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Detection of genetically modified soybean and its product tou-kan by polymerase chain reaction with dual pairs of DNA primers

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Abstract Since genetically modified (GM) crops and foods began to appear in market, the detection of these GM foods has become an important issue. Efficient and reliable methods are urgently needed to analyze the GM content of a large quantity of foods. However, most foods are processed through heating or cooking, and their proteins are denatured. Therefore, DNA rather than protein is the major target for detecting GM foods. In this report, polymerase chain reaction (PCR) was performed with dual sets of DNA primers, which co-amplified the soybean-specific lectin gene and the transgenic petunia transit peptide sequence (CTP) in the 5' end of the 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) gene within the same reaction. PCR with these two sets of primers reacted specifically with lectin gene and CTP, and can detect soybean with GM content less than 1%. PCR detecting both lectin gene and CTP was also applied to a soybean-derived product tou-kan, a traditional oriental food. Results indicated that although DNA was partially degraded in tou-kan, this PCR method was still able to detect the presence of EPSPS gene. However, when the DNA within tou-kan was destroyed badly, neither lectin nor EPSPS genes were detected by the PCR suggested here. Finally, an examination procedure for the Roundup Ready soybean was suggested according to the results of PCR with dual pairs of DNA primers. The proposed PCR method has proved to be a reliable and efficient method for detecting the GM content of the processed foods from Roundup Ready soybean.

Keywords Genetically modified soybean · Tou-kan · Polymerase chain reaction

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Introduction

Genetically modified (GM) foods are derived from transgenic crops with foreign genes, and are now widely available in markets around the world. Owing to the concern of consumers, the labeling of GM foods has become mandatory in many countries. The threshold limits for the proportion of GM within foods to be labeled with GM foods are 0.9% for the EU [1], and 5% for Japan [2] and Taiwan [3]. Several methods have been developed for detecting GM crops. However, most foods are highly processed from crops, and proteins are denatured or destroyed during preparation. Hence, it is inadequate to use protein-based methods to detect GM foods. On the contrary, DNA can stand high heat, and becomes a major target for analyzing GM foods.

Polymerase chain reaction (PCR) using DNA as a template has been established for the detection of GM foods. Quantitative PCR such as competitive PCR [4–7] is complicated and time-consuming method, while real time PCR [6, 8–11] is expensive [9]. Qualitative PCRs, such as simple PCR and duplex PCR, without appropriate control may result in the occurrence of false positive and false negative results [12]. In particular, DNA from processed foods may not be intact, PCR from partially degraded DNA may increase the uncertainty for distinguishing between GM and non-GM foods. Accurate and efficient methods for analyzing the various GM foods and additives are still needed to perform large screening of foods.

Soybean has been processed into various products such as bakery products, infant formula, complex meat, and tofu. Hence, the purpose of this study is to develop reliable procedures to detect GM soybean and its processed products. The target GM soybean is the Roundup Ready soybean, which is glyphosate resistant due to the incorporation of a 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) gene with the petunia transit peptide sequence (CTP) in the 5' end. Both lectin, a soybean-specific gene, and CTP, the transgenic sequence, were analyzed by PCR with dual pairs of DNA primers at the same reaction. Furthermore, the detection of the lectin gene was treated as an internal control

during PCR test, and both false positive and false negative results from PCR can be distinguished. This PCR procedure with duo sets of primers is an easy and efficient method, and can be used to screen GM foods in large quantity.

Materials and methods

Materials

Both non-GM (MT21) and GM (Roundup Ready) soybeans were kindly provided by the American Soybean Association of Taiwan.

DNA isolation

DNA of soybeans and their products was purified by the method described by Taylor and Powell [13]. This method for isolating DNA was further modified in the following manner. Soybeans and their products (1 g) were ground in liquid N₂, and then 2 ml of hexadecyltrimethyl-ammonium bromide (CTAB) buffer (20 g/l CTAB, 1.4 M NaCl, 0.1 M Tris-HCl, pH 8.0, and 20 mM EDTA) was added to 1 g of the sample powder. After incubation at 60°C for 10 min, the samples were centrifuged. The supernatant was transferred to a new tube, and extracted with an equal volume of chloroform. The upper phase had the CTAB precipitation buffer (5 g/l CTAB and 0.04 M NaCl) added, and stayed in room temperature for 60 min. After another centrifugation, the precipitant was dissolved by 2 M NaCl, and extracted by an equal volume of phenol:chloroform:isoamyl alcohol (24:23:1). The upper phase was added with 0.6 volume of isopropanol, and DNA was precipitated by centrifugation. The quantity of DNA was estimated by a spectrophotometer, while its quality was analyzed by agarose gel.

