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Applicability of the *Chymopapain* Gene Used as Endogenous Reference Gene for Transgenic Huanong No. 1 Papaya Detection

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The virus-resistant papaya (*Carica papaya* L.), Huanong no. 1, was the genetically modified (GM) fruit approved for growing in China in 2006. To implement the labeling regulation of GM papaya and its derivates, the development of papaya endogenous reference gene is very necessary for GM papaya detection. Herein, we reported one papaya specific gene, *Chymopapain (CHY)*, as one suitable endogenous reference gene, used for GM papaya identification. Thereafter, we established the conventional and real-time quantitative PCR assays of the *CHY* gene. In the *CHY* conventional PCR assay, the limit of detection (LOD) was 25 copies of haploid papaya genome. In the *CHY* real-time quantitative PCR assay, both the LOD and the limit of quantification (LOQ) were as low as 12.5 copies of haploid papaya genome. Furthermore, we revealed the construct-specific sequence of Chinese GM papaya Huanong no. 1 and developed its conventional and quantitative PCR systems employing the *CHY* gene as endogenous reference gene. This work is useful for papaya specific identification and GM papaya detection.

KEYWORDS: *Carica papaya* L.; *Chymopapain* gene; conventional PCR; real-time quantitative PCR; endogenous reference gene

INTRODUCTION

The global area of genetically modified (GM) crops continuously increased since 1996, reaching 125 million hectares by the end of 2008 (*1*). As consumers are concerned for the safety of GM food and feed, more than 50 countries and areas issued a number of genetically modified organisms (GMOs) labeling regulations. To implement GMOs labeling regulations, the accurate and reliable GMOs detection and quantification techniques are necessary. Several DNA-based analysis techniques are being used widely because of its high specificity and sensitivity, especially for the polymerase chain reaction (PCR) technique. The TaqMan real-time PCR employing one specific hybridized probe has been accredited for GMOs quantification by International Organization for Standardization (ISO) and which has been widely used for determining the presence of GMOs in processed food and feed samples (*2*−*4*). In real-time quantitative PCR analysis of GMOs, the initial amounts of GM and non-GM DNA templates can be quantified by the standard curves and GM contents (%) can be calculated by the ratios of specific GMOs target sequence with respect to species-specific endogenous reference gene sequence (*4*). Consequently, validation of an appropriate endogenous reference gene for each GM plant is necessary for GMOs analysis as well as identification of plant ingredients in the mixed samples.

According to the requirement of GMOs quantification, one endogenous reference gene should have three typical characters such as species specificity, no allelic variation among various cultivars, and low or stable copy number in haploid genome (*5*). Until now, much effort has been made in obtaining reference genes of different crops for the detection of GMOs, for instance, the *Adh1*, *invertase 1*, *Zein*, and *Hmga* genes for maize (*6*), the *Lectin* (*7*) and *β-actin* (*8*) genes for soybean, the *Acc1* (*9*) and *PKABA1* (*10*) genes for barley, the *Hmg I*/*Y* (*11*) and *BnACCg8* (*12*) genes for rapeseed, the *SPS* (*13*), *Oryzain β* (*9*), and *Gos 9* (*9*) genes for rice, the *LAT52* gene for tomato (*14*), and the *Sad1* gene for cotton (*15*, *16*) et al. Furthermore, the conventional and quantitative PCR methods of *LAT52* and *SPS* have been validated by international collaborative ring trials (*17*, *18*).

Papaya (*Carica papaya* L.), one of the most important economic fruit crops, has been widely cultivated in tropical and subtropical lowland regions for its nutritional benefits and medicinal applications. The papaya ringspot virus (PRSV) can virtually eliminate papaya yield. GM PRSV-resistant papayas,

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SunUp and Rainbow, have already been commercialized in the United States (Hawaii) in 1998 (*19*). In China, a GM virus-resistant papaya variety, Huanong no. 1, was developed by the introduction of the papaya ringspot virus replicase *(NIb)* gene (GenBank no. AF469604) and approved for commercialization in 2006. The exogenous DNA insert of Huanong no. 1 papaya contains the *NIb* gene cassette and the *neomycin phosphotransferase II* (*NptII*) gene cassette. In the *NIb* gene cassette, the *NIb* gene was regulated by *cauliflower mosaic virus* (*CaMV*) 35S promoter and *Agrobacterium tumifaciens* nopaline synthase (NOS) terminator. Thus, the development of an ideal papaya endogenous reference gene and its specific detection method for GM papaya identification and quantification is necessary.

Herein, we first selected four papaya genes (*pectinesterase*, *SPG1*, *CHY*, and *NPR1* genes) as candidate genes through BLAST analysis via the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). Then, we confirmed that the papaya *CHY* gene encoding a major cysteine endopeptidase with 218 amino acid residues (*20*, *21*), is a good candidate that could be used as one papaya endogenous reference gene for further GM papaya analysis. Thereafter we designed the species-specific primers and probe for the *CHY* gene and established its conventional and quantitative PCR systems. Furthermore, we revealed the construct-specific sequence of GM papaya Huanong no.1 and developed the construct-specific real-time PCR methods.

MATERIALS AND METHODS

Plant Materials. Papaya fruits of 10 different papaya varieties, such as Meizhonghong, Shuizhonghong, Lingnan no.5, and You 8 from Hainan, China, Taiwan no.5 from Taiwan, China, Hongrou from Thailand, Sinta and RedLady from the Philippines, Wilder and Blue Solo from Hawaii, USA, were purchased from Taiwan and Shanghai, China. Other 15 plant samples of different species such as 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*), wheat (*Triticum aestivum*), soybean (*Glycine max*), potato (*Solanum tuberosum*), pepper (*Capsicum annuum*), tomato (*Lycopersicon esculentum*), and tobacco (*Nicotiana tabacum*), were collected by our laboratory. Transgenic Huanong no. 1 papaya leaves containing the papaya ringspot virus replicase *(NIb)* gene and its conventional control were kindly supplied by Prof. Huaping Li of South China Agriculture University (SCAU), China.

