Selection and Validation of Reference Genes for Normalization of RT-qPCR Analysis in Developing and Abiotic-Stressed Tissues of Loquat (*Eriobotrya japonica*)

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Research

Keywords: Loquat, *Eriobotrya japonica*, Reference genes, qRT-PCR, Gene expression, Normalization, Developing tissues, Abiotic-stressed tissues

Posted Date: February 19th, 2020

DOI: <https://doi.org/10.21203/rs.2.23936/v1>

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1 **Selection and Validation of Reference Genes for Normalization of**
2 **RT-qPCR Analysis in Developing and Abiotic-Stressed Tissues of**
3 **Loquat (*Eriobotrya japonica*)**

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15

16 **Abstract**

17 **Background:** Loquat (*Eriobotrya japonica* Lindl.) is a subtropical evergreen fruit tree that
18 produces fruits with abundant nutrients and medicinal components. Confirming suitable reference
19 genes for a set of loquat samples before qRT-PCR experiments is essential for the accurate
20 quantification of gene expression.

21 **Results:** In this study, eight candidate reference genes were selected from our previously
22 published RNA-seq data, and primers for each candidate reference gene were designed and
23 evaluated. The Cq values of the candidate reference genes were calculated by RT-qPCR in 31
24 different loquat samples, including 12 subgroups of developing or abiotic-stressed tissues.
25 Different combinations of stable reference genes were screened according to a comprehensive
26 rank, which was synthesized from the results of four algorithms, including the geNorm,
27 NormFinder, BestKeeper and ΔC_t methods. The screened reference genes were verified by
28 normalizing *EjLGAI* in each subgroup. The obtained suitable combinations of reference genes for
29 accurate normalization were *GAPDH*, *EF1 α* and *ACT* for floral development; *GAPDH*, *UBCE* and
30 *ACT* for fruit setting; *EF1 α* , *GAPDH* and *eIF2B* for fruit ripening; *ACT*, *EF1 α* and *UBCE* for
31 leaves under heat stress; *eIF2B*, *UBCE* and *EF1 α* for leaves under freezing stress; *EF1 α* , *TUA* and
32 *UBCE* for leaves under salt stress; *ACT*, *EF1 α* and *eIF2B* for immature pulp under freezing stress;
33 *ACT*, *UBCE* and *eIF2B* for immature seeds under freezing stress; *EF1 α* , *eIF2B* and *UBCE* for
34 both immature pulp and seeds under freezing stress; *UBCE*, *TUB* and *TUA* for red-fleshed fruits
35 under cold-storage stress; *eIF2B*, *RPS3* and *TUB* for white-fleshed fruits under cold-storage stress;
36 and *eIF2B*, *UBCE* and *RPS3* for both red- and white-fleshed fruits under cold-storage stress.

37 **Conclusions:** This study obtained different combinations of stable reference genes for accurate

38 normalization in twelve subgroups of developing or abiotic-stressed tissues in loquat. The use of
39 the three most stable reference genes could increase the reliability of future quantification
40 experiments.

41 **Keywords:** Loquat, *Eriobotrya japonica*, Reference genes, qRT-PCR, Gene expression,
42 Normalization, Developing tissues, Abiotic-stressed tissues

43

44 **Background**

45 Loquat (*Eriobotrya japonica* Lindl.) is a subtropical evergreen fruit tree with fruit that is edible,
46 providing abundant nutrients, and has medicinal uses, relieving coughs and asthma[1]. Many
47 studies have focused on the functions of specific genes in loquat. Quantifying gene transcript
48 abundance is a frequently used and effective strategy to predict gene functions and determine
49 targets for loquat functional research[2-4]. An understanding of the gene expression characteristics
50 of developing and abiotic-stressed tissues of loquat will improve the breeding of loquat cultivars
51 with high quality and stress tolerance.

52 Reverse transcription–quantitative real-time PCR (RT-qPCR) is an established benchmark
53 technique that is widely used for quantifying the abundance of steady-state mRNA in biological
54 samples because of its simplicity, specificity, sensitivity, wide dynamic range, potential for high
55 throughput and accuracy [5, 6]. In addition, it is considered to be the most accurate and reliable
56 method and often serves to validate data obtained by other methods, such as cDNA microarray or
57 RNA-seq [7]. Absolute quantification and relative quantification are two primary methods used to
58 present RT-qPCR data. Absolute expression requires a standard curve to transform the RT-qPCR
59 data to yield an exact copy number, while relative expression presents RT-qPCR data relative to
60 reference genes as a fold change level. In fact, RT-qPCR data often do not need to be presented by
61 using the absolute quantification method, and relative quantification is sufficient [8].

62 A widely used method to present relative gene expression is the $2^{-\Delta\Delta C_t}$ method, which is a
63 convenient way to analyse the relative fold changes in gene expression from RT-qPCR data. This
64 method depends on the use of reference genes to normalize the RT-qPCR data. Housekeeping
65 genes such as *GAPDH* and *Actin* are usually sufficient as reference genes [9]. However, there is no

66 universal reference gene that can be used for RT-qPCR experiments in all cell types and under all
67 conditions. Most experimental designs are restricted to a few different tissues or a few different
68 conditions in the same tissue [10]. Therefore, which reference gene or genes are best for
69 normalization in a group of samples should be confirmed before RT-qPCR experiments are
70 performed [11]. Many algorithms have been developed to screen for suitable reference genes,
71 including the geNorm [12], NormFinder [13], BestKeeper [14] and ΔCt [15] methods. To increase
72 the reliability of screening, the results of multiple algorithms could be combined to obtain a
73 comprehensive result [16, 17].

