Reference Gene Selection for RT-qPCR Assays in Different Tissues of Huperzia Serrata Based on Full-Length Transcriptome Sequencing

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Research

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Abstract

Background

Huperzia serrata (H. serrata) produces various types of effectively 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.

Results

Based on the full-length transcriptome datasets and previous studies, thirteen candidate reference genes were selected in different tissue of H. serrata. Then, two optimal reference genes GAPDHB and HisH2A were confirmed by various analysis softwares. In order to further verify the accuracy of the two reference genes, they were used to analyze the expression patterns of four HupA-biosynthetic related genes (lysine decarboxylas, RS-norcoclaurine 6-O-methyltransferase, cytochrome P45072A1 and copper amine oxidase). The data suggested that the expression trends of HupA-biosynthetic related genes were consistent with them in transcriptome sequencing in different tissue of H. serrata.

Conclusions

This study screened the best reference genes GAPDHB and HisH2A in different tissues of H. serrata, which provides suitable normalization for analyzing the expression of HupA-biosynthetic gene in transcriptional level in H. serrata.

Background

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) [10]. H. serrata has been widely known as a medicinal plant since Chinese scientists discovered Huperzine A (HupA) from it during the 1980s [15]. 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 with AD disease, with relatively few and mild adverse effects [34, 25]. However, H. serrata is scarce in nature and grows very slowly in specialized habitats. Furthermore, the HupA content is very low in H. serrata [20]. 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 results 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 [2, 36]. Real-time quantitative PCR (RT-qPCR) has been widely used in gene expression measurement in transcriptional level. Identification of suitable reference genes (RGs) is pre-requisite for RT-qPCR assays [29, 3]. 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, ubiquitin and so on [21, 9]. However, there is no RG suitable to all biological systems because all previous studies have revealed alterations for housekeeping genes expression. So, researchers reach a consensus that specific RG for a given species and treatment needs to be identified firstly [3]. Unfortunately, the repportorial RGs are not suitable for the research of HupA-biosynthetic genes [35].

In present study, in order to obtain the optimal RGs for researching HupA-biosynthetic, 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, thirteen candidate RGs were selected. Finally, GAPDHB and HisH2A stood out among the thirteen candidate RGs, and became the best combination for normalization in different tissues of H. serrata by the comprehensive analysis of four softwares. Using GAPDHB and HisH2A as RGs, the consistent expression trend was obtained between transcriptome sequencing and RT-qPCR when researched 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). This further verified that GAPDHB and HisH2A were suitable for HupA-biosynthetic gene expression normalization. This work provides suitable RGs for the subsequent research of HupA-biosynthetic in H. serrata.
Methods

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.

HPLC parameters and conditions

HupA were extracted from the different plant tissues as previously described [19, 12]. 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 RT 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.

Candidate RGs selection and primers design

RGs for this study were selected from full-length transcriptome sequencing dates and previous studies where these were found to have stable expression in other plants by using local NCBI-blast (version 2.4.0+). Thirteen candidate RGs (MH560040 · MH560049, MZ042627-MZ042629) were selected (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.

RNA extraction and cDNA synthesis

The total RNA was extracted according to the modified CTAB method [11]. 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.

RT-qPCR analysis

The RT-qPCR reactions were performed with FastStart Universal SYBR GreenMaster (Roche, Basel, Switzerland) on a CFX-96 thermocycling system (Bio-Rad, Hercules, CA, USA). Each RT-qPCR reaction was performed as described previously [24]. PCR amplifications were carried out with the following cycling 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 [16]. Finally, melt curve analyses were done by slowly heating the PCR mixtures from 58 to 95°C. Amplification efficiencies (E) and correlation coefficients (R²) for each primer pair were calculated by LinRegPCR program [26]. 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.

Data analysis of gene expression stability

Three software algorithms: geNorm [29], NormFinder [1], BestKeeper [23] and RefFinder [27] 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^ΔCt (ΔCt = each corresponding Ct value - lowest Ct value). M value, which resents average expression stability, was calculated in geNorm. The candidate RGs showing a higher M value (M>1.5) are not considered for normalization studies [29]. geNorm software was also used to confirm the numbers of RGs with pair wise variation (Vn/Vn+1, n refers to the RGs number) [29]. NormFinder provides the stability value for each gene,
which is a direct measure of the estimated expression variation enabling evaluation of the systematic error introduced when using the
gene for normalization [1]. 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 determined to be those which exhibit the lowest CV and SD (CV ±
SD). The comprehensive ranking order was recommended on the basis of geometric mean (GM) by RefFinder [37].

