



Screening of valid reference genes for real-time RT-PCR data normalization in *Hevea brasiliensis* and expression validation of a sucrose transporter gene *HbSUT3*

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ARTICLE INFO

Article history:

Received 23 December 2010

Received in revised form 21 April 2011

Accepted 22 April 2011

Available online 30 April 2011

Keywords:

Real-time RT-PCR

Gene expression

Normalization

Reference gene

Sucrose transporter *HbSUT3*

Hevea brasiliensis

ABSTRACT

Real-time RT-PCR (RT-qPCR) is a sensitive and precise method of quantifying gene expression, however, suitable reference genes are required. Here, a systematic reference gene screening was performed by RT-qPCR on 22 candidate genes in *Hevea brasiliensis*. Two ubiquitin-protein ligases (*UBC2a* and *UBC4*) were the most stable when all samples were analyzed together. A mitosis protein (*YLS8*) and a eukaryotic translation initiation factor (*eIF1Aa*) were the most stable in response to tapping. *UBC2b* and *UBC1* were the most stable among different genotypes. *UBC2b* and a DEAD box RNA helicase (*RH2b*) were the most stable across individual trees. *YLS8* and *RH8* were most stably expressed in hormone-treated samples. Expression of the candidate reference genes varied significantly across different tissues, and at least four genes (*RH2b*, *RH8*, *UBC2a* and *eIF2*) were needed for expression normalization. In addition, examination of relative expression of a sucrose transporter *HbSUT3* in different RNA samples demonstrated the importance of additional reference genes to ensure accurate quantitative expression analysis. Overall, our work serves as a guide for selection of reference genes in RT-qPCR gene expression studies in *H. brasiliensis*.

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1. Introduction

Gene expression analysis is frequently used to explore biological processes. Steady state levels of individual RNAs can be measured by non-PCR-based methods, such as Northern blotting [1], RNase protection analysis [2] and *in situ* hybridization [3]. However, these techniques are time-consuming and/or require large quantities of RNA for detection. Reverse transcription polymerase chain reaction (RT-PCR) overcomes these limitations and is more rapid, sensitive and specific. Fluorescence-based real-time RT-PCR, also known as reverse transcription-quantitative PCR (RT-qPCR) is presently the most reliable method for detecting low abundance mRNAs. Because of its high sensitivity, lack of post-PCR processing and wide dynamic range [4,5], RT-qPCR can be used to directly compare mRNAs that differ widely in their abundances. It is also easy to use, allows high-throughput production of data and eliminates the need for radioactive isotopes [6].

The accuracy of RT-PCR techniques is challenged by many sources of variation, including sampling errors, template quality

and amplification efficiency [7]. The reliability of RT-qPCR results is highly dependent on the reference genes chosen [8]. The expression level of a reference gene needs to remain constant under varying experimental parameters such as treatments used, organs sampled and experimental pressures applied [7,9,10]. The reference transcripts in plants most frequently used for RT-qPCR include the genes for 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor-1a (*EF-1a*), polyubiquitin (*UBQ*), actin (*ACT*), and α -tubulin and β -tubulin (*TUA* and *TUB*) [6,11–15]. However, many of these reference genes may not be stably expressed under all conditions [9,16–18]. Some reference genes that had been reliably validated in one plant species are not well suited for use in others [19–21]. Therefore, prior validation is required when a reference genes is suggested to be used in a new plant species or in a new experimental condition. Nonetheless, due to lacking studies of systematic prior validation, many researchers simply rely on one or two of these genes, regardless of the plant species and the developmental or physiological state of the materials being analyzed.

Natural rubber (*cis*-polyisoprene) is an important industrial raw material that is primarily produced from a single tropical tree species, the Brazilian rubber tree (*Hevea brasiliensis*, Hevea hereafter) [22]. Rubber synthesis occurs in a highly specialized cell type known as a laticifer, in which this is the main metabolic activity of the cell [23]. The formation and function of laticifers is regulated by the wound signals of jasmonic acid and ethylene, and possibly

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Table 1Candidate reference genes used for gene expression normalization in *H. brasiliensis*.

Gene symbol ^a	NCBI accession no.	blastX search			
		Arabidopsis ortholog locus	Arabidopsis locus description	Similarity (e-value)	Identity (%)
18S	AB268099	AT3G41768	rRNA	0.0	97%
ACT7a *	HQ260674	AT5G09810	ACT7 (ACTIN 7)	0.0	98%
ACT7b *	HQ395755	AT5G09810	ACT7 (ACTIN 7)	0.0	99%
ADF	HM126477	AT5G59890	ADF4 (actin depolymerizing factor 4)	1e–77	85%
ADF4 *	HQ268020	AT5G59890	ADF4 (actin depolymerizing factor 4)	2e–77	82%
UBC2a *	HQ323246	AT2G02760	Ubiquitin-protein ligase (ATUBC2)	2e–102	96%
UBC2b *	HQ323247	At2g02760	Ubiquitin-protein ligase (ATUBC2)	5e–100	93%
UBC1 *	H323245	AT3G52560	Ubiquitin-protein ligase	5e–90	93%
UBC3 *	HQ323248	AT1G78870	Ubiquitin-protein ligase	6e–101	97%
UBC4 *	HQ323249	AT5G25760	Ubiquitin-protein ligase	4e–99	93%
FP	HQ268026	AT5G15710	F-box family protein	2e–126	90%
elf2 *	HQ268024	AT4G27130	Eukaryotic translation initiation factor	5e–69	90%
elf3 *	HQ268025	AT4G27130	Eukaryotic translation initiation factor	7e–68	87%
elf1Aa *	HQ268022	AT5G35680	Eukaryotic translation initiation factor 1A	2e–61	91%
elf1Ab *	HQ268023	AT2G04520	Eukaryotic translation initiation factor 1A	1e–60	95%
RH2a	HQ323242	AJ010456	DEAD box RNA helicase, RH2	0.0	91%
RH2b	HQ323243	AJ010456	DEAD box RNA helicase, RH2	0.0	87%
RH8	HQ323244	AT4G00660	DEAD/DEAH box helicase (RH8)	7e–132	95%
CYP2 *	HQ268021	At2g21130	Cyclophilin (CYP2)	2e–90	82%
ROC3	EU295481	AT2G16600	Cytosolic cyclophilin (ROC3)	7e–90	85%
YLS8 *	HQ323250	AT5G08290	Mitosis protein YLS8	2e–94	99%
PTP	HQ268027	At3g19420	Tyrosine phosphatase	0.0	85%

