Detection of Six Genetically Modified Maize Lines Using Optical Thin-Film Biosensor Chips

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As more and more genetically modified organisms (GMO) are commercialized, efficient and inexpensive assays are required for their quick detection. An event-specific detection strategy based on the unique and specific sequences of integration junctions is useful because of its high specificity. This study developed a system for detecting six GM maize lines (Bt11, Bt176, GA21, MON810, NK603, and T25) using optical silicon thin-film biosensor chips. Aldehyde-labeled probes were arrayed and covalently attached to a hydrazine-derivatized chip surface. Biotinylated PCR amplicons were then hybridized with the probes. After washing and brief incubation with an anti-biotin IgG horseradish peroxidase conjugate and a precipitable horseradish peroxidase substrate, biotinylated PCR amplicons perfectly matched with the probes can be visualized by the color change on the chip surface (gold to blue/purple). This assay is extremely robust, exhibits high sensitivity and specificity, and is flexible from low through moderate to high throughput.

KEYWORDS: GMO; maize; optical thin-film biosensor chip; microarray

INTRODUCTION

DNA-based methods for detecting genetically modified organisms (GMO) are based on the detection of specific DNA fragments genetically engineered into the organisms. At present, most commercial testing is conducted using Polymerase Chain Reaction (PCR) technology (*1*−*3*). In principle, PCR can detect a single target molecule in a complex DNA mixture. However, many diagnostic applications require the simultaneous detection of several targets in samples (*4*, *5*). Therefore, the challenge for PCR is to analyze several targets simultaneously or to detect particular targets that may be present in several GM crops (*6*).

Development of multiplex PCR strategies is generally limited by the complexity of the amplification reaction (*7*−*9*). The number of possible primer pair combinations increases arithmetically with the number of primers present in the reaction, which leads to nonspecific side reactions. These nonspecific events and the variation in amplification efficiencies between amplicons are severe challenges to multiplex PCR (*4*, *5*, *10*). To overcome these problems, methods have been developed in which bipartite primers with both 5′ universal and 3′ specific regions are used in multiplex PCR (*4*). A more uniform amplification can be obtained with such approaches. Primers identical to the universal region can also be added with the bipartite primers or after a few

cycles of the PCR (*4*, *5*, *10*). This further improves the uniformity of the amplifications. However, the presence of the bipartite primers throughout the amplification contributes to primer−dimer formation and distortion of the quantitative results.

Microarray-based technology for detecting many types of molecules is currently under development. It has been developed in recent years for automated rapid screening of gene expression or sequence variation of large numbers of samples (*11*−*13*). The basic idea is that (many) selected probes are bound spotwise in array format to a solid surface, with each spot containing numerous copies of the probe. The array is subsequently hybridized with DNA isolated from the sample of interest that is labeled with a fluorescent marker. During the hybridization phase the labeled fragments will associate with the spotted probes by base-pairing between the cDNA sequences. The longer the stretch of complementary sequences, the stronger will be the bond (*14*). This method was widely applied in many research fields because it is informative and reproducible. However, the expensive equipment needed for scanning the arrays may be beyond the budget of many laboratories, particularly those in the public sector such as trading standards laboratories and public analysts.

As more and more GMO plants are created, PCR procedures must be developed to detect each line or transformation event. Event-specific primers amplify a fragment of a unique junction region between the inserted and plant DNA, which acts as a unique identifier (*15*−*17*). For rapid, efficient, accurate, and inexpensive detection, we developed a method for identifying

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genetically modified (GM) maize lines using the specific junction sequences of GM maize as probes on the surface of optical thin-film biosensor chips. This method combined the advantages of the information content of microarrays and the visiblility of results on biosensor chips. Because of the optical characteristics of the thin-film biosensor chip surface, experimental results of tests for GM markers can be visualized by the unaided human eye without any specific instruments. Therefore, this technology not only provides a high-throughput GMO assay but also eliminates a large initial investment for the purchase of expensive instruments. Moreover, the cost of chemical reagents per GMO analysis is relatively low. Taken together, we have developed a rapid, sensitive, and inexpensive technique that is based on microarrays yet provides data visible to the naked eye for identification of genetically modified maize lines.

