

Detecting genes inserted into genetically modified *Zea mays L.* Yieldgard[®] MON810, Roundup Ready[®] GA21, and Roundup Ready[®] NK603 using multiplex polymerase chain reaction

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The paper describes one of the studies to address the need of researchers, private companies, and Philippine government regulatory units, for protocols to detect transgenes inserted into genetically modified crops. PCR-based detection of a native corn gene and transgenes inserted into three kinds of genetically modified (GM) corn (*Zea mays L.*), including Yieldgard[®] MON810, Roundup Ready[®] GA21, and Roundup Ready[®] NK603, was achieved using primers that amplify fragments of *zein* (native corn gene), *CamV 35S* (cauliflower mosaic virus promoter), *cry1Ab* (gene for the insecticidal protein, Cry1Ab, derived from *Bacillus thuringiensis*), *OTP/m-epsps* (gene for optimized chloroplast transit peptide and the

modified plant enzyme 5-enolpyruvylshikimate-3-phosphate synthase, respectively), *r-act pro* (the rice actin promoter), and *nos* (nopaline synthase transcriptional termination element from *Agrobacterium tumefaciens*). Fragments of three genes *zein* (329-bp), *CamV 35S* promoter (220-bp), and the *cry1Ab* (194-bp) were detected by multiplex PCR from GM corn Yieldgard[®] MON810. Multiplex PCR amplified a fragment of the *OTP/m-epsps* gene (270-bp), in addition to the fragments of the *zein* gene (329-bp) and the *nos* terminator (151-bp) in the GM corn Roundup Ready[®] GA21. In the Roundup Ready[®] NK603 GM corn, multiplex PCR detected fragments of *zein* (329-bp), *camV 35S* promoter (220-bp) and *nos* terminator gene (151-bp).

The study showed that a fragment of the rice actin promoter (408-bp) which could not be amplified in multiplex PCR, was detected in single PCR using DNA template from both Roundup Ready[®] GA21 and Roundup Ready[®] NK603. Multiplex PCR could detect the presence of target gene fragments in DNA extracted from seed samples containing as low as 0.1% (0.1g GM/99.9g non-GM IPBVar1) or 1.0% (1.0g GM/99g non-GM IPBVar1) GM corn seeds.

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INTRODUCTION

Advances in the field of plant genetic engineering allowed the production of improved plant varieties with transgenes that enable them to express specific valuable agronomic traits such as insect resistance and herbicide tolerance in corn. James Clive (2009) reported that out of a total of 158 million hectares planted into maize, 26% are transgenic. Although many GM crops are already available in the global market, their worldwide acceptance has yet to be attained.

Concerns about environmental and public health safety issues have been raised as a result of the commercial propagation of the first transgenic crop (Monsanto Company's Yieldgard® *Bt*-corn) in the Philippines in 2002. The Philippine government's approval of the commercial propagation and importation of several other GM crops after that, has drawn mixed reaction from different stakeholders, leading to requests for government and private services to detect the presence of foreign genes in products of modern crop biotechnology. This study was conducted to come up with a detection protocol for genetically modified corn without the need to use more expensive commercially available detection kits. The same detection approach tested in this study could be applied in developing detection protocols for other genetically modified crops.

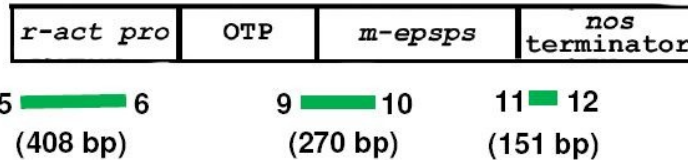
This study reports the detection of transgenes in GM corn Yieldgard® MON810, Roundup Ready® GA21, and Roundup Ready® NK603 (Figure 1). Yieldgard® MON810 or *Bt* corn was developed to be resistant to attack by European corn borer *Ostrinia nubilalis*. This GM corn contains a single copy of the truncated *cry1Ab* gene for the production of the bacterial (*Bacillus thuringiensis*) toxin which renders the plant resistant to Lepidopteran insects. The *CamV 35S* promoter and *hsp70* leader sequences are also present in *Bt* corn but the *nos* 3' termination signal was not integrated into the host genome and was lost through a 3' truncation of the gene cassette (GM Crop Database, 2004). Detection of transgenes from two GM maize lines (GM GA21 and NK603) that were genetically engineered to make the plants tolerant to the herbicide glyphosate (GM Crop Database,

