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Original Article

Identification of reference genes for gene expression normalization in safflower (*Carthamus tinctorius*)

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ABSTRACT

Safflower (*Carthamus tinctorius* L., Asteraceae) is an important oil crop and medicinal plant. Gene expression analysis is gaining importance in the research of safflower. Quantitative PCR has become a powerful method for gene study. Reference genes are one of the major qualification requirements of qPCR because they can reduce the variability. To identify the reference genes in safflower, nine candidate genes of the housekeeping genes were selected from the EST library of safflower constructed by our lab: *CtACT* (actin), *CtGAPDH* (glyceraldehyde 3-phosphate dehydrogenase), *CtE1F4A* (elongation factor 1 alpha), *CtTUA* (alpha-tubulin), *CtTUB* (beta-tubulin), *CtPP2A* (serine/threonine-protein phosphatase), *CtE1F4A* (eukaryotic initiation factor 4A), *CtUBI* (Ubiquitin), and *Ct60S* (60S acidic ribosomal protein). Expression stability was examined by qPCR across 54 samples, representing tissues at different flowering stages and two chemotype of safflower lines. We assessed the expression stability of these candidate genes by employing four different algorithms (geNorm, NormFinder, ΔCt approach, and BestKeeper) and found that *CtUBI* and *Ct60S* were the highly ranked candidate genes. *CtUBI* and *Ct60S* were used as reference genes to evaluate the expression of *CtFAD2-10* and *CtKASII*. Our data suggest *CtUBI* and *Ct60S* could be used as internal controls to normalize gene expression in safflower.

Introduction

Safflower (*Carthamus tinctorius* L., Asteraceae) is a thistle-like, self-compatible, annual, diploid ($2n = 24$) herbaceous crop that thrives in hot, dry climates and survives on minimal surface moisture. The safflower cultivars are distributed from the Mediterranean to the Pacific Ocean at latitudes between 20°S and 40°N, wherever a hot, dry climate suits the crop. In some countries, safflower has become an important crop due to the rich content of edible oil, which has the highest polyunsaturated/saturated ratios of any oil available (Gecgel et al., 2007; Yeilaghi et al., 2012). Safflower is also a valuable medicinal plant. The flowers of safflower can be used for the treatment of cardiovascular and cerebrovascular diseases (Tian et al., 2010; Asgarpanah and Kazemivash, 2013) and the extracts from safflower can be used as anti-inflammatory agents as well (Jun et al., 2011). There are several studies about the genetic variation of safflower cultivars using various molecular DNA markers, such as SNP (Chapman and Burke, 2007), SRAP (Peng et al., 2008), ISSR (Chapman et al., 2009; Golkar et al., 2011), and AFLP (Zhang et al., 2009; Feng et al., 2010; Li et al., 2010). Meanwhile, in the previous study of our lab, we have found that obvious differentiation has occurred in safflower populations from exterior appearance to inner chemical constituent, due to the long natural and artificial selection (Peng et al., 2008; Zhang et al., 2009; Feng et al., 2010; Li et al., 2010). From our conclusion, hydroxysafflor yellow A (HYSA), as the active compound, is the main factor to determine the diversity of safflower (Yang et al., 2011).

As secondary metabolites in safflower, the flavonoids are major components of the extracts from the flowers with medicinal function (Andersen and Markham, 2010; Asgarpanah and Kazemivash, 2013). HSYA, one of the most important flavonoids with a unique presence in the flower petals of safflower, plays a major role in the pharmacological effects of flavonoids (Feng et al., 2013; Sun et al., 2013; Wang et al., 2013). The flowering process of safflower is a complex development associated with the biosynthesis of flavonoids, particularly the color change (Tanaka et al., 2010). In safflower seeds, the identification and initial characterization of the FAD2 gene family with eleven members provides an insight into the principal determinants of synthesis of linoleic acid in safflower seed oil (Cao et al., 2013; Liu et al., 2013). And the *CtFAD3* enzyme activity is important for fatty acid desaturation in safflower flower (Guan et al., 2014). The understanding of the expression of

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the key genes will help investigate the mechanism involved in the biosynthesis of flavonoids and fatty acids.

Quantitative real-time PCR (qPCR) is a routine tool with high sensitivity and specificity for the quantification of gene expression (Gachon et al., 2004). When we study the gene expression, the reference genes can be used in the normalization of experimental variations, such as the amount of material, extraction of RNA, efficiency of reverse transcription, and so on (Silver et al., 2006; Gutierrez et al., 2008; Bustin et al., 2009; Guénin et al., 2009). It is important to normalize the expression of the target gene in order to obtain reliable and accurate results by using the reference genes. Housekeeping genes are often considered as being constantly expressed and are always used as internal standards. But many researches showed that the expression of housekeeping genes varies in different materials and even in the same materials during different treatments (Thellin et al., 1999; Schmidt and Delaney, 2010). So the evaluation of reference genes in different plant tissues during biotic or abiotic stress is necessary for expression studies.

