J. Verbr. Lebensm. (2012) 7:63–70
DOI 10.1007/s00003-011-0748-6

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

METHODS

Development of an event-specific detection method for genetically modified rice Kefeng 6 by quantitative real-time PCR

Patrick Guertler · Ingrid Huber · Sven Pecoraro · Ulrich Busch

Received: 10 February 2011 / Accepted: 12 October 2011 / Published online: 6 December 2011

Abstract The genetically modified (GM) rice Kefeng 6 has gained resistance against several rice pests by inserting the *cpti* and *cry1Ac* genes. As this transgenic line is not approved for import, processing and cultivation in the European Union (EU), sensitive and specific detection methods need to be available to monitor any illegal presence of Kefeng 6 in food products within the EU. The aim of this study was to develop and validate an event-specific detection method by means of quantitative real-time PCR (qPCR) for the detection of Kefeng 6 in foodstuff. A primer pair and hydrolysis probe were designed according to the right border junction sequence of the transgene. The qPCR assay was validated according to the ENGL/EURL-GMFF guidelines for GMO testing and is presented according to the MIQE guidelines. The in-house validation process resulted in a limit of detection of 5 DNA copies of the transgene with confidence intervals (95 %) between 0.07 and 0.52, a PCR efficiency of 105 % and a correlation coefficient (R^2) value of 0.9997. The specificity of the assay was tested by end-point PCR, gel electrophoresis and subsequent sequencing of the PCR products. By testing DNA of several GM and non-GM crops, cross reactivity of the assay was not observed. Further, 35 food products were analyzed for the presence of Kefeng 6 by means of the event-specific detection method. For 9 out of 35 samples, PCR products for Kefeng 6 DNA were observed.

Keywords Genetically modified rice · Quantitative real-time PCR · Kefeng 6 · Event-specific · Validation

P. Guertler (✉) · I. Huber · S. Pecoraro · U. Busch
Bavarian Health and Food Safety Authority, Veterinärstr. 2, 85764 Oberschleißheim, Germany
e-mail: patrick.guertler@lgl.bayern.de

1 Introduction

In 2010, genetically modified (GM) plants were cultivated by 15.4 million farmers on estimated 148 million hectares worldwide (James 2010) with soybean, maize, rapeseed and cotton being the most prominent GM crops. Nevertheless, rice (*Oryza sativa*) is the most important food crop, especially in developing countries. Rice yields in China have increased since the 1980s, but the growth rate of the rice-consuming population is increasing faster than the rice yields. Furthermore, significant rice yield losses from 24 to 41 % per year are caused by rice insect pests like the yellow stem borer (*Scirpophaga incertulas*), the Asiatic stem borer (*Chilo suppressalis*) or the rice leaffolder (*Cnaphalocrocis medinalis*) (Savary et al. 2000; Chen et al. 2011; Ye et al. 2001). Therefore, insect resistance (Saha et al. 2006; Ye et al. 2001) as well as herbicide tolerance (Datta et al. 1992; Xiao 2009) or nutritional alteration (Ye et al. 2000; Paine et al. 2005) have been incorporated into rice plants by genetic modification in order to enhance rice yield and plant protection. Insect resistance conferred by *cry* genes from the ubiquitous *Bacillus thuringiensis* (Bt) is the most important trait in GM rice. However, only three GM rice variants (LL06, LL62, LL601) are approved for commercial cultivation in the USA (CERA 2010) so far. In China, the world's major rice producer, biosafety certificates for commercial production of the Bt rice lines Huahui No. 1 and Shanyou 63 were issued by China's Ministry of Agriculture in 2009 (Chen et al. 2011). Furthermore, several GM rice variants have been tested in field and environmental release trials in China. In addition, two Bt rice lines, Bt63 and Kemingdao, entered pre-production trials

in 2001 (Tu et al. 2000; Pray et al. 2006; Babekova et al. 2009). Pre-production trials were also performed using the Bt rice named Kefeng 6. In Kefeng 6, three gene expression cassettes were incorporated by biolistic transformation. The *cpti* (*cow pea trypsin inhibitor*) gene controlled by a rice actin promoter as well as the *cry1Ac* gene controlled by a maize ubiquitin promotor and the *hygromycin phosphotransferase* (*hpt*) gene were transferred into this GM rice line resulting in broad insect resistance. Hygromycin resistance is used as selective marker (Chen et al. 2006; Rong et al. 2005).

