DETECTION OF THE GENETICALLY MODIFIED SOYBEANS IN PROCESSED FOODS

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INTRODUCTION

Genetically modified (GM) foods and now widely available on global markets. Because of consumer concern over these crops, the labeling of GM foods has become mandatory in many countries, include most countries in Asia. The purpose of this study was to develop reliable procedures to detect transgenic soybean s in processed foods. The processing of soybeans includes heating and/or fermentation. During this process, most of the proteins in the soybean are denatured. Therefore, relatively heat-resistant materials, such as DNA, are the main target for detecting GM soybeans in processed foods. Polymerase chain reaction (PCR) is a sensitive method of analyzing DNA. It is considered the most important method of detecting GM soybeans in processed foods. The target transgenic soybean is Roundup Ready, a soybean which is resistant to the weed killer glyphosate. The resistance is the result of the incorporation of a 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS) gene.

MATERIALS AND METHODS

Materials

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

DNA purification

The method used to purify the DNA from soybean and its products follows that described by Murray and Thompson (1980) and Taylor and Powell (1982) method was modified in the following manner. Soybean and its products were ground to a fine powder. Then 15 ml of CTAB (hexadecyltrimethyl-ammonium bromide) buffer (20 g/L CTAB, 1.4 M NaCl, 0.1 M Tris-HC1 pH 8.0, and 20 mM EDTA) were added to 1 gram of the sample powder. The samples were incubated at 60°C for 60 min, and then centrifuged. The supernatant was transferred to a new tube, and extracted with an equal volume of chloroform. The upper phase was added with CTAB precipitation buffer (5 g/L CTAB and 0.04 M NaC1), and the sample was kept at room temperature for 60 min. After another centrifugation, the precipitant was dissolved by 2 M NaCl, and extracted by phenol:chloroform:isoamyl alcohol (24:23:1). The upper phase was added with 0.6 volume of isopropanol, and DNA was precipitated by centrifugation.

PCR reaction

PCR was performed by GeneAmp 9700 (Perkin-Elmer), GeneAmp PCR System 2400 (Perkin-Elmer), or Rapid Cycler (Idaho Techology). Each sample contained 1 PC

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buffer (10 mM Tris-HC1 pH 8.8, 1.5 mM $MgC1_2$, 50 mM KC1, and 0.1% Triton X-100), 0.2 mM dNTP, 0.5 – 0.8 M primer (each), 1 unit of DNA polymerase (DyNAzyme), and various amounts of DNA template, as indicated by each reaction. Real-time PCR was run on a LightCycler (Roche), and PCR products were labeled with SYBR green 1 for detection. Binding positions and characteristics of DNA primers for PCR reactions are listed in Table 1.

RESULTS AND DISCUSSION

GM and non-GM soybeans

Soybean seeds were germinated, and glyphosate was applied to distinguish the GM soybeans from the non-GM soybeans. All plantlets from non-GM soybeans did not survived the glyphosate treatment. This plantlets from non-GM soybeans did not survived the glyphosate treatment. This indicates that soybeans which are non-GM do not contain any glyphosate resistance gene. This result was further confirmed by the use of PCR with amplified genomic DNA from non-GM soybeans. The EPSPS DNA fragment could not be detected in this reaction. In contrast, 95% of the GM soybeans were found to contain EPSPS DNA.

Effect of heat on DNA templates for PCR testing

Most soy-derived products are processed by heating. This heating may affect the quality of the DNA template for PCR detection. Soybean DNA was purified, and individual samples were heated at 100°C for 10, 20, 30, 60, 120, and 180 min, respectively. Another series of samples were heated at 121°C for 10, 20 and 30 min, respectively. The quality of the DNA of the samples was then analyzed, using PCR reactions to detect the integrity of the lectin gene. DNA templates of all treatments could be analyzed by LeC primers, which produce a 318 bp-DNA fragment after the PCR reaction (Fig. 1). LeD primers, which generate 444 bp DNA in PCR reaction, cannot detect DNA templates treated at 121°C over 20 min (Fig. 2). Furthermore, the 628 bp-DNA fragment cannot be revealed by primers LcE after DNA templates processed at 100°C over 120 min or 121°C over 10 min (Fig. 3). These results indicate that heating process does damage DNA template for PCR reaction, but PCR generating the smaller DNA fragment still can detect the presence of DNA.

