Selection and Validation of Reference Genes for Gene Expression Studies Using Quantitative Real-Time PCR in Prunus Necrotic Ringspot Virus-Infected Cucumis sativus

Zhenfei Dong 1,2, Binhui Zhan 1,* and Shifang Li 1,*

1 State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China; dongzhenfeiqaz@163.com
2 Department of Fruit Science, College of Horticulture, China Agricultural University, Beijing 100193, China
* Correspondence: binhoiuzhan@126.com (B.Z.); sfli@ppcaas.cn (S.L.)

Abstract: Several members of the genus Ilarvirus infect fruit trees and are distributed worldwide. Prunus necrotic ringspot virus (PNRSV) is one of the most prevalent viruses, causing significant losses. Cucumis sativus can be infected by several ilarviruses, leading to obvious symptoms, including PNRSV, which suggests that cucumbers could be good hosts for the study of the pathogenesis of ilarviruses. Real-time quantitative PCR is an optimal choice for studying gene expression because of its simplicity and its fast and high sensitivity, while its accuracy is highly dependent on the stability of the reference genes. In this study, we assessed the stability of eleven reference genes with geNorm, NormFinder, ∆Ct method, BestKeeper, and the ranking software, Reffinder. The results indicated that the combined use of EF1α and F-BOX was the most accurate normalization method. In addition, the host genes AGO1, AGO4, and RDR6 were selected to test the reliability of the reference genes. This study provides useful information for gene expression analysis during PNRSV infection and will facilitate gene expression studies associated with ilarvirus infection.

Keywords: reference genes; real-time PCR; gene expression; Cucumis sativus; virus-infected

1. Introduction

Gene expression analysis is one of the important and efficient approaches to investigating gene function and revealing phenotypic differences in biological research [1]. Microarray analysis [2], transcript profiling [3], and real-time quantitative polymerase chain reaction (RT-qPCR) [4] are common methods used to identify gene expression patterns and compare RNA levels at transcriptional levels among different populations. In particular, RT-qPCR has been widely utilized in the quantification of the expression levels of single or several genes because of its simplicity, speed, sensitivity, accuracy, and relatively low cost [5]. RT-qPCR has also been used to evaluate the accuracy of microarray analysis and transcriptome data [6]. The selection of appropriate reference genes to ensure the accuracy of transcript normalization is both a prerequisite and the most prominent challenge in the performance of RT-qPCR analyses [7]. The use of reference genes that are not stable has a significant impact on the results, even leading to different conclusions [8]. The ideal reference genes are usually stably expressed across all the compared samples or groups, regardless of the experimental conditions, tissue differences, or treatments [9]. However, there is no universal gene that can satisfy all of the requirements [10]. Some housekeeping genes, including ACTIN, TUBULIN, UBQUITIN, and GAPDH, are the most commonly used reference genes in RT-qPCR tests; however, they vary substantially under different conditions [11–13]. A growing body of research indicates that it is critically important to evaluate the stability of the candidate reference genes experimentally for accurate quantification analysis case-by-case, which depends on specific species, the sample or tissue type, different treatments, experimental conditions, etc. [14,15].
Recently, a number of tools have been developed to conduct the comparison of stability analyses of candidate genes and help to select the most suitable reference genes. The free online analytical programs, geNorm (http://medgen.ugent.be/~jvdesomp/genorm, accessed on 8 December 2021) [16], NormFinder (http://www.mdl.dk/publications/normfinder.htm, accessed on 10 December 2021) [17], and BestKeeper (http://www.gene-quantification.de/bestkeeper.html, accessed on 15 December 2021) [18] are usually used in combination based on different algorithms. Other tools, such as Reffinder (http://blooge.cn/Reffinder/, accessed on 20 December 2021) [19] and RankAggreg [20], can be used to calculate the ranks of candidate genes. These tools have been successfully used to validate reference genes in different plant species, such as Nicotiana tabacum [21], Eleusine indica [22], Conyza bonariensis [23], Conyza canadensis [23], Alopecurus myosuroides [13], Sorghum bicolor [24], Avena fatua [25], Salix matsudana [26], and Lilium spp. [27], under a variety of conditions, including biological and abiotic stress.