MATERIALS AND METHODS

Maize Materials. Certified Reference Materials (CRMs) produced by the European Union (EU) Joint Research Center, Institute for Reference Materials and Measurements (IRMM), were purchased from Fluka, Buchs, Switzerland. Six GM maize lines [Bt11, Bt176, developed by Novartis Seeds Inc. (Greensboro, NC), GA21, MON810, NK603, developed by Monsanto Co. (St. Louis, MO), and T25, developed by AgrEvo (Canada Inc.)] were used. Nontransgenic maize was purchased from the local market in Beijing, China.

DNA Extraction. Plant genomic DNA was extracted and purified using the DNA extraction kit developed by Tiangen Biotech Co. Ltd. (Beijing, China) following the protocol provided by the manufacturer. DNA concentrations were calculated from the absorbance at 260 nm measured with a NanoDrop Instrument (NanoDrop Technologies, Wilmington, DE). Samples were diluted to 100 ng/μL. The quality of DNA samples was analyzed by agarose gel electrophoresis and then by the ability to amplify the endogenous maize ZEIN gene by PCR (forward primer, 5′gACATTgTggCATCATCATTT3′; reverse primer, 5′AgTgCgACCCA-TATTCCAg3′; PCR amplicon length, 277 bp).

PCR Conditions. PCR reactions were conducted according to the method described by Su et al. (7) modified as follows: PCR reactions were carried out in 50 μL reaction mixtures [containing 1× PCR buffer, 2 mmol/L MgCl$_2$, 0.1 mmol/L dNTP, 0.2 μmol/L of each primer, 1 U of rTaq enzyme (TaKaRa, Japan), and 100 ng of DNA template] for 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s in an ABI 2720 Thermal Cycler (Applied Biosystems), with preheating at 94 °C for 5 min and post-PCR incubation at 72 °C for 5 min. The PCR products were then quantified and sized by electrophoresis on agarose gels (2%, w/v). Gels were photographed with a GelDoc XR system (Bio-Rad).

Oligonucleotide Synthesis. Oligonucleotides were synthesized by Invitrogen (Shanghai, China). Published sequences available in the GenBank database, U.S. patents, and published papers (18) were used to design PCR primers and probes that bind the regions flanking the junctions between the inserted foreign genes and the recipient maize genomes. The genetic elements located in the six GM maize lines are shown in **Figure 1**. The sequences of PCR primers and probes for the detection of the GM maize lines are listed in **Table 1**. The reverse primers for PCR were synthesized with biotin at their 5′ ends for subsequent detection. The probes have 10 deoxyadenosine residues that constitute a "spacer" with an aldehyde group modification at their 5′ ends for conjugating to amino groups on the chip surface, followed by 40 nucleotides complementary to the corresponding target sequence.

Optical Thin-Film Biosensor Chips. Thin-film biosensors are capable of transducing specific molecular interactions into signals that can be visualized by the unaided human eye because chemicals deposited on the thin-film surface by enzymatic catalysis alter the wavelength of light reflected by the optical layer, resulting in a perceived color change on the surface (19, 20). The biosensors used here were prepared following the procedure described by Zhong et al. (19).

Standard Assay Protocol. Aldehyde-labeled oligonucleotide probes were spotted by manual pipet or a robotic pipetting device (Biodot AD3200), placing 250 or 40 nL per spot, respectively, onto biosensor chips from 1 μM oligonucleotide stocks in 0.1 M sodium phosphate buffer,
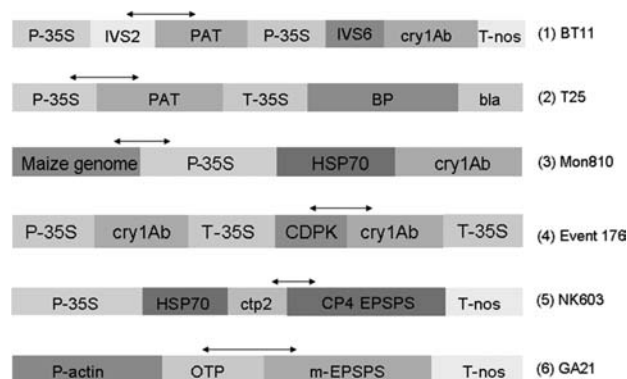
Figure 1. Schematic diagrams of the PCR primer pairs designed to detect six GM maize lines, (1) BT11, (2) T25, (3) MON810, (4) Event 176, (5) NK603, and (6) GA21. Arrows indicate the junctions that the primer pairs amplify.

