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Analytical Methods

Real-time PCR method for detection of the transgenic rice event TT51-1

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ABSTRACT

The insect-resistant transgenic rice event TT51-1 (synonym BT63) has been found illicitly planted and distributed for years although it has never been approved for commercial cultivation in any country up to now. The purpose of this study was to establish a detection method that is specific for this transformation event. The event-specific PCR method produces an amplicon of 120 basepairs (bp) based on the revealed 3′ junction sequence with a limit of detection (LOD) and a limit of quantification (LOQ) being approximately 5 and 10 initial template copies, respectively. Two mixed rice samples with known TT51-1 contents were used to verify the developed real-time PCR system, from which the expected results were observed.

1. Introduction

Rice (*Oryza sativa*) is one of the most important food crops in the world. In Asian countries, two thirds of food calories are provided by rice. The stem borer causes considerable yield losses of rice in Asia and pesticides have been used broadly to control this serious pest. To address this issue, transgenic insect-resistant rice has been developed to reduce pesticide application and to increase rice production through resistance to yellow stem borers and leaffolders (Tu et al., 1998, 2000).

TT51-1 (synonym BT63) is an insect-resistant transgenic rice transformation event harbouring a hybrid Cry1Ab/Ac gene regulated by the rice actin 1 gene promoter and the nopaline synthase (NOS) terminator (Tu et al., 1998, 2000, 2003). Although transgenic rice TT51-1 shows great potential in rice production, it has not yet been approved for either human consumption or safe for the environment up to now. According to the regulations of China, the European Union and many other countries, food and feed containing genetically modified (GM) material cannot be sold in the market unless the associated GM material has been authorised, following a rigorous safety assessment (European Food Safety Authority, 2009; Food Standards Agency, 2009a, 2009b; Herrera, 2007; State Council of China, 2001). Material from unauthorized GM organisms in food or feed is not permitted at any level (Chief Medical Officer of the Russian Federation, 2007; European Commission, 2003a, 2003b; Herrera, 2007; Matsuoka, 2001; Ministry of Agriculture and Forestry of the Republic of Korea, 2000; Ministry of Agriculture of the People's Republic of China, 2002).

Since 2005, the presence of adventitious insect-resistant rice containing transformation event TT51-1 has been found and reported many times in the market place (GeneWatch UK, 2008). In 2006 it was also found in baby food sold in Beijing, Guangzhou and Hong Kong, China. In late 2006, TT51-1 was found for the first time in Europe and 10 cases of products contaminated with GM rice were reported by European governments (Austria, France, UK and Germany) (GeneWatch UK & Greenpeace International, 2008). In 2007, GM rice was found in 10 imports to Europe (Cyprus, Germany, Greece, Italy and Sweden). The European Commission has now adopted emergency measures (commencing 15 April, 2008) requiring compulsory certification for the imports of Chinese rice products that could contain the unauthorized GM rice transformation event TT51-1 (European Commission, 2008a, 2008b).

In order to monitor the presence of TT51-1, validated detection methods are urgently needed. In 2006, Mäde, Degner, and Grohmann (2006) first reported a construct-specific method to detect this event. However, this construct-specific method cannot distinguish this event from others derived from the same transformation construct. In order to distinguish different transformation events derived from the same insert, PCR methods based on the junction fragments are required because the insertion site and the associated DNA rearrangement is unique for each transformation event (Miraglia et al., 2004; Røning, Vaïtilingom, Berdal, & Holst-Jensen, 2003).

The aim of this research was to establish qualitative and quantitative PCR methods for the event-specific detection of TT51-1.

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2. Materials and methods

2.1. Plant materials

Genuine seeds of GM rice (*O. sativa*) pure line containing transformation event TT51-1 and two other GM insect-resistance rice cultivars were provided by the Center of Science and Technology Development, Ministry of Agriculture of the People's Republic of China (Beijing, China).

Non-transgenic seeds of *O. sativa*, *Arabidopsis thaliana*, *Brassica napus*, *B. rapa*, *B. oleracea*, *B. juncea*, *Glycine max*, *Zea mays* and *Gossypium hirsutum* were collected by our laboratory (Wuhan, China).

Seeds of TT51-1, non-transgenic *O. sativa* and *Z. mays* were sown and cultivated in a greenhouse. The fresh leaves were collected for DNA extraction.

