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Development of Multiplex and Quantitative PCR Assay to Detect Genetically Modified Roundup Ready Soybean in Foods

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ABSTRACT

The objective of this study was to develop a qualitative detection method for genetically modified (GM) soybeans using the multiplex PCR technique. Potential applications for using the developed detection method to analyze GM material in soya and its products were also evaluated. Four primers for the detection of transferred genes in Roundup Ready soybean artificially synthesized for this study included 35sP (Cauliflower mosaic virus 35S promoter), nosT (*Agrobacterium tumefaciens* nopaline synthase terminator), and 35sP/CTP (*Petunia hybrida* EPSPS chloroplast transit peptide). In addition, Lec (Lectin) primers were used to detect soybean species specificity. The results showed using either 35sP or 35sP/CTP as a primer obtained a detection limit of 0.01% (w/w) and using either primer nosT or multiplex PCR with 35sP/CTP obtained a detection limit of 0.1% (w/w). Furthermore, the transferred genes in Roundup Ready soybeans were confirmed through the isolation and sequencing of their genes. By using the multiplex PCR method developed in this study, we detected GM soybean material in 14 of the 21 soybean products obtained from the open market for this study. In addition, samples with 20%, 10%, 5% and 1% GM-soya and 5% GM-soya standard were quantitatively analyzed with SYBR Green I, with an R^2 of 0.9683 when regression analysis was applied. Our results showed that, in addition to readily detecting GM soybean material, the multiplex PCR method reduced detection time and costs. The multiplex PCR method proposed in this paper may offer a useful tool to detect and monitor GM foods.

Key words: multiplex PCR, genetically modified organism (GMO), Roundup Ready soybean

INTRODUCTION

The growing world population threatens many countries and regions with food shortages. According to the Sixth World Food Survey[1] conducted by the United Nations' Food and Agriculture Organization (FAO) about 0.8 billion people in our world are undernourished. World food stocks have fallen to a level now that provides a safety margin of only 60 days. Therefore, resolving the problem of how to improve world food productivity is an urgent issue. The application of biotechnology, especially genetic engineering technology, is surely an important tool available for use in improving food productivity. The development of genetic recombination technology offers an opportunity to control food processing effectively and provides an innovative tool for the food industry to improve food quality and food product development[2]. Already, many genetically modified (GM) foods have been approved for marketing. The wide-scale use of several insect-resistance and herbicide tolerant transgenic crops in field cultivation and commercial production indicate that GM food development is now progressing at a rapid pace. Currently, the United States has taken the leading position in the research, development and commercialization of GM foods. To date, more than 50 GM crops have been approved for commercial production in that country[3]. Data from the Organization for Economic Cooperation and Development (OECD)[4] show that, at present, the GM crops being field-tested the most include soybeans, tomatoes, corn, potatoes, wheat, cotton, rice, and tobacco.

Roundup Ready soybean[5] and Bt maize[6], introduced into agricultural production in 1996, are gaining increasing shares of the planted acreage in the United States. Statistical data indicates that 99% of GM crops worldwide are grown in four countries. These are the United States (with 68% of the total), Argentina (with 11.8%), Canada (with 6%), and China (with 3%). GM soybeans account for 63% of global GM crop production[7]. According to import data published by the Council of Agriculture, Executive Yuan, ROC[8], Taiwan imports approximately two million tons of soybeans every year. Of this total, more than 95% is from the United States. In light of this, we estimate that approximately 50% of soybeans on the market in Taiwan are GM soybeans.

The currently available methods by which to analyze GM foods can be divided into three categories. Transgene identification and quantification can be done using either the Southern blot or polymerase chain reaction (PCR) test; transgenic expression protein detection can be done using an Enzyme-linked immunosorbent assay (ELISA), Western blot, or Lateral flow strip assay[9]. The third category of enzyme activity measurement, can be done as well, although it is not widely performed.

