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Validation of a genus-specific gene; *TPS*, used as internal control in quantitative Real Time PCR of transgenic cotton

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Abstract

Identification of genes with invariant levels of gene expression is a prerequisite for validating transcriptomic changes accompanying development. Ideally expression of these genes should be independent of the morphogenetic process or environmental condition.We report here the validation of internal control gene i.e.*TPS* (trehalose 6-phosphate-synthase) in cotton (*Gossypium* spp), using TaqMan system in quantitative Real Time PCR (qRT-PCR). The Gene expression was tested in five different *G. hirsutum* cultivars including Coker 312, Acala SJ, ZETA 2, Taghva, Neishabour and a diploid wild type*; G. barbadense*. Identical amplicons were obtained within these cultivars. No amplifications was achieved when DNA samples from barley (*Hordeum vulgare*), maize (*Zea mays*), rice (*Oryza sativa*), Fig (*Ficus carica*), pistachio (*Pistacia vera*), yew (*Taxus baccata*), wheat (*Tirticum aestivum*), rose (*Rosa hybrida*) and soybean (*Glycine max*) were used as template. Therefore, it was confirmed that the primers and probes designed in this study were specific for the identification and quantification of internal control gene. These results reveal the possibility of using the *TPS* gene as an internal control in cotton. In another word, this gene would be a suitable candidate as a reference gene in examination of gene expression, detection of transgenic cotton and determining e the zygosity as well as the copy number of the transgene.

Keywords: *Gossypium* spp, trehalose 6-phosphate-synthase gene (TPS), endogenous reference gene, RT-PCR, transgene.

Abbreviations: BHQ (black hold quencher), *TPS* (trehalose 6-phosphate-synthase), TET (tetrachloro-6-carboxyfluorescein).

Introduction

Real Time PCR is the most sensitive and flexible method for measuring gene expression. This can be efficiently used for comparison of relative levels of transcripts' abundance and detection of differentially expressed genes under different conditions.

Data obtained by gene expression methods can be strongly affected by different factors such as RNA extraction procedures, and cDNA synthesis. To tackle this obstacle, usually the level of gene expression will be compared with the expression level of a house keeping gene as an internal control. This is necessary that previous identified such genes, which can then be reliably used to normalise relative expression of genes of interest (Izaskun *et al*, 2010). Cotton (*Gossypium* spp.) is the most important fiber crop in the world and has been situated the target of various genetic modifications to improve its agronomic traits and fiber quality in the last decade. When new transgenic cotton plants are obtained, one early and essential step is their molecular characterization, such as determining transgene copy number and gene expression level. Development of endogenous reference gene for detection transgenic plant and gene expression is the basic step in risk assessment. Currently more than 17 million ha transgenic cotton exists and this cultivation area is anticipated to grow increasingly. Developing quantitative and precise instructions such as real time PCR in order to detect and identify such products is necessary (James, 2010).

At present, the most creditable and widely used methodology that enables the primary detection of transgenic plants and their molecular characterization is the conventional PCR method. Among the PCR detection methods, fluorescence quantitative real-time (RT)-PCR is considered to be an easy, useful and accurate method (Zhang *et al*. 2003). Using this method, a target gene (transegene) can be quantified by plotting a standard curve using known quantities of an endogenous gene and the consequent extrapolation based on the linear regression line. In order to make theses PCR procedures more standardized, the target sequences and plant genus-specific endogenous reference genes should be detected. This system requires both specific primers to the transgene and genus-specific primers complementary to an endogenous reference gene.

So far, much effort has been made to obtain the reference gene of different plants for the primary detection of GMOs and their molecular characterization. Several plants internal reference genes have been reported. For examples reference genes *arezein* or *invertase* 1 for maize (Valtilingom *et al*. 1999; Zimmermann *et al.* 1998), *lectin* or *hsp* (heat-shock protein), for soybean (Duijn *et al*. 1999), *BnACCg8,cruciferin* or *HMGI/Y* for rapeseed (James *et al*. 2010; Weng *et al*. 2005), *LAT52* for tomato (Yang *et al*. 2005b) and *SPS* for rice (Ding *et al* .2004). However, there are only a few reports on an endogenous reference gene for GM cotton identification and quantification (Yang *et al*. 2005a).

