bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1 # Selection and Validation of Reference Genes
2 # Desirable for Gene Expression Analysis by qRT-PCR
3 # on Seed Germination of *Castanea henryi*

4 Bin Liu[1,2], Yuting Jiang[1,2], Ruqiang Lin[1,2], Yuanfang Xiong[1,2], Shuzhen Jiang[1,2], Hui
5 Lian[1,2], Xuedie Liu[1,2], Zhong-Jian Liu[2*] and Shipin Chen[1,2*]
6
7 [1] College of Forestry, Fujian Agriculture and Forestry University, Fuzhou, China;
8 [2] Key Laboratory of National Forestry and Grassland Administration for Orchid
9 Conservation and Utilization at College of Landscape, Fujian Agriculture and
10 Forestry University, Fuzhou, China
11
12 [*] Corresponding author
13 E-mail: zjliu@fafu.edu.cn (Z-J.L.) and fjcsp@126.com (S.C)
14

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

15 **Abstract**

16 Seed germination is the beginning of the plant's life cycle, and seed biology is one
17 of the most extensively researched areas in plant physiology, however, *Castanea*
18 *henryi* as an important seed plant, the stable internal reference gene during
19 germination is not clear. In this study, seven candidate genes (TUA, TUB, TIF, UBC,
20 RPL21, RPL30, RPL34) were screened out from transcriptome data, we analyzed the
21 expression of seven candidate reference genes in *C. henryi* at different germination
22 stages with RT–qPCR, and using common algorithms including NormFinder, geNorm
23 and BestKeeper to evaluate the candidate genes stability. The results showed that
24 RPL34 and RPL30 were selected as the most stable genes by NormFinder; TIF was
25 the most stable gene identified by BestKeeper; RPL34 and RPL21 were the most
26 stable genes ranked by geNorm, and TUB was the most unstable gene identified by all
27 of the three software. The RPL34 gene was used as the reference gene, to detected the
28 expression trend of two starch synthetase genes SS1 and SS2 during germination by
29 RT–qPCR, the results of RT–qPCR and transcriptome sequencing were basically
30 consistent, which verified the stability of RPL34 candidate gene. Our result is not
31 only showed functional genes for germination of *C. henryi* seeds and provide useful
32 guidelines for the selection of reliable reference genes for the normalization of RT–
33 qPCR data for germination of seed plants.

34 **Keywords: *Castanea henryi*, Reference gene, Seed germination, RT–qPCR**

35

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

36 **Introduction**

37 Seed germination is the basis of plant formation and is also the strongest period of
38 life activity in all life periods of a plant, not surprisingly that seed biology is one of
39 the most extensively researched areas in plant physiology [1,2]. Real-time
40 fluorescence quantitative PCR (RT–qPCR) is a nucleic acid quantitative technology
41 developed on the basis of qualitative PCR technology. By adding specific fluorescent
42 genes to the PCR reaction system, the expression of the target gene is accurately and
43 quantitatively analyzed [3]. It has the characteristics of quantitative accuracy, high
44 sensitivity, strong specificity and wide applicability, and is widely used in molecular
45 biology, transgenic products, food safety testing, genetics and the research of mining
46 new genes and their functions [4–7]. However, the relative quantitative results of RT–
47 qPCR are affected by the quantity and quality of RNA, primer specificity, PCR
48 reaction conditions, etc. [8–10]. To monitor the process of RT–qPCR, it is very
49 important to select appropriate internal reference genes. Internal reference gene refers
50 to the gene used as internal reference in gene research, usually housekeeping gene,
51 which is involved in the process necessary for cell survival and expressed at a
52 relatively stable and constant level. It needs to be corrected by internal reference gene
53 in detecting the change of target gene expression level, and its stability has a decisive
54 impact on the accuracy of RT–qPCR results [7,11,12]. The stable expression of the
55 internal reference gene should be relatively stable in different tissues and organs,
56 different environments and different stress body conditions [12–14]. In the process of
57 plant RT–qPCR analysis, some relatively stable housekeeping genes, such as tubulin
58 (TUB), actin (ACT), ubiquitin conjugating enzyme (UBC), transcription initiation
59 factors (TIF), and ribosomal protein (RP), were selected as the internal reference
60 genes.

61 Fagaceae is the main tree species of tropical, subtropical and temperate forests in
62 the northern hemisphere, and also the most important dominant species of
63 angiosperms. Many Fagaceae plants are important economic plants and dual-use
64 plants [15,16]. *Castanea henryi* belongs to the Castanea of Fagaceae. Castanea is

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

65 widely distributed between East Asia and East North America, and is used to produce
66 wood, tanning agents and food [17,18]. *C. henryi* has important economic value,
67 including starch, soluble sugar, protein, lipids and 18 kinds of amino acids, 8 of which
68 are necessary for human body, and its nutritional value is higher than that of flour, rice
69 and potatoes [19–21], which can not only be used as staple food [22], but also as
70 traditional Chinese medicine [23]. Actin was selected as the internal reference gene
71 [24,25] in the study of cloning related genes of starch branching enzyme and
72 expression related genes of somatic embryogenesis in *Castanea mollissima*. Chen et
73 al. [26] analyzed the expression stability of EF1α, TUA, TUB, UBQ, 18S rRNA and
74 actin in different tissues and organs of *C. mollissima*. Germination is an important
75 biological process in the growth and development, however, the genes stably
76 expressed in different stages of the germination of *C. henryi* seeds have not been
77 discussed. In this study, the expression levels of 7 candidate genes were analyzed by
78 RT–qPCR using 4 seeds in different stages of the germination of *C. henryi.* Three
79 software were used to evaluate the expression stability of candidate internal reference
80 genes, and selected the internal reference genes stably expressed during the
81 germination. Combining with the data of transcriptome sequencing, the expression
82 level of two starch synthesis genes in the process of germination of *C. henryi* was
83 analyzed and compared to further verify the stability of the internal reference genes
84 and provide theoretical basis for the molecular mechanism of germination of *C.*
85 *henryi*.
86

