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GENETICS AND GENOMICS

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Validation of a cotton-specific gene, *Sad1*, used as an endogenous reference gene in qualitative and real-time quantitative PCR detection of transgenic cottons

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Abstract Genetically modified (GM) cotton lines have been approved for commercialization and widely cultivated in many countries, especially in China. As a step towards the development of reliable qualitative and quantitative PCR methods for detecting GM cottons, we report here the validation of the cotton (*Gossypium hirsutum*) endogenous reference control gene, *Sad1*, using conventional and real-time (RT)-PCR methods. Both methods were tested on 15 different *G. hirsutum* cultivars, and identical amplicons were obtained with all of them. No amplicons were observed when DNA samples from three species of genus *Gossypium*, *Arabidopsis thaliana*, maize, and soybean and others were used as amplified templates, demonstrating that these two systems are specific for the identification and quantification of *G. hirsutum*. The results of Southern blot analysis also showed that the *Sad1* gene was two copies in these 15 different *G. hirsutum* cultivars. Furthermore, one multiplex RT-quantitative PCR employing this gene as an endogenous reference gene was designed to quantify the *Cry1A(c)* gene modified from *Bacillus thuringiensis* (Bt) in the insect-resistant cottons, such as Mon531 and GK19. The quantification detection limit of the *Cry1A(c)* and *Sad1* genes was as low as 10 pg of genomic DNA. These results indicat that the *Sad1* gene can be used as an endogenous reference gene for both qualitative and quantitative PCR detection of GM cottons.

Keywords *Gossypium hirsutum* · *Stearoyl-Acyl Carrier Protein desaturase* gene · Endogenous reference gene · Genetically modified organism · Conventional and real-time PCR

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Introduction

Since the first transgenic tomato, named Flavr Savr, was approved for commercialization, an increasing number of transgenic plants have been created and produced that carry different agronomic traits (Kok and Kuiper 2003). Globally, the area cultivated with transgenic crops has increased from 1.7 million hectares in 1996 to 67.7 million hectares in 2003 (James 2003). In China, six types of genetically modified (GM) plants have been approved for commercialization since 1997; these include two types of insect-resistant cotton, sweet pepper and tomato, long shelf-life tomato and color-altered petunia (Huang et al. 2002; http://www.libnet.sh.cn). The area now cultivated in GM cottons, such as cotton (*Gossypium hirsutum*) vars. Mon531, SGK321 and GK19, has increased to 2.8 million hectares in 2003. Moreover, GM soybean, maize and rapeseed products are also being imported from other countries into China.

Since 2001, pressure from public consumers expressing their concerns about GM crops has led China to require labeling for the presence of GM organisms (GMOs) in foods. In May 2001, the State Council of the People's Republic of China issued new biosafety guidelines—the Agricultural GMO Safety Regulations. These regulations have been supplemented by three detailed implementation guidelines and executed since March 20, 2002. Seventeen types of GM crops and their derived products, including GM cotton seeds, require labeling in China according to these guidelines (Order of the Ministry of Agriculture of P. R. China, 2002, Nos. 8–10; Order of the State Council of P. R. China, 2001, No.304).

At present, the most creditable and widely used methodology that enables detection of GMOs that is necessary following this labeling policy is the PCR method. This method is not only used for identifying GM products but also for quantification purposes because of its simplicity, specificity and sensitivity (Meyer 1995; Pietsch et al. 1997; Saiki et al. 1988). In order to make these PCR procedures more standardized, the target sequences and plant species-specific endogenous reference genes should be detected. Among the PCR detection methods, fluorescence quantitative real-time (RT)-PCR is considered to be an easy, useful and accurate quantitative method (Bonfini et al. 2002; Zhang et al. 2003). One unique feature of the fluorescence quantitative real-time PCR technique is that the amplification of the target DNA sequence can be traced during the whole reaction by indirect monitoring of the product formation through the fluorescence. Using this method, a target gene can be quantified by preparing a standard curve from known quantities of additional endogenous gene and extrapolation from the linear regression line. This system requires both primers to be specific for the transgene and that the species-specific primers be complementary to an endogenous reference gene. In practice, accurate relative quantification might be achieved by a combination of two absolute quantitative reactions: one for the GMO-specific gene and the other for plant endogenous reference gene (Ahmed 2002).

