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ORIGINAL RESEARCH

Reference gene selection for real-time quantitative PCR assays in different tissues of *Huperzia serrata* based on full-length transcriptome sequencing

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Abstract

Huperzia serrata (*H. serrata*) produces various types of effective lycopodium alkaloids, especially Huperzine A (HupA), which is a promising drug for the treatment of Alzheimer's disease. Numerous studies focused on the chemistry, bioactivities, toxicology, and clinical trials of HupA; however, the public genomic and transcriptomic resources are very limited for *H. serrata* research, especially for the selection of optimum reference genes. Based on the full-length transcriptome datasets and previous studies, 10 traditional and three new candidate reference genes were selected in different tissue of *H. serrata*. Then, two optimal reference genes *GAPDHB* and *HisH2A* were confirmed by four analysis methods. In order to further verify the accuracy of the two reference genes, they were used to analyze the expression patterns of four HupA-biosynthetic genes (lysine decarboxylas, RS-norcoclaurine 6-O-methyltransferase, cytochrome P45072A1, and copper amine oxidase). The data suggested that the expression pattern of HupA-biosynthetic genes was consistent with them in transcriptome sequencing in different tissue of *H. serrata*. This study identified that *GAPDHB* and *HisH2A* provides the reliable normalization for analyzing the HupA biosynthetic gene expression in different tissues of *H. serrata* on the transcriptional level.

KEYWORDS
Huperzia serrata, Huperzine A, reference gene, RT-qPCR

1 | INTRODUCTION

Huperzia serrata (*H. serrata*) belongs to the *Huperzia* genus, Lycopodiaceae order. The whole plant of *H. serrata* has been used as a medicine in China to treat different kinds of ailments, including bruises, strains, swelling, rheumatism, schizophrenia, myasthenia gravis, and fever since 739 (during the Tang Dynasty) (Ferreira et al., 2016). *H. serrata* has been widely known as a medicinal plant since Chinese scientists isolated Huperzine A (HupA) from it during the 1980s (Liu et al., 1986). HupA is a promising candidate drug for treating Alzheimer's disease (AD), it could improve cognitive function, daily living activity and global clinical assessment in patients for AD disease, with relatively few and mild adverse effects (Qian & Ke, 2014; Yang et al., 2013). However, *H. serrata* is a scarce species and grows very slowly in specialized habitats. Furthermore, the HupA content is very low in *H. serrata* (Ma et al., 2007). At present, the

rapidly growing demand has put *H. serrata* resources on the brink of extinction. Although a lot of efforts have been focused on artificial culture and tissues culture for *H. serrata* production, the outcomes were unsatisfactory.

Now, researchers try to improve HupA content by studying the gene information of HupA biosynthesis. However, the public genomic and transcriptomic resources are very limited. Only two papers focused on transcriptomic resources (André et al., 2010; Yang et al., 2017). Real-time quantitative PCR (RT-qPCR) has been widely used in gene expression measurement on transcriptional level. Identification of suitable reference genes (RGs) is pre-requisite for RT-qPCR assays (Bansal et al., 2015; Vandesompele et al., 2002). Many housekeeping genes have been used as RGs under different experimental conditions, such as actin, tubulin, elongation factor (*EF*), 18S ribosomal RNA (*18S* rRNA), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), histone, and ubiquitin (Fei et al., 2018). However, there is no RG suitable to all biological systems based on previous studies. So, researchers reach a consensus that specific RG for a given species and treatment needs to be identified first (Bansal et al., 2015). Unfortunately, the previously reported RGs are not suitable for the research of HupA-biosynthetic genes in our materials (Yang et al., 2019).

In present study, in order to obtain the optimal RGs for studying HupA-biosynthesis, we detected the concentration of HupA and carried out full-length transcriptome sequencing for the different tissues of *H. serrata*. Based on full-length transcriptome sequencing data and previous studies, 13 candidate RGs were selected. Finally, *GAPDHB* and *HisH2A* stood out among the 13 candidate RGs and became the best combination for normalization in different tissues of *H. serrata* by four analysis methods (geNorm, NormFinder, BestKeeper, and RefFinder). Using *GAPDHB* and *HisH2A* as RGs, the expression profile of four HupA-biosynthetic genes, lysine decarboxylase (*LDC*), RS-norcoclaurine 6-O-methyltransferase (*MET*), cytochrome P45072A1 (*CYP*), and copper amine oxidase (*CAO*) showed the similar expression trend between transcriptome sequencing and RT-qPCR. This result further confirmed that *GAPDHB* and *HisH2A* were suitable for HupA-biosynthetic gene expression normalization. This work provides suitable RGs for the subsequent research of HupA-biosynthesis in *H. serrata*.

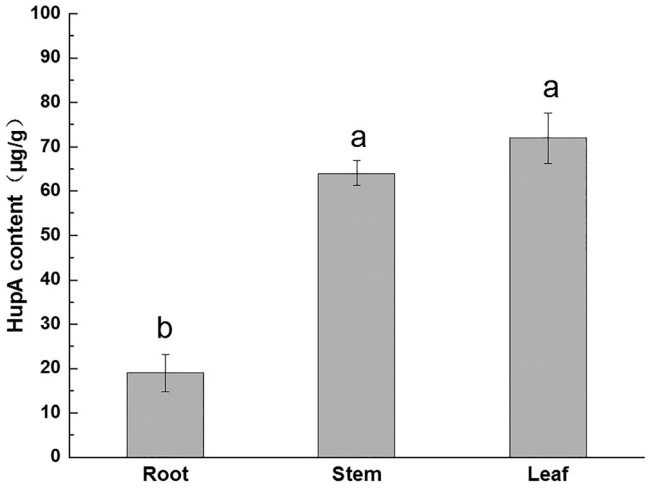

FIGURE 1 The content of HupA in different tissues of *H. serrata*. The mean and standard deviation were calculated by three independent biological replicates

2 | RESULTS

2.1 | The Hup A content analyses

HPLC-UV was performed to detect the HupA content in *H. serrata*. Typical chromatograms from HupA standard and three tested samples are shown in Figure S1, indicating that HupA was well separated from different tissues. A linear relationship exists between the peak area (measured at 308 nm) and the concentration of HupA in the sample injected into the HPLC. The results showed that there was obvious difference between the HupA content in different tissues. The highest HupA concentration (72 μg/g) was obtained in the leaves of *H. serrata*. The lowest content (19 μg/g) of HupA was found in root tissues of *H. serrata* (Figure 1).

2.2 | The screening of candidate RGs

Given the variability of Hup A concentrations in different tissues, the root, stem, and leaf samples were collected and applied to the full-length transcriptome sequencing by Nanopore. After assembly, 43,443 unigenes were retrieved. CPM (counts per million) is the index for measuring the expression of unigenes. Based on the CPM value analysis, 19,400 genes were obtained with similar and stably expression in three tissues. Combining with reported literatures, 10 traditional and three new candidate RGs were chosen. The three new candidate RGs were annotated as hypothetical or uncharacterized proteins by NCBI Nr database, more importantly, they were never used as RGs before. The detail information of total 13 candidate RGs was showed in Table 1.

2.3 | Verification of the primer specificity and RT-qPCR amplification efficiency

The primer information of 13 candidate RGs was given in Table 2. Each primer pair was designed outside of the conserved domains to secure the specificity (Figure S2). Initially, the agarose gel electrophoresis yielded a specific fragment of expected size (Figure S3). Further, the melting curve in the RT-qPCR reaction showed the single peak for each primer pair indicating absence of non-specific amplification (Figure S4). For all primer pairs, the amplification efficiencies were spanning from 90.4% to 103.6%, and the correlation coefficient (R^2) were greater than .990 (Table 2). Taken together, these results indicated each primer pair was specificity and the RT-qPCR assays were highly efficient.

