Electronic Journal of Biotechnology 35 (2018) 48–56

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

A whole-transcriptome approach to evaluate reference genes for quantitative diurnal gene expression studies under natural field conditions in *Tamarix ramosissima* leaves

Xia Yan [a,b], Chaoju Qian [a], Xiaoyue Yin [a], Xingke Fan [a], Xueyong Zhao [a,c], Menghe Gu [d], Tao Wang [d], Xiao-Fei Ma [a,*]

[a] Key Laboratory of Stress Physiology and Ecology in Cold and Arid Regions of Gansu Province, Cold and Arid Regions Environmental and Engineering Research, Chinese Academy of Sciences, Lanzhou 730000, China
[b] Key Laboratory of Inland River Ecohydrology, Cold and Arid Regions Environmental and Engineering Research, Chinese Academy of Sciences, Lanzhou 730000, China
[c] Naiman Desertification Research Station, Cold and Arid Regions Environmental and Engineering Research, Chinese Academy of Sciences, Lanzhou, 730000, China
[d] Key Laboratory of Desert and Desertification, Cold and Arid Regions Environmental and Engineering Research, Chinese Academy of Sciences, Lanzhou 730000, China

ARTICLE INFO

Article history:
Received 13 September 2017
Accepted 7 August 2018
Available online 24 August 2018

Keywords:
Desert plants
Desert restoration
Drought adaptation
Fragile ecosystem
Functional analysis
Gene expression
qPCR
Reference gene
RNA-seq
Tamarix ramosissima
Water transport

ABSTRACT

Background: Tamarix ramosissima is a desert forest tree species that is widely distributed in the drought-stricken areas to sustain the fragile ecosystem. Owing to its wide usage in the desert restoration of Asia, it can be used as an ecophysiological model plant. To obtain reliable and accurate results, a set of reference genes should be screened before gene expression. However, up to date, systematical evaluation of reference genes has not been conducted in *T. ramosissima*.

Results: In this study, we used eigenvalues derived from principal component analysis to identify stable expressed genes from 72,035 unigenes from diurnal transcriptomes under natural field conditions. With combined criteria of read counts above 900 and CV of FPKM below 0.3, a total of 7385 unigenes could be qualified as candidate reference genes in *T. ramosissima*. By using three statistical algorithm packages, *geNorm*, *NormFinder*, and *BestKeeper*, the stabilities of these novel reference genes were further compared with a panel of traditional reference genes. The expression patterns of three aquaporins (AQPs) suggested that at least UBQ (high expression), EIF4A2 (low expression), and GAPDH (moderate expression) could be qualified as ideal reference genes in both RT-PCR and RNA-seq analysis of *T. ramosissima*.

Conclusions: This work will not only facilitate future studies on gene expression and functional analysis of genetic resources of desert plants but also improve our understanding of the molecular regulation of water transport in this plant, which could provide a new clue to further investigate the drought adaptation mechanism of desert plant species under harsh environments.

How to cite: Yan X, Qian C, Yin X, et al. A whole-transcriptome approach to evaluating reference genes for quantitative diurnal gene expression studies under natural field in *Tamarix ramosissima* leaves. Electron J Biotechnol 2018;35. https://doi.org/10.1016/j.ejbt.2018.08.004.

1. Introduction

Gene expression analysis is increasingly important in many fields of biological research. Understanding gene expression patterns is expected to provide insights into complex regulatory networks and helps to identify genes that are relevant to new biological processes. Plants fluctuate diurnally for approximately 24 h under diurnal signals such as light–dark or temperature cycles with diverse biological activities and physiological output [1,2]. Most transcripts were diurnally expressed following those signals in plants. In the dicot model *Arabidopsis thaliana*, this number amounts to approximately 80% [3,4,5,6]. In rice, poplar, maize, tomato, and soybean, similar results were observed [7,8,9,10]. In addition to rhythmic changes, complex and noise environmental signals also affect gene expression. Many studies have dissected these signals under artificial constant conditions to further investigate the contribution of environmental factors to the molecular mechanism in plants [2,11,12]. However, many results showed discrepancy with those obtained under natural field conditions, which is even more serious in transgenic crop plants [13,14,15,16]. Natural conditions are increasingly taken into

* Corresponding author.
E-mail address: maxiaofei@lzb.ac.cn (X.-F. Ma).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2018.08.004

consideration to meet the required arguments in modern agriculture and ecology [17,18].

qPCR is the best method for quantifying gene expression owing to its simplicity, accuracy, and low cost. To obtain reliable and accurate expression levels, one or a set of reliable reference genes with low variation in expression across diverse sample types is a prerequisite [19,20,21,22,23,24]. Usually, a handful of selected reference genes such as actin (*ACT*), elongation factor 1 alpha (*EF-1α*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and ubiquitin (*UBQ*) are commonly used without any validation. Unfortunately, these traditional reference genes can exhibit surprisingly high expression variance in some species or under different environmental conditions [19,25,26,27,28,29]. Consequently, bias of expression data occurred, or incorrect conclusions were drawn [30]. Many statistical methods for analyzing expression variability have been developed, including *geNorm*, *BestKeeper*, and *NormFinde*r [23,31,32], but these programs can only analyze the expression data from a handful of selected genes from a small *priori* set of genes. With the development of sequence technology, reference genes from a genome-wide background selected by high-throughput technologies have prevailed. For example, microarray has been used in *A. thaliana*, *Eucalyptus*, and soybean [26,33,34]; RNA-seq has been used in other plant species [35,36,37,38]. The genes selected by high-throughput technologies are generally better than commonly used reference genes after validation with experiments [39]. At present, with advantages such as fast output, inexpensive, and minimal variation across technical replicates, RNA-seq is more attractive than microarray and is more widely used to select reference genes [21,40,41,42,43].

A large number of studies on systematic validation of reference genes have been reported in classic plants such as *Arabidopsis* [26], rice [44], poplar [45], soybean [46], wheat [47], barley [48], tomato [49], grape [50], and potato [51]. Some stability evaluation of orthologous genes of these plants was also reported in nonmodel plant species [52]. These studies were limited to specific contexts and generally unsuitable for any context, including any considerable alteration in different biological samples [19,29]. Thus, selection of a series of new reference genes on nonmodel plants was widely developed, for example, in bamboo [53], peach [54], *Caragana intermedia* [55], eggplant [56], *Mimulus* [20], andromonoecious *Taihangia rupestris* [22], watermelon [40], lettuce [12], and *Reaumuria songarica* [57].

Tamarix ramosissima is a typical halophytic plant. It can sequester high concentrations of sodium and other salts in their above-ground tissue and can secrete these concentrated salts through specialized salt glands to the surface of leaves or shoots [58,59]. It is also a desert forest tree species widely distributed in the drought-stricken areas, with annual precipitation less than 200 mm in North China [60]. With the pursuit to understand the desertification of Asia, the mechanism by which *T. ramosissima* adapts to the harsh environment since millions of years has attracted increasing ecological and physiological interests in recent years. Some studies have investigated the relationship between gene expression and water balance in other species of *Tamarix* [61,62,63,64,65]. The results may lead to bias regarding the stability of the reference genes. In addition, studies have revealed that water from leaves has been regarded as an important subsidiary to mitigate the deleterious effects of soil water deficits [66,67,68]; aquaporins (AQPs) in leaves are responsible for absorbing water from moisture in air at night in *Tamarix* [65]. On the basis of the experiments, a diurnal transcriptome of *T. ramosissima* under natural field conditions has been profiled to reveal the molecular mechanism of foliar water uptake. There is an urgent need to develop a set of candidate reference genes to finely analyze gene expression in response to water availability [69]. In addition, there is no study to date on the systematic evaluation of reference genes in *T. ramosissima*.