2004) was also performed. In GM corn GA21, the gene for enolpyruvylshikimate-3-phosphate synthase (*epsps*) was modified through site-directed mutagenesis, resulting in a modified enzyme (mEPSPS) that is insensitive to inactivation by glyphosate. Maize line GA21 also contains the gene for the optimized chloroplast transit peptide (OTP, that allows subcellular targeting of mEPSPS protein into the chloroplast) and the *nos* 3' termination sequence. The GM corn NK603 contains two adjacent expression cassettes for a *cp4* 5-enolpyruvylshikimate-3-phosphate synthase gene (*cp4 epsps*), one under the regulation of a rice actin promoter while the other is regulated by a *CamV 35S* promoter. NK603 also contains the chloroplast transit peptide (CTP2; isolated from *Arabidopsis thaliana epsps*) and the *nos* terminator. Multiplex PCR has been used in detecting transgenes including the *CamV 35S* promoter, *nos* terminator, deltaendotoxins *cry1Ab* and *cry9*, phosphonothricin acetyl transferase, neomycin phosphotransferase II (*nptII*), and CP4/*m-epsps* (Vollenhofer et al. 1999; Chiueh et al. 2001 and 2002; Matsuoka et al. 2002). This study was focused on using multiplex PCR to detect three types of genetically modified corn seeds and experiments were conducted with the following specific objectives for each variety of GM corn used: select primers that target transgenes inserted into GM corn from among the primers reported in the literature, optimization of conditions for multiplex PCR using selected primers, sequence analysis of amplicons to verify the amplification of target genes, and the determination of detection

Yieldgard MON810



Roundup Ready GA21



Roundup Ready NK603

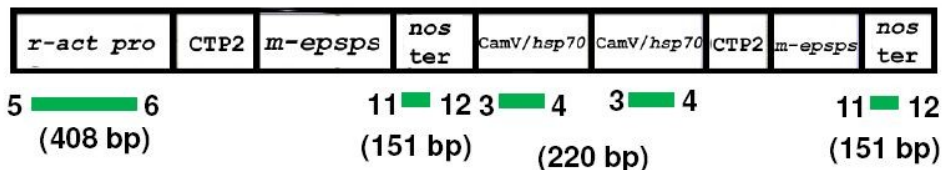


Figure 1. Schematic diagram of transgenes inserted into GM corn showing the relative annealing sites of primers and sizes of amplicons

Table 1. Primers used to detect native corn gene *zein* and transgenes inserted in GM corn

Code	Primers	Sequence	Target gene	Reference/ Source
1	ZeO1	TGCTTGCATTGTTGCTCTCCTAG	<i>Zein</i> (329 bp)	Studer et al. 1997
2	ZeO2	GTCGCAGTGACATTGTGGCAT		
3	CMO1	CACTACAAATGCCATCATTGCGATA	<i>CamV 35S</i> (220 bp)	Matsuoka et al. 1999
4	CMO2	CTTATATAGAGGAAGGGTCTTGCGA		
5	RAct5	ATCTTTGGCCTTGGTAGTTTG	<i>Rice actin promoter (r-act pro, 408 bp)</i>	Matsuoka et al. 2002
6	JRA2	GGATTCAAATTCTACCCAC		This study
7	HSO1	AGTTTCCTTTTGTGCTCTCCT	<i>hsp70/cry1Ab</i> (194 bp)	Matsuoka et al. 2000
8	CRO1	GATGTTTGGGTTGTTGCCAT		
9	OTP5	ACGGTGGAAGAGTTCAATGTATG	<i>OTP/m-epsps</i> (270 bp)	Matsuoka et al. 2000
10	EPS3	TCTCCTTGATGGGCTGCA		
11	NOS ter 3-5	GTCTTGCATCATTATCATATAAATTTCTG	<i>nos terminator</i> (151 bp)	Matsuoka et al. 1999
12	NOS ter 3-3	CGCTATATTTGTTTCTATCGCGT		

limit by getting the percent GM ground seeds (grams GM ground seeds/100 grams total ground seed) needed to detect the transgenes using multiplex PCR.

