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Characterisation of the Roundup Ready soybean insert

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Abstract In this article we describe the isolation and characterisation of the junction between insert DNA and plant DNA in the transgenic Roundup Ready soybean line event 40-3-2. Our results establish that during integration of the insert DNA several rearrangements occurred at the 3′ NOS junction and that the genomic plant DNA at the pre-integration site may have been rearranged. These findings highlight the utility of characterising junction regions to fulfil the request for information regarding which DNA sequences have been incorporated in commercialised transgenic lines. Furthermore, the characterisation of junction regions is, in our opinion, the method of choice to support method development for detection and identification of plant biotechnology-derived products.

Keywords Roundup Ready soybean · Junction fragment · Rearrangements · GMO · Identification

Introduction

Since labelling of foods containing genetically modified ingredients is mandatory in all European member states [1], the demand for reliable and easy to perform detection and identification methods for genetically modified organisms (GMOs) is high. As a governmental research institute involved in the development of strategies to detect and identify GMOs we analyse DNA sequences which flank the insert DNA in order to develop GMO event specific identification methods. Up to now most qualitative as well as quantitative GMO analysis methods make use of the polymerase chain reaction (PCR) to amplify and detect regulatory sequences that are frequently used in transgenic constructs, for example promoters, terminators and so on [2–6]. As a consequence, methods that amplify a unique sequence within the inserted DNA only allow detection of the presence of GMOs. Other methods are required for the identification of GMOs in food. It is clear that a primer pair that covers the junction between the insert DNA and the plant DNA will enable us to discriminate between different GMOs containing the same insert and to identify different GMOs containing different copy numbers of the same insert. As such, event specific primer pairs are the primers of choice in quantitative GMO analysis [7].

In Europe as well as in the United States a technical dossier containing all the information available and of importance to allow experts to judge the safety of a GMO has to be submitted in order to obtain authorisation to market a GMO[1, 8, 9]. Any new and relevant information that becomes available with regard to the technical dossier or to the risk assessment study has to be brought to the attention of the competent authorities. Here we show that the information obtained by characterising the junction region between plant DNA and insert DNA can be used to check the accuracy of the technical dossier or to complete already existing information. Padgette et al. [10] described earlier that the Roundup Ready (RR) single genetic insert, that was introduced into soybeans by the particle acceleration method, contains a portion of the cauliflower mosaic virus (CaMV) 35S promoter, the Petunia hybrida 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) chloroplast transit peptide (CTP), the CP4 EPSPS coding sequence and a portion of the 3′ non-translated region of the nopaline synthase gene terminator. However, up to now no information has

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been available regarding the exact end-points of the insert DNA and the sequences following those end-points for RR soybean event 40-3-2.

We are interested in a more detailed characterisation of the insertion event, especially as the analysis of the transgene organisation in transgenic rice and oat that were transformed using particle bombardment showed that intact transgene copies are frequently accompanied by rearranged and truncated transgene fragments [11, 12]. Therefore, both regions adjacent to the end-points of the insert DNA of RR soybean event 40-3-2 and part of the pre-integration site were isolated using anchored PCR [13, 14] and adapter ligation PCR [15]. The anchored PCR approach is an AFLP related method that is based on a transposon display procedure [16]. Initially the DNA is digested using a restriction enzyme of choice. This restriction of the DNA is followed by a ligation step, during which an adapter is ligated to the restriction fragments. Subsequently a touchdown PCR reaction using an adapter primer and a radioactively labelled anchor primer – a primer that is homologous to the end region of the insert DNA – will amplify the junction fragment. In contrast to the transposon display method there is no selective pre-amplification step during anchored PCR. Once the junction fragment is isolated and re-amplified the sequence of the fragment can be determined. The adapter ligation PCR approach on the other hand amplifies the junction region using a nested PCR approach combining adapter primers and anchor primers after ligation of adapters to the generated restriction fragments.

Materials and methods

DNA isolation

Genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen) starting with 20 mg of ground soybeans. Wild type DNA from the soybean cultivar A5403 was used as negative control DNA during all experiments.

