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Suitable internal control genes for qRT-PCR normalization in cotton fiber development and somatic embryogenesis

TU LiLi, ZHANG XianLong†, LIU DiQiu, JIN ShuangXia, CAO JingLin, ZHU LongFu, DENG FengLin, TAN JiaFu & ZHANG CunBin

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China

The mechanisms of cotton fiber development and somatic embryogenesis have been explored systematically with microarray and suppression subtractive hybridization. Real-time RT-PCR provides the simultaneous measurement of gene expression in many different samples, with which the data from microarray or others can be confirmed in detail. To achieve accurate and reliable gene expression results, normalization of real-time PCR data against one or several internal control genes is required, which should not fluctuate in different tissues during various stages of development. We assessed the gene expression of 7 frequently used housekeeping genes, including 18S rRNA, *Histone3*, *UBQ7*, *Actin*, *Cyclophilin*, *Gbpolyubiquitin-1* and *Gbpolyubiquitin-2*, in a diverse set of 21 cotton samples. For fiber developmental series the expression of all housekeeping genes had the same down tendency after 17 DPA. But the expression of the *AGP* gene (arabinogalactan protein) that has high expression level at the later fiber development stage was up-regulated from 15 to 27 DPA. So the relative absolute quantification should be an efficient and convenient method for the fiber developmental series. The expression of nonfiber tissues series varied not so much against the fiber developmental series. And three best control genes *Histone3*, *UBQ7* and *Gbpolyubiquitin-1* have to be used in a combinated way to get better normalization.

cotton, fiber development, housekeeping genes, internal control, real-time PCR, somatic embryogenesis

Quantitative real-time PCR (qRT-PCR) analysis has become a common, rapid and reliable method for the detection and quantification of mRNA transcription levels of the genes of interest. It is safer, and more sensitive and accurate than the Northern blot, and works with a minimal amount of starting material. A problem is that the experimental procedure could lead to severe misinterpretation of the results by different quantities and qualities of starting RNA, efficiencies of retrotranscription from RNA to cDNA, and efficiencies of PCR[1], for which relative quantification is a commonly adopted strategy. The target concentration in each sample is calculated relative to another gene transcript, a housekeeping gene, and the result is expressed as a target/reference ratio. Housekeeping genes, such as 18S rRNA, *ubiquitin*, *actin*, *tubulin*, *histone* and glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) were chosen because of their known or expected roles in basic cellular processes (cell structure maintenance or primary metabolism) and the least variation in their expression under various conditions. However, some housekeeping genes vary considerably since biological systems are dynamic and constantly changing in response to their environment[2−4]. In order to choose the appropriate control genes, statistical algorithms, such as geNORM and BestKeeper have been

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†Corresponding author (email: xlzhang@mail.hzau.edu.cn)
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developed for the evaluation of best suited reference gene(s) for normalization of qRT-PCR data[1,5]. And various housekeeping genes have been evaluated for stable expression in various conditions and organisms[6−11]. Especially in *Arabidopsis* a set of superior reference genes were identified by genome wide analysis of the whole-genome gene chip data for transcript normalization[4].

Cotton is an important economic crop and the leading natural fiber used in textile production worldwide. The mechanism of the fiber development is the focus of scientists, for which the expression analysis of the genes related to fiber developments is one of the important techniques[12−16]. In addition, as the initial basis of cellular and genetic engineering, somatic embryogenesis plays an important role in cotton genetic transformation. The development of cotton somatic embryos exhibits an extraordinarily dynamic program of gene activity in the course of cell differentiation, organ formation and maturation by analysis with suppression subtractive hybridization (SSH) and macroarray[17]. For the further detailed study of an interesting gene, qRT-PCR analysis is the prima choice of researchers. The problem is that researchers continue to rely on one gene as the internal control in this analysis, without an adequate validation of the stability of expression of these genes under the conditions assayed in cotton. In this paper, we will report the validation of housekeeping genes to identify the most suitable internal control gene(s) for normalization of real-time PCR data obtained with different tissues/organs of cotton at different somatic embryogenesis and fiber developmental stages.

