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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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.

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Introduction

Cucumber (*Cucumis sativus* L.) is one of the most widely cultivated vegetable crops in the world. Grafted cucumber plants are popular because they are more resistant to soil-borne diseases, show increased tolerance to abiotic stress, improved mineral nutrition uptake and use, and increased fruit yield and quality (Huang et al;2014). A cucumber scion is usually grafted onto pumpkin (*Cucurbita moschata*Duch.) rootstock (Huang et al., 2014; Lee et al., 2010).

Grafting conveys advantages over each individual parent plant, but the resulting plant is also more complicated than the parents. The graft union is a critical part of combining the scion and rootstock. Connecting them correctly results in successful grafting and the establishment of complex communication between rootstock and scion. Physiological and biochemical studies of cucumber–pumpkin grafted plants have been carried out for several decades (Ahn et al., 1999; Yang et al., 2006; Haroldsen et al., 2012; Li et al., 2014), however, there have been few studies analyzing gene function, transcription or expression in cucumber–pumpkin grafted plants, as most pumpkin genes were unknown. Now, the entire cucumber (http://cucurbitgenomics.org/organism/2) (Huang et al., 2009) and pumpkin (http://cucurbitgenomics.org/organism/9) (Sun et al., 2017) genomes have been published, enabling further studies on the molecular biology of these species.

Gene expression analysis is fundamental to elucidate the molecular mechanisms underlying various biological processes (Bustin et al., 2005). qRT-PCR is the most common technique used to study gene expression because of its high sensitivity, accuracy, specificity, cost-effectiveness and reproducibility (Bustin et al., 2002; Nolan et al., 2006; Derveaux et al., 2010). However, 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). To avoid this, stable reference genes should be used to normalize the gene expression data. Appropriate reference genes should be systematically evaluated across various environments (varieties, tissues, experimental treatments and developmental stages) before being applied to qRT-PCR analysis (Bustin et al., 2009; Guenin et al., 2009; Sgamma et al., 2016). However, there have not previously been any systematic studies performed on grafted cucumber plants to determine reliable reference genes.

Common reference genes like *ACT* (*actin*), *TUA* (*tubulin*), *CYP* (*cyclophilin*), *UBI-1* (*ubiquitin*), and *EF-α* (*elongation factor*) are considered to be stably expressed in various plants (Duan et al., 2017; Obrero et al., 2011; Tashiro et al., 2016; Niu et al., 2017) and have been used for gene expression studies in cucumber (Wang et al., 2009, Migocka and Papierniak, 2011; Warzybock and Migocka, 2013). 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 been successfully applied to both cucumber and pumpkin in specific environments, including powdery mildew, salinity, cold, dehydration, H_2O_2, and abscisic acid (ABA) treatments (Berg et al., 2015;Cao et al., 2017; Reda et al., 2018). Unfortunately, there is no single confirmed reference gene exhibiting uniform and stable expression under different experimental conditions.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.Therefore, it is necessary to identify one or more reference genes under different experimental conditions prior to carrying out gene expression studies (Duan et al., 2017).

In this study, traditional reference genes from published research and new ones based upon their coefficients of variation (CVs) and expression intensity in our RNA-seq data from cucumber–pumpkin grafted plants at different stages were selected for further analysis. Twelve genes were investigated in this study, eight traditional reference genes, *ACT*, *CYP*, *CACS*, *TUA*, *TIP41*(*tonoplast intrinsic protein*), *F-Box* (*F-box protein*), *RPL36Aa*, and *PP2A*, and four new genes screened by RNA-seq analysis, *UBC* (*Ubiquitin conjugating enzyme*), *ARF* (*ADP-ribosylation factor-like protein*), *LEA26* (*Late-embryogenesis abundant protein 26*), and *40SRPS8* (*40S ribosomal protein S8*). These genes were evaluated to validate their use as stable reference genes for qRT-PCR in different organs, at different stages, in different varieties and under stress conditions in cucumber, pumpkin, and cucumber–pumpkin grafted plants. To determine the appropriate reference genes, four statistical tools were used to evaluate the accuracy of these candidate genes: the ΔCt method (Silver et al., 2006), geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004). Comprehensive stability rankings were generated by RefFinder (Xie et al., 2012). Additionally, the genes csa*CYCD3;1* (Csa2G356610), csa*RUL* (Csa3G895630), cmo*RUL* (CmoCh15G013320) and cmo*PIN* (CmoCh15G009810), which are thought to be related to graft union healing in grafted cucumber(*Table S1*), were investigated as a case study to evaluate the effectiveness of the reference genes identified in this study.In our study, *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. The results obtained in this study will be useful in many further gene expression analyses in cucumber, pumpkin, and their grafted plants.

