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Identification of suitable reference genes in mangrove *Aegiceras corniculatum* under abiotic stresses

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Abstract Gene expression studies could provide insight into the physiological mechanisms and strategies used by plants under stress conditions. Selection of suitable internal control gene(s) is essential to accurately assess gene expression levels. For the mangrove plant, *Aegiceras corniculatum*, reliable reference genes to normalize real-time quantitative PCR data have not been previously investigated. In this study, the expression stabilities of five candidate reference genes [glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), 18SrRNA, *β-Actin*, 60S ribosomal protein L2, and elongation factor-1-A] were determined in leaves of *A. corniculatum* treated by cold, drought, salt, heavy metals, and pyrene and in different tissues of *A. corniculatum* under normal condition. Two software programs (geNorm and NormFinder) were employed to analyze and rank the tested genes. Results showed that *GAPDH* was the most suitable reference gene in *A. corniculatum* and the combination of two or three genes was recommended for greater accuracy. To assess the value of these tested genes as internal controls, the relative quantifications of *CuZnSOD* gene were also conducted. Results showed that the relative expression levels of *CuZnSOD* gene varied depending on the internal reference genes used, which highlights the importance of the choice of suitable internal controls in gene expression studies. Furthermore, the results also confirmed that *GAPDH* was a suitable reference gene for qPCR normalization in *A. corniculatum* under abiotic stresses. Identification of *A. corniculatu*m reference gens in a wide range of experimental samples will provide a useful reference in future gene expression studies in this species, particularly involving similar stresses.

Keywords Mangrove plants · *Aegiceras corniculatum* · Quantitative real-time PCR · Reference gene · Normalization

Introduction

Mangroves are a group of plant communities that distributed in tropical and subtropical coasts with characteristics of high salinity and anaerobic soil (Tomlinson 1994). With the intervention of human activities, mangrove plants are more and more threatened by pollutants such as heavy metals and polycyclic aromatic hydrocarbon (PAH) (Marchand et al. 2006; Tam et al. 2009), as well as global climate change (e.g., sea-level rising and cold events) (Gilman et al. 2008). Numerous investigations have focused on identification and expression analyses of stress-responsive genes from mangroves, with the aim of better understanding the stress-resistant mechanisms of mangroves (Fu et al. 2005; Jithesh et al. 2006; Zhang et al. 2007; Huang and Wang 2009, 2010; Basyuni et al. 2010; Peng et al. 2013).

Quantitative real-time PCR (qPCR) is currently one of the most powerful and sensitive techniques for rapid and

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reliable quantification of gene expression. Despite qPCR has many advantages, such as high sensitivity, high specificity and broad quantification range, qPCR also has its pitfalls. The accurate and reliable results quantified by qPCR rely on the use of appropriate normalization techniques. Since the use of internal reference gene takes into account variation introduced by initial sample amount, RNA integrity and enzymatic efficiencies, it has become one of the most popularly used methods for normalization (Remans et al. 2008). The expression of reference gene(s) used as internal control should not be affected by experimental conditions or tissue types; otherwise, it may lead to erroneous results (Udvardi et al. 2008). However, several studies have demonstrated that the expression levels of many so-called reference genes, such as the *18S* rRNA, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *β-Actin*, cyclophilin (*Cyp*), elongation factor-1-A (*EF1A*) and the 60S ribosomal protein L2 (*rpl2*) may differ greatly among different tissue types, species or in response to various stresses (Czechowski 2005; Migocka and Papierniak 2010; Xu et al. 2011). Recognizing the importance of reference genes in normalization of qPCR data, valuation of suitable reference genes has been conducted in various plants, such as *Arabidopsis thaliana* (Remans et al. 2008), *Cucumis sativus* (Migocka and Papierniak 2010), and *Populous* (Xu et al. 2011), under specific conditions. Hitherto suitable internal controls for gene expression studies have not been defined for mangroves.

