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ARTICLE

Evaluation of the Impacts of Different Nuclear DNA Content in the Hull, Endosperm, and Embryo of Rice Seeds on GM Rice Quantification

Donger Liu, Jie Shen, Litao Yang,* and Dabing Zhang*

GMO Detection Laboratory, SJTU-Bor Luh Food Safety Center, School of Life Science and Biotechnology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, People's Republic of China

Rice (*Oryza sativa*) is a main staple food in the world, and several genetically modified (GM) rice events have been approved for commercialization. To accurately quantify GM contents in rice derived products, we have evaluated the variation of seed DNA density and nuclear DNA content in the hull, endosperm, and embryo of rice seeds from 19 cultivars, as well as their impacts on GM rice quantification. Rice endosperm DNA accounts for 73.71% of total seed DNA, whereas the hull and embryo DNAs account for 3.98% and 22.31%, respectively. Two formulas were established to describe the relationship between GM content on the basis of weight ratio (GM_{wt}%) and that on the basis of haploid genome copy number ratio (GM_{hg}%) for the samples containing heterozygous GM rice seeds. These two equations were well confirmed in quantification of the heterozygous GM rice TT51-1 seeds containing the GM allele from a female parent or that from a male parent. This work is useful for accurate quantification of GM rice using reference materials containing the heterozygous GM rice seed powder.

KEYWORDS: Rice; DNA content; haploid genome; TT51-1; reference material

INTRODUCTION

Rice is one very important cereal crop and a primary food in the world. It has been globally cultivated in more than 100 countries (*1*). With the great advances in plant genetic modification, more and more GM rice events have been developed (*2*,*3*). Currently, several GM rice events have been approved for commercialization, such as Events CL121, CL141, and CFX51 from BASF Inc. and Event LLRICE62 from Bayer CropScience (*4*). In China, several GM rice events with various traits have also been developed, and one GM rice event was approved for commercialization in 2009 (*5*−*8*). However, the public is still concerned about the environment and food safety of genetically modified organisms (GMOs). Therefore, many countries have issued laws and regulations to label the GMOs and their derivates (*9*−*12*).

An accurate analysis method of GM content is essential for the execution of GMO labeling regulations. The quantitative real-time PCR technique has been widely used in GMO analysis, such as MON863, TC1507, T45, and Oxy235, etc. (*13*−*15*). Currently, two units are used to express the GM percentage in GMO quantification, one is expressed as the percentage of GM haploid genome DNA copy number relative to the target taxon specific DNA copy number (GM_{hg}%) in the European Union (EU) since 2004 (*9*). The other is expressed as the percentage of GM component weight relative to the total weight of GM and the corresponding non-GM component (GM_{wt}%) in some countries, such as Japan and Korea, etc. (*10*,*11*). The inconsistent results often occur during GM content calculation by converting the DNA copy number ratio to the weight ratio. This is because of the impact from many biological factors, such as whole seed DNA density, tissue DNA contents, and tissue genetic structures, etc. (*16*).

One rice seed mainly consists of the embryo and endosperm enclosed by a pair of the hull. The endosperm accounts for more than half of the total seed's weight, while the weight of an embryo is relatively smaller. It has been reported that rice embryos are made of small and dense cells, whereas most of the rice endosperm cells are larger and vacuolated (*17*). These facts lead to the possibility that the DNA proportions of the hull, embryo, and endosperm are different from each other and that the DNA proportions of one tissue vary among different rice cultivars. Therefore, we assume that the genome copy numbers per mass unit varied among different rice cultivars. The endosperm genome DNA is triploid, containing one paternal haploid and two maternal haploid, the embryo genome DNA is diploid with a maternal haploid and a paternal haploid, and the hull genome DNA is diploid and of maternal origin. Thus, a hemizygous GM rice seed might have a different transgene copy number, which relies on the transgenic DNA origins and the tissue DNA contents. As we know, the certified reference materials (CRMs) used for GMO quantification are mainly made of hemizygous GM seeds and non-GM seed powder. The GM content of a CRM is expressed in the form of GM_{wt}%. If a CRM contains hemizygous GM seed powder, its GM_{hg}% might not be equal to the GM_{wt}%. In GMO quantification by PCR-based methods, GM content is expressed in the form of GM_{hg}%. If the CRM containing hemizygous GM seed powder is used for quantification for GM rice using PCR-based methods, the influence from