87 **Results**

88 **Candidate gene and RNA quality**

89 The average value, standard deviation and coefficient of variation were calculated
90 according to the expression data of internal reference genes in four stages of
91 germination of *C. henryi*. Through the comparison of variation coefficients, seven
92 internal genes were screened out: TUA (tubulin alpha)、TUB (tubulin beta)、TIF
93 (translation initiation factor 4A)、UBC (ubiquitin-conjugating enzyme E2D)、

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

94 RPL21 (large subunit ribosomal protein L21e)、RPL30 (large subunit ribosomal
95 protein L30e) and RPL34 (large subunit ribosomal protein L34e) (Table 1)。Primer
96 premier 5.0 was used to design primers, and the results are shown in Table 2. The
97 results of agarose gel electrophoresis showed that the total RNA of the four periods
98 had clear and intact bands of 28S and 18S, and there was no tail phenomenon in the
99 band, indicating that total RNA had no degradation or degradation and had good
100 integrity; the total RNA was not degraded (Figure 1). The results of ultramicro
101 spectrophotometer show that the a260/A280 value is between 1.85–2.20, and the
102 concentration is between 250–800 ng/μL, it shows that the RNA extracted is of high
103 purity, free of protein and inorganic salt pollution, which can meet the needs of
104 subsequent experiments (Table 3).
105
106 **Candidate internal reference genes amplification efficiency and linear analysis**
107 The amplification efficiency and linear relationship of the internal reference genes
108 were calculated by RT–qPCR with the mixture of four stages of chestnut kernel
109 cDNA as template, diluted 5 times and diluted five times into four gradients. The
110 results showed that the dissolution curves of the seven internal reference genes were
111 single signal peaks, there was no obvious heteropeak before TM (Figure 2), and the
112 amplification efficiency was between 90% and 105% (Table 4), indicating that the
113 amplification specificity of each pair of primers was good, there was no primer dimer
114 and non-specific amplification in the amplification process, which could be used for
115 subsequent RT–qPCR analysis.
116
117 **Analysis of stable expression of candidate internal reference genes**
118 The results of CT value analysis showed that the 7 candidate genes had some
119 changes in different stages of germination of *C. henryi*, and the trend was similar. The
120 lower CT value of UBC indicates the higher expression abundance, and the higher CT
121 value of tub indicates the lower expression level of tub. It can be inferred that RPL21,
122 RPL30, RPL34, TIF and UBC can be used as candidate genes. According to the

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

123 analysis of BestKeeper software, the expression stability of 7 candidate genes is TIF >
124 TUA > RPL21 > RPL34 > RPL30 > UBC > TUB (Figure 3 and Table 5), from which
125 it can be seen that TIF is the most stable one among the seven candidate internal
126 reference genes. According to the analysis results of GeNorm software, the expression
127 stability of the seven candidate internal reference genes is RPL34 / RPL21 > TIF >
128 RPL30 > UBC > TUA > TUB (Figure 3 and Table 5), which shows that RPL34 and
129 RPL21 are better than other candidate internal reference genes in terms of stability,
130 combined with standard deviation According to the analysis results, $V_{2/3}$ is less than
131 1.5, so the combination of RPL34 and RPL21 can be selected; NormFinder software
132 analysis results show that the expression stability of the seven candidate genes is
133 RPL34 / RPL30 > UBC > TIF > RPL21 > TUA > TUB (Figure 3 and Table 5). The
134 results showed that RPL34 was stable and could be selected as the best internal
135 reference gene for gene expression analysis during seed germination of *C. henryi*.
136

137 **Validation of RPL34 gene**

138 The expression level of two starch synthetase genes (SS1, SS2) in the germination
139 of *C. henryi* was analyzed by RT–qPCR with RPL34 as the internal reference, and
140 compared with the transcriptome data. The results showed that in the transcriptome
141 data, SS1 and SS2 decreased sharply from T01 to T02, while in T02 to T04, the
142 expression of SS1 continued to increase, while the expression of SS2 changed less.
143 RPL34 was used as the internal reference gene for real-time fluorescence quantitative
144 detection. The expression trend of SS1 and SS2 genes was basically consistent with
145 the results of transcriptome sequencing (Figure 4). The results showed that RPL34
146 screened from 7 candidate genes was stable in the germination process of *C. henryi*,
147 and it was suitable to be used as an internal reference gene.
148

149 **Discussion**

150 RT–qPCR technology is widely used in the study of gene expression verification.
151 The selection of stable internal reference genes is an important condition to ensure the

