Reference genes selection for qRT-PCR assays in *Stellera chamaejasme* under abiotic stresses and hormone treatments based on transcriptome datasets (#21713)

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Reference genes selection for qRT-PCR assays in *Stellera chamaejasme* under abiotic stresses and hormone treatments based on transcriptome datasets

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Background. *Stellera chamaejasme* Linn, one kind of important poisonous plants of China grassland, has a certain toxicity to human and livestock. The rapid expansion of *S. chamaejasme* has been greatly damaged the grassland ecology and consequently seriously endangered the development of animal husbandry. In order to draft efficient prevention and control measures, it becomes more urgent to carry out research on its adaptive and expansion mechanisms in different unfavorable habitats at the genetic levels. qRT-PCR is considered to be the widely used technique for gene expression analysis in transcript level, which needs reference genes (RGs) as the endogenous control for data normalization. However, little research on the selection of RGs for gene expression data normalization in *S. chamaejasme* have been thoroughly investigated. **Method.** In this study, 10 candidate RGs, namely *18S*, *60S*, *CYP*, *GAPCP1*, *GAPDH2*, *EF1B*, *MDH*, *MON1*, *TUA1*, and *TUA6*, were singled out from the transcriptome database of *S. chamaejasme* and their expression stability under three abiotic stresses (Drought, Cold, Salt) and three hormone treatments (ABA, GA, ETH) were estimated by using geNorm, NormFinder, and BestKeeper. **Result.** Our results showed that *GAPCP1* and *EF1B* were the best combination for three abiotic stresses, whereas *TUA6* and *MON1*, *TUA1* and *CYP*, *GAPDH2* and *60S* were the top two choices for ABA, GA and ETH treatment, respectively. Moreover, *GAPCP1* and *60S* were assessed to be the best combination for all samples. Instead, *18S* was the least stable RG for use as internal controls for all experimental subsets. The expression patterns of two target genes (*P5CS2* and *GI*) further verified that the RGs we selected were suitable for gene expression normalization. **Discussion.** This work is the first attempt to comprehensively estimate the stability of RGs in *S. chamaejasme*, which will provide suitable RGs for high-precision normalization in qRT-PCR analysis, thereby making it more convenient to analyze gene expression under these experimental conditions.

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ABSTRACT

Background. *Stellera chamaejasme* Linn, one kind of important poisonous plants of China grassland, has a certain toxicity to human and livestock. The rapid expansion of *S. chamaejasme* has ~~been~~ greatly damaged the grassland ecology and consequently seriously endangered the development of animal husbandry. In order to draft efficient prevention and control measures, it becomes more urgent to carry out research on its adaptive and expansion mechanisms in different unfavorable habitats at the genetic levels. qRT-PCR is considered to be the widely used technique for gene expression analysis in transcript level, which needs reference genes (RGs) as the endogenous control for data normalization. However, little research on the selection of RGs for gene expression data normalization in *S. chamaejasme* have been thoroughly investigated.

Method. In this study, 10 candidate RGs, namely *18S*, *60S*, *CYP*, *GAPCP1*, *GAPDH2*, *EF1B*, *MDH*, *MON1*, *TUA1*, and *TUA6*, were singled out from the transcriptome database of *S. chamaejasme* and their expression stability under three abiotic stresses (Drought, Cold, Salt) and three hormone treatments (ABA, GA, ETH) were estimated by using geNorm, NormFinder, and BestKeeper.

Result. Our results showed that *GAPCP1* and *EF1B* were the best combination for three abiotic stresses, whereas *TUA6* and *MON1*, *TUA1* and *CYP*, *GAPDH2* and *60S* were the top two choices for ABA, GA and ETH treatment, respectively. Moreover, *GAPCP1* and *60S* were assessed to be the best combination for all samples. Instead, *18S* was the least stable RG for use as internal controls for all experimental subsets. The expression patterns of two target genes (*P5CS2* and *GI*) further verified that the RGs we selected were suitable for gene expression normalization.

Discussion. This work is the first attempt to comprehensively estimate the stability of RGs in *S. chamaejasme*, which will provide suitable RGs for high-precision normalization in qRT-PCR analysis, thereby making it more convenient to analyze gene expression under these experimental conditions.

INTRODUCTION

Stellera chamaejasme Linn (Thymelaeaceae), a perennial herb and dominant plant of grassland desertification, is native to the northern and southwestern regions in China (Tseng, 1999; Sepulveda-Jimenez et al., 2005). The whole plant is toxic, and its main toxicity compo[illegible]n is isochamaejasmin which can make cattle, sheep and other livestock poisoning and even death (Shi & Wei, 2016). The rapid spread of *S. chamaejasme* could speed up the process of grassland desertification and also poisoned a large number of livestock in pasturing areas, causing great damage and loss to the local grassland ecology and livestock husbandry (Shi & Wei, 2016). Thus, it is of fundamental importance to elucidate the mechanisms of rapid spread and stress adaptation of *S. chamaejasme*. However, limited genome sequence information is available, which greatly hinders stress functional gene discovery, ultimately resulting in a slow improving advancement of prevention and control measures. For the above reasons, our group established the local transcriptome data for *S. chamaejasme* sedlings at five different stages[illegible]0 mM NaCl treatment for 0 h, 3 h, 12 h, 24 h, 72 h, three biological replicates) using Illumina HiSeq 4000 sequencing platform. After transcriptome sequencing and data analysis, fragments per kilobaseof exon per million fragments mapped (FPKM) converted from RSEM (RNA-Seq by Expectation Maximization) were taken for estimating the unigenes expression, which in some cases existed a

few false-positive results, and its accuracy and reliability need to be evaluated and ectified with Quantitative real-time PCR (qRT-PCR).

qRT-PCR, is considered to be one of the most widely-applied technology to detect the expression levels of selected genes in many different samples (Huggett et al., 2005), attributed to its relatively accurate quantification, simplicity, specificity, high sensitivity and high throughput capacity (Qi et al., 2016; Wang et al., 2016a). In the relative quantitative method of qRT-PCR data processing application, the choice of internal gene is particularly important, and the small changes of the reference genes(RGs) stability will significantly influence the accuracy of the relative expression of target genes (Dheda et al., 2005). Generally speaking, an ideal RG should be a kind of endogenous genes that cannot change with all tested tissues or experiment conditions (Derveaux, Vandesompele & Hellemans, 2010; Li et al., 2016a; Wang et al., 2016b). In organism cells, some endogenous housekeeper genes with consistent relatively expression are often to be as reference genes (Taylor et al., 2016).

Housekeeping genes (HKGs) generally refers to a class of highly conserved genes that have basic functionality in biochemistry metabolism in organisms (Fiume & Fletcher, 2012), which normally express at relatively constant rates across different tissues (Warrington et al., 2000; Paolacci et al., 2009). However, several studies have found that their expression levels in varying degrees existed differences because of different tissues, developmental stages, or experimental conditions (Thellin et al., 1999; Nicot et al., 2005; Wu et al., 2016). Therefore, it is necessary to select stably expressed HKGs to be as RGs before they are utilized for normalizing the target genes expression by qRT-PCR (Guenin et al., 2009; Gong et al., 2016).

