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Reliable Detection and Identification of Genetically Modified Maize, Soybean, and Canola by Multiplex PCR Analysis

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Multiplex PCR procedures were developed for simultaneously detecting multiple target sequences in genetically modified (GM) soybean (Roundup Ready), maize (event 176, Bt11, Mon810, T14/25), and canola (GT73, HCN92/28, MS8/RF3, Oxy 235). Internal control targets (invertase gene in corn, lectin and β-actin genes in soybean, and cruciferin gene in canola) were included as appropriate to assess the efficiency of all reactions, thereby eliminating any false negatives. Primer combinations that allowed the identification of specific lines were used. In one system of identification, simultaneous amplification profiling (SAP), rather than target specific detection, was used for the identification of four GM maize lines. SAP is simple and has the potential to identify both approved and nonapproved GM lines. The template concentration was identified as a critical factor affecting efficient multiplex PCRs. In canola, 75 ng of DNA template was more effective than 50 ng of DNA for the simultaneous amplification of all targets in a reaction volume of 25 μL. Reliable identification of GM canola was achieved at a DNA concentration of 3 ng/μL, and at 0.1% for GM soybean, indicating high levels of sensitivity. Nonspecific amplification was utilized in this study as a tool for specific and reliable identification of one line of GM maize. The primer cry1A 4-3′ (antisense primer) recognizes two sites on the DNA template extracted from GM transgenic maize containing event 176 (European corn borer resistant), resulting in the amplification of products of 152 bp (expected) and 485 bp (unexpected). The latter fragment was sequenced and confirmed to be *Cry1A* specific. The systems described herein represent simple, accurate, and sensitive GMO detection methods in which only one reaction is necessary to detect multiple GM target sequences that can be reliably used for the identification of specific lines of GMOs.

KEYWORDS: GMO; transgene; multiplex PCR; simultaneous amplification profiling; nonspecific amplification; soybean; maize; canola

INTRODUCTION

Genetically modified (GM) or transgenic crops have been developed in an attempt to improve food quality and solve some of the problems associated with commercial agriculture, including disease and weed management. The global area in which GM crops are grown is increasing. Between 1996 and 2001, the global area of GM crops increased more than 30-fold, from 1.7 million hectares in 1996 to 52.6 million hectares in 2001 (*1*). The main GM crops are soybean (63% of the global area), maize (19%), cotton (13%), and canola (5%) (*1*). Herbicide tolerance is the dominant trait in GM crops, followed by insect resistance. Herbicide tolerant Roundup Ready (Monsanto) soybean is the most common transgenic line of soybean planted, whereas several lines of GM maize and canola, with differing traits, have been approved and are planted in North America.

Consumer concerns about GM foods have affected food regulation policies worldwide and have prompted the development or changes in GM food labeling legislation in many countries. Implementation of any labeling policy will require the development of reliable detection methods for genetically modified organisms (GMOs). The polymerase chain reaction (PCR) is one of the most commonly used methods for identifying the presence of GMOs (*2*, *3*). Most PCR protocols for GMO detection involve reactions that amplify a single target (*3*). Multiplex PCR is a variation of the conventional technique in which two or more targets are simultaneously amplified in the same reaction (*4*). This approach has the potential for greater reliability, flexibility, and cost reduction.

Several studies have recently described the use of multiplex PCR as a rapid and convenient screening assay for the detection of GMOs. Permingeat et al. (*5*) developed a multiplex PCR which simultaneously detects two distinct transgenes, *CryIA(b)* and *pat*, from any of four different lines of GM maize. A more specific approach was employed by Matsuoka et al. (*6*). They developed a multiplex PCR system in which five lines of GM maize were distinguished by utilizing specific primers designed