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Each method listed above has advantages and shortcomings. All can be applied in unprocessed raw materials detection activities relatively successfully. Testing methods that detect proteins and measure enzyme activity can not be employed on fermented products in which the protein may already have been denatured. Due to DNA fragmentation, highly processed products cannot be accurately tested using the PCR method. Therefore, the detection techniques for highly processed products must be improved to meet GM food labeling testing requirements. Although several techniques are applicable, PCR analysis remains the most popular and most commonly used test for GM food analysis[9]. The GM product detection primers are designed according to the sequences of regulatory and structural genes on transgenes[10]. The different methods used to confirm PCR reaction products include restriction enzyme analysis, Southern blot, nested PCR, and nucleic acid sequencing[11]. Some alternative methods recently have been proposed, including the chromatographic, near infrared, and biochip methods[11].

Biosafety and ecological issues are the two GM food topics most often debated in international forums. However, consumer acceptance will be critical in order to the success of GM in the marketplace. As concerns remain regarding potential safety issues with GM food, consumers demand a right to know the GM content of food products they buy and expect food labeling to indicate this information.

Because food testing is the basis of a sound food labeling program, a reliable testing method is essential to ensure food labeling criteria are met. In Taiwan, an effective GM food testing and labeling protocol is needed urgently. On February 22, 2001, the Department of Health in Republic of China declared that any food with raw materials containing more than 5% GM soybean or maize (by weight) must be labeled as "genetically modified" or "containing genetically modified organisms". Conversely, food items that are free of GM raw materials were permitted to bear a label stating "non-genetically modified" or "not genetically modified"[12]. In view of such, it is important to develop a reliable testing method for GM food detection.

In this study, a Multiplex PCR identification method was developed and evaluated for potential application on commercial GM soy products. Several primers specific to the transgene and species trait gene in Roundup Ready GM soybean were designed for multiple PCR detection. This method is expected to be of direct benefit to GM food testing and labeling efforts underway in Taiwan.

MATERIALS AND METHODS

I. *Reagents*

Chloroform, isoamylalcohol, and isopropanol were purchased from Merck Co. (Darmstadt, Germany). Cetyltriammonium bromide (CTAB) was obtained from Sigma (Louis, MO, USA), and Agarose was obtained from BMA Co. (Rockland, ME, USA).

II. *Equipment*

GeneAmp PCR system Model 2400 was the product of Perkin-Elmer Co. (Branchburg, NJ, USA). GeneAmp PCR System Model 5700 was from Applied Biosystems (Foster City, CA, USA).

III. *Materials*

The Roundup Ready GM soybeans (Monsanto Co., USA) were kindly given by American Soybean Association's representative in Taipei. The non-GM soybeans, Kaushung No. 10, were supplied by the Tainan District Agricultural Research and Extension Station. Other commercially available soy products were purchased on the open market. GM and non-GM soybeans were ground with a blender and mixed in different ratios to obtain 100% (w/w), 20%, 5%, 1%, 0.5%, 0.1%, and 0% concentrations of GM-soya. A 5% GM-soya standard was purchased from Fluka Co. (Buchs SG, Switzerland).

IV. *PCR Primers*

Four sets of primers as listed in Table 1 were designed. They were 35sP, nosT, 35sP/CTP, and Lec specific to 35S promoter, NOS terminator, EPSPS and Lectin genes in Roundup Ready GM soya, respectively. These primer pairs were synthesized by GENSET company (Singapore). Nucleic acid primers were designed using Seq Web and Vector NTI Suite software designed by the National Health

Table 1. Primer sequences and size of amplification products

Primer	Gene	Sequence 5'-3'	Amplicon (bp)
35sP-F	35S promoter	AAAGATGGACCCCCACCCAC	143
35sP-R		GAGGAAGGGTCTTGCGAAGG	
nosT-F	NOS terminator	CTGTTGCCGGTCTTGCGATGAT	189
nosT-R		CCGCGCGCGATAATTTATCCTAG	
CTP-R	*Petunia hybrida* CTP[a]	GGGTTTGTATCCCTTGAGCCATG	256[b]
Lec-F	Lectin	AAGGTTGACGAAAACGGCACCC	175
Lec-R		GAAAGAAGGCAAGCCCATCTGC	

[a]*Petunia hybrida* CTP: *Petunia hybrida* EPSPS chloroplast transit peptide.

[b]The amplicon includes the last 202 bp of the 35S promoter and the first 54 bp of the *N*-terminus of the *Petunia hybrida* CTP gene.

