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Characterization of the Exogenous Insert and Development of Event-specific PCR Detection Methods for Genetically Modified Huanong No. 1 Papaya

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Genetically modified (GM) papaya (*Carica papaya* L.), Huanong No. 1, was approved for commercialization in Guangdong province, China in 2006, and the development of the Huanong No. 1 papaya detection method is necessary for implementing genetically modified organism (GMO) labeling regulations. In this study, we reported the characterization of the exogenous integration of GM Huanong No. 1 papaya by means of conventional polymerase chain reaction (PCR) and thermal asymmetric interlaced (TAIL)-PCR strategies. The results suggested that one intact copy of the initial construction was integrated in the papaya genome and which probably resulted in one deletion (38 bp in size) of the host genomic DNA. Also, one unintended insertion of a 92 bp truncated *NptII* fragment was observed at the 5′ end of the exogenous insert. Furthermore, we revealed its 5′ and 3′ flanking sequences between the insert DNA and the papaya genomic DNA, and developed the event-specific qualitative and quantitative PCR assays for GM Huanong No. 1 papaya based on the 5′ integration flanking sequence. The relative limit of detection (LOD) of the qualitative PCR assay was about 0.01% in 100 ng of total papaya genomic DNA, corresponding to about 25 copies of papaya haploid genome. In the quantitative PCR, the limits of detection and quantification (LOD and LOQ) were as low as 12.5 and 25 copies of papaya haploid genome, respectively. In practical sample quantification, the quantified biases between the test and true values of three samples ranged from 0.44% to 4.41%. Collectively, we proposed that all of these results are useful for the identification and quantification of Huanong No. 1 papaya and its derivates.

KEYWORDS: Event-specific PCR; Huanong No. 1 papaya; integration flanking sequence; TAIL-PCR; genetically modified organisms

INTRODUCTION

Because of the rapid development of modern agricultural biotechnology, more than 150 genetically modified (GM) plant events with new agronomic traits have been approved for commercialization (*1*). Since 1996, the global area of GM crops has consistently increased, reaching 125 million hectares at the end of 2008 (*2*). However, because of the consumers' concern regarding the safety of genetically modified organisms (GMOs), more than 50 countries and areas have issued the GMO labeling regulations with various labeling thresholds. In China, seventeen kinds of products from five GM crops were required to be labeled since 2003 (*3*).

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The development and application of reliable analytical methods for GM food and feed product detection are essential to fulfill the labeling regulations. A polymerase chain reaction (PCR) technique based on nucleic acid analysis has been widely used for GMO detection because of high efficiency, sensitivity, and stability (*4*−*6*). On the basis of the different transgenic elements, four PCR strategies, such as screening and gene-, construct-, and event-specific PCR, have been developed and used for GMO detection (*7*). Event-specific PCR has been used as the dominating method for GMO analysis because of its high specificity. It can specifically distinguish different transformation events based on the unique 5′ or 3′ integration flanking sequences. Up to now, a number of event-specific PCR detection methods for GM events have been developed and validated (*8*−*13*).

Papaya is an edible melon-like fruit enriched with vitamins and certain enzymes and is planted widely in tropical and subtropical lowland regions, such as the Americas, Mexico, Brazil, South

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Table 1. Oligonucleotide Primers and Probes Used in This Study

PCR system	primer name	sequence (5′-3′)	target	amplicon (bp)	ref
TAIL-PCR assay	5-GMP-SP1R	CGACAACTTGTGCAACACTTTCCT	*Nlb*		this work
	5-GMP-SP2R	TCCCATCAGCGGACTAAAGAAAG			
	5-GMP-SP3R	CCAGGTAACTACGGAAGAGGG			
	3-GMP-SP1F	AATGGATGAATTGGAGGC	*Nlb*		this work
	3-GMP-SP2F	GATTCGCCTGAGCTACTGGTGT			
	3-GMP-SP3F	ATTGAATCCTGTTGCCGGTCTT	T-NOS		
	NPT-SP1F	TTGGCTACCCGTGATATTGCTG	*NptII*		this work
	NPT-SP2F	TGACCGCTTCCTCGTGCTTTAC			
	NPT-SP3F	GCCTTCTTGACGAGTTCTTCTG			
	NP1R	ATCCAGATCCGGTGCAGATTAT	P-NOS		this work
	NP2R	ATGGAACGTCAGTGGAGCATTT			
	NP3R	AACGGCTTGTCCCGCGTCATC			
	3-GMP-SP4F	CCTTTAGGGTTCCGATTTAGTGC	T-NOS/ papaya genome		this work
	3-GMP-SP5F	TCGCCCTGATAGACGGTTTTTC			
	3-GMP-SP6F	CCAAACTGGAACAACACTCAACC			
	AD1	NTCGASTWTSGWGTT			(*19*)
	AD2A	NGTCGASWGANAWGAA			
	AD3	WGTGNAGWANCANAGA			
	SAD11	NTCAGSTWTSGWGWT			
	AD10	TTGIAGNACIANAGG			
	AD20	TCTTICGNACITNGGA			
	W4	AGWGNAGWANCANAGA			
qualitative PCR assay	5PA-test-1F	ATCTATAATGCCACGATGACG	papaya genome	1300	this work
	3PA-test-2R	AGGAAAAGAGATGGTGTGAAC			
	P-NOS-TF	CGACAATCTGATCATGAGCGGAG	P-NOS/P-35S	1938	this work
	N35S-TR	CCCTTATTAGCGTTTGCCATCTTT			
	Chy-1F	ACGCTAAACTTATCCTATGA	*CHY* gene	281	(*18*)
	Chy-2R	TTGTCATTCACTCTACTTGC			
	HN-F	ATTGACGAGTACAAGGAGACGC	5′ integration flanking sequence	285	this work
	HN-R	CTCCGCTCATGATCAGATTGT			
TaqMan real-timePCRassay	Q-Chy-1F	CCATGCGGATCCTCCCA	*CHY* gene	73	(*18*)
	Q-Chy-2R	CATCGTAGCCATTGTAACACTAGCTAA			
	Q-Chy-P	FAM-TTCCCTTCAT-(BHQ1)-CCATTCCCACTCTTGAGA			
	qHN-F	GACGAGTACAAGGAGACGCC	5′ integration flanking sequence	174	this work
	qHN-R	GTTGTCACTGAAGCGGGAAG			
	qHN-P	FAM-TGGCTGCTATTGGGCGAATCAACTAC-BHQ1			

