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98-278-01p

October 1, 1998

Ms. Rebecca A. Bech
Assistant Director
USDA, APHIS, Scientific Services
4700 River Rd. Unit 133
Riverdale, MD 20737-1237

Re: Petition for Determination of Nonregulated Status: In Vigor® Hybrid Canola Transformation Events MS8 and RF3

Dear Ms. Bech:

AgrEvo USA Company herein submits a Petition for Determination of Nonregulated Status for In Vigor® Hybrid Canola Transformation Events MS8 and RF3.

This petition requests a determination from USDA/APHIS that In Vigor® Hybrid Canola Transformation Events MS8 and RF3, and any progeny derived from breeding other canola lines with events MS8 and RF3, no longer be considered regulated articles under 7 CFR Part 340. This petition contains a full statement explaining the factual grounds why In Vigor® Hybrid Canola Transformation Events MS8 and RF3 should not be regulated under 7 CFR 340.6. This petition does not contain any trade secrets or confidential business information (CBI) and is so marked.

Please find enclosed the following documents:

Two (2) official copies of the Petition for Determination of Nonregulated Status: In Vigor® Hybrid Canola Transformation Events MS8 and RF3, including Appendicies 1-5.

Four (4) copies for APHIS reviewers as requested in The User's Guide for Petitions, November 1996

AgrEvo USA Company
Little Falls Centre One, 2711 Centerville Road, Wilmington, DE 19808, Telephone: (302) 892-3000, Fax: (302) 892-3013

10/5/98
Kay

Do not hesitate to contact me at 302-892-3034, phone; or, 302-892-3099, fax, with any questions regarding this petition.

Your consideration of this petition is greatly appreciated.

Sincerely,

Vickie Forster

Vickie Forster
Registration Specialist, Biotechnology

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Petition for Determination of Nonregulated Status: InVigor® Hybrid Canola Transformation Events MS8/RF3

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

Submitted by:

Vickie Forster
Registration Specialist, Regulatory Affairs-Biotechnology

AgrEvo USA Company
Little Falls Centre One
Centerville Road
Wilmington, DE 19808
Telephone: 302-892-3034
FAX: 302-892-3099

Contributors:
Inge Huybrechts, Bert Uijtewaal, Klaus Trinks, Marc de Beukeleer, Tom Schuler, Kevin Thorsness

September 30, 1998

Contains No Confidential Business Information

STATEMENT

Plant Genetics Systems (America), Inc. and AgrEvo USA Company are affiliated companies under common ownership by AgrEvo GmbH, Frankfurt, Germany.

SUMMARY

AgrEvo USA Company herewith submits a Petition for Determination of Nonregulated Status to The Animal and Plant Health Inspection Service (APHIS) for Hybrid Canola (*Brassica napus*) Transformation Events MS8 and RF3. AgrEvo requests a determination from APHIS that Transformation Events MS8 and RF3, and their hybrid combination MS8 x RF3 (a.k.a. MS8/RF3), as well as any progeny derived from crosses of events MS8 and RF3 with traditional or transgenic canola varieties which have also received a Determination of Nonregulated Status, no longer be considered regulated articles under 7 CFR Part 340. Events MS8 and RF3 are considered regulated articles because they were transformed using the plant pest *Agrobacterium tumefaciens* as well as certain sequences from *A. tumefaciens.*

The genetic modifications are aimed at the introduction of a new type of pollination control system, enabling the production of truly hybrid oilseed rape varieties. The PGS' hybridization system in oilseed rape, is based on two oilseed rape events: a male sterile oilseed rape event, designated as MS8, or its progeny, and a fertility restorer oilseed rape event, designated as RF3, or its progeny; and the hybrid combination. In addition to the inserted genes for male sterility (*barnase*) and fertility restoration (*barstar*), the *bar* gene has been inserted to allow for selection during breeding, and in the commercial phase, resistance to the broad spectrum herbicide glufosinate-ammonium.

Southern blot analyses show that events MS8 and RF3 each contain a single, stably integrated insert of the *barnase-bar* gene construct and *barstar-bar* gene construct, respectively. Southern blot analyses also indicate that the incorporation has been limited to DNA sequences contained within the T-DNA borders. The *bar* gene's expression product is the phosphinothricin-N-acetyl transferase (PAT) enzyme. The PAT enzyme catalyzes the conversion of glufosinate-ammonium to an inactive form, thereby conferring resistance to the herbicide. The *bar* gene in MS8 and RF3 has been isolated from the soil bacterium *Streptomyces hygroscopicus. Barnase* and *barstar* were derived from *Bacillus amyloliquefaciens*. The *barnase-bar* and *barstar-bar* gene constructs were introduced into canola plant material using disarmed *A. tumefaciens*.

Glufosinate-ammonium is currently registered in the United States as a herbicide for both non-crop and crop uses. It is registered as FINALE® for non-crop uses, and it is registered as RELY® for use on trees, nuts and vines, REMOVE® for seed propagation use, currently on corn and soybean, and as LIBERTY® for crop use, currently on corn and soybean. Registration and tolerance extension for use of LIBERTY on canola is pending at the Environmental Protection Agency. Glufosinate-ammonium is biodegradable, has no residual activity, and has very low toxicity for humans and wild fauna.

Transformation Events MS8 and RF3 and their hybrid combination MS8 x RF3, have been field tested in the United States in 1997, at two locations under permit 97-035-05r. MS8 and RF3 are currently being evaluated in the field in 1998 at fourteen (14) locations under notifications 98-064-38n, 98-064-35n, 98-064-33n, 98-168-04n, and 98-064-31, as well as permit 98-119-01r.

Events MS8 and RF3 have also been extensively field tested in Canada, and on October 21, 1996, were authorized for unconfined release into the environment and use as livestock feed by Agriculture Canada (DD 96-17, included here as Appendix 1). Events MS8 and RF3 have also been field tested in Chile, Japan, Europe (United Kingdom, Belgium, Germany, France) and Australia. The 90/220 Part C application for MS8 and RF3 has been approved by the Rapporteur EU Member State, Belgium, on January 2, 1997, and an Article 21 Committee vote is scheduled regarding EU clearance for 1998.

Transformation Events MS8 and RF3 have been selected for commercial development. They have been crossed with commercially available traditionally derived canola lines and cultivars. The primary transformation events MS8 and RF3 and their progeny are collectively referred to as MS8, RF3 and, or MS8 x RF3 or MS8/RF3, respectively, in this petition.

Consultation with the Food and Drug Administration (FDA) has been completed on September 16, 1998, regarding the food and feed safety of events MS8 and RF3. In their final consultation letter, the FDA have stated that, "(MS8/RF3 canola) does not raise issues that would require premarket review or approval of FDA". (See Appendix 1). In conjunction with the conclusion of the FDA consultation, the USDA, APHIS, have issued an opinion letter on September 23, 1998, regarding the fact that "these lines should not pose a plant pest risk" when imported into the United States for the purpose of commercial crushing. (See Appendix 1). On March 12, 1997, Health Canada also approved the human safety of varieties derived from transformation Events MS8 and RF3. (See Appendix 1).

As is presented in this application for Determination of Nonregulated Status, there are no indications to anticipate that the insertion of the male sterility gene construct (*bar* and *barnase* gene derived from the plasmid pTHW107 in MS8, and/or the restorer of fertility gene construct (*bar* and *barstar* gene derived from the plasmid pTHW118 in RF3 would have any negative impact on the environment.

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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 are unfavorable to the petition.

Vickie Forster

Vickie Forster
Registration Specialist, Regulatory Affairs-Biotechnology

AgrEvo USA Company
Little Falls Centre One
2711 Centerville Road
Wilmington, DE 19808
Telephone: 302-892-3034
FAX: 302-892-3099

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ACRONYMS AND SCIENTIFIC TERMS

AAFC: Agriculture and Agri-Food Canada

***bar*:** bialaphos resistance gene (origin *Streptomyces hygroscopicus*)

DNA: dioxyribonucleic acid

PAT: phosphinothricin-N-acetyltransferase

PCR: polymerase chain reaction

mRNA: messenger ribonucleic acid

RNA: ribonucleic acid

T-DNA: transferred DNA

Ti-DNA: tumor inducing DNA

Ti-plasmid: tumor inducing plasmid

USCA: United States Canola Association

I. Rationale for Development of Hybrid Canola derived from the Transformation Events MS8 and RF3

The major target of oilseed rape breeders is creating higher-yielding oilseed rape (*Brassica napus* L. *oleifera*) varieties. The most effective method to obtain this goal is the use of F1 hybrids, since hybrid oilseed rape varieties are estimated to yield 20-25% more seeds than the best open-pollinated oilseed rape varieties. Additionally, the uniformity of the F1 hybrids is an advantage in commercial oilseed rape production, facilitating both harvesting and marketing. Since oilseed rape is a crop capable of both self-pollination (70%) and cross-pollination (30%). A pollination control system is required to produce 100% F1 hybrid seeds.

Male sterility has been widely used in breeding programs of many different crops as a tool to ensure cross-pollination. Though naturally occurring male sterile oilseed rape plants have been used to a certain extent to develop hybrids, side effects displayed by these male sterile plants and the lack of an associated morphological marker which could allow the male sterility trait to be followed more easily in the offspring, prevented the efficient use of these plants. A novel approach was undertaken to develop male sterile oilseed rape plants by introducing a ribonuclease (*barnase*) gene which is expressed exclusively in the tapetum, the cell layers surrounding the pollen sac, during anther development. Expression of *barnase* in this tissue blocks pollen development and results in a male sterile plant.

The *barstar* gene (ribonuclease inhibitor) has also been introduced into oilseed rape, and is used to develop the restorer line of the hybrid system. Upon crossing a male sterile and a restorer line, the fertility of the oilseed rape progeny will be restored because the barstar protein will inactivate the barnase enzyme.

Additionally, the *bar* gene, because it allows for an efficient selection in both the transformation/regeneration process and in the field, was closely linked to both the chimeric *barnase* and *barstar* gene constructs. In addition to selection, the expressed *bar* gene in the form of phosphinothricin-N-acetyltransferase (PAT) allows for very effective postemergent weed control with the glufosinate-ammonium-containing herbicide, LIBERTY®.

II. Biology of the Crop Brassica napus L.

In lieu of a discussion here on the biology of *Brassica napus L.*, AgrEvo hereby cite the OECD paper, "A Consensus Document of the Biology of *Brassica napus L.* (Oilseed rape) OECD Series on the Harmonization of Regulatory Oversight in Biotechnology No. 7 (1997). Citation of this document is permitted under amendment 97-5 of the USDA Users Guide for Preparing and Submitting a Petition for Genetically Engineered Plants, available November 1996.

Whilst the above-referenced OECD paper gives a thorough discussion of all biological aspects of *Brassica napus* as a crop, it does not discuss specific behavior of *Brassica napus* in the United States, and the potential for gene transfer to sexually compatible relatives in the United States. In order to address this issue, AgrEvo gathered firsthand information from knowledgeable state personnel in the individual states where commercial canola (*Brassica napus*) is grown. This information was presented in Petition 97-205-01p, Petition for Determination of Nonregulated Status: Glufosinate Tolerant Canola Transformation Event T45, and is included here as Appendix 2.

From the information gathered, Table 1, on the following page, was developed listing sexually compatible species with *Brassica napus*, and the potential for gene transfer under field conditions in the United States.

Table 1: Outcrossing Potential of *B. napus* with Related Species in the United States

Summary of interspecific crossing results under field conditions between various *Brassicaceae* member species and ***B. napus*** (pollen donor).

Pollen Recipient	Occurs in Agriculturally unmanaged areas	State (>1% of U.S. canola production)[1]	Field Hybrids Produced?	Fertility of Hybrids	Reference
B. napus	Yes	CA	Yes	normal	Bing et al., 1991
B. rapa	Yes	AL, CO, GA, ID, MN, MN, ND, OR, SD, WA	Yes (0.7-1.3%) Yes (56-93%)	< 10% viable 21-86% pollen viable	Bing et al., 1991 Jorgensen & Anderson, 1994
B. juncea	Yes	AL, CO, GA, ID, MN, MN, ND, OR, SD, WA	Yes (0.1-0.3%)	< 10% pollen viable	Bing et al., 1991 Calgene, 1994
B. nigra (black mustard)	Yes	AL, CO, GA, ID, MN, MN, ND, OR, SD, WA	Yes (extremely low numbers);	male sterile	Bing et al., 1991 Calgene, 1994 Brown et al., 1994
B. oleracea[2] (cabbage family)	No	CA	No	n/a	Calgene, 1994 Kerlan et al., 1992 Downey, 1992
B. carinata[2]	No		No	n/a	Calgene, 1994
B. elongata	Yes	NV	No	n/a	Calgene, 1994
B. tournefortii	Yes	CA	No	n/a	Calgene, 1994
B. adpressa, syn. Herschfeldia incana (hoary mustard)	Yes	CA, NV, OR	Yes (extremely low numbers)	mostly sterile	Lefol et al., 1991 Eber et al., 1994
Raphanus raphanistrum (wild radish)	Yes	AL, CO, GA, ID, MN, MN, ND, OR, SD, WA	Yes (0.2%) Yes (but only under sp. circumstances)	very low (0.16 seeds/ plant) very low (4-14%)	Baranger, et al., 1995 Eber et. al., 1994
Sinapis arvensis syn. *B. kaber* (wild mustard)	Yes	AL, CO, GA, ID, MN, MN, ND, OR, SD, WA	No	n/a	Lefol et al., 1994 Lefol et al, 1996 Bing et al., 1991 Bing et al., 1995
Sinapsis alba syn. *B. hirta*	Yes	AL, CO, GA, ID, MN, MN, ND, OR, SD, WA	No	n/a	Calgene, 1994 Warwick, 1993
Diplotaxis muralis	Yes	CA, OR, SD	No	n/a	Ringdahl, 1987 Calgene, 1994

n/a = not assessed

[1] Warwick, 1993.
[2] In North America, does not naturally occur in the wild and is not taken to seed.

III. Description of the Transformation System Used

To obtain the male sterile, MS8, and fertility restorer, RF3, oilseed rape lines, the vector system as described by Deblaere et al. (1985, 1987) has been used. Plasmids pTHW107 and pTHW118 were used to engineer male sterility and restoration of fertility, respectively. Tables 2 and 3, and Figures 1 and 2, give an overview of the origin of the sequences of the designed vectors used to obtain the respective MS8 and RF3 oilseed rape lines. The donor organisms only function were to be the source of the described sequences, and were not actively involved in the modification process.

A transformation method as described by De Block et al. (1989), was used to incorporate the T-DNA of the plasmids pTHW107 and pTHW118 into a maturity group 00 oilseed rape variety (Drakkar) (hypocotyls). Drakkar is a common variety in the canola growing regions of western Canada and Europe.

A. tumefaciens mediated transformation is one of the most widely used systems for introducing foreign genes into plants. Progress in adapting the *A. tumefaciens* Ti-plasmid for the delivery of foreign DNA into plant cells and obtaining intact transformed plants, depended upon the following advances in understanding the molecular biology of the natural crown gall disease caused by oncogenic *Agrobacterium* strains: the development of chimeric genes that function as selectable markers; the construction of convenient intermediate vectors for introducing foreign genes into *A. tumefaciens* cells; and, improved transformation and tissue culture procedures that enable facile regeneration of transformed plants (Schell et al., 1983; Deblaere et al., 1987; Rogers et al., 1988; Walden et al., 1990; Corbin et al.,1991; Fincham et al, 1991). A number of plant transformation vectors, capitalizing on the experimental observations that the DNA transferred to the plant cell is defined by the 25bp T-DNA border repeats, and that the transfer and integration into the plant genome does not require the presence of any of the genes encoded by the T-DNA, have been developed (Leemans et al., 1982; Deblaere et al., 1985; Deblaere et al., 1987; Klee et al., 1987; Goodman et al., 1987).

Table 2: Nucleotide sequence of the DNA comprised between the T-DNA border repeats of pTHW107 and origin of the different sequences

Nucleotide sequence			Origin of the sequence
#	1-25	:	Right border repeat from the TL-DNA from pTiB6S3 (Gielen et al., 1984).
#	26-97	:	Synthetic polylinker derived sequences
#	309-98	:	The 3'untranslated end from the TL-DNA gene 7 (3'g7) of pTiB6S3 (Velten and Schell., 1985; Dhaese et al., 1983).
#	310-330	:	Synthetic polylinker derived sequences
#	882-331	:	The coding sequence of the bialaphos resistance gene (*bar*) of *Streptomyces hygroscopicus* (Thompson et al., 1987). On the N-terminal, two codons of the wild type *bar* coding region have been substituted for the codons ATG and GAC respectively.
#	2608-883	:	The promoter from the atS1A ribulose-1,5-biphosphate carboxylase small subunit gene from *Arabidopsis thaliana* (PSsuAra) (Krebbers et al.,1988).
#	2609-2658	:	Synthetic polylinker derived sequences
#	2919-2659	:	A 260 bp TaqI fragment from the 3' untranslated end of the nopaline synthase gene (3'nos) from the T-DNA of pTiT37 and containing plant polyadenylation signals (Depicker et al., 1982).
#	2920-3031	:	3'untranslated region downstream from the *barnase* coding sequence of *B. amyloliquefaciens*. (Hartley et al., 1988)
#	3367-3032	:	The coding region of the *barnase* gene from *Bacillus amyloliquefaciens* (Hartley, 1988)
#	4877-3368	:	The promoter region of the anther-specific gene TA29 from *Nicotiana tabacum* (Seurinck et al., 1990). The promoter comprises the 1.5 kb of the sequence upstream from the ATG initiation codon.
#	4878-4921	:	Synthetic polylinker derived sequences.
#	4922-4946	:	Left border repeat from the TL-DNA from pTiB6S3 (Gielen et al., 1984).

The complete nucleotide sequence for pTHW107 can be found in De Beuckeleer, M. (1996a)

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Figure 1. Map of plasmid pTHW107, indicating the T-DNA, origins of replication and prokaryotic selective markers

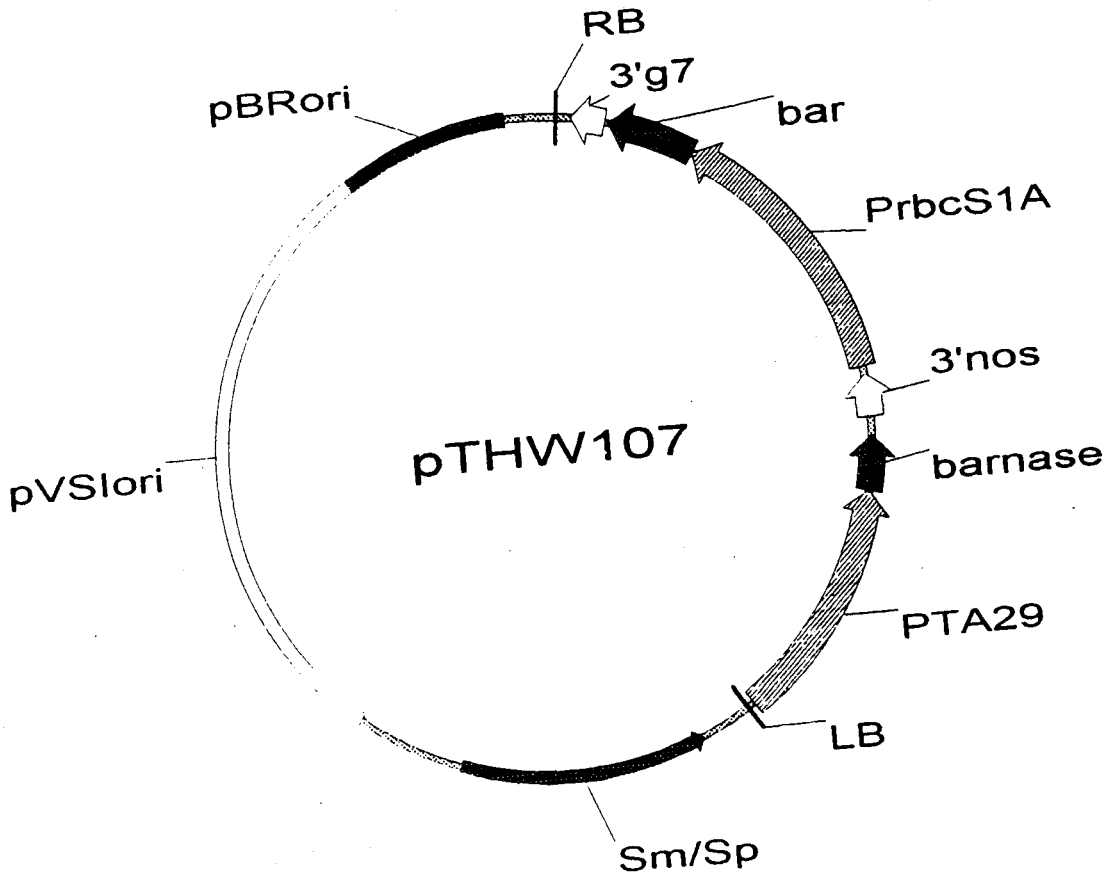
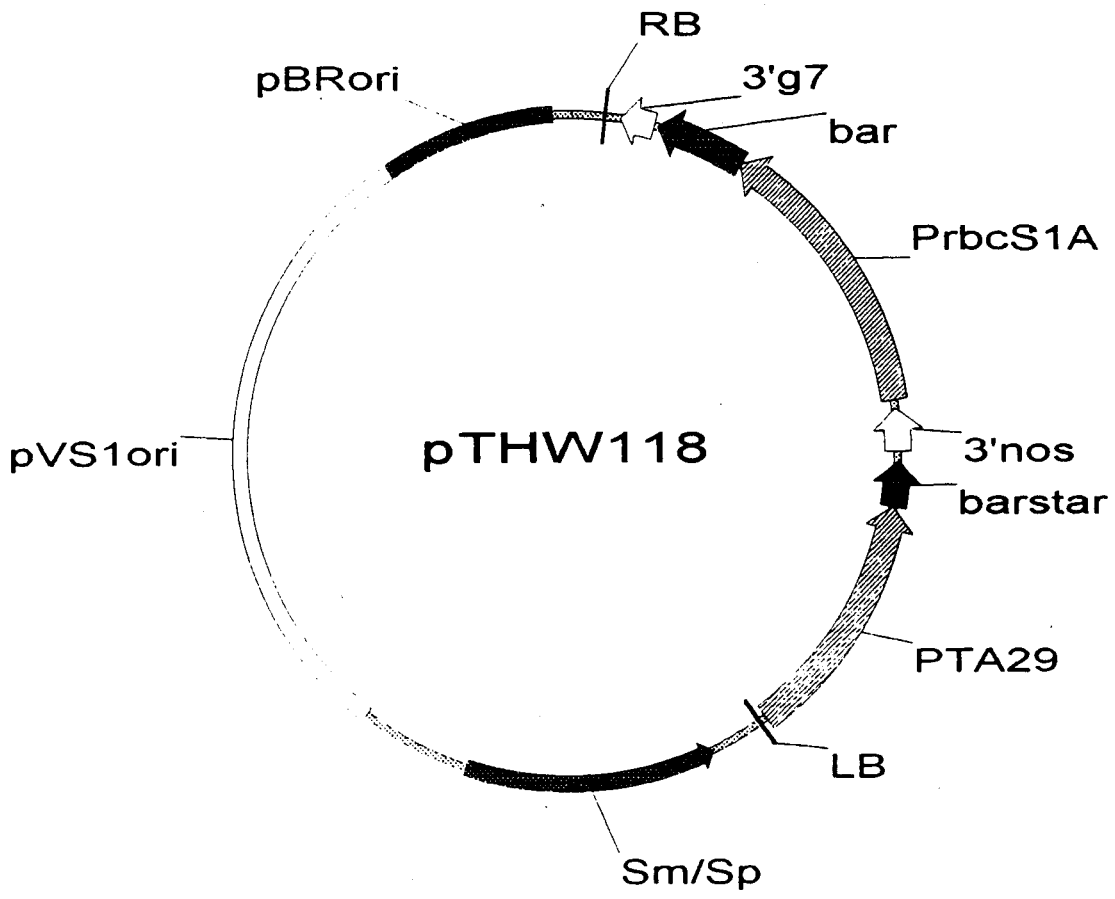


Table 3: Nucleotide sequence of the DNA comprised between the T-DNA border repeats of pTHW118 and origin of the different sequences

Nucleotide sequence		Origin of the sequence
# 1-25	:	Right border repeat from the TL-DNA from pTiB6S3 (Gielen et al., 1984).
# 26-53	:	Synthetic polylinker derived sequences.
# 54-90	:	Residual sequence from the TL-DNA at the right border repeat (Gielen et al., 1984).
# 91-97	:	Synthetic polylinker derived sequences.
# 309-98	:	The 3'untranslated end from the TL-DNA gene 7 (3'g7) of pTiB6S3 (Velten and Schell., 1985; Dhaese et al., 1983).
# 310-330	:	Synthetic polylinker derived sequences.
# 882-331	:	The coding sequence of the bialaphos resistance gene (*bar*) of *Streptomyces hygroscopicus* (Thompson et al., 1987). The N-terminal two codons of the wild type *bar* coding region have been substituted for the codons ATG and GAC respectively.
# 2608-883	:	The promoter from the atS1A ribulose-1,5-biphosphate carboxylase small subunit gene from *Arabidopsis thaliana* (PSsuAra) (Krebbers et al.,1988).
# 2609-2658	:	Synthetic polylinker derived sequences.
# 2919-2659	:	A 260 bp TaqI fragment from the 3' untranslated end of the nopaline synthase gene (3'nos) from the T-DNA of pTiT37 and containing plant polyadenylation signals (Depicker et al., 1982).
# 2920-2940	:	synthetic polylinker derived sequences.
# 2941-2980	:	3'untranslated region, downstream of the coding region of *barstar* from *B. amyloliquefaciens*. (Hartley, 1988)
# 3253-2981	:	The coding region of the *barstar* gene from *Bacillus amyloliquefaciens*.
# 4763-3254	:	The promoter region of the anther-specific gene TA29 from *Nicotiana tabacum* (Seurinck et al., 1990). The promoter comprises the 1.5 kb of the sequence upstream from the ATG initiation codon.
# 4764-4808	:	Synthetic polylinker derived sequences.
# 4809-4833	:	Left border repeat from the TL-DNA from pTiB6S3 (Gielen et al., 1984).

The complete nucleotide sequence for pTHW118 can be found in De Beuckeleer, M. (1996a).

Figure 2. Map of plasmid pTHW118, indicating the T-DNA, origins of replication and prokaryotic selective markers



IV. Open Reading Frames and Associated Regulatory Sequences

The male sterile MS8 oilseed rape line contains the PSsuAra-*bar*-3'g7 - PTA29-*barnase*-3'nos gene construct. The fertility restorer RF3 oilseed rape line contains the PSsuAra-*bar*-3'g7 - PTA29-*barstar*-3'nos gene construct. The donor organisms (*A. thaliana, S. hygroscopicus, N. tabacum* and *B. amyloliquefacies*) only function were to be the source of the described sequences, and were not actively involved in the modification process. None of the introduced genes (*bar*, *barnase*, *barstar*) has any inherent plant pest characteristics or poses a risk to plant health when introduced into the modified plants. A detailed description of each inserted T-DNA piece of material follows.

IV. a. PSsuAra-bar-3'g7

This sequence contains the promoter PSsuAra isolated from *Arabidopsis thaliana*, an herbaceous plant belonging to the *Brassicaceae* family. The PSsuAra promoter regulates the expression of the *bar* gene isolated from the bacterium *Streptomyces hygroscopicus*. The *bar* gene is expressed most actively in green tissues. Polyadenylation signals are provided by the 3' end of gene 7 of *A. tumefaciens*. (Van den Broeck et al., 1985; Thompson et al., 1987; Krebbers et al., 1988; De Almeida et al., 1989).

I.V. b. PTA29-barnase-3'nos

The promoter TA29 of *Nicotiana tabacum* (a *Solanaceae* plant) regulates the expression of the *barnase* gene isolated from the bacterium *Bacillus amyloliquefaciens*. The TA29 promoter limits the activity of the *barnase* gene to a specific tissue (the tapetum cells of the pollen sac) as well as it limits the time the gene is expressed (only when flowering during anther development). The tapetum is one of the specialized tissues of the anther. The anther is the organ in which male reproductive processes take place. The tapetum itself plays an important role in the development and maturation of the pollen grains. It has been observed male sterility systems seem to interfere with cell differentiation and/or functioning of the tapetum, indicating that this tissue is essential for the production of functional pollen grains. It has been documented that the specificity of the TA29 promoter is primarily confined to the tapetal cell layer of *B. napus*. The sequence also contains the 3'end of the nopaline synthase gene of *A.tumefaciens*. (Depicker et al., 1982; Hartley, 1988; Kaul, 1988; Koltunow et al., 1990; Seurinck et al., 1990; De Block et al., 1992; Goldberg et al., 1993).

I.V. c. PTA29-barstar-3'nos :

The promoter TA29 of *N. tabacum* regulates the expression of the *barstar* gene of *B. amyloliquefaciens*. *Barstar* inhibits the function of *barnase*, and thus restores fertility to the hybrid canola plant. This sequence contains also the 3'end of the nopaline-synthase (*nos*) gene of *A. tumefaciens* which functioned as a terminator. (Ellis and Murphy, 1981; Hartley, 1988; Koltunow et al., 1990; Seurinck et al., 1990).

I.V. d. Functions and scope of applications of the genes *bar*, *barnase* and *barstar* in transgenic oilseed rape

Male sterility has been widely used in breeding programs of many different crops as a tool to ensure cross-pollination. Though naturally occurring male sterile oilseed rape plants have been used to a certain extent to develop hybrids, side effects displayed by these male sterile plants and the lack of an associated morphological marker which could allow the male sterility trait to be followed more easily in the offspring, prevented the efficient use of these plants. A novel approach was undertaken to develop male sterile oilseed rape plants by introducing a ribonuclease (*barnase*) gene which is expressed exclusively in the tapetum, the cell layers surrounding the pollen sac, during anther development. Expression of *barnase* in this tissue blocks pollen development and results in a male sterile plant. The *barstar* gene (ribonuclease inhibitor) has also been introduced into oilseed rape, and is used to develop the restorer line of the hybrid system. Upon crossing a male sterile and a restorer line, the fertility of the oilseed rape progeny will be restored because the barstar protein will inactivate the barnase enzyme by forming a one-to-one complex with it. Additionally, the *bar* gene, which is an intrinsic part of the system, because it allows for an efficient selection in both the transformation/regeneration process and in the field, was closely linked to both the chimeric *barnase* and *barstar* gene constructs.

IV. d. i. The bar gene

The *bar* gene encodes the enzyme phosphinothricin-N-acetyl transferase (PAT). PAT acts by transferring the acetyl group of Acetyl Co-A to the herbicide glufosinate-ammonium. Acetylation of glufosinate-ammonium renders it inactive. In the active form, glufosinate-ammonium interferes in the GOGAT (glutamine-2-oxoglutarate) cycle of plants by inhibition of glutamine synthetase. This leads to the accumulation of ammonia and causes plant death. The *bar* gene has been isolated from *S. hygroscopicus*, a bacterial microorganism that has been discovered to produce an antibiotic, bialaphos. Bialaphos or its synthetically derived compound glufosinate-ammonium, is a herbicide with a novel mode of action described above (Bayer et al., 1972; Sadaaki Mase, 1984; Murakami et al., 1986; De Block et al., 1987; Thompson et al., 1987; Wild et al., 1987).

The integration of the *bar* gene in the hybrid system is fundamental to the oilseed rape hybrid system (De Block et al., 1989; Mariani et al., 1990; Mariani et al., 1992). It enables the use of glufosinate ammonium as a selective agent at the *in vitro* stage, and as mentioned in the previous paragraph, it confers resistance to glufosinate-ammonium, when glufosinate-ammonium is used as a broad spectrum herbicide in field tests or in commercial growing fields. Since the *barnase-bar* and *barstar-bar* gene constructs are physically linked to the *bar* gene construct, the genes will segregate as a single locus. As a consequence, the male sterile line and the restorer line can be maintained through crossing with wild type plants followed by the application of the herbicide. This enables identification of the parental lines (male sterile and fertility restoration line) independent of the developmental stage of the plant. As a result, the male sterile *Brassica napus* plants can be selected before they flower, and the male fertility restorer lines can be identified without prior testing of their restorer capacity (by test crosses with male sterile lines and subsequent scoring of the restoration capacity).

I.V. d. ii. The barnase gene

In flowering plants, pollen formation is a highly regulated developmental process that occurs in the anther. One of the tissues of the anther, the tapetum, plays a vital nutritive role during and after pollen formation. Defects in the tapetal function are therefore considered as the primary causes of male sterility (Kaul, 1988). By selectively destroying tapetal cells, naturally occurring male sterile plants could be mimicked. Since it has been shown that genes under the control of the TA29 promoter are specifically expressed in the tapetal tissues of oilseed rape plants, the a-specific ribonuclease enzyme, encoded by *barnase* under the control of the TA29 promoter, was inserted into the plant genome as a tapetum cell-lethal enzyme. The activity of this protein was proven to be detrimental for tapetal RNA and thus for its cell function. Introduction of the *barnase* gene, expressed under the control of the tapetum specific TA29 promoter, therefore results in male sterility of the transformed plants (Hartley, 1989; Drews et al., 1989; Mariani et al., 1990; De Block et al., 1993).

In crops where seeds or fruits are not the harvested product, male sterile plants can be crossed with any pollinator line to produce hybrid seeds. In contrast, in crops where seeds or fruits are harvested from the hybrid and where cross-pollination is not efficient, it is required to restore full male fertility in the hybrid offspring. If the hybrid crop is fully fertile, it will assure an optimal yield of the crop for the oilseed rape grower.

I.V. d. iii. The barstar gene

The existence of Barstar as the bacterial protein inhibitor of Barnase (Hartley, 1989), has facilitated the development of strategies for male fertility restoration of male sterile oilseed rape (Mariani et al., 1990; Mariani et al., 1992). By introducing the *barstar* gene under the control of the TA29 promoter into the oilseed rape genome, so-called male fertility restorer lines could be developed. The *barstar* encoded protein can form a one-to-one complex with the *barnase* encoded ribonuclease, rendering the latter inactive. Crossing the male fertility restorer lines with

male sterile (pTA29-*barnase*) oilseed rape, therefore results in the co-expression of the *barnase* and *barstar* genes in the tapetum of the F_1 progeny. The tapetum cell layer of the hybrid plant will develop in a normal fashion and plants are again male fertile (Mariani et al., 1992).

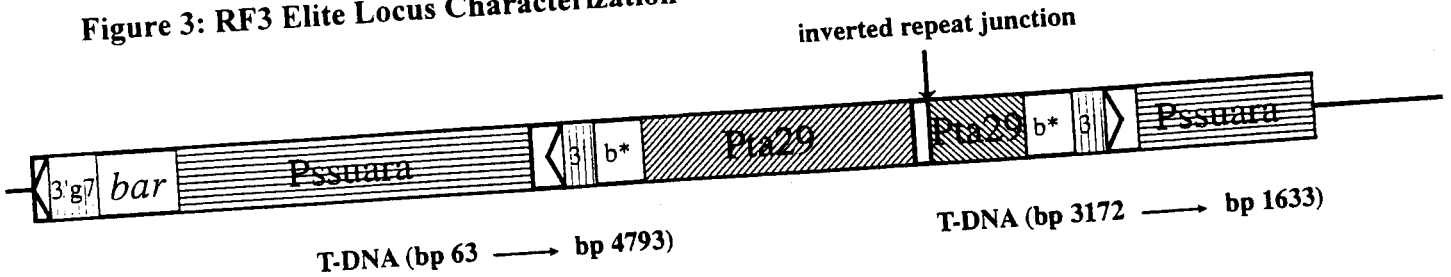
V. Genetic Analysis and Agronomic Performance

V. a. PCR and Southern Gel Analysis of Events MS8 and RF3

V. a. i. PCR Analysis

Based on PCR analysis and cloning of the PCR sequences of the expected inserts (De Beukeleer, M., 1996b), it was determined that, as expected, for MS8 plants one copy of T-DNA had been inserted into the plant genome in a single locus. PCR results also showed that the RF3 elite locus carries one T-DNA copy arranged in an inverted repeat structure with a second, incomplete T-DNA copy (see Figure 3). The inverted repeat is centered on the left T-DNA boundary with the second copy junction-point 458 bp upstream of the barstar ATG initiation codon. The second copy (2079 bp) includes a functional part of the promoter region of the anther specific gene TA29, the coding region of the *barstar* gene, 3'nos and a non-initiation codon of the *bar* gene.

Figure 3: RF3 Elite Locus Characterization



Furthermore, PCR analysis demonstrated that the integrated DNA is restricted to the DNA comprised between the T-DNA border repeats. The sequences from the plasmid vector outside the T-DNA border repeats are not present in the MS8 and RF3 transformants.

V. a. ii. Southern Analysis

In order to conclusively confirm the PCR results Southern analysis was conducted. Southern Analysis was performed according to Doyle and Doyle (1987). Genomic DNA was prepared from leaf tissue of an MS8 plant carrying the gene for male sterility (*barnase*), and from an RF3 plant carrying the gene for fertility restoration (*barstar*), respectively, and compared with total plasmid DNA. The respective DNA were digested with several restriction enzymes (see Table 4) then probed with different fragments of the transferred DNA in order to make comparisons between plasmid and genomic DNA fragments. The comparative analysis is based on the determination of the sizes of the respective hybridizing bands. For this reason restriction digests were chosen which produce restriction fragments between 0.8 and 3.4 kb. Within this range, restriction fragments can be sized within a resolution of 0.1 kb. The use of different probes comprising the *bar* and *barstar* genes and SsuAra and TA29 promoters allows visualization of different fragments of the T-DNA. Digest and probe combinations were chosen in such a manner

that the entire T-DNA region was covered. Plasmid DNA was used as the positive control and leaf tissue DNA from the nontransgenic parent canola variety, Drakkar, was used as the negative control.

Table 4 gives an overview of the different digests performed on total genomic DNA from the male sterile line (MS8) and the plasmid vector pTHW107, and on the total genomic DNA of the fertility restorer line (RF3) and the plasmid vector pTHW118. Four probes have been used in the hybridizations. The expected fragments fragment sizes are given in Table 4.