Research Institute. The position of primers in the template is shown in Figure 1.

V. *DNA Extraction and Purification*

A CTAB method adopted from Fred *et al*. (1994)[13] was modified for use in this study. The sample (2g), mixed with liquid nitrogen, was ground into powder form and placed into a 15-mL centrifuge tube. A mixture including 8 mL of CTAB solution I (CTAB 2% (w/v); Tris-HCl 100 mM pH 8.0; EDTA 20 mM pH 8.0; NaCl 1.4 M), 10 μL of protease K (10 mg/mL), and 5 μL of RNase A (10 mg/mL) was prepared, heated at 65˚C for 1 hr, and centrifuged at 8000×g for 15 min. The upper layer was collected and extracted with 1-fold volume of chloroform/isoamylalcohol (24/1, v/v) solution. A one-fold volume of CTAB solution II (CTAB 10% (w/v); NaCl 0.7 M) was then added and incubated at 65˚C for 30 min, after which the solution was centrifuged and the suspension discarded. The precipitate was dissolved in a 1.2 M NaCl solution, mixed with 0.6-fold isopropanol and then kept in an ice-bath for 15 min. The resulting solution was then centrifuged and the suspension was disregarded. The precipitate was washed with 80% alcohol twice and dried at 65˚C for 2 min. The extracted DNA was quantitatively standardized to 100 μg/mL and monitored by O.D. 260/280 nm prior to proceeding with necessary testing for this study.

VI. *Analysis of PCR and its Products*

(I) *Qualitative PCR*

The final 25 μL volume of PCR contained 200 μM dNTPs, 25 μg/mL primer, 2 units AmpliTag Gold® polymerase (Perkin-Elmer), and 5 μL of 10× reaction buffer (Perkin-Elmer) with 1.5 mM Mg^{2+}. A 5 μL quantity of DNA template was added to this PCR and, after centrifugal mixing, the mixture was placed in a PCR reactor. The following program was used for PCR reaction: at 95˚C for 5 min; 95˚C for 30 sec; 61˚C for 30 sec (at 62˚C for multiplex PCR reaction); at 72˚C for 30 sec (40 cycles reaction in total); 72˚C for 5 min. The resultant PCR products were then analyzed using 2.5% agarose gel electrophoresis.

(II) *Quantitative PCR*

The following reagents were used for our quantitative PCR: DNA template (50 ng/μL); 1× SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) – which included 1× SYBR Green buffer, 5 mM $MgCl_2$, 200 μM dNTP (dATP, dCTP, dGTP, dTTP), 8% glycerol, 0.3 units AmpliTaq Gold® DNA polymerase and SYBR Green I Dye; and 250 nM 35sP-F/CTP-R primer. Reactions were performed under the following conditions: 95˚C for 10 min, 95˚C for 15 sec, and 60˚C for 1 min. A total of 45 cycles was performed.

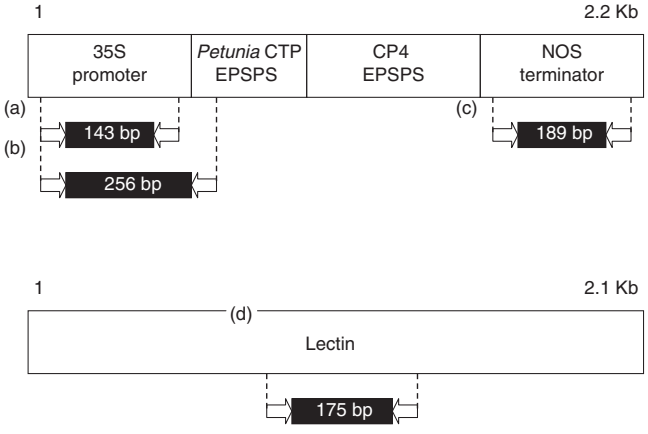

Figure 1. Designed primers used in this investigation. The position of primers in the template are indicated by ⇨. Amplification of the initial template using (a) set of primers yielded a 143-bp PCR product. (b), (c) and (d) primer sets yielded 256-bp, 189-bp and 175-bp PCR products, respectively.

