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Development, Optimization, and Single Laboratory Validation of an Event-Specific Real-Time PCR Method for the Detection and Quantification of Golden Rice 2 Using a Novel Taxon-Specific Assay

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Supporting Information

ABSTRACT: In this study, we developed, optimized, and in-house validated a real-time PCR method for the event-specific detection and quantification of Golden Rice 2, a genetically modified rice with provitamin A in the grain. We optimized and evaluated the performance of the taxon (targeting rice Phospholipase D α2 gene)- and event (targeting the 3′ insert-to-plant DNA junction)-specific assays that compose the method as independent modules, using haploid genome equivalents as unit of measurement. We verified the specificity of the two real-time PCR assays and determined their dynamic range, limit of quantification, limit of detection, and robustness. We also confirmed that the taxon-specific DNA sequence is present in single copy in the rice genome and verified its stability of amplification across 132 rice varieties. A relative quantification experiment evidenced the correct performance of the two assays when used in combination.

KEYWORDS: *in-house validation, Golden Rice 2 (GR2), quantitative real-time PCR (qPCR), genetically modified organism (GMO), endogenous taxon-specific assay, event-specific method*

INTRODUCTION

The Golden Rice project was initiated by scientists from the Swiss Federal Institute of Technology and the University of Freiburg with the aim of addressing a serious public health problem, vitamin A deficiency, which affects those populations who consume predominantly rice as food and have limited access to vegetables and dairy and meat products.[1] Because no rice cultivars produce β-carotene, a precursor of vitamin A, in the endosperm, recombinant DNA technologies were used to insert the genes necessary for its synthesis.[2] Golden Rice 2 (GR2) was ready in 2005,[3] and the next step, undertaken by the International Rice Research Institute in the Philippines (IRRI) and national research institutes in India, was the transfer of the GR2 trait into local varieties by classical breeding. While conducting field trials of the introgressed GR2 varieties, IRRI became aware of the need to develop an event-specific method for the detection and quantification of GR2. Collaboration for this purpose was set up between IRRI and the Molecular Biology and Genomics (MBG) unit of the Institute for Health and Consumer Protection (IHCP) of the European Commission Joint Research Centre (JRC). The polymerase chain reaction (PCR), and in particular quantitative real-time PCR (qPCR), has become the technique of choice for the determination of the genetically modified organism (GMO) content of a sample,[4,5] and on the basis of its experience with the validation of GMO detection methods,[6−10] the MBG unit accepted to develop, optimize, and in-house validate a qPCR detection method for GR2. With qPCR methods, the GMO target is quantified in percentage of the amount of DNA from the same species present in the sample, by means of a taxon-specific assay and an assay targeting the GMO. Different types of assays, with different levels of specificity, can be used to target a GMO: element-, construct-, or event-specific.[11] Event-specific assays, which target a DNA sequence unique to a single GMO, are generally the method of choice for GMO quantification. The taxon-specific assay, targeting an endogenous gene, is very important for correct quantification.[11−13] To act as a reliable reference for GMO quantification, a taxon-specific assay must be specific; its sequence must be present in single copy in the plant's genome and stably amplified across different varieties of the target species.[11,14] Different rice taxon-specific assays were available for GMO analysis at the time when this project was started: ppi Phosphofructokinase (ppi-PPF),[15] RBE4[16] and gos9,[17−19] sucrose phosphate synthase (SPS),[20,21] and Phospholipase D (PLD) developed by Bayer.[22] However, two studies evidenced the presence of technical problems for most of those assays, confirmed by additional bioinformatics analyses performed by the MBG Unit of the IHCP, which in many cases led to method underperformance with respect to amplification efficiency or assay specificity.[23,24] In view of this, together with IRRI, the MBG Unit of the IHCP thus decided to develop and validate in-house a new taxon-specific assay for rice, designed on the PLD α2 gene and named PLD-GR, and an event-specific assay for GR2. Primers and probes for the two assays were designed by IRRI, while

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bioinformatics analyses, assay optimization, characterization, and in-house validation were conducted by the MBG Unit of the IHCP with the samples provided by IRRI. The characterization and validation of the two assays were performed in accordance with the European Network of GMO Laboratories (ENGL) minimum performance requirements (MPR) for analytical methods of GMO testing,[14] the requirements of the Codex Alimentarius for methods for detection, identification and quantification of specific DNA sequences,[25] and taking into account International Union of Pure and Applied Chemistry (IUPAC) harmonized guidelines for single-laboratory validation of methods of analysis.[26]

MATERIALS AND METHODS

Bioinformatics Analyses. Bioinformatics analyses were conducted on the JRC Central Core DNA Sequence Information System (CCSIS)[27] using PLD gene sequence (GenBank accession number NM_001064552.1) and sequence data for event Golden Rice 2-R (GR2-R) provided by IRRI for the two PLD-GR taxon-specific assays (Set1 and Set2) and the GR2 event-specific assays (LB-01 and RB-BS), respectively. Similarity searches were performed with BLASTN 2.2.15:[28] both primer sequences and the corresponding amplicon were used as query to scan the CCSIS DNA set, the plant sequence data section of the EMBL database, the NCBI nucleotide sequence and the rice TIGR databases, the vector sequences from synthetic (syn) division of GenBank, the NCBI patent nucleotide sequence, and the NCBI UniVec databases (web addresses available in the Supporting Information).

Genomic DNA and Plant Materials. Frozen leaves of non-GM rice (*Oryza sativa* ssp. Japonica, cv. Kaybonnet) and GM rice (homozygous for GR2-R, GR2-E, GR2-G, GR2-L, GR2-T, or GR2-W GR2 transformation events, cv. Kaybonnet), and genomic DNA extracted from 131 non-GM rice varieties (complete list available in Table S1, Supporting Information) were provided by IRRI. DNA from sorghum (*Sorghum vulgare*), barley (*Hordeum vulgare*), wheat (*Triticum durum*), triticale (*x Triticosecale*), and rye (*Secale cereale*) was extracted from ground seeds or leaves at the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF). Genomic DNA from non-GM soybean (*Glycine max*), rapeseed (*Brassica napus*), cotton (*Gossypium hirsutum*), sugar beet (*Beta vulgaris*), potato (*Solanum tuberosum*), and from the GM events listed in "Genomic DNA and plant materials", Supporting Information, was prepared by the EU-RL GMFF.

Genomic DNA Extraction and Evaluation of Quality and Quantity. Barley, rice, rye, sorghum, triticale, and wheat genomic DNA was extracted with either a CTAB DNA extraction method (modified from ISO 21571[29]) or a phenol-chloroform method optimized for rice leaves (modified from Sambrook and colleagues[30]). All extracted DNAs were tested for integrity using a 1% (w/v) agarose gel electrophoresis and for purity by assessing the presence of PCR inhibitors as described by Zel and colleagues[31] and the absence of contamination with the GR2-R event. DNA concentration was determined fluorimetrically with the Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes, Eugene, U.S.) with a five-point standard curve ranging from 1 to 500 ng/mL with a Biorad (Hercules, U.S.) VersaFluor fluorometer. Only pure, noninhibited high molecular weight DNA was used.

