Identification And Evaluation of Reference Genes For *Cynanchum Auriculatum* Under Various Stress Conditions

Zhi-Peng Zhu
Yancheng Teachers University
Jian-Xiang Yu
Yancheng Teachers University
Ke-Xin Wu
Yancheng Teachers University
Qin-Yi Xu
Yancheng Teachers University
Yi-Jun Kang
Yancheng Teachers University
Miao Sun (nixbio@sci.edu.rs)
Yancheng Teachers University
Min Shen
Yancheng Teachers University

Research Article

Keywords: Cynanchum auriculatum, Reference gene, qRT-PCR, Abiotic stress

Posted Date: January 10th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1196107/v1

Abstract

Baishouwu (*Cynanchum auriculatum*) is a kind of critical Chinese herbal medicine. However, compared with the studies of other Chinese herbal medicines, the screening study on the reference genes of *C. auriculatum* is still the blank. Deterioration of the natural environment severely affects the growth and development of *C. auriculatum*. This study screened and identified suitable reference genes of *C. auriculatum* under various stress conditions. Based on qRT-PCR, geNorm, NormFinder, BestKeeper, and RefFinder were used for the expression stability evaluation of 12 potential reference genes from *C. auriculatum*. The ranking table showed that optimal reference genes included *EF2* and *SAMDC* (heat stress), *CYP* and *TUB-β* (cold stress), *TUB-α* and *GAPDH* (drought stress), *SAMDC* and *TUB-α* (waterlogging stress), along with *EF2* and *ACT7* (salt stress). These results also demonstrated that under different abiotic stresses, suitable reference genes of plants should be selected for qRT-PCR analysis.

1. Introduction

Baishouwu (*Cynanchum auriculatum*) belongs to the family of *Asclepiadaceae*, which is a kind of precious traditional Chinese herbal medicine, mainly distributed in Binhai of Jiangsu Province, China [1,2]. Many bioactive substances, such as intronic polysaccharides, C_{21} steroids, and acetophenone, have been determined in *C. auriculatum* [3–5]. Multiple pharmacological effects have been proved in *C. auriculatum* [6], including hepatoprotective [7,8], enhancing immunity [9], inhibiting cell carcinogenesis [10–12], antitumor [13,14], and hypolipidemic [15]. Up to now, the reference genes of various Chinese herbal plants, including *Panax ginseng* [16], *Poria cocos* [17], and *Coptis Chinensis* [18], have been analyzed and identified by the ICG knowledgebase (http://icg.big.ac.cn), while the reference gene study of *C. auriculatum* is still the blank.

qRT-PCR has been widely used for determining the expression pattern of the target gene at the transcription level [19–21]. Nevertheless, the reliability of qRT-PCR data is affected DNA concentration, primer sequence, and reference gene stability [22]. In plants, common reference genes include Actin (*ACT*), 18S ribosomal RNA (*18S rRNA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), alpha-tubulin/beta-tubulin (*TUB-α/TUB-β*), elongation factor 1/ elongation factor 2 (*EF1/EF2*), cyclophilin (*CYP*), and others [23,24]. Meanwhile, reference gene expression stability differs in different species [25], tissues [26], biological processes [27,28], and environmental conditions [29]. Thus, to ensure the reliability of qRT-PCR data, the identification of stably expressed reference genes according to certain experimental conditions is essential.

Here, we identified 12 potential reference genes from our published *C. auriculatum* transcriptomic database (NCBI accession No. SRX10889362), and gene expression analysis of *C. auriculatum* under various stress conditions was performed with qRT-PCR. Four sorts of software, geNorm [30], NormFinder [31], BestKeeper [32], and RefFinder [33], were applied for gene stability assessment in *C. auriculatum*. The 12 selected genes included 60S ribosomal protein L18-2 (*60s18*), 60S ribosomal protein L13-1 (*60s13*), Tubulin alpha-3 chain (*TUB-α*), Tubulin beta-1 chain (*TUB-β*), Membrane-anchored ubiquitin-fold protein 3 (*MUB3*), Actin-7 (*ACT7*), Elongation factor 1-alpha 1 (*EF1*), Elongation factor 2 (*EF2*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Eukaryotic translation initiation factor 3 subunit I (*EIF*), Peptidyl-prolyl cis-trans isomerase *CYP*95 (*CYP*), and S-adenosylmethionine decarboxylase proenzyme (*SAMDC*). In addition, *CaWRKY1* expression was analyzed for standardized pattern analysis. WRKY is an essential component of the signaling network that regulates plant biological processes [34] and responds to abiotic stress [35]. In summary, we figured out appropriate reference genes for *C. auriculatum* and promoted the study of metabolic mechanisms in *C. auriculatum*.

2. Methods

2.1 Plant material and abiotic stress conditions

The experimental samples were collected from one-month-old Baishouwu (*C. auriculatum*, cv 'Binwu No. 1') seedlings cultured in Yancheng Xinyang Experimental Station, Jiangsu Province, China. The growth condition of 22 °C/20 °C (light/dark) and 16 h/8 h (light/dark) was applied to the seedlings. The seedlings were cultured under various abiotic stress conditions of heat (42 °C treatment), cold (4 °C treatment), salt (200mM NaCl solution treatment), waterlogging (Keep the water surface 10cm above the soil surface), and drought (20% PEG6000 solution treatment) stresses. The leaf samples of *C. auriculatum* were obtained at four-time points (0, 6, 12, and 24 h), and stored at -80 °C for further research. Three or more biological replicates were made at every acquisition point for each sample.

2.2 *C. auriculatum* RNA extraction and cDNA preparation

To extract total RNA from *C. auriculatum*, TaKaRa MiniBEST Universal RNA Extraction Kit (Catalog number: 9767, purchased from TaKaRa Biotechnology (Dalian) Co., Ltd.) was used in this study. The RNA quality and quantity were measured by Thermo Scientific™ NanoDrop Lite (Catalog number: ND-LITE-PR, purchased from China Thermofisher Scientific Co., Ltd.). After that, the cDNA was prepared according to the manual of *EasyScript*® Reverse Transcriptase (Catalog number: AE101-02, purchased from Beijing Transgen Co., Ltd.)

2.3 Reference gene screening and specific primer information

Twelve potential reference genes were screened out from the *C. auriculatum* full-length transcriptomic database (NCBI accession No. SRX10889362) uploaded by our research group, including *60s18*, *60s13*, *TUB-α*, *TUB-β*, *MUB3*, *ACT7*, *EF1*, *EF2*, *GAPDH*, *EIF*, *CYP*, and *SAMDC.*

The nucleotide sequences of specific primers were designed based on the NCBI-Primer-Blast tool and DAMAN 7.0, and the primers were synthesized by Nanjing Genscript Biotechnology Co., Ltd.