74 The screening of suitable reference genes has been carried out in many species. Tong *et al.* found
75 that *TEF2*, *UBQ10* and *RP II* were the three most suitable reference genes for peach samples [18].
76 Chen *et al.* reported that the *RPS2* and *UBQ2* genes were the most suitable reference genes across
77 all tested banana samples, and different banana sample sets should select different suitable
78 reference gene(s) [19]. Perini *et al.* concluded that *MDH*, *SAND*, *THFS*, *TMp1* and *WD40* were the
79 best reference genes for RT-qPCR in various apple tissues [20]. In loquat, the *Actin* gene has been
80 widely used to normalize gene expression under different experimental conditions, such as fruit
81 pulp coloration, floral initiation, fruit cell division [16], fruitlets under freezing stress [21], and
82 postharvest fruits under chilling stress[22]. However, Su *et al* revealed that the *Actin* gene was not
83 always suitable for detecting expression in all samples. *RPL4*, *RPL18*, *HIS3* and *TUA3* were the four
84 most stable reference genes across all of the tested loquat samples, including fruits, ovules and seeds,
85 floral tissues and vegetative tissues, while the *Actin* gene was the sixth choice [16]. Therefore, suitable
86 reference genes should be further confirmed for other developing or abiotic-stressed tissues for
87 which screening of reference genes was not performed.

88 In this study, eight candidate reference genes were selected from our previously published
89 RNA-seq data, and primers for each candidate reference gene were designed and evaluated.
90 Moreover, the Cq values of the candidate reference genes were calculated by RT-qPCR in 31
91 different loquat samples, including 12 subgroups of developing or abiotic-stressed tissues. Then,
92 the Cq values were analysed using four algorithms (geNorm, NormFinder, BestKeeper and ΔCt),
93 and the four results were synthesized to obtain a comprehensive rank to confirm the suitable
94 reference genes for each subgroup. Finally, to verify the results, the expression abundance of
95 *EjLGAI* in each subgroup was normalized to the screened reference genes.

96 **Materials and Methods**

97 **Plant Materials**

98 The loquat cultivars ‘Zaozhong No. 6’ (ZZ6), ‘Jiefangzhong’ (JFZ) and ‘Baili’ (BL) were used and
99 prepared according to our previously published methods with some modification[1, 3]. Loquat
100 seedlings and mature trees were both grown in an outdoor nursery and managed by conventional
101 agronomic methods. For seedlings, loquat scions of the ZZ6 cultivar were collected from mature
102 loquat trees and grafted to one-year-old loquat seedlings that were cultured in cloth bags (25 cm in
103 diameter and 20 cm in height) filled with nursery soil in winter. The next summer, the grafted
104 container seedlings were treated under heat (40 °C; Lh I: 0 h, Lh II: 1 h, Lh III: 2 h), freezing
105 (-3 °C; Lf I: 0 h, Lf II: 1 h, Lf III: 2 h) and salt (NaCl; Ls I: 0 mM, Ls II: 100 mM, Ls III: 200 mM)
106 stress. After each abiotic stress treatment, the 3rd-5th leaves of each grafted container seedling were
107 individually sampled and mixed. When the floral tissues developed, the floral meristems (Fd I),
108 buds (Fd II) and flowers (Fd III) were successively sampled and grouped as floral development
109 samples. After that, flowers in full bloom (Fs I), fruitlets at 15 d after full bloom (DAF) (Fs II),

110 and fruitlets at 30 DAF (Fs III) were collected and grouped as fruit setting samples. Grafted
111 container seedlings with 60 DAF young fruits were exposed at 12 °C for 5 d of cold acclimation
112 and then treated with freezing stress (6 h; Ff I: 25 °C, Ff II: -1 °C, Ff III: -3 °C). The pulp and
113 seeds of young fruits were sampled. For mature trees, the flowers were removed except those in
114 full bloom, which were defined as 0 DAF, in the red-fleshed cultivar JFZ and white-fleshed
115 cultivar BL. When the fruits reached 40 DAF, four uniform fruits were reserved and bagged per
116 cluster. The fruit samples were peeled and rapidly seeded before being frozen in liquid nitrogen.
117 The fruit ripening samples were sampled from the JFZ cultivar at 70 DAF (Fr I), 90 DAF (Fr II:),
118 110 DAF (Fr III), and 130 DAF (Fr IV). For the postharvest cold-storage samples, the mature
119 fruits (125 DAF) of two cultivars were stored at 4 °C and sampled after 0 d, 14 d and 28 d
120 (red-fleshed fruits: Jcs I: 0 d, Jcs II: 14 d, Jcs III: 28 d; white-fleshed fruits: Bcs I: 0 d, Bcs II: 14 d,
121 Bcs III: 28 d). Three biological replicates were performed for each sample. For the samples from
122 seedlings, each replicate was sampled and mixed from three grafted container seedlings. For the
123 samples from mature trees, each replicate contained the samples collected from five bagged fruit
124 clusters. All samples were frozen in liquid nitrogen, ground and mixed with an A11 mill (IKA,
125 Germany), and stored at -80 °C. The plant materials and sampling processes are also shown in Fig.
126 1.

127 **RNA extraction, cDNA synthesis and RT-qPCR**

128 Total RNA was extracted using the E.Z.N.A Plant RNA Kit (Omega, USA) with DNase I digestion.
129 The cDNAs were synthesized from RNAs that passed quality control by using the PrimeScript™
130 RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Japan). The RT-qPCR experiments
131 were carried out with CFX Connect (Bio-Rad, USA) and SYBR *Premix Ex Taq* (Tli RNaseH Plus)

132 (Takara, Japan) as previously described [1]. Three technological repeats were performed for each
133 biological replicate. All kits were operated according to the manufacturer's instructions. The
134 obtained Cq values were used for further data analysis.

