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A Multiplex PCR System for the Screening of Genetically Modified (GM) Maize and the Detection of 29 GM Maize Events Based on Capillary Electrophoresis

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Abstract: The detection of genetically modified (GM) maize events is an inevitable necessity under the strict regulatory systems of many countries. To screen for GM maize events, we developed a multiplex PCR system to specifically detect 29 GM maize events as well as the cauliflower mosaic virus 35S promoter, the *Agrobacterium tumefaciens nos* terminator, the *Streptomyces viridochromogenes pat* gene, and the endogenous *zSSIIb* maize reference gene. These targets were divided into five panels for screening and event-specific detection by multiplex (10-plex, 7-plex, 7-plex, 4-plex, and 5-plex) PCR. All amplification products were separated and visualized by fluorescence capillary electrophoresis (CE). By taking advantage of the high resolution, multiple fluorescence detection, and high sensitivity of CE, our system was able to identify all targets simultaneously with a limit of detection of 0.1%. The accurate identification of specific amplification peaks from different GM maize materials by CE confirmed the specificity of the system. To verify the practical applicability of this system, we analyzed 20 blind samples. We successfully identified five MON810, four TC1507, and three MIR162 samples. The detection of concomitant elements also verified the accuracy of this approach. Our system can, therefore, be used for the screening and detection of GM maize events. The system, which is easy to use, facilitates high-throughput detection with the help of a high-throughput platform and automated identification software. Multiplex PCR coupled with CE is, thus, very suitable for the detection of genetically modified organisms (GMOs) with a large number of detection targets. Additional multiplexed electrophoretic targets can be easily incorporated as well, thereby increasing the usefulness of this system as the number of GMO events continues to increase.

Keywords: genetically modified (GM) maize; multiplex polymerase chain reaction (PCR); capillary electrophoresis (CE)

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1. Introduction

Genetic engineering boosts crop productivity and ensures food security by improving traits, such as insect resistance and herbicide tolerance in crops. At present, 538 genetically modified (GM) events of 32 crops have been approved in 72 countries and regions. A total of 190 million hectares of GM crops were planted in 29 countries in 2019, which corresponds to a more than a 100-fold increase in the 24 years since 1996. GM maize is the main GM

crop, with 244 GM events approved in 35 countries and regions, accounting for 45% of the total number of GM events. A total of 60.9 million hectares of GM maize were planted in 2019, accounting for 32% of the area planted in GM crops [1]. The production of GM crops such as GM maize is increasing, and commercialization activities are intensifying. Many countries have developed and implemented regulatory frameworks and related regulations, such as labeling systems, for the cultivation and commercial production of GM crops [2]. In many countries and regions, GM labeling is mandatory [3–5]. The GM content threshold is set at 0.9% in the European Union and Turkey and at 5% in Japan; in China, labeling is required if the product contains any genetically modified organisms (GMOs) from the approved list [6–10]. In the current state of strict regulation, high-throughput methods for screening and detection of GMOs have become an inevitable necessity for relevant industries and regulatory authorities.

The polymerase chain reaction (PCR) method, the most commonly used molecular detection technique, is also the standard approach for detection of GMOs [11–15]. The two most widespread PCR methods are PCR combined with agarose gel electrophoresis (AGE) and real-time PCR (qPCR). Along with the number of GM events, the number of targets to be detected is increasing. To improve detection throughput and efficiency, researchers have turned to multiplex systems, including multiplex PCR and multiplex assays. Multiplex PCR allows the simultaneous amplification of several targets in a single reaction [15–17]. Multiplex electrophoresis detects the amplification products of different primers within a single lane. Multiple fluorescence detection channels allow simultaneous detection of multiple fluorescence signals. On this basis, many laboratories have proposed different multiplex protocols for GM maize detection, such as selection of several screening elements to form triplex or tetraplex PCR assays [17,18], construction of 10-target grouped multiplex PCR with grouped multiplex electrophoresis [19], formation of event-specific multiplex PCR for four GM maize events [20], development of two- or triplex qPCR [16,21], formation of a six-plex qPCR analysis by reuse of detection channels [22], and construction of five sets of multiplex qPCRs for multi-well detection of 20 events [23]. Unfortunately, the resolution of AGE and the number of qPCR detection channels are limited [24,25], resulting in a restricted number of multiplex targets. Neither of these two common methods can, therefore, be used to construct multiplex systems for a large number of targets. An alternative method, capillary electrophoresis (CE), is well suited to meet the demand for high-throughput multi-target detection. CE uses primers with a fluorescent tag at the 5′ end to detect amplicons of a specific length with a fluorescent marker. CE has high resolution and can easily distinguish amplification peaks differing by only 1 bp [26]. In addition, this method is able to detect a wide range of fluorescence signals. Aside from its high resolution, CE is simple to operate, can detect numerous target sequences simultaneously, and is easily automated [26]. Consequently, this technique is an excellent choice for high-throughput GMO detection needs.

