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Original Article

Visual DNA Microarray Coupled with Multiplex-PCR for the Rapid Detection of Twelve Genetically Modified Maize

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Abstract We herein developed a visual DNA microarray system coupled with multiplex PCR (m-PCR) to rapidly detect twelve genetically modified maize (GMM). The microarray comprised short oligonucleotide probes complimentary to the specific gene region for twelve different GMM. The m-PCR products annealed to the microarray probe were reacted with streptavidin-alkaline phosphatase conjugate and nitro blue tetrazolium/5-bromo-4-chloro-3′-indolylphosphate, p-toluidine salt (NBT/BCIP), resulting in blue spots that are easily visualized by unaided eyes for qualitative analysis. To ensure the reliability of this method, positive and negative hybridization controls were used in DNA microarray. Commercial GM materials (GMM: Bt176, Bt11, MON810, GA21, T25, MON88017, NK603, MON863, MON89034, DAS-59122-7, TC1507, MIR604; GM cotton: (MON1445, MON15985); GM soybean (Monsanto Roundup Ready soybean 40-3-2)) and non-GM materials were identified by this method and further confirmed by PCR and sequencing. The results showed that each probe consistently identified its corresponding GMM target very quickly and in a cost-effective and more time efficient way. The limit of detection is 0.5% for Bt176, Bt11, T25, MON88017, DAS59122-7, MON89034 and 1% for MON810, MIR604, GA21, MON863, NK603, TC1507. This method is advantageous because of rapid detection, cost-effectiveness and ease of use. These high specificity and sensitivity results demonstrate the feasibility of using this method in routine analysis of GMOs.

Keywords: Visual detection, Multiplex PCR, Genetically modified maize, Microarray, Biochip

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Introduction

Since the introduction of genetically modified organisms (GMO) in the food chain, the debate over the safety of GM foods has never been stopped. In order to protect the consumer's right to know, several countries, including China, Japan and many members of European Union, have set up food-labeling laws that incorporate limits for the reason of safety concerning GM crops. Thus, food authenticity is presently a subject of great concern to food authorities, as the incorrect labeling of foodstuffs can represent a commercial fraud. To meet these challenges, the novel or refinement of existing analytical methodologies for the verification of any GM foods labeling statements is indispensable[1,2]. At present, the two most prevalent methods for GMO detection rely mainly on antibody-based immunoassay[3] or DNA-based PCR[4,5], while the latter is a prefer alternative to immunoassay due to its high stability and to its presence in most biological tissues[6-8]. However, PCR-based methods, especially for multiplex PCR when it is used to amplify two or more DNA fragments simultaneously, are not an accurate method due to its difficulty in the discrimination of PCR products by the following gel electrophoresis[9,10]. In recent years, the combination of multiplex PCR and DNA microarray for the detection of GMO is reported[11-14]. Although DNA microarray technology makes a remedy for the shortage of PCR-based methods, the

existing DNA microarray methodology has some disadvantages. In most microarray formats, substrate was made of glass, which is fragile. The glass substrates are often stained with fluorophore, and the signal has to be measured by expensive instruments, such as epifluorescence confocal microscopy or a microarray scanner. In addition, such experiment put a high demand for the operator due to the complicated analysis of fluorescence data. Therefore, the refinement of existing DNA microarray technology is required. In this paper, the DNA probe was immobilized onto a 75 × 25 mm polystyrene substrate via a mature technique of UV cross-linking, which overcomes the fragile shortage of glass substrate. In addition, precipitation staining methods were introduced to the microarray technology. In the system, biotin-labeled PCR products are firstly captured by the specific probe on the microarray during hybridization. Then the streptavidin conjugated alkaline phosphatase (Strep-AP) in the staining reagent binds to the biotinylated site in the hybridization complexes. The chromogenic precipitation resulting from the reaction of 5-bromo-4-chloro-3′-indolylphosphate, p-toluidine salt (BCIP) in the colorimetric developing reagent and Strep-AP produces a blue-colored precipitate at the site of enzymatic activity. Nitro blue tetrazolium (NBT) acts as a co-precipitant agent for the BCIP reaction, forming a dark blue, precisely localized precipitate thus helps to visualize positive spots on the microarray. We here constructed a visual DNA microarray and applied it to detect the transgenic maize, reducing the need for expensive fluorescence detection facilities and the sophisticated technician.

