JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 183−190 **183**

Validation of a Tomato-Specific Gene, *LAT52*, Used as an Endogenous Reference Gene in Qualitative and Real-Time Quantitative PCR Detection of Transgenic Tomatoes

LITAO YANG,[†,§] AIHU PAN,[#] JUNWEI JIA,[#] JIAYU DING,[#] JIANXIU CHEN,[§] HUANG CHENG,[§] CHENGMEI ZHANG,[#] 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

Toward the development of reliable qualitative and quantitative Polymerase Chain Reaction (PCR) detection methods of transgenic tomatoes, one tomato (*Lycopersicon esculentum*) species specific gene, *LAT52*, was selected and validated as suitable for using as an endogenous reference gene in transgenic tomato PCR detection. Both qualitative and quantitative PCR methods were assayed with 16 different tomato varieties, and identical amplified products or fluorescent signals were obtained with all of them. No amplified products and fluorescent signals were observed when DNA samples from 20 different plants such as soybean, maize, rapeseed, rice, and *Arabidopsis thaliana* were used as templates. These results demonstrated that the amplified *LAT52* DNA sequence was specific for tomato. Furthermore, results of Southern blot showed that the *LAT52* gene was a single-copy gene in the different tested tomato cultivars. In qualitative and quantitative PCR analysis, the detection sensitivities were 0.05 and 0.005 ng of tomato genomic DNA, respectively. In addition, two real-time assays employing this gene as an endogenous reference gene were established, one for the quantification of processed food samples derived from nontransgenic tomatoes that contained degraded target DNA and the other for the quantification of the junction region of *CaMV35s* promoter and the *anti-sense ethylene-forming enzyme* (*EFE*) gene in transgenic tomato Huafan No. 1 samples. All of these results indicated that the *LAT52* gene could be successfully used as a tomato endogenous reference gene in practical qualitative and quantitative detection of transgenic tomatoes, even for some processed foods derived from transgenic and nontransgenic tomatoes.

KEYWORDS: ***Lycopersicon esculentum*; *LAT52*; endogenous reference gene; GMO detection; qualitative and quantitative PCR**

INTRODUCTION

Since the first transgenic tomato, FLAVR SVAR, was approved for commercialization, transgenic techniques have been well developed (*1*), and many more genes have been introduced into plants, producing more and more genetically modified organisms (GMOs) with novel traits (*2*). During the seven-year period from 1996 to 2003, the global area of transgenic crops has increased 40-fold, from 1.7 million ha in 1996 to 67.7 million ha in 2003 (*3*). In China, the first commercialized transgenic plant was Huafan No. 1 tomato, in which an anti-sense ethylene-forming enzyme (*EFE*) gene to delay the ripening period was introduced (*4*). Up to now, six genetically modified (GM) crops, two kinds of insect-resistant cottons, virus-resistant sweet pepper, cauliflower mosaic virus (CMV) resistant tomato, shelf-life-altered tomato, and color-altered petunia, have been approved for commercialization in China (*5*−*7*). Moreover, GM soybean, maize, and rapeseed products are being imported from other countries to China. Pressure from consumer groups and public demands have led China to require labeling for the presence of GMOs in foods since 2001.

In conjunction with the labeling policy, PCR methods are considered to be the most common DNA detection method for identifying the presence of GMOs (*8*). These methods are very sensitive, and very small aliquots of vegetal material are required for the analysis. PCR methods are used not only for the identification of GM products but also for quantification purposes (*9*). To make the screen procedure more normative, one should detect the target sequences and the plant species-specific

* Author to whom correspondence should be addressed (telephone/fax +86 21 62208750; e-mail zdb3000@yahoo.com.cn).
[†] Shanghai Jiao Tong University
[§] Nanjing University.
[#] Shanghai Academy of Agricultural Sciences.

10.1021/jf0493730 CCC: $30.25
Published on Web 12/21/2004

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

PCR system	name	orientation	sequence (5′- - - 3′)	amplicon size (bp)
endogenous conventional and real time	Lat1	sense	AGACCACGAGAACGATATTTGC	92
	Lat2	antisense	TTCTTGCCTTTTCATATCCAGACA	
	Lp	probe	CTCTTTGCAGTCCTCCCTTGGGCT	
qualitative PCR for amplification of hybridization probe	Lat3	sense	AAAACTCAGCGAGAACCT	946
	Lat4	antisense	GGAACTTATCATCCCATA	
exogenous conventional and real time	E1	sense	TGTGATATCTCCACTGACGTAAGG	148
	E2	antisense	TTTAAATATAGCAATCTATGTATCGACTCTAG	
	Ep	probe	CAATCCCACTATCCTTCGCAAGACCCTTCCT	

endogenous reference gene. Among the PCR detection methods, fluorescence real-time PCR is considered to be an easy-to-use and accurate quantitative method (*9*−*11*). One unique feature of this PCR technique is that the amplification of target DNA sequence can be followed during the whole reaction by indirect monitoring of the product formation. 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 the primers specific for the transgene and the species-specific primers complementary to an endogenous reference gene. In practice, accurate relative quantification might be achieved by a combination of two absolute quantification reactions: one for the GMO-specific gene and the second for the plant endogenous reference gene (*9*, *12*).

For this purpose, much effort has been made to obtain the reference gene of different plants. Several endogenous reference genes of plants have been reported, for instance, the *zein* or *invertase1* genes for maize (*13*−*16*), *lectin* or *hsp* (heat-shock protein) genes for soybean (*13*, *17*, *18*), the *SPS* gene for rice (*19*), and *BnACCg8*, *cruciferin*, or *HMG I/Y* genes for rapeseed (*20*−*22*). However, there is no report about the endogenous reference gene for detection GM tomatoes. To our knowledge, seven GM tomato varieties have been authorized for commercialization in globe acres to date, that is, 351 N from Agritope Inc., 8338 and 5345 from Monsanto Co., 1345-4 from DNA Plant Technology Corp., FLAVR SAVR from Calgene Inc., B, Da, and F from Zeneca Seeds, and Huafan No. 1 from Huazhong Agriculture University, China, in which one *EFE* gene expression cassette containing one *CaMV35s* promoter, one *anti-sense EFE* gene, and one *NOS* terminator was inserted into the tomato genome with the new agronomic character of long shelf life (*5*, *21*, *23*).

