



Full paper

Selection and validation of endogenous reference genes for RT-qPCR normalization in different stresses and tissues of the tiger milk mushroom, *Pleurotus tuber-regium*

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ABSTRACT

Pleurotus tuber-regium is a sclerotium-producing mushroom with pharmacological and nutritional value. Many researches applied molecular biological strategies that allow analyzing numerous genes expressed in response to different growth stresses or tissues. In order to capture accurate and reliable results in gene expression studies, it is necessary to select appropriate internal reference genes. They must have relatively uniform expression in the majority of tested samples, thereby reducing the impact of the sample itself on the RT-qPCR results. The selection and validation of 14 candidate reference genes, including *α-tub*, *β-tub*, *γ-tub*, *GAPDH*, *Tif-5a*, *Tef-1α*, *ATPase*, *acyl-CoA*, *U2ribo*, *SIG*, *Wlp*, *VtpAsE*, *E3upl*, and *Sdh*, were carried out for gene expression analysis in *P. tuber-regium* mycelia in response to different temperatures, drought levels, and salinity shifts, fruitbody, and sclerotium. Four statistical algorithms, NormFinder, geNorm, BestKeeper, and RefFinder, were recruited to evaluate the transcription stability of candidate reference genes. The RT-qPCR expression stability analysis indicated that the *E3upl* and *Tif-5a* were the most stable expressed genes among all experimental samples, so the combination of these two reference genes is suitable for the analysis of gene expression patterns in *P. tuber-regium*.

Keywords: reference gene, stability evaluation, transcription abundance

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1. Introduction

The basidiomycete *Pleurotus tuber-regium* (Fr.) Singer, known as tiger milk mushroom, is a tropical and subtropical sclerotium-producing mushroom (Oso, 1977). *Pleurotus tuber-regium* is of popularity and economic importance due to the nutritional value and bioactive compounds around the world especially in China, Africa, and the United States (Wong & Cheung, 2008; Liu et al., 2018a). Previous studies have focused on the research of *P. tuber-regium* reproduction (Fasidi & Ekuere, 1993; Okhuoya & Etugo, 1993; Bamigboye, Oloke, & Dames, 2019), nutrition (Oyetayo & Akindahunsi, 2005; Ohiri, 2018), pharmacological value (Wu, Lu, Jiang, Li, & Huang, 2014; Okolo, Orisakwe, & Siminialayi, 2018), and molecular biology (Huang, Lin, Cheung, & Wu, 2011; Liu et al., 2018b). With the completion of the diploid genome sequencing (Lam et al., 2018) and the construction of *Agrobacterium tumefaciens*-mediated transformation system (Liu et al., 2018b) in *P. tuber-regium*, there is an increasing attention on the molecular biological research of important functional genes as it provides

necessary information about fungal development and metabolite synthesis.

Currently, gene expression analysis is one of the most important fields in molecular functional research. One of the most widely used techniques to determine gene expression is reverse transcription quantitative polymerase chain reaction (RT-qPCR), due to its high accuracy, specificity and reproducibility (Derveaux, Vandensompele, & Hellemans, 2010). Thereinto, appropriate reference genes are necessary to standardize RT-qPCR results, and they must be validated in analyses to minimize changes in the quantity and quality of RNA, reverse transcription efficiency in the PCR steps (Fleige & Pfaffl, 2006; Derveaux et al., 2010). Reference genes, used to normalize mRNA fractions, are often referred to as housekeeping genes which should not vary their expression during development, among tissues or cells, or in response to experimental treatments (de Oliveira et al., 2012).

Although several genes have been routinely used and reported as reference genes in mushroom molecular biology studies, the reference genes for *P. tuber-regium* have not yet been reported. Many studies make use of the housekeeping genes without proper validation of their presumed stability, based on the assumption that they would be constitutively expressed due to their roles in basic cellular processes (de Oliveira et al., 2012). To obtain a precise

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quantification of gene expression, it is necessary to catch hold of stabilized reference genes for the different conditions being researched in *P. tuber-regium*. In this study, 14 candidate reference genes from the transcriptomic data and commonly used reference genes were screened and verified for gene expression research in *P. tuber-regium* different tissues and mycelia which grown under stress treatments of different temperatures, water deficit, and salinity shifts. Four different statistical algorithms were used to obtain a combination of reference genes that can be used for prospective *P. tuber-regium* gene expression studies. To our knowledge, this is the first report on selection and validation of reference genes for RT-qPCR data normalization in *P. tuber-regium*.

2. Materials and methods

2.1. Strain and culture situations

The *P. tuber-regium* strain ACCC 50657, provided by the Laboratory of Food Microbiology, Huazhong Agricultural University, was maintained and sub-cultured on Petri dishes with PDA medium (potato 200 g/L, glucose 20 g/L, agar 20 g/L) in the darkness at room temperature and 32 °C, respectively.

