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Selection of suitable reference genes for real-time qPCR gene expression in cauliflower under abiotic stress and methyl jasmonate treatment

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Abstract

Cauliflower is one of the most important popular vegetables in China. Cauliflower (*Brassica oleracea* L. var. *botrytis*) produces glucosinolates, which are secondary metabolites that have anti-cancer properties. The choice of suitable reference genes (RGs) for gene expression studies has a significant effect on experimental outcomes. In this study, we selected 15 candidates as RGs and analyzed them under heat, cold, drought, and salt stresses and methyl jasmonate (MeJA) treatment. The precision of the real-time quantitative polymerase chain reaction data was assessed using four methods (*geNorm*, *NormFinder*, *BestKeeper*, and *ΔCt*). The results revealed that *EF1-β* and *ACT7*; *ACT1*, *EF1-β*, and *TUB6*; *TUA2* and *EF1-β*; *HIS* and *ACT7*; and *ACT7* and *ACT3* were the most stable combinations of genes for heat stress, cold stress, drought stress, salt stress, and MeJA treatment, respectively. The expression stability of the RGs fluctuated under different experimental conditions. Choosing the optimal RG for the specific experiment is therefore essential. This work provides relevant information for further gene expression studies on cauliflower and other closely related species.

Keywords: *Brassica oleracea* var. *botrytis*, cold, drought, gene expression, heat, methyl jasmonate, salinity.

Introduction

Cauliflower (*Brassica oleracea* L. var. *botrytis)*, an annual herbaceous crop belonging to the cruciferous vegetables, is an important and widely-grown vegetable worldwide (Giuffrida *et al*. 2018). It is an excellent source of phenolics, ascorbic acid, vitamins B1, B2, and B3, folic acid, tocopherols, and dietary fibre (Mashabela *et al*. 2018, Nerdy 2018, Sun *et al*. 2018, Thorwarth *et al*. 2018). Medical research has revealed that a diet rich in cauliflower can lower the risk of cancer (Bergès *et al*. 2018, Kalisz *et al*. 2018). Cauliflower contains glucosinolates, a class of secondary plant metabolites; their hydrolyzed products have anti-carcinogenic properties (Oda *et al*. 2019). The findings of multiple studies have indicated that environmental stresses can increase the accumulation of glucosinolates (Jousef *et al*. 2018, Oda *et al*. 2019), but the glucosinolate content in most cauliflower plants is very

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Abbreviations: ABA - abscisic acid; ACT - actin; ACT1 - actin-1-like; ACT2 - actin-2-like; ACT3 - actin-like protein; ACT7 - actin 7; Ct - the cycle threshold values; CV - coefficients of variance; EF1-α - elongation factor 1-alpha; EF1-β (elongation factor 1-beta); eIF4A-1 - eukaryotic initiation factor 4A-1; FBA5 - fructose- bisphosphate aldolase 5; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; HIS -histone H1-like; HKGs - highly conserved housekeeping genes; KIN - protein kinase; MeJa - methyl jasmonate; PEG - polyethylene glycol; qPCR - real-time quantitative polymerase chain reaction; R^2 - correlation coefficient; RGs - reference genes; SAMDC - S-adenosyl-L-methionine decarboxylase; SD - standard deviation; TUB - tubulin-β; TUA - tubulin-α; UBQ -polyubiquitin; Vn/n+1 - the average pairwise variation.
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low. Exploring the biosynthesis pathway of glucosinolates in cauliflower under different stress conditions such as heat, cold, and drought may provide further insights into increasing glucosinolate content. Analyzing the expression of key functional genes may help us to understand the metabolic pathways and regulatory mechanisms of cauliflower under various stresses.

The real-time quantitative polymerase chain reaction (qPCR) method is an effective experimental method for analyzing the expression of functional genes under abiotic stresses in cauliflower on account of its high sensitivity, specificity, good repeatability, and high throughput to target genes (Zhang *et al.* 2017, Nguyen *et al.* 2018, Sun *et al.* 2019, Wang *et al.* 2019). To obtain accurate gene expression data, it is important to limit the deviation of expression due to factors such as primer design, cDNA transcription, and PCR amplification (Huggett *et al.* 2005, Nolan *et al.* 2006, Expósito-Rodríguez *et al.* 2008, Liu *et al.* 2018). Using internal control reference genes (RGs) for the normalization of target gene expression is a method commonly used to correct deviations (Bustin *et al.* 2010). The ideal RG is expressed consistently among various cells, tissues, organs, developmental stages, and environmental stresses.