2.4 qRT-PCR analysis of potential reference genes from *C. auriculatum*

qRT-PCR amplification was carried out according to the manual of SuperReal PreMix Kit (Catalog number: FP215-01, purchased from Beijing Tiangen Biotech Co., Ltd.). The amplification program of the melting curve was set from 60.0 °C to 95.0 °C, increasing by 1.0 °C per 15 s, to determine the specific qRT-PCR reaction product. Besides, qRT-PCR effects in this study were detected by electrophoresis on 1.5 % agarose gel. At the same time, standard curves were obtained by quantitatively analyzing the amplification efficiency (En) and correlation coefficient (R^2) of selected genes.

2.5 The determination of *C. auriculatum* reference gene stability

Circulation threshold (CT) values were used to evaluate *C. auriculatum* gene stability under various conditions. GeNorm [30], NormFinder [31], and BestKeeper [36] were applied for the stability analysis of selected genes. GeNorm evaluates gene expression stability with M value, and also suggests the quantity of reference gene in combination with Vn/n+1. Compared with geNorm, NormFinder uses a different algorithm to rank the stability of potential genes. Through calculating the standard deviation (SD) and co-variation (CV) of CT value, BestKeeper provides the ranking of gene expression stability. Finally, to obtain the overall ranking from the above results, we used the online software RefFinder (http://blooge.cn/RefFinder/), which integrates the above computing programs based on the raw CT value, to comprehensively analyze the expression stability of selected genes [33].

2.6 The verification of suitable reference genes from *C. auriculatum*

To validate the reliability of the ranking table produced by the four algorithms, the top two stable genes, and their combination, and the last two stable genes recommended by RefFinder were selected from different groups (heat stress, cold stress, drought stress, waterlogging stress, salt stress, and all samples). According to the $2^{-\Delta\Delta CT}$ method [37], their expression levels were used to make relative comparisons with the transcription factor *CaWRKY1*. Three technical repetitions were applied in each sample.

3. Result

3.1 Verification of PCR product specificity and determination of CT value validity

The specificity of PCR products was detected by gel electrophoresis. It could be observed that each PCR product revealed a single band in agarose gel (Fig. S1). Besides, the melting curve of each gene showed a single peak (Fig. S2), which indicated that the primers of reference genes had strong specificity. According to the amplification efficiency (En) (ranged from 91.81% (*EF1*) to 103.04% (*60s18*)) and correlation coefficient (R^2) ($R^2 > 0.99$), the results complied with the standard of qRT-PCR (Table 1).

Under various abiotic stresses, the expression levels of selected reference genes diversified greatly across all samples of *C. auriculatum*. As shown in Fig. 1, the ultimate value of CT was 31.66, and the minimum value was 13.18. The expression abundance of *EF1* was the highest, and the maximum, minimum, and median values of CT were 38.64, 24.04, and 31.88, respectively. The maximum, minimum, and median CT values of *EF1* expression were 21.10, 17.91, and 20.29, respectively. In addition, the reference genes showed significant variable expression. Compared with the other genes, *SAMDC* and *TUB-α* had a relatively narrow CT value range, indicating that they were more stable in term. These results also showed that for *C. auriculatum,* it is essential to select corresponding reference genes under certain conditions.

3.2 GeNorm analysis

GeNorm evaluates gene stability by calculating the M value. M value less than 1.5 usually represents the stable expression of the gene. M values and the ranking list of the 12 selected genes under various stresses were provided by geNorm (Fig. 2), and M values across all samples were lower than 1.5. Under heat stress, the expression of *SAMDC*, *CYP*, and *ACT7* was the most stable (Fig. 2A). Under cold stress, the face of *ACT7*, *MUB3*, and *SAMDC* was the most stable (Fig. 2B). Under drought stress, *TUB-α*, *GAPDH*, and *EF2* were observed as the most stably expressed (Fig. 2C). Under waterlogging stress, *TUB-α*, *SAMDC*, and *60s13* were most stably expressed (Fig. 2D). Under salt stress, the expression of *SAMDC*, *ACT7*, and *TUB-α* was the most stable (Fig. 2E). In conclusion, *ACT7* showed high stability under heat, cold, and salt stresses. Under both drought and waterlogging stresses, the expression of *TUB-α* was relatively more stable. *SAMDC* had the highest stability under heat, cold, waterlogging, and salt stresses. *MUB3* was stably expressed only under cold stress. Besides, in all samples, *ACT7*, *TUB-α*, and *GAPDH* were identified as stably expressed genes (Fig. 2F).

To find out the appropriate number of selected genes, geNorm software introduced paired variation analysis to calculate the Vn/n+1 value [38]. In this study, the Vn/n +1 was less than 0.15 under heat, cold, drought, waterlogging, and salt stress, which indicated that the n of gene combinations should be 2. In all samples, the paired variation was 0.165 for V2/3, and 0.136 for V3/4 (Fig. 3), indicating that adding a third gene would obviously affect the analysis result. Therefore, the best reference gene combination in all samples of *C. auriculatum* was *ACT7*, *TUB-α*, and *GAPDH*.

3.3 NormFinder analysis

To better analyze the stability of the 12 selected genes from *C. auriculatum*, NormFinder software was applied for further study. The data showed that, under heat stress, the most stable genes were *EF2* (0.243), *TUB-β* (0.243), and *SAMDC* (0.359) (Fig. 4A). Under cold stress, the expression of *TUB-β* (0.107), *CYP* (0.355), and *GAPDH* was more stable (0.469) (Fig. 4B). Under drought stress, the expression of *GAPDH* (0.165), *TUB-α* (0.217), and *EF2* (0.290) was more stable (Fig. 4C). The top three genes stably expressed were *SAMDC* (0.084), *TUB-α* (0.139), and *CYP* (0.395) under waterlogging stress, and *EF2* (0.284), *Elf* (0.307), and *60s13* (0.499) under salt stress (Fig. 4D-E). Besides, among all samples, *EF2* (0.048), *GAPDH* (0.048), and *CYP* (0.258) were the most stable (Fig. 4F). In addition, the stability ranking lists provided by NormFinder and geNorm were inconsistent.

