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Identification and Quantification of Three Genetically Modified Insect Resistant Cotton Lines Using Conventional and TaqMan Real-Time Polymerase Chain Reaction Methods

LITAO YANG,[†,‡] AIHU PAN,[§] KEWEI ZHANG,[†] JINCHAO GUO,[†] CHANGSONG YIN,[†] JIANXIU CHEN,[‡] CHENG HUANG,[‡] AND DABING ZHANG*,[†,§]

School of Life Science and Biotechnology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, People's Republic of China, Department of Biological Science and Technology, Nanjing University, 22 Hankou Road, Nanjing 210093, People's Republic of China, and Key Laboratory of Agricultural Genetics and Breeding, Agro-biotech Research Center, Shanghai Academy of Agricultural Sciences, 2901 Beidi Road, Shanghai 201106, People's Republic of China

As the genetically modified organisms (GMOs) labeling policies are issued in many countries, qualitative and quantitative polymerase chain reaction (PCR) techniques are increasingly used for the detection of genetically modified (GM) crops in foods. Qualitative PCR and TaqMan real-time quantitative PCR methods to detect and identify three varieties of insect resistant cotton, i.e., Mon531 cotton (Monsanto Co.) and GK19 and SGK321 cottons (Chinese Academy of Agricultural Sciences), which were approved for commercialization in China, were developed in this paper. Primer pairs specific to inserted DNAs, such as *Cowpea trypsin inhibitor* (*CpTI*) gene of SGK321 cotton and the specific junction DNA sequences containing partial *Cry1A(c)* gene and *NOS* terminator of Mon531, GK19, and SGK321 cotton varieties were designed to conduct the identified PCR assays. In conventional specific identified PCR assays, the limit of detection (LOD) was 0.05% for Mon531, GK19, or SGK321 in 100 ng of cotton genomic DNA for one reaction. Also, the multiplex PCR method for screening the three GM cottons was also established, which could save time and cost in practical detection. Furthermore, a real-time quantitative PCR assay based on TaqMan chemistry for detection of insect resistant gene, *Cry1A(c)*, was developed. This assay also featured the use of a standard plasmid as a reference molecule, which contained both a specific region of the transgene *Cry1A(c)* and an endogenous stearoyl-acyl carrier protein desaturase (*Sad1*) gene of the cotton. In quantitative PCR assay, the quantification range was from 0.01 to 100% in 100 ng of the genome DNA template, and in the detection of 1.0, 3.0, and 5.0% levels of three insect resistant cotton lines, respectively, all of the relative standard deviations (RSDs) were less than 8.2% except for the GM cotton samples with 1.0% Mon531 or GK19, which meant that our real-time PCR assays involving the use of reference molecule were reliable and practical for GM insect resistant cottons quantification. All of these results indicated that our established conventional and TaqMan real-time PCR assays were applicable to detect the three insect resistant cottons qualitatively and quantitatively.

KEYWORDS: Genetically modified organisms (GMOs); insect resistant cotton; multiplex PCR; real-time PCR; *CpTI* gene; *Cry1A(c)* gene

INTRODUCTION

Since the first genetically modified (GM) tomato "FLAVR SVAR" was approved for commercialization in 1994, transgenic techniques have been well-developed (*1*). Up to now, hundreds of GM plants have been authorized for commercialization in many countries. In the year 2003, the production of transgenic crops reached 67.7 million ha; the main transgenic crops are soybean (41.4 million ha), maize (15.5 million ha), and cotton (7.2 million ha), and the principal traits are herbicide tolerant and insect resistant (*2*). Rice and wheat crops are currently under development (*3*).

Cotton (*Gossypium hirsutum*), an important economic crop for textile industry, also is the food and feed source in many areas of the world, especially for the feed source. However, damage caused by insect pests, especially by the bollworm (*Helicoverpa armigera* Hubner), is a major factor in the loss of the world's cotton crops, despite the use of protective

* To whom correspondence should be addressed. Tel and Fax: +86 21 34201073. E-mail: zhangdb@sjtu.edu.cn.
[†] Shanghai Jiao Tong University.
[‡] Nanjing University.
[§] Shanghai Academy of Agricultural Sciences.

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measures such as chemical pesticides. In view of this, insect resistance has been genetically engineered into crops such as corn to control insect damage and reduce the need for traditional chemical pesticides (*4*). Since Plant Genetics System in Belgium reported the development of transgenic *Bacillus thuringiensis* (Bt) tobacco plants for the first time, some kinds of Bt gene, i.e., *Cry1A(b)*, *Cry1A(c)*, and *Cry3A*, have been transferred to many plants such as cotton, tomato, and tobacco by the scientists of Monsanto, Agricetus, and the Biotechnological Center of Chinese Academy of Agricultural Sciences (CAAS) (*5*). Up to now, there are three insect resistant cotton varieties, i.e., Mon531, GK19, and SGK321, which have been approved for commercial planting in China. One copy of the integrated full synthesized *Cry1A(c)* gene was inserted into the Coker 312 cotton genome, and then the Mon531 variety was developed by Monsanto Co. (*6*). GK19 was developed by introducing an artificially synthesized *Cry1A(b+c)* gene expression cassette, and SGK321 was developed with the cowpea trypsin inhibitor (*CpTI*) gene and the artificially synthesized *Cry1A(b+c)* expression cassettes (*7*−*9*).

Although the GM plants have been authorized for commercialization in many countries, there are still several controversial issues existing, such as biosafety, environment risk, and ethical concerns. Moreover, present science cannot dispel the doubts completely. Therefore, detection and labeling of GM foods are required in more than 30 countries or regions. Moreover, the difference also exists in countries about the detailed requirements of labeling policies regarding GM foods. For instance, the labeling threshold is defined as 0.9% in EU (*10*), 3% in Korea (*11*), and 5% in Japan (*12*). The labeling of GM foods is not compulsory in the United States and Canada (*13*). In China, 17 kinds foods derived from five different kinds of plants should be labeled, such as tomato seeds, ketchup, soybean flour, soybean oil, maize oil, rapeseed seeds, and cotton seeds (*14*).

In conjunction with these labeling policies, qualitative and quantitative polymerase chain reaction (PCR) methods are the most widely used for GMOs identification and quantification. The qualitative and quantitative PCR detection systems for most of the authorized events for commercialization have been established and used in practical GMOs analysis, such as GM soybean (GTS 40-3-2) (*15*−*18*), GM maize (T25, Event 176, Mon810, Mon863, Bt11, GA21, and NK603) (*16*−*23*), GM canola (GT73, HCN92/28, MS8/RF3, and Oxy 235) (*24*, *25*), GM papaya (*26*), GM potato (NewLeaf, NewLeaf Y, and NewLeaf Plus) (*27*, *28*), and GM tomato (FLAVR SVAR and Huafan No. 1) (*29*, *30*). Although cotton is not considered a significant food crop for human and only the cotton oil is used as the feed or food materials, moreover, it is difficult to detect transgenes in the processed oil using PCR methods because of the degraded DNA fragments in the processed samples, such as cotton oil. However, to suffice to the requirement of GM cotton labeling regulation in China, the qualitative and quantitative PCR systems for detection of the three commercial planted insect resistant cotton lines in China should be established.

