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ORIGINAL ARTICLE

Validation of suitable reference genes for quantitative gene expression analysis in *Tripterygium wilfordii*

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Abstract

Validation of suitable reference genes is critical in quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Suitable and reliable reference genes for the normalization of gene expression data are characterized by high gene expression stability across tissues and different experimental conditions. This study evaluated the gene expression stability of ten reference genes commonly used in *Arabidopsis thaliana* for their suitability in qRT-PCR analysis in *Tripterygium wilfordii* Hook.f. The orthologous sequences of these ten candidate genes were identified from *T. wilfordii* transcriptomic data (Project No. SRX472292). Five algorithms including GeNorm, NormFinder, BestKeeper, ΔCt, and RefFinder were used to assess the gene expression stability of these putative reference genes in different plant tissues and different stress conditions. The results identified *ACTINT7* and *TBP* as the most suitable reference genes across all samples. The gene expressions of *TwHMGR* (3-hydroxy-3-methylglutaryl coenzyme A reductase, KU246037.1) and of *TwDXR* (1-deoxy-D-xylulose-5-phosphate reductoisomerase, KJ174341.1) were investigated to validate the suitability of the reference genes. The validation analysis confirmed the suitability of *ACTINT7* and *TBP* as the best reference genes for elucidating secondary metabolite biosynthesis pathway in *T. wilfordii*. In summary, this study identified the most suitable and reliable reference genes for future qRT-PCR-based studies in *T. wilfordii*.

Keywords *Tripterygium wilfordii* · qRT-PCR · Reference gene · Gene expression

Introduction

Tripterygium wilfordii Hook.f. is a herb used in Traditional Chinese Medicine which has a diverse range of bioactivities and thus, has been used in the treatment of an array of ailments including inflammation, systemic lupus erythematosus and rheumatoid arthritis for hundreds of years [1–3]. About 415 secondary metabolites were isolated from *T. wilfordii*, mainly terpenoids and alkaloids [4]. Triptolide, a main diterpenoid epoxide, has been reported to have immunosuppressive and anticancer activities [5–7]. Sesquiterpene pyridine alkaloids (such as wilforgine, wilforine, wilfortrine and wilfordine) have immunosuppressive, antitumor-promoting and cytotoxic, antiviral and anti-inflammatory properties [8–12]. Traditionally, the secondary metabolites such as terpenoids and alkaloids are obtained by extraction from the root of *T. wilfordii*. However, *T. wilfordii* is not widely cultivated and the content of secondary metabolites in natural plants is far below demand. Therefore, it is necessary to understand the secondary metabolites biosynthetic pathway for producing the compound through synthetic biology strategies.

The selection of target genes for the genetic manipulation of plants towards the production of bioactive metabolites often relies on quantitative transcription reverse polymerase chain reaction (qRT-PCR) analysis [13]. The qRT-PCR data of target genes is normalized against that of reference genes that, in turn, must be characterized by high levels of stability of gene expression, across different developmental stage and different experimental settings. This data normalization procedure mitigates many sources of experimental

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errors associated with the qRT-PCR method [14, 15]. Ideal reference genes should be stable and reliable throughout the diverse experimental conditions. However, no single reference genes have been shown to be universally constant during development and diverse experimental conditions. Furthermore, many studies have shown that traditionally used popular housekeeping genes used to normalize gene expression vary considerably under different conditions [16–21].

To elucidate biosynthesis pathway of bioactive metabolites, accurate quantification of key genes expression is needed which will help us to understand gene function in secondary metabolite biosynthesis. qRT-PCR has become the preferred method due to its high sensitivity, specificity, and accuracy in detecting the target gene expression. In previous studies, many reference genes were used to study the secondary metabolites biosynthesis for *T. wilfordii*, including elongation factor 1-α (*EF-1α*) [22–25], *ACTIN7* [26], *β-Actin* [27–31], polypyrimidine tract-binding protein (*PTB*) [32].

Until now, no systematic validation of reference genes has been performed in *T. wilfordii*. Therefore, we evaluated the expression stability of ten genes (*PP2A, TBP, TUB, 26S, SAND, GAPDH, TIP41, eIF-4α, EF-1α,* and *ACTINT7*) among the tissues of *T. wilfordii* and under a range of abiotic stresses, such as drought, salinity, and methyl jasmonate (MeJA) and salicylic acid (SA) in *T. wilfordii* hairy roots by qRT-PCR. To validate the reliability of the reference genes, the expression levels of the *TwHMGR* and *TwDXR*, which encode for enzymes that participate in the isoprenoid biosynthesis pathways in *T. wilfordii*, were used to assess the performance of the newly selected reference genes to confirm their suitability for qRT-PCR analysis in *T. wilfordii*.

Materials and methods

Plant materials and experimental treatments

Tripterygium wilfordii plants were grown in the greenhouse of Northwest A&F University which transferred from a natural growing in Fujian Province of China. 0.8–1 g of roots, stems and leaves were collected randomly each from six cottage plantlets with the same growth and development status, each tissue part was repeated three times. The abiotic stress treatments were performed on hairy roots (HRs) of *T. wilfordii*. The culture of HRs was carried out as described previously [33]. Stock solutions of MeJA and SA were prepared and added to the final media (0.1 mM) [34]. The samples were collected from plants exposed to different stress treatments after 0, 3, 6, 9, 12, and 24 h for follow-up analysis. Salinity stress was induced by the addition of NaCl to the media to the final concentration of 150 mM. Drought stress was induced by the addition of PEG 8000 (20% w/v) to the media [35]. The different plant tissues were collected, immediately frozen in liquid nitrogen, and kept at − 80 °C for further analysis. All the treatments were gathered in three biological replications.

RNA extraction for cDNA synthesis

RNA isolation was performed according to Miao et al. with minor modifications [36]. The extraction buffer contained the following: 2 M NaCl, 200 mM Tris (pH 8.0), 25 mM EDTA, 2% CTAB (w/v), 2% PVPP (w/v) as well as 5% β-mercaptoethanol (v/v). RNA concentration and purity were measured using an M200 Pro-multimode reader (Tecan, Switzerland). RNA integrity was further assessed by electrophoresis using 1% agarose gels. For each sample, about 300 ng of total RNA was treated with gDNA Eraser (Takara, Japan) and used to make cDNA with PrimeScript™ RT (Takara, Japan).

qRT-PCR conditions

The qRT-PCR analysis was performed in an iCycler iQ™ Real-Time PCR Detection System (BioRad, USA). Each reaction contained nuclease-free water (9.5 µL, Takara, Japan), SYBR Premix Ex Taq™ II (12.5 µL), forward primer (1 µL, 10 µM) and reverse primer (1 µL, 10 µM), and cDNA (1 µL, unquantified). The cycling condition was: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 52 °C for 30 s and 72 °C for 30 s followed by 95 °Cfor 10 s. Controls for reverse transcription were included in the PCR reactions to reveal any DNA contamination. Each PCR analysis was performed with three biological replicates and three technical replicates and the mean values were used for the analysis. Amplification efficiency (E) and correlation coefficient (R^2) were of primers were calculated by standard curve method, and all the samples were diluted five times. The E value is calculated by formula (% Efficiency = $(10^{[-1/\text{slope}]} - 1) \times 100\%$) [37].