For the most part, highly conserved housekeeping genes (HKGs) are stably expressed to maintain basic cellular activities under various conditions and stresses in plants (Li *et al.* 2016b). Therefore, HKGs, or the genes that are stably expressed in cells, for instance, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *UBQ* (polyubiquitin), *TUB* (tubulin-β), *TUA* (tubulin-α), *EF1-α* (elongation factor 1-alpha), *EF1-β* (elongation factor 1-beta), *eIF4A-1* (eukaryotic initiation factor 4A-1), and *ACT* (actin), are widely used for gene expression analysis under different abiotic and biotic stresses in different plant species (Xu *et al.* 2014, Wang *et al.* 2016, Duan *et al.* 2017, Li *et al.* 2020a, Yu *et al.* 2020). The same HKGs cannot be used in all studies. Among monocotyledons, for example, GAPDH has the most stable expression for hormone ethylene treatment in *Stellera chamaejasme* (Liu *et al.* 2018b) but is not appropriate for studies on *Brachypodium distachyon* (Hong *et al.* 2008). In dicotyledons, *UBQ10* is the most suitable gene in *Populus ussuriensis* under drought, cold, and salt stress, and abscisic acid (ABA) treatment (Wei *et al.* 2020). However, *UBQ10* has the lowest stability in *Glehnia littoralis* treated with salt stress, drought stress, ABA, and MeJA (Li *et al.* 2020a). In herbaceous plants, *TUB* is not a suitable gene for studies on *Moringa oleifera* under drought stress (Deng *et al.* 2016), but in *Brassica rapa* ssp. *pekinensis*, *TUB* is the most stable gene during flower bud development (Xu *et al.* 2014). In studies on cruciferous vegetables, *TUA* exhibits the most stable expression in different tissues of radish hybrids (Duan *et al.* 2017), while in *Isatis indigotica*, *TUA* is the most unstable gene under nitrogen treatment (Qu *et al.* 2019). In a study of *Brassica*, during the development of the stigma for non-heading Chinese cabbage SI plants in non-heading Chinese cabbage, *ACT* gene expression is the most stable (Wang *et al.* 2016), whereas, during the various stages of stem enlargement in mustard stems, *ACT* is thought to be the least reliable RG (Li *et al.* 2020b). Since no RGs are stably expressed among different plants, there is a need to screen for suitable internal RGs in different plant species.

There has been some preliminary progress in the selection of RGs in cauliflower (Sheng *et al.* 2016, Randhawa *et al.* 2008). However, many studies have noted that the expressions of RGs are not stable under different experimental conditions (Huang *et al.* 2014, Arya *et al.* 2017, De Andrade *et al.* 2017, Zhang *et al.* 2017). For example, *TUA6* shows the most stable expression in ABA experiments on *Stellera chamaejasme*, but *TUA6* is the most unstable gene under ethylene treatment (Liu *et al.* 2018b). *GAPDH* is the most suitable RG during the pistil development of radish, while ubiqutin-conjugating enzyme (*UBC*) expression is more stable under various biotic and abiotic stresses (Duan *et al.* 2017). Therefore, under specific conditions, it is important to use suitable internal RGs to guarantee accurate results in gene expression analyses. To obtain more accurate results, two or more internal RGs are often used to calibrate target genes (Nguyen *et al.* 2018, Wang *et al.* 2019). In this study, it was necessary to validate the best combination of internal parameters for the accuracy of the real-time qPCR findings. Our findings will promote studies on the involvement of cauliflower genes in glucosinolate biosynthesis and metabolic pathways under abiotic stress and hormone treatments.

In this work, 15 candidate RGs, including *ACT1* (actin-1-like), *ACT2* (actin-2-like), *ACT3* (actin-like protein), *ACT7* (actin 7), *TUA2* (alpha-tubulin 2), *TUB6* (tubulin beta-6), *EF1-α*, *EF1-β*, *GAPDH*, *HIS* (histone H1-like), *KIN* (protein kinase), *eIF4A-1*, *FBA5* (fructose-bisphosphate aldolase 5), *SAMDC* (S-adenosyl-L-methionine decarboxylase), and *UBQ* were selected based on the cauliflower transcriptome database to detect suitable internal control genes for normalizing gene expression under a set of different experimental factors, such as heat, cold, drought, and salt stresses and MeJA treatment. The stability of the candidate RGs was evaluated by means of four algorithms: *GeNorm* (Vandesompele *et al.* 2002), *NormFinder* (Andersen *et al.* 2004), *BestKeeper* (Pfaffl *et al.* 2004), and the *ΔCt* method (Schmittgen and Livak 2008). To further verify the stability of the selected genes, the expression of the target gene, *WRKY3*, was measured. Finally, the most accurate RGs for each factor can be useful for further research in gene expression analysis in cauliflower and other closely-related species.

Materials and methods

Plants and experiments: This experimental work used the seeds of *Brassica oleracea* L. var. *botrytis* cv. Qingnong 65. Like many vegetables, cauliflower prefers a mild climate. Its optimum growth temperature is 8 - 24 °C, and the suitable temperature for flower ball growth is 15 - 18 °C. It grows slower when the temperature is below 8 °C, and when the temperature is over 25 °C, the flower ball is small and the quality is poor (Wang 2012). The seeds were washed three times with distilled water and

placed in a flask containing (1/3 the volume) of water. The flask was then shaken in a shaker at 150 rpm at a constant temperature of 25 °C. The water was changed every day. When the seeds germinated, they were sown to soil in an incubator under a 12-h photoperiod, day/night temperatures of 25/20 °C, an irradiance of 300 μmol(photons) m^{-2} s^{-1}, and relative humidity of 60 %. When the seedlings were six weeks old, we conducted different treatments.

