Event-Specific Qualitative and Quantitative PCR Detection Methods for Transgenic Rapeseed Hybrids MS1×RF1 and MS1×RF2

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Except for the events RT73, MS8, RF3, and T45, event-specific detection methods for most commercialized genetically modified (GM) rapeseed varieties have not been established, and as a result, the enforcement of genetically modified organism labeling policies has been hindered. The genetically modified rapeseeds, MS1×RF1 and MS1×RF2, are 2 of 11 approved GM-rapeseed varieties for commercialization. In this study, the right border junction fragments between the gene construct and the rapeseed genome of events RF1, RF2, and MS1 were isolated using the commercially available GenomeWalker technology. Homology analysis indicated that the gene construct of RF1 integrated upstream of the nuclease gene, and that of the RF2 and MS1 inserted into the exon region of a gene encoding for an unknown protein. The event-specific primer pairs and corresponding probes were designed on the basis of the revealed right border junction fragments. Then, we successfully developed the identification and quantification methods for the gene-stacked hybrids MS1×RF1 and MS1×RF2 using those primers and probes. The relative limit of detection in the qualitative polymerase chain reaction (PCR) was 0.013% for the RF2 and MS1 assays using 100 ng of rapeseed DNA per reaction and 0.13% for the RF1 assay. The absolute limit of detection in the quantitative PCR was approximately one to two initial copies for each of the three event-specific assays. The evaluation of the real-time PCR assays revealed that the qualitative and quantitative methods developed by focusing on the gene-stacked hybrids MS1×RF1 and MS1×RF2 were highly specific, sensitive, and suitable for samples with a low quantity of DNA.

KEYWORDS: Transgenic rapeseed; event-specific detection; quantification PCR; gene-stacked hybrid; male sterile; fertility restorer

INTRODUCTION

Four major genetically modified (GM) crops are currently being grown worldwide, with soybeans being the most prevalent (60%), followed by maize (24%), cotton (11%), and rapeseeds (5%) (*1, 2*). Rapeseed (*Brassica napus*) is one of the most studied plants for genetic engineering; until now, at least 11 GM rapeseed varieties have been approved for commercial production. Transgenic rapeseed varieties, such as MS1×RF1, MS1×RF2, MS8×RF3, and GT73, have been commercially cultivated only in Canada and in the United States but have been exported to other countries including China, Japan, and Mexico (*3*).

With the commercialization of genetically modified organisms (GMOs), the use of GMOs as food and in food products is becoming more and more widespread; many countries or regions have successfully instituted market-specific labeling laws which stipulate when a product containing approved GMO traits must be labeled as such (*4, 5*). For example, the EU regulations 1829/2003 and 1830/2003 stipulate that a product must be labeled when an approved GMO trait threshold of 0.9% is reached (*6, 7*), while Korea allows for 3% (*8*), and 5% is allowed in Japan and Taiwan (*9, 10*). Additionally, the implementation of a zero-tolerance policy in China disallows unapproved GM varieties from entering the market. Enforcing the labeling laws requires the development and the validation of GMO detection methods for foods and feed that are sensitive, reliable, standardized, specific, and quantitative. The European Union has supported several research programs, such as the "QPCRGMOFOOD" project and the "development of methods to identify foods produced by means of genetic engineering" (SMT4-CT96–2072), mainly aimed at developing detection methods for GMOs (*11–13*).

Currently, the most commonly used DNA-based detection methods involve amplification of a specific DNA fragment by the polymerase chain reaction (PCR) technique, which can be categorized into four levels of specificity: (*1*) screening methods, (*2*) gene-specific methods, (*3*) construct-specific methods, and

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Table 1. Oligonucleotide Primers and TaqMan Probes

PCR system	primer name	sequence (5′–3′)	location
GenomeWalker PCR	AP1[a]	GTAATACGACTCACTATAGGGC	adapter
	AP2[a]	ACTATACCCCACGCGTGGT	adapter
	RB1	GGATCCCCCGATGAGCTAAGCTAGC	3′g7 terminator
	RB2	GCTTGGACTATAATACCTGACTTG	3′g7 terminator
	LB1	AGATTCCTTGAAGTTGAGTATTGGC	3′ocs terminator
	LB2	AAAACCAACGGCTCAGACTTACCAG	3′ocs terminator
Reamplification PCR	Grf1	TTCAGATTTGACCAAGAGGGA GTA	rapeseed genome
	Grf2	TACCAATGTTCCTGCCTTCCA	rapeseed genome
	Gms1	ACGGTGGTTTCTGTCTTACTCG	rapeseed genome
RF1 qualitative and quantitative PCR	RF1R	GGTGACTACACGCGACTCAT	rapeseed genome
	RF1F	AGACCTCAATTGCGAGCTTTCTAAT	gene construct
	RF1P	CATCCTCACCCAGTCAGCATCATCAC	junction site of RF1
RF2 qualitative and quantitative PCR	RF2R	ATCGACGTATATATAGCTGTGCCAG	rapeseed genome
	RF2F	CTGTGGTCTCAAGATGGATCATTAA	gene construct
	RF2P	CTCACCGGCCAAATTCGCTCTTAGCCG	junction site of RF2
MS1 qualitative and quantitative PCR	MS1R	ATCTCTGGTTAAACATTCCATCTTTG	rapeseed genome
	MS1F	CGAGCTTTCTAATTTCAAACTATTCGG	gene construct
	MS1P	TGGATAGGTTCTTCAGCATCATCACACC	junction site of MS1
PCR for endogenous gene	FatA primer 1[b]	GGTCTCTCAGCAAGTGGGTGAT	junction between intron and exon of FatA
	FatA primer 2[b]	TCGTCCCGAACTTCATCTGTAA	exon of FatA
	FatA probe[b]	ATGAACCAAGACACAAGGCGGCTTCA	exon of FatA

[a] Provided by the BD GenomeWalker Universal Kit. [b] Developed by Monsanto Company.

(*4*) event-specific methods (*14, 15*). Since different GMOs may share several genetic elements and sometimes even contain the same transfer DNA (T-DNA) (e.g., the two distinct GM-maize Bt10 and Bt11 and GM-rapeseed RF1 and RF2), the gene-specific and construct-specific methods cannot distinguish among the different GMOs (*16, 17*). Event-specific methods offer the highest levels of specificity since their detection target is a unique junction located at the integration locus between the inserted DNA and the recipient genome (*12, 17–19*). PCR-based quantification is the most commonly used technology for the quantification of GMOs, and real-time PCR methods combine event-specific and reference gene assays into estimating GMO content. This approach provides the most accurate and reproducible method for detecting the presence of transgenic traits in grains and oilseeds (*15*).