In this study, we used eigenvalues derived by using the mathematical method and principal component analysis (PCA) to identify stable expressed genes from 72,035 unigenes from diurnal transcriptomes in *T. ramosissima* under natural field conditions. With combined criteria of read counts above 900 and FPKM (fragments per kilobase per million reads) coefficient of variation (CV) below 0.3, a total of 7385 unigenes were selected as candidate reference genes in *T. ramosissima*. Three statistical algorithm packages, *geNorm* [31], *NormFinder* [23], and *BestKeeper* [32], were used to compare stability between a set of novel reference genes from these 7385 unigenes and a panel of traditional reference. Further validation was quantified on the expression pattern of three AQPs against three representations of the reference genes *UBQ* (high expression), *EIF4A2* (low expression), and *GAPDH* (moderate expression) with RT-PCR and RNA-seq, respectively. This work will provide information for future studies about not only gene expression and functional analysis of the genetic resources in desert plants but also the molecular regulation of foliar water uptake in *T. ramosissima*, which could provide a new clue to us to understand the genetic mechanism of desert plants in their adaptation to drought environments.

2. Material and methods

2.1. Ethics statement

T. ramosissima is a desert tree species widely distributed in Jingtai County, Gansu Province, and other arid regions; it has not been included in any list of endangered or protected species. Before collecting the samples, oral permission was obtained from the local management of forestry after sending introduction letters from the CAREERI (Cold and Arid Regions Environmental and Engineering Research Institute, Chinese Academy of Sciences).

2.2. Plant material and cDNA preparation

RNA was extracted from at least 5 g of leaves harvested from one *T. ramosissima* plant from 6:00 AM to 24:00 PM on 25 June in 2013, and it served as samples for both RNA-seq and qPCR. RNA concentration and integrity were assessed using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, DE). RNA with high quality isolated from leaves of at least three branches was mixed equally for replicate experiments.

Part of the RNA sample was used to construct the CDNA library by using the Illumina HiSeq platform. Expression levels in FPKM were determined for a total of 72,035 unigenes with a mean length of 831 bp and N50 length of 1494 bp (unpublished data).

Part of the RNA sample was used for RT-PCR analysis. For each sample, 1 μg of total RNA was reverse transcribed in a 20 μl reaction volume with oligo(dT) primers, using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas/Thermo Fisher Scientific). The cDNAs were diluted as 1:10 with nuclease-free water and stored at −20°C.

2.3. Reference gene and AQP gene selection

As low-expression genes would make poor qPCR reference genes owing to the difficulties in detecting and quantifying their expression, genes with expression levels lower than 5 FPKM in any of the eight transcriptomes were excluded from further stability analyses. Eigenvalues derived by using the mathematical method with PAST software were used to determine the comprehensive value, which indicated the common expression pattern along time series. Genes with slight or changed curve during the time series were selected to meet the requirements of a reference gene. To further support selection with eigenvalues, read counts and CV of FPKM were included. Genes with both a mean of read counts above 500 and a CV below 0.3 were considered as stably expressed [26,52]. According to nonredundant (Nr) annotation, homologs of the traditional reference genes used in previous diurnal studies [12,22,70] were also included.

Table 1
RT-PCR primer sequences and annotation information of aquaporin genes.

GeneID	Forward primers (5′–3′)	Reverse primers (5′–3′)	Gene name	Annotation
Unigene046477	GCTTGAGGATGGGAGGACTT	TTGATCATAACAGCGCGTGG	*UBQ*	Ubiquitin [*Arabidopsis thaliana*]
Unigene042278	TGCAGAGGAAGCCATGAGAA	GACACACCGGTTGCTTTTCT	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase [*Arabidopsis thaliana*]
Unigene023196	CACTGAACCCCAAAAGAGCC	TTCATCTGTGTTTGCGTGGG	*ELIF4A2*	Eukaryotic initiation factor 4A-2-like [*Solanum tuberosum*]
Unigene045840	AGGGAGGGAGAGAGAGAAGG	CGCTGTTCCTAATGGCTGTC	*PIP12*	Aquaporin PIP12 [*Arabidopsis thaliana*]
Unigene021922	TTCCCAAGATACCCACACCC	GCGGAGTTCATAGCTACCCT	*PIP21*	Aquaporin PIP21 [*Arabidopsis thaliana*]
Unigene028225	GGGAGAGGGAAAAGGGTCAT	ACGCGCCTAGGTGAAGTAAG	*PIP14*	Aquaporin PIP14 [*Tamarix* sp. ZDY-001908]

For AQP genes, keyword searches were carried out for putative protein encoding sequences annotated as “aquaporin,” “PIP,” “TIP,” “NIP,” “SIP,” “intrinsic,” “channel,” “transmembrane,” and “nodulin-like” on the National Center for Biotechnology Information (NCBI) Nr protein database with notation E-values less than 1e−14. Unique cDNAs from three AQP genes (*PIP12*, *PIP21*, and *PIP14*) were translated in the GENSCAN web server (http://genes.mit.edu/GENSCAN.html) to inspect conserved features such as NPA motifs. In addition, cDNAs with a length of more than 900 bp and FPKM of more than 500 were also considered.

2.4. qPCR experiments

Primers of each reference gene and AQP genes were designed using the platform OligoPerfect Designer (Invitrogen), with an optimal melting temperature of 60°C, optimal GC content of 50%, optimal primer size of 20 bp, and product size of between 100 and 300 bp (Table 1). qPCR experiments were performed using qTOWER2.0 (JENA, Germany) with a 20 μl reaction system volume. Each reaction mixture contained 10 μl of DyNAmo Flash SYBR Green qPCR Kit Master Mix (Thermo Fisher Scientific), 0.5 μl each of forward and reverse primers, 0.2 μl of F-402 buffer, and 2 μl of cDNA synthesized from total RNA. The PCR program involved initial denaturation for 5 min at 95°C, followed by denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C for 40 cycles.

2.5. Statistical analysis

Three different types of Microsoft Excel-based software, *geNorm*, *NormFinder*, and *BestKeeper*, were used to rank the expression stabilities of reference genes across all experimental sets. The candidate reference genes were ranked using *geNorm* according to the expression stability value M, which was calculated for all reference genes investigated (the lower the M value, the higher is the expression stability of the gene) [31]. According to the *geNorm* program, the reference gene with an M value below 1.5 is considered to be stably expressed, and a lower M value represents more stable expression. The *NormFinder* program is a Visual Basic application tool for Microsoft Excel used to determine the expression stabilities of reference genes that ranks all reference gene candidates according to intra- and inter-group variations and combines both results into a stability value for each candidate reference gene [32]. According to the stability value for each reference gene, ranking order was arranged from the smallest to the largest, with the lower stability value indicating the higher stability. The *BestKeeper* program identifies the most stably expressed genes depending on the coefficient of correlation to the *BestKeeper* index, which is the geometric mean of the candidate reference gene. *BestKeeper* also calculates the standard deviation (SD) and CV of all candidate reference genes [32]. Genes with SD >1 are considered unacceptable [71]. Thus, with the *BestKeeper* program, the best reference genes should be the most stable ones, which should have the lowest CV and SD [32]. Genes with an SD [±CP] value below 1.0 are considered to be stably expressed. The Venn method was used to overcome data overflow for *BestKeeper* software.

