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Applicability of a novel reference molecule suitable for event-specific detections of maize NK603 based on both 5′ and 3′ flanking sequences

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ABSTRACT

Plasmid molecule based reference material (RM) has been shown to be a good alternative as the calibrator for genetically modified organisms (GMOs) identification and quantification, while most of the currently developed plasmid RM can only be used for one specific target detection. In this study, a flexible plasmid RM pNK containing three DNA fragments, i.e. 5′ and 3′ event-specific sequences of maize NK603 and endogenous gene *zSSIIb*, was developed. We have proved that pNK is suitable for using as a calibrator in both 5′ and 3′ event-specific detection of maize NK603, compared with that of genuine genomic DNA. The limit of detection (LOD) was 10 copies of pNK DNA in conventional PCR assays. The absolute LOD and limit of quantification (LOQ) in quantitative PCR assays were 5 and 25 copies. The standard curves targeting to *zSSIIb*, 5′ and 3′ event-specific sequences based on pNK DNA showed high reaction efficiency and good linearity. Also, low bias and variations were obtained in practical samples quantification using pNK as the calibrator. These results demonstrated that the developed pNK is flexible and suitable for identification and quantification of maize NK603, as a preferable substitute of RM from the plant raw material.

1. Introduction

Deficiency of certified reference materials (CRMs) for GMO analysis limits the implementation of GMOs monitoring and supervising. Although the Institute for Reference Materials and Measurements and American Oil Chemists' Society has developed more than 40 kinds of CRMs for GMOs detection, it still could not keep up with the quick development of GM plants. By the end of 2008, nearly 150 GM events have been planted in 25 countries and areas in the global area (James, 2009). Therefore, governments and institutions have to pay more attentions to the investigation and development of CRMs for GMOs detection.

Due to the high sensitivity and specificity, DNA based PCR methods have become the most widely used approach for GMO identification and quantification. Till now, hundreds of PCR methods targeting the inserted exogenous genes, universal elements or event-specific regions have been reported and used for GMO analysis, including several 5′ and 3′ event-specific PCR assays for GM maize NK603 (Dong et al., 2008; Huang & Pan, 2004; Mazzara, Cordeil, & Van den Eede, 2004; Yang, Shen, et al., 2007; Yang, Guo, et al., 2007). CRM is essential for the enforcement of the detection method (Elenis, Kalogianni, Glynou, Ioannou, & Christopoulos, 2008). There are three types of CRMs for GMOs detection for the particular analytical targets, such as Matrix-CRM, protein-CRM and DNA-CRM (Trapmann & Emons, 2005). Matrix-CRM is derived from the blending of plant raw materials, such as the seeds or partial vegetables of GM event and the relevant non-GM plant by specified mass fraction (Broothaerts et al., 2007). DNA-CRM is a group of CRMs presented in the form of DNA, including genomic DNA and plasmid DNA. Protein-CRM is the plant entire proteins usually from the processing of the raw materials or tissues. As a new type of DNA-CRM, plasmid molecule based RM has been recently accepted and used as the calibrator in quantitative real-time PCR systems for GMO quantification (Corbisier et al., 2007; Yang et al., 2005). Because of the advantages of plasmid molecule based RM, such as easy to obtain high quantity and quality DNA, low costs in plasmid DNA production and simplicity in operation, more than 20 kinds of plasmid RMs have been developed by the end of 2008 (Kuribara et al., 2002; Mattarucchi, Weighardt, Barbati, Querci, & Van den Eede, 2005; Rodríguez-Lázaroa et al., 2007; Weighardt et al., 2004). However, most of the plasmid RMs was designed to contain only one exogenous gene or transgenic fragment.

To facilitate the application of plasmid RMs in GMO analysis, a novel flexible plasmid RM pNK suitable for both 5′ and 3′ event-specific detections of GM maize NK603 was constructed in this

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Table 1
List of primers and probes used in PCR analysis.

Object	Target	Primer (probe) name	Sequence (5′–3′)	Amplicon (bp)	Reference
Construction of plasmid	5′ and 3′ junction region of NK603	C-NK603-1F	CCG GAA TTC AAA CTG CAG TTA TTT TGG ACT ATC CCG ACT CT	292	This paper
		C-NK603-2R	CAG AGA AAA GAT CGG CTC ATG CCT TGT ATA CGC GAT GTA ACA CCT A		
		C-NK603-3F	AGG TGT TAC ATC GCG TAT ACA AGG CAT GAG CCG ATC TTT TCT CTG G	211	
		C-NK603-4R	GGA AGA TCT CTT ACC TTT GTT TTA TTT TGG AC		
	zSSIIb	C-zSSIIb-1F	AAA CCA TGG AGT GCG GTG AAG CCA GAG C	257	This paper
		C-zSSIIb-2R	AAA ACT AGT GCA AAG CAC CCA CGA CAT C		
Event-specific analysis	3′ flanking sequence of NK603	NK603-1F	ATG AAT GAC CTC GAG TAA GCT TGT TAA	108	Yang, Shen,. et al., 2007
		NK603-2R	AAG AGA TAA CAG GAT CCA CTC AAA CAC T		
		Q-NK603-2P	FAM- AGC TTG GT(TAMRA)A CCA CGC GAC ACA CTT CCA C		This paper
	5′ flanking sequence of NK603	Q-NK603-1F	CGG CCA GCA AGC CTT GTA	110	Yang, Guo, et al., 2007
		Q-NK603-2R	CGA CTC TCT TCT CAA GCA TAT GAA TG		
		Q-NK603-1P	FAM- CGG CCG CGT T(TAMRA)AA CAA GCT TAC TCG A		
Endogenous gene analysis	*zSSIIb*	zSSIIb-1F	CGG TGG ATG CTA AGG CTG ATG	88	Yang, Guo, et al., 2007
		zSSIIb-2R	AAA GGG CCA GGT TCA TTA TCC TC		
		Q-zSSIIb-P	HEX- TAA GGA GCA CTC GCC GCC GCA TCT G-TAMRA		