2.2. DNA extraction

For the screening of primers and probes, real-time PCR optimisation and standard curve development, genomic DNA was extracted and purified from young leaves of *O. sativa* and *Z. mays* following a cetyltrimethylammonium bromide (CTAB) based protocol (Porebski, Bailey, & Baum, 1997).

For the sample detection experiments, genomic DNA was extracted and purified from seeds with the DNA Extraction Kit for GMO Detection Ver. 2.0 (Takara, Shiga, Japan).

DNA concentrations were estimated with a spectrophotometer Lambda 25 (Perkin Elmer, Ames, IA, USA) and further characterised by agarose gel electrophoresis, ethidium bromide staining and quantification with Quality One software (Bio-Rad, Hercules, CA, USA).

2.3. Primers and probes

The structure of the transgenic locus in TT51-1 rice was clearly elucidated using a GenomeWalker Universal Kit (Clontech, Mountain View, CA, USA). The whole exogenous DNA sequence has been submitted to Genbank under accession number EU880444. The primers and probe used in the event-specific PCR assay were designed according to this recovered sequence.

The primers and probes used in this research were synthesised by Sangon (Sangon, Shanghai, China) and are listed in Table 1.

2.4. Qualitative PCR conditions

In the qualitative PCR assay 100 ng of genomic DNA was used as template in a volume of 50 μL. The reaction mixture contained 1× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 200 μM each dNTP, 1.5 mM $MgCl_2$, 100 nM each primer, and 2 unit Hot Start Taq (Takara, Shiga, Japan). The PCR amplifications were carried out on a GeneAmp® 9700 thermal cycler using the following program: a 94 °C initial denaturation step for 2 min; followed by 40 cycles of 15 s at 94 °C (denaturation), 30 s at 60 °C (annealing), 30 s at 72 °C (extension) and terminated with an extension at 72 °C for 2 min. The PCR products were size separated using electrophoresis on 2% agarose gel and stained with ethidium bromide for visualisation.

2.5. Quantitative PCR conditions

The real-time quantitative PCR reactions were performed for the phospholipase D (PLD) gene and the 3' junction of the event TT51-1 separately on a fluorometric thermal cycler (DNA Engine Opticon® 2 Continuous Fluorescence Detector, MJ Research, Waltham, MA, USA). Fluorescence signals were monitored and analyzed by the software Opticon Monitor® 2 Version 2.02 (MJ Research, Waltham, MA, USA).

The real-time PCR assay for the PLD gene was performed in a volume of 25 μL containing 20 ng genomic DNA as template, 1× TaqMan® buffer (50 mM KCl, 10 mM Tris–HCl, 10 mM EDTA, pH 8.3), 4.5 mM $MgCl_2$, 200 nM each primer KVM159 and KVM160, 200 nM probe TM013, 300 μM each dATP, dCTP and dGTP (Fermentas, Vilnius, Lithuania), 600 μM dUTP (Fermentas, Vilnius, Lithuania), 0.2 unit UNG Amperase Uracil N-glycosylase (UNG, Applied Biosystems, Foster City, CA, USA) and 1.25 units AmpliTaq™ Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA).

The real-time PCR assay for the 3′ junction of the Event TT51-1 was performed in a volume of 25 μL containing 20 ng genomic DNA as template, 1× TaqMan® buffer (50 mM KCl, 10 mM Tris–HCl, 10 mM EDTA, pH 8.3), 4.5 mM $MgCl_2$, 800 nM each primer TT511 V and TT511G, 400 nM probe TT511P, 400 μM each dATP, dCTP and dGTP (Fermentas, Vilnius, Lithuania), 800 μM dUTP (Fermentas, Vilnius, Lithuania), 0.2 unit UNG Amperase Uracil N-glycosylase (UNG, Applied Biosystems, Foster City, CA, USA) and 1.25 units AmpliTaq™ Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA).

All real-time PCR reactions were carried out with the same program as follows: a pre-digest step of 50 °C for 2 min, an initial denaturation and UNG deactivation step 95 °C for 10 min; followed by 50 cycles of 15 s at 94 °C (denaturation), 1 min at 60 °C (annealing and extension), and fluorescence measurements after annealing and extension.

3. Results and discussion

3.1. Primers/probes design and event-specific detection of event TT51-1

Because the 5′ flanking DNA sequence and the exogenous vector fragments were both repeated in direct or inverted manners in event TT51-1 (Genbank accession number EU880444), the event-specific PCR assays were designed using the 3′ junction sequence. Primer TT511 V was designed to anneal with the inserted DNA sequence of event TT51-1, and primer TT511G was located in the flanking rice genome, as shown in Fig. 1.

