Selection of Reference Genes for Quantitative Gene Expression Studies in *Platycladus orientalis* (Cupressaceae) Using Real-Time PCR

Ermei Chang1, Shengqing Shi1, Jianfeng Liu1, Tielong Cheng2, Liang Xue1, Xiuyan Yang1, Wenjuan Yang1, Qian Lan1, Zeping Jiang1*

1State Key Laboratory of Tree Genetics and Breeding, Research Institute of Forestry, Chinese Academy of Forestry, Beijing, People’s Republic of China, 2Sci-tech Management Division, Chinese Academy of Forestry, Beijing, People’s Republic of China

Abstract

*Platycladus orientalis* is a tree species that is highly resistant, widely adaptable, and long-lived, with lifespans of even thousands of years. To explore the mechanisms underlying these characteristics, gene expressions have been investigated at the transcriptome level by RNA-seq combined with a digital gene expression (DGE) technique. So, it is crucial to have a reliable set of reference genes to normalize the expressions of genes in *P. orientalis* under various conditions. To this end, we selected 10 reference genes candidates from transcriptome data of *P. orientalis*, and examined their expression profiles by qRT-PCR using 29 different samples of *P. orientalis*, which were collected from plants of different ages, different tissues, and plants subjected to different treatments including cold, heat, salinity, polyethylene glycol (PEG), and abscisic acid (ABA). Three analytical software packages (geNorm, Bestkeeper, and NormFinder) were used to assess the stability of gene expression. The results showed that ubiquitin-conjugating enzyme E2 (*UBC*) and alpha-tubulin (*aTUB*) were the optimum pair of reference genes at all developmental stages and under all stress conditions. *ACT7* was the most stable gene across different tissues and cold-treated samples, while *UBQ* was the most stably expressed reference gene for NaCl- and ABA-treated samples. In parallel, *aTUB* and *UBC* were used singly or in combination as reference genes to examine the expression levels of *AtNAC2* in plants subjected to various treatments with qRT-PCR. The results further proved the reliability of the two selected reference genes. Our study will benefit future research on the expression of genes in response to stress/senescence in *P. orientalis* and other members of the Cupressaceae.

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* E-mail: jiangzp@caf.ac.cn

† These authors contributed equally to this work.

Introduction

Quantitative real-time PCR (qRT-PCR) allows sensitive, specific, and reproducible quantification of nucleic acids [1], and it has been widely used to analyze mRNA in different organisms, transgenic and gene experiments, and to identify parasitic organisms [2–5]. However, there are substantial variations in RNA stability, quantity, purity, and the efficiency of reverse transcription (RT) and polymerase chain reactions (PCR) [6]. Therefore, it is important to select a suitable reference gene and to use a set of standardized experimental conditions to accurately quantify gene expression by qRT-PCR; otherwise, it may give inaccurate results [7–14].

Reference genes are those that are constitutively expressed and are required for cellular survival. They include genes that encode products with functions in maintaining cell wall structure and primary metabolism. Some examples of reference genes include 18S ribosomal RNA (*18S rRNA*), actin (*ACT*), tubulin (*TUB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), polyubiquitin (*UBQ*), and elongation factor 1-a (*EF1a*) [15,16,17]. Some of them, such as *EF1a*, *ACT2*, and *TUA4*, do not satisfy certain basic requirements for use as an internal control in *Arabidopsis thaliana* [16] and tomato [17]. Recently, some novel reference genes that show highly stable expression were identified from analyses of microarray data sets from *A. thaliana*. These new reference genes include *SAND* and *TIP41*, which encode a SAND family protein and a TIP41-like family protein, respectively [16]. Both of these reference genes outperform the classical ones; for example, *CAC* and *TIP41* were also among the most stably expressed genes in tomato. A recent study demonstrated that even some stress-related genes can serve as reference genes in some experiments, such as those encoding *SKP1*/Ask-interacting protein 16 (*SKP16*), metalloprotease (*MTP*), RNA polymerase subunit (*RPB*), and F-box protein (*F-box*) [18–22]. Therefore, it is important to select a suitable reference gene with a constant level of expression under certain experimental conditions and among various species [16].

To date, many stable reference genes have been screened in both model and crop species, such as *A. thaliana* [23], rice [24,25],...
**Table 1. Descriptions of candidate genes from *Platycladus orientalis* for qRT-PCR.**

| Gene symbol | Gene name | Arabidopsis homolog locus | Primer sequence (5’–3’) | Size (bp) | PCR efficiency |
|-------------|-----------|--------------------------|--------------------------|-----------|----------------|
| GAPDH       | Glyceraldehyde-3-Phosphate dehydrogenase | AT1G79530 | GAGATCCATGGGGTGATTITG | 150       | 99.2%          |
| ACT7        | Actin 7   | AT5G09810 | AAACCAAAACATAGGGGCATC  | 152       | 101.1%         |
| aTUB        | Alpha-tubulin | AT5G19770 | GAGGATTCACCATGTTTCC   | 205       | 103.6%         |
| bTUB        | Beta tubulin | AT1G20010 | GTGCTGACCGAGCCGAAAT   | 197       | 101.6%         |
| UBC         | Ubiquitin-conjugating enzyme E2 | AT3G57870 | CCACTACCTCAGGGATGCAAG  | 108       | 99.2%          |
| UBQ         | Ubiquitin 10 | AT5G20620 | ATGCATCGCCAGCATCACA   | 120       | 101.9%         |
| EF1a        | Elongation factor 1-alpha | AT1G07940 | TCCTGCCTGAGATGTGTTTT  | 144       | 98%            |
| DNAJ        | DanJ-like protein | AT3G44110 | TGATCGTGGGCAGCCGCGA  | 133       | 98.9%          |
| SAND        | Sand family protein | AT2G28390 | GGGAATGCCCATGGCGGAG  | 134       | 95.5%          |
| CAC         | Clathrin adaptor complexes | AT1G56590 | ACTGGGAGAAGTAAATGCAG  | 100       | 104.2%         |
| NAC         | NAC domain protein | AT5G39610 | AGGAAGAGAGGAAGGCGAAG    | 169       | 104.3%         |

Note: All reference gene sequences from transcriptome data of *Platycladus orientalis* were searched with BLAST using sequences of *Arabidopsis thaliana* in GenBank. Sequences of candidate housekeeping genes and NAC domain protein gene are provided in the Supporting Information.

