Journal of Food and Drug Analysis, Vol. 10, No. 1, 2002, Pages 25-33 藥物食品分析 第十卷 第一期

Study on the Detection Method of Six Varieties of Genetically Modified Maize and Processed Foods

LIH-CHING CHIUEH*, YEN-LING CHEN AND DANIEL YANG-CHIH SHIH

National Laboratories of Foods and Drugs, Department of Health, Executive Yuan, 161-2, Kuen Yang Street, Nankang 115, Taipei, Taiwan, R.O.C.

(Received: May 2, 2001; Accepted: September 27 , 2001)

ABSTRACT

To detect and identify six varieties of genetically modified (GM) maize, polymerase chain reaction (PCR) assays were performed in the present study. The effect of heat treatment on the detection of the genes by the PCR methods was investigated. Maize samples were subjected to 100˚C and 121˚C heat treatment for different time intervals, and PCR tests were then conducted by using primers specific for the maize *zein* gene. Results revealed that the *zein* gene still could be detected by the PCR after heating at 100˚C for 120 min and heating at 121˚C for 30 min. In addition, the establishment of PCR assays on the identification of the six varieties of GM-maize was also evaluated. Primers specific for the inserted genes in the Event 176 (Novartis), Bt11 (Novartis), MON810 (Monsanto), T25 (AgrEvo), CBH-351 (AgrEvo), and GA21 (Monsanto) GM-maize were used to conduct the PCR assays. The detection limits for these GM-maize crops is less than 0.1% (w/w). For further understanding on whether commercially available maize materials and processed foods are mixed with GM-maize, corn middling pellets, canned whole kernel maize, frozen whole kernel maize, corn tortilla and potato chips samples were collected from the markets, and detected by the PCR methods developed in this study. Results showed that five to six varieties of GM-maize were detected in the corn middling pellets and corn tortilla, whereas GM-maize of the MON810 variety as well as the T25 variety were detected in canned and frozen whole kernel maize samples. Potato chips only contained GM-maize of the MON810 variety. The results of this study demonstrate that the PCR analysis is suitable for detecting maize materials and products, and that commercially available maize materials and products in Taiwan are usually mixed with different varieties of GM-maize. Meanwhile, the major product of GM-maize is the MON810 variety.

Key words: PCR, GM-maize

INTRODUCTION

In recent years, the techniques of genetic modification have made significant advancements. A total of 18 varieties of genetic modified (GM) maize have already been registered with the 2000 OECD (Organization for Economic Cooperation and Development)[1]. The major traits of GM-maize are insect resistance and herbicide tolerance[1,2,3]. Research continues for improving the following characteristics: (1) adaptability to harsh environmental factors such as drought and/or high salt-, high heavy metal-containing soil; (2) alternations of compositions such as enhancement of specific amino acids (e.g., lysine, methionine, tryptophan) and changing the protein and fatty acid compositions[3]. In 2000, the United States and China were ranked as the top two leading countries in maize production, representing 43% and 18%, respectively, of the global maize production[1]. The United States and Canada, on the other hand, were the major countries for GM-maize production. According to 2000 statistics, the US and Canada produced 7.95 and 0.5 million hectares of GM-maize, respectively, accounting for 25% and 50% of their total maize production[3]. Of the GM-maize cultivated by the US in 2000, 72% was insect-resistant, 24% was herbicide-tolerant, and 4% carried both characteristics[3]. In Asia, China started to cultivate insect-resistant GM-maize during the same year[3]. Statistical data from Taiwan's Council of Agriculture shows that approximately 600 tons of maize are imported from the US to Taiwan annually, of which about 30% is GM-maize[4].

Currently, the insect-resistant traits of GM-maize were achieved by insertion of toxic protein genes of the Bt-series. Based on the genes inserted, the commercialized insect-resistant GM-maize can be categorized into two major types[1], namely, YieldGard (Cry1A) and StarLink (Cry9C). Those belong to Cry1A type include Bt11, Event176, and MON810, which were inserted with Cry1Ab insect-resistant gene. Additionally, DBT418 of the Cry1A type was inserted with Cry1Ac insect-resistant gene, different from the other GM-maize of the category. CBH-351 is by far the only Cry9C type, which differs from the Cry1A type at their toxic protein binding sites. CBH-351 was invented by the European Aventis Company which has both the insect-resistant and herbicide-tolerate traits. This variety of GM-maize was cultivated in the US during 1998-2000. In 2000, there was approximately 13 hectares of farmland cultivating the CBH-351 GM-maize. However, according to Aventis, only about 0.5% of the US maizeland cultivated is of the CBH-351 variety[5]. The most controversial point about this particular variety is that the toxic protein expressed by the inserted insect-resistant gene *cry9C* may result in food allergy to humans. Other commercialized herbicide-tolerate GM-

* Author for correspondence. Tel:02-26531273; Fax:02-26531268; E-mail:clc1025@nlfd.gov.tw

maize varieties are classified into two types[1], i.e. glyphosate-tolerate and glufosinate ammonium-tolerate based on the inserted herbicide-tolerate genes. Examples are the GA21 and NK603 varieties for the former, and the Bt11, Event176, CBH-351 and T25 varieties for the latter.