Combined with the TaqMan® probe TT511P spanning the 3′ junction, the primer pair TT511G/TT511V could be used for both

Table 1
Primers and fluorogenic probes used in this research.

Purpose	Name	Sequence (5′–3′)	Amplicon size (bp)	Specificity
PCR analysis of PLD gene	KVM159	TGGTGAGCGTTTTGCAGTCT	74	Rice genome
	KVM160	CTGATCCACTAGCAGGAGGTCC		
	TM013[a]	TGTTGTGCTGCCAATGTGGCCTG		
Event-specific PCR analysis of TT51-1	TT511 V	AGAGACTGGTGATTTCAGCGGG	120	Transgene
	TT511G	GCGTCCAGAAGGAAAAGGAATA		Rice genome
	TT511P[a]	ATCTGCCCCAGCACTCGTCCG		Junction site

[a] The probes were labelled with 5′-6-carboxy-fluorescein (FAM) and 3′-Black Hole Quencher 1 (BHQ1).

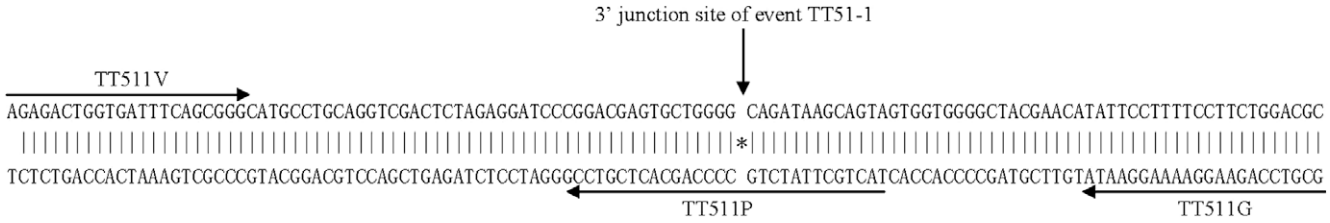

Fig. 1. Sequence of the 3′-integration junction regions and the location of the primers and the TaqMan® probes used for the event-specific detection of the Event TT51-1. The junction sites between host DNA and the insert are indicated by an asterisk. The arrows indicate the sequences and directions of the primers and probe.

qualitative and quantitative PCR detection of the specific event TT51-1, yielding a product of 120 bp. The size of the event-specific PCR product is small enough to meet the requirements of ISO, CRL-GMFF and the labelling policies of most countries (ISO, 2005; Weighardt, 2007).

The PLD gene was used as the endogenous reference gene. The primers and probe for the PLD gene were synthesized according to a validated protocol of CRL-GMFF (CRL-GMFF, 2008).

To ensure the specificity of the designed event-specific primer pair, the genomic DNA from different transgenic and non-transgenic rice cultivars was used as template for PCR assays. The expected amplicons of the endogenous reference gene PLD were detected in all rice samples. The primer pair TT511V/TT511G could amplify specific fragments from TT51-1, but there was no amplification observed from the other two GM insect-resistance or non-transgenic rice cultivars. In further tests with non-rice genomic DNA of *A. thaliana*, *B. rapa*, *B. oleracea*, *B. juncea*, *G. max*, *Z. mays* and *G. hirsutum* as templates, no PCR product was observed with the event-specific primer pair.

3.2. Quantitative analysis of TT51-1

After the optimisation of Mg^{2+}, dNTPs/dUTP and primers/probe concentrations, the TaqMan® real-time PCR assay systems were established.

The standard curve method was used in this study for the quantification assays, because it is superior to the ΔCt methods when the two targets do not amplify with the same efficiency in the PCR (Taverniers, Van Bockstaele, & De Loose, 2004). The haploid genome size of rice was estimated to be 430 Megabasepairs (Mbp) (Arumuganathan & Earle, 1991), corresponding to a weight of 0.47 pg. Therefore, the copy number of the haploid rice genome was calculated by the weight of the rice DNA divided by 0.47 pg.