Five experiments were set up: *1*) for the heat stress, plants were transferred to an incubator set at 38 °C; *2*) for the cold stress, plants were kept in an incubator at 4 °C (Zeng *et al*. 2021); *3*) for the drought stress, plants were watered with 20 % polyethylene glycol (PEG) 6000 solution; *4*) for the salt stress, 200 mM of NaCl was added to the soil; and *5*) for the hormone treatment, 100 μM MeJA was sprayed onto the treated plants. Leaf samples were collected after 0, 3, 6, 12, and 24 h of each treatment (Liu *et al.* 2018). Triplicate samples were collected in different treatments, frozen in liquid nitrogen, and stored at -80 °C for further testing.

RNA extraction and cDNA synthesis: The RNA extraction was conducted according to the manual in the general plant RNA extraction kit (*Bio-Tek*, Beijing, China). The integrity of the proposed RNA was detected by 1 % (m/v) agarose gel electrophoresis. The concentration and purity of the RNA were measured by a *Nanodrop ND-1000* spectrophotometer (*Thermo Scientific Company*, Wilmington, CA, USA). The cDNA was synthesized using *PrimeScriptTM II 1st Strand cDNA Synthesis* kit according to the manufacturer's instructions (*TaKaRa*, Kusatsu, Japan). The amount of RNA per sample was determined according to the extracted RNA concentration for ensuring a consistent mRNA content in each sample. The cDNA concentrations of all of the samples were examined using the *Nanodrop ND-1000* spectrophotometer for further experiments.

Candidate reference gene selection and primer design: We selected 15 RGs according to research performed in other plants, especially research on related species (Liu *et al.* 2013, Xu *et al.* 2014, Nadai *et al.* 2015, Martins *et al.* 2016, Wang *et al.* 2016, Liu *et al.* 2018b, Li *et al.* 2020b, Zeng *et al*. 2021). The sequences of the 15 genes *ACT1, ACT2, ACT3, ACT7, TUA2, TUB6, EF1-α, EF1-β, GAPDH, HIS, KIN, eIF4A-1, FBA5, SAMDC,* and *UBQ* were obtained from the genome sequencing database of cauliflower (unpublished database). We cloned the 15 candidate genes and verified them by sequencing. Using a *BLAST* search in *NCBI*, cauliflower homologs of RGs were obtained (Table 1 Suppl.). Based on the criterion of the primer design, we designed primers for real-time qPCR using *Premier 5.0* software (Table 1 Suppl.). Primers were used in the following procedure: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s; 72 °C extension for 7 min. The 20 mm^3 of reaction system included: 2 mm^3 of 10× PCR buffer (including Mg^{2+}), 1 mm^3 of primer F (10 μM), 1 mm^3 of primer R (10 μM), 1 mm^3 of dNTP (10 μM), 0.2 mm^3 of *Taq* polymerase (5 U), 2 mm^3 of cDNA, and 12.8 mm^3 of ddH_2O. Their gene description, GenBank ID, sequences, amplicon length, amplicon characteristics, and the correlation coefficient (R^2) are presented in Table 1 Suppl.

Reverse transcription RT-qPCR: The RT-qPCR was conducted using the *SYBR Green Premix Ex TaqTM* kit (*TaKaRa*) and the *7500 Real Time System* instrument according to the manufacturer's instructions (*ABI*, Foster City, CA, USA). Each sample was tested three times. The 10 mm^3 of reaction system included 5 mm^3 of 2×*SYBR Premix Ex TaqTM*, 0.4 mm^3 of Primer F (10 μM), 0.4 mm^3 of Primer R (10 μM), 0.2 mm^3 of *ROX Reference Dye* (10 μM), 2 mm^3 of cDNA, and 2 mm^3 of ddH2O. The PCR amplification reaction procedure was as follows: 95 °C for 30 s; followed by 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. After amplification, the melting curve was analyzed. The dissociation curve program was as follows: 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s.

Validation of reference gene stability: The *WRKY3* gene is a transcription factor involved in the physiological activities of cauliflower during different experimental conditions. The WRKY3 plays an important regulatory role in plant tolerance to various stress conditions (Tang *et al.* 2018). The *WRKY3* gene GenBank ID was MN 315265, the upstream sequence was 5'-CTCTGTCACGGTCCTATTTG-3', and the downstream sequence was 5'-TAGCGAGGAAGTTGGTAATG-3'. The expression patterns of *WRKY3* in samples of cauliflower under five stress conditions were tested using the best-chosen combination of RGs and the most unstable RGs. Each test was repeated three times.

