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Collaborative Ring Trial of the Papaya Endogenous Reference Gene and Its Polymerase Chain Reaction Assays for Genetically Modified Organism Analysis

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ABSTRACT: The papaya (*Carica papaya* L.) *Chymopapain* (*CHY*) gene has been reported as a suitable endogenous reference gene for genetically modified (GM) papaya detection in previous studies. Herein, we further validated the use of the *CHY* gene and its qualitative and quantitative polymerase chain reaction (PCR) assays through an interlaboratory collaborative ring trial. A total of 12 laboratories working on detection of genetically modified organisms participated in the ring trial and returned test results. Statistical analysis of the returned results confirmed the species specificity, low heterogeneity, and single-copy number of the *CHY* gene among different papaya varieties. The limit of detection of the *CHY* qualitative PCR assay was 0.1%, while the limit of quantification of the quantitative PCR assay was ∼25 copies of haploid papaya genome with acceptable PCR efficiency and linearity. The differences between the tested and true values of papaya content in 10 blind samples ranged from 0.84 to 6.58%. These results indicated that the *CHY* gene was suitable as an endogenous reference gene for the identification and quantification of GM papaya.

KEYWORDS: *collaborative ring trial, Carica papaya, Chymopapain, endogenous reference gene, qualitative and quantitative PCR*

INTRODUCTION

Following the fast development and application of recombinant DNA techniques in modern agriculture over the past two decades, more than 190 genetically modified (GM) events of 25 different crops have been developed and approved for commercialization worldwide. By the end of 2012, 170 million ha of GM crops have been planted globally.[1] Because of the concern of consumers about the safety of GM food and feed, more than 50 countries and regions have issued series of legislation and regulations for the control and labeling of genetically modified organisms (GMOs). For instance, GM foods must be labeled at thresholds of 0.9% in the European Union (EU), 3% in Korea, 5% in Japan, and 0% in China.[2−5] To implement the GMO labeling regulations and accelerate the standardization of GMO analysis methods at the global level, several countries and organizations, including the Chinese Ministry of Agriculture (MOA), the Community Reference Laboratory for GM Food and Feed (CRL-GMFF), and the International Organization for Standardization (ISO), have taken remarkable efforts to validate and standardize several GMO detection methods.[6] Two public databases, GMO detection database (GMDD) and GMOMETHODS, were also established for the collection and exchange of developed and validated GMO detection methods.[7,8]

To validate a developed detection method, the interlaboratory collaborative ring trial is a commonly accepted practice, in which a developed method is tested and evaluated by multiple GMO testing laboratories. Recently, some GMO detection methods have been validated and recommended as ISO standards, such as the qualitative and quantitative PCR assays for the rice *SPS* endogenous reference gene and the tomato *Lat52* endogenous reference gene,[9,10] the quantitative real-time PCR assays of the *CaMV35S* promoter and *NOS* terminator,[11−14] the qualitative PCR assays for the *FMV35s* promoter and RT73 canola event,[15] and the quantitative PCR assay of *ctp2-cp4epsps* and *bar* genes.[16] Meanwhile, most of the event-specific quantitative PCR assays of the GM events approved by the EU have been validated by the European Network of GMO Laboratories, Joint Research Centre (JRC-ENGL).[8]

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In GMO detection, endogenous reference genes are regarded as the gold standards for the identification of species and quantification of the total amount of genomic DNA.[6] Generally, one ideal endogenous reference gene for GMO analysis should have three typical characteristics, which are the species specificity, low heterogeneity among different varieties, and consistent low copy number in the genome.[17] A great amount of effort has been spent on developing and evaluating endogenous reference genes of different crops. For instance, the *Invertase I*, *Zein*, *zSSIIb*, *Adh1*, and *Hmg-A* genes were developed as endogenous reference genes in maize, and the assays of *zSSIIb* and *Zein* were validated to be suitable in GM maize detection.[18] The *PLD*, *SPS*, *GOS9*, and *ppi-PPF* genes and their PCR assays were used for GM rice analysis, and the *SPS* assay was recommended after comprehensive evaluation.[19] The *Waxy-1*, *PKABA1*, *ALMT1*, and *ACC1* genes were used in GM wheat analysis, and the *Waxy-1* assay was shown to exhibit high amplification performance and specificity in common wheat.[6,20] Some of these endogenous reference genes and their assays have been validated through collaborative ring trials, such as the rice *SPS* gene and tomato *Lat52* gene.[9,10,21,22]