The seeds of GM maize MON863, GTS 40−3−2 soy, and MON1445 cotton were developed and kindly supplied by Monsanto Co. (St. Louis, MO). The seeds of GM maize Bt11 were kindly supplied by Syngenta Seeds, Inc. (Basel, Switzerland). GM canola Oxy-235 was kindly supplied by Bayer CropScience Co. (Monheim, Germany). GM Huafan no. 1 tomato was kindly supplied by Huazhong Agriculture University, China.

DNA Extraction and Purification. The plant genomic DNA samples used for conventional and real-time quantitative PCR analysis were extracted and purified using the mini-plant genomic DNA extraction kit (Shanghai Ruifeng Agro-tech Co. Ltd., Shanghai, China) according to the manufacturer's manual. The concentration and quality of the purified DNA samples were measured and evaluated using the NanoDrop 1000 UV/vis spectrophotometer (NanoDrop Technologies Inc., USA). Also, the quality of DNA was further checked by 1% (w/v) agarose gel electrophoresis in 0.5× TBE with ethidium bromide staining.

Oligonucleotide Primers and Probes. Oligonucleotide primers and probes used in this study were designed using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA) and listed in **Table 1**. The primers Chy-1F/2R, Q-Chy-1F/2R, and probe Q-Chy-P for endogenous reference gene assay were designed based on the *CHY* gene sequence (GenBank no. AY803756). The primers GM-pp-1F/2R, Q-GM-pp-1F/2R, and probe Q-GM-pp-P for construct-specific assay of GM Huanong no. 1 papaya were designed according to our revealed construct-specific sequence between *CaMV35s* promoter and the *NIb* gene of papaya exogenous insertion. Primer pairs PFL-1F/2R for the *PFL* gene (GenBank no. DQ054794) and Chy-1F/2R for the *CHY* gene were used for estimating the copy number of the *CHY* gene in haploid papaya genome. The TaqMan probes were labeled with the fluorescent reporter dye 6-carboxy-fluorescein (FAM) on the 5′ end. To increase the fluorescent intensity, the fluorescent quencher dye was labeled on the T-base of the probes within internal positions with black-hole quencher 1 (BHQ1) for Q-Chy-P or 6-carboxy-tetramethyl-rhodamine (TAMRA) for Q-GM-pp-P according to the description of Proudnikov et al. (*22*). All of the primers and probes were synthesized by Invitrogen Co., Ltd. (Shanghai, China).

Conventional PCR Conditions. Conventional PCR assays were carried out in a 25 μL final volume on a PTC-100 thermocycler machine (MJ Research, Waltham, MA). Each reaction mixture contained the following reagents: 1 × PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM each dNTP, 400 nM each primer, and 1.5 units of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China), and 5 μL genomic DNA. Conventional PCR amplified programs were as follows: 5 min of predenaturation at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C, and a final extension of 7 min at 72 °C. The PCR amplified products were analyzed by 2% (w/v) agarose electrophoresis in 0.5× TBE with ethidium bromide staining.

Real-Time Quantitative PCR Conditions. Real-time quantitative PCR assays were carried out on a fluorometric thermal cycler Rotor-Gene 3000A (Corbett Research, Australia) with final volume of 25 μL.

In TaqMan real-time PCR assays, reactions were consisted of the following reagents: in addition to 1× quantitative PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 200 μM of each dNTP, 1.5 units of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China), 5 μL of genomic DNA sample, for the *CHY* gene assay: 6 mM $MgCl_2$, 500 nM each primer and 100 nM TaqMan probe or for construct-specific assay, 5 mM $MgCl_2$, 400 nM each primer and 120 nM TaqMan probe. Real-time PCR reactions were performed with the following program: 10 min of predenaturation at 95 °C, followed by 45 cycles of 15 s at 95 °C, 60 s at 60 °C. The Fluorescent signal was monitored during every PCR cycle at the extension step.

In real-time PCR assays using SybrGreen I dye, the PCR reagents included: 1× quantitative PCR buffer, 200 μM each dNTP, 400 nM each primer, 2.5 mM $MgCl_2$, 0.25× SybrGreen I dye (Generay Biotech Co., Ltd., Shanghai, China), 1.5 units of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China), and 5 μL of genomic DNA solutions. PCR reactions were carried out with the following program: 10 min of predenaturation at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 58 °C for the *CHY* gene assay or 62 °C for the *PFL* gene assay, and 20 s at 72 °C. The fluorescent signal was collected at the step of extension. After PCR amplification, the PCR products were slowly heated back to 95 °C at a rate of 0.2 °C/s, and T_m curve analysis was performed using the Rotor-Gene analysis software (Corbett Research, Australia). Obtained fluorescence signals were continuously monitored during the slow warming-up gradient and showed a decreasing curve with a sharp fluorescence drop near the denaturation temperature. Plotting the negative derivate of the fluorescence over temperature versus the temperature ($-dF/dT$ versus T) generated peaks from which the T_m of the products were calculated.

All the quantitative PCR reactions were repeated three times and each time with three parallels. The quantitative PCR data was analyzed with the Rotor gene 3000 software version 6.0 (Corbett Research, Australia).

Selection of Candidate Endogenous Reference Genes. In our previous study (*11*, *13*−*15*), the module for selecting candidate endogenous reference genes has been proposed as follows: (i) the bioinformatics analysis for primary selection, (ii) experimental validation of three typical characters, such as the species specificity, no allelic variation among various cultivars, and low or stable copy number in haploid genome, (iii) development of the conventional and quantitative PCR systems of candidate genes, and (iv) use the endogenous reference gene and its systems for practical GM samples analysis. Herein we selected several papaya genes as the candidate endogenous reference genes through BLASTN analysis at GenBank database (http://www.ncbi.nlm.nih.gov/BLAST), such as *pectinesterase*, *spg1*, *CHY*, and *NPR1* gene.