TABLE 1 Description of candidate RGs and target genes

Unigene gene ID	Accession number	Length (bp)	Gene symbol	Gene name	Homolog locus	Root_CPM	Stem_CPM	Leaf_CPM	*E* value[a]
EVM0025399	MH560040	1041	*Actin4*	Actin-related protein 4	KM496528	24.88	39.46	30.90	1e−147
EVM0014845	MH560041	1704	*Actin7*	Actin-related protein 7-like	XM_024546532.1	20.90	24.57	31.48	0
EVM0006291	MH560042	2444	*EF1dt*	Elongation factor 1-delta 2-like	XM_004507134.3	205.15	193.46	160.29	1e−78
EVM0021957	MH560043	3747	*EFTS*	Elongation factor Ts family protein	NM_119050.3	1.79	4.28	2.53	0
EVM0027572	MH560044	2137	*a-tub3*	Tubulin alpha-3 chain	XM_020240093.1	16.55	18.42	23.00	0
EVM0033890	MH560045	1698	*GAPDHB*	Glyceraldehyde-3-phosphate dehydrogenase B	NM_001302308.1	36.73	44.16	35.66	0
EVM0008093	MH560046	1008	*HisH3.3*	Histone H3.3 isoform X1	XM_017375185.1	77.28	62.50	75.11	2e−91
EVM0031551	MH560047	1098	*HisH2A*	Histone H2A-III-like	XM_024521943.1	64.68	81.59	50.83	3e−37
EVM0033477	MH560048	906	*UBQ1*	Ubiquitin-NEDD8-like protein RUB1	XM_007048546.2	65.34	89.70	52.61	2e−89
EVM0000551	MH560049	948	*UBQ11*	Ubiquitin 11	NM_001203752.2	314.46	421.33	369.47	2e−73
ONT.10684	MZ042629	817	10684	Uncharacterized protein	XP_010251846	117.85	116.38	111.58	2e−17
EVM0022608	MZ042627	2036	22608	Hypothetical protein	PTQ36569	34.00	29.033	32.86	5e−99
EVM0017784	MZ042628	1605	17784	Uncharacterized protein	XP_002972660	3.15	2.55	3.68	0
EVM0027909	GO914645	637	*LDC*	Lysine decarboxylase	GO914645	2.20	47.53	53.52	9e−128
EVM0017005	GO914756	1682	MET	(RS)-norcoclaurine 6-O-methyltransferase	GO914756	2.82	13.46	10.48	0
EVM0022835	GO914428	1975	CYP	Cytochrome P450 72A1	GO914428	.30	21.94	30.97	0
EVM0024797	JN247732	2640	*CAO*	Copper amine oxidase	JN247732	4.11	13.86	39.70	0

[a]*E* value represents high homology of candidate RGs with stable reference genes in other plants using the local Blast program.

TABLE 2 Selected candidate RGs and target genes, primers, and amplicon characteristics

Name	Sequence 5′ to 3′	Amplicon size (bp)	Product Tm (°C)	*E* (%)	R^2
Actin4-RT-F	TGTCTCTAAAGTTTCTTGTAGCACC	174	77.5	99.6	.997
Actin4-RT-R	GCACAGCGGACAAGACTCTG				
Actin7-RT-F	AACCCTTATCTGTAGGCTTCTTG	139	78.5–79	95.7	.998
Actin7-RT-R	TCATACACTGCACGTCAGGTAG				
EF1dt-RT-F	GACTGAGCAAATAAGAGGGG	213	75.5–76	91.7	.997
EF1dt-RT-R	CGATATTGCGGCTTTTAACA				
EFTS-RT-F	AAGTATCCTAATACAGGGTTGG	158	75–75.5	92.5	.997
EFTS-RT-R	AAAATTTGGTCTATCGCG				
a-tub3-RT-F	AAATCCAAACAATATGTATGAACAA	210	80–80.5	94.8	.997
a-tub3-RT-R	GCCAAGGGTTTCAATCTTCTA				
GAPDHB-RT-F	GCAAAGTATATGAAGATTAGGCTC	161	76.5–77.5	91.3	.998
GAPDHB-RT-R	GCGTCCACCAACGAACA				
HisH3.3-RT-F	AACTTGGTCTTGCTATGAAACTAAC	212	77–77.5	90.4	.996
HisH3.3-RT-R	CAAGTCAAGAAACTCAACACGA				
HisH2A-RT-F	CTTCCTGCTTTCATCACTTT	270	75.5–76	92.2	.994
HisH2A-RT-R	GTCCCCAAGCCTTACATT				
UBQ1-RT-F	GTGGCGGTCACTTATAGAGAG	130	73–73.5	97.3	.997
UBQ1-RT-R	CTTCTGTAGTTCTGACATCAGTAAA				
UBQ11-RT-F	TCTGAAATGTCGCTTATCCG	174	76.5–77	97.4	.995
UBQ11-RT-R	TCTGTTGGCGTCATTTGTTAG				
10684-RT-F	GCGCTTGATAAGTCACATGCTAC	253	76	94.3	.995
10684-RT-R	GAAAAAAAAGATTGCCATAATAAGG				
22608-RT-F	TGGCGAATTTAGAGGGCAAT	198	77.5	95.1	.998
22608-RT-R	CCTCAAGCCCATTTTAATTTCTCT				
17784-RT-F	AATATGCCCACAGGGTCACC	212	75	93.8	.996
17784-RT-R	GTTGACCACTTGGCTTCCTACC				
LDC-F	GTATAGCCAATTACTCTATCCTCC	155	73–73.5	91.6	.997
LDC-R	GTAACACCCATCCATTGTAGC				
MET-F	GCTCATCTCTGGGACATGG	237	80.5	98.2	.996
MET-R	ACCCGACACTGAATCCTCTAT				
CYP-F	AAGAGTCAGGCTCTACTGTGC	151	78–79	91.4	.998
CYP-R	GCAAGGAAGAACGTCGAGA				
CAO-F	CAAAGCTTGGAATTACGCTT	174	81	94.7	.997
CAO-R	TTATATGTCTTGGCTAGTGTAAATG				

Note: *E* represents amplification efficiencies, and R^2 represents correlation coefficient of RGs.

2.4 | Expression profiles of candidate RGs

The expression profiles of RT-qPCR products were shown in Figure 2. The results illustrated that the mean Ct values of all RGs ranged from 24.04 to 29.43. Lower Ct value indicates the higher expression abundance, conversely means the lower expression abundance. *EF1dt* and *UBQ1* were highly expressed with mean Ct values between 24.04 and 24.08, whereas *EFTS* was the least expressed gene on account of it's the highest mean Ct value (29.43). All candidate genes showed expression variability in different samples. *GAPDHB* and *EFTS* showed relatively smaller variation (<2 cycles), whereas others like *UBQ11* had higher expression variation (3.07 cycles). The results indicated that there was still variable expression even for relative stable housekeeping genes.