The methodologies for the identification of GM foods can be divided into 3 categories[2]. Among those, the polymerase chain reaction (PCR) is the most widely applied method, which can be used for both qualitative and quantitative analyses[6]. The PCR method for GM-maize detection has been utilized by European and Japanese scientists for qualitative analysis of the Event176, Bt11, MON810, T25, GA21, and CBH-351 varieties[7-11]. Commercially available GM-maize detection kits of the PCR, Strip, or ELISA methods have even more diversity and are from various areas of the world, including manufacturers from American, European, and Asian countries[12-16]. Many countries have developed standard methods for the detection of GM foods in order to comply with labeling regulations. Germany, for instance, has published methods for the detection of the Event176, MON810, Bt11, and T25 GM-maize. Japan has also developed PCR-based methods for GM-maize of the Event176, MON810, Bt11, CBH-351, GA21, and T25 varieties[10-12]. Further, to meet the needs of the crop market, the US GIPSA (Grain Inspection, Packers and Stockyards Administration) set up a reference laboratory in 2000 to evaluate GMO detection methods and to certify laboratories with detection capabilities[17].

To date, literature published in Taiwan regarding the detection of GM-maize and the maize-containing raw materials of the Event176, MON810, Bt11, and T25 varieties all employed PCR as the primary methods[18]. As to the detection of other GM-maize varieties, or to the investigation of GM contents of processed food mixed with GM-maize, there are still gaps for further studies. In Taiwan, the food labeling requirements for GM food were promulgated in February 2001, which include voluntary and mandatory reporting. The voluntary part of the requirements has been implemented since 2001, and the mandatory part of the requirements is to be implemented from 2003 in 3 stages over a period of 3 years depending on the extent of processing of the products. Those which require labeling are materials and products of soybean and maize that contain more than 5% (wt) of GMO. As a consequence, the present study emphasized the establishment and revision of the PCR detection methods for 6 GM-maize varieties, and also the evaluation of using commercially available immuno-assay kits for the detection of the CBH-351 GM-maize. In addition, the effects of heat treatment on the PCR detection of GM-maize were also investigated in the present study. Finally, the established methods were applied in the detection of market available maize materials and products, in order to preliminarily demonstrate the picture of GM distribution in such foods in Taiwan. Meanwhile, the methods assessed in the present study would become the fundamentals for the establishment of routine tests of GMO and to be referenced by the food labeling requirements in Taiwan.

MATERIALS AND METHODS

I. Chemicals

Chloroform, isopropanol, and tris (hydroxymethyl) aminomethane (Tris-base) were purchased from the Merck Co. (Darmstadt, Germany). Hexadecyltrimethyl-ammonium bromide (CTAB) was obtained from the Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Agarose was the product of Amresco (Solon, Ohio, U.S.A.).

II. *GM-Maize References*

The following 6 types of GM-maize were employed in the present study as the references: Event176 (Novartis, Greensboro, NC, U.S.A.), Bt11 (Novartis, Greensboro, NC, U.S.A.), MON810 (Monsanto, St. Louis, MO, U.S.A.), T25 (LibertyLink) (Aventis, AgrEvo, Berlin, Germany), GA21 (Monsanto, St. Louis, MO, U.S.A.), and CBH-351(Aventis, AgrEvo, Berlin, Germany). The characteristics are listed in Table 1.

III. *Commercial DNA Extraction and Purification Kits*

Wizard Minipreps DNA Purification Resin (Promega, Madison, WI, U.S.A.), DNeasy Plant Mini Kit, and DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany) were used for the extraction and purification of DNA.

IV. *Equipments*

PCR thermal controller model PTC-100 characterized by a programmable thermal controller was the product of MJ Research (Water Town, Mass., U.S.A.).

V. *PCR Primers and Reagents*

Eight pairs of PCR primers were employed in the present study as follows: the primers CDPK-cry, CM03-PA01, and CM03-CBH02, respectively, were for the detection of the regulatory sequence-structure genes of *cryIA(b)*, *pat* and *cry9C* located within the Event176, T25, and CBH-351 GM-maize. The primers Bt11/1-5'-cryIA/1-3', HS01-cry, and GA21/1-5'-GA21/1-3', respectively, were for the detection of structure genes of the alcohol dehydrogenase No.6 intron sequence-*cryIA(b)*, *hsp70* No.1 intron sequence-*cryIA(b)*, and maize optimized transit peptide sequence-*m-epsps* located within the Bt11, MON810, and GA21 GM-maize. The primer T25/1-5'-T25/1-3' was for the detection of the *pat* structure-terminal sequence gene located within the T25 GM-maize. The primer ZE was for the detection of the *zein* gene of maize. These primers were synthesized by TIB Molbiol (Berlin, Germany). The DNA Polymerase kit was purchased from PROtech Technologies, Inc. Detailed information regarding these primers are listed in Table 2.

