Selection of reference genes for quantitative real-time PCR analysis in cucumber (*Cucumis sativus* L.), pumpkin (*Cucurbita moschata* Duch.) and cucumber–pumpkin grafted plants (#32264)

First revision

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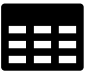

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Selection of reference genes for quantitative real-time PCR analysis in cucumber (*Cucumis sativus* L.), pumpkin (*Cucurbita moschata* Duch.) and cucumber–pumpkin grafted plants

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Background: Quantitative real-time PCR (qRT-PCR) is a commonly used high-throughput technique for mRNA transcription studies. Accurate evaluation of gene expression depends on the use of optimal reference genes. Cucumber–pumpkin grafted plants, made by grafting a cucumber scion onto pumpkin rootstock, are superior plants to either parent, as grafting conveys many advantages. To date, many reliable reference genes have been identified in both cucumber and pumpkin, but none have been obtained for cucumber–pumpkin grafted plants.

Methods: In this work,12 candidate reference genes, which including 8 traditional genes and 4 novel gens analyzed by our transcriptome data, were selected to assess their expression stability. Their expression in 25 samples, including 3 cucumber and 3 pumpkin samples from different organs, and 19 cucumber–pumpkin grafted samples from different organs, conditions and varieties, were analyzed by qRT-PCR, and the stability of their expression was assessed by the comparative ΔCt method, geNorm, NormFinder, BestKeeper, and RefFinder.

Results: The results showed that the most suitable reference gene depended on the organs, conditions and varieties. *CACS* and *40SRPS8* were the most stable reference genes in all samples in our research. *TIP41* and *CACS* had the most stable expression in different cucumber organs, *TIP41* and *PP2A* were the optimal reference genes in pumpkin organs, and *CACS* and *40SRPS8* were also the most stable in all grafted cucumber samples. However, the optimal reference gene varied under different conditions. *CACS* and *40SRPS8* were the best combination of genes in different organs of cucumber–pumpkin grafted plants, *TUA* and *RPL36Aa* were the most stable in the graft union under cold stress, *LEA26* and *ARF* had the most stable expression in the graft union during the healing process, *TIP41* and *PP2A*were the most stable across different varieties of cucumber–pumpkin grafted plants. *LEA26*, *ARF* and *LEA26+ARF* were further verified as reference genes by analyzing the expression levels of *csaCYCD3;1*, *csaRUL*, *cmoRUL*and *cmoPIN* in the graft union at different time points after grafting.

Discussion: This work is the first to identify the appropriate reference genes in grafted cucumber plants and provides useful information for the study of gene expression and molecular mechanisms in cucumber–pumpkin grafted plants.

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Abstract

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38 different organs of cucumber–pumpkin grafted plants, *TUA* and *RPL36Aa* were the most stable
39 in the graft union under cold stress, *LEA26* and *ARF* had the most stable expression in the graft
40 union during the healing process, *TIP41* and *PP2A* were the most stable across different varieties
41 of cucumber–pumpkin grafted plants. *LEA26*, *ARF* and *LEA26+ARF* were further verified as
42 reference genes by analyzing the expression levels of *csaCYCD3;1*, *csaRUL*, *cmoRUL* and
43 *cmoPIN* in the graft union at different time points after grafting.
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45 plants and provides useful information for the study of gene expression and molecular
46 mechanisms in cucumber–pumpkin grafted plants.

47 Introduction

48 Cucumber (*Cucumis sativus* L.) is one of the most widely cultivated vegetable crops in the
49 world. Grafted cucumber plants are popular because they are more resistant to soil-borne
50 diseases, show increased tolerance to abiotic stress, improved mineral nutrition uptake and use,
51 and increased fruit yield and quality (Huang et al;2014). A cucumber scion is usually grafted
52 onto pumpkin (*Cucurbita moschata*Duch.) rootstock (Huang et al., 2014; Lee et al., 2010).
53 Grafting conveys advantages over each individual parent plant, but the resulting plant is also
54 more complicated than the parents. The graft union is a critical part of combining the scion and
55 rootstock. Connecting them correctly results in successful grafting and the establishment of
56 complex communication between rootstock and scion. Physiological and biochemical studies of
57 cucumber–pumpkin grafted plants have been carried out for several decades (Ahn et al., 1999;
58 Yang et al., 2006; Haroldsen et al., 2012; Li et al., 2014), however, there have been few studies
59 analyzing gene function, transcription or expression in cucumber–pumpkin grafted plants, as
60 most pumpkin genes were unknown. Now, the entire cucumber
61 (http://cucurbitgenomics.org/organism/2) (Huang et al., 2009) and pumpkin
62 (http://cucurbitgenomics.org/organism/9) (Sun et al., 2017) genomes have been published,
63 enabling further studies on the molecular biology of these species.
64 Gene expression analysis is fundamental to elucidate the molecular mechanisms underlying
65 various biological processes (Bustin et al., 2005). qRT-PCR is the most common technique used
66 to study gene expression because of its high sensitivity, accuracy, specificity, cost-effectiveness
67 and reproducibility (Bustin et al., 2002; Nolan et al., 2006; Derveaux et al., 2010). However,
68 some non-specific variations can cause errors resulting in unreliability of the qRT-PCR data,
69 such as variability in RNA quality, cDNA synthesis and concentration, PCR procedures, and
70 efficiency of amplification (Delporte et al., 2015). To avoid this, stable reference genes should be
71 used to normalize the gene expression data. Appropriate reference genes should be
72 systematically evaluated across various environments (varieties, tissues, experimental treatments
73 and developmental stages) before being applied to qRT-PCR analysis (Bustin et al., 2009;
74 Guenin et al., 2009; Sgamma et al., 2016). However, there have not previously been any
75 systematic studies performed on grafted cucumber plants to determine reliable reference genes.
76 Common reference genes like *ACT* (*actin*), *TUA* (*tubulin*), *CYP* (*cyclophilin*), *UBI-1* (*ubiquitin*),
77 and *EF-α* (*elongation factor*) are considered to be stably expressed in various plants (Duan et al.,

78 2017; Obrero et al., 2011; Tashiro et al., 2016; Niu et al., 2017) and have been used for gene
79 expression studies in cucumber (Wang et al., 2009, Migocka and Papierniak, 2011; Warzybock
80 and Migocka, 2013). The genes *UFP* (*ubiquitin*), *EF-1A* (*elongation factor*), *PRL36aA* (*60S*
81 *ribosomal protein L36a/L44*), *PP2A* (*protein phosphatase*) and *CACS* (*clathrin adaptor*
82 *complexes medium submit family protein*) have provide the best strategy for reliable
83 normalization in different experimental sets in zucchini (*Cucurbita pepo*) (Obrero et al., 2011),
84 and these reference genes have been successfully applied to both cucumber and pumpkin in
85 specific environments, including powdery mildew, salinity, cold, dehydration, H_2O_2, and abscisic
86 acid (ABA) treatments (Berg et al., 2015;Cao et al., 2017; Reda et al., 2018). Unfortunately,
87 there is no single confirmed reference gene exhibiting uniform and stable expression under
88 different experimental conditions.For example, *ACT* as one of the most frequently used reference
89 genes in many plants, but were the least stable in short-term treatment of cucumber with plant
90 regulators or salt, osmotic or oxidative stress (Migocka and Papierniak, 2011), the unstable
91 reference genes may lead to inaccurate results.Therefore, it is necessary to identify one or more
92 reference genes under different experimental conditions prior to carrying out gene expression
93 studies (Duan et al., 2017).
94 In this study, traditional reference genes from published research and new ones based upon their
95 coefficients of variation (CVs) and expression intensity in our RNA-seq data from cucumber–
96 pumpkin grafted plants at different stages were selected for further analysis. Twelve genes were
97 investigated in this study, eight ~~traditional~~ reference genes, *ACT*, *CYP*, *CACS*, *TUA*,
98 *TIP41*(*tonoplast intrinsic protein*), *F-Box* (*F-box protein*), *RPL36Aa*, and *PP2A*, and four new
99 genes ~~screened~~ by RNA-seq analysis, *UBC* (*Ubiquitin conjugating enzyme*), *ARF* (*ADP-*
100 *ribosylation factor-like protein*), *LEA26* (*Late-embryogenesis abundant protein 26*), and
101 *40SRPS8* (*40S ribosomal protein S8*). These genes were evaluated to validate their use as stable
102 reference genes for qRT-PCR in different organs, at different stages, in different varieties and
103 under stress conditions in cucumber, pumpkin, and cucumber–pumpkin grafted plants. To
104 determine the appropriate reference genes, four statistical tools were used to evaluate the
105 accuracy of these candidate genes: the ΔCt method (Silver et al., 2006), geNorm (Vandesompele
106 et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004).
107 Comprehensive stability rankings were generated by RefFinder (Xie et al., 2012). Additionally,
108 the genes csa*CYCD3;1* (Csa2G356610), csa*RUL* (Csa3G895630), cmo*RUL*
109 (CmoCh15G013320) and cmo*PIN* (CmoCh15G009810), which are thought to be related to graft
110 union healing in grafted cucumber(*Table S1*), were investigated as a case study to evaluate the
111 effectiveness of the reference genes identified in this study.~~In our study, *CACS* and *40SRPS8*~~
~~112 were the most stable reference genes in all samples in our research. *TIP41* and *CACS* had the~~
~~113 most stable expression in different cucumber organs, *TIP41* and *PP2A* were the optimal~~
~~114 reference genes in pumpkin organs, and *CACS* and *40SRPS8* were also the most stable in all~~
~~115 grafted cucumber samples.~~ The results obtained in this study will be useful in many further gene
116 expression analyses in cucumber, pumpkin, and their grafted plants.

117 **Materials & Methods**

118 **Plant Materials and Treatments**
119 Cucumber (*Cucumis sativus* L.) and pumpkin (*Cucurbita moschata*Duch.) were planted in an
120 artificial chamber at the farm of the Institute of Vegetables and Flowers, Chinese Academy of
121 Agricultural Sciences, Beijing, China at a temperature of 28°C/20°C (day/night) with a
122 photoperiod cycle of 12/12 h and 60%–70% relative humidity. Cucumber variety 'Zhongnong
123 No. 26' was used as the scion and pumpkin variety 'Jinxinzhen No. 5' was used as the rootstock.
124 Seeds of the scion and rootstock were sown in 50-cell and 32-cell polystyrene
125 trays(54cm*28cm*5cm), respectively, containing commercial organic substrates
126 (Vpeatmoss:Vvermiculite:Vperlite = 1:1:1). The environmental conditions for germination were
127 25–28°C and 85%–90% relative humidity. The pumpkin seeds were sown three days before the
128 cucumber seeds. When cotyledons of the scion were fully open and the first true leaf of the
129 rootstock started to develop (9–10 d after sowing), the plants were grafted using the hole
130 insertion grafting method as previously described(Miao et al., 2018) (*Fig S1*). Autografts were
131 carried out for both cucumber and pumpkin, as well as cucumber–pumpkin heterografts. The
132 grafted seedlings were maintained at a temperature of 30°C/22°C (day/night), a constant
133 humidity of 95%–100% and a dim light of 50 PPFD for the first 5 days, then the light density
134 was slowly increased from 50 to 500 PPFD and the humidity was decreased from 95% to 60%,
135 while the other environmental conditions were unchanged. For the autograft cucumber and
136 pumpkin plants, samples of the leaves, stems and roots were harvested when the seedlings had
137 two true leaves. For cucumber grafted onto pumpkin, samples of the leaves, the stem of the
138 scion, the graft union (*Fig1*), the stem of the rootstock, and the roots were harvested. For the cold
139 stress experiment, when the grafted cucumber had two leaves, seedlings were exposed to
140 temperatures of 12°C in a chamber, and samples of the graft union were harvested at 0, 5, 12 and
141 24 h of stress treatment. To investigate the graft union healing process, samples of the graft
142 union were harvested 0, 3, 6, 9 and 15 d after grafting. For experiments with varieties, cucumber
143 varieties 'Xintaimici' and 'Zhongnong No. 26' were used as scions and pumpkin varieties
144 'Zhongguonangua No. 26', 'Jinxinzhen No. 5' and 'Huofenghuang' were used as rootstocks. The
145 graft combinations were 'Xintaimici–Zhongguonangua No. 26', 'Xintaimici–Jinxinzhen No. 5',
146 'Xintaimici–Huofenghuang', 'Zhongnong No. 26–Jinxinzhen No. 5', and 'Zhongnong No.26–
147 Huofenghuang'. Graft unions were harvested whengrafted plants had two true leaves. For each
148 treatment, three independent biological replicates were achieved. All samples were immediately
149 frozen in liquid nitrogen and stored at −80°C.
150 **RNA Isolation and cDNA Synthesis**
151 The RNAprep Pure Plant Plus Kit (Tiangen, Beijing, China) was used for total RNA extraction.
152 Genomic DNA was eliminated from the total RNA using RNase-free DNase I. The RNA
153 integrity was confirmed by 1.0% agarose gel electrophoresis. RNA concentrations were
154 determined by NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA),
155 samples with an A260/A280 ratio of 1.8-2.2 and an A260/A230 ratio > 2.0 were used for further
156 analyses. First-strand cDNA synthesis was performed using a FastQuant cDNA Synthesis kit
157 (Tiangen, Beijing, China) according to the manufacturer's instructions.

