Identification of valid reference genes for quantitative RT-PCR in *Caragana microphylla* under salt and drought stresses

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Abstract *Caragana microphylla* is a leguminosae plant and grows mainly in semi-arid areas of northwest China and Mongolia. However, the lack of studies on *C. microphylla* reference genes limits the accurate understanding of the molecular biology mechanisms in this crop under abiotic stresses. In this study, we selected nine candidate genes from salt-treated *C. microphylla* transcriptome data and evaluated their stability by using geNorm, NormFinder, BestKeeper, and RefFinder in salt and drought conditions. In addition, the relative expressions of Delta 1-pyrroline-5-carboxylate synthase 2 (*P5CS2*) and Catalase 2 (*CAT2*) were examined to confirm the stability of the candidate reference genes. As a results, glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPC2*) and 26S proteasome regulatory subunit (*RPN5*) were the most stable in both salt and drought treatments. The relative expression of *P5CS2* and *CAT2* also showed more stable levels in normalization by *GAPC2* and *RPN5* than the most unstable gene, *Ubiquitin 4 (UBQ4)*. Therefore, it is believed that these candidate reference genes selected and validated in our study could be used to study the molecular biological study of response to salt and drought stress in *C. microphylla*.

Keywords Reference genes · Gene expression · *Caragana microphylla* · qRT-PCR · Salt stress · Drought stress

Introduction

The genus *Caragana* has more than 100 species and belongs to the family of Leguminosae. The genus *Caragana* mainly grows in arid and semi-arid areas and is known to comprise ecologically important plants because they reduce wind erosion and enhance water conservation (Wang et al. 2006; Zhang et al. 2006). Some studies investigated the mechanism of response to abiotic stress using biochemical and molecular biological experiments in *Caragana* because the genus *Caragana* have been known to resist salt and drought (Zhang et al. 2006; Wang et al. 2011). Moreover, in order to explore the extensive response genes depending on abiotic stresses, Yang et al. (2014) selected the reference genes for reverse transcription polymerase chain reaction (RT-PCR) and Li et al. (2016) performed transcriptome analysis by using salt—and drought—treated *Caragana korshinskii*.

Quantitative real-time PCR (qRT-PCR), which is a highly sensitive, accurate, reproducible and rapid execution method, is usually used for gene expression analysis under different organs or tissues and environment conditions (Czechowski et al. 2005; Nolan et al. 2006). Therefore, selecting reference genes that are stably expressed under different conditions for the normalization of gene expression is necessary for accurate qRT-PCR analysis (Huggett et al. 2005; Hruz et al. 2011). The reference genes under various tissues and environment conditions have been reported for many plants (Czechowski et al. 2005; Galli et al. 2015; Hossain et al. 2019). Reference genes of *Caragana intermedia* and *Caragana korshinskii*, other

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species belonging to the genus *Caragana*, were reported (Zhu et al. 2013; Yang et al. 2014), but no study has selected candidate reference genes in *C. microphylla* under various abiotic stresses by qRT-PCR. The identification of reference genes is critical for future molecular biological experiments of *C. microphylla*, which is resistant to drought and is an important ecological and industrial resource.

In this study, we validated the expression stability of the following nine candidate reference genes in *C. microphylla* under salt and drought treatments. For the accuracy of sensitive qPCR analysis, normalization by stable reference gene is very important. Therefore, the stability of the nine genes including *UBQ4*, *GAPC2* (GAPDH isoform), eukaryotic initiation factor 4 (*EIF4*), ADP-ribosylation factor (*ARF*), cytosolic NADP⁺-dependent isocitrate dehydrogenase (*CDH*), heat shock protein 70 family protein (*BIP*), *RPN5*, phosphoglycerate kinase family protein (*PGK*), and actin depolymerizing factor 3 (*ADF3*) were assessed to use these genes in molecular biological studies of salt- and drought-treated *C. microphylla*. Consequently, the most stable reference genes were identified for the normalization of qRT-PCR data in *C. microphylla* under salt and drought stresses. The results of this study provide optimal reference genes in salt- and drought-treated *C. microphylla* and can be used for molecular biological studies related to the plant's response to abiotic stress.