In this paper, we report the specific primers and probe for the tomato *LAT52* gene and PCR cycling conditions suitable for the use of this sequence as an endogenous reference gene in both qualitative and quantitative PCR assays. The *LAT52* gene encodes a putative protein of 18 kDa that is cysteine rich and has an N-terminal hydrophobic region with characteristics similar to those of the eukaryotic secretory signal sequence (*24*, *25*). We used qualitative and real-time quantitative PCR assay to validate its species specificity, test the detection sensitivity, and also set up reliable qualitative and quantitative detection systems for transgenic Huafan No. 1 tomato samples and two processed foods.

MATERIALS AND METHODS

Materials. The seeds of 16 different tomato (*Lycopersicon esculentum*) cultivars, Dongnong No. 704, Shenfen No. 2, Maohong No. 1, Jifan No. 4, Zaokuai, Xifen No. 3, Lichunhongza No. 9, Zaofen, Zhongsu No. 5, Hongza No. 10, R144, Nongyou No. 301, Shuangfu, Shennong No. 2, Jiafen No. 1, and Yashu No. 6, were provided by the Horticulture Research Institute of the Shanghai Academy of Agriculture Science, China; all 16 different tomato cultivars were from different ecological areas with high degrees of variability (Prof. Zhu, personal communication), such as from Japan, Israel, The Netherlands, the United States, southern China, northern China, and western China. Other samples of 20 different species, *Sorghum vulgare* Pers, *Trifolium repens*, *Lolium perenne*, barley (*Hordeum vulgare*), cotton (*Gossypium hirsutum*), maize (*Zea mays*), rice (*Oryza sativa*), canola (*Brassica napus*), wheat (*Triticum aestivum*), *Arabidopsis thaliana*, sunflower (*Helianthus annuus*), soybean (*Glycine max*), *L. chinense* Mill, *Solanum melongena* L., potato (*Solanum tuberosum*), sweet pepper (*Capsicum annuum* L. var. *grossum*), pepper (*Capsicum annuum*), *Merrill L. esculentum* var. *cerasiforme*, *Solanum muricacun*, and tobacco (*Nicotiana tabacum*), were collected by our laboratory. Transgenic Huafan No. 1 tomato containing the *anti-sense EFE* gene was developed by Huazhong Agriculture University (HZAU), China, and was purchased from HZAU, and nontransgenic tomato, tomato fruits, tomato ketchup, and tomato juice were purchased from local markets in Shanghai, China.

DNA Extraction and Purification. The DNAs used for qualitative and quantitative real-time PCR detection were extracted and purified from 5 g of plant material or detected samples using the DNA extraction kit developed by Shanghai Ruifeng Agro-tech Co. Ltd. (Shanghai, China). The plant genomic DNA used for Southern blot analysis was extracted and purified from 20 g of dried seeds or fresh leaves according to the CTAB method (*24*). The quality of DNA was evaluated from the 260/280 and 260/230 nm UV absorption ratios and by agarose gel electrophoresis.

Southern Blot. Ten micrograms of DNA from each of the 16 different tomato cultivars (Dongnong No. 704, Shenfen No. 2, Maohong No. 1, Jifan No. 4, Zaokuai, Xifen No. 3, Lichun, Zaofen, Zhongsu No. 5, Hongza No. 10, R144, Nongyou No. 301, Shuangfu, Shennong No. 2, Jiafen No. 1, and Yashu No. 6) was completely digested with *Bam*HI and *Eco*RI, respectively. The digested DNAs were resolved in an 0.8% agarose gel electrophoresis and then transferred onto a nitrocellulose membrane, which was purchased from Gene Co., Ltd. (Shanghai, China). A 946 bp DNA fragment (from 798 to 1743 bp, Genbank No. 19263) amplified with primer pair Lat3/Lat4 of the *LAT52* gene was used as the hybridized probe. This DNA fragment was labeled by α-[^{32}P]-dCTP using Random primer DNA labeling kit ver. 2 from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Hybridization was performed at 62 °C for 24 h, and the filter was washed at room

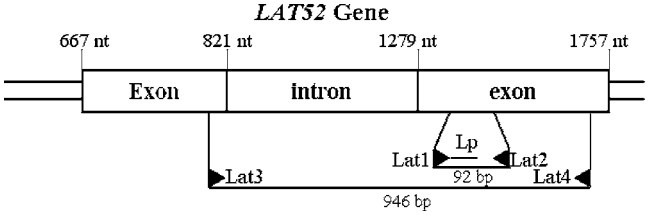

Figure 1. Schematic diagram illustrating the amplification fragments of *LAT52* gene and the positions of PCR primers: primer pairs Lat1/Lat2 were employed for qualitative PCR, primers Lat1/Lat2 and probe Lp for quantitative PCR analysis, and primer pairs Lat3/Lat4 for amplification of the Southern blot probe.