Results and Discussion

Optimization of the Experimental Condition

The whole detection system described here mainly involves two steps: m-PCR and chip hybridization. It is necessary to optimize all reaction procedures to ensure the reliability of this detection system. The m-PCR technique could simultaneously amplify multiple target sequences in a single reaction, which can greatly save time and is cost-effective. In this study, each set of 13 primer pairs can effectively amplify the specific gene fragments in a single-plex PCR reaction (Data not shown). However, with the increasing of primer pairs set, the efficiency of m-PCR was affected greatly. Thirteen primer pairs were divided into two tubes in this study, although the primer pairs can be up to >13 within one tube in some application[16]. After optimization of primer sets combination, specific gene fragments can be effectively amplified by two tubes of m-PCR, of which there is no non-specific band occurs in the following agar gel electrophoresis.

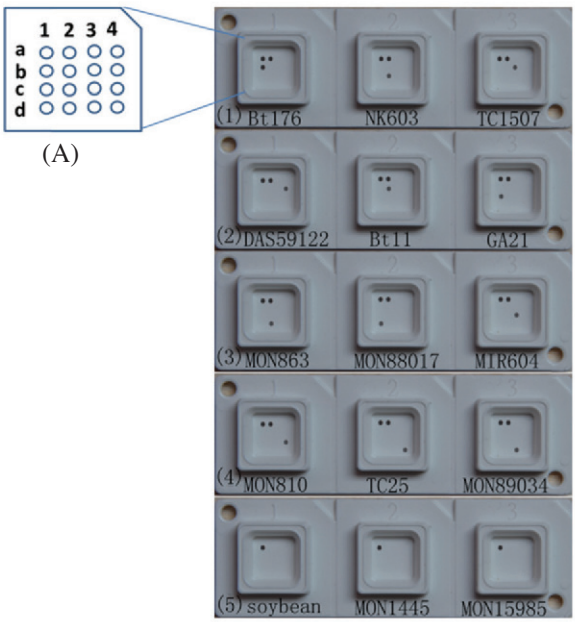

Fig. 1. (A) Probe pattern on microarray. Dots indicate the position of each probe. a1: hybridization positive control; a2: zein; a3: negative control; a4: blank; b1: Bt176; b2: Bt11; b3: TC1507; b4: DAS59122-7; c1: GA21; c2: NK603; c3: MIR604; c4: MON810; d1: MON88017; d2: MON863; d3: MON89034; d4: TC25. (B) Detection and results on the microarray.

To avoid the cross-hybridization reaction, probes were strictly selected by a web-based tool unique probe selector program, and its use and application were described in detailed in references[15,17,18]. All probes were normalized to a calculated annealing temperature of 63 ± 1°C. In addition, to supervise the whole detecting process, the stringent quality control was set up using EV71 probe and poly (T)30 probe as positive and negative control, respectively.

Evaluation of Method

To confirm the prepared GM maize chip, several experiments were conducted to evaluate the reliability, reproducibility and limit of detection. Every experiment was repeated three times. For reliability test, commercial GM maize (Bt176, Bt11, MON810, GA21, T25, MON88017, NK603, MON863, MON89034, DAS-59122-7, TC1507, and MIR604), GM soybean (Monsanto Roundup Ready soybean 40-3-2), GM cotton (MON1445, MON15985) and non-GMO were identified by this method (Figure 1).

As shown in Figure 1, each of twelve GM maize can be effectively detected by this method, while other GM and non-GM materials have no signal occurring except for the hybridization positive control. Furthermore, the identified results of GMMs from DNA microarray were consistent with the one from sequencing of PCR products, which indicating that the method is highly reliable (Data not shown).

To further investigate the ability of multiplex detection, the m-PCR reaction was performed by using of mixed genomic DNA (group 1:TC1507, T25, MON88017, DAS59122-7, MON89034, Bt11; group 2: Bt176, MON810, MIR604, GA21, MON863, NK603) as template. The amplicons were identified by prepared DNA microarray (Figure 2).

