LWT - Food Science and Technology 87 (2018) 169–176

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

A high-throughput multiplex tandem PCR assay for the screening of genetically modified maize

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ARTICLE INFO

Article history:
Received 27 April 2017
Received in revised form
18 July 2017
Accepted 19 August 2017
Available online 23 August 2017

Keywords:
Multiplex tandem PCR
Genetically modified maize
Screening

ABSTRACT

Genetically modified organisms (GMO) are increasingly gaining acceptance, which has led to an increasing demand for high-throughput methods for their detection. This study describes development of a high-throughput and low-cost multiplex tandem PCR (MT-PCR) assay for the screening of transgenic maize of 14 targets, containing 7 genetically modified (GM) common screening elements, 6 transgenic maize events and a maize reference gene, using SYBR Green I for quantification. The 14-target high-throughput multiplex tandem PCR assay successfully amplified products from single-event samples with a GM maize DNA amount less to 0.001 ng, and displayed high specificity. This assay can efficiently be used for screening GM maize in foods and feeds.

1. Introduction

A large number of GMOs are available on the market in many countries in recent decades. According to the International Service for the Acquisition of Agri-Biotech Applications (ISAAA), 29 species and 489 transgenic varieties had been developed and commercialized worldwide by 2016 (C. James, 2017). Remarkably, Maize has acquired the largest range of approval all over the world, with 229 transgenic varieties approved by 29 countries. Several countries have implemented mandatory labeling for foods derived from transgenic plants such as European Union (EU) member countries, regulating that products containing more than 0.9% GMO material must be labeled according to the current regulations. However, unauthorized GMOs (UGMs) are occasionally released into the markets. To protect the consumer rights, it is necessary for high capacity monitoring methods for detecting GM maize.

GMO detection is based on finding either the inserted foreign gene (containing insert junction sequence) or the protein that is specifically expressed in transgenic plants (Kamle & Ali, 2013). The protein-based methods are laborious and less sensitive, usually not suitable for processed products which may cause denaturation of the proteins (Pobozy, Filaber, Koc, & Garcia-Reyes, 2013). In comparison, the DNA-based PCR methods are more widely applicable. In recent years, numerous strategies for the high-throughput DNA-based GMO detection methods have been described. Among them, multiplex PCR which can simultaneously amplify multiple DNA targets in one reaction, is the basic strategy for monitoring of multiple GM content (Germini et al., 2004; D.; James, Schmidt, Wall, Green, & Masri, 2003).

Shrestha et al. has developed a multiplex PCR for simultaneously detection of eight lines of GM maize, but it is time-consuming and throughput-limiting because the PCR product need further agarose electrophoresis analysis to confirm the results (Shrestha, Hwu, Wang, Liu, & Chang, 2008). To improve the capacity of multiplex PCR, new strategies that combined with multiplex PCR were developed, such as capillary gel electrophoresis (CGE) or microarray hybridization (Basak, Ehtesham, Sesikeran, & Ghosh, 2014; Nadal, Coll, La Paz, Esteve, & Pla, 2006; Schmidt et al., 2008; J.; Xu et al., 2007). However, this improvement is also limited by the

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http://dx.doi.org/10.1016/j.lwt.2017.08.061
0023-6438/

capacity of multiplex PCR in single tube. In order to increase the multiplicities of multiplex PCR, several high-throughput improved assays were developed for the detection of GMO based on multiplex PCR, such as universal primer multiplex PCR (UP-*M*-PCR), multiplex micro droplet PCR implemented capillary gel electrophoresis (MPIC), multiplex amplification on a chip with readout on an oligo microarray (MACRO) (Guo et al., 2011; Shao et al., 2014; W.; Xu et al., 2012). These methods can detect multiple targets simultaneously, eg. about 15 targets by UP-*M*-PCR, 24 targets by MPIC and 91 targets by MACRO. However, quantitative analysis cannot be achieved by these methods.

Real-time PCR is a powerful quantitative and qualitative detection method that has been used widely in GMO detection (Hernandez et al., 2003; Jacchia et al., 2015). For the purpose of high-throughput detection of GMO, various multiplex real-time PCR methods were developed (Huber et al., 2013; Köppel, Sendic, & Waiblinger, 2014; Samson, Gullí, & Marmiroli, 2013). However, because of the intrinsic interference and competition between primers, the number of target DNA fragments that can be simultaneously amplified in multiplex PCR reactions is limit.