MATERIALS AND METHODS

Materials. Genuine seeds of GK19 and SGK321 were developed and kindly provided by Prof. Sandui Guo (CAAS). Genuine seeds of Mon531 were developed and kindly provided by Monsanto Co. (St. Louis, MO). Nontransgenic cotton seeds of variety 9404 and other crops (soybean and maize) were purchased from local market in Shanghai.

DNA Extraction and Purification. Plant genomic DNA was extracted and purified using the Plant DNA Mini-Prep Kit (Shanghai Ruifeng Agro-tech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. One hundred milligrams of grounded plant materials mixed with 500 μL of buffer A was transferred in a 1.5 mL tube and incubated for 10 min at 65 °C. Then, it was centrifuged at 13400*g* for 10 min, and the resulting supernatant was collected, deposited with 500 μL of buffer B, mixed for 30 s, and then centrifuged at 13400*g* for 10 min. The deposit was dissolved in 100 μL of buffer C at 37 °C for 5 min, and then, the solution was transferred in silica-based DNA binding column. The solution was centrifuged at 5900*g* for 1 min, and then, the silica column was washed in turn with wash buffer I and wash buffer II two times, respectively. At the last step, genomic DNA was eluted with water from the centrifugal column. Plasmid DNA was extracted and purified using Plasmid Mini Kit (Watson Biotechnologies, Inc., Shanghai, China) according to the manufacturer's instructions. The quantity of DNA samples was calculated using absorbance measurements at 260 nm wavelength.

Selection of Cotton Endogenous Reference Gene, *Sad1*. To assess the identification and quantification of cotton DNA, one endogenous reference gene was necessary, which should be species specific, low copy number, and exhibit low heterogeneity among cotton cultivars (*31*). We have validated that *Sad1* gene (Genbank no. AJ132636) was suit for using as an endogenous reference gene (*32*). *Sad1* gene has two copies per cotton haploid genome and encodes *stearoyl-acyl carrier protein desaturase*, which introduces a cis double bond between carbons 9 and 10 of C18 fatty acids to produce the monounsaturated oleic acid and regulates the extent of unsaturation of membrane lipids and seed oils in cotton plants (*33*, *34*).

Oligonucleotide Primers and Probes. Sequences of oligonucleotide primers and TaqMan fluorescent probes employed in this study were listed in **Table 1** and designed using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA), and the detailed position of primers and probes is shown in **Figure 1**. The probe of endogenous *Sad1* gene was labeled with the fluorescent reporter dye 5-hexachloro-fluorescein (HEX) on the 5′-end and exogenous *Cry1A(c)* gene with 6-carboxy-fluorescein (FAM) on the 5′-end. The fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) was located on the 3′-end of the probes. The primer pair of M1F/2R was designed to amplify a 577 bp DNA fragment containing both the 3′-end of *Cry1A(c)* gene and the 5′-end of *NOS* terminator based on the reported sequence from U.S. patent (U.S. 2003/0024005A1, Seq no. 15, and U.S. 2004/0045054A1, Seq no. 5 and 6) for the detection of Mon531 (*34*, *35*), and the 3′-end sequence of *Cry1A(c)* gene in Mon531 cotton was different from those of GK19 and SGK321 cotton. The G1F/2R primer pair was employed for detection of the synthesized *Cry1A(b+c)* gene and *NOS* terminator inserted into GK19 and SGK321 cottons (Chinese patents, Pub. no. 1134981, **Figure 2** and Pub. No. 1219586, Seq no. 2) (*7*). The SG1F/2R primer pair was used to amplify the specific *CpTI* gene of SGK321 (Chinese patent, Pub. no. 1219586, Seq no. 4) (*8*). Through aligning the *Cry1A(c)* sequence of Mon531 cotton with the synthesized *Cry1A(b+c)* sequences of GK19 and SGK321 cottons, one DNA fragment with the length of ~1800 bp with 86% identities in the three cotton lines was obtained and used for designing the PCR systems for qualitative and quantitative detection of all of the three insect resistance cotton lines (**Figure 2**), and then, the primer pairs (C1F/2R and C3F/4R) and TaqMan probe Cp of *Cry1A(c)* gene were designed and employed in this paper. The primer pairs (S1F/2R and S3F/4R) and TaqMan probe Sp of *Sad1* gene were used as the endogenous control (*31*). All of the primers and fluorescent probes were synthesized and purified by Shanghai Shenyou Co. Ltd. (Shanghai, China).

Construction of a Standard Plasmid as Reference Molecule. A standard plasmid for reference molecule with an endogenous *Sad1* gene and an exogenous *Cry1A(c)* gene fragment was cloned by overlapping PCR method (*22*), and the primer pairs SM1F/2R (123 bp of *Sad1* gene) and SM3F/4R (133 bp of *Cry1A(c)* gene) employed to clone those two fragments were designed based on the amplified sequences of *Sad1* gene and *Cry1A(c)* gene real-time PCR assays, respectively. The PCR amplicons were obtained using two rounds PCR. In the first round PCR, the amplicons of *Sad1* gene and *Cry1A(c)* gene were amplified with primers SM1F/2R and SM3F/4R (**Table 1**) separately. Then, the amplicons of *Sad1* gene and *Cry1A(c)* gene were purified with Gel Extraction Mini Kit (Watson Biotechnologies, Inc., Shanghai,