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Table 1. Primer Sequences

primer	target	orientation	sequence	length (bp)	ref
bac1	*β*-actin gene	sense	GATCCTCCAATCCAGACACTG	319	Promega
bac2		antisense	GAACTATGAATTACCTGATGG		
lecMP1	soybean lectin gene	sense	GGGTGAGGATAGGGTTCTCTG	210	this study
lecMP2		antisense	GCGATCGAGTAGTGAGAGTCG		
sttmf3a	*cp4-epsps*	sense	GCAAATCCTCTGGCCTTTCC	145	*20*
sttmr2a		antisense	CTTGCCCGTATTGATGACGTC		
35SFZMP1	*P35S*	sense	CCGACAGTGGTCCCAAAGATG	158	this study
35SFZMP2		antisense	AGAGGAAGGGTCTTGCGAAGG		
nosFZMP1	*NOS ter*	sense	GAATCCTGTTGCCGGTCTTG	125	this study
nosFZMP2		antisense	GCGGGACTCTAATCATAAAAACC		
Ivr-F	maize invertase gene	sense	CCGCTGTATCACAAGGGCTGGTACC	226	*21*
Ivr-R		antisense	GGAGCCCGTGTAGAGCATGACGATC		
NPTII-3	NPTII gene	sense	GAGGCTATTCGGCTATGACT	271	*22*
NPTII-4R		antisense	AAGGTGAGATGACAGGAGAT		
cryIA 4-5′	*cryIA(b)*	sense	GGACAACAACCCMAACATCAAC	152	*14*
cryIA 4-3′		antisense	GCACGAACTCGCTSAGCAG		
patF2	*pat*	sense	GAAGGCTAGGAACGCTTACG	262	*5*
patR2		antisense	GCCAAAAACCAACATCATGC		
barF2	*bar*	sense	GCACAGGGCTTCAAGAGCGTGGTC	177	this study
barR2		antisense	GGGCGGTACCGGCAGGCTGAA		
PL-1C	*trnL*	sense	CGAAATCGGTAGACGCTACG	387 (canola)	*23*
PL-2D		antisense	GGGGATAGAGGGACTTGAAC		
cruMPF1	canola cruciferin gene	sense	TGGCTAAAGGTACGTGAATCTG	258	this study
cruMPR1		antisense	CTCTCCCCATAAGACCTTCTCC		
goxF3	*gox* 247	sense	TAAGGCACTCCGTAACCTCATC	509	this study
goxR2		antisense	TGTGGTATCCACGTTCGGTATC		
bx3	*oxy*	sense	ACTTTCAAAGCAGCCGCTCTT	459	*7*
bx4		antisense	CACCCGAACCAACGCTAAGTTT		
bnaseMPF1	*barnase*	sense	ATCAAAAGGGAACCTTGCAGAC	202	this study
bnaseMPR1		antisense	CTGATAATGGTCCGTTGTTTTG		
patF1	*pat*	sense	GATATGGCCGCGGTTTGTGAT	186	this study
patR1		antisense	TTCCAGGGCCCAGCGTAAG		

exclusively for individual GM lines. Demeke et al. (*7*) developed a multiplex PCR assay in which three lines of GM canola were simultaneously detected. Two of the GM lines were detected using primers that target sequences within the functional transgenes, while the other line was detected less specifically using primers for promoter and terminator sequences.

Ideally, a multiplex system capable of detecting common GMO targets (promoter, terminator, and/or common transgene sequences) that is also capable of identifying specific lines would be user-friendly and simplify the process of GMO detection and identification. In this study, a reliable DNA extraction protocol is combined with several multiplex PCR procedures to develop relatively simple methods for the detection of nine of the most common transgenic crops (four maize lines, four canola lines, and one soybean line) approved for consumption in Canada and the United States. We describe three qualitative multiplex PCR systems (one each for soybean, maize, and canola), demonstrate sensitivity and broad spectrum detection, and describe the use of simultaneous amplification profiling (SAP) as a simple and reliable tool for identifying specific GM maize lines. This approach has the potential to easily distinguish approved GM lines from nonapproved GM lines.

MATERIALS AND METHODS

Plant Material. Dried powder from non-GM and GM (Monsanto Roundup Ready, GTS 40-3-2) soybeans, and certified reference material containing GM Roundup Ready soybean, 0−5% (IRMM-410R certificate, sample 7386, Fluka Chemie, Retieseweg, Belgium), were used for the soybean analyses. Seeds from four lines of GM maize (insect resistant Bt11 and 176 from Novartis, insect resistant Mon810 from Monsanto, and herbicide resistant T14/T25 from Aventis) and freeze-dried leaves of four lines of GM canola (herbicide resistant GT73 from Monsanto, herbicide resistant HCN92/28 from Aventis, male sterile MS8/RF3 and MS1/RF2 from Aventis, and bromoxynil resistant Oxy 235 from Aventis) as well as seeds and leaf tissue of nontransgenic maize (G4030 isoline) and canola (Westar), respectively, were used for the analyses. All of the transgenic lines that were used have human food safety approval in Canada (*8*) and the United States (*9*).

