Genetics and Molecular Research

In-depth analysis of internal control genes for quantitative real-time PCR in *Brassica oleracea* var. *botrytis*

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Genet. Mol. Res. 15 (3): gmr.15038348
Received December 28, 2015
Accepted March 11, 2016
Published July 15, 2016
DOI http://dx.doi.org/10.4238/gmr.15038348

ABSTRACT. Quantitative reverse-transcription PCR (qRT-PCR) is a versatile technique for the analysis of gene expression. The selection of stable reference genes is essential for the application of this technique. Cauliflower (*Brassica oleracea* L. var. *botrytis*) is a commonly consumed vegetable that is rich in vitamin, calcium, and iron. Thus far, to our knowledge, there have been no reports on the validation of suitable reference genes for the data normalization of qRT-PCR in cauliflower. In the present study, we analyzed 12 candidate housekeeping genes in cauliflower subjected to different abiotic stresses, hormone treatment conditions, and accessions. geNorm and NormFinder algorithms were used to assess the expression stability of these genes. *ACT2* and *TIP41* were selected as suitable reference genes across all experimental samples in this study. When different accessions were compared, *ACT2* and *UNK3* were found to be the most

suitable reference genes. In the hormone and abiotic stress treatments, *ACT2*, *TIP41*, and *UNK2* were the most stably expressed. Our study also provided guidelines for selecting the best reference genes under various experimental conditions.

Key words: Cauliflower; Reference genes; geNorm; NormFinder

INTRODUCTION

Quantitative real-time PCR (qRT-PCR) is considered a versatile technique for the rapid and reliable quantification of gene transcript levels. Because of its specificity, sensitivity, and capacity for high throughput, qRT-PCR offers a broad range of advantages over standard methods like semi-quantitative PCR and northern blot analysis. Therefore, it is the most reliable method for the absolute and relative quantification of gene transcription levels (Gachon et al., 2004). To accurately quantify gene expression, many variables must be considered, including the quality and quantity of the initial sample, primer design, RNA quality, and amplification efficiencies (Mahoney et al., 2004). A suitable normalization technique is essential for obtaining biological reliable data. Among the many approaches proposed thus far, the selection of appropriate reference genes has been the most frequently used method of normalizing gene expression and reducing potential experimental errors generated in the process of qRT-PCR analysis (Guénin et al., 2009).

Generally, a suitable reference gene should be stably expressed constitutively across treatments, tissues, and developmental stages. A number of endogenous housekeeping genes, such as tubulin (*TUB*), actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 18S ribosomal RNA (*18S rRNA*), ubiquitin (*UBQ*), and elongation factor 1-α (*EF1-α*), have been widely used to normalize gene expression data (Nicot et al., 2005). In many cases, these reference genes were used without proper validation, on the supposition that they are constantly expressed because of their role in basic biological processes. However, this concept is misleading since the expression of these genes has been reported to be mutative under different experimental conditions (Tricarico et al., 2002; Gutierrez et al., 2008). Therefore, the systematic validation of appropriate reference genes is a critical step in qRT-PCR analysis to ensure the reliability of experimental data.

In recent years, an increasing number of reports have focused on the systematic validation of reference genes for plants, such as *Arabidopsis thaliana* (Czechowski et al., 2005; Remans et al., 2008; Hong et al., 2010; Dekkers et al., 2012), wheat (Paolacci et al., 2009; Hua et al., 2014), rice (Jain et al., 2006; Li et al., 2010; Bevitori et al., 2014), lentil (Saha and Vandemark 2013), soybean (Jian et al., 2008; Libault et al., 2008; Hu et al., 2009; Kulcheski et al., 2010; Le et al., 2012; Nakayama et al., 2014), tomato (Dekkers et al., 2012), potato (Nicot et al., 2005; Castro-Quezada et al., 2013), berry (Reid et al., 2006), peach (Tong et al., 2009), coffee (Barsalobres-Cavallari et al., 2009), and tobacco (Schmidt and Delaney, 2010). However, there have been few reports on the selection and validation of suitable reference genes for qRT-PCR analysis in *Brassica* crops (Qi et al., 2010; Chen et al., 2010; Chandna et al., 2012; Xu et al., 2014).

