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 to measure mRNA transcript levels. The accuracy of this 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 to either parent plant, as grafting conveys many advantages. However, although many reliable reference genes have been identified in both cucumber and pumpkin, none have been obtained for cucumber–pumpkin grafted plants.

Methods: In this work, 12 candidate reference genes, including 8 traditional genes and 4 novel genes identified from our transcriptome data, were selected to assess their expression stability. Their expression levels 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 varied dependent on the organs, conditions, and varieties. *CACS* and *40SRPS8* were the most stable reference genes for all samples in our research. *TIP41* and *CACS* showed the most stable expression in different cucumber organs, *TIP41* and *PP2A* were the optimal reference genes in pumpkin organs, and *CACS* and *40SRPS8* were the most stable genes 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* showed the most stable expression in the graft union during the healing process, and *TIP41* and *PP2A* were the most stable across different varieties of cucumber–pumpkin grafted plants. The use of *LEA26*, *ARF* and *LEA26+ARF* as reference genes were further verified 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 report of 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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19 technique to measure mRNA transcript levels. The accuracy of this evaluation of gene

20 expression depends on the use of optimal reference genes. Cucumber–pumpkin grafted plants,

21 made by grafting a cucumber scion onto pumpkin rootstock, are superior to either parent plant,

22 as grafting conveys many advantages. However, although many reliable reference genes have

23 been identified in both cucumber and pumpkin, none have been obtained for cucumber–pumpkin

24 grafted plants.

25 **Methods:** In this work, 12 candidate reference genes, including 8 traditional genes and 4 novel

26 genes identified from our transcriptome data, were selected to assess their expression stability.

27 Their expression levels in 25 samples, including 3 cucumber and 3 pumpkin samples from

28 different organs, and 19 cucumber–pumpkin grafted samples from different organs, conditions,

29 and varieties, were analyzed by qRT-PCR, and the stability of their expression was assessed by

30 the comparative ΔCt method, geNorm, NormFinder, BestKeeper, and RefFinder.

31 **Results:** The results showed that the most suitable reference gene varied dependent on the

32 organs, conditions, and varieties. *CACS* and *40SRPS8* were the most stable reference genes for

33 all samples in our research. *TIP41* and *CACS* showed the most stable expression in different

34 cucumber organs, *TIP41* and *PP2A* were the optimal reference genes in pumpkin organs, and

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36 optimal reference gene varied under different conditions. *CACS* and *40SRPS8* were the best

37 combination of genes in different organs of cucumber–pumpkin grafted plants, *TUA* and

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

Introduction

48   Cucumber (*Cucumis sativus* L.) is one of the most widely cultivated vegetable crops in the
49   world. Grafted cucumber plants are popular due to their greater resistance to soil-borne diseases,
50   increased tolerance to abiotic stress, improved mineral nutrition uptake and use, and increased
51   fruit yield and quality (Huang et al., 2014). A cucumber scion is typically grafted onto pumpkin
52   (*Cucurbita moschata* Duch.) rootstock (Lee et al., 2010; Huang et al., 2014). Grafting conveys
53   advantages over the properties of each individual parent plant, but the resulting plant is also more
54   complex than the parents. The graft union is the successful combination of the scion and
55   rootstock, allowing the establishment of complex communication between rootstock and scion.
56   There have been many physiological and biochemical studies of cucumber–pumpkin grafted
57   plants (Ahn et al., 1999; Yang et al., 2006; Haroldsen et al., 2012; Li et al., 2014), however, there
58   have been few studies analyzing gene function, transcription, or expression in cucumber–
59   pumpkin grafted plants, as few pumpkin genes were identified. Now, the entire cucumber
60   (http://cucurbitgenomics.org/organism/2) (Huang et al., 2009) and pumpkin
61   (http://cucurbitgenomics.org/organism/9) (Sun et al., 2017) genomes have been published,
62   enabling further studies on the molecular biology of these species.
63   Gene expression analysis is fundamental to elucidate the molecular mechanisms underlying
64   various biological processes (Bustin et al., 2005), and qRT-PCR is the most common technique
65   used to study gene expression because of its high sensitivity, accuracy, specificity, cost-
66   effectiveness, and reproducibility (Bustin et al., 2002; Nolan et al., 2006; Derveaux et al., 2010).
67   However, non-specific variations can cause errors resulting in unreliability of the qRT-PCR data,
68   such as variability in RNA quality, cDNA synthesis and concentration, PCR procedures, and
69   efficiency of amplification (Delporte et al., 2015). To avoid these problems in analyzing results,
70   stable reference genes should be used to normalize the gene expression data. Appropriate
71   reference genes should be systematically evaluated across various environments (varieties,
72   tissues, experimental treatments, and developmental stages) before being used as controls in
73   qRT-PCR analysis (Bustin et al., 2009; Guenin et al., 2009; Sgamma et al., 2016). However,
74   there are no previous reports of systematic studies performed on grafted cucumber plants to
75   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 most 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 been used for reliable normalization in different
83   experimental sets in zucchini (*Cucurbita pepo*) (Obrero et al., 2011), and these reference genes
84   have been successfully applied to both cucumber and pumpkin in specific environments,
85   including powdery mildew, salinity, cold, dehydration, H_2O_2, and abscisic acid (ABA)
86   treatments (Berg et al., 2015; Cao et al., 2017; Reda et al., 2018). Unfortunately, no single
87   reference gene has been confirmed that exhibits uniform and stable expression under different
88   experimental conditions. For example, *ACT* is a frequently used reference genes in many plants,
89   but showed variant expression during short-term treatment of cucumber with salt, osmotic, or
90   oxidative stress (Migocka and Papierniak, 2011). Overall, it is necessary to identify one or more
91   reference genes that show stable expression under different experimental conditions prior to
92   carrying out gene expression studies (Duan et al., 2017).
93   In this study, traditional reference genes from published research and new genes based upon their
94   coefficients of variation (CVs) and expression intensity in our RNA-seq data from cucumber–
95   pumpkin grafted plants at different stages were selected for further analysis. Twelve genes were
96   investigated in this study, eight commonly used reference genes, *ACT, CYP, CACS, TUA, TIP41*
97   (*tonoplast intrinsic protein*), *F-Box* (*F-box protein*), *RPL36Aa*, and *PP2A,* and four new genes
98   identified by RNA-seq analysis, *UBC* (*Ubiquitin conjugating enzyme*), *ARF* (*ADP-ribosylation*
99   *factor-like protein*), *LEA26* (*Late-embryogenesis abundant protein 26*), and *40SRPS8* (*40S*
100   *ribosomal protein S8*). These twelve genes were evaluated to validate their use as stable
101   reference genes for qRT-PCR in different organs, at different stages, in different varieties, and
102   under stress conditions in cucumber, pumpkin, and cucumber–pumpkin grafted plants. To
103   determine the appropriate reference genes, four statistical tools were used to evaluate the
104   accuracy of these candidate genes: the ΔCt method (Silver et al., 2006), geNorm (Vandesompele
105   et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004).
106   Comprehensive stability rankings were generated by RefFinder (Xie et al., 2012). Additionally,
107   the expression of *CYCD3;1*, *RUL* and *PIN*, genes that are thought to be related to graft union
108   healing in Arabidopsis (Melnyk et al., 2018), were investigated as a case study to evaluate the
109   effectiveness of the reference genes identified in this study. The results obtained in this study
110   will be useful in future gene expression analyses in cucumber, pumpkin, and their grafted plants.

111   **Materials & Methods**

112   **Plant Materials and Treatments**

113   Cucumber (*Cucumis sativus* L.) and pumpkin (*Cucurbita moschata* Duch.) were planted in an
114   artificial chamber at the farm of the Institute of Vegetables and Flowers, Chinese Academy of
115   Agricultural Sciences, Beijing, China at a temperature of 28°C/20°C (day/night) with a
116   photoperiod cycle of 12/12 h and 60%–70% relative humidity. Cucumber variety 'Zhongnong
117   No. 26' was used as the scion and pumpkin variety 'Jinxinzhen No. 5' was used as the rootstock.