Effect of fermentation on the DNA of soybean

Fermentation is an important process in the manufacture of soybean products. During fermentation, most of the DNA is destroyed in order to detect the small amount of soybean DNA left in soybean after prolonged fermentation, nested PCR was used. Nested PCR was performed with two successive PCR tests (Zimmermann 1998). The first PCR amplified a larger DNA fragment, using soybean genomic DNA as the template. The second PCR amplified a small DNA fragment, using the PCR product from the first PCR as

Table 1. Characteristics of DNA primers used for PCR reactions

Name of primer pair	Binding position	Expected length of PCR product
LeC	Lectin gene	318 bp
LeD	Lectin gene	444 bp
LeE	Lectin gene	682 bp
RR02, RR01	35S[1]-EFSPS[2]	509 bp
RR04, RR05	35S-EFSPS	180 bp
LEE, LEX	Lectin gene	270 bp
35S3, EPB2	35S-EFSPS	150 bp

1 35S is the 35S promoter
2 EPSPS is 5-enol-pyruvyl-shikimate-3-phosphate synthase gene, which is the transgenic gene in Roundup Ready soybean

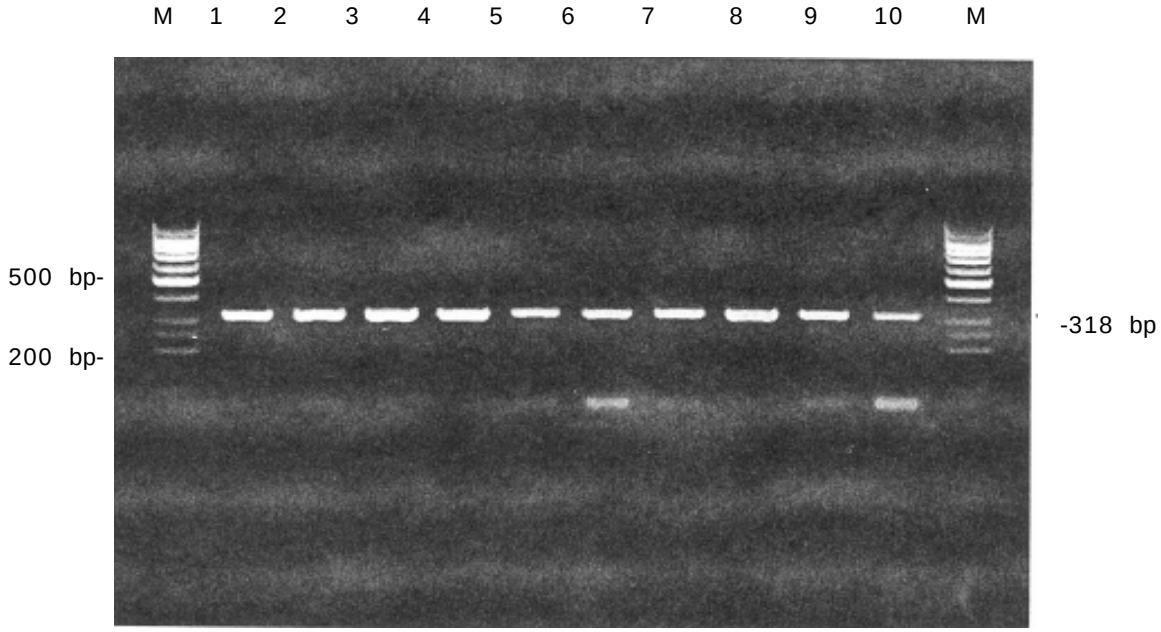

