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ORIGINAL PAPER

Reference Gene Selection for Gene Expression Studies Using Quantitative Real-Time PCR Normalization in *Atropa belladonna*

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Abstract Quantitative PCR (qPCR) is a powerful tool for measuring gene expression levels. Accurate and reproducible results are dependent on the correct choice of reference genes for data normalization. *Atropa belladonna* is a commercial plant species from which pharmaceutical tropane alkaloids are extracted. In this study, eight candidate reference genes, namely 18S ribosomal RNA (*18S*), actin (*ACT*), cyclophilin (*CYC*), elongation factor 1α (*EF-1α*), β-fructosidase (*FRU*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), phosphoglycerate kinase (*PGK*), and beta-tubulin (*TUB*), were selected and their expression stabilities studied to determine their suitability for normalizing gene expression in *A. belladonna*. The expression stabilities of these genes were analyzed in the root, stem, and leaf under cold, heat, NaCl, UV-B, methyl jasmonate, salicylic acid, and abscisic acid treatments using geNorm, NormFinder, and BestKeeper. The statistical algorithms indicated that *PGK* was a reliable gene for normalizing gene expression under most of the experimental conditions. The pairwise value analysis showed that two genes were sufficient for proper expression normalization, except when analyzing gene expression in heat-treated roots. However, the choice of the second reference gene depended on specific conditions. Finally, the relative expression level of the *PMT* gene of *A. belladonna* was detected to validate the selection of *PGK* a reliable reference gene. In summary, our results should guide the selection of appropriate reference genes for gene expression studies in *A. belladonna* under different organs and abiotic stress conditions.

Keywords *Atropa belladonna* · Gene expression · Normalization · qPCR · Reference gene

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Introduction

Atropa belladonna is a commercial plant species that is widely used for extracting pharmaceutical tropane alkaloids (TAs), including hyoscyamine and scopolamine, which are used as anticholinergic agents (Oksman-Caldentey 2007). Scopolamine is useful and valuable because of its high physiological activities and lack of side effects (Wang et al. 2011). There is strong demand for scopolamine worldwide; however, the supply of scopolamine is limited because of the low abundance of scopolamine in *A. belladonna* (less than 0.1 %

DW) (Yang et al. 2011). Metabolic engineering is one method that could be used for the genetic modification of the biosynthetic pathway for scopolamine in *A. belladonna*. Metabolic engineering of the scopolamine biosynthetic pathway in *A. belladonna* would include gene cloning, gene expressing analysis, and functional identification of genes of interest. Gene expression analysis is a fundamental approach to reveal the correlation between target metabolite accumulation and the expression of genes of interest. In *A. belladonna*, scientists have used northern blotting and semi-quantitative reverse transcriptase PCR (RT-PCR) to analyze gene expression levels (Fukami et al. 2002; Suzuki et al. 1999a, b). However, northern blotting is time-consuming and semi-quantitative RT-PCR is not very precise compared with quantitative real-time PCR (qPCR).

qPCR is a useful technique for comparing the expression profiles of target genes in different tissues, cell types, or treatments, and is also used to validate high-throughput gene expression profiles (Gachon et al. 2004; Crismani et al. 2006). The qPCR technique was introduced in 1996 and represents a significant progression from qualitative PCR, with advantages of high sensitivity, distinct specificity, rapidity, and accuracy (Mackay 2004; Valasek and Repa 2005). qPCR analysis is widely used because it can detect very low quantities of a target transcript with very high specificity owing to the high temperature used when annealing the specific primers to the target sequence during PCR (Guenin et al. 2009). Recently, to standardize qPCR experiments, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were established (Bustin et al. 2009).

Despite its numerous advantages, this method has however several issues, with one of the most important being the normalization (Demidenko et al. 2011). There are several strategies for establishing normalization; however, the most popular technique is the use of reference genes (Huggett et al. 2005). Choosing a suitable reference gene can improve the sensitivity and repeatability of the target gene expression analysis. An ideal reference gene is expressed at a level that is independent of the experimental conditions to which the subject has been exposed (Bustin 2002). Traditionally, reference genes have been chosen from among housekeeping genes (Li et al. 2010; Chen et al. 2011). The most frequently used reference genes in plant gene expression studies are actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-tubulin (*TUB*), and 18S ribosomal RNA (*18S*) (Brunner et al. 2004; Czechowski et al. 2004; Jain et al. 2006). If expression of the reference gene is altered by the experimental conditions, the results obtained may be incorrect (Dheda et al. 2005). Therefore, the use of appropriate reference genes for normalization is fundamental to qPCR experiments. Unfortunately, there is no universal reference gene that is expressed at a constant level under all conditions in all tissues (Lee et al. 2002; Czechowski et al. 2005). In gene expression analysis, using a single reference gene for normalization is appropriate for some experiments (Jain et al. 2006); however, the use of a single reference gene does not always comply with test requirements (Vandesompele et al. 2002). Therefore, the use of two or more reference genes for accurate normalization is required. Generally, to obtain more authentic results, gene expression analysis should be carried out to validate reference gene(s), and the most stably expressed reference genes should then be chosen to normalize the expressions of target genes.

Generally, expression stability is evaluated using three different Microsoft Excel-based algorithms: geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and BestKeeper (Pfaffl et al. 2004). The levels of gene expression are determined as the number of cycles needed for the PCR amplification to reach a quantification cycle (C_q) fixed in the exponential phase of PCR reaction (Walker 2002). For geNorm and NormFinder, the raw C_q values are transformed into the required data input format. The maximum expression level (the lowest C_q value) of each gene is used as a control and is set to the value of 1. Relative expression levels are then calculated from the C_q values using the formula: $2^{-\Delta Cq}$, (ΔC_q= minimum C_q value−each corresponding C_q value). BestKeeper analysis is based on untransformed C_q values (Pfaffl et al. 2004). geNorm is a Visual Basic Application applet for Microsoft Excel that ranks the genes expression stability using the *M* value. When the *M* value is below the threshold of 1.5, the gene with the lowest *M* value is considered as the most stably expressed gene, while the gene with the highest *M* value has the least stable expression. To determine the optimal number of reference genes, geNorm calculates the pairwise variation V_n/V_{n+1}. The pairwise variation (*V*) between two sequential normalization factors contains an increasing number of genes. Based on the genome biology data, Vandesompele et al. proposed 0.15 as a cut-off value for *V*, below which the inclusion of an additional control gene is not required; that is, if $V_n/V_{n+1}<$ 0.15, it is not necessary to use $\geq V_{n+1}$ reference genes as internal controls (Vandesompele et al. 2002). Similar to geNorm, the NormFinder algorithm ranks the reference genes according to a parameter, the stability value. The reference gene with the lowest stability value is that with the most stable expression, and the gene with the highest stability value has the least stable expression. NormFinder can also analyze gene expression stability using a mathematical model that focuses on the inter-group and intra-group variation (Andersen et al. 2004). BestKeeper, an Excel-based tool, uses the average C_q value of each duplicate reaction (without conversion to quantity) to analyze the stabilities of candidate reference genes (Zhao et al. 2011). BestKeeper evaluates the stabilities of candidate reference genes based on the coefficient of correlation to the BestKeeper index, which is the geometric mean of the C_q values of all candidate reference genes (Zhao et al. 2011; Pfaffl et al. 2004). BestKeeper also calculates the reference

genes expression levels, where the first estimate of the reference genes expression stability has already been performed, based on the inspection of calculated variations (standard deviation (SD) and coefficient of variance (CV) values). According to the variability observed, reference genes can be ordered from the most stably expressed, exhibiting the lowest CV values, to the least stably expressed, and exhibiting the highest CV values (Pfaffl et al. 2004). A gene with an SD greater than 1 is considered unsuitable as a reference gene (Migocka and Papierniak 2010).

