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Development of a Multiplex Event-specific PCR Assay for Detection of Genetically Modified Rice

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Global rice supplies have been found contaminated with unapproved varieties of genetically modified (GM) rice in recent years, which has led to product recalls in several of countries. Faster and more effective detection of GM contamination can prevent adulterated food, feed and seed from being consumed and grown, minimize the potential environmental, health or economic damage. In this study, a simple, reliable and cost-effective multiplex polymerase chain reaction (PCR) assay for identifying genetic modifications of TT51-1, Kemingdao1 (KMD1) and Kefeng6 (KF6) rice was developed by using the event-specific fragment. The limit of detection (LOD) for each event in the multiplex PCR is approximately 0.1%. Developed multiplex PCR assays can provide a rapid and simultaneous detection of GM rice.

Keywords: multiplex polymerase chain reaction, genetically modified rice, detection, event-specific

Introduction

The global area of GM plants has a 100-fold increase from 1.7 million hectares in 1996 to over 175 million hectares in 2013 (James 2013). The genetic compositions of genetically modified products (GMPs) have been altered by a gene modification technique to gain new characteristics, like herbicide tolerance, insect resistance or modified nutritional composition (Arun et al. 2013). Rice (*Oryza sativa*) is one of the most important food crops in the world, especially in many Asian countries. Worldwide, more than 3.5 billion people have rice for more than 20% of their daily calorie intake (Seck et al. 2012), therefore, the development of genetically modified rice is of high priority. The herbicide resistance and insect-resistant GM events are the most advanced GM rice. GM rice TT51-1, KMD1 and KF6 are three of the most well-known events (Chen et al. 2012). TT51-1 is an insect-resistant transgenic rice event harboring a hybrid *cry1Ab/Ac* gene driven by rice *actin* 1 gene promoter and the nopaline synthase (NOS) terminator, which was granted

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the safety certificate by China in 2009 (Tu et al. 2003; Lu 2010). The transgenic Chinese Bt rice KMD1, derived from the commercial japonica rice variety Xiushui11 (XS11) by *Agrobacterium* transformation with a synthetic *cry1Ab* gene (Babekova et al. 2008; Liu et al. 2012), is resistant to eight lepidopteran pest species like the yellow stem borer, the striped stem borer and the rice leaf folder, which has the potential to be approved in China (Babekova et al. 2009). KF6 contains two insect-resistant genes, *cry1Ac* gene under the control of the maize ubiqutin promoter and *CpTI* (cowpea trypsin inhibitor) gene (Rong et al. 2005).

Unlike other major crops, the commercial cultivation of GM rice is authorized only in a very limited number of countries (James 2010). Three herbicide-resistant GM rice varieties (LLRICE06, LLRICE62 and LLRice601) were approved for commercial cultivation in the USA (CERA 2010). Biosafety certificates were issued for the Bt rice lines Huahui No. 1 and Bt Shanyou 63 ("Bt63") by China's Ministry of Agriculture in 2009 (Chen et al. 2011). Furthermore, a number of other GM rice varieties were also under development in China (Kluga et al. 2013), but commercial production of GM rice is still not permitted in China. In the following years also in Iran GM rice could be commercialized on a larger scale (Ghareyazie 2012). Until today, no GM varieties of rice have been commercialized at a large scale, but global rice supplies have been found contaminated with unapproved varieties of GM rice in recent years. Illegal cultivation and trade of GM rice in China was reported in 2005 (Zi 2005). Rice contaminated with unauthorized LLRICE601 has been found across the world in 2006 (GM Contamination Register 2007). Since 2006, trace amounts of several unapproved Chinese GM rice (e.g. TT51-1 (synonym BT63), KMD1 and KF6) in food products on the EU market have also been notified to the rapid alert system for food and feed (RASFF) (see the notifications at https://webgate.ec.europa.eu/rasff-window/portal/). Following the repeated RASFF notifications of GM rice from China, the EU implemented a new regulation concerning rice from China, replacing decision 2008/289/EC (Bt63 rice). Decision No. 2011/884/EU which became effective since January 11th 2012, requires systematic screening for genetic modifications of rice products from China that are intended for the European market (The Health and Consumers Directorate-General of the European Commission 2012). Lately, genetic modifications in *Basmati* rice originated from Pakistan or India have been detected for the first time in products placed on the EU market in autumn 2011 (Reiting et al. 2013).

