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Development of Genomic DNA Certified Reference Materials for Genetically Modified Rice Kefeng 6

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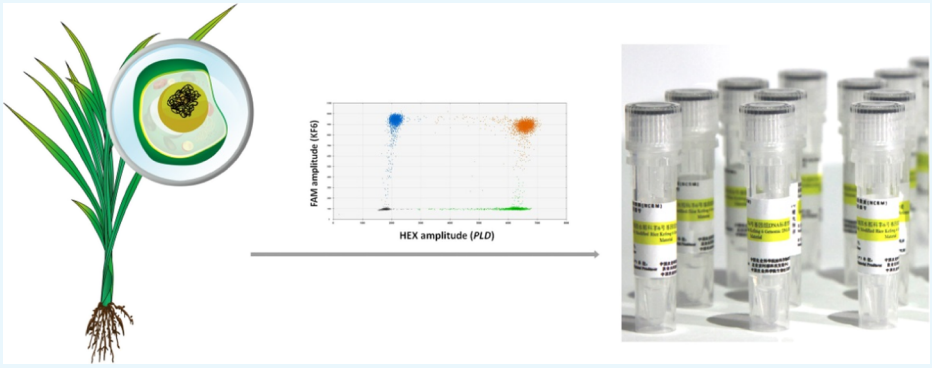

ABSTRACT: The application of certified reference materials (CRMs) to genetically modified organism (GMO) detection is essential for guaranteeing the accuracy, comparability, and traceability of quantitative results over time and among laboratories. Clean leaves from GM rice Kefeng 6 were used as raw materials to develop a batch of genomic DNA (gDNA) CRMs. The optimized KF6/*PLD* duplex digital PCR was used for collaborative characterization of Kefeng 6 gDNA CRMs by eight qualified laboratories; this batch of gDNA CRMs was certified for two property values, namely, copy number ratio and copy number concentration, which were 1.03 ± 0.04 and (1.60 ± 0.11) × 10^5 copies/μL, respectively. The gDNA CRMs displayed good between-vial homogeneity when the minimum sample intake of 2 μL was taken into account. Stability studies indicated that the gDNA CRMs should be transported below 25 °C, and cold chain transport was recommended. Shelf life was assessed to be at least 12 months, and when using gDNA CRMs, freeze−thaw should not exceed 10 cycles. Compared to the available gDNA CRMs in the market, this batch of gDNA CRMs has accurate property values with combined uncertainties, providing user-friendly calibrators for GM rice Kefeng 6 inspection and monitoring. The development and characterization of Kefeng 6 gDNA CRMs contribute to the establishment of a copy number-based reference system for GMO detection.

INTRODUCTION

With the development and application of genetically modified organisms (GMOs), many countries have implemented labeling policies that food or feed containing or consisting of approved GMO ingredients must be labeled if their GMO content exceeds the fixed threshold value such as 0.9% in the EU, 3% in Korea, and 5% in Japan.[1,2] The enforcement of GMO labeling policies requires the establishment of harmonized quantification methods, as well as to produce certified reference materials (CRMs) to calibrate and control the quantitative results.[3] Real-time quantitative polymerase chain reaction (PCR) is currently recognized as the gold standard for quantifying GMO content in test samples.[4] The real-time quantitative PCR is a relative quantitative technique with dependence on RMs with a precise property value.[5,6] Appropriate CRMs are critical to ensure the accuracy, reliability, and comparability of measurement results over time and location because the quantitative results can be traceable to SI units or international recognized measurement standards by establishing traceability to a reference system.[7−9]

The available CRMs for GMO detection include powder CRMs with certified values in mass fraction, genomic DNA (gDNA) CRMs, and plasmid DNA CRMs certified for copy number ratios.[10] These three types of CRMs are all adopted for GMO quantification by different laboratories. The certified expression unit of CRMs used for calibration determines the measurement units that may be used; if the mass fraction-

