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Interlaboratory Transfer of a PCR Multiplex Method for Simultaneous Detection of Four Genetically Modified Maize Lines: Bt11, MON810, T25, and GA21

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The number of cultured hectares and commercialized genetically modified organisms (GMOs) has increased exponentially in the past 9 years. Governments in many countries have established a policy of labeling all food and feed containing or produced by GMOs. Consequently, versatile, laboratory-transferable GMO detection methods are in increasing demand. Here, we describe a qualitative PCR-based multiplex method for simultaneous detection and identification of four genetically modified maize lines: Bt11, MON810, T25, and GA21. The described system is based on the use of five primers directed to specific sequences in these insertion events. Primers were used in a single optimized multiplex PCR reaction, and sequences of the amplified fragments are reported. The assay allows amplification of the MON810 event from the 35S promoter to the *hsp* intron yielding a 468 bp amplicon. Amplification of the Bt11 and T25 events from the 35S promoter to the *PAT* gene yielded two different amplicons of 280 and 177 bp, respectively, whereas amplification of the 5′ flanking region of the GA21 gave rise to an amplicon of 72 bp. These fragments are clearly distinguishable in agarose gels and have been reproduced successfully in a different laboratory. Hence, the proposed method comprises a rapid, simple, reliable, and sensitive (down to 0.05%) PCR-based assay, suitable for detection of these four GM maize lines in a single reaction.

KEYWORDS: Multiplex PCR; GMO; Bt11; MON810; T25; GA21; maize

INTRODUCTION

The area of land used for the cultivation of genetically modified plants has been steadily increasing in the past 9 years with an estimated global area of 81 million hectares grown in 2004 (*1*). This has been accompanied by a considerable increase in the diversity of genetically modified organisms (GMO) currently approved worldwide. Despite progressive introduction into the market, the use of GMO in food products has caused great concern among consumers. In response, national governments have established a series of mandatory rules for regulation of GMO use in food products (*2*−*4*). However, adequate detection tools are required in order to enforce these regulations (*5*). Desirable characteristics for an ideal GMO detection technique are a high specificity, strong reproducibility, excellent efficiency and sensitivity, and detection limits of at least 0.9%. In this regard, the most accepted GMO detection methods are based on DNA amplification-based techniques such as PCR (*6*) because they meet the above requirements, and benefit from the relatively high stability of the DNA, even under extreme conditions encountered during the processing of some food products (*7*). PCR-based GMO detection relies on the specific amplification of part of the inserted transgene. PCR assays have been described for the detection of Flavr Savr tomato from Zeneca (*8*), Liberty Link canola from AgrEvo (*9*), "Maximizer" 176 (*10*−*12*) and Bt11 (*13*) maize lines from Syngenta Seeds, GTS 40-3-2 soybean line (*14*−*17*) or MON810 (*18*, *19*), GA21 (*20*) and NK603 (*21*) maize lines from Monsanto, and StarLink maize from Aventis Crop Science (*22*). PCR-based GMO detection has become so widespread that several independent methods are currently available for some modification events (*23*). Multiplex PCR methods, based on simultaneous amplification of multiple sequences in a single PCR reaction, are also beginning to be applied. These methods save considerable time and effort by decreasing the number of reactions required to assess the possible presence of GMOs in a food sample (*24*). A

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Table 1. PCR Primers Used for the Amplification of GMO Maize Lines

GMO	target	name	sense	sequence	ref
MON810/Bt11/T25	*35Sp-CaMV*[a]	*35Saf2*	forward	5′-TGATGTGATATCTCCACTGAC G-3′	*34*
MON810	Hsp70 intron	*hsp508*	reverse	5′-CGGCAAGTAATCAGCACAG-3′	this work
Bt11/T25	*PAT*[b]	*patB*	reverse	5′-GCTGCTGTAGCTGGCCTAAT CT-3′	*35*
GA21	pDPG434[c]	*GA141F*	forward	5′-GGATCCCCCAGCTTGCAT-3′	*20*
	act-r P[d]	*GA212R*	reverse	5′-TTTGGACTATCCCGACTCTCT TCT-3′	*20*