VII. *Sequencing Analysis*

The PCR products were produced using 35sP-F and NosT-R as primers. The DNA in these products was extracted and purified with a Gene-Spin™ kit (Poster Co. Taiwan), and cloned using a pGEM-T Easy carrier system (Promaga Company, USA) prior to sequencing. Cloning instructions noted in the pGEM-T Easy carrier system operation manual were followed. T4 DNA ligase was used to catalyze the linkage of PCR products to the pGEM-T Easy carrier. The result was then incubated at ambient temperature for 1 hr to facilitate transformation into *E. coli* DH 5α cells. The plasmid DNA was extracted using a QIAprep Spin Miniprep kit (QIAGEN Company, Germany). Sequencing was performed at the National Chung Hsing University Biotechnology Center.

RESULTS AND DISCUSSION

I. *DNA Extraction*

The CTAB method is widely used to extract DNA from soybeans and maize[14,15]. Soy and maize products are rich in protein and polysaccharides – substances that readily conjugate with CTAB and can be removed by phenol and chloroform[16]. As DNA in those products can be purified effectively using this process, the CTAB method was chosen in light of the range of products it could analyze. We used a simplified version of Fred *et al*.'s CTAB method[13] for DNA extraction in this study. Modifications made included (1) an increase in CTAB concentration in the CTAB solution II in order to enhance the conjugation efficiency of protein and polysaccharides in soy products and (2) a one time extraction using chloroform and isoamylalcohol (replacing a two tier extraction process

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using phenol and chloroform) in order to prevent protein residues remaining in the phenol as well as to minimize procedures. In this study, protease K and RNase were added during DNA extraction to catalyze protein and RNA degradation.

II. *Qualitative Determination of GM Soybeans*

The objective of this test was to detect four genes (Lectin, 35S promoter, EPSPS, and NOS) present in Roundup Ready soybean by multiplex PCR. For this test, the target gene EPSPS and species trait gene Lectin were amplified and tested.

1. Singleplex PCR: An initial analysis was performed using singleplex PCR to test the specificity and sensitivity of designed primers. The target fragments were designed to be less than 400 bp due to the predominance of fragments of that size in processed foods[9]. The results, shown in Figure 2, indicate that designed primers yielded satisfactory results in terms of specificity and sensitivity. The lectin gene is a specific gene found in soybeans, detectable in both GM and non-GM soybeans. As shown in Figure 2(A), a 175 bp fragment of the target gene from a soybean with Lec primer is clearly visible and can be readily applied to the screening of food samples that contain soybeans. Figure 2 (B) shows the results of PCR analysis on GM soybeans of different GM concentrations detected using the 35sP primer. The target 143 bp gene fragment is clearly visible. Fragment brightness patterns, correlating positively with the level of GM material concentration in the fragment, conform to our initial expectation. A problem with the appearance of non-specific fragments at the top may be resolved by adjusting PCR reaction conditions in future tests. By using 35sP PCR, we were able to reach a level of sensitivity of 0.01% -- less sensitive than that achieved by Jankiewicz *et al.*[17] (reporting a sensitivity of 0.005% with 35S promoter), comparable to that achieved by Vollenhofer *et al.*[18] (0.01%), and more sensitive than that reported by Lin *et al.*[19] (0.1%). Figure 2(C) shows test results for GM soybeans of varying levels of GM concentration as detected by nosT primer. The target gene, a 189 bp fragment, also demonstrated lower brightness with reduced GM content levels. The obvious primer-dimmer, appearing at the bottom of this figure, indicates that this primer was not ideally designed. The 0.1% sensitivity achieved with nosT PCR was, while less than the level of 0.01% attained by Vollenhofer *et al.*[18], was more sensitive than the 0.5% attained by Lin *et al.*[19]. Figure 2(D) shows PCR analytical results for GM soybeans of varying