2.6. Validation of reference genes

To confirm the reliability of the candidate reference genes, the relative expression profiles of three AQP genes (*PIP12*, *PIP21*, and *PIP14*) were evaluated against *UBQ, GAPHD*, and *ACT* by using RT-qPCR

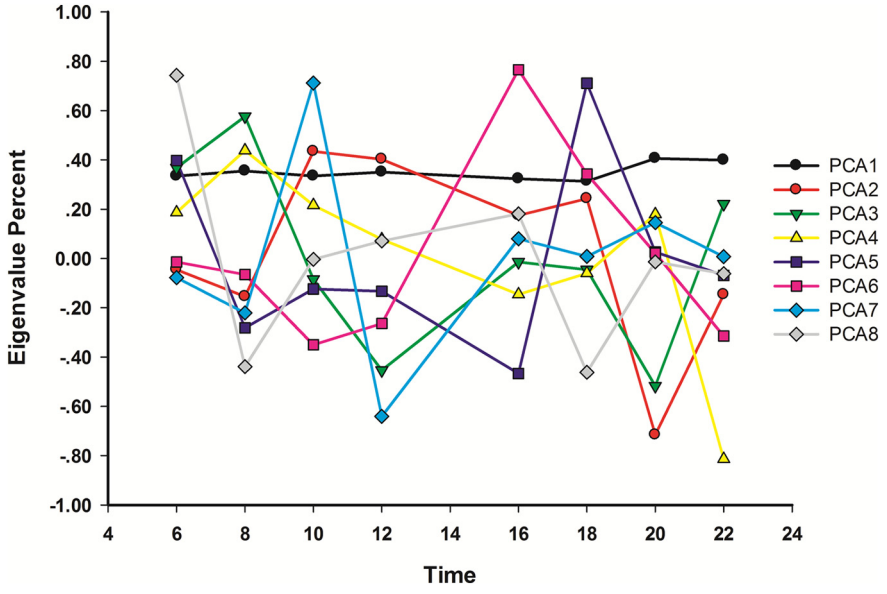

Fig. 1. PCA for reference genes in *Tamarix*.

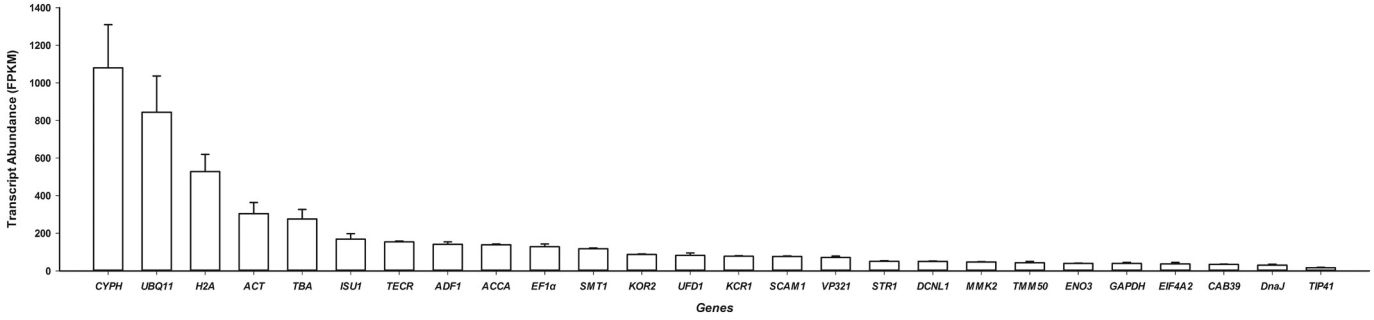

Fig. 2. Boxplot of 26 candidate reference genes.

by the $2^{-\Delta\Delta CT}$ method. Pearson correlation coefficient (PCC) was used to determine consistency of the reference genes. Comparison of expression patterns between RT and RNA-seq further validated the consistency of the genes.

3. Results

3.1. Selection of candidate reference genes

For the diurnal transcriptomes (4:00, 8:00, 12:00, 16:00, 20:00, and 24:00) in *T. ramosissima* under natural field conditions, eigenvalues derived by using the mathematical method were obtained from 72,035 unigenes with PAST software (Fig. 1). The common expression pattern showed that eigenvalues derived from most PCAs accumulated gradually and then declined with a clear-cut maximum, whereas genes in the PCA1 cluster showed a slight peak or no peak during the time series, which suggested that those genes have to meet the requirements of the reference genes.

Combined with average read counts above 500 and CV <0.3, a total of 7385 genes from the PCA1 cluster were obtained (Table S1), including 10 traditional reference genes: Actin (*ACT*), elongation factor 1-alpha (*EF1α*), ubiquitin (*UBQ*), alpha-tubulin (*TUA*), eukaryotic initiation factor 4A-2 (*EIF4A2*), cyclophilin (*CYCL*), *TIP41-like* protein (*TIP41*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), histone H2A (*H2A*), and DNAJ-like protein (*DNAJ*). Additionally, six novel reference genes from [22], including actin-depolymerizing factor 3 (*ADF3*), ubiquitin fusion degradation protein 1 (*UFD1*), iron–sulfur cluster assembly protein (*ISP*), thiosulfate/3-mercaptopyruvate sulfurtransferase (*THS*), transmembrane protein 50 (*TMP50*), and vacuolar protein sorting-associated protein 32 (*VAP32*) were also evaluated to test their common stability under diurnal natural field conditions. To find specific novel reference genes in *T. ramosissima*, the following top 10 genes with lower CV were also selected: cytosolic enolase 3 (*ENO3*), Mo25 family protein isoform 1 (*CAB39*), very long-chain enoyl-CoA reductase (*TECR*), cycloartenol-C-24-methyltransferase (*SMT1*), Acetyl Co-enzyme a carboxylase carboxyltransferase alpha subunit (*ACCA*), DCN1-like protein 1 (*DCNL1*), very long-chain 3-oxoacyl-CoA reductase 1 (*KCR1*), secretory carrier-associated membrane protein 2-like (*SCAM1*), mitogen-activated protein kinase kinase kinase (*KOR2*), and mitogen-activated protein kinase (*MMK2*) (Table S1). In summary, 26 potential candidate reference genes in *T. ramosissima* were evaluated (Table 2). The average transcript abundance (FPKM) of each gene ranged from 16.506 (*TIP41*) to 1045.969 (*CYPH*), and the CV value varied from 0.0285 (*ACCA*) to 0.2297 (*H2A*) (Table 2). *CYPH*, *UBQ11*, *H2A*, *ACT*, and *TBA* were expressed relatively high across the time series (Fig. 2). Further, another 10 novel genes from *T. ramosissima* were also listed in the moderate expression group, among which *TECR*, *SMT1*, and *ACCA* ranked on the top list.

Table 2
FPKM for 26 candidate reference genes across time series.

Gene	4 h	8 h	12 h	15 h	16 h	20 h	22 h	24 h
ACT	304.79	304.45	310.89	279.78	356.32	276.28	198.73	398.24
EF1α	127.81	139.02	128.55	121.62	150.76	119.65	109.52	151.89
UBQ11	640.90	873.03	1099.33	762.69	954.11	815.23	536.19	1042.23
CYPH	980.72	948.16	1095.76	1083.95	1176.56	1077.27	598.15	1407.18
TBA	256.56	256.47	272.26	347.41	314.78	279.95	387.40	249.43
GAPDH	40.24	43.18	40.41	36.56	39.32	37.94	53.46	36.68
TIP41	16.75	17.39	16.16	15.54	16.90	18.41	18.03	12.87
DnaJ	33.53	31.98	28.15	27.24	31.74	28.77	42.81	28.76
EIF4A2	31.53	29.86	37.61	43.19	53.74	37.29	32.67	37.06
H2A	535.87	532.29	466.37	567.09	424.34	515.42	523.38	734.94
ADF1	138.50	141.45	141.47	149.25	140.63	139.83	132.10	174.65
UFD1	92.05	81.23	81.29	74.31	83.66	89.75	63.59	105.51
ISU1	171.26	170.44	143.19	134.37	198.01	166.54	105.64	179.50
STR1	52.46	51.62	51.09	48.93	48.87	56.15	44.19	50.79
TMM50	47.91	39.98	35.72	38.41	36.48	46.25	50.13	55.04
VP321	66.24	60.56	78.10	70.85	70.59	73.17	71.29	85.69
ENO3	41.41	39.92	39.44	41.28	39.06	43.06	38.86	39.88
CAB39	35.36	32.31	33.84	34.77	33.53	35.85	32.73	35.41
TECR	160.20	145.96	154.67	150.58	152.78	154.84	158.72	152.50
SMT1	121.17	111.68	112.74	118.13	118.29	121.70	111.05	116.65
ACCA	139.82	140.02	135.29	132.76	138.48	138.91	137.46	146.31
DCNL1	49.38	49.33	51.39	51.11	49.83	53.97	47.65	50.81
KCR1	77.33	79.22	76.65	73.00	81.60	78.05	76.95	80.20
SCAM1	78.73	74.48	72.70	77.13	77.74	75.68	79.59	71.75
KOR2	92.24	85.51	90.11	88.24	83.26	86.38	83.33	87.36
MMK2	47.12	46.64	48.89	48.57	51.24	46.86	46.19	47.14