3.4 BestKeeper analysis

Based on CV and SD values, BestKeeper ranked the expression stabilities of the 12 selected genes from *C. auriculatum*, and the ranking list was shown in Table 2. Under heat stress, *MUB3* (0.64 ± 0.09), *TUB-α* (1.09 ± 0.30), and *ACT7* (3.06 ± 0.62) expressed more stable than other genes. Under cold stress, the expression *ACT7* (1.89 ± 0.39), *MUB3* (1.99 ± 0.28), and *SAMDC* (2.95 ± 0.61) was more stable. The expression of *MUB3* (2.31 ± 0.33), *ACT7* (3.16 ± 0.63), and *SAMDC* (4.43 ± 0.85) were the most stable under drought stress. Under waterlogging stress, the most stable genes included *MUB3* (0.46 ± 0.07), *ACT7* (0.93 ± 0.19), and *TUB-α* (1.73 ± 0.46). Under salt stress, *MUB3* (0.47 ± 0.07), *TUB-α* (0.99 ± 0.27), and *SAMDC* (2.01 ± 0.44) expressed more stably. Meanwhile, in all samples, *MUB3* (0.72 ± 0.10), *ACT7* (1.57 ± 0.32), and *TUB-α* (1.62 ± 0.44) were most stably expressed.

3.5 RefFinder analysis

Three types of software had been applied in the stability evaluation of the 12 selected genes. However, because of the algorithm-specific preference, the software produced different results. Therefore, the software RefFinder, which integrates the above results, was applied to make an overall ranking of the 12 selected genes among all samples. According to the ranking result produced by RefFinder, the expression of *EF2*, *CYP*, *TUB-α*, *SAMDC*, and *EF2* was more stable under heat, cold, drought, waterlogging, and salt stress, respectively. Meanwhile, *EF2* was listed as the optimal gene among all candidate genes. Besides, except for drought and salt stresses, *60s18* was the most unstable gene (Table 3).

3.6 Stability verification of selected genes associated with *CaWRKY1*

To validate the stabilities of potential genes obtained by comprehensive analysis, the top two and the bottom two genes in the ranking list (Table 3) were picked out to quantify the expression of *CaWRKY1*. It could be obtained that, under heat stress, compared with the top two genes and their combination, the relative expression level of *CaWRKY1* reached a peak (5.23,5.31, and 5.25) at 12h. At the same time, compared with the bottom genes, the relative expression level of *CaWRKY1* increased or decreased differently (Fig. 5A). Under cold, drought, waterlogging, salt stresses, and different stresses (Fig. 5B-F), compared with the top two genes and their combinations, the changing trends of *CaWRKY1* relative expression level were similar. In addition, compared with the bottom genes of the ranking table, the expression-changing trends of *CaWRKY1* were significantly different from the former. Therefore, these results indicated that the data obtained from the RefFinder algorithm was accurate and reliable.

4. Discussion

Comparative analysis of gene expression in different responses to stress is a frequent method to reveal the mechanism of plant response to stress [39,40]. Improper application of reference genes often leads to the misunderstanding expression of target gene [41]. qRT-PCR is a tool commonly used in the modern gene research field [42]. Researchers often use this technique to screen out stable reference genes that are not affected by tissues, development, and biological status [43,44]. A review of previous research results shows that such ideal reference genes rarely exist. The expression of stable genes under one experimental condition may be biased or even fluctuate greatly under other treatments [45,46]. In *Zea mays*, *ZmEIF4A* and *ZmACT2* were the most stable genes in response to cold and heat stresses, whereas *ZmTUB-β* and *ZmGAPDH* were the most stably under drought stress and salt stresses, respectively [47]. As shown in Fig. 1, it found that all potential reference genes could not maintain stable expression levels under different stresses, which further demonstrated that for *C. auriculatum*, it was essential to screen out appropriate reference genes under specific abiotic stresses.

Up to now, the multi-level research on *C. auriculatum* reference genes is still blank. Here, geNorm, NormFinder, and BestKeeper were applied to determine the expressions of 12 potential genes in the leaf of *C. auriculatum* under heat, cold, drought, waterlogging, and salt stresses. Interestingly, results in this study were the same as the results of *Glycine max* [48] and *Brassica juncea* [24], the stability of *ACT7* and *EF1* was not excellent in most sample groups. It is generally considered that the method of simultaneously amplifying multiple genes in a gene family is a strategy to improve the reliability of qRT-PCR data [49,50]. However, in ginseng, the expression of *ACT* was stable, while *ACT11* word was volatile [16]. Similar to this, we also found that the expression level of *60s13* + *60s18* significantly differed from the pair of *EF1* and *EF2* (Table 3).

By calculating the M value, GeNorm evaluated the stability of each gene across all samples [30]. Through variance analysis and quantitative analysis, NormFinder directly evaluates the stability of genes under different treatments [31]. Under various conditions, BestKeeper evaluates the gene expression stability by counting CV values and SD values [32]. According to the analysis of the above programs, RefFinder calculates geometrical gene means to provide the comprehensive ranking table [33]. Related studies have proved that this software all bring specific errors [51]. In *Vaccinium virgatum*, the gene *SAND* ranked 1st for stability as assessed by NormFinder, but 4th and 5th in geNorm and BestKeeper, respectively [52]. Based on geNorm and NormFinder, *EF2* was one of the most stable genes, but the result of BestKeeper was inconsistent (Table 2). This result should be due to it that BestKeeper, the excel-based algorithm, uses the method of pairwise correlation to determine housekeeping genes stability [53,54], while geNorm [30] and Normfinder [31] identify gene stability by analyzing the paired variation between two samples. Therefore, the accuracy of BestKeeper analysis was considered to be improved. To reduce the evaluation error which was caused by differences in algorithms, RefFinder software was applied for comprehensive ranking in this study, and *EF2* and *GAPDH* were listed as appropriate reference genes for further standardized analysis (Table 3).

Besides, through determining the expression profiles of transcription factor *CaWRKY1*, the expression of the most stable genes and unstable genes provided by RefFinder was investigated. Compared with the most stable genes (*EF2*, *SAMDC*, *CYP*, *TUB-β*, *TUB-α*, *GAPDH*, *ACT7*,) and their combinations (*EF2* + *SAMDC*, *CYP* + *TUB-β*, *TUB-α* + *GAPDH*, *SAMDC* + *TUB-α*, *EF2* + *ACT7*, *EF2* + *GAPDH*), *CaWRKY1* had a high expression abundance and similar expression pattern. But when compared with the most unstable genes (*EF1*, *60s18*, *60s13*, *Elf*, *EF2*, *TUB-β*), the expression abundance and expression pattern of *CaWRKY1* showed the opposite trend (Fig. 5). These results also illustrated the importance of choosing appropriate reference genes for analyzing the expression profile changes of target functional genes.

