Reference gene selection for qRT-PCR in *Caragana korshinskii* Kom. under different stress conditions

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Abstract *Caragana korshinskii* Kom., which is widely distributed in the northwest China and Mongolia, is an important forage bush belonging to the legume family with high economic and ecological value. Strong tolerance ability to various stresses makes *C. korshinskii* Kom. a valuable species for plant stress research. In this study, suitable reference genes for quantitative real-time reverse transcription PCR (qRT-PCR) were screened from 11 candidate reference genes, including *ACT*, *GAPDH*, *EF1α*, *UBQ*, *TUA*, *CAP*, *TUB*, *TUB3*, *SKIP1*, *SKIP5-1* and *SKIP5-2*. A total of 129 samples under drought, heat, cold, salt, ABA and high pH treatment were profiled, and software such as geNORM, NormFinder and BestKeeper were used for reference gene evaluation and selection. Different suitable reference genes were selected under different stresses. Across all 129 samples, *GAPDH*, *EF1α* and *SKIP5-1* were found to be the most stable reference genes, and *EF1α+SKIP5-1* is the most stable reference gene combination. Conversely, *TUA*, *TUB* and *SKIP1* were not suitable for using as reference genes owing to their great expression variation under some stress conditions. The relative expression levels of *CkWRKY1* were detected using the stable and unstable reference genes and their applicability was confirmed. These results provide some stable reference genes and reference gene combinations for qRT-PCR under different stresses in *C. korshinskii* Kom. for future research work, and indicate that CkWRKY1 plays essential roles in response to stresses in *C. korshinskii*.

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Abbreviations

qRT-PCR	Reverse transcription quantitative real-time PCR
ACT	Actin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
EF1α	Elongation factor 1-alpha
UBQ	Ubiquitin
TUA	Alpha-tubulin
CAP	Adenylyl cyclase-associated protein
TUB	Beta-tubulin
TUB3	Tubulin beta-3
SKIP1	SKP1/ASK-interacting protein 1
SKIP5-1/ SKIP5-2	SKP1/ASK-interacting protein 5

Introduction

Caragana korshinskii Kom. is a cultivated leguminous shrub belonging to the legume. It widely distributed in the arid and semi-arid regions of northwest China and Mongolia [39, 40]. It is an important forage grass, industrial feedstock and reforestation species with multiple economic and ecological value [12, 15]. As a very strong stress resistant species, it was reported to tolerate drought, cold, saline and other abiotic stresses [36, 37, 40]. In recent years, several stress-related genes were studied in *C. korshinskii* Kom. [32–34]. In order to better understand the stress tolerance mechanisms of this species, intensive study of the expression patterns of many stress-related genes were urgently required.

Gene expression analyses can help us to thoroughly investigate the complex biological processes, increasing our understanding of signaling and metabolic pathways that underlie environmental responses and development [11]. In the past, Northern blotting and semi-quantitative reverse transcription-PCR were widely used in gene expression studies, but reverse transcription quantitative real-time PCR (qRT-PCR) with high sensitivity and specificity is being widely accepted more and more recently [5, 29]. The stable expression of reference genes used in qRT-PCR significantly impacts the accuracy of data analysis, but the stable ideal reference gene expressed in all tissues or organs, at different developmental stages, under various physiological states and stress conditions is not available. Therefore, screening for suitable reference gene is critical for normalization of the gene expression data [7, 14, 22]. Many studies on reference gene selection in plants under different condition have been reported, such as Arabidopsis [24], tobacco [26], cotton [2], soybean [35], banana [4], poplar [38], rice [16], wheat [28] and potato [3], etc. To our knowledge, there is no any report about systematic evaluation of reference genes in *C. korshinskii* Kom. for qRT-PCR so far. *Caragana intermedia*, another species belonging to the Caragana genus, was reported in 2013, but the candidate reference genes they selected were completely different from those used in our study [43].

A total of 11 candidate reference genes were chosen in our research, including 1 each encoding either the actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor 1-alpha (*EF1α*), ubiquitin (*UBQ*), alpha-tubulin (*TUA*), cyclase associated protein gene (*CAP*), or 2 genes encoding the beta-tubulins (*TUB* and *TUB3*) and 3 genes encoding the F-box protein (*SKIP1*, *SKIP5-1*, and *SKIP5-2*). The expression stability of these reference genes under different stresses were analysed by softwares geNORM, NormFinder and BestKeeper. After comprehensive analysis, the most stable reference gene for normalization of gene expression under different treatments, such as drought, heat, cold, salt, ABA and high pH were identified. Finally, the expression analysis of *CkWRKY1*, a gene encoding a member of WRKY transcription factor family, was detected to confirm the reliability of the selected reference genes.