Table 1. Primers and Fluorogenic Probes Used for Qualitative and Quantitative PCR System

target	name	sequence (5′- - -3′)	specificity	amplified length (bp)	ref
Mon531	M1F	GATACGGTGAGGGTTGCG	*Cry1A(c)* gene	577	*6*
	M2R	AAGTCATAGTTAAATAGCCCGATA	*NOS* terminator		
GK19 and	G1F	CTTCACTCGGTAACATCGT	*Cry1A(c)* gene	346	*7, 8*
SGK321	G2R	ATGGGTTTTTATGATTAGAGTCC	*NOS* terminator		
SGK321	SG1F	CACTAAATCAATACCTCCTCAA	*CpTI* gene	172	*8*
	SG2R	TTACTCATCATCTTCATCCCT			
Cry1A(c)	C1F	ACTGGCTTGGAGCGTGTCTG	*Cry1A(c)* gene	409	*6–8*
	C2R	GTTGTTGTGGAGCGGCGTTT			
Sad1	S1F	CGAAGTGCTACAAAGACCCC	*Sad1* gene	125	*32*
	S2R	TTTCCCACGAAGCCCAAT			
Cry1A(c)	C3F	TACTTGGTGGAGAACGCATTGAA	*Cry1A(c)* gene	122	*32*
	C4R	GATGTCAACTAGTCCGAGAACGAA			
	Cp	FAM CACCTGGCACGAACTCGCTGAGCATAMRA			
Sad1	S3F	CCAAAGGAGGTGCCTGTTCA	*Sad1* gene	107	*32*
	S4R	TTGAGGTGAGTCAGAATGTTGTTC			
	Sp	HEX TCACCCACTCCATGCCGCCTCACATAMRA			
construction	SM1F	CCAAAGGAGGTGCCTGTTCA	*Sad1* gene	123	this work
of pSadCry	SM2R	AGTATCCTCCTTGAGGTGAGTCAGAATGTTGTTC			
	SM3F	TCAAGGAGGATACTTGGTGGAGAACGCATTGA	*Cry1A(c)* gene	133	this work
	SM4R	GATGTCAACTAGTCCGAGAACGAA			

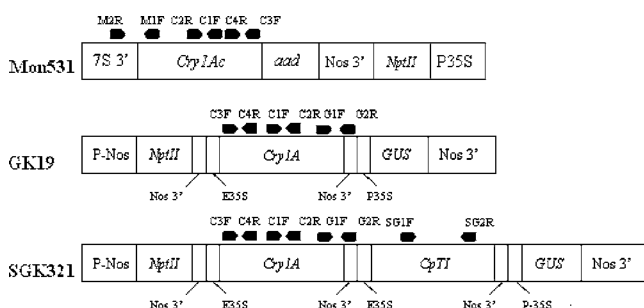

Figure 1. Schematic diagram of PCR strategy for qualitative and quantitative detection of three insect resistant cotton lines. The foreign genes are shown in the squared box. The arrows and arrowheads indicate the location and direction of each primer, respectively.

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                     9        18        27        36
GK19      ATG GAC TGC AGG CCA TAC AAC TGC TTG AGT AAC CCA
SGK321    ATG GAC TGC AGG CCA TAC AAC TGC TTG AGT AAC CCA
Mon531    AAC GAA TGC ATT CCA TAC AAC TGC TTG AGT AAC CCA
                    45        54   C3F  63        72
GK19      GAA GTT GAA G[TA CTT GGT GGA GAA CGC ATT GAA]ACC
SGK321    GAA GTT GAA G[TA CTT GGT GGA GAA CGC ATT GAA]ACC
Mon531    GAA GTT GAA G[TA CTT GGT GGA GAA CGC ATT GAA]ACC
                    81        90        99        108
GK19      GGT TAC ACT CCC ATC GAC ATC TCC TTG TCC TTG ACA
SGK321    GGT TAC ACT CCC ATC GAC ATC TCC TTG TCC TTG ACA
Mon531    GGT TAC ACT CCC ATC GAC ATC TCC TTG TCC TTG ACA
                    117       126  Cp   135       144
GK19      CAG TTT C[TG CTC AGC GAG TTC GTG CCA GGT]GCT GGG
SGK321    CAG TTT C[TG CTC AGC GAG TTC GTG CCA GGT]GCT GGG
Mon531    CAG TTT C[TG CTC AGC GAG TTC GTG CCA GGT]GCT GGG
                    153  C4R  162   168
GK19      [TTC GTT CTC GGA CTA GTT GAC ATC]
SGK321    [TTC GTT CTC GGA CTA GTT GAC ATC]
Mon531    [TTC GTT CTC GGA CTA GTT GAC ATC]
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Figure 2. Partial sequences of the artificial *Cry1A(c)* gene among Mon531, GK19, and SGK321 cotton. Differences in DNA sequences from others are shown by a shaded box. Boxed regions were the primer pair C3F/C4R and TaqMan probe Cp used in real-time PCR analysis.

China). In the second round PCR, amplicons of *Sad1* gene and *Cry1A(c)* gene were connected using primers SM1F/4R using the first round PCR amplicons [*Sad1* and *Cry1A(c)*] as templates. All PCR amplifications were performed in 50 μL reaction volumes with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of dATP, dGTP, dCTP, and dTTP (TaKaRa Biotechnology Co., Ltd., Dalian), 800 nM each primer, 1 unit *Pfu* DNA polymerase (Promega Corp., Shanghai, China), and 20 ng of Mon 531 genomic DNA in first round PCR or purified first round PCR amplicons in second round PCR. All PCR amplifications were run in PTC-100 thermocycler (MJ Research, Waltham, MA) with the program as follows: one step of 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 58 °C, and 60 s at 72 °C; one step of 7 min at 72 °C.

After the second round PCR, one unit of Taq DNA polymerase (TaKaRa biotechnology Co., Ltd., Dalian) was added into the PCR mixture and incubated at 72 °C for 30 min to add an adenosine (A) residue to the 3′-end of the integrated PCR amplicon (*36*). Then, the integrated PCR amplicon was purified with Gel Extraction Mini Kit (Watson Biotechnologies, Inc., Shanghai, China) and ligated into pMD18-T vector. Sequencing analysis of the cloned DNA was performed using the ABI PRISM 3730 Genetic Analyzer (Applied-Biosystems) by Shanghai BioAsia biotechnology Co., Ltd. (Shanghai, China).

Conventional PCR Conditions. In conventional PCR assays, all of the amplifications were carried out in 30 μL volume reactions, with 100 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM each dNTP, 0.8 μM each primer, and 2.5 units of Taq DNA polymerase (TaKaRa biotechnology Co., Ltd., Dalian). The multiplex PCR conditions were the same as described above, except for the primer concentration as 0.8 μM SG1F/2R, 0.4 μM M1F/2R, 0.4 μM of G1F/2R, and 0.2 μM S1F/2R instead. All of the conventional PCR amplifications were performed in PTC-100 Thermalcycler (MJ Research, Watertown, MA) with the program as follows: one step of 7 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 58 °C, and 60 s at 72 °C; one step of 7 min at 72 °C. Amplification products were electrophoresed in 2% agarose gels for approximately 30 min at 100 V and stained with EtBr for visualization. All conventional PCR was repeated three times, each time with three repetitions.

