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EXPERTISE REPORT OF THE GROUP OF EXPERTS MANDATED BY THE BIOSAFETY ADVISORY COUNCIL

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EVALUATION OF NOTIFICATION C/BE/96/01 UNDER DIRECTIVE 2001/18/EC, FOR THE PLACING ON THE EU MARKET OF GENETICALLY MODIFIED OILSEED RAPE, ELITE EVENTS MS8, RF3 AND HYBRIDS THEREOF OF PLANT GENETIC SYSTEMS, PRESENTLY BAYER CROPSCIENCE

Scientific coordination by the Service of Biosafety and Biotechnology
Secretariat of the Biosafety Advisory Council
Scientific Institute of Public Health

1. INTRODUCTION: OVERSIGHT PROCEDURE

In 1996, the dossier C/BE/96/01 'A new hybridisation system in oilseed rape *(Brassica napus* L.). Application for consent to market genetically modified organisms (MS8xRF3)' was submitted to the Belgian Competent Authority (CA) by Plant Genetic Systems (PGS), presently Bayer CropScience within the framework of Part C of Directive 90/220/EEC.

The elite events MS8, RF3 and MS8xRF3 have been tested in the field on several sites in Europe and commercially released in different non-EU countries. Since 1998 the elite events MS8, RF3 and MS8xRF3 have been grown in Canada. In the Decision Document 96-17, the Agriculture and Agri-Food Canada (AAFC) has determined that the plants with novel traits have no altered environmental interactions when compared to currently commercialised rapeseed varieties in Canada or pose concerns for the safety of livestock consuming feed derived from the plants with the novel traits (URL: www.inspection.gc.ca/english/plaveg/pbo/dd/dd9617e.shtml). In the US the Animal and Plant Health Inspection Service (APHIS) has determined on December 23rd, 2002 that canola transformation events MS8 and RF3 do not present a plant pest risk and are therefore no longer considered regulated articles (URL: www.aphis.usda.gov/brs/dec_docs/9827801p_det.htm). On July 25th, 2003 the Australian Regulator issued a license to Bayer CropScience approving the commercial release of RF3 and MS8 in the Australian cropping system (URL: www.ogtr.gov.au/ir/dir021.htm).

The hybrid system MS8xRF3 shows strong homology with the hybrid systems MS1xRF1 and MS1xRF2 of PGS.
The new transgenic lines MS8 and RF3 were obtained using the same oilseed rape variety as for MS1, RF1 and RF2, but transformation vectors were used in which the T-DNA was limited to the desired genes and devoid of any antibiotic resistance gene. The MS1xRF1 and MS1xRF2 hybrid systems have been described in detail in the notifications C/UK/94/M1/1 and C/F/95/05-01A&B.
Notification C/UK/94/M1/1 has been approved by the commission decision 96/158/EC on February 6th, 1996 for growing and seed production. The UK consent was given on February 28th, 1996. A report reviewing the notification and considering in detail the risk assessment made by the Advisory Committee on Releases to the Environment (ACRE) is available on the website of the Department for Environment, Food and Rural Affairs (DEFRA) (URL: www.defra.gov.uk/environment/gm/regulation/pgs/index.htm) as well as an updated version (URL: www.defra.gov.uk/environment/acre/advice/advice03.htm).
The notifications C/F/95/05-01A (MS1xRF1) and C/F/95/05-01B (MS1xRF2) have been approved for all uses (growing, seed production, import, industrial processing and feed) respectively by the commission decisions 97/392/EC and 97/393/EC on June 6th, 1997. The French consents however were not issued.
Oil derived from MS1xRF1 and MS1xRF2 has been notified under Article 5 of the Novel Food Regulation (EC) 258/97 on June 10th, 1997.

The risk assessment of the initial dossier, that complied with the Directive 90/220/EEC at that time, was carried out by the experts of the Scientific Committees 'Transgenic plants' and 'Novel feed/food' of the Belgian Biosafety Advisory Council during the meetings of December 10th and 17th, 1996. After receiving a positive advice of the Biosafety Advisory Council and the consent of the Minister of Agriculture supporting the placing on the market of genetically modified (GM) oilseed rape

MS8xRF3, the dossier was sent to the European Commission (EC) and distributed to the other Member States (MS).

During 60 days the EC and the other MS had the opportunity to comment on the dossier. Most remarks related to the absence of a monitoring plan, which was considered necessary to check the long-term effects of the herbicide use, the outcrossing of transgenes and the potential establishment of multiple herbicide-tolerant plants, and to the absence of a labelling proposal. Questions were also raised about the lack of a Novel food legislation (at that time) for the evaluation of the GM seeds for human use. The supplementary information handed in by the notifier as an answer to the objections and questions of the MS, were evaluated by the experts of the Scientific Committees 'Transgenic plants' and 'Novel feed/food' of the Biosafety Advisory Council on July 4th, 1997. Although not requested under Directive 90/220/EC, the Belgian experts asked AgrEvo (previously PGS) to design a monitoring plan and guidelines for agricultural practices. Moreover, a list of institutes already involved or that would be involved in the biosafety research of MS8xRF3, as well as an overview of the biosafety research topics were requested.

In May 1998, the Scientific Committee on Plants (SCP) of the EC gave a favourable opinion on the MS8, RF3 and MS8xRF3 lines notified by AgrEvo (URL: www.europa.eu.int/comm/food/fs/sc/scp/out09_en.html). The SCP concluded that there is no evidence to indicate that the placing on the market of GM oilseed rape MS8xRF3, with the purpose to be used as any other oilseed rape, is likely to cause adverse effects on human health and the environment. The SCP also expressed the need for an agricultural code of practice and a post-market monitoring plan.

Following the positive advice of the SCP, the notifier kept the notification C/BE/96/01 up-to-date and continued to provide the latest available data and information on the GM oilseed rape events. A 'Stewardship plan for post-market guidance and monitoring' was set up and discussed during the meeting of the Biosafety Advisory Council on March 19th, 1999.

On May 28th, 1999, the EC sent out a proposal for a commission decision (ref: XI/565/97-rev.2) approving the placing on the market of oilseed rape MS8xRF3. Following requests by different MS, the vote was postponed.

On October 14th, 1999, the notifier submitted an overview on the biosafety research carried out in the framework of notifications C/DE/96/5 and C/BE/96/01 (document C005583), including a monitoring plan and a labelling proposal. On November 9th, 1999, an update of the document (document C005938) was submitted to the EC. The documents were presented during the meetings of the Regulatory Committee (according to Article 21 of Directive 90/220/EEC) on October 29th, 1999 and March 9th, 2000. Once again the vote was postponed.

Oil derived from MS8xRF3 was notified under Article 5 of the Regulation (EC) 258/97 on October 21st, 1999.

Awaiting the resumption of the procedure and considering the new Directive 2001/18/EC, Aventis CropScience updated the dossier to the provisions thereof. The notifier submitted a new set of documents. The 'Update 2001' contained: an environmental risk assessment (ERA) confirming the

conclusions of the ERA of the original notification dossier (1996) but providing better funded scientific evidence, the requested agricultural guidelines, a monitoring plan consisting of two sections, a case-specific monitoring and a general surveillance, a detection and identification protocol, and a labelling proposal. As a result of more detailed requirements concerning molecular characterisation and public information (URL: www.biosafety.be/TP/SNIFs/PubDosC-BE-96-01.pdf) at Belgian level, this information package was extended with a number of documents providing further molecular details on the events MS8 and RF3 and with a public dossier.
The additional information was evaluated during the meetings of the Scientific Committee 'Transgenic Plants' of the Biosafety Advisory Council of September 27th, 2001 and February 7th, 2002. After having modified the documents according to the remarks and suggestions of the Belgian experts, the Scientific Committee 'Transgenic plants' approved the different documents.

On January 16th, 2001 and on basis of Article 35 of the Directive 2001/18/EC, Bayer CropScience submitted the update of notification C/BE/96/01 to the Belgian CA. The update included the information required under Article 13, except for the information as requested by Article 13.2 sections (c), (d) and (g). The notifier provided the additionally demanded information to the CA on August 5th, 2003 and on October 6th, 2003 the notifier also provided the bio-informatic analyses of the inserts as demanded in the Belgian guidelines on molecular characterisation (URL: www.biosafety.be/NF/GuidanceNotes/Documents/Chapter2_MGC.pdf). The Belgian experts and Service of Biosafety and Biotechnology (SBB) approved the complementary information.

The assessment report of the updated notification was discussed during the meeting of the Biosafety Advisory Council of October 8th, 2003 and found to lack information according to Annex II D2 of the Directive needed for a complete environmental risk assessment. Awaiting the additional information the clock was stopped. The notifier provided the additional information on January 12th, 2004.