1 Materials and methods

1.1 RNA extraction and cDNA synthesis

Pima 3-79 plants (*Gossypium barbadense*) were cultivated in an experimental field (Wuhan, China) using normal farming practices. The bolls were tagged on the day of anthesis. All fibers of Pima 3-79 at different developmental stages were removed carefully from ovules and immediately immersed into liquid nitrogen. Tissues, such as leaves, flowers, and ovules were harvested from plants in the field and placed in liquid nitrogen. The materials of different developmental stages during Coker 201 somatic embryogenesis were isolated and collected[17,18]. All the frozen materials were preserved at −70℃ before use. Total RNA was isolated from the collected tissues using a modified guanidine thiocyanate method [19]. Purified RNA was treated with DNase I (Promega) according to the manufacturer's instructions. RNA integrity was checked on 1.4% (wt/vol) agarose gel containing ethidium bromide. Nucleic acid concentrations were checked using Eppendorf DU800 spectrophotometer (Eppendorf AG). Only the RNA samples with an A_{260}/A_{280} ratio between 1.9 and 2.1 and an A_{260}/A_{230} ratio greater than 2.0 were used for the analysis. For cDNA synthesis, 3 μg of each RNA was mixed with 1 μL of 500 μg/mL Oligo-$dT_{(15)}$ primer or 500 μg/mL random primer (Promega), 1 μL of 10 mmol/L dNTP mix, and DEPC-water to a volume of 12 μL. The reaction was heated at 65℃ for 5 min and quick chilled on ice. A master mix (8 μL) that contained 4 μL RT buffer, 2 μL 0.1 mol/L dithiothreitol, 40 U of RNasin® Ribonuclease Inhibitor (Promega), and 200 U of Superscript Ⅲ RT (Invitrogen) was added to each reaction. After incubating tubes with random primers at 25℃ for 10 min, the reactions were incubated at 42℃ for 1 h, and then at 75℃ for 15 min. Each cDNA was diluted to 300 μL and stored at −20℃.

1.2 PCR primer design

Several common housekeeping genes and a fiber preferentially expressed gene *AGP* were selected for expression analysis (Table 1). Primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) and Primer Premier 5.0 with melting temperatures (Tm) of 58－62℃. A majority of the primer pairs targeted a single gene within a given gene family but the actin.

1.3 Two-step qRT-PCR analysis of transcript concentrations

The PCR mixture contained 1 μL of diluted cDNA (corresponding to 10 ng of starting amount of total RNA), 10 μL of 2×SYBR Green PCR Master Mix (Applied Biosystems, USA), and 200 nmol/L of each gene-specific primer in a final volume of 20 μL PCRs with no template controls were also performed for each primer pair. The real-time PCRs were performed using ABI Prism 7000 Sequence Detection System and software (PE Applied Biosystems, USA). All the PCRs were performed under the following program: 2 min at 50℃, 10 min at 95℃, followed by 40 cycles of 15 s at 95℃ and 1 min at 60℃ in 96-well optical reaction plates (Applied Biosystems, USA). The specificity of amplicons

Table 1 Housekeeping genes and their primer sequences used for real-time PCR analysis

Gene name	Accession no.	Primer sequence	Amplicon length (bp)
Actin	AY305733	^{569}5′-ATCCTCCGTCTTGACCTTG-3′ ^{783}5′-TGTCCGTCAGGCAACTCAT-3′	215
UBQ7[a)]	DQ116441	^{157}5′-GAAGGCATTCCACCTGACCAAC-3′ ^{354}5′-CTTGACCTTCTTCTTCTTGTGCTTG-3′	198
Gbpolyubiquitin-1[b)]	AY375335	^{579}5′-AGCTCGGATACGATTGATAACG-3′ ^{744}5′-GAAGACGAAGAACAAGGGGAAG-3′	166
Gbpolyubiquitin-2[b)]	EE592464	^{587}5′-CAAGGAGGGTATCCCCCCA-3′ ^{711}5′-GCAGCCGCAACACCAAGTG-3′	125
Histone 3	AF024716	^{240}5′-TCAAGACTGATTTGCGTTTCCA-3′ ^{339}5′-GCGCAAAGGTTGGTGTCTTC-3′	100
18S rRNA	L24145	^{333}5′-TGACGGAGAATTAGGGTTCGA-3′ ^{432}5′-CCGTGTCAGGATTGGGTAATTT-3′	100
Cyclophilin	EE592954	^{52}5′-TGGCCTCAAATCCCAAGGT-3′ ^{151}5′-GCAGTGCGAGGAGTGCAAT-3′	100
AGP	DR176757	^{80}5′-TCTCCTCCATACTTCTTGTTC-3′ ^{279}5′-CGACTTGCGTGGCTTTCA-3′	200

