J. Verbr. Lebensm. (2010) 5:185–188
DOI 10.1007/s00003-010-0573-3

Journal für Verbraucherschutz und Lebensmittelsicherheit
Journal of Consumer Protection and Food Safety

GENERELLE ASPEKTE

A testing cascade for the detection of genetically modified rice by real-time PCR in food and its application for detection of an unauthorized rice line similar to KeFeng6

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Eingegangen: 3 February 2010 / Angenommen: 3 February 2010 / Online publiziert: 4 March 2010

Programs for the development of genetically modified (GM) rice as the main staple food for a large segment of the world population are presently ongoing in several parts of the world (Cao et al. 1992; Ignacimuthu et al. 2000; Ye et al. 2000; Datta 2004; Zhang 2007). Although GM rice varieties are already authorized in some countries, no large scale cultivation is reported presently. In the United States, the rice lines LLRICE62 and LLRICE601 are deregulated. There are also reports that a local variety of Bt rice is cultivated on a smaller scale in Iran (James 2005; Sawahel 2005) but this cannot be verified.

In China, GM rice events with insect resistance, disease resistance and quality improvement have been approved for field testing (Wang and Johnston 2007a, b). The final stage of these field tests are production trials which might cover an area up to 13 ha with an isolation distance of 100 m (Anonymus 2009). At present, several GM rice lines are in the process for authorisation for commercial planting (Anonymus 2009; Wang and Johnston 2007a, b; Qiu 2008).

In rice noodles originating from China a GM rice line containing a construct derived from plasmid TT51-1 was detected (Mäde et al. 2006; Akiyama et al. 2007). Chinese authorities traced this rice back to illegal propagation of GM seeds (Anonymus 2009). This GM rice line was called 'Bt 63'. Since 2006 several rice products originating from China were found to contain traces of a DNA construct coding for the δ-endotoxin of *Bacillus thuringiensis* (Bt). These findings have been notified to the European Rapid Alert System for Food and Feed (RASFF) (Commission Decision of 3 April 2008).

Scientific reports (Akiyama et al. 2007), notifications in the RASFF (Anonymus 2009) and analytical results in our laboratories indicated the presence of other GM rice lines in addition to 'Bt 63'. This is traced back to the presence of the CaMV-35S promoter which was not used in 'Bt 63' (Mäde et al. 2006). Akiyama et al. (2007) assumed that the 35S-signals originate from the presence of a GM rice line called 'Kemingdao'. However, they could not verify this due to the lack of authentic reference material.

"Kemingdao" was intensively field trialed (Wu et al. 2003; Huang et al. 2008). Detection methods for GM rice line 'Kemingdao' ('KMD1') expressing the *cry*IA(b) gene driven by a maize ubiquitin promoter were published recently (Babekova et al. 2008, 2009). This line is a progeny of the commercial *japonica* rice variety Xiushui 11 genetically transformed with a *cry*IA(b) gene using the binary vector pKUB (Cheng et al. 1998; Wu et al. 2002). The genetic elements of 'KMD1' are described, but little information is available on other GM rice lines.

Reports of a GM rice line containing two genes coding for insect resistance, a Bt gene under the control of the maize ubiquitin promoter and the *CpTI*

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gene (cow pea trypsin inhibitor) under the control of the rice actin promoter in combination with the hygromycin phosphotransferase (*hpt*) gene under control of the *CaMV-35S* promoter as selectable marker were published recently (Rong et al. 2005, 2007). This GM line was designated as 'KeFeng6'.

At present, the analysis of rice and rice products for GM rice is done by the combination of screening methods followed by construct specific methods for further characterisation, and, if available, by event specific detection systems. The combination of screening and construct specific methods as described by Waiblinger et al. (2009) is a comprehensive tool for the detection of authorized and unauthorized GMO. With regard to GM rice, this approach can be extended by addition of construct specific real-time PCR systems as listed in Table 1.