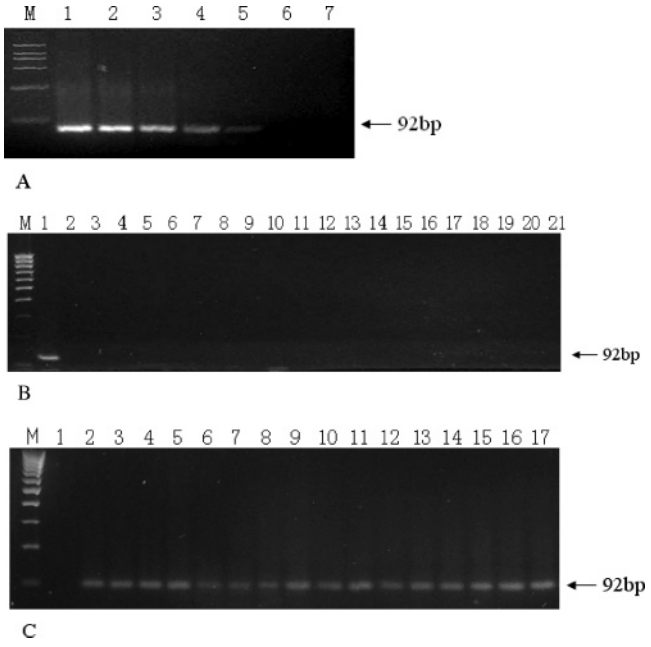

Figure 2. Two percent agarose gel electrophoresis of conventional PCR products amplified with the Lat1/Lat2 primer pair: (**A**) amplification results of serial dilutions of tomato DNA (lanes 1–6 correspond to 500, 50, 5, 0.5, 0.05, and 0.005 ng; lane 7 was a negative control; lane M, DL2000 marker); (**B**) amplification results of DNA from 20 different plant species (lane 1 was a tomato positive control; lanes 2–21, *Sorghum vulgare* Pers, *Trifolium repens*, *Lolium perenne*, *Hordeum vulgare*, *Gossypium hinsutum*, *Zea mays*, *Oryza sativa*, *Brassica napus*, *Triticum aestivum*, *A. thaliana*, *Helianthus annuus*, *Glycine max*, *L. chinense* Mill, *Solanum melongena* L., *Solanum tuberosum*, *C. annuum* L. var. *grossum*, *Capsicum annuum*, *Merrill Lycopersicon esculentum* var. *cerasiforme*, *Solanum muricacun*, and *Nicotiana tabacum*, respectively; lane M, 100 bp DNA ladder; no amplified products were obtained in all 20 different plant species); (**C**) amplification result of DNA from 16 different tomato lines (lane 1 was a negative control; lanes 2–17 correspond to Dongnong No. 704, Shenfen No. 2, Maohong No. 1, Jifan No. 4, Zaokuai, Xifen No. 3, Lichun, Zaofen, Zhongsu No. 5, Hongza No. 10, R144, Nongyou No. 301, Shuangfu, Shennong No. 2, Jiafen No. 1, and Yashu No. 6, respectively; lane M, 100 bp DNA ladder; the identical fragments were obtained from all 16 different tomato cultivars).

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 (*26*). DNA markers (*λ* DNA digested with *Hin*dIII and *Eco*RI) were run on the same gel.

Primers and Probes. PCR primers and TaqMan fluorogenic probes were designed with Primer Express 2.0 software (Applied Biosystems Division of Perkin-Elmer Corp., Foster City, CA) and are listed in **Table 1**. The endogenous probe was labeled with the fluorescent reporter dye 5-hexachloro-fluorescein (HEX) on the 5′ end and the exogenous probe with 6-carboxy-fluorescein (FAM) on the 5′ end. The fluorescent quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) was located on the 3′ end of the probes. The primer pair Lat1/Lat2 was used in qualitative PCR with one 92 bp amplified DNA fragment and was also used in combination with the Lp probe in real-time PCR for the quantification of total tomato DNA quantity; the construct specific detection primer pair E1/E2 of transgenic Huafan No. 1 tomato was employed to amplify one 148 bp junction DNA fragment between the *CaMV35s* promoter and the *anti-sense EFE* gene (*35s-EFE*) in qualitative PCR, and the probe Ep combined with them was employed for quantifying the GM contents of transgenic Huafan No. 1 tomato (*22*), and the primer pair Lat3/Lat4 with 946 bp amplicon was used for amplification of the hybridized probe for Southern blot analysis.

PCR Conditions. Conventional PCR was run in a PTC-100 (MJ Research, Waltham, MA) thermocycler. Each reaction mixture contained 1 × PCR buffer, 0.2 mM dNTP, 1 μM each primer, different DNA samples, and 1.5 units of Taq DNA polymerase; the final reaction volume was 30 μL. All reagents were from TaKaRa Biotechnology Co., Ltd., except for the primers. The amplification reaction ran using the following cycle conditions: denaturing of DNA 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 2% agarose gel electrophoresis in 0.5 × TBE and stained with ethidium bromide.

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. The PCR reaction mixture contained the following ingredients: 1 × PCR buffer, 300 nM primers, and 250 nM probes in an endogenous PCR system or 100 nM primers and 300 nM probes in an exogenous PCR system, 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: 5 min at 95 °C, 50 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. Fluorescence was monitored during every PCR cycle at the annealing step. Data were analyzed with Rotor gene 2000 software version 4.6 (Corbett Research).

