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Short Communication

Selection of reliable reference genes for gene expression studies by quantitative real-time PCR in *Anoectochilus roxburghii*

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Abstract

RT-qPCR is a sensitive, efficient and reliable method for gene expression studies and reference gene expression stability was essential for RT-qPCR. Since previous studies showed that no reference gene could exhibit changeless expression pattern in all experiments, this study determined the gene expression stability of 11 reference genes across various treatments in *Anoectochilus roxburghii*. The results indicated that expression stability of genes varied considerably under different treatments. The genes, *GAPDH*, *EF1*α and *ACT1* emerged as the most reliable reference genes.

Key words: *Anoectochilus roxburghii*, geNorm, NormFinder, reference gene

The quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR) was a sensitive, efficient and reliable method for gene expression studies and introduction of reference gene was necessary for normalization of the RT-qPCR results. However, the expression stability of frequently used reference genes has been questioned by Demidenko et al. (2011). Thus, selection of stable reference gene(s) was prerequisite before RT-qPCR analysis.

Anoectochilus roxburghii (Orchidaceae) has great value as ''the king of orchid'' and there was no study about selection of reliable reference genes in this plant. This study was to evaluate the stability of the 11 reference genes in *A. roxburghii* gene expression, including 18S rRNA (*18S*), *GAPDH*, actin1 (*ACT1*), actin2 (*ACT2*), ubiquitin (*UBQ*), elongation factor 1A (*EF1*α), elongation factor 1B (*EF1*β), 5.8S rRNA (*5.8S*), ribulose-1, 5-bisphosphate carboxylase (*rbcL*), ribosomal protein S12 (*RPS12*) and L-Orn N(5)-oxygenase (*PSBA*).

The sequences were downloaded from the NCBI database, and the Primer 3 was used to design primers (Supplementary Table S1). *A. roxburghii* were treated with high temperature (42^{o}C), low temperature (4^{o}C), polyethylene glycol (PEG) (42% PEG), flooding, high salinity (300 mM NaCl), lead acetate (100 μM), and darkness. After plant materials were prepared, RNA extraction, RT-qPCR, and Lin-RegPCR analysis (Fig. 1) were performed in order (Ramakers et al. 2003).

The GeNorm was used to calculate gene expression stability (M) of the 11 tested genes (Supplementary Table S2). The lowest M value indicates the most stably expressed genes (Liu et al. 2016). *GAPDH* and *ACT1* exhibited the highest stable expression pattern in all samples. With the treatment of PEG and lead acetate, *EF1*α and *rbcL* showed the most stable expression. *GAPDH* and *EF1*α were the most stable genes after high temperature treatment. Under low temperature and darkness stress *ACT2* showed the highest stable expression. *PSBA* expressed highest stability in flooding and high salinity treatment. The optimal number of genes was calculated by geNorm with a cut-off value of 0.15 (Fig. 2). In all samples, the pairwise variation V2/3 was 0.155, a little higher than 0.15 which indicated two

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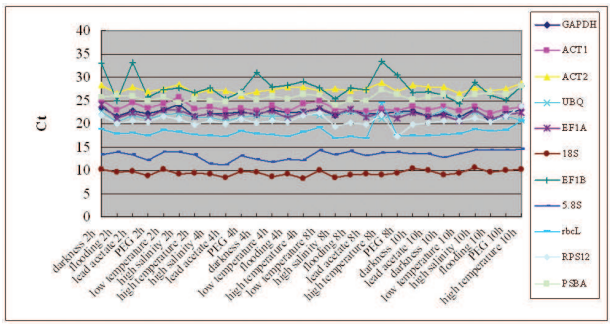

Fig. 1. RT-qPCR Ct values for reference genes.

best genes were enough to normalize the output of RT-qPCR in majority experiment and it would be more accurate for the analysis of results, if the third reference gene was introduced. Whether the third reference gene is introduced depended on the experimental accuracy requirement and experimentation cost. This phenomenon also exists in high temperature treatment and high salinity treatment. On the other hand, in those cases of V2/3 < 0.15, such as in PEG, lead acetatet, low temperature, darkness and flooding treatment, the purpose of normalization with the top two reference

Fig. 2. Determination of the optimal number of reference genes. Pairwise variation calculated by geNorm to determine the minimum number of reference genes for accurate normalization in all the samples (A), PEG Stress (B), lead acetate treatment (C), low temperature treatment (D), high temperature treatment (E), high salinity treatment (F), darkness stress (G), flooding stress (H)

Table 1. Three most stable genes selected base on combined analysis by geNorm and NormFinder

Rank	All samples	PEG	lead acetate	low temp.	high temp.	high salinity	darkness	flooding
1	EF1α	18S	EF1α	UBQ	EF1α	rbcL	GAPDH	RPS12
2	GAPDH	rbcL	rbcL	ACT2	GAPDH	18S	5.8S	18S
3	ACT1	EF1α	RPS12	PSBA	ACT2	EF1α	ACT2	rbcL

genes should be sufficient.