Quantitative PCR Conditions. Real-time PCR assays were carried out in a fluorometric thermal cycler (Rotor-Gene 2000; Corbett Research, Australia) with a final volume of 25 μL. Fluorescence was monitored during every PCR cycle at the annealing step. Reactions contained either 100 nM endogenous gene primers (S3F/4R) or 300 nM exogenous gene primers (C3F/4R) and 200 nM endogenous (Sp) or 400 nM exogenous (Cp) probes for both the separate and the duplex detection assay, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 400 μM each of dATP, dGTP, and dCTP, 800 μM dUTP, 1.5 units of Taq DNA polymerase, 0.2 unit of Amperase Uracil N-glycosylase (UNG), and 6.5 mM $MgCl_2$. Real-time PCR reactions ran with the following procedures: 2 min at 50 °C and 10 min at 95 °C, 50 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. Data were analyzed with Rotor gene 2000 software version 5.0 (Corbett Research,

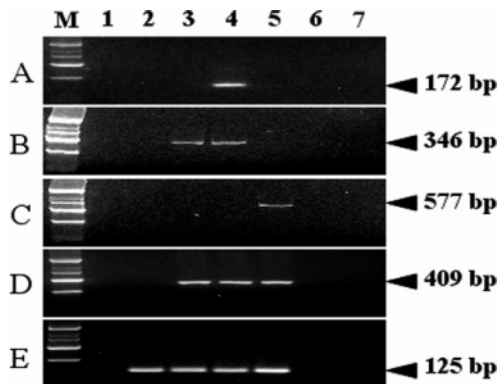

Figure 3. Specificity of the primer pairs designed for the three lines of insect resistant cottons. Arrowheads indicate the expected PCR amplification products. The primer pairs for detection of *CpTI* gene of SGK321 (**A**), junction region between *Cry1A(c)* and NOS of GK19 or SGK321 (**B**), junction region between *Cry1A(c)* and *NOS* of Mon531 (**C**), identical DNA fragment of *Cry1A(c)* gene of three insect resistant cottons (**D**), cotton endogenous gene, *Sad1* (**E**), respectively, were used. Lanes 2–7: amplification of cotton DNAs from non-GM cotton, GK19, SGK321, Mon531, non-GM soybean, and non-GM maize, respectively. Lane 1: no template control (NTC). M: DL2000 DNA marker.

Australia). All of the PCR reactants were purchased from Roche Molecular Biochemicals (Shanghai, China) except for primers and probes.

Five serial diluted concentrations (3, 30, 300, 3000, and 30000 copies per reaction, respectively) of standard plasmid DNA were diluted by salmon testis DNA solution (5 ng/μL) and used as reference molecules for the construction of standard curves. Salmon testis DNA (5 ng/μL) was used as a no-template control (NTC). All of the real-time PCR was performed three times with triple replication each time.

Determination of GMO Contents. According to the principle of standard curves optimization, optimal standard curves were obtained for the real-time PCR assays. Ct values were used to determine the amount of total DNA using the endogenous *Sad1* gene PCR system and the amount of transgenic DNA using the *Cry1A(c)* gene PCR system based on the standard curves. Sample GMO contents were determined by the ratio of transgenic DNA copy numbers to total DNA copy numbers with application of coefficient values (C_V) (*23*).

RESULTS AND DISCUSSION

Qualitative PCR Analysis of Three Insect Resistant Cottons. In the three Bt cotton lines, the artificial synthetic sequence of Mon531 *Cry1A(c)* gene was not completely same with the native *Cry1A(c)* gene from *B. thuringiensis* bacteria although their amino acid sequences were same. In GK19 and SGK321, the artificial synthetic *Cry1A* gene sequence consisted of a partial *Cry1A(c)* sequence and a partial *Cry1A(b)* sequence. Primer pairs were designed to screen and identify three insect resistant cotton lines according to the recombinant expression cassette structures (**Figure 1**). Primer pair C1F/2R [409 bp amplified fragment of *Cry1A(c)*] was employed to detect insect resistant cottons. Primer pair M1F/2R [577 bp amplified fragment of *Cry1A(c)-NOS*] was specific for Mon531. The combination of primer pairs G1F/2R [346 bp amplified fragment of *Cry1A(c)-NOS*] and SG1F/2R (172 bp amplified fragment of *CpTI*) was used to identify GK19 and SGK321; only one target amplified fragment (346 bp) of G1F/2R was obtained in GK19, and two target amplified fragments (346 bp and 172 bp) were obtained in SGK321. These primer pairs could be used to discriminate the single line from others successfully (**Figure 3**). As shown in **Figure 3A−C**, each primer pair could be used to amplify these specific fragments in Mon531, GK19, or SGK321 cotton, respectively, and no amplification was observed from non-GM cotton and other plants, such as soybean and maize.

The *Cry1A(c)* gene, conferring resistance to the bollworm, has a partially identical DNA sequence among the three insect resistant cotton lines. To discriminate transgenic insect resistant cotton lines from non-GM cotton lines or unknown cotton samples quickly, we designed the primer pair C1F/2R to amplify similar regions of *Cry1A(c)* gene in these three insect resistant cottons, and one fragment (409 bp) could be detected in all three insect resistant cottons (**Figure 3D**).

To make the developed PCR systems normatively and creditably, the positive control of endogenous reference gene is very important and necessary. We have validated four novel endogenous reference genes suitable for GMOs qualitative and quantitative PCR detection, i.e., the *sucrose phosphate synthase* (*SPS*) gene of rice (*38*), *high mobility group protein I/Y* (*HMG I/Y*) gene for rapeseed (*39*), *Lat52* gene of tomato (*40*), and *stearoyl-acyl carrier protein desaturase* (*Sad1*) gene for cotton (*32*). According to the certified results that *Sad1* gene was specific in different species, low heterogeneity among cotton cultivars and two copies in haploid cotton genome (*32*), the primer pair of S1F/2R for endogenous *Sad1* gene was selected and successfully used to amplify a 125 bp fragment from cotton DNA, whereas no fragment was amplified from other crops, such as soybean and maize (**Figure 3E**). The species specificity of those primers (C1F/2R, M1F/2R, G1F/2R, SG1F/2R, and S1F/2R) was also tested using other plant genomic DNAs as templates, such as rice, wheat, canola, etc., and similar results were obtained (data not show).

Because the degraded or low quantity DNAs derived from GMOs often occurred in practical detection, the higher sensitivity of PCR detection system was important and necessary. In qualitative PCR, the test sensitivity may be embodied by the limit of detection (LOD). The absolute limit is the lowest number of initial template copies that can be detected and quantified, the relative limit refers to the lowest percentage of GMO relative to the species (e.g., cotton) that can be detected and quantified, and the practical limit is the functional limit of the sample during an analysis. To test the limits of the established qualitative detection (LODs), the DNA mixture was prepared with each of three GM cotton lines and non-GM cotton line at various levels such as 0.01, 0.05, 0.1, 0.5, 1.0, 3.0, and 5.0%. The test results in three repetitions showed that the target fragment was detected from all of the levels tested except for 0.01% level, which meant that the lowest tested level was 0.05%, which approximates to 34 haploid genome copies according to the genome sizes of cotton (**Figure 4A−D**); these LODs were similar to those of GM soybean and GM maize (*41*, *42*), which indicated the established qualitative PCR detection systems of three insect resistant cottons were suited for the practical detection of GM cotton samples.

