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Event-Specific Detection of Seven Genetically Modified Soybean and Maizes Using Multiplex-PCR Coupled with Oligonucleotide Microarray

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With the increasing development of genetically modified organism (GMO) detection techniques, the polymerase chain reaction (PCR) technique has been the mainstay for GMO detection. An oligonucleotide microarray is a glass chip to the surface of which an array of oligonucleotides was fixed as spots, each containing numerous copies of a sequence-specific probe that is complementary to a gene of interest. So it is used to detect ten or more targets synchronously. In this research, an event-specific detection strategy based on the unique and specific integration junction sequences between the host plant genome DNA and the integrated gene is being developed for its high specificity using multiplex-PCR together with oligonucleotide microarray. A commercial GM soybean (GTS 40-3-2) and six GM maize events (MON810, MON863, Bt176, Bt11, GA21, and T25) were detected by this method. The results indicate that it is a suitable method for the identification of these GM soybean and maizes.

KEYWORDS: Genetically modified organism; event-specific; integration junction sequence; oligonucleotide microarray; multiplex-PCR

1. INTRODUCTION

In the past decade, biotechnology has been widely used in modern agriculture and related industries, and hundreds of genetically modified organisms (GMOs) have been approved for commercialization worldwide. From 1996 to 2006, the global area dedicated to GM crops increased 60-fold, from 1.7 million hectares in 1996 to 102.0 million hectares in 2006, with an increasing proportion located in developing countries. In 2006, the planted area of GM soybean was 58.6 million hectares with the ratio of 57% in global biotechnology area, followed by GM maize (25.2 million hectares at 25%) (*1*). The event that has received regulatory approval in most countries is herbicide tolerant soybean event GTS 40-3-2 with 21 approvals (EU = 25 counted as 1 approval only), followed by insect resistant maize (MON 810) and herbicide tolerant maize (NK603), both with 18 approvals, and insect resistant cotton (MON 531/757/1076) with 16 approvals worldwide (*1*). In 2004 and 2005, 18 GM events from the United States and Canada were approved for commercialization in China, that is, one GM soybean (GTS 40-3-2), eight GM maizes (Bt11, Bt176, GA21, T25, NK603, MON810, MON863, and TC1507), seven GM canolas (Ms1Rf1, Ms1Rf2, Ms8Rf3, GT73, T45, Oxy235, and Topas 19/2), and two GM cottons (MON531 and MON1445) (*2*).

However, as more GM foods, food ingredients, and additives are introduced from the field to the market and table, several controversial issues are being discussed, such as food safety, environment risk, and ethical concerns, resulting in more than 40 countries and areas have issued GMO labeling regulations for protecting the consumers' authority. For instance, the labeling threshold was defined as 0.9% in the European Union (*3*), 3% in Korea (*4*), and 5% in Japan (*5*, *6*). In China, 17 GM products must be labeled, such as maize seeds, maize oil, tomato seeds, ketchup, soybean seeds, soybean oil, rapeseed seeds, and cotton seeds (*7*).

To execute the labeling requirements, the PCR technique has become the main technique for GMO detection, and the PCR detection strategies which discriminate the GM- and non-GM-derived DNA varieties are divided into four levels, that is, screening and gene-, construct-, and event-specific PCR detection. The screening method is associated with a particular risk of yielding false positives. Gene-specific and construct-specific methods are more specific, but may cause false positives when the same gene or construct is integrated into other GMOs with variable copy number. To overcome this, a line or transformation event-specific PCR method should be performed (*8*, *9*). Up to now, some event-specific quantitative PCR methods for a few GM crop events, compared with the total commercialized GM

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Table 1. Primers/Probes of the Special Junction Site Sequences To Be Detected

