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Suitable reference genes for real-time quantitative PCR in *Salsola laricifolia* under five abiotic stresses

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Abstract

Salsola laricifolia, a typical C₃-C₄ intermediate desert plant, is an important for understanding gene evolution and mechanisms for drought resistance. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a preferred choice for gene expression studies, but it requires stable reference genes for normalization. Therefore, we tested the expression stability of five candidate reference genes in *S. laricifolia*: *EF1α* (elongation factor 1-α), *ACT* (actin), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *TUB* (tubulin), and *18S* (18S ribosomal RNA). The expressions were tested in different tissues and under five stresses caused by abscisic acid (ABA), NaCl, NaHCO₃, darkness, and osmotic stress (polyethylene glycol 6000, PEG). Four commonly used software programs (*geNorm*, *NormFinder*, *BestKeeper*, and *RefFinder*) were used. The results show the following most stable reference genes: *GAPDH* for ABA and dark treatments; *EF1α* for NaCl, PEG; and all samples; *TUB* for NaHCO₃; and *18S* for the controls. The *ACT* was not ranked first in any group, and was the least stable reference gene under the dark, NaHCO₃, and PEG. Moreover, pairwise analysis by the *geNorm* algorithm shows that two best reference genes were *18S* and *EF1α* for the controls, *GAPDH*, and *18S* for the ABA and dark treatments, *EF1α* and *TUB* for the NaCl treatment, *TUB* and *18S* for the NaHCO₃ treatment, *EF1α* and *GAPDH* for the PEG treatment, and *EF1α* and *18S* for all samples. The reference genes for RT-qPCR in *S. laricifolia* identified in our study will facilitate future work on targeted gene expression.

Additional key words: abscisic acid, C₃-C₄ intermediate species, NaCl, NaHCO₃, polyethylene glycol, RT-qPCR normalization.

Introduction

Gene expression analysis has contributed to better understanding the characteristics and functions of targeted genes. There are three commonly used methods for analyzing gene expression: northern blotting, semi-quantitative reverse transcription-PCR, and reverse transcription quantitative real-time PCR (RT-qPCR) (Bustin *et al.* 2009). Among these, RT-qPCR is a preferred choice because of its high sensitivity, accuracy, high-throughput capability, and low-cost (Bustin 2000, Huggett

et al. 2005). However, it is necessary to utilize a normalization method to correct for the variations resulting from experimental procedures such as the quality and quantity of the mRNA template, reverse transcription efficiency, and PCR efficiency (Vandesompele *et al.* 2002, Udvardi *et al.* 2008, Guénin *et al.* 2009). The RT-qPCR data must be normalized with at least one stable reference gene (Chen *et al.* 2011). Some reports have even suggested that at least two reference genes should be combined

Submitted 15 May 2018, last revision 28 November 2018, accepted 29 November 2018.

Abbreviations: *18S* - 18S ribosomal RNA; ABA - abscisic acid; ACT - actin; cDNA - complementary DNA; Cq values - quantification cycles; CV - coefficient of variance; *CYC* - cyclophilin; *EF1α* - elongation factor 1-α; *GAPDH* - glyceraldehyde-3-phosphate dehydrogenase; ICGs - internal control genes; KLCs - Kranz-like cells; M value - the expression stability value; PEG - polyethylene glycol 6000; RPL - ribosomal phosphoprotein large; RPS - ribosomal protein S; RT-qPCR - reverse transcription real-time quantitative PCR; TIP41 - TIP41-like family protein; TUB - tubulin; UBI - ubiquitin, $V_{n/n+1}$ - pairwise variation.

Acknowledgements: This work was supported by the Natural Science Foundation in China (grant Nos. 31670339 and 31770227) and the Special Funds for the Young Scholars of Taxonomy of the Chinese Academy of Sciences (No. ZSBR-007). The first two authors contributed equally to this work.

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together to normalize the RT-qPCR data (Hong *et al.* 2008).