The targets for PCR-based GMO tests can be grouped into at least four categories contained screen-specific, gene-specific, construct-specific and event-specific (Zhang and Guo 2011). The event-specific PCR targets the unique and specific junction sequences between the transgenic insert and the plant genome DNA (Yang et al. 2007). Some event-specific PCRs have been accredited or validated for the nucleic acid detection by official bodies/reference laboratories, and are currently the most widely used DNA detection methods for determining the presence of GMOs content in processed food and feed samples for the high specificity (Dong et al. 2008).

In order to monitor the presence of TT51-1, KMD1 and KF6, which are found contaminating food products not only in China but as well as in EU and have been reported many times, simple and effective detection methods are needed urgently. Real-time PCR

assays and loop-mediated isothermal amplification (LAMP) assays for KMD1, TT51-1 and KF6 have been reported (Babekova et al. 2009; Wu et al. 2010; Cao et al. 2011; Chen et al. 2012). However, multiplexing can provide a cost effective diagnostic assay for GM detection with higher throughput and less consumption of samples and reagents as compared to simplex assays (Bahrdt et al. 2010; Kim et al. 2013; Kim et al. 2014). Our goal is to develop a simple, reliable and cost-effective multiplex PCR assay for simultaneous detection of genetic modifications of TT51-1, KMD1 and KF6 rice to meet the above monitoring requirement.

Materials and Methods

Plant materials

GM rice (TT51-1, KMD1, KF6) and Non-GM rice (New Liangyou6, Liangyou6326, Y Liangyou1, Zaoxian788) were collected by our laboratory.

Methods

DNA extraction and purification

DNA extraction was carried out following the cetyl-trimethylammoniumbromide (CTAB) protocol (Dellaporta et al. 1983). The purity and concentration of extracted DNA were measured and analyzed by an ultraviolet (UV) spectrophotometer (BioPhotometer plus, Eppendorf). The quality of the extracted DNAs was evaluated from the ratio of UV absorptions at 260/280 and 260/230 nm wavelengths.

PCR primers

A total of four sets of primer pairs were designed for multiplex PCR to detect and distinguish three varieties of GM rice. The rice sucrose phosphate synthase (*SPS*) gene (Accession no. U33175) as an endogenous reference gene has been applied in the national standards of China and undergone an international collaborative study (Ding et al. 2004; Jiang et al. 2009). The primers TT51-F/R, KMD1-F/R and KF6-F/R were designed based on the revealed integration junction region sequences plated between the rice genome and integrated foreign genes of TT51-1 (Accession no. EU880444.1), KMD1 (Accession no. EU980363.1) (Babekova et al. 2009), KF6 (Accession no. HM124449) (Wang et al. 2011). The sequences of the oligonucleotide primers used in this study were listed in Table 1. All oligonucleotide primers used were synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China).

PCR conditions

All PCR reactions were carried out in thermal cycler (Eppendorf Mastercycler® ep gradient S). The multiplex PCR reaction mixture in 25 μL volumes contained 100 ng of

Table 1. Primers used in this study

Primer	Sequence (5′-3′)	Targets	Amplicon length (bp)
TT51-F	CGACTCTAGAGGATCCCG	T-DNA	200
TT51-R	CTCTGAGGATCCAAACAAGGC	Genome	
KMD1-F	AAGCGTCAATTTGTTTACACC	T-DNA	175
KMD1-R	CCTGTCAGTCTCGTCAGCAA	Genome	
KF6-F	CCTATGATGTTATCCCATGC	Genome	151
KF6-R	AAAACGTCCGCAATGTGT	T-DNA	
SPS-F	ATCTGTTTACTCGTCAAGTGTCATCTC	SPS	287
SPS-R	GCCATGGATTACATATGGCAAGA	SPS	

genomic DNA, 2.5 μL of 10× buffer (Takara, Dalian, China), 200 μM of each dNTP (Takara), 1.5 mM of $MgCl_2$, and 0.65 units of Hot Start Taq polymerase (Takara). The concentration of primers for each target gene from 0.2 μM to 12 μM was adjusted independently until the PCR products showed similar intensities after agarose gel electrophoresis. Final concentrations of primers (SPS-F/R 0.25 μM, TT51-F/R 10 μM, KMD1-F/R 0.54 μM, KF6-F/R 5.6 μM) were included in each reaction. The reaction conditions of multiplex PCR were the following: initiation step of 3 min at 94 °C followed by 50 cycles of 30 s at 94 °C, 30 s at 61 °C and 30 s 72 °C, final extension at 72 °C for 7 min. The concentration of primer pairs was 0.4 μM in the conventional simplex PCR. The thermal cycling condition was set as 3 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C, final extension at 72 °C for 7 min. All PCR products were separated and identified based on the length of the amplified DNA fragments after electrophoresis in a 3% agarose gel in 1×TAE buffer [40 mM Tris–acetate (pH 8.0), 1 mM EDTA] with ethidium bromide (EB) (Sigma, US), evaluated by the gel imaging system (Gel Dox XR system, Bio-Rad). The 50 bp DNA ladder (Takara) was used as a size standard for amplified DNA fragments. Sequencing of PCR products was performed by Sangon Biotechnology Co. Ltd. (Shanghai, China).