158 **Candidate Reference Gene Selection and Primer Design**
159 Eight traditional candidate reference genes (*ACT, CYP, CACS, TUA, TIP41, F-Box, PRL36Aa*
160 and *PP2A*) from published studies on cucumber, pumpkin,chicory, buckwheat, Lettuce and
161 mangrove treewere selected (Wan et al., 2010; Obrero et al., 2011;Delporte et al., 2015;
162 Demidenko et al. 2011; Borowski et al., 2014;Saddheet al., 2018). For new candidate reference
163 genes, we analyzed our transcriptomic data from the graft union.Graft union of cucumber-
164 pumpkin were respectively harvested at 0, 3, 6, 9 days after grafting, three biological replicates
165 were performed for each time point. In total 18 transcriptomelibraries, 132.7G raw reads were
166 obtained, at the least 91.4% of the reads were mapped to the reference sequence, and assemble
167 into 32852 and 47906 transcripts of cucumber and pumpkin, respectively. 20782 unigene of
168 cucumber with the average length of 4.1kb were obtained, while 27187 unigene with average
169 length of 4.4kb were generated(data do not show). The genes with the most constant expression
170 levels were defined as candidate reference genes (De Jong et al., 2007). We calculated the mean
171 expression value, standard deviation, and coefficients of variation (CVs) based on the raw RNA-
172 seq data, and CVs = standard deviation of RPKM/average of RPKM. Based on the requirements
173 CV ≤ 0.2 and 300≤RPKM≤ 500 (Duan et al., 2017), we selected new reference genes by
174 removing overabundant genes with low expression levels.With requirements of evalue e-5,
175 weused BLAST to determine the proteinsencoded by cucumber and pumpkin genes,
176 respectively(https://blast.ncbi.nlm.nih.gov/Blast.cgi), and then filtered the BLAST results based
177 on an identity ≥ 90 and an overlap ratio > 0.5 (between query and target). This resulted in ten and
178 seven genes of cucumber and pumkin, respectively, which may be suitable as reference genes. A
179 comparison of the relationship between cucumber and pumpkin by homology analysis is shown
180 in *Table S2*. Finally, *UBC, ARF, LEA26* and *40SRPS8* were selected as candidate reference genes
181 based on preliminary experiments of single PCR product in agarose gel electrophoresis (data do
182 not show). Based on the conserved sequence of these genes between cucumber and pumpkin,
183 primers were designed using Primer Premier 5.0 software with the following parameters: a
184 melting temperature (Tm) of 50–60°C, a primer length of 17–25 bp, and a product size of 70–
185 260 bp (http://www.premierbiosoft.com/)(*Table 1*). Amplification of a single PCR product in 1%
186 agarose gel electrophoresis and a single peak of the melting curve in qRT-PCR were used to
187 ensure the specificity of the primers for the candidate reference genes.
188 **qRT-PCR Assay**
189 qRT-PCR was performed on an Agilent Stratagene Mx3000P Real-Time PCR machine (Agilent
190 Stratagene, USA) using SYBR® Premix Ex Taq™ (TliRNaseH Plus) (TaKaRa, Dalian, China).
191 Each 20 µl reaction mixture contained 2 µl of cDNA template, 0.4 µl of each primer, 0.4 µl of
192 ROX dye, 10 µl of 2× SYBR Premix Ex Taqand 6.8 µl of ddH_2O. The qRT-PCR reaction
193 conditions were as follows: 94°C for 30 s, 40 cycles of 94°C for 5 s, then 60°C for 34 s. A
194 melting curve was determined by increasing the amplification temperature from 60–95°C, with a
195 temperature increment of 0.5°C every 5 s. All samples were performed with three technical
196 replicates, and samples without template were used as a control. The amplification efficiencies
197 for each primer and the regression coefficients (R^2) were evaluated using five-fold dilutions of

198 pooled cDNA (1/5, 1/25, 1/125, 1/625, 1/3125) that were diluted using EASY dilution solution
199 (Takara, Japan).
200 **Gene Expression Stability Analysis**
201 To evaluate the expression levels of each reference gene, we drew boxplots of the Ct values for
202 the 12 candidate reference genes (*Fig 2*). Four statistical tools, the ΔCt method (Silver et al.,
203 2006), geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and
204 BestKeeper (Pfaffl et al., 2004), were used to evaluate the stability of the 12 candidate reference
205 genes at various treatment durations. The raw Ct values of the reference genes were transformed
206 into the correct input files according to the requirements of the software. Finally, a
207 comprehensive ranking of the reference genes was generated using RefFinder (Duan et al.,
208 2017).
209 **Validation of Reference Gene Stability**
210 To confirm the reliability of the selected reference genes, the relative expression levels of three
211 genes involved in xylem development were measured during graft union healing in grafted
212 cucumbers(*Table S1*). Samples of the graft union of cucumber–pumpkin grafted plants were
213 harvested at 0, 1, 3, 6, 9 and 15d after grafting. The most stable reference genes (*LEA26,ARF* and
214 *LEA26+ARF*), and the least stable reference gene (*PP2A*) ranked by RefFinder were used for
215 normalization. Comparative gene expression levels of csa*CYCD3;1* (Csa2G356610), csa*RUL*
216 (Csa3G895630), cmo*RUL* (CmoCh15G013320) and cmo*PIN* (CmoCh15G009810) were
217 calculated using the $2^{-\Delta\Delta Ct}$ method. Three technical replicates were performed for each biological
218 sample.
219 ## Results
220 **Evaluation of Primer Specificity and Amplification Efficiency**
221 Eight genes used traditionally (*ACT*, *CYP*, *CACS*, *TUA*, *TIP41*, *F-Box*, *PRL36Aa* and *PP2A*) and
222 four potentialreference genes (*UBC*, *ARF*, *LEA26* and *40SRPS8*) were selected for qRT-PCR
223 analysis. To validate the primer specificity, specific bands in cucumber, pumpkin and grafted
224 cucumber were checked by 1% agarose gel electrophoresis (*Fig S2*). The product lengths were
225 consistent with the expected lengths, and a single sharp peak was observed in the melting curves
226 for cucumber, pumpkin and grafted cucumber (*Fig S3, S4A*). In our study, amplification
227 efficiency (E) ranged from 0.86 to 1.13 with the correlation coefficients (R^2) of the standard
228 curve varying from 0.986 to 0.999 (*Table 1, Fig S4B, S5*).
229 **Expression Levels and Variations in Candidate Reference Genes**
230 The transcript abundances of the 12 candidate reference genes were assessed by the Ct values
231 from the qRT-PCR incucumber, pumpkin and grafted cucumber.As shown in *Fig 2*,the Ct values
232 for the 12 candidate reference genes in all samples ranged from 16.98 to 31.71, and the mean Ct
233 values were 19.04, 18.35, 23.235, 20.795, 20.655, 26.695, 20.785, 24.785, 20.26, 21.775, 20.8
234 and 21.085 for *ACT*, *CYP*, *CACS*, *TUA*, *TIP41*, *F-Box*, *PRL36Aa*, *PP2A*, *UBC*, *ARF*, *LEA26* and
235 *40SRPS8*, respectively.
236 **Expression Stability Analysis of Candidate Reference Genes**

237 To evaluate the stability of the 12 candidate reference genes in our study, theΔCt method,
238 geNorm, NormFinder, BestKeeper, and RefFinder were used. The 12 candidate reference genes
239 were divided into eight groups in different treatments: organs of cucumber, pumpkin, and
240 cucumber–pumpkin grafted plants under normal conditions were termed Cos, Pos, and Gos,
241 respectively. Graft union samples of cucumber–pumpkin grafted plants under low temperatures
242 were termed GLgs, graft union samples of cucumber–pumpkin grafted plants during the healing
243 process were termed Ggs, graft union samples of different varieties of cucumber–pumpkin
244 grafted plants were termed Ggvs, all cucumber–pumpkin grafted plant samples were termed
245 GoAll, and all samples in our study were termed All.
246 The ΔCt method ranks the stability of expression of tested genes by comparing the relative
247 expression of gene pairs within each sample (Silver et al., 2006). As was shown in Table 2,
248 *TIP41* were most stable reference gene in the Cos, Pos, and Ggvs samples, while *TIP41* was the
249 lowest stable reference gene in the GLgs samples. *CACS* were most stable reference gene in the
250 Gos, GoAll, and All samples, *TUA* and *LEA26* were ranked as the most stable reference genes in
251 the GLgs and Ggs samples, respectively.
252 The BestKeeper program identifies potential reference genes by calculating the coefficients of
253 variation (CVs) and the standard deviation (SD) of the Ct values, where lower CVs and SD
254 indicate higher stability (Pfaffl et al., 2004). For the Cos and GoAll samples, *CACS* were
255 identified as the most stable gene, and for the Pos and Ggvs samples, *TIP41* was the most stable.
256 *CYP* was the most stable gene in the Gos samples, but exhibited as the lowest ranking for the
257 Ggvs samples. Similarly, *TUA* was the most stable gene in the GLgs samples, while was also the
258 lowest stable gene in the Pos samples. *ARF* and *LEA26* were ranked as the most stable reference
259 gene in the Ggs and All the samples, respectively. *PP2A* were the lowest stable reference gene in
260 most of samples with the BestKeeper analysis, including the Gos, Ggs, GoAll, and All samples
261 (*Table 2*).
262 NormFinder ranks the stability of tested genes based on inter- and intragroup variations in
263 expression across different sample groups, with lower values indicating higher stability
264 (Andersen et al., 2004). *TIP41* with the stability values of 0.084, 0.153, 0.203 was the most
265 stable gene in the Cos, Pos, and Ggvs samples, respectively. *40SRPS8* and *CACS* were the two
266 most stable genes and *PP2A* was the lowest stable in the Gos, GoAll, and All samples. For the
267 GLgs samples, *TUA* were most stable, while it ranked as the lowest reference gene in the Pos
268 samples. The stability of *LEA26* were best in the Ggs samples according to the NormFinder
269 analysis (*Table 2*).
270 The geNorm software determines the gene expression stability using M-values based on the
271 average pairwise variation of all candidate genes(Vandesompele et al., 2002). *TIP41and*
272 *40SRPS8, CACS and ARF, CYP and UBC, UBC and ARF, PP2A and ARF, ARF and 40SPRS8,*
273 were the two most stable genes in the Cos, Gos, GLgs, Ggs, Ggvs, and GoAll samples,
274 respectively. *CACS* and *40SRPS8* were identified as the most stable reference genes with M-
275 values of 0.093 and 0.582 respectively in the Pos and All samples. In addition, the optimal
276 number of reference genes for normalizing the gene expression arejudged by calculating the

277 pairwise variation (Vn/Vn+1) by geNorm algorithm, and Vn/Vn+1<0.15 indicates that the
278 optimal number of reference genes equal to the value of n to use as reference gene
279 (Vandesompele et al., 2002). In our study, the values of V_2/V_3 of all experimental samples was
280 less than 0.15, indicated that 2 reference genes would be sufficient for gene normalization under
281 these experimental conditions (*Fig 3*).
282 RefFinder considers the ΔCt method, geNorm, NormFinder and BestKeeper rankings to provide
283 a comprehensive ranking of the most stable genes (Xie et al., 2012). *TIP41* was the most stable
284 reference gene in the Cos, Pos, and Ggvs samples, *CACS* was ranked as the most stable gene in
285 the Gos, GoAll, and All samples. *TUA* was the most the stable in the GLgs samples, while it was
286 also the lowest reference gene in the Pos samples. *LEA 26* and *ARF* were the two most stable
287 reference genes in the Ggs samples. *PP2A* was the lowest stable reference gene in the Gos, Ggs,
288 GoAll and All samples according the RefFinder analysis (*Table 2*).
289 **Validation of the Selected Reference Genes**
290 To confirm the stability of the selected reference genes, the expression levels of *csaCYCD3;1*
291 (Csa2G356610), *csaRUL*(Csa3G895630), *cmoRUL*(CmoCh15G013320), and
292 *cmoPIN*(CmoCh15G009810), which are possibly important during the graft union healing
293 process(*Table S1*), were examined using*LEA26*, *ARF*, *LEA26+ARF*, and *PP2A* as reference
294 genes for normalization. RefFinder analysis had shown that *LEA26* and *ARF* were the most
295 suitable reference genes and *PP2A* was the least suitable reference gene in the graft union during
296 the healing process (*Table 2, S3*).
297 The expression patterns of *csaCYCD3;1*, *csaRUL*, *cmoRUL* and *cmoPIN* showed similar changes
298 when *LEA26*, *ARF* or *LEA26+ARF* were selected as the reference genes for normalization(*Fig4*).
299 The expression levels of *csaCYCD* and *csaRUL* were significantly downregulated at 3 d and 6 d
300 compared to 1 d after grafting. However, these values were markedly higher when *PP2A* was
301 selected for normalization. Compared to 0 d after grafting, *cmoPIN* expression was clearly
302 downregulated at the graft junction at 6 d, 9 d and 15 d after grafting when using *LEA26*, *ARF*, or
303 *LEA26+ARF* as reference genes, while this value was ~~abnormally~~ upregulated when *PP2A* was
304 used as a reference for normalization. Similarly, *cmoRUL* expression levels were extremely
305 upregulated at the graft junction 6 d after grafting when using *PP2A* as the reference gene
306 compared to the levels determined using the most stable reference genes (*LEA26*, *ARF* and
307 *LEA26+ARF*).

