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ORIGINAL PAPER

Event-specific quantitative detection of genetically modified wheat B72-8-11 based on the 3′ flanking sequence

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Abstract Various PCR technologies have been developed for the execution of genetically modified organism (GMO) labeling policies of which an event-specific PCR detection method based on the flanking sequence of exogenous inserted DNA is the key trend in GMO detection due to its high specificity. In this study, 3′ flanking sequence between the host wheat DNA and the exogenous integrated gene construct of pHMW1Dx5 vector in transgenic wheat B72-8-11 was revealed by means of adaptor PCR, thus the fragment with the length of 210 bp was obtained, including a 144 bp unknown wheat genome DNA sequence. The event-specific quantitative PCR primers were designed based upon the revealed 3′ flanking sequence, and a SYBR Green I real-time PCR assay was subsequently applied. In the quantitative SYBR Green I real-time PCR assay, the LOD and LOQ were 10 and 20 haploid genome copies, respectively. In addition, three mixed wheat B72-8-11 samples with known contents were detected using this established quantitative PCR system, and the ideal results indicated that the developed event-specific quantitative PCR detection method could be used for identification and quantification of B72-8-11 wheat and its derivates.

Keywords Genetically modified organisms · Wheat B72-8-11 · Genome walker · Flanking sequence · Event-specific quantitative detection

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Introduction

Nowadays, genetically modified organisms (GMOs) have been widely developed and applied in agriculture, and the global hectarage of biotech crops have increased more than 100-fold from 1.7 million hectares in 1996 to over 175 million hectares in 2013—this makes biotech crops the fastest adopted crop technology in recent history [1]. To allow consumer freedom of choice, more than 50 countries and areas have regulated the authorization, labeling, and compliance control of GMOs [2]. Therefore, the detection and identification of GMOs in food have become important issues for all the subjects involved in food control [3, 4]. Since labeling of foods containing GMO ingredients is mandatory in some countries, the demand to develop easy, accurate, sensitive, and reliable detection methods for GMOs is very high [5].

PCR technology-based GMO detections can be grouped into at least four categories, such as screening-, gene-, construct-, and event-specific PCR [2]. The four PCR detection systems were developed based on the four different kinds of target DNA fragments of exogenous integration corresponding to their level of specificity. Each category corresponds to the composition of the DNA fragment that is amplified in the PCR. The flanking sequence is the only unique signature of a transformation event (within the limitations of present day technology), which is the junction

Table 1 The primers used in the genome walker and event-specific quantitative PCR detection

Primer type	Primer name	Sequence 5′–3′	Reference
Adaptor primers	AP1	GTAATACGACTCACTATAGGGC	Genome Walker™ Universal Kit
	AP2	ACTATAGGGCACGCGTGGT	
	NF1 (GSP1)	AGATTGAATCCTGTTGCCGGTCTTGCGATG	This work
	NF2 (GSP2)	AGATGGGTTTTTATGATTAGAGTCCCGCAA	
B72-8-11 event-specific primers	qES-HMW F	AGTCCCGCAATTATACAT	This work
	qES-HMW R	GCGTAATACGACTCACTAT	
Endogenous gene primers	qwx012 F	GTCGCGGGAACAGAGGTGT	[22]
	qwx012 R	GGTGTTCCTCCATTGCGAAA	

at the integration locus between the recipient genome and the inserted DNA. This junction is the target of category of event-specific method. Currently, the event-specific PCR method has been the key trends for GMO identification and quantification because of its high specificity based on the flanking sequence [6, 7]. Also, the qualitative or quantitative event-specific detection methods have been used in detection of other GM crops, such as the soybean [8, 9], maize [2, 10–16], rice [17], Carnation Moonlite [18], and so on.

