

Project Name	Application of GMO Safety Certificate (import) for Canola Event Ms1Rf2 with Gene <i>barnase</i> and <i>barstar</i>
Applicants	Robert Yeh
Submission Date	2002/03/21
Notes	It is for FFP approval which was submitted in Year 2002



M-422971-01-1

Appendix 1

OCDE/GD(97)63 Series on Harmonization of Regulatory Oversight in Biotechnology
No.7 CONSENSUS DOCUMENT ON THE BIOLOGY OF BRASSICA NAPUS L.
(OILSEED RAPE)

Appendix 2

C000155 Description of vector pTTM8RE and pTVE74RE

Appendix 3

OSR-MS1-01 MS1 Discriminating PCR Protocol
OSR-RF2-02 RF2 Discriminating PCR Protocol

Appendix 4

Exporting country approval letter

项目编号：

项目类别：

农业转基因生物安全评价

申 报 书

转 barnase 雄不育基因及其 barstar 育性恢复基因双低杂交油菜 Ms1Rf2
安全证书申请

(2002 年 3 月 21 日填报日期)
中华人民共和国农业部制

农业转基因生物安全评价 申 报 书

项目名称：

转 barnase 雄不育基因及其 barstar 育性恢复基因双低杂交油菜 Ms1Rf2 安全证书
申请

申请单位：

安万特（中国）投资有限公司 Aventis (China) Investment Co., Ltd.
北京市朝阳区 100022 东三环南路 2 号航华科贸中心二号楼 7 层

申请人：

1. Mr. Jean Broue (总经理)
2. 叶天勋博士 (技术顾问)

地址：

1. 北京市朝阳区 100022 东三环南路 2 号航华科贸中心二号楼 7 层 (北京邮政信箱 2393)。
2. 天津市 300061 河西区紫金山路澳隆花园 A 区 1 门 402 室

邮政编码：

1. 100022
2. 300061

电话：

1. 010-65685588 分机 500
2. 022-28358471, 28358477

传真：

1. 010-65668422
2. 022-28369292

e-mail：

1. jean.broue@aveintis.com
2. robert.yeh@aventis.com

填报日期：2002 年 3 月 21 日

填 写 说 明

1、在填写申报书之前，应认真阅读《农业转基因生物安全管理条例》、《农业转基因生物安全评价管理办法》、《农业转基因生物进口安全管理方法》等有关法规，了解相关的要求、标准和技术规范。

2、此申报书同样适用于实验研究和中间试验的报告，但名称分别改为“农业转基因生物试验研究报告书”和“农业转基因生物中间试验报告书”，有些不适用的条款可以不用填写。例如：实验研究的报告书就不用填写试验方案。

3、申报书内容应当包括以下部分：目录、申请表、项目内容摘要、工作目的和意义、国内外相关研究的背景资料、农业转基因生物安全评价、试验方案、相关附件资料、申报单位农业转基因生物安全小组审查意见、申报单位审查意见、所在省（市、自治区）的农业行政主管部门的审查意见。

填写申请表时，申请实验研究、中间试验、环境释放和生产性试验按表 2 填写；申请农业转基因生物安全证书按表 3 填写。

申报书中安全评价、试验方案和相关附件资料部分依照附录 I、II、III 要求逐条填写。

4、申报书应当用中文填写，一式十份，一律使用 A4 纸，正文用小四宋体打印，标准字间距和单倍行距，并提供软盘或光盘。对于不符合要求的申报书，不予受理。

5、申请者可以注明哪些资料属于机密并说明理由。

6、对于已批准过的环境释放或生产性试验，在试验结束后，若同一转基因生物在原批准地点以相同规模重复试验，在申报时“安全性评价”部分可省略。

7、受理时间：每年两次，截止日期分别为 3 月 31 日和 9 月 30 日。

8、受理单位：农业部科技发展中心。地址：北京市朝阳区麦子店街 18 号楼。
邮政编码：100026。收款单位：农业部科技发展中心；开户行：农行北京朝阳支行；
帐号：873—9732。

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表 2:
试验申请表

项目名称					
项目阶段	<input type="checkbox"/> 实验研究	<input type="checkbox"/> 中间试验	<input type="checkbox"/> 环境释放	<input type="checkbox"/> 生产性试验	(选 √)
项目来源					
供试转基因生物概况					
类别	<input type="checkbox"/> 动物 <input type="checkbox"/> 植物 <input type="checkbox"/> 微生物 (选 √)				
受体生物	中文名			学名	
	分类学地位			安全等级	
目的基因	名称			供体生物	
	生物学功能				
载体			供体生物		
标记基因			供体生物		
报告基因			供体生物		
转基因方法			基因操作类型	1 2 3 (选一)	
转基因生物安全等级			转基因生物产品安全等级		
试验起止时间					
试验地点					
试验规模					
申报人					
姓名		性别		出生年月	
学历		专业技术职务			
工作单位及地点					
何时何地曾从事何种基因工程工作					
项目主要参加人员					
姓名	年龄	学历	职称	单位	在本项目中的分工

表 3:
农业转基因生物安全证书申请表

项目名称		转 barnase 雄不育基因及其 barstar 育性恢复基因双低杂交油菜 Ms1Rf2 安全证书申请				
项目来源						
转 基 因 生 物 概 况	类别	动物 <input type="checkbox"/> 植物 <input checked="" type="checkbox"/> 微生物 <input type="checkbox"/> (选 √)				
	转基因生物名称		MS1RF2 双低油菜			
	受体生物	中文名	油菜(甘蓝型)	学名	<i>Brassica napus L. Oleifera</i>	
		分类学地位	十字花科芸薹属	安全等级	安全等级 I	
	目的基因	名称	1. barnase 2. barstar 3. bar	供体生物	1. 解淀粉芽孢杆菌 <i>Bacillus amyloliquefaciens</i> 2. 解淀粉芽孢杆菌 <i>Bacillus amyloliquefaciens</i> 3. 吸水链霉菌 <i>Streptomyces hygroscopicus</i>	
		生物学功能	1. RNA nuclease (RNA 核酸酶) 2. 抑制 barnase 的表达 3. 抗除草剂草氨膦			
	载体	pTTM8RE(MS1) pTVE74RE(RF2)	供体生物	根瘤农杆菌 <i>Agrobacterium tumefaciens</i>		
	标记基因	neo 基因	供体生物	大肠杆菌 <i>Escherichia coli</i>		
	报告基因	bar 基因	供体生物	无		
	转基因方法	农杆菌转染法	基因操作类型	1 <input checked="" type="checkbox"/> 2 <input type="checkbox"/> 3 (选一)		
转基因生物安全等级	安全等级 I					
转基因生物产品安全等级	安全等级 I					
试验概况	中间试验情况	转基因生物名称及编号				
		试验的时间、地点和规模				
		转基因生物名称及编号				
		批准文号				
		批准时间、地点和规模				
		转基因生物名称及编号				
	批准文号					
	批准时间、地点和规模					

(续前)

拟申请使用范围(省、自治区、直辖市)								
拟申请使用年限		5年						
申请单位概况	单位名称	安万特(中国)投资有限公司			法人代表	Dr. Ruediger Barth		
	地址及联系方法	北京市朝阳区东三环南路2号航华科贸中心二号楼7层 北京邮政信箱2393 电话: 010-65685588 传真: 010-65668422 emial: ruediger.bARTH@aventis.com						
研制单位概况	单位名称	Bioscience Regulatory Affairs			法人代表	Volkert Sjut		
	地址及联系方法	Aventis CropScience GmbH, Hoechst Works, South Gate, K-607 65926 Frankfurt-am-Main, Germany Tel: 00 49 69 305 12075, Fax: 00 49 69 305 30949 Volkert.sjut@aventis.com						
	主要完成人							
	姓名	Robert MacDonald		性别	男	出生年月	1964年4月19日	
	学历	农学及环境生物博士			专业技术职务	全球法规部经理		
	何时何地曾从事何种基因工程工作	于1991年11月在加拿大萨斯克彻温省若季那市赫司特公司						
	参与完成人							
	姓名	年龄	学历	职称	单位		在本项目中的分工	
	Marc De Beuckeler	49	工业工程师	研究员	生物技术产品特性的鉴定		分子生物	
	Johan Botterman	44	博士	经理	小麦及油菜生物技术转基因性状研发		分子生物及细胞生理	
Christiane Opsomer	50	医学士	研究员	小麦及油菜生物技术转基因性状研发		分子生物		
Greta De Both	41	农学士	经理	育种及生产部		育种及生产		

(注: 进口转基因生物直接申请安全证书的, 本表“试验概况”一栏不填写)

农业部科技发展中心基因安全管理处

电话: 64191432

农业部农业转基因生物安全管理办公室

电话: 64193077 64193022

二 项目内容摘要：

本文的主要内容是关于转基因甘蓝型油菜 (*Brassica napus*) 的最终加工食品（主要是油料）和饲料（主要是谷粉）。安万特作物科学公司的杂交系统不是用于改变油菜品种的成分和/或用途。转基因的目的在于引入新型的授粉控制系统，使其能够获得真正的油菜杂交品种。

安万特作物科学公司首先申请两个主要的双低油菜品种以及该两组合。另外有一个恢复系 Rf2 (以前为 B94-2) 也被证实可用这个恢复系是和 Rf1 一样利用农样菌浸染法转化，转育而成，它包含了源自于 pTVE47RE 相同的嵌合基因构造。因为关于 Rf2 的食品或饲料相关的安全分析均相同于 Ms1 与 Rf1 的安全分析，这份报告材料只是提供一些关于新 Rf2 品系及其 Ms1 及 Rf2 的杂交品种额外的新信息。

在开发这种杂交系统的过程中，安万特作物科学公司及世界上一些独立的研究专家和法规机构已经证明 Ms1 系和 Rf2 系对环境是安全的。甘蓝型油菜 (*Brassica napus*)。由这些亲本杂交所生产的 F1 (子一代) 杂交油菜品种已在加拿大的主要产区商品化的种植。这种产品，或商品化生产在大田里所收获的 F2 (子二代) 种子，将在本国加工或以原料的方式出口做为加工使用。油菜油料和油菜粉是其主要的产品，加工后可以分别用于人类食用和动物饲料。由于国际农产品贸易的进行，在不久的将来中国也会有这种经 Ms1Rf2 原料加工得到的食品和饲料。

油菜加工产品在食品和饲料中的应用非常普遍：油料既可以单独食用也可以用作其它食品的添加成分，谷粉则可以不同比例加入饲料中。正如本文所述，没有迹象显示，MS1 和/或 RF2 的遗传修饰对由转基因甘蓝型油菜 (*Brassica napus*) 加工而成的食品和饲料的安全性有任何负面影响。根据本文件，加拿大、美国和日本的行政当局已经承认源于 MS1 和 RF2 或这两个品系杂交系的油料以及饲料产品可以安全地用于食品和饲料。

三 工作目的和意义：

安万特作物科学公司开发了一种新型杂交系统，可以广泛应用于各种农作物。这种新型杂交系统已经被成功地应用于甘蓝型油菜 (*Brassica napus* L. *oleifera*)。

油菜育种者的主要目的是获得高产量的甘蓝型油菜品种 (*Brassica napus* L. *oleifera*)。实现此目标的最有效办法是使用 F1 代杂交种，因为杂交油菜品种预计可以比最好的常规品种产量提高 20–25%。另外，F1 代杂交种的一致性有利于油菜的商品化生产，既有助于提高产量又有助于促进销售。

因为油菜既可以自花授粉 (70%)，又可以异花授粉 (30%)，需要利用授粉控制系统才能得到 100% 的 F1 代杂交种子。与其它农作物的杂交系统一样，安万特作物科学公司的油菜杂交系统也包括两部分：雄性不育系和育性恢复系。雄性不育系阻止自花授粉，因此可以保证得到 100% 真正的杂交种子。育性恢复系使杂交种子自身在田间完全可育。

雄性不育系作为保证异花授粉的有效工具已广泛应用于不同的农作物。油菜的雄性不育系的构建采用了新的方法，即转入了核糖核酸酶 (*barnase*) 基因。这种基因特异性表达在绒毡层组织，这是一种在花药发育过程中包围花粉囊的细胞层。*barnase* 基因在这种组织中的表达阻断了花粉的发育，从而产生雄性不育植株。

barstar 基因 (核糖核酸酶抑制物) 也被转入，用于构建杂交系统的育性恢复系。将雄性不育系和育性恢复系杂交，油菜后代的育性将得到恢复，因为 *barstar* 蛋白可使 *barnase* 酶失活。

bar 基因是杂交系统中的内在元件，因为它直接决定了转化/再生过程和田间的选育效率。此外，*bar* 基因的表达产生了抗草氨膦的油菜，从而可在转基因作物田中使用除草剂进行杂草防治。

在主要种植区加拿大农民们目前正在种植 F1 (子一代) 甘蓝型油菜 (*Brassica napus*) 杂交品种。由这些亲本杂交所生产的 F1 (子一代) 杂交油菜品种已商品化的种植。这种产品，或商品化生产在大田里所收获的是 F2 (子二代) 种子，将在本国加工或以原料的方式出口做为加工使用。。油菜油料和油菜粉是其主要的产品，加工后可以分别用于人类食用和动物饲料。这些油菜品种是“双低”品种，即油料中芥子酸含量低和加工油菜粉中的硫甙含量低，从而提高了品质。由于国际农产品贸易的进行，在不久的将来中国也会有经 Ms1Rf2 原料加工得到的食品和饲料。

四 国内外研究的相关资料；

从 1991 年开始，安万特作物科学公司对这种新型的杂交系统进行了户外田间实验的评估，显示了其可行性和与科学预测的一致性，并对农艺性状，花的表型，雄性不育和育性恢复性状的稳定性和确认其不会减产等方面进行了研究。在欧洲和北美的几个国家进行了多处田间实验。田间实验结果证明在选择合适的油菜雄性不育系和育性恢复系的基础上可以得到可靠的油菜杂交系统。

1994 年加拿大首次批准以 MS1 和 RF2 油菜品种为基础的杂交系统上市，随后欧洲、日本和美国也批准了它们的上市。1995 年，上市正式开始。从 1996 年起，育种的重点集中于雄性不育系 MS8 和育性恢复系 RF3 上，并已经向上述国家递交了申请。

五. 安全性评价

1 受体植物安全性评价

1.1 受体植物的背景资料:

1.1.1 学名、俗名和其它名称;

Brassica napus L. oleifera, Metzg., / 油菜、芸苔，菜籽，油菜籽。

1.1.2 分类学地位;

十字花科芸苔属

1.1.3 实验用受体品种（或品系）名称;

源于春油菜（阿根廷）品系的常规品种和/或杂交品种。关于这些品种的详细信息可以登陆www.canola-council.org

1.1.4 是野生种还是栽培种;

尽管油菜品种主要是栽培种，但是野生种油菜同样存在。

1.1.5 原产地及引进时间;

在欧洲和亚洲都已经发现野生种油菜，但是准确的发源地（或者是地中海地区，或者是北欧地区）尚不清楚。公元前 2000 年到 1500 年的一些梵语著作直接描述了油菜的形态。古代亚洲和地中海地区的一些居民就有用菜籽油进行照明的记录，后来又被用作食用油。早期的记录显示油菜籽早在 3000 多年前的印度就已有种植。然后又在公元前被引入中国和日本。

1.1.6 用途;

菜籽油和菜籽油粕是主要的油菜产品，经过加工后分别可以用于人类食用和动物饲料。

1.1.7 在国内的应用情况;

在国内油菜籽油是良好的食用油，营养价值高，含有大量脂肪酸和多种维生素，如维生素 A、维生素 B 和维生素 E 等。

菜籽油在食品工业上应用普遍。还是重要的工业原料，在冶金、机械、

橡胶、化工、纺织、油漆、制肥皂、油墨、造纸、皮革、医药等方面都有重要用途。随着工业的发展，菜籽油在加工利用上也在向广度和深度发展，几乎所有工业部门都不同程度地使用脂肪酸制品。

油菜生产提供大量油脂和油饼，有很高的经济价值。菜籽饼含氮 4.6%，磷 2.48%，钾 1.4%，以及其它多种营养元素，是很好的肥料。菜籽饼含蛋白质 35%-47%，以及各种氨基酸，还含有粗脂肪、纤维素、矿物质和多种维生素；油菜饼粕除去硫代葡萄糖甙后，是家畜的优质饲料。油菜秸秆、角壳也可沤制成有机肥料，供作物吸收利用，对培养地力也有良好的作用。

丽油菜在作物轮作复种中占有重要地位。油菜地腾茬早、地力肥、土壤理化性好。油菜茬的土壤有效氮、磷养分高，是水稻、棉花、麦类及其它作物的优良前作物。由于油菜适应性强，又是新垦地、休闲地、盐碱地的一种良好的先锋作物。油菜生产过程中还有不少落花、落叶、根茬等，供给土壤大量的有机物和氮、磷、钾等营养元素。油菜的根系能分泌某些有机酸，溶解土壤中的难溶性磷素，供给作物吸收利用。

I. 1.8 对人类健康和生态环境是否发生过不利影响；

油菜栽培品种被称作“双低”品种，由它加工生产的油料芥子酸含量低，菜籽油粕的硫甙含量低。油菜不会对植物、动物或人类产生危害，美国食品和药物管理局在 1985 年 1 月 28 日将油菜归为“普遍安全”的作物。自从菜籽油和菜籽油粕加工的产品被使用以来，还没有关于引起不利影响的报道。

1. 1.9 从历史上看，受体植物演变为有害植物（如杂草等）的可能性；

在种植这种作物的国家，还从来没有关于油菜危害植物的报道。这是一种不具有竞争性和侵袭性的栽培作物。同其它栽培作物一样，油菜自生植物可以在下一年的作物轮种中发现，形成暂时性的群体。但是，油菜从来没有被认为是一种危害严重的杂草。这种植物在生长早期不和杂草竞争，因为它生长缓慢，需要花很长时间才能覆盖地表。还有，这种作物并不十分抗旱，与其它栽培作物相比，它对硫元素的营养需求最高。而且，进口的转基因原料只作为加工用原料。因此，这种植物在自然界中演变成有害植物的可能性非常小。

1. 1.10 是否有长期安全应用的记录。

油菜在全世界范围内的栽培已经具有 3000 多年的历史。油菜作物在加拿大的栽培已经进行了 20 多年，还没有关于对人类健康或环境产生不利影响的报导。菜籽油和饲料从来没有影响过食品/饲料的安全性。与其它食用油相比，菜籽油的临床和流行病学研究更加深入。这些研究集中于菜籽油对人类健康的影响，结果显示无芥子酸菜籽油对人类健康具有安全性。此外，

根据研究，美国食品和医药管理局在 1985 年将油菜列为普遍安全（Generally Recognised As Safe, GRAS）的农作物。油菜籽的副产物，菜籽油粕，被认为是一种高蛋白的动物营养。硫甙含量的减少和加工技术的优化提高了菜籽油粕的价值。根据营养含量，为每类动物都有提供最大用量水平的建议。详细情况可以查询www.canola-council.org

1.2 受体植物的生物学特性：

1.2.1 是一年生还是多年生；

油菜是一年生作物。

1.2.2 对人类及其它生物是否有毒，如有毒，应说明毒性存在的部位及其毒性的性质；

油菜对人类和其它生物均不具有毒性。

1.2.3 是否有致敏原，如有，应说明致敏存在的部位及其致敏的特性；

油菜不含有任何已知的致敏原。

1.2.4 繁殖方式是有性繁殖还是无性繁殖，如为有性繁殖，是自花授粉还是或是异花授粉或常异花授粉；是虫媒传粉还是风媒传粉；

油菜进行有性繁殖，通常通过 70% 自花授粉来繁殖。传粉方式既有虫媒传粉，又有风媒传粉。

1.2.5 在自然条件下与同种或近源种的异交率；

油菜的种内远交可以发生，距离花粉源 1 米的种内远交发生率报道为 2%。然后随距离加大呈指数下降。油菜和亲缘杂草种在农田条件下发生种间杂交的可能性也已经被报道，在最适条件下与下述 5 种植物可能发生种间杂交：白菜型油菜 (*Brassica rapa*)，芥菜型油菜 (*Brassica juncea*)，*Hirschfeldia incana*，萝卜型油菜 (*Raphanus raphanistrum*)，和 *Sinapis arvensis*，杂交可能性按顺序依次降低。自然条件下，基因流动到野生亲缘种的频率非常低。远交频率决定于几个因素：比如远交的方向，风的方向，与花粉源的距离，农田地形，授粉器的有无，花的颜色和性别相容性。进口的转基因原料不会释放到环境中，并且只被用作加工用原料。因此，在自然界发生远交的可能性很小。风险性评估的详细情况，参考www.defra.gov.uk/environment/acre/pgs/01.htm