Fig. 1. Effect of heat on DNA template for PCR reaction: The LeC Primer

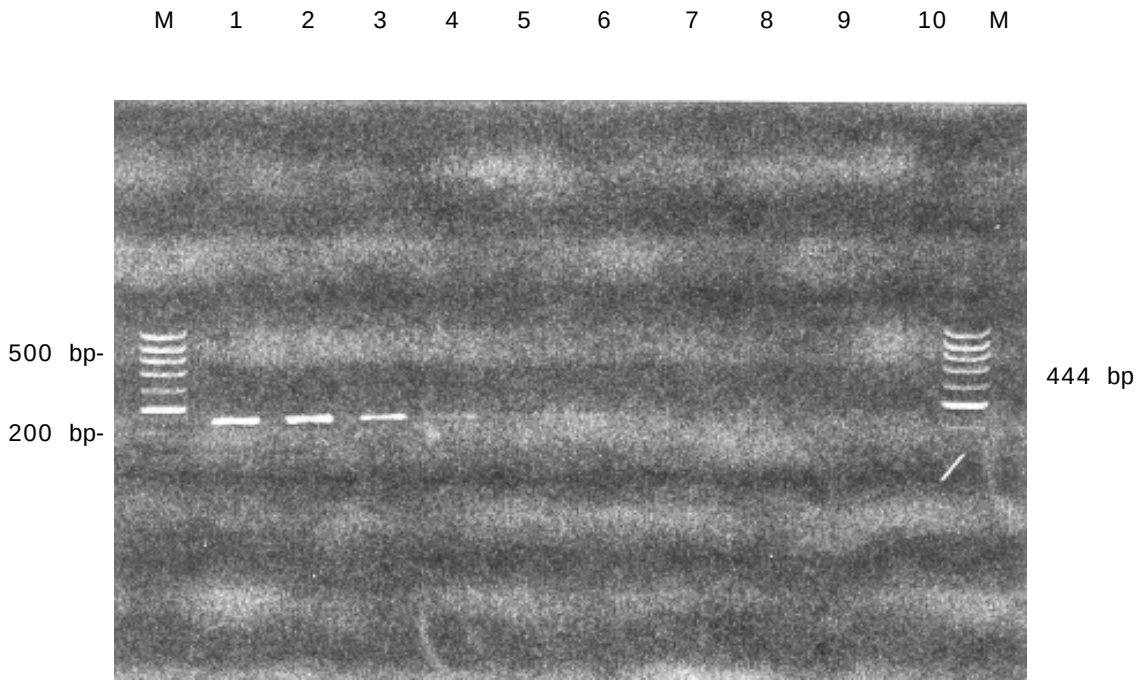

Fig. 2. Effect of heat on DNA template for PCR reaction: The LeD Primer

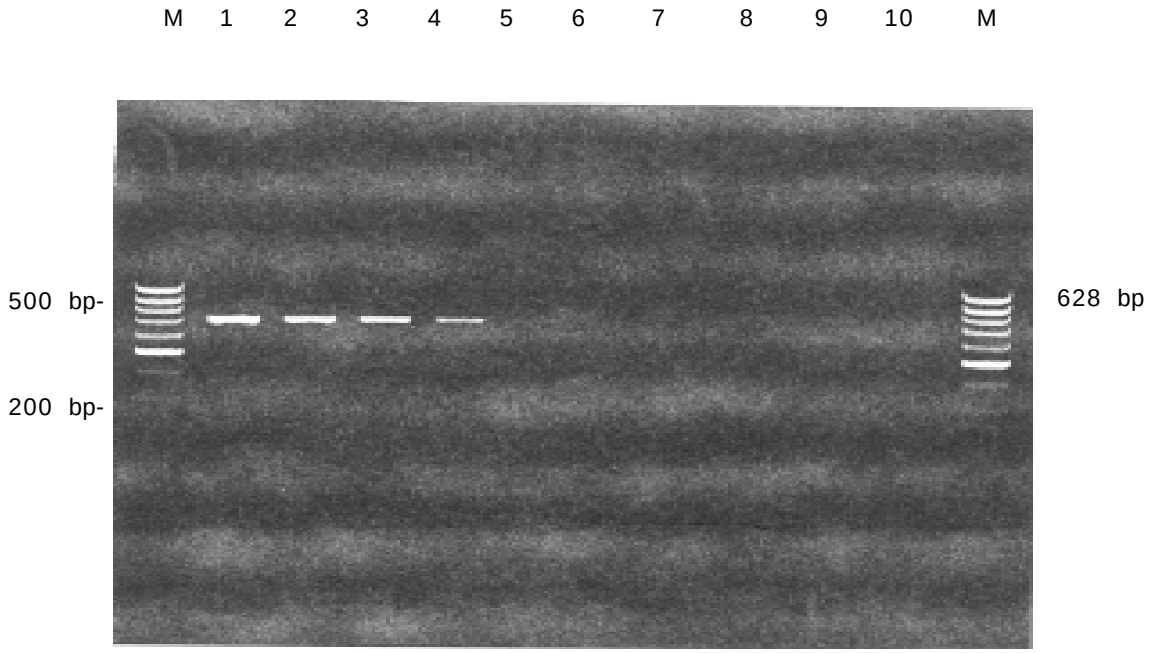

Fig. 3. Effect of heat on DNA template for PCR reaction: The LeF Primer

Separate samples of purified soybean DNA were heated at 100°C for 10 (lane 2), 20 (lane 3), 30 (lane 4), 60 (lane 5), 120 (lane 6), and 180 (lane 7) min, and 121°C for 10 (lane 8), 20 (lane 9) and 30 (lane 10) min. The control soybean DNA which was not heated is shown in lane 1. M is 50 bp DNA ladder. After the heat treatment, DNA was analyzed by PCR with LeC (Fig. 1), LeD (Fig. 2) and LeD (Fig. 3) primer sets. The characteristics of the DNA primers used are listed in Table 1.

the PCR template (Fig. 4).

First, the sensitivity of the nested PCR was examined. Varying amounts of DNA from Roundup Ready soybean were inspected by nested PCR (Fig. 5). Results showed that nested PCR can detect the soybean DNA template in quantities as small as less than 0.01 ng, which is about 10 copies of the soybean genome.

