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ORIGINAL PAPER

Identification and evaluation of reference genes for normalization in quantitative real-time PCR analysis in the premodel tree *Betula luminifera*

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Abstract *Betula luminifera* is a commercial tree species that is emerging as a new model system for tree genomics research. A draft genomic sequence is expected to be publicly available in the near future, which means that an explosion of gene expression studies awaits. Thus, the work of selecting appropriate reference genes for qPCR normalization in different tissues or under various experimental conditions is extremely valuable. In this study, ten candidate genes were analyzed in *B. luminifera* subjected to different abiotic stresses and at various flowering stages. The expression stability of these genes was evaluated using three distinct algorithms implemented using geNorm, NormFinder and BestKeeper. The best-ranked reference genes varied across different sample sets, though *RPL39*, *MDH* and *EF1a* were determined as the most stable by the three programs among all tested samples. *RPL39* and *EF1a* should be appropriate for normalization in N-starved roots, while the combination of *RPL39* and *MDH* should be appropriate for N-starved stems and *EF1a* and *MDH* should be appropriate in N-starved leaves. In PEG-treated (osmotic) roots, *MDH* was the most suitable, whereas EF1a was suitable for PEG-treated stems and leaves. *TUA* was also stably expressed levels in PEG-treated plants. The combination of *RPL39* and *TUB* should be appropriate for heat-stressed leaves and flowering stage. For reference gene validation, the expression levels of *SOD* and *NFYA-3* were investigated. This work will be beneficial to future studies on gene expression under different abiotic stress conditions and flowering status in *B. luminifera.*

Keywords Reference genes · Gene expression · *Betula luminifera* · Abiotic stresses · Premodel tree

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Introduction

Betula luminifera is a deciduous tree, a member of the *Betulaceae* family, and is widely distributed in the 14 provinces of China. Its good wood properties, beautiful texture and fast-growing character make it widely used in solid wood furniture and flooring. Furthermore, *B. luminifera* is highly adaptable to barren soil, thus it was proposed as a priority timber species for afforestation (Huang et al. 2014). In addition to its commercial traits, *B. luminifera* has great potential for use as a species for forest genetic studies, exemplified by its small genome size (approximately 450 Mb) and a comparatively short juvenile period. Most lines of *B. luminifera* begin flowering at

1.5 years old, and it has the potential to be a model for woody plants that usually have long juvenile periods. It may also shorten the genetic improvement period for tree breeding. Most tree plantations are located on barren lands where the available nitrate is extremely low, limiting plant growth (Bigelow and Canham 2007). Moreover, extreme weather conditions, such as high temperature and drought, occur frequently in these areas and may affect plant development (Guenin et al. 2009). For plants, the subtle evolutionary changes that accompanied the process of plant domestication are exemplified by a series of genes in response to these abiotic stresses (Mittler and Blumwald 2010).

Gene expression analysis is a fundamental approach for functional genomics studies, which can be used to estimate complicated gene regulatory networks (Obrero et al. 2011). Currently, reverse transcription quantitative real-time PCR (RT-qPCR), extremely sensitive, efficient and reproducible method, is frequently used to quantify gene transcript levels and to analyze gene abundance among different plant tissues, developmental stages and abiotic stresses (Chen et al. 2011; Hong et al. 2010; Wan et al. 2010). However, a number of variables are involved in the accuracy of RT-qPCR. Thus, it is indispensable to adopt a normalization step to avoid bias. The most useful approach for normalization is to apply one or several reference genes, whose expressions are presumed to be stable in both control and experimental conditions (Guenin et al. 2009). Extensive transcriptomics mining and experimental data have shown that there is no such universal reference gene that can be used in all plant tissues under all experimental conditions (Chen et al. 2011). Statistical algorithms have been developed recently but, surprisingly, this robust approach is under-utilized in plants. Instead, putative housekeeping genes tend to be used as references without any proper validation, which may lead to misinterpretation of some results (Guenin et al. 2009).

Studies involving the selection of reference genes have increased, but mainly focus on model and important crop species, such as *Arabidopsis* (Hong et al. 2010), rice (Bustin et al. 2009; Udvardi et al. 2008), cucumber (Wan et al. 2010), soybean (Jian et al. 2008), wheat (Paolacci et al. 2009), and poplar (Brunner et al. 2004). There has been no systematic analysis for the selection of reference genes for RT-qPCR in *B. luminifera*, which may adversely impact molecular research of this species. In this study, ten candidate reference genes were selected to normalize the results of gene expression studies under nitrate starvation, osmotic stress and high temperature conditions and at differing flowering stages using three algorithms: geNorm, NormFinder and Bestkeeper (Udvardi et al. 2008).

Materials and methods

Plant materials and treatments

The G49-3 line of *B. luminifera* was used. The seedlings were maintained on half strength Murashige and Skoog (1/2MS) medium with 30 g L^{-1} sucrose and solidified with 6 g L^{-1} agar, pH adjusted to 5.9, with 16 h light/8 h dark.

For experimental treatments, the top three internodes of seedlings were placed on bridges made of filter-paper in glass tubes, which were filled with 40 mL of half solid (3 g L^{-1} agar) 1/2MS medium. After 1 month of growth, only uniformly developed plants were used in subsequent experiments. For the nitrate starvation treatment, the original medium was suctioned out and displaced by non-nitrate medium, and K^+ concentration was adjusted by the addition of KCl for 1, 2 or 10 days. For osmotic treatment, plants were placed in PEG6000 (15 %) for 1, 2 or 5 days. For heat stress treatment, seedlings were grown at 45 °C for 0.5, 1, 2 or 4 h. For the analysis of flowering stages, both male and female inflorescences were sampled every 3 days.