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*Brachypodium distachyon* [26], wheat [27], barley [28], soybean [29,30], tomato [31], potato [32], sugarcane [33] and poplar [34]. However, no suitable internal controls for gene expression studies have been defined for *Platycladus orientalis*, which limits further studies on this species at the transcriptome level.

*P. orientalis*, as a member of the Cupressaceae, is used extensively as a medicinal ingredient and as an ornamental landscape plant that can tolerate a wide range of environmental extremes [35,36]. Many individuals of this species have lifespans of one to several thousands of years in China [37]. However, why do some trees, such as *P. orientalis*, live for so long? This question has received much attention in recent years [38,39]. The long lifespan and hardiness of *P. orientalis* make it an ideal material in which to study expression patterns of genes related to stress responses and longevity of tree species. In our previous research, the transcriptome and digital expression profiles were compared between young and old trees of *P. orientalis* by RNA-seq combined with the digital gene expression (DGE) technique (data not shown). To further elucidate the excellent genetic traits of *P. orientalis*, further research is required to analyze expression of particular genes at various developmental stages and under certain stress conditions. However, several studies have shown that the transcription levels of genes vary considerably under various experimental conditions [40,41,42]. Therefore, it is urgent to identify a set of stable reference genes for further analyses of gene expression profiles in *P. orientalis*.

In this study, we used qRT-PCR to examine variations in the expressions of 10 candidate reference genes, including 8 traditional housekeeping genes and 2 novel reference genes selected from transcriptome data of *P. orientalis*. Then, we compared their stabilities across a large set of *P. orientalis* samples representing different developmental stages, organs, and stress treatments using statistical and graphical methods. The results demonstrated that the expressions of the selected genes showed different degrees of variations among samples. Furthermore, we examined the expression of a NAC transcription factor, a homolog of the stress- and senescence-responsive gene *AtNAC2* [43–47], in *P. orientalis* subjected to various stress treatments, using *UBC* and *aTUB* in combination as internal control genes. This work will benefit future studies on gene expression in *P. orientalis* and other members of the Cupressaceae.

**Results**

**Expression Profiles of Reference Genes**

We selected 10 candidate reference genes, including 8 traditional genes (*GAPDH, ACT7, aTUB, bTUB, UBC, UBQ, EF1a and DNAJ*) and 2 novel candidate reference genes (*SAND* and *CAC*) (Table 1, Text S1). The stability of gene expression was determined by quantifying the mRNA level by qRT-PCR. For each gene, we calculated the cycle threshold (Ct) value, which represents the cycle at which a significant increase of the PCR product occurs. In general, this is marked by the middle of the
GeNorm Analysis

We analyzed gene expression stabilities of the 10 reference genes in all of the designated conditions as described by Vandesompele et al. [13] using geNorm software. GeNorm automatically calculates the average expression stability value (M) as the average pairwise variation (V) of a particular gene with all other control genes and determines the V values with all other control genes as the standard deviation of the logarithmically transformed expression ratios [26]. The gene with the lowest M value is that with the most stable expression, while the gene with the highest M value has the least stable expression. As shown in Figure 2, we analyzed data from seven sets of treatments. When all the results from all 29 samples of *P. orientalis* were combined, aTUB and UBC showed the lowest M value (0.64) and CAC showed the highest M value (1.31). Therefore, aTUB and UBC had the most stable expressions, and CAC the least stable expression (Fig. 2A). Among the tissues of different ages, the most stably expressed genes were UBC and aTUB, while CAC was the least stably expressed, consistent with the pattern observed across all samples (Fig. 2B). For the different organs, the DNAJ and ACT7 genes showed the greatest stability of expression, and CAC showed the least stable expressions (Fig. 2C). Similarly, the stabilities of reference genes varied among samples under different stress treatments. As shown in Figure 2D, SAND and ACT7 were the most stably expressed under cold stress, and EF1α the least stably expressed. In heat-treated samples, UBC and aTUB were expressed more stably than the other eight reference genes, while EF1α was the least stably expressed (Fig. 2E). Under NaCl stress, DNAJ and UBC were the most stably expressed genes, while EF1α and CAC were the least stably expressed genes (Fig. 2F). In contrast, under PEG stress, aTUB and EF1α were the most stably expressed genes and ACT7 was the most variable (Fig. 2G). The exogenous application of ABA had the least effect on expressions of UBC and UBQ, and the greatest effect on expression of EF1α (Fig. 2H). The geNorm analysis indicated that EF1α and CAC were the least stably expressed reference gene, although EF1α was stably expressed in PEG-treated samples, which is consistent with its roles in stress responses and development. Overall, all of the tested reference genes showed relatively high stability with low M values of less than 1.35, which is below the default limit of M = 1.5. Evaluation of all expression data revealed that UBC and aTUB were the most stably expressed genes; therefore, these may be suitable reference genes for analyses of gene expression in a wide variety of tissue types, developmental stages, and stress treatments in *P. orientalis* (Fig. 2).