Taking advantage of multiplex PCR combined with CE, we developed a GM maize detection system that synchronizes screening with an event-specific method. We constructed an event-specific multiplex system for 29 GM maize events and, at the same time, added element and gene targets to cover more than 98% of GM maize events. Among the 29 GM maize events collected in our laboratory, 21 have received a Chinese Agricultural GMOs Safety Certificate, and 11 were developed in China. Because of their high frequencies, the cauliflower mosaic virus 35S promoter (*P-CaMV 35S*), the *Agrobacterium tumefaciens nos* terminator (*T-nos*), and the *Streptomyces viridochromogenes pat* gene (*pat*) were also selected for screening. The chosen targets were divided in our multiplex complex system into 5 multiplex PCR panels, and 33 targets containing the endogenous *zSSIIb* maize reference gene could be identified simultaneously in the same electrophoresis lane. The practical applicability of our method was confirmed by testing 20 blind samples. Our system can help screen GM maize components and is able to detect 29 GM maize events in one step. By using multiplex PCR combined with CE and data processing software, we have been able to develop a multi-target high-throughput GMO detection system.

2. Materials and Methods

2.1. Samples

To develop our detection system, we used the following 29 GM maize seed powders: 3272, 4114, 5307, 59122, Bt11, Bt176, Bt506, BVLA430101, C0010.1.1, C0010.1.3, C0010.3.1, C0030.2.5, C0030.3.5, CC-2, DAS-40278-9, GA21, G1105E-823C, GH5112E-117C, MIR162, MIR604, MON810, MON863, MON87427, MON88017, MON89034, T25, TC1507, VCO-01981-5, and SK12-5. The GMO mass content of MON810 was 2% (*w*/*w*), and that of the others was 1% (*w*/*w*). GM maize powders of Bt11, Bt176, MON810, MON863, and TC1507 were purchased from the Institute for Reference Materials and Measurements, and the remaining ones were purchased from the Development Center of Science and Technology, Ministry of Agriculture and Rural Affairs, China. The non-GM maize 'Zhengdan 958' was collected by our laboratory.

2.2. DNA Extraction

DNA was extracted using a commercial kit (ComWin Biotech, Beijing, China) according to the accompanying instructions manual. The concentration and quality of extracted DNA were measured on a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Shanghai, China), and DNA integrity was examined by agarose gel electrophoresis (8 g/L). DNAs were uniformly diluted to a concentration of 20 ng/μL before use. The DNA concentration of MON810 was diluted to 1% (*w*/*w*) with a solution of 'Zhengdan 958' maize DNA at the same concentration. The presence of amplification-inhibitory substances in the extracted DNA was checked by amplification of the maize endogenous reference gene (*zSSIIb*).

2.3. Synthesis of Fluorescently Labeled Primers

Primer sequences were based on the standards issued by the Ministry of Agriculture and Rural Affairs of China and are detailed in Supplementary Table S1. Primers were synthesized and then labeled at the 5′ end with blue (6-carboxy-fluorescein, FAM) or red (Carboxy-X-Rhodamine, ROX) fluorescent dyes (Tianyi Huiyuan Biotech, Beijing, China). Upstream and downstream primers were mixed in equal amounts and diluted to a concentration of 20 μM for PCR.

2.4. PCR System and Procedures

For the simplex PCR system, amplifications were performed in 20-μL reaction volumes containing 2 μL of template DNA, 0.25 μL of primers, and 10 μL of 2 × *Taq* Plus Master Mix (Vazyme Biotech, Nanjing, China).

For the multiplex PCR system, the 33 targets were divided into five multiplex PCR panels as follows: (i) Bt176, Bt506, C0010.1.1, C0010.3.1, DAS-40278-9, G1105E-823C, GH5112E-117C, MON810, MON87427, and VCO-01981-5; (ii) 5307, C0010.1.3, CC-2, GA21, MIR162, MON863, and MON89034; (iii) 3272, 4114, Bt11, C0030.3.5, SK12-5, T25, and TC1507; (iv) BVLA430101, C0030.2.5, MIR604, and MON88017; and (v) 59122, *zSSIIb*, *P-CaMV 35S*, *T-nos*, and *pat*. Except for the amount of added primers, which ranged between 0.06 and 0.5 μL per primer pair, the quantities of reaction components were the same as in the simplex PCR system. The following primer concentrations were used: 0.06 μM for the endogenous reference gene; 0.1 μM for C0010.1.1, GH5112E-117C, and MON863; 0.15 and 0.2 μM for BVLA430101 and C0030.2.5, respectively; 0.25 μM for 5307, Bt11, Bt176, Bt506, C0010.1.3, C0030.3.5, CC-2, GA21, MIR162, MIR604, MON810, T25, *P-CaMV 35S*, *T-nos*, and *pat*; and 0.5 μM for all other targets.

The PCR cycling protocol was as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 60 °C for 35 s, and 72 °C for 45 s, with a final extension of 72 °C for 10 min and then held at 4 °C.

2.5. Capillary Electrophoresis

Prior to CE, 2 µL of PCR product (10-fold diluted in the case of simplex PCR amplification), 10 µL of deionized formamide, and 0.1 µL of GeneScanTM-500 LIZ standard (Thermo Fisher Scientific) were mixed, incubated at 95 °C for 5 min, and stored at 4 °C.

