DUPLEX POLYMERASE CHAIN REACTION (PCR) FOR THE SIMULTANEOUS DETECTION OF *CRYIA(B)* AND THE MAIZE UBIQUITIN PROMOTER IN THE TRANSGENIC RICE LINE KMD1

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ABSTRACT

This study describes a combination of screening and gene-specific methods for the detection of GMO in food and feed. Using specific primers, the maize ubiquitin promoter and the cryIA(b) gene, which encodes for a delta-endotoxin transferring insect resistance, can be detected simultaneously by a duplex PCR in samples containing the transgenic rice line Kemingdao1 (KMD1). This method is also suitable for the screening for Bt11 maize.

Keywords: GMOs, transgenic rice, screening, gene-specific methods, KMD1, *cryIA(b),* maize ubiquitin promoter, Bt11

Introduction

The cultivation of genetically modified plants has increased to an extend of over 100 million ha worldwide in 2006 (11). Although several transgenic varieties of maize, soybean, cotton, and oilseed rape have been approved in the European Union for cultivation or processing (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm), European farmers cultivate only about 100.000 ha with transgenic plants (http://www.transgen.de/). Nevertheless, the amount of food and feed products made from or containing genetically modified organisms (GMOs) imported in the EU is steadily increasing.

The deliberation and commercialization of GMOs in the EU has to follow the strict European legislative frame in terms of approval and labelling. For this reason, appropriate detection methods for the presence of GMOs in different samples are needed. The analytical tools used for GMO quantification have to be specific, sensitive and reliable. Different approaches are used for qualitative and quantitative PCR analysis (10). Detection methods for GMOs approved in Europe are available from the Community Reference Laboratory (CRL) (http://gmo-crl.jrc.it/statusofdoss.htm). However, the majority of the worldwide cultivated transgenic crops is not approved in the EU. The consumer protection demands that unapproved GMOs do not enter the EU market. Efforts should therefore also be focused on the development of methods for the detection of non-approved GMOs like Papaya, Bt10 maize or LL601 rice which were detected by European food control authorities in food samples (2, 13, 17).

An artificial gene construct in transgenic plants is basically composed of several elements like a promoter, the newly introduced gene and a terminator. Usually the target sequence for screening and/or detection of a GMO is a part of a modified gene construct (e.g. promoter, gene, terminator) or the junction region between the inserted gene and the host genome. According to the target sequences there are four categories of PCR with increasing specificity: screening methods, gene-specific methods, construct-specific and event-specific methods (10). Most of the commercially cultivated transgenic plants were transformed with constructs containing the Cauliflower Mosaic Virus (CaMV) 35S promoter (P-35S) and/or a 35S terminator (T-35S) from CaMV or a nopaline synthase terminator (T-Nos) from *Agrobacterium sp.* (1).

The non-authorised transgenic Chinese Bt-rice line Kemingdao1 (KMD1) investigated in this study is currently tested in large field tests in China (16, 18). In order to establish a screening method for the detection of this Bt-rice variety, we developed a sensitive and reliable duplex PCR system for the simultaneous amplification of the *cryIA(b)* gene, which is used in several transgenic plants for insect resistance, and the maize ubiquitin promoter, by which the *cryIA(b)* gene in KMD1 is driven (6, 19). This method proved to be suitable also for the detection of Bt11 maize.

Materials and Methods

Plant material. Seeds of the transgenic rice line Kemingdao1 (KMD1) and the non-modified isogenic line Xiushui 11 (X11) were kindly provided by Prof. Dr. Qingyao Shu (Zhejiang University, Hangzhou, China). KMD1 was derived from the commercial japonica rice variety Xiushui 11 (X11) by *Agrobacterium* transformation. The gene construct contains a synthetic *cryIA(b)* gene from *Bacillus thuringiensis* under the control of a maize ubiquitin promoter, the regulatory elements P-35S from CaMV and T-nos from *Agrobacterium tumefaciens* and the selection marker genes nptII (neomycin phosphotransferase gene) and hpt (hygromycin phosphotransferase gene) linked in tandem. KMD1 is homozygous for the transgene and showes one single insertion of the gene construct (8, 13, 16).

TABLE 1

Primers for the detection of the *cryIA(b)* gene and the maize ubiquitin promoter in KMD1 and Bt11

Primer	Primer sequence 5'-3'	Length of amplicon (bp)	Source
cry1A(b)2_for	CTT GGT GGA GAA CGC ATT GAA	74	This work
cry1A(b)2_rev	CTG AGC AGA AAC TGT GTC AAG GA		
Pubi_for	CTC TAG ATC GGC GTT CCG GT	140	This work
Pubi_rev	TCA GAA CGT GTC TGA CGT ACA GG		

In order to determine the applicability of the method, DNA reference materials from other transgenic and non-transgenic plant species (Fluka and CRL) were also tested (**Table 2**).