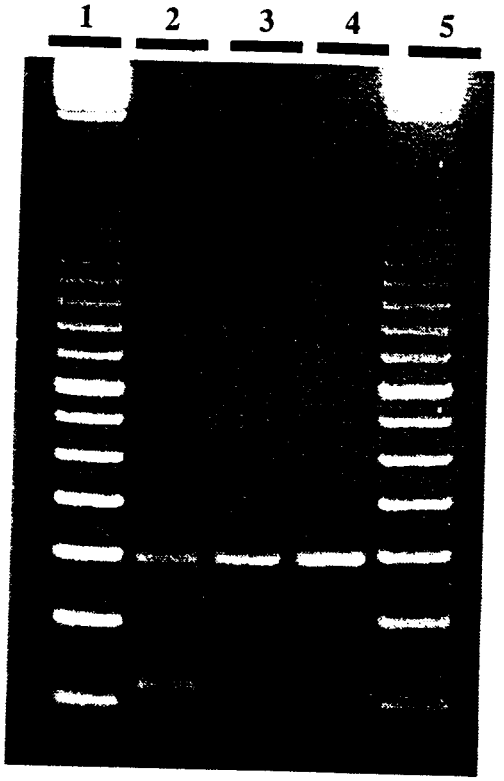
Figures 5 (a., b. and c.) and 6 (a., b., c. and d.) are copies of autoradiograms of Southern gel hybridizations of different probe-digest combinations. Southern analysis conclusively demonstrated that the primary transformants MS8 and RF3 and their respective progeny contain a single locus of the chimeric *barnase-bar* and *barstar-bar* genes, respectively. By comparing the observed fragment sizes with the expected fragment sizes (see individual restriction maps for a given probe hybridizing with MS8 or RF3, respectively) it was confirmed that for MS8 there is one copy of the inserted T-DNA into the plant genome. The expected fragment sizes given in Table 4 for the plasmid pTHW107 and on the restriction maps in Figures 5.a., 5.b. and 5.c. agree with what is shown on the Southern blots. For RF3 it was confirmed that one copy was inserted into the plant genome, but also that a second, incomplete copy had been inserted as well. For the fragments hybridizing with pSsuAra, TA29 and *barstar*, there is an extra fragment detected in the RF3 genome over and above what is in the plasmid pTHW118 T-DNA (see Table 4). Figures 6.a., 6.c. and 6.d. show this unambiguously. The second copy includes a functional part of the promoter region of the anther specific gene TA29, the coding region of the *barstar* gene, 3'*nos* and a non-functional part of the PSsuAra with the T-DNA sequence ending 750 bp upstream of the ATG initiation codon of the *bar* gene.

By a combination of segregation data, (section V. c.) Southern blot analyses and PCR results, it has been determined conclusively that all the genes of the T-DNA are inserted at a single locus, which segregates in Mendelian fashion, for both the primary MS8 transformant and its progeny as well as for the RF3 primary transformant and its progeny.

V. a. iii. PCR Analysis for RF3 Elite Locus Identification

Primers were used to construct a probe which was capable of RF3 elite locus identification (De Beuckeleer, M., 1996b). Figure 4 below demonstrates that unambiguous identification of the RF3 elite locus is possible using the PCR procedure as developed by DeBeuckeleer, M. (1996b). Lane 2 contains RF3 plant DNA and has a band occurring at approximately 75 bp. Neither the other fertility restorer event (RFx, lane 3), nor the control (nontransgenic, Drakkar, lane 4) plant has this unique band. This analysis is used for routine quality checks and identification purposes.

Figure 4: RF3 Elite Locus Identification



1. **MW marker (100bp ladder)**
2. **Rf3**
3. **Rfx**
4. **Control**
5. **MW marker (100bp ladder)**

Table 4: Overview of Restriction Digests, Probes and sizes of the Expected Respective Fragments Hybridizing with the Probes. For observed sizes see respective restriction maps in Figures 4a, 4b, 4c and 5a, 5b, 5c and 5d, respectively.

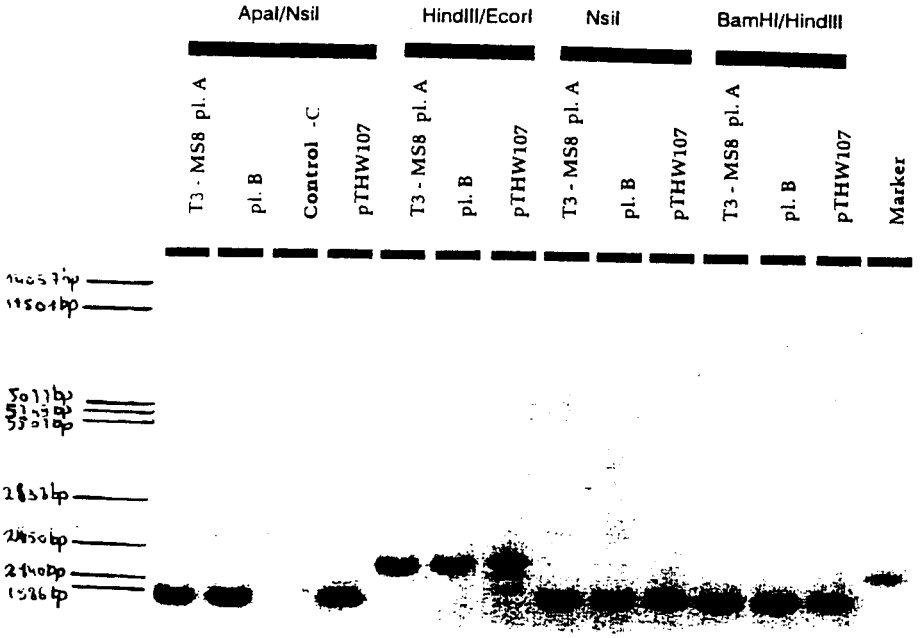
Male Sterile (MS8) Derived Lines (pTHW107 T-DNA)

	Expected Fragments hybridizing with:		
Digest	**Bar**	**pSsuAra**	**TA29**
ApaI/NsiI	712 bp + RB fragment	712 bp + 1423 bp	1966 bp
HindIII/EcorI	RB fragment	1665 bp	2266 bp
NsiI	RB fragment	RB fragment + 1423 bp	1966 bp
BamHI/HindIII	652 bp	1955 bp	1966 bp

Fertility Restorer (RF3) Derived Lines

	Fragments hybridizing with:							
Digest	**bar**		**PSSuAra**		**TA 29**		**Barstar**	
	RF3 (OBSERVED)	**pTHW118 plasmid (EXPECT)**	**RF3 (OBSERVED)**	**pTHW118 plasmid (EXPECT)**	**RF3 (OBSERVED)**	**pTHW118 plasmid (EXPECT)**	**RF3 (OBSERVED)**	**pTHW118 plasmid (EXPECT)**
APa I/NsiI	RB frag. + 712bp	RB frag. + 712bp	712bp+1423bp+LB frag.	712bp+ 1423 bp	1849bp +1070bp	1849bp	1849bp +1070bp	1849bp
HindIII/EcorI	RB frag.	RB frag.	1665bp+ LB frag.	1665 bp	1845bp + 790bp	1845 bp	1845bp + 790bp	1845 bp
NsiI	RB frag.	RB frag.	RB frag.+1423bp + LB frag.	RB frag. + 1423 bp	1849bp + 1070bp	1849 bp	1849bp + 1070bp	1849 bp
BamHI/HindIII	652bp	652bp	1955 bp+ LB frag.	1955 bp	1835bp + 800 bp	1835 bp	1835bp + 800 bp	1835 bp

Figure 5a: MS8 Plant Genomic DNA, TA29 Probe. Genomic DNA from leaf tissue was prepared and compared to total plasmid DNA. The respective DNA was digested with restriction enzymes - see Table 4 - then probed with TA29 DNA fragment. 2.5µg plant DNA was loaded onto gels. Positive control = total plasmid DNA. Negative control = nontransgenic parent Drakkar. 3rd generation plants (And B), plasmid pTHW107, and control (nontransgenic) lanes are marked below.



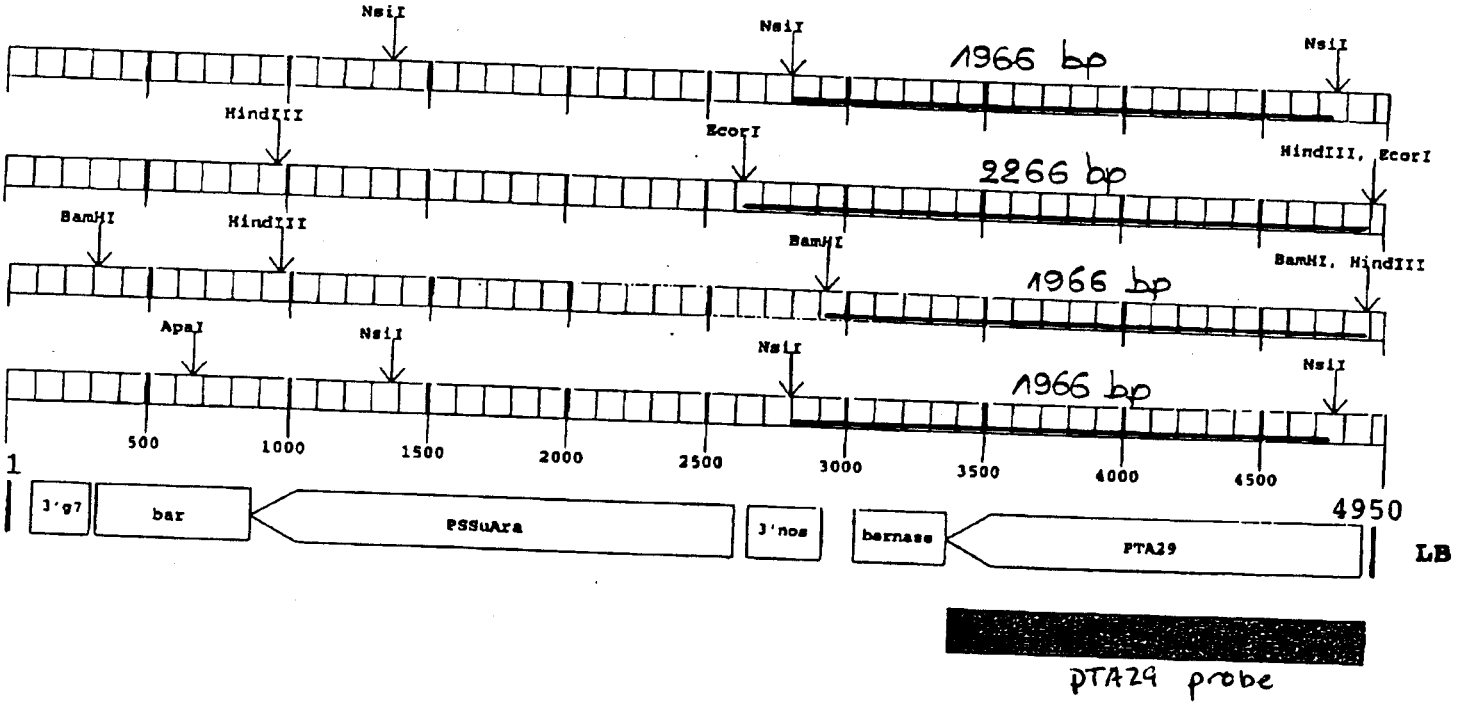
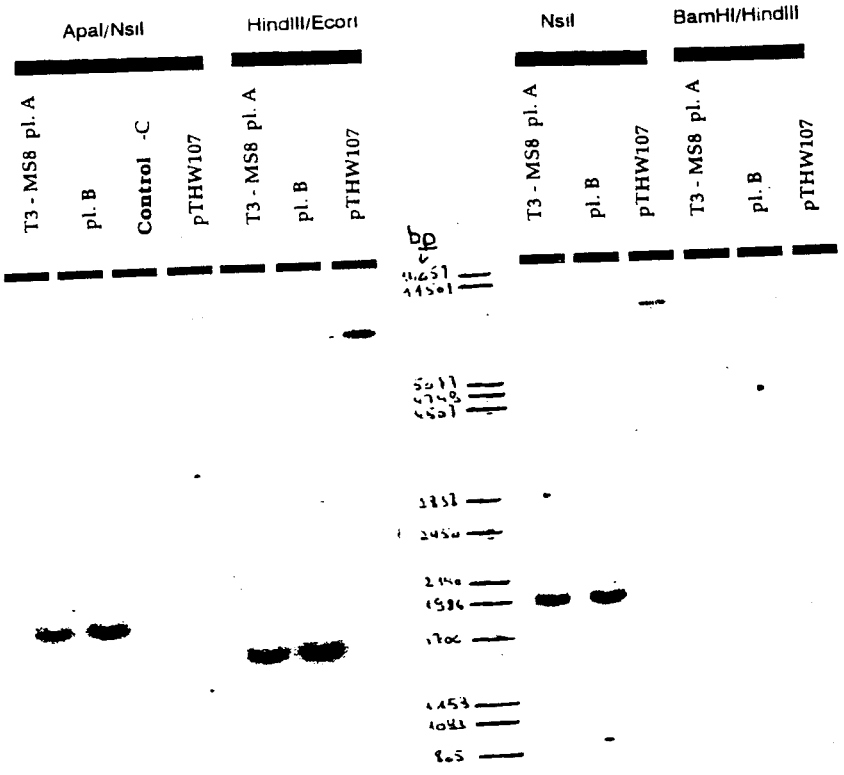


Figure 5b: MS8 Plant Genomic DNA, *bar* Probe. Genomic DNA from leaf tissue was prepared and compared to total plasmid DNA. The respective DNA was digested with restriction enzymes - see Table 4 - then probed with *bar* DNA fragment. 2.5µg plant DNA was loaded onto gels. Positive control = total plasmid DNA. Negative control = nontransgenic parent Drakkar. 3rd generation plants (And B), plasmid pTHW107, and control (nontransgenic) lanes are marked below.



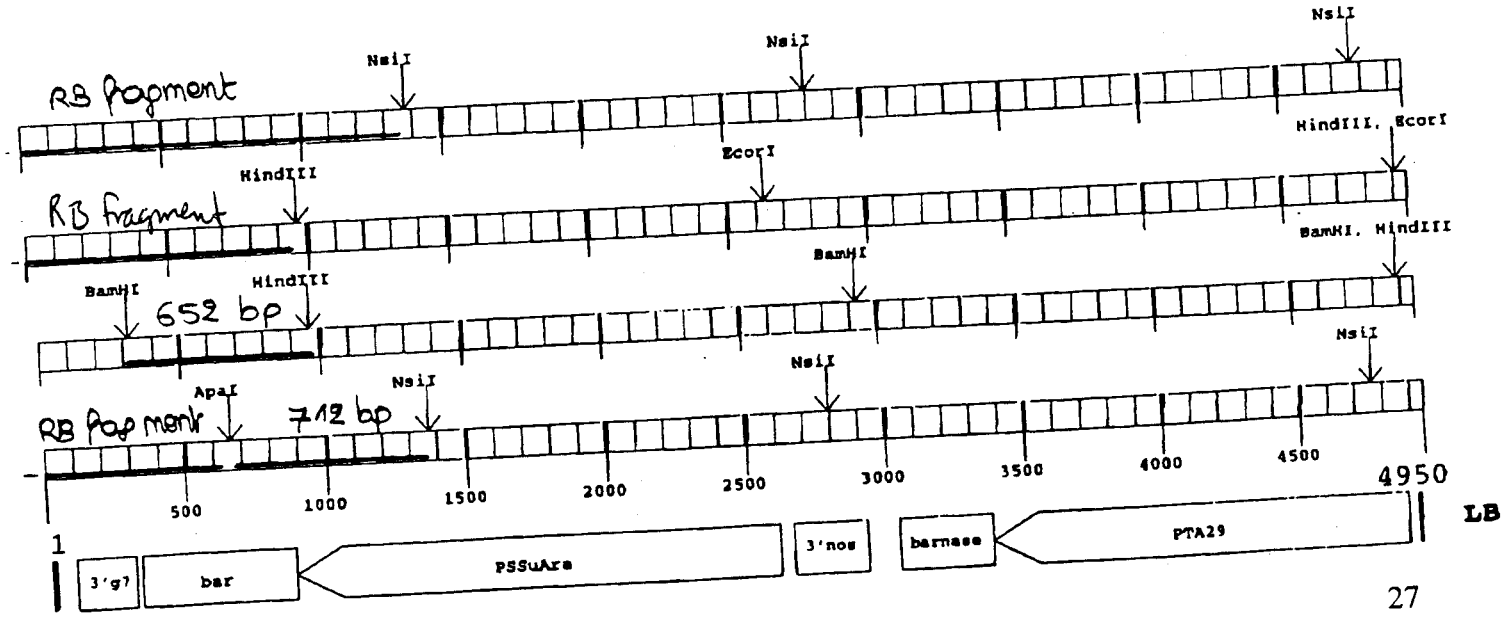


Figure 5c: MS8 Plant Genomic DNA, pSsuAra Probe. Genomic DNA from leaf tissue was prepared and compared to total plasmid DNA. The respective DNA was digested with restriction enzymes - see Table 4 - then probed with pSsuAra DNA fragment. 2.5µg plant DNA was loaded onto gels. Positive control = total plasmid DNA. Negative control = nontransgenic parent Drakkar. 3rd generation plants (And B), plasmid pTHW107, and control (nontransgenic) lanes are marked below.

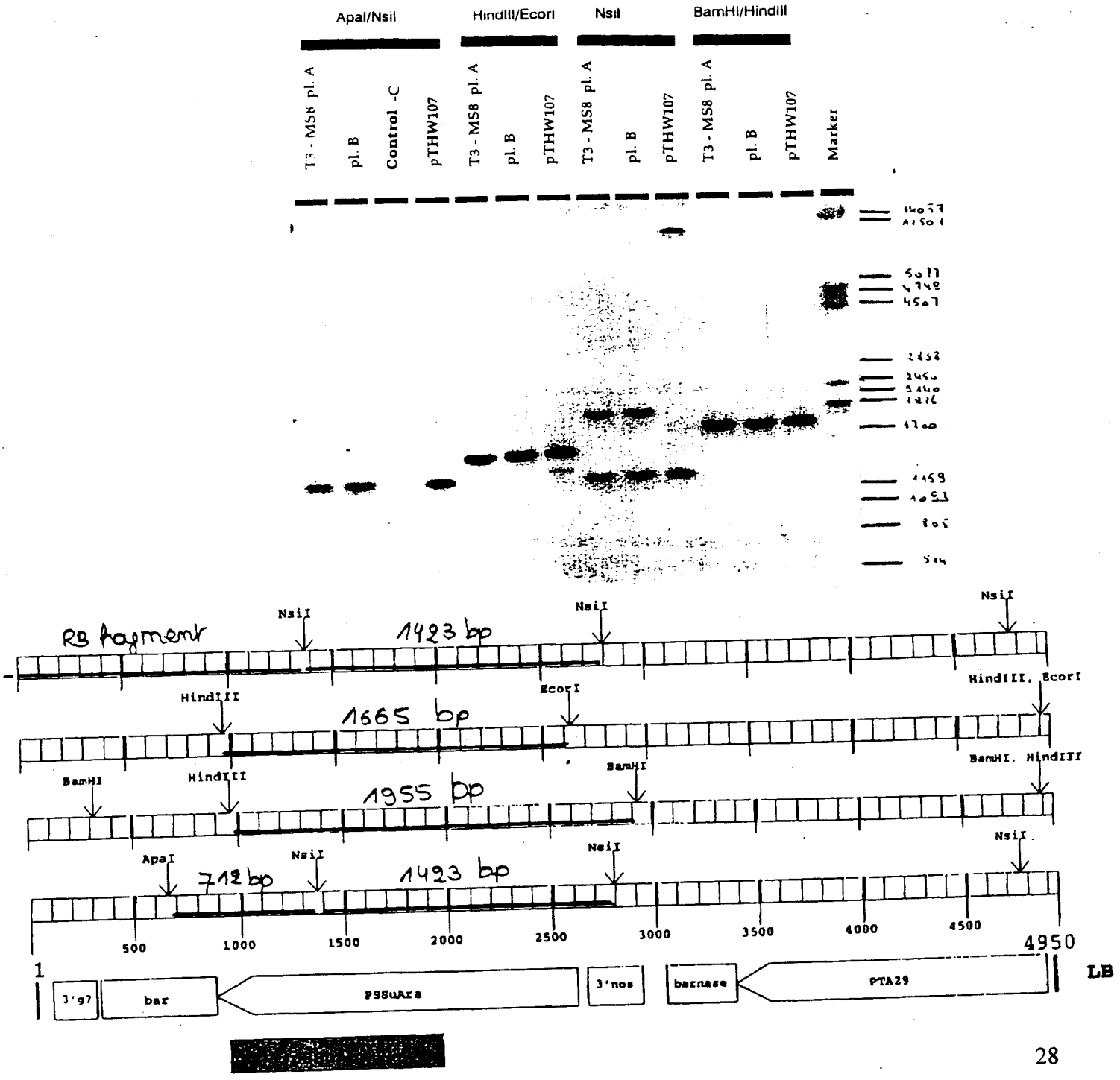
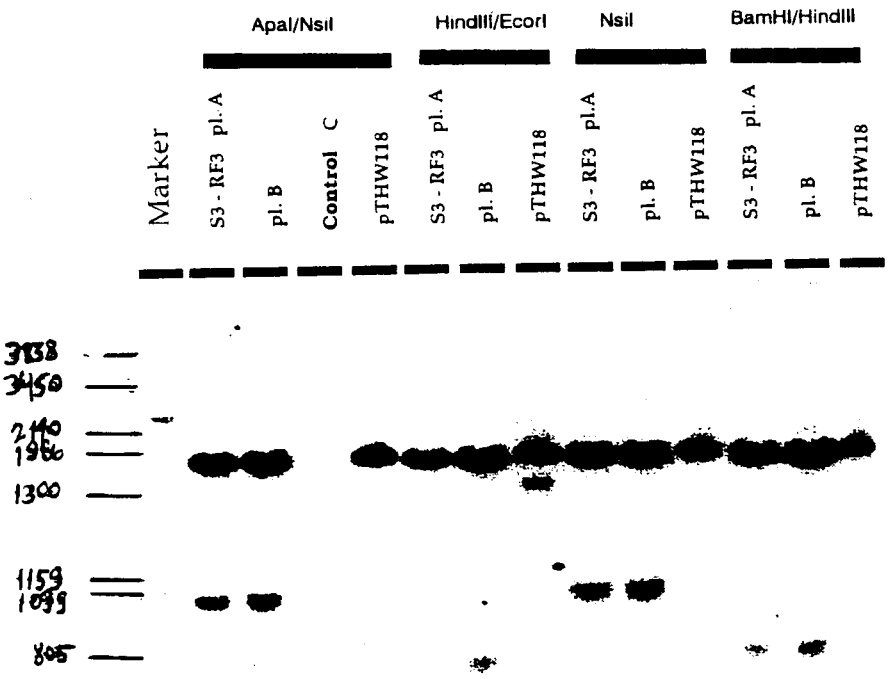
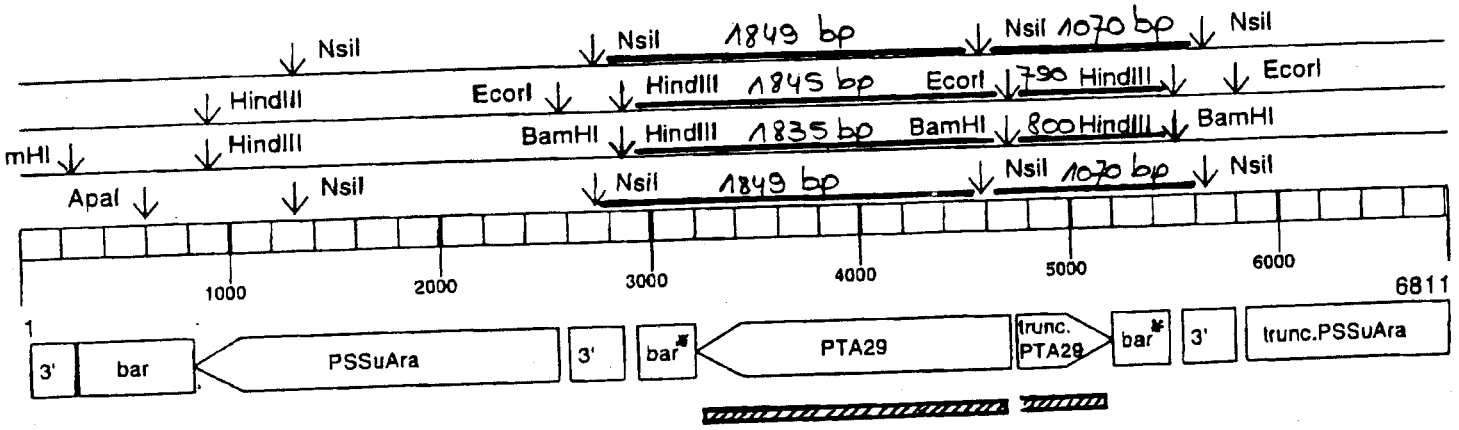


Figure 6a: RF3 Plant Genomic DNA, TA29 Probe. Genomic DNA from leaf tissue was prepared and compared to total plasmid DNA. The respective DNA was digested with restriction enzymes - see Table 4 - then probed with TA29 DNA fragment. 2.5µg g plant DNA was loaded onto gels. Positive control = total plasmid DNA. Negative control = nontransgenic parent Drakkar. 3rd generation plants (And B), plasmid pTHW118, and control (nontransgenic) lanes are marked below.

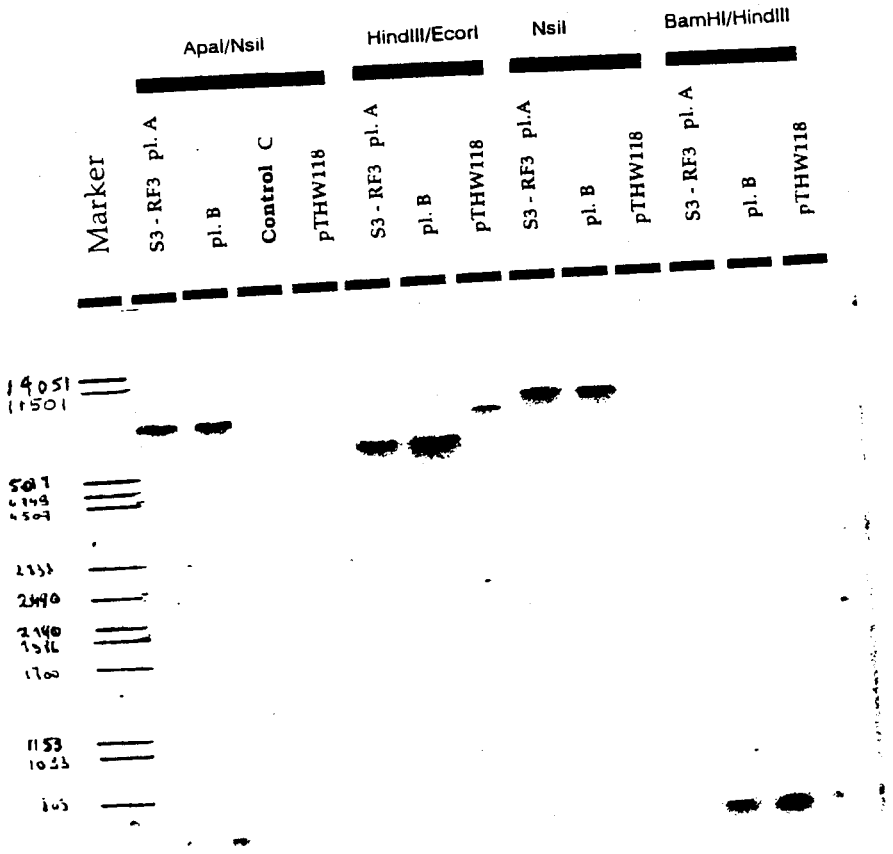


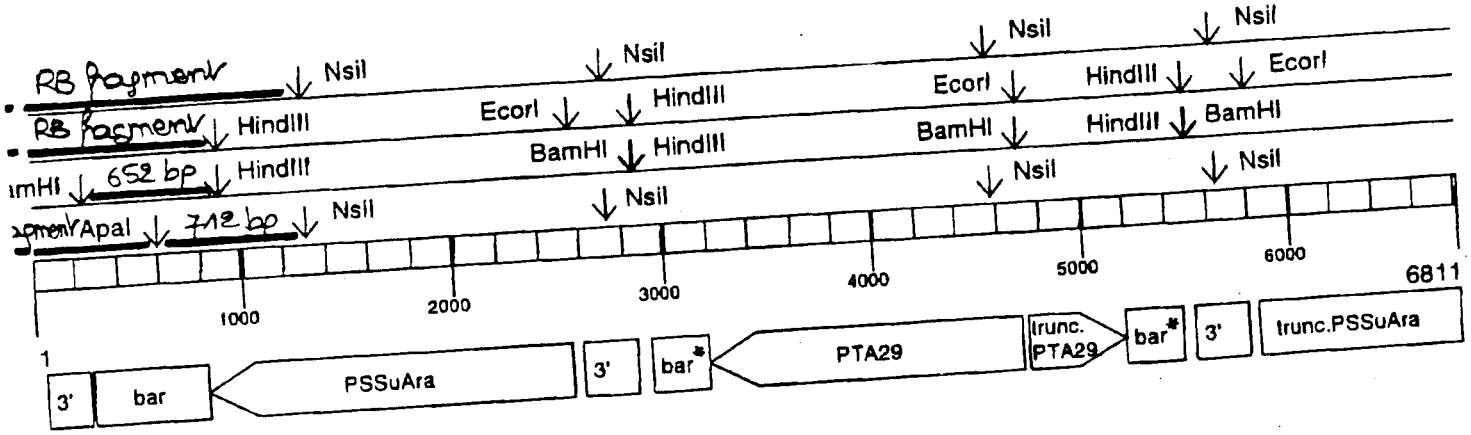


PTA29 probe

AgrEvo®

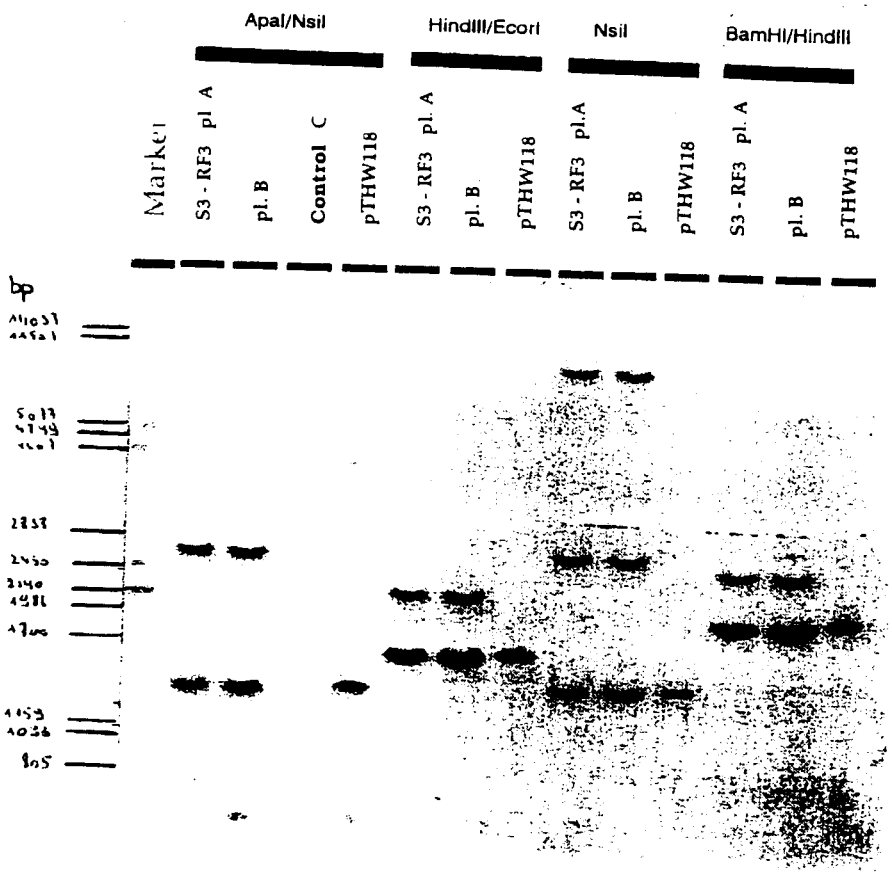
Figure 6b: RF3 Plant Genomic DNA, *bar* Probe. Genomic DNA from leaf tissue was prepared and compared to total plasmid DNA. The respective DNA was digested with restriction enzymes-see Table 4- then probed with *bar* DNA fragment. 2.5µg plantDNA was loaded onto gels. Positive control = total plasmid DNA. Negative control = nontransgenic parent Drakkar. 3rd generation plants (And B), plasmid pTHW118, and control (nontransgenic) lanes are marked below.

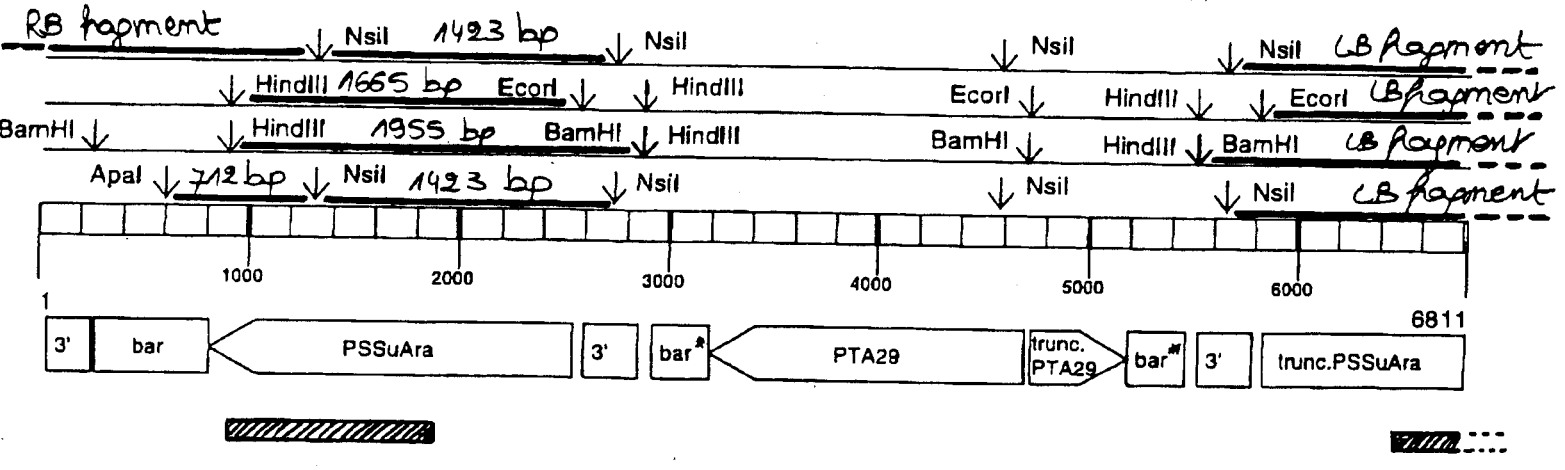




Bar probe

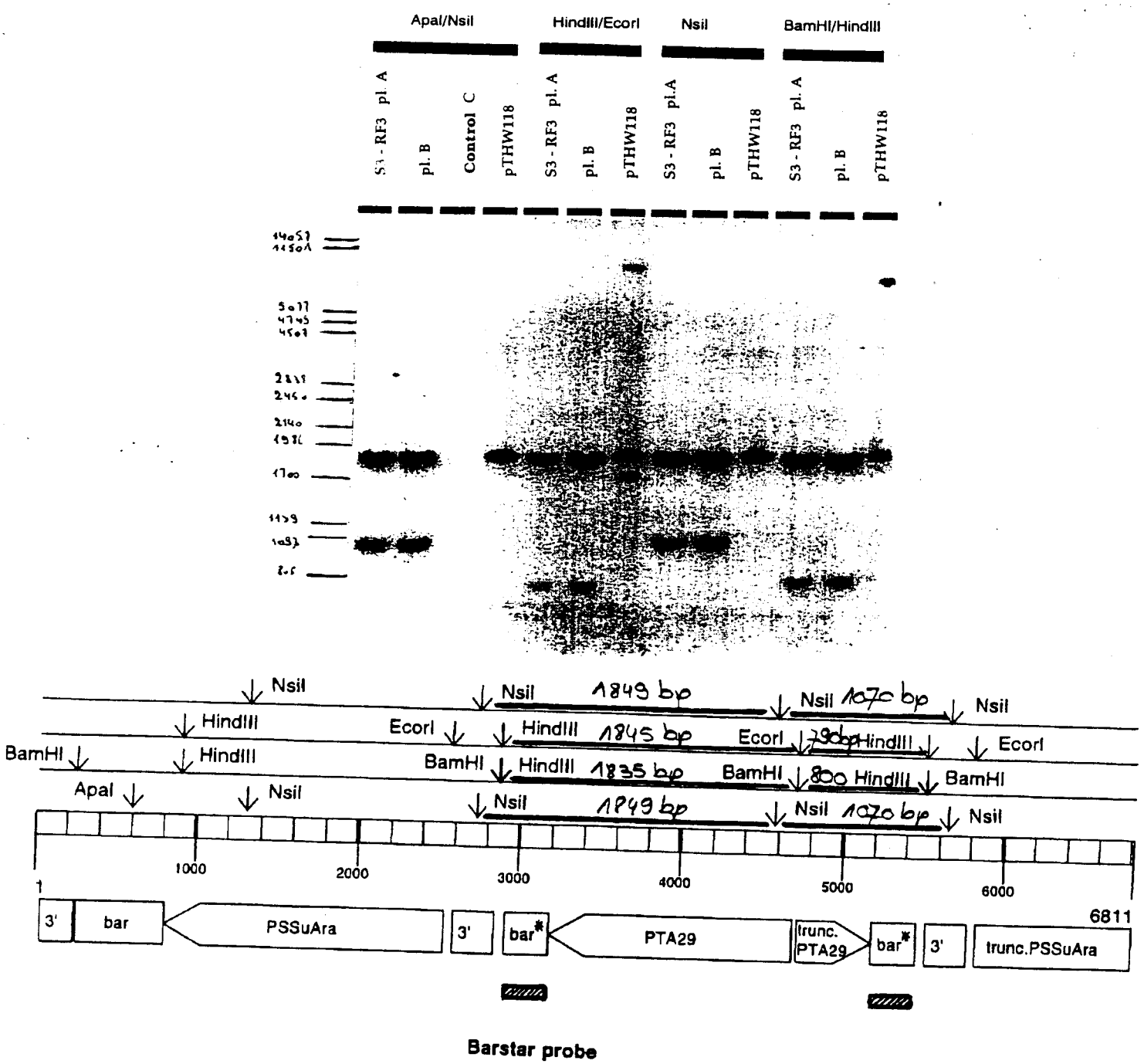
Figure 6c: RF3 Plant Genomic DNA, PSsuAra Probe. Genomic DNA from leaf tissue was prepared and compared to total plasmid DNA. The respective DNA was digested with restriction enzymes - see Table 4 - then probed with pSsuAra DNA fragment. 2.5µg plant DNA was loaded onto gels. Positive control = total plasmid DNA. Negative control = nontransgenic parent Drakkar. 3rd generation plants (And B), plasmid pTHW118, and control (nontransgenic) lanes are marked below.





PSSuAra probe

Figure 6d: RF3 Plant Genomic DNA, Barstar Probe. Genomic DNA from leaf tissue was prepared and compared to total plasmid DNA. The respective DNA was digested with restriction enzymes - see Table 4 - then probed with *barstar* DNA fragment. 2.5µg plant DNA was loaded onto gels. Positive control = total plasmid DNA. Negative control = nontransgenic parent Drakkar. 3rd generation plants (And B), plasmid pTHW118, and control (nontransgenic) lanes are marked below.