Conclusion

From *C. auriculatum*, 12 potential reference genes were screened out, and under various abiotic stresses, their expression stabilities were calculated and analyzed. Based on the results provided from four algorithms, the expression of *EF2* and *GAPDH* was most stable in all samples. Besides, the most stable genes included *EF2* and *SAMDC* (heat stress), *CYP* and *TUB-β* (cold stress), *TUB-α* and *GAPDH* (drought stress), *SAMDC* and *TUB-α* (waterlogging stress), and *EF2* and *ACT7* (salt stress). In addition, *60s18* showed low expression stabilities under various abiotic stresses, so it should not appear in the stress resistance research of *C. auriculatum*. Besides, the comparison of *CaWRKY1* with the most stable genes and the most unstable genes guaranteed the reliability of the ranking table. In summary, this study is helpful to elucidate the molecular mechanism of *C. auriculatum* in resistance to abiotic stress, which is of great significance for the cultivation and breeding of *C. auriculatum*.

Declarations

Author contributions

Zhi-Peng Zhu: Conceptualization, Experiment, Software, Visualization, Writing-original draft preparation. Jian-Xiang Yu: Experiment, Methodology Writing-original draft preparation. Ke-Xin Wu: Methodology, Experiment. Qin-Yi Xu: Methodology Experiment. Yi-Jun Kang: Conceptualization, Methodology, Resources. Miao Sun: Conceptualization, Methodology, Resources, Writing-reviewing and modifying, Funding acquisition.

Declaration of Competing Interest

The authors declare that they do not have any competing financial or commercial interest that represents a conflict of interest in connection with this paper.

Statement

The authors confirm that the experimental research and field studies on plants, including the collection of plant material, comply with the relevant institutional, national, and international guidelines and legislation.

Acknowledgements

The research was supported by the Open Project of Jiangsu Key Laboratory for Bioresources of Saline Soils (JKLBS2016002).

References

1. Han, E. H., *et al.* Development of molecular markers, based on chloroplast and ribosomal DNA regions, to discriminate three popular medicinal plant species, Cynanchum wilfordii, Cynanchum auriculatum, and Polygonum multiflorum. *Mol. Biol. Rep.* **43**, 323–332. https://doi.org/10.1007/s11033-016-3959-1 (2016).
2. Qi, L. W., *et al.* Structural characterization of pregnane glycosides from Cynanchum auriculatum by liquid chromatography on a hybrid ion trap time-of-flight mass spectrometer. *Rapid Commun. Mass Spectrom.* **23**, 2151–2160. https://doi.org/10.1002/rcm.4125 (2009).
3. Sun, Y., *et al.* An application of high-speed counter-current chromatography for separation and purification of bungeiside-A, bungeiside-B and baishouwubenzophenone from Cynanchum bungei Decne. *Phytochem. Anal.* **22**, 526–531. https://doi.org/10.1002/pca.1313 (2011).
4. Li, J., *et al.* Constituents of the roots of Cynanchum bungei Decne. Isolation and structures of four new glucosides, bungeiside-A, -B, -C, and -D. *Chem. Pharm. Bull. (Tokyo)* **40**, 3133–3137. https://doi.org/10.1248/cpb.40.3133 (1992).
5. Lu, Y., *et al.* Three new steroidal glycosides from the roots of Cynanchum auriculatum. *Molecules* **16**, 1901-1909. https://doi.org/10.3390/molecules16021901 (2011).
6. Han, L., *et al.* Ethnobotany, Phytochemistry and Pharmacological Effects of Plants in Genus Cynanchum Linn. (Asclepiadaceae). *Molecules* **23**, 1194. https://doi.org/10.3390/molecules23051194 (2018).
7. Zhang, W., *et al.* Biotransformation and metabolic profile of caudatin-2,6-dideoxy-3-O-methy-beta-d-cymaropyranoside with human intestinal microflora by liquid chromatography quadrupole time-of-flight mass spectrometry. *Biomed. Chromatogr.* **29**, 1715–1723. https://doi.org/10.1002/bmc.3484 (2015).
8. Shan, L., *et al.* Gastroprotective effect of a traditional Chinese herbal drug "Baishouwu" on experimental gastric lesions in rats. *J. Ethnopharmacol.* **107**, 389–394. https://doi.org/10.1016/j.jep.2006.03.022 (2006).
9. Qin, J. J., *et al.* C21-steroidal glycosides and sesquiterpenes from the roots of Cynanchum bungei and their inhibitory activities against the proliferation of B and T lymphocytes. *Fitoterapia* **124**, 193–199. https://doi.org/10.1016/j.fitote.2017.11.014 (2018).
10. Ding, Y. F., *et al.* Baishouwu Extract Suppresses the Development of Hepatocellular Carcinoma via TLR4/MyD88/NF-kappaB Pathway. *Front Pharmacol* **10**, 389. https://doi.org/10.3389/fphar.2019.00389 (2019).
11. Wang, D. Y., *et al.* [Apoptosis induced by the C21 sterols in Baishouwu and its mechanism of action in hepatoma]. *Yao Xue Xue Bao* **42**, 366–370. https://pubmed.ncbi.nlm.nih.gov/17633201 (2007).
12. Wang, Y. Q., *et al.* A C 21 -Steroidal Glycoside Isolated from the Roots of Cynanchum auriculatum Induces Cell Cycle Arrest and Apoptosis in Human Gastric Cancer SGC-7901 Cells. *Evid. Based Complement. Alternat. Med.* 2013, 180839. https://doi.org/10.1155/2013/180839 (2013).
13. Peng, Y. R., *et al.* Antitumor activity of C-21 steroidal glycosides from Cynanchum auriculatum Royle ex Wight. *Phytomedicine* **15**, 1016–1020. https://doi.org/10.1016/j.phymed.2008.02.021 (2008).