Results

Specificity of the multiplex PCR

We designed event-specific primers targeting three GM rice events, TT51-1, KMD1 and KF6. In order to evaluate and verify the specificity of primer pairs designed for multiplex PCR, the performance of each individual pair of primers in the conventional simplex PCR was carried out using the genomic DNA templates from GM and non-GM materials as follows: GM rice TT51-1, GM rice KMD1, GM rice KF6 and non-GM rice. PCR products were observed under ultraviolet light after electrophoresis. As shown in Fig. 1, endogenous gene *SPS* was normally amplified. The expected amplifications were specifically amplified from each target GM rice (TT51-1, KF6 and KMD1), but not those from

the non-GM rice, indicating the high specificity of the primer pairs. The three target amplifications from GM rice were identified by DNA sequencing analysis and found to have the correct target sequences (Fig. 2).

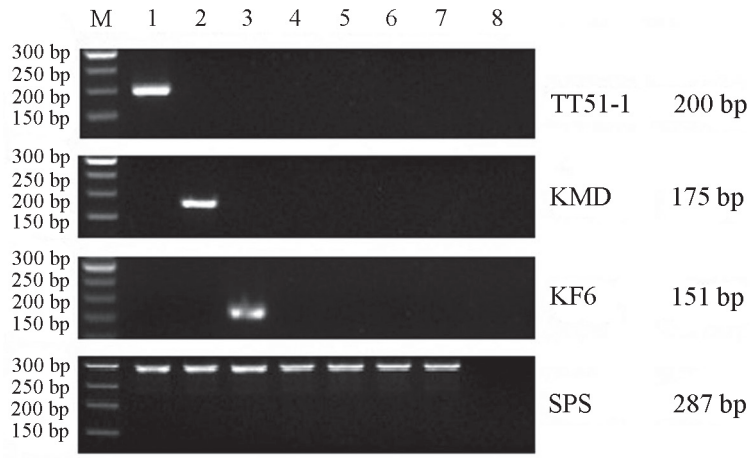

Figure 1. A conventional simplex PCR assay analysis of the specificity of primer pairs designed for multiplex PCR. Lane M, 50 bp DNA ladder; Lane 1, TT51-1; Lane 2, KMD1; Lane 3, KF 6; Lanes 4–7, Non-GM rice (New Liangyou 6, Liangyou 6326, Y Liangyou 1, Zaoxian 788); Lane 8, environmental control, no template

A

1 CGACTCTAGAGGATCCCGGACGAGTGCTGGGGCAGATAAGCAGTAGTGGTGGGGCTACGA
61 ACATATTCCTTTTCCTTCTGGACGCTACCACTCATATGTTCCAAAATTACAAATTTGTCC
121 TTTGTATTTGTTGCAATTTTCATGTAAGAAATCCAACGAGGCTCTGTTTTTTTTTATTGG
181 CCTTGTTTGGATCCTCAGAG

B

1 CCTATGATGTTATCCCATGCTAATGTATCCAGAGCGATGGCTTGCTTTGAGCACTCTAAT
61 TTCTTCAAAGTAACGGCGCCGGAGGCACGACCCGGCCAGTTAAGGCCAGGAGCGCATCTG
121 ATCCAACTTAATAACACATTGCGGACGTTTT

C

1 AAGCGTCAATTTGTTTACACCACAATATATCCCGAGATGGGCAGGCATATCGGCGTACGC
61 ACGCAGCCCGGTGAGACCCGCCGCAGTTGGAGCGCGCATCGCCATCGCCGCGAGCCCGCG
121 AAGTCCACGGCGCCCTCGTCGGCGGAACACCCCAGTTGCTGACGAGACTGACAGG

Figure 2. Flanking sequences of the transferred DNA flanking region and the inserted region of the GMO genome from three GM rice PCR products. A, 3′ flanking sequences of TT51-1, the PCR product is 200-bp long. B, 5′ flanking sequences of KMD, the PCR product is 175-bp long. C, 3′ flanking sequences of KF6, the PCR product is 151-bp long. The primers are underlined. The rice genome sequence is shaded