Reference genes are usually housekeeping genes expressed universally in all cells (Bustin 2002) such as *elongation factor 1- α* (*EF1 α*), *actin* (*ACT*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and *tubulin* (*TUB*). However, these traditional reference genes are not always stably expressed under different conditions (Xiao *et al.* 2015, Chen *et al.* 2017). Even for the same plant, reference genes may show different stability patterns under different stresses (Li *et al.* 2015, Xiao *et al.* 2015). Therefore, it is necessary to establish suitable reference genes for each species and tested them under certain experimental conditions in order to ensure their stability (Li *et al.* 2012, 2013, Zhao *et al.* 2016, Acevedo *et al.* 2017, Chen *et al.* 2017). So far, suitable reference genes have been selected in a variety of model plants, such as *Arabidopsis thaliana* (Czechowski *et al.* 2005), rice (Kim *et al.* 2003), and tomato (Expósito-Rodríguez *et al.* 2008), as well as in crops, such as cotton (Wang *et al.* 2013), wheat (Paolacci *et al.* 2009), soybean (Jian *et al.* 2008); fruit trees, such as apple (Perini *et al.* 2014) and banana (Chen *et al.* 2011); flowers, such as rose (Klie and Debener 2011) and petunia (Mallona *et al.* 2010); grasses, such as *Eleusine indica* (Chen *et al.* 2017) and *Salicornia europaea* (Xiao *et al.* 2015); woody plants, such as *Eremosparton songoricum* (Li *et al.* 2012) and poplar (Brunner *et al.* 2004); and a moss *Syntrichia caninervis* (Li *et al.* 2015). The development of next-generation sequencing technologies can be used to explore the genetic resources of more plants (Hong *et al.* 2010, Demidenko *et al.* 2011, Feng *et al.* 2013). To evaluate the most stable reference genes of a particular species under given experimental conditions, several statistical algorithms, such as *geNorm* (Vandesompele *et al.* 2002), *NormFinder* (Andersen *et al.* 2004), *BestKeeper* (Pfaffl *et al.* 2004), and *RefFinder* (Xie *et al.* 2012) have been used.

Salsola laricifolia (Chenopodiaceae), is a C₃-C₄ intermediate desert species (Wen and Zhang 2015b), which is mainly distributed from east Kazakhstan and Kyrgyzstan to northern China and Mongolia (Wen *et al.* 2014). The C₃-C₄ intermediate species have anatomical and photosynthetic characteristics intermediate between

those of C₃ and C₄ plants (Ueno 2011). For example, *S. laricifolia* has near-continuous and distinctive Kranz-like cells (KLCs), a relatively low CO₂ compensation point (30.4 $\mu\text{mol mol}^{-1}$), mitochondria in KLCs primarily occurring along the centripetal wall, and, importantly, P-protein glycine decarboxylase is located in the KLCs (Wen and Zhang 2015b). The C₃-C₄ species are of a special interest because they may help to elucidate C₄ evolution and its mechanisms for reduction of photorespiration (Edwards and Ku 1987). Until now, no comprehensive study has been performed to evaluate stable reference genes for C₃-C₄ intermediate species in response to abiotic stresses. The β -*ACT* has been utilized as an RT-qPCR reference gene in *S. laricifolia* under soil drought (Wen and Zhang 2015a). Therefore, a systematic selection of stable reference genes for *S. laricifolia* is necessary and will be helpful for molecular studies using RT-qPCR in the future.

In 2017, a knowledge base of internal control genes (ICGs) for RT-qPCR normalization has been established (http://icg.big.ac.cn/index.php/Main_Page) for a variety of species (Sang *et al.* 2017a). The top 10 ICGs are mostly traditional reference genes: *EF1 α* , *ACT*, *ubiquitin* (*UBI*), *GAPDH*, *ribosomal phosphoprotein large* (*RPL*), *TUB*, *ribosomal protein S* (*RPS*), *18S ribosomal RNA* (*18S*), *cyclophilin* (*CYC*), and *TIP41-like family protein* (*TIP41*). In the ICG database, more than 70 species have adopted *EF1 α* and *ACT* as reference genes; 50 to 70 species have chosen *UBI* and *GAPDH*; 30 to 50 species have adopted *RPL* and *TUB*; and less than 30 species have chosen *RPS*, *18S*, *CYC*, and *TIP41*. After considering the top 10 ICG ranking and the total number of species adopting them, we have selected commonly preferred candidate reference genes (*EF1 α* , *ACT*, *GAPDH*, *TUB*, and *18S*). The expression stabilities of these five candidate genes were evaluated in *S. laricifolia* subjected to non-stress (control) conditions and to polyethylene glycol 6000 (PEG), NaCl, NaHCO₃, abscisic acid (ABA), and dark treatments, and data were analyzed by the four most commonly used algorithms (*geNorm*, *NormFinder*, *BestKeeper*, and *RefFinder*) to determine sets of candidate reference genes suitable for gene expression studies in this species.