Materials and methods

Plant materials and treatments

C. microphylla were grown under controlled conditions (22 °C/21 °C and 50%/50% humidity on a 16 h light/8 h dark cycle) in a growth chamber. For salinity treatment, the one-month-old seedlings were dipped in 50, 300, and 500 mM NaCl for 1 h. For drought treatment, the one-month-old seedlings were not watered for 2, 6, and 12 days. The samples were represented by three biological replicates under each treatment for validating the reference genes.

RNA isolation and cDNA synthesis

Total RNA was extracted from whole seedlings of *C. microphylla* treated with each condition by using a TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Japan). The OD260/280 ratio was determined using a DS-11 spectrophotometer (DeNovix, USA) and was used for assessing the RNA quality and purity. The integrity of the purified RNA was checked by 1.5% agarose gel

electrophoresis. cDNA product for qRT-PCR was synthesized by PrimeScriptTM 1st strand cDNA Synthesis Kit (Takara, Japan) using approximately 1 µg total RNA. The cDNAs were diluted 1:15 with RNase Free water prior to the qRT-PCR analyses.

Selection of the candidate reference genes and designed primers for qRT-PCR

Nine candidate reference genes and two target genes were selected from the *C. microphylla* transcriptome data PRJNA628566 submitted in NCBI. Primers were designed based on the sequences of the 11 genes using Primer3plus (<https://primer3plus.com/cgi-bin/dev/primer3plus.cgi>) with the following criteria: GC content 45–65%, optimal T_m 57–63 °C, primer length 18–22 bp, and amplicon length 70–240 bp. The melting curves and amplification efficiency from the slope of the standard curve during qRT-PCR and amplification size of electrophoresis were used to validate the specificity of the selected primer set. The amplification efficiency of the primer set was generated using the slope of the standard curve according to the equation $E = [5^{-1/\text{slope}} - 1] \times 100$ (Arezi et al. 2003).

qRT-PCR conditions and statistical analyses for the expression of candidate reference genes

qRT-PCR reactions were performed by a modified protocol based on Luna Universal qPCR master mix (New England Biolabs, USA) manufacturer's protocol and Magnetic Induction Cycler qRT-PCR system (Bio molecular systems, Australia). Amplification was performed as follows: 95 °C for 60 s followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The melting curves were analyzed at 60–95 °C after 40 cycles. All reactions were performed in biological triplicate and technical triplicate. The cycle threshold (C_t) was calculated automatically.

The stability of the candidate reference genes was analyzed by using three Microsoft excel-based algorithm programs: geNorm (ver 3.0) (Vandesompele et al. 2002), NormFinder (ver 0.953) (Andersen et al. 2004), BestKeeper (ver 1.0) (Pfaffl et al. 2004) and RefFinder (<https://www.heartcure.com.au/reffinder/?type=reference>) (Xie et al. 2012), which is a web-based program to compare and rank the candidate reference genes. The analyses were performed by following each program protocols.

Table 1 List of primer sequences and related information for 11 genes from transcriptome data of *Caragana microphylla*

Unigene	Accession number	Annotated gene name	Gene description	Primer sequence (Fw)	Primer sequence (Rv)	TM (°C)	Product size (bp)	Efficiency (%)
Caragana1SL000231t003	MN894839	PGK	Phosphoglycerate kinase family protein	GAGAACTGACACCGAGGG	GGCCGTGGAGAAAAGTAGGAG	84.82	187	109.5
Caragana1SL012204t009	MN894840	UBQ4	ubiquitin 4	ACTCGCCCTCTATCTTCCGT	AACCCAGTTGCCAACCTCTT	79.82	76	90.8
Caragana1SL031144t001	MN894841	ARF	ADP-ribosylation factor	CCAAACGAACACGAGGAGGA	GGCAGCATCAAGACCTACCA	82.03	189	89.6
Caragana1SL014059t001	MN894842	EIF4	eukaryotic initiation factor 4	TCTCCACACTTTTGCCTCC	CGTTTGCCAGGTCGTTGAAA	84.09	159	84.9
Caragana1SL008803t001	MN894843	CDH	cytosolic NADP + - dependent isocitrate dehydrogenase	CCACGAAGGGCAAAATGAGC	AAAATCAACGTCGCCAACCC	82.68	100	101.8
Caragana1SL000839t002	MN894844	BIP	luminal binding protein	TTGAGCGGATGGTTCGTGAA	TTGTGGCCAGCTTGTCTTT	82.38	142	96.6
Caragana1SL000083t005	MN894845	GAPC2	glyceraldehyde-3-phosphate dehydrogenase C2	TTGTTGAGGGCCTGATGACC	AGCTCTTCCACCTCTCCAGT	83.54	95	91.9
Caragana1SL012499t001	MN894846	RPN5	regulatory particle non-ATPase subunit 5A	GGCACCATGATCCAATGC	TTCTGCTGCCTTTTTCACCCA	81.66	205	93.2
Caragana1SL017867t001	MN894847	ADF3	actin depolymerizing factor 3	TGCCTTCCTGCTGATGAGTG	GGCACGGCTTTTGAACACAT	84.21	240	87.4
Caragana1SL003865t006	MN894848	P5CS2	delta 1-pyruvate-5-carboxylate synthase 2	CGGGCTTCCTTTTCCTCCTTT	GTCCTTTGGGTGTCCTCCTG	82.78	118	96.7
Caragana1SL012836t004	MT495796	CAT2	catalase 2	GCATGGACAACACGTTCTGG	AGCGCCTTCAAACTCTCCTTT	83.30	170	112.5