Validation of RGs

The primer of four Hup A-biosynthetic genes LDC (GO914645) MET (GO914756), CYP (GO914428) and CAO (JN247732) [17, 33, 28] were
designed using the Primer 5.0. The combination of the top two best ranked RGs and worst ranked RGs were used to standardize the
expression of two target genes. The target gene expression data was normalized using the geometric mean values calculated for the RG
pairs [29]. Relative expression level and fold change were determined using the comparative $2^{ΔΔCt}$ method [22]. One-way analysis of
variance was performed using SPSS software (Version 16.0, SPSS Inc., Chicago, IL, USA).

Results And Discussion

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 has good peak shape and is 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 for the HupA content in different tissues. The highest HupA content (72 μg/g) was found in the leaves of H.
serrata. The lowest content (19 μg/g) of HupA was found in root tissues of H. serrata (Figure 1).

The screening of candidate RGs

In consideration of the varied difference of Hup A concentrations in different tissues, the root, stem and leaf samples were collected and
proceeded the full-length transcriptome sequencing by Nanopore. After assembly, 43,443 unigenes were retrieved. CPM is the index for measuring the expression of unigenes. Based on the CPM value and reported literatures, ten traditional RGs and three new
RGs candidate were choosed. The three new RGs candidate had stable expression in full-length transcriptome sequencing. They were
notated hypothetical or uncharacterized proteins by NCBI Nr database, furthermore, they were not used as RGs before. The three new
RGs candidate as following: 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). The detail information of total thirteen candidate RGs was showed in Table 1.

Verification of the primer specificity and RT-qPCR amplification efficiency

The primer information of thirteen candidate RGs was given in Table 2. Each primer pair was designed except the conserved domains to
ensure the specificity (Figure S2). Initially, the agarose gel electrophoresis yielded a specific fragment of expected size (Figure S3A, S3B
and S3C). Further, the melting curve analysis in the RT-qPCR reaction showed the single peak for each primer pair indicating an absence of
non-specific product 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 0.990 (Table 2). Taken together, these results indicated each primer pair was specificity
and the RT-qPCR assays were highly efficient.

Expression profiles of candidate RGs

The expression profiles of RT-qPCR products for all experimental samples are shown in Figure2. 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 profiles. EF1dt and UBQ1 were highly expressed with mean Ct values between 24.04 and 24.08 while EFTS was the least
expressed gene on account of its highest mean Ct value (29.43). All candidate genes showed expression variability in different samples as
evident from a wide range of Ct values. Genes such as GAPDHB and EFTS showed relatively smaller variation (< 2 cycles), while others like
UBQ11 had the highest expression variation (3.07 cycles). The results indicated that there was still variable expression even for relative
stable housekeeping genes.
**geNorm analysis**

To identify the most stable RG, geNorm algorithm calculated the average expression stability values (M values) of each RG. As Figure 3 shown, each M value was less than 1.5, which suggested the appropriateness of all RGs for normalization consideration in different tissues of *H. serrata*. Concretely analyzing, EF1dt, HisH2A and GAPDHB were the most stable genes in each *H. serrata* samples, while HisH3.3 and EFTS were the least stable genes in each *H. serrata* samples.

**NormFinder analysis**

NormFinder evaluates each RG according to the stability value. Lower stability value indicates more stable gene expression, and vice versa. As shown in Table 3, GAPDH and HisH2A were obviously stable in all samples, and EFTS (highest stability value = 0.210) was the least stable gene. For the root samples, HisH2A and a-tub3 were most stable, and the EFTS (stability value = 0.506) still was the least stable gene. Whereas GAPDH and His2A were the most stable gene and HisH3.3 (stability value = 0.358) was the least one in stem. In leaf tissues, the most stable RGs were EF1dt, HisH2A and, GAPDH, meanwhile, the least stable RG was Actin7 (stability value = 0.364). Overall, with NormFinder analysis, GAPDH, HisH2A and EF1dt were the most stable genes, while EFTS and HisH3.3 were the least stable genes in different tissues of *H. serrata*.