Electrophoresis was performed using an ABI 3730XL DNA analyzer (Thermo Fisher Scientific). After pre-electrophoresis at 15 kV for 2 min and then 2-kV electrical injection for 10 s, the separation was carried out at 15 kV for 20 min.

The generated data were analyzed using SSR Analyser software developed by the Maize Research Center of Beijing Academy of Agriculture and Forestry Sciences [27].

2.6. Specificity Analysis

Simplex PCR amplification of each single-event sample was performed using 1 of 29 pairs of GM maize event-specific primers. Amplification products were then mixed and subjected to 29-plex electrophoresis to determine the specificity of each pair of event-specific primers under simplex PCR.

The 33 targets were divided into five multiplex PCR panels (see Section 2.4 for details). Single-event samples were grouped accordingly, and multiplex PCR was performed on each sample. The amplification products were then mixed and subjected to 10-plex, 7-plex, 7-plex, 4-plex, 5-plex, and 33-plex electrophoretic analysis.

2.7. Limit-of-Detection (LOD) and Blind-Sample Analyses

To determine LODs, positive DNAs of Bt11, Bt176, MON810, MON863, and TC1507 were diluted with the same concentration of 'Zhengdan 958' maize DNA to create different DNA contents (*w*/*w*). The dilution gradient was 1%, 0.7%, 0.4%, 0.1%, 0.07%, 0.04%, and 0.01%, with three replicates performed per concentration.

The multiplex system was tested for practical applicability using 20 blind samples.

3. Results

3.1. Collection and Molecular Characterization of Gm Maize Events

We collected information on 259 GM maize events worldwide from the GM Approval Database website and other websites [28–30]. We classified the 259 events into 67 single events and 192 stacked events (i.e., those in which two or more events were compounded). Identification of the 67 single events would guarantee the ability to identify the stacked ones. On the basis of single-event analysis, we thus developed a GM multiplex system for 33 targets consisting of 29 event-specific assays, 3 element screening assays, and 1 maize endogenous reference gene assay. Most of the selected events were GM events with import demand in China or developed in that country. For the screening of elements, we selected three screening targets (*P-CaMV 35S*, *T-nos*, and *pat*) because of their high frequency in the remaining single events. Primer sequences were developed according to currently published detection standards based on AGE detection. The selected targets covered 64 single events, i.e., a total of 256 single and stacked GM maize events. Three events were not covered by this system: LY038, HCEM485, and C0030.2.4. LY038 and HCEM485 are, respectively, high-lysine and glyphosate-herbicide-resistant maize events; the promoters and terminators of their inserts are all maize in origin, and no *pat* gene is present. Information on the C0030.2.4 event is not publicly available. The system we developed covers more than 98% of the 259 GM maize events, and almost all events can be identified in a single step.

3.2. Specificity Analysis of Fluorescently Labeled Primers

Prior to construction of the multiplex system, the specificity of the fluorescently labeled primers was confirmed by simplex PCR (Figure 1). The DNA quality of different samples was consistent and superior, with good integrity and no amplification inhibition. Each primer pair amplified a specific peak, with a normal peak pattern, a fixed amplification fragment size, and no other nonspecific peaks. These results indicate that the primers

designed for AGE could be directly converted to fluorescent CE primers simply by adding fluorescent dyes, a process that had a success rate of 100% in this study. We also found that the size of the amplified fragments of some primers differed slightly—by no more than 3 bp—from that of the standard reference fragment (Supplementary Table S2). This deviation may be due to differences between fluorescence CE and AGE. The addition of fluorescent dyes affects the migration rate of DNA during electrophoresis. This effect varies by fragment; however, the effect is directional and non-random, and the ultimate amplified fragment size of each target is constant and does not change with repeated experiments. We also confirmed this principle in replicate experiments. We set up a series of panels for the 33 targets in SSR Analyser [27] to enable the software to automatically acquire reads of the targets corresponding to the amplification peaks.

