

**Petition for Determination of Nonregulated Status for Roundup
Ready® Canola (*Brassica napus*)
Line RT73**

97-350U

Submitter:

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July, 1998

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CONTAINS NO CONFIDENTIAL INFORMATION

**Petition for Determination of Nonregulated Status for
Roundup Ready® Canola (*Brassica napus*) Line RT73**

SUMMARY

Monsanto Company submits this Petition for Determination of Nonregulated Status to the Animal Plant Health Inspection Service (APHIS) for canola which is tolerant to Roundup® herbicide. This petition requests a determination from APHIS that the Roundup Ready® canola line RT73, any progenies derived from crosses between this line and other canola varieties, and any progeny derived from crosses of this line with transgenic canola varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under regulations in 7 CFR part 340.

Weed management is a critical component to maximize yields and retain a high-quality harvest, free of weed seeds. The use of canola plants containing the Roundup Ready® gene would enable the farmer to utilize Roundup® herbicide for effective control of weeds during the growing season and to take advantage of this herbicide's environmental and safety characteristics.

Roundup Ready® canola line RT73, also referred to as RT73, has been transformed with the plasmid PV-BNGT04, which contains the enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene from *Agrobacterium* sp. and a modified glyphosate oxidoreductase (*goxv247*) gene based on the *gox* gene from *Ochrobactrum anthropi* sp. These genes and the proteins they produce have been fully characterized. The CP4 EPSPS gene in RT73 is the same as the gene imparting Roundup tolerance in Roundup Ready® cotton line 1445 and Roundup Ready® soybean line 40-3-2, previously deregulated by USDA. Canola RT73 is tolerant to Roundup through the expression of both the CP4 EPSPS and GOXv247 proteins. Canola RT73 has been extensively field tested in Canada, Europe and the United States over the last six years. Testing in the United States was conducted under USDA permits and notifications (Appendix 1).

Monsanto obtained food, feed and environmental regulatory approvals for Roundup Ready canola line RT73 in Canada in March, 1995. Commercial launch of RT73 occurred in 1996 under the trade name Roundup Ready®, with over 450,000 Canadian acres planted in 1997. Food and environmental approvals were obtained in Japan in September, 1996. Monsanto also completed the consultation process with the U.S. Food and Drug Administration in September, 1995 and obtained a finding of no concern. In

addition, the U.S. EPA granted a tolerance exemption for CP4 EPSPS on August 2, 1996 and for GOX on October 9, 1997. Monsanto has submitted (April, 1998), and will obtain, prior to commercial launch in the United States, a registration from the U.S. EPA for use of Roundup for over-the-top application on Roundup Ready canola.

Data and information for RT73 transformed with the plasmid vector PV-BNGT04 are provided to demonstrate that this canola line and its progeny are no more likely to become weeds than traditional canola varieties and are unlikely to increase the weediness potential of any cultivated plant or native wild species. In addition, this line does not exhibit plant pathogenic properties and exhibits no toxicity to non-target organisms, including those organisms beneficial to agriculture.

Therefore, Monsanto Company requests a determination from APHIS that Roundup Ready canola line RT73, any progenies derived from crosses between this line and other canola varieties, and any progeny derived from crosses of this line with transgenic canola varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under regulations in 7 CFR part 340.

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CERTIFICATION

The undersigned certifies that, to the best of his knowledge and belief, this petition includes all data, information, and views relevant to the matter, whether favorable or unfavorable to the position of the undersigned, which is the subject of the petition.

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List of Abbreviations

<i>aad</i>	Gene encoding adenyltransferase conferring spectinomycin and streptomycin resistance
bp	basepair
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase from <i>Agrobacterium</i> sp. strain CP4
CTP	chloroplast transit peptide
ELISA	enzyme-linked immunosorbant assay
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
<i>gox</i> /GOX	glyphosate oxidoreductase gene/protein from <i>Achromobacter</i> sp. strain LBAA
<i>goxv247</i>	a variant of the <i>gox</i> gene
GOXv247	a variant of the GOX protein
LB	left border of T-DNA
OSR	oilseed rape
Mha	million hectares
PEP	phosphoenolpyruvate
PCR	polymerase chain reaction
RB	right border of T-DNA
RBDO	refined, bleached, deodorized oil
RT73	Roundup-tolerant canola line 73
Spc/Str	Phenotype with resistance to spectinomycin and streptomycin conferred by the <i>aad</i> gene
S3P	shikimate-3-phosphate
SSU	small subunit

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I. RATIONALE FOR THE DEVELOPMENT OF ROUNDUP READY® CANOLA

The use of canola containing Roundup Ready® genes would enable the farmer to utilize Roundup herbicide for effective control of weeds during the growing season and to take advantage of this herbicide's environmental and safety characteristics. Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. Glyphosate has excellent environmental features, such as rapid soil binding (resistance to leaching) and biodegradation (which decreases persistence), as well as extremely low toxicity to mammals, birds and fish (Malik *et al.*, 1989). In addition, glyphosate is one of the commercially available herbicides classified by the EPA as Category E (evidence of non-carcinogenicity for humans) (57 FR 8739).

The use of Roundup Ready canola can positively impact current agronomic practices in canola by:

- Offering the farmer a new, wide-spectrum weed control option.
- Allowing the use of an environmentally acceptable herbicide.
- Increasing flexibility to treat weeds on an "as needed" basis.
- Providing an excellent fit with reduced-tillage systems, which results in increased soil moisture, while reducing soil erosion and fuel use.
- Providing cost-effective weed control.

II. THE CANOLA FAMILY

A thorough review of the taxonomy and biology of the canola family may be found in the Consensus Document on the biology of *Brassica napus* L (Oilseed Rape) in the OECD Series on the Harmonization of Regulatory Oversight in Biotechnology (OECD, 1997). Information not discussed in the OECD document concerning the distribution of sexually compatible species in the U.S. can be found in Sections II.A. and VI.F. The development of canola quality rapeseed has also been previously reviewed (Stefansson, 1983).

In the fall of 1990, U.S. production of canola was quite modest: 24,000 hectares or 59,000 acres. By 1997 U.S. production had increased to 289,000 hectares or 715,000 acres (NASS, 1997). Within ten years, the U.S. production may be as high as 4.5 million hectares/11.1 million acres (Raymer

and Thomas, 1990). The two leading states in canola production are North Dakota and Montana, with significant production also found in Idaho, Minnesota and Washington.

A. Genetic Nature of the Amphidiploid *B. napus*

The origin of the species *Brassica napus* can be traced to natural crossing between two diploid species, *B. oleracea* and *B. rapa*, growing in close proximity, followed by spontaneous chromosome doubling of the hybrid. Amphidiploids, a special case of polyploidy, are formed by mating two species with different genomes and doubling the chromosome number of the hybrid. Such a doubled chromosome configuration would be stable at meiosis and thus allow the new polyploid species to reproduce. Crossing without chromosome doubling results in sterile progeny. Cytological studies of *B. napus* have shown that it contains both the aa and the cc genome, and is an amphidiploid derived from the monogenomic species, *B. oleracea* (cc genome) and *B. rapa* (aa genome), (Mizushima, 1980; U, 1935). Such a crossing/doubling event probably occurred only once for *B. napus*, since it has a discrete center of origin in the Mediterranean area. Both *B. napus* and *B. juncea* have the aa *B. rapa* genome in common (Figure 1).

B. napus is self-compatible and thus, primarily self-pollinating, although some crossing between individual plants of *B. napus* (outcrossing) does occur under field conditions (Huhn and Rakow, 1979; Rakow and Woods, 1987). The pollen is sticky and only transferred physically, through contact between plants or by insects. The only cultivated species sexually compatible with *B. napus* under field conditions are other *B. napus*, *B. rapa*, and *B. juncea*. Several wild mustard species, *B. carinata*, *B. oleracea*, *Sinapis alba* syn *B. hirta* and *B. tournefortii*, require intervention such as emasculation and manual pollination to produce progeny when crossed with *B. napus* under field conditions (OECD, 1997). See section VI.F. for additional discussion of outcrossing potential between *B. napus* and other closely related species.

Figure 1. Genome relationships of some economically important *Brassica* species. After U (1935).

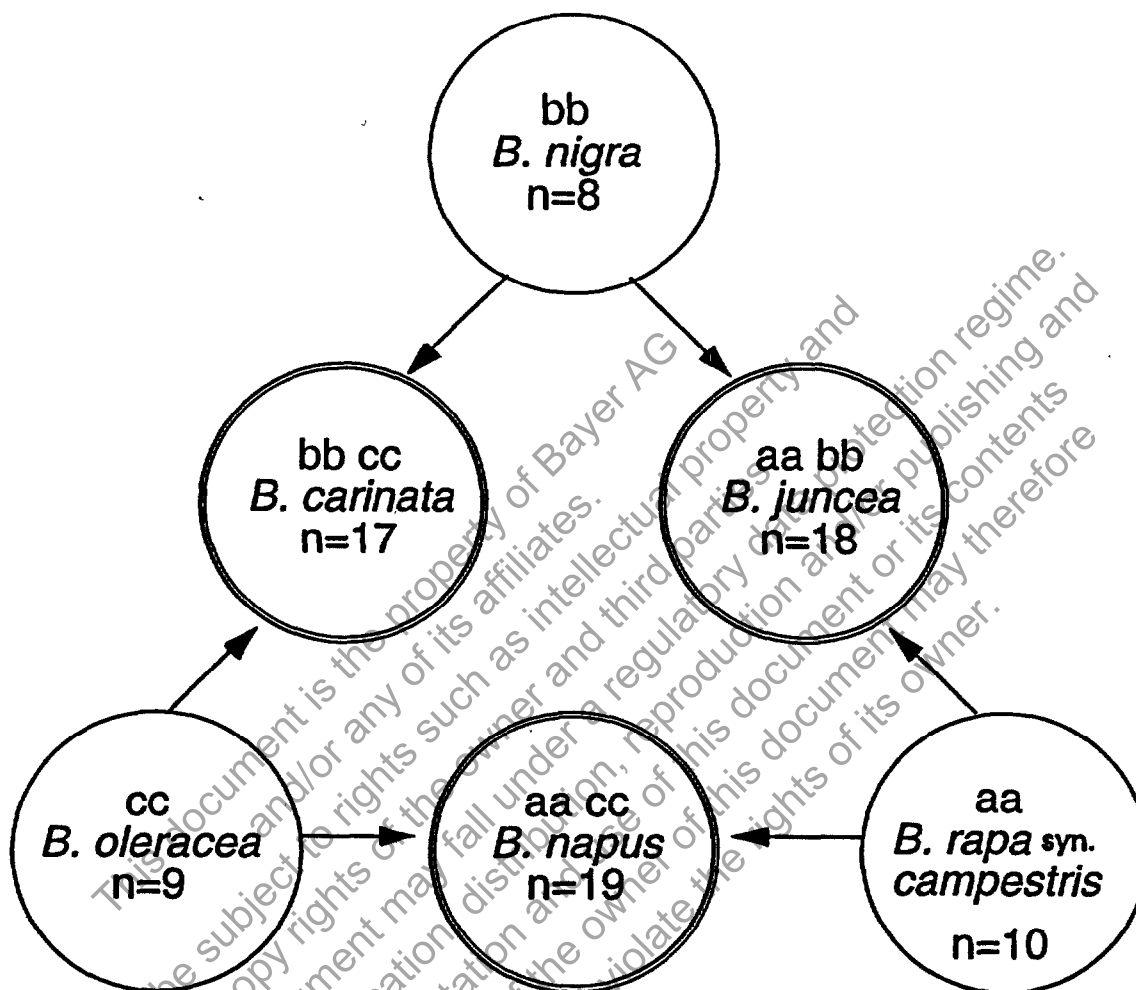


Table 1. Members of the genus *Brassica* found in the U.S. which are sexually compatible with *B. napus* under field conditions, including the common names of cultivated and naturalized or wild forms. (Fribourg *et al.*, 1989; Hortus Third, 1976; Rollins, 1981).

<u><i>Brassica</i> species</u>	<u>cultivated form</u>	<u>naturalized or wild form</u>
<i>B. juncea</i>	brown mustard leaf mustard mustard greens	Indian mustard
<i>B. napus</i>	canola, oilseed rape	wild rape
<i>B. rapa</i>	fodder turnip, turnip rape, canola, rapeseed	field mustard, wild turnip

B. Characteristics of the nontransformed cultivar

Roundup Ready canola line RT73 was selected from plants of the well-known Westar variety of canola (*Brassica napus* L.) (Klassen *et al.*, 1987). Since 1982, this variety has had a history of safe use in the commercial production and breeding of canola. Its pedigree has been published along with 6 year performance data (Klassen *et al.*, 1987). Westar has been a standard, as well as a source of breeding germplasm for many other registered varieties of canola. Thus, there are no safety concerns related to the host plant for Roundup Ready canola line RT73.

III. AGROBACTERIUM TRANSFORMATION METHOD

The disarmed *Agrobacterium tumefaciens* plant transformation system was used to produce Roundup Ready canola line RT73. This delivery system is well documented to transfer and stably integrate T-DNA into a plant nuclear chromosome (White, 1989; Howard *et al.*, 1990). Vector PV-BNGT04 was mobilized into disarmed *A. tumefaciens* strain ABI and selected on spectinomycin and chloramphenicol. Five to six week-old stem sections from Westar canola were used as explant sources, and were infected with the *Agrobacterium* culture. Following co-culture, *Agrobacterium* were killed using a culture media containing the appropriate antibiotics. Explants were later placed on glyphosate selection medium. Developing shoots were excised from an R₀ plant. The positive shoots were grown to maturity, selfed to produce seed and the resulting progeny plants were screened for glyphosate tolerance.

IV. DONOR GENES AND REGULATORY SEQUENCES

A. Plant transformation vector, PV-BNGT04

The plant transformation vector used to produce Roundup Ready canola RT73 was PV-BNGT04. It is a double border vector, and encodes CP4 EPSPS and *goxv247* genes optimized for plant expression. As described in more complete detail in Table 2, the sequence between the left and right border sequences contains the following genetic elements:

Promoter	Transit peptide	Gene	Terminator
P-CMoVb	CTP1	<i>goxv247</i>	E9 3'
P-CMoVb	CTP2	CP4 EPSPS	E9 3'

The plasmid map is shown in Figure 2. All of the clonings performed to construct plasmid PV-BNGT04 were done in non-pathogenic *E. coli* strains derived from *E. coli* K-12 (*E. coli* LE392, JM101, and MM294), commonly used in molecular biology research (Sambrook *et al.*, 1989).

The vector PV-BNGT04 contains well-characterized DNA segments required for selection and replication of the plasmid in bacteria, as well as a right border for initiating the region of DNA transferred into plant genomic DNA. The same constitutive promoter, P-CMoVb, was used to drive expression of both the CP4 EPSPS and *goxv247* genes. A chloroplast transit peptide (CTP) was fused upstream of the N-terminus of CP4 EPSPS and *goxv247* to facilitate import of the newly translated protein into chloroplasts (della-Cioppa *et al.*, 1987). The Arab-SSU1A/CTP1 (CTP1) is a chloroplast transit signal peptide derived from the small subunit of ribulose biphosphate carboxylase of *Arabidopsis thaliana*. The CTP1 DNA sequence encodes an 89 amino acid peptide fused to the N-terminus of mature *goxv247*. The amino acid sequence of CTP1 contains 2 potential Cys-Met (cysteine-methionine) cleavage sites upstream of the fusion. The AEPSPS/CTP2 (CTP2), fused to CP4 EPSPS, is the *A. thaliana* EPSPS CTP. The CTP2 DNA sequence encodes a 77 amino acid peptide fused to the N-terminus of mature CP4 EPSPS. The amino acid sequence of CTP2 contains only one Cys-Met cleavage site at the point of the fusion.

Outside the borders, the vector PV-BNGT04 (Figure 2) contains bacterial origins of replication (*ori-V*, *ori-322*) and the *aad* selectable marker gene that provides resistance to spectinomycin and streptomycin antibiotics. These elements were not transferred to canola RT73. Lastly, well-defined restriction sites are present in PV-BNGT04 (Figure 2). These sites enable characterization of the genetic elements in canola RT73.

Figure 2. Plasmid Map of PV-BNGT04.

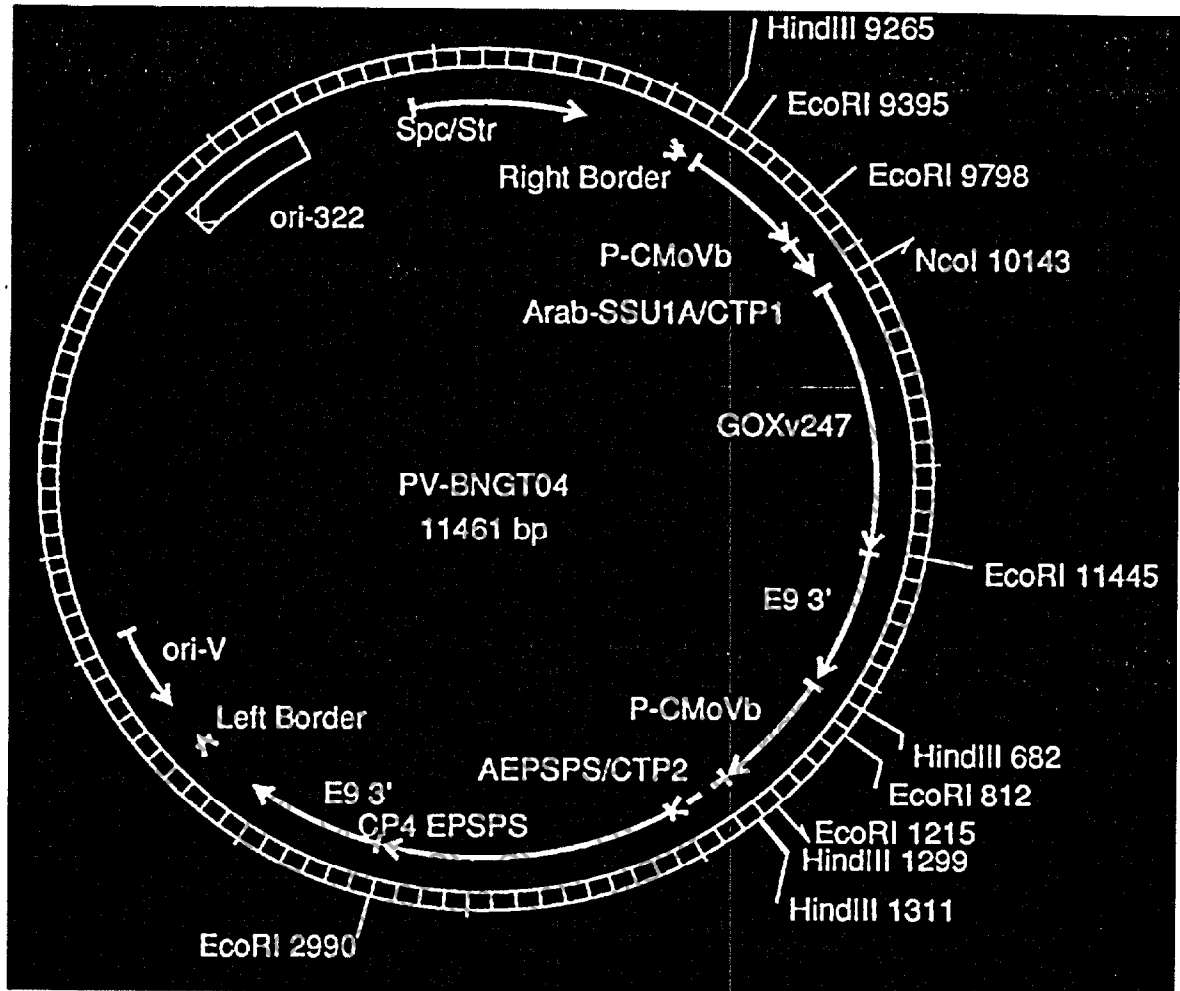


Table 2. Summary of the Genetic Elements in Plasmid Vector PV-BNGT04.

<u>Genetic Element</u>	<u>Function (Reference)</u>
Right Border	A 25 nucleotide direct repeat that acts as the initial point of DNA transfer into plant cells, originally isolated from pTiT37 (Depicker <i>et al.</i> , 1982).
<i>P-CMoVb</i>	The 35S promoter from a modified figwort mosaic virus (Gowda <i>et al.</i> , 1989; Richins <i>et al.</i> , 1987; Sheperd <i>et al.</i> , 1987).
<i>Arab-SSU1A/CTP1</i>	The N-terminal of the small subunit 1A of the ribulose-1,5-bisphosphate carboxylase chloroplast transit peptide from <i>Arabidopsis</i> (Timko <i>et al.</i> , 1988).
<i>goxu247syn</i>	A synthetic glyphosate oxidoreductase (<i>gox</i>) gene variant number 247 based on the glyphosate oxidoreductase (<i>gox</i>) gene isolated from <i>Ochrobactrum anthropi</i> strain LBAA (Appendix 2, [redacted] <i>et al.</i> , 1994; and Appendix 3, [redacted] <i>et al.</i> , 1994).
<i>E9 3'</i>	The 3' end of the pea <i>rbcs</i> E9 gene which provides the polyadenylation sites for the <i>goxu247</i> and CP4 EPSPS genes (Coruzzi <i>et al.</i> , 1984; Morelli <i>et al.</i> , 1985).
<i>AEPSPS/CTP2</i>	The N-terminal chloroplast transit peptide sequence from the <i>Arabidopsis</i> EPSPS gene (Klee <i>et al.</i> , 1987).
CP4 EPSPS <i>syn</i>	The synthetic 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) gene based on the sequence from <i>Agrobacterium</i> sp. strain CP4 (Padgett <i>et al.</i> , 1996; see Appendix 4).
Left Border	Isolated from the octopine Ti plasmid, pTiA6, and contains the 25 bp direct repeat sequence that delimits the DNA transferred (Barker <i>et al.</i> , 1983).
<i>ori-V</i>	The vegetative origin of replication that permits plasmid replication in <i>Agrobacterium</i> . It was originally isolated from plasmid RK2 (Rogers <i>et al.</i> , 1987).
<i>ori-322</i>	A plasmid replication origin which permits propagation of DNA in bacterial hosts such as <i>E. coli</i> . (Sutcliffe, 1979).
<i>aad (Spc/Str)</i>	The bacterial gene encoding the Tn7 AAD 3' adenylyltransferase conferring spectinomycin and streptomycin resistance to bacterial cells (Fling <i>et al.</i> , 1985).

B. CP4 EPSPS gene

Two genes were introduced into Roundup Ready canola line RT73 that confer tolerance to glyphosate: CP4 EPSPS and *goxv247*. The CP4 EPSPS gene, encoding the protein 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), was originally obtained from *Agrobacterium* sp. strain CP4 and is identical to the CP4 EPSPS gene in Roundup Ready cotton line 1445 and soybean line 40-3-2 which have received determinations of non-regulated status from USDA (USDA, 1995; USDA, 1994).

The protein, CP4 EPSPS, coded for by the CP4 EPSPS gene, catalyzes the reaction of shikimate-3-phosphate (S3P) with phosphoenolpyruvate (PEP) into 5-enolpyruvylshikimate-3-phosphate (EPSP), as do other EPSPSs, a step in the production of aromatic amino acids via the shikimate pathway (Herrmann, 1983; Haslam, 1974). Unlike EPSPSs found in plants, CP4 EPSPS is highly insensitive to inhibition by glyphosate, the active ingredient in Roundup® herbicide. The CP4 EPSPS protein represents one of many different EPSPSs found in nature (Schulz *et al.*, 1985), is highly tolerant to inhibition by glyphosate and has high catalytic efficiency, compared to most EPSPSs (Barry *et al.*, 1992; Padgett *et al.*, 1991).