^a Excluding the genes for 18S, ADF and ROC3, the genes listed were cloned in this study, including the full-length cDNAs for 14 genes (asterisk) and partial cDNAs for 5 genes.

involves reactive oxygen species [24–26]. The cytoplasm of laticifers, known as latex, contains up to 90% rubber by dry weight and is expelled and harvested as a commercial product when the trunk bark is regularly cut (tapped) [23,27]. In addition to genetic factors (clone or variety), the latex yield of *Hevea* is significantly affected by the method of exploitation, including the length of the cut, the number and frequency of tappings, and ethylene stimulation by the application of Ethrel [28].

In *Hevea*, gene expression studies are fundamental to understand the molecular basis of rubber productivity. A literature search using the keywords “*Hevea*” and “gene expression” produced 38 hits in Highwire+Pubmed (publication period 2001–2010); and 27 of these hits were relevant *Hevea* gene expression studies. In eight of these studies (29.6%), RT-qPCR was used to investigate the expression of genes of interest while the other studies used conventional RT-PCR or Northern blotting. Only a single reference gene was used in each of the RT-qPCR studies, such as 18S rRNA [29–32], the 40S ribosomal protein [33], actin [34,35] or GAPDH [36,37], and no preliminary validation was performed except in one study where the relative stabilities of an actin gene and a ubiquitin gene were compared [35]. In the present study, we compared the performance of 22 candidate reference genes across a large set of biological samples representing various tissues, hormone treatments, tappings, individual trees and genotypes of *Hevea*. The candidate genes were selected because their orthologs have been validated in other plant species [18,19,38,39] and included genes encoding 18S rRNA, two actins (ACT7a and ACT7b), two actin depolymerizing factors (ADF and ADF4), five ubiquitin-protein ligases (UBC1, UBC2a, UBC2b, UBC3 and UBC4), one F-box family protein (FP), four translation initiation factors (elf1Aa, elf1Ab, elf2 and elf3), three DEAD-box RNA helicases (RH2a, DRH2b and RH8), one cyclophilin (CYP2), one cytosolic cyclophilin (ROC3), one yellow leaf-specific protein (YLS8) and one tyrosine phosphatase (PTP). Moreover, the selected suitable reference genes were used to normalize the expression of a previously characterized sucrose transporter gene *HbSUT3*, which plays an active role in loading sucrose to the rubber-producing cells-laticifers [32]. The goal was to provide reliable internal controls for gene expression studies in *Hevea* under different conditions.

2. Materials and methods

2.1. Plant materials

Unless otherwise mentioned, the Brazilian rubber trees (*Hevea*) selected for this study were growing at the experimental plantations of the Rubber Research Institute of the Chinese Academy of Tropical Agricultural Sciences (Danzhou, Hainan). The experimental site has a latitude of 19°32′47″N, a longitude of 109°28′30″E, and an elevation of 144 m. To study the expression of reference genes across different tissues, samples of root, trunk bark, latex, mature leaf, flower and seed were collected from 10-year-old trees of clone Reyan7-33-97, which had been regularly tapped for 2 years in a half spiral at 3-day intervals with no stimulation system (S/2, d/3). The tissues were immediately frozen in liquid nitrogen and stored at –80 °C until required. This type of tree was also used to examine the effects of Ethrel (2-chloroethylphosphonic acid, an ethylene releaser), abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) on the expression of reference genes. To analyze the expression stability of reference genes in response to tapping and among individual trees, 8-year-old virgin (previously untapped) trees were selected. To investigate the expression stability of reference genes across different genotypes, four *Hevea* clones (PR107, RRIM600, Reyan8-79 and Reyan7-33-97) and nine Amazonian wild *Hevea* accessions (codes: 86-7, 87-62, 87-68, 87-86, 87-96, 89-4, 89-11, 94-35, 94-57) were used [40].

2.2. Field experiments

To determine the effect of Ethrel on the expression of reference genes, 1.5% Ethrel was applied to five groups of five *Hevea* trees at 0, 3, 12, 24 and 60 h before tapping as described previously [32]. The hormones 1.5% JA, 200 μM SA and 200 μM ABA were applied in a similar fashion as Ethrel at four time points (0, 3, 12 and 24 h) before tapping.

To investigate the expression of reference genes among different *Hevea* genotypes, ten trees of each of the four clones (PR107, RRIM600, Reyan8-79 and Reyan7-33-97) and two to three trees of each of the nine *Hevea* wild accessions were sampled [40]. To

Table 2
Reference gene primer sequences and amplicon lengths characteristics.