2. SUBJECT OF THE NOTIFICATION

2.1 SCOPE

The notification covers:

- Growing of oilseed rape in the European Union (EU) for production and multiplication of parental lines seeds and hybrid seeds, including official testing in the perspective of the listing on national and common catalogues according to the Directives 69/208/EEC, 70/457/EEC and 98/95/EC.

- Import of seeds from non-EU countries.

- Processing of the harvested seeds into human food (only oil[1]), animal feeding stuffs (meal remaining after oil extraction) and industrial products (bio-fuel, lubricants, cosmetics, paints, etc.).

[1] Oil derived from MS8xRF3 for human consumption has been notified under Article 5 of the Regulation (EC) 258/97 on October 21st, 1999.

2.2 CONDITIONS FOR MARKETING/LABELLING

The proposed period for the consent is 10 years. The placing on the market of the hybrid system will be in two phases. The first phase will include variety development, monitoring accompanying the placing on the market, and demonstration activities. In a second phase the full commercial release is foreseen, but phase one activities will be continued also after commercialisation.

The proposed seed bag label of the GMOs will contain the following information:

- the trade name (InVigor),
- the Seedlink and LibertyLink logos,
- the statement "this product contains Genetically Modified Organisms",
- a reference to the EC decision authorising the commercialisation of the GMOs,
- the unique identifier of MS8 and RF3,
- a notice that a registration is necessary for spraying the GM crop with the compatible herbicide,
- a notice about the way of controlling volunteers in subsequent crops by conventional agricultural methods other than the use of glufosinate ammonium,
- the name and address of the breeder and/or distributor.

3. ASSESSMENT OF THE SUBMITTED DATA AND DOCUMENTS

3.1 INFORMATION REGARDING THE RECIPIENT ORGANISM

Brassica napus L. is a member of the subtribe *Brassicinae* of the tribe *Brassiceae* of the Cruciferous (Brassicaceae) family, sometimes referred to as the mustard family. The spring and winter forms of the oil-yielding oleiferous rape 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) forms the subject of the notification.

Oilseed rape is grown as a winter annual in regions where winter conditions do not result in too low temperatures, which would kill the plants. The winter biotype typically requires vernalisation before the onset of stem elongation, raceme development, flowering and seed set. The spring biotype of *B. napus* requires no vernalisation prior to flowering. This biotype is typically lower yielding than the winter annual type, but requires considerably less time to complete its life cycle.

More information on the biology of oilseed rape including 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 is discussed in the consensus document on oilseed rape of the Organisation for Economic Co-operation and Development (OECD) (URL: www.olis.oecd.org/olis/1997doc.nsf/LinkTo/ocde-gd(97)63).

There are many years of experience with the cultivation and processing of oilseed rape as a raw material for animal feed. The genetic modifications of MS8 and RF3 relate to agronomic properties and do not have the objective of modifying its nutritional properties or modifying the use of oilseed rape as a raw material.

3.2 DESCRIPTION OF GENETICALLY MODIFIED ORGANISMS

The transgenic plants are:

- The male sterile oilseed rape line MS8 and progeny obtained through traditional breeding crosses with non-transgenic rape (*Brassica napus* L. spp. oleifera). Line MS8 contains a *barnase* gene, (origin *Bacillus amyloliquefaciens*) coding for a ribonuclease, that is only expressed in the tapetum cells during anther development and results in lack of viable pollen and male sterility, and a *bar* gene (origin *Streptomyces hygroscopicus*) coding for a phosphinotricin acetyl transferase (PAT) used as a selectable marker for tolerance to herbicides containing glufosinate ammonium. The *bar* gene is driven by a plant promoter that is active in all green tissues of the plant.

- The fertility restoration line oilseed rape RF3 and progeny obtained through traditional breeding crosses with non-transgenic rape (*Brassica napus* L. spp. oleifera). Line RF3 contains a *barstar* gene (origin *Bacillus amyloliquefaciens*), coding for an inhibitor of the Barnase protein, that is only expressed in the tapetum cells during anther development and leads to restoration of fertility after crossing to the male sterility line, and a *bar* gene (origin *Streptomyces hygroscopicus*) coding for PAT used as a selectable marker for tolerance to herbicides containing glufosinate ammonium. The *bar* gene is driven by a plant promoter that is active in all green tissues of the plant.

- The hybrid seeds derived from traditional crossings between the parental lines MS8 and RF3.

3.3 ASSESSMENT OF THE MOLECULAR DATA

3.3.1 Plasmid used for transformation

The male sterile line MS8 was obtained by means of *Agrobacterium* mediated transformation using plasmid pTHW107. This plasmid contains the *barnase* gene derived from *Bacillus amyloliquefaciens* and the *bar* gene derived from *Streptomyces hygroscopicus*. The *barnase* gene is under regulation of the tapetum specific promoter pTA29 isolated from *Nicotiana tabacum* and the 3' nopaline synthase (nos) gene of *A. tumefaciens*. The *bar* gene is regulated by the pSsuAra promoter isolated from *Arabidopsis thaliana* and by the 3' end of the T-DNA gene 7 of *A. tumefaciens* (3'g7).

The transgenic fertility restorer line RF3 was obtained using plasmid pTHW118. Plasmid pTHW118 contains a *barstar* gene (derived from *B. amyloliquefaciens*) under regulation of the pTA29 promoter and the nos terminator, and the same *bar* cassette as described for MS8.

3.3.2 Characterisation of the insert and flanking regions

Central in the Belgian recommendations was the determination of the sequence of the insert and the flanking regions, including 500 bp of the plant DNA.

The Flemish Agricultural Research Centre (CLO, Ghent, Belgium) independently evaluated the molecular data of MS8xRF3 oilseed rape. In its evaluation, emphasis was put on the analysis of the sequence structure of the junction regions. The experimental data were in agreement with the data in the dossier.

In line RF3, the transgene construct is integrated in a single genetic locus (see Fig. 1). Molecular characterisation of the T-DNA locus for line RF3 shows that besides an intact T-DNA copy, a truncated T-DNA copy is present in an inverted repeat orientation around the left border junction (3' end of the T-DNA). A fragment of 807 bp of the host genome has been determined at the junction with the T-DNA right border. At the left border, the second incomplete T-DNA ends up within the pSsuAra promoter and is flanked by 816 bp from the host genome. This fragment is also present at the 5' end upstream from the right border. The duplication is followed by 459 bp of the host genome. Both flanking sequences were found in the parental line (see Fig.1). BLASTn analysis showed that the 3' end of the insert of RF3 showed high similarity with *Arabidopsis thaliana* DNA, and meaningful sequence similarities with *Brassica oleracea* sequences could be found for the 5' end.

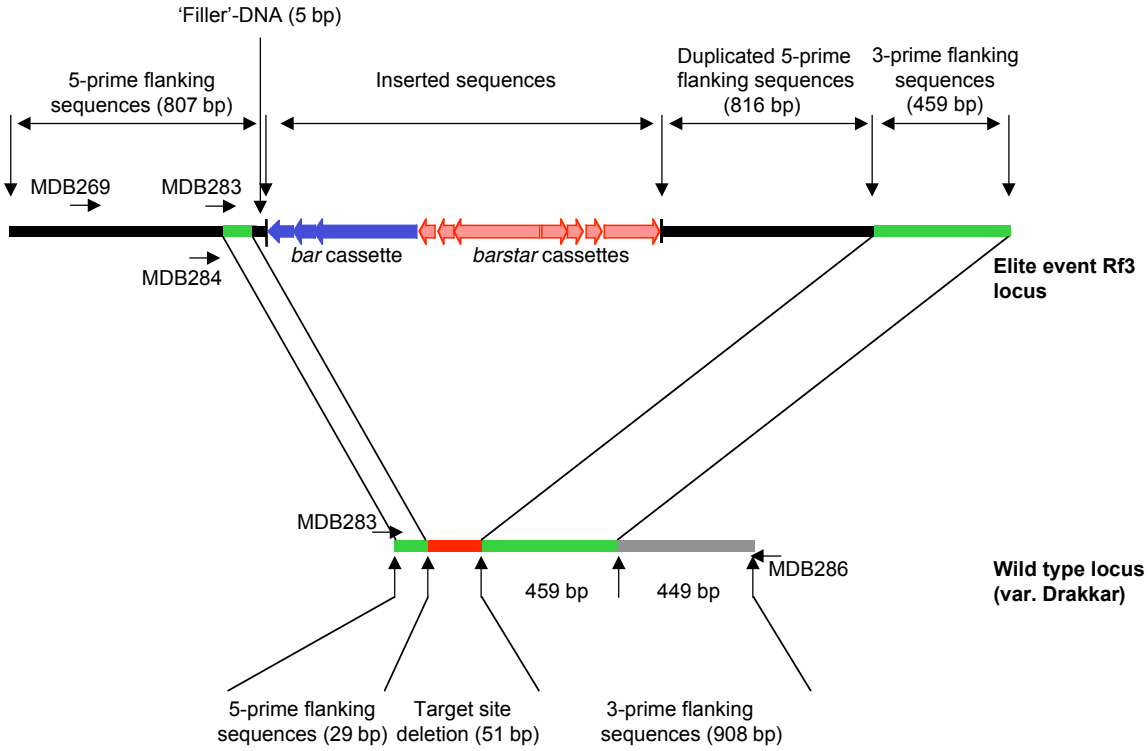

Figure 1: Physical map (not drawn to scale) of the insert of event RF3 and schematical representation of the alignment of the RF3 transgene locus and the wild type locus.