2.5 | geNorm analysis

geNorm algorithm calculates the M values to evaluate the expression stability of each RG. The lower M value indicates the more stable gene expression, and vice versa. As Figure 3 shown, each M value was less than 1.5, which demonstrated all candidate RGs were

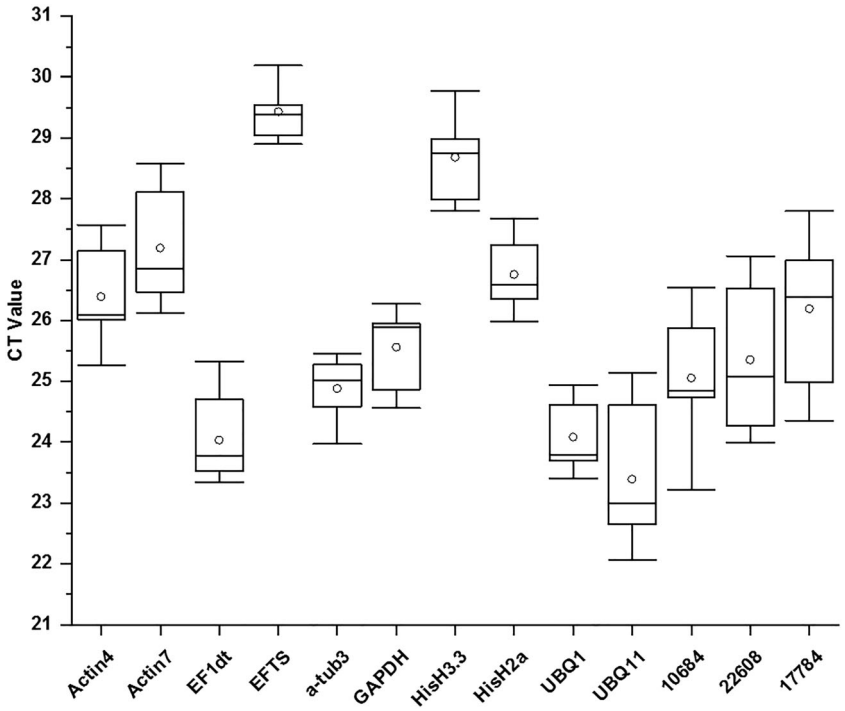

FIGURE 2 Distribution of Ct values for 13 candidate reference genes in different tissues of *H. serrata*. Lines across the boxes denote the medians. The box represents the 25th and 75th percentile. The top and bottom whisker caps depict the maximum and minimum values, respectively. The white dots represent mean Ct values

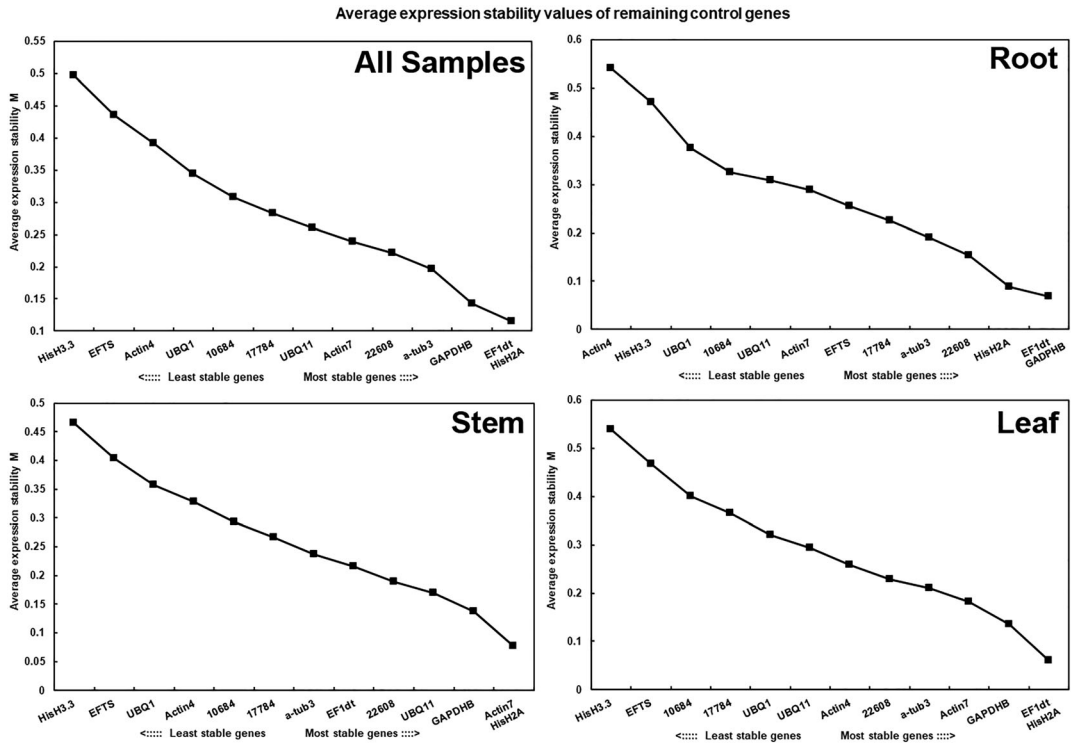

FIGURE 3 Average expression stability value (M) and ranking of the 13 candidate reference genes analyzed by geNorm. (a) All samples. (b) Root. (c) Stem. (d) Leaf. The least stable genes are listed on the left, whereas the most stable genes are exhibited on the right

suitable for normalization in different tissues of *H. serrata*. Among them, *EF1dt*, *HisH2A* and *GAPDHB* were the most stable genes in each *H. serrata* samples, whereas *HisH3.3* and *EFTS* were the least stable genes.

2.5.1 | NormFinder analysis

NormFinder evaluates the stability of each RG via the stability value. The smaller stability value, the more stable gene, and vice versa. As

TABLE 3 Expression stability of candidate RGs as calculated by Normfinder

	All		Root		Stem		Leaf	
Rank	Gene name	Stability value	Gene name	Stability value	Gene name	Stability value	Gene name	Stability value
1	*GAPDHB*	.030	*HisH2A*	.042	*GAPDHB*	.045	*EF1dt*	.014
2	*HisH2A*	.044	*a-tub3*	.046	*HisH2A*	.096	*HisH2A*	.017
3	*EF1dt*	.060	*GAPDHB*	.086	*EF1dt*	.131	*GAPDHB*	.024
4	*22608*	.069	*Actin4*	.108	*22608*	.156	*22608*	.064
5	*a-tub3*	.077	*22608*	.108	*10684*	.174	*UBQ1*	.083
6	*10684*	.077	*UBQ1*	0.109	*a-tub3*	.189	*10684*	.089
7	*Actin4*	.078	*10684*	.113	*UBQ11*	.228	*17784*	.089
8	*17784*	.094	*UBQ11*	0.116	*17784*	.241	*Actin4*	.090
9	*UBQ1*	.104	*17784*	.151	*Actin7*	.257	*a-tub3*	.165
10	*UBQ11*	0.109	*EF1dt*	.167	*Actin4*	.280	*HisH3.3*	.217
11	*Actin7*	.160	*Actin7*	.211	*EFTS*	.281	*UBQ11*	.224
12	*HisH3.3*	.166	*HisH3.3*	.287	*UBQ1*	.348	*EFTS*	.305
13	*EFTS*	.210	*EFTS*	.506	*HisH3.3*	.358	*Actin7*	.364