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

152 accuracy of the results [7,27–29]. Ideally, the expression amount of the internal
153 reference gene is relatively stable in different development stages, different tissues
154 and different stress conditions of the sample, but the stability is not absolute. At
155 present, no single fixed internal reference gene can meet all experimental
156 requirements, so it is necessary to select a relatively stable internal reference gene
157 according to the actual experimental conditions [30–32]. It was found that ubiquitin
158 converging enzyme (UBC), actin (ACT) and phospholipase A22 (PLA) were the most
159 stable internal reference genes during *Plukenetia volubilis* seedling and flower
160 development, while UBC *30S ribosomal protein S13*（RPS13）and RNA polymerase
161 II subunit (RPII) were the most stable during seed development [33]. The conclusion
162 of research on *Xanthoceras sorbifolia* is that UBQ and EIF-4α are the most stable in
163 the period of sex differentiation, while EF-1α and EF-1α are the most stable in
164 different organs [34]. Under different stress conditions, the stable expression genes of
165 *Caragana nipponica* were screened, it was found that the combination of UNK2,
166 SAND family protein (SAND) and elongation factor 1-α (EF–1α) was the most stable
167 under salt stress, the combination of TIP41-like family protein (TIP41) and protein
168 photosphatase 2A (PP2A) was the most stable under PEG treatment, and the
169 combination of sand, PP2A and TIP41 was the most stable under heat stress, while the
170 combination of SAND and EF-1 α was the most stable under cold stress [35]. In order
171 to study the expression of related genes in the process of somatic cell development
172 and fruit formation of *C. mollissima*, actin was selected as the internal reference gene
173 for the experiment, GAPDH was selected as the internal reference gene in the study of
174 oxalate oxidase (oxo) gene expression in the process of leaf necrosis caused by
175 *Cryptosporidium parasitica* in *C. dentata,* and Chen found that actin and EF1 α had
176 the best stability in different tissues and organs of *C. mollissima*, [24–26,36].
177 Seed germination is an important stage in the life cycle of plants, it is one of the
178 urgent problems to screen the relatively stable internal reference genes during
179 germination of *C. henryi*. In this paper, based on the annotation analysis of
180 transcriptome sequencing data and the preliminary comparison of the expression

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

181 stability of several kinds of housekeeping genes, seven candidate genes (TUA, TUB,
182 TIF, UBC, RPL21, RPL30 and RPL34) were selected. Combined with the real-time
183 fluorescence quantitative technology, three software geNorm, NormFinder and
184 BestKeeper were used for further stability analysis and comparison. In the research
185 process of *Amygdalus persica*, *Lolium multiflorum* and *Ping'ou Hybrid Hazelnut*
186 (*C.heterophylla* × *C.avellana*), the internal reference genes were screened based on
187 the transcriptome sequencing data and further combined with the analysis software
188 [37-39]. GeNorm, NormFinder and BestKeeper are the most commonly used analysis
189 software in the current screening of internal reference genes. The evaluation of
190 stability of internal reference genes by the three is based on different algorithms and
191 evaluation indicators, which may lead to inconsistent analysis results. Among them,
192 the analysis results of geNorm and NormFind based on the relative expression Q
193 value are relatively close, while the results of the BestKeeper analysis may be slightly
194 different from the first two which based on CT value [40,41]. In the screening of
195 young bulb internal reference genes in different tissues, hybrids and flower
196 development of Lycoris, it was found that the analysis results of geNorm and
197 NormFinder software were close, while the analysis results of BestKeeper software
198 were significantly different [42]. In addition, similar situations occurred in the internal
199 reference screening of *Siraitia grosvenorii*, *Ampelopsis grossedentata* and Citrus [43–
200 45].

201 In this study, the expression stability of 7 candidate genes in the seeds of *C. henryi*
202 during germination was analyzed and compared. The results of geNorm showed that
203 RPL34 and RPL21 had the best stability, the results of NormFinder showed that
204 RPL34 and RPL30 had the best stability, while the BestKeeper's analysis results show
205 that TIF has the best stability and RPL34 has the general stability. TUB had the worst
206 stability in the three analysis results. Ribosomal protein (RP) is the main component
207 of ribosome, which plays an important role in protein biosynthesis in cells. Ribosomal
208 protein includes large subunit ribosomal protein and small subunit ribosomal protein
209 [45]. Comparing the stability of ten kinds of internal reference genes in different

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

210 development stages of peanut seed samples and different tissues, it was found that the
211 stability of alcohol dehydrogenase class III (ADH3) was the best, followed by 60S
212 ribosomal protein L7 (60s) [47]. TUB gene plays an important role in maintaining cell
213 structure. It is used as a reliable internal reference gene in switchgrass and peach
214 research. However, in this study, the stability of TUB gene is poor in the germination
215 process of *C. henryi*. This result is similar to that of potato and Soybean [48–51].
216 Based on the above results, RPL34 has the best stability in the germination process of
217 *C. henryi*, which is suitable for studying the molecular mechanism of starch
218 metabolism during seed germination. The stability of RPL 34 during the germination
219 of *C. henryi* was further confirmed by the expression of SS1 and SS2.
220

221 **Conclusion**

222 In this study, through the analysis of the expression stability of seven candidate
223 genes (TUA, TUB, TIF, UBC, RPL 21, RPL30 and RPL34) in the germination
224 process of *C. henryi*, RPL34 gene was screened out which was stably expressed in
225 different stages of the germination process, and the expression stability of TUB was
226 poor, which was not suitable to be the internal reference gene in the germination
227 process of *C. henryi*. RPL34 gene was used as the reference gene to analyze the
228 relative expression of SS1 and SS2 genes in different germination stages of *C. henryi*.
229 The results of RT–qPCR and transcriptome sequencing were basically the same: the
230 expression of SS1 and SS2 was the highest in the first stage, decreased rapidly in the
231 second stage, and changed little in the latter two stages. In order to improve the
232 accuracy of RT–qPCR experiment in the germination process of *C. henryi*, a suitable
233 internal reference gene was screened.
234

235 **Materials and methods**

236 **Plant materials**

237 The *C. henryi* variety "Youzhen" was planted in the research center of Youcha,
238 Fujian agricultural and Forestry University. From December 2019 to January 2020,

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

239 samples were taken every five days. According to the difference between the time
240 interval and kernel morphology, four periods were selected: 0 d, 10 d, 20 d and 30 d
241 for the study, and the transcriptome sequencing was carried out. During sampling, the
242 seed shell and exotesta were peeled off quickly. The whole plant was washed and
243 dried with pure water, and then put into a 50 ml centrifuge tube, frozen with liquid
244 nitrogen, and stored in an ultra-low temperature refrigerator at -80 °C for future use.
245 Three biological repeats were taken from each period.
246