135 **Candidate reference gene selection and primer verification**

136 Eight candidate reference genes were selected from our previously published RNA-seq data [1],
137 including Actin (*ACT*), Elongation factor 1- α (*EF1 α*), Translation initiation factor eIF-2B
138 subunit α (*eIF2B*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Ribosomal protein
139 S3 (*RPS3*), Tubulin α chain (*TUA*), Tubulin β -1 chain (*TUB*), and Ubiquitin-conjugating
140 enzyme E2 (*UBCE*). The RT-qPCR primers were designed by NCBI Primer-BLAST
141 (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) with the following parameters: annealing
142 temperature (T_m) of 56-64 °C (optimal 60 °C), primer length of 18-22 bp (optimal 20 bp), GC
143 contents of 40–60% (optimal 50%), and product size of 80-300 bp. Detailed information on the
144 primers is listed in Table 1. The primer specificities were evaluated by melt curve analysis and
145 electrophoretic analysis. After that, the amplification efficiency (AE) and correlation coefficient
146 (R^2) of each pair of primers were calculated according to the Schmittgen and Livak method [8].
147 The AE values of the qualified pairs of primers should lie between 90% and 110%, and the R^2
148 should be greater than 0.98.

149 **Stability analysis of candidate reference genes**

150 The Cq values of the candidate reference genes in different samples were calculated by RT-qPCR
151 and exhibited by boxplots. The expression stability of each candidate reference gene was
152 individually analysed using the geNorm [12], NormFinder [13], BestKeeper [14] and ΔC_t [15]
153 methods, and ranked according to the results of the four algorithms. The overall rank order of the

154 candidate reference genes was established by calculating the geometric mean of the rank orders
155 derived from the four algorithms. A lower rank number represented a higher stability in both the
156 rankings of the four individual algorithms and the overall rank [16, 17].

157 **Validation of screened reference genes**

158 The validation of the screened reference genes was performed according to Su's methods [16]
159 with some modifications. The cloned loquat gene *EjLGA1* was used to verify the expression
160 stabilities of the screened reference genes. The expression pattern of *EjLGA1* was analysed by
161 RT-qPCR (forward primer: 5'- ATGCTGTCTATTTTGATCGAAAAT -3'; reverse primer: 5'-
162 TCACAATAAACCGGCCTCG -3') [22] and calculated using the $2^{-\Delta\Delta C_t}$ method [8]. The
163 geometric mean of the three most stable reference genes and that of the three most unstable
164 reference genes were used to normalize the expression of *EjLGA1*. The expression patterns of
165 *EjLGA1* when normalized to the three most stable and unstable reference genes were analysed to
166 obtain the Pearson correlation coefficient (PCC) in each subgroup. $0 \leq |PCC| < 0.2$ was a very weak
167 correlation, $0.2 \leq |PCC| < 0.4$ was a weak correlation, $0.4 \leq |PCC| < 0.6$ was a moderate correlation,
168 $0.6 \leq |PCC| < 0.8$ was a strong correlation, and $0.8 \leq |PCC| < 1$ was a very strong correlation.

169 **Results**

170 **Verification of specific primers for selected candidate reference genes**

171 Eight candidate reference genes (*ACT*, *EF1 α* , *eIF2B*, *GAPDH*, *RPS3*, *TUA*, *TUB*, and *UBCE*)
172 were selected to design the specific primers. The T_m of the designed primers ranged from
173 60.65 °C to 63.44 °C, the GC content ranged from 47.62% to 60.00%, and the product size ranged
174 from 82 bp to 259 bp. The melt curve analysis of the RT-qPCR for each primer exhibited a single
175 specific melt peak, and the electrophoretic analysis of RT-qPCR products showed a single specific

176 electrophoretic band with the expected size. The AE values of the designed primers ranged from
177 91.11% to 103.76%, and the R^2 values ranged from 0.9807 to 0.9989. (Table 1 and Fig. 2). These
178 results suggested that the eight pairs of primers could be used for the expression stability analysis
179 of eight candidate reference genes.

180 **Expression stability of candidate reference genes**

181 The expression stability of eight candidate reference genes was investigated by RT-qPCR in 93
182 loquat samples (including biological replicates) that were separated into four groups, including
183 tissue development (group I), leaves under abiotic stress (group II), immature fruitlets under
184 freezing stress (group III), and postharvest fruits under chilling stress (group IV). Each group
185 included three subgroups as follows: Group I included floral development stages (A), fruit setting
186 stages (B) and fruit ripening stages (C); Group II involved leaves under heat (D), freezing (E) and
187 salt (F) stress; Group III comprised immature pulp (G), immature seeds (H) and immature pulp
188 and seeds (I). Group IV contained red-fleshed fruits (J), white-fleshed fruits (K) and red- and
189 white-fleshed fruits (L). The C_q value distributions of the candidate reference genes in the 4
190 groups and 12 subgroups are displayed in Fig. 3.

191 Across all samples, the minimum C_q value was 19.550 (*UBCE*), while the maximum C_q value
192 was 36.081 (*TUB*). The mean C_q values ranged from 24.532 (*EF1 α*) to 29.014 (*ACT*), and the
193 variation levels of the C_q values varied from 8.689 (*EF1 α*) to 13.706 (*TUB*). In contrast, the
194 variation levels of the C_q values fluctuated from 2.579 (*EF1 α*) to 6.058 (*TUB*) in subgroup A,
195 1.722 (*UBCE*) to 6.412 (*TUB*) in subgroup B, 1.910 (*EF1 α*) to 4.417 (*UBCE*) in subgroup C,
196 1.806 (*UBCE*) to 4.591 (*TUB*) in subgroup D, 1.631 (*UBCE*) to 3.824 (*TUB*) in subgroup E, 5.932
197 (*EF1 α*) to 12.671 (*TUB*) in subgroup F, 2.615 (*UBCE*) to 6.121 (*RPS3*) in subgroup G, 2.010

198 (*UBCE*) to 4.004 (*TUB*) in subgroup H, 3.808 (*UBCE*) to 8.464 (*RPS3*) in subgroup I, 2.908
 199 (*UBCE*) to 5.566 (*ACT*) in subgroup J, 2.490 (*eIF2B*) to 5.052 (*TUA*) in subgroup K, and 4.279
 200 (*TUB*) to 6.232 (*ACT*) in subgroup L (Fig. 3). The results suggested that *EF1α* had a high
 201 expression level and *ACT* had a low expression level. In addition, the variation levels of the Cq
 202 values were very high across all the samples, but the variation was significantly narrower in each
 203 subgroup, which indicated that the expression levels of these candidate reference genes were more
 204 stable within subgroups than across all samples. Therefore, screening suitable reference genes in
 205 each subgroup was reasonable.