118 Seeds of the scion and rootstock were sown in 50-cell (55 cm^3/cell) and 32-cell (32cm^3/cell)
119 polystyrene trays, respectively, containing commercial organic substrates
120 (Vpeatmoss:Vvermiculite:Vperlite = 1:1:1). The environmental conditions for germination were
121 25–28°C and 85%–90% relative humidity. The pumpkin seeds were sown three days before the
122 cucumber seeds. When cotyledons of the scion were fully open and the first true leaf of the
123 rootstock started to develop (9–10 d after sowing), the plants were grafted using the hole
124 insertion grafting method as previously described (Miao et al., 2018) (*Fig S1*). Autografts were
125 carried out for both cucumber and pumpkin, as well as cucumber–pumpkin heterografts. The
126 grafted seedlings were maintained at a temperature of 30°C/22°C (day/night), a constant
127 humidity of 95%–100% and a dim light of 50 PPFD for the first 5 days, then the light density
128 was slowly increased from 50 to 500 PPFD and the humidity was decreased from 95% to 60%,
129 while the other environmental conditions were unchanged. For the autograft cucumber and
130 pumpkin plants, samples of the leaves, stems and roots were harvested when the seedlings had
131 two true leaves. For cucumber grafted onto pumpkin, samples of the leaves, the stem of the
132 scion, the graft union (*Fig1*), the stem of the rootstock, and the roots were harvested. For the cold
133 stress experiment, when the grafted cucumber had two leaves, seedlings were exposed to
134 temperatures of 12°C in a chamber, and samples of the graft union were harvested at 0, 5, 12 and
135 24 h of stress treatment. To investigate the graft union healing process, samples of the graft
136 union were harvested 0, 3, 6, 9 and 15 d after grafting. For experiments with varieties, cucumber
137 varieties 'Xintaimici' and 'Zhongnong No. 26' were used as scions and pumpkin varieties
138 'Zhongguonangua No. 26', 'Jinxinzhen No. 5' and 'Huofenghuang' were used as rootstocks. The
139 graft combinations were 'Xintaimici–Zhongguonangua No. 26', 'Xintaimici–Jinxinzhen No. 5',
140 'Xintaimici–Huofenghuang', 'Zhongnong No. 26–Jinxinzhen No. 5', and 'Zhongnong No.26–
141 Huofenghuang'. Graft unions were harvested when grafted plants had two true leaves. For each
142 treatment, three independent biological replicates were achieved. All samples were immediately
143 frozen in liquid nitrogen and stored at −80°C.
144 **RNA Isolation and cDNA Synthesis**
145 The RNAprep Pure Plant Plus Kit (Tiangen, Beijing, China) was used for total RNA extraction.
146 Genomic DNA was eliminated from the total RNA using RNase-free DNase I. The RNA
147 integrity was confirmed by 1.0% agarose gel electrophoresis. RNA concentrations were
148 determined by NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA),
149 samples with an A260/A280 ratio of 1.8-2.2 and an A260/A230 ratio > 2.0 were used for further
150 analyses. First-strand cDNA synthesis was performed using a FastQuant cDNA Synthesis kit
151 (Tiangen, Beijing, China) according to the manufacturer's instructions.
152 **Candidate Reference Gene Selection and Primer Design**
153 Eight commonly used reference genes (*ACT, CYP, CACS, TUA, TIP41, F-Box, PRL36Aa* and
154 *PP2A*) from published studies on cucumber, pumpkin, chicory, buckwheat, lettuce and mangrove
155 tree were selected (Wan et al., 2010; Obrero et al., 2011; Delporte et al., 2015; Demidenko et al.
156 2011; Borowski et al., 2014; Saddhe et al., 2018). For new candidate reference genes, we
157 analyzed our transcriptomic data from the graft union. Graft union of cucumber-pumpkin were

158   respectively harvested at 0, 3, 6, 9 days after grafting, three biological replicates were performed
159   for each time point. In total 18 transcriptome libraries, 132.7G raw reads were obtained, at the
160   least 91.4% of the reads were mapped to the reference sequence, and assemble into 32,852 and
161   47,906 transcripts of cucumber and pumpkin, respectively. 20,782 unigene of cucumber with the
162   average length of 4.1kb were obtained, while 27,187 unigene with average length of 4.4kb were
163   generated (*Table S1*). The genes with the most constant expression levels were defined as
164   candidate reference genes (De Jong et al., 2007). We calculated the mean expression value,
165   standard deviation, and coefficients of variation (CVs) based on the raw RNA-seq data, and CVs
166   = standard deviation of RPKM/average of RPKM. Based on the requirements CV ≤ 0.2 and
167   300≤RPKM≤ 500 (Duan et al., 2017), we selected new reference genes by removing
168   overabundant genes with low expression levels. With requirements of evalue e-5, we used
169   BLAST (Basic Local Alignment Search Tool) to determine the proteins encoded by cucumber
170   and pumpkin genes, respectively (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and then filtered the
171   BLAST results based on an identity ≥ 90 and an overlap ratio > 0.5 (between query and target).
172   This resulted in ten and seven genes of cucumber and pumpkin, respectively, which may be
173   suitable as reference genes. A comparison of the relationship between cucumber and pumpkin by
174   homology analysis is shown in *Table S2*. Finally, *UBC, ARF, LEA26* and *40SRPS8* were selected
175   as candidate reference genes based on preliminary experiments of single PCR product in agarose
176   gel electrophoresis (*Fig S2*). Based on the conserved sequence of these genes between cucumber
177   and pumpkin, primers were designed using Primer Premier 5.0 software with the following
178   parameters: a melting temperature (Tm) of 50–60°C, a primer length of 17–25 bp, and a product
179   size of 70–260 bp (http://www.premierbiosoft.com/) (*Table 1*). Amplification of a single PCR
180   product in 1% agarose gel electrophoresis and a single peak of the melting curve in qRT-PCR
181   were used to ensure the specificity of the primers for the candidate reference genes.
182   **qRT-PCR Assay**
183   qRT-PCR was performed on an Agilent Stratagene Mx3000P Real-Time PCR machine (Agilent
184   Stratagene, USA) using SYBR® Premix Ex Taq™ (TliRNaseH Plus) (TaKaRa, Dalian, China).
185   Each 20 µl reaction mixture contained 2 µl of cDNA template, 0.4 µl of each primer, 0.4 µl of
186   ROX dye, 10 µl of 2× SYBR Premix Ex Taqand 6.8 µl of ddH$_2$O. The qRT-PCR reaction
187   conditions were as follows: 94°C for 30 s, 40 cycles of 94°C for 5 s, then 60°C for 34 s. A
188   melting curve was determined by increasing the amplification temperature from 60–95°C, with a
189   temperature increment of 0.5°C every 5 s. All samples were performed with three technical
190   replicates, and samples without template were used as a control. The amplification efficiencies
191   for each primer and the regression coefficients (R^2) were evaluated using five-fold dilutions of
192   pooled cDNA (1/5, 1/25, 1/125, 1/625, 1/3125) that were diluted using EASY dilution solution
193   (Takara, Japan).
194   **Gene Expression Stability Analysis**
195   To evaluate the expression levels of each reference gene, we drew boxplots of the Ct values for
196   the 12 candidate reference genes. Four statistical tools, the ΔCt method (Silver et al., 2006),
197   geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper

198 (Pfaffl et al., 2004), were used to evaluate the stability of the 12 candidate reference genes at
199 various treatment durations. The raw Ct values of the reference genes were transformed into the
200 correct input files according to the requirements of the software. Finally, a comprehensive
201 ranking of the reference genes was generated using RefFinder (Duan et al., 2017).
202 **Validation of Reference Gene Stability**
203 To confirm the reliability of the selected reference genes, the relative expression levels of three
204 genes involved in xylem development were measured during graft union healing in grafted
205 cucumbers (*Table S3*). Samples of the graft union of cucumber–pumpkin grafted plants were
206 harvested at 0, 1, 3, 6, 9 and 15d after grafting. The most stable reference genes (*LEA26, ARF*
207 and *LEA26+ARF*), and the least stable reference gene (*PP2A*) ranked by RefFinder were used for
208 normalization. Comparative gene expression levels of csa*CYCD3;1* (Csa2G356610), csa*RUL*
209 (Csa3G895630), cmo*RUL* (CmoCh15G013320) and cmo*PIN* (CmoCh15G009810) were
210 calculated using the $2^{-\Delta\Delta Ct}$ method. Three technical replicates were performed for each biological
211 sample.
212 # Results
213 **Evaluation of Primer Specificity and Amplification Efficiency**
214 To validate the primer specificity of the 12 candidate reference genes in our study, the specificity
215 of the PCR reactions was subjected to 1% agarose gel electrophoresis (*Fig S2*). The product
216 lengths were consistent with the expected lengths, and a single sharp peak was observed in the
217 melting curves for cucumber, pumpkin, and grafted cucumber (*Fig S3, S4A*). Additionally, the
218 amplification efficiency (E) ranged from 0.86 to 1.13, with the correlation coefficients (R^2) of
219 the standard curve varying from 0.986 to 0.999 (*Table 1, Fig S4B, S5*).
220 **Expression Levels and Variations in Candidate Reference Genes**
221 The transcript abundances of the 12 candidate reference genes were assessed based on the Ct
222 values from the qRT-PCR for different kinds of samples. As shown in *Fig 2*, the Ct values for
223 the 12 candidate reference genes ranged from 16.98 to 31.71, and the mean Ct values were
224 19.04, 18.35, 23.26, 20.80, 20.66, 26.70, 20.79, 24.79, 20.26, 21.78, 20.8 and 21.09 for *ACT*,
225 *CYP*, *CACS*, *TUA*, *TIP41*, *F-Box*, *PRL36Aa*, *PP2A*, *UBC*, *ARF*, *LEA26* and *40SRPS8*,
226 respectively.
227 **Expression Stability Analysis of Candidate Reference Genes**
228 The stabilities of the 12 candidate reference genes in our study were evaluated separately using
229 the ΔCt method, geNorm, NormFinder, BestKeeper, and RefFinder. The 12 candidate reference
230 genes were divided into eight groups of different treatments: organs of cucumber, pumpkin, and
231 cucumber–pumpkin grafted plants under normal conditions were termed Cos, Pos, and Gos,
232 respectively. Graft union samples under low temperatures were termed GLgs, graft union
233 samples during the healing process were termed Ggs, graft union samples of different varieties of
234 cucumber–pumpkin grafted plants were termed Ggvs, all cucumber–pumpkin grafted plant
235 samples were termed GoAll, and all samples in our study were termed All.
236 **ΔCt method analysis.** The ΔCt method ranks the stability of expression of tested genes by
237 comparing the relative expression of gene pairs within each sample (Silver et al., 2006). As