As of now, there is no available internal control gene for qRT-PCR data normlization has been characterized and identified in *S. chamaejasme*, so we were unable to carry out the study of transcriptome sequencing result verification, expression patterns analysis of salt or more stress-related genes, nor further clarify its spread mechanism. To solve the problem, in our study, we selected 10 candidate RGs based on the local salt *S. chamaejasme* transcriptome database (unpublished data) and then determined their expression profiles in five different stages under various abiotic stresses (Drought, Salt, and Cold) and three hormone treatments (Abscisic Acid, ABA; Gibberellin, GA; Ethephon, ETH) by qRT-PCR and further evaluated their expression stabilities using three popular software packages**:** geNorm (Vandesompele et al., 2002), NormFinder (Andersen, Jensen & Orntoft, 2004) and BestKeeper (Pfaffl et al., 2004). The 10 candidate genes were 18S ribosomal RNA (*18S*), 60S ribosomal RNA (*60S*), cyclophilin (*CYP*), elongation factor 1-beta (*EF1B*), glyceraldehyde-3-phosphate dehydrogenase of plastid 1 (*GAPCP1*), glyceraldehyde-3-phosphate dehydrogenase 2 (*GAPDH2*), malate dehydrogenase (*MDH*), Monensin sensitivity 1 (*MON1*), alpha-tubulin 1 (*TUA1*), alpha-tubulin 6 (*TUA6*). Two target genes, Delta 1-pyrroline-5-carboxylate synthetase 2 (*P5CS*2), which encodes a crucial enzyme in the proline synthesis pathway under stress conditions by exercising the activity of glutamate 5-kinase and glutamate-5-semialdehyde dehydrogenase (Strizhov et al., 1997), and GIGANTEA (*GI*), a circadian regulated gene whose protein product has not only been shown to regulate photoperiodic flowering and various developmental processes but has been implicated in mediating the cold stress response (Cao, Ye & Jiang, 2005; Li et al., 2017), were used to verify the selected RGs.

MATERIALS AND METHODS

Plant Materials and Stress Treatments

S. chamaejasme seeds were collected from Qilian, Qinghai province. After peeled, seeds were treated with 98% H_2SO_4 for 9 – 11 min, then rinsed for 30 min with running water and planted into individual pots (14.5 × 14.5 × 6.5 cm) filled with nutrition soil, vermiculite and perlite (6 : 1 : 1). Germinated seeds were kept growing 7 weeks and then were transferred to the nurseries potted with double-layered filter paper for 3 days adaptation cultivation. All these nursery pots were put in the artificial climate chamber with a temperature of 25 ± 2℃ during the day and 15 ± 2℃ at night, a relative humidity of 50 – 55%, and an illumination intensity of 300 μmol $m^{-2}s^{-1}$ (14/ 10h, day/ night). Three pots of 7-week-old seedlings (three biological replicates) with consistent growth status for each group were chosen and treated by abiotic stresses and hormone treatments.

For drought and salt treatments, 20% PEG-6000 (w/v, Sangon, China) and 300 mM NaCl (Sangon, China) were applied to irrigate the seedlings respectively. For cold stress, all other things being equal, seedlings in the nursery pots were shift to another artificial climate chamber at 4℃. For hormone treatments, the leaves were sprayed with 0.1 mM ABA (Reddy et al., 2016; Wan et al., 2017) or 0.1 mM GA (Li et al., 2016b), or 1.5 mM ETH (Wu et al., 2016). Seedlings were irrigated or sprayed every 12 hours during the course of the experiment. Complete seedlings were carefully collected at 0h, 3h, 12h, 24h, 48h after treatments and immediately frozen in liquid nitrogen, and stored at –80℃ refrigerator until total RNA isolation.

Total RNA Isolation and 1st Strand cDNA Synthesis

Approximately 100 mg seedlings of each sample was used for total RNA isolation with a TRNzol reagent kit (TIANGEN, China). The concentration, ratio of 260/280 and 260/230 of RNA samples were detected with the Nano Drop ND-1000 Spectrophotometer (Nano Drop Technologies, USA) and the integrity of all RNA samples were verified by 1.0% (w/v) agarose gel electrophoresis (AGE). Subsequently, for Reverse Transcription PCR (RT-PCR) and qRT-PCR, total 3.0 μg RNA was used to synthesize the 1st strand cDNA by reverse transcription following the specification (Roche, USA) in a 20 μl reaction system. At last, the cDNA diluted 50 folds with ddH_2o was severed as the templete for PCR amplication.

Candidate RGs Selection and Primers Design

Ten candidate RGs from the local *S. chamaejasme* transcriptome database were selected by using local NCBI-blast (version 2.4.0+). Sequences of these genes were used to design qRT-PCR primers using Primer 5.0, Oligo 7.60 and Beacon Designer 8.20 software with the following criteria: melting temperature (Tm) of 50 – 65℃, primer lengths of 17 – 25 bp, GC contents of 45 – 55% and product lengths of 90 – 300 bp. The specificity of all selected primer pairs were observed via RT-PCR and each gene fragmentation was underpinned by 2.0% (w/v) AGE and sequenced to ensure its reliability.

RT-PCR and qRT-PCR Analysis

In order to confirm each primer specificity we designed, we performed RT-PCR in 25 μl system using Bio-Rad C1000 PCR system (Bio-Rad, USA). The reaction system was as follows: 2.5 μl Ex Taq buffer, 2 μl dNTP, 0.125 μl TaKaRa Ex Taq (TaKaRa, China), 2.0 μl of cDNA template, 0.5 μl of forward primers (10 μM), 0.5 μl of reverse primer (10 μM) and 17.375 μl of sterilized water. The RT-PCR reactions parameters: 95℃ for 3 min, 40 cycles at 95℃ for 30 s, 58℃ for 30 s, 72℃ for 20 s, and 72℃ for 5 min. The amplification products were evaluated by 2.0% (w/v)

AGE. To further confirm that the amplicon was corresponded with what we desired, target products contained in agarose gel were recycled using TIANgel Midi Purification Kit (TIANGEN, China) and then sequenced.

qRT-PCR reactions were carried out with the Fast Start Universal SYBR GreenMaster (Roche, Germany) on a Bio-Rad CFX96 Real-Time PCR system (Bio-Rad, USA) in accordance with the manual. Reactions were conducted at 95℃ for 3 min as an initial activation, followed by 40 cycles at 95℃ for 10 s, 58℃ for 10 s, and 72℃ for 20 s. After 40 cycles, the melting curves ranging from 58℃ to 95℃were performed to check the specificity of the amplicons. Three technical replicates were analyzed for each biological sample, and the final Ct values for each set of samples were the average of three biological replicates. The mean amplification efficiency (E) of each primer pair was calculated by the LinRegPCR program (Ruijter et al., 2009; Zhuang et al., 2015; Vavrinova, Behuliak & Zicha, 2016; Wu et al., 2016).

Data Analysis of Gene Expression Stability

Three different types of statistical tools: geNorm (version 3.5), NormFinder (version 0.953) and BestKeeper (version 1.0), were applied to rank the expression stability of the RGs across all experimental sets. For geNorm and NormFinder, the raw Ct values calculated by the CFX equipment™ software were converted into the relative quantities by the formula $2^{-\Delta Ct}$ (ΔCt = each corresponding Ct value – lowest Ct value) for gene expression profiling. For BestKeeper, the raw Ct values and amplification efficiencies estimated by the LinRegPCR program were used to calculate the coefficient of variation (CV) and the standard deviation(SD). The RG with the lowest CV ± SD value was identified to be the most stable gene. geNorm software was also used to determine the proper RG numbers by using pair wise variation (Vn/Vn+1, n refers to the RGs number) between two sequential normalization factors.

Validation of Reference Genes

To detect the concrete manifestation of the comprehensive ranking orders of the RGs from this study, the optimum RGs combination, the most stable RG and the least RG were used to atandardize the expression of two objective genes namely *P5CS2* and *GI* across abiotic stresses and hormone treatments, respectively. Furthermore, their expression levels under salt stress were also in comparison with the FPKM values in *S. chamaejasme* transcriptome database.