Digestion and adapter ligation

Templates for the anchored PCR and adapter ligation PCR reactions were prepared as follows. Genomic DNA was digested using one of the six following restriction enzymes: MseI, MboI, BfaI, TaqI, EcoRI or HindIII. 250 ng of genomic DNA was digested with 2.5 U restriction enzyme in 25 µl reaction buffer (10 mM Tris-HCl pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate) for 2 h at 37°C, except for TaqI restriction that needed an incubation of 2 h at 65°C. In the latter case, the restriction mix was covered with mineral oil. Inactivation of the restriction enzyme by heating (70°C) the samples for 15 min was required only when MboI or EcoRI were used. After digestion, 25 µl of reaction buffer containing 50 pmol adapter in the case of MseI, MboI, BfaI or TaqI digestion and 5 pmol in the case of EcoRI or HindIII digestion, 1 U T4-ligase and 9.6 pmol ATP was added to the tube. This mixture was incubated for 2 h at 37°C. The structure of the adapters and the adapter primers that were used, was as described elsewhere [17, 18,15].

Anchored PCR reactions

All anchored PCR reactions were performed according to Theuns et al. [14]. The position of the anchor primers is presented in Fig. 1, a sequence overview is given in Table 1.

Adapter ligation PCR

The adapter ligation PCR that was used, was a modification of the protocol described by Spertini et al. [15]. Some adjustments were made in order to optimise the protocol for working with soybean. A first PCR amplification was performed as follows: 8 µl of ligation reaction, 1X PCR-buffer (10 mM Tris-HCl pH 8.3; 50 mM KCl and 1.5 mM MgCl2), 400 µM dNTP's, 0.25 µM primer AP1 and 0.25 µM of the anchor primer (Fig. 1; Table 1) were mixed in a total volume of 50 µl. The PCR profile for this first PCR amplification was as described [15] except that the second amplification step from this first PCR amplification was lengthened from 32 to 37 cycles. Spertini and co-workers suggested lengthening the second amplification step when working with DNA derived from species with large genomes. Secondary PCR amplifications were conducted with 1 µl of a 10-fold dilution of the primary PCR products. The PCR mixture that was used during secondary PCR amplifications was similar to that used during the first PCR amplification, except for the use of primer AP2 and a nested anchor primer (Fig. 1; Table 1). The second amplification step of the second PCR was lengthened from 22 cycles to 30 cycles. In some

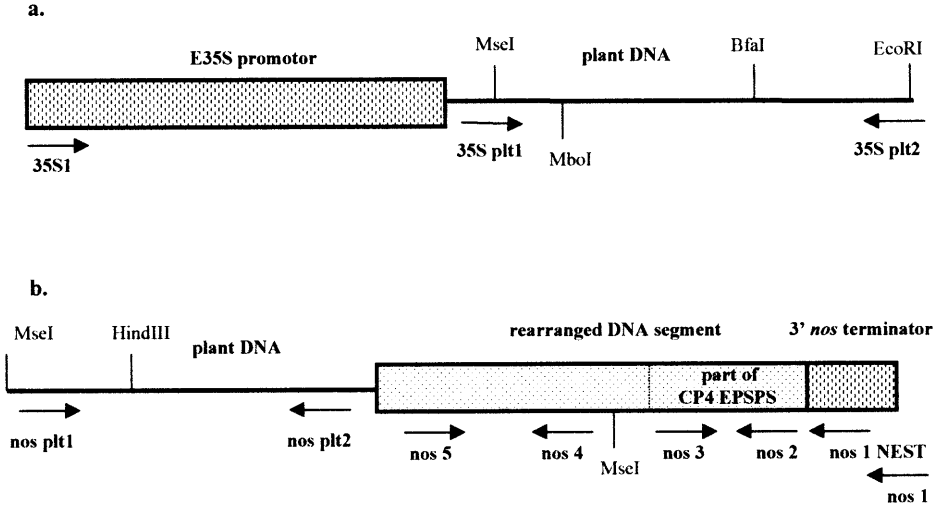

Fig. 1 Schematic representation of the amplified junction fragments (not drawn to scale). **a** structure of the amplified 35S promotor/plant DNA junction. **b** structure of the amplified 3′NOS/plant DNA junction. The position of the primers that were used to verify the structure of the junction fragments is depicted using arrows. Only those restriction sites that were important to amplify the junction fragments are presented.

