1 **Reference genes for qRT-PCR normalisation in different tissues,** 2 **developmental stages and stress conditions of *Hypericum perforatum***

3 Wen Zhou¹; Lei Yang¹; Yan Sun¹; Qian Zhang¹; Bin Li¹; Bin Wang^{1,2}; Lin Li¹; Donghao Wang¹;
4 Shiqiang Wang¹; Zhezhi Wang^{1*}

5 ¹National Engineering Laboratory for Resource Development of Endangered Crude Drugs in
6 Northwest China, Key Laboratory of the Ministry of Education for Medicinal Resources and Natural
7 Pharmaceutical Chemistry, College of Life Sciences, Shaanxi Normal University, Xi'an, 710119,
8 China

9 ²College of Chemistry, Biology and Materials Science, East China University of Technology,
10 NanChang, 330013, P. R. China

11 * Corresponding author:

12 E-mail address: zzwang@snnu.edu.cn (ZW)

13 **Abstract**

14 *Hypericum perforatum* is a widely known medicinal herb used mostly as a remedy for depression
15 because of its abundant secondary metabolites. Quantitative real-time PCR (qRT-PCR) is an
16 optimized method for the efficient and reliable quantification of gene expression studies. In general,
17 reference genes are used in qRT-PCR analysis because of their known or suspected housekeeping
18 roles. However, their expression level cannot be assumed to remain stable under all possible
19 experimental conditions. Thus, the identification of high quality reference genes is very necessary for
20 the interpretation of qRT-PCR data. In this study, we investigated the expression of fourteen

candidate genes, including nine housekeeping genes and five potential candidate genes. Additionally, the *HpHYPI* gene, belonging to the PR-10 family associated with stress control, was used for validation of the candidate reference genes. Three programs were applied to evaluate the gene expression stability across four different plant tissues, three developmental stages and a set of abiotic stress and hormonal treatments. The candidate genes showed a wide range of Ct values in all samples, indicating that they are differentially expressed. Integrating all of the algorithms and evaluations, *ACT2* and *TUB-β* were the most stable combination overall and for different developmental stages samples. Moreover, *ACT2* and *EF1-α* were considered to be the two most applicable reference genes for different tissues and for stress samples. Majority of the conventional housekeeping genes exhibited better than the potential reference genes. The obtained results will contribute to improving credibility of standardization and quantification of transcription levels in future expression research of *H. perforatum*.

Introduction

An increasing number of studies on the gene expression levels in plants have been carried out in order to understand the signalling and metabolic pathways underlying the developmental processes involved in plant development and growth, as well as in plant responses to biotic and abiotic stresses [1-4]. Methods for detecting gene expression include northern blot, gene chips, semi-PCR, RNase protection analysis and qRT-PCR. qRT-PCR because of its high sensitivity, specificity, reproducibility and accuracy has become a very popular and effective method for detecting and quantifying gene transcription levels [5-7]. Reliable quantification of gene expression levels by qRT-PCR analysis requires the standardization and fine-tuning of several parameters, such as the amount of initial sample, RNA recovery and integrity, enzymatic efficiency of cDNA synthesis and PCR amplification, and the overall transcriptional activity of the tissues or cells analysed [5-9]. The expression stability of frequently used reference genes also cannot be neglected. Therefore,

normalization for transcript levels of test genes is essential for minimizing technical differences for different samples and experimental conditions [9]. More importantly, selection of stable reference gene(s) is prerequisite before qRT-PCR analysis.

A appropriate reference gene is considered to be not affected by the experimental conditions and also shouldh maintained at a invariable levle among samples [10]. Actually, the transcription levels of the traditional reference genes and even some housekeeping genes (HKGs) can vary between different types of tissue and under different treatment conditions [11]. Therefore, a growing number of research have been published on the analysis and evaluation of the stability of internal reference genes in plant tissues under different conditions. For *Gentiana macrophylla*, *SAND1* and *EF-1 α 4* were found to be the most suitable overall; *GAPC2* and *SAND1* certified to be the best reference genes for the roots under abiotic stresses, while *SAND1* and *EF-1 α 4* were screened out from the leaves of stressed plants [12]. For the tung tree, *ACT7*, *UBQ*, *GAPDH*, and *EF-1 α* were the four optimal reference genes in all samples and developing seeds. *ACT7*, *EF-1 β* , *GAPDH*, and *TEF1* were the top four candidate genes for different tissues [13]. Consequently, different reference genes should be applied in different experimental materials and conditions.. In addition, the reference genes with lower stability may lead to a more erroneous understanding of qRT-PCR [14] and mask the true nature of gene expression [15,16].

Hypericum perforatum L. (commonly known as St. John's wort) is a widely known medicinal herb used mostly as a remedy for depression [17]. Pure compounds essentially isolated from *H. perforatum*, namely, naphthodianthrone and phloroglucinols, are proven to possess anti-depressive, anti-cancer, anti-viral, anti-inflammatory activities and other activities [18,19]. Xanthones and flavonoids have also been identified in extracts from this plant [20]. To date, a limited number qRT-PCR studies focusing on *H. perforatum* has been published. Isabel et al. studied the stability of 11 candidate reference genes analysed in *H. perforatum* plants only subjected to cold and heat stresses,

revealing TUB being the most stable gene in both experimental conditions [21]. Therefore, it is very necessary to screen suitable reference genes in different tissues of *H. perforatum* under different experimental conditions. Ribosomal RNA and some HKGs usually served as reference genes, such as actin (*ACT*), tubulin (*TUB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and polyubiquitin (*UBQ*) [22-26], whereas many studies have revealed that the most commonly used HKGs are not always reliable in different experimental samples [27-30]. So a evaluation for screening out the optimal HKGs in different species is essential.

This study aimed to assess the expression stabilities of fourteen reference genes in fifteen experimental samples by qRT-PCR, including 9 traditional HKGs and 5 potential reference genes: *GAPDH*, actin (*ACT2*, *ACT3*, *ACT7*), ubiquitin-conjugating (*UBC2*), elongation factor (*EF-1a*), tubulin (*TUB-α*, *TUB-β*), cyclophilin (*CYP1*), polyketide synthase (*PKS1*), glutamate semialdehyde aminomutase (*GSA*), SAND family protein (*SAND*), ribosomal protein L (*RPL13*) and protein phosphatase 2A (*PP2A*). These genes were selected from the *H. perforatum* genome sequencing data in our lab. Three mathematical programs, geNorm [31], NormFinder [32] and BestKeeper [33], were applied to analyse the raw Ct values and to figure out the relatively stable expressed genes. All of the raw Ct values need to be converted to ΔCt (ΔCt = each corresponding average Ct value - minimum Ct value) in NormFinder and geNorm algorithms, while there is no need in BestKeeper [34]. The selected reference genes were detected by using a target gene *HpHYP1* belonging to the Pathogenesis related class-10 (PR-10) family [35,36].

