JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Simultaneous Detection of Eight Genetically Modified Maize Lines Using a Combination of Event- and Construct-Specific Multiplex-PCR Technique

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To fulfill labeling and traceability requirement of genetically modified (GM) maize for trade and regulation, it is essential to develop an event-specific detection method for monitoring the presence of transgenes. In pursuit of this purpose, we systematically optimized and established a combined event- and construct-specific multiplex polymerase chain reaction (mPCR) technique for simultaneous detection of 8 GM maize lines. Altogether 9 sets of primers were designed, including six that were event-specific for Event176, Bt11, TC1507, NK603, MON863, and Mon810; two that were construct-specific for T25 and GA21, and one for an endogenous zein gene. The transgene in each GM maize line and the endogenous zein gene could be clearly detected and distinguished according to the different sizes of PCR amplicons. The limit of detection (LOD) was approximately 0.25% (v/v), although the detection can be as sensitive as 0.1% as demonstrated by the International Seed Testing Association (ISTA) proficiency test. This study further improves the current PCR-based detection method for GM maize. The method can be used in an easy, sensitive, and cost and time effective way for the identification and quality screening of a specific GM maize line.

**KEYWORDS: Genetically modified (GM) maize (*Zea mays* L.); event-specific; construct-specific; multiplex-PCR (mPCR); transgene integration sequence**

## INTRODUCTION

Modern agricultural biotechnology has opened a new avenue in the development of genetically modified (GM) plants with enhanced insect or herbicide resistance, abiotic stress tolerance, or nutritional improvement. To date, 21 genetically modified crops including maize (*Zea mays* L.) have been commercialized and approved by the European Union (EU) and another 26 countries for food, feed, or seed production (*1*). GM crop production area has increased dramatically from 1.7 million hectares in 1996 to 102 million hectares in 2006 (*1*) and is expected to continue to grow. Among GM crops, GM maize now accounts for 17% (25.2 million hectares) of the global maize production area, including maize for food, feed, ethanol (biofuel), and other industrial purposes (*1*).

Despite the potential benefits of GM crops, perceived environmental risks associated with GM crops have been a matter of public concern. Therefore, many countries have implemented a regulatory framework for risk assessment of the safety aspects relevant to genetically modified organisms (GMOs), such as their possible impact on ecological food chains and species diversity. For consumers to make informed choices regarding the level of GMO content in supermarket foods, most countries have begun to set up labeling thresholds for GM-derived products. Specific package labeling is required to indicate whether the tolerance level of GMO content within an agricultural product is above regulated standards. However, the labeling thresholds are not the same for all countries. In the EU and Russia, the maximum allowed is 0.9%, whereas in Australia, New Zealand, Brazil, and Saudi Arabia, it is 1%, in South Korea 3%, and in Canada, Japan, Philippines, Thailand, Indonesia, and Taiwan, 5% (*2*).

In order to enforce a sound labeling, monitoring, and traceability system for GM crops, it is necessary to develop sensitive and accurate GM detection methods (*3*). Currently, the two methods employed are either protein- or DNA-based. In protein-based testing methods, GM-derived products are detected according to the proteins produced by the introduced trait genes, through interaction with its specific antibody, using enzyme linked immunosorbent assays (ELISA). However, this method is limited to fresh and unprocessed foods. In DNA-based testing methods, a specific transgene target sequence is used to detect GMOs by PCR. In contrast to the protein detection method, DNA-based analysis can detect event-specific GM varieties. For most samples, including seeds, raw materials, processed foods or feeds, and partially degraded materials, the sensitivity and reproducibility of PCR has led to the DNA-based

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10.1021/jf800501z CCC: $40.75 
Published on Web 09/04/2008

PCR methods becoming routinely and widely used in the detection of GMOs (*4*).

