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Detection and characterization of recombinant DNA in the Roundup Ready® soybean insert

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Abstract

The genetically modified (GM) Roundup Ready® soybean event GTS 40-3-2 contains the bacterial gene 5-enol-pyruvylshikimate-3-phosphate synthase. A 534 bp rearrangement of the DNA in the 3′ region flanking the functional insert likely occurred as a consequence of the insertion event. The structure of the DNA surrounding the insertion has not been fully characterized. A semi-nested PCR method identified the rearranged soybean DNA in samples of raw, partially processed and highly processed food at a level of 0.01%. The 534 bp rearranged segment contained a contiguous portion at least 238 bp long that was also identified in non-GM soybeans. Specific combinations of semi-nested PCR primers differentiated GM and non-GM soybean DNA. These studies confirm that the rearranged DNA originates from the soybean genome and does not involve the introduction of non-soybean genetic material.
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1. Introduction

The Roundup Ready® soybean event GTS 40-3-2 was approved for environmental, food and animal feed use in the United States in 1994 (Payne, 1994). The use of herbicide-tolerant soybean varieties has grown consistently since then and they are now produced on 36.5 million hectares (James, 2002). The transgenic strain was produced by transformation of the non-transgenic soybean line A5403 with the 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene linked to other transcription and processing control elements by the particle acceleration method.

The ability to identify and differentiate transformed and non-transformed plants has become a global activity due to the existence of a wide range of statutory limitations on the acceptability of transformed plant strains for food, feed and environmental use. Nucleic acid based detection methods currently offer the greatest sensitivity in terms of identifying specific genetically modified organisms (GMOs). Methods that amplify a unique nucleotide sequence within inserted DNA allow detection of GMOs (Windels, Taverniers, Depicker, Van Bockstaele, & De Loose, 2001). In contrast, amplification of the junction between insert DNA and the plant DNA enables the discrimination of different GMOs containing the same insert and the identification of different GMOs containing different copy numbers of the same insert (Windels et al., 2001). Rearrangement of genomic DNA at the site of insertion of foreign DNA has been described previously (Bhattramakki et al., 2002; Nevers & Saedler, 1977; Risseeuw, Offringa, Franke-van Dijk, & Hooykaas, 1995). The isolation and characterization of the junction between insert DNA and plant DNA for event GTS 40-3-2 has been described previously (Windels et al., 2001). These authors found that the 3′ region flanking the insert had undergone extensive rearrangement and identified the presence of additional DNA sequences. Researchers from Monsanto Company (St. Louis, MO, USA) confirmed the existence of a 534 bp segment within the EPSPS-plant junction region ("junction fragment") of GTS 40-3-2 in further studies and agreed that the most likely

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source of the extraneous flanking DNA was a rearrangement of genomic soybean DNA (Anon., 2002). However, the flanking DNA sequence was not found in a search of public sequence databases (Windels et al., 2001). Researchers at Monsanto Company identified 455 of the 534 bp rearranged portion of the flanking DNA sequence in their proprietary soybean database (Anon., 2002; Goley, Reiser, Cavato, Beazley, & Lirette, 2002). The presence of the rearranged DNA in commercially available foodstuffs has not been described previously.

The benefits and implications of GM crops on human and animal health are widely disputed (Cantani & Micera, 2001; Goodyear-Smith, 2001; Sears et al., 2001). The precise molecular identity of the insert and its flanking regions was not presented to regulatory authorities at the time of applying for product approval and registration. Approval was thus granted without a complete knowledge of the origin, function and stability of the rearranged region. More detailed molecular analyses have subsequently been submitted to the relevant authorities for examination, and no agency has modified its registration of GTS 40-3-2 as a result of the new information (Anon., 2002).

The purpose of this study is to further characterize the rearranged DNA in the 3′ region flanking the functional insert in GTS 40-3-2 and to demonstrate the feasibility of producing a highly sensitive routine assay for the identification of the rearranged DNA segment in a variety of food products.