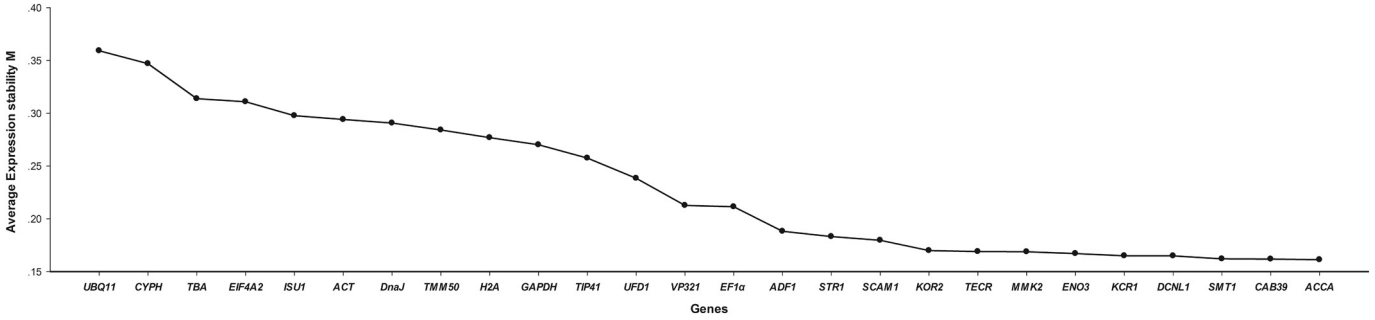

Fig. 3. Gene expression stability and ranking of 26 reference genes calculated using *geNorm*. The most stable genes are on the left and the least stable genes are on the right.

3.2. Expression stability of candidate reference genes with geNorm analysis

Results from *geNorm* analysis showed that all the top 10 genes have M values lower than 1.5, which suggested their acceptable stability in the diurnal expression (Fig. 3). In this study, *ACCA*, *CAB39*, *SMT1*, *DCNL1*, and *KCR* were the top five genes with low M values, which belonged to the novel specific group from *T. ramosissima*. Compared to these five genes, the traditional reference genes, namely, *UBQ11*, *CYPH*, *TBA*, *EIF4A2*, and *ACT* have less stable M values. The stability ranking of the tested genes was plotted according to the M value shown in Fig. 3.

3.3. NormFinder analysis

By using *NormFinder* analysis, *UFD1* was ranked in the first place for expression stability, followed by *ADF1*, *EF1α*, *VP321*, and *ACCA*, which suggested to be the top five stable internal reference genes across the time series (Table 3). *UBQ* and *CYPH* were listed as the least stability genes, although these two genes had high FPKM, which suggests these genes can be more detectable and easy perform for RT-PCR.

3.4. BestKeeper analysis

Data of the top 15 genes obtained using three software programs (CV, *geNorm*, and *NormFinder*) were input into Venny 2.1.0 (Fig. 4).

Table 3
Expression stability of the reference genes calculated using *NormFinder* software.

Gene name	Stability value	Standard error
ACT	40.633	11.877
EF1α	5.796	7.950
UBQ11	183.410	49.172
CYPH	218.011	58.368
TBA	65.130	18.029
GAPDH	21.712	7.681
TIP41	17.853	7.031
DnaJ	21.060	7.562
EIF4A2	13.968	6.571
H2A	86.558	23.587
ADF1	4.766	8.981
UFD1	2.011	18.425
ISU1	19.055	7.218
STR1	14.675	6.633
TMM50	17.375	6.961
VP321	10.725	6.484
ENO3	16.125	6.794
CAB39	15.349	6.703
TECR	18.819	7.180
SMT1	15.222	6.689
ACCA	13.753	6.554
DCNL1	14.992	6.665
KCR1	14.784	6.644
SCAM1	18.947	7.201
KOR2	15.685	6.741
MMK2	15.277	6.695

Finally, data of 10 candidate genes were entered into *BestKeeper* software (Table 4). The stability ranking order of the 10 genes was as follows: *CAB39*, *ENO3*, *MMK2*, *DCNL1*, *KCR1*, *STR1*, *KOR2*, *ACCA*, *SMT1*, and *ADF1*, which suggested little discrepancy with *NormFinder* analysis. Further data processing using PCC and regression analysis (r) showed that *ACCA* exhibited the lowest correlation with *KCR1* ($r = 0.845$, p-value $= 0.008$), which could be selected as a less likely combination reference system.

In summary, *ACCA*, *KCR1*, *SMT1*, and *DCNL1* shared on the top rank of four analysis (CV, *geNorm*, *NormFinder*, and *BestKeeper*), which is a potential candidate reference gene in *T. ramosissima*. *GAPDH*, *DnaJ*, *H2A*, *ACT*, *CYPH*, and *UBQ11* expression was relatively high; however, their stability was less than the novel candidate reference genes.

3.5. Reference gene validation

From stability analysis with three software (*geNorm*, *NormFinder*, and *BestKeeper*), a total of 26 genes were found to be very stable during the diurnal variation, which was well consistent with the definition of the internal reference gene. However, from the CV analysis with *past* software, *UBQ* had the highest transcript level,

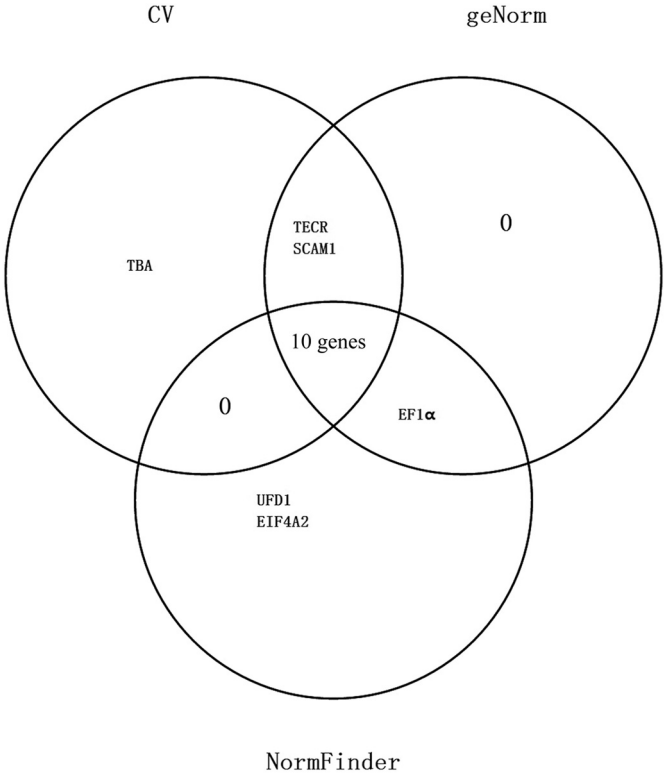

Fig. 4. Venn diagram using CV *geNorm* and *NormFinder*.

Table 4
Expression stability of the reference genes calculated using *BestKeeper* software.

