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Detection of Four Types of Genetically Modified Maize by Polymerase Chain Reaction and Immuno-Kit Methods

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ABSTRACT

To detect and identify four types of genetically modified (GM) maize, a polymerase chain reaction (PCR) assay and an immuno-assay kit were performed in this study. Primers specific to inserted genes in the Event 176 GM maize (Novartis company), Bt11 (Novartis company), MON810 (Monsanto company) and Liberty (AgrEvo company) were used to conduct the PCR assay. Four pairs of primers, namely, CDPK-cry (Event176), IV01-cry (Bt11), HS01-cry (MON810) and CM03-PA01 (Liberty) were used to identify the GM-maize. Results showed that the limit of detection for GM-maize were 0.01%, 0.1%, and 1% (w/w) as using HS01-cry, CDPK-cry and IV01-cry, and CM03-PA01 primers, respectively. The GM-maize references as well as 20 food-grade maize samples were also tested using a commercial immuno-kit. Three GM-maize references, Event176, Bt11 and MON810, and 6 corn-middling pellet samples were tested as positives; while 14 popcorn samples were negative. To confirm these results, the 20 food-grade maize samples were further tested by the PCR method. Two types of GM-maize were detected in popcorn samples; while 3~4 types of GM-maize were detected in the samples of corn middling pellets. The results of this study demonstrate that using PCR method is capable of differentiating four types of GM-maize from non-GM products; while the commercial immuno-kit can only be used for screening purpose. All samples, with positive or negative results as tested by immuno-kit, need to be further confirmed by the PCR method. The results of this study also reveal that marketed maize products are usually mixed with different types of GM-maize.

Key words: GM-maize, PCR, Immuno-kit

INTRODUCTION

In recent years, gene modification techniques have been greatly improved worldwide. Some insect-resistant and herbicide-tolerant genetically modified crops (GM-crops) have been successfully developed and widely cultivated. The US is presently the most advanced country in R&D and commercialization of GM-crops. So far, there are more than 50 GM-crop products approved in the US[1]. Cultivation of GM-crops in Taiwan has not yet been employed; however, field trials for GM-papayas and GM-tomatoes have been permitted[2]. In total, 18 types of GM-maize have been approved for commerce globally as of 2000[1]. The major traits of GM-maize are insect resistance, herbicide tolerance, and male-sterility[1, 3, 4]. Research has continued to improve the following characteristics of GM-maize: (1) adaptability to harsh growing conditions, such as drought and soil with high salt or heavy metal content; (2) alteration of compositions, such as increasing specific amino acids (lysine, methionine, tryptophan), and altering protein and fatty acid composition[4]. The US and China were ranked as the top two leading countries in maize production in 1998 with 42% and 21% of global maize production, respectively[1]. In 1998, the global area for GM-maize production was concentrated in the US and Canada with 95.6% and 3.8% production, respectively[4]. GM-maize cultivated in the US is mostly insect-resistant (accounts for 80%), and some types are herbicide-tolerant or others (accounts for 20%)[4]. In 1999, the areas for cultivating GM-maize in the US were further expanded to 10 million hectares; accounting for 31% of total maize production in that year[5]. Statistical data from the ROC Council of Agriculture shows that Taiwan imports about 6 million tons of maize annually, and 96% is from the US[2]. Based on this data, about 30% of commercial maize in Taiwan is estimated to be GM-maize.

Methods for the identification of GM food can be divided into 3 categories[3]. The first category includes nucleotide-based amplification methods, such as polymerase chain reaction (PCR). The second category includes protein-based methods, such as enzyme-linked immunosorbent assay (ELISA). The third category is based on the detection of enzymatic activities. Every detection method has its own specificity and limitations. For example, the detection of enzymatic activity method is not recommended for processed foods, where proteins may be denaturalized. The methods based on PCR are not suitable for detection of highly processed foods because DNA fragments in foods could be broken into pieces. Among the 3 categories, PCR is the most popular method used worldwide. Using the PCR method to identify GM products, a primer is designed based on the regulatory sequence or structural gene in the inserted gene fragment[3]. These designed primers possess some specific characteristics and can be used for product screening and product-specificity detection[3]. The PCR products need to be further confirmed by the following method: nucleic acid sequencing, endonuclease mapping, and probe hybridization

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[3]. The PCR method is not only used for identification of GM-products, but also for quantification purposes[6]. In addition to the PCR method, the ELISA method is mostly applied for screening tests and, in some cases, for quantification purposes[7]. However, the PCR method is recommended for further confirmation of GMO types.