Aegiceras corniculatum is a dominant mangrove species growing in the tidal zone of South China, and has been found to be highly tolerant to various abiotic stresses, such as salt and heavy metals (Burchett et al. 2006; Tam et al. 2009). Since *A. corniculatum* was considered to represent an excellent model system to study the stress-resistant mechanism of mangroves, many studies focus on gene expression analyses based on qPCR have been conducted in *A. corniculatum* (Fu et al. 2005; Huang and Wang 2010). In these studies the *18S* rRNA or *β-actin* was commonly used as internal control. Since fluctuant stabilities of *18S* rRNA or *β-actin* have been reported in many other plants under abiotic stresses (Remans et al. 2008; Migocka and Papierniak 2010), it is necessary to validate suitable reference genes for *A. corniculatum*.

With the aim to identify suitable internal reference gene(s) for normalization of qPCR data, the present study report the validation of five candidate reference genes (*GAPDH*, *18S* rRNA, *β-Actin*, *rpl2*, and *EF1A*) in *A. corniculatum* under various abiotic stresses (cold, drought, salt, heavy metals, and pyrene) and among different tissue types. Two software programs (geNorm and NormFinder) were employed to analyze and rank the tested genes. And in order to assess the value of these genes as internal controls in expression studies, the relative quantifications of Cu–Zn superoxide dismutase (*CuZnSOD*) gene were also conducted.

In the paper, it was the first attempt to validate a set of reference genes of mangroves. The results of this study will contribute to accurate and reliable normalization of qPCR data in this species in the future.

Materials and methods

Plant materials and treatments

Propagules of *A. corniculatum* were collected from Dongchong, Shenzhen, China. Seedlings were planted in clean sand and fertilized with 1/2 Hoagland nutritive medium every day. Four-month-old plantlets were subjected to experiments. All the results were obtained from at least two independent experiments on three plantlets. To evaluate the expression stabilities of genes among different tissues (leaves, stems, and roots) were randomly harvested on three plantlets and immediately frozen in liquid nitrogen until RNA extraction. To evaluate the expression stabilities of genes in response to cold, leaves were collected on three plantlets at 0, 2, 24 and 48 h after chilling (5 °C). To evaluate the expression stabilities of genes in response to drought, leaves were collected at 3 days, on three plantlets grown in solutions containing 10 or 20 % (w/v) PEG 6000, respectively. To evaluate the expression stabilities of genes in response to salt, leaves were collected at 3 days, on three plantlets grown in solutions containing NaCl (0, 15 or 30 g l^{-1}). To evaluate the expression stabilities of genes in response to PAH, leaves were collected at 3 days, on three plantlets grown in sand media containing pyrene (10 or 30 μg g^{-1}). To evaluate the expression stabilities of genes in response to heavy metals, leaves were collected at 3 days, on three plantlets grown in solutions with heavy metals (Cd 20 mg l^{-1} or Pb 60 mg l^{-1}). Harvested leaves were dropped immediately into liquid nitrogen and stored at −80 °C until RNA extraction.

Primer design

The sequences of *18S* rRNA, *β-Actin*, *GAPDH* and *CuZnSOD* were obtained from GenBank database (Table 1). The EST sequence of *EF1A* and *rpl2* in *A. corniculatum* were obtained by homologous clone using degenerate primers (Table 2). Multi-alignment showed that the two genes display high similarity to their homologous genes in *Arabidopsis*. The sequences of the two genes were deposited to GenBank (Accession number: Ac*EF1A,* JQ396426 and Ac*rpl2,* JQ396427). Six primer pairs for real-time qPCR were designed by Oligo 7.0. The primer

Table 1 The housekeeping genes used in this study

Symbol	Name	Function	Accession number
β-Actin	β-Actin	Cytoskeleton	DQ884963
EF1A	Elongation factor-1-A	Translation	JQ396426
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis enzyme	DQ884962
rpl2	Ribosomal protein L2	Involved in the association of the ribosomal subunits, tRNA binding and peptidyl transfer	JQ396427
18S rRNA	18S ribosomal RNA gene	Ribosomal subunit	FJ976669

CuZnSOD is the gene of interest

Table 2 Primers sequences for amplification of *EF1A* and *rpl2* homologous from *Aegiceras corniculatum*

Gene symbol	Primer sequence (5′ → 3′)
Dg-EF1A-F	RGCTGACTGTGCNRTBC
Dg-EF1A-R	RTDCCAATRCCACCRAT
Dg-rpl2-F	TTCAARTCCCAYACNCAC
Dg-rpl2-R	TGRGGATGCTCMACDGG

sequences, sizes of the amplified bands and the expected melting temperature of the six genes were listed in.