Roots, stems and leaves were collected from plants subjected to nitrate starvation and osmotic stress. For the high temperature treatment, only leaves were collected. Three biological replicates were sampled for all treatments, and these materials were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent use.

RNA isolation, purification and cDNA synthesis

Total RNA was extracted from collected samples using Trizol reagent, according to the manufacturer's instructions (Invitrogen). RNA samples were treated with RNase-free DNase (Promega) to remove remaining DNA and the integrity was examined using 1 % agarose gel electrophoresis. RNA concentration and purity was determined using a NanoDrop ND-2000 spectrophotometer (Thermo-Scientific, Wilmington, DE, USA). RNA samples with an absorbance ratio at OD260/280 between 1.8 and 2.2 and OD 260/230 of ~2.0 were used for further analysis. Complementary DNA (cDNA) was synthesized using the Primer Script RT reagent kit (TaKaRa Biotechnology, Dalian, China) following the manufacturer's instructions in a final volume of 20 μL using 1 μg of total RNA. The final cDNA products were diluted fivefold prior to use in qPCR.

Reference gene selection, primer design and amplification efficiency

To identify suitable reference genes for *B. luminifera*, ten candidates that are frequently used or newly identified in

other plant species were selected (Supplementary Table 1). These candidates included such traditional reference genes as large ribosomal subunit 39 (*RPL39*), polyubiquitin 10 (*UBQ10*), ribosomal protein S24 (*S24*), β-tubulin (*TUB*), malate dehydrogenase (*MDH*), elongation factor 1-*a* (*EF1-a*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *a*-tubulin (*TUA*), actin7 (*ACT*), and a novel identified stable reference gene of *Arabidopsis*, the TIP-41 like protein (*TIP41*).

To obtain *B. luminifera* orthologous sequences of the candidates, BLASTN searches were conducted using *Arabidopsis* genes as queries on *B. luminifera* unigenes in the transcriptomic data. PCR primers were designed using Oligo 7.0 software (Molecular Biological Insights, Colorado Springs, CO, USA) with melting temperatures of 60 ± 1 °C and amplicon lengths of 100–200 bp (Table 1, Supplementary Table 2). Specific products and amplicon lengths of all primer pairs were verified using 2.0 % agarose gel electrophoresis (Supplementary Fig. 1). The amplification efficiency of each primer pair was calculated using a standard curve generated from a serial dilution of cDNA (1, 10, 10^2, 10^3, 10^4 dilutions). Mean quantification cycle (C_q) values of each tenfold dilution were plotted against the logarithm of the cDNA dilution factor. The amplification efficiency (E) for each gene was calculated using the slope of a linear regression model (Pfaffl 2001) using the C_q values and the following equation: $E = [10^{(1/-\text{slope})} - 1] \times 100$ %.

qPCR and statistical analyses

The transcript abundance for each of the ten genes was analyzed by qPCR (CFX96, BioRad, Hercules, USA) using SYBR Premix EX Taq (TaKaRa Biotechnology, Dalian, China) in a volume of 10 μL, following the manufacturer's instructions. Thermal conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. The dissociation curve was obtained by heating the amplicon from 60 to 95 °C, and the melting curve had a single peak (Supplementary Fig. 2). All qPCR reactions were performed in technical triplicate. In this work, we followed the minimum information for publication of qPCR experiments (MIQE) (Bustin et al. 2009). The stability of candidate reference genes across all of the experimental conditions was analyzed using three Microsoft Excel-based software packages: geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004). All three software packages were used according to their manuals; all other multiple comparisons were performed using SPSS 17.0 (IBM, Armonk, NY, USA).

Table 1 Description of candidate reference genes and primer sequences used for qPCR

Gene name	Abbreviation	GenBank accession	*Arabidopsis* homolog	Primer sequence	PCR efficiency (%)
Large ribosomal subunit 39	*RPL39*	KP245805	AT4G31985	ATGCCGTCGCACAAGACCTT CCAGGCTCATCCACCTTAGAAC	103.6
Polyubiquitin 10	*UBQ10*	KP245806	AT4G05320	ACATTCAGAAGGAGAGCACC CCACCTCAAGTGTAATTGTCTTGC	97.1
Tonoplast intrinsic protein	*TIP41*	KP245807	AT4G34270	GCCTACACACGAGCCACT ATTCCATTCCCCAGACTATGAC	103.2
Ribosomal protein S24	*S24*	KP245808	AT3G04920	TCCAAGGGCTCTTAAAATGATGCT CAGACGTAACAAGCCAACACCAC	102.0
β-Tubulin	*TUB*	KP245809	AT1G75780	AGGCTTAACACACAAGTTCTGCT CACCCAGACACAGGCAATACG	97.9
Malate dehydrogenase	*MDH*	KP245810	AT1G04410	CTCATGCCTCACTTGAATGCTCGT AGCTCCTCCATCATTAACAGTTCC	91.05
Elongation factor 1-α	*EF1a*	KP245811	AT5G60390	ACTGGAAATTTTGAGGAAGCTAC AAGTTCCCTGCACAACTCAAC	94.9
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	KP245812	AT1G13440	AGACCCATTAGAGGACTTCTGC ACATAGGCCCTAGACAATCAGC	119.4
α-Tubulin	*TUA*	KP245813	AT5G19780	ACTAACCTTGTACCATATCCTCGT GGCTCAAATACTGCACTTGTGA	103.4
Actin7	*ACT*	FJ410442	AT5G09810	GCCAACAGAGAAAAGATGACTC TCACCAGAATCCAGCACAATAC	104.1

Results

Expression profiling of candidate reference genes

To determine the expression profiles of the candidate reference genes across all of the experimental samples, we ran an RT-qPCR, and the raw quantification cycle (C_q) values were calculated. The expression levels of candidates showed a relatively wide range of expression levels and variation, as shown in the box and whisker plots (Fig. 1). *MDH* expression was the most abundant with the lowest mean C_q value (22.68), while *S24* had the lowest level of expression and its average C_q value was 31.23. All genes showed expression variation among the tested samples. The lowest expression variation was observed in *RPL39* with a coefficient of variation (CV) of 3.59 %, while *TIP41* was the most variable across all tested samples, with a CV of 9.25 %.