Research on the PCR method for detection of GM-maize has been extensively carried out. In 1997, 29 laboratories in 13 European countries performed a collaborative trial study for detection of GM-maize. Results showed that using a PCR method with 35S-promoter primer was capable of detecting as low as 2% of Event176 GM-maize in maize flour [8]. Some German researchers (Ehlers *et al.* and Hupfer *et al.*) have reported that the selected primers derived from *ivr1, cry IA(b), bar, 35S-bar,* and *ampR* genes can be successfully used to detect Event176 GM-maize[9, 10]. In 2000, the PCR method for detection of the specificity of 4 GM-maize, Event176, Bt11, MON810, and Liberty was developed in Japan. The limit of detection for Bt11 was reported to be 0.01% and for other 3 types was 0.05%[11].

In compliance with the labeling regulation for GM foods, some countries have proposed the official method for GM food tests. Germany has established 6 methods for detection of 6 products: soybean, maize, potato, yogurt, fermented sausage, and tomato[12]. To meet crop market demand the US GIPSA (Grain Inspection, Packers and Stocklyards Adminstration) set up a reference laboratory in 2000 to evaluate the GMO detection method and certify related laboratories[13]. So far, the PCR and ELISA kits for GM-maize detection are commercially available[7, 14].

The PCR method for GM-maize detection developed in Europe is mainly designed for Event176 and Bt11 GM-maize detection [8, 9, 10]. Japan has developed the PCR method for detection of 4 imported types of GM-maize[11]. In commerce, the most popular ELISA kit for GM-maize detection is Trait Bt1 Lateral Flow Test and Bt Cry1Ab Test Kit produced by Strategic Diagnostics Inc. (SDI, USA)[7, 14]. In Taiwan, a report regarding the feasibility of detecting Event176 GM-maize and Roundup Ready GM-soybean by a PCR method has been published[15]. However, the PCR detection of other types of GM-maize, immno-assay kit detection, and investigation of the GM-maize in commercial maize materials have not been well studied in Taiwan. In view of an urgent issue regarding the GM food labeling aroused in Taiwan, the purpose of this study was to establish a PCR method to detect 4 types of GM-maize and to evaluate the feasibility of commercial immuo-assay kits. Application of the established methods for the detection of GM-maize in commercial maize materials was also conducted, in order to preliminarily reveal the status of using GM-maize in Taiwan. In addition, the established method could provide useful information for related authorities to regulate GM food labeling, and could be a base method to set up an official method for detection of GM products.

MATERIALS AND METHODS

I. *Reagents*

Chloroform and isopropanol were purchased from Merk (Darmstadt, Germany). Hexadecyltrimethyl-amoniumbromide (CTAB) was obtained from Sigma (St. Louis, MI, USA) and Agarose was purchased from Amresco (Solon, OH, USA).

II. *GM-maize Reference*

The following 4 types of GM-maize were used in this study: Event176 (Novartis, Greensboro, NC, USA), Bt11 (Novartis, Greensboro, NC, USA), MON810 (Monsanto, St. Louis, MO, USA), and T25 (Liberty) (AgrEvo, Berlin, Germany). Their characteristics are listed in Table 1.

III. *Commercial Immuno-assay Kits*

Trait Bt1 Lateral Flow Test (Strip) and Bt CryIAb Test Kit (ELISA) were made by Strategic Diagnostics Inc., (Newark, U.S.A.).

IV. *Equipment*

PCR thermal controller model PTC-100 with programmable thermal controller was purchased from MJ Research Co. (Water Town, MA, U.S.A.).

V. *PCR Primers and Reagents*

Six pairs of PCR primers as listed in Table 2 were used in this study. The primer CDPK-cry was used to detect the regulatory sequence-structure gene of *cryIA(b)* gene in Event176 GM-maize. The primer IV01-cry was specific to the maize alcohol dehydrogenase No.6 intron sequence-*cryIA(b)* structure gene in Bt11 GM-maize. The primer HS01-cry were used to detect maize *Hsp70* No.1 intron sequence-*cryIA(b)* structure gene in MON810 GM-maize, and the primer CM03-PA01 was for detection of the regulatory sequence-structure gene of *pat* gene in Liberty GM-maize. The primer CM01-CM02 was derived from 35S-promoter of cauliflower mosaic virus. The primer ivr was the primer of Invertase gene. The above 6 primers were synthesized by TIB Molbiol Co. (Berlin, Gernamy). DNA polymerase kit was obtained from PROtech Technologies, Inc.

