JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 3789−3794 **3789**

Rapid and Reliable Detection and Identification of GM Events Using Multiplex PCR Coupled with Oligonucleotide Microarray

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To devise a rapid and reliable method for the detection and identification of genetically modified (GM) events, we developed a multiplex polymerase chain reaction (PCR) coupled with a DNA microarray system simultaneously aiming at many targets in a single reaction. The system included probes for screening gene, species reference gene, specific gene, construct-specific gene, event-specific gene, and internal and negative control genes. 18S rRNA was combined with species reference genes as internal controls to assess the efficiency of all reactions and to eliminate false negatives. Two sets of the multiplex PCR system were used to amplify four and five targets, respectively. Eight different structure genes could be detected and identified simultaneously for Roundup Ready soybean in a single microarray. The microarray specificity was validated by its ability to discriminate two GM maizes Bt176 and Bt11. The advantages of this method are its high specificity and greatly reduced false-positives and -negatives. The multiplex PCR coupled with microarray technology presented here is a rapid and reliable tool for the simultaneous detection of GM organism ingredients.

KEYWORDS: GMO; oligonucleotide microarray; multiplex PCR; Roundup Ready soybean

INTRODUCTION

The production of genetically modified (GM) crops is increasing rapidly, while the detection and identification of genetically modified organisms (GMOs) are receiving global attention. In 2002, China approved new regulations on the production and use of GMO products. Under these new rules, labeling of grains and foodstuffs containing transgenic material is mandatory. Herbicide-tolerant Roundup Ready (RR) soybean, i.e., the glyphosate-resistant soybean (Monsanto), is the most common transgenic line of soybean planted. Glyphosate resistance results from the incorporation of an *Agrobacterium* (*sp.*strain CP4) derived 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS) gene. Several studies have recently described the application of RR soybean as the model material for setting up new methods for the detection of GMOs in grains and foodstuffs (*1*, *2*).

To detect RR soybean, one of the most commonly authorized methods currently is the polymerase chain reaction (PCR). Such method usually uses primers that recognize regions of the 35S promoter in the cauliflower mosaic virus (CaMV) and regions of *Agrobacterium tumefaciens* nopaline synthase (NOS) terminator. However, this screening method generally has significant risks of obtaining false-positives (*3*), as these two DNA elements are natural DNA sequences present in fresh vegetables and in their growing environment. Furthermore, such a gene-specific method normally targets the DNA region harboring a transgenic gene, for example, the EPSPS gene that codes for tolerance against a specific herbicide. Because the specific gene can be incorporated in various independent transformation events and may be integrated with regulatory elements (promoters and terminators) into various GMOs, the detection of such a gene by PCR cannot tell whether the GMO is authorized or not. Such drawbacks of the method could be eliminated by the so-called event-specific method which targets the junction between the inserted functional DNA and the recipient genome that is unique to the GMO and not present in nature (*4*).

Multiplex PCR assay, unlike the conventional one, was developed to simultaneously amplify two or more gene products in a single reaction (*5*). It was reported by Matsuoka et al. (*6*) that, by utilizing sequence-specific primers, a multiplex PCR system could simultaneously detect five lines of GM maize. A multiplex PCR assay was also developed by James et al. (*7*) to detect multiple target sequences in different GM crops, including soybean, maize, and canola. These studies demonstrated that the multiplex PCR system is a simple, reliable, and efficient method to specifically identify GMOs.

Agrose gel electrophoresis has been the commonly used technique to assess results from multiplex PCR, but oligonucleo-

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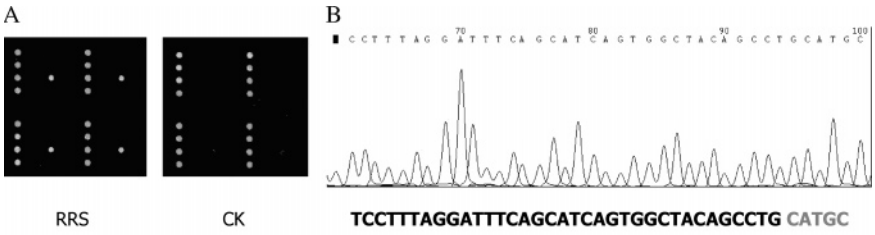
10.1021/jf048368t CCC: $30.25
Published on Web 04/20/2005



