DOI: 10.1007/s10535-016-0670-y BIOLOGIA PLANTARUM 61 (3): 463-472, 2017

# Selection of reference genes for quantitative real-time PCR in *Casuarina equisetifolia* under salt stress

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## Abstract

Real time quantitative PCR (qPCR) is widely used in gene expression analysis for its accuracy and sensitivity. Reference genes serving as endogenous controls are necessary for gene normalization. In order to select an appropriate reference gene to normalize gene expression in *Casuarina equisetifolia* under salt stress, 10 potential reference genes were evaluated using real time qPCR in the leaves and roots of plants grown under different NaCl concentrations and treatment durations. *GeNorm*, *NormFinder*, and *BestKeeper* analyses reveal that *elongation factor 1-alpha* (*EF1α*) and *ubiquitin-conjugating enzyme E2* (*UBC*) were the most appropriate reference genes for real time qPCR under salt stress. However, *β-tubulin* (*βTUB*) and *actin 7,* which were widely used as reference genes in other plant species, were not always stably expressed. The combination of *EF1α*, *UBC*, *uncharacterized protein 2*, *DNAJ homolog subfamily A member 2*, and glyceraldehyde-3-phosphate dehydrogenase should be ideal reference genes for normalizing gene expression data in all samples under salt stress. It indicates the need for reference gene selection for normalizing gene expression in *C. equisetifolia*. In addition, the suitability of reference genes selected was confirmed by validating the expression of *WRKY29-like* and *expansin-like B1*. The results enable analysis of salt response mechanism and gene expression in *C. equisetifolia*.

*Additional key words*: *ACT*, *EF1a*, *DNAJ*, *GAPDH*, *U2*, *UBC*.

## Introduction

*Casuarina equisetifolia* belongs to the family *Casuarinaceae* and is predominantly a monoecious species. It is extensively cultivated in coastal areas and on limestone soils near the shores of tropical and subtropical regions for sand stabilization, soil rehabilitation, and as shelter belts (Zhong *et al.* 2010). *C. equisetifolia* seedlings survive in a 500 mM NaCl solution (Tani and Sasakawa 2003), suggesting its high salt tolerance. As woody plants, *C. equisetifolia* shows a long life span and ability to cope with excess salt for extended periods of time. Specific genes mediate salt response in *C. equisetifolia*. Gene expression analysis is a powerful tool to elucidate the possible mechanisms mediating salt stress and to identify the relevant signal transduction pathways for further prediction of genes with biological functions. Real time quantitative PCR (qPCR) was used extensively to study altered gene expression with a high sensitivity and specificity. However, its reliability depends on stably expressed reference genes (Van Guilder *et al.* 2008, Bustin *et al.* 2009). During the past decade, many stable reference genes were screened in plant species such as *Arabidopsis thaliana* (Czechowski *et al*. 2005), rice (Jain *et al*. 2006), *Brachypodium distachyon* (Hong *et al*. 2008), wheat (Paolacci *et al.* 2009), soybean (Libault *et al.* 2008), tomato (Løvdal and Lillo 2009), and poplar (Xu *et al.* 2011). However,

*Submitted* 25 March 2016. *last revision* 1 July 2016, *accepted* 12 July 2016.

*Abbreviations*: ACT - actin 7; βTUB - β-tubulin; Cq - quantification cycle; DNAJ - DNAJ homolog subfamily A member 2; EF1α - elongation factor 1-alpha; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; GST - glutathione-S-transferase; M - gene expression stability value; MDH - malate dehydrogenase; qPCR - quantitative PCR; U1 - uncharacterized protein 1; U2 - uncharacterized protein 2; UBC - ubiquitin-conjugating enzyme E2.

*Acknowledgments*: We are grateful to the Ministry of Science and Technology of China (2013AA102705) and the Fundamental Research Funds for central public welfare research institutes (RITFYWZX201304) for financial support. We are also grateful to Dr. Zhang Yong (the Research Institute of Tropical Forestry, Chinese Academy of Forestry, Guangzhou, China) for providing experimental material.

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appropriate reference genes have yet to be identified in *C. equisetifolia*, which prevents further studies investigating gene response to salt stress.

Therefore, this study assessed the stability of 10 candidate reference genes in *C. equisetifolia* grown under salt stress. These genes included eight traditional housekeeping genes, such as *ubiquitin-conjugating enzyme E2 (UBC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), DNAJ homolog subfamily A member 2 (DNAJ), elongation factor 1-alpha (EF1α), glutathione-S-transferase (GST), β-tubulin (βTUB), actin 7 (ACT),* and *malate dehydrogenase (MDH),* which were used as reference genes in other plant expression studies. These eight candidate reference genes were evaluated for stable expression under five salt treatments in the *C. equisetifolia* root [SRP064226 in *NCBI*]. Further, two novel reference genes, *uncharacterized protein 1* (*U1*) and *uncharacterized protein 2* (*U2*), were selected from the transcriptome data of *C. equisetifolia* owing to their stable expression in roots under different durations of salt exposure (SRP064226 in *NCBI*). The gene sequences were obtained using the assembly of the *C. equisetifolia* root transcriptome sequenced by *Illumina high-seq* technology. The stability across a large set of samples representing different salt concentrations and treatment durations was compared using statistical and graphical methods. Further, the expressions of *WRKY29-like* and *expansin-like B1* in *C. equisetifolia* subjected to salt stress were examined. This study lays the foundation for further research into gene expression and functional annotation in *C. equisetifolia.*

