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Analytical Methods

# A multiplex degenerate PCR analytical approach targeting to eight genes for screening GMOs

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ABSTRACT

Currently, the detection methods with lower cost and higher throughput are the major trend in screening genetically modified (GM) food or feed before specific identification. In this study, we developed a quadruplex degenerate PCR screening approach for more than 90 approved GMO events. This assay is consisted of four PCR systems targeting on nine DNA sequences from eight trait genes widely introduced into GMOs, such as *CP4-EPSPS* derived from *Acetobacterium tumefaciens* sp. strain CP4, phosphinothricin acetyltransferase gene derived from *Streptomyces hygroscopicus* (*bar*) and *Streptomyces viridochromogenes* (*pat*), and *Cry1Ab*, *Cry1Ac*, *Cry1A(b/c)*, *mCry3A*, and *Cry3Bb1* derived from *Bacillus thuringiensis*. The quadruplex degenerate PCR assay offers high specificity and sensitivity with the absolute limit of detection (LOD) of approximate 80 target copies. Furthermore, the applicability of the quadruplex PCR assay was confirmed by screening either several artificially prepared samples or samples of Grain Inspection, Packers and Stockyards Administration (GIPSA) proficiency program.



## 1. Introduction

In the past two decades, the recombinant DNA technology has been widely used in modern agriculture. The planting area of genetically modified (GM) crops has reached 134 million hectares at the end of 2009, and a total of 155 GM events have been authorized for food and feed production in 57 countries (James, 2010). Specially, several unauthorized or unknown GMOs and their derived products are possibly present in market owing to the asynchronous authorization of GM crops worldwide or unintentional contamination of seed lots with unauthorized GMOs (Prins et al., 2008; Ruttink et al., 2010), such as Bt10 and LLRice601 (EC decision on Bt10: 2005/317/EC, 2005; EC decision on LL601: 2006/601/EC, 2006). To protect the authority of consumers, food products, and ingredients containing GMOs which content exceeds certain threshold value are required to be labelled in many countries. As a consequence, detection and identification of GMOs in food becomes a challenging issue for complete compliance with the traceability and labelling regulations (Holst-Jensen, 2009). According to the conventional GMO analytical procedure (Holst-Jensen, Ronning, Lovseth, & Berdal, 2003; Ruttink et al., 2010), a large-scale and cost-efficient screening assay based on the amplification of the universal elements and selectable marker genes is frequently adapted for GMO detection.

Polymerase chain reaction (PCR) is the dominating analytical approach for GMO detection (Holst-Jensen, 2009), several singleplex or multiplex PCR assays have been reported and used for the detection and identification of the authorized, unauthorized, or unknown GM events (Bahrdt, Krech, Wurz, & Wulff, 2010; Dörries, Remus, Grönewald, Grönewald, & Berghof-Jäger, 2010; Grohmann, Brünen-Nieweler, Nemeth, & Waiblinger, 2009; Guo et al., 2009; Liu et al., 2009; Lu, Lin, & Pan, 2010; Mano et al., 2009a; Randhawa, Chhabra, & Singh, 2009). Although increasing development of the number of GM plant events, the herbicide and insect resistances are the major traits in the GM crops (not including the stocked GM crops), occupying 62% and 15% of the global biotech area, respectively (James, 2010). Most herbicide-tolerant GM crops contain the *CP4-EPSPS* gene derived from *Acetobacterium tumefaciens* sp. strain CP4, and phosphinothricin acetyltransferase gene from *Streptomyces hygroscopicus* (*bar*) or from *Streptomyces viridochromogenes* (*pat*), while most insect-resistant GM crops contain the *Bacillus thuringiensis* (*B.t.*) family genes derived from *B. thuringiensis*, such as *Cry1Ab, Cry1Ac, Cry1A*(*b*/*c*), *mCry3A*, and *Cry3Bb1* (AGBIOS

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GM Crop Database; GMDD). Thus, the development of screening assays targeting to these exogenous trait genes will be of great interest. Based on the available public databases (AGBIOS GM Crop Database; Dong et al., 2008; GMDD; NCBI Entrez Nucleotide database), we found that the nucleotide sequences encoding the same protein have variations in different GM events, such as the sequences of *CP4-EPSPS* gene in GM soybean event GTS 40-3-2 (*CP4-EPSPS1*, GenBank No. I43998) and MON89788 (*CP4-EPSPS2*, GenBank No. GV597339); the sequences of *Cry1Ab* gene in Bt176 (*Cry1Ab1*, GenBank No. I41419), Bt11 (*Cry1Ab2*, GenBank No. GV597352) and MON810 (*Cry1Ab3*, GenBank No. AR127583). Several PCR approaches reported for amplifying of these herbicide-tolerant or insect-resistant genes are mainly specific to the single target sequence (Dörries et al., 2010; Grohmann et al., 2009; Lu et al., 2010; Mano et al., 2009a; Randhawa et al., 2009), which are time-/cost-consuming or give rise to false-negative identification.