To this end, much effort has been made to obtain the reference gene of different plants for the detection of GMOs, and several endogenous reference genes of plants have been reported. Examples of such genes are the *zein* or *invertase1* genes for maize (Studer et al. 1997; VaÏtilingom et al. 1999; Vollenhofer et al. 1999; Zimmermann et al. 1998), the *lectin* or *hsp* (heat-shock protein) genes for soybean (Meyer et al. 1996; Duijn et al. 1999), the *BnACCg8*, *cruciferin* or *HMGI/Y* genes for rapeseed (Hernández et al. 2001; James et al. 2003; Weng et al. 2005), the *LAT52* gene for tomato (Yang et al. 2005) and the *SPS* gene for rice (Ding et al. 2004). However, there are only a few reports on a endogenous reference gene for GM cotton identification and quantification (Rangwala and Ye 2002; Jeanna et al. 2003; Beazleyn et al. 2004). GM cottons are an important economic crop in China and are not only used for fibre production, but the seeds are also used as the main oil and protein resources in food and in the foodstuff industry. To our knowledge, nine GM cotton varieties have been authorized for commercialization to date—the 15958, MON1445/1698 and MON531/757/1076 lines from the Monsanto company (St. Louis, Mo.), the 31807/31808 and BXN lines from Calgene (Davis, Calif.), 19-51A from DuPont Canada Agricultural Products, LLCotton25 from Bayer CropScience (http://www.agbios.com/dbase.php) and two kinds insect-resistant cotton from the Chinese Academy of Agricultural Sciences (CAAS)—GK19 and SGK321. GK19 was developed by introducing artificially synthesized *Cry1A(b+c)* gene expression cassette, and SGK321 with the *Cowpwa typsin inhibitor* (*CpTI*) gene and the artificial synthesized *Cry1A(b+c)* gene expression cassettes (Guo et al. 1996).

In this paper, we report the specific primers and the probe for cotton (*G. hirsutum*), the *stearoyl-acyl carrier protein desaturase* (*Sad1*) gene, and the PCR cycling conditions that are suitable for the use of the specific sequence as an endogenous reference gene in both qualitative and quantitative PCR assays. The *Sad1* gene encodes a stearoyl-acyl carrier protein desaturase in *G. hirsutum*. We have validated its species specificity and tested its detection sensitivity using both conventional and TaqMan PCR methods. In addition, a reliable duplex quantitative RT-PCR system for detecting the artificially synthesized *Cry1A(c)* gene of insect-resistant cotton was set up using the *Sad1* gene as an endogenous gene.

Materials and methods

Materials

The seeds of three *Gossypium* species, including *G. barbadense*, *G. arboretum* and *G. herbaceum*, and 15 different *G. hirsutum* cultivars, such as Zhongminsuo 27, Coker 312, Sumian 14, Sumian 15, Xuzhou 142, Lumian 11, Chuanmian 243, Chouyou 1, Yumian 22, Jimian 17, Wanmian 13, Jinmian 16, Simian 3, Liaomian 14 and Xiangzamian 2, were provided by CAAS. The seeds or leaves of 16 different plants—barley (*Hordeum vulgare*), maize (*Zea mays*), rice (*Oryza sativa*), canola (*Brassica napus*), wheat (*Triticum aestivum*), sunflower (*Helianthus annuus*), soybean (*Glycine max*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), *Arabidopsis thaliana*, sisal (*Agave sisalana*), flax (*Linum usitatissimum*), *Hibiscus syriacus*, *Abelmoschus esculentus* and Althaea officinalis—were collected by our laboratory. Insect-resistant cotton GK19 containing the artificial synthesized partial *Cry1A(b)* and *Cry1A(c)* genes developed by CAAS was provided by Prof. Sandui Guo, and Mon531 harboring the modified *Cry1A(c)* gene developed by the Monsanto Company was purchased from the local seed market. The reference materials containing 0%, 0.5%, 1%, 2%, 5%, 10% (w/w) of GK19 dried cotton flours were prepared by our laboratory for construction of the calibration curves. Two mixed samples with known contents of GM cotton seeds or flours were also prepared and analyzed to determine the accuracy of the duplex TaqMan RT-PCR detection system using the *Sad1* gene as the endogenous reference gene.

Table 1 Primers and TaqMan probes used for conventional and RT-PCR systems

PCR system	Name	Orientation	Sequence (5′–3′)	Length (bp)
Endogenous conventional and real-time PCR	S1F	Sense	CCAAAGGAGGTGCCTGTTCA	20
	S2R	Anti-sense	TTGAGGTGAGTCAGAATGTTGTTC	24
	SP	Probe	TCACCCACTCCATGCCGCCTCACA	24
Exogenous RT-PCR	Bt1F	Sense	TACTTGGTGGAGAACGCATTGAA	23
	Bt2R	Anti-sense	GATGTCAACTAGTCCGAGAACGAA	24
	BtP	Probe	CACCTGGCACGAACTCGCTGAGCA	24

DNA extraction and purification

The DNAs used for conventional and RT-PCR analysis were isolated and purified from 1 g of plant materials or mixed samples using the Plant DNA Mini-Prep kit developed by the Shanghai Ruifeng Agro-tech Co. (http://www.bio-safety.com). The plant genomic DNAs used for the Southern blot analysis were extracted from 10 g dried seeds or fresh leaves according to the method CTAB (Sambrook and Russell 2001). The quality and quantity of DNA was estimated using a spectrophotometer based on the 260/280-nm and 260/230-nm UV absorption ratios and analyzed by 1% agarose gel electrophoresis.