As an important economical crop that contains a wide range of health-promoting phytochemicals, papaya (*Carica papaya* L.) is widely planted in tropical and subtropical lowland zones, such as America, Brazil, Argentina, Southeast Asia, and South Africa. However, the infection by *Papaya* ringspot virus (PRSV) has caused tremendous loss of papaya yields worldwide in the past century.[23] To manage PRSV infections, several transgenic papaya lines were developed using recombinant DNA techniques, and two GM events (55-1 and Huanong No. 1) have been approved for commercialization in the United States and China, respectively.[24,25] According to the GMO labeling regulations, it is necessary to detect and monitor GM papaya and their derivatives. Recently, two papaya genes, *Chymopapain* (*CHY*) and *Papain*, were developed as endogenous reference genes for GM papaya detection.[26−28] Nevertheless, these two genes and their corresponding PCR assays were not fully validated, leaving a gap in standardizing these genes and their assays in GM papaya analysis.

Herein, we designed and organized a collaborative ring trial to validate the previously reported papaya *CHY* endogenous reference gene and its qualitative and quantitative PCR assays. The ring trial included 12 laboratories and was conducted according to the ISO standard (ISO 5547:1994) and the IUPAC protocol for collaborative ring trials.[29] The analyses of the returned results showed that the *CHY* gene was an ideal papaya endogenous reference gene and its PCR assays were suitable for GM papaya analysis.

MATERIALS AND METHODS

Plant Materials. The seeds of eight wild-type papaya varieties, Xiaobai, Suizhonghong, Baipi, Qingpi, Suoluo, Suoluo No. 2, Weizhi, and Risheng, were kindly collected and supplied by the Chinese Academy of Tropical Agricultural Sciences (CATAS). The transgenic Huanong No. 1 papaya seeds were kindly supplied by South China Agriculture University (SCAU). The other 14 plant species used in this study were purchased from local markets in Shanghai, including longan (*Dimocarpus longana* L.), banana (*Musa paradisiaca* L.), mango (*Mangifera indica* L.), sweet orange [*Citrus sinensis* (L.) Osbeck], lemon [*Citrus limon* (L.) Burm.f], cotton (*Gossypium hirsutum*), maize (*Zea mays*), rice (*Oryza sativa*), rapeseed (*Brassica napus*), soybean (*Glycine max*), potato (*Solanum tuberosum*), pepper (*Capsicum annuum*), tomato (*Lycopersicon esculentum*), and tobacco (*Nicotiana tabacum*).

Five mixed papaya samples were prepared in duplicate by mixing the seed powder of Risheng papaya and rice based on different mass to mass ratios in the following procedure. Dried papaya and rice seeds were homogenized separately with a SPEX CertiPrep 6870 freezer/mill (SPEX CertiPrep, Inc., Metuchen, NJ), and the ground powder was dried; 0.1000, 0.0500, 0.0100, 0.0050, and 0.0010 g of dried papaya seed powder and 9.9000, 9.9500, 9.9900, 9.9950, and 9.9990 g of dried rice seed powder were weighed using a Sartorius BS 224S Balance (readability of 0.0001 g) and mixed to make 1.0, 0.5, 0.1, 0.05, and 0.01% (papaya/rice) mixtures, respectively. When the two powders were mixed, each sample was added to a 50 mL grinding vial simultaneously, and liquid nitrogen was subsequently added to the freezer/mill for 10 min. After the vial had been removed from the freezer/mill, it was kept at room temperature for 20 h without the vial cap being opened. When the vial reached room temperature and was free of condensation on the tube surface, the powder was packaged into a new sample vial. Four papaya varieties (Xiaobai, Qingpi, Risheng, and Suizhonghong) were randomly selected and used for the contribution of real-time PCR standard curves and preparation of blind samples.

DNA Extraction and Purification. Genomic DNA used for conventional qualitative and real-time quantitative PCR assays was extracted and purified with a silica column-based DNA extraction kit (Ruifeng Agro-tech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The quality and quantity of purified DNAs were measured and evaluated using 1.0% agarose gel electrophoresis and ultraviolet spectrometric equipment (NanoDrop 1000), respectively. Four calibration DNAs at 10.0, 5.0, 3.0, and 1.0 ng/μL were prepared from a 50 ng/μL stock solution. This 50 ng/mL DNA stock solution was also used to prepare blind samples. Salmon sperm DNA (10 ng/μL) was used as the negative control, and the Suizhonghong papaya genomic DNA was used as the positive control.