V. b. Mendelian Inheritance

In order to evaluate the inheritance and stability of the inserted genes of MS8 and RF3, backcrosses of the respective primary transformants were made with nontransgenic control lines.

V. b. i. MS8:

Evaluation of the Mendelian inheritance and expression of the chimeric *barnase-bar* gene construct in different genetic backgrounds of spring oilseed rape was made by backcrossing T_1 (MS8) plants into different spring oilseed rape lines. These lines are designated as SOSR-C1, SOSR-C2, SOSR-C3, SOSR-C4, SOSR-C5 and SOSR-C6. Seeds were grown in the greenhouse. Three week old transgenic seedlings were sprayed with glufosinate-ammonium (200ga.i./L). The number of surviving plants was recorded. If the locus is segregating as a single entity, then according to the laws of Mendel, half of the progeny should be resistant to the herbicide. This is what was observed in the F_1, BC_1 and BC_2 generations (see Table 5).

Table 5: MS8 Segregation Results

Plant Material	Total Number of Seedlings	Number of Seedlings Survinging glufosinate-ammonium Treatment	Expected segregation ratio	Observed segregation ratio	Chi^2
Male sterile (MS8) Plants					
T_1 (MS8)	176	93	1:1	83/93	(0.28) NS
F_1 plants					
F_1 (MS8 x SOSR-C1)	112	62	1:1	50/62	(0.64) NS
F_1 (MS8 x SOSR-C2)	111	49	1:1	62/49	(0.65) NS
F_1 (MS8 x SOSR-C3)	119	58	1:1	61/58	0.02) NS
F_1 (MS8 x SOSR-C4)	115	66	1:1	49/66	(1.42) NS
F_1 (MS8 x SOSR-C5)	114	68	1:1	46/68	(2.12) NS
F_1 (MS8 x SOSR-C6)	117	71	1:1	46/71	(2.91) NS
BC_1 plants					
BC_1 (MS8 x SOSR-C1)	113	56	1:1	56/57	(0) NS
BC_1 (MS8 x SOSR-C2)	116	57	1:1	59/57	(0.02) NS
BC_1 (MS8 x SOSR-C3)	108	50	1:1	58/50	(0.30) NS
BC_1 (MS8 x SOSR-C4)	112	55	1:1	57/55	(0.02) NS
BC_1 (MS8 x SOSR-C5)	116	46	1:1	70/46	(2.48) NS
BC_2 plants					
BC_2 (MS8 x SOSR-C1)	97	44	1:1	53/44	(0.33) NS
BC_2 (MS8 x SOSR-C2)	96	55	1:1	41/55	(1.02) NS
BC_2 (MS8 x SOSR-C3)	105	53	1:1	52/53	(0.02) NS
BC_2 (MS8 x SOSR-C4)	110	52	1:1	58/52	(0.16) NS
BC_2 (MS8 x SOSR-C5)	119	58	1:1	61/58	(0.02) NS

NS = not significant according to Fischer Chi^2 test

The resulting data showed that the chimeric *barnase-bar* gene construct was stably inherited in the different genetic backgrounds of spring oilseed rape tested.

V. b. ii. RF3:

Evaluation of the Mendelian inheritance and expression of the chimeric *bar/barstar* gene construct in different genetic backgrounds of spring oilseed rape was made by backcrossing S_1 (RF3, homozygous) plants into a spring oilseed rape line. The line is coded SOSR-C7. Seeds were grown in the greenhouse. Three week-old transgenic seedlings were sprayed with glufosinate-ammonium (200g a.i./L). The number of surviving plants was recorded. If the locus is segregating as a single entity, then according to the laws of Mendel, all of the progeny of the

F_1 generation should be resistant to the herbicide, and one-half of the BC_1 and BC_2 generations should be resistant to the herbicide. Indeed this is what was observed. (See Table 6).

Table 6: RF3 Segregation Results

Plant Material	Total Number of Seedlings	Number of Seedlings Survinging glufosinate-ammonium Treatment	Expected segregation ratio	Observed segregation ratio	Chi^2
F_1 plants					
F_1 (RF3 x SOSR-C6)	185	90	1:1	95/90	(0.26) -
BC_1 plants					
BC_1 (RF3 x SOSR-C7)	120	64	1:1	56/64	(0.27) NS

NS = not significant according to Fischer Chi^2 test

This data demonstrates that the chimeric *bar/barstar* gene construct was stably inherited in the different genetic backgrounds of spring oilseed rape tested.

V. c. Expression of Inserted Genes: *barnase, barstar* and *bar*

In order to demonstrate the expression of the introduced transgenes in the male sterile, MS8, and fertility restorer, RF3, progenies, Northern blot analysis of messenger RNA was conducted. Leaf tissue, dry seed, pollen and flower bud tissue were analyzed. Figures 7 – 10 show the results of hybridization in the different tissues. Figure 6 is hybridzation results of RF3 mRNA probed with *barstar*. Figure 9 is hybridization results of RF3 mRNA probed with *bar*. Figures 8 and 10 are the dilution sequences of *in vitro* synthesized RF3 mRNA complementary to the probe used. The control mRNA samples have had 5µg control (nontransgenic) leaf mRNA added to them.

The method given in Appendix 3 was used to quantify mRNA expression of the *bar*, *barnase* and *barstar* transgenes. Only detectable levels of transgene expression, in the range of the linear regression of the control dilution series, were quantified by ImageQuant of Molecular Dynamics.

MS8 Results: The mRNA levels of bar in leaves and flower buds varied between 0.03 pg and 0.22 pg/µg total RNA. In the dry seed samples, no bar mRNA signal (LOD = 0.1 pg/µg total RNA) was detected. No barnase mRNA signals were detected (LOD = 0.1 pg/µg total RNA) in the tissues tested. This was expected since barnase is driven by the tapetum specific TA29 promoter. Results are summarized in Table 7.

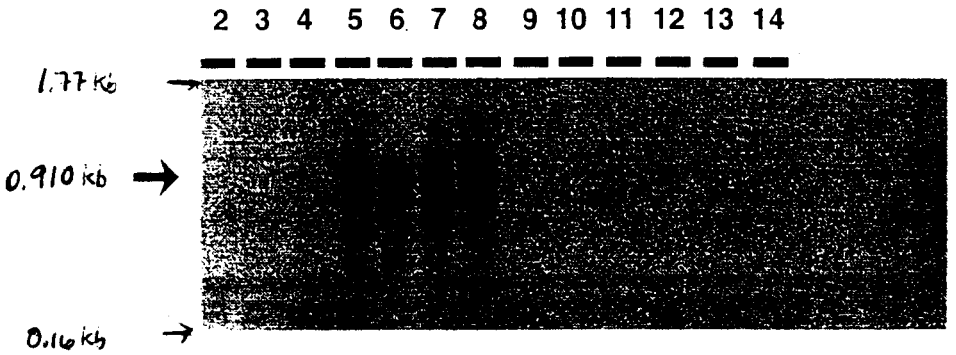
RF3 Results: The mRNA levels of bar in leaves and flower buds vary between 0.2 and 1.1 pg/µg total RNA (LOD = 0.05 pg/µg total RNA). In the pollen and dry seed samples, no bar mRNA signals were detected (LOD = 0.05 pg/µg total RNA). Barstar mRNA is only detectable in the flower bud mRNA samples with a level that varies between 1.2 pg and 2.4 pg/µg total

RNA (LOD = 0.1 pg/µg total mRNA). This is expected since the *barstar* gene is driven by the tapetum specific TA29 promoter. Results are summarized in Table 7.

C

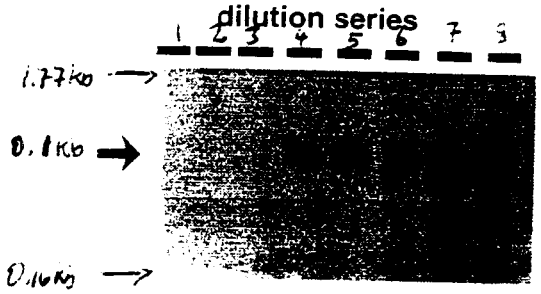
V. f. i. Northern Blot Analysis of Transgene Expression

Figure 7: RF3 mRNA Hybridization with the probe *barstar*



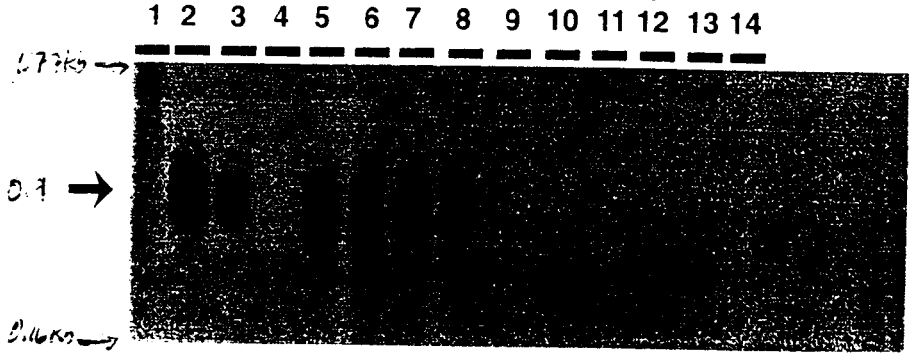
Lane	Sample	Plant no.	Tissue	µg RNA loaded
1	MW (0.16 – 1.77 kb RNA ladder, Life Technologies, Inc.)			
2	RF3	S3-A	Leaf	5µg
3	RF3	S3-B	Leaf	5µg
4	control		Leaf	5µg
5	RF3	S3-A	Flower buds 2mm	5µg
6	RF3	S3-A	Flower buds 3 mm	5µg
7	RF3	S3-B	Flower buds 2mm	5µg
8	RF3	S3-B	Flower buds 3 mm	5µg
9	control		Flower buds 2mm	5µg
10	control		Flower buds 3 mm	5µg
11	RF3	S3	pollen	5µ g
12	control		pollen	5µg
13	RF3	S3	dry seed	5µg
14	control		dry seed	5µg

Figure 8: Dilution series. *In vitro* synthesized mRNA hybridized with the complementary probe (*barstar*). Control samples have had 5µg leaf mRNA (nontransgenic) added to them.



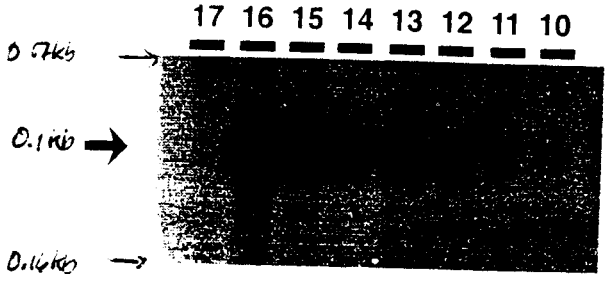
Lane	Sample	µg RNA loaded
1	MW (0.16 – 1.77 kb RNA ladder, Life Technologies, Inc.)	
2	control	0.25pg
3	control	0.5pg
4	control	1 pg
5	control	2 pg
6	control	4pg
7	control	8pg
8	control	16pg

Figure 9: RF3 mRNA Hybridization with the probe *bar*



Lane	Sample	Plant no.	Tissue	µg RNA loaded
1	MW (0.16 – 1.77 kb RNA ladder, Life Technologies, Inc.)			
2	RF3	S3-A	Leaf	5µg
3	RF3	S3-B	Leaf	5µg
4	control		Leaf	5µg
5	RF3	S3-A	Flower buds 2mm	5µg
6	RF3	S3-A	Flower buds 3 mm	5µg
7	RF3	S3-B	Flower buds 2mm	5µg
8	RF3	S3-B	Flower buds 3 mm	5µg
9	control		Flower buds 2mm	5µg
10	control		Flower buds 3 mm	5µg
11	RF3	S3	pollen	5µg
12	control		pollen	5µg
13	RF3	S3	dry seed	5µg
14	control		dry seed	5µg

Figure 10: Dilution series. *In vitro* synthesized mRNA probed hybridized with the complementary probe (*bar*). Control samples have had 5µg leaf mRNA (nontransgenic) added to them.



Lane	Sample	g RNA loaded
1	MW (0.16 – 1.77 kb RNA ladder, Life Technologies, Inc.)	
2	control	0.25pg
3	control	0.5pg
4	control	1 pg
5	control	2 pg
6	control	4pg
7	control	8pg
8	control	16pg

Table 7: Summary of Barnase and Bar mRNA Expression Results of MS8 and RF3 as detected by Northern Analysis

Total RNA	Transgene Expression (pg/µg total RNA)		Total RNA	Transgene Expression (pg/µg total RNA)	
	bar pGembar/SP6	barnase pVE113/SP6		bar pGembar/SP6	barstar pVE113/SP6
MS8-T3 leaf A	0.03 pg	n.d.	RF3-S3 leaf A	1.1 pg	n.d.
MS8-T3 leaf B	0.22 pg	n.d.	RF3-S3 leaf B	0.2 pg	n.d.
MS8-T3 flower buds 2mm A	0.14 pg	n.d.	RF3-S3 flower buds 2mm A	0.46 pg	1.54 pg
MS8-T3 flower buds 2mm B	0.11 pg	n.d.	RF3-S3 flower buds 2mm B	0.52 pg	1.3 pg
MS8-T3 flower buds 3mm A	0.19 pg	n.d.	RF3-S3 flower buds 3mm A	0.38 pg	1.22 pg
MS8-T3 flower buds 3mm B	0.03 pg	n.d.	RF3-S3 flower buds 3mm B	0.34 pg	2.4 pg
MS8-T3 dry seed	n.d.	n.d.	RF3-S3 dry seed	n.d.	n.d.
			RF3-S3 pollen	n.d.	n.d.
LOD (pg/ g total RNA)	0.1	0.1	LOD (pg/ g total RNA)	0.05	0.1

n.d. = no signal detected
pGembar/SP6 and pVE113/SP6 = plasmids used for preparation of RNA probes. See Appendix 3

V. c. ii. PAT Expression

Expression of the PAT enzyme in leaf tissue and seeds of the MS8 and RF3 events was measured using spectrophotometric assay. The method used is given in Appendix 3. PAT activity is quantified by measuring enzyme kinetics. The method is based on the generation of free coenzyme A (CoA) sulfhydryl groups during the transfer of the acetyl group of the glufosinate-ammonium molecule. The reaction of the reduced CoA with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) yields a molar equivalent of free 5-thio-2-nitrobenzoic acid with a molar extinction coefficient of 13,6000 at 412 nm. Color intensity of the sample of interest is measured against a standard curve.

Table 8: PAT activity in Seed

Sample	Flour (g)	Protein (mg/mL)	PAT (µg/mL)	PAT/protein (µg/mg)
Control (a)	0.020	3.5	0.03	0.01
Control (b)	0.010	2.1	-0.12	-0.06
				Mean: -0.03
MS8 (a)	0.045	6.1	0.03	0.00
MS8 (b)	0.048	6.4	0.19	0.03
MS8 (c)	0.046	6.1	0.50	0.08
				Mean: 0.04
RF3 (a)	0.043	1.8	0.03	0.02
RF3 (b)	0.052	2.4	0.50	0.21
RF3 (c)	0.070	2.9	0.19	0.06
				Mean: 0.10

Table 9: PAT activity in Leaves

Sample	Protein (mg/mL)	PAT (µg/mL)	PAT/protein (µg/mg)
Control (a)	1.96	0.87	0.44
Control (b)	1.20	0.46	0.38
Control (c)	1.06	0.25	0.24
Control (d)	0.76	0.19	0.25
Control (e)	0.65	0.34	0.52
			Mean: 0.37
MS8 (a)	0.34	0.12	0.35
MS8 (b)	0.64	0.12	0.19
MS8 (c)	0.84	0.24	0.29
MS8 (d)	0.41	0.26	0.64
MS8 (e)	0.53	0.42	0.79
MS8 (f)	0.64	0.50	0.79
			Mean: 0.51
RF3 (a)	0.84	1.11	1.32
RF3 (b)	1.02	0.74	0.73
RF3 (c)	1.76	2.04	1.16
RF3 (d)	0.84	1.42	1.69
RF3 (e)	1.86	2.82	1.52
RF3 (f)	1.70	2.66	1.56
			Mean: 1.33

These measurements demonstrated that the introduction of the bar gene does not convey specific PAT activity above background acetyl-transferase activity in seed extracts. This is in line with the expectation that the pSsuAra promoter directs expression to green plant tissue. Determination of PAT activity in green leaves did reveal PAT activity .

V. d. Verification of the Agronomic Performance of MS8 and RF3

The agronomically important characteristics of MS8 and RF3 and their hybrid offspring were evaluated over several seasons in a field evaluation program in Europe (Belgium, France and The United Kingdom) and in Canada (Saskatchewan). A detailed description of the evaluation is given in subsequent subsections and the general conclusions were:

- The phenotype of the selected male sterile (MS8) and fertility restorer (RF3) canola plants was comparable to commercial varieties except for the male sterile trait,
- Glufosinate-ammonium segregation data for the MS8 and RF3 plants confirmed that the transgenes were inserted at a single locus,
- Treatment with glufosinate-ammonium did not influence plant growth and flowering,
- Male sterility of the MS8 line was maintained throughout the growing season,
- Restorer capacity of the RF3 line was satisfactory, and
- Seed quality parameters were comparable to controls.

Transformation Events MS8 and RF3 were field tested in the United States in 1997, at two locations under permit 97-035-05r (Cass Co., ND and Dane County, WI). MS8 and RF3 are currently being evaluated in the field in 1998 at fourteen (14) locations under notifications 98-064-38n (Polk Co., MN); 98-064-35n (Pierce Co., Nelson Co., Towner Co., Ramsey Co., Foster Co. (2 locations), and Cavalier Co. (2 locations), ND); 98-064-33n (Waushara Co., and Door, Co., WI); 98-168-04n (Columbia Co., WI); and 98-064-31 (Madison Co., ID), as well as permit 98-119-01r (Cass Co., ND). Agronomic characteristics and performance of the hybrid MS8/RF3 and the nontransgenic parent (Drakkar) were recorded and compared. In all trials MS8/RF3 exhibited the same agronomic behavior as Drakkar regarding seed germination rates, plant stand, plant vigor, flowering times and vigor, deleterious effects, disease and pest resistance/susceptibility. See Appendix 5 for complete field release termination reports and their conclusions.

V. d. i. Evaluation of the agronomic performance of the male sterile MS8 and fertility restorer RF3 oilseed rape lines and their hybrid progeny

Different phenomena (somaclonal variation, position effects and pleiotropic effects) may influence the agronomic performance of new transformants and their progeny. To evaluate and check the expression of agronomic important features of the male sterile MS8 and fertility restorer RF3 oilseed rape line, several field experiments were carried out over several growing seasons. During selection and development of lines and during the period of testing the feasibility of the new hybrid system, no abnormalities were observed for the MS8 and RF3 progeny in comparison with transgenic control plants (lines designated as MS1, RF1 or RF2 [3])

[3] Lines designated as MS1, RF1 and RF2 contain the *barnase-bar* and *barstar-bar* chimeric gene constructs, respectively. MS1, RF1 and RF2 have received approval for Unconfined Release by Agriculture and Agri-Food Canada and have been approved by USDA for import to crush, only, and are relevant to this petition in that they were used as positive control plants for agronomic performance evaluation of MS8 and RF3.

and the non-transgenic control cultivar, Drakkar, except for the predicted effects of the inserted genes. The absence of major changes of agronomic and developmental characteristics of the selected male sterile and fertility restorer oilseed rape line provide additional evidence that the engineered trait and the engineering process have not modified the oilseed rape plants to a significant extent in comparison with normal oilseed rape cultivars.

V. d. i. a. Germination, crop establishment and plant vigor of the transgenic oilseed rape in comparison with non-transgenic oilseed rape

Germination of nontransformed parent and transformed (MS8 and RF3) oilseed rape, has been compared under agronomic circumstances as well as in a number of germination tests. The introduction of the chimeric genes *barnase-bar* and *barstar-bar*, respectively, has not changed the ability of the oilseed rape to germinate and to survive. Under field conditions, transgenic and non-transgenic seedlings germinated at about the same time after sowing. Thereafter, both transgenic and non-transgenic oilseed rape developed evenly, and uniform plant stands were established. Plant height and plant vigor of the MS8 and RF3 plants and their (MS8 x RF3) restored hybrid combination, were comparable to the nontransgenic oilseed rape. No different susceptibility to temperature, humidity, desiccation, light or other ecological stress factors from that of other oilseed rape cultivars has been observed from planting to harvest. Disease resistance and lodging resistance of the plants were comparable.

V. d. i. b. Evaluation of herbicide resistance levels and segregation data for the herbicide tolerance phenotype of MS8 and RF3 plants and their (MS8 x RF3) hybrid combination

To test the tolerance level of the male sterile MS8 and fertility restorer RF3 oilseed rape line, glufosinate-ammonium was sprayed on the transgenic plants at different rates in a number of locations. The male sterile MS8 and fertility restorer RF3 oilseed rape line and their hybrid combinations have shown an adequate resistance level both under greenhouse and field conditions. The MS8 line consistently showed a normal 1:1 segregation pattern upon application of glufosinate-ammonium herbicide.

V. d. i. c. Flowering data of the male sterile MS8 and fertility restorer RF3 oilseed rape line, their progeny and their hybrid combination

During all field trials, MS8 and RF3 plants, their respective progenies and their hybrid combination derived from the same non-transgenic cultivar started flowering at about the same time when evaluated under the same environmental conditions. The male sterile MS8 oilseed rape plants often showed a flowering delay in comparison with the non-transgenic control variety. In general, a maximum flowering delay of three days has been observed for MS8. Considering the overall flowering results, no significant differences in flowering date of the transgenic versus the non-transgenic oilseed rape were obtained.

Tested under different environmental conditions, the male sterile MS8 oilseed rape line showed a 1:1 segregation ratio of male sterile versus male fertile oilseed rape plants when crossed with wild type plants. In different environments, the MS8 oilseed rape line appeared to be consistently male sterile. The male sterile MS8 plants appeared to be male sterile with minimal side effects on flower morphology. Nectaries in male sterile oilseed rape flowers developed normally. There are no indications that the male sterile plants have a greater tendency towards bud abortion than the male fertile ones or the parental cultivars. Normal insect activity was observed.

The fertility of the fertility restorer RF3 plants was similar to the control cultivar (Drakkar). Flower phenotype was normal at all sites. Normal insect activity was observed. When evaluating the flower phenotype of restored hybrid combinations, a high restoration level was observed. The male fertile hybrids were phenotypically normal with no side effects on flower and vegetative morphology, bud abortion or attractiveness to pollinating insects. The observed level of restoration is adequate to ensure a reliable and efficient hybrid oilseed rape seed production.

V. d. i. d. Yield data of MS8 and RF3 oilseed rape lines, their progeny and their hybrid combination

Yield capacity of the male sterile MS8 and fertility restorer RF3 line and their hybrid combination has in general not been changed. Though under suboptimal conditions, completely male sterile oilseed rape plants may yield less than the control plants, equivalent yield data were obtained as soon as good pollination conditions were created. This confirmed that the yield capacity of the male sterile plants had not changed.

V. d. i. e. Verification of oil and seed quality of MS8 and RF3 oilseed rape

To screen for negative pleiotropic interactions or unintended metabolic effects that may occur in the transgenic male sterile MS8 or fertility restorer RF3 oilseed rape plants, oil quality and seed quality analyses were performed. Special attention was given to naturally occurring oilseed rape compounds such as erucic acid and glucosinolates. Quality analysis of the MS8 and RF3 plants and their hybrid combination were carried out on samples taken at different locations, during several years and from several generations. No major changes in oil and protein content and composition were observed for the transgenic oilseed rape lines in comparison to the control oilseed rape cultivar. As the original variety is a double zero variety, the erucic acid level was not expected to be higher than 0.05% of the total oil composition. Results of the oil analysis confirmed these expectations.

V. d. i. f. Conclusion of Agronomic Performance of MS8 and RF3 Canola

The *B. napus* male sterile MS8 and fertility restorer RF3 oilseed rape line have been selected out of many other transformants for :

- their normal phenotypic morphology,
- acceptable level of glufosinate-ammonium resistance linked to the desired flower phenotype,
- predictable segregation pattern,
- normal agricultural performance,
- stability of the male sterile flower phenotype throughout the growing season,
- stability of the expression in different genetic backgrounds,
- if applicable : capacity to restore the fertility of the male sterile plants,
- confirmation of the absence of yield penalties,
- normal oil and seed quality

as observed under different environmental conditions, during several years and over many generations. Therefore, it can be concluded that after gene insertion, no significant effects on the plant phenotype or metabolism have been observed.

The fact that no undesirable phenotypic traits and no detectable significant effects on the agronomic performance of the transgenic oilseed rape plants were observed, gave a first indication of the non-altered oilseed rape plant metabolism.

V. e. Disease and Pest Resistance Characteristics

To evaluate disease and pest resistance characteristics of MS8 and RF3 and their hybrid combination, field trials were conducted during the 1994 and 1995 field seasons in Canada and during the 1995 field season in Europe. Disease and pest resistance and susceptibility were also recorded during observations of field releases in the United States in 1997 and 1998 (see Appendix 5 for complete termination reports). The results are summarized here.

V. e. i. 1994 Saskatchewan, Canada

Over the summer of 1994 (Saskatchewan, Canada), the following insects were observed feeding in both the transgenic and non-transgenic plots in the same numbers:

- Aphids (*Brevicoryne brassicae* L.), were observed during flowering and shortly thereafter.
- Honey bees (*Apis mellifera*)
- Leaf cutter bees (*Megachile rotunda*), and
- Cabbageworms (*Pieris* spp.), were observed in mid-August.

A constant population of various flies, wasps and mosquitoes in both the transgenic and non-

transgeic canola plots was observed. Populations were similar in the transgenic versus the non-transgenic plots. In 1994, the province-wide populations of flea beetles, Diamond-back moths, Blister beetles and *Lygus spp.* Were all low, so that it was not surprising that these were not present in the test plots.

V. e. ii. 1995 Saskatchewan, Canada

Over the summer of 1995 (Saskatchewan, Canada), the following insects were observed feeding in both the transgenic and non-transgenic plots in the same numbers:

- Aphids (*Brevicoryne brassicae* L.), were observed during flowering and shortly thereafter.
- Honey bees (*Apis mellifera*)
- Leaf cutter bees (*Megachile rotunda*), and
- Diamondback Moth larvae (*Plutella xylostella*)
- Bertha Armyworm (*Mamestra configurata*)
- Blister Beetles

Saskatchewan had a heavy infestation of Diamondback Moths and Bertha Armyworms during the 1995 season. These pests were present in high populations throughout the canola growing area of the province. The rate of infestation in the test plots was similar to that of the commercial canola growing areas.

V. e. iii. 1994 Belgium

Over the summer of 1995 (Belgium), the following insects were observed feeding in both the transgenic and non-transgenic plots in the same numbers:

- Fleabeetles (high infestation)
- Aphids (*Brevicoryne brassicae* L.), and
- Pollen Beetles.

The field plots were also periodically hosts to numerous populations of honey bees, flies and bumble bees.

During observation of transgenic and nontransgenic plots during these field seasons, no difference in disease and/or insect susceptibility was recorded. MS8 and RF3 plants, as well as their hybrid offspring were as susceptible, or as resistant as the nontransgenic control plants to different pathogenic agents. Pest resistance variability was more significant between the different oilseed rape cultivars than the variability observed within the transgenic and nontransgenic counterpart.

V. e. iii. 1997 and 1998 United States

During the growing seasons of 1997 and 1998, insect observations were made during field releases of MS8/RF3 plants in the major canola growing regions of the United States. No incidences of fleabeetles or diamondback moths were observed. This is due in large part to the application of Counter at seeding. Honeybees were observed at all test sites with the same population numbers in both the transgenic and nontransgenic parent plots. See Appendix 5 for complete termination reports of 1997 and 1998.

During observation of transgenic and nontransgenic plots during these field seasons, no difference in disease and/or insect susceptibility was recorded. MS8 and RF3 plants, as well as their hybrid offspring were as susceptible, or as resistant as the nontransgenic control plants to different pathogenic agents. Pest resistance variability was more significant between the different oilseed rape cultivars than the variability observed within the transgenic and nontransgenic counterpart.

V. f. Characteristics of MS8/RF3 hybrid canola expressing glufosinate-ammonium resistance and current uses of herbicides on canola

Canola acreage in the United States has grown exponentially in the last few years. In 1997 approximately 733,000 acres of canola were planted in the U.S., with 590,000 being planted in North Dakota; 120,000 in Minnesota, and 13,000 in Montana, Idaho and Washington. Georgia planted close to 2000 acres of winter oil seed rape (USCA, 1998).

The recommended label application rate of glufosinate-ammonium to glufosinate-ammonium resistant canola is of 20-34 oz./acre, allowing 68 oz./season in 1-2 applications to control many of the major weeds such as, green and yellow foxtail, wild oats, kochia, redroot pigweed, common lambsquarters and wild buckwheat in canola. These weeds can significantly decrease canola yields. Non glufosinate-ammonium resistant canola is still very susceptible to treatment with glufosinate-ammonium, and glufosinate-ammonium resistant canola is highly susceptible to treatment with other herbicides in the phenoxy class, glyphosate and sulfonyl ureas. Products currently registered for use on canola are Treflan® (trifluralin), Assure® II (quizalofop), Poast® (sethoxydim), Stinger® (Section 18 registration in 1997-1999, clopyralid-monoester salt) and Herbicide 273® (endothall)[3]. Treflan is effective on yellow and green foxtail, and redroot pigweed, widely considered the most prevalent weeds in canola. Treflan is also used a preplant herbicide. Stinger is effective on Canada thistle and perennial sowthistle (considered noxious weeds in North Dakota). All products currently registered for use on canola are for control of primarily grassy weeds. Glufosinate-ammonium is effective on both grass weeds and broadleaf

[3] Treflan®and Stinger®are registered trademarks of Dow AgroSciences, Inc. Assure® II is a registered trademark of the E.I. DuPont de Nemours Company. Poast® is a registered trademark of BASF, Inc. Herbicide 273® is a registered trademark of Elf Atochem, Inc.

weeds such as foxtails and kochia. Glufosinate-ammonium will also provide control of trifluralin resistant foxtail which is becoming an increasing weed pest in canola in North Dakota and Minnesota. Glufosinate-ammonium will give growers a 'wait and see option' of herbicide application. With glufosinate-ammonium, a grower can plant his canola and wait to see which weeds emerge before making 1-2 'over the top' applications of glufosinate-ammonium. This capability could decrease the need for preplant hebicides.

VI. Environmental Consequences of the Introduction of MS8/RF3 Canola

VI. a. MS8RF3 hybrid canola expressing glufosinate-ammonium resistance

Environmentally desirable features of the introduction of glufosinate-ammonium resistant canola:

- Could aid in development of minimum-till practices which could result in reduced soil erosion
- Less drift of herbicide onto adjacent fields than other products currently used in canola
- Glufosinate-ammonium is less likely to lead to the development of resistant weeds than other herbicides currently used
- The introduction of glufosinate-ammonium is compatible with Integrated Pest Management Strategies, allowing growers to apply on an as-needed basis and could lead to reduced use of preemergence herbicides
- Glufosinate-ammonium is quickly degraded to CO_2 and H_2O in soil and water; there are no carryover issues.

VI. a. i. Appearance of glufosinate-ammonium resistant weeds

Glufosinate-ammonium has been used on hundreds of thousands of acres (AgrEvo have no firm numbers) in Europe for well over 10 years in the formulated product Basta® as a broad-spectrum non-selective weed control tool. As of today, no reports of development of resistance to glufosinate-ammonium resistance have been reported.

VI. a. ii. Weediness of MS8/RF3

MS8/RF3 canola, expressing the PAT enzyme and thus conferring resistance to the otherwise nonselective herbicide glufosinate-ammonium, will not present any increased potential for weediness than its parent nontransgenic canola. In this petition AgrEvo/PGS have submitted evidence to indicate a lack of weedy nature of this transformed canola under agricultural conditions. Seed germination, seed production, pest and disease resistance characteristics, seed dormancy and sensitivity to herbicides other than glufosinate-ammonium are the same for MS8/RF3 as for nontransgenic canola. "Canola, *B. napus* is not considered a weed in the United States, despite its ability to volunteer and escape from cultivated fields. *B. napus* is not listed as a weed in Weed Science Society of America. The environmentally relevant introduced trait, resistance to glufosinate-ammonium, is unlikely to increase weediness of this canola unless glufosinate-ammonium is the only alternative for control of the plant. Such an alteration, because it does not confer any pest resistance or alter reproductive biology or change any physiology related to survival, does not confer a competitive advantage favoring the canola plants over

unmodified varieties. To increase weediness of the canola plant there would have to be selection pressure on glufosinate-ammonium resistant canola (Tiedje et al., 1989; Office of Technology and Assessment, 1988) (USDA, 1988)." In termination reports of 1997 (97-035-05r) and 1998 (98-064-38n, 98-064-35n, 98-064-33n, 98-164-04n, 98-064-31n and 98-119-01r) AgrEvo has presented data to show that MS8/RF3 canola is as readily controlled with non-glufosinate-ammonium herbicides as is nontransformed canola (see Appendix 5). The expression of the *barstar* and *barnase* genes result in fertility restoration and weediness assessment given in the preceding sentences applies to the expression products of these genes as they would for any other fertile canola.

VI. a. iii. Vertical transfer of the introduced genes

"Whereas intra-specific crosses between *B. napus* cultivars occur readily, interspecific crosses between *B. napus* and related species occur with varying degrees of success and are influenced greatly by the direction of the cross. Even where there is a possibility of the hybridization between *B. napus* and a related species growing in the vicinity of a release poor vigor and high sterility in the hybrids will generally mean that hybrids and their progeny will not survive in either an agricultural or natural habitat. (Scheffler and Dale, 1994) (USDA, 1998)."

"The potential of a gene movement, at a very low level, from *B. napus* to other *Brassica spp.* Such as *B. juncea* or *B. rapa*, will be subject to the availability of the target organism and the reduced fertility of the hybrids. *B. napus* can cross with *B. rapa* (under co-cultivation 1.3% hybrid seed was formed) and produce hybrids of much reduced fertility (MacDonald, R., 1994). *B.napus* can also cross at low frequency with *B. juncea* (under field co-cultivation 4.7% hybrid seed formed) and these hybrids can produce a small amount of seed and fertile progeny (Bing, 1991) (USDA, 1998)." Should gene transfer occur, the possibly integrated *barstar-bar* and *barnase-bar* gene constructs will not give any fitness advantage due to the lack of selection pressure for these expressed traits in nature.

"Gene movement is also possible to other members of the *Brassicaceae*, e.g. *Herschfeldia incana* (*Brassica adpressa*) and *Raphanus raphanistrum*. Gene movement is at extremely low levels, and as with members of the genus *Brassica*, it is unlikely that the gene that codes for glufosinate-ammonium tolerance would confer competitive advantage in these species unless glufosinate-ammonium is used for control." (USDA, 1998)

VI. a. iv. Horizontal transfer of the introduced genes

Movement of transgenes from genetically engineered plants to microorganisms has been suggested as a risk if such plants are released into the environment. As initially stated in the USDA's Interpretive Ruling on Calgene, Inc. Petition for Determination for Nonregulated Status of FLAVR SAVR®Tomato (USDA, 1992), and subsequently repeated in other USDA determination documents, "There is no published evidence for the existence of any mechanism, other than sexual crossing" by which genes can be transferred from a plant to other organisms.

As summarized in these determination documents, evidence suggests that, based on limited DNA homologies, transfer from plants to microorganisms may have occurred in evolutionary time over many millennia. Even if such transfer were to take place, transfer of the bar gene to a microbe would not pose a plant pest risk. Genes encoding both PAT enzymes and acetyl transferases are found in microbes in nature. Moreover, the naturally occurring *bar* gene is derived from a soil microbe (*S. hygroscopicus*).

VI. a. v. Potential impact on nontarget and beneficial organisms

In all field trials of MS8 and RF3 canola and their hybrid, MS8/RF3, in Canada in Europe, no deleterious or harmful effects on beneficial organisms such as honeybees or earthworms were observed. Moreover, there is no reason to anticipate any harmful effects or significant impact of MS8/RF3 canola on beneficial organisms or endangered or threatened species due to the agricultural cultivation of MS8/RF3. "Neither the PAT enzyme or the *bar* gene exhibit any toxic properties" (USDA, 1998).

VI. a. vi. Impact of agricultural use of MS8/RF3 canola expressing glufosinate-ammonium resistance outside the United States

In their EA of Petition 97-205-01p, Petition for Determination of Nonregulated Status: Glufosinate Tolerant Canola, Transformation Event T45, APHIS concluded that, "(we) (have) not identified any impacts on the environment that might be relevant to glufosinate-(ammonium) tolerant canola or follow from the unconfined cultivation of these canola lines in the United States and its territories, or abroad. In addition to the assurance provided by the analysis leading APHIS to a finding of no significant impact for the introduction of this canola, it should be noted that all the considerable, existing national and international regulatory authorities and phytosanitary regimes that currently apply to introductions of new canola cultivars internationally apply equally to those covered by this determination" (USDA, 1998). This same conclusion applies to MS8/RF3 canola that expresses resistance to glufosinate-ammonium.

VII. Adverse Consequences of the Introduction of InVigor Hybrid MS8RF3 Canola

No evidence or data has been demonstrated which would indicate any adverse consequences to the environment, humans or livestock by the introduction of MS8/RF3 canola that expresses resistance to glufosinate-ammonium.

VII. Statement of Grounds Unfavorable

No unfavorable information and data has been demonstrated for MS8/RF3 glufosinate-ammonium resistant canola.

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IX. Appendicies

1. Decision Documents regarding MS8/RF3 Canola from FDA, Agriculture Canada, and Health Canada

2. Outcrossing of *B. napus* to sexually compatible relatives: Data from States and Literature

3. Methodologies for Northern Analyses for Bar, Barnase and Barstar

4. Methodology for Determination of Level of PAT Expression

5. Termination Reports for 1997 and 1998 U.S. Field Releases

Appendix 1: Decision Documents regarding MS8/RF3 Canola from FDA, Agriculture Canada, and Health Canada

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

SEP 16 1998

Vickie Forster
AgrEvo USA Company
2711 Centerville Road
Wilmington, DE 19808

Dear Ms. Forster:

This is in regard to AgrEvo's consultation on genetically modified canola that you initiated with the Agency on May 29, 1998, specifically transformation events MS8 and RF3. According to AgrEvo, the canola line MS8 has been modified to express the male sterile gene (barnase) and the herbicide glufosinate-ammonium resistance gene (bar). MS8 is used to produce F1 hybrids. The canola line RF3 has been modified to contain the fertility restorer gene (barstar) and the herbicide glufosinate-ammonium resistance gene (bar). Upon crossing MS8 with RF3, the fertility of the oilseed rape progeny will be restored. The use of these two lines allows for the production of seed that is 100% hybrid, 100% fertile, and 100% glufosinate tolerant.