Southern blot

Ten micrograms of each genomic DNA from different non-transgenic *G. hirsutum* cultivars was completely digested with *Eco*RI and *Pst*I, respectively. The digested DNAs were resolved by 0.8% agarose gel electrophoresis and then transferred onto a nitrocellulose membrane, which was purchased from the Gene Company (http://www.genehk.com). A 107-bp DNA fragment (from 4,834 bp to 4,940 bp; Genbank No. AJ132636) amplified with S1F/S2R of the *Sad1* gene was used as the hybridized probe. This DNA fragment was labeled by α-[^{32}P]-dCTP using the Random Primer DNA Labeling kit ver. 2 (TaKaRa Biotechnology; http://www.takara.com.cn). Hybridization was performed at 62°C for 24 h, and the filter was washed at room temperature with 2× SSC/0.1% SDS and 1× SSC/0.1% SDS for 10 min each and at 60°C with 0.2× SSC/0.1% SDS for 30 min (Sambrook and Russell 2001). DNA markers (λDNA digested with *Hin*dIII and *Eco*RI) were run on the same gel.

Primers and probes

The PCR primers and TaqMan fluorescent probes designed with PRIMER EXPRESS 2.0 software (Applied Biosystems, Foster City, Calif.; http://www.appliedbiosystems.com.cn) are listed in Table 1. The TaqMan probe of the endogenous reference gene *Sad1* was labeled with the fluorescent reporter dye 5-hexachloro-fluorescein (HEX) on the 5′ end and the exogenous 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 pairs S1F/S2R were used not only in conventional PCR but also in TaqManRT-PCR combined with the SP probe specific for the endogenous reference *Sad1* gene. In order to set up the RT-PCR system for the detection of insect-resistant cottons commercialized in China—for example, Mon531 and GK19—their inserted modified *Cry1A(c)* DNA sequences were obtained from U.S. Patent and Chinese Patents (Guo et al. 1996; Beazleyn et al. 2004). The deduced homologous DNA sequence of the two modified *Cry1A(a)* genes was selected to design primer pairs Bt1F/Bt2R and TaqMan probe BtP (Fig. 1).

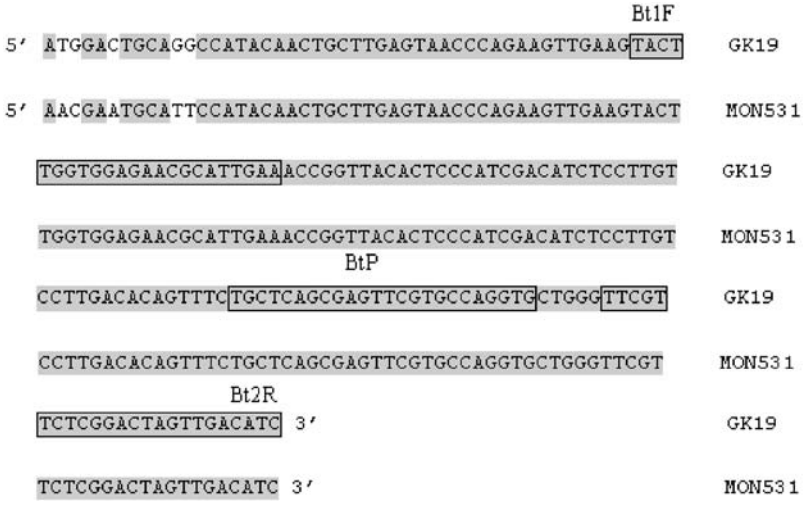

Fig. 1 Partially uniform sequences of the artificial *Cry1A(c)* gene from Mon531 and GK19. The uniform *Cry1A(c)* sequences of Mon531 and GK19 are *shaded*, and the *boxed* regions are the primer pair Bt1F/Bt2R and TaqMan probe BtP used in duplex RT-PCR analysisx

Optimization of PCR conditions

Conventional PCR was carried out in a PTC-100 thermocycler (MJ Research, Waltham, Mass.;http://www.mjr.com). Each reaction mixture contained 1× PCR buffer, 0.2 m*M* dNTP, 1 µ*M* each primer, 5 ng each DNA sample and 1.5 U *Taq* DNA polymerase, and the final reaction volume was 30 µl. All PCR reagents were purchased from the TaKaRa Biotechnology except for the primers. The amplification reaction was run according to the following program: one cycle of denaturing at 94°C for 10 min, 35 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C and a final extension at 72°C for 5 min. Amplified products were analyzed by 3% agarose gel electrophoresis in 0.5× TBE and stained with ethidium bromide. The results were evaluated by direct observation on a UV transilluminator and then recorded using a computer imaging system.

RT PCR assays with TaqMan chemistry were carried out in a fluorometric thermal cycler Rotor-Gene 2000 (Corbett Research, Mortlake, Australia; http://www.corbettresearch.com) in final volumes of 25 µl. Fluorescence was monitored during every PCR cycle at the annealing step. Reactions contained either 100 n*M* endogenous gene primers (S1F and S2R) or 300 n*M* transgenic primers (Bt1F and Bt2R), and 200 n*M* endogenous (SP) or 400 n*M* exogenous (BtP) probes for both the separate and duplex detection assay, 1× PCR buffer, 400 µ*M* each of dATP, dGTP, dCTP, 800 µ*M* dUTP, 1.5 U *Taq* DNA polymerase, 0.2 U Amperase uracil *N*-glycosylase (UNG) and 6.5 m*M* $MgCl_2$. RT-PCR reactions were run according to the following procedure: one cycle of 2 min at 50°C, 10 min at 95°C; 50 cycles of 30 s at 94°C, 40 s at 60°C and 30 s at 72°C. The data was analyzed with ROTOR GENE 2000 software ver. 4.6 (Corbett Research). All primers and fluorescent probes were synthesized and purified by the Shanghai Shenyou (http://www.sybio.com.cn), and the other RT-PCR reactants were purchased from Roche Molecular Biochemical, Indianapolis, Mo.; http://www.biochem.roche.com).