Multiplex PCR method is an effective strategy for this complexity (Randhawa et al., 2009; Yang et al., 2005), however, the level of multiple PCR amplification is usually limited to a maximum of 10 targets simultaneously amplified in a single reaction (Dahl, Gullberg, Stenberg, Landegren, & Nilsson, 2005). Multiplex PCR with the lower level of amplification and the larger-scale coverage are being developed and will be one of the main aims for GMO detection and identification. The degenerate multiplex PCR employing degenerate primers can be used for simultaneous amplification of the highly homologous gene sequences (Digiaro, Elbeaino, & Martelli, 2007; Najafabadi, Torabi, & Chamankhah, 2008), which offers many advantages over those conventional PCR approaches, such as the need for a minimal number of analysis, covering a large-scale of targets, and more cost-efficient. In this study, we proposed a routine screening strategy employing multiplex degenerate PCR to reduce the level of multiple PCR amplification in a single reaction. Four sets of degenerate primers targeting to the *CP4-EPSPS*, *bar*, *pat*, *mCry3A*, *Cry3Bb1*, *Cry1Ab*, *Cry1Ac*, and *Cry1A*(*b*/*c*) genes, were designed and a quadruplex degenerate PCR method was developed for testing these genes. The results showed that this established quadruplex PCR assay is a time-and cost-efficient as well as sensitive approach for GMO detection and monitoring the presence of GMO in food.

## 2. Materials and methods

### 2.1. Plant materials

The GM events used in this study included eleven GM maize events, four GM soybean events, seven GM canola events, five GM cotton events, two GM rice events, one GM papaya event, one GM tomato event and one GM sugar beet event as detailed in Table 1. Such a matrix table combined with the screening approach allows the effective identification of the potential presence of GMOs (Querci, Van den Bulcke, Zel, Van den Eede, & Broll, 2010). The seeds of NK603, MON810, MON863, MON88017, RT73, GTS40-3-2 (RRS), MON89788, MON1445, MON88913, MON15985, and MON531 were kindly supplied by Monsanto Co. The seeds of Bt176, Bt11, MIR604, and GA21 were kindly supplied by Syngenta Seeds, Inc. The seeds of Topas19/2, MS8 × RF3, T45, A2704-12, A5547-127, LLCotton25, and T25 were kindly supplied by Bayer CropScience Co. The seeds of DAS59122 and TC1507 were kindly supplied by DOW AgroSciences LLC. The seeds of MS1 × RF1, MS1 × RF2, and OXY-235 were kindly supplied by DOW AgroSciences LLC. The seeds of GM rice (Huahui-1 and Bt63) and GM tomato (Huafan-1) were kindly supplied by Huazhong Agricultural University, China. The non-GM seeds (maize, soybean, canola, cotton, and rice) purchased from local market (Shanghai, China) were checked for absence of any GM events and used in this study.

Six corn and four soybean samples from April 2010 proficiency program organized by Grain Inspection, Packers and Stockyards Administration (GIPSA) were used as the practical samples for the developed assays. The corn samples contained various combinations and concentrations of the following transgenic traits: T25, CBH351, MON810, GA21, Bt-176, Bt11, NK603, Herculex, MON863, Herculex RW, MIR 604 (Agrisure RWTM), Event 3272, or no events (i.e., negative corn sample). The soybean samples were non-transgenic soybeans, or contained the transgenic glyphosate-tolerant soybeans (GTS 40-3-2) and/or the glufosinate ammonium tolerant soybeans (A2704-12).

### 2.2. DNA extraction

Dry seeds were ground with a SPEX 6870 Freezer/Mill (Spex SamplePrep, LLC., NJ, USA). The ground materials were stored at −20 °C for DNA extraction. The plant genomic DNA was extracted and purified using the mini-plant genomic DNA extraction kit (Shanghai Ruifeng Agro-tech Co., Ltd., Shanghai, China) according to the manufacturer's manual. The concentration and quality of the purified DNA samples were evaluated using the NanoDrop 1000 UV/Vis Spectrophotometer (Thermo Scientific) and 1% agarose gel electrophoresis.

### 2.3. Test samples preparation

In this study, none GM event used in this study was confirmed to contain all eight target genes. For this reason, three mixed genomic DNA solutions (hereafter referred as mA, mB, and mC) solutions were prepared. In the mA solution, the DNA solutions from MON88017 and Bt11 were mixed and diluted to contain approximately 2000 copies of the *Cry3Bb1*, *CP4-EPSPS1*, *pat*, and *Cry1Ab2* gene per microlitre considering about 2.6 pg per haploid genome in the case of maize (Arumuganathan & Earle, 1991). In the mB solution, the DNA solutions from MON88017 and Bt176 were mixed and diluted to contain approximately 2000 copies of the *Cry3Bb1*, *CP4-EPSPS1*, *bar*, and *Cry1Ab1* gene per microlitre. In the mC solution, the DNA solutions from MIR604, RT73, T45, and Bt11 were mixed and diluted to contain approximately 2000 copies of the *mCry3A*, *CP4-EPSPS2*, *pat*, and *Cry1Ab3* gene per microlitre according to the corresponding genome size (Arumuganathan & Earle, 1991). For the evaluation of the LOD of quadruplex degenerate PCR assay, series of mixed DNA solutions were prepared by diluting mA, mB, and mC, respectively, to final concentrations equivalent to 2000, 400, 80, 16, 4, and 2 copies of each target gene per microlitre. In addition, 14 practical samples (samples A–N) were also prepared based on the mass/mass ratio to test the applicability of quadruplex degenerate PCR assay (Table 2). The expression of GM content of each prepared sample was indicated as of GMO ingredient based on each species. The sample A contained non-GM seed power and served as a negative control.