There has been no report concerning selection and estimation of reference genes in *A. belladonna*. In this study, eight potential reference genes: *18S*, *GAPDH*, *ACT*, *TUB*, phosphoglycerate kinase (*PGK*), elongation factor 1α (*EF-1α*), cyclophilin (*CYC*), and β-fructosidase (*FRU*) were used to identify reliable reference genes for normalization of real-time qPCR data obtained from different organs of *A. belladonna*, and under treatments with various environmental conditions, including cold, heat stress, methyl jasmonate (MeJA), salicylic acid (SA), abscisic acid (ABA), NaCl, and UV-B.

Materials and Methods

Plant Materials and Treatments

A. belladonna plants were grown in culture pots at 25±1 °C under a photoperiod of 16 h of light per day. The plants were subjected to the different treatments when they had been growing for 35 days. Two groups of *A. belladonna* were grown at 4 °C (cold) and 42 °C (heat), respectively, for 24 h; the controls were grown at 25±1 °C. Another experimental group was treated with 100 mM NaCl, whereas the control was treated with water, both for 24 h. Further groups were treated separately with three plant hormones: ABA, SA, and MeJA, which were purchased from Sigma (MO, USA), and dissolved in ethanol (EtOH) to a final concentration of 10 μM ABA (Rothe et al. 2001), 1 mM SA (Kang et al. 2004), and 0.1 mM MeJA (Kang et al. 2004); their controls were treated with EtOH vehicle only. The diluted solutions were sprayed on both sides of the leaves until the liquid dripped from the leaves. The salt-stressed group was irrigated with 100 m NaCl for 24 h. A further group was treated with UV-B (0.15 $W \cdot m^{-2}$) for 3 h. At least three replicates of each group were performed. The fourth and fifth plant leaves, all roots and whole stems were collected, immediately frozen in liquid nitrogen, and kept at −80 °C for further analysis.

Total RNAs Extraction

Total RNAs were extracted from prepared materials using the RNA simple total RNA Kit (TIANGEN, China) and treated with RNase-free DNaseI (TaKaRa, Japan). RNA concentration was assessed by AstraGene Haste Nucleic acid/Protein Analyser (UK). Only samples with OD260/280 ratio within the range of 1.9–2.1 and OD260/230 ratio in the range of 2.0–2.6 were included in further analysis. The integrity of the RNA was further checked by 1.2 % agarose gels electrophoresis.

Cloning of Reference Genes and qPCR Primer Design

The sequences of the eight candidate reference genes were not available in the public NCBI GenBank database; therefore, the genes had to be cloned. Degenerate primers were designed based on the homologous genes of other species of the Solanaceae for cloning and sequencing of the partial cDNA sequences of the genes. The amplified sequences were submitted and deposited in GenBank (for accession numbers, see Table 1). The qPCR primers for *18S*, *GAPDH*, *ACT*, *TUB*, *PGK*, *EF-1α*, *CYC*, and *FRU* (Table 1) were designed based on the sequenced fragments. Beacon designer 7.0 (http://www.premierbiosoft.com/molecular_beacons/) was used to design the primers. The amplification efficiency (*E*) and correlation coefficients (R^2) of the primers were tested.

Quantitative Real-Time PCR

A sample of each RNA (800 ng) was reverse transcribed to cDNA with random primers and an Oligo dT Primer using the PrimeScript® RT reagent Kit Perfect Real Time (TaKaRa, Japan) for quantitative RT-PCR analysis on a iQ™5 instrument (Bio-Rad, USA). The qPCRs were performed in a 20-μL volume, containing 10 μL SYBR Green solution with 0.5 U ExTaq™ (TaKaRa, Japan), forward and reverse primer at 0.4 μM final concentration, 200 ng of cDNA, and RNase-free H_2O. The qPCR conditions comprised an initial denaturation of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, the appropriate melting temperature for 20 s, and 72 °C for 20 s. At the end of the cycle process, the temperature was raised from 60 to 95 °C at a rate of +0.5 °C/s to derive the necessary melting curve. Three technical replicates of the real-time PCR analysis were performed for each biological replicate.

Validation of Reference Genes

The expression of the *A. belladonna* putrescine *N*-methyltransferase gene (*AbPMT*) has been studied intensively (Suzuki et al. 1999a, b). Therefore, the expression level of *AbPMT* was detected to validate the reference genes. According to the statistical results, *PGK* was a reliable reference gene in most cases; *CYC* was the least reliable one in organs; and *FRU* was the least reliable one in plant hormone treatments. *PGK*, *CYC*, and *FRU* were used in the validation tests. The primers for detecting *AbPMT* expression were listed in Table 1.

Table 1 Candidate reference genes and target genes used in this study

Gene name	Accession number	Function	Primer sequence (5′ to 3′) (forward/reverse)	Amplicon length (bp)	Melting temperature (°C)	*E* (%)	R^2
18S	KC881109	40S ribosome subunit RNA component	ATGATAACTCGACGGATCGC CTTGGATGTGGTAGCCGTTT	169	56	99.6	0.999
ACT	JX154681	Cytoskeleton contraction	TTGTGTTGGACTCTGGTGATGG CCGTTCAGCAGTGGTGGTG	167	60	98.3	0.999
CYC	JX154678	Protein folding, RNA splicing	CCCAGGGTTCATGTGTCAAGG ATTAGTTCCAGGTCCAGCATTAGC	154	59	97.1	0.997
FRU	JX154680	Hydrolysis sucrose	AGACTCAAACGGGCTGGACAC GCCTCAACCCAATACCACAATCC	170	60.4	102.5	0.998
EF-1α	JX154677	Protein translation	TGAAGAATGGTGATGCTGGTATGG CTTCTGAGCAGCCTTGGTGAC	191	59.5	91.1	0.998
GAPDH	JX154679	Glucose metabolic process	GCTGCTTCATTCAACATCATTCC ACAGTAAGGTCCACAACAGAAAC	140	56.5	96.6	0.999
PGK	JX154676	Glucose metabolic process	TCGCTCTTGGAGAAGGTTGAC CTTGTCGGCAATCACTACATCAG	195	59.5	97.4	0.999
TUB	JX154675	Structural constituent of cytoskeleton	CTTGCTGTTTGAGATTCCCTGGTC CGCACATCATATTCTTGGCATCCC	195	59	99.7	0.996
PMT	AB018570	Putrescine *N*-methyltransferase	CCTACTTACCCTACTGGTGTTATC GCGAAAGATGGCAAAATAAAAGC	178	57	95.3	0.998

E amplification efficiency, R^2 correlation coefficients

Data Acquisition and Statistical Analysis

Three widely used statistical algorithms were used to analyze the gene expression data: geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and BestKeeper (Pfaffl et al. 2004). In the validation of the selected reference genes, one-way ANOVA was used to analyze the results statistically.