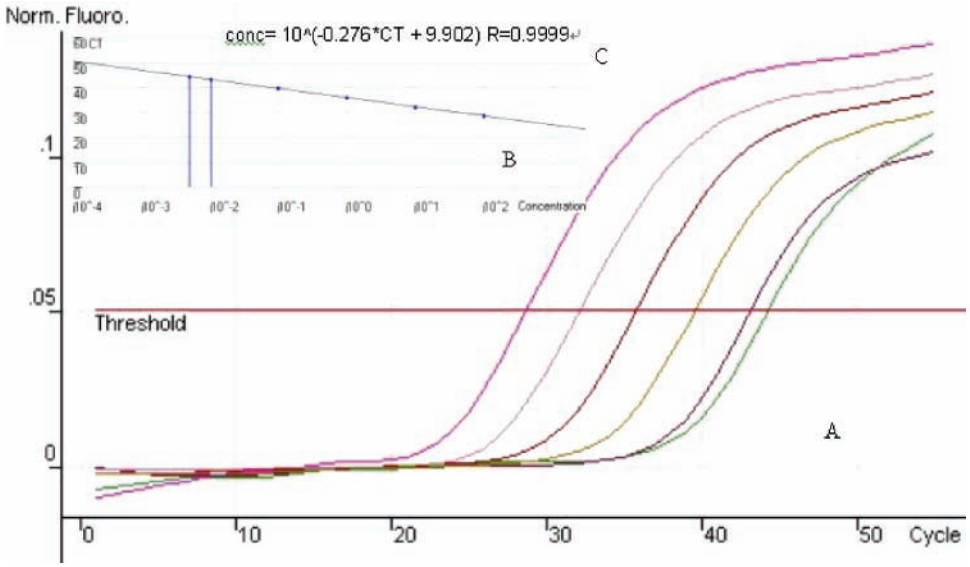

Figure 3. Sensitivity analysis of quantitative detection of real-time PCR: (**A**) amplification plot generated by serial dilutions of tomato DNA corresponded to 100, 10, 1, 0.1, 0.01, 0.005, and 0.001 ng; (**B**) standard curve generated from the amplification data given in (**A**); (**C**) equation and fit of (B).

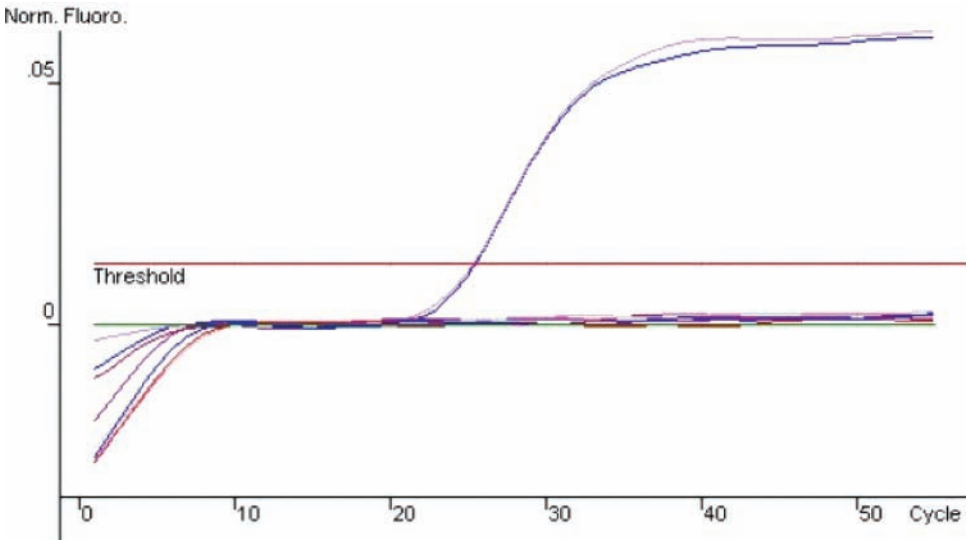

Figure 4. Specificity analysis of tomato *LAT52* gene in real-time PCR: amplification plot generated from 20 different plants described in **Figure 2B** and tomato seeds; no fluorescence signal was detected in the 20 different plants' PCR reactions.

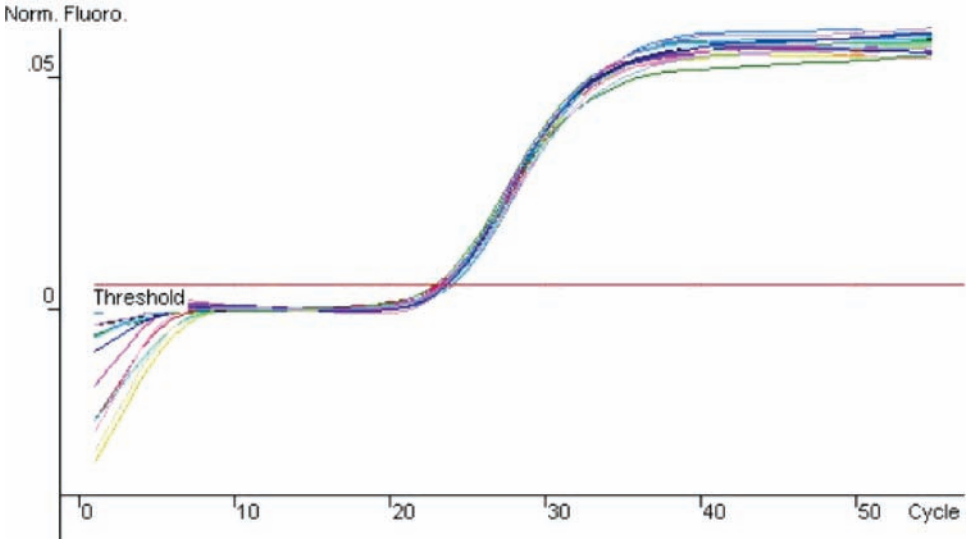

Figure 5. Allelic variation analysis of *LAT52* gene among tomato cultivars: amplification plot generated from 16 different tomato cultivars described in **Figure 2C**; the fluorescent signal with approximated intensity was detected from the 16 different tomato cultivars' PCR reactions.

Table 2. Reproducibility of the Tomato *LAT52* Gene with TaqMan Assay

DNA amount (ng/reaction)	Ct value for reaction					
	1	2	3	mean	SD	CV
100.000	27.09	27.16	27.34	27.20	0.11	0.14
10.000	30.27	30.57	30.47	30.44	0.12	0.09
1.000	33.79	33.96	34.18	33.98	0.16	0.14
0.100	37.45	37.41	37.87	37.58	0.21	0.17
0.010	41.60	41.50	41.55	41.55	0.04	0.13

All primers and fluorescent probes were synthesized and purified by Shanghai Shenyou Co. Ltd. (Shanghai, China), and the other real-time PCR reactants were purchased from Roche Molecular Biochemical (Shanghai, China).