Wheat is not only the most important crop in the world, but also widely used in food processing and as feed for livestock [19]. At present, the wheat B72-8-11 line has been confirmed by large scale testing of grain grown in field trials [20]. The B72-8-11 was obtained by particle bombardment co-transformation of the plasmid vectors pAHC25 and pHMW1Dx5 [21]. Although the molecular characterization of the two plasmids has been described in the paper of Zhang et al. [22], the flanking sequence of B72-8-11 is not same to that of B73-6-1 wheat because of the different insertion site and integration pattern. To B72-8-11 wheat, event-specific qualitative PCR for B72-8-11 has been established by Zhang et al. [23], but event-specific quantitative PCR method has not been developed. In this study, the 3′ integration flanking sequences of B72-8-11 were revealed by adaptor PCR, and the event-specific quantitative PCR analysis method was established based on the 3′ flanking sequence of B72-8-11 wheat.

Materials and methods

Plant materials

GM wheat B72-8-11 and B73-6-1 were kindly supplied by Heilongjiang Entry-Exit Inspection and Quarantine Bureau. GM soybean (GTS 40-3-2, A2704-12, and MON89788), GM maize (MON810, Bt176, and MON863), GM cotton (MON531, MON1445, and LLCOTTON25), GM rice (TT51-1 rice and "Kefeng 6"), and GM canola (MS1, RF3, and MS8) were provided by Chinese Academy of Agricultural Sciences.

Primers

The genome walker-specific primers NF1 (GSP1, the gene-specific primers for primary PCR) and NF2 (GSP2, the gene-specific primer for secondary PCR) (Table 1) used in adaptor PCR were designed based on the sequence of the pHMW1Dx5 plasmid, and the adaptor primers AP1 and AP2 (Table 1) were supplied by Genome Walker™ Universal Kit (Clontech, USA). The event-specific quantitative PCR primers (qES-HMW F/R) used for B72-8-11 wheat detection were designed with Primer Premier 5 (PREMIER Biosoft Int., USA) based on the flanking sequence of the pHMW1Dx5 vector (one of the two co-transformation vectors), and this primer pair was employed for SYBR Green I quantitative PCR. Here, a wheat housekeeping endogenous waxy gene, wx012, was selected to be used as an internal control, and qwx012 F/R were employed for wx012 gene quantitative PCR [22]. All primers were synthesized and purified by BGI (Beijing Genomics Institute, China), and the primers sequence are shown in Table 1. The primer locations within the B72-8-11 3′ flanking sequence are shown in Fig. 1.

Construction of genome walker libraries

Total genomic DNA was isolated from seeds by the Plant Genome DNA Extraction Kit (Tiangen Biotech Co. Ltd, China). The quantity and quality of DNA were measured with a NanoDrop ND-2000 spectrometer (Thermo Scientific, USA). The genome walker libraries were constructed using Genome Walker™ Universal Kit (Clontech, USA). B72-8-11 wheat genomic DNA (2 μg) was digested overnight at 37 °C using 80 units of *Pvu* I, *Dra* I, *Stu* I, and *Eco*R V, respectively, in a total volume of 100 μL and was successively purified with the phenol/chloroform extraction protocol. The digested genomic DNAs were ligated to genome walker adaptor as follows: 1× ligation buffer, three units of T4 DNA ligase, 1.9 μL genome walker adaptor