You submitted a summary of your safety and nutritional assessment of the AgrEvo hybrid canola containing transformation events MS8 and RF3. These communications informed FDA of the steps taken by AgrEvo to ensure that this product complies with those legal and regulatory requirements that fall within FDA's jurisdiction. Based on the safety and nutritional assessment you have conducted, it is our understanding that AgrEvo has concluded that the canola lines are not materially different in composition, safety, or other relevant parameters from canola currently on the market, and that they do not raise issues that would require premarket review or approval of FDA. All materials relevant to this consultation have been placed in a file that has been designated BNF 0057 and will be maintained by the Office of Premarket Approval.

Based on the information AgrEvo has presented to FDA, we have no further questions concerning the AgrEvo hybrid canola containing transformation events MS8 and RF3 at this time. However, as you are aware, it is AgrEvo's continued responsibility to ensure

that foods the firm markets are safe, wholesome, and in compliance with all applicable legal and regulatory requirements.

Sincerely yours,

/ Laura M. Tarantino /for

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

United States
Department of
Agriculture

Marketing and
Regulatory
Programs

Animal and
Plant Health
Inspection
Service

4700 River Road
Riverdale, MD 20737

Ms. Vickie Forster, Registration Specialist
AgrEvo USA Company
Little Falls Centre One
2711 Centerville Road
Wilmington, DE 19808

SEP 23 1998

Dear Ms. Forster:

I am writing in response to your letter of June 26, 1998, in which you requested an opinion on the regulatory authority of Animal and Plant Health Inspection Service (APHIS) with respect to your company's male sterile canola (*Brassica napus*) varieties derived from transformation events MS8/RF3. As you describe in you letter, transformation events MS8 and RF3 each have been transformed with the glufosinate herbicide tolerance gene (*bar*, encoding phosphinothricin acetyltransferase) which serves as a selectable marker gene. In addition, transformation event MS8 has been engineered with the *barnase* gene which confers male sterility (non-viable pollen). Transformation event RF has been engineered with the *barstar* gene which restores fertility.

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, for the express purpose importation for processing, APHIS will not consider the varieties derived from transformation events MS8/RF3, and progeny canola seed, imported into the United States 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 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 Agriculture 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 notes the copies of correspondence included with your letter which indicate that the U.S. Food and Drug Administration, Office of Premarket Approval, Center for Food Safety and Applied Nutrition, has completed their review of these canola lines and concurred that they do not raise issues that would require a premarket review.

5. APHIS 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.

6. The three new traits introduced into these two canola lines: *barnase* (encoding a ribonuclease), *barstar* (encoding a highly specific inhibitor of *barnase*), and *bar* (encoding phosphinothricin acetyltransferase) have not altered the canola's plant pest risk potential. The use of phosphinothricin acetyltransferase, which confers tolerance to the herbicide glufosinate, 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.

I 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 under APHIS regulations found under 7 CFR Part 340. This opinion includes canola transformation events MS8 and RF3 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 other than processing, 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. David S. Heron at Area Code (301) 734-5141.

Sincerely,

Arnold Foudin
for

Rebecca Bech
Assistant Director
Scientific Services
Operational Support
Plant Protection and Quarantine

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

Tunney's Pasture
Ottawa, Ontario
K1A 0L2

March 12, 1997

Ms. Barb Fowler
Regulatory Affairs Manager
Plant Genetic Systems (Canada) Inc.
104-111 Research Drive
Saskatoon, Saskatchewan
S7N 3R2

Dear Ms. Fowler:

This will refer to the Novel Food Submission concerning transgenic canola (*Brassica napus*) lines derived from a new hybridization system, MS8 (male sterility) and RF3 (fertility restorer). Officers of the Health Protection Branch have reviewed the information that Plant Genetic Systems (Canada) Inc. provided for assessment of the acceptability of oil from these canola lines for sale as human food in Canada.

According to the submitted information, the procedure used in developing the subject MS8 male sterile line involved the introduction of the *barnase* gene, encoding a specific extracellular ribonuclease (RNase), from the bacterium *Bacillus amyloliquefaciens*. The subject RF3 fertility restorer line was developed by the introduction of the *barstar* gene, encoding the specific inhibitor of the barnase enzyme, from the bacterium *Bacillus amyloliquefaciens*. In addition, the *bar* gene, which codes for the enzyme phosphinothricin acetyl transferase (PAT) resulting in tolerance to phosphinothricin, and originally isolated from the soil organism *Streptomyces hygroscopicus* is included in both lines as a means of detecting transformed plants. A cross of the MS8 and RF3 lines results in fully fertile progeny with 100% hybrid seed and which contain the following novel constituents:

.../2

Canada

(1) the *barnase* gene;
(2) the specific extracellular ribonuclease enzyme encoded by the *barnase* gene;
(3) the *barstar* gene;
(4) the specific inhibitor of the barnase enzyme encoded by the *barstar* gene;
(5) the *bar* gene; and,
(6) the enzyme phosphinothricin acetyl transferase which is encoded by the *bar* gene.

Based on our evaluation of the submitted data, we have no objection to the sale of refined canola oil from canola lines derived from the hybridization system involving the MS8 and RF3 lines described in the notification as human food in Canada.

It should be noted that this opinion is solely with respect to the suitability for sale as human food of refined canola oil from lines derived from the subject hybridization system. It is the continuing responsibility of Plant Genetic Systems (Canada) Inc. to ensure that its products are in compliance with all applicable statutory and regulatory requirements.

Please note that we are providing our colleagues in Agriculture and Agri-Food Canada (AAFC) with a copy of this letter in regard to that Department's responsibility respecting variety registration, animal feeds, environmental release and labelling issues. We are also providing our colleagues in the Pest Management Regulatory Agency (PMRA) with a copy of this letter for their information.

Yours truly,

George M. Paterson

George M. Paterson, Ph.D.
Director General
Food Directorate

c.c. Dr. A. MacKenzie, AAFC
Dr. C. Franklin, PMRA

Agriculture and Agri-Food Canada
Food Production and Inspection Branch
Plant Products Division

Agriculture et Agroalimentaire Canada
Direction générale, Production et inspection des aliments
Division des produits végétaux

Decision Document DD96-17

Determination of Environmental Safety of Plant Genetic Systems Inc.'s (PGS) Novel Hybridization System for Rapeseed (*Brassica napus* L.)

This Decision Document has been prepared to explain the regulatory decision reached under the guidelines Dir94-08 *Assessment Criteria for Determining Environmental Safety of Plants with Novel Traits* and its companion document Dir94-09 *The Biology of* Brassica napus *L. (Canola/Rapeseed)* and the guidelines Dir95-03 *Guidelines for the Assessment of Livestock Feed from Plants with Novel Traits.* Agriculture and Agri-Food Canada (AAFC), specifically the Plant Biotechnology Office of the Plant Products Division, with input from the Plant Health Risk Assessment Unit, has evaluated information submitted by Plant Genetic Systems Inc. (PGS). This information is in regard to a rapeseed hybridization system comprising two transgenic parental lines, MS8 and RF3, and their hybrid MS8 x RF3. AAFC has determined that these plants with novel traits do not present altered environmental interactions or pose concerns for the safety of livestock consuming feed derived from the PNT when compared to currently commercialized rapeseed varieties in Canada.

Unconfined release into the environment and use as livestock feed of MS8, RF3 and MS8xRF3 is therefore authorized. Any other *B. napus* lines and intra-specific hybrids resulting from the same transformation events, and all their descendants, may also be released, provided no inter-specific crosses are performed, provided the intended use is similar, provided it is known following thorough characterization, that these plants do not display any additional novel traits and provided that the resulting lines can be shown to be substantially equivalent to currently grown rapeseed, in terms of their potential environmental impact and livestock safety.

Please note that, while determining the environmental and livestock feed safety of plants with novel traits is a critical step in the commercialization of these plant types, other requirements still need to be addressed, such as the evaluation of food safety (Health Canada) and Variety Registration (AAFC).

(publié aussi en français)

December 2, 1996

This bulletin is published by the Plant Products Division, Agriculture and Agri-Food Canada. For further information, please contact the Plant Biotechnology Office or the Feeds Section at:

Plant Products Division
Food Production and Inspection Branch
59 Camelot Drive
Nepean, Ontario K1A 0Y9

Telephone: (613) 952-8000
Facsimile: (613) 992-5219

Table of Contents

I. Brief Identification of Plants with Novel Traits (PNT's)

Designation(s) of the PNT's: Male sterile line: MS8 (DBN230-0028)
Fertility restorer: RF3 (DBN212-0005)
Hybrid line: MS8 x RF3

Applicant: Plant Genetic Systems (Canada) Inc. (PGS)

Plant Species: *Brassica napus* L.

Novel Traits: **MS8:** male sterility; glufosinate ammonium (herbicide) tolerance

RF3: fertility restoration; glufosinate ammonium (herbicide) tolerance

Trait Introduction Method: *Agrobacterium tumefaciens*-mediated transformation

Proposed Use of PNT's: Production of *B. napus* for seed oil for human consumption and seed oil and meal for livestock feed. These materials will not be grown outside the normal production area for canola in Canada.

II. Background Information

Plant Genetic Systems Inc. has developed a novel *B. napus* oilseed rape hybridization system. This system, derived from the *B. napus* variety "Drakkar," involves the use of two parental lines. The first parental line (MS8) is male sterile; does not produce viable pollen grains; and cannot self-pollinate. The second parental line (RF3) codes for specific restoration of the male sterility coded by the first parental line. When the two lines are crossed, the progeny is one hundred per cent true hybrid, and since fertility is restored, the hybrid plants are fully fertile and produce seed. These lines are similar to the lines MS1 and RF1 authorized for unconfined release by AAFC on April 28 1995, as explained in Decision Document DD95-04.

To date, attempts to develop hybridization systems in oilseed rape by traditional methods have not been completely successful for commercial applications. Potential benefits are that F_1 hybrids of oilseed rape are estimated to potentially yield 20-25% more than open-pollinated varieties, and their uniformity facilitates harvesting and marketing.

The development of the MS8 and RF3 lines was based on recombinant DNA technology by the introduction of bacterial genes into the *B. napus* variety "Drakkar."

Nuclear male sterility of the first parental line results from the localized production of an RNAse (*barnase*) in a specific anther cell layer, and at a specific stage in anther development. Fertility restoration in the hybrid line is obtained through insertion, in the second parental line of a gene coding for *barstar*, a specific inhibitor of the enzyme *barnase*. A gene conferring tolerance to the herbicide glufosinate ammonium was inserted in both lines, coding for phosphinothricin acetyltransferase, an enzyme that inactivates glufosinate ammonium through acetylation. Herbicide tolerance was introduced as a field selection trait to obtain 100% hybrid seed.

These materials have been field tested in Canada under confined conditions in Saskatchewan (1994-96), Alberta (1996), Manitoba (1995, 96) and Ontario (1996).

PGS has submitted data to AAFC on the identity of each of MS8, RF3 and MS8xRF3: detailed descriptions of the modification method, data and information on the inserted DNA and the gene insertion site, the role of the inserted genes in donor organisms, the role of regulatory sequences in donor organisms, their molecular characterization and full nucleotide sequences.

The novel proteins were identified and characterized, including their potential toxicity to non-target organisms, potential for allergenicity, and levels of expression in the plant. A number of relevant scientific publications were referrenced.

Agronomic characteristics such as seed dormancy, vegetative vigour, seed production, time to maturity, flowering period, male and female fertility, and disease and insect susceptibilities were compared to those of unmodified *B. napus* counterparts.

The Plant Biotechnology Office of the Plant Products Division, AAFC, has reviewed the above information, in light of the assessment criteria for determining environmental safety of plants with novel traits, as described in the regulatory directive Dir94-08:

- potential of the PNT's to become weeds of agriculture or be invasive of natural habitats,
- potential for gene flow to wild relatives whose hybrid offspring may become more weedy or more invasive,
- potential for the PNT's to become plant pests,
- potential impact of the PNT's or their gene products on non-target species, including humans, and
- potential impact on biodiversity.

The feed section of the Plant products Division, AAFC, has also reviewed the information submitted by PGS in light of the assessment criteria for determining safety and efficacy of livestock feed, as described in the regulatory directive Dir95-03 *Guidelines for the Assessment of Livestock Feed from Plants with Novel Traits:*

- potential impact on livestock, and
- potential impact on livestock nutrition.

III. Description of the Novel Traits

1. Nuclear Male Sterility

- The male sterility gene encodes the barnase ribonuclease (RNAse). Male sterility is caused by the production of this enzyme at a specific stage during anther development in the tapetum cell layer of the anther. The RNAse affects RNA production, disrupting normal cell functioning and arresting early anther development.

- The gene is linked to an anther-specific promoter, and the enzyme was detected only in early stages of development of the tapetum cell layer of anthers. It was not detected in other plant tissues.

- The *barnase* gene was isolated from *Bacillus amyloliquefaciens*, a common soil bacterium frequently used as a source for industrial enzymes. The enzyme is therefore naturally occurring in the soil. More generally, ribonucleases are very commonly found in various organisms including bacteria and plants.

- The full nucleotide sequence of the gene was provided. Barnase is a small single-domain protein, containing no disulfide bonds, metalion cofactors or other non-peptide components. It unfolds completely into an inactive form when heated. When subjected to comparative analyses using a database of polypeptide sequences, the enzyme amino acid sequence did not show significant homology with other proteins present in the database other than bacilli ribonucleases. No resemblance with potential toxins or allergens was observed.

- The gene and its associated regulatory sequences are the same as those of the line MS1 that was authorized for unconfined release by AAFC on April 28, 1995 (please see DD95-04).

2. Fertility Restoration

- The fertility restoration gene codes for the barstar enzyme. This enzyme is a ribonuclease inhibitor and specifically inhibits the barnase RNAse. Barnase and its inhibitor barstar form a one-to-one complex, in which the RNAse is inactivated. The barnase-barstar complex is very stable in the absence of a denaturant and the inhibition is very specific.

- The *barstar* gene was isolated from *Bacillus amyloliquefaciens*, a common soil bacterium frequently used as a source for industrial enzymes. The enzyme is therefore naturally occurring in the soil. More generally, ribonuclease inhibitors are very commonly found in various organisms including bacteria and plants.

- The gene is linked to an anther-specific promoter, and the enzyme is only produced at a specific stage during anther development in the tapetum cell layer of the anther. It was not detected in other plant tissues.

- The full nucleotide sequence of the gene was provided. Barstar is a small single-domain protein that unfolds completely into an inactive form when heated. When subjected to comparative analyses using the FASTDB algorithm of Intelligenetics with three databases of polypeptide sequences, the enzyme amino acid sequence did not show significant homology with other proteins present in the databases. No resemblance with potential toxins or allergens was observed.

- The gene and its associated regulatory sequences are identical to those of the line RF1 that was authorized for unconfined release by AAFC on April 28, 1995 (please see DD95-04).

3. Glufosinate Ammonium Tolerance

- Phosphinothricin (PPT), the active ingredient of glufosinate ammonium, inhibits glutamine synthetase, which results in the accumulation of lethal levels of ammonia in susceptible plants within hours of application.

- The phosphinothricin tolerance gene engineered into MS8 and RF3 codes for PPT-acetyltransferase (PAT). This enzyme detoxifies phosphinothricin by acetylation into an inactive compound. PAT has extremely high substrate specificity for L-PPT and dimethylphosphinothricin (DMTT), but cannot acetylate L-PPTs analog L-glutamic acid, D-PPT, nor any protein amino acid. Expression levels of PAT varied from 0.04 mg/g (f.w.) of protein in seeds, to 1.80 mg/g (f.w.) of protein in leaves.

- The gene was isolated from *Streptomyces hygroscopicus*, an aerobic soil actinomycete. The PAT enzyme is therefore naturally occurring in the soil. More generally, acetyltransferases are ubiquitous in nature.

- A plant derived coding sequence expressing a chloroplast transit peptide was co-introduced with the gene. This peptide facilitates the import of the newly translated enzyme into chloroplasts. The PAT enzyme was detected in leaves, but not in flower buds or seeds.

- The nucleotide sequence of the gene was provided. When subjected to comparative analyses using the FASTDB algorithm of Intelligenetics with three databases of polypeptide sequences, the enzyme amino acid sequence did not show significant homology with other proteins present in the databases, except with other phosphinothricin acetyltransferases originating from different organisms. No resemblance with potential toxins or allergens was observed.

- The gene and its associated regulatory sequences were identical to those of the lines MS1 and RF1 that were authorized for unconfined release by AAFC on April 28, 1995 (please see DD95-04).

4. Development Method

- *Brassica napus* cultivar "Drakkar" was transformed using a disarmed non-pathogenic *Agrobacterium tumefaciens* vector. The vector contained the transfer DNA (T-DNA) region of an *Agrobacterium* plasmid from which disease-causing genes were removed and replaced with the genes of interest. The T-DNA portion of the plasmid is known to insert randomly into the plant's genome and the insertion is usually stable, as was shown to be the case in MS8 and RF3.

5. Stable Integration into the Plants' Genomes

- The data provided clearly showed that there was no incorporation of any coding region from outside the T-DNA borders and that only one copy was integrated at a single insertion site.

- The insertion site was very well characterized and determined to be located in the *B. oleracea* portion of the amphidiploid *B. rapa/B. oleracea* genome of *B. napus* for RF3, and in the *B. rapa* genome of *B. napus* for MS8.

- Segregation was predictable over all generations observed and showed that transformation resulted in integration at one single dominant locus.

- Comparisons between the original transformants and derived lines several generations away from these transformants show no difference in the presence and expression of the genes nor in the insertion site.

IV. Assessment Criteria for Environmental Safety

1. Potential of the PNT's to Become Weeds of Agriculture or be Invasive of Natural Habitats

AAFC has evaluated data submitted by PGS on the reproductive and survival biology of MS8, RF3 and resulting hybrids. It was determined that germination, vegetative vigour, flowering period, time to maturity and seed production of both transgenic lines were within the normal range of expression of characteristics in unmodified *B. napus* counterparts. These lines have no specific added genes for cold tolerance or winter survival. Flowers of the MS8 line have undeveloped anthers, slightly smaller petals and do not produce fertile pollen, but nectar production remains unchanged and normal insect pollination was observed. Seed morphology, size, and average seed weight did not change, indicating that seed dispersal potential was not altered.

Based on the submitted information, AAFC has determined that MS8, RF3 and MS8xRF3 did not show any change in resistance or susceptibility to major *B. napus* pests and pathogens (e.g., blackleg, sclerotinia, flea beetles, diamondback moth larvae). The lines were tested in several countries, and showed no differences in agronomic performance when compared to unmodified counterparts under the same conditions.

The biology of *B. napus*, described in Dir94-09, shows that unmodified plants of this species are not invasive of unmanaged habitats in Canada. Information provided by PGS shows that MS8, RF3 and their hybrids were not different from their counterparts in this respect. Published data showed that seed survival of similar transgenic *B. napus* seeds expressing kanamycin resistance and glufosinate ammonium tolerance was significantly lower than seed survival of unmodified counterparts, when seeded at a variety of unmanaged locations. Glufosinate ammonium is not used in normal crop rotation cycles, and resistance is therefore not an issue of concern in weed management control. Glufosinate ammonium resistant *B. napus* volunteer plants can easily be managed by mechanical means and other available chemicals used to control *B. napus*.

The above considerations, together with the fact that the novel traits have no intended effect on weediness or invasiveness, led AAFC to conclude that MS8, RF3 and their hybrid progeny have no altered weed or invasiveness potential compared to currently commercialized *B. napus* varieties.

Note: A longer term concern, if there is general adoption of several different crop and specific herbicide weed management systems, is the potential development of crop volunteers with a combination of novel resistances to different herbicides. This could result in the loss of the use of these herbicides and any of their potential

benefits. Therefore, agricultural extension personnel, in both the private and public sectors, should promote careful management practices for growers who use these herbicide tolerant crops to minimize the development of multiple resistance.

2. **Potential for Gene Flow to Wild Relatives Whose Hybrid Offspring May Become More Weedy or More Invasive**

The MS8 line is male sterile and will therefore not pollinate any other plants. Although these plants can act as pollen recipients, their progeny will also be male sterile and will not produce pollen. The RF3 and hybrid plants displayed normal reproductive characteristics. *Brassica napus* plants are known to outcross up to 30% with other plants of the same species, and potentially with plants of the species *B. rapa*, *B. juncea*, *B. carinata*, *B. nigra*, *Diplotaxis muralis*, *Raphanus raphanistrum*, and *Erucastrum gallicum* (Dir 94-09). Studies show that gene flow is most likely to occur with *B. rapa*, the other major canola species, and an occasional weed of cultivated land especially in the eastern provinces of Canada.

The genes coding for male sterility and fertility restoration do not confer any ecological advantage to potential hybrid offspring of MS8 or RF3 plants. If glufosinate ammonium tolerant individuals arose through interspecific or intergeneric hybridization, the novel traits would confer no competitive advantage to these plants unless challenged by glufosinate ammonium. This would only occur in managed ecosystems where glufosinate ammonium is used for broad spectrum weed control, e.g., in the cultivation of plant cultivars developed to exhibit glufosinate ammonium tolerance and in which glufosinate ammonium is used to control weeds. As with glufosinate ammonium tolerant *B. napus*, these herbicide-tolerant individuals, should they arise, would be easily controlled using mechanical and other available chemical means. Hybrids, if they developed, could potentially result in the loss of glufosinate ammonium as a tool to control these species. This, however, can be minimized by the use of sound crop management practices.

The above considerations led AAFC to conclude that gene flow from the transgenic lines or their hybrids to canola relatives is possible, but would not result in increased weediness or invasiveness of these relatives.

3. **Altered Plant Pest Potential**

The intended effects of the novel traits are unrelated to plant pest potential and *Brassica napus* is not a plant pest in Canada (Dir94-09). In addition, agronomic characteristics, insect and disease susceptibilities, and qualitative and quantitative composition of MS8, RF3 and MS8xRF3 were shown to be within the range of values displayed by currently commercialized *B. napus* varieties, leading to the conclusion that plant pest potential was not inadvertently altered. AAFC therefore concurs with the conclusion that plant pest potential of these plants has not been inadvertently altered.

4. Potential Impact on Non-Target Organisms

The detailed characterization of each novel gene and resulting protein, as summarized in Part III of the present document, led to the conclusion that they do not result in altered toxicity or allergenicity properties. The *barnase* and *barstar* proteins are only produced in the tapetum cell layer of anthers at a specific developmental stage. Potential toxicity of these proteins was previously evaluated (please see DD95-04).

Based on the above, AAFC has determined that the unconfined release of the MS8, RF3 and their hybrid progeny will not result in altered impacts on interacting organisms, including humans, compared with currently commercialized counterparts.

5. Potential Impact on Biodiversity

The transgenic lines and their hybrids have no novel phenotypic characteristics which would extend their use beyond the current geographic range of canola/rapeseed production in Canada. Since potential outcross species are only found in disturbed habitats, transfer of novel traits would not have an impact on unmanaged environments.

AAFC has therefore concluded that the potential impact on biodiversity of MS8, RF3 and derived hybrids is equivalent to that of currently commercialized rapeseed lines.

V. Assessment Criteria for Use as Livestock Feed

1. Anti-nutritional Factors

Glucosinolate and erucic acid content of seed meal and oil of the transformed and hybrid lines was determined at several locations, representing a variety of conditions. The analysis revealed no differences in glucosinolate content in the meal, between the PNT's and the corresponding non-transformed line, at all sites. The reported values were also within the acceptable range for conventional canola, except at one location in Canada, where levels were elevated in both the PNT's and the corresponding control, as a result of drought induced stress. Erucic acid content of the oil was substantially equivalent to the non-transformed controls and within the acceptable range for conventional canola.

2. Nutritional Composition of PNT's

No statistical differences in the nutritional composition, i.e., crude protein, crude fat, crude fibre, ash and gross energy content, were noted between the whole seed, processed meal or oil, derived from MS8, RF3 and their resulting hybrids, when compared to the non-transformed controls or conventional canola cultivars. These results collectively demonstrate that the introduction of the novel traits into these lines and their presence in the resulting hybrids did not affect the composition or nutritional quality of the canola cultivar. Accordingly, MS8, RF3 and their hybrids are judged to be substantially equivalent to conventional canola varieties.

VI. Regulatory Decision

Based on the review of data and information submitted by PGS, and through comparisons of the transgenic lines with unmodified *B. napus* counterparts, AAFC has concluded that neither the novel genes nor their resulting gene products and associated novel traits confer any intended or unintended ecological advantage to either MS8, RF3 or MS8xRF3. Should these traits be transferred through outcrossing to related plants, these also would result in no ecological advantage.

Based on the review of data submitted to the Feed Section of the Plant Products Division, AAFC concludes that the novel genes introduced into lines MS8 and RF3 and their corresponding traits do not raise any concerns regarding livestock safety or the nutritional composition of this line. Canola oil and meal are currently listed in Schedule IV of the Feeds Regulations and are, therefore, approved for use in livestock feeds in Canada. As lines RF3, MS8 and their resulting hybrid have been assessed and found to be substantially equivalent to traditional canola varieties, these lines and their byproducts are considered to meet the present feed definitions and are approved for use as livestock feed ingredients in Canada.

If at any time, PGS becomes aware of any information regarding risk to the environment, or risk to animal or human health, that could result from release of these materials in Canada, or elsewhere, PGS must immediately provide such information to AAFC. On the basis of such new information, AAFC may re-evaluate the potential impact of the release and re-evaluate its decision.

Unconfined release into the environment and feed use of MS8, RF3 and MS8xRF3 is therefore authorized. Any other *B. napus* lines and intra-specific hybrids resulting from the same transformation events, and all their descendants, may also be released, provided no inter-specific crosses are performed, provided the intended use is similar, provided it is known that these plants do not display any additional novel traits and provided that the resulting lines can be shown to be substantially equivalent to currently grown rapeseed, in terms of their potential environmental impact and livestock feed safety.

Please note that, while determining the environmental an livestock feed safety of plants with novel traits is a critical step in the commercialization of these plant types, other requirements still need to be addressed, such as for the evaluation food safety (Health Canada) and Variety Registration (AAFC).

Appendix 2: Outcrossing of *B. napus* to sexually compatible relatives: Data from States and Literature

In the United States, in 1994, ten (10) states accounted for 98.3% of the total canola-planted acreage. These states and percentages were:

Alabama (2.2%)
Colorado (1.7%)
Georgia (5.1%)
Idaho (12.5%)
Minnesota (9.3%)
Montana (15.1%)
North Dakota (38.7%)
Oregon (3.5%)
South Dakota (1.6%)
Washington (8.6%)

In order to find out which weedy species in each of these ten states could present outcrossing concerns with canola, representatives from each of the ten states listed above were contacted. Following conversations with knowledgeable representatives from each of these states, AgrEvo has received the information given below about weeds/plants in each state with which *B. napus* could potentially outcross. Table 1 (Section II.) of the document summarizes weeds/plants that occur in the major (>1%) canola growing states of the U.S. and with which *B. napus* (canola) can outcross, their resulting hybrid fertility characteristics and literature references.

Alabama: On October 17, 1996, Dr. Glen Wehtje of Auburn University, Department of Agronomy and Soils, (334) 844-4100, informed AgrEvo that there are only two (2) weeds in Alabama which could interbreed with canola: wild mustard (*B. kaber* L.) and wild radish (*Raphanus raphanistrum* L.).

Colorado: On April 22, 1997, Dr. Duane Johnson of Colorado State University, Department of Soil and Crop Sciences, (970) 491-6517, informed AgrEvo that weeds in Colorado which could interbreed with canola are *B. nigra*, *B. juncea*, *B. rapa*, *B. hirta*, and *B. kaber*. None are exceptionally prevalent in Colorado with the exception of *B. nigra*.

Georgia: On October 17, 1996, Mr. Tom Kowalski, Director Entomology and Pesticide Division, Georgia Department of Agriculture, (404) 651-9486, informed AgrEvo that he knows of no weeds growing in Georgia which could outcross with *B. napus*.

Idaho: On April 22, 1997, Dr. Rogelio Vega of Division of Plant Industries, Idaho Department of Agriculture, (208) 332-8620, informed AgrEvo that although there are several *Brassica* species produced in Idaho, only wild mustard (*B. kaber* L.) is of concern. No plant/weed with which canola can interbreed is considered noxious in Idaho.

Minnesota: Charles G. Dale, Supervisor of the Seed and Noxious Weed Section of the Minnesota Department of Agriculture, (612) 296-6123, forwarded to AgrEvo the Minnesota Noxious Weeds Bulletin. As discussed in the overview, wild mustard (*B. kaber*), is the only species related to *B. napus* which is considered a weed.

Montana: On April 28, 1997, Dr. Barbara Mullen, Weed Specialist, Montana Department of Agriculture, Agricultural Sciences Division, (406) 444-2944, faxed a list of the wild *Brassica* species which are recognized as established in Montana and with which *B. napus* can outcross. Dr. Mullen verbally informed AgrEvo that the weed of greatest outcrossing concern is *B. kaber*.

North Dakota: On May 1, Dr. Bill Barker of the North Dakota State University Agronomy Department, (701) 231-7222, informed AgrEvo that wild *Brassica* species occurring in North Dakota with which *B. napus* can interbreed are wild mustard (*B. kaber*), wild radish (*Raphanus raphanistrum* L.), white mustard (*B. hirta*), Indian mustard (*B. juncea*), wild turnip (*B. campestris*) and black mustard (*B. nigra*). In addition Mr. Cliff Nygard, Burleigh County Weed Officer, North Dakota Department of Agriculture, forwarded the North Dakota Noxious Weed Law and Regulations which lists problematic weeds in North Dakota. There are no weeds on this list which have the potential to interbreed with canola.

Oregon: On April 28, 1997, Dr. Dan Ball, Hermiston Agriculture and Research Extension Center, (541) 278-4186, said that in Oregon the most prevalent weed and, therefore, the greatest concern for outcrossing with *B. napus* is wild mustard, *B. kaber*.

South Dakota: On May 8, 1997, Dr. Leon Reggie, South Dakota State University Agronomy Extension, (605) 688-4600, informed AgrEvo that the weed/plant species which present the greatest outcrossing concern with *B. napus* is wild mustard (*B. kaber*).

Washington: On June 6, 1997, Tom Wessells, State Pathologist, Plant Services Division, Washington Department of Agriculture, (509) 786-9275, informed AgrEvo that weedy species occurring naturally in Washington with which *B. napus* could outcross are wild mustard (*B. kaber*), white mustard (*B. hirta*) and *B. rapa*.

California: On April 28, 1997, Dr. Steve Kafka, (916) 752-8108, told AgrEvo that several wild mustards and radishes occur in California.

Although California grows <1% of the total canola acreage for production in the United States (336 acres in 1994), California does grow other *Brassica* species, such as *B. olrecea* in agriculturally managed areas for crop production, and does grow canola for seed production. Therefore experts in California were consulted regarding the possible impact of *B. napus* to outcross with relatives in California.

Following are synopses from literature regarding the potential for outcrossing to and gene introgression, and their subsequent consequences, into the species listed in Table 1 (Section II) of the document.

Brassica napus

MacDonald, R., 1996, Glufosinate Tolerant Canola: (N-acetyl-L-phosphinothricin: metabolic product) Canola Lines pHoe 4/Ac. Environmental Safety Assessment Background Volume 1, Basis for Selectivity.

Self-pollination characteristics of T45 canola (*B. napus*) were no different than self-pollination of nontransgenic canola varieties. Findings of low outcrossing (0.6% beyond 4 m) were observed under field conditions.

Brassica rapa* syn. *Brassica campestris

Jørgensen, Rikke and Bente Andersen, 1994, Spontaneous Hybridization between Oilseed Rape (*Brassica napus*) and Weedy *B. campestris* (*Brassicaceae*): A risk of growing genetically modified oilseed rape, Am. J. 81, 1620-1624.

Research completed in Denmark has shown that under field conditions, where *B. rapa* has long been cultivated, that it has become a persistent weed because proper weed management practices have not been followed. *Brassica rapa* is not grown commercially in the U.S. due to lower yields and its tendency to cultivate weed banks due to a prolonged seed dormancy. AgrEvo/PGS have no plans to introduce a transgenic *B. rapa* hybrids into the U.S. for commercial canola production due to the associated commercial disadvantages in comparison with *B. napus*. In *B. napus* production, the introgression of herbicide tolerant genes does occur where the two species are in close proximity and flowering periods overlap. This is not a surprising result, since these two species have been shown to outcross and produce hybrids of <10% fertility. (Bing et al., 1991).

Indian/brown mustard (*Brassica juncea*)

Calgene, Inc., 1994, Petition for Determination of Nonregulated Status for Laurate Canola (*Brassica napus*).

"*B. napus* is capable of acting as the pollen donor in crosses with *B. juncea*, cultivated as Indian or brown mustard although fertility of the hybrids is less than 10% (Bing, 1991; Dhillon et al., 1985; Heyn, 1977; Roy, 1980). Under field conditions in western Canada with *B. napus* and *B. juncea* interplanted, an average of 4 hybrid seed per plant (4.7% of seeds tested) were produced on the maternal *B. juncea* plants. Many of these F1 plants were completely infertile and produced no seed, 50% produced only 5 seed, 10% produced up to 25 seed and the remainder produced intermediate amounts of seed (6 to 15 seed per plant) under open pollinating conditions

in a greenhouse (Bing, 1991). Using herbicide tolerant *B. napus* as the pollen parent, 0.3% and 0.1% of seed were hybrid in two years of field trials. Fertility of the hybrids was very low, but actual values were not given (Bing, 1991). The distribution of naturalized *B. juncea* is sparse (although widespread) throughout temperate North America." (Calgene, 1994)

No published reprints of natural field hybrids being formed were found.

black mustard (*Brassica nigra*)

Calgene, Inc., 1994, Petition for Determination of Nonregulated Status for Laurate Canola (*Brassica napus*).

"Crosses (of *B. napus*) with *B. nigra* under field conditions produced either no hybrids (Baranger, et al., 1992) or were produced in very low numbers and were male sterile (Bing, 1991)." (Calgene, 1994).

Brown, A.P. Brown, J. Thill, D. C., Brammer, T. A., Nair, H.S., 1995, Gene Transfer between Canola (*Brassica napus* and *Brassica campestris*) and related weed species. Proceedings GCIRC 9th International Rapeseed Congress, Cambridge, 4, 1040-1043.

Brown et al. (1995) attempted crosses in the greenhouse to wild mustard (*Sinapis arvensis*, syn. *B. kaber*) and black mustard (*B. nigra*) pollinating immature buds with pollen from glufosinate tolerant canola. No fertile hybrids were made, however the authors proposed bridge crosses across the *Brassica* genomes as a potential means to introgress the glufosinate-ammonium reistance gene into related species. The work published by Bing, Doweny and Rakow (1991) and Bing (1995) showed that such introgression did not occur under field conditions in Western Canada.

wild radish (*R. raphanistrum*)

Baranger A., Chevre A.M., Eber F.; Renard M., 1995, Effect of Oilseed Rape Genotype on the Sponaneous Hybridization Rate with a Weedy Species- An Assessment of Transgene Dispersal. Theoretical and Applied Genetics, V91, N6-7:956-963.

Westar T5 from Plant Genetics Systems was crossed into 5 male sterile lines, all with the Ogura cytoplasm (derived from *Raphanus raphanistrum*). The resulting hybrid seed gave rise to male sterile plants, as Westar does not carry the restorer gene for fertility. The canola plants were interplanted with wild radish (*R. raphanistrum*) and seed was set by pollen from the wild radish and a canola field some distance away. The resulting seed was in two sizes, large seed from the rapeseed pollinations and small seed from wild radish pollinations. The small seed were triploid and produced mostly sterile plants (86% to 96% of the plants). Under normal conditions, male sterile plants would be planted with male fertile plants in the adjacent row. Thus, rapeseed pollen would be much more abundant and the likelihood of pollination by wild radish would be

extremely remote.

Therefore, based on the observations of Baranger, et al., 1995, it can be concluded that the likelihood of introgression of the transgene into populations of wild radish is extremely low because:

1) Crosses are only possible in the field under special circumstances; when pollen from the wild radish can successfully pollinate a male sterile canola using the Ogura cytoplasm (derived from wild radish). Hybrid seed production fields are planted with a large supply of pollinator plants and care is taken to isolate a seed production field from contaminating weeds,
2) The fertility of the resulting triploid plants is reduced,
3) The resulting triploid plants must survive in the field in subsequent generations, and backcross into the existing populations of wild radish. The triploid chromosome structure will make such backcrossing difficult, and
4) The only selective advantage would be resistance to the herbicide.

wild mustard (*Sinapis arvensis* L., syn. *Brassica kaber*)

Lefol E., Danilou V., Darmency H., 1996, Predicting Hybridization between Transgenic Oilseed Rape and Wild Mustard. Field Crops Research, V45, N1-3:153-161.

Quote from abstract: "No hybrid was found among 2.9 million seeds produced by wild mustard grown in a garden in the presence of a herbicide-resistant transgenic cultivar." The herbicide resistant rapeseed was glufosinate tolerant, supplied by Plant Genetics Systems. Wild mustard is (*Sinapis arvensis* syn *B. kaber*)

Bing, D.J., Downey, K., and Rakow, G.F.W., 1995, An Evaluation of the Potential of Intergeneric Gene Transfer between *Brassica napus* and *Sinapis arvensis*. Plant Breeding, V115:481-484.

To summarize this article: the likelihood of introgression of the transgene into populations of wild mustard is nil because crosses between canola and wild mustard do not occur under field conditions.

Brown, A.P. Brown, J. Thill, D. C., Brammer, T. A., Nair, H.S., 1995, Gene Transfer between Canola (*Brassica napus* and *Brassica campestris*) and related weed species. Proceedings GCIRC 9th International Rapeseed Congress, Cambridge, 4, 1040-1043.

Brown et al. (1995) attempted crosses to wild mustard (*Sinapis arvensis*, syn. *B. kaber*) and black mustard (*B. nigra*) pollinating immature buds with pollen from glufosinate tolerant canola in the greenhouse. No fertile hybrids were made, however the authors proposed bridge crosses across the *Brassica* genomes as a potential means to introgress the glufosinate tolerant gene into related species. The work published by Bing, Doweny and Rakow (1991) and Bing (1995)

showed that such introgression did not occur under field conditions in Western Canada.

cabbage family (*Brassica olracea*)

Kerlan, M.C., Chevre, A.M., Eber, F., Baranger, A. and Renard, M., 1992. Risk assessment of outcrossing of transgenic rapeseed to related species: I. Interspecific hybrid production under optimal conditions with emphasis on pollination and fertilization. Euphytica 62: 145-153.

Downey, R.K., Biosafety of Transgenic Oilseed *Brassica* Species, 1992, Proceedings of 2nd International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms, Goslar, Germany.

B. oleracea was not identified by Dr. Keith Downey as a potential recipient of *B. napus* pollen under field conditions (Downey, 1992). Neither did the USDA recognize *B. oleracea* as a potential recipient of *B. napus* pollen under field conditions (USDA, 1994).