a) For this pair of primers, please see ref. [15]; b) the two pairs of primers were designed using Primer Premier 5.0

was verified by melting curve analysis (60℃ to 95℃) after 40 cycles and agarose gel electrophoresis. Each assay included (in triplicates) a standard curve of six serial dilution point of the bulked cDNA sample in a range of 50, 25, 10, 5, 2, 1 ng of the starting amount of the total RNA; a no-template control and 10 ng of each test cDNA.

1.4 Analysis of gene expression stability and Northern blot analysis

The Microsoft excel file of raw expression values for the tested genes in different samples was imported into geNORM to analyze the gene expression stability[1]. After denaturing at 65℃ for 15 min, 20 μg of total RNA in the loading buffer from each sample was loaded on a 1.2% agarose-formaldehyde gel for electrophoresis in the MOPS buffer. The fractionated RNA samples were then transferred to nylon membranes (Amersham), which were immobilized in an oven at 80℃ for 2 h and stored at –20℃. Then the filters were hybridized with an [α-^{32}P] dCTP-labeled probe at 42℃ for at least 20 h; the solution included 50% hybridization solution (5×SSC, 0.1% SDS, 50 mmol/L Tris-HCl, 10 mmol/L EDTA, 1×Dehart's) and 50% N,N-dimethylformamide. The washing procedure was as follows: wash 1 (2× SSC, 0.1% SDS), 42℃, 5 min; wash 2 (2× SSC, 0.1% SDS), 42℃, 5 min; wash 3 (2×SSC, 0.1% SDS), 42℃, 20 min. The Prime-a-Gene labeling system (Promega, USA) was used to generate probes. The hybridized membranes were exposed to Imagingplate (Fuji Photo Film Co., Ltd., Japan) for 12 h. The signals of filters were scanned using PhosphorImager SI (Molecular Dynamics, USA) and analyzed with the software Multi-Gauge Version 3.0 (Fuji).

2 Results

To evaluate the stability of the expression of housekeeping genes, seven pairs of primers were designed for six commonly used housekeeping genes in cotton (Table 1). The primers for *actin* were designed from conserved domain of 14 cotton actin genes searched from the GenBank (AY305724－AY305737), with which the qRT-PCR product could present expression level of the total family. The expression stabilities of these 7 housekeeping genes were assessed by real-time PCR in a set of 21 tissue samples divided into two series. One is the non-fiber tissues series, which included 7 samples representing different somatic embryogenesis stages: hypocotyl, hypocotyl 7 d (hypocotyls cultured on the medium for 7 d), non-embryogenic callus, embryogenic callus, globular-shaped embryo, cotyledonary embryo, regenerated plantlet[17]; two protoplast samples: protoplast just isolated and protoplast 48 h (protoplast cultured on the medium for 48 h)[20]; and four common tissues/organs: leaf, bud, flower, anther. Another series included 8 samples representing various fiber developmental stages of Pima 3-79: –4－–1, 5, 7, 10, 15, 17, 21, and 27 DPA. Initially, cDNAs were synthesized with random primers. It was difficult to subtract the baseline value for 18S rRNA primers with the cDNA templates (10 ng of starting amount of total RNA) and 200 nmol/L

primers (data not shown). Furthermore the rRNA transcription of the later stage fiber was also changed obviously (Figure 1(d)). So the further analysis did not include 18S rRNA and the cDNAs were synthesized with the Oligo-$dT_{(15)}$. The transcriptions of the six housekeeping genes: *Histone3*, *UBQ7*, *Actin*, *Cyclophilin*, *Gbpolyubiquitin-1* and *Gbpolyubiquitin-2*, varied among the different tissues (Figures 1(a) and (b); Figure 2).