Results

Primer Efficiency and C_q Variation

The sequences of the reference genes are essential for detecting their gene expression levels by qPCR. Unfortunately, the sequences of the eight candidate reference genes were not available. Therefore, we isolated and sequenced partial sequences of the eight reference genes (*18S*, *ACT*, *GAPDH* and *TUB*, *PGK*, *EF-1α*, *CYC*, and *FRU*), which have been used as reference genes in other plant species. The sequences were deposited in GenBank with accession numbers shown in Table 1 and used for qPCR analysis. The amplification of each candidate produced a single peak in the melting curve, which demonstrated that the primer pairs used for qPCR were gene specific. The amplification efficiencies of the standards ranged from 91.1 to 102.5 % (Table 1), which matched the expected amplification efficiencies between compared genes (Zhao and Fernald 2005). Furthermore, the standard curves demonstrated good correlation coefficients (R^2), which ranged from 0.996 to 0.999 (Table 1).

The C_q data were obtained from the PCR amplification of each reference gene with its own gene-specific primer pair. To reveal variations in transcript levels of the candidate genes, the average C_q was calculated across all samples (Fig. 1). The eight reference genes showed different levels of transcription. The *18S* gene had the lowest C_q value (mean C_q=7.45± 0.725), which corresponded to the highest transcription level among the eight reference genes; while *FRU* had the highest C_q value (21.39±1.036), making it the least abundant reference gene. The transcription levels of *ACT*, *CYC*, *EF-1α*, *GAPDH*, *PGK*, and *TUB* were moderate, with C_q values ranging from 14.10 to 18.44.

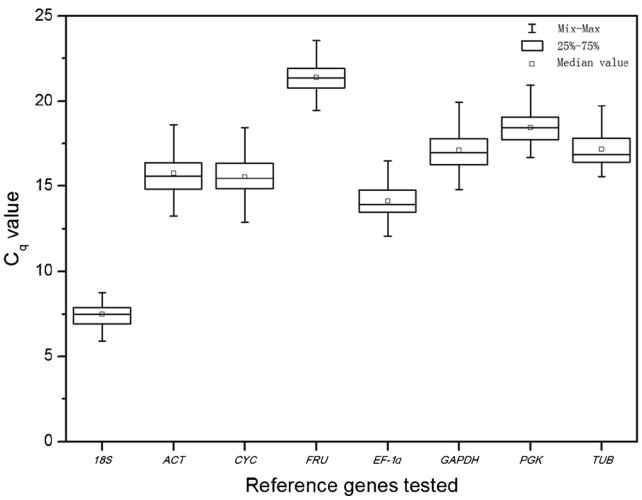

Fig. 1 Box chart showing the C_q variation of each candidate reference gene among all samples

Expression Stability of the Candidate Reference Genes geNorm Analysis

We analyzed the gene expression stabilities of the eight reference genes in all of the designated conditions using the geNorm software established by Vandesompele et al. (2002). When all the results from all samples of *A. belladonna* were combined, *ACT* and *PGK* had the lowest M value and *FRU* had the highest *M* value. Therefore, *ACT* and *PGK* were the most stably expressed, and *FRU* was the least stably expressed (Fig. 2a). For the different organs including leaf, stem, and root, *GAPDH* and *FRU* showed the most stable expression, and *CYC* showed the least stable expression (Fig. 2b). *PGK* had the lowest *M* value in leaves, even under heat or cold treatment, which suggested that *PGK* was an ideal reference gene for qPCR analysis of leaves under temperature treatment (Fig. 3i, k). *ACT* had the lowest *M* value in roots treated with heat or cold, which suggested that *ACT* was an ideal reference gene for qPCR analysis of roots under temperature treatment (Fig. 3j, l).

Under treatment with plant hormones, the candidate reference genes showed different expression patterns. *ACT* and *PGK* had lower *M* values than the other genes in the leaves of *A. belladonna* respectively treated with plant hormones MeJA, SA, and ABA (Fig. 4o, q, s). Thus, *ACT* and *PGK* were the most stably expressed reference genes. When the data collected from the plant hormone treatments in leaves were combined, *ACT* and *PGK* still showed lower *M* values (Fig. 4m). *CYC* and *TUB* had the lowest *M* values in plant-hormone-treated roots, which suggested that *CYC* and *TUB* were the most stably expressed reference genes among the eight candidate reference genes (Fig. 4p, r, t). *CYC* and *TUB* also had the lowest *M* value when the data collected from roots treated with plant hormones were combined. Combining the results for leaves and roots, *PGK* was the most stably expressed reference gene under hormone treatments (Fig. 4m, n).

During salt stress caused by NaCl, *TUB* had the lowest *M* value in leaves and roots (Fig. 2c, d), which suggested that *TUB* could be used as a reference gene in qPCR analysis of salt-treated leaves and roots. Furthermore, *GDPDH* had the second lowest *M* value of all genes, meaning that it could also be considered as a authentic reference gene during salt treatment (Fig. 2c, d). During treatment with UV-B, *PGK* and *18S*