[a] *35Sp-CaMV*: 35S promoter from cauliflower mosaic virus. [b] *PAT*: phosphinothricin acetyltransferase gene. [c] pDPG434: plasmid used for GA21 transformation. [d] *act-r* P: actin rice promoter.

number of multiplex PCR-based assays have been published for GM maize detection (*25*−*27*) but these have not proven their transferability to other laboratories. Here, we describe a new multiplex PCR assay aimed at detecting four of the most widely cultivated maize GMOs authorized in Europe. This system uses a common primer directed to the 35S promoter region present in three out of the four analyzed transgenes, to reduce primer complexity, and to circumvent differences in the rate of amplification due to small differences in primer annealing efficiency. Reproducibility of the method was verified after application in a different laboratory (BioGEVES, France), with identical results obtained by both groups. The method we describe is simple, reliable, efficient, and sensitive and offers a cost-effective alternative for routine GMO identification in food product analysis.

MATERIALS AND METHODS

DNA Isolation. Powdered certified reference materials (CRMs) of Bt176, Bt11, and MON810 maize lines, and Roundup Ready soybean were obtained from the Institute for Reference Materials and Measurements (IRMM) and commercialized by Fluka Chemie GmbH (Buchs, Switzerland). GA21 and StarLink DNAs were purchased from Fluka, and T25 DNA was kindly provided by Aventis. Genomic DNA was isolated from 0.1 g of these samples using a hexadecyltrimethylammonium bromide (CTAB)-based protocol (*28*) and subsequent purification through QIAquick minicolumns (QIAGEN, GmbH, Germany). DNA concentration was quantified using a spectrophotometer GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany).

Multiplex PCR. PCR primers used for amplification of the Bt11, MON810, T25, and GA21 maize transgenic lines are shown in **Table 1**. These primers were designed using the Primer Express v1.5 software (Applied Biosystems, Foster City, CA) and yielded PCR products of 468 (Bt11), 280 (MON810), 177 (T25), and 72 (GA21) bp, respectively. Oligonucleotides were purchased from MWG-Biotech AG (Ebensburg, Germany). PCR reactions were performed using reagents from Applied Biosystems (Applied Biosystems−Roche Molecular Systems Inc., Branchburg, NJ) in a 50-μL reaction mixture containing the following: 1 × buffer II (10 mM Tris-HCl pH 8.3, 50 mM KCl), 6 mM $MgCl_2$, 400 μM dNTPs, 0.5 μM *35Saf2* primer, 0.2 μM *hsp508* primer, 0.4 μM *patB* primer, 0.15 μM *GA141F* primer, 0.15 μM *GA212R* primer, 1 unit of Ampli*Taq* Gold DNA polymerase, 0.2 units of AmpErase uracil *N*-glycosylase (UNG), and 100 ng of template DNA. PCR amplification reactions were run in a GeneAmp 9600 PCR (Perkin-Elmer) device, according to the following conditions: 2 min at 50 °C followed by 10 min at 95 °C and 40 cycles of 20 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C, with a final extension step of 5 min at 72 °C. The PCR products were resolved by electrophoresis through 2% (w/v) agarose gels and visualized by ethidium bromide staining of the gel. To evaluate the detection limits, PCR was performed as described above, using mixtures of DNA with different ratios of each GMO. Amplified fragments were extracted from the gel and cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI). DNA inserts in this vector were sequenced using the Big Dye Terminator Cycle Sequencing kit and an ABI PRISM 377 DNA sequencer (Applied Biosystems Division of Perkin-Elmer Corp., Foster City, CA).