Table 1 Sequence of the primers used in this study. For a schematic representation of the position of the different primers see Fig. 1

Primer name	Sequence (5′->3′)
35S1 primer	GAT AGT GGG ATT GTG CGT CA
35S plt1 primer	GGT TCC CTA TGT TTA TTT TAA CCT G
35S plt2 primer	CCT TCA ATT TAA CCG ATG C
nos 1 primer	AGC GCG CAA ACT AGG ATA AA
nos 1 NEST primer	GCG CGG TGT CAT CTA TGT TA
nos 2 primer	CTT CAT GTT CGG CGG TCT
nos 3 primer	AAT CGT AGA CCC CGA CGA G
nos 4 primer	TGG GAA ATT TTA GCG AGA TTA T
nos 5 primer	TCT GGG AGA AGC AGT TAC TTA
nos plt2 primer	GAT CGG AGA AGA ACT GTT TGA
nos plt1 primer	GAG AAC TAC CTT CTC ACC GCA TT

cases two consecutive PCR cycles were not enough to generate an amount of amplified fragment that could be detected on agarose gel using ethidium bromide staining. In these cases a third PCR cycle was performed. Tertiary PCR amplifications were performed on 1µl of the secondary PCR products. The same PCR mixture and primers were used as for the secondary PCR amplifications. The PCR profile was as follows: cycles 1–5: 25 s at 94°C and 3 min at 67°C followed by 35 additional cycles with the same denaturation temperature and an annealing/extension step of 60°C for 3 min. The PCR profile ended with a final extension step at 60°C for 7 min. 5 µl of the third PCR amplification product was analysed by means of 1.5% agarose gel electrophoresis and visualised with ethidium bromide.

Isolation of junctions

In order to isolate the correct junction from a dried polyacrylamide gel we superimposed the autoradiogram upon the gel. In this way the autoradiogram could be used as a template to find the fragment of interest on the gel. The desired junction was cut out of the gel using a sharp scalpel. The junction DNA was eluted from the gel slice by incubating the slice in 200 µl of water for 1 h on ice. During this incubation the solution was mixed 3-4 times. After a centrifugation of 10 min at maximum speed, 150 µl of the eluate was collected and could be used for further processing.

Junction fragments that were obtained by adapter ligation PCR were eluted from agarose gels by using the Qiaquick Gel Extraction Kit(Qiagen). All reactions were performed according to the manual.

Reamplification and sequencing of junctions

To reamplify each junction the same primers were used as those used to generate the junction fragment. The reamplification PCR was conducted using the following conditions: 1 µl of the eluted junction fragment, 1X PCR-buffer (10 mM Tris-HCl pH 8.3; 50 mM KCl and 1.5 mM MgCl2), 250 µM dNTP's, 0.4 µM anchor primer and 0.4 µM adapter primer. Water was added to a final volume of 50 µl. The PCR cycle profile used was the same as the one used during the anchored PCR. Cycle 1: 25 s at 93°C, 30 s at 65°C and 60 s at 72°C; cycle 2–13: 25 s at 93°C, 30 s at an annealing temperature 0.7°C lower than for each previous cycle, starting at 64.3°C, 55 s at 72°C; cycle 14–40: 25 s at 93°C, 30 s at 56°C and 55 s at 72°C. After completion of the PCR program, the concentration of the excised fragments was estimated on a 1.5% agarose gel, and the solution was concentrated to 25 ng/µl by means of ethanol precipitation. Subsequently the junctions were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems). The junctions were sequenced in both directions by using, on the one hand, the anchor primer and, on the other hand, the adapter primer as the sequencing primers. An ABI Prism 377 DNA Sequencer and matching software were used to separate the sequencing reactions and to analyse the sequence information.

BLAST analysis of amplified junction fragments

Homology analysis of the amplified junction fragments was done by performing a BLAST search against the GenBank sequence database [19].