Housekeeping genes, such as actin (Bas et al., 2004), elongation factor 1 alpha (Schmidt and Delaney, 2010; Li et al., 2012), alpha-tubulin (Jarosova and Kundu, 2010; Wan et al., 2011), beta-tubulin (Jarosova and Kundu, 2010), serine/threonine-protein phosphatase (Liu et al., 2012), eukaryotic initiation factor 4A (Silveira et al., 2009), ubiquitin (Infante et al., 2008), and 60S acidic ribosomal protein (Le et al., 2012), are commonly used as reference genes for gene expression studies. There are studies which have used reference genes for the normalization of gene expression in safflower (Li et al., 2012; Cao et al., 2013). But these reference genes were not evaluated for qPCR data in safflower tissues. In this study, nine housekeeping genes namely *CtACT* (actin), *CtGAPDH* (glyceraldehyde 3-phosphate dehydrogenase), *CtE1F4A* (elongation factor 1 alpha), *CtTUA* (alpha-tubulin), *CtTUB* (beta-tubulin), *CtPP2A* (serine/threonine-protein phosphatase), *CtE1F4A* (eukaryotic initiation factor 4A), *CtUBI* (Ubiquitin), and *Ct60S* (60S acidic ribosomal protein) were selected as candidate reference genes from our constructeded transcriptome data of safflower. At the same time, two representative chemotype of safflower varieties, with-HSYA (yellow flower) and without-HSYA (white flower) were selected to identify reference genes. The expression of these genes in the bract, ovary, stem, leaf, calyx, petal (I–IV, four different stages of development during flowering) of two safflower lines were analyzed using four different algorithms, that is, geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), ΔCt approach (Silver et al., 2006), and BestKeeper (Pfaffl et al., 2004). Our study will help to achieve more accurate and reliable results in a wide variety of safflower samples.

Materials and methods

Plant material

The seeds of two lines of safflower, *Carthamus tinctorius* L., Asteraceae (ZHH0082 with yellow flower and Xin Honghua NO.7 with white flower) from the Chinese populations were cultivated in the field at the medicinal botanical garden of Second Military Medical University. The samples (bract, ovary, stem, leaf, calyx, petal (I–IV)) were collected from three different flowering plants (biological triplicates) and immediately frozen in liquid nitrogen. All 54 samples were then stored at −70 °C for RNA extraction.

Total RNA extraction

Total RNA was isolated using the RNA Extraction Plant Mini Kit (LifeFeng, Shanghai, China) according to the protocol provided by the manufacturer. RNA concentration and quality were measured with the NanoDrop spectrophotometer (NanoDrop Technologies) and agarose gel electrophoresis. Only the RNA samples with A_{260}/A_{280} ratios between 1.9 and 2.1 and A_{260}/A_{230} ratios greater than 2.0 were used for cDNA synthesis.

First strand cDNAs synthesis

First strand cDNAs were synthesized according to the manufacturer's instructions of TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Total RNA (1 μg), suitable volumes of H_2O, and 1 μl anchored Oligo (dT) 18 primer (0.5 μg/μl) were mixed and incubated at 65 °C for 5 min followed by cooling on ice. The reverse transcriptase reactions were started after adding 10 μl 2× TS reaction mix, 1 μl Enzyme Mix, and 1 μl gDNA Remover at 42 °C for 45 min. And then the mixture was heated for 5 min at 85 °C for inactivating the enzymes. All cDNA samples were diluted 1:10 with RNase-free water before being used as templates in the qPCR analysis.

Q-PCR

Nine sequences were selected as candidate reference genes from the petal EST libraries of *C. tinctorius* (Table 1). The qPCR primers were designed using the Beacon Designer v8.0 software. Amplicon lengths varied from 75 to 200 bp, with melting temperatures (Tm) varying between 52 °C and 60 °C and primer lengths between 18 and 22 bp. Primer pairs were tested for specificity by qPCR, followed by a dissociation curve and agarose gel electrophoresis (Fig. S1). PCR reactions were performed in 96-well plates with the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA) and ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA). The CFX96 was used to obtain the qPCR data of nine candidate genes and ABI 7500 was used to obtain the qPCR data of *CtFAD2-10* (KC257456, F-CTTTACCGTATGGCTTTAG, R-GTGTGTTGAAGGTATGTG), *CtKASII* (KC257458, F-GACAGGTTTATGCTCTAC, R-CAATCAGAACTCCACATC), and two stable reference genes. Each reaction comprising 20 μl of the following was prepared as follows: 0.3 μm forward primer, 0.3 μm reverse primer, 10 μl 2× TranStartTM Top Green qPCR SuperMix (TransGen Biotech, Beijing, China), 2 μl cDNA, 0.4 μl passive reference dye II (only used in ABI system), and suitable volumes of H_2O. The thermocycling conditions were set at 95 °C for 30 s, followed by 40 cycles of 10 s at 95 °C for template denaturation, 15 s at Tm for annealing, and 20 s (30 s for ABI system) at 72 °C for extension and fluorescence measurement. Afterwards, the dissociation curve was obtained by heating the amplicon from 60 °C to 95 °C and reading at each 0.5 °C increase (0.2 °C for ABI system). Three technical replicates were performed for each PCR reaction.