1.2.6 育性 (可育还是不育, 育性高低, 如果不育, 应说明属何种不育类型);

F1 受体植物完全可育。由转基因 Ms1 系和 Rf2 系的 F1 代杂交系的种子 F2 发育而成的植株称为 F2 植株, 而 F2 再生植株经分离其中有约 10% 的雄性不育植株。这属于基因工程改造的细胞核雄性不育。而商品化生产在大田里所收获的是 F2 (子二代) 种子。更详细的情况参考本文的第 3 部分。

1.2.7 全生育期;

Ms1Rf2 为春油菜从种子到种子的全生育期是 90 到 110 天。

1.2.8 在自然界中生存繁殖的能力, 包括越冬性, 越夏性及抗逆性等;

如果是埋藏较深的情况, 油菜的种子在土壤中可以休眠保存几年。对土壤的耕种通常会终止其休眠状态。种子的生存能力受土壤条件的影响, 如温度和湿度。春油菜的抗旱性不佳。

1.3 受体植物的生态环境;

I.3.1 在国内的地理分布和自然环境;

油菜是我国主要油料作物之一, 分布极为广泛, 几乎遍及全国各地。北至黑龙江省大兴安岭地区, 南迄海南岛, 西起新疆维吾尔自治区克孜勒苏柯尔克孜自治州的乌恰县, 东抵沿海各省, 从平原到海拔 4630m 的西藏高原都有油菜栽培。由于我国幅员辽阔, 各地自然条件悬殊, 因而分布在全国范围内的油菜播种期和收获期也有很大差别。构成我国油菜品种和栽培制度、栽培技术的多样性、复杂性。从全国范围来说, 一年四季都可以看见油菜在田野生长, 自 3 月至 10 月均有油菜播种和收获。

我国冬油菜种植面积约占全国油菜面积的 90%, 分布在上海、江苏、浙江、安徽、北湖南、江西、福建、广东、广西、河南、山东、云南、贵州等省(市、区), 以及四川雅安地区以东, 陕西、甘肃、山西、河北等省南部地区, 辽宁省东南角, 新疆伊犁河套。此外, 西藏自治区局部河谷也有种植。以长江流域及其支流、太湖、鄱阳湖、洞庭湖冲积平原以及四周的低山丘陵地区最为集中, 其中安徽省种植面积最大, 四川省总产量最高。

我国春油菜种植面积约占全国油菜面积的 10%, 分布在西藏、青海、四川西部、甘肃六盘山、内蒙古阴山至大、小兴安岭、黑龙江北部以及新疆阿尔泰地区等高寒山地和寒冷地带。其中以青海省较为集中, 其发展速度也较快。

此外，我国还有春种夏收，夏种秋收的春夏种油菜。春夏复种油菜在我国种植的历史不长，零星分布在冬季温度低，夏季温度较高，热量条件较好，油菜既不能越冬，也不能过夏的中温带，如青海省东部、河西走廊、陇中、河套平原、山西省西北部等山间盆地、河套平原川水地带、辽宁全省、黑龙江省南部以及新疆准噶尔和塔里木盆地四周农区。华北平原及长江中下游江北一带也有少量地区利用早春低温进行春种夏收作为填闲或补灾而栽培。

春播夏收一般于临近解冻时播种生育期短的油菜品种，套种玉米或复种水稻、谷子、糜子、早熟玉米、油用向日葵、蔬菜等作物，全国曾一度达到数百万亩以上。由于油菜生育期短，生产技术条件要求较高，且产地春旱严重，近几年随着农作物布局的调整，种植面积显著缩小，但仍保持着一定面积。

夏种秋收油菜为的是充分利用秋季光、热、水资源，增收一季；或是春、夏复种一年收两季油菜；或是在青稞、小麦后复种。

1.3.2 生长发育所要求的生态环境条件，包括自然条件和栽培条件的改变对对其地理分布区域和范围影响的可能性；

尽管油菜在大多数土壤类型中都可以很好的生长，其最适土壤是没有严重硬化并妨碍种子发芽的壤土。在泥炭土和重质粘土中栽培的油菜可以获得较高的产量。在包括澳大利亚、中国、欧洲和美国在内的许多国家油菜都可以很好地生长。尽管土壤类型和天气条件在这些地区变化很大，基本的原则同样适用：油菜是冷季节作物，需要充足的水分才可以生长良好。中国的自然条件和栽培条件不会影响进口油菜的地理分布和范围。

1.3.3 是否为生态环境中的组成部分；

在油菜的农业种植区，如中国，油菜是生态环境的组成部分。

1.3.4 与生态环境中其它植物的生态关系，包括生态环境的改变对这种（些）关系的影响以及是否会因此而产生或增加对人类健康和生态环境的不利影响；

生态环境中能够与油菜相互作用的其它植物是一些野生的亲缘种。由于它们与杂草的竞争不利，油菜通常生长在杂草相对较少的农田里。杂草可以对油菜的产量和质量产生不利的影响。与油菜相比，杂草种子通常含油量较低，游离脂肪酸和叶绿素含量较高。许多杂草种子来源于十字花科，油的芥子酸和硫甙的含量很高。分级系统将油菜根据质量分为不同的等级。保证加工者能使大多数油菜籽符合可接受的质量标准。生态环境的变化能通过影响杂草的生长而影响到油菜的质量。由于油菜不具有侵袭性，并且

由于它是中国的一种主要栽培作物，进口的油菜不会对生态环境产生不利影响。详细情况参考网站 www.canola-council.org/。

1.3.5 与生态环境中其它生物（动物和微生物）的生态关系，包括生态环境的改变对这种（些）关系的影响以及是否会因此而产生或增加对人类健康和生态环境的不利影响；

由于进口的原料只用于加工，生态环境的改变不会影响油菜和其它生物（动物和微生物）的关系。

1.3.6 对生态环境的影响及其潜在的危险程度；

由于进口的原料只用于加工，不会对生态环境产生影响。

1.3.7 涉及到国内非通常种植的植物物种时，应该描述该植物的自然生境和有关其天然捕食者、寄生物、竞争物和共生物的资料；

油菜是中国最重要的经济油料作物之一。

1.4 受体植物的遗传变异：

1.4.1 遗传稳定性；

商品化油菜品种具有遗传稳定性。通过几代的回交保证了其性状的一致性。只有显示具有稳定性才可以登记到官方的品种目录。

1.4.2 是否有发生遗传变异而对人类健康或生态环境产生不利影响的资料；

没有资料显示遗传变异对人类健康或生态环境产生过不利影响。

1.4.3 在自然条件下与其它植物种属进行遗传物质交换的可能性；

在种植油菜的国家，存在通过异花授粉与其它油菜品种发生遗传物质交换或基因流动（见第 1.2.5），这可能会在某些需要高纯度遗传物质的特殊性状方面降低种子的质量。自然条件下，基因流动到野生亲缘种的几率很低，种间杂交适应性与亲本相比通常要小，遗传信息稳定渗入到杂草物种的基因组中的事件很难发生。而且，进口的转基因原料只用作加工材料。因此，自然界中发生遗传物质转移到其它物种的可能性非常小。更多详情请登陆 www.defra.gov.uk/environment/acre/pgs/01.htm。

1.4.4 在自然条件下与其它生物（例如微生物）进行遗传物质交换的可能性；

转 barnase 雄不育基因及其 barstar 育性恢复基因双低杂交油菜 Ms1Rf2 安全证书申请

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在非贫瘠土壤中，没有实验表明可以发生从植物到微生物的水平基因转移。对此转移机制的理解也不是很清楚。大部分已知细菌不能被自然转化，在正常条件下还没有发生转移的现象，也没有发现植物基因在细菌中的表达（Morrison, M., 1996）。而且，转基因原料只被用作加工用原料。此外，没有证据显示植物的完整基因可以被转移并表达在哺乳动物细胞中（Morrison, M., 1996）。这些与转化过程相关的细菌在自然条件下存在于多种土壤中，目前还没有观察到有某些物种基因的水平转移。

1.5 受体植物的监测方法和监控的可能性

在过去 50 年中收集的关于主要的谷类和油类作物中的杂草监测信息为监测油菜提供了有用的监测工具。例如，Saskatchewan 杂草监测系统用于监测转基因经济作物（MacDonald 和 Deschamps, 1998）。其它专家正在提出生态监测和指南，用来监测经济作物对环境的影响。例如，加拿大的生态监测和评估网络（Ecological Monitoring and Assessment Network, EMAN）（<http://eqb-dqe.cciw.ca/eman>）。然而，由于进口的转基因原料将只用于加工用原料，因此在自然界中繁殖的可能性非常小，并且由于应用的范围有限，没有必要进行农田监测活动。

1.6 受体植物的其它资料。

无。

1.7 根据上述评价，参照本办法第十一条有关标准划分受体植物的安全等级。

安全等级 I。

2 基因操作的安全性评价

安万特作物科学公司的杂交系统中，雄性不育系由 RNase (*barnase*) 在花发育过程的特定阶段，在花药特定细胞层的定向表达产生。携带 *barnase* 基因的雄性株系称为 Ms1 (原名 B91-4)。育性恢复通过雄性不育系 Ms1 和编码 *bBarstar* 蛋白的基因表达株系的杂交而获得，*barstar* 蛋白是 *barnase* 的特异性抑制剂。携带 *barstar* 基因的雌性恢复系称为 Rf2 (B94-2)。

为了能够进行早期的体外选择和后期的利用除草剂在大田中筛选，*barnase* 和 *barstar* 基因都与卡那霉素抗性基因 (neo) 和草氨膦抗性基因 (bar) 相连接。Rf2 因为所有关于 Ms1 的特性均与 Ms1Rf1 的申请材料所描述相同，这份报告材料只是提供一些关于新 Rf2 品系及其 Ms1 及 Rf2 的杂交品种额外的新信息。

2.1 转基因植物中引入或修饰性状和特性的描述。

- *neo* 基因，由弱组成型启动子 (P_{Nos}) 操纵，卡那霉素抗性提供了早期体外转化材料的选择。
- *bar* 基因，主要在绿色组织中表达，由 P_{SsuAra} 启动子操纵，在农田中有效利用对草氨膦除草剂 (Liberty[®]) 的抗性对选择转化材料。
- *barstar* 基因，由 P_{T29} 启动子操纵，对植株的表型没有影响。*barstar* 编码的蛋白是 Rnase 源的 *barnase* 基因的特异性抑制剂。当两者在同一植株表达时，*barstar* 干扰 *barnase*，可以防止诱导产生雄性不育，从而恢复植株的育性。

2.2 实际插入或删除序列的以下资料：

2.2.1 插入序列的大小和结构，确定其特性的分析方法；

杂交系统中雄性不育系 Ms1 和育性恢复系 Rf2 中插入序列的遗传基础在文献中已有详细的论述，其基本原理是聚合酶链式反应 (Polymerase Chain Reaction, PCR) 和 Southern blot 分析。

- 将基因单拷贝插入单基因座。
- 只有 T-DNA 端边序列之间的 T-DNA 能够被转化，并整合到植物基因组中。
- 转化体可以通过转基因的典型分子整合模式进行鉴定。
- 转化体可以根据其基因型进行鉴定；通过 Southern blot 和 PCR 技术可以对转基因株系进行准确的鉴定。用 PCR 方法可以进行常规质量检测和鉴定。关于这种技术的详细描述见附件 4。PCR 产物的典型分布附图见附图 1 和附图 2。

2.2.2 删减区域的大小和功能；

在植株的发育阶段，在不同地点对不同品种进行田间实验，比较 Ms1, Rf2, Ms1Rf2 杂交品种和野生型非转基因分离系的农艺性状。除了引入了雄性不育和草氨膦抗性之外，在整个植株发育过程中没有发现其它表型，包括产量、种子发芽率和传代发生变化。在植物形态学方面，除了雄性不育花药以外，其它方面也完全相似。此外，原种和加工后种子的成分分析也没有显示转基因植物和非转基因植物有任何不同。

2.2.3 目的基因的核苷酸序列和推导的氨基酸序列；

bar, 和 *barstar* 和 *neo* 基因的核苷酸序列和推导的氨基酸序列见附图 2，转 *barnase* 雄不育基因及其 *barstar* 育性恢复基因双低杂交油菜 Ms1Rf2 安全证书申请

3, 4。

2.2.4 插入序列在植物细胞中的定位（是否整合到染色体、叶绿体、线粒体，或以非整合形式存在）及其确定方法；

通过孟德尔遗传规律对不同代表现型及基因型的观察可以确定，转基因被插入到基因组中。

2.2.5 插入序列的拷贝数。

转基因都以单拷贝插入。

2.3. 目的基因与载体构建的图谱，载体的名称、来源、结构、特性和安全性包括载体是否有致病性以及是否可能演变为有致病性

pTVE74RE 质粒载体的设计(见附图 5)是用于获得 B. Napus 育性恢复系 Rf2。这质粒载体来源于土壤根瘤农杆菌 *Agrobacterium tumefaciens*, 其中引起植物根瘤病的致瘤基因被删除。将目的基因插入 25bp 的 T-DNA 端边序列。这些剔除了瘤基因的去毒载体没有致病的危险，只有左右端边序列之间的 DNA 序列才被整合到植物基因组中。这两个载体的序列特点已经完全清楚。

2.4. 载体中插入区域各片断的资料：

2.4.1 启动子和终止子的大小、功能及其供体生物是名称；

pTVE74RE 质粒在 T-DNA 端边重复序列之间含有 3 个嵌合基因。嵌合基因由启动转录的启动子序列，目的基因编码序列和来自 T-DNA 提供转录—终止信号和 PolyA 加尾功能的 3' 端非翻译区组成。

携带 *barstar* 嵌合基因的 pTVE74RE 质粒表示为 PTA29-*barstar*-3' nos。*barstar* 基因编码 barnase 抑制酶，称之为 *barstar*。

pTVE74RE

- 启动子： 花药组织特异基因 TA29，来自烟草 (*Nicotiana tabacum*)，其启动子区域包括 ATG 起始密码子上游序列。大小：1510bp。
- 编码区： 编码来自于解淀粉芽孢杆菌 *Bacillus amyloliquefaciens* 的成熟 *barstar* 蛋白，大小为 272bp。
- 终止子： 来自于解淀粉芽孢杆菌 *B. amyloliquefaciens* *barstar* 基因 3' 端非翻译区的序列，大小为 39bp。

2.4.2 标记基因和报告基因的大小、功能及其供体生物的名称；

pTVE74RE 质粒均携带有产生抗生素卡那霉素 kanamycin 抗性和除草剂草氨膦抗性的基因。这些基因分别表示为 PNos-neo-3'ocs 和 PSsuAra-tp-bar-3'g7。neo 基因编码新霉素磷酸转化酶 II neomycin phosphotransferaseII (APH(3')II) (NPTII)，可以产生对氨基糖苷 aminoglycoside 类抗生素如卡那霉素 kanamycin, geneticin(G418) 和新霉素 neomycin 的抗性。bar 基因编码 PPT 乙酰基转化酶 (PAT)，这种酶可以消除 PPT 的毒性，活化草氨膦除草剂的有效成分

PNos-neo-3'ocs

- 启动子： 土壤根瘤农杆菌 *Agrobacterium tumifaciens* pTiT37 的 T-DNA 中胭脂碱合成酶基因的启动子。大小：404bp。
- 编码区： neo 基因上编码新霉素磷酸化酶 II neomycin phosphotransferaseII 的编码区。该序列对应于大肠杆菌 *Escherichia coli* 的 Tn5 序列。大小：977bp
- 终止子： 胭脂碱合成酶基因的 3' 非翻译区 (3'ocs) 末端，来自土壤根瘤农杆菌 *Agrobacterium tumifaciens*。大小：875bp。

PSsuAra-tp-bar-3'g7

- 启动子： atS1A 核酮糖 -1,5- 磷酸 氢 盐 羧化 酶 ribulose-1,5-biphosphate carboxylase 小亚基基因的启动子，来自拟南芥 *Arabidopsis thaliana* (PSsuAra)。大小：1726bp。
- 编码区： 吸水链霉菌 *Streptomyces hygroscopicus* 的 bar 基因的编码区。大小：551bp。
- 终止子： TL-DNA 基因 7 的 3' 端加尾信号 (3' g7)，来自土壤农杆菌 *Agrobacterium tumifaciens*。大小：211bp。

2.4.3 其它表达调控序列的名称及其来源 (如人工合成或供体生物名称);

核基因编码参与叶绿体中的代谢过程多数蛋白。它们都在细胞质中合成，然后被输送到这些细胞器。这些蛋白必须要通过叶绿体膜才能达到基质空间。这些蛋白以较大的前体蛋白形式合成，其氨基末端为转运肽 (transit peptide, tp)。转运肽在通过叶绿体膜运输过程中被剪切掉。转运肽在将蛋白转移到叶绿体中的过程中是必需和足够的。如果删除哪怕一小段转运肽，都将阻止蛋白的转运。还有实验显示，如果将目的基因与植物基因的转运肽序列相融合时，嵌合基因的编码产物可以被导向叶绿体。PssuAra-tp-bar-3'g7 嵌合体中的转运肽核苷酸序列是从拟南芥 *Arabidopsis thaliana* 分离出来的一个大小 164bp 的片段，。

2.5 转基因方法。

产生油菜杂交系统的转基因方法源于土壤根瘤农杆菌 *Agrobacterium tumifaciens* 基因转化系统。该系统可以使位于 25bp 大小的 T-DNA 端边序列中间的功能基因在植物基因组中进行简单整合。De Block 等人在 *neo* 和 *bar* 选择标记基因的基础上已经建立了有效的转基因方法 (1989)。他们描述的这种方法已经用于将 pTTM8RE 和 pTVE74RE 中的 T-DNA 整合到野生型的油菜品种 Drakkar。

2.6 插入序列表达的资料:

2.6.1 插入序列表达的器官和组织, 如根、茎、叶、花、果、种子等;

barstar 基因特异表达在油菜转化体 Rf2 花粉囊胚周围的绒毡层细胞。详细的 Northern blot 分析表明, 在叶、种子和花粉中都没有侦测到该基因的 mRNA。

嵌合 *bar* 基因不会在除了绿色组织之外的器官中表达。*bar* 基因产物 (mRNA 或蛋白或蛋白活性) 可以在叶、绿色花蕾中检测到, 种子中也有痕量存在。*neo* 基因由弱组成型启动子操纵。其 mRNA 在各种组织中都检测不到存在, 只在叶组织中可以检测到 NPTII 活性阳性信号。

2.6.2 插入序列的表达量及其分析方法;

转入基因的表达情况用 Northern blot 分析定量检测 mRNA, 用 ELISA 方法定量检测 PAT 蛋白。PAT 和 NPTII 酶的活性分别用分光光度 PAT 检测法和放射性反应产物定量分析法进行测定, 测定的结果如下:

***bar* 基因:** 对于 Rf2 转化体, 叶中 *bar* 基因的 mRNA 的含量是 0.8 到 1.6pg/ μ g 总 RNA。我们也在 1, 2, 3mm 花蕾中检测到了 *bar* 基因的 mRNA(0.1 到 0.2pg/ μ g 总 RNA)。所有其它检测的组织和对照都是转化阴性。这些结果通过 ELISA 实验对不同组织中 PAT 蛋白的检测得到了佐证。痕量的 PAT 蛋白可以在种子中检测到, 其浓度很接近于检测灵敏度 (>0.001% 总抽提蛋白)。