2. Materials and methods

2.1. Food samples

Raw, partially processed and extensively processed food samples known to contain the GTS 40-3-2 event and samples that did not contain soybean-derived materials were obtained from a variety of commercial sources.

2.2. Reference standards

Genetically modified (GM) soybean event GTS 40-3-2 (Roundup Ready®, Monsanto Company, IRMM-410S-3), GM maize Bt11 (Syngenta Seeds, Inc., Basel, Switzerland), Event 176 (NaturGard™ KnockOut™, Syngenta Seeds, Inc.) and MON810 (Yieldgard®, Monsanto Company) and non-genetically modified soybean and maize powders were obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) in standardized aliquots containing 0%, 0.1%, 0.5% and 2% GM material, respectively. GM standards below 0.1% were prepared in-house by mixing appropriate quantities of the GM and non-GM standard material.

2.3. Primer sequences

Primers for standard and semi-nested PCR (22F, 46F, 46R, 47F, and 47R) were selected from GenBank sequence AJ308515. Primer 22F was identical to the sequence published previously (Windels et al., 2001). Primer sequences and their coordinates with respect to AJ308515 are shown in Table 1. Primers were obtained from Gibco BRL (Tseun Wan, Hong Kong SAR, China). The relationship between template, primers and PCR products is shown in Fig. 1.

2.4. DNA extraction

DNA was extracted using an in-house method. Food samples were homogenized by grinding to a fine powder in a blender. Liquid samples were lyophilized (FTS Systems, Inc., New York, USA) until dry before DNA extraction. Duplicate samples of about 400 mg lyophilized powder were added to screw-capped microcentrifuge tubes. Extraction buffer A (0.86 ml) comprising 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA-Na, 1% SDS was added and mixed thoroughly. Guanidine–HCl (5 M, 0.1 ml) was added and mixed thoroughly. To this, 40 μl 20 mg/ml proteinase K solution (Invitrogen Corp., Carlsbad, CA, USA) was added and mixed thoroughly. The mixture was incubated in a 60 °C water bath for 3 h with occasional mixing. The suspension was cooled to room temperature for 5 min and centrifuged (14,000 rpm × 10 min). The colourless supernatant was removed and filtered through a 0.2 μm syringe filter. An equal amount of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) was added to the filtrate, mixed thoroughly and centrifuged

Table 1
Primer sequences used in this study

Primer	Nucleotide sequence (5′–3′)	Co-ordinates w.r.t. GenBank AJ308515
22F	GCG CGG TGT CAT CTA TGT TA	2–21
46F	AAG CGC ATC ATG CTG GGA AAT T	295–316
46R	TCC GAT CAT TCT GGG AGA AGC A	645–624
47F	CAG GGT CAT TTG TTG AAG ATA G	571–592
47R	ACG AGA AGC TAT ATG TAG ATG C	808–787