Materials and methods

Plant material and different treatments

The seeds of *C. korshinskii* Kom. were collected from Chifeng County, Inner Mongolia, China. The seeds were sewn in pots containing a soil mixture (rich soil: vermiculite, 1:1, v/v) and grown at conditions with 22 °C, 16-h light/8-h dark. One-month-old seedlings were used in the following experiments.

For cold and heat treatments, seedlings were placed at 4 or 45 °C respectively for 0.5, 1, 3, 6, 12, 24 and 48 h. For drought, salinity, high pH and ABA treatments, the seedlings were removed from soil slightly, then washed clean with tap water. In drought treatment, the seedlings were placed on filter paper in the initial conditions for 0.5, 1, 3, 6, 12, 24 and 48 h. For salinity, high pH and hormonal treatments, the seedlings were dipped in solution of 200 mM NaCl, pH 10 solution (adjusted by NaOH) and 100 μM ABA for 0.5, 1, 3, 6, 12, 24 and 48 h respectively. The seedlings without any treated were used as control. The shoots of all samples was collected and frozen immediately in liquid nitrogen for RNA extraction. Each of the samples contained three seedlings respectively and every treatment was repeated three times independently unless otherwise stated.

Totally 129 samples were used in our study, including 126 samples treated by drought, heat, cold, salinity, high pH and ABA respectively and 3 samples as controls.

### RNA isolation and cDNA synthesis

Total RNA was extracted from all samples with Trizol Regent (Invitrogen, USA), and then treated with RNase-free DNase (Ambion). The purity and integrity of RNA extracted were detected by $A_{260/280}$ ratios and electrophoresis on 1 % agarose gel. One microgram of total RNA was reverse transcribed into cDNA with the M-MLV reverse transcriptase kit using oligo (dT) 18 primers (TaKaRa) according to the manufacturer's instructions. The cDNA acquired by reverse transcription was diluted to 16 folds for qRT-PCR.

Primer design and qRT-PCR

The sequences of the candidate reference genes were obtained from the GeneBank, which were submitted by our

Table 1 Details of primer and amplicon lengths for the reference genes tested in this study

Gene name	GenBank number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon length (bp)	Efficiency
ACT1	FJ485727	TGACAGGATGAGCAAGGAGA	GTTGGAAGGTGCTGAGGGA	131	1.988 ± 0.043
GAPDH	KC679843	GCATTCCGTGTACCTACTCCCA	CAACATCCTCGTCGTGTATCCA	151	1.935 ± 0.018
TUA	KC679847	ATTCTAAGAGAGGGCATTTGTCCAC	TTCATCATCACCAACACCATCAC	143	1.937 ± 0.044
TUB	KC679849	TTACAATGAGGCTTCTGGTGGA	AGACGAAGTTATCAGGCGCGAA	125	1.942 ± 0.029
UBQ	KC679848	AAAAAAGGGTACAAGAGTTGGACAAGG	TAACCATAACAGTCGGGGGAAC	136	1.934 ± 0.017
TUB3	KC679850	TCAGGTGTGTCATTCTTTTGGGTG	GGTGAACAGAGAGAGTGGCATTG	173	1.986 ± 0.044
SKIP5-1	KC679845	GCTTCCTTCCCCCTATTCCAG	AAAACACCAGGTTCAGATAAGTCTTTG	133	1.942 ± 0.030
SKIP5-2	KC679846	GTATTGAAGGAGGTGCGAAAGC	AAACAACAAATGATAATAACACATAGGC	166	1.935 ± 0.019
SKIP1	KC679844	GGGTGTCCTAACCTTGAGTTCTTG	CATATAGTCTCCAATGTCCATACCTTTC	172	1.937 ± 0.030
CAP	KC679841	GGGAAGGATTGCGGTATGAGTA	TCGACATGATTTGGGTCTTTGTT	119	1.990 ± 0.054
EF1α	KC679842	CAAAAAGTCCCCTCGTTGTCTC	AGCAATCGTTCTTCCTAATGATCTAA	142	1.971 ± 0.024
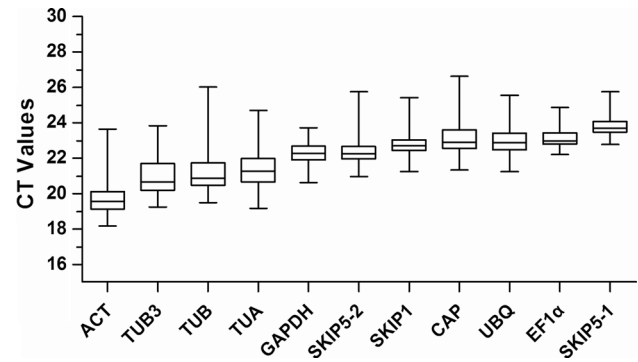
Fig. 1 The range of CT values of 11 reference genes in *C. korshinskii* Kom. across all 129 samples. The *box* represents the 25th and 75th percentiles of data. *Whiskers* represent the maximum and minimum values. *Horizontal line within the box* represents the median CT value

lab previously. According to these sequences, Primer Premier 5 was used to design the primers for qRT-PCR. The primers followed the rules which describe by Udvardi et al. [29].