Data analysis

The PCR efficiency shown in Table 1 was calculated for each candidate gene with LinRegPCR program (Ruijter et al., 2009). The raw fluorescence data, *Ct* values, and the relative quantity (ΔCt) values of candidate genes were generated with the CFX manager software (BioRad). The *Ct* values of *CtFAD2-10*, *CtKASII*, and two stable reference genes were calculated using the 7500 software v2.06 (ABI). The data obtained were converted into correct input files and analyzed using geNorm, NormFinder, ΔCt approach, and BestKeeper. In Fig. 1, normalized *CtFAD2-10* and *CtKASII* expressions were calculated using the geometric means of *CtUBI* and *Ct60S Ct* values of each sample.

Table 1
Nine candidate reference genes and their primer sequences for qPCR.

Gene identification/gene description	*E* value ID (%)	Primer sequence	Amplicon size	Amplification efficiency	GeneBank Accession Number	Tm
*Ct*ACT	0.0	F-ACTGGTGTTATGGTAGGA	89	1.85	KJ634809	60
actin	100	R-GGATACTTCAAGGTAAGGATA				
*Ct*GAPDH	0.0	F-GTTGTGGACTTAACCGTAA	78	1.83	KJ634805	53
glyceraldehyde 3-phosphate dehydrogenase	93	R-TTCTGATTCCTCCTTAATAGC				
*Ct*EF1	0.0	F-CCAAGAGACCATCAGACAA	76	1.88	KJ634806	58
elongation factor 1 alpha	98	R-GGCACAGTTCCAATACCA				
*Ct*TUA	0.0	F-CTACACCAACCTCAATCG	91	1.89	KJ634803	58
α-tubulin	99	R-AGTCACATCCACATTCAAG				
*Ct*TUB	0.0	F-GGAAGAGGAGTATGATGA	93	1.88	KJ634802	54
β-tubulin	99	R-AATGGCAGTTGAGATTAC				
*Ct*PP2A	0.0	F-GAGAACC*T*GATGTAACGAGAC	80	1.89	KJ634804	58
serine/threonine-protein phosphatase	95	R-ACCACCAAGCAAGCAATC				
*Ct*E1F4A	0.0	F-CGCTGATTACATTAAGATG	90	1.85	KJ634807	54
eukaryotic initiation factor 4A	98	R-AATTGGAAGATATCGTAGAT				
*Ct*UBI	$3e^{-87}$	F-TCACTTATGTTTACCAGAA	150	1.85	KJ634808	54
Ubiquitin	98	R-GCTTTCAATTTCAACTCA				
*Ct*60S	$9e^{-165}$	F-CATCCATTATCCAACAATC	92	1.86	KJ634810	54
60S acidic ribosomal protein	88	R-AAGAGTAATCAGTCTCCA				

Results

Selection of putative reference genes for qPCR experiments

In order to find the best reference genes in safflower, nine housekeeping genes, *CtACT*, *CtGAPDH*, *CtE1F4A*, *CtTUA*, *CtTUB*, *CtPP2A*, *CtE1F4A*, *CtUBI*, and *Ct60S*, were selected as putative reference genes (Table 1). These genes were used to BLAST search against a *C. tinctorius* EST (expressed sequence tag) library constructed by our laboratory from the petal during flowering. The housekeeping gene commonly used as reference gene is the 18S rRNA. In this study, however, the *Ct* values of 18S in the safflower tissues were at least 10 cycles higher than the *Ct* values of the other candidate genes (data not shown). Such high *Ct* values make 18S unsuitable for use as a reference gene (Li et al., 2012). The dissociation cures of amplicons of these nine candidate genes exhibited a single peak showed that the primer pairs were specificity, which were also verified by agarose gel electrophoresis results (Fig. S1). The expression of these genes in bract, ovary, stem, leaf, calyx, petal (I–IV, four different stages of development during flowering, Fig. 2, Table S1) was analyzed. Since flowering begins in the outer circle of florets and progresses centripetally toward the center of the capitulum, the four stages of petals can be found in one capitulum. The reason we differentiate the four stages of petals is that the compounds have significant variation during the development of the *C. tinctorius* flowers (Salem et al., 2011) and the gene expressions are different as well (Mallona et al., 2010). In our study, a total of 54 samples (two lines of safflower (ZHH0082 and Xin Honghua NO.7), nine organs, three biological replicates) were used to evaluate the stability of putative reference genes. The significant difference between the ZHH0082 and Xin Honghua NO.7 was the color of flowers. The flowers of ZHH0082 were yellow and the flowers of Xin Honghua NO.7 were white.

Expression stability of putative reference genes via differential statistical analyses

The cycle threshold (*Ct*) values profiling of candidate genes in different organs and safflower lines are shown in Fig. 3. The mean of each of the nine candidate genes in 54 samples are shown in Table S2. In this study, geNorm, NormFinder, Δ*Ct* approach and BestKeeper were used to analyze the stability of gene expression.

GeNorm calculated the gene expression stability value *M* (the average pairwise variation of a particular gene with all other

Table 2
Ranking of the candidate reference genes according to their values calculated by geNorm, NormFinder, Δ*Ct* approach and BestKeeper in AS, Y and W.