MATERIALS AND METHODS

Ground seeds of GM insect-resistant Yieldgard® MON810 and two GM herbicide-resistant corn, Roundup Ready® GA21 and Roundup Ready® NK603 were provided by Monsanto, USA. IPBVar1 corn seeds purchased from the Institute of Plant Breeding in UP Los Baños were used as the source of non-transgenic control samples.

Preparation of corn seeds

Ground corn seed samples containing different weight percentages (100%, 10%, 1%, 0.5%, 0.3%, and 0.1% GM seeds) of transgenic corn Yieldgard® MON810, Roundup Ready® GA21, and Roundup Ready® NK603 were prepared. For example, 100% GM samples contained only ground GM seeds, 10% GM preparation contained 10g ground GM seeds plus 90g non-GM IPBVar1 corn seeds, 1.0% GM sample contained 1.0g ground GM seeds and 99.0g IPBVar1 seeds. Only the non-GM ground PBVar1 seeds were used for 0% GM.

Ten grams of corn seeds were sterilized by rinsing with sterile distilled water twice, incubating the seeds for 45 minutes in a 100-mL solution containing 2.75% (w/v) sodium hypochlorite or 50% of commercially available Zonrox (Green

Cross, Inc., Parañaque City, Philippines) and 200 mL Joy detergent (Procter & Gamble Philippines, Inc., Makati City, Philippines), and rinsing in sterile distilled water four times. Sterilized seeds were incubated at 60°C until dehydrated. Homogenization of the seeds was performed using a Braun Model Type 4184 homogenizer (Braun: Naucalpan, Mexico) after which ground samples were sieved prior to use for DNA extraction.

DNA extraction from seeds

The Nucleospin® Plant Kit (BD Biosciences: California, USA) was used to extract DNA from corn seed samples following manufacturer's protocol. Visualization of DNA extracts was done by resolving aliquots of DNA extracts in agarose gel electrophoresis, subsequent staining with ethidium bromide, and observation under ultraviolet (UV) light in a UV transilluminator (UVP: California, USA).

Selection of PCR primers that target a native corn gene and transgenes

Eleven primers reported in previous studies and one primer designed in this study were used in multiplex PCR detection of transgenes from GM corn (Table 1). One of the primer pairs targets *zein* gene (a native *Zea mays* gene) and the other primers target transgenic genes including promoters such as cauliflower mosaic virus promoter (*CamV 35S*) and rice actin promoter (*r-act pro*), a transcription terminator from *Agrobacterium tumefaciens*, the nopaline synthase gene (*nos*), the gene for the optimized transit peptide and modified maize enolpyruvylshikimate-3-phosphate synthase (*OTP-mepsps*), and the *cry1Ab* delta-endotoxin gene from *Bacillus thuringiensis* subs. *kurstaki* (Table 1). A new primer (JRA2, Table 1) was designed in this study and was used in combination with primer RAct5 to amplify a fragment of the rice actin promoter. All primers were synthesized by Genset Singapore Biotech Pte Ltd (Singapore, Singapore).

PCR detection of native corn gene and transgenes

Preliminary single PCR experiments tested the amplification of target genes using selected primers with transgenic or non-transgenic *Zea mays* DNA template. Final concentrations of the PCR components were as follows: PCR buffer, 1X; MgCl₂, 3 mM; dNTPs, 0.2 mM each; primers, 0.2 µM each; recombinant *Taq* Polymerase, 0.05U/reaction, and 50 ng of corn DNA template. The following PCR conditions were used: initial denaturation at 94°C and 5 minutes, denaturation at

94°C and 1 minute, annealing at 34°C and 45 seconds, extension at 72°C and 1 minute, and final extension at 72°C and 3 minutes. The cycling number was 50 for all reactions.

Multiplex PCR with all the primers in one reaction mixture and DNA template from three corn samples (one at a time) was performed using the following concentrations of reagents 1X PCR buffer, 2.0 mM MgCl₂, 0.3M dNTPs; 1.0 mM for each primer; 0.05 U/mL of Platinum *Taq* High Fidelity Polymerase (Invitrogen Life Technologies: California, USA), and 200 ng of DNA template. PCR conditions were as follows: initial denaturation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 40 seconds, and final extension at 68°C and 3 minutes. The number of cycles for all reactions was 40.