The CP4 EPSPS gene from *Agrobacterium* sp. strain CP4 has been completely sequenced and encodes a 47.6 kD protein consisting of a single polypeptide of 455 amino acids. The bacterial isolate, CP4, was identified by the American Type Culture Collection as an *Agrobacterium* species. There is no human or animal pathogenicity known from *Agrobacterium* species, nor is the EPSPS gene a determinant of *Agrobacterium* plant pathogenesis. The amino acid sequence of CP4 EPSPS is given in Figure 3.

The original gene sequence from *Agrobacterium* was modified to create a synthetic gene which allows for higher expression in plants. Bacterial genes, like those from *Agrobacterium*, have several features that reduce their ability to function efficiently in plants. These features include potential polyadenylation sites that are often rich with A+T nucleotides, a higher G+C nucleotide percentage than that frequently found in dicotyledonous plant genes, concentrated stretches of G and C nucleotide residues, and codons that may not be found frequently in dicotyledonous plant genes. This high G+C nucleotide percentage in the CP4 EPSPS gene from *Agrobacterium* sp. could result in the formation of strong hairpin structures that may affect expression or stability of the RNA. Therefore, a plant-preferred version of this gene was synthesized and used in vector PV-BNGT04 without affecting the functional activity of the expressed CP4 EPSPS protein.

Figure 3. Deduced amino acid sequence of CP4 EPSPS.

1	MSHGASSRPA	TARKSSGLSG	TVRIPGDKSI	SHRSFMFGGL	ASGETRITGL
51	LEGEDVINTG	KAMQAMGARI	RKEGDTWIID	GVGNGGLLAP	EAPLDFGNAA
101	TGCRLTMGLV	GVYDFDSTFI	GDASLTKRPM	GRVLNPLREM	GVQVKSEDGD
151	RLPVTLRGPK	TPTPITYRVP	MASQVKSASV	LLAGLNTPGI	TTVIEPIMTR
201	DHTEKMLQGF	GANLTVETDA	DGVRTIRLEG	RGKLTGQVID	VPGDPSSTAF
251	PLVAALLVPG	SDVTILNVLM	NPTRTGLILT	LQEMGADIEV	INPRLAGGED
301	VADLRVRSST	LKGVTVPEDR	APSMIDEYPI	LAVAAFAEG	ATVMNGLEEL
351	RVKESDRLSA	VANGLKLVGV	DCDEGETSLV	VRGRPDGKGL	GNASGAAVAT
401	HLDHRIAMSF	LVMGLVSENP	VTVDDATMIA	TSEPEFMDLM	AGLGAKIELS
451	DTKAA				

C. *goxv247* gene

A variant of the *gox* gene, cloned from *Ochrobactrum anthropi*¹ strain LBAA, was also inserted to provide tolerance to glyphosate. The variant *gox* gene, *goxv247*, encodes the glyphosate-metabolizing enzyme glyphosate oxidoreductase (GOXv247) (Hallas *et al.*, 1988; Barry *et al.*, 1992). The proteins GOX and the GOXv247 variant of the same enzyme are >99% identical, differing by 3 amino acids out of more than 400. The substitution of the histidine residue at position 334 with arginine effects a ten-fold lowering of the apparent K_m ² (app K_m) for glyphosate in GOXv247 (Barry *et al.*, 1994; see Appendix 3), and thus enhances the efficiency of glyphosate degradation. GOX was isolated from *Ochrobactrum anthropi* strain LBAA, and catalyzes the breakdown of glyphosate into aminomethylphosphonic acid (AMPA) and glyoxylate (Barry *et al.*, 1994; see Appendix 2). This degradation effectively inactivates the herbicide and enables canola RT73 to grow when treated with Roundup® herbicide.

As with the CP4 EPSPS gene above, the *gox* gene sequence from *Ochrobactrum anthropi* was modified to create a synthetic gene which allows for higher expression in plants. The amino acid sequence of GOXv247 is compared to that of GOX in Figure 4. It is readily seen that the two are >99% identical. The differences between the two forms of GOX are shown in bold in Figure 4.

¹ A previous designation was *Achromobacter* sp. strain LBAA.

² The Michaelis-Menton constant, K_m , is a measure of the affinity of a particular substrate for an enzyme. The lower the K_m , the higher the affinity for the enzyme.

[AEPSPS/CTP2] (Gowda *et al.*, 1989; Richins *et al.*, 1987; Klee *et al.*, 1987). Likewise, the *goxv247* gene was fused to the N-terminal chloroplast transit peptide sequence of the small subunit 1A ribulose-1,5-bisphosphate carboxylase gene from *Arabidopsis*; [Arab-SSU1A/CTP1] (Timko *et al.*, 1988). The current literature on transit peptides supports a model whereby the CTP is degraded rapidly and completely by proteases after transport of the precursor protein has occurred. Thus, after a "pre-" protein (containing the CTP amino-terminal extension) reaches the chloroplast or plastid stroma, the CTP is cleaved and degraded (Bartlett *et al.*, 1982) leaving only a "mature" protein. Therefore, the "mature" (not containing the CTP) CP4 EPSPS and GOXv247 proteins are the only introduced proteins present in canola RT73.

V. GENETIC ANALYSIS AND AGRONOMIC PERFORMANCE

A. Characterization of Inserted Genetic Material Including Insert Stability

As described in Part III-A, Roundup Ready canola line RT73 was generated using *Agrobacterium tumefaciens* mediated transformation with the plasmid PV-BNGT04. This vector (Figure 2) contains two functional segments: the T-DNA containing the CP4 EPSPS and *goxv247* genes bounded by the Right and Left Borders and the plasmid backbone containing the bacterial origins of replication and selectable marker. DNA analyses were performed to characterize the inserted DNA in terms of:

- insert number (number of integration events)
- copy number (number of T-DNA copies at a particular genetic locus)
- insert integrity (gene size, composition and linkage)

The characterization was performed by PCR and Southern blot analyses on genomic DNA isolated from the leaf tissue of the control and transgenic canola generated in field tests from 1992 (Mullis and Faloona, 1987; Southern, 1975). A similar glyphosate-tolerant canola line, designated GT200, was included in the molecular characterization studies which were conducted, but this line is not the subject of this petition.

Molecular analyses performed on RT73 demonstrate that only a single copy of the T-DNA was inserted into the genomic DNA of Westar at a single location to produce RT73 and that the plasmid backbone sequences, including the bacterial marker gene *aad*, were absent from DNA of RT73 (Table 3). PCR analysis was conducted to demonstrate that the border sequences of the T-DNA were the endpoints of the DNA insert, as further evidence that only the T-DNA sequences are present in the DNA of RT73. Southern blot analysis

performed on DNA from the R₃ and R₅ generations showed the same patterns, demonstrating structural stability of the inserted DNA. Finally, the presence of the single insert was confirmed by inheritance data showing the glyphosate tolerance phenotype was inherited as a single dominant Mendelian trait. Details of these analyses follow.

Table 3. Summary of Genetic Elements Found in Roundup Ready Canola RT73

<u>Genetic Element</u>	<u>Line RT73¹</u>
CMoVb	+
<i>goxu247</i>	+
CP4 EPSPS	+
E9 3'	+
<i>aad</i> (Spc/Str)	-
<i>ori-V</i>	-
<u>Number of Loci</u>	<u>1</u>

¹ + indicates the genetic element is present;
 - indicates the genetic element is not present

1. Insert Number and Copy Number

Analyses performed on DNA derived from leaves of R₃ canola RT73 plants demonstrate that only a single copy of the DNA was inserted into the genomic DNA of Westar at a single location to produce Roundup Ready canola line RT73. Genomic DNA isolated from RT73 leaf tissue and Westar was digested with *SpeI*, a restriction enzyme that does not cut inside the plasmid used in transformation. Since *SpeI* does not cut within PV-BNGT04, the number of bands present in this Southern blot correspond to the number of loci where plasmid DNA has been inserted into the plant genomic DNA. The positive control on these blots was the transformation vector PV-BNGT04 cut with *EcoRI*. The resulting blot was probed with ³²P-labelled plasmid PV-BNGT04. Figure 7 shows one very high molecular weight band in all lanes including the Westar control lane. This band represents cross-hybridizing sequences found naturally in all canola lines. Blots using intact transformation vectors containing the plasmid backbone often show some background hybridization with plant genomic DNA. A second higher molecular weight band is seen in lane 3 containing RT73. This single distinctive band in RT73 indicates that the T-DNA integrated at a single locus in each line. The presence of a single insert was confirmed by inheritance data showing the glyphosate tolerance phenotype was inherited as a single dominant Mendelian trait (see below).

2. Insert Composition and Structure

Only the genetic elements responsible for the glyphosate tolerance proteins and resultant phenotype were detected in canola line RT73. This insert (Figure 2, 6) contains the CMoVb promoter, the *Arabidopsis* small subunit CTP, the *goxv247* variant gene, the pea E9 3' terminator, a second copy of the CMoVb promoter, the *Arabidopsis* EPSPS CTP, the CP4 EPSPS gene, and a second copy of the pea E9 3' terminator.

Genetic Elements

In order to identify the genetic elements present in line RT73, Southern blot analyses were done. The positive control on these blots was the transformation vector PV-BNGT04. The negative control was the untransformed parental line Westar. Genetic element-specific probes for *goxv247*, CP4 EPSPS gene, *oriV/ori322* region and the *aad* gene were utilized as shown in Table 5 and Figure 6. Plasmid and genomic DNA was cut with *EcoRI* unless otherwise noted in figure legends. There are 6 *EcoRI* sites within PV-BNGT04, which all occur between the left and right border sequences, as illustrated in Figure 6. A second glyphosate-tolerant canola line, designated GT200, was included in the molecular characterization studies, but this line is not the subject of this petition.

a. GOXv247 coding sequences: Southern blot analysis was performed using genomic DNA extracted from leaf tissue from line RT73 and the parental negative control line Westar. All DNAs were cut with *EcoRI*. The blot was probed with a ³²P-labelled fragment containing a full-length copy of *goxv247* (Figure 6, Probe 1). In Figure 8, Panel A, a single band of approximately 1650 bp, the predicted size of *goxv247*, is observed in lanes 1 and 4 containing PV-BNGT04 and RT73 DNA. The *goxv247* band in the RT73 lane migrates slightly slower than in the plasmid control lane due to matrix effects of the abundant genomic DNA in the RT73 lane. No hybridizing band is observed in the negative control lane containing Westar.

c. CP4 EPSPS coding sequences: Southern blot analysis was performed in a similar manner to that described for *goxv247*. The blot was probed with a ³²P-labelled fragment containing a full-length copy of CP4 EPSPS (Figure 6, Probe 2). Figure 8, Panel B, shows a band of approximately 1775 bp, the predicted size of CP4 EPSPS, in line RT73 as well as in the positive control lane containing PV-BNGT04. The CP4 EPSPS band in the RT73 lane migrates slightly slower than in the plasmid control lane due to matrix effects of the abundant genomic DNA in the RT73 lane. No hybridizing band is observed in the negative control lane containing Westar.

d. Ori-322, ori-V and *aad* (Str/Spc) sequences: Southern blot analyses were performed in a similar manner to that described for *goxv247*. Separate

blots were prepared for the *ori-322* and *ori-V* region of the backbone and for the region containing the bacterial marker gene *aad*. Blots were either probed with a ^{32}P -labelled fragment containing the bacterial origins of replication (*ori-322* and *ori-V*) (Figure 6, Probe 3) or fragment containing the *aad* (Str/Spc) gene (Figure 6, Probe 4). Figure 9, Panel A, shows a band of approximately 6400bp in the positive control lane 1 containing PV-BNGT04, the predicted size of the fragment containing the origin of replication. No band was observed in lanes 2 and 3 containing Westar and RT73, respectively. Similarly in Figure 9, Panel B, a single band of approximately 6400kb was apparent in the positive control lane, but no band was observed in either the Westar or RT73 lanes. Therefore, it is concluded that neither origins of replication nor the bacterial marker gene *aad* are present in RT73.

e. Characterization of the right and left borders: There are two DNA sequences of 25bp each within the plasmid used in the transformation of RT73 that are defined as the "right border" and the "left border." The right border from *Agrobacterium* functions as the initiation site of the transfer of the DNA into the plant genomic DNA. The left border functions as the termination site of that transfer. PCR analysis was conducted to demonstrate that the border sequences of the inserted DNA were the endpoints of the DNA insert and to provide further evidence that only the T-DNA sequences are present in the DNA of RT73.

PCR primers from the region just inside the borders and just outside the borders (see Figure 5) were used with template DNA derived from RT73. DNA from the Westar variety served as negative control and an appropriate plasmid vector, PV-BNGT03 (a plasmid vector identical to PV-BNGT04, except for the presence of the unmodified *gox* gene in place of the *gox* variant gene *goxu247*) served as a positive control.

Right border: The right border (RB) sequence is located from map position 9207 to 9231 (Figure 2). If the T-DNA has been inserted intact into RT73, it is predicted that a product will be produced with primer #1 plus primer #2 and primer #1 plus primer #3 using RT73 DNA as template since all of these sequences are located within the T-DNA. If the RB delimits the inserted T-DNA, no product would be expected with primer #1 and primer #4 with RT73 DNA as template, as primer #4 sequence falls outside the T-DNA. The primers used in characterization of the right border are illustrated in Figure 5, panel A.

Using PV-BNGT03 DNA as template, the primers should give products of the following sizes: $1 + 2 = 343\text{bp}$; $1 + 3 = 567\text{bp}$; and $1 + 4 = 683\text{bp}$. If the functionality of the right border has been maintained, no product should be produced when using RT73 DNA as template with primers $1 + 4$. The results

of the PCR using the above primer combinations with PV-BNGT03, RT73, GT200 (another Roundup Ready canola line that is not being commercialized and is not the subject of this petition), and Westar templates, are shown in Figure 10.

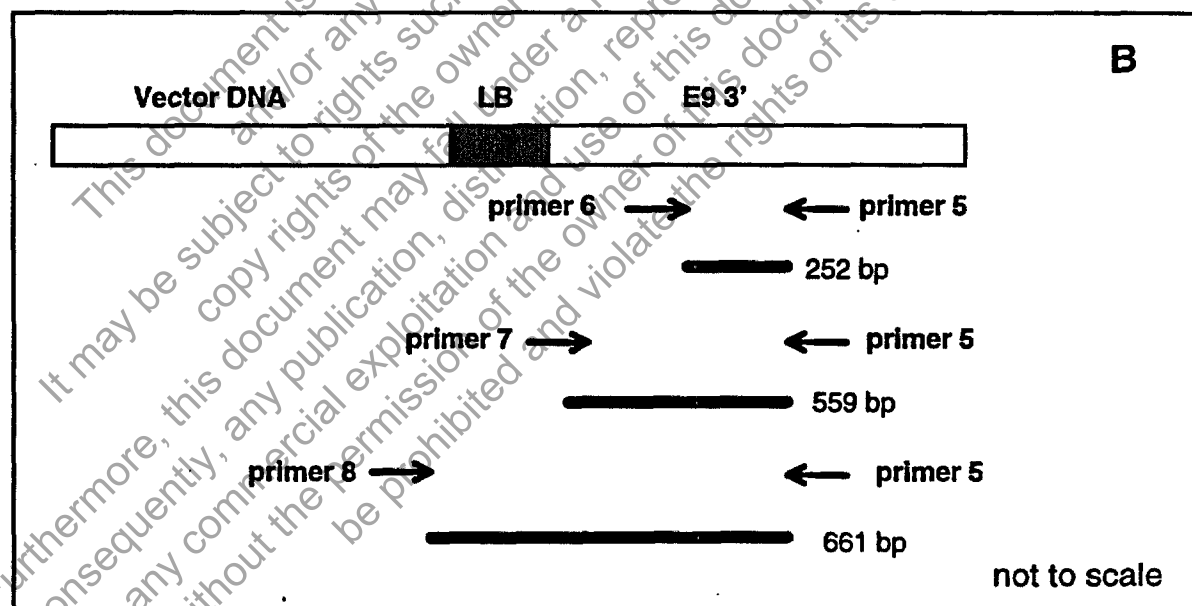
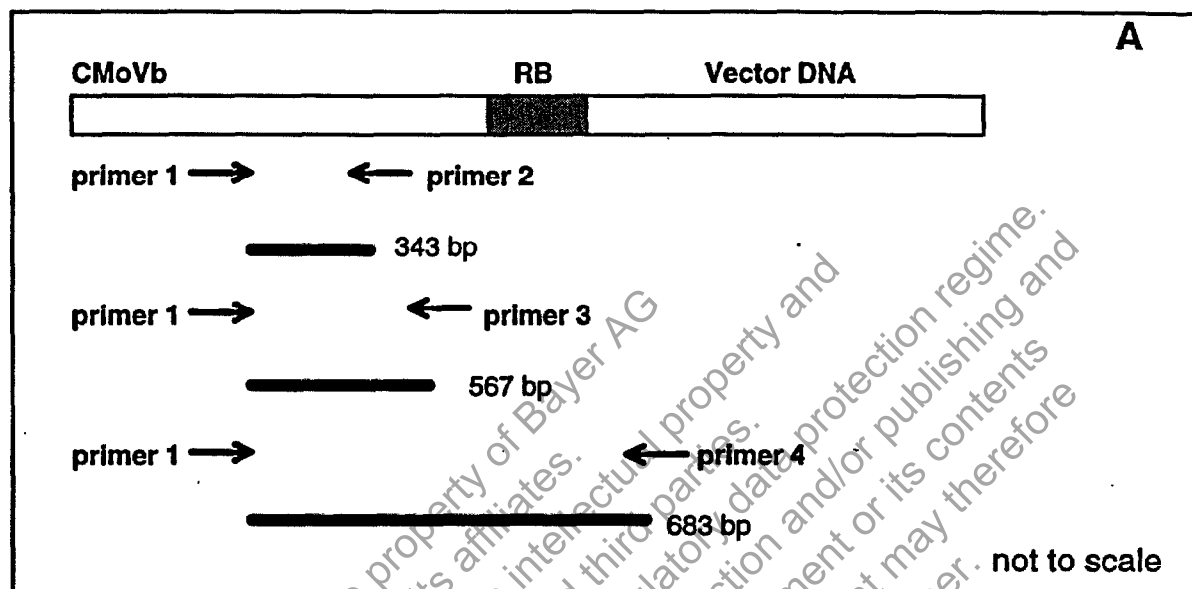
The PV-BNGT03 template shows products of 343bp, 567bp, and 683bp as expected. Canola RT73 has a product of 343bp with primers 1 + 2 and a product of 567bp with primers 1 + 3. RT73 did not yield an amplified product when primers 1 + 4 were used. No bands were observed in the Westar control with any of the combinations of the primers. These results establish that integration of the plasmid DNA did not proceed outside of the right border.

Left border: The left border (LB) sequence is located from map position 3994 to 4017 (Figure 2). If the T-DNA has been inserted intact into RT73, it is predicted that a product will be produced with primer #5 plus primer #6 and primer #5 plus primer #7 using RT73 DNA as template, since all of these sequences are located within the T-DNA. If the LB delimits the inserted T-DNA, no product would be expected with primer #5 and primer #8 with RT73 DNA as template, as primer #8 sequence falls outside the T-DNA. The primers used in characterization of the left border are illustrated in Figure 5, panel B.

Using positive control PV-BNGT03 DNA as template, the primers should give products of the following sizes: $5 + 6 = 252\text{bp}$; $5 + 7 = 559\text{bp}$; and $5 + 8 = 661\text{bp}$. If the functionality of the left border has been maintained, no product should be produced when using RT73 DNA as template with primers 5 + 8. The results of the PCR using the above primer combinations PV-BNGT03, RT73, GT200 (another Roundup Ready canola line that is not being commercialized and is not the subject of this petition), and Westar templates, are shown in Figure 11.

The PV-BNGT03 template shows products of 252bp, 559bp, and 661bp as predicted. Canola RT73 has a product of 252bp with primers 5 + 6 and a product of 559bp with primers 5 + 7. RT73 did not yield an amplified product when primers 5 + 8 were used. No bands are observed in the Westar control with any of the combinations of the primers. These results establish that integration of the plasmid DNA did not proceed outside of the left border.

Figure 5. Illustration of PCR Primers Used to Characterize the Right and Left Border Regions.



Solid bars indicate the predicted PCR product size for the primer pair used.

3. Stability of Inserted DNA

Structural stability of the inserted DNA was determined using Southern blot analysis performed on DNA from the R₃ generation and R₅ generation of canola RT73. DNA from Westar and RT73 from the R₃ generation or R₅ generation were digested with *EcoRI* and probed with either the *goxv247* or CP4 EPSPS coding regions or the E9 3' gene terminator region. Plasmid PV-BNGT04 was used as a positive control. Each of the blots exhibited identical banding patterns in the RT73 R₃ or R₅ generation (Figure 12), showing physical stability of the inserted and surrounding canola genomic DNA.

4. Mendelian Inheritance

The physical stability of the genetic insert conferring resistance to glyphosate in RT73 is consistent with inheritance data in BC1F2 progeny of crosses between traditional canola lines and RT73 that consistently segregate 3 tolerant to 1 susceptible. This segregation ratio establishes that the RT73 insert behaves as a single dominant gene that is inherited in a Mendelian fashion (Table 4). The glyphosate tolerance phenotype and Mendelian transmission have been consistent over more than five generations of canola RT73.

Table 4. Segregation of Glyphosate-Tolerant Canola Obtained in BC1F2 Crosses Using RT73.

Cross	Observed		Expected		Chi-square (X ²) value
RT73 x CV1	Tolerant	187	Tolerant	183	0.350
	Susceptible	57	Susceptible	61	
RT73 x CV2	Tolerant	162	Tolerant	164	0.123
	Susceptible	57	Susceptible	55	
RT73 x CV3	Tolerant	223	Tolerant	224	0.005
	Susceptible	75	Susceptible	74	

Tabular value at one degree of freedom and a five percent level of significance = 3.84
CV1, CV2 and CV3 designate non-transgenic canola lines

Summary

As clearly demonstrated, the only genes present on PV-BNGT04 which are present in Roundup Ready canola line RT73 are CP4 EPSPS and *goxv247*. No genetic elements from outside of the right and left borders of the T-DNA were transferred into the genomic DNA of the Roundup Ready canola line RT73. This conclusion was drawn from the following types of molecular data: 1) the positive detection of fragments containing the CP4 EPSPS and *goxv247* genes by Southern analysis; 2) the lack of *ori-322* and *ori-V* signals by Southern analysis; and 3) the lack of PCR fragments produced using PCR

primer pairs, one of which is located within the T-DNA and the other located just beyond either of the right or left border sequences. The stability of the inserted DNA has been demonstrated both by molecular characterization by Southern analysis of R₃ and R₅ generation of RT73 and observation of simple Mendelian inheritance over many generations.

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Figure 6. Schematic Diagram of PV-BNGT04 Showing the Probes Used in the Molecular Characterization of Roundup Ready Canola Line RT73.