gene	name	sequence (5′-3′)	length (bp)	ref
GTS 40-3-2	forward primer	TGCCATCATTGCGATAAAGGAAA	256	GenBank AY596948
	reverse primer	TAGAGTCAGCTTGTCAGCGTGTC		
	probe	AGATGGACCCCCACCCACGAGGAGC		
maize MON810	forward primer	ATCTTGTGCTGATGAAGGTATGT	139	GenBank AF434709
	reverse primer	CAATAAAGTGACAGATAGCTGGG		
	probe	TTAACATCCTTTGCCATTGCCCAGC		
maize Bt176	forward primer	AACTGGCATGACGTGGGTTTCTGG	209	GenBank AJ878607
	reverse primer	TCCGTGGGCGTGGTATCGACTTT		
	probe	CACCGAGATCTGATGTTCTCTCCTC		
maize Bt11	forward primer	ACATTTAATACGCGATAGAAAAC	286	GenBank AY123624
	reverse primer	ACACCTACAGATTTTAGACCAAG		
	probe	TATGTTACTAGATCTGGGCCTCGTG		
maize GA21	forward primer	TGTAGTTGTTGGCTGTGGTGGAA	159	GenBank AJ878608
	reverse primer	CTTACCTTTGTTTTATTTTGGAC		
	probe	AGGAAGTGCAGCTCTAGAACTAGTG		
maize T25	forward primer	TGACGCACAATCCCACTATCCTT	149	GenBank AY629235
	reverse primer	GCTGCTGTAGCTGGCCTAATCTC		
	probe	CTCTAGAGTCGACATGTCTCCGGAG		
maize MON863	forward primer	ATGGGAACTCTTGGCTGGAAACA	254	*20*
	reverse primer	GAAGTGACAGGTAGGATCGGAAA		
	probe	CACCCCAAAGTGTACCAAACTTTCC		

events, have been developed, such as GTS 40-3-2 soybean (*10*−*12*), MON531 and MON1445 cotton (*13*), GT73 canola (*14*), MON810 maize (*15*, *16*), Bt11 maize (*17*, *18*), GA21 maize (*14*, *19*), MON863 maize (*20*, *21*), Bt176 maize (*14*), T25 maize (*22*), NK603 (*23*), and CBH351 (*24*).

But these methods have an obvious limit that they can only detect one gene at a time. Even for multiplex-PCR, since the limit of ordinary electrophoresis, it can only detect four to five genes simultaneously. For the GMOs, especially for the samples such as admixture material, we should detect ten genes simultaneously to find out the GMO events (*25*). Oligonucleotide microarray shows great advantages to detect ten or more target genes at the same time in theory, and this method can give us the result with high accuracy and sensitivity.

In this article, we developed a new method to identify the different GMO events based on unique and specific integration junction sequences between the host plant genome DNA and the integrated gene, using multiplex-PCR together with oligonucleotide microarray.

2. MATERIALS AND METHODS

2.1. Materials. Genuine seeds of GM maize lines MON863, MON810, GA21, and GM soybean (GTS 40-3-2) were developed by Monsanto Co. Genuine seeds of GM maize lines Bt11 and Bt176 were developed by Syngenta Seeds, Inc. Genuine seeds of GM maize line T25 were developed by Aventis CropScience Co. The GM soybean sample GTS 40-3-2 and the GM maize samples MON810, Bt176, Bt11, GA21, NK603, and MON863 were purchased from Fluka Co., and the other GM maize sample (T25) was supplied by the developer. Conventional soybean and maize were purchased from a local market in Shanghai, China.

2.2. DNA Extraction. CTAB method was used for genomic DNA extraction (*26*). Double distilled H_2O was added to dissolve the DNA. The DNA concentration was measured by absorbance at 260 nm, and the final concentration was 20 ng/μL. It was stored at −20 °C for further use.

2.3. Design of Primers and Probes. According to the unique and specific integration junction sequences between the host plant genome DNA and the integrated gene, a pair of primers, one from the host plant genome DNA, another from the integrated genome, was designed by Prime Premier Software. Primers were used to amplify target genes in a multiplex-PCR system, so PCR primers were selected to meet the following criteria: (1) be able to analyze degraded genomic DNA from the highly processed product (each amplification spans 100−300 bp); (2) have a melting point between 55 and 60 °C; (3) be compatible in the PCR mixture. The corresponding probe was also designed to the junction site, just covering both the host plant genome DNA and the integrated gene (**Table 1**). Meanwhile, in order to detect whether this new method is accurate or efficient, positive control primers and probe (18S rRNA) were designed. The other primers and probes to promoter and terminator were also included (**Table 2**). The HPLC-purified primers and probes were synthesized by TAKARA Corporation. Each probe contains a 5′-amine group and a 10-mer poly-dT space. The amine group enables the probe to covalently attach to the aldehyde-activated slides. The poly-dT10 is used as a link-arm to facilitate the hybridization between PCR products and homologous probe.

