JOURNAL OF
EMERGING INVESTIGATORS

Article

PCR technology for screening genetically modified soybeans

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SUMMARY

Genetically modified (GM) crops involve transferring a specific stretch of DNA into the plant's genome and giving it new or different characteristics. Since the first industrial application of transgenic crops in the United States in 1996, GM crops are becoming increasingly common in the market. At present, not all crops in the Chinese market are clearly marked as to whether they are GM crops or not. In order to protect consumer's option to select their preferred products, we decided to conduct experiments to identify soybeans on the market. We hypothesized that soybeans on the market with unknown origin were GM soybeans. Then we established a detecting method using genetic technology and screened the soybeans. In this study, we used GTS 40-3-2 as a positive control, which contains genomic DNA, foreign DNA enolpyruvylshikimate-3-phosphate synthase (EPSPS) and other exogenous elements, and GTS 40-3-2 SOYA BEAN (blank) as negative control, which contains genomic DNA only. To amplify the foreign DNA, we optimized the polymerase chain reaction (PCR) procedure by attempting different annealing temperatures to establish the PCR detection method. According to the electrophoresis results, in the samples from a local store, only genomic DNA lectin could be amplified, which indicated the samples were non-GM soybeans. Our study not only provides a detection method but also lays a foundation for testing soybean and processed soybean products of unknown origin.

INTRODUCTION

Since the first genetically modified (GM) food was developed in United States in 1996 , GM technology has been rapidly applied and developed in agricultural production (1). The global area for GM crops has continued to grow for the ninth consecutive year, from 1.7 million hectares in 1996 to 191 million hectares in 2019 (2). Genetically modified organisms are a group of organisms that have had their genomes altered using genetic engineering techniques (3). GM food has many advantages like herbicide-tolerance or insect-resistance, as well as a high production rate. However, GM food also poses some environmental and human safety concerns.

The widespread use of transgenic soybean benefits from the rapid development of transgenic technology, which introduces and integrates the desired genes into the genome of host organisms to improve the original traits of organisms or give them new beneficial traits. Since traditional breeding can only perform gene transfer on individuals of the same species, transgenic technology breaks the barrier of natural hybridization between different species and expands the range of available genes. The most prominent feature of GM crops is obtaining new exogenous genes, so that the crops get new traits. One of the methods used to transfer the exogenous DNA is through a bacterium or virus. For GM crops, the bacterium most frequently used is called *Agrobacterium tumefaciens* (4). First, the gene of interest is transferred into the bacterial plasmid. Then, the recombinant plasmid is inserted into the genome of the plant cells. Because of the presence of this exogenous DNA, GM crops can be identified through genetic sequencing.

Among the available methods to detect GM crops, PCR is generally accepted as the most sensitive and reliable method for analyzing the presence of exogenous DNA. PCR involves three processes (5,6). Firstly, in the denaturation step, the double-stranded template DNA is heated to separate into two single strands. Secondly, the DNA is annealed, where the complementary template DNA and primers are combined according to the base pairing principle in a lowered temperature. Thirdly, in the extension step, the temperature is adjusted to a proper temperature to produce a new DNA double-strand. The primers used for PCR are designed according to the exogenous DNA. If there is exogenous DNA in the crop's genome, the primers will attach to the specific location on the genomic DNA via hydrogen bonding. Thus, the exogenous DNA can be amplified and detected. Although several PCR primer pairs for GM crops analysis have been developed and published, the range of application of many primers is still limited. Also, many GM crops have no published primer pairs suitable for reliable identification or quantification.

The majority of GM crops have been transformed with constructs containing the Cauliflower Mosaic Virus 35S promoter (CaMV 35S) and the *A. tumefaciens* nopaline synthase terminator (t-NOS) (7). Consequently, PCR methods targeting CaMV 35S and t-NOS have been widely used for detecting GM crops. However, this method can only determine whether the crop is genetically modified, not which genes were transferred into the genome. Thus, PCR methods targeting the gene of interest are more specific than methods targeting elements in the transformed plasmid. The vast majority of commercially available GM soybeans are glyphosate-resistant (7,8). Glyphosate kills common soybean plants along with weeds. In the 1980s, Monsanto researchers cloned and obtained the glyphosate-resistance gene (EPSPS) from petunia plants and introduced the EPSPS gene into the

soybean genome to produce a soybean resistant to glyphosate (9). GM soybean GTS 40-3-2 expresses the EPSPS gene, which decreases the binding affinity for glyphosate, thereby conferring increased tolerance to glyphosate herbicide.