C

18S rRNA	35S	NOS	Bar	NptII
18S rRNA	Lectin	Napin	PG	Invertase
18S rRNA	35S/PLANT	35S/CTP	CP4/CTP	NOS/CP4-EPSPS
18S rRNA	CP4-EPSPS	K4	SPOTTING	CryIA(b)

Figure 1. Results of microarray hybridization and the corresponding sequence map. (**A**) Microarray hybridization result from the CP4/CTP amplified fragment. (**B**) Sequence profile of the CP4/CTP PCR products depicting the structure of the CP4/CTP probe. The sequence homologous to CP4-EPSPS is depicted in red capital letters. (**C**) Oligonucleotide array format.

tide microarrays offered a more discriminating means to examine reaction products for specific sequences. 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. To detect the presence of the genes of interest in a sample, genomic DNA isolated from the sample was amplified by PCR and hybridized to the array. The presence of the genes of interest in the sample will result in the hybridization of their sequences with their probes on the microarray and can be detected by a fluorescence imaging system. The resulting patterns and relative intensities on the microarray will show whether the tested GMOs carrying the detected genes should be attributed to approved GM varieties (*8*−*10*).

In this paper, we designed and evaluated a rapid and simple method by means of coupling multiplex PCR with a low-density oligonucleotide microarray to detect RR soybean. Furthermore, by using this method, GM maizes (Bt176, Bt11) can be precisely detected using sequence-specific primers. We also discussed the inclusion of various probes and their effects on increasing the specificity in detecting GMOs.

MATERIALS AND METHODS

Plant Material. Dried powder from GM soybean (Monsanto RR soybeans GTS 40-3-2) from GeneScan Inc. (Hong Kong) and non-GM soybean from the Laboratory of Food Biotechnology, China Agricultural University, were used for the soybean analysis. Seeds from non-GM and two lines of GM maize (insect-resistant Bt11 and Bt176 from Novaritis), dried non-GM rapeseed, and herbicide-resistant RT73 from Monsanto, and frozen tomato fruit from non-GM and anti-ACS (inhibiting ethylene biosynthesis) from our own laboratory were used for the analysis

DNA Extraction. DNA extractions from soybean powder, ground corn, rapeseed, and frozen tomato were performed using the CTAB (cetyltrimethylammonium bromide) methods with some modifications (*11*). The plant material was ground in a mortar with liquid nitrogen when necessary. A 100-mg portion of plant material and 300 μL of CTAB extraction solution [3% CTAB (w/v); 100 mM Tris-HCl, pH 8; 20 mM ethylenediaminetretraacetic acid (EDTA); 1.4 M NaCl] were incubated at 65 °C for 30 min. After phenol/chloroform extraction, DNA was precipitated and dissolved in 300 μL of TE solution. A 1-μL aliquot of RNAse (10 mg/mL) was added and incubated for 2 h at 37 °C. Finally, DNA was precipitated, rinsed with 70% ethanol, and dissolved in 30−50 μL of sterile distilled water (ddH_2O) and stored at −20 °C.

The DNA concentration in samples was measured by the UV absorption at 260 nm, and the quality of DNA was evaluated from the 260 to 280 nm UV absorption ratios (DU 640, Beckman coulter). Samples with a ratio of 1.6−1.9 were used in the following assays.

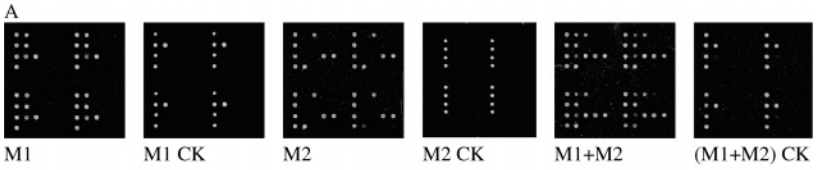
Oligonucleotides Design and Synthesis. Several primers either designed by ourselves or taken from the literature were used to detect RR soybean. Previously, Padgette et al. (*12*) described the RR soybean single genetic insert structure, and Windels et al. (*13*) identified the exact end points of the insert DNA and the soybean sequences following those end points in the RR soybean genome. Based on this, an oligonucleotide microarray (40mer) which contains 17 probes recognizing the structure gene and other related genes was designed. The array format has been described in Figure 1, Figure 2, and Figure 3. The primers chosen for the multiplex PCR, the amplicon lengths, and their specific sequences are presented in Table 1, and the corresponding probe sequences are presented in Table 2.