Sample Preparation. Samples for optimization and in-house testing of the taxon- and event-specific assays were prepared diluting DNAs in 0.1 × TE. Samples for the relative quantification were prepared in haploid genome equivalents (hge) ratio by mixing GM (GR2-R event) and non-GM (cv. Kaybonnet) rice DNA. Standard curve samples were prepared by serial dilution; test samples were prepared independently. Genome sizes taken into account for the calculation of hge contents are listed in "Genome Sizes", Supporting Information.

Specificity. For the taxon-specific assay (Set1 and Set2), all of the species mentioned in the section Genomic DNA and Plant Materials (except carnation) were tested with 200 ng DNA/reaction; reactions were performed in triplicate, and the resulting Cqs were averaged. Rice DNA was included as positive control at 100 ng/reaction, with six replicates. For the event-specific assay (RB-BS set), the events listed in "Genomic DNA and Plant Materials", Supporting Information, were tested, as well as non-GM DNA from the same species tested with the taxon-specific assay, with a DNA amount corresponding to 200 000 hge/reaction, up to a maximum of 200 ng of DNA. Reactions were performed in triplicate, and the resulting Cqs averaged. The DNA amount used for each event/species and the GM material used are reported in "Specificity", Supporting Information. DNAs used for the tests were previously checked for the absence of inhibition as described in ref 31 with a suitable taxon-specific assay.

Stability of the Taxon-Specific Assay. The variability of the Cqs obtained with the PLD-GR taxon-specific assay (Set1) with a fixed DNA concentration was evaluated on 131 rice varieties, selected by IRRI as representative of global genetic diversity, and the Kaybonnet variety. The variability between plates was maintained as low as possible by performing all qPCR experiments on the same day and the same instrument; the same baseline and threshold were used for the analysis of the results of all plates. The list of the varieties tested, subspecies they belong to, and the obtained Cq are available in Table S1, Supporting Information. The genomic DNA of each variety was diluted to 25 ng/reaction, corresponding to 50 000 hge; reactions were performed in triplicate, and the resulting Cqs were averaged.

Dynamic Range and LOQ. A set of standard curve and test samples was prepared for the PLD-GR (Set1) and one for the GR2 (RB-BS set) assay, with non-GM and GR2-R DNA, respectively. Table S2, Supporting Information, reports the hge and DNA content of the standard curve and the test samples. Each standard curve sample was analyzed in three plates in triplicate, for a total of nine replicates, and each test sample was measured in three plates with eight replicates, for a total of 24 replicates. The hge content of the test samples was estimated by interpolation from the standard curve constructed with the standard curve samples. The limit of quantification (LOQ) of the two assays was evaluated concurrently.

LOD. The limit of detection (LOD) of the two assays was first assessed with 10 replicates over a range of 3−50 hge/reaction, on the samples described in Table S3, Supporting Information. The 0.1, 0.5, and 1 hge samples were included in the test to verify the correctness of the dilution, by evaluating the number of negative replicates obtained. Samples D1−D9 were prepared by serial dilution of non-GM rice DNA or GR2-R GM DNA for the taxon- and event-specific assays, respectively; the 0.5 hge sample was prepared and assessed only for the event-specific assay. To reach the 95% level of confidence, on the basis of the pilot test, two hge levels were selected for each assay, which were then tested, together with the 0.1 and 1 hge samples, with 60 replicates. For the PLD-GR taxon-specific assay, the 4.5 and 3 hge levels were selected, for the GR2 event-specific assay, the 6 and 4 hge levels. The LOD was set at the lowest concentration yielding at least 59 positive results.[32,33]

Robustness. The robustness of the PLD-GR (Set1) and the GR2 (RB-BS set) assays was tested by introducing variations in the volume of reaction mix per reaction (21 μL or 19 μL instead of 20 μL), the concentration of Mastermix (±20%), the concentration of primers or probe (±30%), the annealing temperature (±1 °C), and the qPCR equipment: Roche (Basel, Switzerland) LightCycler (LC) 480, Stratagene (La Jolla, CA) Mx3005P, Applied Biosystems (ABI, Waltham, U.S.) 7500. RSD_r and bias were evaluated on quantification of three test samples chosen to cover the full dynamic range of the GR2 assay (from 40 to 200 000 hge) and almost all that of the PLD-GR assay (from 30 to 200 000 hge). Test samples were quantified in repeatability conditions, with 3 replicates over one run for each condition. Standard curve and test samples were prepared with non-GM DNA for the taxon-specific assay and with GM (GR2-R) DNA for the event-specific assay (Table S4, Supporting Information).

Relative Quantification. Three test samples with a GM % ranging from 0.1% to 4.5% were prepared on the basis of their hge content and quantified with two qPCR runs and 6 replicates for each GM-level on each plate, with the PLD-GR (Set2) and the GR2 (RB-BS set) assays,

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Table 1. Position, Amplicon Size, Target, and Sequence Information for the Four Primers/Probe Sets Tested in This Study[a]

assay	sequence (5′–3′)	target	amplicon size (bp)	position (bp)
GR2 (LB-01)	For- CCGAGCTGCGAGAAGGAGTA	GR2-R event, left border - plant, DNA junction	120	518–637
	Rev-CGTTTCCCGCCTTCAGTTTA			
	Probe-CGACCTGCAGAAACCCACCACCA			
GR2 (RB-BS)	For-TGGCCGTATCCGCAATGTG	GR2-R event, right border - plant, DNA junction	121	9541–9660
	Rev-GCTGCGCCTCTAACCAGGTAT			
	Probe-TCGATATGATTCCTTCATCGAGCGAGC			
PLD-GR (Set1)	For-GGCAAGAACAACACCATTGACA	*Oryza sativa* PLD α 2 gene sequence (GenBank NM_001064552.1)	124	Exon 2 (1635–1758)
	Rev-CGGCTTTCCATGCAAATGA			
	Probe-ATTCGCCGCGCCAAGAACTTCA			
PLD-GR (Set2)	For-GGCAGCTCATTTGCATGGA	*Oryza sativa* PLD α 2 gene sequence (GenBank NM_001064552.1)	121	Exon 2 (1734–1854)
	Rev-CAAAACGCTCACCAGCTTCA			
	Probe-ACATTGAGGCGTTGCATCTGATTCCC			

[a]bp, base pairs.