*To whom correspondence should be addressed. Tel: +86 21 34204869. Fax: +86 21 34204869. E-mail: yylltt@gmail.com (L.Y.); zhangdb@sjtu.edu.cn (D.Z.).

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Table 1. Primer Pairs and TaqMan Probes for Quantitative PCR

target	primer name	sequence (5′-3′)	amplicon (bp)	ref
TT51	TT51-1-F	AGAGACTGGTGATTTCAGCGGG	120	*19*
	TT51-1-R	GCGTCCAGAAGGAAAAGGAATA		
	TT51-1-P	FAM-ATCTGCCCCAGCACTCGTCCG-B HQ1		
SPS	SPS-F	TTGCGCCTGAACGGATAT′	81	*20*
	SPS-R	CGGTTGATCTTTTCGGGATG		
	SPS-P	FAM-TCCGAGCCGTCCGTGCGTC- TAMRA		

the different nuclear DNA content of rice seed tissues should be considered. Likewise, if the analyte contains hemizygous GM seed material, the influence should also be considered. Obviously, the different nucleic DNA content and origin of the hull, endosperm, and embryo may affect the accuracy of GMO quantification using the nucleic-acid based methods. In maize, the DNA content of the embryo and endosperm had been measured, and its impact on GMO quantification had been evaluated by Trifa and Zhang in 2004 (*18*), and this understanding is of great help for GM maize seed quantification. However, few studies on the DNA content of the hull, endosperm, and embryo of rice seed and its impact on GM rice quantification have been reported.

In this study, we analyzed the whole seed DNA density and DNA proportions of the hull, endosperm, and embryo of rice seed from 19 different cultivars, and established two equations to estimate the GM content in rice samples considering the influence from the different tissue DNA content of rice seed during GM rice seed quantification.

MATERIALS AND METHODS

Plant Materials. Seeds of 19 different nontransgenic rice cultivars and one transgenic rice event TT51-1 were used in this study. The nontransgenic materials included ten indica cultivars (Fuhui838, Gangchangai, Minghui63, II-32B, 9311, CP71, CP78, CP83, CP86, and CP96) and nine japonica rice cultivars (Guihuahuang, Ribenqing, Balila, Jingduxu, Nongken57, Nongken58, JP149, JR36, and JR52). The transgenic materials included two kinds of hemizygous TT51-1 seeds, one containing the GM allele from the female parent and the other containing the GM allele from the male parent. Transgenic rice event TT51-1 with the insect-resistant trait was produced by inserting a hybrid *cry1Ab*/*Ac* gene under the control of the rice *Actin1* gene promoter and the nopaline synthase (NOS) terminator to Minghui63 genome (*6*−*8*). The GM rice TT51-1 and its corresponding non-GM rice Minghui63 were planted in Hubei province and were provided by Huazhong Agriculture University, China. The other 18 different nontransgenic rice cultivars were planted in Shanghai and were provided by Shanghai Academy of Agricultural Sciences, China. All of the seeds were harvested from July, 2008, to September, 2008, dried and stored in 4 °C before testing.

Preparation of Powders of the Hull, Endosperm, and Embryo of Rice Seeds. Seeds of each cultivar were soaked in *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) extraction buffer [20 g/L CTAB; 1.4 M NaCl; 0.1 M tris[hydroxymethyl]-aminomethane (TRIS); 20 mM ethylenediaminetetraacetic acid (EDTA); pH 8.0] for 24 h. Hulls, endosperms, and embryos of individual seeds were separated from soaked seeds. The separated tissues were lyophilized for 36 h and weighed. Dry tissues were ground into powder using SPEX 6870 Freezer/Mill (SPEX SamplePrep, USA) in liquid nitrogen.