206 The Cq values of the candidate reference genes were first analysed by four different algorithms
 207 and then synthesized to gain overall rankings, which were used to confirm the expression
 208 stabilities of the reference genes. In subgroup A, the three most stable reference genes were
 209 *GAPDH*, *EF1α* and *ACT*, while the three most unstable reference genes were *RPS3*, *TUA* and *TUB*.
 210 In subgroup B, the three most stable reference genes were *GAPDH*, *UBCE* and *ACT*, while the
 211 three most unstable reference genes were *eIF2B*, *RPS3* and *TUB*. In subgroup C, the three most
 212 stable reference genes were *EF1α*, *GAPDH* and *eIF2B*, while the three most unstable reference
 213 genes were *ACT*, *TUB* and *UBCE* (Table 2). In subgroup D, the three most stable reference genes
 214 were *ACT*, *EF1α* and *UBCE*, while the three most unstable reference genes were *GAPDH*, *RPS3*
 215 and *TUB*. In subgroup E, the three most stable reference genes were *eIF2B*, *UBCE* and *EF1α*,
 216 while the three most unstable reference genes were *TUA*, *RPS3* and *TUB*. In subgroup F, the three
 217 most stable reference genes were *EF1α*, *TUA* and *UBCE*, while the three most unstable reference
 218 genes were *eIF2B*, *RPS3* and *TUB* (Table 3). In subgroup G, the three most stable reference genes
 219 were *ACT*, *EF1α* and *eIF2B*, while the three most unstable reference genes were *TUB*, *TUA* and

220 *RPS3*. In subgroup H, the three most stable reference genes were *ACT*, *UBCE* and *eIF2B*, while
 221 the three most unstable reference genes were *RPS3*, *EF1α* and *TUB*. In subgroup I, the three most
 222 stable reference genes were *EF1α*, *eIF2B* and *UBCE*, while the three most unstable reference
 223 genes were *TUA*, *TUB* and *RPS3* (Table 4). In subgroup J, the three most stable reference genes
 224 were *UBCE*, *TUB* and *TUA*, while the three most unstable reference genes were *RPS3*, *GAPDH*
 225 and *ACT*. In subgroup K, the three most stable reference genes were *eIF2B*, *RPS3* and *TUB*, while
 226 the three most unstable reference genes were *EF1α*, *ACT* and *TUA*. In subgroup L, the three most
 227 stable reference genes were *eIF2B*, *UBCE* and *RPS3*, while the three most unstable reference
 228 genes were *EF1α*, *GAPDH* and *ACT* (Table 5). Obviously, all the candidate reference genes could
 229 be among either the three most stable or the three most unstable reference genes in different
 230 subgroups, demonstrating that there were no reference genes suitable for all subgroups.

231 **Validation of screened reference genes**

232 To verify the screened reference genes, the expression patterns of *EjLGAI* were analysed by
 233 RT-qPCR and normalized by using the geometric mean of the most three stable reference genes as
 234 well as the most three unstable reference genes in the 12 subgroups. The PCC values of the
 235 relative expression abundance of *EjLGAI* were calculated. As shown in Fig. 4, the expression
 236 patterns of *EjLGAI* normalized by the three most stable and unstable reference genes were
 237 positively correlated. The PCC values showed very strong correlation in 7 subgroups (B, D, E, F,
 238 H, J, and K), strong correlation in 2 subgroups (G and L) and moderate correlation (A, C and I) in
 239 3 subgroups. In the moderate correlation subgroups, the use of unstable reference genes resulted in
 240 a difference in expression patterns of *EjLGAI* compared to the use of stable reference genes. In
 241 addition, although the relative expression patterns of *EjLGAI* normalized to stable and unstable

reference genes were similar in the very strong and strong correlation subgroups, normalization using stable reference genes could result in a smaller deviation in most samples, which was beneficial for reducing errors (Fig. 4). Thus, our results indicated that stable reference genes could provide more reliable results.

Discussion

Loquat (*Eriobotrya japonica* Lindl.) is a subtropical evergreen fruit tree that produces fruit with abundant nutrients and medicinal components [1]. Gene expression quantification by using RT-qPCR with the relative quantification $2^{-\Delta\Delta C_t}$ method has been widely performed in loquat in different tissues or organs and under different stress conditions, such as pulp coloration, floral initiation, fruit cell division, fruitlets under freezing stress and postharvest fruits under chilling stress. The *Actin* gene was the most commonly used reference gene and has been widely used to normalize gene expression in various works [16, 21, 22]. In fact, the use of reference genes is the most effective and appropriate method for the problem of the normalization step in RT-qPCR [7, 10]. However, it has been reported that there is no universal reference gene that could be used for all RT-qPCR experiments [10], and the most stable reference genes should be confirmed before RT-qPCR experiments [11]. The screening of suitable reference genes has therefore been carried out in many species [18-20]. A recent study in loquat supported these opinions and found that *RPL4*, *RPL18*, *HIS3* and *TUA3* were the four most stable reference genes across different tissues or organs of loquat, while *Actin* was the sixth choice [16]. Therefore, it is worth further evaluating suitable reference genes in other developing or abiotic-stressed tissues.