14. Wang, X. J., *et al.* Antitumor evaluation and multiple analysis on different extracted fractions of the root of Cynanchum auriculatum Royle ex Wight. *J. Sep. Sci.* **40**, 3054–3063. https://doi.org/10.1002/jssc.201601415 (2017).
15. Jiang, H. W., *et al.* Potential hypoglycemic effect of acetophenones from the root bark of Cynanchum wilfordii. *Nat. Prod. Res.* **33**, 2314–2321. https://doi.org/10.1080/14786419.2018.1443100 (2019).
16. Wang, M., *et al.* Validation of Suitable Reference Genes for Quantitative Gene Expression Analysis in Panax ginseng. *Front Plant Sci.* **6**, 1259. https://doi.org/10.3389/fpls.2015.01259 (2015).
17. Zhang, X., *et al.* Selection and validation of reference genes for normalization of quantitative real-time reverse transcription PCR analysis in Poria cocos (Schw.) Wolf (Fuling). *Chin. Med.* **11**, 8. https://doi.org/10.1186/s13020-016-0079-8 (2016).
18. Chen, D. X., *et al.* The chromosome-level reference genome of Coptis chinensis provides insights into genomic evolution and berberine biosynthesis. *Hortic. Res.* **8**, 121. https://doi.org/10.1038/s41438-021-00559-2 (2021).
19. Bustin, S. A. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* **29**, 23–39. https://doi.org/10.1677/jme.0.0290023 (2002).
20. Derveaux, S., *et al.* How to do successful gene expression analysis using real-time PCR. *Methods* **50**, 227–230. https://doi.org/10.1016/j.ymeth.2009.11.001 (2010).
21. Davoren, P. A., *et al.* Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer. *BMC Mol. Biol.* **9**, 76. https://doi.org/10.1186/1471-2199-9-76 (2008).
22. Robledo, D., *et al.* Analysis of qPCR reference gene stability determination methods and a practical approach for efficiency calculation on a turbot (Scophthalmus maximus) gonad dataset. *BMC Genomics* **15**, 648. https://doi.org/10.1186/1471-2164-15-648 (2014).
23. Li, X., *et al.* Selection of optimal reference genes for qRT-PCR analysis of shoot development and graviresponse in prostrate and erect chrysanthemums. *PLoS ONE* **14**, e0225241. https://doi.org/10.1371/journal.pone.0225241 (2019).
24. Dixit, S., *et al.* Evaluation of suitable reference genes in Brassica juncea and its wild relative Camelina sativa for qRT-PCR analysis under various stress conditions. *PLoS ONE* **14**, e0222530. https://doi.org/10.1371/journal.pone.0222530 (2019).
25. Sun, Y., *et al.* Pseudogenes as weaknesses of ACTB (Actb) and GAPDH (Gapdh) used as reference genes in reverse transcription and polymerase chain reactions. *PLoS ONE* **7**, e41659. https://doi.org/10.1371/journal.pone.0041659 (2012).
26. Song, H., *et al.* Selection and validation of reference genes for measuring gene expression in Toona ciliata under different experimental conditions by quantitative real-time PCR analysis. *BMC Plant Biol.* **20**, 450. https://doi.org/10.1186/s12870-020-02670-3 (2020).
27. Garrido, J., *et al.* Identification and validation of reference genes for RT-qPCR normalization in wheat meiosis. *Sci. Rep.* **10**, 2726. https://doi.org/10.1038/s41598-020-59580-5 (2020).
28. Paolacci, A. R., *et al.* Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. *BMC Mol. Biol.* **10**, 11. https://doi.org/10.1186/1471-2199-10-11 (2009).
29. Qian, J., *et al.* Selection and Evaluation of Appropriate Reference Genes for RT-qPCR Normalization of Volvariella volvacea Gene Expression under Different Conditions. *Biomed. Res. Int.* 2018, 6125706. https://doi.org/10.1155/2018/6125706 (2018).
30. Vandesompele, J., *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034. https://doi.org/10.1186/gb-2002-3-7-research0034 (2002).
31. Andersen, C. L., *et al.* Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496 (2004).
32. Czechowski, T., *et al.* Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiol.* **139**, 5–17. https://doi.org/10.1104/pp.105.063743 (2005).
33. Xie, F., *et al.* miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol. Biol.* **80**, 75–84. https://doi.org/10.1007/s11103-012-9885-2 (2012).
34. Rushton, P. J., *et al.* WRKY transcription factors. *Trends Plant Sci.* **15**, 247–258. https://doi.org/10.1016/j.tplants.2010.02.006 (2010).
35. Jiang, J., *et al.* WRKY transcription factors in plant responses to stresses. *J. Integr. Plant Biol.* **59**, 86–101. https://doi.org/10.1111/jipb.12513 (2017).
36. Pfaffl, M. W., *et al.* Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **26**, 509–515. https://doi.org/10.1023/b:bile.0000019559.84305.47 (2004).
37. Schmittgen, T. D., *et al.* Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* **3**, 1101–1108. https://doi.org/10.1038/nprot.2008.73 (2008).
38. Chapman, J. R., *et al.* With Reference to Reference Genes: A Systematic Review of Endogenous Controls in Gene Expression Studies. *PLoS ONE* **10**, e0141853. https://doi.org/10.1371/journal.pone.0141853 (2015).
39. Boava, L. P., *et al.* Selection of endogenous genes for gene expression studies in Eucalyptus under biotic (Puccinia psidii) and abiotic (acibenzolar-S-methyl) stresses using RT-qPCR. *BMC Res. Notes* **3**, 43. https://doi.org/10.1186/1756-0500-3-43 (2010).
40. Fei, X., *et al.* Expression Stabilities of Ten Candidate Reference Genes for RT-qPCR in Zanthoxylum bungeanum Maxim. *Molecules* **23**, 23(24):802. https://doi.org/10.3390/molecules23040802 (2018).
41. Li, D., *et al.* Identification and Evaluation of Reference Genes for Accurate Transcription Normalization in Safflower under Different Experimental Conditions. *PLoS ONE* **10**, e0140218. https://doi.org/10.1371/journal.pone.0140218 (2015).

42. Wong, M. L., *et al.* Real-time PCR for mRNA quantitation. *Biotechniques* **39**, 75–85. https://doi.org/10.2144/05391RV01 (2005).
43. Bustin, S. A., *et al.* Quantitative real-time RT-PCR–a perspective. *J. Mol. Endocrinol.* **34**, 597–601. https://doi.org/10.1677/jme.1.01755 (2005).
44. Zheng, T., *et al.* Reference gene selection for qRT-PCR analysis of flower development in Lagerstroemia indica and L. speciosa. *PLoS ONE* **13**, e0195004. https://doi.org/10.1371/journal.pone.0195004 (2018).
45. Brunner, A. M., *et al.* Validating internal controls for quantitative plant gene expression studies. *BMC Plant Biol.* **4**, 14. https://doi.org/10.1186/1471-2229-4-14 (2004).
46. Liu, D., *et al.* Identification of reference genes for transcript normalization in various tissue types and seedlings subjected to different abiotic stresses of woodland strawberry Fragaria vesca. *Sci. Hortic.* **261**, 108840. https://doi.org/10.1016/j.scienta.2019.108840 (2020).
47. Lin, Y., *et al.* Validation of potential reference genes for qPCR in maize across abiotic stresses, hormone treatments, and tissue types. *PLoS ONE* **9**, e95445. https://doi.org/10.1371/journal.pone.0095445 (2014).
48. Hu, R., *et al.* Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. *BMC Mol. Biol.* **10**, 93. https://doi.org/10.1186/1471-2199-10-93 (2009).
49. Reid, K. E., *et al.* An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biol.* **6**, 27. https://doi.org/10.1186/1471-2229-6-27 (2006).
50. Charrier, B., *et al.* Expression profiling of the whole Arabidopsis shaggy-like kinase multigene family by real-time reverse transcriptase-polymerase chain reaction. *Plant Physiol.* **130**, 577–590. https://doi.org/10.1104/pp.009175 (2002).
51. Hibbeler, S., *et al.* Housekeeping genes for quantitative expression studies in the three-spined stickleback Gasterosteus aculeatus. *BMC Mol. Biol.* **9**, 18. https://doi.org/10.1186/1471-2199-9-18 (2008).
52. Deng, Y., *et al.* Selection of reference genes for RT-qPCR normalization in blueberry (Vaccinium corymbosum x angustifolium) under various abiotic stresses. *FEBS Open Bio.* **10**, 1418–1435. https://doi.org/10.1002/2211-5463.12903 (2020).
53. Szabo, A., *et al.* Statistical modeling for selecting housekeeper genes. *Genome Biol.* **5**, R59. https://doi.org/10.1186/gb-2004-5-8-r59 (2004).
54. Haller, F., *et al.* Equivalence test in quantitative reverse transcription polymerase chain reaction: confirmation of reference genes suitable for normalization. *Anal. Biochem.* **335**, 1–9. https://doi.org/10.1016/j.ab.2004.08.024 (2004).

