101	76.3	81	85.5	75	81
Drakkar	75.0	80	84	73	80
Drakkar 1/2	76.5	80	87	76	80
Local check 1	75.0	81	82.5	62	79
Local check 2	75.0	77.5	84	71	77
Local check 3			82.5		

B91-4 was flowering significantly longer than the checks in Can1.

Table FBN9301$_6$ Fertile/Sterile segregation in stability trial (mean of 4 reps) :

Genotype	B	F	UK	USA	Can07	Sweden	Average
B91-4	0/100	0/100	0/100	1/99	0/100	0/100	**0/100**
B93-101	100/0	100/0	100/0	100/0	100/0	100/0	**100/0**
B91-4 x B93-101	100/0	99/1	100/0	99/1	99/1	94/6	**98/2**

Table FBN9301$_7$ Number of plants with seed set / number of pods with normal seed set

Genotype	B	UK	USA	Can07	Sweden
B91-4	10/0	7/3	0/0	3/0	20/not determined

Table FBN9301$_8$ Number of days to maturity

Genotype	Can01	Can07	Sweden
B91-4	129	103	134
B93-101	123	100	133
B91-4 x B93-101	122	100	132
Drakkar	123	99	131
Drakkar 1/2	124.5	101	131
Local check 1	106	86	129
Local check 2	107	96	127
Local check 3	106		

Remark : in B, UK, F and USA no important differences in maturity were observed between the different entries

Table FBN9301$_9$ Seed yield (kg/ha)

Genotype	B	F	UK	USA	Can01	Can07	Sweden
B91-4	2873-	1647--	2030--	2259	1401-	2821	1668
B93-101	3009	1832	2340	2069	1679	3127	1643
B91-4 x B93-101	3264	1920	2540	2733	1539	2733	1644
Drakkar	3323	1961	2550	2144	1709	2979	1616
Drakkar 1/2	2620--	1461--	2200--	2000	1608	2983	1629
Local check 1	4206**	2882**	2090--	2408	1574	2538	2120**
Local check 2	3355	2699**	2350	2599	1622	1356--	2070**
Local check 3					1525		
C.V. %	8.6	7.4	5.2	11.6	17.07	11.4	7.5

- means significantly lower than control (Drakkar) at the 0.05 level (-- at the 0.01 level)
* means significantly higher than control (Drakkar) at the 0.05 level (** at the 0.01 level)

FBN9301 Conclusions

Basta-segregation of all transgenic lines was as expected. The male sterile line was somewhat less vigorous than all other entries. It also needed 0 to 3 days more to start flowering than Drakkar, whereas the restorer line and the combination flowered as early as Drakkar (maximum 1 day delay). The male sterile trait was stable over all the different environments and throughout the flowering period. This was confirmed by the results on seed set in isolation bags. Whereas an isolation of a fertile plant could yield 400 well filled pods, only a maximum of 20 pods were found on the male sterile plants. Few were filled with non-viable seeds.

Restoration level in the B91-4 x B93-101 combination was adequate, i.e. more than 95% if the plants were fertile.

The male sterile lines needed 3 to 7 days more to mature, when compared to Drakkar, and this only in the canadian and scandinavian locations.

In most of the locations yield of the male sterile lines was significantly lower than the one of Drakkar, but similar to the one of Drakkar sown at 1/2 density. This observation was not surprising since the male sterile plant density was also half of the normal oilseed rape density as a consequence of the phosphinothricin selection. In addition, the male sterile plants completely rely on external pollination. No differences in yield were found for the restorer and restored combinations in any of the locations.

EXPERIMENT FBN9302 **Phosphinothricin tolerance used as selectable marker in production schemes**

Authorization numbers : BIOT/93/M03 (Gent, Belgium)
93-PGS-CAN-01..04 (Saskatoon, Canada)

Responsible : ir G. De Both, PGS Product Development Manager Oilseeds
T. Schuler, PGS Oilseed rape breeder

FBN9302 Goal of the experiment

Evaluation of phosphinothricin tolerance as selectable marker in production schemes.

FBN9302 Plant material

Table FBN9302 Origin of the plant material

Plant material	Origin of the plant material
Drakkar	Cultivar
B91-4	T_3 generation of B91-4
B93-101 (homozygous)	S_3(B93-101)

FBN9302 Methods

The field trial was designed as a 8x3 factorial split plot (by genotype) (main factor phosphinothricin rate) and contained two replicates. The trial contained 48 plots of 6 m by 1.8 m (6 rows).

Seeds were sown conform to normal agricultural practices (seed density of approximately 90 seeds per square meter). Plants were sprayed at the four leaves stage with phosphinothricin at the following rates :

0 l/ha (Spraying liquid volume = 500 l/ha)
0.625 l/ha (0.125%)
0.125 l/ha (0.25%)
2.5 l/ha (0.5 %)
5 l/ha (1 %)
10 l/ha (2 %)
20 l/ha (4 %)
40 l/ha (8 %)

The number of plants before and after phosphinothricin treatment was recorded. Flowering dates (beginning and end) and the number of male sterile and male fertile plants per plot were scored. Any differences in plant growth, vigor or morphology were noted. Plots were harvested with a plot combine. The obtained seeds were cleaned and weighed and yield was determined. Seed quality analysis (oil and glucosinolate content) were carried out.

FBN9302 Results and conclusions

FBN9302 Results

Table FBN9302$_1$ Percentage plants that survived the phosphinothricin-treatment

Plant material	Percentage of plants that survived the phosphinothricin treatment at the different rates							
	0	1/8	1/4	1/2	1	2	4	8
Drakkar Belgium	100	61	-	0	0	0	0	0
Canada	100	56	50	0	0	2	1	0
B91-4 Belgium	100	93	45	45	34	44	44	42
Canada	100	73	56	62	53	42	47	50
B93-101 Belgium	100	100	100	100	100	100	100	100
Canada	100	100	100	100	100	100	99	100

Remark : Although most susceptible plants did not survive the treatment, regrowth and late germination has been observed after this assessment.

Table FBN9302$_2$ Vigour* of the male sterile and fertility restorer oilseed rape under different concentrations of phosphinothricin applications

Plant material	Vigour of the plants at different phosphinothricin rates							
	0	1/8	1/4	1/2	1	2	4	8
Drakkar Belgium	4.0	2.0	1.5	1.5	1.0	1.0	-	-
Canada	7.5	8.5	5.5	4.5	2.5	1.0	1.0	1.0
B91-4 Belgium	3.5	2.5	3.0	2.5	3.0	2.0	2.0	1.5
Canada	8.0	6.5	8.0	8.0	7.0	7.5	8.5	8.0
B93-101 Belgium	4.5	4.5	5.0	5.0	4.0	4.0	2.5	2.0
Canada	8.5	8.0	7.5	9.0	8.0	8.5	7.5	9.0

* : Remark : scale Belgium : 1 to 5 : 1=bad, 5=good, observations done just before flowering
scale Canada : 1 to 10 : 1=bad, 10=good, observations done just before flowering

Table FBN9302$_3$ Plant height (cm) (measured at end flowering) of the male sterile and fertility restorer oilseed rape under different concentrations of phosphinothricin applications

Plant material	Plant height at different phosphinothricin rates							
	0	1/8	1/4	1/2	1	2	4	8
Drakkar Belgium	145	142	128	117	101	96	-	-
Canada	140	134	114	100	77	65	62	62
B91-4 Belgium	146	149	150	145	147	144	142	134
Canada	134	127	126	126	124	129	125	121
B93-101 Belgium	150	152	152	153	148	146	143	139
Canada	137	134	135	135	130	135	125	126

Table FBN9302$_4$ **Number of days to flower for the male sterile and fertility restorer oilseed rape under different concentrations of phosphinothricin**

Plant material	Number of days to flower after different phosphinothricin rates							
	0	1/8	1/4	1/2	1	2	4	8
Drakkar Belgium	49	50	50	50	50	NF	NF	NF
Canada	63	63.5	70	87.5	87.5	NF	NF	NF
B91-4 Belgium	50	50	50	50	49	50	50	50
Canada	62.5	63	62	62.5	62.5	63	63	63
B93-101 Belgium	49	49	49	49	50	49	49	49
Canada	63.5	63	63.5	62	63	63	63.5	64

NF : No flowering during observation period

Table FBN9302$_5$ **Number of days to end of flowering of the male sterile and fertility restorer oilseed rape under different concentrations of phosphinothricin**

Plant material	Number of days to end flowering after different phosphinothricin rates							
	0	1/8	1/4	1/2	1	2	4	8
Drakkar Belgium	76	78	81	NE	NE	NF	NF	NF
Canada	84.5	88.5	89.5	NE	NE	NF	NF	NF
B91-4 Belgium	76	77	78	79	78	79	79	81
Canada	86	87.5	87.5	89.5	89.5	90	89	90
B93-101 Belgium	76	76	76	76	76	76	77	77
Canada	85.5	83.5	85.5	85.5	87	87	87.5	87.5

NF : No flowering during observation period
NE : Plants did not end flowering before end of trial

Table FBN9302$_6$ **Flower phenotype segregation ratio**

Plant material	Percentage male fertile plants after different phosphinothricin rates							
	0	1/8	1/4	1/2	1	2	4	8
Drakkar Belgium	100	100	100	100	100	-	-	-
B91-4 Belgium	58	38	14	0	0	0	0	0
B93-101 Belgium	100	100	100	100	100	100	100	100

Table FBN9302$_7$ Yield (kg/ha at 9% moisture)

Plant material	Yield (kg/ha) after different phosphinothricin rates							
	0	1/8	1/4	1/2	1	2	4	8
Drakkar Belgium	3411	2882	1534	1701	313	-	-	-
Canada	1392	1743	1202	1407	702	-	-	-
B91-4 Belgium	3415	3003	2984	3054	3124	3097	2891	3102
Canada	1305	1215	1778	1533	1425	1433	1426	1779
B93-101 Belgium	3683	3140	3489	3372	3267	3482	3292	3717
Canada	1622	1411	1530	1974	1416	1728	1502	1983

Table FBN9302$_8$ Seed quality analysis (oil %) of the male sterile and fertility restorer oilseed rape under different concentrations of phosphinothricin

Plant material	Oil % different phosphinothricin rates							
	0	1/8	1/4	1/2	1	2	4	8
Drakkar Belgium	43.44	43.07	42.98	42.05	38.61	-	-	-
Canada	50.21	46.63	49.68	45.61	41.98	-	-	-
B91-4 Belgium	43.48	44.21	42.88	43.38	43.66	43.75	43.63	44.25
Canada	50.56	50.81	44.73	45.28	48.76	46.87	45.11	42.61
B93-101 Belgium	44.12	42.85	43.42	44.02	43.26	42.53	43.99	43.47
Canada	46.88	45.82	47.65	47.68	49.18	44.17	46.93	43.92

Table FBN9302$_9$ Seed quality analysis (glucosinolate content) of the male sterile and fertility restorer oilseed rape under different concentrations of phosphinothricin

Plant material	Glucosinolate (µmol/g) different phosphinothricin rates							
	0	1/8	1/4	1/2	1	2	4	8
Drakkar Belgium	10.74	11.88	10.91	14.52	14.66	-	-	-
Canada	13.00	14.95	20.05	17.10	19.65	-	-	-
B91-4 Belgium	13.88	13.37	15.24	13.65	15.36	13.02	14.55	12.97
Canada	15.10	14.15	13.00	22.20	22.80	24.65	25.90	15.95
B93-101 Belgium	10.82	10.30	10.49	10.51	10.65	11.23	10.25	11.08
Canada	15.50	16.70	16.90	12.50	18.45	19.60	16.10	14.45

FBN9302 Conclusions

The indicated field rate for Basta for the control of larger weeds is 7.5 - 10l/ha. Within this program, selection levels are standard determined at 5l/ha. The data obtained on the control variety confirm the presence of a natural tolerance level to phosphinothricin. The effect of a lower dosage does not result in a complete removal of non-transgenic plants. At higher doses, plants are clearly affected (see observations on vigor), but manage to survive and continue development, leading to smaller stressed plants. If no selection pressure is available, these plants can survive even up to 2 times the experimental field dose (see control variety in Table $FBN9302_3$). However, when the transgenic plants are competing, doses up to 1/4 of the experimental field doses can be tolerated (see Table $FBN9302_6$).

For the restorer and male sterile line, phosphinothricin-segregation was as expected, except for the lowest rate (1/8x), which did not give a complete selection (Table $FBN9302_1$).

Both the male sterile and restorer line are not influenced by the phosphinothricin-sprays, as can be deduced from the observations on vigor and plant height (Table $FBN9302_2$ and Table $FBN9302_3$).

Date of start flowering of both the male sterile and the restorer line was not affected by any of the phosphinothricin-treatments. The male sterile line prolonged flowering when sprayed at higher rates. The length of flowering period of the restorer line was only slightly affected (Table $FBN9302_4$ and Table $FBN9302_5$).

Fertile/sterile segregation was as expected for the 1/2 rate and all higher rate treatments (Table $FBN9302_6$).

No yield penalties were observed for the restorer line at the different spray-rates. In Belgium, the male sterile line showed a higher yield in the non-treated plots than in the non-sprayed, but this difference was not significantly different. In Canada, no differences were found (Table $FBN9302_7$).

Seed quality characteristics are not changed by any of the phosphinothricin-treatments (Table $FBN9302_8$ and Table $FBN9302_9$).

EXPERIMENT FBN9316 **Basta tolerance evaluation of the B91-4 male sterile and the B93-101 restorer line**

Authorization numbers : BIOT/93/M03 (Gent, Belgium)
93-PGS-CAN-01..04 (Saskatoon, Canada)

Responsible : ir G. De Both, PGS Product Development Manager Oilseeds
T. Schuler, PGS Oilseed rape breeder

FBN9316 Goal of the experiment

Basta tolerance evaluation of the B91-4 male sterile line and the B93-101 restorer line : to assess the effect of various field rates of Basta on the oilseed rape plants.

FBN9316 Plant material

Table FBN9316 Origin of the plant material

Plant material	Origin of the plant material
Drakkar	Cultivar
B91-4	T_3 generation of B91-4
B93-101 (homozygous)	S_3(B93-101)

FBN9316 Methods

The field trial was designed as a 6x5 factorial split plot (by genotype) (main factor Basta rate) and contained four replicates. The trial contained plots of 6 m by 1.8 m (6 rows). The male sterile plots were surrounded by pollinating plots containing Drakkar or the restorer. In Belgium, the Drakkar plots were not sprayed with Basta.

Seeds were sown conform to normal agricultural practices (seed density of approximately 90 seeds per square meter). Plants were sprayed at the four leaves stage with Basta at the following rates :

0 l/ha (Spraying liquid volume = 500 l/ha)
2.5 l/ha (0.5 % conc)
5 l/ha (1 %)
10 l/ha (2 %)
20 l/ha (4 %)

The number of plants before and after Basta treatment was recorded. Flowering dates (beginning and end) and the number of male sterile and male fertile plants per plot were scored (Belgium). Any differences in plant growth, vigour or morphology were noted. Plots were harvested with a plot combine. The obtained seeds were cleaned and weighed and yield was determined.

FBN9316 Results and conclusions

FBN9316 Results

Table FBN9316$_1$ Number of plants per square meter before and after Basta-treatment

Plant material	Number of plants before Basta				Percentage of plants that survived the Basta application			
	1/2x	1x	2x	4x	1/2x	1x	2x	4x
B91-4 Belgium	67.3	71.3	64.8	75.3	51.8	44.8	44.1	49.2
Canada	39.5	33.0	37.3	57.0	88.0	46.0	51.7	52.2

Table FBN9316$_2$ Vigour* of the male sterile and fertility restorer oilseed rape under different concentrations of Basta applications

Plant material	Plant vigour* after different Basta rates				
	0x	1/2x	1x	2x	4x
Drakkar Belgium	4.00				
Canada	5.75				
B91-4 Belgium	3.75	2.75	2.50	2.33	2.25
Canada	5.25	5.50	3.50	3.25	3.25
B93-101 Belgium	4.25	4.00	3.75	4.00	4.00
Canada	5.25	5.50	5.25	5.50	5.25

* : Remark : scale Belgium : 1 to 5 : 1=bad, 5=good, observations done just before flowering
scale Canada : 1 to 10 : 1=bad, 10=good, observations done just before flowering

Table FBN9316$_3$ Plant height (cm) of the male sterile and fertility restorer oilseed rape, observations done at the end of flowering

Plant material	Plant height (cm) after different Basta rates				
	0x	1/2x	1x	2x	4x
Drakkar Belgium	136				
Canada	136				
B91-4 Belgium	137	138	137	133	134
Canada	136	127	130	126	125
B93-101 Belgium	138	136	135	136	134
Canada	136	135	136	133	130

Table FBN9316$_4$ **Days to flower for the male sterile and fertility restorer oilseed rape**

Plant material	Days to flower after different Basta rates				
	0x	1/2x	1x	2x	4x
Drakkar Belgium	48.3				
Canada	62.0				
B91-4 Belgium	49.0	49.8	49.5	50.0	49.8
Canada	61.0	61.0	61.8	61.0	60.5
B93-101 Belgium	49.5	49.5	48.8	49.3	48.5
Canada	62.0	61.8	62.3	62.3	61.8

Table FBN9316$_5$ **Days till end of flowering for the male sterile and fertility restorer oilseed rape**

Plant material	Days till end of flowering after different Basta rates				
	0x	1/2x	1x	2x	4x
Drakkar Belgium	75.4				
Canada	85.0				
B91-4 Belgium	75.5	77.3	77.0	77.0	77.5
Canada	85.5	86.3	89.0	89.5	89.0
B93-101 Belgium	76.0	75.8	76.3	76.3	76.0
Canada	86.5	86.0	86.8	86.8	86.5

Table FBN9316$_6$ **Percentage fertile oilseed rape plants in the male sterile and fertility restorer oilseed rape plots in Belgium**

Plant material	Percentage fertile oilseed rape plants after different Basta rates				
	0x	1/2x	1x	2x	4x
Drakkar Belgium	100				
	55.1	0.5	0	0	0
B93-101 Belgium	100	100	100	100	100

Table FBN9316$_7$ Yield (kg/ha at 9% moisture)

Plant material	Basta rate 0x	1/2x	1x	2x	4x
Drakkar Belgium	3148				
Canada	1627				
B91-4 Belgium	2978	3019	3114	2693	2857
Canada	1323	1358	1358	1022	1043
B93-101 Belgium	3050	3060	3138	3091	2825
Canada	1621	1801	1799	1957	1730

FBN9316 Conclusions

Basta segregation
Basta segregation results (average over 4 reps, 2 locations) show a segregation ratio (resistant/susceptible plants) as expected.

Vigour
The restorer line seems to be not affected by the Basta-treatment. The male sterile line shows some effect : in Belgium, B91-4 looses 1 to 1.5 on the 1 to 5 scale, in Canada, B91-4 looses 2 points on the 1 to 10 scale (Table FBN9316$_2$).

Plant height
Plant height is not significantly influenced by the different Basta-treatments. Plant heights are comparable for the different genotypes over the range of concentrations used.
Yet in Canada, the male sterile line showed some effect of the herbicide spray : the treated plants were shorter (Table FBN9316$_3$).

Start of flowering
In Belgium, flowering of the male sterile line is retarded with maximum 1 day due to the Basta-treatments. Flowering of the restorer line was not influenced by the Basta treatment.
In Canada, flowering of none of the transgenic lines seemed to be retarded by the different Basta-treatments and none of them flowered later than Drakkar (Table FBN9316$_4$). .

Days till end of flowering
In Belgium, flowering of the treated male sterile line was prolonged with about 2 days, when compared to the non-treated genotype. Days till end of flowering of the restorer line was not influenced by the Basta treatment.
In Canada, flowering of the treated male sterile line was prolonged with 3 to 4 days, when compared to the non-treated genotype. Days till end of flowering of the restorer line was not influenced by the Basta treatment (Table FBN9316$_5$).

Male sterile/male fertile segregation ratio
Half of the normal Basta-rate still seems to give a nearly perfect selection (98 %). From normal dose on, the selection is perfect (Table FBN9316$_6$)..

Yield

Yield of the male sterile line seems to be affected by the higher Basta-rates (2x and 4 x normal dose). The different Basta-treatments did not affect the yield of the restorer lines. At the contrary, B93-101 performed somewhat better when treated (Table $FBN9316_7$), which could be attributed to an additional weed control aspect of the selective treatment.

Appendix VIII

Monitoring Program to Assess the Occurrence and Fate of SeedLink Canola Volunteers Following the 1999 Growing Season in Western Canada

Aventis Crop Science

Monitoring Program to Assess the Occurrence and Fate of SeedLink Canola Volunteers Following the 1999 Growing Season in Western Canada

2000 REPORT
October 2, 2000

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Aventis Crop Science Report Number
ACC00-03

Study Initiation Date
April 15, 2000

Proposed Study Termination Date
September 30, 2000

ABSTRACT

In 2000, the first year of a survey to assess the occurrence and fate of glufosinate tolerant canola volunteers following the 1999 growing season in Western Canada was conducted.
Seedlink (glufosinate tolerant hybrid canola) was commercialised in Western Canada in 1997 and was cultivated on greater than 500,000 hectares in 1999. The survey was conducted at 7 sites where glufosinate tolerant *Brassica napus* Seed Link canola was grown in 1999 in Saskatchewan and Manitoba. In addition, non-cropped areas and transportation routes in the vicinity of some of the survey sites were monitored. A total of four sites where non-transgenic canola was grown were included in the survey for comparison purposes. Volunteer canola plants were effectively controlled by the weed control program employed by the producer regardless of whether they were transgenic or not. The growers postemergent herbicidal weed control proved to be the most critical component for the control of volunteer control regardless of whether the volunteers were herbicide tolerant or not. Very few canola volunteers were found growing in non-cropped areas such as fencelines or ditches along the margins of fields where canola was grown in 1999. Canola plants were found growing in ditches along roads and rail beds in the vicinity of the survey sites. A follow up survey to be conducted in 2001 to further assess the ecological significance of these populations. Based on the first year of the survey, no evidence was found to suggest that Seed Link canola behaves any differently in managed and unmanaged environments than does standard, non-transgenic canola. The results from this survey are consistent with those reported earlier by (Deschamps et al, 1998) which reported on the persistence and control of glufosinate tolerant canola volunteers (LibertyLink).

1.0. INTRODUCTION

In the development of glufosinate tolerant canola, the potential for the crop to become a weed problem has been expressed as a concern Agriculture and Agri-Food Canada (AAFC), in assessing the environmental impact of glufosinate tolerant canola, have concluded that the herbicide tolerance trait has not bestowed any adaptive advantage on glufosinate tolerant canola in the absence of the herbicide as a selection pressure.

The potential for the glufosinate tolerance trait to outcross into wild species related to *Brassica napus* creating a new herbicide tolerant weed has also been expressed as a concern. Experiments conducted by AgrEvo and others have shown that *Brassica napus* will indeed outcross with related species (MacDonald and Manning, 2000). However, the frequency of outcrossing is extremely low and the viability or fertility of hybrids is very poor. In addition, the very low numbers of fertile hybrids can be controlled chemically by many other active ingredients or mechanically by cultivation. Given the above, AAFC has concluded that while gene flow from glufosinate tolerant canola to relatives is possible, it would not result in increased weediness or invasiveness of these relatives.

Despite the considerable body of evidence to the contrary, the concern that widespread production of Seed Link canola will result in a new weed problem remains. As part of our commitment to product stewardship Aventis Crop Science initiated in 2000 a survey of 1999 glufosinate tolerant canola production fields, adjacent non-agricultural areas, and transportation routes to document the occurrence and fate of *Brassica napus* volunteers. This is an interim report summarising the results of the first year of the survey.

2.0. OBJECTIVES

1. To assess the occurrence and fate of Seed Link *Brassica napus* canola volunteers in an agronomic environment.

2. To assess the occurrence and fate of Seed Link *Brassica napus* canola volunteers in a non-agronomic environment

3.0. METHODS

3.1. Overview.

The project was separated into 3 specific modules focusing on:
1) Cultivated areas including the fields in which Seedlink hybrid canola was grown in 1999,
2) Non-agricultural areas in the vicinity of 1999 Seed Link fields such as shelterbelts, fence lines, sloughs,
3) Transportation routes.

The first module was conducted at specified intervals over the growing season (detailed below). Modules 2 and 3 were conducted later in the growing season when seed bearing plants could be identified. The rationale behind delaying the assessments for Modules 2 and 3 was that from an ecological point of view, the only individuals of concern were those that successfully reproduced resulting in the potential for re-establishment of the population in the next season.

3.2. Module 1: Cultivated areas.

The approach chosen to assess the occurrence and fate of glufosinate tolerant *Brassica napus* canola and related weedy species was to systematically scout and sample 1999 Seed Link fields and adjacent fields throughout the 2000 growing season.

Seven (7) sites were selected where glufosinate tolerant canola (Seed Link) was grown in 1999 (Figure 2). To provide a comparison, additional four- (4) sites where non-transgenic canola was produced in 1999 were surveyed in a similar manner.

The specific survey methods were similar to those used to obtain the annual weed surveys published in the province of Saskatchewan (Douglas and Thomas, 1985) with a few modifications indicated below. Each field was surveyed using a set pattern. The survey began 100 paces along the

edge of the field and 100 paces into the field. At this point an inverted W pattern was used with five counts done per arm of the W, giving 20 counts (Figure 1). Each quadrat was placed approximately 20 paces apart. Figure 2 shows the pattern walked. The number of volunteers was counted in a quadrant 0.25 m^2 at each of the 20 locations in the pattern. One pattern was done for every 40 acres at a site.

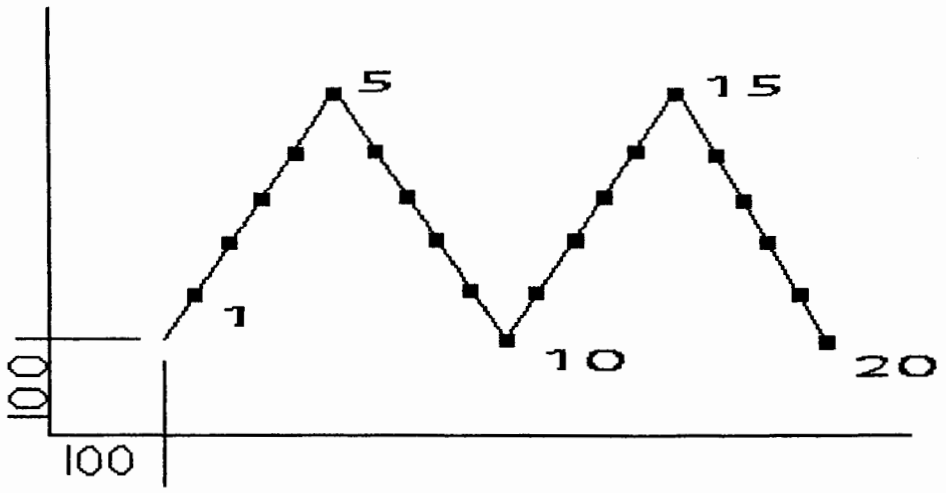


Figure 1

Assessments were taken at the following times:

1) Early spring prior to cultivation and seeding (early May),
2) Following tillage and/or premergent herbicide
3) Following crop emergence (June),
4) Following herbicide application (July),
4) After crop flowering (mid-August).

3.3. Module 2: Non-agricultural areas in the vicinity of 1999 Seed Link fields.

Appropriate non-agricultural areas in the vicinity of 1999 Seed Link fields were identified during the site selection process. Late in the growing season when *Brassica* and related species are in the reproductive stage, the non-crop areas were surveyed for their presence. The survey area was not extended more than 20 m from the margin of the field, as it is unlikely for seed to be spread a greater distance by wind or harvest operations. Five field margins were surveyed. Two along fields that were seeded to conventional canola in 1999 and three along fields that were

seeded to Seed Link hybrid canola in 1999. The area in the ditches that had not been cultivated was surveyed. The margin along one entire side of the field was the distance surveyed, this distance varied from 130 m to 450 m.

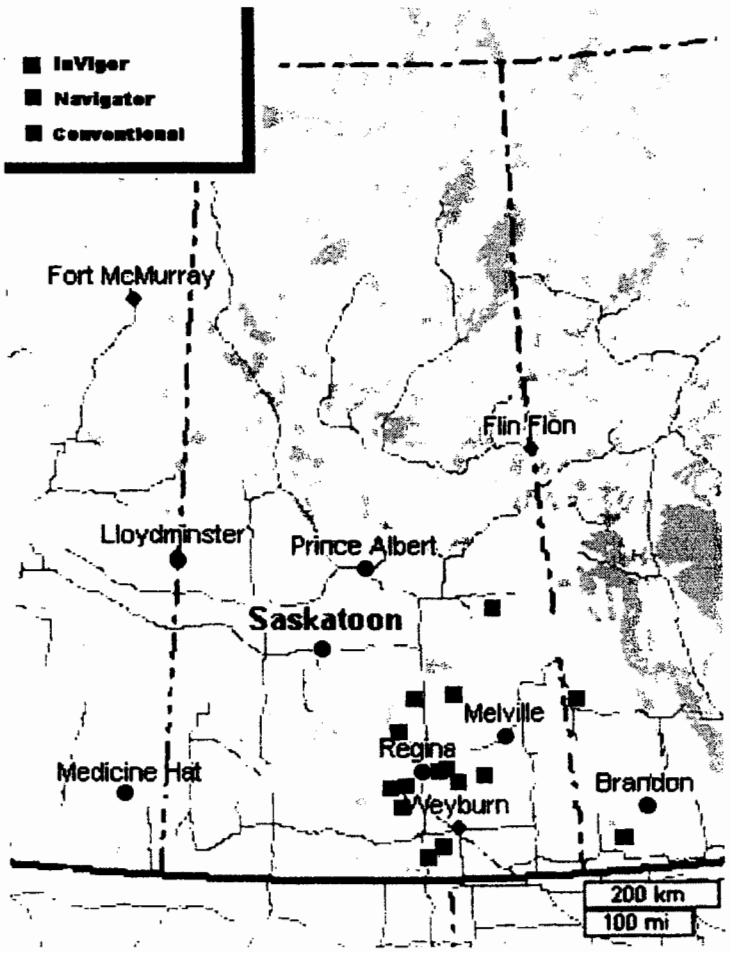
3.4. Module 3: Transportation routes.

Non Agricultural Environment:

Non agricultural areas such as roadsides and railway sides were chosen around elevators. The survey was conducted in late July to early September to ensure that only plants that would reach maturity would be counted. The method of surveying the volunteer canola in these areas differed according to the number of plants present and the level of difficulty encountered travelling through the survey area. If many plants were present, only periodic 100-m intervals were sampled. If there were relatively few plants present every plant was counted and sampled. If it was difficult to access sections of the survey area than periodic 100-m intervals were sampled and counted. Green tissue samples were taken in the field chilled and transported to the laboratory for evaluation by PCR to determine the identity of the harvested samples.

The areas chosen for roadside assessment were near the towns of Indian Head and Vibank. At Indian Head, two miles of railway side and 4.5 miles of grid roadside were surveyed. At Vibank, three miles of railway side, three miles of grid roadside, and three miles of highway side were surveyed.

Figure 2 shows the locations chosen.