VI. *DNA Preparation and Purification*

A CTAB method for sample extraction and purification reported by Lipp *et al.* in 1999[8] was adopted in this study. The sample (25 mg) and deionized water (100 μL) were mixed and kept at 65˚C for 1 hr followed by spiking 500 μL of CTAB buffer solution and keeping at 65˚C for another 30 min. The resulting solution was centrifuged at 12000 rpm for 10 min. The supernatant was collected, spiked with 200 μL of chloroform, vortex-mixing for 30 sec, and then centrifuged at 11500 rpm for 10 min. The supernatant was collected, spiked with 2-fold CTAB precipitation solution, kept at room tem-

Table 1. Characteristics of Event176, Bt11, MON 810 and Liberty GM-Maize (modified from http://www.bats.ch.)

Tradename	Characteristic	Inserted gene		
		Promoter	Structure	Terminator
Event176 (Novartis)	ECB[a] (GA)[b]	1) P-PEPC[c] P-CDPK[d] 2) P-35S[e] 3) bacterial	1) two synthetic, truncated *cryIA(b)* [f] 2) *bar*[g] 3) *bla*[h]	1) I9[i], T-35S[j]
Bt11 (Novartis)	ECB GA	1) P-35S with IVS6-int. [k] 2) P-35S with IVS2-int. [l]	1) synthetic *cryIA(b)* 2) synthetic *bar*	1) nos 3' [m] 2) nos 3'
MON810 (Monsanto)	ECB	1) P-35S with *hsp70*-int.1[n]	1) synthetic *cryIA(b)*	1) nos 3'
Liberty (AgrEvo)	GA	1) P-35S 2) bacterial	1) synthetic *bar* 2) *bla* (incomplete)	1) T-35S 2) bacterial

[a] ECB: european corn borer (lepidopteran insect resistant).
[b] GA: phosphinothricin (glufosinate ammonium) herbicide tolerant, GA as selective trait.
[c] P-PEPC: green tissue-specific phosphoenolpyruvate carboxylase (PEPC) promoter from maize.
[d] P-CDPK: pollen-specific calcium-dependent protein kinase (CDPK) promoter from maize.
[e] P-35S: promoter from the cauliflower mosaic virus.
[f] *cryIA(b)*: delta-endotoxin from *Bacillus thuringiensis* subsp. *kurstaki.*
[g] *bar:* gene coding for a phosphinothricin acetyltransferase from *Streptomyces hygroscopicus.*
[h] *bla:* beta-lactamase gene; conveys resistance to beta-lactam antibiotics; from Tn3.
[i] I9: intron 9 from corn PEPC gene.
[j] T-35S: terminator of the 35S gene from the cauliflower mosaic virus.
[k] IVS6-int.: the No.6 intron sequence from maize alcolhol dehydrogenase 1 gene (*adh 1*-IS).
[l] IVS2-int.: the No.2 intron sequence from *adh 1*-IS.
[m] nos 3': terminator of nopaline synthase gene from *Agrobacterium tumefaciens.*
[n] *hsp70*-int. 1: the No.1 intron sequence from maize *hsp70* gene (heat-shock protein).

perature for 60 min, and centrifuged at 12000 rpm for 5 min. The supernatant was collected again and each of 350 μL of 1.2 M NaCl solution and chloroform were then added to the supernatant. The resulting solution was then vortex mixing for 30 sec and centrifuged at 12000 rpm for another 10 min. The supernatant was discarded and 0.6-fold isopropanol was then added to precipitate DNA. The resulting solution was centrifuged at 11500 rpm for 10 min and the supernatant was discarded. The precipitant was washed with 500 μL of 70% alcohol and centrifuged at 11500 rpm for 10 min. The supernatant was discarded and the DNA precipitant was then collected and dissolved in 100 μL of deionized water.

VII. *PCR Reactions and Product Analysis*

PCR reagent was prepared by mixing 21 μL of deionized water with 10-fold PCR buffer solution (5 μL) containing 1.5 mM Mg^{2+} (made by PROtech Technologies, Inc.), 8 μL of dNTP (200 μM) (made by PROtech Technologies, Inc.), 2 μL of DNA polymerase (2.5 unit/μL) (made by PROtech Technologies, Inc.), and 2 μL of each primer (100 μM). PCR reaction was performed by spiking 10 μL of DNA extracts into a centrifugation tube where the PCR reagent was then added. The DNA in centrifugation tube was incubated in a PCR thermocycler under the following program: 95˚C for 5 min followed by 95˚C for another 20 sec, 57˚C for 40 sec (57˚C for ivr primer; while 60˚C for CM01-CM02, IV01-cry, HS01-cry, and CM03-PA01 primers, and 63˚C for CDPK-cry primer), 72˚C for 1 min (in total, 40 cycles of above program was performed), and finally at 72˚C for another 3 min. The PCR products were analyzed using a 2% agarose gel electrophoresis.