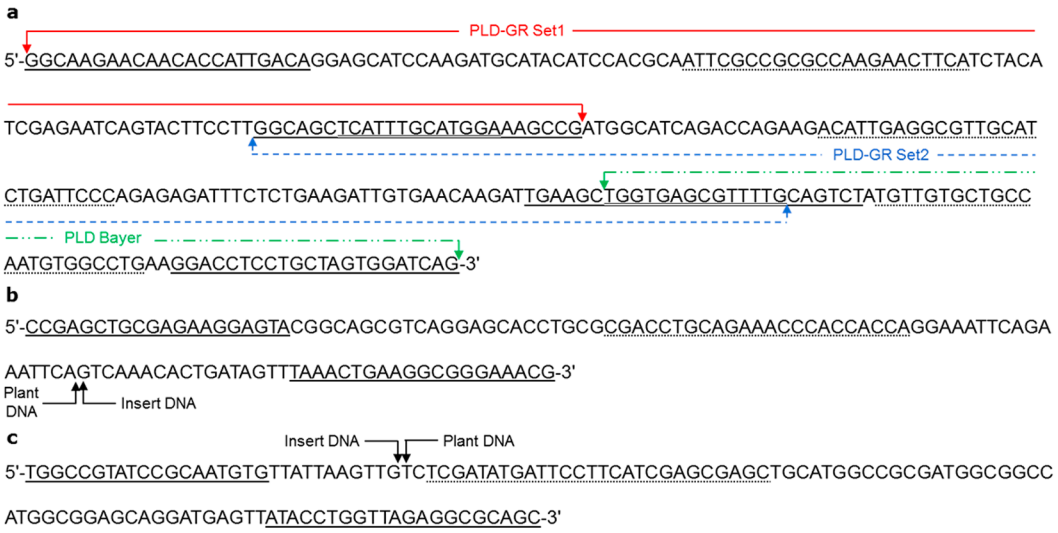

Figure 1. Amplicon sequence of PLD taxon-specific assays and GR2 event-specific assays. (a) PLD-GR Set1 and Set2, and PLD taxon-specific assay developed by Bayer;[22] (b) GR2 LB-01 primers/probe set; (c) GR2 RB-BS set. PLG-GR Set1 and Set2 overlap of 25 bp at their 5′ and 3′ ends, respectively; PLD-GR Set2 and PLD from Bayer overlap of 14 bp at their 5′ and 3′ ends, respectively; GR2 LB-01 set covers the 3′ plant-to-insert DNA junction; GR2 RB-BS set covers the 5′ insert-to-plant junction. Primer sequences are underlined with a single line, or a double line if two primers overlap, probe sequences are underlined with a dotted line; the junction between plant and transgenic insert DNA is indicated with arrows in GR2 amplicons; PLD-GR Set1 amplicon is indicated with a solid, red line, PLD-GR Set2 amplicon with a blue, broken line, and PLD amplicon developed by Bayer with a green broken and dotted line.

with a five-point standard curve with a GM % content ranging from 5% to 0.07%. The GR2 hge and PLD hge content of the standard curve and test samples were included in the dynamic range of the two assays. Hge and DNA content, dilution factor (standard curve only), and GM % of the standard curve and test samples are reported in Table S5, Supporting Information.

PCR Primers and Probes. Primers and TaqMan probes sequences were designed by IRRI using Primer Express Software (Life Technologies, Carlsbad, CA) and selected by the EU-RL GMFF on the basis of bioinformatics analyses. Primers were also tested against known plant genomes using the ePCR prediction tool.[34] The selected TaqMan probes are labeled with 6-carboxyfluorescein (FAM) fluorophore at 5′ and with tetramethylrhodamine (TAMRA) quencher at 3′. The sequence, annealing position of the primers and probes, and length of the obtained amplicons are reported in Table 1. Primers were purchased from Eurofins MWG operon (Ebersberg, Germany) and probes from ABI UK (Warrington, United Kingdom). The primers/probe concentration for PLD-GR taxon-specific assay and for GR2 event-specific assay was optimized by testing different primers/probe concentrations on a serial 4-fold dilution of DNA starting from 200 ng of non-GM DNA/reaction to 0.78 ng/reaction for the taxon-specific assay and from 100 to 0.39 ng of GM (GR2-R event) DNA/reaction for the event-specific assay. An optimal primer concentration was first selected, keeping the probe at 150 nM and testing the primers from 150 to 450 nM by 100 nM increases; probe concentrations tested ranged from 100 to 250 nM by 50 nM increases, with primers at 350 nM. The selection criteria were: profile and level of fluorescence of the amplification curves at different DNA concentrations, presence/absence of inhibition, and slope of the dilution. The final selection was 300 nM primers and 150 nM probe for the two primers/probe pairs tested for each of the two assays.

Real-Time PCR. The optimized qPCR reaction mixture for all four primers/probe sets tested contained the following reagents: 1x TaqMan universal master mix (ABI), 300 nM primer forward, 300 nM primer reverse, 150 nM probe, 5 μL of DNA sample, and nuclease free water (Promega, Madison, WI) up to a volume of 25 μL/reaction. All qPCR runs, except where specified for the robustness test, were performed with ABI 7900 platform (Life Technologies, Carlsbad, CA) with the following thermal profile: 50 °C for 2 min; 95 °C, 10 min; 45 cycles of 95 °C, 15 s and 60 °C, 60 s; fluorescence data were acquired at the end of each cycle.

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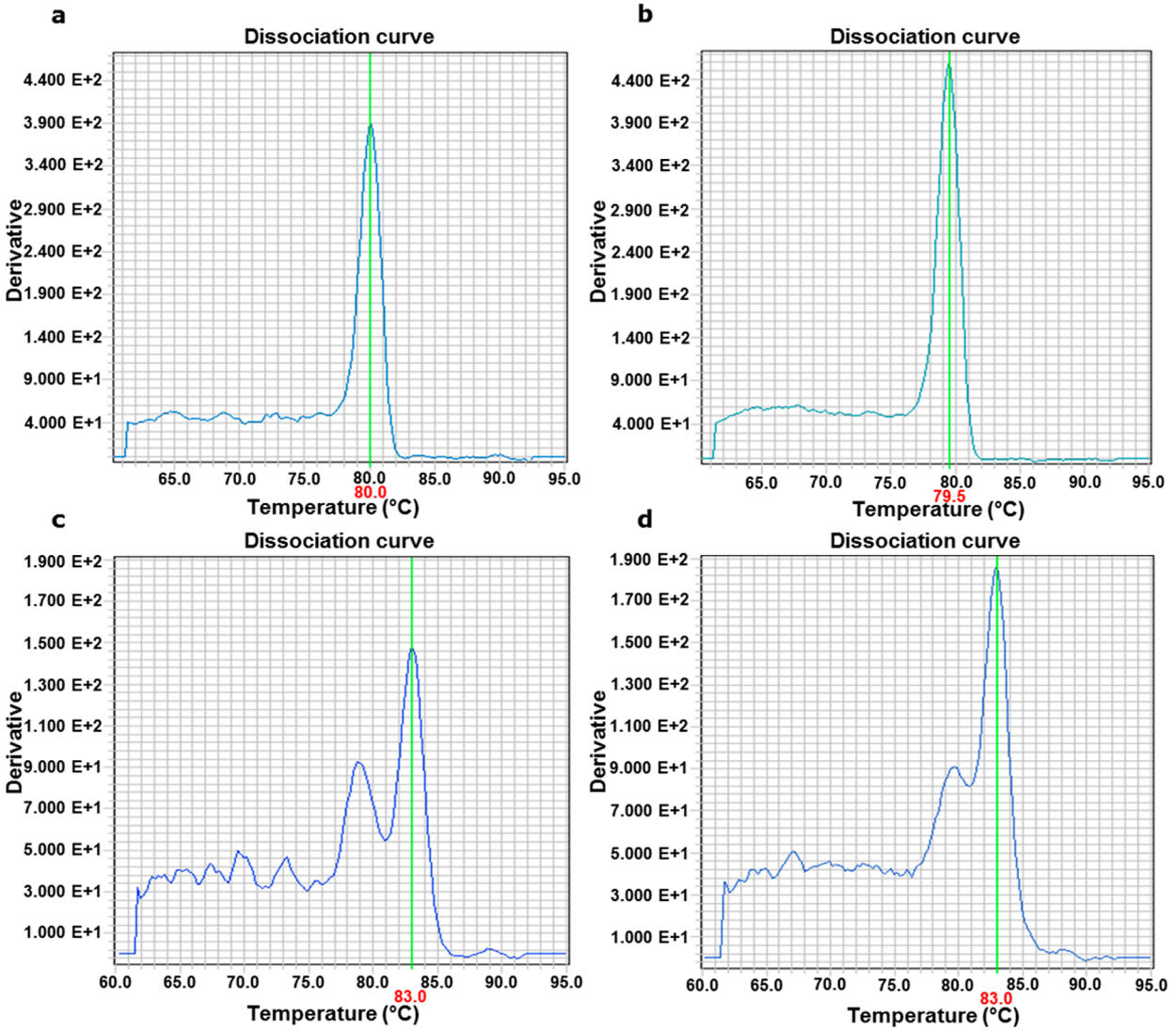