308 ## Discussion

309 qRT-PCR is the most powerful method for detecting transcriptomic data and studying the
310 underlying molecular mechanisms (Niu et al., 2017). Appropriate reference genes are required to
311 ensure the accuracy of the qRT-PCR results. There has recently been research into the selection
312 of optimal reference genes in cucumber and pumpkin (Wan et al., 2010;Obrero et al., 2011;
313 Warzybock and Migocka, 2013), however, there have been no studies on the selection of the
314 optimal reference genes for cucumber–pumpkin grafted plants. Grafting assembles the scion and
315 rootstock into a plant that often have a massive advantage over their parents, there were
316 substances exchange between scion and rootstock, includingwater, sugars, hormones, RNAs and

317 proteins (Melnyk, 2017),so research into cucumber–pumpkin grafted plants is necessary to
318 identify the optimal reference genes. Therefore, we selected some published traditional reference
319 genes (*ACT*, *CYP*, *CACS*, *TUA*, *TIP41*, *F-Box*, *PRL36Aa* and *PP2A*) that are expressed in
320 cucumber or pumpkin. We also selected four novel genes (*UBC*, *ARF*, *LEA26* and *40SRPS8*)
321 from our transcriptomic data on graft union healing in cucumber–pumpkin grafted plants, and
322 primers were designed based on the conserved sequence of the genes between cucumber and
323 pumpkin.
324 The ΔCt method, BestKeeper, NormFinder, geNorm, and RefFinderare five software programs
325 and methods that are commonly used for identifying reference genes (Scarabel et al., 2017; Duan
326 et al., 2017). In our study, two genes were sufficient for reliable normalization when all samples
327 were considered by geNorm analysis (*Fig3*). The ΔCt method, NormFinder, geNorm and
328 RefFinder programs all suggested the same least suitable reference genes, differing from the
329 rankings obtained by BestKeeper.For instance, *F-Box* was ranked as the least stable gene in Cos
330 samples by ΔCt method, NormFinder, geNorm and RefFinder programs analysis, while
331 BestKeeper identified *UBC* as the lowest stable in the Cos samples.This is in concordance with a
332 study by Niu et al (2017) where the rankings obtained by BestKeeper were also different from
333 those obtained by geNorm and NormFinder. The most suitable reference gene differed between
334 the five algorithms, six of the traditional reference genes (*TIP41*, *CACS*, *ARF*, *UBC*, *CYP* and
335 *PP2A*) and two novel reference genes (*LEA26* and *40SRPS8*) were identified as the optimal
336 reference genes in different samples by different software analysis in our study. the
337 comprehensive evaluation by RefFinder used data from the other four computational methods,
338 and this ranking showed that only *TIP41*, *CACS*, *TUA* and *LEA26* were the most suitable
339 reference genes in different samples of cucumber, pumpkin, and cucumber–pumpkin grafted
340 plants.
341 *TIP41* is a tonoplast intrinsic protein that functions as a *PPA2* activator in plants, and has been
342 identified as the most suitable reference gene in *Cucumis sativus*(Wan et al., 2010),
343 *Cichoriumintybus*(Delporte et al., 2015), and *Papaver rhoeas*(Scarabel et al., 2017). In our study,
344 *TIP41* was regarded as one of the most stable reference genes in cucumber, pumpkin, and at the
345 graft union of different varieties of grafted cucumber plants. But for the Gos samples, the *TIP41*
346 were ranks as the relative lower stable (*Table 2*), this indicated the normal grafted plants is
347 different from scion and rootstock in molecular levels.Surprisingly, *TIP41* was ranked as the
348 least stable reference gene in the graft union of cucumber–pumpkin grafted plants at low
349 temperatures. Reference gene stability can vary under different experimental treatments (Bustin
350 et al., 2005).Reid et al (2006) showed that *TIP41* is an inadequate reference gene during berry
351 development. Similarly, *TUA* was regarded as the most stable reference gene in the graft union
352 under cold stress, while it was also the least suitable reference gene in pumpkin organs by
353 RefFinder analysis(*Table 2*). In cucumber, *TUA* was considered a highly stable gene when
354 different cucumber tissues were treated with abscisic acid, salicylic acid, and methyl jasmonic
355 acid (Wan et al., 2010), however, *TUA* also had some limitations as a stable reference gene in
356 cucumber under conditions of salt, osmotic stress, and high or low temperature (Wan et al.,

357 2010; Migocka and Papierniak 2011). *CACS* encodes the clathrin adaptor complex subunit which
358 links clathrin to receptors in vesicles(Migocka and Papierniak 2011). As this gene participates in
359 a basic intracellular transport process, *CACS* has been recommended as an optimal reference
360 gene at different developmental stages and under varying environmental conditions in
361 *Arabidopsis thaliana* (Czechowski et al., 2005), buckwheat (*Fagopyrumesculentum*)
362 (Demidenko et al. 2011), and Lettuce (*Lactuca sativa*)(Borowski et al., 2014). In cucumber,
363 *CACS* was ranked as the best reference gene under different nitrogen nutrition conditions
364 (Warzybok and Migocka 2013), heavy metal stress, and on deprivation and/or readdition of
365 different nutrients (N, C, P and S) (Migocka and Papierniak 2011). Additionally, a novel
366 reference gene, *LEA26* (*Late Embryogenesis Abundant protein 26*)*,*is not currently regarded as a
367 reference gene in any species, and *LEA26* protein is related to abiotic stress tolerance, especially
368 desiccation tolerance in *Arabidopsis* (Dang et al., 2014). In our study, *LEA26*was recommended
369 as the most stable reference gene in theGgs. However, *LEA26* was also identified as the lowest
370 stable in the GLgs samples by BestKeeper analysis and relative lower stable in the Pos sample.
371 The all results also showed it was very necessary to validatereliable reference genes prior toqRT-
372 PCR analysis under detailed experimental conditions.
373 To validate the availability of the identified reference genes, the expression levels of *csaRUL*,
374 *csaCYCD3;1*,*cmoRU*L, and *cmoPIN* in the cucumber-pumpkin graft union healing process were
375 normalized by the two most stable reference genes and the lowest stable gene. The results
376 showed that *LEA26*and *ARF*may be the best candidate reference gene for the normalization of
377 gene expression in the graft union healing process, and the use of inappropriate reference genes
378 may lead to inaccurate results, hence it is extremely important to identity suitable reference
379 genes for making sure the reliable qRT-PCR data for target gene expression.

380 **Conclusions**

381 Grafting also assemble desirable roots and shoots to generate chimeras that are more vigorous,
382 more pathogen resistant, and more abiotic stress resistant (Melnyk, 2017). To our knowledge,
383 cucumber, pumpkin and their grafted plants weresimultaneously used as samples for the first
384 time to identify the optimal candidate reference genes in our study. 12 candidate reference genes
385 were validated in different organs, conditions, species of cucumber, pumpkin and their grafted
386 plants using five software tools-ΔCt method, BestKeeper, NormFinder, geNorm and RefFinder.
387 The results showed that *TIP41* and *CACS* had the most stable expression in different cucumber
388 organs, *TIP41* and *PP2A* were the optimal reference genes in pumpkin organs, and *CACS* and
389 *40SRPS8* were also the most stable in all grafted cucumber samples. This work will be helpful in
390 future studies on gene function and molecular mechanisms in cucumber–pumpkin grafted plants
391 and other closely related species.

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395 ## References

396 **Ahn, S. J., Im, Y. J., Chung, G. C., Cho, B. H., and Suh, S. R.** (1999). Physiological
397 responses of grafted-cucumber leaves and rootstock roots affected by low root
398 temperature. *Scientia Horticulturae* 81, 397-408. Doi:10.1016/S0304-4238(99)00042-4.
399 **Andersen, C. L., Jensen, J. L., Ørntoft, T. F.** (2004). Normalization of real-time quantitative
400 reverse transcription-PCR data: a model-based variance estimation approach to identify genes
401 suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research* 64,
402 5245-5250. Doi:10.1158/0008-5472.CAN-04-0496.
403 **Berg, J. A., Appiano, M., Martínez, M. S., Hermans, F. W., Vriezen, W. H., Visser, R. G.,**
404 **Bai, L. Y., Schouten, H. J.** (2015). A transposable element insertion in the susceptibility gene
405 *CsaMLO8* results in hypocotyl resistance to powdery mildew in cucumber. *BMC Plant*
406 *Biology* 15, 243. Doi:10.1186/s12870-015-0635-x.
407 **Borowski, J. M., Galli, V., da Silva Messias, R., Perin, E. C., Buss, J. H., e Silva, S. D. D. A.,**
408 **Rombaldi, C. V.** (2014). Selection of candidate reference genes for real-time PCR studies in
409 lettuce under abiotic stresses. *Planta* 239(6), 1187-1200.Doi:10.1007/s00425-014-2041-2.
410 **Bustin, S. A**. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-
411 PCR): trends and problems. *Journal Molecular Endocrinology* 29, 23-
412 39.Doi:10.1677/jme.0.0290023.
413 **Bustin, S. A., Benes, V., Nolan, T., and Pfaffl, M. W.** (2005). Quantitative real-time RT-PCR–
414 a perspective. *Journal of Molecular Endocrinology* 34, 597-601. Doi: 10.1677/jme.1.01755.
415 **Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M.,**
416 **Vandesompele, J.** (2009). The MIQE Guidelines: Minimum Information for Publication of
417 Quantitative Real-Time PCR Experiments.*Clinical chemistry* 55, 611-
418 622.Doi:10.1373/clinchem.
419 **Cao, H., Wang, L., Nawaz, M. A., Niu, M., Sun, J., Xie, J., Kong, Q., Huang, Y., Cheng, F.,**
420 **Bie, Z.** (2017). Ectopic expression of pumpkin nac transcription factor cmnac1 improves
421 multiple abiotic stress tolerance in Arabidopsis. *Frontiers in Plant Science* 8, 2052.
422 Doi:10.3389/fpls.2017.02052.
423 **Melnyk, C. W.** (2017). Connecting the plant vasculature to friend or foe. *New*
424 *Phytologist*, 213(4), 1611-1617.Doi:10.1111/nph.14218.
425 **Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., Scheible, W. R.** (2005). Genome-
426 wide identification and testing of superior reference genes for transcript normalization in
427 Arabidopsis. *Plant Physiology* 139, 5-17. Doi:10.1104/pp.105.063743.
428 **Dang, N. X., Popova, A. V., Hundertmark, M., and Hincha, D. K.** (2014). Functional
429 characterization of selected LEA proteins from Arabidopsis thaliana in yeast and in
430 vitro. *Planta*240, 325-336. Doi:10.1007/s00425-014-2089-z.
431 **De Jonge, H. J., Fehrmann, R. S., de Bont, E. S., Hofstra, R. M., Gerbens, F., Kamps, W.**
432 **A., De Vries, E. G., Van der Zee, A. G., TeMeerman, G. J., ArjaterElst.** (2007). Evidence
433 based selection of housekeeping genes. *PLoS ONE* 2 e898. Doi:10.1371/journal.pone.0000898.

434 **Delporte, M., Legrand, G., Hilbert, J. L., Gagneul, D.** (2015). Selection and validation of
435 reference genes for quantitative real-time PCR analysis of gene expression in
436 Cichoriumintybus. *Frontiers in Plant Science*6, 651. Doi:10.3389/fpls.2015.00651.
437 **Demidenko, N.V., Logacheva, M. D., Penin, A. A.** (2011). Selection and validation of
438 reference genes for quantitative real-time PCR in buckwheat (Fagopyrumesculentum) based on
439 transcriptome sequence data. *PLoS ONE* 6, e19434. Doi:10.1371/journal.pone.0019434.
440 **Derveaux, S., Vandesompele, J., Hellemans, J.** (2010). How to do successful gene expression
441 analysis using real-time PCR. *Methods* 50, 227-230. Doi:10.1016/j.ymeth.2009.11.001.
442 **Duan, M., Wang, J., Zhang, X., Yang, H., Wang, H., Qiu, Y., Li, X**. (2017). Identification of
443 optimal reference genes for expression analysis in Radish (*Raphanus sativus* L.) and its relatives
444 based on expression stability. *Frontiers in Plant Science*8, 1605. Doi:10.3389/fpls.2017.01605.
445 **Guénin, S., Mauriat, M., Pelloux, J., Van Wuytswinkel, O., Bellini, C., Gutierrez, L.** (2009).
446 Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental
447 conditions-specific, validation of references. *Journal of Experimental Botany* 60, 487-493.
448 Doi:10.1093/jxb/ern305.
449 **Haroldsen, V., Szczerba, M. W., Aktas, H., Lopez, J., Odias, M. J., Chi-Ham, C. L.,**
450 **Labavitch, J. M., Bennett, A. B., Powell, A. L.** (2012). Mobility of transgenic nucleic acids and
451 proteins within grafted rootstocks for agricultural improvement. *Frontiers in Plant Science* 3, 39.
452 Doi:10.3389/fpls.2012.00039.
453 **Huang, S., Li, R., Zhang, Z., Li, L., Gu, X., Fan, W., Lucas, W.,Wang, X., Xie, B., Ni, B.,**
454 **Ren, Y., Zhu, H., Li, J., Lin, K., Jin, W., Fei, Z., Li, G., Staub, J., Kilian, A., van der**
455 **Vossen, E., Wu, Y., Guo, J., He, J., Jia, Z., Ren, Y., Tian, G., Lu, Y., Ruan, J., Qian, W.,**
456 **Wang, M., Huang, Q., Li, B., Xuan, Z., Cao, J., Wu, Z., Zhang, J., Cai, Q., Bai, Y., Zhao,**
457 **Y., Han, Y., Li, Y., Li, X., Wang, S., Shi, Q., Liu, S., Cho, W., Kim, J., Xu, Y., Heller-**
458 **Uszynska, K., Miao, H., Cheng, Z., Zhang, S., Wu, J., Yang, Y., Kang, H., Li, M., Liang, H.,**
459 **Ren, X., Shi, Z., Wen, M., Jian, M., Yang, H., Zhang, G., Yang, Z., Chen, R., Liu, S., Li, J.,**
460 **Ma, L., Liu, H., Zhou, Y., Zhao, J., Fang, X., Li, G., Fang, L., Li, Y., Liu, D., Zheng, H.,**
461 **Zhang, Y., Qin, Y., Li, Z., Yang, G., Yang, S.,Bolund, L., Kristiansen, K., Zheng, H., Li, S.,**
462 **Zhang, X., Yang, H., Wang, J., Sun, R., Zhang, B., Jiang, S., Wang, J., Du, Y., Li, S.** (2009).
463 The genome of the cucumber, *Cucumis sativus* L. *Nature Genetics*41, 1275. Doi:10.1038/ng.475.
464 **Huang, Y., Kong, Q. S., Chen, F., Bie, Z. L.** (2014). The history, current status and future
465 prospects of vegetable grafting in China. *International Symposium on Vegetable Grafting* 1086,
466 31-39. Doi:10.17660/ActaHortic.2015.1086.2.
467 **Lee, J. M., Kubota, C., Tsao, S. J., Bie, Z., Echevarria, P. H., Morra, L., Oda, M.** (2010).
468 Current status of vegetable grafting: Diffusion, grafting techniques, automation. *Scientia*
469 *Horticulturae*.127, 93-105. Doi:10.1016/j.scienta.2010.08.003.
470 **Li, H., Liu, S. S., Yi, C. Y., Wang, F., Zhou, J., Xia, X. J.,Shi, K., Zhao, Y. H., Yu, J. Q.**
471 **(2014).**Hydrogen peroxide mediates abscisic acid-induced HSP 70 accumulation and heat
472 tolerance in grafted cucumber plants. *Plant and Cell Environment* 37, 2768-2780.
473 Doi:10.1111/pce.12360.