Two step quantitative real-time RT-PCR

Total RNA was isolated using plant total RNA extract kit (Tiangen, Invitrogen). The concentration and purity of total RNA was determined using a NanoDrop ND-2000C spectrophotometer (Thermo Scientific, USA). Retrotranscription of RNA (1 μg) was performed with PrimeScript® RT reagent Kit (Takara, Dalian, China). The concentration of cDNA was determined and then diluted to 20 ng·μl^{-1}. qPCR was performed in a 15 μl of 2× SYBR® Premix Ex Taq™ II (Takara, Dalian, China) mixture, with 200 ng of cDNA and 350 nM of each primer. Three replicates of each PCR were run in iCycler iQ5 real-time PCR detection system (Bio-Rad, CA, USA) using a program as the following: 1 min denaturation at 95 °C, then 45 cycles of 5 s at 95 °C, 15 s at 57/60 °C and 20 s at 72 °C. All PCRs displayed with efficiencies between 95 and 105 %. Specific amplified products were confirmed by the melting curves (Fig. 1).

Data analysis

Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (Cp). To evaluate the stability of these candidate reference gene(s), two free Excel-based tools, geNorm and Normfinder, were used in this study. NormFinder (Andersen et al. 2004) evaluate the systematic error (SE) introduced when a gene is used for normalization. GeNorm (Vandesompele et al. 2002) determined the expression stability as well as the optimal number of reference genes. The relative expression of *CuZnSOD* was calculated according to Pfaffl (2001) using a unique reference gene or the combination of reference genes suggested by geNorm.

Results

Variations of housekeeping genes

The RNA transcription profiles of the five housekeeping genes and *CuZnSOD* were shown in Fig. 2. The crossing point (Cp) values were fluctuated from 12.25 to 31.26. The *β-Actin* and *rpl2* showed relatively low expression levels, while *18S* rRNA showed the highest expression level. These genes displayed relative stable expression levels except in leaves treated by heavy metals Cd and Pb (Table 3).

Expression stability of candidate reference genes

NormFinder analysis

As shown in Table 4, NormFinder evaluate the SE introduced when a gene is used for normalization; and the gene with the lowest SE is assigned as the best reference gene. According to NormFinder analysis, the *GAPDH* gene had a lowest SE value in drought (0.194) and salt experiments (0.267). The *EF1A* gene was ranking as the most stable gene in Pyrene treatments with a lowest value of 0.148 and in heavy metal experiments (0.335). The *rpl2* was ranking as the most stable gene among tissue types under normal condition and in cold experiment. The *18S* rRNA was ranking as the least stable gene among different tissues, and in cold or heavy metal treatments. When all samples were considered, the *EF1A* had the lowest SE value (0.344), followed by *GAPDH* (0.399) and *rpl2* (0.407).

geNorm analysis

geNorm provides a measure of gene expression stability (M), which is the mean pairwise variation between an