Materials and Methods

Plant materials

The *H. perforatum* seeds ($2n=2x=16$) were germinated on a seedling bed in the glasshouse (25 ± 2 °C, natural lighting, 60–80% humidity). Whole plant tissues were collected at the one-month-old

(1M), two-month-old (2M), three-month-old (3M) and six-month-old (6M) stages. The different tissue samples (leaf, flower, stem and root) were taken from two-year-old plants ($2n=2x=16$). Three-month-old seedlings were treated with abiotic stress and hormonal treatments, including 10 μ M salicylic acid (SA), 200 μ M methyl jasmonate (MeJA), 100 μ M abscisic acid (ABA), 1 mM AgNO₃ (Ag), 200 μ M CuSO₄ (Cu), 100 mM NaCl (Na), low temperature (4 °C) and wounding (W). The stress samples were acquired severally after 6 h of the corresponding treatments, and the control groups were collected following non-treatment. The collected plant samples are . All samples were collected in three replicates and frozen in liquid nitrogen immediately and then stored at -80 °C.

Total RNA isolation and cDNA synthesis

Total RNA was extracted using the Polysaccharide and Polyphenols Plant Quick RNA Isolation Kit (centrifugal column type; Waryong, Beijing, China). The genomic DNA was digested with RNase-free DNase I (TaKaRa, Japan). The total RNA was quantified by absorbance at A_{260}/A_{280} nm and A_{260}/A_{230} nm with a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). A 1% (p/v) agarose gel was run to visualize the integrity of the RNA. Only RNA samples with a A_{260}/A_{280} wavelength ratio between 1.9 and 2.1 and an A_{260}/A_{230} ratio close to 2.0 were used for cDNA synthesis. A quantity of 1.0 μ g DNA-free total RNA was used to synthesize first-strand cDNAs with a PrimeScript RT Reagent Kit (TaKaRa, China) in a 20 μ L volume. All cDNA samples were diluted (1:30) with DNase/RNase-free deionized water for qRT-PCR.

Selection of reference genes and primer design

We performed genomic sequencing of *H. perforatum* using Illumina paired-end, 10X Genomics linked reads and PacBio SMART (unpublished). The transcriptome sequencing of *H. perforatum* for the roots, stems, leaves and flowers assisted annotation. Fourteen candidate genes, including 9 traditional HKGs (*GAPDH*, *ACT2*, *ACT3*, *ACT7*, *EF1- α* , *UBC2*, *TUB- α* , *TUB- β* and *CYP1*) and 5

potential reference genes (*PKSI*, *GSA*, *RPL13*, *SAND* and *PP2A*), were selected for investigation in order to identify the most stably expressed reference genes. To ensure the accuracy of the reference gene predictions, we first screened the candidate genes according to the genome annotation of each, which was assigned based on the best match of the alignments using Blastp to SwissProt, KEGG, NR and TrEMBL databases. Then the coding sequences of the fourteen chosen genes were used as queries for BLAST orderly through the TAIR (<http://www.arabidopsis.org/>) for further ensuring accuracy. Finally the sequences with the highest homology with Arabidopsis were shown in Table 1. The primers of all the genes were designed using GenScript (<https://www.genscript.com>) with a melting temperature between 59 and 61 °C, a primer length of 20 - 25 bp, and an amplicon length of 70 - 180 bp. The descriptions of the candidate reference genes, primer sequences and qRT-PCR amplification efficiencies are presented in Table 1.

qRT-PCR conditions and analysis

PCR reactions were performed on the Roche LightCycler 96 system using SYBR® Master Mix. Reactions were done in 20 µl volumes in triplicate containing 5 µL of 30-fold diluted synthesized cDNA, 10 µL SYBR® Master Mix, 0.4 µL 10 mM forward primer, 0.4 µL 10 mM reverse primer and 4.2 µL DNase/RNase-free deionized water. The cycling conditions included 95 °C for 30 s, 45 cycles of 95 °C for 5 s and 60 °C for 30 s, followed by a final melting curve analysis. Each reaction was set up with a negative control group and conducted in three technical replicates. According to calculate the amplification efficiency from 10-fold continuous dilution of the cDNA for each gene, the standard curves were constructed to get the correlation coefficients (R^2) and slope values. So the corresponding PCR amplification efficiencies (E) could be calculated ($E = (10^{-1/\text{slope}} - 1) \times 100$ [37]).

Assessment of expression stability

The expression stability of the genes was analysed with three different Visual Basic applets, GeNorm [30,38], NormFinder [39, 40] and BestKeeper [41,42]. GeNorm derives a stability measure (M-value), via a stepwise exclusion of the least stable gene, and creates a stability ranking [43]. Genes with $M < 1.5$ are generally considered stable reference genes [44,45]. This measure is based on the principle that the expression ratio of two ideal control genes should be identical in all samples; thus genes with the lowest M-value are the most stably expressed [46]. NormFinder uses an ANOVA-based model to estimate intra- and inter-group variation within tissues or treatments ("groups" in NormFinder terminology) to assess the expression stability [47]. For GeNorm and NormFinder, the raw Ct values needed to be converted to relative quantities (Q) by the formula $Q = 2^{-\Delta Ct}$, in which $\Delta Ct = \text{each average Ct value} - \text{minimum Ct value}$ [48]. In BestKeeper, the coefficient of variance (CV) and the standard deviation (SD) were calculated by the Ct values, with the lower CV and SD indicating higher stability [49]. All other statistical analyses were calculated by GraphPad Prism 6.0 [50], and the level of statistical significance was assessed using * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Validation of reference gene stability

Previous studies showed that the *HpHYPI* (JF774163) gene is classified as a member of the plant PR-10 (pathogenesis-related class 10) gene family. Members of the PR-10 family are ubiquitous plant proteins associated with stress control [51]. The relative expression of *HpHYPI* gene in different tissues were measured and standardized by using the most stable and unstable candidate genes as internal reference to verify the reliability of the selected genes according to the $2^{-\Delta\Delta Ct}$ method [52]. Three technical replicates were performed for each biological sample.

