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Note

Optical Detection of Specific Genes for Genetically Modified Soybean and Maize Using Multiplex PCR Coupled with Primer Extension on a Plastic Plate

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A novel DNA microarray method to detect one line of genetically modified (GM) soybean and five lines of GM maize was developed using multiplex PCR coupled with primer extension on a plastic plate. Multiplex PCR products were applied on an extension primer-immobilized plate and the spots corresponding to the DNA sequences were visualized. This method is a rapid and simple way to detect GM soybean and GM maize optically.

Key words: arrayed primer extension; genetically modified organism; multiple primer extension; oligonucleotide microarray

The numbers and uses of genetically modified organisms (GMOs) have increased in recent years, but controversy continues to surround their increased distribution and use due to concerns about food safety, environmental risks, and ethical issues. Many countries and regions have issued GMO labeling regulations, for example thresholds of 5% in Japan.[1)] The primary means of GMO detection to determine whether such labeling requirements have been met is the polymerase chain reaction (PCR) technique, but this method has an obvious limitation in that it can detect only one gene at a time. Even for multiplex PCR, due to the limit of the separation of the amplified products for ordinary electrophoresis, it can detect only five to six genes simultaneously.

Oligonucleotide microarrays show great advantages in the detection of many target genes simultaneously. In fact, microarrays have been applied to simultaneous detection of GMOs.[2–7)] These microarrays are based on the hybridization of labeled target oligonucleotides to probes on the chip, but the hybridization method is generally time-consuming, especially the hybridization step itself. In addition, many of these microarrays require laser scanning. On the other hand, primer extension methods on a microarray, such as the arrayed primer extension method and the multiple primer extension method, are used mainly for mutation and single nucleotide polymorphism analysis.[8,9)] The extension method is relatively quick and can be adapted to optical detection.[10)] This method was recently used for rapid identification of bacteria.[11)]

In this study, we adapted this rapid, simple method to detect target DNA sequences optically and simultaneously, as shown in Fig. 1. We developed a method of identifying different GMO events, one line of genetically modified (GM) soybean (Roundup Ready soybean, RRS) and five lines of GM maize (Event176, Mon810, Bt11, GA21, and T25). The method is based on specific integration junction sequences between the host plant genome DNAs, and it uses multiplex (8-plex) PCR together with primer extension on a plastic plate.

Conventional soybean and maize seeds were purchased from a local market in Hyogo, Japan, and were ground to a powder (AM-3, Nihon Seiki Seisakusho, Tokyo). The powdered certified reference materials of RRS, Event176, Mon810, Bt11, and GA21 were obtained from the Institute for Reference Materials and Measurements (Geel, Belgium) and commercialized by Fluka (Buchs, Switzerland). DNA was extracted from powder samples using a silica membrane-type kit (DNeasy Plant Mini Kit, Qiagen, Hilden, Germany) according to a previously reported procedure.[12)] The concentration of DNA was calculated from the absorbance at 260 nm, as measured with a UV spectrophotometer. On the other hand, T25 DNA was purchased from Generon (Modena, Italy). Samples (1% and 5%) of T25 were prepared by mixing T25 DNA with non-GM maize DNA. A GM maize mixture containing 1% each of Event176, Mon810, Bt11, GA21, and T25 was also prepared by mixing GM maize DNAs with non-GM maize DNA.

The PCR primer sets for taxon specific sequences in soybean (the lectin gene, *Le1*) and maize (the starch synthase IIb gene, *SSIIb*), and construct specific GM sequences, *RRS*, *Event176*, *Mon810*, *GA21*, and *T25*, had been used to quantify GMOs using real-time PCR (Table 1).[12)] The set for *Bt11* has also been reported.[5)] The primers were synthesized by Texas Genomics Japan (Tokyo). A 25-μl reaction mixture contained 12.5 μl, QuantiTect Multiplex PCR Master Mix (Qiagen),