Materials & Methods

Plant Materials and Treatments

Cucumber (*Cucumis sativus* L.) and pumpkin (*Cucurbita moschata*Duch.) were planted in an artificial chamber at the farm of the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China at a temperature of 28°C/20°C (day/night) with a photoperiod cycle of 12/12 h and 60%–70% relative humidity. Cucumber variety 'Zhongnong No. 26' was used as the scion and pumpkin variety 'Jinxinzhen No. 5' was used as the rootstock. Seeds of the scion and rootstock were sown in 50-cell and 32-cell polystyrene trays(54cm*28cm*5cm), respectively, containing commercial organic substrates (Vpeatmoss:Vvermiculite:Vperlite = 1:1:1). The environmental conditions for germination were 25–28°C and 85%–90% relative humidity. The pumpkin seeds were sown three days before the cucumber seeds. When cotyledons of the scion were fully open and the first true leaf of the rootstock started to develop (9–10 d after sowing), the plants were grafted using the hole insertion grafting method as previously described(Miao et al., 2018) (*Fig S1*). Autografts were carried out for both cucumber and pumpkin, as well as cucumber–pumpkin heterografts. The grafted seedlings were maintained at a temperature of 30°C/22°C (day/night), a constant humidity of 95%–100% and a dim light of 50 PPFD for the first 5 days, then the light density was slowly increased from 50 to 500 PPFD and the humidity was decreased from 95% to 60%, while the other environmental conditions were unchanged. For the autograft cucumber and pumpkin plants, samples of the leaves, stems and roots were harvested when the seedlings had two true leaves. For cucumber grafted onto pumpkin, samples of the leaves, the stem of the scion, the graft union (*Fig1*), the stem of the rootstock, and the roots were harvested. For the cold stress experiment, when the grafted cucumber had two leaves, seedlings were exposed to temperatures of 12°C in a chamber, and samples of the graft union were harvested at 0, 5, 12 and 24 h of stress treatment. To investigate the graft union healing process, samples of the graft union were harvested 0, 3, 6, 9 and 15 d after grafting. For experiments with varieties, cucumber varieties 'Xintaimici' and 'Zhongnong No. 26' were used as scions and pumpkin varieties 'Zhongguonangua No. 26', 'Jinxinzhen No. 5' and 'Huofenghuang' were used as rootstocks. The graft combinations were 'Xintaimici–Zhongguonangua No. 26', 'Xintaimici–Jinxinzhen No. 5', 'Xintaimici–Huofenghuang', 'Zhongnong No. 26–Jinxinzhen No. 5', and 'Zhongnong No.26–Huofenghuang'. Graft unions were harvested whengrafted plants had two true leaves. For each treatment, three independent biological replicates were achieved. All samples were immediately frozen in liquid nitrogen and stored at −80°C.

RNA Isolation and cDNA Synthesis

The RNAprep Pure Plant Plus Kit (Tiangen, Beijing, China) was used for total RNA extraction. Genomic DNA was eliminated from the total RNA using RNase-free DNase I. The RNA integrity was confirmed by 1.0% agarose gel electrophoresis. RNA concentrations were determined by NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), samples with an A260/A280 ratio of 1.8-2.2 and an A260/A230 ratio > 2.0 were used for further analyses. First-strand cDNA synthesis was performed using a FastQuant cDNA Synthesis kit (Tiangen, Beijing, China) according to the manufacturer's instructions.