Sensitivity of the multiplex PCR

To develop a reliable, cost-effective and rapid multiplex PCR assay, factors which could influence the detection need to be considered. These factors include the sequence, specificity, concentration and annealing temperature of the primers, etc. Among these factors, the development of suitable primers is much more critical in multiplex PCR than in conventional PCR. In this study, primer concentrations were optimized in order to avoid common constraints like non-specific product amplification or cross-amplification reactions and generate specific PCR products with equal efficiency. The concentration of primers for each target gene from 0.2 μM to 12 μM was adjusted independently until the PCR products showed similar intensities after agarose gel electrophoresis. Figure 3 showed the results of multiplex PCR using the optimized conditions. Final concentration of primers (SPS-F/R 0.25 μM, TT51-F/R 10 μM, KMD1-F/R 0.54 μM, KF6-F/R 5.6 μM) were included in the same PCR run. In optimized multiplex PCR the expected target sequences for each GM rice event and the *SPS* gene were specifically amplified and showed similar intensities. These PCR results indicate that this method is sufficient to distinguish three GM rice events.

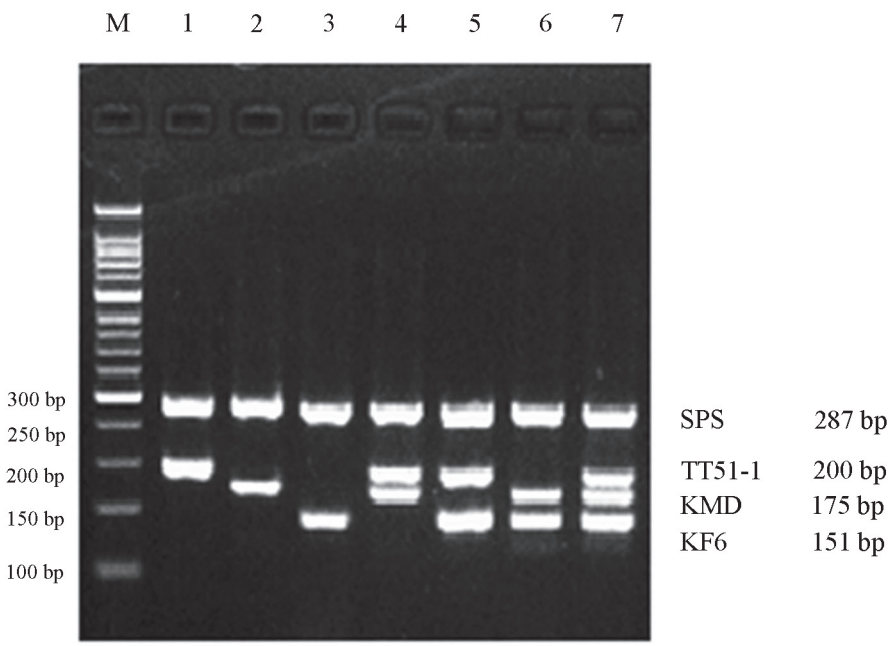

Figure 3. Multiplex PCR amplified from GM rice. Lane M, 50 bp DNA ladder; Lane 1, *SPS* and *TT51-1*; Lane 2, *SPS* and *KMD*; Lane 3, *SPS* and *KF6*; Lane 4, *SPS*, *TT51-1* and *KMD*; Lane 5, *SPS*, *TT51-1* and *KF6*; Lane 6, *SPS*, *KMD* and *KF6*; Lane 7, *SPS*, *TT51-1*, *KMD* and *KF6*

Limit of detection (LOD)

To determine the limit of detection (LOD) of GM rice using this established multiplex PCR assay, a dilution series of rice gDNA mixture was prepared from three GM rice events and pure non-GM rice to be used as templates at various levels. The final relative GM contents were 10%, 5%, 1%, 0.5%, 0.1%, 0.05% and 0.01% (mass/mass ratio) for each event of the three GM rice events, respectively. Three GM rices were mixed at the ratio 1:1:1 in mass. As shown in Fig. 4, the results revealed that the LOD value of this multiplex PCR is approximately 0.1%.