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based powder CRMs are used for quantification, the measurement results are expressed in mass fractions, whereas using the copy number ratio-based CRMs, the measurement results should be expressed in copy number ratios.[11−13] The mass fraction- and copy number ratio-based reference systems have different metrological traceability chains, and the property value of mass fraction powder CRMs is characterized by accurate weighing of raw materials using calibrated balances, with the SI unit kg as the top of the traceability chain. The property value of copy number ratios should be characterized by a metrologically higher-order reference method, whereas the real-time quantitative method, which relies on RMs to convert signals to a concentration or an amount of initial DNA target, is unsuitable for measuring copy number ratios of CRMs. The lack of metrological reference methods has hampered the establishment of a copy number-based reference system. The emerging digital PCR (dPCR) can achieve absolute quantification of copy number of DNA molecules without relying on the application of CRMs,[14,15] thereby providing a potential metrological higher-order reference method for copy number characterization. During characterization by dPCR, the use of a CRM with precisely defined quantity of template as a quality control is crucial to evaluate the accuracy of measurements.[16] dPCR may be utilized as a primary method when factors influencing measurements are sufficiently defined.[17−19]

Currently, the PCR technique is best suited for quantification of GMO content in food or feed products; however, it can only measure the copy number of amplifiable DNA targets in a PCR system and is unable to directly measure the mass of GMO and non-GMO powders.[6,20] Although, the expression unit for GMO content has been stipulated to be expressed in mass fraction in EU,[21] we think that for GMO quantification, the copy number-based reference system is more in line with the PCR principle and the development of the PCR technology.

The gDNA CRM is a major type of GM CRM that belongs to the copy number-based reference system. The American Oil Chemist's Society (AOCS) is the main producer of gDNA CRMs that are produced using clean plant leaves as raw materials, and a total of 19 gDNA CRMs are now in the market. gDNA CRMs are certified as "pure, homozygous" or "pure, heterozygous" but not in terms of copy number or copy number ratio. The emergence of dPCR offers a well-characterized reference method for precisely measuring DNA copy number independent of RMs. dPCR has been used for characterizing the copy number or copy number ratio of gDNA CRMs or pDNA CRMs.[22−24]

GM rice Kefeng 6 is an insect-resistant variety developed by the Chinese Academy of Sciences (Beijing, China) and Fujian Academy of Agricultural Science (Fuzhou, China). In this study, GM rice Kefeng 6 was used as a raw material to produce gDNA CRMs, and dPCR was employed to determine copy number and copy number ratio of Kefeng 6 gDNA CRMs.

RESULTS

Identification of Kefeng 6 Plants. Before planting, the authenticity of Kefeng 6 seeds was identified by event-specific real-time PCR assays targeting Kefeng 6, TT51-1, KMD, G6H1, Golden Rice 2, and LLRICE62. Only Kefeng 6 event-specific real-time PCR assays yielded typical amplification curves, whereas other event-specific assays did not produce any amplification curve. The test results showed that the testing seeds contained Kefeng 6 event and the level of other GM rice events is below 3% at 95% of probability (n = 200). At the early tillering stage of rice, the genotype of Kefeng 6 plants was analyzed one by one using the Kefeng 6 event-specific method together with the insert site-specific method targeting insert sites in the recipient genome. The schematic diagram of genotype identification is shown in Figure S1. The genotype identification results demonstrated that a total of 14 Kefeng 6 plants were homozygous (Figure S2), and the copy number ratio between Kefeng 6 event and *phospholipase D gene* (*PLD*) reference gene is theoretically equal to 1.0. The clean leaves from homozygous plants were used as raw materials for producing gDNA CRMs.