Candidate Reference Gene Selection and Primer Design

Eight traditional candidate reference genes (*ACT, CYP, CACS, TUA, TIP41, F-Box, PRL36Aa* and *PP2A*) from published studies on cucumber, pumpkin,chicory, buckwheat, Lettuce and mangrove treewere selected (Wan et al., 2010; Obrero et al., 2011;Delporte et al., 2015; Demidenko et al. 2011; Borowski et al., 2014;Saddheet al., 2018). For new candidate reference genes, we analyzed our transcriptomic data from the graft union.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 transcriptomelibraries, 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(data do not show). The genes with the most constant expression levels were defined as candidate reference genes (De Jong et al., 2007). We calculated the mean expression value, standard deviation, and coefficients of variation (CVs) based on the raw RNA-seq data, and CVs = standard deviation of RPKM/average of RPKM. Based on the requirements CV ≤ 0.2 and 300≤RPKM≤ 500 (Duan et al., 2017), we selected new reference genes by removing overabundant genes with low expression levels.With requirements of evalue e-5, weused BLAST to determine the proteinsencoded 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). This resulted in ten and seven genes of cucumber and pumkin, respectively, which may be suitable as reference genes. A comparison of the relationship between cucumber and pumpkin by homology analysis is shown in *Table S2*. Finally, *UBC, ARF, LEA26* and *40SRPS8* were selected as candidate reference genes based on preliminary experiments of single PCR product in agarose gel electrophoresis (data do not show). 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 and a single peak of the melting curve in qRT-PCR were used to ensure the specificity of the primers for the candidate reference genes.

qRT-PCR Assay

qRT-PCR was performed on an Agilent Stratagene Mx3000P Real-Time PCR machine (Agilent Stratagene, USA) using SYBR® Premix Ex Taq™ (TliRNaseH Plus) (TaKaRa, Dalian, China). Each 20 µl reaction mixture contained 2 µl of cDNA template, 0.4 µl of each primer, 0.4 µl of ROX dye, 10 µl of 2× SYBR Premix Ex Taqand 6.8 µl of ddH$_2$O. The qRT-PCR reaction conditions were as follows: 94°C for 30 s, 40 cycles of 94°C for 5 s, then 60°C for 34 s. A melting curve was determined by increasing the amplification temperature from 60–95°C, with a temperature increment of 0.5°C every 5 s. All samples were performed with three technical replicates, and samples without template were used as a control. 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 (Takara, Japan).

Gene Expression Stability Analysis

To evaluate the expression levels of each reference gene, we drew boxplots of the Ct values for the 12 candidate reference genes (*Fig 2*). Four statistical tools, the ΔCt method (Silver et al., 2006), geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004), were used to evaluate the stability of the 12 candidate reference genes at various treatment durations. The raw Ct values of the reference genes were transformed into the correct input files according to the requirements of the software. Finally, a comprehensive ranking of the reference genes was generated using RefFinder (Duan et al., 2017).

Validation of Reference Gene Stability

To confirm the reliability of the selected reference genes, the relative expression levels of three genes involved in xylem development were measured during graft union healing in grafted cucumbers(*Table S1*). Samples of the graft union of cucumber–pumpkin grafted plants were harvested at 0, 1, 3, 6, 9 and 15d after grafting. The most stable reference genes (*LEA26,ARF* and *LEA26*+*ARF*), and the least stable reference gene (*PP2A*) ranked by RefFinder were used for normalization. Comparative gene expression levels of csa*CYCD3;1* (Csa2G356610), csa*RUL* (Csa3G895630), cmo*RUL* (CmoCh15G013320) and cmo*PIN* (CmoCh15G009810) were calculated using the $2^{-\Delta\Delta Ct}$ method. Three technical replicates were performed for each biological sample.