***barstar* 基因:** 对于 Rf2 转化体, 我们在 2mm 花蕾中检测到了 *barstar* 基因的 mRNA 是介于 0.8 到 1.6pg/ μ g 总 RNA。所有其它组织和对照 mRNA 检测均为阴性

***Neo* 基因:** 其 mRNA 不能在任何组织中检测到, 只是在对 NPTII 活性进行检测时, 可以在叶组织中检测到阳性信号。

2.6.3 插入序列表达的稳定性;

- 嵌合基因对 Drakkar 基因组的稳定整合通过对雄性不育和育性恢复性状转 barnase 雄不育基因及其 *barstar* 育性恢复基因双低杂交油菜 Ms1Rf2 安全证书申请

的标准孟德尔遗传实验而验证。通过对 Rf2 转化体子代以及 Ms1Rf2 育性恢复杂交组合的分离模式的仔细测试，表明已经发生了稳定的物理整合。

- 对 40 多个不同品种的春油菜和冬油菜进行了复杂交和回交实验，收集了草氨膦除草剂的分离数据和花的分离数据。没有发现任何新性状包括表型和基因型的丢失。一旦整合入不同的遗传背景，嵌合基因都按照预测的方式表达。

- 为了回答雄性育性恢复系在不同环境条件下生长是否稳定的问题，设计了田间实验。结果显示，*neo*, *bar* 和 *barstar* 基因整合到育性恢复系及其 Ms1Rf2 的 B. napus 杂交组合的基因组中后，它们的表达在生长季节表现稳定。

2.7 根据上述评价，参照本办法第十二条有关标准划分基因操作的安全类型。 安全等级 1。

3 转基因植物的安全性评价

3.1 转基因植物的遗传稳定性

嵌合基因对 Drakkar 基因组的稳定整合通过对育性恢复性状的标准孟德尔遗传实验而验证。通过对 Rf2 转化体子代及其育性恢复的 Ms1Rf2 杂交组合的分离模式的仔细测试，表明已经发生了稳定的物理整合。

转基因的特殊分子整合模式通过对几代植株的 Southern blot 杂交分析检测，没有观察到与初始模式的偏离现象。

综合这些数据，可以肯定转基因植物具有遗传稳定性。

3.2 转基因植物与受体或亲本植物在环境安全性方面的差异：

3.2.1. 生殖方式和生殖率；

Ms1 植株只会在接受其它植株的育性花粉时才能繁育。通过杂交产生的子代只有一半具有 Ms1 等位基因并具有育性。另一半将具有非转基因育性。通过 Ms1 和 Rf2 的杂交，由于它们含有 *barstar* 基因，其子代 Ms1Rf2 的育性将恢复，因为 *barstar* 蛋白可以使 barnase 酶失活。*barstar* 基因的整合和表达不会使受体植物具有任何比野生型非转基因植物更好的选择优势。在过去 10 年的几处田间实验表明 Rf2 的繁殖率与非转基因野生型植株相当。如果把进口的 F2 种子种在田间，将产生大约 10% 的雄性不育系和 15% 的非转基因后代。

3.2.2. 传播方式和传播能力

自然界中的传播能力不会比任何其它常规的油菜品种高。油菜是一种一年
转 barnase 雄不育基因及其 *barstar* 育性恢复基因双低杂交油菜 Ms1Rf2 安全证书申请

生植物，自然界中的唯一传播方式是通过产生种子，它不能进行无性繁殖。

3.2.3 休眠期

发芽能力（检测休眠的关键参数）和转基因油菜种子发芽能力的进化与非转基因油菜没有不同。

3.2.4 适应性

转基因植株和非转基因植株在适应性和成长结实方面没有显示任何不同。

3.2.5 生存竞争能力

在不用草氨膦处理时，Rf2 及 Ms1Rf2 的生存竞争能力没有显示出和任何常规油菜品种的不同。

3.2.6 转基因植物的遗传物质向其它植物、动物和微生物发生转移的可能性；

在种植油菜的国家，通过和其它异花授粉的油菜植物而发生的遗传物质交换或基因流动存在（见 1.2.5），结果在需要遗传物质高纯度的某些性状方面降低了质量。在自然条件下，基因流动到野生亲缘种的几率很低，种间杂交的适应性通常比亲本小，而且在杂草中稳定的基因渗入现象很难发生。详情请登陆www.defra.gov.uk/environment/acre/pgs/01.htm。Ms1Rf2 中发生基因流动的可能性不比受体非转基因油菜高。雄性不育系更能够限制基因流动的发生。而且，进口的转基因原料只用作加工用原料。因此，在自然条件下，遗传信息转移到其它植物的可能性几乎不存在。

3.2.7 转变成杂草的可能性；

在种植农作物的国家，还没有关于油菜是一种危害严重的杂草的报道（见 1.1.9）。它是一种栽培作物，其特点是不具有竞争性和侵袭性。研究表明，Ms1Rf2 中的基因插入不会改变植株的性状（性状。种子的大小，发芽能力，再生能力，等），而使其易于演变为杂草。在过去的 10 年中，欧洲和加拿大启动了不同的研究项目，比较抗除草剂杂交系和常规油菜在建群和生根方面的能力。这些研究的结果肯定了以下几点：

1. 在农业群落（农田和农田隔离带，通过施加除草剂具有潜在的选择压力），抗除草剂（杂交）油菜的抗性不会持久：

- 抗除草剂（杂交）油菜不会比常规非转基因油菜产生更多的再生植物问题。
- 标准农业耕作规程提供了足够的转基因油菜/油菜再生植物的

控制手段。

2. 在自然群落中（缺少选择压力），抗除草剂（杂交）油菜不会变得更加具有侵袭性，因为在缺少通过施加除草剂产生的选择压力的情况下，抗除草剂（杂交）油菜没有选择优势。

进口的转基因油菜只用作加工用原料，Ms1Rf2在自然界演变为杂草的可能性极小。

3.2.8. 抗病虫转基因植物对靶标生物和非靶标生物的影响，包括对环境中有益和有害生物的影响

对于抗草氨膦（杂交）转基因油菜，没有靶标生物。

由安万特作物科学公司或独立的科学家进行的所有研究都表明，没有观察到抗除草剂（杂交）油菜对非靶标生物有任何影响。其理由有：

- 研究专家证实，从遗传修饰植物到细菌的水平基因转移，尽管在理论上难以完全排除，但实际上很难发生（见 1. 4.4）。即使发生从抗除草剂（杂交）油菜的基因转移，也不会产生影响，因为转移的基因不会提供选择优势。
- 安万特作物科学公司或独立研究者/合作者进行的所有研究都证实，转基因油菜对蜜蜂（吸引力，行为，传粉活动，死亡率，等）和地面生活的以油菜为食的节肢动物都不会产生影响。
- 对鸟类和哺乳类没有观察到有任何影响。

而且，进口的转基因原料将只作为加工用原料。因此，这种原料影响自然界中非靶标生物的可能性非常低。

3.2.9 对生态环境的其它有益或有害作用；

在开发 Ms1Rf2 抗除草剂杂交材料的过程中，已经考虑了下述几个方面的问题：

- 油菜在生化循环中并没有起到关键作用。
- 抗除草剂（杂交）油菜产生的 PAT 酶非常专一，唯一的酶解物是草氨膦有效成份 PPT。
- 对除草剂残留监测的田间实验和对 *Rhizobacterial flora* 的研究表明抗除草剂（杂交）油菜没有对其产生影响。
- 根据上述情况，安万特作物科学公司推断，抗除草剂（杂交）油菜在生态环境中不会产生有利或有害的影响。

3.3. 转基因植物与受体或亲本植物在对人类健康影响方面的差异：

转 barnase 雄不育基因及其 barstar 育性恢复基因双低杂交油菜 Ms1Rf2 安全证书申请

油菜不直接用于消费。菜籽油和油菜籽粕是其主要加工产品，分别可以用于人类食用和动物饲料。没有迹象表明插入的转基因会改变由转基因油菜加工的食品和饲料对人类健康的影响，也不会改变间接使用由转基因油菜加工的食物的安全性。

3.3.1. 毒性

在开发 Ms1Rf2 的过程中，已经考虑了下述几个方面的问题：

- 没有资料显示转入的新蛋白质 (PAT, barnase, barstar, or NPTII) 对人类有毒性。
- 没有理由怀疑转基因 Ms1 和 Rf2 及其后代种子的质量由于嵌合 *barnase* 和 *barstar* 基因的整合而改变。*barnase* 和 *barstar* 蛋白只在特定的组织中（花药的绒毡层）的特定时期（花粉发育）表达，因此暴露于人类的可能性很小。*bar* 基因只在油菜的绿色组织中表达。PAT 蛋白只在种子中痕量存在，其活性不会检测到。*neo* 基因受弱组成型启动子操纵，*neo* 基因的 mRNA 没有在转基因油菜中检测到。此外，转入的转基因不会在转基因的花粉中检测到，因此不会在来自转基因作物的蜂蜜中存在。
- 这些新蛋白是高度专一的酶类，不会和除专一底物之外的其它物质结合。
- 表达相关蛋白的植株部分的喂养实验显示，与用亲本原料喂养相比，不会产生任何负面影响。用抗除草剂杂交材料 Ms1Rf2 全种子喂养兔子的消化实验显示，没有产生毒性问题。
-

由于早在 1990 年就已经开发，没有科学数据报告显示抗除草剂（杂交）油菜对人类健康具有潜在的影响，尽管世界上的不同国家（包括在加拿大的销售）的种植者已经使用转基因油菜品种超过 5 年。转基因植物原料的安全性与亲本 Drakkar 和其它油菜品种的安全性完全相同。

3.3.2. 敏感性；

没有专门研究测试携带 *neo*, *bar*, *barnase* 和/或 *barstar* 基因的转基因油菜的食物过敏性。菜籽油是人类消费的唯一源自油菜的产品。尽管有许多关于食物引起过敏反应的报道，但是油类是个例外。由于转基因菜籽油的成分和质量与非转基因菜籽油没有不同，没有理由认为转基因菜籽油会产生过敏反应。此外，菜籽油不含有任何蛋白质，没有理由怀疑转入的新蛋白质存在于油中。

没有专门研究测试转基因油菜花粉的过敏性：

- 没有理由怀疑插入的基因在转基因油菜的花粉中存在，因为在转基因油菜的花粉中，我们没有检测到任何 *neo*, *bar*, *barnase* 和 *barstar* 基因 mRNA 的信号。
- 目前还没有关于插入蛋白过敏性的报道，例如，NPTII 酶已经被证实不会对已知的食物过敏原产生促进作用。

没有迹象显示 NPTII, PAT, barnase 和 barstar 蛋白可能会导致过敏反应：

- 利用计算机对 4 种转基因 DNA (PAT, NPTII, barnase 和 barstar) 序列进行了同源多肽的搜索。PAT, NPTII, barnase 和 barstar 多肽和 HIVAA7, PIR42 和 Swiss-Prot30 数据库中的多肽序列的同源性很低，因此很可能不会具有重要意义。
- 许多安万特作物科学公司员工和承包者天天在温室或田间接触转基因植物。尽管他们肯定吸入了较多量的转基因花粉，没有特别的证据显示油菜杂交系统会引起或改变专业或日常暴露者对油菜花粉过敏反应的强度。尽管所有的安万特作物科学公司员工每年接受体检，没有迹象显示过敏反应发生改变。

转基因植物原料的安全性同亲本 Drakkar 植物和其它油菜品种原料的安全性相同，

3.3.3. 抗营养因子

没有迹象显示转入的蛋白质具有抗营养作用，遗传修饰也没有改变天然油菜的抗营养因子。

3.3.4. 营养成分

根据环境和遗传条件的不同，油菜籽中菜籽油和蛋白质的数量变化很大。此外根据类似的条件，菜籽油脂肪酸的成分和种子中硫甙的含量也有明显变化。因此，转基因油菜籽油和蛋白质的含量，脂肪酸的成分和硫甙的含量与非转基因油菜及其它生长在不同环境条件下的经济油菜品种是相似的。

由安万特作物科学公司和一家专业实验室 (ECCA NV) 进行了常规的种子质量分析。转基因 (杂交) 油菜的所有成分 (油和蛋白质，脂肪酸构成谱和硫甙含量) 与非转基因品种也是相似的 (见表 1, 2, 3, 4, 5)。

雄性不育基因和育性恢复基因的插入没有改变种子中芥子酸和硫甙的含量，也没有因草氨膦的施加而改变种子的质量 (见表 6, 7, 8)。

类似的研究显示，由转基因油菜间接获得的产品 (如蜂蜜) 与由常规油菜得到的产品 (如蜂蜜) 没有不同。

3.3.5. 抗生素抗性;

消耗 NPTII 蛋白的影响的详细研究显示这种蛋白对动物和人类没有毒性。像大多数其它蛋白一样，NPTII 酶在消化道迅速被降解，而且，实验还显示食入这种酶不会影响氨基糖苷 aminoglycoside 类抗生素的使用效果 (Fuchs *et al.*, 1993 a&b)。这个细致的毒性实验通过 NPTII 酶对临床使用某些氨基糖苷 aminoglycoside 类抗生素效果的影响进行判断。而且，*neo* 基因的 mRNA 在所有的组织中都没有检测出 (种子、叶、花粉、花蕾)。基因转化到人类细胞的可能性非常小 (见 2.4.4)，并且即使发生这种情况，也没有影响。

3.3.6 对人体和食品安全性的影响;

自从 90 年代这种技术的早期开发以来，Ms1Rf2 原料的种植生产消费还没有发生对人类健康和食品安全的不利影响。至今，许多加拿大、欧洲和澳大利亚的种植者一直在种植含有转基因 Ms1 和/或 Rf2 的油菜品种，包括经济生产田。没有关于影响人类和食品安全的报道。

3.4. 根据上述评价，参照本办法第十二条有关标准划分基因操作的安全类型。

安全等级 1

4 转基因植物产品的安全性评价

4.1 生产加工活动对转基因植物安全性的影响;

由 POS 植物公司 (Pilot Plant Corporation) 对种子和转基因油菜/非转基因油菜加工油进行详细的分析评价。对含有雄性不育基因和育性恢复基因的转基因杂交油菜种子的加工功效工业规程进行并完成。转基因油菜和非转基因油菜在加工特点和成分 (如，油，蛋白质，维生素，矿物质) 方面没有显著的不同。(表 9, 10, 11)。

所有的分析均属于适用于油菜的典型分析。结果表明，转基因油菜种子可以在加工前同其它非转基因油菜种子一起进行收集。转基因油菜产品使用的数量和范围没有不同，也没有特别的说明。

4.2. 转基因植物产品的安全性

由于经济性食用油的质量要求不允许存在油提取纯化过程中的任何蛋白质，需要在油加工过程中通过一些步骤保证去除酶类物质。考虑到我们不能在转

基因油菜种子中检测到新引入的蛋白质，或只能检测到痕量 PAT 蛋白，并且由于油菜种子在工业加工过程中经受的极端条件（如，高温），在油菜籽中存在完整蛋白或酶活性的可能性几乎不存在。安万特作物科学公司对油菜种子按照仿效欧洲和加拿大工业规程的步骤提取的不同组分进行了研究，以便监测转基因序列和蛋白的存在。数据显示，在油菜籽饼中只能检测到痕量的 PAT 蛋白，在粗制油中没有检测到油菜籽粕萃取成分。转基因序列只能在油菜籽饼、萃取法制造的油菜籽粕和过滤残余物中检测到。核酸序列在精制过程中继续分解。油中的几种成分如脂肪酸或叶绿素可以影响油的稳定性。由于菜籽油没有由于基因改造而发生变化，从转基因油菜和传统非转基因油菜获得菜籽油没有稳定性方面的差别。

4.3. 转基因植物产品和转基因植物在环境安全性方面的差异；

如第 2 部分和第 3I 部分所述，转基因植物产品和转基因植物在环境安全性方面没有差异，它们对于环境都是安全的。转入的新蛋白的性质明确，功能特异，没有毒性或过敏反应。插入基因组中的序列不会改变植物的一般性状如农艺性状或种子的全部成分，包括营养成分和天然毒性成分。因此，由转基因 Ms1Rf2 种子加工获得产品与常规产品相比没有变化。

4.4 转基因植物产品和转基因植物在对人类健康影响方面的差异；

转基因油菜和油菜的部分，如粗原料，通常不直接用于人类食用，因此不会对人类产生直接影响。唯一的例外是种植者直接在田间暴露于转基因植物。然而，尽管 Ms1Rf2 油菜品种已经种植了大约 10 年，开始是田间实验后来是经济生产种植田，到目前为止还没有关于其不利影响的报导。只有由油菜加工得到的产品如菜籽油和菜籽油粕用于食品和饲料。这些产品最容易影响人类的健康。但是，转基因 Ms1Rf2 菜籽油和菜籽油粕同常规产品相比没有变化。在消费菜籽油和菜籽油粕的国家（如加拿大和日本），也没有关于其影响人类健康的报告。因此，转基因 Ms1Rf2 植物和产品对于人类健康的影响没有不同：两者都没有影响。

4.5 根据上述评价，参照本办法第十二条有关标准划分基因操作的安全类型。

安全等级 1.