Preparation of Kefeng 6 gDNA CRMs. Large-scale gDNA was extracted from clean leaves of homozygous Kefeng 6 plants. The extracted DNA concentration was measured to be 370 ng/μL using the PicoGreen method. gDNA was diluted to a concentration of about 100 ng/μL using 0.1× TE buffer. Gel electrophoresis of gDNA revealed a single, clear band without smear, indicating that the extracted DNA did not degrade into fragments during the extraction. The OD230, OD260, and OD280 of the gDNA solution at wavelengths of 230, 260, and 280 nm were repeatedly measured six times using the ultraviolet (UV) spectrophotometric method, and the average value of OD260/OD280 was calculated to be 1.82, within the range of 1.8−1.9, and that of OD260/OD230 was 2.14, which is higher than 2.0. The measurement results indicated that the extracted DNA only contained trace of impurities such as RNA, protein, salts, and organic reagents. To exclude the presence of PCR reaction inhibitors in the DNA extract, real-time PCR was conducted to evaluate the amplification efficiencies of the PCR assays. The serial dilution series of 100, 20, 4, 0.8, and 0.16 ng/μL were used as calibrants to perform both Kefeng 6 event-specific and *PLD* real-time PCR assays. The amplification efficiencies were calculated to be 98.5 and 97.3% based on the slope of standard curves for Kefeng 6 event-specific assay and *PLD* assay, respectively. The PCR amplification efficiency test indicated that there was no inhibitor in the DNA solution. The extracted gDNA was verified to have structural integrity and contain no inhibitor and was suitable for preparing gDNA CRMs.

Before dispensing into vials, the DNA solution must be analyzed to guarantee that the DNA had been uniformly dissolved in the TE solution. The OD260 of the DNA sample from different positions was measured during the mixing process; F test results demonstrated that after 36 and 48 h of mixing, the OD260 values did not show a significant difference between various DNA samples (Table S1). Therefore, the liquid gDNA achieved good homogeneity after 36 h of mixing. The concentration of 9 DNA subsamples taken at 48 h was measured by KF6/*PLD* duplex ddPCR, and the average of 9 measurements was computed to be 1.69×10^5 copies/μL, which was used as the concentration of the DNA stock solution that was used for subsequent analysis. The DNA solution was dispensed to minimum packing units (100 μL/tube), and a total of 500 tubes were dispensed.

Homogeneity Study. Homogeneity includes within-unit homogeneity and between-unit homogeneity. For the between-unit homogeneity test, a total of 15 vials were randomly selected from the whole batch of gDNA CRMs, and three subsamples were taken from the top, middle, and bottom of each vial.[28] The Kefeng 6 event-specific and *PLD* real-time quantitative PCR methods were used to measure the copy

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Table 1. Primers and Fluorogenic Probes Used for Conventional, Real-Time PCR, and dPCR Assays

PCR system	target	Name	sequence (5′−3′)	amplicon size (bp)
insert site specific PCR	KF6 insert site	IS-F	TCGATTGGTCACATGGTCCA	297
		IS-R	CTCACGTTCTTTTGCCTCCC	
event-specific PCR	KF6 flanking sequence	QK6-5F	GTGGTATCCTACCTCTCCCAGC	86
		QK6-5R	CGCAGCATGGTTCTCAGTACA	
		QK6-5P	FAM-TACCGCCGTGTGCCCGTGTC-BHQ	
reference gene PCR	*PLD*	KVM159	TGGTGAGCGTTTTGCAGTCT	68
		KVM160	CTGATCCACTAGCAGGAGGTCC	
		TM013	HEX-TGTTGTGCTGCCAATGTGGCCTG-BHQ	

number concentration and copy number ratio of gDNA CRMs. The measured results are shown in Figure S3. The values of copy number concentration and copy number ratio for these within-vial and between-vial samples were analyzed using the *F* test, and both the calculated *F* values of copy number concentration and copy number ratio were significantly less than the critical $F_{0.05(14,30)}$ value of 2.04 (Table 2). Statistical

Table 2. Results of the Homogeneity Study

parameter	copy number concentration	copy number ratio
mean	1.59×10^5	1.003
MS_{within}	6.85×10^7	0.00138
$MS_{between}$	7.70×10^7	0.00105
F	0.89	1.32
$F_{0.05(14,30)}$	2.04	2.04
Conclusion	homogeneous	Homogeneous
$u_{bb,rel}$	0.016	0.01

analysis results did not detect any significant vial-to-vial variation. The within-unit heterogeneity determines the minimum intake of an aliquot that is representative for the whole unit. Approximately, 2 μL of DNA solution was taken for between-unit homogeneity testing, which revealed acceptable repeatability and good between-unit homogeneity. The minimum sample intake was determined to be 2 μL. The within-unit heterogeneity does not contribute to the uncertainty of the certified value because the minimum sample intake is respected. The relative uncertainty contributed by between-unit homogeneity was assessed following ISO guide 35,[29] the uncertainty of copy number concentration was estimated to be 0.016, and the uncertainty of copy number ratio was 0.01.

