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Validity Assessment of the Detection Method of Maize Event Bt10 through Investigation of Its Molecular Structure

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In March 2005, U.S. authorities informed the European Commission of the inadvertent release of unauthorized maize GM event Bt10 in their market and subsequently the grain channel. In the United States measures were taken to eliminate Bt10 from seed and grain supplies; in the European Union an embargo for maize gluten and brewer's grain import was implemented unless certified of Bt10 absence with a Bt10-specific PCR detection method. With the aim of assessing the validity of the Bt10 detection method, an in-depth analysis of the molecular organization of the genetic modification of this event was carried out by both the company Syngenta, who produced the event, and the European Commission Joint Research Centre, who validated the detection method. Using a variety of molecular analytical tools, both organizations found the genetic modification of event Bt10 to be very complex in structure, with rearrangements, inversions, and multiple copies of the structural elements (*cry*1Ab, *pat*, and the *amp* gene), interspersed with small genomic maize fragments. Southern blot analyses demonstrated that all Bt10 elements were found tightly linked on one large fragment, including the region that would generate the event-specific PCR amplicon of the Bt10 detection method. This study proposes a hypothetical map of the insert of event Bt10 and concludes that the validated detection method for event Bt10 is fit for its purpose.

KEYWORDS: Feed; food; genetically modified organism; GM; GMO; PCR; genomic library; Southern blot analysis; event-specific detection method

INTRODUCTION

Marketing of food and feed in the European Union (EU) is strictly regulated, and in case of an appearance of an unauthorized genetically modified (GM) food or feed, the European Commission (EC) can take measures to ensure that unauthorized food and feed, the safety of which has not yet been assessed by the authorities, will not be consumed. The discovery of GM event Bt10 authorized neither within the United States (U.S.) nor in the EU, was such an unexpected case. Event Bt10 had inadvertently entered the U.S. market in 2001, and its presence was reported by Syngenta once discovered in the United States. As soon as the EC was informed in March 2005 about the potential dissemination of event Bt10 to the EU through the grain channel, emergency procedures were taken (*1*). Import of two specific maize products from the United States (maize gluten and brewers grain for animal feed) was prohibited in the EU unless certified as Bt10 free, and a Bt10-specific detection method was hence needed for control purposes.

Validated detection methods for GM food and feed are a prerequisite for biotech companies in order to place their products on the European market (*2*). In the U.S. authorization process biotechnology companies are required to present a GMO detection method, which can be either protein- or DNA-based, for use in testing raw ingredients (*3*). The EU, however, asks for the GM detection method to be event specific (*4*). Hence, in practice, only DNA-based detection techniques are considered in the EU. The event specificity of the method implies the generation of a PCR amplicon, based on a region covering the junction of the transgene with the flanking plant genome. Therefore, an understanding of the number of transgenic insert(s), their organization, and their stability in sibling plants

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is important and relevant to the design and reliability of any DNA-based detection method.

Event Bt10 presented a unique challenge in that very little was known about its genetic modification at the time it was released. A Bt10 event-specific detection method was quickly developed by the company GeneScan Europe based upon the available information. This method was submitted to the Joint Research Centre (JRC) and in-house validated by the Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) and was demonstrated to be specific for event Bt10. Syngenta was requested to release to the European Food Safety Authority (EFSA) information about the safety characteristics of event Bt10 and its distinction from the closely related event Bt11. Data on the organization of the Bt10 insert, as well as on the stability of the insert in sibling plants and over generations, was necessary to assess the occurrence/avoidance of false-negative results in screenings for event Bt10 using the validated method. In the absence of a complete set of molecular data at the time of emergency, the JRC started an investigation of the molecular structure. Numerous efforts in obtaining molecular data, by both Syngenta and the JRC, indicated that event Bt10 carried a complex insert.

The literature reports several studies of plant genome rearrangements such as in *Arabidopsis* (*5*), but there are only a few cases in which reorganizations of commercial GM events have been described. Windels et al. (*6*) reported on rearrangements of the 3′ *nos* gene junction of the Roundup Ready soybean. Studies on the characterization of inserts of GM- events (maize, soybean, rape) can also be found on the Belgian Biosafety Server (*7*).

This paper is focused on the findings that led to the final conclusion of the organization of the event B10 insert and reliability of the detection method.

MATERIALS AND METHODS

Material. Ground seed material from GM event Bt10 (inbred line JHID829BB) and a near-isogenic line (JHID829) were used for DNA extractions. Seeds of the same lines were also grown under environmentally controlled growth conditions, and leaves from V2−V4 plants were harvested for subsequent DNA extractions.

DNA Extractions. DNA was extracted from ground seed material using the NucleoSpin Food kit (Macherey-Nagel, Germany). Two methods of genomic DNA extraction from plants were used. The method of Thomas et al. (*8*) was employed in Syngenta with the following modifications: 8 g of leaf tissue was processed, 10 mL of buffer B was used, NaOAc was added after the chloroform/isoamyl extraction step to a final concentration of 300 mM, 60 μg of RNase was added and incubated for 30 min, 0.7 volume of isopropanol was used for precipitation, and the DNA pellets were washed with 70% EtOH. Alternatively, the extraction was carried out in the EC-JRC with the maize DNA preparation method of Dellaporta et al. (*9*). The DNA concentration was determined with a fluorometer using Quant-iI TM Picogreen dsDNA reagent (Invitrogen, Carlsbad, CA).