VI. *Commercial Immuno-assay Kits*

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Table 1. Characteristics of Event176, Bt11, MON 810, T25, GA21 and CBH-351 GM-Maize (modified from http://www.bats.ch. and http://www.agbios.com)

Tradename	Characteristic	Inserted gene		
		Promoter	Structure	Terminator
Event176 (Novartis)	ECB[a] (GA)[b]	1) P-PEPC[c] P-CDPK[d] 2) P-35S[e]	1) two synthetic, truncated *cryIA(b)*[f] 2) *bar*[g]	1) I9[h], T-35S[i]
Bt11 (Novartis)	ECB GA	1) P-35S with IVS6-int.[j] 2) P-35S with IVS2-int.[k]	1) synthetic *cryIA(b)* 2) synthetic *bar*	1) nos 3'[l] 2) nos 3'
MON810 (Monsanto)	ECB	1) P-35S with *hsp*70-int.I[m]	1) synthetic *cryIA(b)*	1) nos 3'
T25 (AgrEvo)	GA	1) P-35S	1) synthetic *bar*	1) T-35S
GA21 (Monsanto)	Gly[n]	1) Pr-act with OTP [o]	1) *m-epsps*[p]	1) nos 3'
CBH-351 (AgrEvo)	ECB GA	1) P-35S 2) P-35S	1) truncated *cry9C*[q] 2) *bar*	1) 35S poly (A) signal[r]

[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] I9: intron 9 from corn PEPC gene.
[i] T-35S: terminator of the 35S gene from the cauliflower mosaic virus.
[j] IVS6-int.: the No. 6 intron sequence from maize alcohol dehydrogenase 1 gene (*adh 1*-IS).
[k] IVS2-int.: the No. 2 intron sequence from *adh 1*-I.
[l] nos 3': terminator of nopaline synthase gene from *Agrobacterium tumefaciens.*
[m] *hsp70*-int. 1: the No. 1 intron sequence from maize *hsp70* gene (heat-shock protein).
[n] Gly: glyphosate herbicide tolerant.
[o] Pr-act 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 peptide sequence of the N-terminal upstream region of ribulose-1,5- bisphosphate carboxy-lase derived from maize and sunflower.
[p] *m-epsps*: point mutated epsps gene derived from maize.
[q] *cry9C*: delta-endotoxin from *Bacillus thuringiensis* subsp. *tolworthi* BTS02618A.
[r] 35S poly (A) signal: 35S poly (A) signal from the cauliflower mosaic virus.

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	(19)
HS01-cry	AGT TTC CTT TTT GTT GCT CTC CT	*hsp70*/sense		
CR01	GAT GTT TGG GTT GTT GTC CAT	*cryIA(b)*/anti-sense	194	(10)
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	(10)
T25 1-5'	GCC AGT TAG GCC AGT TAC CCA	*pat*/sense		
T25 1-3'	TGA GCG AAA CCC TAT AAG AAC CCT	35S terminator/anti-sense	149	(11)
Bt11 1-5'	CCA TTT TTC AGC TAG GAA GTT C	*adh1*-1S IVS6/sense		
cryIA 1-3'	TCG TTG ATG TTK GGG TTG TTG TCC	*cryIA(b)*/anti-sense	110	(11)
GA21 1-5'	ACG GTG GAA GAG TTC AAT GTA TG	OTP/sense		
GA21 1-3'	TCT CCT TGA TGG GCT GCA	*m-epsps*/anti-sense	270	(11)
CM03	CCT TCG CAA GAC CCT TCC TCT ATA	CaMV/sense		
CBH02	GTA GCT GTC GGT GTA GTC CTC GT	*cry9C*/anti-sense	170	(12)
ZE01	TGC TTG CAT TGT TCG CTC TCC TAG	*Ze 1*/sense		
ZE02	GTC GCA GTG ACA TTG TGG CAT	*Ze 1*/anti-sense	329	(20)

Trait Bt9 Corn Grain Test Kit (Strip) and Bt9 Maize Test Kits (ELISA) were made by Strategic Diagnostics Inc. (Newark, U.S.A.).

VII. *Pretreatment of Maize*

Ten grams of fresh sweet maize produced locally in

Taiwan were measured and 100 mL of distilled water was added. The samples were heated to 100˚C for 0, 3, 10, 20, 30, 60, or 120 min, or to 121˚C for 0, 10, 15, 20, 30, or 60 min. The heat-treated samples were drained of excess humidity and placed in oven set at 55˚C until completely dry. The dried samples were then ground to powder for analysis.

VIII. *DNA Preparation and Purification*

The CTAB method published by Lipp *et al*. in 1999[7] was adapted for the preparation and purification of DNA as follows: 25 mg of the heat-treated sample were measured and 100 μL of deionized water was added. The mixture was kept at 65˚C for an hour followed by the addition of 500 μL of CTAB buffer (CTAB 20 g/L, NaCl 1.4 M, Tris-base/HCl 0.1 M and Na_2-EDTA 20 mM ， pH 8.0) and allowing for reaction at 65˚C for 30 min, then centrifuged at 16000 x g for 10 min. The supernatant was collected, 200 μL of chloroform was added, and vortexed for 30 sec and then centrifuged at 12000 ×g for 10 min. After collecting the supernatant and adding 2-fold of CTAB precipitation solution (CTAB 5 g/L, NaCl 0.04 M), the mixture was kept at room temperature for 60 min, and centrifuged at 14500 ×g for 10 min. The resulting supernatant was removed. Three hundred fifty μL of each NaCl (1.2 M) and chloroform were added, vortexed for 30 sec, and centrifuged at 12000 ×g for 10 min. The supernatant was again collected, 0.6-fold isopropanol was added, and allowed to stand for 30 min to participate DNA. The solution was centrifuged at 15000 ×g for 30 min, and the supernatant was discarded. The precipitant was washed with 500 μL of alcohol (70%, v/v) and centrifuged at 15000 ×g for 10 min. The resulting supernatant was removed and the precipitated DNA was washed with 100 μL of deionized water.

