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The Selection of Reference Genes for a Comparison of the Biosynthesis of Flavoids from *L. confusa* and *L. japonica*

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Research Article

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ABSTRACT

The stability of 8 candidate reference genes in 4 tissues of *L. confusa* and *L. japonica* were evaluated by using four algorithms to assess the validity of reference genes for their use in accurate normalization of qRT-PCR data. The 8 candidate reference genes are *β-TUB, eIF-4a, ACT11, eEF-1α, GAPDH, UBQ10, 18S* and *25S*, and the 4 tissues were leaves, green buds, white buds and white flowers. Results showed that the most stable genes selected by the four algorithms of these 8 genes are slightly different for these tissues, but the least stable genes were the same in each case. *ACT11, eIF-4a* and *β-TUB* are the three most stable genes as selected by geNorm, *β-TUB* is the most stable as judged by Delta CT and Normfinder, and GAPDH by Bestkeeper. *18S* and *25S* were the two least stable genes as judged by all the four algorithms. To verify the validity of these selected genes by the four methods, the comparative expression level of *FS* Ⅱ gene in these 8 tissues were normalized with them. Similar expression profiles of *FS* Ⅱ were seen with the most stable genes selected by the four methods and the highest was found in the leaves and the lowest in the flowers of *L. confusa*. But they are apparently different from the profiles when subjected to normalization by the least two stable genes: *18S* and *25S*. This demonstrated that apporatative reference genes are indispensable for gene profile assays, and these recommended genes by the four algorithms are all reliable. This is the first report on the selection of candidate reference genes in *Lonicera* species and this will provide a more precise comparsion of gene expression in the biosynthesis pathway of flavoids between *L. confuse* and *L. japonica*.

INTRODUCTION

Lonicerae japonicae flos (Jinyinhua), the dry bud and the flower of Lonicera, is the traditional Chinese medicine for the clearing heat and detoxication, dispelling wind and heat, protecting liver and resistance of poison [1]. *L. confusa*, which has similar pharmacological effects to *L. japonicae* is not considered a good plant source of jinyinhua because it has a low content of luteoloside [2-5]. The difference in luteoloside content between the two species is likely to result from differences in the enzymes involved in biosynthetic pathways of flavoids. Comparison analysis of gene expression between these two species is a fundamental step to answer these questions.

The two most commonly used approaches for gene expression analysis at the transcriptional level are microarrays and quantitative reverse-transcription PCR (qRT-PCR). qRT-PCR, with its advantages of speed, sensitivity, reproducibility and wide dynamic range, is becoming the method of choice to provide simultaneous measurement of gene expression in many different samples for limited number of genes. At present, it is widely applied to molecular medicine, biological sciences, microbiology and diagnosis [6-9]. The purity and integrity of RNA, the efficiency of reverse transcription and other factors can influence the

accuracy of quantitative results [9-11], and it is therefore essential to normalize and adjust the data of qRT-PCR with a reference gene. In theory, these reference genes should remain constant in the cells of different tissues or when tissues are subjected to different treatments. Thus, the genes involved in basic cellular processes such as 18S rRNA, ubiquitin, actin, tubulin and GAPDH were often used as reference genes. But several studies have shown that no gene has an absolutely stable expression; it is only comparatively constant for a certain cell type or under certain conditions [12-14]. Thus, it is imperative to estimate the validity of reference genes for the accurate normalization of qRT-PCR data.

Recognizing the importance of internal controls for qRT-PCR, some statistical algorithms were developed for the evaluation of possible reference genes in a given biological sample. GeNorm, the first reported software, calculates the expression stability value by the average pairwise variation of a particular gene with all other genes [15]. NormFinder estimates the stability value according to the intra- and inter-group variation [16]. BestKeeper determines the rank of housekeeping genes using pair-wise and Poisson correlation analyses of all pairs of candidate genes as well as the target gene [17]. Delta CT ranked the stability of genes using the Standard Deviation of the Mean ΔCt of one gene with all the other genes.

The expression levels of genes relative to the biosynthesis of flavoids have been studied in different *Lonicera* species [18]. Unfortunately, identification of reference genes was not carried out. In this study, the suitability as internal controls of 8 housekeeping genes were evaluated in the tissues of *L. japonica* and *L. confusa*, which are often used as reference genes in plants. Our work will make the subsequent gene expression analysis between the two species more accurate.