Stability Study. Stability is used for describing the changes in the property value of CRMs over time, usually including short-term stability and long-term stability. This batch of gDNA CRMs consists of DNA solutions, and the gDNA CRMs need to be repeatedly frozen and thawed, and thus, their freeze−thaw stability is a concern. The optimal transport conditions of CRMs can be determined by performing a short-term stability test, and the shelf time of CRMs can be evaluated using a long-term stability test. The gDNA CRMs sealed in vials are usually protected from moisture, light, and radiation during transport and storage. The factors of temperature and time are mainly assessed in a CRM stability study. The stability studies were conducted using an isochronous design, and all samples were analyzed by performing Kefeng 6 event-specific and *PLD* real-time quantitative PCR assays under repeatability conditions. The quantified results are shown in Figures S4 and S5. The measurement values of three samples per storage time at the same temperature showed good repeatability with relative standard deviations (SDs) ranging from 0.21 to 3.71%.

For short-term stability studies, the CRMs were evaluated by analyzing 3 tubes stored at 4, 25, and 37 °C for 1, 2, and 4 weeks. The property values of gDNA CRMs did not show obvious changes at 4 and 25 °C with the extension of storage time, whereas at 37 °C, the gDNA copy number concentration significantly increased over time (Figure S4) because of evaporation of the aqueous solution resulting from high temperature. The *t*-test results indicated that the slopes of regression curves (β_1) were not significantly different from zero at 4 and 25 °C at the 95% confidence level, but the slopes were significant at 37 °C (Table 3). Short-term stability assessment demonstrated that the property values of gDNA CRMs remained stable when transported below 25 °C for 4 weeks. The gDNA CRMs are suggested to be best transported in a cold chamber to guarantee the stability of property values of gDNA CRMs.

For long-term stability, the CRMs were evaluated by analyzing 3 tubes stored at 4 and −20 °C for 1, 2, 4, 6, and 12 months. The *t*-test results did not reveal a significant slope

Table 3. Results of the Short-Term and Long-Term Stability Study[a]

test	property value	temperature (°C)	mean	β_1	$s(\beta_1)$	$t_{0.95,n-2}$	$t_{0.95,n-2} \cdot s(\beta_1)$	conclusion
short-term stability	copy number concentration	4	158,300	−391	1591	4.30	6844	stable
		25	159,100	−456	684		2944	stable
		37	255,900	48,196	7356		31,652	instable
	copy number ratio	4	1.004	0.0033	0.0036		0.0150	stable
		25	1.000	−0.0023	0.0016		0.0070	stable
		37	0.991	−0.0107	0.0034		0.0150	stable
long-term stability	copy number concentration	4	158,700	197	110	2.78	305	stable
		−20	159,600	−184	100		279	stable
	copy number ratio	4	1.007	−0.0017	0.0012		0.0032	stable
		−20	0.999	0.0011	0.0006		0.0016	stable

[a] β_1 the slope of the regression line, $s(\beta_1)$ SD of β_1.

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at 4 and −20 °C at the 95% confidence level (Table 3). We observed that the gDNA CRMs can be stably stored at −20 °C for at least 12 months and the gDNA CRMs can be stored at 4 °C to prevent DNA degradation caused by repeated freeze−thaw cycles when the gDNA CRMs are frequently used in short term. The relative uncertainty of copy number concentration introduced by instability at −20 °C was estimated to be 0.0076, and the relative uncertainty of copy number ratio was 0.0069.

Using gDNA CRMs involves repeated freeze−thaw cycles, which in turn may influence their stability. We observed an increase in copy number concentration of gDNA CRMs with increasing number of freeze−thaw cycles (Figure S6). The *t*-test results indicated that there was a significant increase in copy number concentration values after 10 freeze−thaw cycles (Table 4); this result is consistent with that of the plasmid CRM of ERM-AD623.[22] The evaporation caused by frequent opening may be responsible for the observed increase in gDNA copy number concentration. We thus recommend that gDNA CRMs should not be exposed to more than 10 freeze−thaw cycles during use.