Africa, and Southeast Asia. However, one important problem for papaya planting is the papaya ringspot virus (PRSV), which causes disastrous damage to papaya harvests (*14*). Thus, modern agricultural biotechnology is widely applied in the development of GM papayas with enhanced virus resistance. The GM PRSV-resistant papaya events, SunUp and Rainbow, were first planted commercially in Hawaii, United States in 1998 (*15*) and have been available in the markets in United States and Canada (*16*). In China, one new virus-resistant papaya, Huanong No. 1, containing the papaya ringspot virus replicase (*NIb*) gene was developed by South China Agriculture University (SCAU) and has been approved for planting and commercialization in Guangdong province, China since 2006 (*17*). The exogenous insert DNA of Huanong No. 1 papaya includes two synthetic gene expression cassettes. One is the *NIb* gene cassette (GenBank No. FJ490192), and the *NIb* gene is regulated by *cauliflower mosaic virus* 35S promoter (P-35S) and *Agrobacterium tumifaciens* nopaline synthase terminator (T-NOS). Another is the *neomycin phosphotransferase II* (*NptII*) gene cassette with the *NptII* gene regulated by *Agrobacterium tumifaciens* nopaline synthase promoter (P-NOS) and T-NOS. However, the information on the molecular characterization of Huanong No. 1 papaya is not comprehensive, and no available method could be used for GM Huanong No. 1 papaya detection.

In this study, we characterized the organization of the insert DNA fragment and its flanking regions in GM Huanong No. 1 papaya. Furthermore, the qualitative and real-time quantitative PCR methods employing the *chymopapain* (*CHY*) gene as the endogenous reference gene were developed based on the revealed junction sequence between the papaya genome and the 5′ exogenous insert DNA.

MATERIALS AND METHODS

Plant Materials. GM Huanong No. 1 papaya and its conventional control were kindly supplied by Professor Huaping Li of South China Agriculture University (SCAU), China. Seven nontransgenic papaya varieties (Meizhonghong, Shuizhonghong, Taiwan No.5, Sinta, RedLady, Wilder and Blue Solo) were purchased from Taiwan and Shanghai, China. The seeds of MON863 maize, Roundup Ready Soybean, and MON1445 cotton were developed and kindly supplied by Monsanto Company. The seeds of GM maize Bt11 were developed and kindly supplied by Syngenta Seeds, Inc. The genomic DNA of GM canola Oxy-235 was supplied by Bayer CropScience Company. The seeds of GM tomato Huafan No. 1 were supplied by Huazhong Agriculture University, China.

DNA Extraction and Purification. The plant genomic DNA was 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 NanoDrop 1000 UV/vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE., USA). Also, the quality of DNA was evaluated by 1% (w/v) agarose gel electrophoresis in 0.5×TBE with GelRed staining.

Oligonucleotide Primers and Probes. Oligonucleotide primers and probes used in this study were designed using the Primer Express software