RESULTS

Selection of Candidate RGs and Target Genes

After comparing the reported RGs in other species with the local transcriptome database of *S. chamaejasme* by local BLast program, ten RGs and two target genes were singled out to perform the gene normalization studies. The results showed that the E value of each blast gene indicated a high homology. The untranslated region (UTR) of these full-length unigene sequences were used to design the specific primers for RT-PCR and qRT-PCR. The unigene ID, NCBI accession number, gene symbol, gene name, homolog locus of 10 candidate RGs and two target genes, and E value compared with homologous genes were enlisted in **Table 1**.

Verification of Primer Specificity and qRT-PCR Amplification Efficiency

The specificity of each primer was tested by 2.0% AGE, sequencing and melting curves analysis, which presented the expected amplicon length (**Supplemental figure 1)** and the single peak melting curves (**Supplemental figure 2)**. The primer sequences, amplicon size, product Tm, amplification efficiencies, and other relevant information were showed in **Table 2**. The amplification product length of PCR varied from 94 bp to 267 bp. Tm for all PCR products were spanning from 76.0℃ for *MDH* to 83.5℃ for *GAPCP1*. The E-values of these genes were between 1.824 (*MDH*) to 1.930 (*GAPDH2*), and the linear correlation coefficients (R^2) varied from 0.994 (*MON1*) to 0.998 (*CYP*). In conclusion, we had every reason to believe that all of these specificity and efficiency estimates of the amplification were reliable enough for further analysis.

Expression Profiles of Candidate RGs

The boxplot analysis about Ct value of different reference genes in all experimental samples was performed using origin 2017 software (**Fig. 1**). Granpuic results demonstrated that the mean Ct values of ten candidate RGs presented a relatively wide field, from 19.26 to 30.76. *60S* showed the least expression variations while *18S* exhibited the highest variations with the Ct value ranging from 15.58 to 22.59. Since Ct values are negatively related to gene expression levels, the smaller the Ct value, the higher the gene expression level. As **Fig. 1** showed, *18S* was counted as the highest-expressed RG for its lowest mean Ct value (15.58). Instead, *GAPDH2* showed the lowest expression level on account of its supreme mean Ct value (32.58).

Analysis of Gene Expression Stability

geNorm analysis. geNorm calculats the gene expression stability measure M value as the average pairwise variation V for that RG and other tested RGs (Vandesompele et al., 2002). The smaller the M value means the more stable the gene, and vice versa. In our study, the M values of the 10 candidate RGs of *S. chamaejasme* calculated by geNorm software were below 1.5 in all experimental settings(**Fig. 2**), suggesting that the genes should be considered relatively stable. As described in **Fig. 2A, Fig. 2B and Fig. 2C**, *GAPCP1* and *EF1B* under drought stress, *GAPCP1* and *60S* under cold stress, and *EF1B*and *60S* under salt stress were ranked to be the most stable RGs with the lowest M value of 0.07,0.05 and 0.19. At the same time, in ABA(**Fig. 2D**), GA (**Fig. 2E**), and ETH (**Fig. 2F**) treatment groups, *MON1* and *TUA6*, *TUA1* and *CYP*, *GAPDH*2 and *60S* were considered to be the most stable genes for the lowest M value of 0.21, 0.22 and 0.19, respectively. In addition, ~~in the context of~~ all sample set (**Fig. 2G**), *GAPCP1* and *CYP* were suggested as the top two stable RGs. On the contrary, *18S* was always the latest stable gene in all sets except ETH treatment, in which *TUA6* was the least stable gene.

NormFinder analysis. NormFinder provides a stability value for each gene by analyzing expression data obtained through qRT-PCR, which is a direct measurement for estimating the expression variation when the gene was used for normalization (Dheda et al., 2005). The sort orders based on the stability values calculated by NormFinder (**Table 3**) were similar to those determined by geNorm. The stability ranking results in cold stress and GA treatment subsets were completely consistent with the results determined through geNorm, meanwhile *TUA6* and *18S* were still the two latest stable genes among ETH treatment and the rest of treatments. For cold stress group, *GAPCP1* and *EF1B* were the most two stable RGs (also ranked first by geNorm). For salt stress group, *GAPCP1* and *TUA1* were the two most stable RGs, which was totally different with the geNorm result. For All samples, ABA-treated and ETH-treated subsets,

NormFinder suggested that *GAPCP1* and *60S*, *TUA1* and *MON1*, *GAPDH2* and *60S* were the top two stable RGs, respectively, which were not exactly the same as geNorm analysis results.

BestKeeper analysis. BestKeeper evaluates the RGs expression stability by calculating the CV and SD of the average Ct values. The lower the CV value means that the more stable the RGs expression, and candidates RGs with SD values greater than 1.0 are adjudged unstable and should be avoided for gene expression normalization (Guenin et al., 2009). As shown in **Table 4**, in the drought stress and all samples subsets, *TUA1* with the lowest CV ± SD value of 0.52 ± 0.16 and 0.53 ± 0.16, was considered to be the most stable RG. In the cold stress, salt stress and ABA treatment subsets, *EF1B* with the lowest CV ± SD values of 1.16 ± 0.31, 1.35 ± 0.36 and 1.04 ± 0.27 respectively, was identified as the best RG. In the GA treatment subset, *TUA6* had the lowest CV ± SD values of 0.82 ± 0.22, and showed the most stability. In ETH treatment subset, BestKeeper suggested *GAPDH2* as the most stable RG with the lowest CV ± SD values of 0.68 ± 0.18. Additionally, only a few genes have a SD value greater than 1.0, indicated most of candidates RGs were relatively stable. Except for the ETH treatment subset, the most unstable RGs among all experimental settings were *18S*, which was the same as the results of geNorm and NormFinder.

Determination of Optimal Number of RGs

At the suggestion of the geNorm Service tool, the critical value Vn/Vn+1 to determin the optimal RGs number for qRT-PCR normalization is 0.15, below which the inclusion of an additional RGs is not required (Vandesompele et al., 2002). As **Fig. 3** showed, the V2/3 values of all experimental groups were less than 0.15, which indicated that two RGs combination would be sufficient to used for normalization.

Comprehensive Stability Analysis of RGs

Table 5 and **Fig. 4** summarized and ranked the determination results got from geNorm, NormFinder and Bestkeeper programs. Based on the analysis results, *GAPCP1* and *EF1B* were the most stable RGs under three abiotic stresses, thus *TUA6* and *MON1*, *TUA1* and *CYP*, *GAPDH2* and *60S* could be the best RGs combination under ABA, GA and ETH treatment, respectively. Still, *18S* was the most unstable RG among all experimental conditions.

Reference Gene Validation

Verification results in **Fig. 5** turned out that the relative expression levels of *P5CS2* under different experimental conditions tend to be the same when normalized using the best RGs combination or only the most stable RG. However, different expression patterns generated and the expression levels of *P5CS2* and *GI* were overestimated when the least stable gene *18S* was selectd to be RG for normalization. Similar conclusions were also reached in **Fig. 6**.The maximal expression level of *GI* under drought **(Fig. 6A)**, cold **(Fig. 6B)**, salt **(Fig. 6C)**, ABA **(Fig. 6D)** and ETH **(Fig. 6E)** treatment had taken place prominent changes compared with the control group using the combinations of RGs, which was 4.66-fold, 29.22-fold, 2.10-fold, 6.45-fold and 2.45-fold higher than that of the control group, while it did not show a significant differences under GA treatment.

In particular, as shown in **fig. 5C**, under salt treatment, when the RGs combination (*GAPCP1* and *EF1B*) was selected to carry out the normalization, the gene expression of *P5CS2* gradually increased from 0h and reached the maximizing at 24 h, and then began a slight decline at 48h. In

the same way, the expression levels of *GI* increased at first, then decreased and kept a lower level till 48 h (**Fig. 6C**). For sure, the expression trends of *P5CS2* and *GI* in the first 24 hours were generally consisitent with that of RNA-seq (**Supplemental figure 3**). This further validated the accuracy and reliability of our experimental results.