5 参考文献

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六. 试验方案

一般的农艺性状的观察可以依据以下的栽培条件：

1. 甘蓝型油菜 (*Brassica napus* 品种) 在北半球种植时，在 4/5 月份播种，8/9 月份收获。从播种到收获的历期根据种植地区和种植品种的不同而变化。一般来说，春油菜需要 90 到 110 天的成熟期。
2. 油菜的播种密度通常为 5-7 千克/公顷。在田间实验中，种子用播种机播于土地中，土地经浅耕、松土和平整以形成种床。7 千克/公顷的播种密度大约是一行每米播种 26 粒种子。油菜种子很小，应浅播于结实而潮湿的土中。

但在考虑所进口作为加工的转基因农产品可能会在如内陆运输途中的撒落，机械的混杂或误当成种子使用而萌芽再生，于是可以规划为以下三种试验方案来测试所进口转基因农产品在上述万一的情况下被释放到环境中对生态环境的影响：

1. 生存竞争力试验
 - 在试验室中先测定转基因油菜之发芽率；
 - 将转基因农作物每隔二个月均匀撒播在一定面积的荒地上；
 - 调查该荒地原有物种的种类、分布、数量以及该转基因农作物的存活率及两者间的消长；
 - 并在隔年当天再调查是否有再生转基因农作物之存活以及与原物种间消长的关系。
2. 与近缘物种异交程序试验
 - 以条播方式，每隔一行转基因农作物种植一行花期相近的当地近缘栽培品种；
 - 分别收获这些近缘种的种子，干燥，保存；
 - 隔年再以条播的方式将以上所收获的种子分别播种；
 - 并在齐苗期后一星期喷施除草剂 glufosinate 400gr, ai/公顷，水量 300 公升/公顷。
3. 目标基因随花粉漂移的试验
 - 在小于 2500m²(50X50m) 的旱田里，在其中心位置以播种方式种植不小于 25m²(5X5) 的转基因油菜，并在其四周种植当地推广而且花期相近的非转基因油菜，而且以东南西北方向点标点 A、B、C、D；
 - 油菜完全成熟后分别在距中心点向东南西北方向每隔 10 公尺的位置收获 50 株非转基因油菜装入网袋并以 A1, A2, A3, A4, A5 加以标示，晒干脱粒保存；
 - 隔年当天可将以上所收获的种子以条播的方式分别播种；
 - 在齐苗期后一星期喷施除草剂 glufosinate 400gr, ai/公顷，水量 300 公升/公顷。

七 相关附件资料

附件 1:

关于甘蓝型油菜（BRASSICA NAPUS L.）生物学的一致性文件：经济合作及发展组织（OECD）环境健康和安全出版物，生物技术的行政监督协调系列文件，第 7 号，1997。这份文件描述了欧洲油菜的生物学，尤其详细描述了与转基因油菜品种的环境安全评价有关的方面。

附件 2:（机密）

载体 pTVE74RE 的描述：本资料对用于构建 Rf2 株系的载体中的所有遗传元件做了详细描述。

附件 3:（机密）

PCR 检测鉴定方法：基于 DNA 技术的方法可以通过检测特定的核酸序列对转基因植株进行检测和鉴定。检测和鉴定转基因植株的方法也包括在附件中。

OSR-RF@-02 (No PGS0009) 文件：B94-2 (RF2) 的 PCR 检测技术资料。

附件 4:

批准文件：这份附件包括了安万特作物科学公司从各种法律机构得到的转基因植株 Ms1Rf2 的商业运作批准文件的一份拷贝。

八 本单位农业转基因小组审查意见；

油菜是一种人们熟知的作物，几个世纪以来被用于食品和饲料。转基因油菜是一种“双低”品种，由此生产的油料其芥子酸含量和加工油菜粉中硫甙含量均低，从而提高了品质。转入杂交系统的遗传元件已了解得非常清楚，且有非常详细的文献报道。它们可以稳定地整合到基因组中。对于引入的蛋白质的特点也了解得非常清楚，它们具有非常专一的活性，并且可以稳定表达。引入的这些基因的产物不具有毒性。由 Ms1Rf2 加工的食品/饲料产品也没有毒性。转基因油菜种子及产品的成分完全和常规油菜种子及产品相似。转入的蛋白质在油料中检测不到，只有痕量的 PAT 蛋白在种子中可以检测到。Ms1Rf2 来源的株系或杂交品系和常规油菜具有同样重要的意义。

根据上述评估，可以得出结论，经雄性不育系 Ms1、育性恢复系 Rf2 及其杂交品系 ($\text{Ms1} \times \text{Rf2}$) 加工的食品和饲料产品与目前商品化的油菜产品实质上是等同的。此外，提供的数据显示，遗传背景（不仅局限于传统的杂交育种技术）或草氨膦的施用都不会影响产品质量的标准参数。并且没有资料显示食品/饲料的使用或成分的变化与引入的杂交系统相关。因此，建议这些产品进行商品化生产。

九 本单位审查意见

源自 Ms1、Rf2 或 Ms1Rf2 的 农业转基因油菜的安全等级均为一级。banase 蛋白、barstar 蛋白、PPT 乙酰基转化酶 (PAT)、NPTII 在油料产品中都不会检测到，只有痕量的 PAT 蛋白会在油菜原产品中可被检测到。在田间比较来自 Ms1 和 Rf2 或 Ms1Rf2 杂交的油菜与常规油菜品种的区别，发现除了农业转基因油菜抗除草剂草氨膦和具有雄性不育性状外，两者没有明显的差别。Ms1 和 Rf2 油菜与常规的油菜相比，在重要的营养成分方面，也基本相当，不必担心它对营养和健康有不利影响。

5. 安全性评价之附图及附表:

图 1.: 双低油菜 Rf2 基因 PCR 产物的典型分布:

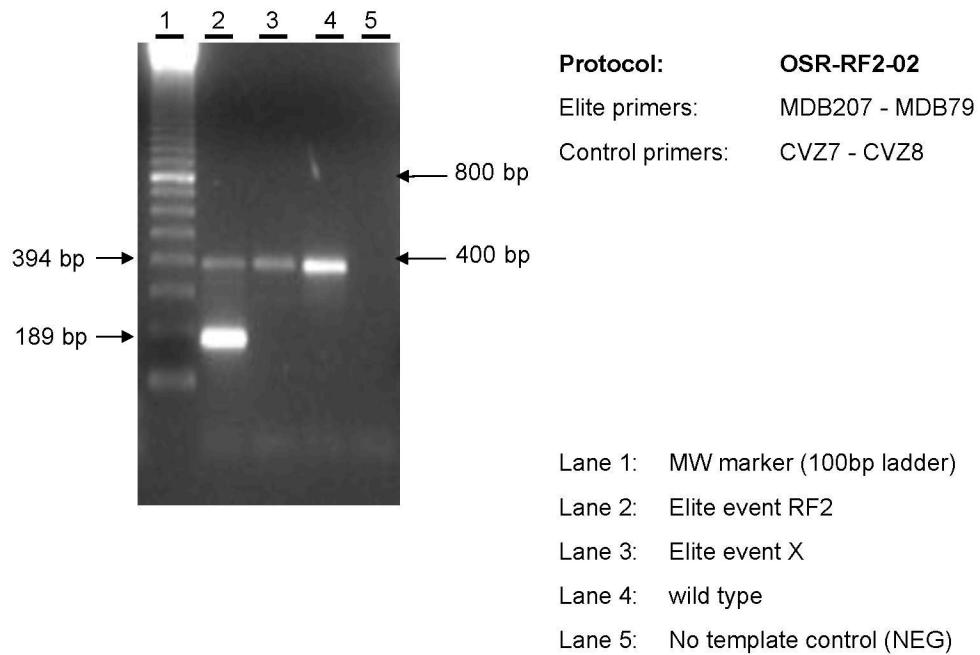


图 2: bar 基因序列

核苷酸序列:

1 atggacccag aacgacgccc ggccgacatc cgccgtgccca ccgaggcgga
catgccggcg
61 gtctgcacca tcgtcaacca ctacatcgag acaagcacgg tcaacttccg
taccgagccg
121 caggaaccgc aggagtggac ggacgacctc gtccgtctgc gggagcgcta
tccctggctc
181 gtcgccgagg tggacggcga ggtcgccggc atcgccctacg cggggccctg
gaaggcacgc
241 aacgcctacg actggacggc cgagtcgacc gtgtacgtct ccccccgcca
ccagcggacg
301 ggactgggct ccacgctcta cacccacctg ctgaagtccc tggaggcaca
gggcttcaag
361 agcgtggtcg ctgtcatcg gctgccaac gacccgagcg tgcgcatgca
cgaggcgctc
421 ggatatgccc cccgcggcat gctgccccg gccggcttca agcacggaa
ctggcatgac
481 gtgggtttct ggcagctgga cttcagcctg ccgttaccgc cccgtccgg
cctgcccgtc
541 accgagatct ga

氨基酸序列:

1 mdperrpadi rrateadmpa vctivnhyie tstvnfrtep qepqewtdl
vrlrerypw
61 vaevdgevag iayagpwkar naydwtaest vyvsprhqrt glgstlythl
lksleaqgf
121 svvaviglpn dpsvrnheal gyaprgmlra agfkhnwhd vgfqwldfs
pvpprpvlpv
181 tei

图 3: barnase 基因序列

核苷酸序列:

1 atgaaaaaaag cagtcattaa cggggaacaa atcagaagta tcagcgacct
ccaccagaca
61 ttgaaaaagg agcttgcct tccggaatac tacggtaaa acctggacgc
tttatggat
121 tgtctgaccg gatgggtgga gtacccgctc gttttggaat ggaggcagtt
tgaacaaagc
181 aagcagctga ctgaaaatgg cgccgagagt gtgcttcagg ttttccgtga
agcgaaagcg
241 gaaggctgca acatcaccat catacttct taa

氨基酸序列:

1 mkkavingeq irsisdlhqt lkkelalpey ygenldalwd cltgwveypl vlewrqfeqs
61 kqltengaes vlqvfreaka egcditiils

图 4: neo 基因序列

核苷酸序列:

1 atgatcatgt ggattgaaca agatggattg cacgcagggtt ctccggccgc
ttgggtggag
61 aggctattcg gctatgactg ggcacaacag acaatcggtc gctctgatgc
cggcgtgttc
121 cggctgttag cgcaaaaaaa cccggttttt tttgtcaaga ccgacctgtc
cggtgccctg
181 aatgaactgc aggacgaggc agcgccgtta tcgtggctgg ccacgacggg
cgttccctgc
241 gcagctgtgc tcgacgttgt cactgaagcg ggaaggact ggctgctatt
gggcgaagtg
301 ccggggcagg atctcctgtc atctcacctt gctcctgccc agaaagtatc
catcatggct
361 gatgaatgc ggcggctgca tacgcttgat ccggctacct gcccattcga
ccaccaagcg
421 aaacatcgca tcgagcgagc acgtactcgg atggaagccg gtcttgcga
tcaggatgat
481 ctggacgaag agcatcaggg gctcgccca gccgaactgt tcgcccaggct
caaggcgcc
541 atgcccgacg gcgaggatct cgtcgacc catggcgatg cctgctgcc
aatatcatg
601 gtggaaaatg gccgctttc tggattcatc gactgtggcc ggctgggtgt
ggcggaccgc
661 tatcaggaca tagcggtggc taccgtgat attgctgaag agcttggcgg
cgaatggct
721 gaccgcttcc tcgtgctta cggtatcgcc gctcccgatt cgcaagcgat cgccttcat
781 cgccttctt acgagttctt ctga

氨基酸序列:

1 mimwieqdgl hagspaawve rlfgydwaqq tigcsdaavf rlsaqgrpv

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fvktndlsgal
61 nelqdearl swlattgvpc aavldvtea grdwllgev pgqdllssh
apaekvsima
121 damrllhtld patcpfdhqa khrierartr meaglvdqdd Ideehqqlap
aelfarlkar
181 mpdgedlvvt hgdaclpnim vengrfsgfi dcgrlgvadr yqdialatrd
iaeelggewa
241 drflvlygia apdsqriafy rlldeff

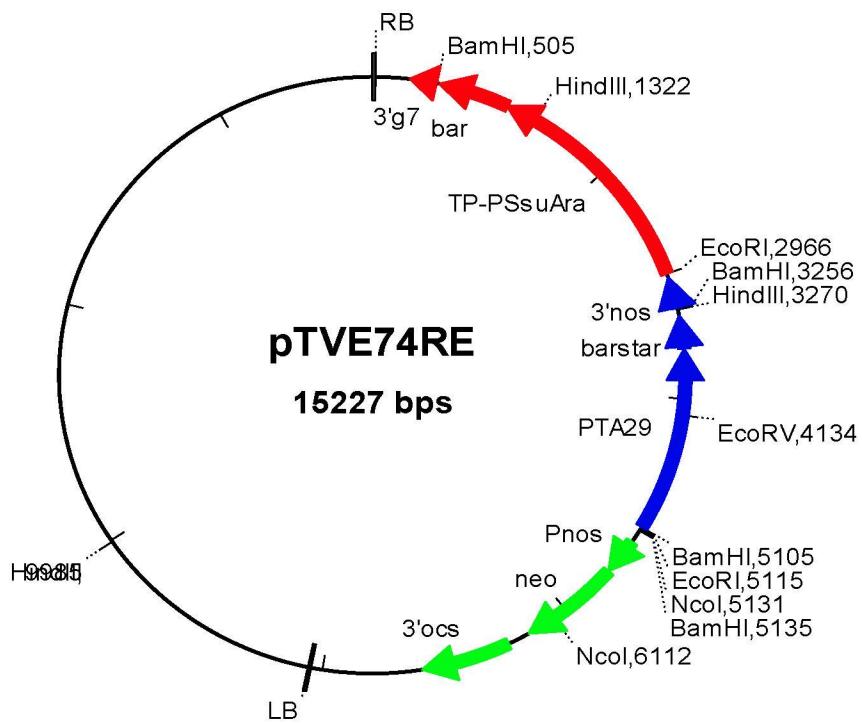


图 5: TVE74RE 质粒构建图

pTVE74RE 质粒
与 T-DNA 两端在重复序列相接的不同核苷酸的位置来源及其说明与参考文献

核苷酸的位置	方位	说明及文献
1-25		PTiB6S3 右端 TL-DNA 重复序列 (Gielen et al. (1984) The EMBO Journal 3: 835-846) .
26-283		在 TL-DNA 右端重复序列的残余序列。该结合点是位于 TL-DNA 的 HpaI 酶切点。
284-290		人工合成多接点序列
502-291	顺时针	一段 212EcoRV-ClaI 片段其中包括植物 poly(A) 信号以及获得从 pTiB6S3 质粒上的 TL-DNA 3' 端末翻译片段。(Velten and schell. (1985) Nucleic Acids Research 13: 6981-6998; Dhaese et al. (1983) The EMBO Journal 3: 835-846).
503-523		人工合成多接点序列
1075-524	顺时针	源自吸水链霉菌 (<i>Streptomyces hygroscopicus</i>) 的 bar 基因的编码序列 (Thompson et al. (1987) The EMBO Journal 6: 2519-2523)
2966-1076	顺时针	启动子源自拟兰芥位于 SIA (ribulose-1,5-biphosphate carboxylase) 小单位基因, 该启动子片段包含该 SIA ATG 密码子上游 1.7kb 的片段 (Krebbers et al., 1988 Plant Molecular Biology 11: 745-759) 以及这段过渡的多肽 (tp) 序列用来标定叶绿体。
2967-2995		人工合成多接点序列
3256-2996	顺时针	260 bp 的 TaqI 片段来自于 pTiT37 质粒 T-DNA 上 3' 端未翻译胭脂碱合成基因 (3' nos) 以及包含植物 poly(A) 信号。(Depicker et al. (1982) Journal of Molecular and Applied Genetics 1: 561-573).
3257-3276		人工合成多接点序列
3277-3316		解淀粉芽孢杆菌 <i>Bacillus amyloliquefaciens</i> 的 barnase 基因 3' 端未翻译的序列
3589-3317	顺时针	源自于 <i>Bacillus amyloliquefaciens</i> 编码成熟 barnase 区域 (Hartley (1988) Journal of Molecular Biology 202: 913-915)。
5100-3590	顺时针	烟草 (<i>Nicotiana tabacum</i>) 的花药特异表达基因 TA29 的启动子区域。该 PTA29 启动子包括了 ATG 启始密码子的上游 1.5kb 的序列。(Seurinck et al. (1990) Nucleic Acids Research 18: 3403) .
5101-5136		人工合成多接点序列
5137-5541	顺时针	土壤农杆菌 (<i>Agrobacterium tumefaciens</i>) (Pnos) pTiT37 质粒的 T-DNA 上的胭脂碱基因启动子 (Pnos); G 该 Pnos 启动子序列由 Depicker et al. (1982). 描述
5542-6519	顺时针	Neo 基因的编码新霉素磷酸转化酶 (Neomycin phosphotransferaseII) 的一段序列, 这个序列如 Beck et al. (1982) 所描述的相定于大肠杆菌的 Tn5 序列。它的 neo 编码区上的 ATG 启示区密码子已被结合序列所取代 (by Reiss et al. (1984)) 从 Tn5 的 neo 编码区下游序列有 171bp。
6520-7395	顺时针	章鱼碱合成基因 (3' ocs) 3' 端未翻译序列, 这相当于章鱼碱合成基因的 706 bp 的 PvuII 片段 (Gielen et al., 1984)。PvuII 片段已克隆在 Tn5 序列的 SmaI 位点, 在

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		3' ocs 的 Tn5 序列下游有一额外的 169bp 的片段。
7396-7399		人工合成多接点序列
7400-8089		TL-DNA 左端的重复序列的残余序列。接合点是在 TL-DNA 的前面 BgIII 位点上。
8090-8114		PTiB6S3 质粒的 TL-DNA 的左端重复序列 (Gielen et al. (1984) The EMBO Journal 3:835-846) .

表 1. 转基因油菜籽育性恢复系 RF1 和 RF2 及 MS1xRF1 及 MS1xRF2 组合与非转基因对照组合在不同区域不同世代、含油量 (%) 的最小和最大值

季节	含油量 (百分含量, %)				
	对照组	RF2	RF1	MS1xRF2	MS1xRF1
1993 (8 个区域)	39.3-49.0	38.8-48.7	38.4-48.6	37.5-49.2	38.2-47.4
1994 (5 个区域)	39.0-53.0	38.7-51.7	38.2-51.9	38.3-52.3	38.8-51.4

表 2. 转基因油菜籽育性恢复系 RF1 和 RF2 及 MS1xRF1 及 MS1xRF2 组合与非转基因对照组合在不同区域不同世代、蛋白质含量 (%) 的最小和最大值

季节	蛋白质含量 (在种子中的百分含量, %)				
	对照组	RF2	RF1	MS1xRF2	MS1xRF1
1993 (8 个区域)	23.6-28.9	24.0-28.0	24.0-28.0	24.0-28.0	24.0-28.0
1994 (6 个区域)	17.3-26.3	19.9-26.2	17.3-27.0	17.6-27.0	18.6-26.4

表 3. 对照组品种及育性恢复 PGS 杂交种 (含 MS1 雄性不育和 RF1 或 RF2 育性恢复构建基因) 在一系列加拿大环境下的硫甙含量的最小和最大值

项目	硫甙含量 ($\mu\text{mol/g}$ 无油菜籽粕)		
	对照品种	基于 MS1 和 RF1 的杂交种	基于 MS1 和 RF2 的杂交种

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1993 (5个区域)	10.5-35.4	10.4-25.8	16.7-25.5
1994 (2个区域)	5.6-25.0	16.7-24.8	8.2-27.2

表 4. 非转基因对照组 及对照品种， RF2， RF1，杂交组合：MS1xRf2 及 MS1xRF1， 以及一些育性恢复菜籽杂交种（含 MS1 雄性不育及 RF1 或 RF2 育性恢复构建基因（分别表示为 H1 和 H2）在所有试验区的两个不同年份中油菜籽的脂肪酸含量(占总量的百分数，%)最小和最大值

记录	油的组成 (占总量的百分数, %) : 极限值										
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C20:2	C22:0	C22:1
油菜标准值	3-6	<0.5	1-3	50-66	18-28	6-14	<0.5	1	traces	<0.5	<1
对照组	3.7-4.4	0.2-0.3	1.4-2.4	61.6-71.5	13.5-19.3	6.1-10.7	0.5-0.7	0.9-1.3	0.0-0.0	0.1-0.4	0.0-0.0
RF2	3.6-4.3	0.0-0.5	1.4-2.4	62.4-69.8	15.6-19.5	5.7-10.0	0.5-0.7	1.1-1.3	0.0-0.4	0.2-0.4	0.0-0.0
RF1	3.8-4.4	0.0-0.3	1.6-2.5	62.3-68.3	15.3-18.1	6.5-12.0	0.5-0.7	1.0-1.5	0.0-0.0	0.0-0.5	0.0-0.0
MS1x RF2	3.6-4.4	0.2-0.3	1.5-2.4	61.8-68.9	15.6-19.1	6.0-10.4	0.5-0.7	0.9-1.3	0.0-0.0	0.2-0.4	0.0-0.0
MS1xRF1	3.8-4.5	0.2-0.5	1.5-2.3	61.9-67.8	15.9-19.0	6.7-11.3	0.5-0.7	0.9-1.4	0.0-0.0	0.1-0.4	0.0-0.0
其它对照品种	3.6-4.9	0.2-0.4	1.2-2.0	54.3-67.8	16.7-24.1	6.3-13.1	0.0-0.8	0.0-2.6	0.0-0.0	0.0-0.5	0.0-1.6
H1	3.4-4.5	0.1-0.6	1.3-2.1	57.4-69.3	14.0-21.8	6.4-12.7	0.4-0.8	1.0-1.8	0.0-0.0	0.2-0.5	0.0-0.4
H2	3.4-4.4	0.2-0.6	1.3-2.1	57.5-69.7	14.3-21.6	5.9-12.2	0.4-0.8	1.0-2.3	0.0-0.0	0.1-0.5	0.0-0.7

表 5. 雄性不育系 MS1 油菜籽、育性恢复油菜籽系 RF2 和 RF1 及非转基因对照组在喷洒了不同浓度除草剂草氨膦后的种子质量分析。草氨膦的喷洒量按下列比例进行 (0 1/ha (喷洒液体量 = 500 l/公顷); 5 1/公顷 (1%); 20 1/公顷 (4%)。在杂交油菜籽计划范围内, 选择标准水平为 51/公顷.