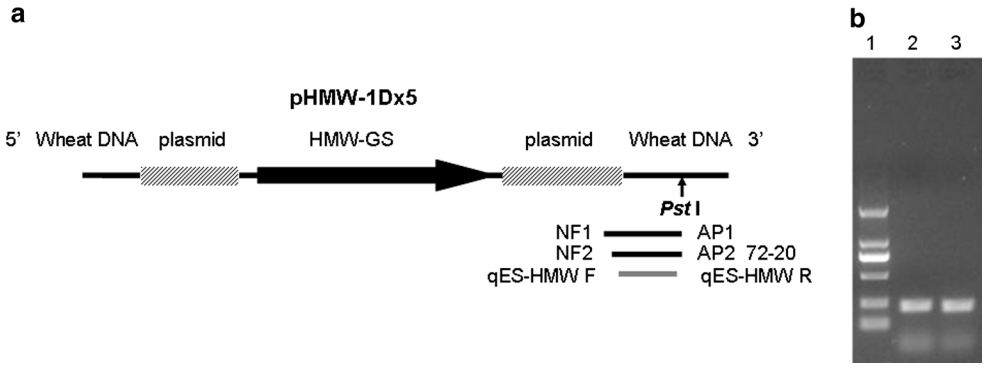

Fig. 1 Schematic diagram of exogenous insert and amplification result of 3′ flanking sequence in GM wheat B72-8-11. **a** Schematic diagram of exogenous insert in GM wheat B72-8-11. "72–20" was amplified in the nested PCR using *Pst* I digested DNA as template with the primers AP2/NF2. The primer pair of qES-HMW F/R was used to amplify the GM wheat B72-8-11 event-specific fragment. **b** Amplification result of the genome walker of 3′ flanking sequence in GM wheat B72-8-11. 1, DL2000 marker; 2 and 3, product of 72–20 with primers of AP2/NF2 (210 bp)

(25 μM), 4 μL digested/purified DNA, incubated at 16 °C overnight. The reaction system was incubated at 70 °C for 5 min to stop the reactions, and then 72 μL ddH_2O was added to the mixture. Amplification products were electrophoresed in 2 % agarose gels for approximately 20 min at 100 V.

Amplification of 3′ flanking sequence

The 3′ flanking sequence of B72-8-11 wheat was identified by the adaptor PCR method described in Genome Walker™ Universal Kit. The experiment was performed with a nested PCR.

The nested PCR was formed by two adaptor PCRs. The first adaptor PCR was performed in a total volume of 20 μL containing 1× Advantage 2 PCR Buffer, 0.25 mM dNTP mix, 0.2 μM AP1/NF1, 1× Advantage 2 Polymerase Mix (Clontech, USA), and 1 μL adaptor ligation of B72-8-11 wheat genomic DNA. The PCR program for the primary adaptor PCR consisted of seven cycles of 25 s at 95 °C followed by 3 min at 72 °C and the following amplification cycles: 95 °C 25 s, 67 °C 3 min for 32 cycles, last step of 7 min at 67 °C. Second adaptor PCR amplification was carried out in a total volume of 20 μL, containing 1× Advantage 2 PCR Buffer, 0.25 mM dNTP mix, 0.2 μM AP2/NF2, 1× Advantage 2 Polymerase Mix, and 1 μL of the 50-fold dilution of the primary PCR products. The PCR program for the second adaptor PCR consisted of five cycles of 25 s at 95 °C followed by 3 min at 72 °C and the following amplification cycles: 95 °C 25 s, 67 °C 3 min for 25 cycles, last step of 7 min at 67 °C. Amplification products were electrophoresed in 2 % agarose gels for approximately 20 min at 100 V.

The amplified fragments in the adaptor PCR amplifications were purified and cloned into pMD18-T vector (TaKaRa, Japan). Fully sequencing analysis of the PCR products was performed by BGI.

Quantitative PCR assay

For event-specific quantitative PCR assay, primers qES-HMW F/R leading to a 182 bp product were amplified. PCR reactions contained 1× SYBR® Premix Ex Taq™ II (TaKaRa, Japan), 200 nM primers, 1× ROX Reference Dye, and 1 μl of the DNA solution. Quantitative PCR was performed using the following program: 30 s 95 °C, 40 cycles of 5 s at 95 °C and 31 s at 60 °C. After PCR amplification, Tm curve analysis condition was as follows: the PCR products were heated to 95 °C during 15 s, cooled at 60 °C for 20 s, and then slowly heated to 95 °C at a rate of 0.2 °C/s. The SYBR Green I assays were performed on Applied Biosystems 7300 Sequence Detection System (ABI, USA). Standard curve for event-specific and wx012 assays was established with five dilutions of DNA from B72-8-11 wheat. Genomic DNA of 100 % B72-8-11 wheat was diluted with ddH_2O to final concentrations equivalent to 100,000, 10,000, 1,000, 100, 20, and 10 copies of haploid genomes/μL; 1 μL of diluted DNA sample was added, and all reactions were repeated three times, each time with triple parallels for each template DNA.