4.0. RESULTS AND DISCUSSION

4.1. Module 1: Cultivated areas.

The 2000 field surveys where Seed Link canola was grown in 1999 revealed similar results to that found in surveys of standard canola fields (Figure 3). The following trends were common to both situations:

(1) There was a relatively high density of volunteer canola plants present early in the spring prior to any field operations in 2000;

(2) Initial soil disturbance while effective at controlling already emerged canola volunteers results in the emergence of an even greater number of volunteers as indicated by counts after tillage

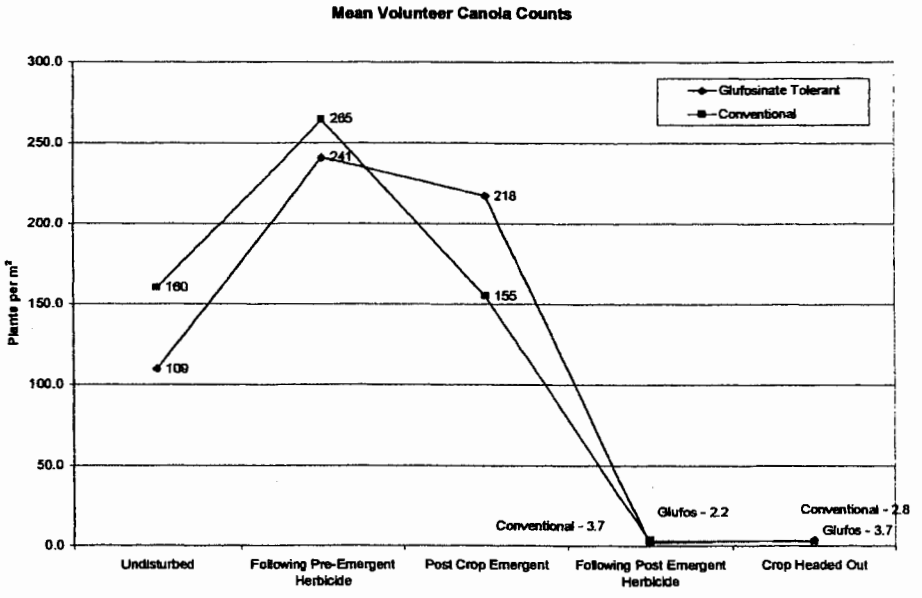
and crop emergence the time of survey taken following crop emergence;

(3) The post-emergent weed control program employed by the producer was effective in controlling canola volunteers present in the 2000 crop; the herbicide application reduced glufosinate tolerant volunteer canola counts from 218 to 2.2 plants per m2. The conventional canola showed a volunteer reduction from 155 to 3.7 plants per m^2.

(4) Few canola volunteers germinated late in the season following postmergence control

(5) There was no substantial difference between the 1999 Seed Link sites and the five non-transgenic sites used for comparison.

Figure 3



Two conclusions can be made based on the first year of the survey. In cropland situations, glufosinate tolerant canola cv. "Seed Link" appears to be no more weedy than standard canola varieties. This is in agreement with the earlier reported studies conducted by AgrEvo Canada Inc. (Deschamps and MacDonald, 1998). Of note is the fact that the herbicide program employed was entirely under the control of the producer at each site. No advice or special recommendations were given. The significance of this fact is that it demonstrates that volunteer herbicide resistant canola can be controlled using cultural and chemical practices that producers are currently familiar with. Secondly it can be

concluded that the growers postemergent weed control program was largely responsible for the control of volunteers canola in crop as the population continued to increase up to the time of postemergent herbicide application.

4.2. Module 2: Non-agricultural areas in the vicinity of 1999 Seed Link fields.

Unlike cultivated areas, adjacent non-agricultural areas are often unmanaged and therefore represent an opportunity for Seed Link escapes, if present, to establish and perpetuate the population. Canola seed can be disseminated to these areas by a variety of mechanisms: Canola windrows can actually be blown across or off of a field in a strong wind; or during harvest, the small fraction of seed that is not collected in the grain tank but is blown out the back of the combine with the canola straw and chaff can be spread into non-crop areas.

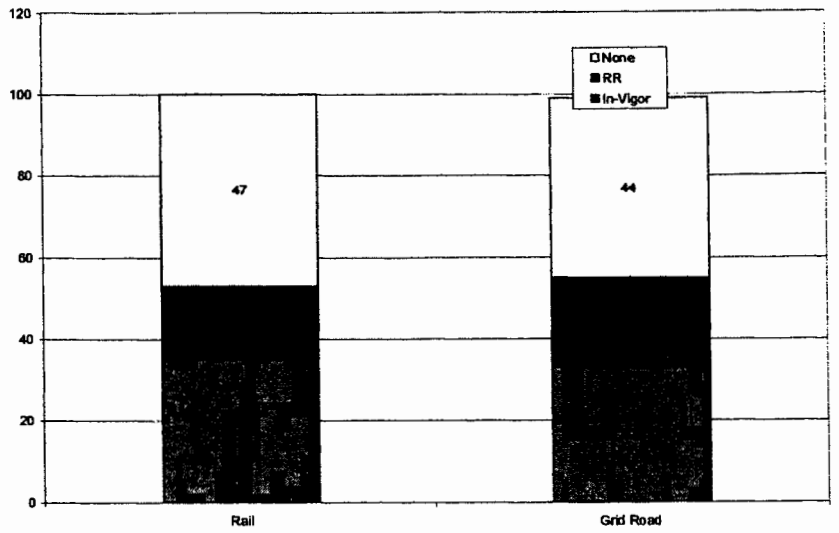
Canola volunteers growing in non-crop areas in field margins where Seed Link was grown in 1999 occurred infrequently in 2000. In total approximately 1300 m of field margins were surveyed for the presence of Brassica volunteers. No Brassica volunteers were detected in the field margins of any of the surveyed locations. In all cases field margins were dominated by perennial grasses leaving little opportunity for invasion by Brassica species. This result is consistent with previously reported results, which found that Brassica napus had limited invasive ability of feral areas where perennial plants presented significant competition.

4.3. Module 3:Transportation routes.

Although canola can and does grow in ditches, the probability of any plants producing seed to perpetuate the population is likely small. This survey was planned for late in the growing season so that any plants found would be well into the reproductive stage. This proved not to be the case. That is, nearly all of the plants sampled were still at the vegetative stage. Most ditches along rural roads in Saskatchewan are mowed periodically over the course of the summer. It is likely that mowing prevents plants from growing to maturity. However, mowing also may permit plants to establish as no plants were found in non-agricultural areas that did not have dome form of disturbance. Those found at the time of the survey were either very young, late germinating seedlings or plants that had been cut and were regenerating stems and leaves from axillary buds. Plants that are not yet flowering by September or October would not likely produce viable seed before the first killing frost and would not therefore perpetuate the population. Surveys of transportation routes were limited to two areas surrounding local grain terminals in the towns of Indian Head and Vibank Saskatchewan.

Indian Head:

In three miles of railroad leading away form the local grain elevator there were 287 volunteer canola plants. The plant were all limited to the interface between the gravel of the rail road bed and the tall grass of the rail right of way. Plants grew in a narrow band at the interface and were predominately located on the North side of the tracks. No plants were located either directly on the rail tracks or in the tall grass along the sides of the rail line. Approximately 5% of the plants were tissue sampled and analysed by PCR to determine the presence of herbicide tolerance alleles. The samples showed that the Barstar allele, which is a component of Seedlink canola, was present in six plants sampled. The Round-up Ready allele was in approximately 18% of the plants sampled and the remainder were not identified as either Roundup Ready or Seed Link. Plants located along the rail bed were flowering and would likely produce and shed seed however, their habitat would be limited to the interface between the grass and the rail bed.



A total of 7000 m of roadside was surveyed in the Indian Head area. A total of 13 volunteer canola plants were located in the ditches along the surveyed roadsides. All of the plants found were sampled for PCR analysis. The samples showed that 54% of the harvested plants contained the BarStar allele and therefore were SeedLink canola. The Round-up Ready allele was present in 15 % of the plants sampled. The volunteers growing on the roadsides were found mostly in grass that had been mowed. Tall roadside grass seemed to have hindered the growth of any volunteer canola.

Vibank:

At Vibank all of the canola plants that were found were sampled because of a low number of volunteers present. A total of 29 canola plants were identified. The samples showed that four of the plants were Seed Link. The Round-up Ready allele was present in eight plants. Sixteen of the plants were not Seed Link, Round-up Ready. The volunteer canola grew in the gravel of the railbed here unlike at Indian Head. No canola was counted outside of the gravel area surrounding the tracks.

Along the highway, the samples showed two glufosinate tolerant or Seed Link plants. The Round-up Ready allele was present in 9 plants. Two of the sampled plants were not glufosinate tolerant or Round-up Ready. Most of these plants grew on cracks in the shoulder of the highway; the plants were small and usually grew to a height of only 2-3 inches and were not likely to produce seed.

Along the grid road all 14 plants sampled were not glufosinate tolerant or Round-up Ready
All of these plants grew in mowed areas or in the margin between the grass and the gravel.

Clearly, *Brassica napus* volunteers can be found along roadsides and at the edge of railbeds in Western Canada.

Based on the presented results no specific type of canola appears to predominate in these environments as several different types of herbicide tolerant canola were identified in these environments. The distribution of the different types of canola cultivars in these environments is most likely influenced by the selection
of which lines e local farmers choose to cultivate.

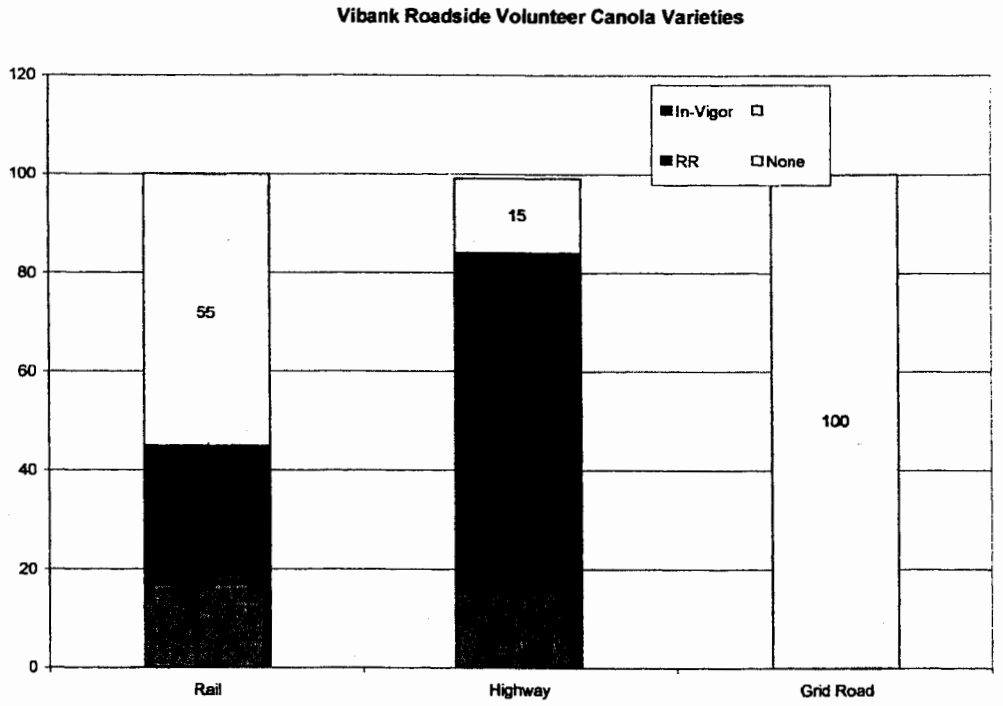


Figure 5.

Based on the results of the first year of this survey, no evidence was found to suggest that glufosinate tolerant hybrid canola behaves any differently in managed and unmanaged environments than does standard, non-transgenic canola.

5.0. REFERENCES

Douglas, D.W. and A.G. Thomas. 1986. Weed survey of Saskatchewan mustard, lentil and dry pea crops, 1985. Weed Survey Series Publication 86-2, Agriculture Canada, Regina, SK. 157 pp.

MacDonald, R. L. and R. J. A. Deschamps. 1998. Bringing Monitoring Concept in Practice. Fifth International Symposium on Biosafety Results from Field Trials of Transgenic Plants. Braunschweig, Germany.

Appendix IX

Molecular Characterization Reports for Transformation Events MS1/RF1/RF2

PLANT GENETIC SYSTEMS N.V.

A NEW HYBRIDIZATION SYSTEM IN OILSEED RAPE (*BRASSICA NAPUS*)

Annex

TABLE OF CONTENTS

PART 1 : ANNEX

Part 1. Annex VI Detailed description of experiments carried out to identify, select and characterize the B94-2 fertility restorer oilseed rape line

Part 1. Annex VI.1. General introduction to the experiments carried out in the laboratory, the greenhouse and the field to evaluate the *barstar* chimeric gene as inserted in the transgenic line B94-2

As was outlined in the Table 5 of Part 1.VI. (*General outline of the experiments carried out to identify, select and characterize the transgenic fertility restorer oilseed rape line B94-2*) and as will be described in detail in the following pages, Plant Genetic Systems has carried out many laboratory, greenhouse and field experiments to evaluate the *Brassica napus* fertility restorer B94-2 line over many generations (Table[VI] 1.) and under different environmental conditions (Part 1.VI. Table 5.). Special attention was given to the molecular characterization of the plants, their morphology, the linkage between the level of PPT tolerance and flower phenotype, the performance of the selectable marker, the normal agricultural performance, the stability of the flower phenotype throughout the season, the stability of the expression in different genetic backgrounds, the capacity to restore the fertility of the male sterile plants, the yield of the selected lines and the oil and seed quality.

Statistical analyses were performed on raw data. When analyzing segregations, a 2 x 2 test for independence was performed using, appropriate to the sample size, the Chi^2 test, the Fisher's exact test and/or the Yates correction. Complex data sets were subjected to analysis of variance (ANOVA) and in particular cases Least Significant Differences (LSD) were calculated according to Sokal, R.R. and Rohlf, F.J. (1981). (Biometry. 2nd edition. Freeman W.H. and company, New York, 859p)

Part 1. Annex VI.2. General regulatory procedures and safety measures taken into account when carrying out laboratory, greenhouse and field trials

Laboratory and greenhouse trials were carried out under contained use procedures, meaning that no viable transgenic material left the PGS facilities. Field experiments were carried out under inspection of the national governments, federal and/or provincial authorities of each country (e.g. Belgium, France, Sweden, United Kingdom, Canada, USA, Argentina) in which the experiment was performed. Every preparation of a trial included the submission of an application file to the competent authorities. Submitted data included information on the unmodified and modified oilseed rape, the used transformation procedure, the trial sites, the trial designs and protocols, monitoring and intentions for public consultations. Applications were based on the *EEC90/220 Directive Format*, the Canadian *Ag Canada Guidelines* or the *USDA-APHIS 2000 Format.*

Though all field trials were carried out following normal agricultural practices, safety measures were taken into account not only for regulatory reasons but also to ensure better reliability of our results :

- seeds were transported from the lab to the different locations in break-proof packages by a special courier or by PGS personnel; upon arrival, they were registered, identified and stored to avoid any mixture with other seed lots;
- during the preparation of the field trials, the experimental seed lots were treated with phytosanitary products as required by normal agricultural practices, weighed and divided for seeding the individual plots;
- seed bags were unequivocally labelled before the local responsible took them to the trial site;
- if sowing machines were used, it was guaranteed that the equipment was cleaned and controlled for the presence of any remaining seed; surplus transformed seeds were collected and returned for secure storage to the farm office;
- as it is imperative by the nature of oilseed rape, all trials were allowed to flower openly as to produce seeds;
- border plots were added at both ends of the experimental blocks; guard rows were surrounding the entire experiment resulting in a degree of biological containment; the material grown in the border rows was considered as experimental material; in some designs an additional border of fallow land was included to allow normal field work;
- to protect the crop from bird damage cannons and/or bird netting have been installed in some cases;
- the personnel responsible for the execution of the experiment ensured that all seeds were harvested; though seeds were harvested just before complete maturity, small quantities of seeds could have fallen on the ground; unrecoverable seeds were allowed to germinate whereafter the plants were destroyed with a total herbicide; in all cases, the area of 'accidental release' was marked for further monitoring; the remaining plant material was destroyed by cultivation and left on the site;
- the bulk quantity of the harvested seed was either immediately destroyed by heating and/or burning or bagged in polyethylene bags for further analysis; none of the harvested products was used for commercial feed or food production; however, some larger batches could be required for additional safety tests.

Part 1. Annex VI.3. Basic characterization of the fertility restorer B94-2 oilseed rape line in the laboratory

Part 1. Annex VI.3.1. Detailed description of routinely used molecular techniques :
- Southern hybridization
- Polymerase Chain Reaction

Part 1. Annex VI.3.2. Primary characterization of the fertility restorer B94-2 line in the laboratory

Part 1. Annex VI.3.3. Detailed characterization of the fertility restorer B94-2 line in the laboratory

Part 1. Annex VI.3.1. Detailed description of routinely used molecular techniques :

- Southern hybridization
- Polymerase Chain Reaction

SOUTHERN HYBRIDIZATION PROCEDURE

Introduction

Total genomic DNA is isolated from plant tissue according to Dellaporta et al. (1983). A fraction of the isolated DNA is digested with appropriate restriction enzymes and the digested DNA fragments are separated by electrophoresis in agarose. After a depurination step, the fragments are denatured and transferred to nylon filters. The DNA fragments attached to the membranes are hybridized with P^{32}-labeled purified DNA fragments. Subsequently, the membranes are washed and the hybridizing bands are visualized by autoradiography. Based on the mobility of the respective fragments, results are interpreted.

Preparation of total genomic DNA (according to Dellaporta et al. (1983). Plant Molecular Biology Reporter, 1, 3, 19-21)

- Weigh 0.5 g of leaf tissue, freeze in liquid nitrogen, grind with morter and pestle, and transfer the powder into a 30 ml Oak Ridge tube.
- Add 15 ml extraction buffer (100 mM Tris.HCl pH 8, 50 mM EDTA, 500 mM NaCl, 10mM β mercaptoethanol).
- Mix well.
- Add 1 ml 20% SDS, vortex and incubate at 65°C for 10 min.
- Add 5 ml 5 M potassium acetate, vortex and leave at 0°C for 20 min.
- Spin tubes at 25000 x g (gravitation constant) for 20 min (13000 rpm in Sorvall SA 600 rotor). Pour supernatant trough Miracloth filter (Calbiochem) into a clean 30 ml tube, containing 10 ml isopropanol. Mix and incubate at -20°C for 30 min .
- Pellet the DNA at 20 000 x g for 15 min.
  Gently pour off the supernatant and dry pellets by inverting the tubes on paper towels for 10 min.
- Redissolve DNA pellets with 700 µl of TE20 buffer (50mM Tris.HCl pH8, 20mM EDTA), and transfer to an Eppendorf tube.
- Add 2.5 µl RNase (10 mg/ml) and incubate for 10 min at 37°C.
- Spin tubes for 10 min in an Eppendorf centrifuge to remove insoluble debris.
- Transfer the supernatant to a new eppendorf tube and add 75 µl 3 M sodium acetate and 500µl isopropanol. Mix well and pellet the DNA for 30 seconds in an eppendorf centrifuge.
- Wash pellets with 80% ethanol, dry and redissolve DNA in 100 µl TE buffer (10 mM Tris.HCl pH 8, 1 mM EDTA).
- Determine the concentration of the DNA by measuring the UV absorbance at 260 nm.
- Use the required genomic DNA amount to set up the digestion.

Restriction digests of total genomic DNA

- Mix together in an eppendorf tube :
  10 µg of genomic DNA
  4 µl 10 x RE buffer
  20 units of restriction enzyme
  H_2O to 40 µl
- Incubate digest overnight in an oven at recommended temperature.

composition of 10 x RE buffer

- 100 mM Tris.HCl pH 8
- 50 mM $MgCl_2$
- 60 mM β mercaptoethanol
- 1 mM EDTA
- 1 mg/ml BSA

RE 50 - 0.5 M NaCl
RE 100 - 1 M NaCl
RE 150 - 1.5 M NaCl

Separation of the restriction fragments on agarose gels

- Prepare 1% agarose gel in TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8 with acetic acid), containing 0.3 µg/ml Ethidium Bromide.
- Pour the gel into the gelsupport and let solidify.
- Add 5 µl of loading dye to the digested DNA samples and load the gel. Include a marker (lambda DNA digested with restriction endonuclease PstI on the gel
- Run the gel at an electric currency of 20 mA overnight .
- Cover the gel with Saran-wrap after the samples have migrated about 1 cm into the gel.

Blotting of the restriction fragments on nylon membranes

- After electrophoresis is completed, cut the gel from the support and photograph the gel. Place a transparent ruler alongside the gel so that the migration distance of the fragments can be read directly from the photograph.
- Blot the separated DNA fragments on Nylon membrane by capillary transfer or by vacuum transfer.
  * Depurination of the gel : put the gel in 0.25 M HCl until the dyes have changed colour.
  * Alkali transfer : transfer is done in 0.4 M NaOH (for capillary blotting) or 1 M NaOH (for vacuum blotting)
  * Membrane : Hybond - N+
  * Duration of transfer : minimum 3 hours to overnight for capillary transfer; 1 hour for vacuum transfer.
  * Rince the membrane briefly in 2 x SSC buffer (20 x SSC = 3M NaCl + 0.3M Sodium Citrate), wrap in Saran-wrap and store at 4°C.

Purification of fragments for probe preparation

- Digest +/- 20 µg of the plasmid DNA with the appropriate restriction enzyme as to generate the desired double stranded DNA fragment.
- Separate the DNA fragments on a 1% Low Melting Agarose gel, prepared in TAE buffer and containing 0.3 µg/ml Ethidium bromide.
- After electrophoresis is completed, cut the desired fragment from the gel with a scalpel. Put the gel slice in an Eppendorf tube.
- Add an equal volume of TE buffer (10mM Tris.HCl pH8, 1mM EDTA) to the gel

slice.

- Melt the gel slice in a 65°C waterbath for 10 min.
- Preheat an equal volume of phenol (equilibrated with TE buffer) 30 sec. at 65°C.
- Add the phenol to the melted gel slice and put the mixture on an Eppendorf shaker for 15 min.
- Centrifuge for 10 min in an Eppendorf centrifuge to separate the two phases.
- Transfer the water phase to a new Eppendorf tube and extract for a second time with an equal volume of phenol.
- Precipitate the DNA from the water phase with 0.1 volume of 5 M Sodium perchlorate and 1 volume of isopropanol.
- Pellet the precipitated DNA by spinning for 15 min in an Eppendorf centrifuge.
- Dry pellets and redissolve in 50 µl of TE.
- Measure the concentration of the DNA solution and dilute with H_2O, to a concentration of 25 ng/µl.

Labeling the DNA fragment with radioisotope (according to Feinberg and Vogelstein (1983) Analyt. Biochem., 132, 6-13 and Feinberg and Vogelstein (1984). Analyt. Biochem., 137, 266)

- Mix 25 ng DNA fragment + H_2O (total volume = 12 µl) in an Eppendorf.
- Denature the DNA fragment for 5 min in a boiling waterbath and cool quick in ice-water.
- Add to the tube :

  18 µl LS buffer (*)
  1 µl 5 mg/ml BSA (DNAse free)
  3 µl 1 mM dATP, dGTP, dTTP
  4 µl αP^{32} dCTP (specific activity = 3000 mCi/mmole)
  2 µl Klenow DNA Polymerase (5U/µl)

  40 ul

- Leave at room temperature for 5 hrs.
- Remove the unincorporated nucleotides by purifying the labeled DNA fragment over a BIO-RAD biospin-30 column.

* Composition of the LS buffer:
Mix together:

- 25 µl 1M HEPES pH 6.6
- 25 µl TM buffer (250 mM Tris.HCl pH 8, 25 mM $MgCl_2$, 50 mM β-Mercaptoethanol)
- 7 µl OL (45 O.D. units Hexamers /ml TE - PL Biochemicals)

Hybridization of the membrane with the labeled probe

- Make up a hybridization solution :

  6 x SSC (20 x SSC = 3 M NaCl + 0.3 M Sodium citrate)
  5 x Denhardt's solution(100 x Denhardt's = 2% BSA + 2% Ficoll + 2% PVP)
  0.5% (w/v) SDS
  20 µg/ml denatured sonicated Herring Sperm DNA

- Prehybridize the membrane at 65°C for minimum 1 hour.

- Denature the labeled probe by heating for 5 min. at 95°C.
- Replace the hybridization solution and add the denatured probe (do not exceed a probe concentration of 20 ng/ml)
- Hybridize at 65°C overnight
- Upon hybridization, wash the filters as follows:
  * 15 min in 6 x SSC
  * 30 min in 2 x SSC / 0.1% SDS
  * 30 min in 0.1 x SSC / 0.1% SDS
- Remove excess of washing solution from the membrane (probed membranes may not dry out after hybridization) and wrap in Saran-wrap.

Autoradiography

- Put the membrane, wrapped in Saran-wrap into the X-ray cassette , between two Kodak intensifying screens.
- Expose a Kodak X-ray film to the membrane for an appropiate time period at -70°C.
- Develop film in a X-ray film processor.

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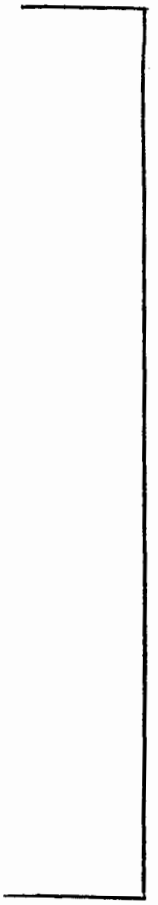

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Part 1. Annex VI.3.3. Detailed characterization of the B94-2 fertility restorer line in the laboratory

* Detailed characterization of the selected lines

- L03 Determination if *Agrobacteria* are present in the transgenic oilseed rape lines
- L04 Stability of integration of the T-DNA over a number of generations via Southern blot analysis
- L05 Confirmation that the inserted DNA corresponds to the plasmid T-DNA
- L06 Characterization of the T-DNA integration via molecular analysis of the regions flanking the T-DNA
- L07 Molecular analysis of the target-site-deletion present on the homologous chromosome
- L08 Homology search between plant DNA sequences and the B94-2 target sequences
- L09 Expression of the introduced genes via Northern analysis and evaluation of occurrence of cryptic gene expression
- L10 Assay of NPTII over a number of generations
- L11 Assay of PAT activity over a number of generations

* Conclusion

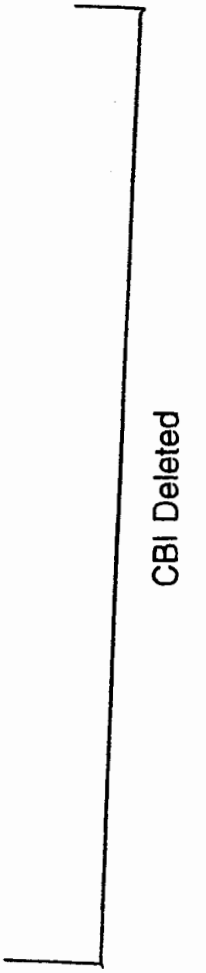
- The descendants of the B94-2 transformant are free of *Agrobacterium tumefaciens*.
- There have been no rearrangements of the T-DNA upon insertion in the plant genome.
- The inserted T-DNA remains genetically stable in distinct generations
- The insertion site has been characterized.
- There is no indication of the insertion of the T-DNA in a functional gene.
- The expression of the inserted T-DNA is limited to the introduced coding sequences. There is no indication of expression of other newly inserted sequences (cryptic genes).
- NPTII and PAT activity of the transgenic lines have been assessed. The level of activity remains constant over the tested generations.

POLYMERASE CHAIN REACTION (PCR) PROCEDURE

Preparation of Plant Genomic DNA

The rapid extraction of small amounts of plant genomic DNA suitable for PCR analysis is done according to the method described by Edwards et al. (K. Edwards et al., Nucleic Acids Research, **19** (6), page 1349, 1991).

- Collect samples for PCR analysis (usually leaf tissue) by using the lid of a Eppendorf tube to pinch out a disc of material into the tube.
- Macerate the tissue with a plastic pestle at room temperature, without buffer for 5 to 15 sec.
- Add 400 µl extraction buffer (EB: 200 mM Tris HCl pH 7.5, 250 mM NaCl, 25mM EDTA, 0.5% SDS). The mixture can be left at room temperature until all samples have been extracted (> 1 hour).
- Centrifuge the extracts for 1 minute at max. speed and transfer 300 µl of the supernatant to a fresh Eppendorf tube.
- Mix with 300 µl isopropanol and leave a room temperature for 2 minutes.
- Centrifuge at max. speed for 5 minutes.
- Dry pellet and dissolve in 100 µl water.
- Centrifuge for 2 minutes and transfer supernatant to a new Eppendorf tube.
- Use 5 µl of this sample in a 50 µl PCR reaction.



EXPERIMENT L01 : Primary evaluation of a number of original fertility restorer transformants

Authorization number : Contained use

Responsible : Dr C. Mariani, PGS NV Project leader Nuclear Male Sterility and Restored Fertility
M. De Beuckeleer, PGS NV Senior Researcher

L01 Goals of the experiment

Primary evaluation of some additional original candidate fertility restorer oilseed rape transformants on laboratory scale :

- screening the transformants for copy number of their gene constructs,
- characterization of the transformants by specific bonds crosshybridizing with the probes.

L01 Plant material

All *Agrobacterium* mediated transformation experiments with the vector pTVE74RE containing the fertility restorer gene *barstar* (see 1.III.), were conducted in spring oilseed rape (SOSR), variety Drakkar.

L01 Methods

Shoots regenerated on selective media. All the plantlets going to be transferred to the greenhouse, were first analysed for the presence of the chimeric gene and the number of the insertions by Southern blot hybridization.

Total DNA was isolated from 1 g of shoot tissue according to Dellaporta et al. (1983). DNA was prepared from each individual transformant and from an untransformed plant of the same cultivar Drakkar. Southern analyses were performed according to the general protocol as given in Annex VI.3.1. [ ] CBI Deleted

L01 Results and conclusions

All plants have been analysed genotypically for the presence of the chimeric *barstar* gene (Table L01). Several transformants, including transformant B94-2, contained a single copy integration of the gene of interest. All individuals were identified by their hybridization pattern (see example in Figure 1.).

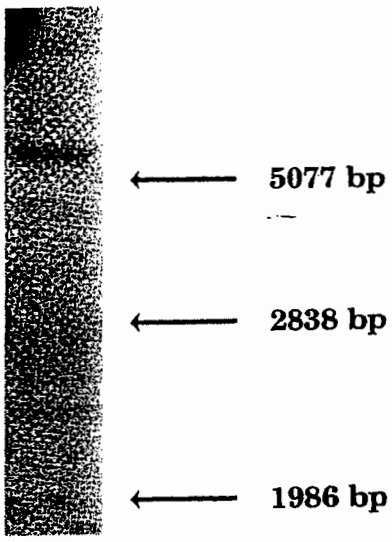


Figure 1. Autodiagram from a Southern blot carrying total genome DNA from B94-2

Table L01. Primary characterization of the fertility restorer Drakkar transformants

Transformation code number	Copy number by Southern analysis	RFLP bands by Southern analysis kb
B94-2	1	5.20

EXPERIMENT L02 : Molecular analysis of the integration of the chimeric gene constructs in the transgenic fertility B94-2 line

Responsible : M. De Beuckeleer, PGS NV Senior Researcher

L02 Goals of the experiment

Molecular analysis of the integration of the chimeric gene constructs in the transgenic fertility restorer B94-2 line.

L02 Plant material

Greenhouse grown plants of the S_1 progeny of B94-2 line and of an untransformed plant of the same cultivar were analyzed in addition to the male sterile B91-4 line, the fertility restorer B93-101 line and some other transgenic lines.

L02 Results and conclusions

L02 Results

Three oligonucleotides were designed based on the nucleotide sequence of pTTM8RE and pTVE74RE at the left border repeat to be used as primers in DNA amplification. Two primers are localized within the transferred region comprised between the border repeats, whereas the other primer is localized immediately outside from the left border repeat.
It was expected that upon proper recognition of the left border repeat, using total genomic DNA as template, an amplified fragment would be obtained using[ ] as pair of primers, whereas no amplified fragment should be obtained using[ ] as pair of primers. To verify the proper reaction conditions for DNA amplification, the same pair of primers were also used in reactions with total plasmid DNA as template. The amplification reactions indicate that no sequences beyond the left border repeat have been transferred to the plant genome.

L02 Conclusions

The molecular analysis showed that sequences outside from the T-DNA left border repeat are not co-transferred with the chimeric gene constructs comprised between the border repeats.