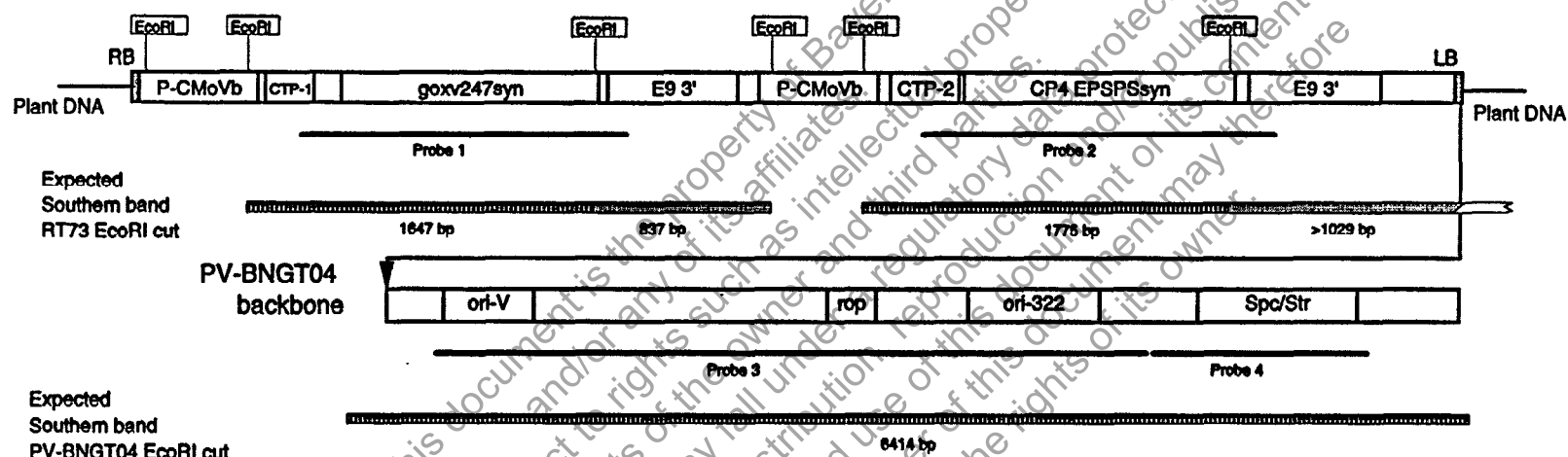
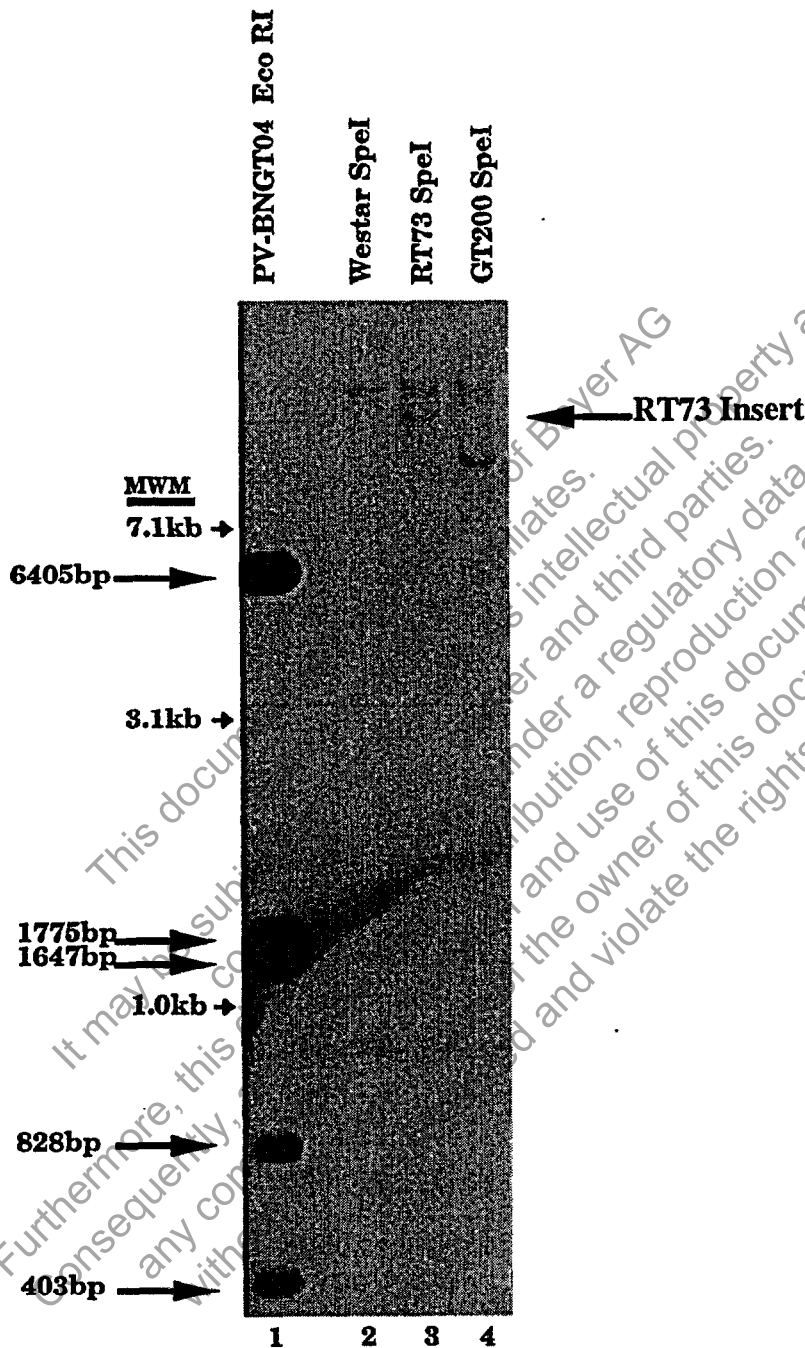


Table 5. Description of Probes Used in Molecular Characterization of Roundup Ready Canola Line RT73.

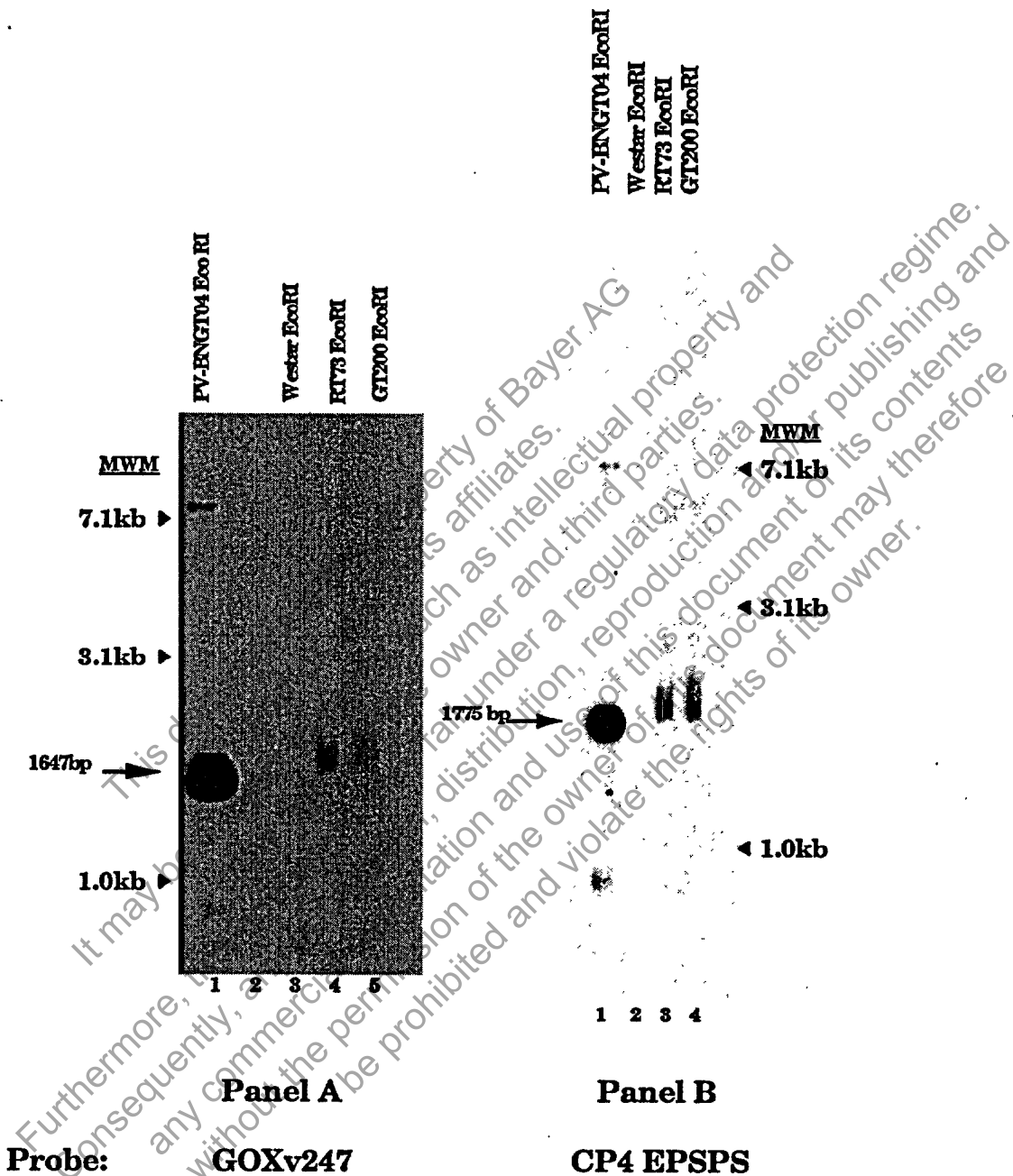
Genetic Element	Source	Probe Number	Restriction Sites	Size of Probe
<i>goxv247 syn</i>	pMON17062	Probe 1	BglII/KpnI	1.324 Kb/full-length
CP4 EPSPS <i>syn</i>	pMON17209	Probe 2	BamHI/SphI	1.393 Kb/full-length
<i>oriV/ori322</i>	pMON977	Probe 3	PvuI	4.144 Kb/full-length
<i>aad</i> (Spc/Str)	pMON5723	Probe 4	BamHI	1.189 Kb/full-length

Figure 7. Southern Blot of Canola Line RT73 to Determine Number of Loci Integrated.



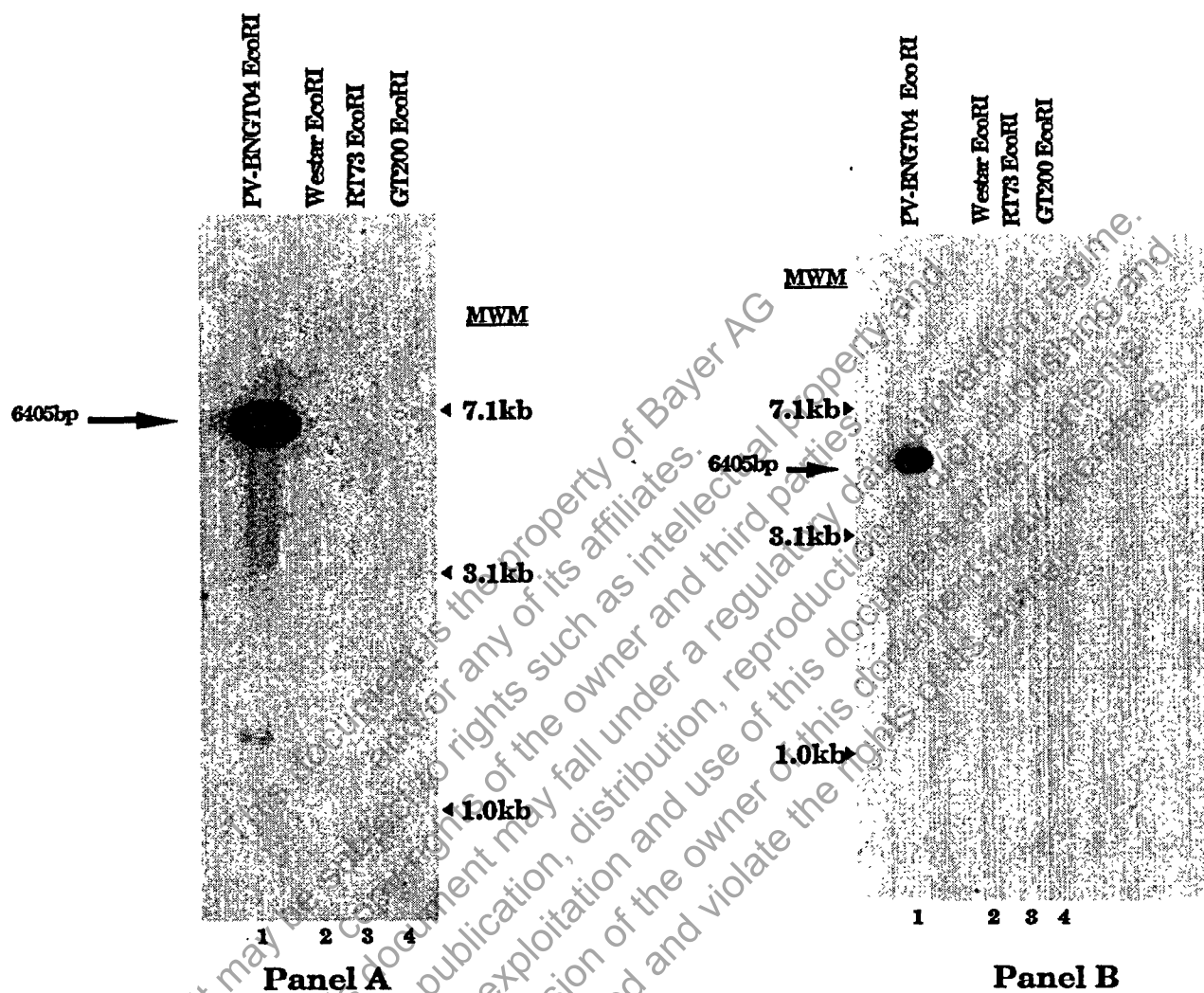
PV-BNGT04 plasmid DNA (lane 1) was digested with EcoRI. Westar control genomic DNA (lane 2), RT73 genomic DNA (lane 3) and GT200 DNA (lane 4) were digested with *SpeI*. Each lane represents 100 pg plasmid DNA or 5 µg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with a ³²P-labelled PV-BNGT04 plasmid DNA and subjected to autoradiography.

Figure 8. Southern Blot Probed for GOXv247 and CP4 EPSPS in Canola Line RT73.



PV-BNGT04 plasmid DNA (lane 1), Westar control genomic DNA (lane 2), RT73 genomic DNA (lane 3) and GT200 DNA (lane 4) were digested with *EcoRI*. Each lane represents 100 pg plasmid DNA or 5 µg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with ³²P-labelled DNA from the GOX coding region for panel A or ³²P-labelled DNA from the CP4 EPSPS coding region for panel B and subjected to autoradiography.

Figure 9. Southern Blot Probed with ori-v and ori-322 or *aad* (Spc/Str) in Canola Line RT73.



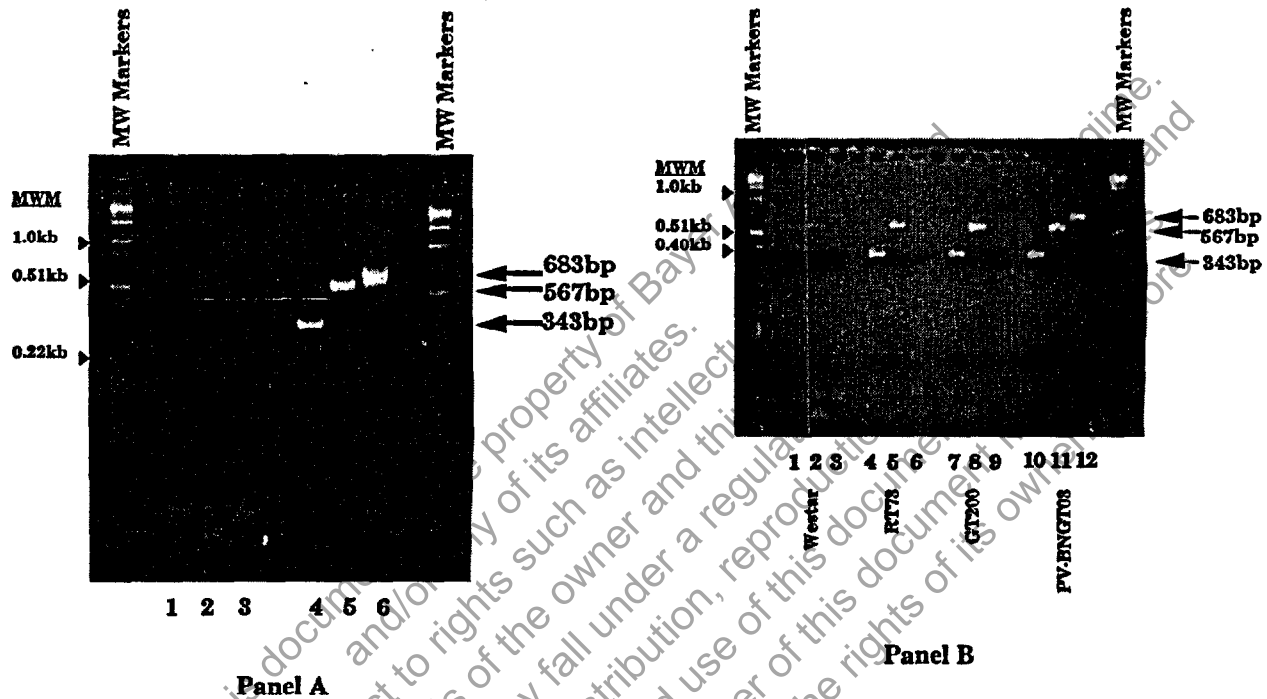
Probe: ori-322/Ori-V

aad

PV-BNGT04 plasmid DNA (lane 1), Westar control genomic DNA (lane 2), RT73 genomic DNA (lane 3) and GT200 DNA (lane 4) were digested with *EcoRI*. Each lane represents 100 pg plasmid DNA or 5 µg of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membrane was probed with a ³²P-labelled DNA fragment containing ori-322 and Ori-V for panel A or ³²P-labelled DNA from the *aad* (Spc/Str) coding region for panel B and subjected to autoradiography.

Figure 10. PCR Analysis of the Right Border in Canola Line RT73.

Genomic DNA from Canola Westar control, lines RT73 and RT200 were analyzed by PCR to determine the integrity of the right border. The positive control was PV-BNGT03 plasmid DNA which is equivalent to PV-BNGT04 in that it contains identical elements including CMoVb promoter adjacent to the right border and plasmid backbone sequence.



The predicted product sizes are shown below:

Primer 1 + primer 2 = 343bp. Primer 1 + primer 3 = 567bp. Primer 1 + primer 4 = 683bp.

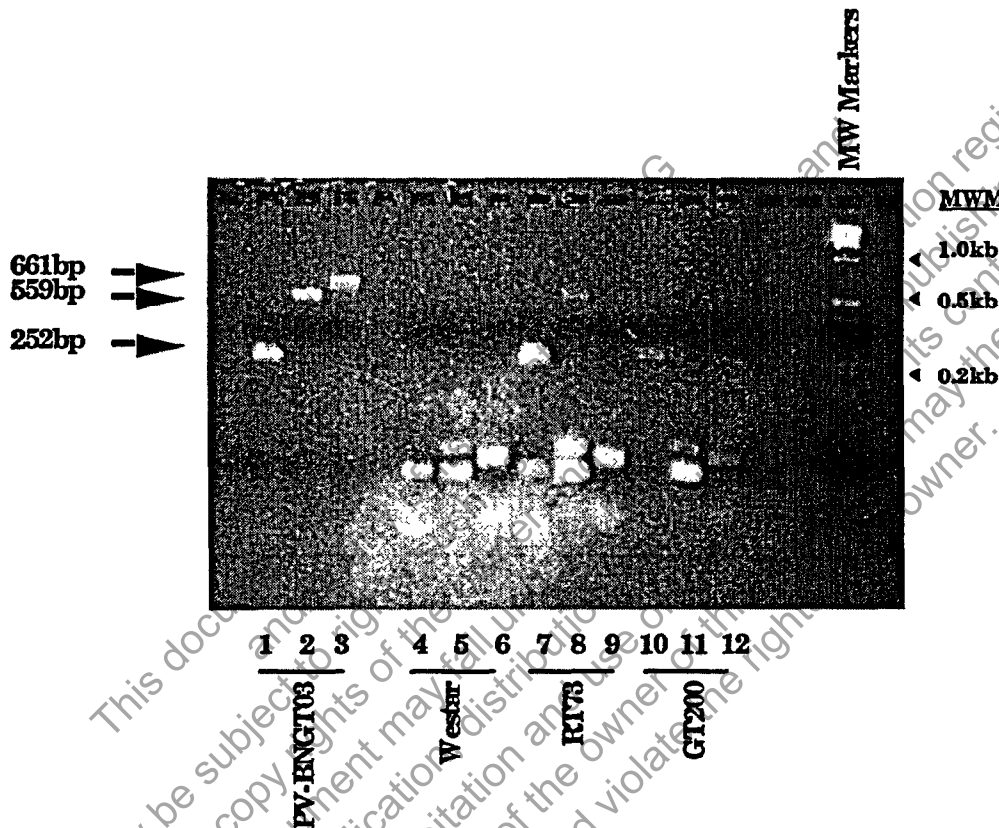
Panel A: Lanes 1, 2 and 3 have Westar genomic DNA as template. Lanes 4, 5, and 6 have PV-BNGT03 plasmid DNA for template. Lanes 1 and 4 have primers 1 + 2; lanes 2 and 5 have primers 1 + 3; lanes 3 and 6 have primers 1 + 4.

Panel B: Lanes 1, 2, and 3 have Westar DNA for template. Lanes 4, 5, and 6 have RT73 genomic DNA as template. Lanes 7, 8, and 9 have genomic GT200 DNA as template. Lanes 10, 11, and 12 have PV-BNGT03 plasmid DNA as template. Lanes 1, 4, 7, and 10 have primers 1 + 2. Lanes 2, 5, 8, and 11 have primers 1 + 3. Lanes 3, 6, 9, and 12 have primers 1 + 4. Faint bands seen in the Westar control (lanes 1, 2, 3 in Panel B) are due to contamination of this reaction sample with PV-BNGT03 DNA. A second Westar genomic DNA sample was used as PCR template to produce the results in Panel A where no amplified bands are observed in lanes 1, 2 and 3.

Reactions were done in 100µl total volume containing 100pg of each primer, 500ng template DNA (50 ng plasmid DNA), dNTP's at 200 µM, 10 units of Taq® Polymerase (Perkin-Elmer Norwalk, CT). The PCR amplification cycle consisted of 94°C denaturation for 1.5 min, 65°C annealing for 1.5 min., and a 72°C extension for 1.5 min. The cycle was repeated 24 times. Products were separated on a 3% agarose gel and visualized by ethidium bromide. The lower bands at the bottom of the gel are unused oligonucleotides.

Figure 11. PCR Analysis of the Left Border in Canola Line RT73.

Genomic DNA from Canola Westar control, lines RT73 and GT200 were analyzed by PCR to determine the integrity of the left border. The positive control was PV-BNGT03 plasmid DNA which is equivalent to PV-BNGT04 in that it contains identical elements including E9 3' adjacent to the left border and plasmid backbone sequence.