Nowadays, there are more and more GM foods in the supermarkets, and different people hold different views on them. People wonder what the advantages and disadvantages of GM food, and whether the benefits outweigh the risks. For those who are in favor of GM food, they think that GM technology can bring food with useful and new traits. Like improving the qualities of certain crops and producing safer food. It has been reported research into GM crops can create safer GM crop varieties by reducing levels of adverse substances such as mycotoxins, alkaloids and glucosinolates (10). Another benefit of GM food is increased yield while reducing the use of pesticides, improving plant adaptation to unfavorable environments (11). Herbicide-resistant and pest-resistant transgenic varieties as well as antiviral and fungus-resistant crops have been developed (12). While people who are against GM are concerned that GM food will have bad effects on people's health. It is likely that these exogenous genes may lead to cancer risks, allergenic potential and antibiotic resistance. Other potential risks associated with the use of GM food are environmental risks and threaten to biodiversity (13). For example, in the case of GM organisms, where an exogenous gene has been inserted into an organism, this network of genes is disturbed by the integration and expression of the exogenous gene (14).

Currently, not all GM commodities in the Chinese markets are clearly labeled, especially crops. In order to protect consumer's option to select their preferred products, we decided to establish detection method to identify soybeans on the market. We hypothesized that soybeans on the market with unknown origin were GM soybeans. Then we built the screening method using PCR technology and tested a positive control for GM soybean, negative control for non-GM soybean, and three samples with unknown origins with a variety of PCR primers to determine whether the samples were GM soybeans. The PCR screening protocol presented in this study should provide a very useful tool for routine GMO detection in food.

RESULTS

DNA Extraction of positive and negative control

Using the Plant Genomic DNA Kit, we extracted high-quality DNA from the samples. The purity of the extracted DNA was crucial for downstream analysis. Through electrophoresis we demonstrated that the DNA was the expected size (**Figure 1**). We assessed the purity through spectrophotometry based on the A260/280 was 1.8 and 2.0. Additionally, we determined the DNA concentration was high enough for subsequent PCR analyses with 205.2 ng/μL and 46.19 ng/μL for positive and negative control soybeans, respectively (**Table 1**).

The establishment of PCR detection method

Specificity of the Designed Primer Pairs

We used the primer pairs of lectin, CaMVp35S, and tNOS to conduct the primary screening of GM soybean. The lectin primers differentiated soybean from other grasses such as maize, barley, rice, and wheat. CaMVp35S and tNOS are universal primer pairs that can distinguish GM soybean from non-GM soybean. The construct of GM GTS 40-3-2 contained

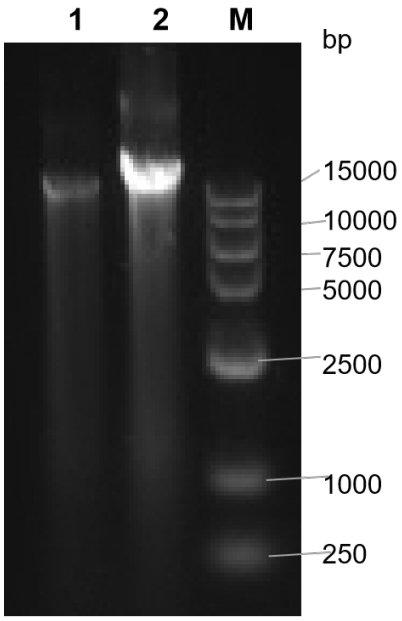

Figure 1: Genome of control soybean detected on 1% agarose gel. M:DL15000 DNA Maker; 1: negative control; 2: positive control.

Control group	260/280	Mean concentration (ng/μL)
positive control	1.8	205.20
negative control	2.0	46.19

Table 1: Purity and Concentration of Genome DNA from controls.

both CaMV35S promoter and NOS terminator genes. CP4-EPSPS primers targeting the EPSPS gene more specifically made it possible to identify the GM gene. The electrophoresis results suggested that these primers for the GTS 40-3-2 GM soybean were suitable for detecting the foreign DNA without amplifying the nonspecific bands.

PCR procedure

To amplify the foreign DNA, different annealing temperatures were tested to determine the optimum PCR protocol. We identified the optimum annealing temperature for each exogenous fragment. For genomic DNA lectin, PCR amplification was successful at all the temperatures from 55°C to 63°C in both positive and negative control soybeans (**Figure 2A**). Exogenous DNA CaMVp35S was amplified at all the temperatures from 55°C to 63°C only in positive control soybean (**Figure 2B**). tNOS was amplified at 55°C, 56.4°C and 58.3°C only in positive control soybean (**Figure 2C**). Cp4 EPSPS was amplified at 62.7°C only in positive control soybean (**Figure 2D**).

