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Detection of Genetically Modified Soybeans by PCR Method and Immunoassay Kits

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ABSTRACT

The suitability of detecting genetically modified (GM) soybeans by polymerase chain reaction (PCR) and immunoassay kits is determined. Primers specific for inserted genes in the Roundup Ready™ soybean (RRS, Monsanto company) were applied. Four pairs of primers, namely, 35S (35S-promoter, originated from cauliflower mosaic virus), NOS (nopaline synthase-terminator, derived from *Agrobacterium tumefaciens*), EPSPS (5-enolpyruvylshikimate-3-phosphate synthase, obtained from *A. tumefaciens* strain CP4) and LE (endogenous gene lectin) were used to identify the GM soybeans. The detection limit of PCR was 0.1% (w/w) GM soybeans when primers 35S and EPSPS were used, and 1% when primers NOS were used. All soybean samples were evidenced by LE primer-PCR as soybean products. Results of Immunoassays by two kinds of kits, strip and ELISA were matched with PCR's, and feasibility of quantitation detecting GM soybeans is determined among 0~2.5%. The data further revealed that the PCR method and immunoassay kits can sufficiently differentiate GM soybeans from non-GM products.

Key words: GM soybeans, PCR, immunoassay

INTRODUCTION

As of March 2001, 3 types of genetically modified-soybeans (GM-soybeans) have been approved for commerce globally. One of them was approved by the European Union and two were approved by Japan; while the US approved all 3 types of GM-soybeans[1]. The major traits of GM-soybeans are herbicide tolerance and high oleic acid content[1, 2]. Research has continued to improve the characteristics of GM-soybeans with low saturated fatty acid and high stearic acid content[3]. According to statistical data from the ROC Council of Agriculture, Taiwan imports about 2 million tons of soybeans annually more than 95% from the US[4]. Based on this data, about 50% of commercial soybeans in Taiwan are estimated to be GM-soybeans. In 1995, the global soybean production was 123.65 million tons and the US produced 47.3% of them. In 1999, the area for cultivating GM-soybeans in the US was 18.4 million hectares; accounting for 65% of total soybean production in that year. Roundup Ready™ soybean (RRS) is the major brand among those GM-soybeans cultivated in the US[2]. Its major trait is herbicide tolerance. The regulated gene of inserted recombination gene in RRS is mainly composed of a 35S-promoter (from cauliflower mosaic virus) and a NOS-terminator (a terminator of nopaline synthase gene from *Agrobacterium tumefaciens*). A CP4EPSPS gene (5-enolpyrunylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* strain CP4) is a structure gene of inserted recombination gene in RRS.

Methods for the identification of GM food can be divided into 3 categories[5]. In the first category are nucleotide-based amplification methods including polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), fingerprinting techniques (such as RFLP, AFLP, and RAPD), probe hybridization, "self-sustained sequence replication" (3SR), and "Q replicase amplification". The second category involves protein-based methods including one-dimensional SDS gel electrophoresis, two-dimensional SDS gel electrophoresis, Western-blot analysis and ELISA. The third category is based on the detection of enzymatic activities. Every detection method has its own specificity and limitations. The detection using an enzymatic activity method is not recommended for processed foods, where proteins may be denaturized. The methods based on PCR are not suitable for detection of highly processed foods because DNA fragments in foods could be broken into pieces[5]. 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. These designed primers possess some specific characteristics and can be used for two purposes: product screening and product-specificity detection[5]. The PCR products need to be further confirmed by the following method: nucleic acid sequencing, endonuclease mapping, and probe hybridization[5]. The PCR method is not only used for identification of GM-products, but also for quantification purposes[6]. In compliance with the labeling regulation for GM foods, several countries in Europe such as Germany and Switzerland have extensively developed PCR methods for both identification and quantification purposes on GM food detection[6]. In 1997, 29 laboratories in 13 European countries performed a collaborative trial study for detection

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of GM-maize and GM-soybeans. Results showed that using a PCR method with 35S-promoter primer was capable of detecting as low as 2% of Roundup Ready™ GM-soybeans in soy flour and Event176 GM-maize in maize flour[7]. An official process using the PCR method for GM food detection has been developed by some European countries[6]. Food products are screened by using 35S-promoter-PCR. The products with positive reaction are further confirmed by product-specific primer-PCR. According to the literatures, several PCR primers have been selected for GMO products screening[7] or product-specificity detection[8-10]. In this study, those PCR primers were tested for both screening and product-specificity detection to study the feasibility of the PCR method on the identification and detection of GM-soybeans. An ELISA kit, which is commercially available for Roundup Ready ™ soybean detection[11], was also tested in this study. In European Union, an inter-laboratories trial for confirmation of protein detection has been carried out. For example, 38 laboratories in 13 European countries performed a collaborative study in an immunoassay study. Results showed a 99% confidence interval in qualitative analysis (of 2% detection limit) and repeatability/reproducibility with 7% RSD_r and 10% RSD_R in quantitative analysis (< 2%) were obtained[12].