## Materials and methods

*Casuarina equisetifolia* J.R. & G.Forst. clone A8 seedlings were cultured in a growth chamber at a 16-h photoperiod, an irradiance of 200 μmol $m^{-2}$ $s^{-1}$, day/night temperatures of 24/18 °C, and a relative humidity of 70 % for 8 weeks. Healthy and vigorous seedlings were selected and the plants were transferred to turf soil and irrigated with water containing NaCl at various concentrations (0, 200, and 400 mM) every 2 d. Four weeks later, the plants showed a clear growth inhibition at 400 mM NaCl. However, the plants showed no visible symptoms of toxicity, except for a slight growth reduction at 200 mM NaCl. Therefore, the plants grown in water for two weeks were transferred to 200 mM NaCl solutions for 0, 1, 6, 24, and 168 h. Leaves and roots were harvested and stored at -80 °C. All the samples were collected and processed in sets of three replicates.

The total RNA was extracted from collected roots and leaves using an RNeasy plant mini kit (*Qiagen*, Hilden, Germany) according to the manufacturer's instructions. The RNA samples were treated with RNase-free *DNaseI* (*Qiagen*) to eliminate residual genomic DNA. The purity and concentration of RNA in the samples were evaluated using a *NanoDrop 2000* spectrophotometer (*Thermo Fischer Scientific*, Wilmington, DE, USA). Samples with concentrations greater than 100 mg $cm^{-3}$ and an absorbance $A_{260}/A_{280}$ ratio greater than 1.8 were used for cDNA synthesis. Next, 1.0 μg of the total RNA was used as a template in real time reactions with *SuperScript III* reverse transcriptase (*Invitrogen*, Carlsbad, USA), and cDNA products were diluted 25-fold for real time qPCR.

The sequences of 10 reference genes and 2 salt stress response genes including *WRKY29-like* and *expansin-like B1* were acquired from the assembly of the *C. equisetifolia* root transcriptome sequenced using *Illumina high-seq* technology (SRP064226 in *NCBI*). Gene primers were designed using the *Primer 3* software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). All the primer pairs were initially tested by standard PCR using the *Premix Ex Taq* (*TaKaRa*, Tokyo, Japan). The presence of a single amplification product of an expected size for each gene was verified by electrophoresis on a 2.5 % (m/v) agarose gel. The real time qPCR reactions were carried out in 96-well blocks with a *7500* real-time PCR system *Applied Biosystems* (Foster City, USA) using a *SYBR® Premix Ex Taq*™ kit (*TaKaRa*) under conditions recommended by the manufacturer (95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 34 s). A dissociation curve was obtained by heating the amplicon from 60 °C to 95 °C. All the qPCR reactions were carried out in triplicate. The final quantification cycle (Cq) values included the mean of nine values (biological triplicate, each in technical triplicate). Meanwhile, NTC (non-template controls) and a reverse transcription negative control were used for each sample.

To select the best reference gene, the stability of mRNA expression of each candidate gene was statistically analyzed with three different types of software: *GeNorm* (Vandesompele *et al.* 2002), *NormFinder* (Andersen *et al.* 2004), and *BestKeeper* (Pfaffl *et al.* 2004). Quantities of standard RNA were prepared by diluting cDNA (1/5, 1/25, 1/125, 1/625, and 1/3125; each gene sample in triplicate). Only quantification cycle (Cq) values less than 40 were used to calculate correlation coefficients ($R^2$ values) in *Microsoft Excel 2007*.

## Results

To identify the best reference genes for *C. equisetifolia* gene expression, 10 genes including *UBC, GAPDH, DNAJ, EF1α, GST, βTUB, ACT, MDH, U1*, and *U2* were selected as candidates in real time qPCR assay based on *SYBR green* detection (Table 1). The efficiency plots for each of the candidate genes yielded a linear correlation coefficient ($R^2$) > 0.97. The specificity of the amplified products was confirmed by the presence of a single band with the expected size for each primer pair in electrophoresis, and single-peak melting curves of the PCR products were identified. Raw expression across all samples revealed a variation between the reference genes (Fig. 1). Quantification cycle values of the 10 genes ranged from 17.7 to 32.9, the majority of these values ranged between 20 and 25. The individual reference genes showed different expression ranges across all studied samples. A narrow range of variation in gene expression contributed to a stable gene expression in different samples. The *UBC*, *EF1α, DNAJ*, and *U2* showed minor variations in expression (below 7 cycles) among the reference genes, whereas *ACT*, *U1*, and *GST* exhibited a much higher variation (above 10 cycles). Further, the wide expression range of the 10 test genes confirms that no single gene was expressed constantly in all the samples. Therefore, selection of a reliable reference gene for normalizing gene expression under salt stress in *C. equisetifolia* was imperative.