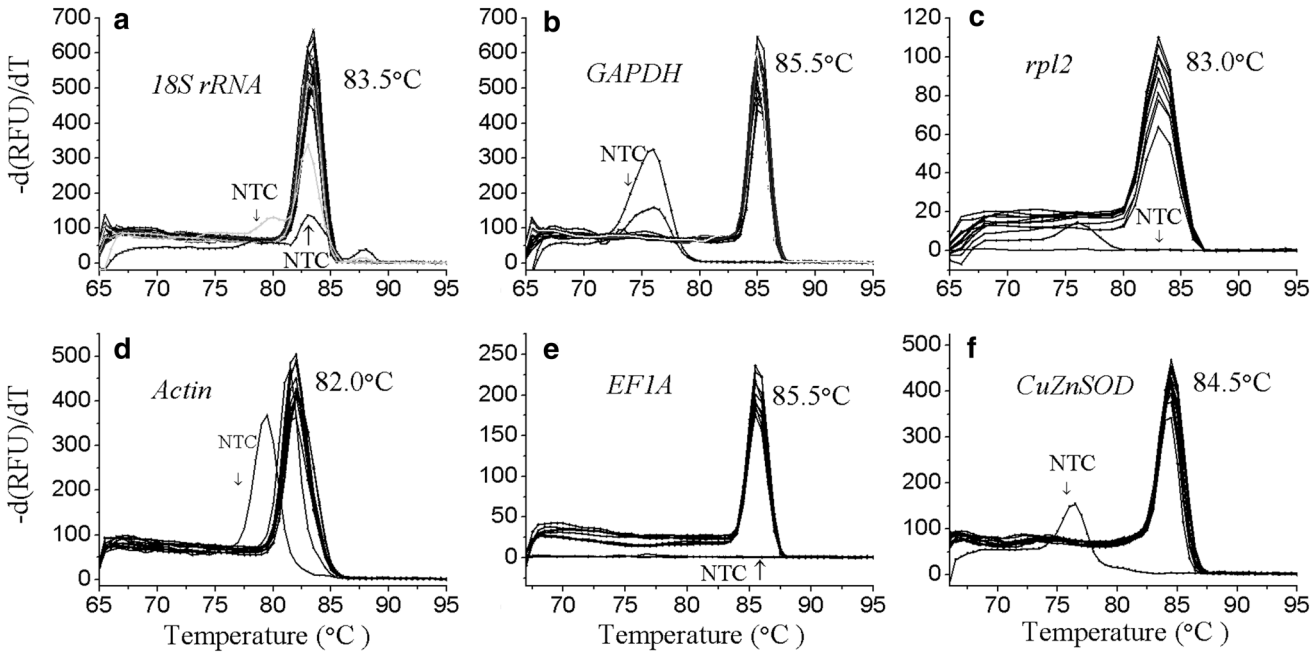

Fig. 1 Melting curve analysis of the five candidate reference genes and interest gene *CuZnSOD*