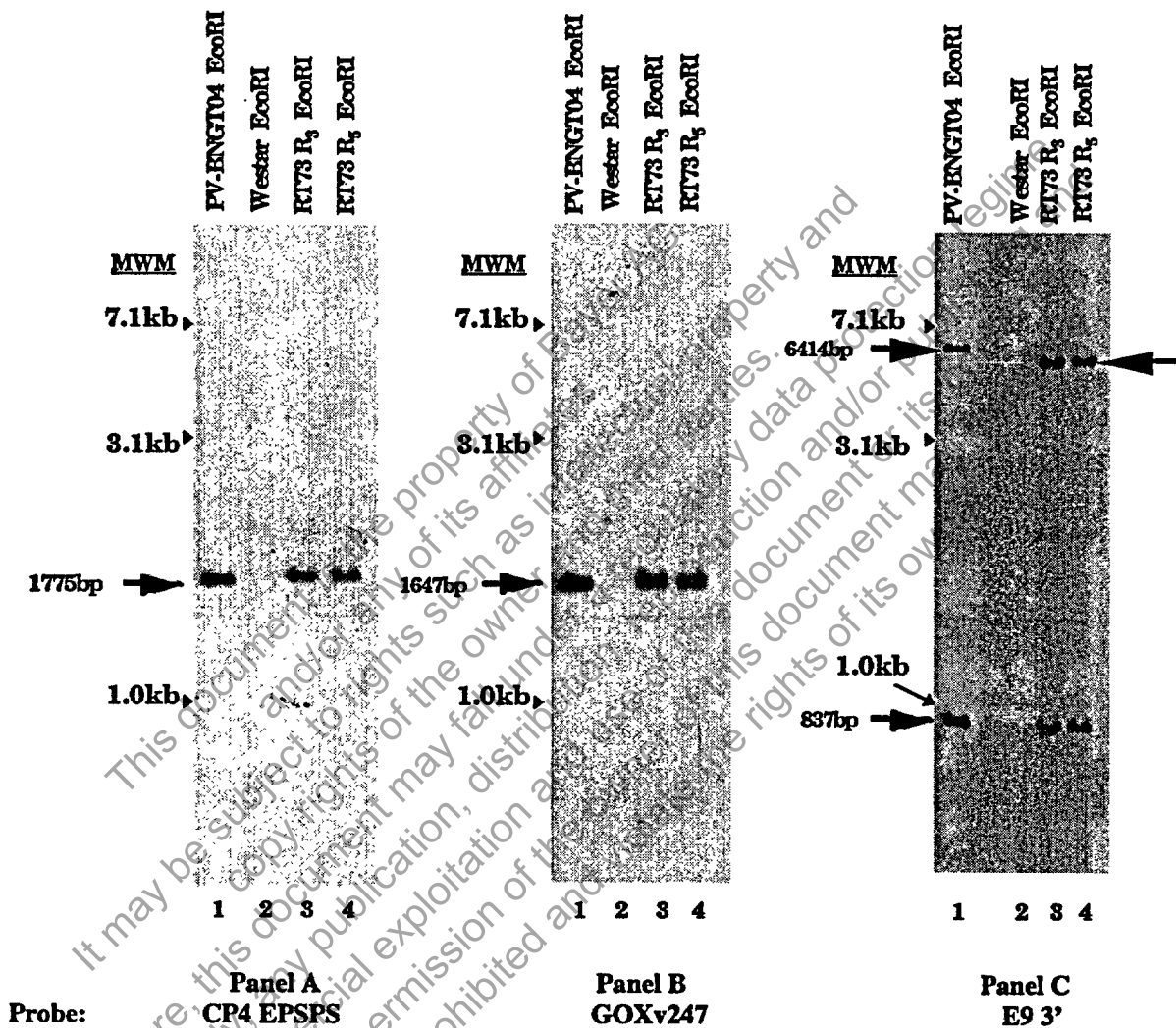


The predicted product sizes are shown below:

Primer 5 + primer 6 = 252bp. Primer 5 + primer 7 = 559bp. Primer 5 + primer 8 = 661bp.

Lanes 1, 2, and 3 have PV-BNGT03 plasmid DNA for template. Lanes 4, 5, and 6 have genomic Westar control DNA as template. Lanes 7, 8, and 9 have genomic RT73 DNA as template. Lanes 10, 11, and 12 have genomic GT200 DNA as template. Lanes 1, 4, 7, and 10 have primers 5 + 6. Lanes 2, 5, 8, and 11 have primers 5 + 7. Lanes 3, 6, 9, and 12 have primers 5 + 8. Reactions were done in 100µl total volume containing 100pg of each primer, 500ng template DNA (50 ng plasmid DNA), dNTP's at 200 µM, 10 units of Taq® Polymerase (Perkin-Elmer Norwalk, CT). The PCR amplification cycle consisted of 94°C denaturation for 1.5 min, 65°C annealing for 1.5 min., and a 72°C extension for 1.5 min. The cycle was repeated 24 times. Products were separated on a 3% agarose gel and visualized by ethidium bromide. The lower bands at the bottom of the gel are unused oligonucleotides.

Figure 12. Southern Blot Analysis of R₃ and R₅ Generation DNA from Canola Line RT73.



PV-BNGT04 plasmid DNA (lane 1), Westar control genomic DNA (lane 2), RT73 R₃ genomic DNA (lane 3) and RT73 R₅ genomic DNA (lane 4) were digested with *EcoRI*. Each lane represents 100 pg plasmid DNA or 5 µg (10 µg in Panel C) of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to nylon membranes. The membranes were probed with a ³²P-labelled DNA fragments containing the CP4 EPSPS coding region for Panel A or ³²P-labelled DNA from the *gox* coding region for Panel B and ³²P-labelled DNA from the E9 3' region for Panel C and then subjected to autoradiography.

B. Expression of the Inserted Genes

To thoroughly characterize canola RT73, the levels of CP4 EPSPS and GOXv247 proteins were determined in leaf and seed tissue from three Canadian field sites in 1992, in seed at 4 Canadian field sites in 1993 and in leaf and seed in 6 European (2 each in Belgium, UK and France) field sites in 1995. Expression of the CP4 EPSPS and GOX proteins is constitutive with both proteins being detectable at low levels in leaves and seed. This is as expected when using the *CMoVb* promoter (Sheperd *et al.*, 1987).

Expression levels of CP4 EPSPS and GOXv247 proteins in canola plant tissues were measured by a validated enzyme linked immunosorbent assay (ELISA) described below.

CP4 EPSPS ELISA

A double antibody indirect enzyme-linked immunosorbant assay (ELISA) was developed and validated for detection of CP4 EPSPS. Levels of CP4 EPSPS were determined by extrapolation from the logistic curve fit of the purified *E. coli* produced CP4 EPSPS standard curve. In brief, 96-well polystyrene plates were coated with purified goat anti-CP4 IgG. Canola tissue samples were ground in buffer and added to the antibody-coated wells alongside a range of pure CP4 standards in buffer for quantitation of CP4 in canola seed extracts or CP4 standards in buffer plus Westar leaf extract for quantitation of CP4 in canola leaf extracts. Plates were incubated for 4 hours allowing antigen capture by the surface bound antibodies. The plates were washed and a second antibody, rabbit anti-CP4 IgG, was added to the wells and incubated overnight. After washing the wells, donkey anti-rabbit IgG conjugated to alkaline phosphatase was added to each well. Following incubation and washing, alkaline phosphatase substrate (pNPP) is added to each well. Wells containing CP4 and hence, the goat-rabbit-donkey antibody sandwich, turn yellow. Quantitation of sample CP4 concentration was accomplished by extrapolation from the logistic curve fit of the CP4 standard curve (range of 0.09 - 0.75 ng/well). The assay recognizes the native CP4 EPSPS, but had no interference from endogenous EPSPS.

GOX ELISA

A direct double antibody sandwich (DAS) enzyme-linked immunosorbant assay (ELISA) was developed and validated to adequately quantitate the levels of GOX proteins. This ELISA uses a purified polyclonal antibody from goat (goat anti-GOX IgG) immobilized on 96-well plates to complex with GOX. The initial complex was then captured by a second antibody (goat anti-GOX-AP IgG) conjugated to alkaline phosphatase (AP). Development of the AP with pNPP, an AP substrate, yields a soluble yellow product. The optical

density was monitored using a spectrophotometric plate reader. Levels of GOX in samples were ultimately determined using the four parameter logistic equation to fit the standards which were obtained from an *E. coli* expression system. The ELISA was validated after running experiments that addressed extraction efficiency, the overall variability of the assay and the stability of GOX towards storage in leaf and seed tissue preparations.

In the 1992 trial, analysis of CP4 EPSPS and GOXv247 proteins in leaf tissue from RT73 gave mean expression levels of 0.034 µg/mg tissue (fresh weight) and 0.108 µg/mg tissue (fresh weight), respectively (Table 6). There was no evidence of an increase or decrease in leaf expression of CP4 EPSPS and GOXv247 over time. Analyses of seed gave mean levels of CP4 EPSPS and GOXv247 proteins of 0.049 µg/mg tissue (fresh weight) and 0.154 µg/mg tissue (fresh weight), respectively. These expression levels are relatively low, accounting for less than 0.02% and 0.07% of the total protein in the seed for CP4 EPSPS and GOXv247, respectively.

The data obtained in 1993 for expression in seed and in 1995 for seed and leaf was in good agreement with the values observed in the initial 1992 trials. Seed in 1993 had a range of expression for CP4 EPSPS in RT73 of 0.018 to 0.047 µg/mg tissue with a mean expression of 0.028 µg/mg tissue. The range of expression for GOXv247 was 0.108 to 0.334 µg/mg tissue, with a mean expression level of 0.194 µg/mg tissue. No leaf tissue was analyzed for expression level in the 1993 field trials. In the 1995 European study, analysis of CP4 EPSPS and GOXv247 proteins in leaf tissue from RT73 gave mean expression levels of 0.027 µg/mg tissue (fresh weight) and 0.133 µg/mg tissue (fresh weight), respectively. Analyses of seed gave mean levels of CP4 EPSPS and GOXv247 proteins of 0.028 µg/mg tissue (fresh weight) and 0.211 µg/mg tissue (fresh weight), respectively.

No detectable CP4 EPSPS or GOXv247 was measured in Westar seed tissue from any of the trials.

Table 6. CP4 EPSPS and GOXv247 Protein Expression in RT73 Canola Seed

Tissue Type		CP4 EPSPS Protein (µg/mg tissue fwt)			GOXv247 Protein (µg/mg tissue fwt)		
RT73		1992 ¹	1993 ²	1995 ³	1992 ¹	1993 ²	1995 ³
Leaf	mean:	0.034		0.027	0.108		0.133
	range:	0.022-0.037	n.a. ⁵	0.016-0.070	0.071-0.161	n.a. ⁵	0.082-0.247
Seed	mean:	0.049	0.028	0.028	0.154	0.194	0.211
	range:	0.044-0.051	0.018-0.047	0.017-0.037	0.109-0.203	0.108-0.334	0.122-0.313
Westar ⁴							
Leaf Seed	Leaf	ND	n.a. ⁵	ND	ND	n.a. ⁵	ND
	Seed	ND	ND	ND	ND	ND	ND

¹ Values for leaf and seed samples in 1992 from 3 locations in Canada. CP4 EPSPS analyses were done on single sample extracts, n=3 for leaf, n=3 for seed. GOX analyses were done on single sample extracts, run at two loadings, n=6 for leaf, n=6 for seed.

² Values for seed samples in 1993 from 4 locations in Canada. CP4 EPSPS analyses were done on single sample extracts at two loadings, n=8. GOX analyses were done on single sample extracts, duplicate runs at two loadings, n=16.

³ Values for leaf and seed samples in 1995 from 6 locations in Europe. CP4 EPSPS analyses were done on single sample extracts, run at two loadings, n=12 for leaf, and single loadings for seed, n=6 for seed. GOX analyses were done on single sample extracts, run at three loadings, n=18 for leaf, and two loadings for seed, n=12 for seed.

⁴ In each analysis, Westar samples were used as a negative control. Values for Westar samples were beneath a calculated limit of detection (LOD). The LOD is determined by computing the mean and the standard deviation for Westar control wells in ELISA. The LOD is then the mean plus three standard deviations. ND - not detected.

⁵ n.a. - not available.

C. Disease and Pest Resistance Characteristics

Roundup Ready canola line RT73 transformed with the plasmid vector, PV-BNGT04, was tested in replicated trials in two years of Canadian field trials in 1992 and 1993 in 22 locations, as well as US field trials in 1996 and 1997 in 23 locations under notifications acknowledged by the USDA (Appendix 1). Detailed monitoring for growth and development characteristics and disease and insect susceptibility of the transformed canola versus nontransgenic control plants was performed approximately every two weeks during the growing season. USDA final reports for the trials conducted in 1996 have been submitted; however, final reports for 1997 field trials will not be submitted until the required year of observation following planting has been completed.

Plots of the Roundup Ready canola line RT73 and Westar control plants were visually checked for the appearance of possible disease symptoms such as spotted leaves, leaf necrosis, stunted or distorted plants and wilting, which are indicative of, but not limited to, diseases such as: sclerotinia white mold (*Sclerotinia sclerotiorum*), powdery mildew (*Erysiphe communis*) and blackleg (*Phoma lingam*) (Auld *et al.*, 1989). Detailed quantitative monitoring of blackleg infestation was conducted, due to the significance of this disease in canola. Major insect pests of canola monitored were; flea beetles (*Phyllotreta* spp.), aphids (*Brevicoryne brassicae* L. and *Liaphis erysimis*) and cabbage seed-pod weevils (*Ceutorhynchus assimilis*).

Based on the results of the field monitoring program, there were no significant differences observed in disease or pest resistant characteristics between canola RT73 and the nontransgenic control (Appendix 7). The overall blackleg infestation rating for canola RT73 was 3.95, compared to 4.11 for the control variety, Westar. This difference of 4.6% was not statistically significant and falls within the range of variability of selections from Westar (1993 Canadian Co-Op tests - Report on Co-Operative Canola/Rapeseed Test 1993).

D. Compositional Analyses

Monsanto Company has completed consultation with the FDA following their policy, "Foods Derived from New Plant Varieties" on the food safety of Roundup Ready canola line RT73. Studies were carried out to compare the nutritional constituents of canola seed, refined, deodorized, bleached (RDB) oil and toasted meal from canola RT73 with seed, RDB oil and toasted meal from Westar control plants grown, processed and analyzed under the same conditions. The study demonstrated that canola RT73 seed and processed fractions of the seed are not significantly different from the nontransgenic

control seed and processed fractions. Based on this compositional information and the criteria provided in the food policy, FDA granted a finding of "No Concern" for Roundup Ready canola RT73 in September, 1995 (US Food and Drug Administration, 1995).

E. Toxicants

In addition to analyses for nutrients, canola RT73 was monitored for two antinutritional components: erucic acid in canola oil and glucosinolate content in canola seed.

Erucic acid is a mono-unsaturated, 22-carbon fatty acid (C22:1) that is a natural constituent of rapeseed. High erucic acid rapeseed oil (levels >2%) has been shown to have cardiopathic potential in experimental animals (Dupont *et al.*, 1989). Data obtained in 1992 and 1993 Canadian field trials show levels of erucic acid were well below the limits allowed for human consumption. Fatty acid profiles, including determinations of erucic acid levels, were determined using standard methodology (AOAC, 1990). The means and ranges of erucic acid content in canola RT73 were 0.24% (0.1-0.5, n=7) in 1992 and 0.04% (0-0.1%, n=4) in 1993.

Glucosinolates are derived biosynthetically from amino acids, with over 100 structural types having been identified (Sorensen, 1990). Numerous feeding studies with high and low glucosinolate varieties of rapeseed in swine, cattle, poultry and rats have noted a correlation between toxic effects as indicated by growth performance, reproduction, goitrogenicity, liver hypertrophy and hemorrhage and palatability and the levels of glucosinolates in the meal (Fenwick, 1989). In *B. napus* and *B. rapa* breeding programs and varietal registration tests, nine unique glucosinolates are closely monitored (Table 7).

Table 7. Commonly Detected Glucosinolates in Canola.

Structure Name	Trivial Name
allylglucosinolate	sinigrin
but-3-enylglucosinolate	gluconapin
2-hydroxybut-3-enylglucosinolate ^a	progoitrin
pent-4-enylglucosinolate	glucobrassicinapin
2-hydroypent-4-enylglucosinolate	napoleiferin
4-methylthiobutylglucosinolate	glucoerucin
5-methylthiopentylglucosinolate	glucoberteroin
4-hydroxyindol-3-ylmethylglucosinolate ^a	4-hydroxyglucobrassicin
Indol-3-ylmethylglucosinolate	glucobrassicin

^a The two glucosinolates that account for >70% of the total glucosinolate content in canola.

Because of the importance of maintaining canola quality, canola RT73 seed and toasted meal was analyzed for glucosinolates using standard methods of the Co-Op Test in both 1992 and 1993 (International Organization for Standardization, 1992). The analytical method used is included in Appendix 5. Means determined for the total alkyl and indolyl glucosinolates, as compared to the levels from the nontransgenic Westar controls are shown in Table 8 for both years. While it is apparent that the average level of alkyl glucosinolates in canola RT73 is consistently equal to or greater than the mean value for Westar nontransgenic controls, all individual values are well below the 30 $\mu\text{mole/g}$ commercial limit. In addition, statistical analysis of the data demonstrates that glucosinolate levels in canola RT73 will not exceed the 30 μmole limit at a 95% confidence level. The variation observed is typical for canola lines selected from the Westar variety, the parental variety for Roundup Ready canola RT73 (Downey opinion letter in Appendix 6). The levels of alkyl glucosinolates in canola RT73 are also well below the harvest survey values for commercially produced No. 1 Canadian canola in 1992 and 1993 (17 and 14 $\mu\text{mol/g}$, respectively) (DeClercq *et al.*, 1992, 1993). Thus there is no meaningful difference in glucosinolate levels between canola RT73 and Westar canola. Furthermore, the levels of the alkyl glucosinolates are well below the limits established for the safe use of meal derived from canola seed as an animal feed.

Table 8. Glucosinolate Levels in Roundup Ready Canola RT73 from 1992 and 1993 Canadian Field Trials.

		$\mu\text{mole/g}$ defatted meal		
		Mean ¹	Range	N
Alkyl				
RT73				
	1992	14.8	10.7-19.8	3
	1993	10.6	8.0-12.9	4
Westar				
	1992	8.8	6.2-11.4	7
	1993	8.7	6.7-11.1	4
Indolyl				
RT73				
	1992	10.8	9.2-11.6	3
	1993	11.5	10.9-12.0	4
Westar				
	1992	11.4	9.8-13.4	7
	1993	11.5	11.0-12.5	4

¹ - single samples were prepared and analyzed in quadruplicate in 1992 and triplicate in 1993. Replicates were averaged. The mean values are an average of the means of replicate analyses of the same sample.

F. Agronomic Performance

Roundup Ready canola RT73 was evaluated for agronomic performance in US field trials in 1996, 1997 and 1998 (ongoing) under permits or notifications acknowledged by the USDA-APHIS (Appendix 1), as well as in Canadian variety trials in 1992 and 1993.

Typical observations in the Canadian trials included relative emergence, vegetative growth, flowering time, days to maturity, yield and shattering. Canola RT73 was determined to be agronomically comparable to Westar and other nontransgenic commercial varieties (Appendix 7). Germination tests of seed of canola RT73 and Westar from Roundup treated and untreated 1992 variety trials were conducted at the Agriculture Canada seed quality testing laboratory in Saskatoon, Saskatchewan. Germination percentages were 98% for RT73 treated with Roundup and 99% for RT73 untreated and Westar, demonstrating high germination and essentially no difference between transgenic canola and nontransgenic controls. These findings, along with

data collected on volunteers observed in the year following RT73 planting indicate that there is not significant difference in dormancy between canola RT73 and the parental line, Westar (Appendix 7).

In February 1995 the Western Canadian Canola and Rapeseed Recommending Committee (WCCRRC) recommended RT73 for conditional registration on the basis of its suitable agronomic performance under Canadian conditions. Following this decision, canola RT73 was grown commercially in Canada on 50,000 acres in 1996 and 450,000 acres in 1997, with excellent agronomic performance comparable to other commercial varieties.

VI. ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION

A. The Herbicide Glyphosate

Glyphosate (N-phosphonomethyl-glycine) (CAS Registry #s 1071-83-6, 38641-94-0), is the active ingredient in the herbicide Roundup®. It is a non-selective, foliar-applied, broad-spectrum herbicide with no soil activity. Glyphosate is highly effective against the majority of annual and perennial grass and broad-leaf weeds. It is widely used because of its broad-spectrum, lack of carryover and favorable environmental and safety characteristics. The primary mode of action of the herbicide is competitive inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP synthase), an enzyme in the shikimate pathway of amino acid biosynthesis (Steinrücken and Amrhein, 1980). This aromatic amino acid pathway is not present in mammalian metabolic pathways (Cole, 1985). Glyphosate is rapidly bound to the soil, thus resisting leaching. It is readily degraded by soil microorganisms which decreases persistence. And it has relatively low toxicity to mammals, birds and fish (Malik, *et.al.*, 1989). There have been no reports of groundwater contamination problems with glyphosate (Goldburg *et al.* 1990). Glyphosate is classified by the EPA as Category E (evidence of non-carcinogenicity for humans) (57 FR 8739).

B. Current Uses of Herbicides in Canola

Weed control options in canola are very limited, compared to major row crops with greater acreage. The most commonly used herbicides in canola are Treflan, Poast and Assure II. Treflan is a preplant incorporated herbicide which provides control of many annual grass weeds. Poast and Assure II are postemergent herbicides which also control many annual grass weeds.

Many broadleaf and perennial weed species are not controlled by currently-available herbicides. Difficult weeds that infest canola fields are mustard species, field pennycress, wild buckwheat, Canada thistle and perennial sow thistle. As a result, mechanical cultivation or crop rotation is relied upon to provide control of a significant number of weeds in canola. All of these weeds can be controlled by Roundup.

C. Agronomic Practices with Roundup Ready Canola

Roundup Ready canola RT73 has been demonstrated to be substantially equivalent to the parent Westar. It has been and continues to be crossed into adapted varieties by traditional breeding methods. Roundup Ready canola RT73 varieties will be grown in the same geographic regions and with the same practices as current varieties.

In addition, Roundup Ready canola RT73 offers canola farmers several new options. Farmers will have the opportunity to manage their crop with a broad-spectrum herbicide that controls annual and perennial grass and broadleaf species. This will allow flexibility in timing of field operations and may allow planting of canola in fields that were previously considered to be too weedy. Control of broadleaf weeds in canola is critical to yield and oil quality, even when weeds are present at low levels in a field. Roundup provides improved control of a range of broadleaf weeds. Roundup Ready canola gives the farmer the option of direct-seeding or no-till with canola. This will have a positive effect on soil conservation and crop establishment. In conventional tillage systems, Roundup Ready canola offers the option of replacing a preplant incorporated herbicide with Roundup, thus reducing overall tillage. In some cases, planting can be done earlier because the grower does not have to wait for the first flush of weeds before working the fields. In areas with warmer summer temperatures, this early planting helps avoid high temperatures during flowering, which can reduce yields.