238 shown in Table 2, *TIP41* was the most stable reference gene in the Cos, Pos, and Ggvs samples,
239 while *TIP41* was the lowest stable reference gene in the GLgs samples. *CACS* was the most
240 stable reference gene in the Gos, GoAll, and All samples, and *TUA* and *LEA26* were ranked as
241 the most stable reference genes in the GLgs and Ggs samples, respectively (*Table 2, S4*).
242 **BestKeeper analysis.** The BestKeeper program identifies potential reference genes by
243 calculating the coefficients of variation (CVs) and the standard deviation (SD) of the Ct values,
244 where lower CVs and SD indicate higher stability (Pfaffl et al., 2004). For the Cos and GoAll
245 samples, *CACS* was identified as the most stable gene, and *TIP41* was the most stable gene in the
246 Pos and Ggvs samples. *CYP* was the most stable gene in the Gos samples, but was the lowest
247 ranking gene in the Ggvs samples. Similarly, *TUA* was the most stable gene in the GLgs
248 samples, but was the lowest stable gene in the Pos samples. *ARF* and *LEA26* were ranked as the
249 most stable reference gene in the Ggs and All samples, respectively. *PP2A* was the reference
250 gene with the lowest stability in most samples, including the Gos, Ggs, GoAll, and All samples
251 (*Table 2, S4*).
252 **NormFinder analysis.** NormFinder ranks the stability of tested genes based on inter- and
253 intragroup variations in expression across different sample groups, and lower values indicate
254 higher stability (Andersen et al., 2004). *TIP41* had stability values of 0.084, 0.153, and 0.203,
255 making it the most stable gene in the Cos, Pos, and Ggvs samples, respectively. *40SRPS8* and
256 *CACS* were the two most stable genes and *PP2A* was the gene with the lowest stability in the
257 Gos, GoAll, and All samples. For the GLgs samples, *TUA* was the most stable, but was ranked as
258 the lowest stability reference gene in the Pos samples. The stability of *LEA26* was best in the Ggs
259 samples (*Table 2, S4*).
260 **geNorm analysis.** The geNorm software determines gene expression stability using M-values
261 based on the average pairwise variation of all candidate genes (Vandesompele et al., 2002).
262 *TIP41 and 40SRPS8, CACS and ARF, CYP and UBC, UBC and ARF, PP2A and ARF, ARF and*
263 *40SPRS8,* were the two most stable genes in the Cos, Gos, GLgs, Ggs, Ggvs, and GoAll samples,
264 respectively. *CACS* and *40SRPS8* were identified as the most stable reference genes with M-
265 values of 0.093 and 0.582 in the Pos and All samples, respectively (*Table 2, S4*). In addition, the
266 optimal number of reference genes was determined using the geNorm algorithm based on
267 pairwise variation (Vn/Vn+1). A value of Vn/Vn+1<0.15 indicates that the optimal number of
268 reference genes equal to a value of n is sufficient for its use as a reference gene (Vandesompele
269 et al., 2002). In our study, the values of V_2/V_3 of all experimental samples were less than 0.15,
270 which indicated that two reference genes would be sufficient for gene normalization under these
271 experimental conditions (*Fig 3*).
272 **RefFinder analysis.** RefFinder considers the results from the ΔCt method, geNorm, NormFinder
273 and BestKeeper to provide a comprehensive ranking of the most stable genes (Xie et al., 2012).
274 *TIP41* was the most stable reference gene in the Cos, Pos, and Ggvs samples, *CACS* was ranked
275 as the most stable gene in the Gos, GoAll, and All samples. *TUA* was the most stable in the GLgs
276 samples, but was also the lowest reference gene in the Pos samples. *LEA 26* and *ARF* were the

277 two most stable reference genes in the Ggs samples. *PP2A* was the lowest stable reference gene
278 in the Gos, Ggs, GoAll, and All samples (*Table 2, S4*).
279 **Validation of the Selected Reference Genes**
280 To confirm the stability of the selected reference genes, the expression levels of four graft union
281 healing-related genes were examined by normalization to the levels of *LEA26*, *ARF*,
282 *LEA26+ARF*, and *PP2A* as reference genes (*Table S3*). RefFinder analysis showed that *LEA26*
283 and *ARF* were the most suitable reference genes and *PP2A* was the least suitable reference gene
284 in the graft union during the healing process (*Table 2, S4*).
285 The expression patterns of *csaCYCD3;1*, *csaRUL*, *cmoRUL,* and *cmoPIN* showed similar
286 changes when *LEA26*, *ARF,* or *LEA26+ARF* were selected as the reference genes for
287 normalization (*Fig 4*). The expression levels of *csaCYCD* and *csaRUL* were significantly
288 downregulated at 3 d and 6 d compared to 1 d after grafting. However, these values were
289 markedly higher when *PP2A* was selected for normalization. Compared with the sample 0 day
290 after grafting, *cmoPIN* expression was clearly downregulated at the graft junction at 6 d, 9 d, and
291 15 d after grafting when using *LEA26*, *ARF*, or *LEA26+ARF* as reference genes, butthe
292 expression pattern was completely different when *PP2A* was used as a reference. Similarly,
293 compared with the sample 0 day after grafting, cmo*RUL* expression levels increased 3.99, 3.31,
294 and 3.43 times at the graft junction 3 days after grafting when using *LEA26*, *ARF,* and
295 *LEA26+ARF* as reference genes, respectively, but the change in expression was calculated as a
296 12.70-fold increase if the level of *PP2A* was used for transcript normalization.
297 # Discussion
298 qRT-PCR is a powerful method for detecting transcriptomic data and studying underlying
299 molecular mechanisms (Niu et al., 2017). Appropriate reference genes are required to ensure the
300 accuracy of qRT-PCR results. There has been research into the selection of optimal reference
301 genes in cucumber and pumpkin (Wan et al., 2010; Obrero et al., 2011; Warzybock and
302 Migocka, 2013), however, there have been no studies on the selection of the optimal reference
303 genes for cucumber–pumpkin grafted plants. Grafting assembles the scion and rootstock into a
304 plant with advantages over the parent plants. This is significant exchange of materials between
305 scion and rootstock, includingwater, sugars, hormones, RNAs, and proteins (Melnyk, 2017), so it
306 is highly likely that the optimal reference genes for cucumber–pumpkin grafted plants may differ
307 from the optimal reference genes for cucumber or pumpkin. To test this, we selected some
308 commonly used reference genes that are previously published (*ACT*, *CYP*, *CACS*, *TUA*, *TIP41*,
309 *F-Box*, *PRL36Aa* and *PP2A*) and are known to be expressed in cucumber or pumpkin. We also
310 selected four novel genes (*UBC*, *ARF*, *LEA26* and *40SRPS8*) from our transcriptomic data on
311 graft union healing in cucumber–pumpkin grafted plants, and primers were designed based on
312 the conserved sequence of the genes between cucumber and pumpkin.
313 The ΔCt method, BestKeeper, NormFinder, geNorm, and RefFinderare five software programs
314 and methods that are commonly used to identify reference genes (Scarabel et al., 2017; Duan et
315 al., 2017). In our study, two genes were sufficient for reliable normalization when all samples
316 were subjected to geNorm analysis (*Fig 3*). The ΔCt method, NormFinder, geNorm, and