### 2.4. Alignment of nucleotide sequences from trait genes detected in this study

The structure and sequence information of recombinant DNA in GM crops was obtained from publicly available resources (AGBIOS GM Crop Database; GMDD; Grohmann et al., 2009; Mano et al., 2009b; NCBI Entrez Nucleotide database). The nucleotide sequences of *CP4-EPSPS*, *bar*, *pat*, *mCry3A*, *Cry3Bb1*, *Cry1Ab*, *Cry1Ac*, and *Cry1A*(*b*/*c*) genes introduced into GMOs were retrieved from GMDD (GMDD, 2010) and GenBank (NCBI Entrez Nucleotide database, 2010). The alignment analysis of related nucleotide sequences was performed using the software (Vector NTI Advance 10). The homologous genes or genes encoding a particular protein family used for alignment analysis were: (i) *mCry3A* in MIR04, and

**Table 1**
Matrix description of the GM events used in this study.

| Species | GM events | *mCry3A* | *Cry3Bb1* | *bar* | *pat* | *Cry1Ab* | *Cry1Ac* | *Cry1A(b/c)* | *CP4-EPSPS* |
|---|---|---|---|---|---|---|---|---|---|
| Maize | Bt 176 | | | X | | X (2*) | | | |
| | Bt 11 | | | | X (1) | X (1) | | | |
| | MON 810 | | | | | X (1) | | | |
| | TC1507 | | | | X (1) | | | | |
| | DAS59122 | | | | X (1) | | | | |
| | T 25 | | | | X (3/1) | | | | |
| | MIR 604 | X (1) | | | | | | | |
| | MON 88017 | | X (1) | | | | | | X (1) |
| | MON 863 | | X (1) | | | | | | |
| | NK 603 | | | | | | | | X (2) |
| | GA 21 | | | | | | | | |
| Soybean | GTS 40-3-2 | | | | | | | | X (1) |
| | MON 89788 | | | | | | | | X (1) |
| | A 2704-12 | | | | X | | | | |
| | A 5547-127 | | | | X (1) | | | | |
| Canola | RT 73 | | | | | | | | X (1) |
| | T 45 | | | | X (1) | | | | |
| | Topas19/2 | | | | X (1) | | | | |
| | MS1 × RF1 | | | X (1) | | | | | |
| | MS1 × RF2 | | | X (1) | | | | | |
| | MS8 × RF3 | | | X (1) | | | | | |
| | OXY-235 | | | | | | | | |
| Cotton | MON 531 | | | | | | X (⩾1) | | |
| | MON15985 | | | | | | X (⩾1) | | |
| | MON 1445 | | | | | | | | X (1) |
| | MON 88913 | | | | | | | | X (2) |
| | LLCotton 25 | | | X | | | | | |
| Rice | Bt 63 | | | | | | | X | |
| | Huahui-1 | | | | | | | X | |
| Papaya | Huanong No.1 | | | | | | | | |
| Tomato | Huafan-1 | | | | | | | | |
| Sugar beet | H7-1 | | | | | | | | X (1) |

* The corresponding number of target copy in the GM events based on the AGBIOS database. "X" indicates the corresponding target is present in the GM event; only the trait genes detected in this study were listed in this table.

**Table 2**
Details of test samples for the quadruplex degenerate PCR assay.

| Test samples | Sample composition* | Targets |
|---|---|---|
| A | 20.0% for each of non-GM rice, maize, soybean, canola, and cotton | / |
| B | 2.0% MON88017, 2.0% Bt176 | *Cry3Bb1*, *CP4-EPSPS*, *bar*, *Cry1Ab* |
| C | 5.0% MON863, 2.0% Bt176, 0.5% NK603 | *Cry3Bb1*, *CP4-EPSPS*, *bar*, *Cry1Ab* |
| D | 1.0% RRS, 2.0% Bt11 | *CP4-EPSPS*, *pat*, *Cry1Ab* |
| E | 1.0% MON863, 3.0% T25, 2.5% 810 | *Cry3Bb1*, *pat*, *Cry1Ab* |
| F | 1.0% MIR604, 1.0% MON89788, 1.5% Bt63 | *mCry3A*, *CP4-EPSPS*, *Cry1A(b/c)* |
| G | 5.0% MON88913, 2.0% MON531 | *CP4-EPSPS*, *Cry1Ac* |
| H | 0.5% MON863, 0.5% NK603, 0.5% T25, 0.5% MON810 | *Cry3Bb1*, *CP4-EPSPS*, *pat*, *Cry1Ab* |
| I | 1.0% MIR604, 0.5% RT73, 1.0% MON531 | *mCry3A*, *CP4-EPSPS*, *Cry1Ac* |
| J | 2.0% MON88017, 5.0%MON810 | *Cry3Bb1*, *CP4-EPSPS*, *Cry1Ab* |
| K | 2.0% MON89788, 2.0% Topas19/2, 5.0% Bt63 | *CP4-EPSPS*, *bar*, *Cry1A(b/c)* |
| L | 1.5% MON863, 2.0% Bt176 | *Cry3Bb1*, *bar*, *Cry1Ab* |
| M | 1.0% RRS, 1.0% MON1445, 0.5% NK603, 1.0% RT73 | *CP4-EPSPS* |
| N | 2.0% MON863, 1.0% RRS, 3.0% Bt11 | *Cry3Bb1*, *CP4-EPSPS*, *pat*, *Cry1Ab* |
| C1 | 0.1% TC1507, 0.1% BT176, 1% T25, 1% MON810, 2% NK603, 0.5%MIR604, 1% CBH351, 0.1% GA21, 2% DAS59122, 0.1% 3272. | *mCry3A*, *CP4-EPSPS*, *bar*, *pat*, *Cry1Ab* |
| C2 | 0.8% TC1507, 0.1% BT176, 0.8% T25, 0.1% MON810, 0.4% MON863, 1.5% MIR604, 0.4% GA21, 0.1% DAS59122. | *mCry3A*, *Cry3Bb1*, *bar*, *pat*, *Cry1Ab* |
| C3 | 0.5% BT176, 1% BT11, 0.5% MON810, 0.8% MIR604, 0.4% GA21, 0.2% 3272. | *mCry3A*, *bar*, *pat*, *Cry1Ab* |
| C4 | Non-GM maize negative control | / |
| C5 | 1.5% BT176, 0.1% BT11, 2% T25, 2% MON810, 1% NK603, 0.1% CBH351, 0.1% GA21, 0.5% 3272. | *CP4-EPSPS*, *bar*, *pat*, *Cry1Ab* |
| C6 | 1.5% TC1507, 0.1% BT176, 2% BT11, 0.5% T25, 0.1% MON810, 0.8% MON863, 1% GA21, 0.1% DAS59122, 1% 3272. | *Cry3Bb1*, *bar*, *pat*, *Cry1Ab* |
| S1 | Non-GM soybean negative control | / |
| S2 | 1.5% RRS | *CP4-EPSPS* |
| S3 | 0.2% A2704-12, 0.2% RRS | *pat*, *CP4-EPSPS* |
| S4 | 0.1% A2704-12. | *pat* |