DNA Extraction. Ground maize seeds, powdered soybean, and freeze-dried canola leaf tissue were used as starting material for isolating DNA. The DNeasy Plant Mini kit (Qiagen, Mississauga, ON) was used according to the manufacturer's instructions with some modifications. Plant material (100 mg), buffer AP1 (500 μL), and 5 μL of RNase A (100 mg/mL) were placed in a 2 mL tube containing glass beads and then disrupted using a Hybaid RiboLyser cell disrupter (Hybaid Ltd., Ashford, U.K.). Samples were centrifuged, and the supernatant was transferred to new tubes. After the samples had been washed, DNA was eluted with 200 μL of preheated (65 °C) sterile distilled water, followed by the addition of 20 μL of 3 M sodium acetate (pH 5.2) and 220 μL of cold 2-propanol. DNA was precipitated at −80 °C for 30 min and then centrifuged. The pellets were washed with cold 70% ethanol, dried, and dissolved in 50 μL of sterile distilled water.

The DNA concentration in samples was determined by measuring the UV absorption at 260 nm, and the quality of DNA was evaluated from the 260 nm to 280 nm UV absorption ratios (GeneQuant II, Pharmacia Biotech, Cambridge, U.K.). Samples with a ratio of 1.7−1.8 were used in all assays. Pure DNA has an A_{260}/A_{280} ratio of 1.7−1.9 (DNeasy Plant Mini Kit Handbook, Qiagen).

Oligonucleotide Primers and Multiplex PCR Conditions. Oligonucleotide primers were synthesized by Invitrogen, Life Technologies (Burlington, ON). Primer sequences and references, where appropriate, are listed in **Table 1**. Various parameters were assessed initially to identify the optimal conditions for multiplex PCR. These parameters included $MgCl_2$ concentration (0.25, 0.5, 0.75, 1.0, 1.5, 3.0, and 4.5 mM), PCR buffer concentration (1.0×, 1.5×, 2.0×, 2.5×, and 3.0×), primer concentration (0.1−1.0 μM), dNTP concentration (0.2−0.6 mM), extension time (30−90 s), annealing temperature (55−61 °C), and number of cycles (25−40).

Multiplex PCR was carried out using a Stratagene (La Jolla, CA) Robocycler Gradient 40 temperature cycler under the following

conditions. For soybean, the reaction volume of 50 μL contained 100 ng of genomic DNA, 2× Taq Extender Buffer (Stratagene), 1.5 mM $MgCl_2$, 0.45 mM dNTP, primers (0.1 μM lecMP1, lecMP2; 0.3 μM 35SFZMP1, 35SFZMP2; 0.6 μM sttmf3a, sttmr2a; and 0.8 μM bac1, bac2, nosFZMP1, nosFZMP2), 5.0 units of Taq Polymerase (Invitrogen), and 5.0 units of Taq Extender (Stratagene) PCR additive. Cycling conditions were as follows: denaturing of DNA at 95 °C for 5 min; 35 cycles of 30 s at 95 °C, 1 min at 59 °C, and 30 s at 72 °C; and a final extension at 72 °C for 10 min. For maize, the reaction volume of 25 μL contained 50 ng of genomic DNA, 1× PCR buffer with $(NH_4)_2SO_4$ (Fermentas, Burlington, ON), 1.5 mM $MgCl_2$, 0.2 mM dNTP, primers (0.2 μM Ivr-F, Ivr-R; 0.3 μM barF2, barR2; 0.4 μM patF2, patR2; and 0.5 μM cry1A4-5′, cry1A4-3′), 2.5 units of Taq Polymerase (Invitrogen), and 2.5 units of Taq Extender (Stratagene) PCR additive. Cycling conditions were as follows: denaturing of DNA at 95 °C for 5 min; 35 cycles of 30 s at 95 °C, 1 min at 61 °C, and 30 s at 72 °C; and a final extension at 72 °C for 10 min. For canola, the reaction volume of 25 μL contained 75 ng of genomic DNA, 1× PCR buffer with $(NH_4)_2SO_4$ (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM dNTP, primers (0.1 μM PL-1C, PL-2D; 0.12 μM goxF3, goxR2; 0.16 μM patF1, patR1; 0.2 cruMPF1, cruMPR1; and 0.6 μM bx3, bx4, bnaseMPF1, bnaseMPR1), 2.5 units of Taq Polymerase (Invitrogen), and 2.5 units of Taq Extender (Stratagene) PCR additive. Cycling conditions were the same as for maize.