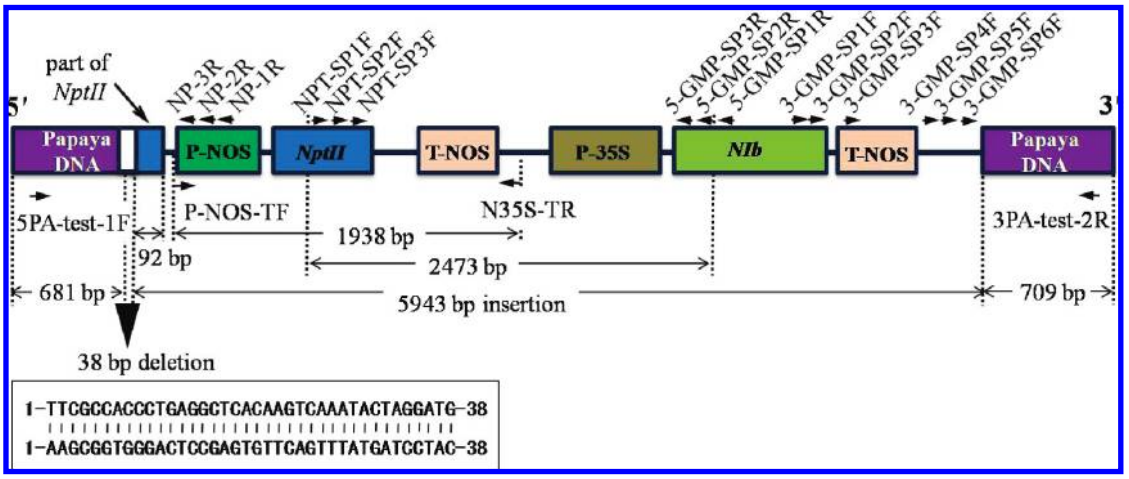

Figure 1. Schematic charaterization of the exogenous insert in GM Huanong No. 1 papaya. GM Huanong No. 1 papaya has a 5943 bp insertion during the transcription process that resulted in a 38 bp deletion. The positions of conventional PCR and TAIL-PCR primers are depicted with arrows.

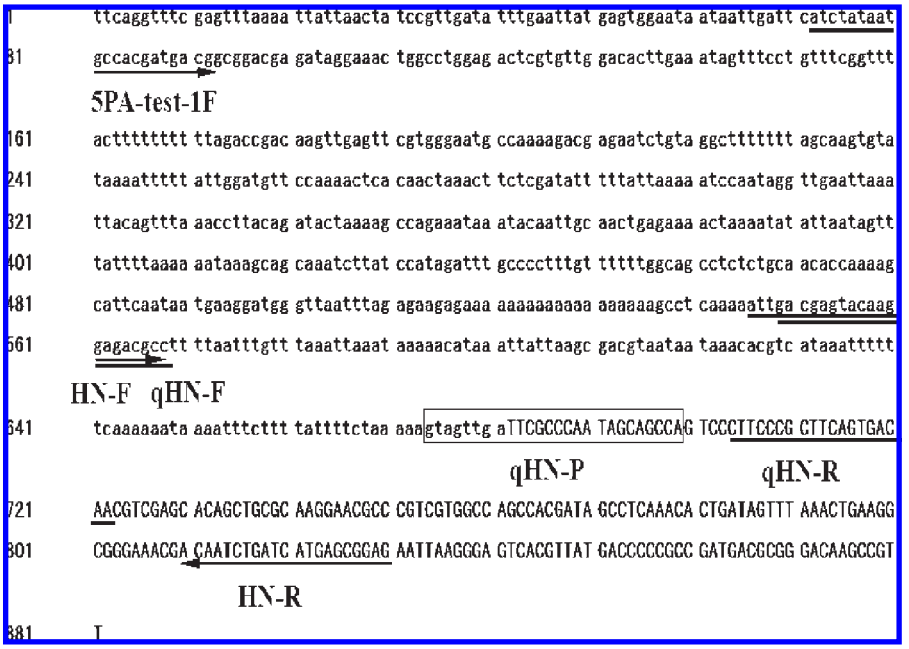

Figure 2. Representation of revealed 5′ integration flanking sequence between the exogenous insert and the host papaya genome of Huanong No. 1 papaya. Lowercase letters represent the flanking papaya genomic sequence, and capital letters show the sequence of exogenous insert DNA. An arrow indicates the primer used for qualitative PCR amplification. Primers and probe used for quantitative PCR amplification are underlined and boxed, respectively.

version 3.0 (Applied Biosystems, Foster City, CA) and listed in **Table 1**. The primers Chy-1F/2R, Q-Chy-1F/2R, and probe Q-Chy-P of papaya endogenous reference gene, *CHY*, were used in this study (*18*). Seven arbitrary degenerate primers (AD-1, AD-2A, AD-3, SAD11, AD10, AD20, and W4) were used for analyzing the exogenous insert and its flanking sequence by means of thermal asymmetric interlaced PCR (TAIL-PCR) (*19*). Five sets of target-specific primers for TAIL-PCR were designed on the basis of the sequence presented in the exogenous insert of Huanong No. 1 papaya, and the detailed locations were shown in **Figure 1**. The event-specific primers HN-F/R, qHN-F/-R, and TaqMan probe HN-P were designed on the basis of the revealed 5′ integration flanking sequence (**Figure 2**). In the TaqMan probe, the fluorescent reporter dye 6-carboxyfluorescein (FAM) was labeled on the 5′ end, and the fluorescent quencher dye Black Hole Quencher 1 (BHQ1) was labeled on the 3′ end. All of the primers and probes were synthesized by Invitrogen Co., Ltd. (Shanghai, China).