Currently, the event-specific identification and quantification methods for GM maize, GM soybeans, and GM cotton have been established including GM-maize Bt11 (*18, 20*), Mon810 (*19, 21*), CBH-351 (*22*), GA21 (*23*), Bt176 (*24*), Mon863 (*25, 26*), and so forth; GM-soybean GTS-40-3-2 (*17, 27, 28*); and GM-cotton Mon531, Mon1445 (*29*), and Mon15985 (*30*). For GM rapeseed, only the event-specific quantification methods for GT73, T45, MS8, and Rf3 have been developed (*24, 31–35*), and the event-specific detection methods for other GM-rapeseed cultivars such as MS1×RF1 and MS1×RF2 have not been reported.

The gene-stacked rapeseed MS1×RF1 is derived from a cross between the MS1 line and the RF1 line, and the MS1×RF2 hybrid is derived from a cross between the MS1 line and the RF2 line (*36, 37*). The RF1 and RF2 lines were transformed from the same plasmid, that is, pTVE74RE (*38*). The MS1 is a transgenic male sterile line with the foreign gene *barnase* for male sterility and *bar* for glufosinate tolerance. RF1 and RF2 are transgenic fertility restorer lines which came from different transgenic events, and all contain the foreign genes *bastar* for fertility restoration and *bar* for glufosinate tolerance. The aim of this study is to explore the flanking sequence of the foreign DNA in genomes of the male sterile lines MS1 and the fertility restorer lines RF1 and RF2, using the GenomeWalker strategy, in order to establish event-specific identification and quantification methods for these events and their derivative hybrids.

MATERIALS AND METHODS

Plant Materials. Genuine seeds of the GM-rapeseeds (*Brassica napus*) MS1×RF1, MS1×RF2, MS8×RF3, T45, Topas, and OXY235 were kindly provided by Bayer CropScience; the seeds of GT73 were provided by Monsanto Company; the seeds of the non-GM rapeseed Zhongyou 821, *Brassica rapa*, *Brassica oleracea*, *Arabidopsis thaliana*, and *Glycine max* (soybean) are stored by our own laboratory; the seeds of *Zea mays* (maize), *Oryza sativa* (rice), and *Gossypium hirsutum* (cotton) were purchased from a local market in Wuhan, China.

DNA Extraction and Purification. The seeds used for the DNA extraction were germinated in a greenhouse. Large-scale genomic DNA was isolated from 4 g of young leaves according to the protocol of Saghai-Maroof with minor modifications (*39*). DNA samples from rapeseed seeds were extracted using the DNA Extraction Kit for GMO Detection Version 2.0 (Takara, Shiga, Japan). The DNA purification and concentration were quantified using the UV/vis spectrophotometer Lamda 25 (Perkin Elmer, U.S.A.). Concentrations were further assessed by agarose gel electrophoresis and ethidium bromide staining. UV-fluorescent emission was recorded and quantified using the Quantity One software (Bio-rad Laboratories Inc., Hercules, CA).

Isolation of Junctions in Events MS1, RF1, and RF2. The BD GenomeWalker Universal Kit (Clone Tech.) was used to isolate the junctions between the inserted T-DNA and the rapeseed genome in the transgenic rapeseeds MS1×RF1 and MS1×RF2 according to the BD GenomeWalker Universal Kit User Manual. Two gene-specific primers (GSPs) at the left and right borders of the T-DNA were designed, respectively. The MS1, RF1, and RF2 lines were transformed using the same Ti-plasmid except for the different target genes. Then, the degenerate nested primers at the left border and right border of T-DNA were designed to isolate the genome sequences flanking the inserted T-DNA region. Two GSP primers, LB-1 and LB-2, located at the left border of the T-DNA, were designed to anneal at the 3′ OCS terminator, and two GSPs, RB-1 and RB-2, at the right border of T-DNA, were designed to match the 3′g7 terminator. The sequences and locations of the GSPs are shown in **Table 1** and **Figure 1** in detail.

According to the Kit User Manual described, slight modifications of the nested PCR reaction profile for the rapeseed genome were made as follows: The primary PCR reaction was performed in a 50 μL reaction volume containing 1 μL of the DNA library as a template, 1 × BD Advantage 2 PCR Buffer, 200 μM dNTP's, 0.2 μM primer AP1, 0.2 μM GSP1 primer (LB-1 or RB-1), 1 μL of BD Advantage 2 Taq DNA Polymerase, and water, as needed, to establish a final volume of 50 μL. The PCR cycle profile entailed the following: 3 min at 94 °C, 45 cycles of 15 s at 94 °C, 3 min at 68 °C, and terminal elongation for 7 min at 68 °C. Secondary PCR amplifications were conducted with 1

Figure 1. Schematic diagram of the inserted T-DNA region of gene construct for events RF1, RF2, and MS1. The arrows show the location and orientation of primers. Adjacent solid lines represent the rapeseed genome.

μL of a 50-fold dilution of the primary PCR products. The secondary PCR reaction volume was similar to that used during the first PCR amplification, except for the use of the primer AP2 and a nested primer GSP2 (LB-2 or RB-2). The second amplification step of the secondary PCR was decreased from 45 cycles to 25 cycles. A total of 5 μL of the secondary amplification product was detected on agarose gel using ethidium bromide staining. If the visible bands were amplified, the PCR products were purified and subsequently cloned into the pZErO-2 vector (Invitrogen, Carlsbad, CA) through the use of the *Eco*RV restriction enzyme sites for sequencing. Then, a homology analysis and a BLASTn search were performed using the sequencing data as the query.

Primers and TaqMan Probes. For each transgenic event, using the Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA), one primer annealing in the rapeseed genome DNA was designed in combination with the primer RB-2 to reamplify the junction fragment (**Table 1**). The primer pair RB2/Grf1 (591 base pair [bp] amplicon) was employed to reamplify the junction fragment of the RF1 event; RB2/Grf2 (594 bp amplicon) was used for the RF2 event, and RB2/Gms1 (713 bp amplicon) was used for the MS1 event. The sequences of the primers are shown in **Table 1**. The position of the primers was depicted using arrows in **Figure 1**.