MATERIALS AND METHODS

I. *Reagents*

Chloroform and isopropanol were purchased from Merck (Darmstadt, Germany). Hexadecyltrimethyl-ammoniumbromide (CTAB) was obtained from Sigma (St. Louis, MI, USA) and Agarose was purchased from Amresco (Solon, OH, USA). Strip kit for Trait RUR Lateral Flow Test and 6 ELISA Soya test kit were obtained from Strategic Diagnostics Inc (SDI, Newark, DE, USA).

II. *GM Soybean Reference*

Roundup Ready™ soybeans (RRS) with 0%, 0.1%, 0.5%, 1%, 2%, and 5% (w/w) GMO contents were obtained from Monsanto (USA) and full-fat soy flour standard prepared by Leatherhead Food RA was purchased from Strategic Diagnostics Inc (SDI, Newark, DE, USA).

III. *Equipment*

PCR thermal controller model PTC-100 with programmable thermal controller was purchased from MJ Research Co. (Water Town, MA, USA). ELISA detector was made by MicroStation, Molecular Devices (USA).

IV. *PCR Primers and Reagents*

Four pairs of PCR primers, synthesized by TIB Molbiol (Berlin, Germany) as listed in Table 1, were used in this study. These primers were 35S, NOS, EPSPS and LE, which were specific to 35S-promoter, NOS-terminator, EPSPS structure gene region, and lectin gene, respectively, in RRS.

V. *DNA Preparation and Purification*

A CTAB method for sample extraction and purification reported by Lipp *et al*. in 1999[7] was adopted in this study. Test sample (25 mg) was extracted with CTAB, precipitated, treated with chloroform, and precipitated with isopropanol to obtain a purified DNA matrix.

VI. *PCR Reactions and Product Analysis*

The PCR reagent was prepared by mixing 25 µL of deionized water with DynaZyme DNA kit, which was composed of 10-fold PCR buffer solution (5 µL) containing 1.5 mM Mg^{2+}, 1.5 µL of dNTP (200 µM), 2.5 unit of DNA polymerase, and 1 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 (for 35S, NOS, EPSPS, and LE primers), 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.

VII. *Immunoassay*

The immunoassay kit used in this study is a product of SDI. This kit was designed based on the principle of enzyme-linked immunosorbent assay (ELISA). A monoclone antibody which is specific to herbicide-tolerant CP4EPSPS protein in RRS was produced and immobilized to the test well of ELISA plate. Combined with monoclone antibody, polyclone

Table 1. Primers used in this study

Primer	Sequence 5'-3'	Gene	Amplicon (bp)	Reference
35S-1	GCTCCTACA AATGCCATC A	35S		
35S-2	GATAGTGGGATTGTGCGTCA	promoter	195	(7)
NOS-1	GAATCCTGTTGCCGGTCTTG	NOS		
NOS-3	TTATCCTAGTTTGCGCGCTA	terminator	180	(7)
EPSPS-B1	TGATGTGATATCTCCACTGACG			
EPSPS-B2	TGTATCCCTTGAGCCATGTTGT	CP4EPSPS[a]	172	(10)
LE103	GCCCTCTACTCCACCCCCATCC			
LE104	GCCCATCTGCAAGCCTTTTTGTG	Lectin	118	(10)

[a]CP4EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase from *A. tumefaciens* strain CP4.

antibody, and horseradish-peroxidease (HRP), an ELISA detection system was thus set up and a HRP product, 3, 3', 5, 5'-tetramethylbenzidine (TMB) was generated after reaction. The procedures for sample treatment and detection described in operation manual of immunoassay kit were followed, and the testing results were generated after detected and calculated by ELISA detector and microsoft^{TM} Excel software, respectively. The Strip kit was also supplied by SDI. Soy samples were ground and reacted with Strip kit for about 5-20 min. The color was then developed and the results were obtained by reading the visible color bands.