To prepare sclerotia and fruitbodies samples, the culture substrates composed of cottonseed shell 500 g, bran 15 g, gypsum 2.5 g, lime 2.5 g, and H₂O 700 mL were made. The 300 g sterilized culture bags inoculated with 5–10 g matrix covered with vigorous hyphae were incubated at 32 °C for 15 d to make the hyphae overgrown with culture substrates. Then, the sclerotia and fruitbodies were induced on culture substrate covered with nutrient soil (approximately 2.5 L) in the plastic vase (approximately 5.5 L) with round bottom at room temperature with proper humidity. The sclerotia and fruitbodies were grown randomly. The sclerotia were harvested when the diameter reached about 1 cm. The fruitbodies of *P. tuber-regium* were divided into pilei and stipes. To prepare mycelia samples, the PDA medium supplemented with mannitol or NaCl were made. The hyphae grown on PDA medium covered with cellophane in advance were harvested when they were 8 d old under different experimental conditions. 12 samples were selected for this assay, including sclerotia, the stipes of fruitbodies, the pilei of fruitbodies, and 9 mycelia samples grown on PDA medium under different temperatures (25 °C, 28 °C, 32 °C), water deficit (mannitol: 0.1 M, 0.2 M, 0.3 M, 0.4 M), and salinity shifts (NaCl: 0.086 M, 0.171 M).

2.2. Extraction of RNA isolation and cDNA synthesis

The RNA of *P. tuber-regium* was extracted by means of KKFast Plant RNAPure Kit (Zomanbio, Beijing, China) according to the manufacturer's instructions. The quantity and purity of RNA were measured in a NanoPhotometer-N60 (Implen, Munich, German), meanwhile the quality and integrity of the RNA were verified using electrophoresis with 1.0% agarose gel. The OD260/OD280 of the extracted RNA ranged from 1.8 to 2.2, and agarose gel electrophoresis had clear three bands that could be used for subsequent experiments. The cDNA was synthesized by reverse transcription reaction from 1 µg of RNA isolation according to the PrimerScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China).

2.3. Candidate reference gene selection and primer design

Novel candidate reference genes were selected based on differentially expressed genes (DEGs) profiles between newly formed sclerotia (approximately 1 cm diam) and mycelia grown on culture

substrates for 12 d in *P. tuber-regium*. The RNA-seq data used in this study were acquired by the Illumina HiSeq and three biological replicates were performed (unpublished data). The Cufflinks software was employed to estimate gene expression levels by using FPKM values (fragments per kilobase of exon per million fragments mapped) (Trapnell et al., 2010). In addition, six genes commonly used as reference genes mentioned in other literatures were also employed in this study.

Primers used in this study were designed using Primer 3 plus online website (<http://primer3plus.com/cgi-bin/dev/primer3plus-package.cgi>), and the specificity of the product was verified using 1.0% agarose gel electrophoresis and RT-qPCR melting curves. The PCR products were within 80–150 bp range and each primer pair was anchored within exon-exon junction. The relevant information of all primers was listed in Table 1.

2.4. RT-qPCR

The total volume of RT-qPCR reactions was 10 µL, containing 5 µL of TB Green™ Premix Ex Taq™ II (TaKaRa), 0.2 µL (10 µM) of each primer (forward and reverse), 1 µL of cDNA (dilution 1:10 previously defined) and 3.6 µL of ultrapure water. The reactions were performed in QuantStudio™ 6 Flex System (Thermo Fisher Scientific, Waltham, USA) using the following amplification parameters: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, with insertion of the melting curve from 72 to 95 °C, with a heating rate of 1.6 °C/s and a continuous fluorescence measurement. All reactions were performed with three technical repetitions and two biological replications with negative controls.

2.5. Amplification efficiencies

To acquire the PCR amplification efficiencies (E) of each primer pair, standard curves were calculated using a 5-fold serial diluted pMD18-T plasmid (TaKaRa). Every candidate reference gene amplified by PCR was constructed into the plasmid. The E values was ranged (Radonić et al., 2004) and listed in Table 1.

2.6. Data analyses

To select suitable reference genes, three software was recruited to calculate the stability: geNorm (Vandesompele et al., 2002), NormFinder (Andersen, Jensen, & Ørntoft, 2004), and BestKeeper (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004). In addition, RefFinder (<https://www.heartcure.com.au/reffinder>) which is a comprehensive web-based tool that integrates the three applets was also applied to produce a comprehensive ranking of the candidate reference genes.

3. Results

3.1. The selection of candidate reference genes

In this study, the constitutively expressed candidate genes were screened using the following standards: (1) the FPKM values range from 200 to 5000 in all of the tested samples; (2) FPKM value in the sclerotia sample/ FPKM in the mycelia sample approximately equal to 1; (3) P value < 0.5 for each candidate gene; (4) the genes can be annotated in the databases including NR, Swiss-Prot, KOG, KEGG, GO, Pfam, TrEMBL. We filtrated and evaluated the candidate genes of interest with fold-change close to one, indicating stability of transcript levels under those conditions. They are *Transitional endoplasmic reticulum ATPase (ATPase)*, *Short/branched*

Table 1 Candidate genes, primers, product lengths, and amplification efficiencies in the RT-qPCR analysis.