RESULTS AND DISCUSSION

Design and Optimization of the PCR. A new PCR-based multiplex assay was developed to simultaneously amplify specific DNA sequences of all four GMOs, using primers designed on specific regions of these transgenes. Specificity of the method was determined by sequencing the amplified DNA fragments (Bt11, AY629236; MON810, AY326434; T25, AY629235; GA21, AY255709) (**Figure 1**). A primer complementary to the 35S cauliflower mosaic virus (CaMV) promoter sequence, present in MON810, Bt11, and T25 GM maize lines, was used for amplification of these transgenes. Events Bt11 and T25 share identical 35S promoter and phosphinotrycin amino transferase (*PAT*) coding regions, and therefore primers complementary to the 35S promoter (forward) and *PAT* gene (reverse) are able to amplify both transgenes. However, the presence of the *Adh1* enhancer in the Bt11 construct leads to a larger size amplicon that allows it to be distinguished from the T25 amplicon. This 35S forward oligonucleotide is also capable of priming the 35S promoter in the MON810 construct, when used in combination with a reverse primer complementary to the *hsp*70 intron. To detect event GA21, a specific primer pair was used, targeting the 5′ region of the GA21 construct within the plasmid vector used for transformation (pDPG434) and the *actin* rice (*act*-r) promoter. Annealing positions of all these primers are indicated in **Figure 2**.

Primers were chosen to have annealing temperatures (T_a) of 57 ± 5 °C, and they were designed to yield amplicon sizes of less than 500 bp. In this way, poor results associated with shearing of the DNA during food processing procedures were minimized and strong bias against larger amplicons was reduced in the multiplex reaction. Additionally, differences in the size of the amplicons were such that the unambiguous identification of PCR fragments in agarose gels was allowed. Similar amplicon lengths have been indeed reported in other studies, i.e., amplicon lengths from 104 to 447 bp (*26*), from 79 to 508 bp (*29*), from 110 to 270 bp (*30*), or from 111 to 526 bp (*31*), and shown to be suitable for this sort of analysis.

Annealing temperatures and amplicon sizes for each primer pair were as follows: 52.9 °C/62.1 °C (*35af2*/*patB*) and 468 bp for Bt11, 52.9 °C/52.9 °C (*35af2*/ *hsp508*) and 280 bp for MON810, 52.9 °C/62.1 °C (*35af2*/*patB*) and 177 bp for T25, and 58.2 °C/61.0 °C (*GA141F*/*GA212R*) and 72 bp for GA21. Multiplex conditions were subsequently optimized for primer and $MgCl_2$ concentrations, with optimal conditions given in Materials and Methods.

Specificity of the Multiplex PCR Assay. The specificity of the multiplex GMO assay was analyzed by amplification of genomic DNA extracted from all four GM targets as well as from four nontemplate GMOs. Multiplex PCR reactions performed with the two forward and three reverse primers on each individual GM target or a combination of all four DNA templates successfully amplified the expected products (**Figure**