RESULTS AND DISCUSSION

As of 2000, 3 types of GM-soybeans had been approved by the US government for commerce; however, Roundup Ready^{TM} soybean (RRS) is the only GM-soybean widely cultivated[6]. In this study, RRS with different GMO contents was selected and its GMO product-specific gene and recombinant protein were detected using PCR and immunoassay. The purpose of this study was to evaluate the feasibility of the PCR method and immunoassay kit on GM food detection.

I. *Detection of RRS Reference by PCR Method*

Two primers, 35S and NOS as listed in Table 1, which are specific to 35S-promoter and NOS-terminator of herbicide-tolerant CP4EPSPS inserted gene in RRS, were selected for PCR analysis[7]. In 1997, 22 out of 28 commercial GM-crops were found to contain 35S-promoter or NOS-terminator in their inserted genes. Theoretically, using the primers specific to the above two genes for PCR analysis allows the identical PCR products to be amplified and therefore the GMO in foods can be detected by using this method[7]. It has been shown that the PCR products with size 195 bp (from 35S) and 180 bp (from NOS) can be obtained from the RRS containing 0.1%, 0.5%, 1%, 2%, or 5% GMO, but no PCR product is found from regular soybean (0% GMO content)[13]. Our previous study showed that the PCR analysis with 35S and NOS primers could detect as low as 0.1% and 1% GMO, respectively, in GM-soybean reference. The 35S- and NOS-PCR analysis conducted by a 29 laboratory collaborative study in Europe showed that the detection limit for GMOs in GM-soybeans was 2%. A possible false negative reaction could happen when less than 2% of GMO exists in the GM-soybeans products[7]. In Switzerland, the labeling of GM foods with GMO content higher than 1%, is required[6]. Thus, the detection technique developed in our laboratory allowing as low as 0.1% GM-soybeans to be detected (using 35S promoter) is capable of meeting the European standard[6]. According to the results of our study and other literature[7], the products amplified by 35S-primer-PCR from GM-soybeans are quantitatively more than those amplified by the NOS-primer-PCR[13]. The mechanism is unclear. A diverging sensitivity possibly exists between these two primers[14]. A CTAB method used for DNA extraction in this study has been reported to yield a higher quality DNA extract but lower in DNA recovery[15]. According to Lipp *et al*.[7], 100-mg of GM-soybeans or GM-maize is required to perform a PCR test. However, we have found that a sample of 25 mg is enough to conduct the same test.

The primers used for detection of specific traits of RRS were specific to the structure gene of herbicide-tolerant CP4EPSPS inserted gene[10]. A PCR product less than 350 bp was suggested to be suitable for GMO detection[5]. A primer capable of producing 172 bp PCR product (Table 1) is the first primer used for GM-soybeans detection. It was therefore selected in our previous study. The results showed that a PCR product with 172 bp appeared after PCR reaction of GM-soybeans containing different GMO contents (0.1%, 0.5%, 1%, 2%, and 5%), but no 172 bp product was found in the negative control sample[13]. The detection limit reached 0.1% demonstrating this method is capable of performing a routine analysis. The test samples were also confirmed to be soybean products by using a Lectin gene primer[10], an endogenous gene of soybean[13].

Four primers tested in this study were capable of generating the PCR products with a size less than 300 bp. Based on this character, they are considered to be suitable for detection of raw materials of GMO products and may also be used to detect processed GMO products. Among those 4 primers, NOS primer is less sensitive in GMO detection. The primers of 35S and EPSPS are recommended to be used for screening and specific traits detection of GM-soybeans products; while the LE primer, which is routinely used for confirmation of the endogenous gene, is recommended for product-specificity detection.

II. *Application of Immunoassay Kit on the Detection of RRS Reference*

Two references were used in this study. One was Certified Reference Materials (CRM), which was produced by Fluka and certified by The Institute for Reference Materials and Measurement (IRMM, Geel, Belgium). The other was full-fat (FF) soy flour made by SDI, namely SDI-FF. A positive reaction was obtained when CRM with more than 0.5% GMO content and SDI-FF with more than 0.3% GMO content were tested, revealing the detection limit the Strip kit is about 0.3%. Similar results were observed using the ELISA kit, which demonstrated the detection limit of 0.1% and 0.3% for CRM and SDI-FF, respectively, as shown in Figure 1A. Theoretically, ELISA method could generate the detection limit far lower than 0.1% for CRM and 0.3% for SDI-FF if the reference with much lower GMO content is available, since its detection is via a spectrophotometric measurement. Two calibration curves with significant difference in the slope at the range of 0%~5% GMO content for CRM and 0%~2.5% GMO content for SDI-FF versus OD_{450} were plotted as shown in Figure 1 B1 and B2. Regression lines were $Y_{CRM}= 0.3339X_{CRM} + 0.3558$ with $R^2_{CRM}= 0.9188$ and $Y_{SDI-FF}= 0.5495X_{SDI-FF} + 0.2044$ with $R^2_{SDI-FF}= 0.9226$, respectively. This difference could be due to an un-parallel concentration range used for calibration. As omitting the point at