**BestKeeper analysis**

The stability standard deviation (SD) and its relationship to the BestKeeper index were considered as two important evaluation criteria in BestKeeper analysis [23]. The results showed that each RG had a SD value < 1.0, which indicated that the candidate RGs were relatively stable for RT-qPCR normalization. In present, GAPDH, EF1dt and HisH2A were the top three ranked genes with lowest CV ± SD values in all samples, stem and leaf tissues (Table 4). In root samples, the top three ranked genes were HisH2A, UBO11 and a-tublin. Rather, HisH3.3 was deemed to the least RG with the highest CV ± SD value (27.35 ±0.21 and 28.66 ±0.20) in all samples and stem tissues, while EFTS (in root tissues) and Actin7 (in leaf samples) showed the least stable expression. Taken together, with BestKeeper analysis, GAPDH, EF1dt and HisH2A were the most stable genes, while HisH3.3 and EFTS were the least stable genes in different tissues of *H. serrata*.

**RefFinder analysis**

Although the results (geNorm analysis, NormFinder and BestKeeper analysis) were similar, it was not strictly consistent. Therefore, we performed overall evaluate using RefFinder to recommend a comprehensive ranking of the most stable genes in diverse tissues (Table 5). In root tissues, the final ranking calculations based on the RefFinder found HisH2A (GM = 1.67), GAPDH (3.33) and a-tub3 (3.33) were the best genes. For stem samples, the top three stable RGs were GAPDH (1.67), EF1dt (2.00) and HisH2A (3.67), while EF1dt (1.33), HisH2A (2.00) and GAPDH (2.33) in leaf samples. Across all samples, the top three stable RGs were GAPDH (1.67), HisH2A (1.67) and EF1dt (2.33). On the other hand, HisH3.3 and EFTS were ranked as the two least stable genes (Table 5).

**Optimal Number of RGs for Normalization**

Though a single and stable RG is sufficient for quantifying gene expression, the use of more than one RG for effective normalization of gene expression data is suggested [29]. Based on the geNorm software, the optimal number of RGs needed for normalization was determined by pairwise variation (Vn/n+1). In our data, the all pairwise variation of V2/3 values were lower than 0.15 (Figure 3), which suggested that the combination of the two most stable RGs was optimum for normalization.

Together with RefFinder analysis, GAPDH and HisH2A were the best combination for normalization in different tissues of *H. serrata*.

**RG Validation**

To demonstrate the utility of identified stable RGs, four HupA-biosynthetic related genes LDC, MET CYP and CAO were selected in *H. serrata*. For the purpose of comparison, expression values of target genes were normalized with respect to the most stable gene pair (GAPDH and HisH2A) and the least stable gene pair (EFTS and HisH3.3) in *H. serrata* different tissues. When normalized using the most stable genes, the transcription levels of LDC, MET, CYP and CAO were (over 2 –fold) in the tissues of leaf, stem and root were compared, and the expression trend was consistent with that of transcriptome sequencing dates (Figure 4). By contrast, when normalized using the
least stable genes, the transcription level of MET and CYP were not up-regulated (less than 2 –fold) 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. This expression trend was not consistent with that of transcriptome sequencing dates. In all, the expression of the most stable gene pair was more reliable than the least stable gene pair.

Discussion

H. serrata has received extensive concern due to produce biologically active lycopodium alkaloids, especially Hupa [7]. Hupa was found to possess potent acetylcholine esterase inhibition (AChEI) and had been clinically exploited for the treatment of Alzheimer’s disease. More studies are focused on the isolation and identification of compounds and endophytic bacteria [31], but little on the transcriptional level. Especially for the selection of optimum reference genes, little research has been reported [35]. So, in present study, we screened and selected the optimal RGs based on full-length transcriptome sequencing and previous researches in different tissues of H. serrata. By the analysis of four softwares and the verification of four Hupa-biosynthetic genes (LDC, MET, CYP and CAO), we obtained two optimal reference genes GAPDH and HisH2A for studying Hupa-biosynthetic related genes. This study provides suitable normalization for analyzing the expression of Hupa-biosynthetic gene. In addition, we found the expression trend of Hupa-biosynthetic genes were similar with the trend of Hupa content 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 constant 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-biosynthetic. Based on the CPM value of transcriptome sequencing and reported literatures [32, 8, 14, 5], ten traditional RGs and three new RGs candidate were chosen (Table 1). Especially for three new RGs candidate, they had stable expression in full-length transcriptome sequencing but they were not used as RGs before.

The primer specificity is the primary condition of RT-qPCR. The ideal primers which should cross intron regions to avoid genomic contamination in cDNA samples and cannot be set in conservative domain. Firstly, each primer pair was designed except 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 non-specific amplification for each primer pair. Furthermore, the E value of PCR varied from 90.4–103.6%, and all of the R² were greater than 0.990 (Table 2), which were similar to previous literatures [16]. In conclusion, these results indicated each primer pair was specificity and the RT-qPCR assays were highly efficient.