Several biological facts prevent such gene flow and potential for environmental consequence:

1) hybrids may be formed only under laboratory conditions (manual pollinations and embryo rescue) between *B. napus* and *B. oleracea* , (Kerlan et al., 1992),
2) crosses between *B. napus* and *B. oleracea* are especially difficult when *B. napus* is the pollen parent (Kerlan et. al., 1992 reported 0.002-0.0067 plants produced per fertilized ovary using hand pollination and embryo rescue techniques, and
3) there is little opportunity for field crossing since *B. oleracea* is not naturalized in North America and geographic isolation is used for the production of seed (Kerlan et al., 1992).

***B. carinata*, *B. elongata*, wild turnip (*B. tournefortii*), white mustard (*Synapis alba*)**

Calgene, Inc., 1994, Petition for Determination of Nonregulated Status for Laurate Canola (*Brassica napus*).

Warwick, S.I., 1993. Guide to the Wild Germplasm of Brassica and Allied Crops, part IV. Agriculture Canada Research Branch Technical Bulletin, 17E, 19.

"Crosses between *B. napus* and *B. carinata* would be possible in the field (although very unlikely due to incompatibility, Fernandez-Serrano et al., 1991; Kerlan et al., 1992; Downey et al., 1980) except that neither species occur in the wild (are naturalized) in the U.S. Standard isolation practices prevent hybrid production. There is no significant production of *B. carinata* anywhere in the U.S. The vegetable *Brassicas* (e.g. broccoli) are not taken to seed intentionally, except in geographically isolated seed production areas." (Calgene, 1994)

B. elongata is not cultivated in the U.S. nor do naturalized forms occur. (Calgene, 1994; Warwick, 1993).

B. tournefortii is not cultivated in the U.S. (Calgene, 1994). No crosses between *B. napus* and *B. tournefortii* have been documented in literature (Calgene, 1994; Warwick, 1993).

No field hybridization between *B. napus* and *Synapis alba* (*B. hirta*) has been documented (Warwick, 1993). Manual hybridization was attempted with no success (Calgene, 1994).

wild radish and hoary mustard

Lefol, E., Dantelou, V., Darmarcy, H., Boucher, F., Maillet, J. and Renard, M., 1995, Gene Dispersal from Transgenic Crops. I. Growth of Interspecific Hybrids between Oilseed Rape and the Wild Hoary Mustard. Journal of Applied Ecology. V32: 803-808.

Research in France has shown that field hybrids can be made under special circumstances between male sterile *B. napus* and hoary mustard (*Herschfeldia incana* syn. *B. adpressa*) a weed of Mediterranean regions. Hoary mustard is found as an occasional weed in North America in roadside and waste areas of California, Oregon and Nevada. It is not likely to be in the proximity of commercial canola production. (Warwick, 1993).

Eber, F., Tanguy, X., Chevre. A.M., Baranger, A., 1994. Spontaneous Hybridization between a male Sterile Oilseed Rape and two Weeds. Theoretical and Applied Genetics, V88 N3-4:362-368.

Eber et al., 1994, used the two weeds hoary mustard (*Herschfeldia incana* syn. *B. adpressa*) and wild radish (*Raphanus raphanistrum*). The male sterile rapeseed was the Ogura cytoplasm (derived from *Raphanus raphanistrum*).

To quote from the discussion section of this paper (p. 367):
"The R1 interspecific hybrids produced were vigorous and well adapted to natural conditions, but some difficulties arose for the BC1 seed production, particularly with the diploid species as the recurrent parent. It seems that it is difficult to return to the diploid level, which is in agreement with the results of Bing et al. (1991). Even if that difficulty could be overcome, gene introgression will depend on chromosome rearrangement in the 2x genome."

"We have demonstrated that interspecific crosses can occur using male-sterile rapeseed. However, we may expect that the pollen competition due to the co-cultiviation of a male-fertile rapeseed variety will result in rare pollinations involving wild species, except where the female parent flowers earlier than the male parent."

The likelihood of introgression of the transgene into populations of hoary mustard is nil in the U.S. because:

1) Hoary mustard does not grow in the same location as canola which is grown for production in

the United States. Hoary mustard (*H. incana*, syn. *B. adrepressa*) grows in ditches and roadside areas of California, Nevada and Oregon. It does not occur in the canola producing areas of these states (Warwick, 1993),

2) In the possible cases of hybrid seed production in the Imperial Valley of California where hoary mustard may be present, the opportunity for hybridization is extremely small due to the management practices of seed production, such as isolation distances of several meters (AgrEvo internal communication), and

3) Introgression of the transgene into the hoary mustard population is not likely due to chromosome incompatibilities. (Eber et al., 1994).

Diplotaxis muralis

Calgene, Inc., 1994, Petition for Determination of Nonregulated Status for Laurate Canola (*Brassica napus*).

"Crosses of *B. napus* with *Diplotaxis muralis* have only been reported from laboratory studies (Ringdahl et al., 1987; Salisbury, 1988). Field crosses with *D. muralis* are extremely unlikely since it is not a common agricultural weed (based on a description of distribution in Rollins, 1980; also, the species is not listed in the Weed Control Manual, 1992). Further, *D. muralis* is highly self-compatible and most fertilization is complete before emasculation (Ringdahl et al., 1987), which is normally done 24-48 hours before the flower would open." (Calgene, 1994).

Appendix 3: Methodologies for Southern Analyses and Northern Analyses

SOUTHERN HYBRIDIZATION PROCEDURE

Introduction

Total genomic DNA is isolated from plant tissue according to Doyle et al. (1987). 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 genomic plant DNA from fresh tissue - CTAB Method (according to Doyle, J.J. and Doyle, J.L. - 1987 - *Phytochem. Bull. 19*, 11)

This original protocol has been optimized for Brassica napus.

- Preheat CTAB extraction buffer[1] to 65°C in a water bath.
- Put 50 to 300 mg fresh leaf tissue in an eppendorf tube and freeze in liquid nitrogen.
- Grind the frozen tissue to a fine powder with a prechilled eppendorf pestle. Do not allow the material to defreeze. Put the eppendorf back in the liquid nitrogen untill all samples are processed.
- Directly add 1 ml of preheated CTAB extraction buffer to the tube. Vortex untill all powder is in suspension.
  Incubate the tube at 65°C for 90 min , with occasional mixing (every 15 min).
  The plant material is floating in the beginning, and will sink to the bottom after some time .
- Let the samples cool at room temperature for 10 minutes (transfer to a 2.2 ml eppendorf tube)
- Extract once with 450 µl chloroform/isoamylalcohol (24/1) and mix tubes by inverting for approximately 5 min.
- Spin in an eppendorf centrifuge at room temperature for 10 min at 7000 rpm.
- Transfer aqueous phase to a new 2.2 ml eppendorf tube.
- Precipitate the DNA with 900 µl isopropanol and mix well. At this stage, large strands of nucleic acids are visible.
- Spin the tubes for 30 sec at 13000 rpm. If the precipitated DNA is rather flocculent, spin for 10 min at 7000 rpm.
- Wash the pellets with 500 µl 76% EtOH, 0.2 M NaOAC. Leave the DNA in the solution for about 20 min.
- Remove wash solution and replace with 500 µl 76% EtOH, 10 mM NH_4OAC. Leave the DNA for 10 min in the solution.
- Remove the last wash solution (spin if necessary) and allow the DNA to dry in a heat block at 37°C.
- Dissolve the DNA in 40 µl TE.
- Analyse 1 µl of the sample on a 1% TBE agarose gel to determine the DNA concentration. Compare the amount of DNA in the sample to 50 ng , 100 ng and 250 ng of lambda DNA loaded on the same gel[2] .
- Use an appropriate amount of DNA for PCR or RFLP analysis[3]

Notes :

1. CTAB extraction buffer: **freshly prepared**

100 mM Tris.Cl pH 7.5	10 ml 1M Tris.Cl pH 7.5
1.4 M NaCl	28 ml 5M NaCl
20 mM EDTA	4 ml 0.5M EDTA
	mq H_2O up to 100 ml
+ 2% CTAB	+ 2 g CTAB
(add freshly before use, dissolve at 65 C)	

* Tris/NaCl/EDTA buffermix cannot be kept for longer than 2 weeks at RT.
* CTAB extraction buffer with CTAB should not be saved.

2. Yields are almost between 4 and 10 µg of genomic DNA/100 to 200 mg fresh leaf material.
3. The genomic DNA is highly susceptible to restricion enzymes.

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.

Methods for the analysis of messenger RNA

The following procedure has been used to demonstrate the expression of the introduced transgenes in the male sterile and fertility restorer progenies. The same procedure was used to analyze the eventual occurrence of cryptic gene expression.

1. Extraction and purification of total RNA

Total RNAs are isolated according to Jones *et al.*(Jones D., Dunsmuir P & Bedbrook J., The EMBO Journal, **4**, 2411-2418,1985).

- Grind 1 to 2 grams of tissue to a fine powder in liquid nitrogen.
- Add 9 ml of NTES buffer (0.1M NaCl, 0.01M Tris-HCl pH 7.5, 1mM EDTA, 1% SDS) and 6 ml of phenol/chloroform/isoamylalcohol (24:24:1).
- Vortex intensively (approximately 10 min.) in 50 ml Falcon tubes.
- Transfer to a DEPC-treated 30 ml Corex tube and centrifuge in the HB4 Sorvall rotor

at 8000 rpm for 10 min.

- Take the aqueous phase, add 1/10 volume of 2M NaOAc and add 2 volumes ethanol.
- Mix well and keep at least 1 hour at -20°C.
- Pellet the precipitate at 8000 rpm for 10 min. (HB4, Corex tubes).
- Rinse the pellet with 70% ethanol.
- Dissolve the pellet in 2 ml water. Spin 5 min. at 5000 rpm (HB4 rotor) to sediment impurities.
- Transfer supernatant to a 15 ml Corex tube and add 2 ml 4M Lithium Acetate or 4M Lithium Chloride.
- Leave on ice for at least 3 hours (preferable over night).
- Pellet the precipitate as above and dissolve the pellet in 1.8ml water. Add 0.2ml 2M NaOAc pH 4.8 and add 2 volumes ethanol.
- Mix well and keep at least 1 hour at -20°C.
- Pellet the precipitate as above and rinse pellet with 70% ethanol and invert the tubes to dry the pellet.
- Finally dissolve the pellet in 100 to 500µl water.

This method is scaled down for the extraction of RNA from pollen and flower buds. The material is crushed with a plastic pestle in the presence of extraction buffer and phenol in an Eppendorf tube. All subsequent handlings are performed in Eppendorf tubes.
For quantifying the amount of RNA, spectrophotometric readings are taken at a wavelength of 260 nm. An OD of 1 corresponds to 40µg/ml RNA.

2. *In vitro* synthesis of control RNA transcripts

For the synthesis of control RNA transcripts (used as positive hybridization controls and for the quantification of the hybridization signals), all four ribonucleoside triphosphates are used. During "cold" transcription reactions, substrate levels are not limiting and the synthesis continues longer, producing higher amounts of RNA.

Templates

A. Plasmids for preparing RNA probes

pVE113: *barnase-barstar* in pGem1 vector (see Figure 1)
- HindIII digested pVE113 DNA transcribed with T7 DNA polymerase gives sense *barstar/barnase* RNA transcripts.
- EcoRI digested pVE113 DNA transcribed with SP6 DNA polymerase gives anti-sense *barstar/barnase* RNA transcripts.

pGemBar: *bar* in pGEM2 vector (see Figure 2)
- EcorI digested pGemBar DNA transcribed with T7 DNA polymerase gives sense *bar* RNA transcripts.
- HindIII digested pGemBar DNA transcribed with SP6 DNA polymerase gives anti-sense *bar* RNA transcripts.

B. PCR amplification of DNA templates for in vitro RNA synthesis

For the analysis of occurrence of cryptic gene expression, we amplified specific T-DNA fragments, by means of PCR, to serve as templates for in vitro RNA synthesis.

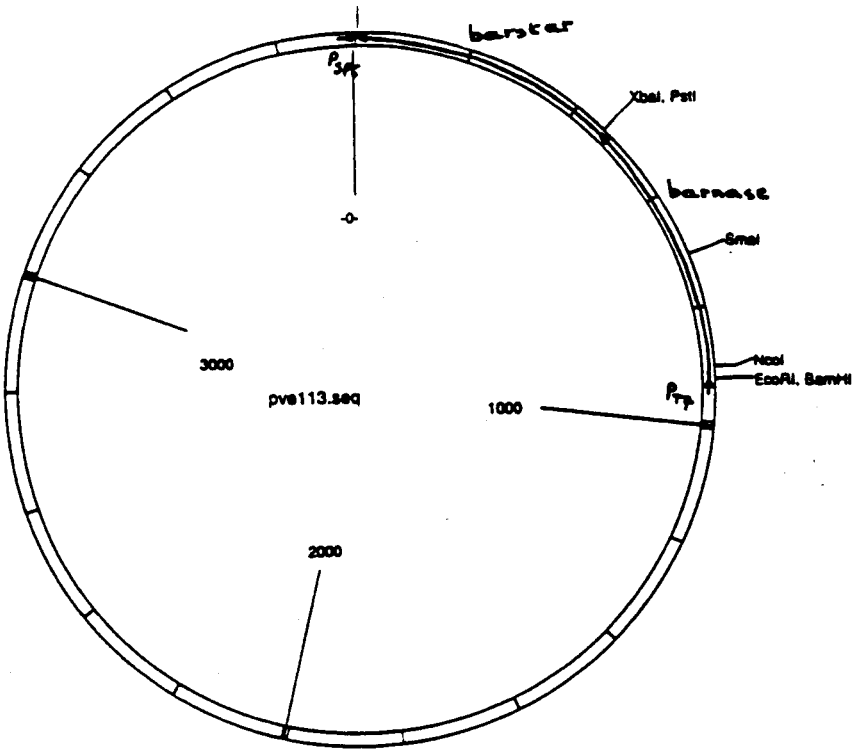


Figure 1. pVE113 : *barnase-barstar* in pGem1 vector

Spliced PGEM2.SEQ 1:28, PVE 5:575, PGEM2.SEQ 29:2869 into PGEM3.SEQ
3440 Base Pairs

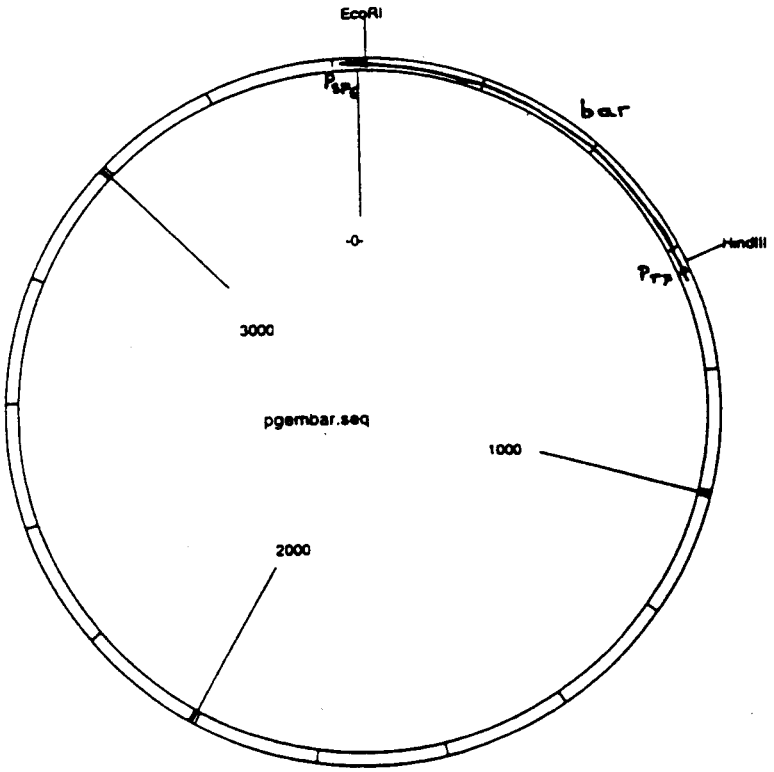


Figure 2. pGemBar : *bar* in pGem2 vector

For every template, two primers are designed : an upstream primer which comprises the T7 promoter (including the 6 nucleotides GGGAGA that are present at the 5' end of transcripts) adjacent to specific T-DNA sequences and a downstream primer which comprises the SP6 promoter (including the 6 nucleotides GAATAC that are present at the 5' end of transcripts) adjacent to specific T-DNA sequences (see Figure 3).

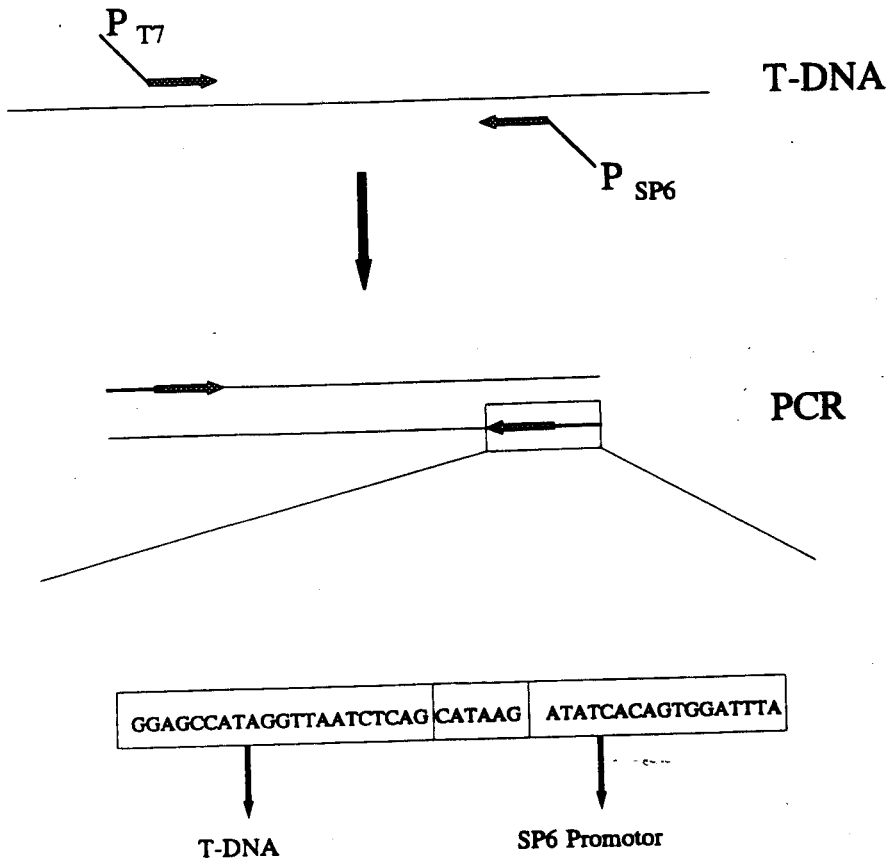
The sequences of the different synthesized primers can be found in Table 1. Amplified fragment lengths and the region of the T-DNA they cover, can be found in Table 2.

PCR is carried out by using the thermostable Vent DNA polymerase (New England Biolabs, Inc.). This polymerase contains a 3' ---> 5' proofreading exonuclease activity, resulting in much higher fidelity of base incorporation compared to Taq DNA polymerase.

25ng of EcoRV linearized pTHW118 DNA or pTHW107 DNA and 30pmoles of upstream primer and downstream primer were mixed in a 50µl PCR reaction containing 10mM KCL, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl (pH8.8 at 25°C), 2mM $MgSO_4$, 0.1% Triton-X-100, 200µM of each deoxyribonucleoside triphosphate and 1 unit of Vent DNA polymerase. DNA amplification occurred during 25 cycles.

Thermocycling profile:	4 min. at 95°C
Followed by:	30 sec. at 95°C
	30 sec. at 57°C
	45 sec. at 75°C
	For 5 cycles
Followed by:	5 sec. at 92°C
	30 sec. at 60°C
	45 sec. at 75°C
	For 20 cycles
Followed by:	10 min. at 75°C

The synthesized fragments were checked on agarose gels. After phenol-chloroform extractions, the fragments were precipitated, washed and subsequently dissolved in water. The concentration of the DNAs was spectrophotometrically measured.
For the primers MDB172-173 and MDB170-171, the elongation temperature in the PCR reaction was 57°C instead of 60°C. Some primer pairs required optimization of Mg^{2+} levels (for primer pair VDS67-VDS68, 6mM $MgSO_4$ was found to be optimal).



Outline for the generation of specific T-DNA fragments for use in the in vitro transcription of RNA probes (The oligonucleotide sequence shown is MDB173)

In vitro synthesis

- Mix the following components in the given order in a microfuge tube at room temperature :

DEPC-treated water	up to 50µl volume
Template DNA	4µg
10x Transcription buffer	5µl
0.5M DTT	1µl
RNAse inhibitor (25 units/µl)	2µl
NTP mix (2.5 mM each)	10µl
DNA- dependent RNA polymerase	1µl

(10x Transcription buffer : 400mM Tris-HCl pH 7.5 at 37°C, 60mM $MgCl_2$, 20mM spermidine and 50mM NaCl)

- Incubate at 37°C for 120 minutes.
- Add 1µl 10x Transcription buffer, 8µl NTP mix and 1µl polymerase. Incubate of another 120 minutes at 37°C.
- The template DNA is removed by treatment with DNAse I for 10 minutes at 37°C.
- The synthesized RNA transcripts are extracted with phenol-chloroform and purified from unincorporated nucleotides on a Bio-Spin® 30 chromatography column (Bio-Gel P-30 polyacrylamide gel, Bio-Rad), equilibrated with DEPC-treated water.
- The concentration is spectrophotometrically measured.
- 1µg of the synthesized RNA transcripts are checked on a 1.5% agarose-formaldehyde gel.

3. Fractionation of RNA

The RNA is separated according to size by electrophoresis through a denaturing agarose gel containing formaldehyde.

The gels are prepared by melting agarose (1.5% final concentration) in water, cooling it to 60°C, adding 10x MOPS buffer (0.2M MOPS, 0.05M NaOAc pH7.0 and 0.01M EDTA) and formaldehyde to give a final concentration of 1x and 2.2M respectively.
Cast the gels in a chemical hood and allow the gel to set at least for 30 min. at room temperature.

Samples are prepared by mixing the following in a sterile microfuge tube:

- RNA (5µg or 10µg)	x µl
- 10x MOPS buffer	2 µl
- formaldehyde	3.5 µl
- formamide	10 µl
- Ethidium bromide (1mg/ml)	1 µl

Note: The control RNA dilutions are complemented with 5µg control leaf RNA

Incubate the samples for 15 minutes at 55°C and then chill them on ice. Add 2 µl of sterile

DEPC-treated dye (50% glycerol, 0.5% bromophenol blue and 0.5% xylene cyanol FF). Run the gel submerged in 1x MOPS buffer at ±5 V/cm.
To avoid unnecessary inhalation of volatile formaldehyde vapours, documentation of the fractionation of the RNA is done after the transfer and fixation of the RNA to the nylon membrane.

Loading sequence of the gels:

Gel A:	Line	Plant N°	Tissue	µg RNA loaded
1.	MW (0.16 - 1.77 kb RNA ladder, Life Technologies Inc.)			
2.	MS8	T3-A	leaf	5µg
3.	MS8	T3-B	leaf	5µg
4.	control		leaf	5µg
5.	MS8	T3-A	flower buds 2mm	5µg
6.	MS8	T3-A	flower buds 3mm	5µg
7.	MS8	T3-B	flower buds 2mm	5µg
8.	MS8	T3-B	flower buds 3mm	5µg
9.	control		flower buds 2mm	5µg
10.	control		flower buds 3mm	5µg
11.	MS8	T3	dry seed	5µg
12.	control		dry seed	5µg
13 →18.	Control RNA dilution series (*in vitro* synthesized RNA complementary to the probe used): 0.25pg - 0.5pg - 1pg - 2pg - 4pg and 8pg. These control RNA samples are complemented with 5µg control leaf RNA.			

Gel B:

1.	MW (0.16 - 1.77 kb RNA ladder, Life Technologies Inc.)
2 → 8.	Control RNA dilution series (*in vitro* synthesized RNA complementary to the probe used): 0.25pg - 0.5pg - 1pg - 2pg - 4pg - 8pg - 16pg. These control RNA samples are complemented with 5µg control leaf RNA.
10 →16. to	Control RNA dilution series (*in vitro* synthesized RNA complementary the probe used) : 0.25pg - 0.5pg - 1pg - 2pg - 4pg - 8pg - 16pg. These control RNA samples are complemented with 5µg control leaf RNA.
17.	MW (0.16 - 1.77 kb RNA ladder, Life Technologies Inc.)

Gel C:	Line	Plant N°	Tissue	µg RNA loaded
1.	MW (0.16 - 1.77 kb RNA ladder, Life Technologies Inc.)			
2.	RF3	S3-A	leaf	5µg
3.	RF3	S3-B	leaf	5µg
4.	control		leaf	5µg
5.	RF3	S3-A	flower buds 2mm	5µg
6.	RF3	S3-A	flower buds 3mm	5µg
7.	RF3	S3-B	flower buds 2mm	5µg
8.	RF3	S3-B	flower buds 3mm	5µg
9.	control		flower buds 2mm	5µg
10.	control		flower buds 3mm	5µg
11.	RF3	S3	pollen	5µg
12.	control		pollen	5µg
13.	RF3	S3	dry seed	5µg
14.	control		dry seed	5µg

4. Transfer of denatured RNA to nylon membranes

The RNAs are transferred immediately after electrophoresis from the agarose to nylon membranes (Hybond-N, Amersham) by capillary elution.

- Fill a glass dish with blotting buffer (20x SSC = 3M NaCl, 0.3M Sodium citrate, pH7). Make a platform and cover with a Whatman 3MM filter paper wick, saturated with buffer.
- Place the gel on the wick and avoid trapping air bubbles beneath it. A sheet of Hybond-N membrane, cut to the exact size of the gel, is placed on top of the gel. Avoid trapping bubbles beneath the membrane.
- Place a sheet of Whatman 3MM cut to size and wetted with blotting buffer, on top of the Hybond-N membrane.
- Surround the gel with Saran Wrap foil to prevent the blotting buffer being absorbed directly into the paper towels above.
- Place a stack of absorbent paper towels on top of the 3MM paper.
- Place a glass plate on top of the paper towels and a 0.5 - 1 Kg weight on top. Allow the transfer to proceed for 12 to 20 hours.
- After blotting carefully dismantle the setup. Before removing from the gel, mark the membrane with a pencil to allow later identification of the tracks.
- The samples are fixed to the membrane by baking in an oven at 80°C for 2 hours.

Documentation of the fractionation of the RNA is done at this stage. The image is acquired, processed and copied to thermal paper using the Foto/Analyst ™ Visionary imaging system from FOTODYNE (CCD camera: charge-coupled device) (see figure 4).

5. *In vitro* synthesis of RNA probes

Single-stranded RNA probes of high specific activity are prepared by using either plasmid vectors containing polycloning sites downstream from powerful promoters derived from the *Salmonella typhimurium* bacteriophage SP6 or from the *E. coli* bacteriophage T7 or by either using PCR generated templates with 5' extensions containing the sequences from the before mentioned promoters.

In vitro labeling

- Mix the following components in the order given in a microfuge tube at room temperature:

DEPC-treated water	up to 20 µl total volume
Template DNA	500 ng
10x Transcription buffer	2 µl
NTP mix (-UTP), 2.5mM each	3 µl
1mM UTP	1 µl
0.2M DTT	1 µl
RNAse inhibitor (25 units/µl)	1 µl
[α-^{32}P]UTP (20mCi/ml)	5 µl
Bacteriophage DNA-dependent RNA polymerase (7-12 units/µl)	1 µl

(10x Transcription Buffer : 400mM Tris-HCl pH7.5 at 37°C, 60mM $Mgcl_2$, 20mM spermidine and 50mM NaCl)

- Mix the reagents by gentle tapping.
- Incubate the reaction for 1 hour at 40°C (SP6 RNA polymerase) or 37°C (T7 RNA polymerase).
- Add 1 µl RNAse inhibitor and 1 µl of RNAse-free pancreatic DNAseI (20 units/µl). Mix and incubate for 15 min. at 37°C.
- Analyze 0.5 µl on a 6% denaturing acrylamide gel.
- The rapid removal of unincorporated nucleotides from the labeling reaction is done by using Bio-spin® 30 chromatography columns (Bio-Gel P-30 polyacrylamide gel, Bio-Rad).

6. Hybridization and autoradiography

- The filters are prehybridized for 1-2 hours in a hybridization oven using 10ml prehybridization buffer (for 3 filters of 14cm x 19cm) at 65°C.
  Prehybridization buffer: 50% formamide, 5x SSC, 5x Denhardt's, 0.5% SDS and 100µg/ml carrier DNA at 65°C.

  (20x SSC: 3M NaCl, 0.3M Sodium citrate, pH7)
  (100x Denhardt's solution: 2% (w/v) BSA, 2% (w/v) ficoll and 2% (w/v) Polyvinylpyrrolidone)

- Remove the prehybridization buffer.
- Add fresh prehybridization buffer supplemented with the denaturated radiolabeled probe to the hybridization tube and continue the incubation over night.
- Wash the filters for 5 min. in 6x SSC, followed by 2-3 washes of 20-30 minutes each in 2x SSC, 0.1% SDS and 1 wash of 10-20 minutes in 0.1x SSC, 0.1% SDS.
- Establish an autoradiography by exposing the filter for 3 up to 96 hours to X-ray film at -70°C with an intensifying screen. The shorter exposures are performed for accurate quantification and for reproduction of the results. The longer exposures are performed to assure the absence of any signals in control samples or in the analysis of occurrence of cryptic gene expression.
- Reproduction of the results in this document is done by using the iphoto deluxe software (U-lead Systems, Taipei, Taiwan, ROC) and the Harvard Graphics Software.
- After the exposure, the membranes are stripped to remove the probes. For this purpose a 0.5% SDS solution is boiled. Membranes are submerged in this solution and allowed to cool to room temperature.
- To check that the probe was removed completely, an autoradiograph for the normal exposure time was established.
- Subsequently, the filters can be prehybridized and hybridized with a new probe.

Appendix 4: Methodology for Determination of Level of PAT Expression

Measurement of PAT activity

Responsible : A. van Vliet, PGS Researcher

Goal of the experiment

Quantification of the amount of phosphinothricin-acetyl-transferase (PAT) in leaves and seeds of the male sterile MS8 and fertility restorer RF3 oilseed rape line.

Material and methods

L10.1. Preparation of *Brassica napus* seed extracts

- Seeds of the non-transgenic control cultivar
- Seeds of T_3(MS8)
- Seeds of S_3(RF3)

Seeds were transferred to an on ice cooled mortar, crushed and ground with a pestle. To x grams of flour, 0.5 mL standard extraction buffer (SEB) was added and after 15 minutes of vigorous agitation, supernatans were collected by centrifugation in an Eppendorf centrifuge for 15 minutes at maximum speed.

Control seed extractions were made in duplicate while extractions of seed from transgenic plants were done in triplicate. The extracts were immediately analyzed for the presence of PAT after preparation because there is some evidence that PAT is rapidly degraded in *Brassica napus* flour.

L10.2. Preparation of *Brassica napus* leaf extracts

- Leaves of the non-transgenic control cultivar
- Leaves of T_3(MS8)
- Leaves of S_3(RF3)

Leaf samples (-70°C) were ground in Eppendorf tubes with a plastic pestle on liquid nitrogen. Extraction was done as described under L10.1. with 0.5 mL SEB.
From each leaf triplicate extractions were made.

L10.3. Spectrophotometric assay for PAT[1]

PAT activity is quantified by measuring enzyme kinetics. The method is based on the generation of free CoA sulfhydryl groups during the transfer of the acetyl group of PPT. The reaction of the reduced CoA with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) yields a molar equivalent of free 5-thio-2-nitrobenzoic acid with a molar extinction coefficient of 13,600 at 412 nm.

PAT assay protocol

1. Prepare a crude cell extract as described in the appropriate protocol and measure the protein concentration with the Bio-Rad protein microassay procedure, based on the Bradford dye-binding procedure[2].
2. Perform reactions at 37°C in 1mL reaction buffer (100 mM TRIS-HCl, pH 7.5, 0.5 mM acetyl-CoA, 1 mM DTNB, and 0.1 mM PPT) using a recording spectrophotometer equipped with a temperature-controlled cuvette chamber.
3. After establishing a base line rate of DTNB reduction in the presence of plant extract and acetyl-CoA, start the reaction by the addition of substrate.
4. (Δ OD/min$_{extract}$ - Δ OD/min$_{buffer}$)/13.6 represents the micromoles DTNB reduced per minute (1µmol DTNB$_{red}$/min = 1 Unit) at 37°C.

Specific activity of pure PAT measured as written above, is estimated to be 19U/mg.

Solutions

- 0.4 mg DTNB/ml 100mM Tris/Cl pH 7.5
- 9.75 mg PPT/ml miQ
- 20.2 mg AcCoA/ml miQ

Measurement

968 µl DTNB
2 µl PPT
20 µl AcCoA
10 µl sample

List of abbreviations

AcCoA	acetyl coenzyme A
CoA	coenzyme A
DTNB	5,5' Dithiobis (2-nitrobenzoic acid)

1 D'Halluin, K., De Block, M., Denecke, J., Janssens, J., Leemans, J., Reynaerts, A., Botterman (1992) J. Methods in Enzymology, vol 216, 415.

2 Bradford, M. (1976). Anal. Biochem. , 72, 248.

Appendix 5: Termination Reports for 1997 and 1998 U.S. Field Releases

MS8/RF3 USDA Termination Report, 1997

MS8/RF3 canola and its nontransgenic parent Drakkar were field tested at two locations in the U.S. during the 1997 growing season. MS8/RF3 is a hybrid canola (F_1) plant which contains genes for expression of male sterility and fertility restoration. MS8/RF3 is fully fertile. MS8/RF3 also contains the bar gene whose expression product is the PAT enzyme. The PAT enzyme confers resistance to the broad-spectrum herbicide glufosinate-ammonium.

USDA Permit Number: **97-035-05r**
Locations: **Cass County, ND; Dane County, WI**

Planting Conditions: Planting occurred on approximately 0.2 acres at both sites in late May 1997. Weather conditions were warm enough to allow for seed germination in a time frame comparable to commercial fields.

Frequency of Observations: The sites were observed by field station managers or associates several times (6+) during the growing season. Data was recorded at 4 different growing stages from emergence through rosette through flowering to seed set.

Agronomic Data Recorded: Seed germination rates, plant stand, plant vigor, flowering times and vigor, deleterious effects, disease and pest resistance/susceptibility were monitored throughout the growing season. With regard to all of the aforementioned agronomic traits, there was no difference in the transgenic MS8/RF3 canola as compared with the nontransgenic parent canola variety Drakkar. Seed germinated well and grew healthily. Plant stand was good. Flowering times for MS8/RF3 were comparable to those of Drakkar. MS8/RF3 canola demonstrated no greater potential to become a weed than its nontransgenic parent. Mild infestation of sclerotinia was observed at the Cass County site, but it was the same for both the transgenic and nontransgenic parnet line. Other disease (black leg) or insect pests (fleabeetles or diamondback moths) were not observed for either MS8/RF3 or Drakkar.

Resistance to Glufosinate-Ammonium; Susceptibility to other Herbicides: At the Cass County, ND site, herbicide treatment was used. Herbicide treatment was not used in Dane County, WI. MS8/RF3 canola plants exhibited resistance to treatment with glufosinate-ammonium, as expected. The nontransgenic parent, Drakkar, was susceptible to treatment with glufosinate-ammonium. Both MS8/RF3 and the nontransgenic parent were susceptible to other herbicides which control mustards: glyphosate, phenoxys and sulfonyl-ureas.

Effects on Beneficial Organisms: No decreased populations of honeybees were observed on the transgenic canola versus the nontransgenic parent. Honeybees were present at both locations.

Volunteer Monitoring and Mitigation Measures: Volunteers were observed at both locations. Volunteers were destroyed in winter by discing the soil or field cultivation several times and

treatment with glyphosate. No germination of volunteeers were observed in spring 1998.

MS8/RF3 USDA Termination Report, 1998

MS8/RF3 canola and its nontransgenic parent Drakkar were field tested at fourteen locations in the U.S. during the 1998 growing season. MS8/RF3 is a hybrid canola (F1) plant which contains genes for expression of male sterility and fertility restoration. MS8/RF3 is fully fertile. MS8/RF3 also contains the bar gene whose expression product is the PAT enzyme. The PAT enzyme confers resistance to the broad-spectrum herbicide glufosinate-ammonium.

USDA Notification Numbers and Locations: **98-064-38n (Polk Co., MN); 98-064-35n (Pierce Co., Nelson Co., Towner Co., Ramsey Co., Foster Co. (2 locations), and Cavalier Co. (2 locations), ND); 98-064-33n (Waushara Co. and Door, Co., WI); 98-168-04n (Columbia Co., WI); and 98-064-31 (Madison Co., ID)**

USDA Permit Number: **98-119-01r**
Location: **Cass County, ND**

Planting Conditions: Planting occurred on approximately 0.2 – 0.5 acres at all sites in late April to late May 1998. Weather conditions were warm enough to allow for seed germination in a time frame comparable to commercial fields, without the danger of frost.

Frequency of Observations: The sites were observed by field station managers or associates several times (6+) during the growing season. Data was recorded at 4 different growing stages from emergence through rosette through flowering to seed set.

Agronomic Data Recorded: Seed germination rates, plant stand, plant vigor, flowering times and vigor, deleterious effects, disease and pest resistance/susceptibility were monitored throughout the growing season. With regard to all of the aforementioned agronomic traits, there was no difference in the transgenic MS8/RF3 canola as compared with the nontransgenic parent canola variety Drakkar. Seed germinated well and grew healthily. At the Cavalier County site, seed was replanted two weeks following initial planting due to 3" of rain which resulted in a hard crust on the soil and poor germination. This was the same for transgenic and nontransgenic. Following replanting a 1" rainfall resulted in reduced starts. This was observed for both transgenic and nontransgenic plants. Plant stand was good at most sites. On 6/9/98, a crop planted at Ashland County, WI (98-064-33n) was destroyed due to drought and crusting. The plant area is being disced and cultivated repeatedly, followed by spraying with glyphosate, then fallowing. Flowering times for MS8/RF3 were comparable to those of Drakkar. MS8/RF3 canola demonstrated no greater potential to become a weed than its nontransgenic parent. Mild infestation of sclerotinia was observed at the Towner County site, but it was the same for both the transgenic and nontransgenic parent line. Ronilan was sprayed on the canola plants, transgenic and nontransgenic, in mid-July at the Ramsey, Nelson and Towner sites as control and prevention measures for sclerotinia. Other disease (black leg) or insect pests (fleabeetles or diamondback moths) were not observed for either MS8/RF3 or Drakkar at any site. At the

Pierce, Nelson, Towner and Ramsey county sites, Counter®[1] (tebufos) (20G) was applied at seeding to control fleabeetle.

