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SHORT COMMUNICATION

Detection of genetically modified rice: collaborative validation study of a construct-specific real-time PCR method for detection of transgenic Bt rice

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Abstract A collaborative trial study has been conducted for validation of an extraction method and a subsequent real-time PCR for detection of a transgenic Bt rice line ('Bt63') in rice products originating from China. A total of 17 laboratories participated in the study and each laboratory received 16 coded samples comprising of rice grain flours, rice noodle flours and plasmid DNAs. Of the accepted results all Bt63-positive rice grain samples (0.1 or 0.05% w/w) and all rice noodle samples prepared from marketed rice products were detected correctly. The result demonstrates that 'Bt63' rice is detectable even at low relative mass concentrations of 0.05%. The absolute LOD determined with plasmid DNA samples showed to be at least five copies of the 'Bt63' target sequence. The data provided in this study show that the method is fit-for-purpose to inspect Chinese rice products for the presence of EU-unauthorised rice lines carrying the 'Bt63' construct.

Keywords Genetically modified rice · Detection method · Real-time PCR · Collaborative trial · Bt63

Introduction

Since 2006 several rice products originating from China were found to contain genetically modified (GM) DNA of a construct coding for the δ-endotoxin of *Bacillus thuringiensis* (Bt) [1]. These findings have been notified to the rapid alert system for food and feed (RASFF) for information of the regional food and feed control authorities in the EU member states [2]. According to the EU legislation currently in place, no food and feed products derived from GM rice are authorised so far. In order to prevent further imports of unauthorised GM rice, in April 2008 the European Commission adopted a decision on emergency measures concerning unauthorised GM Bt63 rice products originating from China [2]. For detection of Bt63, the EU decision referred to a real-time PCR method targeting the *cry1Ab/cry1Ac* fusion gene and *nos* terminator (*T-nos*) construct present in 'GM Shanyou 63' [4]. Decision 2008/289/EC [2] prescribes that all imported rice products originating from China shall be accompanied by an analytical report based on this construct-specific method. The recommended real-time PCR method in combination with an appropriate DNA extraction method has been in-house validated in several laboratories but is not validated in a multi-laboratory study until now. The results of a recently conducted collaborative validation study are presented in this work.

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Materials and methods

Sample materials

Test samples were prepared using milled rice grains, milled rice noodles and a plasmid DNA containing the target sequences of the Bt63 construct-specific real-time PCR method.

Rice flours were prepared as mass per mass mixtures of milled non-GM rice grains ('Roter Camargue Spitzenreis', controlled organic cultivation, Rapunzel Naturkost, Legau,

Germany) and Bt63 rice grains. 'Bt63' referred rice grains were kindly provided by the Chinese authorities to the EC Joint Research Centre, Ispra. Due to irradiation of the 'Bt63' husked grains the DNA was found partially degraded, but the material appeared suitable for the present qualitative study. The non-GM rice grains were also used to prepare negative test samples. Grinding of materials was done in a laboratory milling device (Grindomix, Retsch, Germany). Milled non-GM rice grains and 'Bt 63' rice grains were first mixed to 0.1% 'Bt63' (mass per mass) content. The 0.1% test sample material was then further mixed with ground non-GM rice to obtain a 0.05% 'Bt63' content.

Rice noodles originating from Thailand and tested negative prior to the collaborative trial were used as negative rice noodle test sample material. Two different samples of rice noodles originating from China were used as 'Bt63' positive material ('Egret Rice Vermicelli', 'Swallow Sailing Rice Sticks'). These materials were derived from spot samples tested positive in previous analyses [7] and kindly provided by Dr. Ralf Reiting (Kassel, Germany) and Dr. Norbert Hess (Hamburg, Germany). All rice noodle test sample materials were homogenized by grinding.

Before subdividing the different test sample materials to 50 coded test portions of 1 g each, the homogeneity was analysed in duplicate PCR experiments with four samples of each material using the extraction protocol and detection methods described in the present study.