Based on the analysis of four softwares (geNorm, NormFinder, BestKeeper and RefFinder), GAPDH and HisH2A stood out among the thirteen candidate RGs, and became the best combination for normalization in different tissues of H. serrata (Table 5). Many studies have shown that GAPDH was most often as relatively stable internal RGs in different tissues and under a variety of experimental conditions [6, 24, 14]. Although histone and elongation factor were be reported as most stable RGs in other species [24], HisH3.3 and EFTs were the most unstable RGs in different tissues of H. serrata. These results suggested that the traditional RG may not suitable for all samples. Interestingly, the performance between the homologous genes was obviously different. HisH3.3 and EFTs were more instable than HisH2A and EF1dt. Similar findings can be found that the expression level of Actin2/7 was more stable than Actin11 in diverse tissues of soybean [13]. These results also stated clearly 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 [18]. However, only two enzymes, LDC and CAO have been proved to participate in the biosynthesis of Hupa [33, 4, 28]. Three enzymes RNorscoclaureine 6-O-methyltransferase (MET) and cytochrome P45072A1 (CYP) [17, 33], type III polyketide synthase (PKS) [30], have been described to be possible involvement of 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 selected. The results showed that when the combination of stable RGs (GAPDH and HisH2A) was used, the consistent expressions trend of LDC, MET, CYP and CAO were obtained between transcriptome sequencing and RT-qPCR (Fig. 5 and Table 1). Conversely, the use of the most unstable RGs (HisH3.3 and EFT3) may lead to declinational results (Fig. 5 and Table 1). The results further verified that GAPDH 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 (Fig. 1), which was consistent with the previous study [31, 18]. Therefore, in order to protect the wild resources, we suggested picking the aboveground parts instead of uprooting the whole plant 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 be in stem and leaf. This result will provide the information for further studying the biosynthesis and transportation of Hupa.
Conclusions

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

Declarations

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Authors’ contributions

YF designed the experiment and analyzed data. FN performed the partial experiment, prepared the first draft. HJ, YW and YF contributed reagents/materials and detected the content of HupA. BG provided plant materials used for experiments and discussed data. WH revised the paper. YW edited the graphs and revised the paper. All the authors approved the final draft.

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Availability of data and materials

All data generated or analysed during this study are included in this published article (and its Additional file 1).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Tables**