pH 7.8. PCR amplicon targets, from 108 to 270 bp in length, at a concentration of 100 fmol each in 100 μL of reaction buffer were denatured and hybridized on the chip for 10 min at 45 °C in hybridization buffer [5× standard saline citrate (SSC) and 5 mg/mL acid-treated caseine (ATC)]. After three washes in 0.1× SSC, the chips were incubated with an anti-biotin IgG−horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch; 1:1000 dilution from a 1 mg/mL stock in a buffer containing 5× SSC/5 mg/mL ATC/10% glycerol) for 5 min in hybridization buffer. After rinsing with 0.1× SSC, 100 μL of tetramethylbenzidine (TMB) formulation from BioFx Laboratories (Owings Mills, MD) was added to the chips and incubated for 5 min at room temperature. The chips were then rinsed in ddH$_2$O, air-dried, and scored by eye. For photos the chips were imaged with a dissection microscope (Olympus, SZX12) fitted with an inexpensive digital camera.

RESULTS

**Strategy for Detecting GMO on a Thin-Film Biosensor Chip.** The procedure for detecting GMO described here relies on hybridization of biotinylated PCR fragments with probes covalently attached to thin-film biosensor silicon chips in a specific array (19). For each gene, we synthesized a forward primer and a reverse primer for PCR as well as a probe for spotting on the chip's surface. The reverse primer carries biotin at the 5′ end for subsequent detection (**Table 1**). The probes were 5′ aldehyde-labeled so they could be covalently attached to the chip surface by their 5′ termini. They had 10 dA as spacer followed by 40 bp of nucleotides corresponding to the sequence between the forward and reward primers of their targets (19, 20). Target DNA hybridization reactions were done simultaneously in a 10 min incubation, during which the forward probes complementarily bound the target amplicons. After a wash with 1× SSC to remove all mismatched molecules, immobilized biotinylated PCR products were detected by incubation with an anti-biotin IgG−HRP conjugate and a precipitable HRP substrate. Precipitate deposition from the substrate resulted in a distinguishable color change from gold to blue/purple on the chip surface, which, depending on the probe spotting and PCR product quantity, could be read either by unaided human eyes or by a simple digital-imaging system.

Defining Sensitivity and Specificity of Biosensor Chips. To establish the suitable probe density for spotting on biosensor chips, we randomly chose two probes, cpt2/EPSPS and OPT/mEPSPS, and diluted them to 0.01, 0.1, 1, and 10 μM and then manually spotted them in a total volume of 250 nL per spot (**Figure 2**, left panel). The chips were hybridized for 10 min at 45 °C with OPT/mEPSPS PCR products at 0, 0.1, 1, 10, and 100 fmol and then washed with 0.1× SSC at room temperature. Probes were visualized by a 5 min incubation with an anti-biotin

Table 1. Sequences of PCR Primers and Capture Probes for the Detection of Six GM Maize Lines