D. Glyphosate-Tolerant Weeds

Today there exist some 109 herbicide-tolerant weed biotypes, with over half of them resistant to the triazine family of herbicides (Holt and Le Baron, 1990; Le Baron, 1991; Shaner, 1995). Resistance to herbicides has usually developed because of the selection pressure exerted by the repeated use of herbicides with a single target site and a specific mode of action, long residual activity with the capacity to control weeds year-long, and frequent applications without rotation to other herbicides or cultural control practices. Using these criteria and based on current use data, glyphosate is considered to be a herbicide with a low risk for weed resistance (Benbrook, 1991). Nonetheless, it has been questioned whether the introduction of crops tolerant to a specific herbicide, such as glyphosate, may lead to the occurrence of weeds

resistant to that particular herbicide. This concern is based on the assumptions that the use of the herbicide will be increased significantly, and possibly that it will be used repeatedly in the same location. However, other increases in glyphosate use over previous years have been more significant than the projected increase associated with the introduction of Roundup Ready crops in the U.S. Although it cannot be stated that evolution of resistance to glyphosate will not occur, the development of weed resistance to glyphosate is considered unlikely because:

1. Weeds and crops are inherently not tolerant to glyphosate, and the long history of extensive use of glyphosate has not resulted in resistant weeds. Glyphosate has been used for over 20 years in various preplant, directed, spot and postharvest weed management systems with no verified cases of weed resistance (Holt *et al.*, 1993; Dyer, 1994). A preliminary report was recently presented that discussed annual ryegrass (*Lolium* sp.) seeds collected from a field that, upon germination, demonstrated a rate-related tolerance to glyphosate (Pratley *et al.* 1996). This observation merits further investigation. Insufficient data were reported to define the factors contributing to the observed phenomenon and Monsanto has entered into a collaborative research agreement with Charles Sturt University of Wagga Wagga in Australia to further investigate these results. Since the source of the 'sensitive' biotype used by Pratley (Pratley *et al.*, 1996) was from a different location than the 'resistant' biotype their genetic relatedness is unclear and additional research to address this question is being initiated.
2. Glyphosate has many unique properties, such as its mode of action (glyphosate is unrelated to triazines and has a differing mode of action from any other herbicide on the market today), chemical structure, limited metabolism in plants, lack of residual activity in the soil and its relatively quick break down by microorganisms in the soil (Malik *et al.*, 1989).
3. Selection for glyphosate resistance using whole plant and cell/tissue culture techniques, including mutagenesis, was largely unsuccessful, and unlikely to be duplicated under normal field conditions. Similarly, the complex genetic transformations required for the development of glyphosate tolerant crops (e.g. modified gene, unique promoters, transit peptide, *etc.*) would be unlikely to be duplicated in nature to yield glyphosate resistant weeds (Bradshaw *et al.*, 1997).

E. Weediness of Roundup Ready Canola

B. napus is not a weedy pest in North America, nor is it listed as a weed by the Weed Science Society of America (1989) or in *Weeds of the United States* (Lorenzi and Jeffrey, 1987). *B. napus* is the only naturalized *Brassica* that is not noted as a pestiferous weed by Rollins (1981). No *Brassica* is noxious

(Federal Noxious Weed Regulation, 7 CFR 360). *B. napus* is not listed as a serious, principal, or common weed in the U.S., Canada, Mexico, or any European or Asian country with a comparable latitude, *i.e.* between 25 and 49° north latitude (Holm *et al.*, 1991). Listed as a common weed only in Finland (60° north latitude) and Kenya (5° north to 5° south latitude), *B. napus* is not a serious or principal weed anywhere in the world (Holm *et al.*, 1991).

Numerous experiments have been conducted and observations made in the extensive field trials conducted worldwide to evaluate the weediness potential of canola RT73. The results of field observations have shown that canola RT73 has no increased potential of becoming a weed relative to unmodified *B. napus* (Appendix 7). Data for dormancy, germination, invasiveness, seed production, pod shattering, overwintering capacity, and adaptation to stress factors all demonstrate canola RT73 is equivalent to Westar, the nontransgenic control. In addition, observations have been recorded that note no agronomic differences in canola RT73 and Westar (Section VI.C.). A slight (approximately 1 day) delay in maturation has been reported for Roundup Ready canola RT73, which is within the variation expected as a selection from Westar. Since *B. napus* is not considered a weed and canola RT73 is substantially equivalent to the nontransgenic control variety, canola RT73 is therefore not expected to have a greater weediness potential.

Brassica napus seed can remain in the soil profile and produce volunteer plants in subsequent crop rotations. Mechanical cultivation can reduce the infestation of volunteer canola plants. In addition, numerous herbicide options are available for each of the major crops in a typical rotation with canola (See Table 9). The number of RT73 volunteers was no different than volunteers of the parental line Westar (Appendix 7), and these glyphosate-tolerant volunteers would be unchanged in their response to existing management practices (cultivation and/or herbicides).

Table 9. Herbicides registered for control of *Brassica* spp. in major crops (13 CPR Guide, 1997)

Soybeans	Classic, Basagran, Blazer, Broadstrike, Canopy, Sencor, Pursuit, Scepter
Wheat	2,4-D, Banvel, Ally, Amber, Buctril, Cyclone, Assert, Harmony Extra, Peak and Canvas.
Corn	Atrazine, 2,4-D, Banvel, Buctril, Broadstrike, Extrazine, Marksman, Permit
Cotton	Caparol, Harmony, Gramoxone

F. Vertical Transfer of New Genes

1. Summary of Literature on Outcrossing

The sexual compatibility of *Brassica napus* with other cultivated species and wild species has been extensively documented in the literature (see reviews by Scheffler and Dale, 1994; U.K. Department of the Environment, 1994) and in applications to market genetically modified canola (Plant Genetic Systems, 1994; Calgene, 1994).

The results of sexual compatibility studies have demonstrated that crosses between *B. napus* and other species occur with varying degrees of difficulty. It should be noted that there are reports of hybridization under open pollination conditions between fully fertile *B. napus* parents with only two species, *B. rapa* (syn. *B. campestris*) and *B. juncea* (Scheffler and Dale, 1994). When male-sterile *B. napus* parents are used, hybrid formation with *Hirschfeldia incana* (syn. *B. adpressa*) and *Raphanus raphanistrum* has been reported at low frequencies (Eber *et al.*, 1994). Under artificial conditions, including manual crosses and ovary culture techniques, additional interspecific hybrids have been produced, but have been shown to be low in fitness and often sterile. The interspecific crosses are more successful when *B. napus* is used as the female parent and when the species have at least one genome in common (Renard *et al.* 1993; OECD, 1997; Scheffler and Dale, 1994).

While crosses with wild species have been demonstrated, the probability of introgression of a gene into wild or weedy populations depends upon the ability of hybrids to survive and reproduce. Hybrids of *B. napus* and wild relatives are generally unfit and not expected to survive in wild populations. This conclusion is summarized in the review by Scheffler and Dale (1994),

"In general, the fertility of F1 hybrids was low, and male sterility was common. The fertility of hybrid plants was increased in some cases when the chromosome number was doubled. Increase in the chromosome number can occur spontaneously, either in the F1 hybrid or in later generations, but can lead to decreased as well as increased fertility. Progeny have been generated in some cases by using the F1 plant as the female parent, and backcrossing to one of its parental types. When one of the parents was diploid, progeny were often obtained only when *B. napus* was used as the female parent in the backcross. Because of this unilateral incompatibility, which has been reported for both the initial interspecific hybridization and subsequent generations, flow of genes is most likely to be from related species into *B. napus* and not the reverse. After several generations of backcrossing under controlled conditions, plants resembling the recurrent backcross parent were obtained from an F1 hybrid between *B. napus* and *B. rapa*. This indicates that, in theory, it might be possible to produce a hybrid plant capable of surviving (at least under agronomic cultural conditions). However, for a plant to reach this stage, the two parental species would have to flower at the same time and be

close enough for pollen transfer to occur. Seeds would have to be produced that are capable of germinating and producing a plant with sufficient fertility to produce progeny. If backcrossing was required to produce the progeny, then a suitable parent would have to be available, and flowering at the same time as the F1 plant. While the possibility can never be discounted, the evidence indicates that it would be a rare occurrence."

This conclusion on the likelihood of hybrid establishment would not be impacted by the presence of a transgene(s), such as those present in canola line RT73.

All of the *Brassica* species currently present in North America have been introduced. Those species that are weedy either escaped from cultivation or were introduced into fields as seed contaminants. The distribution within the United States and weed status of species which are sexually compatible with *B. napus* are described in Table 10. In addition, a survey of the distribution of *Brassica* species within the primary canola growing regions of the United States has been conducted (AgrEvo, 1997). The results of these surveys indicate that *B. napus* is grown in areas where sexually compatible *Brassica* weeds may be present. However, transfer of the glyphosate tolerance trait to sexually compatible plant species will only result in a selective advantage for these species if they are treated with glyphosate in managed or semi-managed environments. Since these weedy *Brassica* species can be effectively controlled by other means, such as by herbicides other than glyphosate and cultivation, the glyphosate tolerance trait, if transferred would offer no selective advantage to related species.

Table 10. Members of the Genus *Brassica* and Some Wild Relatives Found in the Continental United States.

Status as a weed in the U.S. is denoted by S (serious), P (principal), C (common), or X (present, weed status unknown) after Holm *et al.*, (1991). Species denoted by R are considered pestiferous weeds by Rollins (1981). Those denoted by LJ are considered weeds by Lorenzi and Jeffrey (1987). Those considered weeds by the Weed Science Society of America (1989) are indicated by WS. *B. oleracea* and *B. carinata* are not considered to be naturalized.

Species	Weed status	Common name of naturalized form	Distribution of naturalized form
<i>Brassica elongata</i>	R	none	roadside weed of eastern Nevada
<i>B. juncea</i>	X, R, WS	Chinese Mustard Indian Mustard	sparse, but widespread throughout temperate North America, occurs in cultivated and disturbed areas
<i>B. napus</i>	none	rape	sporadic in temperate North America, waste places
<i>B. nigra</i>	C, R, LJ, WS	black mustard	widespread in temperate North America, especially common in the Central Valley of California, sporadic in the more northerly areas of the continent
<i>B. rapa</i> syn. <i>campestris</i>	X, R, LJ, WS	field mustard bird's rape	common and widespread throughout temperate North America, occurs in cultivated and disturbed areas
<i>B. tournefortii</i>	R	wild turnip	roadsides and old fields of the Southwest
<i>Diplotaxis muralis</i>	X, R, WS	sand rocket	widely scattered, waste places, roadsides, abandoned land, heavily grazed grassland, beaches
<i>Hirschfeldia incana</i> syn. <i>B. adpressa</i>	X, R, WS	shortpod mustard Mediterranean mustard	roadsides, ditch banks and waste areas of California and Nevada
<i>Raphanus raphanistrum</i>	C, R, LJ, WS	wild radish, jointed charlock	widely distributed, especially in the eastern North America and in the Central Valley of California
<i>Sinapis alba</i> syn. <i>B. hirta</i>	X, R, WS	white mustard	widespread but sporadic in North America, abundant in some localities
<i>Sinapis arvensis</i> syn. <i>B. kaber</i>	P, R, LJ, WS	wild mustard charlock	abundant throughout the temperate agricultural areas of North America, especially in newly disturbed areas

2. Crossing with Other *Brassica napus* Varieties

Brassica napus is principally a self-pollinating crop which is also able to cross with other plants of the same species. Pollen movement is by means of wind and insects, mainly bees. Wind is not a particularly effective means of cross-pollination, as *B. napus* pollen is fairly heavy and sticky and cannot travel more than a few yards without insect pollinators (Downey and Röbbelen,

1989). Literature information concerning the frequency of intraspecific outcrossing (Becker *et al.*, 1992; Bing *et al.*, 1991; Downey *et al.*, 1991; Downey, 1992; Kapteijns, 1993; Chevre *et al.*, 1992; Kerlan *et al.*, 1992; Metz *et al.*, 1997) is variable in its conclusions, which reflects the fact that pollinator activity, planting density, genotype, weather and distance have an impact on outcrossing (Scheffler and Dale, 1994). Values have been reported as high as 30% (Rakow and Woods, 1987; Downey, 1992; Bing *et al.*, 1991).

Numerous pollen dispersal studies using *B. napus* have been conducted within the framework of the European Commission's Biotechnology Action Program (BAP)³, and BRIDGE⁴ program, and the United Kingdom's PROSAMO⁵ project. Experiments were small to medium scale, and employed a transgenic pollen source (marked with a marker gene) and measured the frequency of transfer of the marker gene to surrounding (non-transgenic) canola plants. Results from the PROSAMO project, employing a 9 meter diameter circle of transgenic rape in a one hectare field, demonstrated that the frequency of outcrossing decreased from 5% at zero distance down to 0.0003% at 47 meters distance (Scheffler *et al.*, 1993). Earlier studies, using a 3m diameter circle of transgenic canola within a 100m diameter circle of canola also demonstrated that outcrossing frequency decreased from 1.5% at 3 meters distance, to non-detectable at 24 and 48 meter distances (Scheffler *et al.*, 1993; Dale *et al.*, 1991; De Greef, 1991). The results from the BRIDGE project, using male sterile canola as the pollen trap, confirmed that outcrossing frequencies to other OSR plants is likely to be minimal.

It can be concluded that *B. napus* pollen dispersal is mainly short distance dispersal, although dispersal may occur over greater distances at a very low frequency. Perhaps most importantly, transgenic *B. napus* lines do not exhibit any greater rates of outcrossing than non-transgenic lines (Downey, 1992).

Canola quality *B. napus* can also cross with other types of cultivated *B. napus*, including rapeseed, fodder rape and rutabaga. Rapeseed can be further divided into industrial or high erucic acid rapeseed (HEAR) and canola, the principle difference being the levels of erucic fatty acid content. Both fodder rape and rutabaga are very minor crops in the United States and are harvested before seed is set. Therefore, the probability of introgression

³ Biotechnology Action Program (1985-1990) - supported by the Directorate for Science, Research and Development (DGXII) of the Commission of the European Communities.

⁴ Biotechnology Research for Innovation, Development and Growth in Europe (1990-1993) - supported by the Directorate for Science, Research and Development (DGXII) of the Commission of the European Communities.

⁵ Planned Release of Selected and Modified Organisms (1991-1993) - supported by a consortium of the U.K Dept. of Trade and Industry, the U.K Agriculture and Food Research Council, and industrial members.

from Roundup Ready canola RT73 into fodder rape or rutabaga is very low. Gene introgression from canola into high erucic acid rapeseed (HEAR) can be deterred using standard agronomic practices for production of that crop. For example, it has always been necessary to isolate *B. napus* canola fields from fields of high erucic acid rapeseed (HEAR) to prevent the canola from producing higher than acceptable levels of erucic acid. The precautions taken for this potential outcrossing should also be successful in preventing the bulk of gene transfer between the two crops.

3. Crossing with *Brassica rapa*

Brassica napus is an amphidiploid derived from hybridization between the diploid species *B. rapa* and *B. oleraceae*. The AA genome of *B. napus* was derived from *B. rapa*. *B. napus* and *B. rapa* are known to be sexually compatible under open pollination conditions (Bing *et al.*, 1991; Downey *et al.*, 1991).

While gene transfer from *B. napus* to *B. rapa* is known to occur under controlled conditions, the likelihood of natural introgression of genes from *B. napus* is lower. When *B. napus* is used as the male parent, many of the seeds produced are non-viable, and in the progeny, both pollen fertility and yields are significantly reduced (Bing *et al.*, 1991; Salam and Downey, 1978; Scheffler and Dale, 1994). The F1 hybrids have an intermediate chromosome number between *B. napus* and *B. rapa* and Salam and Downey (1978) reported that the interspecific progeny would quickly revert to *B. napus*, being the higher chromosome number parent, unless the hybrid is backcrossed to *B. rapa*. In addition, the progeny of the F1 plants backcrossed to *B. rapa* exhibited low fertility and high seedling mortality, making them less competitive than plants with genotypes closer to *B. napus*. Sheffler and Dale (1994) also reported that after several generations of backcrossing under controlled conditions, plants resembling the recurrent backcross parent were obtained from interspecific hybrids between *B. napus* and *B. rapa* (Nwantkiti, 1971; Shiga, 1970), but natural barriers were likely to make this a rare event under natural conditions. A recent study (Mikkelsen *et al.*, 1996) has reported the spontaneous development of fertile hybrids between herbicide-tolerant *B. napus* and *B. rapa* by interspecific backcrossing under field conditions. The field conditions used were not representative of normal agricultural practices, as the weedy *B. rapa* plant were sown at a high density which would not normally exist. Hybrids were then replanted into the same fields to facilitate the generation of stable, backcrossed hybrids. This work and others support the conclusion that hybrids between *B. rapa* and cultivated *B. napus* are possible under field conditions. Any such progeny are easily controlled with cultivation and the use of presently registered herbicides.

4. Crossing with *Brassica juncea*

Brassica juncea is a tetraploid containing genomes A and B. Successful hybridization has been reported under experimental field conditions using mixed stands of *B. napus* and *B. juncea* (Bing *et al.*, 1991). Using *B. napus* as the male parent, hybrid seeds were produced at a frequency of 4.7% of the seeds produced on the *B. juncea* plants. The F1 progeny produced low seed sets (from 0 to 25 seeds per plant) (Bing *et al.*, 1991). In further studies employing herbicide resistance markers in *B. napus*, hybrid seed production frequencies on *B. juncea* were reported as 0.3% and 0.1% in the two years of field trials, and fertility of the hybrids was low (Bing *et al.*, 1991).

There have been numerous reports of hybrids being produced by manual crosses, although seed production is very genotype-dependent (Heyn, 1977; Roy, 1980). Pollen viability of the F1 hybrid plants is generally low (less than 10% (Bing *et al.*, 1991), and fertility of the hybrids is usually less than 10% (Bing *et al.*, 1991; Heyn, 1977; Roy, 1980). In experiments where F1 plants were open pollinated or backcrossed to *B. napus* many of the progeny were found to be largely infertile, although some were highly fertile. F1 plants which were fertile were reported to preferentially revert to the *B. napus* form (Roy, 1980). The reciprocal cross to *B. juncea* was not performed (Roy, 1980).

Hybridization of *B. juncea* with *B. napus* is expected to be lower frequency than with *B. rapa*, since the latter is self-incompatible, whereas *B. juncea* self-pollinates around the time of flowering. The data for cross compatibility between *B. juncea* and *B. napus* also show that hybrids are obtained under controlled conditions to a lesser degree than the *B. rapa* x *B. napus* cross (Downey *et al.*, 1991; Kerlan *et al.*, 1992; Bing *et al.*, 1991).

5. Crossing with Other Brassica and Related Species

Brassica nigra

Although *B. napus* (AACC) and *B. nigra* (BB) have no genomes in common, hybridization is theoretically possible because of the homology between the B and C genomes (Mizushima, 1950). The production of hybrids under field conditions has been unsuccessful (Bing, 1991; Baranger *et al.*, 1992). With manual crosses interspecific hybrids were able to be produced, usually when *B. napus* was used as the female parent, but the hybrid seed and backcrossed progeny exhibited low fertility or sterility and reduced survival characteristics (Scheffler and Dale, 1994).

Other mechanisms involving bridging crosses through *B. rapa* and *B. juncea* were also considered as possible means of gene introgression from *B. napus* to *B. nigra* (Downey *et al.*, 1991; Downey, 1992). There are no reports of hybrid

seed production between *B. rapa* and the weedy species *B. nigra* when *B. rapa* is used as pollinator. The reverse cross under controlled conditions produced seed at very low frequency (1 per 2000 pollinations) and the F₁ plants were much easier to backcross to *B. rapa* (Bing, 1991). Therefore, gene flow is more likely from *B. nigra* to *B. rapa* than the reverse, which is considered unlikely.

Crosses between *B. juncea* and *B. nigra* have been successful at low frequency, particularly if *B. juncea* is used as the seed (female) parent (Bing, 1991). Open pollination of the F₁ progeny and backcrossing to *B. juncea* produced plants with low fertility. Backcrossing was easier with *B. juncea* than with *B. nigra*, and the high chromosome numbers of the hybrid progeny suggest that the offspring of this interspecific cross are likely to revert to the cultivated amphidiploid species (Bing, 1991).

Brassica oleracea

B. oleracea is the other progenitor of *B. napus* and has the C genome in common with *B. napus*. Natural hybridization with *B. oleracea*, however, has not been reported (Scheffler and Dale, 1994). Even with artificial techniques, hybridizations are very difficult to achieve (Ayotte *et al.*, 1987; Chiang *et al.*, 1977; Honma and Summers, 1976; U, 1935), and have been more successful when *B. napus* was used as the seed (female) parent (Kerlan *et al.*, 1992). When *B. napus* was used as the male parent hybrid progeny have been able to be produced but pollen fertility was reduced, and F₂ seeds exhibited reduced survival characteristics (Robbelen, 1966). It is unlikely, therefore, that interspecific hybrids with *B. oleracea* will occur under open pollination conditions.

Brassica carinata

B. carinata is an amphidiploid containing genomes B and C, presumably resulting from a cross between *B. nigra* and *B. oleracea*. Hybrid seeds have been produced by manual crosses with *B. napus*, more successfully when *B. napus* was used as the female parent. Fertility and seed production were generally low (Scheffler and Dale, 1994; Roy, 1980; Nishiyama *et al.*, 1991).

Hirschfeldia incana

Hybrids between *B. napus* and *Hirschfeldia incana* have previously been unsuccessful; however, Chevre *et al.* (1992) and Lefol (1993) reported obtaining hybrids by planting male-sterile *B. napus* and fully fertile *H. incana* in adjacent rows in a field trial and allowing open pollination. A small number of hybrid seeds were produced, and the F₁ plants from these exhibited varying degrees of infertility, but some seeds were produced when the hybrids were backcrossed to *H. incana*.

Raphanus raphanistrum

Manual hybridizations with *Raphanus* species have generally been unsuccessful (Scheffler and Dale, 1994). Kerlan *et al.* (1992) attempted reciprocal manual pollinations between *Raphanus raphanistrum* and *B. napus*, but failed to obtain any viable F1 hybrids after making 200 manual pollinations. Cytological examination of pollen tube growth showed that *R. raphanistrum* pollen tubes did not penetrate the *B. napus* styles. Chevre *et al.* (1992) and Lefol (1993) reported obtaining hybrid seed in a field trial similar to that described for *H. incana*, with adjacent rows of *R. raphanistrum* and male-sterile *B. napus*. Pollen fertility of the F1 hybrids ranged from 0% to 30%. Backcross progeny were produced by open pollination when the F1 was used as the female parent (Eber *et al.* 1994).

More recently, Chèvre *et al.* (1997) reported that introgression was possible between male sterile herbicide-tolerant *B. napus* and *R. raphanistrum* under field conditions. The conditions used were not typical for agricultural settings as crossing was facilitated by planting and maintaining high densities of the weed species *R. raphanistrum*. Hence pollen load was high and crossing was forced by use of male sterile material. The authors concluded that successful hybridization and gene introgression would be rare under normal agricultural conditions. (Chèvre *et al.*, 1997).

Sinapis species

No hybrids between *B. napus* and *Sinapis* species have been produced under field conditions, and interspecific hybrid production under artificial conditions has also proven to be extremely difficult. Heyn (1977) reported hybridization with *S. alba*, but it was not demonstrated that the F₂ or backcrossed progeny were fertile. Attempts to produce hybrids with *S. arvensis* have been unsuccessful under field conditions (Bing, 1991; Lefol 1993; Lefol *et al.*, 1991). Successful crosses have only been reported using artificial techniques and employing *B. napus* as the female parent. The hybrid progeny are always sterile or produce very low seed sets (Bing, 1991; Mizushima, 1950; Inomata, 1988).

Bridging crosses through *B. rapa* and *B. juncea* to *S. arvensis* have also been considered. Gene flow from *B. rapa* to *S. arvensis* has been shown to be very unlikely, since reciprocal crosses under controlled conditions failed to produce any seed (Bing, 1991; Hinata *et al.*, 1974). Hybrids between *B. juncea* and *S. arvensis* were only successful when *B. juncea* was used as the seed parent and the progeny were poorly fertile. Gene flow from *B. juncea* to *S. arvensis* is considered to be highly unlikely.

