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Enclosure 1

Report on the molecular characterisation of the genetic map of event Bt11

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This document contains confidential information

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1. Introduction: Bt11 maize dossiers

Bt11 maize notification C/UK/96/M4/1 of Novartis (Syngenta) has been approved under Directive 90/220/EEC for import and processing since 22 April 1998 (Commission Decision 98/292/EC). The notifications C/F/96/05-10 and C/ES/98/02 for cultivation of the same Bt11 maize are still pending for approval. On 30 November 2000 the EU Scientific Committee on Plants (SCP) gave a favourable opinion for the latter two notifications. Up till now, the Commission has not received an updated version of these two notifications according to the requirements of Directive 2001/18/EC. In February 1999, Bt11 sweet maize has been submitted under Regulation (EC) 258/97 by Novartis and currently the application is still pending. On 13 March 2002 the SCP gave a favourable opinion for this application.

Food and food ingredient products derived from Bt11 crossed with the Northrup King Company inbred line #2044 (maize) were notified under Article 5 of the Regulation (EC) 258/97 on 30 January 1998.

The molecular data of the notification C/F/96/05-10 have been discussed during the meeting of the Belgium Biosafety Advisory Council on 17 February 2003. An overview of the molecular data of event Bt11 presented during this meeting and provided by different scientific institutions, namely CLO (Centrum Landbouwkundig Onderzoek, Melle, Belgium), JRC (Joint Research Centre, Ispra, Italy), TEPRAL (Strasbourg, France) and INRA (Institut National de la Recherche Agronomique, Versailles, France) and found in publications is given below. It must be noted that most of the data provided by the scientific institutions are preliminary and further research is needed for confirmation.

2. Overview on molecular data of event Bt11 (C/F/96/05-10)

2.1. Plasmid used for transformation

The plasmid contains a synthetic truncated *cryIA(b)* sequence, isolated from *Bacillus thuringiensis* ssp. *kurstaki* HDI, and a synthetic *pat* gene, isolated from *Streptomyces viridochromogenes* Tü494 (see Annex 1). Both coding sequences are under the regulation of a 35S promoter sequence derived from cauliflower mosaic virus (CaMV) and the 3' untranslated region of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* (see Fig 1). In addition, the promoter sequences of the *pat* and *cryIA(b)* gene were combined with respectively intron Int II and Int VI derived from the maize alcohol dehydrogenase *adh1S* gene to enhance expression.

The event Bt11 maize was obtained by protoplast transformation with the plasmid pZO1502. In order not to transfer the *bla* gene encoding ampicillin resistance, the plasmid was digested prior to transformation with the restriction enzyme *NotI*.

2.2. Characterisation of the internal DNA sequences

2.2.1. Characterisation of the insert

The whole sequence of the insert of Bt11 was determined by TEPRAL as part of the QPCRGMFOOD program (see Annex 3). The insert consists of a single copy of the vector fragment carrying both the

cryIA(b) and *pat* gene. It was found that rearrangements have taken place into the insert compared to the original insert and that several parts of the plasmid have been truncated or unexpectedly inserted, e.g. t35S sequences (see Fig. 2). The presence of t35S fragments into the insert was confirmed by INRA (see annex 4).

Sequence analysis was also done by the CLO (see annex 5). By PCR analysis using a 35S promoter specific primer in combination with a 3' NOS specific primer, they proved that the DNA segment present in between the two expression cassettes of the Bt11 insert is similar to pUC vector backbone sequence (see Annex 5).

2.2.2. Characterisation of junction regions

Zimmermann *et al.* (2000) showed that adjacent the 5' 35S promoter border of the *cryIA(b)* a maize 180 bp knob-specific repeat sequence is present. In addition, the CLO analysed the sequence that is present between the 35S promoter sequence and the maize plant DNA (see annex 5). They demonstrated that a 1099 bp segment is present between the 35S promoter sequence and the adjacent maize plant DNA. This 1099 bp sequence is homologous to the pUC backbone sequence and contains part of the *lacZ* coding sequence.

The junction region at the 3' NOS terminator border was amplified by the CLO using a 3' NOS specific anchor primer. Using this approach, they amplified a 244 bp junction that contains 149 bp plant DNA. BLAST sequence analysis showed that the plant sequence is similar to the *Zea mays* 180 bp knob associated tandem repeat (GENBANK accession n° AF030934). Independently from the CLO, the 3' NOS border region was also amplified by Rønning *et al.* (2003). The data presented by Rønning and co-workers are in agreement with the experimental data of the CLO. The remaining part of the amplified 3' NOS junction is homologous with pUC backbone sequences (see annex 5).