Materials and methods

In our experiment, the seeds of *Salsola laricifolia* Litv. ex. Drobow were collected from Toli City (Xinjiang, China). Seeds were stored at 4 °C before being germinated on moist paper at room temperature. The seeds were then placed in a growth chamber (a relative humidity of approximately 50 %, day/night temperatures of 25/18 °C, a 14-h photoperiod, and an irradiance of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Then, two-week-old seedlings were transplanted to 9 cm diameter pots with a Hoagland solution. Each pot had a float with eight holes and contained eight plants; the

Hoagland solution was changed weekly. Irradiance was increased to 1 000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Two-month-old plants were subjected to various abiotic stresses. All treatments started at 2 h after the beginning of a photoperiod. The Hoagland solution with 0, 50, 100, 150, and 200 μM ABA or with 0, 50, 100, 150, and 200 mM NaCl or with 0, 15, 30, 45, and 60 mM NaHCO₃ or with 0, 10, 20, 30, 40, 50, and 60 % (m/v) PEG were applied for 4 h. For dark treatment, the pots were covered for 0, 0.5, 1, 2, 6, and 12 h. Leaves and roots were all collected immediately,

frozen in liquid nitrogen, and stored at -80°C until used. Each treatment had four biological replicates.

Total RNA was isolated from 100 mg of frozen leaves using a *TransZol* reagent (*Transgen Biotech*, Beijing, China) (four independent extractions for each treatment) following the manufacturer's instructions. Any contaminated genomic DNA was removed by treatment with an RNase-free DNase kit (*Qiagen*, Valencia, USA). The total RNA concentration and purity were determined using a *NanoDrop ND-2000* spectrophotometer (*Thermo Fisher Scientific*, San Jose, USA). The RNA samples with absorption ratios of $A_{260}/A_{280} = 1.8 - 2.2$ and A_{260}/A_{230} higher than 2.0 were used for subsequent complementary DNA synthesis. The integrity was verified by 1.5 % (m/v) agarose gel electrophoresis. The first-strand cDNA was synthesized using a *Reversal Transcription Reagent* kit (*Takara*, Tokyo, Japan) following the manufacturer's instructions.

Based on the top ten ICG ranking and the total number of species adopting them, we chose five candidate reference genes (*EF1a*, *ACT*, *GAPDH*, *TUB*, and *18S*). The five genes retrieved from the transcriptome database of *S. laricifolia* were cloned, and the obtained sequences were highly homologous to the gene sequences registered by the National Center for Biotechnology Information. Reverse transcription qPCR primers were designed with *Primer Premier v. 5.0* using the following criteria: an amplicon length ranged from 100 to 300 bp and melting temperature (T_m) from 55 to 61 $^{\circ}\text{C}$ (Table 1 Suppl.). All RT-qPCR experiments conformed to the *MIQE* guideline (Bustin *et al.* 2009). The primer specificity was judged by melting-curve analysis and agarose gel electrophoresis of the amplification product. Amplification efficiency was assessed using a standard curve generated by RT-qPCR using a 10-fold dilution series over at least four dilution points that were evaluated in triplicate.

For RT-qPCR, the cDNA was diluted five times and reactions were carried out in 96-well plates with a *CFX96* real-time PCR detection system (*Bio-Rad*, Hercules, USA) using *SYBR Premix Ex Taq™* (*Takara*). The RT-qPCR was conducted in a reaction mixture containing 4 mm^3 of diluted cDNA (1:5), 0.5 mm^3 of each of forward and reverse primers (10 μM), 10 mm^3 of real-time master mix,

and 5 mm^3 of PCR-grade water. Conditions for amplification of RT-qPCR were: polymerase activation at 95 $^{\circ}\text{C}$ for 30 s and 40 cycles, each cycle including 95 $^{\circ}\text{C}$ for 30 s, 55 $^{\circ}\text{C}$ for 10 s, and 72 $^{\circ}\text{C}$ for 15 s. All samples were run with two technical replicates, and three no-template controls (replacing the template product with PCR-grade water) were included in every run to monitor possible DNA contamination.