The extraction and purification of DNA were also performed by commercially available kits: Wizard Minipreps DNA Purification Resin (Promega), DNeasy Plant Mini Kit, or DNeasy Plant Maxi Kit (Qiagen). The operation procedures followed the user's manuals provided by the manufacturers.

IX. *PCR and Product Analysis*

The PCR reagents for each reaction include 21 μL of deionized water, 5 μL of 10-fold PCR buffer consisting of 1.5 mM Mg^{2+} (PROtech Technologies, Inc.), 8 μL of dNTP (200 μM) (PROtech Technologies, Inc.), and 2 μL of DNA polymerase (2.5 unit/μL) (PROtech Technologies, Inc.), and 2 μL of each primer (100 μM). The PCR was carried out by aliquoting DNA 10 μL (approximately 100 ng) into a centrifugation tube where the PCR reagents were placed. The DNA and reaction reagent mixture were then incubated in a PCR thermocycler under the following programs. For HS01-cry, CM03-PA01, ZE01-ZE02, Bt11/1-5'-cryIA/1-3', and T25/1-5'-T25/1-3': initial denaturation at 95˚C for 3 min, followed by 40 cycles of amplification, each consisting of 95˚C for 1 min, 60˚C for 1 min, and 72˚C for 1 min. The synthesis was completed at 72˚C for 7 min. For GA21/1-5'-GA21/1-3': initial denaturation at 95˚C for 3 min, followed by 40 cycles of amplification at 95˚C for 1 min, 57˚C for 1 min, and 72˚C for 1 min. The reaction was concluded at 72˚C for 7 min. For CDPK-cry: initial denaturation at 95˚C for 12 min, followed by 40 cycles of amplification at 95˚C for 30 sec, 63˚C for 30 sec, and 72˚C for 30 sec. The reaction was ended by reacting at 72˚C for 10 min. The assay condition for the primer set CM03-CBH02 was initially denatured at 95˚C for 10 min, then subjected to 40 cycles of amplification, each comprised of 95˚C for 30 sec, 60˚C for 30 sec, and 72˚Cfor 30 sec. The reaction was terminated at 72˚C for 7 min. The PCR products were electrophoresed through 2.0% agarose gel to detect the existence of the targeted DNA bands.

X. *Test of the Commercial Immuno-Assay Kits*

Maize powder containing 0%, 0.01%, 0.075%, and 0.2% (w/w) of CBH-351 genetically modified maize was employed as the standard for the quantitative ELISA kits. The operation manual provided by the manufacturer was followed for the qualitative and quantitative tests.

RESULTS AND DISCUSSION

A total of 18 varieties of GM-maize have been registered with the 2000 Organization for Economic Cooperation and Development (OECD)[1]. The European countries permit the import of GM-maize of the Event176, MON809, MON810, T25, and Bt11 varieties[21]. In March 2001, the Japan Ministry of Health and Welfare published a safety assessment for 9 varieties of GM-maize, including the Event176, MON810, T25, T14, DBT418, GA21, NK603, Bt11 Sweet Corn, and Bt11. Among those, the MON810 is the primary maize variety in the sales market[13]. In Taiwan, the statistical import data on GM-maize has not been readily available, nor has the foreign developed GM-maize filed registration to Taiwanese government claiming to be GMO. Nevertheless, the ROC Department of Health (Taiwan) promulgated food labeling requirements for GMO in February 2001. The CBH-351 variety is only allowed to be use as feed-grade material in the US; however, incidents of contamination of feed-grade GM-maize of CBH-351 to food materials have been reported in the US and Japan. As a result, the objective of the present study was to establish detection methods for GM-maize of CBH-351 and other 5 varieties in order to provide relevant information and to set up detection methods that can be recommended to related authorities and industries. The present study utilized 6 GM-maize varieties as the detection references and employed the PCR method to detect the product-specific genes of each GM-maize to establish and investigate the feasibility of applying of the PCR methods for the identification of GMO. In the meantime, the present study also assessed commercially available immunoassay kits for the CBH-351 GM-maize, and investigated the effects of heating treatment on the PCR detection. Finally, the established methods were applied in the detection of mar-

ket available maize raw materials and products, in order to preliminarily demonstrate the picture of GM distribution in such foods in Taiwan.