Cloning and Sequencing of Target Genes

Each amplicon generated from optimized multiplex PCR was cloned into the pCR[®] 4-TOPO[®] vector using TOPO[®] TA Cloning Kit (Invitrogen Life Technologies: California, USA). Clones containing the expected product size were selected by colony PCR and/or *Eco*R1 digestion according to manufacturer's instruction. Mini-preparations of the recombinant plasmids containing the desired insert were isolated using the Nucleospin Miniprep Kit (BD Biosciences: California, USA) and submitted for sequence analysis and using the ABI 377 Automated Sequencer (Perkin Elmer, USA) of the AMOR laboratory (NIMBB, University of the Philippines Diliman).

Determination of Detection Limits

DNA extracts from known weight percentages of GM corn samples (100%, 10%, 1%, 0.5%, 0.3%, and 0.1% GM seeds) were used as templates for the optimized multiplex PCR. The DNA template extracted from samples containing the smallest weight percentage of GM seeds that generated visible amplicons in agarose gel was determined in order to obtain the transgene detection limit.

RESULTS

The primer for *zein* gene and other transgenes (*CamV 35S* promoter, rice actin promoter, *Bt* protein *cry1Ab*, optimized chloroplast transit peptide, OTP, with modified *m-epsps* and *nos* terminator) that generated expected size amplicons in preliminary single PCR experiments (data not shown) were

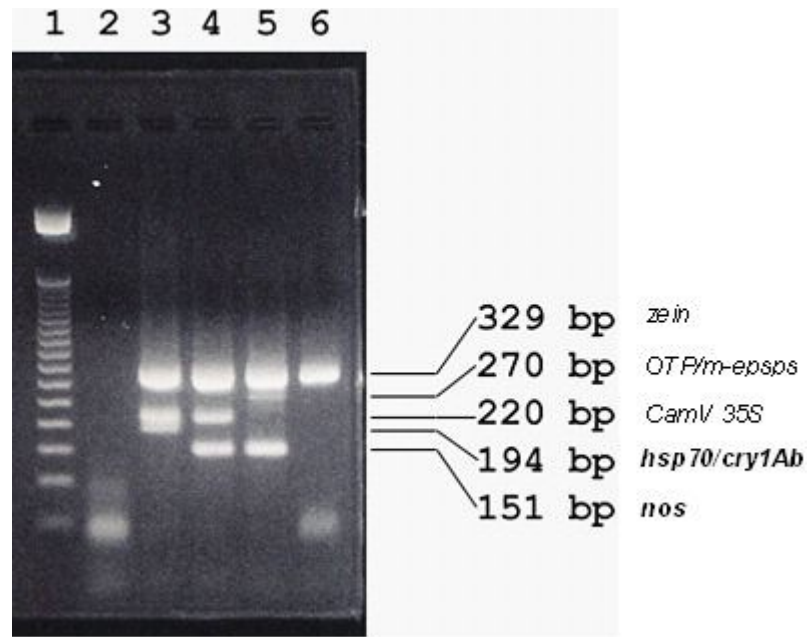


Figure 2. Amplified products of multiplex PCR using *Zea mays* DNA templates and primers targeting *zein* and transgenes. Lane 2, no template control; lanes 3, 4, 5, and 6 are PCR products using Yieldgard[®] MON810, Roundup Ready[®] NK603, Roundup Ready[®] GA21, and non-GM IPBVar1 DNA templates, respectively. Lane 1 is the 50 bp ladder.

selected for multiplex PCR using DNA templates from three GM corn samples (Table 1, Matsuoka et al. 1999, 2000, and 2002; Studer et al. 1997). The relative locations of the primer annealing sites and expected size amplicons are shown in the diagrammatic illustration of GM transgenes (Figure 1).

Multiplex PCR detection of transgenes

The 329-bp *zein* fragment was amplified from all *Zea mays* samples used (Figure 2, lanes 3 to 6). The transgene fragments amplified in the multiplex PCR include a 220-bp *CamV 35S* promoter and a 194-bp *cry1Ab* gene from Yieldgard[®] MON810 (Figure 2, lane 3), the 270-bp *OTP/m-epsps* and 151-bp *nos* terminator fragments from Roundup Ready[®] corn GA 21 (Figure 2, lane 5), and 220-bp *CamV 35S* promoter and 151-bp *nos* terminator fragments from Roundup Ready[®] NK603 corn (Figure 2, lane 4). Although the 408-bp rice actin promoter fragment was amplified in single PCR (Figures 4 and 5) using both Roundup Ready[®] GA21 and NK603 corn DNA template, no band representing that fragment size was amplified in multiplex PCR.