Qualitative PCR Assay. For the *CHY* gene qualitative PCR assay, the previously reported primers that amplify a 281 bp amplicon were adopted; the forward primer sequence was 5′ ATCTACAATCTTG-CTAACCCTA 3′, and the reverse primer was 5′ AGTCATCTTG-AGAATAACCCAC 3′.[26] The qualitative PCR was conducted in a 25 μL reaction mixture containing 20 μL of Qualitative PCR Reaction Master Mix and 5 μL of the genomic DNA. The Qualitative PCR Reaction Master Mix contained the following reagents: 1× PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$], dNTPs (100 μM each), primers (200 nM each), and 1.5 units of Taq DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China). The PCR program was as follows: denaturation for 10 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C; a final PCR product extension of 7 min at 72 °C. The amplified PCR products were analyzed by 2% (w/v) agarose gel electrophoresis in 0.5× TBE buffer with GelRed staining. Each PCR was repeated three times and each time with three replicate reactions.

Quantitative Real-Time PCR Assay. For the *CHY* gene quantitative PCR assay, the forward PCR primer (5′ CCATGCGG-ATCCTCCCA 3′), the reverse primer (5′ CATCGTAGCCATTG-TAACACTAGCTAA 3′), and the probe [5′ FAM-TTCCCTTCAT-(BHQ1)CCATTCCCACTCTTGAGA 3′] were adopted, which amplify a 73 bp fragment.[26] The quantitative PCR was conducted in a 25 μL reaction mixture containing 20 μL of Quantitative PCR Reaction Master Mix and 5 μL of genomic DNA. The Quantitative PCR Reaction Master Mix contained the following: 1× quantitative PCR buffer, quantitative primers (500 nM each), and 200 nM probe. The real-time PCR program was as follows: 94 °C for 10 min, followed by 45 cycles of 94 °C for 15 s and 60 °C for 60 s. The fluorescent signal was monitored during each PCR cycle in the elongation step. The quantitative PCR amplifications were conducted on various fluorescence thermal cyclers, such as a Rotor Gene Q instrument (Qiagen), a Prism ABI 7500 instrument, and Prism ABI 7900 sequence detection system (Applied Biosystems). Each quantitative PCR was repeated three times and each time with three replicates.

Collaborative Ring Trial. The collaborative ring trial was organized by the National Center for Molecular Characterization of

Genetically Modified Organisms at Shanghai Jiao Tong University (NCMCG-SJTU). A total of 12 laboratories were invited and participated in this trial. Each participating laboratory received nine papaya genomic DNA samples denoted F1−F9 (10 ng/μL each), 15 different plant genomic DNA samples denoted E1−E15 (10 ng/μL; E1 is Suizhonghong), 10 mixed papaya/rice DNA samples with five different ratios denoted G1−G10 (10 ng/μL), four purified papaya genomic DNA samples from Xiaobai, Qingpi, Risheng, and Suizhonghong denoted A−D, respectively (50 ng/μL), and 10 blind DNA samples denoted H1−H10. Each participating laboratory also received one positive DNA control denoted P-Chy (10 ng/μL Suizhonghong papaya genomic DNA) and one negative DNA control denoted N-Chy (10 ng/μL salmon sperm DNA solution). Qualitative PCR Reaction Master Mix (3 × 1 mL), Quantitative PCR Reaction Master Mix (6 × 1 mL), and a DNA dilution solution [10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid (EDTA), 2 × 1.2 mL] were also provided.

DNA samples A−D (60 μL each) were used to construct real-time PCR standard curves. Participants were asked to dilute each of these four DNA samples to concentrations of 20, 2, 0.2, 0.02, and 0.002 ng/μL using the supplied DNA dilution buffer {0.1× TE buffer [1 mM Tris-HCl and 0.1 mM EDTA (pH 8.0)]}. Five microliters of each diluted DNA was used for the qualitative and quantitative PCR amplification using the PCR programs described above. All of the DNA samples and reagents were shipped in an insulated box filled with dry ice and ice bags. Within one month of the shipment, all 12 participants sent back their test result reports. Result differences among participating laboratories were statistically analyzed using SPSS version 13.0, which included a least significant difference (LSD) test or Q test.

RESULTS AND DISCUSSION

Applicability of the *CHY* Gene as a Papaya Endogenous Reference Gene. The main purpose of this collaborative ring trial was to validate the applicability of the *CHY* gene as a papaya-specific endogenous reference gene among different laboratories. The tested criteria included species specificity, copy number, and low heterogeneity among different papaya cultivars.

