



Service of Biosafety and Biotechnology

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Report on the molecular characterisation of the genetic map of event Ms8 x Rf3

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1. Introduction: Ms8 x Rf3 canola dossier

Ms8 x Rf3 notification C/BE/96/01 of PGS (Bayer Cropscience) is notified under Directive 90/220/EEC for cultivation, import, seed production and processing into animal feeding stuffs and industrial purposes. On 24 January 2003, the Commission has received an updated version of this notification according to the requirements of Directive 2001/18/EC. Oil derived from Ms8 xRf3 products has been notified under Article 5 of the Regulation (EC) 258/97 on 21 October 1999.

The molecular data of the notification C/BE/96/01 have been discussed during the meeting of the Belgium Biosafety Advisory Council on 6 December 2002. An overview of the molecular data of the event Ms8 x Rf3 presented during this meeting and provided by the applicant and CLO (Centrum Landbouwkundig Onderzoek, Melle, Belgium), is given below.

2. Overview on molecular data of event Ms8 x Rf3

2.1. Plasmid used for transformation

The male sterile Ms8 line was obtained by means of *Agrobacterium* mediated transformation using plasmid pTHW107 (see fig. 1). 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 a tapetum specific promoter pTA29 isolated from *Nicotiana tabacum* and the 3' nopaline synthase (*nos*) gene of *Agrobacterium 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 (see fig. 2). Plasmid pTHW118 contains a *barstar* gene derived from *Bacillus amyloliquefaciens* under regulation of the pTA29 promoter and the *nos* terminator, and the same *bar* cassette as described for pTHW107.

2.2. Characterisation of the insert and flanking regions

According to the applicant's data, in line Rf3, the transgene construct is integrated in a single genetic locus (see annex 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 (see fig. 3). A fragment of 812 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 815 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. Blast analysis showed that the 3' flanking region displays over 88% similarity with an Arabidopsis sequence. Results obtained by CLO confirm the presence of plant DNA at the right border of the insert (see annex 2).

According to the dossier, the Ms8 insertion contains a single T-DNA copy (see annex 1). At the left border junction (3' end of the T-DNA), a 357 bp host sequence was retrieved (see fig. 4). 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. Molecular analysis done by the CLO confirmed that the adjacent DNA is plant DNA (see annex 2). Search in the database showed that part of the 5' flanking region has similarity (over 82%) with Arabidopsis sequences (see annex 1).

Determination of the pre-insertion sites was done by the applicant using DNA isolated from wild type oilseed rape (see annex 1). 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. 3). 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. 4). At the right border junction 3 nucleotides ('filler'-DNA) of unknown origin are inserted at the Ms8 transgene locus. The LB junction-point is the same as the target site breakpoint in the wild type line.

In summary, both for line Rf3 and line Ms8, the transgene construct is integrated in a single genetic locus. The Rf3 transformation event resulted in the insertion of one T-DNA copy arranged in an inverted repeat structure with a second incomplete T-DNA copy. Event Ms8 contains an intact single T-DNA copy. During insertion typical rearrangements have occurred at the pre-insertion site of event Rf3 en Ms8. In both lines, the inserts are flanked by plant DNA showing high similarity with Arabidopsis DNA.

2.3. Comparison of molecular data of Ms8 x Rf3 provided in dossier C/BE/96/01 with data from CLO

Table 1: Comparison of molecular data of event Ms8 x Rf3 according to dossier with data provided by CLO

Dossier [#]	CLO *	Remarks
1 insert of Rf3 T-DNA with an incomplete inverted repeat 5': 812 bp plant DNA flanking the RB sequenced 3': 1274 bp plant DNA flanking the truncated T-DNA sequenced Pre-insertion site sequenced: rearrangements at insertion site (51 bp plant DNA deletion, 5 bp filler, 816 bp plant duplication)	Report confirms data in dossier for the RB, no data available for the truncated LB and the plant DNA rearrangements	Dossier contains very detailed analysis of T-DNA insert, flanking plant DNA sequences and pre-insertion plant DNA, with scheme of transgene locus (see Fig. 3)

[#] see annex 1

* see annex 2

Table 1 (continued)

<p>1 insert of Ms8 T-DNA</p> <p>5': 864 bp plant DNA flanking the RB sequenced</p> <p>3': 357 bp plant DNA flanking the LB sequenced</p> <p>Pre-insertion site sequenced: small rearrangements at insertion site (3 bp filler and 19 bp deletion)</p>	<p>Report confirms data in dossier</p>	<p>Dossier contains very detailed analysis of T-DNA insert, flanking plant DNA sequences and pre-insertion plant DNA, with scheme of transgene locus (see Fig. 4)</p>
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3. Conclusions

The molecular data presented in the dossier C/BE/96/01 fulfil the Belgian requirements concerning molecular data. The sequence of the inserts and the flanking regions, including about 500 bp proven to correspond to plant DNA, are provided in the dossiers. However, bio-informatic analysis should be done to determine the presence of chimaeric open reading frames in the border integration sequences.

Acknowledgements. We would like to thank the experts of the working group 'Molecular Characterisation' of the SBB to contribute to this report.

4. Confidential information

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Figure 1: Plasmid map of pTHW107

Figure 2: Plasmid map of pTHW118

Figure 3: Physical map of the insert of event Rf3

Figure 4: Physical map of the insert of event Ms8

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Annex 1: Molecular data from dossier C/BE/96/01

Annex 2: CLO Report p. 10-11