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Construction of a standard reference plasmid containing seven target genes for the detection of transgenic cotton

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ABSTRACT

Insect resistance and herbicide tolerance are the dominant traits of commercialized transgenic cotton. In this study, we constructed a general standard reference plasmid for transgenic cotton detection. Target genes, including the cowpea trypsin gene *cptI*, the insect resistance gene *cry1Ab/1Ac*, the herbicide tolerance gene *cp4-epsps*, the *Agrobacterium tumefaciens* nopaline synthase (*Nos*) terminator that exists in transgenic cotton and part of the endogenous cotton *SadI* gene were amplified from plasmids pCPT1, pBT, pCP4 and pBI121 and from DNA of the nontransgenic cotton line K312, respectively. The genes *cry1Ab/1Ac* and *cptI*, as well as *cp4-epsps* and the Nos terminator gene, were ligated together to form the fusion genes *cptI-Bt* and *cp4-Nos*, respectively, by overlapping PCR. We checked the validity of genes *Sad1*, *cptI-Bt* and *cp4-Nos* by DNA sequencing. Then, positive clones of *cptI-Bt*, *cp4-Nos* and *Sad1* were digested with the corresponding restriction enzymes and ligated sequentially into vector pCamBIA2300, which contains the CAMV 35S promoter and *nptII* gene, to form the reference plasmid pMCS. Qualitative detection showed that pMCS is a good positive control for transgenic cotton detection. Real-time PCR detection efficiencies with pMCS as a calibrator ranged from 94.35% to 98.67% for the standard curves of the target genes ($R^2 \geqslant 0.998$). The relative standard deviation of the mean value for the known sample was 11.95%. These results indicate that the strategy of using the pMCS plasmid as a reference material is feasible and reliable for the detection of transgenic cotton. Therefore, this plasmid can serve as a useful reference tool for qualitative and quantitative detection of single or stacked trait transgenic cotton, thus paving the way for the identification of various products containing components of transgenic cotton.

1. Introduction

The global area devoted to genetically modified crops (GMC) has increased 100-fold, from 1.7 million hectares in 1996 to 175.2 million hectares in 2013, and the area planted with crops containing stacked traits (e.g., different insect and herbicide resistance genes) has increased to 47.1 million hectares. In China, approximately 4.2 million hectares of Bt cotton, equivalent to approximately 90% of the total cotton growing area, were planted in 2013 (James, 2014).

With the rapid development and deployment of GMC in many parts of the world, it has become increasingly important to pay attention to safety and regulatory issues surrounding genetically engineered products. DNA-based tests, especially PCR, are still the most widely used, effective methods for the detection of products containing

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recombinant DNA. For DNA-level detection, it is necessary to use certified reference materials (CRMs) as positive controls and to avoid false negatives. However, the production of CRMs for different biotech crops can be an extremely complex, expensive and time-consuming process (Debode et al., 2010; D'Andrea et al., 2009). Standardized plasmid molecules are better alternative to CRMs for the detection of GMC due to their ease of preparation and storage, universal applicability and low cost (Yang et al., 2007; Guan et al., 2011; Wang et al., 2011). The application of standardized plasmid molecules as calibrators is becoming essential for the practical detection of GMC (Huang and Pan, 2005). To date, several plasmids have been developed as reference molecules (RMs) (Wang et al., 2009; Scholtens et al., 2010). Plasmids such as ERM-AD413, ERM-AD415, ERM-AD427 and ERM-AD425, which are used for the detection of transgenic corn lines MON810, NK603 and 98140 and the transgenic soybean line 356043, respectively, have been registered as CRMs and commercialized in many regions throughout the world (Corbisier et al., 2007; Jeynov et al., 2011; Caprioara-Buda et al., 2011; Mazoua et al., 2011).

Herbicide tolerance and insect resistance are the dominant traits deployed in cotton. In China, most approved transgenic cotton lines contain the target gene *cry1Ab*/*1Ac*, the cowpea trypsin gene *cptI*, the herbicide tolerance gene *cp4-epsps*, regulatory elements (including the CaMV 35S promoter and the nopaline synthase [nos] terminator [Nos T]) and the selectable marker gene *nptII*. Therefore, in this study, we constructed a new multiplex plasmid RM by integrating seven gene fragments into a single plasmid molecule containing the following: (1) the endogenous cotton gene *Sad1*; (2) the cowpea trypsin gene *cptI*; (3) the CaMV 35S promoter; (4) Nos T; (5) the *cp4-epsps* gene; (6) the *cry1Ab*/*1Ac* gene and (7) the bacterial *nptII* gene for resistance to kanamycin, forming the reference plasmid pMCS. We also assessed the utility of pMCS for the detection of transgenic cotton.