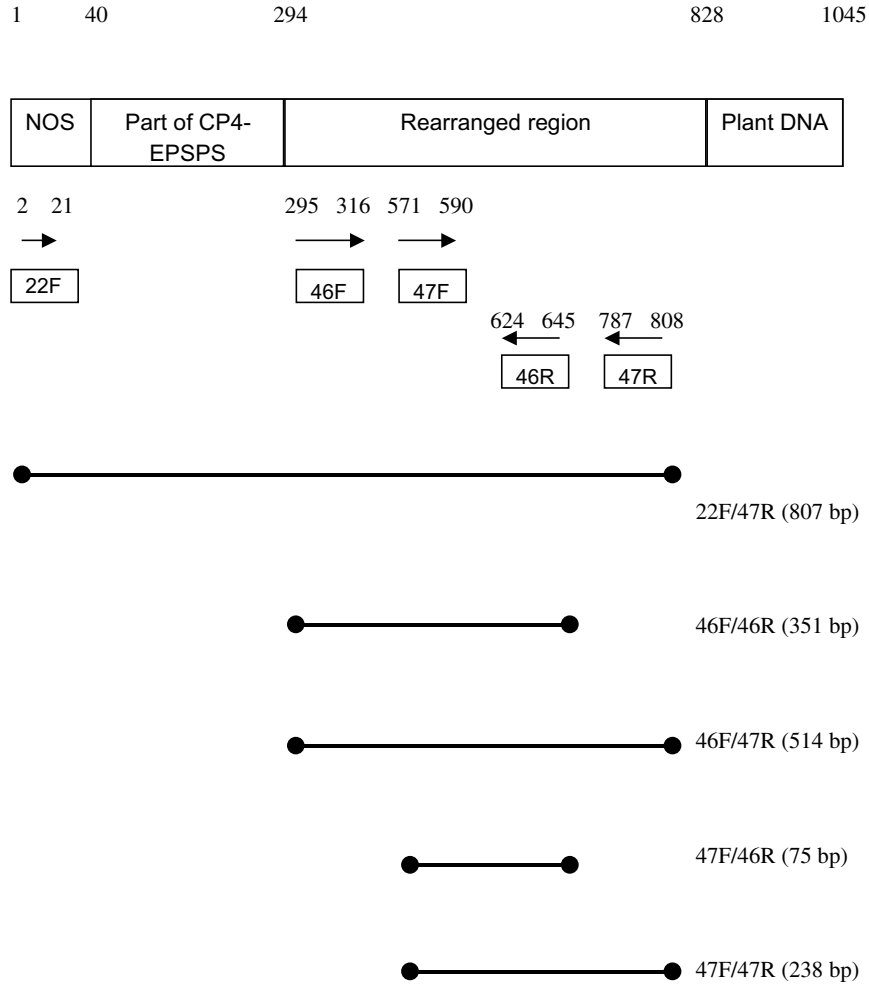

Fig. 1. Relationship of template, primers and PCR products in this study. The figure shows the structure of GenBank sequence AJ308515 (not to scale). The primers are indicated by boxes and arrows indicate the direction of polymerization. The nucleotide coordinates with respect to AJ308515 are indicated above each box. Black lines indicate the relative lengths and positions of the amplification products.

(14,000 rpm × 5 min). The upper layer was retained and an equal volume of chloroform was added, mixed thoroughly and centrifuged (14,000 rpm × 5 min). The upper layer was added to 1 mL DNA purification resin (Promega Corp., Madison, WI, USA) and passed through a mini-column. The column was washed with 2 ml 80% isopropanol and centrifuged (11,000 rpm × 2 min). Diethylpyrocarbonate (DEPC)-treated water (50 μl) warmed to 70 °C was added to the mini-column and incubated at room temperature for at least 2 min before centrifugation (11,000 rpm × 1 min). Absolute ethanol (0.15 ml) was added to the DNA solution and incubated at −80 °C for 30 min. DNA was pelleted by centrifugation (14,000 rpm × 15 min). The supernatant was discarded and the DNA pellet washed twice with 1 ml 70% ethanol. The DNA pellet was air dried and dissolved in 20 μl DEPC-treated water and stored at −20 °C. A portion (4 μl) was used for optical density measurement.

2.5. Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out in a reaction mixture (20 μl) containing 2 μl 10× PCR buffer II (Applied Biosystems, Inc., Foster City, CA, USA), 1.7 μl 25 mM $MgCl_2$ (Applied Biosystems, Inc.), 0.4 μl dNTP (10 mM each of four dNTPs; Promega Corp.), 0.1 μl AmpliTaq Gold (5 units/μl, Applied Biosystems, Inc.), 1 μl primers (10 μM each), 1 μl DNA template (about 100 ng), and 12.8 μl DEPC-treated water. The PCR conditions for the first amplification (using primers 22F and 47R) were: 95 °C for 10 min, 40 cycles of 95 °C for 1.5 min (denaturation), 55 °C for 1.5 min (annealing) and 72 °C for 1 min (extension), followed by 72 °C for 10 min (final extension). The PCR conditions for the second amplification (semi-nested PCR, using primers 46F and 47R) were: 94 °C for 10 min, 40 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing) and 72 °C for 1 min (extension), followed by 72 °C for