Gene	Gene symbol	Orientation	Sequence (5'→3')	Amplification length (bp)	Amplification efficiency (%)	R ²
<i>α-tubulin</i>	<i>α-tub</i>	Forward	TCACGGCTTCGCTTCGTT	114	103.98	0.99445
		Reverse	TGATAGGGGCAAAGGTTCGC			
<i>β-tubulin</i>	<i>β-tub</i>	Forward	TACACGCAGGAGGTATGGA	137	83.59	0.99865
		Reverse	TCGACAGGCTCTCCTCGTA			
<i>γ-tubulin</i>	<i>γ-tub</i>	Forward	CGGTACAGCTGGGCCAAT	116	90.98	0.99945
		Reverse	TCTCCTCCCTCAGTCGCC			
<i>Glyceraldehyde-3-phosphate dehydrogenase</i>	<i>GAPDH</i>	Forward	AGGCTGTGGCAAGGTCA	147	97.17	0.9996
		Reverse	CCTTGACGACGGCCTTGAT			
<i>Translation initiation factor 5a</i>	<i>Tif-5a</i>	Forward	CTGCCCTTCCACCCACAAT	106	85.57	0.9981
		Reverse	ATTGCCATCCGCGTCAT			
<i>Translation elongation factor 1α</i>	<i>Tef-1α</i>	Forward	CGAGCAGGTGTTCAGG	95	86.12	0.99875
		Reverse	TCTTGGAGTCGGAGGCGA			
<i>Transitional endoplasmic reticulum ATPase</i>	<i>ATPase</i>	Forward	CACGCCGTTCTGTATCCGA	85	86.53	0.9993
		Reverse	TTGTTGCCGAAGCCACGA			
<i>Short/branched chain specific acyl-CoA dehydrogenase</i>	<i>acyl-CoA</i>	Forward	TGGATCCCTCAGTGTCCGT	137	79.14	0.99785
		Reverse	CAAGCAAAACGACCCGAGC			
<i>U2 small nuclear ribonucleoprotein A&apos</i>	<i>U2ribo</i>	Forward	ATCGCGAGTGGCTTGTGT	140	82.02	0.99955
		Reverse	TGATAGCGTCGTAGCGAGC			
<i>Succinate/glutarate-semialdehyde dehydrogenase</i>	<i>S/G</i>	Forward	TGGCGGCTTCGACTTTGA	111	88.5	0.9978
		Reverse	TGCATATGGCCGCTCCTT			
<i>WHI2-like protein</i>	<i>Wlp</i>	Forward	ATTTTCAGCGCTGCAG	131	84.17	0.9989
		Reverse	CTTGAAGGCTCCAATGCGC			
<i>V-type proton ATPase subunit E</i>	<i>VtpAsE</i>	Forward	AGAAGAAGCACAAGGCGT	95	94.17	0.9994
		Reverse	TTCTTCGCGGCGGTGAA			
<i>E3 ubiquitin-protein ligase RBX1</i>	<i>E3upl</i>	Forward	AGCAACCAGTGAAGAGTGCA	88	87.44	0.99805
		Reverse	CCTGGTCTTGAGCCAACGT			
<i>Succinate dehydrogenase (ubiquinone) cytochrome b560 subunit</i>	<i>Sdh</i>	Forward	TTCTGATGCCGCAAGT	123	93.35	0.99965
		Reverse	AAGCCCCTTGACCGTGA			

chain specific acyl-CoA dehydrogenase (*acyl-CoA*), *U2 small nuclear ribonucleoprotein A & apos* (*U2ribo*), *Succinate/glutarate-semialdehyde dehydrogenase* (*S/G*), *WHI2-like protein* (*Wlp*), *V-type proton ATPase subunit E* (*VtpAsE*), *E3 ubiquitin-protein ligase RBX1* (*E3upl*), and *Succinate dehydrogenase (ubiquinone) cytochrome b560 subunit* (*Sdh*). In addition, 6 genes used as reference genes in reported literatures, including *α-tubulin* (*α-tub*), *β-tubulin* (*β-tub*), *γ-tubulin* (*γ-tub*), *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *Translation initiation factor 5a* (*Tif-5a*), and *Translation elongation factor 1α* (*Tef-1α*), were also recruited to normalize RT-qPCR data in this research (Zampieri, Nora, Basso, Camassola, & Dillon, 2014; Castanera, López-Varas, Pisabarro, & Ramírez, 2015). The gene name, gene ID, accession number, function, and FPKM in transcriptome samples of each candidate gene were described in Table 2.

3.2. The specificity and amplification efficiency of each primer pair of candidate reference genes

The expression levels of the 14 candidate reference genes were measured under each of the 12 experimental conditions (see Materials and methods). 14 primer pairs were designed for the selected candidate genes, respectively (Table 1). The agarose gel electrophoresis and a melting curve analysis by RT-qPCR were performed to verify the specificity of the primers used in this study (Fig. 1A, B). The PCR amplification efficiencies for the 14 candidate genes varied from 79.14% (*acyl-CoA*) to 103.98% (*α-tub*), and the correlation coefficients ranged from 0.99445 for *α-tub* to 0.99965 for *Sdh* (Table 1).

3.3. The variances of Ct values among 14 candidate reference genes

For visual comparisons of different RNA transcription abun-

dances, the Ct value was analyzed in the samples directly. The variances of Ct values demonstrated a compact distribution in Fig. 2. According to the fluctuation of Ct value, each of candidate gene showed moderate abundance with a limited range between 14.92 and 24.53 in the tested *P. tuber-regium* samples. *γ-tub* demonstrated equivalent levels of gene expression, while the expression of *GAPDH* seemed to be significantly fluctuated by the treatments. The main programs, including geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004) were employed to analyze the 14 candidate reference genes.