Selection of candidate reference genes and primer design

Ten reference genes, frequently used in *Arabidopsis* studies, were investigated in this study: protein phosphatase 2A (*PP2A*), TATA-box binding protein (*TBP*), TUB beta-7 (*TUB*), ribosomal RNA 26S (*26S*), SAND family protein (*SAND*), Glyceraldehyde-3-phosphate Dehydrogenase (*GAPDH*), Tap42-interacting protein (*TIP41*), eukaryotic translation initiation factor 4a − 1 (*eIF-4α*), elongation factor − 1α (*EF-1α*) and Actin 7 (*ACTINT7*) study (Table 1) [38, 39]. The sequences of these Arabidopsis genes were blasted against the root transcriptome (the accession number SRX472292) of *T. wilfordii*. qRT-PCR primers were

Table 1 Candidate reference genes and target genes description and primer sequences

Gene	GeneBank accession number	Primer sequence (5′–3′) forward/reverse	Amplicon length (bp)	Amplicon Tm (°C)	Efficiency (%)	R^2
PP2A	GAVZ01053124	AAGGCCATCAACCACATC/AGGCACGGA CAAGGAACA	148	52	98.2	0.997
TBP	GAVZ01054868	ATCGCAGGAACCAACAAT/ACTGGCGGCTAG GAAGTA	97	52	97.5	0.990
TUB	GAVZ01053165	CATTTGCTGGGTCAGTTC/ATGGTAGGGTTT GCTCCT	81	51	103.7	0.993
26S	GAVZ01034851	TTTGCCGACTTCCCTTGC/TGGTGTCCGATG CGTTCC	144	59	107.6	0.995
SAND	GAVZ01053950	TTGTGACTTGGGCTTGAT/TCTTCCAACCAC CAGCAA	142	51	93.1	0.998
GADPH	GAVZ01033826	CGGTGCTCTGACGTTTCT/ACCCTTTGGCTG TATCCC	113	54	95.8	0.995
TIP41	GAVZ01049133	GCTTCTGTTCCCACTGCT/TTAGACGATGGA CGCCAC	103	53	104.2	0.994
eIF-4α	GAVZ01049152	TCTGCAACAATGCCACCT/AAACTGCTTGAT ACCCTCC	111	52	96.3	0.992
EF-1α	GAVZ01049902	TCCGTCTCCCTCTTCAGG/ACCACCATACCA GGCTTC	97	53	91.8	0.997
ACTIN7	GAVZ01051304	TGCCTGATGGACAGGTTA/AGTTGTAGGTAG TTTCGTGGA	117	52	97.4	0.993
Target gene						
*Tw*HMGR	GAVZ01053357	GGCTTCAAAGTGTTCATTGTT/TTCATCCCC ATTGCGTCA	115	50	102.0	0.991
*Tw*DXR	GAVZ01038138	GCTTTGAATTTGTCGCC/ATCCGCCTGAAT GCTTGA	101	52	96.1	0.994

designed based on the identified orthologous sequences for the ten putative reference genes using Primer Premier 5 software (amplicon length: 97–148 bp, and melting temperature (Tm): 51–59 °C).

Analysis of gene expression stability

Ten selected reference genes were compared regarding the stability of their gene expression with four algorithms: GeNorm, NormFinder, BestKeeper, and the ΔCt. Finally, RefFinder (http://www.fulxie.0fees.us), a comprehensive evaluation platform which integrates the four algorithms mentioned above, was used to produce the final ranks regarding their overall stability.

GeNorm method

The GeNorm algorithm assumes that two suitable reference genes must have a constant gene expression ratio across samples, and therefore, is a measure of variation between a single candidate reference gene and each of the target gene analyzed [40]. Suitable qRT-PCR reference genes must have expression stability values of $M < 1.5$. This algorithm is one of the most commonly used to assess the performance of putative reference genes.

NormFinder method

The NormFinder method measures stability value (SV) of gene expression for reference genes by measuring the variance in gene expression within and between groups [41]. According to the GeNorm method, genes with the lowest SV are less variable in terms of gene expression and thus, better reference genes.

Delta-Ct (ΔCt) method

The Delta-Ct (ΔCt) method [42] is somewhat similar to the GeNorm method because it compares gene expression (ΔCt values) within a group to that of other groups. The reference gene that produces the lowest standard deviation (SD) across all samples is considered the most stable.

BestKeeper method

The BestKeeper method estimates Cq values for all candidate reference genes by measuring the coefficient of

variation (CV) and the standard deviation (SD) for each reference gene. The most stable reference gene will exhibit the lowest Cq values [43].

RefFinder method

RefFinder analyzes the rankings provided by the GeNorm, NormFinder, BestKeeper and ΔCt algorithms and calculates a geometric mean for each gene. The best reference genes are the ones with the lowest geometric mean values [44].

Validation of reference gene analysis

TwHMGR (KU246037.1/GAVZ01053357.1) and *TwDXR* (KJ174341.1/GAVZ01038138.1) encode for enzymes that play a role in the biosynthesis of isoprenoids in *T. wilfordii* [26, 33, 45]. Variation in the transcript levels of these genes were used to validate the performance of the previously identified best reference genes. To evaluate the validity of the selection of reference genes, the expression levels of the *TwHMGR* and *TwDXR* genes were analyzed in different tissues and MeJA-induced abiotic stress treatments. For each experimental condition, the expression of *TwHMGR* and *TwDXR* were normalized using the two best reference genes (*ACTIN7* and *TBP*), and a relatively unstable gene (*TUB*) recommended by RefFinder. RT-qPCR was performed as described above. The average Ct value was calculated from three biological and technical replicates and was used for relative expression analyses. The relative expression of the *TwHMGR* and *TwDXR* gene was subsequently calculated according the $2^{-\Delta\Delta CT}$ method [46]. The expression levels of target genes were assessed using one-way ANOVA using SPSS v.17.0 with a signifcance level set at $P < 0.05$.

Results

Primer specificity and PCR efficiency

The nucleotide sequences of the ten candidate genes were obtained from the *Arabidopsis* genome database and blasted against a *T. wilfordii* transcriptome database (accession number SRX472292, http://www.ncbi.nlm.nih.gov/sra/SRX472292) in order to identify their corresponding homologs to be assessed as putative reference genes for *T. wilfordii*. (Table 1). PCR amplification produced a single band for each primer pair (each gene) as assessed by 2% agarose gel electrophoresis (Fig. 1a). The qRT-PCR melting-curve also showed a single peak corresponding to the expected size of the amplicon (Fig. 1b). These results strongly suggest that each primer pair was able to produce specific PCR amplified products of the target genes. Table 1 summarizes the primer sequences, amplicon length, amplicon temperature, correlation coefficient, and PCR amplification efficiency values. qRT-PCR amplification efficiency varied from 91.8% for *EF-1α* to 107.6% for *26S*, whereas the correlation coefficients varied from 0.990 for *TBP* to 0.998 for *SAND*.