Score	*ACCA*	*ADF1*	*DCNL1*	*ENO3*	*KCR1*	*KOR2*	*MMK2*	*SMT1*	*STR1*	*VP321*
Std. dev. [± CP]	0.02	0.05	0.03	0.03	0.02	0.03	0.03	0.03	0.04	0.06
CV [% CP]	1.89	5.32	2.73	2.83	2.44	2.79	2.66	3.00	4.89	6.93

GAPDH had a moderate transcript level, and *EIF4A2* had the lowest transcript level. In this study, the expression level of three AQP genes (*PIP12*, *PIP21*, and *PIP14*) was normalized against three genes (*UBQ*, *GAPDH*, and *EIF4A2*), respectively, to validate the consistence of reference genes (Fig. 5). PCC values of the diurnal expression level of three AQP genes were calculated against *UBQ*, *GAPDH*, and *EIF4A2*, respectively (Table 5). PCC values showed that the expression level of the three genes against each reference gene was all consistent, with a significant p-value (the lowest PCC was 0.841). For further validation, comparisons between expression data collected by RNA-seq and data collected by RT-PCR were performed; primer details are listed in Table 1. The peak of gene expression occurred almost at 10:00 AM, which is 4 h after the beginning of the light period. At approximately noon, when the light intensity was very strong, the abundance of transcripts declined rapidly. A slight second peak was displayed before dusk. The transcript abundance then decreased slightly to a low level at approximately 20:00 PM (Fig. 5). This clearly shows that the diurnal expression pattern of each AQP gene was similar against the three reference genes.

Table 5
Pearson correlation coefficient (PCC) of aquaporin expression against three reference genes.

PIP12			PIP21			PIP14		
PCC	GAPDH	UBQ	PCC	GAPDH	UBQ	PCC	GAPDH	UBQ
UBQ	0.968 (0.001)	–	UBQ	0.968 (0.001)	–	UBQ	0.968 (0.001)	–
EIF4A2	0.873 (0.005)	0.942 (0.001)	EIF4A2	0.873 (0.002)	0.942 (0.001)	EIF4A2	0.873 (0.002)	0.942 (0.001)

4. Discussion

Owing to limited genetic and sequence information, most reference genes used in nonmodel plant species were ortholog genes from the model plants, mainly from *A. thaliana* [52]. However, numerous studies have reported that expression levels of the selected genes exhibited surprisingly high expression variance in some species or under different environmental conditions [19,25,26,27,28,29]. Consequently, bias of expression data occurred or incorrect conclusions were drawn [30]. Before the development of high-throughput technologies, a handful of genes from a small *priori* set of genes were evaluated. With the advantage of RNA-seq, a large amount of gene expression data had been released, and the assessment of reference genes has been upgraded to the level of complete genome strategy.

T. ramosissima plays an important role in sustaining the fragile ecosystems of arid areas. It has been found to be an alleviation of drought in addition to water being transported from the root in *T. ramosissima* under severe drought conditions [65]. To further investigate the mechanism of adapting to the drought environment, global transcriptome profiling had been conducted in the field at multiple time points. However, to reduce the false discovery rate of high-throughput RNA-seq, the interested genes should be validated against the RT-PCR method, which needs to investigate a set of stable reference genes for normalizing gene expression.

In this study, we have identified a large amount of genes (7385) with slight modulation using *past* software, with criteria average read counts above 500 and CV <0.3 (Table S1). Among them, 10 traditional reference genes, 10 newly discovered genes, and 6 reference genes used in *Taihangia* were included in the modules (Table 2). Although the average transcript abundance (FPKM) varied heavily, the CV value did not change much (from 0.0285 to 0.2297). Stability analyses using *geNorm*, *NormFinder*, and *BestKeeper* also supported that all 26 genes

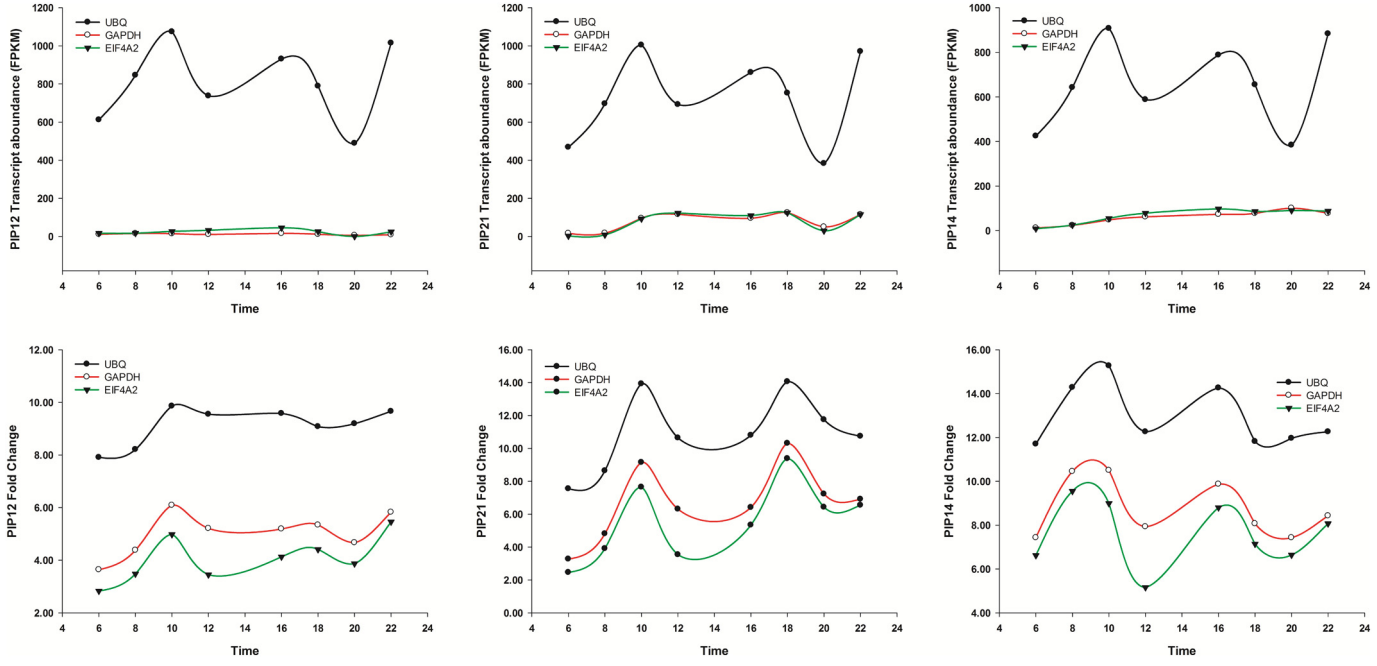

Fig. 5. Diurnal expression pattern comparison between RT-PCR and RNA-seq.

were stable and hence be an acceptable choice of reference genes under diurnal gene expression normalization. Clearly, the ranking order of individual genes was little different with each software. From *geNorm*, the top five stable genes were *ACCA*, *CAB39*, *SMT1*, *DCNL1*, and *KCR*, whereas from *NormFinder*, the genes were *UFD1*, *ADF1*, *EF1α*, *VP321*, and *ACCA*. This is not unexpected because each program employed different algorithm packages and analytical procedures. The data of 10 common genes obtained using *geNorm* and *NormFinder* by Venn analysis were applied to *BestKeeper* software to overcome the drawback of software.

To validate expression stability of candidate genes, the expression pattern of three AQP genes was quantified against three representations of the reference genes *UBQ* (high expression), *EIF4A2* (low expression), and *GAPDH* (moderate expression) with RT-PCR and RNA-seq, respectively. The PCC values were all higher than 0.841 with a significant p-value, which suggested consistence of expression level. According to Table S1, it is not troublesome to select the most stable internal reference genes across diurnal studies in the genus *Tamarix*.

As a halophyte, *T. ramosissima* grows in broad habitats from low soil salinity to salt marshes. It can sequester high concentrations of sodium and other salts in its above-ground tissue and secrete these concentrated salts through specialized salt glands to the surface of leaves or shoots [58,59]. Based on another halophyte *R. songarica*, which is also a species relative to *T. ramosissima*, variation in salinity has little effect on the internal reference gene in different ecotypes [59]. We also evaluated the expression patterns of the candidate reference genes in the transcriptomes of the sister species *Reaumuria trigyna* under stress [72]. We did not find any internal reference gene in the list of differentially expressed genes. Thus, we focused on the diurnal gene expression of *T. ramosissima* under natural conditions without considering salt stress and eco- or geotypes.