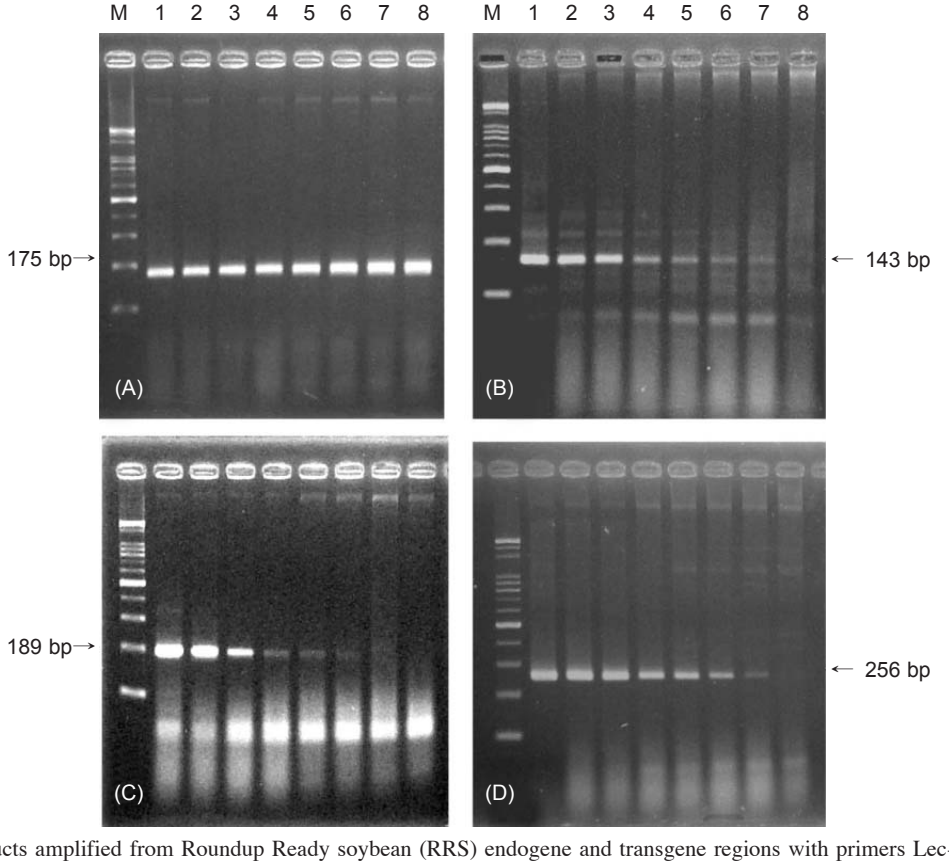

Figure 2. PCR products amplified from Roundup Ready soybean (RRS) endogene and transgene regions with primers Lec-F/R (A), 35sP-F/R (B), nosT-F/R (C) and 35sP-F/CTP-R (D). Lane 1, 100% RRS; lane 2, 20% RRS; lane 3, 5% RRS; lane 4, 1% RRS; lane 5, 0.5% RRS; lane 6, 0.1% RRS; lane 7, 0.01% RRS; lane 8, 0% RRS.

levels of concentration using 35sP-F and CTP-R primers specific to the 35S promoter and Petunia EPSPS CTP transgene fragment. Results show that the level of brightness in the 256 bp target gene fragment also decreased with a decreasing level of GM concentration. The 0.01% sensitivity achieved with 35sP/CTP PCR was as sensitive as that achieved by Vollenhofer *et al.*[18] and Gert *et al.*[20], respectively, in their studies of RRS target transgenic fragments and more sensitive than the 0.1% reported by Lin *et al.*[19]. Our results indicate that a satisfactory level of sensitivity can be obtained using this set of primers.

2. Multiplex PCR: Two sets of primers (35sP/CTP and Lec) were selected to perform this multiplex PCR study. The aims of using multiplex PCR were to method effectiveness at identifying the samples as soya products and at successfully detecting the presence (or absence) of the herbicide tolerant glyphosate EPSPS gene. The analytical results of multiplex PCR using primers 35sP/CTP and Lec on two target genes of fragment lengths 256 bp and 175 bp are shown in Figure 3. 35sP/CTP target gene brightness decreased with decreasing GM constitution content. The 0.1% sensitivity of the multiplex PCR achieved with the 35s/CTP primer was less than the 0.01% achieved through a single-plex PCR using the same primer.