Gene symbol	Forward primer sequence (5'–3') ^a	Reverse primer sequence (5'–3') ^a	PCR efficiency (%)	Amplicon length (bp)
18S	⁹⁶² GCTCGAAGACGATCAGATACC	¹¹⁰⁷ TTCAGCCTTGGCACCATAC	95.90	146
ACT7a	¹⁴⁵³ GGCACTTTGGTACTCAAGTC	¹⁵⁵⁴ GAAGCATCCCAATCACTCTC	99.25	102
ACT7b	¹⁹⁵ GTTGGGATGGGGCAGAAGG	²⁹⁴ TGCTGACAATACCGTGCTCAA	97.90	100
ADF	²¹⁰ GGAGAAGGCAAGCAAGTC	³⁰⁸ GCATATCGGCACTCATCAG	99.80	99
ADF4	²⁸⁵ GTGCCGATATGCTGTCTATGATT	⁴³¹ TCCCTCTTGAACCTGCTTGG	97.05	147
UBC2a	³⁰⁴ CATTTATGCGGATGGAAGCA	⁴²⁸ CAGGGGAGTTGGATTGGA	97.90	125
UBC2b	¹²⁰ CGACCAAGTTTTCAITTCGGGTG	²¹⁴ AGTCTCTTCTTGTCTGGGGTTG	96.90	95
UBC1	⁴ ACTTTGCCCTTGATGCCT	¹⁰⁵ CGCTTAGTTGGGATCTGGT	99.15	102
UBC3	³⁹⁷ CCCTGATATCCACTTTCTG	⁵⁰⁹ ACCATCATGCAACCACTTG	99.55	113
UBC4	²³⁹ TCCTTATGAGGGCGGAGTC	³²⁰ CAAGAACCACCTTGAGGAG	99.15	82
FP	³¹⁹ ATCAGCTTGACGCAGGTTAC	⁴¹⁷ GAAACAGACGCTTCTGAGTG	96.15	99
elF2	¹⁹⁵ CGACCTTTGATCCGTTTGTCT	²⁹² CTTCTACCATTCGCTTGTCT	99.80	98
elF3	²⁰⁸ CGAAACCCAGATTCCTTCTACCT	³⁴⁰ CTGCACAGTAGTCAAGCTCTTTC	98.75	133
elF1Aa	⁴⁷² GCGTGACTATCAGGACGACAA	⁵⁹⁹ CAAGACCTCCAGCAATACCT	97.60	128
elF1Ab	⁸¹ TGGTGTTGGTGTGGTAG	²¹⁵ TATTCCTGCCATCTTCTTG	99.70	135
RH2a	⁷¹³ CCATCACCAGGCTGTATTTTC	⁷⁹⁴ GTAAGTTATTGCTCCGCATCTTC	90.55	82
RH2b	⁸¹⁸ AGGTGGATTGGCTAACTGAG	⁹⁶⁷ GAGCCCAAACATCAGTAGTG	98.20	150
RH8	⁴⁴⁵ TCACAGGGTTGGTAGATCAG	⁵⁴⁹ CCAAGCTCTTGCTCAATCC	95.80	105
CYP2	³⁸⁸ CTGTACTGCCAAGACTGAG	⁴⁷³ CTATGGCCTTCACCATC	96.30	86
ROC3	⁹⁶ ATGGAGCTATTCGCGGATAC	²¹⁶ GGTGGAACTCGATCCTTTG	99.50	121
YLS8	¹⁶³ CCTCGTCGTCATCCGATT	²⁹³ CAGGCACCTCAGTGATGTC	96.90	131
PTP	²⁵² TGCCCTCCAATCAACTG	³³⁷ TGCACCACAACGACATTC	98.40	86

^a The number at the beginning of each primer shows the location of the primer on its GenBank sequence.

explore the variation in reference gene expression with tapping, 10 virgin trees were selected, and the first five consecutive tapplings were sampled. To examine the fluctuations in the expression of the reference genes among individual trees, five virgin trees of the same clone were randomly selected for sampling. After tapping, latex was collected for RNA extraction as described below.

2.3. RNA isolation and cDNA synthesis

RNA was extracted from latex according to Tang et al. [41]. RNA extraction from other *Hevea* tissues was performed according to Tang et al. [32]. Only RNA preparations having an A260/A280 ratio of 1.8 to 2.0 and A260/A230 ratio >2.0 were used for subsequent analysis. RNA integrity was verified by electrophoresis in a 2% formaldehyde denaturing agarose gel and visualized by ethidium bromide staining. Before cDNA synthesis, the RNA samples were treated with RNase-free DNase I (TaKaRa) to eliminate trace contaminating DNA according to the manufacturer's instructions. The failure to amplify an intron-containing genomic fragment of a sucrose transporter gene, *HbSUT3* [32] with the treated RNA sample as the template was used to confirm that the genomic DNA had been completely digested. First-strand cDNA synthesis was performed with 2.5 µg RNA and the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas) with hexamer random primers according to the manufacturer's protocol. The cDNA generated was diluted 20-fold and used as the template in subsequent quantitative real-time RT-PCR.