Determination of the GMO contents of mixed cotton samples

To set up a creditable and sensitive RT-PCR system for quantifying the GMO contents of samples derived from insect-resistant cotton flours, one duplex RT-PCR system was optimized and established employing the *Cry1A(c)* gene and *Sad1* gene.

Following establishment of this duplex RT-PCR system, triplicate amplification reactions were carried out on DNA from the certified reference materials to produce a calibration curve, with the logarithm of insect-resistant cotton concentration being plotted against △Ct ($Ct_{GMO} - Ct_{Sad1}$), where the Ct value can be defined as threshold cycle value (Livak and Schmittgen 2001; Terry and Harris 2001). The equation of this standard curve was then used to determine the percentage of two insect-resistant cotton samples using the △Ct values obtained from triplicate analyses of these matrices.

Results and discussion

Selection of the cotton endogenous reference gene, *Sad1*

Detection and quantification PCR systems for GMOs depend on the use of endogenous reference genes. An endogenous reference gene should be species-specific, have a low copy number in the haploid genome and have a low heterogeneity among cultivars (Hernández et al. 2001). To select a cotton gene suitable for use as an endogenous reference gene for GM cotton detection, we searched the DNA sequences with low copy number, and those candidate sequences were blasted in the database (Genbank). The results of homologous analysis indicated that the sequence of *Sad1* gene (Genbank No. AJ132636) has low homogeneity with the DNA sequences of other plants, such as wheat, maize, barley, tobacco, tomato, rapeseed, rice, sunflower, sisal, flax, *Hibiscus syriacus*, *Abelmoschus esculentus* and *Althaea officinalis* etc. The *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 the C18 fatty acids to produce the mono-unsaturated oleic acid, which in turn regulates the extent of unsaturation of membrane lipids and seed oils in cotton plants (Liu et al. 1996; Harwood 1988). The specific primers and fluorogenic probe based upon this DNA sequence were designed for both conventional and RT-PCR assays, and their positions are shown in Fig. 2.

Sensitivities of conventional and RT-PCR assays for the *Sad1* gene

Following the optimization of the magnesium and primer/probe concentrations, the sensitivity tests of conventional and RT-PCR were performed. In conventional PCR, the cotton genomic DNA concentrations were diluted to correspond to 100, 10, 1, 0.1, 0.05 and 0.01 ng/µl, and a serial dilution of DNAs having concentrations of 100, 10, 1, 0.1, 0.01, 0.001 ng/µl were diluted for the sensitivity test of single RT-PCR. A 1-µl aliquot of DNA template solution was added to each PCR reaction. Conventional PCR allowed the detection of the *Sad1* gene in 50 pg of genomic DNA (Fig. 3a). This sensitivity was similar to that of *lectin*

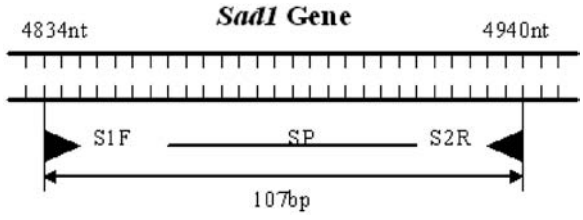

Fig. 2 Schematic diagram illustrating the amplification fragments of the *Sad1* gene and the positions of the PCR primers: primers S1F/S2R were employed for conventional PCR, primers S1F/S2R and TaqMan probe SP were employed for quantitative RT-PCR analysis and the primers S1F/S2R were also used for amplification of the hybridization probe