Table 4. Results of the Freeze−Thaw Stability Study

freeze−thaw cycles	mean of copy number concentration	SD	*t* value	$t_{(0.01,4)}$	conclusion
0	1.58×10^5	5252	−0.88	4.60	stable
2	1.60×10^5	2016	0.82	4.60	stable
4	1.59×10^5	2692	−0.53	4.60	stable
6	1.61×10^5	5037	0.46	4.60	stable
8	1.59×10^5	2901	−0.39	4.60	stable
10	1.60×10^5	2007	0.64	4.60	stable
12	1.77×10^5	5352	7.26	4.60	instable
14	1.89×10^5	6746	9.6	4.60	instable
16	1.91×10^5	6871	10.3	4.60	instable
18	2.03×10^5	8901	10.91	4.60	instable
20	2.11×10^5	8034	14.22	4.60	instable

Value Assignment and Uncertainty Evaluation. The characterization of gDNA CRMs was conducted by eight qualified GMO detection laboratories using KF6/*PLD* duplex ddPCR. The results of collaborative characterization are shown in Table 5. The data were statistically analyzed according to ISO guide 35.[28] Statistical analysis revealed that neither an outlying mean nor outlying SD was identified, and the measured data followed normal distributions. The calculated average of all independent measurement results was taken as the certified value. The certified value of DNA copy number concentration was calculated to be 1.602×10^5 copies/μL, and the certified value of copy number ratio was 1.023.

Table 5. Results of Inter-Laboratories Characterization

	copy number concentration (copies/μL)			copy number ratio		
lab	mean	SD	RSD (%)	mean	SD	RSD (%)
1	1.51×10^5	4077	2.700	1.040	0.008	0.815
2	1.50×10^5	2599	1.734	1.026	0.016	1.567
3	1.69×10^5	4418	2.618	1.015	0.013	1.246
4	1.71×10^5	2704	1.580	1.019	0.010	0.940
5	1.52×10^5	3007	1.983	1.017	0.006	0.585
6	1.69×10^5	3035	1.800	1.024	0.012	1.181
7	1.71×10^5	3534	2.063	1.016	0.010	1.017
8	1.50×10^5	1486	0.994	1.023	0.005	0.485
mean	1.60×10^5	1.023				
SD	3700	0.003				
RSD (%)	2.309	0.282				

The uncertainty components related to the characterization process consist of type A uncertainty and type B uncertainty.[29] Type A uncertainty is evaluated by statistical analysis of measurement data at the required confidence level; the calculated SD of all independent data at 95% confidence level was taken as type A uncertainty of characterization, and type A relative standard uncertainty was 0.0028 for copy number ratio and 0.023 for copy number concentration. Type B uncertainty is determined from non-statistical analysis of factors influencing dPCR measurement such as dilution factor and droplet volume variability. The eight-channel droplet generator cartridge was used to generate droplets for all participating laboratories, and the relative standard uncertainty of droplet volume from a disposable eight-channel droplet generator cartridge was reported to be 0.01.[30] The relative standard uncertainty introduced by DNA dilution was estimated to be 0.0056. The type B relative standard uncertainty was combined to be 0.015 for both copy number ratio and copy number concentration values. The uncertainty of the CRMs (u_{CRM}) consists of uncertainty components from characterization (u_{char}), potential between-unit heterogeneity (u_{bb}), and potential instability during long-term storage (u_{lts}) (Table 6). These different contributions were combined to estimate the expanded relative standard uncertainty of the certified value ($U_{CRM,rel}$) with a coverage factor k ($k = 2$ at 95% confidence level) using the following formula: $U_{CRM,rel} = k \cdot \sqrt{u_{char,rel}^2 + u_{bb,rel}^2 + u_{lts,rel}^2}$.[29] The expanded uncertainty of copy number ratio (U_{CRM}) was estimated to be 0.04, and the expanded uncertainty of copy number concentration (U_{CRM}) was estimated to be 10,165 (Table 6). The certified copy number concentration value was 1.60×10^5 copies/μL, with an expanded uncertainty of 0.11×10^5 copies/μL. The certified copy number ratio value was 1.03, with an expanded uncertainty of 0.04.