According to their level of specificity, PCR-based analysis can be used for screening or gene-, construct-, and event-specific transgene detection (*5*). The screening test usually is associated with common elements of the construct inserted within the GMO, such as the cauliflower mosaic virus *35S*-promoter or the *nos*-terminator of A*grobacterium tumefaciens*. This method is suitable for the initial screening of GMOs but can easily give false results and always needs to be confirmed by either construct- or event-specific PCR. While gene- and construct-specific PCR show higher specificity than DNA-based screening, the application of construct-specific tests to traceability is problematic. Most of the commercialized GM varieties usually share similar genetic elements or constructs that are integrated in different chromosome regions or with variable copy numbers. Thus, construct-specific PCR methods may not be specific and accurate enough for regulatory requirements. Currently, the transgenic event in a GM plant is known to be mediated via nonhomologous recombination with the integration site random. Transgene expression has also been shown to be affected by the so-called positional effect. Therefore, selection for specific transgenic lines or events to ensure high level of transgene expression and confirmation of the desired agronomical traits would be necessary before commercialization of GM crops. An improved method to monitor or trace the presence of a specific transgenic event in a GM cultivar should involve event-specific PCR by using primers located across border sequences between the junctions of the gene construct and the 5′or 3′ flanking genomic DNA sequences. The uniqueness of these sequences is quite important in providing event-specificity and to discriminate different transformants transduced with the same genetic construct.

Several construct-specific multiplex-PCR methods have been developed and adapted as general techniques to detect GM maize (*4, 6*−*8*). A few event-specific PCR methods have been established to monitor one or two specific GM maize lines, including Bt10 (Novartis) (*9*), Bt11(Novartis) (*10, 11*), Bt176 (Novartis, Maximizer maize) (*11*), GA21 (Monsanto) (*11, 12*), MON810 (Monsanto, Yield Gard corn) (*13*), MON863 (Monsanto) (*14*), and NK603 (DOW AgroSciences LLC) (*15*); StarLink (Aventis) (*16*), T25 (AgrEvo, Liberty Link maize) (*4, 6*), and TC1507 (Mycogen (c/o Dow Agroscience) Pioneer (c/o DuPont)) (*17*). Among these commercialized GM maize lines, Mon863 and Mon810 contain the insect-resistant Bt endotoxin gene. The transgenic maize T25, NK603, and GA21 have the herbicide tolerance genes, *PAT* and *EPSPS*, respectively. Event 176, Bt11, and TC1507 contain stacked genes, conferring insect resistance and herbicide tolerance. As far as we know, none of the known event-specific multiplex-PCR methods have been reported to simultaneously detect these GM maize lines in a single reaction tube. Recently, a multiplex-PCR coupled with oligonucleotide microarray method was developed for multiple event-specific detection of GM soybean and maize (*18*). The most recent method for the detection and quantification of transgenes in event-specific GM samples is either by microarray or real-time PCR with a constructed reference plasmid (*19, 20*). Real-time PCR and chip-based technology provide high throughput and sensitivity in GMO detection, but require expensive equipment, complicated procedures, and sophisticated technical support. Therefore, it would be worthwhile to develop an advanced event-specific multiplex-PCR for routine GM testing. This study was designed to modify and further improve current construct- or event-specific multiplex-PCR methods based on the unique 3′-transgeneintegration sequences for the identification and quality screening of specific GM maize lines.

## MATERIALS AND METHODS

**Materials.** Mixed GM maize (*Zea mays* L.) seed samples, including Event176, Bt11, TC1507, NK603, T25, MON863, MON810, and GA21, and soybean (*Glycine max* [L.] Merr. cv. GTS 40-3-2 (glyphosate herbicide resistance) were available from commercial imports into Taiwan. Rice (*Oryza sativa* L.) seeds, variety Tainung-67, were obtained from TARI (Taiwan Agriculture Research Institute). DNA extracted from leaves of a local Taiwan maize cultivar (cv. Tainan No. 5) was used as the standard for non-GM control in this experiment.

**Genomic DNA Isolation, Purity, and Concentration Determination.** GM maize genomic DNA was isolated from 2 to 3-leaved stage seedlings. DNA extraction was carried out using the method of Green et al. (*21*) with little modification. First, a sample of 100−200 mg of maize leaf was ground with liquid nitrogen, then 700 μL of urea extraction buffer (7 M urea, 0.3 M NaCl, 1 M Tris HCl, 0.5 M EDTA, and 20% sarcosine) was added. After mixing with 700 μL phenol/chloroform/isoamyl alcohol (PCI, 25:24:1) and centrifuging for 10 min at 8,000 rpm, DNA was extracted, then precipitated with isopropyl alcohol, and washed with 70% of alcohol. Finally, DNA was dissolved in 50 μL of $dH_2O$ and determined by Biophotometer (Germany) for its quality and quantity.