247 **RNA extraction and cDNA synthesis**

248 Total RNA from the seeds was isolated using the RNAprep Pure Plant Kit
249 (Polysaccharides and Polyphenolics, Tiangen, Beijng, China) according to the
250 manufacturer's instructions. The integrity of the total RNA sample was verified by 1%
251 agarose gel electrophoresis. The purity and concentration of the samples were
252 detected by NanoDrop 2000c ultramicro spectrophotometer (Thermo Scientific,
253 USA), and the A260 /A280 ratio and purity were determined. Three duplicates of total
254 RNA extracts were reverse transcribed for first-strand cDNA synthesis for RT–qPCR
255 using the TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR
256 (One-Step gDNA Removal) (TransGen Biotech, Beijing, China). Finally, it was stored
257 in a refrigerator at -20°C.
258

259 **Selection of internal reference gene and primer design**

260 According to the traditional housekeeping genes and the expression amount of these
261 genes in the transcriptome data of four periods during the germination of *C. henryi*,
262 seven genes with stable expression amount were selected as candidate internal
263 reference genes, namely tub, TUA, RPL 21, RPL30, RPL 34, TIF and UBC. The CDS
264 sequences of 7 genes were introduced into primer 5 design primers. The parameters
265 were set as follows: annealing temperature 60 °C, primer length 20-22 bp. Primer
266 blast in NBCI was used to detect the specificity of primers. The primer sequence was
267 compared with transcriptome data to detect the specificity of primers.

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

268

269 **Amplification efficiency of primers for candidate internal reference genes**

270 Take 2 μL from each sample and dilute it five times to get the mixed sample. Take
271 20 μL mixed sample and dilute it five times to four gradients. That is to say, the final
272 concentration of standard curve template is 24 ng/mL, 4.8 ng/mL, 0.96 ng/mL and
273 0.08 ng/mL respectively, and each reaction is set with three repeats. The RT–qPCR,
274 performed by TransStart® Tip Green qPCR SuperMix (TransGen Biotech, Beijing,
275 China) with the total system was 20 μL, including 2 μL cDNA. The RT–qPCR
276 conditions followed the manufacturer's instructions. The primers were designed using
277 Primer 6.0 software. The thermal cycling protocol was 30 s at 94 °C, then 40 cycles of
278 94 °C for 5 s and 60 °C for 30 s for annealing and extension. Standard curve, slope
279 (k), correlation coefficient (R^2) and amplification efficiency (E) were obtained by data
280 analysis with CT value.

281

282 **RT–qPCR analysis and stability evaluation**

283 According to the RT–qPCR reaction system in 1.4, according to the CT value, we
284 analyzed and compared the expression stability of seven candidate genes in different
285 periods by using the software of geNorm, NormFinder and BestKeeper [52–55].
286 Cycle threshold (CT) in RT–qPCR was converted according to software requirements.
287 Georm software determines the stability according to the average expression stability
288 value (M). The smaller the m value is, the more stable it is. At the same time, it
289 determines the optimal number of internal reference genes according to V_N/V_{N+1}.
290 NormFinder software sequenced the candidate internal reference genes by the stability
291 of gene expression (s). The genes with the lowest s value had the best stability. The
292 best keeper software mainly compares the standard deviation (SD) and Pearson
293 product motion correlation coefficient (R). If the SD value is less than 1, the closer
294 the R value is to one, the more stable the internal reference gene is. The expression
295 level of the selected target gene in the seed germination process of *C. henryi* was
296 quantitatively analyzed and the validity of the internal reference gene was verified.

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

297 The relative expression of the target gene was calculated by $2^{-\triangle\triangle Ct}$ method, and each
298 sample was repeated three times.
299
300 **Author Contributions**
301 Conceptualization, B.L., S.C., and Z-J.L.; methodology, B.L., S.C., and Z-J.L.;
302 software, B.L.; validation, B.L., Y.J., R.L., and Y.X.; formal analysis, B.L.;
303 investigation, B.L., Y.J., R.L., H.L., X.L. and S.J.; resources, B.L., S.C., and Z-J.L.;
304 data curation, B.L., Y.J., R.L., H.L., X.L. and Y.X.; writing—original draft
305 preparation, B.L., Y.J. R.L.,; writing—review & editing, B.L., S.C., and Z-J.L.;
306 visualization, B.L.; supervision, S.C, and Z-J.L.; project administration, B.L., S.C.,
307 and Z-J.L.; funding acquisition, B.L. Z-J.L. and S.C.
308
309 **Funding**
310 This work was supported by the Project of Forestry peak discipline in Fujian
311 Agriculture and Forestry University of China (118/712018007), and the Science and
312 technology innovation special fund of Fujian Agriculture and Forestry University
313 (118/KF2015088).
314
315 **Acknowledgments**
316 The authors would like to thank the Key Laboratory of National Forestry and
317 Grassland Administration for Orchid Conservation and Utilization for supporting this
318 project.
319
320 **Conflicts of Interest**
321 The authors declare there are no conflicts of interest.
322
323
324
325

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

326 **Table 1 Information of seven candidate reference genes**

Gene	Gene ID	Gene annotation	Mean/FPKM	SD	CV
TUA	Che002914	tubulin alpha	15.40	3.55	0.231
TUB	Che010804	tubulin beta	85.80	25.27	0.294
TIF	Che015192	translation initiation factor 4A	218.38	12.00	0.055
UBC	Che026117	ubiquitin-conjugating enzyme E2 D	301.11	25.86	0.086
RPL21	Che002638	large subunit ribosomal protein L21e	680.30	22.62	0.033
RPL30	Che007164	large subunit ribosomal protein L30e	469.41	13.68	0.029
RPL34	Che034253	large subunit ribosomal protein L34e	162.88	5.62	0.035