Table 2
Primer combinations used in this study and expected PCR product size (bp)

Template	Primer pair[a]				
	22F/47R	46F/47R	46F/46R	47F/47R	47F/46R
GM genomic DNA	807	514	351	238	ND
Non-GM genomic DNA	ND	ND	ND	238	ND
807	ND	514	ND	ND	ND
514	ND	ND	351	ND	ND
351	ND	ND	ND	238	ND
238	ND	ND	ND	ND	75

ND, not done.
[a] The expected PCR product size obtained from each template using the indicated primer pairs is shown.

10 min (final extension). PCR products were separated on a 2% agarose gel in 1× TBE under constant current (24 mA) for 45 min followed by visualization with a Gel Doc 2000 gel imaging system (BioRad, Inc., Hercules, CA, USA). Product size was estimated by comparison with a ladder of 50 bp size markers (MBI Fermentas GmbH, Heidelberg, Germany). The various primer combinations used and expected PCR product sizes are shown in Table 2.

2.6. DNA sequencing

The PCR amplified DNA fragments were purified and sequenced from both directions using the same primers used to amplify the DNA. For sequence comparison and identification, the sequences were searched against entries in GenBank using the BLAST program (Altschul, Gish, Miller, Myers, & Lipman, 1990) with default search parameters.

3. Results

3.1. Specificity of junction fragment detection

A portion of the junction fragment was amplified from event GTS 40-3-2 GM soybean using a semi-nested PCR approach utilising a primer complementary to a sequence within the NOS terminator region (22F) and a primer complementary to a sequence within the junction fragment published previously (47R). A band of the expected size (807 bp) was detected (Fig. 2a). The same primers could not amplify a similar fragment from non-soybean material, such as choi sum (Chinese flowering cabbage, *Brassica campestris*), tomato, green peas, rice, wheat and bovine muscle. The 807 bp fragment was sequenced and found to be highly similar (96.5% nucleotide identity) to a segment of the junction fragment sequence AJ308515 (data not shown). The product of the first round of PCR amplification was used to amplify a sub-fragment using a semi-nested PCR approach utilizing primers 46F and 47R. Only the soybean event GTS 40-3-2 gave the expected product size of 514 bp (Fig. 2b). The 514 bp sub-fragment was sequenced and found to be highly similar (98.4% nucleotide identity) to a segment of the junction region sequence AJ308515 (data not shown).

3.2. Detection of junction fragment in soybean products

The semi-nested PCR amplification method was used to differentiate GM and non-GM soybean products. A prominent band of 514 bp was observed only with GM soybeans (Fig. 3a, lane 7). The GM soybean sample also produced a larger band approximately 800–900 bp in size and a similar band was also detected in the positive control sample following the first round of amplification (lane P). A sample of partially processed non-GM soybean flakes produced a band between 500 and 600 bp in size (Fig. 3a, lane 9) and this may be due to non-specific amplification. This band was clearly distinguishable from the 514 bp fragment and did not occur in any of the other soybean-derived samples. Partially processed products derived from GM soybeans did not produce a band of the expected size (Fig. 3a, lanes 10–12).

3.3. Detection of junction fragment in food samples

The semi-nested PCR amplification method was used to identify the junction fragment in commercially available food products. The items selected contained soybean-derived material on the list of ingredients and had all tested positive for the GM event GTS 40-3-2 marker CP4-EPSPS in commercial tests for GM ingredients by qualitative and/or quantitative Taqman PCR (data not shown). A fragment of about 500 bp, consistent with the 514 bp expected size of the PCR product, was observed in most of the samples examined, including meat sausages, soy meal, baked snack foods, tempura flour and soybean curd (Fig. 3b). The fragment could not be amplified from non-GM soybean material and some samples of highly processed foods. Further analysis of meat sausages and soy milk, known to contain GTS 40-3-2 material, revealed that the junction fragment could be amplified from 50% (5/10) of the