Determination of quantification cycle (Cq) values

Quantification cycle values (Cq values) were obtained for the ten candidate reference genes, by performing qRT-PCR amplification reactions across the different *T. wilfordii* Hook.f. tissues and different abiotic stresses in root samples. The mean Cq values ranged from 16.25 to 32.98 across all samples for all the ten candidate reference genes. The *26S* showed the lowest Cq (mean Cq of 18.08), suggesting that this gene had the highest expression level of all compared putative reference genes. *EF-1α* and *ACTIN7* had slightly higher Cq values of 23.75 and 24.21 respectively, suggesting that these genes were also abundant transcripts. *E1F4α, TUB, TBP, SAND,* and *PP2A* had average Cq values between 24.25 and 28.35 and thus, were only moderately expressed. *TIP41* and *GADPH* had the highest Cq (mean Cq of 29.71 and 30.92) suggesting that these genes transcripts were the lowest in abundance. The mean and median Cq values were similar for each gene, especially in the case of *GAPDH* and *TBP* which suggests that the Cq values were evenly distributed. The Cq values of *EF-1α* and *TIP41* were distributed more centrally suggesting less variation across samples (Fig. 2).

Determination of the optimal number of reference genes

The GeNorm algorithm was used to estimate the number of reference genes necessary for accurate data normalization using pairwise variation analysis by measuring the ratio (Vn/Vn + 1) between two sequential normalization factors (NFn and NFn + 1). Figure 3 shows pairwise variation (V) values for the reference genes in which the value is lower than 0.15 as to be optimized. The results show that a combination of two candidate reference genes efficiently normalizes the qRT-PCR data across *T. wilfordii* tissues and different abiotic treatments. According to the GeNorm algorithm, the best combinations were *ACTINT7* + *TBP* across all samples, *TIP41* + *ACTINT7* across all tissues and across MeJA abiotic stress samples, *26S* + *ACTINT7* across SA abiotic stress samples, *TBP* + *eIF-4α* across drought stress samples and *TBP* + *EF-1α* across salinity stress samples.

Gene expression stability analysis of candidate reference genes

The gene expression stability, in different tissue samples and in different stress treatments, was determined for all putative

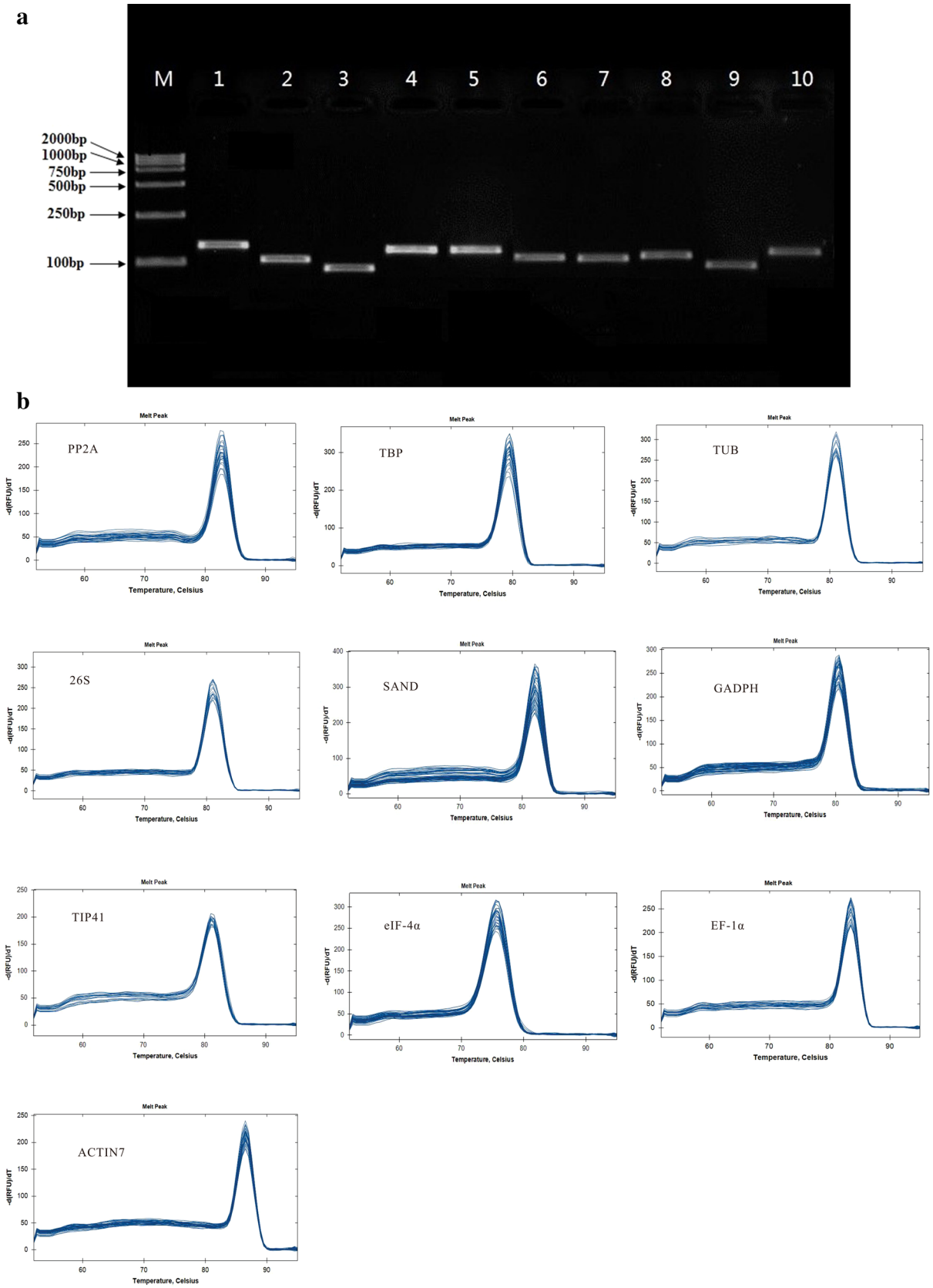


Fig. 1 Amplification products of the 10 genes. **a** Lanes 1–10 are *PP2A, TBP, TUB, 26S, SAND, GADPH, TIP41, eIF-4α, EF-1α and ACTIN7*, respectively.. M: Marker DL2000. **b** Melt curves of the ten reference genes