DNA Extraction and Purification. The modified CTAB method was used to extract the DNA, and the detailed protocol can be described as follows: 80 mg of the hull, 20 mg of the endosperm, or 10 mg of embryo powder was incubated for 30 min at 65 °C in 1 mL of CTAB extraction buffer containing α-amylase (Sigma) (10 μL of a 10 mg/mL solution) and RNase A (Sigma) (10 μL of a 20 mg/mL solution). The mixture was shaken every 10 min during incubation. Twenty microliters of a 40 mg/mL solution of proteinase K (Sigma) was added, and the mixture was further incubated for 40 min at 65 °C. Proteins were extracted with phenol/chloroform/isoamyl alcohol (25:24:1). DNA was precipitated for 1 h on ice with a 0.1 volume of NaOAc (3 M, pH 5.2) and a 0.6 volume of isopropyl alcohol. The pellet was recovered by centrifugation at 15000*g* and 4 °C for 20 min. The pellet was washed with 500 μL of 70% ethanol and dried. The DNA was dissolved for 12 h with 0.1 × TE buffer (1 mM Tris and 0.1 mM EDTA, pH 8.0). Purified DNA quantity was determined using a Quant-iT PicoGreen dsDNA quantification kit (Molecular Probes, Invitrogen, USA) and Fluoroskan Ascent (Thermo Labsystems, USA).

Primers and Probes. The previous reported oligonucleotide primers and TaqMan fluorescent probes for the event-specific fragment of TT51-1 rice and endogenous reference gene *sucrose phosphate synthase* (*SPS*) were used in this study and listed in **Table 1** (*19*, *20*). The primers SPS-F/R combined with the SPS-P probe were used for *SPS* gene detection, and the primers TT51-1-F/R combined with the TT51-1-P probe were used for the exogenous gene of TT51-1 rice. All of the primers and probes were purchased from TaKaRa Company (Dalian, China).

Real-Time PCR Conditions. Real-time PCR assays were carried out in a fluorescent thermal cycler Rotor-Gene 3000A (Corbett Research, Australia) with a final volume of 25 μL. The real-time PCR for the *SPS* gene detection contained the following reagents: 1× PCR buffer, 200 μM each of dNTPs, 400 nM primers, 200 nM TaqMan probes, 1.5 U of *Taq* DNA polymerase, and 3 mM $MgCl_2$ and 5 μL of template DNA samples. The real-time PCR for exogenous gene detection contained the following reagents: 1× PCR buffer, 200 μM each of dATP, dGTP, dCTP, and dTTP, 800 nM primers, 400 nM TaqMan probes, 1.25 U of *Taq* DNA polymerase, and 6 mM $MgCl_2$ and 5 μL of template DNA samples. The real-time PCR profile was 10 min at 94 °C followed by 50 cycles of 15 s at 94 °C and 1 min at 60 °C. The fluorescent signal was monitored during every PCR cycle at the annealing step. Data were analyzed with Rotor gene 3000 software version 6.0 (Corbett Research). All of the PCR reagents were purchased from Biocolor Company (Shanghai, China) except for primers and probes.

Construction of Standard Curves. Standard curves for event-specific and *SPS* assays were established with five dilutions of DNA from 100% TT51-1 material (homozygous GM plant leaf). Five serial diluted concentrations (50, 5, 0.5, 0.1, and 0.05 ng per reaction, respectively) of TT51-1 rice genomic DNA were used for the preparation of standard curves, containing approximately 96150, 9615, 961.5, 192.3, and 96.15 copies of haploid genome per reaction according to the haploid rice genomic DNA quantity (0.52 pg per haploid genome) (*21*). All of the real-time PCR reactions were repeated three times and each time with triple replicates for each template DNA in this study.