Beacon Designer 2.12 software (PREMIER Biosoft International, Palo Alto, CA) was used to design the oligonucleotides. Primer sets with corresponding probes for the event-specific assay were designed for the right border junction fragments between the host DNA and the insert DNA, specifying an optimal melting temperature of about 60 °C for the primers, and about 70 °C for probes. One forward primer located at the insert gene construct, a reverse primer annealed to the rapeseed flanking genomic sequence, and their corresponding probe spanned the integration site of the transgenic construct (**Figure 2**). The probes were labeled with 5′-FAM (6-carboxyfluorescein) and 3′-TAMRA (6-carboxy-tetramethylrhodamine). The primers and probes were synthesized by Sangon (Shanghai, China). The primer sets MS1R/MS1F, RF1R/RF1F, and RF2R/RF2F were used for the qualitative and quantitative PCR assays of the events MS1, RF1, and RF2, separately yielding a 141 bp amplicon, a 99 bp amplicon, and a 138 bp amplicon. The primer pair MS1R/MS1F was combined with the TaqMan probe MS1P for the MS1 real-time PCR assay; RF1R/RF1F was combined with RF1P for the RF1 PCR assay, and RF2R/RF2F was combined with RF2P for the RF2 PCR assay. The acyl-ACP thioesterase (FatA) gene was used as a reference gene for rapeseed; the sequences of the reference-gene-based primers and corresponding probe are described in **Table 1** (*31*).

PCR Reactions. The reamplification PCR was conducted using the following volumes of reagents: 1 μL of the eluted genetically modified rapeseed genomic DNA (100 ng), 1 × PCR-buffer (10 mM Tris-HCl pH 8.3; 50 mM KCl and 1.5 mM MgCl2), 250 μM dNTP's, 0.3 μM primer annealing in the construct and 0.3 μM primer annealing in the genome, and one unit of Taq DNA polymerase (Fermentas). Water

Figure 2. The event-specific PCR assay design for events RF1, RF2, and MS1. **(a)** Partial sequence of the RF1 junction fragment used to design event-specific primer pairs and the TaqMan probe. **(b)** Partial sequence of the RF2 junction fragment used to design event-specific primer pairs and the TaqMan probe. **(c)** Partial sequence of the MS1 junction fragment used to design event-specific primer pairs and the TaqMan probe. Lowercase letters represent the sequence of the T-DNA region, and capital letters show the flanking genomic sequence. Primers and probes used for the PCR assay are bold and underlined.

was added to a final volume of 50 μL. The PCR cycle profile included the following: 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 90 s at 72 °C, and a terminal elongation of 5 min at 72 °C.

The qualitative PCR reactions were all performed in 25 uL reaction volumes containing 100 ng of genomic DNA, 1 × PCR buffer, 2.5 mM MgCl2, 200 μM dNTP's, 0.25 μM primers, and one unit of Taq (Takara, Shiga, Japan). Amplifications were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) under the following conditions: first, denaturation for 2 min at 94 °C; 35 cycles of 20 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C; and terminal elongation for 2 min at 72 °C. The PCR products were size-fractionated using gel elctrophoresis on a 2% agarose gel in 1 × TAE buffer and stained with ethidium bromide. UV-fluorescent emission was recorded using the Quantity One software (Bio-rad Laboratories Inc., Hercules, CA). The reproducibility of PCR patterns was verified by triplicate experiments.

The quantitative PCR reactions were all carried out on a fluorometric thermal cycler (DNA Engine Opticon 2 Continuous Fluorescence Detector, MJ Research, Waltham, MA) in a final volume of 20 μL. Fluorescence signals were monitored and analyzed at the annealing step during every PCR cycle using Opticon Monitor 2 Version 2.02 software.

The real-time PCR reaction system of the RF1 assay contained 1 × PCR TaqMan buffer A; 3.5 mM MgCl2; 400 μM each dATP, dCTP, and dGTP; 800 μM dUTP; 300 nM primers (RF1F/RF1R); 150 nM probe (RF1P); 1.25 units of AmpliTaq Gold DNA polymerase; 0.2 units

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of AmpErase uracil *N*-glycosylase (UNG); and 2 μL of the DNA solution (100 ng).

The real-time PCR system for the RF2 assay contained the following reagents: 1 × PCR TaqMan buffer A; 3.5 mM MgCl$_2$; 400 μM each dATP, dCTP, and dGTP; 800 μM dUTP; 400 nM primers (RF2F/RF2R); 200 nM probe (RF2P); 1.25 units of AmpliTaq GoldTM DNA polymerase; 0.2 units of AmpErase uracil *N*-glycosylase (UNG); and 2 μL of the DNA solution (100 ng).

The real-time PCR reaction volume for the MS1 assay contained 1 × PCR TaqMan buffer A; 3.5 mM MgCl$_2$; 200 μM each dATP, dCTP, and dGTP; 400 μM dUTP; 300 nM primers (MS1F/RF1R); 150 nM probe (MS1P); 1.25 units of AmpliTaq GoldTM DNA polymerase; 0.2 units of AmpErase uracil *N*-glycosylase (UNG); and 2 μL of the DNA solution (100 ng).

The real-time PCR for the FatA gene was performed according to a protocol slightly modified from the Monsanto publication (34 Monsanto). The reaction mixture contained 100 ng of genome DNA as a template; 1 × TaqMan buffer (50 mM KCl, 10 mM Tris-HCl, 10 mM EDTA, pH 8.3); 5 mM MgCl$_2$; 150 nM primer FatA primer 1 and FatA primer 2; 50 nM FatA probe; 200 μM each dATP, dCTP, and dGTP; 400 μM dUTP; 0.2 units of UNG; and 1.25 units of AmpliTaq Gold DNA polymerase in a total volume of 20 μL.

All real-time PCR reactions were carried out using the same program: a predigest step at 50 °C for 2 min, a 95 °C initial denaturation, and a UNG deactivation step for 10 min, followed by 50 cycles of 95 °C denaturation for 15 s, a 60 °C annealing and extending step for 1 min, and a fluorescence measurement after annealing and extending. The real-time PCR was repeated three times, each time with triple-replication by using three reaction wells for each templated DNA.

Calibration Curves. Standard solutions for the RF1 and MS1 assays were prepared by combining purified genomic DNA from MS1×RF1 with that from non-GM rapeseed. Standard solutions for the RF2 and MS1 assays were prepared by combining purified genomic DNA from MS1×RF2 with that from non-GM rapeseed. The final concentrations of MS1×RF1 (MS1×RF2) DNA in the standard solutions were 50, 6.5, 0.65, 0.065, and 0.0065 ng/μL. To prepare standard solutions for the FatA gene, genomic DNA from conventional rapeseeds was serially diluted by salmon sperm DNA to final concentrations of 50, 6.5, 0.65, 0.065, and 0.0065 ng/μL. The concentration of the total DNA in all of the standard solutions was 50 ng/μL. The calibration curves for the amplicons of RF1, RF2, and MS1 were established with the standard solutions described above, and the calibration curves for the FatA amplicon was established with serial dilutions of conventional rapeseed (containing approximately 100 to 0.013 ng of template DNA per PCR reaction).