474 **Migocka, M., Papierniak, A.** (2011). Identification of suitable reference genes for studying
475 gene expression in cucumber plants subjected to abiotic stress and growth regulators. *Molecular*
476 *Breeding*. 28, 343-357. Doi:10.1007/s11032-010-9487-0.
477 **Miao, L., Li, S.H., Bai, L.Q., Ali, A., Li, Y.S., He, C.X., Yu, X.C.** (2019). Effect of grafting
478 methods on physiological change of graft union formation in cucumber grafted onto bottle gourd
479 rootstock. *Scientia Horticulturae* 26, 249-256.Doi:10.1016/j.scienta.2018.09.061.
480 **Mohamed, F.H., El-Hamed, K.E.A., Elwan, M.W.M., Hussien, M.N.E.** (2014). Evaluation of
481 different grafting methods and rootstocks in watermelon grown in Egypt. *ScientiaHorticulturae.*
482 168 (3), 145-150.Doi:10.1016/j.scienta.2014.01.029.
483 **Niu, X., Chen, M., Huang, X., Chen, H., Tao, A., Xu, J., Qi, J.** (2017). Reference gene
484 selection for qRT-PCR normalization analysis in kenaf (Hibiscus cannabinus L.) under abiotic
485 stress and hormonal stimuli. *Frontiers in Plant Science*8, 771. Doi:10.3389/fpls.2017.00771
486 **Nolan, T., Hands, R. E., Bustin, S. A.** (2006). Quantification of mRNA using real-time RT-
487 PCR. *Nature Protocols* 1, 1559. Doi:10.1038/nprot.2006.236.
488 **Obrero, Á., Die, J. V., Román, B., Gómez, P., Nadal, S., González-Verdejo, C. I.** (2011).
489 Selection of reference genes for gene expression studies in zucchini (*Cucurbita pepo*) using
490 qPCR. *Journal of Agricutural Food Chemistry* 59, 5402-5411. Doi:10.1021/jf200689r.
491 **Pfaffl, M. W., Tichopad, A., Prgomet, C., Neuvians, T. P.** (2004). Determination of stable
492 housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–
493 Excel-based tool using pair-wise correlations. *Biotechnology letters* 26, 509-515.
494 Doi:10.1023/B:BILE.0000019559.84305.47.
495 **Reda, M., Golicka, A., Kabała, K., Janicka, M.** (2018). Involvement of NR and PM-NR in NO
496 biosynthesis in cucumber plants subjected to salt stress. *Plant Science*. 267, 55-64.
497 Doi:10.1016/j.plantsci.2017.11.004.
498 **Reid, K. E., Olsson, N., Schlosser, J., Peng, F., Lund, S. T.** (2006). An optimized grapevine
499 RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR
500 during berry development. *BMC Plant Boilogy 6*, 27. Doi:10.1186/1471-2229-6-27.
501 **Saddhe, A. A., Malvankar, M. R., Kumar, K.** (2018). Selection of reference genes for
502 quantitative real-time PCR analysis in halophytic plant *Rhizophoraapiculata*. *PeerJ* 6,
503 e5226.Doi:10.7717/peerj.5226.
504 **Scarabel, L., Milani, A., Panozzo, S., Rasori, A.** (2017). Suitable reference genes for accurate
505 gene expression analysis in Papaver rhoeas under 2, 4-D herbicide stress. *Pesticide biochemistry*
506 *and physiology*, 143, 66-72.Doi:10.1016/j.pestbp.2017.09.008.
507 **Sgamma, T., Pape, J., Massiah, A., Jackson, S.** (2016). Selection of reference genes for diurnal
508 and developmental time-course real-time PCR expression analyses in lettuce. Plant Methods, 12,
509 21. Doi:10.1186/s13007-016-0121-y.
510 **Silver, N., Best, S., Jiang, J., Thein, S. L.** (2006). Selection of housekeeping genes for gene
511 expression studies in human reticulocytes using real-time PCR. *BMC Molecular Biology* 7, 33.
512 Doi:10.1186/1471-2199-7-33.

513 **Sun, H., Wu, S., Zhang, G., Jiao, C., Guo, S., Ren, Y., Zhang, J., Zhang, H.,Gong , G., Jia,**
514 **Z., Zhang, F., Tian, J.,Lucas, W., Doyle, J., Li, H., Fei, Z., Xu, Y.** (2017). Karyotype stability
515 and unbiased fractionation in the paleo-allotetraploid Cucurbita genomes. *Molecular Plant* 10,
516 1293-1306. Doi:10.1016/j.molp.2017.09.003.
517 **Tashiro, R. M., Philips, J. G., Winefield, C. S.** (2016). Identification of suitable grapevine
518 reference genes for qRT-PCR derived from heterologous species. *Molecular Genetics and*
519 *Genomics*, 291(1), 483-492.Doi:10.1007/s00438-015-1081-z.
520 **Van, H., Zhao, Z., Qian, C., Sui, Y., Malik, A. A., Chen, J.** (2010). Selection of appropriate
521 reference genes for gene expression studies by quantitative real-time polymerase chain reaction
522 in cucumber. *Analytical Biochemitry* 399, 257-261. Doi:10.1016/j.ab.2009.12.008.
523 **Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A.,**
524 **Speleman, F.** (2002). Accurate normalization of real-time quantitative RT-PCR data by
525 geometric averaging of multiple internal control genes. *Genome Biology* 3, research0034-1.
526 Doi:10.1186/gb-2002-3-7-research0034.
527 **Warzybok, A., Migocka, M.** (2013). Reliable reference genes for normalization of gene
528 expression in cucumber grown under different nitrogen nutrition. *PLoS ONE* 8, e72887.
529 Doi:10.1371/journal.pone.0072887.
530

Figure 1(on next page)

Graft union of cucumber-pumpkin grafted plants. Red box indicates graft union of cucumber-pumpkin grafted plant, and the upper is scion-cucumber, the lower part is rootstock-pumpkin.

A cucumber cultivar (*Zhongnong No.26*)was used as the scion, a pumpkin cultivar (*Jinxinzhen No.5*) was used as the rootstock. Graft union of cucumber-pumpkin grafted plants 20d after grafting. Red box indicates graft union of cucumber-pumpkin grafted plant, and the upper is scion-cucumber, the lower part is rootstock-pumpkin.

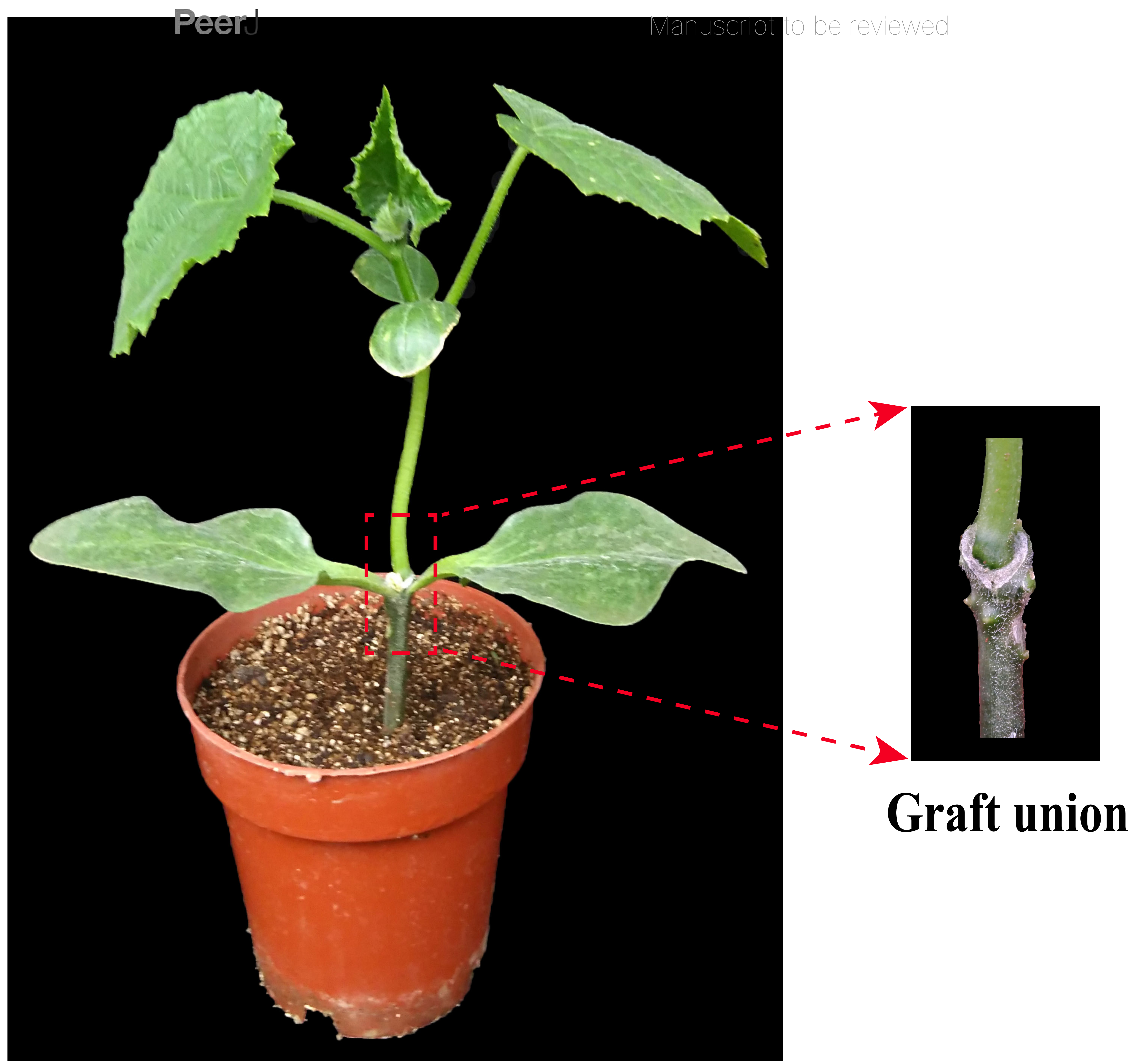
Graft union
Cucumber/pumpkin

Figure 2(on next page)

Ct values of 12 candidate reference genes from the qRT-PCR analysis in all samples. Boxplots show the 25th and 75th percentiles, means, and outliers.

For each reference gene, the line inside the box is the means. The top and bottom line of the box are 75th and 25th percentiles. The circles above or below the box are outliers.

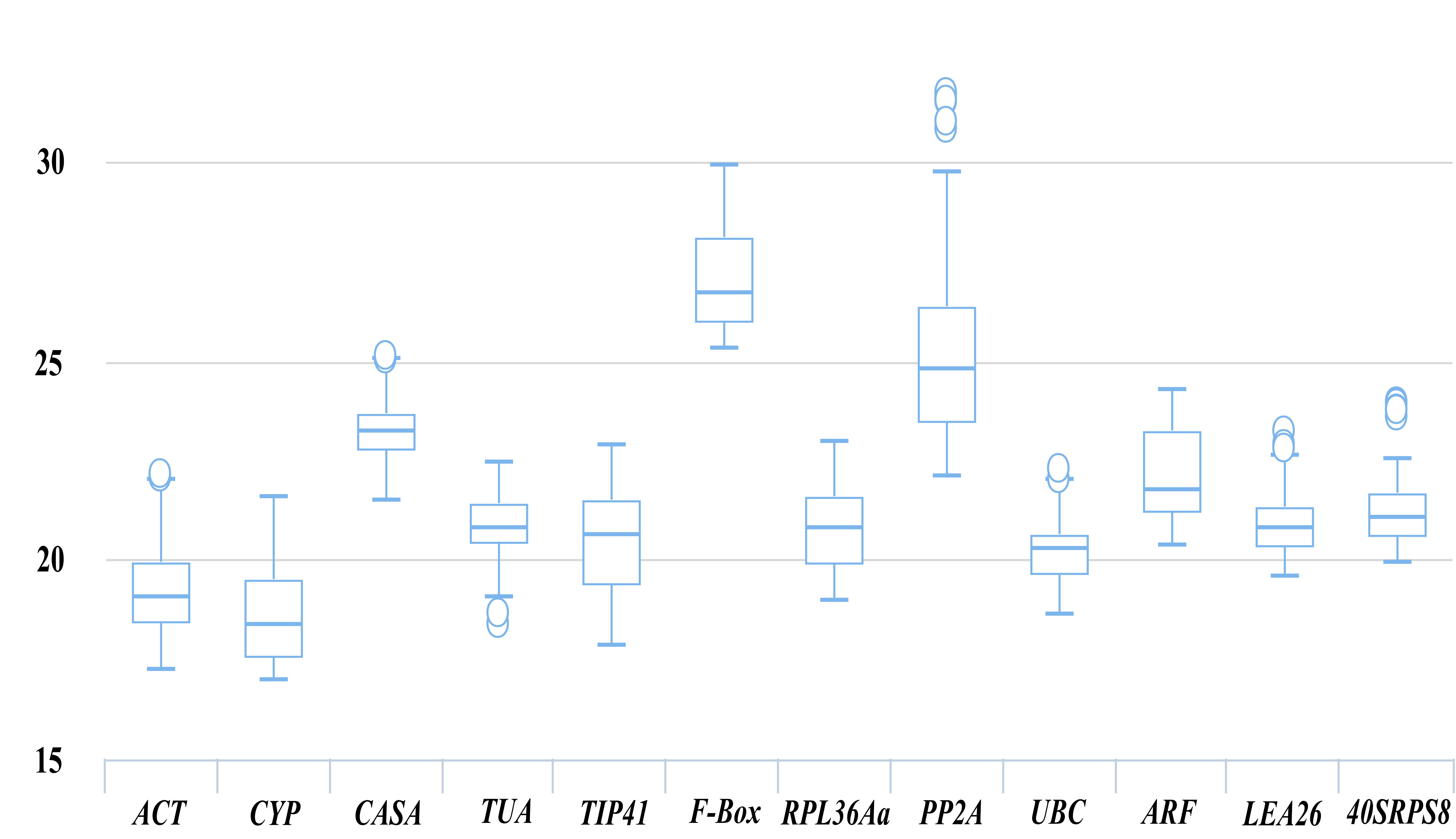
35
30
25
20
15
Ct Values
ACT
CYP
CASA
TUA
TIP41
F-Box
RPL36Aa
PP2A
UBC
ARF
LEA26
40SRPS8

Figure 3(on next page)

Determination of the optimal number of reference genes. Pairwise variation Vn/Vn+1 values caculated by geNorm software. A cut-off of 0.15 (Vn value) is usually applied.