*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  
|----------------|------------------|------------|-------------|-----------|--------------|-----------|----------|----------|--------  
| 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_004507134.3 | 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     | 29.03    | 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)-norcoclarine 6-O-methyltransferase | GO914756    | 2.82      | 13.46    | 10.48    | 0       
| EVM0022835    | GO914428         | 1975       | CYP         | Cytochrome P450 72A1 | GO914428    | 0.30      | 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 (℃) | E (%) | R²* |
|------------|-----------------------------------------|--------------------|----------------|-------|-----|
| Actin4-RT-F | TGTCTCTAAAGTTTCTTGTAGCACC               | 174                | 77.5           | 99.6  | 0.997 |
| Actin4-RT-R | GCACAGCGGCAACAAGACTCTG                  |                    |                |       |     |
| Actin7-RT-F | AACCCCTTATCTGAGGCTTCTTGT               | 139                | 78.5-79        | 95.7  | 0.998 |
| Actin7-RT-R | TCATAACACTGACGTCGTCGGTAG               |                    |                |       |     |
| EF1dt-RT-F  | GACTGAGCAAATAAGAGGCCC                 | 213                | 75.5-76        | 91.7  | 0.997 |
| EF1dt-RT-R  | CGATATTGCGGGCTTTAACA                  |                    |                |       |     |
| EFTS-RT-F   | AAGTATCCTAATAACAGGTTTG                | 158                | 75-75.5        | 92.5  | 0.997 |
| EFTS-RT-R   | AAAATTTGGTCTATCGCG                    |                    |                |       |     |
| a-tub3-RT-F | AAATCCAAACAATATGTATGAACAA             | 210                | 80-80.5        | 94.8  | 0.997 |
| a-tub3-RT-R | GCCAAGGTGTTGCAATCTTCTA                |                    |                |       |     |
| GAPDH-B-RT-F | GCAAAATATATGAAGATAGGCTC            | 161                | 76.5-77.5      | 91.3  | 0.998 |
| GAPDH-B-RT-R | GCCTCCACCAACAGAA                     |                    |                |       |     |
| HisH3.3-RT-F | AACTTTGCTTTGCATGAAACTAAC            | 212                | 77-77.5        | 90.4  | 0.996 |
| HisH3.3-RT-R | CAAGTCAGAAGAATCTCAACAGA             |                    |                |       |     |
| HisH2A-RT-F | CTTCTCGCTTTACATCTTT                  | 270                | 75.5-76        | 92.2  | 0.994 |
| HisH2A-RT-R | GTCCCAAGCTTCAATT                      |                    |                |       |     |
| UBQ1-RT-F   | GTGCGGCTCTTATGAGAG                   | 130                | 73-73.5        | 97.3  | 0.997 |
| UBQ1-RT-R   | CTTCTTGACTTCTGACATGATTTAA            |                    |                |       |     |
| UBQ11-RT-F  | TCTGAAAATGTCCCTATCCG                  | 174                | 76.5-77        | 97.4  | 0.995 |
| UBQ11-RT-R  | TCTGTTGGCTCATTTGTTAG                 |                    |                |       |     |
| 10684-RT-F  | GCGCTTGATAAGTCACTGCTAC               | 253                | 76             | 94.3  | 0.995 |
| 10684-RT-R  | GAAAAAAAAGATTGCCATAATAAGG             |                    |                |       |     |
| 22608-RT-F  | TGGCCGAATTTTAGAGGGAAT                 | 198                | 77.5           | 95.1  | 0.998 |
| 22608-RT-R  | CCTCAAGCCCATTTTTATTCTCT              |                    |                |       |     |
| 17784-RT-F  | AATATGGCCCAAGGGTCACC                 | 212                | 75             | 93.8  | 0.996 |
| 17784-RT-R  | GGTGACCACCTTGGCTTCTAC                |                    |                |       |     |
| LDC-F       | GTATAGCCAATTACTCCTATCCTCC             | 155                | 73-73.5        | 91.6  | 0.997 |
| LDC-R       | GTAACACCACTCCATTTGTCAG               |                    |                |       |     |
| MET-F       | GCTCATCTTCTGGACATG                   | 237                | 80.5           | 98.2  | 0.996 |
| MET-R       | ACCCGACACTGAATCTCTTAT                |                    |                |       |     |
| CYP-F       | AAGAGTCGGGCTCTACTTGCG                | 151                | 78-79          | 91.4  | 0.998 |
| CYP-R       | GCAAGGAAGAAGCCTGAGA                  |                    |                |       |     |
| CAO-F       | CAAAGCTTTGGAATATGCTCTT               | 174                | 81             | 94.7  | 0.997 |
| CAO-R       | TTATATGCTTGGCCTAGTGAAATG            |                    |                |       |     |

* E represents amplification efficiencies and R² represents correlation coefficient of RGs, respectively.
Table 3. Expression stability of candidate RGs as calculated by Normfinder.

| Rank | All Gene name | Stability value | Root Gene name | Stability value | Stem Gene name | Stability value | Leaf Gene name | Stability value |
|------|---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 1    | GAPDH         | 0.030          | HistH2A        | 0.042          | GAPDH          | 0.045          | EF1dt          | 0.014          |
| 2    | HistH2A       | 0.044          | a-tub3         | 0.046          | HistH2A        | 0.096          | HistH2A        | 0.017          |
| 3    | EF1dt         | 0.060          | GAPDH          | 0.086          | EF1dt          | 0.131          | GAPDH          | 0.024          |
| 4    | 22608         | 0.069          | Actin4         | 0.108          | 22608          | 0.156          | 22608          | 0.064          |
| 5    | a-tub3        | 0.077          | 22608          | 0.108          | 10684          | 0.174          | UBQ1           | 0.083          |
| 6    | 10684         | 0.077          | UBQ1           | 0.109          | a-tub3         | 0.189          | 10684          | 0.089          |
| 7    | Actin4        | 0.078          | 10684          | 0.113          | UBQ11          | 0.228          | 17784          | 0.089          |
| 8    | 17784         | 0.094          | UBQ11          | 0.116          | 17784          | 0.241          | Actin4         | 0.090          |
| 9    | UBQ1          | 0.104          | 17784          | 0.151          | Actin7         | 0.257          | a-tub3         | 0.165          |
| 10   | UBQ11         | 0.109          | EF1dt          | 0.167          | Actin4         | 0.280          | HistH3.3       | 0.217          |
| 11   | Actin7        | 0.160          | Actin7         | 0.211          | EFTS           | 0.281          | UBQ11          | 0.224          |
| 12   | HistH3.3      | 0.166          | HistH3.3       | 0.287          | UBQ1           | 0.348          | EFTS           | 0.305          |
| 13   | EFTS          | 0.210          | EFTS           | 0.506          | HistH3.3       | 0.358          | Actin7         | 0.364          |