NormFinder can identify optimal normalization gene(s). Lower expression stability values indicate stable gene expression (Andersen et al. 2004). As shown in Supplementary Table S2, in all samples, *EF1*α showed remarkable stability in its expression levels. *EF1*α also showed the higher stability in lead acetate and high salinity treatments. *ACT1* and *ACT2* were the most stable genes in low temperature and high temperature treatments. In PEG, darkness and flooding treatments, the most stable internal control genes were *18S*, *rbcL*, *RPS12*, respectively. It is notable that *EF1*β was always among the least stable reference genes.

The analysis of geNorm and NormFinder, gene expression was not much of difference. Difference in the ranking of reference genes in different treatments might be caused by different statistical algorithms of different procedures. The data indicated that expression stability of genes varied considerably under different experimental conditions in *A. roxburghii* (Supplementary Table S3). *EF1*α, *GAPDH* and *ACT1* were the three best reference genes for all sample pools by a combination of geNorm and NormFinder (Table 1). In all sample pools, *EF1*β performed poorly and should be cautiously used as a reference gene in RT-qPCR.

Authors' contribution

Conceptualization of research (LH); Designing of the experiments (ZY); Contribution of experimental materials (YB); Execution of field/lab experiments and data collection (LL); Analysis of data and interpretation (XD); Preparation of manuscript (HX).

Declaration

The authors declare no conflict of interest.

Acknowledgment

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Supplementary Table S1. RT-qPCR primer sequences and the characteristics of the resulting amplicons obtained from *Anoectochilus roxburghii*

Gene name	Gene acc. No.	Forward primer(5'-3')	Reverse primer(5'-3')	Promer TM(^{o}C)	Product size(bp)	Product TM(^{o}C)	Efficiency	R^2
GAPDH	JF825421.1	GGACTGGAGAGGTGGAAGAG	GAACCTTCCCAACAGCCTTG	59.9/61.9	80	82.5	1.978	0.996
ACT1	JF825424.1	TTATGCACTCCCTCATGCCA	CGCAGTCGTCGTGAAAGAAT	57.8/57.8	109	82.0	2.071	0.995
ACT2	JF825425.1	CGATCATGAAGTGCGACGTT	CCCTCCAATCCAGACACTGT	57.8/59.9	221	84.5	2.073	0.995
UBQ	JF825423.1	CCCCAGATCAGCAAAGACTG	AAAATCTGCATGCCACCACG	59.9/57.8	130	84.5	2.041	0.995
EF1α	JF825419.1	CCACCACACCCAAGTACTCT	GTCACCCTCGAAACCAGAGA	59.9/59.9	128	80.5	2.045	0.996
18S	JF825422.1	TTCTATGGGTGGTGGTGCAT	CAGGTTGAACTCCGCATAGC	57.8/59.9	112	83.0	2.021	0.995
EF1β	JF825420.1	CATCGAAGCTTGTTCCGGTC	CAGCTCTTGTTCACAGCCAG	59.9/59.9	196	83.0	2.003	0.994
5.8S	GQ396668.1	TCGGCAATGGATATCTTGGC	GATGGTTCACGGGATTCTGC	57.8/59.9	88	82.5	2.028	0.995
rbcL	KF496538.1	ACGTCTGGAAGATCTGCGAA	TGGGCCTTGGAAAGTTTTGG	57.8/57.8	55	77.5	2.050	0.993
RPS12	ALG65729.2	GAACCCTAGATGCTGTCGGA	ATTCAACGCACTAGAACGCC	59.9/57.8	59	80.5	2.028	0.996
PSBA	ALG65685.1	GGGTCGTGAGTGGGAACTTA	TGTGCTCTGCCTGGAATACA	59.9/57.8	75	83.5	2.042	0.996

Supplementary Table S2. Ranking of the 11 candidate reference genes as calculated by geNorm