Tables

Table 1 The information of the 12 *C. auriculatum* reference genes, the nucleotide sequences of specific primers, and the characteristics of PCR products.

Gene symbol	Gene Name	Primer:Forward/reverse	Amplification product size (bp)	standard curve	En	R^2
60s18	60S ribosomal protein L18-2	F:AAACCCTCCGTCCAGAAATCC R:TTCTTGTTCTTGCCTCCGGCC	118	y=-3.3036x+27.367	0.9799	0.9927
60s13	60S ribosomal protein L13-1	F:AGCAAGGCAAAAGAAGGCTG R:AGGATTTGTTCCTGCGACGA	209	y=-3.3446x+28.958	0.9388	0.9968
TUB-α	Tubulin alpha-3 chain	F:ACGTTCCAAGGGCAGTGTTT R:ACGTTCCAAGGGCAGTGTTT	153	y=-3.4847x+29.546	0.9325	0.9993
TUB-β	Tubulin beta-1 chain	F:AGATTTGTTCCCCGTGCTGT R:TCCAGATTGGCCGAAAACGA	117	y=-3.3824x+29.982	0.9625	0.9978
MUB3	Membrane-anchored ubiquitin-fold protein 3	F:GGCTGTGCAAGAGTTGGTTG R:GCAACTTCAGCAACTGGCAA	240	y=-3.2670x+28.105	0.9434	0.9937
ACT7	Actin-7	F:AATGAGAGGTTCCGTTGCCC R:GTTGAACCACCACTGAGCAC	158	y=-3.4122x+29.452	0.9432	0.9949
EF1	Elongation factor 1-alpha 1	F:GGACCCTCTGGGCTTACAAC R:AGTGTGGCAGTCGAGAACAG	255	y=-3.5351x+28.868	0.9267	0.9943
EF2	Elongation factor 2	F:ATTGCTGGTGCTGGTGAACT R:TCACGGAAAGACACGACTGG	110	y=-3.2512x+30.207	0.9318	0.9981
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F:GATAAGGCCGCTGCTCACTT R:AGACCCTCAACAATGCCGAA	206	y=-3.5259x+29.717	0.9706	0.9928
Elf	Eukaryotic translation initiation factor 3 subunit I	F:TCATGGGATTGCCTTCAGCC R:CCCTGCGGACCTTTAAGCAT	97	y=-3.3856x+30.503	0.9519	0.9947
CYP	Peptidyl-prolyl cis-trans isomerase *CYP*95	F:AGAGCATGAGCATTAGCCCC R:ACTCCTGCTCCGCTCTGATA	125	y=-3.3306x+29.878	0.9522	0.9932
SAMDC	S-adenosylmethionine decarboxylase proenzyme	F:GCTTCATTTTCCCTGGTGCTC R:CCTTTCTCCCTGTCCAAACCA	232	y=-3.3452x+27.608	0.9411	0.9966

Table 2 Stability ranking of the 12 *C. auriculatum* reference genes produced from BestKeeper.

Ranking	42°C treatment		4°C treatment		PEG treatment		Flooding treatment		NaCl treatment	
	gene	CV±SD	gene	CV±SD	gene	CV±SD	gene	CV±SD	gene	CV±SD
1	*MUB3*	0.64±0.09	*ACT7*	1.89±0.39	*MUB3*	2.31±0.33	*MUB3*	0.46±0.07	*MUB3*	0.47±0.0
2	*TUB-α*	1.09±0.30	*MUB3*	1.99±0.28	*ACT7*	3.16±0.63	*ACT7*	0.93±0.19	*TUB-α*	0.99±0.2
3	*ACT7*	3.06±0.62	*SAMDC*	2.95±0.61	*SAMDC*	4.43±0.85	*TUB-α*	1.73±0.46	*SAMDC*	2.01±0.4
4	*CYP*	3.40±0.86	*TUB-α*	2.97±0.80	*TUB-α*	4.87±1.25	*SAMDC*	2.20±0.48	*CYP*	2.03±0.5
5	*SAMDC*	3.58±0.73	*CYP*	4.72±1.21	*EF2*	5.80±1.26	*60s13*	2.66±0.77	*ACT7*	3.28±0.6
6	*TUB-β*	5.47±1.50	*GAPDH*	5.45±1.31	*CYP*	6.04±1.45	*EF1*	2.99±1.02	*Elf*	3.58±1.1
7	*EF2*	5.93±1.40	*EF1*	6.90±2.41	*GAPDH*	6.62±1.50	*CYP*	3.31±0.90	*60s13*	6.06±1.8
8	*GAPDH*	7.00±1.72	*TUB-β*	7.11±2.01	*EF1*	5.54±1.90	*Elf*	3.56±1.10	*EF2*	6.58±1.5
9	*60s13*	7.11±2.16	*Elf*	8.79±2.61	*TUB-β*	8.08±2.31	*GAPDH*	4.29±1.03	*60s18*	8.95±2.9
10	*EF1*	7.49±2.43	*60s13*	9.56±2.95	*Elf*	8.57±2.34	*TUB-β*	4.42±1.22	*GAPDH*	9.00±2.1
11	*Elf*	8.22±2.43	*EF2*	9.61±2.35	*60s18*	7.81±2.47	*60s18*	4.61±1.57	*EF1*	9.00±3.0
12	*60s18*	10.56±3.35	*60s18*	11.54±3.63	*60s13*	8.42±2.50	*EF2*	6.29±1.39	*TUB-β*	9.93±2.8

Table 3 Stability ranking of the 12 *C. auriculatum* reference genes produced from RefFinder.