Testing for samples of unknown origin

We purchased the soybean samples 1, 2, and 3 from different sources used for detection at a local store. Spectrophotometer results indicated that the A260/280 was 1.8, 1.8, and 1.9, and the DNA concentration was 40.12 ng/μL, 50.62 ng/μL, and 40.35 ng/μL for samples 1, 2, and 3, respectively (**Table 3**). For PCR detection, the results of three

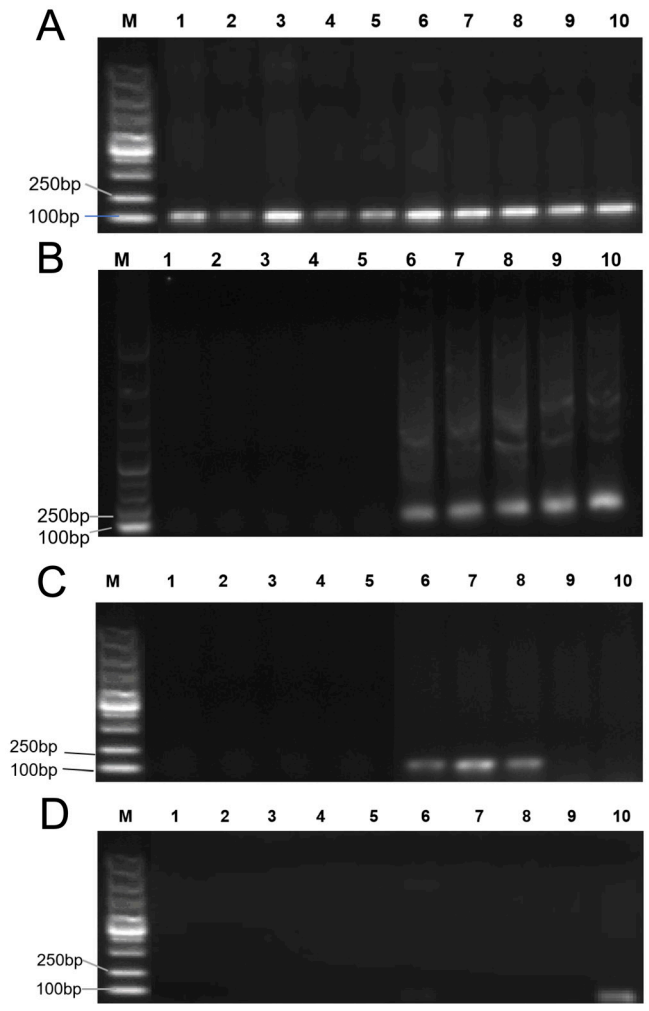

Figure 2: Lectin-, CaMV35s-, tNOS-, and CP4-EPSPS-PCR results. A) Lectin-PCR. 1-5: positive control; 6-10: negative control; 1/6: 55°C, 2/7: 56.4°C, 3/8: 58.3°C, 4/9: 60.5°C, 5/10: 62.7°C. B) CaMV35s-PCR. 1-5: negative control; 6-10: positive control; 1/6: 55°C, 2/7: 56.4°C, 3/8: 58.3°C, 4/9: 60.5°C, 5/10: 62.7°C. C) tNOS-PCR: 1-5: negative control; 6-10: positive control; 1/6: 55°C, 2/7: 56.4°C, 3/8: 58.3°C, 4/9: 60.5°C, 5/10: 62.7°C. D) CP4-EPSPS-PCR: 1-5: negative control; 6-10: positive control; 1/6: 55°C, 2/7: 56.4°C, 3/8: 58.3°C, 4/9: 60.5°C, 5/10: 62.7°C.

trials for each sample were consistent (**Figure 3**). In samples 1, 2, and 3, only genomic DNA lectin was amplified. While in the positive control soybean, all the genes were amplified, and none of the genes besides lectin were amplified in the negative control soybean. These results indicated that the samples were non-GM soybeans.