For the quantification of total rice DNA in a sample, a standard curve for the PLD gene was used, which was developed by using 20 ng for each concentration of a 100–0.0235% dilution series of rice DNA diluted by maize genomic DNA. For the quantitation of the event TT51-1, the standard curve of the 3′ junction sequence was used, which was developed by using 20 ng for each concentra-

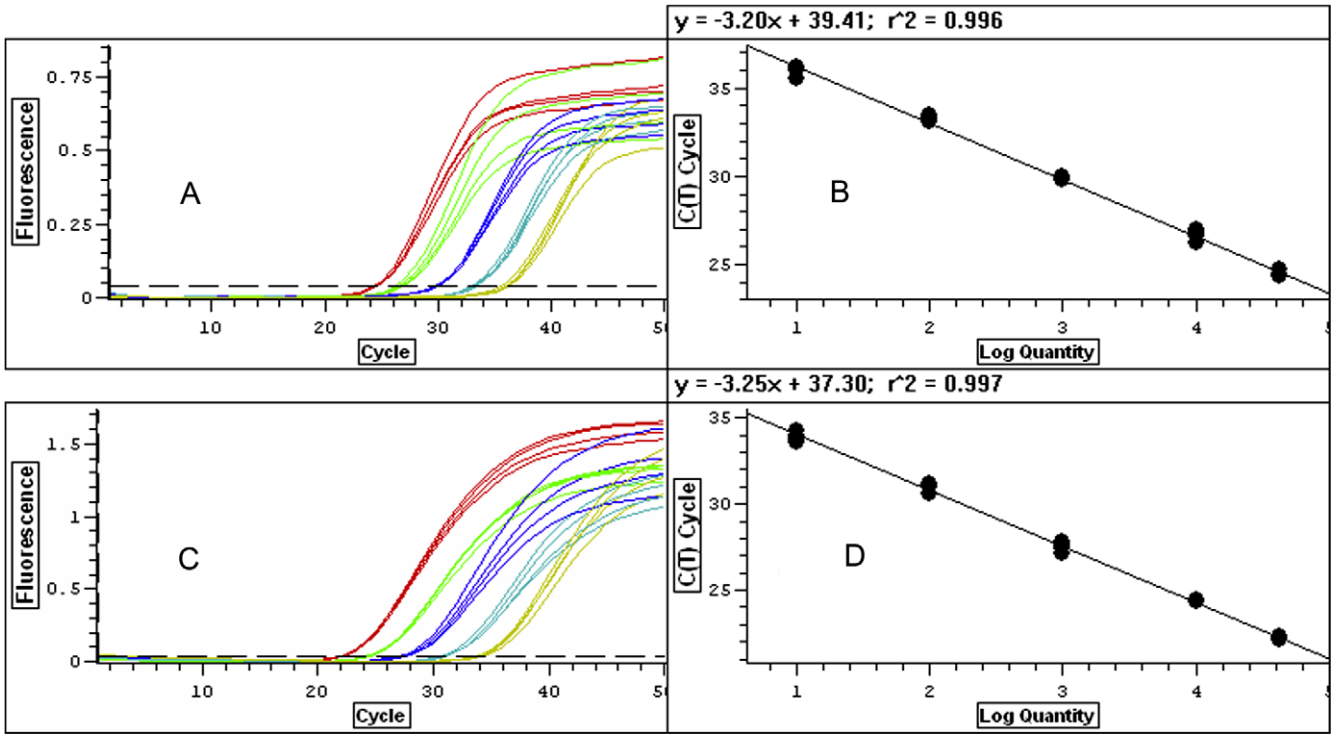

Fig. 2. Amplification and standard curves for the event-specific quantitative PCR method using gradient-diluted TT51-1 genomic DNA as the template. (A) Amplification graph for the PLD gene assay. (B) Standard curve for the PLD gene assay, analyzed using Opticon Monitor® 2 Version 2.02. The quantities of rice genome in each dilution were 42500, 10000, 1000, 100, and 10 copies per reaction, respectively. (C) Amplification graph for the TT51-1 event-specific assay. (D) Standard curve for the TT51-1 event-specific assay, analyzed using Opticon Monitor® 2 Version 2.02. The copy numbers of the transformation event TT51-1 in each dilution were 42,500, 10,000, 1000, 100, and 10 copies per reaction, respectively.

tion of the genomic DNA from TT51-1 diluted by DNA from the non-transgenic rice. The result of each PCR assay was verified by four replications and the assays were repeated four times.