Sequence Analysis of Amplicons from Multiplex PCR

The amplicons corresponding to fragments of transgenes were successfully cloned into pCR[®] 4-TOPO[®] vector using TOPO[®] TA Cloning Kit. When the sequence of each amplicon was subjected to homology search using Basic Local Alignment and Search Tool (Altschul et al. 1990), all *zein* fragments

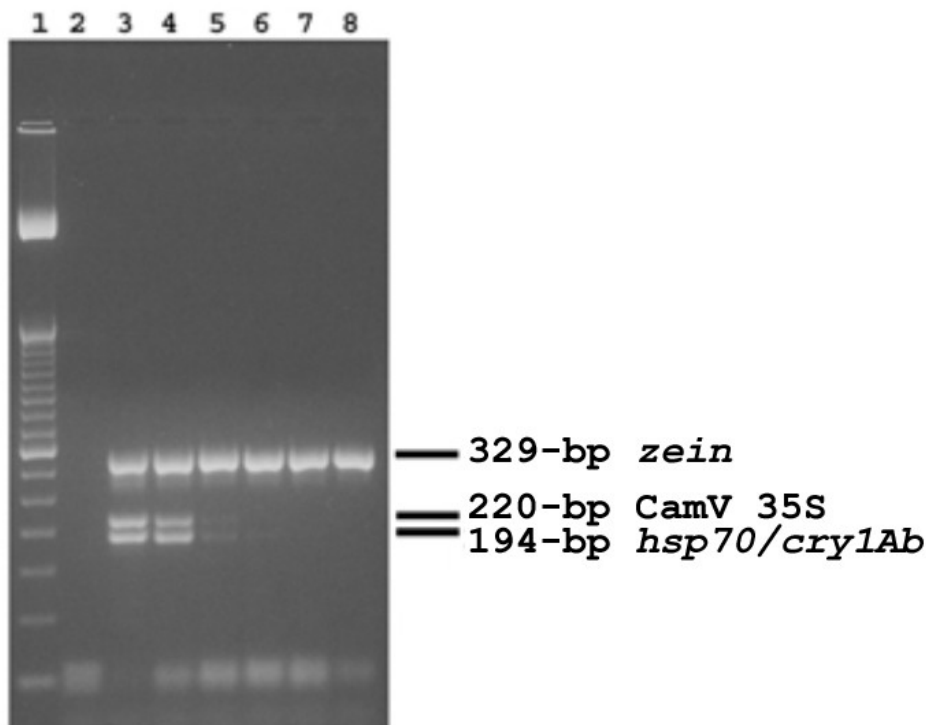


Figure 3. Detection limit of *zein* and two transgenes of Yieldgard® MON810 *Zea mays* by multiplex PCR. PCR products using DNA templates prepared from 100% (lane 3), 10% (lane 4), 1% (lane 5), 0.3% (lane 6), 0.1% (lane 7), and 0% (lane 8) Yieldgard® MON810 *Zea mays* seed mixtures. Lane 2 is the no template negative control. Lane 1 is the 50 bp ladder.

amplified by PCR from the different corn varieties were of the same sequence and were found to be 100% similar to the *Zea mays* 10kD delta zein (GenBank Accession No. AF371266.1). The bases at the 3' end of the 270-bp fragment amplified from Roundup Ready® GA21 corn were found to be 100% similar to *Zea mays* mRNA for EPSPS (GenBank Accession No. X63374.1). The 220-bp *CamV 35S* fragment amplified from Yieldgard® MON810 and Roundup Ready® NK603 corn DNA exhibited 100% sequence similarity with a 218-bp sequence from *CamV* genome (GenBank Accession No. V00141.1). The bases from the 3' end of the 194-bp *cry1Ab* fragment had 100% similarity with *cry1Ac1* (GenBank Accession No. AY126450.1) and the rest of its sequence had 91% similarity with maize heat shock protein 70 exon 2 and pMON9502 (GenBank Accession No. X03697.1). The 151-bp amplicons generated from Roundup Ready® GA21 and NK603 corn exhibited 100% similarity to the *nos* terminator sequence in the *Agrobacterium tumefaciens* Ti plasmid (GenBank Accession No. AJ237588.1).