The MS8 insertion contains a single T-DNA copy (see Fig. 2). At the left border junction (3' end of the T-DNA), a 357 bp host sequence was retrieved. At the right border junction (5' of the T-DNA), an 864 bp host sequence was retrieved. PCR amplification from the parental line showed co-linearity with the sequences found on both sides of the T-DNA insert. Search in the database using BLASTn showed that high similarity with *Arabidopsis thaliana* and *Brassica oleracea* DNA could be found for part of the 5' flanking region, and significant sequence similarities with *Brassica oleracea* DNA for the 3' flanking region.

Determination of the pre-insertion sites was done using DNA isolated from the wild type oilseed rape. Alignment of the wild type sequence with the RF3 transgene locus revealed that a fragment of 51 bp is present at the wild type locus but missing in the transgene locus (see Fig. 1). At the right border junction 5 nucleotides ('filler'-DNA) are inserted. The junction-point of the duplicated 5' plant DNA sequence with the 3' flanking sequence is the same as the target site breakpoint in the wild type line. Alignment of the wild type sequence with the MS8 transgenic locus revealed that 19 bp are missing at the target site (see Fig. 2). At the right border junction 3 nucleotides ('filler'-DNA) of unknown origin are inserted at the MS8 transgene locus. The left border junction-point is the same as the target site breakpoint in the wild type line.

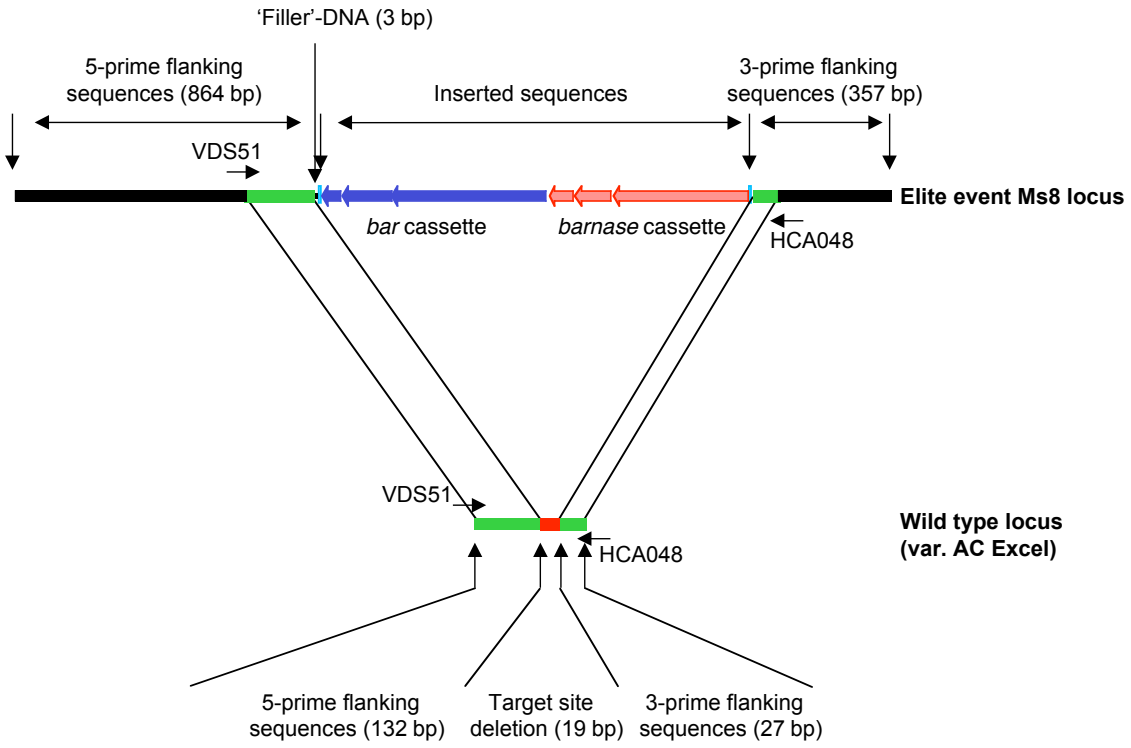

Figure 2: Physical map (not drawn to scale) of the insert of event MS8 and schematical representation of the alignment of the MS8 transgene locus and the wild type locus.

Bio-informatic analyses were performed on the right and left border integration sequences, comprising the flanking sequence and approximately 300 bp insert sequence, of events RF3 and MS8 to confirm the absence of cryptic expression. For each border integration sequence, putative active ORFs were found. It was shown that the ORFs can be assumed as transcriptionally not active, because of the lack of the ATG context consensus sequence, and biologically not functional, because of the lack of a functional promoter and 3' UTR regulatory sequences. Similarity search using BLASTp did not reveal any meaningful sequence similarity of the putative cryptic ORFs with known proteins.

3.3.3 Expression of inserted genes

Expression of the inserted genes was determined in the green tissues (leaves and flower buds) of the MS8 and RF3 plants via the use of Northern Blot analysis. The *bar* gene expression was detected in the leaves and flower buds of MS8 (varying between 0.03 and 0.22 pg/µg total RNA) and RF3 (varying between 0.2 and 1.1 pg/µg total RNA). In MS8, *barnase* gene expression was under the level of detection (detection limit of 0.1 pg/µg total RNA). The *barstar* gene expression in RF3 was only detectable in flower buds (varying between 1.2 and 2.4 pg/µg total RNA).

The expression of the inserted genes was also determined in the reproductive tissues (seed and pollen). Using a PAT-specific ELISA, expression of the *bar* gene was detected in seeds of MS8, RF3 and the hybrid MS8/RF3 at a level of less than 0.001 % of total extractable protein. Using Northern blot analysis, *barstar* gene expression in RF3 seeds and *barnase* gene expression in MS8 seeds were not detectable. This was expected since a tapetum-specific promoter drives both genes. No expression of the *bar* gene could be detected in the pollen of RF3 using Northern Blot analysis (detection limit of 0.05 pg/µg total RNA).

3.3.4 Stability of the insert

Phenotypic analysis showed that the hybrid system in oilseed rape is expressed stably independent of the plant genotype, generation or environment. Via molecular analysis, using segregation and Southern Blot, it was shown that the *barnase* and *barstar* genes were inherited stable in different genetic backgrounds of spring and winter oilseed rape over two to three generations.

3.3.5 Conclusions

From the data supplied by the notifier, it was concluded that the molecular characterisation of events MS8 and RF3 fulfil the Belgian guidelines for molecular characterisation and are sufficient to substantiate that the transgene constructs of MS8 and RF3 are stably integrated in a single locus and that expression is restricted to the genes of interest.

3.4 ASSESSMENT OF THE DETECTION AND IDENTIFICATION PROTOCOL

Reference material (genomic DNA and seeds) for the lines MS8, RF3 and the hybrid derived from the crossing of MS8 and RF3 was used to assess the proposed detection and identification method described in the notification dossier.

The notifier proposed a method for detection/identification of the events MS8, RF3 and the hybrid lines MS8xRF3 based on the 'elite' event-specificity of a junction sequence chosen as analyte. The genetic status, regarding type of cultivar and ploidy, of the non-transgenic reference material used as negative control has not been documented. This could have a concrete influence on the compliance of the method to Article 26(2) if quantitative considerations have to be taken on board.

In order to be able to confirm the specificity of the detection methods for MS8 and RF3, the notifier applied the same proposed method on the genomic DNA of the oilseed rape events MS1, RF1 and RF2. The results indicated that the method does not result in cross-detection for these events. Using public domain protocols and reference material from various GM-maize authorised on the EU market (Mon810, Bt11 and Bt176), the SBB proved the specificity of the method towards these GMOs (see Annex 1). However, the specificity of the method has not been fully established given the lack of

access of enforcement laboratories to reference material from GMOs presently in the pipeline of the EU authorisation procedures or on the world market.

The reliability of the method has not been assessed since this requires a full validation, which is out of the scope of the present assessment.