327

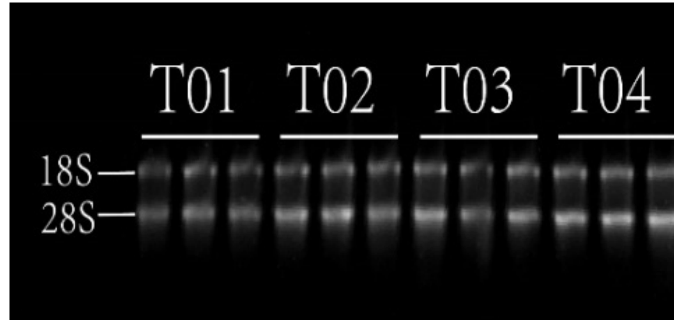
328 **Table 2 Primer sequences of the candidate reference genes and target genes**

Gene	Gene ID		Primer sequence（5'-3'）	Length/bp	Product Length /bp
TUA	Che002914	F	TGAGATGTAGGGTTGCTTTC	20	252
		R	CCTCTTCTGGCATTAGTGGG	20	
TUB	Che010804	F	ACCGTGCCCTTACAGTGCCT	20	378
		R	CCATCTCGTCCATCCCTTCA	20	
TIF	Che015192	F	GGCACTTGGAGACTATCTTGGT	22	147
		R	AGTGACTGTCTTCGCAGCATAT	22	
UBC	Che026117	F	GGATTTACAGAAAGACCCACC	21	110
		R	CTCCAGCATAAGGACTATCAGC	22	
RPL21	Che002638	F	CAACAAGCAGGTGGGTAACAG	21	135
		R	GCCTTTGCCTCAGCCTTCAGT	21	
RPL30	Che007164	F	ACAAGACCGTCCTCAAATCCC	21	117
		R	CAACCTTCGCCAACATAGCAT	21	
RPL34	Che034253	F	GCTACGCCACCAAATCCAATC	21	115
		R	CTCTTGCCAGTTACAGGACACT	22	
SS1	Che013592	F	CCTCTGATGTTGACTCCACCAA	22	168
		R	TTGCTTCACCATCCACCTCTG	21	
SS2	Che036389	F	TCTCTACTCTCGCCTCAGAACC	22	219
		R	ATGGAGTCAACACAGACCTTCG	22	

329

330

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



331

332 **Figure1. The agarose gel electrophoresis of total RNA**

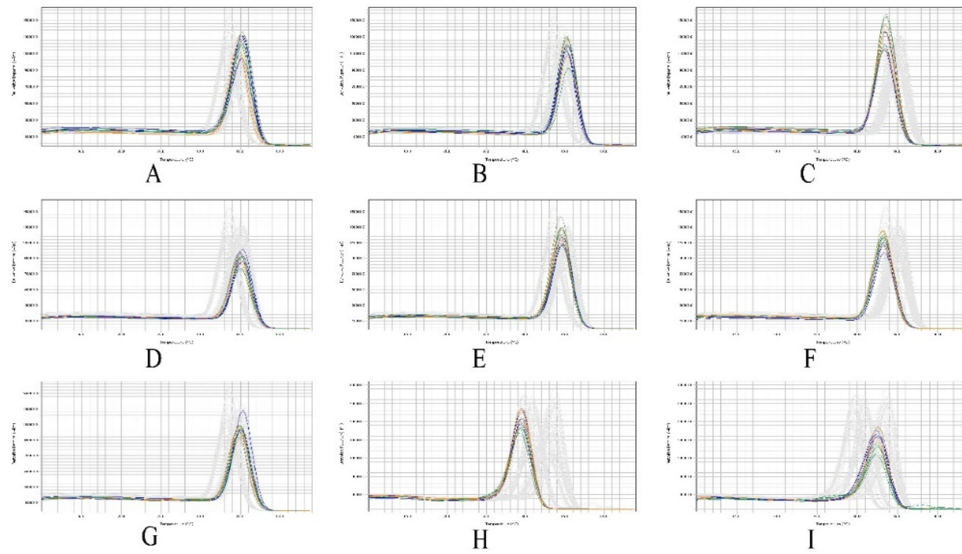
333

334 **Table 3 The quality of total RNA in four periods**

	260/280	Concentration /ng/μL
	1.96	722.87
T01	2.07	308.2
	1.95	769.26
	1.92	759.67
T02	2.13	286.35
	2.08	302.33
	1.91	769
T03	1.89	761.98
	1.84	550.88
	1.9	591.96
T04	2.02	257.7
	2.05	310.05

335

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



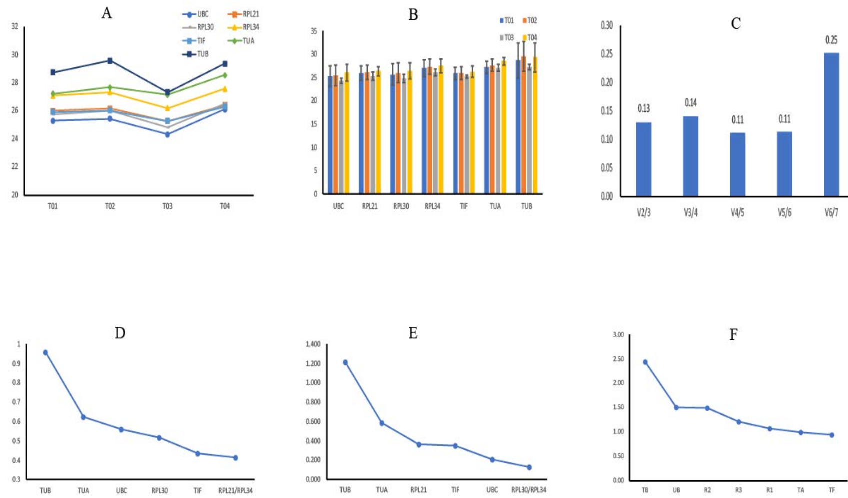
A: TUA; B: TUB; C:TIF; D: UBC; E: RPL21; F: RPL30; I: RPL34; G: SS1; H: SS2