**Preparation of Test Samples.** For the general specificity test, 100 ng/mL (volume/volume, v/v) GM maize DNA was prepared as a positive control sample for all singlet-PCR (100% GM maize) and multiplex-PCR (12.5% GM maize). Also, genomic DNA from non-GM maize and other crop species, such as rice and soybean, were used as negative quality controls. A sensitivity assay was carried out to determine the LOD with DNA templates prepared from seven levels of simulated GM mixture samples containing 0, 0.1, 0.25, 0.3, 0.5, 1.0, and 5% of each of 8 events of GM maize DNA (100 ng/μL) in non-GM maize.

**DNA Sequence Analysis and Oligonucleotide Primer Design.** The structural information of recombinant DNA (r-DNA) in maize including integration sites, gene sequences, and inserted junction regions were from publicly available resources (*4, 6, 9*−*17, 22*−*24*). The construct map for individual GM maize was shown as **Figure 1**. Part of the oligonucleotide primers were newly designed for both GM maize and endogenous control gene using Primer Premier 5 primer design software (PREMIER Biosoft International, Corina Way, Palo Alto, CA, USA). Other primers, namely, bar3/Bt176 plant for Event176 and forward primer T25 1-5′ for Bt11, TC1507, and T25 were the same as previously published (*6, 11*). The G + C content (%) and melting temperature ($T_m$, °C) of designed primers were calculated using BioPHP. Except two construct-specific primers for T25 and GA21 because of the limitation of sequences information, the event-specific primers for six GM maize (Event176, Bt11, TC1507, NK603, MON863, and MON810) in 3′-junction region were used in mPCR. The reverse primer of T25 was located in the pUC18 vector (v) region and GA21 in the 3′-junction region across the m-epsps gene and truncated rice Actin promoter. All primers were synthesized by Purigo Biotech, Inc., Taipei, Taiwan.

**Optimization and Validation of PCR Conditions.** *Singlet-PCR.* The PCR reaction was performed in a final volume of 25 μL with 2.5 μL 10× reaction buffer (with $Mg^{2+}$) containing 100 mM Tris-HCl (pH 8.0 at 25 °C), 500 mM KCl, 15 mM $MgCl_2$, 0.1% (weight/volume, w/v) gelatin, and 1% Triton X-100; 2 μL of 2.5 mM dNTP; 1 μL of oligonucleotide primer (5 μM), 1 μL of template DNA (100 ng/μL), 0.5 μL (5U/μL) of Taq DNA polymerase (GeneTeks BioScience Inc., Taiwan), and 17 μL of $dH_2O$. The reactions were amplified in a GeneAmp PCR System 9700 (Applied Biosystem, USA) with the following PCR program: preincubation at 95 °C for 15 min; then 35 cycles consisting of denaturation at 94 °C for 0.5 min; annealing at 60 °C for 1.5 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis with 2.5% (w/v) agarose gel.

*Multiplex-PCR.* The qualitative assay of the multiplex-PCR reaction was performed according to the manual for the QIAGEN multiplex-PCR Kit. The final volume for the reaction was 25 μL, which contained