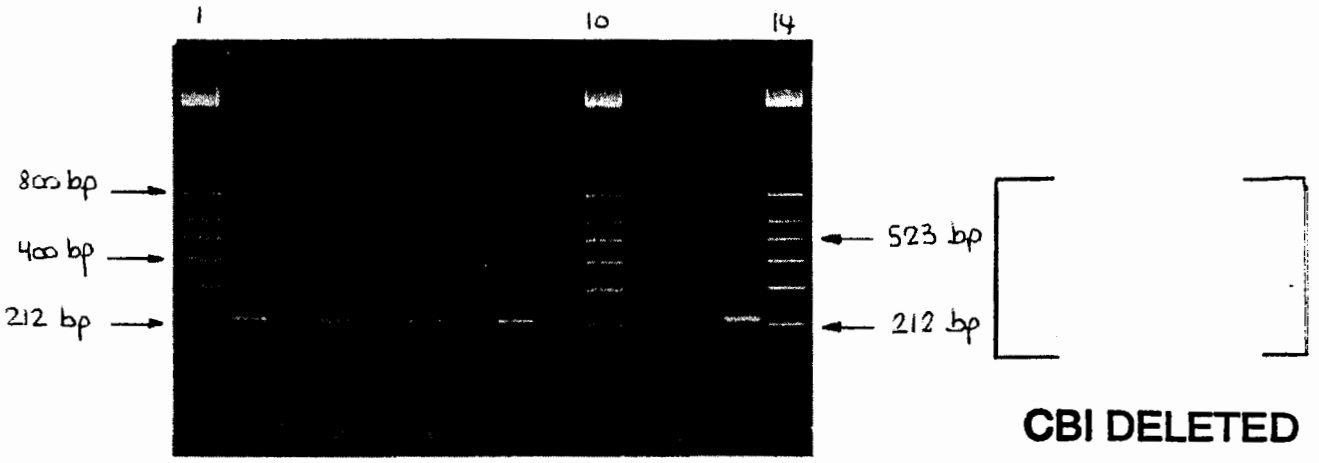


Figure L02. PCR results

DNA was isolated from oilseed rape transformants. Master mixes, containing either primers [redacted] were added to 50 ng genomic DNA. The PCR reaction was thermocycled for 27 cycles. Twenty µl of each PCR reaction was loaded on a 1.5 % agarose gel.

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Loading sequence of the agarose gel:

1. 100 bp MW ladder
2. B91-4
3. B91-4
4. MS3
5. MS3
6. B93-101
7. B93-101
8. **B94-2**
9. **B94-2**
10. 100 bp MW ladder
11. Drakkar control
12. Drakkar control
13. pTTM8RE
14. 100 bp MW ladder

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L03 Determination if *Agrobacteria* are present in the transgenic oilseed rape lines

Responsible : C. Opsomer, PGS nv. Researcher

L03 Goal

In order to determine if *Agrobacteria* containing the Ti-plasmid used for transformation are present in the transgenic oilseed rape lines, a molecular detection procedure has been developed. This method is based on the detection of Ti-plasmid specific sequences (located on the Ti- plasmid beyond the left border repeat) by the PCR amplification method.
To estimate the sensitivity of the method, a reconstruction experiment was done in parallel with the analysis on extracts from the transgenic lines. The reconstruction experiment is based on the mixture of plant extract from a non transgenic line with defined aliquots of an *Agrobacterium* culture.
To increase the sensitivity, PCR amplification was combined with the Southern Blot procedure. An aliquot from fractions not revealing a visible band on agarose gel upon amplification, were further analyzed by hybridization (according to the protocol as described in Part 1. Annex VI.3.1.). The detection limit is set on the dilution which gives a signal above background.

L03 Plant Material

Leaf material from 2 weeks old seedlings was used to make the extracts. At that stage, the developing plant is about 9 cm in height and has 4 small leaves.

L03 Experimental Procedures

1. <u>Extracts screened for detection of the Ti-plasmid</u>

- RF2 (S_3 generation of B94-2) leaf extract (250 mg leaf material)
- Drakkar extract (250 mg leaf material)

2. <u>Reconstruction experiment</u>

Leaf material of a non-transformed Drakkar plant is mixed with varying amounts of *Agrobacterium* cells, containing the Ti-plasmid (from 1.10^7 cells/ 250 mg leaf material to 1.10^2 cells/ 250 mg leaf material) .

- Drakkar + *Agrobacteria*
  - A. 1.10^7 cells/ 250 mg leaf material
  - B. 1.10^6 cells/ 250 mg leaf material
  - C. 1.10^5 cells/ 250 mg leaf material
  - D. 1.10^4 cells/ 250 mg leaf material
  - E. 1.10^3 cells/ 250 mg leaf material
  - F. 1.10^2 cells/ 250 mg leaf material

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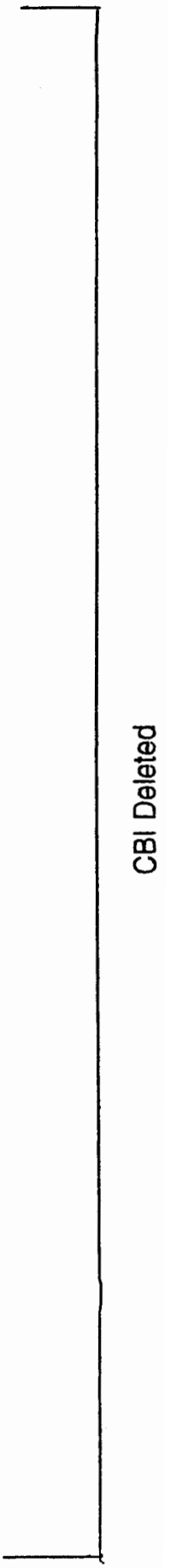

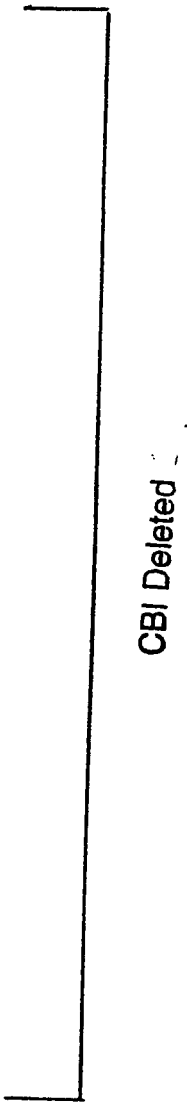

L03 Results and conclusion

No amplification was observed in extracts prepared from Drakkar and B94-2 leaf material. In the reconstruction experiment, we can detect between 1.10^2 and 1.10^3 cells of Agrobacterium added to 250 mg of leaf material.

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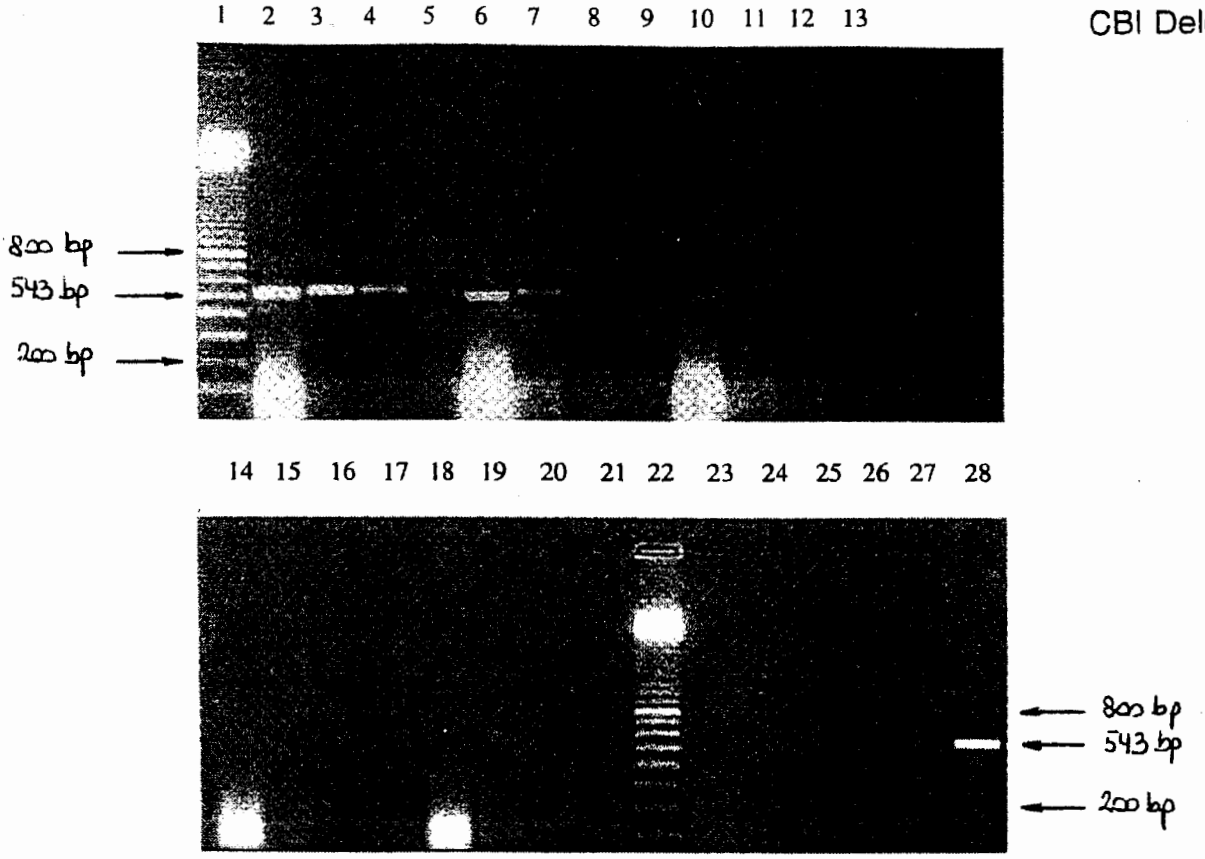

ANNEX VI 3.3

L 03 **Figure 1. Reconstruction Experiment**

250 mg of leaf from wild type Drakkar was mixed with defined amount of *Agrobacterium* cells (dilutions A-F, as specified in 2.) and DNA was extracted.
From each of these DNAs, serial dilutions were made (10^0 - 10^{-3}), and amplified in PCR (primer [redacted] 543 bp)
PCR products are visualized on agarose gel:

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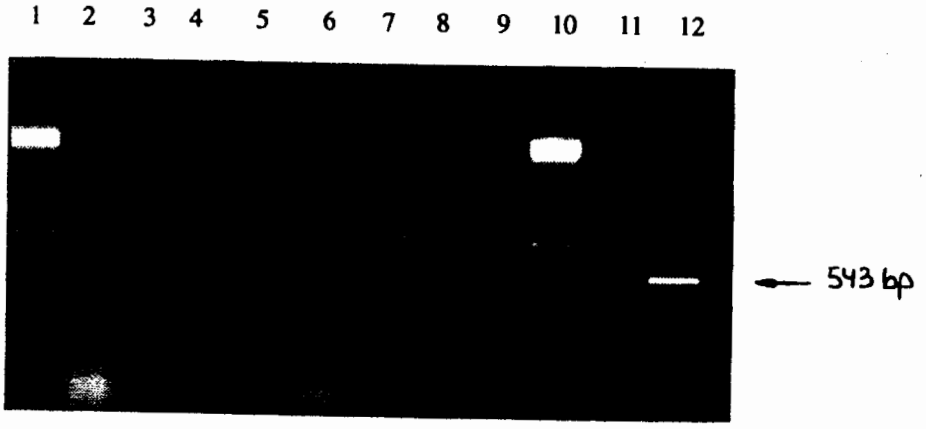
1. 100 bp ladder
2. Drakkar + Agro (A): 20 µl
3. Drakkar + Agro (A): 20 µl 10^{-1}
4. Drakkar + Agro (A): 20 µl 10^{-2}
5. Drakkar + Agro (A): 20 µl 10^{-3}
6. Drakkar + Agro (B): 20 µl
7. Drakkar + Agro (B): 20 µl 10^{-1}
8. Drakkar + Agro (B): 20 µl 10^{-2}
9. Drakkar + Agro (B): 20 µl 10^{-3}
10. Drakkar + Agro (C): 20 µl
11. Drakkar + Agro (C): 20 µl 10^{-1}
12. Drakkar + Agro (C): 20 µl 10^{-2}
13. Drakkar + Agro (C): 20 µl 10^{-3}
14. Drakkar + Agro (D): 20 µl
15. Drakkar + Agro (D): 20 µl 10^{-1}
16. Drakkar + Agro (D): 20 µl 10^{-2}
17. Drakkar + Agro (D): 20 µl 10^{-3}
18. Drakkar + Agro (E): 20 µl
19. Drakkar + Agro (E): 20 µl 10^{-1}
20. Drakkar + Agro (E): 20 µl 10^{-2}
21. Drakkar + Agro (E): 20 µl 10^{-3}
22. 100 bp ladder
23. Drakkar + Agro (F): 20 µl
24. Drakkar + Agro (F): 20 µl 10^{-1}
25. Drakkar + Agro (F): 20 µl 10^{-2}
26. Drakkar + Agro (F): 20 µl 10^{-3}
27. Blanc: 20 µl H_2O
28. positive control: 1 pg pTVE74RE



ANNEX VI 3.3

L03 **Figure 2. Assay for the presence of Agrobacterium cells carrying the pTVE74RE plasmid in transformed Drakkar lines**

250 mg of leaf from wild type Drakkar was mixed with defined amount of *Agrobacterium* cells (dilutions A-F, as specified in 2.) and DNA was extracted.
From each of these DNAs, serial dilutions were made (10^0 - 10^{-3}), and amplified in PCR (primer [redacted]).

PCR products are visualized on agarose gel:

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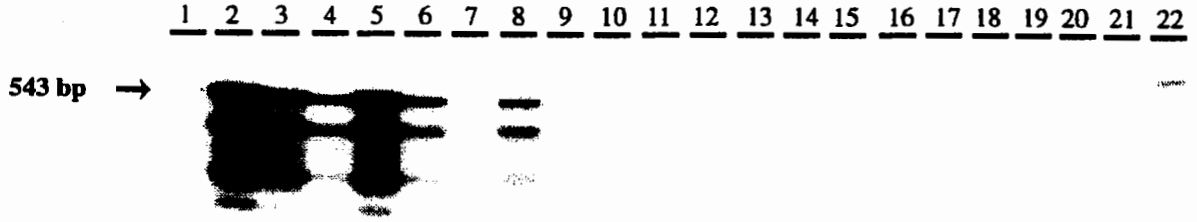
1. 100 bp ladder
2. Drakkar: 20 µl
3. Drakkar: 20 µl 10^{-1}
4. Drakkar: 20 µl 10^{-2}
5. Drakkar: 20 µl 10^{-3}
6. B94-2: 20 µl
7. B94-2: 20 µl 10^{-1}
8. B94-2: 20 µl 10^{-2}
9. B94-2: 20 µl 10^{-3}
10. 100 bp ladder
11. blanc: 20 µl H_2O
12. positive control: 1 pg pTVE74RE



Lo3 **Figure 3. Southern blot hybridisation**

15 µl of PCR amplified fractions, not revealing a visual band on agarose gel (Figure 1. and Figure 2.) are loaded on a 1.5% TBE agarose gel, and blotted on a nylon membrane.
Amplified products are visualised by hybridization with purified PCR fragment, deriving from a PCR reaction with [ ] on pTVE 74RE template.

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lanes 2-12 : amplified fractions from the reconstruction experiment
lanes 13-16 : amplified fractions from Drakkar extract
lanes 17-20 : amplified fractions from B94-2 extract

1. Lambda digested with PstI as marker lane

2. Drakkar + Agro (B) : 10^{-3}
3. Drakkar + Agro (C) : 10^{-2}
4. Drakkar + Agro (C) : 10^{-3}
5. Drakkar + Agro (D) : 10^{-1}
6. Drakkar + Agro (D) : 10^{-2}
7. Drakkar + Agro (D) : 10^{-3}
8. Drakkar + Agro (E) : 10^{0}
9. Drakkar + Agro (E) : 10^{-1}
10. Drakkar + Agro (E) : 10^{-2}
11. Drakkar + Agro (F) : 10^{0}
12. Drakkar + Agro (F) : 10^{-1}

13. Drakkar : 10^{0}
14. Drakkar : 10^{-1}
15. Drakkar : 10^{-2}
16. Drakkar : 10^{-3}

17. B94-2 : 10^{0}
18. B94-2 : 10^{-1}
19. B94-2 : 10^{-2}
20. B94-2 : 10^{-3}

21. Blanc
22. 25 pg purified PCR fragment

L04 Stability of integration of the transforming DNA over a number of generations via Southern analysis

Responsible : Dr J. Botterman, PGS NV Manager Product Development Lab
C. Opsomer, PGS NV Researcher

L04 Goals of the experiment

Analyzing the stability of integration and the inheritance of the introduced chimeric gene constructs in the B94-2 oilseed rape progeny. The experimental approach is based on molecular analysis using the Southern blot procedure.

L04 Plant material

To demonstrate the stability of the integrated gene constructs, a molecular analysis has been performed on the plants from subsequent progenies. Three generations have been tested for each case. The analysis has been performed on two individual plants (A and B) from each progeny. Non-transgenic Drakkar has been used as a negative control.

Table L04 Plant material

Plant material	Notation
B94-2 progeny :	
S_1(B94-2)	P1132
S_3(B94-2)	B0154
BC_2 of B94-2 in Euro SOSR23	BC_2
Drakkar	

L04 Methods

Southern analysis was performed according to the protocol as described in Annex VI.3.1.. Total DNA of the respective plants was isolated from plant tissue according to Dellaporta et al. (1983) and has been digested with [ ]. This enzyme has a single cleavage site in the transferred T-DNA fragment and is localized in the [ ]. Upon hybridization with a [ ] two hybridizing bands overlapping with the genomic DNA towards the right and left integration sites of the transferred DNAs were expected. A schematic representation of the transferred DNA with the genes of interest is shown in Figure 1 (pTVE74RE).

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L04 Results and conclusion

L04 Results

In Figure 1., a schematic representation of the DNA fragment comprised between the T-DNA border repeats of pTVE74RE is given. The localization of the [ ] site within the transferred DNA, the [ ] used as probe and the size of the expected crosshybridizing bands are indicated.

The autoradiogram from a Southern blot on two individual plants (A and B) from different generations (S_1, S_3, BC_2) carrying the gene for restoration of fertility with the hybridization pattern using the [ ] is shown in Figure 2.. As expected, an identical hybridization pattern was found in the respective generations.

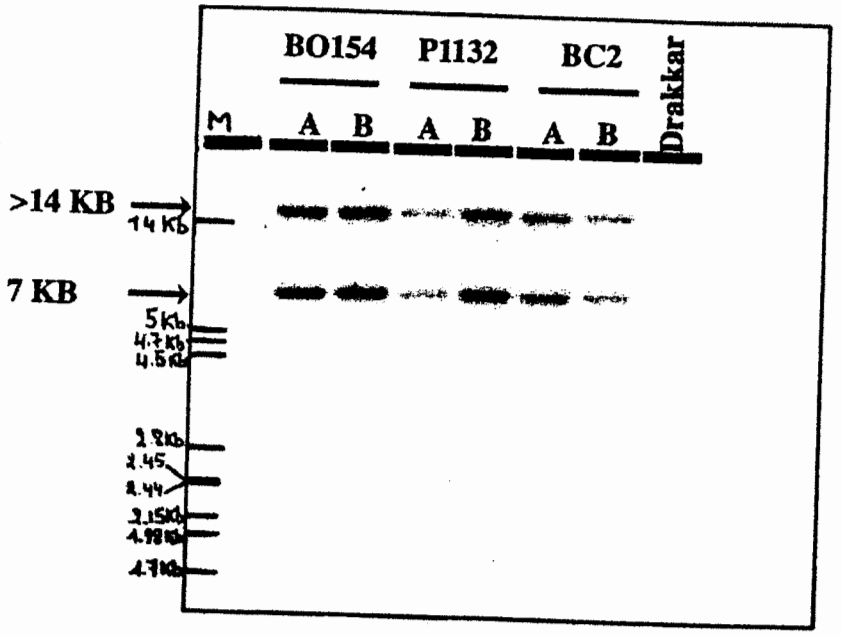


Figure L04-2. Stability analysis B94-2 (Rf2) - Southern blot analysis

DNA was isolated from 2 individual plants from different generations (S1 = P1132, S3 = B0154 and BC2) and one non-transgenic plant (from Drakkar). Ten µg genomic DNA was digested with [ ]

L04 Conclusion

The analysis demonstrates that the transferred DNA is stably inherited in subsequent generations and in different genetic backgrounds.

L05 Confirmation that the transformed DNA corresponds to the plasmid DNA

Responsible : Dr J. Botterman, PGS NV Manager Product Development Lab
C. Opsomer, PGS NV Researcher

L05 Goals of the experiment

To demonstrate that the DNA transferred into the plant genome is identical to the T-DNA region of the plasmid vector, a molecular analysis based on Southern blotting has been performed.

L05 Plant material

Molecular analysis has been performed on plants carrying the construct for restoration of fertility and compared with total plasmid DNA. The analysis has been performed on two individual plants (A and B). Non-transgenic Drakkar plants have been used as negative control.

Table L05 Plant material

Plant material	Notation
B94-2 progeny : S_3(B94-2)	RF2-S_3
Drakkar	

L05 Methods

Southern analysis was performed according to the protocol as described in Annex VI.3.1.. Genomic DNA has been prepared from leaf tissue of plants carrying the gene for restoration of fertility and compared with total plasmid DNA. The experimental approach is based on digesting the respective DNAs with different restriction enzymes, probe with different fragments of the transferred DNA and make a comparison between plasmid and genomic DNA fragments. The comparative analysis is based on the determination of the sizes of the respective hybridizing bands.[

] allows to visualize different fragments of the transferred DNA. Digest and probe combinations were chosen in such a way that the whole transferred region was covered.

L05 Results and conclusion

L05 Results

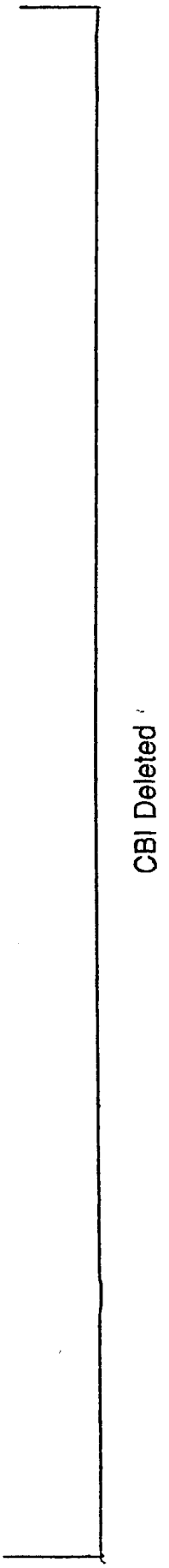
An overview of the results is given in the tables and figures.

Table 1 gives an overview of the different digests performed on total genomic DNA of the restorer line and the plasmid pTVE74RE. Four probes have been used in the hybridizations and the expected fragments based on the plasmid map are indicated. As an example, the schematic representation of the DNA fragment comprised between the right and left border repeat of pTVE74RE with the respective chimeric gene constructs, is outlined in Figure 1a.,1b.,1c. and 1d.. The localization of the respective restriction enzymes used in the consecutive hybrizations are indicated and the crosshybridizing bands with the respective probes are highlighted.

In Figures 2a[ ]some examples of autoradiograms of the different probe-digest combinations are shown, clearly indicating that the plant DNA corresponds to the plasmid DNA.

L05 Conclusion

In conclusion, this analysis demonstrates that the transferred DNA in the plant genome corresponds to the DNA configuration as designed in the plasmid vector.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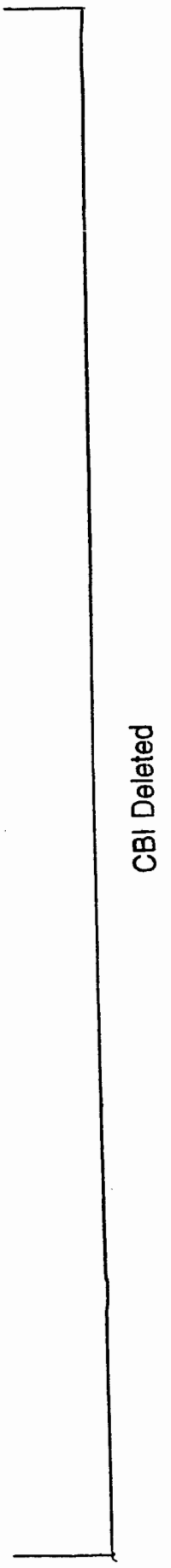

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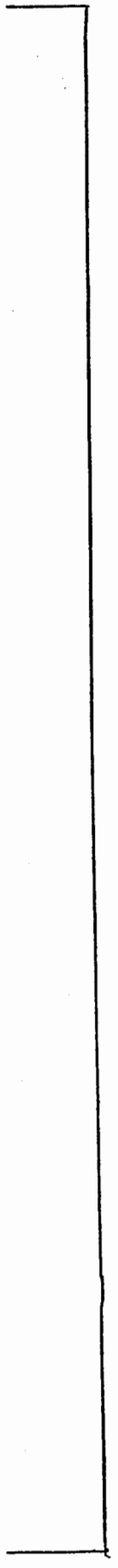

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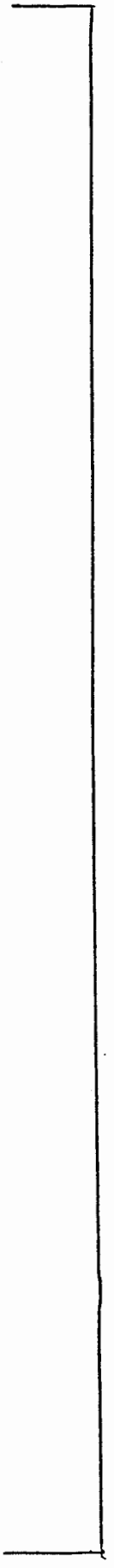

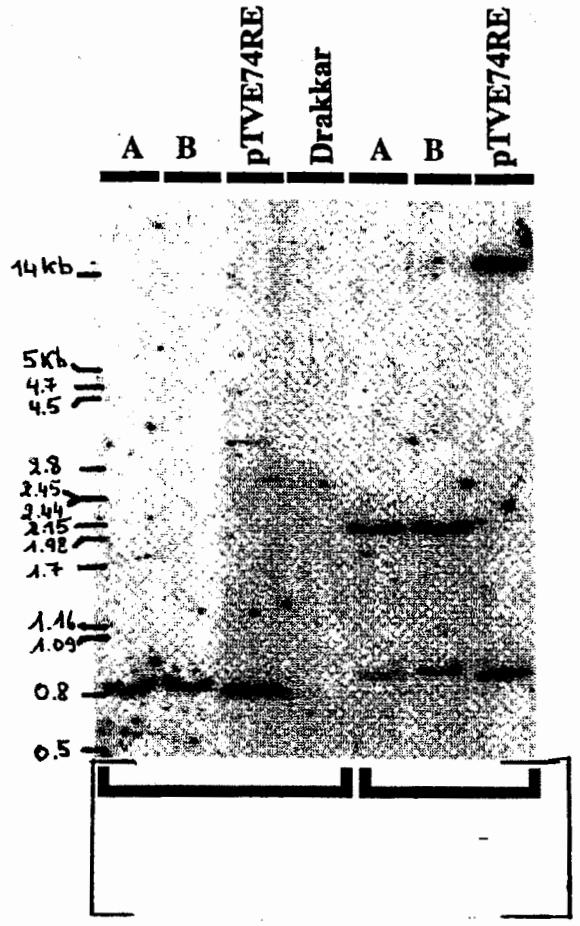

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L05 **Figure 2a. Insert characterization B94-2 (Rf2) - Southern blot analysis**

10 µg genomic DNA, isolated from 2 individual B94-2 plants (A and B), was digested with the indicated restriction enzymes.
The amount of pTVE74RE samples loaded is equivalent to ± 1 copy of the plasmid integrated in 10 µg of genomic oilseed rape DNA.

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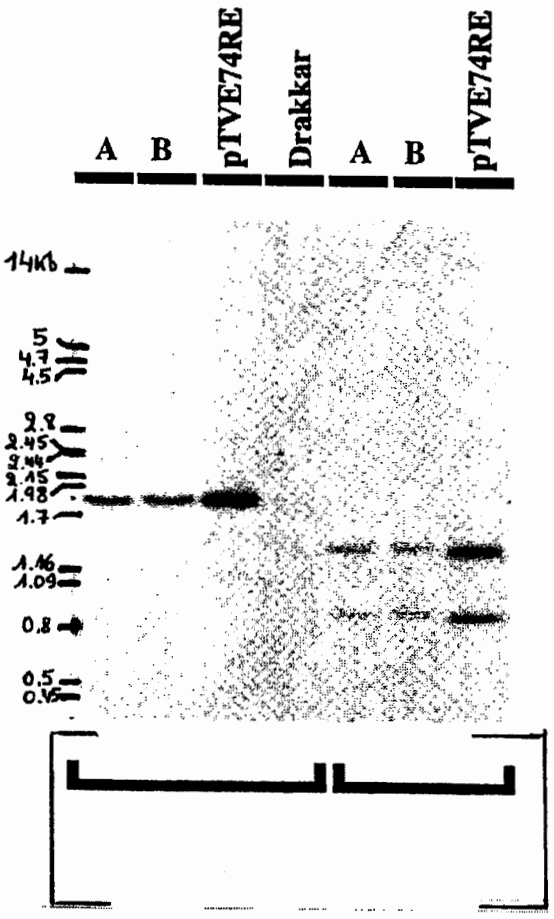

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Lo5 **Figure 2b. Insert characterization B94-2 (Rf2) - Southern blot analysis**

10 µg genomic DNA, isolated from 2 individual B94-2 plants (A and B), was digested with the indicated restriction enzymes.
The amount of pTVE74RE samples loaded is equivalent to ± 1 copy of the plasmid integrated in 10 µg of genomic oilseed rape DNA.

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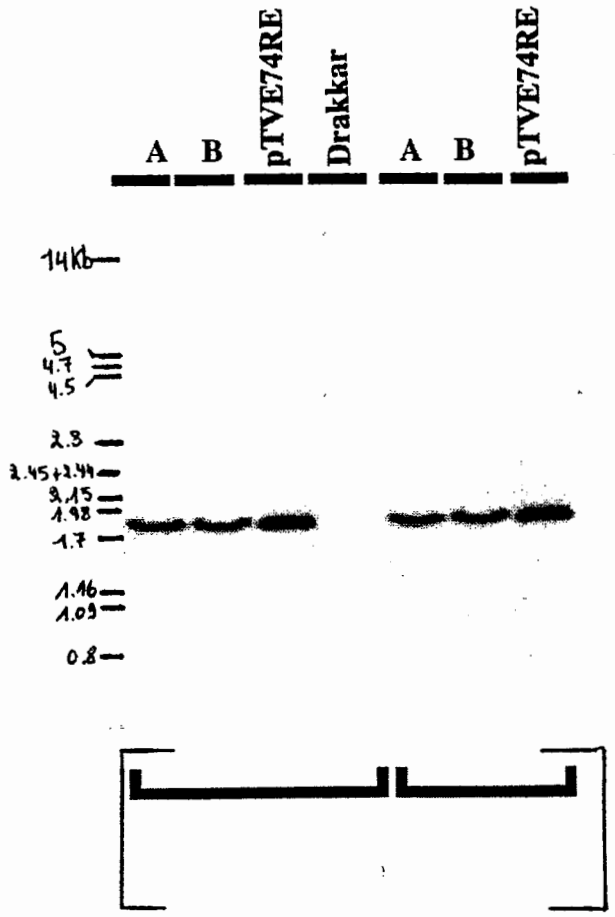

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L 05 **Figure 2c. Insert characterization B94-2 (Rf2) - Southern blot analysis**

10 µg genomic DNA, isolated from 2 individual B94-2 plants (A and B), was digested with the indicated restriction enzymes.
The amount of pTVE74RE samples loaded is equivalent to ± 1 copy of the plasmid integrated in 10 µg of genomic oilseed rape DNA.