Detection Limit of Foreign Genes Inserted into *Zea mays* by Multiplex PCR

Yieldgard® MON810 corn. The presence of the 329-bp *zein* amplicon in multiplex PCR was detected even with samples containing no GM DNA or samples with 100% non-GM IPBVar1 (Figure 3, lane 8) since both GM corn and non-GM corn seeds contain the *zein* gene. The 220-bp *CamV 35S* and 194-bp *hsp70/cry1Ab* amplicons exhibited decreasing band intensities with decreasing percent GM DNA template (Figure 3). Amplification was detected in PCR using DNA extracted from ground corn seeds containing at least 1.0g ground Yieldgard® MON810 plus 99.0g ground non-GM IPBVar1 or with 1.0 % Yieldgard® MON810 (Figure 3, lane 5).

Roundup Ready® GA21 corn.

The multiplex PCR procedure using DNA templates extracted from ground seeds with as low as 1% wt/wt Roundup Ready® GA21 was able to detect all transgene targets *zein*, *OTP/m-epsps*, and *nos* (Figure 4, A). The 151-bp *nos* target was also faintly detected in DNA extracted from 0.1% Roundup Ready® GA21. The 408-bp fragment of the rice

actin promoter was amplified in single PCR using the RAct5 and JRAX primers and Roundup Ready® GA21 DNA template prepared from 10g ground corn with as low as 0.1% wt/wt Roundup Ready® GA21 (Figure 4, B).

Roundup Ready® NK603 corn. The detection limit of the 329-bp *zein* fragment, the transgene fragments 220-bp *CamV 35S* promoter and 151-bp *nos* terminator by multiplex PCR, and the 408-bp rice actin promoter by single PCR, was 0.1% wt/wt Roundup Ready® NK603 (Fig. 5).

DISCUSSION

The selection of primers is crucial for developing optimized multiplex PCR detection of target genes in this study. Multiplex PCR involved the amplification of several target DNA fragments with considerable disparity in sizes. The multiplex PCR using DNA template from Yieldgard® MON810 produced a 194-bp fragment of *cry1Ab* which was not produced in the other two GM corn samples. The 270-bp *OTP/m-epsps* amplicon was produced only in multiplex PCR using Roundup Ready® GA21 template. Based on these results, Yieldgard® MON810 and Roundup Ready® GA21 could be identified and distinguished