PCR reactions

PCR was performed by RapidCycler (Idaho Technology), and contained 1 × PCR buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl₂, 50 mM KCl, and 0.1% Triton X-100), 0.2 mM dNTP, 0.5–0.8 μM primer (each), 1 unit Biotaq DNA polymerase (Bioline, London, UK), and 0.5 μg DNA template. PCR reactions were performed as follows: 5 min at 94°C for denaturation, followed by 35 cycles of 20 s at 94°C, 30 s at 56°C, and 30 s at 72°C. Finally, an additional elongation step of 5 min at 72°C was applied. PCR products were analyzed by electrophoresis with 2% agarose stained with ethidium bromide [14]. Binding positions and characteristics of DNA primers for PCR reactions are listed in Table 1.

Plant materials and glyphosate application

Soybeans were germinated and grown in a controlled environment (16 h/25°C day, 8 h/22°C night, humidity

Table 1 DNA primers used in this work

Name	Sequence (5' to 3')	Binding region
35S3	TTC GCA AGA CCC TTC CTC TA	35S promoter
CTP	TGT ATC CCT TGA GCC ATG TT	CTP ^a
LEE	CGG AAT TCC TCT ACT CCA CCC CCA T	5' of lectin gene
LEX	GCT CTA GAT GGA TCC CAA GAG TTC CG	3' of lectin gene

^aCTP is the petunia transit peptide sequence, which is located at the 5' end of the EPSPS coding region within the Roundup Ready soybean

70%, light 150 μmol photons/m²/s). Glyphosate (2%) was sprayed onto the soybean plants with six to eight fully developed leaves. The leaves were observed 4 to 6 days later to see if they had wilted [15].

Production of tou-kan

The production of tou-kan was described [14], and modified as follows. Soybeans were soaked with water overnight, and the hydrated beans were ground with equal weight of water by blender for 5 min. After being filtrated with a piece of three-layer thin cloth, the soymilk was collected and boiled for 5 min. When the temperature of the soymilk went down to about 73°C, the final 0.4% (w/v) of CaSO₄·H₂O was added with a 250 rpm stirring for 20 s. Then, the boiled soymilk was cooled to room temperature slowly for the formation of soybean curd. The bean curd was transferred to cloth-lined plastic box (12.5 cm × 10.5 cm × 6.5 cm), which contained holes in the bottom and was a holder for blue tip originally. Tou-kan was produced by a constant pressure of about 20 g/cm³ applied to the bean curd in box to squeeze out water at 4°C for 10 h. Tou-kan was then boiled in soy sauce with 1% sugar for 1, 2, 3, and 5 h to become seasoned tou-kan, which was further toasted at 70°C for another 3 h to obtain toasted tou-kan. DNAs of both seasoned and toasted tou-kan were isolated and analyzed by PCR.

Results and discussion

Detection specificity of PCR with dual DNA primer sets

The major target for detecting Roundup Ready soybean is the EPSPS gene transferred into soybean genome. Therefore, the presence of EPSPS gene detected by PCR indicates the presence of GM soybean. However, PCR reaction not resulting in the presence of the EPSPS gene does not imply that the soybean under investigation is non-GM. Several possibilities including impurity of DNA and failure of PCR may also contribute to the negative results, and cause false negative results. Therefore, the detection of the soybean-specific gene, lectin, is treated as a control during PCR. The occurrence of lectin DNA fragment after PCR may reflect

Fig. 1 Specificity examination of DNA primers within PCR. PCR tests were performed with one or two sets of DNA primers. The soybean-specific lectin gene was amplified by primers LEE and LEX, while the transgenic CTP in the 5' end of EPSPS gene was detected by primers 35S3 and CTP. Sequences of primers are listed in Table 1

that DNA purification and PCR reactions are performed well.