2.4. Construction of Oligonucleotide Microarrays. The probes were diluted to a final concentration of 10 μmol/L in spotting solutions (3 × SSC and 0.01% sodium dodecyl sulfate (SDS)). Diluted probes were spotted on aldehyde-activated glass slides. The substrates were made by Shanghai BioStar Genechip Institute. Every probe was spotted five times in a row with 500 μm dot distance (**Figure 1**). Printed arrays were dried in air for a half-hour at room temperature, and then washed in 0.2% SDS and twice in water (5 min each). Finally they were dried in the air at room temperature and stored at 4 °C.

2.5. PCR and Labeling. Target genes were amplified and labeled in a multiplex-PCR system. Amplification reactions were carried out in a total volume of 25 μL on a PTC-100 thermocycler (MJ Research, Inc.). After a great deal of experimentation to optimize the PCR reactions, the end concentrations of PCR components were used as follow: PCR buffer, 1×; $MgCl_2$, 2.5 mmol/L; d(AGT)TP, 0.1 mmol/L each; dCTP, 0.01 mmol/L; Cy5-dCTP, 0.01 mmol/L; primers, 0.3 μmol/L each; Taq enzyme, 2.5 U; UNG enzyme, 0.5 U. PCRs were performed using 10−100 ng of DNA.

PCR cycling conditions were as follows: a 5 min cycle at 94 °C; 35 cycles with 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; and a 7 min cycle at 72 °C.

2.6. Hybridization and Signal Detection. After the completion of PCR, 8 μL of each PCR product was mixed with 2 × hybridization solutions (10 × SSPE, 1% SDS) which had been warmed at 55 °C previously. Then the above mixtures were denatured at 95 °C for 5 min, then 0 °C for 5 min. The mixtures were applied onto the chip under a cover slip. Chips were incubated at 55 °C for 1 h. After incubation, the cover slip was washed off at room temperature in 0.1 × SSC. The slides were then washed in 0.2% SDS, 0.1 × SSC for 5 min followed by 0.1 × SSC and dried at room temperature. The chips were scanned using a Genepix (Axon instruments) confocal scanner, and the fluorescence was quantitated using Genepix 3.0 software.

2.7. Direct Sequenceing Analysis of PCR Products. The PCR products were purified with a PCR products purification kit (Promega).

Table 2. Primers and Probes of Some Ordinary Promoters and Terminators and Positive Control (18s rRNA)

gene	name	sequence (5′-3′)	length (bp)	ref
CaMV35S promoter	forward primer	CCCAGATAAGGGAATTAGGGTTC	134	*27*
	reverse primer	CCCTGGATTTTGGTTTTAGGAAT		
	probe	GAAACCCTTAGTATGTATTTGTATTTGTAA		
NOS terminator	forward primer	TGAATCCTGTTGCCGGTCTT	138	*27*
	reverse primer	AAATGTATAATTGCGGGACTCTAATC		
	probe	GATGATTATCATATAATT		
FMV35S promoter	forward primer	CAGCATTCCAGATTGGGTTCA	172	*27*
	reverse primer	CTTTTGGCTAATGGTTTGGAGAC		
	probe	AAAACCAAGAAGGAACTCCCATC		
NPTII terminator	forward primer	GGAAGGGACTGGCTGCTATTG	157	*27*
	reverse primer	GATGTTTCGCTTGGTGGTCG		
	probe	GCTCCTGCCGAGAAAGTATCC		
Bar	forward primer	GCTCCACGCTCTACACCCAC	181	*27*
	reverse primer	AAACCCACGTCATGCCAGTT		
	probe	CTGTGCCTCCAGGGACTTCA		
CaMV-CP	forward primer	GGGACCAAATTATTGATCTAACCTCT	148	*27*
	reverse primer	TCTCCTGAATCGCTTTCGTCTT		
	probe	TATGACAACTACCGACGACTCGA		
18s rRNA	forward primer	GAGAAACGGCTACCACATCCA	254	*27*
	reverse primer	CGTGCCATCCCAAAGTCCAA		
	probe	CGCGCAAATTACCCAATCCTGACAC		