3.5 ASSESSMENT OF RISKS TO THE ENVIRONMENT

3.5.1 Impact of vertical gene flow

3.5.1.1 Consequences of vertical gene flow outside the field

Vertical gene flow of the transgene(s) occurring through pollen exchange between GM oilseed rape, non-GM oilseed rape, wild relatives and feral oilseed rape plants and through seed spillage during transport (Crawley & Brown, 1995; Norris & Sweet, 2001; Pessel et al., 2001; Eastham & Sweet, 2002; Salisbury, 2002), might have environmental impacts outside the GM oilseed rape field. Moreover, recipient plants, volunteers and feral oilseed rape populations might also act as green bridges and add seeds to the seedbank (Wilkinson et al., 1995).

In most studies, the bulk of cross-pollination has been shown to occur over short distances. Successful pollinations tend to decline rapidly with distance from the source. At distances > 100 m from the source, the dispersion follows a uniform law whereby the percentage of dispersion is maintained at a very low level and does not appear to decrease in any clear manner with the distance (Eastham & Sweet, 2002; Salisbury, 2002).

Till now, several inter-specific hybrids have been described for oilseed rape and its wild relatives, but gene introgression in a wild relative has only been confirmed for *B. napus* - *B. rapa* hybrids (Bing et al., 1996; Hansen et al., 2001; Norris & Sweet, 2002; Warwick et al., 2003; Chèvre et al., 2004).

The transfer of the herbicide-tolerant trait might lead to an enhanced fitness of the recipient plants (oilseed rape crops, wild relatives and feral oilseed rape plants) and make them more invasive and persistent (Tiedje et al., 1989). However, several studies reported that the presence of the herbicide-tolerant trait did not confer a competitive advantage in ecosystems outside the agricultural field, unless the herbicide is applied. In the absence of the herbicide, the herbicide-tolerant plants will not be more invasive and persistent than the untransformed plants (Crawley et al., 1993; 2001; Wilkinson et al., 1995; OECD, 1997; Downey, 1999; Norris et al., 1999; Simpson et al., 1999; Warwick et al., 1999; Norris & Sweet, 2002). As natural vegetation is rarely exposed to herbicides, it is unlikely that the herbicide-tolerant trait will increase invasiveness in such areas (Beckie et al., 2001; Eastham & Sweet, 2002; Salisbury, 2002). The impact of herbicide drift on the establishment and persistence of GM herbicide-tolerant plants in the field margins, however, is not well documented.

No increased fitness is expected to occur from the incorporation of the *barnase* and/or *barstar* genes as these genes leading to male sterility and fertility restoration do not give the recipient plants any selective advantage.

3.5.1.2 Consequences of vertical gene flow in the field

In the field, vertical gene flow of the transgene(s) will occur through the loss of seeds resulting from pod shattering and through pollen exchange between oilseed rape crops, volunteers and wild relatives (Eastham & Sweet, 2002; Salisbury, 2002).

The presence of GM herbicide-tolerant volunteer oilseed rape plants and GM herbicide-tolerant wild relatives in the field might make weed control more difficult and might shift the weed crop balance back to the situation where several compounds and/or less environmentally friendly herbicides have to be applied to control weed infestation (Senior & Dale, 2002; Squire et al., 2003). If farmers are forced to change the type of herbicide or the pattern of application it has the potential for an indirect effect on farmland biodiversity (see 3.5.4 Impact of the herbicide management on farmland biodiversity).

Field trials show that the numbers of GM herbicide-tolerant volunteers in the year following the release of GM herbicide-tolerant oilseed rape seem comparable to, or less than the numbers of volunteers in conventional oilseed rape (Crawley et al., 1993; Hails et al., 1997; Norris et al., 1999; Norris & Sweet, 2001). Although research is ongoing, there is no reason to think that GM herbicide-tolerant volunteers will behave substantially differently than conventional cultivars in the absence of the herbicide (Lutman et al., 2003).

The occurrence of GM multiple herbicide-tolerant plants when different GM herbicide-tolerant oilseed rapes will be grown in proximity without adequate on-farm and off-farm management strategies might be a long-term concern. GM multiple herbicide-tolerant oilseed rape has already been reported in Canada, UK, Germany and France. The control of GM multiple herbicide-tolerant plants might become more difficult and may have an impact on the environment, depending on the type of chosen chemical control (Senior & Dale, 2002; Senior et al., 2002). However, GM single and multiple herbicide-tolerant plants are successfully controlled by the herbicides currently used to control broad-leaved weeds and volunteer oilseed rape within cereal crop rotation (CETIOM, 2000; Senior et al., 2002).

3.5.1.3 Socio-economical consequences of vertical gene flow

Since the potential socio-economic consequences of vertical gene (e.g. compliance to labelling thresholds for the adventitious presence of GM crops in non-GM crops, co-existence measures between the GM and non-GM crops) fall beyond the scope of the competences of the Group of Experts, they are not taken into consideration in the present assessment report.

3.5.2 Impact of horizontal gene transfer on micro-organisms

Horizontal gene transfer refers to the non-sexual gene transfer between organisms, which may belong to unrelated systematic groups. The main environmental concern is that soil micro-organisms might pick up the transgenes from the GM crops grown in the field. However, scientific data indicate that the horizontal gene transfer from GM plants to bacteria is unlikely to occur under normal conditions (Prins & Zadoks, 1994; Nielsen et al., 1998; Gebhard & Smalla, 1999). If such a transfer would occur, the impact on the bacteria will be limited, since the *bar*, *barnase* and *barstar* genes are under the control of plant promoters. These promoter sequences show little or no activity in bacteria. Moreover, the transgenic lines MS8, RF3 and MS8xRF3 are devoid of any antibiotic resistance gene.

3.5.3 Impact of the GM oilseed rape on non-target organisms

3.5.3.1 Impact on honeybees

Bees are considered to be the principal pollinators of oilseed rape, though other insects such as bumblebees, solitary honeybees, pollen beetle and certain dipteran, lepidopteran, hemipteran and coleopteran insects may have a pollinator role as well.

The trait controlling male sterility affects only anther and pollen development: the flower nectaries of MS8, which provide a source of nutrients for pollinators, develop normally. The RF3 plants and the hybrids have normal flower morphology and fertility. Data show that there are no pleiotropic effects of the genetic modification on the attractiveness of the herbicide-tolerant oilseed rape MS8, RF3 and hybrid for honeybees: their foraging activity and foraging preferences remain the same. There is also no evidence that the GM pollen would affect bees any differently than conventional pollen (Picard-Nazou et al., 1997; CETIOM, 2000; Sandoz et al., 2000).

MS8 and RF3 and their hybrid do not contain elevated levels of toxic compounds and therefore, honeybees and other insects that may feed on MS8, RF3 will not be affected in their ability to reproduce or function normally. Studies show that colony health and the mortality rate for honeybees foraging on the transgenic oilseed rape are the same compared to bees foraging on non-transgenic oilseed rape (Picard-Nazou et al., 1995; CETIOM, 2000).

3.5.3.2 Impact on birds, small mammals and humans

Birds and small mammals consume parts of the oilseed rape plants. Field observations carried out by Bayer CropScience indicated that mainly pigeons, sparrows, hares and rabbits show a particular interest in the oilseed rape crop. A bird feeding study and rabbit dietary test did not allow detecting differences in food consumption, behaviour and body weight between the animals fed with seed of transgenic and non-transgenic oilseed rape.

According to the notifier's experience no immediate and/or delayed adverse effects (allergenic reactions) could be detected on humans having come into physical contact with the herbicide-tolerant oilseed rape.

3.5.4 Impact of the herbicide management on farmland biodiversity

Growing transgenic oilseed rape MS8, RF3 and MS8xRF3 will give farmers the opportunity to use glufosinate ammonium as a broad-spectrum herbicide, which might have implications for the environment. Directive 91/414/EEC that addresses the safety assessment of pesticides does not consider the indirect effects of herbicide treatments on the farmland biodiversity through the weed population. The Group of Experts felt that the novel weed management associated with the commercial release of GM herbicide-tolerant crops should be considered as a novel agronomic and management technique and should therefore be addressed under the Directive 2001/18/EC, especially because Directive 2001/18/EC foresees the safety assessment of direct effects of a GMO as well as the indirect, immediate and delayed effects arising from management practices specific to that GMO.

The Farm-Scale Evaluation trial was the first study to measure the indirect effects of herbicide treatments on the farmland biodiversity (macro-fauna, weed flora and food web integrity) through the

weed population (URL: www.defra.gov.uk/environment/gm/fse/index.htm). The FSE results concluded that growing GM herbicide-tolerant spring oilseed rape with the application of cost-effective weed control, as was the case in the FSEs, would result in adverse effects on farmland biodiversity, compared with conventionally managed spring oilseed rape. Following herbicide applications to GM herbicide-tolerant spring oilseed rape crops, there was a 3-fold lower weed biomass and 5-fold lower seed rain compared with conventionally managed spring oilseed rape. This resulted in a 1.3-fold lower return of seeds to the weed seedbank. The FSE results also pointed out that bees, butterflies, common seed-eating carabids and detritivorous invertebrates were found in larger numbers in treatments and crops where there were more forage resources. Since the experimental design of the FSE trial only provides information on 1) the herbicide management associated with 2 types of crops (GM vs. non-GM) and 2) the short-term effects, the results do not allow to make conclusions about 1) the potential impact of the crops themselves and 2) the permanent impacts. One can only speculate on the effects in the long-term and on the effects of a large-scale cultivation of the GM-herbicide tolerant crops.