VIII. *Test for the Commercial Immuno-assay Kit*

MON810 GM-maize with 0%, 0.15%, 0.5%, and 2% GM content were selected for this study. The operation procedure was according to the operation manual of immunoassay kit.

RESULTS AND DISCUSSION

There have been 18 types of GM-maize approved by the US government for commerce as of 2000[(1)]. In Europe, Event176 and Bt11 are the 2 major GM-maize on the market [(5)]. Japan has permitted the import of the following GM-maize: Event176, MON810, Liberty, and Bt11. Among them, MON810 ranked number one in sales market [(11)]. In Taiwan, however, the statistical import data on GM-maize is insufficient. The purpose of this study was to establish some analytical methods for GM-maize detection. Those established methods could be a referral for related authorities or companies, and could possibly be used as official methods in the future. Event176, MON810, Liberty, and Bt11 GM-maize were used as detection references in this study, and the PCR method was used to detect the characteristic genes of the above references. The feasibility of the PCR method on GM food detection was thus evaluated. The commercial available

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immuno-assay kit was also tested in this study and the results could be a reference for a fast detection of GM-maize. In addition, the established method was applied to the detection of the commercial products so as to understand the content of GM-maize in the commercial maize materials.

I. *Detection of 4 Types of GM-maize by PCR Method*

Four primers, CDPK-cry, HS01-cry, IV01-cry, and CM03-PA01, which are specific to the *cryIAb* inserted gene sequence in the 3 types of insect-resistant GM-maize and *pat* inserted gene sequence in herbicide-tolerant Liberty GM-maize, were selected for PCR analysis[11, 16, 17]. Among those selected primers, CDPK-cry covers the partial promoter and structure gene regions in the maize inserted *cryIA(b)* gene. The size of its PCR product is 211 bp[16]; HS01-cry primer covers the partial *hsp70* No.1 intron sequence and *cryIA(b)* structure gene regions in the maize *cryIA(b)* gene with PCR product size of 194 bp[11]; IV01-cry covers the partial alcohol dehydrogenase No.6 intron sequence and *cryIA(b)* structure gene regions in the maize inserted *cryIA(b)* gene with PCR product size of 437 bp [11]; and CM03-PA01 covers the partial promoter and structure gene regions in the inserted *pat* gene with PCR product 231 bp [11, 17]. Four different PCR products with the sizes of 211, 437, 194, and 231 bp were amplified from the product-specific genes of Event176, Bt11, MON810, and Liberty, respectively, as shown in Figure 1B. Thus, 4 types of GM-maize were successfully detected as using the 4 selected primers. It has been shown that the inserted gene region in all 4 types of GM-maize contains 35S-promoter (Table 1). The PCR product with size 220 bp was obtained as amplified by 35S-promoter primer confirming the presence of 35S-promoter in the 4 tested types of GM-maize (Figure 1A). An invertase gene primer, which is specific to maize gene [10], was used to test the maize reference. A PCR product with 226 bp was amplified from maize reference that confirms the maize product was detected (Figure 1C). In 1997, 22 out of 28 commercial GM-crops were found to contain 35S-promoter or NOS-terminator in their inserted genes. Theoretically, the same PCR product could appear when the primers for above 35S-promoter or NOS-terminator are used. Screening GMO products is thus achieved[8]. However, according to our study and studies from other laboratories[8, 11, 15], an unstable result constantly occurred using the 35S-promoter primer to perform a PCR analysis on Event176 GM-maize. The reason is unclear. On the basis of this result, the 35S-promoter primer was not used for the screening test. Product-specific primers were used instead, to confirm the types of GM-maize. Those primers cover the partial regulatory gene region (such as Event176 and Liberty) or intron sequence (such as Bt11 and MON810) and partial structure gene region allowing the partial regulatory gene, intron sequence, and partial structure gene regions in the inserted gene to be identified. The above product-specific primers are suitable for routine analysis. In addition, the ivr primer, which is usually used for the confirmation of a product-specific gene, has shown excellent detection ability