To obtain reliable results from RT-PCR studies, it is recommended that two or more reference genes be used. Therefore, Vandesompele et al. [13] proposed 0.15 as the cut-off value for V, below which the inclusion of an additional control gene is not required; that is, if Vn/n+1 < 0.15, it is not necessary to use ≥ n+1 reference genes as internal controls. The paired variable coefficients (V2/V3) shown in Fig. 3B, C, D, E, F, G, and H indicate that the inclusion of the third reference gene did not contribute significantly to the variation of the normalization factor (V2/3 < 0.15). That is, the two most stable reference genes for each subset would be sufficient for accurate normalization. When all the samples were pooled for analysis, the pairwise variation of V2/3 and V3/4 was greater than 0.15 (0.247 and 0.182, respectively), while that of V4/5 was 0.149 (Fig. 3A), which indicated that four reference genes, UBC, aTUB, GAPDH and UBQ were necessary to normalize gene expression for all the treatments of *P. orientalis* in this study.

BestKeeper Analyses

For analyses using BestKeeper, an Excel-based tool, the average Ct value of each duplicate reaction is used (without conversion to quantity) to analyze the stabilities of candidate reference genes [50]. BestKeeper evaluates the stabilities of candidate reference genes based on the coefficient of correlation to the BestKeeper index, which is the geometric mean of the Ct values of all candidate reference genes [50,51]. BestKeeper also calculates the
Figure 2. Gene expression stability and ranking of 10 reference genes as calculated by geNorm.
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coefficient of variance (CV) and the standard deviation (SD) of the Ct values using the whole data set and all the Ct values are analyzed as a total data set [31]. Reference genes are identified as the most stable genes, as they exhibit the lowest coefficient of variance and standard deviation (CV±SD). Genes that show a SD greater than 1 are considered unacceptable [52]. In this study, UBC and SAND had CV±SD values of 1.58±0.38 and 2.02±0.55, respectively, and showed remarkably stable expression in all the samples. However, GAPDH and bTUB had CV±SD values of 3.18±0.78 and 3.09±0.82, respectively, and showed the least stable expression (Table 2). These results differed to those obtained using geNorm (Fig. 2). For the different ages of P. orientalis, the most stable reference genes (lowest CV±SD) were aTUB and UBC, while UBQ had the highest CV±SD of all the selected genes. BestKeeper analyses indicated that UBC and DNAJ were the most stably expressed, and GAPDH and UBQ were the least stably expressed among different tissue types and PEG-treated samples. UBQ and aTUB were the most stably expressed genes under cold stress; and UBC and aTUB had the lowest coefficient of variance under heat treatment, which was consistent with the results obtained using geNorm and NormFinder. In ABA-treated samples, BestKeeper analysis indicated that the most stable genes

![Figure 3. Determination of the optimal number of reference genes for normalization by pairwise variation (V) using geNorm.](https://doi.org/10.1371/journal.pone.0033278.g003)

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

| Rank | All(A) | Age(B) | Tissue(C) | Cold(D) | Heat(E) | NaCl(F) | PEG(G) | ABA(H) |
|------|--------|--------|-----------|--------|---------|---------|--------|--------|
| 1    | UBC    | aTUB   | UBQ       | aTUB   | GAPDH   | DNAJ    | SAND   |
| 2    | SAND   | UBC    | DNAJ      | aTUB   | UBC     | aTUB    | UBC    | ACT7   |
| 3    | CAC    | SAND   | ACT7      | EF1α   | UBQ     | UBC     | SAND   | UBC    |
| 4    | aTUB   | DNAJ   | aTUB      | DNAJ   | SAND    | DNAJ    | bTUB   | aTUB   |
| 5    | ACT7   | bTUB   | SAND      | UBC    | GAPDH   | SAND    | EF1α   | DNAJ   |
| 6    | EF1α   | ACT7   | EF1α      | ACT7   | ACT7    | bTUB    | aTUB   | bTUB   |
| 7    | DNAJ   | GAPDH  | CAC       | SAND   | EF1α    | UBQ     | CAC    | UBQ    |
| 8    | UBQ    | EF1α   | bTUB      | GAPDH  | CAC     | EF1α    | ACT7   | CAC    |
| 9    | bTUB   | CAC    | UBQ       | bTUB   | DNAJ    | ACT7    | UBQ    | GAPDH  |
| 10   | GAPDH  | UBQ    | GAPDH     | CAC    | bTUB    | CAC     | GAPDH  | EF1α   |
| 11   | CV±SD  | 1.58±0.38 | 1.50±0.35 | 1.50±0.36 | 0.45±0.10 | 0.89±0.22 | 0.43±0.11 | 0.93±0.26 | 0.53±0.14 |
| 12   | CV±SD  | 2.02±0.55 | 2.10±0.51 | 1.59±0.43 | 1.45±0.35 | 1.02±0.21 | 0.43±0.29 | 1.17±0.51 | 0.79±0.10 |
| 13   | CV±SD  | 2.22±0.62 | 2.15±0.59 | 1.60±0.41 | 1.64±0.58 | 1.21±0.26 | 0.44±0.11 | 1.19±0.32 | 0.82±0.20 |
| 14   | CV±SD  | 2.42±0.57 | 2.43±0.69 | 1.73±0.40 | 1.97±0.56 | 1.45±0.39 | 1.01±0.28 | 1.43±0.37 | 1.23±0.29 |
| 15   | CV±SD  | 2.56±0.68 | 2.65±0.70 | 1.75±0.47 | 1.97±0.49 | 1.59±0.40 | 1.28±0.35 | 1.54±0.56 | 1.25±0.35 |
| 16   | CV±SD  | 2.57±0.94 | 2.76±0.73 | 1.87±0.69 | 2.13±0.57 | 1.86±0.50 | 1.81±0.49 | 1.82±0.42 | 1.28±0.34 |
| 17   | CV±SD  | 2.65±0.74 | 2.97±0.73 | 1.90±0.51 | 2.2±0.62 | 2.58±0.95 | 1.99±0.44 | 2.20±0.60 | 1.76±0.38 |
| 18   | CV±SD  | 2.97±0.63 | 3.22±1.16 | 2.22±0.58 | 2.52±0.64 | 3.47±0.96 | 2.21±0.80 | 2.27±0.61 | 1.86±0.51 |
| 19   | CV±SD  | 3.09±0.82 | 3.40±0.95 | 2.65±0.55 | 2.85±0.79 | 3.55±1.00 | 2.48±0.66 | 3.3±0.69 | 1.99±0.49 |
| 20   | CV±SD  | 3.18±0.78 | 3.97±0.85 | 2.92±0.70 | 3.21±0.89 | 3.78±1.02 | 3.73±1.04 | 3.48±0.83 | 2.74±1.01 |