To further evaluate suitable reference genes in loquat, in this work, we constructed a sample pool with 31 samples (each sample had three biological replicates) that could be divided into 4 groups

and 12 subgroups. The sample pool included both developing tissues and abiotic-stressed tissues of loquat. *Su et al.* screened the most stable reference genes in the fruit, floral tissues, ovule, seed and vegetative tissues of loquat [16]. In contrast, we examined 3 subgroups of developing tissues related to the agricultural production of loquat fruits in this work, including floral development, fruit setting and fruit ripening. On the other hand, abiotic stress conditions could cause serious losses to agricultural production worldwide, and temperature, salinity and drought have been the subject of intense research in many plants [23]. Loquat also suffers from many abiotic stresses, such as the exposure of fruitlets to freezing stress and that of postharvest fruits to chilling stress [21, 22]. When the temperature drops below -3°C , freezing stress could seriously damage the fruitlets [21]. In addition, cold storage is the main preservation method for postharvest loquat fruits, but cold storage could result in many chilling injury phenomena, especially lignification. Interestingly, red-fleshed fruits often suffer from lignification due to chilling, while white-fleshed fruits do not [1, 22]. It is important to determine how loquat plants alter their gene expression in response to different abiotic stresses. However, suitable reference genes for loquat under stress conditions have never been reported. Therefore, we also examined 9 subgroups of abiotic-stressed tissues, including leaves under abiotic stress (heat, freezing and salt), immature fruitlets under freezing stress (immature pulp, seeds, and both pulp and seeds), and postharvest fruits under chilling stress (red-fleshed fruits, white-fleshed fruits and both red- and white-fleshed fruits). The expression abundance of a reliable reference gene should be unaffected by experimental factors and show minimal variations in different tissues and different physiological states of the same tissue [24]. It is generally believed that basic metabolism genes, also called housekeeping genes, could perfectly fulfil these requirements. Housekeeping genes are defined as the genes that

286 are crucial for the survival of cells, suggesting that their expression must remain stable and
 287 constant [7]. Suitable reference genes should be chosen from a sufficient number of candidate
 288 reference genes [25]. It has been recommended that at least four potential reference genes should
 289 be selected from the literature and that gene-specific primers should be designed and tested for
 290 these genes [11]. We selected eight candidate reference genes from our previously published
 291 RNA-seq data, including *ACT*, *EF1 α* , *eIF2B*, *GAPDH*, *RPS3*, *TUA*, *TUB*, and *UBCE*. These
 292 candidate reference genes are common housekeeping genes that have been used as candidate
 293 reference genes in many plants [26-29].

294 In theory, the expression of reference genes should be unchanged in each tested sample. However,
 295 in practice, it is important to select genes with the most stable expression or the least deviation
 296 from the mean expression because variations in Cq values are always observed [12]. In our results,
 297 the Cq values were obviously more stable within each subgroup than across all samples. Therefore,
 298 we continued to screen suitable reference genes in each subgroup. To confirm the most stable
 299 reference genes, there are many algorithms for the optimal choice of reference genes, such as the
 300 geNorm [12], NormFinder [13], BestKeeper [14], and Δ Ct [15] methods. However, it has been
 301 found that different algorithms may lead to different rankings of the candidate reference genes [7].
 302 A similar result was also observed in our work. For example, *GAPDH* ranked 3rd (geNorm), 2nd
 303 (NormFinder), 4th (BestKeeper), and 5th (Δ Ct method) in subgroup I. To solve these problems, a
 304 comprehensive approach was presented in which the results of the different algorithms were
 305 combined to obtain aggregated stability rankings [16, 17, 30]. In this approach, at least three
 306 different algorithms should be used [7]. Here, we synthesized the results of four commonly used
 307 algorithms, the geNorm, NormFinder, BestKeeper and Δ Ct methods, to obtain an overall rank

order for each subgroup. Not surprisingly, no reference genes were appropriate for all subgroups. Although the most stable reference gene could be calculated by this comprehensive strategy, a significant bias may still be generated by using one reference gene [31]. To avoid this, at least three reference genes should be used for accurate normalization of qRT-PCR data [25, 31]. In this work, we normalized the qRT-PCR data of the *EjLGA1* gene to verify the screened reference genes. *EjLGA1* encodes the $G\alpha$ subunit of heterotrimeric G protein, which could transduce extracellular signals to intercellular downstream effectors [22]. Our results showed that the usage of the three most stable reference genes to normalize the qRT-PCR data could avoid the underestimation or overestimation of actual expression abundance or reduce the expression deviation from the mean. This result suggested that the combined use of the three most stable reference genes was suitable for the accurate normalization of the twelve experimental subgroups of loquat.

Conclusion

The current study evaluated suitable reference genes in twelve subgroups of developing or abiotic-stressed tissues in loquat. Eight candidate reference genes were selected from our previously published RNA-seq data and evaluated by synthesizing the results of four algorithms (geNorm, NormFinder, BestKeeper and ΔCt methods) to yield an overall rank. Finally, we obtained different combinations of the three most stable reference genes for accurate normalization in twelve subgroups.

Abbreviations

ACT: Actin; *Ef1 α* : Elongation factor 1- α ; *eIF2B*: Translation initiation factor eIF-2B subunit α ; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *RPS3*: Ribosomal protein S3; *TUA*:

330 Tubulin alpha chain; *TUB*: Tubulin beta-1 chain; *UBCE*: Ubiquitin-conjugating enzyme E2.

331 **Authors' contributions**

332 Jincheng Wu supervised this study and revised the manuscript. Shoukai Lin participated in the
333 design and coordination of this study and drafted the manuscript. Shichang Xu and Liyan Huang
334 performed the RT-qPCR experiments and data analysis. Fuxiang Qiu, Yihong Zheng, Qionghao
335 Liu, and Jing Ling prepared the plant materials and carried out the samples collection. Shiwei Ma,
336 Bisha Wu substantively revised the manuscript. All authors read and approved the final
337 manuscript.