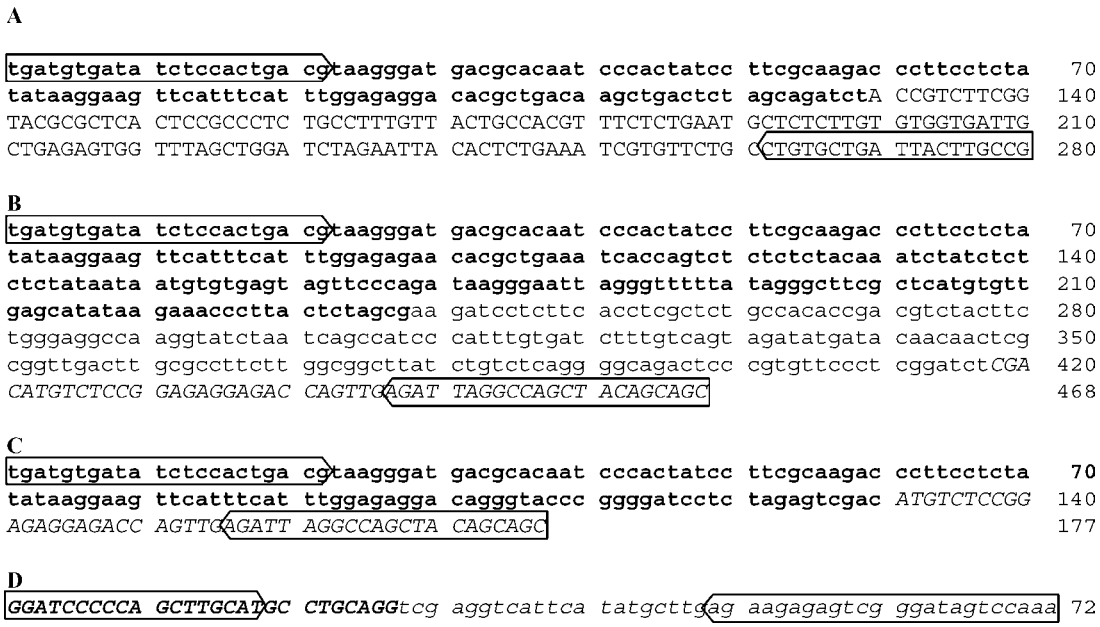

Figure 1. DNA sequences from the MON810 (**A**), Bt11 (**B**), T25 (**C**), and GA21 (**D**) events amplified with the described PCR method. Primers are boxed, with arrows indicating the orientation. Lowercase letters highlighted in bold indicate the sequence of the 35S promoter; capital letters, the *hsp*70 intron; lowercase letters, the *Adh*1 gene; capital letters and italics, the *PAT* gene; capital letters in bold and italics, the plasmid flanking sequence; and lowercase letters in italics, the 5' sequence of the *act*-r promoter.

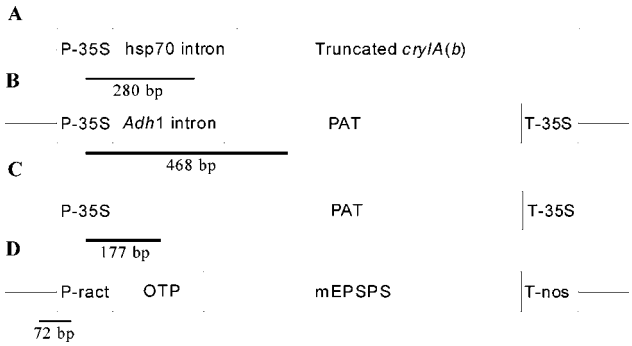

Figure 2. Schematic representation of the MON810 (**A**), Bt11 (**B**), T25 (**C**), and GA21 (D) events. Amplified regions are shown with a line, and fragment sizes are indicated.

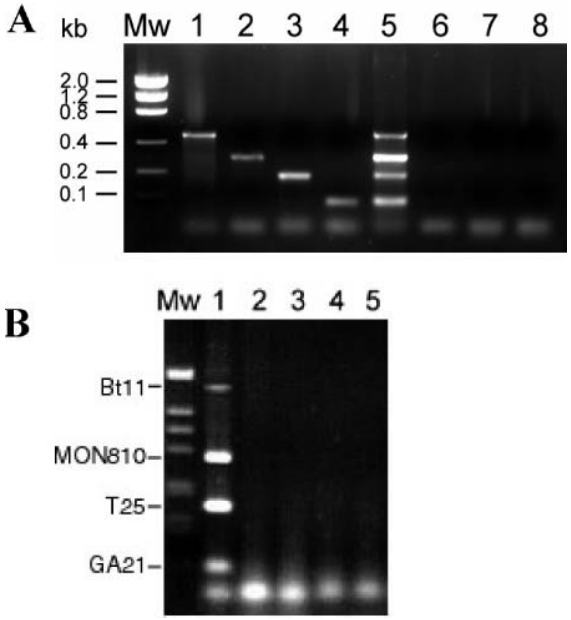

Figure 3. GMO amplification with the multiplex PCR method. (**A**) Amplification with the target maize GMs: lane 1, Bt11 (468 bp); lane 2, MON810 (280 bp); lane 3, T25 (177 bp); lane 4, GA21 (72 bp); lane 5, mixture of the four transgenic DNAs; lane 6, wild-type maize; lane 7, wild-type soybean; lane 8, no template control. (**B**) Control of amplification with nontarget GMOs: lane 1, amplification of the four target GMO; lane 2, Bt176 maize; lane 3, RR soybean; lane 4, RR cotton; lane 5, StarLink maize. 1% dilutions of each of these DNAs were used as template.