Table 1. Collaborative Trial Results of the Species Specificity and Allelic Variation of the *CHY* Gene

	species specificity	low heterogeneity
no. of laboratories	12	12
no. of laboratories submitting results	12	12
no. of samples per laboratory	14	9
no. of accepted results	168	108
no. of positive results	0	108
no. of negative results	168	0
false-positive rate (%)	0	0

Species Specificity of CHY. In a previous study, the low heterogeneity of the papaya *CHY* gene (GenBank entry AY803756) was demonstrated by BLASTN and further tested by employing 14 different plant species that were either evolutionarily related to papaya or frequently found in food and/or feed samples (longan, banana, mango, sweet orange, lemon, rice, tobacco, soybean, rapeseed, tomato, pepper, cotton, potato, and maize). In this collaborative trial, a total of 15 different plant genomic DNAs denoted E1−E15 were tested in a qualitative PCR assay. The genome DNAs and Qualitative PCR Reaction Master Mix were prepared in our laboratory and sent to each participant. Sending DNA samples to the participants prevented possible variation of DNA quality

Table 2. PCR Efficiencies (E) and Square Regression Correlations (R^2) of Four Standard Curves from 12 Participating Laboratories

	sample A				sample B				sample C				sample D			
laboratory	E	SD	R^2	SD	E	SD	R^2	SD	E	SD	R^2	SD	E	SD	R^2	SD
1	0.9729	0.040	0.9999	0.0032	1.0031	0.057	0.9989	0.0018	0.9661	0.039	0.9995	0.0013	1.0267	0.057	0.9987	0.0021
2	0.9647		0.9997		0.9333		0.9994		0.9534		0.9999		0.9191		0.9999	
3	0.9440		0.9993		0.9480		0.9998		0.9178		0.9992		0.9337		0.9999	
4	0.9866		0.9993		1.0300		0.9996		0.9948		0.9995		1.004		0.9996	
5	0.9342		0.9990		1.0792		0.9982		1.0263		0.9965		1.0446		0.9997	
6	0.9665		0.9998		0.9369		0.9998		0.8906		0.9997		1.0319		0.9944	
7	1.0370		0.9939		1.0031		0.9991		0.9715		0.9963		0.8976		0.9941	
8	0.9606		0.9997		0.9077		0.9982		0.9333		0.9978		0.8941		0.9989	
9	0.9742		0.9993		0.9516		0.9994		0.9925		0.9991		0.9629		0.9997	
10	0.8793		0.9894		1.0687		0.9931		0.9824		1.0000		1.0286		0.9998	
11	1.0142		0.9979		0.9213		0.9992		1.0035		0.9976		0.9077		0.9997	
12	0.9852		0.9993		0.9751		0.9975		0.9797		0.9998		0.9802		0.9990	

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Table 3. Quantified Haploid Genome Copy Numbers of Different DNA Dilutions in Four Papaya Cultivar DNA Dilutions in This Trial

copy number	mean A	mean B	mean C	mean D	SD	RSD (%)
250000	237770.8	250607.3	231928.8	223684.9	11324.7	4.80
25000	27162.2	28373.0	26912.7	26582.3	780.6	2.86
2500	2748.1	2710.9	2635.5	2637.0	55.9	2.09
250	255.5	256.7	253.0	234.2	10.5	4.22
25	24.5	26.5	26.0	25.0	0.9	3.58

Table 4. Real-Time PCR Assay of the Allelic Stability of the *CHY* Gene in Four Different Papaya Cultivars

	mean Ct value[a]							
laboratory	A	B	C	D	mean	SD of the mean	difference[b]	difference[c]
1	23.33	23.64	23.44	23.47	23.47	0.13	0.14	0.17
2	24.56	24.44	24.38	24.05	24.36	0.22	0.31	0.20
3	24.50	24.91	24.98	24.87	24.82	0.21	0.31	0.16
4	24.30	24.44	24.40	24.27	24.35	0.08	0.08	0.08
5	23.84	23.90	24.19	23.80	23.93	0.17	0.13	0.25
6	23.62	24.35	23.87	24.71	24.14	0.49	0.52	0.57
7	24.99	25.28	24.82	25.32	25.10	0.24	0.29	0.22
8	23.92	24.37	23.80	24.23	24.08	0.27	0.28	0.29
9	24.21	23.95	24.10	23.68	23.99	0.23	0.30	0.23
10	25.53	25.32	25.01	25.07	25.23	0.24	0.23	0.30
11	25.19	23.99	25.03	24.50	24.68	0.55	0.69	0.51
12	26.49	26.69	25.71	25.62	26.13	0.54	0.51	0.56

[a]A total of 250000 papaya haploid genomic copies were used. [b]The difference between the mean and the smallest Ct value. [c]The difference between the mean and the largest Ct value.