草氨膦喷洒比例	项目	油 (%)	蛋白质 (%)	硫甙 (链烯基 + 吲哚) (μmol/g 种子)	芥子酸 (%)
01/ha	对照组	39.76	25.39	25.74	0.00
	MS1	39.40	25.43	26.44	0.00
	RF1	40.36	25.48	24.95	0.00
	Rf2	39.08	25.50	25.74	0.00
51/ha	对照组	40.26	25.20	25.71	0.00
	MS1	40.15	25.05	26.54	0.00
	RF1	38.90	25.93	26.03	0.00
	Rf2	39.85	25.24	25.85	0.00
201/ha	对照组	39.20	25.07	24.92	0.00
	MS1	39.64	25.29	25.97	0.00
	RF1	38.68	25.91	25.91	0.00
	Rf2	38.69	25.55	25.82	0.00

表 6. 工艺流程分析 (POS 加拿大): 进行的所有分析均在油菜的典型范围之内; 物料平衡和亏损在实验室操作的正常范围之内。

工艺流程	被分析的部分参数	MS1 x RF2
纯种子	水分, % 含油量, % (无改变)	7.26 37.69
除去溶剂后的油菜粕	挥发性物质, % 含油量, % 维生素 E, ppm (无改变) 元素分析, ppm 硫 磷 钼 锌 铜 锰 铁 镁 钙 钾 钴 硒	6.94 0.25 0.69 10,140 13,710 < 0.10 83.70 6.49 74.46 140.5 6,411 7,479 12,680 < 0.02 < 0.50
调合粗油	过氧化值, meq/kg 游离脂肪酸, % 颜色, 1" Lovibond(洛维邦德) 叶绿素, ppm 磷, ppm	0.53 0.47 70+Y7.0R 12.5 424.9
酸性脱胶油	游离脂肪酸, % 磷, ppm	0.31 14.28
精制油	皂, ppm	17.1
水洗油	过氧化值, meq/kg 游离脂肪酸, % 皂, ppm 颜色, 1" Lovibond(洛维邦德) 叶绿素, ppm 磷, ppm	3.47 0.03 7.3 70+Y6.8R 4.81 < 0.2

工艺流程	被分析的部分参数	MS1 x RF2
漂白油	过氧化值, meq/kg	0.34
	游离脂肪酸, %	0.03
	皂, ppm	0.0
	颜色, 1" Lovibond(洛维邦德)	8.6Y0.7R
	叶绿素, ppm	0.00
	磷, ppm	< 0.2

Series on Harmonization of Regulatory Oversight in Biotechnology No.7

**CONSENSUS DOCUMENT ON THE BIOLOGY OF BRASSICA NAPUS L.
(OILSEED RAPE)**

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris

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OECD Environmental Health and Safety Publications

Series on Harmonization of Regulatory Oversight of Biotechnology

No. 7

**Consensus Document on the Biology of *Brassica napus* L.
(Oilseed Rape)**

Environment Directorate
Organisation for Economic Co-operation and Development
Paris 1997

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 29 industrialised countries in North America, Europe and the Pacific, as well as the European Commission, meet to co-ordinate and harmonize policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialized Committees and subsidiary groups composed of Member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's Workshops and other meetings. Committees and subsidiary groups are served by the OECD Secretariat, located in Paris, France, which is organised into Directorates and Divisions.

The Environmental Health and Safety Division publishes complimentary documents in six different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides; Risk Management; Harmonization of Regulatory Oversight in Biotechnology; and Chemical Accidents.** More information about the Environmental Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (see below).

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or contact:

**OECD Environment Directorate,
Environmental Health and Safety Division**

**2 rue André-Pascal
75775 Paris Cedex 16
France**

Fax: (33-1) 45 24 16 75

E-mail: ehscont@oecd.org

FOREWORD

The OECD's Expert Group on Harmonization of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on the development of *consensus documents* that are mutually acceptable among Member countries. These consensus documents contain information for use during the regulatory assessment of a particular product. In the area of plant biosafety, consensus documents are being initiated on the biology of certain crop plants and on selected traits.

This document, which was prepared by Canada as lead country, addresses the biology of the crop plant *Brassica napus* L. (oilseed rape). The OECD's Working Group for Environmental Biosafety of Transgenic Plants reviewed the format of the document at a meeting in Washington, D.C. in October 1995. The document was forwarded to National Co-ordinators for technical comments in January 1996, and subsequently revised.

As part of a joint project with the United Nations Industrial Development Organization (UNIDO) on centres of origin of diversity, this document was reviewed by experts in several countries in central and eastern Europe, northern Africa, and Asia. Relevant comments submitted by these experts have been incorporated.

The Joint Meeting of the Chemicals Group and Management Committee of the Special Programme on the Control of Chemicals has recommended that this document be made available to the public. It is published on the authority of the Secretary-General of the OECD.

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Preamble

OECD Member countries are moving rapidly towards the commercialization and marketing of agricultural and industrial products of modern biotechnology. They have therefore identified the need for harmonization of regulatory approaches to the assessment of these products, in order to avoid unnecessary trade barriers.

In 1993, **Commercialization of Agricultural Products Derived through Modern Biotechnology** was instituted as a joint project of the OECD's Environment Policy Committee and Committee on Agriculture. The objective of this project is to assist countries in their regulatory oversight of agricultural products derived through modern biotechnology – specifically in their efforts to ensure safety, to make oversight policies more transparent and efficient, and to facilitate trade. The project is focused on the review of national policies, with respect to regulatory oversight, that will affect the movement of these products into the marketplace.

The first step in this project was to carry out a survey concentrating on national policies with regard to the regulatory oversight of these products. Data requirements for products produced through modern biotechnology, and mechanisms for data assessment, were also surveyed. The were published in *Commercialisation of Agricultural Products Derived through Modern Biotechnology: Survey Results* (OECD, 1995).

Subsequently, an OECD Workshop was held in June 1994 in Washington, D.C. with the aims of improving awareness and understanding of the various systems of regulatory oversight developed for agricultural products of biotechnology; identifying similarities and differences in various approaches; and identifying the most appropriate role for the OECD in further work towards harmonization of these approaches. Approximately 80 experts in the areas of environmental biosafety, food safety and varietal seed certification, representing 16 Member countries, eight non-Member countries, the European Commission and several international organisations, participated in the Workshop. The *Report of the OECD Workshop on the Commercialisation of Agricultural Products Derived through Modern Biotechnology* was published by the OECD in 1995.

As a next step towards harmonization, the Expert Group on Harmonization of Regulatory Oversight in Biotechnology instituted the development of *consensus documents* that are *mutually acceptable* among Member countries. The purpose of these documents is to identify common elements in the safety assessment of a new plant variety developed through modern biotechnology, in order to encourage information sharing and prevent duplication of effort among countries. These common elements fall into two general categories: the biology of the host species, or crop; and the gene product. This consensus document on the biology of oilseed rape (*Brassica napus* L.) is one of the first in a planned series of such documents.

In reviewing this document, and the biology of other plants, two OECD publications will prove particularly useful. *Traditional Crop Breeding Practices: An Historical Review to Serve as a Baseline for Assessing the Role of Modern Biotechnology* provides information about 17 different crop plants. It includes sections on phytosanitary considerations in the movement of germplasm and on current end uses of the crop plants. There is also a detailed section on current breeding practices. *Safety Considerations for*

Biotechnology: Scale-Up of Crop Plants provides a background on plant breeding, discusses scale dependency effects, and identifies various safety issues related to the release of plants with "novel traits".¹

The safety issues identified in the consensus documents on crop specific biologies are intended to address the potential for gene transfer within the crop plant species, and among related species, and the potential for weediness. They make no attempt to be definitive in this respect, however, as the many different environments in which the crop species may be grown are not considered individually.

This document is a "snapshot" of current information that may be relevant in a regulatory risk assessment. It is intended to be useful not only to regulatory officials as a general guide and reference source, but also to industry and others carrying out research.

In order to ensure that scientific and technical developments are taken into account, Member countries have agreed that these consensus documents will be updated regularly. Additional areas relevant to the subject of each consensus document will be considered at the time of updating.

Users of this document are therefore invited to provide the OECD with new scientific and technical information, and to make proposals for additional areas to be considered. *There is a short, pre-addressed questionnaire for that purpose at the end of this document. The completed questionnaire (or a photocopy) should be returned to the Environmental Health and Safety Division at the address shown.*

¹ For more information concerning these two publications, contact the OECD's Publications Service, 2 rue André-Pascal, 75775 Paris, Cedex 16, France. Fax: (33) (01) 49 10 42 76. Internet: Compte. PUBSINQ@ oecd.org.)

Section I – General Information

This consensus document addresses the biology of the species *Brassica napus* L. Included are general descriptions of this species as a crop plant, its origin as a species, its reproductive biology, its centres of origin, and its general ecology. The ecology of this species is not described in relation to specific geographic regions. Special emphasis has been placed on detailing potential hybridization between *B. napus* and its close relatives, although this discussion is limited to hybridization events which do not require intervention through means such as embryo rescue (i.e. these events could possibly occur in nature, and could result in fertile offspring).

This document was prepared by a lead country, Canada. It is based on material developed in OECD Member countries – for example, for risk assessments or for presentation at conferences and scientific meetings. It is intended for use by regulatory authorities and others who have responsibility for assessments of transgenic plants proposed for commercialization, and by those who are actively involved in these plants' design and development.

The table in the Appendix showing potential interactions of *B. napus* with other life forms during its life cycle was developed **with respect to Canada**. As such, it is intended to serve as an example. Member countries are encouraged to develop tables showing interacting organisms specific to their own geographic regions and environments.

Section II – General Description and Use as a Crop

Brassica napus L. is a member of the subtribe *Brassicinae* of the tribe *Brassicaceae* of the Cruciferous (Brassicaceae) family, sometimes referred to as the mustard family. The name "cruciferous" comes from the shape of its flowers, which have four diagonally opposite petals in the form of a cross. The dark bluish green foliage of *B. napus* is glaucous, smooth or has a few scattered hairs near the margins, and is partially clasping. The stems are well branched, although the degree of branching depends on variety and environmental conditions; branches originate in the axils of the highest leaves on the stem, and each terminates in an inflorescence. The inflorescence is an elongated raceme; the flowers are yellow, clustered at the top but not higher than the terminal buds, and open upwards from the base of the raceme (Musil, 1950).

There are two types of *B. napus*: 1) oil-yielding oleiferous rape, of which one subset with specific quality characteristics is often referred to as "canola" (vernacular name), and 2) the tuber-bearing swede or rutabaga. This document is written for oil-yielding oleiferous rape. The oleiferous type can also be subdivided into spring and winter forms. Sanskrit writings of 2000 to 1500 BC directly refer to oleiferous *B. napus* forms (sarson types) and mustard. Greek, Roman and Chinese writings of 500 to 200 BC refer to rapiferous forms of *B. rapa* (Downey and Röbbelen, 1989). In Europe, domestication is believed to have occurred in the early Middle Ages. Commercial plantings of rapeseed are recorded in the Netherlands as early as the 16th century. At that time rapeseed oil was used primarily as an oil for lamps. Later it came to be used as a lubricant in steam engines.

Although used widely as an edible oil in Asia, only through breeding for improved oil quality, and the development of improved processing techniques, has rapeseed oil become important in western countries. Since the Second World War, rapeseed production in Europe and Canada has increased dramatically as a result of improved oil and meal quality. Modern techniques of plant transformation and genotype identification using isozymes, restriction fragment length polymorphism (RFLP) markers, or random amplified polymorphic DNA (RAPDs) markers will complement classical breeding for the production of other improved lines (Buzzo, 1995). China, India, Europe and Canada are now the top producers, although this crop can be successfully grown in the United States, South America and Australia, where annual production has increased sharply over the last few years.

Today, two species of *Brassica* have commercialized varieties with "double low" characteristics, i.e. low erucic acid content in the fatty acid profile and very low glucosinolate content in the meal, characteristics desirable for high-quality vegetable oil and high-quality animal feed. In North America these species (*B. napus* and *B. rapa*) are considered to be of "canola" quality. *B. napus* is grown as a winter annual in regions where winter conditions do not result in very low temperatures, which would kill the plants. These biotypes typically require vernalisation before the onset of stem elongation, raceme development, flowering and seed set. In North America and northern parts of Europe, a spring biotype of *B. napus* that requires no vernalisation prior to flowering is grown. These biotypes are typically lower yielding than the winter annual types, but require considerably less time to complete their life cycle.

Section III – Agronomic Practices for Oleiferous *B. napus*

The spring-type oleiferous *B. napus*, a cool season crop, is not very drought tolerant. It is widely adapted and performs well under a range of soil conditions, provided that moisture and fertility levels are adequate. Air and soil temperatures influence plant growth and productivity. The optimum temperature for maximal growth and development of spring-type oilseed rape is just over 20°C, and it is best grown between 12°C and 30°C. After emergence, seedlings prefer relatively cool temperatures up to flowering; high temperatures at flowering will hasten the plant's development, reducing the time from flowering to maturity. Among cultivated crop plants, *Brassica* species show the highest nutritional demand for sulphur.

Due to increased awareness of soil conservation issues, minimal or no-till *B. napus* production is advised, in which most of the crop residue and stubble are left on the soil surface to trap snow, reduce snow melt run-off, reduce wind and water erosion of the soil, and increase soil water storage. Reduced tillage techniques, however, are only effective when combined with a good systematic weed control programme. Winter oilseed rape covers the soil for ten to eleven months. It has high nutritional demands in autumn and reduces soil erosion in winter.

Weeds can be one of the most limiting parameters in rapeseed production. The closely related cruciferous weeds, for example wild mustard (*Sinapis arvensis*), stinkweed (*Thlaspi arvense*), shepherd's purse (*Capsella bursa-pastoris*), ball mustard (*Neslia paniculata*), flixweed (*Descurainia sophia*), wormseed mustard (*Erysimum cheiranthoides*), hare's ear mustard (*Coriaria orientalis*), common peppergrass (*Lepidium densifolium*), etc., are often problematic. Spring-type oilseed rape does not compete well with weeds in the early growth stages, as it is slow-growing and slow to cover the ground. Weeds must be controlled early to avoid yield loss due to competition. Although rapeseed crops can be attacked by a number of insect pests, insect control must be carefully designed to reduce unnecessary and costly pesticide applications, the chances of resistance build-up in insects, and damage to honeybees and native pollinating insects. Diseases can be severe in large production areas, and are greatly influenced by cultivation practices and environmental factors, so that disease management programmes are advisable (refer to the table in the Appendix for examples of *B. napus* pests and diseases in Canada).

When the first siliques begin to shatter, *B. napus* can be cut just below the level of the seed pods and swathed. The use of dessicants allows a reduction of shattering, and possibly allows direct combining.

Generally, oilseed rape should not be grown on the same field more often than once every three to four years in order to prevent the build-up of diseases, insects and weeds. Chemical residues from herbicides and volunteer growth from previous crops (including rapeseed crops grown for different oil types) are also important factors to consider when selecting sites, although suitable soil treatments following harvest may considerably reduce the volunteer problem.

Section IV – Centres of Origin/Diversity²

A. Geographic origin of *B. napus*

The origins of *B. napus* (an amphidiploid with chromosome n=19) are obscure, but were initially proposed to involve natural interspecific hybridization between the two diploid species *B. oleracea* (n = 9) and *B. rapa* (syn. *campestris*)³ (n = 10) (U, 1935). Recent evidence (Song and Osborn, 1992), through analyses of chloroplast and mitochondrial DNA, suggests that *B. montana* (n = 9) might be closely related to the prototype that gave rise to both cytoplasms of *B. rapa* and *B. oleracea*. It also suggests that *B. napus* has multiple origins, and that most cultivated forms of *B. napus* were derived from a cross in which a closely related ancestral species of *B. rapa* and *B. oleracea* was the maternal donor. In Europe, it is predominantly the winter form which has become a common yellow crucifer found along roadsides, on waste sites and cultivated ground, on docks, in cities and towns, on tips, and on arable fields and along riverbanks. In the British Isles, it has been naturalised wherever oilseed rape is grown. It is a relatively recent introduction into Canada and the United States, and is described as an occasional weed, escape or volunteer in cultivated fields (Munz, 1968, and Muenscher, 1980). It is found typically in crops, fields and gardens, along roadsides, and on waste sites.

B. Geographic origin of *B. oleracea*

The wild form of *B. oleracea*, a suffrutescent (low, shrubby plant with woody lower parts of stems and herbaceous upper parts) perennial, grows along the coast of the Mediterranean from Greece through to the Atlantic coasts of Spain and France, around the coast of England, and to a limited extent in Helgoland (Snogerup et al., 1990). Typically the wild type is found on limestone and chalk cliffs in situations protected from grazing. Individuals are often found below cliffs in scree, where they grow among other shrubs, and some populations are found on steep grassy slopes. In Helgoland, populations are found on open rocky ground. In Europe and North America, domesticated types have been reported as escapes but do not form self-sustaining populations outside cultivation. *B. oleracea* is a recent introduction into North America.

C. Geographic origin of *B. rapa*

Wild *B. rapa* (subspecies *sylvestris* L.) is regarded as the species from which the ssp. *rapa* (cultivated turnip) and *oleifera* (turnip rape) originated. It is native throughout Europe, Russia, central Asia and the Near East (Prakash and Hinata, 1980), with Europe proposed as one centre of origin. There is some debate as to whether the Asian and Near Eastern types arose from an independent centre of origin in

² This section draws heavily on discussions with, and a review paper prepared by, Dr S.I. Warwick and A. Francis (1994), Centre for Land and Biological Resources Research, Agriculture and Agri-Food Canada.

³ First described as two species by Linnaeus, with *B. rapa* being the turnip form and *B. campestris* the oleiferous form. Metzger in 1933 concluded that these were the same species and chose the name *B. rapa* (Toxeopus et al., 1984).

Afghanistan and then moved eastward as *B. rapa* became domesticated. Prakash and Hinata (1980) suggest that oleiferous *B. rapa* subspecies developed in two places, giving rise to two different races, one European and the other Asian.

Typically, *B. rapa* is found in coastal lowlands, high montane areas (the slopes of high valleys or mountain ranges), and alpine and high sierras. In Canada, where it is a recent introduction, it is found on disturbed land, typically in crops, fields and gardens, along roadsides, and on waste sites (Warwick and Francis, 1994).

D. Geographic origin of *B. montana*

B. montana, possibly a progenitor species of *B. napus* (see above) and also a suffrutescent perennial, originates in the Mediterranean coastal area between Spain and Northern Italy (Snogerup et al., 1990). It is found typically on or below limestone cliffs and rocks, walls, etc., often on disturbed ground. Although usually found in coastal areas and on rocky islets, it has been recorded at an elevation of 1000 m somewhat inland of the coast.

Section V – Reproductive Biology

Under field conditions the fertilization of ovules usually results from self-pollination, although outcrossing rates of 5-30 per cent have been reported (Hühn and Rakow, 1979, and Rakow and Woods, 1987). The pollen, which is heavy and sticky, can be transferred from plant to plant through physical contact between neighbouring plants and by wind and insects. Oilseed rape pollen has been detected in the air above rape fields (Williams, 1984) and beyond the borders of a rape crop (Olsson, 1955); however, the concentration decreases rapidly with increasing distance from the source of the pollen and windborne pollen may make no or only a negligible contribution to long-distance pollination of oilseed rape (Mesquida and Renard, 1982, and McCartney and Lacey, 1991). Timmons et al. (1995), using pollen traps and “bait” plants whose petals had been removed and which had been emasculated, reported airborne pollen at distances up to 2.5 km from commercial plantings of *B. napus*. The “bait” plants also produced some seed at this distance from the commercial oilseed rape, suggesting the airborne pollen might be capable of successful fertilization events.

Pollinating insects, in particular honeybees (*Apis mellifera*) and bumblebees (*Bombus* sp.), play a major role in *B. napus* pollination and are believed to be involved in the transfer of pollen over long distances. Oilseed rape is very attractive to bees because it produces large quantities of nectar and pollen. Williams et al. (1987) reported that “plants in plots caged with bees had their flowers pollinated faster, shed petals sooner, finished flowering earlier and were shorter than plants caged without bees.” *B. napus* pollen is a major food source for bees, and hives are often placed near rapeseed fields during flowering to take advantage of the honey production potential (Marquard and Walker, 1995).