* The expression of GM content was indicated as the mass/mass ratio of GMO ingredient based on each species.

*Cry3Bb1* in MON863 and MON88017; (ii) *CP4-EPSPS* in NK603, MON88017, RT73, MON1445, MON88913, RRS, and MON89788; (iii) *bar* in Bt176 and *pat* in T25, TC1507, Bt11, DAS59122, and T45; (iv) *Cry1Ab* in Bt176, Bt11, and MON810, *Cry1Ac* in MON531, and *Cry1A*(*b*/*c*) in Bt63 and Huahui-1.

### 2.5. Design of degenerate primers for GMO screening

Degenerate primers for aligned sequences were designed according to the results of sequence alignment and should be compatible with each other in the multiplex mixture. The criteria for primer design were the following: (i) the maximum coverage, i.e., the degenerate primers covering a maximum number of known gene sequences; (ii) the minimum degeneracy, i.e., the degenerate primers containing a minimum number of degenerate bases; (iii) the similar annealing temperature between 58 and 63 °C; and (iv) high efficiency and robustness (each amplicon with 100–500 bp in length) (Morisset, Dobnik, Hamels, Zel, & Gruden, 2008; Najafabadi et al., 2008). The primers used in this study were listed in Table 3. All the primers were synthesized by Invitrogen Co., Ltd. (Shanghai, China).

### 2.6. Degenerate PCR conditions

All PCR assays were performed with the final volume of 25 μL. In the singleplex PCR assay, each reaction contained the following reagents: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of the dNTPs, 200 nM each of the primers, 1 U of HS-*Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd.), and DNA template. For the quadruplex PCR assay, each reaction contained the following reagents: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.8 mM $MgCl_2$, 200 μM dNTP, 1× primer mix (50 nM mCry3-F/R, 120 nM mCP4ES-F/R, 140 nM mPAT-F/R, and 140 nm mCry1A-F/R, 1 U of HS-*Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd.), and DNA template. All PCR amplification programs were as follows: 5 min of predenaturation at 94 °C; 5 cycles of 30 s at 94 °C, 30 s at 63 °C and 30 s at 72 °C; 32 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C; and a final extension of 7 min at 72 °C. After the PCR amplification, 10 μL PCR products were analyzed by 2% (w/v) agarose gel electrophoresis. The electrophoresis was run at a constant voltage (130 V) for about 40 min in 0.5× TBE buffer with 1× Gel-red (Biotium, Inc., CA., USA) staining. The gel was scanned by Gel Image System (Tanon Science & Technology Co., Ltd., Shanghai, China).

## 3. Results

### 3.1. Selection of PCR targets and design of primers

The major biotech crops have herbicide tolerance and/or insect resistance traits. According to publicly available database (AGBIOS GM Crop Database, 2010; GMDD, 2010), 35 GM events contain *CP4-EPSPS*, 48 GM events contain *bar* or *pat*, 34 GM events contain *Cry1Ab*, *Cry1Ac*, *or Cry1A*(*b*/*c*), and 13 GM events contain *mCry3A* or *Cry3Bb1* (Supplemental Table S1). More than 90 authorized GM events were shown to at least have one of these trait genes. While the DNA sequences of some target genes (such as, *CP4-EPSPS* or *Cry1Ab*) within GM crops have different variants in different GM events or different target genes (such as, *bar* and *pat*) share high similarity. Therefore, a strategy employing multiplex degenerate PCR was introduced for maximally reducing the number of PCR reactions and saving time and costs associated with GMO analysis. Owing to the degenerate primers may lead to the poor specificity of PCR amplification or the larger difference in PCR amplification efficiency for different targets, the design for multiple degenerate primers is significantly critical, and you should make the primers match and amplify as many of the corresponding sequences as possible. Here, four pairs of degenerate primers were designed to screen *mCry3A*, *Cry3Bb1*, *CP4-EPSPS*, *Cry1Ab*, *Cry1Ac*, *Cry1A*(*b*/*c*), *bar* and *pat* based the principle described in previous paper (Najafabadi et al., 2008) (Table 3, Fig. 1). Additionally, the specificity of these degenerate primer pairs was tested in singleplex PCR.

#### 3.1.1. mCry3-F/R

Primer pair mCry3-F/R was targeted to the conserved regions of the modified insect-resistant gene *mCry3A* introduced into MIR604 and *Cry3Bb1* in MON863, with one amplicon of 444 bp in length. As shown in Supplemental Figure S1A, only one expected 444 bp DNA fragment from *mCry3A* or *Cry3Bb1* gene was obtained in MIR604, MON863, and MON88017, and no amplification product was observed in other Bt events with different Bt family genes (*Cry1Ab*, *Cry1Ac*, *Cry1F*, etc.), other GM crops and no template control (NTC).