Qualitative Analysis of Three Insect Resistant Cottons with Multiplex PCR. Because plant seeds are often mixed with different varieties in practical use, multiplex PCR methods were sometimes used for detection of the several GM materials in one reaction tube economically (*25*). A multiplex system could detect many GMOs target genes (promoter, terminator, and/or common transgene sequences), and that is also capable of identifying specific lines, which would be user friendly and simplify the process of GMOs detection and identification. To achieve this, a simple and efficient multiplex PCR method was developed for the detection and identification of the three insect resistant cottons, in which only one reaction used for detection

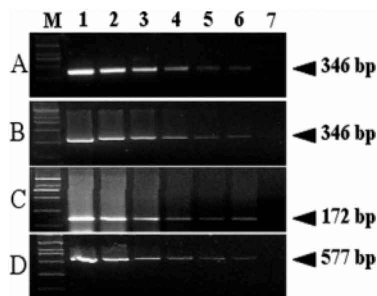

Figure 4. Sensitivity of the primer pairs designed for the three lines of insect resistant cottons. PCR products were amplified from each insect resistant cotton line DNA containing various amounts of insect resistant cotton genomic DNA, for detail, **A**–**D**: amplification of target gene from insect resistant cotton, junction region between *Cry1A(c)* and NOS of GK19 (**A**), junction region between *Cry1A(c)* and NOS of SGK321 (**B**), *CpTI* gene of SGK321 (**C**), and junction region between *Cry1A(c)* and *NOS* of Mon531 (**D**), respectively. Arrowheads indicate the expected PCR amplification products. Lanes 1–7: amplification of 100 ng of cotton DNAs with GM contents of 5, 3, 1, 0.5, 0.1, 0.05, and 0.01%, respectively. Lane M: DL2000 DNA marker.

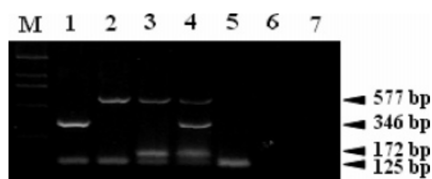

Figure 5. Multiplex PCR analysis of three insect resistant cotton lines. Lanes 1–7 are the results of multiplex PCR with a mixture of specific primer pairs (M1F/2R, G1F/2R, SG1F/2R, and S1F/2R). Arrowheads indicate the expected PCR amplification products. Lanes 1–7: amplification of cotton DNAs from GK19, Mon531, SGK321, a mixture of three insect resistant cotton lines, non-GM cotton, non-GM soybean, and no template DNA. Lane M: DL2000 DNA marker.

Table 2. Profile of Multiplex PCR Amplifications on Insect Resistant Cotton Samples[a]

target samples	*Sad1* S1F/2R (125 bp)	*CpTI* SG1F/2R (172 bp)	*Cry1A(b+c)* + *NOS* G1F/2R (346 bp)	*Cry1A(c)* + *NOS* M1F/2R (577 bp)
non-GM cotton	+	−	−	−
Mon531	+	−	−	+
GK19	+	−	+	−
SGK321	+	+	+	−
mixture of three GM lines	+	+	+	+

[a] +, positive; −, negative.

any of the three lines. In the established multiplex PCR, four primer pairs with different amplified lengths (M1F/2R, G1F/2R, SG1F/2R, and S1F/2R), which have been described in above paragraphs in detail, were employed in one tube for amplification, and the expected amplification products (577, 346, 172, and 125 bp) were present in each of the three lines and the mixture of three insect resistant cotton lines that were tested (**Figure 5** and **Table 2**).

Standard Plasmid instead of Positive Reference Material for Real-Time PCR. After optimization of magnesium and primer/probe concentrations, a highly sensitive and quantitative real-time PCR assay for the accurate measurement of GM cottons was established. In this established real-time PCR system, the identical DNA sequence of *Cry1A(c)* gene among these three cottons described above was used for quantification

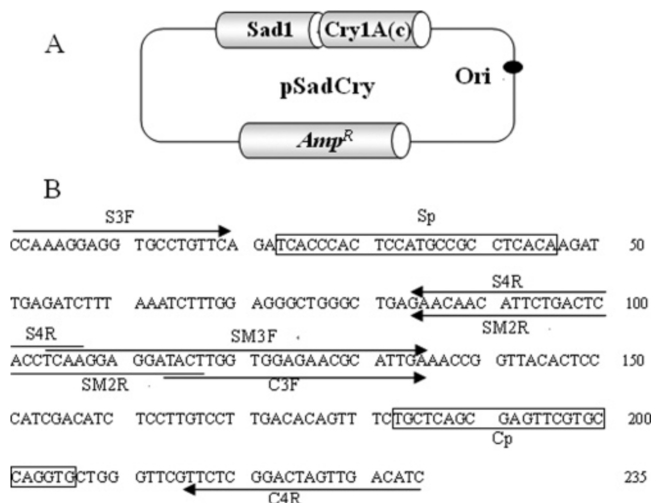

Figure 6. Standard plasmid pSadCry as a reference molecule. (**A**) Schematic diagram of pSadCry. *Amp*^{R}, ampicilin resistance gene; *Sad1*, cotton endogenous gene; *Cry1A(c)*, common gene in three insect resistant cotton lines with partial same DNA sequences. (**B**) Nucleotide sequence of the *Sad1* and *Cry1A(c)* region in pSadCry. The arrows locate primers with direction, and the squared boxes indicate TaqMan probes.

purpose, and the quantity of each insect resistant cotton line could be quantified. In practical GMOs detection, it is difficult to obtain the standard GM plant materials with similar quality and other physical conditions (humidity and growth period, etc). In addition, the reference molecule could be easily produced in bacteria with high quality and quantity, the standard dilution procedure was simplified where few error rates occurred, and many target genes from different GM lines might be integrated into one reference molecule, which decreased the requirement of the standard GM plants (*27*). The plasmid pSadCry for reference molecule was constructed by the tandem integration of two amplicons obtained by using respective primer pairs for *Sad1*, cotton endogenous gene and *Cry1A(c)*, an exogenous gene fragment commonly existed in all three lines of insect resistant cotton (**Figure 6A**). The nucleotide sequence of the integrated fragments in pSadCry was shown in **Figure 6B**.