For the non-fiber tissues series, the expression of the six genes varied not so obviously as that of fiber developmental series (Figure 1(b)). We found that the *Gbpolyubiquitin-2* had a lower expression than the other five genes in the samples but anther, and the dissociation curve showed two peaks when the templates were anther and flower. The Northern blot also confirmed that *Gbpolyubiquitin-2* had the highest expression level in anther (Figure 3). Because the primers of actin were designed from conserved domain, all actin family could be amplified and the total expression level was expected to be stable. But as shown in Figure 1, the RNA transcription level varied obviously and C_T values ranged from 24 to 28.

In order to choose the best housekeeping genes in various tissue samples, geNORM[1] was used. The geNORM is a statistical algorithm that determines the gene stability measure (M) of all the genes under investigation based on the geometric averaging of multiple control genes and mean pairwise variation (V) of a gene from all other control genes in a given set of samples. A low M value is indicative of a more stable expression,

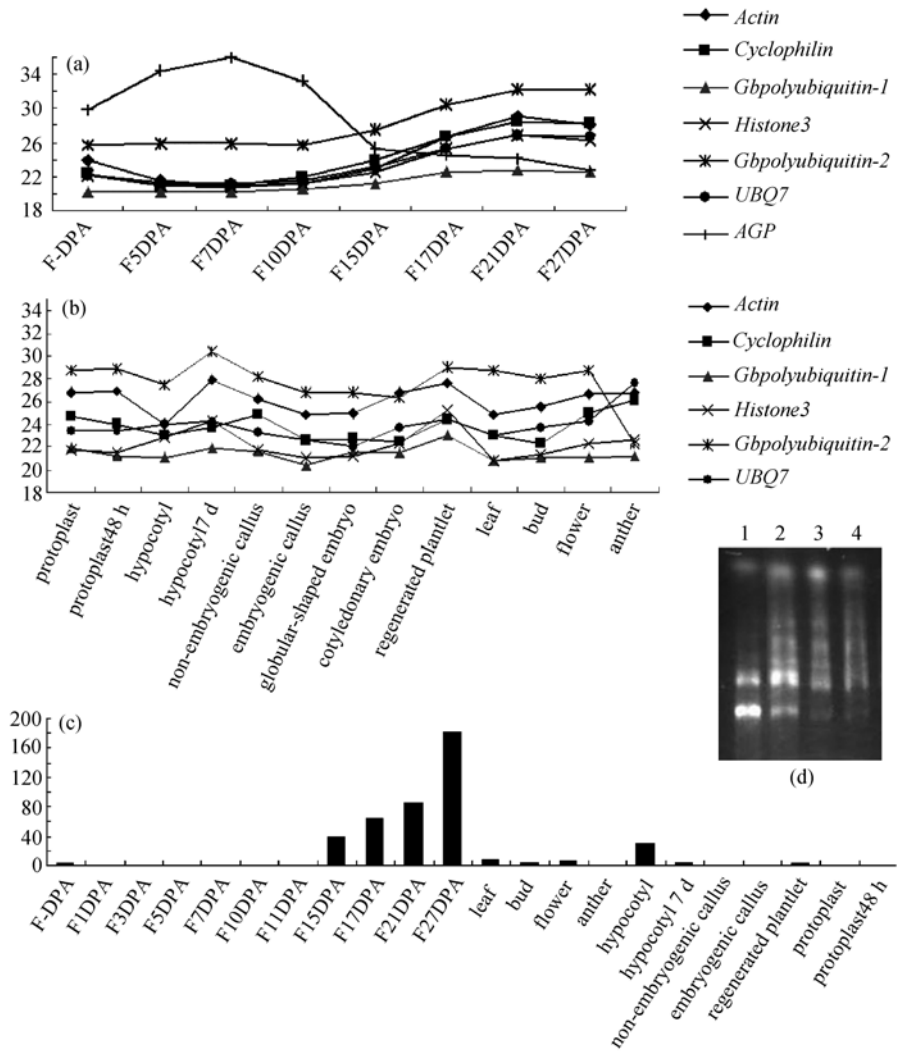

Figure 1 RNA transcription levels of housekeeping genes, presented as C_T value in different samples. (a) The fiber developmental series sampled at different stages (DPA); (b) the non-fiber tissues series, hypocotyl 7 d (hypocotyls cultured on the callus induction medium for 7 d), protoplast 48 h (protoplast cultures on the medium for 48 h); (c) the transcription level of *AGP* in the different tissues and developmental stages; (d) the RNA of the fiber, lanes 1—4: 17 DPA, 21 DPA, 23 DPA, 27 DPA.