I. *Effects of Heat Treatment on PCR Detection*

Each method for the detection of GMO has its own specificity and limitations. Generally speaking, there is little concern for the detection of GMO in raw foods. However, protein in processed foods has undergone denaturation so that immunological or enzymatic assays may not be appropriate for the detection of GM content[2]. PCR is the most popular method in GM detection in the aspects of applicability and universality. Nevertheless, the methods based on PCR are not suitable for the detection of highly processed foods because the DNA might be already fragmented to pieces smaller beyond what can be effectively detected by the PCR method. The present study investigated the effects of different heat treatments on the PCR detection. Fresh maize samples were heated to 100˚C for 0, 3, 10, 20, 30, 60, or 120 min, or to 121˚C for 0, 10, 15, 20, 30, or 60 min. After the heat treatments, the samples were removed of extra humidity and placed into a oven of 55˚C for dehydration. The DNA was then extracted and detected by the PCR method. The primers used were designed according to the *zein* gene of the maize, and the amplification products were in the size of 329 bp. As shown in Figure 1, after heating to 100˚C for 60 min, the quantity of PCR amplification product did not change; however, when the heating duration was extended to 120 min, the quantity of PCR amplification product significantly decreased, although it is still detectable. For groups that were treated to 121˚C, the quantity of PCR amplification products reduced to half of the control (group that was not subjected to 121˚C heat treatment) in the group that was only treated for 10 min. In fact, the quantities of PCR amplification products detectable decreased as the heating duration increased. When the samples were heated to 121˚C for 60 min, there ware no longer any detectable PCR products. These results suggest that the maize DNA in these samples were fragmented to pieces smaller than 329 bp by the heat treatment and as a consequence, can not be detected by the PCR method. Similar results were obtained by German researchers Hupfer *et al*. who heated the maize samples to 100˚C and amplified the products with two sets of primers of the sizes of 1914 and 211 bp. They found that in only 30 min, those samples amplified with the larger primer could not be detected, whereas those amplified with the smaller primer could still be detected even though the heating duration was extended to 105 min[22]. It was recommended that the primer used in the PCR detection of GM foods to yield amplification products in the range of 150 to 300 bp[2]. The PCR primers employed by most of the published literature for quantitative purposes produced amplification products smaller than 150 bp[23]. This can be again extrapolated from the present study. Because the amplification products of primers used in the present study are all smaller than 270 bp, these primers are not only good for the detection of the 6 varieties of GM-maize references, but also suitable for the detection of market available maize materials and processed products.

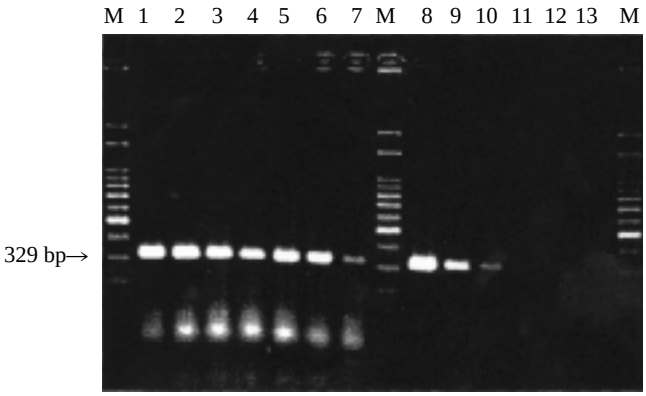

Figure 1. Effect of heat treatment on the detection of the maize-*zein* gene regions by PCR method with primers ZE01/ZE02.
Lane M: 100 bp DNA ladder; lane 1-lane 7: heat treatment with 100˚C, 0 min, 3 min, 10 min, 20 min, 30 min, 60 min, 120 min, respectively; lane 8-lane 13: heat treatment with 121˚C, 0 min, 10 min, 15 min, 20 min, 30 min, 60 min, respectively.

II. *Applications of Commercial Immono-assay Kits on the Qualitative and Quantitative Detection of CBH-351 GM-maize*

There are 18 varieties of GM-maize that have been officially registered thus far[1]. The PCR method and the immuno-assay kits are widely utilized for the differentiation of conventional and GM-maize. Because the PCR method requires enzyme reactions, any factor that would interfere with the enzymatic reactions may in turn affect the PCR reactions. In addition, its operation procedure requires a DNA extraction prior to the PCR detection, which is a complex technique that is not easily acquired by regular industrial laboratories. The purpose of the present study was not only to develop a method for GM food detection, but also to test and recommend some user-friendly commercial immuno-assay kits to provide the related authorities means of rapid screening of GMO. There are at least 4 brands of commercial immuno-assay kits for the detection of CBH-351 GM-maize. Strip, for example, is for the purpose of qualitative identification[24], and ELISA, on the other hand, is for quantitative analysis[25]. In the present study, the Strip kit was selected because of its easy operation procedure and rapid completion time. It takes only 5-10 min to complete a test run and therefore it is a strong candidate for screening and/or quarantine purposes. The Strip immuno-assay kit contain an antibody which is specifically developed for the Bt Cry9C protein expressed by the *cry9C* gene[24]. Based on the inserted insect-resistant genes, the current commercially available insect-resistant GM-maize can be categorized into two series, YieldGard (Cry1A) and StarLink (Cry9C), which differ by the binding sites of the insecticidal proteins[5]. According to information released by the US manufacture Strategic Diagnostics Inc., there are 3 commercial kits currently available for the detection of GM-maize of the Bt1, Bt9C, and

pesticide-tolerate Liberty varieties. Theoretically the Bt1 kit can be used for the detection of GM-maize of the MON810, Bt11, and Event176 varieties. The Bt9C kit is only responsive to the CBH-351 variety. It was found in the present study that the Bt9C kit is only specific to the Bt9C protein-containing CBH-351 variety based on the Strip testing. The specificity and detection limits are the two parameters that need to be considered as selecting immuno-assay kits for the detection of GM-maize. Tests to the detection limit were performed using the CBH-351 GM-maize references with various GM concentrations (0.01, 0.075, and 0.2%). All were tested positive with the limitation of detection 0.01%. Quantitative detection was performed by ELISA kits, also manufactured by the Strategic Diagnostics Inc. on CBH-351 maize with various GM concentrations (0%, 0.01%, 0.075%, and 0.2%). The operation procedure followed the user's manual provided by the manufacture and the obtained correction coefficient of the standard curve was 0.95. Taken together, the tested commercial kits are suitable for the screening and quantitation of maize materials.