Characterization of the Huanong No. 1 Papaya Exogenous Insert and Its Flanking DNA. The inserted DNA and its integrated site of Huanong No. 1 papaya were analyzed by means of conventional PCR and TAIL-PCR methods (*19*, *20*). The TAIL-PCR included three sequential PCR reactions. Primary TAIL-PCR amplification was performed in a total PCR volume of 50 μL per reaction containing 1× LA PCR Buffer II (Mg^{2+} free, TaKaRa), 2.5 mM $MgCl_2$, 400 μM dNTP, 0.4 μM target-specific primer 1, 4 μM arbitrary degenerate primer, 2.5 units of *TaKaRa LA Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd.), and 100 ng of Huanong No. 1 papaya genomic DNA. Secondary TAIL-PCR amplification was carried out in a total PCR volume of 50 μL per reaction containing 1× LA PCR Buffer II (Mg^{2+} free), 2.5 mM $MgCl_2$, 400 μM dNTP, 0.4 μM target-specific primer 2, 4 μM arbitrary degenerate primer, 2.5 units of *TaKaRa LA Taq* DNA polymerase, and 1 μL of primary PCR products. Tertiary TAIL-PCR amplification was carried out in a total PCR volume of 50 μL per reaction containing 1× LA PCR Buffer II (Mg^{2+} free), 2.5 mM $MgCl_2$, 400 μM dNTP, 0.4 μM target-specific primer 3, 4 μM arbitrary degenerate primer, 2.5 units of *TaKaRa LA Taq* DNA polymerase, and 1 μL of secondary PCR products.

All TAIL-PCR reactions were carried out in a PTC-100 thermocycler (MJ Research, Waltham, MA) using the program described in **Table 2**. Parallel reactions with nontransgenic papaya genomic DNA as template

Table 2. Description of the PCR Programs Used in This Study

TAIL-PCR						Qualitative PCR		Quantitative PCR	
1 st-PCR		2 nd-PCR		3 rd-PCR					
94 °C, 1 min; 98 °C, 1 min		94 °C, 30 s; 60 °C, 1 min; 72°C, 2 min	15 cycles	94 °C, 30 s; 65 °C, 1 min; 72 °C, 2 min	15 cycles	94°C, 5 min		94 °C, 10 min	
94 °C, 30 s; 60 °C, 1 min; 72°C, 2 min	5 cycles					94 °C, 30 s;	35 cycles	94 °C, 15 s	45 cycles
94 °C, 30 s; 25 °C, 3 min; 0.4 °C/s to 72 °C; 72°C, 2min	1 cycles	94 °C, 30 s; 60 °C, 1 min; 72 °C, 2 min		94 °C, 30 s; 65 °C, 1 min; 72 °C, 2 min		62 °C, 30 s for event-specific PCR; 58 °C, 30 s for other PCR			
94 °C, 30 s; 60 °C, 1 min; 72°C, 2 min	15 cycles							60 °C, 60 s	
94 °C, 30 s; 60°C, 1 min; 72 °C, 2 min		94 °C, 30 s; 44 °C, 1 min; 72 °C, 2 min		94 °C, 30 s; 44 °C, 1 min; 72 °C, 2 min		72 °C, 30 s			
94 °C, 30 s; 44 °C, 1 min; 72 °C, 2 min									
72 °C, 10 min		72 °C, 10 min		72 °C, 10 min		72 °C, 7 min			

were performed to identify Huanong No. 1 specific PCR products. In all TAIL-PCR amplifications, the secondary and tertiary PCR products were analyzed by 1.5% agarose gel electrophoresis with GelRed staining. The amplified fragments with similar size in these two PCR amplifications were reclaimed and purified with the AxyPrep DNA Gel Extraction Kit (Axygen Scientific, Inc., California, USA). The eluted DNA fragment was cloned into the pMD18-T vector (TaKaRa Biotechnology Co., Ltd.), and three individual clones were selected and subsequently analyzed using the ABI PRISM 3730 Genetic Analyzer by Shanghai Invitrogen Co., Ltd. (Shanghai, China). Then, sequence analysis was determined by the BLAST program in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) or perforemd employing the software Vector NTI Advance 10.

Qualitative PCR Assay. The qualitative PCR assay was run on PTC-100 thermocycler (MJ Research, Waltham, MA) with the final volume of 25 μL. Each reaction contained the following reagents: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, 400 nM each primer, 1.5 units of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd.), and 5 μL of genomic DNA. The PCR amplification program is listed in **Table 2**. The PCR products were analyzed by 2% (w/v) agarose electrophoresis. Each reaction of one test was repeated three times and each time with triple replicates.

Real-Time Quantitative PCR Assay. Real-time quantitative PCR was run on a fluorometric thermal cycler Rotor-Gene 3000 (Corbett Research, Australia) with the volume of 25 μL per reaction. The PCR reaction contained the following reagents: in addition to 1× quantitative PCR buffer (10 mM Tris-HCl (pH 8.3) and 50 mM KCl), 200 μM dNTP, 1.5 units of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd.), and 5 μL of genomic DNA sample; for the *CHY* gene assay, 6 mM $MgCl_2$, 500 nM Q-Chy-1F/2R primers, and 100 nM Q-Chy-P TaqMan probe; for the event-specific assay, 5 mM $MgCl_2$, 400 nM qHN-F/R primers, and 100 nM qHN-P TaqMan probe. Real-time PCR was performed using the program described in **Table 2**. The fluorescent signal was collected during every PCR cycle at the extension step. Data were analyzed with Rotor gene 3000 software version 6.0 (Corbett Research, Australia). The real-time PCR of each test was performed three times and each time with triple replicates.