Test of Sensitivities of Conventional and Real-Time PCR Assays. To determine the sensitivities of established conventional and real-time PCR assays of the *CHY* gene and real-time PCR assay of Huanong no. 1 papaya construct-specific sequence, the Huanong no. 1 papaya genomic

Table 1. Oligonucleotide Primers and Probes Used for Conventional and Quantitative PCR System

PCR system	primer name	sequence (5′−3′)	target	amplicon (bp)
endogenous reference gene assay	Chy-1F	ATCTACAATCTTGCTAACCCTA	*chymopapain*	281
	Chy-2R	AGTCATCTTGAGAATAACCCAC		
	Q-Chy-1F	CCATGCGGATCCTCCCA		73
	Q-Chy-2R	CATCGTAGCCATTGTAACACTAGCTAA		
	Q-Chy-P	FAM TTCCCTTCAT (BHQ1) CCATTCCCACTCTTGAGA		
	Pec-1F	TGATAGTGTTGCTGGAGGTT	*pectinesterase*	314
	Pec-2R	TCCGAGGAAGACGAAGAA		
	Spg1-1F	TGAGTGGTGGCTCTGGAA	*SPG1*	158
	Spg1-2R	GCTGACGCCGTTAGTGGTA		
	Spg1-3F	GCATTAGTATCGGTAGTCTTGG		101
	Spg1-4R	GCTGACGCCGTTAGTGGTA		
	Npr1-1F	TCGCATCCATTAGAGCCT	*NPR1*	262
	Npr1-2R	CATCATAGTCTCCAGCAAGT		
construct-specific assay	GM-pp-1F	ATGACGCACAATCCCACTAT	GM papaya	201
	GM-pp-2R	TCCAGGTAACTACGGAAGAG		
	Q-GM-pp-1F	ATAAGGAAGTTCATTTCATTTGGAGAGA		120
	Q-GM-pp-2R	AACATGCTTCGTGACTAAATTGCTA		
	Q-GM-pp-P	FAM AGCTCACT (TAMRA) CCCTTAAGATTGCCGTGTAACTTATCC		
copy-number estimatingassay	PFL-1F	GACGAACCAGGTGTTTAGATATG	*PFL*	259
	PFL-2R	AATGGCAAGACGAGGATGTG		

DNA was serially diluted with 0.1× TE buffer to final concentrations of 50000, 5000, 500, 50, 5, 2.5, and 0.5 copies of haploid papaya genome/μL according to the 372 Mbp of papaya genome in size (*23*). Consequently, the amounts of temple DNA per reaction corresponded to 250000, 25000, 2500, 250, 25, 12.5, and 2.5 copies of haploid papaya genome in each PCR reaction (5 μL for each reaction). Each DNA dilution was analyzed with triplicates. For testing the sensitivity of the established construct-specific conventional PCR assay, the DNA mixture was prepared from Huanong no. 1 papaya event and non-GM papaya event at various levels such as 0%, 0.01%, 0.05%, 0.1%, 1.0%, 3.0%, and 5.0%.

Construction of Standard Curves for Real-Time PCR Assays. Two types of real-time quantitative PCR systems (TaqMan and SybrGreen I) were employed to quantify GM Huanong no. 1 papaya contents and determine the copy number of the *CHY* gene in haploid papaya genome, respectively. A series of Huanong no. 1 papaya genomic DNA dilutions with final concentrations of 50000, 5000, 500, 50, 5, and 2.5 copies of haploid genome/μL were used as calibrators for *CHY* and construct-specific sequence real-time PCR assays. Another series of Huanong no. 1 papaya genomic DNA dilutions with final concentrations of 25000, 2500, 250, 125, and 25 copies of haploid genome/μL were employed as calibrators in *CHY* and *PFL* SybrGreen I real-time PCR assays. The standard curves were constructed by plotting the C_T values of each reaction against the logarithm of the initial amounts of the papaya genomic DNA.

Estimation of the Copy Number of the *CHY* Gene. Real-time PCR technique has been used for gene copy number determination instead of Southern blotting method because of its high accuracy, time-saving, and high throughput in recently published reports (*24*−*26*). According to the method reported by Weng et al. (*24*), the copy number of the *CHY* gene in six different papaya varieties (conventional control of Huanong no. 1, Taiwan no. 5, Shuizhonghong, Sinta, Wilder, and Huanong no. 1) haploid genome was analyzed by means of SybrGreen I based real-time PCR. For determining the copy number of the *CHY* gene, one reported single-copy gene of papaya, *PFL* (*27*), was selected and used as one internal control. The copy number of the *CHY* gene was directly calculated by the formula .

$$IQ_{chy}/IQ_{pfl} = 10^{[(C_{T,chy} - I_{chy})/S_{chy}] - [(C_{T,pfl} - I_{pfl})/S_{pfl}]} \quad (1)$$

Where the IQ_{chy} and IQ_{pfl} represent the initial quantities of the *CHY* gene and the *PFL* gene, respectively, I_{chy} and I_{pfl} represent the intercepts of the standard curves of the *CHY* gene and the *PFL* gene, respectively, S_{chy} and S_{pfl} represent the slopes of the standard curves of the *CHY* gene and the *PFL* gene, respectively; $C_{T,chy}$ and $C_{T,pfl}$ represent the detected threshold cycles of the same tested sample in the *CHY* quantitative PCR assay and the *PFL* quantitative PCR assay, respectively.