The prepared DNA microarray can simultaneously identify multiple targets in one test without cross hybridization. The signal intensity was varied between spots, which may attribute to different amplification efficiency in m-PCR.

For reproducibility evaluation, the same GM maize sample was identified by the chips prepared in the within and between batches. Each type of test was repeated over 5 times, the prepared chips within and between batches successfully identified the GM sample throughout the whole test, indicating high reproducibility.

(1) (2)

Fig. 2. (1), (2): Microarray hybridization of amplicons from group 1, group 2, respectively.

The detection limit of this method is defined as the ability of the prepared DNA microarray to detect different components in parallel without reciprocal interference and to identify the analysis unambiguously. The limit of detection was determined by using of a series of maize sample with known GM contents (0.0%, 0.1%, 0.5%, 1.0%, and 2.0%), Bt176 as an example was shown in Figure 3. In the present study, hybridization results revealed that the limit of detection is 0.5% for Bt176, Bt11, T25, MON88017, DAS59122-7, MON89034 and 1% for MON810, MIR604, GA21, MON863, NK603, TC1507, which is comparable to that by m-PCR[19] or better than that by existing DNA microarray[13].

In this study, DNA microarray and m-PCR were employed to develop an assay that is capable of simultaneous and specific detection of 12 GMM. The obvious advantages of such combined assay over traditional gel electrophoresis-based m-PCR are that it does not depend on the size of the amplification products for product identification. However, the existing microarray has some limitations: 1) it is fragile for the cause of glass nature; 2) expensive equipment and highly skilled technician required for the using of fluorescence detection; 3) low detection throughput (one sample/one slide), which has limitation for large sample size testing. Comparison to existing DNA microarray, DR. N3 chip has three macro-well on a 75 × 25 mm polystyrene substrate, and probe array was integrated into individual well, i.e. one chip one well. Such design improved the detection throughput comparison with conventional slide microarray. Furthermore, the established experimental condition on this substrate can easily applied to a higher throughput substrate, such as 96 well plates, which is very useful for a test with a large number of samples. In addition, for the sake of easy to use, a colorimetric reaction by alkaline

(0.1%) (0.5%) (1%) (2%)

Fig. 3. Detection limit of DNA microarray.

phosphatase and its substrate (NBT/BCIP) was introduced into this assay. Such design not only facilitate the detection process (especially for qualitative analysis, the positive results can be directly visualized by unaided eyes) but also reduce the requirement of expensive equipment and skilled technicians.

Conclusions

Our m-PCR based visual DNA microarray offers a highly reliable and specific method for detection of twelve GMM. In current study, we only test the samples of seeds and grains; it is not enough for routine GMO detection. How to treat different samples such as food, etc., is still a hard work we will do in the future.

Materials and Methods

Samples

GM maize (Bt176, Bt11, MON810, GA21, T25, MON88017, NK603, MON863, MON89034, DAS-59122-7, TC1507, MIR604) and GM soybean (Monsanto Roundup Ready soybean 40-3-2), GM cotton (MON1445, MON15985), non-GMO were provided by Monsanto (St. Louis, MO, USA), Bayer Crop Science (Monheim am Rhein, Germany) and used to test the feasibility of microarrays as positive and negative controls, respectively. Certified reference materials (CRM; Institute of Reference Materials and Measurements [IRMM], (Fluka Chemie GmbH, Buchs, Switzerland)), were provided as individual vials containing flours at 0.0%, 0.1%, 0.5%,1.0%, 2.0% (% w/w) for the validation experiments to determine limit of detection.

DNA Extraction

Genomic DNA from GM and non-GM plant materials was extracted with the DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The concentrations of extracted DNA were measured with a UV spectrophotometer Beckman DU-640 (Beckman, Corona, CA). The final concentration was adjusted to 10-100 ng/μL for use in PCR. Absorbance at 260 nm, 280 nm and 230 nm was determined to evaluate the quality of extracted DNA.