Prunus necrotic ringspot virus (PNRSV) is a member of the genus Ilarvirus in the family Bromoviridae, which was first identified in peach (Prunus persica L.) in 1941. It is one of the most economically important and prevalent viruses, and mainly infects stone-fruit trees, including peach [28], nectarine (P. persica) [29], almond (P. dulcis) [30], sweet cherry (P. avium) [31], sour cherry (P. cerasus) [32], plum (P. domestica) [33], and apricot (P. armeniaca) [34], causing significant losses; it can also infect apple (Malus domestica) [35], rose (Rosa chinensis) [36], cucumber (Cucumis sativus) [37], hops (Humulus) [38], etc. According to research by Pallas et al., PNRSV infection in Prunus species can reduce bud-take in nurseries, decrease fruit growth by 10–30%, induce fruit-yield losses by 20–60%, and delay fruit maturity [39]. Recently, many studies focused on the development of viral detection techniques and the culture of virus-free propagation materials, which reduced the incidence of PNRSV greatly in the production [40]. However, there is little documented evidence as to its pathogenic mechanism. Cucumber is the natural host of PNRSV and shows symptoms of mosaic leaves and severe stunting once infected (Figure 1) [41]. Furthermore, cucumber can also be infected by other ilarviruses from woody plants, which cause obvious symptoms in laboratory conditions involving agrobacterium-mediated infectious clones, such as apple mosaic virus [42] (ApMV), apple necrotic mosaic virus (ApNMV, data not shown), and prune dwarf virus [43] (PDV). Furthermore, the availability of the complete genomic sequence of cucumber and the successful application of virus-induced gene silencing (VIGS) vectors on cucumber, such as cucumber green mottle mosaic virus (CGMMV)-based [44] and tobacco ringspot virus (TRSV)-based vectors [45], has also contributed to the functional genomics research on cucumber and increased the utilization of cucumber as a model plant in the mechanistic research on ilarviruses from woody plants.

In this study, eleven potential reference genes (GAPDH, L23, PP2A, F-BOX, EF1α, TUA, CYP, CACS, TIP41, UBI-1, and UBI-ep) were selected according to previous research, and we evaluated which of them would be suitable for normalization analysis in specific conditions (at 12 days post-PNRSV-infection) in C. sativus. After the validation of the primer specificity, four programs (geNorm, NormFinder, ∆Ct, and BestKeeper) and the ranking software Reffinder were used to analyze the stability of the candidate reference genes, which indicated that EF1α and F-BOX used in combination were the most accurate normalization method. In addition, the three key host genes associated with the viral infection, AGO1, AGO4, and RDR6, were selected to test the reliability of the reference genes. This study is expected to provide a basis for reference-gene normalization in the context of PNRSV infection in C. sativus in future gene-expression research.
2. Materials and Methods

2.1. Plant Materials and Virus Inoculation

Seedlings of *C. sativus* were planted in a growth room under control conditions (28 °C day and 24 °C night, 16 h light and 8 h dark). The viral inoculation was conducted as previously reported. The PNRSV infectious clones with two vectors (one included RNA1 and RNA2 of PNRSV isolate Pch12, and another included RNA3 of PNRSV isolate Pch12) were transformed into *Agrobacterium tumefaciens* EHA105 and grown in Luria-Bertani medium containing kanamycin (100 mg/L) and rifampicin (25 mg/L). The agrobacterium cells were harvested by centrifugation and resuspended in the infiltration buffer (10 mM MgCl$_2$, 10 mM MES, and 150 µM acetosyringone) [37]. After incubation for 2 h at room temperature, the culture was diluted to an optical density of 1.0 at OD$_{600}$ nm. After mixing at equal volumes, the cultures were agroinfiltrated into the two expanded cotyledons of seedlings that were about 1 week old. The agroinfiltrated plants were grown in the same conditions.