Note: Expression stability and ranking of 10 reference genes as calculated by Bestkeeper in all samples (A), different ages (B), different tissue types (C), cold-treated (D), heat-treated (E), NaCl-treated (F), PEG-treated (G), ABA-treated (H). Descriptive statistics of 10 candidate genes based on their coefficient of variance (CV) and standard deviation (SD) of Ct values were determined using the whole data set, and all Ct values were analyzed as a total data set. Reference genes are identified as the most stable genes (those with the lowest coefficient of variance and standard deviation; CV±SD).
were SAND and ACT7 and the least stable were GAPDH and EF1a. The results obtained from BestKeeper showed slight differences from those obtained from geNorm (Fig. 2).

**NormFinder Analysis**

Similar to geNorm, the NormFinder program is a Visual Basic application tool for Microsoft Excel used to determine expression stabilities of reference genes [53]. As in the geNorm method, the gene with the lowest M value is that with the most stable expression, and the gene with the highest M value has the least stable expression. The results of the NormFinder analysis were showed in Table 3. The NormFinder outputs with and without different sample subgroups showed some common features. The NormFinder analysis ranked UBC and aTUB in the top positions for all the samples, different age samples, and heat-treated samples, while EF1a and CAC were ranked as less stable, consistent with the result obtained from geNorm. Among the different tissues, DNAJ and ACT7 were the most stably expressed with values of 0.093 and 0.101, respectively, while expressions of CAC and bTUB were the least stable. Under cold, ABA, and NaCl treatments, UB was calculated to be the most stably expressed gene, and EF1a was the least stable. Under PEG treatment, bTUB and aTUB were predicted as the best internal controls, while ACT7 and SAND were the least stably expressed genes. The results obtained from NormFinder were highly consistent with those obtained from geNorm (Fig. 2).

**Evaluation of Reference Genes for Determining Age- and Stress-responsive NAC Expression**

To further evaluate the reliability of the top two reference genes aTUB and UBC, NAC transcription factor AtNAC2 (At5g39610; also named ANAC092), which plays a crucial role in protecting plants against abiotic stresses, as well as in leaf senescence [45], was selected to qRT-PCR analysis with aTUB and UBC singly or in combination as reference genes. In our previous study, we found that the NAC domain gene from P. orientalis, a homolog of AtNAC2 in A. thaliana, was differentially expressed between young and old trees by using RNA-seq combined with a DGE technique (data not shown). Therefore, this gene was selected to further evaluate the reliability of the reference genes by qRT-PCR. We selected aTUB and UBC in combination as the reference genes and determined their average expression patterns (Fig. 4). The highest level of NAC expression was in leaves of 3-year-old P. orientalis. The expression of NAC in leaves increased significantly from 20- to 2000-year-old individuals of P. orientalis (P < 0.05) (Fig. 4A), which matches the result obtained from the DGE analysis. NAC showed evidently different expression levels in the tissues, such as seeds, fruits, roots, stems, and leaves (P < 0.05) (Fig. 4B). Cold and heat stress significantly increased the expression of NAC (Fig. 4C) by 8.0 and 5.7 folds, respectively, at 48 h compared with that at 0 h (P < 0.05). The expression profiles of NAC showed similar trends under NaCl, PEG, and ABA treatments, and its abundance increased to maximum levels at 24 h, increasing to 12.9, 5.6 and 12.2 folds compared with that at 0 h, respectively (P < 0.05) (Fig. 4C). In addition, the expression patterns of NAC showed similar trends to those of aTUB and UBC as internal controls either singly or in combination (Fig. 4; Fig. S1). There were no significant differences in the expression patterns of NAC using either aTUB or UBC singly as the internal control (P > 0.05), although it seems that there was a bit of difference between aTUB and UBC at the 48-hour treatments (P = 0.059) (Fig. S1), which further indicated that aTUB and UBC were suitable as reference genes.