6. Summary on Outcrossing

Hybridization with *B. napus* under natural conditions has been demonstrated with the closely related species *B. rapa* (syn. *campestris*) and *B. juncea*. Hybridization has also been demonstrated with other related species using artificial conditions, but it is considered unlikely that natural hybridization with these species can occur. In the event that hybrids between RT73 canola and other Brassica species did occur, current management practices would be effective in controlling these plants.

G. Horizontal Transfer of New Genes

As stated in the USDA's Interpretive Ruling on Calgene, Inc., Petition for Determination of Regulatory Status (FR 57, No.202, pp. 47608-47616, October 19, 1992), "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. Evidence presented in the Calgene petition and supplementary information and summarized in the FR Notice suggests that, based on limited DNA homologies, transfer from plant to microorganisms may have occurred in evolutionary time over many millennia. Even if such transfer were to take place, transfer of an EPSPS gene to a microbe would not pose any plant pest risk. Based on these considerations, transfer to microbes or other living species in nature is extremely unlikely and of no significant consequence from a plant pest point of view.

VII. ADVERSE CONSEQUENCES OF INTRODUCTION

Monsanto Company knows of no unfavorable results or observations associated with Roundup Ready canola RT73 that would result in adverse consequences of introduction. Therefore, on the basis of the substantial potential benefits to the grower, the environment and the consumer, Monsanto requests that Roundup Ready canola RT73 and progeny derived from traditional breeding no longer be regulated under 7 CFR part 340.6.

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_____ and _____ 1994. Isolation and Characterization of a Variant of the Enzyme Glyphosate Oxidoreductase with Improved Kinetic Properties. Monsanto Technical Report MSL-13246, St. Louis. (see Appendix 3)

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**Appendix 1. LIST AND STATUS OF USDA NOTIFICATIONS FOR
ROUNDUP READY CANOLA RT73**

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FIELD TRIAL LOCATIONS FOR GT CANOLA LINE #RT73

USDA #	MONSANTO #	LOCATION OF TRIAL	COUNTY	STATUS
95-279-01R	95-226PR	K-F Seeds, 4307 Fifield Road, Brawley, CA	Imperial	Complete*
96-045-01R	96-018PR	Diamond Valley Road, Dutton, MT	Teton	Complete*
		Route #1, Box 148, Ritzville, WA	Adams	Complete*
		475 Road 1 SW, Waterville, WA	Douglas	Complete*
		HC 2, Box 84, Plaza, ND	Mountrail	Complete*
96-061-02R	96-034PR	3 miles south of Minot, ND on U.S. Hwy 83 on the west side	Ward	Complete*
		3 miles east of Langdon, MD and 1/2 mile south on Hwy 5	Cavalier	Complete*
96-211-01R	96-135PR	K-F Seeds, 4307 Fifield Road, Brawley, CA	Imperial	Complete*
96-274-01R	96-170PR	H&H Seed Company, P. O. Box 1688, Yuma, AZ	Yuma	Complete*
97-022-01R	97-035PR	Qualls Agricultural Lab, Ephrata, WA	Grant	Complete*
		AgroTech Incorporated, Velva, ND	McHenry	Complete*
		Miller Research, Inc., Rupert, ID	Blaine	Complete*
97-024-01R	97-038PR	Hallock Township	Kittson	Trial cancelled
		Red Lake Falls, MN	Red Lake	Trial cancelled
		K. B. Farming, Conrad, MT	Pondera	Complete*
		Western Triangle Agricultural Research Center, Conrad, MT	Pondera	Complete*
		Cut Bank, MT	Glacier	Trial cancelled
		Northern Agricultural Research Center, Harvre, MT	Hill	Complete*
		Kay-Jay Agricultural Services, Fargo, ND	Barnes	Complete*
		Raymond Township	Cass	Complete*
		Farmers Union Oil Company, Cando, ND	Towner	Complete*
		NW 1/4, Sect 29, Township 140 N, Range 105	Golden Valley	Complete*
		Hazelton, ND	Edmonds	Complete*
		North Central Research Experiment Center, Minot, ND	Ward	Complete*
		Williston Research Experiment Station, Williston, ND	Williams	Complete*
		Garfield, WA	Garfield	Trial cancelled
		Colfax, WA	Whitman	Destroyed June '97
		Ricks College, Rexburg, ID	Madison	Complete*
		*Final Report Submitted		

FIELD TRIAL LOCATIONS . JR GT CANOLA LINE #RT73

USDA #	MONSANTO #	LOCATION OF TRIAL	COUNTY	STATUS
97-254-02N	97-259XR	H&H Seed, Yuma, AZ	Yuma	Trial in process
		K-F Seed, Brawley, CA	Imperial	Trial in process
97-254-04N	97-260XR	K-F Seed, Brawley, CA	Imperial	Trial in process
97-324-06N	97-323XR	K-F Seeds, Brawley, CA	Imperial	Trial in process
97-309-03N	97-332XR	University of Georgia, Griffin, GA	Spalding	Plant in Spring '98

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Monsanto

Monsanto Company
700 Chesterfield Parkway North
St. Louis, Missouri 63198
Phone: [REDACTED]

February 16, 1998

[REDACTED]
Animal and Plant Health Inspection Service
Biotechnology and Scientific Services
4700 River Road, Unit 147
Riverdale, Maryland 20737-1237

Subject: Submission of Final Reports

Dear [REDACTED]

Enclosed are the final reports on the following field tests of Roundup Ready™ Canola.

USDA Permit #

Monsanto #

95-279-01R

95-226PR

96-045-01R

96-018PR

96-061-02R

96-034PR

96-211-01R

96-135PR

96-274-01R

96-170PR

97-022-01R

97-035PR

97-024-01R

97-038PR

These experiments are completed and we are submitting the final reports required by regulation.

Should you have any questions concerning these reports, please feel free to contact me at

Sincerely,

[REDACTED]

Regulatory Compliance Coordinator

**1995 CANOLA FIELD RELEASE
USDA # 95-279-01R/MONS # 95-226PR
FINAL REPORT**


Monsanto Company

The purpose of this trial was seed propagation.

Imperial County, CA

Field Monitoring for Disease Susceptibility: Common diseases to canola are alternaria, blackleg and sclerotinia and none of these diseases were detected in the crop.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: The yields of regular open pollinated varieties averaged close to 40 bushels per acre. There was average standability and the variety was easy to swath and harvest. The variety was anchored well in the swath so plants were unable to blow and get into irrigation canals. There was even germination and no stress to the plants during growing season.

Field Monitoring for Weediness Characteristics: Roundup® gave extremely good control of the weeds in the crop.

**1996 CANOLA FIELD RELEASE
USDA # 96-045-01R/MONS # 96-018PR
FINAL REPORT**

[REDACTED]
Monsanto Company

The purpose of the trial was to demonstrate field tolerance of selected glyphosate tolerant lines with various rates of glyphosate.

<u>State</u>	<u>County</u>
MT	Teton
WA	Adam
WA	Douglas
ND	Mountrail

Individual Site Information

Teton County, MT

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. The trial was monitored May 3, June 18, and July 15, 1996.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. The trial was monitored May 3, June 18, and July 15, 1996.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of the transgenic and non-transgenic plants. The trial was monitored May 3, June 18, and July 15, 1996.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from the non-transgenic plants. The trial was monitored May 3, June 18, and July 15, 1996.

Mountrail County, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. The trial was monitored June 12, July 3, July 31, August 28, September 25, October 23 and November 20, 1996.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. The trial was monitored June 12, July 3, July 31, August 28, September 25, October 23 and November 20, 1996.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of the transgenic and non-transgenic plants. The trial was monitored June 12, July 3, July 31, August 28, September 25, October 23 and November 20, 1996.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from the non-transgenic plants. The trial was monitored June 12, July 3, July 31, August 28, September 25, October 23 and November 20, 1996.

Adams County, WA

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of the transgenic and non-transgenic plants.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from the non-transgenic plants.

Douglas County, WA

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of the transgenic and non-transgenic plants.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from the non-transgenic plants.

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**1996 CANOLA FIELD RELEASE
USDA # 96-061-02R/MONS # 96-034PR
FINAL REPORT**


Monsanto Company

The purpose of this trial was to confirm in-house research data by increasing seed volume to evaluate polymer characteristics and composition.

<u>State</u>	<u>County</u>
ND	Ward
ND	Cavalier

Individual Site Information

Ward County, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of the transgenic and non-transgenic plants.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from the non-transgenic plants.

Cavalier County, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of the transgenic and non-transgenic plants.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from the non-transgenic plants.

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**1996 CANOLA FIELD RELEASE
USDA # 96-211-01R/MON # 96-135PR
FINAL REPORT**


Monsanto Company

The purpose of this trial was seed propagation.

<u>State</u>	<u>County</u>	<u>Planting Date</u>	<u>Harvest Date</u>
CA	Imperial	October 18, 1996	April 17, 1997

Imperial County, CA

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. The trial was monitored November 20, 1996, January 5, February 15, and March 10, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. The trial was monitored November 20, 1996, January 5, February 15, and March 10, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of the transgenic and non-transgenic plants. The trial was monitored November 20, 1996, January 5, February 15, and March 10, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was in no way different from non-transgenic plants. The trial was sprayed with Roundup® in November killing less than four percent of the plants. The trial was monitored November 20, 1996, January 5, February 15, and March 10, 1997.

**1996 CANOLA FIELD RELEASE
USDA 96-274-01R/MONS # 96-170PR
FINAL REPORT**


Monsanto Company

The purpose of this trial was seed propagation.

<u>State</u>	<u>County</u>
AZ	Yuma

Yuma County, MT

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of the transgenic and non-transgenic plants.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from the non-transgenic plants.

**1997 CANOLA FIELD RELEASE
USDA # 97-022-01R/MONS # 97-035PR
FINAL REPORT**

Monsanto Company

The purpose of this trial was to supply data on the glyphosate residue levels that will likely result in or on canola raw agricultural commodities as a result of the application of Roundup Ultra herbicide according to label directions for current use plus the topical applications afforded by and use of Roundup Ready™ canola plants.

<u>State</u>	<u>County</u>	<u>Planting Date</u>	<u>Harvest Date</u>
WA	Grant	May 13, 1997	August 25, 1997
ND	McHenry	May 14, 1997	August 21, 1997
ID	Blaine	May 13, 1997	August 16, 1997

Individual Site Information

Grant County, WA

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. The trial was monitored June 9, July 2 and August 22, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. The trial was monitored June 9, July 2 and August 22, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of the transgenic and non-transgenic plants. All plants were normal and vigorous. The trial was monitored June 9, July 2 and August 22, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from the non-transgenic plants. Germination was normal. The trial was monitored June 9, July 2 and August 22, 1997.

McHenry County, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. The trial was monitored May 26, June 19, July 15, and August 21, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. The trial was monitored May 26, June 19, July 15, and August 21, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of the transgenic and non-transgenic plants. The trial was monitored May 26, June 19, July 15, and August 21, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from the non-transgenic plants. The trial was monitored May 26, June 19, July 15, and August 21, 1997.

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Blaine, ID

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. The trial was monitored June 9, July 11, August 9, and August 19, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. The trial was monitored June 9, July 11, August 9, and August 19, 1997.

Field Monitoring for Plant Growth Characteristics: There were differences in the general appearance and growth of the transgenic and non-transgenic plants. Monitoring June 9, 1997 revealed 35% speckling of leaves in the transgenic lines. Monitoring August 19, 1997 revealed that 100% of the transgenic plants were shorter. Seed yield was good. The trial was monitored June 9, July 11, August 9, and August 19, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was slightly different from the non-transgenic plants. Monitoring August 19, 1997 revealed more weeds in the non-transgenic plants. The trial was monitored June 9, July 11, August 9, and August 19, 1997.

**1997 CANOLA FIELD RELEASE
USDA # 97-024-01R/MONS # 97-038PR
FINAL REPORT**



Monsanto Company

These were efficacy studies trials and demo trials.

<u>State</u>	<u>County</u>	<u>Planting Date</u>	<u>Harvest Date</u>
ID	Rexburg	5/15/97	8/25/97
MN	Kittson	Cancelled	
MN	Red Lake	Cancelled	
MT	Pondera	5/7/97	8/27/97
MT	Pondera	5/9/97	9/8/97
MT	Glacier	Cancelled	
MT	Hill	5/6/97	7/10/97
ND	Barnes	5/17/97	8/18/97
ND	Cass	5/29/97	9/23/97
ND	Towner	5/24/97	7/31/97
ND	Golden Valley	5/13/97	8/22/97
ND	Edmonds	5/9/97	8/22/97
ND	Ward	5/9/97	8/19/97
ND	Williams	5/12/97	8/22/97
WA	Garfield	Cancelled	
WA	Whitman	4/16/97	6/19/97

Individual Site Information

Pondera County, MT

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. There were no diseases during this trial. Plants looked normal and healthy. Trials were monitored June 9, July 2, August 12, and September 8, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Insects were not a problem. Trials were monitored June 9, July 2, August 12, and September 8, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Plants grew and looked normal. Trials were monitored June 9, July 2, August 12, and September 8, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Plants germinated and grew normally. Trials were monitored June 9, July 2, August 12, and September 8, 1997.

Pondera County, MT

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. 0-5% of the transgenic plants experienced Black leg while 5-100% of the non-transgenic plants experienced Black leg. Trials were monitored May 15, June 15, July 7, August 12, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored May 15, June 15, July 7, August 12, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored May 15, June 15, July 7, August 12, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored May 15, June 15, July 7, August 12, 1997.

Hill County, MT

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored June 6 and July 7, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored June 6 and July 7, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored June 6 and July 7, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored June 6 and July 7, 1997.

Barnes County, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored June 7, June 14, June 20, July 1, July 10, and August 5, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored June 7, June 14, June 20, July 1, July 10, and August 5, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Monitoring July 1, 1997 revealed that Blister Beetles were in 10% of the transgenic plants while 0% were in the non-transgenic plants. Trials were monitored June 7, June 14, June 20, July 1, July 10, and August 5, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored June 7, June 14, June 20, July 1, July 10, and August 5, 1997.

Cass County, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Monitoring occurred June 18, July 11, August 12 and September 4, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Monitoring occurred June 18, July 11, August 12 and September 4, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Monitoring occurred June 18, July 11, August 12 and September 4, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Monitoring occurred June 18, July 11, August 12 and September 4, 1997.

Towner County, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Trials were monitored June 17, July 15 and July 23, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored June 17, July 15 and July 23, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored June 17, July 15 and July 23, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored June 17, July 15 and July 23, 1997.

Golden Valley, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. No diseases were present in either crop. Trials were monitored June 17, June 27, July 23, and August 22, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Trials were monitored June 17, June 27, July 23, and August 22, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Trials were monitored June 17, June 27, July 23, and August 22, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Trials were monitored June 17, June 27, July 23, and August 22, 1997.

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Edmonds County, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants.

Monitoring occurred June 14, June 23, July 23, and August 22, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Monitoring occurred June 14, June 23, July 23, and August 22, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Monitoring occurred June 14, June 23, July 23, and August 22, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Monitoring occurred June 14, June 23, July 23, and August 22, 1997.

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Ward County, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Disease was not present on either plants. Monitoring occurred June 16, July 14, and August 19, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. One hundred percent of both plants experienced flea beetle pressure. Monitoring occurred June 16, July 14, and August 19, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Monitoring occurred June 16, July 14, and August 19, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Monitoring occurred June 16, July 14, and August 19, 1997.

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Williams County, ND

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Disease was not present in either plants. Monitoring occurred June 3, June 27, July 28, and August 22, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. One hundred percent of both groups experienced moderate flea beetle pressure. Monitoring occurred June 3, June 27, July 28, and August 22, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Monitoring occurred June 3, June 27, July 28, and August 22, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Monitoring occurred June 3, June 27, July 28, and August 22, 1997.

Whitman County, WA

Field Monitoring for Disease Susceptibility: The transgenic plants did not have a higher incidence of disease than the non-transgenic plants. Monitoring occurred June 18, 1997.

Field Monitoring for Insect Susceptibility: The transgenic plants did not have a higher incidence of non-target insect species than the non-transgenic plants. Monitoring occurred June 18, 1997.

Field Monitoring for Plant Growth Characteristics: There were no differences in the general appearance and growth of transgenic and non-transgenic plants. Monitoring occurred June 18, 1997.

Field Monitoring for Weediness Characteristics: Germination of transgenic plants was not different from non-transgenic plants. Monitoring occurred June 18, 1997.

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Appendix 2. Study Summary: Cloning and expression in *Escherichia coli* of the glyphosate-to-aminomethylphosphonic acid degrading activity from *Achromobacter* sp. strain LBAA.

MSL-13245 Abstract

Date: May 9, 1994

Title: Cloning and expression in *Escherichia coli* of the glyphosate-to-aminomethylphosphonic acid degrading activity from *Achromobacter* sp. strain LBAA.

Authors: [REDACTED]

Abstract: The conversion of glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate is the primary route for the degradation of glyphosate, the active ingredient of the herbicide Roundup®, in soils and other environments. Little is known about the protein(s) involved in this conversion and there have been no reports of cell-free conversion of glyphosate-to-AMPA. Using a genetic approach, in which recombinant clones were identified by the ability of AMPA-degrading *E. coli* to use glyphosate as a P source, we have cloned the coding region responsible for this activity. The activity is encoded by a soluble 45 kD protein.

Appendix 3. Study Summary: Isolation and characterization of a variant of the enzyme glyphosate oxidoreductase with improved kinetic properties.

MSL-13246 Abstract

Date: May 9, 1994

Title: Isolation and characterization of a variant of the enzyme glyphosate oxidoreductase with improved kinetic properties.

Authors: [REDACTED]

Abstract: The enzyme glyphosate oxidoreductase (GOX) carries out the conversion of the herbicide glyphosate to aminomethyl phosphonate and glyoxylate. This step is the primary route for the degradation of glyphosate in the soil and other environments. The gene for GOX has been cloned from *Achromobacter* sp. strain LBAA and expressed in *E. coli*. The cloned enzyme shows activity on only a very few substrates, primarily glyphosate and iminodiacetic acid. To increase our understanding of the enzyme and its activity, the *gox* gene was mutagenized and screened for the production of variants with improved enzyme activity. One variant, containing five nucleotide differences that result in three amino changes, was characterized in detail. The $appK_m$ for both substrates has been reduced approximately 10-fold. The improvements in enzyme activity are all attributable to one of the changed amino acids and the effect of different substitutions at this position on the enzyme activity and kinetics has been investigated.

Appendix 4. Study Summary: Purification, Cloning and Characterization of a Highly Glyphosate-tolerant 5-Enolpyruvylshikimate-3-phosphate Synthase from *Agrobacterium* sp. strain CP4

MSL-12738 Abstract

Date: 10/9/93

TITLE

Purification, Cloning and Characterization of a Highly Glyphosate-tolerant 5-Enolpyruvylshikimate-3-phosphate Synthase from *Agrobacterium* sp. strain CP4

AUTHORS

[REDACTED]

ABSTRACT

5-Enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants and microorganisms, is the biological target enzyme of glyphosate, the active ingredient of Roundup® herbicide. Expression *in planta* of glyphosate-tolerant EPSPSs has proven to be an effective mechanism for conferring glyphosate-tolerance to crop plants. We now wish to report the purification, cloning and expression in *E. coli* of EPSPS from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). Based on steady-state kinetic analysis, CP4 EPSPS exhibits very high-level glyphosate tolerance ($\text{app}K_i[\text{glyphosate}]=2.7\text{mM}$), while retaining a very low $\text{app}K_m(\text{PEP})$ ($12\text{ }\mu\text{M}$), comparable to that of wild-type plant EPSPSs. CP4 EPSPS has the highest $\text{app}K_i(\text{glyphosate})/\text{app}K_m(\text{PEP})$ ratio, 227, of any EPSPS described to date, while the $\text{app}K_m(\text{S3P})$ is approximately $1\text{ }\mu\text{M}$. CP4 EPSPS has approximately 50-60% similarity to previously described EPSPSs, and numerous active site residues are conserved relative to other EPSPSs. The kinetic data collected supports the use of the CP4 EPSPS gene obtained herein for the development of glyphosate-tolerant crops.

Appendix 5. GLUCOSINOLATE ANALYSIS METHODOLOGY
ISO Determination of glucosinolates

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**Rapeseed — Determination of glucosinolates
content —**

**Part 1:
Method using high-performance liquid
chromatography**

Graines de colza — Dosage des glucosinolates —

Partie 1: Méthode par chromatographie liquide à haute performance



Reference number
ISO 9167-1:1992(E)

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 9167-1 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 2, *Oleaginous seeds and fruits*.

ISO 9167 consists of the following parts, under the general title *Rapeseed — Determination of glucosinolates content*:

- *Part 1: Method using high-performance liquid chromatography*
- *Part 2: Method using X-ray fluorescence spectrometry*

Annex A of this part of ISO 9167 is for information only.

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Rapeseed — Determination of glucosinolates content —

Part 1:

Method using high-performance liquid chromatography

1 Scope

This part of ISO 9167 specifies a method for the determination of the content of the different glucosinolates in rapeseeds (colza) using high-performance liquid chromatography.

NOTES

1 This method does not determine glucosinolates which are substituted on the glucose molecule, but these compounds are of little importance in commercial rapeseed.

2 A rapid method for the determination of glucosinolates content using X-ray fluorescence spectrometry is the subject of ISO 9167-2.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 9167. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 9167 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 664:1990, *Oilseeds — Reduction of laboratory sample to test sample.*

ISO 665:1977, *Oilseeds — Determination of moisture and volatile matter content.*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods.*

3 Principle

Extraction of glucosinolates by methanol, then purification and enzymatic desulfatation on ion-exchange resins. Determination using reversed-phase high-performance liquid chromatography (HPLC) with elution gradient and ultraviolet detection.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and water complying with grade 2 of ISO 3696.

4.1 Methanol, HPLC grade, 70 % (V/V) solution.

4.2 Sodium acetate, 0,02 mol/l at pH 4,0.

4.3 Sodium acetate, 0,2 mol/l solution.

4.4 Imidazole formate, 6 mol/l solution.

Dissolve 204 g of imidazole in 113 ml of formic acid in a 500 ml one-mark volumetric flask. Make up to the mark with water.

4.5 Internal standard, use either sinigrin monohydrate (potassium allylglucosinolate monohydrate, $M_r = 415,49$) (see 4.5.1) or, for rapeseed (cultivated or self-propagated) in which sinigrin is present naturally, glucotropaeolin (benzylglucosinolate, potassium salt, $M_r = 447,52$) (see 4.5.2).

For rapeseed with a low glucosinolate content ($< 20 \mu\text{g/g}$), reduce the internal standard concentration (1 mmol/l to 3 mmol/l) in 4.5.1 and 4.5.2.1.

Sinigrin monohydrate

4.5.1.1 Sinigrin monohydrate, 5 mol/l solution.

Dissolve 207,7 mg of potassium allylglucosinolate monohydrate in water in a 100 ml one-mark volumetric flask. Make up to the mark with water.

The solution thus prepared may be stored in a refrigerator at approximately 4 °C for up to a week or in a freezer at -18 °C for a longer period.

4.5.1.2 Sinigrin monohydrate, 20 mmol/l solution.

Dissolve 831,0 mg of potassium allylglucosinolate monohydrate in water in a 100 ml one-mark volumetric flask. Make up to the mark with water.