PCR Conditions. Primers were synthesized at Microsynth (Switzerland). Qualitative PCR was carried out using AmpliTaq Gold TAQ polymerase (Applied Biosystems). PCR conditions were applied as recommended by the manufacturers, taking into account the annealing temperatures of the primers. Long-template PCR was employed with the kit Expand Long Template PCR (Roche Applied Science, Italy) according to the manufacturer's instructions. PCR fragments for DNA sequence analysis were isolated from the gel and/or purified with the Qiaquick PCR purification kit (Qiagen, Germany). The primer sequences were as indicated in **Table 1**.

Genomic Library Construction. Genomic DNA was digested with the enzymes *Bam*HI, *Sau*3AI, *Hind*III, and *Eco*RI+*Hin*dIII, respectively (New England Biolabs). For *Bam*HI and *Sau*3AI partial digestions, samples were removed at nine time points starting from 2 to 20 min, pooled, and then size fractionated via agarose gel electrophoresis. Fragments of 6−10 kb or 3 kb were selected for library construction and isolated from the gel using Gelase (Epicenter) and cloned with the Lambda ZAP Express Vector system (Stratagene), according to the manufacturer's protocol. The library was screened with ^{32}P-dCTP labeled fragments: *cry*1Ab and *pat*. Inserts of positive clones were subcloned to the pBK-CMV plasmid.

Table 1. Primer Sequences

primer name	DNA sequence
1002	CCACTTGGTAAAGTAGTCAGTGGC
3168	CGCCTCCATCCAGTCTATTA
bldiaR1	GGAAGCTAGAGTAAGTAGTTC
bldiaR2	TGAGTAAACTTGGTCTGACAG
pR1	TCTGGTCTTCTGACGAGCTC
pR2	GGTTACTCAAGCAGTTGTATG
pR3	CGTAATCATGGTCATAGCTGT
cry1R	CATAGAGAGGAAAGGTAAACTC
cry2R	ATCACCTCCTGTGAAGCCTG
cry3R	CTTAACTATGCGGCATCAGAG
MPR	CCACTTGCTTTGAAGACGTG
PatR2	GACTCAGATCTGGGTAACTG
NR2	ATCTAGTAACATAGATGACAC
blaF2	AGGAAGAGTATGAGTATTCAAC
blaR1	GGTCTGACGCTCAGTGGAAC
patIntF	CCACATTGTACACACATTTGCT

DNA Sequence Analysis. PCR fragments and clones were sequenced with the ABI 3730 XL analyzer using the ABI BigDye 3.1 chemistry via primer walking on both strands. Alternatively, PCR fragments were sequenced at Microsynth. Sequence analysis was done using the Phred, Phrap and Consed package from the University of Washington or Lasergene software (DNAstar). Accession numbers are as follows: EU363764 (fragment obtained with primers 1002 and 3168); EU363765 (fragment obtained with primers blaF2 and blaR1); EU363766 (fragment obtained with primers patIntF and 3168); EU363767 (fragment obtained with primers patIntF and bldiaR1); EU363768 (fragment obtained with primers patIntF and pR3).

Southern Analysis. Genomic DNA was digested overnight according to the conditions recommended by the suppliers of the restriction enzymes (New England Biolabs; Roche Applied Science, Italy). The samples were run overnight in 0.8% agarose TAE gels containing ethidium bromide (0.5 μL/mL). Subsequently the gels were photographed on an UV-transilluminator. The DNA in the gel was depurinated and transferred by capillary blotting to a membrane, according to the manufacturer's instructions (GE Healthcare, USA; GE Healthcare, Italy) and finally covalently linked to the membrane with a UV Stratalinker (Stratagene) or, alternatively, on a UV transmission box. Prehybridization, hybridization, and washing of the membranes were carried out with the chemicals provided by and according to the instructions of either Sigma Chemical (USA) or GE Healthcare (Italy). The probes were labeled by random priming with ^{32}P-dCTP using the Rediprime II DNA labeling kit (Amersham Biosciences). Finally, the membranes were subjected to autoradiography.

The DNA fragments used as probes covered the entire coding regions of the respective genes *cry*1Ab (1848 bp), *pat* (552 bp), and *amp* (861 bp).

RESULTS

PCR Analysis of the Presumed Bt10 Insert. Events Bt10 and Bt11 had been generated by Syngenta via transformation with the plasmid pZO1502, carrying the genes *cry*1Ab, *pat*, and *amp* (see **Figure 1**). Prior to transformation, the plasmid had been digested with *Not*I to exclude the *amp* gene from the transformation. Early on in the Bt10 investigations, it was determined that event Bt10 had inherited the *amp* gene. Therefore, the presumed structure of the insertion of Bt10 could be a contiguous cluster containing the *cry*1Ab and *pat* genes, flanked by the *amp* gene (see **Figure 2A**). The presence of the

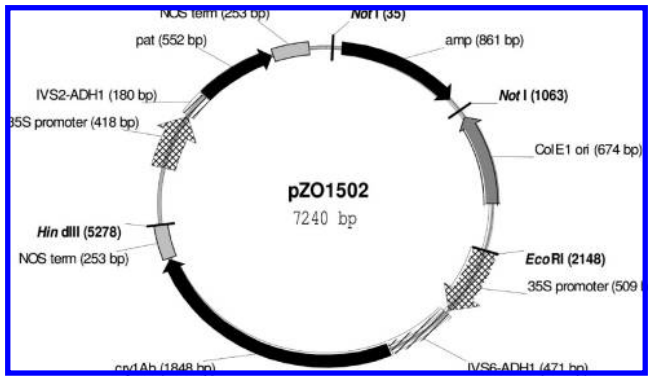

Figure 1. Map of the plasmid pZO1502, used for the transformation and generation of event Bt10. The *cry*1Ab, *pat*, and *amp* regions and relevant restriction sites are indicated.