III. *Quantitative Determination of GM Soybeans*

Quantitative PCR results for the 5% GM-soya standard and GM-soya products of differing GM-soya content (20%, 10%, 5%, and 1%), using SYBR Green I, are shown in Figure 4. The Ct value for 5% GM-soya standard was 19.01. Ct values for self-prepared GM-soya with 20%, 10%, 5%, and 1% GM-soya contents were 14.00, 17.52, 18.94, and 21.67, respectively. The similar Ct values obtained for the self-prepared 5% GM-soya and 5% standard (18.94 and 19.01, respectively) indicates the high level of precision with which the self-prepared GM-soya was made. Regression analytical data gave an R^2 value of 0.9683. Because SYBR Green I is a non-specific fluorescent dye, the formation of primer-dimmer could potentially affect test results. This phenomenon was observed using a Dissociation curve, as shown in Figure 4(C). The melting point of regular PCR products is in the range of 80~90°C, while the melting point of the primer-dimmer ranges between 70~80°C. A small peak appeared at 76°C, indicating that the primer-dimmer was produced with 35sP-F/CTP-R primers (Figure 4), which could negatively affect testing precision.

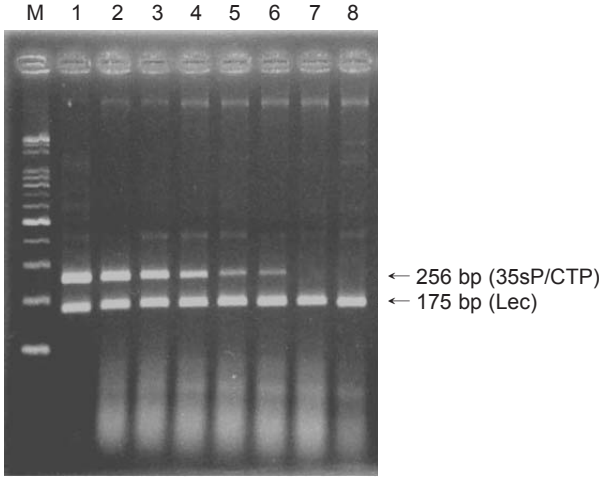

Figure 3. Multiplex PCR products amplified from RRS endogene and transgene regions with primers Lec-F/R and 35sP-F/CTP-R. Lane 1, 100% RRS; lane 2, 20% RRS; lane 3, 5% RRS; lane 4, 1% RRS; lane 5, 0.5% RRS; lane 6, 0.1% RRS; lane 7, 0.01% RRS; lane 8, 0% RRS.