Extraction of genomic DNA. Rice grains from 100% transgenic line KMD1 were mechanically disrupted with sterile pestle and mortar. DNA was extracted with the Wizard Kit (Promega) according to the manufactorer´s protocol. Purity and quantity of the extracted DNA were measured by UV-Spectrophotometer (Ultrospec 3000, Pharmacia Biotech) and PicoGreen®-Analysis (Tecan, Genios Plus).

Polymerase chain reaction. The samples were first screened by PCR for the *cryIA(b)* gene and the maize ubiquitin promoter in separate reactions. The PCR primers for the screening are listed in **Table 1**. Primers were designed using FastPCR 4.0 (Institute of Biotechnology, University of Helsinki, Finland). The design of the *cryIA(b)* primers is based on a sequences cited in (8). The primers for the amplification of the maize ubiquitin promoter are based on gene bank sequences (Accession numbers >EM_PL:U29159 and CS543085). PCR was performed in a total volume of 25 µl with 12.5 µl HotStarTaq MasterMix (Qiagen), 0.4 µM of each primer and 50 ng DNA according to the following temperature programme: heat activation of polymerase 15 min at 95 °C; 40 cycles with 30 sec at 95 °C, 30 sec at 60 °C, 45 sec at 72 °C, and a final elongation with 3 min at 72 °C. The UV visualisation of the PCR products was performed on a 2 % Agarose gel after 20 min staining with Ethidium bromide.

The reactions of the duplex PCR were performed in a final volume of 25 µl, using 0.625 units GoTaq Polymerase (Promega), 5 µl 5x Green GoTaq Reaction Buffer (Promega), 0.5 µl dNTP (10 mM each; Eppendorf), 0.4 µM of the Pubi primers, 0.32 µM of the cry1A(b)2 primers, and 2 µl of template DNA corresponding to 20 ng DNA with the same cycling parameters as the simplex PCR.

Results and Discussion

In this study, we established a fast and efficient screening method for two elements of a gene construct present in the non-authorised transgenic Chinese Bt rice line KMD1 and in Bt11 maize.

The transgenic *cryIA(b)* gene and its promoter, the maize ubiquitin promoter, can simultaneously be amplified by duplex PCR. In samples of KMD1 and Bt11, the cryIA(b)2 and the Pubi primers revealed PCR fragments of 74 bp and 140 bp in length, respectively. The conventional isogenic rice line X11 showed no amplification signal of the *cryIA(b)* gene as expected. The Pubi primers amplify a fragment of the maize ubiquitin promoter which is naturally present in all maize varieties, but was also used in the transformation of the GM rice line KMD1. Therefore the fragment of the maize ubiquitin promoter could be amplified in all tested transgenic and non-transgenic maize varieties as well as with the rice line KMD1. Other transgenic plant species showed no amplification signal of the maize ubiquitin promoter. Apart from KMD1 and Bt11, also the insect resistant Bt63 rice and the Bt cotton variety MON-15985-7 showed a signal of the *cryIA(b)* gene (**Table 2**). This result is in accordance with the available information about the genetic constructs (1, 9). Although other transgenic plants like the maize lines Bt176, MON810 or MON863 also contain a synthetic gene for the expression of the *Bacillus thuringiensis* d-endotoxin, the cryIA(b)2 primers did not reveal a PCR signal. For the transformation of insect resistant plants a number of different *cry* genes or truncated sequences are used so that varying PCR primers are necessary for the detection of these variants (4).

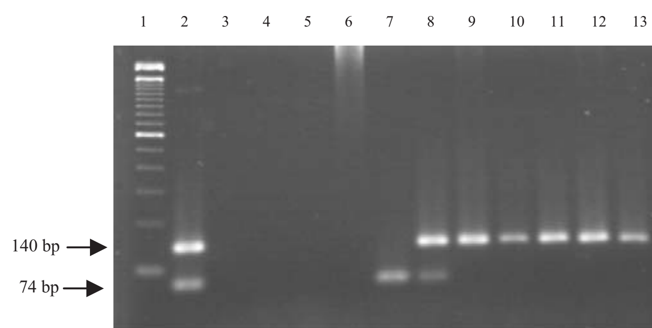

Fig. 1. Duplex PCR with *cryIa(b)2* and Pubi primers with different rice and maize samples