Results

Evaluation of Primer Specificity and Amplification Efficiency

Eight genes used traditionally (*ACT*, *CYP*, *CACS*, *TUA*, *TIP41*, *F-Box*, *PRL36Aa* and *PP2A*) and four potentialreference genes (*UBC*, *ARF*, *LEA26* and *40SRPS8*) were selected for qRT-PCR analysis. To validate the primer specificity, specific bands in cucumber, pumpkin and grafted cucumber were checked by 1% agarose gel electrophoresis (*Fig S2*). The product lengths were consistent with the expected lengths, and a single sharp peak was observed in the melting curves for cucumber, pumpkin and grafted cucumber (*Fig S3, S4A*). In our study, amplification efficiency (E) ranged from 0.86 to 1.13 with the correlation coefficients (R^2) of the standard curve varying from 0.986 to 0.999 (*Table 1*, *Fig S4B, S5*).

Expression Levels and Variations in Candidate Reference Genes

The transcript abundances of the 12 candidate reference genes were assessed by the Ct values from the qRT-PCR incucumber, pumpkin and grafted cucumber.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.

Expression Stability Analysis of Candidate Reference Genes

To evaluate the stability of the 12 candidate reference genes in our study, theΔCt 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 arejudged 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 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*).

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*).

Validation of the Selected Reference Genes

To confirm the stability of the selected reference genes, the expression levels of *csaCYCD3;1* (Csa2G356610), *csaRUL*(Csa3G895630), *cmoRUL*(CmoCh15G013320), and *cmoPIN*(CmoCh15G009810), which are possibly important during the graft union healing process(*Table S1*), were examined using*LEA26*, *ARF*, *LEA26+ARF*, and *PP2A* as reference genes for normalization. RefFinder analysis had shown that *LEA26* and *ARF* were the most suitable reference genes and *PP2A* was the least suitable reference gene in the graft union during the healing process (*Table 2, S3*).

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(*Fig4*). The expression levels of *csaCYCD* and *csaRUL* were significantly downregulated at 3 d and 6 d compared to 1 d after grafting. However, these values were markedly higher when *PP2A* was selected for normalization. Compared to 0 d after grafting, *cmoPIN* expression was clearly downregulated at the graft junction at 6 d, 9 d and 15 d after grafting when using *LEA26*, *ARF*, or *LEA26+ARF* as reference genes, while this value was abnormally upregulated when *PP2A* was used as a reference for normalization. Similarly, *cmoRUL* expression levels were extremely upregulated at the graft junction 6 d after grafting when using *PP2A* as the reference gene compared to the levels determined using the most stable reference genes (*LEA26*, *ARF* and *LEA26+ARF*).

Discussion

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, includingwater, 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 RefFinderare 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 (*Fig3*). 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), *Cichoriumintybus*(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 (*Table 2*), 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 (*Fagopyrumesculentum*) (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 theGgs. 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 validatereliable reference genes prior toqRT-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

Grafting also assemble desirable roots and shoots to generate chimeras that are more vigorous, more pathogen resistant, and more abiotic stress resistant (Melnyk, 2017). To our knowledge, cucumber, pumpkin and their grafted plants weresimultaneously 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.

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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.