Fig. 2 shows the standard curves for the endogenous reference gene PLD and for the event TT51-1. Based on the slope of the standard curve derived from the TT51-1 genomic DNA dilution series, this event-specific quantitative system had almost 100% efficiency. There was good agreement between the amount of template and the threshold of cycle (Ct) values with the coefficient of determination (R^2) of 0.997 for the four replicates, which was significantly higher than the minimum requirement of 0.98 (Taverniers et al., 2004). This indicates that the real-time assay established in this study is suitable for the quantification of TT51-1 DNA.

Although the standard curves were derived from serially diluted samples and showed good agreement with the template, ΔCt values between different dilutions still did not completely match the theoretical values (3.32 for 10-fold, 2.32 for 5-fold, 2.09 for 4.25-fold and 1 for 2-fold) (Table 2). These theoretical values were calculated based on a PCR efficiency of 100%. In practice, PCR efficiency cannot be completely equivalent to the theoretical value because of interferences from reagents, reaction conditions and operation. Upon repetition with the increasing sample volumes and number of repeats, these random errors will cancel each other out. Therefore, in spite of these minor errors a good standard curve was still generated on the basis of these data (Fig. 2 and Table 2).

The repeatability of the real-time methods was analyzed using the serially diluted standards as described above. The standard deviation (SD) and relative standard deviation (RSD) of four parallel experiments, and the standard deviation (SD^r) and relative standard deviation (RSD^r) of repeatability were calculated based on the data from four parallel reactions and four repeats of the assays. With the reduction of the template from 42,500 to 10 copies, the average Ct values of the event-specific assay increased from 22.115 (SD = 0.123) to 33.809 (SD = 0.323) (Table 2). At the same time the RSD^r value of three replications ranged from 0.558% to 1.074% (Table 2). Based on these data, the established quantitative PCR assays have good repeatability and are suitable for the practical measurement of TT51-1 DNA from GM samples with a template copy number ranging from 42,500 to 10.

However, with a further reduction in copy number, a noticeable variation in Ct values was observed and some samples had no observable PCR amplifications (Table 2). In order to determine the limit of detection (LOD) and limit of quantitation (LOQ) of the real-time PCR method, a DNA dilution series containing 10, 5 and 1 copies (copy) of TT51-1 genomic DNA per reaction was analyzed in 16 replicate real-time PCR analyses (Fig. 3). As expected the ability to detect TT51-1 DNA decreased with a decreasing number of template copies. When the template level was 10 or 5 copies, positive signals could be detected in all 16 replicates. However, when the template copy number was 1, the amplicons could only be detected in 6 reactions. This phenomenon can be explained with the Poisson distribution model. Based on these data, the 95% confidence interval of the detection limits was calculated to be 2.5–4.5 copies for the event-specific quantitative assay established in this study. From this result, the LOD for the event TT51-1 was estimated to be 5 copies, or 5 copies of TT51-1 haploid genomic DNA. Although all the data ranging from 42,500 to 10 copies was found to be concordant with the standard curve, the SD value increased with the decreasing levels of template. When the template content was as low as 5 copies, the Ct values of replicate reactions had a deviation exceeding 1. Considering all of the data generated by these experiments, it was estimated that the LOQ of the event-specific quantitative detection of the event TT51-1 was 10 copies. Taking all of this into account, it is safe to conclude that as low as 0.023% of TT51-1 genomic DNA in 20 ng of total rice genomic DNA samples could be detected and quantified by the method presented in this study.

Table 2
Repeatability of real-time PCR assays employing TT51-1 DNA as reference.