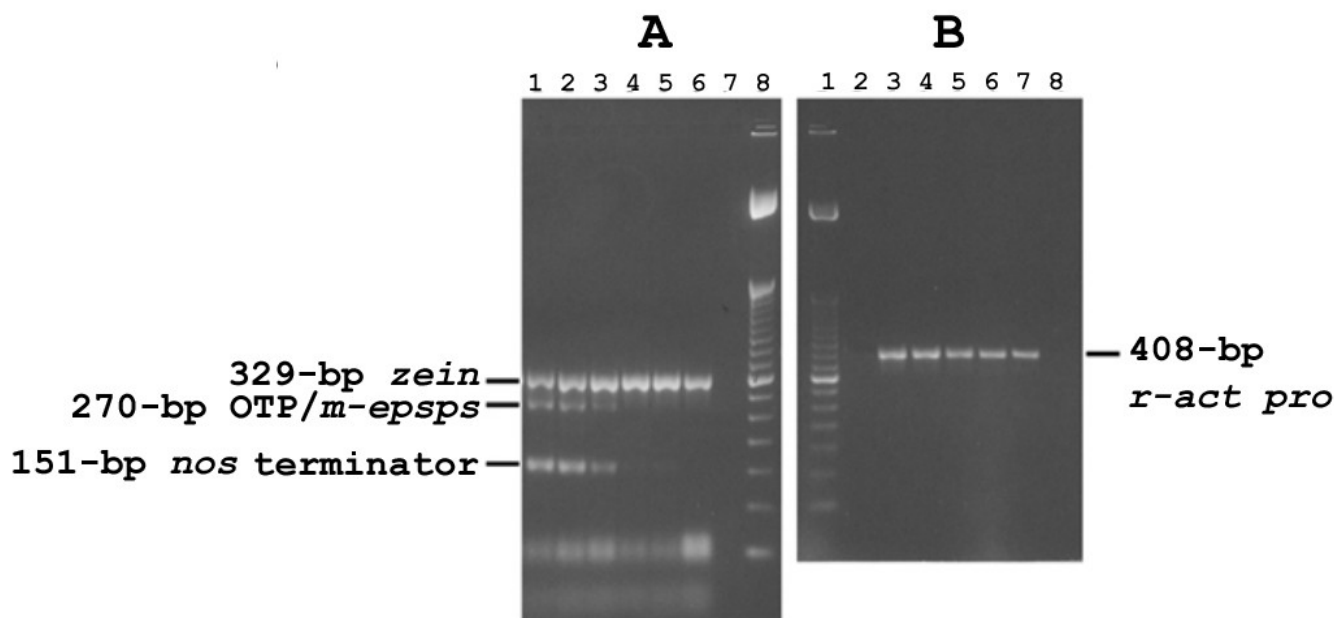


Figure 4. Detection limit of *zein* and three transgenes of Roundup Ready® GA21 *Zea mays* by PCR. A, multiplex PCR profile using primers targeting *zein*, *OTP/m-epsps*, *CamV 35S promoter*, *hsp70/cry1Ab*, and *nos terminator* and DNA templates from 100% (lane 1), 10% (lane 2), 1% (lane 3), 0.3% (lane 4), 0.1% (lane 5), and 0% (lane 6) Roundup Ready® *Zea mays* seed mixtures. Lane 7 is the no template negative control and lane 8 is the 50 bp ladder. B, PCR using a pair of primers targeting the rice actin promoter and DNA templates prepared from 100% (lane 3), 10% (lane 4), 1% (lane 5), 0.3% (lane 6), 0.1% (lane 7), and 0% (lane 8) Roundup Ready® GA21 *Zea mays* seed mixtures. Lane 2 is the negative control and lane 1 is the 50 bp ladder for both A and B.

from each other and from the Roundup Ready® NK603 based on the 194-bp *cry1Ab* and 270-bp *OTP/m-epsps* markers, respectively. Both the 220-bp fragment of *CamV 35S promoter* and the 151-bp *nos* fragments were amplified in multiplex PCR of Roundup Ready® NK603 and resulted in a profile different from the other two GM corn. The *nos* amplicon in the NK603 profile was not amplified from Yieldgard® MON810 because the *nos* 3' termination signal was not integrated into the Yieldgard® MON810 host genome and was lost through a 3' truncation of the gene cassette (GM Crop Database, 2004). The 270-bp *OTP/m-epsps* fragment that is not present in the NK603 profile was amplified from Roundup Ready® GA21.

The expected 408-bp rice actin promoter fragment was amplified in single PCR but was not amplified in the multiplex PCR for both Roundup Ready® GA21 and Roundup Ready® NK603. When annealing sites of primers targeting two different fragments are in close proximity, amplification of one or both targets may be compromised. It is possible that the binding of the polymerase on adjacent primer-template complex for the *OTP/m-epsps* may have blocked the annealing of the rice actin promoter primer to its target site and may have prevented the primer extension.

In this study, the detection limit is described as the smallest

percent of GM corn seeds (wt/wt) used for DNA extraction that produced DNA template resulting in amplification of target genes. Weight percentage of GMO in a sample cannot be measured directly by DNA or protein analysis of samples, thus the weight percentage of GMO detected by the procedure is assumed to be equivalent to the relative amounts of a typical GMO DNA sequence or protein present in the sample. *Zea mays* transgene fragments amplified by multiplex PCR had detection limits in the range of 0.1% to 1.0% GM weight percentage. Band intensities of the transgene fragments decreased with decreasing weight percentage.