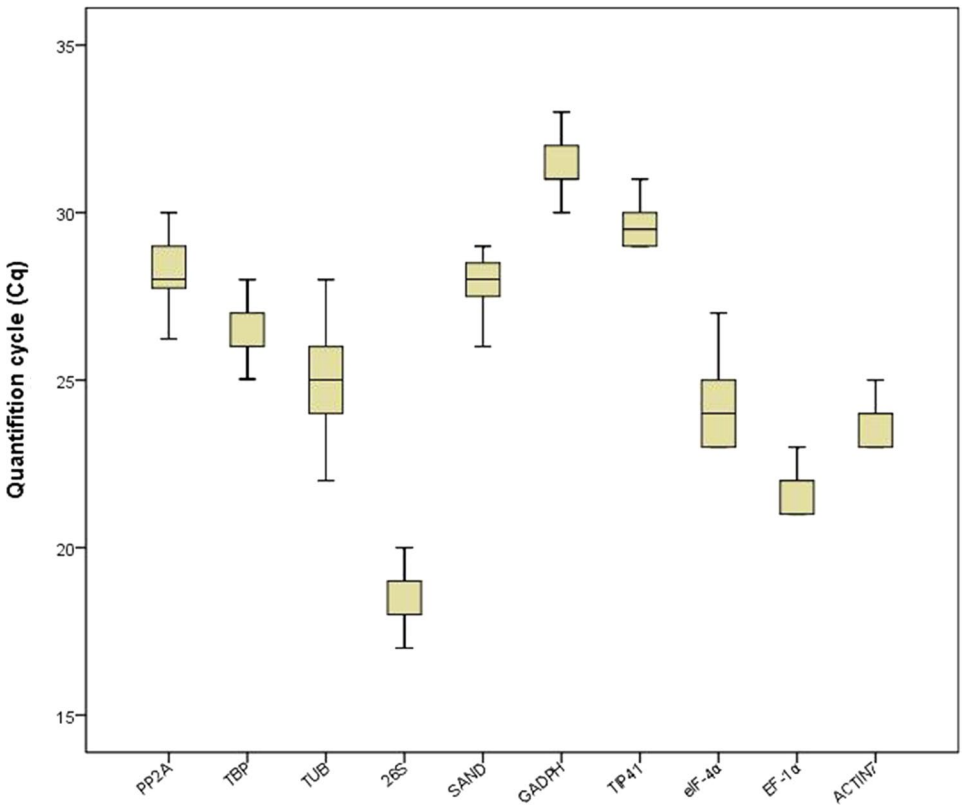
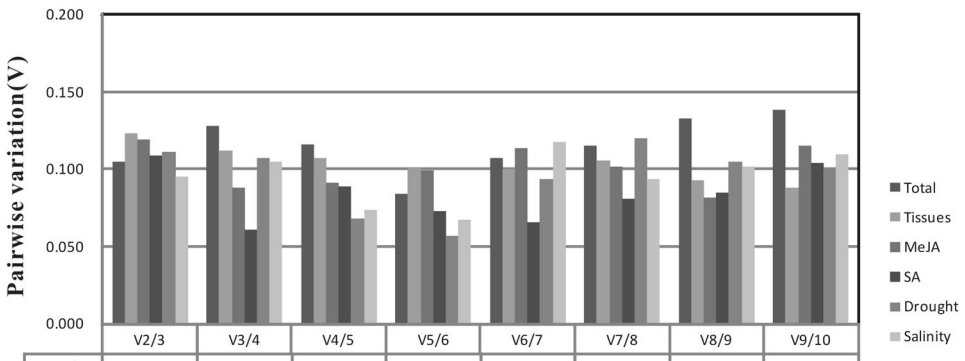


Fig. 2 Box and whisker plot displaying the Cp values for the candidate reference genes in all samples. The lower and upper edges of the box represent the 25th and the 75th percentiles, respectively; the line inside the box indicates the median value; the whiskers represent the maximum and minimum values



	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8	V8/9	V9/10
Total	0.105	0.128	0.116	0.084	0.107	0.115	0.133	0.138
Tissues	0.123	0.112	0.107	0.101	0.100	0.106	0.093	0.088
MeJA	0.119	0.088	0.091	0.099	0.114	0.102	0.082	0.115
SA	0.109	0.061	0.089	0.073	0.066	0.081	0.085	0.104
Drought	0.111	0.107	0.068	0.057	0.094	0.120	0.105	0.100
Salinity	0.095	0.105	0.074	0.067	0.118	0.094	0.102	0.110

Fig. 3 Determination of the optimal number of reference genes for normalization by pairwise variation (V) using GeNorm

reference genes and analyzed using GeNorm, NormFinder, ΔCt, and BestKeeper. Finally, RefFinder summarized the results of the previously listed algorithms.

GeNorm analysis

Figure 4 summarizes the stability scores for the ten putative genes across all samples as estimated by GeNorm. *ACTINT7* and *TBP* had the highest level of stability, whereas *eIF-4α* was the least stable gene across samples. Across tissues, *TIP41* and *ACTINT7* had the highest stability levels, while *EF-1α* showed the least stable expression across tissues. The most stable genes across samples submitted to the MeJA abiotic stress were *TIP41* and *ACTINT7*, whereas *TUB* was the least stable. Under SA abiotic stress treatment, *ACTINT7* and *26S* showed the highest stability, whereas *eIF-4α* showed the least stable expression. *TBP* and *eIF-4α* had the highest stability in the drought stress group, whereas *TBP* and *EF-1α*