Although the expression patterns of the three AQP genes in *T. ramosissima* were consistent, the expression level for each gene was apparently higher by RNA-seq than that by the RT-PCR method. In addition to systematic error from the sequencing concentration of RNA, water absorption from air moisture at night attributed to the high expression of AQP genes [65]. As a major channel to control water content within plant [73,74,75], AQP isoforms direct water transport by different expression [76,77] under different physiological and environmental stresses [78]. Much work has focused on the differential expression from the root or stress response [69,79,80,81, 82,83,84,85]; few papers reported their regulation of foliar water uptake [86,87]. Previous studies have revealed that *PIP21*, one of the isoforms of AQPs was responsible for water uptake through the leaf at night [65]. In this study, two other genes (*PIP12* and *PIP14*) were included into the regulation system of foliar water uptake. From another halophyte, *Kochia sieversiana*, results showed that some paralogs of AQPs were regulated by low- or high-salinity stresses, whereas some were regulated only by low-salinity stress [88]. Considering potassium ($K+$), one of the important signals of gene expression involved in ion balance, the water uptake was suggested to be a complex network. Future studies should consider the mechanism of osmotic homeostasis in halophytes. Nevertheless, this study provided some indication with regard to the gene level to support absorption of moisture from air as a subsidiary to alleviate drought in arid regions.

5. Conclusion

To our knowledge, this study was the first to systematically analyze reference genes in *T. ramosissima* against diurnal oscillations. With the evaluation of expression stability using *past* software, *geNorm*, *Norm-Finder*, and *BestKeeper*, the genes *ACCA*, *KCR1*, *SMT1*, and *DCNL1* were ranked as the top 26 genes, which were suggested to be potential candidate reference genes in *T. ramosissima*. However, the genes *GAPDH*, *DnaJ*, *H2A*, *ACT*, *CYPH*, and *UBQ11* showed relatively high expression, which made them easier to be detected or quantified by RT-PCR; their stability was less than that of the novel candidate reference genes. In addition, we provided a diurnal transcript pattern of AQPs in the field and referred a possible additional mechanism for drought adaptation in *T. ramosissima* through regulating AQPs in leaves to absorb moisture in the air. This study will speed up functional analysis of special genetic resources and serve a solid foundation for study in *Tamarix* species under natural conditions.

Conflicts of interest

The authors declare no conflict of interest.

Financial support

This work was supported by the National Key Research and Development Program of China (Grant Nos. 2016YFC0500903 and 2017FY100200), the Natural Science Foundation of China (NSFC, Grant Nos. 31370395 and 31500266), and Gansu Science and Technology Supporting Project (Grant No. 1604NKCA049).

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2018.08.004.

References

[1] Cronn R, Dolan PC, Jogdeo S, et al. Transcription through the eye of a needle: Daily and annual cyclic gene expression variation in Douglas-fir needles. BMC Genomics 2017;18(1):558. https://doi.org/10.1186/s12864-017-3916-y.
[2] Kim SW, Lee SK, Jeong HJ, et al. Crosstalk between diurnal rhythm and water stress reveals an altered primary carbon flux into soluble sugars in drought-treated rice leaves. Sci Rep 2017;7(1):8214. https://doi.org/10.1038/s41598-017-08473-1.
[3] Harmer SL, Hogenesch JB, Straume M, et al. Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 2000;290(5499):2110–3. https://doi.org/10.1126/science.290.5499.2110.
[4] Edwards KD, Anderson PE, Hall A, et al. *FLOWERING LOCUS C* mediates natural variation in the high-temperature response of the *Arabidopsis* circadian clock. Plant Cell 2006;18(3):639–50. https://doi.org/10.1105/tpc.105.038315.
[5] Covington MF, Maloof JN, Straume M, et al. Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol 2008;9(8):R130. https://doi.org/10.1186/gb-2008-9-8-r130.
[6] Michael TP, Mockler TC, Breton G, et al. Network discovery pipeline elucidates conserved time-of-day-specific *cis*-regulatory modules. PLoS Genet 2008;4(2):e14. https://doi.org/10.1371/journal.pgen.0040014.
[7] Facella P, Lopez L, Carbone F, et al. Diurnal and circadian rhythms in the tomato transcriptome and their modulation by cryptochrome photoreceptors. PLoS One 2008;3(7):e2798. https://doi.org/10.1371/journal.pone.0002798.
[8] Khan S, Rowe SC, Harmon FG. Coordination of the maize transcriptome by a conserved circadian clock. BMC Plant Biol 2010;10:126. https://doi.org/10.1186/1471-2229-10-126.
[9] Filichkin SA, Breton G, Priest HD, et al. Global profiling of rice and poplar transcriptomes highlights key conserved circadian-controlled pathways and *cis*-regulatory modules. PLoS One 2011;6(6):e16907. https://doi.org/10.1371/journal.pone.0016907.
[10] Marcolino-Gomes J, Rodrigues FA, Fuganti-Pagliarini R, et al. Transcriptome-wide identification of reference genes for expression analysis of soybean responses to drought stress along the day. PLoS One 2015;10(9):e0139051. https://doi.org/10.1371/journal.pone.0139051.
[11] Marcolino-Gomes J, Rodrigues FA, Fuganti-Pagliarini R, et al. Diurnal oscillations of soybean circadian clock and drought responsive genes. PLoS One 2014;9(1):e86402. https://doi.org/10.1371/journal.pone.0086402.
[12] Sgamma T, Pape J, Massiah A, et al. Selection of reference genes for diurnal and developmental time-course real-time PCR expression analyses in lettuce. 2016;12:21. https://doi.org/10.1186/s13007-016-0121-y.
[13] Izawa T, Mihara M, Suzuki Y, et al. Os-*GIGANTEA* confers robust diurnal rhythms on the global transcriptome of rice in the field. Plant Cell 2011;23(5):1741–55. https://doi.org/10.1105/tpc.111.083238.
[14] Izawa T. Physiological significance of the plant circadian clock in natural field conditions. Plant Cell Environ 2012;35(10):1729–41. https://doi.org/10.1111/j.1365-3040.2012.02555.x.
[15] Kudoh H. Molecular phenology in plants: *In natura* systems biology for the comprehensive understanding of seasonal responses under natural environments. New Phytol 2016;210(2):399–412. https://doi.org/10.1111/nph.13733.
[16] Nishio H, Buzas DM, Nagano AJ, et al. From the laboratory to the field: Assaying histone methylation at *FLOWERING LOCUS C* in naturally growing *Arabidopsis halleri*. Genes Genet Syst 2016;91(1):15–26. https://doi.org/10.1266/ggs.15-00071.