DISCUSSION

qRT-PCR is currently viewed as a powerful technique to quantify the target genes expression, whose accuracy directly depends on the stability of the internal genes. The use of inappropriate RGs for normalization of qRT-PCR data will lead to deviation in the results (Shivhare & Lata, 2016). In this study, three programs, geNorm, NormFinder and Bestkeeper had been used to select the optimum RGs for six different experimental conditions. As described in results above, three kinds of data analysis software detected different RGs as stable internal control for qRT-PCR normalization based on different mathematical methods or parameters. Ten potential RGs exhibited differential stability in response to different stresses, just taking ABA treatment for example, in this experimental subset, geNorm software ranked *MON1* as the head of RGs, thus NormFinder regarded *TUA1* as the most stable RG. But the meantime, Bestkeeper identified *EF1B* as the best RG according to its lowest CV value. This meant that three kinds of software generated three different results and which solution should we choose. Our study made a comprehensive analysis for this and gave the ultimate stability ordering result by ranking the geometric means of three software analysis results, which was a common strategy for evaluating expression stability of RGs reported in previous scientific papers.

It must be clear that there are no universal RGs that are stably expressed under all biological materials and/or trial conditions. Our experimental results also absolutely confirmed this point. *EF1B* catalyzes the exchange of Guanosine diphosphate (GDP) bound to the G-protein, elongation factor 1-alpha (*EF1A*), for guanosine triphosphate (GTP), an important step in the elongation cycle of the protein biosynthesis. According to the comprehensive ranking results, *EF1B* was ranked in the upstream of result order and had better expression stability across three abiotic stresses more than those in three hormone treatments. Other RGs stability sorts also presented varied adaption of different degrees due to changes of processing conditions.

The expression stability of two homologous RGs: *TUA1* and *TUA6,* were estimated in our study. According to the above analysis result, the stability ranking of *TUA1* was always in front of *TUA6* for all conditions except ABA treatment in which *TUA6* exhibited a batter express stability. Nevertheless, It is notable that the homologous RGs showed different rank order in each subset, and in most case, *TUA1* showed better expression stability than *TUA6*. For the homolog genes, they have similar coding sequence from different gene loci. This means that we need to ensure primer specificity when we amplified these homogenous sequences from the gene families. In our study, primer sets were designed in the UTR to avoid the conserved domain, which to a large extent enabled the gene specific amplification.

18S is also a frequently-used HKG which can be widely used for normalization in qRT-PCR analysis, but at some point it is not suitable as a RG for its excessive high expression level. Our analysis results suggested that *18S* was the most unatable in all experiment groups. In comparation with ~~the~~ the best RGs combination and the most stable RG, when *18S* was selectes as RG to validate the expression of two target genes *P5CS2* and *GI*, their expression pattern were significantly overestimated, suggesting over up to 160.05 fold of gene expression which was

consistent with findings in *Oxytropis ochrocephala* (Zhuang et al., 2015) and *rice* (Bevitori et al., 2014).

Two target genes *P5CS2* and *GI* have been used to verify the stability of selected RGs for gene expression normalization. (Strizhov et al., 1997) said that expression of *Arabidopsis thaliana P5CS* (*AtP5CS*) was tissue-specific and could be regulated by salinity, drought and ABA. The same experimental results had been reproducted in our experiments. But the meantime, we also found that *P5CS* could be efficiently expressed during later period of cold stress, which may be a supplement to previous findings. The induction mechanism remains to be further studied. (Cao, Ye & Jiang, 2005) had reported that *GI* was induced by cold stress, but not by salt, mannitol, and ABA. In contrast, (Park, Kim & Yun, 2013) claimed that GI as a transitory regulator of salt overly sensitive (Sepulveda-Jimenez et al., 2005) pathway activity whose presence or amount connects flowering to salt stress condition, and (Riboni et al., 2016) revealed that ABA affects flowering through two independent regulatory mechanisms: the activation of *GI* and constant (*CO*) functions upstream of the florigen genes and the down-regulation of suppressor of overexpression of CO1 (*SOC1*) signalling. Our findings indicated that the gene expression of *GI* not only changed under salt stress and cold stress, but also undergone a significant change under drought and ETH treatments. We have reasons to believe that these mechanisms will be revealed with the experiment further in-depth.

There is no doubt that it is necessary to select suitable RGs or/and RGs combination for gene normalization studies to get more accurate and reliable results. Combined with all the validation results above, we can observed that in most cases, *P5CS2* and *GI* showed similar response patterns when normalized by the RGs combination and the most stable RG, but some differences still sank. Unfortunately, we could not tell which kind of choice was better for normalization. However, in order to eliminate the small variations caused by technical protocols in qRT-PCR, two or more RGs are often required to correct for non-specific experimental variation (Thellin et al., 1999; Bustin et al., 2009). In this study, two RGs combination, whose V2/3 value were less than 0.15 across all experimental subsets in line with the geNorm software results, can completely meet the requirements of normalization.

CONCLUSONS

This current study represents the first attempt to comprehensively analyze the stability of RGs for use as the internal control in qRT-PCR analysis of target genes expression in *S. chamaejasme* under three abiotic stresses and three hormone treatments by combining results from three different methods. The results fully indicate that the stability of the identical gene was not exactly the same under different treatments, and the stability ranking of RGs caculated by three parameters are not identical under the same treatment. As a result, it makes sense to carry out a comprehensive analysis against the results of three procedures. Moreover, it may be a better choice to select the combination of two or more RGs to be an effective internal control for further improving the accuracy and reliability of gene expression normalization under different stresses. In conclusion, this study will provide a guideline to select a valid RG combination that can ensure more accurate qRT-PCR based gene expression quantification and a basic data to facilitate future molecular studies on gene expression in *S. chamaejasme* and the other Thymelaeaceae species (Che et al., 2016).

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Table 1(on next page)

Description of candidate reference genes and target genes.

Unigene Gene ID	Accession number	Gene symbol	Gene name	Homolog locus	E value
>c73334.graph_c0	MG516523	*18S*	18S ribosomal RNA	AH001810.2	1e-105
>c68075.graph_c0	MG516524	*60S*	60S ribosomal RNA	KJ634810.1	0.0
>c71629.graph_c0	*MG516525*	*CYP*	Cyclophilin	JN032296.1	2e-123
>c70757.graph_c0	MG516526	*EF1B*	Elongation factor 1-beta	XM_013599463	9e-138
>c67520.graph_c0	MG516527	*GAPCP1*	Glyceraldehyde-3-phosphate dehydrogenase of plastid 1	NM_106601.4	0.0
>c74212.graph_c0	MG516528	*GAPDH2*	Glyceraledehyde-3-phosphate dehydrogenase 2	KM370884.1	0.0
>c70711.graph_c1	MG516529	*MDH*	Malate dehydrogenase	HQ449567.1	0.0
>c72957.graph_c1	MG516530	*MON1*	Monensin sensitivity 1	NM_128399.4	0.0
>c60567.graph_c0	MG516531	*TUA1*	Alpha-tubulin 1	AT1G64740.1	0.0
>c65147.graph_c0	MG516532	*TUA6*	Alpha-tubulin 6	AT4G14960.2	0.0
>c57696.graph_c0	MG516533	*P5CS2*	Delta 1-pyrroline-5-carboxylate synthetase 2	AT3G55610.1	0.0
>c73625.graph_c0	MG516534	*GI*	GIGANTEA	KR813315.1	0.0

Table 2(on next page)

Selected candidate RGs and target genes, primers, and amplicon characteristics.