Results

Primer specificity and expression level analysis of candidate reference genes

The gene names and abbreviations, accession numbers, primer sequences, amplification efficiencies, amplicon sizes, T_m values and molecular functions are listed in **Table 1**. The amplification efficiencies ranged from 92.5% (*RPL13*) to 109.5% (*TUB-α*), the T_m values varied from 81.2 °C (*PP2A*) to 87.8 °C (*CYP1*), and the amplicon sizes were between 76 bp (*GAPDH* and *PKSI*) and 170 bp (*TUB-β*). Furthermore, primer specificities were detected by melting curves (**Fig 1**) and a single band indicated the correct size of each pair. The raw CT values of different genes ranged from 21.2 to 38.2 (**Fig 2**). Data were analysed within experiments and were divided into four groups: tissues from two-year-old plants (TS: R, S, L, and F), developmental stages seedlings (SG: 1M, 2M, 3M, and 6M), three-month-old seedlings exposed to abiotic stresses (ST: SA, MeJA, ABA, Cu, Ag, Na, 4 °C and W) and a combination of all experimental conditions (TT). The fourteen selected genes' expression levels in the four groups were shown in Fig. 2. Among them, UBC2 (SG) performed the highest Ct value (30.33) , but GAPDH (TS) represented the opposite, which indicated the levels of their expression.

Gene symbol	Gene name	Accession No.	Amplicon size (bp)	Primer sequence U/L [5'–3']	T _m (°C)	Efficiency (%)
<i>ACT2</i>	actin 2	MK054303	101	Fw: AGGAGTCCCTCCACGACCAC Rv: GCCGTTGTGTACCGGGTAGG	83.6	97.6
<i>ACT3</i>	actin 3	MK106364	139	Fw: ATCCTTCCCACGGTGGTTGC Rv: CAATCGCCTCGTCGCCTACA	88.2	102.9
<i>ACT7</i>	actin 7	MK106365	131	Fw: GAGCAGCAGCAGGTCGACAA Rv: ACCCACTCGAGCCCAGTGTA	83.0	107.6
<i>CYP1</i>	cyclophilin	MK106359	163	Fw: AGGGATCCAGCTTCCACCGT Rv: GCGTTGGCCATGGAGAGGAT	87.8	96.2
<i>EF1-α</i>	elongation factor 1-alpha	MK106356	121	Fw: TGGAGGCTCTCCCTGGTGAC	85.8	105.6

				Rv: AAGTTGGCAGCCTCCTTGGC		
<i>GAPDH</i>	glyceraldehyde-3-phosphate dehydrogenase A subunit	EU301783	76	Fw: AGGCCTCCCACCTCCTCAAG Rv: GGTTGACAGGGTTGCGGTCA	84.7	105.1
<i>TUB-α</i>	alpha tubulin	MK106362	132	Fw: TGCTGCGGTTGCCACCATTA Rv: CGCTGCACCTTTGCAAGATCG	84.8	109.5
<i>TUB-β</i>	beta tubulin	MK106361	170	Fw: CGACGGGAGTGACAGCCTTG Rv: CGACCACATCGCTCGTCTCC	86.5	108.6
<i>UBC2</i>	ubiquitin-conjugating enzyme 2	MK106357	103	Fw: AGGAGGAGGCGCCTTTGAGA Rv: CGCAAGAGCCGGTCCATTCA	84.5	98.2
<i>GSA</i>	glutamate-1-semialdehyde 2,1-aminomutase	KJ624985	78	Fw: GGTGTCAGGATGGCGGTGTC Rv: GGGCTGGCCACCAACTGATT	85	94.4
<i>PKS1</i>	polyketide synthase 1	EF186675	76	Fw: ACGGACGCTGCCATCAA Rv: ACATAACCGTGTACCTTGTC	82.5	98.3
<i>PP2A</i>	protein phosphatase 2A	MK106360	93	Fw: GGCAAGTGCCCAGACACCAA Rv: CAGCGCCACTAGCAGCGTAA	81.2	95.5
<i>RPL13</i>	ribosomal protein L	MK106363	151	Fw: CAGCGCTGGATGTTTCGAGGA Rv: TGGTGGAAGCCAACCTCCCA	84.5	92.5
<i>SAND</i>	SAND family protein	MK106617	111	Fw: CTCCCTCGCACTGGGACAAC Rv: AAGACCAGCAGGACCACCCA	83.2	97.1

174 **Table 1.** Description of the candidate reference genes, primer sequences and qRT-PCR amplification
175 efficiencies in *H. perforatum*.

176 **Figure 1. Melting curves for the candidate reference genes.** The melting temperature was shown
177 by plotting the negative derivative of fluorescence relative to Celsius temperature $-(d/dT)$.

Figure 2. Average Ct values for the candidate reference genes. (A) Different tissues; (B) developmental stage seedlings; (C) stress-treated seedlings; (D) data from all experimental conditions combined. A line across the box is depicted as the median. The box indicates the 25th and 75th percentiles; whisker caps represent the maximum and minimum values; and dots represent outliers.

Stability of candidate reference genes

GeNorm is requested to calculate a normalization factor from the geometric mean of the genes to distinguished the most stably expressed gene. The M-values is defined as the average pairwise variation of a particular gene with all other potential reference genes. In GeNorm, the threshold for eliminating a gene was set as $M < 1.5$, the lower the M value, the higher the stability [53]. The expression stability ranking of the fourteen reference genes was arranged in the TT samples as follows: *TUB-β* > *ACT2* > *CYP1* > *EF1-α* > *TUB-α* > *SAND* > *UBC2* > *ACT7* > *PP2A* > *GSA* > *PKS1* > *RPL13* > *GAPDH* > *ACT3*. *ACT2* and *ACT3* were the genes with the lowest and highest M values in the TS, SG and ST groups. In brief, *ACT2* and *TUB-β* were the optimal two reference genes for pooled samples that included all treatments. *ACT7*, *RPL13* and *GAPDH* were determined to be the relatively unstable reference genes in the majority of samples (**Table 2, Fig 3**). The Genorm pairwise variation (V) values between ranked genes (V_n/V_{n+1}) were determined for the optimal number of reference genes to be used in the quantitative analysis experiments. A cut-off of 0.15 (V_n value) is usually applied [53]. The $V_{2/3}$ values for all of the experimental sets in *H. perforatum* were lower than the cut off threshold of 0.15 (**Fig 4**), which indicated that the combination of two reference genes can accurately standardize these samples. In other words, *ACT2* and *TUB-β* was the optimum selection as the reference gene combination for qRT-PCR analysis in all of the experimental sets.