Evaluation of Event-Specific Real-Time PCR Assay. To evaluate the event-specific real-time PCR assay of Huanong No. 1 papaya, a series of Huanong No. 1 papaya genomic DNA dilutions, corresponding to the concentrations of 50000, 5000, 500, 50, 5, and 2.5 copies of haploid genome/μL considering 0.4 pg per haploid genome in the case of papaya (*21*), were used for testing the repeatability, assessing the limits of detection and quantification (LOD and LOQ), and constructing the calibration curve. In each reaction, 5 μL of diluted DNA sample was added, and all reactions were performed three times and each time with triple replicates.

RESULTS AND DISCUSSION

Characterization of Exogenous Insert in GM Huanong No. 1 Papaya. *Exogenous Gene Expression Cassettes*. To reveal the detailed information of the organization of the transgenic cassette in GM Huanong No. 1 papaya, two sets of target-specific TAIL-PCR primers (5-GMP-SP1R/2R/3R and NPT-SP1F/2F/3F) were first designed and individually used in combination with seven arbitrary degenerate primers (AD-1, AD-2A, AD-3, SAD11, AD10, AD20, and W4) to clone the exogenous insert DNA sequence of Huanong No. 1 papaya. The nested target-specific primer set of 5-GMP-SP1R/2R/3R was designed on the 5′ end of the *NIb* gene, and the primers NPT-SP1F/2F/3F were targeted to the 3′ end of the *NptII* gene (**Figure 1**).

In the TAIL-PCR analysis using the Huanong No. 1 DNA as template, one DNA fragment 1553 bp in length was obtained employing the primer pair 5-GMP-SP3R and AD2A, and another 1346 bp DNA fragment was obtained using the primer pair NPT-SP3F and AD2A. Sequencing analysis indicated that both of these two amplified DNA fragments contained an overlapping region with 576 bp in length. Thus, the amplified DNA fragment between the NPT-SP3F and 5-GMP-SP3R was expected to be 2323 bp in length. Also, we proposed that the *NptII* gene cassette was located upstream of the *NIb* gene cassette. Alternatively, one unique DNA fragment 2473 bp in length was also amplified with primer pair NPT-SP1F and 5-GMP-SP2R in conventional PCR using Huanong No. 1 DNA as a template, and the sequencing result further confirmed this transgenic cassette, as shown in **Figure 1**.

To isolate the whole sequence of the exogenous insert DNA of Huanong No. 1 papaya, another primer pair (P-NOS-TF and N35S-TR) was designed. The primer P-NOS-TF was located at the 5′ end of the *NptII* cassette, and primer N35S-TR was located at the junction between the NOS terminator of the *NptII* cassette and the CaMV35s promoter of the *NIb* cassette. One expected 1938 bp DNA fragment was amplified from Huanong No. 1 papaya genomic DNA, and the sequencing and homology analysis indicated that the 1938 bp DNA fragment contained the intact *NptII* gene cassette. Likewise, we haved revealed the *NIb* gene cassette sequence and submitted it to GenBank database with the accession number of FJ490192. Thereafter, we deduced the structure of the integrated expression casstte and its DNA sequence based on the following three DNA sequences, i.e., 1938 bp from P-NOS-TF and N35S-TR, 2323 bp between the

Table 3. Description of the Exogenous Insert of Huanong No. 1 and Its Flanking Regions

section	position no. of the nucleotide on the 7333 bp sequence	length (bp)	description of genetic elements	homology to Genbank no. (position of corresponding bp)
A	1−681	681	papaya genomic sequence	
B	682−773	92	sequence of the truncated *NptII* gene	AF485783 (3125−3034)
C	774−816	43	transgenic vector sequence	AF485783 (2476−2518)
D	817−1123	307	*Agrobacterium tumifaciens* nopaline synthase (NOS) promoter	AF485783 (2519−2825)
E	1124−1135	12	transgenic vector sequence	AF485783 (2826−2837)
F	1136−1930	795	*neomycin phosphotransferase II* (*NptII*) gene	AF485783 (2838−3632)
G	1931−2319	389	transgenic vector sequence	AF485783 (3633−4021)
H	2320−2575	256	*Agrobacterium tumifaciens* nopaline synthase (NOS) terminator	AF485783 (4022−4277)
I	2576−3271	696	transgenic vector sequence	AF485783 (4278−4973)
J	3272−4006	835	*cauliflower mosaic virus* 35S promoter	AF485783 (4974−5808)
K	4007−4024	18	transgenic vector sequence	AF485783 (5809−5826)
L	4025−5726	1602	papaya ringspot virus replicase (*Nlb*) gene	FJ490192 (438−2039)
M	5727−5748	22	transgenic vector sequence	FJ490192 (2040−2061)
N	5749−6001	253	*Agrobacterium tumifaciens* nopaline synthase (NOS) terminator	AF485783 (7727−7979)
O	6002−6624	623	transgenic vector sequence	AF485783 (7980−8602)
P	6625−7333	709	papaya genomic sequence	

NPT-SP3F and 5-GMP-SP3R, 2317 bp of the *NIb* cassette (GenBank no. FJ490192). The combined DNA sequence information in Huanong No. 1 is listed in **Table 3**.