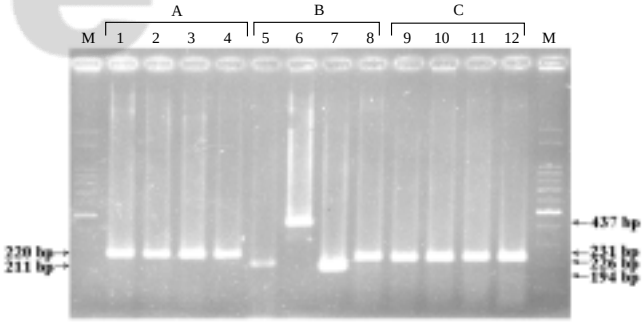

Figure 1. PCR produts amplified from 35S-promoter, product-specific gene and invertase gene regions of Event 176, Bt11, MON810 and Liberty GM-maize with primers CM01/02 (A), CDPK-cry03/04, IV01-CR01, HS01-CR01, CM03-PA01 (B) and ivrl-1/2 (C), respectively.
Lane M: 100 bp DNA ladder; lanes 1,5 & 9: Event176 GM-maize; lanes 2,6 & 10: Bt11 GM-maize; lanes 3, 7 & 11: MON810 GM-maize; lanes 4, 8 & 12: Liberty GM-maize; lanes 1-4: with primers CM01/02; lane 5: with primers CDPK-cry03/04; Lane 6: with primers IV01-CR01; Lane 7: with primers HS01-CR01; land 8: with primers CM03-PA01; lanes 9-12 with primers ivrl-1/2.

on maize-specific gene.

The detection limit of using PCR method was also studied. Results showed that a PCR product with 211 bp was generated from different contents (0.1, 0.5, 1, 2, and 5%) of Event 176 GM-maize reference, but no 211-bp product was found in the negative control sample as shown in Figure 2A. The detection limit of using CDPK-cry primer was 0.1%. This is the same result as reported by other researchers[15]. Since 0.01, 0.1, 1, and 5% GMO contents of 3 other types of GM-maize, which were used as references for detection limit study, are not commercially available, they were prepared by diluting the DNA extracts of 100% GM-maize with sterile water. The detection limits of using other 3 primers, IV01-cry, HS01-cry, and CM03-PA01 were 0.1, 0.01, and 1% (w/w), respectively (Figure 2A, B, and C). The results found in this study are in accordance with those reported data show-

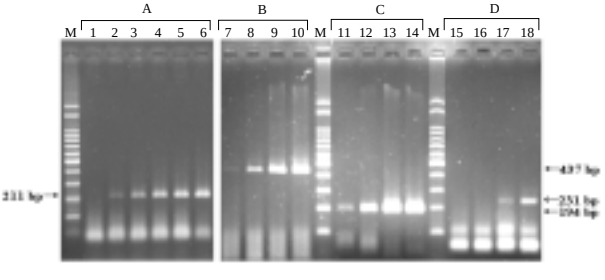

Figure 2. PCR products amplified from product-specific gene regions of Event176, Bt11, MON810 and Liberty GM-maize with primers CDPK-cry03/04 (A), IV01-CR01 (B), HS01-CR0 (C) and CM03-PA01 (D), respectively.
Lane M: 100 bp DNA ladder; lanes 1-6: 0%, 0.1%, 0.5%, 1%, 2% & 5% Event176 GM-maize, respectively, with primers CDPK-cry03/04; lanes 7-10: 0.01%, 0.1%, 1% & 5% Bt11 GM-maize, respectively, with primers IV01-CR01; lanes 11-14: 0.01%, 0.1%, 1% & 5% MON810 GM-mazie, respectively, with primers HS01-CR01; lanes 15-18: 0.01%, 0.1%, 1% & 5% Liberty GM-maize, respectively, with primers CM03-PA01.

ing that a diverging sensitivity could exist among different primers[19]. The PCR method developed in this study allows as low as 0.1~1% GM-maize to be detected indicating this method is sensitive enough to fit in with the GMO labeling regulation set by Europe (1%)[4] and Japan (5%)[20].