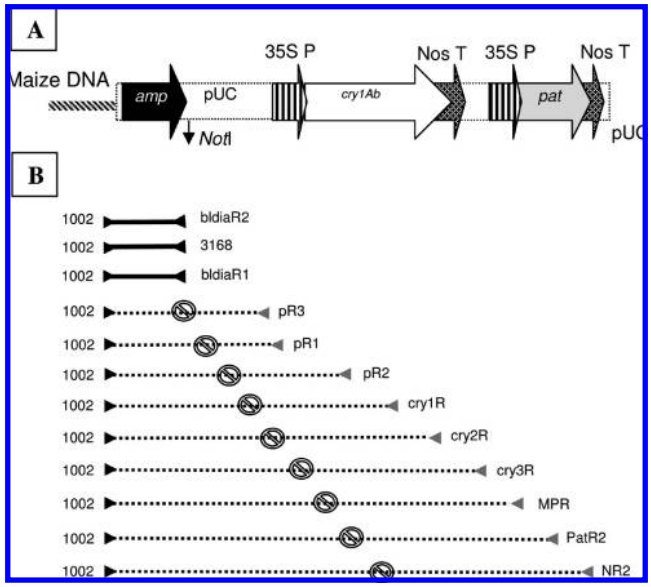

Figure 2. (**A**) Map of the presumed Bt10 insert. The different elements are indicated. (**B**) Long-template PCR analysis. Indicated with arrows are the primers and their positioning versus the Bt10 insert, in bold lines are PCR products obtained, in dotted lines and marked with the international "prohibited" sign are PCR products not obtained.

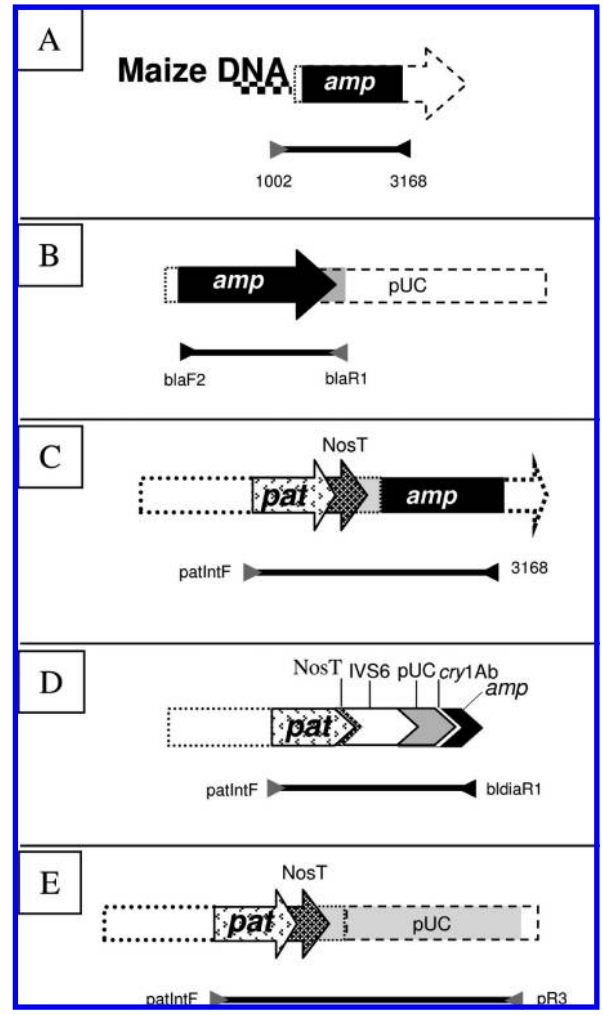

Figure 3. PCR fragments isolated from event Bt10, with indication of the parts that show DNA sequence similarity to the elements of the pZO1502 transformation plasmid.: (**A**) fragments 1002 and 3168; (**B**) fragments blaF2 and blaR1; (**C**) fragments patIntF and 3168; (**D**) fragments patIntF and bldiaR1; (**E**) fragments patIntF and pR3. Indicated with arrows are the primers, with bold lines the obtained PCR products, and with dotted lines the presumed entire gene.

amp gene was the only documented difference between the events Bt10 and Bt11 based upon the data that existed in 2005.

The Bt10-specific detection method, developed by GeneScan Europe was reported to yield a 117 bp amplicon. In the context of assessing the Bt10-specific detection method, this amplicon was analyzed for its DNA sequence. The event Bt10-specific amplicon was composed of one part of maize genomic DNA and another part of *amp* sequence. Therefore, the Bt10 region that would yield the event-specific PCR amplicon was thought to cover a region 5′ upstream of the construct plus part of the *amp* gene. Physical linkage of the *amp* gene to the rest of the construct had to be demonstrated as this was important information for the specificity of the Bt10 detection method. Therefore, a PCR analysis (regular PCR and long-template PCR) was employed, using a Bt10-specific forward primer (based on the 5′ flanking region to the *amp* gene) in combination with well-defined reverse primers based on the DNA sequence of the *amp* gene, the pUC vector, the *cry*1Ab gene, and the *pat* gene (see **Figure 2B**). PCR products of the expected size were obtained with only the first three primer pairs (1002 and bldiaR2; 1002 and 3168; 1002 and bldiaR1). Once the reverse primer was located beyond the *Not*I site, PCR amplicons could not be produced, regardless of the positioning of the reverse primer to the template. The absence of these PCR products could indicate that the *amp* gene was not in close proximity to the other parts of the insert, being *cry*1Ab and *pat*, or the presumed map was incorrect.