V1 to V12 stand for the variation in candidate reference genes ranked based on their stability, which V1 is the variation for the most stable and V12 is the least stable gene. Cos: organs of cucumber; Pos: organs of pumpkin; Gos: organs of cucumber-pumpkin; GLgs: graft union of cucumber-pumpkin under low temperature stress; Ggs: graft union of cucumber-pumpkin in healing process; Ggvs: graft union of different varieties of cucumber-pumpkin; GosAll: all grafted cucumber samples; All, all samples.

$V_{2/3}$ $V_{3/4}$ $V_{4/5}$ $V_{5/6}$ $V_{6/7}$ $V_{7/8}$ $V_{8/9}$ $V_{9/10}$ $V_{10/11}$ $V_{11/12}$

Pairwise Variation(V)

0.25
0.2
0.15
0.1
0.05
0

Cos Pos Gos GLgs Ggs Ggvs GoAll All

Figure 4 (on next page)

Relative expression levels of *csaCYCD3;1*(A), *cmoPIN* (B), *csaRUL*(C), *cmoRUL*(D) using different reference genes at the graft union at 0, 1, 3, 6, 9, 15d after grafting.

The two most suitable reference genes (*LEA*, *ARF*), their combination (*LEA26+ARF*), and the least stable reference gene (*PP2A*) by RefFinder analysis were used for expression normalization. Bars represent the means and standard deviations of three biological replicates.

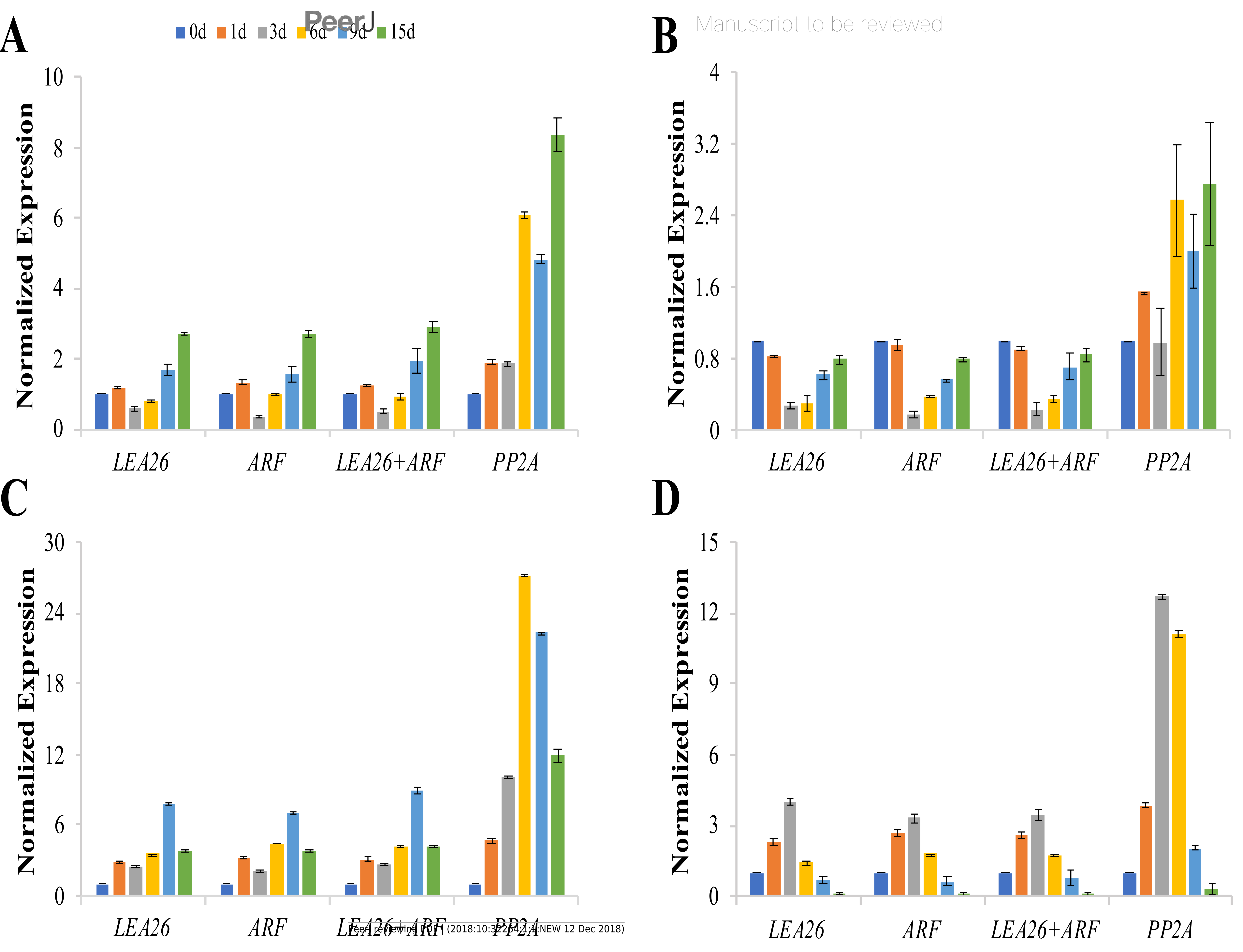
A
B
C
D
0d 1d 3d 6d 9d 15d
Normalized Expression
LEA26
ARF
LEA26+ARF
PP2A

Table 1(on next page)

Description of the candidate references, primer sequences and RT-PCR amplification efficiencies in cucumber, pumpkin, and grafted cucumber/pumpkin.

Description of the candidate references, primer sequences and RT-PCR amplification efficiencies in cucumber, pumpkin, and grafted cucumber/pumpkin.

1 **Table 1 Description of the candidate references, primer sequences and RT-PCR amplification efficiencies in cucumber, pumpkin, and grafted**
2 **cucumber/pumpkin.**

Gene	Accession number (NCBI)	Annotation	Gene ID in cucumber	Forward primer (5'-3')	Reverser primer (5'-3')	Amplification length	Tm(℃)	RT-qPCR efficiency		
								Cucumber	Pumpkin	Cucumber/pumpkin
ACT	AB010922	Actin (ACT)	Csa6G484600	TCTCCGTTTGGACCTTGC	ATTTCCCGTTCGGCAGT	99	83.2	0.88	1.05	0.86
CYP	AY942800	Cyclophilin	Csa7G009740	TTTCATGTGCCAGGGAGG	AGCCAATCGGTCTTAGCG	189	88.1	0.99	1.05	1.05
CACS	GW881874	Clathrin adaptor complex subunit (CACS)	Csa3G902930	TGGGAAGATTCTTATGAAGTGC	CTCGTCAAATTTACACATTGGT	171	84.2	1.02	0.95	1.00
TUA	AJ715498	Alpha-tubulin (TUA)	Csa4G000580	TCAGCGGCAAGGAAGATG	GCGGATTCTGTCCAAGCA	92	83.7	1.03	0.87	1.00
TIP41	GW881871	TIP41-like family protein	Csa7G071610	TGGGAGGATTGCGAGGAGA	AAGTGATATGCCATTGTCAGC	117	81.6	0.97	1.08	1.13
F-BOX	GW881870	F-box/kelch-repeat protein	Csa5G642160	TGGTTCATCTGGTGGTCTTG	TTAGCTGCCTCTGCTGATTG	131	84.3	1.08	0.93	0.90
PRL36Aa	HM594174	60S ribosomal protein L36a/L44	Csa3G653380	AAGATAGTCTTGCTGCACAGGG	AACACGGGCTTGGTTTGA	79	83.3	0.97	0.95	0.99
PP2A	HM594171	protein phosphatase 2A regulatory subunit A	Csa5G608520	GAAGCTGTAGGACCTGAACCA	AGCCGCTGCAATACGAAC	96	84.6	1.07	1.13	0.91
UBC	-	-	Csa3G358610	GTCACCATTCATTTTCCTCCG	GGGCTCCACTGCTCTTTCA	131	83.9	1.04	1.07	1.12
ARF	-	-	Csa5G524710	CTGCTGGAAAGACCACGAT	GACCACCAACATCCCATACA	132	83.5	1.02	1.12	1.03
LEA26	-	-	Csa2G151040	CGTTGACTTACCCATCACCTTC	GCGTGTAGTACCACCCTCTTTA	163	85.5	1.00	1.06	0.98
40SRPS8	-	-	Csa6G382970	ACTCGACACTGGAAACTACTCG	CCTGAACAACGGCACTCTT	134	85.1	0.87	1.03	1.01

3
4
5

Table 2 (on next page)

Overall ranking of the candidate reference genes in eight groups by ΔCtmethod, BestKeeper, NormFinder, geNorm, and RefFinder.

Overall ranking of the candidate reference genes in eight groups by ΔCtmethod, BestKeeper, NormFinder, geNorm, and RefFinder.

1 **Table 2 Overall ranking of the candidate reference genes in eight groups by ΔCt method, BestKeeper, NormFinder, geNorm, and RefFinder.**

Method	1	2	3	4	5	6	7	8	9	10	11	12
Ranking Order of candidate reference genes in different organs of cucumber plants (Better--Good--Average)												
Delta CT	*TIP41*	*CACS*	*40SRPS8*	*TUA*	*PP2A*	*CYP*	*UBC*	*RPL36Aa*	*ACT*	*ARF*	*LEA26*	F-Box
BestKeeper	*CACS*	*TIP41*	*40SRPS8*	*PP2A*	*TUA*	*RPL36Aa*	*CYP*	*ARF*	*F-Box*	*ACT*	*LEA26*	*UBC*
Normfinder	*TIP41*	*CACS*	*40SRPS8*	*PP2A*	*TUA*	*CYP*	*ARF*	*RPL36Aa*	*UBC*	*ACT*	*LEA26*	*F-Box*
geNorm	*TIP41 | 40SRPS8*		*CACS*	*TUA*	*PP2A*	*CYP*	*UBC*	*ACT*	*ARF*	*LEA26*	*RPL36Aa*	*F-Box*
Recommended comprehensive ranking	*TIP41*	*CACS*	*40SRPS8*	*PP2A*	*TUA*	*CYP*	*RPL36Aa*	*ARF*	*UBC*	*ACT*	*LEA26*	*F-Box*
Ranking Order of candidate reference genes in different organs of pumpkin plants (Better--Good--Average)												
Delta CT	*TIP41*	*PP2A*	*UBC*	*F-Box*	*ARF*	*CACS*	*CYP*	*ACT*	*40SRPS8*	*RPL36Aa*	*LEA26*	*TUA*
BestKeeper	*TIP41*	*UBC*	*PP2A*	*F-Box*	*ARF*	*CYP*	*ACT*	*CACS*	*LEA26*	*40SRPS8*	*RPL36Aa*	*TUA*
Normfinder	*TIP41*	*PP2A*	*UBC*	*F-Box*	*ARF*	*CYP*	*ACT*	*CACS*	*40SRPS8*	*RPL36Aa*	*LEA26*	*TUA*
geNorm	*CACS | 40SRPS8*		*RPL36Aa*	*PP2A*	*TIP41*	*UBC*	*ACT*	*CYP*	*F-Box*	*ARF*	*LEA26*	*TUA*
Recommended comprehensive ranking	*TIP41*	*PP2A*	*UBC*	*CACS*	*F-Box*	*40SRPS8*	*ARF*	*CYP*	*ACT*	*RPL36Aa*	*LEA26*	*TUA*
Ranking Order of candidate reference genes in different organs of cucumber/pumpkin grafted plants (Better--Good--Average)												
Delta CT	*CACS*	*40SRPS8*	*ARF*	*CYP*	*TUA*	*RPL36Aa*	*UBC*	*TIP41*	*LEA26*	*F-Box*	*ACT*	*PP2A*
BestKeeper	*CYP*	*RPL36Aa*	*40SRPS8*	*ARF*	*CACS*	*LEA26*	*UBC*	*TUA*	*ACT*	*TIP41*	*F-Box*	*PP2A*
Normfinder	*40SRPS8*	*CACS*	*ARF*	*TUA*	*CYP*	*RPL36Aa*	*TIP41*	*F-Box*	*UBC*	*LEA26*	*ACT*	*PP2A*
geNorm	*CACS | ARF*		*40SRPS8*	*CYP*	*RPL36Aa*	*TUA*	*UBC*	*LEA26*	*ACT*	*TIP41*	*F-Box*	*PP2A*
Recommended comprehensive ranking	*CACS*	*40SRPS8*	*ARF*	*CYP*	*RPL36Aa*	*TUA*	*UBC*	*LEA26*	*TIP41*	*F-Box*	*ACT*	*PP2A*
Ranking Order of candidate reference genes in graft union of cucumber/pumpkin plants under low temperature (Better--Good--Average)												
Delta CT	*TUA*	*CACS*	*RPL36Aa*	*F-Box*	*40SRPS8*	*CYP*	*ARF*	*ACT*	*UBC*	*LEA26*	*PP2A*	*TIP41*
BestKeeper	*TUA*	*RPL36Aa*	*CACS*	*CYP*	*40SRPS8*	*F-Box*	*ACT*	*ARF*	*UBC*	*PP2A*	*TIP41*	*LEA26*
Normfinder	*TUA*	*CACS*	*RPL36Aa*	*F-Box*	*40SRPS8*	*ARF*	*ACT*	*CYP*	*UBC*	*LEA26*	*PP2A*	*TIP41*
geNorm	*CYP | UBC*		*40SRPS8*	*RPL36Aa*	*TUA*	*ACT*	*CACS*	*F-Box*	*ARF*	*LEA26*	*PP2A*	*TIP41*
Recommended comprehensive ranking	*TUA*	*RPL36Aa*	*CACS*	*CYP*	*40SRPS8*	*UBC*	*F-Box*	*ACT*	*ARF*	*LEA26*	*PP2A*	*TIP41*
Ranking Order of candidate reference genes in graft union during healing process (Better--Good--Average)												
Delta CT	*LEA26*	*F-Box*	*TIP41*	*40SRPS8*	*RPL36Aa*	*ARF*	*UBC*	*CACS*	*TUA*	*ACT*	*PP2A*	*CYP*
BestKeeper	*ARF*	*TIP41*	*F-Box*	*40SRPS8*	*RPL36Aa*	*ACT*	*LEA26*	*UBC*	*CYP*	*CACS*	*TUA*	*PP2A*
Normfinder	*LEA26*	*F-Box*	*40SRPS8*	*TIP41*	*RPL36Aa*	*ARF*	*UBC*	*CACS*	*TUA*	*ACT*	*PP2A*	*CYP*
geNorm	*UBC | ARF*		*F-Box*	*LEA26*	*TIP41*	*RPL36Aa*	*40SRPS8*	*CACS*	*TUA*	*ACT*	*PP2A*	*CYP*
Recommended comprehensive ranking	*LEA26*	*ARF*	*F-Box*	*TIP41*	*40SRPS8*	*UBC*	*RPL36Aa*	*CACS*	*ACT*	*TUA*	*CYP*	*PP2A*
Ranking Order of candidate reference genes in graft union of different varities of grafted plants (Better--Good--Average)												
Delta CT	*TIP41*	*PP2A*	*UBC*	*ARF*	*40SRPS8*	*RPL36Aa*	*LEA26*	*CACS*	*ACT*	*TUA*	*F-Box*	*CYP*
BestKeeper	*TIP41*	*LEA26*	*PP2A*	*ARF*	*UBC*	*RPL36Aa*	*ACT*	*40SRPS8*	*CACS*	*F-Box*	*TUA*	*CYP*
Normfinder	*TIP41*	*UBC*	*PP2A*	*40SRPS8*	*RPL36Aa*	*ARF*	*LEA26*	*ACT*	*CACS*	*TUA*	*F-Box*	*CYP*
geNorm	*PP2A | ARF*		*TIP41*	*40SRPS8*	*RPL36Aa*	*UBC*	*CACS*	*LEA26*	*ACT*	*F-Box*	*TUA*	*CYP*
Recommended comprehensive ranking	*TIP41*	*PP2A*	*ARF*	*UBC*	*40SRPS8*	*LEA26*	*RPL36Aa*	*CACS*	*ACT*	*F-Box*	*TUA*	*CYP*
Ranking Order of candidate reference genes in all samples in grafted cucumber/pumpkin plants (Better--Good--Average)												
Delta CT	*CACS*	*40SRPS8*	*LEA26*	*UBC*	*ARF*	*TUA*	*F-Box*	*ACT*	*RPL36Aa*	*TIP41*	*CYP*	*PP2A*
BestKeeper	*CACS*	*LEA26*	*40SRPS8*	*TUA*	*UBC*	*RPL36Aa*	*ARF*	*ACT*	*F-Box*	*CYP*	*TIP41*	*PP2A*
Normfinder	*CACS*	*40SRPS8*	*UBC*	*TUA*	*LEA26*	*ARF*	*F-Box*	*ACT*	*RPL36Aa*	*TIP41*	*CYP*	*PP2A*
geNorm	*ARF | 40SRPS8*		*CACS*	*LEA26*	*TUA*	*UBC*	*ACT*	*F-Box*	*TIP41*	*RPL36Aa*	*CYP*	*PP2A*
Recommended comprehensive ranking	*CACS*	*40SRPS8*	*LEA26*	*ARF*	*UBC*	*TUA*	*F-Box*	*ACT*	*RPL36Aa*	*TIP41*	*CYP*	*PP2A*
Ranking Order of candidate reference genes in all samples (Better--Good--Average)												
Delta CT	*CACS*	*40SRPS8*	*ARF*	*UBC*	*TUA*	*LEA26*	*F-Box*	*ACT*	*TIP41*	*RPL36Aa*	*CYP*	*PP2A*
BestKeeper	*LEA26*	*CACS*	*UBC*	*TUA*	*40SRPS8*	*RPL36Aa*	*CYP*	*ARF*	*ACT*	*TIP41*	*F-Box*	*PP2A*
Normfinder	*CACS*	*40SRPS8*	*ARF*	*TUA*	*F-Box*	*UBC*	*LEA26*	*ACT*	*TIP41*	*RPL36Aa*	*CYP*	*PP2A*
geNorm	*CACS | 40SRPS8*		*ARF*	*LEA26*	*UBC*	*TUA*	*ACT*	*F-Box*	*TIP41*	*RPL36Aa*	*CYP*	*PP2A*
Recommended comprehensive ranking	*CACS*	*40SRPS8*	*LEA26*	*ARF*	*UBC*	*TUA*	*F-Box*	*ACT*	*RPL36Aa*	*TIP41*	*CYP*	*PP2A*