In addition, to test the sensitivity of *LAT52* gene qualitative and quantitative PCR assays, tomato genomic DNA solutions were diluted corresponding to 500, 50, 5, 0.5, 0.05, and 0.005 ng/μL in the qualitative PCR system, and another series of DNA solutions with concentrations of 100, 10, 1, 0.1, 0.01, 0.005, and 0.001 ng/μL was used for real-time PCR. One microliter of tomato genomic DNA solution was added to each PCR reaction.

RESULTS AND DISCUSSION

Selection of the Tomato Endogenous Reference Gene, *LAT52*. The detection and quantification systems of GMOs depend on the use of endogenous reference genes. An endogenous reference gene should be species specific, with a low copy number in the genome, and exhibits low heterogeneity among cultivars (*20*). To select one tomato gene suitable for use as an endogenous reference gene for GMO detection, we searched the DNA sequences with a low copy number and then blasted those candidate sequences in the database (Genbank). Results of homologous analysis indicated that the sequence of the tomato *LAT52* gene (Genbank No. 19263) has low homogeneity with DNA sequences from other plants, such as *A. thaliana*, wheat, maize, barley, tobacco, soybean, rapeseed, rice, sunflower, pepper, Merrill, *L. esculentum* var. *cerasiforme*, and *S. muricacun*. Therefore, we chose the DNA sequence that encodes a heat-stable, glycosylated, cysteine-rich *LAT52* protein that is necessary in pollen development for further tests (*24*, *25*). The specific primers and fluorogenic probe of this DNA sequence were designed for both qualitative and real-time quantitative PCR assays; their positions are shown in **Figure 1**.

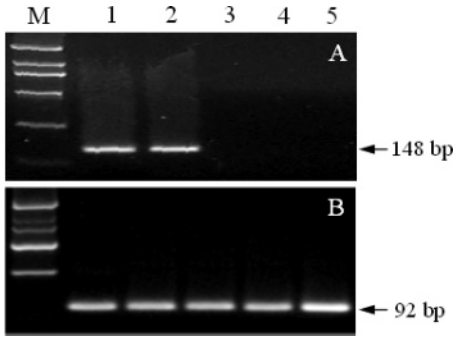

Figure 6. Conventional PCR detection of mixed Huafan No. 2 tomato samples and tomato processed foods: (**A**) *35s-EFE* gene; (**B**) tomato endogenous reference gene *LAT52*; lane M, DL2000 DNA molecular marker; lane 1, mixed Huafan No. 1 tomato leaves; lane 2, mixed Huafan No. 1 tomato seeds; lane 3, tomato juice; lane 4, tomato ketchup; lane 5, tomato fruits. PCR-amplified products were electrophoresed on 2% agarose gel. Arrowheads indicate the expected length of PCR-amplified fragments.

Sensitivities of Qualitative and Quantitative Real-Time PCR Assays. In the sensitivity test, qualitative PCR allowed detection in 50 pg of genomic DNA (**Figure 2A**). These data were similar to those of *lectin* or *10-kDa-zein* used in GMO qualitative detection (*13*, *27*). In the real-time PCR system, we could detect the amplified fluorescent signal with as little as 5 pg of tomato template DNA (**Figure 3**). On the basis of the tomato genome size of 0.74 pg per haploid genome (*28*, *29*), the sensitivity of the *LAT52* real-time system corresponds to an average of seven copies of the haploid genome of tomato.

Reproducibility of *LAT52* Gene Real-Time PCR. To test the reproducibility of the quantification PCR system, we generated the standard curve three times, each time with three parallel repetitions using five tomato DNA dilutions ranging 10-fold from 100 ng/μL to 10 pg/μL as templates. In real-time PCR, the PCR cycle number at which fluorescence reaches a threshold value is used for quantitative measurement; this cycle number is called the cycle threshold (Ct), and it is inversely proportional to the starting amount of target DNA. In the reproducibility test, the Ct values ranged from 27.20 to 41.55,