II. *Application of Commercial Immuno-assay Kits on the Detection of 4 GM-maize References*

Eighteen types of GM-maize are now commercially available[1]. The PCR method and the immuno-assay kits are widely used for identification of regular and GM-maize in most countries. The PCR method requires an enzyme reaction. Any factors, which can interfere with enzyme reactions, may affect PCR reaction. In addition, its operation procedure requires a DNA extraction prior to PCR reaction. This technique may be too difficult and complicated for most companies or laboratories to operate. The purpose of this study was not only to develop a GM food detection method but also to recommend and test the commercial immuno-assay kits to provide a rapid detection method for related authorities. So far, only 2 immuno-assay kits for GM-maize detection are commercially available: the Strip[14] and ELISA kits[7]. The former is used for identification; while the latter is used for quantification purposes. In this study, the Strip kit was selected because its operation procedure is easy and rapid. It took only 5~10 min to complete a test run and therefore is suitable for a routine inspection. The Strip kit contains an antibody, which is specific to Bt protein expressed by the *cryIA(b)* gene [14]. The GM-maize MON810, Bt11, and Event176 are all inserted with a *cryIA(b)* gene[1]. They should contain the Bt protein and show a positive reaction on Strip kit detection. However, based on the research information from the kit supplier, the quantity of expression Bt protein in the above 3 types of GM-maize varies. That could result in a different intensity on immuno-assay. The original design of Strip kit is for detection of the Bt protein expressed by inserted *cryIA(b)* gene in MON810 GM-maize[14]. Our study showed that MON810, Bt11, and Event176 GM-maize showed a positive reaction on the Strip immuno-assay; while Liberty GM-maize was negative. Liberty GM-maize was inserted with the *pat* herbicide-tolerant gene. Its negative reaction on the Strip kit was predicable. The specificity and detection limit are the 2 parameters that need to be considered as selecting the immuno-assay kits for GM-maize detection. Basically, the Strip kit shows an excellent specificity on Bt protein detection. To ensure its specificity on GM-maize, other crops including red beans, green beans, soybeans, and black beans were also tested in this study. A negative reaction was observed when the above crops were tested by using the Strip kit, indicating that the test crops were not mixed with GM-maize. The detection limit test was performed using the MON810 GM-maize with 0.15, 0.5, and 2% GM contents as references. Results showed that a weak positive reaction was observed in only 2% of the GM-maize. The detection limit of using the Strip kit was thus determined to be 2%.

III. *Inspection of Marketed Food Grade-maize*

The PCR and immuno-assay methods for detection of 4 types of GM-maize references have been established based on the results described above. For further understanding of whether the marketed food grade-maize was mixed with GM-maize and the feasibility of these established methods on commercial product detection, 14 popcorn and 6 corn middling pellet samples were collected from local markets and detected using the developed methods in this study. The Strip kit was at first used for the screening test. All 14 popcorn

Table 2. Primers used in this study

Primer	Sequence 5'-3'	Specificity	Amplicon (bp)	Reference
CDPK-cry 03	CTC TCG CCG TTC ATG TCC GT	CDPK-pro/sense		
CDPK-cry 04	GGT CAG GCT CAG GCT GAT GT	*cryIA(b)*/anti-sense	211	(16)
IV01	GGT ACA GTA CAC ACA CAT GTA T	*adh1*-1S/sense		
cry-CR01	GAT GTT TGG GTT GTT GTC CAT	*cryIA(b)*/anti-sense	437	(11)
HS01	AGT TTC CTT TTT GTT GCT CTC CT	*hsp70*/sense		
cry-CR01	GAT GTT TGG GTT GTT GTC CAT	*cryIA(b)*/anti-sense	194	(11)
CM03	CCT TCG CAA GAC CCT TCC TCT ATA	CaMV/sense		
PA01	AGA TCA TCA ATC CAC TCT TGT GGT G	*pat*/anti-sense	231	(11,17)
CM01	CAC TAC AAA TGC CAT CAT TGC GAT A	CaMV/sense		
CM02	CTT ATA TAG AGG AAG GGT CTT GCG A	CaMV/anti-sense	220	(18)
ivr1-1	CCG CTG TAT CAC AAG GGC TGG TAC C			
ivr1-2	GGA GCC CGT GTA GAG CAT GAC GAT C	Invertase	226	(10)

Table 3. Results of marketed food grade-maize containing GM-maize detected by Immuno-Kit and PCR methods

Sample	Total No.	Immuno-Kit	PCR			
		Strip	Event176	MON810	Bt11	Liberty
		+ / -	+ / -	+ / -	+ / -	+ / -
Popcorn	14	0 (14)	2 (12)	1 (13)	0 (14)	0 (14)
Corn middling pellets	6	6 (0)	6 (0)	6 (0)	5 (1)	6 (0)

+: means positive by Immuno-Kit or PCR method.