3A). No amplification product was obtained with wild-type maize or soybean DNA (**Figure 3A**) or with template DNA from nontarget GMOs such as maize Bt176 (**Figure 3B**), which does not include any of these target sequences in the transgene. Likewise, an amplification product was not obtained with template DNA from the maize StarLink or the soybean and cotton Roundup Ready events, which contain 35S promoter sequences but not the *hsp70* or *PAT* targets (**Figure 3B**). This PCR multiplex assay therefore allows specific detection of the Bt11, MON810, T25, and GA21 maize lines out of complex combinations of these DNAs, using a single amplification reaction followed by amplicon size analysis in an agarose gel.

Limit of Detection of the Multiplex PCR Assay. The sensitivity of the multiplex assay was measured using genomic DNA templates corresponding to each individual target GMOs or a mixture of all four DNAs. In both instances, four concentrations of the GMOs (calculated with respect to wild type DNA) were used: 0.05% (yielding approximately 20 copies of target DNA per PCR reaction), 0.1% (40 copies), 0.5% (200 copies), and 0.9% (360 copies), in 100 ng of maize genomic DNA, which corresponds to approximately 36 630 haploid maize genome copies (*32*). PCR reactions were run as described above, and the amplicons were resolved by agarose gel electrophoresis and ethidium bromide staining of the gel. A limit of detection (LOD) of 0.1% (40 haploid genome copies) was determined for event Bt11 with the larger amplicon size. Detection limits for events MON810, T25, and GA21 were less than 0.05% (20 haploid genome copies), with similar detection limits determined for reactions run on each DNA alone, or when DNAs from all

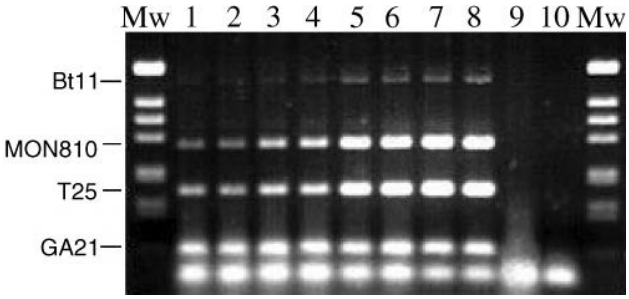

Figure 4. Analysis of the limit of detection of the qualitative multiplex PCR using a mixture of four different dilutions of each GM DNA (Bt11, MON810, T25, and GA21): lanes 1 and 2, 0.05%; lanes 3 and 4, 0.1%; lanes 5 and 6, 0.5%; and lanes 7 and 8, 0.9%; lanes 9 and 10, non-GM maize.

four events were present in the reaction. Consequently, this multiplex PCR assay is adequate for unambiguous detection and identification of the MON810, T25, GA21, and Bt11 GM maize lines. More importantly, the LODs for these events were significantly lower than those established by the currently approved GMO regulations, i.e., Commission Regulation (EC) No. 1829/2003 (*3*), which makes this multiplex assay suitable for detection of any of these four GMOs in routine analysis.

Following the recommendations of the European Network of GMO Laboratories (*33*) we tested the performance of the multiplex PCR assay in a different laboratory (BioGEVES, France) from the developer one (CSIC, Spain), to evaluate transferability of the assay as a first step toward a full interlaboratory trial required for validation. The method performed equally well in the receiver laboratory as in the developer one, showing identical sensitivities (see **Figure 4**). This result thus proved the capacity of the method to be transferred to other users, its validation in ring trials therefore being recommended.

In conclusion, the increasing demand from consumers to know the origin and composition of food products in the market demands the development and implementation of sensitive and accurate methods for GMO detection. This report presents a robust, easy, highly sensitive, and rapid multiplex PCR assay for detection of four different GMOs: Bt11, MON810, T25, and GA21. The method proved to be 100% specific for events Bt11, MON810, and GA21. In the case of T25, constructs identical identical to those used for this transformation event were used in the transformation of other crops. Among these, only commercialization of canola and cotton has been approved (www.agbios.com). Therefore, presence of a 177 bp amplicon would be indicative of the presence of any of these three GMOs, the final identification event being performed during the subsequent quantification methods. The simplicity, ease, and cost-effective nature of this assay qualifies it as a highly advantageous method to detect these four transgenes in routine laboratory analysis. This method thus represents a first qualitative screen for the absence/presence of the transgenes in food samples, determining the need of subsequent application of quantification methods specific to each of these GMOs.

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