Figure 2. Melting curves of the candidate reference genes and target genes

Table 4 The amplification efficiency of candidate internal reference genes primers and target gene primers

Gene	Gene ID	K	R^2	amplification efficiency /E
TUA	Che002914	2.37	0.997	0.97
TUB	Che010804	2.29	0.996	1.02
TIF	Che015192	2.46	0.999	0.93
UBC	Che026117	2.31	0.996	1.01
RPL21	Che002638	2.36	0.999	0.98
RPL30	Che007164	2.45	0.998	0.93
RPL34	Che034253	2.36	0.997	0.98
SS1	Che013592	3.230	0.997	0.969
SS2	Che036389	3.186	0.995	0.956

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



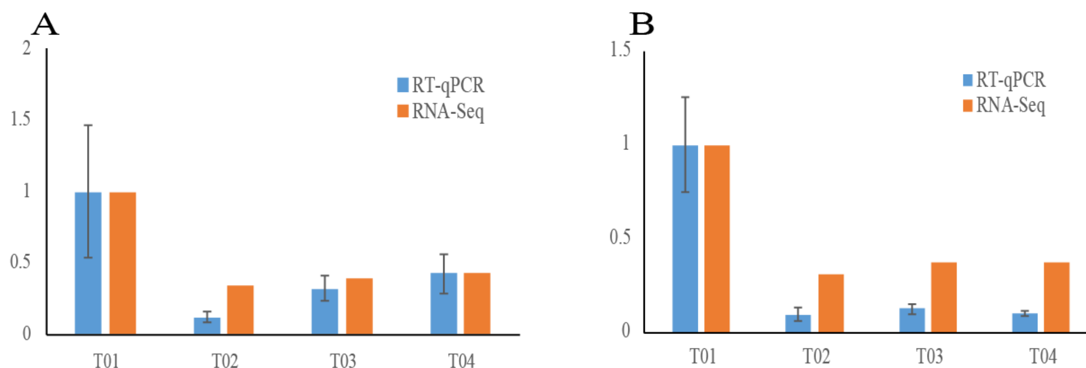
A: Line chart of CT value distribution; B: Column chart of CT value distribution; C: geNorm result; D: geNorm stability analysis; E: Normfinder stability analysis; F: Bestkeeper stability analysis

Figure 3. The evaluation of candidate reference genes stability from Ct value、GeNorm、Normfinder and Bestkeeper

Table 5 The evaluation of candidate reference genes stability from Bestkeeper、GeNorm and Normfinder

	Bestkeeper		GeNorm		Normfinder	
	SD	Rank	M	Rank	S	Rank
UBC	1.50	6	0.5614	4	0.209654	2
RPL21	1.07	3	0.4159	1	0.36629	4
RPL30	1.49	5	0.5173	3	0.131927	1
RPL34	1.22	4	0.4159	1	0.131927	1
TIF	0.94	1	0.435	2	0.349297	3
TUA	0.99	2	0.6238	5	0.587422	5
TUB	2.44	7	0.9577	6	1.216812	6

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



355

356 A: SS1 RNA-Seq result; B: SS1 RT–qPCR result; C: SS2 RNA-Seq result; D: SS2 RT–qPCR result.

357 **Figure 4. The result of target gene's RT–qPCR and transcriptome sequencing**

358

359 **References**

360 **1.** Li Z, Peng Y, Zhang X Q, *et al*. Exogenous spermidine improves seed germination
361 of white clover under water stress via involvement in starch metabolism,
362 antioxidant defenses and relevant gene expression [J]. Molecules, 2014, 19 (11):
363 18003-18024.
364 **2.** Bewley J D. Seed Germination and Dormancy [J]. The plant Cell, 1997, 9, 1055-
365 1066.
366 **3.** Giulietti A, Overbergh L, Valckx D. An overview of real-time quantitative PCR:
367 applications to quantify cytokine gene expression [J]. Methods, 2010, 25 (4): 386-
368 401
369 **4.** Wang Y Q, Xue X H. Research progress and application of real-time fluorescence
370 quantitative PCR [J]. Bulletin of Biology2016, 51(2): 1-6.
371 **5.** Huang Z X, Wang F J, Jiang H, et al. Comparison of Cadmium-Accumulation-
372 Associated Genes Expression and Molecular Regulation Mechanism between Two
373 Rice Cultivars (Oryza sativa L.subspecies japonica) [J]. The Crop Journal,
374 2014,40(04),581-590.
375 **6.** Cui H Q, Feng N J, Sun F D, et al. Regulation of DTA-6 by Abscission Cellulase
376 and GmAC Gene Expression in Flowers and Pods of Soybean [J]. The Crop Journal,
377 2016, 42(1): 51-57.
378 **7.** Huggett J, Dheda K, Bustin S, *et al*. Real-time RT-PCR normalisation; strategies
379 and considerations [J]. Genes and Immunity, 2005, 6(4): 279-284.
380 **8.** Peters I R, Helps C R, Hall E J, *et al*. Real-time RT-PCR considerations for efficient
381 and sensitive assay design [J]. Immunological Methods, 2004, 286 (1/2): 203—217.
382 **9.** Liu X T, Wang S L, Xue J Q,et al. Selection of Reference Genes for Quantitative
383 Real-Time PCR in Different Tissue and Organ of Barbadoslily [J]. Horticultural
384 Plant Journal, 2018, 45(5): 919-930.
385 **10.** Dheda K. Validation of housekeeping genes for normalizing RNA expression in
386 real-time PCR[J]. Biotechniques, 2004, 37(1):118-9.
387 **11.** Bustin S A. Quantification of mRNA using real — time reverse transcription
388 PCR(RT-PCR): trends and problems [J]. Molecular Endocrinology, 2002, 29(1): 23-

