RESEARCH NOTE

Evaluation of suitable reference genes for gene expression studies in *Lycoris longituba*

SHAO JIE CUI[1], QIU LING HE[2], YING CHEN[1] and MIN REN HUANG[1]*

[1]*College of Forest Resources and Environments, Nanjing Forestry University, Nanjing 210037, People's Republic of China*
[2]*College of Agriculture and Biotechnology, Huajiachi Campus, Zhejiang University, Hangzhou 310029, People's Republic of China*

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Introduction

An appropriate choice of reference genes is prerequisite to accurate quantification of gene expression with quantitative real-time polymerase chain reaction (qRT-PCR). In this study, 13 candidate reference genes were selected to study the expression stability of qRT-PCR normalization in different tissues and floral development of *Lycoris longituba*.

Flowers are the most attractive parts of most angiosperms, and many studies of floral development have been conducted on floral morphogenesis, senescence mechanism of flower and modification of flower colour. During recent years, transcription factors (TFs) have been found to play important regulatory roles in floral development (Broun 2004; Lemmetyinen *et al.* 2004; Koes *et al.* 2005), such as the well-known ABCDE model (Theissen 2001), genes from the MADS family of TFs control floral organ identity within each whorl of the flower by activating downstream genes. Measuring gene expression in different tissue types and developmental stages is of fundamental importance in TFs functional research. In last few years, quantitative real-time PCR (qRT-PCR) has become the favoured tool in gene expression analysis based on determining the starting template copy number with accuracy and high sensitivity over a wide dynamic range. However, the accurate and reliable result of qRT-PCR will be affected with many reasons, different amounts and quality of RNA, efficiency of retrotranscription from RNA to complementary DNA (cDNA) or PCR efficiency. In qRT-PCR experiments, reference genes are used as controls to normalize the data by correcting for differences in quantity of template and PCR efficiency, a perfect reference gene is one that does not exhibit changes in expression between samples from various tissue or developmental stages. Therefore, an appropriate choice of reference genes is prerequisite to qRT-PCR experiments.

Lycoris longituba is a perennial, bulbiferous, monocotyledonous, herbaceous plant in the genus of *Lycoris*, Amaryllidaceae family. A unique biological characteristic of this plant is that its leaves come out in spring and die in early summer, followed by flowering. Its vegetative growth and reproduction are discrete, which is rare in angiosperm (Cui *et al.* 2004). Besides the unique flowering characteristic, *L. longituba* also exhibits a great deal of diversity in both flower colour and flower form, making it a suitable model for the study of floral development. Recently, we have identified 338 putative TFs from more than 30,000 expressed sequence tags (ESTs) sequenced from the floral tissue of *L. longituba*, and recognized 51 of the TFs as being potentially flower-specific by comparing their expression in leaf and flower (He *et al.* 2010). To further accurately analysis these TFs expression in different tissues and different phases of floral development by qRT-PCR, a systematic selection of reference genes was performed. Thirteen commonly used reference genes in qRT-PCR were considered: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tubulin-alpha (*TUBα*), elongation factor 1-alpha (*EF1α*), tubulin-beta (*TUBβ*), actin (*ACT*), ubiquitin (*UBQ*), ubiquitin-conjugating enzyme (*UBC*), histone H3 (*HIS*), eukaryotic translation initiation factor (*eIF*), 60S ribosomal protein (*60S RP*), 40S ribosomal protein (*40S RP*), 18S ribosomal RNA (*18S RNA*), and cyclophilin (*CYP*). The *L. longituba* EST database was used to search for available sequences for the 13 candidate reference genes.

*For correspondence. E-mail: njmrhuang@njfu.edu.cn.

Keywords. *Lycoris longituba*; reference genes; gene expression; floral development; normalization.