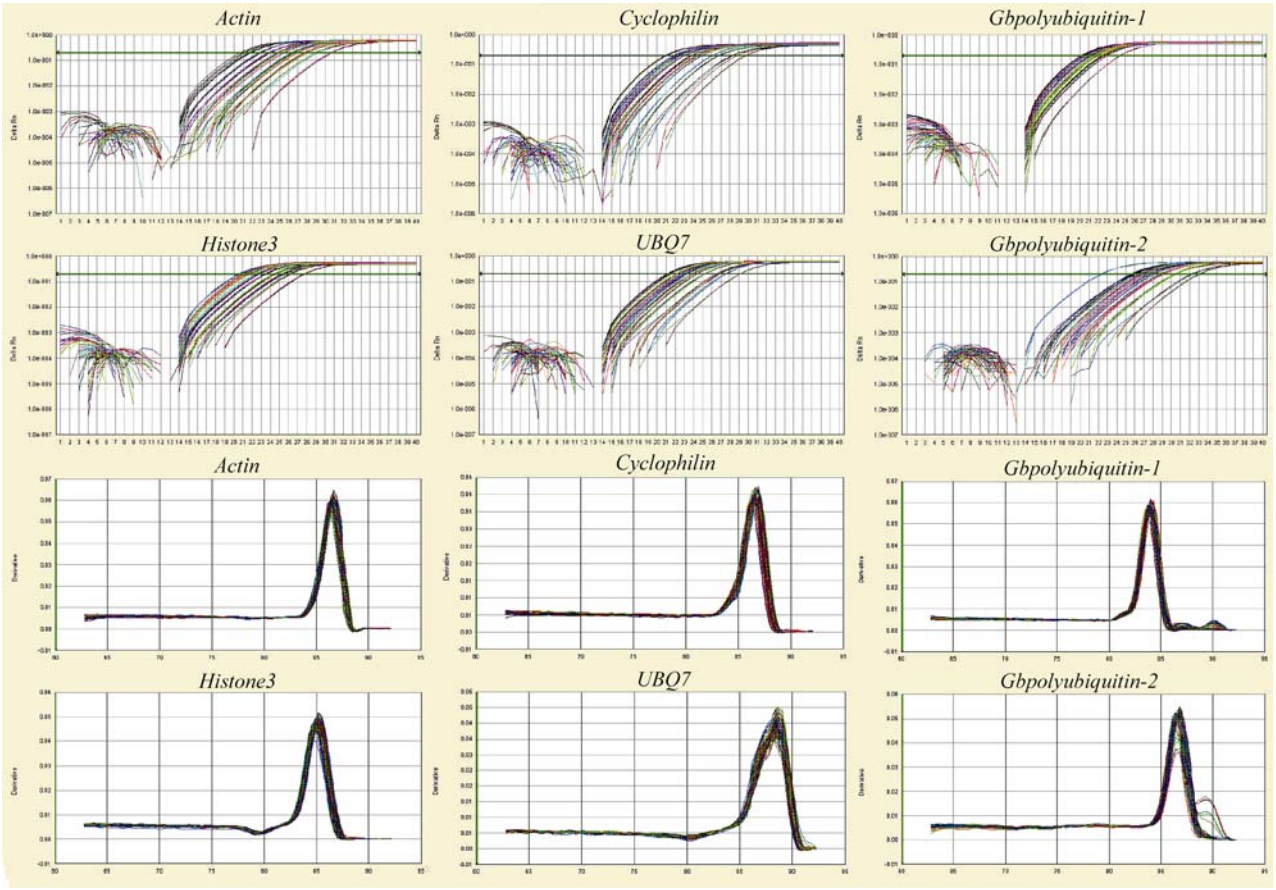

Figure 2 Amplification plots and the dissociation curves of 6 genes in 21 cDNA samples, the green horizontal line represents the threshold fluorescence at which the C_T value was determined.