The reliability of PCR systems for primary detection and quantification of GMOs in order to determine the transgene copy number and zygosity level

depends on the availability of an appropriate endogenous reference gene. Reference genes should be species- or genus-specific, and exhibit a low level of heterogeneity among cultivars.

Therefore, this study was set to search for an appropriate cotton gene to be used as an endogenous reference gene for PCR amplification. Moreover, specific primers and probe for that particular cotton gene were designed, and the quantitative real-time PCR conditions were optimized. Finally, the specificity of the primers and probes designed to various species including cotton was tested using TaqMan PCR method.

Materials and methods

To select an appropriate cotton gene, *TPS*, to be used an endogenous reference gene for PCR amplification, all gene databanks (GenBank-Gene-nucleotide) were searched for sequences of a low copy number within the genome.

The seeds of five different cotton (*G.hirsutum*) cultivars, including Coker 312, Acala SJ, ZETA 2, Taghva, Neishabour and one diploid wild cultivar (*G.barbadense*) were provided by the Agricultural Biotechnology Research Institute of Iran (ABRII).The seeds or leaves of 9 different plants including barley (*Hordeum vulgare*), maize *(Zea mays),* rice *(Oryza sativa*), wheat *(Tirticum aestivum)*, Fig (*Ficusc arica*), pistachio (*Pistaci avera*), yew (*Taxus baccata*), Rose (*Rosa hybrida*) and soybean *(Glycine max)* were collected from a number of agriculture research institute across Iran. Transgenic cotton (Coker, Line 61), containing a synthetic *cry1Ab* gene developed by ABRII was also used (Tohidfar *et al*. 2008).

DNA extraction and purification

Plant genomic DNA used for real-time-PCR were extracted from 1g fresh leaves using the CTAB method (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.

All oligonucleotides for TaqMan were designed by Beacon Designer 7.0.The internal oligonucleotide probe specific to the candidate gene chosen were labeled at the 5′ end with TET (tetrachloro-6-carboxyfluorescein), whereas 3′ end was labeled with the BHQ (black hole quencher).

Optimization of PCR conditions

Following PCR program was carried out for TaqMan polymerase chain reaction: 2 min at 95 °C, 30 cycles; 8 s at 94 °C, 20 s at 60 °C and 1min at 72 °C.

The concentrations of the all primers were adjusted at 200 nM. Real-time detection of fluorescence emission was performed using Real Time PCR machine (Model Rotor Gene 3000, Corbett Research, Australia). To set up a reliable and sensitive real-time PCR system for identification of zygosity level of transgenic cotton, one duplex Real-time PCR system was optimized and

established using *cry1A(b)* and the selected candidate gene. Then the standard calibration curve was plotted using logarithm of insect-resistant cotton concentration against ΔCt $Ct_{TRANSGENIC}-Ct_{TPS}$), by duplicate amplification of reference DNA; Ct value can be defined as the threshold cycle value (Livak and Schmittgen 2001; Terry and Harris 2001).

Results and discussion

Selection of an endogenous reference gene for cotton

To select an appropriate cotton gene to be used an endogenous reference gene for PCR amplification, all gene databanks were investigated for sequences of a low copy reference gene within the genome, to produce a cotton-specific product. The several candidate genes selected were then blasted in the database to determine their homology. The results of homologous analysis indicated that the sequence of *TPS* gene (with accession No.AY628140) had the lowest homogeneity with the DNA sequences of the other plants. The *TPS* gene has one copy per *Gossypium* genome (AADD) and encodes trehalose-6-phosphate-synthase enzyme in the trehalose biosynthesis pathway (Javadi *et al*. 2007; Zimmermann *et al* 2004; Eastmond *et al*. 2003).

Following the reference gene selection and based on the full sequence of the *TPS* gene, an appropriate forward primer (655 to 677 bp, corresponding to the *TPS* gene sequence in the GenBank with the accession number of AY628140) and a reverse primer (731 to 754 bp), as well as a specific probe (681 and 704) were designed by Beacon Designer 7.90 software. The designed primer pair successfully amplified a 100 bp fragment. The details of the designed primers and probe are presented in Table 1. Finally, the specificity of the primers and the probe for *TPS* gene sequence when used in quantitative real- time PCR assays was confirmed (Figure 1).