To assess the limit of detection, a plasmid containing a 717 bp long fragment of the *cryIAc* and *nos* terminator region was used (kindly provided by Eurofins/GeneScan, Freiburg, Germany). Plasmid DNA was linearised with *Xmn*I and diluted to concentrations of 4 and 1 copy/µL. Copy numbers were calculated on basis of the size of the plasmid and DNA concentration measurements using the PicoGreen method. Plasmid DNA dilutions were stabilised with 10 ng/µl eukaryotic genomic DNA.

Collaborative trial

The collaborative trial was organised by the Federal Office of Consumer Protection and Food Safety (BVL) and the German working group "Development of methods for identifying foodstuffs produced by means of genetic engineering techniques". 17 laboratories from Germany, Austria and Switzerland participated in the study. Each laboratory received 1 gram of the following coded test portions: two portions of negative rice flour, two portions of 0.1% Bt63 rice flour, two portions of 0.05% Bt63 rice flour, two portions of negative rice noodle flour, two portions of positive 'Rice Vermicelli' rice noodle flour and two portions of 'Swallow Sailing Rice' rice noodle flour. Additionally, each participant received three coded DNA solutions containing four copies, one copy or no copy per microliter of the 'Bt63' plasmid, respectively.

To allow the laboratories training with the extraction procedure, each participant received two positive control sample materials containing 'Bt63' positive rice noodle flour which should be analysed before the analyses of the coded test samples. In addition, extracted DNA of the GM rice sample FR0502519 [4] was provided as positive control DNA, the concentration was adjusted to result a Ct-value of ~30 in the Bt63 detection system. Test samples were shipped at +4 °C and stored at −18 °C until the analysis.

DNA extraction

For DNA extraction a modified CTAB protocol was used [4, 5]. In the collaborative study, 1 g of each test sample, 5 mL CTAB extraction buffer and 10 µL proteinase K (20 mg/mL) were incubated overnight at 65 °C. After centrifugation at 2,000×*g* for 10 min, 1 mL of the clear supernatant were transferred into a new reaction tube. All following steps were carried out as described [4], except that the final DNA extract was resolved in a volume of 100 µL.

Real-time PCR

Real-time PCR was performed using the primers org1 and org2 with the probe orgp for detection of the *gos9* target taxon sequence present in rice [4, 6, 8]. The primers T51F and T51R and the probe T51p were used to detect the *cry1Ac/T-nos* construct identified in 'Bt63' rice [4, 8]. Reactions were carried out in universal mastermix (Applied Biosystems) and corresponding primer and probe concentrations [4] with 5 µL of the undiluted DNA solutions. Different real-time PCR instruments were used by the participants (ABI 7000, 7500, 7900; MX3005P, Rotorgene 3000).

Results and discussion

Seventeen laboratories took part in the collaborative study and returned results in the given timeframe of 3 weeks. Thirteen participants returned correct results for all samples, while four laboratories delivered no results for few samples or reported difficulties to detect any rice DNA (Table 1). None of the participating laboratories reported specific problems to perform the DNA extraction and PCR analysis with the positive control samples. One laboratory recommended addition of an α-amylase incubation step for all samples in order to reduce the viscosity caused by high starch content. Another laboratory reported that addition of

Table 1 Collaborative trial study results obtained with rice grain and rice noodle flour samples

Year of collaborative trial	2008
Number of laboratories	17
Number of laboratories submitting results	17
Number of rice samples per laboratory	12
Number of accepted results	191[a]
Number of samples containing 'Bt63' rice	129
Number of samples non-GM rice	62
False-positive results	1 (1.6%)
False-negative results	0 (0%)

[a] One laboratory reported leakage of four samples during the overnight incubation and therefore these test samples could not be analysed; for a total of five test samples no rice DNA was detectable in three different laboratories. One laboratory reported for five test samples (two rice grain and three rice noodle samples) unusual low *gos9* Ct-values ($Ct \leq 14$) and therefore results for these test samples were excluded in the evaluation

RNAse A to all extracted DNA solutions reduced the RNA content and thereby distinctively improved their PCR results. In summary, these observations point out that appropriate training and experience is necessary before establishing a new DNA extraction method in an analytical laboratory.