TABLE 4 Expression stability of candidate RGs as calculated by BestKeeper

	All			Root			Stem			Leaf		
Rank	Gene	CV	SD	Gene	CV	SD	Gene	CV	SD	Gene	CV	SD
1	*GAPDHB*	7.48	.07	*HisH2A*	4.99	.05	*GAPDHB*	5.36	.05	*GAPDHB*	8.38	.07
2	*HisH2A*	9.71	.09	*UBQ11*	6.14	.06	*EF1dt*	10.46	.09	*EF1dt*	9.59	.09
3	*EF1dt*	11.83	0.10	*a-tub3*	7.96	.07	*HisH2A*	10.83	.09	*HisH2A*	1.19	.09
4	*a-tub3*	12.44	0.11	*22608*	8.06	.167	*a-tub3*	15.35	.12	*a-tub3*	10.63	.09
5	*22608*	12.67	.24	*UBQ1*	8.28	.07	*22608*	15.61	.21	*22608*	10.69	.23
6	*10684*	12.97	.18	*GAPDHB*	8.57	.08	*UBQ11*	16.02	.14	*10684*	10.73	.18
7	*UBQ1*	13.07	0.11	*10684*	9.17	.21	*10684*	16.21	.20	*Actin4*	10.78	0.10
8	*17784*	13.14	.19	*17784*	9.84	.19	*Actin7*	16.86	0.13	*17784*	11.68	.22
9	*UBQ11*	13.19	0.11	*Actin4*	9.94	.09	*17784*	16.95	.17	*UBQ1*	12.17	0.10
10	*Actin4*	14.44	.12	*Actin7*	13.63	0.11	*EFTS*	17.20	.15	*EFTS*	14.85	0.13
11	*Actin7*	16.84	0.13	*EF1dt*	14.68	.12	*Actin4*	18.36	.14	*UBQ11*	16.04	0.13
12	*EFTS*	20.13	.17	*HisH3.3*	25.81	.19	*UBQ1*	20.46	.16	*HisH3.3*	19.47	.17
13	*HisH3.3*	27.35	.21	*EFTS*	26.12	.20	*HisH3.3*	28.66	.20	*Actin7*	20.31	.16

Note: CV and SD represent coefficient of variation and standard deviation, respectively.

shown in Table 3, *GAPDHB*, *HisH2A* and *EF1dt* were the lowest stability value almost in every sample, whereas *EFTS*, *HisH3.3*, and *Actin7* were the highest stability value. The stability of some genes was variable, such as *a-tub3* and *UBQ1*. *a-tub3* (stability value = .046) was the one of the top three stable genes in root samples, but outside of top three in other samples. *UBQ1* (stability value = .348) was the lowest stable genes in stem samples, but it was relative stable in other samples. Overall, by NormFinder analysis, *GAPDHB*, *HisH2A* and *EF1dt* were the most stable genes, whereas *EFTS*, *HisH3.3*, and *Actin7* were the least stable genes in different tissues of *H. serrata*. This result was similar with geNorm analysis.

2.6 | BestKeeper analysis

BestKeeper evaluates the expression stability of RGs via the CV and SD. A lower CV value indicates more stable expression (Pfaffl et al., 2004). The analytic results showed that *GAPDHB*, *EF1dt* and *HisH2A* were the top three genes with lowest CV ± SD values in all, stem and leaf samples except in root samples (Table 4). In root samples, the top three genes were *HisH2A*, *UBQ11* and *a-tublin*. However, *HisH3.3* was the least stable RG with higher CV ± SD value (27.35 ± .21, 25.81 ± .19, 28.66 ± .20 and 19.47 ± .17) in every samples. Taken together, by BestKeeper analysis, *GAPDHB*, *EF1dt* and

HisH2A were the most stable genes, whereas *HisH3.3* was the least stable gene.

2.7 | RefFinder analysis

Although the results (geNorm analysis, NormFinder, and BestKeeper analysis) were similar, it was not strictly consistent. Therefore, we evaluated the comprehensive rank using RefFinder (Table 5). In root tissues, the final ranking calculations based on the RefFinder found *HisH2A* (GM = 1.67), *GAPDHB* (3.33) and *a-tub3* (3.33) were the best genes. For stem samples, the top three stable RGs were *GAPDHB* (1.67), *EF1dt* (2.00) and *HisH2A* (3.67), whereas *EF1dt* (1.33), *HisH2A* (2.00), and *GAPDHB* (2.33) in leaf samples. Across all samples, the top three stable RGs were *GAPDHB* (1.67), *HisH2A* (1.67) and *EF1dt* (2.33). On the other hand, *HisH3.3* and *EFTS* were the least stable genes (Table 5).

2.8 | Optimal number of RGs for normalization

One single and stable RG is sufficient for quantifying gene expression, however, more than one RG for effective normalization is more suggested (Vandesompele et al., 2002). Based on geNorm analysis, the optimal number of RGs was determined by pairwise variation Vn/n + 1. The threshold of Vn/n + 1 is 0.15. In our data, the pairwise variation of V2/3 values was lower than 0.15 (Figure 4), which suggested that two RGs were optimum number.

TABLE 5 Expression stability ranking of the 13 candidate RGs by RefFinder

	All		Root		Stem		Leaf	
Rank	Gene	GM	Gene	GM	Gene	GM	Gene	GM
1	*GAPDHB*	1.67	*HisH2A*	1.67	*GAPDHB*	1.67	*EF1dt*	1.33
2	*HisH2A*	1.67	*GAPDHB*	3.33	*EF1dt*	2.00	*HisH2A*	2.00
3	*EF1dt*	2.33	*a-tub3*	3.33	*HisH2A*	3.67	*GAPDHB*	2.33
4	*a-tub3*	4.33	*22608*	4.00	*22608*	4.67	*22608*	5.00
5	*22608*	4.67	*UBQ11*	6.33	*a-tub3*	5.67	*a-tub3*	6.00
6	*10684*	6.67	*EF1dt*	7.33	*UBQ11*	5.67	*Actin4*	7.33
7	*17784*	8.00	*UBQ1*	7.33	*Actin7*	6.00	*UBQ1*	7.67
8	*UBQ1*	8.67	*17784*	7.67	*10684*	7.00	*10684*	7.67
9	*UBQ11*	8.67	*10684*	8.00	*17784*	8.33	*17784*	8.00
10	*Actin4*	9.33	*Actin4*	8.67	*Actin4*	10.33	*UBQ11*	10.00
11	*Actin7*	9.33	*Actin7*	9.67	*EFTS*	11	*Actin7*	10.00
12	*EFTS*	12.33	*EFTS*	11	*UBQ1*	11.67	*EFTS*	11.33
13	*HisH3.3*	12.67	*HisH3.3*	12	*HisH3.3*	13	*HisH3.3*	11.67

Note: GM represents geometric mean.

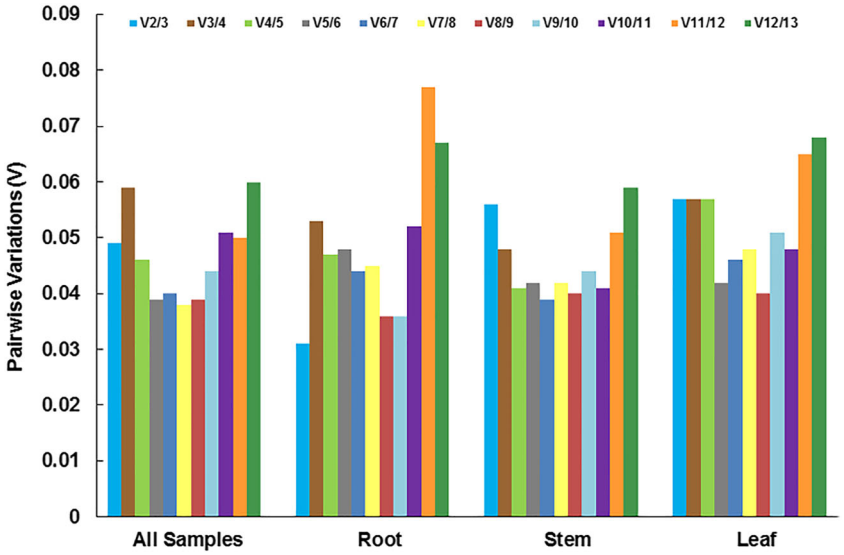

FIGURE 4 Pairwise variation (Vn/n + 1) of 13 candidate reference genes calculated by geNorm. The threshold of determining the optimal number of RGs for qRT-PCR normalization is .15

2.8.1 | Comprehensive stability analysis of RGs

Table 6 was used to compare the results of four analysis methods (geNorm, NormFinder, BestKeeper, and RefFinder). The ranking of stability based on four analysis methods were basically consistent. By comprehensive analysis, *GAPDHB* and *HisH2A* were the best combination for normalization in different tissues of *H. serrata*. On the other hand, *HisH3.3* and *EFTS* were the least stable genes.