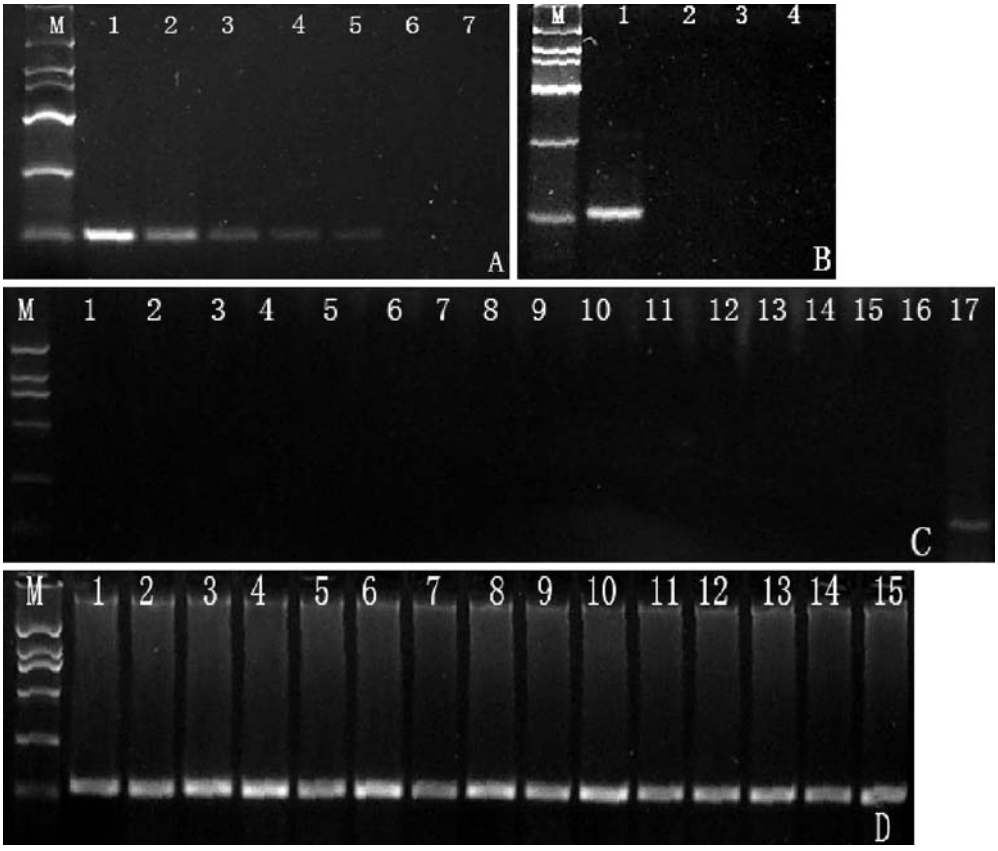

Fig. 3 Three percent (3%) agarose gel electrophoresis of conventional PCR products amplified with the S1F/S2R primer pair. **a** Amplification result of serial dilutions of cotton DNA (*lanes 1-6* correspond to 100 ng, 10 ng, 1 ng, 0.1 ng, 0.05 ng and 0.01 ng, *lane 7* negative control). **b** Amplification of DNA from different *Gossypium* species. *Lanes 1–4 G. hirsutum, G. barbadense*, *G. arboretum* and *G. herbaceum*, respectively; no amplified products were obtained in these *Gossypium* species except for *G. hirsutum*. **c** Amplification result of DNA from 16 different plant species. *Lanes 1–16* correspond to *Hordeum vulgare*, *Zea mays*, *Oryza sativa*, *Brassica napus*, *Triticum aestivum*, *Helianthus annuus*, *Glycine max*, *Solanum tuberosum*, *Lycopersicon esculentum*, *Nicotiana tabacum*, *Arabidopsis thaliana*, *Agave sisalana*, *Linum usitatissimum*, *Hibiscus syriacus*, *Abelmoschus esculentus* and *Althaea officinalis*, respectively; *lane 17* is a cotton positive control. No amplified products were obtained in all 16 different plant species. **d** Amplification result of DNA from 15 different *G. hirsutum* cultivars. *Lanes 1–15* correspond to Zhongminsuo 27, Coker 312, Sumian 14, Sumian 15, Xuzhou 142, Numian 11, Chuanmian 243, Chouyou 1, Yumian 22, Jimian 17, Wanmian 13, Jinmian 16, Simain 3, Liaomian 14 and Xiangzamian 2, respectively; identical fragments were obtained from all 15 different *G. hirsutum* cultivars. *Lane M* (**a–d**) is the DL2000 DNA Marker

or 10-kDa *zein* that was used in the qualitative detection of GMOs (Studer et al. 1997; Wurz et al. 1999). In the RT-PCR assay, PCR products could be amplified with as little as 10 pg of cotton genomic DNA (Table 2). This sensitivity corresponds to an average of seven copies per haploid genome of cotton based on the cotton genome size of 3 pg per haploid genome (Arumuganathan and Earle 1991).

Table 2 Sensitivity test of the *Sad1* gene with single TaqMan RT-PCR

Amount of DNA (ng/reaction)	Ct value for reaction			Mean	Standard deviation
	1	2	3		
100.000	21.41	21.61	21.76	21.59	0.14
10.000	25.60	25.85	25.71	25.72	0.10
1.000	30.51	30.72	30.40	30.54	0.13
0.100	33.29	33.23	33.30	33.27	0.03
0.010	38.01	37.71	37.95	37.89	0.15
0.001	–	–	–	–	–

Species specificity of the cotton *Sad1* endogenous reference gene

To test the species specificity of the *Sad1* endogenous reference gene, 50-ng aliquots of genomic DNA from 19 different plant species that were either evolutionarily related to *G. hirsutum* or frequently found in foodstuff were amplified as templates in both conventional and real-time PCR reactions. These included *Gossypium* species such as *G. barbadense*, *G. arboretum* and *G. herbaceum*; other species tested were barley (*Hordeum vulgare*), maize (*Zea mays*), rice (*Oryza sativa*), canola (*Brassica napus*), wheat (*Triticum aestivum*), sunflower (*Helianthus annuus*), soybean (*Glycine max*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*) tobacco (*Nicotiana tabacum*), *Arabidopsis thaliana*, sisal (*Agave sisalana*), flax (*Linum usitatissimum*), *Hibiscus syriacus*, *Abelmoschus esculentus* and *Althaea officinalis*. No amplified fragments were observed with any of these species tested other than *G. hirsutum* in both conventional (Fig. 3b and c) and TaqMan