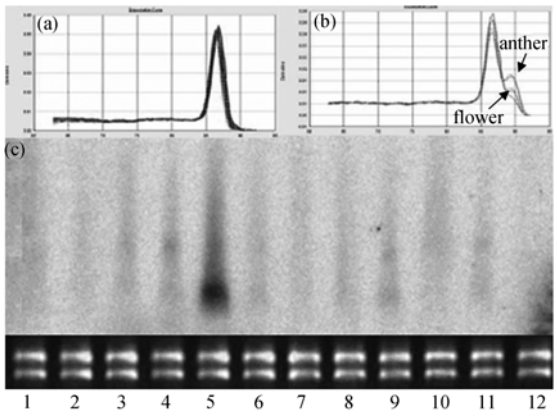

Figure 3 The dissociation curve of *Gbpolyubiquitin-2* and the expression patterns of *Gbpolyubiquitin-2* by RNA gel blot analysis. Twenty micrograms of total RNA of *Gossypium barbadense* cv. Pima 3-79 tissues were loaded. (a) The qRT-PCR with all sample cDNA except anther and flower; (b) the qRT-PCR with cDNA of anther and flower; (c) lanes 1—12: root, leaf, bud, petal, anther, pistil, 0 DPA ovule, 1 DPA ovule, 5 DPA fiber, 10 DPA fiber, 15 DPA fiber, 20 DPA fiber.

hence an increase in the suitability of particular gene as a control gene. With cDNA of the anther as the template, the amplicons were not specific and transcription of *Gbpolyubiquitin-2* was obviously high in anther (Figure 3). The average expression stability value (*M*) of *Histone3* and *UBQ7* was the least, and that of *actin* was the highest (Figure 4(a)). Vandesompele et al. [1] proposed 0.15 as a cutoff value for the pairwise variation below which the inclusion of an additional control gene is not required. They also annotated that 0.15 value must not be taken as a too strict cutoff. In most cases, ignoring the graph of determination of the optimal number of control genes for normalization and 'just' using the 3 best control genes is a valid normalization strategy, and results are much more accurate and reliable in normalization than by the use of only one single control gene. In this series the pairwise variation was higher than 0.15 (0.29, Figure 4(b)), and three best control genes *Histone3*, *UBQ7* and *Gbpolyubiquitin-1* had to be used together to get better normalization.

For fiber developmental series, all housekeeping genes varied, and the expression level declined quickly after 17 DPA. The expression of *Gbpolyubiquitin-1* had the least variation against the other five genes. As the