Results and discussion

Characterisation of the 35S promotor/plant DNA junction (EMBL accession# AJ308514)

Initially we used the anchored PCR technique to amplify the junction between the 35S promoter region and the plant DNA. An anchor primer homologous to the 35S promotor region was designed. The position of the 35S1 primer is shown in Fig. 1. The anchor primer was used in six different anchored PCR reactions. Each reaction was performed on digested RR soybean DNA as well as on digested untransformed soybean DNA. The latter was used as a negative control. Using this strategy we succeeded in isolating and sequencing four different junction fragments spanning the junction between the 35S promoter and the plant DNA: a 35S/MseI fragment (234 bp), a 35S/MboI fragment (249 bp), a 35S/BfaI fragment (283 bp) and a 35S/EcoRI fragment (390 bp) (results not shown). A possible explanation for the absence of a 35S/TaqI and a 35S/HindIII fragment could be that the amplified junction fragments are too large to be detected on our polyacrylamide gel electrophoresis system. Subsequently, we analysed the sequence of the four amplified junction fragments. In order to verify the sequence data we reasoned that the sequences of the 35S/MboI, the 35S/BfaI and the 35S/EcoRI fragment should be an extension of the sequence of the 35S/MseI fragment. Indeed this was the case.

Since the 35S/EcoRI fragment was the longest fragment, it was most valuable for further analysis. Homology analysis made clear that the 35S/EcoRI fragment is made up of 181 bp of non-insert DNA and 209 bp of insert DNA (Fig. 2). As could be expected, the insert DNA is identical to the sequence of the CaMV 35S promotor. To verify if the 181 bp of non-insert DNA could be considered plant DNA we conducted a plant DNA-test-PCR reaction with a primer pair, the 35S plt1/35S plt2 primer pair (Table 1), that was located within this 181 bp sequence. The exact position of the primers that were used to establish whether plant DNA was present at the 35S/EcoRI junction is shown in Fig. 1. Since the expected PCR product was detected when using untransformed soybean DNA as template DNA we can conclude that the non insert DNA is homologous to a soybean plant DNA sequence.

Fig. 2 Representation of the sequence of the amplified junction fragments. **a** sequence of the 35S junction fragment and **b** sequence of the 3′ NOS junction fragment. The insert DNA sequence is depicted in capitals, the plant DNA sequence in lower case, for the 3′NOS junction we used bold typing to indicate the part of the junction that is a part of the CP4 EPSPS coding sequence and the rearranged DNA segment is presented in slanted typing.

a.

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   1 GATAGTGGGATTGTGCGTCATCCCTTACGTCAGTGGAGATATCACATCAATCCACTTGCT  60
  61 TTGAAGACGTGGTTGGAACGTCTTCTTTTTCCACGTGCTCCTCGTGGGTGGGGGTCCATC 120
 121 TTTGGGACCACTGTCGGCAGAGGCATCTTCAACGATGGCCTTTCCTTTATCGCAATGATG 180
 181 GCATTTGTAGGAGCCACCTTCCTTTTCCAtttgggttccctatgtttattttaacctgta 240
 241 tgtatgatcttattttgaatgaaatgcaataagttatttctagtaaaaaaaaataaacat 300
 301 ttgatagaaacaaattaaagcatgcaaaaataactcattagcatcggttaaattgaaggg 360
 361 tttgaataatttgcacaaggttctgaattc 390
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b.