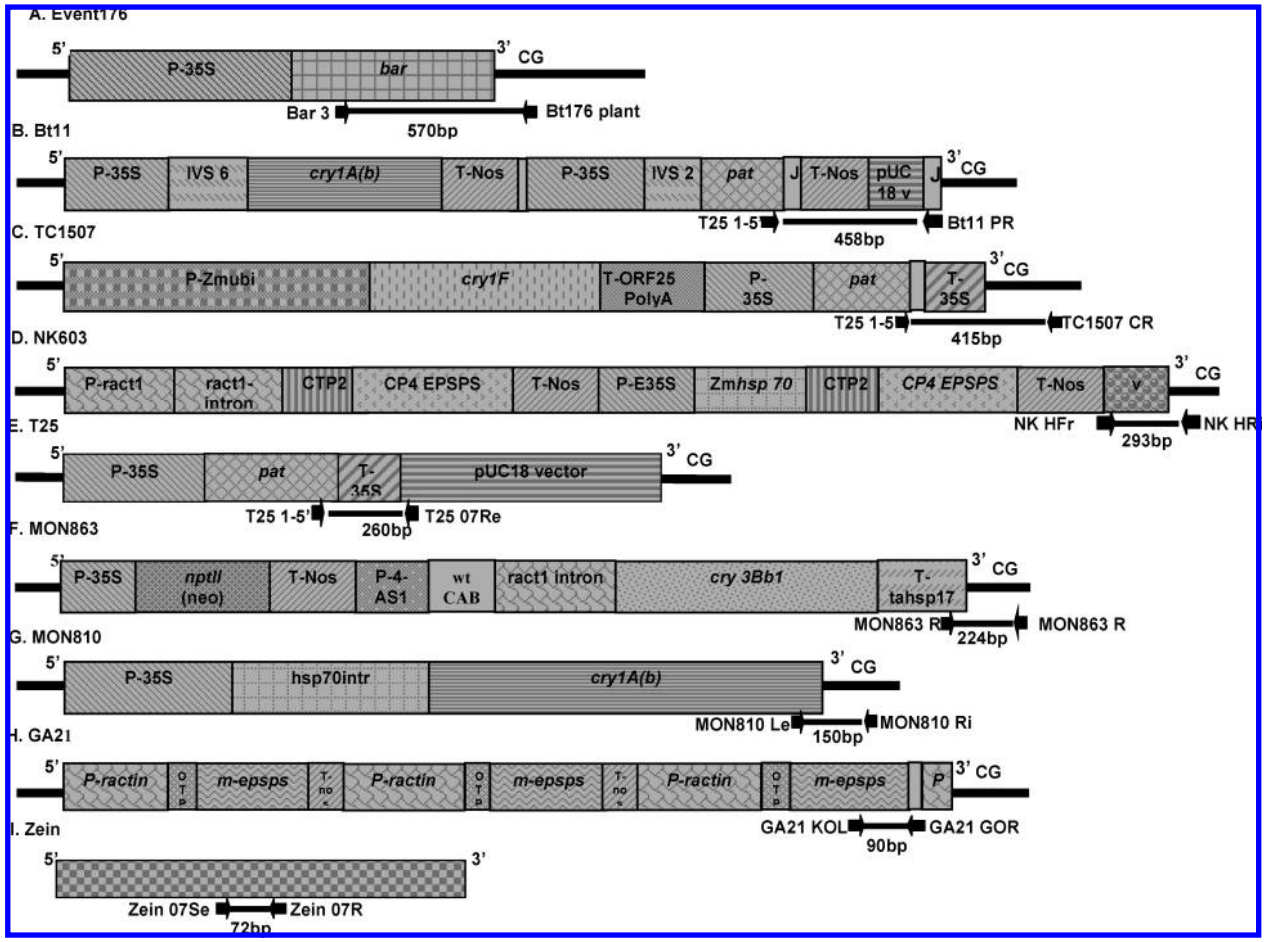


**Figure 1.** Linear construct map of recombinant DNA, showing the position of primers designed for multiplex-PCR, amplicon region of different eight GM maize lines, and the endogenous zein gene.

12.5 $\mu$L of 2× QIAGEN Multiplex PCR Master Mix; 3.2 $\mu$L of 16 premixed primer of different concentrations (0.2 $\mu$M for the primers of Event176, Bt11, TC1507, T25, MON863, GA21, and Zein; 0.1 $\mu$M for MON810, and 0.3 $\mu$M for the NK603); 2.5 $\mu$L of Q-solution; 100 ng of genomic DNA; and 5.8 $\mu$L of RNase-free water. An extra 10% PCR solution, as mentioned in QIAGEN, was not prepared to reduce the cost of PCR. Instead, 0.7 $\mu$L of $H_2O$ was added per reaction to compensate for this. The reactions were amplified in a GeneAmp PCR System 9700 (Applied Biosystem, USA) using the following PCR program: preincubation at 95 °C for 15 min; 35 cycles consisting of denaturation at 94 °C for 0.5 min; annealing at 60 °C for 1.5 min; and extension at 72 °C for 1.0 min, followed by a final extension at 72 °C for 10.0 min. The PCR products were analyzed by electrophoresis using a 3% (w/v) agarose gel. All of the PCR products were sequenced and confirmed (Genomics BioSci & Tech Corp, Taipei, Taiwan).

**International Proficiency Test of GM Maize.** Twelve GM maize samples received from ISTA were qualitatively analyzed in this experiment. After multiplex-PCR analysis, results were sent to the ISTA for the purpose of performance evaluation. The accuracy and sensitivity of the qualitative assay for GM maize were assessed by comparing our data with ISTA's results.