Although the weed management might be easier with a GM herbicide-tolerant spring oilseed rape, growing the crop more frequently in a rotation is not likely to occur owing to a number of plant diseases. It continues to be difficult to predict how quick (if at all) the novel herbicide management will endanger the survival of farm weed species. In any crop rotation the abundance of weed species varies cyclically because each crop has a characteristic weed population. However, the application of a (very successful) novel herbicide management might increase fluctuations in the weed abundance. Apart from the application of a new herbicide, several other factors affect farmland biodiversity, e.g. the time and frequency of application of a herbicide, band spraying versus spraying the whole field, the establishment and maintenance of uncropped and unsprayed crop margins, the length of the crop rotation cycles, the sequence of crops within the rotation, the tillage system and the land use. Moreover, growing a crop with a minimum of weeds is considered as a good agricultural practice, since weeds compete with the crop and have negative effects on the yield performance and the quality of the yield.

If agricultural weeds become less abundant in fields and if one continues to strive (both for economical as for food safety and food quality reasons) for clean fields, uncropped fields and/or field margins and untreated field margin strips might be the only physical habitats to preserve these plants and the whole chain of living organisms living on and from these plants. Within these fields, margins or strips, the agricultural weeds and the accompanying fauna may find a place to survive, provided that the farmers allow them to.

There is a need to produce scientific data underpinning this hypothesis. If, scientific data support the hypothesis, the question raises who should be responsible to ´restore´ the biodiversity: the technology provider (the notifier) or the technology user (the farmer) or both? The question is important, since next to the potential positive effects on biodiversity, any reduction in cropped area or any reduction in cleanliness has economic consequences that might disable herbicide-tolerant crops. Expanding this rationale, one comes to the conclusion that any new technique affecting biodiversity should be confronted with these questions.

Extrapolating the findings for GM herbicide-tolerant spring oilseed rape to GM herbicide-tolerant winter oilseed rape might be difficult, as the conclusions made for GM herbicide-tolerant spring

oilseed rape do not necessarily apply to winter GM herbicide-tolerant oilseed rape.

3.5.5 Final conclusions

Taking the previous findings into account and considering that farmers will comply with the agricultural guidelines of the notifier (see 3.6 Agricultural guidelines), there is no reason to think that MS8, RF3 or MS8xRF3 oilseed rape will behave substantially differently than conventional cultivars in the absence of glufosinate ammonium. Actually there are no indications of health risk or of jeopardising the wild flora and fauna outside the fields upon cultivation of MS8, RF3 or MS8xRF3 oilseed rape. MS8, RF3 or MS8xRF3 do not show a change in persistence or invasiveness; have no selective advantage except when treated with glufosinate ammonium; have no impact on non-target organisms feeding on the plants and have no health effects on people coming into contact and/or handling the transgenic material. However, according to the FSE trials, GM herbicide-tolerant spring oilseed rape offers the opportunity to keep the fields cleaner than fields cropped with non-GM herbicide-tolerant varieties (and their appropriate weed management) resulting in the short-term into a probable decline of the crop accompanying weed population and all organisms living on these weeds. If these wildlife forms are to be preserved, compensating actions are necessary and one should find out which agricultural practices and risk management strategies can be proposed. One should also define who will be responsible for compensating provisions: the technology provider, the technology user or both of them.

On the long-term one can expect difficulties with oilseed rape volunteers tolerant to herbicide that was the target of the genetic transformation and with the potential development of resistant or tolerant weeds.

3.6 AGRICULTURAL GUIDELINES

3.6.1 Introduction

The safe and sound integration of herbicide-tolerant oilseed rape into the European agriculture will require significant on-farm and off-farm stewardships efforts. The education of farmers, seed suppliers and other operators concerned in the management of herbicide-tolerant oilseed rape will thereby be crucial. To give practical guidance to the farmer and seed suppliers on the handling of the seed, crop and product, in order to limit the occurrence of physical mixing (mixing of transgenic with non-transgenic seeds) and biological mixing (cross-pollination), to avoid negative impacts on the environment (see 3.5 Assessment of risks to the environment) Bayer CropScience drafted the agricultural guidelines for growing MS8xRF3 oilseed rape.

It is important to note that the guidelines are not only based on Bayer CropScience's own experience, but also on the existing codes of good agricultural practices and SCIMAC guidelines for growing herbicide-tolerant crops in UK (URL: www.scimac.org.uk).

Due to the non-binding character of the agricultural guidelines, farmers will not legally be bound to follow the guidelines. To avoid no compliance with the agricultural guidelines, the document was improved by explaining "why" certain measures need to be taken and "why" certain recommendations need to be followed. The possible consequences of failing to meet the recommendations of the agricultural guidelines are explained. By giving this explanation, farmers will be aware of the possible risks and problems involved and stimulate them to follow the recommendations.

3.6.2 Proposed management measures by Bayer CropScience

3.6.2.1 Measures to limit physical admixing

Farmers and seed suppliers are recommended to store the transgenic seeds separately, to keep the seeds in their original packaging and to retain the label with the seeds. The seed drilling and harvesting equipment must be thoroughly cleaned before use. After use and before leaving the field, drills and harvesting machinery must also be cleaned to prevent any spills in non-agricultural areas. Spillage of seed must be prevented when travelling to and from the field and during intermediate storage of the harvested crop. In case seed spillage occurs, potential volunteers must be controlled by appropriate management measures.

3.6.2.2 Measures to limit biological admixing

Cross-pollination must be minimised by respecting appropriate separation distances between oilseed rape fields. In this context neighbour-to-neighbour communication (e.g. about the seeding intentions) is stimulated. The appropriate distances are the subject of current research and of modelling efforts.

To limit the occurrence of herbicide-tolerant volunteer oilseed rapes in subsequent crops, the guidelines recommend limiting seed spillage during harvest and promoting germination of the shed seeds. The farmer must carefully choose the moment of harvest and properly adjust settings and speed of the harvesting equipment. Deep soil inversion treatments must be avoided for at least 3 weeks after harvest in order to maximise germination of shed seeds. To destroy the volunteers emerging in the following crops within a rotation cycle, a list of the suitable herbicide active ingredients that can be used to control glufosinate ammonium tolerant volunteer oilseed rape, is provided**.** Records of on-farm activities must be kept, which will allow farmers to know when the transgenic oilseed rape was grown, to adequately consider subsequent cropping/rotation and to give information on the history of the field, which is needed before deciding on when to grow herbicide-tolerant oilseed rape since the presence of oilseed rape seed in the seed bank must be considered.

3.7 MONITORING PLAN

The monitoring plan incorporates (a) a case-specific monitoring focusing on potential adverse effects identified in the ERA and (b) a general surveillance for unanticipated adverse effects.

The case-specific monitoring plan, to be undertaken by Bayer CropScience itself, will focus on the establishment of herbicide tolerance, be it through herbicide-tolerant volunteers or wild Brassicaceae relatives. The notifier aims:

1) to confirm that growing of MS8xRF3 oilseed rape does not lead to additional or unmanageable volunteer problems as compared to non-transgenic oilseed rape and that the risk management strategies are effective.
2) to characterise the presence of wild relative species susceptible to outcross with oilseed rape and to assess the transfer and establishment of the herbicide tolerance trait in such populations.

To fulfil these goals 20 locations in France, Germany and UK will be surveyed during a period of 3 to 5 years, until a full rotation cycle is completed. Each location comprises of a pair of fields lying in

close vicinity to each other. One field will contain the transgenic oilseed rape hybrid MS8xRF3, the other non-transgenic oilseed rape. For each field, 20 quadrates (1m x 1m) will be monitored in the field and 20 in the field border.

The inspections of the selected fields will be done thrice during the growing season. The number of oilseed rape volunteers and wild relatives will be counted. The spread of the herbicide tolerance trait will be assessed. At the end of each growing season an *interim* report will be prepared and at the end of the monitoring, a final report will be submitted to the EC.

The general surveillance monitoring will focus on the occurrence of adverse effects of MS8xRF3 oilseed rape, which were not anticipated in the ERA, including potential long-term environmental impacts.

Bayer CropScience proposes the general surveillance monitoring to be carried out co-operatively with already existing networks active in plant variety testing, plant protection and in other fields such as agriculture, ecology, biodiversity and the influence on animal and human health. The different institutes/networks involved will define special targets depending on their own interests (risks, benefits, economy, environment, ecology, impact of agricultural practices, etc.) to elaborate programs for the general surveillance.