Table 5. Variations of the Quantification Results of Papaya DNAs among the 12 Laboratories

	A			B			C			D		
copy number	mean	SD	RSD (%)	mean	SD	RSD (%)	mean	SD	RSD (%)	mean	SD	RSD (%)
250000	237770.8	65607.0	27.59	250607.3	62723.3	25.03	231928.8	64112.1	27.64	223684.9	51619.2	23.08
25000	27162.2	5466.7	20.13	28373.0	6413.8	22.61	26912.7	5408.0	20.09	26582.3	6257.5	23.54
2500	2748.1	660.2	24.02	2710.9	600.5	22.15	2635.5	513.1	19.47	2637.0	683.4	25.92
250	255.5	77.9	30.5	256.7	83.3	32.44	253.0	67.7	26.74	234.2	66.5	28.4
25	24.5	7.9	32.19	26.5	8.8	33.38	26.0	8.70	33.39	25.0	8.4	33.8

Table 6. LODs of the Qualitative PCR Assay of the *CHY* Gene

relative concentration ($W_{papaya/papaya+rice}$) (%)	1.0	0.5	0.1	0.05	0.01
no. of laboratories	12	12	12	12	12
no. of laboratories submitting results	12	12	12	12	12
no. of samples per laboratory	2	2	2	2	2
no. of samples	24	24	24	24	24
no. of samples that yielded positive results	24 (100%)	24 (100%)	23 (95.8%)	12 (50%)	8 (33.3%)

and yields from DNA extraction among different laboratories. All participants were asked to perform the qualitative PCRs using commonly available PCR machines, and each PCR needed to be repeated three times. All the results were expressed as positive or negative detection of papaya DNA. The results could be accepted only when the positive control could generate the 281 bp fragment, and the negative and no-template control (NTC) generated no DNA fragment.

The returned results from 12 participating laboratories are listed in Table 1 and statistically analyzed. In assays of the total of 168 non-papaya DNA samples (12 participating laboratories and 14 samples for each laboratory), no amplified DNA fragment was obtained, indicating that the *CHY* gene was species-specific for papaya.

Low Heterogeneity of the CHY Gene among Different Papaya Cultivars. In this collaborative ring trial, the low heterogeneity of the papaya *CHY* gene was validated using nine different papaya cultivars from different geographic and phylogenic origins, including Xiaobai, Suizhonghong, Baipi, Qingpi, Suoluo, Suoluo No. 2, Weizhi, Risheng, and Huanong No. 1. The corresponding genomic DNAs of these papaya cultivars were denoted F1−F9 and sent to each laboratory. In all of the 108 DNA assays (9 × 12), the expected 281 bp DNA fragment of the *CHY* gene was observed (Table 1), indicating the low heterogeneity of the *CHY* gene among different papaya cultivars.

Single-Copy Number of the CHY Gene among Different Papaya Cultivars. The papaya *CHY* gene has been demonstrated to be a single-copy papaya gene by real-time

Table 7. Efficiencies and Square Regression Correlations of the Quantitative PCR Assay

laboratory	efficiency	SD	RSD (%)	R^2	SD	RSD (%)
1	0.992	0.028	2.83	0.9993	0.0006	0.055
2	0.943	0.020	2.16	0.9997	0.0002	0.024
3	0.936	0.013	1.44	0.9996	0.0004	0.035
4	1.004	0.019	1.88	0.9995	0.0001	0.014
5	1.021	0.062	6.07	0.9984	0.0014	0.138
6	0.956	0.059	6.19	0.9984	0.0027	0.269
7	0.977	0.059	6.09	0.9959	0.0024	0.243
8	0.924	0.029	3.18	0.9987	0.0008	0.084
9	0.970	0.017	1.80	0.9994	0.0003	0.025
10	0.990	0.082	8.25	0.9956	0.0052	0.524
11	0.962	0.055	5.71	0.9986	0.0010	0.101
12	0.980	0.004	0.42	0.9989	0.0010	0.099