Figure 2. Melting curve profile of (a) PLD-GR taxon-specific assay, Set1; (b) PLD-GR taxon-specific assay, Set2; (c) GR2 event-specific assay, LB-01 set; and (d) GR2 event-specific assay, RB-BS set. A single peak is present in PLD-GR Set1 and Set2 assays melting profile; a weaker peak (lower melting temperature, putative primer dimers) and a stronger peak (higher melting temperature, specific amplification) are present in the GR2 LB-01 and RB-BS assays melting profile, the weaker peak being more evident in the LB-01 assay melting profile.

Melting curve analysis, Agilent microfluidic chip assay, sequencing, and digital PCR methods are described in the Supporting Information.

Data Analysis. Standard curves were generated by plotting the Cq values measured for the calibration points against the logarithm of the DNA hge content and by fitting a linear regression line into these data. The hge content of the test samples was then estimated by interpolation from the standard curve. Experiments performed on ABI 7900 qPCR equipment were analyzed with the SDS 2.4 software (Life Technologies, Carlsbad, CA); the baseline was set manually three cycles before the cycle number at which the threshold line crosses the first amplification curve; the threshold was set manually in the area where the amplification profiles appeared linear and there was no fork effect between replicates of the same sample. MIQE guidelines were taken into consideration when applicable to the present work.[35] Robust mean calculation and cluster analysis are described in the Supporting Information.

Performance Criteria. Performance parameters of the PLD-GR and GR2 assays were evaluated in accordance with ENGL MPR.[14] Specificity, dynamic range, accuracy, PCR efficiency, linearity, LOQ, LOD, and robustness were evaluated, as well as copy number and stability of the PLD-GR taxon-specific assay. Accuracy was evaluated on the basis of two parameters: trueness and precision. Trueness was expressed as mean percent bias (difference between the mean value of test results and the accepted reference value divided by the latter). Precision was expressed as relative repeatability standard deviation (RSD_r) in percent relative to the accepted reference value. Amplification efficiency was calculated from the slope of the standard curve using the formula: efficiency = $((10^{(-1/\text{slope})}) - 1) \times 100$. Linearity was expressed as coefficient of determination of the standard curve obtained by linear regression analysis. The criteria set for each of these parameters are discussed throughout the text.

RESULTS

Bioinformatics Analyses. The two sets of primers and probes for the PLD-GR taxon-specific assay (see Table 1 for primers and probes sequence and location) produced alignments only with exon 2 of the PLD α2 gene in *Oryza sativa* and with sequences from *Oryza glaberrima*, African rice (data not shown). Set1 and Set2 PCR amplicons overlap for 25 bp and Set2 overlaps for 14 bp with the amplicon of the PLD assay developed by Bayer (Figure 1a).[22] The two sets of primers and probes for the event-specific assay produced alignments with no other sequence than the GR2-R event. The LB-01 set is

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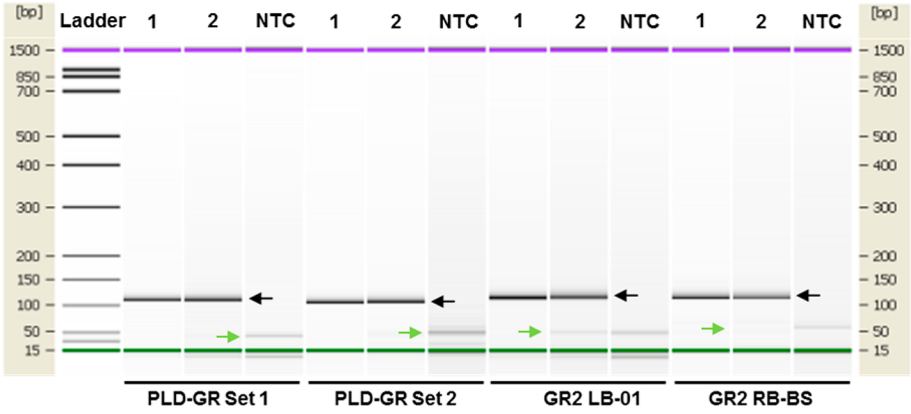

Figure 3. Virtual gel representation of capillary electrophoresis of the amplification products of PLD-GR taxon-specific assay Set1 and Set2, and GR2 event-specific assay LB-01 and RB-BS sets. PLD-GR Set1 and Set2 assays show one specific amplification product (black arrow, pointing left) for the two template DNA concentrations loaded (1, 40 000 hge; 2, 156 hge), and a lower band of putative primer dimers (green arrow, pointing right) only in the no template control (NTC). GR2 LB-01 and RB-BS sets show one specific amplification product for the two template DNA concentrations loaded and primer dimers in the no template control and with a weak (LB-01 set) and very weak band (RB-BS set) also in the amplification with lower template DNA concentration. bp, base pairs.

designed across the 5′ plant−insert DNA junction, the RB-BS set across the 3′ insert−plant DNA junction (Figure 1b,c).