To test the range of quantization, five levels of concentration of the reference molecule were set to 3, 30, 300, 3000, and 30000 copies per reaction for the calibration of insect resistant cotton specific real-time PCR. According to the genome sizes of the reference molecule and the cotton (*41*), we considered that the range of copy numbers from 3 to 30000 of the reference molecule was sufficient to quantify GMOs from 0.01 to 100% in the 100 ng of the template for one reaction. The range was supposed to be sufficient to quantify GMOs because of the quantified threshold values of labeling regulations in the EU, Korea, and Japan.

Six dilutions of reference molecule (corresponding to 0, 3, 30, 300, 3000, and 30000 copies each reaction) were used to establish calibration curves for the exogenous *Cry1A(c)* and endogenous *Sad1* gene. The similar PCR reaction efficiencies between *Cry1A(c)* and *Sad1* gene [0.98 of *Cry1A(c)* PCR assay and 0.96 of *Sad1*] indicated that GM contents could be calculated using these two PCR assays by means of relative quantitative method. The square regression coefficients (R^2) were 0.9964 and 0.9969 for the *Cry1A(c)* and *Sad1* amplicons, respectively. Good linearity between copy number and fluorescence values (Ct) visualized in the calibration curves in **Figure 7** indicated that these assays combined with reference molecule established in this paper were well-suited for quantitative measurements.

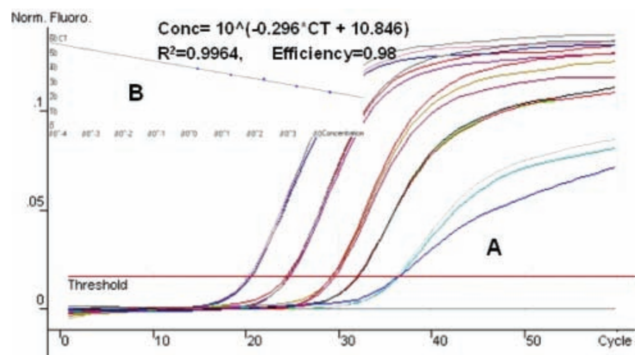

Figure 7. Amplification plots and standard curves for real-time PCR. (**A**) Amplification curves (six serial dilutions, from 0 to 30000 copies of pSadCry, respectively) were generated for detection of insect resistant cotton lines (300 nM C3F/4R primers and 400 nM Cp probe), and each curve corresponds to three replicates. The horizontal line indicates the threshold line determined for drawing up the standard curve. (**B**) Parameters of the regression line through data points are indicated within the plot.

Table 3. Repeatability and Reproducibility of Real-Time PCR Assays Employing *pSadCry* as a Reference Molecule[a]

true copy no.	1	mean Ct 2	3	mean copy no.	RSD_r[b]	RSD_R
			Cry1A(c)			
30000	21.47	21.4	21.57	30755.30	2.9	5.8
3000	24.96	24.86	24.95	2942.34	8.0	3.8
300	28.21	28.55	28.33	282.13	3.1	11.5
30	31.6	31.64	31.39	32.30	17.4	9.4
3	35.14	35.72	35.21	2.40	10.2	20.2
			Sad1			
30000	21.55	21.76	21.49	28575.91	6.1	9.4
3000	24.79	24.76	24.87	2896.90	16.8	3.8
300	28.03	28.01	27.72	313.90	7.2	12.2
30	31.17	31.05	31.45	29.70	16.1	13.6
3	34.61	35.09	34.52	2.41	17.0	19.6

[a] RSDr, repeatability relative standard deviation (RSDr); RSD_R, reproducibility relative standard deviation. [b] RSD (relative standard deviation) values were calculated by dividing the standard deviation by mean value and are given in %.

To validate the reproducibility and repeatability of these assays, five reference molecule dilutions (corresponding to 3, 30, 300, 3000, and 30000 copies each reaction) were employed in established PCR assays and performed three times with triplicate reactions each time. The repeatability relative standard deviation (RSD_r) and reproducibility relative standard deviation (RSD_R) of the reference molecule pSadCry were calculated by using data of triplicate reactions and three replications, respectively. The RSD_r values ranged from 2.9 to 17.4% (**Table 3**), and RSD_r values were slight except for the lower concentrations of reference molecule. The RSD_R values ranged from 3.8 to 20.2% (**Table 3**). All of the above results indicated that the pSadCry was successfully used as a standard material for quantification of insect resistant cottons.

Measurement of C_Vs. According to the previous report (*23*), the ratios of introduced DNA and endogenous sequence in each genuine seed were calculated and defined as C_V. GMO amounts (%) of unknown sample were calculated according to defined C_V. We determined the C_V of each insect resistant cotton with three times, and each time with triple replicated reactions, and then, the mean value was decided as C_V. **Table 4** showed the C_V of insect resistant cotton lines tested, and the values are 0.60, 0.44, and 1.12 for Mon531, GK19, and SGK321, respectively.

Table 4. Coefficient Values of PCR Systems for Each Insect Resistant Cotton Line

GM line	target gene	copy no. mean 1	mean 2	mean 3	mean copy no.	mean C_V	SD[a]	RSD[b]
Mon531	*Cry1A(c)*	504.71	562.86	455.66	507.74	0.60	0.0065	4.3
	Sad1	850.71	892.28	783.90	842.30			
SGK321	*Cry1A(c)*	902.05	1041.49	864.73	936.09	1.12	0.0268	9.5
	Sad1	886.22	844.93	768.03	833.06			
GK19	*Cry1A(c)*	376.50	424.89	324.10	375.16	0.44	0.0118	10.7
	Sad1	794.66	923.21	839.19	852.35			

[a] SD, standard deviation. [b] Experiments were repeated three times, and triplicate reactions were repeated one time.

Table 5. Accuracy and Precision Statistics for Quantitative Methods

GM line	true value (%)	accuracy mean GMO (%)	bias true value (%)	precision SD[a]	RSD[b]
	1.0	0.743	−25.67	0.11	15.1
Mon531	3.0	2.787	−7.11	0.23	8.2
	5.0	5.167	3.33	0.25	4.7
	1.0	0.833	−16.67	0.10	11.7
GK19	3.0	3.313	10.44	0.24	7.2
	5.0	5.423	8.47	0.19	3.6
	1.0	1.157	15.67	0.09	7.6
SGK321	3.0	3.227	7.56	0.08	2.4
	5.0	4.710	−5.8	0.22	4.7

[a] SD, standard deviation. [b] Experiments were repeated three times, and triplicate reactions were repeated one time.