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   1 TGCGCGGTGTCATCTATGTTACTAGATCGGGGATCGATCC**CCCACCGGTCCTTCATGTTC** 60
  61 **GGCGGTCTCGCGAGCGGTGAAACGCGCATCACCGGCCTTCTGGAAGGCGAGGACGTCATC** 120
 121 **AATACGGGCAAGGCCATGCAGGCCATGGGCGCCAGGATCCGTAAGGAAGGCGACACCTGG** 180
 181 **ATCATCGATGGCGTCGGCAATGGCGGCCTCCTGGCGCCTGAGGCGCCGCTCGATTTCGGC** 240
 241 **AATGCCGCCACGGGCTGCCGCTTGACCATGGGCCTCGTCGGGGTCTACGATTTC***AAGCGC* 300
 301 *ATCATGCTGGGAAATTTTAGCGAGATTATAAGTATCTTCCTGGGGATCTCTGCTGTTACT* 360
 361 *GGTGAATAGTGAGACAGAGTCTTCTGAGCTCATAGGATAAAATAAATTATAATTAGTAAA* 420
 421 *TTTTTTAATTAAATAAATCAATTACTTCATAAATAATTTTTTTTATAGAATATGTTGACA* 480
 481 *TTCTAGCCGGATATAGAACTAATGTAAAGAAACCTTAAAAATTTTGTTTGGAAGAATATG* 540
 541 *TTATTGAAAGACAAATCTAATTAAGTTTATCAGGGTCATTTGTTGAAGATAGGAAACCTT* 600
 601 *CAGCAATTTGAATATTAAGTAACTGCTTCTCCCAGAATGATCGGAGTTTCTCCTCCTGCT* 660
 661 *ATTACATGAGCAAAAATAAAAAATAAATAAAAGATAAGATTAAGCTTCAACATGTGAAGG* 720
 721 *AGTAGTACACTCACCAGTGACCCTAATAGGCAACAGCATGAAAAAAAATAAAAAAGAATA* 780
 781 *AAAATAGCATCTACATATAGCTTCTCGTTGTTAGAAAAACAAAACTAT*ttgggatcggag 840
 841 aagaactgtttgaggcgaatggcctggtcgtcgcggccatcgtcgagaagttcgtgaaga 900
 901 agctcgaatgcggtgagaaggtagttctcttccaacagaaagttcaccacgcaattgcac 960
 961 agcgaagatctctccacgtccattttctctctctgtctctgatcttaagccattcattca 1020
1021 agacaagacaagagaagagaagaga 1045
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Characterisation of the 3′NOS/plant DNA junction (EMBL accession# AJ308515)

The junction between the 3′NOS region and the plant DNA was characterised using adaptor ligation PCR. Since no junction fragment connecting the 3′NOS region and the plant DNA could be amplified using one adaptor-aided PCR reaction, different sequential adaptor ligation PCR reactions had to be performed in order to reach the plant DNA.

The first adaptor-aided PCR reaction that we performed made use of nested anchor primers homologous to the 3′NOS region (Fig. 1; Table 1). The amplified fragment was sequenced and we tested if the DNA adjacent to the 3′NOS sequence was plant DNA. This experiment was done in the same way as described for the 35S junction fragment. A PCR reaction using a primer pair situated in the unknown DNA gave the expected fragment when using RR soybean DNA as a template, however when untransformed soybean DNA was used the expected amplification product could not be detected. This experiment shows that the DNA segment immediately adjacent to the 3′NOS region is not unaltered soybean plant DNA. For each fragment that was amplified during the sequential adaptor-aided PCR reactions, we tested if the amplified DNA showed homology to a soybean plant DNA sequence. All the primer pairs used to look for plant DNA homology are indicated in Fig. 1. The sequences of these primers is presented in Table 1. In the third sequential adaptor ligation PCR a fragment was amplified for which similarity to a soybean plant DNA sequence was proven using the plant DNA-test-PCR with the nos plt1/nos plt2 primer pair. After combining all sequence data for the different amplified fragments, we obtained a 1045 bp junction fragment at the 3′NOS region. This junction fragment is shown in Fig. 1. The next question that had to be answered was if the DNA segment that is present between the 3′NOS region and the plant DNA is homologous to a known sequence. A BLAST search gave no valuable homologies. When