2

Rebuttal letter

Reviewer 1 (Anonymous)
Basic reporting
Well done.
Minor:
Lines 58/59: Formatting error
Revise：Formatting had been in corrected way.

Line 112: Please add the authors for all species you mention in the complete text not only for Cucumis sativus.
Formatting of the of the reference genes (in Italics) has to be carefully checked in the complete manuscript. There are more formatting issues that need to be carefully corrected.
Revise: pumpkin (*Cucurbita moschata*) -------- pumpkin (*Cucurbita moschata* Duch.)
I have carefully checked the formatting of the reference genes in the complete manuscript and revised them. And I also checked carefully other formatting issues in the total manuscript and revised them.

Reference in line 486 is not in alphabetical order. There is a disproportionally high number of references published by Asian/Chinese authors. There are many more relevant publications from authors from all over the world. Please carefully address this point.
Revise: All references have been listed in alphabetical order. I have checked all references in my manuscript, and adjust or replace them because the content is partly rewritten. At last, total 40 reference articles are cited, 9 references published by Chinese authors, 3 references published by Korea authors, 1 from Japan, 1 from Indian, the rest of references published by not Asian authors. When citing these references, I select them basing on content, not the authors' country, so it is hard to keep a reasonable geographical proportion to cite.

Experimental design
Abstract
The abstract provides all information in a condensed way.

Introduction
In the introduction many studies mentioning many putative reference genes are listed. What could be added is a more detailed compilation of the problems

that might occur when the reference genes chosen are not experimentally verified. This aspect is still not taken into account in many publications which often leads to the uncritical application of Actin as a reference gene for many different cultivation conditions.

Revise: I have added the more detailed compilation of the problems that might occur when the refence genes chosen are not experimentally verified as follows:

For example, *ACT* as one of the most frequently used reference genes in many plants, but were the least stable in short-term treatment of cucumber with plant regulators or salt, osmotic or oxidative stress (Migocka and Papierniak, 2011), the unstable reference genes may lead to inaccurate results.

I also mentioned this problem in the introduction, as follows:

some non-specific variations can cause errors resulting in unreliability of the qRT-PCR data, such as variability in RNA quality, cDNA synthesis and concentration, PCR procedures, and efficiency of amplification (Delporte et al., 2015).

Material & Methods

Line 154/155: Please be more specific about the unpublished data. It might be more scientifically meaningful to publish the unpublished data set together with the analysis of the reference genes submitted in this manuscript.

Revise: Graft union of cucumber-pumpkin were respectively harvested at 0, 3, 6, 9 days after grafting, three biological replicates were performed for each time point. In total 18 transcriptome libraries, 132.7G raw reads were obtained, at the least 91.4% of the reads were mapped to the reference sequence, and assemble into 32852 and 47906 transcripts of cucumber and pumpkin, respectively. 20782 unigene of cucumber with the average length of 4.1kb were obtained, while 27187 unigene with average length of 4.4kb were generated. The research about the graft union healing is based on this study about reference genes, but in the reference genes study, its research content consists of different species, organs, varieties and stress treatment, not only including graft union. They have different emphases and different research scope, as you say "the uncritical application of Actin as a reference gene for many different cultivation conditions" should be avoided, hence it is necessary to publish independent this study about reference genes for providing theoretical support for others' study quickly and professionally.

Results

The paragraph starting in line 222 is very long and need to be structured. In additions some sentences could be written in a clearer way. Please carefully improve this paragraph for better readability.

Revise: I have rewritten the results, which is divided into 6 paragraphs, including background, the ΔCt method analysis, BestKeeper analysis, NormFinder analysis, geNorm analysis and RefFinder analysis, as follows:

Expression Stability Analysis of Candidate Reference Genes

To evaluate the stability of the 12 candidate reference genes in our study, the ΔCt method, geNorm, NormFinder, BestKeeper, and RefFinder were used.

The 12 candidate reference genes were divided into eight groups in different treatments: organs of cucumber, pumpkin, and cucumber–pumpkin grafted plants under normal conditions were termed Cos, Pos, and Gos, respectively. Graft union samples of cucumber–pumpkin grafted plants under low temperatures were termed GLgs, graft union samples of cucumber–pumpkin grafted plants during the healing process were termed Ggs, graft union samples of different varieties of cucumber–pumpkin grafted plants were termed Ggvs, all cucumber–pumpkin grafted plant samples were termed GoAll, and all samples in our study were termed All.

The ΔCt method ranks the stability of expression of tested genes by comparing the relative expression of gene pairs within each sample (Silver et al., 2006). As was shown in Table 2, TIP41 were most stable reference gene in the Cos, Pos, and Ggvs samples, while TIP41 was the lowest stable reference gene in the GLgs samples. CACS were most stable reference gene in the Gos, GoAll, and All samples, TUA and LEA26 were ranked as the most stable reference genes in the GLgs and Ggs samples, respectively.

The BestKeeper program identifies potential reference genes by calculating the coefficients of variation (CVs) and the standard deviation (SD) of the Ct values, where lower CVs and SD indicate higher stability (Pfaffl et al., 2004). For the Cos and GoAll samples, CACS were identified as the most stable gene, and for the Pos and Ggvs samples, TIP41 was the most stable. CYP was the most stable gene in the Gos samples, but exhibited as the lowest ranking for the Ggvs samples. Similarly, TUA was the most stable gene in the GLgs samples, while was also the lowest stable gene in the Pos samples. ARF and LEA26 were ranked as the most stable reference gene in the Ggs and All the samples, respectively. PP2A were the lowest stable reference gene in most of samples with the BestKeeper analysis, including the Gos, Ggs, GoAll, and All samples (Table 2).

NormFinder ranks the stability of tested genes based on inter- and intragroup variations in expression across different sample groups, with lower values indicating higher stability (Andersen et al., 2004). TIP41 with the stability values of 0.084, 0.153, 0.203 was the most stable gene in the Cos, Pos, and Ggvs samples, respectively. 40SRPS8 and CACS were the two most stable genes and PP2A was the lowest stable in the Gos, GoAll, and All samples. For the GLgs samples, TUA were most stable, while it ranked as the lowest reference gene in the Pos samples. The stability of LEA26 were best in the Ggs samples according to the NormFinder analysis (Table 2).

The geNorm software determines the gene expression stability using M-values based on the average pairwise variation of all candidate genes (Vandesompele et al., 2002). TIP41and 40SRPS8, CACS and ARF, CYP and UBC, UBC and ARF, PP2A and ARF, ARF and 40SPRS8, were the two most stable genes in the Cos, Gos, GLgs, Ggs, Ggvs, and GoAll samples, respectively. CACS and 40SRPS8 were identified as the most stable reference genes with M-values of 0.093 and 0.582 respectively in the Pos and All samples. In addition, the optimal number of reference genes for normalizing the gene expression are judged by calculating the pairwise variation (Vn/Vn+1) by geNorm algorithm, and Vn/Vn+1<0.15 indicates that the optimal number of reference genes equal to the value of n to use as reference gene (Vandesompele et al., 2002). In our study, the values of V2/V3 of all experimental samples was less than 0.15, indicated that 2 reference genes would be sufficient for gene normalization under these experimental conditions (Fig. 3).

RefFinder considers the ΔCt method, geNorm, NormFinder and BestKeeper rankings to provide a comprehensive ranking of the most stable genes (Xie et al., 2012). TIP41 was the most stable reference gene in the Cos, Pos, and Ggvs samples, CACS was ranked as the most stable gene in the Gos, GoAll, and All samples. TUA was the most the stable in the GLgs samples, while it was also the lowest reference gene in the Pos samples. LEA 26 and ARF

were the two most stable reference genes in the Ggs samples. PP2A was the lowest stable reference gene in the Gos, Ggs, GoAll and All samples according the RefFinder analysis (Table 2).

Discussion

Part of the discussion is a repetition of aspects mentioned already in the other parts. Please try to reduce and be more specific with respect to your data.

Revise: I also try my best to reduce the repetition parts and be more specific with respect to my data, but some parts are necessary in the discussion. The red parts as follows:

qRT-PCR is the most powerful method for detecting transcriptomic data and studying the underlying molecular mechanisms (Niu et al., 2017). Appropriate reference genes are required to ensure the accuracy of the qRT-PCR results. There has recently been research into the selection of optimal reference genes in cucumber and pumpkin (Wan et al., 2010; Obrero et al., 2011; Warzybock and Migocka, 2013), however, there have been no studies on the selection of the optimal reference genes for cucumber–pumpkin grafted plants. Grafting assembles the scion and rootstock into a plant that often have a massive advantage over their parents, there were substances exchange between scion and rootstock, including water, sugars, hormones, RNAs and proteins (Melnyk, 2017), so research into cucumber–pumpkin grafted plants is necessary to identify the optimal reference genes. Therefore, we selected some published traditional reference genes (*ACT*, *CYP*, *CACS*, *TUA*, *TIP41*, *F-Box*, *PRL36Aa* and *PP2A*) that are expressed in cucumber or pumpkin. We also selected four novel genes (*UBC*, *ARF*, *LEA26* and *40SRPS8*) from our transcriptomic data on graft union healing in cucumber–pumpkin grafted plants, and primers were designed based on the conserved sequence of the genes between cucumber and pumpkin.

The ΔCt method, BestKeeper, NormFinder, geNorm, and RefFinder are five software programs and methods that are commonly used for identifying reference genes (Scarabel et al., 2017; Duan et al., 2017). In our study, two genes were sufficient for reliable normalization when all samples were considered by geNorm analysis (Fig. 3). The ΔCt method, NormFinder, geNorm and RefFinder programs all suggested the same least suitable reference genes, differing from the rankings obtained by BestKeeper. For instance, *F-Box* was ranked as the least stable gene in Cos samples by ΔCt method, NormFinder, geNorm and RefFinder programs analysis, while BestKeeper identified *UBC* as the lowest stable in the Cos samples. This is in concordance with a study by Niu et al (2017) where the rankings obtained by BestKeeper were also different from those obtained by geNorm and NormFinder. The most suitable reference gene differed between the five algorithms, six of the traditional reference genes (*TIP41*, *CACS*, *ARF*, *UBC*, *CYP* and *PP2A*) and two novel reference genes (*LEA26* and *40SRPS8*) were identified as the optimal reference genes in different samples by different software analysis in our study. the comprehensive evaluation by RefFinder used data from the other four computational methods, and this ranking

showed that only *TIP41*, *CACS*, *TUA* and *LEA26* were the most suitable reference genes in different samples of cucumber, pumpkin, and cucumber–pumpkin grafted plants.