Sequencing Analysis of the *CHY* Gene. To reveal the detailed sequence information of the *CHY* gene in different papaya cultivars, we amplified the specific fragment of the *CHY* gene with Chy-1F/2R primers employing KOD Plus DNA polymerase PCR system (TOYOBO Co., Ltd.). The amplified fragments from different papaya cultivars were purified with the AxyPrep DNA Gel Extraction Kit (Axygen Scientific, Inc., CA) and analyzed using the ABI PRISM 3730 Genetic Analyzer (Shanghai Invitrogen Co., Ltd. Shanghai, China). Then, the obtained sequences were aligned using the software (Vector NTI Advance 10) and used for designing the primers and TaqMan probe.

RESULTS AND DISCUSSION

Selection of Candidate Endogenous Reference Genes in Papaya. Generally, an ideal taxon specific reference gene for GMOs detection should be species specificity, no allelic variation among various cultivars, and low or stable copy number of haploid genome (*2*). To select one suitable endogenous reference gene for papaya detection, we first selected some candidates using bioinformatics approaches. Initially, we searched several published papaya genes with low copy number from NCBI database, then sequences homologies of these genes were determined via the BLAST program in the GenBank database. As a result, four candidate genes with low homology in other papayas and plant species were selected, such as *pectinesterase* (GenBank no. Y07899), *SPG1* (GenBank no. Y07900), *chymopapain* (*CHY*) (GenBank no. AY803756), and *NPR1* (GenBank no. AY550242). On the basis of the specific region of each gene, sequence-specific primers were designed (**Table 1**). Then the conventional PCR assays of these four genes were performed for further validation.

We tested them for species specificity and allelic variation using genomic DNAs from different plant species and papaya cultivars as templates (data not shown). As to the *pectinesterase* gene, we obtained nonexpected fragments in several papaya cultivars (Hongrou, Sinta) and other plant species (tobacco and rice),

indicating that the *pectinesterase* region may not be suitable for endogenous reference gene. Also nonspecific fragments were observed in the test of the *SPG1* gene in both designed PCR assays employing primer pair Spg-1F/2R and Spg-3F/4R. Furthermore, we obtained the nonspecific DNA fragment from maize cultivars using the *NPR1* PCR assay. While in the test of the *CHY* gene, only the expected fragment from papaya was obtained and no detectable signal among other plant species.

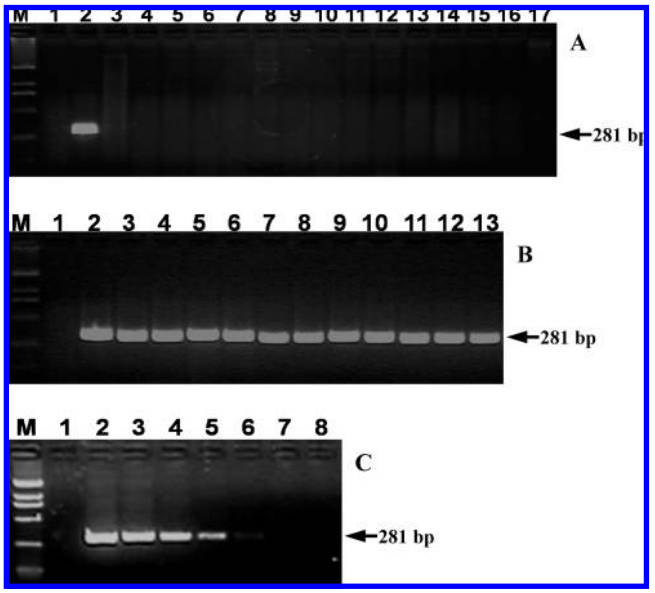

Figure 1. Agarose gel electrophoresis of conventional PCR products amplified with the Chy-1F/2R primer pair. (**A**) Amplification results of DNA from 16 different plant species. Lane 1: NTC; lanes 2−17: GM Huanong no. 1 papaya, longan, banana, mango, orange, lemon, wheat, maize, rice, tobacco, soybean, rapeseed, tomato, pepper, cotton, and potato; lane M: DL2000 marker. (**B**) Amplification results of DNA from different papaya cultivars. Lane 1: NTC; lanes 2−13: GM Huanong no. 1 papaya, conventional control, Meizhonghong, Shuizhonghong, Lingnan no. 5, You 8, Taiwan no. 5, Hongrou, Sinta, RedLady, Wilder, and Blue Solo; lane M: DL2000 marker. (**C**) Amplification results of serial dilutions of papaya DNA. Lane 1: NTC; lanes 2−8 corresponded to 250000, 25000, 2500, 250, 25, 12.5, and 2.5 copies of papaya haploid genom; lane M: DL2000 marker.

These results indicated that the *CHY* DNA fragment is likely suitable to be used as the papaya endogenous reference gene.

Validation of the *CHY* Gene. *Species Specificity*. To test the species specificity of the amplified *CHY* DNA fragment, both conventional and real-time PCR reactions were performed employing 40 ng genomic DNA from 15 different plant species that are either closely related to papaya or frequently consumed plant materials, such as 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*), wheat (*Triticum aestivum*), soybean (*Glycine max*), potato (*Solanum tuberosum*), pepper (*Capsicum annuum*), tomato (*Lycopersicon esculentum*), and tobacco (*Nicotiana tabacum*). No amplified products or fluorescent signals were observed in any of these tested species except for papaya in both conventional (**Figure 1A**) and real-time PCR (**Table 2**). These results demonstrated that the *CHY* endogenous reference gene is species-specific for papaya.