NormFinder with lower values indicating higher stability, is another Excel application, which ranks the candidate genes from their minimal combined inter- and intra-group variation of expression.

Table 3 shows the expression stability values of the four data sets calculated by NormFinder. *ACT2*, *TUB-β* and *EF1-α* were the top three suitable genes for normalization, while *ACT3* was at the end of the rankings in all groups. *ACT7* ranks at the top of expression stability in the ST group, but it is very unstable in other groups. The stability order of the candidate genes calculated by NormFinder is nearly the same as that calculated by GeNorm, but this conclusion did not exclude a small difference. For example, *ACT2* was identified as the most stable reference gene in the TS group with the GeNorm analysis, while its stability rankings placed it third within the NormFinder analysis.

BestKeeper is also an Excel-based program that is able to compare the expression levels of up to ten target genes, each in up to a hundred biological samples [33]. The CV and SD of the fourteen candidate genes computed by BestKeeper are represented in **Table 4**. The smaller the SD and the CV, the better the stability of the internal reference genes. When $SD > 1$, the expression of the reference gene was unstable [33]. The results revealed that *ACT2* ($SD = 0.303$, $CV = 1.121$) and *TUB-β* ($SD = 0.551$, $CV = 2.145$) were the top two most stable genes for the TT samples. *TUB-β* was ranked first in the TS samples and *PKS1* showed the lowest stability. *ACT2* was still the most stable gene in the SG and ST samples. Unlike the GeNorm and NormFinder rankings, the stability of *RPL13* was ahead in BestKeeper analysis and was relatively stable under all tested conditions.

Rank	Total		Tissue		Stage		Stress	
	Gene	M-value	Gene	M-value	Gene	M-value	Gene	M-value
1	<i>TUB-β</i>	0.818	<i>ACT2</i>	0.861	<i>ACT2</i>	0.639	<i>ACT2</i>	0.474
2	<i>ACT2</i>	0.890	<i>EF1-α</i>	0.867	<i>TUB-β</i>	0.665	<i>EF1-α</i>	0.501
3	<i>CYP1</i>	0.895	<i>TUB-β</i>	0.882	<i>CYP1</i>	0.670	<i>CYP1</i>	0.504
4	<i>EF1-α</i>	0.936	<i>CYP1</i>	0.921	<i>TUB-α</i>	0.737	<i>ACT7</i>	0.506
5	<i>TUB-α</i>	0.946	<i>TUB-α</i>	0.934	<i>GSA</i>	0.738	<i>TUB-β</i>	0.506
6	<i>SAND</i>	0.952	<i>ACT7</i>	1.009	<i>EF1-α</i>	0.748	<i>TUB-α</i>	0.531
7	<i>UBC2</i>	1.005	<i>UBC2</i>	1.070	<i>PP2A</i>	0.750	<i>GSA</i>	0.559
8	<i>ACT7</i>	1.009	<i>PP2A</i>	1.126	<i>GAPDH</i>	0.753	<i>PKS1</i>	0.599
9	<i>PP2A</i>	1.059	<i>SAND</i>	1.167	<i>SAND</i>	0.762	<i>SAND</i>	0.639
10	<i>GSA</i>	1.086	<i>GSA</i>	1.181	<i>UBC2</i>	0.862	<i>UBC2</i>	0.642

11	<i>PKSI</i>	1.087	<i>PKSI</i>	1.199	<i>ACT7</i>	0.886	<i>GAPDH</i>	0.645
12	<i>RPL13</i>	1.089	<i>RPL13</i>	1.229	<i>PKSI</i>	0.940	<i>PP2A</i>	0.650
13	<i>GAPDH</i>	1.151	<i>GAPDH</i>	1.339	<i>RPL13</i>	1.205	<i>RPL13</i>	0.700
14	<i>ACT3</i>	1.216	<i>ACT3</i>	1.404	<i>ACT3</i>	1.284	<i>ACT3</i>	0.772

Table 2. Ranking and M-values of the candidate reference genes calculated by geNorm.

Rank	Total		Tissue		Stage		Stress	
	Gene	Stability Value	Gene	Stability Value	Gene	Stability Value	Gene	Stability Value
1	<i>TUB-β</i>	0.246	<i>EF1-α</i>	0.090	<i>ACT2</i>	0.048	<i>ACT2</i>	0.141
2	<i>ACT2</i>	0.332	<i>TUB-β</i>	0.248	<i>TUB-β</i>	0.048	<i>ACT7</i>	0.179
3	<i>CYP1</i>	0.346	<i>ACT2</i>	0.323	<i>TUB-α</i>	0.190	<i>EF1-α</i>	0.194
4	<i>EF1-α</i>	0.398	<i>CYP1</i>	0.371	<i>EF1-α</i>	0.205	<i>TUB</i>	0.195
5	<i>TUB-α</i>	0.445	<i>ACT7</i>	0.462	<i>CYP1</i>	0.251	<i>CYP1</i>	0.205
6	<i>SAND</i>	0.453	<i>UBC2</i>	0.521	<i>GSA</i>	0.304	<i>TUA</i>	0.247
7	<i>ACT7</i>	0.511	<i>GSA</i>	0.620	<i>GAPDH</i>	0.308	<i>GSA</i>	0.262
8	<i>UBC2</i>	0.532	<i>PP2A</i>	0.644	<i>PP2A</i>	0.325	<i>PKSI</i>	0.311
9	<i>PKSI</i>	0.562	<i>GAPDH</i>	0.653	<i>SAND</i>	0.337	<i>SAND</i>	0.335
10	<i>PP2A</i>	0.586	<i>PKSI</i>	0.663	<i>UBC2</i>	0.487	<i>UBC2</i>	0.341
11	<i>RPL13</i>	0.594	<i>SAND</i>	0.687	<i>RPL13</i>	0.491	<i>RPL13</i>	0.353
12	<i>GSA</i>	0.602	<i>TUB-α</i>	0.710	<i>ACT7</i>	0.540	<i>GAPDH</i>	0.375
13	<i>GAPDH</i>	0.668	<i>ROL13</i>	0.789	<i>PKSI</i>	0.771	<i>PP2A</i>	0.401
14	<i>ACT3</i>	0.688	<i>ACT3</i>	0.934	<i>ACT3</i>	1.297	<i>ACT3</i>	0.462

Table 3. Ranking and expression stability values of the candidate reference genes calculated by NormFinder.