The solution thus prepared may be stored in a refrigerator at approximately 4 °C for up to a week or in a freezer at -18 °C for a longer period.

4.5.1.3 Purity check

Use one or more of the following three tests:

- C analysis using the method specified in this of ISO 9167;
- analysis of the intact sinigrin by HPLC (ion-pair technique);
- analysis of the desulfated and silylated sinigrin by gas chromatography.

For each test, the chromatogram shall show only one major peak representing at least 98 % of the total peak area.

Confirm the purity by determining the quantity of glucose released after hydrolysis with myrosinase (thioglucoside glucosylhydrolase, EC 3.2.3.1). Measure the glucose by enzymatic means. The use of a commercially available test kit facilitates the determination. Take into account any free glucose present; this is determined in the same way but without addition of myrosinase. The molar concentration of glucose measured should be at least 98 % of the molar concentration of the sinigrin solution tested.

4.5.2 Glucotropaeolin

NOTE 3 Glucotropaeolin is sometimes difficult to separate from other natural minor glucosinolates.

4.5.2.1 Glucotropaeolin, 5 mmol/l solution.

Dissolve 233,8 mg of glucotropaeolin in water in a 100 ml volumetric flask. Make up to the mark with water.

4.5.2.2 Glucotropaeolin, 20 mmol/l solution.

Dissolve 895,0 mg of glucotropaeolin in water in a 100 ml volumetric flask. Make up to the mark with water.

4.5.2.3 Purity check

Check the purity in accordance with the procedure described in 4.5.1.3.

4.5.2.4 Response factor

Verify that the response factors of glucotropaeolin, in comparison with sinigrin, correspond to those indicated in 9.2.

4.6 Mobile phases

4.6.1 Eluant A: water, purified by passing it through an activated charcoal cartridge (e.g. Norgenic Millipore[®] system) or water of equivalent purity.

4.6.2 Eluant B: acetonitrile, HPLC grade, 20 % (V/V) solution in purified water. The concentration may be modified in relation to the column used.

4.7 Ion-exchange resin, use either 4.7.1 or 4.7.2.

4.7.1 DEAE Sepharose CL-6B[®] suspension, available commercially ready for use, or an equivalent product.

4.7.2 DEAE Sephadex A25[®] suspension, prepared as follows.

Mix 10 g of DEAE Sephadex A25 resin (or an equivalent resin) in excess 2 mol/l acetic acid solution. Leave to settle. Add 2 mol/l acetic acid until the volume of the liquid is equal to twice the volume of the sediment.

Norgenic Millipore system is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 9167 and does not constitute an endorsement by ISO of this product.

Sepharose and Sephadex are examples of suitable products available commercially. This information is given for the convenience of users of this part of ISO 9167 and does not constitute an endorsement by ISO of these products.

4.8 Sulfatase, *Helix pomatia* type H1 (EC 3.1.8.1), having an activity of greater than 0,5 units of activity per millilitre of purified sulfatase solution.

Purify, test and dilute the sulfatase in accordance with the method described in 4.8.1 to 4.8.4.

4.8.1 Preparation of ion-exchange columns

Cut five Pasteur pipettes (5.8) 7 cm above the neck and place a glass wool plug (5.8) in the neck. Place the pipettes vertically on a stand and add to each a sufficient quantity of ion-exchange resin (4.7) such that, once the water has drained off, a volume of 500 µl of resin is obtained.

Pour 1 ml of the imidazole formate solution (4.4) into each pipette and rinse twice with 1 ml portions of water.

4.8.2 Purification

Weigh, to the nearest 0,1 mg, 25 mg of *Helix pomatia* type H1 (4.8), dissolve it in 2,5 ml of water and transfer 500 µl of this solution to each of the columns prepared in 4.8.1. Wash each column with 1,5 ml of water and discard the effluent. Then add 1,5 ml of the sodium acetate solution (4.3) and collect the eluates from the five columns in a test tube.

Concentrate the eluates by filtration using a Millipore PTGC 11K25³⁾ immersion filter until approximately 100 µl of liquid remains (sulfatase with a molar mass in excess of 5 000 is not removed). Add 2,5 ml of water and concentrate once more by filtration until approximately 100 µl of liquid remains. Dilute to 2,5 ml with water and store the purified sulfatase in a freezer at -18 °C in small amounts in order to allow defrosting of the amount necessary for immediate use.

4.8.3 Test of the sulfatase activity

4.8.3.1 Preparation of a 0,15 mmol/l sinigrin solution, buffered to pH 5,8.

Prepare three solutions in succession as follows:

- transfer 1 ml of acetic acid to a 500 ml one-mark volumetric flask and make up to the mark with water;
- transfer 1 ml of ethylene diamine to a 500 ml one-mark volumetric flask and make up to the mark with water;
- mix 73 ml of solution a) with 40 ml of solution b) and adjust the mixture to pH 5,8 using solution a) or solution b) as appropriate.

Pour 3 ml of the 5 mmol/l sinigrin solution (4.5.1.1) into a 100 ml one-mark volumetric flask and make up to the mark with solution c).

4.8.3.2 Test of activity

Using a pipette, transfer 2 ml of the buffered sinigrin solution (4.8.3.1) into the reference and measuring cells of the spectrometer (5.3) adjusted to a wavelength of 229 nm with a cell temperature of 30 °C. At time $t = 0$, add 50 µl of purified sulfatase (4.8.2) to the measuring cell and immediately switch on the recorder. Stop the recorder when the absorbance no longer varies (A_0), plot the tangent to the point $t = 0$ and measure its gradient $\Delta A/\Delta t$.

The activity of the sulfatase (i.e. the production of 1 micromole of desulfated sinigrin per minute at 30 °C and pH 5,8), expressed in units of activity per millilitre of sulfatase solution, is equal to

$$\frac{\Delta A}{\Delta t} \times \frac{V}{\Delta \epsilon} \times \frac{1000}{50} \times 10^3$$

where

$\Delta A/\Delta t$ is the gradient of the tangent to the point $t = 0$, in absorbance units per minute;

V is the volume, in litres, of the reacting medium (i.e. $2,05 \times 10^{-3}$ l);

$\Delta \epsilon$ (approximately $1\,500\text{ l}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$) is the difference between the molar extinction coefficients of sinigrin and of desulfosinigrin at 228 nm, i.e.

$$\Delta \epsilon = \frac{A_0}{l \cdot c}$$

where

A_0 is the difference between the absorbance at equilibrium of the desulfated sinigrin and the absorbance at time $t = 0$;

l is the path length of the cell, in centimetres (i.e. 1 cm);

c is the concentration of desulfated sinigrin at equilibrium, in moles per litre, i.e.

$$c = \frac{0,15 \times 10^{-3} \times 0,95 \times 2}{2,05} \\ = 1,39 \times 10^{-5} \text{ mol/l}$$

0,95 is the yield at equilibrium of the desulfatation of the sinigrin.

3) Millipore PTGC 11K25 is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 9167 and does not constitute an endorsement by ISO of this product.

natively, the activity of the sulfatase may be used using the following simplified formula, the activity is given by the expression

$$\frac{\Delta A \times 5,7}{\Delta A_0}$$

5.4 Dilution

Using a pipette, transfer 1 ml of purified sulfatase (4.8.2) to a 10 ml one-mark volumetric flask. Make up to the mark with water and mix.

Divide the solution into small quantities and store in freezer at -18°C .

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 High-performance liquid chromatograph, suitable for obtaining an elution gradient and control of the temperature of the column at 30°C , connected to an ultraviolet detector permitting measurements to be made at a wavelength of 229 nm.

5.2 Chromatography column for HPLC, type C₁₈ or C₈, of particle size less than or equal to $5\text{ }\mu\text{m}$, for sample⁶

Hrosorb RP18 column, $< 5\text{ }\mu\text{m}$ (150 mm x 4,6 mm)

Spherisorb ODS2 column, $< 5\text{ }\mu\text{m}$ (250 mm x 4 mm; 250 mm x 5 mm)

Novapak C18 column, $4\text{ }\mu\text{m}$ (150 mm x 4 mm)

Uchrospher RP8 column, $< 5\text{ }\mu\text{m}$ (125 mm x 4 mm)

Nucleosil C18 column, $< 5\text{ }\mu\text{m}$ (200 mm x 4 mm)

The performance of the column should be checked regularly, preferably using a reference sample of colza desulfoglucosinolate⁶. In particular, the column shall not degrade 4-hydroxyglucobrassicin, an important but relatively unstable glucosinolate.

New columns shall be subjected to preliminary conditioning in accordance with the manufacturer's instructions so that reproducible results can be obtained.

5.3 Double-beam spectrometer, capable of operating in the ultraviolet region of the spectrum, and at a controlled temperature of 30°C , equipped with quartz cells of path length 1 cm and a recording system.

5.4 Microgrinder, for example a coffee mill.

5.5 Centrifuge, suitable for use with the tubes (5.6), capable of obtaining a centrifugal acceleration of $5\text{ }000g$.

5.6 Polypropylene tubes, of 6 ml capacity.

5.7 Water-bath or other heating apparatus, capable of maintaining a temperature of $75^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

5.8 Glass wool

5.9 Pasteur pipettes, 150 mm long, and a suitable stand, or any other appropriate apparatus.

6 Sampling

Sampling should have been carried out in accordance with ISO 542.

If large non-oleaginous foreign bodies have been separated before the reduction of the laboratory sample, allowance shall be made for this in the calculation.

7 Preparation of the test sample

Reduce the laboratory sample in accordance with ISO 664.

If the seeds have a moisture and volatile matter content in excess of 10 % (m/m), dry them using a current of air at approximately 45°C .

The impurities level is generally 2 % (m/m). If sinigrin is found in the sample, carry out the test on pure seed and analyse the impurities separately.

Determine the moisture and volatile matter content of the test sample in accordance with ISO 665.

If the seeds have been treated, wash them with dichloromethane.

Grind the seeds in the microgrinder (5.4) for 20 s. Mix the meal and then grind for a further 5 s.

⁶ The examples given are suitable products available commercially. This information is given for the convenience of this part of ISO 9167 and does not constitute an endorsement by ISO of these products.

⁶ Reference samples of colza desulfoglucosinolate may be obtained from the Community Reference Bureau.

8 Procedure

8.1 Test portion

Label two tubes (5.6) A and B and transfer 200 mg, weighed to the nearest 0.1 mg, of the prepared test sample (clause 7) to each tube.

8.2 Extraction of glucosinolates

8.2.1 Place the tubes in the water-bath or other heating apparatus (5.7) set at 75 °C and leave for 1 min. Add 2 ml of boiling methanol solution (4.1) and then immediately add

- to tube A, 200 µl of 5 mmol/l internal standard solution (4.5.1.1), and
- to tube B, 200 µl of 20 mmol/l internal standard solution (4.5.1.2).

8.2.2 Continue heating at 75 °C for a further 10 min, shaking the tubes at regular intervals. Mix the contents of each tube and then centrifuge at an acceleration of 5 000g for 3 min. Transfer the supernatant liquid from each tube to two other tubes (5.6) labelled A' and B'.

8.2.3 Add to the two tubes containing the solid residue 2 ml of boiling methanol solution (4.1) and reheat for 10 min in the water-bath or other heating apparatus (5.7) set at 75 °C, shaking the tubes at regular intervals.

Centrifuge for 3 min and then add the supernatant liquid from the two tubes to the respective supernatant liquids retained in 8.2.2.

8.2.4 Adjust the volume of the combined extracts to approximately 5 ml with water and mix.

These extracts may be kept for 2 weeks if stored in the dark in a freezer at -18 °C.

8.3 Preparation of ion-exchange columns

Cut the required number of Pasteur pipettes (5.9), i.e. one pipette per combined extract, so as to leave a volume of 1.2 ml above the neck and place a glass wool plug (5.8) in the neck of each pipette. Place the pipettes vertically on a stand.

Transfer 0.5 ml of a well-mixed suspension of ion-exchange resin (4.7) to each pipette and allow to settle.

Rinse the pipettes with 2 ml of the imidazole formate (4.4) and then twice with 1 ml portions of water.

8.4 Purification and desulfatation

8.4.1 Carry out the operations given in 8.4.2 to 8.4.5 for each combined extract.

8.4.2 Transfer 1 ml of the extract (8.2.4) to a prepared column (8.3) without disturbing the resin surface and allow to drain. Add two 1 ml portions of the sodium acetate buffer (4.2), allowing the buffer to drain after each addition.

8.4.3 Add to the column 75 µl of diluted purified sulfatase solution (4.8.4). Leave to act overnight at ambient temperature.

8.4.4 Place a tube (5.7) under the column to collect the eluate.

Elute the desulfo glucosinolate obtained with two 1 ml portions of water, allowing the water to drain after each addition.

8.4.5 Mix the eluate well. If not used immediately for chromatography, the eluate may be stored in the dark in a freezer at -18 °C for up to 1 week.

8.5 Blank test

If required (see 9.3), carry out a blank test using the same procedure on a test portion taken from the same test sample, but omitting the sinigrin internal standard solution in order to detect and quantify any sinigrin present in the test portion.

8.6 Chromatography

8.6.1 Adjustment of the apparatus

Adjust the chromatograph to give:

a flow-rate of the mobile phase (4.6), depending on the nature of the column (see 8.6.2), of generally of the order of 1 ml/min,

a column (5.2) temperature of 30 °C, and

a detection wavelength of 229 nm.

8.6.2 Analysis

Operating in accordance with the instructions for the apparatus, inject into the chromatograph not more than 50 µl of the desulfo glucosinolate solution obtained in 8.4.4.

Use an elution gradient appropriate to the column employed.

NOTES

4 The following elution gradients are given as examples.

— sorb RP18 column, $\leq 5 \mu\text{m}$ (150 mm \times 4,6 mm)

— pass 100 % of eluant A (4.6.1) for 1 min

— apply a linear elution gradient over 20 min until 0 % of eluant A and 100 % of eluant B (4.6.2) are obtained

— apply a linear elution gradient over 5 min until 100 % of eluant A and 0 % of eluant B are obtained

— pass 100 % of eluant A for 5 min to establish equilibrium

— Uchrospher RP8 column, $\leq 5 \mu\text{m}$ (125 mm \times 4 mm)

— pass 100 % of eluant A for 2 min 30 s

— apply a linear elution gradient over 18 min until 0 % of eluant A and 100 % of eluant B are obtained

— pass 100 % of eluant B for 5 min

— apply a linear elution gradient over 2 min until 100 % of eluant A and 0 % of eluant B are obtained

— pass 100 % of eluate A for 5 min to establish equilibrium

— gradient profiles may be modified to give optimum results according to the columns used.

8.3 Examination of chromatograms

Take into account only those peaks having an area greater than 1 % of the sum total of the peak areas.

The order of elution of the peaks with a type C₁₈ column and a suitable elution gradient (see the examples given in 8.6.2) is generally as shown in figure 1.

9 Expression of results

9.1 Calculation of the content of each glucosinolate

The content of each glucosinolate, expressed in micromoles per gram of dry matter of the product, is equal to

$$\frac{A_i}{A_s} \times \frac{n}{m} \times K_0 \times \frac{100}{100 - w}$$

where

A_i is the peak area, in integrator units, corresponding to desulfo-glucosinolate;

A_s is the peak area, in integrator units, corresponding to desulfosinigrin;

K_0 is the response factor of desulfo-glucosinolate (9.2);

m is the mass, in grams, of the test portion;

n is the quantity, in micromoles, of internal standard added to the tube in 8.2;

w is the moisture and volatile matter content, expressed as a percentage by mass, of the test sample.

If it is desired to express the result relative to a specified moisture and volatile matter content w_0 [e.g. $w_0 = 9 \%$ (m/m)], multiply the result obtained for dry matter (as above) by

$$\frac{100 - w_0}{100}$$

9.2 Response factors

The following response factors shall be adopted:

NOTE 6 These response factors have been determined experimentally and have been fixed by consensus between the various laboratories who took part in the test; they may need to be revised in due course.

1	Desulfo-glucolberin	1,07
2	Desulfo-progoltrin	1,09
3	Desulfo-epi-progoltrin	1,09
4	Desulfo-sinigrin	1,00
5	Desulfo-glucoraphanin	1,07
6	Desulfo-glucanapoleiferin	1,00
7	Desulfo-glucosylsin	1,07
8	Desulfo-glucanapin	1,11
9	Desulfo-4-hydroxyglucobrassicin	0,28
10	Desulfo-glucobrassicinapin	1,15
11	Desulfo-glucotropaeolin	0,95
12	Desulfo-glucobrassicin	0,29
13	Desulfo-glucanasturtin	0,95
14	Desulfo-4-methoxyglucobrassicin	0,25
15	Desulfo-glucobrassicin	0,20
16	Other desulfo-glucosinolates	1,00

9.3 Calculation of the total glucosinolate content

The total glucosinolate content, expressed in micromoles per gram of dry matter of the product, is equal to the sum of the contents of each glucosinolate (the corresponding peak area of which is greater than 1 % of the sum total of the peak areas).

If the difference between the total glucosinolate content results using both concentrations satisfy the requirements for repeatability (see 10.2), there is no

contamination of the internal standard. In this case take as the result the arithmetic mean of the two determinations.

10 Precision

10.1 Results of Inter-laboratory test

An inter-laboratory test, carried out at the international level in 1988, in which 11 laboratories participated, each of which carried out two determinations on each sample, gave the statistical results (evaluated in accordance with ISO 5725) shown in table 1.

Table 1 — Statistical results of inter-laboratory test

Sample	Repe- seed A	Repe- seed B	Repe- seed C	Repe- seed D
Number of laborato- ries retained after eliminating outliers	11	11	11	11
Mean glucosinolate content ($\mu\text{mol/g}$ dry matter)	20,6	14,1	4,9	25,6
Standard deviation of repeatability, s_r	1,7	0,6	0,3	0,8
Coefficient of vari- ation of repeatability	8,5 %	4,4 %	6,7 %	3,3 %
Repeatability, $2,83s_r$	4,9	1,7	0,9	2,4
Standard deviation of reproducibility, s_R	3,4	2,5	1,5	2,4
Coefficient of vari- ation of reproduc- ibility	17 %	18 %	31 %	9,4 %
Reproducibility, $2,83s_R$	9,6	7,1	4,4	6,8

10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, should not be greater than $2 \mu\text{mol/g}$ for glucosinolate contents less than $20 \mu\text{mol/g}$, and $4 \mu\text{mol/g}$ for glucosinolate contents within the range $20 \mu\text{mol/g}$ to $35 \mu\text{mol/g}$.

10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than $4 \mu\text{mol/g}$ for glucosinolate contents less than $20 \mu\text{mol/g}$, and $8 \mu\text{mol/g}$ for glucosinolate contents within the range $20 \mu\text{mol/g}$ to $35 \mu\text{mol/g}$.

11 Test report

The test report shall specify the method used and the result obtained. It shall also mention all operating details not specified in this part of ISO 9167, or regarded as optional, together with details of any incidents which may have influenced the result.

The test report shall include all information necessary for the complete identification of the sample.

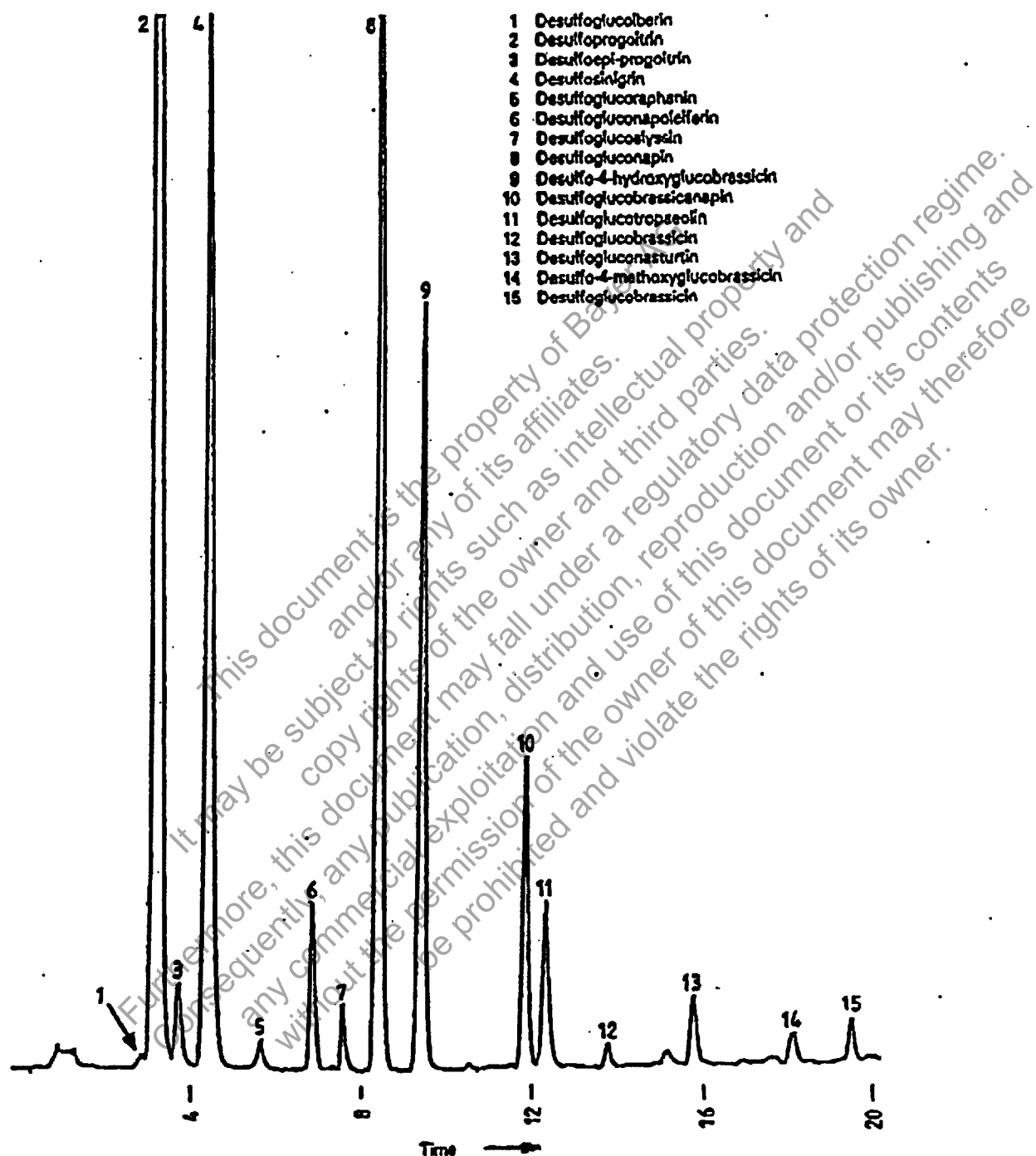


Figure 1 — Example of a typical chromatogram

Annex A (Informative)

Bibliography

- [1] ISO 542:1990, *Oilseeds — Sampling*.
- [2] ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests*.

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Descriptors: agricultural products, plant products, oilseeds, rapeseeds, chemical analysis, determination of content, glucosinolate, High performance liquid chromatography.