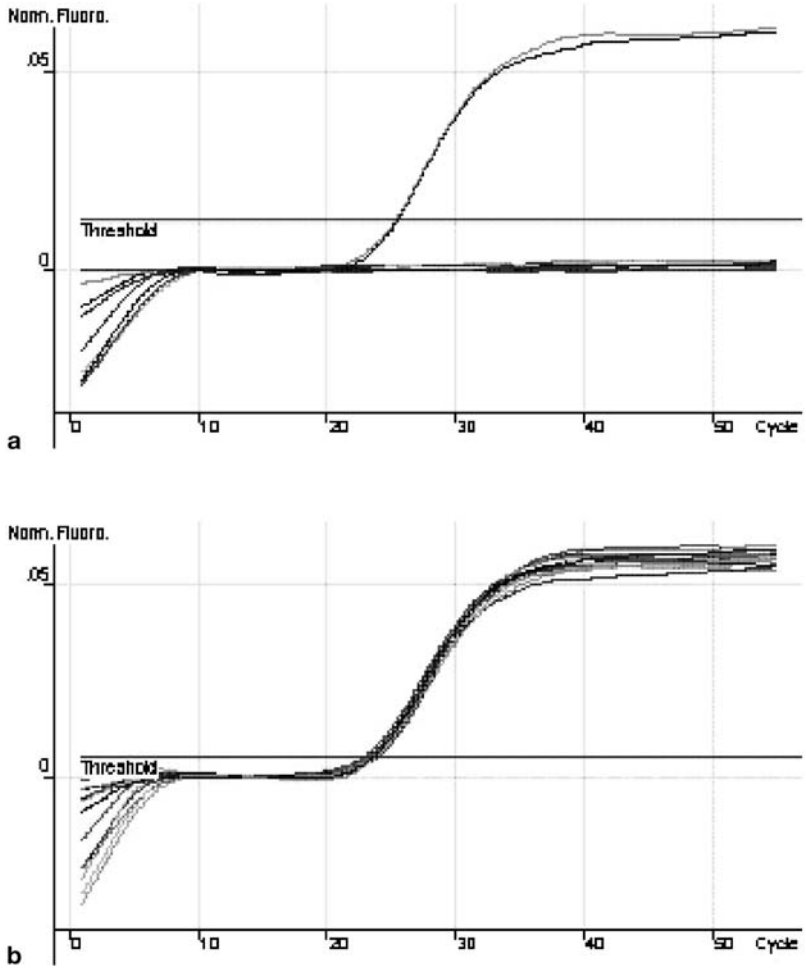

Fig. 4 Species specificity and allelic variation analysis of the *Sad1* gene with the TaqMan RT-PCR assay. **a** Specificity analysis of cotton *Sad1* gene in TaqMan RT-PCR. The amplification plot was generated from 19 different plant species described in Fig. 3 and from *G. hirsutum* seeds. No fluorescence signal was detected in these 19 different plant species PCR reactions except for *G. hirsutum* seeds. **b** Allelic variation analysis of *Sad1* gene among *G. hirsutum* cultivars. The amplification plot was generated from 15 different *G. hirsutum* cultivars described in Fig. 3d. The fluorescent signal with approximated intensity was detected from 15 different cotton cultivars PCR reactions

RT-PCR (Fig. 4a). Amplicons were not observed when *G. barbadense*, *G. arboretum* or *G. herbaceum* DNA was amplified as template, respectively. These three species are closely related to *G. hirsutum* and belong to the same taxonomic genus. Our results demonstrate that the *Sad1* gene is highly specific for *G. hirsutum* in both the conventional and RT-PCR analysis.

Allelic variation of the *Sad1* gene among *G. hirsutum* cultivars

An ideal endogenous reference gene should not exhibit allelic variation among varieties of the same species, while it should present a consistent low copy number in the different cultivars. To investigate whether different *G. hirsutum* cultivars exhibit allelic variation within the amplified *Sad1* sequence, we performed both conventional and RT-PCR using a fixed amount of 20 ng DNA obtained from 15 different *G. hirsutum* cultivars that originated from several climatic regions (for example, North America, Asia, the Yangtze River, Yellow River and Pearl River Valley in China). As shown in Fig. 3d, identically amplified fragments were obtained from all of the cultivars tested in conventional PCR. In RT-time PCR analysis, similar Ct values (from 23.00 to 24.11, Fig. 4b) were obtained in triplicate amplifications with the genomic DNA of the *G. hirsutum* cultivars. These results indicate that there were no major sequence differences among the different cultivars in this amplified region. The small amount of variation in the Ct values of the RT-PCR analysis also showed that the copy number of the *Sad1* gene was consistent among the tested *G. hirsutum* cultivars.

