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Establishment of a Multiplex-PCR System for Identification of Genetically Modified Maize Events

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Abstract: More and more genetically modified (GM) crops have been approved for various commercial purposes in the world for high yield and resistance against abiotic and biotic factors. On the other hand, GM crops have also created public fear about ecology and human health. It is necessary to establish a high throughput cost-effective detection platform for large-scale GM crops. In the present study, the multiplex PCR system was developed to amplify specific maize sequences from plant DNA (IVR and ZSSIIb) and genetic control elements CaMV 35S, NOS terminator and Bt11 (IVS2/PAT) gene for the detection of GM maize. The results showed that the multiplex PCR system was reliable, sensitive and rapid for detection of mass GM maize.

Key words: GM crops · Genetic control · Maize · Multiplex PCR · Orthogonal experiment design

INTRODUCTION

Since the industrialization of the first genetically modified (GM) crop in 1996, the transgenic technology has become an effective method for the development of GM maize with improved insect resistance, herbicide and drought tolerances. So far, more than 24 GM crops have been approved and produced for various commercial purposes in around 25 countries [1].

With enormous social and economic returns of GM crops, its protection has attained worldwide attention. GM crop protection mainly focused on genetic pollution and damage to human health. The first scenario occurred in 1996 when the 2S albumen protein from Brazil nut was transferred into soybean, which created an IgE mediated response towards GM soybean [2]. There was also report that liver, pancreas and testes function were disturbed in mice fed GM soybean [3].

Several countries including China have set up food-labeling laws to provide freedom to consumers for the selection of GM organisms and conventional products [4]. This requirement makes it necessary to develop qualitative and quantitative methods for detection of GM organisms. DNA-based PCR is the most frequently used technique in GM organism detection because of its high stability [5, 6].

Multiplex PCR put forward by Chamberlian *et al.* [7]. So far, multiplex PCR has been successfully applied to biological researches for example, pathogenic identification and detection, genetic disease diagnosis, gene deletion and polymorphism analysis [8-10]. Now-a-days, multiplex PCR technique has also been extensively applied to detect GM crops [6, 11-16]. The objective of the present study was to develop a feasible multiplex PCR protocol for qualitative screening and large-scale detection of GM maize.

MATERIALS AND METHODS

Plant Materials: All the plant materials, including Shen502 with Bt11(IVS2/PAT) gene, K162 without Bt11(IVS2/PAT) gene and F_2 plants of Shen502×K162, were obtained from Maize Institute, Shenyang Academy of Agricultural Sciences, Shenyang, China.

PCR Primers: The sequences of oligonucleotide primers used in this study to detect the Caulifower Mosaic Virus 35S promoter (CaMV 35S) and the *Agrobacterium tumefaciens* NOS terminator (NOS terminator), Bt11(IVS2/PAT), IVR and ZSSIIb genes are listed in Table 1. The primers were synthesized by Shaibaisheng Biotechnologies, Inc. (Beijing, China).

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Table 1: List of Oligonucleotide primers

Gene	Sequence (5'-3')	Amplicon (bp)	Reference
CaMV 35S	5'-TCA TCC CTT ACG TCA GTG GAG-3 5'-CCA TCA TTG CGA TAA AGGAAA-3'	165	SN/T, 2003
NOS	5'-GAA TCC TGT TGC CGG TCT TG-3' 5'-TTA TCC TAG TTT GCG CGC TA-3'	180	SN/T, 2003
IVS2/PAT	5'-CTG GGA GGC CAA GGT ATC TAA T-3' 5'-GCT GCT GTA GCT GGC CTA ATC-3'	189	SN/T, 2003
ZSSIIb	5'-CGG TGG ATG CTA AGG CTG ATG-3' 5'-AAA GGG CCA GGT TCA TTA TCC TC-3'	88	Wu, H. B., *et al*. 2009
IVR	' 5'-CCG CTG TAT CAC AAG GGC TGG TAC C-3' 5'-GGA GCC CGT GTA GAG CAT GAC GAT C-3'	226	SN/T, 2003

Table 2: The variable of orthogonal experimental design for duplex PCR

Run No.	dNTP concentration (µl)	Taq polymerase concentration (U)	Primer 1[a] concentration (µmol/L)	Primer 2[b] concentration (µmol/L)	anneal temperature (¡æ)
1	1.0	0.12	0.20	0.20	54
2	1.0	0.13	0.22	0.22	56
3	1.0	0.14	0.24	0.24	58
4	1.0	0.15	0.26	0.26	60
5	1.1	0.12	0.22	0.26	58
6	1.1	0.13	0.24	0.24	60
7	1.1	0.14	0.26	0.22	54
8	1.1	0.15	0.20	0.20	56
9	1.2	0.12	0.24	0.22	60
10	1.2	0.13	0.26	0.20	58
11	1.2	0.14	0.20	0.26	56
12	1.2	0.15	0.22	0.24	54
13	1.3	0.12	0.26	0.24	56
14	1.3	0.13	0.24	0.26	54
15	1.3	0.14	0.22	0.20	60
16	1.3	0.15	0.20	0.22	58

[a]primer 1= CaMV 35S.
[b]primer 2= NOS.