Some of the targets and/or genes studied in this project include the *epsps* gene [encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium tumefaciens* strain CP4 (CP4 EPSPS), for herbicide tolerance], the *pat* gene (encodes the enzyme phosphinotricin-*N*-acetyltransferase from *Streptomyces hygroscopicus*, for herbicide tolerance), the *bar* gene (encodes phosphinotricin-*N*-acetyltransferase from *Streptomyces viridochromogenes*, a homologue of the *pat* gene), the *barnase* gene (encodes a ribonuclease from *Bacillus amyloliquefaciens*), the *oxy* gene (encodes a nitrilase enzyme from the bacterium *Klebsiella pneumoniae*, the enzyme that hydrolyzes oxynil herbicides), and the *gox* gene (encodes the enzyme glyphosate oxidase from the bacterium *Ochrobactrum anthropi* and confers resistance to the herbicide glyphosate).

In all cases, amplification products were electrophoresed in 3% (w/v) TBE agarose gels, containing 0.1 μg/mL ethidium bromide, for 1 h at a constant voltage (100 V). Each experiment was repeated at least three times.

Cloning and Sequencing PCR Products. The 485 bp fragment associated with PCR analysis (using primers cry1A 4-5′ and cry1A 4-3′) of the maize event 176 was excised from a 3% agarose gel and purified using Bio-Rad's DNA purification kit (catalog no. 732-6010). The PCR fragment was generated using *Taq* DNA polymerase, ligated into the pCR(R)2.1 TOPO vector, and cloned using the TOPO TA cloning kit as directed by the supplier (Invitrogen, Carlsbad, CA). Plasmids with DNA inserts were sequenced using a Long Read IR 4200 automated sequencer (LI-COR Biotech, Lincoln, NE).

RESULTS AND DISCUSSION

Multiplex PCR Detection of GM Maize. A simple and efficient multiplex PCR method was developed for the detection and identification of GM maize in which only one reaction is necessary to detect any of the following four lines of transgenic maize: event 176, T14/T25, Mon810, and Bt11. Four primer pairs that generate amplification products of different lengths were added to the reaction mixture. Primer pairs cryIA 4-5′ and cryIA 4-3′ (152 bp product), patF2 and patR2 (262 bp product), and barF2 and barR2 (177 bp product) were used to detect the above-mentioned four lines of GM maize (**Figure 1**). The other primer pair, Ivr-F and Ivr-R (226 bp product), targeting the endogenous invertase gene, was used to confirm the presence of amplifiable maize DNA; the expected fragment of 226 bp was present in all maize samples that were tested, including non-GM maize. The expected amplification products were present in each of the lines that were tested (**Table 2** and **Figure 1**). All reactions produced the expected products except for a 485 bp fragment associated with the event 176 line (**Figure 1**, lane 2). After single-locus PCR with the aforementioned four primer pairs had been performed on all four lines of maize, the 485 bp fragment was observed only with the cry1A 4-5′ and cry1A 4-3′ primer pair and only associated with the event 176 line (data not shown). Sequence analysis of this DNA fragment confirmed it as being specific for the *Cry1A(b)* insect toxin gene. Analysis of the primers against the maize event 176 sequence (*10*) in a sequence analysis program revealed the reasons for the presence of the expected 152 bp product as well as the unexpected 485 bp product. The first 10 of 19 nucleotides at the 3′ end of the antisense primer cry1A 4-3′ aligned perfectly with the event 176 sequence 467 nucleotides downstream from the sense primer cry1A 4-5′ binding site, resulting in a second PCR product of 485 bp. This is consistent with previous studies that have shown that under the right conditions amplification is possible with primers that are 63−83% complementary, with 5−10 perfectly matched bases at the 3′ end of the primer (*11*). Interestingly, agarose gel analysis of the multiplex PCR amplification products consistently showed this 485 bp fragment migrating at a rate that was faster than expected (**Figure 1**, lane 2). Deng et al. (*12*) described potential size differences between multiplex and single-locus PCRs. In this study, the difference in the migration rate was not due to a difference in size. This was confirmed by gel purifying, cloning, and sequencing the fragment which was exactly 485 nucleotides. The purified fragment also migrated as expected when analyzed separately by agarose gel electrophoresis. Its migration rate in multiplex PCR analysis is obviously affected by the three bands preceding. Also, migration of the invertase gene-associated band (226 bp) in lane 2 of **Figure 1** is distorted when compared to the corresponding band in lanes 1 and 3−5 of **Figure 1**. The *Cry1A(b)* specific primers used in this study were described and used by Matsuoka et al. (*13*), but they did not report the presence of a second PCR product when using the same primer