Rank	All samples (AS)				Yellow (Y)				White (W)			
	geNorm (*M*)	NormFinder (SV)	Δ*Ct* approach	BestKeeper	geNorm (*M*)	NormFinder (SV)	Δ*Ct* approach	BestKeeper	geNorm (*M*)	NormFinder (SV)	Δ*Ct* approach	BestKeeper
1	*Ct*UBI	*Ct*60S	*Ct*60S	*Ct*PP2A	*Ct*60S	*Ct*UBI	*Ct*60s	*Ct*PP2A	*Ct*ACT	*Ct*PP2A	*Ct*UBI	*Ct*PP2A
	0.776	0.061	0.671		0.774	0.063	0.670		0.756	0.062	0.643	
2	*Ct*60S	*Ct*ACT	*Ct*ACT	*Ct*UBI	*Ct*UBI	*Ct*60s	*Ct*ACT	*Ct*UBI	*Ct*TUB	*Ct*EF1	*Ct*TUB	*Ct*UBI
	0.777	0.066	0.673		0.781	0.070	0.672		0.759	0.072	0.649	
3	*Ct*ACT	*Ct*UBI	*Ct*UBI	*Ct*60S	*Ct*TUB	*Ct*ACT	*Ct*TUB	*Ct*E1F4A	*Ct*GAPDH	*Ct*ACT	*Ct*60S	*Ct*60S
	0.788	0.068	0.681		0.788	0.081	0.683		0.767	0.092	0.651	
4	*Ct*TUB	*Ct*GAPDH	*Ct*TUB	*Ct*TUB	*Ct*ACT	*Ct*E1F4A	*Ct*UBI	*Ct*60S	*Ct*UBI	*Ct*60S	*Ct*ACT	*Ct*TUB
	0.837	0.076	0.724		0.804	0.081	0.689		0.770	0.113	0.660	
5	*Ct*E1F4A	*Ct*EF1	*Ct*E1F4A	*Ct*E1F4A	*Ct*EF1	*Ct*TUB	*Ct*GAPDH	*Ct*TUB	*Ct*60S	*Ct*GAPDH	*Ct*E1F4A	*Ct*E1F4A
	0.839	0.078	0.736		0.814	0.085	0.748		0.773	0.123	0.699	
6	*Ct*GAPDH	*Ct*E1F4A	*Ct*GAPDH	*CtACT*	*Ct*E1F4A	*Ct*GAPDH	*Ct*EF1	*Ct*ACT	*Ct*E1F4A	*Ct*TUA	*Ct*GAPDH	*Ct*GAPDH
	0.845	0.085	0.748		0.815	0.088	0.772		0.780	0.137	0.700	
7	*Ct*EF1	*Ct*TUA	*Ct*EF1	*Ct*GAPDH	*Ct*GAPDH	*Ct*EF1	*Ct*PP2A	*Ct*EF1	*Ct*PP2A	*Ct*UBI	*Ct*PP2A	*Ct*ACT
	0.851	0.127	0.785		0.878	0.103	0.863		0.856	0.144	0.766	
8	*Ct*PP2A	*Ct*TUB	*Ct*PP2A	*Ct*TUA	*Ct*PP2A	*Ct*PP2A	*Ct*E1F4A	*Ct*GAPDH	*Ct*EF1	*Ct*E1F4A	*Ct*EF1	*Ct*TUA
	0.975	0.127	0.818		1.021	0.148	0.869		0.880	0.146	0.781	
9	*Ct*TUA	*Ct*PP2A	*Ct*TUA	*Ct*EF1	*Ct*TUA	*Ct*TUA	*Ct*TUA	*Ct*TUA	*Ct*TUA	*Ct*TUB	*Ct*TUA	*Ct*EF1
	1.104	0.138	0.957		1.130	0.168	0.974		1.077	0.154	0.926	
RankAggreg[a]	*Ct*60S/*Ct*UBI/*CtACT*				*Ct*UBI/*Ct*60S/*Ct*TUB				*Ct*UBI/*Ct*60S/*Ct*TUB			

[a] Top three ranked genes were shown.