Rank	Total			Tissue			Stage			Stress		
	Gene	SD[±CP]	CV	Gene	SD[±CP]	CV	Gene	SD[±CP]	CV	Gene	SD[±CP]	CV
1	<i>ACT2</i>	0.303	1.121	<i>TUB-β</i>	0.184	0.677	<i>TUB-β</i>	0.294	0.996	<i>ACT2</i>	0.181	0.692
2	<i>TUB-β</i>	0.551	2.145	<i>RPL13</i>	0.520	2.106	<i>ACT2</i>	0.329	1.514	<i>EF1-α</i>	0.231	0.857
3	<i>CYP1</i>	0.597	2.308	<i>CYP-1</i>	0.766	2.963	<i>GSA</i>	0.454	2.020	<i>CYP-1</i>	0.274	1.070
4	<i>TUB-α</i>	0.606	2.650	<i>TUB-α</i>	0.798	3.291	<i>RPL13</i>	0.585	3.098	<i>TUB-β</i>	0.281	1.103
5	<i>RPL13</i>	0.610	2.423	<i>SAND</i>	0.870	3.819	<i>GAPDH</i>	0.610	2.258	<i>RPL13</i>	0.285	1.167
6	<i>EF1-α</i>	0.628	2.099	<i>ACT7</i>	0.925	3.640	<i>EF1-α</i>	0.610	2.378	<i>PKS-1</i>	0.319	1.678
7	<i>ACT7</i>	0.633	2.452	<i>ACT2</i>	0.943	3.641	<i>SAND</i>	0.695	2.771	<i>TUB-α</i>	0.356	1.499
8	<i>PP2A</i>	0.644	2.655	<i>EF1-α</i>	0.958	3.787	<i>CYP1</i>	0.793	3.036	<i>SAND</i>	0.357	1.550

9	<i>SAND</i>	0.763	3.248	<i>GAPDH</i>	0.976	4.580	<i>TUB-α</i>	0.803	3.540	<i>ACT3</i>	0.362	1.440
10	<i>UBC2</i>	0.770	4.005	<i>ACT3</i>	0.985	3.656	<i>UBC2</i>	0.833	3.211	<i>PP2A</i>	0.378	1.471
11	<i>PKSI</i>	0.831	3.498	<i>PP2A</i>	0.995	4.151	<i>PKS-I</i>	0.855	3.732	<i>ACT7</i>	0.399	1.705
12	<i>GSA</i>	0.893	3.928	<i>GSA</i>	1.013	5.071	<i>PP2A</i>	0.859	3.539	<i>UBC2</i>	0.436	1.457
13	<i>GAPDH</i>	0.947	4.803	<i>UBC2</i>	1.165	3.841	<i>ACT7</i>	0.873	4.605	<i>GSA</i>	0.471	2.090
14	<i>ACT3</i>	1.039	3.989	<i>PKS-I</i>	1.393	5.655	<i>ACT3</i>	1.888	7.201	<i>GAPDH</i>	0.562	2.909

220 **Table 4.** Ranking of the candidate reference genes and their expression stability calculated by
221 BestKeeper.

222 **Figure 3.** Average expression stability (M-value) of 14 candidate reference genes calculated by
223 geNorm. (A) Different tissues; (B) developmental stage seedlings; (C) stress-treated seedlings; (D)
224 data from all experimental groups combined. A lower average expression stability (M-value)
225 indicates more stable expression.

226 **Figure 4.** Pairwise variation (V) calculated by geNorm to determine the optimal number of
227 reference genes in different tissues, developmental stage seedlings, stress-treated seedlings and
228 all experimental groups combined. The average pairwise variations V_n/V_{n+1} were analysed
229 between the normalization factors NF_n and NF_{n+1} to indicate the optimal number of reference genes
230 in different samples.

231 Reference gene validation

232 In order to test the reliability of the results, the relative expression patterns for the target gene
233 *HpHYPI*, belonging to the PR-10 family [35,36] and associated with stress control, were evaluated
234 by different internal control genes in the roots, stems, leaves and flowers. Through the present
235 research results, the top two stable genes (*ACT2* and *TUB-β*) and the most unstable gene (*ACT3*)
236 were used as internal controls. As shown in **Fig 5**, when normalized using *ACT2* as the reference
237 genes, transcript abundance of *HpHYPI* was upregulated compared with the result in the root
238 samples. When *ACT2* and *TUB-β* (as identified by geNorm) both served as the internal references,

the expression patterns are similar with *ACT2*. When normalization was based on *TUB-β* alone, the expression level of *HpHYP1* was still up-regulation. The only difference was that the expression level in stems was lower than that in the leaves, but a significant difference was not apparent. In general, when stable reference genes including *ACT2*, *TUB-β* and their combination were used as internal parameters, *HpHYP1* had the highest expression level in flowers. When normalized by the less stable gene *ACT3*, the target genes in all tissues were expressed at lower levels but showed significantly upregulation ($P < 0.001$). Thus, selection of inappropriate reference genes can lead to over- or under-estimation of the relative transcript level, which might lead to a biased result.

Figure 5. Relative expression levels of *HpHYP1* in different tissues (root, stem, leaf, and flower) used for normalization using the most stable reference genes or a combination and the least stable genes. Error bars show mean standard error calculated from three biological replicates. The statistical level was assessed with * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Discussion

qRT-PCR is a very convenient and efficient method and widely used for gene expression analysis [8,15]. The reliability of qRT-PCR data will be greatly improved by the inclusion of a reference gene with a transcription level that is invariable across different experimental conditions [8]. In many published studies, some common reference genes especially the housekeeping genes have been verified in many species, such as in *Arabidopsis thaliana* [46,54], *Poa pratensis* [54], *Salvia miltiorrhiza* [55], *Cucumis sativus* [56], *Gentiana macrophylla* [37], and *Isatis indigotica* [57]. But selecting appropriate reference genes for *H. perforatum* in different experimental conditions have not yet been conducted, which might be due to the fact that genomic information for *H. perforatum* is very limited in NCBI. With the rapid development of whole-genome sequencing technology, deciphering the genomes of medicinal herbs is a vital step in understanding and improving their

medicinal value. For that reason, we completed the assembly of the first high-quality sequence of the *H. perforatum* genome. Therefore, the reported literature on *H. perforatum* [58,59] combined with the obtained genomic data laid the foundation for selecting reference genes.