An internal system is in place to report adverse effects and to determine what needs to be done. In case any adverse effect on human health and the environment is observed, the institute/network involved will forward the information to Bayer CropScience. The notifier will be responsible for informing the EC and CA(s) of the MS.

The Belgian experts and the Group of Experts are not aware of all the current agricultural practices and regional differences of the countries in which the monitoring program will be applied (France, UK and Germany). For this reason the case-specific monitoring plan was approved on behalf of the potential comments of the concerned MS of the EU that will be consulted during the authorisation procedure.

The proposed general surveillance will allow to cover all agricultural and environmental aspects and to make it possible to record any adverse affect. Till now, however, the notifier did not provide a detailed description of the tasks and the kind of data that will be collected by the different networks. It is not clear if programs are already put in place by the different networks. The Group of Experts recommends the notifier to supply relevant information after the different networks have elaborated their programs for the general surveillance.

3.8 FEED AND FOOD SAFETY ASSESSMENT

3.8.1 Substantial equivalence

The initial dossier covers processing for animal feeding stuffs (meal remaining after oil extraction) and oil production for human consumption. The Novel food legislation was awaited to approve the oil for human consumption.

To confirm the substantial equivalence of the oil and meal derived from the transgenic oilseed rape and its non-transgenic counterpart, compositional studies were done. Data are provided on the content of oil, fatty acids, including erucic acid, glucosinolates, vitamin E, phytic acid, crude fibre, amino acid profile and ash of the seeds. The ranges of these molecules were proven to be equivalent in transgenic and non-transgenic oilseed rape seeds.

There are no indications that the *barnase* and *barstar* genes are expressed in the seeds. The PAT protein was detected in low amounts in the seeds. Taking into account that oilseed rape meal was proven to be substantially equivalent to commercially available products, and that *in vitro* and *in vivo* tests have proven the safety of PAT, it was concluded that there is no significant risk to livestock following ingestion of the MS8xRF3 meal.

The conclusions of the Ministry of Agriculture Fisheries and Food (MAFF, UK now replaced by the Food Standards Agency) concerning the substantial equivalence of oil derived from the genetically modified oilseed rape were approved at Belgian level.

3.8.2 Residue assessment

In 1996, questions were raised concerning the lack of toxicological tests for the metabolites and residues derived from glufosinate ammonium herbicides. At that time, the use of glufosinate ammonium as a total herbicide was authorised in Belgium, but not the use of the herbicide on herbicide-tolerant plants. For this reason, AgrEvo handed in a dossier for the broadening of the use of the herbicide 'Liberty' under the Directive 91/414/EEC. The risk assessment of the herbicide use and thus the toxicity of glufosinate ammonium and metabolites were assessed by the Belgian Health Council within the framework of the plant protection products Directive 91/414/EEC. End 2000, the Health Council gave a positive opinion for the use of glufosinate ammonium on herbicide-tolerant plants. The final authorisation of the Ministry is not yet granted.

3.8.3 Indirect food use of honey

The adventitious presence of transgenic DNA in honey will be difficult to avoid. It is recognised that pollen may be present in small amounts (up to 2 %) in honey, which raises the question on the 'indirect food use' of oilseed rape pollen in honey. In the dossier, food safety concerns associated with the presence of transgenic material in honey were taken into account. The studies indicated that the presence and expression of transgenes in the transgenic oilseed rape do not pose an increased risk to human health or the environment when compared to non-GM oilseed rape varieties. Nor herbicide residues, nor PAT protein, nor Barnase and Barstar proteins were found in unprocessed honey and pollen. No adverse effects are to be expected from a safety assessment point of view.

3.8.4 Conclusions

Based on these data, there are no reasons to assume that the Bar, Barnase and Barstar protein will have a toxic or allergenic effect upon incidental consumption or as use of feed of MS8, RF3 or MS8xRF3. Neither are there reasons to assume that the reaction and/or degradation products of the catalytic activity of these proteins will have a toxic effect.

4. CONCLUSIONS AND RECOMMENDATIONS BY THE GROUP OF EXPERTS

4.1 CONCLUSIONS

The documents submitted by Bayer CropScience are in accordance with the provisions of Directive 2001/18/EC and include all the necessary information to carry out a safety assessment. Based on the current state of knowledge on the events MS8, RF3 and MS8xRF3 oilseed rape, the assessment of the notification by the Group of Experts has lead to the conclusion that no adverse effects to human health or the environment are to be expected from the placing on the market of the transgenic oilseed rape MS8, RF3 and MS8xRF3 provided that appropriate risk management strategies are associated to the commercial release and that the recommendations made are respected (see 4.2 Recommendations).

To allow safe and sound integration of MS8, RF3 and MS8xRF3 oilseed rape in Europe the notifier proposed agricultural guidelines giving guidance to farmers and seed suppliers. Since the experts considered that the guidelines would be crucial in limiting vertical gene flow and its potential consequences, it was strongly recommended that the practical mitigation measures as described in the guidelines would be followed.

Based on the scientific data provided by the FSE results, short-term impacts on the biodiversity in the fields, namely a decline in crop accompanying weed population and all organisms living on these weeds, can be expected. If these wildlife forms in the field are to be preserved, compensating actions are necessary.

Moreover, it was proposed that the effectiveness of the proposed risk management strategies in limiting vertical gene flow (and its potential consequences) and in preserving farmland biodiversity should be assessed in the post-market monitoring plan. In addition, an independent farmers' audit to monitor compliance with the agricultural guidelines should be foreseen on a regular basis.

The experts also mentioned that effects in the long-term of a large-scale cultivation of MS8, RF3 and MS8xRF3 and its associated herbicide regime remain hard to predict. Based on scientific evidence, one can on the long-term expect difficulties with herbicide-tolerant weeds and volunteers. The potential impacts on the (farmland) biodiversity due to the change in weed management to control these weeds, remain unpredictable, since the potential impacts not only depend on the herbicide used, but also on agricultural practices and habitat resources outside the field.

4.2 RECOMMENDATIONS

4.2.1 Detection/identification method

The Group of Experts is of the opinion that the assessment of the detection/identification method should be further pursued by the EC. The method proposed is qualitative and its specificity has been partially checked using reference material from commercialised GMOs as competitors. The specificity versus other oilseed rape events is presently very limited and should be followed up in the future as a function of the evolution on the European market. The reliability of the method has been assessed neither by the notifier nor by the reviewers. This should be taken into consideration, perhaps as an item for the post-marketing monitoring.

4.2.2 Monitoring plan

The post-market monitoring plan should also assess and determine the effectiveness of the proposed risk management strategies in limiting vertical gene flow and its potential consequences and in preserving farmland biodiversity. Moreover, an independent farmers' audit to monitor compliance with the agricultural guidelines should be foreseen on a regular basis.

The Group of Experts recommends that a more detailed description of the tasks and the kind of data that will be collected by the different networks involved in the general surveillance should be provided.

4.2.3 Agricultural guidelines

Taking the importance of the agricultural guidelines into account, the Group of Experts recommends that the agricultural guidelines should be translated in the national or local language(s) of each MS of the EU. The guidelines should be readable and understandable by all the operators (e.g. farmers, seed suppliers) that will handle the seed, crop and product of MS8, RF3 and MS8xRF3. A widely comprehensible language should be used. Moreover, the agricultural guidelines should be adapted to the recommendations made by the Member States of the European Union because of differences in agricultural practices and landscape and be continuously updated according to the new available scientific information.

Given that the occurrence of spontaneous inter-specific crossings among oilseed rape, *Brassica rapa* and *Brassica juncea* crops has already been reported, the Group of Experts recommends that each farmer growing *B. napus*, *B. juncea* or *B. rapa* crops in the neighbourhood of the transgenic oilseed rape field is aware of the agricultural guidelines.
Considering that seeds will be imported and transported and that seed spillage might occur, feral oilseed rape plants might establish and persist outside the fields (e.g. on roadsides, railways, wastelands, points of import). For this reason, the Group of Experts recommends that every operator should be informed on the appropriate management measures to be taken in the case of accidental seed spillage and the establishment of feral oilseed rape populations. It should also be made clear to whom these events must be reported. Where seed spillage occurs, the agricultural guidelines should recommend that the seeds must be swept or shovelled into sealed containers. In addition, seed spills should be recorded and monitored in subsequent years in order to allow appropriate control.

To limit seed spillage during transport and the potential establishment of feral herbicide-tolerant oilseed rape population, the agricultural guidelines should recommend transporting the seeds in closed and sealed containers.

To limit the development of multiple herbicide-tolerant plants through vertical gene transfer of the herbicide-tolerant trait, the agricultural guidelines should recommend that:

- the cultivation of transgenic oilseed rape with tolerance to other herbicides in the same or nearby rotation must be avoided,
- oilseed rape must not be grown on the same land more than one year in four.