2.9 | RG validation

To demonstrate the utility of identified stable RGs, four HupA-biosynthetic related genes *LDC*, *MET CYP*, and *CAO* were selected. For comparison, expression values of target genes were normalized with the most stable gene pair (*GAPDHB* and *HisH2A*) and the least stable gene pair (*EFTS* and *HisH3.3*) in different tissues of *H. serrata*. When normalized with the most stable genes, *LDC*, *MET*, *CYP*, and *CAO* had tissue specific expression (over twofold) in leaf. Furthermore, the expression trend was consistent with that of transcriptome sequencing dates (Figure 5**)**. By contrast, when normalized using the least stable genes, the expression trend was not consistent. The transcription level of *MET* and *CYP* were not up-regulated (less than twofold) in stem and leaf tissues. The transcription level of *LDC* was down-regulated (0.77-fold) in stem tissues, and the *CAO* was down-regulated (0.67-fold) in leaf tissues. In all, the expression of the most stable gene pair was more reliable than the least stable gene pair.

3 | DISCUSSION

H. serrata has received extensive concern due to its ability to produce lycopodium alkaloids, especially HupA (Christenhusz et al., 2011). HupA was found to possess potent acetylcholine esterase inhibition (AChEI) and had been clinically exploited for the treatment of AD. More studies were focused on the isolation and identification of compounds and endophytic bacteria (Wu et al., 2017), but little on the transcription of HupA-biosynthetic genes. Especially for the selection of optimum reference genes, little research has been reported. TATA binding protein (TBP) and GAPDH were identified as reference genes for *H. serrata* in Yang et al.'s (2019) report. Unfortunately, TBP gene could not be amplified when we study the expression of HupA-biosynthetic genes in our materials. So, in the present study, we screened and selected the new optimal RGs based on full-length transcriptome sequencing and previous researches. Based on the four analysis methods, we obtained two optimal reference genes *GAPDHB* and *HisH2A* for studying HupA-biosynthetic genes, and their reliability was confirmed via testing the expression profile of four HupA-biosynthetic genes (*LDC*, *MET*, *CYP*, and *CAO*). This study provides suitable normalization for analyzing the expression of HupA-biosynthetic gene. In addition, we found the expression trend of HupA-biosynthetic genes correlated with the HupA production in different tissues of *H. serrata*. This result will provide the information for further studying the biosynthesis and transportation of HupA.

In general, the expression level of RGs should be constantly stable in any physical conditions. However, there is no RG suitable to all biological systems. We had to screen the most suitable RGs for studying the HupA-biosynthesis. Based on the CPM value of transcriptome sequencing and reported literatures (Chen et al., 2019; Dudziak et al., 2020; Wu et al., 2020), 10 traditional and three new candidate RGs were chosen (Table 1**)**. Especially for three new candidate RGs, they had stable expression in full-length transcriptome sequencing but have not been used as RGs before. This is a new attempt distinguishing from previous studies. Thirteen candidate genes were also enough to ensure the experimental accuracy.

The primer specificity is the primary condition of RT-qPCR. The ideal primers should cross intron regions to availably avoid genomic contamination and cannot be set in conservative domain. First, each primer pair was designed outside of the conserved domains (Figure S2). Subsequently, the products of each primer pair were detected by agarose gel electrophoresis (Figure S3) and melting curves (Figure S4). The results indicated that there were no primer dimers and nonspecific amplification for each primer pair. Furthermore, the *E* value of PCR varied from 90.4% to 103.6%, and all of the R^2 were larger than .990 (Table 2), which were similar to previous literatures (Liu et al., 2018). In conclusion, these results indicated each primer pair had specificity and the RT-qPCR assays were highly efficient.

Based on four analysis methods (geNorm, NormFinder, BestKeeper, and RefFinder), *GAPDHB* and *HisH2A* stood out among the 13 candidate RGs, and became the best combination for normalization in different tissues of *H. serrata* (Table 5). Many studies have shown that *GAPDH* was often applied as stable RG in different tissues or under various experimental conditions (Chen et al., 2017; Zhuang et al., 2015). Especially for *H. serrata*, GAPDH also was proved as stable RG by Yang et al. (2019). TBP was also identified as stable RG for *H. serrata* by Yang et al. (2019). Unfortunately, TBP gene could not be amplified in our materials. This result indicated that expression level of housekeeping gene was different even in the same species and same tissues. Histone and elongation factor were reported as most stable RGs in other species (Zhuang et al., 2015); however, *HisH3.3* and *EFTs* were the most unstable RGs in different tissues of *H. serrata* (Table 6). Interestingly, *HisH2A* and *EF1dt* were the top three stable RGs (Table 6). Similar findings were in previous studies. The expression level of *Actin2/7* was more stable than *Actin11* in diverse tissues of soybean (Jian et al., 2008). These results stated that the expression level and stability of RGs from the same gene family may be different in the same samples. Taken together, the results further proved the necessity for screening suitable RGs in different tissues of *H. serrata*.

A proposed biosynthesis pathway for HupA and related lycopodium alkaloids was reported (Ma & Gang, 2005). However, only two enzymes, *LDC* and *CAO* have been proved to participate in the biosynthesis of HupA (Bunsupa et al., 2012; Sun et al., 2012; Xu et al., 2017). Three enzymes *RS*-norcoclaurine 6-*O*-methyltransferase (*MET*) and cytochrome P45072A1 (*CYP*) (Luo et al., 2010; Xu et al., 2017), type III polyketide synthase (*PKS*) (Wang et al., 2016),

TABLE 6 Expression stability ranking of the 13 candidate RGs by four analysis methods