Optimization and Characterization of the PLD-GR and GR2 Assays. We optimized and characterized the PLD-GR taxon-specific and the GR2-R event-specific qPCR assays independently. We defined the optimal concentration of primers and probes as described in the Materials and Methods, which resulted to be 300 nM primers and 150 nM probe for all sets. We characterized the PCR products of the four primers/probe sets with melting curve analysis, capillary electrophoresis, and amplicon sequencing. The melting temperature of the PLD-GR assay ranged, depending on the amount of DNA in reaction, from 79.7 to 80.3 °C with an average of 80.0 °C for Set1 and from 79.2 to 79.9 °C with an average of 79.5 °C for Set2; the profiles of the dissociation curves were indicative of specific amplification (Figure 2a,b). Capillary electrophoresis showed the presence of a single band of the expected size for both sets, on a clean background (Figure 3). The sequences generated on both strands of PLD-GR Set1 and Set2 amplicons showed 100% identity with PLD α2 gene, further confirming the specificity of the amplification. For the GR2 assay, the melting temperature ranged from 82.5 to 83.0 °C with an average of 82.8 °C for the LB-01 set and from 82.5 to 83.0 °C with an average of 82.9 °C for RB-BS primer set. The melting profiles of the two sets were indicative of specific amplification, although they showed peaks consistent with the formation of putative primer-dimers, more evident for the LB-01 set (Figure 2c,d). Capillary electrophoresis showed the presence of a single band of the expected size for both sets, and smaller bands barely visible, with a size consistent with primer-dimers (Figure 3). The sequences obtained for GR2 LB-01 and RB-BS amplicons showed 100% identity with the sequence of the GR2 event provided by IRRI. Because both melting curve analysis and capillary electrophoresis indicated that the formation of primer dimers was less evident for GR2 RB-BS primer set as compared to LB-01 primer set, we chose the RB-BS set for further analyses.

Specificity. We tested the specificity of the PLD-GR and GR2 assays experimentally, verifying their amplification with genomic DNA from other species and/or events. PLD-GR Set1 assay amplified only rice DNA, while PLD-GR Set2 produced unspecific amplification, with high Cqs, with DNA from barley, wheat, and rye (Table S6, Supporting Information). We thus selected Set1 primers and probe for further analyses, and discarded Set2. We tested the specificity of the GR2 assay on the same species listed for the PLD-GR assay and on DNA from all of the GM events, which were authorized or in the course of authorization in the EU at the moment of testing, plus all GR2 transformation events, rice events LL62 and LL601, and carnation event IFD-25958. We did not observe any unspecific amplification. A detailed list of the DNAs and the DNA amount tested is reported in the Materials and Methods and as Supporting Information, and the list of the species and events tested with the obtained Cqs is available in Table S7, Supporting Information.

Copy Number and Stability of the PLD-GR Taxon-Specific Assay. We performed the digital PCR experiment as described in the Supporting Information, and obtained a mean ratio between event-specific and taxon-specific amplification of 1.033, which, given that the 95% confidence interval of the mean spans around one, is not significantly different from the expected ratio of one (Table 2). We thus confirmed that the PLD-GR amplicon is present in single copy in the haploid genome of the transformed variety.

Table 2. Results of Digital PCR Analysis Conducted with the GR2 and PLD-GR Assays on GM DNA, 100% GM for GR2 Event

mean ratio (GR2/PLD-GR)	1.033
standard deviation	0.106
RSD$_r$, %	10.230
standard error of the mean	0.027
upper 95% CI of the mean	1.090
lower 95% CI of the mean	0.977

We then assessed the stability of the PLD-GR assay as described in the Materials and Methods. The results of the test are presented in Figure 4, where the Cq value obtained for each variety (Kaybonnet plus 131 varieties) is plotted, together with the overall mean, median, and robust mean (i.e., a mean whose estimate is less influenced by potential outliers in the data), and ±0.5 Cq intervals. According to ENGL MPR,[14] the range of

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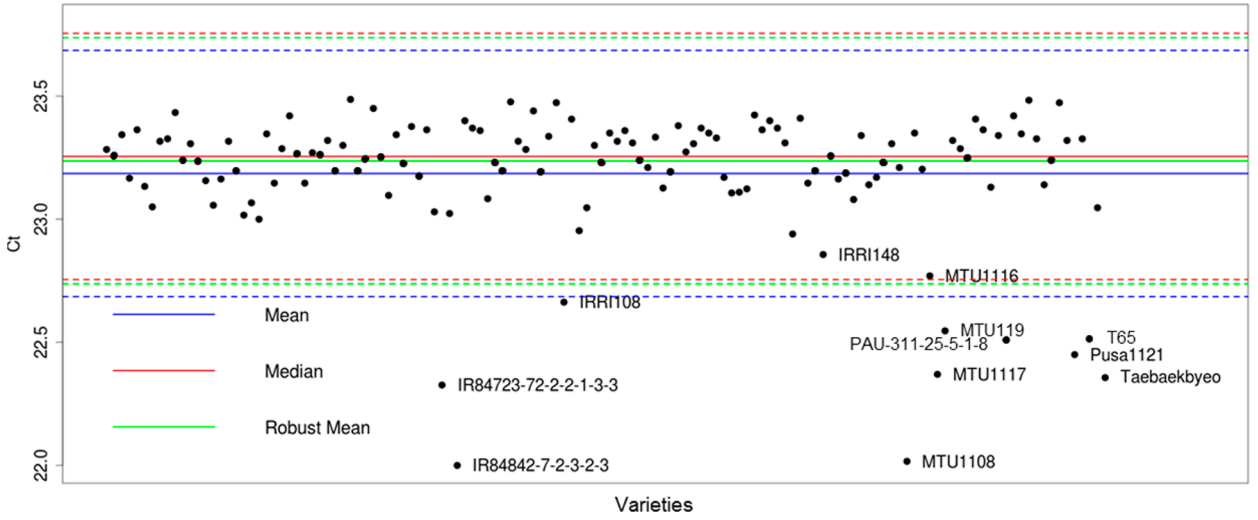

Figure 4. Results of the stability test for PLD-GR taxon-specific assay (Set1). The average Cq value obtained for each variety is plotted along the *x* axis; the mean of all measured Cq values (blue line), the median (red line), and the robust mean (green line) are represented; broken lines represent the mean (blue), median (red), and robust mean (green) plus or minus 0.5 Cq. Most varieties (122 out of 132) are distributed within ±0.5 Cq from the mean/median/robust mean. Twelve varieties, labeled with a text string, were identified as a cluster distinct from the other varieties.