However, illegal cultivation and trade of GM rice in China was reported in 2005 (Zi 2005), which might have led to the finding of Kefeng 6 in food samples in the Netherlands in 2010 (rapid alert system for food and feed, RASFF No. 2010.0336). In the EU, Kefeng 6 is not approved for import, processing and cultivation or its use as food and feed. In conclusion, traces of Kefeng 6 in food- and feedstuff are considered as illegal (EC 2003a, b). Thus, there is a need for sensitive and reliable detection methods in order to monitor the potential presence of Kefeng 6 in products imported to the European market. Reiting et al. (2010) reported a construct-specific detection method for this GM rice line. However, in case of transformation events derived from the same insert, it cannot be distinguished between these events by using construct-specific detection methods. Only an event-specific detection method can clearly define the event being processed in food products.

The aim of this study was to develop and in-house validate an event-specific detection assay for the GM rice Kefeng 6 according to guidelines for GMO testing and assay validation (ENGL 2009). The presentation of the data shall follow the instructions outlined in the MIQE guidelines (Bustin et al. 2009). The MIQE guidelines are a compendium that gives an overview on essential information that should be provided when publishing a PCR assay. This should enable other working groups to relate to this method and to compare it with similar methods.

Further, this novel assay was tested for its use in the official surveillance for the potential presence of DNA from Kefeng 6 in several routine samples.

2 Materials and methods

2.1 Sample materials

For the development and in-house validation of the event-specific detection assay for Kefeng 6 rice, reference material was provided by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF, Italy).

Reference material provided by IRMM (MON810), Fluka (DAS-59122, GA21, MON89788, NK603, non-transgenic maize, non-transgenic soybean), ERM (GTS 40-3-2), AOCS (LL62), ENGL (LL601, non-transgenic rice) and the Bavarian Health and Food Safety Authority (GT73, non-transgenic rapeseed, KMD1, Bt63) were used to ensure the specificity of the developed assay.

2.2 DNA extraction and pre-amplification

For the assay development, a pre-amplification step with Kefeng 6 reference material was performed applying the GenomiPhi DNA Amplification Kit (Amersham Biosciences, Germany) according to the manufacturer's instructions. This step was carried out due to the limited availability of the reference material for Kefeng 6. The assay validation was performed using Kefeng 6 reference material.

The CTAB-extraction method was applied for the extraction of DNA from reference material of several GM plants. Briefly, 200 mg of homogenized sample material was weighed, transferred in a 2 mL tube and 1,000 μL CTAB buffer (2 % CTAB, 1.4 M NaCl, 0.1 M Tris, 20 mM EDTA, pH 8.0) was added. Subsequently, 20 μL RNase A (10 mg/mL) was added and the sample was vortexed and incubated for 30 min at 65 °C. Afterwards, 20 μL Proteinase K (20 mg/mL) was added and vortexed. The mixture was incubated for at least three hours at 65 °C. Then a centrifugation step was performed at 14,500×*g* for 10 min. The clear upper phase was transferred into a new tube and a CTAB precipitation buffer (0.5 % CTAB, 40 mM NaCl, 50 mM Tris, pH 8.0) was added in a volume of two times the supernatant, followed by an incubation step of 60 min at room temperature. After centrifugation at 14,500×*g* for 5 min, the supernatant was discarded and the pellet dissolved in 350 μL of 1.5 M NaCl solution. Then 350 μL chloroform was added and the sample was mixed for 30 s, followed by a centrifugation step at 14,500×*g* for 10 min. The upper aqueous phase was transferred into a new tube. 2 μL glycogen (20 mg/mL) and one volume of 2-propanol (pre-cooled at −20 °C) were added and mixed. Subsequent centrifugation at 17,000×*g* for 15 min resulted in a pellet and the supernatant was discarded. The pellet was washed using EtOH (70 %, pre-cooled at −20 °C), followed by centrifugation at 17,000×*g* for 5 min. The supernatant was discarded and the pellet was dried at room temperature for

20 min. By adding 100 μL elution buffer (1 M Tris–HCl, pH 9.0, pre-warmed at 65 °C), the pellet was dissolved.

For food samples, obtained on the local market, the extraction method was up-scaled for a sample material of 2 g.