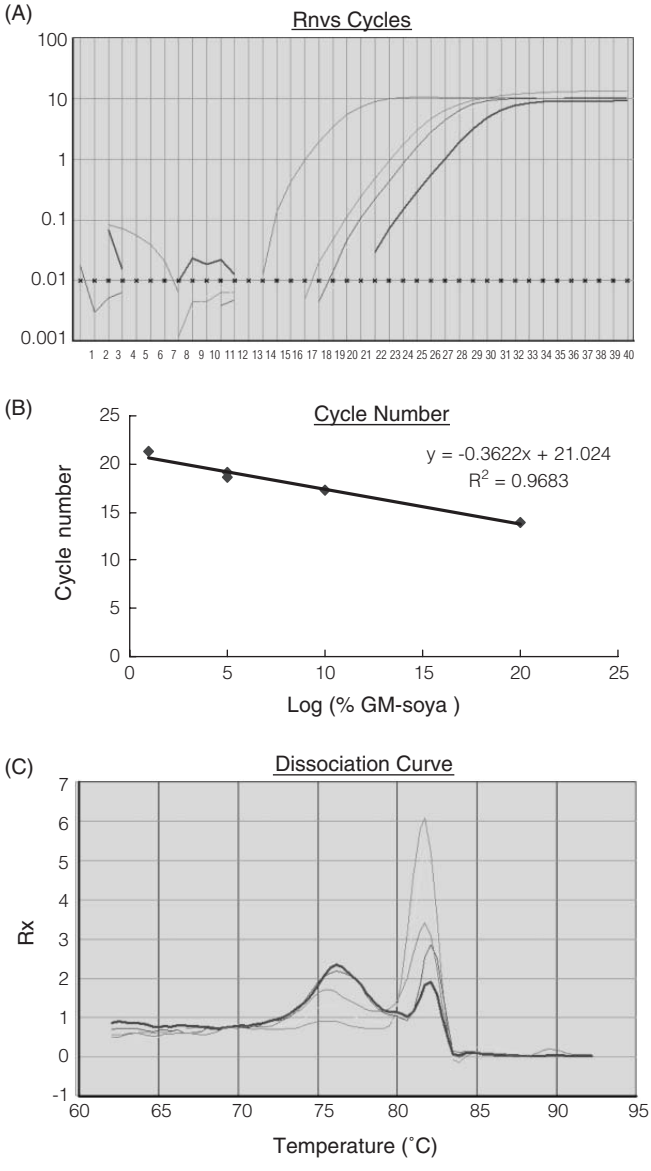

Figure 4. Real-time PCR of the 35sP-F/CTP-R fragment from Roundup Ready soybeans, using SYBR Green I. Amplification plot (A), standard curve (B) generated by 20%, 10%, 5% and 1% GM-soya content, using the SYBR Green I detection system, showing an R^2 value of 0.9683 and dissociation curve (C).

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IV. *Detection of Commercial Products*

In this study, 22 soya products were obtained from the Taichung area in central Taiwan. Due to over-processing, no DNA from Gon-Yen miso could be extracted or detected using the modified CTAB method, leaving 21 samples valid for testing and analysis. Results show that the modified CTAB method was capable of extracting more DNA from samples. Table 2 lists the multiplex PCR detection results for GM soybeans in processed foods. Products nos. 3, 4, 5, 6, 7, 9, 10, 11, 12, 15, 18, 19, 20, and 21 tested positive for GM material, while products nos. 1, 2, 8, 13, 14, 16, and 17 tested negative. In total, 14 of the 21 samples were tested as containing GM material. Seven of the 21 were tested GM-free. Among those samples tested GM-free, nos. 1, 2, and 8 were labeled as "non-GM" foods by the manufactures. Products nos. 16 and 17 were highly processed soya products. Based on detection results, the multiplex PCR method developed in this study should be viewed as a valid tool with which to conduct qualitative analyses on commercialized soya products.

V. *Verification of PCR Products*

The PCR products were sequenced in order to verify the amplified PCR products. The 2.2 kb transgenic fragments of GM soybeans (including 35S promoter, EPSPS and NOS target genes) were transferred into a pGEM-T Easy vector, which was subsequently transformed into *E. coli* DH5α. Target plasmid fragments were then extracted and sequenced. Results showed the DNA sequence of PCR products comparable to the sequence expected.

Benefit of the modified CTAB method developed for this study includes simplified operational procedures and its suitability for use in the extraction of DNA from different soya products. Another advantage of the modified CTAB method is its lower cost. Using two primers specific to 35sP/CTP and Lec for multiplex PCR detection could determine the presence in samples of GM soybean and herbicide tolerant glyphosate EPSPS genes simultaneously. Results demonstrated that high specificity and a level of sensitivity as low as 0.1% could be attained by using the two above-mentioned primers in multiplex PCR detection. This study demonstrates that the multiplex PCR as developed is capable of detecting GM material in food products and can be a beneficial tool for GM food analysis and inspection. This study is recommended as an important reference for both government regulatory authorities and industry.

Table 2. Detection of genetically modified soybeans in processed foods with multiplex PCR

Sample no.	Sample	Detection by multiplex PCR	Labeling status
1	Soybean milk	-	L[a]
2		-	L
3		+	N[b]
4	Tofu Pudding	+	N
5		+	N
6	Tofu	+	N
7		+	N
8		-	L
9	Dried tofu	+	N
10		+	N
11		+	N
12	Vegetarian ham	+	N
13		-	N
14		-	N
15	Miso	+	N
16	Fermented soybean curd	-	N
17		-	N
18	Fermented tofu	+	N
19		+	N
20	Soybean powder	+	N
21		+	N

[a]Labeled (product labeled as "non-GMO").
[b]Not labeled.

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