Furthermore, the efficiency was calculated based on the slope of the line ($E = 10^{slope}$), considering an ideal value range ($1.8 \geq E \leq 2$), this experiment was repeated three times [20].

The cDNA was amplified using SYBR Premix ExTaq (TaKaRa), with a Roche LightCycler 480 Real Time PCR system. The thermal cycling program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 15 s. The melting curves were analyzed at 60–95 °C after 40 cycles. Each RT-qPCR analysis was performed in triplicate.

Stability analysis of reference genes

Stability analysis of reference genes were performed by geNORM [31], NormFinder [1] and BestKeeper [21], which are the most widely used reference gene screening software for qRT-PCR. The CT values were calculated by LightCycler 480 software and the data were imported into geNorm v3.5, NormFinder and BestKeeper analysis tools, which were used as described in their manuals. Considering the three analytic results obtained by geNorm, NormFinder and BestKeeper together, the most stable reference genes under different stresses for qRT-PCR were selected from *C. korshinkii* Kom..

Expression analysis of *CkWRKY1* under different stresses

A WRKY transcription factor encoding gene, *CkWRKY1* (GeneBank number JX987095) was previously cloned from *C. korshinkii* Kom. in our lab. In order to confirm the
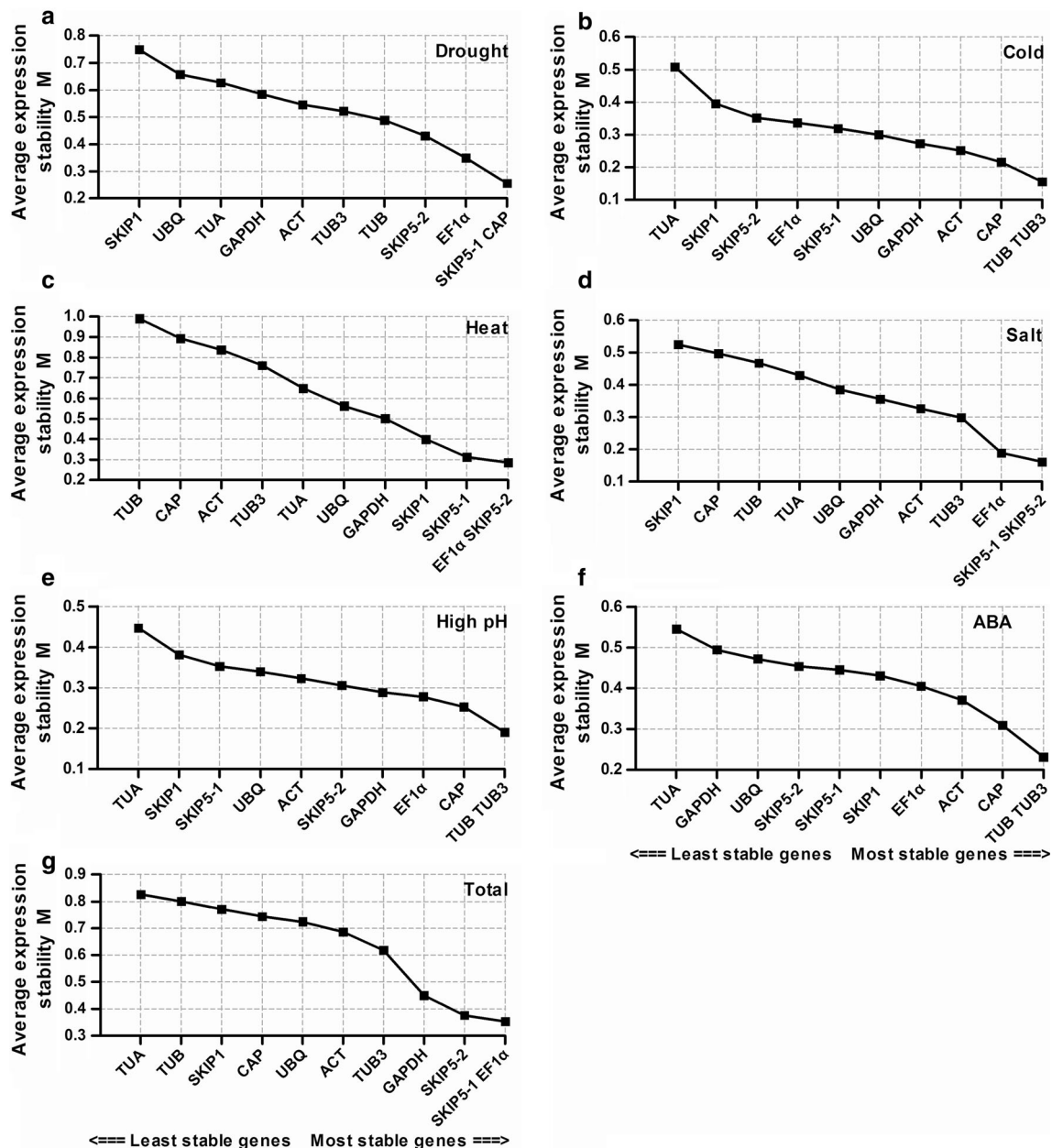
Fig. 2 The average expression stability values (*M*) of the candidate reference genes analyzed by the geNorm in *C. korshinskii* Kom. Samples under different treatments. **a** Drought treatment, **b** cold treatment, **c** heat treatment, **d** salt treatment, **e** high pH treatment, **f** ABA treatment, **g** total