Oligonucleotide probes were designed by Mprobe (*14*) software based on the published DNA sequences. The NOS/CP4-EPSPS probe consists of two parts: the 5′CTAGATCGGGGATCGATCC3′ sequence belongs to T-NOS, and the 5′CCCACCGGTCCTTCATGTTC3′ sequence belongs to the CP4-EPSPS gene. The 35S/CTP probe consists of two parts: the 5′GCTGA3′ sequence belongs to P-35S; the 5′CAAGCTGACTCTAGCAGATCTTTCAAGAATGGCAC3′ sequence belongs to CTP-EPSPS. For the event-specific structure, the junction between the 35S promoter gene and soy genome DNA (35S/PLANT) was included in this study. The amplified product (187bp) contains P-35S and soybean plant DNA sequences. The TGGAAAAGGAAGG sequence is homologous to the P-35S sequence, and the CAGGTTAAAATAAACATAGGGAACCCAAA sequence is homologous to the soybean plant DNA sequence.

For signal detection, the primers were fluorescent (Cy3) labeled at their 5′ ends. The probes were synthesized with the 3′ end amino-modified to have a primary NH2 group for the immobilization onto aldehyde-coated slides. The NH_2 group was linked by a polyethlene glycol spacer to a specific probe sequence that was 40 nucleotides long (except 35S/PLANT,NOS/CP4-EPSPS 2 probes which were a little different in length) with its sequence complimentary to the amplified PCR product that was labeled with fluorescence. Oligonucleotide primers were synthesized by using automatic DNA synthesizer (ABi 391A). All transgenic genes in RR soy and other reference genes chosen in this study are as follows: cauliflower mosaic virus (CaMV) 35S promoter (35S); the 3′ nontranslated region of the nopaline synthase gene (NOS); the phosphinothricin-*N*-acetyltransferase gene (Bar); 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS); chloroplast transit peptide (CTP); neomycin phosphotransferase II gene (NptII).

PCR Conditions. An asymmetric PCR method was used to generate single-stranded DNA target segments. The ratio of forward primer to

A

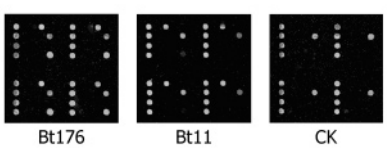


B

18S rRNA	35S	NOS	Bar	NptII
18S rRNA	Lectin	Napin	PG	Invertase
18S rRNA	35S/PLANT	35S/CTP	CP4/CTP	NOS/CP4-EPSPS
18S rRNA	CP4-EPSPS	K4	SPOTTING	CryIA(b)

Figure 2. Microarray hybridization results of two sets of multiplex PCR amplification of Roundup Ready soybean (**A**). Oligonucleotide array format (**B**).

A



B

18S rRNA	35S	NOS	Bar	NptII
18S rRNA	Lectin	Napin	PG	Invertase
18S rRNA	35S/PLANT	35S/CTP	CP4/CTP	NOS/CP4-EPSPS
18S rRNA	CP4-EPSPS	K4	SPOTTING	CryIA(b)

Figure 3. Microarray hybridization results of multiplex fluorescence PCR product for detecting and identifying Bt176 and Bt11 maizes (**A**). Oligonucleotide array format (**B**).