Allelic Variation of the CHY Gene among Various Papaya Cultivars. The allelic variation of the *CHY* gene among 12 different papaya cultivars from China (7 cultivars), Thailand (1 cultivar), Philippines (2 cultivars) and USA (2 cultivars) were tested using both conventional and real-time PCR. The results of conventional PCR were shown in **Figure 1B**, and only the expected PCR amplicon (281 bp in size) with identical size and relative intensity was obtained from these 12 cultivars. In the real-time PCR analysis, the similar fluorescent signal (data not shown) and C_T values ranging from 22.34 to 23.08 were obtained in these 12 papaya cultivars (**Table 2**). The slight variation of C_T values might be from pipetting inaccuracy error or the quality of template genomic DNAs. These results indicated that the *CHY* gene has no allelic variation among the different papaya cultivars and the copy number of the *CHY* gene in the tested papaya cultivars is also consistent.

The Copy Number Estimation of CHY Gene. The copy number of one endogenous reference gene is critical for GMOs quantification. Herein six different papaya cultivars were used to estimate the copy number of the *CHY* gene in papaya haploid genome by means of real-time PCR method employing *PFL* as control. The standard curves of *PFL* and *CHY* were established employing five papaya genomic DNA dilutions, i.e., 25000, 2500, 250, 125, and 25 copies of haploid genome/μL, respectively. Each

Table 2. Species Specificity and Allelic Variation of the *CHY* Gene among Various Papaya Cultivars

	C_T value						C_T value				
plant species[a]	mean1	mean2	mean3	mean	SD	plant species[c]	mean1	mean2	mean3	mean	SD
Huanong no. 1 papaya	20.14	20.03	20.19	20.12	0.08	Huanong no. 1 papaya	22.54	22.79	22.73	22.69	0.13
Longan	ND[b]	ND	ND			conventional control[d]	22.37	22.47	22.63	22.49	0.13
banana	ND	ND	ND			Meizhonghong	23.08	22.46	22.8	22.78	0.31
mango	ND	ND	ND			Shuizhonghong	23.10	22.84	22.95	22.96	0.13
sweet orange	ND	ND	ND			Lingnan no. 5	22.50	22.58	22.59	22.56	0.05
lemon	ND	ND	ND			You 8	23.08	22.68	22.68	22.81	0.23
cotton	ND	ND	ND			Taiwan no. 5	22.50	22.34	22.39	22.41	0.08
maize	ND	ND	ND			Hongrou	23.00	22.85	22.92	22.92	0.07
rice	ND	ND	ND			Sinta	23.04	22.89	22.91	22.95	0.08
rapeseed	ND	ND	ND			RedLady	22.86	22.57	22.60	22.68	0.16
wheat	ND	ND	ND			Wilder	22.84	22.80	22.75	22.80	0.05
soybean	ND	ND	ND			Blue Solo	22.72	22.95	22.92	22.86	0.13
potato	ND	ND	ND								
pepper	ND	ND	ND								
tomato	ND	ND	ND								
tobacco	ND	ND	ND								

[a] 40 ng genomic DNA was used as template. [b] no fluorescent signals were detected; [c] 10 ng genomic DNA was used as template. [d] host papaya of Huanong no. 1 papaya.

Table 3. Copy Number of the Papaya *CHY* Gene Estimated by Real-Time Quantitative PCR

	CHY assay			*PFL* assay				
papaya varieties	linear regression equation of standard curve	R^2	C_T value of tested sample[a]	linear regression equation of standard curve	R^2	C_T value of tested sample[a]	IQ_{chy}/IQ_{pfl}[a]	estimated copy numbers of *CHY*
conventional control of Huanong no. 1	$y = 29.44 - 3.357x$	0.999	21.42 ± 0.04	$y = 30.44 - 3.362x$	0.999	22.41 ± 0.13	0.99 ± 0.08	1
Taiwan no. 5	$y = 29.45 - 3.360x$	0.999	21.40 ± 0.11	$y = 30.06 - 3.257x$	0.999	22.21 ± 0.15	0.97 ± 0.04	1
Shuizhonghong	$y = 29.97 - 3.419x$	0.999	21.70 ± 0.16	$y = 30.27 - 3.302x$	0.999	22.29 ± 0.08	1.01 ± 0.07	1
Huanong no. 1	$y = 29.64 - 3.405x$	0.999	21.41 ± 0.05	$y = 30.53 - 3.412x$	0.999	22.37 ± 0.12	1.07 ± 0.08	1
Sinta	$y = 29.68 - 3.323x$	0.999	21.78 ± 0.15	$y = 30.53 - 3.346x$	0.999	22.52 ± 0.09	0.96 ± 0.03	1
Wilder	$y = 30.04 - 3.391x$	0.999	21.91 ± 0.07	$y = 30.37 - 3.301x$	0.999	22.40 ± 0.08	0.97 ± 0.08	1

[a] Data are expressed as mean ± SD. Each reaction was analyzed with three times and each time with triple replication.