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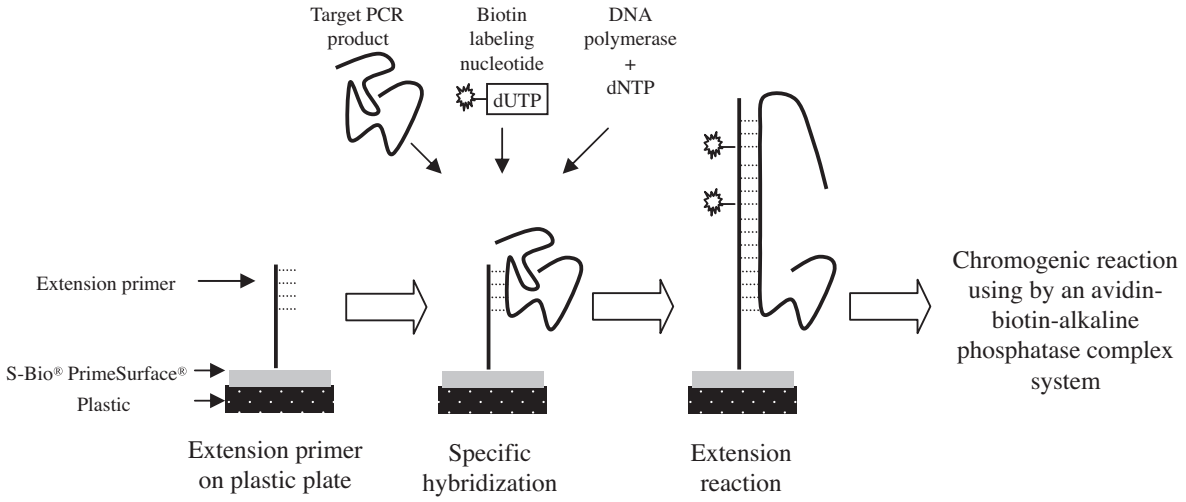

Fig. 1. Process for Primer Extension on a Plastic Plate.
An extension primer is immobilized on the S-Bio® PrimeSurface®-treated plastic plate. Target PCR product amplified by multiplex PCR hybridizes to the primer. Extension and incorporation of biotin-dUTP are carried out by DNA polymerase. The extension reaction is optically detected by an avidin-biotin-alkaline phosphatase complex system.

Table 1. Forward and Reverse Primers Used in Multiplex (8-plex) PCR and Extension Primers Used in an Extension Reaction on a Plastic Plate

Target	Orientation	Sequence (5′-3’)	Reference
Le1	Forward	GCCCTCTACTCCACCCCCA	12
	Reverse	GCCCATCTGCAAGCCTTTTT	12
	Extension[a]	AGCTTCGCCGCTTCCTTCAACTTCAC	12
RRS	Forward	CCTTTAGGATTTCAGCATCAGTGG	12
	Reverse	GACTTGTCGCCGGAAATG	12
	Extension[a]	CGCAACCGCCCGCAAATCC	12
SSIIb	Forward	CTCCCAATCCTTTGACATCTGC	12
	Reverse	TCGATTTCTCTCTTGGTGACAGG	12
	Extension[a]	GCAATGCAAAACGCAACGAGTGGGGG	This study
Event176	Forward	TGTTCACCAGCAGCAACCAG	12
	Reverse	ACTCCACTTTGTGCAGAACAGATCT	12
	Extension[a]	TCGATGTGGTAGTCGGTCACGTCGG	12[b]
Mon810	Forward	GATGCCTTCTCCCTAGTGTTGA	12
	Reverse	GGATGCACTCGTTGATGTTTG	12
	Extension[a]	TTGTTGTCCATGGCCGCTTGGTATCT	12[b]
Bt11	Forward	ACATTTAATACGCGATAGAAAAC	5
	Reverse	ACACCTACAGATTTTAGACCAAG	5
	Extension[a]	TATGTTACTAGATCTGGGCCTCGTG	5
GA21	Forward	GAAGCCTCGGCAACGTCA	12
	Reverse	ATCCGGTTGGAAAGCGACTT	12
	Extension[a]	CGGCCATGCACCGGATCCTT	12[b]
T25	Forward	GCCAGTTAGGCCAGTTACCCA	12
	Reverse	TGAGCGAAACCCTATAAGAACCCT	12
	Extension[a]	TGCAGGCATGCCCGCTGAAATC	12

[a]Modification with amino linkers at the 5′ end due to immobilization of the extension primer on a plastic plate.
[b]Using the complement sequence of the reference.

0.05 μM each of primer for *SSII*, *Event176*, and *Bt11*, 0.1 μM each of primer for *Le1*, *RRS*, *Mon810*, and *T25*, 0.5 μM each of primer for *GA21*, a 100-ng DNA sample, and sterilized water to make up the final reaction volume. Eight-plex PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal cycle program was as follows: 2 min at 50 °C and 15 min at 95 °C, followed by amplification of DNA for 35 cycles of 60 s at 94 °C and 60 s at 61 °C. The PCR products were used for the primer extension reaction.