Table 1. The primers and TaqMan probe used for Real Time-PCR.

PCR system	Name	Orientation	Sequence(5′-3′)	Length (bp)
Endogenous real-time PCR	TPS F	Sense	*5′-ACGAACTTTC CCATTTCCTTT CG-3′*	23
	TPS R	Anti-Sense	*5′-CGAGAAGGA GATACTTACTT GCAG-3′*	24
	TPS P	Probe	*TET-5′-CCCACAAATC CGCCTCTCCT CCGC-3′-BHQ*	24

Sensitivity of the real-timePCR assay for the *TPS* gene

Following the optimization of the magnesium and primer/probe concentrations, the sensitivity tests of Real Time-PCR was also performed (Figure 1). A serial dilution of the cotton genomic DNA was prepared at the concentrations of 100, 10 and 1 ng/ l in

order to determine the sensitivity of the real-time PCR assay for the selected reference gene; TPS. A 2 micro liter aliquot of the DNA template solution was added to the PCR reaction. In the real-time PCR assay, cotton genomic DNA as little as 20 ng resulted in a successful amplification and a consequent detection of the *TPS* gene in genomic DNA (Table 2).

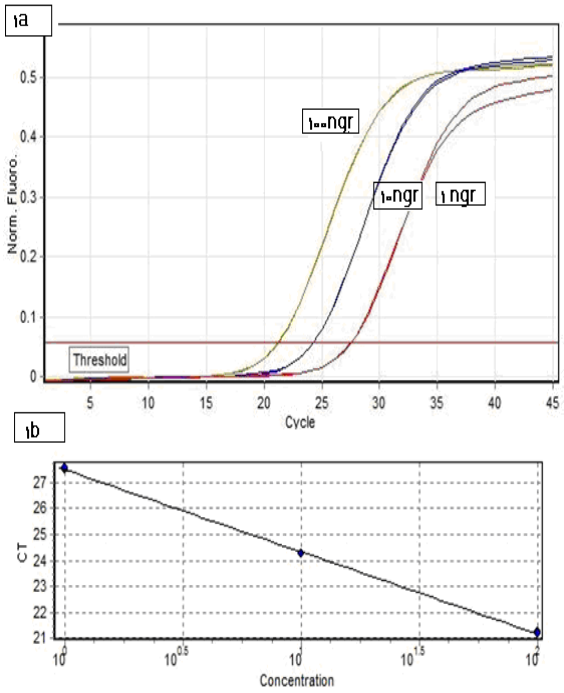

Figure 1. Real-time PCR amplification and the standard curve established for *TPS* gene .**1a** amplification plot of serial dilutions of cotton DNA (100 ng, 10ng, 1ng of cotton DNA) using the *TPS*-specific primers and probe described (shown in Table 1). Each sample was run in duplicate. 1b Standard curve obtained for the same samples. Correlation coefficient was 0.999 and reaction efficiency was 1.095. The calculated C_t values were plotted versus the log of each starting quantity.

Table 2. Sensitivity test of the *TPS* gene with single TaqMan RealTime-PCR.

Amount of DNA (ng/reaction)	Ct value reaction		Mean	Standard deviation
	1	2		
100.000	21.24	21.19	21.21	0.025
10.000	24.25	24.24	24.24	0.007
1.000	27.49	27.56	27.52	0.035

Genus specificity of the cotton *TPS* gene as an endogenous reference gene

To test the genus specificity of the *TPS* gene as an endogenous reference gene, 20-ng aliquots of genomic DNA extracted from 4 different plant genera that were either evolutionarily related to *Gossypium* spp. or frequently found as foodstuff were used as templates in real-time PCR reactions. These included barley (*Hordeum vulgare*), maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), Fig (*Ficus carica*), pistachio (*Pista ciavera*), yew (*Taxus baccata*), cut rose (*Rosa hybrida*) and soybean (*Glycine max*). Except for *Gossypium* spp, no amplification was observed when the DNA extracts of the other above-mentioned genera were tested (Figure.2a). The results demonstrate that the *TPS* gene is highly specific to *Gossypium* spp. when used in RealTime-PCR analyses. It should be mentioned that Sothern blot analysis has revealed that the *Gossypium* genome (AADD) contains only a single copy of TPS (Kosmas *et al,* 2006).