[17] Tang W, Lin YJ. Field experiment of transgenic cry1Ab insect resistant rice. Hereditas 2007;29(8):1008–12. https://doi.org/10.1360/yc-007-1008.
[18] Bravo-Almonacid F, Rudoy V, Welin B, et al. Field testing, gene flow assessment and pre-commercial studies on transgenic *Solanum tuberosum* spp. *tuberosum* (cv. Spunta) selected for PVY resistance in Argentina. Transgenic Res 2012;21(5): 967–82. https://doi.org/10.1007/s11248-011-9584-9.
[19] Zhang Y, Han X, Chen S, et al. Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in *Salix matsudana* under different abiotic stresses. Sci Rep 2017;7:40290. https://doi.org/10.1038/srep40290.
[20] Stanton KA, Edger PP, Puzey JR, et al. A whole-transcriptome approach to evaluating reference genes for quantitative gene expression studies: A case study in *Mimulus*. G3 Genes Genomes Genet 2017;7(4):1085–95. https://doi.org/10.1534/g3.116.038075.
[21] Pombo MA, Zheng Y, Fei Z, et al. Use of RNA-seq data to identify and validate RT-qPCR reference genes for studying the tomato-*Pseudomonas* pathosystem. Sci Rep 2017;7: 44905. https://doi.org/10.1038/srep44905.
[22] Li W, Zhang L, Zhang Y, et al. Selection and validation of appropriate reference genes for quantitative real-time PCR normalization in staminate and perfect flowers of andromonoecious *Taihangia rupestris*. Front Plant Sci 2017;8:729. https://doi.org/10.3389/fpls.2017.00729.
[23] Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 2004;64. https://doi.org/10.1158/0008-5472.can-04-0496.
[24] Thellin O, Zorzi W, Lakaye B, et al. Housekeeping genes as internal standards: Use and limits. J Biotechnol 1999;75(2–3):291–5. https://doi.org/10.1016/s0168-1656(99)00163-7.
[25] Brunner AM, Yakovlev IA, Strauss SH. Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol 2004;4:14. https://doi.org/10.1186/1471-2229-4-14.
[26] Czechowski T, Stitt M, Altmann T, et al. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol 2005;139(1):5. https://doi.org/10.1104/pp.105.063743.
[27] Dheda K, Huggett JF, Bustin SA, et al. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 2004;37(1):112–9. https://doi.org/10.2144/04371rr03.
[28] Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. Biotechniques 2000;29(2):332–7.
[29] Hu R, Fan C, Li H, et al. Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. BMC Mol Biol 2009; 10:93. https://doi.org/10.1186/1471-2199-10-93.
[30] Gutierrez L, Mauriat M, Guenin S, et al. The lack of a systematic validation of reference genes: A serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol J 2008;6(6):609–18. https://doi.org/10.1111/j.1467-7652.2008.00346.x.
[31] Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3(7). Research0034.1 . https://doi.org/10.1186/gb-2002-3-7-research0034.
[32] Pfaffl MW, Tichopad A, Prgomet C, et al. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. Biotechnol Lett 2004;26(6):509–15. https://doi.org/10.1023/B:BILE.0000019559.84305.47.
[33] Libault M, Thibivilliers S, Bilgin DD, et al. Identification of four soybean reference genes for gene expression normalization. Plant Genome 2008;1(1):44–54. https://doi.org/10.3835/plantgenome2008.02.0091.
[34] de Oliveira LA, Breton MC, Bastolla FM, et al. Reference genes for the normalization of gene expression in *Eucalyptus* species. Plant Cell Physiol 2012;53(2):405–22. https://doi.org/10.1093/pcp/pcr187.
[35] Marioni JC, Mason CE, Mane SM, et al. RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays. Genome Res 2008;18 (9):1509–17. https://doi.org/10.1101/gr.079558.108.
[36] Wang Z, Gerstein M, Snyder M. RNA-Seq: A revolutionary tool for transcriptomics. Nat Rev Genet 2009;10(1):57–63. https://doi.org/10.1038/nrg2484.
[37] Mortazavi A, Williams BA, McCue K, et al. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 2008;5(7):621–8. https://doi.org/10.1038/nmeth.1226.
[38] Nagalakshmi U, Wang Z, Waern K, et al. The transcriptional landscape of the yeast genome defined by RNA sequencing. Science 2008;320(5881):1344–9. https://doi.org/10.1126/science.1158441.
[39] Hruz T, Wyss M, Docquier M, et al. RefGenes: Identification of reliable and condition specific reference genes for RT-qPCR data normalization. BMC Genomics 2011;12: 156. https://doi.org/10.1186/1471-2164-12-156.
[40] Wu W, Deng Q, Shi P, et al. Identification of appropriate reference genes for normalization of miRNA expression in grafted watermelon plants under different nutrient stresses. PLoS One 2016;11(10):e0164725. https://doi.org/10.1371/journal.pone.0164725.
[41] Carmona R, Arroyo M, Jiménez-Quesada MJ, et al. Automated identification of reference genes based on RNA-seq data. Biomed Eng Online 2017;16(Suppl. 1):65. https://doi.org/10.1186/s12938-017-0356-5.
[42] Geng X, Li W, Shang H, et al. A reference gene set construction using RNA-seq of multiple tissues of Chinese giant salamander, *Andrias davidianus*. GigaScience 2017;6(3):1–7. https://doi.org/10.1093/gigascience/gix006.
[43] Hoang VLT, Tom LN, Quek XC, et al. RNA-seq reveals more consistent reference genes for gene expression studies in human non-melanoma skin cancers. PeerJ 2017;5:e3631. https://doi.org/10.7717/peerj.3631.
[44] Jain M, Nijhawan A, Tyagi AK, et al. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Commun 2006;345(2):646–51. https://doi.org/10.1016/j.bbrc.2006.04.140.
[45] Xu M, Zhang B, Su X, et al. Reference gene selection for quantitative real-time polymerase chain reaction in *Populus*. Anal Biochem 2011;408(2):337–9. https://doi.org/10.1016/j.ab.2010.08.044.
[46] Hao QN, Zhou XA, Sha AH, et al. Identification of genes associated with nitrogen-use efficiency by genome-wide transcriptional analysis of two soybean genotypes. BMC Genomics 2011;12:525. https://doi.org/10.1186/1471-2164-12-525.
[47] Demidenko NV, Logacheva MD, Penin AA. Selection and validation of reference genes for quantitative real-time PCR in buckwheat (*Fagopyrum esculentum*) based on transcriptome sequence data. PLoS One 2011;6(5):e19434. https://doi.org/10.1371/journal.pone.0019434.
[48] Bedada G, Westerbergh A, Muller T, et al. Transcriptome sequencing of two wild barley (*Hordeum spontaneum* L.) ecotypes differentially adapted to drought stress reveals ecotype-specific transcripts. BMC Genomics 2014;15:995. https://doi.org/10.1186/1471-2164-15-995.
[49] Lovdal T, Lillo C. Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. Anal Biochem 2009;387 (2):238–42. https://doi.org/10.1016/j.ab.2009.01.024.
[50] Borges AF, Fonseca C, Ferreira RB, et al. Reference gene validation for quantitative RT-PCR during biotic and abiotic stresses in *Vitis vinifera*. PLoS One 2014;9(10): e111399. https://doi.org/10.1371/journal.pone.0111399.
[51] Nicot N, Hausman JF, Hoffmann L, et al. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot 2005;56 (421):2907–14. https://doi.org/10.1093/jxb/eri285.
[52] González-Agüero M, Garcia-Rojas M, Di Genova A, et al. Identification of two putative reference genes from grapevine suitable for gene expression analysis in berry and related tissues derived from RNA-Seq data. BMC Genomics 2013;14: 878. https://doi.org/10.1186/1471-2164-14-878.
[53] Fan C, Ma J, Guo Q, et al. Selection of reference genes for quantitative real-time PCR in bamboo (*Phyllostachys edulis*). PLoS One 2013;8(2):e56573. https://doi.org/10.1371/journal.pone.0056573.
[54] Tong Z, Gao Z, Wang F, et al. Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol 2009;10:71. https://doi.org/10.1186/1471-2199-10-71.
[55] Zhu J, Zhang L, Li W, et al. Reference gene selection for quantitative real-time PCR normalization in *Caragana intermedia* under different abiotic stress conditions. PLoS One 2013;8(1):e53196. https://doi.org/10.1371/journal.pone.0053196.
[56] Gantasala NP, Papolu P, Thakur P, et al. Selection and validation of reference genes for quantitative gene expression studies by real-time PCR in eggplant (*Solanum melongena* L). BMC Res Notes 2013;6:312. https://doi.org/10.1186/1756-0500-6-312.
[57] Yan X, Dong X, Zhang W, et al. Reference gene selection for quantitative real-time PCR normalization in *Reaumuria soongorica*. PLoS One 2014;9(8):e104124. https://doi.org/10.1371/journal.pone.0104124.
[58] Storey R, Thomson WW. An X-ray microanalysis study of the salt glands and intracellular calcium crystals of *Tamarix*. Ann Bot 1994;73(3):307–13. https://doi.org/10.1006/anbo.1994.1036.
[59] Sookbirsingh R, Castillo K, Gill TE, et al. Salt separation processes in the salt cedar *Tamarix ramosissima* (Ledeb.). Commun Soil Sci Plant Anal 2010;41(10):1271–81. https://doi.org/10.1080/00103621003734281.
[60] Yu T, Feng Q, Si J, et al. Patterns, magnitude, and controlling factors of hydraulic redistribution of soil water by *Tamarix ramosissima* roots. J Arid Land 2013;5(3): 396–407. https://doi.org/10.1007/s40333-013-0173-z.
[61] An Y, Wang Y, Lou L, et al. A novel zinc-finger-like gene from *Tamarix hispida* is involved in salt and osmotic tolerance. J Plant Res 2011;124(6):689–97. https://doi.org/10.1007/s10265-011-0403-4.
[62] Gao C, Wang Y, Liu G, et al. Expression profiling of salinity-alkali stress responses by large-scale expressed sequence tag analysis in *Tamarix hispid*. Plant Mol Biol 2008;66 (3):245–58. https://doi.org/10.1007/s11103-007-9266-4.
[63] Gao C, Zhang K, Yang G, et al. Expression analysis of four peroxiredoxin genes from *Tamarix hispida* in response to different abiotic stresses and Exogenous Abscisic Acid (ABA). Int J Mol Sci 2012;13(3):3751–64. https://doi.org/10.3390/ijms13033751.
[64] Ji X, Wang Y, Liu G. Expression analysis of *MYC* genes from *Tamarix hispida* in response to different abiotic stresses. Int J Mol Sci 2012;13(2):1300–13. https://doi.org/10.3390/ijms13021300.
[65] Yan X, Zhou M, Dong X, et al. Molecular mechanisms of foliar water uptake in a desert tree. AoB Plants 2015;7:plv129. https://doi.org/10.1093/aobpla/plv129.
[66] Eller CB, Lima AL, Oliveira RS. Foliar uptake of fog water and transport belowground alleviates drought effects in the cloud forest tree species, *Drimys brasiliensis* (Winteraceae). New Phytol 2013;199(1):151–62. https://doi.org/10.1111/nph.12248.
[67] Fordham MC, Harrison-Murray RS, Knight L, et al. Effects of leaf wetting and high humidity on stomatal function in leafy cuttings and intact plants of *Corylus maxima*. Physiol Plant 2001;113(2):233–40. https://doi.org/10.1034/j.1399-3054.2001.1130211.x.
[68] Breazeale EL, McGeorge WT, Breazeale JF. Moisture absorption by plants from an atmosphere of high humidity. Plant Physiol 1950;25(3):413–9. https://doi.org/10.1104/pp.25.3.413.
[69] Zupin M, Sedlar A, Kidric M, et al. Drought-induced expression of aquaporin genes in leaves of two common bean cultivars differing in tolerance to drought stress. J Plant Res 2017;130(4):735–45. https://doi.org/10.1007/s10265-017-0920-x.
[70] Mallona I, Lischewski S, Weiss J, et al. Validation of reference genes for quantitative real-time PCR during leaf and flower development in *Petunia hybrida*. BMC Plant Biol 2010;10:4. https://doi.org/10.1186/1471-2229-10-4.