The sequences of the extension primers, which were immobilized on a plastic plate (for *Le1*, *RRS*, *Event176*, *Mon810*, *GA21*, and *T25*) were those previously used as TaqMan probes in real-time PCR (Table 1).[12] The sequence of the TaqMan probe for *SSIIb* was modified for use in the primer extension reaction. The modified sequence was checked for specificity by BLAST search. The primer extension sequence for *Bt11* has also been reported.[5] These primers were modified with amino linkers at the 5′ end, and were synthesized and purified on a reverse-phase column by Nippon EGT (Toyama, Japan). The plastic plate was treated with S-Bio® PrimeSurface® (Sumitomo Bakelite, Hyogo, Japan), providing a unique biocompatible phospholipid polymer and a highly active functional ester moiety to bind the attachment site covalently for amino-linked oligonucleotides under alkaline conditions.[13] One microliter of

10 μM 5′-amino-link primer in alkaline solution was spotted onto the plate. After incubation for 3 h, the primer-immobilized plate was treated with 0.1 M sodium hydroxide solution to block the remaining functional ester moieties, and was washed with hot water. The plate was dried in air and stored at 4 °C.

Fifty μl of reaction mixture of primer extension consisted of 5 U TERMIPol DNA polymerase (Solis Biodyne, Tartu, Estonia), 1 × reaction buffer C, 2 mM $MgCl_2$, 50 μg/ml, salmon sperm DNA, 0.05% triton-X 100, 100 μM dATP, 100 μM dGTP, 100 μM dCTP, 65 μM dTTP (each dNTP, Indianapolis, Roche, IN), 35 μM biotin-11-dUTP (Bioron, Ludwigshafen, Germany), 5 μl of PCR products, and sterilized water to make up the final reaction volume. The sample was incubated for 5 min at 95 °C, and then was applied to the extension primer-immobilized plastic plate (preheated to 72 °C). The reaction was allowed to proceed at 72 °C for 3 min under a coverslip. The plate was washed in 0.1% triton X-100 in 50 mM tris buffer (pH 7.5), and then in 50 mM tris buffer (pH 7.5). It was incubated at 37 °C for 20 min with the addition of alkaline phosphatase-conjugated biotin and avidin complex (VectaStain ABC-AP Kit, Burlingame, Vector Laboratories, CA), which was prepared by dilution of A and B reagents with 200 times the volume of 2% BSA and 0.01% salmon sperm DNA in 50 mM tris buffer (pH 7.5) and preincubated for 20 min at room temperature before use. The plate was washed in 0.1% triton X-100 in 50 mM tris buffer (pH 7.5) and then in 50 mM tris buffer (pH 7.5). The plate was incubated at 37 °C for 10 min with the addition of NBT/BCIP solution (Roche). Then it was washed in water and dried in air. The image on the plate was scanned using an MP 600 scanner (Cannon, Tokyo).

The principle of the present method, based on multiplex PCR amplification and identification of the primer extension reaction on a plastic plate, is shown in Fig. 1, but it is necessary to optimize all reaction procedures. In multiplex PCR, the primer sets were tested and the primer concentration and thermal cycle programs were optimized to minimize non-specific amplification and variation in amplification efficiency between the pairs of primers. In the primer extension reaction, the oligonucleotide lengths and orientations of the extension primers were examined, and incubation time and temperature were optimized to minimize cross hybridization and self-extension.

Under optimized conditions, *Le1* and *SSIIb* spots were visible to the naked eye in the non-GM soybean and non-GM maize samples (Fig. 2). Accordingly, detection of these species-specific genes can be used as a positive control. In the samples containing 1% and 5% of GM soybean and GM maize, except for 4.3% GA21, the corresponding spots were visible to the naked eye. In addition, in the mixture sample containing 1% each of the five lines of GM maize, the spots corresponding to *SSIIb* and five lines of GM maize were simultaneously visible. These results indicate that this method can identify GM soybean lines and GM maize lines in seed and grain samples containing 1% GM material. However, spots with a lower signal intensity than those containing 1% GMO, especially *Bt11* and *GA21*, are invisible to the naked eye. In fact, when the DNA amount used for multiplex PCR decreased from 100 ng to 50 ng, the corresponding spot was frequently invisible in the sample containing 1% GA21 (data not shown).