The primers, 35S3 and CTP (Table 1), for analyzing the target gene, EPSPS, were designed to bind 35S promoter and CTP, and produced a 120-bp DNA fragment after PCR reaction. The possibility of false positive for detecting the DNA fragment between 35S promoter and CTP is much lower than that for analyzing 35S promoter alone (data not shown). Moreover, soybean-specific gene, lectin, was analyzed by primers LEE and LEX, and their PCR product was a 254-bp DNA fragment, which was easily distinguished from the 120-bp DNA fragment produced from the 35S3/CTP primer set (Fig. 1). DNA from transgenic soybean was amplified by PCR with primers 35S3/CTP and LEE/LEX sets, respectively, and only one DNA fragment with expected molecular weight was revealed in each reaction (Fig. 1). In addition, when both primer sets, 35S3/CTP and LEE/LEX, were applied to PCR at the same tube, two DNA fragments corresponding to CTP and lectin gene fragments were shown. These results indicated that the presence of both EPSPS and lectin genes from soybean genomic DNA can be examined by dual primer sets 35S3/CTP and LEE/LEX in one PCR reaction specifically.

Detection sensitivity of PCR with dual DNA primer sets

In order to perform sensitivity assay, soybeans with known percentage of GM content were required as standards. Therefore, GM and non-GM-labeled soybeans were examined by the application of glyphosate. Two hundred seeds of each GM and non-GM-labeled soybeans were germinated and applied with glyphosate until they had six to

Fig. 2 PCR examination of soybean grains. The GM-labeled soybeans were germinated within soil for 4 days. The un-germinated soybeans were retrieved from soil, and examined by PCR with dual sets of primers LEE/LEX and 35S3/CTP for detecting lectin gene and the CTP in the 5' end of EPSPS gene, respectively. Primer dimerization of PCR is indicated as dimer, while M is the DNA marker

eight leaves. Not all GM-labeled soybeans with EPSPS gene were glyphosate resistant, while all non-GM-labeled soybeans became wilted 2 days later. PCR with primers 35S3/CTP and LEE/LEX further confirmed that non-GM-labeled soybean were 100% GM free (data not shown). However, only 94% of GM-labeled soybeans germinated, and 90% of the GM-labeled soybeans survived the treatment of glyphosate. Since the glyphosate test cannot be applied to the un-germinating soybeans, the un-germinated GM-labeled soybeans retrieved from soil were examined by PCR with primers 35S3/CTP and LEE/LEX grain by grain (Fig. 2). Results indicated that one out of six un-germinated GM-labeled soybeans were actually GM free (reaction 4 in Fig. 2). PCR result also showed DNA fragment from 35S-CTP region of the EPSPS gene only (reaction 2 in Fig. 2). This may be due to the partial degeneration of soybean DNA in wet soil for 5 days; compared with lectin gene, 35S-CTP DNA region can be easily amplified by PCR with both primers 35S3/CTP and LEE/LEX. Consequently, 95% of the GM-labeled soybeans are really GM soybeans according to the results from glyphosate tolerance assay and PCR test.

DNA of both GM- and non-GM-labeled soybeans was isolated. DNA from the GM-labeled soybean was treated as 95% GM content, and thus the proper amount of GM and non-GM DNA was mixed so that soybean DNA with 0, 0.2, 1, 5, 20, and 95% GM DNA was obtained to perform sensitivity assays. These DNAs were amplified by PCR with primers 35S3/CTP and LEE/LEX. The 120-bp DNA from PCR with primers 35S3/CTP was observed in reaction with as low as 1% GM DNA (Fig. 3), while the 254-bp PCR product from soybean-specific lectin gene was present in every reaction as expected. Moreover, the PCR product from 35S3/CTP was also barely seen in the DNA with 0.2% GM content (Fig. 3). Therefore, the sensitivity of PCR with primers 35S3/CTP and LEE/LEX is good enough to examine GM soybeans.