338 **Funding**

339 This research was funded by the Natural Science Foundation of Fujian Province (2017J01644,
340 2017J01645, 2019J01809), the College Outstanding Young Researchers Cultivation Program of
341 Fujian education department (2017), the Key Research Project of Putian Science and Technology
342 Bureau (2018ZP03, 2018ZP07, 2018ZP08), the Research and Innovation Special Foundation of
343 Putian University (2016CX001), the Scientific Research Project of Putian University (2018064).

344 **Acknowledgements**

345 The English language of this manuscript was edited by American Journal Experts (AJE).

346 **Availability of data and materials**

347 The datasets analyzed during the current study are available from the corresponding author on
348 reasonable request.

349 **Competing interests**

350 The authors declare that they have no competing interests.

351 **Consent for publication**

352 All authors have consented for publication.

353 **Ethics approval and consent to participate**

354 Not applicable.

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439 Table 1 Primer information for candidate reference genes

Gene abbreviati on	Gene name	Primer sequences (5'-3')	Tm (°C)	GC content (%)	Product size (bp)	Application efficiency (%)	Regression coefcient (R ²)
ACT	Actin	F: GAGAAGATTGTCATCACACG	60.65	47.62	187	103.11	0.9807
		R: TGGCGTAGAGGGAAGAACAG	61.66	52.38			
EF1 α	Elongation factor 1-alpha	F: CTTGGTCGCTTTGCTGTGAG	62.09	55.00	82	100.68	0.9965
		R: CGGTTGGCTCCTTCTTCTCC	63.44	60.00			
eIF2B	Translation initiation factor eIF-2B subunit alpha	F: CGGCTGGATGTGACCTGTTC	61.02	60.00	134	102.08	0.9995
		R: TCTGCGGGCCTTCATCGATA	61.11	50.00			
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: TGCCCACTGTTGATGTTTCC	61.91	50.00	103	103.76	0.9989
		R: TTGCCCTCAGACTCCTCCTT	61.30	55.00			
RPS3	Ribosomal protein S3	F: GGCTTTCGTCTTGGTACGTGT	60.87	52.38	125	95.45	0.9921
		R: CCCTTCTCCTTTCCGGGTCT	60.91	60.00			
TUA	Tubulin alpha chain	F: AATCACCAACAGTGCCTTCG	61.10	50.00	129	102.11	0.9965
		R: GCAACAGCAGCATTCACATC	60.43	50.00			
TUB	Tubulin beta-1 chain	F: TCCCTGGTCAACTCAACTCG	61.25	55.00	259	99.11	0.9829
		R: TCGTCAACTTCCTTGGTGCT	60.83	50.00			
UBCE	Ubiquitin-conjugating enzyme E2	F: CTTCCGCCGATCAAGTCAG	60.81	60.00	95	91.11	0.9966
		R: AAGTCCAACAGCAGCCCATG	60.90	55.00			

440

441

442 Table 2 Expression stability of 8 candidate reference genes during tissue development.

Samples	Candidate internal reference gene	geNorm		NormFinder		Bestkeeper		ΔC_q		Overall rank order
		M value	Rank order	Stability value	Rank order	SD[$\pm$ CP]	Rank order	Average of standard deviation	Rank order	
Floral development (Subgroup A)	ACT	0.96	2	0.34	3	1.63	7	0.53	2	3
	EF1 α	1.26	7	0.71	7	0.72	1	0.45	1	2
	eIF2B	0.97	3	0.33	2	1.49	6	0.60	4	5
	GAPDH	0.86	1	0.18	1	1.24	3	0.65	5	1
	RPS3	1.22	5	0.67	5	1.45	4	0.74	6	6
	TUA	1.24	6	0.68	6	1.47	5	0.89	7	7
	TUB	1.37	8	0.82	8	2.09	8	0.91	8	8
	UBCE	1.11	4	0.56	4	0.77	2	0.55	3	4
Fruit setting (Subgroup B)	ACT	0.97	2	0.40	2	0.95	5	0.71	6	3
	EF1 α	1.11	5	0.59	6	0.68	2	0.54	2	3
	eIF2B	1.10	4	0.56	4	1.14	8	0.62	5	6
	GAPDH	0.82	1	0.03	1	0.74	3	0.60	3	1
	RPS3	1.29	8	0.74	8	0.87	4	0.61	4	7
	TUA	1.03	3	0.49	3	1.08	6	0.71	6	5
	TUB	1.23	7	0.70	7	1.12	7	1.23	8	8
	UBCE	1.11	5	0.56	4	0.63	1	0.20	1	2
Fruit ripening (Subgroup C)	ACT	0.39	7	0.77	7	0.17	3	0.66	8	6
	EF1 α	0.94	1	0.14	1	0.15	1	0.36	3	1
	eIF2B	1.09	2	0.40	2	0.17	3	0.34	2	3
	GAPDH	1.11	3	0.45	3	0.16	2	0.30	1	2
	RPS3	1.12	4	0.54	5	0.25	7	0.49	5	5
	TUA	1.13	5	0.53	4	0.21	6	0.46	4	4
	TUB	1.27	6	0.71	6	0.28	8	0.51	6	7
	UBCE	1.64	8	1.01	8	0.19	4	0.64	7	8