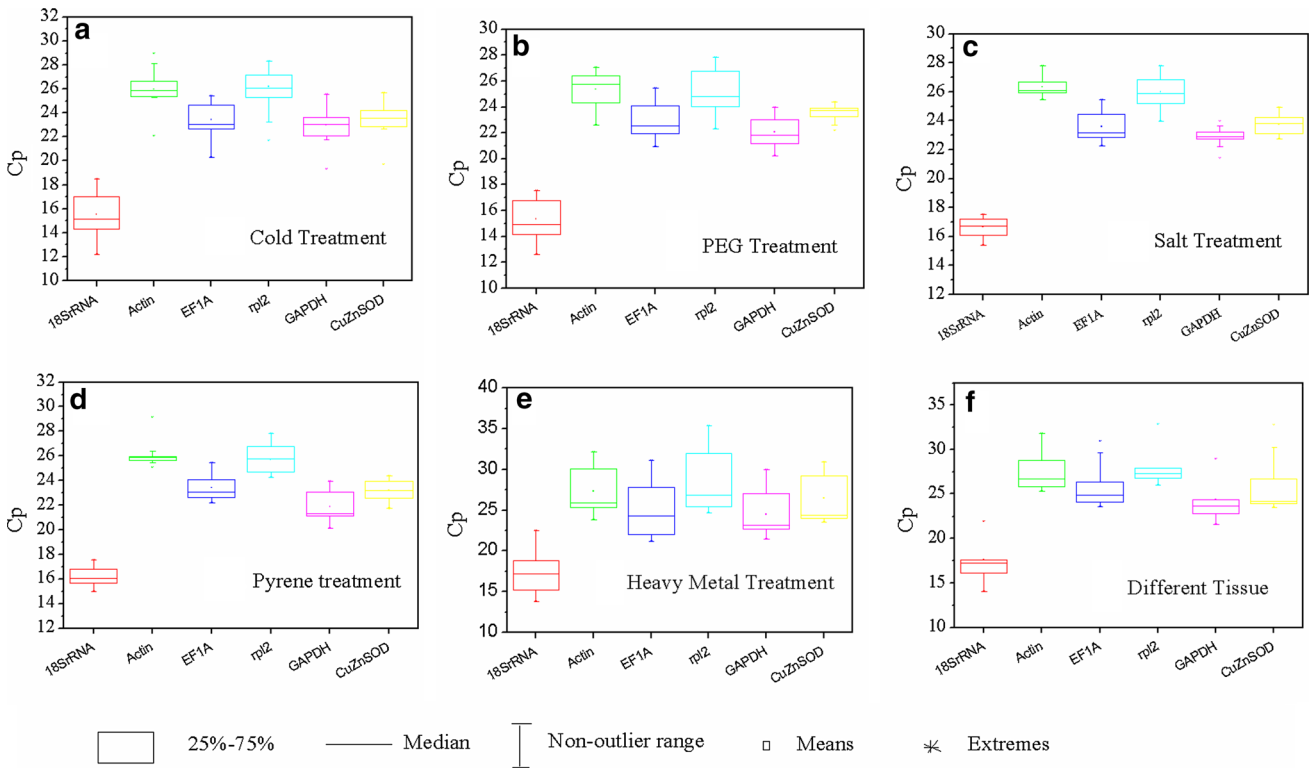

Fig. 2 RT-qPCR Cq values. Cp values for each reference gene and CuZnSOD gene in leaves of *A. corniculatum* after cold (**a**), PEG (**b**), salt (**c**), Pyrene (**d**) or heavy metals (**e**) treatments, and in different tissues of *A. corniculatum* under normal condition (**f**)

individual gene and all other tested control genes. The gene with lowest M is the best reference gene for a given set of samples. According to geNorm analysis (Table 4), the *GAPDH* was ranking as the most stable gene among different tissues, and under cold and pyrene treatments. The *EF1A* had the lowest M value in drought, salt and heavy

Table 3 Primers sequences and real-time PCR efficiencies

Gene symbol	Primer sequence (5′ → 3′)	Length (bp)	Tm (°C)	Efficiency (%)
β-Actin	AGCTCATCGGTGGAGAAGAA	94	82.5	101.8
	GTTGGAACAGGACCTCAGGA			
EF1A	ATGGTGATGCTGGTATGGTTAAGAT	156	85.4	100.5
	CAGTGGGTTCCTTCTTCTCAACGC			
GAPDH	ACACTCTATTACCGCCACA	149	85.6	99.0
	GCTTTCCGTTTAGTTCAGG			
rpl2	CCTTTCGTTACAAGCACCAG	92	82.9	98.5
	CAGATTTGCCTTCTTCCCAC			
18S rRNA	ACCATAAACGATGCCGACCAG	113	83.5	101.5
	TTCAGCCTTGCGACCATACTC			
CuZnSOD	GTGCTCCTGAAGATGAAA	116	85.6	95.5
	ATTAGGTCCAGTAAGTGGTAT			

Table 4 Candidate reference genes ranked according to NormFinder or geNorm

Gene name	Cold				Salt				Drought				Pyrene			
	NormFinder		geNorm		NormFinder		geNorm		NormFinder		geNorm		NormFinder		geNorm	
	Rank	SE	Rank	M	Rank	SE	Rank	M	Rank	SE	Rank	M	Rank	SE	Rank	M
GAPDH	3	0.325	1	0.434	1	0.267	4	0.469	1	0.194	3	0.67	3	0.289	1	0.32
EF1A	2	0.248	4	0.521	3	0.417	1	0.351	2	0.216	1	0.536	1	0.148	2	0.32
rpl2	1	0.138	3	0.472	4	0.338	2	0.365	3	0.367	4	0.735	4	0.349	3	0.35
Actin	4	0.335	2	0.457	5	0.286	5	0.615	5	0.613	5	0.993	5	0.547	5	0.7
*18S*rRNA	5	0.534	5	0.746	2	0.473	3	0.422	4	0.455	2	0.617	2	0.283	4	0.45

Gene name	Heavy metals(Pb/Cd)				Different tissues				All			
	NormFinder		geNorm		NormFinder		geNorm		NormFinder		geNorm	
	Rank	SE	Rank	M	Rank	SE	Rank	M	Rank	SE	Rank	M
GAPDH	2	0.338	4	1.025	3	0.338	1	0.478	2	0.399	3	0.795
EF1A	1	0.335	1	0.902	4	0.355	4	0.602	1	0.344	2	0.785
rpl2	4	0.566	2	0.962	1	0.223	3	0.511	3	0.407	1	0.765
Actin	3	0.432	3	0.984	2	0.278	2	0.501	4	0.446	4	0.909
*18S*rRNA	5	0.719	5	1.247	5	0.531	5	0.818	5	0.575	5	1.009

All when all samples were included in the calculation, *SE* systematic error, *M* measure of gene expression stability

metal experiments. The *rpl2* had the lowest M value when all samples were involved, followed by *EF1A* and *GAPDH*. The *Actin* was always ranking as the least stable gene except in the cold or heavy metal experiments. The *18S* rRNA was always ranking as the least stable gene except in the salt or drought experiments.