Table 3. Results of the Quantification of the Test Samples for the Determination of the Dynamic Range and LOQ of PLD-GR Taxon-Specific Assay and GR2 Event-Specific Assay

PLD-GR				GR2			
expected hge/reaction	measured mean	RSD_r %	bias %	expected hge/reaction	measured mean	RSD_r %	bias %
200 000	170 507	4.2	−14.7	200 000	213 864	10.1	6.9
20 000	20 650	6.5	3.3	20 000	20 647	11.7	3.2
2000	2043	8.8	2.2	2000	1939	8.7	−3.0
200	170	10.4	−14.8	200	184	12.4	−8.0
60	63	17.4	5.4	60	54	21.0	−9.5
50	42	19.8	−15.1	50	49	13.5	−1.6
40	46	16.7	15.5	40	37	20.0	−8.6
30	37	17.8	22.2	30	31	**33.8**	2.9

Table 4. Standard Curve Parameters Obtained in the Experiment for the Determination of the Dynamic Range and LOQ of PLD-GR Taxon-Specific Assay and GR2 Event-Specific Assay

PLD-GR				GR2			
plate	slope	PCR efficiency	linearity	plate	slope	PCR efficiency	linearity
A	−3.59	89.8	0.996	A	−3.50	93.0	0.998
B	−3.55	91.2	0.998	B	−3.55	91.2	0.998
C	−3.56	91.1	0.997	C	−3.56	91.1	0.999
mean	−3.57	90.7	0.997	mean	−3.54	91.8	0.998

variability of Cq values within the taxon should not exceed 1 Cq. As shown in Figure 4, most varieties (120 out 132) were comprised between 22.94 and 23.49 Cq with a maximum distance of 0.55 Cq (unlabeled varieties). To identify possible outlying varieties, we performed cluster analysis, a technique that aims at discovering groups of objects with similar characteristics in a set of data, and we labeled with a text string the varieties that were identified as a distinct cluster. Some of the labeled varieties were however comprised in the mean ±0.5 Cq interval (IRRI148 and MTU1116) or at least in an interval of 1 Cq from the variety with the highest Cq value (IRRI108, MTU1119, T65, and PAU-311-25-5-1-3). Only six varieties (Pusa1121, MTU1117, Taebaekbyeo, IR84723-72-2-2-1-3-3, MTU1108, and IR84842-7-2-3-2-3) were outside that 1-Cq interval. The Cq we measured for those varieties was lower than the accepted range, thus excluding the possibility that sequence variability in the region amplified by the PLD-GR assay would lead to lower PCR efficiency, and thus to a higher Cq value.

Dynamic Range of the Two Assays. We assessed the dynamic range of the two assays as reported in the Materials and Methods. Table 3 reports the hge content, RSD_r, and bias measured for each test sample, for the PLD-GR and the GR2 assays. For the PLD-GR taxon-specific assay, the RSD_r was acceptable between 200 000 and 30 hge per reaction, for the GR2 event-specific assay between 200 000 and 40 hge. Bias was below 25% for all test samples for both the PLD-GR and the GR2 assays. On the basis of these values of RSD_r and bias, and in accordance with ENGL MPR,[14] which establishes that the RSD_r and bias should be below 25% over the whole dynamic range, we defined the dynamic range of the PLD-GR taxon-specific assay to be between 30 and 200 000 hge and between

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Table 5. Results of the Quantification of the Test Samples for the Determination of the Robustness of the PLD-GR Taxon-Specific Assay

	200 000 hge/reaction			2000 hge/reaction			40 hge/reaction		
condition	estimated hge	RSD_r %	bias %	estimated hge	RSD_r %	bias %	estimated hge	RSD_r %	bias %
21 μL of reaction mix	209 046	4.0	4.5	2363	2.2	18.2	41	13.5	1.6
19 μL of reaction mix	180 696	5.4	−9.7	2074	5.7	3.7	32	9.0	−19.7
+20% Mastermix	211 853	2.2	5.9	2202	4.8	10.1	34	8.0	−13.8
−20% Mastermix	190 496	2.0	−4.8	2018	6.0	0.90	31	7.2	−23.4
+30% primers	178 343	24.7	−10.8	2230	5.4	11.5	45	15.8	12.9
−30% primers	182 927	5.3	−8.5	1972	6.0	−1.4	32	9.1	−21.2
+30% probe	187 402	3.0	−6.3	1862	3.3	−6.9	43	22.3	8.2
−30% probe	187 864	0.55	−6.1	2074	2.4	3.7	43	13.9	6.3
+1 °C annealing *T*	188 213	7.8	−5.9	1981	5.4	−0.9	45	3.9	12.7
−1 °C annealing *T*	207 456	2.3	3.7	1877	3.1	−6.2	32	9.7	−19.4
Cycler B (Roche)	176 620	1.7	−11.7	2101	2.7	5.1	34	20.3	−15.5
Cycler C (Stratagene)	233 789	6.2	16.9	2025	4.1	1.3	33	16.9	−16.6
Cycler D (ABI 7500)	181 524	2.8	−9.2	1931	3.9	−3.5	45	6.8	11.7

Table 6. Results of the Quantification of the Test Samples for the Determination of the Robustness of the GR2 Event-Specific Assay

	200 000 hge/reaction			2000 hge/reaction			40 hge/reaction		
condition	estimated hge	RSD_r %	bias %	estimated hge	RSD_r %	bias %	estimated hge	RSD_r %	bias %
21 μL of reaction mix	193 669	2.9	−3.2	1799	4.7	−10.1	30	13.0	−24.4
19 μL of reaction mix	196 258	5.1	−1.9	1956	7.3	−2.2	35	6.3	−11.5
+20% Mastermix	195 887	3.9	−2.1	1696	3.3	−15.2	30	7.2	−23.8
−20% Mastermix	183 955	8.6	−8.0	1570	3.1	−21.5	32	20.6	−20.5
+30% primers	190 938	6.6	−4.5	1844	3.5	−7.8	44	8.7	11.0
−30% primers	196 258	5.1	−1.9	1956	7.3	−2.2	35	6.3	−11.5
+30% probe	187 233	5.5	−6.4	1716	12.2	−14.2	35	8.0	−13.7
−30% probe	209 074	1.4	4.5	1649	0.66	−17.6	31	11.4	−21.4
+1 °C annealing *T*	190 313	3.6	−4.8	1690	11.1	−15.5	30	16.2	−24.9
−1 °C annealing *T*	183 526	3.1	−8.2	1663	3.4	−16.8	37	13.9	−7.4
Cycler B (Roche)	196 018	2.6	−2.0	1871	0.39	−6.5	29	4.2	**-27.9**
Cycler C (Stratagene)	201 041	2.5	0.52	2053	8.3	2.6	39	21.1	−2.2
Cycler D (ABI 7500)	187 506	2.3	−6.2	1976	1.1	−1.2	38	11.6	−5.3

40 and 200 000 hge for GR2 event-specific assay. As reported in Table 4, for the PLD-GR assay and the GR2 assay, slope values were below −3.6; the average PCR efficiency was above 90% and the average R^2 coefficient was above 0.99. These values are within the limits established by ENGL MPR.[14]

LOQ and LOD. According to ENGL MPR,[14] the LOQ of a method should be equal to or below the lowest amount or concentration included in the dynamic range. In this study, we assessed the LOQ together with the dynamic range of the method, and it consequently corresponds to the lowest test sample included in the dynamic range. For the PLD-GR assay, we thus established the LOQ at 30 hge per reaction, with a RSD_r of 17.8% and a bias of 22.2%; for the GR2 assay at 40 hge per reaction, with a RSD_r of 20.0% and a bias of −8.6% (Table 3).