Fermented tofu made from Roundup Ready and non-GM soybeans was made by the natural fermentation in the laboratory. Three species of bacteria, *Bacillus pumilus*, *bacillus subtilis*, and *Bacillus cereus*, were involved in the fermentation process (Lee et al. 1996). Three lots of samples of fermented tofu were heated. One lot was put into boiling water for 10 min, one was heated in a rice cooker for 10 min, and the third was heated in hot oil for 2 min. The NDA of these samples of fermented tofu were then isolated. The nested PCR method was applied with DNA primers which could detect 35S-EPSPS (Fig. 4). All tofu or fermented tofu made from Roundup Ready soybeans shows a 180-bp DNA fragment. In contrast, tofu from non-GM soybeans did not produce this fragment after the PCR reactions (Fig. 6). These results indicates that nested PCR is sensitive enough to detect the presence of GM soybeans as an ingredient of fermented tofu.

Miso is also an important fermented food in which soybeans are a major ingredient. Miso was made from Roundup Ready soybeans and non-GM soybeans in the laboratory. Samples of miso were taken for the nested PCR reaction at 30, 70, 100, 120, 150, 180, and 210 days after the fermentation process had begun. Results (Fig. 7) showed that the 180-bp 35S-EPSPS fragments were detected in the miso up to 150 days after fermentation. However, they were not detected in the miso fermented for more than 180 days. Therefore, a long period fermentation of soybean may damage DNA template so much as to interfere with the PCR reaction.

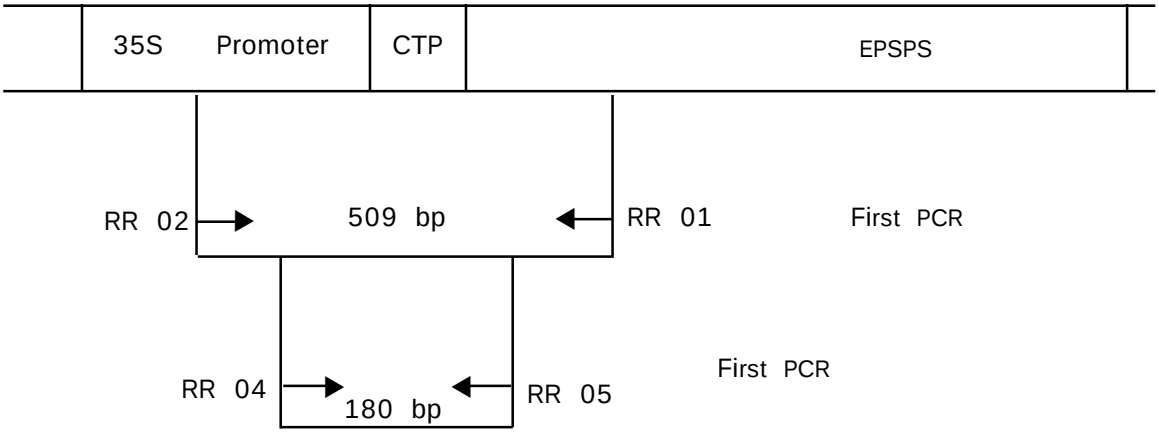

Fig. 4. Schematic representatioin of the nested PCR used to detect DNA fragments in fermented soybeans

Nested PCR was performed using two successive PCR tests. The first PCR amplified a larger DNA fragment, using the soybean genomic DNA as a template. The second PCR test amplified a small DNA fragment, using the PCR product from the first PCR as the PCR template.