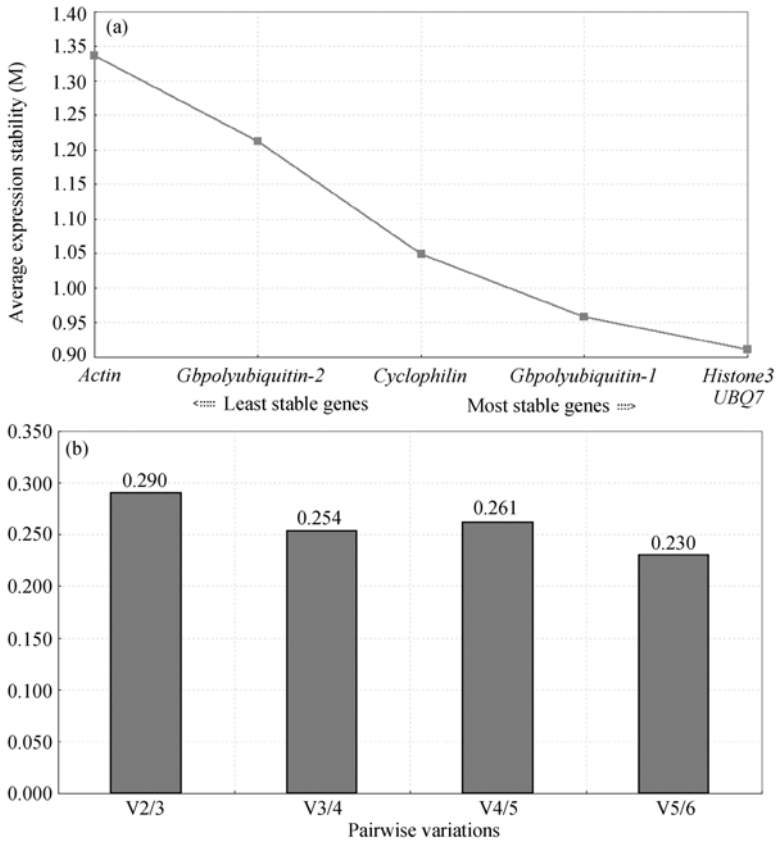

Figure 4 Average expression stability values of control genes (a) and determination of the optimal number of control genes for normalization (b) by geNORM analysis in the non-fiber tissues series.

expressions of all six genes varied greatly, the geNORM was not efficient enough to analyze the fiber developmental series. Because of the same tendency of all the genes, it is necessary to make clear whether the RNA of fiber degraded or the cDNA synthesis was inefficient after 17 DPA. For confirmation, qRT-PCR analysis was performed with an *AGP* gene (arabinogalactan protein) that has a high expression level at the later fiber development stage. The *AGP* was isolated from the SSH library with 20 DPA fibers RNA as the tester and the mixture RNA of non-fiber tissues as the driver. The Northern blot also confirmed that it has a high expression after 20 DPA [21]. In our analysis (Figures 1(a) and (c)) the *AGP* had high a transcription level in the later stage of fiber development (after 15 DPA), which implied that the RNA was integrated and the cDNA synthesis was efficient. It suggested that at the later fiber development stage the expression of 'housekeeping' genes did decline.

3 Discussion

In order to make relative RT-PCR accurate, an appropriate internal control must be determined, which should show minimal changes over the course of an experiment. In the pre-genomic era some housekeeping genes had been selected as controls because of their known or expected roles in basic cellular processes. Unfortunately, not all 'housekeeping' genes have a stable expression under every experimental condition, as numerous studies reported that expression of housekeeping genes can also vary considerably with experimental conditions[2 − 4]. Validations of internal controls have been performed in some human tissues and animal samples, and then it also attracted attention in plants in recent years[11,22−25]. For *Arabidopsis*, the model plant, some superior reference genes were identified by using the large public collection of data from Affymetrix GeneChip experiments[4]. But, for organisms with complicated genome like cotton, public datasets are limited. A majority of studies of cotton in the literature used a unique internal control, such as *Ghpolyubiquitin*, *Histone3*, *UBQ7*, and 18S rRNA[13−16]. Therefore, it is necessary to validate the expression stability of a control gene under specific experimental conditions prior to its use for normalization.

The mechanism of the fiber development and somatic embryogenesis are the focuses in cell differentiation and development in cotton, so we validated the internal controls in the two developmental processes.

Ubiquitin is always reported as a housekeeping gene and used as control in relative qRT-PCR analysis. Ubiquitin is a highly conserved small protein of 76 amino acids functioning in a wide range of cellular processes by degrading physiologically important regulatory proteins[26]. In all eukaryotes examined so far, ubiquitin is encoded by polyubiquitin gene or ubiquitin extension protein gene. The polyubiquitin gene, encoding tandemly repeated multiple ubiquitins, constitutes an ubiquitin gene subfamily. The ubiquitin extension protein gene encodes the other type of ubiquitin fusion protein, ubiquitin monomer followed by one of two nonrelated proteins[27]. Ubiquitin is associated with the regulation of protein turnover in a cell by closely targeting specific proteins for degradation[28]. By regulating protein degradation, cells can quickly eliminate a protein that in turn regulates another function. Most frequently, the ubiquitin tag is used to mark particular proteins for proteolytic elimination, but it can also have nonproteolytic functions[29]. In our study we analyzed two polyubiquitin genes (*Gbpolyubiquitin-1* and *Gbpolyubiquitin-2*) and an ubiquitin extension protein gene (*UBQ7*). The nucleic acid sequences of *Gbpolyubiquitin-1* and *Gbpolyubiquitin-2* were 78% similar and the protein sequences had only several different amino acids. The primers of these two genes were designed using Primer Premier 5.0. Because of the tandemly repeated structure of the polyubiquitin, the primers designing failed by Primer Express 2.0 software. The expression levels of the two polyubiquitin genes and the *UBQ7* were different and *Gbpolyubiquitin-1* had the highest level (Figures 1(a) and (b)). Interestingly we found that *Gbpolyubiquitin-2* had high expression in anther, and the dissociation curve of the PCR product with the anther cDNA as the template had two peaks. The Northern blot confirmed this result. We also found different hybridization bands with other RNA, which possibly were the non-specific hybridization with other ubiquitins of the gene family (Figures 1 and 3). All this confirmed that the polyubiquitins were included in a big family and may suggest that the different members have different roles.