RESUTLS AND DISCUSSION

Characterization of Junctions in Events MS1, RF1, and RF2. The isolation of the junction sequence at the integration sites should be performed using the lines MS1, RF1, and RF2. In this study, the gene-stacked hybrids MS1×RF1 and MS1×RF2 are used as experimental materials to isolate the junctions between inserted DNA and the recipient genome of events MS1, RF1, and RF2 since the parental lines MS1, RF1, and RF2 are not commercialized.

After the nested PCR products were sequenced, it was found that two DNA fragments of 1165 bp (Genbank accession No. EU090198) and 3192 bp (Genbank accession No. EU090199) in length were successively amplified from MS1×RF1 genomic DNA with primer sets AP1/RB1 and AP2/RB2, and two PCR fragments of 1165 bp (Genbank accession No. EU090198) and 2943 bp length (Genbank accession No. EU090197) were also amplified from MS1×RF2 genomic DNA with the primer sets described above. Sequence alignment showed that the two 1165 bp fragments from MS1×RF1 and MS1×RF2 were of the same origin with 100% homology. Since the transgenic hybrids MS1×RF1 and MS1×RF2 have the same female parent MS1 line, it was speculated that the 1165 bp should be the putative
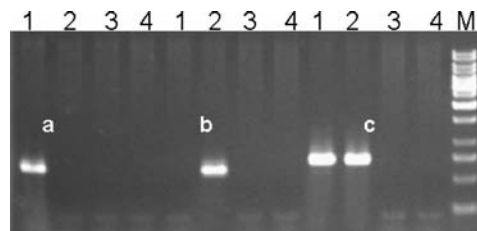

Figure 3. Confirmation of the isolated junction sequences of transgenic events RF1, RF2, and MS1. **(a)** PCR product with primer pair of RB2/Grf1; **(b)** PCR product with primer pair of RB2/Grf2; **(c)** PCR product with primer pair of RB2/Gms1. Sample order: lane 1, MS1×RF1; lane 2, MS1×RF2; lane 3, MS8×RF3; lane 4, zhongyou 821; lane M, 1 kb marker.

junction fragment connecting the right border of T-DNA and the rapeseed DNA in the MS1 event. The 3192 bp fragment only existed in the MS1×RF1 genome, and the 2943 bp fragment only existed in the MS1×RF2 genome. It was shown that the two fragments were the right border junction fragments of events RF1 and RF2, respectively. Several PCR products containing different lengths were amplified from MS1×RF1 and MS1×RF2 genomic DNA with primer pairs AP1/LB1 and AP2/LB2. The sequenced results indicated that the multiple fragments were nonspecific amplifications. The nested PCR reactions were carried out again after the PCR reaction system and program were modified. The left border junction fragments of events MS1, RF1, and RF2 were not obtained. It was speculated that genome rearrangements and a large deletion of the left border may have occurred during the integration of the DNA insert, making it difficult to isolate the left border junction fragments of the three events (*19, 40*).

Sequence alignment between the T-DNA insert sequence and isolated junction fragments suggested that a 3192 bp junction fragment of RF1 contained 330 bp of insert DNA and 2862 bp of noninsert DNA. A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) showed that part of the noninsert DNA had over 80% similarity with *A. thaliana* mRNA (accession numbers NM128588, NM128586, etc.) and a BAC clone (AC002338). The homologous mRNA NM128588 encodes nuclease with the function of DNA repair. Alignment between mRNA and noninsert DNA revealed that the gene construct of RF1 integrated into the upstream region of the nuclease gene. The 2943 bp junction fragment of RF2 contained 264 bp of a T-DNA sequence and 2679 bp of a non-T-DNA sequence displaying similarity to the full length cDNA (BX825005), mRNA (BT021122, NM104451, etc.), and the BAC clones (AC002328 and AL132962) from *A. thaliana*. A total of 66 bp of the T-DNA sequence were deleted during the integration of the construct. The 1165 bp fragment of MS1 is made up of 330 bp of insert DNA and 835 bp of noninsert DNA homologous to *A. thaliana* mRNA (NM101554) and BAC clones (AC026237, AC051629, etc). Alignment data between *Arabidopsis* mRNA and noninsert DNA of MS1 and RF2 suggested that their transgenes may randomly integrate in the ORF of the rapeseed gene homologous to the mRNA of *Arabidopsis*, respectively. But the function of the putative proteins encoded by the interrupted genes is still unknown.

Specific primers annealing in the flanking sequence of T-DNA were designed with the primer RB2 to verify that the above isolated fragments truly span the junctions between the transgenic DNA and the rapeseed DNA. As shown in **Figure 3**, primer set Grf1/RB2 was employed to generate the expected band from only MS1×RF1, and no amplified fragments were obtained from the other two hybrids (MS1×RF2 and MS8×RF3)
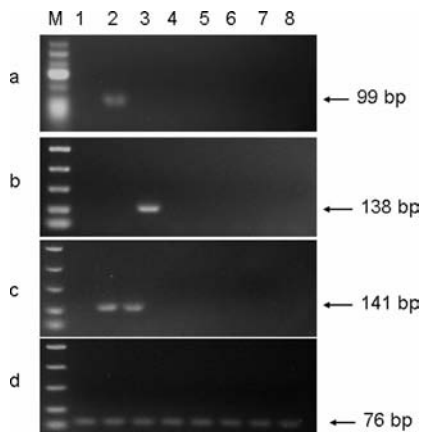
Figure 4. Event-specific detection of seven transgenic rapeseed samples using event-specific qualitative PCR. PCR products were separated through electrophoresis on 2% agarose gel. Arrowheads indicate the expected length of the PCR amplified band. **(a)** The RF1 assay; **(b)** the RF2 assay; **(c)** the MS1 assay; **(d)** the rapeseed endogenous gene FatA. Lane M, 100 bp marker; lane 1–8 correspond to MS8×RF3, MS1×RF1, MS1×RF2, OXY235, T45, Topas, GT73, and nontransgenic rapeseed, respectively.

or the wild-type rapeseed Zhongyou 821. Similarly, primer pair Grf2/RB2 only gave rise to the specific expected band from MS1×RF2. The MS1 male sterile line was the common female parent of MS1×RF1 and MS1×RF2. As expected, specific bands were observed from MS1×RF1 and MS1×RF2 with the primer pair RB2/Gms1, simultaneously. The reamplification fragments were purified and sequenced. The sequencing results and the specificity of amplification further confirmed that the above isolated fragments truly spanned the insert site of the transgenes.