Cauliflower (*Brassica oleracea* L. var. *botrytis*) is an important vegetable crop in the genus *Brassica*. The whole genome of *B. oleracea* was recently sequenced, and there is increasing interest in the functional genomics of cauliflower (Wang et al., 2012). In the present

study, we aimed to assess the most suitable reference gene(s) for qRT-PCR gene expression studies in *Brassica oleracea* L. var. *botrytis*. Based on sequence homology, *B. oleracea* orthologs of the best 12 *A. thaliana* housekeeping genes were identified. We compared the performance of these 12 housekeeping genes across a large number of biological samples, using various tissue types, abiotic stress treatments, and accessions of cauliflower. The geNorm (Garson et al., 2005) and Normfinder (Andersen et al., 2004) software programs were used for statistical analysis.

MATERIAL AND METHODS

Plant material

A doubled haploid of cauliflower (DH_{3-2}) was used for all experiments. Seeds of DH_{3-2} were germinated and grown in chambers under long-day conditions (14-h light/8-h dark) at a temperature of 24°-26°C. The relative humidity in growth chambers was kept at 60-70%.

Abiotic stress treatments

For the drought and salt stress treatments, seedlings at the third-true leaf (TTL) stage were transplanted into pots containing 33 μM PEG-6000 and 300 mM NaCl, respectively, for 4 and 8 h.

For the heat and cold stress treatments, the seedlings at the TTL stage were kept at 4° ± 1°C and 39° ± 1°C, respectively, for 4 and 8 h. After the specified treatment duration, the roots, stems, and leaves were harvested for RNA extraction.

Hormone treatments

Seedlings at the TTL stage were sprayed with solutions of jasmonic acid (100 μM) and gibberellic acid (100 μM). Root, stem, and leaf tissues were harvested 4 and 8 h after the hormone spray.

Different accessions

Leaves were harvested from six DH lines of cauliflower at the TTL stage, including DH_{3-2}, DH_{3-1}, DH_{3-6}, DH_{3-4}, DH_{3-9}, and DH_{3-14}. All samples were promptly frozen in liquid nitrogen and then stored in an ultra-low temperature freezer at -70°C until required.

Total RNA extraction and cDNA synthesis

Total RNA of the samples was extracted using an RNA Simple Total RNA Kit (TaKaRa, Japan) with DNase I (TaKaRa) to eliminate residual genomic DNA, according to the manufacturer protocol. The concentration and purity of the extracted RNA were tested using a NanoPhotometer Spectrophotometer (Germany). Only the RNA samples with an A_{260}/A_{280} ratio of 1.8-2.0 and an A_{260}/A_{230} ratio higher than 2.0 were selected for subsequent analysis. The integrity of the total RNA was evaluated with electrophoresis by 1.2% agarose gel.

The isolated mRNA (100 ng) was reverse-transcribed to synthesize the first-strand cDNA with an SYBR PrimeScript RT-PCR Kit II (TaKaRa). A total of 100 ng RNA was mixed with the primers, incubated at 65°C for 5 min, and then briefly chilled on ice. Next, a transcription mixture was added for reverse transcriptase reaction at 42°C lasting 1 h, and first-strand cDNA was synthesized. Finally, the mixture was incubated at 75°C for 10 min to inactivate the reverse transcriptase. All cDNA samples were diluted 1:20 with PCR-grade water before being used for real-time PCR.

Selection of potential reference genes and primer design

Recently, many superior reference genes have been validated in *Arabidopsis*. Using these reference genes as queries, the corresponding orthologs were obtained from the *B. oleracea* genome gateway (http://brassicadb.org/brad/). The 12 candidate housekeeping genes selected are listed in Table 1, corresponding to the *Arabidopsis* Gene Initiative (AGI) locus, *B. oleracea* homolog locus, primer sequences, and PCR efficiency.