**Table 3. Ranking of candidate reference genes in order of their expression stability as calculated by NormFinder.**

| Rank | All(A) | Age(B) | Tissues(C) | Cold(D) | Heat(E) | NaCl(F) | PEG(G) | ABA(H) |
|------|--------|--------|------------|---------|---------|---------|--------|--------|
| 1    | UBC    | aTUB   | DNAJ       | UBC     | aTUB    | UBC     | bTUB   | UBC    |
| M value | 0.263  | 0.099  | 0.093      | 0.139   | 0.058   | 0.067   | 0.094  | 0.082  |
| 2    | aTUB   | UBC    | ACT7       | ACT7    | UBC     | GAPDH   | aTUB   | UBC    |
| M value | 0.362  | 0.270  | 0.101      | 0.227   | 0.089   | 0.067   | 0.121  | 0.232  |
| 3    | UBQ    | GAPDH  | aTUB       | SAND    | UBQ     | SAND    | DNAJ   | SAND   |
| M value | 0.533  | 0.351  | 0.273      | 0.292   | 0.180   | 0.251   | 0.155  | 0.245  |
| 4    | GAPDH  | bTUB   | UBC        | DNAJ    | SAND    | aTUB    | UBC    | CAC    |
| M value | 0.553  | 0.494  | 0.279      | 0.388   | 0.226   | 0.259   | 0.228  | 0.262  |
| 5    | ACT7   | ACT7   | SAND       | GAPDH   | ACT7    | DNAJ    | EF1a   | DNAJ   |
| M value | 0.591  | 0.594  | 0.312      | 0.402   | 0.495   | 0.352   | 0.237  | 0.264  |
| 6    | bTUB   | UBQ    | EF1a       | UBQ     | GAPDH   | UBQ     | CAC    | ACT7   |
| M value | 0.603  | 0.663  | 0.395      | 0.415   | 0.509   | 0.409   | 0.361  | 0.274  |
| 7    | DNAJ   | DNAJ   | UBQ        | CAC     | CAC     | bTUB    | UBQ    | bTUB   |
| M value | 0.608  | 0.678  | 0.450      | 0.467   | 0.696   | 0.522   | 0.479  | 0.366  |
| 8    | SAND   | SAND   | GAPDH      | aTUB    | DNAJ    | ACT7    | GAPDH  | GAPDH  |
| M value | 0.715  | 0.680  | 0.552      | 0.487   | 0.781   | 0.635   | 0.523  | 0.391  |
| 9    | EF1a   | EF1a   | bTUB       | bTUB    | EF1a    | SAND    | aTUB   |
| M value | 0.852  | 0.810  | 0.572      | 0.665   | 0.795   | 0.757   | 0.547  | 0.519  |
| 10   | CAC    | CAC    | EF1a       | EF1a    | EF1a    | CAC     | ACT7   | EF1a   |
| M value | 1.126  | 0.937  | 0.673      | 1.199   | 0.923   | 0.871   | 0.662  | 0.689  |

Note: Expression stability and ranking of 10 reference genes as calculated by NormFinder in all samples (A), different ages (B), different tissue types (C), cold-treated (D), heat-treated (E), NaCl-treated (F), PEG-treated (G), ABA-treated (H). Lower average expression stability (M value) indicates more stable expression.

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Discussion

In plant molecular biological research, studies on gene expression patterns help us to understand biological processes. The qRT-PCR technique is one of the most common methods to quantify gene expression levels, which is a crucial step in identifying gene function [26]. However, to accurately analyze expression of a particular gene, it is essential to have a reliable method to normalize its expression. Thus, an appropriate internal reference gene is required for reliable quantification of gene expression. Identifying gene function [26]. However, to accurately analyze quantification of gene expression levels, which is a crucial step in expression patterns help us to understand biological processes.

Figure 4. Expression profiles of *P. orientalis* age- and stress-responsive gene expression in transcripts.

To screen for appropriate reference genes suitable for studies on age- and stress-responsive gene expression in *P. orientalis*, we examined the expressions of 10 reference genes from its transcriptome in a set of different conditions. We then analyzed their expressions using three different software packages: geNorm, NormFinder and Bestkeeper. Analysis with geNorm is an easy method to determine the optimal number of stable housekeeping genes for accurate normalization [29], whereas NormFinder and Bestkeeper were used to assess the quality of the ranking obtained by geNorm [54]. Our data showed that the top two positions of reference genes for all samples, for samples of different ages and under PEG stress (Fig. 2; Table 2), *aTUB* was also identified as being stably expressed across various developmental stages of soybean [29] and different tissues of poplar [34]. *DNAJ* showed remarkably stable expression in the different tissues and NaCl-treated samples of *P. orientalis*, which was consistent with an earlier study on *DNAJ* expression in tomato [56]. As noted previously, expression of *ACT7* is relatively weak in soybean, rice, potato, and sugarcane [23–25], and rather variable in *A. thaliana* [16]. In our study, geNorm analysis indicated that expression of *ACT7* was most stable across different tissues and cold-treated samples of *P. orientalis*.

All three software packages indicated that *UBQ*, *bTUB*, and *GAPDH* ranked in middle positions in all samples of *P. orientalis*. In the present study, *UBQ* was expressed stably in NaCl- and ABA-treated samples. *UBQ* also showed very stable expression in *A. thaliana* and *B. distachyon* [26], but was unsatisfactory as a reference gene in soybean [29] and grape [57]. *GAPDH* is one of the most commonly used reference genes to normalize gene expression data in qRT-PCR assays [58]. Here, we found that *GAPDH* was the most stably expressed reference gene in different ages, but was expressed less stably in different tissues of *P. orientalis*.

Previously, *EF1a* was reported to be stably expressed during biotic and abiotic stress in both potato and rice [29,32]. In this study, however, analyses using all three software packages ranked is necessary to validate the expression stability of the control gene under specific experimental conditions prior to its use for normalization [26].

Our data demonstrate that *UBC, aTUB, DNAJ* and *ACT7* were ranked in top positions in all the samples of *P. orientalis* based on the results from the three software packages. Here, *UBC* was found to be one of the most stably expressed genes in all samples of *P. orientalis*, which was consistent with the result in *B. distachyon* [26], whereas it showed less stable expression in *A. thaliana* under heavy metal (Cu and Cd) stress [23], therefore expression levels of reference genes vary among different species [20]. In this study, *aTUB* expression did not vary, or varied little, among tissues of different ages and under PEG stress (Fig. 2; Table 2), *aTUB* was also identified as being stably expressed across various developmental stages of soybean [29] and different tissues of poplar [34]. *DNAJ* showed remarkably stable expression in the different tissues and NaCl-treated samples of *P. orientalis*, which was consistent with an earlier study on *DNAJ* expression in tomato [56]. As noted previously, expression of *ACT7* is relatively weak in soybean, rice, potato, and sugarcane [23–25], and rather variable in *A. thaliana* [16]. In our study, geNorm analysis indicated that expression of *ACT7* was most stable across different tissues and cold-treated samples of *P. orientalis*.