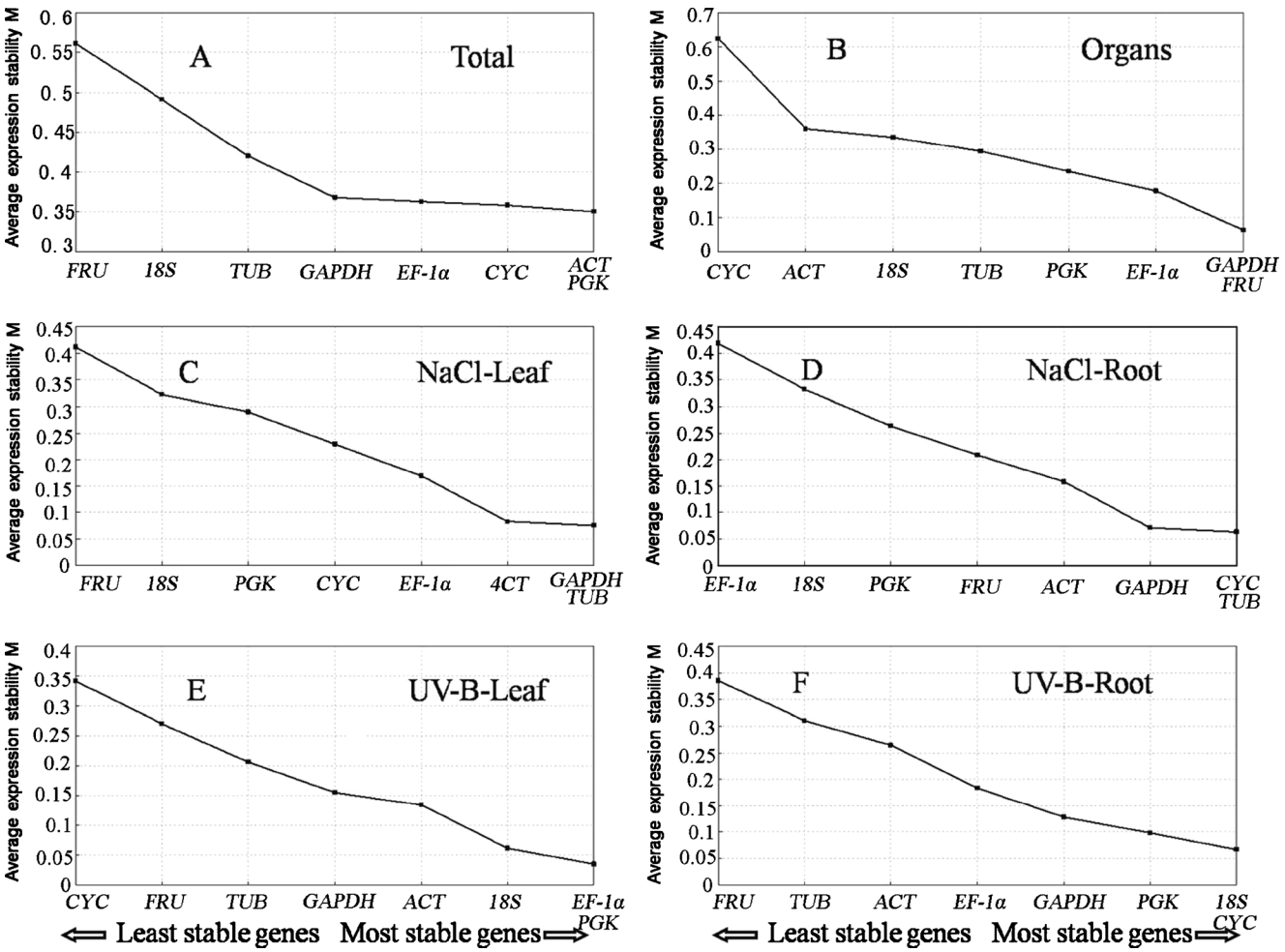

Fig. 2 Average expression stability values (*M*) of the reference genes in leaves and roots under salt and UV stresses, as determined using geNorm

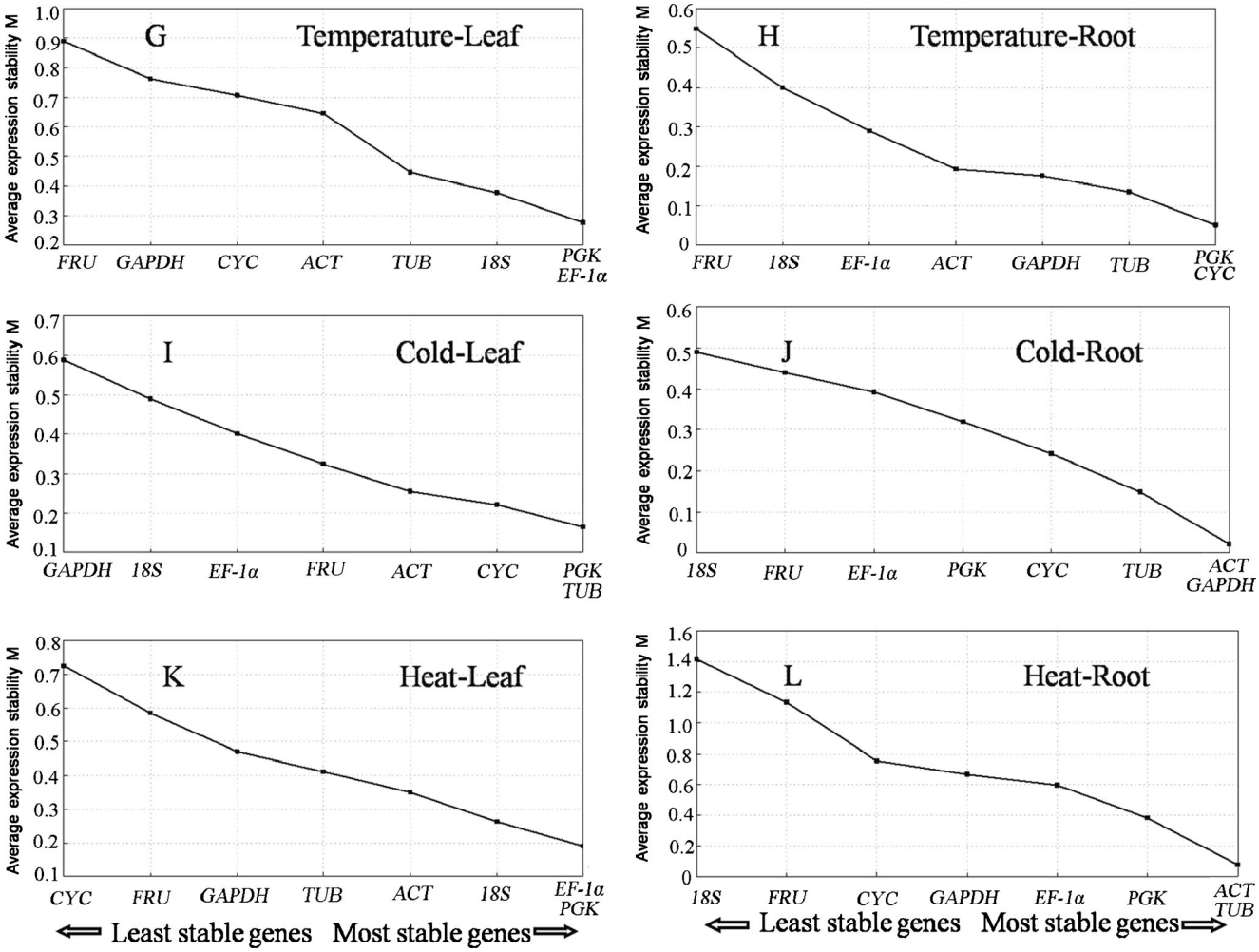

Fig. 3 Average expression stability values (*M*) of the reference genes at different temperatures, as determined using geNorm

had the lowest *M* values, meaning that both of them could be used as reference genes (Fig. 2e, f).

The pairwise variation of $V_{2/3}$ or $V_{3/4}$ in heat-treated roots was higher than 0.15, while that of $V_{4/5}$ was lower than 0.15. This suggested that four reference genes should be used in the qPCR analysis of gene expression in heat-treated roots (Fig. 5). The pairwise variation of $V_{2/3}$ for all the other samples was lower than 0.15, which suggested that two reference genes would be sufficient for qPCR analysis.

NormFinder Analysis

NormFinder calculated the stability value of each gene (Table 2). Reference genes that are more stably expressed are indicated by lower average expression stability values (Ma et al. 2013). When all the results from all samples of *A. belladonna* were combined, *ACT* showed the lowest stability value (0.130), *TUB* and *PGK* showed the next lowest stability values (0.147 and 0.241, respectively) and *CYC* showed the highest stability value (0.405; Table 2). Therefore, *ACT* had the most stable expression, which was consistent with the result given by geNorm (Fig. 2a), and *CYC* the least stable expression. In the different organs, *PGK* showed the greatest stability of expression. *EF-1α* and *TUB* were more stably expressed than *FRU*, *18S*, *ACT*, *GAPDH*, and CYC, and *CYC* showed the least stable expression (Table 2). In the cold/heat treatment in leaves or in roots of *A. belladonna*, *PGK* had the lowest stability value and *FRU* had the highest stability value (Table 2). This meant that *PGK* was the most stably expressed gene under different temperatures in leaves and roots, indicating *PGK* might be the ideal reference gene *FRU* was the least stably expressed gene, indicating that is was not suitable as a reference gene under different temperature treatments. This conclusion was consistent with the result provided by geNorm (Fig. 3g, h).