Table 3: The variable of orthogonal experimental design for triplex PCR

Run No.	dNTP concentration (µl)	Taq polymerase concentration (U)	Primer 1[c] concentration (µmol/L)	Primer 2[d] concentration (µmol/L)	Primer 3[e] concentration (µmol/L)	anneal temperature(¡æ)
1	1.8	0.18	0.20	0.36	0.36	54
2	1.8	0.20	0.22	0.38	0.38	56
3	1.8	0.22	0.24	0.40	0.40	58
4	1.8	0.24	0.26	0.42	0.42	60
5	1.8	0.26	0.28	0.44	0.44	62
6	2.0	0.18	0.22	0.40	0.42	62
7	2.0	0.20	0.24	0.42	0.44	54
8	2.0	0.22	0.26	0.44	0.36	56
9	2.0	0.24	0.28	0.44	0.38	58
10	2.0	0.26	0.20	0.38	0.40	60
11	2.2	0.18	0.24	0.40	0.38	60
12	2.2	0.20	0.26	0.36	0.40	62
13	2.2	0.22	0.28	0.38	0.42	54
14	2.2	0.24	0.20	0.40	0.44	56
15	2.2	0.26	0.22	0.42	0.36	58
16	2.4	0.18	0.26	0.38	0.44	58
17	2.4	0.20	0.28	0.40	0.36	60
18	2.4	0.22	0.20	0.42	0.38	62
19	2.4	0.24	0.22	0.44	0.40	54
20	2.4	0.26	0.24	0.36	0.42	58
21	2.6	0.18	0.28	0.42	0.40	56
22	2.6	0.20	0.20	0.44	0.42	58
23	2.6	0.22	0.22	0.36	0.44	60
24	2.6	0.24	0.24	0.38	0.36	62

cprimer 1=ZSSIIb gene.
[d]primer 2=CaMV 35S.
[e]primer 3=Bt11(IVS2/PAT) gene.

DNA extraction, PCR amplification and electrophoresis: Genomic DNA was isolated from 0.5g samples of two-leaf seedlings maize plants for each parent and F_2 plants by using the cetyltrimethyl ammonium bromide (CTAB). PCR reactions (volume 10μL) contained 10×PCR buffer 1μl, dNTP 1μl, 0.2μmol/L of each dNTP, DNA 100ng, Taq DNA 1U were purchased from Tiangen Biotechnologies, Inc. (Beijing, China). After 5 min of denaturation at 94°C, amplifications were programmed for 34 consecutive cycles, each consisting of 45s at 94°C, 45s at 58°C, 1 min at 72°C and followed by a 10 min extension step at 72°C. After amplification, about 2.5μl of the PCR products mixed with loading buffer and each sample was loaded for electrophoresis in a 2% agarose gel.

Optimization of Multiplex PCR System: By using DNA of Shen502 with Bt11(IVS2/PAT) for optimizing the factors which influence the multiplex PCR, orthogonal experiment design was employed to estimate the appropriate initial value of dNTP concentration, Taq polymerase and primer concentrations and anneal temperature to improve the identification accuracy and efficacy. The parameters for duplex PCR and triplex PCR are listed in Tables 2 and 3, respectively. After amplification, two methods were used for electrophoresis. By first method, 6μl of formamide was mixed in loading buffer [98% formamide, 10 mM EDTA (pH 8.0), 0.025% (W/V) xylene cyanol and 0.025% (W/V) bromophenol blue] and with the PCR products for another 4 min denaturation at 94°C for electrophoresis in 30% polyacrylamide gels. After electrophoresis, the gel was silver-stained and photographed. By second method, formamide was mixed in loading buffer mixture with 2.5μl of the PCR products for each sample for electrophoresis in a 2% agarose gel.

Validation of Multiplex PCR System: F_2 plants of Shen502×K162 were used to validate the accuracy and efficacy of duplex PCR and triplex PCR system, respectively.

RESULTS

GM maize DNA was extracted and purified by using DNA extraction kit. There was 1.8-2.0. ratio of OD260/280. In order to detect GM maize by PCR amplifications, the presence of IVR amplicons from maize DNA was necessary. The results showed that IVR amplicons with 226-bp were present in all the samples (Fig. 1).