Primer pairs for RT-qPCR amplification were designed by Beacon Designer 2.06 with melting temperatures between 54° and 58°C, primer lengths of 18 to 20 bp, GC content of 40 to 55.56%, and amplicon lengths of 113 to 278 bp (**Table S1**). For each primer pair, the amplification efficiency was estimated by a standard curve produced by a 10-fold dilution series of pooled cDNA (1, 10, 10^2, 10^3, 10^4, and 10^5 x dilutions of each gene in triplicate). The PCR amplification efficiency was evaluated by the following equation: $E = [10^{(1/-S)}-1] \times 100\%$, where *S* indicates the slope of the standard curve.

RT-qPCR and data analysis

The real-time PCRs were performed on a 96-well optical plate with an iQ5 machine. About 25 ng template (1:20 diluted cDNA), 1 µL (10 pM) each primer and 12.5 µL 2X SYBR Green PCR Master Mix were mixed in a final volume of 25 µL. The qRT-PCR amplification program was as follows: 95°C for 6 min (denaturation), followed by 35 cycles of amplification for 45 s at 94°C, 40 s at 58°C and 60 s at 72°C. All qRT-PCRs were performed in biological and technical triplicate, and water without mRNA was used as a control in each run for each gene. Two algorithms, geNorm (version 3.5) and NormFinder (version 0.953), were used to evaluate the expression levels of the 12 housekeeping genes. Data from the three biological replicates were calculated separately in the two algorithms.

RESULTS

Identification of primer specificity and PCR amplification efficiency

Prior to the qRT-PCR experiment, primer specificity was determined by agarose gel electrophoresis. For each primer pair, a single PCR product was amplified at the expected size. The analysis of the melting curve also confirmed the specific amplifications of these 12 primer pairs by the presence of a single peak. Amplification efficiency was evaluated, with values ranging from 96 to 104% (Table 1).

Table 1. *Brassica oleracea* candidate reference gene descriptions and comparison with *Arabidopsis*.

Gene symbol	Gene name	AGI	*B. oleracea* orthologs	Primer sequence (5'-3')	Amplicon length (bp)	PCR efficiency (%)
UBQ10	Polyubiquitin 10	AT4G05320	Bol030585	CATCCAGAAAGAATCAACTC TGCTGGTCTGGAGGAATAC	171	98.1
EF-1α	Elongation factor-1α	AT5G60390	Bol001470	TGCCAACTTCACATCCCAG ACCAGCATCACCATTCTTC	190	101.2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	AT1G13440	Bol010398	AGGAATCTGAGGGCAAGCT CCATTCGTTGTCATACCAC	164	97.7
ACT2	Actin 2	AT3G18780	Bol003004	GAATCCACGAGACAACATAT AGGGAAGCAAGAATGGAAC	229	99.1
UBC	Ubiquitin conjugating enzyme	AT5G25760	Bol022331	TTATGAAGGCGGAGTGTTT TGAACCCTCTCGCATCTC	278	103.2
UBC9	Ubiquitin conjugating enzyme 9	AT4G27960	Bol021156	TGCCCTCACTATCTCCAAG TGTTCTTGTCTGTCTTGTAC	113	98.5
UNK1	Hypothetical protein	AT4G33380	Bol017536	TTACACACCACAAAGAGAGT TACAACACCTGAAACCCATG	275	101.6
UNK2	Hypothetical protein	AT2G32170	Bol006419	AAGGATGGAGGAGTTTGGAT TTCTCTCTTTCTCAATCTCG	151	96.4
TIP41	TIP41-like family protein	AT4G34270	Bol013651	ATTTGGCTGCTCTTTCACTT AAATCGTAAGAGGAGAAACC	146	104.3
PDF2	Protein phosphatase 2A subunit A3	AT1G13320	Bol042758	CAACCCTCACTATCTGTAC ATCGTCTTCTCTACAACCG	240	97.4
UNK3	Hypothetical protein	AT4G26410	Bol039624	ATTGCTTGAGAGGTCGGCT GCAATCCTCTTCTGTCTC	225	99.7
PTB	Polypyrimidine tract-binding protein	AT3G01150	Bol002171	GGCAATAGCGAAAGAAGCAT ACCTGCGTATTGAGTTTGC	177	98.4

AGI = *Arabidopsis* Gene Initiative locus.