To test rearrangements of the presumed Bt10 insert, PCR experiments were carried out with selected primer combinations that could indicate the proximity or absence of linkage of certain elements in the Bt10 insert (**Figure 3**). For example, primer combinations were tested that should give indications if the *amp* gene was possibly located in the near vicinity of the pUC region or the *amp* gene in the near vicinity of the *pat* gene. Five PCR amplicons were obtained and submitted for DNA sequence analysis. **Figure 3** shows the individual organization of these five amplicons. One amplicon revealed the junction of the *amp* gene with 5′ upstream maize genomic sequence (**Figure 3A**); a second amplicon consisted of the full *amp* gene, linked to pUC sequence (**Figure 3B**); two amplicons contained a portion of the *pat* gene linked to either an *amp* gene or pUC vector sequence (**Figure 3C**,**E**); and one amplicon contained several truncated portions of various elements that constituted the

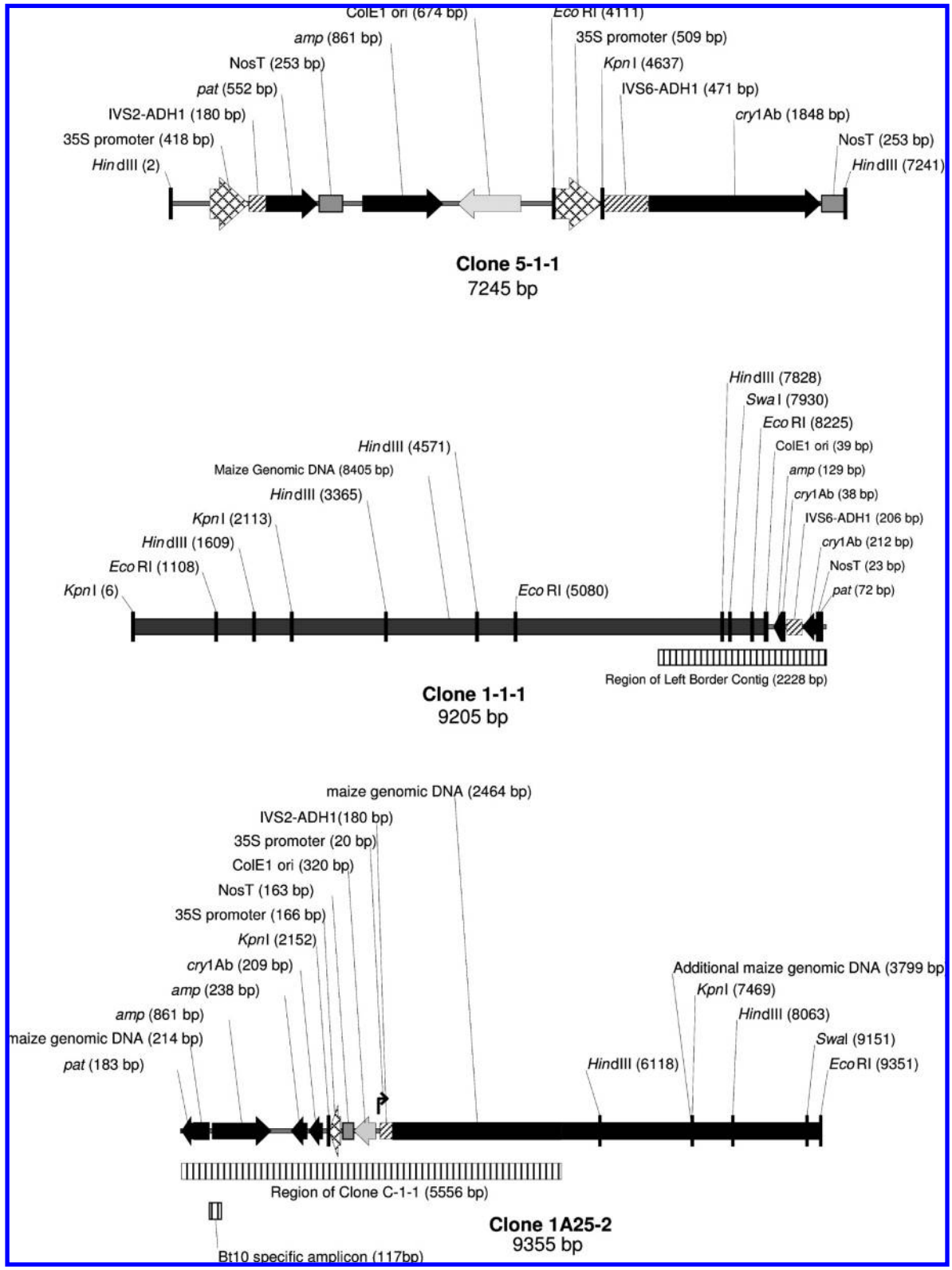

Figure 4. Map of identified Bt10 clones 5-1-1, 1-1-1, and 1A25-2. The elements, internal to pZO1502, are indicated with arrows. Maize DNA is shown in dark gray color. Relevant restriction sites are indicated.

pZO1502 plasmid (*pat*, pUC, *cry*1Ab, *amp*) (**Figure 3D**). Moreover, the small elements within this particular amplicon (**Figure 3D**) were found with different positions and orientations versus each other when compared to the presumed contiguous insertion of the structural elements *amp*, *cry*1Ab, and *pat*, revealing a complex insert of which portions appeared rearranged when compared to the expected organization as present in the transformation plasmid pZO1502. These data also indicated that more than one copy of *cry*1Ab, *pat*, and *amp*,, either full or partial, was present in event Bt10.