To assess the stability of the five candidate genes, three publicly available software programs *geNorm*, *NormFinder*, and *BestKeeper* were used (Vandesompele *et al.* 2002, Anderson *et al.* 2004, Pfaffl *et al.* 2004). Additionally, a web-based tool *RefFinder* (<http://150.216.56.64/referencegene.php#>) was selected to generate a comprehensive ranking for the candidate reference genes by comparing the results obtained using three programs. The quantification cycles (C_q values) of all reference genes used in *geNorm* and *NormFinder* were converted into relative quantities according to a formula $2^{-\Delta C_t}$ (ΔC_t - the corresponding C_q value - minimum C_q) (Livak and Schmittgen 2001). In the *geNorm* program, two parameters including the expression stability value (M value) and the pairwise variation ($V_{n/n+1}$) were used to assess the appropriate reference genes. The recommended threshold of the M value is below 1.5 (Vandesompele *et al.* 2002). The $V_{n/n+1}$ was analyzed to determine the optimal number of reference genes for accurate normalization. The cut-off value was proposed to be 0.15. If $V_{n/n+1} < 0.15$, the minimal number of best reference genes for accurate normalization should be n ; otherwise it should be $n + 1$ (Vandesompele *et al.* 2002). The *NormFinder* program calculated the expression stability of the reference genes by combining the intra- and inter-group variations. The lowest stability value indicates the most stable expression within the gene set examined. Raw C_q values were directly analyzed by the *BestKeeper* and *RefFinder* programs. *RefFinder* generated the final overall ranking based on the geometric mean of the weights of every gene calculated by each program.

All statistical tests were performed with *SPSS v. 19.0* (*SPSS Inc.*, Chicago, USA) using a one-way analysis of variance (*ANOVA*).

Results

Total RNA from 256 different plant materials was extracted. The RNA quality was checked by 1.5 % (m/v) agarose gel electrophoresis. All selected samples showed both 28S and 18S ribosomal RNA bands with a density ratio of about 2.0 (Fig. 1 Suppl.). The A_{260}/A_{280} ratio ranged from 2.0 to 2.2, and the A_{260}/A_{230} ratio was higher than 2.0 for all selected samples. The concentration of total RNA ranged from 245 to 986 $\mu\text{g g}^{-1}$ (f.m.). All sequences of the reference genes were reconfirmed by the PCR

results. All primer sets were *BLAST* searched against the non-redundant database in *NCBI* (*primer-BLAST* program) as well as the local *S. laricifolia* transcriptome data in order to verify their specificity. Primers for five sequences were confirmed for amplification specificity by gel electrophoresis (Fig. 2 Suppl.). Then, the designed primers were detected by qPCR. For each gene, the melting curve had a single peak (Fig. 3 Suppl.). A PCR amplification efficiency for five reference genes varied from 95.3 for

ACT to 104.1 for *18S*, and correlation coefficient (R^2) values ranged from 0.9862 to 0.9991 over 10^5 times cDNA dilution (Table 1 Suppl.).

The expressions of five candidate reference genes were determined as Cq values. According to the box-plot, the Cq values of the five reference genes ranged from 5.93 to 33.57. The mean Cq values for the reference genes ranged from 8.29 to 29.65. The average Cq value of all the test samples was 20.76 cycles (Fig. 1). The *GAPDH* had the

highest median Cq value (28.97) indicating a relatively low expression. In contrast, the gene *18S* was highly expressed compared with other reference genes (median Cq value = 8.57), corresponding to a high expression. Even for the same reference gene, gene expression varied across different *S. laricifolia* samples. The results indicate that no individual gene showed a constant expression under all the experimental conditions.

Fig 1. Expressions of reference genes tested under different experimental conditions. A - Controls; B - abscisic acid; C - dark stress; D - NaCl; E - NaHCO₃; F - polyethylene glycol 6000; G - average of all samples (for each stress, it contained the data across all of the concentrations). The box-plot contains the mean, interquartile range, non-outlier range, and outlier, $n = 4$. *ACT* - actin; *TUB* - tubulin; *EF1a* - elongation factor 1-alpha; *GAPDH* - glyceraldehyde-3-phosphate dehydrogenase; *18S* - 18S ribosomal RNA.