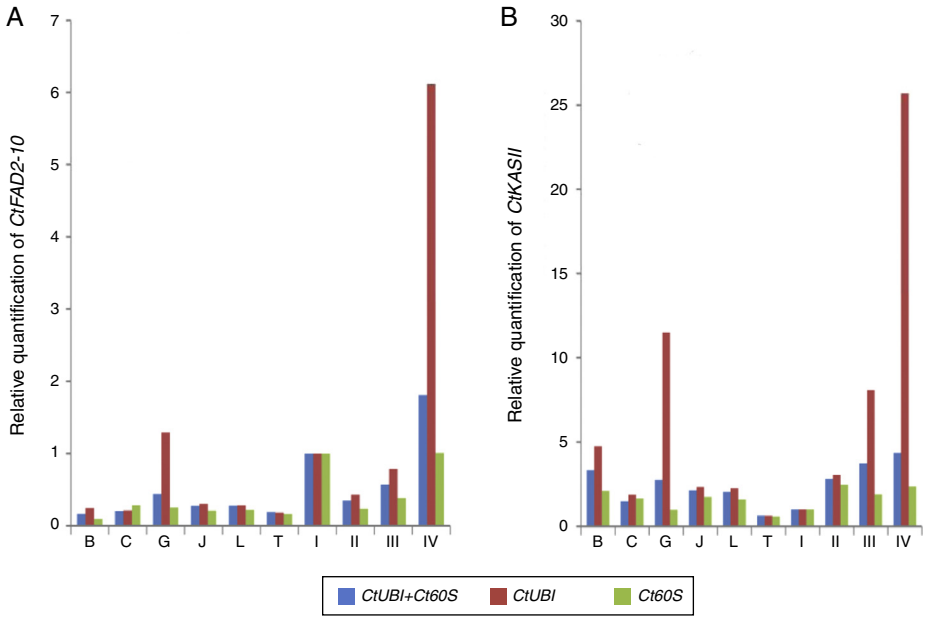

Fig. 1. Relative quantification of *CtFAD2-10* and *CtKASII* expression using different internal controls analyzed by the $2^{-\Delta\Delta Ct}$ method in all samples. Relative quantification of *CtFAD2-10* and *CtKASII* expression were detected using *CtUBI* and *Ct60S* both individually and in combination. The relative expression of petal I was set to 1. (A) *CtUBI*, *Ct60S* and the geometric average of *CtUBI* and *Ct60S* were used as internal controls for *CtFAD2-10* expression. (B) *CtUBI*, *Ct60S* and the geometric average of *CtUBI* and *Ct60S* were used as internal controls for *CtKASII* expression. B, bract; C, calyx; L, leaf; O, ovary; S, stem; I–IV, petal I–IV.

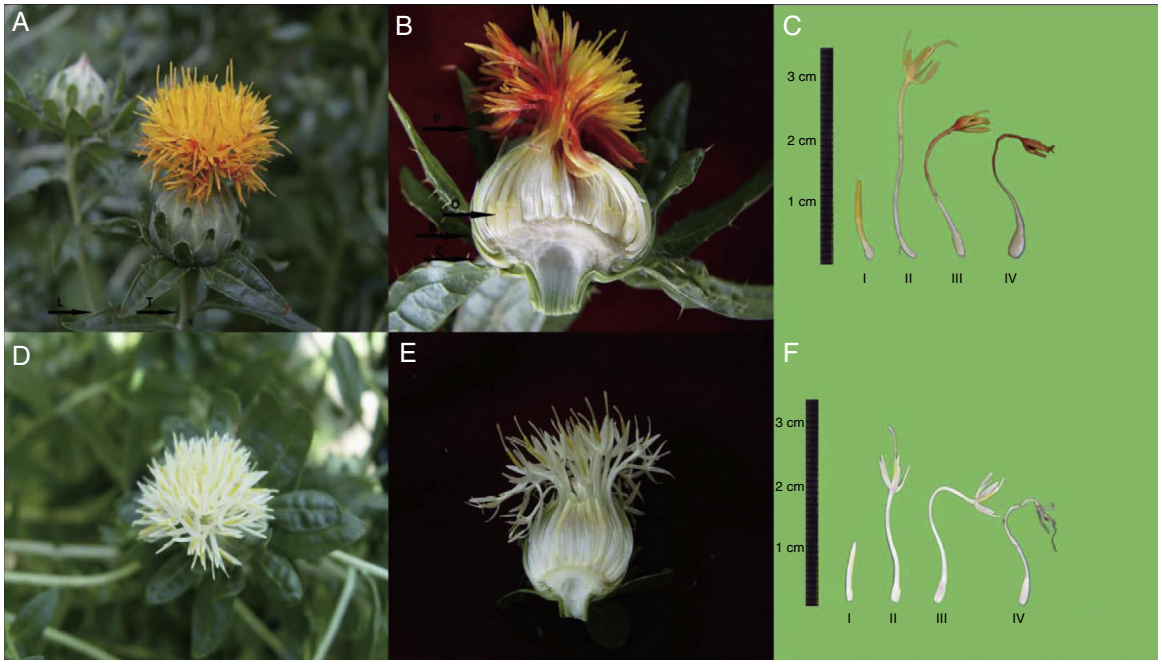

Fig. 2. Two lines of safflower. (1) A, B, C are the ZHH0082 (Yellow); (2) D, E, F are the Xin Honghua NO.7 (White); (A, D) capitulum of safflower; (B, E) longitudinal section of capitulum; (C, F) four different stages of the petal during flowering. Black arrows point to the samples collected from safflower. B, bract; O, ovary; T, stem; L, leaf; C, calyx; P, petal.

control genes) for a reference gene. The lower the *M* value, the more stably expressed the gene is. NormFinder used a model-based approach for identifying the optimal normalization gene(s). The intra- and intergroup variations were calculated and included in the gene expression stability values. Genes with the lowest values had the most stable expression. GeNorm and NormFinder used the linear scale expression quantities, which can be calculated from the *Ct* values by using a standard curve or comparative CT method (Schmittgen and Livak, 2008). ΔCt approach compared the ΔCt variation of pairs of housekeeping genes within individual samples to identify the reference genes. The genes with lower ΔCt variation were stably expressed. BestKeeper, an Excel-based tool, determined the most stably expressed genes based on the variation (SD and SV values) and repeated pairwise correlation analysis. The rank-ordered genes calculated by these four algorithms were further analyzed by RankAggreg (Pihur et al., 2009), which calculated footrule distances and obtained the consensus rank list of genes by means of Cross-Entropy Monte Carlo algorithm. Six groups (AS, Y, W, F, YF, WF) were analyzed by these algorithms and the results are shown in Tables 2, 3 and Fig. S2.