	Rank												
Method	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
A. Ranking order for all samples													
geNorm	HisH2A	EF1dt	GAPDHB	a-tub3	22608	Actin7	UBQ11	17784	10684	UBQ1	Actin4	EFTS	HisH3.3
NormFinder	GAPDHB	HisH2A	EF1dt	22608	a-tub3	10684	Actin4	17784	UBQ1	UBQ11	Actin7	HisH3.3	EFTS
BestKeeper	GAPDHB	HisH2A	EF1dt	a-tub3	22608	10684	UBQ1	17784	UBQ11	Actin4	Actin7	EFTS	HisH3.3
RefFinder	GAPDHB	HisH2A	EF1dt	a-tub3	22608	10684	17784	UBQ1	UBQ11	Actin4	Actin7	EFTS	HisH3.3
B. Ranking order in root samples													
geNorm	GAPDHB	EF1dt	HisH2A	22608	a-tub3	17784	EFTS	Actin7	UBQ11	10684	UBQ1	HisH3.3	Actin4
NormFinder	HisH2A	a-tub3	GAPDHB	Actin4	22608	UBQ1	10684	UBQ11	17784	EF1dt	Actin7	HisH3.3	EFTS
BestKeeper	HisH2A	UBQ11	a-tub3	22608	UBQ1	GAPDHB	10684	17784	Actin4	Actin7	EF1dt	HisH3.3	EFTS
RefFinder	HisH2A	GAPDHB	a-tub3	22608	UBQ11	EF1dt	UBQ1	17784	10684	Actin4	Actin7	EFTS	HisH3.3
C. Ranking order in stem samples													
geNorm	HisH2A	Actin7	GAPDHB	UBQ11	22608	EF1dt	a-tub3	17784	10684	Actin4	UBQ1	EFTS	HisH3.3
NormFinder	GAPDHB	HisH2A	EF1dt	22608	10684	a-tub3	UBQ11	17784	Actin7	Actin4	EFTS	UBQ1	HisH3.3
BestKeeper	GAPDHB	EF1dt	HisH2A	a-tub3	22608	UBQ11	10684	Actin7	17784	EFTS	Actin4	UBQ1	HisH3.3
RefFinder	GAPDHB	EF1dt	HisH2A	22608	a-tub3	UBQ11	Actin7	10684	17784	Actin4	EFTS	UBQ1	HisH3.3
D. Ranking order in leaf samples													
geNorm	HisH2A	EF1dt	GAPDHB	Actin7	a-tub3	22608	Actin4	UBQ11	UBQ1	17784	10684	EFTS	HisH3.3
NormFinder	EF1dt	HisH2A	GAPDHB	22608	UBQ1	10684	17784	Actin4	a-tub3	HisH3.3	UBQ11	EFTS	Actin7
BestKeeper	GAPDHB	EF1dt	HisH2A	a-tub3	22608	10684	Actin4	17784	UBQ1	EFTS	UBQ11	HisH3.3	Actin7
RefFinder	EF1dt	HisH2A	GAPDHB	22608	a-tub3	Actin4	UBQ1	10684	17784	UBQ11	Actin7	EFTS	HisH3.3

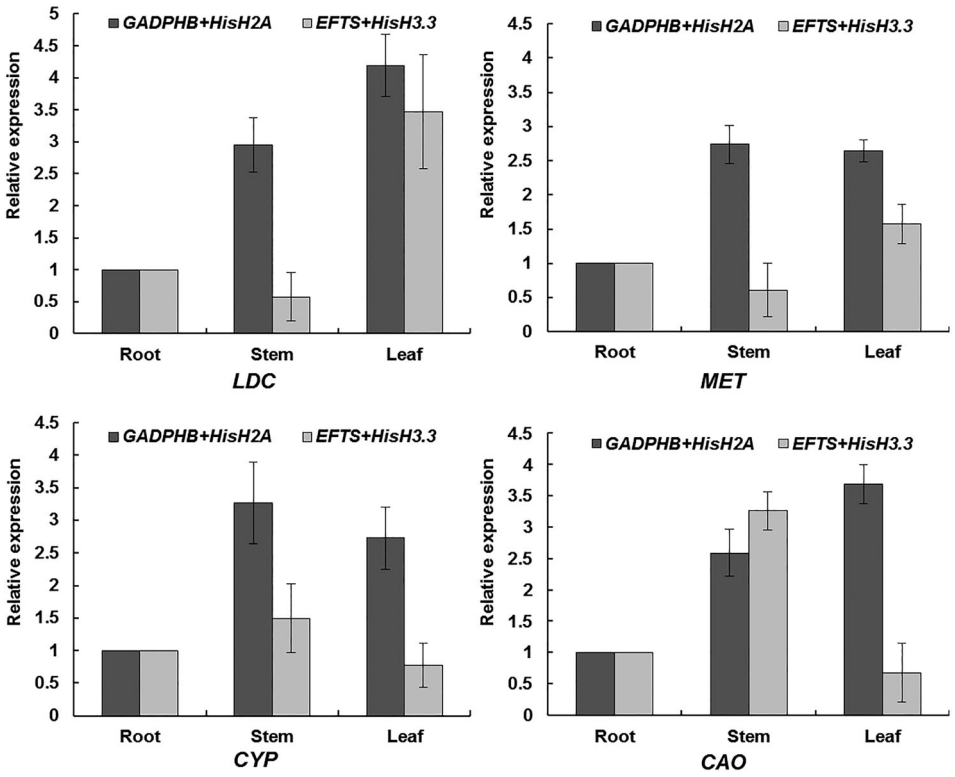

FIGURE 5 Relative expression levels of four HupA-biosynthetic genes in different tissues normalized by the most stable and unstable combination. The expression level of *LDC*, *MET*, *CYP* and *CAO*. The relative expression was calculated using the comparative threshold method ($2^{-\Delta\Delta CT}$). The transcription levels of target gene in the root were set to one. Bars represent the standard error from three biological replicates

have been described to possibly involve in the biosynthesis of HupA. In order to verify the accuracy of the stable RGs identified in this paper, four HupA-biosynthetic genes, *LDC*, *MET*, *CYP*, and *CAO* were tested. The results showed that using the combination of stable RGs (*GAPDHB* and *HisH2A*), the consistent expressions trend of *LDC*, *MET*, *CYP*, and *CAO* were obtained between transcriptome sequencing and RT-qPCR (Table 1 and Figure 5). Conversely, using the most unstable RGs (*HisH3.3* and *EFTS*) may lead to declinational results (Table 1 and Figure 5). The results further proved that *GAPDHB* and *HisH2A* were suitable for gene expression normalization, especially for HupA-biosynthetic genes. In addition, we tested the content of HupA in different tissues. The results indicated the content of HupA in root was obviously lower than that in stem or leaf (Figure 1), which was consistent with the previous studies (Ma & Gang, 2005; Wu et al., 2017). Therefore, in order to protect the wild resources, we suggest picking the aboveground parts instead of uprooting the whole plan when digging *H. serrata*. In addition, we found the expression trend of HupA-biosynthetic genes were similar with the trend of HupA content in different tissue of *H. serrata*, which indicated that the biosynthesis of HupA may happen in stem and leaf. This result will provide the information for further studying the biosynthesis and transportation of HupA.

4 | CONCLUSIONS

In the present study, based on full-length transcriptome sequencing data and four analysis methods, we obtained two optimal reference genes *GAPDHB* and *HisH2A* from thirteen candidate reference genes in different tissue of *H. serrata*. The expression patterns of four HupA-biosynthetic genes *LDC*, *MET*, *CYP*, and *CAO* further verified that *GAPDHB* and *HisH2A* were suitable for the normalization of HupA-biosynthetic genes. This work provides suitable RGs for the subsequent research of HupA-biosynthetic and transportation in *H. serrata*.

5 | MATERIALS AND METHODS

5.1 | Plant materials

H. serrata plants were collected from Hanzhong, Shaanxi, China (107°09′/32°30′), in March 2018. All materials used in this study were identified by phytotaxonomist. The plants were rinsed carefully by running water. Root, stem, and leaves were collected in liquid nitrogen and were immediately frozen at −80°C for RNA extraction. Other plant materials were dried at 60°C and powdered for determining HupA content.