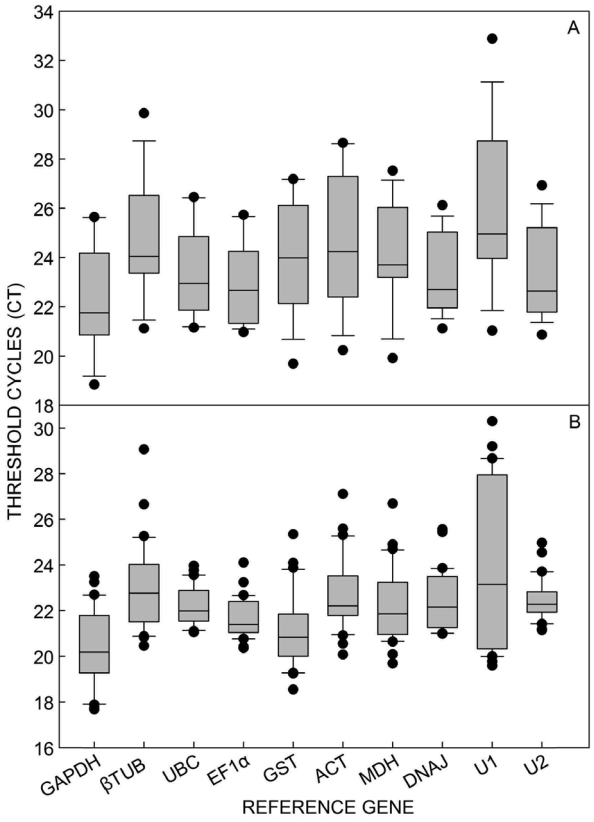


Fig. 1. Absolute CT values of following quantitative PCR of samples. *A* - Leaves and roots treated with different NaCl concentrations ($n$ = 18); *B* - Leaves and roots treated with 200 mM NaCl at different treatment duration ($n$ = 30). Each *box* indicates 25/75 percentiles. *Whisker caps* represent 10/90 percentiles. The median is depicted by *lines* and *dots* represent outliers of the $10^{th}$/$90^{th}$ percentiles. GAPDH - glyceraldehyde-3-phosphate dehydrogenase, βTUB - β-tubulin, UBC - ubiquitin-conjugating enzyme E2, EF1α - elongation factor 1-alpha, GST - glutathione-S-transferase, ACT - actin 7, MDH - malate dehydrogenase, DNAJ - DNAJ homolog subfamily A member 2, U1 - uncharacterized protein 1, U2 - uncharacterized protein 2.

*GeNorm*, *NormFinder*, and *BestKeeper* were used to analyze the stability of the tested genes in *C. equisetifolia* leaves and roots. *GeNorm* assumes that the expression ratio of two ideal reference genes is stable in a given sample set. The gene expression stability value (M) is calculated for all genes under investigation and a lower M value indicates a higher stability (Vandesompele *et al.* 2002). Under different NaCl concentrations, *DNAJA2* and *EF1α* were the most stable genes with values of 0.482, whereas *ACT7* and *U1* were the least stable with values ranging from 0.944 to 1.16 (Fig. 2*A*). Under the different salt duration treatments, *UBC* and *EF1α* were the most

stable genes, whereas *GST* and *U1* were the least (Fig. 2*B*). When all samples were analyzed together, *UBC* and *EF1α* with an M value of 0.436 were the most stable, whereas *GST* and *U1* were the least stable (Fig. 2*C*). In addition, almost all of the tested reference genes (except *U1*) showed a relatively high stability with M values less than the default limit of M ≤ 1.5.

In this study, pairwise variation (Vn/Vn+1) between the sequential normalization factors (NF) (NFn and NFn+1) was calculated using *GeNorm* to determine the optimal number of reference genes. It was unnecessary to include an additional reference gene when the cut-off value was less than 0.15 according to recommendations of Vandesompele *et al.* (2002). As shown in Fig. 1 Suppl., when all samples were considered together to determine the optimal number of reference genes, the pairwise variations in the V2/3, V3/4, and V4/5 values were 0.187, 0.16, and 0.202, respectively, whereas the pairwise variation in the V5/6 value was 0.148. The results indicate that *EF1α*, *UBC*, *U2*, *DNAJ*, and *GAPDH* were ideal reference genes for normalizing gene expression in all the samples. However, analysis of the pairwise variation with the different salt concentration treatments reveals that three reference genes *EF1α*, *DNAJ*, and *U2* were sufficient to normalize the expression of target genes. Under the different salt duration treatments, the five genes (*EF1α*, *UBC*, *U2*, *DNAJ*, and *GAPDH*) were required for normalization. Therefore, the three to five most stably expressed genes, mentioned above for each type of samples, were selected as reference genes.