Resistance to Glufosinate-Ammonium; Susceptibility to other Herbicides: At the Nelson, Towner, Pierce, Ramsey and Cass County, ND sites, herbicide treatment was used. Herbicide treatment was not used in Waushara, Columbia and Door Counties, WI. MS8/RF3 canola plants exhibited resistance to treatment with glufosinate-ammonium, as expected. The nontransgenic parent, Drakkar, was susceptible to treatment with glufosinate-ammonium. Both MS8/RF3 and the nontransgenic parent were susceptible to other herbicides that control mustards: glyphosate, phenoxys and sulfonyl-ureas.

Effects on Beneficial Organisms: No decreased populations of honeybees were observed on the transgenic canola versus the nontransgenic parent. Honeybees were observed at all locations.

Volunteer Monitoring and Mitigation Measures: Observations for volunteers will be made throughout the fall and winter. Appropriate measures such as discing the soil or field cultivation several times and treatment with a herbicide to which canola plants are highly susceptible will be taken.

[1] Counter® is a registered trademark of American Cyanamid Company.

AgrEvo

98-278-01p
1 Amendment

Susan Koehler, Ph.D.
USDA/APHIS Scientific Services
4700 River Road, Unit 147
Riverdale, MD 20737-1237

November 17, 1998

SUBJECT: Deficiency letter dated **October 30, 1998**, regarding petition **98-278-01p**

Dear Dr. Koehler,

Please find attached additional information in response to your letter regarding petition **98-278-01p**.

The information has been given so that each numbered point of your letter is addressed individually. A copy of your letter is attached immediately following this cover letter for reference.

It is our intent to answer all questions fully herein, however, if you have additional questions, please do not hesitate to contact me at telephone (302) 892-3034, fax (302) 892-3099.

Sincerely,

Vickie Forster

Vickie Forster
Regulatory Affairs-Biotechnology

Attachments

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11/19/98
MKB

PCR and Southern Gel Analysis

USDA Question 1, Part 1:

Additional information has been requested to demonstrate that the Sm/Sp coding region has not been integrated into the plant genome.

AgrEvo Response:

PCR analyses were conducted on plant DNA of both the MS8 and RF3 events. The 800 bp flanking regions (see Attachment 1) of the inserted T-DNA were cloned and sequenced. The EMBL nucleic acid sequence databank was searched (3/96) using the Ifind® sequence data search program (IntelliGenetics, Inc., Mountain View, CA). The *Agrobacterium tumefaciens* Ti-plasmid is included in the EMBL databank. 514,109 sequences were compared. The scores were sorted by optimizing score and subsequently aligned to the query sequence. No significant homologies were found, thus indicating no material (including any part of the Sp/Sm coding region) outside the Left and Right borders, respectively, was inserted into the plant genome.

USDA Question 1, Part 2:

The text of Figure 4, PCR analysis for RF3 elite locus identification, does not include the amount of DNA loaded, PCR primers used (what the sequence is designed to amplify), positive control and methods.

AgrEvo Response:

This analysis is used as a check and is not relevant to this petition. Therefore, no further data is provided here.

USDA Question 2:

Figures 5.b. and 5.c. are missing expected bands, 652 bp and 712 bp for 5.b., and 712 bp for 5.c. In addition, molecular weight markers for 5.a., 5.b., and 5.c. are in small hand-written print and are very hard to read.

AgrEvo Response:

New Southern blots for 5.a., 5.b., and 5.c. are attached here (Attachment 2) with bands and molecular weight markers more clearly identified.

Inheritance of Transgene Traits

USDA Question *Bar*:

The data in Table 6 does not demonstrate what is described in the text on pp 34-35. Also BC_2 data is not included in the table. It appears that a line is missing and/or data has been incorrectly entered. Please explain.

AgrEvo Response:
Please see a corrected version of Table 6 included here as Attachment 3.

USDA Question *Barnase*:

No data was provided to support the statement that the male sterility trait segregated 1:1.

AgrEvo Response:

Please see a report of a study (FBN 9413), included here as Attachment 4, for data regarding segregation of MS8. Table FBN 9413_1, row 3, indicates approximately 50% were male sterile, indicating a 1:1 segregation ratio. No statistical analyses were performed. Please note Basta® is the registered product name of glufosinate-ammonium for non-selective use in Europe. Basta® is a registered trademark of AgrEvo.

USDA Question *Barstar*:

Some data should be provided to indicate that the fertility is fully restored in the hybrid progeny of the MS8 x RF3 cross.

AgrEvo Response:

Please see Attachment 5 for yield data and proof of restoration based on the high yield levels of the MS8 x RF3 hybrid.

Expression of Transgenes and Agronomic Performance

USDA Question Bar, part 1:

It is unclear whether the individual values given in Tables 8 and 9 represent the average of duplicate or triplicate readings or whether they are individual readings from the same source material. It should also be specified for the PAT assays and Northern analysis whether the RF3 plants are homozygous or heterozygous for the insert.

In Table 7, two (2) values are below (0.03 pg) the LOD given (0.1 pg/ug).

AgrEvo Response:

Each value represents a seed or leaf sample respectively, from a single plant. The MS8 plants measured were heterozygotes; the RF3 plants were homozygous. The relative value of the PAT protein is indeed quite low in MS8 plant material analyzed when compared to RF3 plants. Whether this is a result of the fact that MS8 plants were treated with glufosinate- ammonium when the plants were young in order to eliminate segregating fertile plants in this population is not clear. No statistical analyses have been performed.

Table 7 should read as is given in Attachment 6.

USDA Question Bar, part 2:

Some attempt should be made to link the level of PAT expression seen in enzyme assays to the concentration of glufosinate-ammonium applied to the plants for segregation studies to the recommended label rate of LIBERTY® for field application.

AgrEvo Response:

Basta was applied at a rate of approximately 5kg/acre, or 10 times the highest recommended label rate for LIBERTY on canola. Plants are clearly tolerant to glufosinate-ammonium at PAT levels expressed.

USDA Question Barnase and Barstar, part 1:

There is no mention of enzyme analysis for Barnase and Barstar. The Canadian decision document refers to enzyme data. This data would be most useful.

AgrEvo Response:

No enzyme data was submitted to the Canadian authorities. This is a misunderstanding or a misrepresentation of the Canadian decision document.

USDA Question Barnase and Barstar, part 2:

Barnase analysis appeared to be complicated by the fact that it encodes a ribonuclease that might be degrading the mRNA since none was detected.

AgrEvo Response:

The paragraph titled 'MS8 Results', on page 35 is misleading. The last sentence of the paragraph should be replaced with the following: The *barnase* gene is only expressed in the tapetal layer within the flower bud because the promoter driving gene expression, TA29, is specific for the tapetum only. The reason no Barnase mRNA was detected in the tapetum is because the ribonuclease is self degrading.

USDA Question Barnase and Barstar, part 3:

No Northern analysis was conducted on the hybrid which might have demonstrated the presence of the Barnase messenger RNA or protein.

AgrEvo Response:

Evidence that Barnase is being expressed is from phenotypic observation – no anther development was observed and recorded by field personnel in over 100 observations over several years (1994-1998).

USDA Question Barnase and Barstar, part 4:

The lane numbers and legend for Figure 10 do not match, and the molecular weight markers for Figure 8 and 10 appear to be incorrectly labeled.

AgrEvo Response:

Figure 8 is correct. A corrected Figure 10 is included here as Attachment 6.

Disease and Pest Resistance Characteristics and Impacts on Nontarget and Beneficial Organisms

USDA Question:

On how many acres were the Canadian and Belgian field trials conducted? How were the observations for nontarget and beneficial organisms made?

Why would the *barnase* and *barstar* genes not be expected to impact beneficial organisms or threatened and endangered species?

AgrEvo Response:

No figure is available for acreages of field trials in Europe.
Canola has been grown in Canada at 41 sites during 1997-1998 on a total acreage of approximately 140.

Barnase is a gene for male sterility. Neither *barnase* nor its expression product, the protein Barnase, would negatively impact beneficial organisms or threatened or endangered species because it is expressed in the tapetal layer of the flower bud and it is only expressed for a very short time. Male sterility in and of itself would not be expected to be harmful to the above mentioned species.

Barstar is a gene for fertility restoration. It has been observed in field trials over several seasons in Europe and North America that no unusual or aberrant observations have been made of foraging honey or bumble bees, such as decreased populations. Populations and foraging patterns of honey and bumble bees were equivalent in plots of MS8, RF3 and the hybrid combination, MS8 x RF3 plants, to those in nontransgenic plants. No unusual behavior of birds or small mammals visiting MS8, RF3 and/or MS8 x RF3 plots has been observed or recorded. These observations lead to the conclusions that the *barstar* gene and its expression product, the protein Barstar, have no negative impact on the above mentioned organisms.

Attachment 1:

Verification that no vector material outside left and right borders was transferred into the plant genome for constructs pTHW107 (MS8) and pTHW107 (RF3)

MS8 - Proof of absence of sequences derived from the 'vector'-part of the construct.

Responsible De Beuckeleer Marc, Senior Researcher
Lecleir Machteld, Technician

Study completed November 3, 1997
Study no **PGS,MDB,03.11.1997,MS8**

Testing facility Plant Genetic Systems NV
Jozef Plateaustraat 22
B-9000 Gent, Belgium
Phone: (32)(9)235.84.11
Telefax: (32)(9)224.06.94
Email: pgs@pgsgent.be

Goal of the experiment

The aim of the experiment is to demonstrate, by means of Southern blot analysis, the absence of sequences derived from the 'vector'-part of the pTHW107 plasmid.

Plant material

Southern blot analysis has been performed on genomic DNA isolated from MS8 plants carrying the male sterility gene. Genomic DNA isolated from a non-transgenic plant has been used as control.

Analysis strategy

The vector sequences outside the T-DNA borders of pTHW107 comprises the following structural elements:

- the plasmid core comprising the origin of replication from the plasmid pBR322 for replication in *Escherichia coli* and a restriction fragment comprising the origin of replication from the *Pseudomonas* plasmid pVS1 for replication in *Agrobacterium tumefaciens*
- a selectable marker gene conferring resistance to streptomycin and spectinomycin for propagation and selection of the plasmid in *Escherichia coli* and *Agrobacterium tumefaciens*
- a barstar gene with regulatory signals for expression in *E.coli*.

All parts of the pTHW107 vector are used as DNA probes in Southern blot analysis:a 1213 bp fragment (MLD001-MLD002) comprising the origin of replication from pBR322, a 4645 bp fragment (MDB469-MLD004) comprising the origin of replication from pVS1 and a barstar gene with regulatory signals for expression in *E.coli*, and a 1915 bp HindIII fragment comprising the Sm/Sp selectable marker gene.

Southern Blot Analysis

Method

Southern blot analysis has been performed using probes covering the complete 'vector'-part of the transforming plasmid.
Total genomic DNA was isolated from leaf tissue according to Dellaporta et al. (1983, Plant Molecular Biology Reporter, 1, vol.3, p. 19-21). 10 µg of genomic DNA was digested with selected restriction enzymes, applying concentration, buffers and temperature according to the conditions proposed by the manufacturer. Upon termination of digestion, DNA fragments were separated by agarose gel-electrophoresis. The separated DNA fragments were transferred upon denaturation, through capillary force from the agarose gel to a Nylon membrane. Vector DNA templates were labelled using the 'Rediprime DNA labelling system' from Amersham International Inc. Hybridization and washing steps were carried out according to Sambrook et al. (Molecular cloning, a laboratory manual). After the washing was completed, autoradiography was established by exposing the membrane to a sheet of Kodak X-Omat AR film.

Assigned positive and negative controls to a Southern blot analysis

Since the analysis presented in this study draws upon negative evidence (i.e. the evidence on absence of specific DNA sequences), the results can only be used as a valid proof in case the correct controls have been used.

- As a DNA positive control (POS 1) we used HindIII digested genomic DNA prepared from a non-transgenic plant, supplemented with total pTHW107 plasmid DNA, digested with HindIII.
- As a DNA positive control (POS 2) we used HindIII digested genomic DNA prepared from a non-transgenic plant, supplemented with total pTHW100 plasmid DNA (intermediate vector: this vector has an artificial T-region consisting of the left and right border sequences of the TL-DNA from pTiB6S3 and multilinker cloning sites allowing the insertion of chimeric genes between the T-DNA border repeats and was used to construct the pTHW107 plasmid), digested with HindIII.
- Genomic DNA prepared from a non-transgenic plant is used as a DNA negative control (NON). When no hybridizing fragments are observed, this indicates that there is no background hybridization in a transgenic genomic DNA sample.
- The Nylon membrane was sequentially hybridized with the two 'vector' probes.

Loading order of the agarose gel

1. Phage Lambda DNA - PstI
2. MS8 undigested
3. MS8 - EcoRI
4. MS8 - EcoRV
5. wild-type control DNA - HindIII
6. wild-type control DNA + 0.1 copies pTHW100 - HindIII
7. wild-type control DNA + 1 copy pTHW100 - HindIII
8. wild-type control DNA + 0.1 copies pTHW107 - HindIII
9. wild-type control DNA + 1 copy pTHW107 - HindIII
10. Phage Lambda DNA - PstI

9

Probes

- Some of the DNA templates for probe preparation were synthesized by means of PCR, using the Expand™ High Fidelity PCR system (Boehringer Mannheim). The primers used for amplification of specific fragments are listed in table 1. One ng of linearized pTHW107 plasmid (EcoRV digest) and 20 pmoles of upstream and downstream primers were mixed in a 50 µl PCR reaction containing 3.2 mM Tris-HCl (pH7.5), 16 mM KCl, 0.16 mM DTT, 16 µM EDTA, 0.08% Nonidet® P40 (v/v), 8% glycerol (v/v), 200 µM of each deoxyribonucleoside triphosphate and 8 units enzyme mix. The reaction mixture was covered with 50 µl mineral oil. For each specific amplification 10 individual 50 µl PCR reactions were set up.

  Thermocycling conditions used:

  4 min. at 95°C

  followed by: 1 min. at 95°C
  1 min. at 57°C
  2 min. at 68°C
  for 5 cycles

  followed by: 15 sec. at 95°C
  45 sec. at 60°C
  2 min. at 68°C
  for 22 cycles

  followed by: 10 min. at 68°C

  On completion of the PCR, samples were pooled, dried down and resuspended in 75 µl water. Pooled samples were loaded on a preparative 1% agarose gel (1 x TAE buffer). On completion of the electrophoresis, the size of the amplified fragment was verified and the fragment was cut from the gel with a scalpel. The DNA was recovered from the gel slice using the Microcon 50 concentrators (Amicon). The concentration of the recovered DNA sample was estimated by loading a small amount of the sample on a 1% agarose gel and comparing band intensities with a know standard (Low DNA mass ladder, Life technologies).

Table 1: Primers used to amplify vector sequences of pTHW107

Primers	Sequence (5' --> 3')	Position in pTHW107
MLD001		bp 105 --> bp 85
MLD002		bp 11565 --> bp 11586
MDB469		bp 6949 --> bp 6968
MLD004		bp 11595 -->11573

To avoid confusion between bases, a lower-case 'g' is used to clearly differentiate between 'g' and 'C'.

- The Sm/Sp DNA template used for probe preparation was isolated from the intermediate vector pTHW100 by restriction enzyme digesting.

  pTHW100 HindIII - XbaI 1907 bp fragment (Sm/Sp):

  10 µg pTHW100 DNA was digested overnight with HindIII and XbaI (New England Biolabs; applying concentration, buffers and temperature according to the conditions proposed by the manufacturer of the enzymes). One µg of the digest was checked on a 1% agarose gel. The digest resulted in 3 fragments having the expected sizes: 5465 bp, 1907 bp and 450 bp. The rest of the digest was loaded on a preparative 1% agarose gel and the 1907 bp fragment was cut from the gel with a scalpel. The DNA was recovered from the gel slice using the Microcon 50 concentrators (Amicon). The concentration of the recovered 1907 bp fragment was estimated by loading a small amount of the sample on a 1% agarose gel and comparing band intensities with a know standard (Low DNA mass ladder, Life technologies).

Table 2: Probes used for Southern blot analysis

Probe	Features	Position in pTHW107	Length
MLD001-MLD002	ori pBR322	bp 105 --> bp 11565	1213 bp
MDB469-MLD004	ori pVS1 + barstar	bp 11595 --> bp 6949	4645 bp
1907bp HindIII/XbaI	Sm/Sp	bp 5109 --> bp 7016	1907 bp

Data interpretation

Data from transgenic plant DNA samples will not be acceptable unless the DNA positive controls (POS1 and POS2) show the expected hybridizing fragments.
Lanes with transgenic plant DNA samples not showing visible hybridizing fragments, indicate that the corresponding plant from which the genomic DNA template was prepared has not inherited the sequences assayed for.

Results

For the molecular verification of the absence of pTHW107 vector sequences, a number of overlapping probes were prepared (see figure 1 and table 2).

With the three probes used, the assigned negative control showed no background hybridization and the assigned positive controls showed hybridizing fragments of the expected sizes (see figures 2, 3 and 4).

With the MLD001-MLD002 probe (ori pBR322), no hybridizing fragments could be observed in the lanes with transgenic plant DNA samples (see figure 2, lanes 2, 3 and 4). This probe hybridized to the expected 5915 bp HindIII fragment of pTHW100 and the 6815 bp HindIII fragment of pTHW107 (see figure 2, lanes 6, 7, 8 and 9).

With the MDB469-MLD004 probe (ori pVS1 + barstar), no hybridizing fragments could be observed in the lanes with transgenic plant DNA samples (see figure 3, lanes 2, 3 and 4). This probes hybridized to the expected pTHW100 and pTHW107 HindIII fragments (see figure 3, lanes 6, 7, 8 and 9).

With the 1907 bp HindIII/XbaI probe (Sm/Sp), no hybridizing fragments could be observed in the lanes with transgenic plant DNA samples (see figure 4, lanes 2, 3 and 4). This probe hybridized to the expected 1915 bp HindIII fragment of pTHW100 and pTHW107 (see figure 4, lanes 7 and 9).

Conclusion

By means of Southern blot analysis, we demonstrated that there are no sequences from the 'vector'-part of the transforming pTHW107 plasmid integrated in elite event MS8. The reliability of the method was validated by the controls used in every analysis.

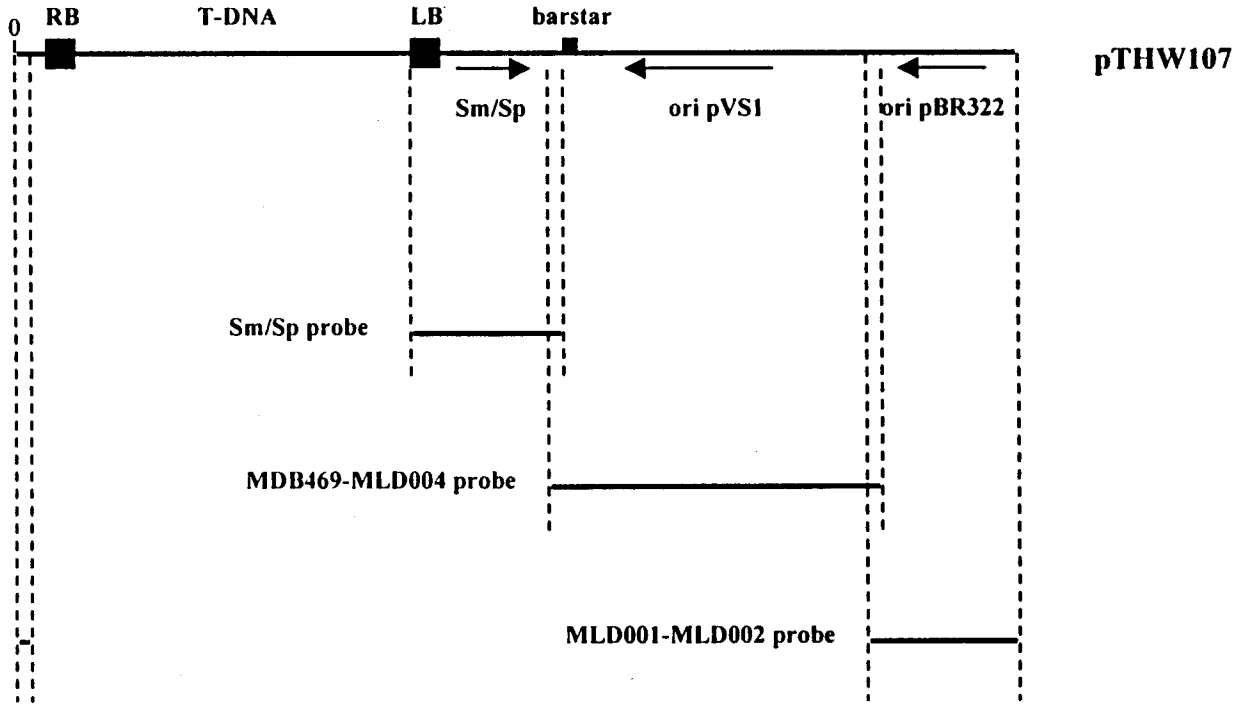

Figure 1: Schematic drawing of the Southern blot analysis strategy

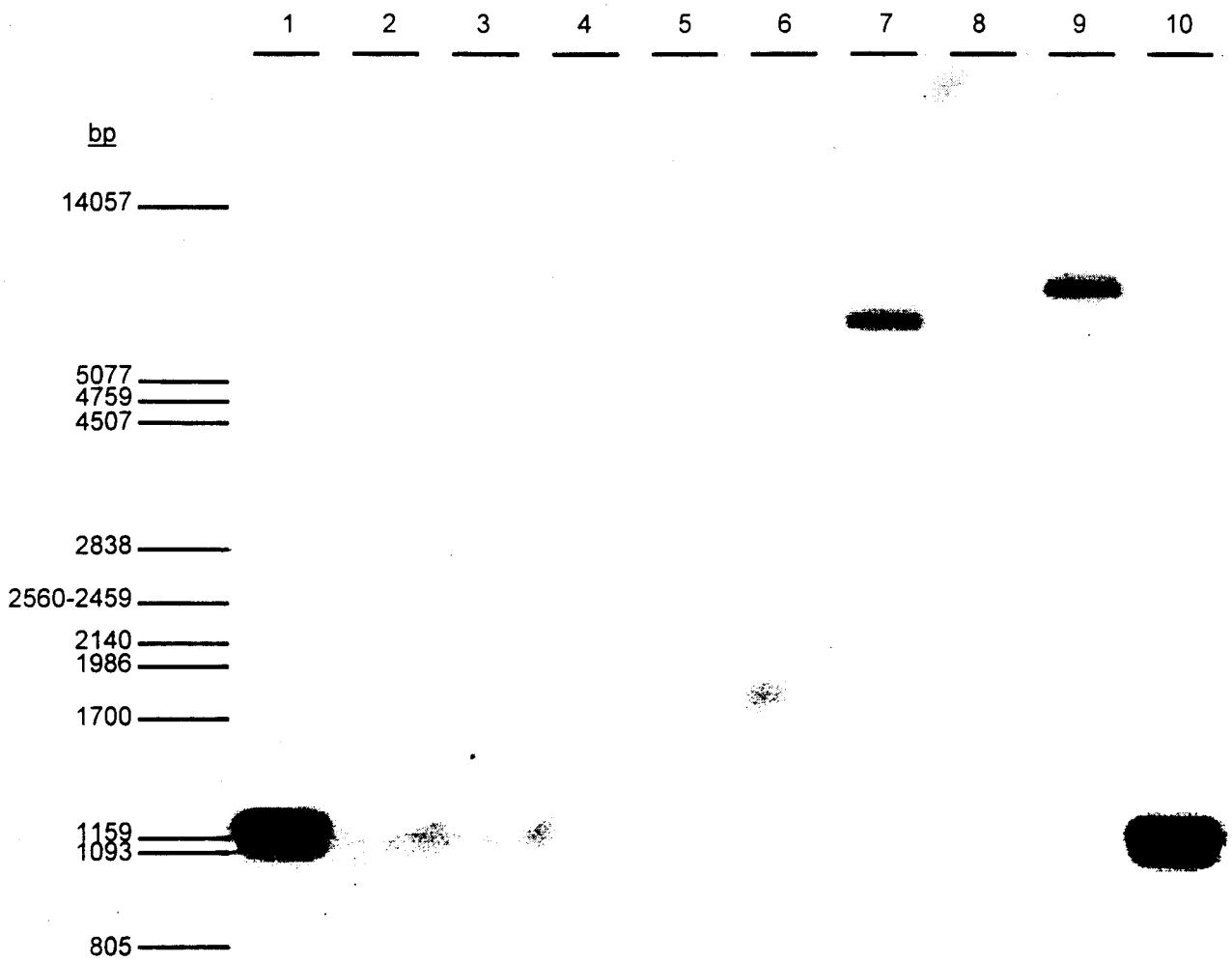

Figure 2: **Southern blot analysis - MLD001-MLD002 probe**

probe: 1213 bp MLD001-MLD002 fragment (ori pBR322)
Lane 1. Phage Lambda DNA: PstI
Lane 2. MS8 DNA: undigested
Lane 3. MS8 DNA: EcoRI
Lane 4. MS8 DNA: EcoRV
Lane 5. Wild-type control DNA: HindIII
Lane 6. Wild-type control DNA + 0.1 copy pTHW100: HindIII
Lane 7. Wild-type control DNA + 1 copy pTHW100: HindIII
Lane 8. Wild-type control DNA + 0.1 copy pTHW107: HindIII
Lane 9. Wild-type control DNA + 1 copy pTHW107: HindIII
Lane 10. Phage Lambda DNA: PstI

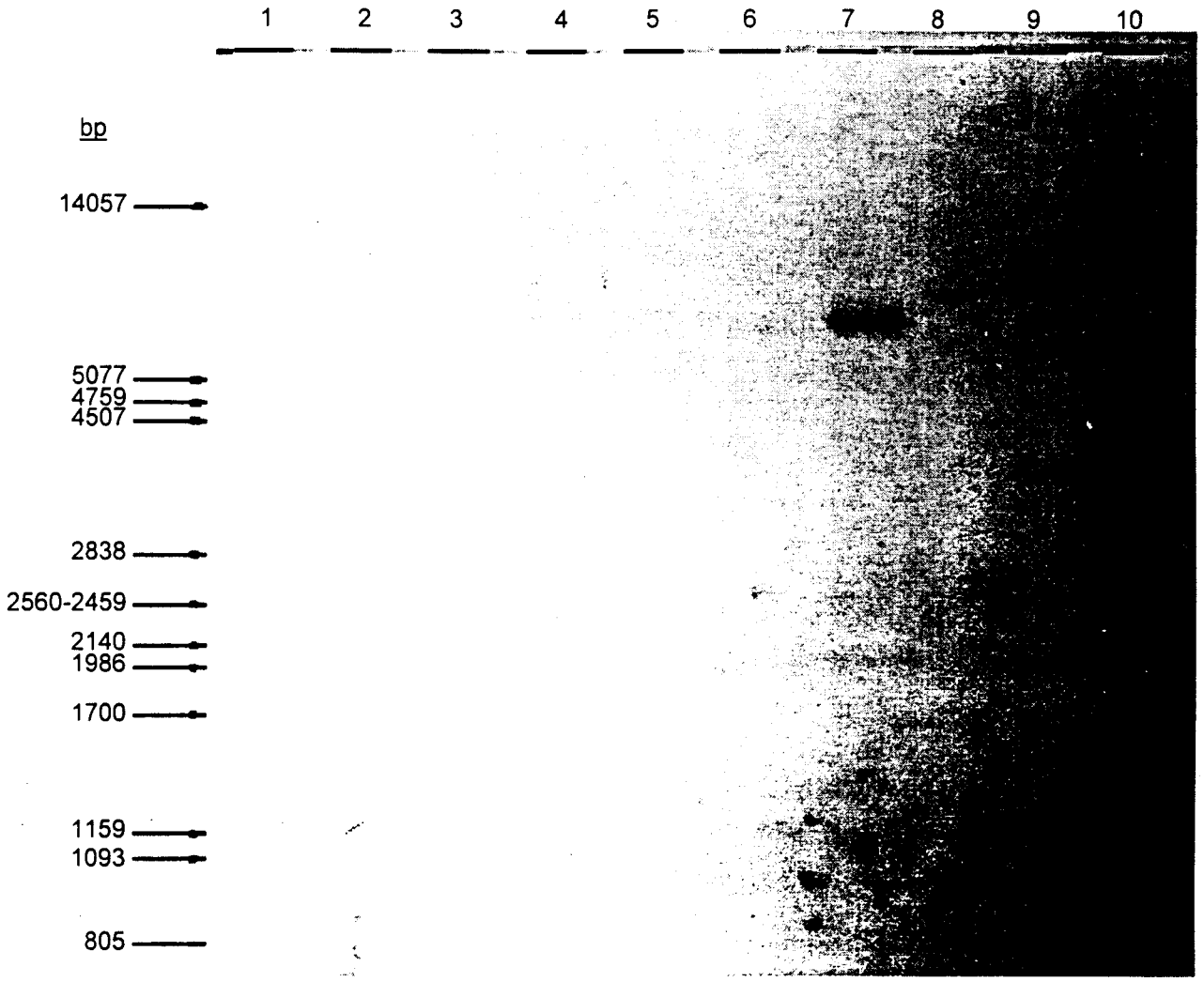

Figure 3: **Southern blot analysis - MDB469-MLD004 probe**

probe: 4645 bp MDB469-MLD004 fragment (ori pVS1+barstar)
Lane 1. Phage Lambda DNA: PstI
Lane 2. MS8 DNA: undigested
Lane 3. MS8 DNA: EcoRI
Lane 4. MS8 DNA: EcoRV
Lane 5. Wild-type control DNA: HindIII
Lane 6. Wild-type control DNA + 0.1 copy pTHW100: HindIII
Lane 7. Wild-type control DNA + 1 copy pTHW100: HindIII
Lane 8. Wild-type control DNA + 0.1 copy pTHW107: HindIII
Lane 9. Wild-type control DNA + 1 copy pTHW107: HindIII
Lane 10. Phage Lambda DNA: PstI

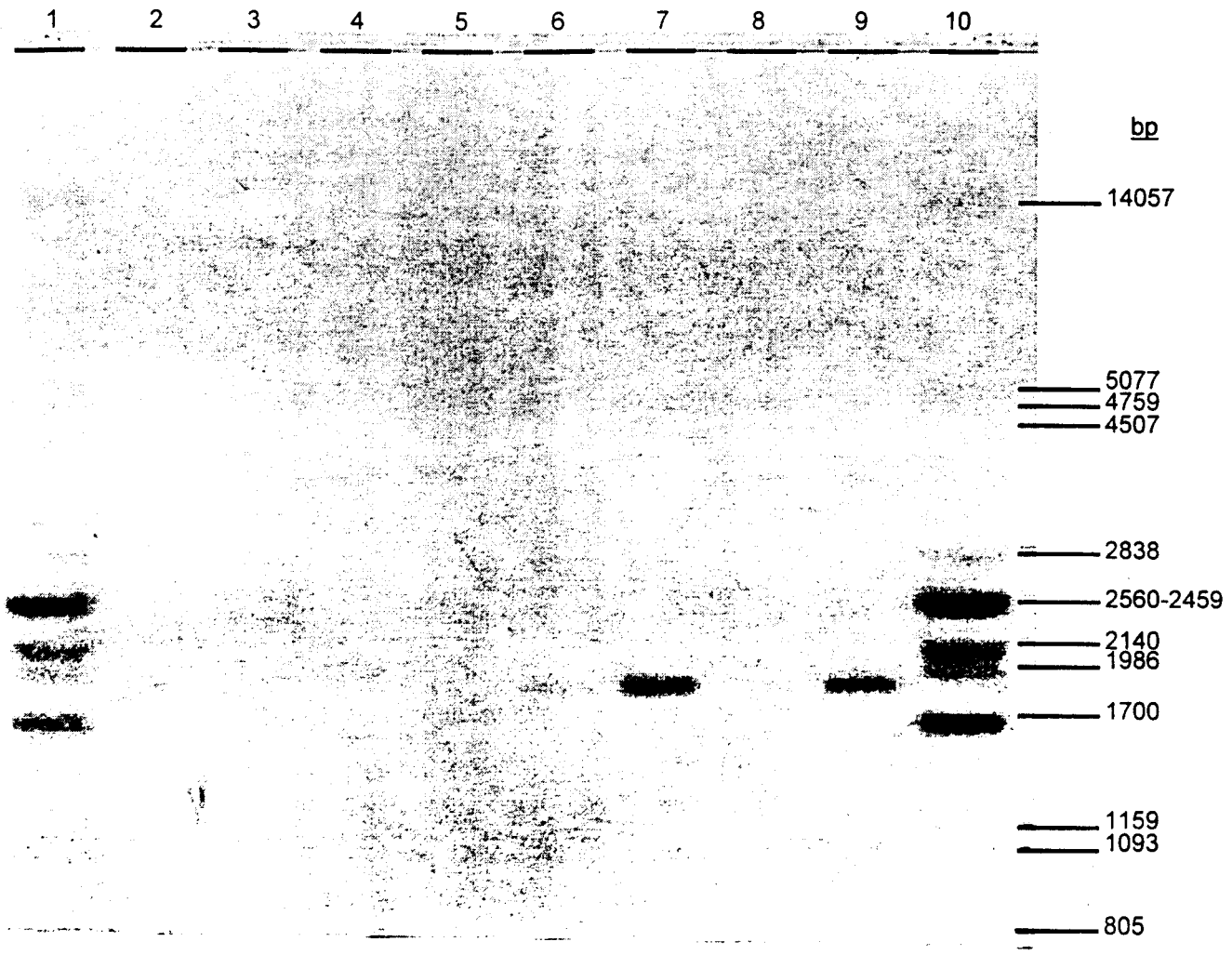

Figure 4: **Southern blot analysis - Sm/Sp probe**

probe: 1907 bp HindIII-XbaI fragment (Sm/Sp)
Lane 1. Phage Lambda DNA: PstI
Lane 2. MS8 DNA: undigested
Lane 3. MS8 DNA: EcoRI
Lane 4. MS8 DNA: EcoRV
Lane 5. Wild-type control DNA: HindIII
Lane 6. Wild-type control DNA + 0.1 copy pTHW100: HindIII
Lane 7. Wild-type control DNA + 1 copy pTHW100: HindIII
Lane 8. Wild-type control DNA + 0.1 copy pTHW107: HindIII
Lane 9. Wild-type control DNA + 1 copy pTHW107: HindIII
Lane 10. Phage Lambda DNA: PstI

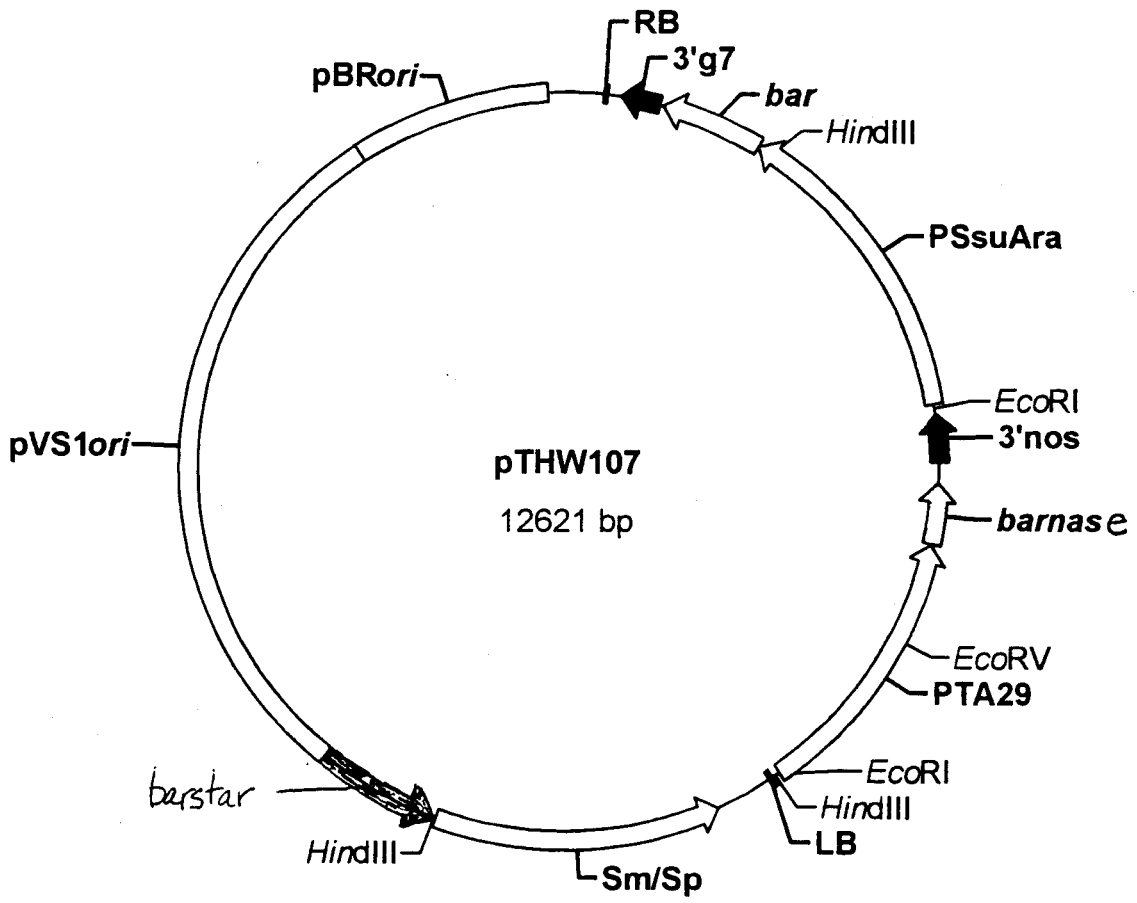

Figure 1: Circular plasmid map of construct pTHW107 indicating restriction sites of EvoRI, EcoRV and HindIII

RF3 - Proof of absence of sequences derived from the 'vector'-part of the construct.

Responsible De Beuckeleer Marc, Senior Researcher
Lecleir Machteld, Technician

Study completed November 3, 1997
Study no **PGS,MDB,03.11.97,RF3**

Testing facility Plant Genetic Systems NV
Jozef Plateaustraat 22
B-9000 Gent, Belgium
Phone: (32)(9)235.84.11
Telefax: (32)(9)224.06.94
Email: pgs@pgsgent.be

Goal of the experiment

The aim of the experiment is to demonstrate, by means of Southern blot and PCR analysis, the absence of sequences derived from the 'vector'-part of the pTHW118 plasmid.

Plant material

SB and PCR analysis has been performed on genomic DNA isolated from RF3 plants carrying the restorer of fertility gene. Genomic DNA isolated from a non-transgenic plant has been used as control.