Table 4. Expression stability of candidate RGs as calculated by BestKeeper.

| Rank | All Gene | CV b | SD b | Root Gene | CV | SD | Stem Gene | CV | SD | Leaf Gene | CV | SD |
|------|----------|------|------|-----------|----|-----|-----------|----|-----|-----------|----|-----|
| 1    | GAPDH    | 7.48 | 0.07 | HistH2A   | 4.99| 0.05| GAPDH     | 5.36| 0.05| GAPDH     | 8.38| 0.07|
| 2    | HistH2A | 9.71 | 0.09 | UBQ11     | 6.14| 0.06| EF1dt     | 10.46| 0.09| EF1dt     | 9.59| 0.09|
| 3    | EF1dt   | 11.83| 0.10 | a-tub3    | 7.96| 0.07| HistH2A   | 10.83| 0.09| HistH2A   | 10.19| 0.09|
| 4    | a-tub3  | 12.44| 0.11 | 22608     | 8.06| 0.167| a-tub3    | 15.35| 0.12| a-tub3    | 10.63| 0.09|
| 5    | 22608   | 12.67| 0.24 | UBQ1      | 8.28| 0.07| 22608     | 15.61| 0.21| 22608     | 10.69| 0.23|
| 6    | 10684   | 12.97| 0.18 | GAPDH     | 8.57| 0.08| UBQ11     | 16.02| 0.14| 10684     | 10.73| 0.18|
| 7    | UBQ1    | 13.07| 0.11 | 10684     | 9.17| 0.21| 10684     | 16.21| 0.20| Actin4    | 10.78| 0.10|
| 8    | 17784   | 13.14| 0.19 | 17784     | 9.84| 0.19| Actin7    | 16.86| 0.13| 17784     | 11.68| 0.22|
| 9    | UBQ11   | 13.19| 0.11 | Actin4    | 9.94| 0.09| 17784     | 16.95| 0.17| UBQ1      | 12.17| 0.10|
| 10   | Actin4  | 14.44| 0.12 | Actin7    | 13.63| 0.11| EFTS      | 17.20| 0.15| EFTS      | 14.85| 0.13|
| 11   | Actin7  | 16.84| 0.13 | EF1dt     | 14.68| 0.12| Actin4    | 18.36| 0.14| UBQ11     | 16.04| 0.13|
| 12   | EFTS    | 20.13| 0.17 | HistH3.3  | 25.81| 0.19| UBQ1      | 20.46| 0.16| HistH3.3  | 19.47| 0.17|
| 13   | HistH3.3| 27.35| 0.21 | EFTS      | 26.12| 0.20| HistH3.3  | 28.66| 0.20| Actin7    | 20.31| 0.16|

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

Table 5. Expression stability ranking of the ten candidate RGs by RefFinder.
| Rank | All | Root | Stem | Leaf |
|------|-----|------|------|------|
|      | Gene | GM  | Gene | GM  | Gene | GM  | Gene | GM  |
| 1    | GAPDH | 1.67 | HisH2A | 1.67 | GAPDH | 1.67 | EF1dt | 1.33 |
| 2    | HisH2A | 1.67 | GAPDH | 3.33 | EF1dt | 2.00 | HisH2A | 2.00 |
| 3    | EF1dt | 2.33 | a-tub3 | 3.33 | HisH2A | 3.67 | GAPDH | 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 |

c. GM represents geometric mean.

**Figures**
Figure 1

The content of Hup A in different tissues of *H. serrata*. The mean and standard deviation were calculated using the data from three independent biological replicates.
Figure 2
Distribution of Ct values for ten 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 thirteen candidate reference genes analyzed by geNorm. (A) All samples. (B) Root. (C) Stem. (D) Leaf. The least stable genes are listed on the left, while the most stable genes are exhibited on the right.
Figure 4

Pairwise variation (Vn/n+1) of ten candidate reference genes calculated by geNorm. The cut-off value to determine the optimal number of RGs for qRT-PCR normalization is 0.15.
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, 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.

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