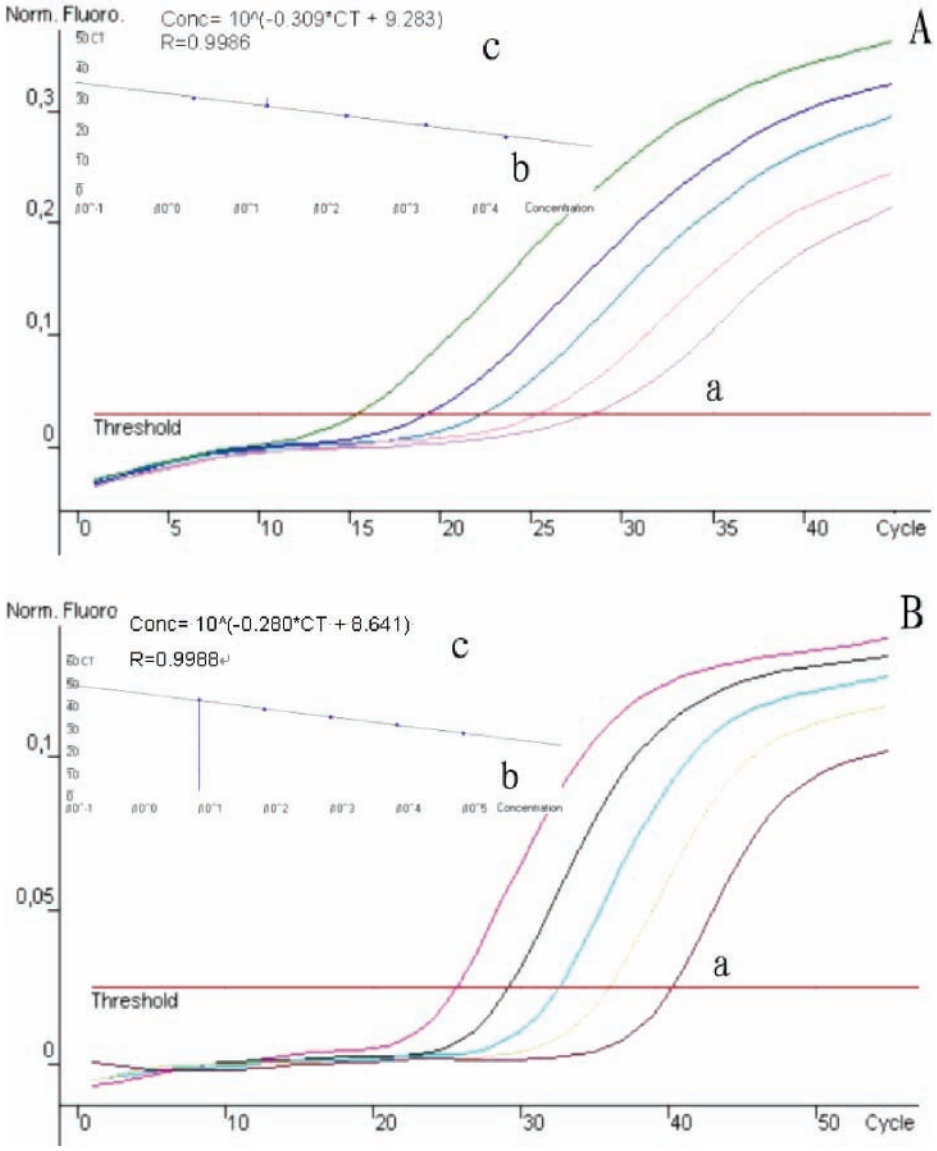

Figure 7. Constructed standard curves of endogenous and exogenous real-time PCR systems: (**A**) standard curve of *35s-EFE* gene quantification [**a**, amplification plot generated by known amounts of DNA with *35s-EFE* gene real-time PCR system (E1/E2 and Ep oligonucleotide); **b**, standard curve from the data given in **a**; **c**, equation and fit of **b**]; (**B**) standard curve of *LAT52* gene real-time PCR system for total tomato DNA quantification with primer pair Lat1/Lat2 and probe Lp. For details, see the description of (**A**).

Table 3. Determination of Known GM Content Tomato Sample

sample	probe dye	Ct value for reaction 1	2	3	mean	SD	% GM contents
Endogenous *LAT52* Gene							
sample (1%)	HEX	28.34	28.38	28.21	28.31	0.09	
sample (0.5%)	HEX	28.23	28.29	28.31	28.28	0.04	
Exogenous *35s-EFE* Gene							
sample (1%)	FAM	32.11	32.35	32.26	32.24	0.12	1.10
sample (0.5%)	FAM	33.3	33.42	33.19	33.30	0.12	0.51

the standard deviation (SD) values ranged from 0.04 to 0.21, and the coefficient of variation (CV) values ranged from 0.09 to 0.17 (**Table 2**). The little variation of SD and CV values derived from these tests indicated that the real-time detection system worked stably and reliably.

Species Specificity of the *LAT52* Gene. To test the species specificity of the amplified *LAT52* DNA fragment, we ran both qualitative and real-time PCR reactions on 20 ng of template DNA from 20 different plant species that were either evolutionarily related to tomato or frequently used as transgenic plant materials. These included *S. vulgare* Pers., *T. repens*, *L. perenne*, barley (*H. vulgare*), cotton (*G. hirsutum*), maize (*Z. mays*), rice (*O. sativa*), canola (*B. napus*), wheat (*T. aestivum*), *A. thaliana*, sunflower (*H. annuus*), soybean (*G. max*), and more related plants in evolution from solanaceae such as *L. chinense* Mill, *S. melongena* L., potato (*S. tuberosum*), sweet pepper (*C. annuum* L. var. *grossum*), pepper (*C. annuum*), *Merrill L. esculentum* var. *cerasiforme*, *S. muricacun*, and tobacco (*N. tabacum*.). No amplified products were observed with any of these tested species other than tomato in conventional PCR (**Figure 2B**) and real-time PCR (**Figure 4**). These results demonstrated that the *LAT52* gene was highly specific for tomato in both conventional and real-time PCR analyses.

Allelic Variation of the *LAT52* Gene among Tomato Cultivars. An ideal endogenous reference gene should not exhibit allelic variation among varieties of the same species; meanwhile, it should be present as a consistent low copy number in the different varieties. To investigate whether different tomato cultivars exhibit allelic variation within the amplified *LAT52* sequence, we performed both qualitative and real-time PCR on a fixed amount of 20 ng of DNA from 16 different tomato cultivars. As shown in **Figure 2C**, identical PCR products were obtained from all of the tested cultivars in conventional PCR. Real-time PCR analysis performed with triplicates of DNA from these tomato cultivars exhibited similar Ct values (**Figure 5**). These results indicated that there were no major sequence differences among the different cultivars in this amplified region. The little variation of the Ct values of real-time PCR analysis also showed that the copy number of the *LAT52* gene was consistent among the tested tomato cultivars.

Copy Number of *LAT52* Gene. To analyze the copy number of the *LAT52* gene in tomato, we performed Southern blot analysis in which tomato genomic DNA was completely digested with *Eco*RI or *Bam*HI hybridized with a 946 bp *LAT52* fragment. A single hybridization band was obtained in the digested DNA from 16 tomato cultivars (data not shown), which indicated that the *LAT52* gene was a single copy in the tomato genome.