2.2. Total RNA Isolation and First-Strand cDNA Synthesis

Total RNA was extracted from the new leaves of mock-inoculated or PNRSV-inoculated cucumber seedlings 12 days post-inoculation (dpi), using TRIzol reagent (TianGen, Beijing, China), according to the manufacturer’s instructions. One ug of total RNA was treated with DNase I and then a synthesis of first-strand cDNA was performed with random primer and M-MLV reverse transcriptase, using the PrimeScriptTM RT reagent Kit with gDNA Eraser (Perfect Real Time) (RR047A, Takara, Dalian, China). The mixture was incubated at 37 °C for 30 min and 85 °C for 5 s. The resulting cDNA can be stored at −80 °C for long-term use.

2.3. Selection of Candidate Reference Genes and Primer Design

Eleven widely used reference genes (*GAPDH*, *L23*, *PP2A*, *F-BOX*, *EF1α*, *TUA*, *CYP*, *CACS*, *TIP41*, *UBI-1*, and *UBI-ep*) were selected for this study. The reference sequences of these genes can be obtained from the NCBI database, and the accession numbers are listed in Table 1. The primer pairs of *GAPDH*, *L23*, *PP2A*, and *F-BOX* were designed using DNAMAN software, version 5.0 (Lynnon Biosoft, Quebec, QC, Canada). The conditions were set with the following parameters: melting temperature of 58–62 °C, GC content of 40–60%, primer length of 20–25 nt, and amplicon length of 80–250 bp. The specific primers
of EF1a, TUA, CYP, CACS, TIP41, UBI-1, and UBI-ep were based on previous studies. The primer sequences and the associated parameters are listed in Table 1. The specificity of primers was evaluated by PCR amplification using cucumber cDNA as template. The amplified products were subjected to 2% agarose gel electrophoresis and sequenced to verify the targeted gene.

Table 1. Primers used in this study. Tm, annealing temperature; E: PCR amplification efficiency; R²: correlation coefficient.

| Gene      | Full Name                | Accession Number | Sequence (5’-3’) | Amplicon Length (bp) | Tm (°C) | E (%)       | R²     | References |
|-----------|--------------------------|------------------|------------------|----------------------|---------|-------------|--------|------------|
| GAPDH     | Glyceraldehyde-3-phosphate dehydrogenase | NM_001305758 | F: CATCAAGGAAACATGACTCCAC, R: TAAAGAACGAGGAAACAAAGC | 161      | 58         | 95.21   | 0.9928   | This study |
| LF3       | L3                        | XM_004140527    | F: ACAAGTTTCCCATCTAATC, R: GAAGACCATGACTCCAC    | 211      | 58         | 93.92   | 0.9946   | This study |
| PP2A      | F-box protein             | XM_004146712    | F: TGGAAACACTCCAGACCTCT, R: GGACCAACGCCGAGATTTTC | 224      | 58         | 94.37   | 0.9992   | This study |
| F-BOX     | F-box protein             | XM_011885920    | F: AGACCTCTATCTGATATGC, R: GCAACGACTAATCTCCCG | 166      | 58         | 96.24   | 0.9925   | This study |
| EFL1a     | Elongation factor 1-a     | DQ341381        | F: ATCGAATGTATGCCTCCGC, R: GGATCCAATGGTATGC   | 174      | 58         | 92.10   | 0.9958   | Wan et al., 2012 [46] |
| TUA       | α-Tubulin                 | AJ715498        | F: CACCTTCTGACACCTAGA, R: TCACACGCTGAAAGATGGA | 106      | 58         | 95.74   | 0.9938   | Wan et al., 2010 [47] |
| CYP       | Cyclophilin               | AP942800        | F: GCGAAGTACACAGGATCC, R: CAATCCCTCAACGCTTGCAC | 88       | 58         | 95.02   | 0.9984   | Wan et al., 2010 [47] |
| CACS      | AP-2 complex              | GT030018        | F: GACCTCTGTCGTCATATGAC, R: TCAACCTCCAGAGTGGA | 158      | 58         | 93.12   | 0.9924   | Warzybok et al. 2013 [48] |
| TIP41     | TIP41-like family protein | GW881871        | F: CACACGGATGATGGATGATTAC, R: GCGACCTTCATCCTCATATAAG | 221      | 58         | 91.99   | 0.9928   | Wan et al., 2011 [47] |
| UBI-1     | Ubiquitin-like protein    | AF104391        | F: CCAAGACCAACGCAACAGAC, R: AGTACCTCTGTATCATCGGG | 143      | 58         | 93.29   | 0.9990   | Wan et al., 2010 [47] |
| UBI-ep    | Ubiquitin extension protein | AY372537    | F: CACCAGACCCAGAAGAATGC, R: TAAACCTCTTACACACGAC | 220      | 58         | 92.93   | 0.9955   | Warzybok et al. 2013 [48] |
| AGC1      | Argonaute 1               | XM_011661337    | F: GGCTCTCTGACACCCACAC, R: GGCTCTCTGACACCCAC | 245      | 58         | 94.79   | 0.9987   | Warzybok et al. 2013 [48] |
| AGC4      | Argonaute 4               | XM_011655229    | F: GGCTCTCTGACACCCACAC, R: TGGCTCTCTGACACCCAC | 223      | 58         | 94.73   | 0.9982   | Warzybok et al. 2013 [48] |
| RDR6      | RNA dependent             | XM_011650266    | F: TCTCAGACCCAGACCCACAC, R: TGGCTCTCTGACACCCAC | 228      | 58         | 95.93   | 0.9961   | Warzybok et al. 2013 [48] |