usefulness of the validated candidate reference genes in qRT-PCR, the transcript level of *CkWRKY1* was quantified under different treatments using the most stable reference gene and the most unstable gene we selected. The $2^{\Delta\Delta CT}$ method [18, 27] was used to evaluate the relative expression level of *CkWRKY1* in all samples. The following primers were used in qRT-PCR analysis:

F-CkWRKY1-rt: TACAATACAACATACCAAATAG GGGG
R-CkWRKY1-rt: CCTATAGCATGGAAGACTGAGC TGAG

Results

PCR specificity and efficiency analysis of the candidate reference genes

The specificity of the amplification was confirmed by agarose gel electrophoresis (Supplementary Fig. 1, Online Resource) and melting curve analysis (Supplementary Fig. 2, Online Resource).

The GenBank numbers of the 11 candidate reference genes, the primer sequences and the amplicon sequences l
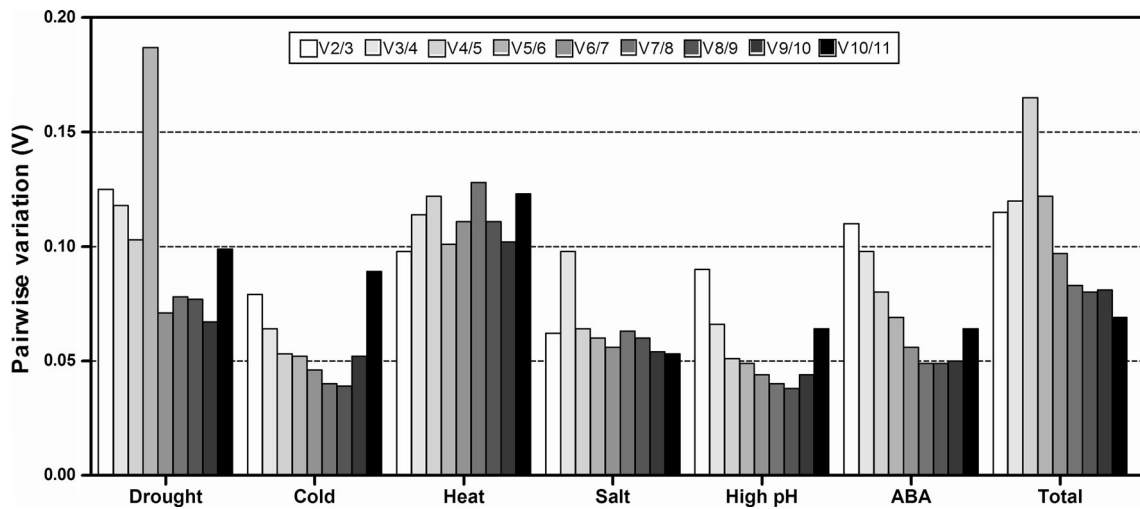
Fig. 3 Pairwise variation (V) analysis of the candidate reference genes. The pairwise variation (Vn/Vn + 1) was analyzed between the normalization factors Vn and Vn + 1 by the geNorm software to determine the optimal number of reference genes required for qRT-PCR data normalization

Table 2 Ranking of the candidate reference genes according to their stability estimated by NormFinder