2.4. Selection and cloning of candidate reference genes

To acquire the *Hevea* orthologs of reference genes that had previously performed well in other plant species [18,19,38,39], publicly available *Hevea* ESTs were downloaded and used with our previously established latex EST library (Tang et al., unpublished data) to create local databases. All of the databases were screened by employing the Blast algorithm [42] with *Arabidopsis* candidate reference genes as query sequences. The EST sequences recovered allowed us to assemble contigs containing 22 uni-gene sequences; three of these genes were previously available in GenBank: 18S rRNA, an actin depolymerizing factor (ADF) and a cytosolic cyclophilin (ROC3). To facilitate subsequent PCR primer design, the full-length or partial cDNAs of the other 19 genes were

cloned by RT-PCR and rapid amplification of cDNA ends (RACE), and the sequences were deposited in GenBank (Table 1).

2.5. Design of PCR primers and evaluation of amplification efficiency

Primers were designed with Primer Premier 5.0 software (PREMIER Biosoft International, www.PremierBiosoft.com/faq.html) using the following criteria: primer lengths of 18–24 bp, melting temperatures of 59–61 °C, and amplicon lengths of 80–150 bp. For each primer pair, the amplification efficiency was calculated from the slope of a linear regression model [4]. Melting curve analysis and gel electrophoresis were used to confirm the specificity of the amplification (Supplemental Figs. 1 and 2). The amplified bands were cloned and sequenced.

2.6. RT-qPCR and data analysis

RT-qPCR was performed using the generated cDNA as the template with the fluorescent dye SYBR-Green (TaKaRa) and a LightCycler 2.0 system (Roche Diagnostics, Germany) according to the manufacturer's protocol. Each 20 µl reaction mix contained 2 µl template, 10 µl 2× SYBR Premix and 0.3 µl (0.15 µM) each primer. The reaction program was the following: an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 5 s at 94 °C, 20 s at 60 °C

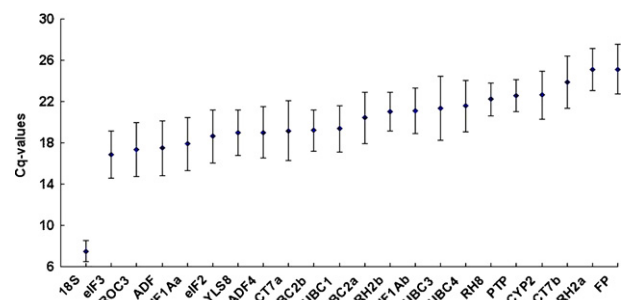


Fig. 1. Expression levels of the candidate reference genes in different experimental samples. The scatter plots illustrate the expression levels of the candidate reference genes in the examined *Hevea* samples ($n = 46$). Values are given as the quantification cycle numbers (Cq) of RT-qPCR. Bars indicate standard deviations.