MATERIALS AND METHODS

Materials

Four tissues including leaves, green buds, white buds and white flowers, were respectively collected from *L.confusa* grown in Xincheng County of Guangxi Province, and *L. japonica* grown in Qufu County of Shandong Province at the beginning of the flowering season in 2014. The samples of leaves, green buds, white buds and white flowers of *L. confusa* are referred sequentially to as FL, FG, FW and FF in this paper. Correspondingly, the same tissues of *L. japonica* are referred to as LL, LG, LW and LF respectively.

RNA isolation and first strand cDNA synthesis

Total RNA was extracted from these 8 tissues by Trizol method (Invitrogen, USA). RNA was treated with RNase-free DNase I according to the manufacturer's instruction (Takara, Japan). RNA integrity was detected by 1% agarose gel electrophoresis, and its purity and concentrations were measured by ultraviolet spectrophotometer (Shimadzu, Japan). All RNA samples were adjusted to an equal concentration, measured and adjusted again to homogenize RNA input in the subsequent cDNA synthesis reaction. First Strand cDNA was synthesized by AMV First Strand cDNA Synthesis Kit (Sangon, China) and then diluted 8-fold to serve as the template of PCR.

Primer design and real-time PCR

Gene-specific primers were designed using Primer Primier 5.0 software with the amplicon lengths of 70-250 bp and melting temperatures between 57-61℃. The primers sequences are given in **Table 1**. The PCR mixture contained 2 μL of diluted cDNA, 10 μL of 2X SybrGreen PCR Master Mix, 200 nM of each gene-specific primer in a final volume of 20 μL. The real-time PCR was performed employing LightCycler480 Software Setup (Roche, Swiss) and ABI SybrGreen PCR Master Mix (2X) kit (ABI, USA). All the PCRs were performed on under following conditions: 3 min for 95℃, and 40 cycles of 15 s for 95℃ and 40 s for 60℃. The specificity of amplicons was verified by melting curve analysis (65-90℃) after 40 cycles. Primer efficiencies were calculated based on a standard curve generated using a 10-fold dilution series at 5 dilution points that were measured in triplicates. Expression levels of each sample were calculated based on three technical replicates. Negative controls with water instead of cDNA were included for each gene target assayed.

Table 1. Description of reference genes and *FS* Ⅱ gene, as well as the primer sequences for qRT-PCR.

Gene name	Tentative annotation	Primer sequence (5'-3')	Length(bp)
18S	18S Ribosomal RNA	F:5'CGAGACCTCAGCCTGCTAACTA 3' R:5'CCAGAACATCTAAGGGCATCAC 3'	130 bp
25S	25S ribosomal RNA	F: 5' GTCGGGTTGTTTGGGAATG 3' R: 5' CGGTACTTGTTTGCTATCGGT 3'	90 bp
UBQ10	Ubiquitin 10	F:5' TCAGCAGAGGCTTATTTTCGC 3' R:5'CGTCTTTCCCGTTAGGGTTTTA 3'	148 bp
β-TUB	Beta-tubuli	F: 5' CGTGTATGTGACCACCTCAAACT 3' R:5'CCTGGAAGGATATTAGCAGTAGTCT 3'	167 bp
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: 5' GCATCTGGTAGACAAGAAGGCT 3' R:5'TGTGAAGAGCGAGTCAGTAAATGT3'	124 bp
ACT11	Actin 11	F: 5' CTGGTGTTATGGTTGGGATGG 3' R: 5' GATACCTCTTTTGGATTGGGCT 3'	71 bp

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eEF-1α	Eukaryotic elongation factor 1-alpha	F:5'GATTGGAGGCATTGGAACTGT3' R:5'AGTGACTACCATACCAGGCTTGA 3'	73 bp
eIF-4a	Eukaryotic initiation factor 4a	F:5'CAACTATTCTGGTGCTGCTGATT3' R:5'AATGCACTCAAACTCCTCTCACTAT3'	78 bp
FS Ⅱ	flavone synthase II	F:5' CGTAAACACTCCGCTGCCATT 3' R:5'TGGTCCTAATGGGGAGAAAGTG 3'	151 bp

Data analysis

To analyze the gene expression stability, the geNorm, Normfinder, Delta CT and BestKeeper (http://www.leonxie.com/referencegene.php) software were used. The comparative $2^{\Delta\Delta Ct}$ method was used to evaluate the relative quantities of each amplified products.