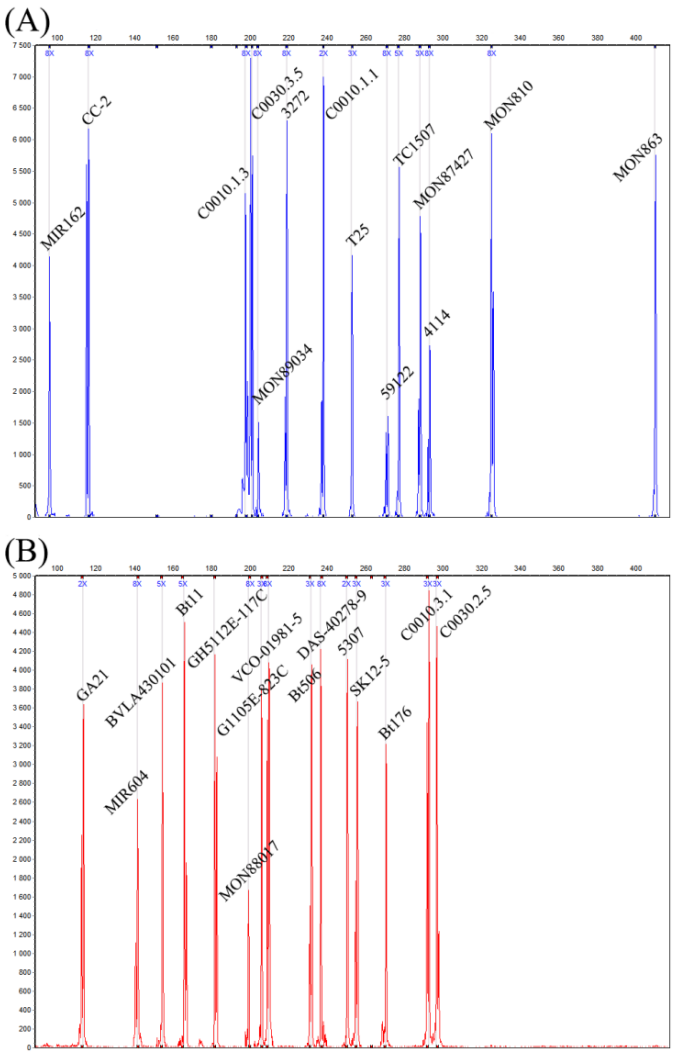

Figure 1. Capillary electrophoretograms from simplex PCR and multiplex electrophoresis assays. The x- and y-axes represent amplified fragment length and fluorescence intensity, respectively. (**A**,**B**) Results obtained using FAM-labeled (**A**) and ROX-labeled (**B**) primers.

3.3. Specificity Analysis of the Multiplex System

Using fluorescence CE, we successfully constructed a GM multiplex assay system for 33 targets, which were divided into five multiplex PCR panels (see Section 2.4 for details). To ensure the relative consistency of amplification peak heights of the 1% GM samples (i.e., the amplification efficiency of the targets), we optimized the primer concentrations in each system by increasing the concentration of primers with low peak heights and decreasing the concentration of primers with high amplification efficiencies in the multiplex system.

We also determined that amplification of the endogenous reference gene using a primer concentration of 0.06 μM resulted in a peak height similar to those of the other samples. Multiplex PCR was performed for each single event, and different amplification products within the same multiplex panel assay were mixed and electrophoretically separated. As shown in Figure 2A–E, each multiplex assay resulted in a normal pattern of panel-specific peaks of the expected fragment sizes. These specific peaks and fragment sizes were consistent with the results of simplex PCR, thus confirming our previous observations that the sizes of the amplified fragments were constant and distinguishable. When the products of the five multiplex PCR panel assays were mixed and subjected to electrophoresis, the targets were still correctly identified (Figure 2F). The 33 GM targets could be identified in a single electrophoresis well. By exploiting the high resolution of CE in combination with the automatic identification function of SSR Analyser, we had achieved our goal: automating the identification of 33 different targets within the same electrophoresis well.

3.4. LOD Analysis

We determined the LODs of Bt11, Bt176, MON810, MON863, and TC1507 (Table 1). In all cases, the minimum event content to obtain a 100% detection rate within three repetitions was 0.1%. In addition, all tested samples containing GM events were identified at least once within each set of three replicates at a given concentration, that is, samples with a minimum GM content of 0.01% could also be detected. The lowest GM contents detected in all three replicates varied between 0.01% and 0.1% for each event. According to European Commission–Joint Research Centre instructions, all GM maize are heterozygous. The GM parents of Bt11, Bt176, and MON810 are male, whereas those of MON863 and TC1507 are female. Given that the copy number ratio of MON810 with a 2% mass ratio is 0.77% and assuming that the DNA content of one haploid maize genome is 2.725 pg [19,31], we calculated the corresponding copy numbers of Bt11, Bt176, MON810, MON863, and TC1507 as 0.57 (0.01%), 5.65 (0.1%), 2.26 (0.04%), 6.32 (0.07%), and 9.03 (0.1%), respectively. The calculated copy number of Bt11 was therefore less than 1. To verify the reliability of the assay, we analyzed samples with Bt11 contents of 0.1 and 0.01%. We detected Bt11 events in 46 of 48 0.1% samples and 23 of 48 0.01% samples, which corresponds to detection rates of 95.8% and 47.9%, respectively. Consequently, samples with a 0.01% GM content are identifiable with a certain probability, whereas samples with a 0.1% GM content are almost certainly detectable. The LOD of a DNA sequence is the lowest DNA content that can be detected with reasonable statistical certainty, i.e., with a probability of detection of at least 95% [16]. The LOD of Bt11 with this method is, therefore, 0.1%, i.e., the copy number is 5.65. We can assume that the LODs of other events are approximately 5.65 copies as well. Taking into account that the detection of Bt11 is the most sensitive, we can also assume that the LOD is approximately 5 to 10 copies. In conclusion, events with a 0.1% GM content were detected almost every time, which proves that our method is sufficiently sensitive to meet the GM labeling requirements of most countries.