III. *Detection of 6 Varieties of GM-maize References by the PCR Method*

Our laboratories set up the PCR method for the detection of 4 GM-maize references[18]. The aim of the present study was to find the proper primers for the Bt11 and T25 varieties, and to develop detection methods specific for GM-maize of the GA21 and CBH-351 varieties. The detection methods for GM-maize of the Event176 and MON810 varieties by the PCR technique, including the designs of product-specific primers and test conditions adapted from Hupfer[19] and Matsuoka[10], have been verified and employed by our laboratories[18]. The primer used by our laboratories for the amplification of the Bt11 GM-maize yielded product of 437 bp, is not suitable for detecting processed products,.thus, another primer with product specificity, namely, Bt11/1-5'-cryIA/1-3', was chosen for the present study[11]. This specially designed primer covers portions of the alcohol dehydrogenase No. 6 intron sequence and the structural gene regions of the insect-resistant *cryIA(b)* gene sequence inserted in Bt11 GM-maize whose PCR amplification product is in the size of 110 bp. Additionally, the GM-maize of the Bt11, Event176, and T25 varieties are all inserted with the herbicide-tolerate *pat* gene sequence. Among these, the *pat* gene sequence in the Event176 is only for selective traits[1] and the expressed gene products are not sufficient to withstand herbicide. However, its gene sequence has 65% homology with the *pat* gene sequence of the T25 GM-maize[10]. On the other hand, when tested with the T25 product-specific primer CM03-PA01, the sizes of the PCR amplification products of the Bt11 and T25 GM-maize were 522 bp and 231 bp, respectively[10]. As a result, primer T25/1-5' — T25/1-3', which partially covers the structural gene sequence and the terminal gene sequence of the *pat* inserted herbicide-tolerate gene, was selected to run an additional test for the T25 GM-maize variety, and the PCR amplification product was 149 bp[11]. When this primer was used, only the T25 variety could be detected with the PCR product, due to the different *pat* terminal sequences in the Bt11 (nos 3') and in the T25 (T-35S) varieties (Table 1). The primer selected for the glyphosate-tolerate GA21 GM-maize was GA21/1-5'-GA21/1-3' which partially covers the optimized transit peptide sequence region and the *m-epsps* structural gene region of the inserted *m-epsps* glyphosate-tolerate gene sequence with the PCR amplification product of 270 bp[11]. The primer selected for the CBH-351 variety was CM03-CBH02 which is specific for the regulatory sequence-structure gene region with the PCR amplification product of 170 bp[12].

The PCR amplification products of the 6 GM-maize references obtained by the individual product-specific primers were as follows: 211 bp for the Event176, 110 bp for the Bt11, 194 bp for the MON810, 149 bp for the T25, 270 bp for the GA21, and 170 bp for the CBH-351 varieties (Figure 2). Hence, the 6 selected primers successfully detected the 6 varieties of GM-maize references. These product-specific primers cover part of the regulatory gene region (e.g. Event176 and CBH-351), terminal sequence (e.g. T25), intron sequence (Bt11 and MON810), optimized transit peptide (OTP) sequence (e.g. GA21), or partial of the structural gene region allowing the regulatory, termination, intron, OTP, or structural gene sequences in the inserted gene to be identified. The above primers are thereby suitable for routine analysis of GM-maize. The detection limit tests were performed by extracting the DNA of the GM-maize followed by detecting on the diluted DNA. The results were found to be 0.01% for the MON810 and CBH-351 varieties, and 0.1% for the T25, Bt11, Event176, and GA21 varieties.