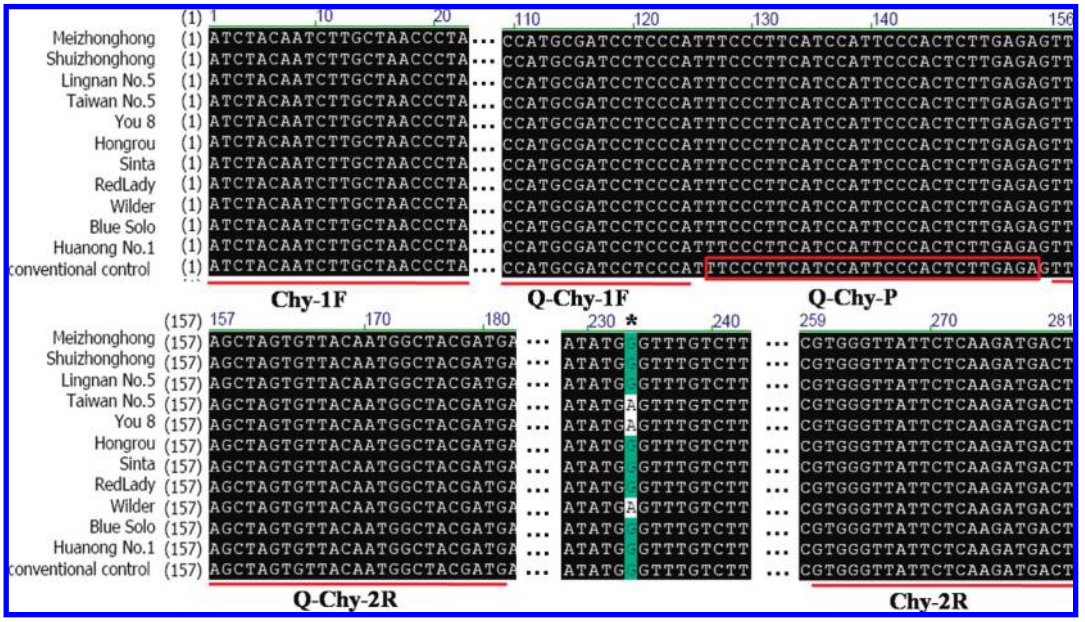

Figure 2. Alignment of the partial nucleotide sequences amplified employing the primers Chy-1F/2R in 12 different papaya varieties. The suspension points represent the part of identical nucleotide sequences in all sequences. Asterisk above the alignment indicates the position of nucleotide variation. Primers and probe used for conventional and quantitative PCR amplification are indicated by underlines and box, respectively.

reaction was repeated three times and each time with three parallels. The results showed that both PCR efficiencies of *PFL* and *CHY* PCR assays were above 0.97, the squared regression coefficient (R^2) values of all standard curves were 0.999 (**Table 3**). The high PCR reaction efficiency and the good linearity between logarithm of initial DNA quantities and cycler threshold (C_T) indicated that the established real-time PCR systems of *PLF* and *CHY* are suitable for quantitative analysis. The C_T values of tested samples were listed in **Table 3**, and the copy number of the *CHY* gene in six papaya cultivars was calculated according to the formula based on the slopes (S_{chy} and S_{pfl}) and intercepts (I_{chy} and I_{pfl}) of the *CHY* and *PFL* gene standard curves. The calculated results indicated that the *CHY* gene is single-copy gene in haploid papaya genome in six different papaya cultivars. Together with the results of less variation among different papaya cultivars, we believed that the *CHY* gene is one single copy in different papaya cultivars.

Sequencing Analysis of the *CHY* Gene. Because the single nucleotide polymorphism (SNP) in primer and probe might affect the efficiency, sensitivity, and quantified accuracy of the conventional and quantitative PCR methods, we isolated and sequenced the specific DNA fragment of the *CHY* gene with primer pair Chy-1F/2R in 12 different papaya cultivars to make sure whether sequence variation occurs within the PCR amplification region. The obtained DNA fragments (281 bp) of the *CHY* gene from these 12 papaya cultivars were aligned, and results indicated that there is one single nucleotide polymorphism (SNP) at position 233 of the 281 bp region amplified from the *CHY* gene (**Figure 2**). On the basis of the coincident sequence, we designed the specific primers (Q-Chy-1F/2R) and probe (Q-Chy-P) for developing conventional and quantitative PCR methods of *CHY*.

Conventional PCR Method of the *CHY* Gene. The conventional PCR method was established based on the primer pair Chy-1F/2R, and its limit of detection (LOD) was tested using one series of papaya genomic DNA dilutions (with final concentrations of 50000, 5000, 500, 50, 5, 2.5, and 0.5 copies of haploid genome/μL) as templates. These results showed that the expected amplicon with 281 bp in size was obtained in all the dilutions except for the levels of 2.5 and 0.5 copies/μL (**Figure 1C**), demonstrating the LOD of the *CHY* conventional PCR assay was as low as 25 copies of haploid papaya genome.

TaqMan Real-Time PCR Method of the *CHY* Gene. After optimizing the concentrations of primer pair Q-Chy-F/R, probe Q-Chy-P, and Mg^{2+}, the TaqMan real-time PCR method for the *CHY* gene was established. The standard curve, repeatability, and reproducibility were estimated by employing the papaya genomic DNA dilutions with final concentrations of 50000, 5000, 500, 50, 5, and 2.5 copies of haploid genome/μL. The LOD and LOQ were tested by employing the papaya genomic DNA dilutions with final concentrations of 50000, 5000, 500, 50, 5, 2.5, and 0.5 copies

of haploid genome/μL. All the quantitative PCR reactions were repeated three times and each time with three parallels.

Standard Curve. To evaluate the accuracy of the *CHY* real-time PCR method, the standard curve was constructed and shown in **Figure 3**. The PCR efficiency was 1, and the squared regression coefficient (R^2) of the standard curve was 0.9995. The high PCR reaction efficiency and the good linearity between logarithm of DNA quantities and cycler threshold (C_T) indicated that the *CHY* real-time PCR assay is suitable for papaya genomic DNA quantification.

Repeatability and Reproducibility. Repeatability and reproducibility were evaluated using the described papaya genomic DNA dilutions (50000, 5000, 500, 50, 5, and 2.5 copies of haploid genome/μL). The standard deviation (SD_r) and relative standard deviation (RSD_r) of repeatability were calculated according to the mean copy numbers from three replications performed by one researcher; standard deviation (SD_R) and relative standard deviation (RSD_R) of reproducibility were calculated from the mean copy numbers from three replications performed individually by three researchers, respectively. The RSD_r values of the *CHY* gene real-time PCR ranged from 0.72% to 6.00%, and the RSD_R values ranged from 1.34% to 4.77% (**Table 4**). All the results of the repeatability and reproducibility tests indicated that the *CHY* quantitative PCR assay works reliably.