## RESULTS AND DISCUSSION

**Internal Sample Control and Event-Specific Primer Design.** Eight events of GM maize (*1, 22*−*24*) and the zein gene (M23537, a 10 kDa storage protein from maize) as an endogenous reference control were used for GM maize analysis (*24*−*26*). *Zein* is specific to maize and teosinte (*8, 9, 26*) and can still be detected after heating at 100 °C for 120 min or 121 °C for 30 min (*27*). To design event-specific primers for multiplex-PCR, the cross-border DNA sequences between 3′-transgene and host plant DNA were analyzed, and those specific flanking regions were selected. To ensure the detection specificity, the forward primer from each pair was specifically annealed to an internal region of each foreign gene construct, and the reverse primer to the flanking genomic sequence region of maize. Thus, nonspecific PCR products, derived from foreign DNA and maize genome, cannot be amplified. All oligonucleotide primers were checked with a public database (i.e., NCBI BLAST search) and confirmed that no match with an unintended DNA sequence was found. The primer pairs used in this study and the corresponding GeneBank accession number of target genes or PCR amplicons are listed in **Table 1**.

**Primer Specificity Validation for Singlet-PCR.** To test whether primer specificity did indeed correspond to individual GM lines, all of the primer sets were used for singlet-PCR test in series. The efficiencies of all of the primer sequences were individually examined using DNA templates from different GM and non-GM maize lines, as well as other crops (rice and soybean). The results showed that each individual primer set for the corresponding GM maize was able to amplify a specific target band. No PCR products were found from either non-GM maize or other crop species (data not shown). Sequencing results revealed that the DNA fragment of 570 bp was identical to the partial sequence of the truncated BAR expression cassette and plant junction region in event176 from 1 to 570 bp (*24*) and that the 458 bp DNA fragment was identical to the partial coding sequence of the phosphinothricin *N*-acetyl transferase (Pat) gene in Bt11 from 53−410 bp (*11*). A single 415 bp fragment was identical to the 35 S promoter and maize genomic DNA

**Table 1.** List of Primer Sets and Amplified Junction Sequences in Multiplex-PCR Analysis of Eight GM Maize Lines[a]

| GM Maize | primer name | primer sequence (5′−3′) | amplicon size (bp) | orientation (specificity) | reference |
|---|---|---|---|---|---|
| Event176 | bar3 | AAGCACGGTCAACTTCCGTAC | 570 | S (*bar*) | AJ878607 |
| | Bt176 plant | TCGACTTTATAGGAAGGGAGAGG | | A (MG) | |
| Bt11 | **T25 1−5′** | GCCAGTTAGGCCAGTTACCCA | 458 | S (*pat*) | AY123624 |
| | Bt11 PR | CAAAAATCCAAGAATCCCTCCAT | | A (MG+J+v) | |
| TC1507 | **T25 1−5′** | GCCAGTTAGGCCAGTTACCCA | 415 | S (*pat*) | *17* |
| | TC1507 CR | CAAGATCAAGCGGAGTGAGG | | A (MG) | |
| NK603 | NK HFr | CGGTACCAAGCTTTTATAATAGTAG | 293 | S (T-nos+v) | *15* |
| | NK HRi | CTAGTCTGTTATGGTTCGAG | | A (MG) | |
| T25 | **T25 1−5′** | GCCAGTTAGGCCAGTTACCCA | 260 | S (*pat*) | DQ156557 |
| | T25 07Re | GAATTCGAGCTCGGTACCCCTGG | | A (vector) | |
| MON863 | Mon863 F | GTAATCGGCTAATCGCCAAC | 224 | S (T-tahsp17) | X13431; AY506529 |
| | Mon863 R | GCCAGTTCATTGCGAGTACA | | A (MG) | |
| MON810 | Mon810 Le | CACCACAGCCACCACTTCT | 150 | S (*cry1Ab*) | AY326434. |
| | Mon810 Ri | AGGAAAAGCTATTGTAAAGCCAAA | | A (MG) | |
| GA21 | GA21 KOL | GCTGCCAAAAGAGCTGTAG | 90 | S (*m-epsps*) | AJ878608 |
| | GA21 GOR | GATCCACTAGTTCTAGAGCTGCAC | | A (*rac+J+m-epsps*) | |
| Zein | Zein 04Se | GCTTGCCAGCTTGATGGCGT | 72 | S (*zein*) | M23537 |
| | Zein 07R | GGCATCGTCTGAAGCGGTAAGG | | A (*zein*) | |

[a] S, sense orientation; A, antisense orientation; bar, phosphinotricin-*N*-acetyltransferase *(pat)* gene from *Streptomyces viridochromogenes*; pat, a homologue of phosphinotricin-*N*-acetyltransferase gene from *Streptomyces hygroscopicus*; J, junction; v, vector; T-nos, terminator-nontranslated polyadenylation signal of nopaline synthase from *Agrobacterium tumefaciens*; T-tahsp17, 3′-terminal untranslated region of wheat heat shock protein *17.3*; cry1Ab, synthetic delta endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki*; mepsps, point mutated epsps gene from maize; rac, rice *Actin* promoter; MG, maize genome.