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

389 29.
390 **12.** Thellin O, Zorzi W, Lakaye B, *et al*. Housekeeping genes as internal standards: Use
391 and limits [J]. J Biotechnol, 1999,75:291–295.
392 **13.** Zhang J P, Wang Y Q. Selection of reference genes for quantitative real-time PCR
393 during anther development of *Caulokaempferia coenobialis* (Zingiberaceae) [J].
394 Guihaia2018,38(01):76-83.
395 **14.** Yang J, Lu Y B, Chi M H, et al. Analysis on stability of reference genes in different
396 developmental stages of seeds from *Carthamus tinctorius* [J]. Chinese Herbal
397 Medicines,2017, 48(9): 1845-1850.
398 **15.** Zhou Z K. Fossils of the Fagaceae and their implications in systematics and
399 biogeography [J]. Acta Phytotaxonomica Sinica, 1999, 37(4):369-385.
400 **16.** Zhou L, Xu M, Yang C R, *et al*. The Advance of Chemical Components and
401 Bioactivity of Fagaceous Plants [J]. Nat Prod Res Dev, 2012, 24:260-273.
402 **17.** Wen J. Evolution of eastern Asian and eastern North American disjunct
403 distributions in flowering plants [J]. Ann Rev Ecol Syst, 1999, 30:421–455.
404 **18.** Wu B, Zhang X D, Wu XD. New lignan glucosides with tyrosinase inhibitory
405 activities from exocarp of *Castanea henryi* [J]. Carbohydrate Research, 2012,
406 355:45-49.
407 **19.** Xu J. The effect of low-temperature storage on the activity of polyphenol oxidase
408 in *Castanea henryi* chestnuts [J]. Postharvest Biol. Technol, 2005, 38:91–98.
409 **20.** Feng J L, Yang Z J, Chen H. Advances in *Castanea henryi* [J]. Subtropical
410 Agriculture Research, 2009, 5:237-241.
411 **21.** Fan X M, Yuan D Y, Tang J, *et al*. Sporogenesis and gametogenesis in chinese
412 chinquapin (*Castanea henryi*(skam) rehder & wilson) and their systematic
413 implications [J]. Trees, 2015, 29(6):1713-1723.
414 **22.** Useful Temperate Plants Database, Available online:
415 http://temperate.theferns.info/plant/Castanea+henryi
416 **23.** Li, S.Z. Compendium of Material Medica [M]. *Liaoning National Publishing*
417 *House*. 1999. 1123–1127.
418 **24.** Chen L K, Lu D, Wang T, et al. Identification and expression analysis of starch
419 branching enzymes involved in starch synthesis during the development of chestnut
420 (*Castanea mollissima* Blume) cotyledons [J]. PLoS One, 2017,12(5): e0177792.
421 **25.** Lu D, Wei W, Zhou W, et al. Establishment of a somatic embryo regeneration
422 system and expression analysis of somatic embryogenesis-related genes in Chinese
423 chestnut (*Castanea mollissima* Blume) [J]. Plant Cell Tiss Org Cult, 2017, 130 (3):
424 601–616.
425 **26.** Chen G S, Li J T, Liu Y, et al. Selection and validation of reference genes for
426 quantitative RT-PCR analysis in *Castanea mollissima* [J]. Plant Physiology Journal,
427 2019, 55(03):158-166.
428 **27.** Vandesompele J, De Preter K, Pattyn F, *et al*. De Paepe AFS: Accurate
429 normalization of realtime quantitative RT-PCR data by geometric averaging of
430 multiple internal control genes [J]. Genome Biology. 2002, 3: research0034.1-
431 research0034.11.
432 **28.** Pfaffl M W, Tichopad A, Prgomet C, *et al*. Determination of stable housekeeping
433 genes， differentially regulated target genes and sample integrity: BestKeeper-
434 Excel-based tool using pair-wise correlations [J]. Biotechnology Letters, 2004，
435 26(6):509－515.
436 **29.** Andersen C L, Jensen J L, Orntoft T F. Normalization of real-time quantitative