DNA concentrations were measured using the Quant-iT PicoGreen technology according to the manufacturer's instructions (Invitrogen, USA). Extracted DNA samples were either stored at −20 °C or directly used for analysis.

2.3 End-point PCR

A primer pair (Kef6 forward: 5′-TGGATCAGATTGTC GTTTCCCGCCTT-3′, Kef6 reverse: 5′-CAAGAAGCAAG CTGAGGCAAACAAGCT-3′, synthesized by Metabion, Germany) was designed based on the Kefeng 6 right border junction sequence [Accession number HM124448 (Wang et al. 2011)] of the insert using the NCBI primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Optimal primer conditions were checked by means of an end-point temperature gradient PCR.

The qualitative end-point PCR was performed using 20 ng template DNA, amplifying a 128 bp fragment, which spans the crossing from the recombinant DNA insert and the genomic rice DNA at the right border (3′ junction) as shown in Fig. 1. The PCR master mix contained 1× HotStarTaq Master Mix (Qiagen GmbH, Germany) and 0.5 μM of each primer. PCR grade water was added to a final volume of 25 μL. The following cycling conditions were used: initial enzyme activation at 95 °C for 15 min, denaturation at 95 °C for 45 s, annealing at variable temperatures (54, 57, 60 and 63 °C) for 45 s, extension at 72 °C for 45 s and final extension at 72 °C for 5 min. 40 PCR cycles were performed. PCR products were separated using gel electrophoresis and visualized by ethidium bromide staining. PCR products were subsequently sequenced using a 3130 Genetic Analyzer (Applied Biosystems, USA) in order to assess the specificity of the assay (BigDye Terminator Sequencing Kit, Applied Biosystems, USA).

2.4 Quantitative real-time PCR

QPCR was performed using the TaqMan technology (ABI 7900HT, Applied Biosystems, USA). The qPCR master mix consisted of 0.2 μM forward primer (Kef6 forward) and reverse primer (Kef6 reverse), 0.2 μM of the hydrolysis probe (Kef6 hydrolysis probe: 5′-FAM-AGGGCGATTGCTGGCGAGGC-TAMRA-3′, synthesized by Metabion, Germany), 1× Applied Biosystems Universal MasterMix and 5 μL template DNA. PCR grade water was added to a final volume of 25 μL. The following cycling conditions were used: 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. DNA from Kefeng 6 served as a positive control, whereas water was used as a non-template control (NTC).

The assay was in-house validated according to the "Minimum Performance Requirements for Analytical methods for GMO Testing" (ENGL 2009) and results are presented as outlined in the MIQE guidelines. DNA from Kefeng 6 was applied in a serial dilution for determining the standard deviation (SD), the confidence intervals $\left(CI : \bar{x} \pm 1.96 \frac{SD}{\sqrt{n}}\right)$ and the limit of detection (LOD) of the qPCR assay. The LOD is defined as the lowest copy number of the target that can be reliably detected (ISO24276:2006 2006). Copy numbers between 1 and 10,000, which were calculated according to the haploid genome size of 430 Mbp for rice (Arumuganathan and Earle 1991), were applied in 12 PCR replicates. According to these calculations, 0.47 pg genomic DNA from Kefeng 6

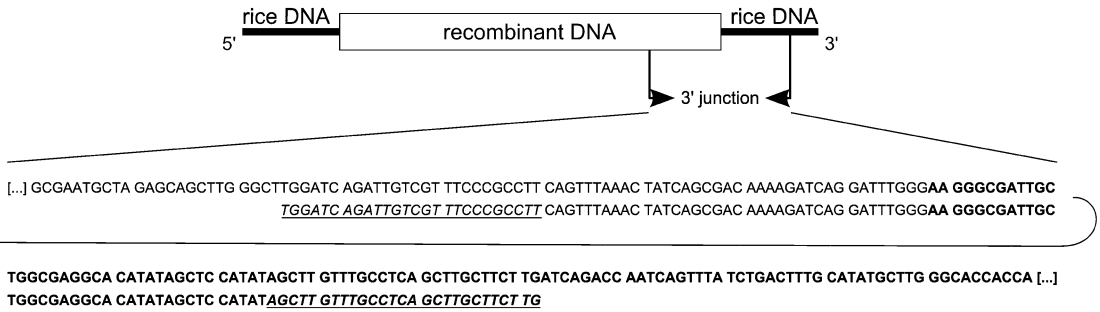

Fig. 1 Primer positions (*italic and underlined*) and amplicon sequence of the event-specific qPCR assay for the detection of the genetically modified Kefeng 6 rice. The upper sequence shows the 3′ junction sequence of the inserted DNA in Kefeng 6 as reported by Wang et al. (2011). *Normal uppercase* indicates the recombinant DNA, whereas *bold uppercase* indicates the genomic rice DNA

corresponds to one haploid rice genome (Wu et al. 2010).