Given that reliance on a single mode of herbicide control exerts a high selective pressure on the weed population and volunteers, which may lead to the development of resistance to the concerned herbicide and that rotating active ingredients reduces resistance development, the agricultural guidelines should recommend that:

- the glufosinate ammonium must be used in combination with other herbicides during the same rotation cycle,
- the use of different crops tolerant to glufosinate ammonium must be avoided in the same rotation cycle,
- integrated weed management systems should be applied.

Given that agricultural weeds become less abundant in fields where glufosinate ammonium is applied and that the environmental disbenefits of very clean fields might be judged unacceptable, uncropped fields and/or field margins and untreated field margin strips might be the only physical habitats to preserve these plants and the whole chain of living organisms living on and from these plants. Within these fields, margins or strips, the agricultural weeds and the accompanying fauna may find a place to survive, provided that the farmers allow them to. For this reason the agricultural guidelines should recommend farmers to preserve field margins (hedges, hedgerows, grassy borders, small woodlots and others), leave field margins unsprayed and limit herbicide drift. The agricultural guidelines should also recommend farmers to use band spraying if the negative impact of cleanliness is to be reduced.

To limit any selective pressure of the glufosinate ammonium on plants outside the field (e.g. herbicide-tolerant feral oilseed rape plants, wild relatives in field margins), the agricultural guidelines should recommend farmers to avoid herbicide drift in field margins according to the current principles of good agricultural practices.

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6. ANNEXES

6.1 ANNEX 1: QUALITATIVE PRE-VALIDATION OF THE METHOD OF DETECTION/IDENTIFICATION

Scientific Institute of Public Health

Section of Biosafety and Biotechnology

Dr.W. Moens

Concerns: Annex to notification C/BE/96/01

Title: Establishment of a qualitative pre-validation method submitted by Bayer CropScience, in the frame of notification C/BE/96/01 as foreseen by article 4§6 of Directive 2001/18/EC, for detection and/or identification of the oilseed rape events MS8, RF3 and MS8xRF3.

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Introduction

Directive 2001/18/EC, noteworthy articles 4§6, 26 (2), Annex IIIA II.C.2.f-g and Annex IV, paragraph A.7 requires from the applicant a method proposal allowing the detection and/or identification of the concerned GMO and its progeny, to be placed on the market for any proposed uses.

The Belgian Scientific Committee "Transgenic Plants" of the Biosafety Advisory Council, within the framework of the Biosafety Advisory Council, proposed guidelines for the molecular/biochemical detection/identification/characterization of GMO events (URL: http://ww.biosafety.be/NF/GuidanceNotes/Documents/Chapter2_MGC.pdf).

The detection/identification of a specific GMO within a certain matrix needs careful evaluation of the choice of analyte adapted to the specific matrix.

Over the past years, there has been an international agreement to target event-specific sequences of GMOs. The preferred method to date has been the Polymerase Chain Reaction (PCR) technology. PCR, designed in 1987, has been widely accepted as a sensitive and efficient method to trace and identify selected genomic sequences.

Objective of this study

As an initial assessment of the proposed detection/identification method, this study aims at checking whether the PCR primers targeting the oilseed rape (OSR) events MS8, RF3 and/or two derived hybrids, behave as expected in a standard enforcement laboratory. Evaluation will be performed under Standard Operating Procedures (SOPs) of qualitative PCR technology recognized by ISO 17025 norms. If not available, a descriptive protocol using working reference material delivered by the company will be performed and a full overview of the concerned experimentations will be delivered.

Materials and methods

Reception of genomic DNA and seeds as working reference material

Genomic DNA purified from OSR *Brassica napus* elite events MS8 (10 µg), *Brassica napus* RF3 (10 µg), two *Brassica napus* hybrid events MS8XRF3 (2 x 5 µg) and a non-transgenic *Brassica napus* sample (wild type, 2 x 5 µg) were supplied by the Aventis CropScience company on September 12th, 2001 to the Scientific Institute of Public Health, Service of Biosafety and Biotechnology (SBB). The company also supplied the SBB with 100 seeds of each elite event MS8 and RF3.

Genetically modified maize seeds Bt11 (Novartis), Bt176 (Novartis), Mon810 (Monsanto) and wild type maize seeds were supplied by the different companies. These plant varieties were used in this study to further assess the specificity of the present OSR detection method provided by Bayer CropScience.

Extraction and purification of genomic DNA:

DNA of 200 mg of maize seeds were extracted according to the CTAB (cetyltrimethylammonium bromide) procedure as described in the SOP 15/F/II/2002 applied in the Section of Biosafety and Biotechnology.

Primers:

Primers were synthesized *de novo* (Amersham Pharmacia, Belgium) on basis of the regulatory dossier as summarized in table 1.

Table 1: primer names used in this study and length of related amplicons

Endogenous control	OSR elite event RF3	OSR elite event MS8
Primer set: CVZ7 - CVZ8 Amplicon of 394 bp.	Primer set: MDB268 - MDB193 Amplicon of 215 bp.	Primer set: VDS51 - MDB201 Amplicon of 274 bp.

Polymerase Chain Reaction (PCR):

The PCR tests were carried out in a Perkin Elmer thermocycler "GenAmp PCR system 9600". The composition of the PCR mixtures and the thermocycling profiles were according to method provided in the C/BE/96/01 notification.

Agarose gel electrophoresis:

The PCR amplicons were loaded on a 2,5% agarose gel - TBE 1X. The 123 bp DNA ladder from Gibco BRL was used to estimate the size of the amplicons.

Results

1. **Do the Company's PCR primers, protocol and working reference material allow to detect the proposed GMO sequences?**

Conditions of acceptability:

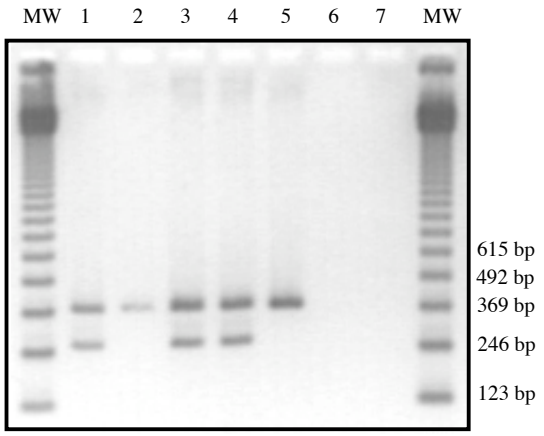
The PCR primers are presented as being specific for detection of transgenic elite events MS8 or RF3 DNA from related working standard material if and only if:

- The primers aimed at detecting the said GMO are able to trigger the amplification of amplicons of the expected sizes from the positive test DNA (DNA from elite events MS8, RF3 or the MS8XRF3 hybrids): 215 bp for the elite event RF3, 274 bp for the elite event MS8.
- The same said primers are unable to trigger such amplification in the DNA from negative control samples.
- An amplicon of 394 bp is amplified from the OSR endogenous gene in the transgenic and non-transgenic control samples.

Gel electrophoresis:

Results from three independent test experiments are summarized in figures 1 A and 1B.

A



B

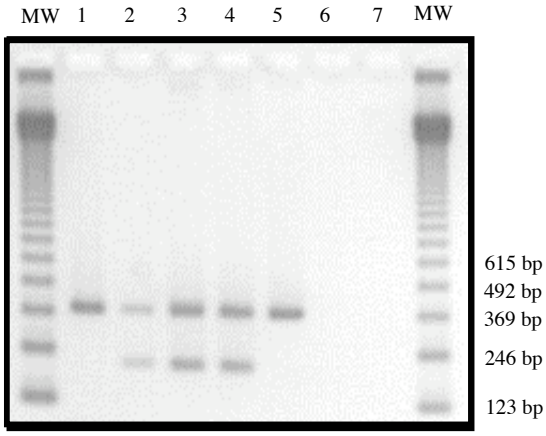


Figure 1:

A. PCR products resulting from genomic DNA amplification with specific primers for the OSR elite event MS8 (Primers VDS51 - MDB201 - fragment of 274 bp) and specific primers for the OSR endogenous gene (Primers CVZ7 - CVZ8 - fragment of 394 bp).

B. PCR products resulting from genomic DNA amplification with specific primers for the OSR elite event RF3 (Primers MDB268 - MDB193 - fragment of 215 bp) and specific primers for the OSR endogenous gene (Primers CVZ7 - CVZ8 - fragment of 394 bp).

Lane 1: MS8 event, lane 2: RF3 event, lanes 3 and 4: two hybrid of MS8 and RF3 events, lane 5: non-transgenic oilseed rape, lanes 6 and 7: negative PCR control.