Fig. 3 Sensitivity of PCR with dual sets of DNA primers. Genomic DNAs were purified from GM and non-GM soybeans separately, and the proper amount of these DNAs was mixed so that DNAs containing 0, 0.2, 1, 5, 20, and 95% GM content were obtained. PCR tests were performed with primers LEE and LEX to detect soybean-specific lectin gene fragment, and primers 35S3 and CTP to analyze the transgenic CTP in the 5' end of EPSPS gene. M is the DNA marker

GM content for processing of soybean to its product tou-kan

Tou-kan is a traditional food, and an important protein source for oriental countries. Tou-kan is always treated with soy sauce, and may be further toasted. Therefore, the GM content of soybean during the processing to seasoned tou-kan was monitored by PCR. GM-labeled soybean was first processed to become tou-kan [16]. Seasoned tou-kan was made by placing tou-kan in soy sauce with 1% sugar for 1, 2, 3, and 5 h, and was further toasted for another hour. DNAs of both seasoned and toasted tou-kan were isolated and analyzed by PCR with dual sets of primers LEE/LEX and 35S3/CTP (Fig. 4). PCR result indicates that both EPSPS and lectin genes were only present in tou-kan without any further treatment and that boiled in soy sauce

Fig. 4 GM content for the processing of soybean to its product tou-kan. Soybean was first processed to become tou-kan, which was further boiled in soy sauce for 1 h (r1), 2 h (r2), 3 h (r3), and 5 h (r5). Then, the cooked tou-kan was toasted at 70 °C for another 3 h (r1t, r2t, r3t, r5t). Their DNAs were then isolated and analyzed by PCR with primers LEE/LEX and 35S3/CTP to detect lectin gene and CTP, respectively. PCR reactions without DNA template (no DNA) were also included as controls. Primer dimerization of PCR is indicated as dimer

Fig. 5 PCR examination of commercial tou-kan. **A** Qualities of DNAs isolated from soybean and tou-kan. DNA was isolated by modified CTAB method as described in Materials and Methods section. Intact and degraded DNA templates are indicated. **B** PCR products of soybean-derived tou-kan. DNAs as in (A) were amplified by PCR with primers LEE/LEX and 35S3/CTP to detect lectin gene and CTP, respectively. Dimer is primer dimerization of PCR, and M is the DNA marker

for only 1 h. Also, tou-kan boiled in soy sauce for more than 1 h and all toasted tou-kan did not show the existence of EPSPS and lectin genes. These results indicate that DNA in soybean products was destroyed so heavily that even PCR cannot detect the presence of EPSPS and lectin genes once tou-kan was boiled over 1 h in soy sauce or toasted for 3 h.

Primer dimers with molecular weight of less than 100 bp were observed in Figs. 2, 4, and 5. The formation of primer dimers was related to the sequence of primers involved in PCR. The partial binding of primers by themselves was amplified by PCR, and resulted in the DNA fragments with molecular weight below 100 bp. Primer dimers were present when PCR with primers LEE and LEX in the absence of soybean DNA was performed (reactions 1 and 3 in Fig. 4). However, primer dimerization did not occur for PCR with primers 35S3 and CTP alone (reaction 2 in Fig. 4). Furthermore, the presence of primer dimers may be related to the degree of DNA degradation. Primer dimers were present in the reaction with partially degraded soybean DNA whose lectin gene cannot be amplified (reaction 2 in Fig. 2 and reactions 4–6 in Fig. 5), but absent in the

reactions whose DNA was degraded badly and that both EPSPS and lectin genes could not be detected (reactions 7–13 in Fig. 4). Conclusively, primers LEE and LEX may be the cause for the formation of primer dimers during PCR reaction.

Detection of commercial tou-kan

The assay condition developed above for GM soybean products was applied to commercial tou-kan. Tou-kan and several seasoned tou-kans were purchased from market place. DNAs of these tou-kan and seasoned tou-kans were purified as described in Materials and Methods section, and shown in Fig. 5A. DNAs from GM- and non-GM-labeled soybeans were also analyzed as controls, and displayed intact forms with high molecular weights. However, DNAs of all these tou-kan were degraded (Fig. 5A). The DNA length of plain tou-kan ranged from 600 to 150 base pairs (reaction 3 in Fig. 5A), while those for both seasoned tou-kans were from 450 to 100 base pairs (reactions 4 and 5 in Fig. 5A). Furthermore, DNA of the dry seasoned tou-kan was degraded badly, and was 200–80 base pairs in length (reaction 6 in Fig. 5A). These results indicated that processing of soybean to tou-kan degraded genomic DNA, and baking may heavily destroy DNA.