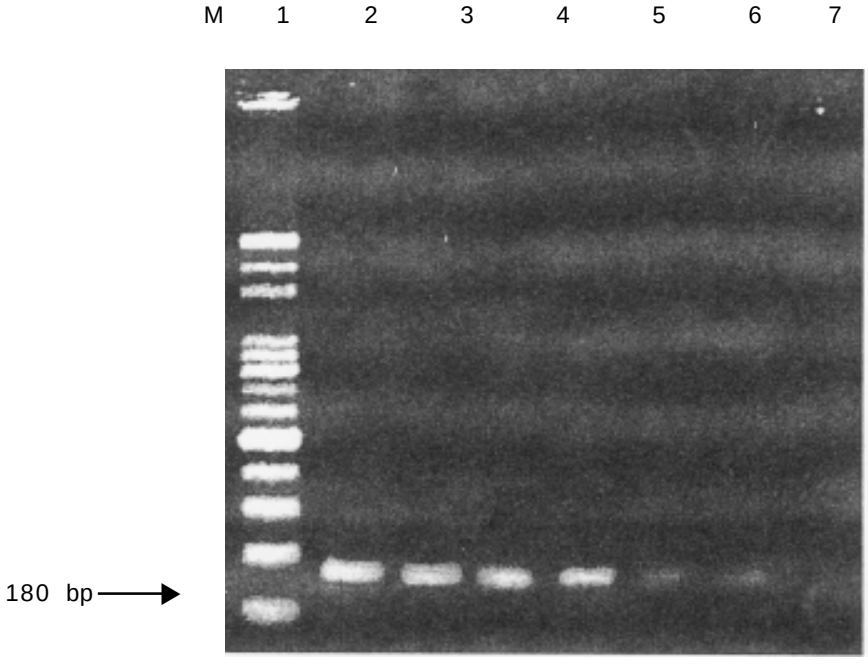

Fig. 5. Examination of the sensitivity of the nested PCR

Nested PCR used to examine 1 ng (lanes 1 and 2), 0.1 ng (lanes 3 and 4), and 0.01 ng (lanes 5 and 6) of Roundup Ready soybean. The procedure for the nested PCR is outlined in Fig. 2. Lane 7 is the negative control, which does not contian the DNA template.

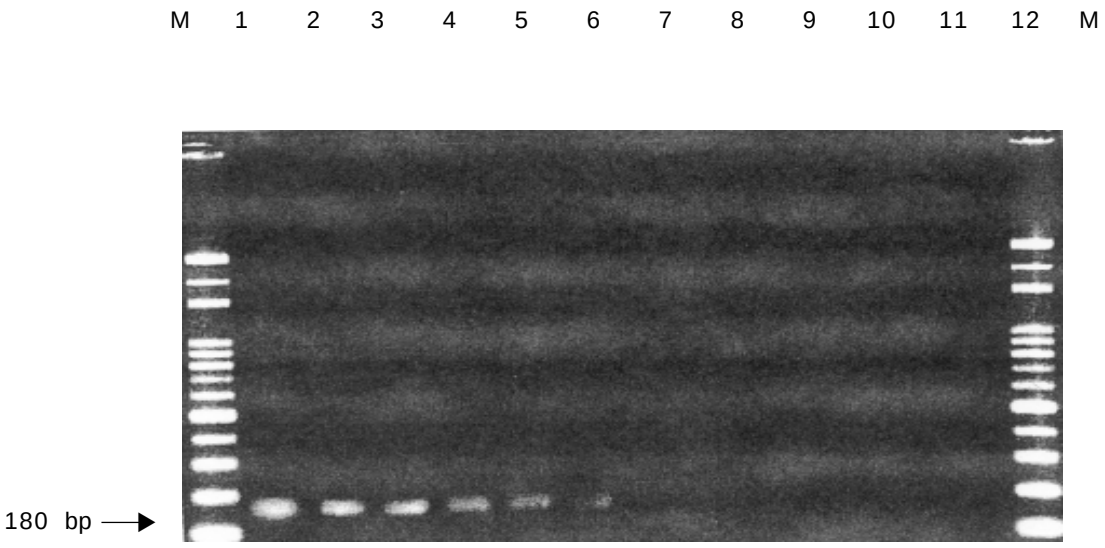

Fig. 6. Detection of the GM content of fermented tofu by nested PCR

Fermented tofu from Roundup Ready and non-GM soybeans was made in the laboratory. When the samples of fermented tofu were ready, they were into either boiling water for 10 min, a rice cooker for 10 min, or hot oil for 2 min. The DNA was analyzed by nested PCR (Fig. 2). Lanes 1 to 6 are from Roundup Ready soybean, while lanes 7 to 12 are from non-GM soybean. Lanes 1 and 7 are the PCR results from unprocessed soybeans. Lanes 2 and 8 are the PCR results from tofu. Lanes 3 and 9 are the PCR results from fermented tofu without any heating treatment. Lanes 4 and 10 are the PCR results from fermented tofu treated with boiling water for 10 min. Lanes 5 and 11 are the PCR results from fermented tofu placed in a rice cooker for 10 min. Lanes 6 and 12 are the PCR results from fermented tofu put in hot oil for 2 min. M is the DNA marker.