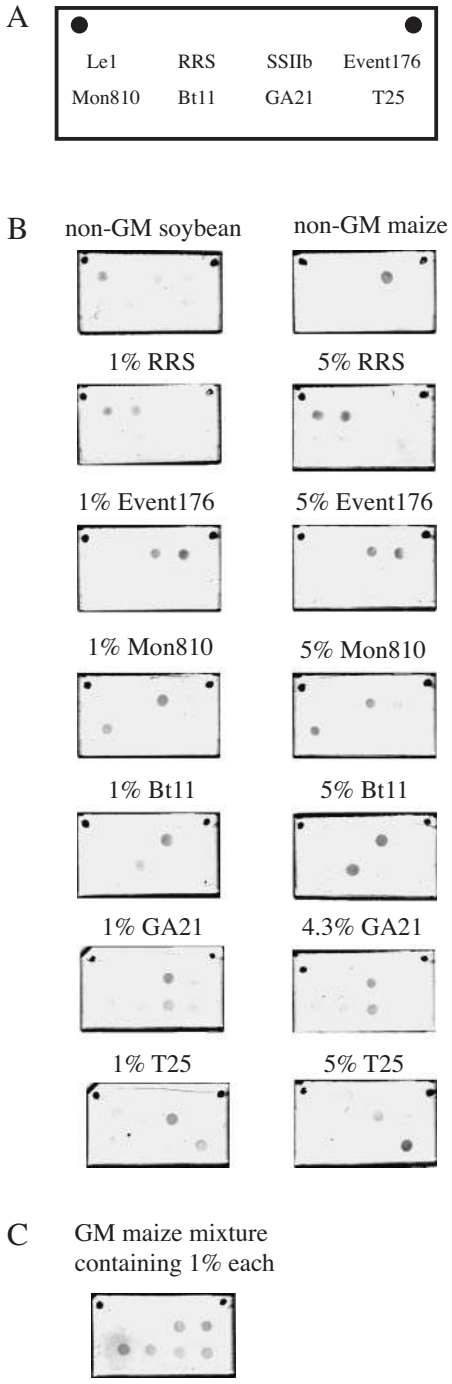

Fig. 2. Detection of GMO Genes Using Multiplex (8-plex) PCR Coupled with Primer Extension.

A, Layout of the extension primer-immobilized plastic plate. The sequences of the primer are given in Table 1. B, Image of the primer extension reaction of non-GM soybean, GM soybean (Roundup Ready soybean), non-GM maize and GM maize (Event176, Mon810, Bt11, GA21, and T25). The GMO contents of the samples are 1 and 5%, except for 4.3% GA21. In the case of T25, the percentages indicate the percentages of GM DNA relative to non-GM DNA. C, Image of the primer extension reaction of the GM maize mixture containing 1% each of Event176, Mon810, Bt11, GA21, and T25. The percentages indicate the percentages of GM DNA relative to non-GM DNA. The positive signals appear as gray spots. The dots in the upper corners denote the position of the plate.

In previous reports, the detection limits of microarrays for GMO were 0.1%.[2–4] These amplification processes were the four separated PCR,[3] PCR amplifi-

cation with a tag primer in tag-labeled samples prepared by polymerase reaction with a bipartite primer containing the tag sequence,[2] and nucleic acid sequence-based amplification (NASBA) in promoter-conjugated samples prepared by polymerase reaction with a bipartite primer containing the promoter sequence.[4] These methods are sensitive and quantitative, but are time-consuming and require more manipulation. On the other hand, the amplifying and fluorescent-labeling system using multiplex PCR detected 0.5% and 1% of GMOs.[5,6] The method had low sensitivity, but was rapid and simple. The present method also used multiplex PCR amplification and optically detected samples containing 1% GM soybean and GM maize. In addition, microarrays for GMO detection in all previous reports were based on the hybridization of labeled target oligonucleotide to a probe on the chip.[2–7] The hybridization times were from 1 h to 18 h as compared with the time of primer extension reaction in the present method of 3 min.

In conclusion, we have developed a new detection method for GMOs using multiplex PCR coupled with a primer extension method in a microarray. This rapid and simple method should be useful for optical identification of GM soybean and GM maize.

Acknowledgments

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