Validation of reference genes for accurate normalization of gene expression with quantitative real-time PCR in *Haloxylon ammodendron* under different abiotic stresses

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Abstract *Haloxylon ammodendron* plays an important role in maintaining the structure and function of the entire ecosystem where it grows. No suitable reference genes have been reported in *H. ammodendron* plants to date. In this study, a total of 8 reference genes (*18S*, *ACT1*, *ACT7*, *UBC18*, *TUA5*, *GAPDH*, *EF-1 α* and *UBQ10*) were selected from the available transcriptome database, and the expression stability of these 8 candidate genes was validated under different abiotic stress with three different statistical algorithms (geNorm, NormFinder and BestKeeper). The results produced from different models were in agreement with each other essentially: *18S* and *TUA5* were the most stable genes under drought stress, *18S*, the most stable gene under heat stress and mechanical damage, *ACT7* and *UBC18*, stable under salt stress while *TUA5* and *GAPDH* expressed constantly under mechanical damage, and *ACT1* expressed steadily under cold conditions. Expression

profiles of several stress response genes, including *FT-5*, *FT-9*, *DREB2A* and *DREB2C*, were further confirmed with various candidate reference genes. None of the candidate genes showed a constant expression among all tested samples. Hence, it's essential to use more than one reference gene in order to guarantee the accuracy of quantitative real-time PCR. The results of this study will contribute to the accuracy and reliability in transcripts quantification, which is of significance to transcription-based studies and applications in this important shrub *H. ammodendron*.

Keywords *Haloxylon ammodendron* · Reference genes · Abiotic stress · Expression stability

Introduction

The *Haloxylon* plants in Chenopodiaceae family are a kind of shrubs or small trees which best survive in fixed dune, semi-fixed dune, saline soil and Gobi desert in temperate and subtropical zones of Asia and Africa (25–48°10'N, 5–110°30'E) (Pyankov et al. 1999). The researchers have to date identified 11 species in *Haloxylon* plants, of which there are only two species (*Haloxylon ammodendron* and *H. persicum*) distributed in the desert and semi-desert areas of northwest China (Yu et al. 2012). *H. ammodendron*, known as the “Desert Guardian”, is one of the major species for desert afforestation in China. It plays an irreplaceable role in maintaining the structure and function of the ecosystem in arid areas (Sheng et al. 2005; Yu et al. 2012) due to its outstanding capacity to resist high temperature and drought. It also helps reducing the wind speed and improving the microclimate of the forest, thus facilitating the growth of other plants (Long et al. 2014). *H. ammodendron* has evolved many xeromorphic

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characteristics. For instance, its leaves are degenerated into scales and the annual succulent branches take over the function of assimilation (Yu et al. 2012). These type of branches are usually referred to as assimilation branches (Yu et al. 2012) (Supplementary Fig. 1).

The adaptation of *H. ammodendron* to the harsh environment such as drought and high temperature indicates its unique adaptability to these stresses, and some functional gene expression may play an important role in this case. With the help of transcriptome technique, many genes functioning in stress tolerance have been mined in *H. ammodendron* (Long et al. 2014). However, the molecular mechanism for such an adaptability of *H. ammodendron* to environmental stress has not been reported.

Quantitative real-time PCR (qRT-PCR) is a method commonly used to determine gene expression at the mRNA level with its high sensitivity, reproducibility and specificity (Bustin 2002; Derveaux et al. 2010). The use of the internal reference gene is considered as the most appropriate and standardized method as it may effectively correct the difference between the RNA starting amount and the reverse transcription efficiency (Bao et al. 2016; Zhang et al. 2016). Thus, it is critical to find suitable reference genes for normalizing the expression of stress resistance genes in *H. ammodendron*.

The ideal reference genes which can be used exclusively for different types of samples should be expressed stably in all cells under any physiological conditions (Ma et al. 2016; Li et al. 2017; Perez et al. 2017). However, in the plants of same species, no reference gene can be stably expressed with the change of experimental conditions, and a reference gene suitable under one experimental condition is not necessarily suitable under another experimental condition; and in the plants of different species, the same reference gene may be expressed differently under the same stress (Volkov et al. 2003). Therefore, it is very important to select the stable reference gene(s) under specific experimental conditions. The plant-originated reference genes most frequently used in qRT-PCR include *actin* (*ACT*) (Maroufi et al. 2010), *tubulin* (*TUB*) (Wan et al. 2010), *ubiquitin* (*UBQ*) (Chen et al. 2011), *elongation factors* (*EF*) (Yuan et al. 2014), *ubiquitin-conjugating enzyme* (*UBC*) (Ma et al. 2016), *18S ribosomal RNA* (*18S*) (Amil-Ruiz et al. 2013) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (Jain et al. 2006), etc.