To obtain a reliable dataset of the optimal reference genes for the six sample groups, the expressions of five candidate genes were analyzed and ranked using *geNorm*, *NormFinder*, *BestKeeper*, and *RefFinder* individually. In the *geNorm*, two parameters including the M value and $V_{n/n+1}$ were used to assess the appropriate reference genes. Each candidate reference gene had an M value less than 1.5 (Table 2 Suppl.). In the control, *18S* ranked as the most stable gene, whereas *TUB* was considered the least stable gene. Under the treatments with ABA and darkness, *GAPDH* was the most stable gene, whereas *ACT* was least

stable. For all the tested samples and the conditions of NaCl and PEG treatments, the most stable gene was the same (*EF1a*). The *TUB* ranked as the most stable gene under the NaHCO₃ treatment set. The *ACT* was considered the least stable gene (Table 2 Suppl.). The RT-qPCR data were classified into seven groups (all samples, ABA, dark, NaCl, NaHCO₃, PEG, and controls), their pairwise variation ($V_{2/3}$) being 0.028, 0.019, 0.032, 0.015, 0.015, 0.029, and 0.125, respectively (Fig. 2). Based on the rule of pairwise variation (Vandesompele *et al.* 2002), two of the best reference genes were used to determine the

optimal number of reference genes for each group: *18S* and *EF1a* for the control group, *GAPDH* and *18S* for the dark treatment, *EF1a* and *TUB* for the NaCl treatment, *TUB* and *18S* for the NaHCO₃ treatment, *EF1a* and *GAPDH* for the PEG and ABA treatments, and *EF1a* and *18S* for all samples (Table 2 Suppl.).

According to the *NormFinder*, a lower stability value indicates the most stable expression for RT-qPCR (Andersen *et al.* 2004). The results of stable value ranking are shown in Table 2 Suppl. for each group. The most and least stable reference genes were the same as in the results evaluated by *geNorm* with the exception of the least stable gene in the ABA group (*ACT* determined by *geNorm*, whereas *18S* by the *NormFinder*).

Fig 2. Pairwise variation (V_n/V_{n+1}) analysis for selecting optimal number of reference genes in normalization of *Salsola laricifolia* with the *geNorm* algorithm.

Discussion

In this work, we have focused on *S. laricifolia*, an important C₃-C₄ intermediate desert plant (Wen and Zhang 2015b). We have used RT-qPCR analysis to search for stable reference genes as their knowledge in *S. laricifolia* lagged behind that for other major plant groups.

The housekeeping genes *ACT*, *18S*, and *GAPDH*, are frequently used for RT-qPCR normalization (Sang *et al.* 2013). However, some studies suggest that new reference genes could be more stably expressed than these ones under specific conditions (*e.g.*, Czechowski *et al.* 2005, Libault *et al.* 2008, Li *et al.* 2015, Liu *et al.* 2017). Therefore, the selection of candidate reference genes is important. The ICG is a useful base of reference genes for RT-qPCR normalization in many species including both model and non-model organisms (Sang *et al.* 2017a). Taking into account the top 10 ICG ranking and the total number of species adopting them, we chose five preferred candidate reference genes for this study (*EF1a*, *ACT*, *GAPDH*, *TUB*, and *18S*).

We compared analyses done by *geNorm* and *NormFinder* and found these programs consistent in the ranking of the reference genes under most treatments except under the ABA. Under the ABA treatment, the most

In *BestKeeper*, the coefficient of variance (CV) and the standard deviation (SD) of candidate genes were calculated to evaluate the stability of each group. The lowest SD and CV values suggest the most stable reference gene (Chang *et al.* 2012). The most stable reference gene based on *BestKeeper* analysis was the same as with *geNorm* and *NormFinder* in the control, dark, and PEG treatments. In the ABA set, *EF1a* was the most stable gene (SD = 0.56), whereas *18S* was the least stable gene (SD = 1.51). For NaCl treatment, *ACT* was the most stable gene (SD = 0.42), whereas *GAPDH* was the least stable gene (SD = 0.82). For the NaHCO₃ group, *18S* was the most stable gene (SD = 0.26), whereas *GAPDH* was the least stable gene (SD = 0.69).