Rank	All samples		PEG		lead acetate		low temperature		high temperature		high salinity		darkness		flooding	
	Gene	Value	Gene	Value	Gene	Value	Gene	Value	Gene	Value	Gene	Value	Gene	Value	Gene	Value
1	GAPDH	0.495	EF1α	0.248	EF1α	0.111	ACT2	0.205	GAPDH	0.398	18S	0.174	ACT2	0.300	rbcL	0.133
1	ACT1	0.495	rbcL	0.248	rbcL	0.111	UBQ	0.205	EF1α	0.398	PSBA	0.174	5.8S	0.300	PSBA	0.133
2	EF1α	0.516	18S	0.323	RPS12	0.180	PSBA	0.263	ACT2	0.678	rbcL	0.358	GAPDH	0.411	18S	0.301
3	UBQ	0.631	GAPDH	0.388	ACT2	0.275	rbcL	0.418	PSBA	0.737	RPS12	0.424	ACT1	0.471	RPS12	0.447
4	ACT2	0.705	PSBA	0.456	PSBA	0.303	RPS12	0.463	5.8S	0.852	EF1α	0.490	UBQ	0.526	ACT1	0.567
5	18S	0.763	UBQ	0.506	18S	0.359	ACT1	0.489	18S	0.886	UBQ	0.539	PSBA	0.561	ACT2	0.656
6	5.8S	0.846	ACT1	0.543	UBQ	0.409	GAPDH	0.506	ACT1	0.916	ACT2	0.607	rbcL	0.600	5.8S	0.737
7	PSBA	0.901	5.8S	0.600	GAPDH	0.455	18S	0.558	RPS12	0.977	GAPDH	0.657	18S	0.626	UBQ	0.835
8	RPS12	0.954	ACT2	0.646	ACT1	0.501	EF1α	0.627	UBQ	1.044	ACT1	0.729	RPS12	0.657	EF1α	0.891
9	rbcL	1.046	RPS12	0.899	5.8S	0.581	5.8S	0.717	EF1β	1.409	EF1β	0.800	EF1α	0.680	GAPDH	0.946
10	EF1β	1.281	EF1β	1.217	EF1β	1.034	EF1β	0.831	rbcL	1.658	5.8S	0.882	EF1β	1.130	EF1β	1.059

Supplementary Table S3: Ranking of the 11 candidate reference genes as calculated by NormFinder

Rank	All samples		PEG		lead acetate		low temperature		high temperature		high salinity		darkness		flooding	
	Gene	Value	Gene	Value	Gene	Value	Gene	Value	Gene	Value	Gene	Value	Gene	Value	Gene	Value
1	EF1α	0.351	18S	0.103	EF1α	0.163	ACT1	0.108	ACT2	0.170	EF1α	0.105	rbcL	0.134	RPS12	0.135
2	ACT2	0.367	GAPDH	0.124	rbcL	0.173	UBQ	0.181	PSBA	0.350	rbcL	0.109	GAPDH	0.145	ACT1	0.269
3	GAPDH	0.395	UBQ	0.150	PSBA	0.203	rbcL	0.206	EF1β	0.352	RPS12	0.228	EF1α	0.158	18S	0.333
4	18S	0.442	rbcL	0.217	RPS12	0.273	GAPDH	0.242	GAPDH	0.434	UBQ	0.309	RPS12	0.199	ACT2	0.379
5	ACT1	0.455	PSBA	0.233	ACT1	0.282	RPS12	0.242	5.8S	0.612	18S	0.366	ACT1	0.286	UBQ	0.454
6	PSBA	0.522	EF1α	0.238	ACT2	0.282	PSBA	0.276	ACT1	0.732	GAPDH	0.385	18S	0.404	EF1α	0.546
7	UBQ	0.522	ACT1	0.317	18S	0.303	ACT2	0.319	RPS12	0.756	ACT2	0.421	5.8S	0.425	rbcL	0.571
8	5.8S	0.632	5.8S	0.414	UBQ	0.324	18S	0.486	18S	0.776	PSBA	0.505	ACT2	0.451	GAPDH	0.632
9	RPS12	0.635	ACT2	0.444	GAPDH	0.382	EF1α	0.507	UBQ	0.936	ACT1	0.651	UBQ	0.524	5.8S	0.662
10	rbcL	0.806	RPS12	1.451	5.8S	0.558	5.8S	0.780	EF1β	1.741	EF1β	0.715	PSBA	0.543	PSBA	0.669
11	EF1β	1.535	EF1β	1.805	EF1β	2.110	EF1β	0.903	rbcL	1.800	5.8S	0.773	EF1β	2.168	EF1β	1.009