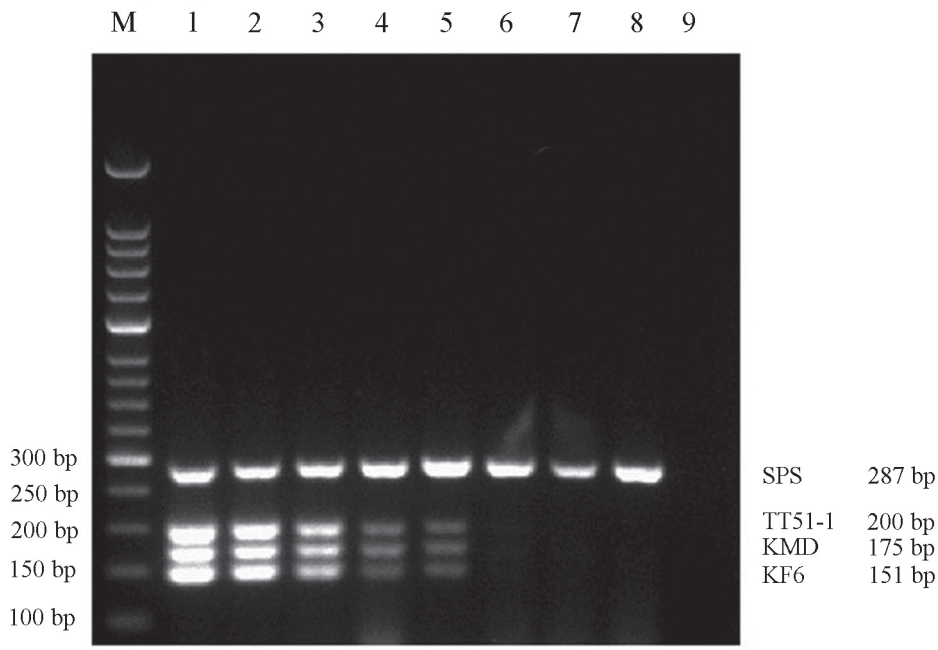

Figure 4. LOD analysis of the multiplex PCR. Lane M: marker (50 bp DNA Ladder); Lanes 1–7: 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, and 0.01% of a mixture of GM rice varieties (TT51-1, KMD and KF6); Lane 8: non-GM rice; Lane 9: no template

Discussion

For assured food safety and quality of GM crops and to address consumer concerns, efficient analytical methodologies must be available for GM testing (Rodríguez-Lázaro et al. 2007; Kamle et al. 2010). Hence, in order to monitor the potential present of illegal GM rice, the simple and effective detection methods are very urgent and much required. The introduction of a foreign gene into the DNA of an organism can be unambiguously detected at the DNA level (Heller 2003; Kamle et al. 2010). Hence, PCR-based transgene detection in GM crops remains the most sensitive method (Shrestha et al. 2010). The

variant of PCR method, multiplex PCR method can be simultaneously performed of multiple event-specific sequences with more than one pair of primer sets in single-tube reactions and considered time-saving, low-cost and effective, as compared to the standard simplex PCR systems (Shrestha et al. 2010; You 2014). Multiplex PCR assays of different GMPs have been developed, such as GM soybean (Kim et al. 2009; Kim et al. 2013), GM cotton (Demeke et al. 2002; Kim et al. 2008; Yang et al. 2005), GM canola (Kim et al. 2007), and GM maize (Kim et al. 2014). In this study, multiplex PCR assay was developed for the qualitative detection of three GM rice genes amplified concurrently in a single PCR tube.

Primer design is critical in the multiplex PCR assay for the simultaneous amplification of multi-target DNA sequences to ensure specificity and sensitivity and to avoid cross-reactions (Lee et al. 2014). As the number of primers increases, the interaction effects also increase and may result in the formation of amplification artifacts, such as primer-dimers, truncated DNA fragments, non-specific bands or an absence of bands (Shrestha et al. 2010). Although specific primers amplify target DNA sequences, they do so with the differential amplification rates (Kim et al. 2009). Taking into account these factors, lengths of multiple sets and melting temperatures (Tm) of primers must be similar (Kwon et al. 2014; Lee et al. 2014). To establish a multiplex PCR method for simultaneous detection of three events of GM rice, we designed event-specific primers targeting to three GM rice events, namely, TT51-1, KMD1 and KF6. Tm of primer pairs was maintained in the range of 49–62 °C in order to avoid the generation of nonspecific amplification. The optimal annealing temperature was found to be 61 °C in the multiplex PCR. The specificity of designed primer pairs was individually assessed by a simplex PCR assay. Only PCR products of the expected lengths were amplified from each target DNA, and no obvious non-specific signals were present in non-GM rice. Three events of GM rice were successfully detected by multiplex PCR using all the primer pairs.

In conclusion, we successfully developed a simple, reliable and cost-effective multiplex PCR assay for identifying genetic modifications of TT51-1, KMD1 and KF6 rice by using the event-specific fragment. The limit of detection (LOD) for each event in the multiplex PCR is approximately 0.1%. The developed multiplex PCR method was so highly specific and sensitive and cost effective that it is useful to monitor GM rice in the markets around the world.

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