DISCUSSION

The labeling threshold of early legislations was commonly understood as the mass percentage; Institute for Reference Materials and Measurements (IRMM) produced a series of powder CRMs by blending GM seed powder and non-GM counterparts at different mass fractions, certified for their mass fractions with SI unit kg as expression unit. For gravimetric mixture of GM powder and non-GM powder, the mass fraction value is demonstrated to be significantly different from the copy number ratio because of the factors of raw material purity, tissue ploidy, parental origin, and different DNA extractability between GMO powder and non-GM powder.[31−33] The inconsistency between the expression units of mass fraction and copy number ratio makes it impossible for the powder CRMs to accurately deliver their property value of mass fraction to final measurements using the PCR technique. To maintain the consensus between the measurement unit of CRMs and legal requirements, the Joint Research Centre (JRC) Technical Reports released in 2017 specified that the measurement unit of GMO content must be expressed in mass fraction, and the measurement results expressed in copy

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Table 6. Results of Certified Value and Uncertainty Evaluation

property value	certified value	$u_{char,rel}$		$u_{bb,rel}$	$u_{lts,rel}$	$U_{CRM,rel}$ (k = 2.0)	U_{CRM} (k = 2.0)
		$u_{type\ A,rel}$	$u_{type\ B,rel}$				
copy number ratio	1.02	0.003	0.015	0.010	0.0069	0.039	0.04
copy number concentration (copies/μL)	1.60×10^5	0.023	0.015	0.016	0.0076	0.065	0.11×10^5

number ratio must be converted into mass fraction using a conversion factor (CF).[34] To facilitate the mutual conversion of these two measurement units, the EU has established and published a CF for each event.[35]

The real-time quantitative PCR technique uses gDNA extracted from powder CRMs with exact known concentration as calibrants to calibrate the measurement results. Before being used for calibration, the concentration of gDNA extracted from powder CRMs is usually measured by common analytical methods such as UV absorption method, fluorescence staining, and diphenylamine methods. However, these analytical methods are easily disturbed by trace impurities of tRNA, proteins, and reagents in the extracted DNA,[36] making it very difficult to obtain accurate DNA mass concentrations of extracted gDNA. Moreover, the used DNA extraction methods are unable to eliminate plastid DNA contamination (e.g., chloroplast DNA and mitochondrial DNA) from nuclear DNA, and the presence of plastid DNA induces overestimation of DNA concentrations using analytical methods. In this study, the copy number concentration of gDNA CRMs was collaboratively measured to be 1.60×10^5copies/μL, corresponding to about 72 ng of rice DNA based on the C value of rice haploid genome expressed in picograms (0.45 pg).[37] The obtained DNA concentration by duplex dPCR was significantly less than the value of about 100 ng/μL that was measured using the PicoGreen method. The different measurement results demonstrate that the commonly used DNA concentration measurement methods usually overestimate the DNA concentration because of the presence of plastid DNA in the DNA solution. The copy number of target DNA is usually calculated based on the DNA mass concentration and the size of the haploid genome that varies considerably between different varieties of the same species, for instance, the C value of maize ranges from 2.35 to 6.30 pg.[6] Owing to the above factors, it is impossible to obtain the accurate copy number concentration of a DNA reference by the commonly used DNA concentration measurement method. A reference with an inaccurate property value must transfer an inaccurate value to the final PCR results.

The pure gDNA CRMs developed in this study were assigned two property values of copy number ratio and copy number concentration by collaborative characterization using KF6/*PLD* duplex ddPCR. Compared to the pure gDNA CRMs of AOCS, the genotype of the raw materials for producing gDNA CRMs was identified to be homozygotes, and Kefeng 6 gDNA CRMs are certified for accurate property values with combined uncertainties. The gDNA CRMs can be used to calibrate the quantitative results, to validate the performance of Kefeng 6-related analytical methods, to assess the absolute limit of detection and limit of quantification, and to assess the performance of laboratories in proficiency testing schemes. Kefeng 6 gDNA CRMs can be directly used in preparing serial dilutions with exact copy number concentrations without measuring the DNA concentration. The application of gDNA CRMs would transfer copy number values to the final measurement results, thus forming the copy number-based reference system different from the mass fraction-based reference system. The dPCR, an absolute quantification method for nucleic acid molecules, would thus be the mainstream technology for GMO detection. We conclude that the copy number-based reference system will be more in line with the further developments in the PCR technology. The development of Kefeng 6 gDNA CRMs contributes to the establishment of a copy number-based reference system for GMO detection.