Evaluation of expression stability

geNorm, NormFinder and BestKeeper were used to aid in the evaluation of suitable reference genes from the candidates. These three programs evaluate gene expression stability using distinct algorithms. The expression stability of ten candidate reference genes was evaluated in 34 cDNA samples, including roots, stems and leaves of seedlings under nitrogen starvation and PEG treatment, leaves under heat stress and flowers at different developmental stages. Altogether, the results obtained from geNorm, NormFinder, and BestKeeper were similar with regard to the best candidate reference gene for expression normalization.

geNorm analysis

The stability of potential reference genes was first examined using geNorm, in which the gene expression stability (M) of ten candidates was calculated. This program uses an *M* value below a threshold of 1.5 to identify reference genes with stable expression (Vandesompele et al. 2002). The lower the *M* value, the higher the gene expression stability. Figure 2 shows the ranking of the tested genes according to their expression stability in *B. luminifera* using data from four sets of treatments. When all of the samples were analyzed together, *MDH* and *RPL39* were the most stable genes, while *TIP41* and *S24* were the least stable genes. In N-starved roots (NR), *UBQ10* and *ACT* had the most stable expression levels, while in N-starved stems (NS), *RPL39* and *MDH* had the highest expression stability. In N-starved leaves (NL), the best reference genes were *MDH* and *EF1a*. *S24* was the least stable gene in all N-starved tissues tested. In the PEG-treated plants, *MDH* and *EF1a* were the most stably expressed genes in roots and stems, respectively, while *EF1a* and *GAPDH* were the most stable genes in leaves, with *MDH* as the next most stable. *S24* and *TIP41* were the most unstable genes under osmotic stress. *GAPDH* and *TUB* were the most stable genes under heat stress, while *S24* and *TIP41* were the least stable genes. In flowers, *RPL39* and *TUB* were the most stable genes in the various developmental stages, while *S24* and *TUA* showed the lowest expression stability.

To determine the optimal number of reference genes, geNorm performs a stepwise calculation of the pairwise variation (V_n/V_{n+1}) between sequential normalization factors. It is suggested that the inclusion of an additional reference gene is not required when the variation value is below 0.15 (Vandesompele et al. 2002). In our study, six of the eight experimental samples (three N-starved tissues, PEG-treated leaves, heat stress and flowering stage), $V_{2/3}$ was already below the cutoff value, suggesting that using the two most stable reference genes is sufficient for reliable gene expression normalization of target genes. While in PEG-treated roots and stems, the recommended variation value was $V_{5/6}$ and $V_{3/4}$, respectively, indicating that five and three genes are recommended for data normalization, respectively (Fig. 3).

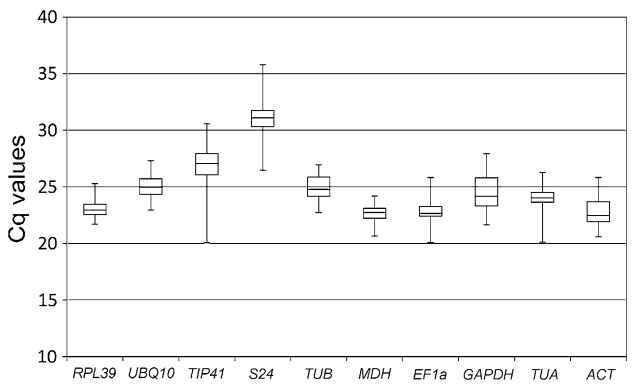


Fig. 1 Expression levels of each candidate reference gene across all samples. A *line across the box* depicts the median. *Boxes* indicate the interquartile. *Whiskers* represent the maximum and minimum values

NormFinder analysis

NormFinder uses a model-based approach to rank the candidate reference genes and generate a stability measure in which a lower value indicates increased stability in gene expression (Andersen et al. 2004; Zhu et al. 2013). In N-starved roots, the most stable reference genes were *TUB* and *MDH* (0.071 and 0.133, respectively) (Table 2). In the N-starved stems, *TUA* (0.033) and *TIP41* (0.046) showed the highest expression stability. *S24* was always the least stable gene. *RPL39*, *UBQ10* and *MDH* were the most stable reference genes under osmotic stress, although their rankings varied slightly among tissues. These results were