Rank	Drought	Cold	Heat	Salt	High pH	ABA	Total
1	*CAP* (0.198)	*SKIP5-1* (0.186)	*UBQ* (0.245)	*EF1α* (0.15)	*EF1α* (0.099)	*EF1α* (0.174)	*EF1α* (0.45)
2	*SKIP5-1* (0.211)	*CAP* (0.196)	*SKIP1* (0.567)	*SKIP5-1* (0.198)	*TUB* (0.174)	*ACT* (0.297)	*GAPDH* (0.498)
3	*EF1α* (0.218)	*SKIP5-2* (0.224)	*GAPDH* (0.607)	*SKIP5-2* (0.214)	*CAP* (0.21)	*SKIP1* (0.308)	*ACT* (0.522)
4	*GAPDH* (0.416)	*EF1α* (0.237)	*TUB3* (0.646)	*ACT* (0.275)	*TUB3* (0.236)	*TUB3* (0.309)	*SKIP5-1* (0.535)
5	*SKIP5-2* (0.471)	*ACT* (0.257)	*SKIP5-1* (0.682)	*TUB3* (0.282)	*GAPDH* (0.242)	*SKIP5-2* (0.361)	*UBQ* (0.549)
6	*TUA* (0.539)	*GAPDH* (0.27)	*TUA* (0.694)	*GAPDH* (0.342)	*SKIP5-2* (0.253)	*TUB* (0.365)	*TUB3* (0.552)
7	*ACT* (0.582)	*TUB* (0.288)	*ACT* (0.718)	*UBQ* (0.39)	*SKIP5-1* (0.321)	*CAP* (0.384)	*CAP* (0.574)
8	*UBQ* (0.59)	*UBQ* (0.299)	*EF1α* (0.741)	*TUA* (0.475)	*ACT* (0.325)	*SKIP5-1* (0.406)	*SKIP5-2* (0.628)
9	*TUB* (0.598)	*TUB3* (0.3)	*SKIP5-2* (0.801)	*TUB* (0.523)	*UBQ* (0.335)	*UBQ* (0.408)	*SKIP1* (0.732)
10	*TUB3* (0.703)	*SKIP1* (0.522)	*CAP* (0.863)	*CAP* (0.559)	*SKIP1* (0.426)	*GAPDH* (0.484)	*TUA* (0.736)
11	*SKIP1* (1.079)	*TUA* (0.979)	*TUB* (1.336)	*SKIP1* (0.57)	*TUA* (0.694)	*TUA* (0.696)	*TUB* (0.778)

The numbers in parentheses are stable values

Table 3 Ranking of the candidate reference genes according to their stability calculated by BestKepper

Rank	Drought	Cold	Heat	Salt	High pH	ABA	Total
1	*GAPDH*	*SKIP5-2*	*GAPDH*	*SKIP5-2*	*EF1α*	*EF1α*	*SKIP5-1*
2	*TUA*	*EF1α*	*SKIP5-1*	*EF1α*	*SKIP5-1*	*ACT*	*EF1α*
3	*SKIP5-1*	*SKIP5-1*	*SKIP1*	*SKIP5-1*	*SKIP5-2*	*SKIP1*	*GAPDH*
4	*CAP*	*SKIP1*	*EF1α*	*GAPDH*	*SKIP1*	*SKIP5-2*	*SKIP5-2*
5	*UBQ*	*UBQ*	*UBQ*	*UBQ*	*ACT*	*GAPDH*	*SKIP1*
6	*EF1α*	*ACT*	*SKIP5-2*	*ACT*	*GAPDH*	*UBQ*	*UBQ*
7	*SKIP1*	*CAP*	*TUA*	*TUB3*	*TUB*	*SKIP5-1*	*ACT*
8	*SKIP5-2*	*GAPDH*	*TUB3*	*TUA*	*CAP*	*TUB3*	*CAP*
9	*ACT*	*TUB3*	*ACT*	*SKIP1*	*UBQ*	*CAP*	*TUA*
10	*TUB*	*TUB*	*CAP*	*TUB*	*TUB3*	*TUB*	*TUB3*
11	*TUB3*	*TUA*	*TUB*	*CAP*	*TUA*	*TUA*	*TUB*

Table 4 Expression stability of the reference genes estimated by geNorm, NormFinder and BestKepper

Different treatment	The most stable reference genes estimated by geNorm, NormFinder and BestKepper	Most stable combination of two genes estimated by geNorm	The least stable gene
Drought	*SKIP5-1*[a,b,c], *CAP*[a,b,c], *KIP5-2*[a,b], *GAPDH*[b,c], *EF1α*[a,b]	*SKIP5-1, CAP*	*SKIP1*[a,b], *TUB*[c]
Heat	*GAPDH*[a,b,c], *SKIP1*[a,b,c], *SKIP5-1*[a,b,c], *UBQ*[b,c], *EF1α*[a,c]	*SKIP5-2, EF1α*	*TUB*[a,b,c]
Cold	*CAP*[a,b], *ACT*[a,b], *SKIP5-1*[b,c], *SKIP5-2*[b,c]	*TUB, TUB3*	*TUA*[a,b,c]
NaCl	*EF1α*[a,b,c], *SKIP5-1*[a,b,c], *SKIP5-2*[a,b,c], *TUB3*[a,b]	*SKIP5-1, SKIP5-2*	*CAP*[c], *SKIP1*[a,b],
High pH	*EF1α*[a,b,c], *CAP*[a,b], *TUB*[a,b], TUB3[a,b]	*TUB, TUB3*	*TUA*[a,b,c]
ABA	*EF1α*[a,b,c], *ACT*[a,b,c], TUB3[a,b], SKIP1[b,c], *SKIP5-2*[b,c]	*TUB, TUB3*	*TUA*[a,b,c]
Total	*GAPDH*[a,b,c], *EF1α*[a,b,c], *SKIP5-1*[a,b,c], *SKIP5-2*[a,c]	*EF1α, SKIP5-1*	*TUA*[a], *TUB*[b,c]