Housekeeping genes like those participated in cell structure maintenance (*ACT* and *TUB*) or basic cellular processes (*UBC* and *CYP*) remain widely applied, but their expression can vary between different types of tissue (normal and pathological samples) and under different treatment conditions (drugs and chemicals) [60,61]. Thus, normalization with multiple reference genes is becoming popular and standard in plant research [33,53]. In this work, fourteen potential reference genes were chosen, including nine traditional housekeeping genes and five potential reference genes. The study represents an effort to identify and systematically compare fourteen potential reference genes. We assessed the expression patterns of these genes in different tissues, different developmental stages and under various abiotic stress treatments to screen for the most stable housekeeping genes for qRT-PCR analyses. As shown in Fig. 2, No one has a constant CT value, which shows how important it is to evaluate the most suitable reference gene for normalizing expression under all detection conditions in *H. perforatum*.

The analytical procedures applied in our research based on statistical algorithm to identify the stability of reference genes are commonly used by researchers to select the best reference genes [53,62-65]. Furthermore, a comparison of the different algorithms of reference genes contributes a better evaluation and reduces the risk of artificial selection of co-regulated transcripts [66]. The arithmetic of NormFinder and geNorm is almost the same, but GeNorm analysis is used to determine not only the most stable reference genes but also the optimal number of the gene combinations. In the present study, when the data were taken together to figure out the optimal number of reference genes, the pairwise variation of V2/3 values for all of the experimental sets was lower than the cut off threshold of 0.15 (Fig. 4). Thus, these results show that the best combination (*ACT2* and *TUB-β*)

should be both involved to improve the accuracy of the quantitative expression analysis in *H. perforatum*. For the BestKeeper program, it can worked out the best suited standards in accordance with SD and CV values [33], out of ten candidates including reference and target genes.

In our research, GeNorm and NormFinder create similar rankings for stability values, while BestKeeper programs always get different rankings. For example, BestKeeper ranked RPL13 as the most stable, as it was relatively stable under all tested conditions. In contrast, the expression stability value of the RPL13 gene in GeNorm and Normfinder was very low. There were also some previous papers revealing similar difference between BestKeeper and other methods [54,67]. Homologous genes are widely used as reference genes for gene expression analysis. For example, *ACT6*, *ACT8*, and *ACT7* were selected as internal controls for stress treatments in *Fortunella crassifolia* [33]; *UBC19*, *UBC22*, and *UBC29* were selected as reference genes in the context of the relevant experimental conditions in *Isatis indigotica* [57]; and *EF1A2a*, *EF1A2b*, and *EF1A1a1* were the best reference genes under all tested conditions in *Glycine max* [68]. Nevertheless, as can be seen in our results, the three homologous genes (*ACT2*, *ACT3* and *ACT7*) exhibited totally different expression levels when used for the normalization of qRT-PCR, especially *ACT2* and *ACT3* (Table 2, Table 3, Table 4 and Fig. 3). Therefore the paralogous genes have similar structures, but their expression levels are entirely different in gene expression quantification [57].

In summary, our results support that the selection of reference genes has a significant impact on the normalized gene expression data in qRT-PCR experiments. We have investigated the expression of fourteen candidate reference genes across a large number of *H. perforatum* samples in an effort to identify those most stable genes for normalizing gene expression. Finally, a combination of the two genes *ACT2* and *TUB-β* provided the best internal controls for transcript normalization across experimental conditions in this study.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31670299) and the Fundamental Research Funds for the Central Universities (1301031470, GK201806006).

Author contributions

WZ managed and carried out the experimental design. LY and YS sowed the seeds, directed the abiotic stress treatments and collected samples. WZ and LY screened the candidate reference genes and designed the primers. QZ, BW and LL performed the qRT-PCR. WZ drafted the manuscript, and BL revised it. All authors discussed and commented on the manuscript.

Reference

1. Morenorisueno MA, Norman JMV, Moreno A, Zhang J, Ahnert SE, et al. (2010) Oscillating Gene Expression Determines Competence for Periodic Arabidopsis Root Branching. *Science* 329: 1306-1311.
2. Hiroataka Y, Hiroyuki F, Tomohito A, Akio O, Tsukasa N, et al. (2010) Gene expression analysis in cadmium-stressed roots of a low cadmium-accumulating solanaceous plant, *Solanum torvum*. *Journal of Experimental Botany* 61: 423-437.
3. Di W, Pan Y, Zhao X, Zhu L, Fu B, et al. (2011) Genome-wide temporal-spatial gene expression profiling of drought responsiveness in rice. *Bmc Genomics* 12: 149.
4. Dechorgnat J, Patrit O, Krapp A, Fagard M, Danielvedele F (2012) Characterization of the Nrt2.6 Gene in *Arabidopsis thaliana*: A Link with Plant Response to Biotic and Abiotic Stress. *Plos One* 7: e42491.

- 329 5. Bustin SA (2002) Bustin, S. A. Quantification of mRNA using real-time reverse transcription PCR
330 (RT-PCR): trends and problems. J Mol Endocrinol. Journal of Molecular Endocrinology 29: 23-
331 39.
- 332 6. Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR – a perspective.
333 Journal of Molecular Endocrinology 34: 597-601.
- 334 7. Nolan T, Hands RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. Nature
335 Protocols 1: 1559.
- 336 8. Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalisation; strategies and
337 considerations. Genes & Immunity 6: 279-284.
- 338 9. Udvardi MK, Czechowski T, Scheible WR (2008) Eleven golden rules of quantitative RT-PCR.
339 Plant Cell 20: 1736-1737.
- 340 10. Bustin SA, Mueller R (2005) Real-time reverse transcription PCR (qRT-PCR) and its potential
341 use in clinical diagnosis. Clinical Science 109: 365-379.
- 342 11. Leal MF, Astur DC, Debieux P, Arliani GG, Silveira Franciozi CE, et al. (2015) Identification of
343 Suitable Reference Genes for Investigating Gene Expression in Anterior Cruciate Ligament
344 Injury by Using Reverse Transcription-Quantitative PCR. Plos One 10: e0133323.
- 345 12. He Y, Yan H, Hua W, Huang Y, Wang Z (2016) Selection and Validation of Reference Genes for
346 Quantitative Real-time PCR in Gentiana macrophylla. Frontiers in Plant Science 7: 945.
- 347 13. Tong ZG, Gao ZH, Wang F, Zhou J, Zhang Z (2009) Selection of reliable reference genes for
348 gene expression studies in peach using real-time PCR. BMC Molecular Biology,10,1(2009-07-
349 20) 10: 1-13.