Expression stability of candidate reference genes

The expression stability of the 12 candidate genes in 42 diverse samples was evaluated by qRT-PCR assay with SYBR Green detection. Threshold cycle (Ct) value was used to determine the expression levels of these genes. Overall, the transcripts of these 12 candidate genes exhibited broad variability. The mean Ct values ranged from 17 to 32, with most falling between 20 and 25 in all detected samples (Figure 1). Among the 12 candidate reference genes, *GAPDH* had the highest expression level, with a mean Ct of 21.8, and *PTB* had the lowest expression level (mean Ct of 28.6). However, *PTB* showed the least variation in expression, with a coefficient of variation value of 9.07%, while *GAPDH* (12.99%) and *EF-1α* (12.72%) showed the most variation across all detected samples (Figure 1).

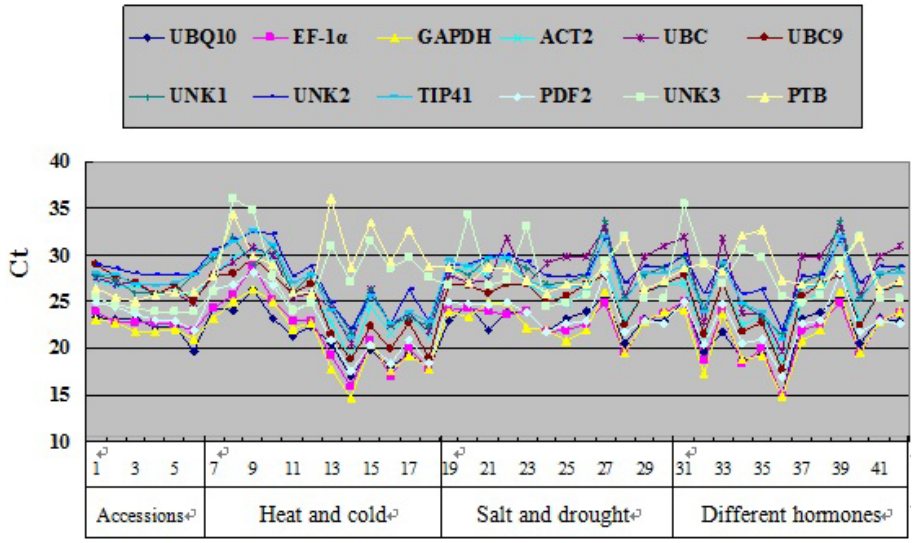


Figure 1. Expression levels of candidate reference genes across experimental samples. Values are given as qRT-PCR quantification cycle numbers (Ct values).

geNorm analysis

The geNorm software was used to calculate the expression levels of the 12 candidate reference genes and rank them by expression stability value (M). The M value is the average pairwise variation of a particular gene with all the other control genes, which is inversely related to the level of gene expression. A gene with a higher M value has a less stable expression. The geNorm software also suggested a threshold M value of 1.5; values below this number indicated stable expression.

Figure 2 shows the M values of all genes as calculated by the geNorm algorithm. Most of the genes had an M value below 1.5, except *PTB* and *UNK3*, which had M values a little higher than 1.5 for the hormone and heat/cold stress treatments and across all tested sample pools. However, the gene with the most stable expression varied among the sample sets. *GAPDH* and *ACT2* showed the most stable expression levels under heat/cold and hormone stress treatments. When the 42 samples were pooled, *GAPDH* and *ACT2* remained the two most stably expressed genes. Under salt and drought stress treatments, the expression levels of *TIP41* and *UNK3* showed the most stability, and *PTB* the least stability. In response to the different accessions, *UNK2* and *UNK3* showed the most stable expression levels and *UNK1* the least stable level. Overall, the expression levels of these 12 candidate reference genes exhibited a large variation among the different sample sets. Therefore, it appears necessary to choose one or more suitable reference gene(s) to normalize gene expression for obtaining reliable biological data.