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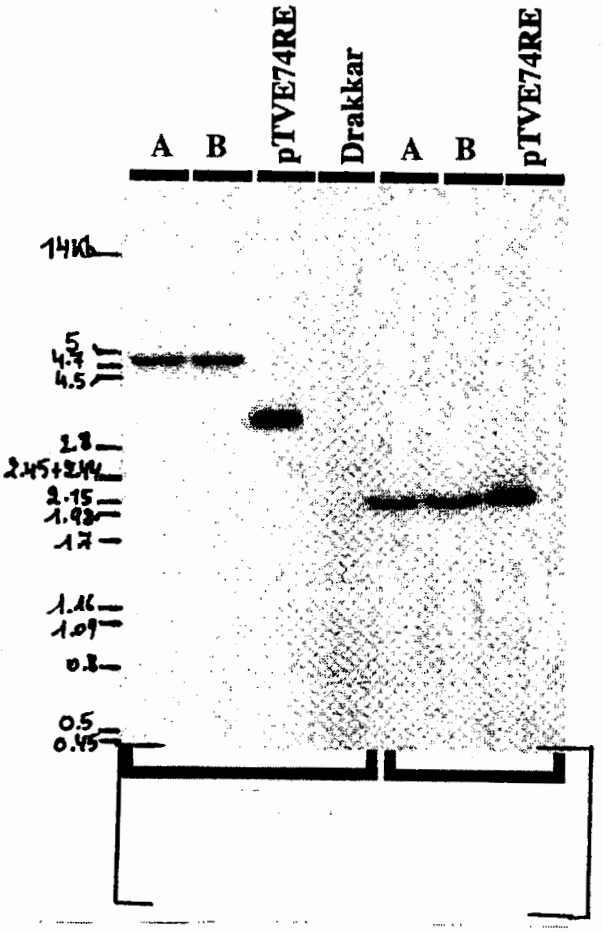

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Los **Figure 2d. Insert characterization B94-2 (Rf2) - Southern blot analysis**

10 µg genomic DNA, isolated from 2 individual B94-2 plants (A and B), was digested with the indicated restriction enzymes.
The amount of pTVE74RE samples loaded is equivalent to ± 1 copy of the plasmid integrated in 10 µg of genomic oilseed rape DNA.

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EXPERIMENT L06 **Characterization of the T-DNA integration via molecular analysis of the regions flanking the T-DNA**

Responsible : M. De Beuckeleer, PGS NV Senior Researcher

L06 Goal

Characterization of the T-DNA integration via molecular analysis of the regions flanking the T-DNA.

L06 Plant material

Molecular analysis has been performed on plants carrying the gene for restoration of fertility (Table L06). Non-transgenic Drakkar plants have been used as negative control.

Table L06 Plant material

Plant material	Notation
B94-2 progeny :	
S_3(B94-2)	RF2-T_3

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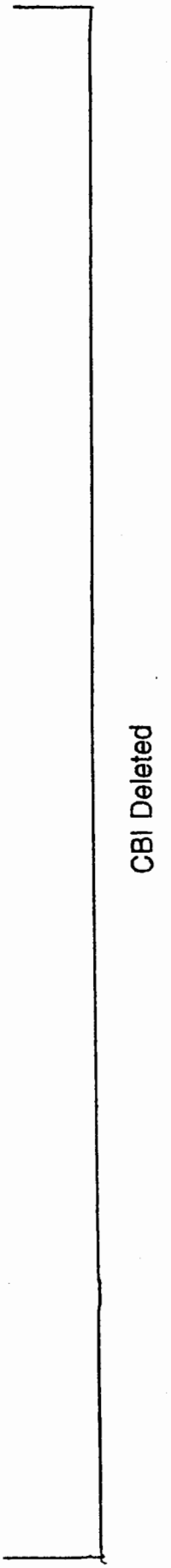
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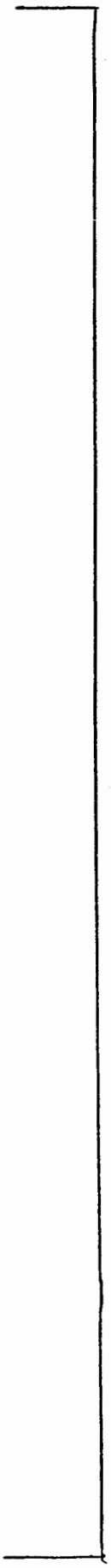

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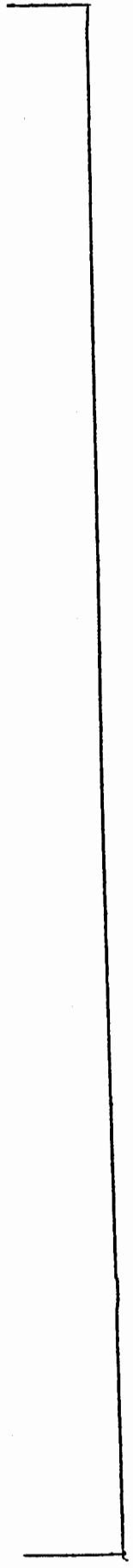

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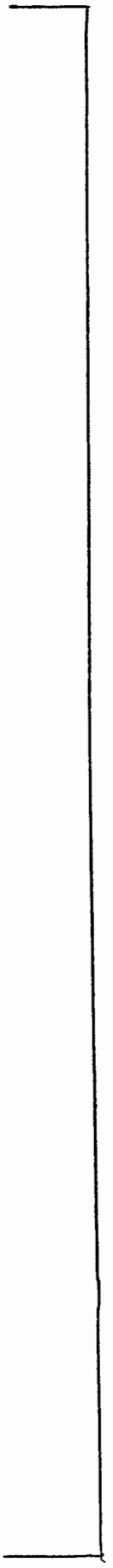

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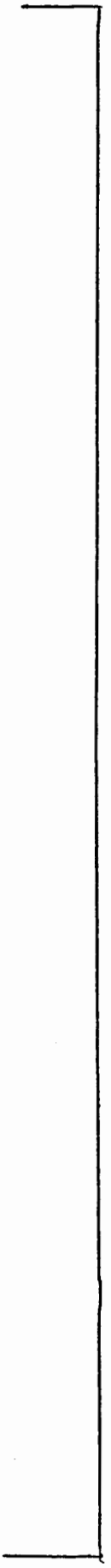


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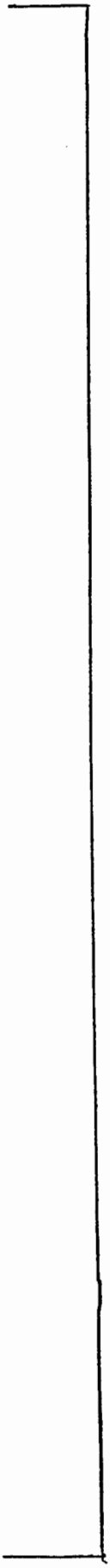

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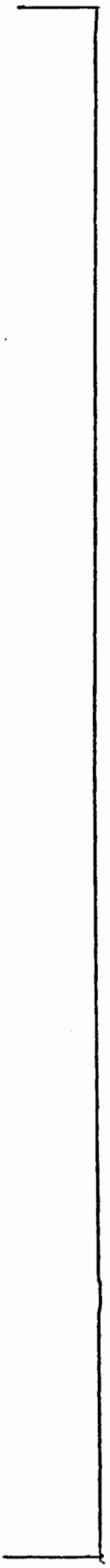

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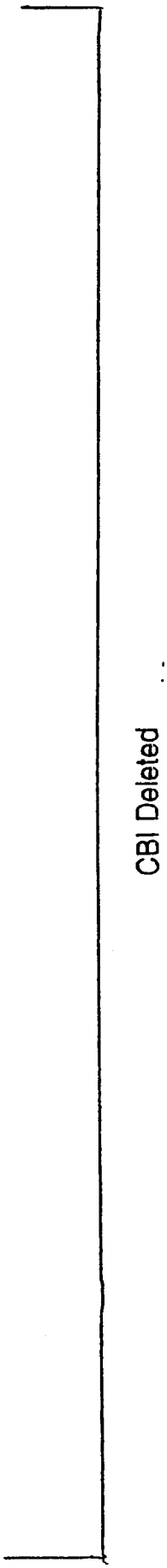

EXPERIMENT L08 Homology search between plant DNA sequences and the B94-2 target sequence

Responsible : M. De Beuckeleer, PGS NV Senior Researcher

L08 Goal

Homology search between plant DNA sequences and the B94-2 target sequences

L08 Plant material

Molecular analyses have been performed on plants carrying the gene for restoration of fertility.

Table L08 Plant material

Plant material	Notation
B94-2 progeny : S_3(B94-2)	RF2-T_3

L08 Method and results

A spliced sequence, composed of sequencing results from different amplifications [redacted]

[redacted] This sequence was used to do a homology survey with published plant DNA and protein sequences.

We performed a nucleotide sequence database and a peptide sequence database search through the BLAST network service. Computation was performed at the National Centre for Biotechnology Information (NCBI, Rockville Pike, Bethesda, U.S.A.). This search was performed on 94-05-16. 175.544 DNA sequences (187.813.237 letters in total) were searched. Only 1 high-scoring segment pair was found. When analysing this sequence no significant homology was detected. For the search in the peptide databases, both strands of the query sequence were translated in all 6 reading frames. 117.877 sequences were searched (33.481.105 letters in total). 157 sequences producing high-scoring segment pairs were found. When analysing these sequences, no significant homologies were found.

Nucleotide sequence databases searched: PDB (Brookhaven Protein Data Bank), GenBank(R) Release 82.0, GenBank(R) cumulative daily updates, EMBL Data Library Release 38.0 and EMBL Data Library cumulative daily updates. Protein sequence databases searched : Brookhaven Protein Data Bank, SWISS-PROT Release 28.0, PIR Release 40.0 (complete), SWISS-PROT cumulative weekly update, CDS translations from GenBank(R) Release 82.0 and Cumulative daily updates to the major release of genpept.

L08 Conclusion

No significant homologies between the plant DNA and B94-2 target sequences have been recorded.

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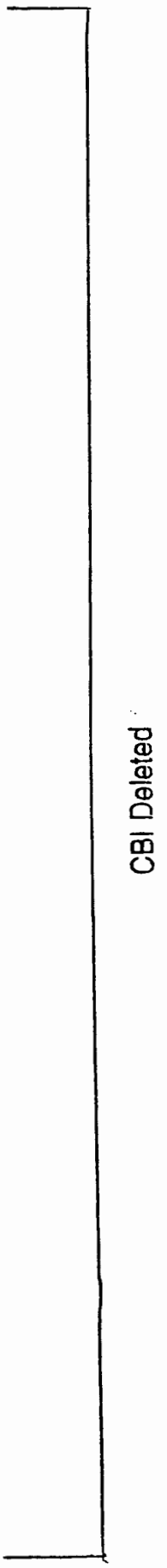

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L09 Expression of the introduced transgenes and analysis of the possible occurrence of cryptic gene expression

Responsible : M. De Beuckeleer, PGS NV Senior Researcher

L09 Goals of the experiment

To demonstrate the expression of introduced transgenes in the male sterile and fertility restorer oilseed rape progenies and to analyze the possible occurrence of cryptic gene expression.

L09 Plant material

Molecular analysis has been performed on plants carrying the gene for restoration of fertility. Non-transgenic Drakkar plants have been used as negative control.

Table L09 Plant material

Plant material	Notation
B94-2 progeny : T_3(B94-2)	RF2-T_3

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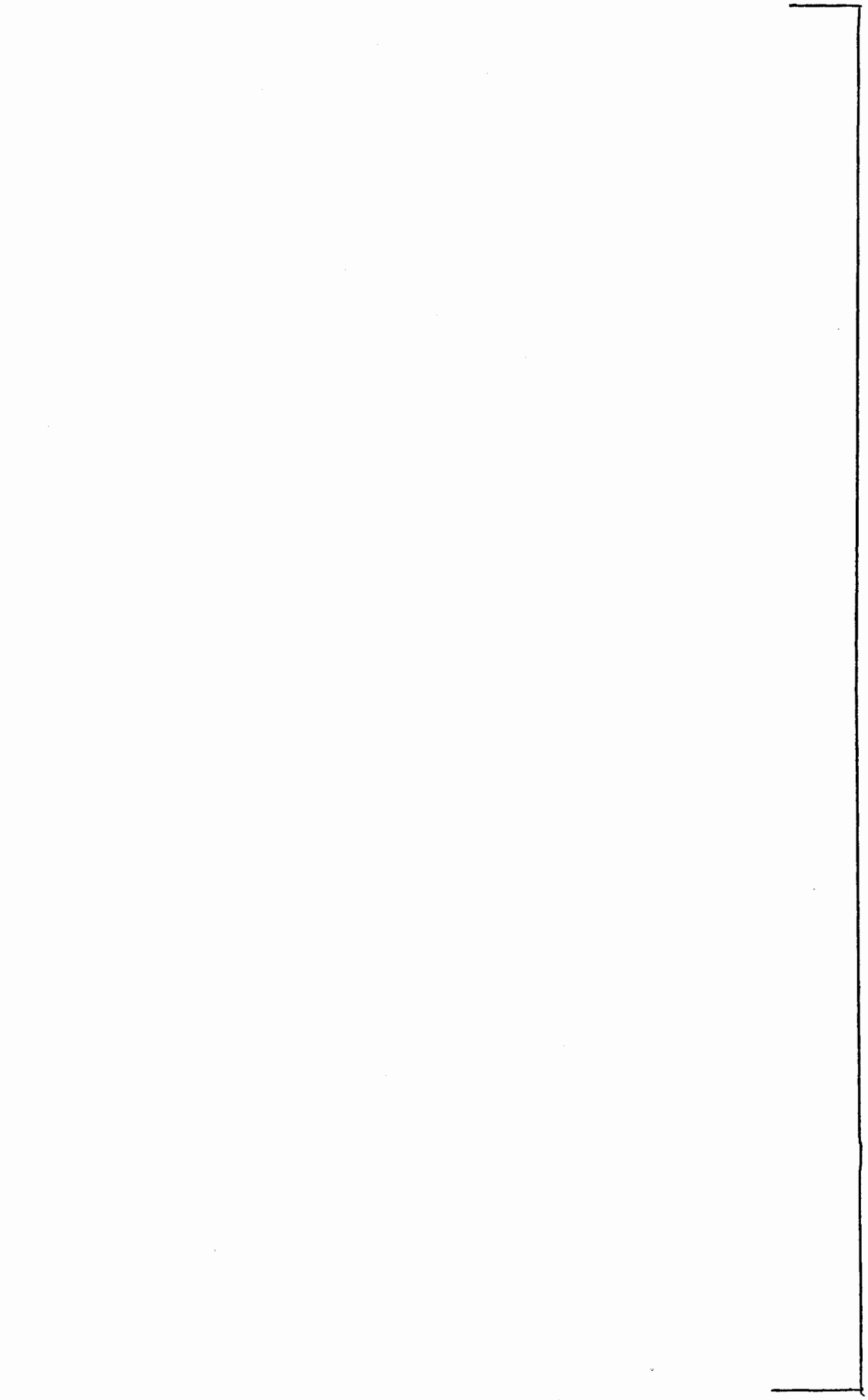

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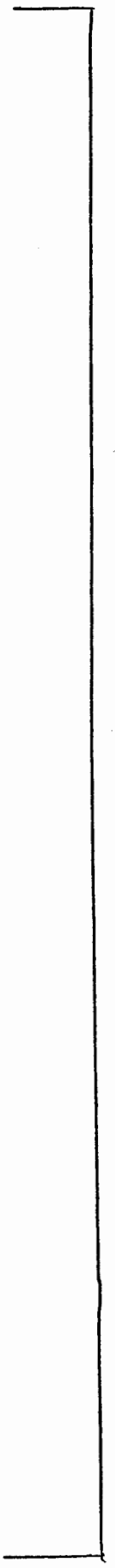

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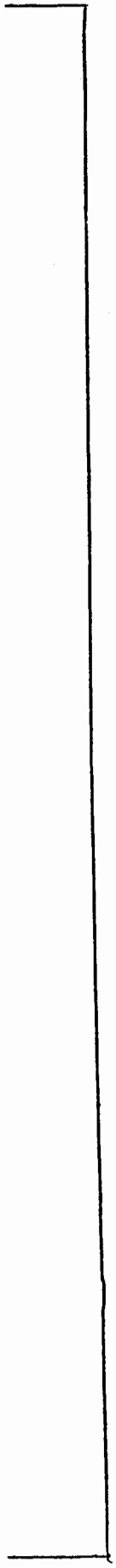

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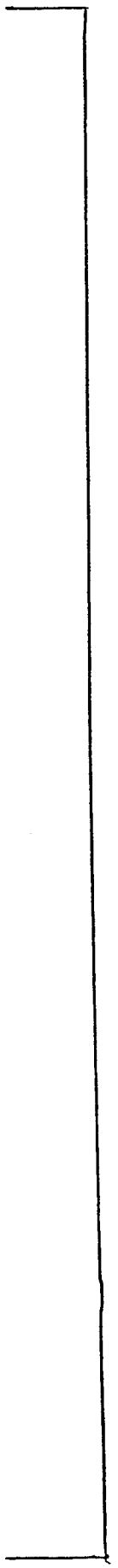

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L09 Results and conclusions

Transgene expression

Bar

The detected bar mRNA levels in leaves are between 0.8 and 1.6pg/µg total RNA. We also detected bar mRNA in 1mm, 2mm and 3mm flower buds (between 0.1 and 0.2pg/µg total RNA). All other tested tissues and controls are negative (< 0.2pg/µg total RNA) (see Figure 1).

Barstar

We detect barstar mRNA in 2mm and 3mm flower buds. All other tissues and controls are negative (<0.1pg/µg total RNA) (see Figure 1). Estimated barstar mRNA levels are between 0.4 and 0.8pg/µg total RNA in 2mm flower buds (± 0.2 pg/µg total RNA in 3mm flower buds).

Neo

We were unable to demonstrate any neo mRNA signals in the RF2 transformant using 5µg total RNA/lane. The detection limit for this hybridization was 0.1pg/µg total RNA.

Cryptic gene expression

Bar - Barstar - Neo

Using sense RNA probes of the specified transgenes, we were unable to detect any hybridization signals whatsoever. Detection limits are indicated in the tables.

Right Border T-DNA [redacted] CBI DELETED

The in vitro synthesized [redacted] of both fragments were used together in a hybridization experiment (the same was done with the [redacted]). We were unable to detect any hybridization signals with both set of probes. Detection limits are indicated in the table.

Left Border T-DNA [redacted] CBI DELETED

Both [redacted] were used together in the hybridization (the same was done with the [redacted]). We were unable to detect any hybridization signals (see Figure 2). Detection limits are indicated in the table.

Plant DNA sequence at the insertion site [redacted] CBI DELETED

The in vitro synthesized [redacted] were used in two hybridizations to check for cryptic expression. We were unable to detect any hybridization signals (see Figure 3). Detection limits are indicated in the table.

Numbers on the presented figures (Figure 1.) correspond to the below indicated numbers:

Plant material

1. Leaf RNA (plant RF2 A)
2. Leaf RNA (plant RF2 B)
3. Leaf RNA Drakkar control plant
4. 1mm Flower bud RNA (plant RF2 A)
5. 2mm Flower bud RNA (plant RF2 A)
6. 3mm Flower bud RNA (plant RF2 A)
7. 1mm Flower bud RNA (plant RF2 B)
8. 2mm Flower bud RNA (plant RF2 B)
9. 3mm Flower bud RNA (plant RF2 B)
10. 2mm Flower bud RNA Drakkar control plant
11. 3mm Flower bud RNA Drakkar control plant
12. Seed RNA RF2
13. Seed RNA Drakkar control plant
14. Pollen RNA RF2
15. Pollen RNA Drakkar control plant
16. Blank
17. MW marker: 0.16 - 1.77Kb RNA ladder (GIBCO-BRL)

5µg total RNA was loaded/lane (exception: on the gel that was used for the barstar detection, we loaded 10µg total RNA/lane).

Control RNA dilution series :

A dilution series ranging from [ ] of the complementary, in vitro synthesized, RNA is loaded on a 1.5% agarose formaldehyde gel in the presence of 5µg Drakkar leaf RNA.

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Loading example for barstar [ ]
Numbers on the presented figure correspond to the below indicated numbers.

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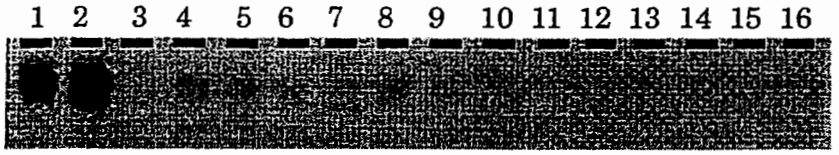
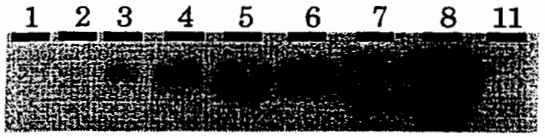
11. MW marker: 0.16 - 1.77Kb RNA ladder (GIBCO-BRL)

Transgene Expression

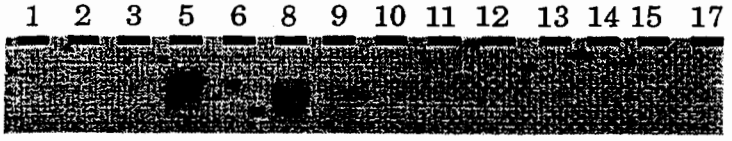
1. bar

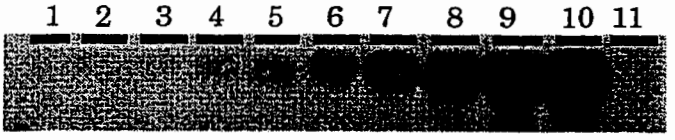




Dilution series

2. barstar





Dilution series

Figure 1

The typical loading arrangement of the gels (5µg total RNA/lane) (Figure 2. and 3.) (sample numbers on the presented figures correspond to the numbers indicated below).

Plant material :

1. MW marker: 0.16 - 1.77Kb RNA ladder (GIBCO-BRL)
2. Leaf RNA (plant RF2 A)
3. Leaf RNA (plant RF2 B)
4. Leaf RNA Drakkar control plant
5. 1mm Flower bud RNA (plant RF2 A)
6. 2mm Flower bud RNA (plant RF2 A)
7. 3mm Flower bud RNA (plant RF2 A)
8. 1mm Flower bud RNA (plant RF2 B)
9. 2mm Flower bud RNA (plant RF2 B)
10. 3mm Flower bud RNA (plant RF2 B)
11. 2mm Flower bud RNA Drakkar control plant
12. 3mm Flower bud RNA Drakkar control plant
13. Seed RNA RF2
14. Seed RNA Drakkar control plant
15. Pollen RNA RF2
16. Pollen RNA Drakkar control plant
17. Blank
18. MW marker: 0.16 - 1.77Kb RNA ladder (GIBCO-BRL)

Control RNA dilution series :

A dilution serie ranging from 0.5pg to 64pg of the complementary, in vitro synthesized, RNA is loaded on a 1.5% agarose - formaldehyde gel in the presence of 5µg Drakkar leaf RNA.

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Cryptic Gene Expression

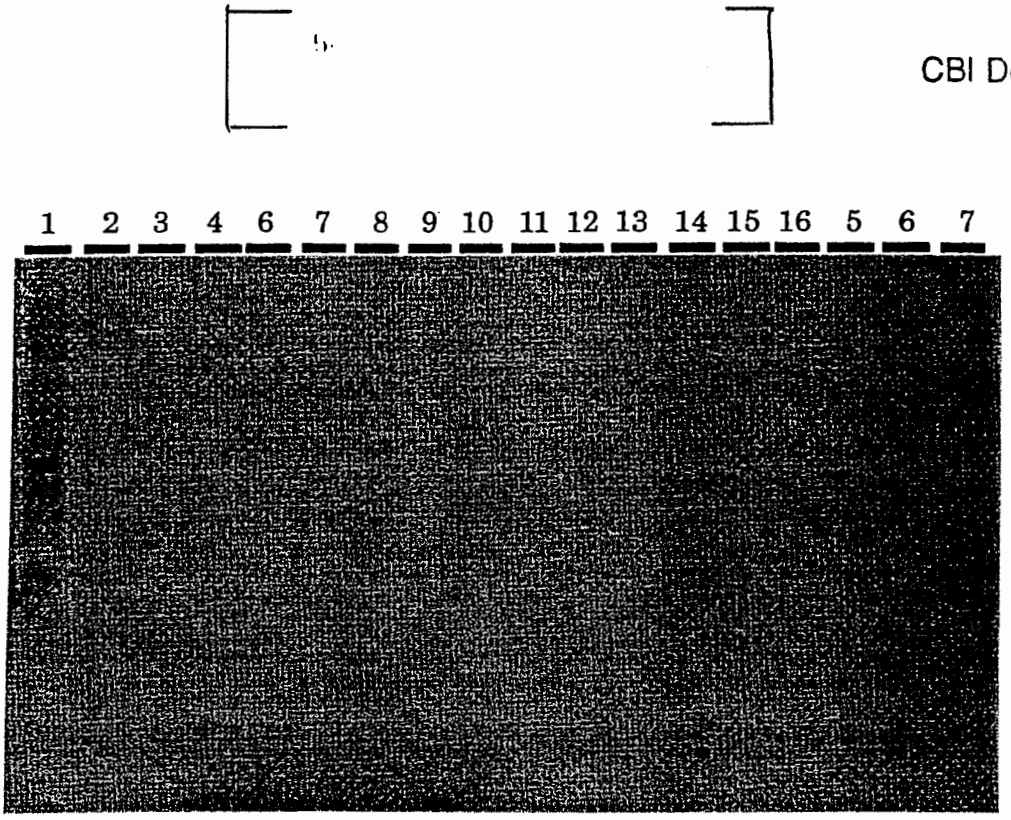

Figure 2.

Cryptic Gene Expression

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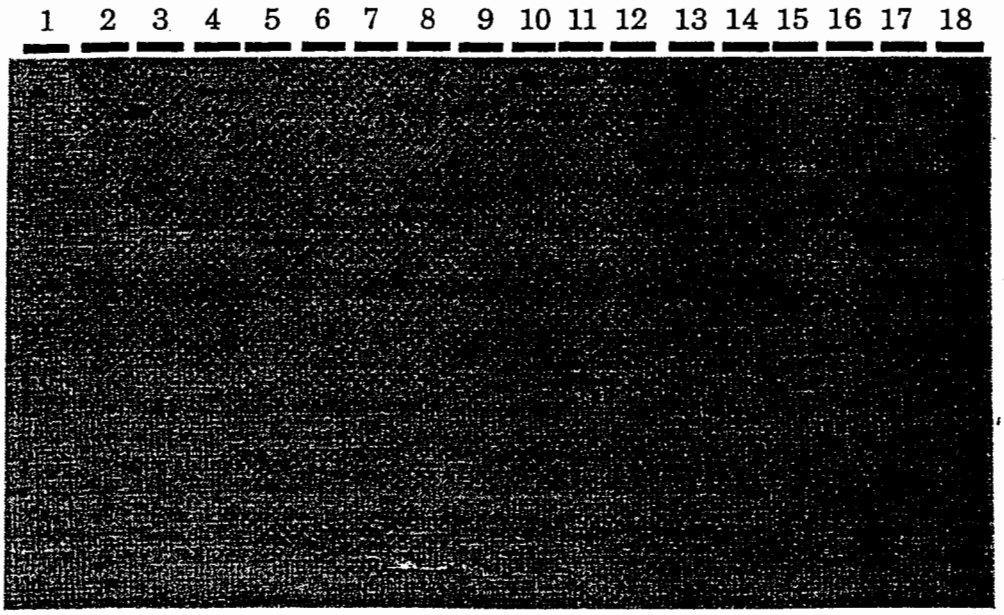


Control dilution series

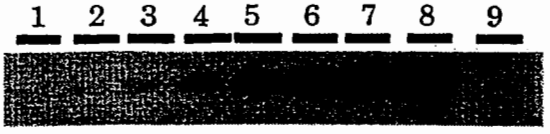
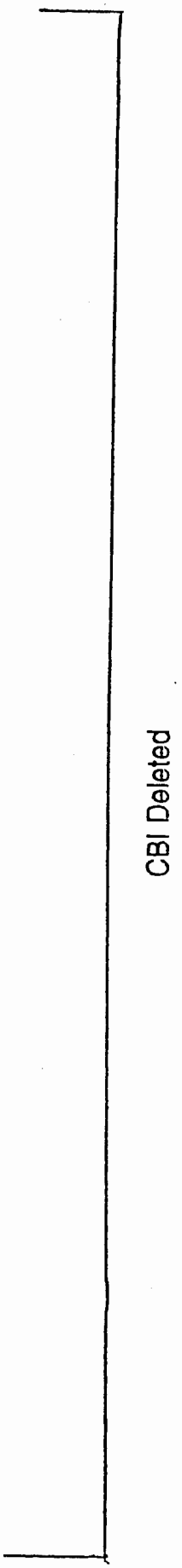


Figure 3.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L10 NPTII activity over a number of generations

Responsible : A. De Sonneville, Senior Technician

L10 Goal of the experiment

The NPTII enzymatic assay was used to evaluate the expression and inheritance of the *neo* gene over a number of B94-2 generations.

L10 Plant material

The different plant entries from the progeny of the B94-2 fertility line (2 different plants per entry, further indicated as A and B) are outlined in the Table below.

Plant material	Notation
B94-2 progeny :	
S_1(B94-2)	RF2-S_1
S_3(B94-2)	RF2-S_3
BC_2 of B94-2 in Euro SOSR23	RF2-BC_2

L10 Methods

The assay is based on the electrophoretic separation of the NPTII protein from plant endogenous phosphorylating enzymes by a non-denaturing gel electrophoresis and detection of its enzymatic activity by in situ phosphorylation of kanamycin in the presence of radioactively labeled ATP. Both kanamycin and (γ-^{32}P)ATP acting as substrates are embedded in an agarose gel placed on the polyacrylamide gel containing the separated proteins. After the enzymatic reaction, the phosphorylated kanamycin is transferred to P81 phosphocellulose ion exchange paper and the radiolabelled kanamycin is visualized by autoradiography. An overview of the different steps in the enzymatic assay are described. Details of the different steps and solutions used are described in the mentioned references (Reiss et al.(1984). The EMBO Journal, 3, 3317-3322. Reynaerts et al. (1987). In : Gelvin, S.B., Schilperoort, R.A. (eds). Plant molecular biology manual. Kluwer, Dordrecht, The Netherlands, Sect. A9, p 1-16).

Extraction of plant material

100 mg fresh plant tissue is homogenized in extraction buffer on ice and transferred into a microcentrifuge tube. The cell debris is pelleted by centrifugation in an Eppendorf centrifuge. The supernatans is transferred to another tube and the protein concentration in the crude extract is determined by Biorad assay with bovine serum albumine as standard. Protein concentration in all samples is adjusted with extraction buffer.

Enzymatic assay

Loading buffer is added to an aliquot of 100 µg total protein which is separated by electrophoresis through a 10% non-denaturing polyacrylamide gel. The gel is washed in distilled water and equilibrated in reaction buffer. The gel is transferred onto a glass plate and overlaid with a 1% agarose gel containing 30 µg/ml kanamycin sulphate and 100 µCi (γ-^{32}P)ATP in reaction buffer. After incubation, the gel sandwich is covered with a sheet of Whatman P81 phosphocellulose paper. Upon incubation, the phosphorylated kanamycin is bound to the P81 paper. Subsequently, the P81 paper is washed, dried and exposed to a X-ray film.

L10 Results and conclusions

As shown in Figure $L10_1$, a typical band is identified corresponding to the reaction product. The presence of the band is consistent in the different stages of the tested material. This indicates that the *neo* gene is inherited and stably expressed over a number of generations.