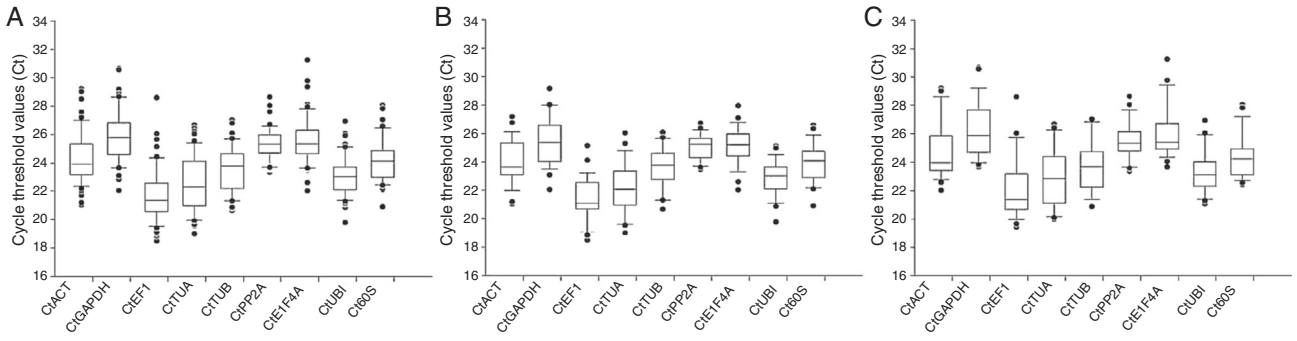

Fig. 3. *Ct* values of candidate reference genes. Expression data showed the *Ct* values (black spots) of each gene. The vertical boxes represent the 25th and 75th percentiles with error bars (whiskers). The lines across the boxes are depicted as the median. (A) All samples of safflower, (B) samples of ZHH0082 (Yellow), and (C) samples of Xin Honghua NO.7 (White).

In AS (all 54 samples of the two lines of safflower), *CtUBI*, *Ct60S*, and *CtACT* were the three most stably expressed candidate genes calculated by geNorm, NormFinder and ΔCt. In the list of BestKeeper, the top three were *CtPP2A*, *CtUBI*, and *Ct60S*. The most stably expressed gene identified by BestKeeper was *CtPP2A*, which had the highest variation in the results from other methods (geNorm ranked 8, NormFinder ranked 9, and ΔCt ranked 8). The top three aggregate order calculated by RankAggreg was *Ct60S*/*CtUBI*/*CtACT* in AS (Table 2).

The results of AS were analyzed by segregating into two subgroups, Y and W. The Y group included 27 samples collected from ZHH0082 (Yellow) and W contained 27 samples from Xin Honghua NO.7 (White). The four algorithms produced similar ranking lists in AS and Y. But there were more differences between two lines, Y and W. In Y, *Ct60S* was the first, second, and the first best-ranked gene in the geNorm, NormFinder, and ΔCt approach, respectively. However, *Ct60S* was ranked at fifth, fourth, and third in W. In the analysis of NormFinder, the position of *CtPP2A* had remarkable variation between Y and W, which was the eighth in Y but the first in W. Although there were discrepancies among the orders produced by these algorithms in the two lines, the aggregate orders were the same, namely *CtUBI*/*Ct60S*/*CtTUB* (Table 2).

The results analyzed by algorithms were different in all Flowers (F), Yellow-Flower (YF, flowers of ZHH0082), and White-Flower (WF, flowers of Xin Honghua NO.7). According to the results of NormFinder, the *CtE1F4A* was the second most stably expressed gene in F, seventh in YF, and first in WF. In particular, *CtE1F4A* was the most stably expressed gene in YF calculated by the geNorm, NormFinder, and ΔCt approach. The upgraded positions of *CtE1F4A* resulted in changing of the aggregate orders in SA, F, and YF. But the order in WF was *Ct60S*/*CtUBI*/*CtACT*, which was the same as that in AS (Table 3).

It has been suggested that the use of two or more reference genes for RT-qPCR studies might generate more reliable results (Vandesompele et al., 2002). To determine the optimal number of genes required for accurate normalization, pairwise variations V_n/V_{n+1} were calculated based on the normalization factor (NF_n and NF_{n+1}) values according to the geNorm algorithm. If the V_n/V_{n+1} of n genes were below the cut-off value, the additional housekeeping gene ($n+1$) was not necessary for reliable normalization. The cut-off value of pairwise variations V_n/V_{n+1} was set at 0.15. In the results of the six groups analyzed, at least four reference genes can be used for accurate normalization in AS ($V_2/V_3 = 0.173$, $V_3/V_4 = 0.176$), Y ($V_2/V_3 = 0.193$, $V_3/V_4 = 0.175$) and F ($V_2/V_3 = 0.193$, $V_3/V_4 = 0.183$). In W ($V_2/V_3 = 0.22$) and YF ($V_2/V_3 = 0.203$), the best number was three. Two genes were sufficient for expression normalization in WF ($V_2/V_3 < 1.5$) (Fig. 4).