Further, the cross reactivity of the developed assay was checked by amplification of DNA isolated from GM plant material (maize: DAS-59122, GA21, MON810, NK603; rapeseed: GT73; soybean: MON89788, GTS 40-3-2; rice: LL601, LL62; all 1 % GMO content; rice: Bt63, KMD1, 0.1 % GMO content) and non-transgenic plants (maize, rapeseed, rice; all 0 % GMO content). In order to illustrate the robustness of the assay, the same dilution series as used for the determination of the SD and the CI was analysed by qPCR applying different cyclers (LightCycler 480, Roche Applied Science, Mannheim, Germany and Agilent Mx3000P, Agilent Technologies, Waldbronn, Germany). The same cycling conditions as described above were used for the LightCycer 480 and the Agilent Mx3000P. On the LightCycer 480, data were assessed by applying the 2nd derivative maximum method. All data are shown as C_q values (C_q = cycle of quantification) (Bustin et al. 2009).

In order to compare this new assay with established construct-specific detection assays, the following constructs were amplified as outlined by Reiting et al. (2010): *P35S-hpt*, *pubi-cry* and *cpti-nos*.

2.5 Surveillance of food and feed for DNA from Kefeng 6

The suitability of the new qPCR assay for the monitoring of DNA from Kefeng 6 in food and feed was tested by analysing 35 rice samples (analysed in duplicates) from the market. These samples consisted of rice flakes, rice flour, rice cakes, rice noodles, grain rice and infant cereals containing rice. Further, rice samples were kindly provided by the Hessen State Laboratory (Kassel, Germany, Dr. R. Reiting) and the Authority for Social Affairs, Family, Health and Consumer Protection, Institute for Hygiene and the Environment (Hamburg, Germany, Dr. G. Näumann). DNA was extracted as mentioned above. For each sample, two sub-samples were analysed in duplicates.

3 Results

3.1 End-point PCR

The optimal annealing temperature of the new designed primer pair was determined by using an end-point temperature gradient PCR followed by agarose gel electrophoresis. PCR analysis revealed an optimal annealing temperature of 60 °C. Unspecific products were not observed. All PCR products were sequenced and showed no difference from the target sequence published by Wang et al. (2011) (data not shown).

3.2 Quantitative real-time PCR

A serial dilution of Kefeng 6 DNA was analysed by qPCR. The results are illustrated in Table 1. SD and the CI were calculated based on the C_q values. According to the results, a SD between 0.12 and 0.92 and CI (95 %) of 0.07 to 0.52 were determined. The PCR efficiency E was calculated as 105 % by applying the equation $E = 10^{\frac{-1}{slope}} - 1 \times 100\,\%$ (calibration curve: $y = -3.2145x + 38.336$). The correlation coefficient R^2 was 0.9997 (Fig. 2).

DNA samples from several GM crops were analysed to assess the cross reactivity of the developed Kefeng 6 assay. Specific amplification was not observed for any GM as well as for non-GM crops (0 % maize, rapeseed, maize, and soybean) as shown in Table 2. A positive and specific result was obtained for DNA from Kefeng 6 which served as a positive control.