In conclusion, these data provide evidence that the Bt11 insert is integrated in the *Zea mays* 180 bp knob associated tandem repeat locus. At the 35S promoter border an extensive piece (1099 bp) of pUC backbone DNA was observed between the plant DNA and the 35S promoter, while at the 3' NOS border only a small stretch of pUC backbone DNA is present.

2.2.3. Comparison of molecular data of Bt11 provided in dossier C/F/96/05-10 with data from other sources

Table 1: Comparison of molecular data of Bt11 according to dossier with data from other sources

Dossier	Other Sources*	Remarks
<p>transgene insert (single copy)</p> <p>p35S-intron-<i>pat</i>-tnos/ p35S-intron-<i>cryIA(b)</i>-tnos (6.349-bp <i>NotI</i> fragment)</p>	<p>transgene insert</p> <p>primary insert with rearrangements, truncations and unexpected insertions</p>	<p>-In the dossier a 134 bp deletion is observed at p35S regulating <i>pat</i> gene expression.</p> <p>-According to TEPRAL it is not certain if only one copy of the insert is present</p>
<p>linker DNA: no information</p>	<p>linker DNA: homology with pUC18</p>	

Table 1 (continued)

Dossier	Other Sources	Remarks
<u>5' end</u> : 1.1 kb of pUC18 present (954 bp proven by PCR)	<u>5' end</u> : 1.1 kb of pUC18 present (1.099 bp proven by PCR and BLAST analysis); followed by plant DNA (homology with 180 bp knob specific repeat sequence)	
<u>3' end</u> : no information	<u>3' end</u> : pUC18 backbone, followed by plant DNA (homology with 180 bp knob specific repeat sequence)	

*CLO, JRC, TEPRAL, INRA and scientific publications (see references)

3. Detection and identification of event Bt11 (C/F/96/05-10)

Although no identification and detection protocol is provided in the dossier C/F/96/05-10, Rønning *et al.* (2003) published a validated event-specific PCR assay for detection and quantitation of Bt11 maize.

4. Cross-pollination between Bt11 and Bt176

Preliminary data of INRA show that a set of primers designed on the edge fragment of Bt176 amplifies sequences from both Bt176 and Bt11 (see annex 4). These data were obtained on six different Bt11 plant seeds received by Syngenta. They suggest that the presence of a fragment of Bt176 DNA might be the result of an initial contamination of Bt11 by Bt176.

5. Conclusions

There are still uncertainties concerning the molecular data provided in the dossier C/F/96/05-10: rearrangements in the insert and truncations of parts of the insert might have occurred. Therefore, the sequence of the insert should be further checked together with the number of inserts.

Since it has been shown that unexpectedly t35S fragments are present in the primary insert, it should be clarified where these sequences come from. It should be determined whether they originate from the vector used for transformation or any other source.

The molecular data presented in the dossier C/F/96/05-10 do not fulfil the Belgian requirements concerning molecular data. The sequence of the insert, together with the sequence of the flanking regions should be provided. In addition, the flanking regions of the insert should be analysed for the presence of chimaeric open reading frames.

It must be noted that the same Bt11 event as in notification C/F/96/05-10 (and C/ES/98/02) has been submitted for approval under Regulation (EC) 258/97, implicating that the molecular data for both dossiers are similar (see Annex 1 & 2). However, one cannot entirely exclude that backcrosses of the

original event with a maize line for feed purposes or sweet maize, might give rise to rearrangements at molecular level.

6. References

Rønning, SB., Vařtilingom, M., Berdal, KG. and Holst-Jensen, A. (2003) Event specific real-time quantitative PCR for genetically modified Bt11 maize (*Zea mays*). *Eur. Food Res. Technol.*, DOI 10.1007/s00217-002-0653-4.

Zimmerman, A., Lüthy, J. and Pauli, U. (2000) Event specific transgene detection in Bt11 corn by quantitative PCR at the integration site. *Lebensm. Wiss. u. Technol.* **33**, 210-216.

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7. Confidential information

Figures:

Figure 1: Plasmid map of pZO1502

Figure 2: Physical maps of characterised inserts of Bt11

List of annexes:

Annex 1: Molecular data from dossier C/F/96/05-10

Annex 2: Molecular data from Novel Food dossier Bt11 sweet maize

Annex 3: Sequence of insert of Bt11

Annex 4: INRA Report on Bt176 and Bt11

Annex 5: CLO Report on Bt11