In the leaves treated with plant hormones, *PGK* and *ACT* had the lowest stability values (0.073 and 0.078, respectively), which suggested that both *PGK* and *ACT* were the most stably

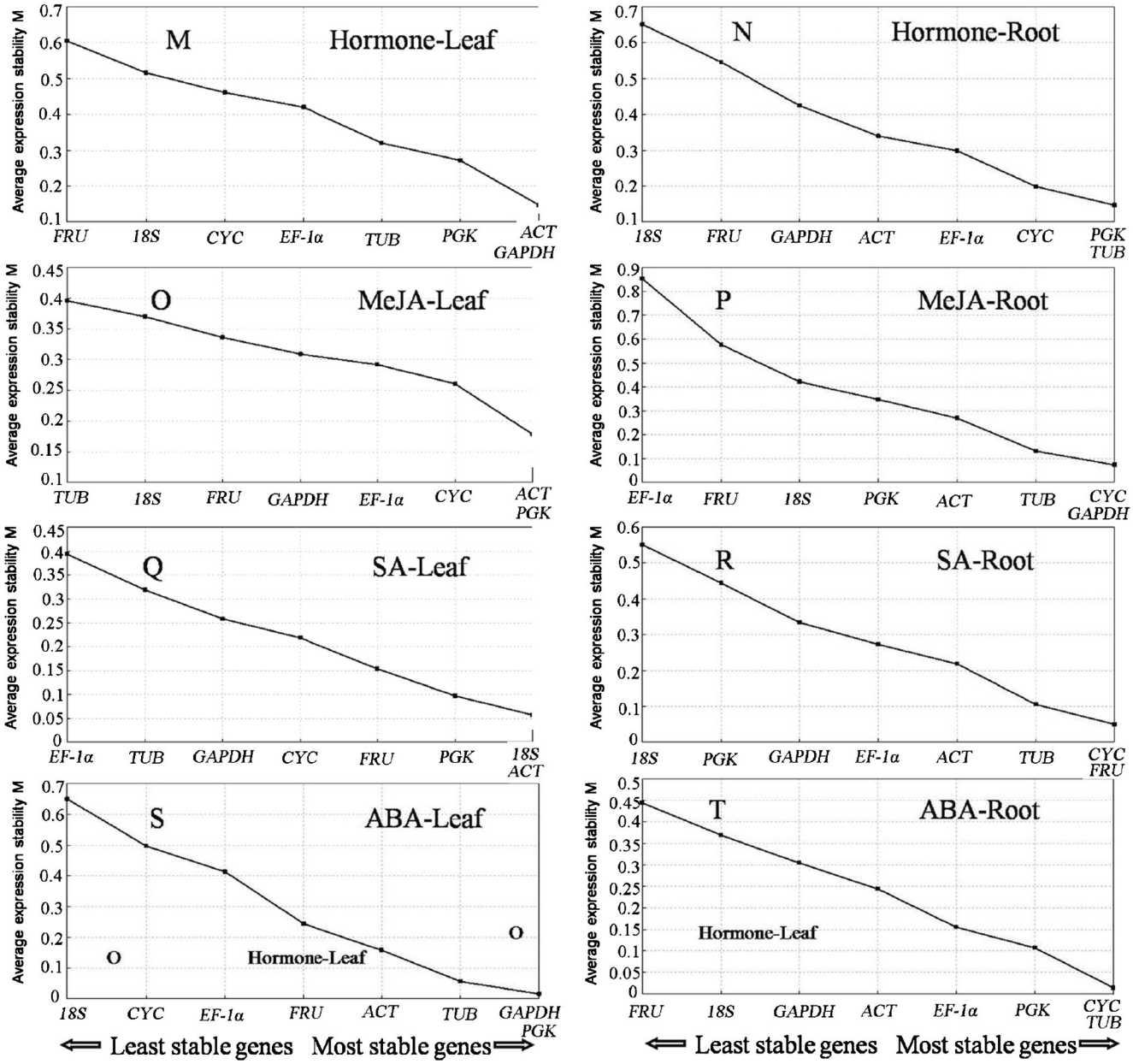

Fig. 4 Average expression stability values (*M*) of the reference genes in different tissues, as determined using geNorm

expressed genes; *FRU* was the least stably expressed gene, according to its highest stability value (Table 2). The results of NormFinder were very similar to those provided by geNorm (Fig. 4m). In the plant-hormone-treated roots of *A. belladonna*, *PGK* and *TUB* had the same low stability values (0.050), and *FRU* had the highest stability value (0.622). According to the stability values, *PGK* and *TUB* were the most stably expressed genes that could be selected as the suitable reference genes in plant-hormone-treated roots. The results from NormFinder were the same as those from geNorm (Fig. 4n).

In NaCl-stressed leaves of *A. belladonna*, *GAPDH* and *TUB* showed the lowest stability values, suggesting stable expression, while the high stability value of *FRU* indicated that its expression was the least stable. These results agreed with those of geNorm (Fig. 2c). In NaCl-treated roots, *ACT* and *TUB* had the lowest stability values, suggesting they were more stably expressed genes. *EF-1α* was the least stably expressed gene with the highest stability value. These results were very similar to those of geNorm (Fig. 2d).

In the leaves treated with UV-B, NormFinder analysis showed that *PGK* and *EF-1α* had the same low stability value (0.012) and *CYC* had the highest stability value (0.359). These results suggested that *PGK* and *EF-1α* were the most stably expressed genes and *CYC* was the least stably expressed gene. The same results were provided by geNorm (Fig. 2e). In the

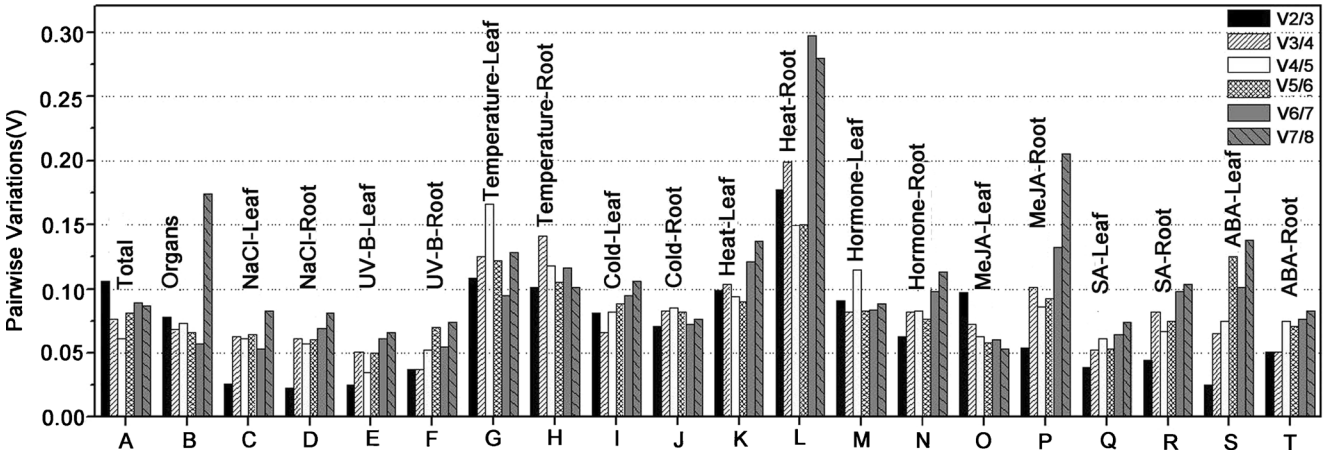

Fig. 5 Determination of the optimal number of reference genes for normalization by pairwise variation using geNorm

roots treated with UV-B, *GAPDH* and *18S* showed the most stable expression and *FRU* showed the least stable expression, which were very similar to the results provided by geNorm (Fig. 2f).