#### 3.1.2. mCP4ES-F/R

Primer pair mCP4ES-F/R were designed according to the region of *CP4-EPSPS* from RRS and MON89788 with higher similarity, and expected to be used for screening *CP4-EPSPS* gene in GM crops, such as, NK603, MON88017, RRS, MON89788, RT73, MON1445, and MON88913. The 5′ end region of the *CP4-EPSPS* gene in NK603, MON88017, and RRS were identical to that of (*CP4-EPSPS1*) (GenBank No. I43998). MON1445, MON88913, MON89788, and RT73 contained identical 5′ end regions of *CP4-EPSPS2* (GenBank No. GV597339). In the specificity testing, only one expected 333 bp DNA fragment was obtained from NK603, MON88017, RRS, MON89788, RT73, MON1445, H7-1, and MON88913, and no amplification product was observed in other herbicide-tolerant GM crops and NTC (Supplemental Figure S1B).

#### 3.1.3. mPAT-F/R

Alignment analysis showed that the nucleotide sequences of synthetic *pat* gene in TC1507, DAS59122, T25, and T45 were identical to that of Bt11 (GenBank No. AY629236) and with 66% simi-

**Table 3**
List of degenerate primers used in this study.

| Target | Primer name | Sequence (5′–3′)* | Amplicon (bp) | Ref. |
|---|---|---|---|---|
| *mCry3A*; *Cry3Bb1* | mCry3-F | **M**CTTCCTGAACACCATCTGGC | 444 | This work |
| | mCry3-R | CGGTGTACT**SY**TGGGTCAGCTTC | | |
| *CP4-EPSPS* | mCP4ES-F | ACGGTGA**Y**CGTCTTCC**M**GTTAC | 333 | This work |
| | mCP4ES-R | GAACAAGCA**R**GGC**M**GCAACCA | | |
| *bar*; *pat* | mPAT-F | GAAGGC**WM**G**S**AACGC**Y**TACGA | 262 | This work |
| | mPAT-R | CCA**R**AAACC**M**AC**R**TCATGCCA | | |
| *Cry1Ab*; *Cry1Ac*; *Cry1A*(*b*/*c*) | mCry1A-F | TT**Y**CTGCT**S**AGCGAGTTCGT | 200 | This work |
| | mCry1A-R | GC**R**TAGATTTGGTA**S**AG**R**TTGCT | | |

* Italic boldface letters represent the degenerate nucleotides.