TIP41 is a tonoplast intrinsic protein that functions as a *PPA2* activator in plants, and has been identified as the most suitable reference gene in *Cucumis sativus* (Wan et al., 2010), *Cichorium intybus* (Delporte et al., 2015), and *Papaver rhoeas* (Scarabel et al., 2017). In our study, *TIP41* was regarded as one of the most stable reference genes in cucumber, pumpkin, and at the graft union of different varieties of grafted cucumber plants. But for the Gos samples, the *TIP41* were ranks as the relative lower stable, this indicated the normal grafted plants is different from scion and rootstock in molecular levels. Surprisingly, *TIP41* was ranked as the least stable reference gene in the graft union of cucumber–pumpkin grafted plants at low temperatures. Reference gene stability can vary under different experimental treatments (Bustin et al., 2005). Reid et al (2006) showed that *TIP41* is an inadequate reference gene during berry development. Similarly, *TUA* was regarded as the most stable reference gene in the graft union under cold stress, while it was also the least suitable reference gene in pumpkin organs by RefFinder analysis (Table 2). In cucumber, *TUA* was considered a highly stable gene when different cucumber tissues were treated with abscisic acid, salicylic acid, and methyl jasmonic acid (Wan et al., 2010), however, *TUA* also had some limitations as a stable reference gene in cucumber under conditions of salt, osmotic stress, and high or low temperature (Wan et al., 2010; Migocka and Papierniak 2011). *CACS* encodes the clathrin adaptor complex subunit which links clathrin to receptors in vesicles (Migocka and Papierniak 2011). As this gene participates in a basic intracellular transport process, *CACS* has been recommended as an optimal reference gene at different developmental stages and under varying environmental conditions in *Arabidopsis thaliana* (Czechowski et al., 2005), buckwheat (*Fagopyrum esculentum*) (Demidenko et al. 2011), and Lettuce (*Lactuca sativa*)(Borowski et al., 2014). In cucumber, *CACS* was ranked as the best reference gene under different nitrogen nutrition conditions (Warzybok and Migocka 2013), heavy metal stress, and on deprivation and/or readdition of different nutrients (N, C, P and S) (Migocka and Papierniak 2011). Additionally, a novel reference gene, *LEA26* (Late Embryogenesis Abundant protein 26), is not currently regarded as a reference gene in any species, and *LEA26* protein is related to abiotic stress tolerance, especially desiccation tolerance in *Arabidopsis* (Dang et al., 2014). In our study, *LEA26* was recommended as the most stable reference gene in the Ggs. However, *LEA26* was also identified as the lowest stable in the GLgs samples by BestKeeper analysis and relative lower stable in the Pos sample. The all results also showed it was very necessary to validate raliable reference genes prior to qRT-PCR analysis under detailed experimental conditions.

To validate the availability of the identified reference genes, the expression levels of *csaRUL*, *csaCYCD3;1*, *cmoRU*L, and *cmoPIN* in the cucumber-pumpkin graft union healing process were normalized by the two most stable reference genes and the lowest stable gene. The results showed that *LEA26* and *ARF* may be the best candidate reference gene for the normalization of gene expression in the graft union healing process, and the use of inappropriate reference genes may lead to inaccurate results, hence it is extremely important to identity suitable reference genes for making sure the reliable qRT-PCR

data for target gene expression.

Conclusions

This important part of paper needs to be more carefully arranged and completely rewritten because of incomplete sentences and repetitions.

Revise: Indeed, the conclusion is needed to rewritten, I am sorry to waste your time to review the old version, the new version as follows:

Grafting also assemble desirable roots and shoots to generate chimeras that are more vigorous, more pathogen resistant, and more abiotic stress resistant (Melnyk, 2016). To our knowledge, cucumber, pumpkin and their grafted plants were simultaneously used as samples for the first time to identify the optimal candidate reference genes in our study. 12 candidate reference genes were validated in different organs, conditions, species of cucumber, pumpkin and their grafted plants using five software tools-ΔCt method, BestKeeper, NormFinder, geNorm and RefFinder. The results showed that *TIP41* and *CACS* had the most stable expression in different cucumber organs, *TIP41* and *PP2A* were the optimal reference genes in pumpkin organs, and *CACS* and *40SRPS8* were also the most stable in all grafted cucumber samples. This work will be helpful in future studies on gene function and molecular mechanisms in cucumber–pumpkin grafted plants and other closely related species.

Supplementary

The supplemental data files need meaningful legends, best summarized in a legend file. The note in Table S1 is not clear and the sentence and the described fast hast to be described in more detail.

Revise: The legends have been revised as follows (the red):

Figure S1. Illustrations of hole insertion grafting methods process in cucumber grafted on pumpkin. A cucumber cultivar (Zhongnong No.26) was used as the scion, a pumpkin cultivar (Jinxinzhen No.5) was used as the rootstock. The rootstocks were sown 2–3 d earlier than scions (6–7 d after sowing). When cotyledons of the scion were fully opened and the first true leaf of the rootstock started to develop (9–10 d after sowing) plants grafted as previously described (Mohamed et al., 2014). A hole on the upper portion of the rootstock hypocotyls was made, and then the growing point of the rootstock were removed with a razor blade. The scion was cut on a 30˚-60˚ on both sides of the hypocotyls, then made the scion insert into the hole made in the rootstock quickly, and the cut surfaces were matched together and held with a grafting clip (*Fig S1*).

Figure S2. Amplification of single PCR product of the expected size for 12 candidate reference genes using cucumber (A), pumpkin (B), cucumber-pumpkin grafted plants. Lines: 1-10, *ACT*, *CYP*, *CACS*, *TUA*, *TIP41*, *F-Box*, *PRL36Aa*, *PP2A, UBC*, *ARF*, *LEA26*, *40SRPS8*, M represents a 2000bp DNA marker. Amplified fragments of 12 candidate genes on 1% agarose gel.

Based on the conserved sequence of these genes between cucumber and pumpkin, primers were designed using Primer Premier 5.0 software with the following parameters: a melting temperature (Tm) of 50–60°C, a primer length of 17–25 bp, and a product size of 70–260 bp

(http://www.premierbiosoft.com/) (*Table 1*). Amplification of a single PCR product in 1% agarose gel electrophoresis.

Figure S3. Melting curves of 12 candidate reference gene in cucumber (A) and pumpkin (B).

The Fluorescence changes (X-axis) was plotted versus the reaction temperature of qRT-PCR (Y-axis). A single peak of the melting curve in qRT-PCR were used to ensure the specificity of the primers for the candidate reference genes.

Figure S4. Melting curves (A) and Standard curves (B) of 12 candidate reference genes in cucumber-pumpkin grafted plants. Melting curves of 12 candidate reference gene in cucumber (A) and pumpkin (B).

The Fluorescence changes (Y-axis) was plotted versus the reaction temperature of qRT-PCR (X-axis). A single peak of the melting curve in qRT-PCR were used to ensure the specificity of the primers for the candidate reference genes (*Figure S4A*). The amplification efficiencies for each primer and the regression coefficients (R^2) were evaluated using five-fold dilutions of pooled cDNA (1/5, 1/25, 1/125, 1/625, 1/3125) that were diluted using EASY dilution solution, the Ct values changes (Y-axis) was plotted versus the initial quality of qRT-PCR (X-axis). The linear regression equation of every primer was also showed in *Figure S4B*.

Figure S5. Standard curves of 12 candidate reference genes in cucumber (A) and pumpkin (B). The amplification efficiencies for each primer and the regression coefficients (R^2) were evaluated using five-fold dilutions of pooled cDNA (1/5, 1/25, 1/125, 1/625, 1/3125) that were diluted using EASY dilution solution. The Ct values changes (Y-axis) was plotted versus the initial quality of qRT-PCR (X-axis. The linear regression equation of every primer was also showed in *Figure S5*.

Figure 1. Graft union of cucumber-pumpkin grafted plants. A cucumber cultivar (Zhongnong No.26) was used as the scion, a pumpkin cultivar (Jinxinzhen No.5) was used as the rootstock. Red box indicates graft union of cucumber-pumpkin grafted plant, and the upper is scion-cucumber, the lower part is rootstock-pumpkin.

Figure 2. Ct values of 12 candidate reference genes from the qRT-PCR analysis in all samples. Boxplots show the 25th and 75th percentiles, means, and outliers. For each reference gene, the line inside the box is the means. The top and bottom line of the box are 75th and 25th percentiles. The circles above or below the box are outliers.

Figure 3. Determination of the optimal number of reference genes. Pairwise variation Vn/Vn+1 values caculated by geNorm software. A cut-off of 0.15 (Vn value) is usually applied. V1 to V12 stand for the variation in candidate reference genes ranked based on their stability, which V1 is the variation for the most stable and V12 is the least stable gene. Cos: organs of cucumber; Pos: organs of pumpkin; Gos: organs of cucumber-pumpkin; GLgs: graft union of cucumber-pumpkin under low temperature stress; Ggs: graft union of cucumber-pumpkin in healing process; Ggvs: graft union of different varieties of cucumber-pumpkin; GosAll: all grafted cucumber samples; All, all samples.

Validity of the findings

The data are well presented and the description and conclusion based on the data are plausible and evident.

Comments for the Author

The authors carry out a very broad study investigating 12 different reference genes. There are no technical concerns on the execution of this study. However, this paper is a very technical paper as the authors also state in their cover letter. My major concern is that it could be part as a supplement of a publication that deals already with a real biological research question instead of being published by its own.

Answer: When we wanted to observe some genes expression which were important in the graft union healing process of cucumber-pumpkin grafted plants, we found there no appropriate reference genes of cucumber-pumpkin published, so we carried out this experiment to select the optimal reference genes in cucumber-pumpkin grafted plants. At the beginning, we wanted to put it as a supplement of our graft union healing research, but we decided to publish this as a independent part, the reason as follows:

Firstly, emphasis is different. Graft union healing research revealed the mechanism of healing in heterograft cucumber-pumpkin grafted plants, while the study of selecting reference genes mainly provided the stable reference gene in cucumber, pumpkin and their grafted plants under different conditions in our study. As a independent part to publish, it will help the demanders to find it easily and quickly.

Secondly, research scope is different. The study of selecting reference gene is a systematic and comprehensive research about identifying stable reference genes, it not only includes graft union healing treatment, but also includes different organs, low temperature stress, and different species.

Thirdly, we had to admit this is a very technical paper, but it also has a great application value. It would be helpful in future studies on gene function and molecular mechanisms in cucumber–pumpkin grafted plants and other closely related species. As you say “the uncritical application of Actin as a reference gene for many different cultivation conditions”, this should be avoided, so the study of the reference genes is meaningful.

Reviewer 2 (Anonymous)

Basic reporting

Li et al. reports the optimal reference gene to normalize the expression data for qRT-PCR in cucumber, pumpkin and cucumber-pumpkin grafted plants by four statistical tools. Eight candidate genes were tested under various conditions, and most constant expression genes were defined as candidate reference genes. This study is a well-organized and thorough to identify optimal reference genes. However, the current manuscript is missing several important requirements for data presentation and methodology explanation. I provide major and minor points to revise.

Experimental design

No problem.

Validity of the findings

There is no large impact but meaningful.

Comments for the Author

Li et al. reports the optimal reference gene to normalize the expression data for qRT-PCR in cucumber, pumpkin and cucumber-pumpkin grafted plants by four statistical tools. Eight candidate genes were tested under various conditions, and most constant expression genes were defined as candidate reference genes. This study is a well-organized and thorough to identify optimal reference genes. However, the current manuscript is missing several important requirements for data presentation and methodology explanation. I provide major and minor points to revise.

<Major comments>

1. In my view, I do not find any citation of Figs 3 and 4 in the text. Please remove or cite them in the text.

Revise: I have added the citation of Fig.3 and Fig.4 in the text as follows:

In our study, the values of V_2/V_3 of all experimental samples was less than 0.15, indicated that 2 reference genes would be sufficient for gene normalization under these experimental conditions (Fig.3).

The expression patterns of *csaCYCD3;1*, *csaRUL*, *cmoRUL* and *cmoPIN* showed similar changes when *LEA26*, *ARF* or *LEA26+ARF* were selected as the reference genes for normalization (Fig.4).

2. Figure titles and legends are not written in proper manner. Some parts seems to be just copied, then titles include detailed panel information and legends are missing information needed to understand all presented figure panels. Please consider to revise them.