14. Gutierrez L, Mauriat M, Guénin S, Pelloux J, Lefebvre JF, et al. (2010) The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant Biotechnology Journal* 6: 609-618.
15. Brattelid T, Levy FO (2011) Quantification of GPCR mRNA Using Real-Time RT-PCR: Humana Press. 165-193 p.
16. Bustin SA, Nolan T (2004) Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J Biomol Tech* 15: 155-166.
17. Butterweck V (2003) Mechanism of action of St John's wort in depression : what is known? *Cns Drugs* 17: 539.
18. Birt DF, Widrechner MP, Hammer KDP, Hillwig ML, Wei JQ, et al. (2009) Hypericum in infection: identification of anti-viral and anti-inflammatory constituents. *Pharmaceutical Biology* 47: 774-782.
19. Caraci F, Crupi R, Drago F, Spina E (2011) Metabolic drug interactions between antidepressants and anticancer drugs: focus on selective serotonin reuptake inhibitors and hypericum extract. *Current Drug Metabolism* 12: -.
20. Schröder J (1997) A family of plant-specific polyketide synthases: facts and predictions. *Trends in Plant Science* 2: 373–378.
21. Velada I, Ragonezi C, Arnholdt-Schmitt B, Cardoso H (1932) Reference Genes Selection and Normalization of Oxidative Stress Responsive Genes upon Different Temperature Stress Conditions in *Hypericum perforatum* L. *Plos One* 9: e115206.

- 370 22. Willems E, Leyns L, Vandesompele J (2008) Standardization of real-time PCR gene expression
371 data from independent biological replicates. *Analytical Biochemistry* 379: 127-129.
- 372 23. Imai T, Ubi BE, Saito T, Moriguchi T (2014) Evaluation of reference genes for accurate
373 normalization of gene expression for real time-quantitative PCR in *Pyrus pyrifolia* using different
374 tissue samples and seasonal conditions. *Plos One* 9: e86492.
- 375 24. Llanos A, François JM, Parrou JL (2015) Tracking the best reference genes for RT-qPCR data
376 normalization in filamentous fungi. *BMC Genomics*,16,1(2015-02-14) 16: 71.
- 377 25. Goulao LF, Fortunato AS, Ramalho JC (2012) Selection of Reference Genes for Normalizing
378 Quantitative Real-Time PCR Gene Expression Data with Multiple Variables in *Coffea* spp. *Plant*
379 *Molecular Biology Reporter* 30: 741-759.
- 380 26. Costa MD, Duro N, Batista-Santos P, Ramalho JC, Ribeiro-Barros AI (2015) Validation of
381 candidate reference genes for qRT-PCR studies in symbiotic and non-symbiotic *Casuarina glauca*
382 *Sieb. ex Spreng.* under salinity conditions. *Symbiosis* 66: 21-35.
- 383 27. Thellin O, Zorzi W, Lakaye B, Borman BD, Coumans B, et al. (1999) Housekeeping genes as
384 internal standards: use and limits. *Journal of Biotechnology* 75: 291-295.
- 385 28. Selvey S, Thompson EW, Matthaei K, Lea RA, Irving MG, et al. (2001) Beta-actin--an
386 unsuitable internal control for RT-PCR. *Molecular & Cellular Probes* 15: 307-311.
- 387 29. Ohl F, Jung M, Xu C, Stephan C, Rabien A, et al. (2005) Gene expression studies in prostate
388 cancer tissue: which reference gene should be selected for normalization? *Journal of Molecular*
389 *Medicine* 83: 1014-1024.

30. Vandesompele J, De KP, Pattyn F, Poppe B, Van NR, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* 3: research0034.0031.
31. Kidd M, Nadler B, Mane S, Eick G, Malfertheiner M, et al. (2007) GeneChip, geNorm, and gastrointestinal tumors: novel reference genes for real-time PCR. *Physiological Genomics* 30: 363-370.
32. Wang Q, Ishikawa T, Michiue T, Zhu BL, Guan DW, et al. (2012) Stability of endogenous reference genes in postmortem human brains for normalization of quantitative real-time PCR data: comprehensive evaluation using geNorm, NormFinder, and BestKeeper. *International Journal of Legal Medicine* 126: 943-952.
33. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (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.
34. Li T, Wang J, Lu M, Zhang T, Qu X, et al. (2017) Selection and Validation of Appropriate Reference Genes for qRT-PCR Analysis in *Isatis indigotica* Fort. *Frontiers in Plant Science* 8.
35. Košuth J, Katkovčinová Z, Olexová P, Čellárová E (2007) Expression of the hyp-1 gene in early stages of development of *Hypericum perforatum* L. *Plant Cell Reports* 26: 211.
36. Assavalapsakul W, Panyim S (2012) Molecular cloning and tissue distribution of the Toll receptor in the black tiger shrimp, *Penaeus monodon*. *Genetics & Molecular Research* 11: 484.

- 410 37. Radonić A, Thulke S, Mackay IM, Landt O, Siebert W, et al. (2004) Guideline to reference gene
411 selection for quantitative real-time PCR. Biochemical & Biophysical Research Communications
412 313: 856-862.
- 413 38. Schlotter YM, Veenhof EZ, Brinkhof B, Rutten VP, Spee B, et al. (2009) A GeNorm algorithm-
414 based selection of reference genes for quantitative real-time PCR in skin biopsies of healthy dogs
415 and dogs with atopic dermatitis. Veterinary Immunology & Immunopathology 129: 115-118.
- 416 39. Zhang X, Xu ZC, Xu J, Ji AJ, Luo HM, et al. (2016) Selection and validation of reference genes
417 for normalization of quantitative real-time reverse transcription PCR analysis in *Poria cocos*
418 (Schw.) Wolf (Fuling). Chinese Medicine 11: 1-17.
- 419 40. Die JV, Roman B, Obrero A (2011) Selection of Reference Genes for Gene Expression Studies in
420 Zucchini (*Cucurbita pepo*) Using qPCR. J Agric Food Chem 59: 5402-5411.
- 421 41. Rhinn H, Marchand-Leroux C, Croci N, Plotkine M, Scherman D, et al. (2008) Housekeeping
422 while brain's storming Validation of normalizing factors for gene expression studies in a murine
423 model of traumatic brain injury. BMC Molecular Biology 9: 62-62.
- 424 42. Rotenberg D, Thompson TS, German TL, Willis DK (2006) Methods for effective real-time RT-
425 PCR analysis of virus-induced gene silencing. Journal of Virological Methods 138: 49-59.
- 426 43. Zhang Q, Li H, Fan C, Hu R, Fu YF (2009) Evaluation of putative reference genes for gene
427 expression normalization in soybean by quantitative real-time RT-PCR. BMC Molecular
428 Biology,10,1(2009-09-28) 10: 93-93.
- 429 44. Tong Z, Gao Z, Fei W, Zhou J, Zhen Z (2009) Selection of reliable reference genes for gene
430 expression studies in peach using real-time PCR. BMC Molecular Biology 10: 1-13.