IV. *Inspection of Marketed Food Grade-Maize*

The PCR and immuno-assay methods for the detection

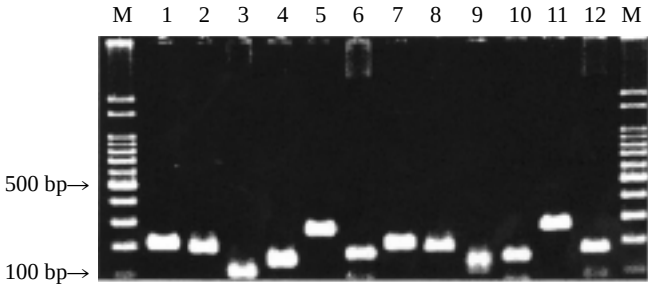

Figure 2. PCR products amplified from product-specific gene regions of six lines of GM-maize with primers cry03/04 (Event 76), HS01/CR01 (MON810), Bt11/1-5'-cryIA/1-3' (Bt11), T25/1-5'-T25/1-3' (T25), GA21/1-5'-GA21/1-3' (GA21) and CaM03/CBH02 (CBH-351), respectively.
Lane M: 100 bp DNA ladder; lane 1: Event176 GM-maize; lane 2: MON810 GM-maize; lane 3: Bt11 GM-maize; lane 4: T25 GM-maize; lane 5: GA21 GM-maize; lane 6: CBH-351 GM-maize; lane 7-lane 12: corn middling pellets (sample No. 96) tested with primers cry03/04 (Event176); HS01/CR01 (MON810): Bt11/1-5'-cryIA/1-3' (Bt11); T25/1-5'-T25/1-3' (T25); GA21/1-5'-GA21/1-3' (GA21) and CaM03/CBH02 (CBH-351), respectively.

of the CBH-351 GM-maize, and the PCR method for the detection of the 6 GM-maize references were established based on the aforementioned results. For further understanding of whether the marketed food grade-maize contained GM-maize and the feasibility of these developed methods on the detection of commercial products, 14 popcorn and 30 corn middling pellet samples were collected from local markets and were detected with the methods set up in the present study. The samples were screened with the Strip kit for qualification of the existence of the CBH-351 GM-maize. Those tested positive were followed by the ELISA kit for quantitation. As shown in Table 3, 9 of the tested corn middling pellet samples contained a minute quantity of CBH-351 GM-maize with concentrations in the range of 0.01 to 0.2% (w/w). All of the popcorn samples tested negative. Of the 3 animal feed-grade maize samples, 2 were detected to contain 0.08% and 0.14% (w/w) of CBH-351 GM-maize, respectively. These data were confirmed by the PCR to ensure the GM-maize detected were indeed the CBH-351 variety, as shown by the immuno-assay kits.

The marketed maize samples tested above were also analyzed for the existence of other 5 GM-maize varieties. Of the 14 popcorn samples, two were detected to contain the Event176. Of the 30 corn middling pellet samples, seven were detected to contain 6 varieties, twenty-one were detected to contained 5 varieties, and the remaining two were detected to contain 3 varieties of GM-maize (Table 4 and Figure 2). The results indicated that different batches of maize raw materials can possibly be mixed with various GM-maize, and the varieties of GM-maize mixed in a given sample can be up to six. However, whether the results of the highly mixed varieties were due to farming of stacked varieties of maize[26], contaminated by maize pollen of different varieties, or mixed-up after harvest could not be determined by the present study. According to a market survey conducted in Japan, GM-maize imported from the US include 5 major varieties. In order of quantity they are the MON810, T25, GA21, Event176, and Bt11. Among those, the MON810 is the primary variety, accounting for approximately 80% of the quantity of GM-maize imported to Japan, which is consistent with the data reported by the present study. Other research literatures have noted that the GM-maize produced in the US was mixed with the conventional maize immediately after harvest, then sold as a Dent-corn product as food or feed[9]. In addition to the maize samples described above, the present study also tested one raw material sample from US-imported cargo. All of the six varieties were detected in this particular sample (data not shown).

Table 3. Results of marketed maize containing CBH-351 GM-maize detected by Immuno-Kit and PCR methods

Sample	Total No.	Results (Positive No.)		
		Strip	ELISA[a]	PCR
Popcorn	14	0	0	0
Corn middling pellets	30	9	9	9
Corn tortilla	21	0	0	0
Feed corn	3	2	2	2
Total	68	11	11	11

[a] The concentration of positive samples containing CBH-351 GM-maize detected by ELISA is between 0.01% – 0.2%.

V. *Detection of Marketed Processed Maize Products*

Because there has been no researcher or related authorities proceeding in methodology development or conducting detection of GM-maize on market available processed maize products, the present study employed the methods established herein to detect frozen whole kernel maize, canned whole kernel maize, potato chips, and corn tortilla samples. The results showed that four of the canned whole kernel maize samples and five frozen whole kernel maize samples contained 2 varieties of GM-maize (MON810 and T25). One of the canned whole kernel maize sample contained the MON810 variety. None of the samples were detected to contain the Bt11, GA21, and Event176 varieties (Table 4). The PCR products can be further confirmed by some tests such as nucleic acid sequencing, endonuclease mapping, and probe hybridization[2]. However, there is no absolute need for these additional tests to be done. The present study first confirmed by the nucleic acid sequencing but the results were not perfect. The reason was speculated to be due to the samples were collected from processed products, not from materials made of a single variety of GM-maize. Some modifications were practiced to serve as a double-checking means, and to get an insight on the percentage of the GM-maize content in the products. Briefly, canned or frozen whole kernel maize

Table 4. Identification of genetically modified-maize in raw and processed food by PCR methods

Sample	Total No.	PCR					
		Event176	MON810	Bt11	T25	GA21	CBH-351
		+ / –	+ / –	+ / –	+ / –	+ / –	+ / –
Pop corn	14	2/12	0/14	0/14	0/14	ND	0/14
Corn middling pellets	30	30/ 0	30/ 0	29/ 1	27/ 3	28/ 2	9/21
Canned whole kernel corn	5	0/ 5	5/ 0	0/ 5	4/ 1	0/ 5	ND
Frozen whole kernel corn	5	0/ 5	5/ 0	0/ 5	5/ 0	0/ 5	ND
Potato crisps	10	0/10	10/ 0	0/10	0/10	0/10	ND
Corn tortilla	21	15/ 6	21/ 0	21/ 0	17/ 4	18/ 3	0/21
Total	85	47/38	71/14	50/35	53/32	46/25	9/56