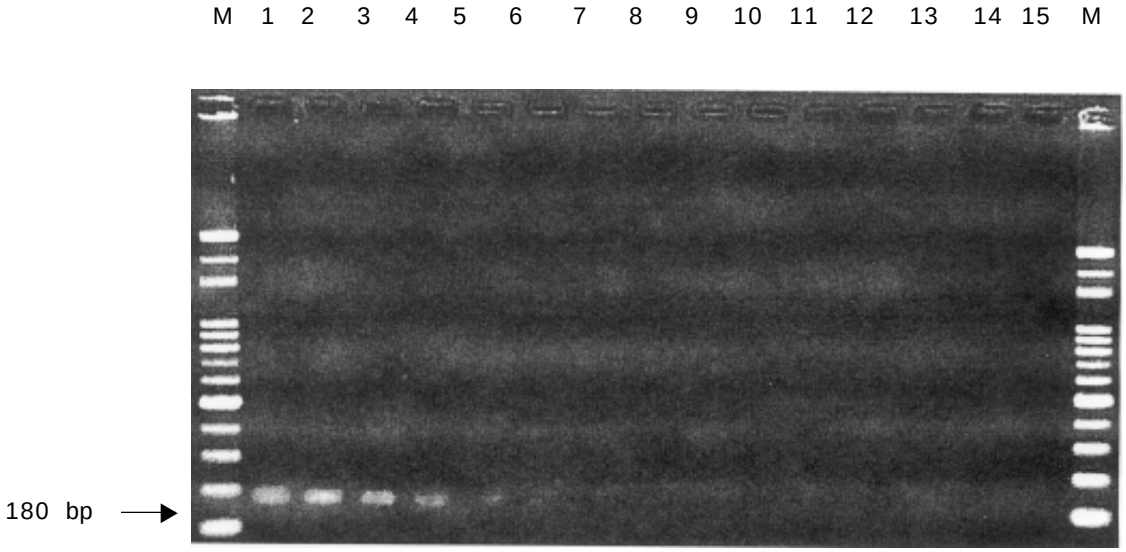

Fig. 7. Detection of the GM content of miso by nested PCR

Separate samples of miso were made from Roundup Ready and non-GM soybeans in the laboratory. Miso samples were taken out to test by nested PCR reaction (Fig. 2) at 30, 70, 100, 120, 150, 180, and 210 days after the fermentation process was begun. Lanes 1 to 9 are from Roundup Ready soybean, while lanes 10 to 15 are from non-GM soybean. Lanes 1 and 10 are the PCR results from soybeans only. Lanes 2 and 11 are the PCR results from the semi-processed miso. Lane 3 is the PCR result from niso which had been fermented for 30 days, Lane 4 from miso which had been fermented for 70 days and Lane 5 from miso fermented for 100 days. Lanes 6 and 12 are the PCR results from miso which had been fermented for 120 days. Lanes 7 and 13 are the PCR results from miso which had been fermented for 150 days. Lanes 8 and 14 are the PCR results from miso which had been fermented for 180 days. Lanes 9 and 15 are the PCR results from miso which had been fermented for 210 days. M is the DNA marker.

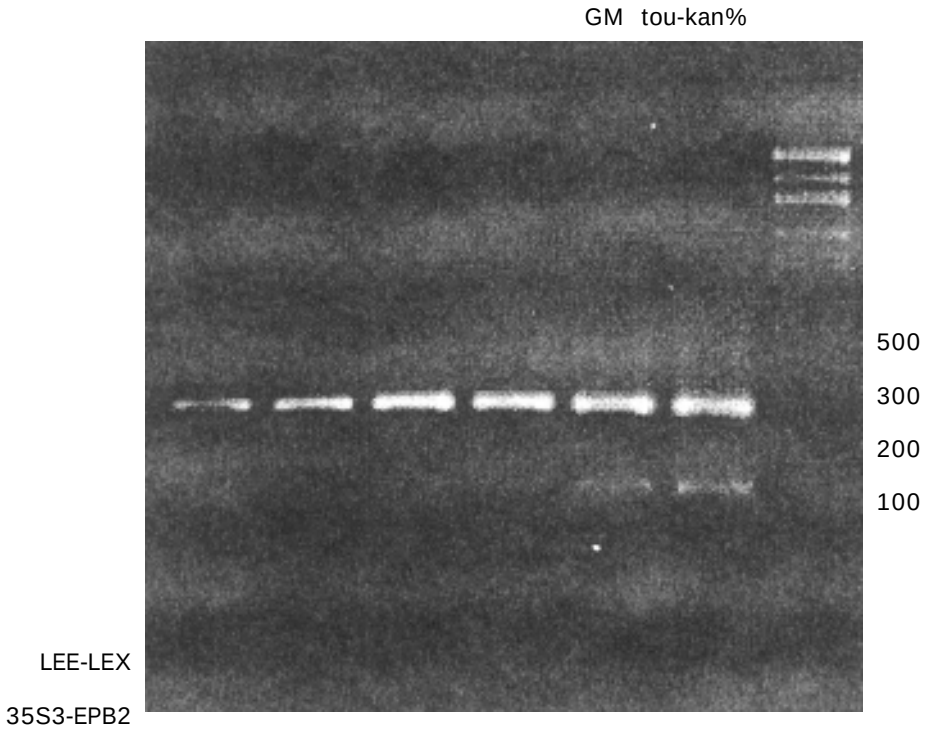

Fig. 8. Detection of the GM content of tofu-gan (dried tofu) by multiplex PCR

Tofu-kan, a dried, hard beancurd, was made in the laboratory. Different samples contained 0, 0.2, 1, 5, 20, and 95% of Roundup Ready soybean mixed wityh non-GM soybeans. After the DNA in the tofu-gan had been purified, multiplex PCR reactions were applied uisng LEE and EPB2 primer sets to detect. The characteristic gene of ordinary soybeans, lectin, and the glyphosate resistance gene of GM soybeans EPSPS, respectively.