5.2 | HPLC parameters and conditions

HupA were extracted from the different plant tissues as previously described (Ishiuchi et al., 2013; Ma et al., 2005). After the plant material was dried and milled, 100 mg each of powdered plant tissues was extracted by adding 2% (2:100, w/v) aqueous tartaric acid (5 ml) for overnight and then sonicating for 2 h at 25°C. Centrifuging for 30 min at room temperature and the upper extraction solution was filtered

into a 1.5 ml measuring flask through a 0.45-μm filter. Finally, the filtered solutions (10 μl) were injected into the HPLC system (LC-20AT, Shimadzu, Japan) for detection of HupA content. Each experiment comprised three independent biological replicates. The details are as follows: The elution conditions: (flow rate, 0.8 ml/min; column temperature, 28°C; injection volume, 10 μl; detection, the detection was performed at the wavelength of 308 nm). The mobile phase was methanol/acetonitrile/0.08-M ammonium acetate (pH = 6) (10:30:60). Chromatography was performed on a C-18 column (Hypersil ODS2, China) of 250 × 4.6 mm dimensions and 5-μm particle size.

5.3 | Candidate RGs selection and primers design

Candidate RGs for this study were selected from full-length transcriptome sequencing dates and previous studies. First, candidate genes were screened based on the stable CPM value in root, stem, and leaf samples. More appropriate genes were chosen by combining previous studies in numerous candidate genes. Because housekeeping genes have been used as RGs in many previous studies, 10 commonly used housekeeping genes were chosen in numerous candidate genes. Furthermore, three never reported genes were chosen as the candidate genes. The three new RGs candidate are as follows: ONT.10684 represented the high expression level (CPM over 100), EVM0022608 was the middle level (CPM 29–34), and EVM0017784 was the low level (CPM less than 5). Thirteen candidate RGs (MH560040–MH560049, MZ042627–MZ042629) were selected at last (Table 1). Gene specific primers for each RG were designed using the Primer 5.0. Conserved domains of RGs were evaluated, and the primer binding positions were presented. Initially, primer specificity was verified by RT-qPCR and confirmed with 2% (w/v) agarose gel electrophoresis and melting curve.

5.3.1 | RNA extraction and cDNA synthesis

The total RNA was extracted according to the modified CTAB method (Gasic et al., 2004). RNA samples were treated with DNase I (Ambion, Waltham, MA, USA) to remove any DNA contamination. Using cDNA synthesis kit (Roche, Basel, Switzerland), first strand cDNA was prepared with 3-μg RNA as manufacturer's instructions.

5.4 | RT-qPCR analysis

The RT-qPCR reactions were performed with FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) on a CFX-96 thermocycling system (Bio-Rad, Hercules, CA, USA). Each RT-qPCR reaction was performed as described previously (Zhuang et al., 2015). PCR amplifications were carried out by the following conditions: one cycle at 95°C (180 s), followed by 40 cycles of denaturation at 95°C (30 s), annealing at 58°C for 10 s and extension at 72°C for 20 s (Liu et al., 2018). Finally, melt curve analyses were done by slowly heating the PCR mixtures from 58 to 95°C. Amplification efficiencies (*E*) and correlation coefficients (R^2) for each primer pair were calculated by LinRegPCR program (Ruijter et al., 2009). In the negative control group, RT-qPCR was performed using water instead of cDNA as the template. Three technical replicates were analyzed for each biological sample, and each experiment comprised three independent biological replicates.

5.5 | Data analysis of gene expression stability

Four analysis methods: geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and RefFinder (Silver et al., 2006) were applied to determine the stability of RGs. For geNorm and NormFinder, the raw Ct values were converted into the relative quantities using the formula $2^{-\Delta Ct}$ (ΔCt = each corresponding Ct value − lowest Ct value). *M* value was calculated the average expression stability, in geNorm algorithms. The candidate RGs showing a higher *M* value (*M* > 1.5) are not considered for normalization studies (Vandesompele et al., 2002). geNorm software was also used to confirm the best numbers of RGs with Vn/Vn + 1 (n refers to the RGs number) (Vandesompele et al., 2002). NormFinder can provide the stability value for each gene (Andersen et al., 2004). The smaller stability value, the more stable gene, and vice versa. For BestKeeper, the raw Ct values and amplification efficiencies were used to calculate the coefficient of variation (CV) and standard deviation (SD). The most stable genes are the lowest CV and SD (CV ± SD). The comprehensive ranking order was recommended on the basis of geometric mean (GM) by RefFinder (Zhang et al., 2018).

5.6 | Validation of RGs

The primer of four HupA-biosynthetic genes *LDC* (GO914645), *MET* (GO914756), *CYP* (GO914428), and *CAO* (JN247732) (Luo et al., 2010; Sun et al., 2012; Xu et al., 2017) were designed using the Primer 5.0. The combination of the two best and worst RGs were used to standardize the expression of four HupA-biosynthetic genes. The target gene expression data were normalized using the geometric mean values calculated for the RG pairs (Vandesompele et al., 2002). Relative expression level and fold change were determined using the comparative $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). One-way analysis of variance was performed using SPSS software (Version 16.0, SPSS Inc., Chicago, IL, USA).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Yanping Fu conceived and designed the experiments. Yanping Fu and Fei Niu performed the experiments, analyzed the data, and prepared figures and/or tables. Fei Niu prepared the first draft. Hui Jia and Yanli Wang contributed reagents/materials and detected the content of HupA. Bin Guo contributed reagents/materials/analysis tools. Yahui Wei conceived and designed the experiments and reviewed drafts of the paper. All the authors approved the final draft.

REFERENCES

Andersen, C. L., Jensen, J. L., & Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research*, *64*, 5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496

André, S., Sun, Y., Song, J., Wu, Q., Sun, C., Li, Y., Luo, H., & Chen, S. (2010). Comparison of 454-ESTs from *Huperzia serrata* and *Phlegmariurus carinatus* reveals putative genes involved in lycopodium alkaloid biosynthesis and developmental regulation. *BMC Plant Biology*, *10*, 209. https://doi.org/10.1186/1471-2229-10-209

Bansal, R., Mittapelly, P., Cassone, B. J., Mamidala, P., Redinbaugh, M. G., & Michel, A. (2015). Recommended reference genes for quantitative PCR analysis in soybean have variable stabilities during diverse biotic stresses. *PLoS ONE*, *10*, e0134890. https://doi.org/10.1371/journal.pone.0134890

Bunsupa, S., Katayama, K., Ikeura, E., Oikawa, A., Toyooka, K., Saito, K., & Yamazaki, M. (2012). Lysine decarboxylase catalyzes the first step of quinolizidine alkaloid biosynthesis and coevolved with alkaloid production in leguminosae. *Plant Cell*, *24*, 1202–1216. https://doi.org/10.1105/tpc.112.095885

Chen, C., Wu, J., Hua, Q., Tel-Zur, N., & Qin, Y. (2019). Identification of reliable reference genes for quantitative real-time PCR normalization in pitaya. *Plant Methods*, *15*, 70. https://doi.org/10.1186/s13007-019-0455-3

Chen, X., Mao, Y., Huang, S., Ni, J., & Wu, L. (2017). Selection of suitable reference genes for quantitative real-time PCR in *Sapium sebiferum*. *Frontiers in Plant Science*, *8*, 637. https://doi.org/10.3389/fpls.2017.00637

Christenhusz, M. J. M., Zhang, X. C., & Schneider, H. (2011). A linear sequence of extant families and genera of lycophytes and ferns. *Phytotaxa*, *19*, 7–54. https://doi.org/10.11646/phytotaxa.19.1.2

Dudziak, K., Sozoniuk, M., Szczerba, H., Kuzdraliński, A., Kowalczyk, K., Brner, A., & Nowak, M. (2020). Identification of stable reference genes for qPCR studies in common wheat (*Triticum aestivum* L.) seedlings under short-term drought stress. *Plant Methods*, *16*, 1–8. https://doi.org/10.1186/s13007-020-00601-9