All three software packages indicated that *UBQ, bTUB*, and *GAPDH* ranked in middle positions in all samples of *P. orientalis*. In the present study, *UBQ* was expressed stably in NaCl- and ABA-treated samples. *UBQ* also showed very stable expression in *A. thaliana* and *B. distachyon* [26], but was unsatisfactory as a reference gene in soybean [29] and grape [57]. *GAPDH* is one of the most commonly used reference genes to normalize gene expression data in qRT-PCR assays [58]. Here, we found that *GAPDH* was the most stably expressed reference gene in different ages, but was expressed less stably in different tissues of *P. orientalis*. *bTUB* was reported to perform poorly as a reference gene in grape and potato [32,58], while in this study, NormFinder analysis indicated that *bTUB* showed highly stable expression in PEG-treated samples of *P. orientalis*.
EF1a in the bottom positions. Similarly, the novel reference gene CAC was the lowest ranked gene in analyses from geNorm and NormFinder in all samples. These results indicated that EF1a and CAC were both unsuitable reference genes in all  P. orientalis samples. Additionally, the novel reference gene SAND was not the best choice to analyze  P. orientalis gene expression over a wider range of conditions, although it was stably expressed under cold stress in  P. orientalis. However, SAND and CAC were the recommended reference genes for studies on development in berry [30] and tomato [31], respectively. Therefore, to investigate the transcript stability of the commonly used reference genes and to identify novel and superior reference genes, it is necessary to collect as many data as possible about gene expression in different organisms, organs, and experimental conditions.

To further validate the applicability of the screened reference genes, we analyzed the expression of the NAC domain gene, a homolog AtNAC2 from A. thaliana, in  P. orientalis. AtNAC2 has recently been discovered as a central regulator of age-dependent and salt-promoted senescence in A. thaliana [45]. In this study, we examined the expression of NAC using a combination of UBC and aTUB as reference genes. The result showed that NAC exhibited a leaf age-dependent expression pattern with low to moderate expression in 20-year-old tree leaves, and significantly high expression in leaves of 1000- and 2000-year-old individuals of  P. orientalis (P < 0.05). This pattern of expression was highly similar to that obtained from the DGE profile (data not shown), and the changes in the expression patterns of NAC under all the designated treatments (Fig. 4) showed similar trends to those of aTUB and UBC under the same conditions (Fig. S1). However, because subtle changes in expression are of critical importance in some experiments, it may not be suitable to use a single reference gene [13]. Therefore, to understand the molecular mechanisms underlying the stress tolerance and longevity of  P. orientalis, it would be helpful to use two or more reliable reference genes for normalization of expression of genes of interest.

Above all, we identified 10 reference genes that were suitable for normalization of qRT-PCR data obtained from  P. orientalis samples of different ages, from different tissues, and from plants subjected to various exogenous treatments. Evaluations using geNorm, NormFinder and BestKeeper indicated that the two most suitable reference genes in  P. orientalis were aTUB and UBC, while the two least suitable reference genes were EF1a and CAC. To obtain the most reliable results from gene expression studies of  P. orientalis, it is recommended that two or more reference genes are used as internal controls for relative gene quantification.

Materials and Methods

Plant Materials and Treatments

The trees sampled in this study were 20-, 100- and 1000-year-old individuals of  P. orientalis growing in similar conditions in Zhongshan Park, Beijing. Nine-month-old and 3-year-old seedlings of  P. orientalis were cultivated in pots with soil in the greenhouse at the Chinese Academy of Forestry, Beijing. For salt-, osmotic-, and ABA-treatments, 9-month-old seedlings were carefully pulled out from pots, washed cleanly with tap water, and placed in the solutions of NaCl (200 mM); PEG6000 (10%), and ABA (150 μM), respectively, for 0, 12, 24, and 48 h in the greenhouse. For the cold- and heat-treatments, the seedlings in pots were placed at 4°C or 40°C, respectively, in chambers with a 14-h light/10-h dark photoperiod for 0, 12, 24, and 48 h. For the collection of tissues of trees in 20-, 100-, 1000-, and 2000-year-old, fresh leaves were collected from the branches in different directions in June and August 2011. Different tissues including leaves, roots, stems, fruits, and seeds were collected from 5-year-old seedlings. Leaves from 9-month-old seedlings were collected from the whole seedlings subjected to various treatments, immediately frozen in liquid nitrogen and stored at -80°C. Samples above were collected from 3 trees to give 3 replicates.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from treated samples using a Column Plant RNAout kit (TIANDZ, CHINA), and then genomic DNA and polysaccharides were eliminated using RNase-free DNase I (TIANDZ, CHINA) kits, respectively. The purity of the total RNA extracted was determined using a NanoDrop DU8000 spectrophotometer. RNA samples with an absorbance ratio at OD260/280 between 1.9 and 2.2 and OD260/230 = 2.0 were used for further analyses. RNA integrity was verified by 2% agarose gel electrophoresis and ethidium bromide staining. Samples with 218/18S ribosomal RNA between 1.5 and 2.0 and without smears on the agarose gel were used for the following experiment. First-strand cDNA was synthesized from 600 ng total RNA in a volume of 20 μl using the PrimeScript® RT reagent kit (TaKaRa, Japan) according to the manufacturer’s protocol. cDNA was diluted 7.5× before quantification and determinations of quantity and quality.

Quantitative Real-time RT-PCR

The primers for the 10 reference genes from the transcriptome of  P. orientalis were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). All primer pairs were initially tested via standard RT-PCR using the Premix Ex Taq (TaKaRa, Japan) and a single amplification product of the expected size for each gene was verified by electrophoresis on a 3% agarose gel and staining with ethidium bromide. qRT-PCR reactions were carried out in 96-well blocks with an Applied Biosystems 7500 Real-Time PCR system using SYBR® Premix Ex Taq™ (TaKaRa, Japan) in a 20 μl reaction volume (containing 2 μl cDNA reaction mixture, 10 μl 2×SYBR Premix Ex TaqTM, 0.4 μl ROX Reference Dye II, and 0.4 μl each primer). The reaction conditions were those recommended by the manufacturer (30 s at 95°C, 40 cycles of 95°C for 5 s, and 60°C for 34 s). The dissociation curve was obtained by heating the amplicon from 60 to 95°C. All qRT-PCR reactions were carried out in technical and biological triplicate. The final threshold cycle (Ct) values were the mean of nine values (biological triplicate, each in technical triplicate).