Multiplex PCR

Multiplex PCR amplifies more than one DNA fragment in one reaction, and can thus detect and analyze several genes at the same time (Masuelli *et al.* 2000). In particular, the involvement of the internal standard gene in multiplex PCR increases the accuracy and avoids possible mistakes. Tofu-gan the hard bean curd, was made with a mixture of non-GM and Roundup Ready soybeans, so that different mixtures contained 0, 0.2, 1, 5, 20, and 95% of Roundup Ready soybean, respectively. After the DNA of the to-gan samples had been purified, the multiplex PCR reactions were applied with LEE and EPB2 primer sets, in order to detect separately the characteristic soybean gene, lectin, and the glyphosate resistance gene, EPSPS. The lectin gene was detected in all tou-gan samples (Fig. 8), indicating that they are all soybean products. The EPSPS gene was detected only when the to-gan contained more than 1% Roundup Ready soybean (Fig. 6). This indicates the detection limits of multiplex PCR.

Multiplex PCR was also used to analyze commercial tofugan, seasoned tofu-gan, and seasoned dry tofu-gan. DNA templates of these different kinds of processed tofu were purified, and all proved to be degraded (Fig. 7A). After these DNA templates were amplified by multiplex PCR, the presence of EPSPS fragments in all the samples of processed commercial tofu-gan indicates that they were made from Roundup Ready soybean (Fig. 7B). Surprisingly, the soybean characteristic gene, lectin, was not detected in the dry seasoned tou-kan. This may indicate that the process to generate dry seasoned tou-kan destroyed DNA so heavily that PCR was unable to amplify lectin gene. Therefore, the multiplex PCR is a reliable detection procedure for analyzing GM soybean products.