Due to limited genome sequence information, no applicable reference genes have been explored from *H. ammodendron* yet. Based on our previous transcriptome analysis, 8 commonly used reference genes (*18S*, *ACT1*, *ACT7*, *UBC18*, *TUA5*, *GAPDH*, *EF-1 α* and *UBQ10*) in *H. ammodendron* were cloned. In order to obtain the most suitable reference genes, expression assays were performed in diverse abiotic-stressed samples of *H. ammodendron* by

using BestKeeper, geNorm and NormFinder. This study provides evidences on reference gene selection based on their expression levels under particular conditions, which will benefit the future studies related to *H. ammodendron* as well as other species of *Haloxylon* genus.

Materials and methods

Plant materials and treatments

Cultured 1-year seedlings of *H. ammodendron* from a nursery in Jimsar, Xinjiang were separately transplanted in a greenhouse [$30 \pm 2/20 \pm 2$ °C (day/night), $900\text{--}1260 \mu\text{mol/m}^2 \text{ s}$] and ready for experimental use (Supplementary Fig. 1). Except for *H. ammodendron* seedlings treated under drought stress, all the other stressed *H. ammodendron* seedlings were sampled at 6 time points: 0, 4, 8 (or 6), 12, 24, and 72 h after treatments. After each stress treatment, the assimilation branches were cut off from stress treated *H. ammodendron* seedlings. For mechanical damage treatment, the assimilation branches of *H. ammodendron* seedlings were cut with a sterilized blade, and preserved immediately. For low temperature treatment, *H. ammodendron* seedlings were grown at 4 °C with 12/12 h day/night light cycle. During salt treatment, *H. ammodendron* seedlings were irrigated with 200 mM NaCl. For high temperature treatment, *H. ammodendron* seedlings were grown at 45 °C with 12/12 h day/night light cycle. For drought treatment, *H. ammodendron* seedlings were dried by decreasing soil moisture content to 15, 12, 9, 6 or 3% and then assimilation branches were sampled while the assimilation branches sampled at 20% soil moisture content were taken as the control. All assimilation branches were sampled from three different plants as biological replicates. Moreover, all the assimilation branch samples were snap frozen in liquid nitrogen and stored at -80 °C for total RNA isolation.

Total RNA isolation and cDNA synthesis

200 mg of each assimilation branch sample was collected for total RNA isolation. Total RNA was isolated using Trizol (Life Technologies, USA). RNA concentration and purity were determined by spectrophotometer (Eppendorf, Germany). RNA integrity was confirmed by electrophoresis using 1.0% agarose gel. Total RNA isolated from assimilation branch samples were diluted at a concentration of $1 \mu\text{g}/\mu\text{L}$ and were used for the qRT-PCR experiments. The first-strand cDNA was synthesized from Dnase-free total RNA using M-MLV reverse transcriptase (Transgen, China).

Sequence retrieving and cloning of the candidate reference genes

Sequence information of 8 candidate genes (*18S*, *ACT1*, *ACT7*, *UBC18*, *TUA5*, *GAPDH*, *EF-1 α* and *UBQ10*) was retrieved from our *H. ammodendron* transcriptome data. Based on sequence information, primers (Supplementary Table 1) for cloning full length coding domains were designed using the Primer Premier 5 software. Amplified full length coding domains of candidate genes were confirmed by sequencing. Sequences and accession numbers could be obtained from NCBI database (Table 1).

Primer designing and qRT-PCR

Primers were designed by using Primer Premier 5. All primer sequences are listed in Supplemental Table 1 (for full-length cloning and qRT-PCR) and Table 1 (for qRT-PCR). The amplification efficiency (E) and correlation coefficients (R^2) of the primers for qRT-PCR were calculated (Reddy et al. 2015). qRT-PCR reactions were carried out on 7500 Fast (ABI, USA), in 96-well optical reaction plates. Reactions were performed in a total volume of 20 μ L, containing 2 μ L of cDNA (1:10 dilution), 0.4 μ L of each primer (10 mM), 10 μ L of 2 $\times$ SYBR Select Master Mix (Transgen, China) and made to 20 μ L with RNase-free H₂O. The qRT-PCR cycling conditions were as follows: 95 $^{\circ}$ C for 5 min followed by 40 cycles of 15 s at 95 $^{\circ}$ C, annealing (at each pair of primer's specific T_m) for 30 s and fluorescent signal recording for 30 s at 72 $^{\circ}$ C. The melting curve analysis was carried out after 40 cycles to verify the primer specificity by heating from 65 to 95 $^{\circ}$ C with fluorescence measured within 20 min. The relative expression levels were determined by $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) in three biological replicates with three technical repeats.