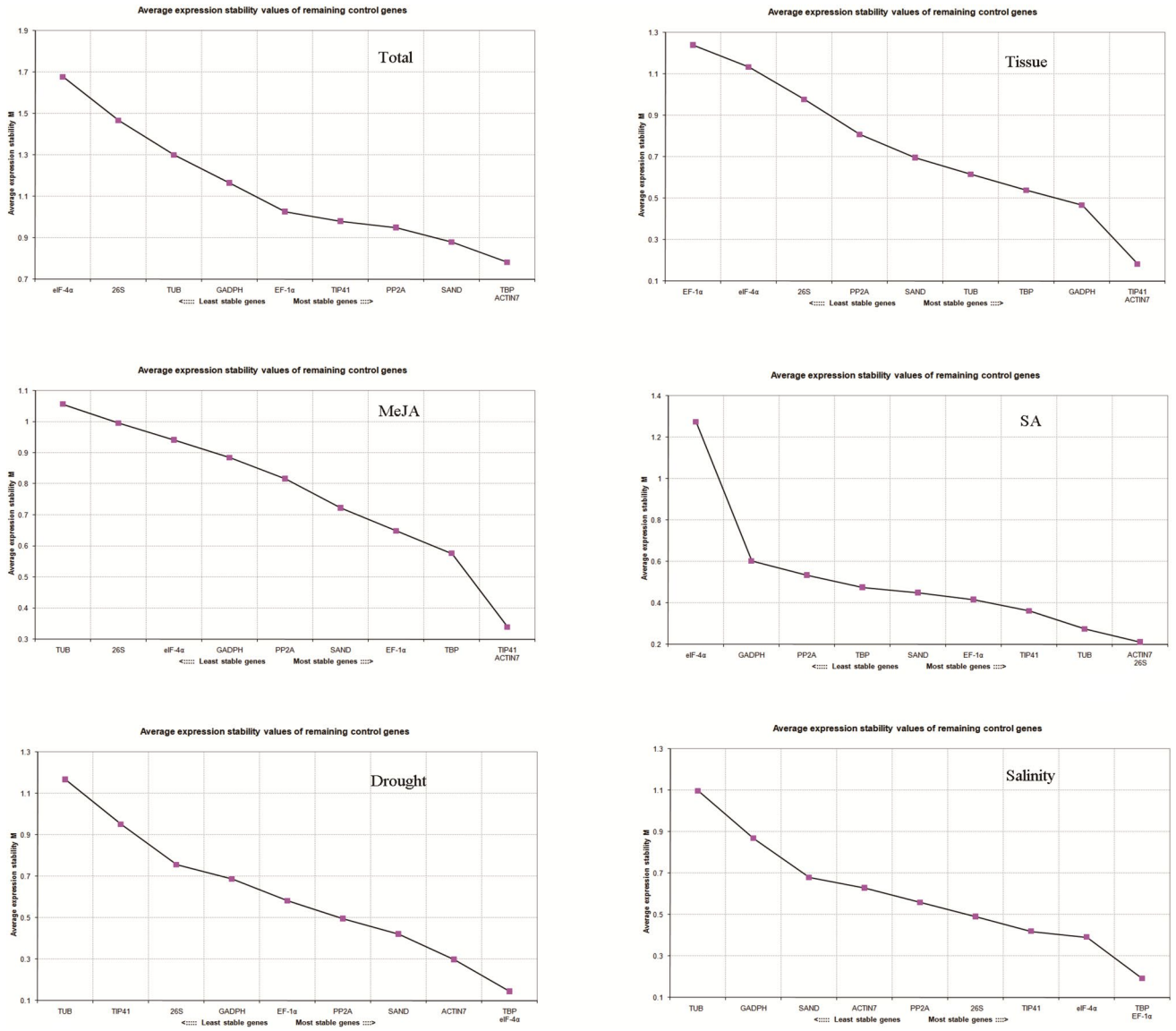


Fig. 4 Expression stability of ten candidate genes as calculated by GeNorm

showed low stability under salinity stress. *TUB* showed the lowest level of gene stability under both drought and salinity abiotic stresses.

NormFinder analysis

The NormFinder analysis differs from that of GeNorm as it uses ANOVA analysis to estimate intra- and inter-group variation in gene expression stability for each reference genes. According to this algorithm, *ACTIN7* and *TBP* showed the highest level of stability in gene expression across all tissues and different abiotic stress treatments. *TIP41* and *ACTINT7* showed the highest stability across tissue samples. *EF-1α* and *TIP41* were the most stably expressed genes in samples submitted to MeJA-induced abiotic stress whereas in samples submitted to SA-induced abiotic stress *ACTIN7* and *TBP* had the highest stability. Finally, *TBP* and *eIF-4α* showed high levels of stability under drought stress, whereas *TBP* and *EF-1α* were the best reference genes under salinity stress (Table 2).

Delta-Ct (ΔCt) analysis

In the ΔCt analysis, the reference gene that produces the lowest gene pair standard deviation (SD) across all samples is most stable and therefore the most suitable reference gene. The ten candidate reference genes were ranked from the highest to the lowest levels of stability of gene expression according to the ΔCt algorithm (Table 3). Overall, *ACTINT7* had the highest level of stability in gene expression across all

Table 2 Ranking of ten candidate reference genes under different experimental conditions in order of their expression stability calculated by NormFinder

Rank	Total		Tissue		MeJA		SA		Drought		Salinity	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	ACTIN7	0.285	TIP41	0.0513	EF-1α	0.120	ACTIN7	0.129	TBP	0.046	TBP	0.051
2	TBP	0.430	ACTINT7	0.063	TIP41	0.333	TBP	0.173	eIF-4α	0.050	EF-1α	0.066
3	EF-1α	0.465	SAND	0.166	ACTINT7	0.338	SAND	0.290	SAND	0.130	ACTIN7	0.289
4	TIP41	0.541	PP2A	0.287	TBP	0.396	TIP41	0.293	ACTIN7	0.210	eIF-4α	0.293
5	PP2A	0.593	26S	0.371	SAND	0.467	PP2A	0.298	PP2A	0.369	TIP41	0.378
6	SAND	0.629	TBP	0.532	GADPH	0.505	26S	0.303	EF-1α	0.464	SAND	0.379
7	GADPH	0.831	TUB	0.805	eIF-4α	0.584	EF-1α	0.381	GADPH	0.528	PP2A	0.461
8	TUB	1.102	GADPH	1.217	PP2A	0.711	TUB	0.433	26S	0.657	26S	0.543
9	26S	1.328	eIF-4α	1.234	26S	0.783	GADPH	0.471	TIP41	1.167	GADPH	1.084
10	eIF-4α	1.579	EF-1α	3.222	TUB	0.871	eIF-4α	2.726	TUB	1.361	TUB	1.355

Table 3 Ranking of ten candidate reference genes under different experimental conditions in order of their expression stability calculated by ΔCt

Rank	Total		Tissue		MeJA		SA		Drought		Salinity	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	ACTINT7	0.61	TIP41	0.79	TIP41	0.61	ACTINT7	0.6	TBP	0.63	TBP	0.58
2	TBP	0.64	ACTINT7	0.82	ACTINT7	0.79	26S	0.62	eIF-4α	0.65	EF-1α	0.66
3	TUB	0.70	GADPH	0.93	EF-1α	0.94	TIP41	0.67	ACTIN7	0.77	eIF-4α	0.67
4	PP2A	0.95	26S	0.97	PP2A	0.97	EF-1α	0.71	TIP41	0.82	TIP41	0.71
5	TIP41	1.04	TUB	1.04	TBP	0.99	TUB	0.73	PP2A	0.98	ACTIN7	0.80
6	EF-1α	1.32	TBP	1.06	eIF-4α	1.11	GADPH	0.75	EF-1α	1.04	GADPH	0.86
7	GADPH	1.49	SAND	1.13	26S	1.16	TBP	0.9	GADPH	1.15	SAND	0.89
8	SAND	1.61	EF-1α	1.39	GADPH	1.50	PP2A	1.07	26S	1.43	PP2A	1.05
9	26S	1.87	eIF-4α	1.54	SAND	1.89	SAND	1.09	SAND	1.55	TUB	1.12
10	eIF-4α	1.95	PP2A	1.71	TUB	1.91	eIF-4α	1.21	TUB	1.57	26S	1.36

tissues, and within the SA-induced abiotic stress group. In the MeJA-induced abiotic stress group as well as across different tissues *TIP41* was the most stably expressed, whereas *TBP* was the best reference gene under the drought and salinity stresses.

BestKeeper analysis

BestKeeper estimates expression stability by measuring the standard deviation (SD) across samples. A gene with SD > 1 is considered unacceptable for a reference gene. The SD values of all genes as estimated by BestKeeper logarithm are shown in Table 4. Across all samples, *TIP41* (0.51) had the lowest SD value, followed by *SAND* (0.55), while *EF-1α* and *eIF-4α* had SD values were higher than 1. Across tissue samples, *TIP41* was considered the most suitable reference genes (0.37), followed by *ACTINT7* (0.60) and *TUB* (0.67). Among the MeJA treated samples, all reference genes had SD values lower than 1.0, the lowest ones obtained for the *TIP41*, *ACTINT7* and *TBP* (0.31, 0.33 and 0.4, respectively). Similarly, under the SA stress treatment, all reference genes had low SD values (SD < 0.52), with the *TBP* (0.21), *PP2A* (0.3) and *GADPH* (0.32) showing the highest levels of stability. *ACTIN7* showed the lowest SD value of 0.09 in the drought treatment group. Under salinity stress, *GADPH* (0.53) and *TIP41* (0.67) were the two most stable genes.