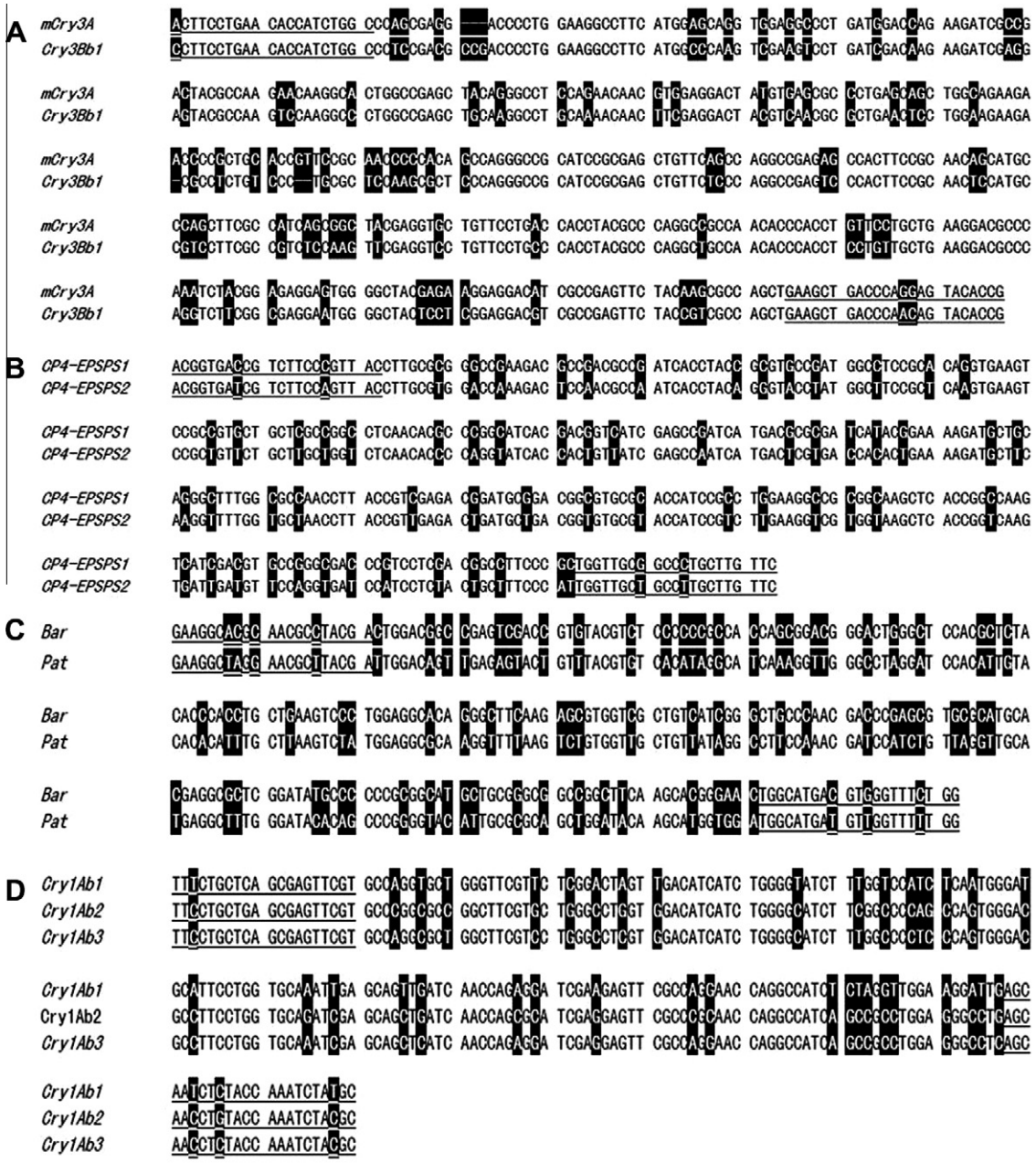


**Fig. 1.** Alignment of the nucleotide sequences for the design of the degenerate primers mCry3-F/R (A), mCP4ES-F/R (B), mPAT-F/R (C), and mCry1A-F/R (D), respectively. Dashes denote gaps introduced to obtain maximum similarity. Variable nucleotides are shown by outline type. Primer sequences for PCR amplification are highlighted with underlines.

larity to the sequence of *bar* in Bt176 (GenBank No. AJ878607). Therefore, the degenerate primer pair mPAT-F/R targeting to the herbicide-tolerant genes *bar* and *pat* were designed on the identical region of the *pat* gene in Bt11 and *bar* in Bt176. As expected, in the singleplex PCR assay, only one 262 bp DNA fragment was obtained from Bt176, MS1 × RF1, MS1 × RF2, MS8 × RF3, LLCotton25, Bt11, TC1507, DAS59122, T25, A2704-12, A5547-127, T45, and Topas19/2, and no amplification product was observed in other herbicide-tolerant GM crops (GA21, NK603, MON88017, RRS, RT73, OXY-235, MON88913, and MON1445) and NTC (Supplemental Figure S1C).

### 3.1.4. mCry1A-F/R

Although the nucleotide sequences of *Cry1Ab* gene introduced into Bt176 (*Cry1Ab1*, GenBank No. I41419), Bt11 (*Cry1Ab2*, GenBank No. GV597352), and MON810 (*Cry1Ab3*, GenBank No. AR127583) were shown to have some sequence variations, the alignment analysis indicated that the nucleotide sequence of the 5′ encoding region of *Cry1Ac* gene (GenBank No. AR656168) in GM cotton MON531 and fused *Cry1A*(*b*/*c*) gene (GenBank No. EU880444) in GM rice Bt63 was identical to that of *Cry1Ab* in GM maize Bt11. Therefore, on the basis of the *Cry1Ab* gene sequences introduced into Bt176, Bt11, and MON810, we designed the mCry1A-F/R primer pair expected to simultaneously amplify *Cry1Ab*, *Cry1Ac*, and *Cry1A*(*b*/*c*) in GM crops. As shown in Supplemental Figure S1D, the designed specific primers could be used for effective amplfying *Cry1Ab*, *Cry1Ac*, or *Cry1A*(*b*/*c*) target sequences introduced into Bt176, Bt11, MON810, MON15985, MON531, Bt63, Huahui-1, etc., yielding one expected 200 bp DNA fragment.

In above four singleplex degenerate PCR assays, DNA amplicons were not observed in negative DNA templates and NTC, and only corresponding products of the expected size in the positive DNA templates were generated. In conclusion, the singleplex degenerate PCR assays employing the four primer pairs are highly specific, and which can potentially be used to establish the multiplex degenerate PCR assay for GMO screening.

### 3.2. Development of quadruplex degenerate PCR assay

To develop the effective quadruplex degenerate PCR assay targeting to the above eight trait genes, the primer concentrations, annealing temperature, and the $Mg^{2+}$ concentrations were optimized. For the primer concentrations, the ratios of various primers were adjusted according to the intensity of amplified DNA fragments in the agarose gel, and the final concentrations of the primers were 50 nM mCry3-F/R, 120 nM mCP4ES-F/R, 140 nM mPAT-F/R, and 140 nM mCry1A-F/R. The significance of different annealing temperature for degenerate PCR was investigated in a gradient PCR from 58 to 65 °C with a gradient of 1 °C. The optimal reaction conditions were performed as follows: five cycles of pre-amplification with annealing temperature at 63 °C, followed by 32 cycles of amplification with annealing at 60 °C. Furthermore, the 1.8 mM $Mg^{2+}$ concentration was shown to be optimal for quadruplex PCR (data not shown).

After the optimization of the quadruplex degenerate PCR assay, the performances of the developed quadruplex degenerate PCR were evaluated. The specificity of the quadruplex degenerate PCR assay was evaluated by testing all available GM events in this study. Forty nanograms genomic DNA extracted from individual GM events (MON88017, MON863, MIR604, NK603, RRS, MON89788, RT73, MON1445, MON88913, H7-1, A2704-12, A5574-127, DAS59122, TC1507, T25, Topas19/2, T45, MS1 × RF1, MS1 × RF2, MS8 × RF3, LLCotton25, Bt176, Bt11, MON810, Bt63, Huahui-1, MON531, Huanong No.1, Huafan-1, GA21, and OXY-235) and mixed non-GM crops (containing equal weights of maize, soybean, canola, cotton, and rice) were used as templates. The presence/absence of each target sequence in GM event was described in Table 1. In addition, the mixed DNA solutions mA and mC were also tested as the positive control. The amplified result was shown in Fig. 2A, only the amplicons of the expected size were amplified with the corresponding GM events, and all expected four amplicons were amplified with the mixed DNA solutions mA and mC. In contrast, DNA amplicons were not observed in the other DNA templates and NTC. The results were consistent with the records in the available GMO database (AGBIOS GM Crop Database; GMDD), suggesting that the established quadruplex PCR assay is specific and reliable for amplifying the all nine targets, unlike some other PCR assays in which each primer is specific to one target sequence (Bahrdt et al., 2010; Lu et al., 2010).