Table 1. Real time quantitative PCR analysis of candidate genes (for explanation of the gene abbreviations see Fig. 1) from *Casuarina equisetifolia*.

| Gene | Acc. number and length | Gene description | *Arabidopsis* ortholog locus | Primer pair (forward/reverse) | Product size and position | $R^2$ |
|---|---|---|---|---|---|---|
| *GAPDH* | comp97785 (594 bp) | glyceraldehyde-3-phosphate dehydrogenase | AT1G79530 | CGGCCTTTTCCTTGTCAGTG<br>GTCAAGGACTCCAATACCCTTCT | 145bp (156-300) | 0.984 |
| *DNAJA2* | comp148822 (1926 bp) | DNAJ homolog subfamily A member 2 | AT3G44110 | CATTGTGCCGTGCTGAAACC<br>TGAAGATTGAGTGTTAACGAGGTG | 132bp (14-145) | 0.992 |
| *EF1α* | comp109215 (516 bp) | elongation factor 1-alpha | AT1G07940 | TGGCATGTACTTGAGGATCACA<br>TGCATTGATGGCACGAAGC | 148bp (11-132) | 0.978 |
| *UBC* | comp131250 (1160 bp) | ubiquitin-conjugating enzyme E2 | AT3G57870 | CCATGGTGTACTCTCTCTGCC<br>TGTTTTGTGATAAGGATTACCCAGA | 147bp (601-747) | 0.994 |
| *GST* | comp143368 (1213 bp) | glutathione-S-transferase | AT1G02930 | GGCACCAGTAACCACAAAAGC<br>CGGTGGCCTATCTGGGGTAT | 132bp (61-192) | 0.982 |
| *U1* | comp168148 (1120 bp) | uncharacterized protein | No hit | TCCATCCATGACTGTTTTGTTTT<br>TGGGTCAAATCTAGCTTGCCT | 143bp (301-443) | 0.972 |
| *U2* | comp135147 (1046 bp) | translationally-controlled tumor protein | No hit | GTGTCAACAACTTGATGTACCTCTT<br>GTGGAGGTGAGGATGAGGGT | 139bp (603-741) | 0.971 |
| *βTUB* | comp156426 (2082 bp) | tubulin beta | AT1G20010 | GGTGCTGGAAACAATTGGGC<br>GACAGTCACAGTTCTCAGCCT | 103bp (410-512) | 0.988 |
| *ACT7* | comp150391 (2020 bp) | actin 7 | AT5G09810 | TCTATGAAGGATATGCCCTCCC<br>GGACAATTTCCCGTTCGGC | 135bp (858-992) | 0.992 |
| *MDH* | comp148486 (1658 bp) | malate dehydrogenase | AT3G47520 | GTTGTTGAGGCTTGCACTGG<br>CAGCTGCATGCTTTTCGAGG | 145bp (453-597) | 0.992 |
| *WRKY29 like* | Comp151289 (1771 bp) | WRKY transcript factor 29 | AT4G23550 | TGGGCACCGAATTCTTTCCA<br>GTCCCAGAAGCAACCATCCA | 80bp(1381 -1460) | 0.994 |
| *expansin-like B1* | Comp149703 (1291 bp) | expansin-like B1 | AT4G17030 | GCGTGTGAAAGTGCCTTGAG<br>TTGAGACATGGGGTTCGCAA | 97bp(1127 -1223) | 0.993 |

The stability of expression was then re-analyzed using the *NormFinder* algorithm that is based on minimal combined inter- and intra-group variations in gene expression for normalization factor (NF) calculation (Andersen *et al.* 2004). Similarly to *GeNorm* analysis, a lower average expression stability indicated a more stable gene expression (Fig. 3). Ranking reference genes showed a minimal difference from that of the *GeNorm* software. When the *C. equisetifolia* seedlings were subjected to various NaCl concentrations and durations of 200 mM NaCl treatment, *GAPDH* always emerged as the most stably expressed gene, whereas it was ranked fourth and sixth by *GeNorm*, respectively. However, *UBC, EF1α*, and *U2* showed a rather stable expression both under varying salt concentrations and under treatment durations, similar to the results of *GeNorm*.

*BestKeeper* analysis is another applet, which was used to analyze raw Cq data to determine the most stable genes based on the standard deviation (SD) and coefficient of variance (CV). Genes with the lowest values of SD were

classified as the most stable (Pfaffl *et al.* 2004). The results were similar to the *GeNorm* and *NormFinder* programs (Fig. 4). The *EF1α, U2*, and *UBC* were always considered the most stable among the 10 genes in all the experimental samples. Similarly, the least stable genes *U1*, *ACT*, and *βTUB* were observed under the different salt duration and NaCl concentration datasets. However, slightly altered rank orders were observed. For example, *GAPDH* was ranked sixth by *BestKeeper*, whereas it emerged as the most stable in *NormFinder* analysis in all the samples.

To obtain appropriate reference genes, ranking orders of candidate genes by *BestKeeper*, *NormFinder*, and *GeNorm* were comprehensively analyzed. In summary, irrespective of the ranking order (Table 2), *EF1α*, *UBC*, and *U2* were the most appropriate reference genes to normalize mRNA levels within the context of different NaCl concentrations and treatment durations. Conversely, *MDH*, *βTUB*, *GST*, *ACT*, and U1 showed relatively low expression stabilities in the leaves and roots of *C. equisetifolia* exposed to salt stress.