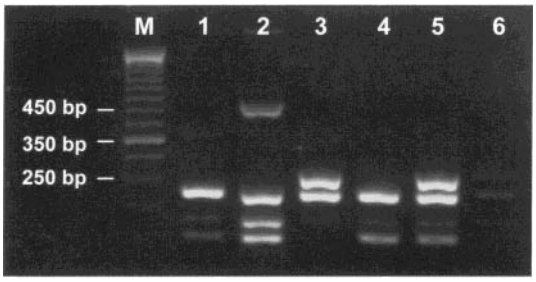

Figure 1. Multiplex PCR detection and identification of four lines of transgenic maize. Amplified DNA fragments of 262, 226, 177, and 152 bp correspond to the *pat*, *invertase*, *bar*, and *cry1A(b)* fragments, respectively: lane M, molecular weight markers (50 bp ladder); lane 1, non-GM maize; lane 2, event 176; lane 3, T14/T25; lane 4, Mon810; lane 5, Bt11; and lane 6, negative control (no DNA).

Table 2. Profile of Multiplex PCR Amplifications on Maize Samples

	invertase Ivr-F, Ivr-R 226 bp[a]	*cryIA(b)* cryIA 4-5′, cryIA 4-3′ 152 bp[a]	*pat* patF2, patR2 262 bp[a]	*bar* barF2, barR2 177 bp[a]
non-GM	+	−	−	−
event 176	+	+	−	+
T14/T25	+	−	+	−
Mon810	+	+	−	−
Bt11	+	+	+	−

[a] Target, primers, and amplicon size.

pair on the event 176 line of maize. However, the PCR cycling conditions they used differed from the ones used in this report. The high G/C content (70%) of the 10-nucleotide binding region of the cry1A 4-3′ primer coupled with these conditions appears to permit binding of the antisense primer in two distinct regions of the event 176 sequence. Under the PCR conditions used in this report, this 485 bp fragment may be used as a distinguishing feature of the event 176 line of GM maize.

The PCR amplification profile of each maize transgenic line that was tested is outlined in **Table 2**. By assessing the combination of targets present or absent in each sample, we may determine its identity. For instance, the presence of the *CryIA(b)* and *pat* targets combined with the absence of the *bar* target in a sample indicates that the sample is Bt11 maize. This system may also be used effectively to detect the existence of unspecified GMOs, including nonapproved lines, since many GM crops contain at least one of the targets detected by the primers used in the reaction. As new GM lines with new traits are approved, this system will need modification to facilitate detection of the new events.

The use of multiplex PCR to detect and identify GM maize has been described previously by Matsuoka et al. (*6*, *13*) and Permingeat et al. (*5*). Matsuoka et al. (*6*) developed a multiplex PCR system which identifies five different lines of GM maize by utilizing event specific primers. While this approach is useful, it precludes the detection of other GM lines. Permingeat et al. (*5*) developed a multiplex PCR which simultaneously detects the *cryIA(b)* and *pat* genes from any of four different lines of GM maize and can conceivably detect the *pat* gene in other GM events, but individual lines or events cannot be identified conclusively. A more beneficial strategy would allow both the identification of specific GM lines and the detection of common targets which simply determine the presence of GMOs. Matsuoka et al. (*14*) reported one such method whereby primer pairs for the detection of several frequently used foreign DNA segments (including transgenes and promoter and terminator regions) were designed and used in single-locus PCR. Different lines of GM maize were distinguished by constructing PCR amplification profiles of each sample tested individually with each pair of primers. In this study, multiple targets were simultaneously amplified to create an amplification profile that allows easy and reliable identification of different lines of GM maize. The procedures described above were used for reliable analysis of processed foods made with Bt 176 corn (cornbread and wheat-free pasta) or spiked with Bt11 and Bt 176 (pasta and corn cereal).