PCR analysis in a previous study.[26] In this study, the copy number of the *CHY* gene among different papaya cultivars was evaluated by real-time PCR assays in all 12 participating laboratories. Each laboratory was asked to construct four separate PCR standard curves using serially diluted genomic DNAs of four papaya cultivars (Xiaobai, Qingpi, Risheng, and Suizhonghong, which were denoted A–D, respectively). The serially diluted genomic DNA concentrations of each papaya cultivar were 20, 2, 0.2, 0.02, and 0.002 ng/μL. The quantity of papaya genomic DNA used in each PCR was 100, 10, 1.0, 0.1, and 0.01 ng, respectively (5 μL each), which equaled approximately 250000, 25000, 2500, 250, and 25 copies of haploid papaya genomic DNA, respectively. The average PCR efficiencies (E) of the four constructed standard curves among 12 laboratories ranged from 0.8793 to 1.0792. The square regression correlations (R^2) of the constructed standard curves from 12 laboratories were all >0.99, with a standard deviation (SD) of <0.0032 (Table 2). The copy numbers of the serially diluted DNA samples (A–D) were calculated using the four constructed standard curves, and the differences in the calculated values among the four papaya cultivars and different laboratories were analyzed statistically. The mean copy numbers of papaya DNA samples A–D calculated in the 12 laboratories are listed in Table 3. The relative standard deviation (RSD) were calculated with values that ranged from 2.09 to 4.80%.

Furthermore, the Ct values of qualitative PCR of the four papaya DNAs (same concentration) from 12 laboratories are listed in Table 4 and analyzed by the LSD test using SPSS version 13.0. The calculated values from the LSD test indicated that no significant difference in Ct was observed with each laboratory (LSD test; $p > 0.05$). The largest difference between the mean and individual Ct values among the 12 laboratories was <1 (0.08–0.69); we believe that the slight variability of Ct values among the 12 laboratories might be attributed to unavoidable experimental errors, including estimation of quantities of DNA, liquid handling, etc. The RSDs of the quantified values of each diluted DNA sample (A–D) among 12 laboratories are listed in Table 5. The RSDs ranged from 19.47 to 33.80%, which are acceptable in GMO analysis.[30] Therefore, all data from different laboratories could be included and demonstrated that the copy number of the *CHY* gene in different papaya cultivars was one copy per haploid genome.

Validation of the *CHY* Gene Qualitative PCR Assay. To determine the sensitivity of the *CHY* gene qualitative PCR assay, the limit of detection (LOD) was evaluated in this ring trial. Ten DNA samples (denoted G1–G10) were sent to each laboratory and used to determine the LOD of the *CHY* gene qualitative PCR assay. The 10 DNA samples were extracted from powders containing a mixture of rice and papaya seeds with papaya contents (w/w) of 1, 0.5, 0.1, 0.05, and 0.01%. The expected 281 bp DNA amplicon was detected from the DNA samples with papaya contents of 1, 0.5, and 0.1% in all 12