PCRs with primers 35S3/CTP and LEE/LEX were performed to analyze DNAs from these tou-kans and soybeans as controls (Fig. 5B). As expected, PCR reaction from non-GM soybeans produced one DNA fragment from lectin gene, while that from GM soybeans showed two DNA fragments from EPSPS and lectin genes (reactions 1 and 2 in Fig. 5B). DNA from plain tou-kan also displayed lectin and CTP DNA fragments produced by PCR, indicating that even DNA of plain tou-kan was degraded but its lectin and EPSPS genes were detectable (reaction 3 in Fig. 5B). However, PCR results from seasoned tou-kans 1 and 2 only produced one DNA fragment from CTP, and the soybean-specific lectin gene was not detected. These results reveal that these seasoned tou-kans still comprised GM content, but their DNAs were partially degraded (reactions 4 and 5 in Fig. 5A) so that the lectin gene cannot be amplified by PCR. Additionally, DNA of seasoned dry tou-kan was destroyed so badly that both DNA fragments of CTP and lectin gene were not obtained (reaction 6 in Fig. 5B). Consequently, it is unlikely to know the DNA content of dry seasoned tou-kan.

According to the results of Figs. 4 and 5, the outcome of DNA lengths after DNA degradation is correlated with its availability for PCR test. When the length of the largest DNA after degradation was still larger than 500 bp, both lectin and EPSPS genes were detectable by PCR, as seen in the DNA from plain tou-kan (reaction 3 in Fig. 5B). While the length of the DNA was smaller than 500 bp but larger than 100 bp after DNA degradation, EPSPS was still detectable by PCR, as seen in the DNA of seasoned tou-kans (reactions 4 and 5 in Fig. 5B). Finally, when the length of the largest DNA fragment after DNA degradation was smaller than 200 bp, both lectin and EPSPS genes could not

Lectin PCR product	EPSPS PCR product	
+	+	It contains Roundup Ready soybean.
+	-	It does not contain Roundup Ready soybean.
-	+	1. DNA is partially degraded, but contains Roundup Ready soybean. 2. It is a false positive result.
-	-	1. DNA is totally destroyed. 2. It is a false negative result.

Fig. 6 Detection results of the Roundup Ready soybean by PCR with dual sets of DNA primers. DNA was purified from soybean and its products, and amplified by PCR with DNA primers LEE/LEX and 35S3/CTP detecting lectin gene and CTP, respectively. A *plus* symbol indicates the appearance of a correct DNA fragment after PCR, while a *minus* symbol represents the absence of the corresponding DNA fragment

be detected by PCR, as seen in the DNA from dry seasoned tou-kan (reaction 6 in Fig. 5B). Therefore, the quality of DNA has a significant impact on the results of PCR.

Examination procedure for Roundup Ready soybean with dual pairs of DNA primers

The dual sets of primers LEE/LEX and 35S3/CTP within PCR detect lectin and EPSPS genes, respectively. The presence and absence of lectin gene and CTP fragments after PCR represent different situations as displayed in Fig. 6. Lectin gene is specific for soybean species, and thus the appearance of lectin DNA fragment after PCR not only indicates that the PCR by itself undergoes well but also points out that the quality of DNA is good for PCR test. Therefore, under the presence of lectin DNA fragment after PCR, the appearance of EPSPS DNA fragment verifies the presence of GM content, while the absence of EPSPS DNA fragment indicates that the sample tested is GM free. On the contrary, if the lectin gene is not detected but the EPSPS gene is present after PCR, it may indicate that DNA is partially degraded, and the sample tested still contains GM content. Otherwise, it is a false positive result, and the PCR fragment needs further treatment of DNA sequencing or restriction enzyme digestion to understand its origin. Finally, the absence of both lectin and EPSPS DNA fragments after PCR indicates that DNA is totally destroyed, or PCR does not occur at all. Then, it is a kind of false negative. Interestingly, the primer dimerization during PCR may occur, and can be used as an indicator that the PCR by itself works well.

Most food goes through a series of processing, and the quality of its DNA becomes questionable for PCR test. Therefore, a reliable and efficient method is required for analyzing the DNA from food materials. In this study, PCR with primers LEE/LEX detects soybean-specific lectin gene, while that with 35S3/CTP amplifies target EPSPS gene at the same time. PCR with dual sets of DNA primers provides much more information than that with a single set

of primers, and is an efficient detection method for large screening. PCR analyzing two genes at the same time also monitors the occurrence of false positive and negative results. Therefore, PCR with dual sets of DNA primers is also a reliable way to analyze GM foods.

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