Results and discussions

Identification of candidate genes for normalization of gene expression

To select the stable candidate reference genes of *C. microphylla* under salt and drought conditions, nine genes were filtered from transcriptome data PRJNA628566 submitted in NCBI, which measured response to salt condition in *C. microphylla* (Table 1). These genes indicated no significant changes ($-0.1 < \log_2 \text{fold change} < 0.1$) in salt condition. Subsequently, we designed the primers for the nine selected genes and two target genes, and verified the primer specificity and amplification efficiency. Electrophoresis on a 1.5% agarose gel using each primer set and melting curves confirmed amplicon sizes and the specificity of qRT-PCR products (Fig. 1 and Supplemental Fig. 1). The values of amplification efficiency ranged from 84.9% (*EIF4*) to 112.5% (*CAT2*) (Table 1).

Fig. 1 Amplicon band size and cycle threshold (Ct) values of candidate reference genes. **a** Amplicon band size via 1.5% agarose gel electrophoresis. Numbers indicate amplicon size of each genes. **b** Mean values of Ct in the candidate reference genes under all experiments. The box graph indicates the interquartile range. The line across the box depicts the median

Quantitative assessment for stable expression of the candidate reference genes

To analyze the expression levels of the candidate reference genes under salt (50, 300, and 500 mM) and drought (2, 6, and 12 days) treatments, the raw Ct values of all samples were obtained by qRT-PCR (Fig. 1). The mean Ct values of the candidate genes ranged from 23.46 (*PGK*) to 28.81 (*EIF4*).

In addition, their stability was analyzed by using geNorm, NormFinder, BestKeeper, and RefFinder (Vandesompele et al. 2002; Andersen et al. 2004; Pfaffl et al. 2004; Xie et al. 2012). In geNorm analysis, *RPN5* and *ADF3* were the most stable reference genes in total treatment (Supplemental Fig. 2). On the other hand, *UBQ4* was the least stable reference gene. In both salt and drought treatments, *UBQ4* was the least stable reference gene. In NormFinder analysis, *GAPC2* and *RPN5* were the most stable reference genes and *UBQ4* was the least stable reference gene in total treatment (Supplemental Table 1). geNorm and NormFinder analyses showed relatively consistent results. In BestKeeper analysis, *EIF4* and *CDH* had the lowest CV $\pm$ SD values in total treatment (Supplemental Table 2). But the SD value was more than 1, and there was no stable reference gene in all treatments except *UBQ4* in drought treatment. BestKeeper generated different results from the other two analyses. There have been many studies which have found inconsistent results from these analyzes for the stability of reference genes (Zhang et al. 2016; Coelho et al. 2019). Although they sometimes generate different results, a more sophisticated evaluation can be performed on the selection of reference genes by using various analyses based on different algorithms.

To obtain reliable results for the expression stabilities of the nine candidate reference genes, the rankings of the four methods were integrated by using the web-based program RefFinder (<https://www.heartcure.com.au/reffinder/?type=reference>) (Xie et al. 2012) (Table 2). In the recommended comprehensive ranking of all treatments, *GAPC2* and *RPN5* were the most suitable reference genes. *UBQ4* was the least stable reference gene in all treatments.