Multiplex PCR Detection of GM Soybean. Several reports describing PCR techniques used to detect GM soybean have been published (*15*−*17*). These involve conventional, single-locus detection by primers directed toward specific promoters, terminators, or transgenes. Hurst et al. (*18*) developed a multiplex PCR which combines a Roundup Ready specific primer pair, amplifying a region spanning the 35S promoter [from cauliflower mosaic virus (CaMV)] and the *cp4-epsps* transgene, and a pair of internal control primers amplifying a portion of the soybean lectin gene. In this study, five primer pairs were identified that allow reliable differentiation of transgenic or GM soybean lines from non-GM lines. When used in multiplex PCR, these primers were capable of simultaneously amplifying DNA products of different lengths, which can be detected by agarose gel electrophoresis. The sttmf3a and sttmr2a primer pair, directed toward the *cp4-epsps* transgene, amplifies a 145 bp DNA fragment; the 35SFZMP1 and 35SFZMP2 primer pair targets the 35S promoter and amplifies a fragment of 158 bp, and the nosFZMP1 and nosFZMP2 primer pair targets the *A. tumefaciens* nopaline synthase (NOS) terminator and amplifies a fragment of 125 bp (**Table 1**). The primer pairs lecMP1 and lecMP2 (targeting the soybean specific lectin gene, 210 bp fragment) and bac1 and bac2 (targeting the β-actin gene, 319 bp fragment) were used as internal controls to confirm the presence of amplifiable soybean DNA and assess the fitness of the procedure. These primers were successfully combined in multiplex PCR to simultaneously amplify all five targets, and the procedure that was developed was capable of reliably distinguishing non-GM soybean from Roundup Ready soybean (**Figure 2**). The sensitivity and robustness of the procedure were demonstrated by the detection of certified reference material containing GM Roundup Ready soybean ranging from 0.1% to 5% GM content (**Figure 2**, lanes 2−6). The bands associated with the 0.1% sample were present but weak, whereas the bands associated with samples containing 0.5−5% GM soybean were clearly visible. Targets *cp4-epsps* transgene, 35S promoter, and NOS terminator were detected in the Roundup Ready soybean, but were absent, as expected, in the non-GM soybean sample (**Figure 2**). The internal control products were observed for both the non-GM and GM samples, ruling out any possibility of false negatives (**Figure 2**). These procedures were also used to detect processed foods with Roundup Ready soybean, including nondairy frozen desserts, biscuits, and soy snack food (GEMMA 21 proficiency panel sample).

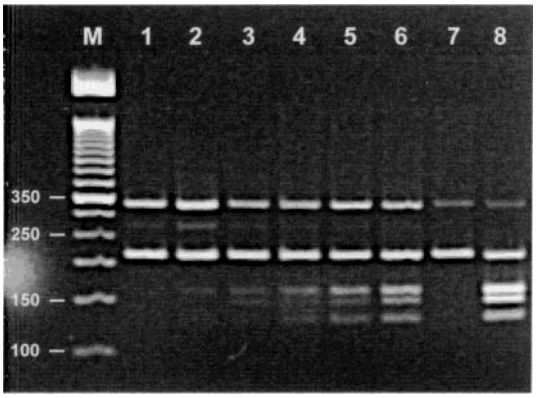

Figure 2. Multiplex PCR for the detection of transgenic soybean and determination of its sensitivity. Amplified DNA fragments of 319, 210, 158, 145, and 125 bp correspond to the β-actin, soybean lectin, P35S, *cp4-epsps*, and NOS fragments, respectively: lane M, molecular weight markers (50 bp ladder); lanes 1–6, 0, 0.1, 0.5, 1.0, 2.0, and 5.0% GMO certified Fluka reference material (IRMM-410R) containing GM Roundup Ready soybean, respectively; lane 7, non-GM soybean; and lane 8, 100% GM Roundup Ready soybean.

A large number of transgenic crop plants contain the CaMV 35S promoter and/or the NOS terminator control elements (*19*). For example, in Canada, 100% of transgenic soy and maize and at least 67% of transgenic canola approved for consumption contain either the CaMV 35S promoter or the NOS terminator control elements in their transgenic constructs (*8*). The set of primers described above for the detection of transgenic soybean contain primer pairs directed toward the CaMV 35S promoter and NOS terminator, so this primer set was assessed for the transgenic detection in crops other than soybean. In preliminary multiplex PCR assays of maize and canola, the bac1 and bac2 primer pair did not consistently amplify the expected 319 bp fragment and so was eliminated from future reactions. GM canola (Oxy 235) and GM maize (Bt11) could be distinguished from their non-GM counterparts using the soybean primer set (excluding the bac1 and bac2 primer pair) (**Figure 3**). Since