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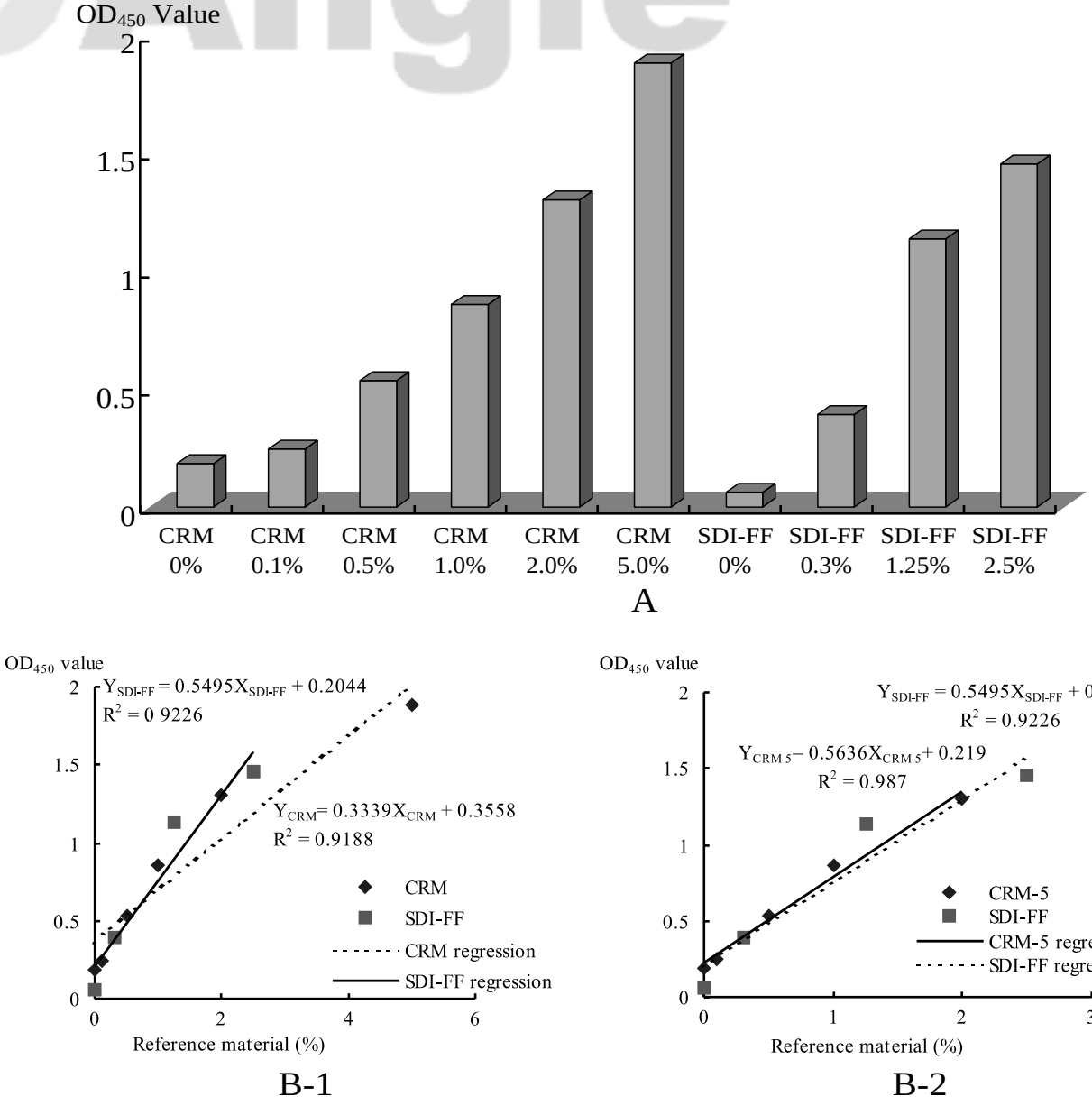

Figure 1. Testing results of CRM and SDI-FF reference materials. A: OD values by ELISA immunoassay kit. B: Linear regressions of two kinds of reference materials.