Primers and Probes Design

A total of 13 primer pairs were used in this study. All primers were designed using the Prime Premier software version 5.0 and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). To establish a multiplex PCR system for simultaneous amplification of target genes in GM maize in processed foods, PCR primers were selected to meet the following criteria: (1) smaller sized amplicons (each amplification spans 100-300 bp); (2) have a melting point between 55 and 60°C; (3) be compatible in the PCR mixture.

Fourteen probes were designed based on the specific gene region using the unique probe selector program (http:array.iis.sinica.edu.tw/ups/). The maize zein protein gene was chosen as an endogenous reference gene. Probe EV71 was used as positive control for the confirmation of hybridization efficacy (the biotin-labeled EV71 PCR amplicon was incorporated in the hybridization buffer supplied with the DR. Chip DIY Kit^{TM}), and thirty poly (A) oligonucleotides were used as the negative control probe [15]. All probes were normalized to a calculated annealing temperature of 63 ± 1°C and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The nucleotide sequences of the primers and probes are shown in Table 1. The reverse primers for PCR were synthesized with a biotin modification at the 5′ end to generate biotinylated PCR amplicons that could react with streptavidin-conjugated alkaline phosphatase (Strep-AP) and NBT/BCIP for colorimetric signaling on the chips. Each probe has an poly (T)15 modification at their 5′ end for immobilization and as a spacer arm.

Target DNA PCR Amplification

The specific gene fragments of GM maize were amplified by two group of multiplex PCR using 13 primer pairs. In brief, m-PCR group-1 was performed in a 30 μL reaction mixture containing 0.3 μL of Taq DNA polymerase (5 U/μL; Promega), 3 μL of 10 × PCR buffer, 1.2 μL of 10 mM dNTP mix, 6 μL of 10 mM $MgCl_2$, 0.2 μL of 10 μM primer Zein-f, Zein-r, TC1507 -f, TC1507-r, T25-f, T25-r; 0.8 μL of 10 μM primer MON88017-f, MON88017-r, DAS-59122-7-f, DAS-59122-7-r, MON89034-f MON89034-r and Bt11-f, Bt11-r, and 1.2 μL of genomic DNA (100 ng/μL), and 10.7 μL sterile H_2O. M-PCR group-2 was carried out with the same components except for primers and the volume of H_2O as below: 0.2 μL of 10 μM primer Zein-f, Zein-r, Bt176-f, Bt176-r, MON810-f, MON810-r, 1.2 μL of 10 μM primer MIR604-f, MIR604-r, GA21-f, GA21-r; 1.6 μL of 10 μM primer MON863-f, MON863-r, and NK603-f, NK603-r, and 5.9 μL sterile H_2O.

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

Table 1. Oligonucleotides used in this study.