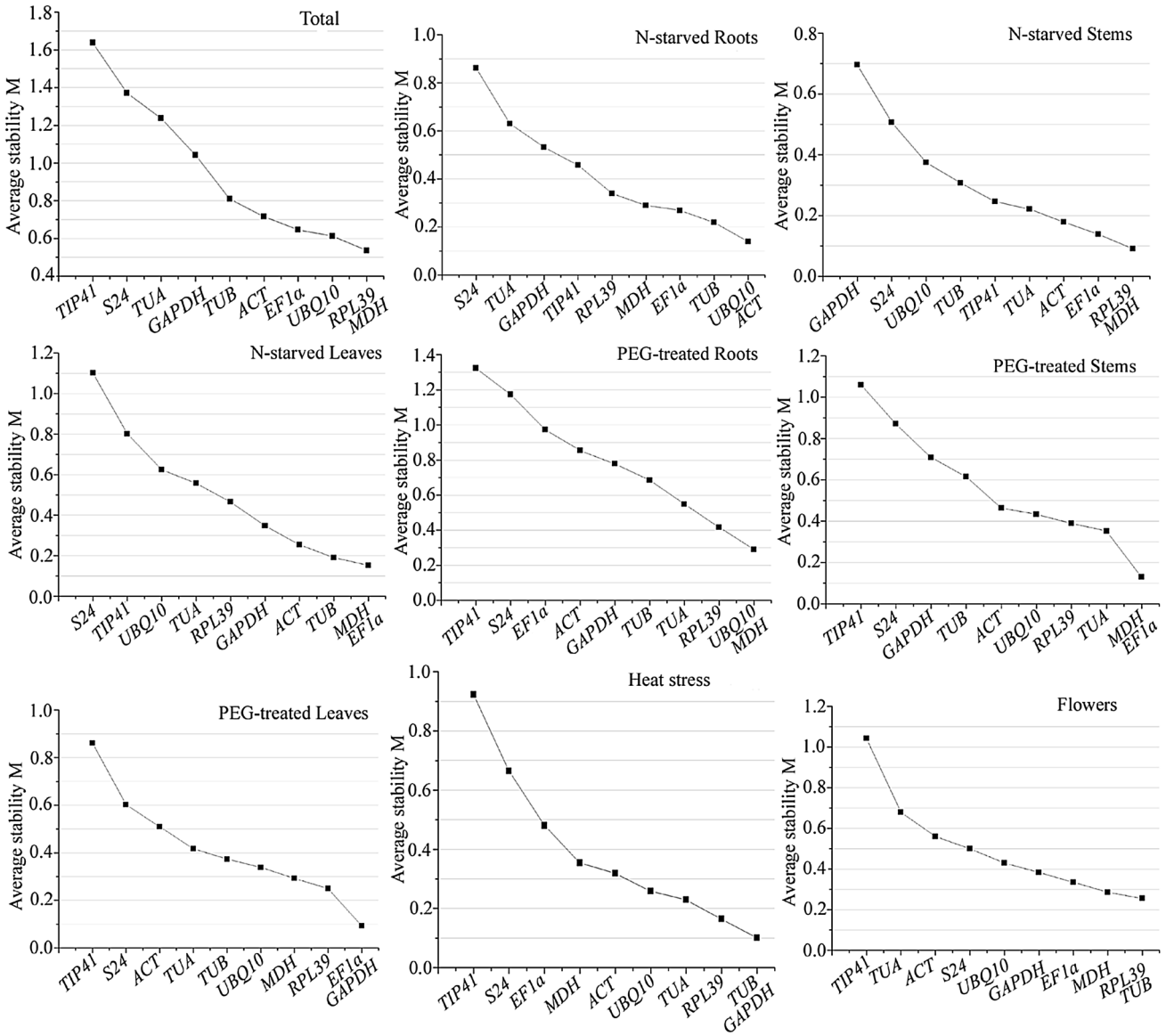
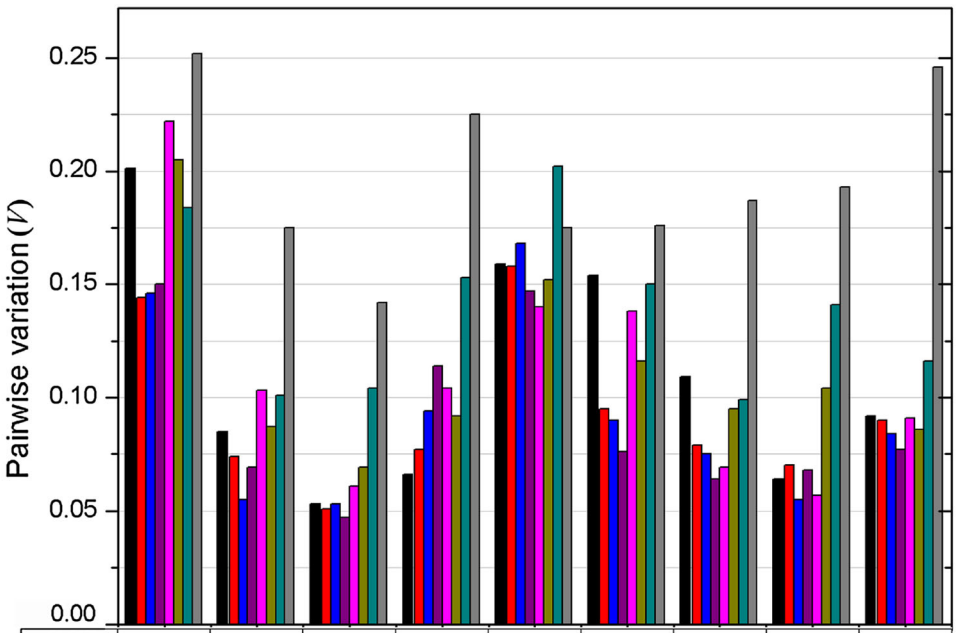


Fig. 2 Gene expression stability and ranking of the ten reference genes as calculated by geNorm. Mean expression stability (*M*) was calculated following stepwise exclusion of the least-stable gene across all treatment groups. The least-stable genes are on the *left*, the most stable are on the *right*

similar to those of geNorm. For heat stress, NormFinder ranked *RPL39* and *TUB* as the most stable reference genes with the same stability value (0.054). *UBQ10* (0.104) and *S24* (0.222) were selected to be the most stable genes in flowers. Considering all treatments, *MDH* and *RPL39* appeared most frequently among the most stable genes, while *TUA* and *TIP41* were always the least suitable reference genes (Table 2).