Ranking	42°Ctreatment	4°C treatment	PEG treatment	Flooding treatment	NaCl treatment	All samples
1	*EF2*	*CYP*	*TUB-α*	*SAMDC*	*EF2*	*EF2*
2	*SAMDC*	*TUB-β*	*GAPDH*	*TUB-α*	*ACT7*	*GAPDH*
3	*CYP*	*MUB3*	*EF2*	*60s13*	*SAMDC*	*ACT7*
4	*TUB-β*	*ACT7*	*CYP*	*CYP*	*Elf*	*TUB-α*
5	*ACT7*	*GAPDH*	*ACT7*	*MUB3*	*CYP*	*CYP*
6	*MUB3*	*TUB-α*	*MUB3*	*GAPDH*	*TUB-α*	*MUB3*
7	*TUB-α*	*SAMDC*	*TUB-β*	*ACT7*	*MUB3*	*SAMDC*
8	*GAPDH*	*EF2*	*60s13*	*Elf*	*60s13*	*Elf*
9	*60s13*	*Elf*	*SAMDC*	*TUB-β*	*GAPDH*	*EF1*
10	*Elf*	*EF1*	*EF1*	*EF1*	*60s18*	*TUB-β*
11	*EF1*	*60s13*	*60s18*	*EF2*	*TUB-β*	*60s13*
12	*60s18*	*60s18*	*Elf*	*60s18*	*EF1*	*60s18*

Figures

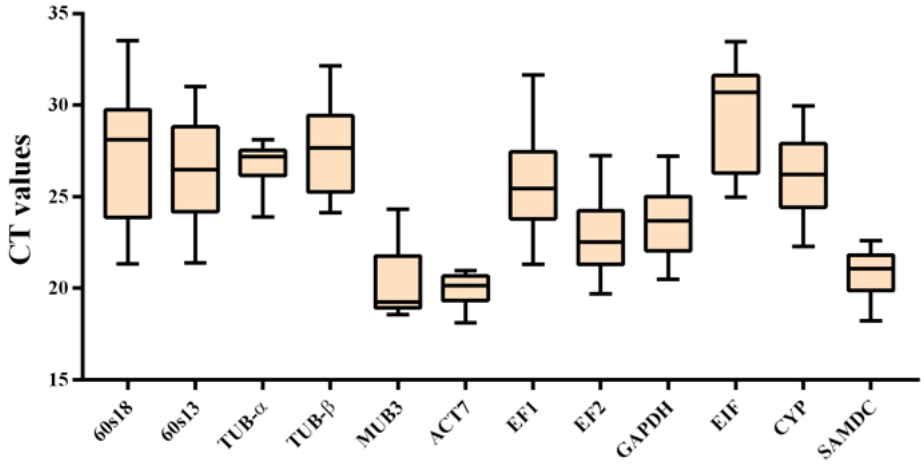

Figure 1

CT value distribution for 12 potential *C. auriculatum* reference genes. The middle line of the box represents the median of CT value. The top boundary of the box corresponds to the maximum value (75th percentiles), and the bottom boundary of the box represents the minimum value (25th percentiles).

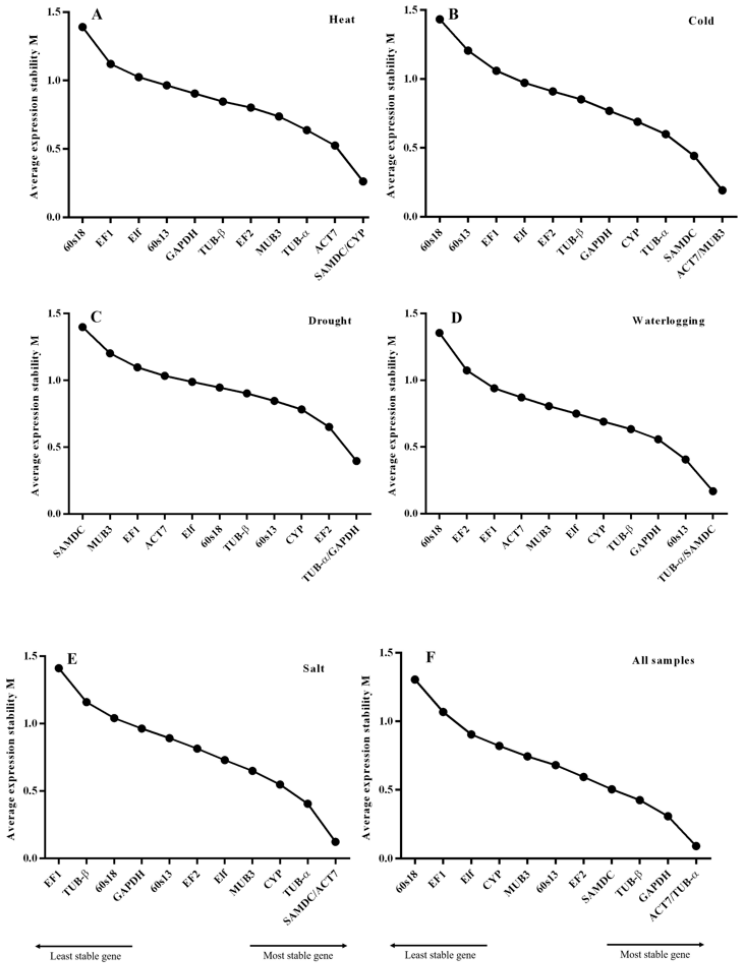

Figure 2

Based on geNorm, the average stability values (M) of the 12 potential genes from *C. auriculatum* under heat stress (A), cold stress (B), drought stress (C), waterlogging stress (D), salt stress (E), and across all samples (F).

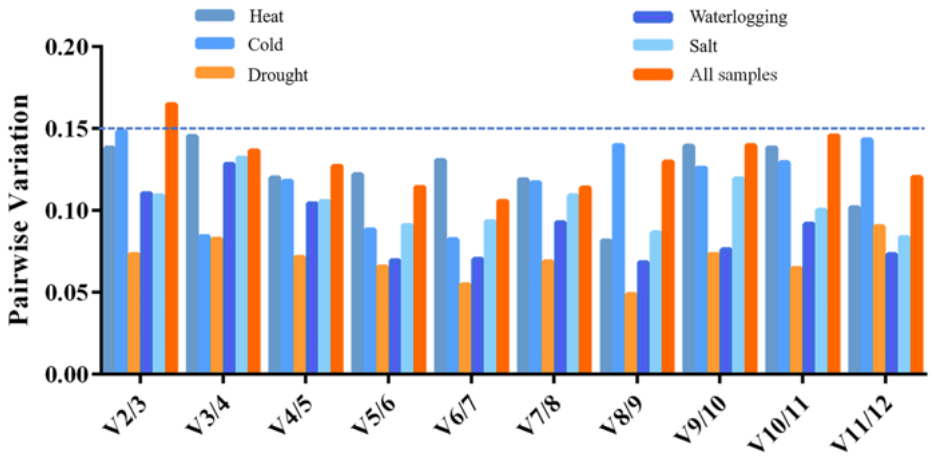

Figure 3

Based on geNorm, pairwise variations of the 12 potential genes from *C. auriculatum* under various stresses. The threshold was set as 0.15, and the value greater than or equal to 0.15 indicated that another reference gene should be added to satisfy the demand of gene expression standardization.