Table 1 Standard deviations and confidence intervals of the real-time PCR assay using different DNA copy numbers

Number of replicates	Number of positive reactions	Number of copies (nominal)	Median C_q values	Standard deviation	Confidence interval (95 %)
12	12	10,000	26.59	0.44	0.25
12	12	5,000	27.47	0.12	0.07
12	12	1,000	29.71	0.19	0.11
12	12	500	30.76	0.29	0.17
12	12	100	32.96	0.46	0.26
12	12	50	33.80	0.34	0.19
12	12	10	36.25	0.92	0.52
12	12	5	37.16	0.74	0.42
12	5	1	37.65	1.14	1.00

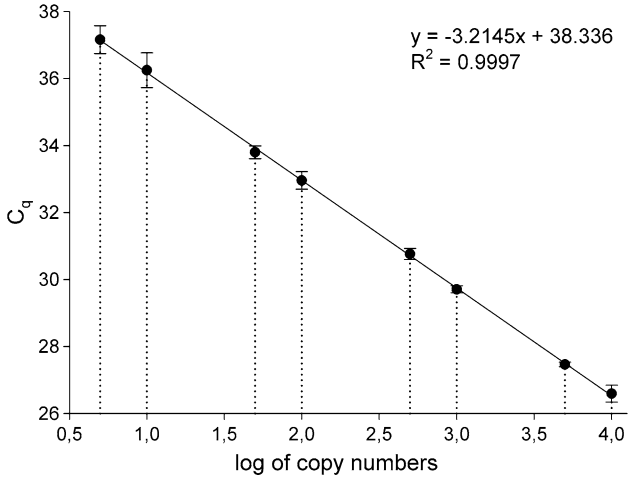

Fig. 2 Calibration curve of the real-time PCR assay using the ABI TaqMan technology (ABI 7900HT). The *error bars* indicate the confidence intervals (95 %) at different log of copy numbers (10,000 to 5)

Table 2 Specificity test of the new qPCR assay

Species	Line/variety	Amplification
Rice	Kefeng 6	+
	LL62	–
	LL601	–
	Bt63	–
	KMD1	–
	Non-GM	–
Maize	MON810	–
	NK603	–
	GA21	–
	DAS-59122	–
	Non-GM	–
Rapeseed	GT73	–
	Non-GM	–
Soybean	MON89788	–
	GTS 40-3-2	–
	Non-GM	–

NTC samples did not result in any amplification signals. All experiments were repeated using two other qPCR systems (LightCycler 480, Agilent Mx3000P) in order to test the assay's robustness. The obtained data show no significant difference between the qPCR systems (Table 3).

When using Kefeng 6 DNA containing as little as 5 copies of the transgene, 12 out of 12 PCR reactions resulted in specific PCR products. Therefore, the LOD of the assay was found to be about 5 copies (Table 1).

To compare the construct-specific detection assay (Reiting et al. 2010) with the newly developed event-specific assay, DNA from Kefeng 6 (0.1 %) was used to amplify a fragment of the right border junction from the transgene to the plant specific DNA as well as fragments of the three following constructs: *35S-hpt*, *pubi-cry* and *cpti-nos*. The qPCR analysis revealed no significant differences between these four assays with respect to the obtained C_q values. A mean C_q value of 34.46 ± 0.61 was obtained for the event-specific assay, whereas the construct-specific assay produced mean C_q values of 34.89 ± 0.36 for *35S-hpt*, 33.20 ± 0.29 for *pubi-cry* and 33.95 ± 0.19 for *cpti-nos* (data are presented as C_q ± standard deviation).

Table 3 Comparison of three different qPCR cyclers

Number of copies (nominal)	Median C_q ± SD ABI 7900HT $n = 16$	Median C_q ± SD Agilent Mx3000P $n = 4$	Median C_q ± SD LightCycler 480 $n = 4$
4,468	28.21 ± 0.19	27.88 ± 0.08	29.27 ± 0.03
2,234	29.36 ± 0.16	28.65 ± 0.06	30.07 ± 0.08
223	32.69 ± 0.37	32.61 ± 0.38	33.56 ± 0.10
45	35.08 ± 0.66	33.90 ± 0.41	35.64 ± 0.39
22	36.61 ± 0.79	35.27 ± 0.59	36.71 ± 0.60

C_q cycle of quantification, *SD* standard deviation

3.3 Surveillance of food and feed for DNA from Kefeng 6

The suitability of the new assay for monitoring the presence of DNA from Kefeng 6 in food products was determined by analysis of 35 samples obtained from the local market. In 9 out of 35 food samples, DNA from Kefeng 6 was detectable (Table 4). All positive samples have been tested positive previously by means of the construct-specific detection assay (Reiting et al. 2010) at the Hessen State Laboratory. DNA from KMD1 and Bt63, as additional negative control, were also analysed and were found negative by applying the event-specific Kefeng 6 assay. Two subsamples for each sample were analysed in duplicates and if analysis of one of these duplicates resulted in an amplification of the event-specific fragment, the sample was considered positive (Waiblinger et al. 2011).