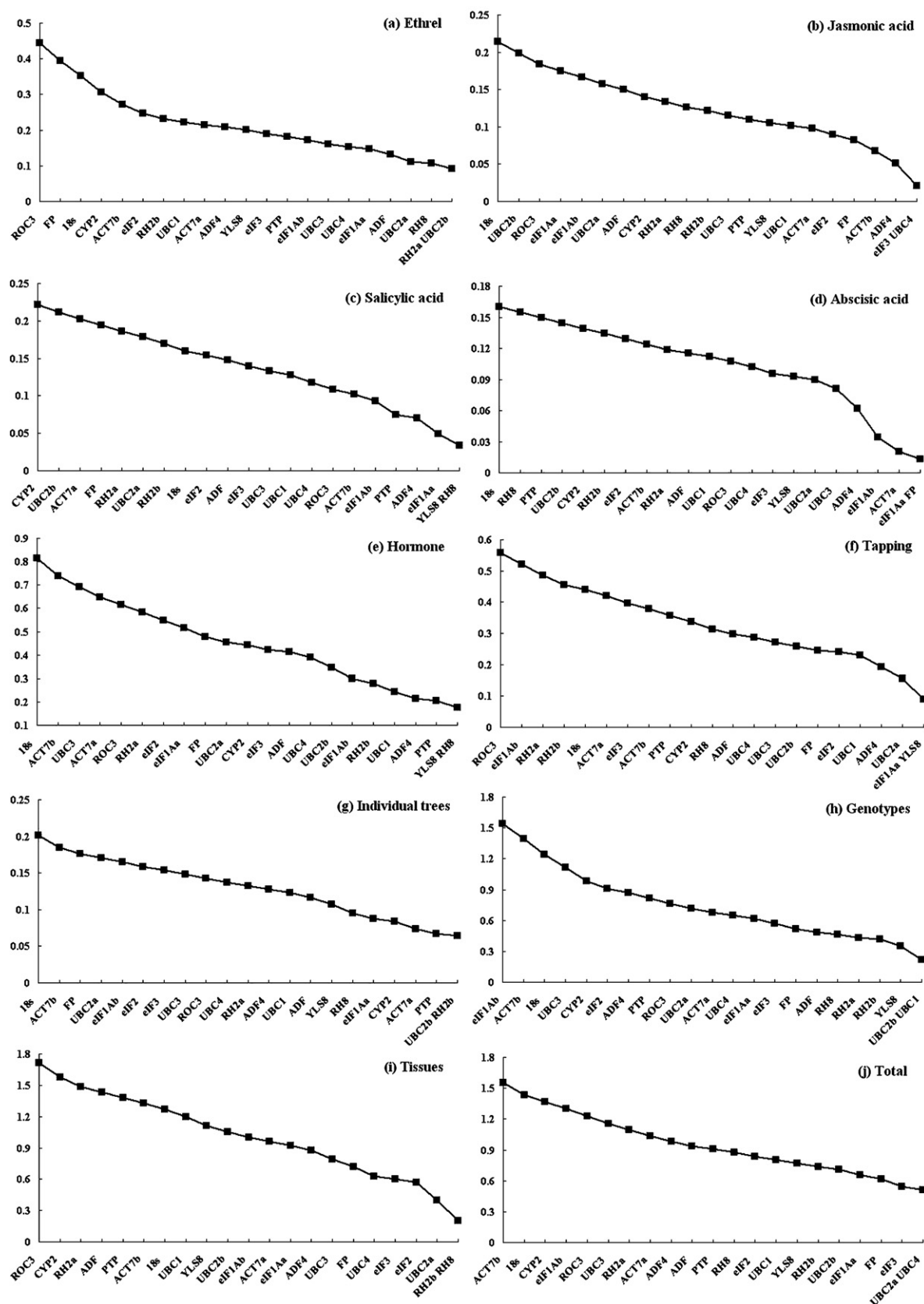


Fig. 2. Average expression stability values (M) of the candidate reference genes. Average expression stability values (M) were determined for the reference genes following stepwise exclusion of the least stable reference genes. The least stable genes are on the left, and the two most stable genes are on the right. (a) Latex samples from Ethrel-treated trees; (b) latex samples from jasmonic acid (JA)-treated trees; (c) latex samples from salicylic acid (SA)-treated trees; (d) latex samples from abscisic acid (ABA)-treated trees; (e) latex samples from hormone-treated trees; (f) latex samples from tapping-treated virgin trees; (g) latex samples from individual trees of the same rubber clone (variety); (h) latex samples of different genotypes; (i) different tissue samples; (j) all plant samples.

and 20 s at 72 °C. The baseline and quantification cycles (Cq) were automatically determined using the LightCycler 4.05 software. No template controls were included for each primer pair, and each PCR reaction was performed in triplicate. The stability of reference gene expression was analyzed by the software packages geNorm (version 3.4, <http://medgen.ugent.be/~jvdesomp/genorm/#download>) [8] and NormFinder (version 0.953, <http://www.mdl.dk/publicationsnormfinder.htm>) [13].

3. Results

3.1. Cloning of Hevea orthologs of reference genes

The full genomic sequence of Hevea is not yet publically available, although the scientists from Universiti Sains Malaysia announced one year ago that the Hevea genome had been successfully decoded (<http://www.researchsea.com/index.php>). Despite its economic importance, few nucleotide sequences from Hevea have been deposited in public databases, and only 13016 ESTs (mostly from latex) and 1846 gene sequences are available in GenBank. These publicly available ESTs and our previously acquired 20126 latex ESTs were used to obtain 22 assembled contigs of candidate Hevea genes. After searching with blastX, only three of the 22 Hevea genes, 18S, ADF and ROC3, were found to have their cDNA sequences already deposited in GenBank. To facilitate the design of RT-qPCR primers for subsequent analysis, the cDNAs of all of the other Hevea genes were cloned, including the full-length cDNAs of 14 genes (ACT7a, ACT7b, ADF4, UBC1, UBC2a, UBC2b, UBC3, UBC4, eIF1Aa, eIF1Ab, eIF2, eIF3, CYP2 and YLS8) and the partial cDNAs of 5 genes (FP, RH2a, DRH2b, RH8 and PTP), and the sequences were deposited in GenBank (Table 1).

3.2. Amplification specificity and expression levels of candidate reference genes

Using the primer pairs selected for each of the 22 candidate genes (Table 2), gene-specific amplification was confirmed by the appearance of a single peak in melting curve analyses following RT-qPCR. Electrophoretic separation of the amplicons produced single fragments of the expected sizes (82–150bp) in all cases, and nucleotide sequencing further confirmed the identity of these amplicons. The amplification efficiencies ranged from 90.6% to 99.8% (Table 2), which were well in the range of 90–105% as suggested acceptable in a RT-qPCR optimization guide from ABgene Limited (<http://www.abgene.com/downloads/QPCR.Optimization.pdf>). To give an overview of the relative abundance of the candidate reference genes in Hevea, the quantification cycle (Cq) values were determined for each gene across all of the experimental samples, and scatter plots were constructed (Fig. 1). As shown in Fig. 1, the Cq values for many reference genes showed rather large SD (more than ±2), which was due to the large number of biological samples used in this work. Similar results have been observed in some pertinent RT-qPCR studies [19,38]. The 22 genes had a wide range of Cq values, ranging from 7.50 for 18S rRNA (the gene with the highest expression level) to 25.14 for FP (the gene with the lowest expression level). The average Cq of all of the other genes was approximately 20 cycles, with most between 18 and 23. Therefore, the 18S transcript was approximately 8000 times more abundant than the average of all of the other genes. The Cq values of different genes within the same gene family varied either greatly, e.g., actins, or very little, e.g., ubiquitin-protein ligases.