GeNorm also measured the pairwise variation to determine the optimal number of reference genes. The value of "Vn/n + 1" represents the effect of adding additional reference genes on the normalization factor for these treatments (Table 5). There was no need to add another control gene if the "Vn/n + 1" value is <0.15. For Pyrene stress, both the $V_{2/3}$ and $V_{3/4}$ were <0.15, which means that two reference genes should be used as control. For different tissue types, as well as the cold and salt stresses, the $V_{3/4}$ was less than 0.15, which means that three reference genes should be used as internal control. For heavy metal and drought stresses, the V values were all more than 0.15, which means that the stabilities of all these candidate references genes may largely fluctuated under these conditions.

CuZnSOD gene expression

When *GAPDH*, *β-Actin* and the combination of three genes (*GAPDH*, *β-Actin* and *rpl2*) were used for normalization,

Table 5 The pairwise variation analysis according to geNorm

Vn/n + 1	Cold	Saline	Drought	Pyrene	Metal	Tissues	All
V2/3	0.153	0.177	0.256	0.131	0.31	0.163	0.240
V3/4	0.131	0.114	0.172	0.128	0.227	0.149	0.231
V4/5	0.209	0.163	0.265	0.211	0.297	0.219	0.204

The value of "Vn/n + 1" represents the effect of adding additional reference genes on the normalization factor for these treatments

All when all samples were included in the calculation

the *CuZnSOD* gene expression increased quickly after 2-h of cold, and then decreased to the control level after 48 h of cold (Fig. 3a). When 18S rRNA, *EF1A* and *rpl2* were used as a unique internal control separately, obvious increase of *CuZnSOD* transcripts after 2 and 24 h of cold were not detected. Moreover, when 18S rRNA was used as a unique control, the CuZnSOD transcripts decreased to below the control level after 48 h of cold (Fig. 3a). Under drought, salt and pyrene stresses, the *CuZnSOD* expression levels were declined to various extents when the best reference genes recommended by geNorm were used as internal control (see Fig. 3b–d). The expression levels were obviously overestimated or underestimated when the least two stable genes (*β-Actin* and *rpl2* in drought treatment, *β-Actin* and *GAPDH* in salt treatment, *β-Actin* and *18S* rRNA in PAH treatment) were used as unique control (see Fig. 3b–d). For heavy metal treatments, the *CuZnSOD* gene expression were seriously inhibited in Pb treated plants and strongly induced in Cd treated plants when the combination of *GAPDH*, *β-Actin* and *EF1A* was used for normalization (Fig. 3e). The use of *18S* rRNA as unique control obviously underestimated the *CuZnSOD* expression level under Cd stress, while the use of *rpl2* significantly overestimated the *CuZnSOD* mRNA transcripts abundance. Under normal condition, the minimum of *CuZnSOD* mRNA transcripts was observed in roots except using *18S* rRNA for normalization (Fig. 3f).

Discussions

Although *18S* rRNA has been frequently used as an internal control in mangroves *A. corniculatum* (Fu et al. 2005; Huang and Wang 2009), our results indicated that *18S* rRNA may be not suitable as reference gene for qPCR normalization in *A. corniculatum* under various stresses. Due to the high abundance of *18S* rRNA compared with target mRNA transcripts, it was difficult to accurately subtract the baseline value in qPCR data analysis (Vandesompele et al. 2002). The expression level of *CuZnSOD* was obviously overestimated when *18S* rRNA was used as a unique internal control under cold and heavy metal experiments (Fig. 3). According to geNorm and NormFinder analysis, the *β-Actin* gene of *A. corniculatum* have poor stability under various abiotic stresses (e.g. drought, salinity and PAH treatment). Qi et al. (2010) also demonstrated that the expression level of *β-Actin* gene may fluctuate largely in Cabbage.