4 Discussion

From our experiences over the past decade, it has been shown that the number of unapproved GM crops found in the EU increases rapidly as a result of

Table 4 Analysis of 35 rice samples by means of the developed event-specific detection assay in duplicates

Rice sample	Description of the sample	Cq_{mean} Kef6	Rice sample	Description of the sample	Cq_{mean} Kef6
RS 13-1	Rice noodles	37.31	RS 64-1	Brown rice	–
RS 13-2	Rice noodles	43.75	RS 64-2	Brown rice	–
RS 27-1	Infant cereal (containing rice)	–	RS 65-1	Infant cereal (containing rice)	–
RS 27-2	Infant cereal (containing rice)	–	RS 65-2	Infant cereal (containing rice)	–
RS 28-1	Basmati rice	–	RS 548-1	Rice noodles	38.30
RS 28-2	Basmati rice	–	RS 548-2	Rice noodles	41.32
RS 29-1	Long grain rice	–	RS 549-1	Rice noodles	39.56
RS 29-2	Long grain rice	–	RS 549-2	Rice noodles	40.19
RS 30-1	Infant cereal (containing rice)	–	RS 556-1	Rice noodles	40.11
RS 30-2	Infant cereal (containing rice)	–	RS 556-2	Rice noodles	38.94
RS 31-1	Infant cereal (containing rice)	–	RS 558-1	Rice noodles	–
RS 31-2	Infant cereal (containing rice)	–	RS 558-2	Rice noodles	–
RS 32-1	Long grain rice	–	RS 563-1	Rice noodles	40.67
RS 32-2	Long grain rice	–	RS 563-2	Rice noodles	41.24
RS 33-1	Rice flour	–	RS 564-1	Rice noodles	–
RS 33-2	Rice flour	–	RS 564-2	Rice noodles	–
RS 34-1	Long grain rice	–	RS 565-1	Rice noodles	37.61
RS 34-2	Long grain rice	–	RS 565-2	Rice noodles	38.89
RS 47-1	Long grain rice	–	RS 566-1	Rice noodles	37.85
RS 47-2	Long grain rice	–	RS 566-2	Rice noodles	37.85
RS 48-1	Long grain rice	–	RS 4781-1	Rice noodles	–
RS 48-2	Long grain rice	–	RS 4781-2	Rice noodles	–
RS 49-1	Rice flour	–	RS 5160-1	Rice noodles	–
RS 49-2	Rice flour	34.96	RS 5160-2	Rice noodles	–
RS 50-1	Jasmin with scented rice	–	RS 5161-1	Rice noodles	–
RS 50-2	Jasmin with scented rice	–	RS 5161-2	Rice noodles	–
RS 51-1	Basmati long grain rice	–	RS 5163-1	Rice cakes	–
RS 51-2	Basmati long grain rice	–	RS 5163-2	Rice cakes	–
RS 52-1	Rice flakes	–	RS 5217-1	Rice noodles	–
RS 52-2	Rice flakes	–	RS 5217-2	Rice noodles	–
RS 53-1	Milk pudding (containing rice)	–	RS 5218-1	Rice noodles	41.02
RS 53-2	Milk pudding (containing rice)	–	RS 5218-2	Rice noodles	–
RS 54-1	Rice flakes	–	RS 5219-1	Rice noodles	–
RS 54-2	Rice flakes	–	RS 5219-2	Rice noodles	–
RS 63-1	Rice noodles	–			
RS 63-2	Rice noodles	–			

C_q cycle of quantification

varying approval processes around the world. In 2004, a GM papaya, which has not been approved in the EU, was detected by the Bavarian Health and Food Safety (Busch et al. 2004). This shows that information on GM crops entering the European market as well as specific and sensitive detection methods need to be available to ensure a proper surveillance of food and feedstuff.