When beehives were placed at the centre of each side of a 1 ha square of non-transgenic *B. napus* plants with a 9 m circle of transgenic plants at the centre, Scheffler et al. (1993) reported outcrossing ranging from 1.5 per cent at 1 m to 0.00033 per cent at 47 m. In a later study using 20 x 20 m plots of transgenic and non-transgenic plants, separated by distances of 200 and 400 m, the space separating the plots being either bare ground or planted with barley (*Hordeum vulgare*), Scheffler et al. (1995) reported the average frequency of hybridization to be 0.0156 per cent at 200 m and 0.0038 per cent at 400 m.

The dynamics of bee-mediated pollen movement depend on the quantity of pollen available (size and density of donor population) and the size and location of the receiving populations, as well as on environmental conditions and insect activity (Levin and Kerster, 1969, Ellstrand et al., 1989, and Klinger et al., 1992). These studies, together with the findings of Scheffler et al. (1993 and 1995), suggest that surrounding an experimental plot of *B. napus* with other plants of the same species flowering synchronously with the experimental plants could decrease the long-distance dispersal of pollen from experimental plants by insects.

Section VI – Cultivated *B. napus* as a Volunteer Weed

As with all crops cultivated and harvested at the field scale, some seed may escape harvesting and remain in the soil until the following season, when it germinates either before or following the seeding of the succeeding crop. In some instances the volunteers may give considerable competition to the seeded crop and cause deterioration in the quality of the crop harvest. In such instances, chemical and/or mechanical control is essential.

The problem of volunteer plants in succeeding crops is common to most field crop species. Much depends on the management practices used in the production of the crop, for example whether the plants have disbursed seed at the time of harvest, the setting of the harvesting equipment, and the speed of the harvesting operation, which will determine whether more or less seed is lost by the harvester. With crops of the *Brassica* family, because of the small seed size and large number of seeds produced by the crop, poor management practices can result in severe volunteer problems in succeeding crops. Suitable soil treatment after the harvest can considerably reduce the volunteer problem.

Section VII – Crosses

A. Inter-species/-genus

In considering potential environmental impact following the unconfined release of genetically modified *B. napus*, it is important to have an understanding of the potential for the development of hybrids through interspecific and intergeneric crosses between the crop and its related species. The development of such hybrids could result in the introgression of the novel traits into these related species, and result in:

- the related species becoming weedy or more invasive of natural ecosystems;
- altered environmental interactions, potentially causing harm to the environment or to human health and safety.

While many interspecific and intergeneric crosses have been made between *B. napus* and its relatives (Prakash and Hinata, 1980, Warwick and Black, 1993, and Scheffler and Dale, 1994), many have necessitated intervention in the form of ovary culture, ovule culture, embryo rescue and protoplast fusion. Reported in **Table 1**, and ranked in order of relative ability to form hybrid progeny when crossed with *B. napus*, are instances reported by Scheffler and Dale (1994) of sexually obtained interspecific and intergeneric crosses with *B. mapus*.

Flowering periods of *B. napus* and these species are critical. For interhybridization events to occur, their flowering periods, which are largely environmentally influenced, must overlap at least partially. To evaluate hybridization potential, it is important to know the flowering chronology of both the cultivated plant and related species; the physical distance between potentially hybridizing species; occurrence of vectors for pollination; and how pollination takes place.

The chromosome numbers of the cultivated species and relatives are also important. Many hybrids fail to occur due to lack of development of the endosperm (tissue resulting from the fertilization of the two polar nuclei of the embryo sac by a male reproductive nucleus). The ratio of maternal and paternal chromosomes must be of 2:1 or higher (Nishiyama and Inomata, 1966). This explains why the direction of crossing is often important. The pollination of a tetraploid female parent by a diploid male usually produces seeds. The reciprocal cross, on the other hand, is sterile. In order to understand existing exceptions, Johnston et al. (1980) proposed the concept of the endosperm balance number (EBN), where the value attributed to a given species is not linked to its chromosome number but to an arbitrary value determined from a successful cross and from the hypothesis that the EBN ratio is 2:1 in the endosperm.

Table 1 Sexually obtained interspecific and intergeneric crosses with *B. napus* (reported by Scheffler and Dale, 1994)

Cross female x male	Progeny	References
<i>B. rapa</i> x <i>B. napus</i>	SH, F1, F2, BcP	Morinaga, 1929 U and Nagamatu, 1933 U, 1935 Bing et al., 1991 Jørgensen and Andersen, 1994 Mikkelsen et al., 1996
<i>B. napus</i> x <i>B. rapa</i>	SH, F1, F2, BcP	Morinaga, 1929 U and Nagamatu, 1933 U, 1935 Bing et al., 1991 Jørgensen and Andersen, 1994 Mikkelsen et al., 1996
<i>B. juncea</i> x <i>B. napus</i>	SH, F1, F2, BcP	Morinaga, 1934 Roy, 1980 Bing et al., 1991 Fernandez-Serrano et al., 1991 Frelo et al., 1995
<i>B. napus</i> x <i>B. juncea</i>	SH, F1, F2, BcP	Morinaga, 1934 Roy, 1980 Bing et al., 1991 Fernandez-Serrano et al., 1991 Frelo et al., 1995
<i>B. oleracea</i> x <i>B. napus</i> <i>B. napus</i> x <i>B. oleracea</i>	F1 F1, F2, BcP	U, 1935 Roemer, 1935 Röbbelen, 1966
<i>B. carinata</i> x <i>B. napus</i>	F1, F2, BcP	Yamagishi and Takayanagi, 1982 Roy, 1980 Fernandez-Escobar et al., 1988
<i>B. napus</i> x <i>B. carinata</i>	F1, F2, BcP	Fernandez-Serrano et al., 1991 U, 1935 Roy, 1980 Fernandez-Escobar et al., 1988
<i>B. nigra</i> x <i>B. napus</i> <i>B. napus</i> x <i>B. nigra</i>	SH, F1, BcP SH, F1, F2, BcP	Fernandez-Serrano et al., 1991 Bing et al., 1991 Heyn, 1977 Bing et al., 1991
<i>B. napus</i> x <i>Hirschfeldia incana</i>	SH, SH(BnMS), F1, BcP	Lefol et al., 1991 Chevre et al., 1992 Eber et al., 1994

(continued on next page)

Table 1 (continued)

Cross female x male	Progeny	References
<i>B. napus</i> x <i>Raphanus raphanistrum</i>	SH, SH(BnMS), F1, BcP	Chevre et al., 1992 Lefol et al., <i>in press</i> Eber et al., 1994
<i>Diplotaxis erucoides</i> x <i>B. napus</i>	F1, BcP	Ringdahl et al., 1987
<i>D. muralis</i> x <i>B. napus</i>	F1, BcP	Ringdahl et al., 1987
<i>B. napus</i> x <i>Erucastrum gallicum</i> *	F1, BcP	Lefol et al., <i>in press</i>
<i>B. napus</i> x <i>Sinapis alba</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>S. arvensis</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>B. fruticulosa</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>B. tournefortii</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>D. tenuifolia</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>Eruca sativa</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>R. rugosum</i>	F1	Heyn, 1977
<i>B. napus</i> x <i>R. sativus</i>	F1	McNaughton and Ross, 1978

Note:

SH = spontaneous hybrids formed without the aid of emasculation and manual pollination transfer;

SH(BnMS) = spontaneous hybrids with male sterile *B. napus* as female parent;

F1 = F1 hybrids produced through intervention of some sort, i.e. emasculation and manual pollination;

F2 = F2 hybrids produced;

BcP = backcross progeny produced.

* This hybridization event not reported by Scheffler and Dale (1994)

Generally, crosses between two species are possible only if the female species has a polyploidy level at least as high as the pollinating male species. Since *B. napus* is tetraploid, it will cross more readily with wild species (diploid) as a female parent (Sikka, 1940, Harberd and McArthur, 1980, and Kerlan et al., 1991). In the case of *Raphanus raphanistrum*, no difference was noted in the direction of crosses (Kerlan et al., 1991); in the case of *Sinapis alba*, the opposite situation occurs (Ripley and Arnison, 1990).

For a trait to become incorporated into a species genome, recurrent backcrossing of plants of that species by the hybrid intermediaries, and survival and fertility of the resulting offspring, will be necessary.

B. Introgession into relatives

The potential hybridization events listed are intended to assist the assessment of the potential for introgression of "novel traits" introduced from cultivated *B. napus* into wild relatives. The first step in this assessment is to determine which, if any, of the potential "mates" of *B. napus* are recorded as present in the geographic region where the cultivation is proposed. Should there be potential wild relative "mates" present, the frequency of hybridization events and the potential for environmental impact should introgression occur would then be considered. Should a trait with positive selective value be introgressed

into wild or weedy populations, the gene may become a permanent part of the gene pool of these populations.

The above listed species are all plants of "disturbed land" habitats. Their success will be dependent on their ability to compete for space with other primary colonizers, particularly other successful weedy plant types. This in turn will depend on how well suited they are to the particular climate, soil conditions, etc. of individual sites. Equal ability of the hybrids to compete among wild populations or in cultivated fields has been shown for *B. napus* and hybrids (Lefol et al., 1995).

C. Interactions with other organisms

The table in the Appendix is intended as an identification guide for categories of organisms which interact with *B. napus*. This table, **representative of Canada**, is intended to serve as an example only. Environmental safety assessors should, on a country-by-country basis, draw up their own lists as a guide for assessing potential effects of the release of genetically modified plants on interacting organisms in their country.

Section VIII – Ecology

B. napus and its progenitors grow in "disturbed land" habitats. In non-managed ecosystems these species may be considered "primary colonizers," i.e. plant species that are the first to take advantage of the disturbed land, where they compete for space with plants of similar types. Unless the habitats are disturbed on a regular basis, for example along the edges of cliffs, rivers, and pathways, populations of these types of plants will be displaced by intermediaries and finally by plants that form climax ecologies, such as perennial grasses on prairies and tree species and perennial shrubs in forests.

In non-natural ecosystems, including along roadsides and on industrial and waste sites as well as cropland, there is potential, because of their "primary colonizing" nature, for ever-present populations of these species to be maintained. It is in such habitats that the species are recorded among the flora of countries where *B. napus* has been introduced as a crop plant. Their success will depend on their ability to compete for space with other primary colonizers, in particular successful weedy types. This, in turn, will depend on how well suited they are to the particular climate, soil conditions, etc. of individual sites.

In crop production systems, poor management practices and insufficient resistance to pod shattering may result in large amounts of *B. napus* seed not being harvested. Especially where there are high crop densities, this may cause volunteer "weed" problems in succeeding crops as well as contamination of such crops with respect to their seed quality.

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Appendix: Potential interactions of *B. napus* with other life forms during its life cycle (Canada)

X indicates the type of interaction between the listed organisms and *B. napus*

Other life forms	Interaction with <i>B. napus</i>			
	Pathogen	Symbiont or beneficial organism	Consumer	Gene transfer
<i>Albugo candida</i>	X			
<i>Alternaria</i> spp.	X			
<i>Botrytis cinerea</i>	X			
<i>Erysiphe</i> spp.	X			
<i>Leptosphaeria maculans</i>	X			
<i>Peronospora parasitica</i>	X			
<i>Plasmiodiophora brassicae</i>	X			
<i>Pseudocercosporella capsellae</i>	X			
<i>Pseudomonas</i> sp.	X			
<i>Pyrenopeziza brassicae</i>	X			
<i>Pythium debaryanum</i>	X			
<i>Rhizoctonia solani</i>	X			
<i>Sclerotinia sclerotiorum</i>	X			
<i>Xanthomonas</i> spp.	X			
<i>Verticillium dahliae</i>	X			
Mychorrhizal fungi		X		
Aster yellow mycoplasma	X			
Cauliflower Mosaic Virus (CaMV)	X			
Beet Western Yellow Virus (BWYV)	X			
Turnip mosaic virus	X			
Soil microbes		X		
Earthworms		X		
Flea beetle			X	
Pollinators		X	X	
Soil insects			X	
Animal browsers (e.g. deer, hare, rabbit)			X	
Birds			X	
Other <i>Brassica napus</i>				X
<i>Brassica rapa</i>				X
<i>Brassica juncea</i>				X
<i>Brassica nigra</i>				X
<i>Raphanus raphanistrum</i>				X
<i>Erucastrum gallicum</i>				X
Others				X

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Title

Description of vector pTTM8RE and pTVE74RE

Author
J. Boterman

Converted to new format on
July 14, 1998

Testing Facility
Plant Genetic Systems NV
Jozef Plateaustraat 22
B-9000 Gent
Belgium

Report ID
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APPROVALS PAGE

Author

Dr J. Botterman

(signature)

July 16 1993

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COMPONENTS OF THE AGROBACTERIUM VECTOR SYSTEM

For plant transformation, the vector system as described by Deblaere et al. (1985) has been used. The plasmids pTTM8RE and pTVE74RE are derived from the octopine Ti-plasmid-derived vector system for gene transfer to plants cells (Deblaere et al., 1985). This vector system consists of two plasmid components : a non-oncogenic Ti-plasmid and an intermediate vector.

The acceptor Ti-plasmid from which the T-region has been deleted is non-oncogenic. This plasmid still carries the *vir* genes required for transfer of T-DNA to the plant genome and is used as an acceptor plasmid for an intermediate vector, carrying the genes of interest between the 25bp border sequences of the octopine T-region. The respective plasmid components are the non-oncogenic Ti-plasmid pGV2260 and the intermediate vector pGV825. Plasmid pGV2260 was derived from the octopine Ti-plasmid pTiB6S3 from which the T-region was deleted and substituted by sequences from the plasmid pBR322 (Deblaere et al., 1985).

The *Agrobacterium tumefaciens* host strain is a rifampicin (Rif) resistant derivative of C58, cured for pTiC58 (C58C1Rif^R) (Van Larebeke et al., 1974). The plasmid pGV825 is derived from pBR322 and is composed of the origin of replication of pBR322 and genes conferring resistance to ampicillin (Ap) and streptomycin/spectinomycin (Sm/Sp) for selection in bacteria (Deblaere et al., 1985). The T-region of pGV825 consists of the DNA regions surrounding the left and right border sequences of the TL-DNA from pTiB6S3 (Deblaere et al., 1985).

Between the T-DNA border repeats, there are still residual sequences left from the TL-DNA: 265 bp at the right border and 689 bp at the left border. The region at the right border is part of the octopine synthase gene which is localized immediately at the right border sequence of the TL-DNA (De Greve et al., 1982). This sequence comprises the octopine synthase upstream activator sequence, but does not contain the TATA box (Leisner et al., 1988; Leisner et al., 1989; Bouchez et al., 1989). The sequence at the left border is part of gene 5 localized immediately at the left border repeat of the TL-DNA (Gielen et al., 1984). A major part of the gene 5 promoter including the putative regulatory boxes such as CCAAT and TATA box are still present. Expression pattern analysis of chimeric gene constructs with this promoter are described by Koncz et al. (1986). Genes under the control of this promoter were expressed in callus tissues and in stems of transformed plants and at barely detectable levels in fully developed leaves. Promoter analysis of gene 5 in plants revealed that its expression was inducible by auxin and confined to the vascular phloem cells. Cis-regulatory elements required for auxin regulation and phloem specific expression of gene 5 were mapped to a 90 bp promoter region (Körber et al., 1991).

Vectors derived from pGV825 contain multilinker cloning sites allowing the insertion of chimeric genes between the T-DNA border repeats (Deblaere et al., 1987). These intermediate plasmid vectors carrying chimeric genes of interest, can be introduced into the acceptor Ti-plasmid pGV2260 by a single homologous recombination, using the Sm/Sp resistance gene as selectable marker for cointegration. The mobilization is based on a triparental mating between an *Escherichia coli* strain carrying the intermediate vector derived from pGV825, the *Agrobacterium* strain C58C1Rif^R (pGV2260) and an *E. coli* strain carrying a mobilization helper plasmid (Van Haute et al., 1983; Deblaere et al., 1987). The resulting *Agrobacterium* strain contains a pGV2260::pGV825 cointegrate plasmid. The structure of the resulting T-region is confirmed by Southern blot hybridization (Deblaere et al., 1985).

For transformation of *Brassica napus*, *Agrobacterium* strains without an ampicillin resistance (Ap^R) phenotype were desired (De Block et al, 1987). For this reason the Ap^R gene carried by pGV2260 was inactivated by insertion of the kanamycin resistance (Km^R) gene from *Tn*903 in the coding region of the Ap^R gene. The *Tn*903-derived Km^R gene was isolated from plasmid pBS8 (Spratt et al, 1986) and inserted into the FspI site of the Ap^R gene of a pGV825-derived vector, yielding pGSV100. This Km^R and streptomycin/spectinomycin resistant (Sm/Sp^R) plasmid

pGSV100 was mobilized to *Agrobacterium* strain C58C1Rif^R(pGV2260) and transconjugants resistant Km, but sensitive to Ap and Sm/Sp were selected for. These recombinants resulted from a double homologous recombination event, whereby the Km^R gene was introduced into pGV2260 and thus knocking out the Ap^R phenotype. The resulting Ti-plasmid was called pGV3000 and is used as an acceptor Ti-plasmid in the same way as pGV2260, except that it now confers resistance to Km instead of Ap. The plasmid pGSV100 and the strain C58C1Rif^R (pGV3000) were used as basis for the design of the T-DNA vectors.

Description of the plasmids pTTM8RE and pTVE74RE

The plasmids pTTM8RE and pTVE74RE comprise three chimeric genes between the T-DNA border repeats. The chimeric genes consist of a promoter sequence for initiation of transcription, the coding sequence of the gene of interest and a fragment containing a 3' untranslated region from a T-DNA gene providing the signals for transcription - termination and polyadenylation. A schematic representation of the intermediate vector pTTM8RE and pTVE74RE is given in Figure 1 and 2. The nucleotide sequence of the DNA fragment comprised between the T-DNA border repeats is completely known. Both plasmids carry chimeric genes conferring resistance to the antibiotic kanamycin and to the herbicide glufosinate-ammonium, respectively. These chimeric genes are denoted as PNos-*neo*-3'ocs and PSsuAra-tp-*bar*-3'g7. Additionally, pTTM8RE carries a chimeric *barnase* gene construct denoted as PTA29-*barnase*-3'nos and pTVE74RE carries a chimeric *barstar* gene construct denoted as PTA29-*barstar*-3'nos. The following description gives an overview of the design of the different gene constructs in order to allow to identify all nucleotide sequences present in the transferred T-DNAs of the plasmid vectors pTTM8RE and pTVE74RE.

The chimeric *bar* gene consists of the promoter from the atS1A ribulose-1,5-biphosphate carboxylase small subunit gene (*ssu*) from *Arabidopsis thaliana*, the *bar* coding sequence and the 3' untranslated fragment of the TL-DNA gene 7. The PSsuAra promoter fragment comprises the 1.7 kb fragment upstream of the atS1A ATG codon and the transit peptide encoding sequence (Krebbers et al., 1988). The *bar* gene contains the complete coding sequence of the *bar* gene as described by Thompson et al. (1987). The 3'g7 fragment is derived from the TL-DNA gene 7 (Velten et al., 1985; Dhaese et al., 1983). The construction of the chimeric PSsuAra-tp-*bar*-3'g7 gene has been described by De Almeida et al. (1989) and can be summarized as follows. The plasmid pATS3 (Krebbers et al., 1988) contains a 1.7 kb EcoRI-SphI fragment which includes the promoter region and the transit peptide encoding sequence of the atS1A *ssu* gene. The plasmid pGSFR2 is derived from pGSFR1 (De Block et al., 1987) and carries the *bar* coding sequence in which an Ncol site has been created at the initiation codon. Accordingly, the second codon of the *bar* gene - AGC (Ser) - has been modified to a GAC (Asp) codon (Botterman et al., 1991). The tp-*bar* fusion was obtained by ligating the filled-in Ncol ends to the SphI ends treated with Klenow DNA polymerase. This yielded a fusion of the *bar* coding sequence at the transit peptide encoding sequence with the same Cys-Met transit peptide cleavage site as present in the wild-type atS1A *ssu* gene. The PSsuAra-tp-*bar* gene was cloned in the polylinker region of pLK56-2 (Botterman et al., 1987) and could be retrieved as a BamHI fragment. In order to have the chimeric gene completed with a 3' untranslated end providing signals for transcription termination and polyadenylation, the BamHI PSsuAra-tp-*bar* fragment was cloned in the BamHI site localized immediately in front of a 3' untranslated end of the TL-DNA gene 7. This yielded the chimeric PSsuAra-tp-*bar*-3'g7 gene construct.