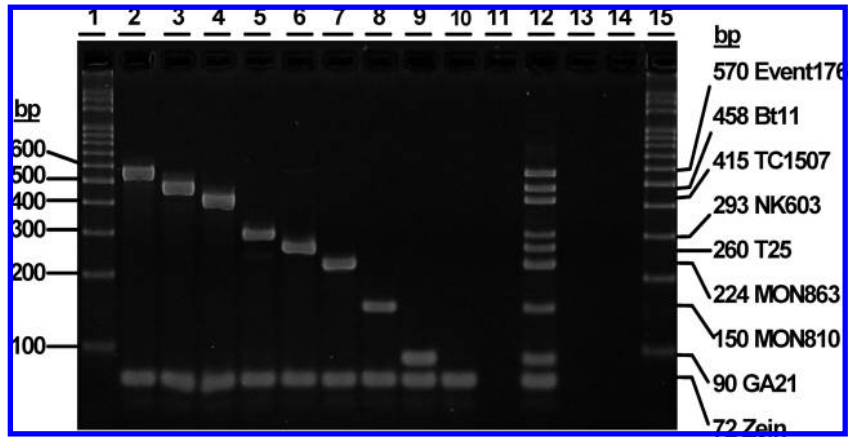


**Figure 2.** Specificity test of multiplex-PCR for eight GM maize lines and the endogenous zein gene. Lanes 1 and 15, 100 bp ladder size marker; lanes 2 to 14, amplicon of PCR product from different GM maize, Event176, Bt11, TC1507, NK603, T25, MON863, MON810, GA21, non-GM maize, negative control, 8 GM mixed, rice, and soybean samples, respectively.

sequence from 606−1020 bp in TC1507 (*17*). The DNA fragment of 293 bp was identical to a sequence of vector backbone and genomic maize DNA in NK603 from 152−444 bp (*15*). Similarly, a DNA fragment of 260 bp was shown to be identical to the sequence in T25 from 525−784 bp (*4*), 224 bp in MON863 from 70−293 bp (*14*), and 150 bp in MON810 from 566−715 bp (*13*). Finally, a 90 bp DNA fragment was identified as the partial sequence for the truncated mEPSPS gene and truncated Actin promotor junction region in GA21 from 17−106 bp (*11, 24*), and a 72 bp DNA fragment from 1283−1354 bp in the *Zea mays* 10-kDa zein gene (*24*). Exact amplicon regions are indicated as bold lines under each event-specific construct in **Figure 1**. In singlet-PCR reactions, very faint unspecific products derived from nonspecific hybridization may sometimes be observed in some GM maize lines. However, these nonspecific products can be made invisible by adjusting different primer concentrations when the multiplex-PCR reaction is performed (**Figure 2**). These results suggest that these primers were effective for the specific detection of their respective targeted events in GM maize and should also work well in a multiplex-PCR system.

**Specificity Test of Multiplex-PCR.** In attempting to set up the combination of event- and construct-specific multiplex-PCR system for detection of the 8 GM maize lines, specificities of all of the designed primers were accessed. Using a QIAGEN multiplex-PCR kit, we first focused on the thermal cycle program and primer concentration for the optimization of PCR parameters. Use of primer concentrations of 0.2 $\mu$M for Event176, Bt11, TC1507, T25, MON863, and GA21; and 0.3 $\mu$M for NK603, and 0.1 $\mu$M for MON810 yielded intense bands for all of the GM samples. No band was observed with water, rice, and soybean samples (**Figure 2**). Clearly, these primer sets were compatible with each other and worked as efficiently in the multiplex-PCR system as in singlet-PCR. This method is expected to be more event-specific and suitable for the traceability of the specific GM maize line, becaue of the position of the primer that mainly corresponded to the 3′-transgene integrated site between insert and plant genomic DNA (except T25 and GA21). This is because nonevent specific methods could cause false positives when the same or similar gene or construct is integrated into other GMOs (*10*).