Table 1. Results of limit-of-detection analysis.

GM Content (% *w*/*w*)	PCR Results														
	Bt11			Bt176			MON810			MON863			TC1507		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.07	+	+	+	+	+	−	+	+	+	+	+	+	+	−	−
0.04	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−
0.01	+	+	+	+	+	−	+	+	−	+	+	−	+	−	−

Three replications (R1, R2, and R3) were performed at each concentration; + and − mean detected or undetected, respectively.

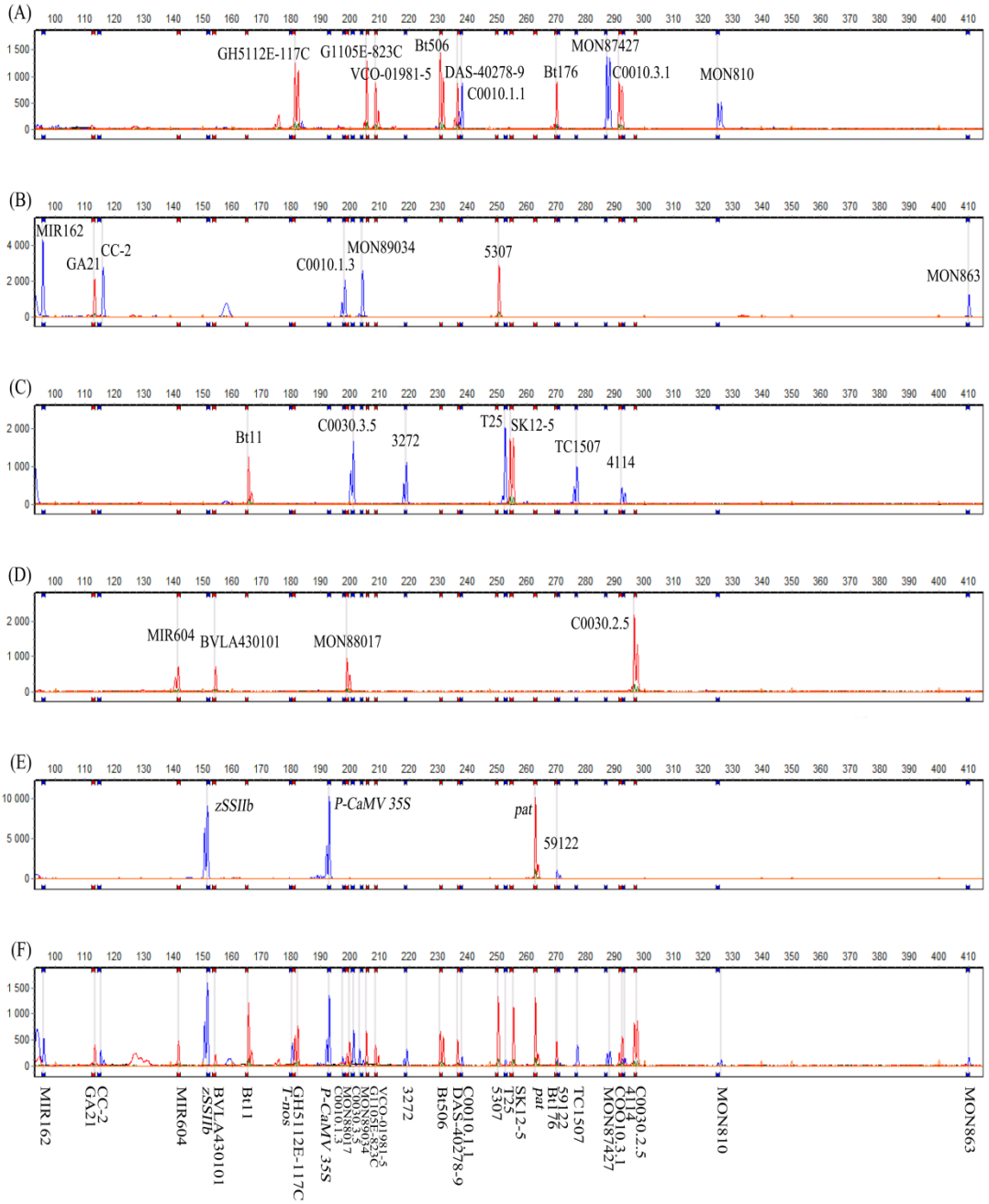

Figure 2. Results of combined capillary electrophoretic analyses of products of five multiplex PCR panel assays. The x- and y-axes represent amplified fragment length and fluorescence intensity, respectively. (**A**–**E**) Capillary electrophoretograms from multiplex PCR assays of panels i to v, respectively. (**F**) Capillary electrophoretogram of combined products from all five assays (mixed 33-plex electrophoresis).

We also compared the sensitivity of simplex PCR with that of multiplex PCR. In the case of Bt11, 47 of the 48 0.1% samples and 30 of the 48 0.01% samples were identified by simplex PCR, which corresponds to detection rates of 97.9%, and 62.5%, respectively. The sensitivity of the multiplex PCR assay system is thus slightly lower than that of simplex PCR, but LODs do no differ significantly between the two methods. Both types of assays can consistently detect target sequences in samples with a GM content of 0.1%.