Data analysis

A threshold fluorescence value of 0.1 was applied to analyze all amplification plots in order to obtain amplification cycle (C_q) values with the SDS version 1.1 software (ABI, USA). The value representing stability and suitability was evaluated respectively using geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004), as described in relevant reports.

Results

Identification of *H. ammodendron* reference genes

Eight candidate reference genes (*18S*, *ACT1*, *ACT7*, *UBC18*, *TUA5*, *GAPDH*, *EF-1 α* and *UBQ10*) were chosen from the *H. ammodendron* transcriptome data and their full-length open reading frames were cloned and sequenced (Supplementary Fig. 2). Their detailed information, including GenBank accession number and putative function description are listed in Table 1.

For qRT-PCR analysis, total RNAs isolated from plant samples (including biological replicates) exhibited high quality. The A260/A280 ratios of extracted RNA samples ranged from 1.95 to 2.12, indicating that RNA samples were pure and protein-free. The amplification efficiencies (E), correlation coefficients (R^2) and the T_m values of all amplification products are listed in Table 1. The specificity of primers had been confirmed by agarose gel electrophoresis and melting curve analysis (Supplementary Fig. 3). The amplification efficiencies (E) of reference genes ranged from 0.95 (*TUA5*) to 1.11 (*UBC18*). The correlation coefficient (R^2) values varied from 0.9944 (*EF-1 α*) to 0.9991 (*18S*) (Table 1). These results show that all primer pairs designed for qRT-PCR analysis were suitable.

The results of expression stability validation showed that *ACT7* had the highest mean C_q value (22.55) in all samples treated under stress (drought, heat, salt, cold and mechanical damage). On the other hand, the lowest mean C_q value was observed for *GAPDH*, followed by *18S* and *UBC18* (18.93, 19.28 and 19.32, respectively). Additionally, the most varied expression levels were observed for *18S*, followed by *UBQ10* and *ACT7*, as shown by the larger whisker taps and boxes comparing with the other genes. The least variation in expression levels was observed in *UBC18*, followed by *EF-1 α* and *GAPDH* (Fig. 1). These results showed none of the candidate reference genes had constant expression levels across all the samples under the above-mentioned stresses (drought, heat, salt, cold and mechanical damage). Therefore, it is of necessity to validate suitable reference gene(s) for accurate normalization of gene expression under different stress conditions in *H. ammodendron*.

Expression stability of the candidate reference genes

Tools are available to evaluate the expression stability of reference gene, such as geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004). In each sample subset, the 8 reference genes were ranked from the most stable to the least stable (Fig. 2). According to Bestkeeper, *18S* was

Table 1 Details of candidate reference genes, primer sequences for qRT-PCR, products size and amplicon characteristics

Gene symbol	Gene name	Accession no.	Primers for qRT-PCR (F/R) (5'–3')	Cellular function	Amplicon length (bp)	Tm (°C)	PCR efficiency	Regression coefficient (R ²)	References
<i>ACT1</i>	Actin 1	KM886609	GAACCCCAAGGCTAACAGAGAAAA CTCACACCATCACCAGAGTCAAGA	Cytoskeleton structure protein	150	85.2	1.02	0.9988	Maroufi et al. (2010)
<i>18S</i>	18S rRNA	KU685539	CTCTGCCCCGTGCTCTGATGAT CCTTGGATGTGGTAGCCGTTTC	Ribosomal RNA	194	89.3	0.97	0.9991	Amil-Ruiz et al. (2013)
<i>TUA5</i>	Tubulin alpha-5	KU685537	CAACTGGCTTCAAAATGTGGTATCA ATCTTCACGGGCTTCAGAAAACTC	Cytoskeleton structure protein	230	87.4	0.95	0.9960	Wan et al. (2010)
<i>ACT7</i>	Actin 7	KU674357	TTGAACCCCAAGGCTAACACAGAG ACGACCAGCAAGATCCAAACGG	Cytoskeleton structure protein	222	87.2	1.05	0.9988	Maroufi et al. (2010)
<i>EF-1α</i>	Elongation factors-1A	KU674358	CGTGAAGGCGACTCGTTAATTG GCTTGGAGACGCTTGACAGGAC	Translation eukaryotic factor	190	84.2	1.14	0.9944	Yuan et al. (2014)
<i>GAPDH</i>	3-Phosphate-glyceraldehyde dehydrogenase	KU674359	AATGCTATTGGCGGGAAGAGAAAG CAAGGATGGAGTCGTATTGAGG	Glycolysis and gluconeogenesis	205	86.9	1.10	0.9952	Jain et al. (2006)
<i>UBC18</i>	Ubiquitin conjugating enzyme 18	KU685538	GATGTCTTCTCATTTCCCTTC ATCGCCGACTCTAAACCTCTAT	Protein degradation	204	83.2	1.11	0.9974	Ma et al. (2016)
<i>UBQ10</i>	Polyubiquitin 10	KU674360	CTGCTTATGGGTGTCAGTTTITA GAGCAAAATAGGCACAAACGAAAG	Protein degradation	192	81.0	0.98	0.9975	Chen et al. (2011)