Data analysis: The stability of the 15 candidate internal RGs was evaluated using *geNorm*, *NormFinder*, *BestKeeper*, and *ΔCt*. The RT-qPCR findings were exported to *Microsoft Excel 2003*, and the cycle threshold (Ct) was converted according to the software requirements. Each software produced a value of the stability for candidate RGs that could be ranked. The final ranking table was generated using the geomean value for the four methods.

Results

The primers for the 15 RGs were confirmed using common PCR. The results suggested that the primers were suitable for real-time qPCR study because the amplified products were between 100 and 250 bp, the primers produced a single band that was the same size as their amplified products, were highly specific and produced no primer dimer (Fig. 1 Suppl.). The melting curves of the 15 candidate RGs were acquired by real-time qPCR amplification. As shown in Fig. 2 Suppl., the results were credible due to their high specificity and the single primer peaks. Hence, the primers were suitable for qPCR.

The cycle threshold values (Ct) confirmed the transcript abundances of the 15 RGs. Higher gene expression was indicated by lower Ct values. The Ct values of the 15 RGs are presented in Table 2 Suppl. and varied from 22.86 to

28.85 (Fig. 1). Apparently, *ACT3* exhibited the lowest expression with a transcript level of 28.85 ± 0.59, whereas *ACT7* possessed the lowest cycle number (22.86 ± 0.53). Most transcript abundances were spread between 23 and 27. Among them, *TUA2*, *GAPDH*, *EF1-β*, *ACT2*, *TUB6*, had low mean Ct values, while *FBA5*, *eIF4A-1*, *ACT1*, *EF1-α*, *KIN,* and *SAMDC* had worse Ct value. More gene expression variation was displayed by the standard deviation (SD) in Fig. 1. The *ACT7* exhibited a narrow variable gene expression range from 21.84 to 23.95 (22.86 ± 0.53), while *FBA5* revealed the greatest variation in expression.

Four different programs, *geNorm*, *NormFinder*, *BestKeeper*, and *ΔCt*, were used to rank and assess the expression stability of these candidate RGs. The RGs were examined by each computational algorithm and ranked from the best to the worst stably-expressed gene.

The tool *geNorm* was utilized to obtain the mean of the expression stability by creating a value M. According to the manual, genes with M values under 1.5 are appropriate RGs, thus the gene with the lowest M value was considered to be the most stable candidate and *vice versa*. As shown in Table 3 Suppl. for heat stress, *ACT3* and *EF1-β* with the lowest *geNorm* M values of 0.273 could be chosen as the most stable RGs, whereas *FBA5* with the highest M value of 1.126 was recognized as the most variably expressed gene. For the cold stress set (Fig. 2), *ACT1* and *TUB6* were ranked as the top-two most stable RGs due to their M values of 0.473, and *UBQ* had the most unstable expression with an M value of 1.105 (Table 3 Suppl.). *EF1-β* and *UBQ* possessed the most stable expression, and *FBA5* had the lowest stability for normalization under drought stress. In the set of samples under salt stress, *ACT7* and *HIS* had the lowest M values, while *FBA5* had the highest M value, indicating that *ACT7* and *HIS* were the top-two most stable genes and *FBA5* was the least stable gene. Under MeJA treatment, *ACT3* and *ACT7* presented optimal expression stability, while *FBA5* was the most unstable.

The *geNorm* program also provided the average pairwise variation (Vn/n+1) to acquire the best quantity of RGs for the most reliable findings. When the cutoff value of Vn/n+1 was lower than 0.15, n RGs were sufficient for real-time qPCR normalization. For treatments subjected to heat, drought, salt, and MeJA, the values of V2/3 were below 0.15, which indicated that the two most stable RG candidates could be used for the best expression stability (Table 4 Suppl., Fig. 3). Unfortunately, under cold stress, the pairwise value of V2/3 was greater than 0.15, and V3/4 was 0.119. Based on the above analysis, more than two reference genes should be used to obtain accurate data in the cold stress experiments.

NormFinder determined the best normalization candidates based on the stability values, which are shown in Table 5 Suppl. A lower stability value indicates more reliable gene expression. The *ACT1* was ranked as the optimal reference gene with the lowest stability value for drought stress among all samples, whereas *EF1-β* was considered to be the most stable gene in the heat stress and the cold stress treatments. The *HIS* and *ACT3* exhibited the most stable expression due to their low stability values under salt stress and MeJA treatment. Except for cold stress, *FBA5* exhibited the most variable expression in all samples. *UBQ* was the least stable gene in the cold treatment.

BestKeeper constructed coefficients of variance (CV) and the standard deviation (SD) for calculating the stability of the 15 candidate genes. More stable expression was indicated by lower CV ± SD values. SDs of more than 1 were not in the suitable range for normalization. As shown in Table 6 Suppl., *ACT3* was the best RG with the minimum (CV ± SD) of 0.62 ± 0.18 under heat stress. The *ACT1* was the best RG under cold stress, *TUA2* was best under drought stress, *TUB6* was best under salt stress, and *ACT2* was best under MeJA treatment.