5%, the slopes of these calibration curves became close. The regression lines turned out to be $Y_{CRM-5}= 0.5636X_{CRM-5} + 0.219$ with $R^2_{CRM-5}= 0.987$ and $Y_{SDI-FF}= 0.5495X_{SDI-FF} + 0.2044$ with $R^2_{SDI-FF}= 0.9226$, respectively. The above data indicated that there is no significant difference in using immunoassay kits to detect different sources of references.

III. *Inspection of RRS in Marketed Soybeans and Other Bean Products*

Test samples including 33 soybean samples and other bean products (red bean, mung bean, black bean, multiflora bean, and rice bean) were collected from traditional markets, supermarkets, and grocery stores in Taipei and Keelung, Taiwan. The 35S-promoter, CP4EPSPS gene, NOS-terminator, and LE product-specific gene in inserted recombinant gene of above test samples were detected using the PCR method. The immunoassay method using the Strip kit was used to detect the CP4EPSPS recombinant protein. The results tested by the PCR method and the Strip test were consistent. All the red bean, mung bean, black bean, multiflora bean, and rice bean samples were tested to be negative as using the PCR and immunoassay methods (by both Strip and ELISA kits). All 33 soybean samples were detected to contain the RRS recombinant gene by the PCR method, and to contain the CP4EPSPS recombinant protein as tested by Strip and the ELISA kits, indicating both the PCR and the immunoassay methods could provide consistent results in RRS detection. An integral result for both recombinant gene and protein was generated using the PCR and immunoassay

methods. Furthermore, detection using ELISA could provide quantitative data and results to show that the GMO contents in all 33 soybean samples were higher than 2.5%.

IV. *Comparison of PCR Method and Commercial Immunoassay Kits*

The PCR and immunoassay methods each have their own specialty in RRS detection. Specificity, precision, speed, and cost are the factors that need to be considered when selecting the detection method. The PCR method has become a basic technique and equipment in life science laboratories. This method is capable of detecting target regions in different test samples using different conditions and primers. To take this study as an example, the regulatory genes (35S-promoter and NOS-terminator) and structure gene (CP4EPSPS gene) regions can be detected individually. Additionally, the same gene region can even be detected using different primers. Thus, not only can the non-specific result be eliminated, but also the detection result could further be confirmed. The PCR method has demonstrated its convenience and flexibility in primer selection. The advantage of using immunoassay kits is they are easy to use. The Strip kit is stable at room temperature. By using a Strip kit, a centrifugation tube is the only device for performing a test and the test result can be obtained in 5~20 min. While the PCR method requires a thermocycler and an electrophoresis system; the ELISA method needs a color-reading instrument to scan the reaction products. The ELISA method needs 4~6 hr to complete one testing and storage of the ELISA kit at 4˚C is necessary. Nevertheless, the ELISA method can achieve a quantitative result in the range of 0~2.5% GMO content. It is easy to use and can efficiently perform a GM food detection. The advantages and instrumentation specialties of using PCR and immunoassay methods are summarized in Table 2. The users' need and purpose of detection determine which method should be used. Precision, speed, and the diversity in sample detection, are the factors that need to be taken into consideration when choosing a suitable method for GM food detection.

Using the PCR method, the regulatory gene is selected to be a target gene[5, 13]. The samples with negative reactions after screening are the samples containing no target gene. The positive samples, which contain the regulatory gene to be tested, need to be further confirmed by testing the specificity of the structure gene and detecting an endogenous gene. The samples with positive reaction are designated as RRS soybeans; while the samples with negative reactions in detection of the structure gene are classified as non-authorized GM-soybeans products. Based on the described above, to perform a PCR method, it is necessary to detect regulatory, structure and endogenous genes before reaching a conclusion. By using the above detection method, the test samples conducted in this study were confirmed to be RRS soybeans. The purposes of using the PCR method for GM food (or RRS in this study) detection are (1) to identify if the test samples are adulterated with the products which contain the artificially inserted genes, and (2) to identify the test samples as soybean products rather than other bean products. The immunoassay is specific to the recombinant proteins in GM food. Its detection is not only on inserted genes but also on the protein expressed by those inserted genes. Therefore, both precision and reliability are improved using immunoassay.