Data Statistical Analysis. All data were statistically analyzed using statistical software SPSS 14.0. The difference of seed DNA density of 19 rice cultivars was analyzed by one-way ANOVA and Duncan's multiple range test. The difference between the experimental tested GMhg% and the expected values for heterozygous TT51-1 seeds was analyzed by Student's *t*-test.

RESULTS AND DISCUSSION

DNA Density of Whole Rice Seed. To analyze the whole seed DNA density, the seeds of 19 rice cultivars were individually ground, and 50 mg of rice seed powder of each cultivar was used to extract the genomic DNA. For each cultivar, three biologically replicated samples were used, and the extracted DNAs were quantified three times for each sample using the PicoGreen method (*22*). The mean values were used. The DNA density of each cultivar was described with the mean value of three randomly repeated samples and listed in **Table 2**. The results show that the quantity of extracted DNA from 1 mg of rice seed powder varies from 0.0855 to 0.1539 μg for the 19 different rice cultivars.

Table 2. DNA Density of Rice Whole Seed from 19 Cultivars

cultivar	DNA density (μg/mg)	CV	significance level (α = 0.05)[a]
Fuhui838	0.1251	0.048	CDE
Gangchangai	0.1265	0.023	CDE
Minghui63	0.0915	0.077	AB
II-32B	0.1141	0.104	CD
9311	0.1087	0.099	BC
CP71	0.0945	0.023	AB
CP78	0.0944	0.107	AB
CP83	0.1288	0.039	DE
CP86	0.1410	0.189	EF
CP96	0.0928	0.024	AB
Guihuahuang	0.1423	0.039	EF
Ribenqing	0.1225	0.102	CDE
Balila	0.1315	0.104	DE
Jingduxu	0.1136	0.085	CD
Nongken57	0.1298	0.041	DE
Nongken58	0.1539	0.060	F
JP149	0.1370	0.063	EF
JR36	0.0855	0.090	A
JR52	0.0882	0.125	A

[a] Nineteen cultivars were divided into six subsets (A, B, C, D, E, and F) on the basis of Duncan's new multiple range test, and rice cultivars with similar seed DNA densities were grouped in the same subset.

The result of one-way ANOVA indicates that the difference of seed DNA density among 19 rice cultivars is significant [F = 12.22, $F_{0.05}$ (18, 38) = 1.88]. All of the data were also analyzed by Duncan's multiple range test, and the results are shown in **Table 2**. In addition, previous investigations on kernel DNA densities of 10 different maize cultivars and 15 soybean cultivars indicated that the significant difference of DNA density was also observed among different cultivars (*18*, *23*).

Considering the different DNA density obtained from each cultivar, the weight ratio of any specific cultivar, whether it is transgenic or not, cannot be simply assumed to be equal to the DNA ratio of that cultivar in mixed rice samples. For instance, 5% (w/w) of JR36 powder mixed in Nongken58 powder corresponds to 2.84% of JR36's genomic DNA to total DNA extracted from the mixture. However, it can be assumed that the GM plant event and its isogenic control have comparable DNA density since the only difference between the original cultivar and the GM cultivar is the transgene fragment. In PCR-based quantitative analysis of GM rice products, the results that are given as DNA copy number ratios could not be automatically converted into weight-to-weight ratios if the cultivars of the mixture have different DNA densities.

Optimization of DNA Extraction Procedure for Different Rice Seed Tissues. DNA extraction is a very important step during GMO quantification. The DNA extraction efficiency might be influenced by three major factors: particle size of the subjected powder (*24*), DNA extraction reagents (*25*), and the quality of the powders subjected to DNA extraction. Our results showed that rice seed powder with the size < 100 μM diameter was suitable for DNA extraction with high extraction efficiency, and the CTAB-based method (*26*) was suitable for using to purify the DNA from rice hull, embryo, and endosperm samples. After comparing the DNA yields of various amounts of starting materials for DNA extraction (**Table 3**), we used 80 mg of hull, 20 mg of endosperm, and 10 mg of embryo powders for DNA extraction in further analyses.