Copy number of *Sad1* gene

To analyze the copy number of the *Sad1* gene in the *G. hirsutum* genome, Southern blot analysis was performed in which cotton genomic DNA was digested with *Eco*RI or *Pst*I, respectively, then hybridized with the amplified 107-bp *Sad1* fragment. Equivalent amounts of *G. barbadense, G. arboretum*, *G. herbaceum*, *A. thaliana*, maize, and soybean DNA, digested with *Eco*RI or *Pst*I, were also analyzed to further assay the species specificity of the S1F and S2R fragments. Two hybridizing bands were detected in the lanes corresponding to *Eco*RI-digested cotton DNA of ten different *G. hirsutum* cultivars, indicating the presence of two copies of the *Sad1* gene per cotton haploid genome (Fig. 5). No cross-hybridization was obtained with

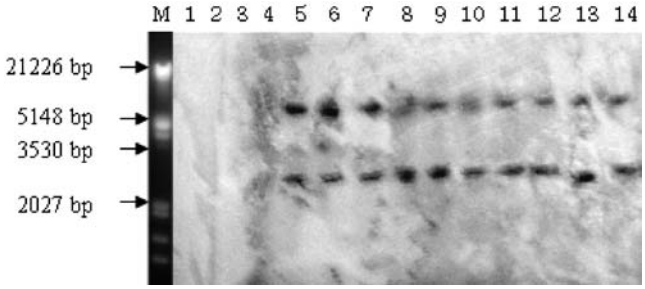

Fig. 5 Southern blot analysis for the copy number of *Sad1* gene in cotton genome. Two hybridizing bands were detected in the lanes corresponding to *Eco*RI-digested cotton DNA of ten different *G. hirsutum* cultivars, which was indicative of the presence of two copies of the *Sad1* gene per cotton haploid genome. No hybridizing band was obtained in the *Eco*RI-digested DNA of *G. barbadense*, *G. arboretum*, *G. herbaceum*, *A. thaliana*. *Lanes 1–4* correspond to *G. barbadense, G. arboretum*, *G. herbaceum*, *A. thaliana*, *lanes 5–14* correspond to Zhongminsuo 27, Coker 312, Sumian 14, Xuzhou 142, Numian 11, Chuanmian 243, Chouyou 1, Yumian 22, Wanmian 13 and Xiangzamian 2, respectively, *lane M* is the λDNA/*Hin*dIII + *Eco*RI Marker. *Arrowheads* indicate the length of partial marker DNA bands

G. barbadense, G. arboretum, *G. herbaceum*, *A. thaliana* and maize DNA, confirming that the S1F and S2R DNA fragments of cotton are specific for *G. hirsutum* (similar results were also obtained from the *Pst*I-digested genomic DNA).

Quantitative detection of insect-resistant cotton samples with duplex RT-PCR system

Following optimization of the magnesium and primer/probe concentrations, the duplex RT-PCR assay was applied to quantify cotton samples containing known percentages of insect-resistant cottons. Reproducibility of the threshold of detection (Ct) measurements was determined using 20 ng of total DNA varying in GK19 cotton contents from 0% to10% (w/w) per sample, repeated in triplicate (Table 3). The average Ct values of the *Sad1* gene varied from 21.05 to 21.37 cycles due to the fixed estimation of DNA content, with a standard deviation of 0.03 to 0.44. For insect-resistant cotton detection, Ct values correlated with the amount of GMO contents in each standard, ranging from 26.56 to 31.26 cycles, with a standard deviation of 0.10–0.39.

Sensitivity was tested on a serial dilutions of DNA extracted from 100% (w/w) dry GK19 cotton flours. The DNA samples were diluted to give a concentration range of 100 ng to 1 pg/μl, each serial dilution was assayed in triplicate per PCR run. The results indicated that the exogenous gene *Cry1A(c)* could be detected using 10 pg cotton DNA in the duplex real-time PCR detection system (Table 4).

The logarithm of GMO concentration was plotted against $\triangle$Ct ($Ct_{GMO} \sum Ct_{Sad1}$) for GMO standards to obtain a calibration plot for the duplex RT-PCR assay, and the calibration line with R^2 values of 0.9975 were obtained (Fig. 6).

Table 3 Reproducibility of the Ct measurements of replicate standards using 20 ng of total DNA varying in GK19 cotton contents from 0% to10% (w/w) using duplex TaqMan RT-PCR

GM content (%)	Ct value for reaction			Mean	Standard deviation
	1	2	3		
Total (*Sad1*)					
0.0	21.18	21.13	21.02	21.11	0.08
0.5	21.32	20.72	21.11	21.05	0.30
1.0	21.59	21.29	20.72	21.20	0.44
2.0	20.88	21.52	21.71	21.37	0.43
5.0	21.23	21.19	21.18	21.20	0.03
10	21.35	21.35	20.99	21.23	0.21
GK19 (*Cry1A(c)*)					
0.0	–	–	–	–	–
0.5	31.34	31.29	31.15	31.26	0.10
1.0	30.21	30.42	29.67	30.1	0.39
2.0	29.06	29.23	29.04	29.11	0.10
5.0	27.56	27.82	27.27	27.55	0.28
10	26.82	26.34	26.52	26.56	0.24

Amplified reactions for two known GMO samples [contents of 1% and 5% (w/w) containing Mon531 and GK19, respectively, were quantified in triplicate with the duplex RT-PCR system. The Ct values for each sample were then applied to the standard calibration graphs which were obtained from the calculated insect-resistant cotton contents (Table 5). In the assay, results of 1.09% and 5.18% GM content were obtained, respectively, for Mon531 and GK19. Comparison of the quantified GMO contents with the known percentage indicated that the established duplex RT-PCR system employing the *Sad1* gene and *Cry1A(c)* gene as endogenous and exogenous gene, respectively, was easily and reliably applied to the samples containing a low quantity of DNA.