Results and discussions

Characterization of the 3′ flanking sequence of exogenous gene (pHMW1Dx5 vector) in B72-8-11 wheat

In B72-8-11 wheat, 14 copies of transgene (HMW) were estimated by real-time PCR (data not shown). So, only one flanking sequence of the 14 insertion/integration sites of foreign gene was determined, we could develop an event-specific detection method for B72-8-11 according to the discovered flanking fragment. The plasmid pHMW1Dx5

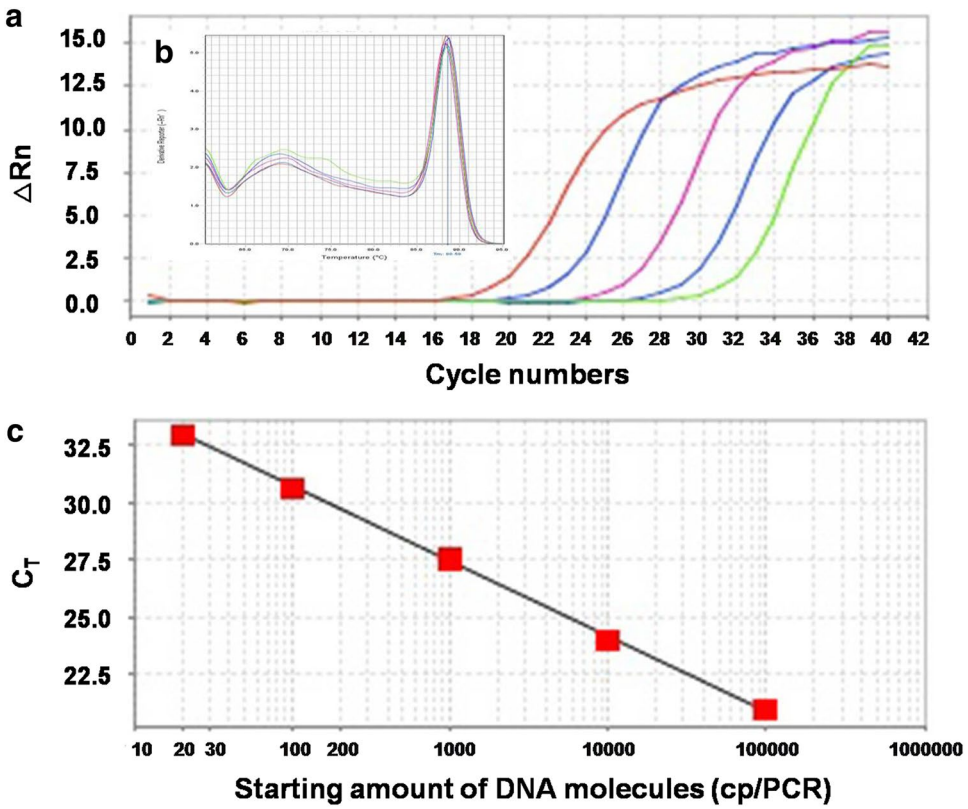

Fig. 2 Amplification plot, melt curve, and standard curve for B72-8-11 event-specific quantitative PCR assay. **a** Amplification plot (serial B72-8-11 DNA dilutions corresponding to 100,000, 10,000, 1,000, 100, and 20 copies of B72-8-11 haploid genomes per reaction as standard samples) was generated for B72-8-11 wheat quantification; **b** melt curve of the standard samples; **c** standard curve of the standard samples