3.5. Practical Applicability

To verify the practical applicability of our system, we analyzed 20 blind samples. In five samples, the endogenous reference gene *zSSIIb* was not detected, thus indicating that no maize component was present. In three samples, no targets other than *zSSIIb* were detected; these samples, therefore, had no GM content. MON810, TC1507, and MIR162 events were, respectively, detected in five, four, and three samples. The MON810-containing samples were also found to harbor *P-CaMV 35S*, while samples with the TC1507 event had detectable *P-CaMV 35S* and *pat*, and those carrying the MIR162 event were determined to include *T-nos* as well. These results, which were expected, confirmed that the samples contained MON810, TC1507, or MIR162 events. Our method can, therefore, quickly identify common GM maize events, and the authenticity of the results can be verified by simultaneous element screening. Other GM maize events not included in the 29-member set can also be screened simultaneously to obtain preliminary information, narrow down the candidate events, and simplify the subsequent identification process. Our system is, therefore, suitable for the screening and detection of samples containing GM maize events.

4. Discussion

GM crops play an increasingly important role in agricultural production. To ensure the safety of food production, different countries have established various regulatory requirements for GM crop seeds and products. The increasing number of GM events and GM crop commercialization activities have resulted in new demands on the timeliness and accuracy of GM detection methods.

Two methods are commonly used to detect GMOs: end-point PCR and qPCR. The first of these methods, end-point PCR, typically relies on AGE, which is plagued by extremely low electrophoresis efficiency, poor experimental stability, and the required use of hazardous reagents. Although some researchers have established multiple electrophoresis detection strategies based on current experimental systems [17–20], AGE can no longer be adapted for use with present-day GM detection requirements. The second GMO detection method, qPCR, suffers from equipment limitations (i.e., usually a maximum of 4- or 5-plex detection systems can be established) and relatively high detection costs. New high-throughput methods are urgently needed. Some new solutions, such as chips and sequencing [32–35], have improved the detection throughput, but the detection period is longer than that of PCR methods; in addition, bioinformatic analysis requirements are increased, which makes these approaches unsuitable for use in rapid detection systems.

Our proposed method combines the advantages of the above-mentioned methods while avoiding their shortcomings. Our method is simple, fast, relatively inexpensive, and facilitates high-throughput, automated detection. In this study, we developed a multiplex detection system based on CE for 33 GM targets. This system can identify 29 GM events in one step within the short time frame of 4 h. With the screening elements and genes targeted with this system, more than 98% of GM maize events are covered. The system is as simple to operate as ordinary PCR. The incorporation of a high-throughput platform and supporting software allows standardized, automated high-throughput detection, supplemented only with simple manual operations and checks, to be realized.

Our new system can be used to accurately, sensitively, and rapidly detect any of 29 GM events, individually or in combination, in a sample. The LOD of our system is approximately 5 to 10 copies; in other words, GMOs with a mass fraction ratio as low as 0.1% can be detected. The sensitivity of our system is thus comparable to that of the detection system developed by Niu et al., [36] and higher than those of Fu et al. and Singh et al., [9,18]. The GM mixing threshold is set at 0.9% in the EU, Russia, and Turkey; 3% in Korea; and 5% in Japan, Indonesia, and Thailand [8,18]. The sensitivity of our system is below all of these thresholds. Our new system is therefore sensitive enough to meet national regulations and can be used internationally. Now that large quantities of GM crops are being commercially cultivated, our system is beneficial for reducing the cost of labeling

GM seeds or agricultural products and is suitable for use by GM regulatory industries, such as those involved in GM import–export trade and agricultural product supervision.

Although only two fluorescent dyes, FAM and ROX, are currently used in our system, the ABI 3730XL DNA analyzer has five fluorescence-color channels. As two fluorescence-color channels are currently unused (the remaining channel is the internal standard channel), the addition of multiplexed electrophoretic targets to the present system would be extraordinarily easy. Given the increasing number of GM maize events, our system will be even more useful in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12030413/s1, Table S1: Primer information; Table S2: Information on amplified fragments based on capillary electrophoresis.