The LOD of a method should be below 25 hge with a level of confidence of 95%, ensuring less than or equal to 5% false negative results.[14] The results of the test are reported in Table S8, Supporting Information: the LOD was at 4.5 hge/reaction for the PLD-GR assay and at 4 hge for the GR2 assay. The 1 hge level had 36.7% and 33.3% negative results, respectively, for the PLD-GR and the GR2 assay, in line with the value of 36% that is expected taking into account the probability of distribution of the target DNA in the 60 replicates.[36]

Robustness. Robustness was evaluated as described in the Materials and Methods. The results of the quantification for the determination of the robustness are illustrated in Table 5 for the PLD-GR assay and in Table 6 for the GR2 assay. The measured RSD_r was below 25% for the three test samples with all of the conditions tested for both the PLD-GR and the GR2 assays, in line with ENGL MPR;[14] bias was within ±25% for all of the conditions and test samples for the PLD-GR assay, while for the GR2 assay there was only one value of bias below −25% for the test sample at the LOQ, with Roche LC 480 qPCR instrument. We also evaluated the efficiency and linearity of the two assays, and the results are reported in Table 7. The values of slope and linearity were within the ENGL MPR[14] for both assays; the PCR efficiency was above 90% for both assays with all conditions, with the exception of the experiments performed with the GR2 assay with a concentration of primers 30% lower and on ABI 7500, for which the efficiency was 89.5% and 89.9%, respectively.

Relative Quantification. The results of the relative quantification are reported in Table 8: for the three test samples, the measured RSD_r ranged from 9.9% to 13.1%, and bias ranged from −6.2% to 5.3%. Both parameters are below 25%. The slope, PCR efficiency, and linearity of the standard curves for the two runs and their average over the two runs are

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Table 7. Standard Curve Parameters Obtained with the Experiment for the Determination of the Robustness of PLD-GR Taxon-Specific Assay and GR2 Event-Specific Assay

	PLD-GR			GR2		
condition	slope	PCR efficiency	linearity	slope	PCR efficiency	linearity
21 μL of reaction mix	−3.46	94.4	0.998	−3.53	91.9	0.999
19 μL of reaction mix	−3.48	93.6	0.999	−3.55	91.1	0.998
+20% Mastermix	−3.44	95.4	0.998	−3.47	94.3	0.998
−20% Mastermix	−3.39	97.1	0.999	−3.53	92.1	0.998
+30% primers	−3.45	95.1	0.998	−3.57	90.5	0.999
−30% primers	−3.44	95.3	0.998	−3.60	**89.5**	0.997
+30% probe	−3.39	97.3	0.999	−3.53	91.9	0.998
−30% probe	−3.44	95.2	0.998	−3.50	93.1	0.998
+1 °C annealing *T*	−3.44	95.1	0.998	−3.51	92.7	0.998
−1 °C annealing *T*	−3.40	97.0	0.998	−3.58	90.2	0.998
Cycler B (Roche)	−3.44	95.2	0.998	−3.55	91.3	0.999
Cycler C (Stratagene)	−3.33	99.5	0.998	−3.53	92.0	1.000
Cycler D (ABI 7500)	−3.40	96.9	1.000	−3.59	**89.9**	0.999

Table 8. Relative Quantification Test Results

expected GM %	measured GM %	RSD_r %	bias %
4.5	4.74	9.9	5.3
0.9	0.90	13.1	0.4
0.1	0.09	10.4	−6.2

reported in Table 9. All parameters are within ENGL MPR[14] for both assays.

Table 9. Standard Curve Parameters Obtained for PLD-GR Taxon-Specific Assay and GR2 Event-Specific Assay in the Relative Quantification Experiment

	PLD-GR taxon-specific assay			GR2 event-specific assay		
	slope	PCR efficiency	R^2	slope	PCR efficiency	R^2
run A	−3.27	102.10	0.999	−3.48	93.77	0.997
run B	−3.45	95.02	0.997	−3.46	94.42	0.998
average	−3.36	98.56	0.998	−3.47	94.10	0.997

DISCUSSION

In this study, we describe the development, optimization, and in-house validation of the two TaqMan qPCR assays that compose the method for the event-specific detection and quantification of GR2 with a new rice taxon-specific assay, PLD-GR. The GR2 event-specific assay amplifies a 121-bp fragment across the 3′ junction between transgenic insert and plant DNA; the new PLD-GR taxon-specific assay amplifies a 124-bp fragment of rice PLD gene. These two assays were validated independently, on hge basis, and their performance was evaluated following European guidelines[14] and taking into consideration IUPAC harmonized guidelines for single-laboratory validation of methods of analysis.[26] All performance criteria adopted in this study satisfy the requirements of the Codex Alimentarius for methods for detection, identification, and quantification of specific DNA sequences.[25]

According to the principle of modularity, different parts of an analytical procedure, for example, DNA extraction and qPCR, can be evaluated and validated independently, provided that specific performance requirements are satisfied for each module.[37−39] In this study, we developed and validated only the qPCR module, independently of the DNA extraction method, in line with the principle of modularity. This approach is also supported by a study that compared the results of 53 collaborative trials for the validation of qPCR methods and indicated that the standard deviation introduced by the DNA extraction step with respect to GM quantification is negligible when compared to the one introduced by the qPCR step.[40] Furthermore, we considered the taxon- and the event-specific assays that constitute the GR2 qPCR method as independent modules, as already discussed in Kagkli et al.[39] This strategy is advantageous because it offers increased flexibility and potentially lowers the cost of method testing and validation, allowing, for example, the use of the same taxon-specific assay in combination with different event-specific assays, without the need to revalidate the combination of assays. A GMO testing laboratory could select the best combination of taxon- and event-specific assays, depending on the availability of PCR reagents or of verified and implemented taxon-specific assays. When a detection method for a new GMO event would have to be developed, the taxon-specific assay would not need to be developed and/or validated a second time. This approach also allows a wider freedom in the GM percentages that can be tested, which are instead limited to the validated dynamic range when the qPCR method is treated as a single module, and on whether to use a delta Cq method or a two-standard curves method. The results of the relative quantification experiment performed with the GR2 method confirmed that the two assays can be used in combination to quantify GR2 relative content, with acceptable levels of trueness and precision (Table 8).