GM rice imported into the EU was screened positive for the presence of DNA from several GM rice lines. Among those GM rice lines was the insect resistant Kefeng 6 rice (Rong et al. 2005). However, the detection of Kefeng 6 DNA in the official food surveillance is still based on a previously published construct-specific detection system (Reiting et al. 2010). Amplification of three fragments from different recombinant DNA constructs need to be performed in order to clearly distinguish Kefeng 6 from other GM rice lines. Therefore, an event-specific detection assay for GM rice Kefeng 6 was developed

and in-house validated for its use in the official food surveillance, resulting in an improvement regarding the specificity, lab work and cost efficiency of the GM food surveillance. Other event-specific detection assays were developed in parallel (Su et al. 2011; Wang et al. 2011). However these assays were developed and optimized for leaves and seed only. The applicability of these assays for the official food surveillance for the presence of GM rice Kefeng 6 in food products was not demonstrated.

Our assay was developed for its use in surveillance of food products and is highly specific for Kefeng 6, as the cross reactivity test revealed no positive amplification signals for any other analysed GM crop. Obtained C_q values were comparable to those of the construct-specific detection system (Reiting et al. 2010) and underlined the suitability of this assay to complement the construct-specific assay in food surveillance. The LOD of 5 or less copies is in the range of other assays used for detecting GMO in foodstuff (Dörries et al. 2010; Guertler et al. 2010; Hernández et al. 2004). The in-house validation process revealed a high PCR efficiency and good robustness of the assay.

By applying this event-specific detection method for analyzing several food and feed samples obtained on the market, the general suitability for the detection of novel DNA from Kefeng 6 in food samples was shown. In 9 out of 35 samples, Kefeng 6 DNA was detected even though the C_q value was high for some samples. Up to now, the evaluation and interpretation of qPCR data is up to the individual laboratory which makes it difficult to compare inter-laboratory data (Waiblinger et al. 2011). The importance of detecting trace amounts is further underlined by a EU regulation (for feed only) adopting a 0.1 percent threshold for low level presence of non-approved GM crops for which an authorisation process is pending or expired (EC 2011).

Our results demonstrate that GM plants, which are not approved in the EU, do enter the European market and that event-specific detection methods need to be available for the official food surveillance authorities in order to detect these non-approved GM plants.

Acknowledgments The authors would like to thank the Bavarian State Ministry of the Environment and Public Health for funding this project. Andrea Harwardt, Krimhilde Posthoff, Ulrike Mulats, Claudia Bujotzek, Melina Mehmedovic, Daniela Sebah, and Karola Grünwald are gratefully acknowledged for their excellent technical work. We thank Dr. Ralf Reiting (Hessen State Laboratory, Kassel, Germany) and Dr. Gabriele Näumann (Authority for Social Affairs, Family, Health and Consumer Protection, Institute for Hygiene and the Environment, Hamburg, Germany) for their co-operation and for providing rice samples.

References

Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:208–218

Babekova R, Funk T, Pecoraro S, Engel K-H, Busch U (2009) Development of an event-specific real-time PCR detection method for the transgenic Bt rice line KMD1. Eur Food Res Technol 228(5):707–716

Busch U, Pecoraro S, Posthoff K, Estendorfer-Rinner S (2004) Erster Nachweis einer gentechnisch veränderten Papaya in Europa - Beanstandung eines in der EU nicht zugelassenen gentechnisch veränderten Organismus. Deut Lebensm Rundsch 100(10):377–380

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55(4):611–622

CERA (2010) GM Crop Database. Center for Environmental Risk Assessment (CERA), ILSI Research Foundation. http://cera-gmc.org/index.php?action=gm_crop_database

Chen M, Shelton A, Ye G-y (2011) Insect-resistant genetically modified rice in china: from research to commercialization. Annu Rev Entomol 56(1):81–101

Chen M, Zhao J-Z, Ye G-Y, Fu Q, Shelton AM (2006) Impact of insect-resistant transgenic rice on target insect pests and non-target arthropods in China. Insect Sci 13(6):409–420

Datta SK, Datta K, Soltanifar N, Donn G, Potrykus I (1992) Herbicide-resistant Indica rice plants from IRRI breeding line IR72 after PEG-mediated transformation of protoplasts. Plant Mol Biol 20(4):619–629

Dörries H–H, Remus I, Grönewald A, Grönewald C, Berghof-Jäger K (2010) Development of a qualitative, multiplex real-time PCR kit for screening of genetically modified organisms (GMOs). Anal Bioanal Chem 396(6):2043–2054