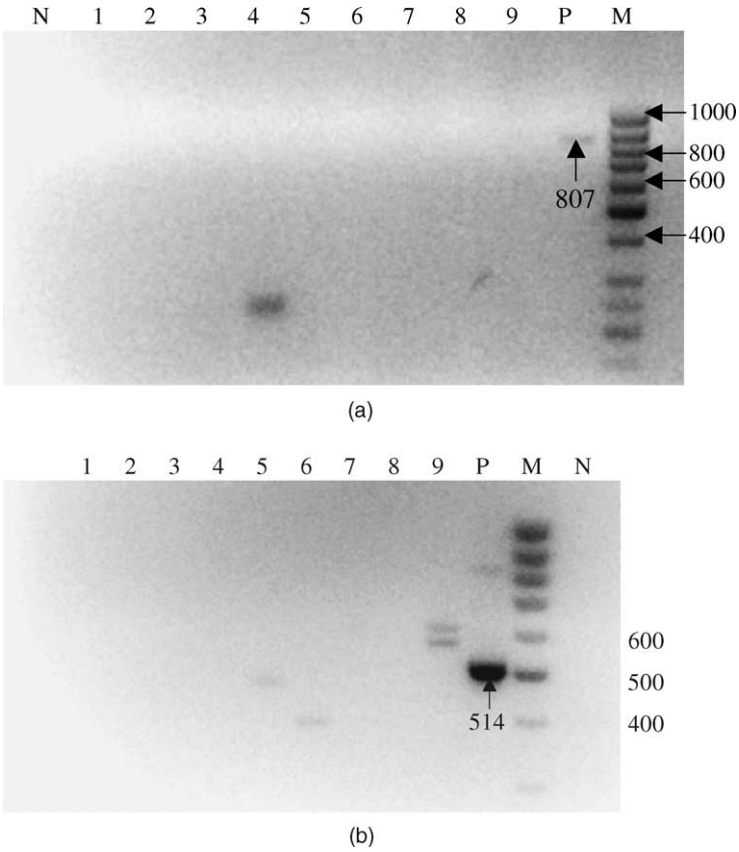

Fig. 2. Amplification of junction fragment DNA from various sources. (a) Primers 22F and 47R were used to amplify an 807 bp fragment from a variety of sources. (b) The 807 bp product from the first round of amplification was used as the template in a second round of amplification using primers 46F and 47R to produce a 514 bp product. In both figures: Lane M, 50 bp marker ladder; Lane P, positive control; Lane N, negative control; Lanes 1–9, choi sum, 1% Bt-11 maize reference standard, 5% Bt-176 maize reference standard, 0.5% MON-810 maize reference standard, tomato, rice, wheat, green peas and bovine muscle.

sausages and other highly processed foods and 50% (3/6) of the soy milk samples (data not shown). The results of the CP4-EPSPS and junction fragment analysis are presented in Table 3.

3.4. Limit of detection

The semi-nested PCR amplification method using primer pairs 22F/47R and 46F/47R were used to determine the limit of detection of the junction fragment. DNA extracted from the 1% IRMM GTS 40-3-2 reference standard (IRMM-410S-3) was serially diluted with DNA extracted from a non-GM soybean reference standard to give 0.1%, 0.01%, 0.001% and 0.0001% standard solutions. The results are shown in Fig. 4. The semi-nested PCR can detect the junction fragment at a level of 0.01%.

3.5. Characterization of the junction fragment

Primers 46F and 47R were used to amplify the 514 bp product directly from GM and non-GM soybean material. The 514 bp product was subjected to a further round of semi-nested PCR amplification using primers 46F and 46R. The expected size of the semi-nested amplification product is 351 bp. As expected, the 351 bp fragment was only amplified from the GTS 40-3-2 soybean material and not from non-GM soybeans (Fig. 5a). Using primers 47F and 47R and the 351 bp product as template in a semi-nested PCR amplification, a fragment of 238 bp could be amplified from both GTS 40-3-2 and non-GM soybean material (data not shown). The 238 bp fragment was sequenced and found to be highly similar (97.5–98.3% nucleotide identity) to a segment within the junction region sequence AJ308515 submitted to GenBank (data not shown).