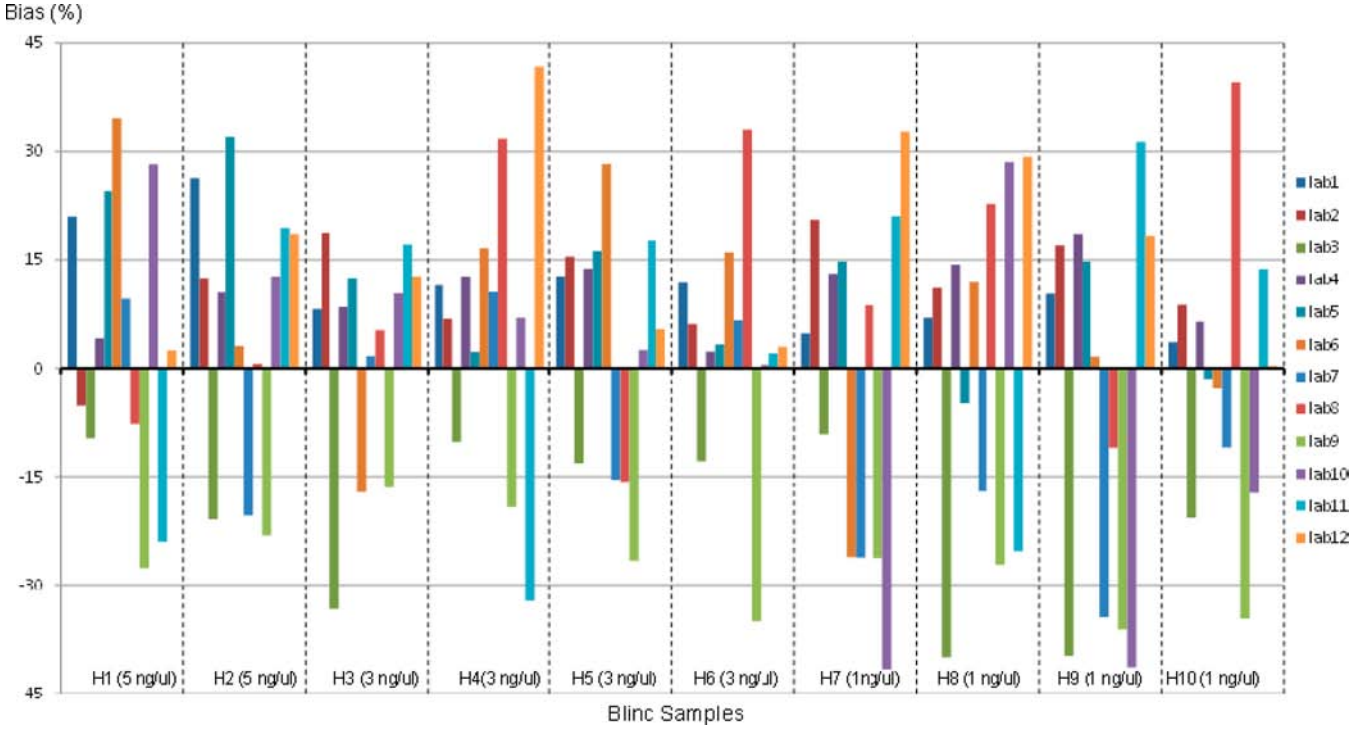

Figure 1. Relative deviation from the true value of 10 blind samples for all participating laboratories.

Table 8. Test Results of Blind DNA Samples Using the Quantitative PCR Assay

	H1 (5.0 ng/μL)	H2 (5.0 ng/μL)	H3 (3.0 ng/μL)	H4 (3.0 ng/μL)	H5 (3.0 ng/μL)	H6 (3.0 ng/μL)	H7 (1.0 ng/μL)	H8 (1.0 ng/μL)	H9 (1.0 ng/μL)	H10 (1.0 ng/μL)
no. of laboratories that returned results	12	12	12	12	12	12	12	12	12	12
total no. of data	108	108	108	108	108	108	108	108	108	108
no. of outliers	1	0	1	1	0	0	4	4	3	2
reason for exclusion					Grubbs' test					
mean value	5.21	5.29	3.07	3.20	3.10	3.09	0.99	1.01	0.96	0.99
SD of repeatability	0.7495	0.821	0.5125	0.8445	0.5715	0.7705	1.023	0.93	1.022	0.706
RSD of repeatability (%)	14.99	16.42	10.25	16.89	11.43	15.41	20.46	18.6	20.44	14.12
SD of reproducibility	1.01	0.93	0.48	0.61	0.51	0.49	0.24	0.23	0.27	0.19
RSD of reproducibility (%)	19.33	17.64	15.74	19.04	16.57	15.69	24.17	23.15	28.13	19.13
bias (absolute)	4.15	5.88	2.29	6.58	3.43	3.06	1.16	0.84	4.26	1.32
bias (%)	4.15	5.88	2.29	6.58	3.43	3.06	−1.16	0.84	−4.26	−1.32

participating laboratories except for one negative result from the 0.1% sample in one laboratory. Eight laboratories detected DNA samples with a papaya content of 0.05%. Five laboratories could detect the 281 bp fragment from the DNA samples with a papaya content of 0.01%. These results demonstrated that the LOD of the *CHY* gene qualitative PCR assay is 0.1% at the 95% confidence level,[30] which is sufficient for GMO detection (Table 6).

Validation of the *CHY* Gene Quantitative PCR Assay. To evaluate the developed quantitative PCR assay of the *CHY* gene, four PCR standard curves were constructed in all laboratories using provided papaya genomic DNA. Five serially diluted DNAs at 100, 10, 1, 0.1, and 0.01 ng (absolute amounts in each PCR mixture) from four papaya varieties (Xiaobai, Qingpi, Risheng, and Suizhonghong) were used. The PCR efficiency, linearity of the quantitative PCR standard curve, limit of detection, repeatability, and reproducibility were determined and used to assess the applicability of the quantitative real-time PCR assay. In addition, 10 blind samples (H1−H10) with three different papaya genomic DNA concentrations (H1 and H2 samples at a concentration of 5.0 ng/μL, H3−H6 at a concentration of 3.0 ng/μL, and H7−H10 at a concentration of 1.0 ng/μL) were tested using the *CHY* gene quantitative PCR assay. The differences between the test values and true values were measured and used to assess the accuracy of this quantitative PCR assay for practical papaya sample quantification.