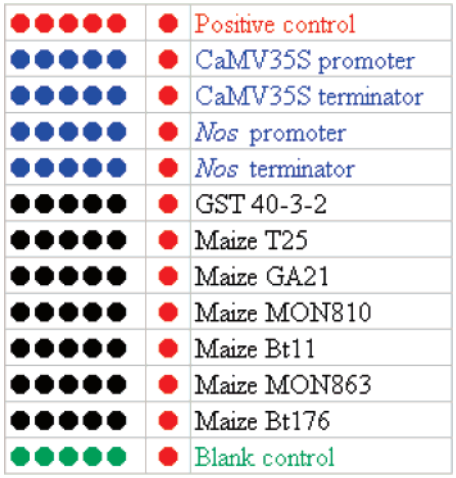

Figure 1. Oligonucleotide microarray format. The red spots mean positive control. The black ones mean probes of the special junction site sequences. The blue ones mean probes of some ordinary promoter and terminator and positive control. The green ones mean blank control.

All PCR products were subjected to direct sequencing by using a DNA sequencer (CEQTM 2000XL DNA analysis system, Beckman) to confirm the validity of the amplified PCR products and to verify whether their sequences were complementary to the probes.

3. RESULTS

3.1. Design of Control Samples. To supervise the whole detecting process, the following control samples were included in addition to the tested samples in each detection: blank of the nucleotide (water control), positive quality control (known GMO samples), and negative quality control (conventional samples) along with the tested samples.

3.2. Threshold Determination of the Positives and Negatives. Theoretically, the positive/negative result can be inferred according to the presence/absence of fluorescent signals of target gene probes. In fact, signal intensities of negative quality control probes can differ substantially and surface conditions. In the present study, we took the average signal intensity of negative quality control probes as the background signal intensity and calculated the ratio of signal intensity of target sequence probes to negative control probe. After a great deal of repeating experiments with known positive and negative samples, we set a ratio of 5:1 as the threshold of positives, and a ratio of 3.5:1 as the threshold of negatives. Basically, when the average ratio was above 5, the sample was defined as positive; when the average ratio was below 3.5, the sample was defined as negative. The sample with the ratio between 3.5 and 5 was considered to be ambiguous and was reprocessed. Every experiment was repeated three times. Using this standard, all of the positive and negative samples can be correctly identified (**Figure 2**).

3.3. Optimization of the Experimental Condition and Validation of the Method. The whole detection system mainly involves two steps, multiplex-PCR and hybridization, which could amplify target sequences, enhance the fluorescent signal intensity, and increase the limit of detection. However, it is necessary to optimize all reaction procedures to ensure the reliability of the detection system. Nonspecific amplification and cross-hybridization would greatly impair the correctness of detection results. Thus, the compatibility of primer sets, the stringent hybridization and washing conditions are important factors that should be modified carefully.

For reproducibility analysis, every sample was detected five times using different batches of chips. The results were consistent throughout the different batches, indicating high reproducibility (data not shown).

The other foreign elements, such as CaMV35S promoter, NOS terminator, NPTII, and Bar, were also designed as positive probes for further confirmation of the result. FMV35S promoter was also used to exclude infection of CaMV virus.

Limits of detection are defined as the ability of a method to detect different components in parallel without reciprocal interference and to identify the analysis unambiguously. The limits of detection of this system for screening event-specific GM crops were evaluated using two kinds of Certified Reference Materials: Roundup Ready soybean GTS 40-3-2 and maize MON810 with known GM contents. Hybridization results were scored on the basis of the ratio of signal intensity of the expected probes to negative quality control probes. In the present study, hybridization results revealed that the GM limit of detection of a probe array for soybean GTS 40-3-2 is 0.5% while for maize MON810 it is 1%. One reason for the difference in limits of detection between soybean GTS 40-3-2 and maize MON810 is probably that the genome size of maize is larger than that of