Actin filaments provide a mechanical support to the cell, determine the cell shape, and enable cell movements. The actin gene family has large and different members expressing in distinct cell lineages in a developmentally regulated fashion[13]. We presumed that the total expression of all gene family should be invariable using the primers designed from the conserved domain that should amplify the all genes (Figure 2). Reid et al.[25] reported that the actin was one of the most relevant reference genes for the development of grapevine berry, but the actin in our study was the most variable in the non-fiber tissues (Figure 4).

For the non-fiber tissues except anther, three best control genes *Histone3*, *UBQ7* and *Gbpolyubiquitin-1* were validated, although considerable fluctuations of the 'housekeeping' genes were found (Figure 1(b)). However, no gene was found stable for fiber developmental series. Unlike other tissues, fiber is a special unicell and the mature fiber is composed of nearly pure cellulose. Fiber development progresses through four overlapping stages[30]: initiation, elongation (primary cell wall synthesis), thickening (secondary cell wall synthesis), and maturation. In the later stage the genes expression profile will change sharply[12]. The total RNA extracted from the later stage fiber was different from the one of the earlier stage fiber. They had obvious ladders ahead of the 18S rRNA, but not like the degraded RNA that just had the smear in the lane (Figure 1(d)). In our study, the transcription levels of the 'housekeeping' genes obviously reduced (Figure 1(a)). The *Ghpolyubiquitin-1* had the least reducing range against the other five genes: *Actin*, *UBQ7*, *Gbpolyubiquitin-2*, *Histone 3*, and *Cyclophilin.* After performing qRT-PCR analysis of the *AGP* using relative absolute quantification, we considered that the reducing transcription levels of the 'housekeeping' genes is not contributable to the degradation of RNA. In fiber the *AGP* did not express before 11 DPA. Only after 15 DPA did the expression level rise up. The *AGP* also preferentially expressed in fiber and had low expression in the hypocotyls (Figure 1(c)). The result was coincident with the Northern blot of Liu et al.[21]. After determining the suitability of four candidate reference genes in maritime pine by qRT-PCR, Goncalves et al.[24] proposed that the combination of a precise method for RNA quantification, internal controls for monitoring RT reaction and PCR efficiency and a robust external standard curve can guarantee a reliable absolute quantification of mRNA transcripts in qRT-PCR. Similar to the results of Goncalves et al.[24], for qRT-PCR analysis of genes related to fiber development, the relative qRT-PCR is not efficient with the commonly reported 'housekeeping'

genes which magnified the results. The relative absolute quantification should be an efficient and convenient method, before which the standard curves should be decided by a series dilution cDNA stocks with unknown real copies of the target gene[31].

In summary, a control gene with a stable expression in some tissues may not be suitable for normalization of gene expression in others under a given set of conditions and needs to be validated before use. Further studies on the testing of more housekeeping genes should be performed to find a suitable one. Just like Arabidopsis, with the accumulation of data from GeneChip or microarray experiments, superior control genes would be found in cotton in the future. But now, based on the limited data, for the non-fiber tissues series, *Histone3*, *UBQ7* and *Gbpolyubiquitin-1* have to be used together in the normalization, and for fiber developmental series, relative absolute quantification is more reliable.

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