(**1**) 100 bp ladder (Invitrogen), (**2**) KMD1, (**3**) X11, (**4**) LL62; (**5**) LL601, (**6**) rice negative control (CRL), (**7**) Bt63 rice, (**8**) Bt11, (**9**) Bt176, (**10**) MON810, (**11**) MON863, (**12**) Bt11 (0%; CRL); (**13**) maize negative control (CRL). Only the samples of KMD1, Bt63 and Bt11 showed a PCR amplification of the *cryIA(b)* gene (74 bp). All maize varieties and KMD1 were positive for the maize ubiquitin promoter (140 bp)

TABLE 2

Amplification of the cryIA(b) gene and the maize ubiquitin promoter in different plant species by duplex PCR

Plant species	Variety/event	cryIA(b)	maize ubiquitin promoter
Rice	KMD1	+	+
	X11	-	-
	LL601	-	-
	LL62	-	-
	Bt63	+	-
Maize (GMO)	Bt11	+	+
	Bt176	-	+
	MON810	-	+
	MON863	-	+
	LY038	-	+
	NK603	-	+
	MIR604	-	+
Maize (non-GMO)	Ronaldinio	-	+
	Gavott	-	+
	Saludo	-	+
	Sileno	-	+
	Amadeo	-	+
	Early Star	-	+
	Moncada	-	+
	Birko	-	+
Soybean	A2704-12	-	-
Oilseed rape	GS40/90	-	-
	Liberator	-	-
	MS8 x RF3	-	-
Sugarbeet	#77	-	-
	H7-1	-	-
	T120-7	-	-
Cotton	1304	-	-
	MON 15985	+	-
Papaya	P1 (Hapaya)	-	-

Table 2. Different plant species tested for the cryIA(b) gene and the maize ubiquitin promoter by duplex PCR. All transgenic and non-transgenic maize samples contain the maize ubiquitin promoter. The amplified sequence of the *cryIA(b)* gene is present in KMD1, Bt63, Bt11, and the two cotton lines 15985 and 1445.

We are presenting a method for a parallel detection of the *cryIA(b)* gene and the maize ubiquitin promoter in the transgenic rice line KMD1. Despite the differences in the transgenic gene construct, also the transgenic Bt11 maize can be detected by this screening method. A clear differentiation of these two lines in case of a positive screening result can be achieved by a subsequent PCR using primers for rice specific (e.g. *gos*9 and *SPS* (5;7)) or maize specific genes (e.g. *Adh*1 and *ivr*1) or the event-specific detection system of Bt11 (http://gmo-crl.jrc.it/statusofdoss.htm). Due to the small size of the resulting PCR fragments (74 bp and 140 bp), traces of KMD1 and Bt11 also can be detected in processed food and feed stuff, where DNA often is strongly fragmented. The sensitivity of the detection method was proven by serial dilutions of the 100% transgenic KMD1 DNA. Positive results were still obtained with DNA dilutions corresponding to 5 copies of the transgene (**Fig. 2**).

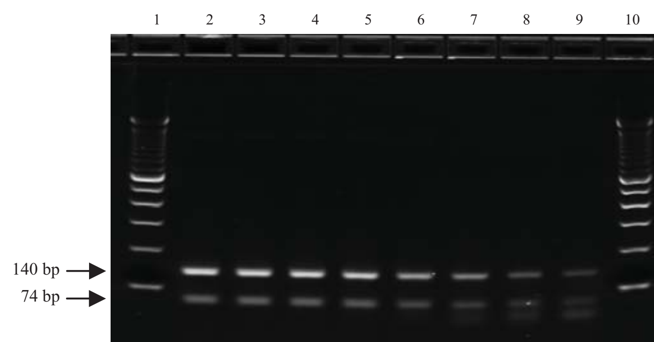

Fig. 2. Sensitivity test of the duplex PCR with serial dilutions of KMD1 containing 50.000 to 5 copies of the transgenic construct

(**1**) 100 bp ladder (Invitrogen), (**2**) 50.000 copies, (**3**) 5.000 copies, (**4**) 1.000 copies, (**5**) 500 copies, (**6**) 50 copies, (**7**) 25 copies, (**8**) 10 copies; (**9**) 5 copies, (**10**) 100 bp ladder (Invitrogen)

Conclusions

The new duplex PCR system presented in this study is based on the simultaneous detection of the *cryIA(b)* gene and the maize ubiquitin promoter. The high specificity and sensitivity of this method indicate that it is a fast and effective screening method for unauthorised KMD1 and Bt11 maize samples in EU food and feed imports. The maize ubiquitin promoter is well suited for rice transformation as it directs high levels of gene expression in rice (3). As an increasing number of insect resistant Bt rice plants driven by the maize ubiquitin promoter is transformed (12, 14) and a contamination of imported rice batches with respective rice grains can not be ruled out, a screening for the *cry* gene and the maize ubiquitin promoter might gain importance in order to detect a greater number of different and potentially unknown GMOs.

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