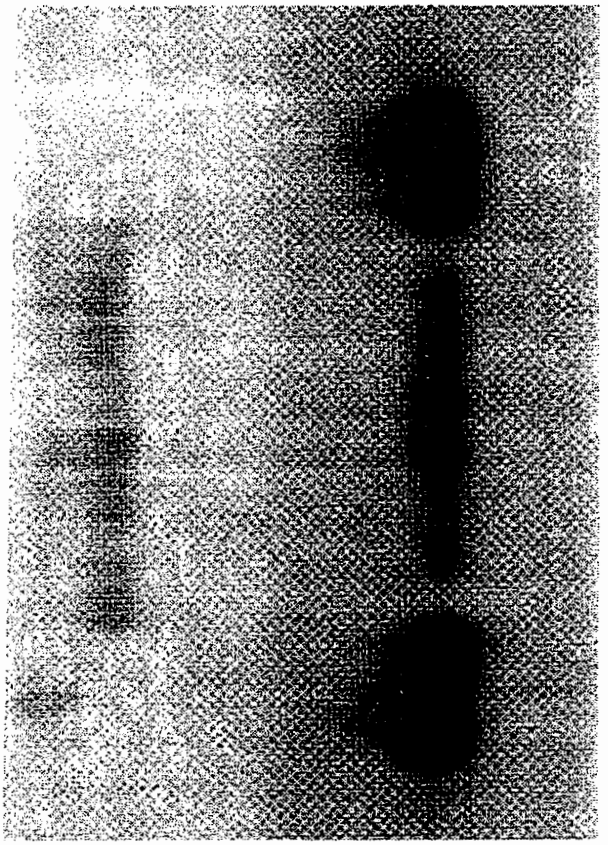

Figure L10$_1$ NPTII assay results

Experiment L11 PAT activity over a number of generations

Responsible : A. van Vliet, PGS NV Researcher

L11 Goal of the experiment

Quantification of the amount of phosphinothricin-acetyl-transferase (PAT) in extracts from leaves of B94-2 fertility restorer oilseed rape plants.

L11 Plant material

Two plants (A and B, stage of flowering) of each entry indicated in the Table below were sampled and analyzed.

Plant material	Notation
B94-2 progeny :	
S_1(B94-2)	RF2-S_1
S_3(B94-2)	RF2-S_3
BC_2 of B94-2 in Euro SOSR23	RF2-BC_2
Drakkar	

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L11 Results and conclusions

L11 Results

Sample		mg prot/ml extract	PAT U/ml extract	µg PAT/ml extract	µg PAT/mg prot
Drakkar control	A	0.7	0.00	0.0	0.0
	B	0.6	0.00	0.0	0.0
RF2-S_1(B94-2)	A	0.5	0.06	0.3	0.7
	B	0.5	0.07	0.4	0.7
RF2-S_3(B94-2)	A	0.7	0.04	0.3	0.4
	B	0.6	0.04	0.3	0.4
RF2-BC_2(B94-2)	A	0.4	0.05	0.3	0.8
	B	0.6	0.10	0.6	0.9

- Protein concentration is measured with the D_c protein assay of Biorad (Lowry method) with Bovine Serum Albumin as standard
- µg PAT is based on an estimated specific activity of 170U/mg PAT

L11 Conclusion

The results clearly indicate that the *bar* gene is inherited and stably expressed over a number of generations.

Part 1. Annex VI.1. General introduction to the experiments carried out in the laboratory, the greenhouse and the field to evaluate the *barnase* and *barstar* chimeric genes as inserted in the transgenic lines B91-4 and B93-101

As was outlined in the Table 5 of Part 1.VI. (*General outline of the experiments carried out to identify, select and characterize the transgenic male sterile and fertility restorer oilseed rape lines B91-4 and B93-101*) and will be described in detail in the following pages, Plant Genetic Systems nv has carried out many laboratory, greenhouse and field experiments to evaluate the *Brassica napus* male sterile B91-4 and fertility restorer B93-101 lines over many generations (Table[VI] 1.) and under different environmental conditions (Part 1.VI. Table 5.). Special attention was given to the molecular characterization of the plants, their morphology, the linkage between level of PPT tolerance and flower phenotype and their segregation patterns, the performance of the selectable marker, the normal agricultural performance, the stability of the flower phenotype throughout the season, the stability of the expression in different genetic backgrounds, the capacity to restore the fertility of the male sterile plants, the yield of the selected lines and the oil and seed quality.

Statistical analyses were performed on raw data. When analyzing segregations, a 2 x 2 test for independence was performed using, appropriate to the sample size, the Chi^2 test, the Fisher's exact test and/or the Yates correction. Complex data sets were subjected to analysis of variance (ANOVA) and in particular cases Least Significant Differences (LSD) were calculated according to Sokal, R.R. and Rohlf, F.J. (1981). (Biometry. 2nd edition. Freeman W.H. and company, New York, 859p)

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Part 1. Annex VI.2. General regulatory procedures and safety measures taken into account when carrying out laboratory, greenhouse and field trials

Laboratory and greenhouse trials were carried out under contained use procedures, meaning that no viable transgenic material left the PGS facilities. Field experiments were carried out under inspection of the national governments, federal and/or provincial authorities of each country (Belgium, France, Sweden, United Kingdom, Canada, USA) in which the experiment was performed. Every preparation of a trial included the submission of an application file to the competent authorities. Submitted data included information on the unmodified and modified oilseed rape, the used transformation procedure, the trial sites, the trial designs and protocols, monitoring and intentions for public consultations. Applications were based on the *EEC90/220 Directive Format*, the Canadian *Ag Canada Guidelines* or the *USDA-APHIS 2000 Format*.

Though all field trials were carried out following normal agricultural practices, safety measures were taken into account not only for regulatory reasons but also to ensure better reliability of our results :

- seeds were transported from the lab to the different locations in break-proof packages by a special courier or by PGS personnel; upon arrival, they were registered, identified and stored to avoid any mixture with other seed lots;
- during the preparation of the field trials, the experimental seed lots were treated with phytosanitary products as required by normal agricultural practices, weighed and divided for seeding the individual plots;
- seed bags were unequivocally labelled before the local responsible took them to the trial site;
- if sowing machines were used, it was guaranteed that the equipment was cleaned and controlled for the presence of any remaining seed; surplus transformed seeds were collected and returned for secure storage to the farm office;
- as it is imperative by the nature of oilseed rape, all trials were allowed to flower openly and to produce seeds;
- border plots were added at both ends of the experimental blocks; guard rows were surrounding the entire experiment resulting in a high degree of biological containment; the material grown in the border rows was considered as experimental material; in most designs an additional border of fallow land was included to allow normal field work;
- to protect the crop from bird damage cannons and/or bird netting have been installed in some cases;
- the personnel responsible for the execution of the experiment ensured that all seeds were harvested; though seeds were harvested just before complete maturity, small quantities of seeds could have fallen on the ground; unrecoverable seeds were allowed to germinate whereafter the plants were destroyed with a total herbicide; in all cases, the area of 'accidental release' was marked for further monitoring; the remaining plant material was destroyed by cultivation and left on the site;
- the bulk quantity of the harvested seed was either immediately destroyed by heating and/or burning or bagged in polyethylene bags for further analysis; none of the harvested products was used for commercial feed or food production; however, some larger batches could be required for additional safety tests.

Part 1. Annex VI.3. Basic characterization of the male sterile B91-4 and fertility restorer B93-101 oilseed rape line in the laboratory

Part 1. Annex VI.3.1. Detailed description of routinely used molecular techniques :
- Southern hybridization
- Polymerase Chain Reaction

Part 1. Annex VI.3.2. Primary characterization of the male sterile B91-4 and fertility restorer B93-101 line in the laboratory

Part 1. Annex VI.3.3. Detailed characterization of the male sterile B91-4 and fertility restorer B93-101 line in the laboratory

Part 1. Annex VI.3.1. Detailed description of routinely used molecular techniques :
- Southern hybridization
- Polymerase Chain Reaction

SOUTHERN HYBRIDIZATION PROCEDURE

Introduction

Total genomic DNA is isolated from plant tissue according to Dellaporta et al. (1983). A fraction of the isolated DNA is digested with appropriate restriction enzymes and the digested DNA fragments are separated by electrophoresis in agarose. After a depurination step, the fragments are denatured and transferred to nylon filters. The DNA fragments attached to the membranes are hybridized with P^{32}-labeled purified DNA fragments. Subsequently, the membranes are washed and exposed to autoradiography to visualize the hybridizing bands. Based on the mobility of the respective fragments, results are interpreted.

Preparation of total genomic DNA (according to Dellaporta et al. (1983). Plant Molecular Biology Reporter, 1, 3, 19-21)

- Weigh 0.5 g of leaf tissue, freeze in liquid nitrogen, grind with morter and pestle, and transfer the powder into a 30 ml Oak Ridge tube.

- Add 15 ml extraction buffer (100 mM Tris.HCl pH 8, 50 mM EDTA, 500 mM NaCl, 10mM β mercaptoethanol).

- Mix well.

- Add 1 ml 20% SDS, vortex and incubate at 65°C for 10 min.

- Add 5 ml 5 M potassium acetate, vortex and leave at 0°C for 20 min.

- Spin tubes at 25 000 x g (gravitation constant) for 20 min (13 000 rpm in Sorvall SA 600 rotor).
  Pour supernatant trough Miracloth filter (Calbiochem) into a clean 30 ml tube, containing 10 ml isopropanol.
  Mix and incubate at -20°C for 30 min .

- Pellet the DNA at 20 000 x g for 15 min.
  Gently pour off the supernatant and dry pellets by inverting the tubes on paper towels for 10 min.

- Redissolve DNA pellets with 700 µl of TE20 buffer (50 mM Tris.HCl pH 8, 20 mM EDTA), and transfer to an Eppendorf tube.

- Add 2.5 µl RNase (10 mg/ml) and incubate for 10 min at 37°C.

- Spin tubes for 10 min in an Eppendorf centrifuge to remove insoluble debris.

- Transfer the supernatant to a new eppendorf tube and add 75 µl 3 M sodium acetate and 500µl isopropanol. Mix well and pellet the DNA for 30 seconds in an eppendorf centrifuge.

- Wash pellets with 80% ethanol, dry and redissolve DNA in 100 µl TE buffer (10 mM Tris.HCl pH 8, 1 mM EDTA).

- Determine the concentration of the DNA by measuring the UV absorbance at 260 nm.

- Use the required genomic DNA amount to set up the digestion.

Restriction digests of total genomic DNA

- Mix together in an eppendorf tube :
  10 µg of genomic DNA
  4 µl 10 x RE buffer (*)
  20 units of restriction enzyme
  H_2O to 40 µl

- Incubate digest overnight in an oven at recommended temperature.

* composition of 10 x RE buffer

- 100 mM Tris.HCl pH 8
- 50 mM $MgCl_2$
- 60 mM β mercaptoethanol
- 1 mM EDTA
- 1 mg/ml BSA

RE 50 - 0.5 M NaCl
RE 100 - 1 M NaCl
RE 150 - 1.5 M NaCl

Separation of the restriction fragments on agarose gels

- Prepare 1% agarose gel in TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8 with acetic acid), containing 0.3 µg/ml Ethidium Bromide.

- Pour the gel into the gelsupport and let solidify.

- Add 5 µl of loading dye to the digested DNA samples and load the gel. Include a marker (lambda DNA digested with restriction endonuclease PstI on the gel

- Run the gel at an electric currency of 20 mA overnight .

- Cover the gel with Saran-wrap after the samples have migrated about 1 cm into the gel.

Blotting of the restriction fragments on nylon membranes

- After electrophoresis is completed, cut the gel from the support and photograph the gel. Place a transparent ruler alongside the gel so that the migration distance of the fragments can be read directly from the photograph.

- Blot the separated DNA fragments on Nylon membrane by capillary transfer or by vacuum transfer.
    * Depurination of the gel : put the gel in 0.25 M HCl until the dyes have changed colour.
    * Alkali transfer : transfer is done in 0.4 M NaOH (for capillary blotting) or 1 M NaOH (for vacuum blotting)
    * Membrane : Hybond - N+
    * Duration of transfer : minimum 3 hours to overnight for capillary transfer; 1 hour for vacuum transfer.
    * Rince the membrane briefly in 2 x SSC buffer (20 x SSC = 3M NaCl + 0.3M Sodium Citrate), wrap in Saran-wrap and store at 4°C.

Purification of fragments for probe preparation

- Digest +/- 20 µg of the plasmid DNA with the appropriate restriction enzyme as to generate the desired double stranded DNA fragment.

- Separate the DNA fragments on a 1% Low Melting Agarose gel, prepared in TAE buffer and containing 0.3 µg/ml Ethidium bromide.

- After electrophoresis is completed, cut the desired fragment from the gel with a scalpel. Put the gel slice in an Eppendorf tube.

- Add an equal volume of TE buffer (10 mM Tris.HCl pH 8 , 1mM EDTA) to the gel slice.

- Melt the gel slice in a 65°C waterbath for 10 min.

- Preheat an equal volume of phenol (equilibrated with TE buffer) 30 sec. at 65°C.

- Add the phenol to the melted gel slice and put the mixture on an Eppendorf shaker for 15 min.

- Centrifuge for 10 min in an Eppendorf centrifuge to separate the two phases.

- Transfer the water phase to a new Eppendorf tube and extract for a second time with an equal volume of phenol.

- Precipitate the DNA from the water phase with 0.1 volume of 5 M Sodium perchlorate and 1 volume of isopropanol.

- Pellet the precipitated DNA by spinning for 15 min in an Eppendorf centrifuge.

- Dry pellets and redissolve in 50 µl of TE.

- Measure the concentration of the DNA solution and dilute with H_2O, to a concentration of 25 ng/µl.

Labeling the DNA fragment with radioisotope (according to Feinberg and Vogelstein (1983) Analyt. Biochem., 132, 6-13 and Feinberg and Vogelstein (1984). Analyt. Biochem., 137, 266)

- Mix 25 ng DNA fragment + H_2O (total volume = 12 µl) in an Eppendorf.

- Denature the DNA fragment for 5 min in a boiling waterbath and cool quick in ice-water.

- Add to the tube :

  18 µl LS buffer (*)
  1 µl 5 mg/ml BSA (DNAse free)
  3 µl 1 mM dATP, dGTP, dTTP
  4 µl αP^{32} dCTP (specific activity = 3000 mCi/mmole)
  2 µl Klenow DNA Polymerase (5U/µl)

  ---
  40 ul

- Leave at room temperature for 5 hrs.

- Remove the unincorporated nucleotides by purifying the labeled DNA fragment over a BIO-RAD biospin-30 column.

* Composition of the LS buffer:
  Mix together:
  - 25 µl 1M HEPES pH 6.6
  - 25 µl TM buffer (250 mM Tris.HCl pH 8, 25 mM $MgCl_2$, 50 mM β-Mercaptoethanol)
  - 7 µl OL (45 O.D. units Hexamers /ml TE - PL Biochemicals)

Hybridization of the membrane with the labeled probe

- Make up a hybridization solution :

  6 x SSC (20 x SSC = 3 M NaCl + 0.3 M Sodium citrate)
  5 x Denhardt's solution (100 x Denhardt's = 2% BSA + 2% Ficoll + 2% PVP)
  0.5% (w/v) SDS
  20 µg/ml denatured sonicated Herring Sperm DNA

- Prehybridize the membrane at 65°C for minimum 1 hour.

- Denature the labeled probe by heating for 5 min. at 95°C.

- Replace the hybridization solution and add the denatured probe (do not exceed a probe concentration of 20 ng/ml)

- Hybridize at 65°C overnight

- Upon hybridization, wash the filters as follows:
    * 15 min in 6 x SSC
    * 30 min in 2 x SSC / 0.1% SDS
    * 30 min in 0.1 x SSC / 0.1% SDS

- Remove excess of washing solution from the membrane (probed membranes may not dry out after hybridization) and wrap in Saran-wrap.

Autoradiography

- Put the membrane, wrapped in Saran-wrap into the X-ray cassette , between two Kodak intensifying screens.

- Expose a Kodak X-ray film to the membrane for an appropiate time period at -70°C.

- Develop film in a X-ray film processor.

POLYMERASE CHAIN REACTION (PCR) PROCEDURE

Preparation of Plant Genomic DNA

The rapid extraction of small amounts of plant genomic DNA suitable for PCR analysis is done according to the method described by Edwards et al. (K. Edwards et al., Nucleic Acids Research, **19** (6), page 1349, 1991).

- Collect samples for PCR analysis (usually leaf tissue) by using the lid of a Eppendorf tube to pinch out a disc of material into the tube.
- Macerate the tissue with a plastic pestle at room temperature, without buffer for 5 to 15 sec.
- Add 400 µl extraction buffer (EB: 200 mM Tris HCl pH 7.5, 250 mM NaCl, 25mM EDTA, 0.5% SDS). The mixture can be left at room temperature until all samples have been extracted (> 1 hour).
- Centrifuge the extracts for 1 minute at max. speed and transfer 300 µl of the supernatant to a fresh Eppendorf tube.
- Mix with 300 µl isopropanol and leave a room temperature for 2 minutes.
- Centrifuge at max. speed for 5 minutes.
- Dry pellet and dissolve in 100 µl water.
- Centrifuge for 2 minutes and transfer supernatant to a new Eppendorf tube.
- Use 5 µl of this sample in a 50 µl PCR reaction.

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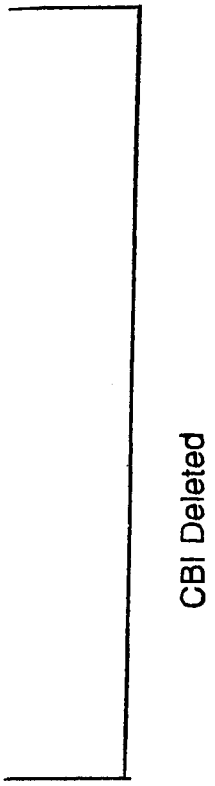

Part 1. Annex VI.3.2. Primary characterization of the male sterile B91-4 and fertility restorer B93-101 line in the laboratory

* Primary characterization of B91-4 and B93-101

- L01 Primary analysis of the original male sterile transformants on laboratory scale
- L02 Primary analysis of the original fertility restorer transformants on laboratory scale
- L03a Molecular analysis of the integration of the chimeric gene in the transgenic fertility restorer B93-101 line
- L03b Molecular analysis of the integration of the chimeric genes in the transgenic male sterile B91-4 and fertility restorer B93-101 lines

* Conclusion

- Primary transformants B91-4 and B93-101 contain a single copy of the chimeric barnase, respectively barstar gene.
- The transformants are characterized unambiguously by RFLP pattern.
- The transfer of the inserted DNA is limited to the sequences comprised between the border repeats.

EXPERIMENT L01 : Primary evaluation of the original male sterile transformants

Authorization number : Contained use

Responsible : Dr C. Mariani, PGS NV Project leader Nuclear Male Sterility and Restored Fertility
M. De Beuckeleer, Senior Researcher

L01 Goals of the experiment

Primary evaluation of the original candidate male sterile oilseed rape transformants on laboratory scale :

- screening the transformants for their gene copy number,
- characterization of the transformants by RFLP bands.

L01 Plant material

All *Agrobacterium* (strain A3135) mediated transformation experiments with the vector pTTM8RE containing the male sterile gene *barnase* (see 1.III.), were conducted in spring oilseed rape (SOSR), variety Drakkar.

L01 Methods

Shoots regenerated on selective medium (see 1.III.). All plantlets that were going to be transferred to the greenhouse were first analysed for the presence of the chimeric gene and the number of the insertions by Southern blot hybridization. Therefore, total DNA was isolated from 1 g of shoot tissue according to Dellaporta et al. (1983). DNA was prepared from each individual transformant and from an untransformed plant of the same cultivar Drakkar. Southern analysis was performed according to the general protocol as given in Annex VI.3.1.[ ]. CBI Deleted

L01 Results and conclusions

All plants have been analysed genotypically for the presence of the chimeric *barnase* containing gene (Table L01). Several transformants, including transformant B91-4, contained a single copy of the gene of interest. Each individual was clearly identified by its RFLP bands (example in Figure 1.).

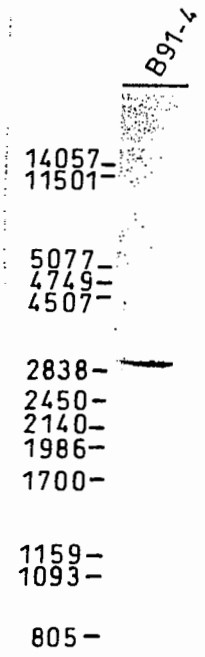

Figure 1. Southern blot of B91-4

Table L01. Primary characterization of the original male sterile Drakkar transformants

Transformation code number	Copy number by Southern analysis	RFLP bands by Southern analysis kb
B91-4	1	3,0

EXPERIMENT L02 : Primary evaluation of the original fertility restorer transformants

Authorization number : Contained use

Responsible : Dr C. Mariani, PGS NV Project leader Nuclear Male Sterility and Restored Fertility
M. De Beuckeleer, Senior Researcher

L02 Goals of the experiment

Primary evaluation of the original candidate fertility restorer oilseed rape transformants on laboratory scale :

- screening the transformants for their gene copy number,
- characterization of the transformants by RFLP bands.

L02 Plant material

All *Agrobacterium* (strain A3147) mediated transformation experiments with the vector pTVE74RE containing the fertility restorer gene *barstar* (see 1.III.), were conducted in spring oilseed rape (SOSR), variety Drakkar.

L02 Methods

Shoots regenerated on selective medium. All these plantlets going to be transferred to the greenhouse, were first analysed for the presence of the chimeric gene and the number of the insertions by Southern blot hybridization.

Total DNA was isolated from 1 g of shoot tissue according to Dellaporta et al. (1983). DNA was prepared from each individual transformant and from an untransformed plant of the same cultivar Drakkar. Southern analysis was performed according to the general protocol as given in Annex VI.3.1.[ ] CBI Deleted

L02 Results and conclusions

All plants have been analysed genotypically for the presence of the chimeric *barstar* containing gene (Table L02). Several transformants, including transformant B93-101, contained a single copy of the gene of interest. All individuals were identified by their RFLP bands (example in Figure 1.).

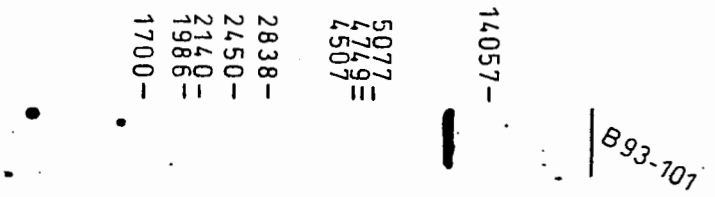

Figure 1. Southern blot of B93-101

Table L02. Primary characterization of the original fertility restorer Drakkar transformants

Transformation code number	Copy number by Southern analysis	RFLP bands by Southern analysis kb
B93-101	1	9,0

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EXPERIMENT L03a : Molecular analysis of the integration of the chimeric gene in the transgenic fertility restorer B93-101 Drakkar line

Authorization number : Contained use

Responsible : Dr J. Botterman, PGS NV Product Development Manager Lab

L03a Goals of the experiment

Molecular analysis of the integration of the chimeric gene in the transgenic fertility restorer S_2 B93-101 Drakkar line :

- determination of the gene integration pattern,
- verification of proper recognition of T-DNA border repeats upon gene transfer to the plant genome.

L03a Plant material

Greenhouse grown plants of the S_2 progeny of the line B93-101 and of an untransformed plant of the same cultivar.

L03a Methods

Total DNA was isolated from 1 gram of shoot tissue according to Dellaporta et al. (1983). For hybridization with probes containing parts of the chimeric genes (*barstar* or *neo* coding region) or parts of the plasmid sequences outside the T-DNA border repeats, total DNA was digested with [ ] separated by electrophoresis in a 0.8% agarose gel, transferred to nylon Hybond-N filters and hybridized with a ^{32}P-labeled purified fragment. Washed filters were exposed by autoradiography. The transferred DNA within the plant genome and the respective probes used is schematically represented in Figure L03$_1$ (probes used for T-DNA transfer; probes used from plasmid sequences outside from the T-DNA border repeats).

To determine the number of integrated copies of the chimeric gene constructs as comprised between the T-DNA border repeats of the plasmid vector pTVE74RE, the total plant DNAs separated on agarose gel were hybridized with two different DNA probes. One fragment comprises part of the nucleotide sequence corresponding with the information [ ] contained between the restriction site chosen for restriction digest [ ] and the right T-DNA border sequence and the other contains nucleotide sequences [ ] contained between the restriction site and the left border (Panel A).

From the Panel A analysis it is not possible to judge if plasmid sequences originating from outside the terminal border repeats are also transferred within the plant genome. To this end, total DNA prepared from the respective plants digested with the same enzyme was also hybridized with DNA fragments comprising the nucleotide sequences as present outside the terminal border repeats in the plasmid pTVE74RE (probes indicated as +RB and +LB in Figure L03$_1$). Hybridization was done with the individual probes.

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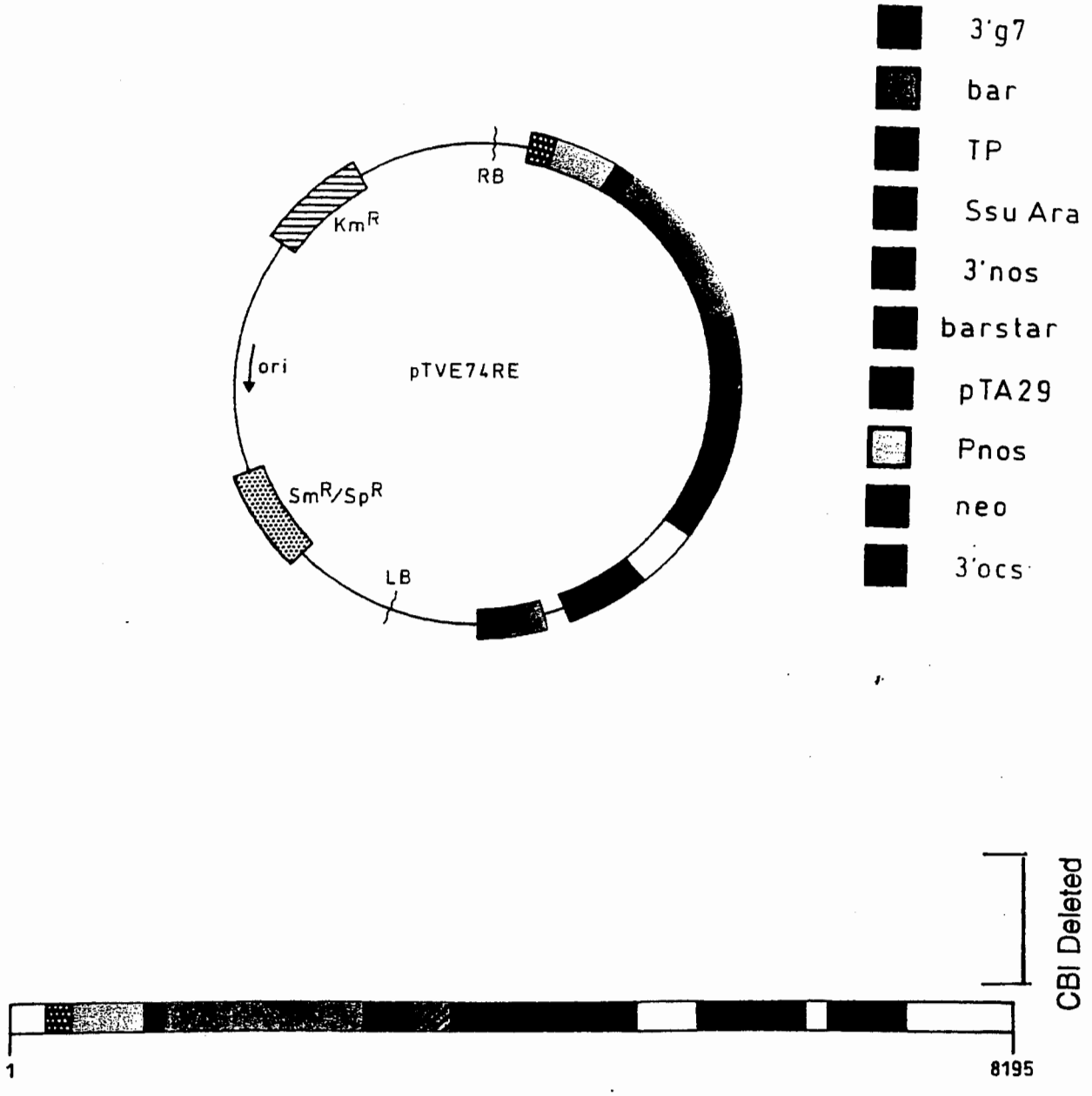
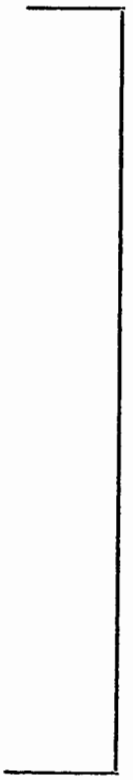


Figure 5. Map of plasmid pTVE74RE



L03a Conclusions

The following conclusions could be drawn from the above described experiment :
- the pattern of gene integration in the plant genome has been analysed by Southern blot;
- one copy of the chimeric gene construct is integrated in B93-101;
- sequences outside the T-DNA border repeats are not co-transferred with the chimeric gene constructs; the latter are comprised between the border repeats.

B 93-101

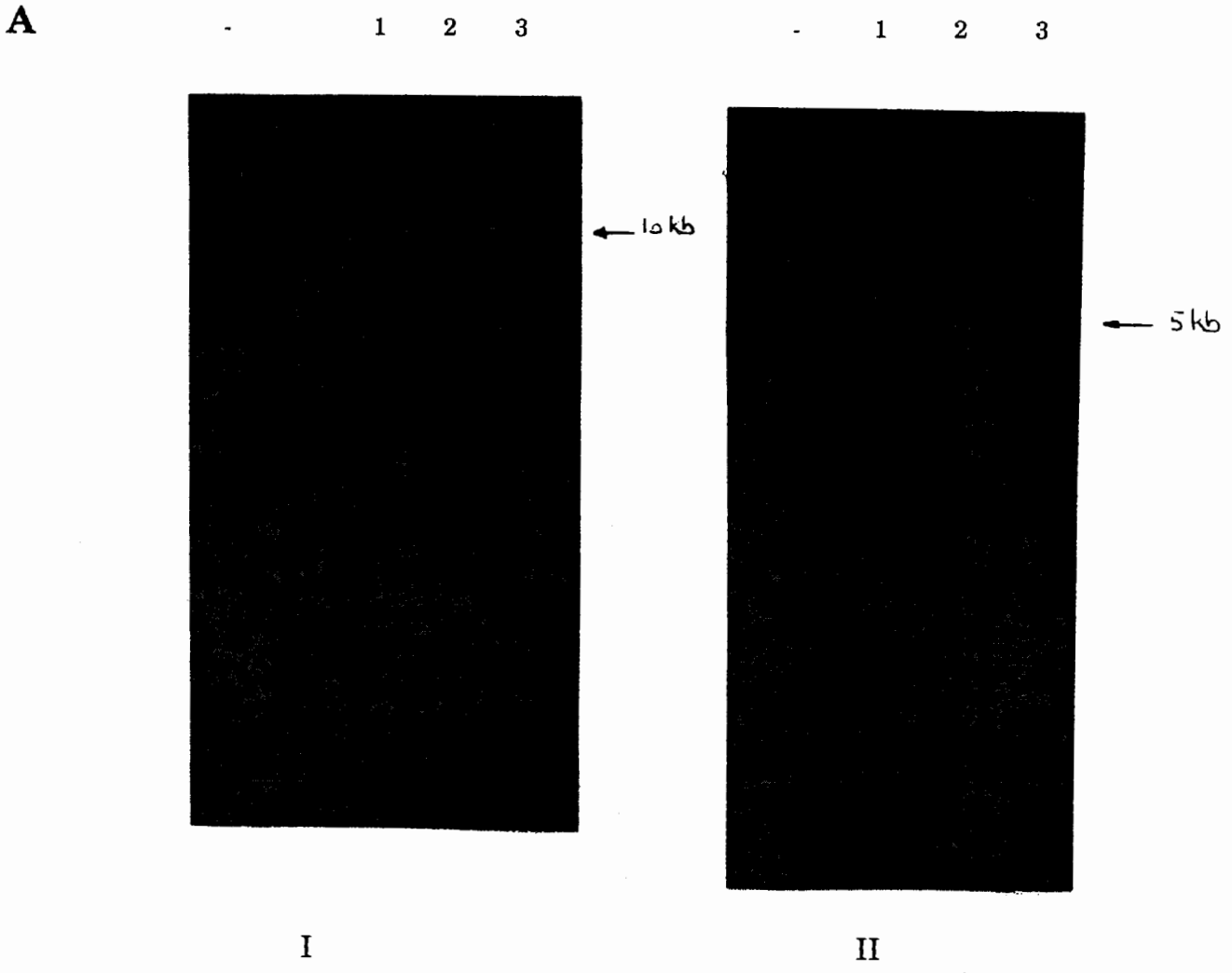


Figure L03a-1. Southern blot analysis B93-101 (Rf1)

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DNA was isolated from 3 individual plants (1, 2 and 3) from the S2 generation. Ten µg of total genomic DNA was digested[ ]

Ten µg total genomic DNA from a non-transgenic plant, digested with[ ]served as the negative control (-).