Table 1. Primer Pairs Used and Amplicon Lengths for Each Target Genes

target	orientation	sequence	length (bp)	ref
18S rRNA	forward	tct gcc cta tca act ttc gat ggt a	137	15
	reverse	aat ttg cgc gcc tgc tgc ctt cct t		
NOS	forward	gc atgacgttat ttatgagatg gg	118	16
	reverse	gac acc gcg cgc gat aat tta tcc		
bar	forward	gca gga acc gca gga gtg ga	264	17
	reverse	agc cgg atg aca gcg acc ac		
Npt II	forward	gga tct cct gtc atc t	173	18
	reverse	gat cat cct gat cga c		
CryIA(b)	forward	acc atc aac agc cgc tac aac gac c	184	17
	reverse	tgg gga aca ggc tca cga tgt cca g		
lectin	forward	gcc ctc tac tcc acc ccc atc c	118	19
	reverse	gcc cat ctg caa gcc ttt ttg tg		
napin	forward	gaa tgg cga aca agc tct tcc tcg	120	20
	reverse	cgg ctg agt ctg tgg cat cat ctt c		
PG	forward	gaa tat caa ggg cac aag	114	21
	reverse	ttt cca ctt tcc cct act		
invertase	forward	ccg ctg tat cac aag ggc tgg tac c	226	17
	reverse	gga gcc cgt gta gag cat gac gat c		
CP4-EPSPS	forward	gca aat cct ctg gcc ttt cc	145	22
	reverse	ctt gcc cgt att gat gac gtc		
35S/PLANT	forward	cag agg cat ctt caa cga	187	this study
	reverse	cat gct tta att tgt ttc tat c		
35S/CTP	forward	tga tgt gat atc tcc act gac g	171	23
	reverse	tgt atc cct tga gcc atg ttg t		
CP4/CTP	forward	gcg ggc cgg ctg ctt gca cc	180	24
	reverse	ccc caa gtt cct aaa tct tca agt		
NOS/CP4-EPSPS	forward	gcg cgg tgt cat cta tgt ta	291	2
	reverse	aat cgt aga ccc cga cga g		

reverse primer (one with the fluorescence labeled) was optimized at different ratios for different primer pairs (data not shown). A final volume of 40 μL was applied to perform the PCR procedures with 1.5 mM $MgCl_2$, 0.1 mM dNTP, 1 $\times$ PCR buffer, and 3 units of Taq polymerase (Cytech). Different concentrations of genomic DNA and primers were used according to the fragments to be amplified.

Table 2. Probe Sequences Used for the Microarray Hybridization

name	sequence (5′–3′)
18S rRNA	ATGGTGGTGACGGGTGACGGAGAATTAGGGTTCGATTCCG
35S	GATGTGATATCTCCACTGACGTAAGGGATGACGCACAATC
NOS	ATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAA
bar	CCACGCTCTACACCCACCTGCTGAAGTCCCTGGAGGCACA
NptII	TGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATG
lectin	TTGCCAGCTTCGCCGCTTCCTTCAACTTCACCTTCTATGC
napin	CGGCAACTCTCGCCTTCTTCTTCCTTCTCACCAATGCCTC
PG	GGTGGCCATAAAATTTGATTGCAGCACAAACTTTCCATGT
invertase	TGGCCATGGTGCCCGATCACCCGTACGACGCCAACGGCGT
35S/PLANT	CAGGTTAAAATAAACATAGGGAACCCAAATGGAAAAGGAAGG
35S/CTP	GCTGACAAGCTGACTCTAGCAGATCTTTCAAGAATGG CAC
CP4/CTP	TCCTTTAGGATTTCAGCATCAGTGGCTACAGCCTGCATGC
NOS/CP4-EPSPS	CTAGATCGGGGATCGATCCCCCACCGGTCCTTCATGTTC
CP4-EPSPS	CGCGAGCGGTGAAACGCGCATCACCGGCCTTCTGGAAGGC
K4(SDHC)	ATTGCCAGAAGACATCCTTACTTTTATGCCCCGGAACTCC
CryIA(b)	GCGACTGGATCAGGTACAACCAGTTCCGCCGCGAGCT GAC

For the soybean reaction, 300 ng of genomic DNA was applied for the PCR reactions of 35S/CTP, lectin, 35S/PLANT, and 18S rRNA. The concentrations of primers are 0.15 μM 35S/CTPpf and 0.5 μM 35S/CTPpr; 0.15 μM lectinpf and 0.5 μM lectinpr; 0.6 μM 35S/PLANTpf and 0.2 μM 35S/PLANTpr; 15 pM 18S rRNApf and 40 pM 18S rRNApr. A 100-ng amount of genomic DNA was applied for the CP4-EPSPS, CP4/CTP, NOS-CP4, NOS, and 18S rRNA. The concentrations of primers are 0.2 μM CP4-EPSPSpf and 0.6 μM CP4-EPSPSpr, 0.5 μM CP4/CTPpf and 0.15 μM CP4/CTPpr, 0.15 μM NOS-CP4pf and 0.5 μM NOS-CP4pr, 0.15 μM NOSpf and 0.4 μM NOSpr, and 15 pM 18S rRNApf and 40 pM 18S rRNApr.