BestKeeper analysis

BestKeeper determines gene expression stability of candidate reference genes based on the correlation coefficient (*r*), standard deviation (SD) and percentage covariance (CV) (de Carvalho et al. 2013). It is vital to emphasize that BestKeeper uses C_q values instead of the raw data (relative quantities) used by geNorm and NormFinder, which may consequently lead to misinterpretation related to lack of correction. Genes with SD greater than 1 are deemed unacceptable. Those that have the lowest coefficient of variance and standard deviation (CV ± SD) are regarded as the most stable reference genes. The results of the BestKeeper analysis are shown in Table 3. In the N-starved roots, *RPL39* and *EF1a* were identified as the most stable reference genes, *TUB* and *TIP41* were the most stable genes in the N-starved stems, while the top ranked



	Total	NR	NS	NL	PR	PS	PL	HT	Flower
V2/3	0. 201	0. 085	0. 053	0. 066	0. 159	0. 154	0. 109	0. 064	0. 092
V3/4	0. 144	0. 074	0. 051	0. 077	0. 158	0. 095	0. 079	0. 070	0. 090
V4/5	0. 146	0. 055	0. 053	0. 094	0. 168	0. 090	0. 075	0. 055	0. 084
V5/6	0. 150	0. 069	0. 047	0. 114	0. 147	0. 076	0. 064	0. 068	0. 077
V6/7	0. 222	0. 103	0. 061	0. 104	0. 140	0. 138	0. 069	0. 057	0. 091
V7/8	0. 205	0. 087	0. 069	0. 092	0. 152	0. 116	0. 095	0. 104	0. 086
V8/9	0. 184	0. 101	0. 104	0. 153	0. 202	0. 150	0. 099	0. 141	0. 116
V9/10	0. 252	0. 175	0. 142	0. 225	0. 175	0. 176	0. 187	0. 193	0. 246

Fig. 3 Pairwise variation analyses of candidate reference genes in different sample sets. Pairwise variation (V_n/V_{n+1}) analyses between the normalization factors (NF_n and NF_{n+1}) were performed using the geNorm software to determine the optimal number of reference genes and carried out for qPCR data normalization in various sample pools. *NR* N-starved roots, *NS* N-starved stems, *NL* N-starved leaves, *PR* PEG treated roots, *PS* PEG treated stems, *PL* PEG treated leaves, *HT* heat stress treatment

genes were geNorm and NormFinder. In the PEG-treated stems and leaves, *MDH* showed the highest expression stability. Under heat stress treatment, *UBQ10* and *TUB* had the most stable expression levels. *UBQ10* was also identified as the most stable gene in flowers. When evaluated across all the experimental samples, *RPL39* and *MDH* ranked in the top positions in all three programs.

Reference gene evaluation

The expression patterns of *SOD* and *NFYA-3* were evaluated using the selected reference genes. In plants, the expression of *SOD* is induced by heat and drought (Alscher et al. 2002; Yin et al. 2008), while transcript levels of *NFYA-3* are induced by N starvation (Czechowski et al. 2005). In this study, expressions of *SOD* in PEG-treated and heat-stressed leaves and *NFYA-3* expression in N-starved roots were assessed. The two novel recommended reference genes (*RPL39* and *MDH*) and the most unstable gene (*TIP41*) were used for normalization. In all tissues tested, similar expression patterns were observed when *RPL39* and *MDH* were used for normalization (Fig. 4). For example, in the N-starved roots, the expression level of *NFYA-3* increased during continuous N-starvation, reaching a peak on day 10. In the heat-stressed leaves, the abundance of *SOD* also increased and reached a peak at 4 h. The expression level of *SOD* was increased in PEG-treated leaves for 24 h, while the abundance decreased after 5 days of continuous PEG-stress. However, when *TIP41* was used for normalization, the expression patterns differed from those of *RPL39* and *MDH*. In the N-starved roots, the transcript level of *NFYA-3* peaked at 24 h and decreased thereafter. In the heat-stressed leaves, the expression level of *SOD* peaked at 2 h, then decreased dramatically at 4 h. The abundance of *SOD* was increased in PEG-treated leaves after 5 days, which differed significantly from that of *RPL39* and *MDH*.

Discussion

Betula luminifera with its desirable wood properties, beautiful texture, fast growth and high adaptability to barren soil, is widely distributed in China and proposed as a priority timber species for afforestation. In addition, *B. luminifera* has considerable potential for use as a model