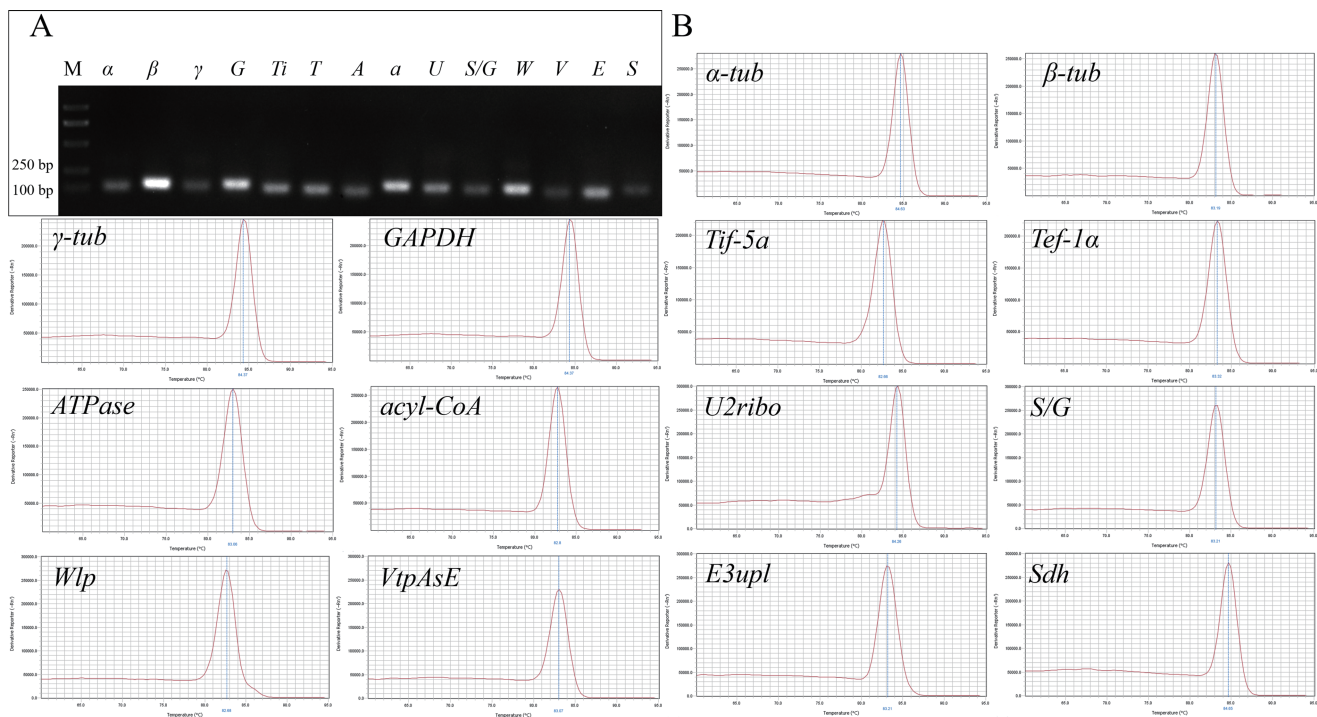
3.4. The prediction of the expression stability of candidate reference genes by geNorm, NormFinder, BestKeeper software

The geNorm tool defined the internal control gene-stability measure *M* as the average pairwise variation of a particular gene with all other control genes (Vandesompele et al., 2002). For all 12 samples tested, *Tif-5a* and *E3upl* were the most stable gene pair, followed by *β-tub*, *ATPase* and *Wlp*. In contrast, *GAPDH* was the least stable candidate gene (Fig. 3). At the same time, the $V_{n/(n+1)}$ value was calculated by geNorm software, and the optimal number of internal reference genes was obtained. In the treatments, data acquired from all 12 samples were analyzed and demonstrated that the *V* value with the inclusion of two genes ($V_{2/3}$) was 0.095, lower than the arbitrary cut off value of 0.15. Therefore, it was enough to select 2 candidate genes as optimal reference gene combination, and the third one was not required for further correction.

The NormFinder analysis strategy provides a direct measure for the estimated expression variation, enabling the user to evaluate the systematic error introduced when using the gene (Andersen et al., 2004). In this analysis, *E3upl* was the most stable internal reference gene, consistent with geNorm analysis, followed by *Sdh*, and the most unstable gene was *GAPDH*.

Table 2 The gene name, gene ID, FPKM in transcriptome samples, and KOG description of each candidate gene used in this study.