Characterization of the Exogenous Integration. To reveal the junction region of the exogenous insert and the host genomic DNA of Huanong No. 1 papaya, we designed nested target-specific primers, 3-GMP-SP1F/2F/3F and NP-1R/2R/3R, for TAIL-PCR analysis. We succeeded in isolating two specific DNA fragments from Huanong No. 1 genomic DNA in TAIL-PCR: an 881 bp DNA fragment from the primers of NP-3R and SAD11 and a 687 bp DNA fragment from primers of 3-GMP-SP3F and AD1. Sequencing and BLASTN analysis showed that this 881 bp DNA fragment included a 65 bp sequence of the NOS promoter, a 43 bp sequence of the transgenic vector, a 92 bp sequence of the truncated *NptII* gene, and 681 bps of an unknown DNA sequence. The 687 bp DNA fragment contained a 218 bp sequence of the NOS terminator and a 469 bp sequence of the transgenic vector, which meant that this cloned 687 bp fragment was from the exogenous insert and contained no papaya genomic DNA. Therefore, we redesigned one nested target-specific primer set (3-GMP-SP4F/5F/6F) based on the sequence of the obtained 687 bp fragment to isolate the 3′ integration flanking sequence. One unique DNA fragment of 945 bp was obtained with the primer pair of 3-GMP-SP6F and SAD11, which consisted of a 236 bp sequence of transgenic vector and a 709 bp unknown DNA sequence.

Since less information is available for the papaya genomic DNA sequence in the Genbank database, we could not make sure that these two unknown DNA sequences (681 bp and 709 bp) were from the host papaya genome. In order to confirm whether these two unknown DNA sequences originated from the papaya genome, we designed two specific primers (5PA-test-1F/3PA-test-2R) based on these two unknown DNA sequences, and performed the conventional PCR using the Huanong No. 1 papaya DNA and its conventional control as templates, respectively. As expected, one unique DNA fragment of 1300 bp was obtained from nontransgenic papaya. While no amplicon was observed in Huanong No. 1 papaya, the possible explanation was that the PCR setup makes it difficult to amplify the putative exogenous insert DNA fragment about 7200 bp in length. Sequencing and alignment analysis revealed that this 1300 bp DNA sequence consisted of three parts: a 610 bp sequence homologous to the TAIL-PCR product amplified using the primers of NP-3R and SAD11, a 652 bp sequence homologous to the TAIL-PCR product amplified using the primers of 3-GMP-SP6F and SAD11, and a 38 bp sequence without sequence homology with the exogenous insert of Huanong No. 1, suggesting that a DNA deletion may have occurred during transformation. Also, we proposed that those two unknown DNA sequences are the host papaya genome DNA.

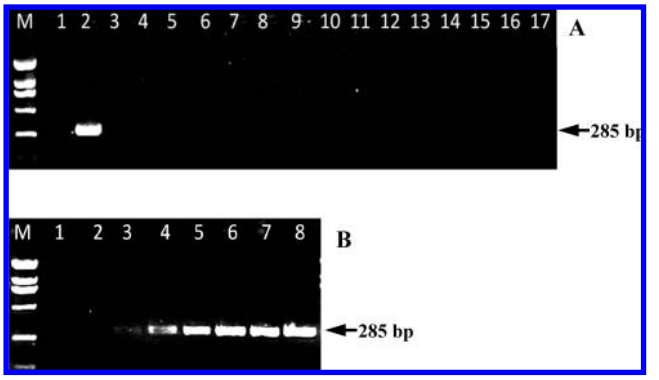

Figure 3. Agarose gel electrophoresis of qualitative PCR products amplified with the HN-F/R primer pair. (**A**) Specificity test of event-specific assay. Lane 1, NTC; lanes 2−17, Huanong No. 1 papaya, Meizhonghong, Shuizhonghong, Taiwan No. 5, Sinta, RedLady, Wilder, Blue Solo, conventional control of Huanong No. 1, Oxy-235 canola, Huafan No. 1 tomato, MON810 maize, MON863 maize, BT11 maize, MON1445 cotton, and RRS, respectively. (**B**) Sensitivity test of event-specific qualitative PCR assay. PCR products were amplified from mixed Huanong No. 1 papaya DNA with different GM content levels. Lane 1, NTC; lanes 2−8, mixed GM papaya samples with GM contents of 0%, 0.01%, 0.05%, 0.1%, 1.0%, 3.0%, 5.0%, respectively; lane M, DL2000 DNA marker.

Accordingly, we confirmed that one intact copy of the initial construction with the *NptII* and the *NIb* gene cassette was integrated in single insertion locus in the papaya genome. Moreover, one unintended partial fragment of the *NptII* gene was observed at the 5′ end close to the site of integration, which probably results in the deletion of the 38 bp host papaya sequence during the integration. It can be also concluded that no major rearrangements at the 3′ end of exogenous insert occur and that papaya DNA is present immediately flanking the 3′ end of the transgenic vector (**Figure 1**).