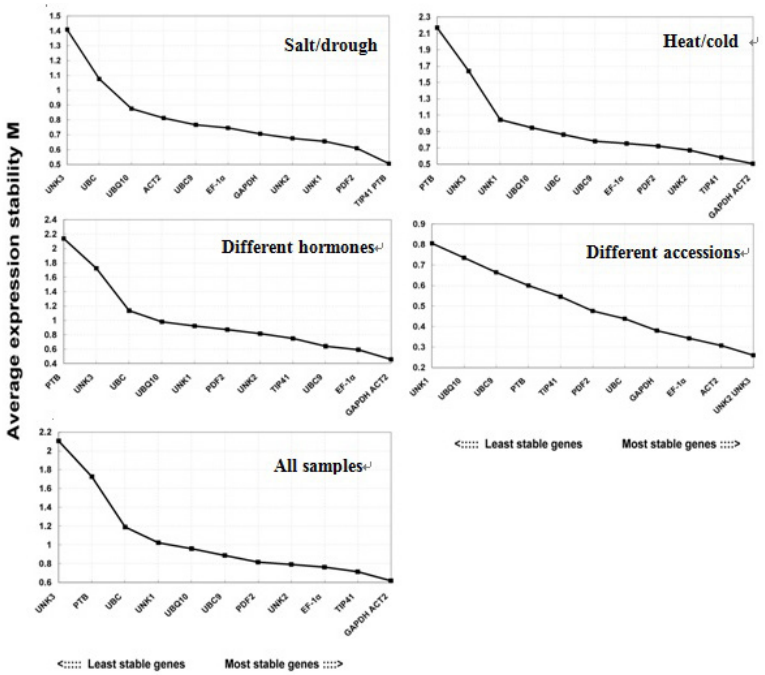
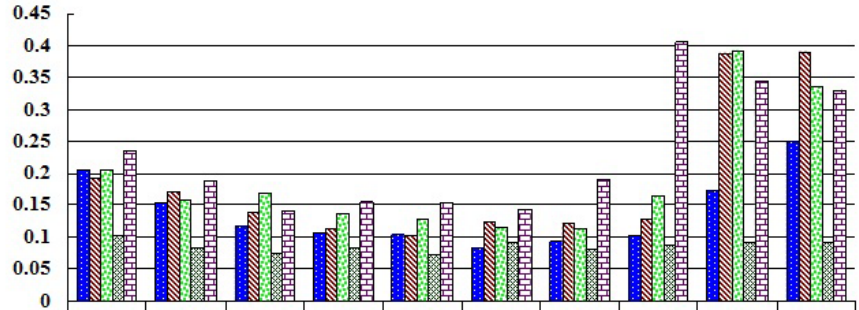


Figure 2. Gene expression stability and ranking of 12 reference genes as calculated by geNorm. Average expression stability (M) following stepwise exclusion of the least stable gene across all the samples within an experimental set. The least stable genes are on the left, and the most stable on the right.

The pairwise variation value was also calculated by the geNorm algorithm. This value was used to determine the optimal combination of reference genes for reliable normalization. The threshold of this value was usually set at 0.15, below which the addition of other reference genes was unnecessary. The results of pairwise variation for each sample set are shown in Figure 3.



	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8	V8/9	V9/10	V10/11	V11/12
Salt/drought	0.207	0.152	0.118	0.106	0.104	0.085	0.093	0.101	0.172	0.25
Heat/Cold	0.193	0.171	0.139	0.113	0.102	0.125	0.122	0.129	0.388	0.39
Different hormones	0.207	0.157	0.168	0.138	0.128	0.116	0.112	0.165	0.393	0.335
Different accessions	0.103	0.084	0.076	0.085	0.073	0.09	0.081	0.087	0.09	0.091
All samples	0.235	0.188	0.141	0.156	0.153	0.143	0.19	0.406	0.343	0.33

Figure 3. Determination of the optimal number of reference genes for effective normalization. The pairwise variation (Vn/Vn+1) between normalization factors NFn and NFn+1 was analyzed by geNorm to determine the optimal number of reference genes.