contains a 8.4 kb genomic fragment including the coding sequence of the Glu-D1-1b (HMW) Gene, 3.8 kb 5′ UTR, and 2.2 kb 3′ UTR, respectively [24]. Primers for genome walker were designed based on the known information. In order to obtain the flanking sequence pHMW1Dx5 vector in B72-8-11, a modified adaptor PCR method based on the 3′ junction characterization method described by Genome Walker Kit user manual was used. Adaptor PCR amplifications were performed with combinations of different primers of the HMW gene using B72-8-11 wheat genomic DNA as template. In the nested PCR, one discrete PCR product named as "72–20" of about 200 bp length was amplified using *Pst* I digested DNA as template with the primers AP1/NF1 and AP2/NF2 (Table 1; Fig. 1a), and the amplification result was shown in Fig. 1b.

The sequencing result of the flanking sequence amplicon showed that one 210 bp DNA fragment encompassing the 3′ flanking sequence was obtained, which included one 144 bp sequence of cloning vector and one 66 bp sequence that was not identical to any sequences (including wheat genome DNA) in NCBI database. Also, the results indicated that this 66 bp fragment was a new recombinant wheat genomic DNA, and this flanking sequence was event-specific sequence to wheat B72-8-11. So, this 3′ flanking sequence of wheat B72-8-11 can be used to design primers for event-specific PCR detection.

Specificity of the developed event-specific quantitative PCR detection method for B72-8-11 wheat

Today, many PCR methods were developed for GM wheat e.g., conventional screening PCR of qualitative PCR [25], quantitative PCR [26] for amplification of the endogenous genes of GAG56D and wx012 and extrinsic genes of ubiquitin promotor, NOS terminator, bar and GUS reported gene, event-specific qualitative PCR and quantitative PCR for wheat B73-6-1 [22], and event-specific qualitative PCR for B72-8-11 [23]. However, no event-specific quantitative PCR method was established to detect B72-8-11 as far as we know.

The primers qES-HMW F/R were designed based on the 3′ flanking sequence described above and employed to establish the event-specific quantitative PCR assay by SYBR real-rime PCR for B72-8-11 wheat (Fig. 1a). In the SYBR Green I method, dissociation protocol results well showed that no eventual primer dimers for primers qES-HMW F/R were generated, and the results of Tm curve analysis was shown in Fig. 2b. When B72-8-11 DNA was used in an event-specific quantitative PCR as template, a single and specific amplification plot was observed as expected, and meanwhile, no amplification was detected using non-GM wheat DNA, B73-6-1 wheat, and other GM crops (GTS 40-3-2, A2704-12, MON89788, MON810,

Table 2 Repeatability of the developed B72-8-11 event-specific quantitative PCR assay

Target copies (copies/PCR)	Ct values			Mean		
	1	2	3	Mean Ct	SD	CV (%)
100,000	20.97	20.85	20.98	20.94	0.072	0.344
10,000	24.05	24.09	24.16	24.10	0.057	0.237
1,000	27.30	27.42	27.27	27.33	0.082	0.302
100	30.34	30.66	30.38	30.46	0.178	0.584
20	32.54	32.94	32.67	32.72	0.209	0.637

Bt176, MON863, MON531, MON1445, LLCOTTON25, TT51-1, "Kefeng 6", MS1, RF3, MS8) as templates in the assay, indicating the specificity of this quantitative PCR. In addition, the templates of all selected GM wheat, maize, soy, rice, cotton, and canola have been confirmed by six primer pairs of different housekeeping genes of the crops, respectively [22].

Construction of standard curve and determination of limit of detection and quantification (LOD and LOQ)

To evaluate the quantitative standard curve, repeatability, and LOD or LOQ of the B72-8-11 event-specific quantitative PCR assay, standard samples should be prepared. Considering the haploid genome, size of wheat was estimated to be 16,000 Mbp, corresponding to a molecular weight of 16.6 pg (assuming that 965 Mb weigh 1 pg) [27], five diluted concentrations of 100,000, 10,000, 1,000, 100, 20, and 10 copies haploid genome (corresponding to about 1,660, 166, 16.6, 1.66, 0.332, 0.166 ng) were used as standard samples for the test of determination of the LOD and LOQ and construction of standard curve (Fig. 2a, c).