To obtain the precise expression stability of the 15 genes, their ranking by the *ΔCt* program is listed in Table 7 Suppl. *ACT7* was suggested to be the most suitable candidate for heat, drought, and salt stress. The *GAPDH* was the most stable for the cold stress group, whereas *TUA2* ranked first for the MeJA treatment. The results, as assessed using the *ΔCt* tool, indicated that *ACT7*, *GAPDH*, and *TUA2* would be stably-expressed RGs.

According to the results of the four methods (*geNorm*, *NormFinder*, *BestKeeper*, and *ΔCt*), the geomean value of each candidate was obtained and used to assess the final rankings of the most stable reference genes. Based on the *geNorm* analysis, two candidate genes were enough for normalization under heat stress, drought stress, salt stress,

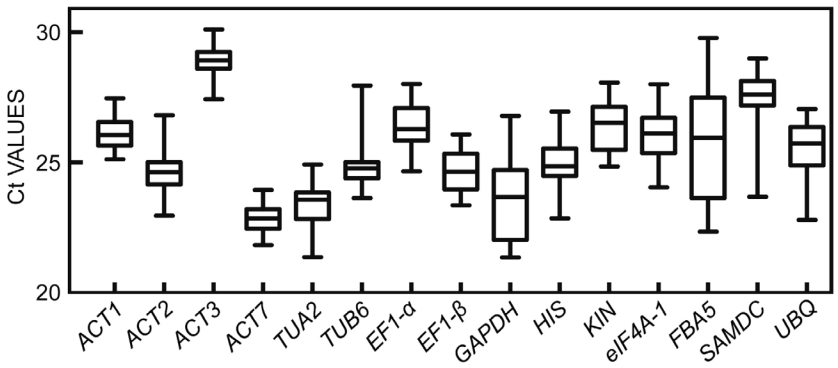

Fig. 1. Ct values of 15 internal control genes in cauliflower under all treatment factors. The line in the box indicates the median values, with the lower and upper sections revealing the first and third quartile. The whiskers represent the value ranges.

and MeJA treatment, whereas three suitable reference genes were sufficient for gene expression analysis in the cold treatment group and overall sample group. Through analysis of the comprehensive results, *EF1-β* and *ACT7* were ranked as the most stable genes for heat stress (Table 8 Suppl.), *TUA2* and *EF1-β* for drought stress, *HIS* and *ACT7* for salt stress, and *ACT7* and *ACT3* for MeJA treatment. Finally, *ACT1*, *EF1-β*, and *TUB6* were chosen for expression normalization under cold treatment. The results confirmed that, except for cold stress, *FBA5* was the most unstable expressed gene.

To validate the stability of the RGs, the expression profiles of the *WRKY3* gene were normalized by the genes selected by the four models under the five abiotic treatment sets. The *WRKY3* gene was differently expressed when the plant suffered from abiotic stresses (Chen *et al.* 2015, Tang *et al.* 2018). As shown in Fig. 4, the best RGs alone or a combination of the most and least stable genes were used to measure the relative expression of *WRKY3* under the tested stresses. The *WRKY3* had similar patterns of expression during the different experimental conditions when a single gene or combination of the best internal RGs was used, while the results presented completely different expression patterns or were overestimated in similar expression patterns when *UBQ* or *FBA5* were applied as calibrators. For example, when the optimal