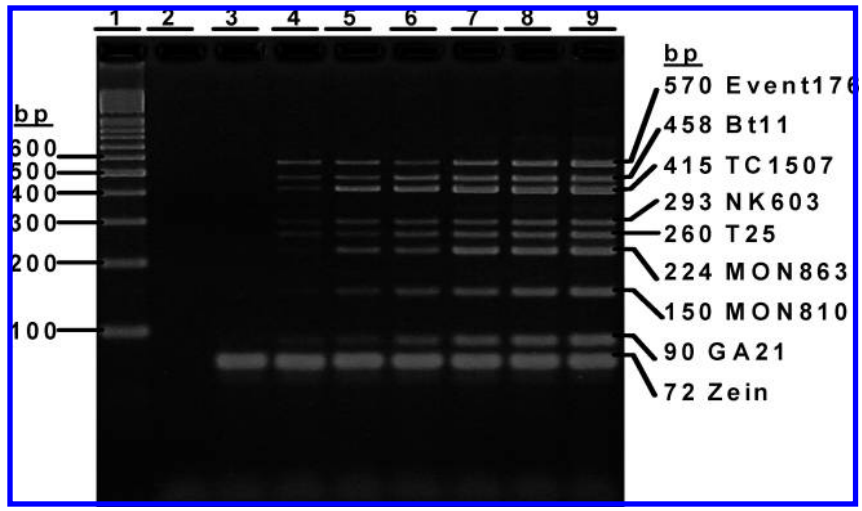


**Figure 3.** Sensitivity test of multiplex-PCR for eight GM maize lines. Lanes 1 to 3: 100 bp size marker, negative control, and non-GM maize. Lanes 4 to 9: PCR amplicon from the simulated 8 GM DNA mixtures containing 0.1, 0.25, 0.3, 0.5, 1.0, and 5% (w/w) of each of the 8 GM maize, respectively.

**Limit of Detection (LOD).** A sensitivity assay was carried out to determine the LOD of event- and construct-specific multiplex-PCR. Seven levels of simulated GM mixture samples ranging from 0 to 5% (0, 0.1, 0.25, 0.3, 0.5 1.0, and 5%) for each of eight events of GM maize DNA (100 ng/$\mu$L) in non-GM maize were prepared as templates. All eight target specific amplicons could be simultaneously detected from GM maize samples as low as 0.25% GM for each of the events, whereas bands with 0.1% were weakly visible (**Figure 3**). This suggests that the GM maize detection limit of our method is sensitive to 0.25%, similar to construct-specific multiplex-PCR (*4*) but more sensitive than multiplex-PCR coupled with oligonucleotide microarray (*28*). This method is sufficiently sensitive to reliably evaluate samples at different threshold labels of GM maize around the world, from 0.9% in EU and Russia to 5% in Canada, Japan, Philippines, Thailand, Indonesia, and Taiwan (*2*). We have carried out single laboratory validation assays at least 5 times for both the specificity and sensitivity assessments based on our technique. Throughout the tests, the results were highly consistent, indicating the stability and reproducibility of this method (data not shown). Hence, this technique can potentially be used as a reference method for GMO detection.

**ISTA Proficiency Test.** Since the preceding results were obtained with homogenously mixed DNA samples, there might be a limitation with a mixture of heterogeneous GM maize leaf samples or in processed food (*4, 29*). Therefore, our next step was to extend this method in detection of GM maize to real field samples, simulated heterogeneous maize samples, and processed GM maize food/feed from market. In order to confirm the applicability of this event-specific method, we participated in the international GM maize proficiency test (participant number 14) using maize samples provided by ISTA. From result PT09, our method was demonstrated to be accurate and able to qualitatively analyze levels of GM maize mixed in these samples, as low as 0.1% either in percentage of mass (processed food) or by kernel number (**Table 2**). To be realistic and in terms of the limit of detection for analysis using our system, we have set our limit of detection to 0.25%, which will detect the most demanding threshold level set by the European Union.

The detection and traceability of GM crops will become more complex and challenging in the future. Regulation is facing a new problem, where GM varieties contain gene stacking or gene pyramiding of input-traits (*30*). Two or more transgenes can be introduced into a plant sequentially with multiple-gene constructs, hybridization between two homozygous transgenic lines, or by retransformation of a new gene into a GM plant. Currently, only two or three novel gene-stacked commercialized GM maize varieties are available on the market. However, recently Monsanto and Dow AgroSciences, LLC have reached an agreement for launching a new project to release the industry's first-ever eight-gene stacked combination in corn for better insect and herbicide resistance. The use of our event-specific PCR detection method should prove useful to monitor samples of multiple gene-stacked GM maize and other crops in the future.