RESULTS

Variation of candidate reference genes

The Ct values of all genes tested, ranged from 16 to 32 in the total dataset **(Figure 1a)**, showing that their expression abundance varied considerably in the different tissues. *eEF-1α* and *eIF-4a* are the two most abundant genes with the least mean Ct values being less than 20. By contrast, UBQ10 and *ACT11* are the least abundance with high Ct values (29.81 and 27.49). Their variation degree of Ct values also varied: UBQ10 and GAPDH have a comparatively narrow variation range, and 18S and 25S have a wide variation range. In addition, the variation degree of Ct for the same gene is different in *L. confusa* **(Figure 1b)** and *L. japonica* species **(Figure 1c)**. Generally, the variation degree of the candidate genes in *L. confusa* is larger than that in *L. japonica*, though their average Ct values varied slightly for the two species. In the tissues samples of *L. confusa*, the Ct value of *eEF-1α* varied least with the respective gene in *L. japonica* being *eIF-4a*. These variable Ct values of the same gene in different tissues of the different species reinforced the significance of the validity of this study which aims to determine the reference genes for the accurate normalization of quantitative PCR.

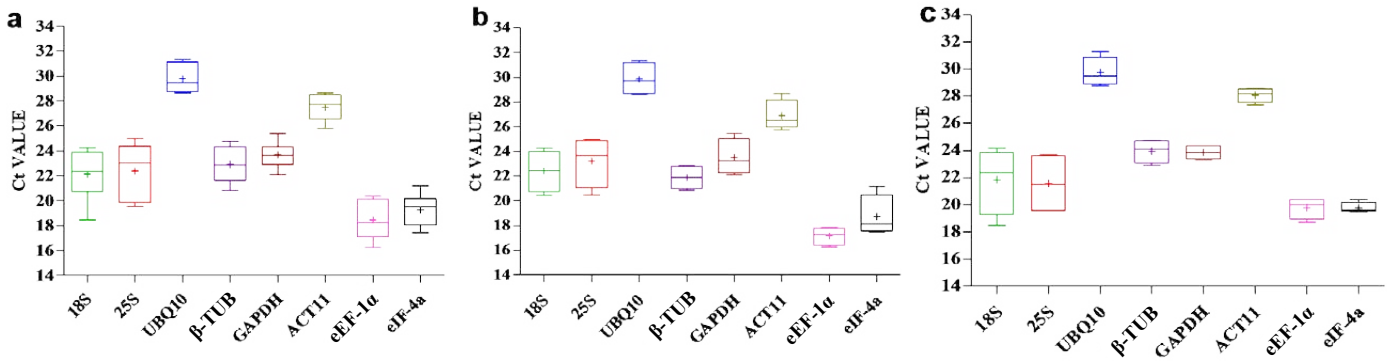

Figure 1. qRT-PCR Ct values for all 8 tested reference genes. Box charts of Ct value for each reference gene in all samples (a), in the samples of *L. confusa* (b), or in the samples of *L. japonica* (c). The vertical lines of the boxes indicate the value ranges. And the horizontal line and the plus in the boxes indicated the first or third quartile and the mean value of the Ct value for each reference gene.

Selection of candidate gene

We used geNorm to analyze the expression stability of the tested genes in these 8 tissues and determine the minimal number of reference genes to be needed in the individual experiment for relative quantities of gene expression. In geNorm, M was defined as the average pairwise variation of a particular gene with all other candidate genes. Usually, a gene with a lower M value is considered to have more stable expression. Results show that the stability of these genes in different samples sets is not identical. When all the tissue samples were taken into account, the M value of *eIF-4a* and *ACT11* was the lowest, and that of *18S* and *25S* are the highest **(Figure 2a).** This demonstrates that *eIF-4a* and *ACT11* are the two most stable genes, and *18S* and *25S* are the least stable genes of the 8 tested genes. However, the most stable genes are *β-TUB* and *eEF-1α* in *L. confusa* **(Figure 2b)** and *β-TUB* and GAPDH in *L. japonica* **(Figure 2c).** Interestingly, *18S* and *25S* were the least stable genes in the samples of *L. confusa* and *L. japonica*.