Gene	Gene ID	Accession number	FPKM value in RNA-seq mycelia samples			FPKM value in RNA-seq sclerotia samples			Function
			Mycelium 1	Mycelium 2	Mycelium 3	Sclerotium1	Sclerotium2	Sclerotium3	
<i>α-tub</i>	evm.TU.contig_3_pilon.401	MW504066	1,637.05	1,981.61	2,022.09	1,391.35	1,424.07	1,786.9	Cytoskeleton
<i>β-tub</i>	evm.TU.contig_38_pilon.186	MW579480	2,089.67	2,061.52	2,212.61	1,735.01	1,698.92	1,954.91	Cytoskeleton
<i>γ-tub</i>	evm.TU.contig_32_pilon.312	MW579481	40.267	50.9676	43.6822	36.9135	38.2139	29.1037	Cytoskeleton
<i>GAPDH</i>	evm.TU.contig_13_pilon.60	MW579482	1,902.2	2,558.2	2,833.49	946.535	604.03	1,577.32	Carbohydrate transport and metabolism
<i>Tif-5a</i>	evm.TU.contig_13_pilon.46	MW625948	2,778.9	2,904.14	3,921.95	1,925.2	1,914.82	2,169.98	Translation, ribosomal structure and biogenesis
<i>Tef-1α</i>	maker-contig_3_pilon-exonerate_protein2genome-gene-52.4	MW625949	7,981.22	7,587.76	8,554.87	3,889.41	2,804.54	5,091.9	Translation, ribosomal structure and biogenesis
<i>ATPase</i>	evm.TU.contig_15_pilon.85	MW625950	452.421	532.817	546.236	486.035	464.005	583.112	Posttranslational modification, protein turnover, chaperones
<i>acyl-CoA</i>	evm.TU.contig_3_pilon.882	MW625951	444.297	432.19	441.913	449.624	416.271	449.464	Lipid transport and metabolism
<i>U2ribo</i>	maker-contig_3_pilon-exonerate_protein2genome-gene-45.6	MW625952	253.134	262.828	347.344	260.039	309.41	270.064	RNA processing and modification
<i>S/G</i>	evm.TU.contig_27_pilon.218	MW625953	277.18	235.686	269.645	294.402	252.925	258.03	Energy production and conversion
<i>Wlp</i>	maker-contig_25_pilon-exonerate_protein2genome-gene-42.2	MW625954	211.321	222.871	225.605	208.97	212.471	220.273	NA
<i>VtpAsE</i>	evm.TU.contig_27_pilon.329	MW625956	299.824	302.765	374.97	348.827	327.401	297.42	Energy production and conversion
<i>E3upl</i>	maker-contig_32_pilon-exonerate_protein2genome-gene-33.2	MW625957	1,105.04	1,059.68	1,866.21	1,392.85	1,356.21	1,173.37	Posttranslational modification, protein turnover, chaperones
<i>Sdh</i>	evm.TU.contig_6_pilon.48	MW625955	428.991	407.464	572.122	336.315	653.744	378.491	Carbohydrate transport and metabolism

**Fig. 1** The specificity verification of pair-wise primers used in this study. A: 1% agarose gel electrophoresis demonstrated the amplification of a single band of the expected size for the 14 reference genes. B: Melting curves of the 14 candidate reference genes showed single peaks.

BestKeeper was the third method used to evaluate the expression stability of the 14 candidate reference genes. Because it can only analyze no more than 10 genes, we chose the top 10 candidates from the geNorm classification as input data. The selected genes were also within the top 10 in the NormFinder ranking besides *acyl-CoA*. The applet determines the optimal housekeeping gene employing the pair-wise correlation analysis of all pairs of candidate genes and calculates the geometric mean of the best suited ones (Pfaffl et al., 2004), and it is usually employed for preliminary filtration. Similar with the results of the previous two methods, the most consistently expressed gene was *E3upL*, followed by

Sdh, the gene with the most erratic expression was *U2ribo*.

3.5. The prediction of best reference gene by online RefFinder analysis

RefFinder compares and ranks the tested candidate reference genes integrating the currently available major computational programs (Xie, Xiao, Chen, Xu, & Zhang 2012). It was demonstrated that *E3upl* was the best reference gene among all tested samples (Fig. 4). In gene expression analysis, using two or more reference genes in the meantime can reduce systematic bias and help to ob-