Efficiency and Linearity of Quantitative Standard Curves. All participants were asked to dilute the DNA standards, run the quantitative PCR assay, and report the Ct values. The Ct values were plotted against the log transformation of the absolute amount of papaya genomic DNA (nanograms) to obtain standard curves. The PCR efficiency (E) was calculated using the equation $E = 10^{-1/\text{slope}} - 1$. The PCR efficiencies ranged from 0.8793 to 1.0792 (Table 2). The square regression correlation (R^2) values were all >0.99 (Table 2). The variation in E and R^2 among four papaya cultivars in 12 laboratories was statistically analyzed (Table 7). The SD and RSD values were acceptable for a standard quantitative PCR assay. The high PCR efficiency and good linearity of the standard curves demonstrated good performance of the *CHY* gene assay with papaya samples.

Limit of Quantification (LOQ). To determine the LOQ of the *CHY* gene quantitative PCR assay, five serial dilutions of genomic DNAs from four different papaya cultivars were employed to construct standard curves. The detection dynamic range of the constructed standard curves was 0.01−100 ng in a 25 μL reaction volume. Within this dynamic range, high PCR efficiency and good standard curve linearity were obtained for all four cultivars in the 12 participating laboratories, and the bias of quantification results of each DNA was within 35%. Thus, the deduced LOQ of the quantitative PCR was as low as 0.01 ng, which equaled 25 copies of haploid papaya genomic DNA, showing that the papaya *CHY* gene quantitative PCR assay is sufficiently sensitive for papaya sample quantification.

Repeatability and Reproducibility of the Quantitative PCR Assay. The repeatability and reproducibility of the *CHY* gene quantitative PCR assay were both evaluated using the same set of papaya genomic DNA dilutions used to construct the standard curves. The standard deviation (SD_r) and relative standard deviation (RSD_r) of repeatability reflected the variation in the results at each DNA dilution level, and the standard deviation (SD_R) and relative standard deviation (RSD_R) of reproducibility reflected the variation in the results among all participating laboratories. All of those values were calculated from three repeats and each repeat with three replicates according to ISO 5725. The RSD_r of the quantitative PCR assay of the *CHY* gene ranged from 8.15 to 9.94%, and the RSD_R ranged from 8.47 to 14.56%, indicating that the established quantitative PCR assay of the papaya *CHY* gene is both stable and reliable.

Quantification of Blind Samples. Ten blind DNA samples (H1−H10) with varying papaya DNA contents were provided to each participating laboratory. Each participating laboratory was asked to analyze the blind samples using the four constructed standard curves. The results are shown in Figure 1 and Table 8. For the statistical analysis, 16 of a total of 1080 quantitative data points (1.48%) were excluded on the basis of Grubbs' test. The biases of the 10 blind samples from all 12 participating laboratories were within the acceptable level of 35%, except for one result from the 3 ng/μL sample and five results from the 1 ng/μL sample. The average bias between the quantified values and true values ranged from 0.84 to 6.58%. For blind sample quantification, the repeatability and reproducibility of the analysis of blind samples were also calculated. The RSD of repeatability ranged from 10.25 to 20.46%, and the RSD of reproducibility ranged from 15.69 to 28.13%. These results indicated that the quantified results of blind samples were accurate and the *CHY* gene quantitative PCR assay was suitable for quantification of papaya samples.

In conclusion, on the basis of the results returned from all the participants in the collaborative ring trial, we validated that the papaya *CHY* gene had three main characteristics necessary for a good endogenous reference gene: species specificity, low

heterogeneity, and single-copy number in different papaya cultivars. In addition, the *CHY* gene qualitative and quantitative PCR assays developed in our laboratory were demonstrated to have high efficiency, high sensitivity, good repeatability and reproducibility, and acceptable accuracy in blind sample quantification. The validation results from the collaborative ring trial showed that the *CHY* gene qualified as a suitable endogenous reference gene in the identification and quantification of transgenic papaya.

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Notes

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