Analysis strategy

The vector sequences outside the T-DNA borders of pTHW118 comprises the following structural elements:

- the plasmid core comprising the origin of replication from the plasmid pBR322 for replication in *Escherichia coli* and a restriction fragment comprising the origin of replication from the *Pseudomonas* plasmid pVS1 for replication in *Agrobacterium tumefaciens*

- a selectable marker gene conferring resistance to streptomycin and spectinomycin for propagation and selection of the plasmid in *Escherichia coli* and *Agrobacterium tumefaciens*

- a barstar gene with regulatory signals for expression in *E.coli.*

All parts of the pTHW118 vector, except for the barstar gene, are used as DNA probes in SB analysis: a 1213 bp (MLD001-MLD002) fragment comprising the origin of replication from pBR322, a 4047 bp (MLD003-MLD004) fragment comprising the

origin of replication from pVS1 and a 1907 bp HindIII-XbaI fragment comprising the Sm/Sp selectable marker gene (see table 2).

Absence of the barstar gene with regulatory signals for expression in *E.coli* is demonstrated by means of PCR (see tables 3 and 4).

Southern Blot Analysis

Method

Southern blot analysis has been performed using probes covering the complete 'vector'-part, except for the barstar gene, of the transforming plasmid.

Total genomic DNA was isolated from leaf tissue according to Dellaporta et al. (1983, Plant Molecular Biology Reporter, 1, vol.3, p. 19-21). 10 µg of genomic DNA was digested with selected restriction enzymes, applying concentration, buffers and temperature according to the conditions proposed by the manufacturer. Upon termination of digestion, DNA fragments were separated by agarose gel-electrophoresis. The separated DNA fragments were transferred upon denaturation, through capillary force from the agarose gel to a Nylon membrane. Vector DNA templates were labelled using the 'Rediprime DNA labelling system' from Amersham International Inc. Hybridization and washing steps were carried out according to Sambrook et al. (Molecular cloning, a laboratory manual). After the washing was completed, autoradiography was established by exposing the membrane to a sheet of Kodak X-Omat AR film.

Assigned positive and negative controls to a Southern blot analysis

Since the analysis presented in this study draws upon negative evidence (i.e. the evidence on absence of specific DNA sequences), the results can only be used as a valid proof in case the correct controls have been used.

- As a DNA positive control (POS) we used HindIII digested genomic DNA prepared from a non-transgenic plant, supplemented with total pTHW100 plasmid DNA (intermediate vector: this vector has an artificial T-region consisting of the left and right border sequences of the TL-DNA from pTiB6S3 and multilinker cloning sites allowing the insertion of chimeric genes between the T-DNA border repeats and was used to construct the pTHW118 plasmid), digested with HindIII.
- Genomic DNA prepared from a non-transgenic plant is used as a DNA negative control (NON). When no hybridizing fragments are observed, this indicates that there is no background hybridization in a transgenic genomic DNA sample.
- The Nylon membrane was sequentially hybridized with the three 'vector' probes.

Loading order of the agarose gel

1. Phage Lambda DNA - PstI
2. RF3 undigested
3. RF3 - EcoRI
4. RF3 - EcoRV
5. wild-type control DNA - HindIII
6. wild-type control DNA + 0.1 copies pTHW100 - HindIII
7. wild-type control DNA + 1 copy pTHW100 - HindIII

Probes

- Some of the DNA templates for probe preparation were synthesized by means of PCR, using the Expand™ High Fidelity PCR system (Boehringer Mannheim). The primers used for amplification of specific fragments are listed in table 1. Oneng of linearized pTHW118 plasmid (EcoRV digest) and 20 pmoles of upstream and downstream primers were mixed in a 50 µl PCR reaction containing 3.2 mM Tris-HCl (pH7.5), 16 mM KCl, 0.16 mM DTT, 16 µM EDTA, 0.08% Nonidet® P40 (v/v), 8% glycerol (v/v), 200 µM of each deoxyribonucleoside triphosphate and 8 units enzyme mix. The reaction mixture was covered with 50 µl mineral oil. For each specific amplification 10 individual 50 µl PCR reactions were set up.

  Thermocycling conditions used:

  4 min. at 95°C

  followed by: 1 min. at 95°C
  1 min. at 57°C
  2 min. at 68°C
  for 5 cycles

  followed by: 15 sec. at 95°C
  45 sec. at 60°C
  2 min. at 68°C
  for 22 cycles

  followed by: 10 min. at 68°C

On completion of the PCR, samples were pooled, dried down and resuspended in 75 µl water. Pooled samples were loaded on a preparative 1% agarose gel (1 x TAE buffer). On completion of the electrophoresis, the size of the amplified fragment was verified and the fragment was cut from the gel with a scalpel. The DNA was recovered from the gel slice using the Microcon 50 concentrators (Amicon). The concentration of the recovered DNA sample was estimated by loading a small amount of the sample on a 1% agarose gel and comparing band intensities with a know standard (Low DNA mass ladder, Life technologies).

Table 1: Primers used to amplify vector sequences of pTHW118

Primers	Sequence (5' --> 3')	Position in pTHW118
MLD001		bp 105 --> bp 85
MLD002		bp 11448 --> bp 11469
MLD003		bp 7430 --> bp 7451
MLD004		bp 11477 -->11456

To avoid confusion between bases, a lower-case 'g' is used to clearly differentiate between 'g' and 'C'.

- The Sm/Sp DNA template used for probe preparation was isolated from the intermediate vector pTHW100 by restriction enzyme digesting.

  pTHW100 HindIII - XbaI 1907 bp fragment (Sm/Sp):

  10 µg pTHW100 DNA was digested overnight with HindIII and XbaI (New England Biolabs; applying concentration, buffers and temperature according to the conditions proposed by the manufacturer of the enzymes). One µg of the digest was checked on a 1% agarose gel. The digest resulted in 3 fragments having the expected sizes: 5465 bp, 1907 bp and 450 bp. The rest of the digest was loaded on a preparative 1% agarose gel and the 1907 bp fragment was cut from the gel with a scalpel. The DNA was recovered from the gel slice using the Microcon 50 concentrators (Amicon). The concentration of the recovered 1907 bp fragment was estimated by loading a small amount of the sample on a 1% agarose gel and comparing band intensities with a know standard (Low DNA mass ladder, Life technologies).

Table 2: Probes used for Southern blot analysis

Probe	Features	Position in pTHW118	Length
MLD001-MLD002	ori pBR322	bp 105 --> bp 11448	1213 bp
MLD003-MLD004	ori pVS1	bp 7430 --> bp 11477	4047 bp
1907bp HindIII/XbaI	Sm/Sp	bp 4992 --> bp 6899	1907 bp

Data interpretation

Data from transgenic plant DNA samples will not be acceptable unless the DNA positive control (POS) shows the expected hybridizing fragments.
Lanes with transgenic plant DNA samples not showing visible hybridizing fragments, indicate that the corresponding plant from which the genomic DNA template was prepared has not inherited the sequences assayed for.

Southern blot results

For the molecular verification of the absence of pTHW118 vector sequences, a number of overlapping probes were prepared (see figure 1 and table 2).

With the three probes used, the assigned negative control showed no background hybridization and the assigned positive control showed hybridizing fragments of the expected sizes (see figures 2, 3 and 4).

With the MLD001-MLD002 probe (ori pBR322), no hybridizing fragments could be observed in the lanes with transgenic plant DNA samples (see figure 2, lanes 2, 3 and 4). This probe hybridized to the expected 5915 bp HindIII fragment of pTHW100 (see figure 2, lanes 6 and 7).

With the MLD003-MLD004 probe (ori pVS1), no hybridizing fragments could be observed in the lanes with transgenic plant DNA samples (see figure 3, lanes 2, 3 and 4). This probes hybridized to the expected pTHW100 HindIII fragment (see figure 3, lanes 6 and 7).

With the 1907 bp HindIII/XbaI probe (Sm/Sp), no hybridizing fragments could be observed in the lanes with transgenic plant DNA samples (see figure 4, lanes 2, 3 and 4). This probe hybridized to the expected 1915 bp HindIII fragment of pTHW100 (see figure 4, lanes 6 and 7).

PCR analysis

Method

The absence of barstar sequences with regulatory signals for expression in *E. coli* was verified by PCR analysis.
Three primer-combinations were used to perform the PCR analysis (see table 3). Primers targeting T-DNA sequences at the RB (MDB185 and MDB251) are included to serve as an internal control.

5µl of diluted isolated plant DNA (50ng) was used in a 50µl PCR reaction containing 10mM Tris-HCl (pH8.3); 50mM KCl; 1.5mM Mg Cl_2; 200µM of each dNTP; 1.25 units Taq DNA polymerase (Pharmacia); 10pmoles of MDB185 and MDB251; 10pmoles of primers MDB469 and MDB470 (or MDB8 and MDB469, or MDB9 and MDB470).
A master mix of reagents (water, buffer, dNTP's, primers and enzyme) was prepared for all samples and then aliquoted to the individual samples. The reaction mixtures were overlayed with 50µl mineral oil and thermocycling is started.

Assigned positive and negative controls to a PCR run

- a DNA positive control (POS): this is a PCR in which the template DNA provided is genomic DNA prepared from a wild-type oilseed rape plant supplemented with one copy of pTHW118 plasmid DNA. Successful amplification of the positive control demonstrates that the PCR was run under conditions which allow for the satisfactory amplification of the target sequences.
- a DNA negative control (NEG): this is a PCR in which no DNA is added to the reaction. When the expected result (no PCR product) is observed this indicates that the PCR enzyme master mix was not contaminated with target DNA.
- a negative control (NON): this is a PCR in which the template DNA provided is genomic DNA prepared from a wild-type oilseed rape plant. When the expected result (no target PCR product) is observed this indicates that there is no detectable background amplification in genomic DNA samples known not to contain the target sequence.

Thermocycling profile

4 minutes at 95°C

Followed by:
1 minute at 95°C
1 minute at 57°C
2 minutes at 72°C
For 5 cycles

Followed by: 30 seconds at 92°C
30 seconds at 57°C
1 minute at 72°C
For 22 cycles

Followed by: 10 minutes at 72°C

Table 3: Primers used for PCR analysis

Primer	Position in pTHW118	Sequence (5' --> 3')
MDB185	bp 345 --> bp 365	
MDB251	bp 519 --> bp 495	
MDB469	bp 6832 --> bp 6851	
MDB470	bp 7387 --> bp 7376	
MDB8	bp 7194 --> bp 7172	
MDB9	bp 6960 --> bp 6851	

To avoid confusion between bases, a lower-case 'g' is used to clearly differentiate between 'g' and 'C'.

Table 4: Primer-combinations used for PCR analysis

Combinations	Fragment lengths	Target sequences
MDB469-MDB470	555 bp	Complete barstar
MDB185-MDB251	174 bp	T-DNA
MDB469-MDB8	362 bp	barstar + downstream sequences
MDB185-MDB251	174 bp	T-DNA
MDB470-MDB9	427 bp	barstar + upstream sequences
MDB185-MDB251	174 bp	T-DNA

Agarose gel analysis

20µl of each PCR sample was separated on a 1.5% agarose gel. A 100bp MW ladder (Pharmacia) was used as a molecular weight marker.

Loading order of the agarose gel (see figure 5)

1. MW marker (100bp ladder Pharmacia)
2. RF3
3. Control, non-transgenic plant (NON)
4. Control, non-transgenic plant + pTHW118 (POS)
5. water (NEG)
6. MW marker (100bp ladder Pharmacia)
7. RF3
8. Control, non-transgenic plant (NON)
9. Control, non-transgenic plant + pTHW118 (POS)
10. water (NEG)

11. MW marker (100bp ladder Pharmacia)
12. RF3
13. Control, non-transgenic plant (NON)
14. Control, non-transgenic plant + pTHW118 (POS)
15. water (NEG)
16. MW marker (100bp ladder Pharmacia)

Lanes 2, 3, 4 and 5: MDB469-MDB470-MDB185-MDB251
Lanes 7, 8, 9 and 10: MDB469-MDB8-MDB185-MDB251
Lanes 12, 13, 14 and 15: MDB470-MDB9-MDB185-MDB251

Data interpretation

Data from transgenic plant DNA samples within a single PCR run and a single PCR cocktail will not be acceptable unless the DNA positive control (POS) shows the expected PCR products, and both the DNA negative control (NEG) and the wildtype DNA control (NON) are negative for PCR amplification.
Lanes showing visible amounts of the expected size PCR product, when compared to the molecular weight marker, indicate that the corresponding plant from which the genomic template DNA was prepared, has inherited the sequence assayed for. Lanes not showing visible amounts of the expected size PCR product, indicate that the corresponding plant from which the genomic DNA template was prepared, has not inherited the sequence assayed for.

PCR results

The assigned negative controls (NEG and NON) both showed the expected results with every primer combination used (no PCR product).
The assigned positive control showed the expected results for each PCR cocktail used: amplification of the targeted vector sequence and of the internal T-DNA fragment.
Primer-pair MDB185-MDB251 amplified the internal T-DNA sequence in the transgenic DNA samples, demonstrating that there is ample DNA of adequate quality in the genomic DNA preparation (see figure 5). Obtained data for transgenic plant DNA samples can therefore be accepted.
The internal T-DNA fragment was amplified in the transgenic DNA samples with the three tested primer combinations. These primer combinations failed to amplify vector sequences in the transgenic DNA samples.

The PCR analysis demonstrates that sequences comprised between bp 2107 and bp 2662 from pTHW100 are not integrated in the RF3 elite event.

General conclusion

By means of Southern blot and PCR analysis, we demonstrated that there are no sequences from the 'vector'-part of the transforming pTHW118 plasmid integrated in elite event RF3. The reliability of both methods was validated by the controls used in every analysis.

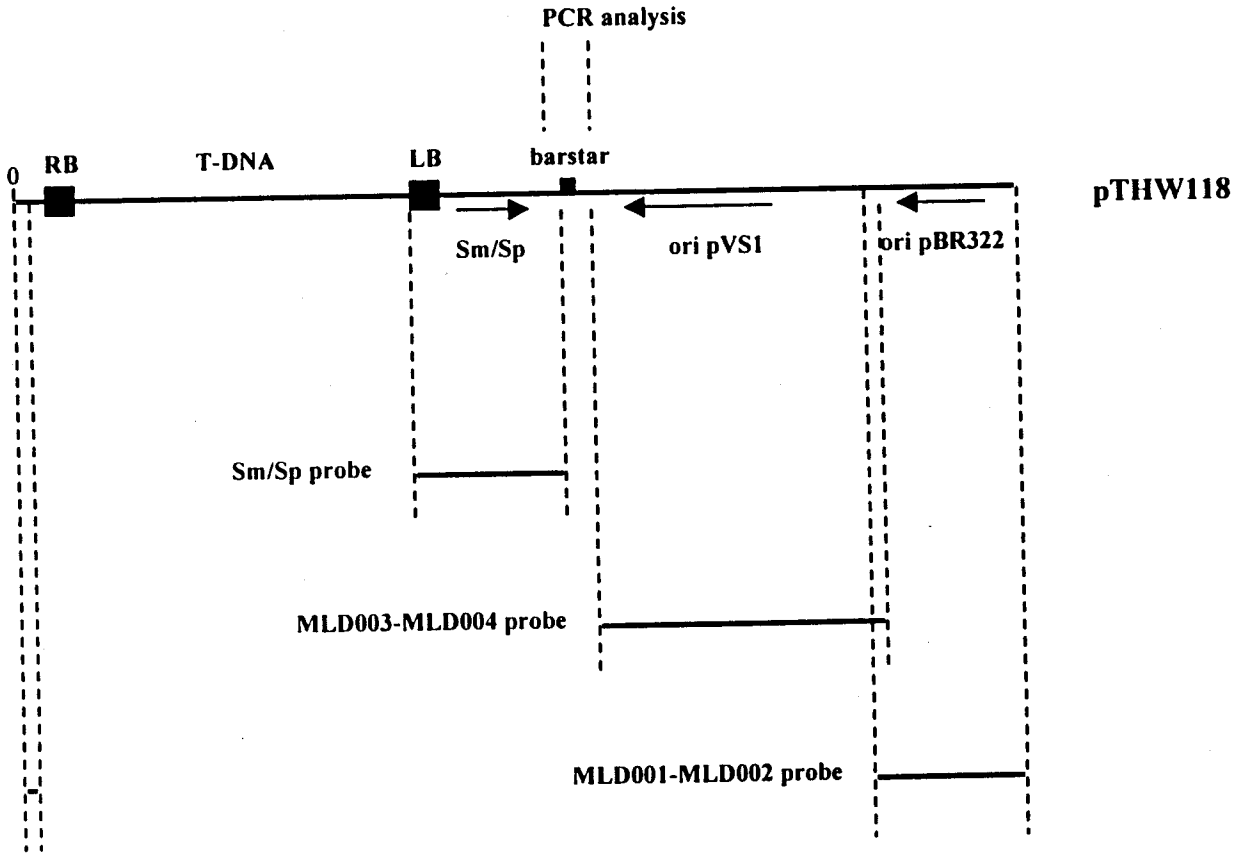

Figure 1: Schematic drawing of the Southern blot and PCR analysis strategy

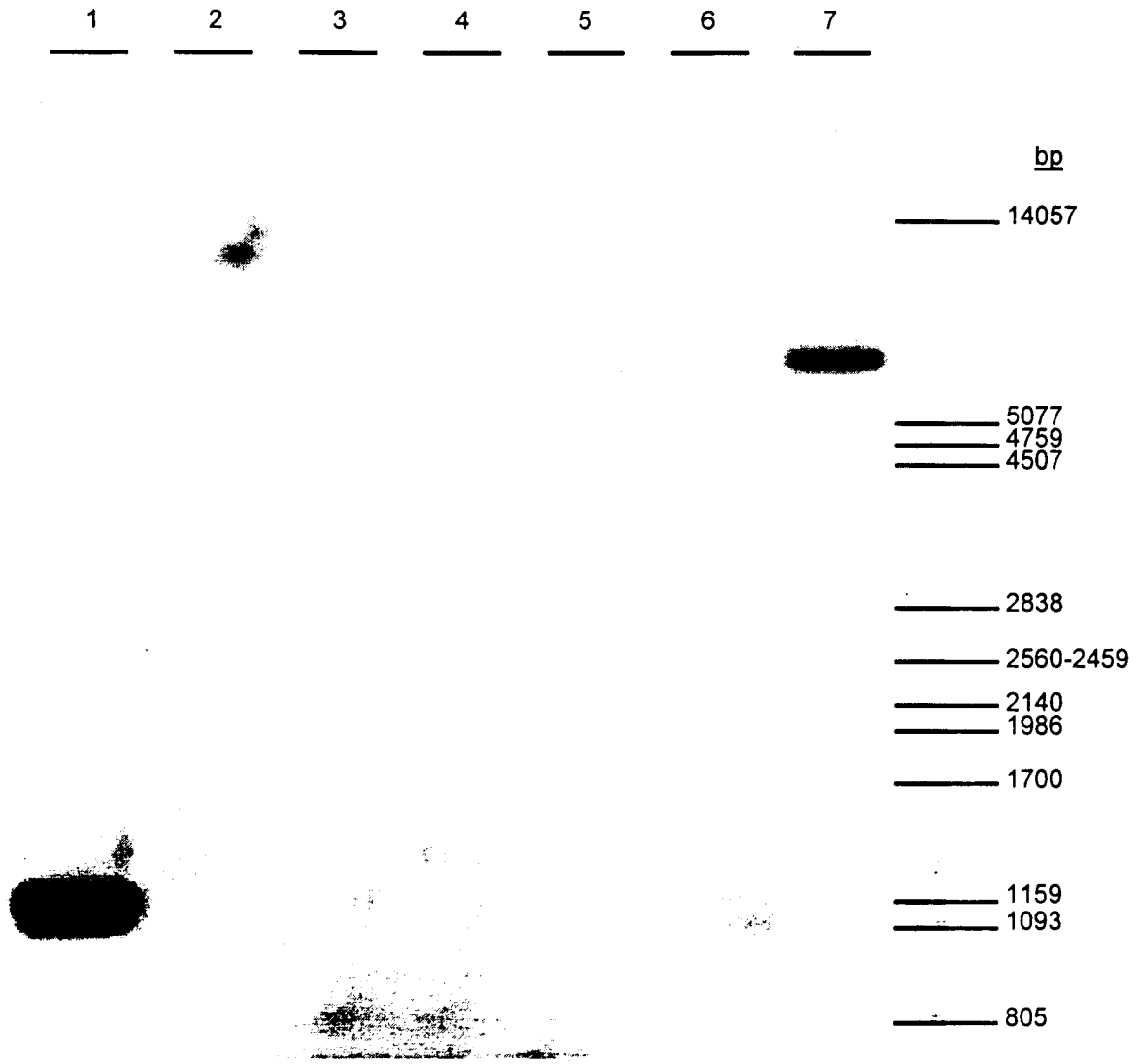

Figure 2: **Southern blot analysis - MLD001-MLD002 probe**

probe: 1213 bp MLD001-MLD002 fragment (ori pBR322)
Lane 1. Phage Lambda DNA: PstI
Lane 2. RF3 DNA: undigested
Lane 3. RF3 DNA: EcoRI
Lane 4. RF3 DNA: EcoRV
Lane 5. Wild-type control DNA: HindIII
Lane 6. Wild-type control DNA + 0.1 copy pTHW100: HindIII
Lane 7. Wild-type control DNA + 1 copy pTHW100: HindIII

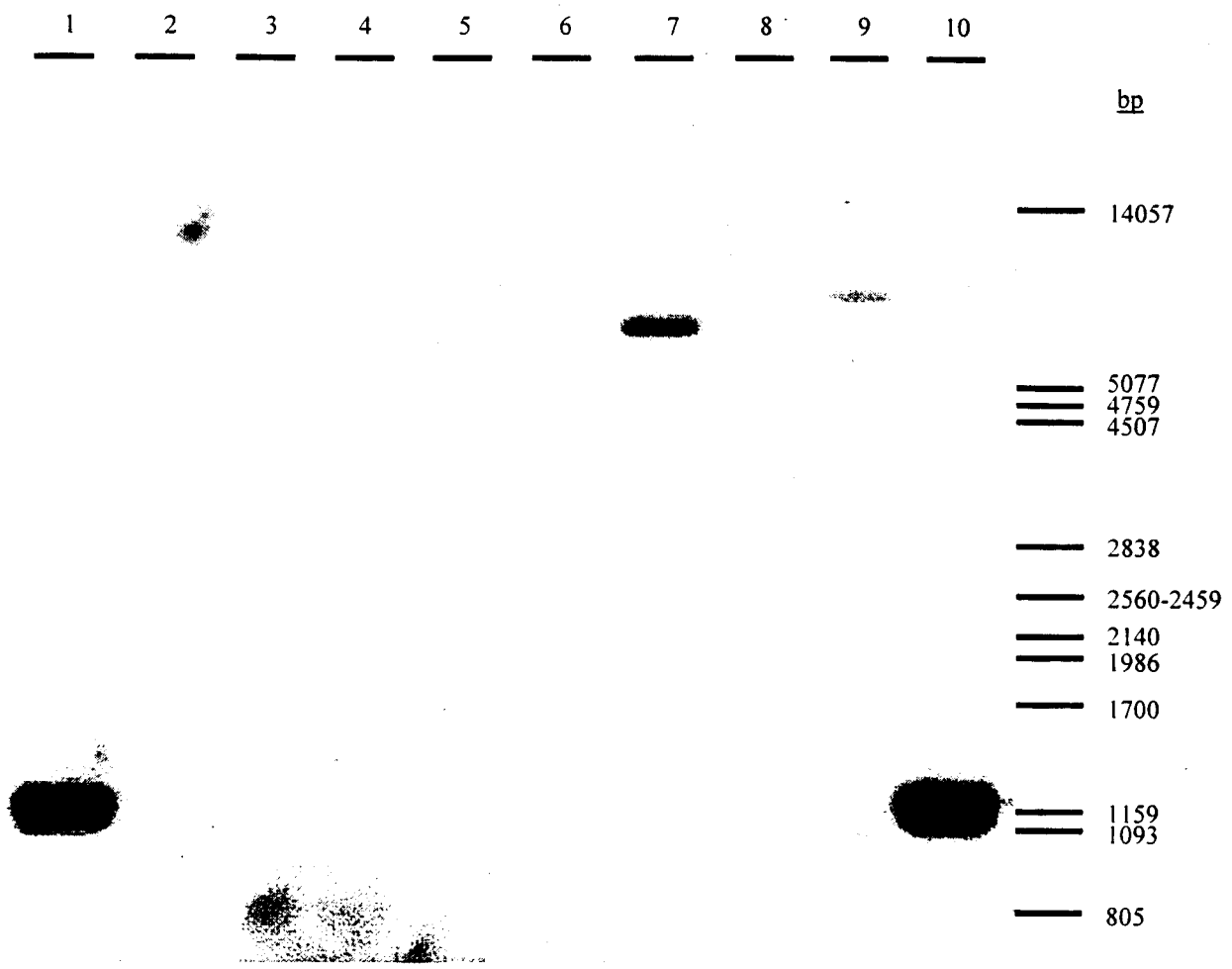

Figure ~~xxx~~ 2 : Rf3 hybridization results

probe : pTHW100, 1213 bp MLD001-MLD002 fragment
Lane 1. Molecular Weight Marker, Lambda DNA-PstI
Lane 2. Rf3 DNA - undigested
Lane 3. Rf3 DNA - EcoRI
Lane 4. Rf3 DNA - EcoRV
Lane 5. Wild Type DNA - HindIII
Lane 6. Wild Type DNA - HindIII + 0.1 copy pTHW100 - HindIII
Lane 7. 1 copy pTHW100 - HindIII
Lane 8. Wild Type DNA - HindIII + 0.1 copy pTHW118 - HindIII
Lane 9. 1 copy pTHW118 - HindIII
Lane 10. Molecular Weight Marker, Lambda DNA-PstI

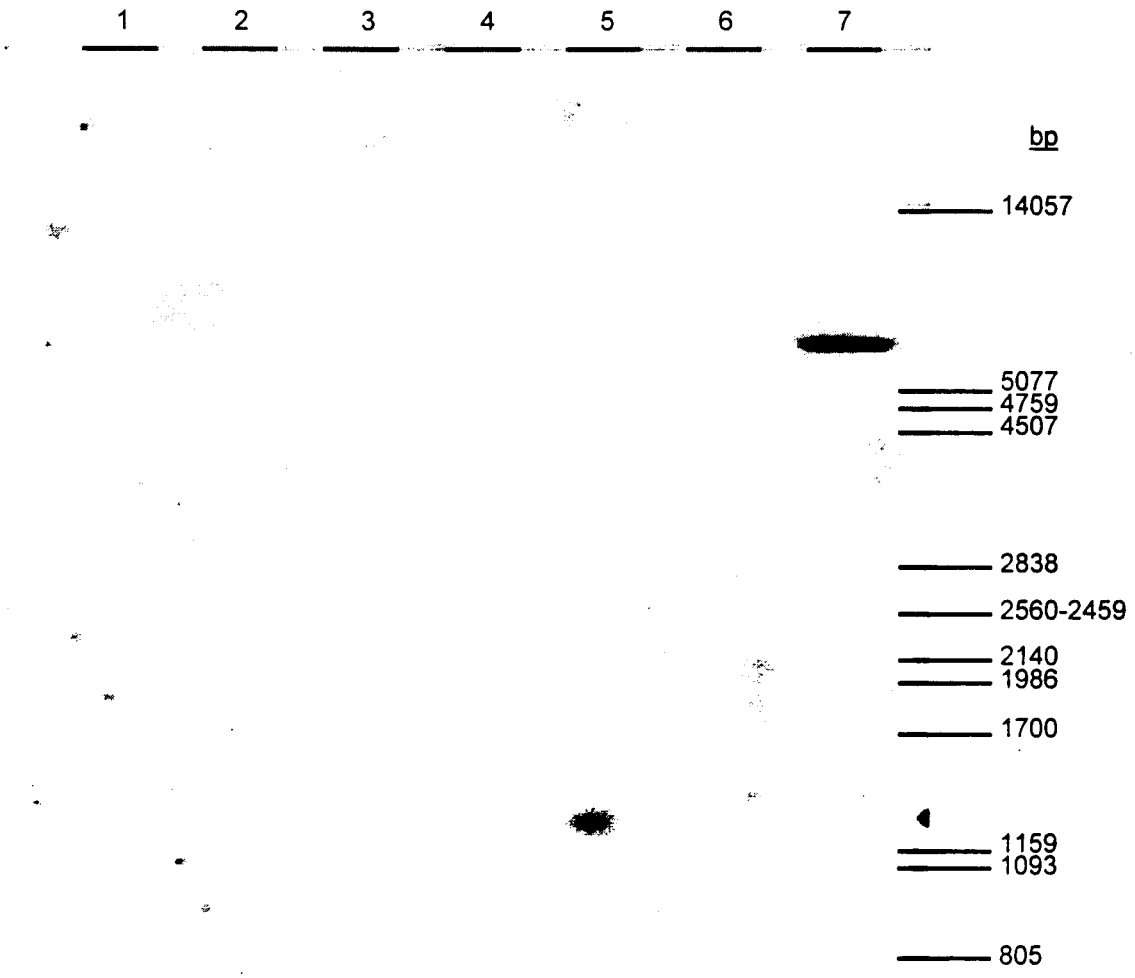

Figure 3: **Southern blot analysis - MLD003-MLD004 probe**

probe: 4047 bp MLD003-MLD004 fragment (ori pVS1)
Lane 1. Phage Lambda DNA: PstI
Lane 2. RF3 DNA: undigested
Lane 3. RF3 DNA: EcoRI
Lane 4. RF3 DNA: EcoRV
Lane 5. Wild-type control DNA: HindIII
Lane 6. Wild-type control DNA + 0.1 copy pTHW100: HindIII
Lane 7. Wild-type control DNA + 1 copy pTHW100: HindIII

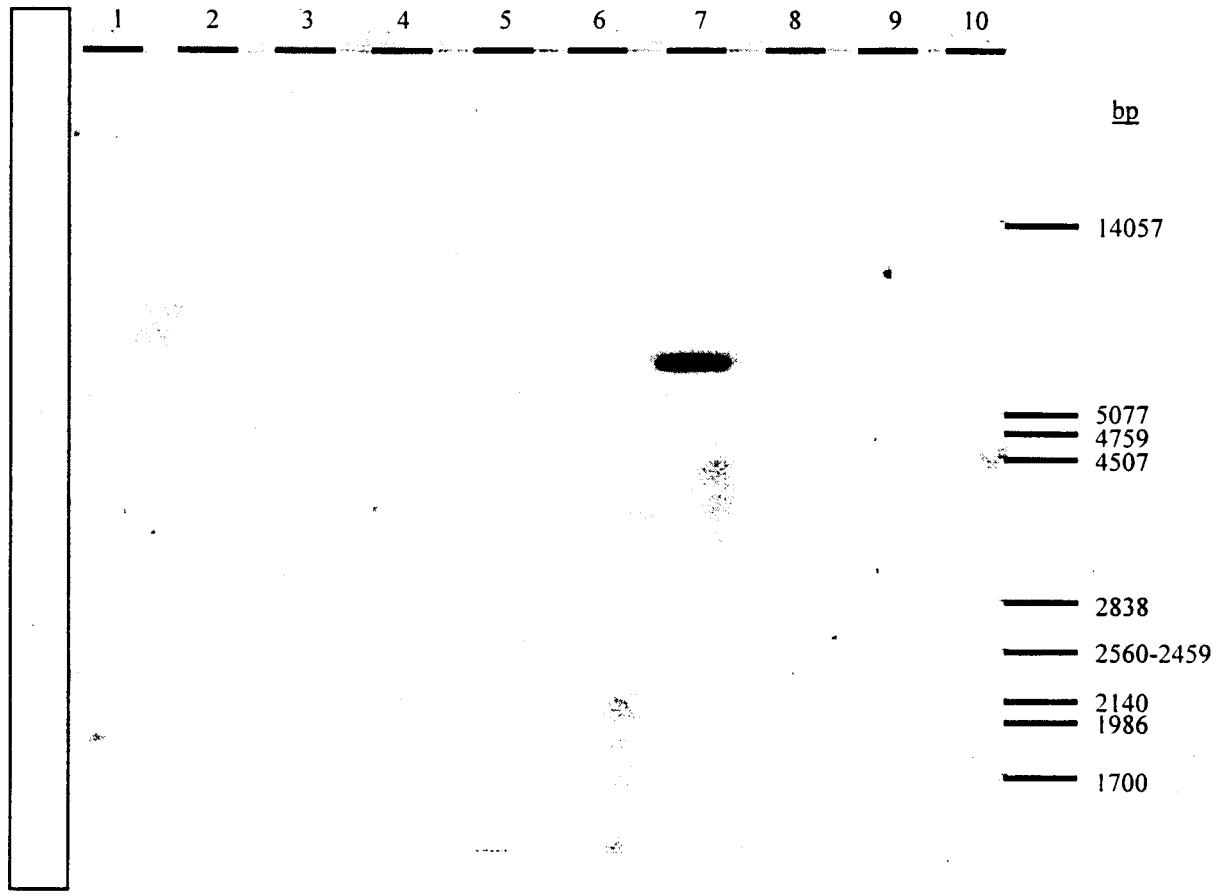

Figure xxx : Rf3 hybridization results

probe : pTHW100, 4047 bp MLD003-MLD004 fragment
Lane 1. Molecular Weight Marker, Lambda DNA-PstI
Lane 2. Rf3 DNA - undigested
Lane 3. Rf3 DNA - EcoRI
Lane 4. Rf3 DNA - EcoRV
Lane 5. Wild Type DNA - HindIII
Lane 6. Wild Type DNA - HindIII + 0.1 copy pTHW100 - HindIII
Lane 7. 1 copy pTHW100 - HindIII
Lane 8. Wild Type DNA - HindIII + 0.1 copy pTHW118 - HindIII
Lane 9. 1 copy pTHW118 - HindIII
Lane 10. Molecular Weight Marker, Lambda DNA-PstI

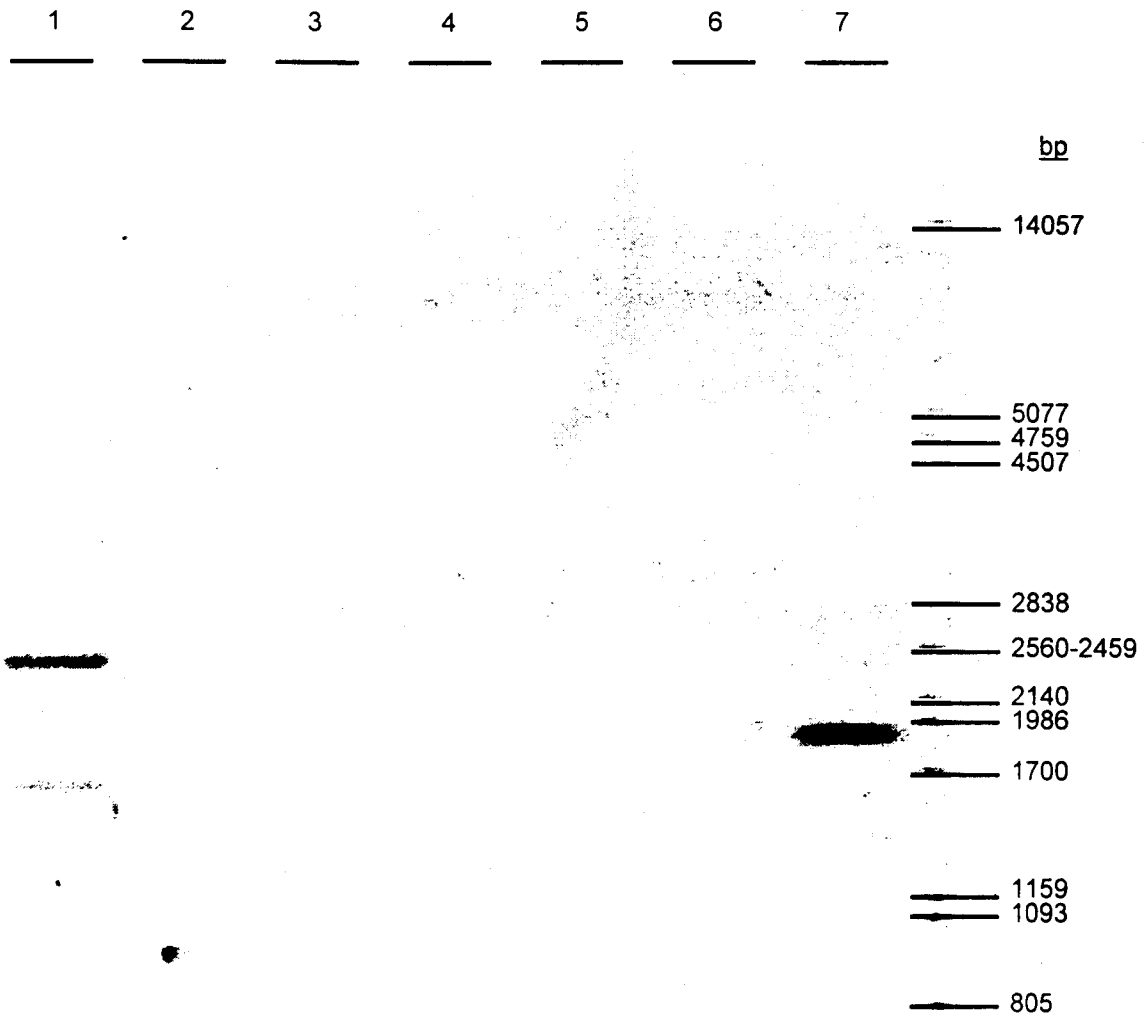

Figure 4: **Southern blot analysis - Sm/Sp probe**

probe: 1907 bp HindIII-XbaI fragment (Sm/Sp)
Lane 1. Phage Lambda DNA: PstI
Lane 2. RF3 DNA: undigested
Lane 3. RF3 DNA: EcoRI
Lane 4. RF3 DNA: EcoRV
Lane 5. Wild-type control DNA: HindIII
Lane 6. Wild-type control DNA + 0.1 copy pTHW100: HindIII
Lane 7. Wild-type control DNA + 1 copy pTHW100: HindIII