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References

1. International Service for the Acquisition of Agri-Biotech Applications. ISAAA Brief 55-2019: Executive Summary. 2019. Available online: https://www.isaaa.org/resources/publications/briefs/55/executivesummary/default.asp (accessed on 10 January 2022).
2. Li, Y.H.; Peng, Y.F.; Hallerman, E.M.; Wu, K.M. Biosafety management and commercial use of genetically modified crops in China. *Plant Cell Rep.* **2014**, *33*, 565–573. [CrossRef]
3. Kamle, M.; Kumar, P.; Patra, J.K.; Bajpai, V.K. Current perspectives on genetically modified crops and detection methods. *3 Biotech* **2017**, *7*, 219. [CrossRef]
4. Lee, D.G.; Park, J.E.; Kim, M.J.; Kim, H.J.; Kim, H.Y. Detection of GM Canola MS11, DP-073496-4, and MON88302 events using multiplex PCR coupled with capillary electrophoresis. *Food Sci. Biotechnol.* **2021**, *30*, 565–570. [CrossRef]
5. Zeng, H.J.; Wang, J.B.; Jia, J.W.; Wu, G.G.; Yang, Q.W.; Liu, X.F.; Tang, X.M. Development of a lateral flow test strip for simultaneous detection of BT-Cry1Ab, BT-Cry1Ac and CP4 EPSPS proteins in genetically modified crops. *Food Chem.* **2021**, *335*, 127627. [CrossRef]
6. Fortunati, S.; Giannetto, M.; Rozzi, A.; Corradini, R.; Careri, M. PNA-functionalized magnetic microbeads as substrates for enzyme-labelled voltammetric genoassay for DNA sensing applied to identification of GMO in food. *Anal. Chim. Acta* **2021**, *1153*, 338297. [CrossRef]
7. Guenthner, J.F.; Johnson, A.J.; Mclntosh, C.S. Seed variety mix: An indicator for GM potato identity preservation. *Am. J. Potato Res.* **2012**, *89*, 172–174. [CrossRef]
8. Turkec, A.; Lucas, S.J.; Karlik, E. Monitoring the prevalence of genetically modified (GM) soybean in Turkish food and feed products. *Food Control* **2016**, *59*, 766–772. [CrossRef]
9. Fu, W.; Wei, S.; Wang, C.G.; Du, Z.X.; Zhu, P.Y.; Wu, X.Y.; Wu, G.; Zhu, S.F. A temperature-tolerant multiplex elements and genes screening system for genetically modified organisms based on dual priming oligonucleotide primers and capillary electrophoresis. *Food Chem.* **2017**, *229*, 396–402. [CrossRef] [PubMed]
10. Liu, H.; Wang, J.B.; Li, P.; Bai, L.; Jia, J.W.; Pan, A.H.; Long, X.Q.; Cui, W.D.; Tang, X.M. Rapid detection of P-35S and T-nos in genetically modified organisms by recombinase polymerase amplification combined with a lateral flow strip. *Food Control* **2020**, *107*, 106775. [CrossRef]
11. Block, A.; Debode, F.; Grohmann, L.; Hulin, J.; Taverniers, I.; Kluga, L.; Barbau-Piednoir, E.; Broeders, S.; Huber, I.; van den Bulcke, M.; et al. The GMOseek matrix: A decision support tool for optimizing the detection of genetically modified plants. *BMC Bioinform.* **2013**, *14*, 256. [CrossRef]