Quantification of CtFAD2-10 *and* CtKASII *expression with stable reference genes*

The expression of *CtFAD2-10*, one of the FAD2 gene family in safflower, was normalized using *KASII* in an early study (Cao et al., 2013). Keto acyl-acyl carrier protein synthase II (*KASII*) also plays a role in the fatty acid biosynthesis in plants. The expression of *KASII* was not evaluated for using as an internal control for normalization. When compared with organs obtained from different individuals, single housekeeping gene RNA levels were not appropriate to be used for normalization of RNA levels (Tricarico et al., 2002). To further verify the suitability of reference genes selected in the present study, *CtFAD2-10* and *CtKASII* expression levels were detected in safflower (Fig. 1). The relative expression data were calculated using $2^{-\Delta\Delta Ct}$ method. The internal control genes were the *CtUBI* and *Ct60S* and the geometric average of *CtUBI* and *Ct60S*.

Its pattern of expression was assessed in all samples (bract, ovary, stem, leaf, calyx, petal (I–IV)). Similar expression patterns were generated when either one or two of the most stable genes were used for normalization (Fig. 1A and B). When normalized the geometric average of *CtUBI* and *Ct60S*, transcript abundance of *CtKASII* gradually increased in different developmental stages of flower, peaking at the Stage IV (Fig. 1B). It suggests that the

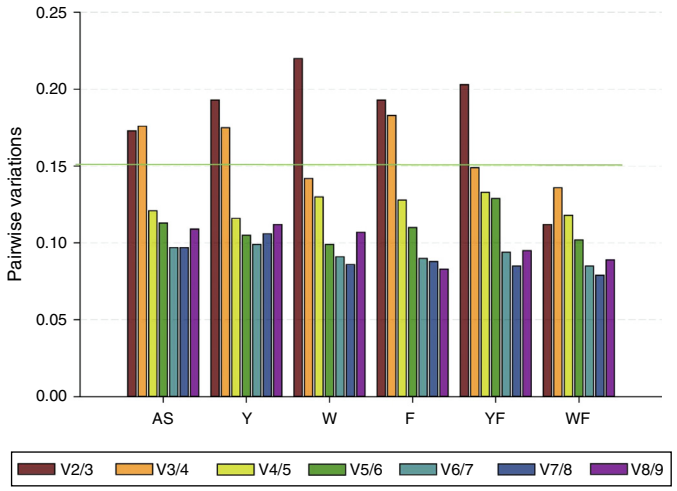

Fig. 4. Optimal number of reference genes for normalization according to geNorm results. Pairwise variation (V_n/V_{n+1}) was measured between the normalization factors NF_n and NF_{n+1}. The inclusion of an additional reference gene was not required below the cut-off value of 0.15 (the chartreuse line). AS, all samples; Y, ZHH0082 (Yellow); W, Xin Honghua NO.7 (White). F, flower; YF, yellow flower; WF, white flower.

Table 3
Ranking of the candidate reference genes according to their values calculated by geNorm, NormFinder, ΔCt approach and BestKeeper in F, YF and WF.

Rank	All flowers (F)				Yellow-Flower (YF)				White-Flower (WF)			
	geNorm (*M*)	NormFinder (SV)	Δ*Ct* approach	BestKeeper	geNorm (*M*)	NormFinder (SV)	Δ*Ct* approach	BestKeeper	geNorm (*M*)	NormFinder (SV)	Δ*Ct* approach	BestKeeper
1	*Ct*60S	*Ct*ACT	*Ct*60S	*Ct*PP2A	*Ct*E1F4A	*Ct*E1F4A	*Ct*E1F4A	*Ct*PP2A	*Ct*60S	*Ct*EF1	*Ct*60S	*Ct*PP2A
	0.705	0.073	0.625		0.727	0.061	0.635		0.604	0.039	0.550	
2	*Ct*E1F4A	*Ct*EF1	*Ct*ACT	*Ct*UBI	*Ct*60S	*Ct*ACT	*Ct*60S	*Ct*UBI	*Ct*E1F4A	*Ct*60S	*Ct*ACT	*Ct*UBI
	0.742	0.086	0.650		0.755	0.071	0.665		0.657	0.050	0.583	
3	*Ct*UBI	*Ct*UBI	*Ct*E1F4A	*Ct*60S	*Ct*TUB	*Ct*GAPDH	*Ct*TUB	*Ct*60S	*Ct*ACT	*Ct*PP2A	*Ct*TUB	*Ct*60S
	0.749	0.097	0.661		0.764	0.079	0.665		0.671	0.060	0.591	
4	*Ct*ACT	*Ct*E1F4A	*Ct*UBI	*Ct*TUB	*Ct*UBI	*Ct*60S	*Ct*ACT	*Ct*E1F4A	*Ct*TUB	*Ct*UBI	*Ct*E1F4A	*Ct*TUB
	0.777	0.097	0.665		0.768	0.098	0.687		0.678	0.060	0.593	
5	*Ct*TUA	*Ct*60S	*Ct*GAPDH	*Ct*E1F4A	*Ct*GAPDH	*Ct*TUB	*Ct*UBI	*Ct*TUB	*Ct*TUA	*Ct*ACT	*Ct*UBI	*Ct*E1F4A
	0.806	0.100	0.707		0.819	0.109	0.692		0.683	0.091	0.594	
6	*Ct*GAPDH	*Ct*GAPDH	*Ct*TUB	*Ct*ACT	*Ct*ACT	*Ct*UBI	*Ct*GAPDH	*Ct*ACT	*Ct*UBI	*Ct*TUA	*Ct*TUA	*Ct*GAPDH
	0.808	0.112	0.709		0.829	0.110	0.697		0.687	0.098	0.637	
7	*Ct*TUB	*Ct*TUB	*Ct*TUA	*Ct*GAPDH	*Ct*EF1	*Ct*EF1	*Ct*EF1	*Ct*EF1	*Ct*PP2A	*Ct*E1F4A	*Ct*PP2A	*Ct*ACT
	0.809	0.123	0.754		0.836	0.130	0.767		0.745	0.126	0.666	
8	*Ct*EF1	*Ct*PP2A	*Ct*PP2A	*Ct*TUA	*Ct*TUA	*Ct*TUA	*Ct*PP2A	*Ct*GAPDH	*Ct*GAPDH	*Ct*GAPDH	*Ct*GAPDH	*Ct*TUA
	0.875	0.127	0.755		0.888	0.146	0.817		0.755	0.158	0.677	
9	*Ct*PP2A	*Ct*TUA	*Ct*EF1	*Ct*EF1	*Ct*PP2A	*Ct*PP2A	*Ct*TUA	*Ct*TUA	*Ct*EF1	*Ct*TUB	*Ct*EF1	*Ct*EF1
	0.885	0.128	0.760		0.981	0.183	0.826		0.900	0.164	0.737	
RankAggreg[a]	*Ct*UBI/*Ct*E1F4A/*Ct*60S				*Ct*E1F4A/*Ct*60S/*Ct*TUB				*Ct*60S/*Ct*UBI/*Ct*ACT			