Name	Forward primer sequences (5′–3′) Reverse primer sequences (5′–3′)	Amplicon Size (bp)	Product Tm[a] (°C)	E	R^2
18S	CTATCCAGCGAAACCACAG CCCACTTATCCTACACCTCTC	122	81.5–82.0	1.918	0.996
60S	TTGTTCGATAGCATCCGTCT ATAAAAGCAAACAACGGAAGCA	170	78.0–78.5	1.836	0.997
CYP	ACATAGTTTGAGGCAACCTAGCAGT TACACCTTCGCAGACAGTCGTT	161	80.0	1.854	0.997
EF1B	GCAGTGAACTCTCCCCAG CCAAACAGGGCATAAAAGAAC	191	78.0–79.0	1.842	0.998
GAPCP1	CCATTAGATCCGTCGCCTGTT TTGTTGGTGGCACTTCTGTAGC	192	83.0–83.5	1.834	0.998
GAPDH2	GTGAAACTGGTCTCCTGGTATG AACCCAGGCAACGCTTATA	115	81.0	1.930	0.998
MDH	CCGCGACTTTGAATAAGCCCAT AACTCAAAATCCTCGTCCCCAA	94	76.0–76.5	1.824	0.997
MON1	CCTGCCAAGATACAATCCCA TTTGTGCTGCCCTAAACGAG	267	80.0–80.5	1.872	0.997
TUA1	GGCACTTTCGAGTTTTCGC CCAGCTTGTCCGATGTGAA	97	79.0–79.5	1.840	0.998
TUA6	GAAGGAATGGAGGAAGGGGAG CAAACACAAGAAAGCGACAAATAAG	165	81.5–82.5	1.837	0.997
P5CS2	TGACTTTATACGGTGGACCAA TCCTCTGTGACAACGCAAT	178	82.5–84.5	1.839	0.997
GI	ATGATTACAGAAACGGAATTAACTCA TAACTCCATGAAGTACCGACAGA	112	79.5–81.0	1.858	0.994

Table 3(on next page)

Expression stability of 10 candidate reference genes calculated by NormFinder.

Rank	Drought	Cold	Salt	ABA	GA	ETH	ALL
1	*GAPCP1*	*GAPCP1*	*GAPCP1*	*TUA1*	*TUA1*	*GAPDH2*	*GAPCP1*
Stability	0.025	0.015	0.089	0.071	0.075	0.048	0.028
2	*EF1B*	*60S*	*TUA1*	*MON1*	*CYP*	*TUA1*	*60S*
Stability	0.052	0.018	0.089	0.072	0.075	0.051	0.031
3	*60S*	*EF1B*	*MON1*	*TUA6*	*GAPCP1*	*GAPCP1*	*CYP*
Stability	0.069	0.060	0.237	0.109	0.096	0.135	0.032
4	*MON1*	*GAPDH2*	*60S*	*CYP*	*MON1*	*MON1*	*MON1*
Stability	0.074	0.076	0.284	0.159	0.103	0.149	0.065
5	*CYP*	*CYP*	*EF1B*	*GAPCP1*	*60S*	*60S*	*TUA1*
Stability	0.245	0.238	0.319	0.188	0.323	0.150	0.130
6	*TUA1*	*MON1*	*CYP*	*60S*	*TUA6*	*MDH*	*EF1B*
Stability	0.316	0.385	0.371	0.201	0.358	0.207	0.163
7	*TUA6*	*TUA6*	*MDH*	*MDH*	*GAPDH2*	*EF1B*	*MDH*
Stability	0.326	0.481	0.447	0.255	0.414	0.251	0.185
8	*GAPDH2*	*TUA1*	*TUA6*	*EF1B*	*EF1B*	*CYP*	*TUA6*
Stability	0.405	0.523	0.726	0.401	0.754	0.359	0.294
9	*MDH*	*MDH*	*GAPDH2*	*GAPDH2*	*MDH*	*18S*	*GAPDH2*
Stability	0.615	0.586	1.286	0.516	0.836	0.486	0.357
10	*18S*	*18S*	*18S*	*18S*	*18S*	*TU 6*	*18S*
Stability	0.999	1.093	1.748	1.272	0.965	0.497	0.556

Table 4(on next page)

Expression stability of 10 candidate reference genes calculated by BestKeeper.

Rank	Drought	Cold	Salt	ABA	GA	ETH	ALL
1	*TUA1*	*EF1B*	*EF1B*	*EF1B*	*TUA6*	*GAPDH2*	*TUA1*
CV ± SD	0.52 ± 0.16	1.16 ± 0.31	1.35 ± 0.36	1.04 ± 0.27	0.82 ± 0.22	0.68 ± 0.18	0.53 ± 0.16
2	*MON1*	*GAPCP1*	*GAPCP1*	*TUA6*	*60S*	*60S*	*EF1B*
CV ± SD	0.89 ± 0.27	1.21 ± 0.37	1.88 ± 0.58	1.06 ± 0.29	1.02 ± 0.26	1 ± 0.26	0.82 ± 0.22
3	*GAPDH2*	*TUA1*	*60S*	*60S*	*TUA1*	*TUA1*	*GAPCP1*
CV ± SD	1.07 ± 0.28	1.39 ± 0.41	1.93 ± 0.5	1.27 ± 0.32	1.76 ± 0.56	1.16 ± 0.35	0.91 ± 0.28
4	*GAPCP1*	*60S*	*CYP*	*CYP*	*CYP*	*GAPCP1*	*60S*
CV ± SD	1.24 ± 0.38	1.4 ± 0.36	2.1 ± 0.61	1.37 ± 0.39	1.78 ± 0.52	1.17 ± 0.35	0.93 ± 0.24
5	*EF1B*	*GAPDH2*	*TUA1*	*MON1*	*MON1*	*MDH*	*TUA6*
CV ± SD	1.27 ± 0.34	1.48 ± 0.38	2.38 ± 0.75	1.45 ± 0.43	1.81 ± 0.56	1.2 ± 0.33	0.99 ± 0.27
6	*60S*	*CYP*	*MON1*	*GAPDH2*	*GAPDH2*	*MON1*	*MON1*
CV ± SD	1.71 ± 0.45	1.77 ± 0.52	2.39 ± 0.74	1.76 ± 0.45	1.9 ± 0.51	1.21 ± 0.36	1.1 ± 0.33
7	*TUA6*	*TUA6*	*TUA6*	*MDH*	*GAPCP1*	*EF1B*	*CYP*
CV ± SD	1.86 ± 0.52	1.87 ± 0.49	2.65 ± 0.74	1.92 ± 0.53	1.92 ± 0.61	1.43 ± 0.37	1.11 ± 0.32
8	*CYP*	*MDH*	*MDH*	*TUA1*	*EF1B*	*TUA6*	*GAPDH2*
CV ± SD	2.07 ± 0.61	2.06 ± 0.57	2.87 ± 0.81	1.95 ± 0.58	3.14 ± 0.85	1.61 ± 0.44	1.26 ± 0.33
9	*MDH*	*MON1*	*GAPDH2*	*GAPCP1*	*MDH*	*CYP*	*MDH*
CV ± SD	3.32 ± 0.94	2.31 ± 0.7	5.02 ± 1.27	2.07 ± 0.63	3.17 ± 0.88	1.62 ± 0.46	1.71 ± 0.48
10	*18S*	*18S*	*18S*	*18S*	*18S*	*18S*	*18S*
CV ± SD	6.33 ± 1.29	8.32 ± 1.63	12.7 ± 2.45	8.26 ± 1.46	6.34 ± 1.32	2.91 ± 0.52	3.56 ± 0.69

Table 5(on next page)

Expression stability ranking of the 10 candidate reference genes.