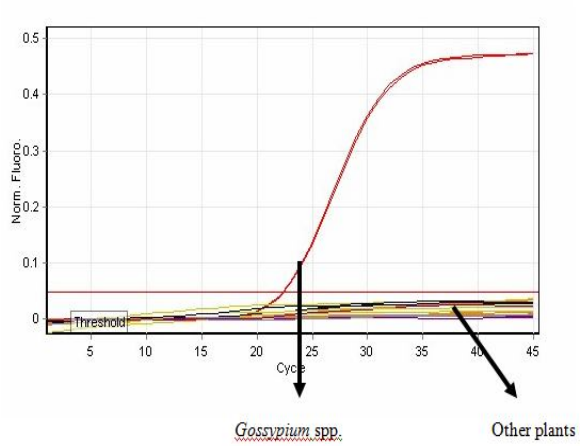

Figure 2.a. Genera specificity and allelic variation analysis of the *TPS* gene with the TaqMan real-time-PCR assay. **(a)** Specificity analysis of cotton *TPS* gene in TaqMan real-time PCR. The amplification plot was generated using 9 different plant genera corresponding to *Hordeum vulgare*, *Zea mays*, *Oryza sativa*, *Triticum aestivum*, *Pistacia vera*, *Taxusbaccata*, *Rosa hybrida* and *Glycine max* and *Gossypium* spp. seeds. No fluorescence signal was detected in these 9 different plants except for *Gossypium* spp.

Allelic variation of the *TPS* gene among *Gossypium* spp. cultivars

An ideal enodgenous reference gene should not exhibit allelic variation among varieties of the same species, while it should present a consistent low copy number in those cultivars (Yang *et al*. 2005 a). In the present study, the presence of allelic variation among a number of varieties of *Gossypium* spp. was investigated. In order to understand whether different *Gossypium* spp. cultivars exhibited allelic variation within the amplified *TPS* gene sequence, real-time PCR was performed using a fixed amount of DNA (20 ng) obtained from 6 different cotton cultivars. In real-time PCR analysis of the genomic DNA of the *Gossypium* spp. cultivars, similar Ct values from 22.40 to 23.65 (Figure 2b) were obtained in duplicate amplifications. These results indicated 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 real-time PCR analysis also confirmed that the copy number of the *TPS* gene was consistent among the tested *Gossypium* spp cultivars.

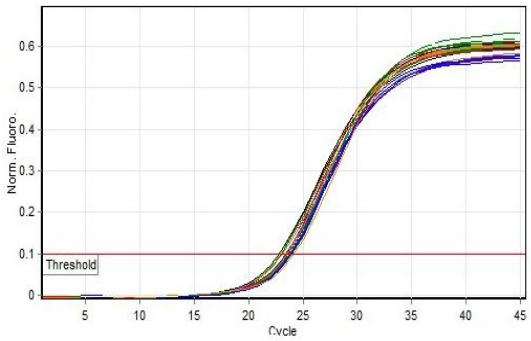

Figure 2.b. Allelic variation analysis of *TPS* gene among *Gossypium spp.*cultivars. The amplification plot was generated using 5 different *G. hirsutum* cultivars and *G. barbadense.*

Zygosity level confirmation of the insect–resistant cotton samples (*cry1A(b)*) using duplex Real-time PCR.

Following the optimization of the magnesium and primer/probe concentrations, the real-time PCR assay (comparative C_t TaqMan assay) was applied in order to confirm the zygosity level of transgenic cotton samples with pre-determined zygosity level using conventional methods. In this method, real-time PCR was performed while the amplification of the transgene of interest