Development of Qualitative PCR. DNA of processed foods is always degraded (i.e., fragment sizes less than 400 bp in highly processed food) and cannot be reliably detected by PCR (*10, 41*). So, it is recommended by ISO 21569 to design primers for the PCR detection of processed foods to yield amplicons in the range of 60–150 bp (*42*). Different primer sets were designed to develop a qualitative PCR test that would target the junction fragments of MS1, RF1, and RF2. Primer pairs that produced a single band with high intensity were chosen to develop the event-specific qualitative PCR assay. We selected primer sets yielding amplicons of 76 bp for the endogenous *FatA* gene and 99, 138, and 141 bp for the RF1, RF2, and MS1 event-specific junctions, respectively.

The specificity of each qualitative PCR assay was assessed by running PCR on 100 ng of template DNA from GM-rapeseed varieties MS1×RF1, MS1×RF2, MS8×RF3, T45, Topas 19/2, OXY235, GT73, and non-GM rapeseed Zhongyou 821. The primer pair of FatA primer 1/FatA primer 2 for the endogenous FatA gene was used to amplify the expected fragments in all GM-rapeseed DNA and Zhongyou 821. No fragment was observed from the reagent blank samples (**Figure 4d**). As shown in **Figure 4**, the expected amplification was observed. The primer set RF1F/RF1R produced the expected amplicon from a sample containing only MS1×RF1 (**Figure 4a**); RF2F/RF2R amplified the expected band from a sample containing only MS1×RF2 (**Figure 4b**), and MS1F/MS1R yielded the expected amplicons from samples containing either MS1×RF1 or MS1×RF2 (**Figure 4c**). We further tested different plant materials, including *B. rapa*, *B. oleracea*, *A. Thaliana*, soybeans, maize, rice, and cotton, and no PCR product was detectable with the three event-specific primer sets (data
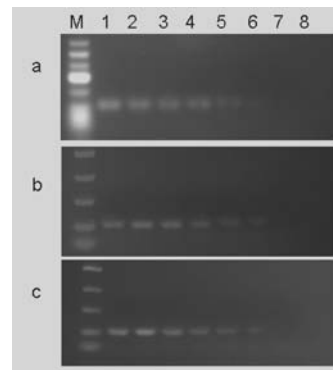

Figure 5. Sensitivity tests of event-specific primer pairs of RF1, RF2, and MS1. **(a)** Amplification of serial dilutions of the MS1×RF1 genome DNA using the RF1 PCR assay; **(b)** PCR reaction with serial dilutions of the MS1×RF2 genome DNA using the RF2 PCR assay; **(c)** PCR reaction with serial dilutions of the MS1×RF1 genome DNA using the MS1 PCR assay. Lane M, 100 bp DNA marker; lanes 1–7 correspond to 100 ng, 50 ng, 13 ng, 1.3 ng, 130 pg, 13 pg, and 1.3 pg MS1×RF1 or MS1×RF2 DNA.

Table 2. DNA Weight Used for Templates and Calculation of Corresponding Target Copy Numbers

total DNA (ng)	containing of MS1RF1 or MS1RF2 (ng)	copy number of MS1 event	copy number of RF1 or RF2 event
100	100	19353	38706
100	50	9676	19353
100	13	2515	5031
100	1.3	251	503
100	0.13	25	50
100	0.013	2.5	5
100	0.0013	0.25	0.5

not shown). The PCR amplification results confirmed that the event-specific PCR systems are highly specific and can accurately discriminate among the three events: MS1, RF1, and RF2. The events MS1 and RF1 in hybrid MS1×RF1 samples were detected, and the MS1 and RF2 in MS1×RF2 samples were detected, simultaneously.

The limit of detection (LOD) was established for the method by diluting DNA containing 100 ng, 50 ng, 13 ng, 1.3 ng, 130 pg, 13 pg, and 1.3 pg of GMO contents into 100 ng of rapeseed DNA per reaction. The results showed that 13 pg (0.013%) of RF2 and MS1 provided a discernable signal in the qualitative assays (**Figure 5b,c**), and 130 pg (0.13%) did so for the RF1 qualitative assay. The common female parent MS1 line of MS1×RF1 and MS1×RF2 is heterozygous, and the RF1 and RF2 lines are homozygous. According to Mendelian inheritance, The MS1×RF1 (MS1×RF2) F1 generation contained the MS1 target sequence in a 1:4 ratio (= 25%) relative to the number of haploid genomes and contained the RF1 (RF2) target sequence in a 1:2 ratio (= 50%) relative to the number of haploid genomes. Considering that 1187 Mb per haploid genome, in the case of rapeseed, corresponds to 1.3 pg (*43*), the copy number of event MS1, RF1, and RF2 per reaction tube was calculated, as shown in **Table 2**. Therefore, the lowest number of copies we were able to detect was estimated to be 2.5 initial template copies for the MS1 assay, 5 initial template copies for the RF2 assay, and 50 initial template copies for the RF1 assay. In practice, when the contents of MS1×RF1 and MS1×RF2 were over 0.13%, reliable results could be obtained upon application of the established event-specific PCR assays.