The *EF1A* gene has been evaluated to be stably expressed in cucumber under salt, osmotic and metal stresses (Migocka and Papierniak 2010) and in cabbage under drought stress (Qi et al. 2010). In this study, *EF1A* was suggested to be the most stable gene under pyrene and metal experiments by NormFinder and the most stable gene under salt, heavy metal and drought experiment by geNorm. Our results also demonstrated that the *rpl2* was the most stable reference gene under cold stress by NormFinder and under salt stress by geNorm, such as found in cabbage (Qi et al. 2010). The small inconsistency between different softwares was due to their different statistical algorithms (Migocka and Papierniak 2010).

Taken together, *GAPDH* gene was suggested to be better internal control than the other four candidate reference genes. *GAPDH* was ranked among the top three best reference genes except for salt treatment according to geNorm. When NormFinder was used, *GAPDH* was identified as the most stable gene under salt and drought stresses. *CuZnSOD* expression analyses also suggested that *GAPDH* was a suitable reference gene in *A. corniculatum*. Although variations of *GAPDH* expression were observed among tissue types of human (Barber et al. 2005), *GAPDH* has been proved to be a suitable reference gene in many plants, such as cabbage (Qi et al. 2010), *Swingle citrumelo* (Carvalho et al. 2010) and *Coffea arabica* (Barsalobres-Cavallari et al. 2009).

Since using single gene could lead to relatively large error, it has been suggested that at least two or three housekeeping genes should be used for normalization (Vandesompele et al. 2002). According to the pairwise variation (V) analysis from geNorm, the geometric mean of multiple reference genes was proposed to normalize the qPCR data. For different tissue types, the geometric mean of three genes (*GAPDH*, *EF1A* and *rpl2*) was suggested to be internal control. Similar results were detected in a study of Barsalobres-Cavallari et al. (2009), which identified the stable expression levels of *GAPDH*, *14-3-3* and *rpl7*