The limit of detection (LOD) of the quadruplex degenerate PCR system was also tested using the series of diluted mA DNA samples. The concentrations for each target gene (*Cry3Bb1*, *CP4-EPSPS*, *pat*, and *Cry1Ab*) in diluted DNA solutions in mA samples were 2000, 400, 80, 16, 4, and 2 copies/μL, respectively, and 5 μL of genomic DNA was used as the template in each quadruplex PCR reaction. The result showed that the all four amplified products could be observed from all levels except for the levels of 4 and 2 target copies/μL (Fig. 2B). For the DNA samples of mB and mC, similar sensitivities of the developed quadruplex degenerate PCR system were detected (data not shown). This suggests that our PCR approach has an absolute LOD value of 80 target copies, similar to some qualitative multiplex-PCR (Lu et al., 2010) but less sensitive than quantitative multiplex PCR (Bahrdt et al., 2010). Furthermore, this approach is sufficiently sensitive and will satisfy labelling requirements for most countries worldwide.

### 3.3. Applicability of the quadruplex degenerate PCR assay for screening the practical samples

The applicability of the developed quadruplex degenerate PCR was evaluated employing the practical GM samples (samples B–N) with various combinations of GM events at different GM contents (%) and one non-GM sample (sample A) as negative control (Table 2). Each sample set was prepared with two replicates. PCR detection was performed in triplicates for each replicate, and total of 100 ng genomic DNA was used as the template in each reaction. As shown in Fig. 3A, the expected specific DNA fragments were amplified only in the corresponding samples.

In addition, we also tested the six corn and four soybean samples of April 2010 proficiency program using the developed assay. In each sample (two replicates), all the corresponding targets were clearly amplified as expected, even as low as 0.1% (BT176 in C1 for *cry1Ab* or A2704-12 in S4 for *pat*) (Fig. 3B). Further, the result

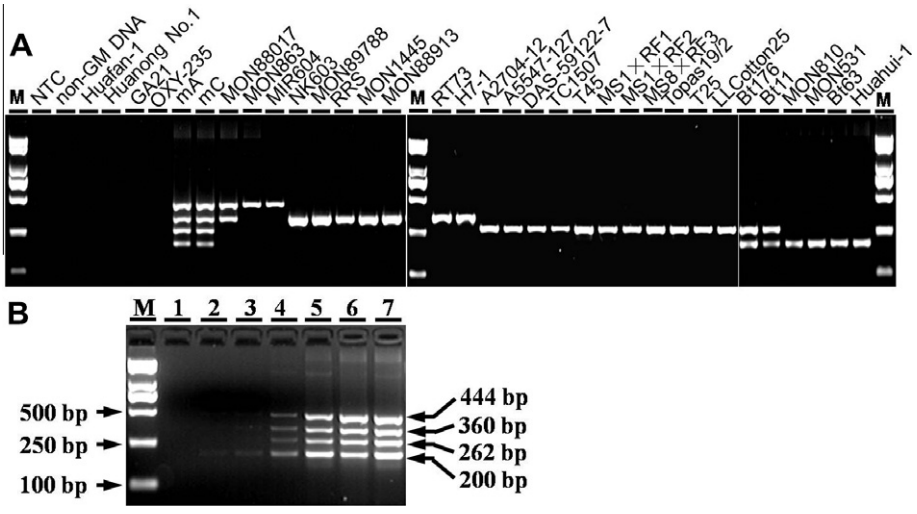


**Fig. 2.** (A) Specificity test of the quadruplex degenerate PCR assay employing the primer pairs mCry3-F/R, mCP4ES-F/R, mPAT-F/R, and mCry1A-F/R. Lane M: DL2000 plus II. (B) Sensitivity test of the quadruplex degenerate PCR assay for simultaneously amplifying all four target sequences. Lane 1: NTC. Lanes 2–7: target sequences corresponded to 10, 20, 80, 400, 2000, and 10,000 copies for each reaction. Lane M: DL2000 plus II.