Statistical Analyses

To select a suitable reference gene, the stability of mRNA expression of each reference gene was statistically analyzed with three different types of Microsoft Excel-based software: geNorm [59], NormFinder [60], and BestKeeper [61]. All three software packages were used according to the manufacturer’s instructions. For geNorm and NormFinder, the raw Ct values were transformed into the required data input format. The maximum expression level (the lowest Ct value) of each gene was used as a control and was set to a value of 1. Relative expression levels were then calculated from Ct values using the formula: 2^-ΔCt, in which ΔCt = each corresponding Ct value−minimum Ct value. The obtained data were further analyzed with geNorm and NormFinder. BestKeeper analyses were based on untransformed Ct values.

Standard curves were generated using Excel software by plotting cycles at threshold fluorescence (Ct) against the logarithmic values of standard RNA amounts. Quantities of standard RNA were prepared by diluting 200 ng cDNA (1, 1/5, 1/25, 1/125, 1/625, 1/3125; each gene in triplicate). Only Ct values of less than 40 were used to calculate correlation coefficients (r² values).
and amplification efficiencies (E) from the given slope generated in Microsoft Excel 2003 according to the equation $E = \left[\frac{5}{1 - \text{slope}}\right] - 1 \times 100\%$. All PCR assays showed efficiency values between 95.05 or 0.05 and 105%.

Differences were scored as statistical significance (ANOVA) and multiple comparisons using the statistical analysis software of SPSS. Differences were scored as statistical significance at the $P < 0.05$ or $P < 0.01$ level.

**Supporting Information**

**Text S1** A list of sequences of candidate housekeeping genes and NAC domain protein gene.

(DOC)

**Figure S1** The expression profile of NAC responsive to aging and stresses in *Platycladus orientalis* (studied by qRT-PCR with UBC and aTUB as reference genes, respectively).

(DOC)

**References**

1. Yoo WG, Kim TI, Li S, Kwon OS, Cho PY, et al. (2009) Reference genes for quantitative analysis on *Chlamydomonas reinhardtii* gene expression by real-time PCR. *Parasitol Res* 104(2): 321–328.
2. Ohdan T, Francisco PB, Sawada T, Hirose T, Teras T, et al. (2005) Expression profiling of genes involved in starch synthesis in sink and source organs of rice. *J Exp Bot* 56: 3329–3344.
3. Ishimaru T, Hirose T, Matsuda T, Goto A, Takahashi K, et al. (2005) Expression patterns of genes encoding carbohydrate-metabolizing enzymes and their relationship to grain filling in rice (*Oryza sativa L*): comparison of caryopses located at different positions in a panicle. *Plant Cell Physiol* 46: 620–628.
4. Baccarese-Henrieo H, Miyoshi K, Hirose T, Tsuchimoto S, Mori M, et al. (2005) Expressions of rice *Oscoxe nic non-ferrimenti* c-terminal protein kinase I genes are differently regulated during the caryopsis development. *Plant Physiol Biochem* 43: 669–679.
5. Narayanan NN, Vasconcelos MW, Grossak MA (2007) Expression profiling of *Oryza sativa* metal homeostasis genes in different rice cultivars yellowing under artificial conditions. *BMC Genomics* 18: 226–233.
6. Demidenko NV, Logacheva MD, Provin AA (2011) Selection and validation of reference genes for quantitative real-time PCR in Buckwheat (*Fagopyrum esculentum*) based on transcriptome sequence data. *PLoS ONE* 6(5): e19451.
7. Tong ZG, Gao ZH, Wang F, Zhou L, Zhang Z (2009) Selection of reliable reference genes for gene expression studies in peach using real-time PCR. *BMC Mol Biol* 10: 71.
8. Kwon MJ, Oh E, Lee S, Roh MR, Kim SE, et al. (2009) Identification of novel reference genes using multiplex expression data and their validation for quantitative gene expression analysis. *PLoS ONE* 4(7): e6162.
9. Laveda T, Lillo C (2009) Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. *Analyst* *Biochem* 367(2): 230–242.
10. Hoemann C, Holze A (2011) Selection of reference genes for normalization of quantitative real-time PCR in cell cultures of *Cyclamen persicum*. *Electron J Biotechnol* 14(1): 12–13.
11. Everaert BR, Boullet GA, Timmermans JP, Vrints CJ (2011) Importance of suitable reference gene selection for quantitative real-time PCR. special reference to mouse myocardial infarction studies. *PLoS ONE* 6(8): e23793.
12. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. * Genome Biol* 3(7): resear ch0034-research0034.11.
13. Tricario C, Pinzani P, Bianchi S, Paglierani M, Distante V, et al. (2002) Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. *Anal Biochem* 309(2): 293–300.
14. Radonic A, Thalke S, Mackay DM, Landl O, Siegent W, et al. (2004) Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun* 313(4): 856–862.
15. Tanaka T, Sato M, Altmann T, Ushbandi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol* 139: 5–17.
16. Yang LT, Pan AH, Jia JW, Ding JY, Chen JX, et al. (2005) Validation of a tomato-specific gene, LAT52, used as an endogenous reference gene in quantitative and real-time quantitative PCR detection of transgenic tomatoes. *J Agric Food Chem* 53(2): 189–190.
17. Chen X, Triska M, Shah S, Westlake RJ (2010) A survey of quantitative real-time polymerase chain reaction internal reference genes for expression studies in *Brassica napus*. *Anal Biochem* 405(1): 138–140.
18. Arico S, Nardelli SM, Brilliante O, Grossi-de-Sa MF, Alves-Ferreira M (2010) Identification and evaluation of new reference genes in *Gaussia nigra* for accurate normalization of quantitative real-time RT-PCR data. *BMC Plant Biol* 10: 49.
19. Barsalobre-Cavallari CF, Severino FE, Falah MP, Maia IG (2009) Identification of susceptible internal control genes for expression studies in *Coffea arabica* under different experimental conditions. *BMC Mol Biol* 10: 1.
20. Die JV, Remon B, Nadal S, Gonzalez-Verdejo CJ (2010) Evaluation of candidate reference genes for expression studies in *Pisum sativum* under different experimental conditions. *Planta* 232(1): 143–153.
21. Lou HL, Chen SM, Wan HJ, Chen FD, Gu CS, et al. (2010) Candidate reference genes for gene expression studies in water lily. *Anal Biochem* 404(1): 100–102.
22. Remans T, Smeets K, Opperdenaker K, Mathijssen D, Vangronsveld J, et al. (2008) Normalization of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. *Planta* 227(6): 1343–1349.
23. Jain M, Nihawans A, Tyagi AK, Khurana JP (2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biosci Biotechnol Biochem* 70(5): 1003–1010.
24. Die JV, Remon B, Nadal S, Gonzalez-Verdejo CJ (2009) Evaluation of candidate reference genes for expression studies in *Pisum sativum* under different experimental conditions. *Planta* 232(1): 143–153.
25. Paolacci AR, Tanarella OA, Porceludo E, Giaffi M (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. *BMC Mol Biol* 10: 11.
26. Faccioli P, Caceri GF, Provero P, Stanca AM, Morcia C, et al. (2007) A combined strategy of “in silico” transcriptional analysis and web search engine optimization allows an age identification of reference genes suitable for normalization in gene expression studies. *PLoS ONE* 3(3): 679–688.
27. Jian B, Liu B, Bi YR, Hou WS, Wu CX, et al. (2008) Validation of internal control for gene expression study in soybean by quantitative real-time PCR. *BMC Mol Biol* 10: 9.
28. Lihault M, Thibaudillers S, Bilgin D, Radwan O, Benitez M, et al. (2008) Identification of four soybean reference genes for gene expression normalization. *BMC Mol Biol* 10: 44.
29. Expo'sito-Rodrı´guez M, Borges AA, Borges-Pe´rez A, Pe´rez JA (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. *BMC Plant Biol* 8: 131.
30. Nicot N, Hassenan JM, Hoffmann L, Evers D (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. *Exp Bot* 56(421): 2907–2914.
31. Iskandar HM, Simpson RS, Casu RE, Bennett GD, Maclean DJ (2004) Identification of suitable reference genes for quantitative real-time polymerase chain reaction analysis of gene expression in sugarcane. *Plant Biol* 22(4): 325–337.
32. Brummer AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. *BMC Plant Biol* 4: 14.