443

444

445 Table 3 Expression stability of 8 candidate reference genes in leaves under abiotic stresses.

Samples	Candidate internal reference gene	geNorm		NormFinder		Bestkeeper		ΔC_q		Overall rank order
		M value	Rank order	Stability value	Rank order	SD[$\pm$ CP]	Rank order	Average of standard deviation	Rank order	
Heat stress (Subgroup D)	ACT	0.69	1	0.23	1	0.65	3	0.47	3	1
	EF1 α	0.77	4	0.35	4	0.97	6	0.30	1	2
	eIF2B	0.75	2	0.34	2	0.74	4	0.55	7	4
	GAPDH	0.80	5	0.41	6	0.59	2	0.50	4	6
	RPS3	0.81	6	0.38	5	0.80	5	0.54	5	7
	TUA	0.76	3	0.34	2	1.01	7	0.54	5	5
	TUB	1.02	8	0.63	8	1.25	8	0.78	8	8
	UBCE	0.86	7	0.48	7	0.43	1	0.36	2	3
Freezing stress (Subgroup E)	ACT	0.63	3	0.24	4	0.52	5	0.29	3	4
	EF1 α	0.64	4	0.30	5	0.46	2	0.30	4	3
	eIF2B	0.56	1	0.17	1	0.46	2	0.27	1	1
	GAPDH	0.73	6	0.36	6	0.46	2	0.32	5	5
	RPS3	0.83	7	0.46	7	0.86	7	0.58	7	7
	TUA	0.64	4	0.21	3	0.61	6	0.50	6	6
	TUB	1.16	8	0.75	8	0.98	8	0.75	8	8
	UBCE	0.57	2	0.19	2	0.37	1	0.27	1	2
Salt stress (Subgroup F)	ACT	1.94	7	1.18	7	1.85	1	1.59	5	4
	EF1 α	1.27	2	0.35	2	2.03	2	1.02	1	1
	eIF2B	1.62	5	0.76	5	2.91	7	1.26	4	6
	GAPDH	1.44	4	0.55	4	2.78	6	1.60	6	5
	RPS3	1.82	6	1.03	6	2.11	4	1.64	7	7
	TUA	1.26	1	0.15	1	2.47	5	1.22	3	2
	TUB	2.33	8	1.48	8	3.91	8	2.00	8	8
	UBCE	1.35	3	0.52	3	2.05	3	1.08	2	3

446

447

448 Table 4 Expression stability of 8 candidate reference genes in fruitlets under freezing stress.

Samples	Candidate internal reference gene	geNorm		NormFinder		Bestkeeper		ΔC_q		Overall rank order
		M value	Rank order	Stability value	Rank order	SD[$\pm$ CP]	Rank order	Average of standard deviation	Rank order	
Immature pulps (Subgroup G)	ACT	0.99	3	0.37	1	0.20	1	0.79	5	1
	EF1 α	1.03	4	0.51	4	0.20	1	0.60	2	2
	eIF2B	0.96	1	0.43	2	0.26	6	0.62	3	3
	GAPDH	0.97	2	0.44	3	0.25	5	0.63	4	4
	RPS3	1.53	8	0.86	8	0.28	7	0.83	6	8
	TUA	1.21	6	0.63	6	0.24	4	0.99	8	7
	TUB	1.10	5	0.51	4	0.30	8	0.83	6	6
	UBCE	1.21	6	0.66	7	0.21	3	0.50	1	5
Immature seeds (Subgroup H)	ACT	0.60	1	0.13	1	0.28	6	0.34	1	1
	EF1 α	0.74	5	0.36	5	0.23	4	0.57	6	7
	eIF2B	0.64	2	0.17	2	0.23	4	0.49	5	3
	GAPDH	0.99	8	0.60	8	0.17	1	0.45	3	4
	RPS3	0.97	7	0.56	7	0.17	1	0.58	7	6
	TUA	0.66	3	0.27	3	0.30	7	0.48	4	5
	TUB	0.94	6	0.55	6	0.30	7	0.77	8	8
	UBCE	0.70	4	0.27	3	0.21	3	0.36	2	2
Both immature pulps and seeds (Subgroup I)	ACT	1.24	6	0.64	6	0.13	1	1.29	7	5
	EF1 α	1.06	2	0.49	2	0.14	2	0.81	2	1
	eIF2B	0.96	1	0.32	1	0.16	4	0.91	4	1
	GAPDH	1.09	3	0.49	2	0.16	4	1.02	5	4
	RPS3	1.47	8	0.89	8	0.19	7	1.35	8	8
	TUA	1.26	7	0.69	7	0.14	2	0.84	3	6
	TUB	1.12	5	0.50	4	0.19	7	1.11	6	7
	UBCE	1.10	4	0.53	5	0.16	4	0.60	1	3

449

450 Table 5 Expression stability of 8 candidate reference genes in cold-stored postharvest fruits under cold stress.

Samples	Candidate internal reference gene	geNorm		NormFinder		Bestkeeper		ΔCq		Overall rank order
		M value	Rank order	Stability value	Rank order	SD[$\pm$ CP]	Rank order	Average of standard deviation	Rank order	
Red-flesh fruits (Subgroup J)	ACT	1.26	7	0.78	7	1.81	8	0.88	8	8
	EF1 α	2.31	8	1.57	8	0.83	2	0.55	3	5
	eIF2B	0.91	3	0.25	3	1.22	5	0.83	6	4
	GAPDH	1.10	5	0.55	6	1.45	7	0.84	7	7
	RPS3	0.97	4	0.39	4	1.33	6	0.77	5	6
	TUA	1.14	6	0.42	5	0.61	1	0.54	2	3
	TUB	0.86	1	0.14	1	1.06	4	0.62	4	2
	UBCE	0.90	2	0.14	1	0.85	3	0.49	1	1
White-flesh fruits (Subgroup K)	ACT	1.36	6	0.82	6	1.17	8	0.86	7	7
	EF1 α	1.72	8	1.09	8	0.97	4	0.73	6	6
	eIF2B	1.07	1	0.24	1	0.72	2	0.44	1	1
	GAPDH	1.19	3	0.62	4	0.99	5	0.70	5	4
	RPS3	1.17	2	0.41	2	0.70	1	0.44	1	2
	TUA	1.49	7	0.82	6	1.02	6	1.01	8	7
	TUB	1.21	4	0.50	3	0.79	3	0.49	3	3
	UBCE	1.25	5	0.65	5	1.04	7	0.54	4	5
Both red- and white flesh fruits (Subgroup L)	ACT	1.33	7	0.78	7	0.17	5	0.91	8	8
	EF1 α	2.02	8	1.33	8	0.14	3	0.70	3	6
	eIF2B	1.01	1	0.23	1	0.13	2	0.71	4	1
	GAPDH	1.19	5	0.60	5	0.15	4	0.76	6	7
	RPS3	1.11	2	0.39	2	0.17	5	0.71	4	3
	TUA	1.32	6	0.63	6	0.12	1	0.81	7	5
	TUB	1.17	4	0.45	3	0.17	5	0.68	2	4
	UBCE	1.14	3	0.45	3	0.19	8	0.66	1	2