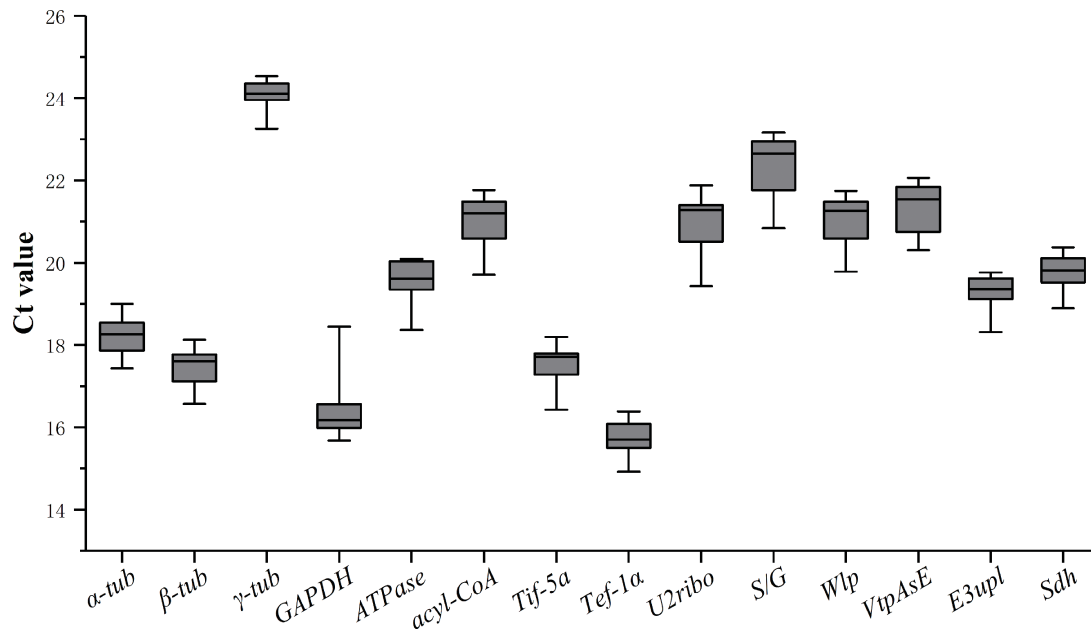


Fig. 2 Variations in cycle threshold (Ct) values of 14 selected candidate reference genes under the 12 experimental conditions.

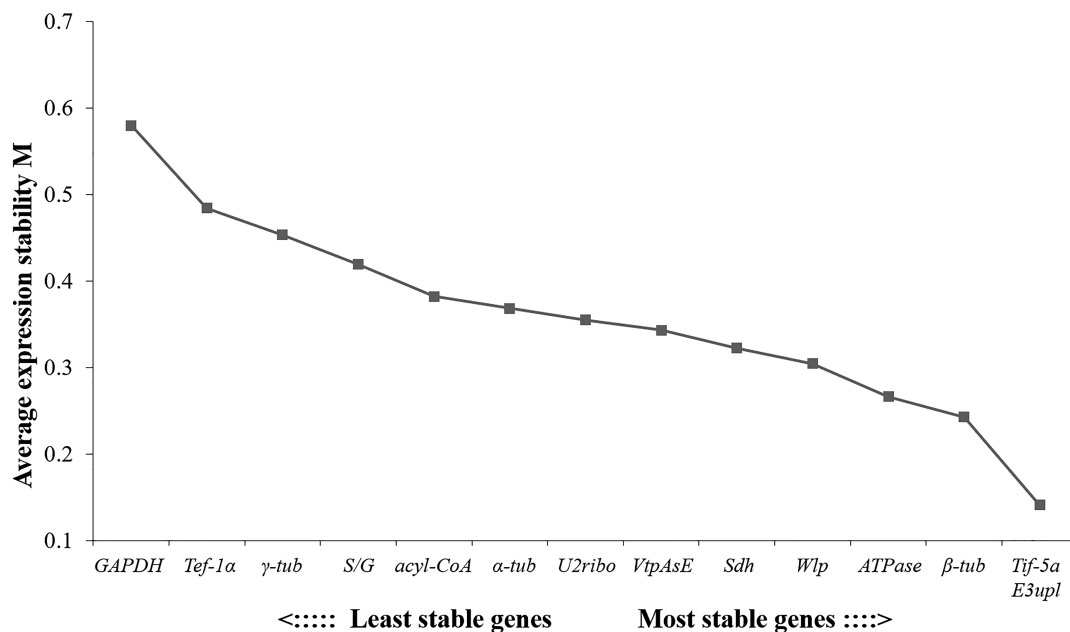


Fig. 3 Average expression stability of the 14 candidate reference genes calculated by the geNorm.

tain more accurate quantitative data of gene expression. Comprehensive analysis of the gene stability revealed that *E3upl* and *Tif-5a* could be used as the optimal reference gene combination among mycelia under different stress responses and different tissues.

3.6. The expression stabilities of candidate reference genes under different conditions

When all 12 samples were considered together, the combination of *E3upl* and *Tif-5a* genes was the most stable for serving as endogenous reference genes by the analyses of four statistical algorithms. Due to RefFinder integrates the currently available major computational programs (geNorm, NormFinder, BestKeeper, and the com-

parative Delta-Ct method) to compare and rank the tested candidate reference genes (Xie et al., 2012), we selected it to detect the expression stabilities of candidate reference genes under four different conditions (Fig. 5). Under different development stages, *β-tub* was the most stable reference gene, while *GAPDH* was considered as the most unstable one (Fig. 5A). For the mycelia samples, *Wlp* showed the most stability under different temperatures (Fig. 5B), *E3upl* showed the most stability under water deficit (Fig. 5C), while *U2ribo* demonstrated the most stability under salinity shifts (Fig. 5D). The *S/G*, *VtpAsE*, and *GAPDH* exhibited the lowest stability, respectively (Fig. 5B–D).