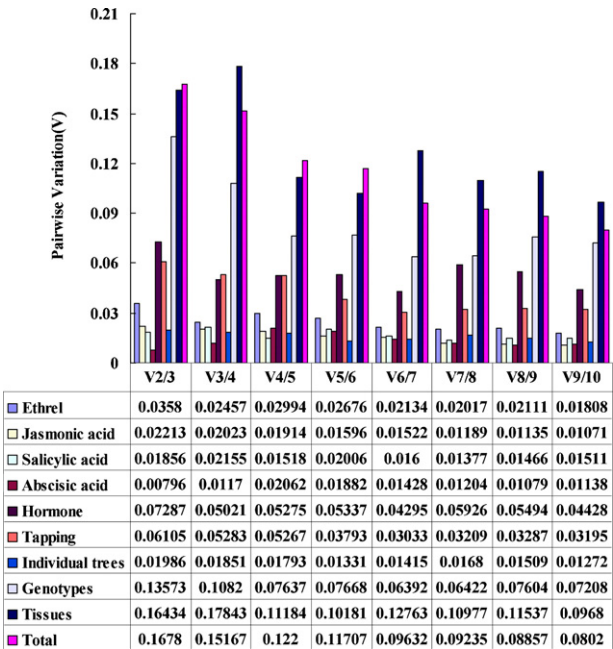


Fig. 3. Optimal number of reference genes required for effective normalization. The pairwise variation (V_n/V_{n+1}) between the normalization factors NF_n and NF_{n+1} was analyzed by the geNorm program to determine the optimal number of reference genes required for RT-qPCR data normalization.

3.3. Gene expression stability analysis

The software geNorm determines the expression stability (M) of each reference gene based on the average pairwise variation between all of the genes tested [8]. Genes with the lowest M value are the most stably expressed, while a higher M value is indicative of less stable expression. Nearly all of the genes had an M value below the geNorm threshold of 1.5, except for a few genes from several experimental conditions (tissue samples, different genotypes, and all plant samples) (Fig. 2). No reference genes performed well for all of the experimental conditions, and the sets of most stable reference genes were different for different experimental conditions. Three genes, YLS8, RH8 and UBC2b were the most stably expressed reference genes, respectively, in three of the ten conditions examined. Overall, UBC2a and UBC4 were the most stable reference genes among all of the sample sets, but ACT7b was the least stable.

To determine the optimal number of reference genes required for normalization, pairwise variation (V) analysis was performed with the software geNorm [8]. A threshold V value of 0.15 was adopted to determine whether the inclusion of an additional reference gene was necessary. Except among samples from different tissues or the complete set of samples, a V2/3 score lower than the threshold of 0.15 was achieved, indicating that the combination of the top two reference genes was sufficient for RT-qPCR data normalization under most experimental conditions (Fig. 3). The evaluation of samples from different tissues gave a V2/3 value of 0.164, suggesting that the top two reference genes were not sufficient for normalization (Fig. 3). The addition of a third gene did not improve the normalization quality because the V3/4 value even deteriorated to 0.178 (Fig. 3). After a fourth gene was included, the V score decreased below the threshold of 0.15–0.112. Therefore, at least four reference genes, i.e., RH2b, RH8, UBC2a and eIF2 would be required to normalize gene expression across samples from different tissues (Figs. 2i and 3). When all of the experimental samples were considered, the combination of the top four reference genes, i.e., UBC2a, UBC4, eIF3 and FP, was necessary for normalization (Figs. 2j and 3).

Table 3

Hevea reference genes for normalization and their expression stability order a calculated by NormFinder.

Stability rank	Performance of reference genes under different conditions									
	Ethrel	JA	SA	ABA	Hormone	Tapping	Individual plants	Genotype	Tissue	Total
1	RH8	eIF2	eIF1Ab	eIF1Ab	eIF1Ab	FP	PTP	RH2b	eIF2	eIF3
2	UBC2a	FP	RH8	UBC2a	UBC4	UBC1	RH8	UBC2b	UBC4	UBC2b
3	UBC3	YLS8	eIF1Aa	UBC3	eIF3	eIF2	YLS8	YLS8	eIF3	RH2b
4	UBC2b	ACT7a	YLS8	FP	RH2b	RH8	ACT7a	UBC1	UBC2a	FP
5	PTP	UBC3	ACT7b	ACT7a	ADF	UBC2a	UBC2b	RH8	UBC2b	UBC4
6	eIF1Aa	PTP	UBC4	eIF1Aa	UBC2b	eIF1Aa	RH2b	ADF	FP	YLS8
7	RH2a	ACT7b	ADF4	ADF4	UBC2a	ADF4	UBC1	RH2a	YLS8	UBC2a
8	eIF3	UBC4	UBC1	YLS8	CYP2	YLS8	ADF4	FP	RH8	RH8
9	ADF	UBC1	PTP	eIF3	YLS8	UBC2b	UBC4	eIF3	RH2b	UBC1
10	UBC4	eIF3	UBC3	RH2a	RH8	CYP2	eIF1Aa	eIF1Aa	UBC3	PTP
11	eIF1Ab	RH2b	ROC3	eIF2	PTP	PTP	RH2a	ROC3	ADF4	eIF1Aa
12	UBC1	ADF4	eIF3	ACT7b	UBC1	ACT7b	ADF	UBC4	UBC1	eIF2
13	YLS8	RH8	UBC2a	ROC3	FP	UBC3	UBC3	ACT7a	eIF1Aa	ADF
14	ACT7a	ADF	RH2b	UBC4	ADF4	eIF3	CYP2	PTP	18s	ADF4
15	ADF4	RH2a	18s	RH2b	eIF2	UBC4	eIF3	UBC2a	ACT7a	ACT7a
16	RH2b	CYP2	RH2a	CYP2	eIF1Aa	ADF	eIF2	ADF4	ACT7b	RH2a
17	eIF2	UBC2a	ADF	UBC1	ACT7a	ACT7a	ROC3	eIF2	PTP	UBC3
18	ACT7b	eIF1Ab	eIF2	ADF	ROC3	18s	eIF1Ab	CYP2	eIF1Ab	ROC3
19	CYP2	ROC3	FP	PTP	RH2a	RH2b	UBC2a	18s	ADF	eIF1Aa
20	18s	eIF1Aa	ACT7a	UBC2b	UBC3	eIF1Ab	FP	UBC3	RH2a	CYP2
21	FP	UBC2b	UBC2b	18s	ACT7b	RH2a	ACT7b	ACT7b	CYP2	18s
22	ROC3	18s	CYP2	RH8	18s	ROC3	18s	eIF1Ab	ROC3	ACT7b