RefFinder analysis

The results from the four previously described algorithms were integrated by the RefFinder algorithm and the results (Table 5). The stability rank of the ten putative reference genes was listed from the highest to the lowest as assessed by RefFinder (Fig. 5). The expression of *TBP* was the most stable under the drought and salinity stress. The most stable gene under MeJA-induced abiotic stress *was TIP41*. The most stable gene under the SA-induced stress and across different tissue samples was *ACTIN7*. Overall, the level of stability of the expression pattern of *ACTIN7* revealed that

Table 4 Ranking of ten candidate reference genes under different experimental conditions in order of their expression stability calculated by BestKeeper

Rank	Total			Tissue			MeJA			SA			Drought			Salinity		
	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV
1	TIP41	0.51	2.16	TIP41	0.37	2.12	TIP41	0.31	1.04	TBP	0.21	0.79	ACTIN7	0.09	0.4	GADPH	0.53	1.72
2	SAND	0.55	2.13	ACTIN7	0.60	1.98	ACTIN7	0.33	1.32	PP2A	0.3	1.07	TBP	0.26	0.99	TIP41	0.67	2.26
3	ACTIN7	0.61	2.83	TUB	0.67	2.83	TBP	0.40	1.55	GADPH	0.32	1.04	eIF-4α	0.3	1.31	eIF-4α	0.7	3
4	26S	0.65	2.22	26S	0.71	2.76	EF-1α	0.46	2.09	ACTIN7	0.32	1.36	SAND	0.36	1.29	EF-1α	0.92	4.22
5	PP2A	0.71	3.01	TBP	0.75	2.73	SAND	0.54	2.00	TIP41	0.39	1.32	EF-1α	0.4	1.87	TUB	0.98	4.08
6	GADPH	0.75	2.65	eIF-4a	0.96	3.91	GADPH	0.55	1.71	eIF-4α	0.39	1.64	PP2A	0.47	1.64	TBP	1.02	3.71
7	TUB	0.81	2.95	SAND	0.98	3.48	PP2A	0.57	2.02	SAND	0.42	1.5	TIP41	0.81	3.47	ACTIN7	1.18	4.82
8	TBP	0.89	2.89	GADPH	1.07	3.79	eIF-4a	0.59	2.41	26S	0.43	2.36	GADPH	0.88	2.92	26S	1.20	4.22
9	EF-1α	1.30	6.99	EF-1α	1.23	5.50	26S	0.87	5.02	EF-1α	0.44	2.03	26S	1.28	6.62	SAND	1.35	4.72
10	eIF-4α	1.35	5.4	PP2A	3.91	14.10	TUB	0.94	3.53	TUB	0.51	0.79	TUB	1.34	4.44	PP2A	2.08	10.3

this gene is the most reliable regarding the normalization of qRT-PCR data in *T. wilfordii,* across all samples.

Expression analysis of *TwHMGR* and *TwDXR* genes for reference gene validation

TwHMGR and *TwDXR* encode for enzymes directly involved in the biosynthesis of isoprenoids in *T. wilfordii* [26, 47]. The relative expression of *TwHMGR* and *TwDXR* were used to validate the suitability of the reference genes across different tissues as well as across root samples in the MeJA-induced abiotic stress group (Fig. 6). We used the RefFinder stability rankings to select the most stable genes (*ACTIN7* and *TBP*) and the least stable gene (*TUB*) for the normalization of qRT-PCR data obtained for *TwHMGR* and *TwDXR.*

For MeJA treatment, *TwHMGR* expression was induced significantly at 3 h and kept to increase until 6 h and then decreased at 24 h, which was normalized by the two most stable reference genes (*ACTIN7* and *TBP*). Furthermore, the expression of *TwHMGR* which was normalized by the two most stable reference gene (*ACTIN7* and *TBP*) was markedly different from the expression level which was calculated using the least suitable reference gene (*TUB*) ($P < 0.05$) (Fig. 6a). Across different tissues, transcript level of *TwHMGR* was the lowest in leaf while it was the highest in root that was normalized by the two most stable reference gene (*ACTIN7* and *TBP*). However, the expression level of *TwHMGR* which was normalized by the two most stable reference gene (*ACTIN7* and *TBP*) was significantly different from the expression level which was calculated using the least suitable reference gene (*TUB*) among different tissues ($P < 0.05$) (Fig. 6b).

The expression level of *TwDXR* began to increase at 3 h, increased until 6 h and then dropped at 24 h that was normalized by the two most stable reference genes (*ACTIN7* and *TBP*). However, the expression level of *TwDXR* normalized by the two most stable reference genes (*ACTIN7* and *TBP*) was significantly different from the expression level calculated using the least suitable reference gene (*TUB*) ($P < 0.05$) (Fig. 6c). Across different tissues, significant difference of *TwDXR* transcript levels was observed. The expression level of *TwDXR* normalized by the two most stable reference genes (*ACTIN7* and *TBP*) was significantly different from the expression level calculated using the least suitable reference gene (*TUB*) ($P < 0.05$) (Fig. 6d).

Discussion

The qRT-PCR analysis is a commonly used method to quantify gene differential expression levels in gene function studies [48]. However, the accuracy of this method depends on the availability of suitable reference genes, which is used

Table 5 Expression stability ranking of the ten candidate reference genes as calculated by RefFinder