317 RefFinder programs all suggested the same least suitable reference genes, but differed from the
318 rankings obtained by BestKeeper. For instance, *F-Box* was ranked as the least stable gene in Cos
319 samples by the ΔCt method, NormFinder, geNorm, and RefFinder programs analysis, while
320 BestKeeper identified *UBC* as the lowest stability gene in the Cos samples. This different result
321 for different methods is consistent with the result of a study by Niu et al. (2017) where the
322 rankings obtained by BestKeeper were also different from those obtained by geNorm and
323 NormFinder. The most suitable reference genes differed between the five algorithms. Six of the
324 traditional reference genes (*TIP41*, *CACS*, *ARF*, *UBC*, *CYP* and *PP2A*) and two novel reference
325 genes (*LEA26* and *40SRPS8*) were identified as the optimal reference genes in different samples
326 using different algorithms for analysis. The comprehensive evaluation by RefFinder used data
327 from the other four computational methods, and this ranking showed that *TIP41*, *CACS*, *TUA,*
328 and *LEA26* were the most suitable reference genes in different samples of cucumber, pumpkin,
329 and cucumber–pumpkin grafted plants.
330 *TIP41* is a tonoplast intrinsic protein that functions as a *PPA2* activator in plants, and has been
331 identified as a suitable reference gene in *Cucumis sativus* (Wan et al., 2010), *Cichorium intybus*
332 (Delporte et al., 2015)*,* and *Papaver rhoeas* (Scarabel et al., 2017). In our study, *TIP41* was a
333 stable reference gene in cucumber, pumpkin, and at the graft union of different varieties of
334 grafted cucumber plants, but *TIP41* was ranked as a gene with relatively lower stable in the Gos
335 samples (*Table 2*). This may suggest that grafted plants are different from the nongraft scion and
336 rootstock at the molecular level. Surprisingly, *TIP41* was ranked as the least stable reference
337 gene in the graft union of cucumber–pumpkin grafted plants at low temperatures. Importantly,
338 reference gene stability can vary under different experimental treatments (Bustin et al., 2005).
339 Reid et al. (2006) showed that *TIP41* is an inadequate reference gene during berry development.
340 Similarly, *TUA* was regarded as the most stable reference gene in the graft union under cold
341 stress, but was ranked as the least suitable reference gene in pumpkin organs by RefFinder
342 analysis (*Table 2*). In cucumber, *TUA* is a highly stable gene when different tissues were treated
343 with abscisic acid, salicylic acid, and methyl jasmonic acid (Wan et al., 2010), however, *TUA*
344 was limited as a stable reference gene in cucumber under conditions of salt, osmotic stress, and
345 high or low temperature (Wan et al., 2010; Migocka and Papierniak, 2011). *CACS* encodes the
346 clathrin adaptor complex subunit, which links clathrin to receptors in vesicles (Migocka and
347 Papierniak, 2011). As this gene participates in a basic intracellular transport process, *CACS* has
348 been used as a reference gene at different developmental stages and under varying environmental
349 conditions in *Arabidopsis thaliana* (Czechowski et al., 2005), buckwheat (*Fagopyrum*
350 *esculentum*) (Demidenko et al. 2011), and lettuce (*Lactuca sativa*) (Borowski et al., 2014). In
351 cucumber, *CACS* was ranked as the best reference gene under different nitrogen nutrition
352 conditions (Warzybok and Migocka, 2013), heavy metal stress, and on deprivation and/or
353 readdition of different nutrients (N, C, P, and S) (Migocka and Papierniak, 2011). One of the
354 novel reference genes, *LEA26* (*Late Embryogenesis Abundant protein 26*)*,* is related to abiotic
355 stress tolerance, especially desiccation tolerance in *Arabidopsis*. *LEA26* has not been evaluated
356 as a reference gene in any species (Dang et al., 2014). In our study, *LEA26* was the most stable

357   reference gene in the Ggs. However, *LEA26* was also identified as the lowest stable in the GLgs
358   samples by BestKeeper analysis and exhibited relatively lower stability in the Pos sample.
359   Overall, the results showed it was necessary to validate reliable reference genes prior to qRT-
360   PCR analysis under detailed experimental conditions.
361   To validate use of the identified reference genes as control genes with unchanging expression
362   levels, the expression levels of *csaRUL*, *csaCYCD3;1*, *cmoRUL,* and *cmoPIN* in the cucumber-
363   pumpkin graft union healing process were normalized by the two most stable reference genes
364   and the least stable gene. The results showed that *LEA26* and *ARF* may be the best candidate
365   reference genes for the normalization of gene expression in the graft union healing process. The
366   use of inappropriate reference genes may lead to inaccurate results, making it extremely
367   important to identity suitable reference genes to increase the reliability of qRT-PCR data for
368   target gene expression.

Conclusions

370   To our knowledge, this is the first report of the simultaneous use of cucumber, pumpkin and their
371   grafted plants as samples to identify optimal candidate reference genes. Twelve candidate
372   reference genes were validated in different organs, conditions, species of cucumber, pumpkin
373   and their grafted plants using five software tools-the ΔCt method, BestKeeper, NormFinder,
374   geNorm and RefFinder. The results showed that *TIP41* and *CACS* showed the most stable
375   expression in different cucumber organs, *TIP41* and *PP2A* were the optimal reference genes in
376   pumpkin organs, *TUA* and *RPL36Aa* were the most stable in the graft union under cold stress,
377   *LEA26* and *ARF* showed the most stable expression in the graft union during the healing process,
378   *TIP41* and *PP2A* were the most stable across different varieties of cucumber–pumpkin grafted
379   plants, and *CACS* and *40SRPS8* were the most stable in all grafted cucumber samples. Our
380   analysis showed that two genes are sufficient for reliable normalization when all samples are
381   considered. This work should facilitate future studies on gene function and molecular
382   mechanisms in cucumber–pumpkin grafted plants and other closely related species.

Acknowledgements

384   We are particularly appreciated to MengmengDuan, Jinglei Wang,and other members in our

385   research group for their kind suggestions to perfect the experiment.

References

387   **Ahn, S. J., Im, Y. J., Chung, G. C., Cho, B. H., and Suh, S. R.** (1999). Physiological
388   responses of grafted-cucumber leaves and rootstock roots affected by low root
389   temperature. *Scientia Horticulturae* 81, 397-408. Doi:10.1016/S0304-4238(99)00042-4.
390   **Andersen, C. L., Jensen, J. L., Ørntoft, T. F.** (2004). Normalization of real-time quantitative
391   reverse transcription-PCR data: a model-based variance estimation approach to identify genes
392   suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research* 64,
393   5245-5250. Doi:10.1158/0008-5472.CAN-04-0496.

394 **Berg, J. A., Appiano, M., Martínez, M. S., Hermans, F. W., Vriezen, W. H., Visser, R. G.,**
395 **Bai, L. Y., Schouten, H. J.** (2015). A transposable element insertion in the susceptibility gene
396 *CsaMLO8* results in hypocotyl resistance to powdery mildew in cucumber. *BMC Plant*
397 *Biology* 15, 243. Doi:10.1186/s12870-015-0635-x.
398 **Borowski, J. M., Galli, V., da Silva Messias, R., Perin, E. C., Buss, J. H., e Silva, S. D. D. A.,**
399 **Rombaldi, C. V.** (2014). Selection of candidate reference genes for real-time PCR studies in
400 lettuce under abiotic stresses. *Planta* 239(6), 1187-1200. Doi:10.1007/s00425-014-2041-2.
401 **Bustin, S. A**. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-
402 PCR): trends and problems. *Journal Molecular Endocrinology* 29, 23-39.
403 Doi:10.1677/jme.0.0290023.
404 **Bustin, S. A., Benes, V., Nolan, T., and Pfaffl, M. W.** (2005). Quantitative real-time RT-PCR–
405 a perspective. *Journal of Molecular Endocrinology* 34, 597-601. Doi: 10.1677/jme.1.01755.
406 **Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M.,**
407 **Vandesompele, J.** (2009). The MIQE Guidelines: Minimum Information for Publication of
408 Quantitative Real-Time PCR Experiments. *Clinical chemistry* 55, 611-622.
409 Doi:10.1373/clinchem.
410 **Cao, H., Wang, L., Nawaz, M. A., Niu, M., Sun, J., Xie, J., Kong, Q., Huang, Y., Cheng, F.,**
411 **Bie, Z.** (2017). Ectopic expression of pumpkin nac transcription factor cmnac1 improves
412 multiple abiotic stress tolerance in Arabidopsis. *Frontiers in Plant Science* 8, 2052.
413 Doi:10.3389/fpls.2017.02052.
414 **Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., Scheible, W. R.** (2005). Genome-
415 wide identification and testing of superior reference genes for transcript normalization in
416 Arabidopsis. *Plant Physiology* 139, 5-17. Doi:10.1104/pp.105.063743.
417 **Dang, N. X., Popova, A. V., Hundertmark, M., and Hincha, D. K.** (2014). Functional
418 characterization of selected *LEA* proteins from Arabidopsis thaliana in yeast and in
419 vitro. *Planta*240, 325-336. Doi:10.1007/s00425-014-2089-z.
420 **De Jonge, H. J., Fehrmann, R. S., de Bont, E. S., Hofstra, R. M., Gerbens, F., Kamps, W.**
421 **A., De Vries, E. G., Van der Zee, A. G., TeMeerman, G. J., ArjaterElst.** (2007). Evidence
422 based selection of housekeeping genes. *PLoS ONE* 2 e898. Doi:10.1371/journal.pone.0000898.
423 **Delporte, M., Legrand, G., Hilbert, J. L., Gagneul, D.** (2015). Selection and validation of
424 reference genes for quantitative real-time PCR analysis of gene expression in
425 Cichoriumintybus. *Frontiers in Plant Science* 6, 651. Doi:10.3389/fpls.2015.00651.
426 **Demidenko, N.V., Logacheva, M. D., Penin, A. A.** (2011). Selection and validation of
427 reference genes for quantitative real-time PCR in buckwheat (Fagopyrumesculentum) based on
428 transcriptome sequence data. *PLoS ONE* 6, e19434. Doi:10.1371/journal.pone.0019434.
429 **Derveaux, S., Vandesompele, J., Hellemans, J.** (2010). How to do successful gene expression
430 analysis using real-time PCR. *Methods* 50, 227-230. Doi:10.1016/j.ymeth.2009.11.001.
431 **Duan, M., Wang, J., Zhang, X., Yang, H., Wang, H., Qiu, Y., Li, X**. (2017). Identification of
432 optimal reference genes for expression analysis in Radish (*Raphanus sativus* L.) and its relatives
433 based on expression stability. *Frontiers in Plant Science* 8, 1605. Doi:10.3389/fpls.2017.01605.