The chimeric *neo* gene consists of the promoter from the T-DNA nopaline synthase gene (PNos), the coding region of the *neo* gene encoding neomycin phosphotransferase II from *Tn*5 and the 3' untranslated end from the octopine synthase gene (3'ocs). The nucleotide sequence of the PNos promoter is described by Depicker et al. (1982). The *Tn*5 sequence comprises the *neo* coding sequences and a part of the *Tn*5 sequence downstream from the *neo* coding region. This sequence corresponds with the sequence as described by Beck et al. (1982). The ATG initiation codon of the *neo* coding region has been substituted for a linker sequence as described by Reiss et al. (1984). The 3' end from the octopine synthase gene corresponds to the 706 bp

PvuII fragment from the octopine synthase gene which has been cloned into sequences originating from *Tn5* downstream from the *neo* coding region. The nucleotide sequence of the PvuII fragment can be found in Gielen et al. (1984). The construction of the chimeric PNos-*neo*-3'ocs gene has been described by Hain et al. (1985).

The plasmid pTTM8RE also contains a chimeric *barnase* construct. The latter consists of a promoter fragment from the tobacco anther-specific gene TA 29 (PTA29), the coding region of the *barnase* gene (*barnase*) and the 3' untranslated end of the nopaline synthase gene (3'nos). The cloning and characterization of the TA29 genomic clone from tobacco has been described by Koltunow et al. (1990) and Seurinck et al. (1990) and is also in detail described within the European Patent Application 89401194.9. A 2.5 kb Clal-Accl fragment carrying the promoter and part of the TA29 coding region was cloned in the polylinker Accl site of pMAC 5-8 (Stanssens et al., 1989). The sequence surrounding the ATG initiation codon - AAAATGGTA - was modified to ACCATGGTA by substituting two A residues for C residues by site directed mutagenesis according to Stanssens et al. (1989). The resulting plasmid was named pMB3 and the 1507 bp Clal-Ncol fragment was denoted as the PTA29 promoter fragment. Subsequently, the PTA29 coding region in pMB3 was deleted and substituted for a 900 bp Asp718-HindIII fragment. The latter comprises the *barnase* coding region as described in Hartley (1988), with an Asp718 site engineered at the start of the coding sequence of the mature *barnase*. The fragment was cloned between Ncol and HindIII sites of PMB3. Before cloning, Asp718 and Ncol sites were rendered blunt end by treatment with Klenow DNA polymerase. This yielded the plasmid pTM8 with a PTA29-*barnase* gene fusion carrying the *barnase* coding region fused at the ATG initiation codon of the TA29 gene.

Subsequently, the EcoRI-XbaI fragment from pTM8 was isolated and ligated to a XbaI-EcoRI fragment from pNOS2. The latter plasmid carries a restriction fragment with the 3'untranslated end of nopaline synthase gene (Depicker et al., 1982) cloned in the polylinker of pUC18 (Yanish-Perron, 1985). Both fragments simultaneously cloned within an EcoRI site of a plasmid vector allows to retrieve the PTA29-*barnase*-3'nos fragment as an EcoRI fragment.

The plasmid pTVE74RE contains a chimeric *barstar* gene construct, which consists of a promoter fragment from a tobacco anther specific TA29 (PTA29) promoter, the coding region of the *barstar* gene (*barstar*) and the 3' untranslated end of the nopaline synthase (3'nos). The design of the PTA29 promoter as a 1.5 kb Clal-Ncol fragment has been described above. In order to fuse the *barstar* coding sequence at the PTA29 promoter fragment, a Clal site was introduced at the ATG initiation codon of the *barstar* coding region (Hartley, 1988). Upon digest with Clal, treatment with Klenow DNA polymerase and digestion with HindIII, a fragment carrying the *barstar* coding region was obtained. This fragment was ligated in pMB3 digested with Ncol and HindIII. The Ncol site was also treated with S1 nuclease in order to have a single ATG codon for translation initiation. This yielded a plasmid with the *barstar* coding region fused to the PTA29 promoter region. From the latter plasmid, an EcoRV-XbaI fragment comprising the PTA29-*barstar* gene was isolated and substituted for the EcoRV-XbaI fragment in a vector carrying the PTA29-*barnase*-3'nos fragment. This yielded a plasmid vector carrying the PTA29-*barstar*-3'nos chimeric gene.

A. MAP OF THE VECTOR pTTM8RE

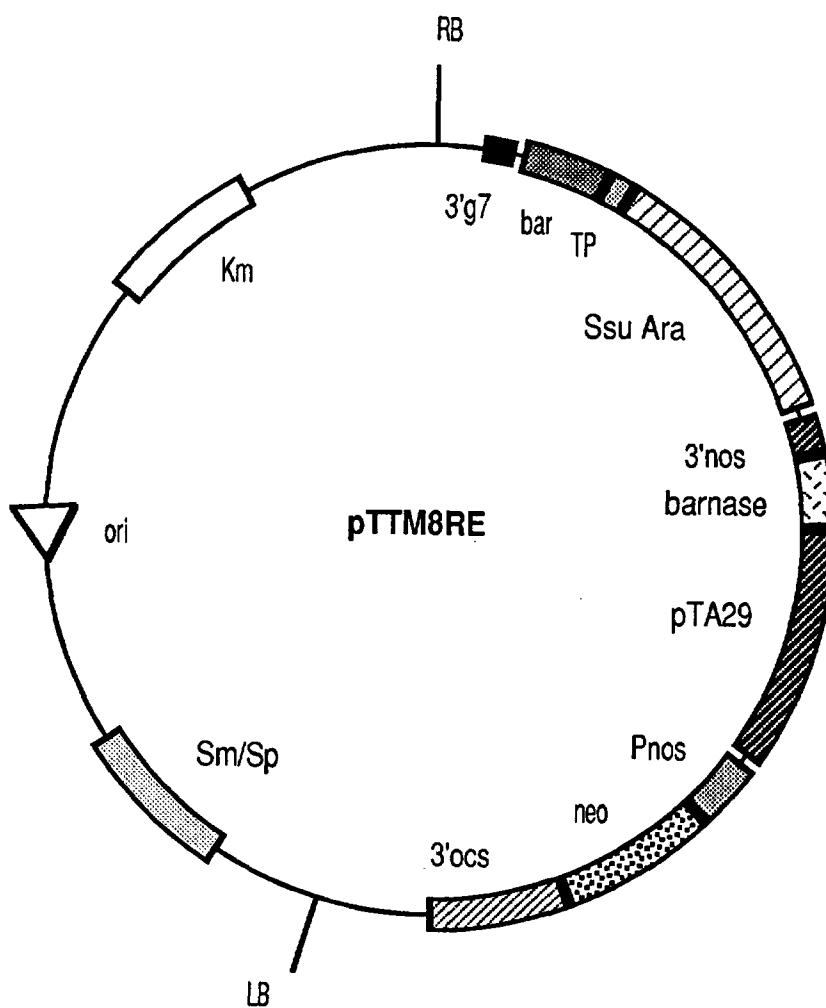


Figure 1. Schematic presentations of the structure of plasmid pTTM8RE, indicating the T-DNA, origins of replication and prokaryotic selective markers

B. MAP OF THE VECTOR pTVE74RE

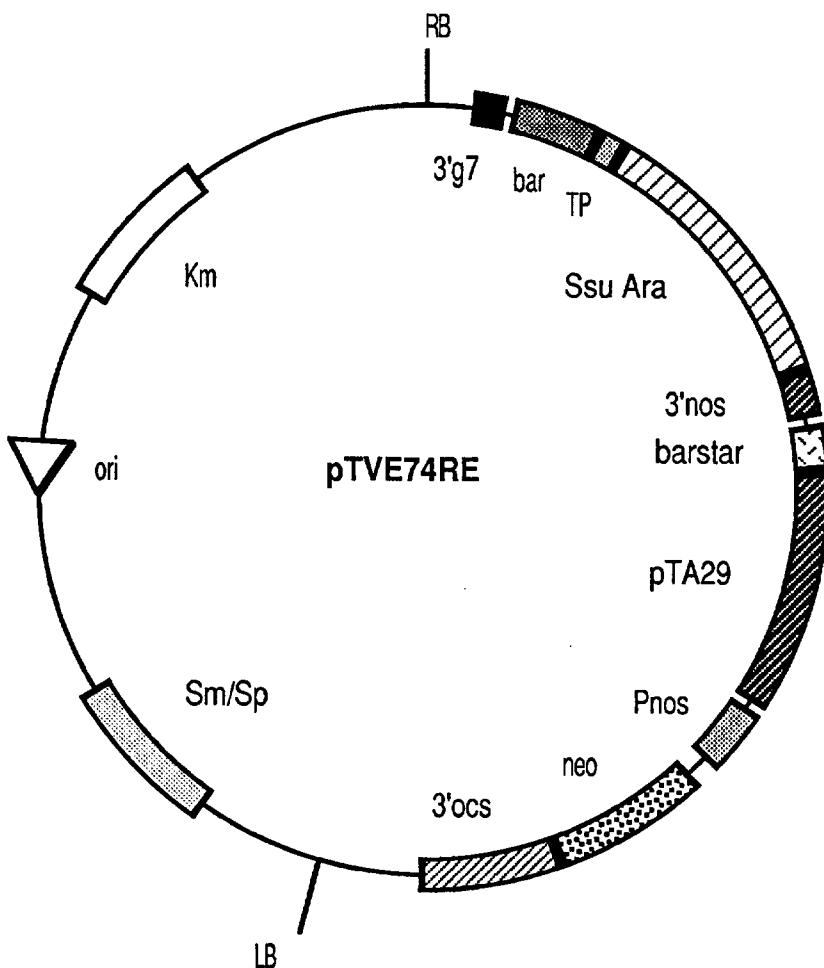


Figure 2. Schematic presentations of the structure of plasmid pTTM8RE, indicating the T-DNA, origins of replication and prokaryotic selective markers

C. DESCRIPTION OF THE GENETIC ELEMENTS

Table 1. Nucleotide positions of the DNA comprised between the T-DNA border repeats of pTTM8RE and origin of the different sequences

nt positions	orientation	Description and references
1-25		Right border repeat from the TL-DNA from pTiB6S3 (Gielen <i>et al.</i> (1984) The EMBO Journal 3: 835-846).
26-283		Residual sequences from the TL-DNA at the right border repeat. The junction is at the HpaI restriction site in the TL-DNA
284-290		Synthetic polylinker derived sequences
502-591	counter clockwise	A 212 EcoRV-ClaI fragment containing plant polyadenylation signals and obtained from the 3'untranslated end from the TL-DNA gene 7 (3'g7) of pTiB6S3 (Velten and Schell. (1985) Nucleic Acids Research 13: 6981-6998; Dhaese <i>et al.</i> (1983) The EMBO Journal 3: 835-846).
503-523		Synthetic polylinker derived sequences
1075-524	counter clockwise	The coding sequence of the <i>bar</i> gene of <i>Streptomyces hygroscopicus</i> (Thompson <i>et al.</i> (1987) The EMBO Journal 6: 2519-2523).
2966-1076	counter clockwise	The promoter from the atS1A ribulose-1,5-biphosphate carboxylase small subunit gene from <i>Arabidopsis thaliana</i> (PSsuAra). The promoter fragment comprises the 1.7 kb fragment upstream from the atS1A ATG codon (Krebbers <i>et al.</i> , 1988 Plant Molecular Biology 11: 745-759)) and the transit peptide (tp) sequence (1240-1076) for targeting to the chloroplast
2967-2995		Synthetic polylinker derived sequences
3256-2996	counter clockwise	A 260 bp TaqI fragment from the 3' untranslated end of the nopaline synthase gene (3'nos) from the T-DNA of pTiT37 and containing plant polyadenylation signals (Depicker <i>et al.</i> (1982) Journal of Molecular and Applied Genetics 1: 561-573).
3257-3272		Synthetic polylinker derived sequences
3273-3367		Sequences from the 3'untranslated end of the <i>barnase</i> gene from <i>B. amyloliquefaciens</i>
3704-3368	counter clockwise	Region encoding mature <i>barnase</i> from <i>Bacillus amyloliquefaciens</i> (Hartley (1988) Journal of Molecular Biology 202:913-915).
5214-3705	counter clockwise	The promoter region of the anther-specific gene TA29 from <i>Nicotiana tabacum</i> . The PTA29 promoter comprises the 1.5 kb of the sequence upstream from the ATG initiation codon (Seurinck <i>et al.</i> (1990) Nucleic Acids Research 18: 3403).
5215-5250		Synthetic polylinker derived sequences
5251-5655	clockwise	The promoter from the nopaline synthase gene from the T-DNA of pTiT37 of <i>Agrobacterium tumefaciens</i> (Pnos); the nucleotide sequence of the Pnos promoter is described by Depicker et al.(1982).
5656-6633	clockwise	The coding sequence from the <i>neo</i> gene encoding neomycin phosphotransferase II. This sequence corresponds with the sequence from <i>Tn5</i> of <i>Escherichia coli</i> as described by Beck <i>et al.</i> (1982). The ATG initiation codon of the <i>neo</i> coding region has been substituted for a linker sequence as described by Reiss <i>et al.</i> (1984). There are 171 bp of the <i>Tn5</i> sequence downstream from the <i>neo</i> coding region present (Beck <i>et al.</i> , 1982).

Table 1 continues on the following page

Table 1 continued

6634-7509	clockwise	The 3' untranslated end from the octopine synthase gene (3'ocs). This corresponds with a 706 bp PvuII fragment from the octopine synthase gene (Gielen et al., 1984). The PvuII fragment has been cloned in the SmaI site of the <i>Tn5</i> sequence. There are an additional 169 bp of the <i>Tn5</i> sequence present downstream from the 3'ocs fragment.
7510-7513		Synthetic linker derived sequences
7514-8202		Residual sequences from the TL-DNA at the left border repeat. The junction is at the former BglII site in the TL-DNA.
8203-8227		Left border repeat from the TL-DNA from pTiB6S3 (Gielen et al (1984) The EMBO Journal 3: 835-846).

Table 2. Nucleotide positions of the DNA comprised between the T-DNA border repeats of pTVE74RE and origin of the different sequences

nt positions	orientation	Description and references
1-25		Right border repeat from the TL-DNA from pTiB6S3 (Gielen <i>et al</i> (1984) The EMBO Journal 3: 835-846).
26-283		Residual sequences from the TL-DNA at the right border repeat. The junction is at the HpaI restriction site in the TL-DNA.
284-290		Synthetic polylinker derived sequences
502-291	counter clockwise	A 212 EcoRV-ClaI fragment containing plant polyadenylation signals and obtained from the 3'untranslated end from the TL-DNA gene 7 (3'g7) of pTiB6S3 (Velten and Schell. (1985) Nucleic Acids Research 13: 6981-6998; Dhaese <i>et al.</i> (1983) The EMBO Journal 3: 835-846).
503-523		Synthetic polylinker derived sequences
1075-524	counter clockwise	The coding sequence of the <i>bar</i> gene of <i>Streptomyces hygroscopicus</i> (Thompson <i>et al.</i> (1987) The EMBO Journal 6: 2519-2523).
2966-1076	counter clockwise	The promoter from the atS1A ribulose-1,5-biphosphate carboxylase small subunit gene from <i>Arabidopsis thaliana</i> (PSSuAra). The promoter fragment comprises the 1.7 kb fragment upstream from the atS1A ATG codon (Krebbers <i>et al.</i> , 1988 Plant Molecular Biology 11: 745-759)) and the transit peptide (tp) sequence (1240-1076) for targeting to the chloroplast
2967-2995		Synthetic polylinker derived sequences
3256-2996	counter clockwise	A 260 bp TaqI fragment from the 3'untranslated end of the nopaline synthase gene (3'nos) from the T-DNA of pTiT37 and containing plant polyadenylation signals (Depicker <i>et al.</i> (1982) Journal of Molecular and Applied Genetics 1: 561-573).
3257-3276		Synthetic polylinker derived sequences
3277-3316		Sequences from the 3'untranslated end of the <i>barnase</i> gene from <i>B. amyloliquefaciens</i>
3589-3317	counter clockwise	The coding region of the <i>barnase</i> gene from <i>Bacillus amyloliquefaciens</i> (Hartley (1988) Journal of Molecular Biology 202:913-915).
5100-3590	counter clockwise	The promoter region of the anther-specific gene TA29 from <i>Nicotiana tabacum</i> . The PTA29 promoter comprises the 1.5 kb of the sequence upstream from the ATG initiation codon (Seurinck <i>et al.</i> (1990) Nucleic Acids Research 18: 3403).
5101-5136		Synthetic polylinker derived sequences
5137-5541	clockwise	The promoter from the nopaline synthase gene from the T-DNA of pTiT37 of <i>Agrobacterium tumefaciens</i> (Pnos); the nucleotide sequence of the Pnos promoter is described by Depicker et al.(1982).
5542-6519	clockwise	The coding sequence from the <i>neo</i> gene encoding neomycin phosphotransferase II. This sequence corresponds with the sequence from <i>Tn5</i> of <i>Escherichia coli</i> as described by Beck <i>et al.</i> (1982). The ATG initiation codon of the <i>neo</i> coding region has been substituted for a linker sequence as described by Reiss <i>et al.</i> (1984). There are 171 bp of the <i>Tn5</i> sequence downstream from the <i>neo</i> coding region present (Beck <i>et al.</i> , 1982).

Table 2. continues on the following page

Table 2 continued

6520-7395	clockwise	The 3' untranslated end from the octopine synthase gene (3'ocs). This corresponds with a 706 bp PvuII fragment from the octopine synthase gene (Gielen et al., 1984). The PvuII fragment has been cloned in the SmaI site of the <i>Tn5</i> sequence. There are an additional 169 bp of the <i>Tn5</i> sequence present downstream from the 3'ocs fragment.
7396-7399		Synthetic linker derived sequences
7400-8089		Residual sequences from the TL-DNA at the left border repeat. The junction is at the former BglII site in the TL-DNA.
8090-8114		Left border repeat from the TL-DNA from pTiB6S3 (Gielen et al (1984) The EMBO Journal 3: 835-846).

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MS1 Discriminating PCR Protocol

Version: OSR-MS1-01	Valid from: 23NOV92	Replaces version: N.A.
Protocol Type: Single seed / Single leaf Agarose gel detection	PGS Number: PGS0016	
Author: Marc De Beuckeleer Biotech Product Characterization Aventis CropScience N.V. J. Plateaustraat 22 B-9000 Gent - Belgium Fax: (32) (9) 224.06.94 Tel: (32) (9) 235.84.37 Email: marc.debeuckeleer@aventis.com	Submitted to: Date:	
MS1 primers:		
MDB96	5'-CTC.TCT.gAC.TTT.TCC.ACC.AAg.g-3'	22-mer
MDB98	5'-TAC.ggC.TAA.gAg.CgA.ATT.Tgg.C-3'	22-mer
Endogenous primers:		
CVZ7	5'-AAC.gAg.TgT.CAg.CTA.gAC.CAg.C-3'	22-mer
CVZ8	5'-CgC.AgT.TCT.gTg.AAC.ATC.gAC.C-3'	22-mer
Amplified fragments:		Annealing temperature: 57 °C
CVZ7-CVZ8:	394 bp (Endo Ctrl)	Thermal Cycling: 30 cycles
MDB96-MDB98:	238 bp (MS1)	Comments: -----
For further help: please contact Marc De Beuckeleer		
Received by: (name)		
Date: / / (signature)		

OBJECTIVE: Provide a protocol for PCR-based confirmation of the presence of the OSR/Canola SeedLink elite event MS1 in plant material.