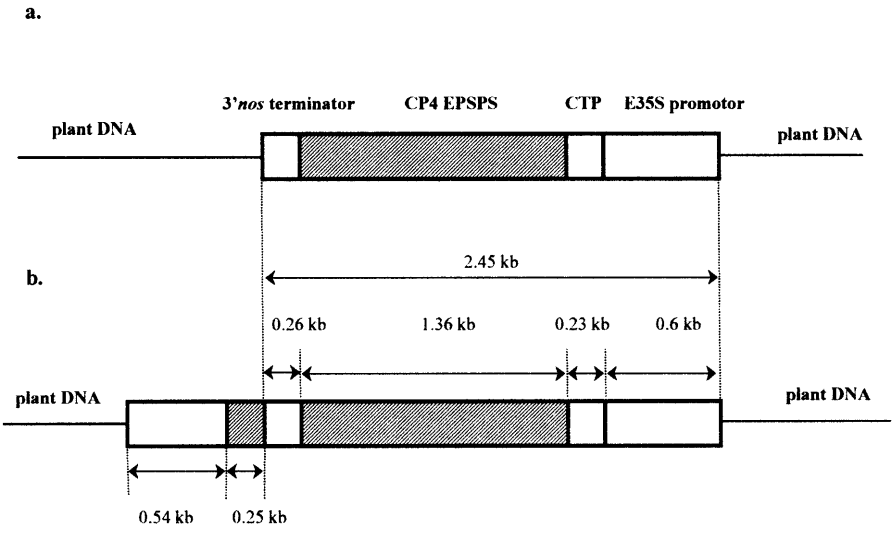

Fig. 3 A comparison between the insert structure as reported by **a** Padgette et al. [10] and **b** the structure of the insert deduced from the results described in this article

the sequence of the intermediate DNA segment was compared to the sequence of the insert DNA we found that a portion of 254 bp of the intermediate DNA segment was similar to a part of the CP4 EPSPS coding sequence (Fig. 2). This 254 bp DNA segment is situated immediately adjacent to the 3′NOS end point. Up to now no sequence homology could be found for the remaining 534 bp present between the plant DNA and the rearranged CP4 EPSPS segment.

To determine the exact starting point of the plant DNA at the 3′NOS junction we performed an anchored PCR reaction using a plant specific primer, the nos plt1 primer, as anchor primer. When using RR soybean DNA as a template this reaction should give a junction fragment, while a plant DNA fragment should be amplified when untransformed soybean DNA is used. Comparing both fragments should allow location of the exact starting point of the plant DNA. Indeed the two fragments were amplified and the exact starting point of the plant DNA could be fixed at position 829 of the 3′NOS junction fragment. In addition we analysed the sequence of the amplified plant DNA segment. First of all we compared the sequence of the amplified plant DNA fragment with the sequence of the plant DNA that is present adjacent to the 35S promotor end point. If no major rearrangements or deletions of the plant DNA have occurred during integration of the insert DNA, we can expect that both sequences should have some degree of similarity. However we could not detect any similarity between both sequences. To verify these results we performed a PCR reaction using a primer pair that consists of the 35S plt2 primer that is situated in the plant DNA adjacent to the 35S region and the nos plt1 primer that is situated in the plant DNA adjacent to the 3′NOS region. This primer pair should enable us to amplify the pre-integration site when using untransformed soybean DNA as template DNA, while the full insert should be amplified when using RR soybean DNA. Since no fragment was amplified when using untransformed soybean DNA, while for the RR soybean DNA the full insert DNA could be amplified, the above described findings are confirmed. An explanation for these findings could be that during integration of the insert DNA a large target site deletion occurred. Since a PCR setup suitable for amplification of DNA templates up to 12 kb was used, we expect a target site deletion of at least 12 kb. However, it is also possible that rearrangements of the plant DNA occurred due to the insertion of the insert DNA.

Taken together the results presented in this paper establish that at the 35S border no major rearrangements occurred during integration of the insert DNA and that plant DNA is present immediately adjacent to the 35S promotor end-point. This in contrast to the junction structure as detected at the 3′NOS junction. Adjacent to the 3′NOS region a 254 bp portion of truncated CP4 EPSPS coding sequence is present. This 254 bp DNA segment is followed by a DNA segment of 534 bp for which no sequence homology could be detected. Finally, adjacent to this unknown DNA segment we detected plant DNA. Furthermore, we can conclude that the target site was not simply disrupted by the insert but that during integration of the insert DNA rearrangements or a large deletion may have occurred.

In our opinion these results indicate that characterisation of junction fragments can offer important information for the accurate description of the insert structure of a transgenic line. Characterisation of junction fragments can be used to check the accuracy of technical dossiers on the one hand and on the other hand this method can be used to complete already existing information. A comparison between the results as reported by Padgette et al. [10] and our findings with regard to the structure of the RR insert is shown in Fig. 3. In the future, problems concerning the inaccurate description of transgene events can be avoided through a detailed characterisation of the transgene plant DNA junctions. Therefore, we welcome the idea that a detailed characterisation of the transgene plant DNA junction and of the plant target locus should be included in the technical dossier that is submitted to the competent authorities. These characterisations of the

junction regions will also give the best information for the development of line specific identification methods, which are at least from a European standpoint of high importance in the context of food safety control.

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