Fig. 1 Expression levels of candidate reference genes among all samples: Lines across the boxes depict the medians. Boxes indicate the interquartile range. Whiskers represent 95% confidence intervals; Stars and circles indicate the presence of outliers

considered the most suitable gene under drought, heat stress and mechanical damage. Meanwhile, *UBC18* and *ACT1* were considered the most suitable genes under cold stress and salt stress. NormFinder analysis showed that *18S* was the most suitable gene under drought and heat stress. *ACT7*, *ACT1* and *GAPDH* were considered being stable under salt stress, cold stress and mechanical damage, respectively. Overall, the results shown in NormFinder were similar to those found in BestKeeper (Table 2). GeNorm analysis showed that the expression profiles of *TUA5* and *UBQ10* were the most constant under drought stress, *18S* under heat stress, *ACT7* and *UBQ10* under salt stress, *ACT1* and *TUA5* under cold stress, and *TUA5* and *GAPDH* under mechanical damage stress.

Based on the expression stability analysis of candidate genes with algorithms geNorm, NormFinder and BestKeeper, we concluded the most stable genes under different stress conditions as follows, *18S* and *TUA5* under drought stress, *18S* under heat stress, *ACT7* and *UBC18* under salt stress, *ACT1* under cold stress, *18S*, *TUA5* and *GAPDH* under mechanical damage (Table 3).

Optimal number of candidate genes for normalization

The optimal number of reference genes was determined by geNorm, which uses the step wise calculation of the pairwise variation (V_n/V_{n+1}) between two sequential normalization factors with 0.15 as the cut-off value (Vandesompele et al. 2002). As shown in Fig. 3, the $V_2/3$ value was lower than 0.15, indicating two reference genes were sufficient for normalizing gene expression under salt

stress and mechanical damage. Three reference genes were sufficient for qRT-PCR analysis under drought and heat stress because the $V_2/3$ value was higher than 0.15 and the $V_3/4$ value lower than 0.15. Seven reference genes were needed for qRT-PCR analysis under cold stress because the $V_6/7$ value was higher than 0.15 and the $V_7/8$ value lower than 0.15.

Validation of reference genes

The expression of *DREB* genes was induced under drought stress in several plants such as *Arabidopsis* (Ding et al. 2013), rice (Oono et al. 2014), wheat (Shavrukov et al. 2016) and moso bamboo (Wu et al. 2015). Abiotic stresses also induce the expression of *FT* gene (Ryu et al. 2011, Manoharan et al. 2016), which regulates flower development and stomatal movement. Therefore, we evaluated relative expression level of *FT-5*, *FT-9*, *DREB2A* and *DREB2C* in *H. ammodendron* subjected to drought stress in order to determine the impact of different reference genes on normalization. According to NormFinder, the most stable gene (*18S*) and the least stable one (*EF-1α*) under drought stress were used as the reference gene control.

As shown in Fig. 4, when *18S* gene is used for normalization, relative expression level of *FT-5*, *FT-9*, *DREB2A* and *DREB2C* were obviously high in comparison to *EF-1α* indicating that unsuitable reference gene led to different relative expression profiles. Moreover, these results indicate the importance of validating reference genes before applying them in experiments.