Method	1	2	3	4	5	6	7	8	9	10
A. Ranking Order under drought stress (Better–Good–Average)										
geNorm	*GAPCP1/EF1B*		*60S*	*MON1*	*TUA1*	*CYP*	*TUA6*	*GAPDH2*	*MDH*	*18S*
Normfinder	*GAPCP1*	*EF1B*	*60S*	*MON1*	*CYP*	*TUA1*	*TUA6*	*GAPDH2*	*MDH*	*18S*
BestKeeper	*TUA1*	*MON1*	*GAPDH2*	*GAPCP1*	*EF1B*	*60S*	*TUA6*	*CYP*	*MDH*	*18S*
Comprehensive ranking	*GAPCP1*	*EF1B*	*MON1*	*TUA1*	*60S*	*GAPDH2*	*CYP*	*TUA6*	*MDH*	*18S*
B. Ranking Order under cold stress (Better–Good–Average)										
geNorm	*GAPCP1/60S*		*EF1B*	*GAPDH2*	*CYP*	*MON1*	*TUA6*	*TUA1*	*MDH*	*18S*
Normfinder	*GAPCP1*	*60S*	*EF1B*	*GAPDH2*	*CYP*	*MON1*	*TUA6*	*TUA1*	*MDH*	*18S*
BestKeeper	*EF1B*	*GAPCP1*	*TUA1*	*60S*	*GAPDH2*	*CYP*	*TUA6*	*MDH*	*MON1*	*18S*
Comprehensive ranking	*GAPCP1*	*EF1B*	*60S*	*GAPDH2*	*CYP*	*TUA1*	*MON1*	*TUA6*	*MDH*	*18S*
C. Ranking Order under salt stress (Better–Good–Average)										
geNorm	*EF1B/60S*		*CYP*	*GAPCP1*	*TUA1*	*MON1*	*MDH*	*TUA6*	*GAPDH2*	*18S*
Normfinder	GAPCP1	*TUA1*	*MON1*	*60S*	*EF1B*	*CYP*	*MDH*	*TUA6*	*GAPDH2*	*18S*
BestKeeper	*EF1B*	*GAPCP1*	*60S*	*CYP*	*TUA1*	*MON1*	*TUA6*	*MDH*	*GAPDH2*	*18S*
Comprehensive ranking	*EF1B*	*GAPCP1*	*60S*	*TUA1*	*CYP*	*MON1*	*MDH*	*TUA6*	*GAPDH2*	*18S*
D. Ranking Order under ABA treatment (Better–Good–Average)										
geNorm	*MON 1/TUA 6*		*CYP*	*60S*	*TUA1*	*MDH*	*GAPCP1*	*EF1B*	*GAPDH2*	*18S*
Normfinder	*TUA1*	*MON1*	*TUA6*	*CYP*	*GAPCP1*	*60S*	*MDH*	*EF1B*	*GAPDH2*	*18S*
BestKeeper	*EF1B*	*TUA6*	*60S*	*CYP*	*MON1*	*GAPDH2*	*MDH*	*TUA1*	*GAPCP1*	*18S*
Comprehensive ranking	*TUA6*	*MON1*	*CYP*	*TUA1*	*60S*	*EF1B*	*MDH*	*GAPCP1*	*GAPDH2*	*18S*
E. Ranking Order under GA treatment (Better–Good–Average)										
geNorm	*TUA1/CYP*		*GAPCP1*	*MON1*	*60S*	*TUA6*	*GAPDH2*	*EF1B*	*MDH*	*18S*
Normfinder	*TUA1*	*CYP*	*GAPCP1*	*MON1*	*60S*	*TUA6*	*GAPDH2*	*EF1B*	*MDH*	*18S*
BestKeeper	*TUA6*	*60S*	*TUA1*	*CYP*	*MON1*	*GAPDH2*	*GAPCP1*	*EF1B*	*MDH*	*18S*
Comprehensive ranking	*TUA1*	*CYP*	*TUA6*	*GAPCP1*	*60S*	*MON1*	*GAPDH2*	*EF1B*	*MDH*	*18S*
F. Ranking Order under ETH treatment (Better–Good–Average)										
geNorm	*GAPDH2/60S*		*TUA1*	*MON1*	*GAPCP1*	*MDH*	*EF1B*	*CYP*	*18S*	*TUA6*
Normfinder	*GAPDH2*	*TUA1*	*GAPCP1*	*MON1*	*60S*	*MDH*	*EF1B*	*CYP*	*18S*	*TUA6*
BestKeeper	*GAPDH2*	*60S*	*TUA1*	*GAPCP1*	*MDH*	*MON1*	*EF1B*	*TUA6*	*CYP*	*18S*
Comprehensive ranking	*GAPDH2*	*60S*	*TUA1*	*GAPCP1*	*MON1*	*MDH*	*EF1B*	*CYP*	*TUA6*	*18S*
H. Ranking Order under ALL stress (Better–Good–Average)										
geNorm	*GAPCP1/CYP*		*60S*	*TUA1*	*EF1B*	*MON1*	*MDH*	*TUA6*	*GAPDH2*	*18S*
Normfinder	*GAPCP1*	*60S*	*CYP*	*MON1*	*TUA1*	*EF1B*	*MDH*	*TUA6*	*GAPDH2*	*18S*
BestKeeper	*TUA1*	*EF1B*	*GAPCP1*	*60S*	*TUA6*	*MON1*	*CYP*	*GAPDH2*	*MDH*	*18S*
Comprehensive ranking	*GAPCP1*	*60S*	*TUA1*	*CYP*	*EF1B*	*MON1*	*TUA6*	*MDH*	*GAPDH2*	*18S*

Figure 1

Distribution of Ct values for ten candidate RGs across all *S. chamaejasme* samples.

Lines across the boxes denote the medians. The box represents the 25th and 75th percentile. The top and bottom whisker caps depict the maximum and minimum values, respectively. The white and black dots represent mean Ct values and potential outliers, respectively.

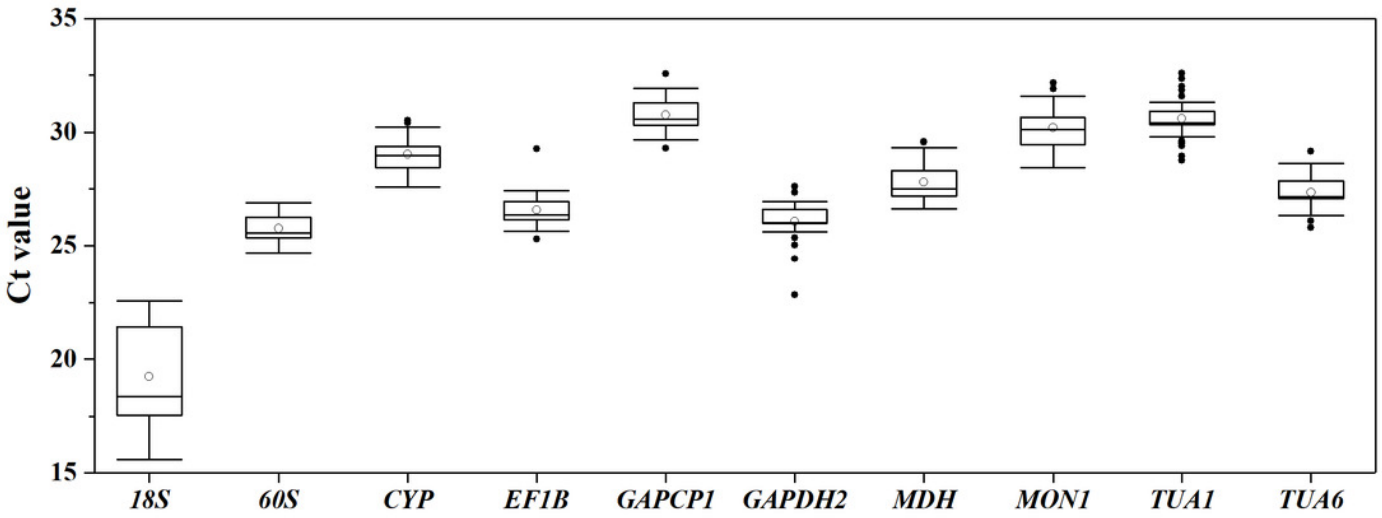


Figure 2

Average expression stability value (M) and ranking of the ten RGs across all treatments calculated using geNorm.