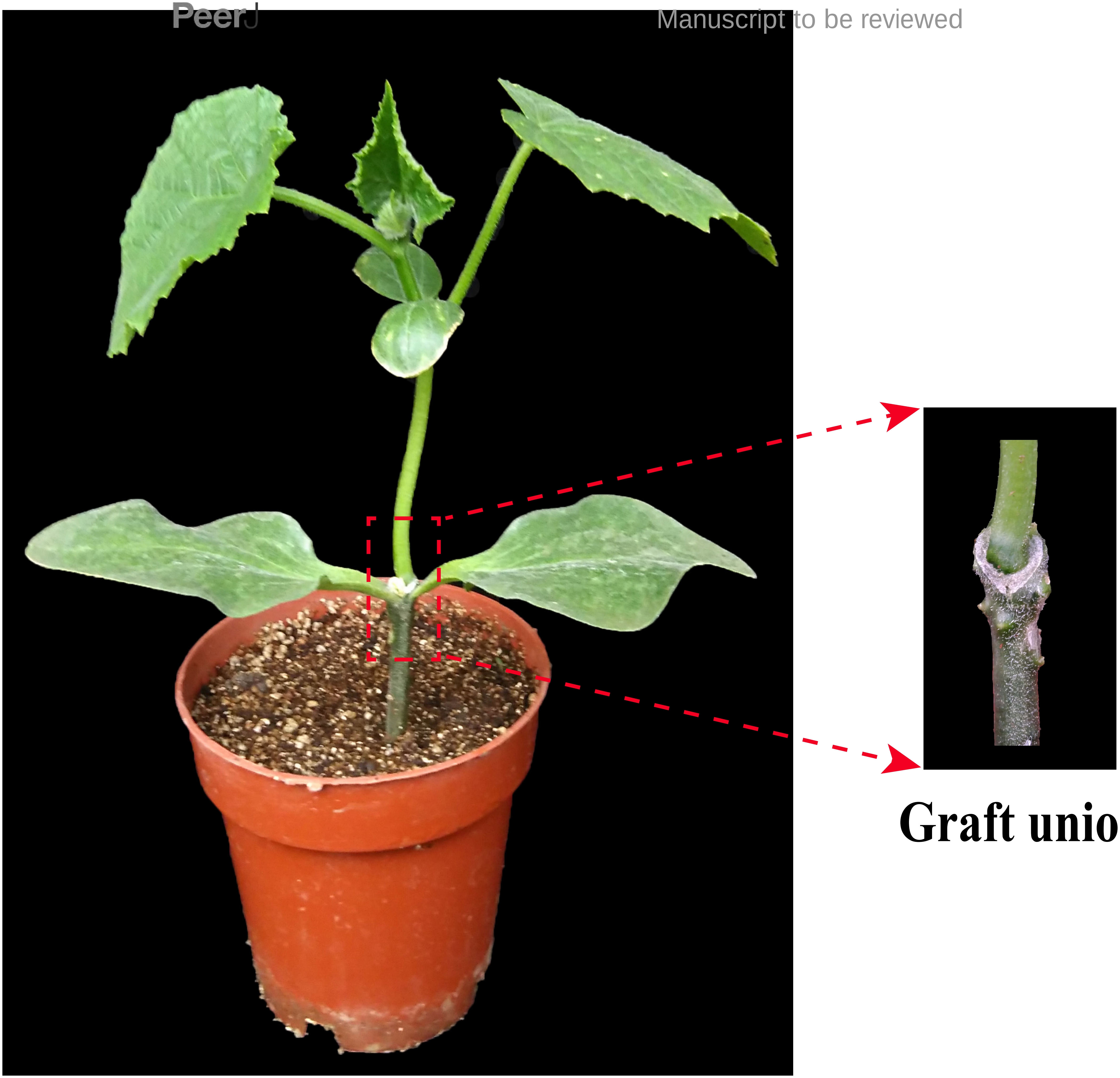
Graft union
Cucumber/pumpkin

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.