- 431 45. Guo Y, Chen JX, Yang S, Fu XP, Zhang Z, et al. (2010) Selection of reliable reference genes for
432 gene expression study in nasopharyngeal carcinoma. 中国药理学报 31: 1487-1494.
- 433 46. Dekkers BJ, Willems L, Bassel GW, van BolderenVeldkamp RP, Ligterink W, et al. (2012)
434 Identification of reference genes for RT-qPCR expression analysis in Arabidopsis and tomato
435 seeds. Plant & Cell Physiology 53: 28-37.
- 436 47. Marchal E, Hult EF, Huang J, Tobe SS (2013) Sequencing and validation of housekeeping genes
437 for quantitative real-time PCR during the gonadotrophic cycle of *Diploptera punctata*. BMC
438 Research Notes,6,1(2013-06-19) 6: 1-10.
- 439 48. Yang Z, Chen Y, Hu B, Tan Z, Huang B (2015) Identification and Validation of Reference Genes
440 for Quantification of Target Gene Expression with Quantitative Real-time PCR for Tall Fescue
441 under Four Abiotic Stresses. Plos One 10: e0119569.
- 442 49. Migocka M, Papierniak A (2011) Identification of suitable reference genes for studying gene
443 expression in cucumber plants subjected to abiotic stress and growth regulators. Molecular
444 Breeding 28: 343-357.
- 445 50. Swift ML (1997) GraphPad Prism, Data Analysis, and Scientific Graphing. Journal of Chemical
446 Information & Modeling 37: 411-412.
- 447 51. Michalska K, Fernandes H, Sikorski M, Jaskolski M (2010) Crystal structure of Hyp-1, a St.
448 John's wort protein implicated in the biosynthesis of hypericin. Journal of Structural Biology 169:
449 161-171.

- 450 52. Kumar V, Sharma R, Trivedi PC, Vyas GK, Khandelwal V (2011) Traditional and novel
451 references towards systematic normalization of qRT-PCR data in plants. Australian Journal of
452 Crop Science 5: 1455-1468.
- 453 53. Vandesompele J, Preter KD, Pattyn F, Poppe B, Roy NV, et al. (2002) Accurate normalization of
454 real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.
455 Genome Biology 3: research0034.0031.
- 456 54. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide
457 identification and testing of superior reference genes for transcript normalization in Arabidopsis.
458 Plant Physiology 139: 5-17.
- 459 55. Espinosa JR, Ayub Q, Chen Y, Xue Y, Tylersmith C (2015) Structural variation on the human Y
460 chromosome from population-scale resequencing. Croatian Medical Journal 56: 194-207.
- 461 56. Yi S, Qian Y, Han L, Sun Z, Fan C, et al. (2012) Selection of reliable reference genes for gene
462 expression studies in *Rhododendron micranthum* Turcz. Scientia Horticulturae 138: 128-133.
- 463 57. Li T, Wang J, Lu M, Zhang T, Qu X, et al. (2017) Selection and Validation of Appropriate
464 Reference Genes for qRT-PCR Analysis in *Isatis indigotica* Fort. Frontiers in Plant Science 8.
- 465 58. Yao L (2011) Molecular cloning and tissue-specific expression of two different chitin deacetylase
466 cDNA sequences from *Mamestra brassicae*. Chinese Journal of Applied Entomology 48: 1417-
467 1424.
- 468 59. Velada I, Ragonezi C, Arnholdtschmitt B, Cardoso H (1932) Reference Genes Selection and
469 Normalization of Oxidative Stress Responsive Genes upon Different Temperature Stress
470 Conditions in *Hypericum perforatum* L. Plos One 9: e115206.

- 471 60. Thorrez L, Deun KV, Tranchevent L, Lommel LV, Engelen K, et al. (2008) Using Ribosomal
472 Protein Genes as Reference: A Tale of Caution. Plos One 3: e1854.
- 473 61. Robinson TL, Sutherland IA, Sutherland J (2007) Validation of candidate bovine reference genes
474 for use with real-time PCR. Veterinary Immunology & Immunopathology 115: 160-165.
- 475 62. Haller F, Kulle B, Schwager S, Gunawan B, Von HA, et al. (2004) Equivalence test in
476 quantitative reverse transcription polymerase chain reaction: confirmation of reference genes
477 suitable for normalization. Analytical Biochemistry 335: 1-9.
- 478 63. Jarošová J, Kundu JK (2010) Validation of reference genes as internal control for studying viral
479 infections in cereals by quantitative real-time RT-PCR. BMC Plant Biology 10: 146-146.
- 480 64. Szabo A, Perou CM, Karaca M, Perreard L, Quackenbush JF, et al. (2008) Statistical modeling
481 for selecting housekeeper genes. Genome Biology 9: 405-405.
- 482 65. Kok JBD, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, et al. (2005) Normalization of
483 gene expression measurements in tumor tissues: comparison of 13 endogenous control genes.
484 Laboratory investigation; a journal of technical methods and pathology 85: 154.
- 485 66. Ayers D, Clements DN, Salway F, Day PJ (2007) Expression stability of commonly used
486 reference genes in canine articular connective tissues. BMC Veterinary Research 3: 1-10.
- 487 67. Rapacz M, Stępień A, Skorupa K (2012) Internal standards for quantitative RT-PCR studies of
488 gene expression under drought treatment in barley (*Hordeum vulgare* L.): the effects of
489 developmental stage and leaf age. Acta Physiologiae Plantarum 34: 1723-1733.

- 490 68. Bansal R, Mittapelly P, Cassone BJ, Mamidala P, Redinbaugh MG, et al. (2015) Recommended
491 Reference Genes for Quantitative PCR Analysis in Soybean Have Variable Stabilities during
492 Diverse Biotic Stresses. Plos One 10: e0134890.

Figure 1. Melting curves for the candidate reference genes. T

Figure 2. Average Ct values for

Figure 3. Average expression stability (M-value) of 14 candidate genes

Figure 4. Pairwise variation (V) calculated by geNorm to determine

Figure 5. Relative expression levels of *HpHYP1* in different tissue