BestKeeper Analysis

The BestKeeper applet calculates gene expression variation for candidate genes based on the C_q values of each candidate gene. The coefficients of variance (CVs) and standard deviations (SDs) are shown in Table 3. When the results from all samples of *A. belladonna* were combined, *PGK* and *ACT* showed the lowest CV±SD value (3.71±0.70 and 4.47±0.95, respectively); *18S* showed the highest CV±SD value (9.06±0.71). Therefore, *ACT* and *PGK* had the most stable expression (Table 3). These results were the same as those provided by geNorm (Fig. 2a) and very similar to those of NormFinder (Table 2). In the different organs, the *TUB* and *EF-1α* genes showed the most stable expression, and *CYC* showed the least stable expression (Table 3). These results were very similar to those obtained using NormFinder (Table 2).

In the cold- and heat-treated leaves or roots, *PGK* was the most stably expressed gene, with the lowest CV value,

Table 2 Ranking of candidate reference genes in order of their expression stability as calculated by NormFinder

Rank Total	1 *ACT*	2 *TUB*	3 *PGK*	4 *18S*	5 *GAPDH*	6 *FRU*	7 *EF-1α*	8 *CYC*
Stability value	0.130	0.147	0.241	0.280	0.289	0.326	0.333	0.405
Organs	*PGK*	*EF-1α*	*TUB*	*FRU*	*18S*	*ACT*	*GAPDH*	*CYC*
Stability value	0.087	0.167	0.194	0.205	0.212	0.223	0.265	0.963
Temperature—leaf	*PGK*	*EF-1α*	*CYC*	*ACT*	*TUB*	*GAPDH*	*18S*	*FRU*
Stability value	0.085	0.229	0.243	0.360	0.375	0.415	0.437	0.787
Temperature—root	*PGK*	*CYC*	*GAPDH*	*EF-1α*	*TUB*	*ACT*	*18S*	*FRU*
Stability value	0.127	0.181	0.217	0.298	0.422	0.435	0.526	0.543
Hormone—leaf	*PGK*	*ACT*	*TUB*	*GAPDH*	*18S*	*CYC*	*EF-1α*	*FRU*
Stability value	0.073	0.078	0.169	0.199	0.319	0.414	0.418	0.480
Hormone—root	*TUB*	*PGK*	*CYC*	*EF-1α*	*ACT*	*GAPDH*	*18S*	*FRU*
Stability value	0.050	0.050	0.139	0.185	0.224	0.315	0.524	0.622
NaCl—leaf	*GAPDH*	*TUB*	*ACT*	*18S*	*PGK*	*EF-1α*	*CYC*	*FRU*
Stability value	0.026	0.032	0.082	0.163	0.177	0.255	0.286	0.458
NaCl—root	*ACT*	*TUB*	*CYC*	*FRU*	*GAPDH*	*PGK*	*18S*	*EF-1α*
Stability value	0.038	0.067	0.092	0.096	0.148	0.304	0.322	0.442
UV-B—leaf	*PGK*	*EF-1α*	*18S*	*ACT*	*GAPDH*	*TUB*	*FRU*	*CYC*
Stability value	0.012	0.012	0.019	0.081	0.122	0.239	0.297	0.359
UV-B—root	*GAPDH*	*18S*	*CYC*	*PGK*	*TUB*	*ACT*	*EF-1α*	*FRU*
Stability value	0.041	0.075	0.015	0.161	0.192	0.218	0.280	0.406

indicating that it was the ideal reference gene; *18S* was the least stably expressed gene with the highest CV value, indicating that it should not be used as the reference gene. In the plant-hormone-treated leaves, *PGK* also showed the most stable expression and the lowest CV value; *18S* also showed the least stable expression and the highest CV value. In the plant-hormone-treated roots, all three algorithms (geNorm (Fig. 4n), NormFinder (Table 2), and BestKeeper (Table 3)) indicated that *TUB* was the most stably expressed gene and represented the ideal reference gene. *FRU* was the least stably expressed genes in the plant-hormone-treated roots of *A. belladonna*.

In NaCl-treated leaf samples, *TUB* and *GAPDH* had the lowest CV values (0.84±0.13 and 1.28±0.22, respectively), which indicated the most stable expression among the eight reference genes, while *18S* was the least stably expressed gene with the highest 'CV value' (Table 3). This result was the same as that obtained by geNorm (Fig. 2c) and very similar to that provided by NormFinder (Table 2). In the roots treated with NaCl, *CYC* was the most stably expressed gene with the lowest CV value, which was the same as the result given by geNorm (Fig. 2d). *18S* and *EF-1α* were the least stably expressed genes with the highest CV values, which were very similar with the results provided by geNorm (Fig. 2d) and NormFinder (Table 2).

In the leaves treated by UV-B, all three algorithms (geNorm (Fig. 2e), NormFinder (Table 2), and BestKeeper (Table 3)) suggested that *PGK* was the most stably expressed gene and *CYC* was the least stably expressed gene. In the UV-B-treated roots, *GAPDH* had the lowest CV value and was the most stably expressed gene, which was consistent with the results from NormFinder (Table 2).

Validation of the Reference Genes

Putrescine *N*-methyltransferase is the first committed-step enzyme in the TAs biosynthesis pathway (Biastoff et al. 2009). The expression of *A. belladonna* PMT gene (*AbPMT*) is root specific, and MeJA treatment did not affect its expression level (Suzuki et al. 1999a, b). Thus, the relative expression level of *AbPMT* was detected to validate the *PGK* gene as a reliable reference gene. The expression of *AbPMT* was detected only in roots (Fig. 6a) when *PGK* was used as the reference gene, and this was consistent with the result reported by Suzuki et al. (1999a, b). The expression of *AbPMT* was also detected only in roots when *CYC* (the least reliable reference gene) was used as the reference gene (Fig. 6b).

The expression of *AbPMT* was not significantly affected by treatment with MeJA (Fig. 6c) when PGK was used as the reference gene. This was consistent with previous Northern blot results (Suzuki et al. 1999a, b). When *FRU* (the least reliable reference gene in plant hormone treatments) was used as the reference gene, the expression of *AbPMT* appeared to be significantly decreased ($P<0.01$) during MeJA treatment (Fig. 6d). This was not consistent with the result reported by Suzuki et al. (1999a, b). Obviously, under these experimental conditions, *PGK* was a reliable reference gene.

Discussion

Studying gene expression is a basic method to understand biological processes, and real-time qPCR is a potent and sensitive tool for detecting gene expression levels. For qPCR to be accurate, the specific PCR conditions and the internal reference genes must be optimized (Nicot et al. 2005). A reliable internal reference gene should not change its expression levels under different experimental conditions (Crismani et al. 2006). Thus, it is important to screen an appropriate internal control or reference gene for quantifying gene expression.