The least stable genes are listed on the left, while the most stable genes are exhibited on the right.

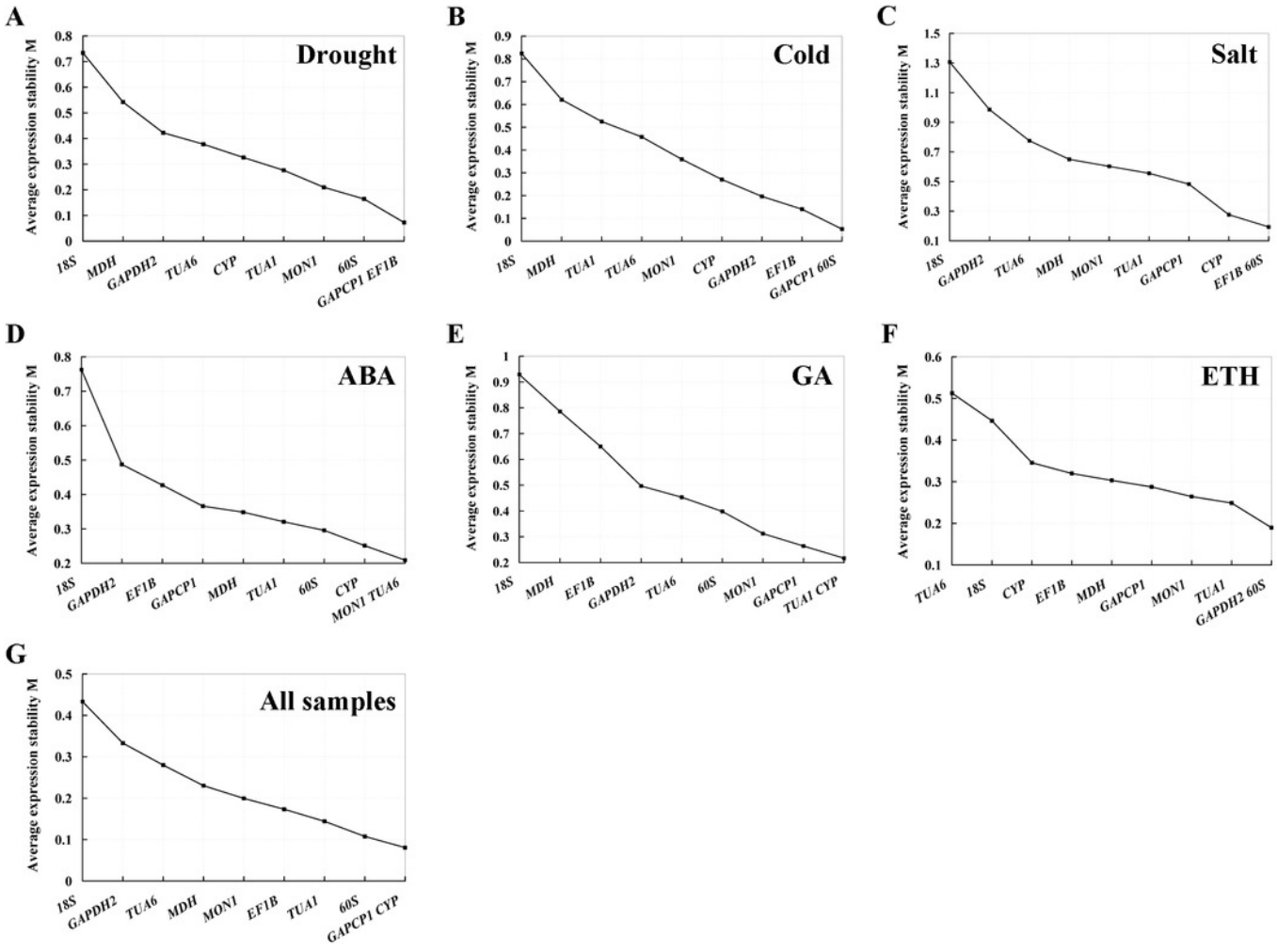
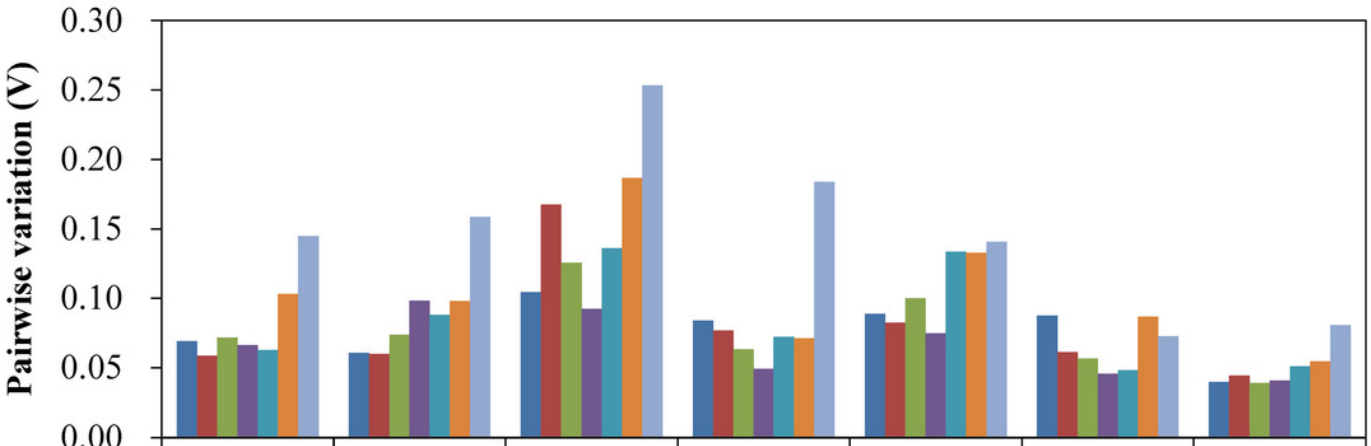


Figure 3

Pairwise variation (Vn/Vn+1) values analysis in all the seven experimental subsets calculated using geNorm.

The cut-off value to determine the optimal number of RGs for qRT-PCR normalization is 0.15.



	Drought	Cold	Salt	ABA	GA	ETH	All
V2/3	0.069	0.061	0.104	0.084	0.089	0.088	0.040
V3/4	0.059	0.060	0.167	0.077	0.082	0.061	0.044
V4/5	0.072	0.074	0.126	0.063	0.100	0.057	0.039
V6/7	0.066	0.098	0.092	0.049	0.075	0.045	0.041
V7/8	0.063	0.088	0.136	0.072	0.133	0.048	0.051
V8/9	0.103	0.098	0.187	0.071	0.133	0.087	0.054
V9/10	0.145	0.159	0.253	0.184	0.141	0.073	0.081

Figure 4

Comprehensive ranking of candidate genes calculated by the geometric mean of three types of rankings (geNorm, NormFinder, and BestKeeper) in each subset.