For the maize reaction, the final reaction volume was 40 μL containing 200 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 1 $\times$ PCR buffer, 3 U Taq polymerase, primers (15 pM 18S rRNApf, 40 pM 18S rRNApr; 0.1 μM NOSpf, 0.4 μM NOSpr; 0.2 μM INVpf, 0.6 μM INVpr; 0.15 μM CryIA(b)pf, 0.5 μM,CryIA(b)pr; 0.15 μM Barpf, 0.5 μM Barpr).

All multiplex PCRs were carried out by using a PCR thermal cycler (PTC-100TM M.J. Research Inc.) under the following conditions: initial incubation at 94 °C for 5 min followed by 35 amplification cycles. The amplification cycles are as follow: denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, and extension for 30 s at 72 °C. A final extension step was carried out for 5 min at 72 °C. The PCR products were analyzed by 2−2.5% agarose gel electrophoresis, containing 0.1 μg/mL ethidium bromide.

DNA Microarrays Construction. The 3′ end amino-modified probes were diluted to a final concentration of 50 μm/L in spotting solutions (3 $\times$ SSC and 0.01% sodium dodecyl sulfate (SDS)). The spotting solutions were subsequently transferred into 96-well plates in the volumes of 6 μL and spotted to aldehyde-coated glass slides with a microarray printer (Cartisan), which deposited 0.5 nL at each spotting site, resulting in spots of 200 nm in diameter. In every matrix, the

array was repeated 4 times and each glass slide contained 10 matrixes. Each probe was spotted 4 times (except 16 times for 18S rRNA) in one matrix. The humidity during spotting was 70%, and the temperature was kept at 23 °C. After spotting, slides were incubated for another 1 h under the same conditions and stored at room temperature for at least 1 day before use. The array formats are shown in Figures 1−3.

Hybridization and Signal Detection. For 2 × multiplex PCR, 2 μL of denatured Cy3-labeled target PCR products was mixed with 8 μL of hybridization solution (6 × SSC, 0.2% SDS), and a 9-μL final volume was transferred to the hybridization area on the glass slide (CSS-100 silylated slides aldehyde, CEI Associate, Inc., Perland, TX). For 4 or 5 × multiplex PCR, 5 μL of Cy3-labeled products was mixed with 5 μL of hybridization solution, and the same final volume was transferred to the glass slide. The slides were incubated in a 42 °C water bath for 40 min in a hybridization chamber. After incubation, slides were washed sequentially in washing solution A (1 × SSC, 0.2% SDS), washing solution B (0.2 × SSC), and washing solution C (0.1 × SSC) for 1 min each time.

The glass slides were then scanned by using the confocal Scanarray GenePix3000 (Axon), with excitation at 540 nm and emission at 570 nm (Cy3). Sixteen-bit TIFF images of 10-μm resolution were analyzed. After subtraction of local background, the average signal intensity of four spots of each probe was used to evaluate the signal intensity (*25*). In this way, we were able to determine objectively whether a spot was truly positive or not. The signal intensity ratio value (positive/background signal intensity) above 4 was considered as a critical limit. It was not considered as a valid signal when either the signal intensity was lower than 400 and/or the signal intensity ratio value was lower than 2.5. The sample was reprocessed if the conditions were not met. Every experiment was repeated three times.

Direct Sequencing Analysis of PCR Products. The PCR reaction was carried out in a final volume of 60 μL, and the PCR products were purified with a PCR products purification kit (Promega). All PCR products were subjected to direct sequencing by using a DNA sequencer (CEQTM 2000XL DNA analysis system, Beckman) to confirm the validity of amplified PCR product and to verify whether their sequences were complementary to the probes.