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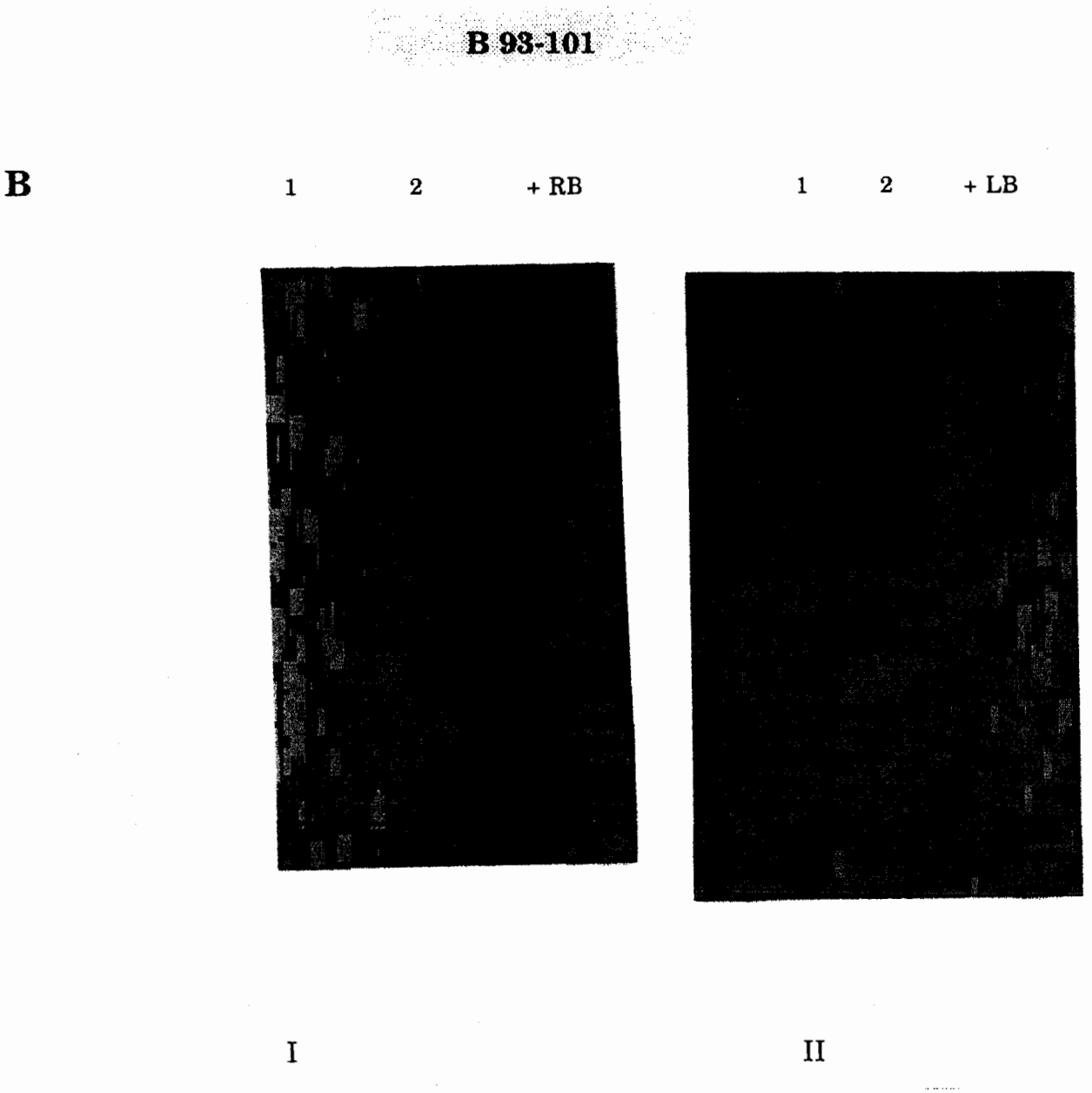

Figure L03a-2. Southern blot analysis B93-101 (Rf1)

DNA was isolated from 2 individual plants (1 and 2) from the S2 generation. Ten µg of total genomic DNA was digested with [redacted] CBI DELETED

The amount of positive controls (+ RB and + LB) loaded on the gel is equivalent to 1 copy integrated in 10 µg of oilseed rape DNA.

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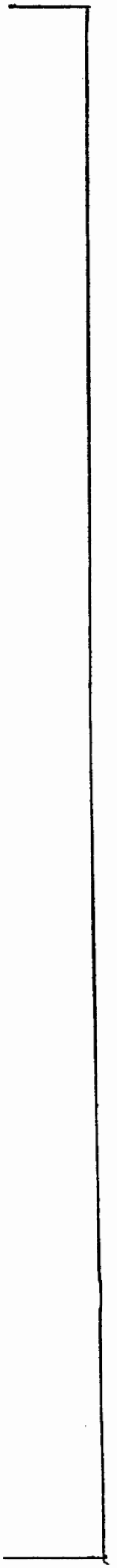

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L03b Results and conclusions

L03b Results

Three oligonucleotides were designed based on the nucleotide sequence of pTTM8RE and pTVE74RE at the left border repeat to be used as primers in DNA amplification. Two primers are localized within the transferred region comprised between the border repeats, whereas the other primer is localized immediately outside from the left border repeat.

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] The amplification reactions indicate that no sequences beyond the left border repeat have been transferred to the plant genome.

L03b Conclusions

The molecular analysis showed that sequences outside from the T-DNA left border repeat are not co-transferred with the chimeric gene constructs comprised between the border repeats.

Agarose Gel :

1. 100 bp MW ladder
2. B91-4
3. B91-4
4. MS3
5. MS3
6. B93-101
7. B93-101
8. RF2
9. RF2
10. 100 bp MW ladder
11. Drakkar control
12. Drakkar control
13. pTTM8RE
14. 100 bp MW ladder

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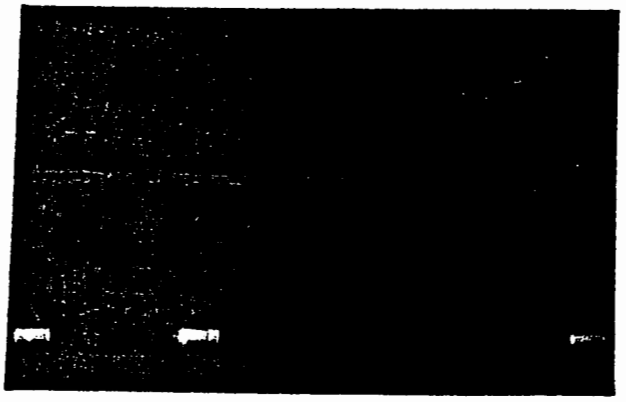

Figure L03b PCR Results

Part 1. Annex VI.3.3. Detailed characterization of the male sterile B91-4 and fertility restorer B93-101 line in the laboratory

* Detailed characterization of the selected lines

- L04 The transformants are free of *Agrobacterium tumefaciens*
- L05 Stability of integration of the T-DNA over a number of generations via Southern blot analysis
- L06 Confirmation that the inserted DNA corresponds to the plasmid T-DNA
- L07 Characterization of the T-DNA integration via molecular analysis of the regions flanking the T-DNA
- L08 Molecular analysis of the target-site-deletion present on the homologous chromosome
- L09 Homology search between plant DNA sequences and the B91-4 and B93-101 target sequences
- L10 Expression of the introduced transgenes via Northern analysis and evaluation of occurrence of cryptic gene expression
- L11 NPTII activity assay over a number of generations
- L12 Spectrophotometric PAT assay over a number of generations
- L13 Identification of the genome on which the genes are inserted.

* Conclusion

- The descendants of the transformants are free of *Agrobacterium tumefaciens*.
- There have been no rearrangements of the DNA upon insertion in the plant genome.
- The inserted DNA remains genetically stable in distinct generations.
- The insertion site has been characterized. The insertion of the chimeric barnase and barstar construct has occurred in the A genome of *Brassica napus* (AACC).
- There is no indication of the insertion of the DNA in a functional gene.
- The expression of the inserted DNA is limited to the introduced coding sequences. There is no indication of expression of other newly inserted sequences (cryptic genes).
- NPTII and PAT activity of the transgenic lines have been assessed. The level of activity remains constant over the tested generations.

L04 Demonstration of the absence of Agrobacteria in the transgenic oilseed rape lines

Responsible : C. Opsomer, Senior Researcher

L04 Goal

In order to confirm the absence of Agrobacteria containing the Ti-plasmid used for transformation of the transgenic oilseed rape lines, a molecular detection procedure has been developed.

This method is based on the detection of Ti- plasmid specific sequences in a plant leaf extract by the PCR amplification method. These sequences are located outside the left border repeats of the Ti-plasmid.

To estimate the sensitivity of the method, a reconstruction experiment is done in parallel with the experiment on plant extract.

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L04 Results and conclusion

No amplification was observed in extracts prepared from Drakkar, B91-4 and B93-101 leaf material.

In the reconstruction experiment, we can detect between 1.10^5 and 1.10^6 cells of Agrobacterium added to 250 mg of leaf material.
Below this limit, we could not amplify the desired fragment.

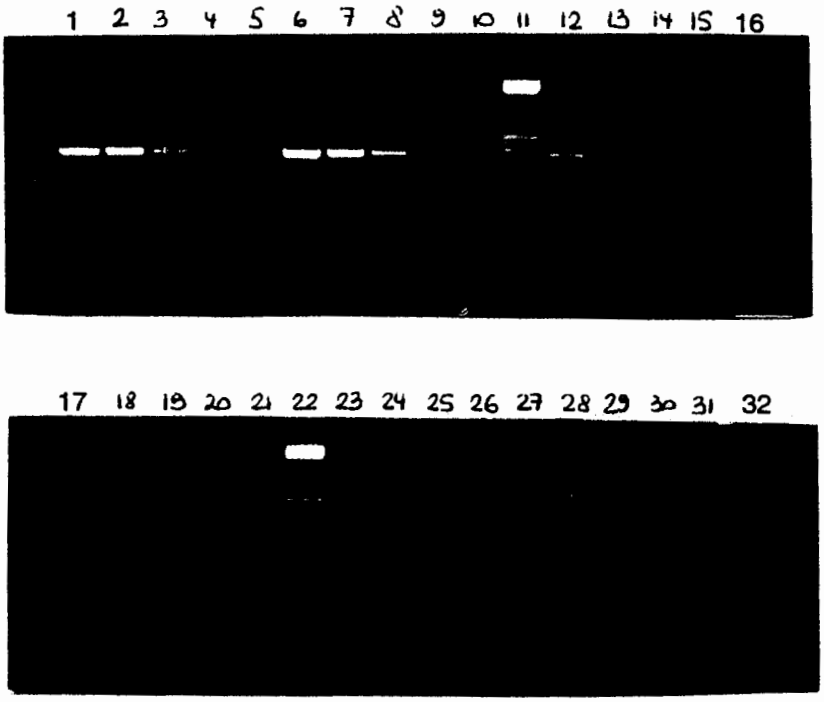

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Figure 1. Reconstruction Experiment

250 mg of leaf from wild type Drakkar was mixed with defined amount of Agrobacterium (dilutions A-F, as specified in 2.) and DNA was extracted.
From each of these DNAs, serial dilutions were made (10^0 - 10^{-4}), and amplified in PCR.

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PCR products are visualized on agarose gel:

1. Drakkar + Agro (A): 20 µl
2. Drakkar + Agro (A): 20 µl 10^{-1}
3. Drakkar + Agro (A): 20 µl 10^{-2}
4. Drakkar + Agro (A): 20 µl 10^{-3}
5. Drakkar + Agro (A): 20 µl 10^{-4}
6. Drakkar + Agro (B): 20 µl
7. Drakkar + Agro (B): 20 µl 10^{-1}
8. Drakkar + Agro (B): 20 µl 10^{-2}
9. Drakkar + Agro (B): 20 µl 10^{-3}
10. Drakkar + Agro (B): 20 µl 10^{-4}
11. 100 bp ladder
12. Drakkar + Agro (C): 20 µl
13. Drakkar + Agro (C): 20 µl 10^{-1}
14. Drakkar + Agro (C): 20 µl 10^{-2}
15. Drakkar + Agro (C): 20 µl 10^{-3}
16. Drakkar + Agro (C): 20 µl 10^{-4}
17. Drakkar + Agro (D): 20 µl
18. Drakkar + Agro (D): 20 µl 10^{-1}
19. Drakkar + Agro (D): 20 µl 10^{-2}
20. Drakkar + Agro (D): 20 µl 10^{-3}
21. Drakkar + Agro (D): 20 µl 10^{-4}
22. 100 bp ladder
23. Drakkar + Agro (E): 20 µl
24. Drakkar + Agro (E): 20 µl 10^{-1}
25. Drakkar + Agro (E): 20 µl 10^{-2}
26. Drakkar + Agro (E): 20 µl 10^{-3}
27. Drakkar + Agro (E): 20 µl 10^{-4}
28. Drakkar + Agro (F): 20 µl
29. Drakkar + Agro (F): 20 µl 10^{-1}
30. Drakkar + Agro (F): 20 µl 10^{-2}
31. Drakkar + Agro (F): 20 µl 10^{-3}
32. Drakkar + Agro (F): 20 µl 10^{-4}

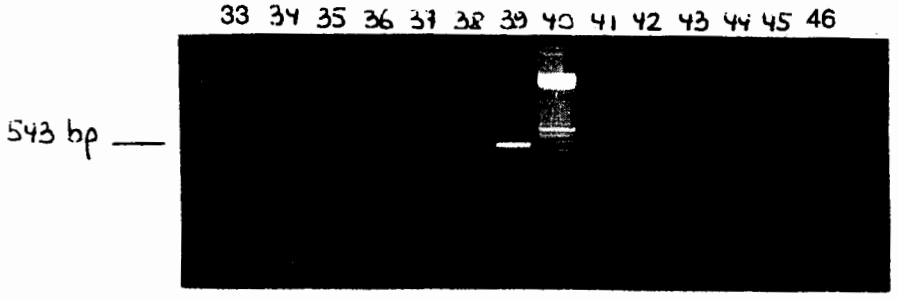

Figure 2. Assay for the presence of Agrobacterium in transformed Drakkar lines

250 mg of leaf from wild type Drakkar was mixed with defined amount of Agrobacterium (dilutions A-F, as specified in 2.) and DNA was extracted.
From each of these DNAs, serial dilutions were made (10^0 - 10^{-4}), and amplified in PCR[ ]
PCR products are visualized on agarose gel:

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33. Drakkar: 20 µl
34. Drakkar: 20 µl 10^{-1}
35. Drakkar: 20 µl 10^{-2}
36. Drakkar: 20 µl 10^{-3}
37. Drakkar: 20 µl 10^{-4}
38. Blanco: 20 µl H_2O
39. Positive control: 1 pg Ti-Plasmid
40. 100 bp ladder
41. B91-4: 20 µl
42. B91-4: 20 µl 10^{-1}
43. B91-4: 20 µl 10^{-2}
44. B93-101: 20 µl
45. B93-101: 20 µl 10^{-1}
46. B93-101: 20 µl 10^{-2}

L05 Stability of integration of the transforming DNA over a number of generations via Southern analysis

Responsible : Dr J. Botterman, PGS Manager Product Development Lab
C. Opsomer, Senior Researcher

L05 Goals of the experiment

Analyzing the stability of integration and the inheritance of the introduced chimeric barnase and barstar gene constructs in respectively the B91-4 and B93-101 oilseed rape progeny. The experimental approach is based on molecular analysis using the Southern blot procedure.

L05 Plant material

To demonstrate the stability of the integrated gene construct, a molecular analysis has been performed on the plants from subsequent progenies. For both the male sterility and restoration of fertility line, 4 generations have been tested for each case. The analysis has been performed on two individual plants (A and B) from each progeny. Non-transgenic Drakkar has been used as a negative control.

Table L05 Plant material

Plant material	Notation
B91-4 progeny :	
T_1(B91-4)	MS1-T_1
T_3(B91-4)	MS1-T_3
BC_4 of B91-4 in Can SOSR2	MS1-BC_4
BC_5 of B91-4 in Euro SOSR6	MS1-BC_5
B93-101 progeny :	
S_1(B93-101)	RF1-S_1
S_3(B93-101)	RF1-S_3
BC_3 of B93-101 in Can SOSR2	RF1-BC_3 (B6907)
BC_3 of B93-101 in Euro SOSR6	RF1-BC_3 (B6923)

L05 Results and conclusion

L05 Results

In Figure 1. and 2., a schematic representation of the DNA fragment comprised between the T-DNA border repeats of respectively pTTM8RE and pTVE74RE is given. The localization of the [ ] site within the transferred DNA, the [ ] and the size of the expected crosshybridizing bands are indicated.

The autoradiogram from a Southern blot on two individual plants (A and B) from different generations (T_1, T_3, BC_4 and BC_5) carrying the male sterility gene and from different generations (S_3, S_3, BC_3) carrying the gene for restoration of fertility with the hybridization pattern using the [ ] is shown in Figure 3. and 4.. As expected, an identical hybridization pattern was found in the respective generations.

L05 Conclusion

In conclusion, this analysis clearly demonstrates that the transferred DNA is stably inherited in subsequent generations and in different genetic backgrounds.

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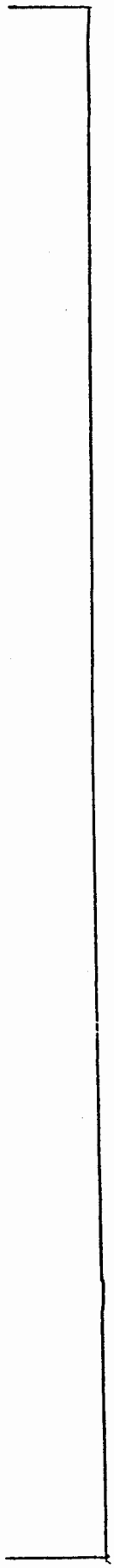
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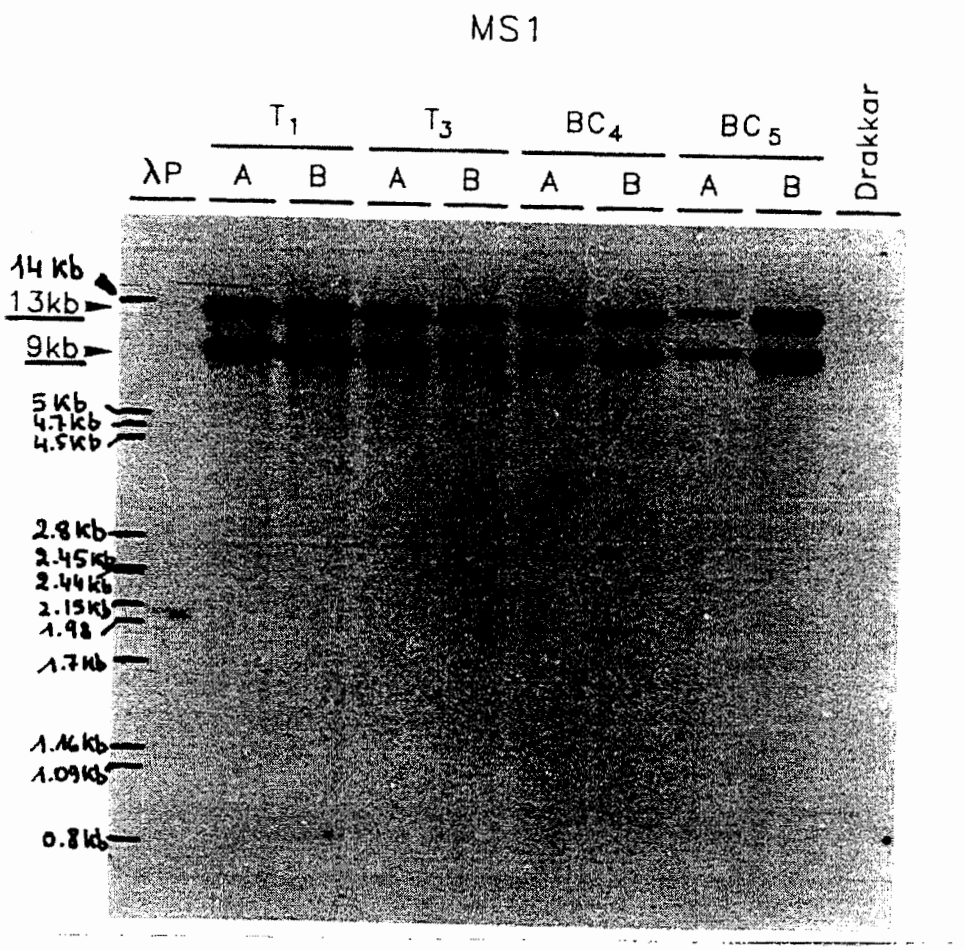

Figure 3. Stability analysis Ms1 - Southern blot analysis

DNA was isolated from 2 individual Ms1 oilseed rape plants (generations T_1, T_3, BC_4 and BC_5).
Ten µg genomic DNA was digested with [redacted]
Ten µg genomic DNA, isolated from a non-transgenic Drakkar plant, were digested with [redacted] and served as a negative control.
Lambda DNA digested with PstI served as a molecular weight marker (sizes given in Kb).

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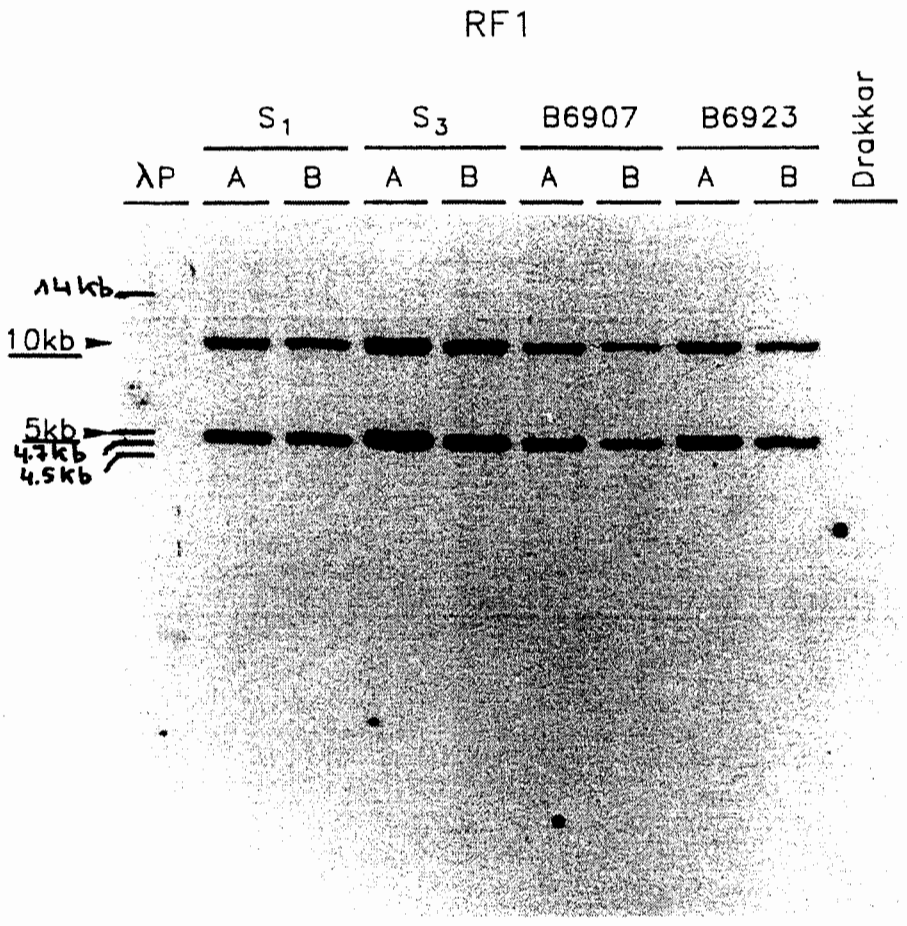

Figure 4. Stability analysis Rf1 - Southern blot analysis

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DNA was isolated from 2 individual Rf1 oilseed rape plants (generations S_1, S_3, B6907 and B6923). Ten µg genomic DNA was digested with [redacted]

Ten µg genomic DNA, isolated from a non-transgenic Drakkar plant, were digested with [redacted] and served as a negative control.

Lambda DNA digested with PstI served as a molecular weight marker (sizes given in Kb).

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L06 Confirmation that the transformed DNA corresponds to the plasmid DNA

Responsible : Dr J. Botterman, PGS Manager Product Development Lab
C. Opsomer, Senior Researcher

L06 Goals of the experiment

To demonstrate that the DNA transferred into the plant genome is identical to the T-DNA region of the plasmid vector, a molecular analysis based on Southern blotting has been performed.

L06 Plant material

Molecular analysis has been performed on the plants carrying the male sterility construct and of a plant carrying the construct for restoration of fertility and compared with total plasmid DNA. The analysis has been performed on two individual plants (A and B) from each progeny. Non-transgenic Drakkar plants have been used as negative control.

Table L06 Plant material

Plant material	Notation
B91-4 progeny : T_3(B91-4)	MS1-T_3
B93-101 progeny : S_3(B93-101)	RF1-S_3

L06 Methods

Southern analysis was performed according to the protocol as described in Annex VI3.1.. Genomic DNA has been prepared from leaf tissue of a plant carrying the male sterility gene and of a plant carrying the gene for restoration of fertility and compared with total plasmid DNA. The experimental approach is based on digesting the respective DNAs with different restriction enzymes, probe with different fragments of the transferred DNA and make a comparison between plasmid and genomic DNA fragments. The comparative analysis is based on the determination of the sizes of the respective hybridizing bands[

]

L06 Results and conclusion

L06 Results

An overview of the results is given in the tables and figures.

Table 1 gives an overview of the different digests performed on total genomic DNA from a male sterile line and the plasmid vector pTTM8RE and on total genomic DNA of a restorer line and the plasmid pTVE74RE. Four probes have been used in the hybridizations and the expected fragments based on the plasmid map are indicated. As an example, the schematic representation of the DNA fragment comprised between the right and left border repeat of pTTM8RE with the respective chimeric gene constructs, is outlined in Figure 1a.,1b.,1c. and 1d.. The localization of the respective restriction enzymes used in the consecutive hybrizations are indicated and the crosshybridizing bands with the respective probes are highlighted.

In Figures 2a., 2b.,2c.,2d., some examples of autoradiograms of the different probe-digest combinations are shown, clearly indicating that the plant DNA corresponds to the plasmid DNA.

L06 Conclusion

In conclusion, this analysis demonstrates that the transferred DNA in the plant genome corresponds to the DNA configuration as designed in the plasmid vector.

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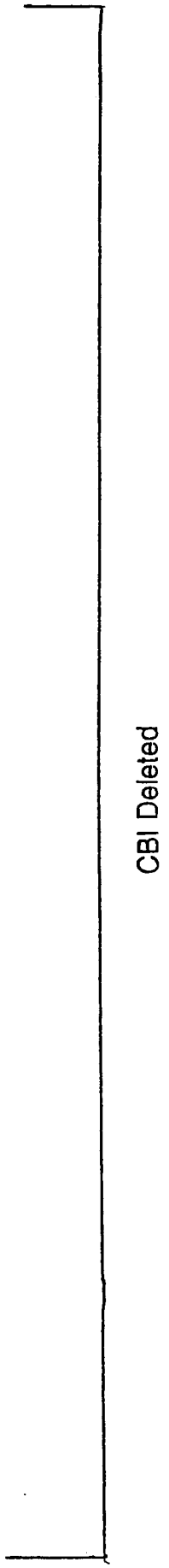
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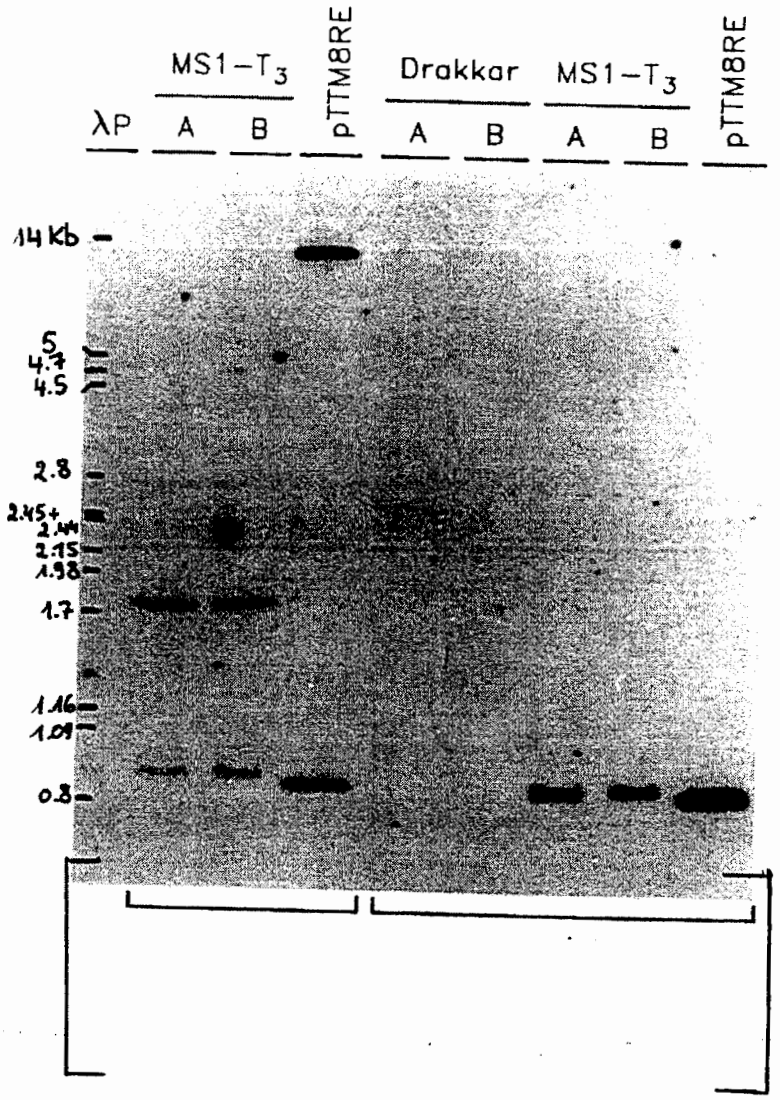

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Figure 2a. Insert characterization Ms1 - Southern blot analysis

DNA was isolated from 2 individual plants from the T_3 generation of Ms1. Ten µg genomic DNA was digested with the indicated restriction enzymes. Ten µg genomic DNA, isolated from non-transgenic Drakkar plants, served as negative controls.
The amount of pTTM8RE samples loaded is equivalent to ± 1 copy of the plasmid integrated in 10 µg of genomic oilseed rape DNA.

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Lambda DNA, digested with PstI, served as the molecular weight marker (sizes given base pairs).