PCR amplification with the specific primers related to Bt11 (IVS2/PAT) maize (Shen502) amplified the IVR, CaMV 35S, NOS terminator, Bt11 (IVS2/PAT) amplicons of sizes 226-bp, 165-bp and 180-bp and 189-bp. GM maize Shen502 amplified CaMV 35S, NOS terminator, IVR and Bt11 (IVS2/PAT) amplicons and the control check K162 only amplified IVR amplicons(data not shown). Theses results showed that Shen502 contained transgenic Bt11 (IVS2/PAT). Part of the PCR products are shown in Fig.2 A and B.

Results for optimized duplex PCR showed that gene combinations CaMV 35S and IVR, CaMV 35S and NOS terminator, NOS terminator and IVR shared the same duplex PCR conditions (Fig. 3A). The amplification results for optimized triplex PCR showed that 30% polyacrylamide gels could detect triplex PCR products with high resolution (Fig. 3B).

The performance of each primer set on GM maize samples was tested before multiplex PCR was conducted. Primer sets targeting CaMV 35S, NOS terminator, ZSSIIb gene, Bt11(IVS2/PAT) gene were independently tested

Fig. 1: Electrophoretic profile of uniplex PCR products of IVR gene: Line 1, DNA ladder; Lines 2-10, IVR amplicons

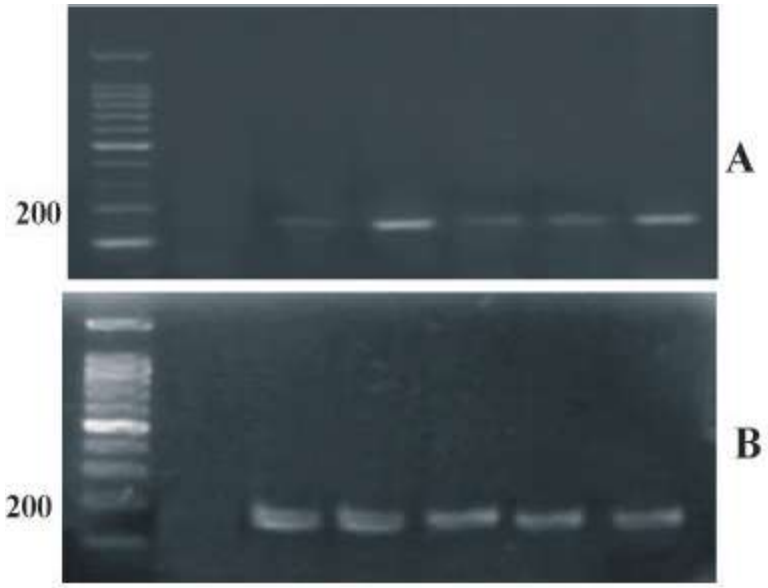

Fig. 2: Electrophoretic profile of uniplex PCR products of genetically modified elements: A: Line 1, 100bp ladder; Line 2, K162; Line 3-7, CaMV 35S amplicons. B: Line 1, 100bp Ladder; Line 2, K162; Line 3-7, Bt11(IVS2/PAT) amplicons.

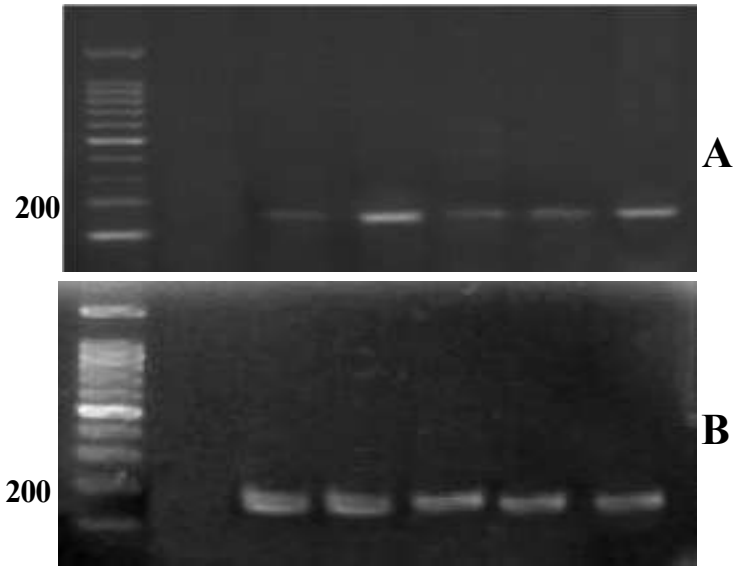

Fig. 3: Electrophoretic profile of multiplex PCR products of genetically modified elements: A: Line 1, 100bp ladder; Line 2, CaMV 35S amplicon; Line 3, NOS amplicon; Line 4, IVR amplicon; Line 5, CaMV 35S amplicon + NOS amplicon; Line 6, IVR amplicon + NOS amplicon; Line 7, CaMV 35S amplicon + IVR amplicon. B: Line 1, ZSSIIb amplicon; Line 2, CaMV 35S amplicon; Line 3, IVS2/PAT amplicon; Line 4-6, ZSSIIb amplicon + CaMV 35S amplicon + IVS2/PAT amplicon