Discussion

Haloxylon ammodendron, a perennial shrub in genus of *Haloxylon* Bunge (Chenopodiaceae), is distributed in northwest China and plays an important role in desert vegetation restoration. *H. ammodendron* adapts to the harsh desert environment with its ability of efficient water utilization and adversity resistance (Yu et al. 2012). By using transcriptome technology, many genes related to environmental stress response have been explored in *H. ammodendron* (Long et al. 2014). In order to understand the biological processes under environmental stresses, it is important to understand gene expression patterns. Among the several methods that help determining gene expression levels, qRT-PCR is the simplest one of high validity, accuracy and sensitivity. In addition, selection of suitable reference genes is vital to obtain reliable and accurate data for analyzing qRT-PCR, and the ideal reference genes should have relatively stable expression, irrespective of the types or physiological conditions of the sample (Crismani et al. 2006). Although, it is critical to highlight expression

Fig. 2 Expression stability of candidate reference genes in various stresses as calculated by BestKeeper, geNorm and NormFinder

stability of reference genes, there is no single reference gene that has a stable expression under all conditions (Czechowski et al. 2005, Radonić et al. 2004; Wei et al. 2013). Therefore, the suitability of reference genes have a great influence on the reliability of the results.

In this study, we cloned 8 reference genes (18S, GAPDH, ACT1, EF-1A, TUA5, ACT7, UBC18 and UBC10) in *H. ammodendron* and validated their expression stabilities. As we concluded, in *H. ammodendron* under drought stress, the most stable gene(s) were 18S and TUA5,

under heat stress, 18S, under salt stress, ACT7 and UBC18, under cold stress, ACT1, and under mechanical damage 18S, TUA5 and GAPDH (Table 3). It is worth noting that much varied expression levels were observed for 18S and ACT7, as shown by the larger whisker taps and boxes comparing with the other genes (Fig. 1), indicating expression levels of 18S and ACT7 were highly unstable across all types of stress (drought, heat, salt, cold and mechanical damage). In other words, although expression levels of 18S and ACT7 were seriously affected by some

Table 2 Expression stability ranks of 8 candidate reference genes in different sets of stressed samples calculated using BestKeeper (BK), geNorm (GN) and NormFinder (NF) methods

Gene	Drought			Heat			Salt			Cold			Mechanical damage		
	BK	GN	NF	BK	GN	NF	BK	GN	NF	BK	GN	NF	BK	GN	NF
<i>18S</i>	1	5	1	1	1	1	8	6	7	5	5	5	1	4	2
<i>ACT1</i>	2	4	7	6	7	5	7	7	8	1	1	1	5	5	4
<i>UBQ10</i>	3	2	3	7	4	3	6	2	2	7	6	6	3	8	5
<i>TUA5</i>	4	1	2	2	8	6	4	8	6	8	2	2	4	1	3
<i>GAPDH</i>	5	6	5	3	2	2	2	3	5	4	8	8	2	2	1
<i>ACT7</i>	6	8	4	5	6	4	3	1	1	3	4	3	7	3	6
<i>EF-1A</i>	7	7	8	4	3	7	5	4	3	6	3	4	6	6	7
<i>UBC18</i>	8	3	6	8	5	8	1	5	4	2	7	7	8	7	8

Table 3 Top 2 stable genes ranked by BestKeeper, geNorm and NormFinder

Experimental sample sets	BestKeeper	geNorm	NormFinder
Drought	<i>18S</i>	<i>TUA5</i>	<i>18S</i>
	<i>ACT1</i>	<i>UBQ10</i>	<i>TUA5</i>
Heat	<i>18S</i>	<i>18S</i>	<i>18S</i>
	<i>TUA5</i>	<i>GAPDH</i>	<i>GAPDH</i>
Salt	<i>UBC18</i>	<i>ACT7</i>	<i>ACT7</i>
	<i>GAPDH</i>	<i>UBQ10</i>	<i>UBQ10</i>
Cold	<i>ACT1</i>	<i>ACT1</i>	<i>ACT1</i>
	<i>UBC18</i>	<i>TUA5</i>	<i>TUA5</i>
Mechanical damage	<i>18S</i>	<i>TUA5</i>	<i>GAPDH</i>
	<i>GAPDH</i>	<i>GAPDH</i>	<i>18S</i>

Fig. 3 Determination of the optimal number of reference genes for geNorm analysis. The pairwise variation (V_n/V_{n+1}) was analyzed for the normalization factors NFn and $NFn + 1$ by geNorm program to determine ($V < 0.15$) the optimal number of reference genes. Threshold value (0.15) is displayed with a dash line

types of stress (for *18S*, salt and cold stresses; for *ACT7*, drought, heat, cold and mechanical damage stresses), *18S* was still the optimal reference gene under drought, heat and mechanical damage stresses, while *ACT7* was the optimal one in *H. ammodendron* under salt and cold stresses as concluded above. These results showed that among the 8 reference genes tested in this study, there was no ideal reference gene(s) suitable to all stress conditions, each reference gene was only applicable to one or several