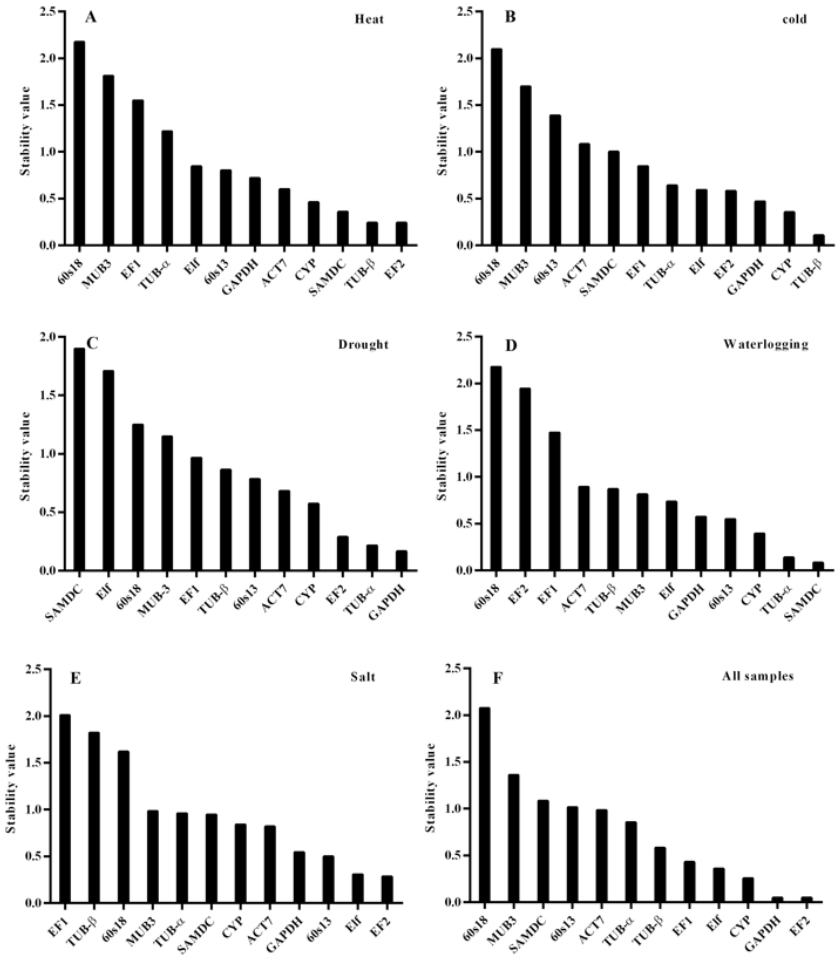

Figure 4

Based on NormFinder analysis, stability value of the 12 potential genes from *C. auriculatum* under heat stress (A), cold stress (B), drought stress (C), waterlogging stress (D), salt stress (E), and across all samples (F).

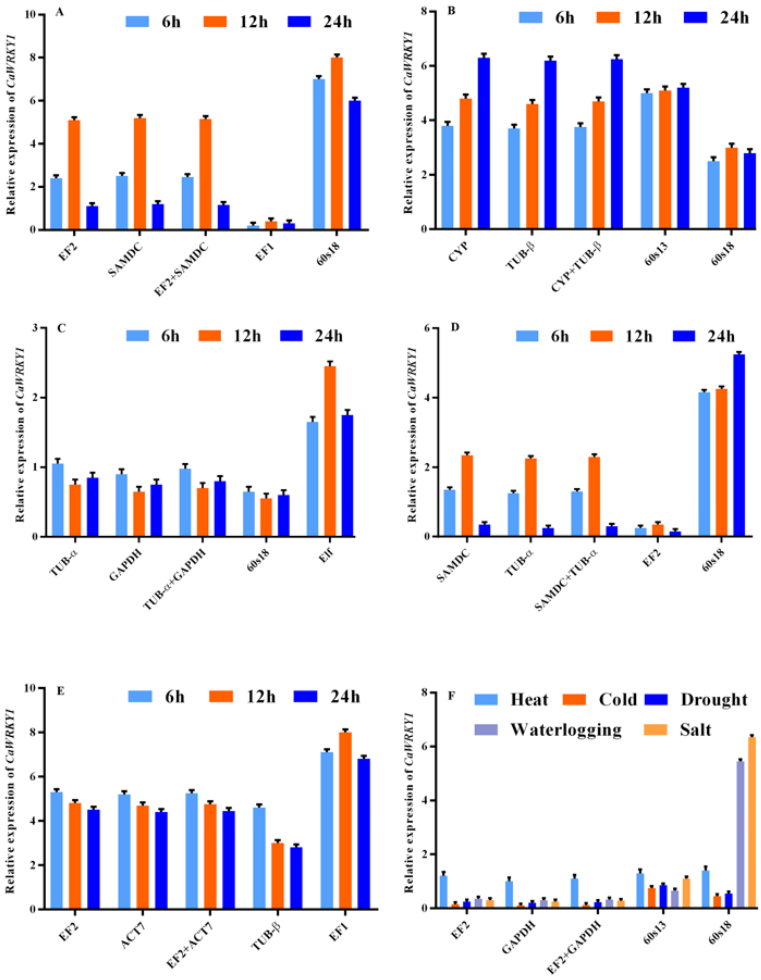

Figure 5

Expression profiles of *CaWRKY1* compared with the most stable (*EF2*, *SAMDC*, *CYP*, *TUB-β*, *TUB-α*, *GAPDH*, *ACT7*, *EF2 + SAMDC*, *CYP + TUB-β*, *TUB-α + GAPDH*, *SAMDC + TUB-α*, *EF2 + ACT7*, *EF2 + GAPDH*) and unstable genes (*EF1*, *60s18*, *60s13*, *Elf*, *EF2*, *TUB-β*) from *C. auriculatum* under heat stress (A), cold stress (B), drought stress (C), waterlogging stress (D), salt stress (E), and across all samples (F).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure.docx