Among the series of samples tested, the expression stability levels of these 12 reference genes showed the smallest differences between samples from different accessions (Figure 3). The V2/3 value for different accessions was 0.103, so *UNK2* together with *UNK3* would be sufficient for the purpose of normalization. In the salt and drought stress comparison, the V4/5 value was 0.118, indicating that four reference genes (*TIP41*, *PTB*, *PDF2*, and *UNK1*) would be needed. However, analysis of the pooled samples and heat/cold treatment showed the suitable reference genes to be *GAPDH*, *ACT2*, *TIP41*, and *UNK1* (V4/5 value = 0.139) and *GAPDH*, *ACT2*, *TIP41*, and *EF-1α* (V4/5 value = 0.141), respectively. Finally, in the evaluation of samples treated with different hormones, five reference genes (*GAPDH*, *ACT2*, *TIP41*, *EF-1α*, and *UBC9*) were suggested (V5/6 value = 0.138).

NormFinder analysis

NormFinder is another algorithm for determining the expression stabilities of candidate reference genes. The expression stability value was calculated by this algorithm for each candidate reference gene based on intra- and inter-group comparison. Table 2 shows the results generated by NormFinder analysis, which differed from those of geNorm. According to NormFinder, *UBC* was the most stably expressed gene under heat/cold treatment, whereas it was ranked eighth by geNorm. Under salt/drought and hormone treatments, *UBC9* and

TIP41 showed the highest level of expression stability, but these two genes ranked eighth and fifth, respectively, in the geNorm analysis. In samples from different accessions, there was little difference between the analyses of NormFinder and geNorm. *ACT2*, *UNK3*, and *EF-1α* were determined by NormFinder to be the three most stably expressed genes, whereas *EF-1α* was ranked fourth by geNorm. In the samples of all experimental conditions, *UNK2* showed the most stable expression, while it was ranked fifth by geNorm. These discrepancies in the results obtained using geNorm and NormFinder were expected because the two programs use completely different statistical algorithms.

Table 2. Expression stability of the reference genes as calculated by the NormFinder software.

Rank	Salt/drought		Heat/cold		Different hormones		Different accessions		All samples	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	UBC9	0.014	UBC	0.017	TIP41	0.014	ACT2	0.004	UNK2	0.013
2	UNK2	0.015	TIP41	0.020	UNK2	0.015	UNK3	0.006	TIP41	0.019
3	UNK1	0.017	UNK2	0.022	PDF2	0.019	EF-1a	0.011	ACT2	0.030
4	EF-1α	0.018	ACT2	0.024	ACT2	0.019	GAPDH	0.011	UNK1	0.031
5	PDF2	0.019	UBQ10	0.026	UNK1	0.032	UNK2	0.014	PDF2	0.033
6	TIP41	0.023	UNK1	0.038	UBC9	0.040	UBC	0.015	UBQ10	0.044
7	ACT2	0.026	PDF2	0.054	EF-1a	0.052	PDF2	0.023	EF-1α	0.048
8	PTB	0.027	UBC9	0.055	UBQ10	0.057	TIP41	0.027	UBC9	0.050
9	GAPDH	0.029	EF-1a	0.062	GAPDH	0.060	UBC9	0.028	GAPDH	0.053
10	UBQ10	0.034	GAPDH	0.064	UBC	0.071	PTB	0.031	UBC	0.062
11	UBC	0.069	UNK3	0.152	UNK3	0.153	UNK1	0.042	PTB	0.143
12	UNK3	0.100	PTB	0.176	PTB	0.157	UBQ10	0.051	UNK3	0.144
12	UNK3	0.144	PTB	0.176	UNK3	0.100	PTB	0.157	UBQ10	0.051

In summary, the results obtained by geNorm and Norm-Finder analysis suggested *ACT2* and *TIP41* as appropriate reference genes across all experimental samples in this study. For different accessions, *ACT2* and *UNK3* were found to be the most suitable reference genes. Under abiotic stress and hormone treatments, *ACT2*, *TIP41*, and *UNK2* were selected for their stable expression levels.