was compared to that of an endogenous gene. By amplifying both genes in the same reaction tube simultaneously, identical conditions were provided. Zygosity level of the transgene of interest was determined by comparing the ΔC_t value of the transgene amplification to that of the endogenous gene. In the comparative Ct assay described in this paper, the trehalose 6-phosphate-synthase gene or *TP*S was used as an endogenous internalcontrol. Molecular, genetic and biochemical analyses indicate the existence of one TPS gene copy per *Gossypium* genome (Kosmas *et al*. 2006). The successand validity of the assay depends on the assurance that both the endogenous and transgene amplification occur at approximately equal efficiencies (Weng *et al*, 2004) (Figure 3). The efficiencies obtained for the transgene and the endogenous internal control gene were 1.18708 and 1.09563, respectively. Comparative Ct values (ΔCt) were calculated by subtracting the Ct values obtained for the TET-labeled endogenous gene reactions fromthe Ct values obtained for the FAM-labeled transgene reaction. subtracting the ΔCts of reference sample and the target. The homozygote nature of the target sample was confirmed using southern blot analysis in our previous study (Yazdanpanah *et al*. 2009). The relative concentration was calculated by the formula as: $2^{-\Delta\Delta Ct}$.

As cotton is an allotetraploid crop, using the amounts of relative concentration and according to Kosmas *et al.* (2006), homozygous plants were detected as those which are having four copy of inserted gene. Delta delta Ct values were then used as an indication of the quantity of transgene copy number relative to that

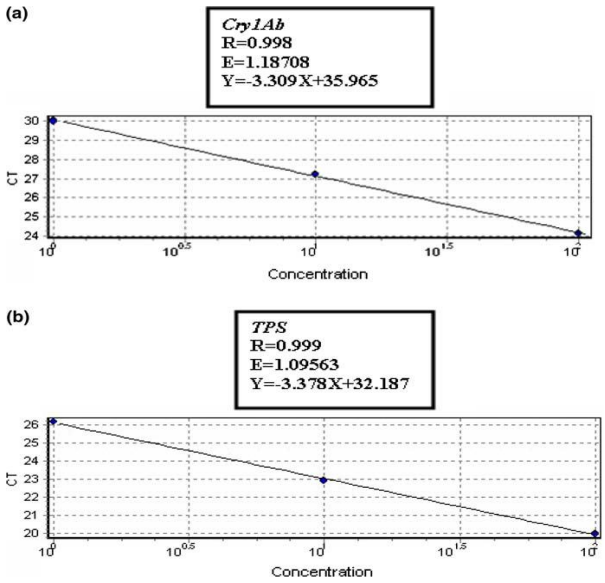

Figure 3. Quantitative real-time PCRs for *cry1Ab* (a) and *TPS* (b) show high efficiencies. The similar slope of curves, Ct = f (-log[DNA]), demonstrates that both PCRs had similar efficiencies.

of the endogenous gene. As for cases where no fluorescence was detected within the duration of the PCR reaction, it was presumed that no amplification occurred due to a lack of the target sequence within the template DNA. TaqMan zygosity assay consistently produced small, negative ΔΔCt values for homozygotes, small, positive ΔΔ Ct values for hemizygotes and large, negative ΔΔCt values for non-transgenic (Table 3) (Prior FA *et al.* 2006) (We calculated the amount of plasmid DNA needed to be mixed with 6 ng of non-transgenic cotton genomic DNA to mimic

different copy numbers of a particular transgene. Variation in the copy number of the samples across different runs of PCR was minimal with coefficient of variation (CV) values being less than 2%. Based on this experiment, the difference between 1, 2, 3 and 4 copies of the transgene could be distinguished.

Table 3. Comparative Ct values (ΔΔCt) for thee zygosity level determination of Cry1Ab cotton progeny.

Replicate	Zygosity	Mean FAM[1]	Mean TET[2]	ΔC_t[3]	$\Delta\Delta C_t$[4]
1	Calibrator	30.69	24.30	6.39	0
	Homo	30.56	24.25	6.31	-0.08
	Hemi	32.73	23.72	9.01	2.62
	Null	0	24.83	-24.83	-31.22
2	Calibrator	30.25	22.10	8.15	0
	Homo	30.10	22.23	7.87	-0.28
	Hemi	31.53	21.91	9.62	1.47
	Null	0	22.73	-22.73	-30.88
3	Calibrator	29.68	21.15	8.53	0
	Homo	29.29	21.22	8.07	-0.46
	Hemi	31.85	20.77	11.08	3.02
	Null	0	21.22	-21.22	-29.75

Table 4 shows the calculated Ct values in order to distinguish 1, 2, 3 and 4 copies. Finally, the results of zygosity estimation by real-time PCR were in line with our previous findings obtained by southern blot analysis (Yazdanpanah et al. 2009).