Genomic Library Construction and Analysis. To elucidate the organization of the Bt10 genomic modification, efforts were made to capture the entire insert of Bt10 on a single genomic

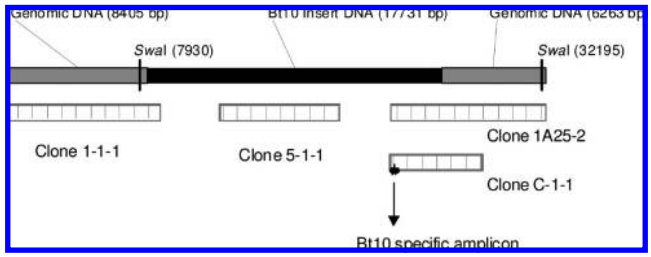

Figure 5. Predicted map of the Bt10 insert. The genomic clones isolated in this study are indicated with boxes. The small asterisk indicates the region that generates the specific PCR amplicon of the Bt10 detection method.

clone. Multiple genomic libraries were constructed (see Materials and Methods) including *Sau*3AI partial digestion, *Bam*HI partial digestion, *Hin*dIII digestion, and *Eco*RI+*Hin*dIII digestion. These libraries were screened with a *cry*1Ab-, *pat*-, or *amp*-specific probe. Several clones (clones 5-1-1, 1-1-1, C-1-1, and 1A25-2) were identified that represented structural elements of the transformation plasmid pZO1502 as well as parts of pZO1502 joined to maize DNA sequences. A graphic illustration of these clones is depicted in **Figure 4**, and their isolation and analysis are described as follows.

Clone 5-1-1 was a representative clone from the *Hin*dIII digestion library and had an insert of 7245 bp. DNA sequence analysis of the insert showed that several intact elements of the pZO1502 vector were present in this clone (*cry*1Ab, *pat*, *amp*, P35S, ColE1, NosT), but not in the same linear order as in the vector pZO1502. In this clone, the *Not*I site located between the *amp* gene and ColEI was lost because a single nucleotide change had disrupted the recognition sequence. The *Not*I site between the *nos* gene terminator, associated with the *pat* gene, and the *amp* gene, however, was maintained.

Clone 1-1-1 was identified from a *Sau*3AI partial digestion library. It carried an insert of 9205 bp. DNA sequence analysis of the insert showed that this clone contained eight different DNA sequence elements, the first of which was homologous to maize genomic sequences. Within this maize genomic sequence a *Swa*I restriction site was identified and utilized in subsequent Southern analysis. The remaining seven elements had sequence homology with elements present in the transformation vector pZO1502. These elements were, however, truncated versions of the elements in pZO1502: the *amp* region, the P35S region, the *pat* region, and the *cry* region. In addition, their positioning versus each other was different from the linear order in pZO1502.

Clone C-1-1 was identified from a *Bam*HI genomic library. Clone C-1-1 carried an insert of 5556 bp, and DNA sequence analysis identified 11 different sequence elements. One element was identified as maize genomic sequence (2.4 kb) and was situated at the 3′ end of the clone The other 10 elements showed DNA sequence homology with elements present in vector pZO1502, most of which were truncated and/or not present as described in the original plasmid. Of particular importance to the Bt10-specific detection method was the identification of a full *amp* gene joined to a maize genomic sequence. This unique junction represents the target sequence of the Bt10-specific detection method. The *Not*I site, supposed to be located between the *amp* gene and ColEI, had not been maintained within this clone.

Clone 1A25-2 was also identified from the *Bam*HI library, and this clone contained the whole clone C-1-1 plus an additional 3.8 kb of maize genomic sequence in the 3′ prolongation of the 2.4 kb maize sequence of clone C-1-1. Within this additional maize genomic sequence a *Swa*I restriction site was identified and utilized in subsequent Southern analysis.

Analysis of the four clones revealed that the Bt10 genetic modification was much more complex then previously thought. The isolation and sequencing of large genomic clones containing the entire Bt10 insert had proven to be problematic. This was likely due to the repetitive nature of the insert, which could result in unstable clones.

A putative map of the locations of the clones identified above was speculated, as shown in **Figure 5**. To verify this predicted map and to provide a better understanding of the nature of event Bt10, Southern analysis was employed (see below). Indicated in this map are the locations of the representative clones described above: clone 1-1-1, which contains a junction between maize genomic DNA and the Bt10 insert; clone 5-1-1, which indicates the presence of a potential head-to-tail concatamer of pZO1502; and clone 1A25-2, which contains the whole clone C-1-1 and which also contains a junction between maize genomic DNA and the Bt10 insert. The predicted map of the Bt10 insert contains gaps. These are regions for which genomic clones have not been isolated to date. The size of these gap regions is hypothetical and based upon cumulative data of Southern analysis (see below).

Verification of the Predicted Map of the Bt10 Insert by Southern Blot Analysis. For the Southern analyses shown in **Figure 6**, DNA was extracted from a pool of 10 Bt10 plants (line JHID829BB) and 10 near-isogenic nontransgenic control plants (line JHID829). The *cry*1Ab, *pat*, and *amp* regions were employed as specific probes. Genomic DNA from both event Bt10 and the nontransgenic control was digested with the following restriction enzymes: *Swa*I, *Eco*RI, *Kpn*I, *Eco*RI+*Kpn*I, and *Eco*RI+*Hin*dIII. On the basis of the predicted map of the Bt10 insert, **Table 2** summarizes the expected and observed hybridization bands that resulted from digestion with the indicated restriction enzymes and probed with either the *cry*1Ab, *pat*, or *amp* region.