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

Rank	Total		N-starved roots		N-starved stems		N-starved leaves		PEG-treated roots	
	Gene	Sta.	Gene	Sta.	Gene	Sta.	Gene	Sta.	Gene	Sta.
1	*MDH*	0.232	*TUB*	0.071	*TUA*	0.033	*Actin*	0.077	*MDH*	0.1
2	*RPL39*	0.406	*MDH*	0.133	*TIP41*	0.046	*TUA*	0.234	*RPL39*	0.119
3	*TUB*	0.447	*UBQ10*	0.156	*EF1a*	0.046	*EF1a*	0.264	*UBQ10*	0.272
4	*EF1a*	0.5	*Actin*	0.264	*MDH*	0.069	*GAPDH*	0.299	*TUA*	0.493
5	*UBQ10*	0.542	*EF1a*	0.31	*RPL39*	0.154	*RPL39*	0.327	*TUB*	0.571
6	*ACT*	0.574	*GAPDH*	0.323	*Actin*	0.173	*MDH*	0.346	*EF1a*	0.584
7	*GAPDH*	0.827	*RPL39*	0.481	*UBQ10*	0.322	*TUB*	0.4	*Actin*	0.679
8	*S24*	0.862	*TUA*	0.503	*TUB*	0.355	*UBQ10*	0.457	*GAPDH*	0.881
9	*TUA*	0.939	*TIP41*	0.52	*S24*	0.69	*TIP41*	0.995	*TIP41*	1.195
10	*TIP41*	1.577	*S24*	1.21	*GAPDH*	0.978	*S24*	1.554	*S24*	1.201

Rank	PEG-treated stems		PEG-treated leaves		Heat stress		Flower	
	Gene	Sta.	Gene	Sta.	Gene	Sta.	Gene	Sta.
1	*UBQ10*	0.047	*RPL39*	0.063	*TUB*	0.035	*UBQ10*	0.104
2	*TUA*	0.095	*UBQ10*	0.076	*RPL39*	0.058	*S24*	0.222
3	*Actin*	0.12	*TUA*	0.124	*GAPDH*	0.074	*Actin*	0.229
4	*EF1a*	0.213	*MDH*	0.133	*ACT*	0.124	*MDH*	0.388
5	*RPL39*	0.234	*GAPDH*	0.236	*TUA*	0.141	*GAPDH*	0.396
6	*MDH*	0.272	*TUB*	0.307	*UBQ10*	0.237	*TUB*	0.431
7	*TUB*	0.624	*EF1a*	0.32	*MDH*	0.241	*EF1a*	0.449
8	*GAPDH*	0.702	*S24*	0.498	*EF1a*	0.518	*RPL39*	0.561
9	*S24*	1.006	*Actin*	0.674	*S24*	0.975	*TUA*	0.574
10	*TIP41*	1.211	*TIP41*	1.289	*TIP41*	1.328	*TIP41*	1.245

tree for forest genetic studies due to its small genome (approximately 450 Mb) and comparatively short juvenile period (1.5 years) (Huang et al. 2014). Furthermore, the availability of abundant transcriptomic sequencing data and a draft genome is underway, which has encouraged many diverse molecular biological studies of *B. luminifera*. qPCR is widely used for accurate and rapid quantification of gene transcript levels, even for genes whose expression levels are low (Zhong et al. 2011) and is more sensitive and simple than northern blotting or semi-quantitative RT-PCR. However, the accuracy of qPCR is strongly influenced by the stability of the reference genes used for normalization. The most commonly used reference genes, such as *actin* and *GAPDH*, were chosen primarily for their known or suspected housekeeping roles in basic cellular processes, assuming that they were always stably expressed. Studies have revealed that there are no reference genes that are applicable to all plant tissues or experimental conditions (Silveira et al. 2009). Therefore, potential reference genes must be studied under different experimental treatments or for specific tissues to avoid erroneous conclusions. In this study, three algorithms (geNorm, NormFinder and BestKeeper) were used to analyze the expression stability of ten candidate genes under different abiotic stresses (N starvation, osmotic, heat) and flowering stages. To our knowledge, this is the first qPCR study of the expression stability of reference genes in *B. luminifera.*

Results obtained in this study show that none of the ten genes analyzed had a constant expression level in all conditions or plant tissues, which is common in plant studies (Gutierrez et al. 2008). Because these three programs are based on different algorithms and analytical procedures, they may give different results. The top stable genes (half of the total) were similar in the three algorithms for each sample set, though slight differences were found in their ranking orders. For example, in N-starved roots, geNorm selected *UBQ10* as the most stable reference gene, while it was ranked third and fourth by NormFinder and BestKeeper, respectively. In N-starved leaves, *GAPDH* emerged as the most stable reference gene using BestKeeper, while it was ranked fourth and fifth by geNorm and NormFinder, respectively. In PEG-treated roots, *TUA* was identified as the most stably expressed gene by BestKeeper, while geNorm and NormFinder

Table 3 Expression stability values of the reference genes calculated by the BestKeeper software