The number of candidate reference genes for normalization was determined by the pairwise variation $V_{n/n+1}$ between the two sequential normalization factors for all samples, which reflected the effect of the additional gene. The result showed that pairwise variation $V_{n/n+1}$ of all three datasets was above 0.15 **(Figure 3).** Taking all the samples together, the pairwise variation $V_{n/n+1}$ decreased to the least value (0.237) when the fourth factor was included, indicating that three normalization factor is sufficient in this dataset. When combined with the above results of the rank of candidate reference genes **(Figure 2a)**, *eIF-4a, ACT11* and *β-TUB* would be the most appropriate the reference genes. Similarly, the pairwise variation of $V_{n/n+1}$ in the samples of *L. confusa* also decreased from 0.346 of $V_{2/3}$ to 0.245 of $V_{3/4}$, the least value of the pairwise variation of $V_{n/n+1}$ in this dataset, the 3 top reference genes in **Figure 2b** should be enough to calculate the genes relative expression level in the tissues of *L. confusa*. They

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are *ACT11, β-TUB* and eEF-1α. For the tissues of *L. japonica*, the pairwise variation $V_{2/3}$ is 0.184, $V_{3/4}$ is 0.214 and $V_{4/5}$ is 0.172, which demonstrates that 4 would be the optimal number of reference genes in the relative quantification of gene expression. However, under the situation of many tested genes and when copy numbers of genes are low, it is inappropriate to use 4 control genes simultaneously. Taking the balance between accuracy and practice, 2 reference genes would be more appropriate for the quantification PCR in the tissues of *L. japonica*. Combining with the rank of tested genes **(Figure 2c)**, the data would suggest that *β-TUB* and GAPDH are the best candidate genes to use.

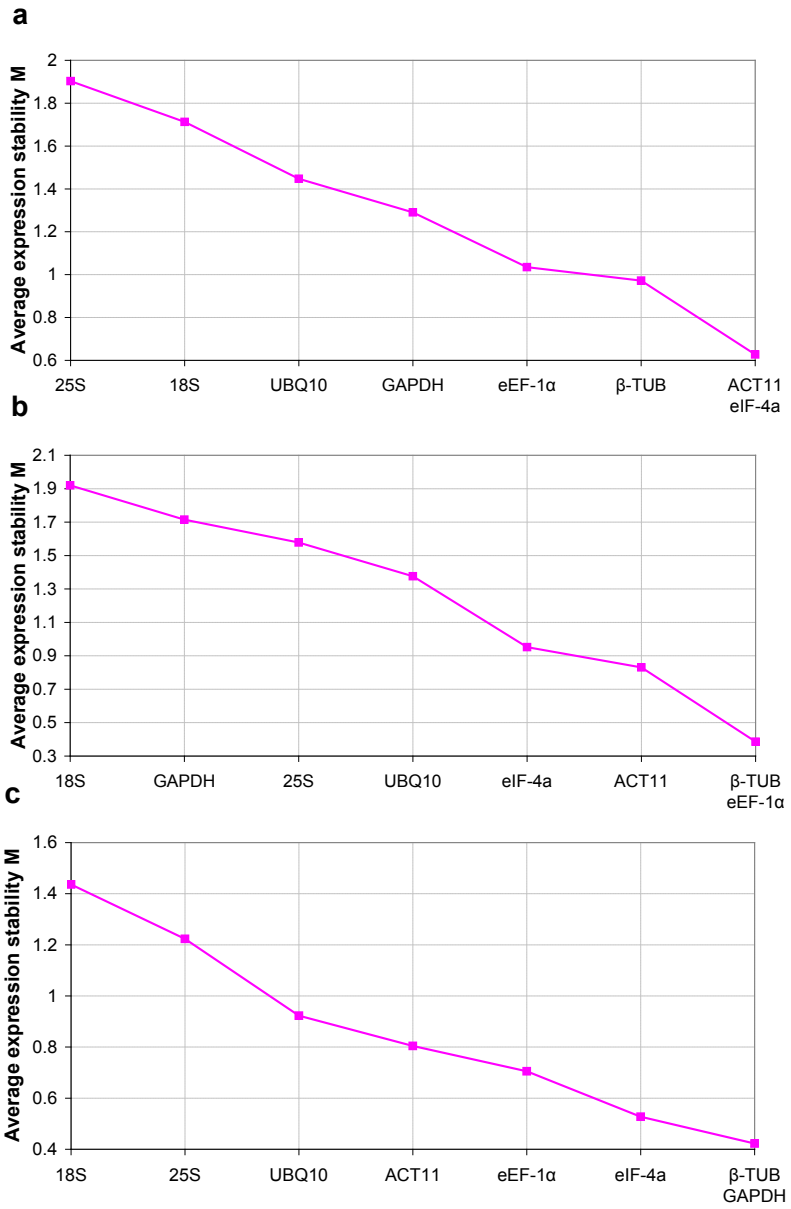

Figure 2. Average expression stability (M) of tested reference genes calcualated by geNorm. Gene expression stability of 8 candidate reference genes were computed with geNorm in all samples (a), in the samples of *L. confusa* (b), and *L. japonica* (c). The higher the M value, the lower the stability.