among F_2 plants of cross Shen502×K162. Then the plants carrying CaMV 35S and NOS terminatorand and the plants carrying CaMV 35S, ZSSIIb gene and Bt11 (IVS2/PAT) gene were selected to validate the accuracy and efficacy of duplex and triplex PCR system, respectively. The results showed that the multiplex PCR system amplified the expected DNA fragments (Fig. 4A, B), suggesting that the optimized multiplex PCR systems in the present study are efficient and can be used to detect transgenic elements in maize.

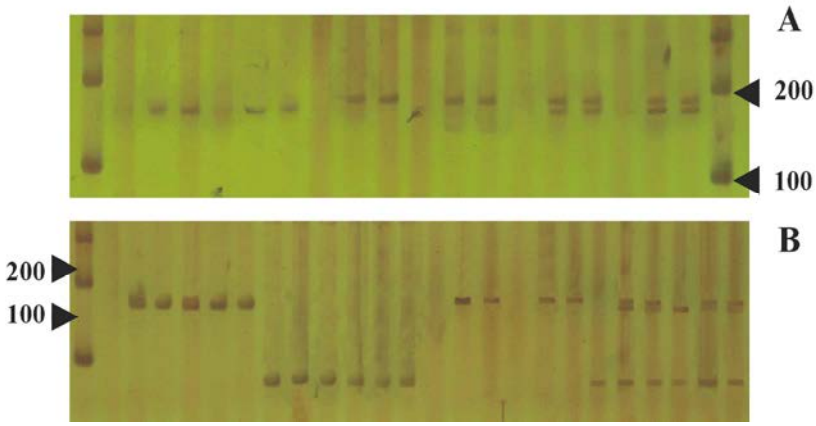

Fig. 4: Electrophoretic profile of multiplex PCR products of genetically modified elements.
A: Line 1 and Line 20, 100bp ladder; Line 2, Line 8 and Line 14, K162; Lines 3-7, CaMV 35S amplicon; Line 9-13, NOSamplicon; Line 15-19, CaMV 35S amplicon+NOS amplicon.
B: Line 1, 100bp ladder; Line 2, Line 8 and Line 14, K162; Line 3-7, CaMV 35S amplicon; Lines 9-13, ZSSIIb amplicon; Line 15-19, IVS2/PAT amplicon£»Line 21-25, ZSSIIb+CaMV 35S+IVS2/PAT amplicons.

DISCUSSION

The large-scale production of GM crops has created public concern about potential ecological contamination and the awareness of consumers' rights. Many countries in the world have set up different legislations for the authorization and labeling of GM organisms in food products. PCR method is being widely used for detection and quantification of GM organisms [4]. However, uniplex PCR only detects one targeting gene, which makes it less suitable for screening purpose. For this reason, multiplex PCR detection systems with combinations of several gene-specific primers have been necessitated for high-accuracy, low-cost, high-throughput detection [17].

DNA purity is a requisite in multiplex PCR. In the present study, host specific internal target (the gene of ZSSIIb or IVR) has been tested in all assays as control to evaluate DNA quality and PCR efficacy, which reduced the risk of false negatives, thereby increased reliability [18]. The difficulty of establishing multiplex PCR system lies in the components of the PCR reaction mixture, especially primer design and reaction efficiency inconsistencies among different primer sets [5, 19].

In the present study, we first began with one primer pair to establish uniplex PCR system. The establishment of duplex PCR system was baesd on the optimized uniplex PCR system and triplex PCR system according to duplex PCR system. The effect of amplicon size on multiplex PCR detection was also investigated. Although 30% polyacrylamide gels were employed, similar amplicon lengths among primer set combinations, for example, the gene combination CaMV 35S with 195-bp amplicon and Bt11(IVS2/PAT) with 189-bp amplicon, were not well detected while using duplex PCR system. Moreover, anneal temperature for each primer in the combinations must be closed while using multiplex PCR. The multiplex PCR system established in the present study would become a high throughput, low-cost and effective detection platform and be helpful for large-scale GM maize detection.

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