+: means positive by PCR method. –: means negative by PCR method.
ND: not determined.

samples were dried in the oven followed by PCR reactions conducted on DNA extracted from a single maize kernel. For example, 24 kernels from the frozen whole kernel maize sample No. 57 were tested, of which 14 tested kernels were found to be the MON810 verity and the other 4 were found to be the T25 variety. Sample No. 57 tentatively contained 75% GM-maize, including 58% (14/24, v/v) and 17% (4/24, v/v) of MON810 and T25 varieties, respectively. Sample No. 36 was canned whole kernel maize, and quantitation data showed that 9 out of the 32 kernels, namely, 28% (9/32, v/v) of the tested kernels, were MON810 variety. No kernel from sample No. 36 was tested and found to be the T25 variety. This data indicated that the T25 GM-maize content was lower than 3% (1/32, v/v) in sample No. 36. Information from German researchers has suggested that the lowest quantity detectable from canned whole kernel maize samples was about 2% (Broll, H., personal communication, 2000); therefore, we suspected that the content of the T25 GM-maize in sample No.36 was in the range of 2% to 3%. The insufficient sample size might explain the inability for T25-containing GM-maize to be detected from this particular sample.

Although potatoes are the major ingredient of marketed potato chips, corn flour and wheat flour were also parts of their components. The existence of GM-maize was investigated in the present study. The results showed that 10 of the tested potato chip samples contained the MON810 GM-maize (Table 4). All of the 21 corn tortilla samples tested positive for the MON810 and Bt11 varieties; whereas none were detected to contain the CBH-351. Fifteen of the 21 samples tested above contained 5 varieties of GM-maize, namely, the Event176, MON810, Bt11, T25, and GA21 (Table 4). Experiences from our laboratories found that for maize raw materials, there is no significant difference between the DNA extracted with CTAB procedure or commercial kits procedures. However, for the processed products, especially for highly processed products such as potato chips, the DNeasy Plant Maxi Kit (Qiagen) is recommended for DNA extraction. In the present study, samples of both maize raw materials and processed maize products were detected to contain the MON810 variety of GM-maize (84%; 71/85). The MON810 variety GM-maize also has the highest content of the GM-maize detected from the maize materials/products. This result is consistent with the market survey conducted in Japan. This confirmed that the MON810 variety being the major GM-maize in the markets of Taiwan and Japan.

Taken together, the present study has successfully established the PCR detection methods for the identification of six varieties of GM-maize. Market-available maize raw materials and processed products were sampled for the investigation of the existence of GM-maize. The results showed that marketed maize products contained various varieties of GM-maize in a mixture form; of those GM-maize varieties detected, the MON810 appeared to be the major variety. Our laboratories are continuously working on the development of quantitative detection methods of GM-maize. As a note concerning the batch of the maize raw materials detected to contain CBH-351 GM-maize, the relative health authority in Taiwan has issued a recall in November 2000, followed by destruction of the recalled materials.

ACKNOWLEDGEMENTS

We would like to thank Dr. Chwen-Ting Wang for her translation.

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檢測六種品系基因改造玉米及其加工製品方法之探討

闕麗卿*　陳彥伶　施養志

行政院衛生署藥物食品檢驗局
台北市南港區昆陽街161-2號

（收稿：May 2, 2001；接受：September 27, 2001）

摘　要

本研究以PCR方法鑑別檢測六種品系基因改造玉米（genetically modified maize，簡稱GM-玉米）。首先探討加熱處理對於PCR檢測之影響，玉米分別經由100˚C及121˚C不同時間熱處理，續抽取DNA，以玉米*zein*基因引子進行PCR檢測，結果發現100˚C處理120 min與121˚C處理30 min仍可檢測出*zein*基因。次為建立六種品系GM-玉米之PCR鑑別檢測方法，針對Event 176（Novartis公司）、Bt11（Novartis公司）、MON810（Monsanto公司）、T25（AgrEvo公司）、CBH-351（AgrEvo公司）及GA21（Monsanto公司）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，其最低檢測濃度皆小於0.1%（w/w）。為瞭解食品玉米原料及加工玉米製品含GM-玉米之狀況，採用前述建立方法測試市售食品玉米原料、冷凍玉米粒、玉米粒罐頭、墨西哥玉米餅及洋芋片等檢體。結果發現玉米碎同件檢體及墨西哥玉米餅同件檢體可分別檢出六種及五種測試品系GM-玉米；冷凍及罐頭玉米粒可檢出MON810及T25二種GM-玉米；洋芋片檢體則含有MON810 GM-玉米。綜合上述，PCR方法適用於玉米原料及其製品之檢測，並得知台灣目前市售玉米原料及其製品含有混合之不同品系GM-玉米，並以MON810 GM-玉米品系為大宗。

關鍵詞：聚合酵素鏈反應，基因改造玉米