MATERIALS AND METHODS

Plant Materials. Seeds of genetically modified rice Kefeng 6 were provided by the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (Beijing, China). The seeds of Kefeng 6 were sown and cultivated in a greenhouse. During the vegetative growth period, leaves were collected for large-scale gDNA extraction.

Primers and Probes. The primers and probes used for Kefeng 6 event-specific detection and quantification were as described elsewhere,[25] and the *PLD* was used as the rice-specific endogenous reference gene.[26] The primer and probe sequences are shown in Table 1, and the 5′ ends of Kefeng 6 and the *PLD* probe were, respectively, labeled with FAM and HEX as reporter dyes, and the 3′ end of both probes was labeled with BHQ as quencher dye. The conventional PCR primers used for identifying homozygous plants were designed using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) (Table 1), and the designing principle of conventional primers for raw material identification is shown in Supporting Information Figure S1.

PCR Assays. Conventional PCR assays were run in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) using an optimized reaction system consisting of 1× PCR buffer, 2.0 mM $MgCl_2$, 200 μM dNTP, 100 nM primers, 0.5 units of DNA polymerase (Fermentas, Vilnius, Lithuania), and 1 μL of each DNA sample, and ddH_20 was added to a final reaction volume of 25 μL. The amplification program was as follows: denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and terminal elongation for 2 min at 72 °C. The PCR products were analyzed on a 1.5% agarose gel in 1× TAE buffer and stained with ethidium bromide.

Real-time quantitative PCR assays were performed using a fluorometric thermal cycler (CFX96 Real-time PCR Detection System, Bio-Rad, Hercules, CA, USA). The reaction system of both Kefeng 6-specific and *PLD* was as follows: 10 μL of 2× TaqMan Universal PCR Master Mix (Takara, Shiga, Japan), 400 nM primers, 200 nM probe, and 2 μL of the DNA template, and ddH_20 was added to a total reaction volume of 20 μL. The reaction program was as follows: denaturation at 95 °C for 2 min; followed by 50 cycles of 95 °C denaturation for 15 s, 60 °C annealing and extension for 1 min. The fluorescence signals were monitored and analyzed using the CFX manager software program (Bio-Rad). The stock Kefeng 6 gDNA solution was serially diluted using 0.1× TE and used

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as calibrants, and standard curves were drawn by plotting DNA amount against Ct values of Kefeng 6 and *PLD*.

KF6/*PLD* duplex droplet dPCR (ddPCR) assays were conducted in a QX200 droplet dPCR system. Each 20 μL of reaction mixture contained 10 μL of 2× ddPCR master mix (Bio-Rad, Pleasanton, CA, USA), and the concentration of the other PCR components was the same as that of real-time PCR assays. Both the 20 μL of reaction mix and 70 μL of the droplet generation oil were loaded into 8-well cartridges to generate droplets using the QX200 droplet generator (Bio-Rad). Then, the generated droplets were transferred into a 96-well plate, sealed, and inserted into a C1000 thermal cycler (Bio-Rad) for amplification using the following conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. After cycling, each sample was incubated at 98 °C for 10 min and then cooled to 4 °C. Then, the plate was transferred to a QX200 droplet reader (Bio-Rad) to read droplet fluorescence data. Data acquisition and analysis were performed using QuantaSoft software (Bio-Rad). Data generated by the QX200 droplet reader were excluded from subsequent analysis when a clog was detected by the Quantasoft software or when a low number of droplets (<10,000) was measured per 20 μL of PCR mixture. After exporting, the data were further analyzed using Microsoft Excel.