Materials and methods

The specific primers of the 13 genes were designed using Oligo 6.0 with melting temperatures of 50–65°C and amplicon lengths of 80–200 bp. Specificity of the amplifications was verified by electrophoresis and at the end of PCR run by dissociation curve analysis. Plant samples of the root, stem, leaf, sepal, petal, stamen, carpel, fruit and six phases of floral development were taken from *L. longituba* growing in an outdoor garden, the tepal length was used to define phase 1 to phase 5 (20 mm, 40 mm, 70 mm, 80 mm, 90 mm) and in phase 6 the flower was beginning to wither. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Samples then were treated with DNase I (TaKaRa, Madison, USA) to remove any genomic DNA. The NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, USA) was used to determine RNA concentration and quality. The quality of RNA was also assessed by agarose gel electrophoresis. First-strand cDNA synthesis was carried out with the PrimeScript^{TM} RT Reagent kit (TaKaRa, Madison, USA). qRT-PCR was performed on the ABI 7500 real-time PCR system (Applied Biosystems, Warrington, USA) using SYBR Premix Ex Taq^{TM} (TaKaRa, Madison, USA), and reactions were performed in triplicates. The mean amplification efficiencies of the primer pairs were estimated using the LinRegPCR 12.5 program (Ruijter *et al.* 2009) and are shown in table 1.

Results and discussion

Cycle threshold (Ct) values for the 13 candidate reference genes tested ranged between 7.36 (*18S RNA*) and 23.49 (*TUBβ*), from the lowest Ct value to the highest Ct value: *18S RNA* < *TUBα* < *UBC* < *eIF* < *EF1α* < *HIS* < *ACT* < *40S RP* < *GAPDH* < *UBQ* < *CYP* < *60S RP* < *TUBβ*, the gene encoding 18S RNA is largely overexpressed compared to the protein coding genes. Ct values and amplification efficiencies were calculated to determine the stability of the selected reference genes by three different visual basic for applications (VBA) applets, geNorm (Vandesompele *et al.* 2002), NormFinder (Andersen *et al.* 2004) and BestKeeper (Pfaffl *et al.* 2004).

GeNorm provided a ranking of the 13 tested genes, based on a gene expression stability measure (M) on the pairwise comparisons of geometric means to minimize outlier effects. Genes with the lowest M values have the most stable expression, and pairwise variations ($V_{n/n+1}$) between consecutively ranked genes were calculated to determine the optimal number of reference genes. Results from all samples identified *eIF* and *HIS* were the most reliable reference genes with the lowest M values, and the top three reference genes (*eIF*, *HIS*, and *ACT*) should be included for qRT-PCR normalization ($V_{3/4} = 0.130$, below the default cut-off value of 0.15). *eIF* and *HIS* also ranked as the most stable genes during different tissues, and the four best-performing genes (*eIF*, *HIS*, *ACT*, and *18S RNA*) were necessary for normalization in different

Table 1. Description of candidate reference genes and primer sequences for qRT-PCR.

Gene ID	GenBank acc. no.	Primer sequence (5′ to 3′)	Annotation	e-Value	Mean PCR efficiency
GAPDH	HQ403589	F: GGTGCCAAGAAAGTTGTCATC R: CATCAATCCCTCAATAATGCC	glyceraldehyde-3-phosphate dehydrogenase (*Zea mays*)	2e-126	1.878
TUBa	HQ403590	F: TGTAGGCGTTCTCTTGACATTG R: GTGGATTCTTGGGTATGGGAC	tubulin-alpha (*Betula pendula*)	9e-110	1.859
EF1a	HQ403591	F: CACTGGGCATCTTATCTACA R: AAGGCAATGTCAATGGTAAT	elongation factor 1-alpha (*Prunus persica*)	0	1.873
TUBb	HQ403592	F: TGATGCTCACCTTCTCCGTC R: CAGCGTTCTCCACAAGTTGAT	tubulin-beta (*Arabidopsis thaliana*)	2e-167	1.875
ACT	HQ403593	F: CTGTATTCCCCAGCATTGTAG R: TCATCACCCACATAAGCATC	actin (*Persea americana*)	0	1.876
UBQ	HQ403594	F: TCACCTTGTCTTGCGTCTTC R: GTTATCAATGGTATCCGAGCC	ubiquitin (*Catharanthus roseus*)	4e-98	1.877
UBC	HQ403595	F: TTCACTTCCCTCCAGACTATCC R: ATGCTCCCGTTGCTATTGAT	ubiquitin conjugating enzyme (*Pisum sativum*)	7e-81	1.875
HIS	HQ403596	F: GCCCAGGACTTCAAGACTGA R: CTCAAAAAGCCCCACAAGGTA	histone H3.2 (*Arabidopsis thaliana*)	3e-70	1.875
eIF	HQ403597	F: CGAAAGAACGGGTACTTAGTCA R: ATGCGGAACATCACAGTTATG	translation initiation factor (*Carica papaya*)	3e-83	1.868
60S RP	HQ403598	F: GCATCCAGGAGCACATTGAT R: GACGACGACCAACACGGTA	60S ribosomal protein L11 (*Elaeis guineensis*)	4e-84	1.873
40S RP	HQ403599	F: GGTTCCGTAAGGCTCAATGTC R: TTGGGTTCAGGTCTGTAAGCA	40S ribosomal protein S5 (*Cicer arietinum*)	1e-89	1.885
18S RNA	HQ403600	F: TTCACCTGTCTGCCCTATCAAC R: TTGGATGTGGTAGCCGTTTCT	18S ribosomal RNA gene (*Beaucarnea recurvata*)	0	1.880
CYP	HQ403601	F: ATCATCAATGGCGGCAAAC R: GCGGAAGTTCTCGGTGGT	cydophilin (*Gossypium hirsutum*)	2e-79	1.866