Validation of the candidate reference genes under salt and drought treatments

To validate the candidate reference genes of *C. microphylla* under salt and drought treatments, the expression level of antioxidant gene *P5CS2* and *CAT2* were analyzed for normalization (Fig. 2). *P5CS2* and *CAT2* were known to be up-regulated in other plants under salt and drought treatments (Chen et al. 2013; Zhu et al. 2014). In the present study, we used the most stable reference genes (*RPN5*, *GAPC2*) and the least stable reference gene

Table 2 Expression stability ranking of the nine candidate reference genes using RefFinder

Method	1	2	3	4	5	6	7	8	9
a. Ranking Order under all treatments (Better–Good–Average)									
Delta CT	GAPC2	RPN5	ADF3	BIP	PGK	ARF	CDH	EIF4	UBQ4
BestKeeper	EIF4	CDH	UBQ4	GAPC2	BIP	RPN5	ARF	PGK	ADF3
NormFinder	GAPC2	RPN5	BIP	ADF3	PGK	ARF	CDH	EIF4	UBQ4
GeNorm	RPN5 ADF3		GAPC2	PGK	BIP	ARF	EIF4	CDH	UBQ4
Recommended comprehensive ranking	GAPC2	RPN5	ADF3	BIP	EIF4	CDH	PGK	ARF	UBQ4
b. Ranking Order under salt treatment (Better–Good–Average)									
Delta CT	RPN5	GAPC2	BIP	PGK	ADF3	ARF	CDH	EIF4	UBQ4
BestKeeper	EIF4	CDH	ARF	RPN5	GAPC2	PGK	BIP	ADF3	UBQ4
NormFinder	RPN5	GAPC2	BIP	CDH	PGK	ADF3	ARF	EIF4	UBQ4
GeNorm	GAPC2 RPN5		ADF3	PGK	ARF	BIP	CDH	EIF4	UBQ4
Recommended comprehensive ranking	RPN5	GAPC2	BIP	CDH	PGK	EIF4	ARF	ADF3	UBQ4
c. Ranking Order under drought treatment (Better–Good–Average)									
Delta CT	ADF3	GAPC2	EIF4	RPN5	BIP	PGK	ARF	CDH	UBQ4
BestKeeper	UBQ4	BIP	EIF4	ADF3	GAPC2	CDH	RPN5	ARF	PGK
NormFinder	ADF3	GAPC2	EIF4	BIP	RPN5	PGK	ARF	CDH	UBQ4
GeNorm	GAPC2 ADF3		RPN5	EIF4	PGK	BIP	ARF	CDH	UBQ4
Recommended comprehensive ranking	ADF3	GAPC2	EIF4	BIP	RPN5	UBQ4	PGK	ARF	CDH

Fig. 2 Relative quantification of *P5CS2* and *CAT2* expression by the candidate reference genes. **a** and **b** indicate salt stress (50, 300, and 500 mM) and drought stress (2, 6, and 12 days) treatment, relatively. Error bars indicate the standard error of the mean

(*UBQ4*) for target gene normalization based on the analysis results. Under salt and drought treatments, the expression

level of *P5CS2* and *CAT2* normalized by the candidate reference genes (*RPN5*, *GAPC2*) showed the similar

expression pattern. In contrast, the least stable reference gene (*UBQ4*) showed a different expression from that of *RPN5* and *GAPC2*. This result demonstrates the validity of the previously performed analyses such as geNorm, NormFinder, and RefFinder.

Conclusions

We selected nine candidate reference genes in *C. microphylla* and validated the stability of them under salt or drought condition. Among them, the *GAPC2* and *RPN5* were stable genes under abiotic stresses in the results of programs and algorithm analyses. They also showed stability when normalizing abiotic stress responsive genes, *P5CS2* and *CAT2*. Therefore, the *GAPC2* and *RPN5* can be used as reference genes under salt and drought conditions in *C. microphylla*.

Authors' contributions All authors contributed to the study conception and design. SYW, and SHK conceived the original research plan; JUN and HN performed the experiments; SJK, JSK, and JEL supervised the experiments; SJK and JUN wrote the article; SJK, JUN, KAL, SYW, and SHK revised the manuscript. All authors have reviewed and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest We declare that we have no conflict of interest.

Availability of data and material We submitted the sequences and related RNA-seq data in NCBI (MN894839, MN894848, MT495796, and PRJNA628566).

Code availability Not applicable.

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