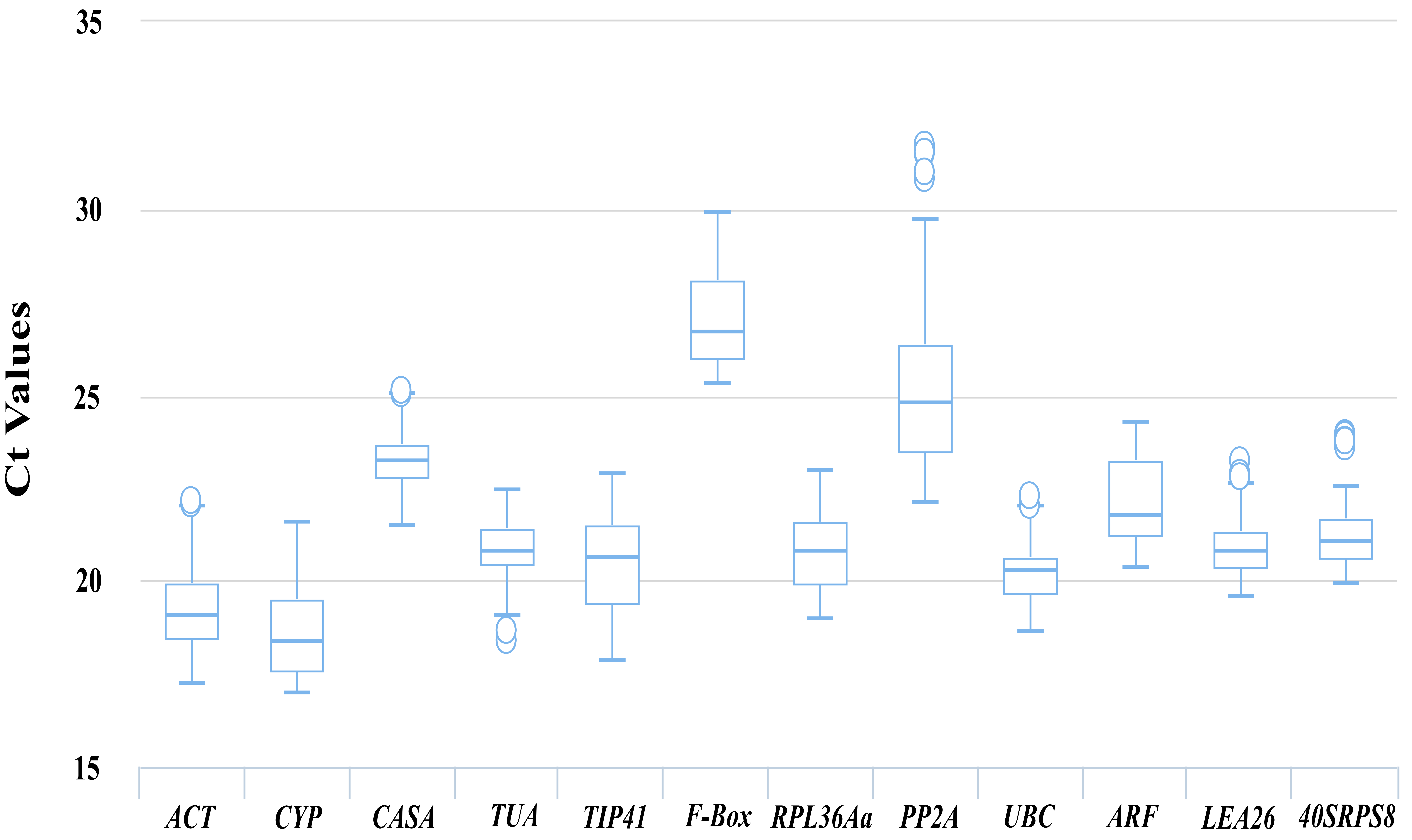
Ct Values
35
30
25
20
15
ACT
CYP
CASA
TUA
TIP41
F-Box
RPL36Aa
PP2A
UBC
ARF
LEA26
40SRPS8

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.