RESULTS AND DISCUSSION

Probe Design and Detection Specificity. In the present study, NOS and NptII (neomycin phosphotransferase) were chosen as screening probes. These probes are applicable to most GM crops approved for food use, and the usages of these probes have become the official methods in several European countries (*23*). CP4-EPSPS from RR soybean, CryIA(b), and Bar genes from Bt176 maize were chosen as reference-specific probes to distinguish different GM crops. Lectin, polygalacturonase (PG), invertase, and napin were chosen as species reference genes for the internal control. Lectin, invertase, and PG are all single-copy genes in soybean, maize, and tomato, respectively. Napin is a storage protein that exists only in rapeseed. Internal control was set up to avoid any false-negative signals. 18S rRNA was also included as an internal control gene because it is universally present in all eukaryotes and can be sensitively detected. To effectively eliminate false-negatives and not inhibit targeted gene amplifications, 18S rRNA primer concentration was optimized as 1/10th of the detected gene concentration in a 2 × multiplex PCR or in the following 4 × or 5 × multiplex PCR reactions. Spotting solution and K4 succinate dehydrogenase complex (SHDC) gene were appointed as negative controls. SHDC is a human-specific gene. Soy, maize, tomato, and rapeseed are four main GM crops approved in the market, so they are suitable as species reference genes.

Three fragments were chosen for the construct-specific genes: junctions between 35S and CTP-EPSPS, between CTP-EPSPS and CP4-EPSPS, and between NOS and CP4- EPSPS. Specific probes were designed to contain two different gene sequences. The sequencing results are consistent with Windels' results (*13*) and with the sequence of the transgenic part of RR soybean from Monsanto (Patent WO 92/04449).

Figure 1A represents the result of microarray hybridization of CP4/CTP amplification products with two pairs of fluorescence-labeled primers. The amplified products were denatured and hybridized to the oligonucleotide microarray. Compared to array format (Figure 1C), specific signals of 18S rRNA and CP4/CTP probes were observed in RR soybean, as expected, while no positive signals, except for the internal control of 18S rRNA, were observed with valid signal in non-GM control. The sequencing result (Figure 1B) accords with our designed sequence. The 5′CATGC3′ sequence is homologous to the CP4-EPSPS gene and the 5′TCCTTTAGGATTTCAGCATCAGTG-GCTACAGCCTG3′ sequence corresponds to the CTP-EPSPS gene. The efficiency of primers in amplifying the target sequences was tested by performing PCRs separately, and the signal intensity was detected by microarray. The results showed that all of the primer pairs successfully amplified the target DNA sequences and that the chosen probes (Table 2) were target-specific. An accurate calibration of the primer concentrations was done to get a comparable signal in the microarray.

Detection of RR Soybean Using Oligonucleotide Microarray. Two sets of the multiplex system were designed and optimized to detect eight different RR soybean genes in the same oligonucleotide microarray. One set called M1 multiplex PCR system included four pairs of primers corresponding to the sequences of 18S rRNA, lectin, 35S/CTP, and 35S/PLANT; another set, M2 multiplex PCR system, included five pairs of primers corresponding to the sequences of 18S rRNA, NOS, CP4-EPSPS, CP4/CTP, and NOS/CP4-EPSPS. To minimize the discrimination in amplification and hybridization, the expected lengths of amplified products were between 100 and 200 bp (except for a few of them), which can be distinguished by electrophoresis.

In the M1 multiplex PCR system, the denatured multiplex PCR products were hybridized with the oligonucleotide microarray (Figure 2). The hybridization results of the microarray demonstrated that homologous probes of 18S rRNA, lectin, 35S/PLANT, and 35S/CTP produced valid signals. The 35S probe also gave a valid signal because the amplified fragment of the 35S/CTP sequence contains the 35S probe sequence. While the probes of 18S rRNA and lectin showed valid signals in non-GM soybean, no valid signals were observed with other corresponding probes. In our experiment, the 35S probe was quite often found to cause false positive signals in non-GM soybean control (data not shown). Such a false positive signal from 35S was due to the fact that its probe sequence is homologous to the amplified fragment of the 35S/CTP sequence. As a result, it was discarded as an independent screening probe in our experimental design.