[a,b] or [c] indicated that stability ranking of the reference gene was estimated by geNorm, NormFinder or BestKepper respectively

are listed in Table 1 and Supplementary Table 1 (Online Resource). As can be seen from Table 1, amplicons length vary from 119 bp (*CAP*) to 173 bp (*TUB3*). The efficiency for the 11 reference genes varied from 1.934 ± 0.017 for *UBQ* to 1.990 ± 0.054 for *CAP*.

Expression profile of the reference genes

The expression of the reference genes was detected by qRT-PCR in 129 samples. The expression levels of 11 reference genes were analysed across all samples with CT values (Fig. 1). The CT values of the reference genes ranged from 18 to 27 in all studied samples, most of them lying between 19 and 26. Among all the candidate genes, *ACT* showed the highest expression level (average CT of 19.8), whereas *SKIP5-1* showed the lowest expression level (the average CT of 23.8). And *EF1α* (2.71 cycles), *GAPDH* (3.12 cycles) and *SKIP5-1* (3.09 cycles) displayed the least gene expression variations in all samples, suggesting they might be the suitable reference genes for qRT-PCR. In contrast, *TUB* (6.57 cycles), *CAP* (5.53 cycles) and *TUA* (5.67 cycles) were the most variable ones among the reference genes examined.

Expression stability analysis of the reference genes

Three different algorithms, including geNorm, NormFinder and BestKeeper were used to evaluate the stability of the 11 candidate reference genes.

When analyzed by geNorm, the stability of reference gene was evaluated by the *M* value [31]. The more stable reference genes have the lower *M* value, conversely, the more unstable the reference gene, the higher the *M* value. The results obtained with geNorm algorithm were presented in Fig. 2. Under drought stress, *SKIP5-1* and *CAP* were the most stable genes with the least *M* value of 0.256, while *SKIP1* was the least stable gene with the high *M* value of 0.748. Under cold, high pH and ABA treatment, *TUB* and *TUB3* were the most stable genes with the *M* values of 0.155, 0.190 and 0.231 respectively. *TUA* was

the least stable gene with the *M* values of 0.508, 0.447 and 0.546 respectively. Under heat treatment, the *M* values of *SKIP5-2* and *EF1α* (0.286) were the lowest, and the *M* value of *TUB* (0.990) was the highest. Under salt treatment, the *M* values of *SKIP5-2* and *SKIP5-1* (0.161) were the lowest, and the *M* value of *SKIP1* (0.525) was the highest. When all samples were analyzed together, *EF1α* and *SKIP5-1* were the most stable genes with an *M* value of 0.353. In contrast, *TUA* was the least stable gene with an *M* value of 0.826.

The V value was used to determine the optimal number of reference genes required in the analysis by geNorm [31]. A threshold V value of 0.15 was adopted to determine whether the inclusion of an additional reference gene was necessary [17]. For example, if V2/3 was lower than 0.15, this indicated that the combination of the two most stable reference genes was sufficient for target gene normalization under most experimental conditions. On the contrary, if V2/3 was higher than 0.15, the combination of the two most stable reference genes was not enough for target gene normalization and the third reference gene must be added into. In this study, all of V2/3 is less than 0.15 (Fig. 3). According to geNorm, the best combinations among the 11 reference genes were *SKIP5-1* and *CAP* under drought stress (V2/3 = 0.125), *TUB* and *TUB3* under cold (V2/3 = 0.079), high pH (V2/3 = 0.090) and ABA treatment (V2/3 = 0.110), *SKIP5-2* and *EF1α* under heat treatment (V2/3 = 0.098), *SKIP5-2* and *SKIP5-1* under salt treatment (V2/3 = 0.062). In addition, *EF1α* and *SKIP5-1* was the best combination of reference genes to normalize gene expression data in all samples (V2/3 = 0.115).