Construction of standard curve

The standard curve was generated by plotting the observed Ct value against the log initial quantities of diluted genomic DNA, and a linear regression line was fitted to these data. The quantitative PCR amplified results showed that the squared regression coefficient (R^2) of the standard curve was 0.999, and the equations of the standard curve was $y = -3.187x + 36.853$ (Fig. 2c). The amplification efficiency of this reaction could be calculated by the following equation: Efficiency $= 10^{(-1/\text{slope})}-1$, and the calculation result of amplification efficiency was 106 % according to the slope of −3.187 of the equation (the average value of the slope of the standard curve should be in the range of $-3.1 \geq \text{slope} \geq -3.6$) [28]. So, the good PCR amplification efficiency signified that this quantitative PCR system was suitable for B72-8-11 genomic DNA quantification. The R^2 coefficient was the correlation coefficient of a standard curve obtained by linear regression analysis (the average value of R^2 shall be ≥0.98) [28]. Thus, the good linearity ($R^2 = 0.999$) between DNA quantities and fluorescence values (Ct) indicated that these systems were well suitable for quantitative measurements in this study.

Repeatability of quantitative PCR assay

Repeatability of the threshold of detection (Ct) measurements was determined using the above standard DNA dilutions repeated in triplicate (Table 2). The repeatability of quantitative PCR assay could be analyzed by the data shown in Table 2. For the quantitative PCR assay for event-specific exogenous detection, the mean Ct values varied from 20.94 to 32.72 with a standard deviation (SD) value from 0.072 to 0.209 and coefficient of variation (CV) value from 0.344 to 0.637 %. These results indicated that these quantitative PCR assays were stable and reliable.

Determination of LOD and LOQ analysis

There are at least three different ways to express detection and quantitation limits, although they all refer to the lowest quantity of the target that reliably can be detected and quantified with a probability of 95 % [29]. The absolute limit is the lowest number of initial template copies that can be detected and quantified. The relative limit refers to the lowest percentage of GMO relative to the species (e.g., maize) that can be detected and quantified, and the practical limit is the functional limit of the sample during the practical analysis. To determine the LOD and LOQ of the established event-specific real-time PCR assay, a series of DNA dilutions containing an estimated average of 100,000, 10,000, 1,000, 100, 20, and 10 copies of the B72-8-11 haploid genome per reaction were tested in three parallel reactions and repeated three times (Table 3). As expected, the ability to detect B72-8-11 wheat decreased with the decreasing genomic DNA copy numbers and ten copies of B72-8-11 genomic DNA had been detected seven times in a total of nine repeated reactions. The results indicated that the LOD value was about ten copies. The data also showed that the SD values of the nine reactions with the same template concentration increased with the decreasing copy number, especially for the dilution with ten copies of the haploid genome. To obtain reliable quantification results

under ideal conditions, approximately 20 initial template copies were required, and we concluded that the LOQ of the event-specific quantitative PCR assay was 20 copies of the haploid genome (Table 3).

For one ideal quantitative PCR assay, a low LOD and LOQ are very important and necessary, especially for the PCR assays of GMO detection. The application of GM labeling differs among countries, mainly in terms of terminology, inclusion, and exclusion criteria as well as threshold levels that trigger labeling. Thresholds to allow a tolerance for the adventitious presence of approved GMOs with mandatory GM labeling range from 0 % (China), 0.9 % (EU and Russia), 1 % (Brazil, Australia, New Zealand, and Saudi Arabia), 3 % (South Korea) to 5 % (Japan, Indonesia, Taiwan, and Thailand). These thresholds, used to trigger mandatory GM labeling, are not based on health and food safety considerations, but rather on consumer perceptions, practical limits of detection and cost implications [30]. In any case, a low LOD and LOQ are desirable in detection assays in order to also allow the detection of few copies of target DNA in processed food and feed.