**Table 2.** Results of International Seed Testing Association (ISTA) Proficiency Test with GM Maize by Multiplex-PCR Analysis

| | % mass GMO[a] (ISTA) | | | | mPCR qualitative result | |
|---|---|---|---|---|---|---|
| sample no. | NK603 | MON 863 | total | % number GMO[b] (ISTA) | NK 603 | MON 863 |
| 1 | 0.08 | 0.00 | 0.08 | 0.1 | + | − |
| 2 | 0.21 | 0.70 | 0.91 | 0.8 | + | + |
| 3 | 0.00 | 0.11 | 0.11 | 0.1 | − | + |
| 4 | 0.10 | 0.00 | 0.10 | 0.1 | + | − |
| 5 | 0.18 | 0.68 | 0.86 | 0.8 | + | + |
| 6 | 0.00 | 0.11 | 0.11 | 0.1 | − | + |
| 7 | 0.00 | 0.00 | 0.00 | 0.0 | − | − |
| 8 | 0.53 | 0.22 | 0.75 | 0.8 | + | + |
| 9 | 0.53 | 0.24 | 0.77 | 0.8 | + | + |
| 10 | 0.40 | 0.41 | 0.81 | 0.8 | + | + |
| 11 | 0.36 | 0.41 | 0.77 | 0.8 | + | + |
| 12 | 0.00 | 0.11 | 0.11 | 0.1 | − | + |

[a] % mass of GMO based on weight. [b] % number GMO based on number of maize kernel; + = GMO present, and − = GMO absent.

In conclusion, we have developed a combination of construct- and event-specific multiplex-PCR method for the detection of eight different GM maize lines. This method is robust, cost-effective, highly specific, and sensitive, as well as rapid. This study has shown that the method has advantages in the detection of specific GM maize line in a routine laboratory test and for establishing a quantification threshold. The method can be considered as an alternative for construct-specific multiplex-PCR or single event-specific PCR in GM maize detection. Meanwhile, this method has potential for the characterization of event-specific gene-stacked GM crops in the future. Finally, further studies of heterogeneous GM maize samples from real field and processed food products are underway.

## ABBREVIATIONS USED

*epsps* gene, encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium tumefaciens* strain CP4 (CP4 EPSPS), for herbicide tolerance; *hsp70*-int. 1, the no. 1 intron sequence from maize *hsp70* gene (heat-shock protein); intron no. 9, from maize phosphoenolpyruvate carboxylase (PEPC) gene; IVS2-int., the no. 2 intron sequence from alcohol dehydrogenase (*adh 1*-I); IVS6-int., the no. 6 intron sequence from maize alcohol dehydrogenase 1 gene (*adh 1*-IS); *NptII*, nopaline synthase gene from *A. tumefaciens*; OTP, optimized transit peptide sequence; $P\bar{4}$-AS1, a promoter that contains four copies of the AS-1 element and a part from the cauliflower mosaic virus (CaMV); P-CDPK, pollen-specific calcium-dependent protein kinase (CDPK) promoter from maize; P-PEPC, green tissue-specific phosphoenolpyruvate carboxylase (PEPC) promoter from maize; P-riceact1 with OTP, rice Actin promoter containing the no. 1 intron optimized transit peptide sequence (OTP); DNA sequence for chloroplast transit peptide synthesized from the information on the peptide sequence of the N-terminal upstream region of ribulose-1, 5- bisphosphate carboxylase derived from maize and sunflower; P-Zmubi, promoter *Zea mays* ubiquitin; T-ORF25 PolyA, 3′ polyadenylation signal from ORF25 (*Agrobacteriu tumefaciens*); UNG, *Amperase uracil N-glycosylase* gene; wt. CAB, 5′-terminal untranslated region of wheat chlorophyll a/b binding the expression of target genes.

## ACKNOWLEDGMENT

We thank Alison A. Powell and Stan Matthews, University of Aberdeen, Scotland, U.K., for their assistance in the use of English. We acknowledge Chwan-Yang Hong, Department of Agricultural Chemistry, National Taiwan University for comments on this work.

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**Received for review February 18, 2008. Revised manuscript received June 5, 2008. Accepted August 12, 2008. This work was supported by funding from Council of Agriculture, Taiwan.**

JF800501Z