Table 2. Ranking of the candidate reference genes according to their stability value using geNorm, NormFinder and BestKeeper analyses.

	geNorm			NormFinder			BestKeeper		
Rank	All samples	Different tissues	Different phases of floral development	All samples	Different tissues	Different phases of floral development	All samples	Different tissues	Different phases of floral development
1	*eIF/HIS*	*eIF/HIS*	*HIS/EF1a*	*eIF*	*18S RNA*	*UBC*	*18S RNA*	*18S RNA*	*UBC*
2	*ACT*	*ACT*	*UBC*	*HIS*	*eIF*	*HIS*	*UBC*	*eIF*	*TUBa*
3	*18S RNA*	*18S RNA*	*eIF*	*ACT*	*HIS*	*EF1a*	*HIS*	*HIS*	*eIF*
4	*60S RP*	*60S RP*	*18S RNA*	*EF1a*	*CYP*	*18S RNA*	*CYP*	*CYP*	*18S RNA*
5	*EF1a*	*CYP*	*TUBa*	*18S RNA*	*ACT*	*eIF*	*eIF*	*UBC*	*60S RP*
6	*CYP*	*EF1a*	*60S RP*	*60S RP*	*60S RP*	*60S RP*	*ACT*	*60S RP*	*HIS*
7	*UBC*	*UBC*	*ACT*	*40S RP*	*EF1a*	*TUBa*	*EF1a*	*EF1a*	*ACT*
8	*40S RP*	*40S RP*	*CYP*	*CYP*	*GAPDH*	*CYP*	*60S RP*	*ACT*	*EF1a*
9	*TUBb*	*GAPDH*	*TUBb*	*TUBb*	*UBC*	*ACT*	*40S RP*	*GAPDH*	*CYP*
10	*TUBa*	*TUBb*	*40S RP*	*UBC*	*40S RP*	*TUBb*	*UBQ*	*40S RP*	*40S RP*
11	*GAPDH*	*TUBa*	*UBQ*	*TUBa*	*TUBb*	*40S RP*	*TUBb*	*TUBb*	*TUBb*
12	*UBQ*	*UBQ*	*GAPDH*	*GAPDH*	*TUBa*	*UBQ*	*GAPDH*	*UBQ*	*UBQ*
13				*UBQ*	*UBQ*	*GAPDH*	*TUBa*	*TUBa*	*GAPDH*

Table 3. Normalizing a couple of transcription factors using two different reference genes.