To test the LOD of the established event-specific PCR assay, DNAs extracted from different concentration of B72-8-11 wheat mixed with non-GM wheat flours were used as templates. The results indicated that the established event-specific quantitative PCR detection assay of GM wheat B72-8-11 was suitable for the practical detection of GM wheat samples. The LODs were similar to those of GM soybean [31] and GM maize [11], which indicated that the established quantitative event-specific PCR detection system of GM wheat B72-8-11 was suitable for the practical detection of GM wheat samples with high specificity and little labor.

Table 3 Amplification data used to determine the absolute LOD and LOQ

Template copies	Signal rate (positive signals)	Mean Ct	SD	RSD (%)
100,000	9/9	20.88	0.046	0.223
10,000	9/9	24.14	0.066	0.274
1,000	9/9	27.36	0.041	0.150
100	9/9	30.42	0.127	0.418
20	9/9	32.76	0.213	0.650
10	7/9	ND	ND	ND

ND not detected

Quantification of GM B72-8-11 mixed samples

Since there are not processed products containing B72-8-11 wheat that is on sale in markets of China, in this study, to test the applicability of B72-8-11, real-time PCR assay for mixed sample detection, three mixed B72-8-11 wheat DNA samples, that named, S1, S2, and S3 with 6.0, 3.0, and 1.0 %, respectively, were artificially prepared by mixing the pure B72-8-11 DNA with non-GM wheat genomic DNA on a genome/genome basis and were used for quantification in developed B72-8-11 event-specific PCR assay. Hundred nanograms of DNA extracted from mixed samples were used as template in one PCR reaction, and each reaction was analyzed in three parallels. As shown in Table 3, three mixed DNA samples (S1, S2, and S3) with wheat B72-8-11 concentrations of 6.0, 3.0, and 1.0 % gave calculated results of 5.93, 3.14, and 1.05 %, respectively (Table 3). The CV value in Table 4 for 0.5 % mixed sample was 9.57 %, and the high value might be caused by the low GMO concentration. The quantified biases from true values of these three samples were 1.22, 4.82, and 4.67 %, respectively. These results showed that the bias values of practical samples were lower than the acceptance threshold of 25 % of one GMO detection method [28, 32], indicating that the developed B72-8-11 quantitative PCR assay was creditable and suitable for the detection and quantification of GM

Table 4 Quantitative analysis of the B72-8-11 mixed samples

True value (%)	Sample		wx012 quantitative assay		Event-specific quantitative assay		Content (%)	Mean content (%)	SD	CV (%)	Bias (%)
			Ct	Copy number	Ct	Copy number					
6.0	S1	1	22.17	125,936	24.54	7,308	5.80	5.93	0.12	1.95	1.22
		2	22.38	106,556	24.74	6,336	5.95				
		3	22.23	120,126	24.55	7,246	6.03				
3.0	S2	1	21.85	162,294	25.03	5,109	3.15	3.14	0.08	2.48	4.82
		2	22.03	141,338	25.26	4,332	3.07				
		3	21.91	154,641	25.07	4,981	3.22				
1.0	S3	1	22.01	143,665	26.88	1,349	0.94	1.05	0.10	9.57	4.67
		2	22.10	132,897	26.72	1,511	1.14				
		3	22.28	115,919	27.00	1,233	1.06				

B72-8-11 wheat and its derivates. The mixed samples test result indicated that quantitative PCR assay established was stable and reliable.

Conclusion

We identified the 3′ flanking sequence of GM wheat B72-8-11 in this study. Based on this sequence, we developed the event-specific quantitative PCR system for the reliable and accurate detection of B72-8-11 wheat. Due to the specificity of the established system, we believed that this method is a new contribution to the labeling system for GMOs, and additionally, the quantitative PCR technique enriched the event-specific detection method for B72-8-11.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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