LOD and LOQ. Generally, the LOD and LOQ are the most important parameters, referring to the lowest quantity of the target that can be reliably detected and quantified with a probability of ≥95% (*28*). In the *CHY* real-time PCR assay, at least 12.5 copies of papaya haploid genomic DNA could be detected and quantified with small RSD values in all nine repeated reactions (**Table 5**). These results indicated that both the LOD and LOQ of the real-time PCR assay were as low as 12.5 copies of haploid genome, showing that the *CHY* gene real-time PCR has sufficient sensitivity for papaya quantification.

Concluding from the results of standard curve, repeatability, reproducibility, LOD, and LOQ of the TaqMan real-time PCR assay for the *CHY* gene, we believed that the established TaqMan real-time PCR assay is applicable for papaya detection.

Construct-Specific PCR Detection of GM Papaya Huanong No. 1. *Qualitative PCR detection.* In this study, the sequence of *NIb* cassette was isolated and sequenced and submitted to the GenBank database (GenBank no. FJ490192). The construct-specific primers (GM-pp-1F/2R) were designed based on the junction sequence of the *CaMV*35S promoter and *NIb* gene to establish the construct-specific conventional PCR assay. As shown in **Figure 4A**, only a 201 bp DNA fragment was observed from Huanong no. 1 papaya and no amplification fragment was detected in other GM plant events (MON863, BT11, Oxy235, RRS, Huafan no. 1, and MON1445), non-GM papaya, and no template control (NTC).

Moreover, to test the LOD of the established construct-specific PCR assay, the mixed DNA samples were prepared from Huanong no. 1 papaya and its conventional control at various levels, such as 0%, 0.01%, 0.05%, 0.1%, 1.0%, 3.0%, and 5.0%. Total genomic DNA (100 ng) was used as a template in each reaction. The results showed that the expected 201 bp target DNA fragment of *CHY* was observed from all levels except for

Table 4. Repeatability and Reproducibility of the *CHY* Gene Quantitative PCR Assays

repeatability test				reproducibility test		
copy no.	mean copy no.	SD_r	RSD_r (%)	mean copy no.	SD_r	RSD_r (%)
250000	261194.7	1875.05	0.72	261359.9	7460.79	2.85
25000	24659.4	268.78	1.09	24929.3	1049.09	4.21
2500	2374.2	81.67	3.44	2366.1	91.85	3.88
250	244.5	5.65	2.31	239.0	11.40	4.77
25	25.5	1.53	6.00	25.8	0.35	1.34
12.5	12.8	0.54	4.20	12.9	0.17	1.35

Table 5. Limits of Detection and Quantification (LOD and LOQ) of *CHY* Gene Quantitative PCR

copy no.	signal rate (positive signals)	mean copy no	SD	RSD (%)
250000	9/9	261555.6	12572.28	4.81
25000	9/9	24949.5	1413.17	5.66
2500	9/9	2369.5	153.65	6.48
250	9/9	244.3	17.11	7.00
25	9/9	25.8	2.02	7.81
12.5	9/9	13.0	1.22	9.46
2.5	0/9			

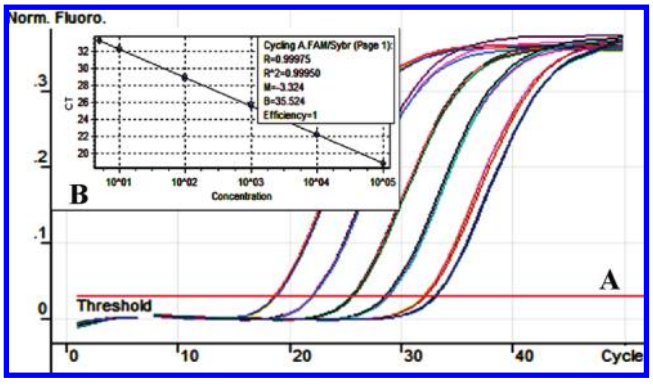

Figure 3. Amplification plots and standard curve for *CHY* DNA real time quantitative PCR. (**A**) Amplification plots generated by serial dilution of papaya DNA correspond to 250000, 25000, 2500, 250, 25, and 12.5 copies of haploid genome with the Q-Chy-1F/2R primer pair and the Q-Chy-P TaqMan probe. (**B**) Standard curve generated from the amplification data given in (A).

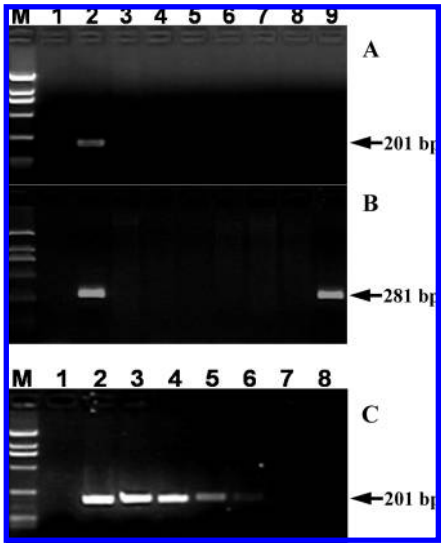

Figure 4. Agarose gel electrophoresis of conventional PCR products. PCR amplified results of Huanong no. 1 papaya construct-specific PCR with the GM-pp-1F/2R primer pair (**A**) and *CHY*-specific PCR with the Chy-1F/2R primer pair (**B**). Lane 1: NTC; lanes 2−9: GM Huanong no. 1 papaya, MON863, BT11, Oxy-235, RRS, Huafan no. 1, MON1445 and non-GM papaya; lane M: DL2000 marker. (**C**) Amplification results of serial dilutions of papaya DNA. Lane 1: NTC; lanes 2−8 corresponded to 5.0%, 3.0%, 1.0%, 0.1%, 0.05%, 0.01% and 0%; lane M: DL2000 marker.