In routine analysis of rice products originating from China, the DNA is extracted using a modified CTAB method as described previously (Mäde et al. 2006) in at least two replicates. To achieve sufficient sensitivity it appears to be important that DNA is added in quantities per reaction which results in C_q values of 22 or below for the rice specific *gos9* system (Hernandez et al. 2005). The target taxon specific real-time PCR is followed by screening for the *CaMV-35S* promoter and the NOS terminator as described in ISO 21570 (2005). Samples with positive screening results require further characterisation. Thus, NOS terminator positive samples are analysed by applying the construct specific method targeting GM rice lines transformed with the plasmid TT51-1 ('Bt 63') as published previously (Mäde et al. 2006). In *CaMV-35S* promoter positive samples, detection systems targeting the GM rice lines LLRICE62, and LLRICE601 are applied (Official Collection of Test Methods 2008; Anonymus 2006a, b) although it is unlikely that these GM lines are present in rice products originating from China. The two GM rice lines 'KMD1' and 'KeFeng6' contain a ubiquitin-promoter driven *cryI*(A)b gene and a *CaMV-35S*- promoter driven *hpt* gene. Thus detection systems of these constructs have to be applied for the analysis 35S-positive samples.

We found three samples in 2008 and 2009 harbouring the *pubi-cry* and *CaMV-35S-hpt* overlapping sequences in addition to the presence of 'Bt 63'. These samples were negative for 'KMD1' using an event-specific real-time PCR method (Babekova et al. 2009). Sequence comparisons of control material from 'KMD1' [kindly provided by Dr. U. Busch, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit] with DNA sequences from these samples showed that there are significant differences in the DNA sequence of the *CaMV-35S-hpt* construct. The conclusion that the samples in question do not contain 'KMD1' was additionally supported by applying a construct specific system targeting overlapping sequences from the *CpTI* gene to the NOS terminator as described for 'KeFeng6'. The positive PCR results with this construct specific system strongly suggests the presence of the GM rice line 'KeFeng6' in these samples. The samples were also tested with the real-time PCR system published by Akiyama et al. (2007) which amplifies an overlapping sequence between *cryIA*(c) sequences and the NOS terminator. This region is different from one present in 'Bt 63' but it shows that the rice line in question does contain a similar hybrid Bt gene consisting of a *cryIA8(b)* 5′-part and a *cryIA(c)* 3′-part. The GM rice line detected by Akiyama et al. (2007) in addition to 'Bt 63' was therefore most likely 'KeFeng6' as well.

Table 1 The oligonucleotides for the construct specific detection systems of genetically modified rice

Construct	Name	Sequence (5′–3′)	Final concentration (nmol/L)
pubi-cry	pubi-F2	ATT TgC TTg gTA CTg TTT CTT TTg TC	300
	cry-rr-R	5′-TTG TTG TCC ATG GAT CCT CTA GAG T-3′	600
	pubi-T	FAM-CTC ACC CTg TTg TTT ggT gTT ACT TCT gCA-BBQ	100
35S-hpt	35S166-F	gCT gAA ATC ACC AgT CTC TCT CTA CA	900
	hpt166-R	TgA gTT CAg gCT TTT TCA TAT CTC AT	600
	35S166-Tm	YAK-TAT CTC TCT CgA gCT TTC gCA gAT CCg g-BBQ	100
cpti-nos	cptiF2	CAA gTC CAg ggA TgA AgA TgA TgA g	900
	tNOS-uni-R	gCT TAA CgT AAT TCA ACA gAA ATT ATA TgA TAA T	600
	tNOS-uni-Tm	FAM-TCg CAA gAC Cgg CAA CAg gAT TC-BBQ	100

The construct pubi-cry is present in both GM rice lines 'KMD1' and KeFeng6, whereas with 35S-hpt and cpti-nos only sequences as described in KeFeng6 are amplified

The identification work and method development described here resulted in a testing cascade for the detection of GM rice as shown in Fig. 1.

These methods were recently applied to routine samples collected from retail shops and wholesalers during official market control in Saxony-Anhalt and Hessen in late 2009. Four of 20 rice noodle samples originating from China were positive for the constructs *pubi-cry*, 35S-*hpt* and *CpTI*-NOS and were verified with the *cryI*A(c)-NOS system according to Akiyama et al. (2007). It is important to note that 'Bt 63' was not detected in these four samples whereas in the reports by Akiyama et al. (2009) and in the samples taken from the market in 2008 and early 2009 'Bt 63' and the new line similar to KeFeng6 were both present.