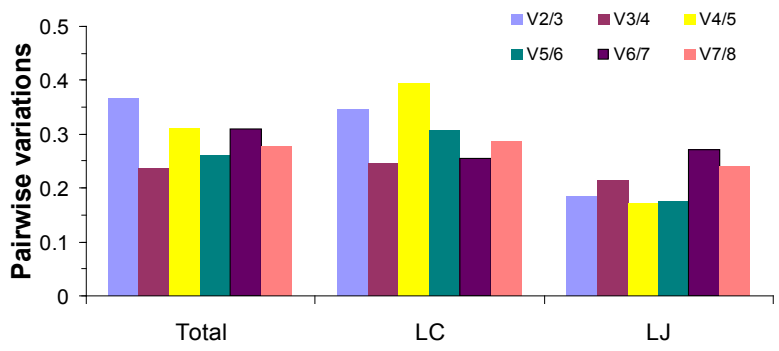

Figure 3. Pairwise variation to determine the optimal number of control genes for accurate normalization. The pairwise variation was calculated by genorm in all samples (Total), in the samples of *L. confusa* (LC), and *L. japonica* (L J).

To minimize the bias generated by the geNorm, the expression data of total samples were also analyzed by other three different statistical approaches to rank the stability of candidate housekeeping genes. These are Delta CT, BestKeeper and Normfinder. The results show that slightly different ranks of candidate genes are derived from the different algorithms **(Table 2).** Among the three algorithms, the rank calculated by Delta CT is the most similar to that with Genorm. *β-TUB* is the best candidate reference genes by the Delta CT and Normfinder, and GAPDH by BestKeeper. Notably, *18S* and *25S* are the least stable genes selected from all these four algorithms.

Table 2. Ranking of the candidate reference genes analyzed by different strategies.

Rank	Delta CT	Genorm	BestKeeper	Normfinder
1	β-TUB	ACT11/eIF-4a	GAPDH	β-TUB
2	eIF-4a	N/A	ACT11	eIF-4a
3	ACT11	β-TUB	UBQ10	GAPDH
4	eEF-1α	eEF-1α	eIF-4a	UBQ10
5	GAPDH	GAPDH	β-TUB	ACT11
6	UBQ10	UBQ10	eEF-1α	eEF-1α
7	18S	18S	18S	18S
8	25S	25S	25S	25S

Validity of selected reference genes

To further evaluate the validity of the reference genes from these four algorithms, we determined the relative expression level of the *FS* Ⅱ gene in whole samples and to examine whether its expression pattern changed with these selected reference genes as normolization factors respectively. The *FS* Ⅱ gene encodes flavone synthase which is responsible for synthesis of luteoloside, an index component that discreaminates *L. japonica* from others within the *Lonicera* species. The results revealed that when the combination of *eIF-4a, ACT11* and β-TUB (selected by geNorm, **Figure 4a**), and *β-TUB* (selected by Normfinder and Delta CT, **Figure 4c**) or GAPDH (selected by Bestkeeper, **Figure 4d**) alone are recruited, the relative expression profiles of the gene in tissues is similar: highest in the leaves, middle in the buds and lowest in flowers for both the species. When *18S* and *25S*, the least stable reference genes were ranked by the four methods, are applied, the *FS* Ⅱ gene relative expression pattern is altered: its expression levels in the leaves and buds of *L. japonica* is similar **(Figures 4f and 4g)**, which is improbable as the total content of luteolin and luteoloside in the leaves is about 10 times higher than that in the buds [19,20]. These results imply that the four methods used are reliable ways to select the housekeeping gene. It is notable that the expression pattern of *FS* Ⅱ in the 8 samples calculated by *eIF-4a* **(Figure 4b)** is accordant with that of the combination of *eIF-4a*, *ACT11* and *β-TUB* **(Figure 4a)**. Thus, *eIF-4a* could also be used as the sole reference gene when gene expression levels in tissues of *L. japonica* and *L. confusa* are measured.