In a separate experiment, primer pair 47F/47R was used to amplify the 238 bp fragment directly from GM and non-GM genomic DNA. The 238 bp fragment derived from this amplification was used as the template in a semi-nested PCR utilizing primers 47F and 46R. The expected size of the semi-nested amplification product is 75 bp (Fig. 5b). These results demonstrate that the junction fragment also contains material common to non-GM soybeans and confirms the notion that the

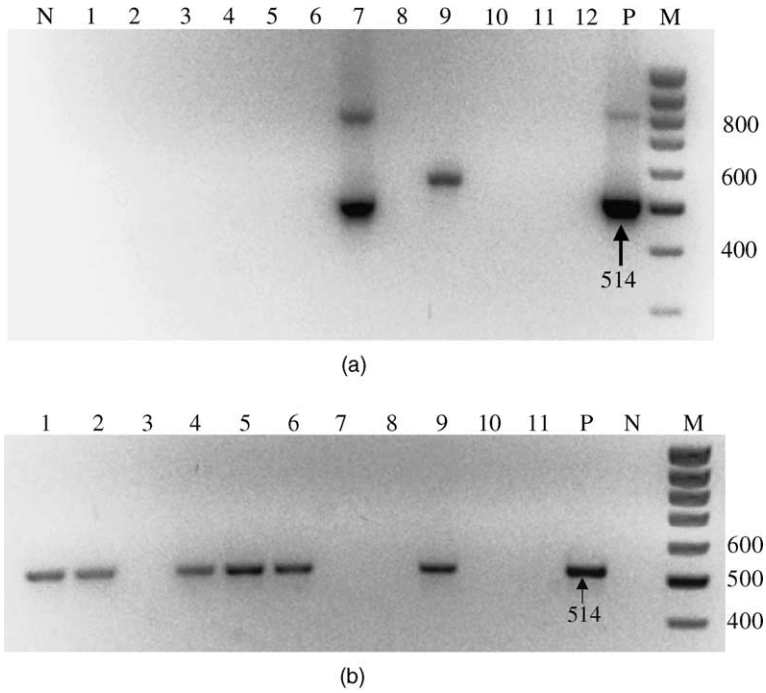

Fig. 3. Detection of junction fragment in soybean products and commercial food products. (a) Detection of junction fragment in soybean products. Primers 46F and 47R were used to generate a 514 bp product from various soybean containing products. The first round of amplification using primers 22F and 47R is not shown. Lane M, 50 bp size ladder; Lane P, positive control; Lane N, negative control; Lanes 1–9, non-GM soybean powder, non-GM soybean seeds, non-GM soybeans, non-GM soybean flakes, non-GM embryo buds, non-GM soybeans, GM soybeans, non-GM soybeans, non-GM soybean flakes. Lanes 10–12, GM soy milk. (b) Detection of junction fragment in commercial food products. Primers 46F and 47R were used to generate a 514 bp product from various commercial food products. The first round of amplification using primers 22F and 47R is not shown. Lane M, 50 bp size ladder; Lane P, positive control; Lane N, negative control; Lanes 1–3, GM sausage; Lanes 4–8, GM fried noodles, GM soybean meal, GM fried snack food, GM tempura flour, GM bean curd/vegetable soup powder; Lane 9, GM soybean; Lane 10–11, non-GM soybeans.

Table 3
Detection of junction DNA fragment in food samples

Sample	Degree of sample processing	No. of samples tested	No. of RR[a] positive samples (%)	No. of RR[a] positive and junction fragment positive samples (%)
Soybeans/soy meal/soy flakes/ tempura flour	Low	14	4/14 (29)	3/4 (75)
Soy milk	Medium	7	6/7 (86)	3/6 (50)
Sausages/noodles/snack food/soup powder	High	10	10/10 (100)	5/10 (50)

[a] RR, Roundup Ready® soybean.

junction fragment region present in GTS 40-3-2 is a rearrangement of genomic soybean DNA.