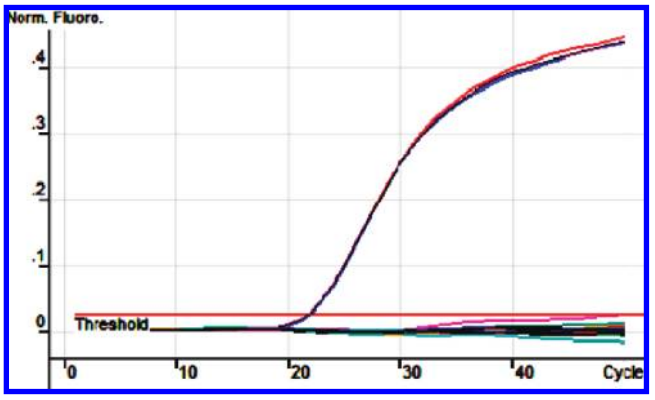

Figure 4. Specificity test of the event-specific real-time PCR assay. Amplification plots generated from 16 different kinds of plants described in **Figure 3** using the qHN-F/R primer pair and the qHN-P TaqMan probe.

Table 4. Limits of Detection and Quantification (LOD and LOQ)

copy number	signal rate (positive signals)	mean C_T value	SD	RSD (%)
250000	9/9	19.79	0.11	0.56
25000	9/9	23.30	0.12	0.53
2500	9/9	26.70	0.16	0.61
250	9/9	30.43	0.20	0.66
25	9/9	33.55	0.21	0.66
12.5	4/9			

Huanong No. 1 Event-Specific Qualitative PCR Assay. On the basis of the 5′ integration flanking sequence, event-specific primers (HN-F/R) were designed to establish the event-specific qualitative PCR assay for Huanong No. 1 papaya. The primer pair HN-F/R located at the papaya genome and insert DNA left border region, respectively, yielding a 285 bp amplicon. The papaya *CHY* gene was selected as the endogenous reference gene, and the primer pair CHY-1F/2R was employed for papaya identification. As expected, in the established event-specific PCR assay, only one expected 285 bp DNA fragment was obtained from Huanong No. 1 papaya, and no amplification product was observed in other GM crop events (MON863, MON810, BT11, Oxy-235, RRS, Huafan No. 1, and MON1445), including nontransgenic papaya and no template control (NTC) (**Figure 3A**). The above results indicated that the qualitative event-specific PCR assay is highly specific for Huanong No. 1 papaya.

To test the LOD of the established Huanong No. 1 PCR assay, the mixed DNA samples were prepared from the Huanong No. 1 papaya event and non-GM papaya at various levels, such as 0%, 0.01%, 0.05%, 0.1%, 1.0%, 3.0%, and 5.0%. In the LOD test, 100 ng of total papaya genomic DNA was used as template in each PCR reaction. The amplified results showed that the expected DNA fragment (285 bp) was observed from all levels except for the samples with 0.0% GM content, indicating that the LOD of the qualitative PCR assay was 0.01% (**Figure 3B**), and the established qualitative PCR detection assay was highly sensitive for practical GM papaya sample detection.

Huanong No. 1 Event-Specific Real-Time PCR Assay. The primers (qHN-F/R) and TaqMan probe (qHN-P) were designed on the basis of the 5′ integration flanking sequence to establish the event-specific real-time PCR assay for Huanong No. 1 papaya. The primers qHN-F and qHN-R located in the papaya genome and insert DNA left border region, respectively. TaqMan probe qHN-R spanned the junction of papaya genomic DNA and left border region of insert DNA, thus providing extra specificity to the detected signal. For quantifying the amount of

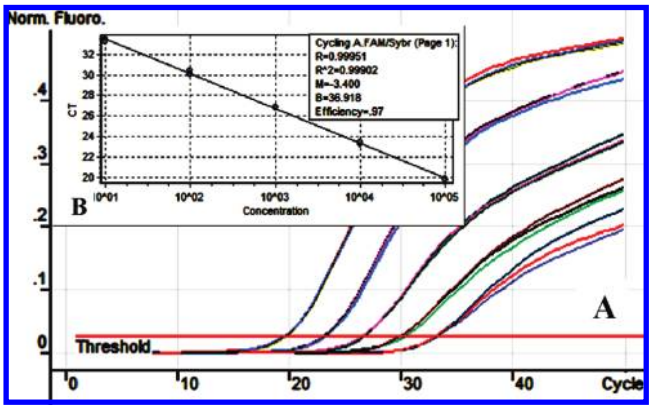

Figure 5. Amplification plots and calibration curve for Huanong No. 1 event-specific real-time PCR assay. (**A**) amplification plots generated by serial DNA dilutions corresponding to 250000, 25000, 2500, 250, and 25 copies of Huanong No. 1 haploid genome, respectively. (**B**) Calibration curve generated from the amplification data given in **A**.

Table 5. Repeatability Test of the Developed Huanong No. 1 Event-Specific Real-Time PCR Assay

	calculated copy no.				repeatability	
copy number	mean 1	mean 2	mean 3	mean copy no.	SD_r	RSD_r (%)
250000	239022.5	268674.2	257604.6	255100.4	14983.63	5.88
25000	25280.0	24386.1	24464.8	24710.3	494.95	2.00
2500	2840.0	2334.3	2570.1	2581.5	253.06	9.80
250	225.0	231.6	217.8	224.8	6.89	3.07
25	25.0	27.6	26.7	26.4	1.30	4.94

the total papaya DNA, the *CHY* real-time PCR assay employing papaya specific primers (Q-Chy-1F/2R) and probe (Q-Chy-P) were used according to the previous report (*18*). The specificity of designed event-specific primers and probe was validated by means of real-time PCR, and several different GM plants (Huanong No. 1 papaya, MON863 maize, MON810 maize, BT11 maize, Oxy-235 canola, RRS, Huafan No. 1 tomato, and MON1445 cotton) and nontransgenic papaya cultivars (Meizhonghong, Shuizhonghong, Taiwan No.5, Sinta, RedLady, Wilder, Blue Solo, and conventional control of Huanong No. 1) were tested. As shown in **Figure 4**, no fluorescent signals were observed in any of these tested species except for GM Huanong No. 1 in real-time PCR, indicating the good specificity of the event-specific real-time PCR assay.