451

Fig. 1 Plant materials and sampling processes. Floral development: Fd I: Floral meristem, Fd II: Bud, Fd III: Flower; Fruit setting: Fs I: Full bloom, Fs II: 15 DAF, Fs III: 30 DAF; Fruit ripening: Fr I: 70 DAF, Fr II: 90 DAF, Fr III: 110 DAF, Fr IV: 130 DAF; Postharvest cold storage: Red-fleshed fruits (125 DAF): Jcs I: 0 d, Jcs II: 14 d, Jcs III: 28 d; White-fleshed fruits (125 DAF): Bcs I: 0 d, Bcs II: 14 d, Bcs III: 28 d; Leaves under heat stress (40 °C): Lh I: 0 h, Lh II: 1 h, Lh III: 2 h; Leaves under freezing stress (-3 °C): Lf I: 0 h, Lf II: 1 h, Lf III: 2 h; Leaves under salt stress (NaCl): Ls I: 0 mM, Ls II: 100 mM, Ls III: 200 mM; Young fruits under freezing stress (6 h): Ff I: 25 °C, Ff II: -1 °C, Ff III: -3 °C; (p/s): pulp/seeds.

462

463 Fig. 2 RT-qPCR primer specificity of the 8 candidate reference genes. A: Melt peaks of 8 primer sets; B: PCR
464 products of 8 primer sets

Fig. 3 Boxplots of the expression levels of the eight candidate reference genes during tissue development and under abiotic stresses. The boxes represent the 25th/75th percentiles. The lines across the boxes show the median. The whiskers indicate the maximum and minimum values. Group I: Tissue development; A: Floral development; B: Fruit setting; C: Fruit ripening. Group II: Leaves under abiotic stress; D: Leaves under heat stress; E: Leaves under freezing stress; F: Leaves under salt stress. Group III: Immature fruitlets under freezing stress G: Immature pulp; H: Immature seeds; I: Immature pulp and seeds. Group IV: Postharvest fruits under cold-storage stress; J: Red-fleshed fruits; K: White-fleshed fruits; L: Red- and White-fleshed fruits.

Fig. 4 The relative expression abundances of EjLGA1 in different samples. The data are normalized to the most two stable reference genes and one unstable reference gene. Group I: Tissue development; A: Floral development; B: Fruit setting; C: Fruit ripening. Group II: Leaves under abiotic stress; D: Leaves under heat stress; E: Leaves under freezing stress; F: Leaves under salt stress. Group III: Immature fruitlets under freezing stress G: Immature pulp; H: Immature seeds; I: Immature pulp and seeds. Group IV: Postharvest fruits under cold-storage stress; J: Red-fleshed fruits; K: White-fleshed fruits; L: Red- and white-fleshed fruits.

Figures

Figure 1

Plant materials and sampling processes. Floral development: Fd I: Floral meristem, Fd II: Bud, Fd III: Flower; Fruit setting: Fs I: Full bloom, Fs II: 15 DAF, Fs III: 30 DAF; Fruit ripening: Fr I: 70 DAF, Fr II: 90 DAF, Fr III: 110 DAF, Fr IV: 130 DAF; Postharvest cold storage: Red-fleshed fruits (125 DAF): Jcs I: 0 d, Jcs II: 14 d, Jcs III: 28 d; White-fleshed fruits (125 DAF): Bcs I: 0 d, Bcs II: 14 d, Bcs III: 28 d; Leaves under heat stress (40 °C): Lh I: 0 h, Lh II: 1 h, Lh III: 2 h; Leaves under freezing stress (-3 °C): Lf I: 0 h, Lf II: 1 h, Lf III: 2 h; Leaves under salt stress (NaCl): Ls I: 0 mM, Ls II: 100 mM, Ls III: 200 mM; Young fruits under freezing stress (6 h): Ff I: 25 °C, Ff II: -1 °C, Ff III: -3 °C; (p/s): pulp/seeds.

Figure 2

RT-qPCR primer specificity of the 8 candidate reference genes. A: Melt peaks of 8 primer sets; B: PCR products of 8 primer sets.

Figure 3

Boxplots of the expression levels of the eight candidate reference genes during tissue development and under abiotic stresses. The boxes represent the 25th/75th percentiles. The lines across the boxes show the median. The whiskers indicate the maximum and minimum values. Group I: Tissue development; A: Floral development; B: Fruit setting; C: Fruit ripening. Group II: Leaves under abiotic stress; D: Leaves under heat stress; E: Leaves under freezing stress; F: Leaves under salt stress. Group III: Immature fruitlets

under freezing stress G: Immature pulp; H: Immature seeds; I: Immature pulp and seeds. Group IV: Postharvest fruits under cold-storage stress; J: Red-fleshed fruits; K: White-fleshed fruits; L: Red- and White-fleshed fruits.

Figure 4

The relative expression abundances of *EjLGA1* in different samples. The data are normalized to the most two stable reference genes and one unstable reference gene. Group I: Tissue development; A: Floral

development; B: Fruit setting; C: Fruit ripening. Group II: Leaves under abiotic stress; D: Leaves under heat stress; E: Leaves under freezing stress; F: Leaves under salt stress. Group III: Immature fruitlets under freezing stress G: Immature pulp; H: Immature seeds; I: Immature pulp and seeds. Group IV: Postharvest fruits under cold-storage stress; J: Red-fleshed fruits; K: White-fleshed fruits; L: Red- and white-fleshed fruits.