DISCUSSION

It has been generally accepted that selection of suitable reference genes is a prerequisite for the success of gene expression profiling. Thus, the stability of various housekeeping gene expression under a given experimental condition must be evaluated. In this study, in order to compare flavoids synthesis in *L. japonica* and *L.confusa*, the expression stability of 8 candidate reference genes in leaves, buds and flowers in the two species were estimated. The rank of their expression stability in these samples calculated by different algorithms was slightly different, but the comparative expression levels of *FS* Ⅱ in these tissues with their own most stable genes as internal control is similar: highest in the leaves and lowest in the flowers. This implied that the four approaches are all reliable for selection of reference genes to normalize the qRT-PCR data.

A small difference was also observed in the expression level of *FS* Ⅱ in FG and FW when multiple genes were used as internal controls as well as when a single gene alone was used. When the combination of eIF-4a, ACT11 and β-TUB was used as reference genes, the expression level of *FS* Ⅱ is higher in FG than FW, but the opposite was seen when *β-TUB* **(Figure 4c)** or GAPDH **(Figure 4d)** were used singly. This is similar to that of SmDXR [21], which shows an expression pattern in tissues of Salvia miltiorrhiza that is slightly different depending on whether ACT and Ubiquitin are used in combination or singly as internal controls.

If *eIF-4a, ACT11* and *β-TUB* are used as internal control, *FS* Ⅱ expression level in FL is 6688 times of that in FF, but it is only 1.67 times in FG of that in FW. As seen in **Figure 4**, *FS* Ⅱ expression level in FL is the highest in all six situations, but its relative amount in FG and in FW changed when we use different reference genes normalized, as shown in **Figures 4a, 4c and 4d.** This implies that when the difference in the expression level in samples is in orders of magnitude, the resultant gene expression calculated with a single gene is also reliable. But when the difference is minute, the normalization with multiple reference genes becomes more accurate. Of course, it is not absolute. As seen in **Figures 4a and 4b**, the expression profiles of *FS* Ⅱ is the same when it is estimated with *eIF-4α* alone and with *eIF-4a, ACT11* and *β-TUB* in combination. Similarly, the expression level of AsC in Aquilaria sinensis didn't vary with GAPDH and TUA in combination or with GAPDH alone [22]. However, when *18S* and *25s*, the least stable genes ranked by all four algorithms, were respectively used as reference genes, the difference of *FS* Ⅱ expression level in leaves and buds of *L. Japonica* could be not detected **(Figure 4e)**, though its expression level in leaves is 12 times of that seen in

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LG and 47 times that seen in LW **(Figure 4a)**. This situation is in agreement with the conclusion of earlier studies, which is that a normalization strategy based on a single gene might lead to erroneous expression profiles [15,23,24].