18S is the 40S ribosome subunit RNA component (Tong et al. 2009) and the *18S* transcript is highly abundant (Brunner et al. 2004). In earlier studies, *18S* was used as an internal reference gene. For example, *18S* was identified as the most reliable reference gene for normalization of real-time PCR data in rice (Kim et al. 2003) and Barley yellow dwarf virus-infected cereals (Jarosova and Kundu 2010). However, in *A. belladonna*, the expression of *18S* was unstable in most cases and it was not a reliable reference gene. Previous research also reported that *18S* had very poor expression stability in peach (Tong et al. 2009), pea (Die et al. 2010), and sunflower (Fernandez et al. 2011). Thus, *18S* was not recommended as a reference gene in *A. belladonna*.

PGK is related to glucose metabolic process (Rubie et al. 2005). *PGK* of tomato was frequently ranked as the more stable reference gene among when treated with Silicon, during *Ralstonia solanacearum* infection and over time (Ghareeb et al. 2011). That study was valuable for our work, because both tomato and *A. belladonna* belong to the Solanaceae family. In the present study, we found that *PGK* was stably expressed in many, but not all, cases. When all the samples were combined together for analysis, geNorm and BestKeeper indicated that *PGK* was the most stable reference gene, and NormFinder showed that *PGK* ranked in position 3. In the temperature-treated roots or leaves of *A. belladonna*, *PGK* was ranked in the top position by all the three software packages. In the hormone-treated leaves, *PGK* also ranked in the top position according to NormFinder and BestKeeper, and ranked at position 2 by geNorm. In the hormone-treated roots, *PGK* ranked in the top position according to NormFinder and geNorm and in position 3 according to BestKeeper. *PGK* was also ranked in the top position in UV-B stressed leaves according to all the three software packages. Taken together, the results strongly suggested that *PGK* was a reliable reference gene for analyzing gene expression in roots

Table 3 Ranking of candidate reference genes in order of their expression stability, as calculated by Bestkeeper

Rank	1	2	3	4	5	6	7	8
Total	*PGK*	*ACT*	*TUB*	*GAPDH*	*EF-1α*	*FRU*	*CYC*	*18S*
CV±SD	3.71±0.70	4.47±0.95	4.61±0.80	5.06±0.87	5.85±0.83	6.05±0.95	6.54±0.95	9.06±0.71
Organs	*TUB*	*EF-1α*	*FRU*	*ACT*	*GAPDH*	*PGK*	*18S*	*CYC*
CV±SD	1.72±0.28	1.76±0.25	1.91±0.43	2.65±0.41	2.78±0.46	3.18±0.57	4.68±0.33	8.00±1.19
Temperature—leaf	*PGK*	*EF-1α*	*FRU*	*TUB*	*CYC*	*ACT*	*GAPDH*	*18S*
CV±SD	1.57±0.30	2.44±0.36	3.04±0.67	3.33±0.58	4.08±0.66	4.34±0.70	4.45±0.80	5.48±0.42
Temperature—root	*PGK*	*CYC*	*ACT*	*TUB*	*EF-1α*	*GAPDH*	*FRU*	*18S*
CV±SD	1.56±0.33	3.23±0.44	4.04±0.64	4.45±0.76	4.78±0.88	5.18±0.86	5.47±0.84	6.75±0.50
Hormone—leaf	*PGK*	*ACT*	*GAPDH*	*TUB*	*CYC*	*FRU*	*EF-1α*	*18S*
CV±SD	1.66±0.31	2.10±0.30	2.27±0.39	2.32±0.39	2.68±0.41	2.91±0.61	3.43±0.64	5.36±0.43
Hormone—root	*TUB*	*EF-1α*	*PGK*	*ACT*	*CYC*	*GAPDH*	*18S*	*FRU*
CV±SD	3.49±0.64	4.31±0.63	4.49±0.86	4.64±0.79	4.79±0.79	4.97±0.83	6.25±0.49	5.81±1.26
NaCl—leaf	*TUB*	*GAPDH*	*CYC*	*EF-1α*	*PGK*	*ACT*	*FRU*	*18S*
CV±SD	0.84±0.13	1.28±0.22	1.48±0.21	1.70±0.24	1.78±0.32	2.86±0.43	2.95±0.63	4.99±0.35
NaCl—root	*CYC*	*GAPDH*	*FRU*	*ACT*	*TUB*	*PGK*	*EF-1α*	*18S*
CV±SD	0.91±0.13	1.09±0.18	1.11±0.24	1.24±0.20	1.77±0.29	1.84±0.32	2.92±0.39	5.56±0.40
UV-B—leaf	*PGK*	*GAPDH*	*EF-1α*	*ACT*	*FRU*	*18S*	*TUB*	*CYC*
CV±SD	1.31±0.25	1.31±0.26	1.40±0.21	1.43±0.22	1.46±0.25	2.07±0.14	2.29±0.40	3.01±0.48
UV-B—root	*TUB*	*PGK*	*ACT*	*18S*	*CYC*	*FRU*	*GAPDH*	*EF-1α*
CV±SD	3.67±0.68	4.67±0.90	4.82±0.83	4.83±0.38	5.68±0.96	5.77±1.21	6.00±1.06	6.24±0.98

CV coefficient of variance, *SD* standard deviation

or leaves treated by temperature or plant hormones. However, *PGK* was not a reliable reference gene in NaCl-treated roots and leaves: none the three software packages ranked *PGK* in the top 4 positions. In tomato and rice, *PGK* also responded to salt stress (Manaa et al. 2011; Nam et al. 2012).

ACT plays a significant role in cytoskeleton contraction (Tong et al. 2009), and has also been widely used as the reference gene. However, it was also frequently reported that *ACT* exhibited highly variable expression levels in humans (Pombo-Suarez et al. 2008), animals (Ingerslev et al. 2006; Goossens et al. 2007; Hibbeler et al. 2008), and plant tissues (Jain et al. 2006). In the present study, both geNorm and NormFinder indicated that *ACT* was an alternative reliable reference gene in *A. belladonna* when all the samples were combined, and BestKeeper supported that *ACT* was the second-most stable reference gene. geNorm, NormFinder, and BestKeeper were used to analyze the expression of *ACT* in *Cichorium intybus* (Maroufi et al. 2010) and faba bean (Gutierrez et al. 2011); the results indicated that *ACT* was the most stably expressed gene under different experimental conditions in *C. intybus* and faba bean. In addition, *ACT* was a stable reference gene in *Coffea* under drought stress (Goulao et al. 2012). However, in under specific experimental conditions, *ACT* was not a suitable candidate reference gene. For example, *ACT* ranked in position 6 in different organs without treatment, and in positions 4 and 6 in temperature-treated leaves and roots, respectively.

EF-1α is associated with protein translation (Thornton et al. 2003), and studies have shown that *EF-1α* was the best reference gene in salmon (Ingerslev et al. 2006) and in several other plants, such as rice, *C. intybus* (Maroufi et al. 2010), potato (Nicot et al. 2005), and ryegrass (Lee et al. 2010). In the untreated different organs of *A. belladonna*, *EF-1α* ranked in position 2 of the eight reference genes according to geNorm, NormFinder, and BestKeeper, indicating that it may be a reliable reference gene for analyzing gene expression in *A. belladonna* organs without treatment.