[71] Migocka M, Papierniak A. Identification of suitable reference genes for studying gene expression in cucumber plants subjected to abiotic stress and growth regulators. Mol Breed 2011;28(3):343–57. https://doi.org/10.1007/s11032-010-9487-0.
[72] Dang ZH, Zheng LL, Wang J, et al. Transcriptomic profiling of the salt-stress response in the wild recretohalophyte *Reaumuria trigyna*. BMC Genomics 2013;14:29. https://doi.org/10.1186/1471-2164-14-29.
[73] Chaumont F, Barrieu F, Wojcik E, et al. Aquaporins constitute a large and highly divergent protein family in maize. Plant Physiol 2001;125(3):1206–15. https://doi.org/10.1104/pp.125.3.1206.
[74] Nada RM, Abogadallah GM. Aquaporins are major determinants of water use efficiency of rice plants in the field. Plant Sci 2014;227:165–80. https://doi.org/10.1016/j.plantsci.2014.08.006.
[75] Chaumont F, Tyerman SD. Aquaporins: Highly regulated channels controlling plant water relations. Plant Physiol 2014;164(4):1600–18. https://doi.org/10.1104/pp.113.233791.
[76] Maurel C, Verdoucq L, Luu DT, et al. Plant aquaporins: Membrane channels with multiple integrated functions. Annu Rev Plant Biol 2008;59:595–624. https://doi.org/10.1146/annurev.arplant.59.032607.092734.
[77] Wudick MM, Luu DT, Maurel C. A look inside: Localization patterns and functions of intracellular plant aquaporins. New Phytol 2009;184(2):289–302. https://doi.org/10.1111/j.1469-8137.2009.02985.x.
[78] Alexandersson E, Fraysse L, Sjovall-Larsen S, et al. Whole gene family expression and drought stress regulation of aquaporins. Plant Mol Biol 2005;59(3):469–84. https://doi.org/10.1007/s11103-005-0352-1.
[79] Kayum MA, Park JI, Nath UK, et al. Genome-wide expression profiling of aquaporin genes confer responses to abiotic and biotic stresses in *Brassica rapa*. BMC Plant Biol 2017;17(1):23. https://doi.org/10.1186/s12870-017-0979-5.
[80] Martins CP, Neves DM, Cidade LC, et al. Expression of the citrus *CsTIP2;1* gene improves tobacco plant growth, antioxidant capacity and physiological adaptation under stress conditions. Planta 2017;245(5):951–63. https://doi.org/10.1007/s00425-017-2653-4.
[81] Clarkson DT, Carvajal M, Henzler T, et al. Root hydraulic conductance: Diurnal aquaporin expression and the effects of nutrient stress. J Exp Bot 2000;51(342):61–70. https://doi.org/10.1093/jexbot/51.342.61.
[82] Henzler T, Waterhouse RN, Smyth AJ, et al. Diurnal variations in hydraulic conductivity and root pressure can be correlated with the expression of putative aquaporins in the roots of *Lotus japonicus*. Planta 1999;210(1):50–60. https://doi.org/10.1007/s004250050653.
[83] Lopez F, Bousser A, Sissoëff I, et al. Diurnal regulation of water transport and aquaporin gene expression in maize roots: Contribution of PIP2 proteins. Plant Cell Physiol 2003;44(12):1384–95. https://doi.org/10.1093/pcp/pcg168.
[84] Sakurai-Ishikawa J, Murai-Hatano M, Hayashi H, et al. Transpiration from shoots triggers diurnal changes in root aquaporin expression. Plant Cell Environ 2011;34(7):1150–63. https://doi.org/10.1111/j.1365-3040.2011.02313.x.
[85] Vandeleur RK, Mayo G, Shelden MC, et al. The role of plasma membrane intrinsic protein aquaporins in water transport through roots: Diurnal and drought stress responses reveal different strategies between isohydric and anisohydric cultivars of grapevine. Plant Physiol 2009;149(1):445–60. https://doi.org/10.1104/pp.108.128645.
[86] Ohrui T, Nobira H, Sakata Y, et al. Foliar trichome- and aquaporin-aided water uptake in a drought-resistant epiphyte *Tillandsia ionantha* Planchon. Planta 2007;227(1):47–56. https://doi.org/10.1007/s00425-007-0593-0.
[87] Laur J, Hacke UG. The role of water channel proteins in facilitating recovery of leaf hydraulic conductance from water stress in *Populus trichocarpa*. PLoS One 2014;9(11):e111751. https://doi.org/10.1371/journal.pone.0111751.
[88] Zhao L, Yang Z, Guo Q, et al. Transcriptomic profiling and physiological responses of *Halophyte Kochia sieversiana* provide insights into salt tolerance. Front Plant Sci 2017;8:1985. https://doi.org/10.3389/fpls.2017.01985.