Using the three probes indicated above, the observed hybridization bands matched the expected hybridization bands for all of the digests carried out (*Eco*RI, *Kpn*I, *Eco*RI+*Kpn*I, and *Eco*RI+*Hin*dIII). The results from the Southern analysis on *Swa*I-digested DNA indicated that the elements *cry*1Ab, *pat*, and *amp* were located on the same *Swa*I fragment of 24 kb. The observation of a *Swa*I site in clones 1-1-1 and 1A25-2 as well as the map of both of these clones indicated that they may present the 5′ and 3′ regions of the event Bt10 insert. These data confirmed the predicted map of the event Bt10 insert and could indicate that the Bt10 insert was present as a single locus contained on a 24 kb *Swa*I fragment.

To analyze if the organization of the Bt10 insert was consistent between sibling Bt10 plants, Southern analyses were performed on DNA extracted from five individual Bt10 plants. In a first set, the DNA of five plants was digested with *Eco*RI+*Hin*dIII. The blot was hybridized with individual probes *cry*1Ab, *pat*, and *amp*. This analysis was expected to result in identical patterns of hybridization bands for each of the plants within a given hybridization. When a *cry*1Ab-specific probe was used, all individual plants resulted in an identical hybridization band pattern (**Figure 7**) and confirmed the previous predicted pattern (**Figure 5**) for this particular restriction digest. A similar observation was made for hybridizations carried out with either the *pat*-specific probe or the *amp*-specific probe (**Figure 7**). A second set of blots was made with either *Swa*I- or *Eco*RI-digested DNA and probed with the three probes individually,

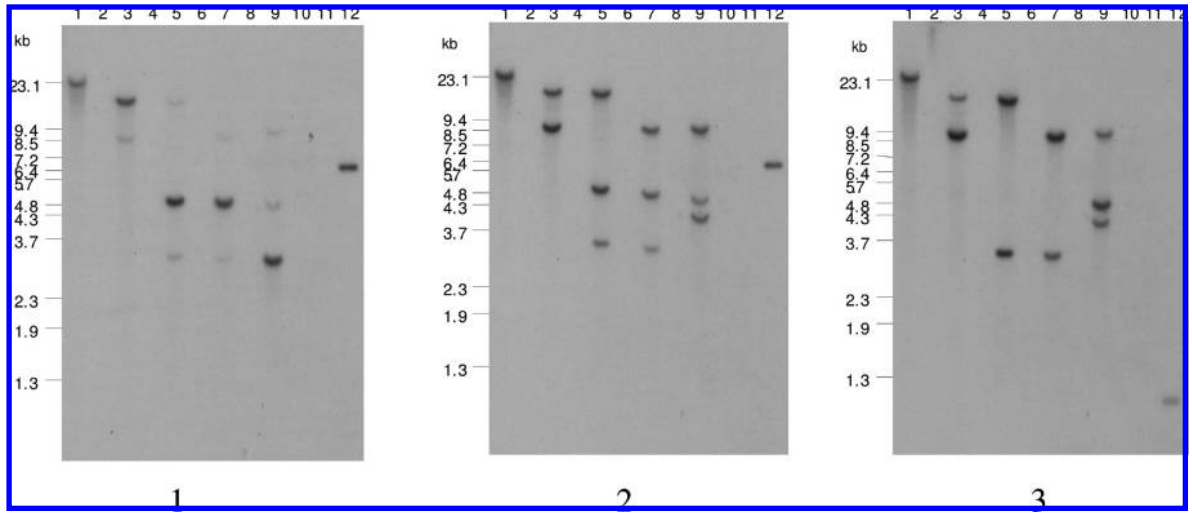

Figure 6. Southern blot analysis of event Bt10 maize genomic DNA of pooled plants was digested with restriction enzymes and, following electrophoresis and transfer to a membrane, hybridized to a *cry*1Ab-specific probe (1), a *pat*-specific probe (2), or an *amp*-specific probe (3). Lanes: 1, *Swa*I-digested event Bt10 DNA; 2, *Swa*I-digested near-isogenic nontransgenic DNA; 3, *Eco*RI-digested event Bt10 DNA; 4, *Eco*RI-digested near-isogenic nontransgenic DNA; 5, *Kpn*I-digested event Bt10 DNA; 6, *Kpn*I-digested near-isogenic nontransgenic DNA; 7, *Eco*RI+*Kpn*I-digested event Bt10 DNA; 8, *Eco*RI+*Kpn*I-digested near-isogenic nontransgenic DNA; 9, *Eco*RI+*Hin*dIII-digested event Bt10 DNA; 10, *Eco*RI+*Hin*dIII-digested near-isogenic nontransgenic DNA; 11, blank; 12, *Not*I-digested pZO1502.

Table 2. Expected Hybridization Fragments, Based on the Predicted Map of the Bt10 Insert and Observed Hybridization Bands in the Southern Blot Analysis[a]

		restriction enzymes				
probe	pattern	*Swa*I	*Eco*RI	*Kpn*I	*Eco*RI+*Kpn*I	*Eco*RI+*Hin*dIII
cry	expected hybridization band	24.3	15.5 8.7	15.3 4.8 3.0	8.7 4.8 3.0	9.2 4.5 4.1 NE 3.1
	observed hybridization band	>20	>12 8.7	>12 4.8 3.0	8.7 4.8 3.0	9.2 4.5 4.1 ND 3.1
pat	expected hybridization band	24.3	15.5 8.7	15.3 4.8 3.0	8.7 4.8 3.0	9.2 4.5 4.1 3.1 NE
	observed hybridization band	>20	>12 8.7	>12 4.8 3.0	8.7 4.8 3.0	9.2 4.5 4.1 3.1 ND
amp	expected hybridization band	24.3	15.5 8.7	15.3 4.8 NE 3.0	8.7 4.8 NE 3.0	9.2 4.5 4.1 3.1 NE
	observed hybridization band	>20	>12 8.7	>12 4.8 ND 3.0	8.7 4.8 ND 3.0	9.2 4.5 4.1 3.1 ND

[a] Fragment size is shown in kb; NE, not expected; ND, not detected.

leading to similar observations (data not shown). This demonstrated a consistent molecular organization of the Bt10 insert between sibling plants. Southern analysis was also carried out on individual Bt10 plants from another seed lot of the same Bt10 line and confirmed that the *amp* and *cry* genes were located on a 24 kb *Swa*I fragment.