The results of the experiments described herein demonstrate that the *Sad1* gene satisfies the criteria for an endogenous reference gene—i.e. species-specificity, low copy number and high homogeneity among *G. hirsutum* cultivars. Meanwhile, the results obtained from the duplex RT-PCR quantitative analyses carried on mixed cotton samples derived from insect-resistant cottons suggest that the reported PCR systems for *Sad1* gene are suitable for practical use in the identification and quantification of GM cottons.

Table 4 Sensitivity test of the *Cry1A(c)* gene with duplex TaqMan RT-PCR

DNA amounts (ng/reaction)	Ct value for reaction			Mean	Standard deviation
	1	2	3		
100.00	19.87	20.00	20.22	20.03	0.14
10.00	24.29	24.46	24.09	24.28	0.15
1.00	29.26	29.08	28.71	29.01	0.23
0.100	31.71	31.47	31.76	31.65	0.13
0.010	35.85	35.53	36.41	35.93	0.36
0.001	–	–	–	–	–

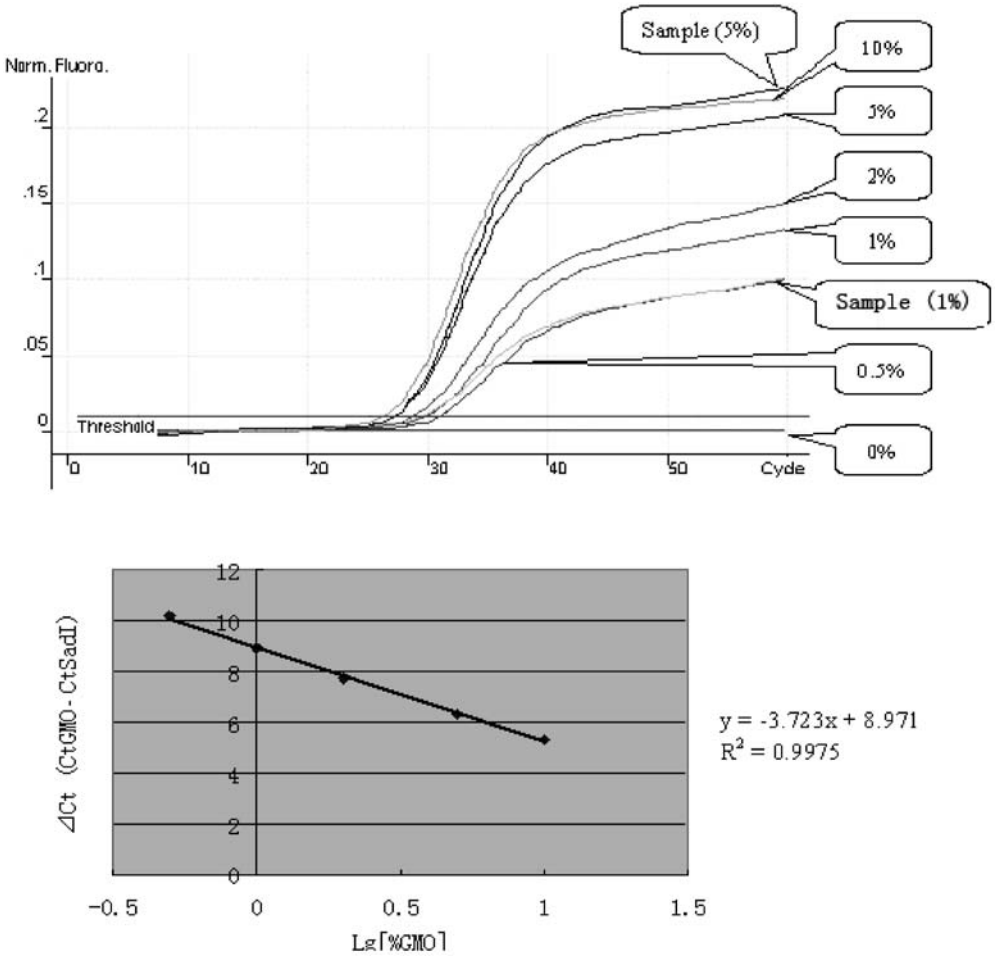

Fig. 6 Amplification plots for insect-resistant cotton detection in duplex RT-PCR employing the endogenous reference gene *Sad1* and exogenous gene *Cry1A(c)*, and the standard curve, plotting log [genetically modified organism (GMO) concentration] versus change in threshold of detection (△Ct)

Table 5 Determination of insect-resistant cotton content in known GMO content samples derived from mixed insect-resistant cotton flours using duplex TaqMan RT-PCR

Sample	GM cotton (%)	*Sad1* Ct (Mean)	*Cry1A(c)* Ct (Mean)	△Ct	Calculated GM cotton (%)
Mon531 cotton mixture	1.0	21.15	29.98	8.83	1.09
GK19 cotton mixture	5.0	21.26	27.57	6.31	5.18

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