DNA Contents of Rice Seed Tissues. To estimate the DNA proportion of the hull, endosperm, and embryo in rice seeds, the seeds of 19 rice cultivars described in the Plant Materials section were used. For each cultivar, 500 seeds were selected randomly and dissected, and the dried hulls, endosperms, and embryos were weighed individually. The weight ratio of each tissue to that of the seed was calculated and shown in **Figure 1**. The mean weight proportion of the hull is 20.05 ± 2.29% among these 19 rice cultivars and 77.68 ± 1.96% for the endosperm and 2.28 ± 0.70% for the embryo.

Table 3. Optimization of Starting Material for DNA Purification from Rice Hull, Endosperm, and Embryo Powder of Cultivar 9311

material	starting weight (mg)	DNA (μg/100 mg)[a]	CV
hull	20	2.19	0.18
	40	3.27	0.07
	80	3.71	0.06
endosperm	10	7.71	0.22
	20	11.81	0.10
	40	10.89	0.09
embryo	5	66.75	0.12
	10	117.24	0.05
	20	101.83	0.09

[a] The average value of three samples for the starting weight is given. The DNA was quantified three times for each sample.

The hull, endosperm, and embryo tissue DNAs were purified from three random samples for each cultivar, and the concentration of each tissue DNA was independently measured three times. The tissue DNA proportions were calculated according to the tissue DNA density and tissue weight ratios. The results were listed in **Table 4**. The average DNA percentage of hull DNA to total seed DNA is 3.98% ranging from 1.59% to 7.93% among 19 cultivars. The average endosperm DNA percentage is 73.71% ranging from 56.01% to 85.06%. The average embryo DNA percentage is 22.31% with the range of 10.42% and 37.40%. These results show that more than half of the total seed DNA is from the endosperm tissue and that the hull tissue contains lower DNA content. The tested results are similar to that of maize and soybean, and almost half of the total DNA originates from the embryo in maize and soybean seeds (*18*, *23*).

Evaluation of the Impact of the Rice Seed Tissue DNA Content on GM Seed Quantification. In this study, one GM rice event TT51-1 was selected and used as a model to evaluate the impact from the different tissue DNA contents of rice seed on GM rice quantification. One GM rice reference material was developed by mixing the TT51-1 hemizygous seeds (the hybrid offspring of a transgenic male and a nontransgenic female) with its corresponding nontransgenic seeds. One hundred nanograms of rice genomic DNAs was extracted from this mixed sample, corresponding to about 192300 copies of the rice haploid genome. Therefore, the copy number of the haploid genome containing the transgene locus can be calculated using the formulas shown below:

$$\mathrm{Nt} = 192300 \times \mathrm{GM_{wt}}\%$$

$$\mathrm{Nt*} = \mathrm{N_{hu}} + \mathrm{N_{en}} + \mathrm{N_{em}}$$

$$\mathrm{N_{hu}} = 0 \times X \times \mathrm{Nt}$$

$$\mathrm{N_{en}} = 1/3 \times Y \times \mathrm{Nt}$$

$$\mathrm{N_{em}} = 1/2 \times Z \times \mathrm{Nt}$$

where Nt is the total haploid genome copies derived from the transgenic rice seeds, Nt* is the haploid genome copies containing the transgene locus, $\mathrm{N_{hu}}$, $\mathrm{N_{en}}$, and $\mathrm{N_{em}}$ are transgenic haploid genome copies originating from the hull, endosperm, and embryo