-: means negative by Immuno-Kit or PCR method.

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samples showed negative reactions; while all 6 corn middling pellet samples showed positive reactions (Table 3). To confirm if the collected samples contain GM-maize, the above samples were further tested using the primers, which are specific to the PCR products of 4 GM-maize, including Event176, MON810, Bt11, and Liberty. Two popcorn samples (No. 22 and No.24) were detected to contain Event176, and No.24 were further detected to contain MON810 as shown in Figure 3B and 4B. Five out of 6 corn middling pellet samples (No. 4, 5, 7, 59, and 65) were found to include 4 types of GM-maize (Event176, MON810, Bt11, and Liberty). While the another 1 (No. 14) sample contained 3 types of GM-maize (Event176, Liberty, and MON810) as shown in Figure 3A, 3B, 4A, and 4B. No.14 and No.65 samples, which were detected to have 3 and 4 types of GM-maize, respectively, were sampled from the same vendor with a different sampling time. The results of this test indicate that different batches of maize raw materials could possibly be mixed with different GM-maize and the mixture could contain up to 4 types of GM-maize. In the US, a Dent-corn product sold as food or feed is a mixture of GM-maize and regular maize (non GM-maize)[11].

All Bt11, Event176, and Liberty GM-maize contain an inserted *pat* herbicide-tolerant gene. However, the *pat* gene in Event176 functions as a selective trait of transplants[1]. Its expression of the herbicide-tolerant property is insufficient, but it shows 65% homology in gene sequence as compared to Liberty[11]. Furthermore, a Liberty product-specific primer (CM03-PA01) was used to test those commercial samples. Results showed that the PCR products with 522 bp and 231 bp were generated from those samples containing Bt11 and Liberty GM-maize as shown in Figure 4A. No Bt11 GM-maize was found in the No.14 sample. Therefore, a PCR product with 522 bp, a product that comes from Bt11 GM-maize, was not found as tested using CM03-PA01 primer (Figure 4A, Lane 4).

The test samples with positive reactions on PCR detection were further tested by using the ELISA kit. The quantification data showed that 8 test samples were tentatively determined to contain GM-maize as follows: No.4 (3.72%), No.5 (3.52%), No.7 (3.55%), No.14 (4.91%), No.22 (0%), No.24 (0%), No.59 (10.42%), and No.65 (9.31%). According to our personal communication with the producer, Carol, A. C., this ELISA kit is designed for detection of the *cryIA(b)* insect-resistant protein in MON810 GM-maize, which was therefore used as a calibration standard. The levels of specificity by using this kit to *cryIA(b)* proteins among MON810, Bt11, and Event176 vary. The ELISA response to Bt11 GM-maize is 3 times more than MON810 GM-maize; while Event176 GM-maize shows only a 5% response rate as compared to MON810 GM-maize. Differentiation of the above 3 types of GM-maize is not achievable by using the ELISA kit. Thus, quantification of GM-maize mixture in commercial products should be sum of the above 3 GM-maize. However, the response of the above 3 GM-maize is not corresponding to 1:1:1. Based on information that about the same ratio of the above 3 types of GM-maize was cultivated in the US in 1999, a factor of 1.25 was suggested to multiply when the data is generated by using the ELISA kit (personal communication with Carol, A. C.). However, in 2000, the cultivation ratio became MON810 (70%), Bt11 (15%), and Event176 (<10%). Event176 GM-maize, which overweighed other types of GM-maize, is now being gradually replaced by new types. Therefore, to update production status and meet market demand, developing a GM food detection method is required. The test samples No. 22 and 24 showed negative results on ELISA detection, but positive on PCR detection, suggesting the PCR method is superior to ELISA in detection limits.

The results of this study demonstrated that using the PCR method is capable of differentiating 4 types of GM-maize from non-GM products; while the Strip kit can only be used for screening purposes. All samples, with positive or negative results as tested by the Strip kit, need to be further confirmed by the PCR method. The commercially available ELISA kit, which is not capable of differentiating the *cryIA(b)* proteins among 3 tested types of GM-maize, is not suggested to detect the maize products containing different