In the M2 multiplex PCR system (Figure 2), five pairs of primers were applied to amplify fragments of 18S rRNA, NOS, CP4/CTP, NOS/CP4, and CP4-EPSPS in a single reaction. The hybridization results showed that their corresponding probes had effective signals in RR soybean. In the non-GM controls, none of these probes showed a valid signal, while the 18S rRNA internal control gave an effective signal. When we mixed the M1 and M2 PCR products together in a ratio of 1:1, the hybridization results showed that there was no change in their homologous probes characterization, except that the signal intensity became slightly lower. In the array design, the 18S rRNA probe was repeated 16 times as an internal control, while other probes were repeated 4 times in each matrix. The results

were very consistent in terms of their reliability and repeatability. Since the designed system includes probes for screening genes, species reference genes, specific genes, construct-specific genes, and event-specific genes, the presence of GM material in RR soybean could only be positively detected when more than six probes simultaneously show effective signals. In the aforementioned DNA-based PCR system, many factors could result in contamination and lead to false-positives. In our system, the chance of eight fragments being contaminated simultaneously is much lower; therefore, it was more specific than the common PCR assays. Also, the main problem on multiplex PCR reaction was the likelihood of generating nonspecific amplification bands, and such nonspecific bands might be indistinguishable from the specific bands due to their size similarities. Since the final detection step in our system was through sequence-specific probe hybridization, such problems were avoided. Therefore, we operated this system in a much simpler way under a relatively nonstringent condition.

Detection of Bt176 and Bt11 Maizes Using Oligonucleotide Microarray. We also applied this nucleotide microarray system coupled with the multiplex PCR to GM maize Bt176 and Bt11 samples to test its specificity and, more importantly, to realize its application in GM and non-GM material detections. We included primers corresponding to the sequences of CryIA(b) and Bar genes as Bt176 maize specific genes and invertase as the species reference gene. As a result, a multiplex system containing five primer pairs, 18S rRNA, invertase, CryIA(b), bar, and NOS, was set up to distinguish Bt176 and Bt11.

According to the information on transgenic maize, Bt11 contains the sequences encoding CryIA(b), PAT, and NOS terminator genes, while Bt176 contains CryIA(b) and bar sequences but not the NOS terminator element. Expected results were observed in Bt176 and in non-GM maize (Figure 3). From Bt11 maize, NOS and invertase signals could be detected, but the CryIA(b) signal was much weaker than that from Bt176, with its intensity being between the valid signal and the invalid signal. Such results can be explained by the fact that the sequences encoding the CryIA(b) genes in Bt176 and Bt11 are different. The CryIA(b) gene introduced to maize does not naturally exist in *Bacillus thuriengenisis* but was synthesized, and there are sequence variations in the synthetic gene sequence among GM maize lines. The chosen primer was optimized for CryIA(b) fragment amplification from Bt176 but not from Bt11. These results showed that our system made for detecting RR soybean was specific and that it could be used for detecting Bt176 and Bt11 maizes as well. To detect many more different lines of GM maize by our system, more diverse probes recognizing specific genes, construct-specific genes, and event-specific genes of different GM maizes could be chosen and included.

Conclusion. In this study, GM plants, Roundup Ready soybean and GM maizes, were chosen as model systems for our research subjects. Coupled with multiplex PCR, an oligonucleotide microarray system was designed to detect Roundup Ready soybean and GM maizes (Bt176 and Bt11). This system contains various probes for GM detection and identification. Our results showed that this system is highly specific, reliable, and easy to operate. It distinguishes different species of GM plants with high specificity and can significantly reduce the possibility of having false-positives and -negatives as compared to other currently used methods. It is also time-efficient because of its ability to detect RR soybean in one microarray. With this method, it is possible to design and add more specific probes, construct-specific probes and event-specific probes to the array for distinguishing more GM varieties, or for distinguishing different GM events in the same species by one microarray.

ACKNOWLEDGMENT

We thank Yu Ding for synthesizing all the primers and probes and Ping Qiao for spotting the microarray. We would also like to thank Dr. Zhongde Wang (Whitehead Institute, Massachusetts Institute of Technology, United States) for his critical reading of the manuscript. This study was financially supported by the National Basic Science Major Program (973) (Grant No. g1999011701) and the National Natural Science Foundation Key Program (Grant No.30430490) (China).

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Received for review September 30, 2004. Revised manuscript received March 18, 2005. Accepted March 18, 2005.

JF048368T