Method	1	2	3	4	5	6	7	8	9	10
Ranking order under total sample (better–good–average)										
geNorm	TBP|SAND		ACTIN7	PP2A	TIP41	EF-1α	eIF-4α	GADPH	26S	TUB
NormFinder	ACTIN7	EF-1α	TBP	TIP41	PP2A	SAND	eIF-4α	GADPH	26S	TUB
Delta CT	ACTIN7	EF-1α	TBP	TIP41	SAND	PP2A	eIF-4α	GADPH	26S	TUB
BestKeeper	ACTIN7	TIP41	EF-1α	TBP	eIF-4α	PP2A	GADPH	SAND	26S	TUB
Comprehensive ranking	**ACTIN7**	**TBP**	**EF-1α**	**TIP41**	**SAND**	**PP2A**	**eIF-4α**	**GADPH**	**26S**	**TUB**
Ranking order under different tissues (better–good–average)										
geNorm	TIP41|ACTIN7		GADPH	TBP	TUB	SAND	PP2A	26S	eIF-4α	EF-1α
NormFinder	ACTIN7	TIP41	GADPH	SAND	TBP	TUB	26S	PP2A	eIF-4α	EF-1α
Delta CT	ACTIN7	TIP41	GADPH	TBP	SAND	TUB	PP2A	26S	eIF-4α	EF-1α
BestKeeper	26S	GADPH	TIP41	ACTIN7	TUB	TBP	eIF-4α	SAND	PP2A	EF-1α
Comprehensive ranking	**ACTIN7**	**TIP41**	**GADPH**	**26S**	**TBP**	**TUB**	**SAND**	**PP2A**	**eIF-4α**	**EF-1α**
Ranking order under MeJA stress (better–good–average)										
geNorm	TIP41|ACTIN7		TBP	EF-1α	SAND	PP2A	GADPH	eIF-4α	26S	TUB
NormFinder	EF-1α	TIP41	TBP	ACTIN7	GADPH	SAND	eIF-4α	PP2A	26S	TUB
Delta CT	EF-1α	TIP41	TBP	ACTIN7	GADPH	SAND	eIF-4α	PP2A	26S	TUB
BestKeeper	ACTIN7	TIP41	GADPH	TBP	EF-1α	PP2A	eIF-4α	SAND	TUB	26S
Comprehensive ranking	**TIP41**	**ACTIN7**	**EF-1α**	**TBP**	**GADPH**	**SAND**	**PP2A**	**eIF-4α**	**26S**	**TUB**
Ranking order under SA stress (better–good–average)										
geNorm	TIP41|ACTIN7		GADPH	TBP	TUB	SAND	PP2A	26S	eIF-4α	EF-1α
NormFinder	ACTIN7	TIP41	GADPH	SAND	TBP	TUB	26S	PP2A	eIF-4α	EF-1α
Delta CT	ACTIN7	TIP41	GADPH	TBP	SAND	TUB	PP2A	26S	eIF-4α	EF-1α
BestKeeper	26S	GADPH	TIP41	ACTIN7	TUB	TBP	eIF-4α	SAND	PP2A	EF-1α
Comprehensive ranking	**ACTIN7**	**TIP41**	**GADPH**	**26S**	**TBP**	**TUB**	**SAND**	**PP2A**	**eIF-4α**	**EF-1α**
Ranking order under drought stress (better–good–average)										
geNorm	TBP|eIF-4α		ACTIN7	SAND	PP2A	EF-1α	GADPH	26S	TIP41	TUB
NormFinder	TBP	eIF-4α	SAND	ACTIN7	PP2A	EF-1α	GADPH	26S	TIP41	TUB
Delta CT	TBP	eIF-4α	SAND	ACTIN7	PP2A	EF-1α	GADPH	26S	TIP41	TUB
BestKeeper	ACTIN7	TBP	eIF-4α	SAND	EF-1α	PP2A	TUB	GADPH	26S	TIP41
Comprehensive ranking	**TBP**	**eIF-4α**	**ACTIN7**	**SAND**	**PP2A**	**EF-1α**	**GADPH**	**26S**	**TIP41**	**TUB**
Ranking order under salinity stress (better–good–average)										
geNorm	TBP|EF-1α		eIF-4α	TIP41	TUB	PP2A	ACTIN7	26S	GADPH	SAND
NormFinder	TBP	EF-1α	ACTIN7	eIF-4α	TIP41	SAND	PP2A	26S	GADPH	TUB
Delta CT	TBP	EF-1α	eIF-4α	TIP41	ACTIN7	SAND	PP2A	26S	GADPH	TUB
BestKeeper	GADPH	TIP41	eIF-4α	EF-1α	TUB	TBP	ACTIN7	26S	SAND	PP2A
Comprehensive ranking	**TBP**	**EF-1α**	**eIF-4α**	**TIP41**	**GADPH**	**ACTIN7**	**TUB**	**26S**	**SAND**	**PP2A**

The bold characters represents the comprehensive rankings calculated by refFinder. The higher the ranking, the more stable the expression of reference genes

to normalize the expression of the target genes. We made an orthologue search analysis using the sequences of ten reference genes of the *Arabidopsis* genome against the *T. wilfordii* root transcriptome database (accession number SRX472292, http://www.ncbi.nlm.nih.gov/sra/SRX472292) (Table 1) to identify putative reference genes in *T. wilfordii*.

The images of the agarose gels of each putative reference gene PCR products as well as the dissociation curves of the qRT-PCR amplification reactions showed that the primers designed for this analysis had good specificity. At the same time, the high value of the R^2 of the standard curve equation as well as the high amplification efficiency values obtained during the qRT-PCR analyses confirmed the high degree of specificity of the primers designed to amplify *T. wilfordii* reference genes.

qRT-PCR data was obtained across different tissues of *T. wilfordii* as well as across samples exposed to different abiotic stress treatments. The stability of gene expression of ten putative reference genes was assessed with five statistical algorithms (GeNorm, NormFinder, BestKeeper, ΔCt, and