**Table S1** The ranking of 10 reference genes and the assembly of the comparisons in different samples of *Platycladus orientalis* as calculated by gNorm, Bestkeeper, and NormFinder.

(DOC)

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**Author Contributions**

Conceived and designed the experiments: ZJ EC SS. Performed the experiments: EC SS. Analyzed the data: EC SS XY. Contributed reagents/materials/analysis tools: JL TC LX XY QL WY. Wrote the paper: EC SS XY.

Reference Genes for *Platycladus orientalis*
35. Li XP, He YP, Wu XJ, Ren QF (2011) Water stress experiments of *Platycladus orientalis* and *Pinus tabulaeformis* young trees. Forest Res 24(1): 91–96 (in Chinese with English abstract).
36. Jiang P, Shi J, Niu PX, Lu Y (2009) Effect on activities of defensive enzymes and MDA content in leaves of *Platycladus orientalis* under naturally decreasing temperature. J Shihezi Univ (Natural Science) 27: 30–33 (in Chinese with English abstract).
37. Zhang YJ, Cong RC, Zhao Q, Zhang GH, Li YH, et al. (2010) Physiological indexes applied to characterize aging old trees. Sci Sin: Sin 46(3): 134–138 (in Chinese with English abstract).
38. Lanner RM (2002) Why do trees live so long? Ageing Res Rev 1(4): 653–671.
39. Pen˜uelas J (2005) A big issue for trees. Nature 437: 965–966.
40. Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, et al. (1999) Housekeeping genes as internal standards: use and limits. J Biotechnol 75(2-3): 291–293.
41. Suzuki T, Higgins PJ, Crawford DR (2000) Control selection for RNA quantitation. Biotechniques 29(2): 332–337.
42. Glare EM, Divjak M, Bailey MJ, Walters EH (2002) β-Acin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. Thorax 57: 765–770.
43. He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, et al. (2005) AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. Plant J 44(6): 903–916.
44. Kim JH, Woo HR, Kim J, Lim PO, Lee IC, et al. (2009) Trivariate feed-forward regulation of age-dependent cell death involving miR164 in *Arabidopsis*. Science 323(5917): 1053–1057.
45. Balazadeh S, Siddiqui H, Allu AD, Matallana-Ramirez LP, Caldana C, et al. (2010) A gene regulatory network controlled by NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. Plant J 62(2): 250–264.
46. Reece E, Harrison E, McHattie S, Hughes I, Hickman R, et al. (2011) High-resolution temporal profiling of transcripts during *Arabidopsis* leaf senescence reveals a distinct chronology of processes and regulation. Plant Cell 23(3): 873–894.
47. McCurley AT, Callard GV (2008) Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. BMC Mol Biol 9: 102.