Due to the lack of reference material the performance characteristics of the newly developed construct specific detection methods cannot be determined and are thus not available yet. This will be done as soon as such material can be obtained and method performance data will be published in a subsequent communication.

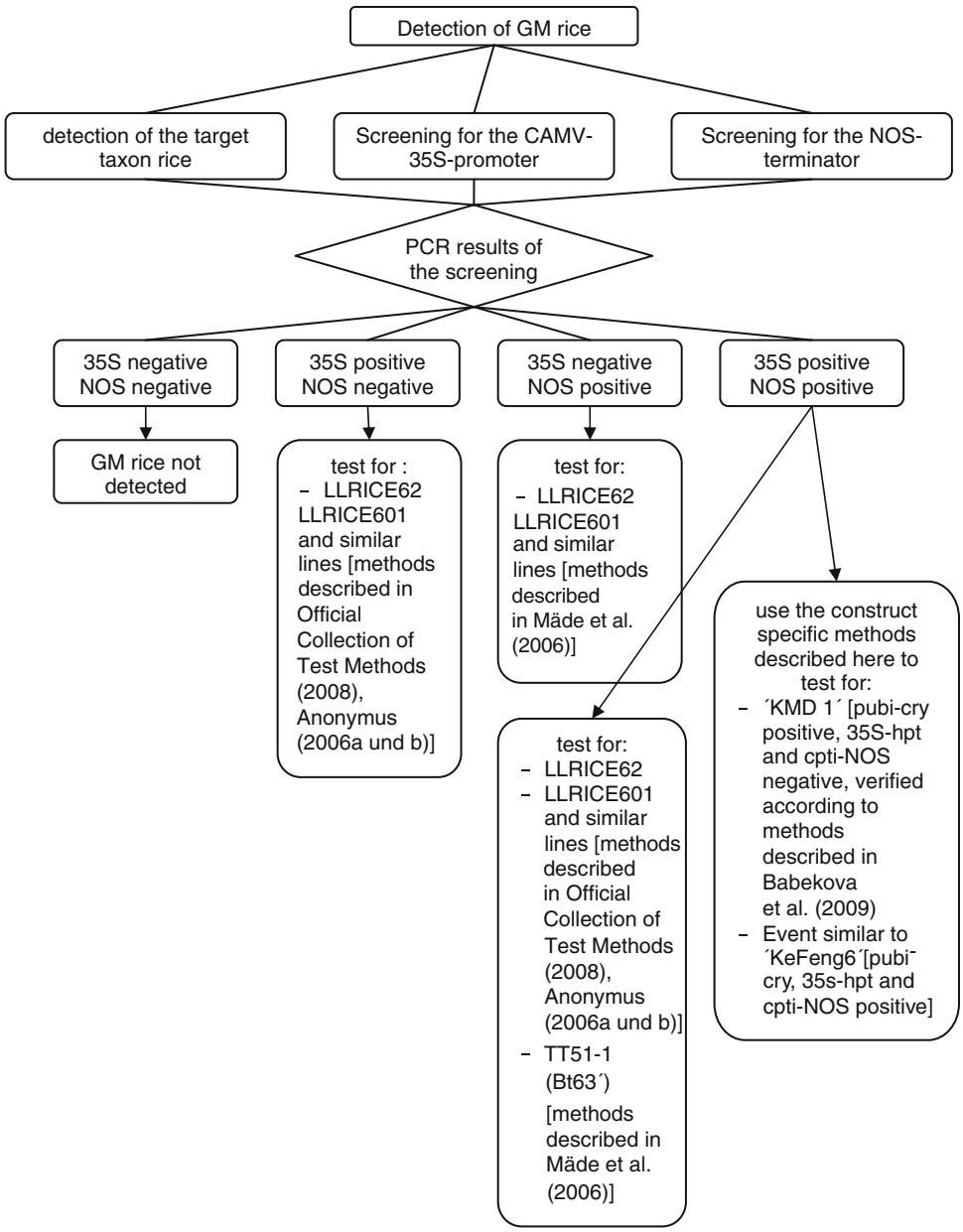

Fig. 1 Testing cascade for the detection of genetically modified rice. Detection systems targeting the several GM rice lines are described in the text

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