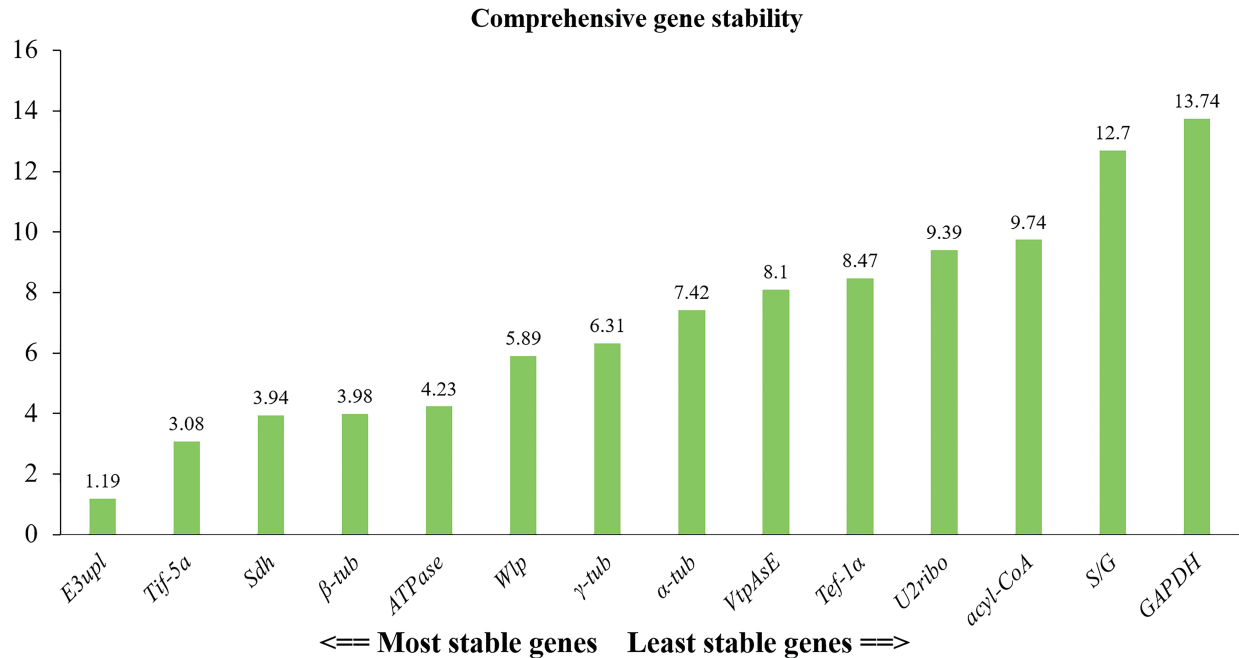


Fig. 4 Comprehensive expression stability of the 14 candidate reference genes calculated by the RefFinder.

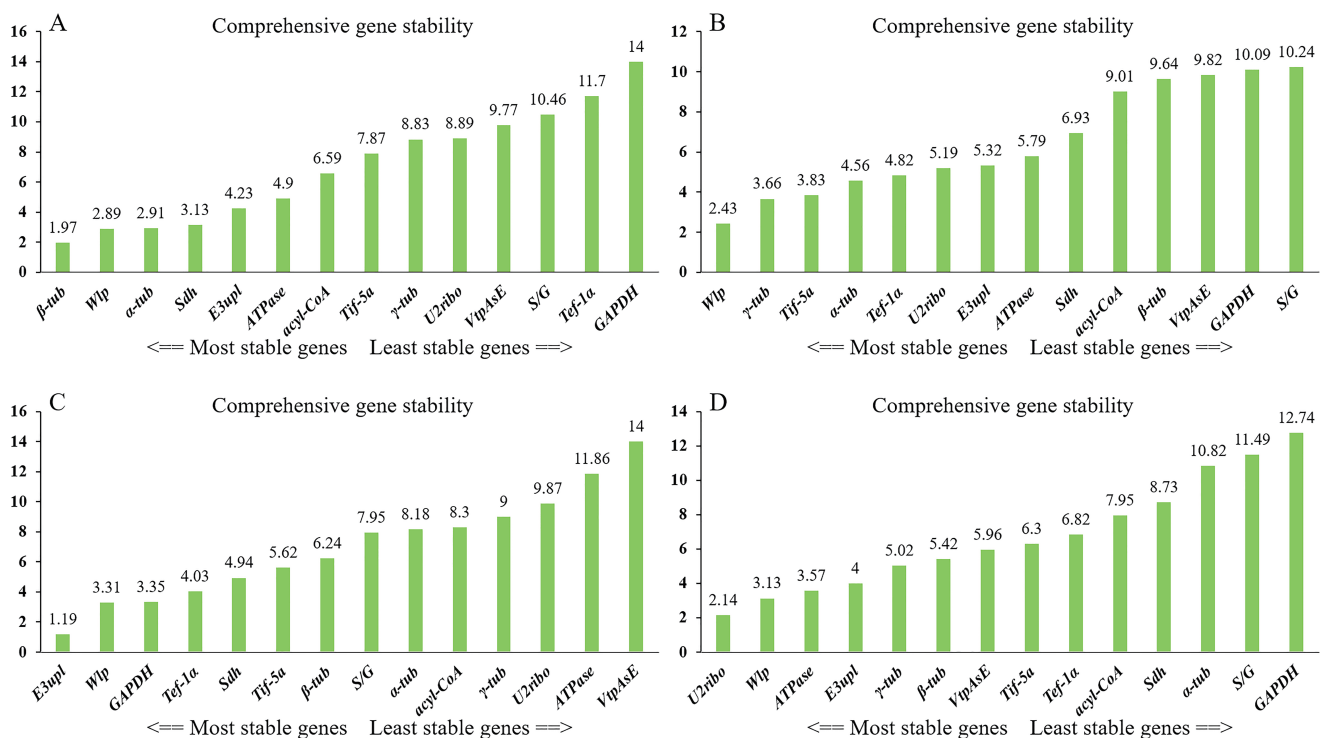


Fig. 5 Expression stability and ranking of candidate reference genes determined by RefFinder. A: Different developmental stages. B: Mycelia under different temperatures. C: Mycelia under water deficit. D: Mycelia under salinity shifts.