LOD and LOQ. Generally, LOD is the lowest amount or concentration of the analytes that can be detected reliably. LOQ is the lowest amount or concentration of the analytes that can be reliably quantified with an acceptable level of precision and accuracy (*22*, *23*). The LOD and LOQ of the established Huanong No. 1 real-time PCR assay were estimated using a series of Huanong No. 1 genomic DNA dilutions with the concentrations of 20, 2, 0.2, 0.02, 0.002, and 0.001 ng/μL (equivalent to 50000, 5000, 500, 50, 5, and 2.5 copies of haploid genome/μL) as templates. Each reaction was repeated three times and each time with three replicates. As shown in **Table 4**, the results indicated that the LOD was as low as 5 pg, corresponding to about 12.5 copies of haploid papaya genome. To obtain reliable quantification results under ideal conditions, at least 10 pg initial template amounts were required, and we concluded that the LOQ was 10 pg, corresponding to 25 copies of the haploid papaya genome.

Construction of the Calibration Curve. Five 10-fold diluted Huanong No. 1 genomic DNA solutions with the concentrations of 20, 2, 0.2, 0.02, and 0.002 ng/μL (equivalent to 50000, 5000,

Table 6. Accuracy and Precision Statistics for Real-Time Methods

	construct amounts × 100/endogenous amounts			accuracy		precision	
true value (%)	mean 1	mean 2	mean 3	mean GMO (%)	bias (%)	SD	RSD (%)
5.0	5.05	5.26	5.35	5.22	4.41	0.15	2.89
3.0	3.30	2.86	2.81	2.99	0.44	0.27	9.04
1.0	1.15	0.90	0.86	0.97	3.05	0.16	16.10

500, 50, and 5 copies of haploid genome/μL) were employed to construct the calibration curve for the Huanong No. 1 real-time PCR assay. The dynamic range of the constructed calibration curves was 0.01−100 ng in total PCR volume of 25 μL per reaction. The PCR efficiency was 0.97, and the squared regression coefficient (R^2) of the calibration curve was 0.999, as shown in **Figure 5**. The high PCR reaction efficiency and the good linear relationship between the logarithm of initial DNA quantities and the threshold cycle (C_T) in the calibration curve indicated that the real-time PCR assay is well suited for quantification.

Repeatability of the Event-Specific Real-Time PCR Assay. Repeatability of the event-specific real-time PCR assay was determined and calculated using a series of papaya genomic DNA dilutions (50000, 5000, 500, 50, and 5 copies of haploid genome/μL). The standard deviation (SD_r) and relative standard deviation (RSD_r) of repeatability were calculated from the mean copy numbers of three times and each time with three replicates. As a result, the RSD_r values ranged from 2.00% to 9.80% (**Table 5**). These data indicated that the established Huanong No. 1 real-time PCR assay is stable and reliable in Huanong No. 1 quantification.

Quantification of Practical GM Papaya Samples. Three mixed Huanong No. 1 papaya DNA samples, that is S1, S2, and S3 with 5%, 3%, and 1%, were artificially prepared by mixing the Huanong No. 1 papaya with non-GM papaya genomic DNA on genome/genome basis. These samples were then analyzed using the Huanong No. 1 event-specific and the *CHY* real-time PCR assays, and the results are shown in **Table 6**. The calculated content of GM papaya has to be normalized with the amount of amplifiable papaya DNA present in the mixed sample. The mean quantitative results of these three DNA samples (S1, S2, and S3) were 5.22%, 2.99%, and 0.97%, respectively. The quantified biases from true values were 4.41%, 0.44%, and 3.05%, respectively. These results showed that the bias of practical samples are lower than the acceptance criterion (< 25%) of the GMO detection method (*24*), indicating that the developed event-specific real-time PCR assay is creditable and accurate for the quantification of GM Huanong No. 1 papaya.

CONCLUSIONS

In this work, we showed the sequences of Huanong No. 1 papya exogenous insert DNA and its flanking fragments. In addition to the expected integration of the insert DNA in this event, a deletion of 38 bp of host genomic DNA adjacent to the insertion site and the unintended insertion of 92 bp from the *NptII* gene at the 5′ end of the insertion site occurred within Huanong No. 1 papaya. Furthermore, on the basis of the revealed 5′ integration flanking sequence, we established the event-specific PCR assays for the identification and quantification of GM papaya Huanong No. 1 and its derivates with high efficiency, acceptable limits of detection, and repeatability. These results demonstrated that our study could be useful for GM Huanong No. 1 papaya detection and labeling.

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