434 **Guénin, S., Mauriat, M., Pelloux, J., Van Wuytswinkel, O., Bellini, C., Gutierrez, L.** (2009).
435 Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental
436 conditions-specific, validation of references. *Journal of Experimental Botany* 60, 487-493.
437 Doi:10.1093/jxb/ern305.
438 **Haroldsen, V., Szczerba, M. W., Aktas, H., Lopez, J., Odias, M. J., Chi-Ham, C. L.,**
439 **Labavitch, J. M., Bennett, A. B., Powell, A. L.** (2012). Mobility of transgenic nucleic acids and
440 proteins within grafted rootstocks for agricultural improvement. *Frontiers in Plant Science* 3, 39.
441 Doi:10.3389/fpls.2012.00039.
442 **Huang, S., Li, R., Zhang, Z., Li, L., Gu, X., Fan, W., Lucas, W.,Wang, X., Xie, B., Ni, B.,**
443 **Ren, Y., Zhu, H., Li, J., Lin, K., Jin, W., Fei, Z., Li, G., Staub, J., Kilian, A., van der**
444 **Vossen, E., Wu, Y., Guo, J., He, J., Jia, Z., Ren, Y., Tian, G., Lu, Y., Ruan, J., Qian, W.,**
445 **Wang, M., Huang, Q., Li, B., Xuan, Z., Cao, J., Wu, Z., Zhang, J., Cai, Q., Bai, Y., Zhao,**
446 **Y., Han, Y., Li, Y., Li, X., Wang, S., Shi, Q., Liu, S., Cho, W., Kim, J., Xu, Y., Heller-**
447 **Uszynska, K., Miao, H., Cheng, Z., Zhang, S., Wu, J., Yang, Y., Kang, H., Li, M., Liang, H.,**
448 **Ren, X., Shi, Z., Wen, M., Jian, M., Yang, H., Zhang, G., Yang, Z., Chen, R., Liu, S., Li, J.,**
449 **Ma, L., Liu, H., Zhou, Y., Zhao, J., Fang, X., Li, G., Fang, L., Li, Y., Liu, D., Zheng, H.,**
450 **Zhang, Y., Qin, Y., Li, Z., Yang, G., Yang, S.,Bolund, L., Kristiansen, K., Zheng, H., Li, S.,**
451 **Zhang, X., Yang, H., Wang, J., Sun, R., Zhang, B., Jiang, S., Wang, J., Du, Y., Li, S.**(2009).
452 The genome of the cucumber, *Cucumis sativus* L. *Nature Genetics*41, 1275. Doi:10.1038/ng.475.
453 **Huang, Y., Kong, Q. S., Chen, F., Bie, Z. L.** (2014). The history, current status and future
454 prospects of vegetable grafting in China. *International Symposium on Vegetable Grafting* 1086,
455 31-39. Doi:10.17660/ActaHortic.2015.1086.2.
456 **Lee, J. M., Kubota, C., Tsao, S. J., Bie, Z., Echevarria, P. H., Morra, L., Oda, M.** (2010).
457 Current status of vegetable grafting: Diffusion, grafting techniques, automation. *Scientia*
458 *Horticulturae*.127, 93-105. Doi:10.1016/j.scienta.2010.08.003.
459 **Li, H., Liu, S. S., Yi, C. Y., Wang, F., Zhou, J., Xia, X. J., Shi, K., Zhao, Y. H., Yu, J. Q.**
460 **(2014).** Hydrogen peroxide mediates abscisic acid-induced *HSP* 70 accumulation and heat
461 tolerance in grafted cucumber plants. *Plant and Cell Environment* 37, 2768-2780.
462 Doi:10.1111/pce.12360.
463 **Livak, K. J., Schmittgen, T. D.** (2001)**.** Analysis of relative gene expression data using real-
464 time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25(4), 402-408.
465 Doi:10.1006/meth.2001.1262.
466 **Migocka, M., Papierniak, A.** (2011). Identification of suitable reference genes for studying
467 gene expression in cucumber plants subjected to abiotic stress and growth regulators. *Molecular*
468 *Breeding*. 28, 343-357. Doi:10.1007/s11032-010-9487-0.
469 **Miao, L., Li, S.H., Bai, L.Q., Ali, A., Li, Y.S., He, C.X., Yu, X.C.** (2018). Effect of grafting
470 methods on physiological change of graft union formation in cucumber grafted onto bottle gourd
471 rootstock. *Scientia Horticulturae* 26, 249-256. Doi:10.1016/j.scienta.2018.09.061.

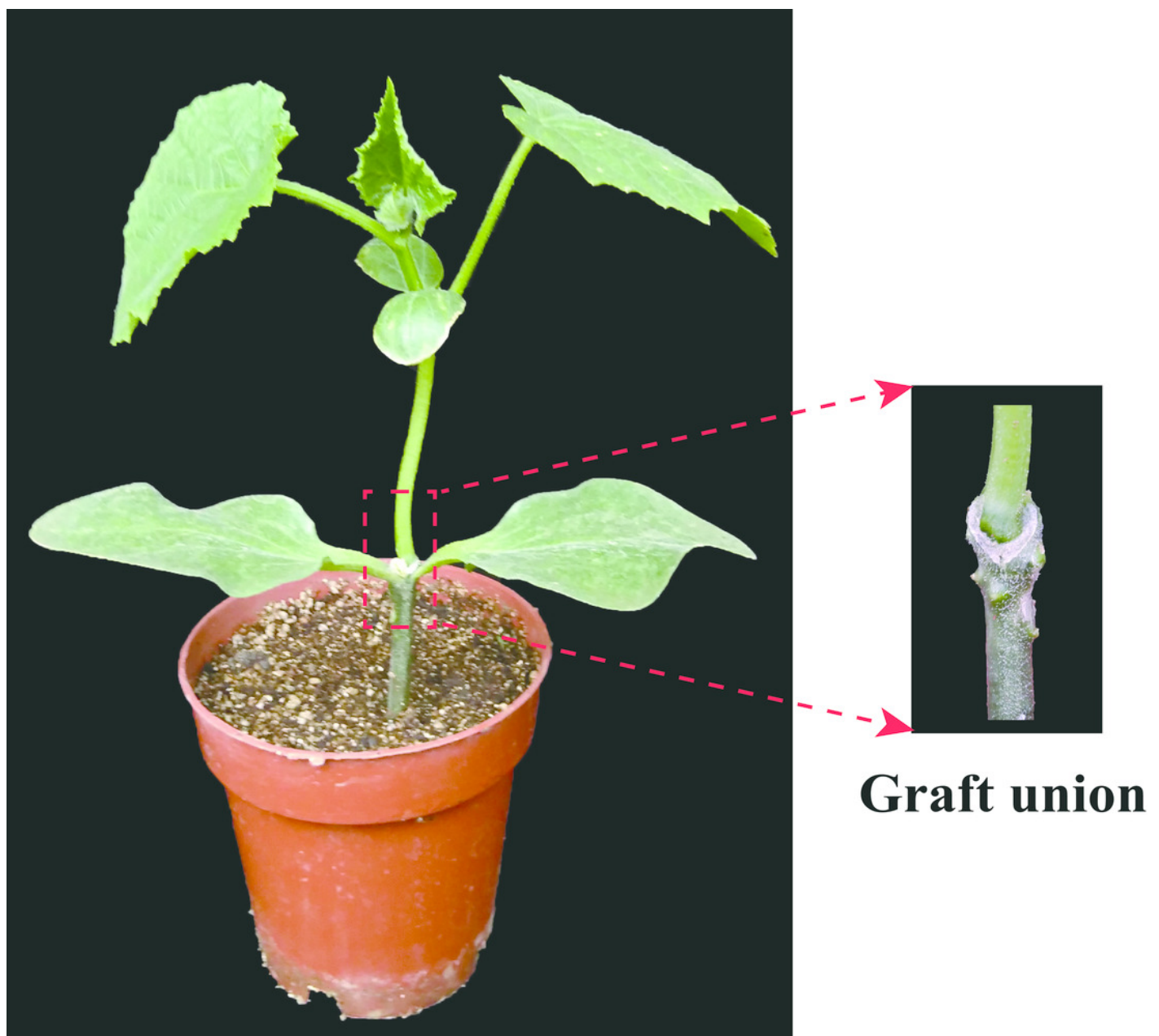
472   **Mohamed, F.H., El-Hamed, K.E.A., Elwan, M.W.M., Hussien, M.N.E.** (2014). Evaluation of
473   different grafting methods and rootstocks in watermelon grown in Egypt. *Scientia Horticulturae.*
474   168 (3), 145-150. Doi:10.1016/j.scienta.2014.01.029.
475   **Melnyk, C. W.** (2017). Connecting the plant vasculature to friend or foe. *New*
476   *Phytologist*, 213(4), 1611-1617. Doi:10.1111/nph.14218.
477   **Melnyk, C. W., Gabel, A., Hardcastle, T. J., Robinson, S., Miyashima, S., Grosse, I.,**
478   **Meyerowitz, E. M.** (2018). Transcriptome dynamics at arabidopsis graft junctions reveal an
479   intertissue recognition mechanism that activates vascular regeneration. *Proceedings of the*
480   *National Academy of Sciences of the United States of America*, 115(10), E2447.
481   Doi:10.1073/pnas.1718263115.
482   **Niu, X., Chen, M., Huang, X., Chen, H., Tao, A., Xu, J., Qi, J.** (2017). Reference gene
483   selection for qRT-PCR normalization analysis in kenaf (Hibiscus cannabinus L.) under abiotic
484   stress and hormonal stimuli. *Frontiers in Plant Science* 8, 771. Doi:10.3389/fpls.2017.00771
485   **Nolan, T., Hands, R. E., Bustin, S. A.** (2006). Quantification of mRNA using real-time RT-
486   PCR. *Nature Protocols* 1, 1559. Doi:10.1038/nprot.2006.236.
487   **Obrero, Á., Die, J. V., Román, B., Gómez, P., Nadal, S., González-Verdejo, C. I.** (2011).
488   Selection of reference genes for gene expression studies in zucchini (*Cucurbita pepo*) using
489   qPCR. *Journal of Agricutural Food Chemistry* 59, 5402-5411. Doi:10.1021/jf200689r.
490   **Pfaffl, M. W., Tichopad, A., Prgomet, C., Neuvians, T. P.** (2004). Determination of stable
491   housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–
492   Excel-based tool using pair-wise correlations. *Biotechnology letters* 26, 509-515.
493   Doi:10.1023/B:BILE.0000019559.84305.47.
494   **Reda, M., Golicka, A., Kabała, K., Janicka, M.** (2018). Involvement of NR and PM-NR in NO
495   biosynthesis in cucumber plants subjected to salt stress. *Plant Science*. 267, 55-64.
496   Doi:10.1016/j.plantsci.2017.11.004.
497   **Reid, K. E., Olsson, N., Schlosser, J., Peng, F., Lund, S. T.** (2006). An optimized grapevine
498   RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR
499   during berry development. *BMC Plant Boilogy* 6, 27. Doi:10.1186/1471-2229-6-27.
500   **Saddhe, A. A., Malvankar, M. R., Kumar, K.** (2018). Selection of reference genes for
501   quantitative real-time PCR analysis in halophytic plant *Rhizophoraapiculata*. *PeerJ* 6, e5226.
502   Doi:10.7717/peerj.5226.
503   **Scarabel, L., Milani, A., Panozzo, S., Rasori, A.** (2017). Suitable reference genes for accurate
504   gene expression analysis in Papaver rhoeas under 2, 4-D herbicide stress. *Pesticide biochemistry*
505   *and physiology*, 143, 66-72. Doi:10.1016/j.pestbp.2017.09.008.
506   **Sgamma, T., Pape, J., Massiah, A., Jackson, S.** (2016). Selection of reference genes for
507   diurnal and developmental time-course real-time PCR expression analyses in lettuce. Plant
508   Methods, 12, 21. Doi:10.1186/s13007-016-0121-y.
509   **Silver, N., Best, S., Jiang, J., Thein, S. L.** (2006). Selection of housekeeping genes for gene
510   expression studies in human reticulocytes using real-time PCR. *BMC Molecular Biology* 7, 33.
511   Doi:10.1186/1471-2199-7-33.