The expression of *WRKY29-like* and *expansin-like B1* genes under salt stress was analyzed using selected reference genes *UBC*, *EF1α*, *U2*, *DNAJ*, and *GAPDH* in

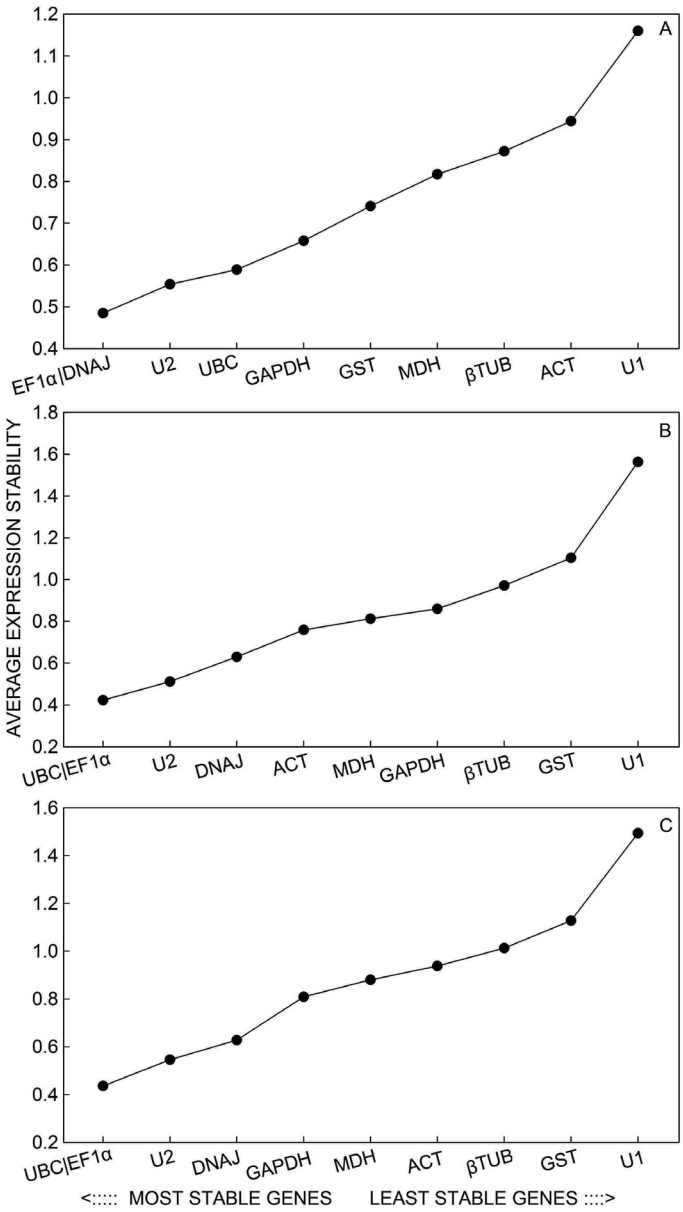


Fig. 2. Stability of average expression and ranking 10 candidate reference genes (for explanation of the gene abbreviations see Fig. 1) calculated by *GeNorm*: *A* - different NaCl concentrations ($n$ =18), *B* - different treatment durations ($n$ = 30), *C* - all samples ($n$ = 48). Lower average expression stabilities (M values) indicate a higher stability.

Table 2. The ranking order of reference genes (for explanation of the gene abbreviations see Fig. 1) using *BestKeeper*, *NormFinder*, and *GeNorm* algorithms.

| | *EF1α* | *UBC* | *U2* | *DNAJ* | *GAPDH* | *MDH* | *ACT* | *βTUB* | *GST* | *U1* |
|---|---|---|---|---|---|---|---|---|---|---|
| *GeNorm* | 1.5 | 1.5 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *NormFinder* | 3 | 2 | 4 | 7 | 1 | 6 | 5 | 9 | 8 | 10 |
| *BestKeeper* | 1 | 3 | 2 | 4 | 6 | 5 | 8 | 7 | 9 | 10 |
| Mean rank | 1.83 | 2.17 | 3 | 5 | 4 | 5.67 | 6.67 | 8 | 8.67 | 10 |