We used the software NormFinder (<http://www.mdl.dk/publicationsnormfinder.htm>) to further confirm the results obtained by the geNorm program. NormFinder is an algorithm based on variance estimation and identifies the optimal normalization gene among a set of candidate genes under a given set of experimental conditions [13]. In contrast to geNorm, NormFinder includes intra- and intergroup variations when calculating the normalization factor (NF). The ranking generated by this approach differed somewhat from that determined by geNorm (Fig. 2 and Table 3). In all of the experimental conditions examined, at least seven of the top ten stable reference genes were the same in both programs, although their ranking orders differed more or less (Fig. 2 and Table 3). When the top five genes identified by both programs were compared, three to four of the genes were the same under most experimental conditions (Fig. 2 and Table 3). Under two experimental conditions (tapping and JA treatment), one to two of the genes remained the same (Fig. 2b and f, and Table 3). For the hormone-treated samples, the top five stable genes (YLS8 & RH8, PTP, ADF4, UBC1) identified by geNorm were completely different to those determined by NormFinder, and their rankings decreased to 9, 10, 11, 14 and 12, respectively, as evaluated by NormFinder (Fig. 2e and Table 3). By comparison, the two software programs were more consistent in ranking the least stable reference genes (Fig. 2 and Table 3). For samples from Ethrel-treated trees, individual plants and the complete sample set, the five least stable genes and their rankings were identical in both software programs (Fig. 2a, g, j and Table 3). In the seven other conditions investigated, the five least stable genes identified in geNorm were confirmed by Normfinder, although their ranking order was slightly altered (Fig. 2b–f, h, i and Table 3).

3.4. Reference gene validation

The effects of tapping and Ethrel treatment on the expression of *HbSUT3*, a Hevea sucrose transporter gene, were analyzed using either 18S rRNA or the selected reference genes for expression normalization (Fig. 4). Previously 18S rRNA was used to quantify expression in *H. brasiliensis* to demonstrate that *HbSUT3* plays an important role in the regulation of sucrose loading into laticifers and subsequent rubber productivity [32]. Both tapping and Ethrel treatment are important measures to stimulate latex production in

Hevea [43,44]. When normalized using 18S rRNA, the expression of *HbSUT3* increased significantly during the first several tapplings (cuts), then declined and fluctuated in subsequent tapplings while maintaining a level significantly higher than that in the initial tapping (Fig. 4a), in agreement with the previous study [32]. Similar

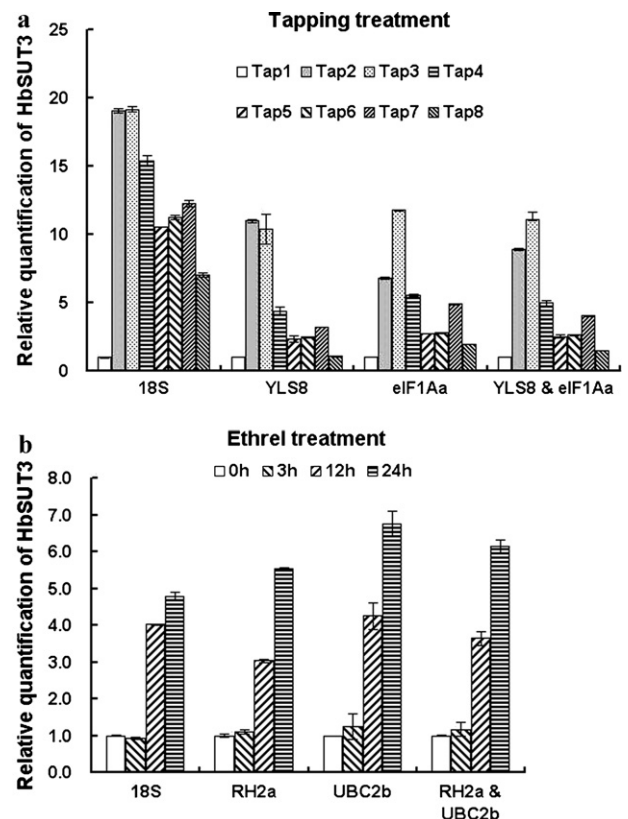


Fig. 4. Relative quantification of *HbSUT3* expression in response to tapping and Ethrel treatment using 18S rRNA or one of two of the most stable reference genes for normalization. (a) The results are presented as the mean fold change in relative expression compared to the first tapping; (b) the results are presented as the mean fold change in relative expression compared to samples at 0 h of Ethrel treatment. Bars indicate the standard deviation of three technical replicates.

expression patterns were obtained when either one or two of the most stable genes (*YLS8* and *eIF1Aa*, as identified by geNorm) were used for normalization (Figs. 4a and 2f). However, the estimated transcript abundance was much lower when normalized against the most suitable reference genes rather than against 18S rRNA, demonstrating that *HbSUT3* expression during tapping was previously overestimated [32]. During Ethrel treatment, the pattern of *HbSUT3* expression was similar when normalized against either 18S rRNA, or one or two of the most stable reference genes (*RH2a* and *UBC2b*), but *HbSUT3* expression was underestimated when 18S rRNA was used for normalization (Figs. 4b and 2a). *HbSUT3* expression appeared to be slightly repressed after 3 h of Ethrel treatment when 18S rRNA was the internal control (Fig. 4b) [32] but was somewhat elevated when the most stable reference genes were used (Fig. 4b). Therefore, the selection of appropriate references is critical for accurately evaluating the expression of a gene of interest.