DISCUSSION

Soybean and corn are the two GM crops with the largest planting area globally and the largest amount of GM crops imported by China (9). Large-scale cultivation of GM crops not only improves the lives of poverty-stricken people in developing countries, but also reduces pesticide use and increases the global vegetation area (9). However, with the expansion of the GM crops planting area, the public began to pay more attention to the safety of the GM crops. The

Target	Name	Sequence (5'→ 3')	Specificity	Length (bp)
CaMV35S	CaMV35S-F	TTGAAGATGCCTCTGCCGACA	p35S	101
	CaMV35S-R	ATTG TGCGTCATCC CTTACGTC	p35S	
tNOS	tNOS-F	GGTCTTGCGATGATTATCATATAATTTCT	tNOS	151
	tNOS-R	AATGTATAATTGCGGGACTCTAA TC	tNOS	
CP4-EPSPS	CP4-EPSPS-F	GGTCTTGCGATGATTATCATATAATTTCT	CP4	180
	CP4-EPSPS-R	AGCCCTGCAG CATCTTTTCCGTA	CP4	
Lectin	Lectin-F	GGTCTTGCGATGATTATCATATAATTTCT	Lectin	118
	Lectin-R	GCCCATCTGCAAGCCTTTTTGTG	Lectin	

Table 2: List of Primers for Qualitative PCR (6).

Sample group	A260/280	Mean concentration (ng/μL)
Sample 1	1.8	40.12
Sample 2	1.8	50.62
Sample 3	1.9	40.35

Table 3: Purity and Concentration of Genome DNA from samples.

inevitable uncertainty make the safety of GM crops become the focus of global concern.

According to the Eurobarometer polls conducted in the twenty-five Member States of the European Union between September 2 and October 6, 2005, when asked to what extent they were worried about genetically modified production food or drinks, 25% of EU citizens answered “very worried” and 37% answered “fairly worried” (15). In addition to concerns about the health and environmental risks of GM food, people have also expressed worries about the unknown future implications of GM technology. Some respondents noted that all testing should be independent and the results published (16). Therefore, it is important to monitor the food chain and promote the labelling of GM foods. Also, it is of great significance to establish appropriate and highly sensitive detection methods for GM foods to ensure that GM ingredients can be controlled within the appropriate range.

There are mainly two methods for transgenic detection: Nucleic acid-based PCR detection and the detection of the expression products of foreign genes, including strip ELISA and protein chip methods (17). Nucleic acid-based detection methods mainly identify the specific exogenous

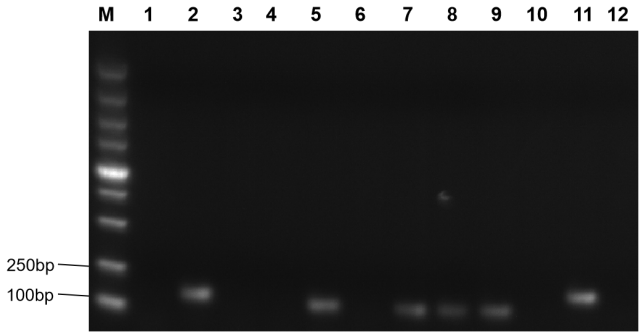

Figure 3: Sample detection. 1-3: CaMV35S, 1: sample, 2: positive, 3: negative; 4-6: tNOS, 4: sample, 5: positive, 6: negative; 7-9: Lectin, 7: sample, 8: positive, 9: negative; 10-12: CP4-EPSPS, 10: sample, 11: positive, 12: negative.

DNA integrated into the genomes of transgenic plants. A key step in PCR detection is the extraction of crop genomic DNA. The kit used in this study successfully extracted genomic DNA from three soybeans of unknown sources, and DNA purity was high. Nevertheless, the kit is not suitable for the genomic extraction of processed soy foods due to highly degraded DNA, such as soy powder and soybean oil. The hexadecyltrimethylammonium bromide (CTAB) method is more suitable for extracting processed soybean DNA (18). Unfortunately, this method involves toxic organic reagents, and we had no access to purchase them, so tests for highly processed soy foods could not be completed.

PCR detection technology has been widely used, and it has become one of the internationally recognized standard techniques for transgenic detection. In our study, we used normal transgenic detection as a qualitative detection method. As early as 2003, qualitative PCR detection has become the industry standard of entry-exit inspection and quarantine system, and now it is widely used to detect transgenic ingredients in import and export commodities (19). To quantify the sample, real-time fluorescence PCR technology would be required, which involves the addition of fluorescent groups into the PCR reaction system, monitoring the PCR process in real-time by the accumulation of fluorescent signals, and finally conducting quantitative analysis on unknown templates through standard curves (20). Due to instrument limitations, we could only carry out qualitative detection through standard PCR.