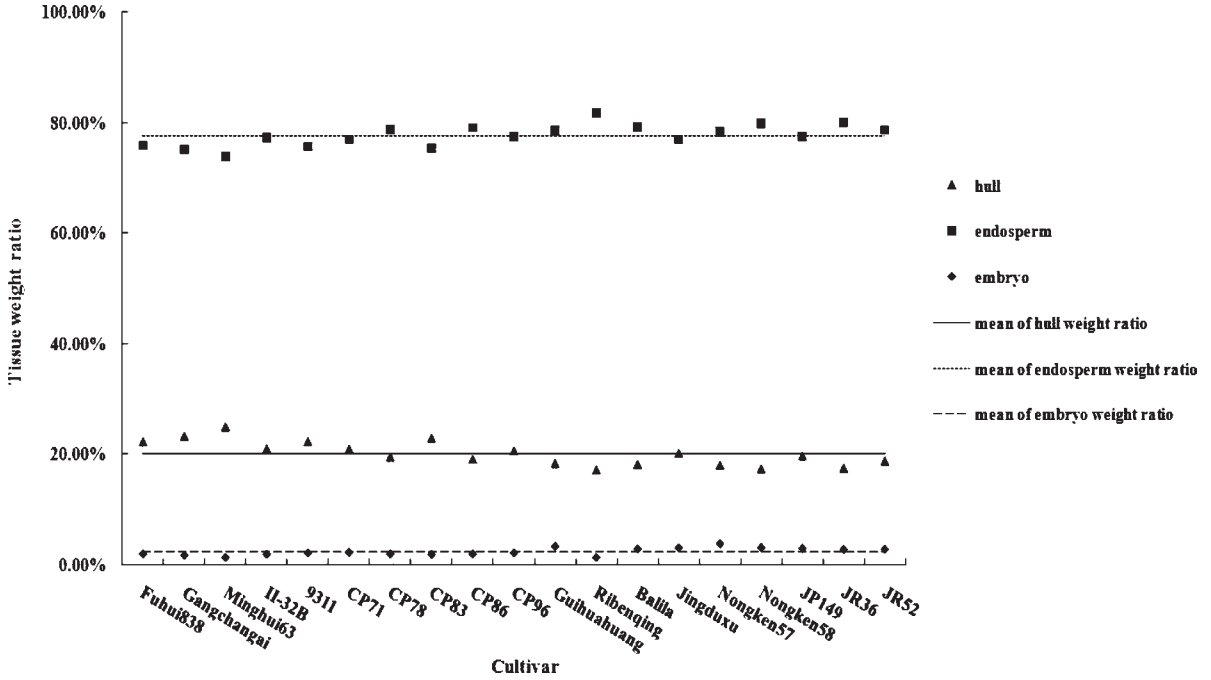

Figure 1. Ratios of hull, endosperm, and embryo weight to the total seed weight.

Table 4. DNA Percentage of Hull, Endosperm, and Embryo Relative to the Total Seed Genomic DNA of 19 Rice Cultivars

	hull		endosperm		embryo	
	mean (%)	SD	mean (%)	SD	mean (%)	SD
Fuhui838	2.47	0.40	78.43	9.12	19.10	0.55
Gangchangai	3.64	0.17	82.40	7.13	13.96	0.36
Minghui63	2.30	0.28	85.06	13.26	12.65	0.91
II-32B	1.59	0.19	80.81	9.07	17.60	1.07
9311	6.82	0.39	73.59	7.38	19.59	1.04
CP71	6.64	0.31	67.28	11.11	26.08	1.96
CP78	2.65	0.72	77.55	14.39	19.80	0.56
CP83	4.06	0.38	79.22	2.02	16.72	0.19
CP86	2.27	0.22	81.13	8.12	16.60	0.26
CP96	4.22	0.43	67.09	1.60	28.69	1.70
Guihuahuang	2.55	0.12	72.31	11.73	25.14	1.09
Ribenqing	7.93	0.23	81.64	8.63	10.42	0.31
Balila	4.90	0.25	71.07	10.56	24.03	0.57
Jingduxu	5.46	0.62	65.07	7.49	29.47	1.28
Nongken57	1.71	0.28	68.36	3.27	29.93	0.66
Nongken58	3.16	0.56	76.76	8.05	20.07	0.50
JP149	4.31	0.50	72.09	15.21	23.60	0.86
JR36	2.35	0.09	64.52	18.72	33.13	1.53
JR52	6.59	0.81	56.01	2.39	37.40	0.71

of the transgenic seeds, respectively, and X, Y, and Z represent the percentages of the hull, endosperm, and embryo DNA among the total seed DNAs, respectively.