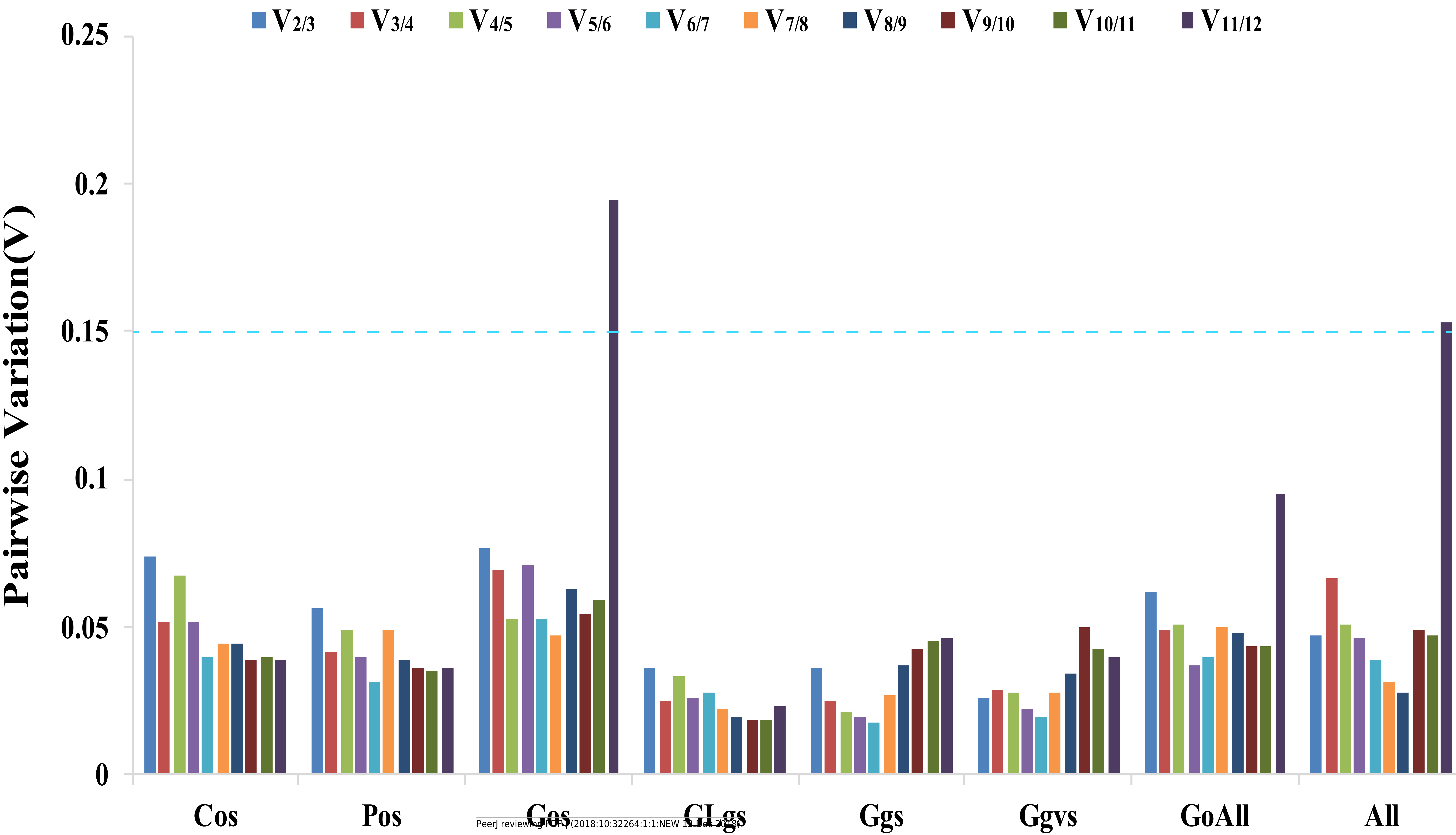
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
V8/9
V9/10
V10/11
V11/12
Pairwise Variation(V)
0.25
0.2
0.15
0.1
0.05
0
Cos
Pos
Gos
GLgs
Ggs
Ggvs
GoAll
All

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.