These results reflect that the C_V is proportional to the copy number of target transgene in the genome. The *Cry1A(c)* gene was integrated into 1−2 loci depending on the GM cotton line, for example, one insert with partial gene cassette at the 3′-end and another insert with a complete gene cassette and a partial gene cassette at the 3′-end in the genome of Mon531 (*6*), a single copy in GK19 and two copies in SGK321, which were similar with the determined C_Vs (*7*, *8*, *43*). The ideal C_V of tetraploid insect resistant cotton for a single copy transgene haploid genome will be 0.5. The discrepancy between experimental and theoretical values might be generated by different PCR efficiencies that resulted from the amounts of the nontargeted sequences in the plasmid and genomic DNA, although any considerable homology to the sequences used for detection by using the DNA database of target crops could not be found (*23*).

Quantitative Analysis of Three Insect Resistant Cottons by Real-Time PCR. Some GM cotton mixtures that contained DNAs of each insect resistant cotton and DNAs of non-GM cotton variety 9409 at different ratios were prepared to evaluate the accuracy and precision of the established real-time PCR method in this study. Before mixing the DNAs, we adjusted the copy number of the *Sad1* gene in each insect resistant cotton line and non-GM cotton genome after measuring the copy number with real-time PCR using reference molecule pSadCry. Test DNA samples containing 1.0, 3.0, or 5.0% of genomic DNA of each insect resistant cotton were employed. Each template was analyzed in three parallels, and the quantitative estimates were computed using the reported method (*23*).

The accuracy of the quantitative method was measured as bias (%) of the tested mean value to the original value. As shown in **Table 5**, as to the sample containing 1.0% GM cotton content of three insect resistant cotton varieties, the bias ranged from −25.67 to 15.67%, the bias ranged from −7.11 to 10.44% to

the sample containing 3.0% GM content, and the bias ranged from −5.80 to 3.33% to the sample containing 5.0% content insect resistant cotton.

The bias existed in our experiment was also similar to the previously reported from maize and soybean samples (*23*). Especially, the bias of the tested samples containing 3 and 5% insect resistant cotton content was less than 10.0% and slightly deviated from true values. The big bias of 1.0% GM samples was probably derived from the low exogenous DNA contents in the samples. These results indicated that the accuracy of this measurement was ideal and credible. Estimation of precision is shown as relative standard deviation (RSD) and standard deviation (SD). The RSD and SD values were calculated from quantified results of the samples containing 1.0, 3.0, and 5.0% GM contents insect resistant cotton, the RSD values were from 7.6 to 15.1% in 1.0% GM contents insect resistant cotton samples, the RSD values were from 2.4 to 8.2% in 3.0% GM cotton containing samples, and the quantification results ranged from 3.6 to 4.7% to the 5.0% GM cotton containing samples. The SD values were from 0.09 to 0.24 in 1.0% GM cotton containing samples, SD values were from 0.08 to 0.24 in 3.0% GM content containing samples and from 0.19 to 0.22 of 5.0% GM cotton containing samples (**Table 5**).

Comparing with the RSD values of the previously reported from maize and soybean samples (*23*), the RSD values of them were accepted except the two tested samples in our experiment with the RSD values of 11.7 and 15.7%, respectively. Thus, concluded from those results, the real-time PCR systems for the three insect resistant cottons quantification established this paper were useful, credible, and practical. However, the established real-time PCR systems were mainly employed to quantify the sum of insect resistant cottons, and it was difficult to quantify each insect resistant cotton line from other insect resistant cottons. Quantification of the three different lines separately, which should use three line specific quantification systems, however, from the recombinant construct information of the three insect resistant cottons (**Figure 1**), few differences existed between GK19 cotton and SGK321cotton, and it was difficult to quantify the GK19 cotton and SGK321 cotton from mixed samples separately by using the construct specific quantified method. So in order to resolve this problem, three event specific quantification systems of these three insect resistant cottons might be efficient, and establishment of these event specific quantification systems will be our next work.

LITERATURE CITED

(1) U.S. Food and Drug Administration. Secondary food additives permitted in food for human consumption; food additives permitted in feed and drinking water of animals; aminoglycoside 3′-phosphotransferase II; final rule. *Fed. Regist*. **1994**, *59*, 26700−26711.

(2) James, C. Global status of commercialized transgenic crops. *ISAAA Briefs* **2003**, *30*.

(3) Gaskell, G.; Bauer, M. W.; Durant, J.; Allum, N. C. Worlds apart? The reception of genetically modified foods in Europe and the U.S. *Science* **1999**, *285*, 384−386.

(4) Vaeck, M.; Reynaerts, A.; Hofte, H.; Jansens, S.; De Beuckeleer, M.; Dean, C.; Zabeau, M.; Van Montagu, M.; Leemans, J.; Vaeck, M.; Reynaerts, A.; Hofte, H. Transgenic plants protected from insect attack. *Nature* **1987**, *328* (2), 33−37.

(5) Zhou, Z.; Zhu, Z. The advances of plant genetic engineering of anti-insect pest. *Adv. Biotechnol*. **1994**, *14* (4), 23.

(6) Monsanto Co. Safety assessment of Bollgard Cotton Event 531. Product safety description; www.monsanto.com, 2002.

(7) Guo, S.; Cui, H.; Xu, Q.; Ni, W. Expressive carrier with coded insect-killing protein fusion gene, and transfer gene plant. Chinese Patent Pub. No. 1134981, 1995.

(8) Guo, S.; Ni, W.; Xu, Q. Expressive carrier with coded two insect-killing protein fusion genes, and transfer gene plant. Chinese Patent Pub. No. 1219586, 1998.

(9) Guo, S.; Cui, H.; Xia, L.; Wu, D.; Ni, W.; Zhang, Z.; Zhang, B.; Xu, Y. Development of bivalent insect-resistant transgenic cotton plants. *Sci. Agric. Sin.* **1999**, *32*, 1−7.

(10) European Commission Regulation (EC) No. 1829/2003 and 1830/2003. *Off. J. Eur. Commun.* **2003**, *L268*, 1−28.

(11) Notification No. 2000-31 Ministry of Agriculture and Forestry of Korea, Seoul, Korea, April 22, 2000.

(12) Notification No. 1775 Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan, Tokyo, Japan, June 10, 2000.

(13) Matsuoka, T. GMO labeling and detection methods in Japan. APEC-JIRCAS Joint Symposium and Workshop on Agricultural Biotechnology, 2001.

(14) Order No. 10 Ministry of Agriculture of the People's Republic of China, Beijing, China, 2002.

(15) Berdal, K. G.; Holst-Jensen, A. Roundup Ready soybean event-specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. *Eur. Food Res. Technol.* **2001**, *213*, 432−438.

(16) Studer, E.; Rhyner, C.; Lüthy, J.; Hubner, P. Quantitative competitive PCR for the detection of genetically modified soybean and maize. *Z. Lebensm. Unters. Forsch. A* **1998**, *207*, 207−213.