Price based on 9 pages

Appendix 6. GLUCOSINOLATE EXPERT OPINION LETTER

[REDACTED] Agriculture and Agri-Food Canada

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Agriculture and
Agri-Food Canada

Research
Branch

Agriculture et
agro-alimentaire Canada

Direction générale
de la recherche

Agriculture Canada
Research Station
107 Science Place
Saskatoon, Sask. S7N 0X2
Tel: [REDACTED]
FAX: [REDACTED]

April 15, 1994

[REDACTED]
Monsanto Agriculture Co.,
700 Chesterfield Village Parkway
Chesterfield, Missouri 63198

FAX: [REDACTED]

Dear [REDACTED]:

RE: Glucosinolate values of the TR Canola

I have reviewed your data with [REDACTED] and [REDACTED], and we note that pretty well all the quality parameters for the transgenic lines RT 200 and RT 73 fall within the range of values recorded for untransformed Westar. However, there appeared to be a tendency for RT 200 and RT 73 to have slightly higher values for the alkyl glucosinolates (averaging 2 to 2.5 μ moles per gram of oil free meal higher when averaged over all trials.) In our opinion, this is a very minor deviation and one which would be expected when single plants are selected from the heterozygous plant population that constitutes the cultivar Westar.

In the late 1980's, [REDACTED] made single plant selections to try and select out of Westar; lines that had the genetic make up to produce no more than 1 or 2 μ moles/g alkyl glucosinolates. It has taken some time to locate these data, but they indicate that in the low alkyl year (unselected Westar 10.8 μ moles) of 1991, they were able to identify single plant lines from Westar that were genetically stable at 6.7 μ moles. Since all the high glucosinolate plants were discarded in the selection process we can not say for certain what the upper level of the glucosinolate range within single plants of Westar would be. However, given our knowledge of biological systems, we can confidently predict that there would be plants within Westar that would produce at least 4 μ moles/g more than the Westar average. We feel very confident in this conclusion, since the evaluation test with low glucosinolate lines was a six rep field test with glucosinolate values determined for each line in all six reps.

It is unfortunate that Monsanto happened to choose plants for transformation that had genotypes capable of producing very slightly more alkyl glucosinolates than the average of the genotypes that make up Westar.

Canada

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There is also agreement among canola researchers here, that an increase of 1 to 2 μ moles in the range of about 6 to 16 μ moles is insignificant as to the quality of the product from such strains, particularly since in no test did the lines approach the maximum acceptable value of 20 μ moles per gram oil free meal.

I hope you find this information useful.

Yours truly,



Research Scientist



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Appendix 7. WEEDINESS POTENTIAL STUDIES

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Weediness Assessment of Roundup Ready Canola RT73

The parental variety Westar is a spring oil seed rape (OSR) or canola variety best suited to Canadian environments. Therefore comparisons between RT73 and the parental variety Westar have been conducted mainly in Canada, since it is the most appropriate environment for the establishment of substantial equivalence. In February 1995 the Western Canadian Canola and Rapeseed Recommending Committee (WCCRRC) recommended RT73 for conditional registration on the basis of its suitable agronomic performance under Canadian conditions. Extensive studies have also been conducted with RT73 and its progeny in Europe and the United States to confirm the conclusion of substantial equivalence under Canadian conditions. On the basis of observations made in all of the tests environments, there were no phenotypic differences, except for tolerance to Roundup, between Westar and RT73. This conclusion is supported by data demonstrating that there are also no compositional differences.

The following section describes data and information related to the biology of RT73 which demonstrates that RT73 is substantially equivalent to its non-modified counterpart in all relevant parameters.

Measurements and observations recorded from 1992 to 1994 were used to compare RT73 and non-modified canola varieties in commercially significant properties, except for tolerance to Roundup® herbicide. Every test was conducted such that a direct comparison to the control, Westar, grown side-by-side at each field site was made.

RT73 was selected from a single seed of the Westar genotype, and it is certain that RT73 inherited only a portion of the genetic variability of the parental variety (See expert letter in Appendix 6). Therefore, additional data was also collected from multiple sites in order to more accurately reflect the range of values (variability) expected for the parental genotype and allow for a reliable comparison with values for RT73.

A. Modes and/or rate of reproduction

i. Flowering period:

Extensive observations recorded by cooperators in 1992, 1993 and 1994 Canadian field trials indicate that the flowering period of RT73 is typical of a variety selected from Westar. As is the case for the parental variety, RT73 flowers from the end of June through the month of July across the Canadian prairies. Thus, there is no significant change in the flowering period of RT73 compared to Westar.

Under northern European conditions in the 1993 and 1994 field seasons flowering of RT73 and Westar also occurred simultaneously, in the period end-May to mid-June. When RT73 was hybridized and backcrossed with winter OSR varieties, the flowering dates of the progeny (mid-April) were also typical for winter OSR under European conditions.

ii. Pollen production and pollen viability:

As a means of determining pollen production and viability for RT73, yields of both RT73 and the parental variety Westar were measured in 1992 and 1993. Based on these results (see Table A6 below for 1993 data), it is concluded that there is no detectable difference in pollen production and viability of RT73 compared to the Westar grown at the same locations.

iii. Self compatibility:

Westar and RT73, like all *Brassica napus* varieties, are self compatible. The program to develop RT73 used this property to generate homozygous plants with the glyphosate tolerance genes. Self-pollination of R₁ and R₂ RT73 plants gave R₂ and R₃ seed, respectively, which were shown to be homozygous for the glyphosate tolerance phenotype. RT73 has been shown by segregation and molecular analyses to have a single insert and single copies of the CP4 EPSPS and *gox* genes. Furthermore, traditional breeding programs designed to introduce the glyphosate tolerance genes into other varieties of *B. napus* have been successful. Based on these facts, we conclude that there are unlikely to be any differences between RT73 and Westar in self compatibility.

iv. Time from seeding to maturity:

Data from the 1993 Canadian trials indicate that RT73 is on average approximately 1 day later in maturation when compared to Westar. This result is consistent with the fact that RT73 is a selection from a single seed of Westar (see above in this Section). Table A5 summarizes the maturity data from a range of Canadian environments. No delays in maturity have been noted in European trials, and the conclusion of no substantial difference has been confirmed in European trials from 1992 to 1994.

v. seed production:

The information provided in Table A6 is from the 1993 Canadian Co-Op Test and is an average of data recovered from 21 diverse test sites. The average yield of 2,000 kg/ha for the cultivar Westar is not atypical because values from some test sites were very low. This variation in performance from test site to site is a result of local weather, soil, disease and pest conditions. It is important to note that these trials are managed under "weed free" conditions, but Roundup was not applied to the plots.

These data show a slightly lower yield from RT73 versus Westar which was attributed to a poor seed source for RT73 from a winter nursery in Chile. Seed of RT73 was bulked in Chile over the winter of 1992 and 1993. To meet deadlines for Co-Op introduction, this seed was harvested earlier than considered optimal. Consequently, the slight yield reduction in the 1993 Co-Op was attributed to the slightly immature seed. Private data from 1992 shows no yield reduction in RT73 relative to Westar. Yield data from Western Canada from 73 side-by-side comparisons of RT73 to other commercial varieties showed a 2.1 bu/ac yield increase for RT73.

Yield trials conducted in Belgium in 1993 and 1994 also demonstrated that under representative European conditions there were no substantial differences in yield between RT73 and Westar.

Table A5. Days to Maturity from the 1993 Canadian Co-Op Test.

Location	Strain or Cultivar (days)	
	RT73	Westar
Lacombe	120	119
Scott	112	110
Durban	107	108
Melfort	114	113
Winnipeg	98	97
Fort Saskatchewan	116	119
Olds	129	126
Yorkton	107	105
Rosebank	99	98
High Level	119	119
Portage la Prairie	94	94
Brandon	102	102
Fort Vermilion	113	111
Saskatoon	112	110
Average	110.1	109.4

B. Dissemination**i. Outcrossing frequency within species:**

There is no *a priori* scientific basis to believe that the outcrossing frequency from RT73 to other *B. napus* varieties will be different to that of Westar. The glyphosate tolerance phenotype has been successfully transferred to other *B. napus* varieties of canola using traditional backcrossing procedures as a part of the breeding programs of several canola seed companies to develop RT73. Based also on the fact that the pollen production and pollen viability (as measured by yield and germination of progeny) and self compatibility are unchanged by the genetic modification, the outcrossing frequency within species is unlikely to be different for RT73 when compared to Westar (See Section IV.F.2).

Table A6. Seed Yield for Test Lines in 1993 Canadian Co-Op Trial.
(Values are 100X kg/ha)

Location	Strain or Cultivar	
	RT73 ¹	Westar
Lacombe	29.5	30.5
Loon Lake	12.8	16.9
Weststock	31.8	31.6
Olds	16.8	17.0
High Level	13.9	12.1
Fort Vermilion	15.2	17.5
Average Short Season Zone	20.0	20.9
Scott	18.7	20.8
Lashburn	21.4	22.0
Durban	20.7	20.0
Melfort	20.7	16.6
Fort Salk.	29.2	33.8
Kelsey	20.5	22.5
Alexandra	37.8	41.5
Yorkton	13.7	13.8
Saskatoon	23.3	24.0
Average Mid Season Zone	22.9	23.9
Thornhill	8.1	7.5
Winnipeg	7.2	9.0
Rosebank	18.8	15.1
Portage	11.9	14.8
Brandon	11.8	18.3
Average Long Season Zone	11.6	12.9

¹ - Seed of RT73 was bulked in Chile over the winter of 1992 and 1993. To meet deadlines for Co-Op introduction, this seed was harvested earlier than considered optimal. Consequently, the slight yield reduction in the 1993 Co-Op was attributed to the slightly immature seed.

ii. Silique shattering and dispersal:

The loss and local dispersal of seed by shattering of mature seed pods is a well known characteristic of canola. Agronomic practices like swathing are routinely used to limit the loss of seed before harvest, however it is inevitable that some seed is lost. The degree of loss to shattering is largely dependent on environmental factors that influence the degree of pod maturity, the degree of moisture present, and physical disturbance of the material prior to harvest.

Brassica seeds do not have any special or specific adaptations to facilitate wide-spread dispersal (they do not blow in the wind or stick to animal fur) so the shattered seed will remain in close proximity to the original site. Since it is accepted that a high proportion of the mature shattered seed can remain viable and will germinate

subsequent to harvest, the degree of shattering can be assessed by counting the volunteers in the subsequent years. It is equally important to note that the volunteers in the subsequent field season result from not only shattering, but also spillage and other mechanisms of seed loss at harvest. Thus, counting volunteers overestimates shattering, but addresses the main issue of potential invasiveness.

Defined test sites which had contained RT73 in 1992 or 1993 were planted in the following year with wheat, barley, barley/rye mixture, occasionally alfalfa and/or left fallow. As expected in the year immediately following a test, some volunteers were observed at some test locations. The numbers of volunteers was highly variable, but no reproducible differences were found upon comparison of RT73 and the Westar control. Furthermore, no differences were evident between plots of RT73 which had been treated and untreated with Roundup herbicide. The data obtained from some selected Canadian test sites is shown below in Table A7.

Table A7. Volunteer Canola Counts Taken in 1993 on 1992 Trial Sites.
(Values are plants/m²)

Location	Westar	RT73 (untreated) ¹	RT73 (treated) ²
Minto ³	10 ^a	12 ^a	13 ^a
Melfort ³	67 ^a	2 ^b	6 ^b
Saskatoon ^{3,4}	no data	292 ^a	388 ^a
Saskatoon ^{3,5}	168 ^a	209 ^a	no data

- ¹ Untreated indicates that Roundup herbicide was not applied to these plants.
- ² Treated indicates that Roundup herbicide was applied to these plots at the 2 to 6 leaf stage at a rate of 0.45 kg a.i./ha.
- ³ Statistical significance for a listed location were determined using Duncan's Multiple Range Test P=0.05. Values followed by the same letter are not significantly different.
- ⁴ Data from plots of a tolerance trial conducted in 1992. No data were available for Westar.
- ⁵ Data from plots of a variety trial conducted in 1992. No data were available for treated RT73.

Although the simple statistical analysis of data at the Melfort site suggest that there was a significant difference in the shattering of Westar when compared with the transgenic materials, it was noted that the Westar control plot was somewhat more mature, which may have resulted in the higher loss of shattered seed. The numbers of volunteers at Saskatoon were much larger because of adverse weather conditions during the harvest period. Although poor weather conditions increased seed loss to shattering and variability of the data obtained, no significant differences were observed between the treatments. Volunteers were monitored in 1994 at five sites, and the results are given in Table A8.

Table A8. Average Volunteer Counts Taken on 1993 Canola Plots in 1994.
(Values are plants/m²)

Location	Westar	RT73 (untreated) ¹	RT73 (treated) ²
Minto ^{3,4}	571.2 ^{a,b}	1063.2 ^b	188.4 ^a
Lethbridge ^{3,5}	119.2 ^a	125.0 ^a	129.0 ^a
Melfort ^{3,4}	67.6 ^{a,b}	85.6 ^a	34.8 ^b
Scott ^{3,6}	166.8 ^a	155.3 ^{a,b}	152.5 ^b
Saskatoon ^{3,7}	863.6 ^a	804.2 ^a	832.2 ^a
Average ³	357.7 ^a	446.6 ^a	267.4 ^a

- 1 Untreated indicates that Roundup herbicide was not applied to these plants.
- 2 Treated indicates that Roundup herbicide was applied to these plots at the 2 to 6 leaf stage at a rate of 0.45 kg active ingredient/ha.
- 3 Statistical significance for a listed location were determined using Duncan's Multiple Range Test P=0.05. Values followed by the same letter are not significantly different.
- 4 Counts were taken from 0.25 m² plot.
- 5 Counts were taken from 0.1 m² plots.
- 6 Counts were taken from 0.5 m² plots.
- 7 Counts were taken from 0.35 m² plots.

Based on two years of monitoring, the evidence shows no difference between Westar and RT73 in its shattering properties. The variability noted at the Minto site (Table A8) was attributed to the topography of the plots. The untreated RT73 plot was on a hill where the snow melted sooner, the soil warmed faster, and the largest flush of volunteers was observed (1063.2 plants/m²). The Westar plot was located midslope and had more counts (571.2 plants/m²) than the treated RT73 plots which were in a depression (188.4 plants/m²). Most importantly, the average values from all locations showed no statistical differences.

A direct measure of seed loss to shattering was also conducted at Minto and Melfort test sites in 1993. At both locations, shattering was evaluated by placing catch pans in plots at the same time as the OSR began to ripen. The results of this test expressed as a percent of Westar are given in Table A9.

Table A9. Results of Shattering Studies Conducted in 1993.
(Values are expressed as % Westar control)

Location	Westar	RT73 (untreated) ¹	RT73 (treated) ²
Minto ³	100 ^a	110 ^a	85 ^a
Melfort ³	100 ^a	53 ^a	66 ^a

1 Untreated indicates that Roundup herbicide was not applied to these plants.

2 Treated indicates that Roundup herbicide was applied to these plots at the 2 to 6 leaf stage at a rate of 0.45 kg a.i./ha.

3 ANOVA statistical analysis was performed indicating no significant difference at each location as indicated by the letter following the value.

Analysis of variance (ANOVA) of data generated by catching seed that fell prior to harvest did not reveal any significant differences. The results for Melfort were variable due to a heavy snowfall and high winds prior to harvest and the uneven maturity of the different test plots at this location. This may also reflect the slight maturity difference between Westar and RT73 (See section A.iv).

On the basis of these results, it can be concluded that there are no differences between RT73 and Westar with respect to silique shattering and dispersal. This conclusion has been confirmed during the monitoring of European trials, where no differences have been noted in the levels of regrowth of RT73 and Westar following the harvesting of the trials.

C. Survivability

i. Germination rate:

Germination tests of seed of Westar and RT73 from Roundup treated and untreated 1992 variety trials were conducted at the Agriculture Canada seed quality testing laboratory in Saskatoon. The results (Table A10) of these tests show that all the seed samples demonstrated high rates of germination and no differences were observed between the RT73 and Westar. These findings also support the conclusion of no differences in dormancy between RT73 and Westar (see below in this Section).

Table A10. Germination Test of Seed from 1992 Trials.

Canola Cultivar or Line	Percent Germination
Westar	99 %
RT73 (untreated) ¹	99 %
RT73 (treated) ²	98 %

1 Untreated indicates that Roundup herbicide was not applied to these plants.

2 Treated indicates that Roundup herbicide was applied to these plots at the 2 to 6 leaf stage at a rate of 0.45 kg a.i./ha.

A more extensive study of germination was conducted by Monsanto Canada in 1994. Known weights of seed from RT73 and four other *B. napus* varieties (Westar, AC Excel, Legend, and Cyclone) were planted at four locations in replicated plots or single rows. Westar seed was 1992 pedigree, while seed of the four other non-modified varieties was obtained from a more recent pool of seed used for the 1994 Canadian Co-Op Tests. The number of plants which emerged were counted, and all values were normalized on a per square meter basis for comparison and analysis. Noted in this test was consistently poor germination of the Westar canola seed (Table A11), which was older seed that had been stored at room temperature. All other materials showed good germination across all locations proving that RT73 is a typical *B. napus* variety as measured by germination (Table A11).

Table A11. Germination Test in 1994 of RT73 Compared with Five *B. napus* Varieties. (Values are plants/m²)

Location	<i>B. napus</i> variety				
	RT73	Westar	AC Excel	Legend	Cyclone
Saskatoon	80	43	84	80	76
Minto	73	48	94	68	66
Melfort	82	51	117	89	109
Scott	94	47	101	96	110
Average ¹	82.3 ^a	47.3 ^b	99.0 ^c	83.3 ^a	90.3 ^{a,c}

1 Statistical significance determined using Duncan's Multiple Range Test $P=0.05$. Averages followed by the same letter are not significantly different.

ii. Vegetative vigor:

In 1993 and 1994, RT73 was evaluated at more than 32 Canadian locations for vegetative vigor. Based on an analysis of documented visual observations 27 of the 32 sites recorded no difference between RT73 and Westar. Of the five documented differences, two (Melfort and LaSalle in 1993) assigned the Westar plots as more vigorous and 3 (Lethbridge and Minto in 1993 and Saskatoon in 1994) noted the RT73 plants to be more vigorous. At only one of the 32 locations (Melfort in 1993) was a consistent difference noted throughout the growing season up to harvest, and the reduced vigor of RT73 was attributed to a slight maturity delay (Section A.iv.). Data on plant height from the 1993 Canadian Co-Op Test further confirms that RT73 is no more vigorous than other *B. napus* canola varieties. The mean height of RT73 plants at 12 locations was 119.9 cm, while Westar was 115.4 cm and the average of all *B. napus* varieties in the Co-Op Test for genetically-modified plants was 119.8 cm.

Observations on the growth of RT73 and Westar under European conditions have been made in trials conducted in Belgium (1993 and 1994), France (1994) and the

U.K (1994). It was confirmed that there were no differences in vigor between RT73 and the parental variety.

It is concluded, based on two years of observations at numerous locations in Canada, and confirmed in northern European locations, that there is no difference in the vegetative vigor of RT73 relative to Westar.

iii. Overwintering capacity as a plant:

All observation of the overwintering capacity of RT73 as a plant indicate that it behaves in a manner consistent with Westar and any other spring canola currently in commerce. Field cooperators have noted that the small amount of seed left in the field after harvest due to shattering and loss germinates prior to winter. These plants, both RT73 and Westar, do not survive to the next season, although some of the seed which has been buried by cultivation may germinate in subsequent years. Like other *B. napus* spring varieties, RT73 is an annual plant which will not survive the harsh winters.

Winter canola plants have been produced by hybridization with RT73 and backcrossing to the winter canola parent. Observations on the winter survival of these plants has confirmed that the glyphosate tolerance trait has been successfully transferred into the winter OSR plants without any negative effects on winter survival.

iv. Seed dormancy:

The principle measure of seed dormancy was to determine volunteer counts in replicated RT73 and Westar plots at multiple locations. Volunteer counts were also taken in 1994 in RT73 and Westar plots from 1992 to assess extended dormancy. These same data are reported above in the discussion related to shattering (Section B.ii.).

There is no evidence to support increased dormancy of RT73 seed as a consequence of the genetic modification or the introduced trait. The results from volunteer counts (Tables A7, A8 and A9) and germination data (Tables A10 and A11), show no obvious differences between RT73 and Westar. The difference noted at Melfort in 1993 (Table A7) can be explained by the 1 day maturity difference between RT73 and Westar (see Section A.iv.), and the atypical growing season in 1992 (35) where a maturity delay was exacerbated by the excessive cold and precipitation close to harvest. The difference noted in germination of Westar in 1993 (Table A11) was attributed to the fact that old seed had to be used to plant all control plots due to the scarcity of this variety. The multi-site data from 1994 (Table A8) shows no statistically significant differences between RT73 and Westar in the number of volunteers observed in the following years. Additionally, no volunteers were observed in 1994 in test plots of RT73 planted at Minto and Lethbridge in 1992. Based on these results, it is concluded that RT73 is not changed in dormancy potential versus the parental canola variety.

v. Adaptations to stress factors:

Introduction of the glyphosate tolerance trait into canola has not resulted in any exceptional or unexpected adaptations to stress factors which would provide RT73 with a selective advantage.

Biotic factors:

RT73 has not demonstrated any observable difference in adaptation to biotic stress factors relative to Westar. Data from the 1993 Canadian Co-Op Tests rank RT73 similarly to the parental variety Westar in blackleg (*Phoma lingam*) disease susceptibility with overall disease ratings of 1.6 and 1.9 on a scale of 0-5, respectively. Like its parental variety, RT73 is highly susceptible to blackleg disease. In a separate experiment in a blackleg disease nursery in 1993, RT73 scored 3.95 out of a possible 5, while Westar was scored at 4.11. Analysis of variance of the ratings showed no difference between RT73 and Westar. It is concluded therefore that RT73 is unchanged in its susceptibility to blackleg disease compared to its parent variety.

Additionally, observations documented over two years of field trials consistently indicate that no differences in disease and insect susceptibility can be seen between RT73 and Westar.

European trials were monitored for the major diseases, including Blackleg or Stem canker (*Phoma lingam*), Light leaf spot (*Cylindrosporium*), Downy mildew (*Peronospora brassicae*), Alternaria (*Alternaria brassicae*), Sclerotinia (*Sclerotinia sclerotiorum*), White leaf spot (*Pseudocercospora*) and others, and insect pests, including Pollen beetle (*Meligethes* sp.), Flea beetle (*Phyllotreta* sp.), *Ceuthorrhynchus* sp., *Baris* sp., *Psylliodes chrysoccephala* and others. In none of the European trials were any differences noted between RT73 and Westar in their sensitivity to pathogens or predators.

It is concluded therefore, that there are no measurable differences in the ability of RT73 to adapt to biotic stress factors.

Abiotic factors:

Observations documented by cooperators over many years of field testing and two years of commercial production demonstrate that there is no difference between RT73 and Westar in its adaptation to abiotic stress factors. No differences have been recorded between RT73 and Westar in their ability to resist drought, heat, and frost.

Herbicidal factors:

The susceptibility of RT73 to herbicides currently used for control of volunteer canola (except glyphosate) was verified in 1994 in Canada, and in Belgium in 1993 and 1994. In Canadian trials, effective control was obtained with all applications of 2,4-D or the sulfonylurea based Refine® (thifensulfuron methyl and tribenuron methyl) herbicide when applied at the 1-3 leaf stages of the crop. Good control was also noted with 560 g active ingredient/ha of 2,4-D at the 3-6 leaf stage. In European trials RT73 was shown to be no different to Westar in its sensitivity to a

range of herbicides, including those commonly used in wheat, barley and sugar beet. Thus, with the exception of glyphosate, RT73 is equivalent to Westar in its susceptibility to the herbicides commonly used to control canola volunteers.

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