maize event	target gene	sequence[a]	fragment size (bp)	reference
Bt11	IVS2/PAT	F: 5′cttctgggaggccaaggtatct3′ R: 5′biotin-gctgctgtagctggcctaatct3′ P: 5′ALD-aaaaaaaaaaaaaaaaaaaaaccatcccatttgtgatctttgtcagtagatatgatacaac	192	AY629236
T25	CaMV35S/PAT	F: 5′agatcatcaatccactcttgtggtg3′ R: 5′biotin-ccttcgcaagacccttcctctata3′ P: 5′ALD-aaaaaaaaaaaaaaaaaaaaagccatatcagctgctgtagctggcctaatctcaactggtc	231	BD378188
Mon810	maize genome/CaMV35S	F: 5′tcgaaggacgaaggactctaacg3′ R: 5′biotin-tccatctttgggaccactgtcg 3′ P: 5′ALD-aaaaaaaaaaaaaaaaaaaaaccattgcccagctatctgtcactttattgtgaagatagtg	170	U.S. Patent 6713259
Event 176	CDPK/CryIA(b)	F: 5′ctctcgccgttcatgtccgt3′ R: 5′biotin-ggtcaggctcaggctgatgt3′ P: 5′ALD-aaaaaaaaaaaaaaaaaaaaatccaacaatggacaacaaccccaacatcaacgagtgcat	211	U.S. Patent 5625136
NK603	ctp2/EPSPS	F: 5′atgaatgacctcgagtaagcttgttaa3′ R: 5′biotin-aagagataacaggatccactcaaacact3′ P: 5′ALD-aaaaaaaaaaaaaaaaaaaaaagggatatcaagcttggtaccacgcgacacacttccactct	108	U.S. Patent 6825400
GA21	OTP/mEPSPS	F: 5′acggtggaagagttcaatgtatg3′ R: 5′biotin-tctccttgatgggctgca3′ P: 5′ALD-aaaaaaaaaaaaaaaaaaaaaccgtgatgatggcctcgtcggccaccgccgtcgctccg	270	U.S. Patent 4940835

[a] F, forward PCR primer. R, reverse PCR primer; P, probe; ALD, aldehyde modification.
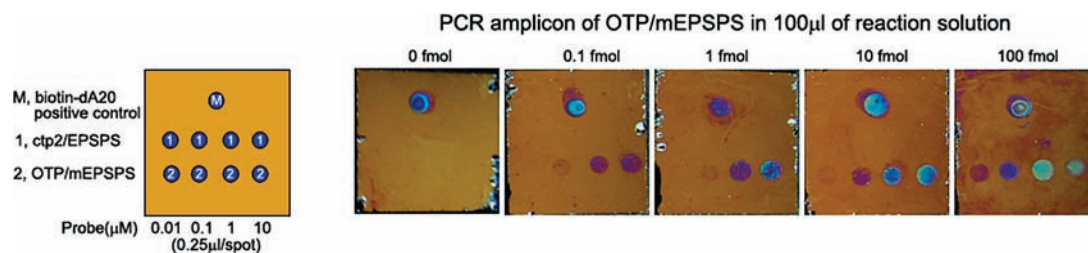
Figure 2. Specificity and sensitivity of GMO detection on a chip with capture probes spotted by hand at various concentrations. Two hundred and fifty nanoliters of probes capturing the ctp2/EPSPS and OTP/mEPSPS genes was spotted by hand at concentrations of 0.01, 0.1, 1, and 10 μM, respectively (left panel). One micromolar M, biotin-dA20, was spotted as positive control. PCR amplicons of OTP/mEPSPS at concentrations of 0, 0.1, 1, 10, and 100 fmol, respectively, in 100 μL of reaction solution were hybridized to five identical chips (right panel).

IgG−horseradish (HRP) conjugate followed by a 5 min incubation with tetramethylbenzidine (TMB). TMB is a precipitable HRP substrate that can increase the thickness of the thin film after reaction, thereby inducing the perceived color change. The chips were then washed, dried, and digitally imaged. Only spots containing OPT/mEPSPS probes, but not EPSPS probes, were detected, indicating the remarkable specificity of this assay (**Figure 2**, right panel). Although the signal intensity decreased with the concentration of target sequence, as little as 0.1 fmol of target was detected when the probe was spotted at 0.1 μM. The signals were detected when the probe concentration was as low as 0.01 μM in the presence of 100 fmol of PCR products. Spotting of probes from stock solutions of more than 1 μM did not increase detection sensitivity; therefore, we adopted 1 μM as our stock concentration for subsequent spotting.