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Table 3. Repeatability and Reproducibility of RF1, RF2, and MS1 Real-Time PCR Assays

DNA amount (ng)	copy number[a]	repeat	Ct value 1	Ct value 2	Ct value 3	mean of Ct values	SD[r]	RSD[r] (%)	mean of all Ct values	SD[R]	RSD[R] (%)
					(a) RF1 Real-Time PCR Assay						
100.000	38706	1	26.672	26.617	26.708	26.666	0.046	0.172	26.627	0.203	0.763
		2	26.807	26.936	26.68	26.808	0.128	0.477			
		3	26.666	26.417	26.138	26.407	0.264	1.000			
13.000	5031	1	29.617	29.553	29.504	29.558	0.057	0.192	29.628	0.077	0.259
		2	29.638	29.482	29.73	29.617	0.125	0.423			
		3	29.645	29.795	29.69	29.710	0.077	0.259			
1.300	503	1	32.603	32.835	32.775	32.738	0.120	0.368	32.716	0.086	0.264
		2	32.664	32.787	32.412	32.621	0.191	0.586			
		3	32.567	32.939	32.863	32.790	0.197	0.599			
0.130	50	1	35.474	35.570	35.740	35.595	0.135	0.378	35.692	0.103	0.288
		2	35.745	35.816	35.483	35.681	0.175	0.492			
		3	35.853	35.316	36.23	35.800	0.459	1.283			
0.013	5	1	39.213	39.458	38.817	39.163	0.323	0.826	38.492	0.674	1.750
		2	38.477	37.912	39.102	38.497	0.595	1.546			
		3	38.36	37.804	37.282	37.815	0.539	1.426			
					(b) RF2 Real-Time PCR Assay						
100.000	38706	1	24.103	24.324	24.306	24.244	0.123	0.506	23.925	0.277	1.156
		2	23.623	23.921	23.783	23.776	0.149	0.627			
		3	23.692	24.164	23.41	23.755	0.381	1.604			
13.000	5031	1	27.338	27.318	27.088	27.248	0.139	0.510	27.287	0.048	0.176
		2	27.284	27.152	27.383	27.273	0.116	0.425			
		3	27.434	27.448	27.141	27.341	0.173	0.634			
1.300	503	1	30.619	31.014	30.351	30.661	0.334	1.088	30.714	0.046	0.150
		2	31.086	30.551	30.606	30.748	0.294	0.957			
		3	30.791	30.617	30.788	30.732	0.100	0.324			
0.130	50	1	33.589	33.787	33.783	33.720	0.113	0.336	33.783	0.113	0.334
		2	33.557	33.539	34.053	33.716	0.292	0.865			
		3	34.042	33.676	34.023	33.914	0.206	0.608			
0.013	5	1	36.410	36.767	37.093	36.757	0.342	0.929	36.768	0.450	1.224
		2	35.575	36.367	37.03	36.324	0.728	2.005			
		3	37.581	36.948	37.142	37.224	0.324	0.871			
					(C) MS1 Real-Time PCR Assay						
100.000	19353	1	25.871	26.001	25.932	25.935	0.065	0.251	26.058	0.114	0.439
		2	25.898	26.206	26.134	26.079	0.161	0.618			
		3	26.148	26.161	26.172	26.160	0.012	0.046			
13.000	2515	1	28.962	28.788	29.025	28.925	0.123	0.424	28.976	0.092	0.317
		2	28.798	29.115	28.849	28.921	0.170	0.589			
		3	28.962	28.957	29.326	29.082	0.212	0.728			
1.300	251	1	31.691	32.024	31.843	31.853	0.167	0.523	32.113	0.287	0.894
		2	31.992	32.036	32.167	32.065	0.091	0.284			
		3	32.365	32.551	32.347	32.421	0.113	0.348			
0.130	25	1	34.613	34.549	34.195	34.452	0.225	0.653	34.415	0.068	0.199
		2	34.564	34.111	34.694	34.456	0.306	0.888			
		3	34.504	34.185	34.319	34.336	0.160	0.467			
0.013	2.5	1	37.673	36.815	37.173	37.220	0.431	1.158	37.014	0.570	1.539
		2	35.46	36.724	36.925	36.370	0.794	2.184			
		3	36.169	37.758	38.426	37.451	1.159	3.096			

Development of Real-Time PCR Assays. The primer sets used for qualitative PCR were selected to establish the MS1, RF1, and RF2 event-specific quantitative PCR assays by comparing the fluorescent signal intensity and amplification repeatability. Røning et al. recommended that an ideal event-specific detection system should consist of one primer located in the transformed construct and one located in the recipient DNA and an event-specific probe that spans the junction (*20*). The event-specific detection system developed in this study contained all of the ideal primers and probe sets and had a high specificity (**Figure 2**). To determine the specificity of the three methods, we amplified several GM-rapeseed varieties (MS1×RF1, MS1×RF2, MS8×RF3, T45, Topas, OXY235, and GT73) and different plant species (*B. rapa*, *B. oleracea*, *A. Thaliana*, soybeans, maize, rice, and cotton). Detectable fluorescent signals were only observed from the DNA of the MS1×RF1 and MS1×RF2 hybrids.