DISCUSSION

Real-time qPCR is a versatile technique for assessing gene expression with high repeatability and throughput (Gachon et al., 2004; Song et al., 2015). For reliable data collection, accurate normalization of gene expression is critical in samples with one or more suitable reference genes. The optimum reference gene should exhibit invariable expression levels in all samples regardless of treatment or tissue.

However, we found no single gene with an invariable expression level under all experimental conditions. Consequently, expression stability should be validated for multiple reference genes under various experimental conditions before their use in qPCR normalization. Professional statistical algorithms have greatly simplified the process of reference gene validation. geNorm and NormFinder are the most commonly used algorithms. They can calculate the expression stability value of candidate reference genes and also determine the optimal number of reference genes for accurate and reliable normalization (Vandesompele et al., 2002; Andersen et al., 2004). In the present study, the expression stability of 12 candidate reference genes was validated under five experimental conditions using 42 diverse samples of cauliflower. To our knowledge, this is the first comprehensive analysis of validation of stably expressed reference genes for qPCR in cauliflower from different accessions and under

different stress treatments.

Our analysis, which was based on the geNorm and NormFinder algorithms, displayed some variation in which reference genes ranked in the top positions. However, both algorithms consistently determined the most unstably expressed gene. For example, in the heat/cold and hormone treatments, *ACT2* and *GAPDH* showed the most stable expression levels in geNorm, but ranked fourth/tenth and fourth/ninth, respectively, in NormFinder. Across the pooled samples, *UNK2* exhibited the highest level of expression stability in NormFinder, but ranked fifth in geNorm. These inconsistencies were expected, since the programs use different statistical algorithms.

geNorm selects the two reference genes with the lowest intra-group expression variation and the highest similarity in expression patterns. In NormFinder, the amount of inter- and intra-group variation is used to rank the genes with the most stable expression (Andersen et al., 2004). Thus, it is understandable that the top reference genes ranked by the two programs were different. Discrepancies between geNorm and NormFinder have also been noted in previous studies (Hong et al., 2008; Wan et al., 2010; Huis et al., 2010).

An increasing number of studies have confirmed that a combination of several reference genes is superior to a single gene for reliable and accurate normalization. However, it is time-consuming and expensive to increase the number of reference genes for normalization. Therefore, the optimum number of reference genes used for normalization should be based on the researcher's considerations. Generally, the two most stably expressed reference genes are effective for reliable normalization. However, in this study, four to five reference genes were required in the most of the sample sets when the geNorm threshold of 0.15 was used. Therefore, this threshold may not be a strict cutoff, and the pairwise variation values may be informative for proposals and suggestions (Hong et al., 2008). Moreover, our analysis also indicated that the selection of suitable reference genes should be based on specific experimental conditions. In the present study, five different optimal reference genes were identified for the five cauliflower sample sets analyzed.

In conclusion, this was the first systematic study to identify and validate appropriate reference genes for data normalization in cauliflower under five experimental conditions, including 42 diverse samples. The results of our analysis with geNorm and NormFinder indicated that *ACT2* and *TIP41* could be considered suitable reference genes across all experimental samples. *ACT2* and *UNK3* were the most appropriate reference genes when different accessions were compared. In samples treated with hormones and abiotic stress, *ACT2*, *TIP41*, and *UNK2* exhibited the most stable expression levels. The results obtained in this paper will facilitate the accurate and sensitive normalization of gene expression in *B. oleracea* and other species of *Brassica*. In the future, we plan to examine multiple cauliflower varieties to see whether there are differences between species in the selection of reference genes, so as to further verify the reliability of the results obtained in this study.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Natural Science Foundation of China (#31201629), the Zhejiang Province for Key Agriculture Development Projects (#2012C12903), the Science and

Technology Department of Zhejiang Province, Zhejiang Academy of Agricultural Sciences, and the Zhejiang Provincial Natural Science Foundation (#LQ13C030002).

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Supplementary material

Table S1. Primer description of the 12 candidate reference genes.