Amplification of Six Target Fragments by PCR. The PCR products for the six maize lines are shown in **Figure 3**. The sizes of PCR products were as predicted (**Table 1**). The results showed that PCR amplification of BT11 maize samples produced one band corresponding to IVS/PAT (**Figure 3**, lane 1), PCR amplification of T25 maize samples produced one band corresponding to CaMV35S/PAT (**Figure 3**, lane 2), PCR amplification of Mon810 maize samples produced one band corresponding to maize genome/CaMV35S (**Figure 3**, lane 3), PCR amplification of

Event 176 maize samples produced one band corresponding to CDPK/CryIA(b) (**Figure 3**, lane 4), PCR amplification of NK603 maize samples produced one band corresponding to EPSPS (**Figure 3**, lane 5), and PCR amplification of GA21 maize samples produced one band corresponding to OTP/mEPSPS (**Figure 3**, lane 6). The DNA targets were successfully amplified by PCR, which showed highly specific amplification.

Chip Design and Detection of Target Genes from Six GM Maize Lines. Microarray techniques were applied to this detection system. Chips spotted as shown in **Figure 4** (upper panel) were used to detect six GM maize lines. M represented the positive control biotin-dA20 that always showed a signal if the chip detection system worked. In this detection system all other genes were negative controls for the detected gene.

Results showed that specific signals were detected from all six GM maize lines (**Figure 4**, lower panel). For example, the IVS/PAT gene targets amplified from the BT11 sample (**Figure 3**, lane 1) were used for hybridization to a biochip and the assay resulted in one set of colored dots (**Figure 4**, lower panel, 1). The other five gene targets showed their own specific sets of colored dots (**Figure 4**, lower panel, 2−6). No false-positive signals were observed among these tests. Our data indicate that one custom-designed chip can be readily used to detect the presence of these six GM maize lines.

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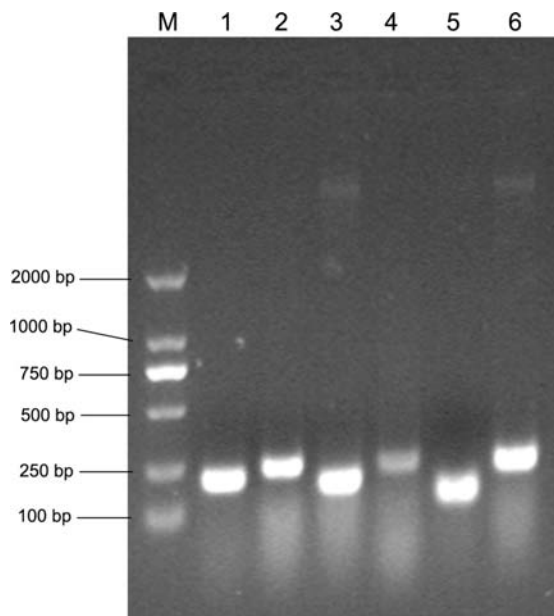
Figure 3. PCR amplification of target DNA fragments from six GM maize lines. PCR products specific for each line were checked by agarose gel electrophoresis. Lanes: M, DNA marker, DL 2000; 1−6, PCR amplicons of integration junctions [1, IVS2/PAT; 2, CaMV35S/PAT; 3, maize genome/CaMV35S; 4, CDPK/CryIA(b); 5, ctp2/EPSPS; 6, OTP/mEPSPS].
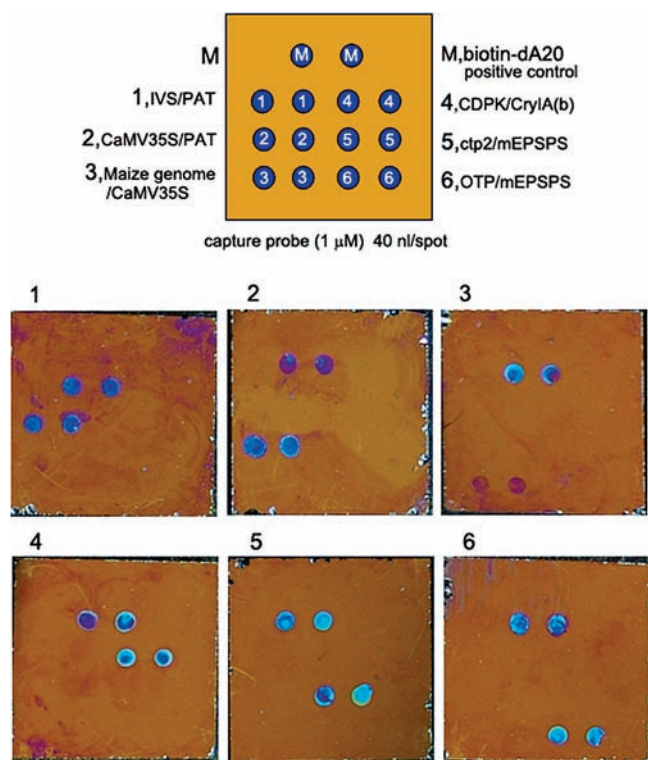
Figure 4. GM maize detection on a chip with capture probes spotted by a computer-controlled dispenser. Each spot comprised 40 nL of 1 μM probe solution. (Upper panel) Capture probes were printed in the order M, biotin-dA20 (positive control and marker); spots 1−6, specific integration junction regions [spot 1, IVS2/PAT; spot 2, CaMV35S/PAT; spot 3, maize genome/CaMV35S; spot 4, CDPK/CryIA(b); spot 5, ctp2/EPSPS; spot 6, OTP/mEPSPS]. (Lower panel) Detection of foreign genes in GM maize products on thin-film biosensor chips. 1−6 represent the detection results of six integration junction regions from Bt11, T25, Mon810, Event 176, NK603, and GA21, respectively.