Rank	Total	N-starved roots	N-starved shoots	N-starved leaves	PEG-treated roots	PEG-treated stems	PEG-treated leaves	Heat stress	Flower
1	*RPL39*	*RPL39*	*TUB*	*GAPDH*	*TUA*	*EF1a*	*MDH*	*UBQ10*	*UBQ10*
CV ± SD	2.73 ± 0.63	0.90 ± 0.20	1.08 ± 0.25	1.30 ± 0.31	1.25 ± 0.30	0.62 ± 0.14	0.30 ± 0.07	1.38 ± 0.34	0.84 ± 0.23
2	*MDH*	*EF1a*	*TIP41*	*EF1a*	*GAPDH*	*MDH*	*RPL39*	*TUB*	*S24*
CV ± SD	2.79 ± 0.63	1.08 ± 0.24	1.10 ± 0.31	1.49 ± 0.34	1.45 ± 0.35	0.95 ± 0.22	0.53 ± 0.12	1.64 ± 0.41	1.08 ± 0.33
3	*TUA*	*Act*	*TUA*	*ACT*	*TUB*	*TUA*	*GAPDH*	*TUA*	*ACT*
CV ± SD	3.03 ± 0.73	1.76 ± 0.38	1.26 ± 0.30	1.73 ± 0.40	1.69 ± 0.44	1.06 ± 0.26	0.93 ± 0.22	1.88 ± 0.46	1.42 ± 0.36
4	*EF1a*	*UBQ10*	*EF1a*	*MDH*	*RPL39*	*RPL39*	*TUA*	*ACT*	*TUB*
CV ± SD	3.40 ± 0.78	1.79 ± 0.44	2.02 ± 0.45	1.75 ± 0.41	2.51 ± 0.57	1.21 ± 0.28	1.10 ± 0.27	1.89 ± 0.42	1.59 ± 0.42
5	*UBQ10*	*MDH*	*RPL39*	*TUB*	*UBQ10*	*UBQ10*	*UBQ10*	*GAPDH*	*EF1a*
CV ± SD	3.48 ± 0.88	1.84 ± 0.41	2.06 ± 0.47	1.79 ± 0.44	2.62 ± 0.66	1.58 ± 0.39	1.13 ± 0.28	1.91 ± 0.42	1.61 ± 0.40
6	*TUB*	*TIP41*	*ACT*	*RPL39*	*MDH*	*ACT*	*EF1a*	*MDH*	*MDH*
CV ± SD	3.75 ± 0.94	1.86 ± 0.50	2.10 ± 0.45	2.39 ± 0.56	2.71 ± 0.61	2.17 ± 0.48	1.32 ± 0.30	2.24 ± 0.48	1.68 ± 0.40
7	*S24*	*TUB*	*MDH*	*UBQ10*	*ACT*	*TIP41*	*TUB*	*RPL39*	*GAPDH*
CV ± SD	3.93 ± 1.23	2.24 ± 0.57	2.16 ± 0.48	3.23 ± 0.82	0.43 ± 0.97	3.29 ± 0.91	1.50 ± 0.37	2.37 ± 0.54	1.85 ± 0.49
8	*ACT*	*GAPDH*	*UBQ10*	*TUA*	*TIP41*	*S24*	*S24*	*EF1a*	*RPL39*
CV ± SD	5.06 ± 1.56	3.00 ± 0.81	2.72 ± 0.66	3.66 ± 0.92	5.16 ± 1.43	3.45 ± 1.06	2.09 ± 0.66	2.63 ± 0.58	2.34 ± 0.57
9	*GAPDH*	*TUA*	*S24*	*TIP41*	*EF1a*	*GAPDH*	*ACT*	*S24*	*TUA*
CV ± SD	5.77 ± 1.41	4.28 ± 1.05	3.48 ± 1.07	4.36 ± 1.25	5.24 ± 1.18	3.45 ± 0.85	2.74 ± 0.64	3.84 ± 1.19	4.31 ± 0.97
10	*TIP41*	*S24*	*GAPDH*	*S24*	*S24*	*TUB*	*TIP41*	*TIP41*	*TP41*
CV ± SD	6.57 ± 1.75	6.17 ± 1.97	3.52 ± 0.87	6.83 ± 2.28	5.85 ± 1.75	3.56 ± 0.88	5.01 ± 1.39	5.76 ± 1.26	6.76 ± 1.74

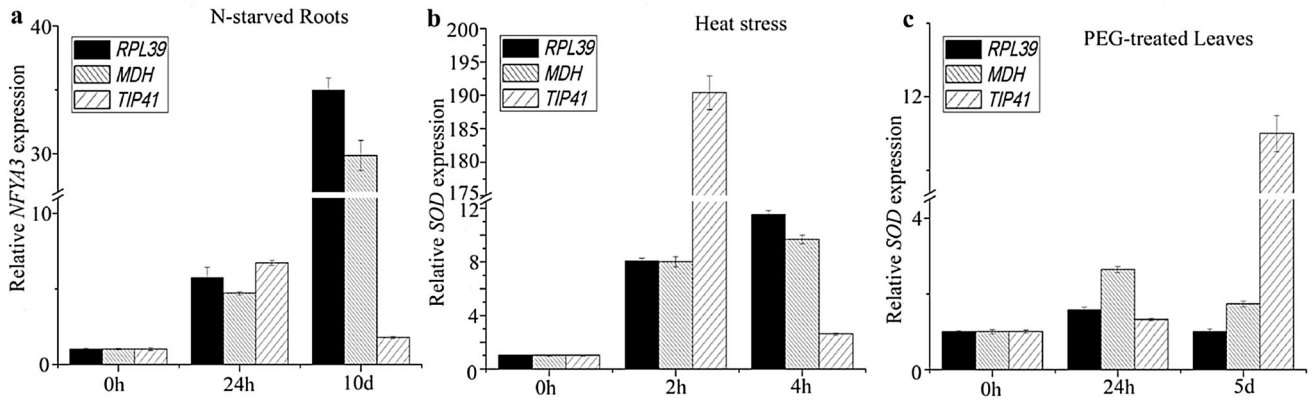


Fig. 4 Expression pattern of *SOD* and ***NFYA-3***. *NFYA-3* expression profile in N-starved roots (**a**). *SOD* expression profile in heat stressed leaves (**b**) and PEG-treated leaves (**c**). Normalized with two stable reference genes (*RPL39* and *MDH*) and the most unstable reference gene (*TIP41*). Values are presented as mean ± SE ($n = 3$)

recommended *MDH*. *EF1a* was classified as the most stable gene in PEG-treated stems and PEG-treated leaves by BestKeeper and geNorm, respectively, but NormFinder presented different results. *RPL39* and *TUB* were selected as the most stable reference genes in heat-stressed leaves, while they were ranked sixth and second by BestKeeper. Finally, considering all the tissues across all of treatments, *RPL39* and *MDH* were identified as the most stably expressed genes by all three algorithms. The results of these three algorithms were considered together when determining suitable reference genes for qPCR normalization.