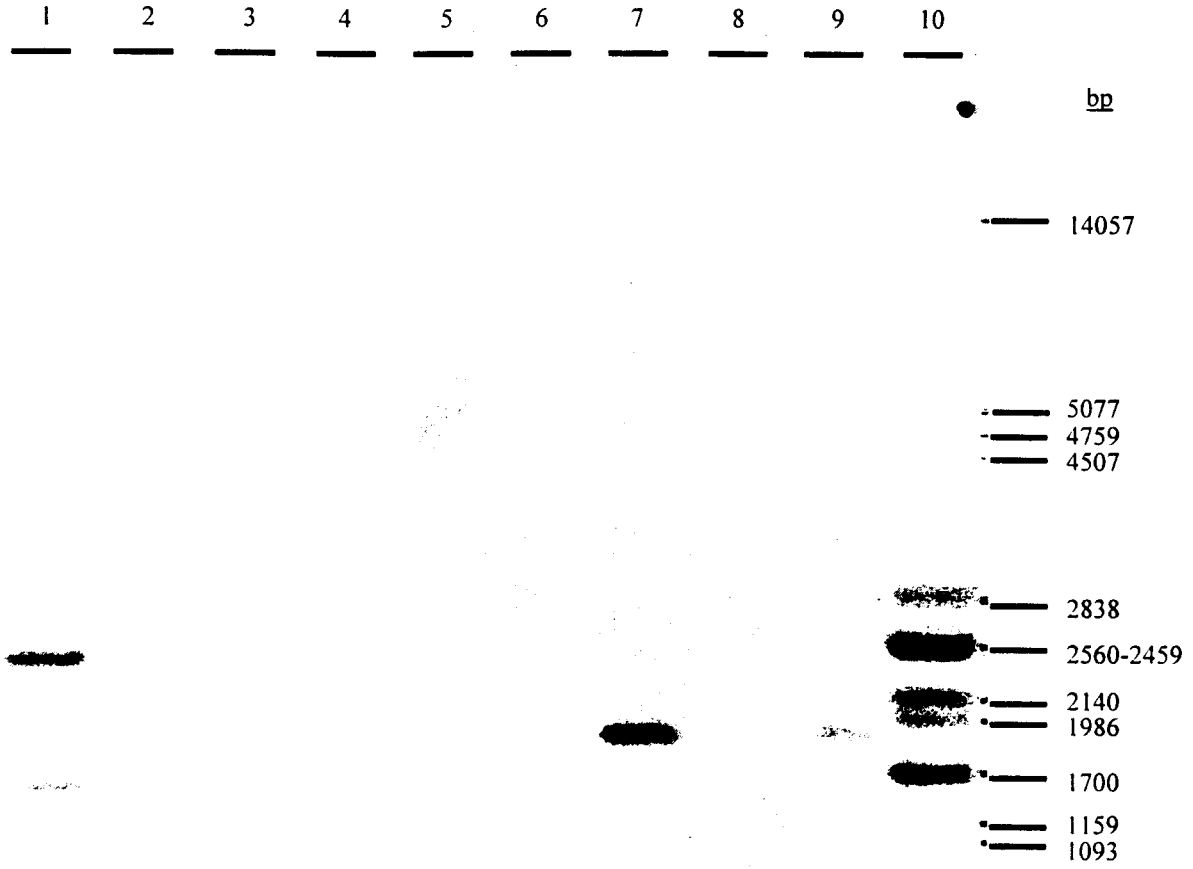

Figure ~~xxx~~ 4 : Rf3 hybridization results

probe : pTHW100, 1907 bp HindIII-XbaI fragment : Sm/Sp probe

Lane 1. Molecular Weight Marker, Lambda DNA-PstI
Lane 2. Rf3 DNA - undigested
Lane 3. Rf3 DNA - EcoRI
Lane 4. Rf3 DNA - EcoRV
Lane 5. Wild Type DNA - HindIII
Lane 6. Wild Type DNA - HindIII + 0.1 copy pTHW100 - HindIII
Lane 7. 1 copy pTHW100 - HindIII
Lane 8. Wild Type DNA - HindIII + 0.1 copy pTHW118 - HindIII
Lane 9. 1 copy pTHW118 - HindIII
Lane 10. Molecular Weight Marker, Lambda DNA-PstI

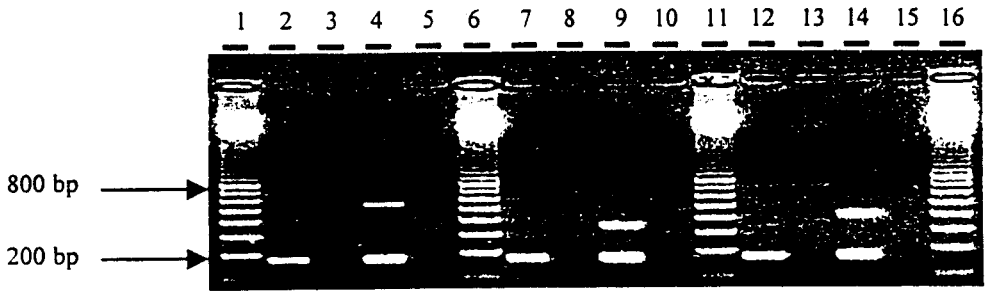

Figure 5: PCR analysis RF3

Lanes 1, 6, 11 and 16 : 100 bp MW marker
Lanes 2, 7 and 12 : RF3 plant DNA
Lanes 3, 8 and 13 : B. napus non-transgenic control (NON)
Lanes 4, 9 and 14 : B. napus non-transgenic control supplemented with pTHW118 plasmid DNA (POS)
Lanes 5, 10 and 15 : water sample (NEG)

Primer combinations used :
Lanes 2, 3, 4 and 5 : MDB469-MDB470-MDB185-MDB251
Lanes 7, 8, 9 and 10 : MDB469-MDB8-MDB185-MDB251
Lanes 12,13, 14 and 15: MDB470-MDB9-MDB185-MDB251

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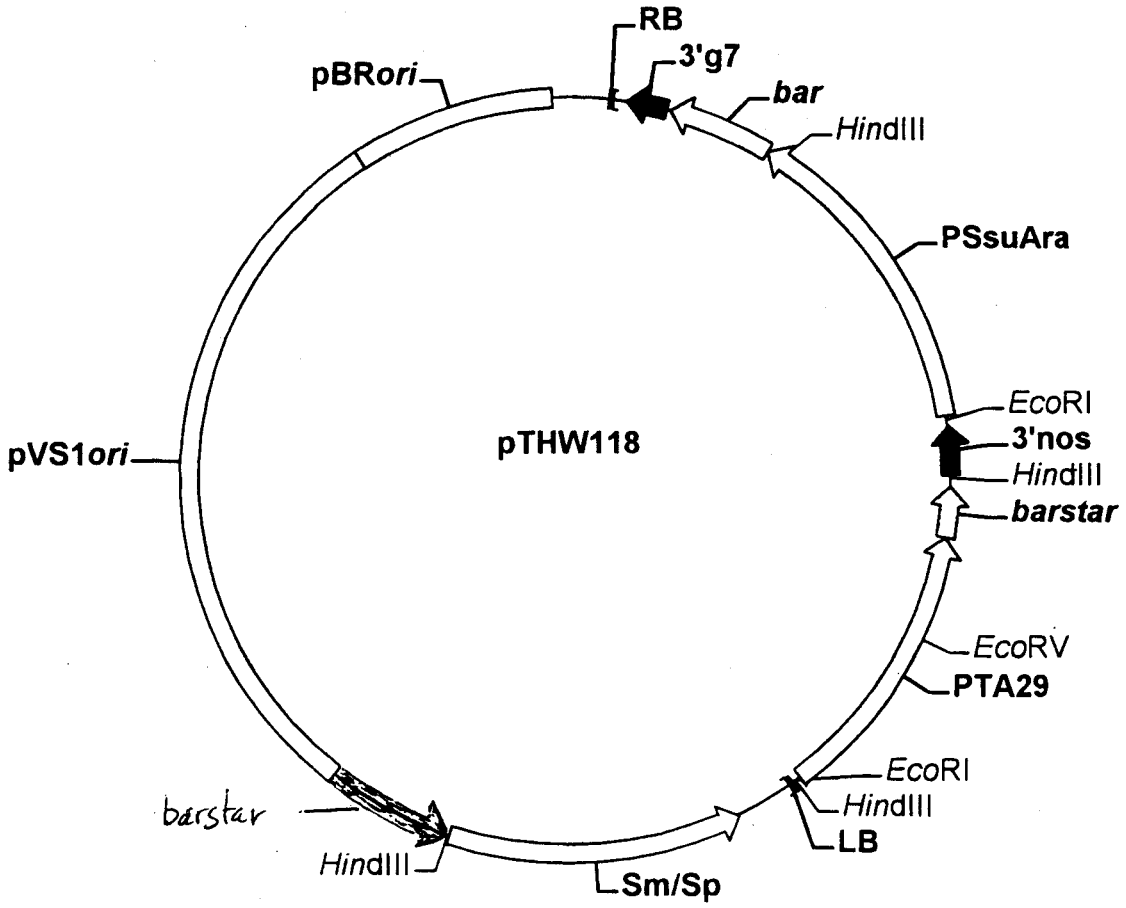
Figure 2: Circular plasmid map of construct pTHW118 indicating restriction sites of EvoRI, EcoRV and HindIII



Attachment 2:

New figures and legends for figures 5.a, 5.b, and 5.c of the original document

Figure 5a: MS8 Plant Genomic DNA, TA29 Probe. Genomic DNA from leaf tissue was prepared and compared to total plasmid DNA. The respective DNA was digested with restriction enzymes - see Table 4 - then probed with TA29 DNA fragment. 2.5µg plant DNA was loaded onto gels. Positive control = total plasmid DNA. Negative control = nontransgenic parent Drakkar. 3rd generation plants (A and B), plasmid pTHW107, and control (nontransgenic) lanes are marked below.

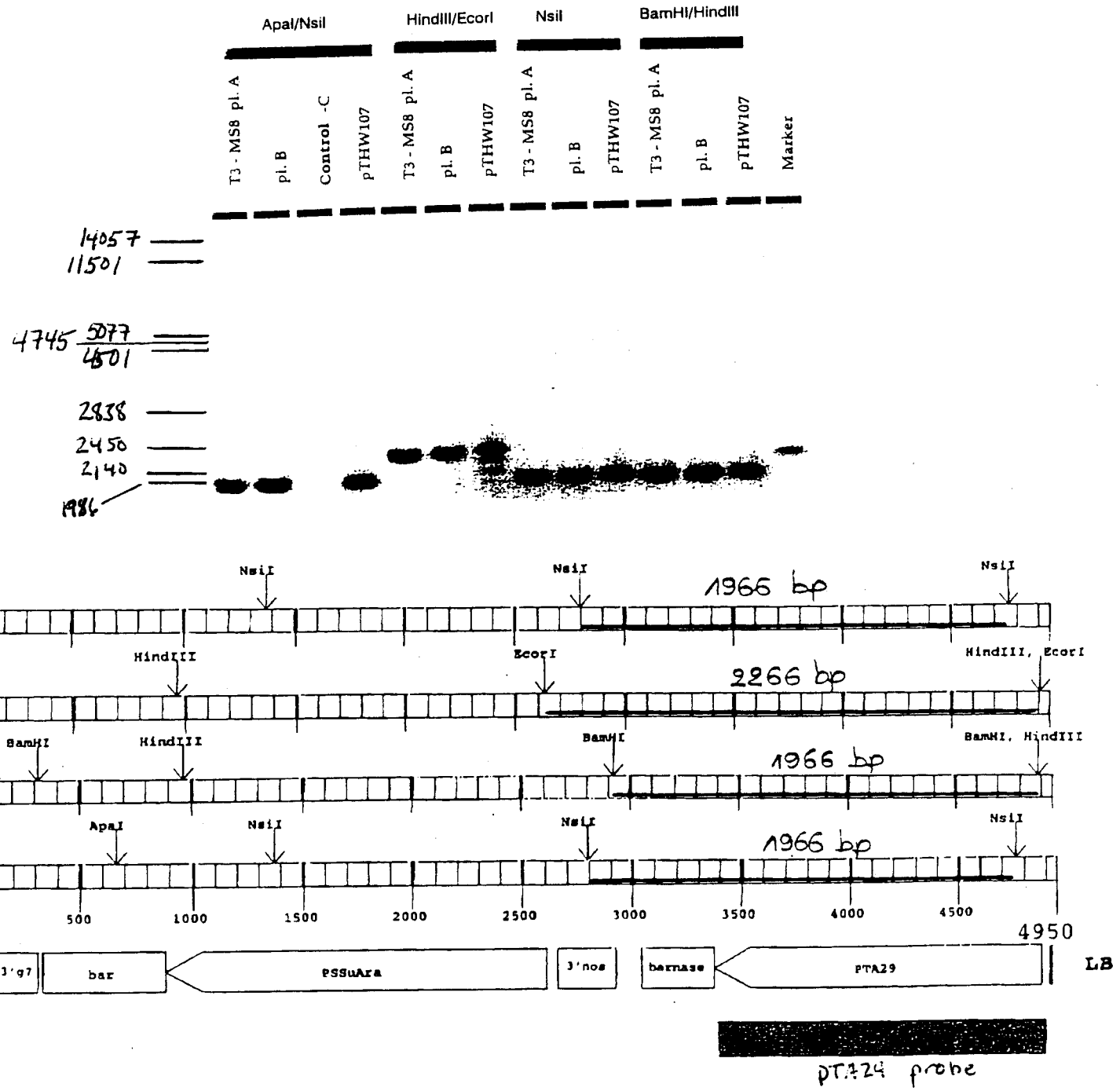
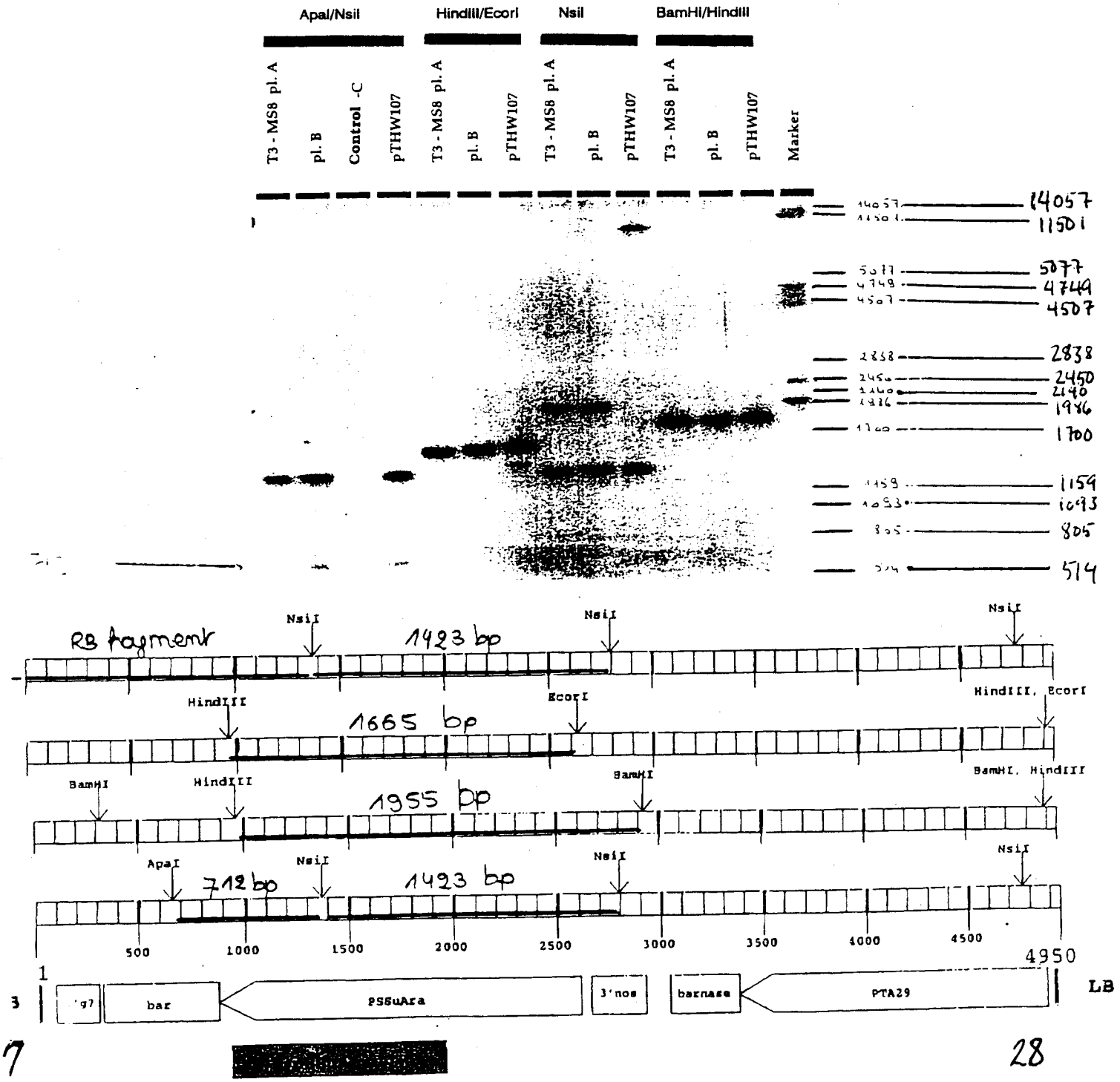


Figure 5c: MS8 Plant Genomic DNA, pSsuAra Probe. Genomic DNA from leaf tissue was prepared and compared to total plasmid DNA. The respective DNA was digested with restriction enzymes - see Table 4 - then probed with pSsuAra DNA fragment. 2.5µg plant DNA was loaded onto gels. Positive control = total plasmid DNA. Negative control = nontransgenic parent Drakkar. 3rd generation plants (A and B), plasmid pTHW107, and control (nontransgenic) lanes are marked below.



Attachment 3:

Table 6 (corrected) of the original document

F_1 generation should be resistant to the herbicide, and one-half of the BC_1 and BC_2 generations should be resistant to the herbicide. Indeed this is what was observed. (See Table 6).

Table 6: RF3 Segregation Results

Plant Material	Total Number of Seedlings	Number of Seedlings Survinging glufosinate-ammonium Treatment	Expected segregation ratio	Observed segregation ratio	Chi^2
F_1 plants					
F_1 (RF3 x SOSR-C6)	95	90	100%	5/90	(0.26) NS
BC_1 plants					
BC_1 (F_1 x SOSR-C7)	120	64	1:1	56/64	(0.27) NS
BC_2 plants					
BC_1 (BC_1 x SOSR-C8)	97	44	1:1	53/44	(0.32) NS

NS = not significant according to Fischer Chi^2 test

This data demonstrates that the chimeric *bar/barstar* gene construct was stably inherited in the different genetic backgrounds of spring oilseed rape tested.

V. c. Expression of Inserted Genes: *barnase, barstar* and *bar*

In order to demonstrate the expression of the introduced transgenes in the male sterile, MS8, and fertility restorer, RF3, progenies, Northern blot analysis of messenger RNA was conducted. Leaf tissue, dry seed, pollen and flower bud tissue were analyzed. Figures 7 – 10 show the results of hybridization in the different tissues. Figure 6 is hybridzation results of RF3 mRNA probed with *barstar*. Figure 9 is hybridization results of RF3 mRNA probed with *bar*. Figures 8 and 10 are the dilution sequences of *in vitro* synthesized RF3 mRNA complementary to the probe used. The control mRNA samples have had 5µg control (nontransgenic) leaf mRNA added to them.

The method given in Appendix 3 was used to quantify mRNA expression of the *bar*, *barnase* and *barstar* transgenes. Only detectable levels of transgene expression, in the range of the linear regression of the control dilution series, were quantified by ImageQuant of Molecular Dynamics.

MS8 Results: The mRNA levels of bar in leaves and flower buds varied between 0.03 pg and 0.22 pg/µg total RNA. In the dry seed samples, no bar mRNA signal (LOD = 0.1 pg/µg total RNA) was detected. No Barnase mRNA signals were detected (LOD = 0.1 pg/µg total RNA) in the leaf, flower bud, seed or dry pollen tissues. This is expected because the specific ribonuclease protein, Barnase, is expected to destroy the tapetum tissues where Barnase is expressed. (The tapetum is located in the flower bud.) Results are summarized in Table 7.

Attachment 4:

Segregation data of MS8 and RF3 from an original study report coded FBN 9413

EXPERIMENT FBN9413 **Primary field evaluation of new male sterile and fertility restorer oilseed rape transformants**

Authorization numbers : B/B/94/W2 (Ophain, Belgium)
94-PGS-CAN-01..04-SK01-08 (Saskatoon, Canada)

Responsible : ir G. De Both, Product Manager Oilseeds

FBN9413 Goal of the experiment

Field evaluation of new male sterile (FBN9413A) and fertility restorer (FBN9413B) oilseed rape lines in comparison with the male sterile MS1 and fertility restorer RF1 and RF2 oilseed rape lines.

FBN9413 Plant material

Though numerous lines were tested, only the MS8 and RF3 line and control material are listed in the table below.

Table FBN9413 Plant material of interest

Plant material
- Nontransgenic control Drakkar
- Transgenic controls : MS1(B91-4) RF1(B93-101), homozygous for the RF1 allele RF2(B94-2), homozygous for the RF2 allele
- T_2(MS8)
- S_2(RF3), homozygous for the RF3 allele
- T_1(MS8) combined with RF1RF1 and combined with RF2RF2 to check the restorability of the new male sterile line
- S_1(RF3) combined with e.g. MS1 to check the restoration capacity of the fertility restorer line

FBN9413 Methods

The field trial design included two replicated plots of 6 m by 1.8 m (6 rows) per entry. Seeds were sown conform to normal agricultural practices. Half of each plot was sprayed at the four leaf stage with Basta (5l/ha), while the other subplot was not Basta treated.

Observations included Basta segregation, Basta tolerance level (effect of spray on vigor, growth, flowering date, 1=bad, 5=good), flower phenotype (1=bad, 3=good), flower segregation and pod set on the main raceme (in segregating NMS plots). Any differences in plant growth or morphology were noted. Plots were harvested. Seed quality parameters were determined.

FBN9413 Results and conclusions

FBN9413 Results

Results of the characterization of the male sterile MS8 and fertility restorer RF3 oilseed rape line are summarized below.

Table FBN9413$_1$ Basta segregation, Basta tolerance and flower segregation data

	MS8	MS1
% Basta tolerant plants Belgium on 1m²: Canada on 0.25m²:	 45 58	 64 50
Basta tolerance level (1-5) on respectively Basta treated/non-treated plants Belgium : Canada :	 4/4 4/3	 4/4.5 4/4
% Sterile plants in non-sprayed subplots Belgium : Canada :	 49 37	 45 26
% Fertile plants in MSx RF1RF1 restored plots Belgium :Canada :	 100 100	 93 89
% Fertile plants in MS x RF2RF2 restored plots Belgium : Canada :	 100 87	 100 99
% Fertile plants in MS x RF3RF3 restored plots Belgium : Canada :	 not tested not tested	 100 100

Table FBN9413$_2$ Plant height (Canada), number of days to maturity (Canada), and number of pods per raceme (Belgium) of the MS8 oilseed rape lines, the MS1 control, and some restored combinations

Plant material	Plant height (cm)	Days to maturity (Basta treated /non-treated plants)	Number of pods per raceme (Fertile/Male sterile segregants)
Control	-	- /107	-
MS1	118	102/105	28.0/28.6
MS8	118	103/104	40.1/33.3
MS8 x RF1	120	100/100	-
MS8 x RF2	110	100/100	-

Table FBN9413$_3$ Yield data (Belgium)

Plant material	Yield data (kg/plot)
FBN9413A	
MS1	3.711
MS8	3.615
MS1 x RF1RF1	3.565
MS8 x RF1RF1	3.686
MS1 x RF1RF1	4.007
MS8 x RF2RF2	3.942
FBN9413B	
RF1RF1	3.333
RF2RF2	3.309
RF3RF3	2.875
MS1 x RF1RF1	3.421
MS1 x RF2RF2	3.412
MS1 x RF3RF3	3.364

Table FBN9413$_4$ Seed quality parameters

Plant material	Oil (%)	Protein (%) in the seeds	Protein (%) in the meal	Glucosinolates (µmol/g seed)	Glucosinolates (µmol/g oilfree meal)
FBN9413A					
MS1	41.73	25.64	43.99	26.81	46.02
MS8	42.19	24.13	41.74	27.13	46.94
MS1 x RF1	40.47	25.82	43.38	26.73	44.92
MS8 x RF1	40.32	25.29	42.39	24.96	41.83
MS1 x RF2	41.55	25.75	44.06	26.33	45.04
MS8 x RF2	41.64	24.66	42.26	26.31	45.09
FBN9413B					
RF1RF1	40.60	26.48	44.58	28.09	47.28
RF2RF2	40.40	26.83	45.01	28.65	48.06
RF3RF3	41.62	26.08	44.66	28.27	48.40
MS1 x RF1	40.31	26.14	43.78	27.60	46.24
MS1 x RF3	41.80	25.16	43.22	26.94	46.27

FBN9413 Conclusions

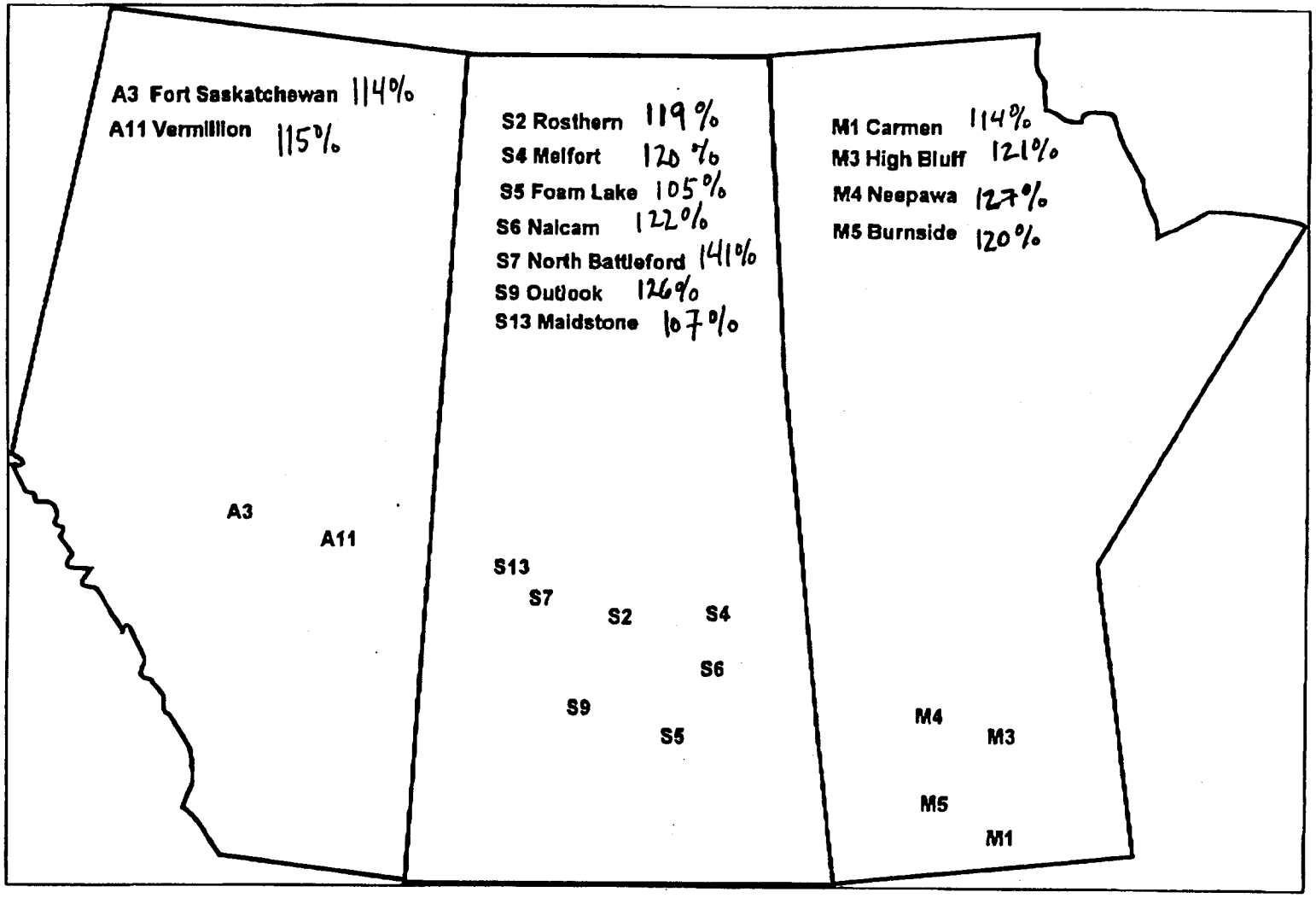
Based on Basta segregation data, Basta tolerance level, flower segregation ratio and restoration by RF1 and RF2, the male sterile MS8(DBN230-0028) oilseed rape line was selected. Within the newly tested candidate fertility restorer oilseed rape lines, RF3 was identified based on its capacity to restore male sterile oilseed rape plants.

Yield data were collected only in Belgium. No significant differences in seed quality data were observed.

Attachment 5:

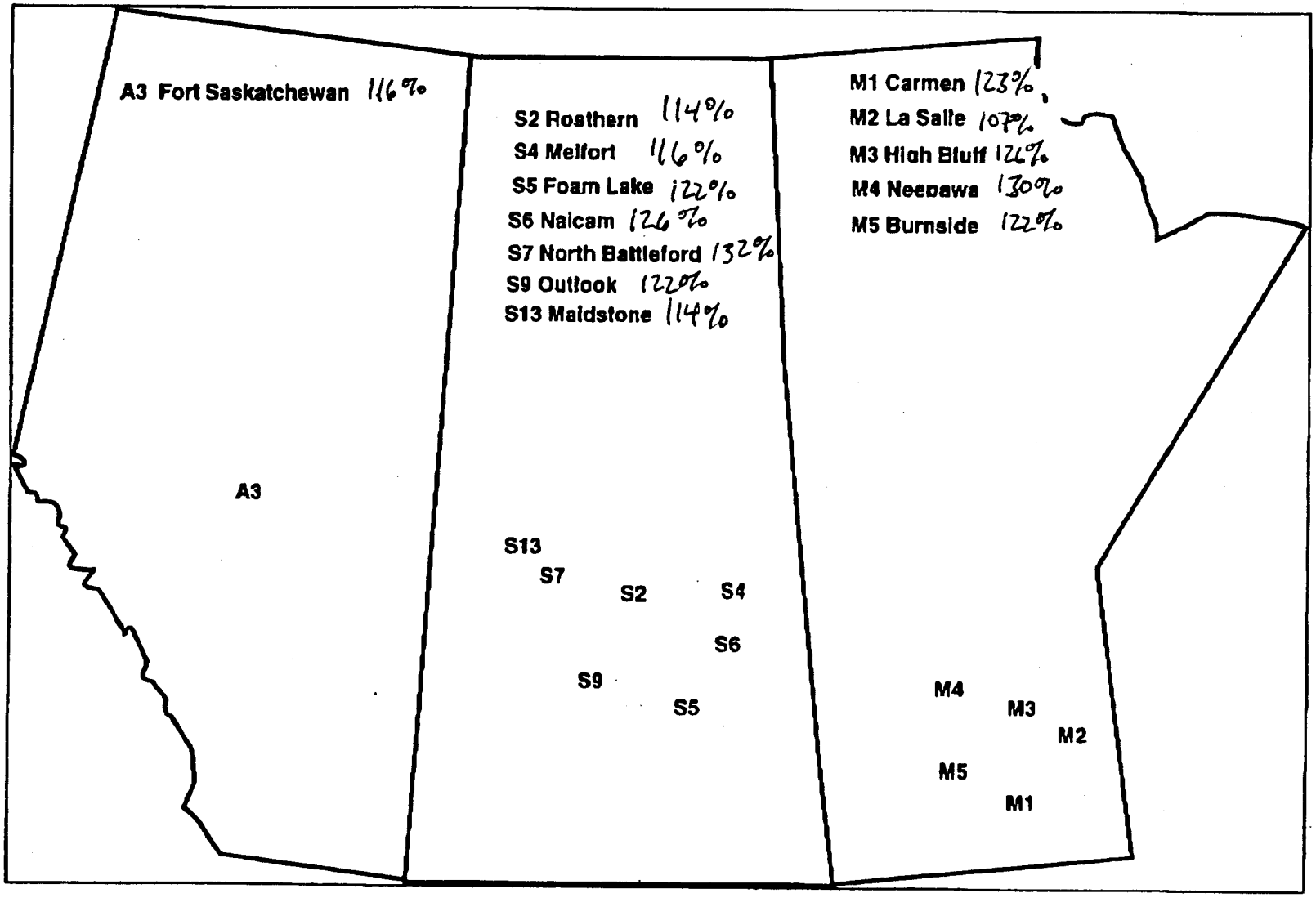
Yield data from 1998 trials in Canada and the US to indicate restoration of MS8 x RF3

N8S53A - Level 3 Co-op MS8 × RF3 Yield Trials, Canada, 1998.



% = average percent yield of MS8 × RF3 plants as compared to an average of hybrid + open pollenated "checks". With the check value = 100%.

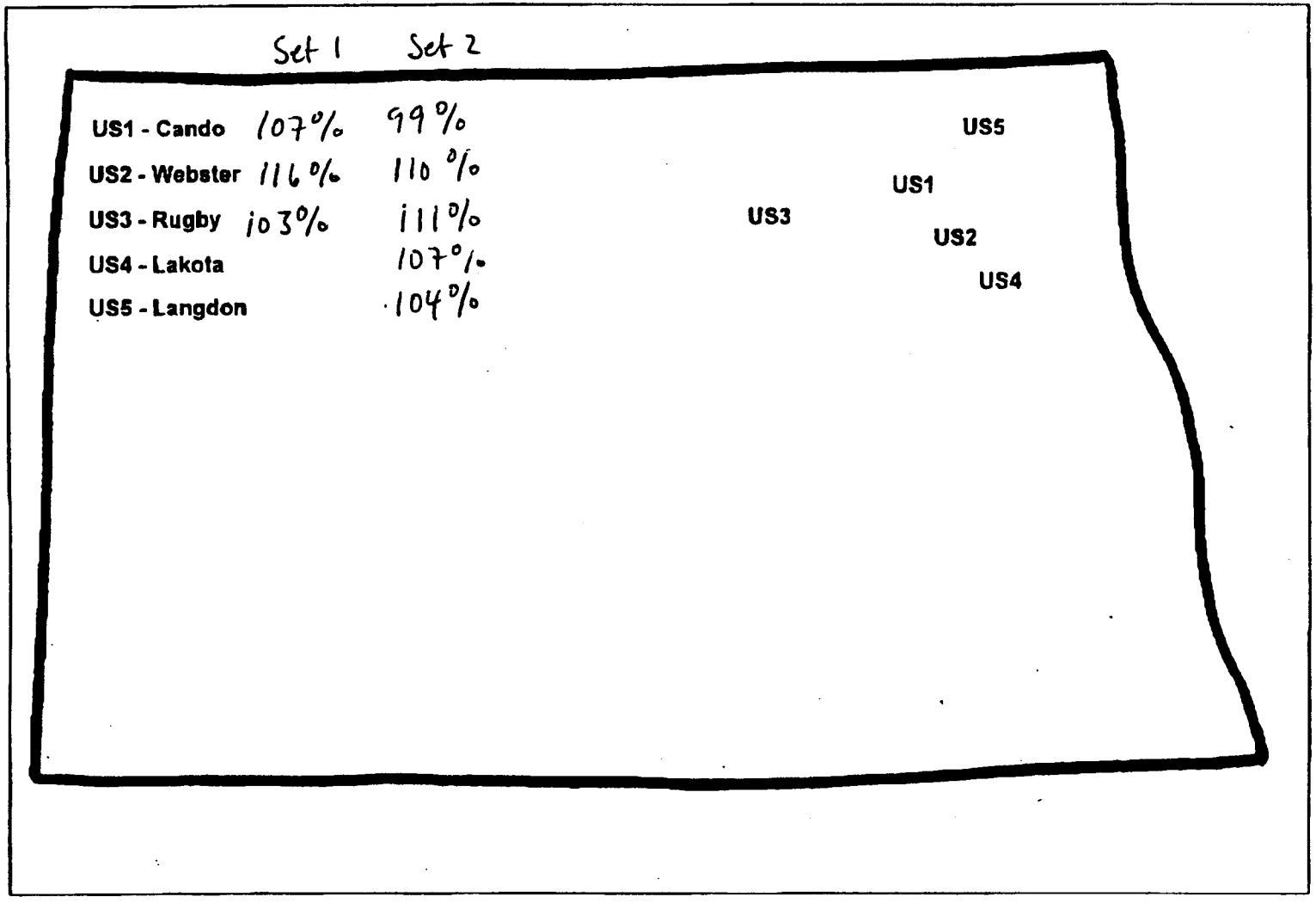
N8S53B - Level 3 Co-op MS8 x RF3 Yield Trials, Canada, 1998.



% = average percent yield of MS8 x RF3 plants as compared to an average of hybrid + open pollenated "checks". With the check value = 100%.

N8S53X - U.S. Level 3 Private Co-ops MS 8 x RF3 Yield Trials, North Dakota, USA, 1998

Results of Two (2) Independendant Sets of Trials

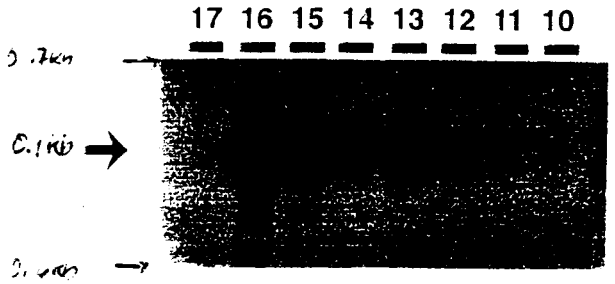


% = average percent yield of MS 8 x RF3 plants as compared to an average of hybrid + open pollinated "checks". With the check value = 100%.

Attachment 6:

Table 7 (corrected) of the original document
Figure 10 (corrected) of the original document

Figure 10: Dilution series. *In vitro* synthesized mRNA probed hybridized with the complementary probe (*bar*). Control samples have had 5μg leaf mRNA (nontransgenic) added to them.



Lane	Sample	g RNA loaded
17	MW (0.16 – 1.77 kb RNA ladder, Life Technologies, Inc.)	
10	control	0.25pg
11	control	0.5pg
12	control	1 pg
13	control	2 pg
14	control	4pg
15	control	8pg
16	control	16pg

Table 7: Summary of Barnase and Bar mRNA Expression Results of MS8 and RF3 as detected by Northern Analysis

Total RNA	Transgene Expression (pg/μg total RNA)		Total RNA	Transgene Expression (pg/μg total RNA)	
	bar pGembar/SP6	barnase pVE113/SP6		bar pGembar/SP6	barstar pVE113/SP6
MS8-T3 leaf A	n.d.	n.d.	RF3-S3 leaf A	1.1 pg	n.d.
MS8-T3 leaf B	0.22 pg	n.d.	RF3-S3 leaf B	0.2 pg	n.d.
MS8-T3 flower buds 2mm A	0.14 pg	n.d.	RF3-S3 flower buds 2mm A	0.46 pg	1.54 pg
MS8-T3 flower buds 2mm B	0.11 pg	n.d.	RF3-S3 flower buds 2mm B	0.52 pg	1.3 pg
MS8-T3 flower buds 3mm A	0.19 pg	n.d.	RF3-S3 flower buds 3mm A	0.38 pg	1.22 pg
MS8-T3 flower buds 3mm B	n.d.	n.d.	RF3-S3 flower buds 3mm B	0.34 pg	2.4 pg
MS8-T3 dry seed	n.d.	n.d.	RF3-S3 dry seed	n.d.	n.d.
			RF3-S3 pollen	n.d.	n.d.
LOD (pg/ug total RNA)	0.1	0.1	LOD (pg/ g total RNA)	0.05	0.1

n.d. = no signal detected
pGembar/SP6 and pVE113/SP6 = plasmids used for preparation of RNA probes. See Appendix 3

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