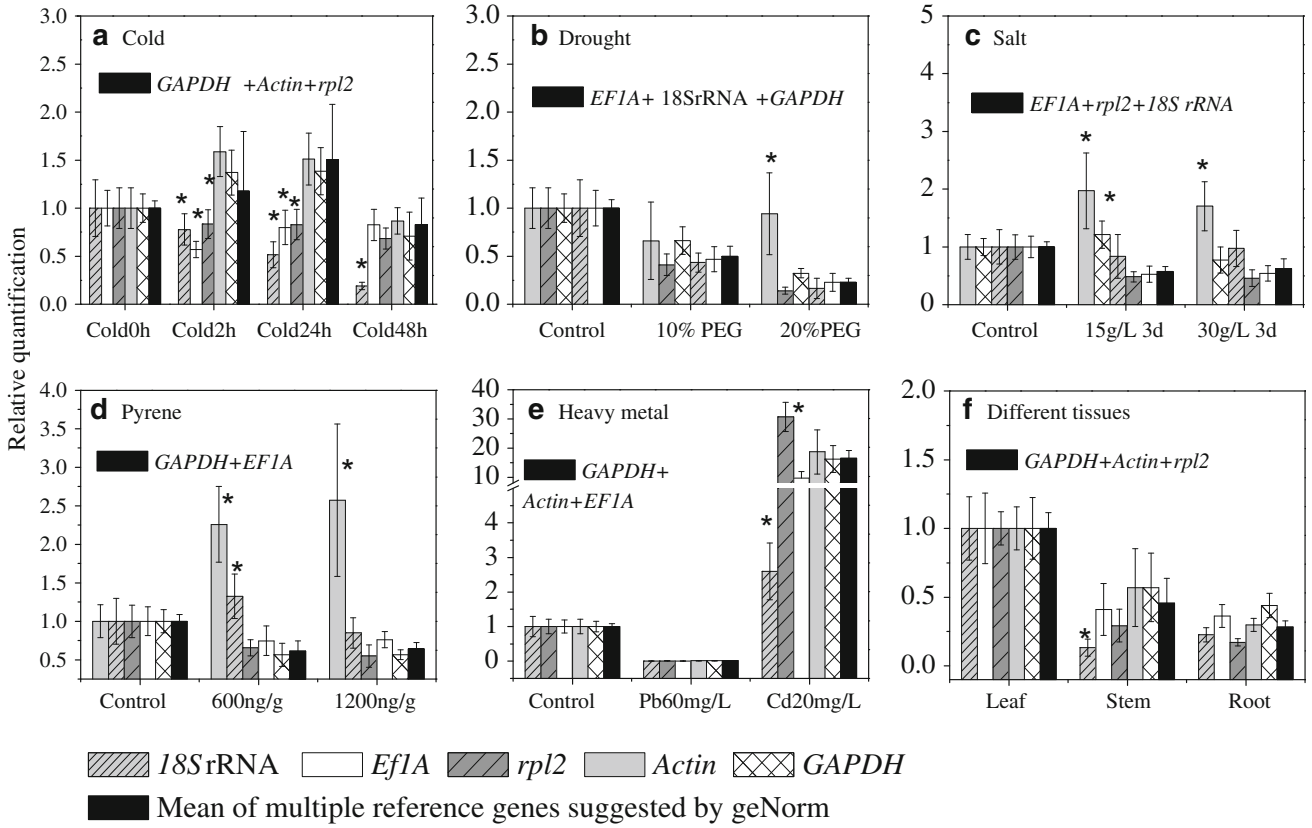

Fig. 3 Relative expression levels of *CuZnSOD* gene in leaves after cold (**a**), drought (**b**), salt (**c**), Pyrene (**d**) or heavy metals (**e**) treatments using different internal controls. Relative expression levels of *CuZnSOD* gene among different tissues under normal condition (**f**) using different internal controls. *Asterisks* indicate significant differences between expression levels of *CuZnSOD* calculated using a unique reference gene ore the combination of the best two or three genes, significance was defined as $p < 0.05$

mRNA in different *C. arabica* tissue types. For the cold, salt or pyrene experiments, geNorm suggested the geometric mean of two or three genes for normalization. The sets of suitable reference genes for the three experiments are as follows: (1) cold stress—*EF1A*, *rpl2* and *GAPDH*; (2) salt stress—*EF1A*, *rpl2* and 18S rRNA; (3) Pyrene stress—*GAPDH* and *EF1A*. Former studies have reported similar suitable reference genes in other plants. For example, *EF1A* and *rpl2* were recommended to normalize qPCR data in potato under cold stress, while *EF1A*, cyclophilin and *rpl2* were suggested for salt stress (Nicot et al. 2005). In *Swingle citrumelo*, *EF1A*, *ADP* ribosylation factor (*ADP*) and *GAPDH* were suggested to be internal control under drought stress (Carvalho et al. 2010). For heavy metal and drought experiments, the V values were all more than 0.15, which means that all these tested genes may be fluctuated largely under these conditions. Therefore, it is necessary to explore metal-stable and drought-stable reference genes in *A. corniculatum* in the future. Relatively higher metal-induced variations of traditional reference gene (e.g. *18S* rRNA, *β-Actin* and *EF1A*) were also observed in *A. thaliana* (Remans et al. 2008).

In the last part of this study, we compared the relative transcript level of a target gene (CuZn-SOD) normalized by each single reference gene and that normalized by the geometric mean of multiple reference genes. As shown in the Fig. 3a, e and f, when *18S rRNA* was used as a unique internal control, the transcript levels of target gene were significantly underestimated. And as shown in the Fig. 3b–d, the target gene expression levels were obviously overestimated when *β-Actin* was used as a unique control. As shown in Fig. 3a–f, the target gene expression levels quantified by *GAPDH* were similar with that quantified by the geometric mean of multiple reference genes. Therefore, taken together of the geNorm and Normfinder analysis, the *GAPDH* gene was suggested as a suitable reference gene for qPCR normalization in *A. corniculatum* under abiotic stresses.

Conclusion

The present work evaluated the suitability of five housekeeping genes as internal control with two Excel-based tools, geNorm and Normfinder. The results evidenced the

stable expression levels of *GAPDH* gene under several abiotic stresses, such as cold, salt and pyrene. Moreover, in order to get more accurate quantification of qPCR date, it is better to use the geometric mean of multiple reference genes as internal control. Finally, the heavy metal-stable and drought-stable reference genes in *A. corniculatum* remain to be explored in the future. Moreover, with the development and use of gene chip technology and transcriptome analysis, more and more novel reference genes that are more stable than traditional reference genes will be identified in further studies.

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Conflict of interest The authors declare that they have no conflict of interest.

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