4. Discussion

Among various technologies, RT-qPCR is considered to be the most appropriated method for gene expression analyses (Xiang et al., 2018). The expression stability of the same reference gene under different conditions is differential among various species. In order to acquire suitable genes for data normalization in *P. tuber-regium*, 14 candidate reference genes were selected from transcrip-

tom data and internal reference genes commonly used in other fungal species, and measured their expression levels by RT-qPCR among 12 samples. The four statistical algorithms (NormFinder, geNorm, BestKeeper, and RefFinder) have been recruited to select and assess candidate reference genes for RT-qPCR data normalization from different tissues, developmental stages, and stress conditions in various filamentous fungi like *Lentinula edodes* (Xiang et al., 2018; Zhao et al., 2018), *P. ostreatus* (Castanera et al., 2015), and

Table 3 Ranking of *Pleurotus tuber-regium* candidate reference genes according to their expression stability, analyzed by geNorm, NormFinder, BestKeeper, and RefFinder for 12 experimental conditions.

Rank	Gene expression stability			
	GeNorm	NormFinder	BestKeeper	RefFinder
1	<i>E3upl/Tif-5a</i>	<i>E3upl</i>	<i>E3upl</i>	<i>E3upl</i>
2	<i>β-tub</i>	<i>Sdh</i>	<i>Sdh</i>	<i>Tif-5a</i>
3	<i>ATPase</i>	<i>Tif-5a</i>	<i>Tif-5a</i>	<i>Sdh</i>
4	<i>Wlp</i>	<i>α-tub</i>	<i>α-tub</i>	<i>β-tub</i>
5	<i>Sdh</i>	<i>ATPase</i>	<i>β-tub</i>	<i>ATPase</i>
6	<i>VtpAsE</i>	<i>Wlp</i>	<i>ATPase</i>	<i>Wlp</i>
7	<i>U2ribo</i>	<i>α-tub</i>	<i>acyl-CoA</i>	<i>γ-tub</i>
8	<i>α-tub</i>	<i>ATPase</i>	<i>Wlp</i>	<i>α-tub</i>
9	<i>acyl-CoA</i>	<i>VtpAsE</i>	<i>VtpAsE</i>	<i>VtpAsE</i>
10	<i>S/G</i>	<i>U2ribo</i>	<i>U2ribo</i>	<i>Tef-1α</i>
11	<i>γ-tub</i>	<i>acyl-CoA</i>		<i>U2ribo</i>
12	<i>Tef-1α</i>	<i>γ-tub</i>		<i>acyl-CoA</i>
13	<i>GAPDH</i>	<i>Tef-1α</i>		<i>S/G</i>
14		<i>GAPDH</i>		<i>GAPDH</i>

Ganoderma lucidum (Xu et al., 2014).

In the current study, the selected 14 candidate reference genes exhibited a wide range of expression profiles under experimental conditions (Fig. 2), demonstrating that no single gene had a consistent expression profile. The expression stability of each candidate reference gene is different according to different test conditions. Each reference gene was ranked with respect to stability using the four tests (Table 3). In general, *E3upl* and *Tif-5a* were identified as the stable gene combination for various stress conditions and different samples by comprehensive statistical analysis, and was also recommended as the best reference gene combination for RT-qPCR data normalization in the tested samples in *P. tuber-regium*. The results demonstrated that there was a relatively good correlation among the results obtained from the four analysis approaches, despite the fact that the fundamental difference in the algorithms implemented by the four software. Particularly, *E3upl*, *Wlp*, and *U2ribo* were the most consistently expressed genes in mycelia under water deficit, different temperatures, and salinity shifts, respectively. While *β-tub* was the most stably expressed gene in different tissues. *GAPDH* was the most unstable gene in different tissues. *S/G*, *VtpAsE*, and *GAPDH* were the most unstable reference genes in mycelia under different temperatures, water deficit, and salinity shifts, respectively. In general, *E3upl* and *Tif-5a* were identified as the stable gene combination for various stress conditions and different samples by comprehensive statistical analysis, and was also recommended as the best reference gene combination for RT-qPCR data normalization in the all samples in *P. tuber-regium*.

5. Conclusions

In summary, the *E3upl* and *Tif-5a* were recommended as the best reference gene combination for RT-qPCR data analyses among the research samples in *P. tuber-regium*. This research provides basic background information with respect to reference gene selection and utilization for RT-qPCR studies in *P. tuber-regium*. The study will contribute to sequencing data and gene function analyses.

Conflicts of Interest

No conflicts of interest among researchers.

Author's contribution

All authors contributed to this study. XS and AM had the ideas for the article. AM guided the experiments. XS performed the experiments, and wrote the manuscript. JW, CM and AM critically reviewed the manuscript.

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