2. Material and methods

2.1. Plant material and reagents

Nontransgenic cotton line K312 is maintained in our laboratory. The 1% reference materials of Bt cotton and herbicide-tolerant cotton were kindly provided by the Jin laboratory at the Biotechnology Research Institute of the Chinese Academy of Agricultural Science. Plasmids pCPT1, pBT, pCP4 and pBI121 were conserved in our laboratory. Vector pCamBIA2300 was purchased from Cambia Company (Australian). The Taq polymerase enzyme, fluorescent quantitative detection kit (SYBR® Premix Ex Taq™, Production code DRR420S), DNA purification kit (Agarose Gel DNA Fragment Recovery Kit Ver.2.0, Production code D301) and T-vector were purchased from TaKaRa (Beijing, China). The general restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Primers were synthesized and purified by Shanghai Sangon Biotech Company. Primers synthesis and DNA sequencing were performed by Shanghai Sangon Biotech Company.

2.2. Construction of the multiplex plasmid DNA reference molecule pMCS

Primers were designed based on the sequences of the target genes *cptI*, *cry1Ab*/*1Ac*, *cp4-epsps*, *Sad1* and Nos T (Table 1). The two primers in the overlapping primer pairs cpt1-F/Bt-R1 and cp4-F/Nos-R1 were complementary to each other. Five restriction enzyme sites, including *Eco*RI, *Kpn*I, *Hin*dIII, *Sal*I and *Bam*HI, were introduced at the ends of the primers for the *cptI*, *cry1Ab*/*1Ac*, *cp4-epsps*, *Sad1* and Nos T amplification products, respectively.

The *cptI* gene was amplified using CPT1-F/CPT1-R as primers and plasmid pCPT1 as template. The *cry1Ab*/*1Ac* gene was amplified using Bt-F/Bt-R as primers and plasmid pBT as template. Fusion PCR was performed to connect *cptI* with *cry1Ab*/*1Ac* using primer pair cpt1-F/Bt-R and the above PCR products. The optimized PCR program was as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 70 s, with a final cycle of 72 °C for 10 min. The fusion gene *cptI-Bt* was ligated into cloning vector pMD18-T and sequenced.

The *cp4-epsps* gene was amplified using CP4-F/CP4-R as primers and plasmid pCP4 as template. Nos *T* was amplified using Nos-F/Nos-R as primers and plasmid pBI121 as template. Fusion PCR was performed to connect *cp4-epsps* with Nos *T* using primer pair cp4-F/Nos-R and the above PCR products. The optimized PCR program was as follows: 94 °C for 5 min, then 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 70 s, followed by a final cycle of 72 °C for 10 min. The fusion gene *cp4-Nos* was ligated into cloning vector pMD18-T and sequenced.

The endogenous upland cotton reference control *Sad1* gene fragment was amplified using Sad1-F/Sad1-R as primers and DNA from nontransgenic cotton line K312 as template. The optimized PCR program was as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 70 s, and a final cycle of 72 °C for 10 min. The amplification product was ligated into cloning vector pMD18-T and sequenced.

In the plant expression vector pCamBIA2300, the plant selectable marker gene *nptII* is driven by the 35S promoter. Therefore, pCamBIA2300 was used as the backbone to construct pMCS. The fusion gene *cptI-Bt* was inserted between the *Eco*R1 and *Kpn*1 sites of pCamBIA2300 to form the intermediate vector p2CB. Subsequently, *SadI* was inserted between the *Hin*dIII and *Sal*I sites of p2CB to form the intermediate vector p2CBS. Finally, the fusion gene *cp4-Nos* was inserted between the *Bam*HI and *Sal*I sites of p2CBS to form the multiplex plasmid reference molecule pMCS.

2.3. Validating the plasmid reference molecule pMCS

PCR amplifications were performed to assess the validity of the plasmid molecule pMCS. The primers and amplification fragment sizes are shown in Table 2. The optimized PCR program was 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s, with a final extension step of 72 °C for 5 min.