4. Discussion

The introduction of genetic variation by recombination is a well-characterized phenomenon in eukaryotes (Cohen & Shapiro, 1980; Pukkila, 1977). In maize, switching genes on and off by insertion and deletion of transposable elements has been studied for decades (Cohen & Shapiro, 1980; Nevers & Saedler, 1977; Scholz, Lorz, & Lutticke, 2001). Often at the site of transposition or insertion, rearrangements in the genetic structure were noted (Bhattramakki et al., 2002; Risseeuw et al., 1995). The precise cause and mechanism of many of these rearrangements is often unclear. Considering the frequency with which such rearrangements are known to occur in economically important food crops, it is surprising that the functional insert in GTS 40-3-2 Roundup Ready® soybeans was not well described until Windels et al.'s study (2001), several years after regulatory approval for the use of this genetically engineered strain had been granted in the United States

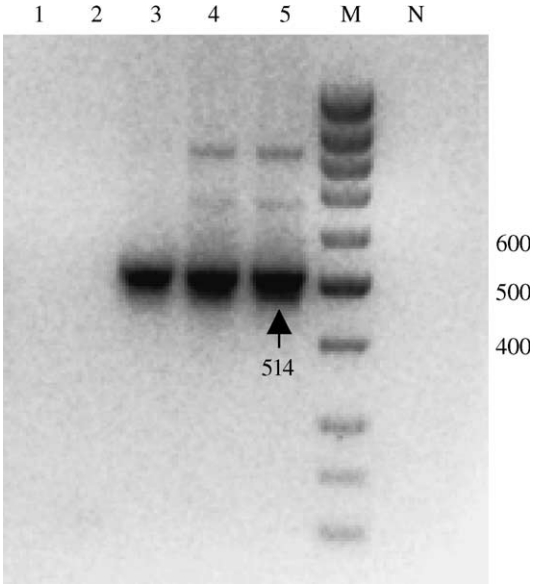

Fig. 4. Limit of detection of the junction fragment. Primers 46F and 47R were used to generate a 514 bp product from IRMM GTS 40-3-2 Roundup Ready soybean standards. The first round of amplification using primers 22F and 47R is not shown. Lane M, 50 bp size ladder; Lane N, negative control; Lanes 1–5, 0.0001%, 0.001%, 0.01%, 0.1% and 1% Roundup Ready soybean reference standards (tenfold dilutions of 1% standard). Arrow indicates position of 514 bp amplification products.