NB: The negative PCR control (master mix PCR reaction with water replacing the sample DNA), which contains no DNA, should not exhibit any PCR product, demonstrating the absence of contamination with target DNA.

Conclusion: the proposed primers are appropriate tools to detect the proposed analytes described as present or absent in the transgenic and non-transgenic OSR background.

2. Is the proposed method able to identify the elite events MS8, RF3 and the MS8XRF3 hybrids?

Identification of the different GMOs:
To specifically identify a GMO, a DNA based method needs to be developed to determine a unique DNA sequence linked to the specific GMO. In most cases, the event-specific sequence is located at the boundary of the novel introduced DNA sequence (the transgenic section) and the plant acceptor genomic DNA. It is the so-called "junction sequence" which is considered as a specific genetic signature for the proposed GMO.
In the present study, two criteria to answer the question concerning the specificity of the proposed identification method are being addressed.

2.1: Do other GMOs interfere with the proposed method?

Conditions of acceptability:

The PCR primers pairs are considered as specific for detection of DNA from OSR elite events MS8 or RF3 as received from the Company if:

- The primers aimed at detecting the targeted GMOs are able to amplify the targeted DNA, giving amplicons of the expected sizes from the positive test DNA (DNA derived from the OSR elite events MS8, RF3): a 215 bp amplicon in the case of the elite event RF3; a 274 bp amplicon in the case of the elite event MS8.
- The same said primers are unable to trigger such amplification in the DNA from a number of transgenic maize lines used in this study.
- An amplicon of 394 bp is amplified from the endogenous gene OSR species and not in the tested transgenic and non-transgenic maize lines.

Gel electrophoresis:

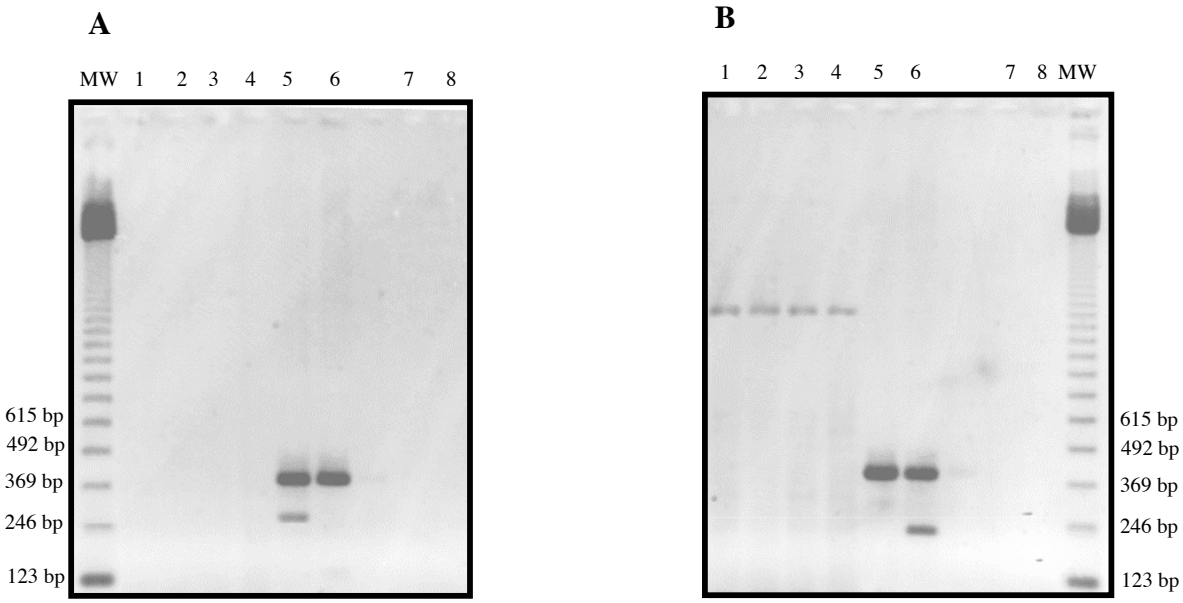


Figure 2:

A. PCR products resulting from genomic DNA amplification with specific primers for OSR elite event MS8 (Primers VDS51 - MDB201 - fragment of 274 bp) and specific primers for the OSR endogenous gene (Primers CVZ7 - CVZ8 - fragment of 394 bp).

B. PCR products resulting from genomic DNA amplification with specific primers for OSR elite event RF3 (Primers MDB268 - MDB193 - fragment of 215 bp) and specific primers for the OSR endogenous gene (Primers CVZ7 - CVZ8 - fragment of 394 bp).

Lane 1: Non-transgenic maize, lane 2: Bt11 maize, lanes 3: Bt176 maize, lane 4:Mon810 maize, lane 5: oilseed rape MS8 event, lane 6: oilseed rape RF3 event, lanes 7 and 8: negative PCR control.

NB: The negative PCR control (master mix PCR reaction with water replacing the sample DNA), which contains no DNA, should not exhibit any PCR product, demonstrating the absence of contamination with target DNA.

Conclusion: the proposed primers are appropriate tools to detect the proposed analytes described as present or absent in the transgenic oil seed rape lines. Neither the analyte for the endogenous oilseed rape gene nor the analyte for the transgenic OSR elite events MS8 or RF3 could be detected in the Bt11, Bt176, Mon810 or non-transgenic maize seeds tested in this study.

2.2: Do other authorized transgenic OSR elite events interfere with the proposed method?

As documented in the Annex 2 of the assessment report C/BE/96/01, no cross-reactivity of the event-specific primers for MS8 or RF3 could be demonstrated when assayed on MS1, RF1 and RF2 oilseed rape events.

Discussion and Conclusions

The notifier has provided a qualitative PCR method to detect three types of genetic markers as criterium to evaluate the presence of their product: one marker is a genomic sequence specific for the junction of the MS8 insert; one marker for a specific sequence of the RF3 insert and one marker allowing to detect an unique gene of the OSR genome. The markers chosen by the notifier to model the analytical detection and identification of lines MS8 and RF3 are clearly and logically derived from the molecular characterization of the two GM lines.

The notifier has provided working standard material for a non-transgenic OSR as negative control and transgenic MS8XRF3 hybrids under the form of genomic DNA. For each of the parental OSR lines MS8 and RF3, material has been provided under the form of genomic DNA and seeds.

The PCR procedure and results obtained by the notifier were reproduced by a local ENGL laboratory. Indeed, the VDS51 and MDB201 primers designed for the specific detection of the MS8 elite event only detected the targeted analyte in those oilseed rape lines with this transgenic event.
The MDB268 and MDB193 primers designed for the specific detection of the RF3 elite event only detected the targeted analyte in those oilseed rape lines, which have inherited this transgenic event.

The specificity of the event-specific primers sets was further demonstrated by the absence of cross-reactivity. The event-specific primer set designated for detecting elite event MS8 does not allow insert amplification in the line FR3, nor does the event-specific primers set for RF3 allow insert amplification within a MS8 background.

In addition, the specificity of the respective primers sets used in this study was tested by PCR on genomic DNA derived from four different maize lines (Bt11, Bt176, Mon810 and wild type). None of the OSR sequences could be amplified in maize.
The notifier further documents no cross-reactivity of the MS8/RF3 elite event-specific primers when assayed on the related MS1, RF1 and RF2 OSR events (see annex 2).

From all these results, it can be concluded that the genetic markers selected by Aventis CropScience are well detected in OSR elite events MS8 and RF3 as well as in examples of their progeny using the proposed method and the working standard material as control.
The specificity of the method was so far successfully challenged by four maize lines as well as by three other transgenic OSR events.

However, there is no general validation of the method. So far, only DNA extracted from unprocessed raw material has been tested. This detection method should also be demonstrated to work using other matrices. Further, the sensitivity of the detection method should still be assayed by testing the presence of the OSR MS8, RF3 elite events or the hybrid MS8XRF3 in mix samples (transgenic OSR in different amount of non-transgenic OSR). Finally, based on the given method, one cannot make a distinction between the hybrid and a mixture of the two parental lines MS8 and RF3.

Moreover, the notifier has further proposed a genetic marker as a marker representative of *Brassica napus*. However, the notifier does not illustrate whether the said marker is ubiquitous in all *Brassica napus* cultivars that the notifier has already or might later cross with the Elite event submitted in the present dossier. In addition, no markers and/or related method are provided to quantify the presence of the submitted GMOs as or in products and derived by-products. Consequently, article 26(2) of the Directive 2001/18/EC cannot be applied and controlled by the Belgian competent authority given the definition of threshold levels in the new Regulations 1829/2003 and 1830/2003. It is suggested to the Member States to commit the Community Reference Laboratory and the European Network of GMO Laboratories defined by annex 1 of the two above mentioned Regulations to clarify this methodological problem together with the validation of sampling methods applied to environmental monitoring of crop cultivation.