DISCUSSION

Analytical methods have been developed for identifying the presence and quantity of GM DNA sequences (*21−23*). The main challenge in the context of GMO detection method development is coping with the increasing number of GMOs worldwide. The method we developed here, optical thin-film biosensor chips for detection of genetically modified maize lines, combines the information-intensive advantages of microarrays and the visibility advantage of biosensor chips, which allow signals to be read directly by unaided human eyes. This method meets the aims of faster, more informative, and user-friendly detection that has the added benefit of requiring fewer reagents.

As described above, precipitates deposited on the thin-film surface by enzymatic catalysis alter the wavelength of light reflected by the optical layer resulting in a perceived color change on the surface. Differing amounts of precipitates will result in differing colors ranging from purple to light blue (*19*). This detection method will become much more powerful if an accurate relationship between the quantities and the colors can be established, because this would enable one-step identification and quantitative measurement of GMO.

Because the number of GMOs is rapidly increasing, the release of GM crops and products has resulted in worldwide public debate (*24−28*). A great number of different strategies and methods are available for testing GM material. However, these are technically demanding, so the result quality depends not only on the methodology and equipment but also on the sampling and the theoretical expertise and practical skills of the examining officers (*1*, *21−23*, *29*). Therefore, easy and high-throughput methods will be essential for the detection of GM crops in the global market. The efficient, accurate, and simple method described above for the detection of GMOs on thin-film biosensor chips has laid a foundation for the future development of chip detection technology. This chip may be further modified to permit the detection of all commercialized GMOs.

ABBREVIATIONS USED

BT11, T25, M810, Event 176, GM, NK603, and GA21, genetically modified maize lines; GMO, genetically modified organism; IRMM, Institute for Reference Materials and Measurements; EPSPS, a region of 5-enolpyruvylshikimate-3-phosphate synthase gene introduced into NK603; PAT, a region of the phosphinothricin-*N*-acetyltransferase gene derived from *Streptomyces hygroscopicus*; CaMV, cauliflower mosaic virus; PCR, Polymerase Chain Reaction; P-35S, 35S promoter region derived from CaMV; T-NOS, terminator region of the nopaline synthase gene derived from *Rhizobium radiobacter*; IVS 2, intervening sequence 2; IVS 6, intervening seguence 6; Cry1Ab, δ-endotoxin; Bla, β-lactamase.

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