Fig. 4 Expression profiles of *FT-5*, *FT-9*, *DREB2A* and *DREB2C* in *H. ammodendron* under drought stress. The best stable gene (*18S*) and the least stable reference gene (*EF-1α*) were used to normalize the expression data. Samples from the control treatment (0 h) were used as reference samples. Stressed samples were collected in 6 h after treatment. The relative expression of each gene was calculated as the mean fold change compared with the control samples in each experiment. Three independent biological samples with three technical replicates were used. At least three plants per treatment were used in each sample

stress conditions. Notably, a suitable reference gene should show a constant expression level in all samples under the same stress condition (Piron Prunier et al. 2016).

Moreover, previous research in soybean proved that *18S* was not suitable as a reference gene under stresses due to its extremely high abundance. But *18S* was one of the most suitable reference genes to study somatic embryo genesis or stimuli reaction of longan (Lin and Lai 2010; Wu et al. 2016) and papaya (Zhu et al. 2012). Under most other conditions, *18S* was either one of the most or least stable genes (Wu et al. 2016). In this study, *18S* ranked as the most stable gene under drought, heat stress or mechanical damage (Tables 2, 3), suggesting its applicability as a reference gene under these abiotic stresses in *H. ammodendron*. In addition, *18S* gene has an expression level dramatically higher than other reference genes (Fig. 1). Wu et al. (2016) concluded that *18S* was not an appropriate reference gene for normalization in qRT-PCR studies involving target genes of mid- or low-level expression. Therefore, *18S* is suitable for normalization of high-

level gene expression under drought, heat stress and mechanical damage in *H. ammodendron*.

Among all tested reference genes, *GAPDH* attracts specific attention because it ranked first or second as the most stable gene with three algorithms BestKeeper, geNorm and NormFinder (Tables 2, 3). Furthermore, *GAPDH* had less variation in expression levels than *18S*, suggesting its higher stability. Hence *GAPDH* should be an appropriate reference gene in substitution for *18S* for normalization in qRT-PCR studies involving target genes with mid-or low-level expression in *H. ammodendron* in the future. *H. ammodendron*, the main pioneer plant and constructive species in desert areas, can tolerate high desert surface layer temperature (HDSLT) (more than 95 °C) (Yu et al. 2012). However, the ability of tolerance to environmental stresses such as HSLT stress is much poor in *H. ammodendron* seedlings, suggesting HDSLT stress tolerance is acquired by heat acclimation in *H. ammodendron*, and its molecular mechanism has not been reported. *18S* and *GAPDH* will become the top two suitable reference genes for normalization in qRT-PCR concerning HSLT stress tolerance in *H. ammodendron*.

Many evidences have shown that greater accuracy of qRT-PCR experiments in plants involves the application of more than one reference gene (Vandesompele et al. 2002; Le et al. 2012). As for the geNorm program, minimal reference genes are applied to calculate the pairwise variation V_n/V_{n+1} between two sequential normalization factors, in order to determine the necessity of adding other reference genes. A large variation indicates the necessity of adding another reference gene for calculation for more accurate normalization (Vandesompele et al. 2002). According to this criterion, the calculation result of $V_{2/3}$ value is lower 0.15, which is the cut-off value subject to the subset of salt and damage stresses, suggesting that no more genes are needed to normalize gene expression in these samples (Fig. 3). However, three reference genes are suggested when performing qRT-PCR analysis using drought and heat stressed samples (Fig. 3). In another sample of the $V_{6/7}$ value under cold stress, even though the value reaches the cut-off value of 0.15, the use of seven reference genes may be reconsidered for cost effectiveness (Fig. 3).

In conclusion, in this study we evaluated 8 candidate reference genes for the normalization of gene expression in *H. ammodendron* under drought, heat, salt, cold and mechanical damage stresses. The results suggested different sets of reference genes should be applied depending on stress conditions. The expression profiles of *FT-5*, *FT-9*, *DREB2A* and *DREB2C* confirmed the importance of validating reference genes in order to obtain accurate qRT-PCR results. This study will contribute to the accuracy and reliability in transcripts quantification, which provides the significance to transcription-based studies and applications

in this important shrub *H. ammodendron* and other close relatives of the *Haloxylon*.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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