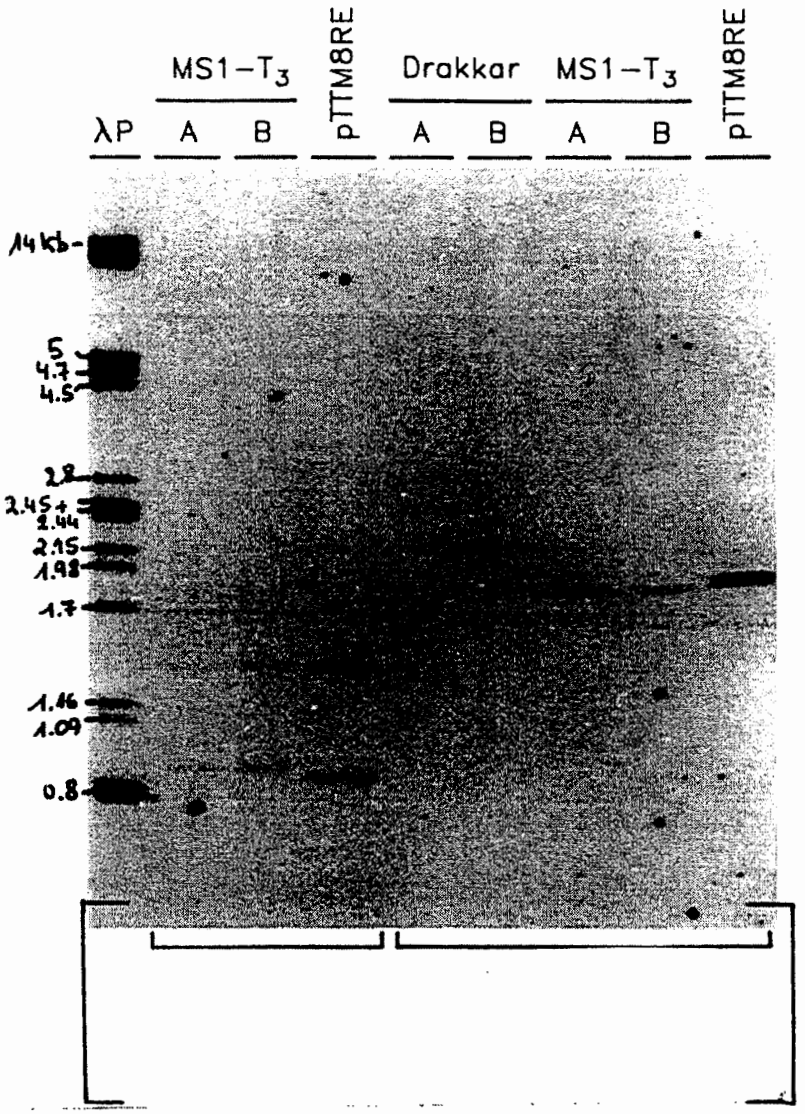

Figure 2b. Insert characterization Ms1 - Southern blot analysis

DNA was isolated from 2 individual plants from the T_3 generation of Ms1. Ten µg genomic DNA was digested with the indicated restriction enzymes. Ten µg genomic DNA, isolated from non-transgenic Drakkar plants, served as negative controls.
The amount of pTTM8RE samples loaded is equivalent to ± 1 copy of the plasmid integrated in 10 µg of genomic oilseed rape DNA.

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Lambda DNA, digested with PstI, served as the molecular weight marker (sizes given base pairs).

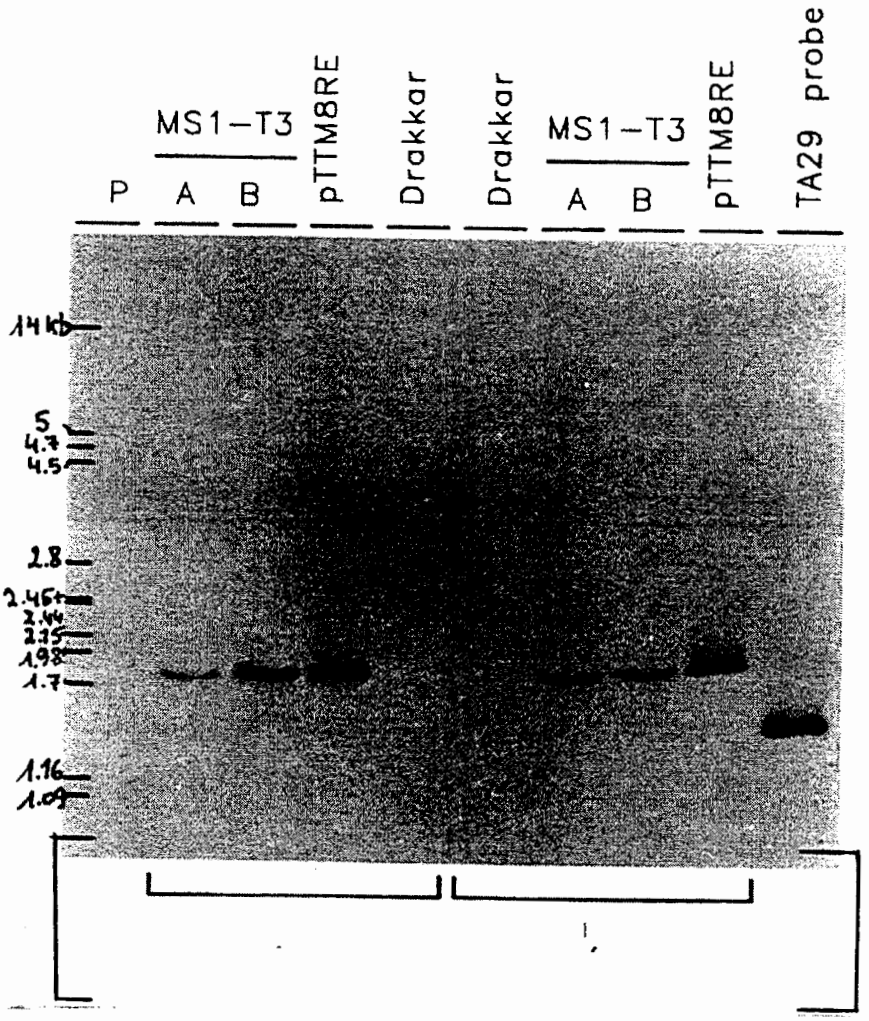

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Figure 2c. Insert characterization Ms1 - Southern blot analysis

DNA was isolated from 2 individual plants from the T_3 generation of Ms1. Ten µg genomic DNA was digested with the indicated restriction enzymes. Ten µg genomic DNA, isolated from non-transgenic Drakkar plants, served as negative controls.
The amount of pTTM8RE samples loaded is equivalent to ± 1 copy of the plasmid integrated in 10 µg of genomic oilseed rape DNA.

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Lambda DNA, digested with PstI, served as the molecular weight marker (sizes given base pairs).

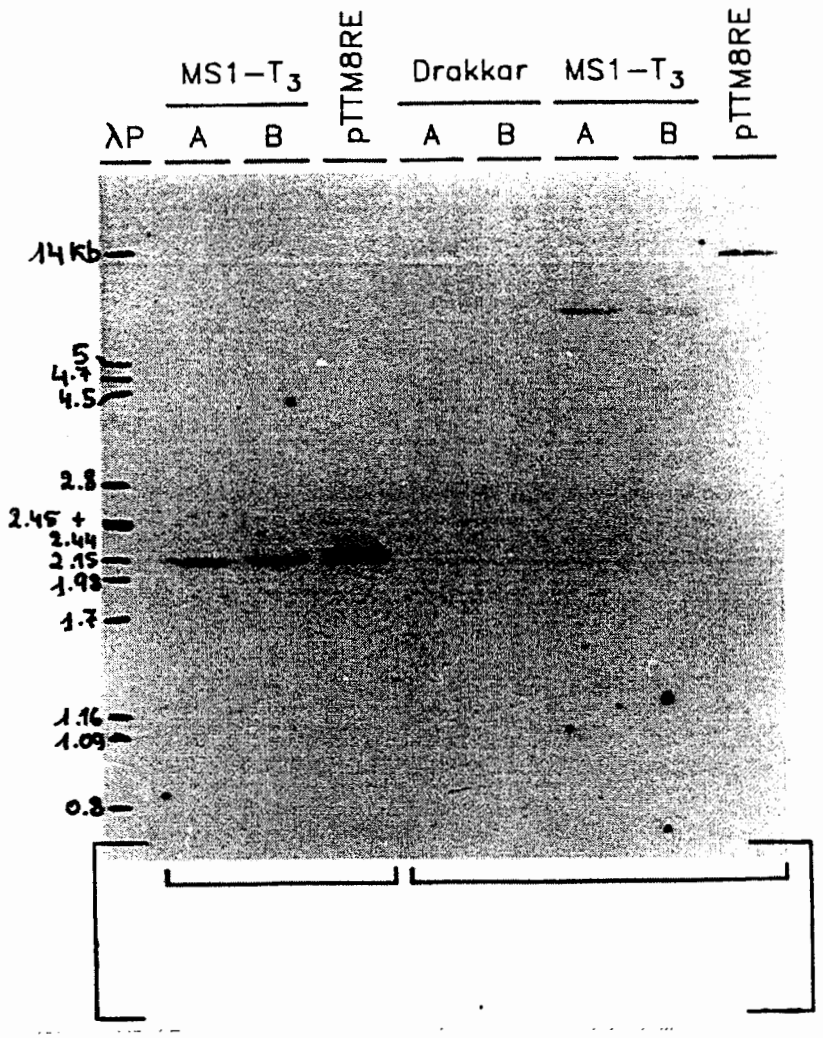

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Figure 2d. Insert characterization Ms1 - Southern blot analysis

DNA was isolated from 2 individual plants from the T_3 generation of Ms1. Ten µg genomic DNA was digested with the indicated restriction enzymes. Ten µg genomic DNA, isolated from non-transgenic Drakkar plants, served as negative controls.
The amount of pTTM8RE samples loaded is equivalent to ± 1 copy of the plasmid integrated in 10 µg of genomic oilseed rape DNA.

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Lambda DNA, digested with PstI, served as the molecular weight marker (sizes given base pairs).

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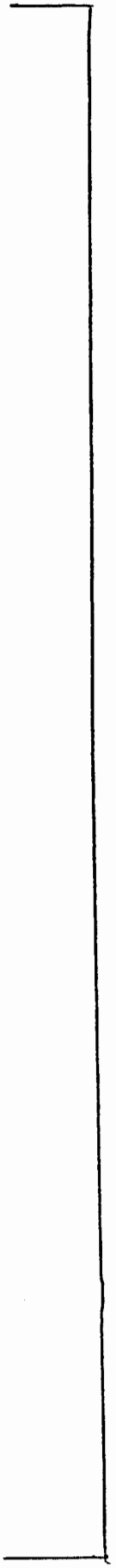

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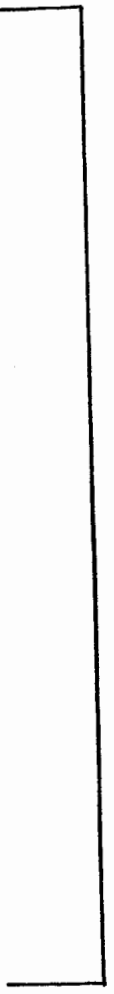

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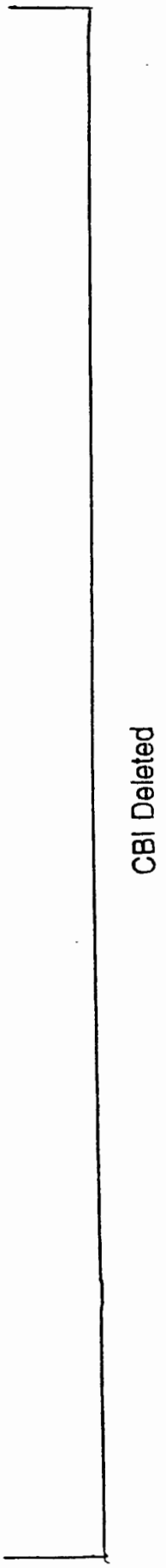

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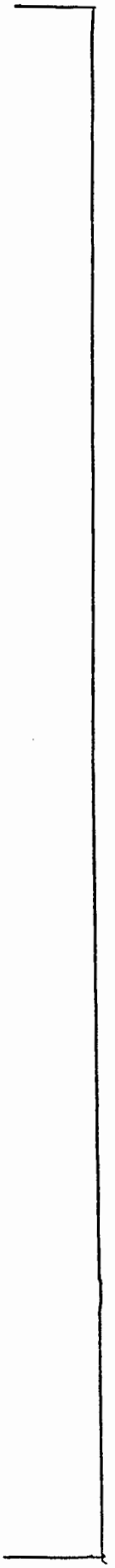

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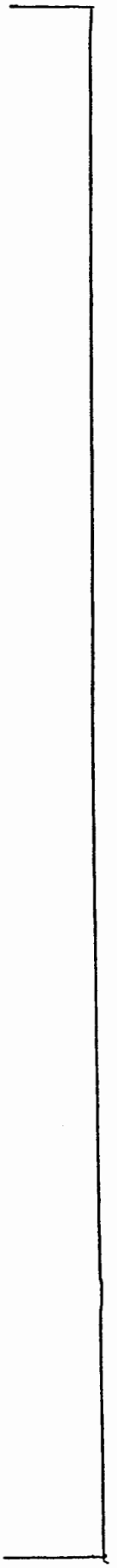

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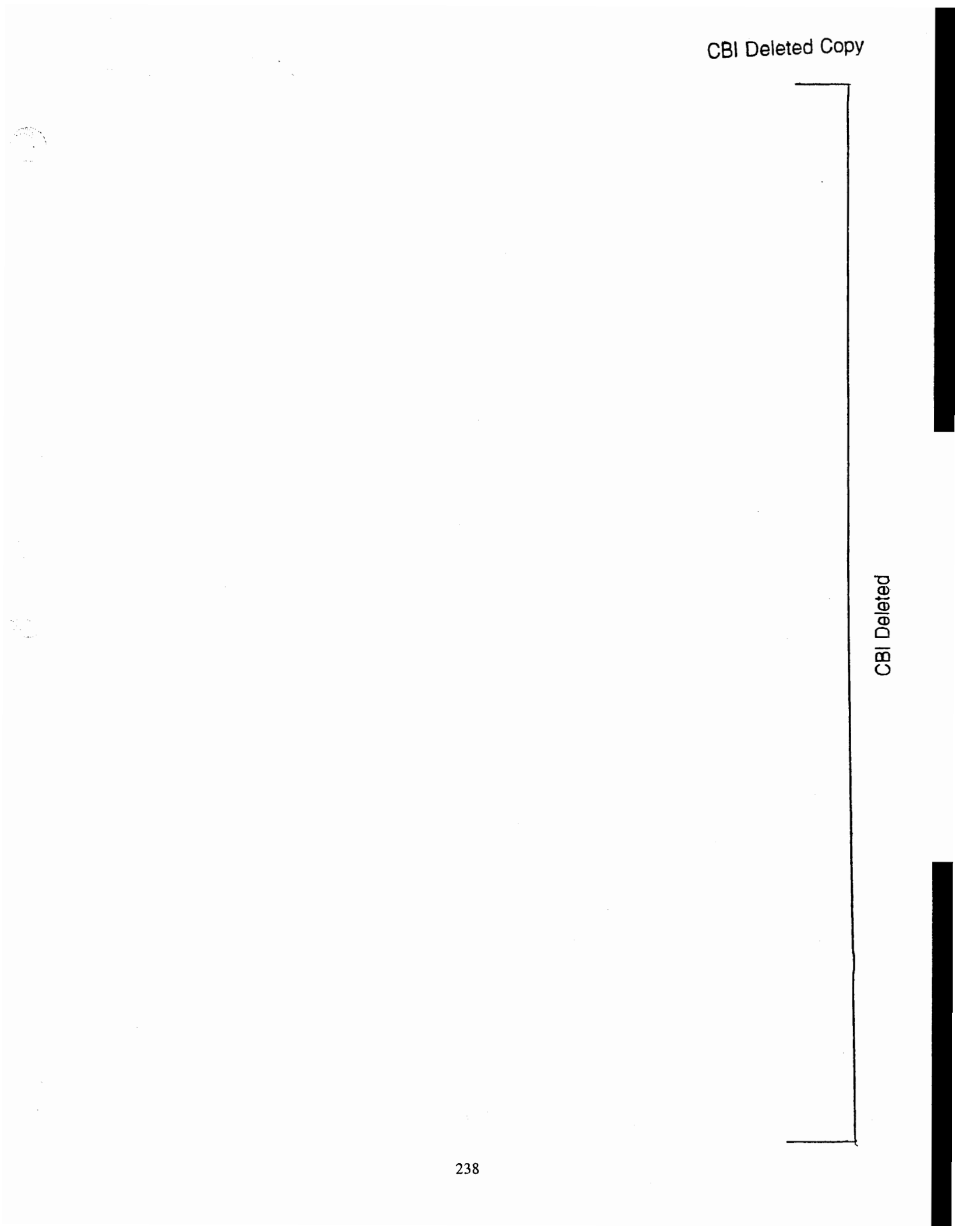

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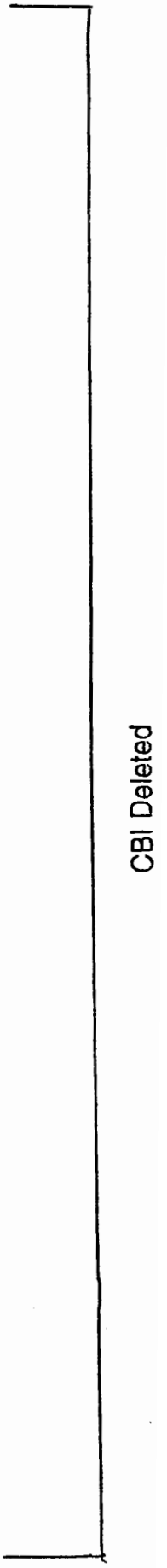

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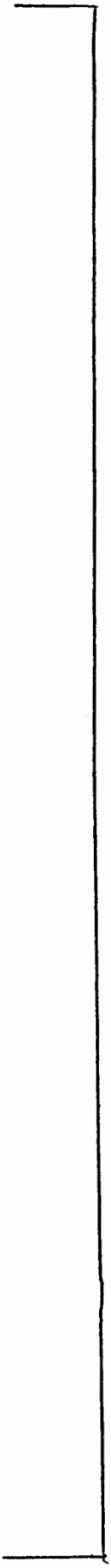
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EXPERIMENT L09 Homology search between plant DNA sequences and the B91-4 and B93-101 target sequences

Responsible : M. De Beuckeleer, Senior Researcher

L09 Goal

Homology search between plant DNA sequences and the B91-4 and B93-101 target sequences

L09 Plant material

Molecular analysis has been performed on plants carrying the male sterility gene and of plants carrying the gene for restoration of fertility.

Table L09 Plant material

Plant material	Notation
B91-4 progeny : T_3(B91-4)	MS1-T_3
B93-101 progeny : S_3(B93-101)	RF1-S_3

L09 Methods, results and conclusions

Following sequence databanks were searched on 93-12-01 using the IntelliGenetics Inc. (Mountain View, California, USA) software:

1.	EMBL 36	EMBL nucleic acid sequence data bank
2.	N-GeneSeq4	Patented nucleic acid sequence data bank
3.	UGenBank 79_36	Sequences unique to the NIH data bank

34309 sequences were compared. The scores were sorted by optimized score and subsequently aligned to the query sequence. No significant homologies were found.

Through the BLAST network service we performed a search in peptide sequence databanks. Computation was performed at the National Centre for Biotechnology Information (NCBI, Rockville Pike, Bethesda, U.S.A.). For this search both strands of the query sequence were translated in all 6 reading frames.
Statistical significance is estimated under the assumption that the equivalent of one reading frame in the query sequence codes for protein and that significant alignments will involve only coding reading frames.
The search was performed on 93-12-09. In total 61.248 sequences were searched.
158 sequences producing high-scoring segment pairs were found. When analysing these sequences no significant homologies were found.

Following sequence databanks were searched on 93-12-01 using the IntelliGenetics Inc. (Mountain View, California, USA) software:

1.	EMBL 36	EMBL nucleic acid sequence data bank
2.	N-GeneSeq4	Patented nucleic acid sequence data bank
3.	UGenBank 79_36	Sequences unique to the NIH data bank

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Statistical significance is estimated under the assumption that the equivalent of one reading frame in the query sequence codes for protein and that significant alignments will involve only coding reading frames.
The search was performed on 93-12-08. In total 61.248 sequences were searched.
114 sequences producing high-scoring segment pairs were found. When analysing these sequences no significant homologies were found.

L10 Expression of the introduced transgenes and analysis of the eventual occurrence of cryptic gene expression

Responsible : M. De Beuckeleer, Senior Researcher

L10 Goals of the experiment

To demonstrate the expression of introduced transgenes in the male sterile and fertility restorer oilseed rape progenies and to analyze the eventual occurrence of cryptic gene expression.

L10 Plant material

Molecular analysis has been performed on plants carrying the male sterility gene and of plants carrying the gene for restoration of fertility. Non-transgenic Drakkar plants have been used as negative control.

Table L10 Plant material

Plant material	Notation
B91-4 progeny : T_3(B91-4)	MS1-T_3
B93-101 progeny : S_3(B93-101)	RF1-S_3


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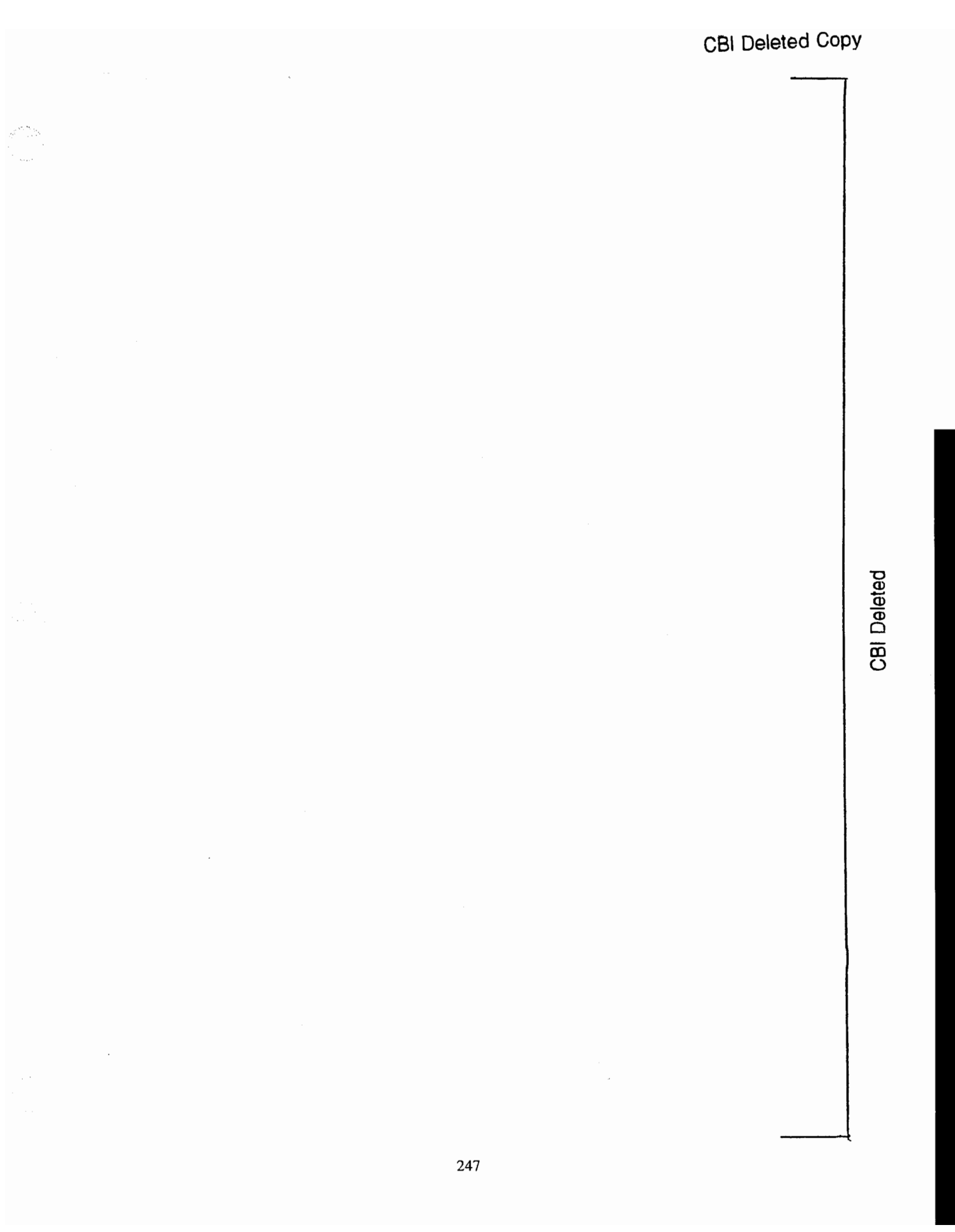
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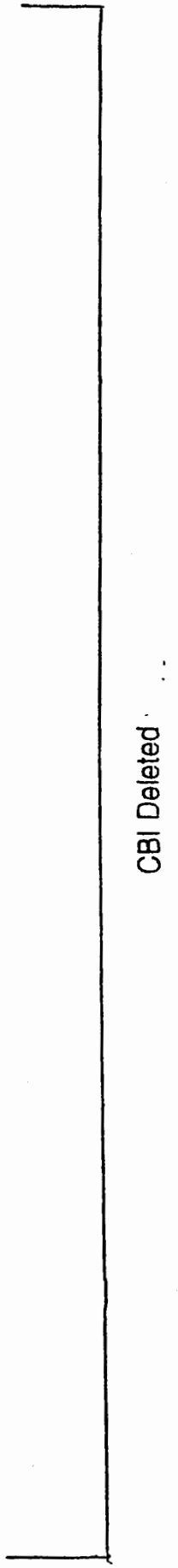
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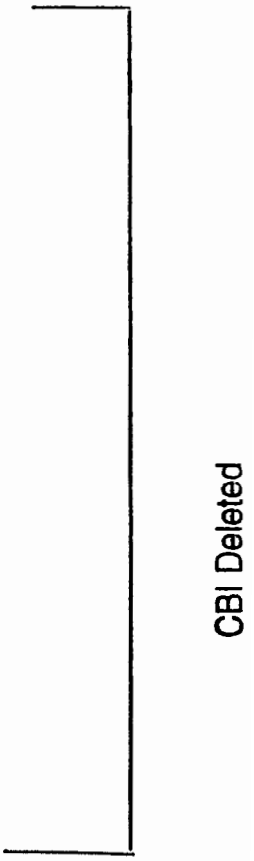

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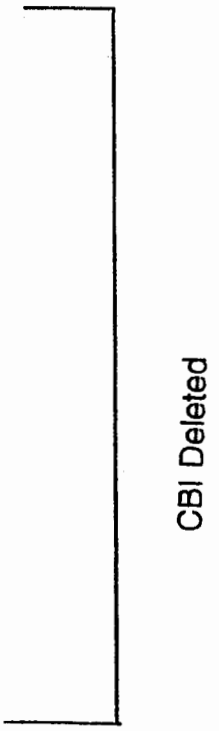
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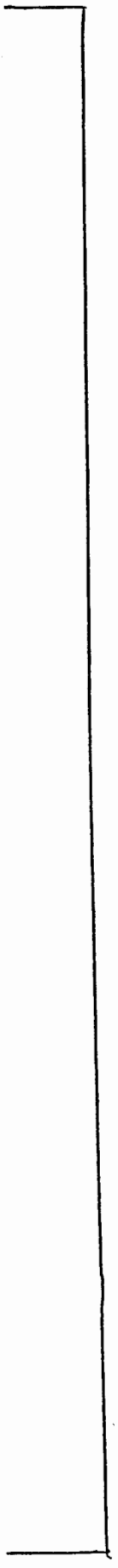

Transgene expression

Bar

For the RF1 transformant, detected bar mRNA levels in leafs are between 0.8 and 1.6pg/µg total RNA. We also detected bar mRNA in 2mm flower buds (between 0.1 and 0.2pg/µg total RNA). For the MS1 transformant, bar mRNA levels are estimated to be ± 0.4pg/µg total RNA. All other tested tissues and controls, are for both transformants negative (< 0.1pg/µg total RNA) (see Figure $L10_1$).

Barstar - Barnase

For the RF1 transformant, we detect barstar mRNA in 2mm flower buds. Plant A gave a very weak hybridization signal : on this particular blot the RNA sample was somewhat degraded. All other tissues and controls were negative (<0.4pg/µg total RNA) (see Figure $L10_1$).
For the MS1 transformant, we couldn't detect any barstar mRNA signals (see Figure $L10_1$). The absence of detectable barstar mRNA in male sterile flower buds is due to the tapetal cell RNA hydrolysis by barnase activity.

Neo

We were unable to demonstrate any neo mRNA signals in the RF1 and MS1 transformants using 10µg total RNA/lane. The detection limit for this hybdridization was 0.1pg/µg total RNA.

The typical loading arrangement of the gels (see Figure $L10_1$):

For Ms1 :

Lane 1. MW marker: 0.16 - 1.77Kb RNA ladder (GIBCO-BRL)
2. Leaf RNA (plant A)
3. Leaf RNA (plant B)
4. Leaf RNA Drakkar control plant
5. 2mm Flower bud RNA (plant A)
6. 3mm Flower bud RNA (plant A)
7. 2mm Flower bud RNA (plant B)
8. 3mm Flower bud RNA (plant B)
9. 2mm Flower bud RNA Drakkar control plant
10. 3mm Flower bud RNA Drakkar control plant
11. Seed RNA Ms1
12. Seed RNA Drakkar control plant

For Rf1 :

Lane 1. MW marker: 0.16 - 1.77Kb RNA ladder (GIBCO-BRL)
2. Leaf RNA (plant A)
3. Leaf RNA (plant B)
4. Leaf RNA Drakkar control plant
5. 2mm Flower bud RNA (plant A)
6. 3mm Flower bud RNA (plant A)
7. 2mm Flower bud RNA (plant B)
8. 3mm Flower bud RNA (plant B)
9. 2mm Flower bud RNA Drakkar control plant
10. 3mm Flower bud RNA Drakkar control plant
11. Seed RNA Rf1
12. Seed RNA Drakkar control plant
13. Pollen RNA Rf1
14. Pollen RNA Drakkar control plant

Control RNA dilution series :

A dilution series ranging from [ ] of the complementary, in vitro synthesized, RNA is loaded on a 1.5% agarose - formaldehyde gel in the presence of 10µg Drakkar leaf RNA.