12. Chen, L.H.; Zhou, J.F.; Li, T.T.; Fang, Z.W.; Li, L.; Huang, G.; Gao, L.F.; Zhu, X.B.; Zhou, X.S.; Xiao, H.F.; et al. GmoDetector: An accurate and efficient GMO identification approach and its applications. *Food Res. Int.* **2021**, *149*, 110662. [CrossRef] [PubMed]
13. Scholtens, I.; Laurensse, E.; Molenaar, B.; Zaaijer, S.; Gaballo, H.; Boleij, P.; Bak, A.; Kok, E. Practical experiences with an extended screening strategy for Genetically Modified Organisms (GMOs) in real-life samples. *J. Agric. Food Chem.* **2013**, *61*, 9097–9109. [CrossRef] [PubMed]
14. Takabatake, R.; Kagiya, Y.; Minegishi, Y.; Yeasmin, S.; Futo, S.; Noguchi, A.; Kondo, K.; Mano, J.; Kitta, K. Development and evaluation of rapid screening detection methods for genetically modified crops using loop-mediated isothermal amplification. *Food Chem.* **2018**, *252*, 390–396. [CrossRef]
15. Wei, S.; Wang, C.G.; Zhu, P.Y.; Zhou, G.B.; Fu, W.; Wu, X.Y. A high-throughput multiplex tandem PCR assay for the screening of genetically modified maize. *LWT—Food Sci. Technol.* **2018**, *87*, 169–176. [CrossRef]
16. Chaouachi, M.; Zellama, M.S.; Nabi, N.; Ben Hafsa, A.; Said, K. Molecular identification of four genetically modified maize (Bt11, Bt176, Mon810 and T25) by duplex quantitative real-time PCR. *Food Anal. Methods* **2014**, *7*, 224–233. [CrossRef]
17. Datukishvili, N.; Kutateladze, T.; Gabriadze, I.; Bitskinashvili, K.; Vishnepolsky, B. New multiplex PCR methods for rapid screening of genetically modified organisms in foods. *Front. Microbiol.* **2015**, *6*, 757. [CrossRef]
18. Singh, M.; Bhoge, R.K.; Randhawa, G. Crop-specific GMO matrix-multiplex PCR: A cost-efficient screening strategy for genetically modified maize and cotton events approved globally. *Food Control* **2016**, *70*, 271–280. [CrossRef]
19. Park, S.B.; Kim, H.Y.; Kim, J.H. Detection of a screening method for the monitoring of 38 genetically modified maize events in food and feed in South Korea. *Food Control* **2017**, *73*, 1459–1465. [CrossRef]
20. Park, J.H.; Seol, M.A.; Eum, S.J.; Kim, I.R.; Lim, H.S.; Lee, J.R.; Choi, W. Development of a multiplex PCR method for identification of four genetically modified maize lines and its application in living modified organism identification. *J. Plant Biotechnol.* **2020**, *47*, 309–315. [CrossRef]
21. Wang, F.J.; Feng, J.L.; Ye, S.D.; Huang, H.N.; Zhang, X.L. Development of a multiplex fluorescence quantitative PCR for detection of genetically modified organisms. *Biologia* **2018**, *73*, 21–29. [CrossRef]
22. Bahrdt, C.; Krech, A.B.; Wurz, A.; Wulff, D. Validation of a newly developed hexaplex real-time PCR assay for screening for presence of GMOs in food, feed and seed. *Anal. Bioanal. Chem.* **2010**, *396*, 2103–2112. [CrossRef]
23. Köppel, R.; Ganeshan, A.; van Velsen, F.; Bucher, T. Five pentaplex real-time PCR systems for the efficient determination of 20 genetically modified maize traits in food. *Eur. Food Res. Technol.* **2017**, *243*, 215–225. [CrossRef]
24. Basak, S.; Ehtesham, N.Z.; Sesikeran, B.; Ghosh, S. Detection and identification of transgenic elements by fluorescent-PCR-based capillary gel electrophoresis in genetically modified cotton and soybean. *J. AOAC Int.* **2014**, *97*, 159–165. [CrossRef]
25. Li, X.F.; Wu, Y.H.; Li, J.; Li, Y.J.; Long, L.K.; Li, F.W. Development and validation of a 48-target analytical method for high-throughput monitoring of genetically modified organisms. *Sci. Rep.* **2015**, *5*, 7616. [CrossRef]
26. Heide, B.R.; Heir, E.; Holck, A. Detection of eight GMO maize events by qualitative, multiplex PCR and fluorescence capillary gel electrophoresis. *Eur. Food Res. Technol.* **2008**, *227*, 527–535. [CrossRef]
27. Jiang, B.; Zhao, Y.K.; Yi, H.M.; Huo, Y.X.; Wu, H.T.; Ren, J.; Ge, J.R.; Zhao, J.R.; Wang, F.G. PIDS: A user-friendly plant DNA fingerprint database management system. *Genes* **2020**, *11*, 373. [CrossRef]
28. International Service for the Acquisition of Agri-Biotech Applications. GM Approval Database. 2021. Available online: https://www.isaaa.org/gmapprovaldatabase/default.asp (accessed on 10 January 2022).
29. Dong, W.; Yang, L.T.; Shen, K.L.; Kim, B.; Kleter, G.A.; Marvin, H.J.P.; Guo, R.; Liang, W.Q.; Zhang, D.B. GMDD: A database of GMO detection methods. *BMC Bioinform.* **2008**, *9*, 260. [CrossRef]
30. Ministry of Agriculture and Rural Affairs of the People's Republic of China. 2022. Available online: http://www.moa.gov.cn/ztzl/zjyqwgz (accessed on 10 January 2022).
31. Arumuganathan, K.; Earle, E.D. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Report.* **1991**, *9*, 208–218. [CrossRef]
32. Yun, Z.Y.; Peng, L.P.; Huo, S.N.; Wu, X.; Zhou, J.; Zhang, H.G.; Li, W.J.; Qi, C.; Huang, G.P. Auto-microfluidic thin-film chip for genetically modified maize detection. *Food Control* **2017**, *80*, 360–365. [CrossRef]
33. Fu, W.; Wang, C.G.; Zhu, P.Y.; Xu, W.J.; Li, X.S.; Zhu, S.F. A universal analytical approach for screening and monitoring of authorized and unauthorized GMOs. *LWT—Food Sci. Technol.* **2020**, *125*, 109176. [CrossRef]
34. EFSA Panel Genetically Modified. Technical Note on the quality of DNA sequencing for the molecular characterisation of genetically modified plants. *EFSA J.* **2018**, *16*, 5345. [CrossRef]
35. Liang, C.J.; van Dijk, J.P.; Scholtens, I.M.; Staats, M.; Prins, T.W.; Voorhuijzen, M.M.; da Silva, A.M.; Arisi, A.C.M.; den Dunnen, J.T.; Kok, E.J. Detecting authorized and unauthorized genetically modified organisms containing *vip3A* by real-time PCR and next-generation sequencing. *Anal. Bioanal. Chem.* **2014**, *406*, 2603–2611. [CrossRef] [PubMed]
36. Niu, C.Q.; Xu, Y.C.; Zhang, C.; Zhu, P.Y.; Huang, K.L.; Luo, Y.B.; Xu, W.T. Ultrasensitive single fluorescence-labeled probe-mediated single universal primer-multiplex-droplet digital polymerase chain reaction for high-throughput genetically modified organism screening. *Anal. Chem.* **2018**, *90*, 5586–5593. [CrossRef] [PubMed]