1. The internal oligonucleotid probe specific for *cry1Ab* labeled at the 5 end with FAM.
2. The probe specific for the endogenous gene *TPS* gene was 5 end-labeled with fluorescent dye TET
3. $\Delta Ct = Ct_{\text{TET-labeled endogenous gene}} - Ct_{\text{FAM-labeled transgene reaction}}$
4. $\Delta\Delta Ct = \Delta Ct_{\text{reference sample}} - \Delta Ct_{\text{target}}$

Table 4. Calculated Ct values in three replications for differentiation between 1, 2, 3 and 4 copies of transgene.

DNA amount(copies)	Ct Value			Cv(%)[a]	SD[b]
	1	2	3		
1	23.16	23.10	23.19	0.2	0.045
2	22.00	21.99	22.35	0.92	0.205
3	21.53	21.29	21.79	1.16	0.250
4	21.09	21.09	21.37	0.76	0.161

[a]Coefficient of variation
[b]Standard deviation

The other reference genes used in cotton include *acp1*, *AdhC*, *Sah7* (Mazzara *et al*, 2006, 2007, 2008). The *Sah7*gene is present in both A-subgenome and D-subgenome, however, the size of the amplicons resulting from A- and D-subgenome differs slightly. However, unlike these reference genes, *TPS* is only present in a single copy in the entire cotton genome (AADD). In order for the *TPS* gene to be practically used as an endogenous reference gene of cotton, the amount of *Cry* transgenic cotton by using duplex quantitative PCR was determined. In this method, *cry1A(b)* and *TPS gene*, were efficiently amplified in a duplex PCR in the same reaction tube. Previous reports showed that the mean PCR efficiency was 101.4%, 90% and 91% for *acp1*, *Sah7*and *adhC* genes, respectively (Mazzara *et al*, 2006, 2007, 2008). In this study, the amplification efficiency of *TPS* gene was obtained at 109.5%.

The results of the experiments described here demonstrate that the *TPS* gene satisfies the criteria considered for an endogenous reference gene such as genera-specificity, low copy number and

high homogeneity among *Gossypium* spp. cultivars.

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اعتبار سنجی ژن TPS به عنوان یک ژن داخلی مرجع در آزمایش های کمی Real Time PCR در پنبه تراریخته

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چکیده

شناسایی ژن هایی با سطح بیان ثابت یکی از پیش نیازهای ارزیابی تغییرات ترانسکرپیتوم است. بیان این ژن ها بطور ایده‌آل نباید با تغییرات شرایطی محیطی و مرفولوژیکی گیاه تغییر کند. در این تحقیق ژن *TPS* (trehalose 6– phosphate synthase) در گیاه پنبه (*Gossypium* spp) توسط تکنیک Taqman Real-time-PCR به روش کمی مورد ارزیابی قرار گرفت. بیان این ژن در ۵ کولتیوار مختلف پنبه که عبارتند از: Coker 312، Acala SJ، ZETA 2، Neishabour Taghva و کولتیوار وحشی دیپلوئید پنبه *G. barbadense* بررسی شد. محصول تکثیر مشخصی برای این کولتیوارها به دست آمد، در صورتیکه هیچ تکثیری در گیاهان جو (*Hordeum Vulgare*) ذرت (*Zea mays*) برنج (*Oryza sativa*) انجیر (*ficus carica*) پسته (*Pistacia vera*) سرخدار (*Taxus baccata*) گندم (*Triticum aestivum*) رز (*Rosa hybrida*) و سویا (*Glycine max*) مشاهده نشد. بنابراین این آزمایش ثابت کرد که پرایمرها و پروب طراحی شده در این مطالعه به منظور شناسایی کیفی و کمی ژن کنترل داخلی *TPS* اختصاصی است. نتایج نشان داد که می‌توان از ژن *TPS* به عنوان ژن داخلی درآزمایش های بیان ژن در پنبه استفاده نمود، به عبارت دیگر این ژن کاندید مناسبی به عنوان ژن داخلی مرجع به منظور تعیین ویژگی های گیاه تراریخته پنبه از نظر هموزیگوسیتی و کپی نامبر است.

کلمات کلیدی: پنبه ، Real Time PCR، ژن داخلی TPS.