In our PCR system, the ratio of template DNA and primer concentration is critical. After the first experiment, we detected many other excessive bands in addition to the specific target bands by electrophoresis. Therefore, in the subsequent experiments, we increased the amount of template DNA to 50 ng and reduced the concentration of primers to 1 μM in one reaction (20 μL). After this adjustment, the electrophoresis results showed that no nonspecific bands were amplified. Simultaneously, some foreign DNA was not amplified even in the positive control soybean genome. Therefore, we continued to optimize the annealing temperature at which the primer and the template bind. Under ideal conditions, the annealing temperature is low enough to ensure effective annealing of the primer with the target sequence, and high enough to reduce non-specific binding (20). We explored the amplification effects of different annealing temperatures, and the results showed that only one specific temperature could amplify specific bands for some foreign genes. Therefore, for a PCR reaction, the concentration of template DNA and primers and the selection of annealing temperature are essential.

We have successfully established the PCR detection method for determining if soybeans contain exogenous DNA, and the samples we tested were non-GM soybeans. Many follow-up experiments could be further conducted, such as using the CTAB method to extract the highly processed soybean genome or exploring the concentration of transgenic components in the soybean genome through real-time fluorescence PCR. Our research offers insights into a deeper understanding of different exogenous gene in GM soybeans and provides a testing method for the identification of GM foods, while better protecting consumers' right to know so that they can choose food that are more suitable for them.

MATERIALS AND METHODS

Soybean Samples

GTS 40-3-2 and GTS 40-3-2 SOYA BEAN (blank) used as positive and negative controls were bought from Shanghai Zhenzhun Biotechnology Co., Ltd. The soybeans used for detection were purchased at a local store.

Oligonucleotide Primers designing

The DNA sequences of the CaMV 35S promoter, the NOS terminator, the Cp4 EPSPS gene, and the soybean lectin gene have been published in the GenBank database (20). The primer pairs were designed based on the DNA sequences and synthesized by Genewise Company (Suzhou, China). The sites of primers and their nucleotide sequences are shown in Table 2.

DNA Extraction

For extraction of genomic DNA from the GTS 40-3-2, GTS 40-3-2 SOYA BEAN (blank), and test samples, the Plant Genomic DNA Kit (Qiagen) was used according to the published protocol. The quality of the extracted DNA was measured using a Microultraviolet spectrophotometer (Unico, UPT-100). The purity of the DNA was determined by the ratio of A260/ A280. To determine the concentration of the extracted DNA, triplicate measurements were taken and the average value was calculated as the concentration. The genomic DNA was analyzed on 1% (w/v) agarose gel and molecular weight marker DL 15000 (Qiagen) was used to estimate size.

The establishment of PCR detection method

PCR was performed to confirm the specificity of the designed primers on genomic DNA extracted from non-GM soybean and GM soybean. Amplification reactions were carried out in a 20 μL total volume on a TC1000-G thermocycler (DLAB, Inc., Beijing). End concentrations of PCR components were as follows: 50 ng DNA template; 1 μM forward and invert primers, each; PrimerSTAR Max DNA Polymerase (Takara), 10 μL/reaction. Different annealing temperatures were tested to determine the optimum PCR procedure. The gradient PCR conditions were as follows: denaturation at 94°C for 10 min, and 25 cycles of 94°C for 30 s, annealing temperatures from 55°C to 63°C for 30 s, extension at 72°C for 30 s, followed by a final extension 72°C for 7 min. The PCR products were analyzed on 1.5% (w/v) agarose gel and molecular weight marker DL 5000 (Takara) was used to estimate size.

Testing for samples of unknown origin

The samples were purchased from three different markets. Samples 1 and 2 were both in bulk without trademark. Sample 3 with brand was from Northeast China. Then the soybeans were wiped with alcohol and placed in the oven for sterilization. Afterwards, they were ground into powder with a mortar. 100 mg of powder was weighed, and the genomic DNA was extracted. CaMVp35S, tNOS and CP4 EPSPS primer pairs were used to verify whether the samples were GM soybeans, and for each set of primers the optimum annealing temperature was identified. Thus, CaMVp35S was amplified at all the temperatures from 55°*C* to 63°*C*; tNOS was amplified at 55°*C*, 56.4°*C*, and 58.3°C ; and Cp4 EPSPS was only amplified at 62.7°*C* . Three trials were carried out for each sample to minimize random errors. Also, soybean powder and oil were tested.

Received: June 7, 2021
Accepted: July 21, 2021
Published: November 16, 2021

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