DNA amount (ng)	Copy number[a]	Repeat	Ct value				Mean of Ct values	SD	RSD (%)	Mean of all Ct values	SD^r	RSD^r (%)
			1	2	3	4						
20	42500	1	21.959	22.075	22.003	22.178	22.054	0.096	0.434	22.115	0.123	0.558
		2	21.924	22.285	22.104	22.153	22.117	0.149	0.675			
		3	22.009	22.193	22.076	22.049	22.082	0.079	0.358			
		4	22.220	22.404	22.128	22.079	22.208	0.143	0.645			
4.70	10000	1	24.332	24.299	24.282	24.209	24.281	0.052	0.214	24.343	0.173	0.711
		2	24.461	24.330	24.455	24.453	24.425	0.063	0.259			
		3	24.218	24.510	24.329	23.922	24.245	0.247	1.017			
		4	24.294	24.705	24.472	24.217	24.422	0.217	0.888			
0.47	1000	1	27.268	27.465	27.636	27.048	27.354	0.254	0.927	27.412	0.154	0.562
		2	27.451	27.478	27.414	27.197	27.385	0.128	0.468			
		3	27.320	27.586	27.456	27.436	27.450	0.109	0.397			
		4	27.277	27.516	27.558	27.492	27.461	0.125	0.457			
0.047	100	1	30.453	31.027	30.860	30.921	30.815	0.251	0.815	30.947	0.237	0.766
		2	30.866	31.162	30.961	31.046	31.009	0.126	0.406			
		3	30.585	30.796	31.007	31.050	30.860	0.214	0.694			
		4	30.916	31.045	31.531	30.918	31.103	0.292	0.939			
0.0047	10	1	33.611	34.156	33.830	33.430	33.757	0.312	0.925	33.809	0.363	1.074
		2	33.447	34.070	33.936	33.510	33.741	0.309	0.915			
		3	33.689	33.649	34.022	33.384	33.686	0.262	0.777			
		4	34.307	34.293	33.246	34.360	34.052	0.538	1.579			
0.00235	5	1	34.256	34.612	35.185	33.810	34.466	0.581	1.686	34.840	0.627	1.798
		2	35.142	35.233	35.097	34.230	34.926	0.467	1.337			
		3	33.708	35.051	35.987	35.161	34.977	0.943	2.697			
		4	35.332	35.490	34.774	34.373	34.992	0.515	1.470			
0.00047	1	1	[b]	[b]	35.410	37.300	36.355	1.336	3.676	36.260	1.080	2.977
		2	[b]	36.221	[b]	[b]	36.221	[b]	[b]			
		3	35.106	37.489	[b]	[b]	36.298	1.685	4.642			
		4	37.224	[b]	[b]	35.070	36.147	1.523	4.214			

[a] Calculated based on an estimated genome size of 430 Mbp.
[b] No data.