Table 6. Repeatability and Reproducibility of construct-specific Quantitative PCR

	repeatability test			reproducibility test		
copy no.	mean copy no.	SD_r	RSD_r (%)	mean copy no.	SD_r	RSD_r (%)
250000	258815.77	1433.29	0.55	258607.84	2423.43	0.94
25000	24098.66	263.20	1.09	24292.61	42.04	0.17
2500	2512.67	29.46	1.17	2456.26	24.16	0.98
250	240.74	10.10	4.19	246.78	12.33	4.99
25	25.90	0.65	2.50	25.68	0.90	3.51

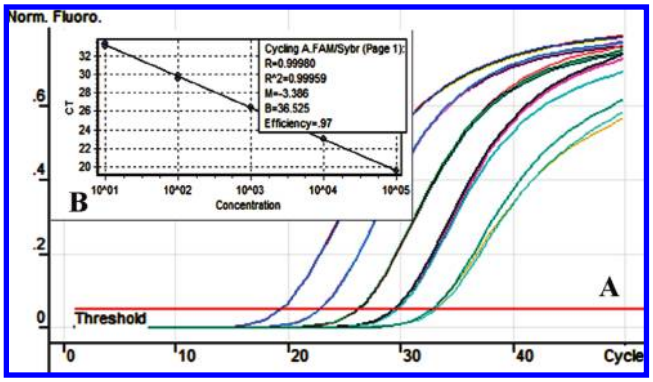

Figure 5. Amplification plots and standard curve for GM Huanong no. 1 papaya construct-specific real-time PCR assay. (**A**) Amplification plots for the construct-specific detection of GM papaya were generated by serial dilutions of GM papaya genomic DNA ranging from 250000 to 25 copies with the Q-GM-pp-1F/2R primer pair and the Q-GM-pp-P TaqMan probe. (**B**) Standard curve generated from the amplification data given in (**A**).

Table 7. Accuracy and Precision Statistics for Quantitative Methods

	construct amounts × 100/ endogenous amounts			accuracy		precision	
true value (%)	mean1	mean 2	mean 3	mean (%)	bias (%)	SD	RSD (%)
5.0	5.27	5.33	5.23	5.28	5.55	0.05	0.87
3.0	2.47	2.53	2.79	2.60	13.47	0.18	6.75
1.0	0.98	0.94	0.88	0.93	6.57	0.05	5.19
0.1	0.081	0.083	0.086	0.083	16.58	0.003	3.13
0	0	0	0	0			

0.01% and 0%, indicating that the LOD of the conventional PCR assay is 0.05%, corresponding to the 125 copies of papaya haploid genome (**Figure 4C**). The above results confirmed that the construct-specific PCR assay is highly specific and sensitive for practical detection of GM papaya samples.

Quantitative PCR Detection. The primers (Q-GM-pp-1F/2R) and TaqMan probe (Q-GM-pp-P) based on the construct-specific sequence were employed to establish the construct-specific quantitative PCR method. Several GM Huanong no. 1 papaya genomic DNA dilutions (with final concentrations of 50000, 5000, 500, 50, and 5 copies of haploid genome/μL) were used for constructing the standard curve and evaluating the repeatability and reproducibility. The amplified results showed that PCR reaction efficiency was 0.97 and the squared regression coefficient (R^2) of the standard curve was 0.999 (**Figure 5**). In the test of repeatability and reproducibility, the RSD_r values of the construct-specific real-time PCR ranged from 0.55% to 4.19% and the RSD_R values ranged from 0.17% to 4.99%, respectively (**Table 6**). Furthermore, the LOD and LOQ of the established construct-specific quantitative PCR assay were tested using a series of Huanong no. 1 genomic DNA dilutions (50000, 5000, 500, 50, 5, 2.5, and 0.5 copies/μL), and the results showed that the LOD and LOQ values were about 12.5 and 25 copies of papaya haploid genome, respectively (data not shown). These results indicated that the established construct-specific real-time PCR assay is accurate and creditable.

Subsequently, five practical samples with known GM Huanong no. 1 contents (5.0%, 3.0%, 1.0%, 0.1%, 0%) were prepared by mixing the pure dried Huanong no. 1 leaves with dried conventional papaya leaves on weight/weight basis. DNA (100 ng) extracted from each sample was used as template in each reaction, and GM contents were calculated based on the standard curves of both the construct-specific PCR assay and the *CHY* PCR assay using relative quantification method. Each sample was analyzed with three times and each time with three parallels. As shown in **Table 7**, the quantified biases (mean tested value vs true value) of tested samples ranged from 5.55% to 16.58%, the RSD values ranged from 0.87% to 6.75%, and the SD values were from 0.003 to 0.18. All the variations from five samples were under the accepted level of 25% for GMOs quantification (*29*), suggesting that the *CHY* gene could be used as the papaya endogenous reference gene, and the established construct-specific PCR systems are also usable for the quantification of practical samples derived from GM Huanong no. 1 papaya.

CONCLUSION

In this paper, we reported the papaya *CHY* gene used as endogenous reference gene for detection and quantification GM papayas and their derivates and developed its conventional and quantitative PCR assays. Furthermore, we revealed the construct-specific sequence of the GM Huanong no. 1 papaya and also established the conventional and quantitative PCR systems employing *CHY* as endogenous reference gene for GM Huanong no. 1 papaya detection. The LOD and LOQ of construct-specific real-time PCR were 12.5 copies and 25 copies of papaya haploid genomic DNA, respectively. All these results demonstrated that the *CHY* gene is one ideal endogenous reference gene for detection of GM papaya, and the developed construct-specific PCR methods of GM Huanong no. 1 papaya will facilitate the enforcement of GMOs labeling in China.

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