Qualitative PCR Analysis of GM Tomato Samples. On the basis of the results validating the *LAT52* gene as an endogenous reference gene for GMO detection, we further employed the established PCR systems for qualitative PCR detection of GM or non-GM samples. In the qualitative PCR detections, the primer pair E1/E2 allowed the amplification of a 148 bp junction DNA fragment between the *CaMv35s* promoter and the *anti-sense EFE* gene (*35s-EFE*) of transgenic Huafan No. 1 tomato and a 92 bp fragment from the *LAT52* gene for the primer pair Lat1/Lat2 amplification. When the purified DNA solutions of tomato samples, that is, mixed Huafan No. 1 tomato seeds, mixed Huafan No. 1 tomato leaves, tomato fruits, tomato ketchup, and tomato juice, were amplified respectively, the expected amplified fragments of the *35s-EFE* gene (**Figure 6A**) were detected in the reactions of Huafan No. 1 tomato samples except for the non-GM tomato samples. The expected amplified fragments of the endogenous reference *LAT52* gene (**Figure 6B**) were obtained in the five samples. These results indicated that our established GM tomato qualitative detection systems employing the *LAT52* gene as the endogenous reference gene could successfully be used in tomato fruit, seed, leaf, and two processed foods.

Quantitative PCR Analysis of Mixed Known and Unknown GM Content Tomato Samples with Endogenous and Exogenous Real-Time PCR Assays. The transgenic tomato

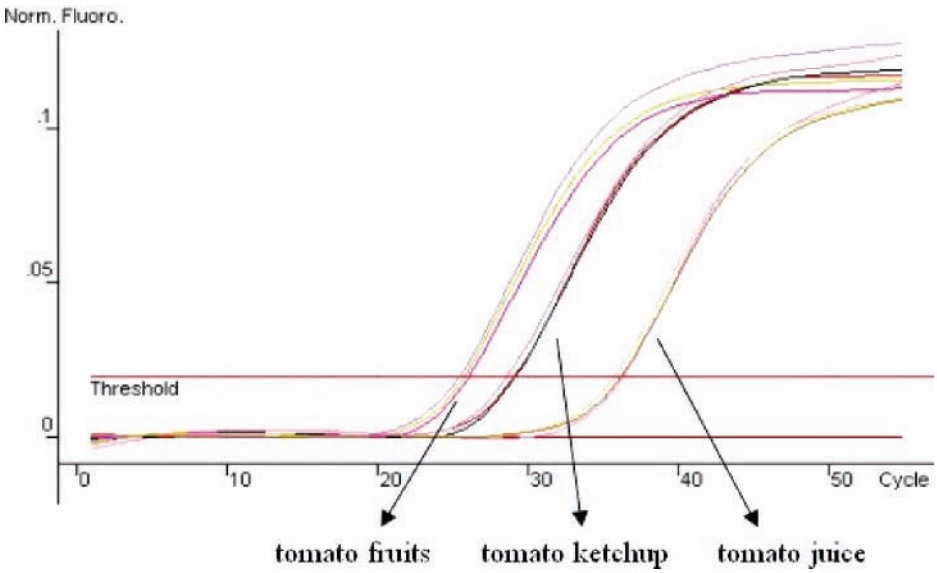

Figure 8. Detection of tomato DNA from tomato fruits, tomato ketchup, and tomato juice using the *LAT52* gene real-time PCR assay with the Lat1/Lat2 primer pair and the Lp TaqMan probe.

samples could be easily quantified using standard curves generated with a scale of diluted DNAs from known percent transgenic tomatoes. For the generation of standard curves, stock DNA from 18.5% transgenic Huafan No. 1 tomatoes was diluted to the following concentrations: 100, 10, 1, 0.1, and 0.01 ng/μL. One microliter of DNA solution was added into the amplification reaction, given 18.5, 1.85, 0.185, 0.0185, and 0.00185 ng for transgenic DNA quantities and 100, 10, 1, 0.1, and 0.01 ng for endogenous DNA quantities, respectively. The PCR efficiencies of the *LAT52* and *35s-EFE* genes were 0.91 and 1.04, respectively, which indicated that these two real-time PCR assays worked normally (*30*). The calculated *R* value of the *35s-EFE* gene standard curve was 0.9988 (**Figure 7A**), that of the *LAT52* gene was 0.9986 (**Figure 7B**). Thus, these two real-time quantitative PCR assays allowed us to quantify GMOs over a very large range of starting DNA quantities.

In quantitative analysis, two mixed known GM content tomato samples (0.5% fresh transgenic Huafan No. 1 leaves and 1% transgenic Huafan No. 1 seed flour) and three non-GM content tomato samples (tomato fruits, tomato ketchup, and tomato juice) were detected and quantified separately according to the optimized standard curves. In the quantification measurement of two known GM contents mixed samples, the quantified results of mixed tomato leaves with 0.5% GM contents were 0.51 and 1.10% contents for the 1% GM content tomato seed flour (**Table 3**). By comparing the quantified results of these two mixed tomato samples with their known GM contents, we found that the quantified results were slightly deviated from their own GM contents, which indicated that the established real-time PCR systems were relatively accurate and creditable.

In the quantitative detection of three non-GM tomato samples, no fluorescent signal was obtained in the *35s-EFE* gene real-time PCR assay, and highly intense fluorescent signals were obtained in the *LAT52* PCR assay instead. Through analysis of the amplified curves (**Figure 8**) of the endogenous reference *LAT52* gene, the mean Ct value of tomato fruits was 25.92, that of tomato ketchup was 28.52, and that of tomato juice was 34.93; the calculated tomato DNA amount of tomato ketchup was 16.829 ng/μL and that of tomato juice, 0.362 ng/μL, according to the generated standard curves (**Figure 7B**). The results proved that the established real-time PCR system was also effective and suitable for the detection of processed foods derived from tomatoes.

Conclusions. In this paper, all of the results demonstrated that the tomato *LAT52* gene accorded with the requirement of the endogenous reference gene, such as species-specificity, low copy number, and high homogeneity among cultivars. Meanwhile, PCR detection results of GM tomato samples and non-GM samples suggested that the reported PCR systems employing the *LAT52* gene as the endogenous gene were suitable for practical use in the qualitative and quantitative monitoring of GM tomatoes.