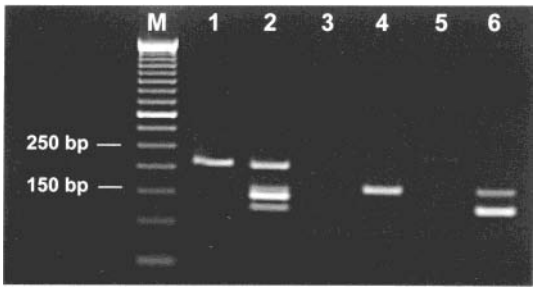

Figure 3. Multiplex PCR identification of transgenic soybean, canola, and maize using primers suitable for broad spectrum detection. Amplified DNA fragments of 210, 158, 145, and 125 bp correspond to the soybean lectin, P35S, *cp4-epsps*, and NOS fragments, respectively: lane M, molecular weight markers (50 bp ladder); lane 1, non-GM soybean; lane 2, Roundup Ready soybean; lane 3, non-GM canola; lane 4, Oxy 235 GM canola; lane 5, non-GM maize; and lane 6, Bt11 GM maize.

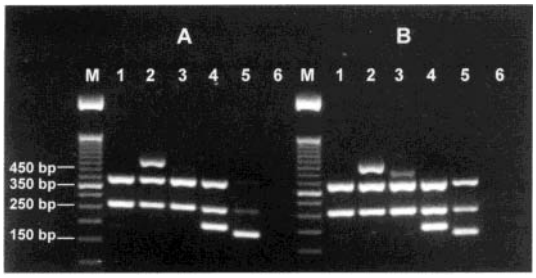

Figure 4. Multiplex PCR identification of four lines of transgenic canola using six primer pairs. The influence of DNA concentration was evaluated using 50 ng/25 μL reaction (A) and 75 ng/25 μL reaction (B). Bands of 509, 459, 378, 258, 202, and 186 bp correspond to the *gox* 247, *oxy*, *trnL*, canola cruciferin, *barnase*, and *pat* fragments, respectively: lane M, molecular weight markers (50 bp ladder); lane 1, non-GM canola; lane 2, GT73; lane 3, Oxy 235; lane 4, MS8/RF3; lane 5, HCN92/28; and lane 6, negative control (no DNA).

the lecMP1 and lecMP2 primers target the soybean specific lectin gene, the lectin specific product (210 bp) was absent from samples of maize and canola. The ability to use a single set of primers for broad spectrum qualitative detection (GM soybean, maize, and canola) saves time and reduces testing costs. It may be possible in the future to modify this procedure to include transgenic maize and canola primers, in addition to the soy primers, creating a method capable of detecting most approved events in Canada.

Multiplex PCR Detection of GM Canola. Primer pairs that generate amplification products of different lengths were added to the canola multiplex reaction mixture (**Figure 4**). However, unlike the maize multiplex system, in which the profile of amplification led to identification, the canola multiplex system utilized primers that detect transgenes specific to the particular GM lines that were tested. Although this system allowed differentiation of MS8/RF3 from the other lines, it does not allow specific identification of MS (male sterile) lines MS1/RF2 and MS8/RF3 (**Figure 5A**, lanes 1 and 2). MS1/RF2 contains the NPTII (neomycin phosphotransferase) gene, whereas MS8/RF3 lacks this transgene; therefore, primers targeting NPTII were used to distinguish these two lines (**Figure 5**). In this case, multiplex PCR followed by a supplemental PCR specific for NPTII was necessary for specific identification of the two male sterile canola lines.

The primer pairs goxF3 and goxR2, bx3 and bx4, bnaseMPF1 and bnaseMPR1, and patF1 and patR1 were used in combination to distinguish the following four lines of genetically modified