Sensitivity and Repeatability of Real-Time PCR Assays. The absolute LODs for the quantitative assays were evaluated on the basis of three parallel real-time PCR analyses using the serial dilutions of genome DNA from gene-stacked hybrids containing 19353, 2515, 251, 25, 2.5, and 0.5 molecules for the MS1 event and 38706, 5031, 503, 50, 5, and 1 molecule for the RF1 and RF2 events per reaction. The experiment revealed that the ability to detect the corresponding events decreased as the initial template copy number decreased. The RF1 and RF2 events can be detected in all three parallel samples down to five copies, while only one of the three parallel samples was positive when using template concentrations as low as one copy; the MS1 event can be detected in three parallel samples down to 2.5 copies, while no parallel sample tested positive when using a template concentration of 0.5 copies (data not shown). These results concur with the Poisson distribution model. The lowest copy number we were able to detect was estimated to one to two initial template copies for the three quantitative PCR assays. Due to the large background of nontarget DNA and lower purity, the number of initial template copies should increase rapidly (*20*) in order to obtain reliable
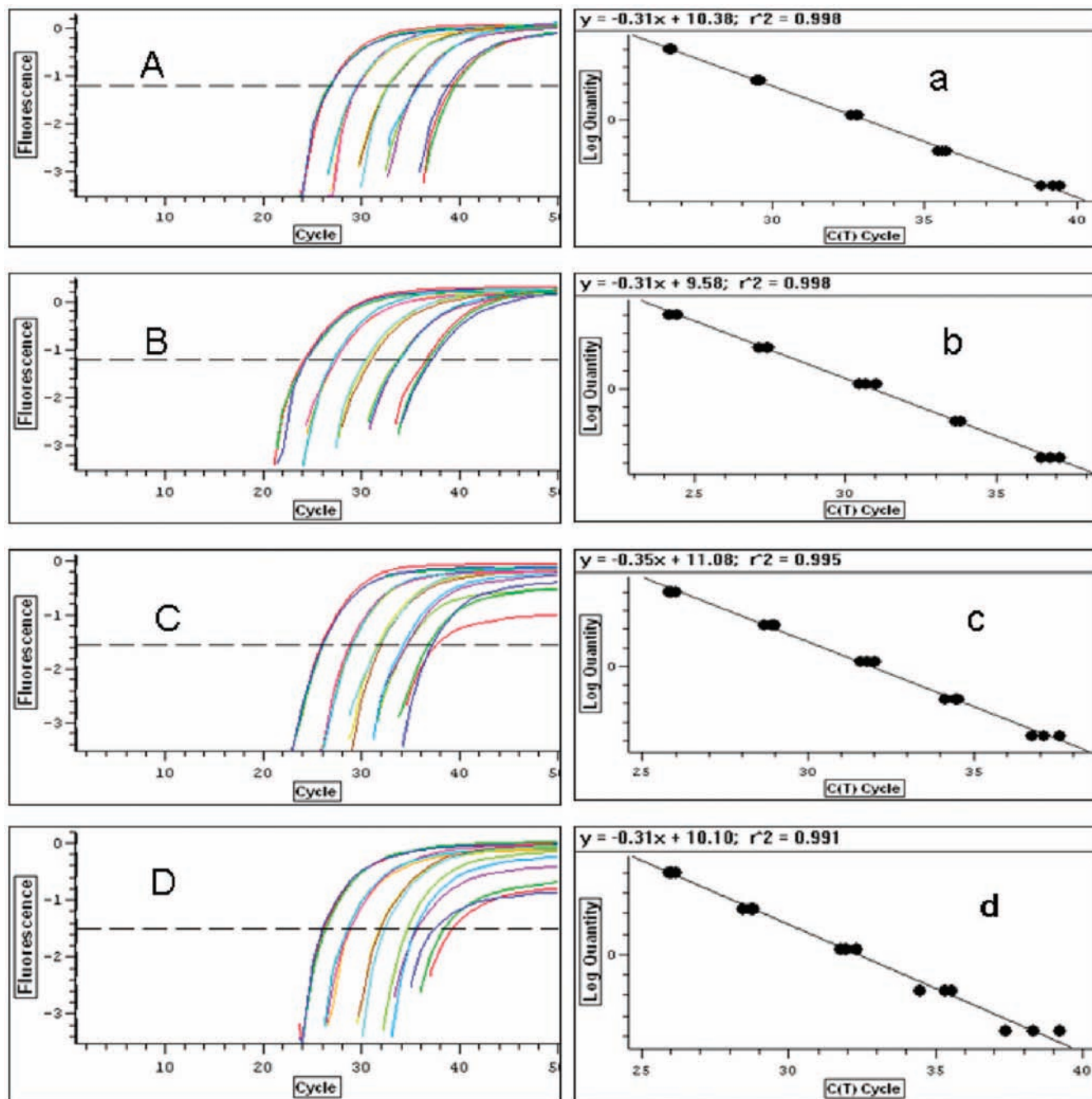
Figure 6. Amplification plots and standard curves for RF1, RF2, MS1, and FatA real-time PCR assays. **(A)** Amplification plot produced by RF1 real-time PCR assay with serial dilutions of MS1×RF1 DNA. **(B)** Amplification plot produced by RF2 assay with serial dilutions of MS1×RF2 DNA. **(C)** Amplification plot produced by MS1 assay with serial dilutions of MS1×RF1 DNA. **(D)** Amplification plot produced by FatA assay with serial dilutions of Zhongyou 821 DNA. Serial dilutions separately contained 100, 13, 1.3, 0.13, and 0.013 ng template DNA. The horizontal line indicates the threshold line which was used for drawing the standard curve. **(a–d)** Representative standard curves generated from the amplification data given in A–D.

quantitative estimates when detecting food and feedstuffs via real-time assays.

Table 3 shows the Ct value of each PCR amplification in detail. For the three event-specific assays, the standard deviation (SD^r) and relative standard deviation (RSD^r) of repeatability and standard deviation (SD^R) and relative standard deviation (RSD^R) of reproducibility were calculated from the data of triplicate reactions and three replications (**Table 3a,b,c**). The values of SD^r and RSD^r indicated that the Ct variations among parallel samples of the same template concentration increase with the decreasing template copies, and SD^R and RSD^R indicated that the reproducibility of the three replications

decreases with the decreasing template copies. The experiment showed that the three real-time PCR assays developed in this study were stable and reliable.

Construction of Calibration Curves. Serial dilutions ranging from 100 ng to 0.013 ng (corresponding to approximately 77412 to 20 rapeseed molecules, 19353 to 2.5 MS1 molecules, and 38706 to 5 RF1 or RF2 molecules per PCR) were used as standard solutions to construct calibration curves for the FatA assay, MS1 assay, and RF1 and RF2 assays (**Figure 6**). The square regression coefficient (R^2) was 0.991 for the *FatA* gene, 0.998 for the RF1 amplicons, 0.998 for the RF2 amplicons, and 0.995 for the MS1 amplicons, as shown in **Figure 6a–d**. All of

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Table 4. Quantification of the GMO Content in Rapeseed Samples Using the RF1, RF2, and MS1 Real-Time PCR Assays

method	Sample	GMO content	experimental			mean	SD	RSD (%)	bias (%)
			1	2	3				
MS1 assay	S1	1.5% MS1 × RF1	1.56	0.93	1.84	1.44	0.47	32.3	3.8
	S2	0.5% MS1 × RF2	0.39	0.35	0.34	0.36	0.03	7.3	28.0
	M1	0.75% MS1 × RF1 + 0.25% MS1 × RF2	0.76	1.19	0.89	0.95	0.22	23.3	5.3
	M2	0.25% MS1 × RF1 + 0.75% MS1 × RF2	0.87	0.98	0.89	0.91	0.06	6.4	8.7
RF1 assay	S1	1.5% MS1 × RF1	1.42	1.95	1.40	1.59	0.31	19.6	6.0
	S2	0.5% MS1 × RF2	0.45	0.42	0.31	0.39	0.07	18.7	21.3
	M1	0.75% MS1 × RF1 + 0.25% MS1 × RF2	0.69	0.73	0.70	0.71	0.02	2.9	5.8
	M2	0.25% MS1 × RF1 + 0.75% MS1 × RF2	0.24	0.21	0.18	0.21	0.03	14.3	16.0
RF2 assay	S3	1.5% MS1 × RF2	1.78	1.57	1.45	1.60	0.17	10.4	6.7
	S4	0.5% MS1 × RF2	0.50	0.49	0.57	0.52	0.04	8.4	4.0
	M1	0.75% MS1 × RF1 + 0.25% MS1 × RF2	0.22	0.26	0.24	0.24	0.02	8.3	4.0
	M2	0.25% MS1 × RF1 + 0.75% MS1 × RF2	0.74	0.75	0.83	0.77	0.05	6.4	3.1

the R^2 values were more than the minimum acceptable value of 0.98 (*44*). Good linearity between the copy number and Ct value was observed in the calibration curves (**Figure 6**), indicating that the quantitative PCR assays were suitable for accurately quantifying the GMO content of the samples.