Validity of the Bt10-Specific Detection Method. The event-specific detection method, developed by GeneScan Europe and in-house validated by the CRL-GMFF, was reported to yield a 117 bp amplicon. The Bt10 genomic region that would yield this PCR amplicon is located in clone 1A25-2 (as shown in **Figure 5**). To verify that this amplicon was indeed derived from the Bt10 insert, Southern analysis was conducted on DNA from five Bt10 plants, employing the amplicon of the Bt10-specific detection method as a probe. Because 23 bp of this 117 bp amplicon-specific probe are complementary to the *amp* gene, it was expected that this probe would cross-hybridize to the previously identified restriction fragments that contain the *amp* gene [**Figures 6**(**3**) and **7**(**3**), *Eco*RI and *Eco*RI+*Hin*dIII digests]. The result of this Southern analysis is shown in **Figure 8**. Bt10 DNA digested with *Eco*RI+*Hin*dIII or *Eco*RI alone and hybridized with the amplicon-specific probe showed three (9.2,

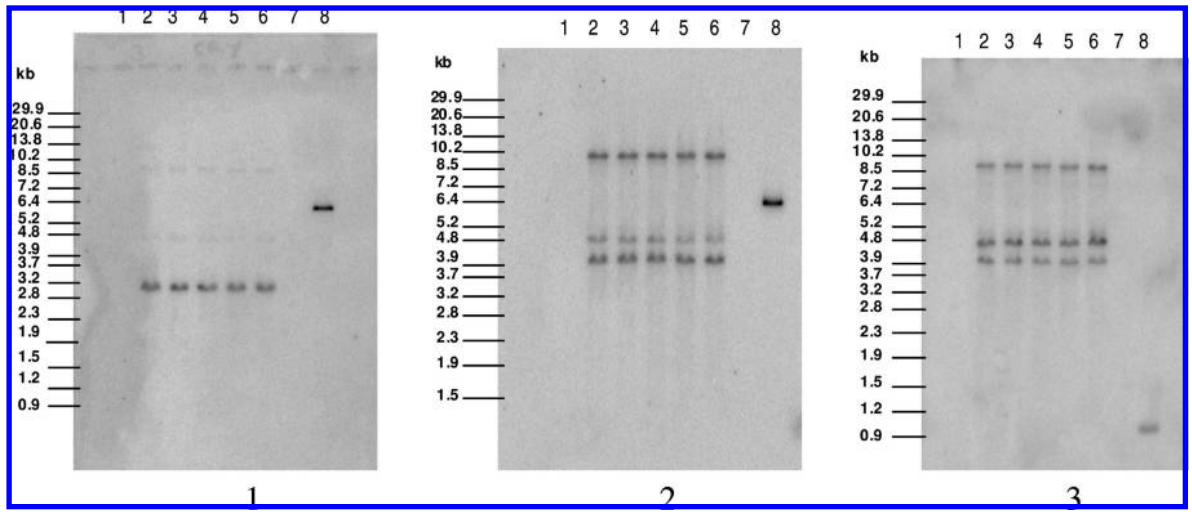

Figure 7. Southern blot analysis of Bt10 individual plants. Maize genomic DNA was *Eco*RI+*Hin*dIII-digested and probed with a *cry*1A*b*-specific probe (**1**), *pat*-specific probe (**2**), and *amp*-specific probe (**3**). Lanes: 1, *Eco*RI+*Hin*dIII-digested near-isogenic nontransgenic DNA; 2−6, *Eco*RI+*Hin*dIII-digested DNA of individual Bt10 plants; 7, blank; 8, *Not*I-digested pZO1502.