According to the formulas above and $X = 3.98\%$, $Y = 73.71\%$, and $Z = 22.31\%$, there is a linear relationship between the $GM_{wt}\%$ and the total haploid genome copies containing the transgene in this reference material, as indicated in the following equation: $Nt^* = 68699.20 \times GM_{wt}\%$. In practical GM TT51-1 rice sample quantification, theoretically there are at least three situations of contaminations in practical samples: (1) sample 1 contains hemizygous GM-seeds generated from the fertilization of a nontransgenic female by the transgenic pollen; (2) sample 2 contains hemizygous GM-seeds generated from the fertilization of a transgenic female by the nontransgenic pollen; and (3) sample 3 contains homozygous GM-seeds. Taking the 5% ($GM_{wt}\%$) GMO contamination, for example, according to the above formulas ($N_{hu} = X \times Nt$; $N_{en} = 2/3 \times Y \times Nt$ for sample 2), the GM haploid genome copy numbers in 100 ng of DNA extracted from the mixture corresponded to 3434.96, 6180.04, and 9615.00 for samples 1, 2, and 3, respectively. The presumed $GM_{hg}\%$ of samples 1, 2, and 3 should be 1.79%, 3.21%, and 5.00%, respectively, as shown in **Table 5**. These results indicate that the deduced values of $GM_{hg}\%$ of the samples containing hemizygous transgenic seeds are lower than that of $GM_{wt}\%$. When the used GM rice reference material was prepared from hemizygous transgenic seeds containing the transgene from the male parent, the theoretical tested values of $GM_{hg}\%$ should be 5.00%, 9.00%, and 14.00% for samples 1, 2, and 3, respectively. According to this deduction, the GM% of the unknown sample will be overestimated in PCR-based quantification if the used matrix reference material was made of hemizygous transgenic seeds and nontransgenic seeds. Clearly, the variation of the DNA content of different seed tissues among different cultivars also affects the quantification of seed samples. Therefore, the impact from the DNA content of different seed tissues should not be ignored during GMOs quantification using PCR-based methods.

Formulas for Estimating the $GM_{hg}\%$ from $GM_{wt}\%$ in Rice Seeds. From the above analysis, we observed that seed tissue DNA content and genetic structures (genome ploidy, GM allele origin, and genotype) greatly influence the accuracy of GM rice quantification using nucleic-acid based methods. The hull DNA originates from a female parent, the endosperm DNA originates from two maternal haploid genomes and one paternal haploid genome, and the embryo originates from one maternal and one paternal haploid genome. Accordingly, the hemizygous transgenic rice seeds would contain different transgenic DNA content if they are generated from different transgenic parents. From our data, the $GM_{hg}\%$ of the sample containing hemizygous GM rice seeds can be calculated using the equations shown below (*18*).

In the case of the GM allele from the female parent

$$GM_{hg}\% = GM_{wt}\% \times (X + 2/3 \times Y + 1/2 \times Z) \qquad (1)$$

Table 5. Calculation of GM_{hg} % on Different Contaminations in Rice Using TT51-1 Reference Materials

situation	5% (GM_{wt}%) sample containing seeds developing from	Nt_{hu}	Nt_{en}	Nt_{em}	Nt*	Nt	GM_{hg} %	GM_{hg} % estimated
1	GM male (♂) × non-GM female (♀)	0.00	2362.41	1072.55	3434.96	9615.00	1.79%	5.00%
2	non-GM male (♂) × GM female (♀)	382.68	4724.81	1072.55	6180.04	9615.00	3.21%	9.00%
3	GM male (♂) × GM female (♀)	382.68	7087.22	2145.11	9615.00	9615.00	5.00%	14.00%