DNA at different concentrations was prepared using a gravimetric protocol to minimize uncertainty due to pipetting. For PCR assays, at least three to four replicates were prepared for each DNA concentration or DNA template.

Extraction and Evaluation of Kefeng 6 gDNA. Large-scale gDNA was isolated from young clean leaves according to the protocol "High-quality DNA Extraction from Rice Leaves".[27] DNA integrity was assessed by performing electrophoresis on a 1% agarose gel in 1× TAE (40 mM Tris, pH 8.5, 1 mM EDTA, and 20 mM acetic acid) and stained with ethidium bromide. The UV-fluorescent emission was recorded using the Quantity One software program (Bio-Rad). The concentration of the extracted DNA was quantified using the PicoGreen dye method (VersaFluor Fluorometer System, Bio-Rad). DNA purity was assessed using the UV spectrophotometric method (NanoDrop2000, Thermo Scientific, Wilmington, DE, USA).[28] The extracted DNA was diluted to a concentration of 100 ng/μL by adding 0.1× TE. The gDNA solution was thoroughly mixed on a shaker at a speed of 150 rpm.

The initial homogeneity was analyzed after 12, 24, 36, and 48 h of mixing. At each time point, three samples were collected from the upper, middle, and bottom layers of the bottle, and a total of nine samples were obtained for analysis, and each sample was tested three times. The optical density (OD) of absorbance maximum at a wavelength of 260 nm (OD260), which is directly related to the concentration of gDNA, was measured using a UV spectrophotometer (NanoDrop2000, Thermo Scientific). The *F* test was applied to analyze the OD260 data of the collected nine samples. After performing initial homogeneity assessment, the homogeneous gDNA solutions were placed in sterile, 2-mL skirted screw-cap self-sealing tubes, and each tube contained approximately 100 μL of Kefeng 6 gDNA.

Homogeneity and Stability Testing. Both Kefeng 6 event-specific and *PLD* real-time quantitative PCR assays were used to assess the homogeneity and stability of gDNA CRMs under repeatability conditions according to general guidelines for the certification of RMs.[29] The copy number concentration of Kefeng 6-specific (C_{KF6}) and *PLD* (C_{PLD}) targets was measured. The copy number ratio between Kefeng 6-specific and *PLD* targets (R) was calculated using the formula $R = \frac{C_{KF6}}{C_{PLD}}$, and the average of copy number concentration of both targets $\left(C = \frac{C_{KF6} + C_{PLD}}{2}\right)$ was calculated as the copy number concentration of DNA solution. Analysis of variance (ANOVA) was performed to evaluate potential between-unit heterogeneity, and short-term and long-term stability were assessed on the basis of the trend of the characteristic values of CRMs over time, and the uncertainty components introduced by heterogeneity and instability were evaluated according to ISO guide 35.[29]

For the freeze−thaw stability study, the characteristic values of CRMs were measured after 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 freeze−thaw cycles. At every cycle, the samples were taken from a −70 °C freezer, naturally thawed at room temperature, and then moved to −70 °C after these had completely melted. At each time, five samples were simultaneously tested under repeatable conditions. *T* tests were performed to evaluate the variance of property values with freeze−thaw cycles.

Collaborative Characterization. Before characterization, an operation protocol was prepared and sent to eight participating laboratories equipped with the ddPCR platform. Two vials of gDNA CRM were mailed to each participant in dry ice, and the primers/probes and related ddPCR reagents were prepared by each participant. The participating laboratories were requested to measure the copy number concentration and copy number ratio of the samples using KF6/*PLD* duplex ddPCR method based on the operation protocol. Each sample was repeatedly measured at least four times, and at least eight independent results were provided for each participant. After measurement, the raw file of dPCR was exported and returned to the CRM producer for statistical analysis according to ISO guide 35.[29]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02274.

Analytical results for initial homogeneity test of CRMs; schematic diagram illustrating the principle of identifying homozygotes; identification results of homozygous KF6 plants; homogeneity test results of CRMs; short-term stability test results of CRMs; long-term stability test of CRMs; and variation in copy number concentration of CRMs with the freeze−thaw cycles (PDF)

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Notes

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