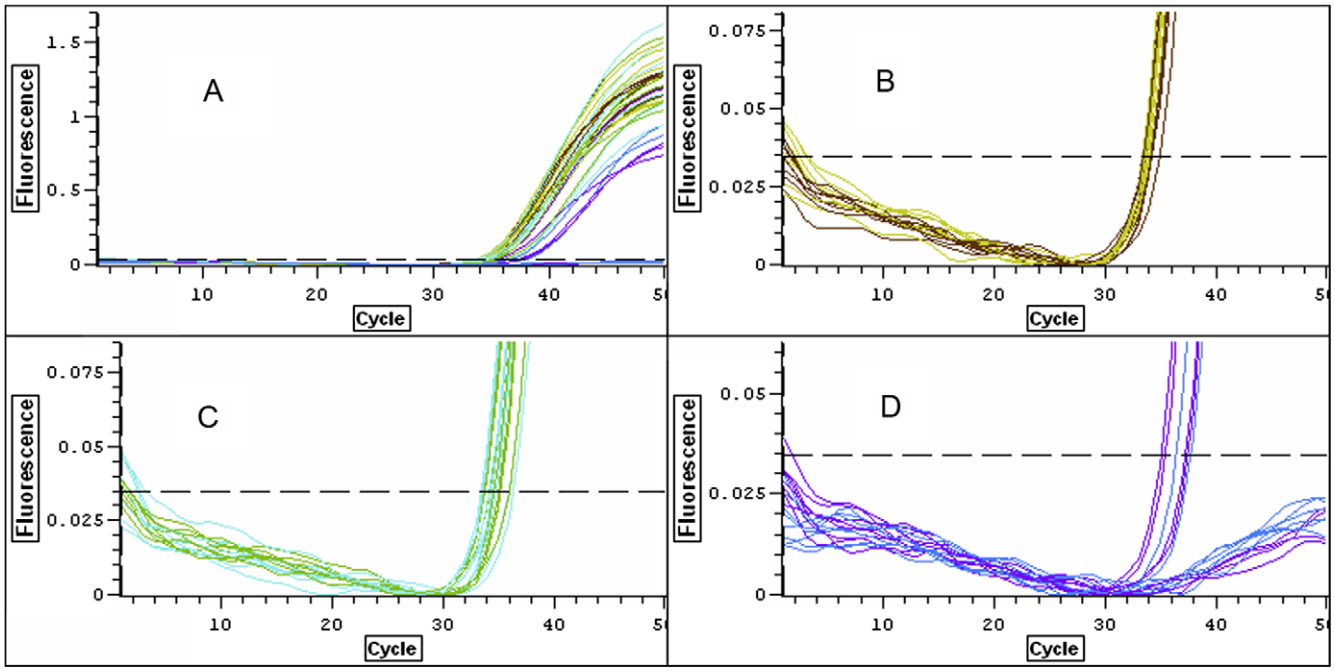

Fig. 3. Estimation of the limit of detection (LOD) of the event-specific quantitative PCR method. (A) Comprehensive amplification graph of 48 replicates with 10, 5 and 1 initial templates. (B) Amplification graph of 16 replicates with 10 initial templates. (C) Amplification graph of 16 replicates with 5 initial templates. (D) Amplification graph of 16 replicates with 1 initial template.

3.3. Validation of the quantitative real-time PCR method

The genomic DNA from mixed samples containing known concentrations of TT51-1 in the non-transgenic rice was used as the PCR template. Sample S1 contained 2% TT51-1 seeds in non-transgenic rice and sample S2 contained 0.5% TT51-1 seeds. The genomic DNA was extracted from these mixed samples and used as template along with the serially diluted standards described above. For each sample and standard about 20 ng of total DNA was used in each of four parallel reactions, and the real-time assays were performed four times. The quantitative estimated results were computed by the software Opticon Monitor® 2 version2.02 based on the standard curves.

The relative content of TT51-1 to total rice DNA (%) was computed as: (mean copy number of TT51-1 of four replicates)/(mean copy number of total rice DNA of four replicates) × 100.

Using the quantitative method based on the 3′ junction of the event TT51-1, no amplification was obtained using the DNA from non-GM rice as the template. For sample S1 the estimated copy numbers of TT51-1 DNA ranged from 870 to 1012 (mean 922, SD 64), and the total rice DNA from 43291 to 50277 (mean 46082, SD 8184). Based on these data the proportion of TT51-1 was calculated to be 2.002% with a bias of 0.111% of the theoretical value (Table 3). For the sample S2 the estimated copy numbers of TT51-1 and total rice DNA were from 193 to 251 (mean 229, SD 27) and from 45898 to 52537 (mean 49856, SD 2822), respectively, and the corresponding proportion of TT51-1 was 0.46% with a bias of 8.007% (Table 3).

From the results described above, deviations of the quantified results from the theoretically true value were observed for every sample. These small errors could be explained by uncertainty in the genome size of rice and possible difference in the genome size of TT51-1 and non-transgenic rice used in this research. The uncertainty in the DNA purity and quantitation after extraction (Corbisier et al., 2005), and the molecular fluctuations with low copy numbers of the initial template are also thought to contribute to the quantitative uncertainty in PCR reactions (Peccoud & Jacob, 1996). Generally speaking, the lower template level resulted in greater uncertainty because of calibration curves properties and the stochastic variations of the PCR amplification procedure (Estalilla, Medeiros, Manning, & Luthra, 2000; Fieller, 1940; Foy & Parkes, 2001). In this research, the copy number of the event TT51-1 was about 1000 in 20 ng 2% mixed sample, and 250 in the 0.5% mixed sample. According to the analysis above, the observed deviations in these measurements are possible and acceptable.

This method has been submitted to the supervision department of China for inter-laboratories validation as a candidate national standard to establish the reliability, accuracy and reproducibility to meet the requirement of ISO 5725 (ISO, 1994). We hope this research provides technological data and method validation for the purpose of detecting and controlling the transgenic rice TT51-1.

Table 3
Accuracy and precision statistics for quantitative method.

Sample	Theoretical (%)	Assay	Experimental (copies)				Mean (copies)	SD (copies)	RSD (%)	Experimental (%)	Bias (%)
			1	2	3	4					
S1	2%	TT51-1	1012	883	926	870	923	64	7	2.002	0.111
		PLD	50277	41764	48994	43291	46082	4184	9.1		
S2	0.50%	TT51-1	249	224	193	251	229	27	11.8	0.46	−8.007
		PLD	45898	52537	50823	50165	49856	2822	5.7		

The assays in this report were optimised for the use of the equipments and chemicals described in this paper, especially for the MJ Research (now Bio-Rad) fluorometric thermal cycler DNA Engine Opticon® 2. Other thermal cycler systems and reagents can be used, but reaction conditions should be verified and re-optimised according to the manufacturer's recommendations.

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