To address the heterogeneity of the results evaluated using *geNorm*, *NormFinder*, and *BestKeeper*, *RefFinder* has been recommended (Xie *et al.* 2012). This would represent a comprehensive ranking of the gene expression stability. The most stable reference gene suggested by *RefFinder* in these seven treatments was the same as in the results evaluated by the *geNorm* and *NormFinder*. The ranking of five reference genes was similar to the *NormFinder*. Based on the results of *RefFinder*, *18S* was the most stable gene in the control (1.57), whereas *TUB* was the least stable gene (4.73). Under treatment with ABA and the dark, *GAPDH* was the most stable gene. For the NaCl, and PEG groups, *EF1a* was the most stable gene, and for the NaHCO₃ group, *TUB* was the most stable gene (1.41), whereas *ACT* was the least stable gene (4.23) (Table 2 Suppl.).

stable reference gene suggested by these two analyses was *GAPDH*, but the gene ranking and the least stable reference gene were different (Table 2 Suppl.). The results from *BestKeeper* analysis were similar to those from *geNorm* and *NormFinder* in the control, dark, and PEG groups, and when all samples were considered. However, there were differences for ABA, NaCl, and NaHCO₃ treatments similarly as has been found in other studies (Petriccione *et al.* 2015, Zhang *et al.* 2017). Based on the above results, a fourth algorithm (*RefFinder*) was used to overcome the differences among *geNorm*, *NormFinder*, and *BestKeeper* to obtain the final choice of reference genes. The genes ranking first were *GAPDH* in ABA and the dark group; *EF1a* in NaCl, PEG, and all samples; *TUB* in the NaHCO₃ group; and *18S* in the control. These have the lowest geometric mean and ranked first in *geNorm*, *NormFinder*, and *BestKeeper* except in the NaCl and NaHCO₃ sets. The ranked first gene was suggested the most stable gene in the corresponding group.

Previous studies suggested that RT-qPCR data must be normalized with at least one stable reference gene (Chen *et al.* 2011), and some reports have even stated that at least two reference genes should be combined to normalize the

RT-qPCR data (Hong *et al.* 2008). Pairwise variation ($V_{n/n+1}$) in *geNorm* was used to assess the appropriate reference genes. The RT-qPCR data were classified into seven groups (all samples, ABA, dark, NaCl, NaHCO₃, PEG, and control), with pairwise variation ($V_{2/3}$) below 0.15 (Fig. 2). Based on the rule of pairwise variation (Vandesompele *et al.* 2002), we suggested two best reference genes as the optimal number of reference genes for each group, the choices being *18S* and *EF1a* for the control, *GAPDH* and *18S* for the ABA and dark treatments, *EF1a* and *TUB* for the NaCl treatment, *TUB* and *18S* for the NaHCO₃ treatment, *EF1a* and *GAPDH* for the PEG treatment, and *EF1a* and *18S* for all samples (Table 2 Suppl.).

Our results show that *EF1a*, ranked first in the top 10 IGCs, was a reliable reference gene in NaCl, PEG, and all samples (Table 2 Suppl.) and ranked second or third in other treatments. The *EF1a* has also been found to be a stable reference gene in *Atractylodes lancea* under drought stress (Sang *et al.* 2017b), *Glycine max* under drought and salinity stresses (Ma *et al.* 2013), *Caragana korshinskii* under heat stress (Yang *et al.* 2014), and creeping bentgrass under cold stress (Chen *et al.* 2015). Only in the

control, *18S* was a reliable reference gene, and only in the NaHCO₃ group, *TUB* was a reliable reference gene. But *18S* and *TUB* exhibited high variations under other experimental conditions (Table 2 Suppl.). The reference gene *ACT*, ranking second in the top 10 IGCs, we found to be the least stable reference gene in the dark, NaHCO₃, and PEG groups (Table 2 Suppl.), which is consistent with *Populus* across developmental stages (Xu *et al.* 2011). However, *ACT* has been found to be a stable gene in *Salicornia europaea* under drought stress and nitrogen treatment (Xiao *et al.* 2015), and in the root of *Cunninghamia lanceolata* (Bao *et al.* 2016). Different studies have yielded inconsistent results because reference genes have been evaluated under different conditions for the same species or in different plant species. Sometimes tested reference genes with the same names actually belong to multigene families. This could be one of the sources of variations in the published studies (Oakley *et al.* 2007). Therefore, it is important to choose suitable reference genes for each species and tested them under certain experimental conditions (Li *et al.* 2012, 2013, Zhao *et al.* 2016, Chen *et al.* 2017).

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