The results evaluated by NormFinder were summarized in Table 2. The reference gene has a lower stability values indicating that the gene is more stable in the results obtained by NormFinder [1]. According to NormFinder, the most stable top three genes are *CAP*, *SKIP5-1* and *EF1α* under drought stress; *SKIP5-1*, *CAP* and *SKIP5-2* under cold stress; *UBQ*, *SKIP1* and *GAPDH* under heat stress; *EF1α*, *SKIP5-1* and *SKIP5-2* under salt stress; *EF1α*, *CAP* and *TUB* under high pH treatment; *EF1α*, *ACT* and

Fig. 4 The relative transcript level of *CkWKY1* under different treatments normalized by the selected reference genes. **a** Drought treatment, **b** heat treatment, **c** cold treatment, **d** salt treatment, **e** high pH treatment, **f** ABA treatment
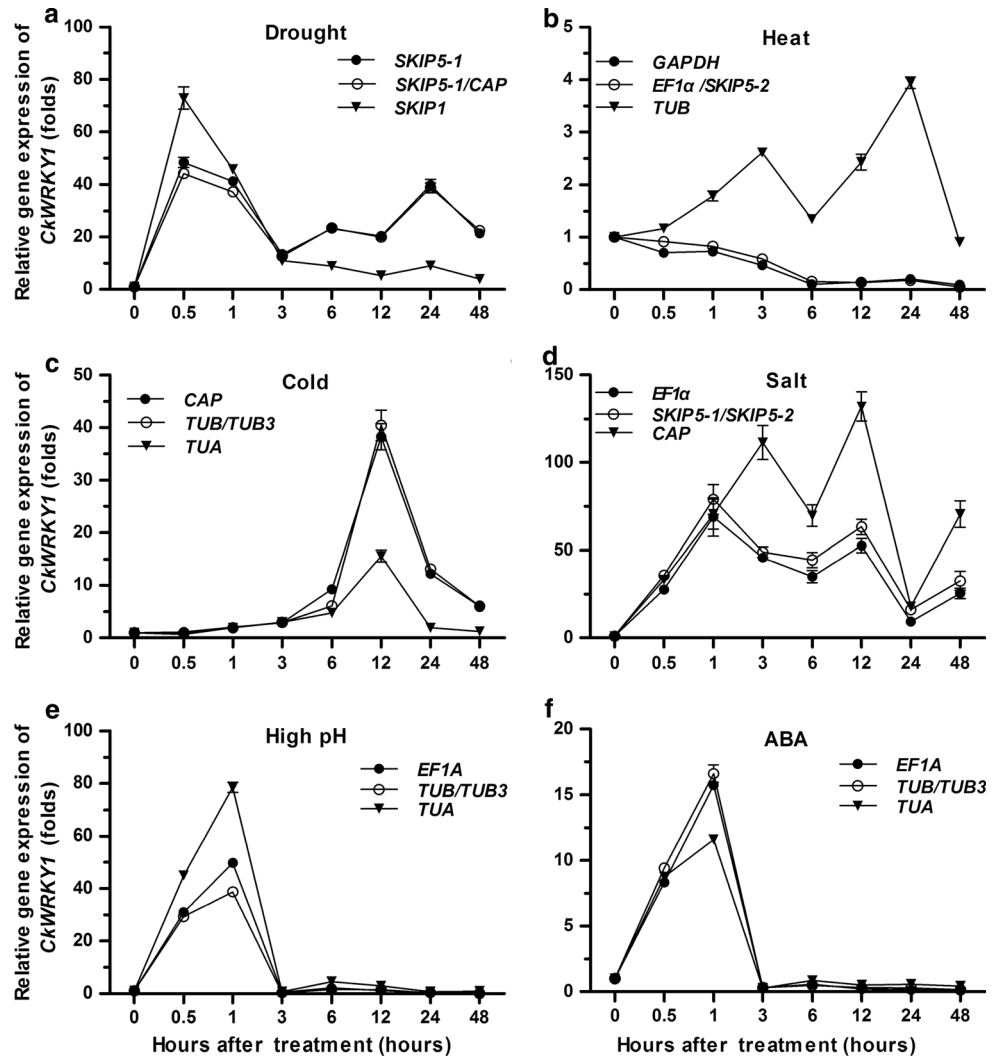
SKIP1 under ABA treatment; and *EF1α*, *ACT* and *GAPDH* under all of these stresses. Compared to the results from NormFinder and geNORM, although there are some differences in ranking, but the most stable top five genes almost consistently shared between two results. We also evaluated the data by BestKeeper [21] to confirm the suitability reference genes evaluated by geNORM and NormFinder. The results are summarized in Table 3.

By comprehensive analysis of these three results obtained by geNORM, NormFinder and BestKeeper, the most stable gene and least stable gene were selected. The most stable five genes using these three or two methods are presented in Table 4.