TF gene ID	GenBank acc. no.	Phase 1 BL 20 mm	Phase 2 BL 40 mm	Phase 3 BL 70 mm	Phase 4 TL 80 mm	Phase 5 TL 90 mm	Phase 6 Withered
UBC as the control gene							
LlTF-MADS-1	GQ165923	1.248	1.761	2.304	3.722	7.595	7.480
LlTF-MADS-5	GQ165954	3.628	5.564	6.118	6.449	15.856	17.160
LlTF-MADS-14	GQ166139	9.273	9.952	9.877	13.501	30.065	24.795
18S RNA as the control gene							
LlTF-MADS-1	GQ165923	1.012	1.238	1.787	3.274	6.386	6.269
LlTF-MADS-5	GQ165954	3.653	4.478	4.952	5.067	16.518	15.649
LlTF-MADS-14	GQ166139	6.233	7.321	8.095	13.205	28.423	21.451

tissues with a $V_{4/5}$ value of 0.121. For different phases of floral development, *HIS* and *EF1a* showed the most stable expression and these two genes should be sufficient for normalization purposes in different phases of floral development with a $V_{2/3}$ value of 0.071.

NormFinder ranks the different candidate reference genes combining the intra-group and inter-group expression variation into a stability value. In this study, different tissues were considered as an experimental group, whereas different phases of floral development were considered as the other group. Ct values and amplification efficiencies were converted to relative quantities using the delta Ct method and entered into NormFinder. The ranking of the NormFinder analysis appeared to be slightly different from what obtained using geNorm. *UBQ*, *GAPDH*, and *TUBα* still occupied the lowest positions, while *eIF* and *HIS* remained the most stable genes when all samples were taken together. *UBC* gave the most stable expression pattern in different phases of floral development, whereas *eIF* and *HIS* were indicated as the top two in different tissues followed by *18S RNA*.

BestKeeper measures candidate reference genes stability by using the inspection of raw Ct values calculated variation (SD [± Ct] and CV [%Ct]), and shows an overall stability in gene expression. Genes with SD values greater than 1 (SD [± x-fold] greater than two-fold) were considered as inconsistent. BestKeeper highlighted *18S RNA* as the least overall variation gene in all samples with an SD of 0.27, which represents an acceptable 1.19-fold change in expression. The analysis also showed *18S RNA* had the lowest Ct value variation with an SD of 0.21 in different tissues, and *UBC* was the most stable candidate genes in different phases of floral development with an SD of 0.20, these results of the BestKeeper analysis were consistent with the result of NormFinder. *UBQ*, *GAPDH* and *TUBα* remained the least three stable genes with the high SD values greater than 1.

Analysis of expression stability using geNorm, NormFinder, and BestKeeper revealed that *eIF*, *HIS*, *UBC*, and *18S RNA* showed good performances overall in all rankings, whereas *UBQ*, *GAPDH* and *TUBα* displayed relatively low expression stability (table 2). Across all the samples, *eIF* and *HIS* were ranked as the top two reference genes

according to both geNorm and NormFinder, therefore, we recommend the use of *eIF* and *HIS* as suitable reference genes for normalization of gene expression across all samples in *L. longituba*. *eIF* and *HIS* also gave the most stable expression pattern in different tissues when evaluated by geNorm, and ranked as the top two genes followed by *18S RNA* in results of NormFind and Bestkeeper. Considering candidate reference genes showed higher expression variation in different tissues, one reference gene (*18S RNA*) may not be sufficient for normalization purposes, besides the expression level of *18S RNA* (Ct = 7.36) was much higher than the appropriate reference genes we suspected (15 < Ct < 25), so *eIF* and *HIS* were a better choice for normalization of gene expression in different tissues of *L. longituba*. For different phases of floral development, *UBC* could be considered as an appropriate reference gene in *L. longituba* as the good performance in the result of three methods (table 3). In recent years, some researches on reference genes selection for qRT-PCR have been reported (Huis *et al.* 2010; Luo *et al.* 2010; Wan *et al.* 2010), however, few studies regarded the choice of reference genes for gene expression in floral development. In this work, we describe the first systematic evaluation of reference genes in different tissues and floral development of *L. longituba* for qRT-PCR normalization, and this result can serve as a resource to help and screen reference genes for accurate and reliable normalization of qRT-PCR analysis in floral development of other plants.

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