TESTED ON: ✓ *Brassica napus*
- *Brassica rapa*
- *Brassica juncea*

A test run, with all appropriate controls, has to be performed before attempting to screen unknowns. The presented protocol might require optimization for components that may differ between labs (template DNA preparation, Taq DNA polymerase, quality of the primers, dNTP's, thermocycler, etc.)

Amplification of the endogenous sequence plays a key role in the protocol. One has to attain PCR and thermocycling conditions that amplify equimolar quantities of both the endogenous and transgenic sequence in a known transgenic genomic DNA template. Whenever the targeted endogenous fragment is not amplified or whenever the targeted sequences are not amplified with the same ethidium bromide staining intensities, as judged by agarose gel electrophoresis, optimization of the PCR conditions is required.

1. Template DNA

Prepared according to Edwards *et al.* (Nucleic Acids Research, 19, p1349, 1991). A brief protocol is presented on page 6.

Sample: leaf punch or a single seed.

Remark: when using DNA prepared with other methods, a test run utilizing different amounts of template should be done. Usually 50 ng of genomic template DNA yields the best results.

2. Assigned positive and negative controls to a PCR run

- Master Mix control (DNA negative control). This is a PCR in which no DNA is added to the reaction. When the expected result, no PCR products, is observed this indicates that the PCR Master Mix was not contaminated with target DNA.
- A DNA positive control (genomic DNA sample known to contain the transgenic sequences). Successful amplification of this positive control demonstrates that the PCR was run under conditions that allow the amplification of target sequences.
- A wildtype DNA control. This is a PCR in which the template DNA provided is genomic DNA prepared from a non-transgenic plant. When the expected result, no amplification of the transgene PCR product but amplification of the endogenous PCR product, is observed this indicates that there is no detectable transgene background amplification in a genomic DNA sample.
- Primers targeting an endogenous sequence are always included in the PCR cocktail. These primers serve as an internal control in unknown samples and in the DNA positive control. A positive result with the endogenous primer-pair demonstrates that there is ample DNA of adequate quality in the genomic DNA preparation for a PCR product to be generated.

3. Primers

MDB96:	5'-CTC.TCT.gAC.TTT.TCC.ACC.AAg.g-3'	22-mer
	MS1 primer	
MDB98:	5'-TAC.ggC.TAA.gAg.CgA.ATT.Tgg.C-3'	22-mer
	T-DNA primer	
CVZ7:	5'-AAC.gAg.TgT.CAg.CTA.gAC.CAg.C-3'	22-mer
	Endogenous primer	
CVZ8:	5'-CgC.AgT.TCT.gTg.AAC.ATC.gAC.C-3'	22-mer
	Endogenous primer	

- To avoid confusion between bases, a lower-case 'g' is used to clearly differentiate between 'g' and 'C'.
- It is recommended that lyophilized primers be reconstituted at concentrations greater than 10 µmolar in TE (10 mM Tris-HCl, pH8.0, 1 mM EDTA) and stored at -20°C. Avoid multiple freeze-thaw cycles of the primer stock: prepare aliquots of the primer with a concentration of 10 pmoles/µl.

4. Amplified fragments

CVZ7 - CVZ8: 394bp (endogenous control)

MDB96-MDB98: 238bp (MS1)

5. PCR conditions

Reaction vessel: 96 well microtiter plate
Prepare one Master Mix / microtiter plate

Components for one 25 µl reaction:

2.5 µl template DNA
2.5 µl 10x Amplification Buffer
0.5 µl 10 mM dNTP's
0.5 µl MDB96 (10pmoles/µl)
0.5 µl MDB98 (10pmoles/µl)
0.25 µl CVZ7 (10pmoles/µl)
0.25 µl CVZ8 (10pmoles/µl)
0.1 µl Taq DNA polymerase (5 units/µl)
water up to 25 µl

- Commercial available Taq DNA polymerases are usually delivered with vials of 10x Amplification Buffer.
- 10x Amplification Buffer: 500 mM KCl, 15 mM MgCl₂, 100 mM Tris-HCl (pH 9.0 at room temperature).

- To set up the PCR, prepare a Master Mix containing all components. Always prepare enough Master Mix for a number of PCR's which is greater than the number of PCR's actually required, in order to account for residual volume in the microfuge tube.
- Maintain the thawed components on ice while preparing the Master Mix. Add *Taq* DNA polymerase just before dispensing the appropriate volume of the Master Mix into the reaction vessels.

6. Thermocycling profile

Thermocycler: MJ Research PTC-200

Profile:

4 min. at 95°C

Followed by: 1 min. at 95°C
1 min. at 57°C
2 min. at 72°C
For 5 cycles

Followed by: 30 sec. at 92°C
30 sec. at 57°C
1 min. at 72°C
For 25 cycles

Followed by: 5 minutes at 72°C

7. Validation of the results

Data from transgenic plant DNA samples within a single PCR run and a single PCR Master Mix will not be acceptable unless:

- the DNA positive control shows the expected PCR products (transgenic and endogenous fragments)
- the DNA negative control is negative for PCR amplification (no fragments)
- the wildtype DNA control shows the expected result (endogenous fragment amplification).

Lanes showing visible amounts of the transgenic and endogenous PCR products of the expected sizes, indicate that the corresponding plant from which the genomic template DNA was prepared, has inherited the transgenic sequence assayed for.

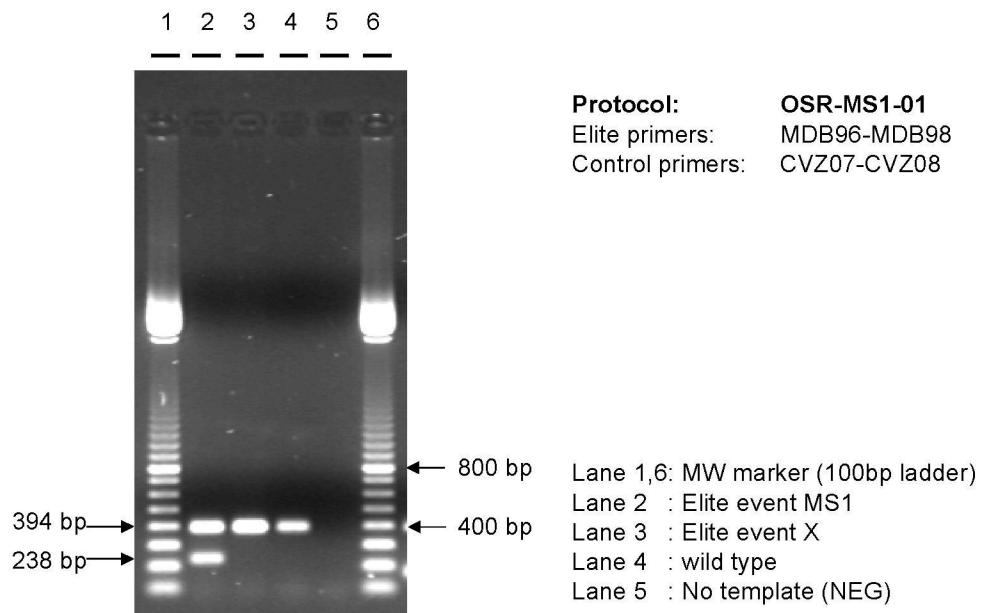
Lanes not showing visible amounts of the transgenic PCR product and showing visible amounts of the endogenous PCR product, indicate that the corresponding plant from which the genomic template DNA was prepared, has not inherited the transgenic sequence assayed for.

Lanes not showing visible amounts of the endogenous and transgenic PCR products, indicate that the quality and/or quantity of the genomic DNA didn't allow for a PCR

product to be generated. These plants cannot be scored. The genomic DNA preparation should be repeated and a new PCR run, with the appropriate controls, has to be performed.

8. Agarose gel analysis

Apply between 10 and 20 μ l of the PCR samples on a 1.5% agarose gel (Tris-borate buffer). Use an appropriate molecular weight marker (e.g. 100bp ladder PHARMACIA).



PCR - Template preparation

1. Preparation of plant genomic DNA for PCR analysis - Leaf samples

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

Reference: K.Edwards et al., NAR vol 19, No 6, page 1349, 1991

2. Preparation of plant genomic DNA for PCR analysis - Seed samples

- Put one seed in a microfuge tube.
- Macerate the seed with a plastic pestle at room temperature, without buffer, for about 15 seconds.
- Add 300 µl extraction buffer (Extraction buffer: 200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Add 300 µl phenol-chloroform. Vortex for 5 seconds. The mixture can be left at room temperature until all samples have been extracted (> 1 hour).
- Centrifuge the extracts for 2 minutes at maximum speed and transfer 250 µl of the supernatant to a new microfuge tube. Mix with 250 µl isopropanol and leave at room temperature for 2 minutes.
- Centrifuge at maximum speed for 5 minutes.
- Dry pellet (e.g. 10 minutes at 37°C), dissolve in 100 µl water.
- Centrifuge for 2 minutes and transfer supernatant to a new microfuge tube.
- Use 5 µl of this sample in a 50 µl reaction.

Aventis CropScience

RF2 Discriminating PCR Protocol

Version: OSR-RF2-02	Valid from: 10JUN96	Replaces version: OSR-RF2-01
Protocol Type: Single seed / Single leaf Agarose gel detection	PGS Number: PGS0009	
Author: Marc De Beuckeleer Biotech Product Characterization Aventis CropScience N.V. J. Plateaustraat 22 B-9000 Gent - Belgium Fax: (32) (9) 224.06.94 Tel: (32) (9) 235.84.37 Email: marc.debeuckeleer@aventis.com	Submitted to:	Date:
RF2 primers:		
MDB207	5'-ggg.TgA.gAC.AAT.ATA.TCg.ACg-3'	21-mer
MDB79	5'-ACC.CTT.gAg.gAA.ACT.ggT.AgC-3'	21-mer
Endogenous primers:		
CVZ7	5'-AAC.gAg.TgT.CAg.CTA.gAC.CAg.C-3'	22-mer
CVZ8	5'-CgC.AgT.TCT.gTg.AAC.ATC.gAC.C-3'	22-mer
Amplified fragments:	Annealing temperature:	57 °C
CVZ7-CVZ8: 394 bp (Endo Ctrl)	Thermal Cycling:	30 cycles
MDB207-MDB79: 189 bp (RF2)	Comments:	- - - - -
For further help: please contact Marc De Beuckeleer		
Received by:(name)		
Date:/./.(signature)		

OBJECTIVE: Provide a protocol for PCR-based confirmation of the presence of the OSR/Canola SeedLink elite event RF2 in plant material.

TESTED ON: ✓ *Brassica napus*
- *Brassica rapa*
- *Brassica juncea*

A test run, with all appropriate controls, has to be performed before attempting to screen unknowns. The presented protocol might require optimization for components that may differ between labs (template DNA preparation, Taq DNA polymerase, quality of the primers, dNTP's, thermocycler, etc.)

Amplification of the endogenous sequence plays a key role in the protocol. One has to attain PCR and thermocycling conditions that amplify equimolar quantities of both the endogenous and transgenic sequence in a known transgenic genomic DNA template. Whenever the targeted endogenous fragment is not amplified or whenever the targeted sequences are not amplified with the same ethidium bromide staining intensities, as judged by agarose gel electrophoresis, optimization of the PCR conditions is required.

1. Template DNA

Prepared according to Edwards *et al.* (Nucleic Acids Research, 19, p1349, 1991). A brief protocol is presented on page 6.

Sample: leaf punch or a single seed.

Remark: when using DNA prepared with other methods, a test run utilizing different amounts of template should be done. Usually 50 ng of genomic template DNA yields the best results.

2. Assigned positive and negative controls to a PCR run

- Master Mix control (DNA negative control). This is a PCR in which no DNA is added to the reaction. When the expected result, no PCR products, is observed this indicates that the PCR Master Mix was not contaminated with target DNA.
- A DNA positive control (genomic DNA sample known to contain the transgenic sequences). Successful amplification of this positive control demonstrates that the PCR was run under conditions that allow the amplification of target sequences.
- A wildtype DNA control. This is a PCR in which the template DNA provided is genomic DNA prepared from a non-transgenic plant. When the expected result, no amplification of the transgene PCR product but amplification of the endogenous PCR product, is observed this indicates that there is no detectable transgene background amplification in a genomic DNA sample.
- Primers targeting an endogenous sequence are always included in the PCR cocktail. These primers serve as an internal control in unknown samples and in the DNA positive control. A positive result with the endogenous primer-pair demonstrates that there is ample DNA of adequate quality in the genomic DNA preparation for a PCR product to be generated.

3. Primers

MDB207:	5'-ggg.TgA.gAC.AAT.ATA.TCg.ACg-3'	21-mer
	RF2 primer	
MDB79:	5'-ACC.CTT.gAg.gAA.ACT.ggT.AgC-3'	21-mer
	T-DNA primer	
CVZ7:	5'-AAC.gAg.TgT.CAg.CTA.gAC.CAg.C-3'	22-mer
	Endogenous primer	
CVZ8:	5'-CgC.AgT.TCT.gTg.AAC.ATC.gAC.C-3'	22-mer
	Endogenous primer	

- To avoid confusion between bases, a lower-case 'g' is used to clearly differentiate between 'g' and 'C'.
- It is recommended that lyophilized primers be reconstituted at concentrations greater than 10 μ molar in TE (10 mM Tris-HCl, pH8.0, 1 mM EDTA) and stored at -20°C. Avoid multiple freeze-thaw cycles of the primer stock: prepare aliquots of the primer with a concentration of 10 pmoles/ μ l.

4. Amplified fragments

CVZ7 - CVZ8: 394bp (endogenous control)

MDB207-MDB79: 189bp (RF2)

5. PCR conditions

Reaction vessel: 96 well microtiter plate
Prepare one Master Mix / microtiter plate

Components for one 25 μ l reaction:

2.5 μ l template DNA
2.5 μ l 10x Amplification Buffer
0.5 μ l 10 mM dNTP's
0.5 μ l MDB207 (10pmoles/ μ l)
0.5 μ l MDB79 (10pmoles/ μ l)
0.25 μ l CVZ7 (10pmoles/ μ l)
0.25 μ l CVZ8 (10pmoles/ μ l)
0.1 μ l Taq DNA polymerase (5 units/ μ l)
water up to 25 μ l

- Commercial available Taq DNA polymerases are usually delivered with vials of 10x Amplification Buffer.
- 10x Amplification Buffer: 500 mM KCl, 15 mM MgCl₂, 100 mM Tris-HCl (pH 9.0 at room temperature).

- To set up the PCR, prepare a Master Mix containing all components. Always prepare enough Master Mix for a number of PCR's which is greater than the number of PCR's actually required, in order to account for residual volume in the microfuge tube.
- Maintain the thawed components on ice while preparing the Master Mix. Add *Taq* DNA polymerase just before dispensing the appropriate volume of the Master Mix into the reaction vessels.

6. Thermocycling profile

Thermocycler: MJ Research PTC-200

Profile:

4 min. at 95°C

Followed by: 1 min. at 95°C
1 min. at 57°C
2 min. at 72°C
For 5 cycles

Followed by: 30 sec. at 92°C
30 sec. at 57°C
1 min. at 72°C
For 25 cycles

Followed by: 5 minutes at 72°C

7. Validation of the results

Data from transgenic plant DNA samples within a single PCR run and a single PCR Master Mix will not be acceptable unless:

- the DNA positive control shows the expected PCR products (transgenic and endogenous fragments)
- the DNA negative control is negative for PCR amplification (no fragments)
- the wildtype DNA control shows the expected result (endogenous fragment amplification).

Lanes showing visible amounts of the transgenic and endogenous PCR products of the expected sizes, indicate that the corresponding plant from which the genomic template DNA was prepared, has inherited the transgenic sequence assayed for.

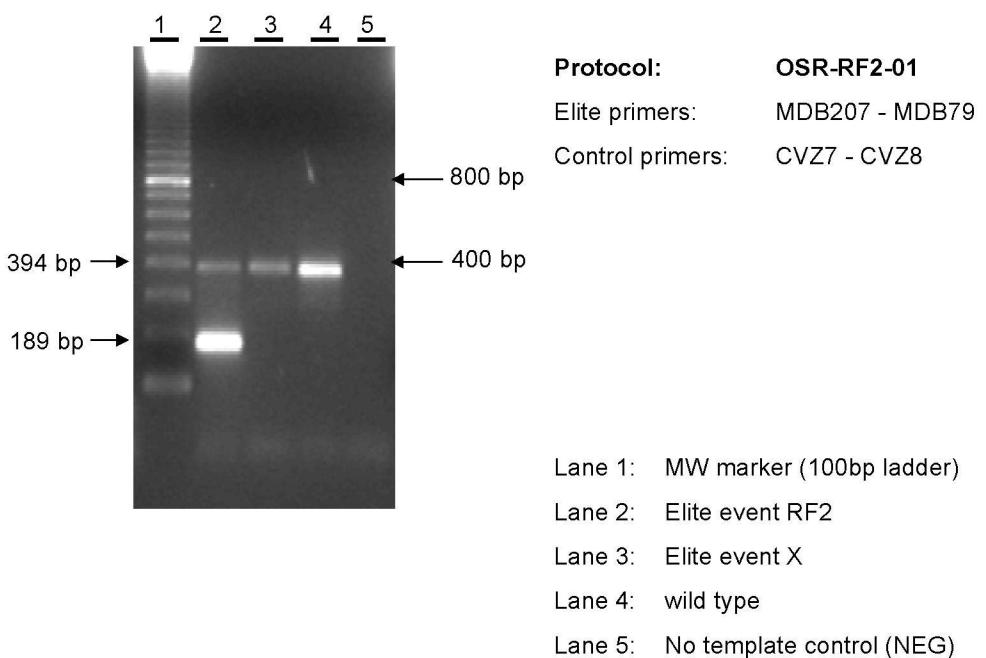
Lanes not showing visible amounts of the transgenic PCR product and showing visible amounts of the endogenous PCR product, indicate that the corresponding plant from which the genomic template DNA was prepared, has not inherited the transgenic sequence assayed for.

Lanes not showing visible amounts of the endogenous and transgenic PCR products, indicate that the quality and/or quantity of the genomic DNA didn't allow for a PCR

product to be generated. These plants cannot be scored. The genomic DNA preparation should be repeated and a new PCR run, with the appropriate controls, has to be performed.

8. Agarose gel analysis

Apply between 10 and 20 μ l of the PCR samples on a 1.5% agarose gel (Tris-borate buffer). Use an appropriate molecular weight marker (e.g. 100bp ladder PHARMACIA).



PCR - Template preparation

1. Preparation of plant genomic DNA for PCR analysis - Leaf samples

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

Reference: K.Edwards et al., NAR vol 19, No 6, page 1349, 1991

2. Preparation of plant genomic DNA for PCR analysis - Seed samples

- Put one seed in a microfuge tube.
- Macerate the seed with a plastic pestle at room temperature, without buffer, for about 15 seconds.
- Add 300 µl extraction buffer (Extraction buffer: 200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Add 300 µl phenol-chloroform. Vortex for 5 seconds. The mixture can be left at room temperature until all samples have been extracted (> 1 hour).
- Centrifuge the extracts for 2 minutes at maximum speed and transfer 250 µl of the supernatant to a new microfuge tube. Mix with 250 µl isopropanol and leave at room temperature for 2 minutes.
- Centrifuge at maximum speed for 5 minutes.
- Dry pellet (e.g. 10 minutes at 37°C), dissolve in 100 µl water.
- Centrifuge for 2 minutes and transfer supernatant to a new microfuge tube.
- Use 5 µl of this sample in a 50 µl reaction.