Recently, a multiplex tandem PCR (MT-PCR) was developed that allows simultaneous detection and analysis of numerous targets (Stanley & Szewczuk, 2005). This method combined reverse transcription PCR, multiplex PCR, nested PCR and real-time PCR as a whole which can be used for the detect and analysis of RNA or DNA targets, especially where only small amounts of starting RNA or DNA are available. Now, this method has been used to detect pathogenic bacteria, virus and fungi (Hazelton, Thomas, Unver, & Iredell, 2013; Lau, Sorrell, Chen, et al., 2008; Lau, Sorrell, Lee, Stanley, & Halliday, 2008; Lau, Stanley, & Sorrell, 2013, pp. 195–201; Mccallum, Mcgregor, & Vanniasinkam, 2013; Szewczuk et al., 2010).

This study describes the development of a low-cost, high-throughput multiplex tandem PCR for the screening of genetically modified maize. The detection targets included 7 screening elements (CaMV35S, FMV35S, NOS, NPTII, Cry1Ab, Bar and Pat), 6 transgenic maize events (NK603, BT11, MON810, MON88017, T25 and TC1507) and a maize reference gene (IVR). Seven screening elements mentioned above have been used for GMO screening as the most common elements, which covered 94% (16/17) approved transgenic maize events in China (Li et al., 2015).

2. Materials and methods

2.1. GMO materials

The GM materials included 10 transgenic maize events (NK603, BT11, MON810, MON88017, T25, TC1507, MON863, BT176, MIR162 and MON89034), 1 transgenic soybean event (GTS 40-3-2), 2 transgenic rapeseed events (MS1 and RT73), and 1 transgenic rice event (TT51-1). These GMO samples were provided by Chinese academy of Inspection and Quarantine. Non-modified maize kernals purchased from local market (Walmart supermarket, Zhichunlujia No. 48, Bejing, China) was checked for the absence of any GM events to be used as a control in this study. All these transgenic samples were identified and collected by the GMO detection laboratories of Chinese academy of Inspection and Quarantine.

2.2. DNA extraction

The seeds of the GMO samples were ground using a SPEX Gene 2010 grinder. DNA was extracted and purified using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's protocol. The concentration and purity of extracted DNA were quantified using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Inc., Montchanin, DE) and adjusted to the concentration using TE buffer (pH 8.0). All DNA samples were stored at −20 °C before use.

2.3. Primer design

In this study, we choose 6 GM maize events, 7 elements for screening and maize endogenous IVR gene as targets for MT-PCR. The gene sequences were based on available sequences deposited in GMDD (Dong et al., 2008). The nucleotide sequences of the primers are shown in Table 1. Schematic diagrams in Fig. 1 shows the location of 6 event-specific outer primers on the flanking region of 6 GM maize event. All primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China).

2.4. Preparation of gene disks with lyophilized inner primers

All 14 pairs of inner primers were added into 96-well plate in triplicate individually to keep the concentration on 0.4 μmol/L in final PCR reaction. These gene disks were then lyophilized for 15 min. Each gene disk can analyze 2 samples which can detect 14 target genes for each sample simultaneously. Gene disks were then stored at 4 °C before use.

2.5. First round (1st) high-throughput multiplex amplification

First round high-throughput multiplex amplification was performed using Multiplex PCR assay kit (TaKaRa, Japan). PCR was reacted in a final volume of 50 μL with the following reagent: 0.1 μmol/L of each outer primer set (up to 14 pairs of outer primers), 25 μL Mix 2, 0.25 μL Mix 1 (5 U/μL), 50 ng DNA template, and sufficient ddH_2O to adjust the volume to 50 μL. First round amplifications were performed on the Mastercycler ep (Eppendorf) and the condition were: 94 °C for 60 s, followed by 10–20 cycles of 94 °C for 30 s, 60 °C for 90 s and 72 °C for 60 s and a final step at 72 °C for 10 min. The PCR product was diluted 100-fold in ddH_2O to reduce the influence of the outer primers to second round amplification.

2.6. Second round (2nd) quantification amplification

The 100-fold diluted PCR product from the 1st round multiplex amplification and gene disk were then used for 2nd round quantification amplification. A final volume of 20 μL was then added to the gene disk containing the lyophilized inner primers with the following reagent: 10 μL SYBR Perfect real-time mix (TaKaRa), 2 μL 100-fold diluted PCR product as template and ddH_2O to adjust the volume to 20 μL. Second round quantification amplification were performed on Roche LightCycler 480 and the condition were: 95 °C for 1 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. Fluorescence was measured at the end of each 72 °C extension step and melting curves were analyzed from 60 °C to 95 °C.

2.7. Optimization of MT-PCR conditions

To determine the cycle number of the 1st round amplification was crucial in the MT-PCR. The MT-PCR with three different cycle numbers (10, 15 and 20) in the 1st round amplification were performed to compare with conventional real-time PCR which only used gene disk without 1st round amplification. BT11 genomic DNA has been used as templates here which contained 6 target genes (BT11, IVR, Pat, CaMV35S, NOS, Cry1Ab). Each gene's Ct value under the conditions of these methods has been analyzed.