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

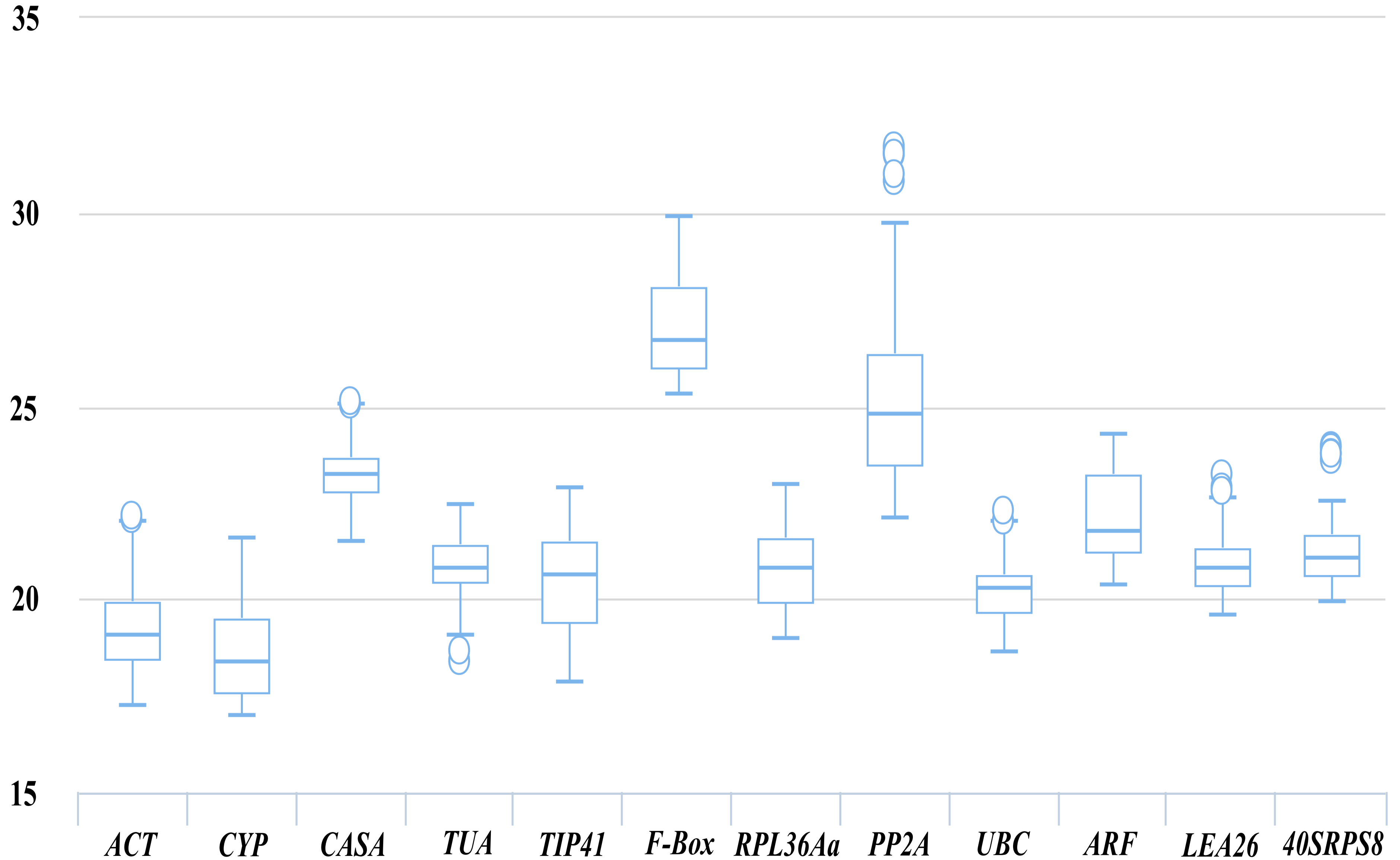
Figure 1

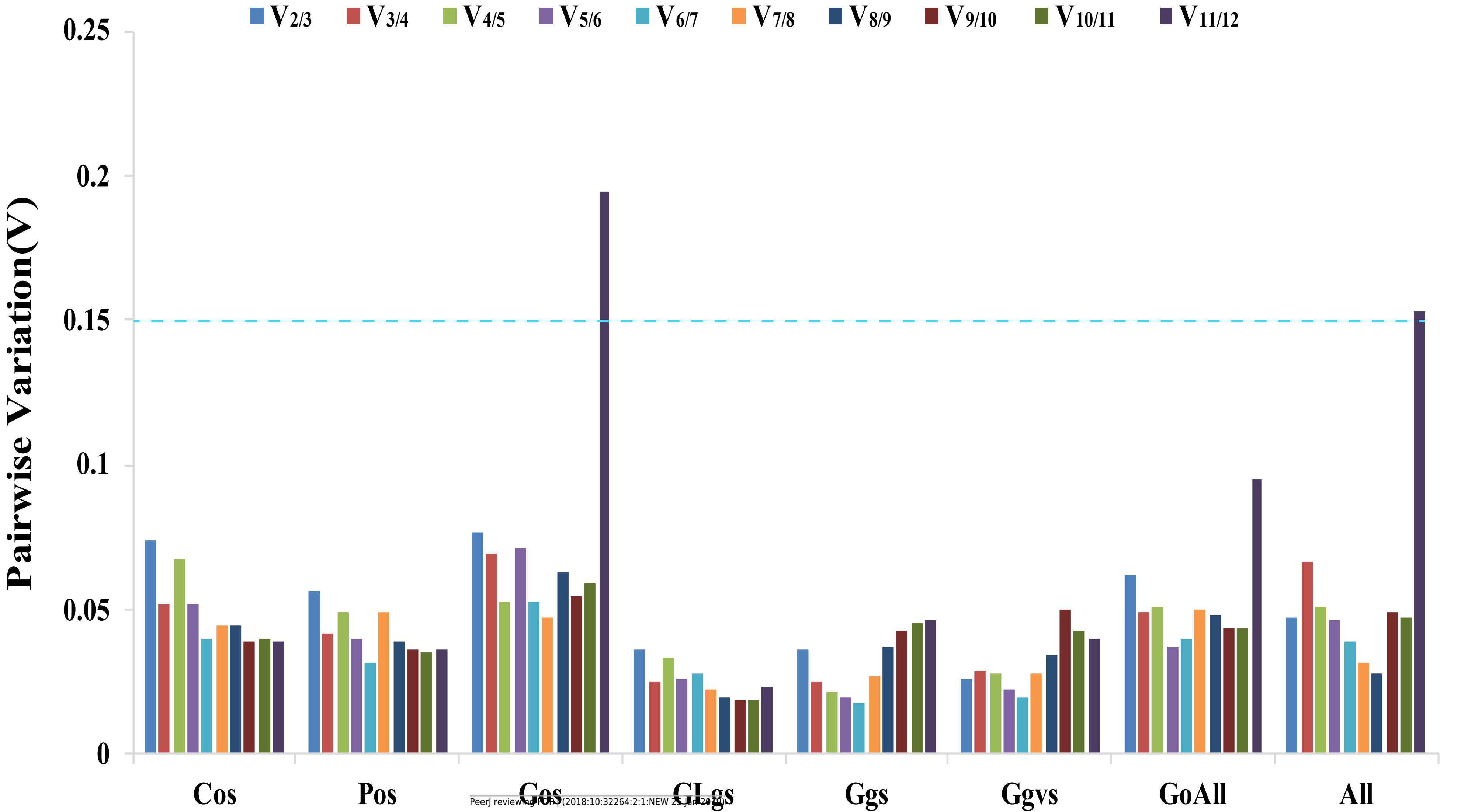
Graft union of cucumber-pumpkin grafted plants.