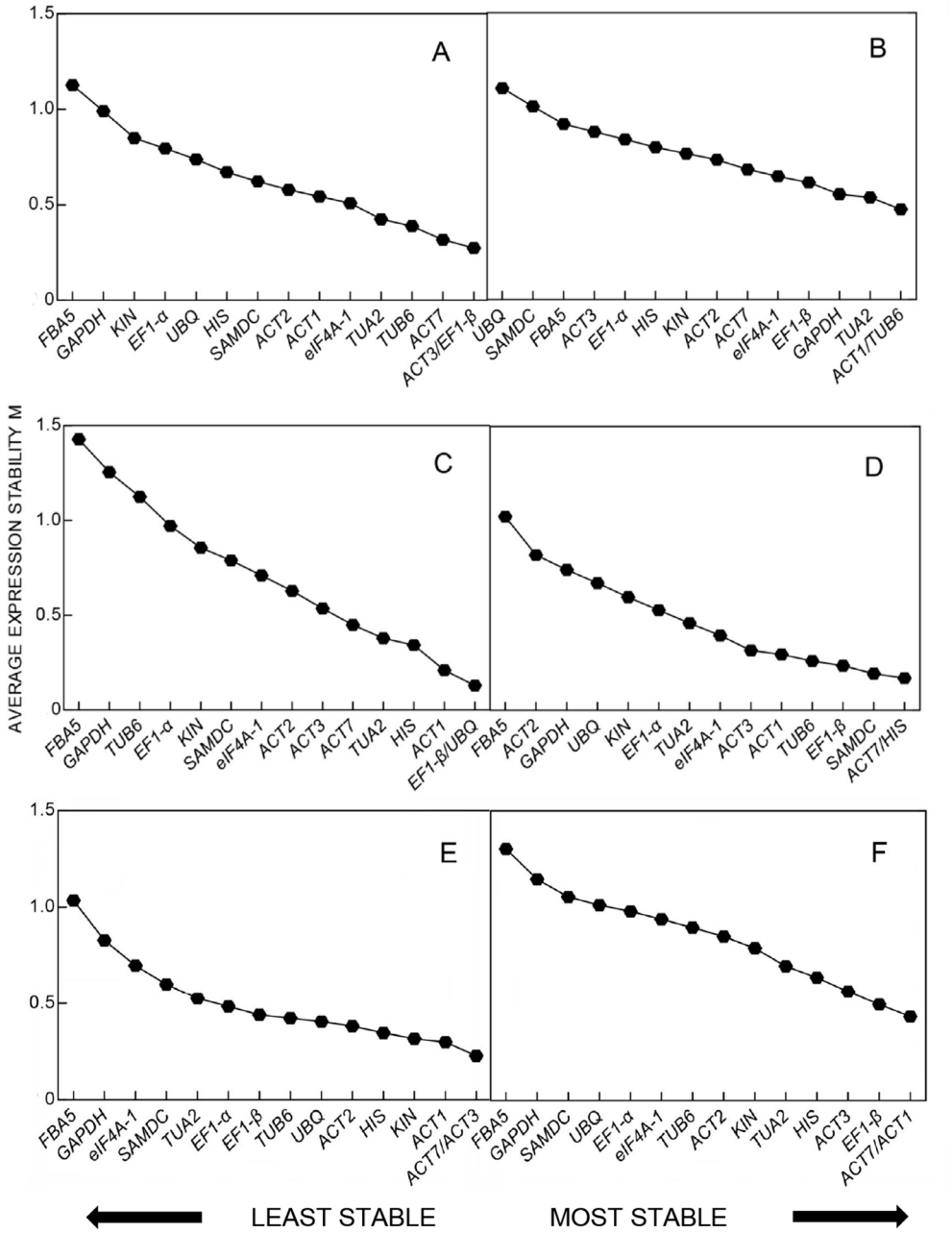

Fig. 2. M-values of the 15 RGs detected by *geNorm*. M: expression stability value. *A* - heat stress (38 °C), *B* - cold stress (4 °C), *C* - drought stress (20 % PEG 6000), *D* - salt stress (200 mM NaCl), *E* - MeJA stress (100 μM) and *F* - all samples.

genes were used in normalization, the *WRKY3* expression was found to reach the highest upregulation at 6 h under the cold stress, whereas the top expression of *WRKY3* was reached at 24 h when the least stable gene, *UBQ*, was used as a calibrator (Fig. 4*B*). For the salt stress group, similar *WRKY3* expression patterns were observed when the most stable genes and the least stable gene *UBQ* were used for normalization. However, in the salt treatment, abnormal upregulation was presented at 3 h when the least stable gene, *FBA5* was used (Fig. 4*D*). Therefore, under given experimental conditions, screening for suitable RGs is essential.

Discussion

Cauliflower is of great research interest due to its ability to synthesize glucosinolates. The qPCR technology is an ideal means to study functional gene expression analysis. However, the reliability of qPCR data depends on the stability of the internal RGs used. It is important to select suitable RGs for different developmental stages, different tissues, different organs, and different experimental conditions (Randhawa *et al.* 2008). To date, there have been few studies on how to choose, use, and verify these genes in cauliflower, which hampers the development of its genetic research. Much research has confirmed that no internal RGs are stably expressed under all experimental conditions (Yu *et al.* 2020, Zeng *et al.* 2021, Zhang *et al.* 2020). The RGs collected from the previous reports were not used arbitrarily for gene expression analysis (Liu *et al.* 2018). The most accurate experimental results can be obtained only by using the most suitable internal reference genes for real-time data.

Traditional HKGs play an important role in the whole life of cells and are therefore considered to be reliable genes. *ACT* genes have often been used as internal RGs in previous studies to reduce experimental deviation. In this study, 15 candidate RGs including *ACT1, ACT2, ACT3, ACT7, TUA2, TUB6, EF1-α, EF1-β, GAPDH, HIS, KIN, eIF4A-1, FBA5, SAMDC*, and *UBQ* were selected from the cauliflower transcriptome database to identify the stable genes. The stability of these genes was examined using four statistical tools (*geNorm*, *NormFinder*, *BestKeeper*, and *ΔCt*). The *ACT7* was the best RG for the MeJA treatment. However, under the heat, cold, drought, and salt stress, *ACT7* was not the most stably expressed gene. Considering previous findings (Deng *et al.* 2016, Li *et al.* 2020a), it was not unexpected that the gene expression indicated discrepancies and fluctuations under different conditions. The *ACT1* was found to be the most suitable gene under cold stress conditions. Though *ACT2* and *ACT3* exhibited stable expressions in the different experiments, they were not as stable as the internal RGs to verify the results of the real-time qPCR. Actin is an important protein in cytoskeleton formation and plays a significant role in plant cell development (Cai *et al.* 2020). In the model plant *Arabidopsis thaliana*, the *ACT* gene family is divided into two categories: one group that encodes for reproductive function genes, which are often expressed in pollen tubes and ovules and contain genes including *ACT1* and *ACT3*, and another group that encodes for vegetative function genes which are mostly expressed in vegetative organs such as leaves, and contains *ACT2*, *ACT7*, and *ACT8* (Kim *et al.* 2003, Borges *et al.* 2012). Despite *ACT* genes being common RGs in cauliflower studies, the stability of different *ACT* genes varies among the sample sets. Stable internal RGs are needed for studying the expression of genes related to glucosinolate biosynthesis under different environmental stresses in cauliflower. Our results revealed that the stability of the RGs was not constant under different treatments. To obtain reliable data with