study. The applicability of pNK used as calibrator in identification and quantification of maize NK603 was also validated in our lab.

2. Materials and methods

2.1. Materials

Seeds of genuine materials of GM maize (*Zea mays*) NK603 were provided by Monsanto Co. and the dry seed powders of GM maize T25, MON810, Bt176, GA21, MON863, Bt11 and TC1507, Roundup Ready® soybean (*Glycine max*) (RRS), GM cotton (*Gossypium herbaceum L.*) MON531 and MON1445 were supplied by the developers. One variety of *Arabidopsis thaliana*, Columbia seeds were purchased from Arabidopsis Biological Resource Center (USA). Conventional maize, barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) materials were purchased from local markets of Shanghai, China.

2.2. Isolation and purification of plasmid DNA and genomic DNA

According to the manufacturer's instructions, plasmid DNA samples were extracted and purified from about 4 mL *E. coli* suspension using Plasmid Mini Extraction Kit (Axygen Scientific, Inc., USA). Genomic DNA samples of maize, cotton, soybean, wheat and barley varieties were isolated and purified from the refined ground powders or from leaves of *Arabidopsis* using Plant DNA Mini-Prep Kit (Ruifeng Agro-tech Co., Ltd., Shanghai, China). The process was carried out with a few modifications, such as the addition of 100 ng/mL proteinase K to Buffer A and the extension of the incubation time to 3 h at 60 °C, instead of 65 °C in the first step. The quality and quantity of the DNA samples were evaluated by UV spectrometry and electrophoresis. DNA copy numbers were calculated according to the mean DNA quantity and the genomic DNA size (Arumuganathan & Earle, 1991; Pan et al., 2006).

2.3. Primers and probes

Primers and TaqMan probes were designed using the primer analysis software Oligo version 6.31 (Molecular Biology Insights, USA) (Table 1, Fig. 1). To construct the plasmid molecule pNK, three fragments from 3′ or 5′ event-specific region and the *zSSIIb* gene were amplified using the primer pairs C-NK603-1F/2R, C-NK603-3F/4R and C-zSSIIb-1F/2R. Primer pairs and probes NK603-1F/2R/Q-NK603-2P and Q-NK603-1F/2R/1P were used for 3′ and 5′ event-specific quantification of maize NK603 according to the previous reports (Yang, Shen, et al., 2007; Yang, Guo, et al., 2007). The amplified products of these two primer pairs had similar lengths (108 bp and 110 bp), which could reduce the effect from amplicon size and facilitate the comparison of two systems. For detection of endogenous gene *zSSIIb* of maize, primer pair zSSIIb-1F/2R and probe Q-zSSIIb-P were used according to the previous report (Yang, Guo, et al., 2007). All the primers and TaqMan probes were synthesized by TaKaRa Biotech Co., Ltd. (Dalian, China).

2.4. Specificity testing

Nine primer pairs specific to six GM maize events MON810, MON863, Bt176, Bt11, T25 and GA21, two GM cotton varieties MON531 and MON1445, and RRS were employed to test the specificity of pNK. The sequences of the primers were from the description of Bulletin of Ministry of Agriculture of the People's Republic of China, Agricultural Industry Standard of the People's Republic of China (Lu, Shen, et al., 2007; Lu, Song, et al., 2007; Luo et al., 2003; Zhang, et al., 2007a, 2007b; Zhang et al., 2007; Zhang, Yang, et al., 2007) and our previous report (Yang et al., 2005).

2.5. Construction of pNK

A plasmid pMaize was firstly constructed to facilitate the construction of a series of plasmid RMs suitable for different GM maize events detection, containing the tandem integration of maize endogenous gene *zSSIIb* sequence with about 250 bp in length and a 2463 bp DNA fragment (Fig. 1A). The 2463 bp sequence was from our previous report (Li et al., 2009). The two DNA fragments were cloned into the vector pBlueScript separately. To meet the requirements of practical use, a tandem recombinant fragment with 485 bp in length, consisting of 3′ (292 bp) and 5′ (211 bp) junction sequences of maize NK603 by overlapping PCR was cloned into pMaize (Fig. 1B–D). Sequence of one isoschizomer *Bgl*II was introduced into the primer C-NK603-4R, resulting in the fusion of the restriction endonuclease sites of *BamH*I and *Bgl*II after cloning. The developed plasmid pNK was confirmed by sequencing by Invitrogen Biotech Co., Ltd. (Shanghai, China).