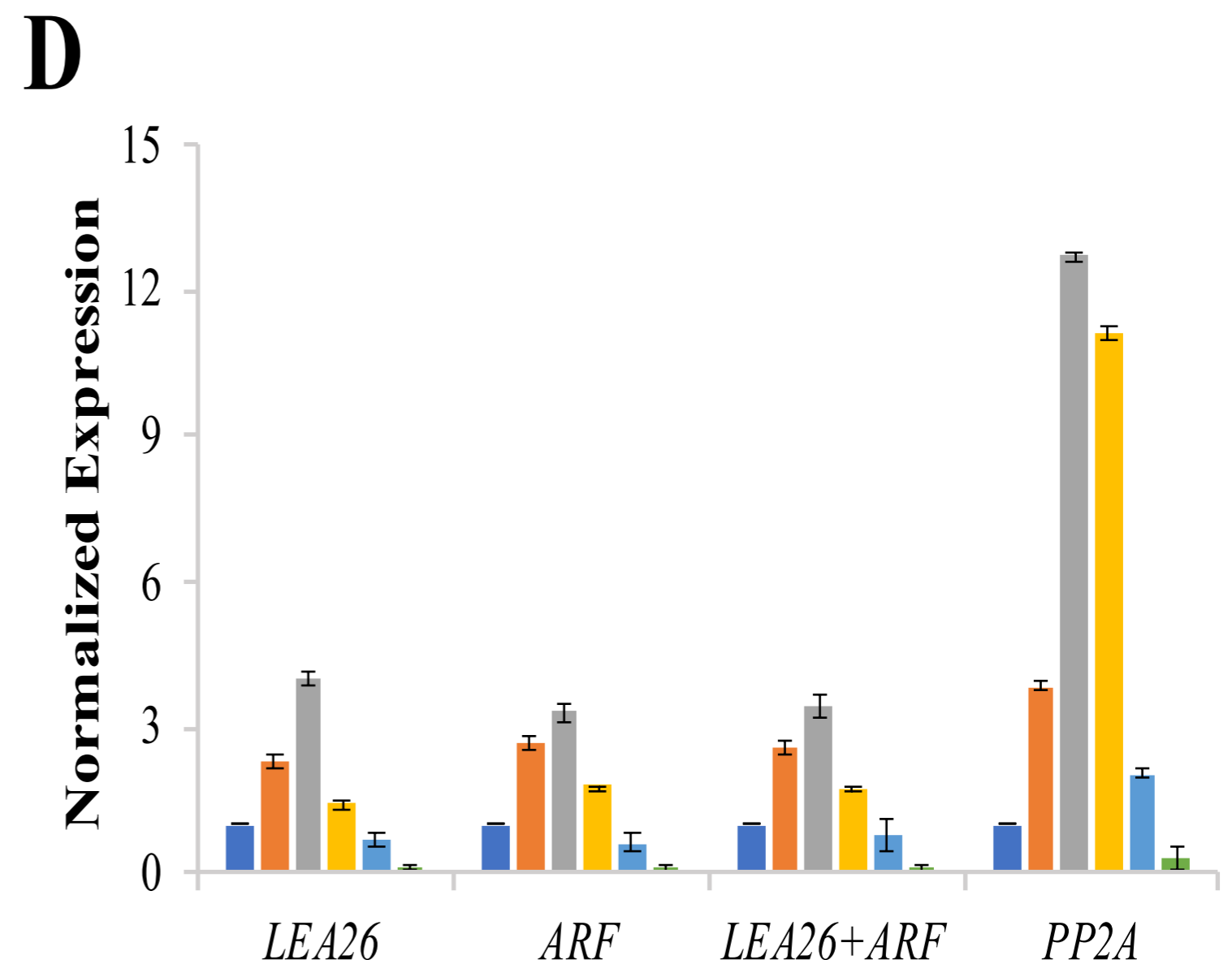
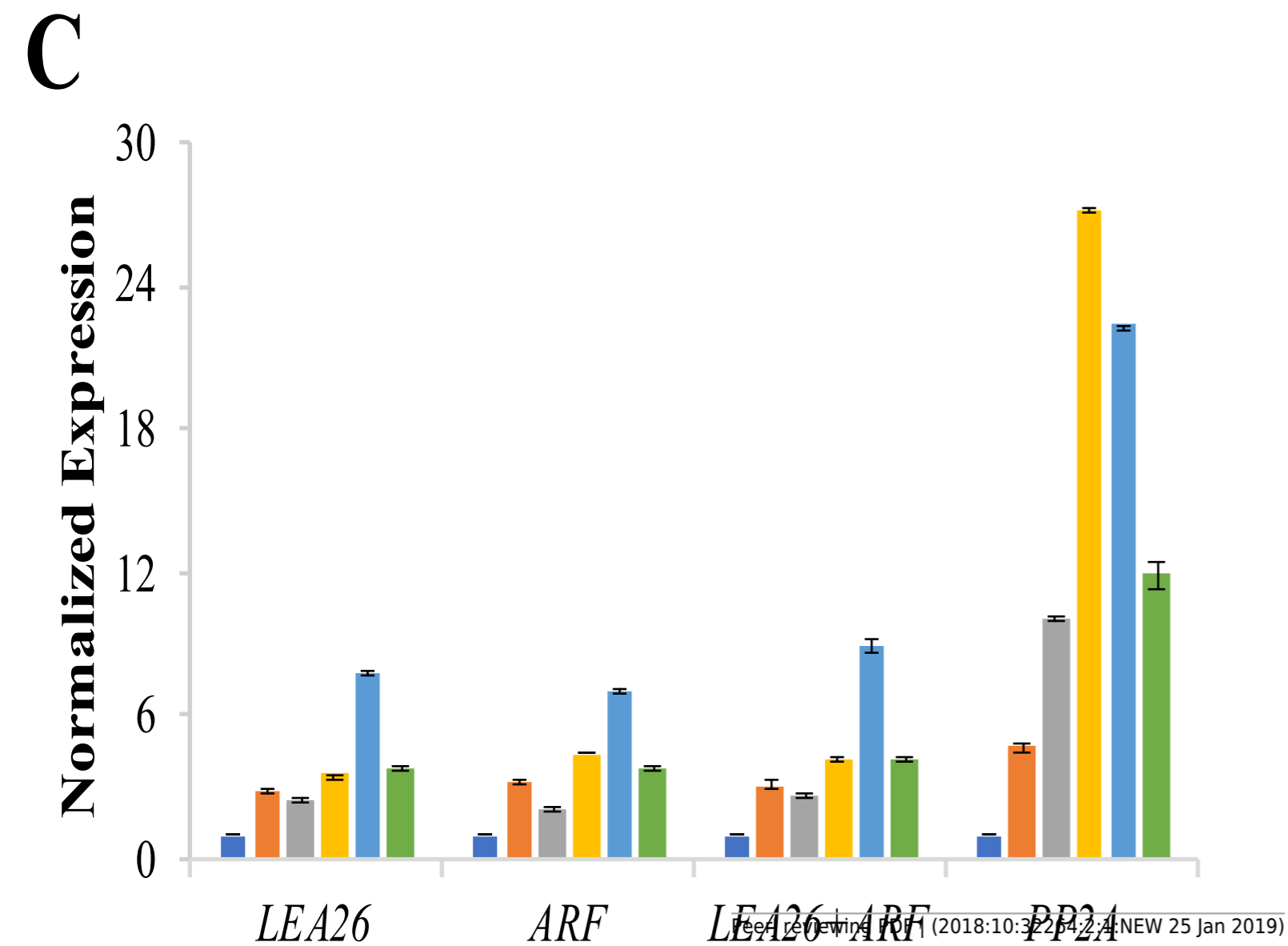
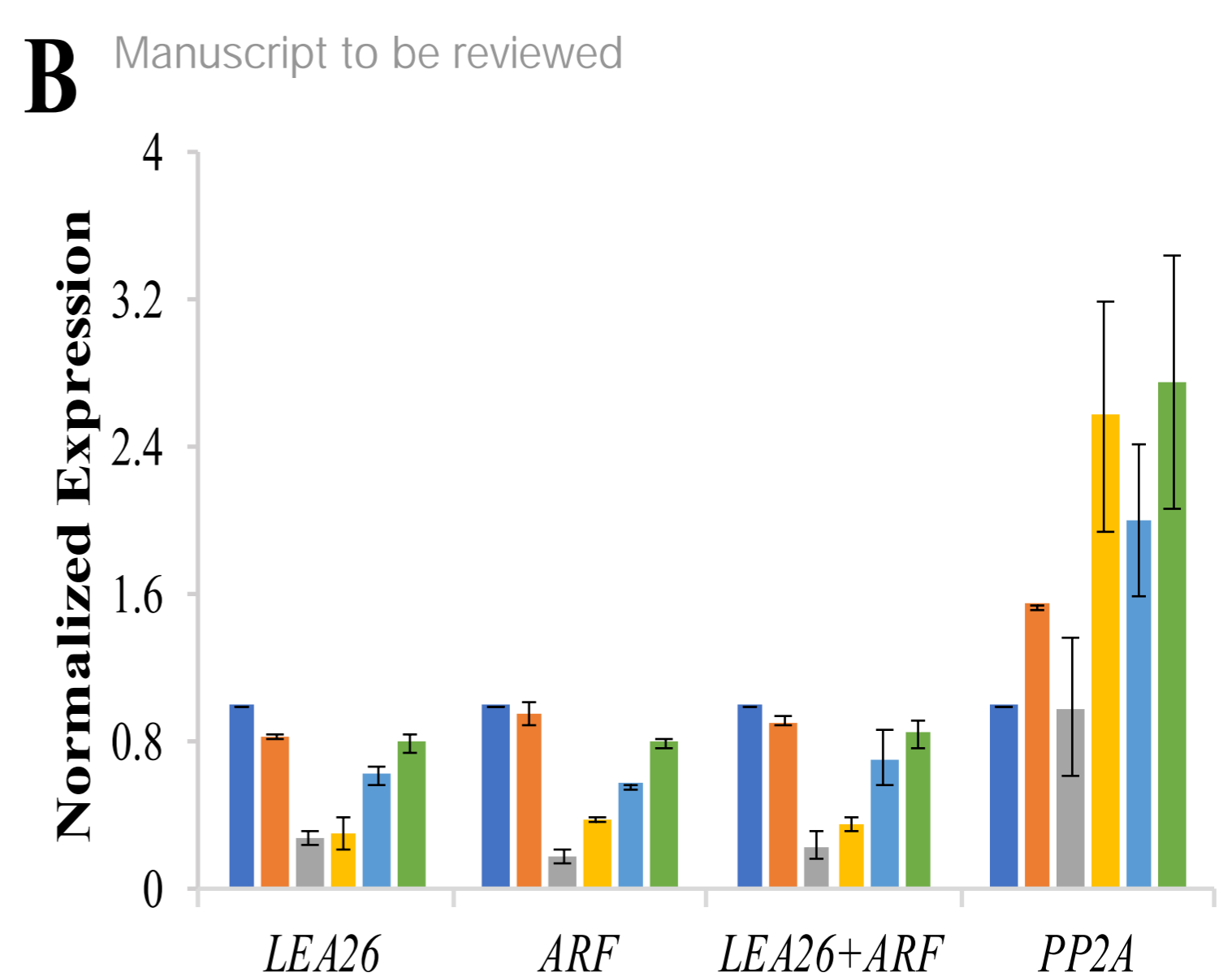
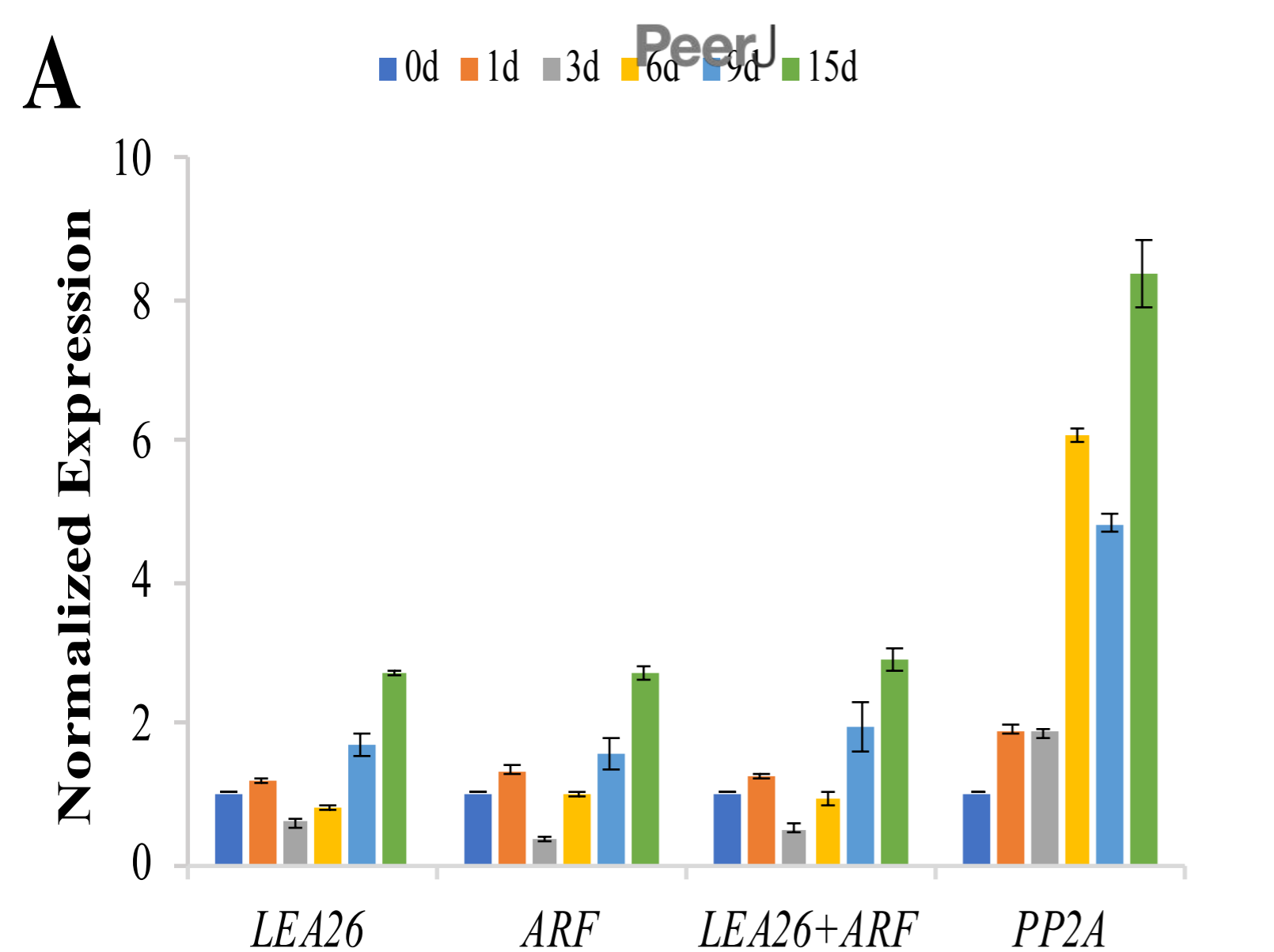
Red box indicates graft union of cucumber-pumpkin grafted plant, and the upper is scion-cucumber, the lower part is rootstock-pumpkin. A cucumber cultivar (*Zhongnong No.26*)was used as the scion, a pumpkin cultivar (*Jinxinzhen No.5*) was used as the rootstock. Graft union of cucumber-pumpkin grafted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

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.

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.

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.

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

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