In the untreated different organs without treatment, *FRU* was the most reliable reference gene according to geNorm. However, *FRU* ranked in positions 3 and 4 according to BestKeeper and NormFinder, respectively. *TUB* is structural constituent of the cytoskeleton (Tong et al. 2009). In leaves of hybrid roses, *TUB* was the least stable reference gene (Klie and Debener 2011). However, *TUB* was a reliable reference gene in poplar (Brunner et al. 2004) and *Eucalyptus* species (de Almeida et al. 2010). In this study, *TUB* showed the highest expression stability in cold/NaCl-treated leaves, according to geNorm. In the plant-hormone-treated roots, *TUB* was the most stable reference gene according to geNorm, NormFinder, and Bestkeeper.

The analysis in geNorm, NormFinder, and BestKeeper showed some differences. For instance, in the combined samples, *PGK* ranked in the top position according to geNorm and BestKeeper, but ranked in position 3 according to

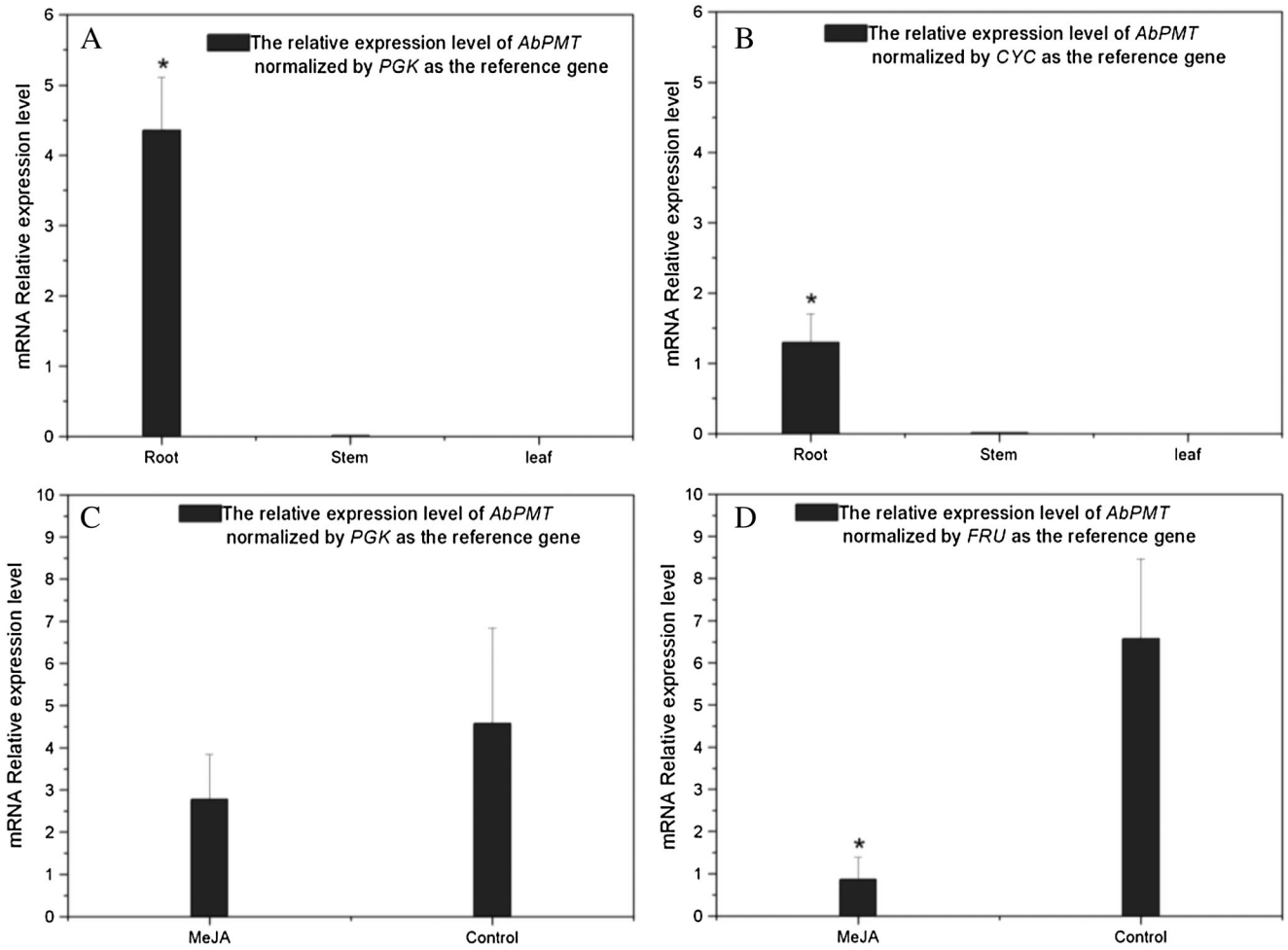

Fig. 6 Validation of the reference genes by detecting the relative expression level of *AbPMT*. *Asterisks* represents a significant difference at the leave of $P<0.01$

NormFinder. Many other studies also found that geNorm, NormFinder, and BestKeeper gave different rankings of gene expression stability for the same gene (Chang et al. 2012; Klie and Debener 2011; Mallona et al. 2010). This apparent divergence probably reflected differences in the statistical algorithms (Andersen et al. 2004; Pfaffl et al. 2004; Vandesompele et al. 2002). The differences in the statistical algorithms of geNorm, NormFinder, and BestKeeper have been discussed in other publications. Our studies also demonstrated that the reference gene rankings in all samples were different from those in subsets. For example, when all samples were considered, *PGK* and *ACT* ranked in the top position according to geNorm; but in different organs, geNORM ranked *GAPDH* and *FRU* in the top position. This suggested that the selection of a reliable reference gene should not solely depend on the results of an all-sample analysis, because all sample analysis includes too many factors. It was necessary to assay the stability of the expression of the candidate genes in a specific biological assay to select suitable reference genes for reliable normalization.

To the best of our knowledge, this article is the first study to describe the assessments of a set of commonly used candidate reference genes in *A. belladonna* that could be used for the normalization of gene expression analysis using qPCR. We tested eight candidate reference genes for their suitability for the normalization of qPCR data in *A. belladonna* root, stem, and leaf samples subjected to treatments with plant hormones, temperatures, NaCl, and UV-B. geNorm, NormFinder, and BestKeeper indicated that *PGK* was a reliable reference gene to normalize gene expression in most of the experimental conditions. The validation of *PGK* as a reliable reference gene was also confirmed by detecting the relative expression level of the *PMT* gene of *A. belladonna*. The pairwise value analysis shows that two genes were sufficient for proper expression normalization, except when analyzing gene expression in heat-treated roots. However, the second reference gene selected according to the specific conditions. In leaves treated with different temperatures, *EF-1α* is more stable. In roots treated with different temperatures, *CYC* expression is relatively stable and is a trusted reference gene. In leaves treated with

different plant hormones, *ACT* is a relatively stable reference gene. In roots treated with different plant hormones, the expression of *TUB* is more stable. In different organs in *A. belladonna*, *EF-1α* a reliable reference gene. In leaves treated with UV-B, the expression of *EF-1α* is stable. In roots treated with UV-B, *CYC* is relatively stable.

Our results not only provide researchers with a shortlist of potential reference genes to use for normalization in qPCR experiments, but may also act as a guide for the selection of appropriate reference genes for gene expression studies in *A. belladonna* in different organs and abiotic stress conditions, or in other plant species under similar treatment conditions.

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