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

437 reverse transcription-PCR data: A model-based variance estimation approach to
438 identify genes suited for normalization, applied to bladder and colon cancer data
439 sets [J]. Cancer Research. 2004, 64 (15): 5245-5250.
440 **30.** Wu J Y, He B, Du Y J, et al. Analysis Method of Systematically Evaluating Stability
441 of Reference Genes Using geNorm, NormFinder and BestKeeper [J]. Modern
442 Agricultural Science and Technology, 2017, (5): 278—281.
443 **31.** Ling D, Salvaterra P M. Robust RT–qPCR Data Normalization: Validation and
444 Selection of Internal Reference Genes during Post-Experimental Data Analysis[J].
445 PLOS ONE, 2011, 6.
446 **32.** Hruz T, Wyss M, Docquier M, et al. RefGenes: Identification of reliable and
447 condition specific reference genes for RT–qPCR data normalization[J]. BMC
448 Genomics, 2011, 12(1):156.
449 **33.** Longjian N, Yan-Bin T, Mao-Sheng C, et al. Selection of Reliable Reference Genes
450 for Gene Expression Studies of a Promising Oilseed Crop, Plukenetia volubilis, by
451 Real-Time Quantitative PCR[J]. International Journal of Molecular Sciences, 2015,
452 16(12):12513-12530.
453 **34.** Wang X, Ao Y, Liu Y, et al. Selection of Reference Genes for Quantitative Real-
454 Time PCR in Xanthoceras sorbifolium Bunge [J/OL]. Molecular Plant Breeding 1-
455 19.
456 **35.** Jianfeng Z, Lifeng Z, Wanfeng L, et al. Reference Gene Selection for Quantitative
457 Real-time PCR Normalization in Caragana intermedia under Different Abiotic
458 Stress Conditions [J]. PLoS ONE, 2013, 8(1): e53196-.
459 **36.** Zhang B, Oakes A D, Newhouse A E, et al. A threshold level of oxalate oxidase
460 transgene expression reducesCryphonectria parasitica-induced necrosis in a
461 transgenic American chestnut (Castanea dentata) leaf bioassay[J]. Transgenic
462 Research, 2013, 22(5):973-982.
463 **37.** Kou X Y, Zhang L, Yang S Z, *et al*. Selection and validation of reference genes for
464 quantitative RT — PCR analysis in peach fruit under different experimental
465 conditions [J]. Scientia Horticulturae, 2017, 225: 195-203.
466 **38.** Liu Q X, Qi X, Yan H D, *et al*. Reference gene selection for quantitative Real time
467 reverse-transcriptase PCR in annual ryegrass (*Lolium multiflorum*) subjected to
468 various abiotic stresses [J]. Molecules, 2018, 23(1):172—184.
469 **39.** Yang D, Li Q, Wang X G, et al. Reference Genes Selection and System
470 Establishment for Real-Time q PCR Analysis in Ping'ou Hybrid Hazelnut (*C.*
471 *heterophylla* Fisch. × *C. avellana* L.) [J]. Scientia Agricultura Sinica, 2017, 50(12) :
472 2399—2410.
473 **40.** Sun P, Hou W H, Chen Q J, et al. Selection of the Reference Genes for Gene
474 Expression Studies in Rehmannia glutinosa by Real-time Quantitative PCR [J].
475 Chinese Agricultural Science Bulletin, 2011, 27(17): 76-82.
476 **41.** Hu R, Qi G, Kong Y, *et al*. Comprehensive analysis of NAC domain transcription
477 factor gene family in *Populus trichocarpa* [J]. BMC Plant Biol, 2010,10.
478 **42.** Jiang T T, Gao Y H, Tong Z K. Selection of Reference Genes for Quantitative Real-
479 Time PCR in Lycoris [J]. Acta Horticulturae Sinica, 2015, 42(6): 1129-1138.
480 **43.** Guo Q Q, Ma X J, Bai L H, et al. Screening of reference genes in Siraitia grosvenorii
481 and spatio-temporal expression analysis of 3-hydroxy-3-methylglutaryl coenzyme

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.27.428382; this version posted January 27, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

482 A [J]. Chinese Traditional and Herbal Drugs, 2014,45(15): 2224-2229.
483 **44.** Wang L H, Pan Y J, Yang L, et al. Validation of internal reference genes for RT–
484 qPCR normalization in 'Guanxi Sweet Pummelo' (*Citrus grandis*) [J]. Journal of
485 Fruit Science, 2013, 30(1): 48-54.
486 **45.** Xu M, Yi H J, Zhao S, et al. Screening and validation of reference genes for
487 quantitative RT-PCR analysis in *Ampelopsis grossedentata* [J]. Chinese Traditional
488 and Herbal Drugs, 2017,48(6): 1192-1198.
489 **46.** Mazahar M, Achala B, Anusree S, et al. Rice Ribosomal Protein Large Subunit
490 Genes and Their Spatio-temporal and Stress Regulation [J]. Frontiers in Plant
491 Science, 2016, 7.
492 **47.** Brand Y, Hovav R. Identification of Suitable Internal Control Genes for
493 Quantitative Real-Time PCR Expression Analyses in Peanut (*Arachis hypogaea*)
494 [J]. Peanut Science, 2010, 37(1):12-19.
495 **48.** Huang L, Yan H, Jiang X, *et al*. Evaluation of candidate reference genes for
496 normalization of quantitative RT-PCR in switchgrass under various abiotic stress
497 conditions [J]. BioEnerg. Res. 2014, 7,1201–1211.
498 **49.** Tong Z, Gao Z, Wang F, *et al*. Selection of reliable reference genes for gene
499 expression studies in peach using real-time PCR [J]. BMC Mol. Biol. 2009, 10.
500 **50.** Reid K, Olsson N, Schlosser J, *et al*. An optimized grapevine RNA isolation
501 procedure and statistical determination of reference genes for real-time RT-PCR
502 during berry development [J]. BMC Plant Biol. 2006, 6.
503 **51.** Hu R, Fan C, Li H, *et al*. Evaluation of putative reference genes for gene expression
504 normalization in soybean by quantitative real-time RT-PCR [J]. BMC Mol. Biol.
505 2009, 10.
506 **52.** Bustin S A. Quantification of Nucleic Acids by PCR. A-Z of Quantitative PCR La
507 Jolla: International University Line Bustin SA, 2004, 5-46.
508 **53.** Wong M L, Medrano J F. Real-time PCR for mRNA quantification [J].
509 BioTechniques 2005, 39:75-85.
510 **54.** Karuppaiya P., Yan X X., Liao W, *et al*. Correction: identification and validation of
511 superior reference gene for gene expression normalization via RT–qPCR in
512 staminate and pistillate flowers of Jatropha curcas- A biodiesel plant [J]. PLoS One,
513 2017, 12(5): e0177039.
514 **55.** Yuan W, Wan H J, Yang Y L. Characterization and Selection of Reference Genes
515 for Real-time Quantitative RT-PCR of Plants [J]. Chinese Bulletin of Botany, 2012,
516 47(4): 427-436.