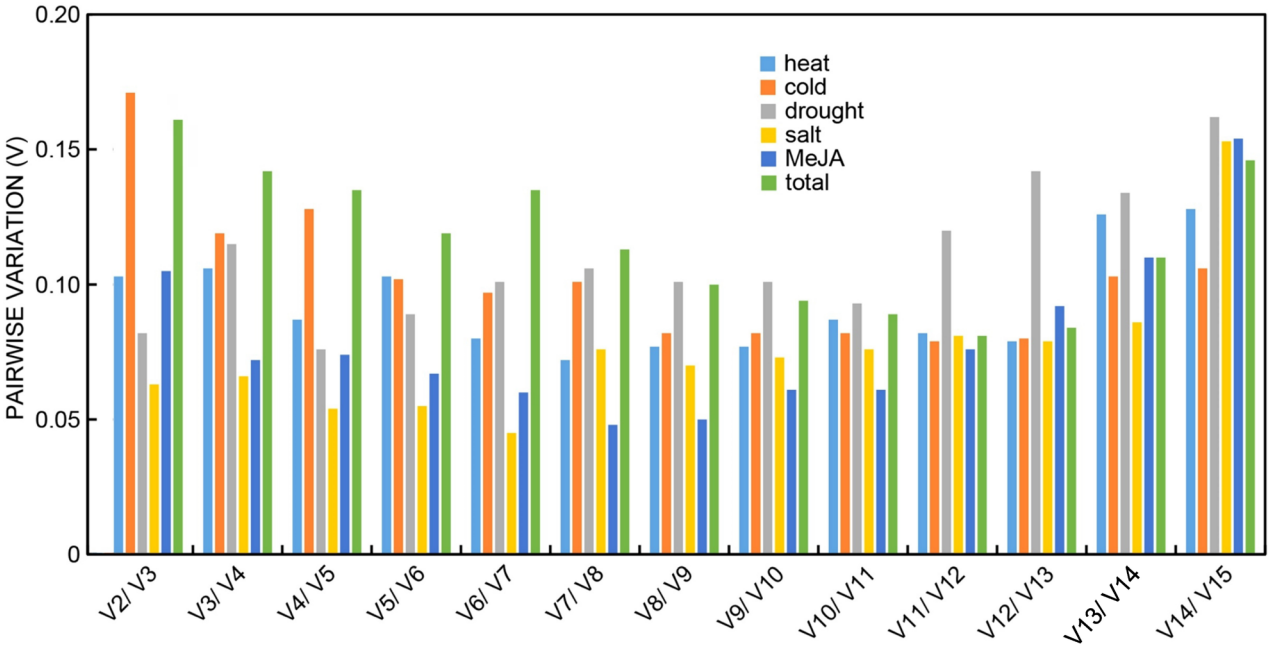

Fig. 3. The most suitable number of RGs in the various tests by *geNorm*.

different experimental factors, it is essential to choose the appropriate stable reference genes.

To evaluate the expressions of the RGs, we used four statistical algorithms (*geNorm*, *NormFinder*, *BestKeeper* software and the *ΔCT* calculations). The rankings yielded by each method were different. The results of this study were in line with the findings in other plants (Xu *et al.* 2014). As different calculation methods were used in each analysis, the calculated best genes and the RG rankings were slightly different. A slight discrepancy in the results