Oligo nucleotide	Sequence 5′-3′	Length (bp)	Reference
Probe			
EV71-P	ATGAAGCATGTCAGGGCTTGGATACCTCG	29	[15]
Zein-P	TTTCCTCTCGTTCTGGCTCTAACTTGGTTC	30	This work
Bt176-P	GATCTGATGTTCTCTCCTCCATTGATGCAC	30	This work
Bt11-P	TTGCCATAGATTACATTCTTGGATTTCTGGTGG	33	This work
TC1507-P	ACATAGACATTAACCCTGAGACTGTTGGACAT	32	This work
DAS-59122-7-P	CAATTCGCCCTATAGTGAGTCGTATTACAATCG	33	This work
GA21- P	AAAGTTCCCAGTTGAGGATGCTAAAGAGGA	30	This work
NK603- P	TGGACTATCCCGACTCTCTTCTCAAGCATA	30	This work
MIR604- P	ATGAGCGGAGAATTAAGGGAGTCACGTTAT	30	This work
MON810- P	GAGGACTTTCGGTAGCCTTCTTTCATTTCC	30	This work
T25-P	TGGCGGAACGACTCAATGACAAGAAATATC	30	This work
MON88017-P	TTGTGTAATCGGCTAATCGCCAACAGATTC	30	This work
MON863-P	TCATTGCGATAAAGGAAAGGCTATCATTCAAGA	33	This work
MON89034-P	TCAACCCAAAGTTTCTTCATGGCACATCTAG	31	This work
Primers		amplicon size (bp)	
Zein-f	CTGGATGAACGGTTAGTTGGAC	177	This work
Zein-r	GAGAAGCCGGTCGAGGTAG		
Bt176-f	CTGCCCGTCACCGAGATC	123	This work
Bt176-r	ATAGGAAGGGAGAGGGAGAGAG		
Bt11-f	GCCATTTATCATCGACCAGAGG	180	This work
Bt11-r	CACACAACCGCCATTTTGC		
TC1507-f	GCGTCGCACAGTGAAAATC	193	This work
TC1507-r	AACCCGAGGATATAGCAAAGC		
DAS-59122-7-f	GCCTCTTGCTCTTCAGGATG	168	This work
DAS-59122-7-r	AAACAAACGGGACCATAGAAGG		
GA21-f	GCTGTAGTTGTTGGCTGTGG	142	This work
GA21-r	GGACTATCCCGACTCTCTTCTC		
NK603-f	TGGACTATCCCGACTCTCTTC	129	This work
NK603-r	ACAGGATCCACTCAAACACTAG		
MIR604-f	GAAGGCGGGAAACGACAATC	114	This work
MIR604-r	GAAGGCGGGAAACGACAATC		
MON810-f	CCACCACAGCCACCACTTC	100	This work
MON810-r	CTCGCAAGCAAATTCGGAAATG		
T25-f	GCTACGACATGATACTCCTTCC	105	This work
T25-r	ATTGCCCTTTGGTCTTCTGAG		
MON88017-f	TACTTGTGTAATCGGCTAATCG	191	This work
MON88017-r	AACTGAAGGCGGGAAACG		
MON863-f	TTCCGATCCTACCTGTCACTTC	110	This work
MON863-r	CGGCAGAGGCATCTTGAATG		
MON89034-f	CATTGCATCCCCGGAAATTATG	199	This work
MON89034-r	CCAGGTGGAGACAGGCTAC		

and 30 s at 72°C; followed by 1 cycle at 72°C for 10 min.

DNA microarray Preparation and Hybridization

20 μM of each DNA probes was spotted onto the specific position on a polystyrene substrate DR. N3 chip (supplied with the DR. Chip DIY Kit™, DR. Chip Biotechnology, Inc., Taiwan) using a contact spotting machine (DR. Fast Spot™; DR. Chip Biotechnology, Inc.). The spotted probes were cross-linked to the surface of substrate by exposing to UV light using a UV cross-linker set at 254 nm, 3 min (Scientz03-II; Ningbo

xinzhi biotechnology co., LTD, China). After successive washing with 0.01 M phosphate-buffered saline (pH7.4), 0.85% (w/v) NaCl (PBS) containing 0.05% Tween 20 (PBST) and MiliQ-water, the microarray was air-dried and stored at 4°C before use.

Hybridization and colorimetric detection were performed using the DR. Chip DIY Kit™ (DR. Chip Biotechnology, Inc.). In brief, PCR products were denatured in boiling water for 5 min, and immediately chilled on ice for 5 min. 15 μL of ice-cold PCR products were mixed with 200 μL DR. Hyb™ Buffer (DR. Chip Biotechnology, Inc.) and transferred to the chip well, incubated at 63°C with vibration for 40 min, and washed twice with wash buffer (DR. Chip Biotechnology, Inc.). The chip was then added to a mixture solution containing 0.2 μL Strep-AP (DR. Chip Biotechnology, Inc.; 0.5 μL/mL in blocking buffer) and 200 μL blocking reagent (DR. Chip Biotechnology, Inc.) and incubated at room temperature (25°C) for 30 min and washed twice again with wash buffer. 4 μL NBT/BCIP and 196 μL detection buffer (DR. Chip Biotechnology, Inc.) were added to the chip well and incubated for 7 min at room temperature in the dark, followed by washing twice with distilled water. Positive hybridization results were indicated on the microarray as blue spots that could be read directly by the naked eye.

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