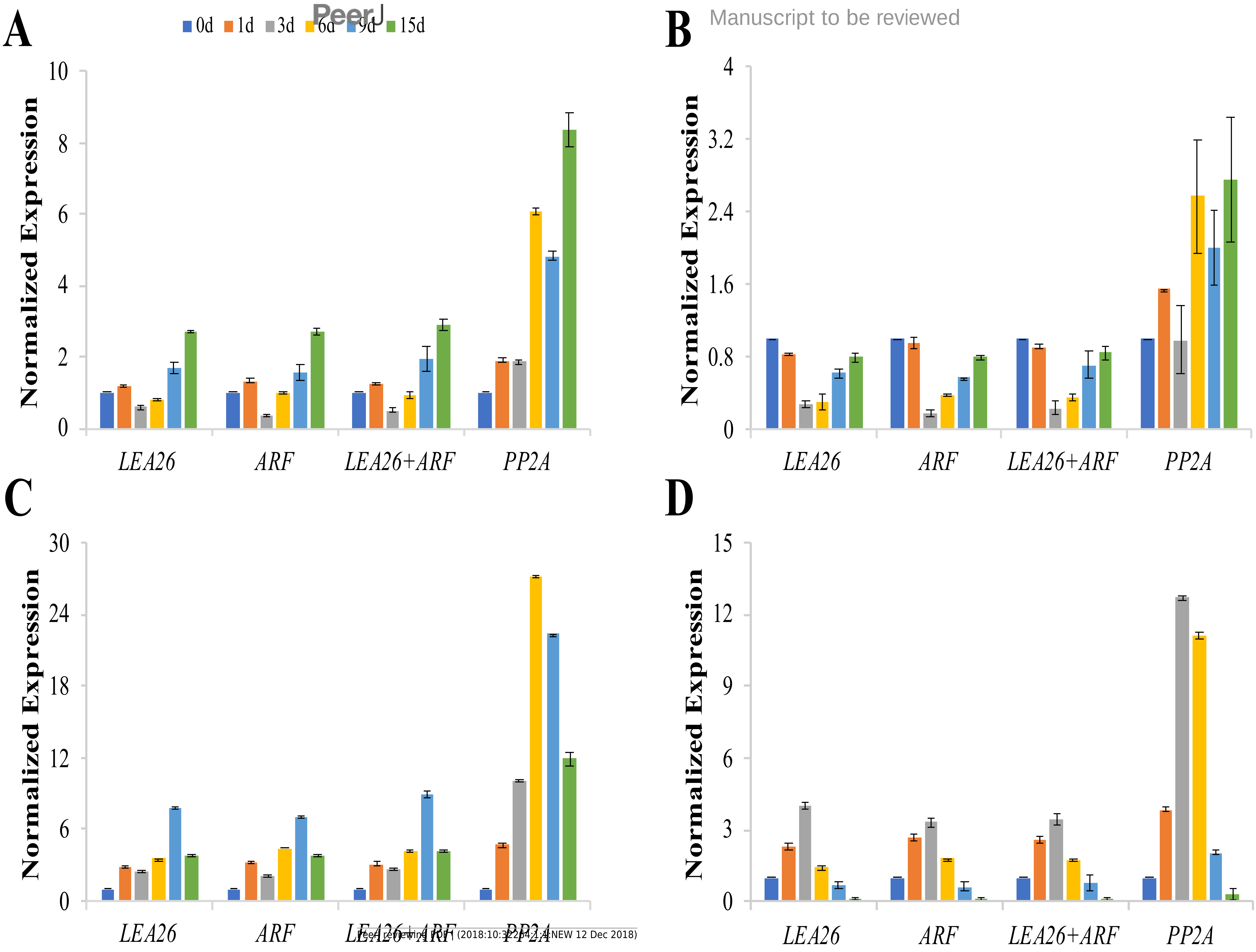
A
0d 1d 3d 6d 9d 15d
Normalized Expression
0
2
4
6
8
10
LEA26
ARF
LEA26+ARF
PP2A
B
Normalized Expression
0
0.8
1.6
2.4
3.2
4
LEA26
ARF
LEA26+ARF
PP2A
C
Normalized Expression
0
6
12
18
24
30
LEA26
ARF
LEA26+ARF
PP2A
D
Normalized Expression
0
3
6
9
12
15
LEA26
ARF
LEA26+ARF
PP2A

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.

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

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.

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*