Revise：I have revised them as your suggestion as follows:
Figure 1. Graft union of cucumber-pumpkin grafted plants. A cucumber cultivar (Zhongnong No.26) was used as the scion, a pumpkin cultivar (Jinxinzhen No.5) was used as the rootstock. Red box indicates graft union of cucumber-pumpkin grafted plant, and the upper is scion-cucumber, the lower part is rootstock-pumpkin.
Figure 2. Ct values of 12 candidate reference genes from the qRT-PCR analysis in all samples. Boxplots show the 25th and 75th percentiles, means, and outliers. For each reference gene, the line inside the box is the means. The top and bottom line of the box are 75th and 25th percentiles. The circles above or below the box are outliers.
Figure 3. Determination of the optimal number of reference genes. Pairwise variation Vn/Vn+1 values caculated by geNorm software. A cut-off of 0.15 (Vn value) is usually applied. V1 to V12 stand for the variation in candidate reference genes ranked based on their stability, which V1 is the variation for the most stable and V12 is the least stable gene. Cos: organs of cucumber; Pos: organs of pumpkin; Gos: organs of cucumber-pumpkin; GLgs: graft union of cucumber-pumpkin under low temperature stress; Ggs: graft union of cucumber-pumpkin in healing process; Ggvs: graft union of different varieties of cucumber-pumpkin; GosAll: all grafted cucumber samples; All, all samples.
Figure S1. Illustrations of hole insertion grafting methods process in cucumber grafted on pumpkin. A cucumber cultivar (Zhongnong No.26) was used as the scion, a pumpkin cultivar (Jinxinzhen No.5) was used as the rootstock. The rootstocks were sown 2–3 d earlier than scions (6–7 d after sowing). When cotyledons of the scion were fully opened and the first true leaf of the rootstock started to develop (9–10 d after sowing) plants grafted as previously described (Mohamed et al., 2014). A hole on the upper portion of the rootstock hypocotyls was made, and then the growing point of the rootstock were removed with a razor blade. The scion was cut on a 30˚-60˚ on both sides of the hypocotyls, then made the scion insert into the hole made in the rootstock quickly, and the cut surfaces were matched together and held with a grafting clip (*Fig S1*).
Figure S2. Amplification of single PCR product of the expected size for 12 candidate reference genes using cucumber (A), pumpkin (B), cucumber-pumpkin grafted plants. Lines: 1-10, *ACT*, *CYP*, *CACS*, *TUA*, *TIP41*, *F-Box*, *PRL36Aa*, *PP2A, UBC*, *ARF*, *LEA26*, *40SRPS8*, M represents a 2000bp DNA marker. Amplified fragments of 12 candidate genes on 1% agarose gel.
Based on the conserved sequence of these genes between cucumber and pumpkin, primers were designed using Primer Premier 5.0 software with the following parameters: a melting temperature (Tm) of 50–60°C, a primer length of 17–25 bp, and a product size of 70–260 bp (http://www.premierbiosoft.com/) (*Table 1*). Amplification of a single PCR product in 1% agarose gel electrophoresis.
Figure S3. Melting curves of 12 candidate reference gene in cucumber (A) and pumpkin (B).
The Fluorescence changes (X-axis) was plotted versus the reaction temperature of qRT-PCR (Y-axis). A single peak of the melting curve in qRT-PCR were used to ensure the specificity of the primers for the candidate reference genes.
Figure S4. Melting curves (A) and Standard curves (B) of 12 candidate reference genes in cucumber-pumpkin grafted plants. Melting curves of 12

candidate reference gene in cucumber (A) and pumpkin (B).
The Fluorescence changes (Y-axis) was plotted versus the reaction temperature of qRT-PCR (X-axis). A single peak of the melting curve in qRT-PCR were used to ensure the specificity of the primers for the candidate reference genes (*Figure S4A*). The amplification efficiencies for each primer and the regression coefficients (R^2) were evaluated using five-fold dilutions of pooled cDNA (1/5, 1/25, 1/125, 1/625, 1/3125) that were diluted using EASY dilution solution, the Ct values changes (Y-axis) was plotted versus the initial quality of qRT-PCR (X-axis). The linear regression equation of every primer was also showed in *Figure S4B*.

Figure S5. Standard curves of 12 candidate reference genes in cucumber (A) and pumpkin (B). The amplification efficiencies for each primer and the regression coefficients (R2) were evaluated using five-fold dilutions of pooled cDNA (1/5, 1/25, 1/125, 1/625, 1/3125) that were diluted using EASY dilution solution. The Ct values changes (Y-axis) was plotted versus the initial quality of qRT-PCR (X-axis. The linear regression equation of every primer was also showed in *Figure S5*.

3. As the current study screened available references which have stable expression patterns under several stressful conditions, the authors could consider to inform such strategy in titles and introduction section.

Revise: I add the main results in the last paragraph of introduction, maybe it increases its repeat. About the titles, I don't get your suggestion well. *CACS* and *40SRPS8* were the most stable reference genes in all samples in our research. *TIP41* and *CACS* had the most stable expression in different cucumber organs, *TIP41* and *PP2A* were the optimal reference genes in pumpkin organs, and *CACS* and *40SRPS8* were also the most stable in all grafted cucumber samples.

<Point-to-point comments>

L167; don't→do not

Revise: don't→do not

L177; H20→H2O

Revise: H20→H_2O

L186-187; This part should be explained in result section.

Answer: I have described this in the section “Expression Levels and Variations in Candidate Reference Genes” of result part.
As shown in Fig. 2, the Ct values for the 12 candidate reference genes in all samples ranged from 16.98 to 31.71, and the mean Ct values were 19.04, 18.35, 23.235, 20.795, 20.655, 26.695, 20.785, 24.785, 20.26, 21.775, 20.8 and 21.085 for *ACT, CYP, CACS, TUA, TIP41, F-Box, PRL36Aa, PP2A, UBC, ARF, LEA26* and *40SRPS8*, respectively.

L208; It is the content of the method. Please move this sentence to the method section.
Revise: I have moved this sentence to the method section.

L224–233 and L310-312; These seem to be the content of the introduction. Please remove or explain them in introduction section.
Revise: I have rewritten the discussion, L224-233 sentences could help to understand the results easily and clearly, so I keep them in the new discussion.

L346; Here, some reference is required.
Revise: I added the reference here. (Migocka and Papierniak 2011)

Gene names/transcripts should be written in italics, eg L77-78, L81-82, and L355. Please check throughout the manuscript and revise them.
Revise: I have checked throughout the manuscript and revised them.

Reviewer 3 (Johannes Fahrentrapp)

Basic reporting

Miao et al. report on the evaluation of a reference gene set for the use in cucumber, Pumkin and cucumber-pumpkin grafted plants. The experimental procedure is sound and well described in good English language (judged as non-native speaker). Results, discussion, tables, figures and supplemental

material are well written and documented. The raw data are shared. Literature is mostly documented (for missing references see below). The whole paper is well structured.
Authors refer to their own unpublished transcriptomic data (L155). This should be publishes before or along with the manuscript.

Experimental design

The experiments are sound and well described with some minor comment detailed below.

Validity of the findings

The findings are valid.

Comments for the Author

I have some comments on different passages of the text:

Title: delete "optimal". There is no proof that these are the best. Only for the current conditions, they may be the most suited reference genes.
Revise: I have deleted “optimal”.

Title (L3): include ...gene expression "data derived from" instead of gene expression in cucumber
Revise: I have revised the title as “Selection of reference genes for quantitative real-time PCR analysis in cucumber (*Cucumis sativus* L.), pumpkin

(*Cucurbita moschata* Duch.) and cucumber–pumpkin grafted plants ”

L45: delete "optimal"

Revise: replace “optimal” by “appropriate”

Introduction: Sentence and reference missing on the MIQE guidelines: Bustin SA et al. (2009) The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments Clin Chem 55:611-622 doi:10.1373/clinchem.2008.112797

Revise: I have added the missing reference in the Introduction.

L52: replace "always" by "usually"

Revise: I have replaced “always” by “usually”

L81-86: Unclear. The mentioned reference genes were used all together or one by one? Explain the "specific environments"

Revise: The mentioned reference genes were used as the most stable reference genes basing on the different treatments.

The genes *UFP* (*ubiquitin*), *EF-1A* (*elongation factor*), *PRL36aA* (*60S ribosomal protein L36a/L44*), *PP2A* (*protein phosphatase*) and *CACS* (*clathrin adaptor complexes medium submit family protein*) have provide the best strategy for reliable normalization in different experimental sets in zucchini (*Cucurbita pepo*) (Obrero et al., 2011), and these reference genes have also been successfully applied to both cucumber and pumpkin in specific environments, including powdery mildew, salinity, cold, dehydration, H_2O_2, and abscisic acid (ABA) treatment (Berg et al., 2015; Cao et al., 2017; Reda et al., 2018;).

84: italics "Cucurbita pepo"

Revise: *Cucurbita pepo*

L92-97: name and function of the mentioned genes

Answer: All the mentioned genes have been named and function briefly just at the first time occur in the manuscript, but the second time we just use their name. *ACT* (*actin*), *TUA* (*tubulin*), *CYP* (*cyclophilin*), *UBI-1* (*ubiquitin*), *EF-α* (*elongation factor*), *UFP* (*ubiquitin*), *EF-1A* (*elongation factor*), *PRL36aA* (*60S ribosomal protein L36a/L44*), *PP2A* (*protein phosphatase*), *CACS* (*clathrin adaptor complexes medium submit family protein*), *TIP41* (*tonoplast intrinsic protein*), *F-Box* (*F-box protein*), *UBC* (*Ubiquitin conjugating enzyme*), *ARF* (*ADP-ribosylation factor-like protein*), *LEA26* (*Late-embryogenesis abundant protein 26*), and *40SRPS8* (*40S ribosomal protein S8*).

L103: replace "gernerated" by "generated"

Revise: generated.

L104-107: provide reference after "...which are thought to be related to graft union healing in grafted cucumber..."

Answer: We referenced the article "Ruonala, R., Ko, D., Helariutta, Y. (2017). Genetic networks in plant vascular development. Annual review of genetics, 51, 335-359.", found CYCD3；1(At4g34160)，RUL(AT5G05160), PIN(At1g73590) play important role in the vascular development in Arabidopsis, we used hmm-search to find the domain in these gene and get the genes in cucumber which have the same domain with the parameters: --noali -E 0.01 --domE 0.01, then we gain the function related genes in cucumber through alignment with arabidopsis genes used blastp software with the filter conditions: -e 0.01, identity >=30 and coverage >=30. Finally, we found the common genes basing the same function. The same methods was used

to find pumpkin genes basing on Arabidopsis genes, we selected the gene (*csaCYCD3；1* (Csa2G356610), *csaRUL* (Csa3G895630), *cmoRUL* (CmoCh15G013320), *cmoPIN* (CmoCh15G013320), *cmoPIN* (CmoCh15G009810)) which had significant changes during the graft union healing process. The RPKM values of these genes covering transcriptomes data of graft union at the 0d , 3d, 6d, 9d after grafting are listed in Table S3.

L117: give size or volume of the pot instead of cell number

Revise： 50-cell and 32-cell polystyrene trays (54cm*28cm*5cm)

L122: ...previously described (Fig S1). Please provide reference.

Revise: Miao, L., Li, S.H., Bai, L.Q., Ali, A., Li, Y.S., He, C.X., Yu, X.C. (2018). Effect of grafting methods on physiological change of graft union formation in cucumber grafted onto bottle gourd rootstock. *Scientia Horticulturae*, 26, 249-256.

L139: replace "seedlings" by "grafted plants"

Revise: grafted plants.

L152: specify "other plants"

Revise: other plants including radish (*Raphanus sativus*) (Duan et al., 2017), chicory(*Cichorium intybus*) (Delporte et al., 2015), buckwheat (*Fagopyrum esculentum*) (Demidenko et al. 2011), and Lettuce (*Lactuca sativa*)(Borowski et al., 2014).

Why did authors not evaluate the "classic" reference genes in their transcriptomic data? This is missing.

Answer: According to our screening criteri (CV ≤ 0.2 and 300≤RPKM≤ 500), we don’t get the classic reference genes. Because the stability of reference genes depends on many factors, especially detailed samples, experimental treatments. So the classic reference genes possibly are not stable in the graft

Commented [FJ(1]: What values did they get?

union healing process, we validate this in the results of ranks order by the five software analysis in the Ggs samples (Table 2), the potential genes *LEA26*, *ARF*, *UBC* were identified as the most stable reference genes by different software analysis, but not the classic genes.

L161: BLAST against what data base?
Answer: With requirements of evalue e-5, we used BLAST to determine the proteins encoded by cucumber and pumpkin genes, respectively (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and then filtered the BLAST results based on an identity ≥ 90 and an overlap ratio > 0.5 (between query and target).

Commented [FJ(2]: There are different databases at NCBI. You should state which of them you used and what method.

L163: rephrase sentence to "This resulted in ten and seven genes of cucumber and pumpkin, respectively, which may be suitable as reference genes"
Revise：I have rephrased this sentence as your suggestion.

L168: provide reference for Primer Premier 5.0 software
Revise：Primer were designed on the website: http://www.premierbiosoft.com/

L174: replace "the" by "an"
Revise: an

L176: give manufacturer of "Premix DimerEraser"
Revise: Replace "Premix DimerEraser" by “Premix Ex Taq”, we use the SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, Dalian, China) in our study, its Code No.is RR420A.

L193: Please provide reference instead of web page

Revise: I have provided reference instead of web page (Duan et al., 2017).

L205: "eight genes used traditionally...and four potential (instead of "new") reference genes..."

Revise: I have revised as your suggestion.

L232: From here onward many abbreviations are used. These should be limited and better explained.

Revise: I have rewritten the results, and tried to avoid these abbreviations, and some abbreviations were necessary.

L241: Explanation of V2/3 value missing. Please add.

Revise：I have added the sentence “the optimal number of reference genes for normalizing the gene expression are judged by calculating the pairwise variation (Vn/Vn+1) by geNorm algorithm, and Vn/Vn+1<0.15 indicates that the optimal number of reference genes equal to the value of n to use as reference gene (Vandesompele et al., 2002)”

L222: The whole section should be shortened and summarized with a table if possible.

Revise: I have rewritten the results, and divided it into 6 parts, including background, the ΔCt method analysis, BestKeeper analysis, NormFinder analysis, geNorm analysis and RefFinder analysis.

L299 & 300: "abnormally" is not a scientific expression. Please replace by proper term.

Revise: have replaced “abnormally” by “extremely”.

L338: replace "," with "."

Revise: have replaced "," with ".".

L364: "Grafted plant is..." has nothing to do with the context of the manuscript. Delete.

Revise： I have deleted them.

L362: Conclusion: Authors did not comment on the importance of validation of reference genes for each experimental setting, organ, or treatment. Please add.

Revise: I have rewritten the conclusion, as follows:

Grafting also assemble desirable roots and shoots to generate chimeras that are more vigorous, more pathogen resistant, and more abiotic stress resistant (Melnyk, 2016). To our knowledge, cucumber, pumpkin and their grafted plants were simultaneously used as samples for the first time to identify the optimal candidate reference genes in our study. 12 candidate reference genes were validated in different organs, conditions, species of cucumber, pumpkin and their grafted plants using five software tools-ΔCt method, BestKeeper, NormFinder, geNorm and RefFinder. The results showed that *TIP41* and *CACS* had the most stable expression in different cucumber organs, *TIP41* and *PP2A* were the optimal reference genes in pumpkin organs, and *CACS* and *40SRPS8* were also the most stable in all grafted cucumber samples. This work will be helpful in future studies on gene function and molecular mechanisms in cucumber–pumpkin grafted plants and other closely related species.

Commented [FJ(3]: "Extremely" isn't better. Give numbers or scales.

Commented [FJ(4]: I think, you should make clear, that the suitability of reference genes depends on treatment, organ and experimental settings and hence need a careful validation each time the settings are changed.

Commented [FJ(5]: Check definition of chimera. A graft is not a chimera.