(17) Lipp, M.; Brodmann, P.; Pietsch, K.; Pauwels, J.; Anklam, E. IUPAC collaborative trial study of a method to detect genetically modified soybeans and maize in dried powder. *J. AOAC Int.* **1999**, *852*, 923−928.

(18) Huang, H. Y.; Pan, T. M. Detection of genetically modified maize MON810 and NK603 by multiplex and real-time polymerase chain reaction methods. *J. Agric. Food Chem.* **2004**, *52*, 3264−3268.

(19) Zimmermann, A.; Lüthy, J.; Pauli, U. Event specific transgene detection in Bt11 corn by quantitative PCR at the integration site. *Lebensm.-Wiss. Technol*. **2000**, *33*, 210−216.

(20) Zhang, Y.; Zhang, D.; Li, W.; Chen, J.; Peng, Y.; Cao, W. A novel real-time quantitative PCR method using attached universal template probe. *Nucleic Acids Res.* **2003**, *31* (20), e123.

(21) Permingeat, H. R.; Reggiardo, M. I.; Vallejos, R. H. Detection and quantification of transgenes in grains by multiplex and real-time PCR. *J. Agric. Food Chem*. **2002**, *50*, 4431−4436.

(22) Matsuoka, T.; Kuribara, H.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. A multiplex PCR method of detecting recombinant DNAs from five lines of genetically modified maize. *J. Food Hyg. Soc. Jpn*. **2001**, *42*, 24−32.

(23) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC Int*. **2002**, *85*, 1077−1089.

(24) James, D.; Schmidt, A. M.; Wall, E.; Green, M.; Masri, S. Reliable detection and identification of genetically modified maize, soybean, and canola by multiplex PCR analysis. *J. Agric. Food Chem.* **2003**, *51*, 5829−5834.

(25) Pan, A.; Zhang, D.; Pan, L.; Chen, J.; Yuan, Z.; Liang, W. A rapid PCR method for detection of Glyphosate resistant transgenic rape. *Agric. Sci. China* **2003**, *36* (7), 856−860.

(26) Wall, E. M.; Lawrence, T. S.; Green, M. J.; Rott, M. E. Detection and identification of transgenic virus resistant papaya and squash by multiplex PCR. *Eur. Food Res. Technol*. **2004**, *219*, 90−96.

(27) Jaccaud, E.; Hohne, M.; Meyer, R. Assessment of screening methods for identification of genetically modified potatoes in raw materials and finished products. *J. Agric. Food Chem*. **2003**, *51*, 550−557.

(28) Rho, J. K.; Lee, T.; Jung, S. I.; Kim, T. S.; Park, Y. H.; Kim, Y. M. Qualitative and quantitative PCR methods for detection of three lines of genetically modified potatoes. *J. Agric. Food Chem.* **2004**, *52*, 3269−3274.

(29) Meyer, R. Detection of genetically engineered plants by polymerase chain reaction (PCR) using the FLAVR SAVR tomato as an example. *Z. Lebensm. Unters. Forsch.* **1995**, *201*, 583−586.

(30) Yang, L.; Shen, H.; Pan, A.; Chen, J.; Huang, C.; Zhang, D. Screening and construct specific detection methods of transgenic Huafan No. 1 tomato by conventional and real-time PCR. *J. Sci. Food Agri.* **2005**, in press.

(31) Hernández, M.; Río, A.; Esteve, T.; Prat, S.; Pla, M. A rapeseed-specific gene, acetyl-CoA carboxylase, can be used as a reference for qualitative and real-time quantitative PCR detection of transgenes from mixed food samples. *J. Agric. Food Chem.* **2001**, *49*, 3622−3627.

(32) Yang, L.; Chen, J.; Huang, C.; Liu, Y.; Jia, S.; Pan, L.; Zhang, D. Validation of a cotton specific gene, *Sad1*, used as an endogenous reference gene in qualitative and real-time quantitative PCR detection of transgenic cottons. *Plant Cell Rep*. **2005**, published online.

(33) Harwood, J. L. Fatty acid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **1988**, *39*, 101−138.

(34) Liu, Q.; Singh, S. P.; Sharp, P. J.; Green, A. G.; Marshall, D. R. Nucleotide sequence of a cDNA from *Gossypium hirsutum* encoding a stearoyl-acyl carrier protein desaturase. *Plant Physiol*. **1996**, *110*, 1436.

(35) Hillyard, J. R.; Roberts, J. K.; Ye, M. Cotton event PV-GHBK04 (757) and compositions and methods for detection thereof. U.S. Patent US2003/0024005 A1, 2003.

(36) Beazley, K. A.; Hillyard, J. R.; Pang, S.; Roberts, J. K. Cotton event PV-GHBK04 (757) and compositions and methods for detection thereof. U.S. Patent US2004/0045054 A1, 2004.

(37) Ido, E.; Hayami, M. Construction of T-tailed vectors derived from a pUC plasmid: A rapid system for direct cloning of unmodified PCR products. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1766−1767.

(38) Ding, J.; Jia, J.; Yang, L.; Wen, H.; Zhang, C.; Liu, W.; Zhang, D. Validation of a rice specific gene, *sucrose phosphate synthase*, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. *J. Agric. Food Chem.* **2004**, *52*, 3372−3377.

(39) Weng, H.; Yang, L.; Liu, Z.; Ding, J.; Pan, A.; Zhang, D. A novel reference gene, *highmobility-group* protein I/Y, can be used in qualitative and real-time quantitative PCR detection of transgenic rapeseed cultivars. *J. AOAC Int*. **2005**, *88*, 577−584.

(40) Yang, L.; Pan, A.; Jia, J.; Ding, J.; Chen, J.; Huang, C.; Zhang, C.; Zhang, D. Validation of a tomato-specific gene, *LAT52*, used as an endogenous reference gene in qualitative and real-time quantitative PCR detection of transgenic tomatoes. *J. Agric. Food Chem.* **2005**, *53*, 183−190.

(41) Arumuganathan, K.; Earle, E. D. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep*. **1991**, *9*, 208−218.

(42) Jankiewicz, A.; Broll, H.; Zagon, J. The official method for the detection of genetically modified soybeans (German Food Act LMBG § 35): A semiquantitative study of sensitivity limits with glyphosate-tolerant soybeans (Roundup Ready) and insect-resistant Bt maize (Maximizer). *Eur. Food Res. Technol.* **1999**, *209*, 77−82.

(43) Li, F.; Guo, S.; Liu, C.; Li, F.; Cui, H.; Zhou, Y.; Li, X. The study on the transformation and selection of insect-resistant cotton harboring double-gene. *Acta Gossypii Sin.* **1999**, *11*, 106−112.

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