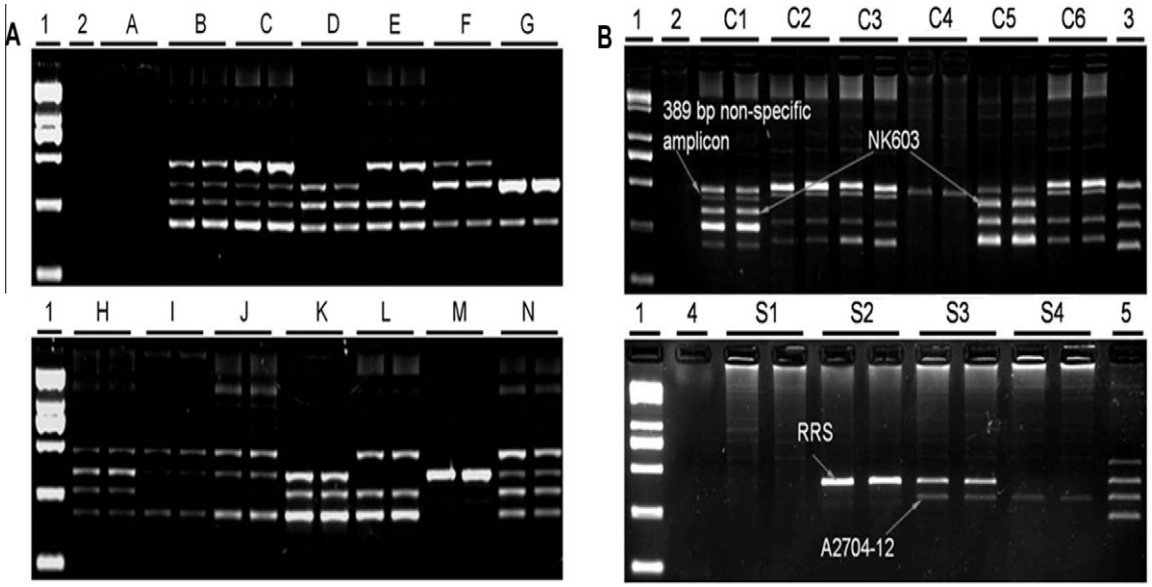


**Fig. 3.** (A) Agarose gel electrophoresis analysis of the quadruplex degenerate PCR assay for the detection of artificially prepared test samples. Lane 1: DL2000 plus II. Lane 2: NTC. Lanes A–N: amplicons were produced from samples A–N as described in Table 2. (B) Agarose gel electrophoresis analysis of the quadruplex degenerate PCR assay for the detection of GIPSA samples. Lane 1: DL2000 plus II. Lanes 2 and 4: NTC. Lanes 3 and 5: mixed genomic DNA solution mC. Lanes C1–C6 and S1–S4: amplicons were produced from samples C1, C2, C3, C4, C5, C6, S1, S2, S3, and S4 as described in Table 2, respectively.

directly showed that C4 and S1 were negative corn and soybean control, respectively, both C1 and C5 samples contained NK603 (*CP4-EPSPS*), both S2 and S3 samples contained RRS (*CP4-EPSPS*), and both S3 and S4 samples contained A2704-12 (*pat*), thus decreasing the time and increasing the efficiency associated with GMO analysis. Unexpectedly, a non-specific DNA fragment between 444 and 333 bp DNA bands was observed in all GIPSA corn samples but not in other maize (Fig. 2A) and soybean samples. Sequencing results showed that the length of PCR product was 389 and more than 200 bp is homologous from corn genome (Supplemental Figure S2), and the reason for this is probably from the specificity of GIPSA corn samples. Accordingly, the amplification results from the practical GM samples and GISPA samples determinate that this assay is still feasible for screening GMOs and their derived products with high sensitivity.

## 4. Discussion

In this work, the multiplex PCR assay was established employing four degenerate PCR primers, and there were some differences and improvements compared with several previously reported papers (Dörries et al., 2010; Grohmann et al., 2009; Mano et al., 2009a; Randhawa et al., 2009), such as the tested targets coverage, the screen strategy based on the exogenous trait genes, and the degenerate primers targeted at the different genes with high homogenous DNA sequences.

In the previously reported multiplex assays, one kind of assay was for quickly screening the existence of the GM contents employing some commonly used elements (promoter, NOS, marker gene, etc.) (Bahrdt et al., 2010; Dörries et al., 2010; Lu et al., 2010; Randhawa et al., 2009); the other assays were specifically identified the detail GM plant events employing the construct-specific sequences or unique event-specific sequences (Nadal, Coll, La Paz, Esteve, & Pla, 2006; Shrestha, Hwu, Wang, Liu, & Chang, 2008). For the screen multiplex assays, 100 approved GMOs can be detected, and which were also used for the routine analysis in several labs. The high specific multiplex assays of different GM events were generally used for concrete identifying of one event, such as the multiplex assay for eight GM corns (Bt11, Event176, GA21, MON810, MON863, NK603, T25, and TC1507) (Shrestha, Hwu, Wang, Liu, & Chang, 2008). Our developed multiplex assay was established employing the four degenerate primer pairs for the commonly used eight exogenous trait genes (*CP4-EPSPS*, *bar*, *pat*, *Cry1Ab*, *Cry1Ac*, *Cry1A*(*b*/*c*), *mCry3A*, and *Cry3Bb1*) in more than 90 GM events. Compared with the previous general screen MPCR assays, the developed assay have the similar coverage and high sensitivity. In addition, the developed MPCR assay was not easily contaminated in routine analysis. However, the screen multiplex PCR assays were particular easily contaminated in routine analysis, because these universal elements were often used during the process of plant transgene and widely existed in GM/non-GM microorganisms.

The purpose for developing the quadruplex degenerate PCR assay is to maximally reduce the number of PCR reactions and save time and costs associated with GMO analysis. In conventional multiplex PCR analysis, each primer pair was aimed to only one target DNA, and which will limit the number of tested targets. The degenerate primer pair targeted at different gene sequences used in this paper showed the following advantages: (i) Only four degenerate primer pairs for the targets of eight genes with nine different DNA sequences; (ii) Covering the more target molecules and GM events; (iii) high-throughput screening of GMO samples including the approved and/or unknown GMO events. Especially, more and more different GM events expressed the same exogenous protein with different modified gene sequences were developed, and which brought more troubles in GMO analysis based on the nucleic acid analytical techniques.

In short, we report the development of an effective quadruplex degenerate PCR assay targeting to commonly used trait genes in GMOs. The quadruplex degenerate PCR assay allows the simultaneous detection of nine different DNA target sequences employing four degenerate primer pairs, and which is expected to detect more than 90 authorized GM events in the world. The developed quadruplex degenerate PCR system reduces the level of multiple PCR amplification, and is a time- and cost-efficient as well as high-throughput assay for GMO screening. Also, the strategy using degenerate primer for analyzing the family genes or different modified DNA sequences of one gene will do favour to improve throughput of MPCR in GMO analysis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.11.096.

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