EC (2003a) Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Official Journal of the European Union L 268/1

EC (2003b) Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. Official Journal of the European Union L 268/24

EC (2011) Regulation (EC) No 619/201 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorization of which has expired. Official Journal of the European Union L166 (9)

ENGL (2009) Definition of minimum performance requirements for analytical methods for GMO testing. http://gmo-crljrceceuropaeu/doc/Min_Perf_Requirements_Analytical_methodspdf

Guertler P, Paul V, Steinke K, Wiedemann S, Preißinger W, Albrecht C, Spiekers H, Schwarz FJ, Meyer HHD (2010) Long-term feeding of genetically modified corn (MON810)—fate of

cry1Ab DNA and recombinant protein during the metabolism of the dairy cow. Livest Sci 131(2–3):250–259
Hernández M, Esteve T, Prat S, Pla M (2004) Development of real-time PCR systems based on SYBR® Green I, Amplifluor(TM) and TaqMan® technologies for specific quantitative detection of the transgenic maize event GA21. J Cereal Sci 39(1):99–107
ISO24276:2006 (2006) Foodstuffs—methods of analysis for the detection of genetically modified organisms and derived products—general requirements and definitions. (http://wwwisoorg/iso/catalogue_detail?csnumber=37125)
James C (2010) Global status of commercialized biotech/GM crops: 2010. ISAAA Brief No. 42:ISAAA. Ithaca, NY
Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Wright SY, Hinchliffe E, Adams JL, Silverstone AL, Drake R (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat Biotechnol 23(4):482–487
Pray CE, Ramaswami B, Huang J, Hu R, Bengali P, Zhang H (2006) Costs and enforcement of biosafety regulations in India and China. Int J Technol Glob 2(1–2):137–157
Reiting R, Grohmann L, Mäde D (2010) 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. J Verbrauch Lebensm 5(2):185–188
Rong J, Song Z, Su J, Xia H, Lu B-R, Wang F (2005) Low frequency of transgene flow from *Bt*/*CpTI* rice to its nontransgenic counterparts planted at close spacing. New Phytol 168(3):559–566
Saha P, Majumder P, Dutta I, Ray T, Roy S, Das S (2006) Transgenic rice expressing *Allium sativum* leaf lectin with enhanced resistance against sap-sucking insect pests. Planta 223(6):1329–1343
Savary S, Willocquet L, Elazegui FA, Castilla NP, Teng PS (2000) Rice pest constraints in tropical Asia: quantification of yield losses due to rice pests in a range of production situations. Plant Dis 84(3):357–369. doi:10.1094/PDIS.2000.84.3.357
Su C, Xie J, Wang X, Peng Y (2011) Integrated structure and event-specific real-time detection of transgenic *cry1Ac*/*SCK* rice Kefeng 6. Eur Food Res Technol 232(2):351–359
Tu J, Zhang G, Datta K, Xu C, He Y, Zhang Q, Khush GS, Datta SK (2000) Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* delta-endotoxin. Nat Biotechnol 18(10):1101–1104
Waiblinger H-U, Graf N, Broll H, Grohmann L, Pietsch K (2011) Evaluation of real-time PCR results at the limit of detection. J Verbrauch Lebensm:1–7
Wang W-X, Zhu T-H, Lai F-X, Fu Q (2011) Event-specific qualitative and quantitative detection of transgenic rice Kefeng-6 by characterization of the transgene flanking sequence. Eur Food Res Technol 232(2):237–305
Wu G, Wu Y, Nie S, Zhang L, Xiao L, Cao Y, Lu C (2010) Real-time PCR method for detection of the transgenic rice event TT51-1. Food Chem 119(1):417–422
Xiao G-y (2009) Recent advances in development of herbicide resistant transgenic hybrid rice in China. Rice Sci 16(3):235–239
Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287(5451):303–305
Ye G-Y, Shu Q-Y, Yao H-W, Cui H-R, Cheng X-Y, Hu C, Xia Y-W, Gao M-W, Altosaar I (2001) Field evaluation of resistance of transgenic rice containing a synthetic cry1Ab gene from *Bacillus thuringiensis* Berliner to two stem borers. J Econ Entomol 94(1):271–276
Zi X (2005) GM rice forges ahead in China amid concerns over illegal planting. Nat Biotechnol 23(6):637