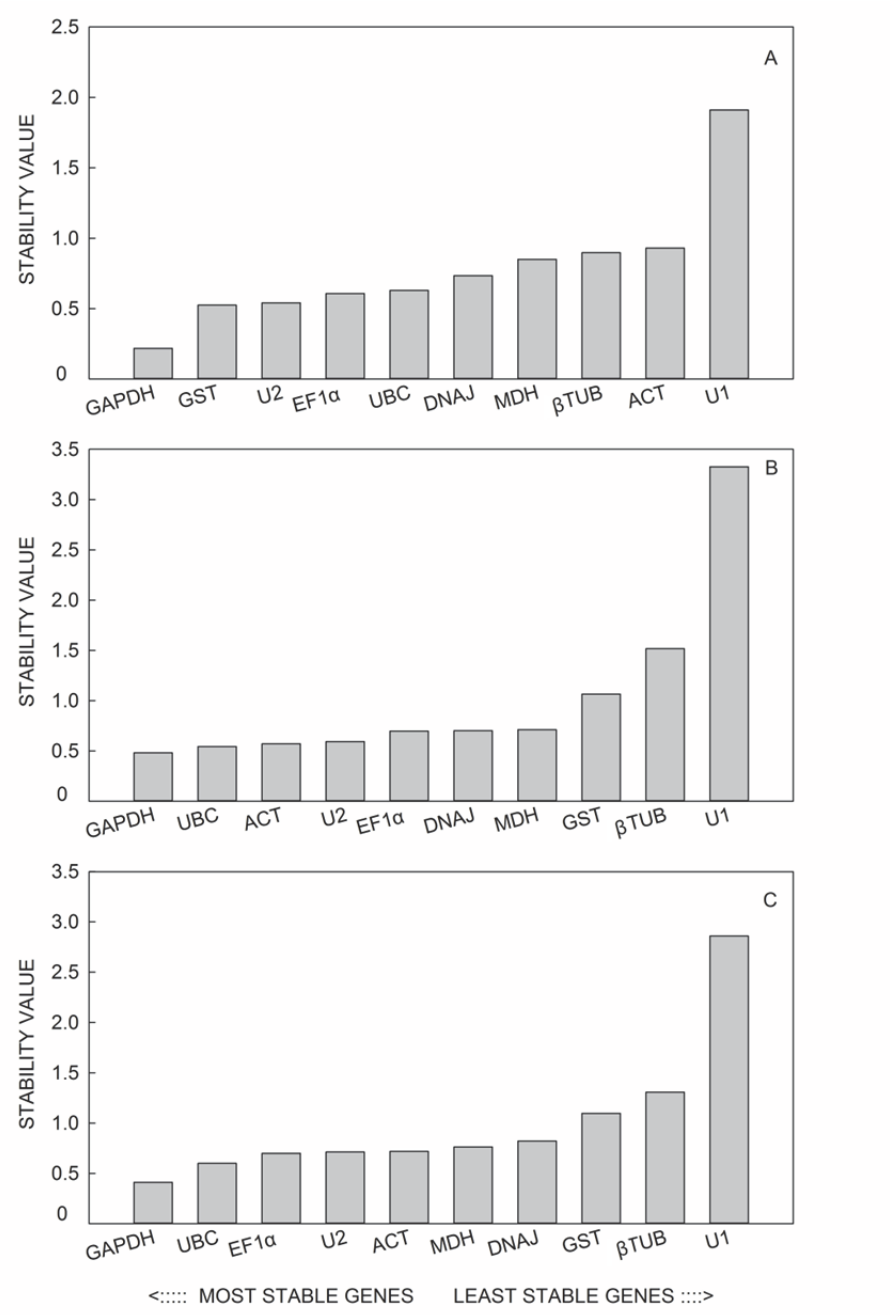


Fig. 3. Stability of *Casuarina equisetifolia* candidate reference genes (for explanation of the gene abbreviations see Fig. 1) using *NormFinder*: *A* - different NaCl concentrations ($n$ = 18), *B* - different treatment durations ($n$ = 30), *C* - all samples ($n$ = 48). A lower stability value indicates more stable expression.

combination (Fig. 5*A*,*B*). The *WRKY29-like* gene was slightly down-regulated in roots with no obvious change in leaves under the increasing NaCl concentration. However, the abundance of *WRKY29-like* mRNA increased rapidly and peaked after 1 h of the treatment in roots and increased dramatically only after 168 h of the treatment in leaves (Fig. 5*A*). The abundance of *expansin-like B1* mRNA was obviously down-regulated with the increasing NaCl concentration in leaves and roots. The *expansin-like B1* expressions were enhanced eventually with the salt treatment and peaked after 168 h (Fig. 5*B*). Further, the expressions of the *WRKY29-like* gene and *expansin-like B1* under the salt stress were analyzed using *UBC* individually (Fig. 5*C*,*D*). The relative expression profiles of the *WRKY29-like* and *expansin-likeB1* genes in leaves and roots showed similar trends when the reference gene *UBC* was used at the different NaCl concentrations and treatment durations. However, the differences in expression of the *WRKY29-like* gene at the different concentrations of NaCl persisted (Fig. 5*C*). Overall, the results show that selection of appropriate reference genes is essential for accuracy and normalization using real time qPCR.