[a] Top three ranked genes were shown.

CtKASII was not a stable gene for gene expression normalization. The expression levels of *CtFAD2-10* were high in petals, especially in the flowering stage IV (Fig. 1A). When only one reference gene was employed, expression profiles of *CtKASII* were similar (Fig. 1B), but differences were evident in estimated transcript abundance, which was higher when normalized against *CtUBI* than against *Ct60S*.

Discussion

Extensive studies are being carried out on the safflower, because it is both an oil crop and a medicinal plant. Furthermore, research on the biosynthesis of these flavonoids and fatty acid metabolism were also reported recently (Cao et al., 2013; Li et al., 2012). Because it is a sensitive, specific, reproducible, and conventional method, qPCR has become an essential tool for gene expression analysis, especially the gene expression of the secondary metabolites pathway(Al-Ghazi et al., 2009).

Although some reference genes have been used for the normalization of gene expression in safflower, but these reference genes were not evaluated for qPCR data in safflower tissues (Li et al., 2012; Cao et al., 2013). Recently, the safflower reference genes were reported in our process of revising, but their transcriptome data were only from the seed and algorithms for the reference genes evaluation were incomplete (Li et al., 2015). In this study, two representative chemotype of safflower lines, with-HSYA (yellow flower) and without-HSYA (white flower), was chosen to identify the safflower reference genes. The nine housekeeping genes were selected as the candidate genes from transcriptome data of different developmental flowering stage of safflower constructed by our lab. The profiling of these genes was carried out using four algorithms and was ranked by RankAggreg. All 54 samples were separated into six groups in order to evaluate the variation of these candidate genes in different lines, organs, and flowers. Although there were different lists of the most stably expressed genes from the results of geNorm, NormFinder, Δ*Ct* approach and BestKeeper, the *CtUBI* and *Ct60S* were in the top three ranked genes according to the RankAggreg analysis in the five groups (except the YF). Our findings were in accordance with the result that *UBQ* (ubiquitin) showed highly stable expression in *Arabidopsis* (Czechowski et al., 2005). Similarly, *UBQ* was the recommended housekeeping gene for normalization in poplar (Brunner et al., 2004) and rice (Jain et al., 2006). However, *UBQ* was not suggested using as internal controls to normalize gene expression in soybean (Jian et al., 2008). As for *60S*, it was found to be the best reference gene in different tissues and under various stress conditions in soybean (Le et al., 2012). Therefore, we used *CtUBI* and *Ct60S* as reference genes and evaluated the expression of *CtFAD2-10* and *CtKASII* in safflower. *CtKASII* had been used as the reference gene to profile the *CtFAD2* gene expression. But according to our study, the *CtKASII* was not suitable for gene expression normalization because its expressions were highly variable.

Our results indicated that the stability of reference gene expression must be validated for each line and the development stages of flowering in safflower. Based on these results, we strongly suggest that *CtUBI* and *Ct60S* should be used as reference genes for gene expression in safflower. The identification of these two stable reference genes will enable accurate and reliable gene expression studies related to functional genomics and metabolomics about safflower.

Ethical disclosures

All authors declare to our manuscript did not involve any ethical issues.

Authors' contribution

XYR and GDD were responsible for the collection of plant sample. LF and TYH developed the analytical methodology. LF and GY were responsible for data analysis. LF and GML designed the study and wrote the manuscript. All the authors read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2016.05.006.

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