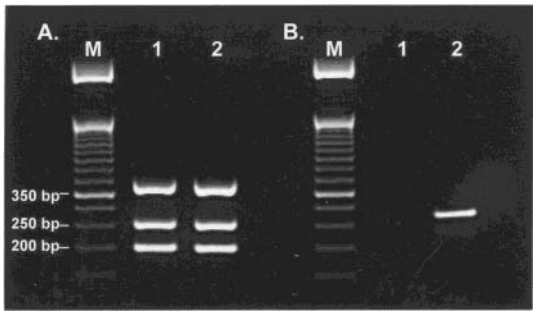

Figure 5. Multiplex PCR identification and differentiation of two male sterile lines of canola. (A) Multiplex PCR showing similar profiles of MS8/RF3 and MS1/RF2 and (B) single-locus PCR with primers NPT11-3 and NPT11-4R specific to the NPT11 gene present in only MS1/RF2: lane M, molecular weight markers (50 bp ladder); lane 1, MS8/RF3; and lane 2, MS1/RF2.

canola: GT73, Oxy 235, MS8/RF3, and HCN92/28 (**Figure 4**). Expected amplification products were present in each of the lines that were tested, and no nonspecific products were detected. The 509 bp fragment in the GT73 line corresponds to the *gox* 247 transgene. The 459 bp fragment in the Oxy 235 line corresponds to the *oxy* transgene. The 202 bp fragment in the MS8/RF3 line corresponds to the *barnase* transgene. The 186 bp fragment in the HCN92/28 line corresponds to the *pat* transgene. Additionally, the primer pairs cruMPF1 and cruMPR1 (258 bp), for the canola specific cruciferin gene, and PL-1C and PL-2D (387 bp), for the chloroplast tRNA-leu gene, confirmed the presence of amplifiable canola and plant DNA, respectively. The expected fragments were present in all samples that were tested, including non-GM canola. In this multiplex system, only one reaction is necessary to detect any of the four lines of transgenic canola mentioned above. The template concentration significantly affects the efficiency of multiplex PCR, and this is demonstrated for canola in panels A and B of **Figure 4** which show the effects of using 50 and 75 ng of template, respectively, for amplification of the various targets.

Aside from our findings, there is only one other published report of a multiplex PCR assay for the detection of GM canola (*7*). Demeke et al. (*7*) developed a multiplex PCR assay in which three lines of GM canola are identified. In this study, four lines of canola [including the three identified by Demeke et al. (*7*)] are reliably detected using a relatively simple multiplex procedure. This robust procedure will facilitate the addition of more primer combinations to identify other approved lines of transgenic canola.

Multiplex PCR procedures that facilitate simple and reliable identification of some of the most common transgenic lines of maize, soybean, and canola were developed. The methods are reproducible since all experiments were repeated at least twice. Host specific internal targets were included in all assays as controls for evaluating DNA quality and PCR efficacy, eliminating any possibility of false negatives, thereby increasing reliability. This is the first study in which simultaneous amplification profiling (SAP) has been described as a reliable tool for identifying transgenic lines of maize. SAP has the ability to identify specific approved GM lines, as well as the added potential of (1) identifying new GM lines containing similar transgenes by simply determining the associated profile (additional primers may or may not be required) and (2) detecting nonapproved lines by their deviant profiles. This definitely saves time and costs of test implementation. This study also conclusively demonstrates that in multiplex PCR, the migration rate

of high-molecular weight bands may be affected by lower-molecular weight bands.

The results obtained from analysis and detection of the Fluka reference material indicate that this primer system may be easily adapted for semiquantitative type analysis of some genetically modified sample matrices such as seed, grain, and flour. Side-by-side PCR analyses may be carried out comparing samples with unknown GM contents with reference standards with known GM contents. The targets identified also may be useful in developing real-time quantitative PCR procedures.

Several factors are important in achieving successful multiplex amplification, and the template concentration, often overlooked, was shown to be a critical factor in some reactions. The combination of sample grinding using the Hybaid Ribolyser cell disrupter and DNA extraction using a modified DNeasy Plant Mini kit protocol simplified and enhanced DNA extraction. This approach also reduced opportunities for cross contamination. The optimum conditions for multiplex PCR appear to depend more on the primer combinations and less on the DNA source. This observation is based on the fact that the primer set and conditions developed for soybean GM detection were also used to reliably detect GM maize and canola.

Procedures are described that allow easy identification of some of the most common GM lines approved for use in Canada and the United States, including four lines of GM maize, four lines of GM canola, and Roundup Ready soybean. Nonspecific reactions are usually seen as undesirable events, but in this study, a nonspecific fragment was identified as a useful and reliable feature for the identification of a transgenic event in maize. The multiplex PCR systems reported in this study are simple, reliable, efficient, and thus easy to implement in any regulatory program aimed at controlling the movement, distribution, and/or use of the GM lines described herein.

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