Table 6. Determination of the Transgenic DNA (GM_{hg}%) Extracted from 100% (w/w) Hemizygous GM Seeds with Different Genetic Structures

	experimentally estimated GM_{hg}%			
type of material	GM_{hg}%	SD	expected GM_{hg}% by eq [1] or [2]	bias (%)[c]
hemizygous from GM ♂	36.86	2.54	35.73[a]/34.68[b]	3.16[a]/6.29[b]
hemizygous from GM ♀	68.57	1.78	64.28[a]/65.32[b]	6.67[a]/4.98[b]

[a] The GM_{hg}% and RSD calculated on the basis of the average value of *X*, *Y*, and *Z* from 19 rice cultivars. [b] The GM_{hg}% and RSD calculated on the basis of the average value of *X*, *Y*, and *Z* from Minghui63. [c] Bias, calculated using the equation: Bias = |experimentally estimated GM_{hg}% − expected GM_{hg}%|/expected GM_{hg}%.

In the case of GM allele from the male parent

$$GM_{hg}\% = GM_{wt}\% \times (1/3 \times Y + 1/2 \times Z) \quad (2)$$

where *X*, *Y*, and *Z* represent the average percentage of the hull, endosperm, and embryo DNA to total DNA.

On the basis of the above equations and the average values of *X*, *Y*, and *Z* from 19 rice cultivars ($X = 3.98\%$, $Y = 73.71\%$, and $Z = 22.31\%$), the expected GM_{hg}% values of two transgenic hybrid lines were calculated. The calculated GM_{hg}% of the transgenic hybrid line from a GM female is 64.28%, and the other transgenic hybrid line from a GM male is 35.73%. On the basis of the value of *X*, *Y*, and *Z* from Minghui63 ($X = 2.30\%$, $Y = 85.06\%$, and $Z = 12.65\%$), the calculated GM_{hg}% of the two transgenic hybrid lines are 65.32% and 34.68%, respectively. The equations can be used to calculate GM rice content expressed in one form according to the GM content expressed in another for the sample containing hemizygous GM rice seed material. The application of the equations depends on the knowledge of the genetic structures of the analyte. The equations are applicable only if the GM component and corresponding non-GM component of the sample under testing have comparable DNA density.

Verification of the Impact of Different Tissue DNA Content and Genetic Structure on GM Rice Seed Quantification. To evaluate the impact of the differences of seed tissues on GMO quantification, the seeds of two hybrid GM TT51-1 rice lines were used and quantified using real-time PCR. One line was a hybrid from the GM female and non-GM male parent, and the other was from the GM male and non-GM female parent. In addition, three random samples were taken from the seed powder of each hybrid line, and the GM_{hg}% of each sample was quantified three times. The standard curves for the TT51-1 event and rice *SPS* endogenous reference gene were constructed using a homozygous GM TT51-1 leaf-derived genomic DNA as the reference material. The constructed standard curves with high linearity, repeatability, and PCR efficiency indicated that the used real-time PCR systems for GM TT51-1 rice were suitable and reliable (data not shown). The quantified results of those samples using the above real-time system were listed in **Table 6**. The tested value of GM_{hg}% of the transgenic hybrid line from the GM female is 68.57% and that of the transgenic hybrid line from the GM male is 36.86%.

By comparing tested GM contents with theoretical values, we found that there were very slight biases (3.16%−6.67%) under the defined acceptable range (<25%). The results of Student's *t*-test indicates that the 95% confidence interval of the mean value of tested GM contents is from 30.56% to 43.16% for the heterozygous TT51-1 line containing the GM allele from the male parent and from 64.15% to 72.99% for the line containing the GM allele from the female parent. All of the theoretical values are under the 95% confidence intervals. There are no significant difference between the tested GM contents and theoretical values at the significant level of 0.05. These results indicate that the developed equations on the basis of DNA contents of seed tissues are applicable. We believe that these two equations are proposed to reduce the bias of quantifying GM rice seed and grain production when the analyte or reference materials contain heterozygous GM rice seed powder.

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