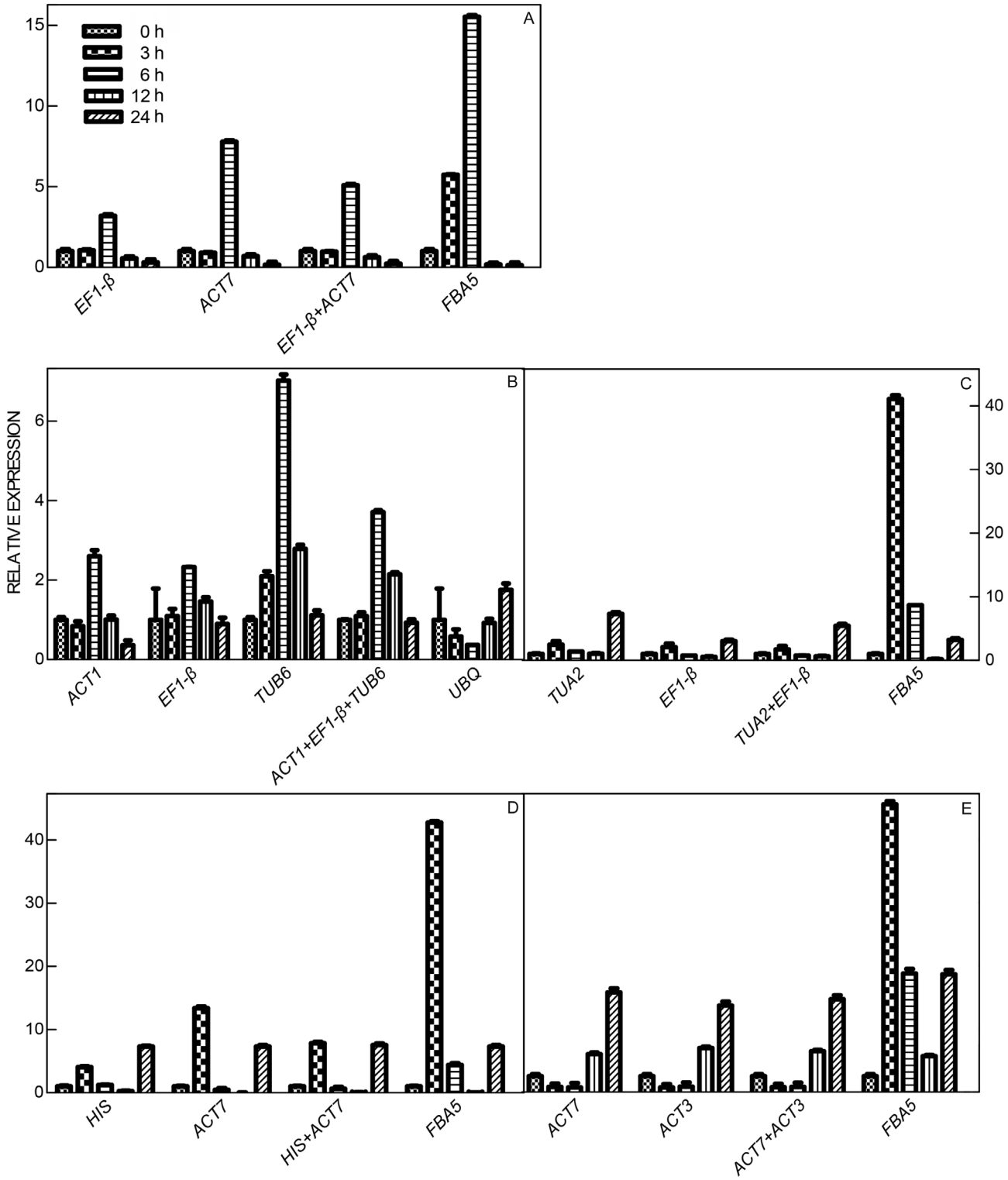

Fig. 4. Gene expression of the *WRKY3* gene in cauliflower for various abiotic factors. *A* - heat stress (38 °C), B - cold stress (4 °C), *C* - drought stress (20 % PEG 6000), *D* - salt stress (200 mM NaCl), *E* - MeJA stress (100 μM).

was acceptable and consistent with previous studies (Li *et al.* 2016a, Shivhare and Lata 2016, You *et al.* 2016). Finally, the outcomes of the four analyses were combined to ascertain the most stable RGs and enhance the reliability of our experimental data by the geomean.

The results showed that none of the genes maintained a constant expression under all experimental conditions. Therefore, two or more internal RGs should be used to attain credible results (Xu *et al.* 2015, Ye *et al.* 2015, Li *et al.* 2017). Using a sufficient quantity of RGs is of particular importance when the aim is to normalize numerous genes and samples. However, using more RGs results in more work that must be tested. Therefore, it is crucial to determine the appropriate number of internal RGs to use in a particular study. Previous studies have indicated that the coefficient of variation (Vn/n+1) in *geNorm* software can confirm the optimal number of RGs. According to the results of this research, we found that 2 - 3 reference genes were sufficient to obtain accurate experimental data. In this study, *EF1-β* and *ACT7* were combined to improve the reliability of test data under heat stress; *ACT1*, *EF1-β*, and *TUB6* were used for cold stress; *TUA2* and *EF1-β* were used for drought stress; *HIS* and *ACT7* were used for salt stress; and *ACT7* and *ACT3* were used for MeJA treatment. Xu *et al*. (2020) also found that 2 - 3 combinations of internal RGs could ensure more accurate results.

Although *ACT* genes can be used as RGs, they cannot be blindly used for credible experimental results under all experimental conditions. In specific experimental samples, screening for appropriate RGs is the key for the study of cauliflower genes. While the RGs we selected may not be the most suitable for other experimental conditions or in different environments, they can be used as a reference in selected cauliflower studies.

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