CONCLUSION

The multiplex PCR procedure described in this study used primers that target the 329-bp *zein*, 270-bp *OTP/m-epsps*, 220-bp *CamV 35S promoter*, 194-bp *cry1Ab* and 151-bp *nos terminator* from GM *Zea mays L.* Yieldgard® MON810, Roundup Ready® GA21, and Roundup Ready® NK603. The procedure did not only detect the presence of the foreign genes but also resulted in profiles that could help distinguish the three GM corn samples from one another. Moreover, the results of the study showed that the multiplex PCR protocol could detect as low as 0.1 to 1.0% (wt ground GM seeds in 100g total wt ground seeds) GM corn seeds. The same approach of primer selection and multiplex PCR optimization can be applied to develop detection

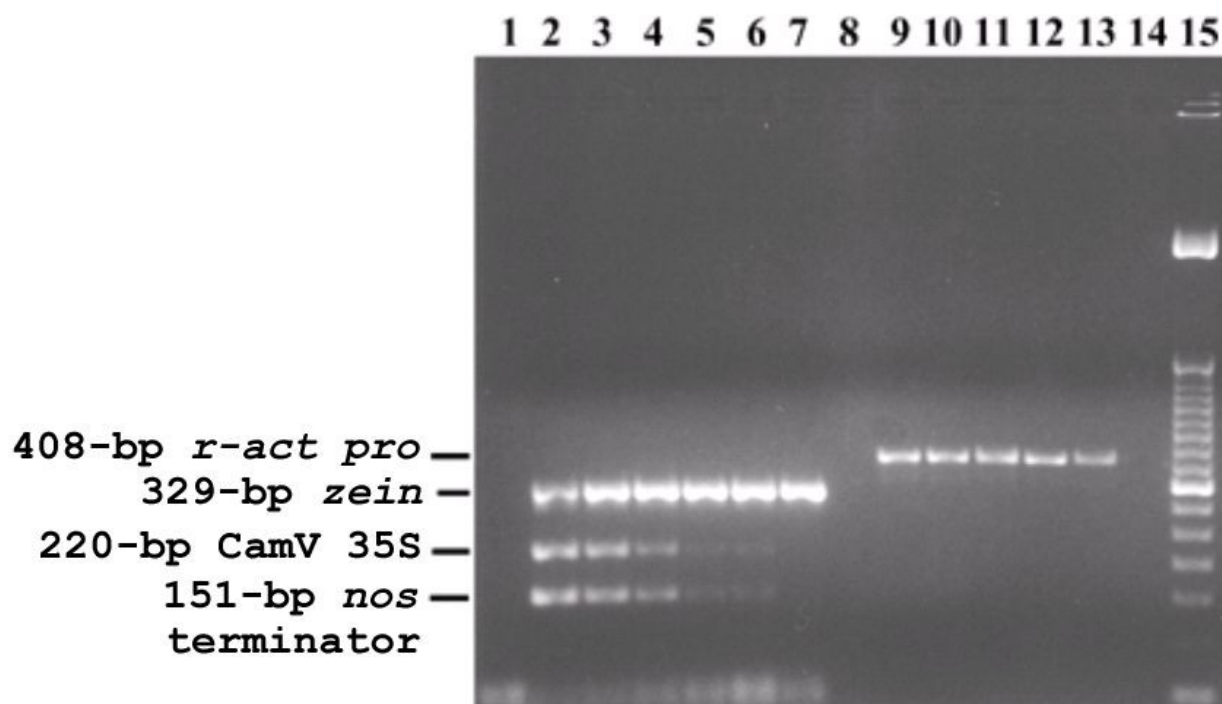


Figure 5. Detection limit of *zein* and three transgenes of Roundup Ready® NK603 *Zea mays* by PCR. Lanes 1 to 7, profile of multiplex PCR products using DNA templates prepared from 100% (lane 2), 10% (lane 3), 1% (lane 4), 0.3% (lane 5), 0.1% (lane 6), and 0% (lane 7) Roundup Ready® NK603 *Zea mays* seed mixtures. Lane 1 is the negative control with no DNA template. Lanes 8 to 14, profile of single PCR using rice actin promoter-targeted primers and DNA templates prepared from 100% (lane 9), 10% (lane 10), 1% (lane 11), 0.3% (lane 12), 0.1% (lane 13), and 0% (lane 14) of Roundup Ready® NK603 *Zea mays* seed mixtures. Lane 8 is the negative control with no DNA template. Lane 15 is the 50 bp ladder.

procedures for transgenes in other genetically modified crops.

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NO CONFLICT OF INTEREST STATEMENT

There is no conflict of interest among authors and institutions and individuals mentioned above in the conduct of this study and the preparation and submission of this manuscript.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Dr. Cynthia T. Hedreyda conceptualized the project and wrote the paper .

Ms. Jennifer L. Roxas did the literature search, conducted the experiments and helped in writing the paper.

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