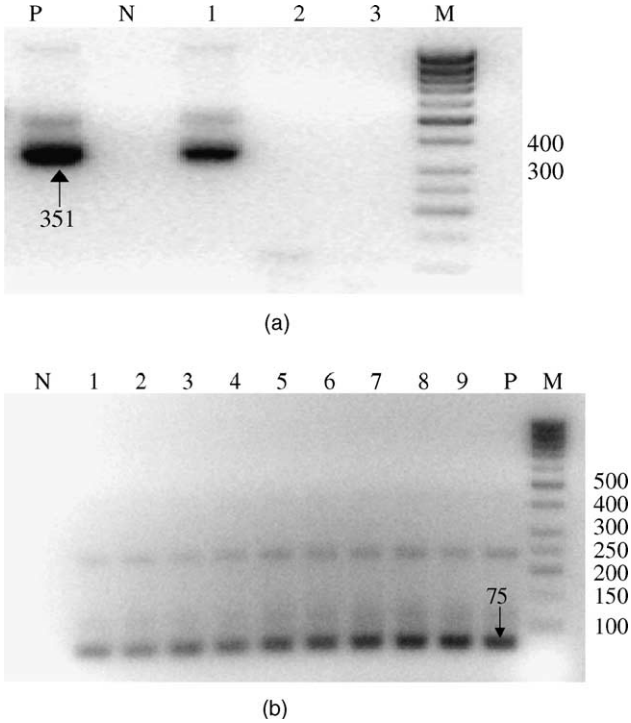

Fig. 5. Further characterization of the junction fragment. (a) Primers 46F and 47R were used to generate a 514 bp product from GM and non-GM soybeans (not shown). The 514 bp product was used as the template in a semi-nested amplification using primers 46F and 46R to produce a 351 bp fragment. The 351 bp fragment was only amplified from the GM material. Lane M, 50 bp size ladder; Lane P, positive control; Lane N, negative control; Lane 1, GM soybean; Lane 2, non-GM soybean; Lane 3, non-GM soybean flakes. (b) Primers 47F and 47R were used to amplify a 238 bp fragment directly from GM and non-GM soybean material (not shown). The 238 bp fragment was used as a template for a further round of amplification using primers 47F and 46R to produce a 75 bp fragment from both GM and non-GM material. Lane M, 50 bp size ladder; Lane P, positive control; Lane N, negative control; Lanes 1–6, non-GM soybean powder, non-GM soybean seeds, non-GM soybeans, non-GM soybean flakes, non-GM embryo buds, non-GM soybeans; Lanes 7, GM soybeans; Lanes 8–9, non-GM soybeans, non-GM soybean flakes.

for food, environmental and animal feed uses (Payne, 1994). The 3′ region flanking the functional gene insert had undergone an extensive rearrangement, involving among other things, partial duplication of the functional insert and deletion of genomic DNA (Windels et al., 2001). It is apparent from the regulatory documents submitted to the United States Department of Agriculture that full disclosure of the molecular characteristics of the functional insert and its flanking genetic material had not been made (Payne, 1994). Subsequent to regulatory approval in the United States, GTS 40-3-2 was approved for use in several countries in South and Central America, Europe, the Asia-Pacific region and Southern Africa. Despite many detailed studies on the GTS 40-3-2 strain prior to regulatory approval, the effects of the rearranged DNA on the stability and function of the GTS 40-3-2 strain, the functional insert, or its effect on other genes and regulatory elements within the plant have not been studied. We have further characterized the functional insert of GTS 40-3-2. Essentially, the junction fragment is a surrogate marker for the GTS 40-3-2 event. We developed a routine qualitative assay to detect the GTS 40-3-2 recombination event. We used this method to identify the rearranged material in a series of processed food products that had tested positive for the presence of Roundup Ready in commercially available GMO screening analyses. The sensitivity of such an assay allowed detection of the rearranged DNA to 0.01% (w/w) with respect to total soy DNA comparable with the detection limit of other GM events. No cross reactivity was observed with non-soy materials (rice, maize, choi sum, potato, tomato, wheat and beef).

Previous studies indicated that 455 bp of the 534 bp junction region corresponded to native soybean DNA (Anon., 2002; Goley et al., 2002). The results of our semi-nested PCR approach to further characterize the functional insert support these claims. In addition, our data indicate that at least 238 bp of the 534 bp rearranged region (~45%) comprises a single contiguous segment of genomic soybean DNA. From sequence comparisons of the amplification products obtained during this study with publicly available databases, it appears that the entire rearranged region consists of soybean DNA and does not contain genomic material from other plant, bacterial or viral sources (data not shown).

Despite being a surrogate marker for the presence of GTS 40-3-2, the junction fragment could not be detected in several samples of food demonstrated to contain Roundup Ready® soybean material from qualitative and quantitative Taqman PCR assays. There was a strong correlation between the degree of processing and the ability to detect the junction fragment. Junction fragment detection was more problematic in medium and highly processed foods, such as soy milk and sausages, compared with raw foods, such as whole soybeans (Table 3). This may be due to template stability. The template for amplification in Taqman PCR is a very small and the resultant PCR product is only 71 bp in length. In contrast, the junction fragment assay is a traditional PCR in which the first round amplification product is 807 bp in length. As extensive food processing is known to lead to DNA degradation via nuclease digestion, denaturation and chemical modification, the probability of obtaining a template intact enough for PCR amplification of an 807 bp product is quite small.

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