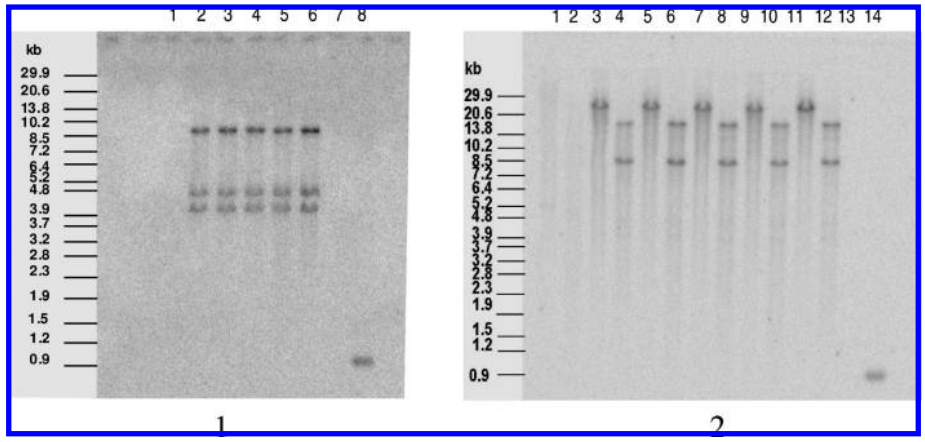

Figure 8. Southern blot analysis (with an amplicon-derived specific probe) of individual Bt10 plants. Maize genomic DNA of individual Bt10 plants was digested with *Eco*RI+*Hind*III (**1**) or either *Swa*I or *Eco*RI (**2**) and hybridized to the Bt10-specific amplicon probe (117 bp). (**1**) Lanes: 1, near-isogenic line; 2−6, individual Bt10 plants; 7, blank; 8, *Not*I-digested pZO1502. (**2**) Lanes: 1, *Swa*I digest of near-isogenic nontransgenic DNA; 2, *Eco*RI digest of near-isogenic nontransgenic DNA; 3, 5, 7, 9, 11, *Swa*I-digested DNA of five individual Bt10 plants; 4, 6, 8, 10, 12, *Eco*RI-digested DNA of five individual plants; 13, blank; 14, *Not*I-digested pZO1502.

4.5, 4.1 kb) and 2 (15.5 and 8.7 kb), respectively, hybridization bands, each of them corresponding to the bands observed for the hybridization with the *amp* probe. In addition, when Bt10 *Swa*I-digested DNA was probed with the same amplicon, it revealed once more a 24 kb *Swa*I fragment. These data confirmed that the PCR amplicon generated by the Bt10 detection method was originating from the Bt10 insert.

DISCUSSION

To assess the validity of the Bt10-specific detection method, designed to allow laboratories to unequivocally detect Bt10, multiple analytical approaches had to be taken. PCR analysis of the 5′ upstream region of the presumed event Bt10 insert showed that the *amp* gene, upon which the Bt10 detection method was partially based, was apparently not directly linked to the *cry*1Ab and *pat* genes as found in the original transformation plasmid. Small PCR fragments that were derived from the Bt10 insert showed a complex nature. Analysis of Bt10 genomic clones provided a better view of larger fragments and confirmed the Bt10 insert to be very complex.

On the basis of the DNA sequence of isolated genomic clones and cumulative Southern blot analyses data, a predicted map of the insert contained within event Bt10 was generated. This map incorporated the identified genomic clones and provided a linear order of these genomic clones within the Bt10 insert. Due to the complex nature of the Bt10 insert, with rearrangements and multiple copies of the ColE1 region, it was not possible to isolate larger clones that would contain the entire insert. Southern blot analyses with individual *cry*1Ab-, *pat*-, and *amp*-specific probes confirmed the predicted map, as the observed hybridization bands matched the expected hybridization bands.

The complexity of the insert was demonstrated via DNA sequence analysis. Several copies of the structural genes *cry*1Ab, *pat*, and *amp*, ranging from one to four copies, were found. These elements were present either as full copies or truncated at either the 5′ or 3′ end, or both. In addition, some of these structural genes were rearranged with respect to the original construct and were interspersed with small maize genomic fragments.

Event Bt10 had been examined very carefully in the light of verifying the accuracy of the Bt10-specific detection method. In this study, Southern blot analysis demonstrated that the Bt10 insert could be described as a single locus present on a *Swa*I fragment of approximately 24 kb. It was demonstrated that this

*Swa*I fragment also contained the region that would generate the Bt10-specific PCR amplicon. With the provided data and within the technical limits of the current state-of-the-art analytical methodology, it is concluded that the Bt10 event-specific detection method is fit for its purposes.

ABBREVIATIONS USED

amp, ampicillin resistance gene; *cry*1Ab, delta endotoxin insect resistance gene; *pat*, phoshinotricine resistance gene; GM, genetically modified; PCR, Polymerase Chain Reaction; EU, European Union; EC, European Commission; JRC, Joint Research Centre; CRL-GMFF, Community Reference Laboratory for GM Food and Feed.

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LITERATURE CITED

(1) Commission Decision of 18 April 2005 on emergency measures regarding the non-authorised genetically modified organism Bt10 in maize products. *Off. J. Eur. Union* **2003**, *L 101* (14).

(2) Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Union* **2003**, *L 268* (1).

(3) Bridges, A. Sampling and Detection Methods for Products of Modern Agricultural Biotechnology in NAFTA Countries. Background paper for International Life Sciences Institute workshop held October 2007, Baltimore, MD.

(4) Regulation (EC) No. 641/2004 of the European Parliament and of the Council of 6 April 2004, on detailed rules for the implementation of Regulation (EC) No 1829/2003 as regards the application for the authorization of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favorable risk evaluation. *Off. J. Eur. Union* **2004**, *L 102* (14).

(5) Windels, P. Integration of exogenous DNA into plant chromosomes. PhD thesis, Ghent University, Belgium, 2004.

(6) Windels, P.; Taverniers, A.; Depicker, A.; van Bockstaele, E.; de Loose, M. Characterization of the Roundup Ready soybean insert. *Eur. Food Res. Technol.* **2001**, *213*, 107–112.

(7) Molecular characterization of the genetic maps of commercial genetically modified plants; available at http://www.biosafety.be/gmcropff/EN/TP/MGC.html.

(8) Thomas, M.-R.; Matsumoto, S.; Cain, P.; Scott, N. S. Repetitive DNA of grapevine: classes present and sequences suitable for cultivar identification. *TAG* **1993**, *86*, 173–180.

(9) Dellaporta, S.-L.; Wood, J.; Hicks, J.-B. Maize DNA miniprep In *Molecular Biology of Plants, A Laboratory Course Manual*; Malmberg, R., Messing, J., Sussex, I., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1985; pp 10−12.

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