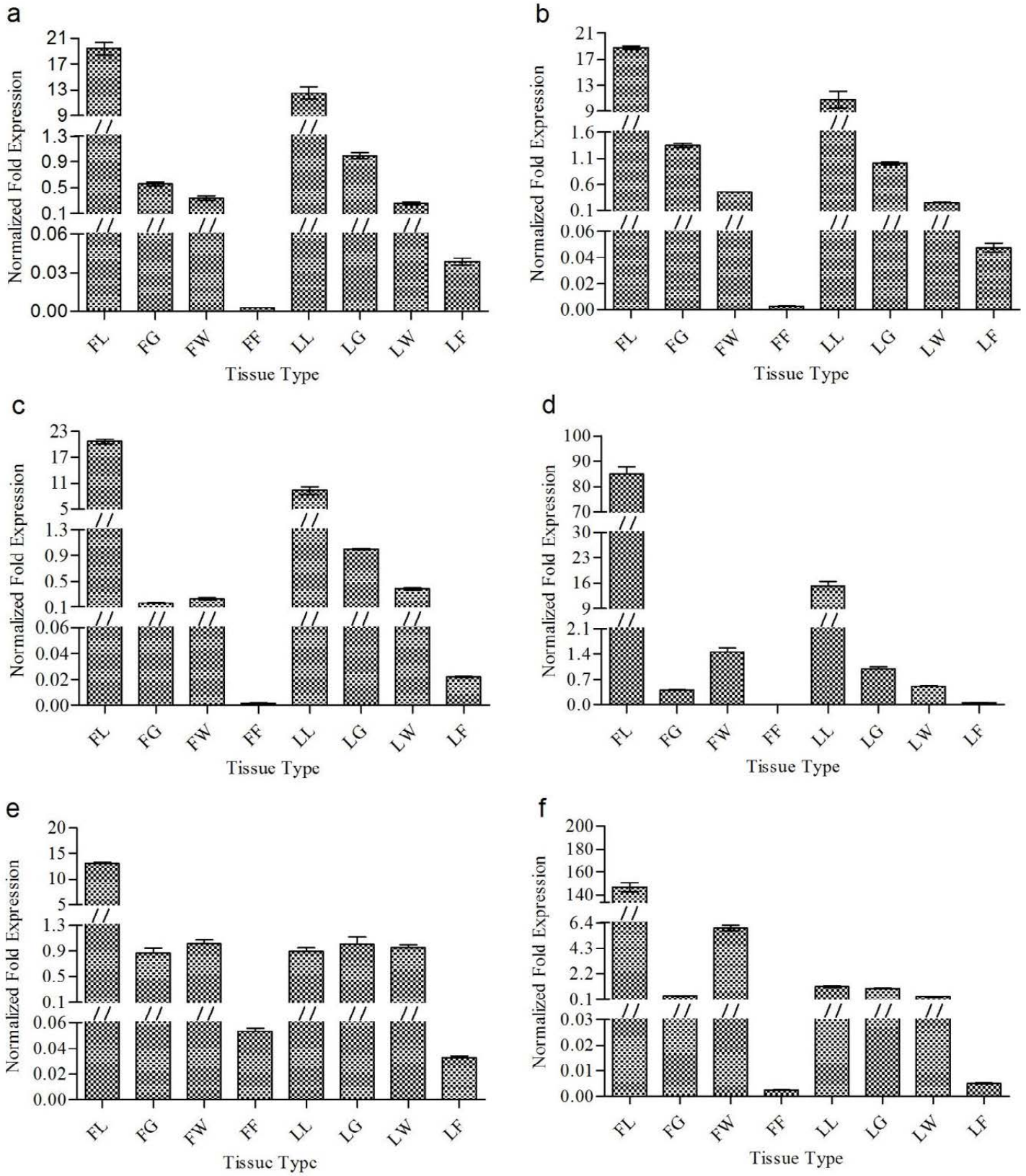

Figure 4. Relative expression levels of *FS* Ⅱ in all the 8 samples normalized by different candidate reference genes. The *FS* Ⅱ expression levels were normalized with individual and combined reference genes. Reference genes used for normalization from a to f are the combination of *eIF-1α, ACT11* and *β-TUB; eIF-4a; β-TUB; GAPDH; 18S* and *25S*.

In Yuan's study [25], the expression level of *FS* Ⅱ in buds of *L. Japonica* was shown to be lower than that of other *Lonicra* species and is higher in buds than in leaves of *L. Japonica*. The results from that study is completely opposite to those found in this study, which is that the expression level of *FS* Ⅱ is higher in buds of *L. Japonica* than in *L. confusa*, and higher in the leaves

than in buds of *L. Japonica*. It is obvious the different conclusion derived is due to the different normalization of qRT-PCR data. Yuan et al. used the *18S* as the reference gene, which is ranked by all four algorithms as the second least stable gene in our study. Yuan's result is similar to our result presented here with the *18S* normalized qRT-PCR data **(Figure 4f)**. This reinforces the significance of assessing the validity of reference genes before their application for normalization of gene expression.

The same housekeeping genes in different species varied in abundance and stability, such as those seen in *L. Japonica* and *L. confusa* **(Figures 1b and 1c)**. The pairwise variation used as the criterion of expression stability of genes could minimize nonspecific errors derived from different treatments or different type cells [15], and therefore is especially appropriate for the comparison of gene expression analysis between different species. Furthermore, the average value would better reflect the true than using a single number in statistics, normalization with the average expression of top most stable genes would make us gain a more objective and accurate result. Based on the above analysis, we suggest that geNorm is the appropriate approach to rank the candidate reference genes for normalization of qRT-PCR data when making a comparison of two species such as *L. Japonica* and *L. confuse*.

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