Fei, X., Shi, Q., Yang, T., Fei, Z., & Wei, A. (2018). Expression stabilities of ten candidate reference genes for RT-qPCR in *Zanthoxylum bungeanum* Maxim. *Molecules*, *23*, 802. https://doi.org/10.3390/molecules23040802

Ferreira, A., Rodrigues, M., Fortuna, A., Falcão, A., & Alves, G. (2016). Huperzine A from *Huperzia serrata*: A review of its sources, chemistry, pharmacology and toxicology. *Phytochemistry Reviews*, *15*, 51–85. https://doi.org/10.1007/s11101-014-9384-y

Gasic, K., Hernandez, A., & Korban, S. S. (2004). RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. *Plant Molecular Biology Reporter*, *22*, 437–438. https://doi.org/10.1007/BF02772687

Ishiuchi, K. i., Park, J. J., Long, R. M., & Gang, D. R. (2013). Production of huperzine A and other Lycopodium alkaloids in Huperzia species grown under controlled conditions and in vitro. *Phytochemistry*, *91*, 208–219. https://doi.org/10.1016/j.phytochem.2012.11.012

Jian, B., Liu, B., Bi, Y., Hou, W., Wu, C., & Han, T. (2008). Validation of internal control for gene expression study in soybean by quantitative real-time PCR. *BMC Molecular Biology*, *9*, 59. https://doi.org/10.1186/1471-2199-9-59

Liu, J. S., Yu, C. M., Zhou, Y. Z., Han, Y. Y., Wu, F. W., Qi, B. F., & Zhu, Y. L. (1986). Study on the chemistry of Huperzine A and B. *Acta Chimica Sinica*, *44*, 1035–1040.

Liu, X., Guan, H., Song, M., Fu, Y., Han, X., Lei, M., Ren, J., Guo, B., He, W., & Wei, Y. (2018). Reference gene selection for qRT-PCR assays in *Stellera chamaejasme* subjected to abiotic stresses and hormone treatments based on transcriptome datasets. *PeerJ*, *6*, 4535. https://doi.org/10.7717/peerj.4535

Luo, H., Sun, C., Li, Y., Wu, Q., Song, J., Wang, D., Jia, X., Li, R., & Chen, S. (2010). Analysis of expressed sequence tags from the *Huperzia serrata* leaf for gene discovery in the areas of secondary metabolite biosynthesis and development regulation. *Physiologia Plantarum*, *139*, 1–12. https://doi.org/10.1111/j.1399-3054.2009.01339.x

Ma, X., & Gang, D. (2005). The Lycopodium alkaloids. *Natural Product Reports*, *21*, 752–772. https://doi.org/10.1039/b409720n

Ma, X., Tan, C., Zhu, D., & Gang, D. R. (2005). Is there a better source of huperzine A than *Huperzia serrata*? Huperzine A content of Huperziaceae species in China. *Journal of Agricultural and Food Chemistry*, *53*, 1393–1398. https://doi.org/10.1021/jf048193n

Ma, X., Tan, C., Zhu, D., Gang, D. R., & Xiao, P. (2007). Huperzine A from Huperzia species-an ethnopharmacolgical review. *Journal of Ethnopharmacology*, *113*, 15–34. https://doi.org/10.1016/j.jep.2007.05.030

Pfaffl, W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, *29*, 2002–2007. https://doi.org/10.1093/nar/29.9.e45

Pfaffl, W., Tichopad, A., Prgomet, C., & Neuvians, P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnology Letters*, *26*, 509–515. https://doi.org/10.1023/B:BILE.0000019559.84305.47

Qian, Z. M., & Ke, Y. (2014). Huperzine A: Is it an effective disease-modifying drug for Alzheimer's disease? *Frontiers in Aging Neuroscience*, *6*, 216. https://doi.org/10.3389/fnagi.2014.00216

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., Hoff, M. J. B. V. D., & Moorman, A. F. M. (2009). Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Research*, *37*, e45. https://doi.org/10.1093/nar/gkp045

Silver, N., Best, S., Jiang, J., & Thein, S. L. (2006). Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Molecular Biology*, *7*, 33. https://doi.org/10.1186/1471-2199-7-33

Sun, J., Morita, H., Chen, G., Noguchi, H., & Abe, I. (2012). Molecular cloning and characterization of copper amine oxidase from *Huperzia serrata*. *Bioorganic & Medicinal Chemistry Letters*, *22*, 5784–5790. https://doi.org/10.1016/j.bmcl.2012.07.102

Vandesompele, J., Preter, K. D., Pattyn, F., Poppe, B., Roy, N. V., Paepe, A. D., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*, 34. https://doi.org/10.1186/gb-2002-3-7-research0034

Wang, J., Wang, X., Liu, X., Li, J., Shi, X., Song, Y., Zeng, K., Zhang, L., Tu, P., & Shi, S. (2016). Synthesis of unnatural 2-substituted quinolones and 1,3-Diketones by a member of type III polyketide synthases from *Huperzia serrata*. *Organic Letters*, *18*, 3550–3553. https://doi.org/10.1021/acs.orglett.6b01501

Wu, S., Fan, Z., & Xiao, Y. (2017). Comprehensive relative quantitative metabolomics analysis of lycopodium alkaloids in different tissues of *Huperzia serrata*. *Synthetic & Systems Biotechnology*, *3*, 44–55. https://doi.org/10.1016/j.synbio.2017.12.003

Wu, Y., Tian, Q., Huang, W., Liu, J., Xia, X., Yang, X., & Mou, H. (2020). Identification and evaluation of reference genes for quantitative real-time PCR analysis in *Passiflora edulis* under stem rot condition. *Molecular Biology Reports*, *47*, 2951–2962. https://doi.org/10.1007/s11033-020-05385-8

Xu, B., Lei, L., Zhu, X., Zhou, Y., & Xiao, Y. (2017). Identification and characterization of L-lysine decarboxylase from *Huperzia serrata* and its role in the metabolic pathway of lycopodium alkaloid. *Phytochemistry*, *136*, 23–30. https://doi.org/10.1016/j.phytochem.2016.12.022

Yang, G., Wang, Y., Tian, J., & Liu, J. P. (2013). Huperzine A for Alzheimer's disease: A systematic review and meta-analysis of randomized clinical trials. *PLoS ONE*, *8*, e74916. https://doi.org/10.1371/journal.pone.0074916

Yang, M., Wu, S., You, W., Jaisi, A., & Xiao, Y. (2019). Selection of reference genes for expression analysis in Chinese medicinal herb *Huperzia serrata*. *Frontiers in Pharmacology*, *10*, 44. https://doi.org/10.3389/fphar.2019.00044

Yang, M., You, W., Wu, S., Fan, Z., Xu, B., Zhu, M., Li, X., & Xiao, Y. (2017). Global transcriptome analysis of *Huperzia serrata* and identification of critical genes involved in the biosynthesis of huperzine A. *BMC Genomics*, *18*, 245. https://doi.org/10.1186/s12864-017-3615-8

Zhang, B., Liu, J., Yuan, G., Chen, X., & Gao, X. (2018). Selection and evaluation of potential reference genes for gene expression analysis in greenbug (*Schizaphis graminum* Rondani). *Journal of Integrative Agriculture*, *17*, 2054–2065. https://doi.org/10.1016/S2095-3119(18)61903-3

Zhuang, H., Fu, Y., He, W., Wang, L., & Wei, Y. (2015). Selection of appropriate reference genes for quantitative real-time PCR in *Oxytropis ochrocephala* Bunge using transcriptome datasets under abiotic stress treatments. *Frontires in Plant Science*, *6*, 475. https://doi.org/10.3389/fpls.2015.00475

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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