ACKNOWLEDGMENT

We thank Prof. Weimin Zhu for providing tomato cultivar seeds.

LITERATURE CITED

(1) Food and Drug Administration (FDA). *Fed. Regist*. **1994**, *59*, 26700−26711.
(2) Einspanier, R. Quantifying genetically modified material in food: searching for a reliable certification. *Eur. Food Res. Technol*. **2001**, *213*, 415−416.
(3) James, C. Preview: Global status of commercialized transgenic crops. *ISAAA Briefs* **2003**, *30*.
(4) Ye, Z.; Li, H.; Liu X. A New Transgenic Tomato Cultivar BIOSCIEN Bred With Antisense *EFE* cDNA. *China Vegetables* **1999**, *1*, 10−12.
(5) Huang, J.; Rozelle, S.; Pray, C.; Wang, Q. Plant biotechnology in China. *Science* **2002**, *295*, 25.
(6) www.libnet.sh.cn/szzy/kepu/2001/01000720zjy.htm; 2004, 1.
(7) www.syinfo.gov.cn; 2004, 1.
(8) 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.
(9) Bonfini, L.; Heinze, P.; Kay, S.; Van den Eade, G. Review of GMO detection and quantification techniques. *EUR 20348 EN*, 2002.
(10) 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.
(11) Terzi, V.; Ferrari, B.; Finocchiaro, F.; Di Fonzo, N.; Stanca, A. M.; Lamacchia, C.; Napier, J.; Shewry, P. R.; Faccioli, P. TaqMan PCR for Detection of Genetically Modified Durum Wheat. *J. Cereal Sci*. **2002**, *37* (2), 157−163.
(12) Farid, E. A. Detection of genetically modified organisms in foods. *Trends Biotechnol*. **2002**, *20*, 215−223.
(13) Vaĭtilingom, M.; Pijnenburg, H.; Gendre, F.; Brignon, P. Real-time quantitative PCR detection of genetically modified Maximizer maize and Roundup Ready soybean in some representative foods. *J. Agric. Food Chem*. **1999**, *47*, 5261−5266.
(14) Studer, E.; Dahinden, I.; Lüthy, J.; Hübner, P. Nachweis des gentechnisch veranderten "Maximizer"-mais mittels der polymerase-kettenreaktion (PCR). *Mitt. Gebiete Lebensm. Hyg*. **1997**, *88*, 515−524.
(15) Zimmermann, A.; Hemmer, W.; Liniger, M.; Lüthy, J.; Pauli, U. A sensitive detection method for genetically modified MaisGard corn using a nested PCR-system. *Lebensm. Wiss. -Technol*. **1998**, *31*, 664−667.
(16) Vollenhofer, S.; Burg, K.; Schmidt, J.; Kroath, H. Genetically modified organism in food-screening and specific detection by Polymerase Chain Reaction. *J. Agric. Food Chem*. **1999**, *47*, 5038−5043.
(17) Terry, C. F.; Harris, N. Event-specific detection of Roundup Ready soya using two different real time PCR detection chemistries. *Eur. Food Res. Technol*. **2001**, *213*, 425−431.
(18) Dujin, G. van.; Biert, R. Van.; Bleeker-Marcelis, H.; Peppelman, H.; Hessing, M. Detection methods for genetically modified crops. *Food Control* **1999**, *10*, 375−378.
(19) 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.
(20) 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.
(21) Delano, J.; 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*, 5839−5834.
(22) Weng, H.; Yang, L.; Ding J.; Liu, Z.; Zhang, D. A novel reference gene, high mobility group protein I/Y, can be used in quanlitative and real-time PCR detection of transgenic rapeseed cultivars. *J. AOAC Int*. **2005**, in press.
(23) www.agbios.com, 2004; 10.
(24) Twell, D.; Wing, R.; Yamaguchi, J.; McCormick, S. Isolation and expression of an anther-specific gene from tomato. *Mol. Gen. Genet*. **1989**, *217*, 240−245.
(25) Muschietti, J. P.; Dircks, L.; Vancanneyt, G.; McCormick S. *LAT52* protein is essential for tomato pollen development: pollen expressing antisense *LAT52* RNA hydrates and germinates abnormally and cannot achieve fertilization. *Plant J*. **1994**, *6*, 321−338.

(26) Sambrook, J.; Russell, D. W. *Molecular Cloning: A Laboratory Manual* (3 Volumes); Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.
(27) Wurz, A.; Bluth, P.; Zeltz, P.; Pfeifer, C.; Willmund, R. Quantitative analysis of genetically modified organism (GMO) in processed food by PCR-based methods. *Food Control*. **1999**, *10*, 385−389.
(28) Arumuganathan, K.; Earle, E. D. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep*. **1991**, *9*, 208−218.
(29) Bernatzky, R.; Tansely, S. D. Methods for detection of single or low copy sequences in tomato on southern blots. *Plant Mol. Biol. Rep*. **1986**, *4*, 37−41.
(30) Alary, R.; Serin, A.; Maury, D.; Jouira, H. B.; Sirven, J. P.; Gautier, M. F.; Joudrier, P. Comparison of simplex and duplex real-time PCR for the quantification of GMO in maize and soybean. *Food Control* **2002**, *13*, 235−244.

Received for review April 19, 2004. Revised manuscript received October 31, 2004. Accepted November 3, 2004. This work was supported by the Fund of National Key Basic Research Developments Program of the Ministry of Science and Technology, China (2001CB109002), the National Transgenic Plant Special Fund (JY03-B-20), the National Natural Science Foundation of China (30370893), and the Shanghai Municipal Committee of Science and Technology (03D.Z.19307, 03D.Z.05032).

JF0493730