Expression analysis of *CkWRKY1* under different treatments

To demonstrate the usefulness of the stable reference genes selected by geNORM, NormFinder and BestKeeper under our conditions, the relative expression level of *CkWRKY1*

was investigated under different stresses (Fig. 4). The most stable reference gene and gene combination (*SKIP5-1* and *SKIP5-1+CAP* under drought stress, *GAPDH* and *EF1α+SKIP5-2* under heat stress, *CAP* and *TUB+TUB3* under cold stress, *EF1α* and *SKIP5-1+SKIP5-2* under salt stress, *EF1α* and *TUB+TUB3* under high pH, *EF1α* and *TUB+TUB3* under ABA treatment) and the least stable reference gene (*SKIP1* under drought stress, *TUB* under heat stress, *CAP* under salt stress, *TUA* under cold, high pH and ABA treatments) were used in this study. The analysis revealed that the expression level of *CkWRKY1* increased after drought, heat, cold, high pH and ABA treatment, inversely, the transcript level of *CkWRKY1* decreased after heat treatment. When using *SKIP5-1* and the combination of *SKIP5-1+CAP* as reference gene(s) under drought stress, the expression of *CkWRKY1* showed similar fluctuation patterns with slight difference. On the contrary, when using *SKIP1* as reference gene, the expression of *CkWRKY1* showed different change patterns compared to *SKIP5-1* and *SKIP5-1+CAP* as reference gene(s). A

similar performance were seen under heat, cold, salt, high pH and ABA treatments (Fig. 4). These results indicate that the stable reference genes selected by our study are reliable for qRT-PCR.

Discussion and conclusion

Although a powerful technology to study gene expression at the transcriptional level, there are several factors that can affect quantification of gene expression by qRT-PCR, such as the quality of the RNA, the efficiency of cDNA synthesis, PCR efficiencies, the methods used for statistical analysis and the reference gene used in normalization [14, 41]. Using different reference gene in qRT-PCR is important to normalization of gene expression analysis. Many research results showed that there was no ideal reference gene stably expressed in all tissues and organs, developmental stages, physiological states and under stress conditions [13]. The stability of the reference gene is relative, different reference gene expression in different conditions is usually not constant, in addition, the reference gene in different species has no absolute versatility [6, 16, 19, 23, 24]. Therefore, the screening of the reference gene for different plants under different conditions is necessary.

The present study selected a set of credible reference genes in *C. korshinkii* Kom. for the normalization of gene expression analysis using RT-qPCR for the first time. We compared 11 potential candidate reference genes for *C. korshinkii* Kom. across 129 biological samples under 6 experimental conditions. The PCR amplification efficiency (Table 1) was estimated by a standard curve and it is high enough for qRT-PCR. The data were analyzed by geNorm to select the stable reference gene and confirmed by NormFinder and BestKepper. Although there are some differences between the three results attained by different methods, but the general trend is consistent (Fig. 2; Table 2 and Table 3). Considering the results analyzed by three methods, the most stable reference genes and the least stable genes were summarized in Table 4. Across all the 129 samples under 6 treatments, *GAPDH*, *EF1α* and *SKIP5-1* are the most stable genes and ranked in top five in three analytical methods, *EF1α+SKIP5-1* is the most stable gene combination, *SKIP1* and *TUB3* are the least stable genes. Similarly, *SKIP5-1* and *CAP* under drought stress, *GAPDH1*, *SKIP1* and *SKIP5-1* under heat stress, *CAP*, *ACT*, *SKIP5-1* and *SKIP5-2* under cold stress, *EF1α*, *SKIP5-1* and *SKIP5-2* under salt stress, *EF1α* and *ACT* under ABA treatment, *EF1α* under high pH stress are the most stable reference genes. It should be noted that there is no single gene that can be ranked in the top five analyzed by three methods. *SKIP5-1+CAP* under drought stress, *SKIP5-2+EF1α* under heat stress, *SKIP5-1+SKIP5-2*

under salt stress, *TUB+TUB3* under cold, ABA and high pH stresses are the most stable gene combinations. *SKIP1* and *TUB3* under drought stress, *TUB* under heat stress, *CAP* and *SKIP1* under salt stress, *TUA* under cold, ABA and high pH stresses are the least stable reference genes.

A *CkWRKY1* gene was previously cloned in our lab from a suppression subtractive hybridization library (SSH) of *C. korshinskii* Kom. under drought stress [39]. In order to illustrate the suitability of the reference genes selected in this study, the relative *CkWRKY1* transcriptional expression levels under different stresses using different reference genes for normalization were compared. The research results show that the least stable reference gene will lead to inaccurate analysis results compared with using the most stable gene or gene combinations used. The WRKY proteins are an important superfamily of transcription factors in plants [9]. Many studies revealed that members in this transcription factor family were involved in signaling pathways in plant resistance to both biotic and abiotic stresses [8, 10, 25, 30]. *CkWRKY1* did respond to a variety of stresses in our study, suggesting that it may play an essential role in defense mechanisms of *C. korshinskii* Kom.

The stable reference genes selected in this study will enable more accurate normalization and quantification of gene expression in *C. korshinskii* Kom. under different stress conditions for qRT-PCR analysis.

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