For the PLD-GR taxon-specific assay, bioinformatics analyses with PLD-GR primer sets and amplicons evidenced that perfect alignments were produced only with rice and African rice sequences. Our bioinformatics analyses showed that also the primers for the PLD assay from Bayer, the SPS, and the gos9 assays[17−22] may potentially amplify sequences from African rice, suggesting that sequence variability between rice and African rice is low, which restricts the possibilities to design primers specific for one of the two species. The production of African rice is however limited, and the color of the African rice grain normally ranges from red to brown, easy to distinguish from rice by eye inspection. The specificity of amplification of the selected event- and taxon-specific assays was confirmed by melting curve analysis, capillary electrophoresis, and amplicon sequencing. The specificity of the GR2 and PLD-GR assays was also verified against the DNA of a number of other species, representative of the most important GM crops and a selection of cereals and grasses; the GR2 assay was additionally tested on DNA from 53 GM events and from five independent GR2 transformation events. The GR2 and PLD-GR assays did not show unspecific amplifications with the species tested. The PLD-GR taxon-specific assay was designed taking into consideration the polymorphisms reported for the region.[41,42] We demonstrated that the newly developed PLD-GR taxon-specific assay is present in single copy in the rice genome of the Kaybonnet variety and that its amplification stability is good

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across the 132 rice varieties tested, belonging to both the indica and the japonica subspecies. The Cq values obtained with DNA from 120 out of 132 varieties were within a range of 0.55 Cq, and only six varieties were outside a range of 1 Cq calculated from the variety with the highest measured Cq value (Table 2, Figure 4, and Table S1, Supporting Information). All of the outlying varieties showed a lower Cq, suggesting either the presence of a second amplicon for the PLD-GR assay or an incorrect quantification of the DNA that was added to the reaction. Both hypotheses were investigated, by sequencing the amplification products of these varieties, and by requantifying their DNA with Picogreen and spettrophotometric methods. However, we did not identify a second amplification product (which seems to exclude the hypothesis of the presence of a second PLD-GR amplicon) and did not obtain significantly different quantification results. We will further investigate these results, but given that the test on the stability of the PLD-GR assay did not indicate the presence of sequence polymorphisms in the primers/probe region negatively influencing the efficiency of amplification, and that less than 5% of the varieties tested were outside the established Cq range, the PLD-GR taxon-specific assay can be classified as having good performance in terms of stability.

We established that the dynamic range of the GR2 event-specific assay is between 200 000 and 40 hge, with the LOQ at 40 hge and the LOD at 4 hge, and the dynamic range of the PLD-GR taxon-specific assay is between 200 000 and 30 hge, with the LOQ at 30 hge and the LOD at 4.5 hge (Table 3 and Table S8, Supporting Information). These numbers are in line with the criteria set in the ENGL MPR document.[14] The RSD_r was generally higher at the lower end of the dynamic range for both assays, a trend common to most calibration systems;[43] the linearity of the standard curves for the event- and taxon-specific assays was in line with that measured for most calibration systems, while PCR efficiency was slightly below the average when compared to data from the EU Database of Reference Methods for GMO Analysis.[44] It has to be taken into consideration, however, that the data available on the EU Database of Reference Methods for GMO Analysis are the results of full validation experiments, while we presented the results of the in-house validation of the two assays.

We optimized the GR2 and the PLD-GR assays on ABI 7900HT qPCR equipment, chosen for being one of the most common qPCR equipment in GMO testing laboratories, and tested the robustness of the two assays on three other common qPCR instruments: Roche LC 480, Stratagene Mx3005P, and ABI 7500. The results of the quantification of three test samples distributed over the whole dynamic range showed that the two assays can be transferred to other qPCR equipment without losing their accuracy; only the sample at the LOQ reported a bias of −27.9% with Roche LC 480 for the GR2 event-specific assay (Tables 5 and 6). It was already observed that deviations from the expected performance occurred at low GM contents with some qPCR methods when Roche LC 480 was employed;[45] in this case, the deviation was small and only at the lower limit of the dynamic range of the assay. However, particular care should be taken when using this platform to perform the GR2 assay. The certification of GMO testing laboratories under ISO 17025[46] requires that validated methods be verified in-house before being implemented for routine analysis; we suggest performing this verification on all of the different qPCR platforms on which the method is planned to be used. The slope, efficiency, and linearity of the standard curves generated with the two assays on the three qPCR instruments were within the performance requirements,[14] with the only exception of the PCR efficiency of the GR2 assay on ABI7500, which was very near to 90%. This small deviation did not influence the quantification results, which showed a RSD_r between 1.1% and 11.6% and bias between −6.2% and −1.2%. We tested the robustness of the two assays also with deliberate and small changes to the reaction conditions and the quantification remained reliable, although as a general trend bias was higher for the samples with lower hge content, especially for the GR2 assay (Tables 5 and 6). It has to be taken into consideration that we tested the GR2 assay at its LOQ; it is thus more likely that changes to the reaction mix or the PCR program have a higher influence at the lower extreme of the dynamic range. The slope, efficiency, and linearity of the standard curves of the two assays were within the performance requirements[14] with the exception of the PCR efficiency of the GR2 assay with 30% lower primer concentration, for which a small decrease in efficiency led to a quantification that was still acceptable, with a RSD_r comprised between 5.1% and 7.3% and a bias between −11.5% and −1.9%. The two assays can thus be considered robust against small deviations from the protocol.

GR2 is intended for cultivation and consumption in developing countries that use rice as a staple crop, with the objective of fighting vitamin A deficiency, and at present its commercialization is not foreseen outside those countries. The humanitarian license that regulates its use and distribution establishes that only national sales of the product are allowed.[1,2,47−49] It cannot however be excluded that this rice will enter the EU, either as a contamination in food imports or as fortified food. In this view, the development of a qPCR detection method for GR2 is important to ensure the traceability of this GM event. The applicability range of the qPCR GR2 detection method that we developed is wide in terms of haploid genome equivalents that can be detected, also as a result of the choice of treating the event and taxon-specific assays that compose the method as independent modules. The method can thus be applied in different regions of the world, notwithstanding the diverse provisions set for the unintended presence of GMOs.[50]

■ ASSOCIATED CONTENT

Supporting Information

List of rice varieties and GR2 transformation events provided by IRRI for this study (Table S1); description of the samples for the determination of the dynamic range, LOQ, LOD, and robustness of the two assays and for the relative quantification test (Tables S2−S5); results of the specificity test for PLD-GR and GR2 assays (Tables S6 and S7); and results of the LOD for the two assays (Table S8). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

ABI, Applied Biosystems; CCSIS, Central Core DNA Sequence Information System; ENGL, European Network of GMO Laboratories; EU-RL GMFF, European Union Reference Laboratory for Genetically Modified Food and Feed; FAM, 6-carboxyfluorescein; GMO, genetically modified organism; GR2, Golden Rice 2; hge, haploid genome equivalents; IHCP, Institute for Health and Consumer Protection; IRRI, International Rice Research Institute; IUPAC, International Union of Pure and Applied Chemistry; JRC, Joint Research Centre; LC, LightCycler; LOD, limit of detection; LOQ, limit of quantification; MBG, Molecular Biology and Genomics; MPR, minimum performance requirements; PLD, Phospholipase D; ppi-PPF, ppi phosphofructokinase; qPCR, quantitative real-time polymerase chain reaction; RSD_r, relative repeatability standard deviation; SPS, sucrose phosphate synthase; TAMRA, tetramethylrhodamine

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