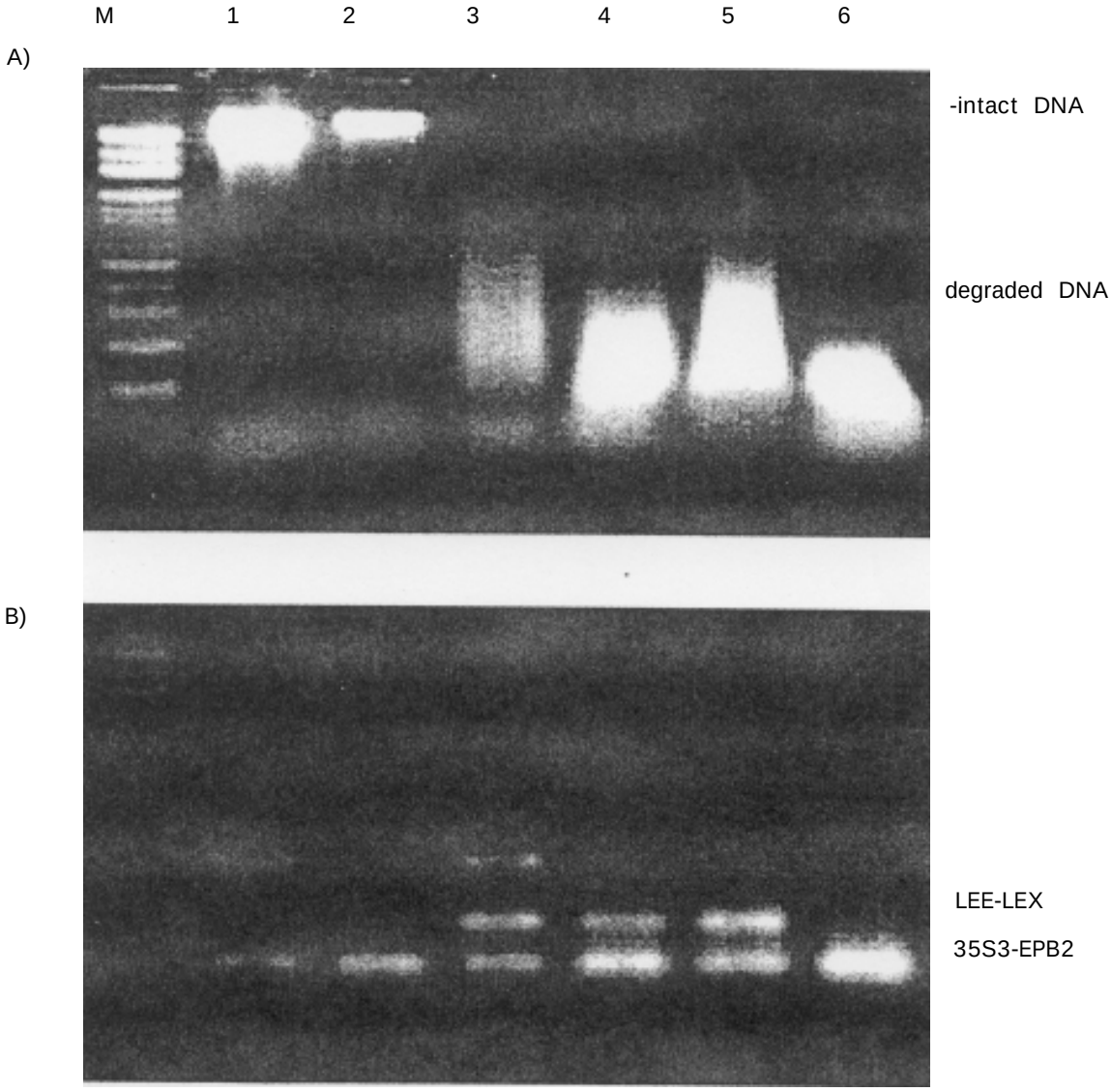

Fig. 9. Detection of the GM content in commercial tofu-gan (dried tofu) by multiplex PCR

Multiplex PCR was used to detect the presence of GM soybeans in commercial tofu-gan, seasoned tofu-gan, and seasoned dried tofu-gan. After DNA templates of these different kinds of tofu-gan were purified (A), the multiplex PCR reactions were applied with LEE and EPB2 primer sets to detect the characteristic soybean gene, lectin, and the glyphosate resistance gene, EPSPS, respectively (B). Lane 1 is the PCR result from non-GM soybean. Lane 2 is the PCR result from Roundup Ready soybean. Lane 3 is the PCR result from commercial tofu-gan. Lane 4 is the PCR result from the first sample of commercial seasoned tofu-gan. Lane 5 is the PCR result from the second sample of commercial seasoned tofu-gan. Lane 6 is the PCR result from commercial season dry tou-kan. M is the DNA marker.

Table 2. Quantification of soybean and its products by real-time PCR

Soybeans and their products	Lectin		EPSPS		Estimated roundup ready content (%)
	Cropy number (photometer)	Copy number (calculation)	Copy number (photometer)	Copy number (calculation)	
5% roundup ready 1/1	80000	79670	4000	4088	4.7
5% roundup ready 1/2	40000	40010	2000	1867	5.3
5% roundup ready 1/4	20000	19880	1000	1009	5.0
5% roundup ready 1/16	5000	5194	250	235	4.8
5% roundup ready 1/32	2500	2431	125	125	5.7
5% roundup ready Soybean milk		39790		2273	5.7
5% roundup ready Soybean milk		37760		1863	4.9
5% roundup ready Hard bean curd		30580		1392	4.6

1 RRS is the roundup ready soybean

Quantitative assay of Roundup Ready soybean

Quantitative assay of Roundup Ready soybean was performed by LightCycler PCR (Roche). PCR products were labeled with SYBR green 1 for detection. DNA from the series dilution of 5% Roundup Ready in non-GM soybean powder was first analyzed. This analysis showed that 4.7 – 5.7% of the calculated value was obtained from the LightCycler PCR machine (Table 2). The 5% of Roundup Ready soybeans in non-GM soybeans was also used to make soybean milk, soy milk film (yuba), and a kind of hard bean curd in the laboratory. Their DNA was purified, and analyzed by quantitative PCR. Results indicate that the value of 4.6 – 5.7% of Roundup Ready soybean was obtained (Table 2). Therefore, the content of Roundup Ready within non-GM soybean as soybean powder or its products can be accurately estimated by quantitative PCR.

CONCLUSION

In this study, soybeans and their products were analyzed by PCR to detect the presence of GM soybeans. Most soy-derived products are generated through a heating process, which damages the DNA template used for the PCR reaction. However, PCR which generates small DNA fragments of only 318 bp can still detect the presence of DNA. Fermentation is also an important part of the process in making soybean products. The DNA of soybean after a long period of fermentation, such as more than 180 days, was seriously damaged. As a result, the gene EPSPS which identifies transgenic soybeans could not be detected by nested PCR. Also, the multiplex PCR amplifies more than one DNA fragment in each reaction, and is a reliable detection procedure for analyzing GM soybean products. Finally, a quantitative assay of Roundup Ready soybean was performed by real-time PCR. Using this

method, it was possible to make an accurate assessment of the percentage of GM soybeans mixed in with non-GM soybeans.

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