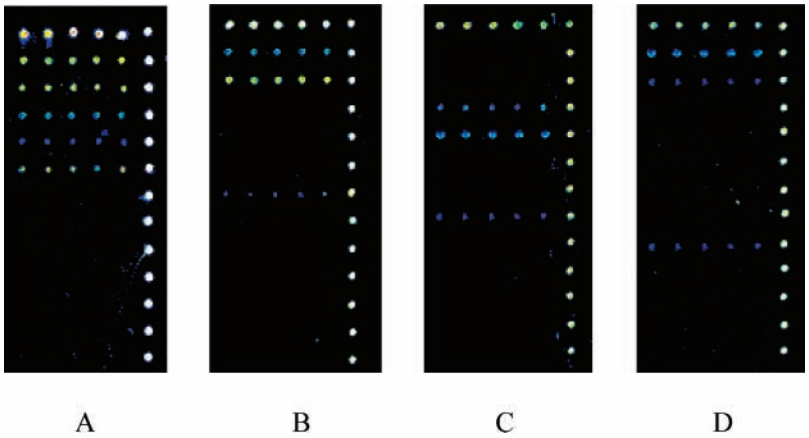

Figure 2. Image of the microarray hybridization. **A**: Image of the microarray hybridization of soybean GTS 40-3-2 1% GMO content. For the positive probes of CaMV35S promoter, CaMV35S terminator, *Nos* promoter, *Nos* terminator, and GST 40–3-2, the sample is from GTS 40-3-2. **B**: Image of the microarray hybridization of maize T25 1% GMO content. For the positive probes of CaMV35S promoter, CaMV35S terminator, and maize T25, the sample is from maize T25. **C**: Image of the microarray hybridization of maize GA21 1% GMO content. For the positive probes of *Nos* promoter, *Nos* terminator, and maize GA21, the sample is from maize GA21. **D**: Image of the microarray hybridization of maize MON810 1% GMO content. For the positive probes of CaMV35S promoter, CaMV35S terminator, and maize MON810, the sample is from maize MON810.

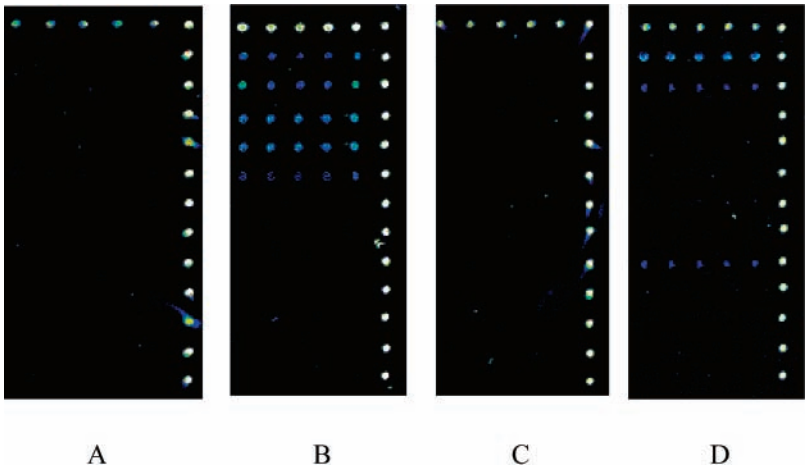

Figure 3. The limits of detection of GTS 40-3-2 and maize MON810. **A**: Image of the microarray hybridization of GTS 40-3-2 0% GMO content. **B**: Image of the microarray hybridization of GTS 40-3-2 0.5% GMO content. **C**: Image of the microarray hybridization of maize MON810 0% GMO content. **D**: Image of the microarray hybridization of maize MON810 1% GMO content.

soybean, and the efficiency of PCR reaction may influence the limit of detection (**Figure 3**).

4. DISCUSSION

PCR has become the main technique for GMO detection. But the target elements are so many that it is difficult to confirm PCR products by conventional methods when multiplex-PCR is used to amplify two or more DNA fragments simultaneously. Oligonucleotide microarray technology can solve this problem because of its high throughput, speediness, and veracity. Theoretically, we can screen ten or even more target genes at the same time in theory. Our results show that oligonucleotide microarray detection method is valid and sensitive. It will be more useful in the detection of GMOs by combination of oligonucleotide microarray and real-time fluorescence quantitative PCR.

Since foreign genes are randomly inserted into the genome of the host plants, the integration junction sequences between the host plant genome DNA and the integrated gene are unique and specific and can be responsible for the special GMO events. Based on this, we design the oligonucleotide microarray containing probes covering the inserted foreign gene and flanking host plant sequences. Since the length of probes is around 20−25 base, one part less than 13 bases of the probe is complementary to the inserted foreign gene and the other part of the probe is complementary to the host plant DNA. This probe will not produce any hybridization signal when inserted foreign DNA or host DNA is alone. Furthermore, the PCR primers are heterogeneous, one from the inserted foreign gene, the other from the host plant DNA. Therefore, foreign DNA and host plant DNA alone will not produce PCR products. This oligonucleotide microarray can correctly identify the GMO lines.

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