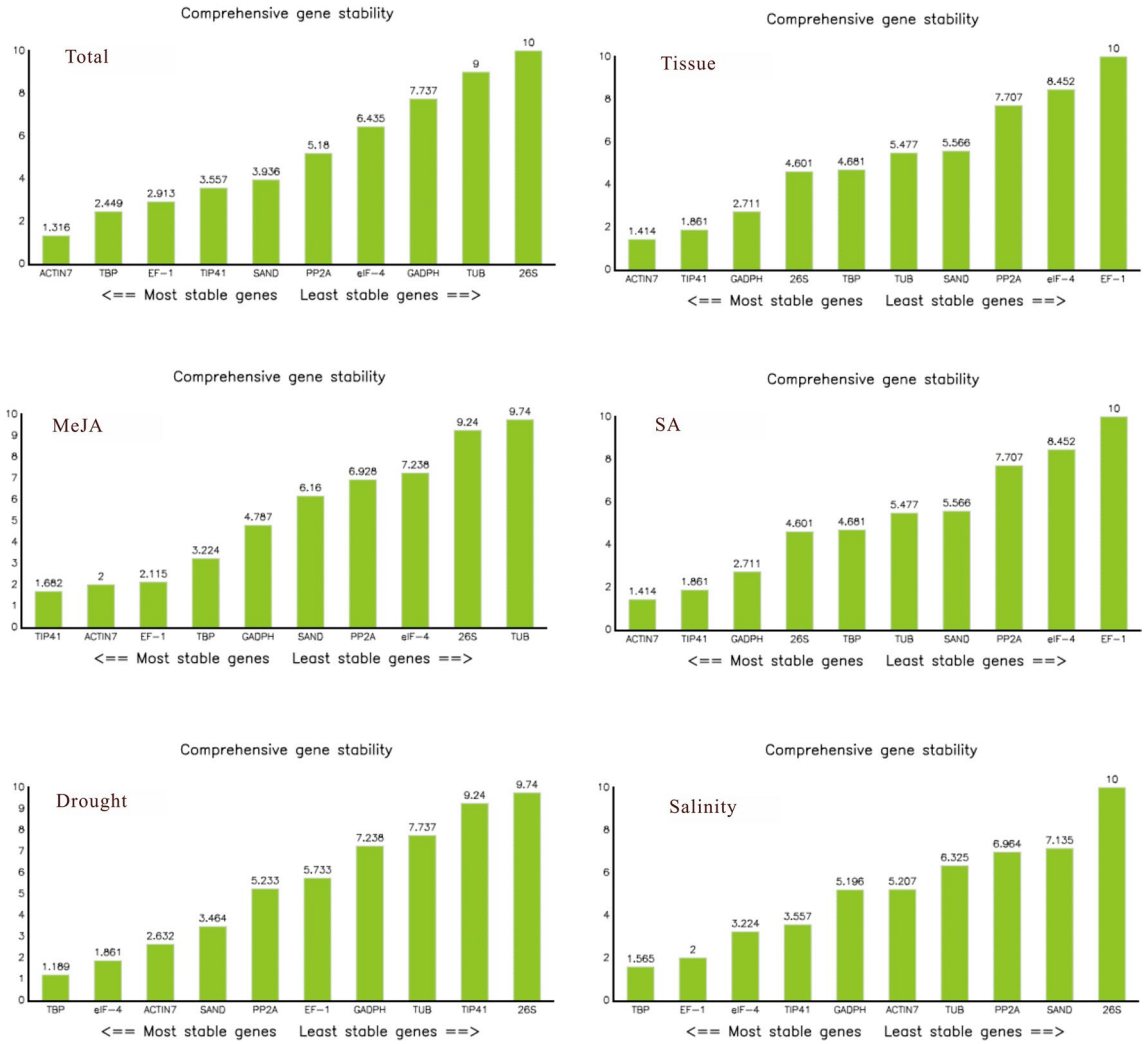


Fig. 5 Expression stability of ten candidate reference genes as calculated by RefFinder

RefFinder). In general, GeNorm, NormFinder, ΔCt, and RefFinder were able to produce consistent stability rank for the putative reference gene (Table 5). The BestKeeper algorithm is based on a different principle as compared to the other algorithms, which might explain why the BestKeeper results differed the most from those of the other algorithms [49]. Finally, we selected the most suitable reference gene(s) as determined by GeNorm, NormFinder, ΔCt, and the RefFinder algorithms.

For validation purposes, we chose *TwHMGR* and *TwDXR* as target genes, which encode for enzymes directly involved in the biosynthesis of isoprenoids in plants. The *HMGR* reductase catalyzes the irreversible conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) into mevalonic acid. This catalysis is a key regulatory step in isoprenoids biosynthesis leading to the biosynthesis of IPP and DMAPP [50, 51]. The (*DXR*) reductoisomerase catalyzes the conversion of *DXP*; a key protein activated upstream of the MEP pathway. Moreover, the addition of MeJA and SA to the media mimics the endogenous signals that occur during the plant response to biotic and abiotic stresses [52]. Moreover, MeJA and SA have also been used as elicitors to enhance the accumulation of secondary metabolites in adventitious roots and hairy roots of *T. wilfordii* Hook. f. [33, 36, 53]. Hence, we chose MeJA to induce an abiotic stress response in the *T. wilfordii* hairy roots in order to evaluate the stability of gene expression. Ideally, the stability of gene expression of a reference gene is not affected by tissue types and experimental conditions. As shown in Fig. 6a, c, *TwHMGR* and *TwDXR* are gradually upregulated by MeJA over the time course of the experiment until the 6 h. This pattern of gene expression was also observed when data was normalized against *ACTIN7* and *TBP*, which had been identified as the most stable reference genes in previous studies [54–56]. Figure 6b and 6d show that the expression trends

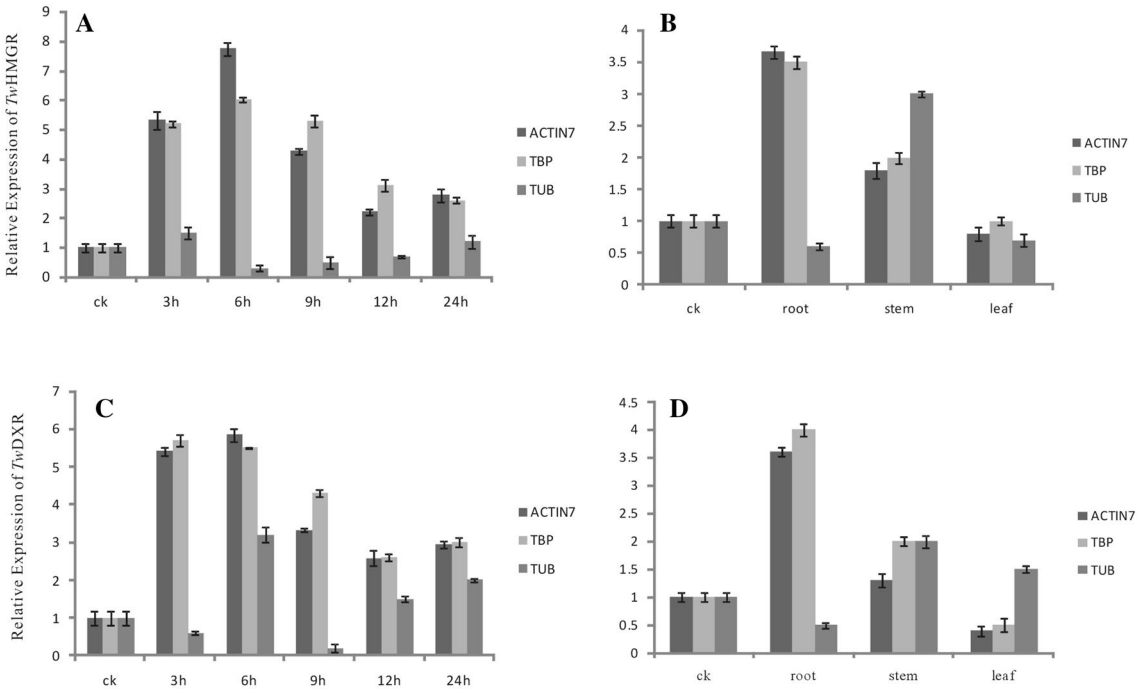


Fig. 6 Relative quantification of *TwHMGR* and *TwDXR* expression using validated reference genes including the most or the least stable reference genes for normalization under different tissues and MeJA treatment experimental sets. **a** *TwHMGR* expression of hairy roots under MeJA treatment after 0 h, 3 h, 6 h, 9 h, 12 h and 24 h; **b** *TwHMGR* expression of different tissues; **c** *TwDXR* expression of hairy roots under MeJA treatment after 0 h, 3 h, 6 h, 9 h, 12 h and 24 h; **d** *TwDXR* expression of different tissues. Bars represent the mean ± SE of three biological replicates

of *TwHMGR* and *TwDXR* in different tissues were identical when *ACTIN7* and *TBP* were used for qRT-PCR data normalization. By contrast, large discrepancies in expression patterns appeared when the least stable reference gene (*TUB*) was used for data normalization.

This study is the first, to our knowledge, to evaluate and validate reference genes for the normalization of qRT-PCR data from different plant tissues and different abiotic stress conditions in *T. wilfordii*. The optimization of the qRT-PCR analysis depends on the identification and validation of reference genes in different tissues, different development stages, and under different experimental conditions. Our study successfully identified the most suitable reference genes for future studies based on qRT-PCR analysis of *T. wilfordii.*

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Author contributions CZ and JZ conceived and designed the study. JZ, YH and BZ performed the experiments. CZ and JZ wrote the paper. CZ, JF, ZM and XZ reviewed and edited the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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