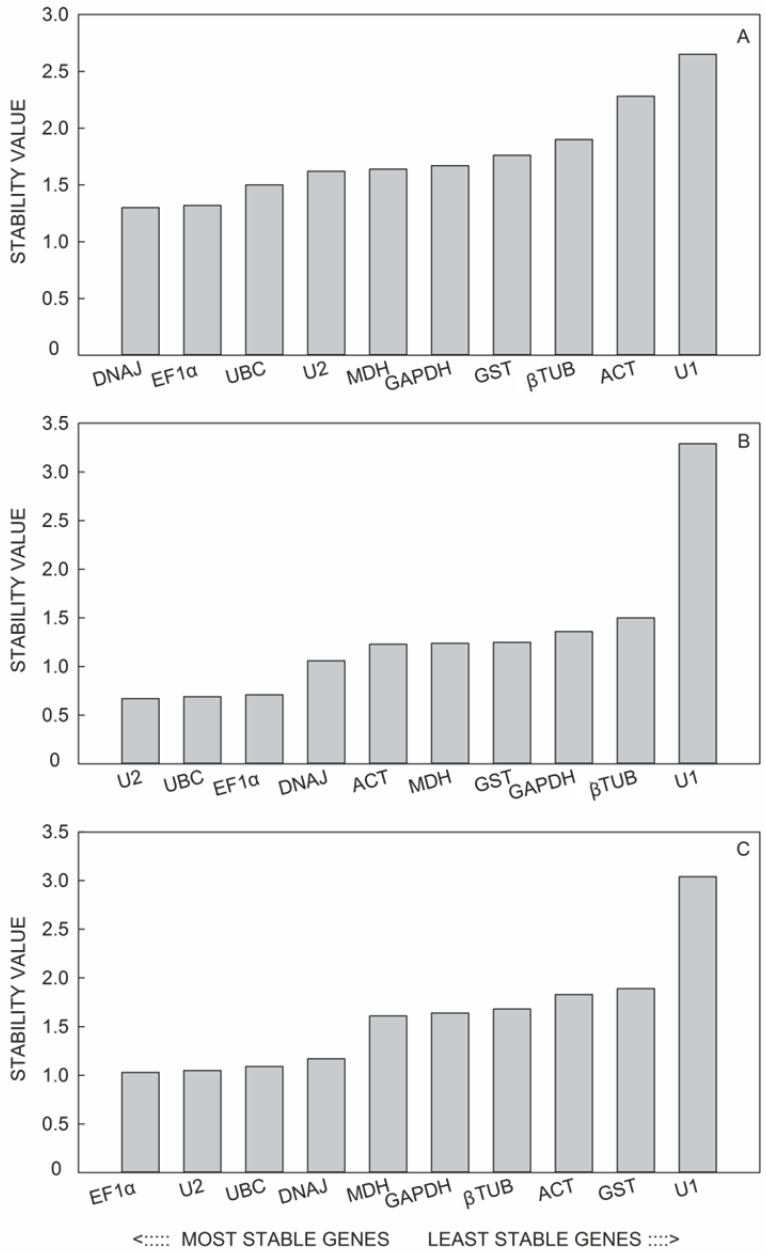


Fig. 4. Stability of *Casuarina equisetifolia* candidate reference genes (for explanation of the gene abbreviations see Fig. 1) using *BestKeeper*: *A* - different NaCl concentrations ($n$ = 18), *B* - different treatment durations ($n$ = 30), *C* - all samples ($n$ = 48). A lower stability value indicates more stable expression.

## Discussion

In plant molecular biology, studies in *C. equisetifolia* mainly focused on gene identification during nitrogen fixation (Obertello *et al.* 2007, Peter *et al.* 2007). No study investigated the response and adaptation of this species to salinity although *C. equisetifolia* seedlings survive in a 500 mM NaCl solution (Tani and Sasakawa 2003). To unravel the complex molecular mechanisms and expression patterns of genes mediating adaption to saline stress, identification of stably expressed reference genes is essential.

Previous studies demonstrated that a single gene is not always expressed stably under all experimental conditions and in all plant species. In this study, *βTUB* and *ACT*, which are frequently used as internal control genes in many experiments (Czechowski *et al.* 2005, De Oliveira *et al.* 2012, Kumar *et al.* 2013, Hu *et al.* 2014), were not stably expressed under the experimental setting using the complementary algorithms (*GeNorm*, *NormFinder*, and *BestKeeper*). The *GAPDH,* which is expressed stably in sugarcane, poplar, and other species, showed a high variation in expression and was not suitable as an internal control (Xu *et al.* 2011, Ling *et al.* 2014). Surprisingly, *U2,* which encoded an unknown protein and was expressed stably in transcriptome analysis (unpublished data), showed comparatively a less variability and acceptable stability. It was also expressed in *Arabidopsis thaliana* and *Eucalyptus grandis* when selecting the best reference gene based on both real time qPCR and microarray (Czechowski *et al.* 2005, De Oliveira *et al.* 2012). These results indicate that a reference gene selected in one organism may not express stably in another organism under a given set of conditions and hence, validation of a housekeeping gene is essential. In this study, several candidate reference genes, such as