Validation of the Event-Specific Assays. Six samples containing well-known MS1×RF1 or MS1×RF2 content (in %) were prepared to evaluate the precision (% RSD) and trueness (% error) of the quantitative methods: sample 1 (S1) with a relative concentration of 1.5% MS1×RF1; S2, 0.5% MS1×RF1; S3, 1.5% MS1×RF2; S4, 0.5% MS1×RF2; mixture 1 (M1), 0.75% MS1×RF1 and 0.25% MS1×RF2; M2, 0.25% MS1×RF1 and 0.75% MS1×RF2. Real-time PCR reactions were performed with DNA extracted from the six samples and the non-GM rapeseed Zhongyou 821, and each template was analyzed in three parallels. No fluorescent signal was detected for the negative control, Zhongyou 821. Ct values of exogenous target and FatA genes were determined for all samples using an identical baseline range with standard solutions. The concentrations of GMO DNA and total rapeseed DNA were then calculated on the basis of the constructed calibration curves of event-specific PCR and FatA PCR. The relative percentage (%) of GMO DNA to total rapeseed DNA was calculated by the following formula: Relative content (%) of GM-rapeseed DNA to total rapeseed DNA = (concentration of GM-rapeseed/concentration of total rapeseed DNA) × 100 (*31*).

The quantitative data of the six samples are shown in **Table 4**. The relative mean values of S1, S2, M1, and M2 were 1.44%, 0.36%, 0.95%, and 0.91% on the basis of the MS1 assay; the quantitative values of S1, S2, M1, and M2 were 1.59%, 0.39%, 0.71%, and 0.21% using the RF1 method; the relative mean values of S3, S4, M1, and M2 were 1.60%, 0.52%, 0.24%, and 0.77% using the RF2 method. We can observe that the quantitative estimates deviated slightly from the true GMO content of the six samples. According to the opinion of Peccoud and Jacob, the quantitative uncertainty in PCR reactions results mostly from the molecular fluctuations occurring when there are low numbers of initial template copies (*45*). In this study, the relative content of GMO DNA to total DNA is ≤5% for all samples. Because the RSD values of the samples were all ≤50%, the quantitative results of test samples can be accepted (*31*). The quantitative results also indicated that the quantitative data become increasingly biased and the trueness decreases with decreasing GMO content in the samples (**Table 4**). For samples M1 and M2, the estimates based on the MS1 method were approximately equivalent to the sum of the estimates derived from the RF1 and RF2 method and in agreement with the true concentrations. Meanwhile, the estimates from S1 and S2 based on the MS1 method were approximately equivalent to those

derived from the RF1 method. As could be concluded, the three event-specific assays can be used simultaneously to quantify the content of gene-stacked hybrids such as MS1×RF1 and MS1×RF2.

Selection of Gene-Stacked Hybrid Quantification Methods. The commercial transgenic rapeseed varieties MS1×RF1 and MS1×RF2 are gene-stacked heterozygous hybrids with the joint presence of MS1 and RF1 (RF2) event-specific sequence motifs. Currently in the marketplace, only MS1×RF1 (MS1×RF2) F_1 hybrid seeds are used for cultivation, and F_2 seeds are utilized for processed materials. Their parental line, MS1, is a heterozygous trait, while RF1 and RF2 are homozygous traits. The MS1×RF1 (MS1×RF2) F_1 seeds contain one MS1 molecule and two RF1 (RF2) molecules per four haploid genomes. The event-specific sequence frequency in F_2 hybrid seeds was the same as in the F1 hybrid seeds by application of the rules of Mendelian inheritance. Although the MS1×RF1 (MS1×RF2) F_2 seeds will be separated into three genotypes, including GM-homozygous, GM-heterozygous, and non-GM (null), we believe that the GM quantity in the MS1×RF1 (MS1×RF2) F2 seeds, without contamination, would correspond to a value of 100%.

The European Commission recommended that the DNA-based unit for expressing the GM material content could best be defined as the ratio of an event-specific sequence to a species-specific reference gene in terms of haploid genomes, and the copy numbers of reference genes are used as estimators of the number of haploid genomes (*16, 46*). Whereas a haploid-genome-based approach may result in underestimations of the quantity of the gene-stacked hybrids MS1×RF1 and MS1×RF2, the estimates from 100% pure MS1×RF1 (MS1×RF2) F1 or F2 seeds would be 25% based on the MS1 genome/rapeseed genome ratio and 50% based on the RF1 (RF2) genome/rapeseed genome ratio. Therefore, for the gene-stacked hybrid, the DNA-based unit for expressing the GM content percentage should be defined as the ratio of an event-specific DNA concentration to a species-specific DNA concentration (*31*). The serial dilutions of 100% MS1×RF1 DNA or MS1×RF2 DNA in non-GM rapeseed DNA and serial dilutions of non-GM rapeseed DNA in salmon sperm DNA were used as reference materials. The "mixed genomic standards" were used to set up the calibration curves of the event-specific assays and the reference gene. Using separate calibration curves, the concentration of the event-specific DNA and total rapeseed DNA for all samples were calculated and were then used to calculate the relative percentages of MS1×RF1 (MS1×RF2) DNA to the total rapeseed DNA. The selected standard curve method was superior to the δ Ct method since the two targets do not amplify in the PCR with the same efficiency (*41*).

GMO detection now encounters a new challenge in that the event-specific detection and quantification methods will not be able to distinguish between a gene-stacked hybrid and a mixture of the parental GMOs (*12*). This study only provided a method whereby the three event-specific assays and the endogenous gene are employed simultaneously to quantify the content of MS1×RF1, MS1×RF2, or the mixture of MS1×RF1 and MS1×RF2 in test samples, ignoring the possibility of a mixture of the parental GMOs, since the parental lines MS1 and RF1 (RF2) for MS1×RF1 and MS1×RF2 do not apply to commercial production. By evaluating the accuracy of the quantification methods (**Table 4**), it was found that the event-specific assays developed on the basis of weight units can be easily applied to the detection of various samples, even for samples with a low quantity of DNA.

ABBREVIATIONS USED

GMO, genetically modified organism; T-DNA, transfer DNA; LOD, limit of detection.

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