4. Discussion

Hevea is a tree species with significant economic and industrial importance. Most of the basic studies in Hevea have focused on the regulation of latex regeneration and rubber yields, which are known to be affected by genetic and exogenous factors, such as tapping and hormone treatments (especially ethylene) [24,25,32,45,46]. In Hevea, 18S rRNA and actin genes are the most frequently used references in RT-qPCR-based gene expression studies [31,32,34,35], but no systematic validation of these genes has been performed. GeNorm and NormFinder analyses demonstrated that 18S rRNA is not appropriate for most experimental conditions (Fig. 2 and Table 3). In fact, 18S rRNA was the least stable genes under three different experimental conditions (JA treatment, hormone treatment and individual plants; Fig. 2b, e, g and Table 3). When all of the experimental samples were considered together, 18S rRNA ranked as the second least stable reference gene (Fig. 2j and Table 3). Actin genes are commonly used reference genes in plants, but their performance is not always satisfactory [39,47,48]. We examined two actin genes, *ACT7a* and *ACT7b*, and found that they performed poorly, ranking as the eighth-least and least stable references, respectively (Fig. 2j and Table 3).

We compared 22 potential reference genes for Hevea across 46 biological samples under 10 experimental conditions. To our knowledge, this is one of the most extensive screenings for reference genes in plants, and is the first systematic attempt to identify reference genes in Hevea. None of the candidate reference genes performed consistently well for all of the experimental conditions investigated, and the best reference genes varied by condition (Fig. 2 and Table 3). Pairwise variation (V) analysis by geNorm demonstrated that a combination of the two most stable reference genes would be sufficient for normalization in most experimental conditions, while at least four reference genes should be used in tissue samples or when comparing all of the samples (Fig. 3). NormFinder was used to confirm the results of the geNorm analysis. The two software programs were more consistent in identifying the least stable reference genes than the most stable ones (Fig. 2 and Table 3). After analyzing the data in other references [39,47,49,50], we concluded that this consistency was a common phenomenon for the two programs. Here, it should be noted that in this work, other than testing the genes on different genotypes, all the other experimental conditions have been done on one Chinese Hevea clone, Reyan7-33-97. The performance of the reference genes may be different between different clones, so a prior validation is suggested to be conducted when the selected references are used in a new clone.

The mitosis protein gene *YLS8* was identified by microarray and RT-qPCR analyses as a novel suitable reference in *A. thaliana*

[19]. *YLS8* performed well in most of our experimental conditions and was the most suitable reference in three conditions (SA, hormone and tapping treatments) (Fig. 2c, e and f). Ubiquitin-protein ligase (ubiquitin-conjugating enzyme) genes are appropriate reference genes in *A. thaliana*, *Brachypodium distachyon*, and *Brachiaria brizantha* [18,39,51]. Five UBC genes, *UBC1*, *UBC2a*, *UBC2b*, *UBC3* and *UBC4*, were selected for this work, and generally performed well in Hevea (Fig. 2 and Table 3). Two of the genes (*UBC2a* and *UBC4*) were identified as the most suitable references when all of the samples were taken together (Fig. 2j). However, the performance of UBC genes varied between each other and between different experimental conditions (Fig. 2 and Table 3). The performances of the four eukaryotic translation initiation factor genes (*eIF2*, *eIF3*, *eIF1Aa* and *eIF1Ab*) differed considerably (Fig. 2 and Table 3).

In Hevea, the rate of sucrose importation into laticifers regulates rubber biosynthesis and latex regeneration [24,28]. In regularly tapped Hevea trees, *HbSUT3* was shown to play an active role in sucrose loading into laticifers and in rubber productivity when the expression of *HbSUT3* was normalized against 18S rRNA [32]. We obtained similar patterns when normalizing the expression of *HbSUT3* with four different normalization factors that included 18S rRNA or the best reference genes identified by geNorm used individually or in combination (Fig. 4). However, the relative quantification of *HbSUT3* varied with the internal control used. The transcript abundance of *HbSUT3* was overestimated when 18S rRNA was used instead of one or two of the selected references (*YLS8* and *eIF1Aa*) to normalize expression during tapping (Figs. 4a and 2f); in contrast, the expression of *HbSUT3* was underestimated by 18S rRNA normalization in Ethrel treatment (Fig. 4f). The patterns of *HbSUT3* expression were similar whether the two most stable references were used individually or in combination. Therefore, one suitable verified reference gene may be sufficient for normalizing data to roughly approximate the expression of a gene of interest. However, additional reference genes (dependent on the geNorm threshold of 0.15) are needed if accurate expression levels among different samples are required.

Acknowledgements

This research was supported partly by the Fundamental Research Funds for Rubber Research Institute, CATAS (Project Number 1630022011022 and XJSYWFZX2010-13), National Natural Science Foundation of China (30760114; 30600386), and Natural Science Foundation of Hainan province (808187;309045).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2011.04.014.

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