Laboratories were requested to perform real-time PCR analyses with each extracted test sample DNA. DNA extracts had to be analysed in single PCR experiments targeting the *cryIAc/T-nos* construct and the *gos9* rice reference gene, respectively. The corresponding Ct-values had to be reported. Of the accepted results all Bt63-positive rice grain and rice noodle samples were detected correctly (Table 1). Only one of 61 Bt63-negative test samples was reported Bt63-positive. The respective laboratory reported a Ct-value of 42.1 for this DNA extract, but in a subsequent second PCR analysis this DNA showed a negative result as was communicated after the end of the collaborative trial.

For the rice grain flour samples a mean *gos9* Ct-value of 22.1 ± 3.3 was measured. Bt63-positive rice grain samples containing 0.1 or 0.05% Bt63 (mass/mass) showed a mean Bt63 Ct of 36.6 ± 2.1. Bt63-positive rice noodle samples showed a slightly higher mean *gos9* Ct of 23.3 ± 3.3. For these samples a mean Bt63 Ct of 35.2 ± 2.0 as calculated from a total of 66 determinations was reported. Comparing the Ct differences (delta Ct of Bt63 vs. *gos9*) observed for the 0.1 and 0.05% Bt63 rice grain flours and those reported for the Bt63-positive rice noodle samples which were taken from rice products placed on the market it appears that the Bt63 content in these materials is in the 0.1% range.

The results of the collaborative trial study demonstrate that 'Bt63' rice is detectable even at low relative mass concentrations of 0.05%. To determine the absolute detection limit of the construct-specific 'Bt63' detection system, tests with three plasmid DNA samples were included in the collaborative trial study. These plasmid DNA samples were analysed in duplicate. Even the calculated number of five copies per reaction of 'Bt63' target sequence was detected in 32 of 34 reactions (mean $Ct = 38.5 \pm 2.0$), which corresponds to 94 % positive results (Table 2). All reactions containing a calculated copy number of 20 were positive when tested with the Bt63 real-time PCR method (mean $Ct = 36.6 \pm 0.9$). As expected, the distribution of the number of target copies in the reaction at a low level follows the Poisson distribution. The results obtained with the plasmid DNA samples also confirmed the in-house validation report of the CRL-GMFF, in which a LOD of at least five copies is given for the Bt63 method [8].

Table 2 Collaborative trial study results obtained with Bt63 plasmid DNA samples

Year of collaborative trial	2008
Number of laboratories	17
Number of 'Bt63' plasmid DNA samples per laboratory	3
Number of determinations per sample DNA	2
Number of positive results/number of determinations of plasmid DNA (0 copies)	0/34
Number of positive results/number of determinations of plasmid DNA (5 copies)	32/34
Number of positive results/number of determinations of plasmid DNA (20 copies)	34/34

In conclusion the data of this collaborative trial study verifies the previous results of the in-house validation carried out in other laboratories and shows that the method is fit-for-purpose to inspect rice products for the presence of unauthorised rice lines carrying the 'Bt63' construct [8]. The DNA extraction procedure used in the study showed to be applicable for rice grains and for rice noodle products by yielding genomic rice DNA of sufficient quality and quantity for PCR analysis.

It should be noted, that the presence of another unapproved transgenic rice line contaminating rice Vermicelli products has been recently reported [9]. These authors showed that another, very similar *cryIAb/cryIAc* fusion gene and *T-nos* genetic construct present in 'GM Shanyou 63' was identified in imported rice products from China. This Bt rice line was further characterised and showed to contain a construct similar to that of the Kemingdao1 line (KMD1), a transgenic rice line currently tested in large field trial tests in China [10, 11]. Further investigations may be necessary in order to evaluate to which extent unauthorised Bt rice products have been already placed on the market.

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