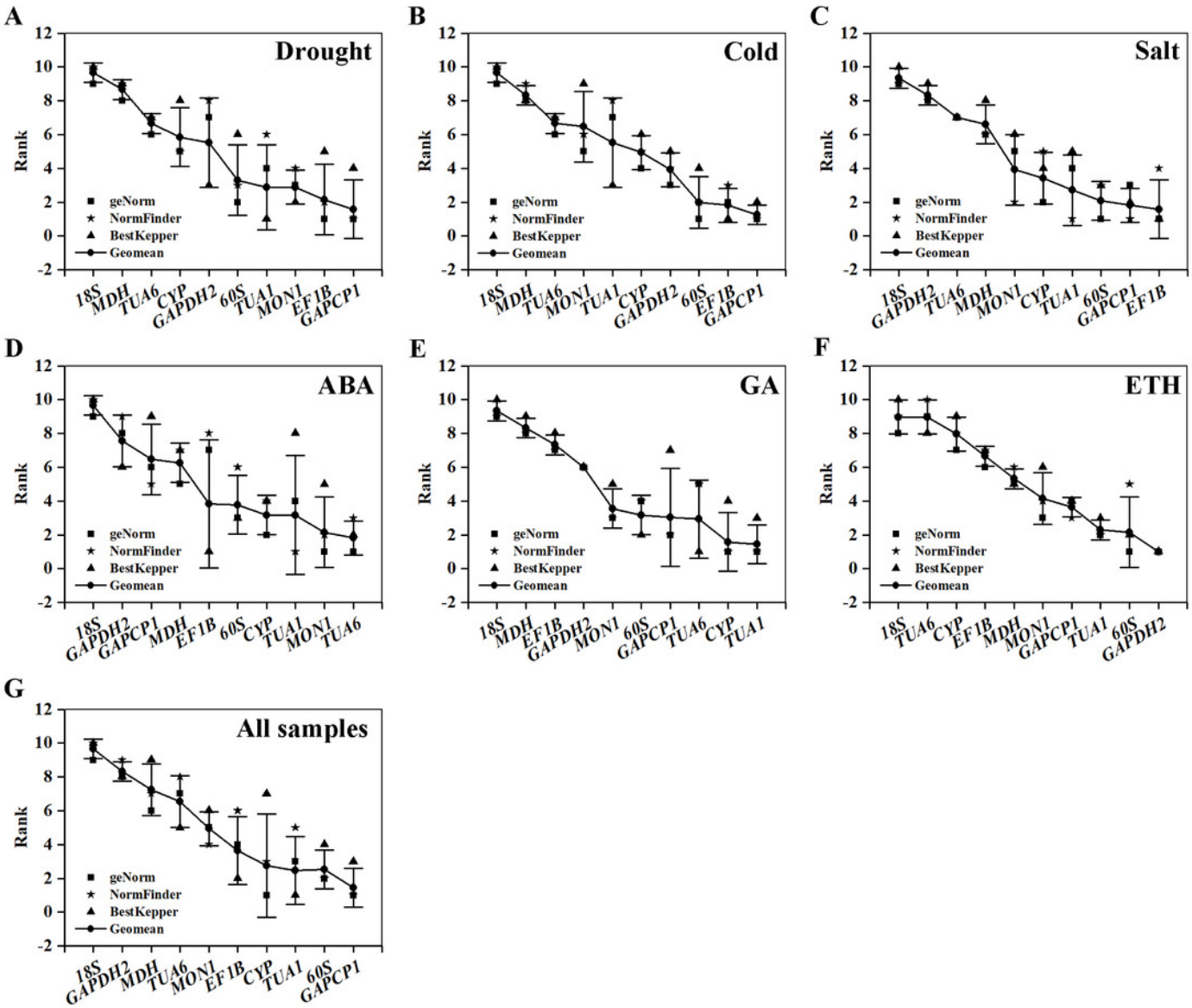


Figure 5

Relative expression levels of *P5CS2* under different experimental conditions normalized by the most stable RGs combination, the most stable gene and the most unstable gene.

(A) Drought stress. (B) Cold stress. (C) Salt stress. (D) ABA treatment. (E) GA treatment. (F) ETH treatment. Bars represent the standard error from three biological replicates.

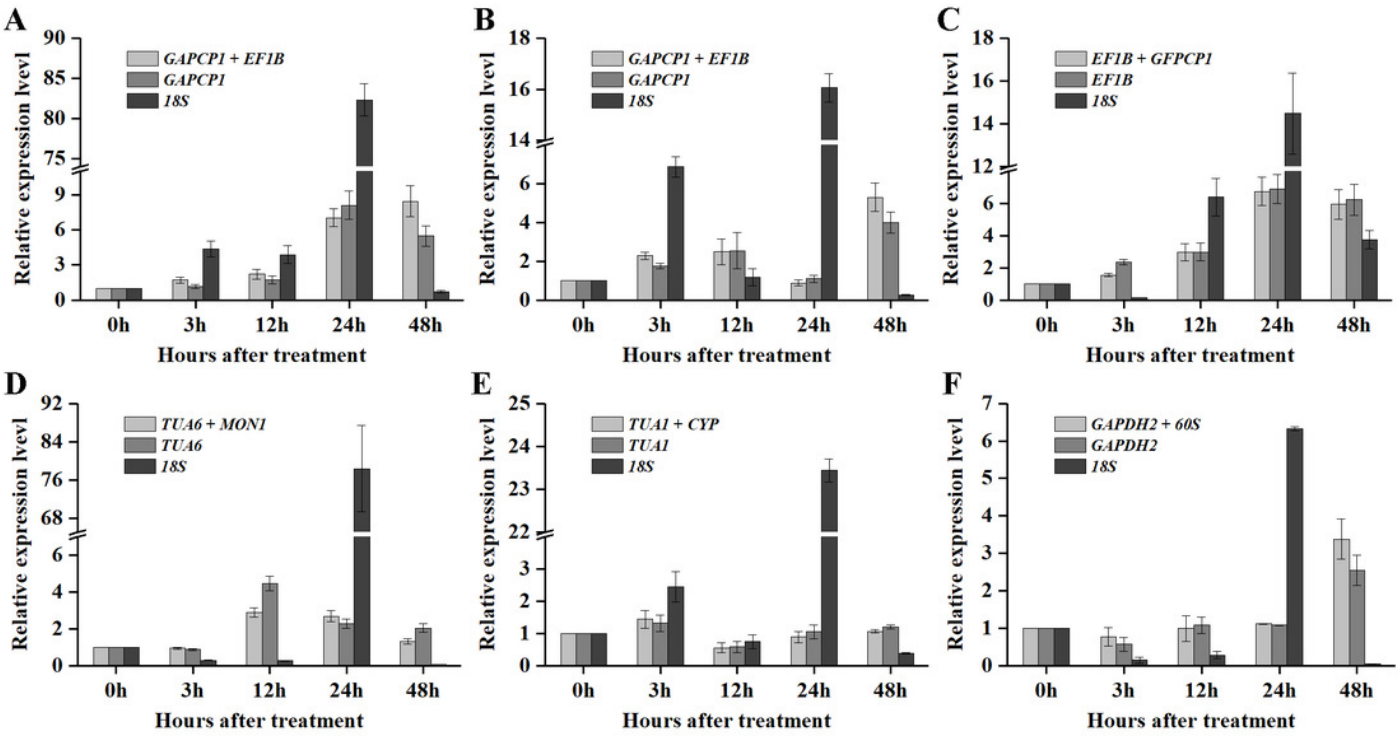


Figure 6

Relative expression levels of *GI* under different experimental conditions normalized by the most stable RGs combination, the most stable gene and the most unstable gene.

(A) Drought stress. (B) Cold stress. (C) Salt stress. (D) ABA treatment. (E) GA treatment. (F) ETH treatment. Bars represent the standard error from three biological replicates.