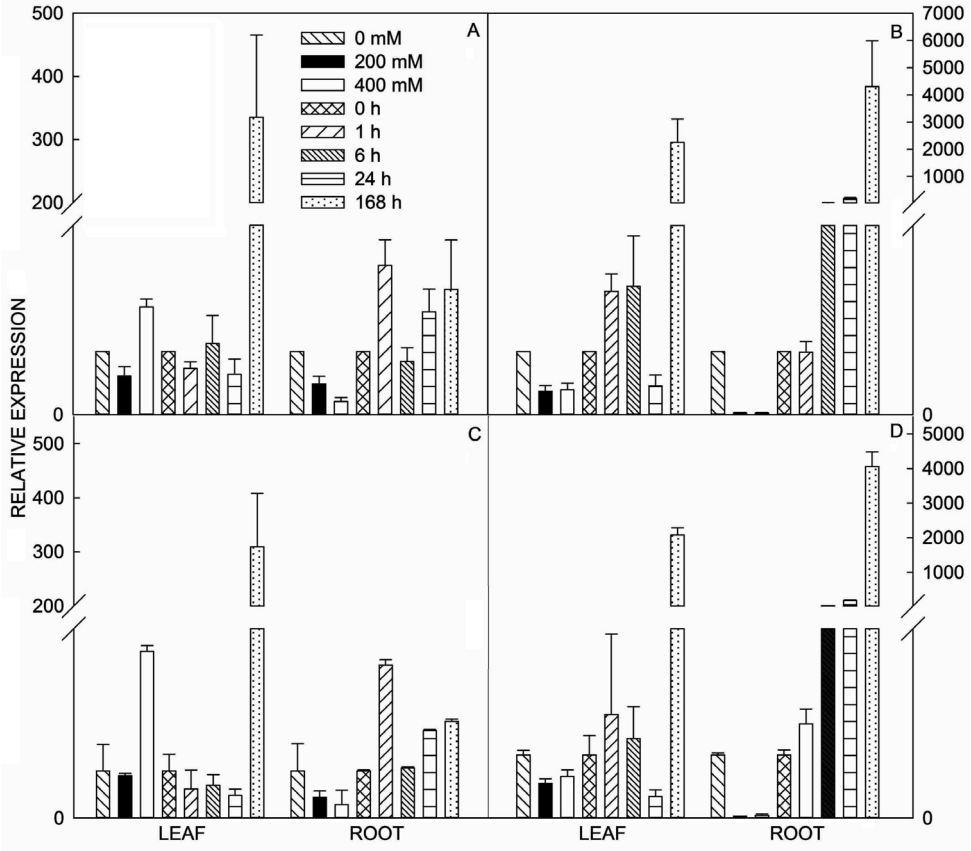


Fig. 5. Quantification of relative expression of *WRKY29-like* and *expansin-like B1* genes using selected reference genes *ubiquitin-conjugating enzyme E2* (*UBC*), *elongation factor 1-alpha*, *uncharacterized protein 2*, *DNAJ homolog subfamily A member 2*, and *glyceraldehyde-3-phosphate dehydrogenase* in combination (*A,B*) and with *UBC* individually (*C,D*). *A,C - WRKY29-like* gene expression at different NaCl concentrations and treatment durations; *B,D - expansin-like B1* gene expression at different NaCl concentrations and treatment durations.

*GST, U1*, and *MDH,* which were expressed stably in roots at different durations of NaCl treatment by previous transcriptome analysis (unpublished data), showed differential expressions in leaves or at different NaCl concentrations. However, *EF1α* and *UBC* performed well in all the three algorithms and therefore were identified as

the most appropriate reference genes, consistent with validated reference genes in other species such as rice, sugarcane, and *Cocos nucifera* (Czechowski *et al.* 2005, Jain *et al.* 2006, Ling *et al.* 2014, Saraiva *et al.* 2014, Xia *et al.* 2013). Further, a single reference gene may not be reliable and two or more internal reference genes are needed for accurate and reliable results. Pairwise variation results in this study were verified. Under different NaCl concentrations, three reference genes were necessary to normalize gene expression. At different salt durations, five genes were required for normalization following stable expression in roots. The results also indicate that many genes including a few housekeeping genes were unstable under salt stress. Therefore, additional internal reference genes were required. Further, ranking by the different softwares varied, especially in the genes ranking the top. The *GAPDH* was the most stable gene when *NormFinder* was used. However, *GeNorm* and *Bestkeeper* ranked *GAPDH* as less stable. *GeNorm* and *BestKeeper* showed more consistency compared with *NormFinder* in ranking the candidate reference genes. This discrepancy in ranking by the three software programs was observed in bamboo (Fan *et al.* 2013) and tung tree (Han *et al.* 2012)), which was mainly attributed to different algorithms.

The *WRKY* family contains nearly 100 members in *Arabidopsis*, some of which appear to mediate plant response to biotic and abiotic stresses (Eulgem *et al.* 2000, Xu *et al.* 2006, Hsu *et al.* 2013, Wang *et al.* 2014). The *WRKY29*/22 is induced in plant response to both bacterial and fungal infection, and its expression in transiently transformed leaves reduces disease symptoms (Asai *et al.* 2002). Expression of *WRKY15* induces endoplasmic reticulum-to-nucleus communication leading to disrupted mitochondrial stress response under salt stress during plant growth (Vanderauwera *et al.* 2012). Many *WRKY* genes are strongly and rapidly induced during response to various abiotic stresses (Hsu *et al.* 2013). In this study, *WRKY 29-like* gene expression was rapidly enhanced in response to salt stress. Expansin represents a cell wall loosening protein and mediates cell wall extensibility. Increasing evidence suggests that expansin plays an essential role in response to salt stress. In a salt-resistant maize cultivar, *β-expansin* transcription is higher than in a sensitive cultivar (Geilfus *et al.* 2010). Meanwhile, the abundances of *β-expansin*, *AtEXP3,* and *AtEXP-B1* transcription vary under salt stress (Buchanan *et al.* 2005, Kwon *et al.* 2008). Expression of *expansin-like B1* was enhanced gradually with increasing NaCl treatment duration in this study, similarly to *EXPA1* and *EXLB1* expressions in *Jatropha curcas* seedlings exposed to salt stress (Zhang *et al.* 2014). These results also indicate the stability and reliability of the selected reference genes.

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