PCR is a kind of enzyme reaction. Any factors that affect an enzyme reaction could also result in a false PCR. These factors include improper preparation of the DNA template, primer and reagents, and existence of interference. A false negative could result from the above factors. A possible false positive reaction can occur if test plant samples are contaminated or infected by the *Cruciferae* group, since the 35S-promoter comes from the Cauliflower mosaic virus[5]. The non-GM plant contains no NOS-terminator, however, a NOS-terminator could exist in the roots of non-GM-plants resulting in a false positive PCR. This is because the NOS-terminator originates from *A. turmefaciens*, which is a microorganism flora existing in soil and could contaminate the roots of the plant[4]. Therefore, to ensure a proper PCR, a positive and a negative control reference is used to minimize a false reaction.

Some problems as follows could arise in developing a PCR or immunoassay method[13]. (1) Processed food: In highly processed or fermented foods, genes could be altered or proteins could be denatured, making detection difficult. (2) Varieties of GM products: a great quantity with different varieties of GM-crops is cultivated. (3) Mixture of GM products: different varieties of GM-crops are mixed either artificially or naturally. (4) Shortage of information regarding the gene sequence of the inserted gene, which could result in difficulty in designing a suitable primer[5, 13]. (5) Trade to countries where GM foods are unregulated could create a problem in GM food detection.

Table 2. Comparisons for PCR method and immunoassay kits in this study

	PCR method	Immunosassay	
		Strip	ELISA
Detection targets	structural 、regulatory and product-specific gene	Recombinant protein	Recombinant protein
Time	1-3 days	5-20 min	4-6 hours
Cost	Low	Medium	High
Instrument	Thermocycler	None	ELISA reader
Application	Qualitation	Qualitation	Quantitation（0-2.5%）
Detection Limit[a]	0.1%	0.3%	0.3%

[a]Detection limits depend on reference materials' concentrations.

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An inter-laboratory study among European countries for performing the precision of ELISA test demonstrated a satisfactory testing result with 99% confidence interval in qualitative analysis (of 1% detection limit) and 7% RSD_r (repeatability) and 10% RSD_R (reproducibility) in quantitative analysis (of 2% GMO content)[12]. However, some proteins in regular food could possibly react with ELISA causing a false reaction. A satisfactory result could also be obtained by modifying the extraction method to improve the extraction efficiency and selecting a proper reference to control cross-reaction as well as minimize the interference interaction. The above ELISA kit was not originally designed for quantitative purposes and only 4 concentration levels, 0%, 0.3%, 1.25%, and 2.5%, of standard references were provided and tested. Therefore, on the basis of this result, the calculation of more than 2 parameters is unpredictable and thus increases the variation in GMO content calculation.

In this study, we have developed a PCR and applied immunoassay kits method for RRS soybean detection. The detection limit could reach as low as 0.3% and 0.1% as using commercial Strip and ELISA kits, respectively. The future work in our laboratory will include evaluation of a DNA extraction method; and testing of different primers, GMOs or processed foods; and other detection methods in addition to the PCR method.

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以PCR方法與市售免疫套組檢測基因改造大豆

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摘　要

以PCR方法與市售免疫套組探討鑑別檢測基因改造大豆之可行性。針對Roundup Ready™基因改造大豆（RRS, Monsanto公司）殖入基因與品種特性基因選定不同引子，進行PCR與ELISA方法檢測。用以鑑別基因改造大豆之引子共四對，分別為35S（針對35S-promoter, 源自cauliflower mosaic virus）、NOS（nopaline synthase-terminator,源自*Agrobacterium tumefaciens*）、EPSPS（5-enolpyruvylshikimate-3-phosphate synthase，源自*A. tumefaciens* strain CP4）及LE（品種特性基因lectin）設計檢測。結果顯示，大豆檢體以35S及EPSPS引子檢測時，其最低檢測量均為0.1%（w/w），NOS則為1%（w/w）；檢體並以LE引子-PCR反應確定均為大豆產品。經以不同免疫檢測套組試驗，條片法較適用於定性之檢測，易於操作、攜帶和判讀；而ELISA方法除可鑑別基因改造大豆外，亦適用於含量在0-2.5%之檢體定量檢測。以市售免疫檢測套組檢測的結果與PCR法檢測的結果相符，均可區分鑑別一般大豆與RRS基因改造大豆。

關鍵詞：PCR，免疫檢測套組，基因改造大豆