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Upper part Lower part

[ ]

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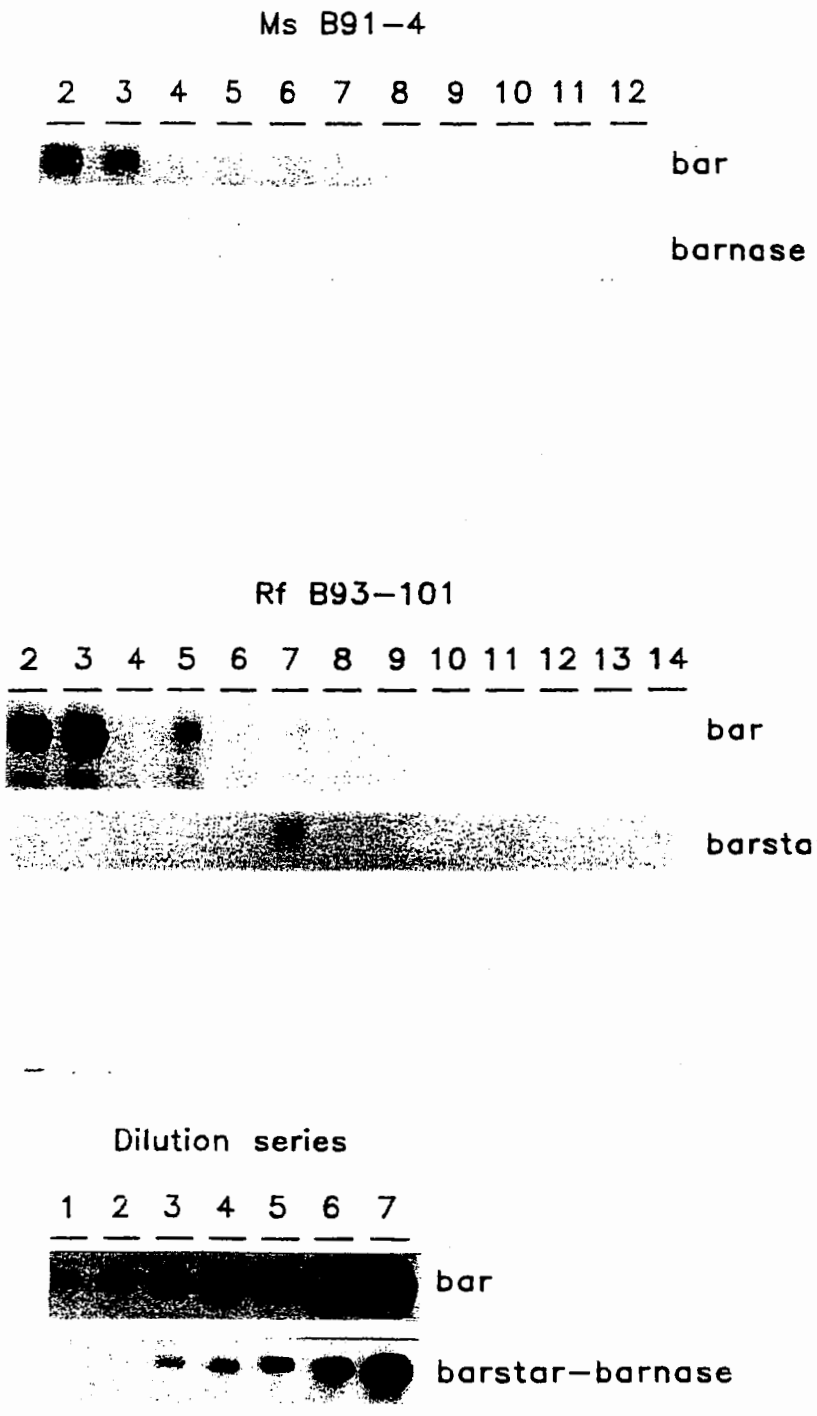
Ms B91–4
2 3 4 5 6 7 8 9 10 11 12
bar
barnase
Rf B93–101
2 3 4 5 6 7 8 9 10 11 12 13 14
bar
barstar
Dilution series
1 2 3 4 5 6 7
bar
barstar–barnase

Figure L10$_1$

Cryptic gene expression

Bar - Barstar/Barnase - Neo

Using sense RNA probes of the specified transgenes, we were unable to detect any hybridization signals whatsoever. Detection limits are indicated in the tables.

Right Border T-DNA [ ] CBI Deleted

The in vitro synthesized [ ] of both fragments were used together in a hybridization experiment (the same was done with the [ ]. We were unable to detect any hybridization signals with both set of probes (see Figure $L10_2$.). Detection limits are indicated in the tables.

Left Border T-DNA [ ] CBI Deleted

Both [ ] were used together in the hybridization (the same was done with the [ ]. We were unable to detect any hybridization signals (see Figure $L10_3$.). Detection limits are indicated in the tables.

The typical loading arrangement of the gels (see Figure $L10_{2 \text{ and } 3}$):

For Ms1 :

Lane 1. MW marker: 0.16 - 1.77Kb RNA ladder (GIBCO-BRL)
2. Leaf RNA (plant A)
3. Leaf RNA (plant B)
4. Leaf RNA Drakkar control plant
5. 2mm Flower bud RNA (plant A)
6. 3mm Flower bud RNA (plant A)
7. 2mm Flower bud RNA (plant B)
8. 3mm Flower bud RNA (plant B)
9. 2mm Flower bud RNA Drakkar control plant
10. 3mm Flower bud RNA Drakkar control plant
11. Seed RNA Ms1
12. Seed RNA Drakkar control plant
13-18. No samples

For Rf1 :

Lane 1. MW marker: 0.16 - 1.77Kb RNA ladder (GIBCO-BRL)
2. Leaf RNA (plant A)
3. Leaf RNA (plant B)
4. Leaf RNA Drakkar control plant
5. 2mm Flower bud RNA (plant A)
6. 3mm Flower bud RNA (plant A)
7. 2mm Flower bud RNA (plant B)
8. 3mm Flower bud RNA (plant B)
9. 2mm Flower bud RNA Drakkar control plant

10. 3mm Flower bud RNA Drakkar control plant
11. Seed RNA Rf1
12. Seed RNA Drakkar control plant
13. Pollen RNA Rf1
14. Pollen RNA Drakkar control plant
15-18. No samples

Control RNA dilution series :

A dilution series ranging from 5pg to 40pg of the complementary, in vitro synthesized, RNA is loaded on a 1.5% agarose - formaldehyde gel in the presence of 10µg Drakkar leaf RNA.

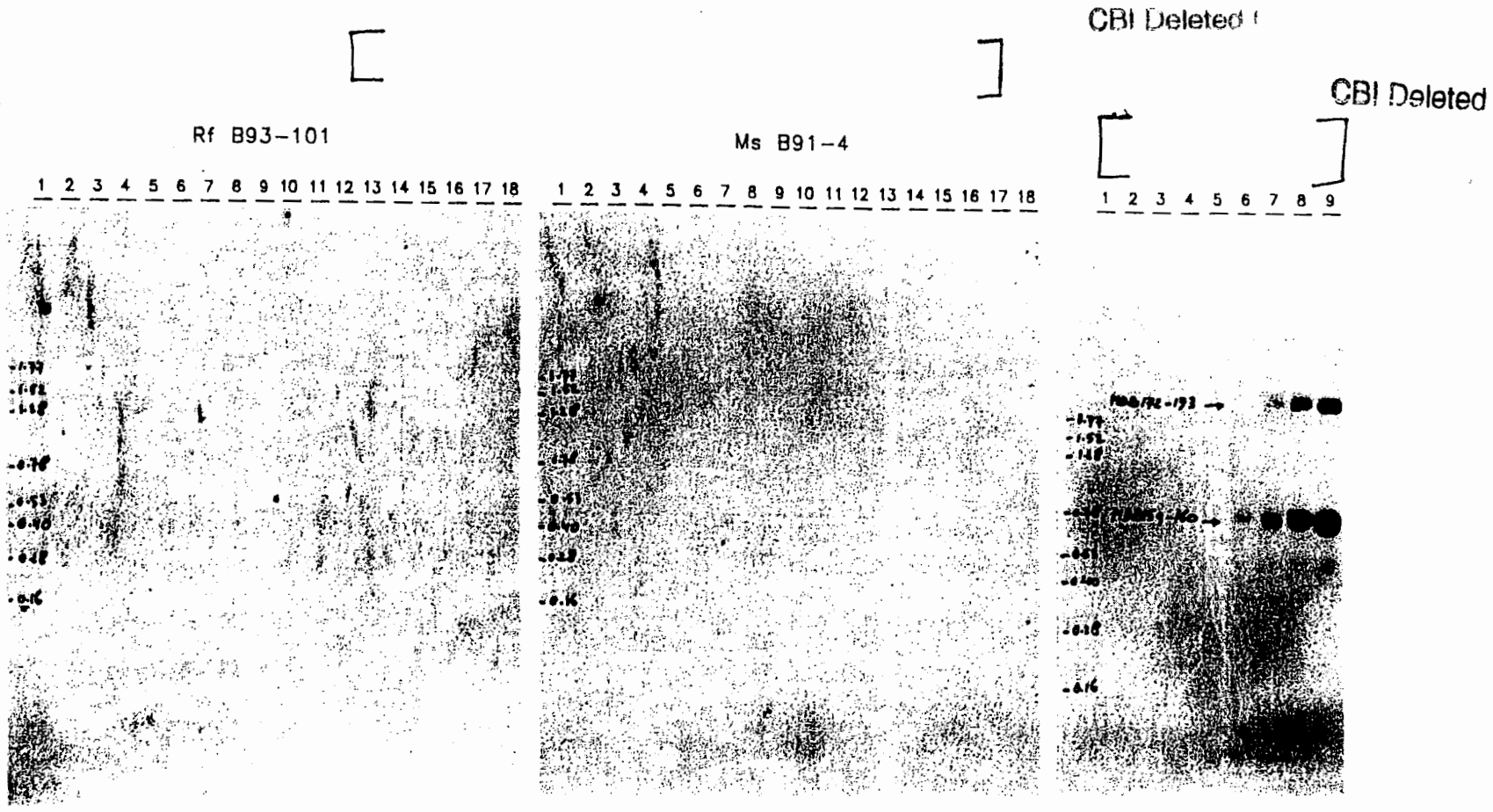

Figure L10$_3$

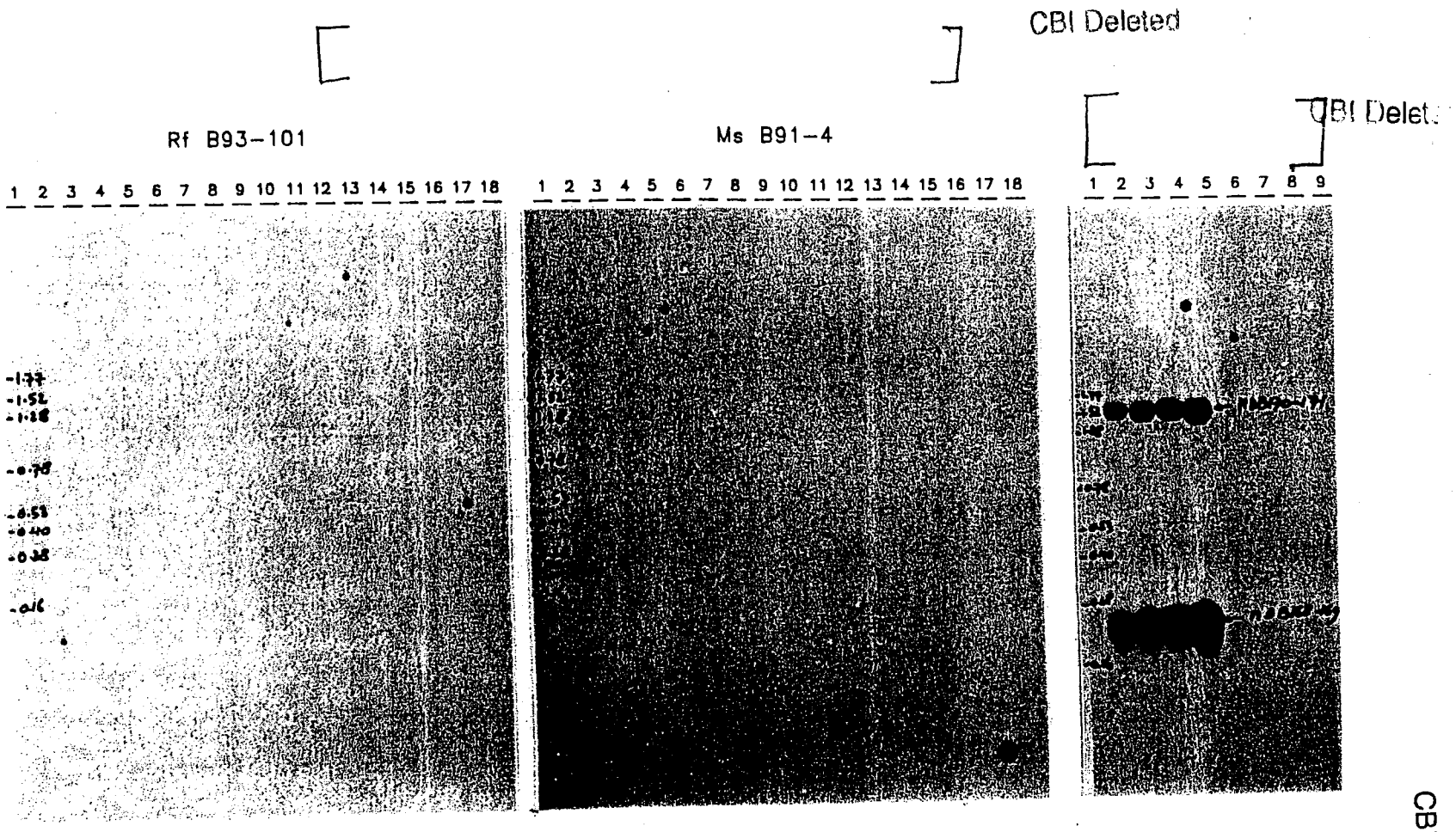

Figure L10$_2$

RF1 transformant B93-101

Total RNA	Transgene expression			Cryptic gene expression						
	bar pGembar/SP6	barstar pVE113/SP6	neo pFM146/SP6	bar pGembar/T7	barstar pVE113/T7	neo pFM147/T7				
2. Leaf (A)	+	-	-	-	-	-	-	-	-	-
3. Leaf (B)	+	-	-	-	-	-	-	-	-	-
4. Leaf Drakkar Control	-	-	-	-	-	-	-	-	-	-
5. 2mm Flower buds (A)	+	(+)	-	-	-	-	-	-	-	-
6. 3mm Flower buds (A)	-	-	-	-	-	-	-	-	-	-
7. 2mm Flower buds (B)	+	+	-	-	-	-	-	-	-	-
8. 3mm Flower buds (B)	-	-	-	-	-	-	-	-	-	-
9. 2mm Flower buds Drakkar Control	-	-	-	-	-	-	-	-	-	-
10.3mm Flower buds Drakkar Control	-	-	-	-	-	-	-	-	-	-
11.Seed	-	-	-	-	-	-	-	-	-	-
12.Seed Drakkar Control	-	-	-	-	-	-	-	-	-	-
13.Pollen	-	-	-	-	-	-	-	-	-	-
14.Pollen Drakkar Control	-	-	-	-	-	-	-	-	-	-
Detection limit (pg/µg total RNA)	0.1	0.4	0.2	0.4	0.2	0.2	0.1 / 0.25	0.1 / 0.1	0.25 / 0.5	0.25 / 0.25

MS1 transformant B91-4

Total RNA	Transgene expression			Cryptic gene expression						
	bar pGembar/SP6	barnase pVE113/SP6	neo pFM146/SP6	bar pGembar/T7	barstar pVE113/T7	neo pFM147/T7				
2. Leaf (A)	+	-	-	-	-	-	-	-	-	-
3. Leaf (B)	+	-	-	-	-	-	-	-	-	-
4. Leaf Drakkar Control	-	-	-	-	-	-	-	-	-	-
5. 2mm Flower buds (A)	-	-	-	-	-	-	-	-	-	-
6. 3mm Flower buds (A)	-	-	-	-	-	-	-	-	-	-
7. 2mm Flower buds (B)	-	-	-	-	-	-	-	-	-	-
8. 3mm Flower buds (B)	-	-	-	-	-	-	-	-	-	-
9. 2mm Flower buds Drakkar Control	-	-	-	-	-	-	-	-	-	-
10.3mm Flower buds Drakkar Control	-	-	-	-	-	-	-	-	-	-
11.Seed	-	-	-	-	-	-	-	-	-	-
12.Seed Drakkar Control	-	-	-	-	-	-	-	-	-	-
Detection limit (pg/µg total RNA)	0.1	0.4	0.2	0.4	0.2	0.2	0.1 / 0.25	0.1 / 0.1	0.25 / 0.5	0.25 / 0.25

L11 NPTII activity assay over a number of generations

Responsible : A. De Sonneville, Senior Technician

L11 Goal of the experiment

The NPTII enzymatic assay was used to evaluate the expression and inheritance of the *neo* gene over a number of generations.

L11 Plant material

The different plant entries from the progeny of the male sterility and restoration of fertility line (2 different plants per entry, futher indicated as A and B) are outlined in the Table below.

Plant material	Notation
B91-4 progeny :	
T_1(B91-4)	MS1-T_1
T_3(B91-4)	MS1-T_3
BC_4 of B91-4 in Can SOSR2	MS1-BC_4 (B0498)
BC_4 of B91-4 in Euro SOSR6	MS1-BC_4 (B4937)
B93-101 progeny :	
S_1(B93-101)	RF1-S_1
S_3(B93-101)	RF1-S_3
BC_3 of B93-101 in Can SOSR2	RF1-BC_3 (B6907)
BC_3 of B93-101 in Euro SOSR6	RF1-BC_3 (B6923)

L11 Methods

The assay is based on the electrophoretic separation of the NPTII protein from plant endogenous phosphorylating enzymes by a non-denaturing gel electrophoresis and detection of its enzymatic activity by in situ phosphorylation of kanamycin in the presence of radioactively labeled ATP. Both kanamycin and (γ-^{32}P)ATP acting as substrates are embedded in an agarose gel placed on the polyacrylamide gel containing the separated proteins. After the enzymatic reaction, the phosphorylated kanamycin is transferred to P81 phosphocellulose ion exchange paper and the radiolabelled kanamycin is visualized by autoradiography. An overview of the different steps in the enzymatic assay are described. Details of the different steps and solutions used are described in the mentioned references (Reiss et al.(1984). The EMBO Journal, 3, 3317-3322. Reynaerts et al. (1987). In : Gelvin, S.B., Schilperoort, R.A. (eds). Plant molecular biology manual. Kluwer, Dordrecht, The Netherlands, Sect. A9, p 1-16).

Extraction of plant material

100 mg fresh plant tissue is homogenized in extraction buffer on ice and transferred into a microcentrifuge tube. The cell debris is pelleted by centrifugation in an Eppendorf centrifuge.

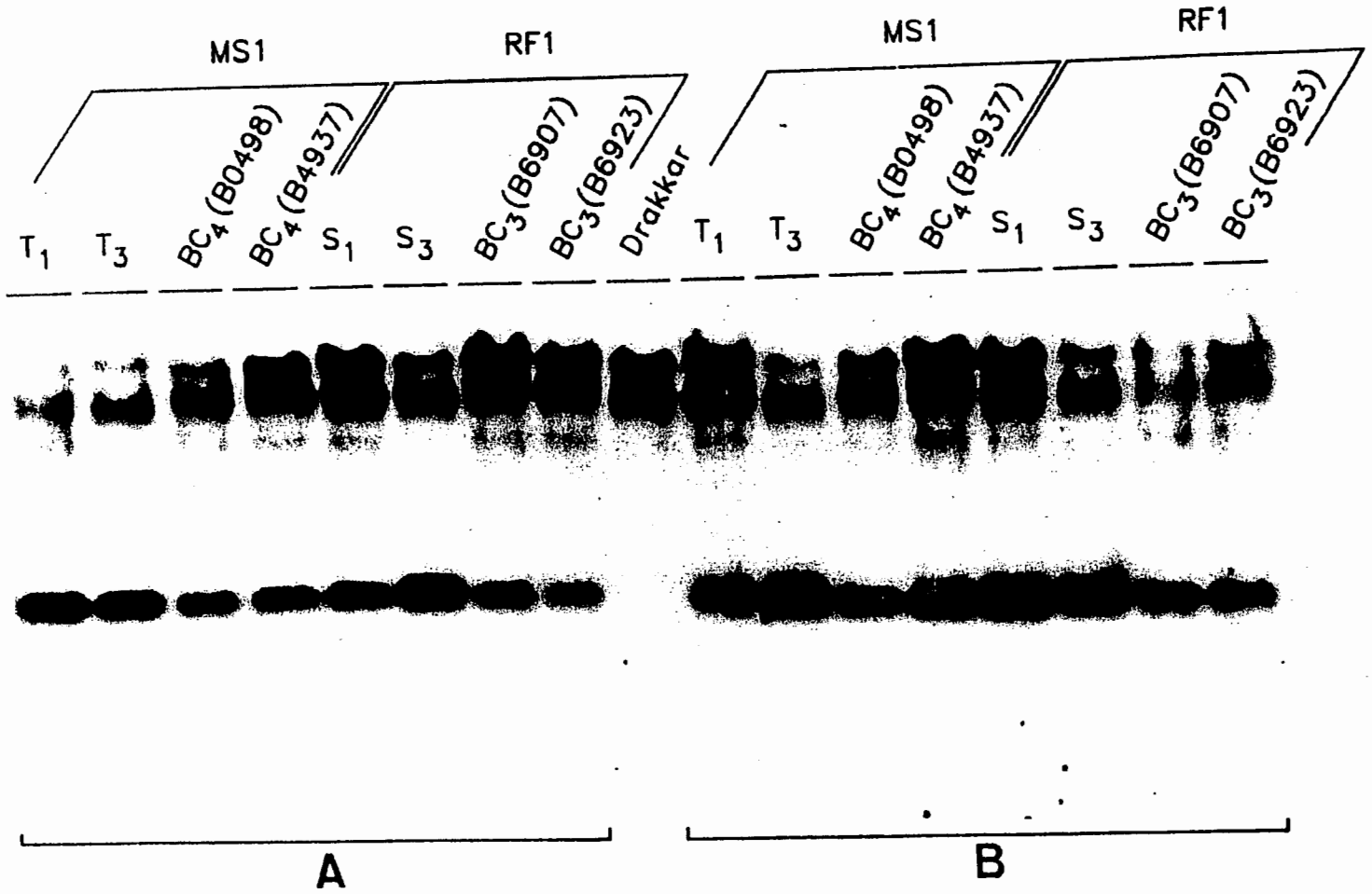

Figure L11$_1$ NPTII assay results

The supernatans is transferred to another tube and the protein concentration in the crude extract is determined by Biorad assay with bovine serum albumine as standard. Protein concentration in all samples is adjusted with extraction buffer.

Enzymatic assay

Loading buffer is added to an aliquot of 100 µg total protein which is separated by electrophoresis through a 10% non-denaturing polyacrylamide gel. The gel is washed in distilled water and equilibrated in reaction buffer. The gel is transferred onto a glass plate and overlaid with a 1% agarose gel containing 30 µg/ml kanamycin sulphate and 100 µCi (γ-^{32}P)ATP in reaction buffer. After incubation, the gel sandwich is covered with a sheet of Whatman P81 phosphocellulose paper. Upon incubation, the phosphorylated kanamycin is bound to the P81 paper. Subsequently, the P81 paper is washed, dried and exposed to a X-ray film.

L11 Results and conclusions

As shown in Figure L11$_1$, a typical band is identified corresponding to the reaction product. The presence of the band is consistent in the different stages of the tested material. This indicates that the *neo* gene is inherited and stably expressed over a number of generations.

Experiment L12 Spectrophotometric PAT assay over a number of generations

Responsible : A. van Vliet, Researcher

L12 Goal of the experiment

Quantification of the amount of phosphinothricin-acetyl-transferase (PAT) in extracts from leaves of male sterile and fertility restorer oilseed rape plants.

L12 Plant material

The different plant entries from the progeny of the male sterility and restoration of fertility line are outlined in the Table below.

Plant material	Notation
B91-4 progeny :	
T_1(B91-4)	MS1-T_1
T_3(B91-4)	MS1-T_3
BC_4 of B91-4 in Can SOSR2	MS1-BC_4 (93B0498)
BC_4 of B91-4 in Euro SOSR6	MS1-BC_4 (B4973)
B93-101 progeny :	
S_1(B93-101)	RF1-S_1
S_3(B93-101)	RF1-S_3
BC_3 of B93-101 in Can SOSR2	RF1-BC_3 (B6907)
BC_3 of B93-101 in Euro SOSR6	RF1-BC_3 (B6923)

L12 Methods

The spectrophotometric PAT assay was largely based on the protocol as described by D'Halluin et al. (1992) (Methods in enzymology, **216**, 415-427).

L12 Results and conclusions

L12 Results

Sample	mg prot/ml	PAT U/ml	µg PAT	µg PAT/mg prot
MS1 T_1(B91-4) A	2.8	0.50	2.9	1.1
B	2.8	0.34	2.0	0.7
MS1 T_3(B91-4) A	3.5	0.35	2.1	0.5
B	2.5	0.22	1.3	0.5
MS1 BC_4(B91-4) (93B0498) A	3.4	0.51	3.0	0.9
B	3.6	0.64	3.8	1.1
MS1 BC_4(B91-4) (B4973) A	2.9	0.82	4.8	1.6
B	2.7	0.67	3.9	1.5
RF1 S_1(B93-101) A	1.7	0.54	3.2	1.9
B	3.6	0.77	4.5	1.2
RF1 S_3(B93-101) A	4.8	1.15	6.8	1.4
B	3.6	0.69	4.1	1.1
RF1 BC_3(B93-101) (B6907) A	3.8	1.01	5.9	1.6
B	2.9	0.64	3.8	1.3
RF1 BC_3(B93-101) (B6923) A	2.4	0.75	4.4	1.8
B	3.0	0.75	4.4	1.5
Drakkar(control)	2.8	0.00	0.00	0.0

- Protein concentration is measured with the D_c protein assay of Biorad (Lowry method) with Bovine Serum Albumin as standard
- µg PAT is based on an estimated specific activity of 170U/mg PAT

L12 Conclusion

The results clearly indicate that the *bar* gene is inherited and stably expressed over a number of generations.

EXPERIMENT L13 : Identification of the genome on which the chimeric gene is located

Responsible : M. De Beuckeleer, Senior Researcher

L13 Goal of the experiment

To identify the genome on which the chimeric barstar allele is located. A similar procedure has been followed to identify the genome on which the barnase allele is located.

L13 Plant material

Homozygous S_2(B93-101) *Brassica napus* (AACC) (RF1RF1), heterozygous B93-101 *Brassica napus* (RF1); *Brassica napus* wild-type: *Brassica juncea* (AABB) wild-type, *Brassica campestris* (AA) wild-type, *Brassica carinata* (BBCC) wild-type and *Brassica oleracea* (CC) wild-type plants.

L13 Methods

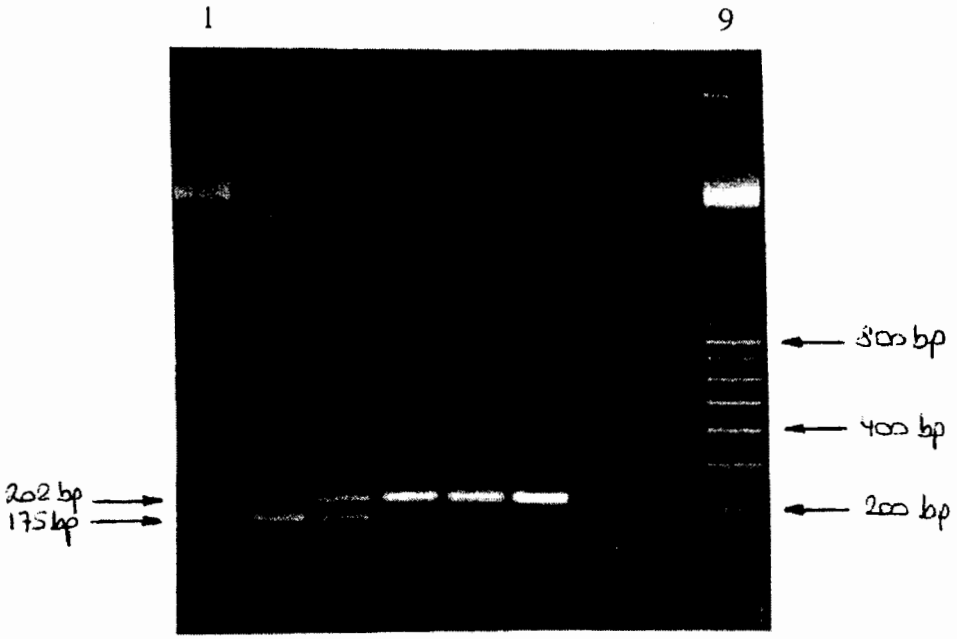

Figure L13. PCR analysis

DNA was isolated from different Brassica species
A master mix, containing primers [ ]was added to 50 µg genomic DNA. The PCR reaction was thermocycled for 27 cycles. Twenty µl of each PCR reaction was loaded on a 1.5 % agarose gel.
Loading sequence of the agarose gel:

1. 100 bp ladder
2. homozygous Rf1 (*Brassica napus*)
3. heterozygous Rf1 (*Brassica napus*)
4. *Brassica napus* wild-type
5. *Brassica campestris* wild-type
6. *Brassica juncea* wild-type
7. *Brassica carinata* wild-type
8. *Brassica oleracea* wild-type
9. 100 bp ladder

Appendix X

Confidential Business Information Justification

Aventis

CONFIDENTIAL BUSINESS INFORMATION JUSTIFICATION

The information claimed as confidential within this application may fall into two categories, namely (1) the genotype/phenotype description and (2) commercial development information. The genotype/phenotype description category includes names and information about the recipient plant, the phenotype of the regulated article, vectors, mode of transformation, gene coding regions, associated regulatory sequences and expressed traits. Commercial development information includes the names and locations of cooperators, collaborators, investigators, and contacts.

This confidential business information justification is submitted by Aventis CropScience USA LP ("Aventis USA"). Aventis USA is made up of the former AgrEvo USA Company and Rhone Poulenc Ag Company. Aventis USA is part of the worldwide Aventis CropScience Group of companies which also includes Aventis CropScience NV (the former Plant Genetics Systems N.V.) and Aventis CropScience GmbH (the former Hoechst Schering AgrEvo GmbH). All of these entities are referred to as Aventis in the statements given below.

GENOTYPE/PHENOTYPE DESCRIPTION

Central to the commercial value of Aventis' biotechnology products is the genetic information that confers the desired traits on the plant product, as well as the technical means by which the desired products have been achieved. Aventis has spent many person years in developing its expertise in the field of plant biotechnology, concurrent with the expenditure of millions of dollars on biotechnology research. In the rapidly growing and highly competitive industry of biotechnology products, Aventis has a leading edge.

Aventis has been working on the development of genetically enhanced plants, particularly those with herbicide tolerance, since the early 1980's and can document the large sums of money spent in research and testing costs. The uniqueness of Aventis' products lies in the transformation and regeneration methods and/or the combination of genetic components in the vectors transferred into the genomes of the recipient plants. The transformation and regeneration methods may be Aventis proprietary methods or available through licensing of other's proprietary methods. The genetic components in these vectors include the coding sequence for the expression of the trait(s), and regulatory sequences such as promoters, enhancers, introns, termination and polyadenylation sequences. In certain cases the recipient plant strain used is tantamount for regeneration and other desired features. Although the information on the transformation methods, recipient plant strains, or on each of these vector components may be in the public domain, the particular combination of the components put together by Aventis is unique and represents a great expenditure of time and money.

Competitors (which include Monsanto/DeKalb, Syngenta, DuPont/Pioneer, Dow Mycogen) of Aventis cannot presently duplicate Aventis' commercially valuable products without going through the painstaking process of trial and error development and testing of many different combinations of genetic information and plant strains. Access to genotype and/or phenotype description information, including the donor organisms and the recipient plant, for Aventis' products would allow competitors to create similar products that would result in a market share loss for Aventis of millions of dollars. By performing simple copy

work, these competitors would avoid the expenditure of dollars, research time and effort used by Aventis to develop its commercial products. Furthermore, the release of genotype and phenotype information would provide competitors with commercially valuable knowledge about particular products that Aventis is planning to commercialize and the likely timeframe for commercialization. Such information would be extremely useful to these companies in developing their own marketing and development strategies.

COMMERCIAL DEVELOPMENT INFORMATION

The disclosure of information about the names of cooperators, collaborators, investigators, research farm on-site personnel or contacts and the location and characteristics of the field experiments will provide Aventis' competitors with invaluable information about Aventis' marketing strategy, and could cause severe harm to Aventis' competitive standing in the industry.

In particular, release of the choice of cooperators and collaborators provides the competition with knowledge about the individuals and organizations that Aventis has found, through experience and investigation, to be most expert. Information on the location and characteristics of the field experiments will directly, or with little effort, provide the identity of the cooperators and collaborators. There is no doubt that competitors would seek to use the services of the entities found most expert by Aventis, and limit or block access of these sources. This could be accomplished by prices for services being increased, or by competitors acquiring exclusive licenses with these individuals and organizations, or by entering into contracts that would essentially tie up the time and facilities of such entities.

Maintaining the good will of the cooperators and collaborators is also a very important consideration for Aventis' success. The release of information that would directly or indirectly identify these entities could cost Aventis considerable good will and the breach of an agreement with the entity concerned. This could lead to the loss of the entity as an expert source. If Aventis is forced to use alternative cooperators and collaborators, it would take time to identify high technical performance, and it would represent a loss of the valuable expertise and understanding built-up with former entities. This, in turn, could result in a delay in bringing products to market, which would cost Aventis sums in to the millions of dollars.

Additionally, the disclosure of information about cooperators and collaborators would provide strong insights into Aventis' marketing strategy by revealing where Aventis is planning to introduce the products, and the schedule for such introduction.