Among the housekeeping genes evaluated in this study, *TIP41* and *S24* genes were previously described as reliable reference genes in *Brassica juncea* across developmental stages (Chandna et al. 2012) and in coffee (Cruz et al.

2009), but they performed poorly in our experiment. Their expression levels were unstable, especially *TIP41* with CV for the C_q values was the highest among all samples, and both of them were transcribed at a relatively low abundance. For these reasons, we excluded these two genes from consideration as reference genes in *B. luminifera*. *GAPDH* is a traditional housekeeping gene used as a reference gene and has been validated in grapevines to be stably expressed during berry development (Park et al. 2012). In this study, *GAPDH* showed stable expression only in PEG-treated leaves. It displayed inappropriate expression variability, limiting its use as an internal control in *Betula*. *TUA* was not a top reference gene, although it was expressed stably under certain conditions (N-starved stems, osmotic stress). Similarly, across different tissues or under distinct hormone treatments in poplar and cucumber, *TUA* was the recommended gene for normalization (Alscher et al. 2002; Vandesompele et al. 2002). In contrast, *TUA* performed poorly across different organs, shading and girdling plus defoliation treatments in litchi (Zhong et al. 2011). *TUB* and *UBQ10* are among the top housekeeping genes used as reference genes in plant science according to Guenin (Guenin et al. 2009). Moreover, *UBQ10* was validated as suitable for normalization in *Arabidopsis* (Czechowski et al. 2005) and tomato (Lovdal and Lillo 2009). In *Musa*, *TUB* can be used for normalization in greenhouse leaf samples (Podevin et al. 2012). In this study, based on the results from the three programs and original C_q values, *UBQ10* can be considered as a stable reference gene in flowers, PEG-treated stems and PEG-treated leaves. For other treatments, fluctuations in C_q values were observed. *UBQ10* was suggested as inappropriate for qPCR normalization in different tissues at different developmental stages in rice (Ding et al. 2004) and soybean (Jian et al. 2008). However, in early studies of *Arabidopsis* and tomato, *UBQ10* showed highly stable expression. In this study, results from all three software analyses showed that *UBQ10* expression varied depending on the experimental condition. Consequently, it should be used with caution as an internal control. Among three potential reference genes, *EF1a* has been validated in cucumber (Wan et al. 2010) and strawberry (Amil-Ruiz et al. 2013) as an optimal normalization factor for gene expression under various conditions and it emerged as the most stably expressed gene in N -starved plants in our study. *MDH* and *RPL39* are not commonly used in qPCR as reference genes, but they showed highly stable expression under almost every condition in this study. *MDH* was more highly ranked by geNorm under N-starvation conditions. In a previous analysis of this gene in grapevines, it did not perform well (Park et al. 2012). In contrast, similar result to ours were observed in coffee, where *MDH* was identified as the most stable reference gene in N starvation and heat stress (de Carvalho et al. 2013). *RPL39* was ranked in the top position by the different algorithms in N starvation, osmotic and heat stresses. In melon (Kong et al. 2014) and sweet potato (Park et al. 2012), *RPL* was found to be a suitable reference gene. In summary, according to the results of the three statistical algorithms, *RPL39*, *MHD* and *EF1a* were the most stably expressed genes, and these genes can be applied to almost all treatments examined in this work, while *TIP41* and *S24* were the most variable. Additionally, *TUB* can be used as a reference gene in N-starved roots and stems and *TUA* can be used in N-starved leaves and stems. In PEG-treated roots and leaves, *TUA* and *GAPDH* can be used for normalization, while in PEG-treated stems and PEG-treated leaves, *UB10* is a good choice. In heat stress treatments, *TUB* was also a good choice.

To demonstrate the need for accurate relative quantification using suitable reference genes, we determined the expression levels of *SOD* and *NFYA-3*. SOD is an important antioxidant in plants and the gene encoding SOD is induced by heat and drought stresses (Alscher et al. 2002; Yin et al. 2008). *NFYA-3* is a transcription factor that responds to N starvation (Czechowski et al. 2005; Zhao et al. 2011). In this study, expression of *SOD* in PEG-treated and heat-stressed leaves and *NFYA-3* expression in N-starved roots were assessed. Similar expression patterns were observed using the two new recommended genes (*RPL39* and *MDH*) for normalization (Fig. 4). However, a distinctly different pattern was obtained when *TIP41* was used as the reference gene. For example, when *RPL39* and *MDH* were used for normalization, the expression level of *NFYA-3* increased under continuous N-starvation, reaching a peak on day 10 in N-starved roots, whereas the transcript level of *NFYA-3* peaked at 24 h and decreased significantly by day 10 using *TIP41* as the reference gene. These results demonstrated that the utilization of an unsuitable reference gene could lead to inaccurate results and even opposite expression patterns. Therefore, candidate genes that are unstable under different abiotic stresses (N starvation, osmotic, heat) and flowering stages are not recommended for normalization in *B. luminifera.*

Conclusions

This is the first work regarding the selection of reliable reference genes for transcriptional studies in *B. luminifera* under N starvation, osmotic and heat stresses, and at different flower developmental stages. These results can be used as a guide for the selection of reference genes during the analysis of gene expression. This will be beneficial to future studies that require more accurate gene expression quantification in *B. luminifera*.

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