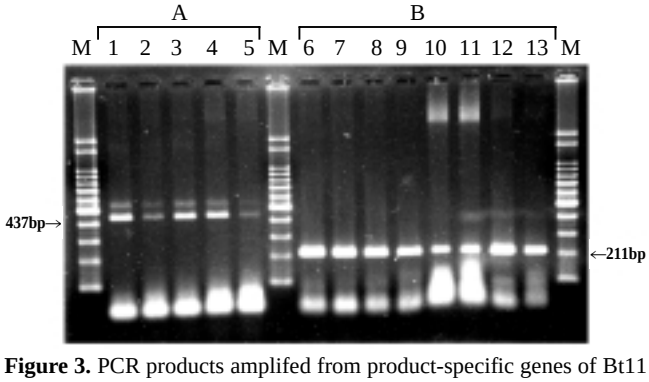

Figure 3. PCR products amplifed from product-specific genes of Bt11 and Event176 GM-maize with primers IV01-CR01 (A) and CDPK-cry03/04 (B), respectively.
Lane M: 100 bp DNA ladder; lanes 1-5: corn middling pellets (sample No. 4, No. 5, No. 7, No. 59, No. 65); lanes 6-9 and lanes 12-13: corn middling pellets (sample No. 4, No. 5, No. 7, No. 14, No. 59, No. 65); lanes 10-11: ppopcorn (sample No. 22 & No. 24).

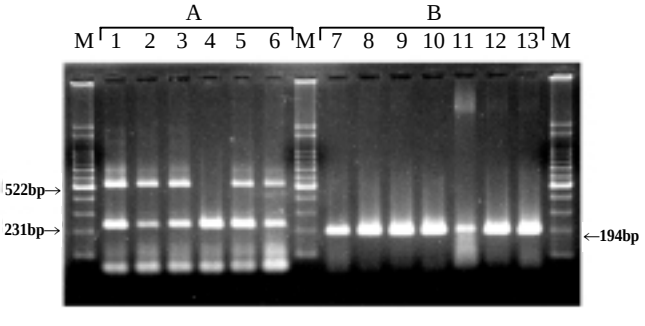

Figure 4. PCR products amplified from product-specific genes of Liberty and MON810 GM-maize with primers CM03-PA01 (A) and HS01-CR01 (B), respecitvely.
Lane M: 100 bp DNA ladder; lanes 1-6: corn middling pellets (sample No. 4, No. 5, No. 7, No. 14, No. 59, No. 65); lanes 7-10 and lanes 12-13: corn middling pellets (Sample No. 4, No. 5, No. 7, No. 14, No. 59, No. 65); lane 11: popcorn (sample No. 24).

GM-maize mixture. The results of this study also reveal that commercial maize products are usually mixed with different types of GM-maize. The future work in our laboratory will focus on the method development for quantification and detection of low-processed GM food.

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以聚合酵素鏈鎖反應（PCR）方法及市售免疫套組檢測四種基因改造玉米

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摘　要

本研究以PCR方法及市售免疫套組探討鑑別檢測四種基因改造玉米。針對Event 176 (Novartis公司)、Bt11 (Novartis公司)、MON810 (Monsanto公司)及Liberty (AgrEvo公司)四種GM-玉米之殖入基因分別選定具產品特異性引子，進行PCR方法檢測。用以鑑別GM-玉米之產品特異性引子共四對，分別為CDPK-cry (Event176 GM-玉米)、IV01-cry (Bt11 GM-玉米)、HS01-cry (MON810 GM-玉米)及CM03-PA01 (Liberty GM-玉米)。結果顯示，GM-玉米參考品以HS01-cry引子檢測時，其最低檢測量為0.01% (w/w)，CDPK-cry及IV01-cry引子均為0.1% (w/w)，CM03-PA01引子則為1% (w/w)。GM-玉米參考品分別再以市售免疫套組測試，Event176、Bt11及MON810三種GM-玉米皆呈現陽性反應。為瞭解食品玉米原料含GM-玉米之狀況，首先以市售免疫套組方法測試食品玉米原料等檢體共二十件，結果發現供作爆米花之玉米粒檢體十四件皆呈陰性反應，玉米碎檢體六件則為陽性反應；進一步再以PCR方法測試該二十件檢體，發現供作爆米花之玉米粒檢體可檢出二種GM-玉米品種，而玉米碎同件檢體，則可檢出四種GM-玉米品種或三種GM-玉米品種。綜合上述，本報告所使用之PCR方法能區分一般與四種基因改造玉米，市售免疫套組僅能供作檢體篩選用途，套組呈陽性或陰性反應之檢體，皆建議再配合PCR方法以確認與鑑別GM-玉米品種。同時，本研究得知玉米原料含有混合之不同品種GM-玉米。

關鍵詞：聚合酵素鏈鎖反應(PCR)，免疫套組，基因改造玉米