2.4. RT-qPCR Analysis

After the validation of primer specificity, RT-qPCR reactions were carried out in 20 µL volume containing 10 µL of TB Green Premix Ex Taq II (Tli RNaseH Plus) (2×), 0.8 µL of each primer (10 µM), 0.4 µL of ROX Reference Dye II (50×), 2 µL of template (the diluted cDNA of different samples), and 6 µL of distilled water. Every treatment contained at least three biological replicates with three technical replicates in clear 96-well or 384-well plates. The RT-qPCR analyses were performed in the Applied Biosystems QuantStudio 6 Flex Real-Time PCR systems (Thermo fisher scientific, Waltham, MA, USA). The PCR program was set as follows: 95 °C for 30 s (denaturation), followed by 40 cycles of 5 s at 95 °C, 20 s at 58 °C, and 35 s at 72 °C, before, finally, dissociation stage was added to generate a melting curve (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s) to verify the specificity of PCR amplification. The E and R² were calculated from the raw data using LinRegPCR software (Academic Medical Centre, Amsterdam, the Netherlands).

2.5. Data Analyses for Expression Stability

Four tools, based on different algorithms, were selected to evaluate the stability of the 11 candidate reference genes, as follows: geNorm, NormFinder, ΔCt, and BestKeeper. The Ct values were directly used. The geNorm software, which is based on the principle that the expression ratio of two reference genes should be constant in all samples regardless of different groups, calculates the gene-expression M-value as the average pairwise variation of one gene with the other candidate genes. The cut-off value of 1.5 is used to assess the stability of the reference genes and the gene with lowest M-value refers is the most stable. In addition, geNorm calculates the optimal number of reference genes required
for accurate normalization by pairwise variation (V_{n/n+1}). The value of V_{n/n+1} < 0.15 indicates that no extra reference genes are required for normalization. NormFinder is used to rank the stability of the reference genes by the parameters of stability value (SV) within and among groups. Reference genes with the lowest SVs are considered the most stable reference gene. The \( \Delta C_t \) method identifies the potential reference genes by comparing the relative expression between gene pairs. BestKeeper analyses determine the most stable reference genes with the lowest coefficient of variation (CV) and the lowest relative standard deviation (SD). The candidate genes with SD higher than 1.0 are considered inconsistent and are cut off. The \( r \) and \( p \)-value are also important parameters to be considered. RefFinder is an additional web-based tool, which was used to comprehensively rank the order of the 11 candidate genes.

2.6. Relative Quantification of AGO1, AGO4, and RDR6

The EF1\( \alpha \) and F-BOX, the two most stably expressed genes, were used in combination as reference genes for the relative quantification of AGO1, AGO4, and RDR6. The geometric mean of the Ct values of two reference genes (multiplying the Ct values of the two reference genes and then taking the square root) was used as the reference Ct value for each biological replicate. The mock-inoculated samples were used as control and the relative quantifications of AGO1, AGO4, and RDR6 in PNRSV-inoculated samples were calculated by \( 2^{-\Delta \Delta C_t} \) method. The experiments were replicated three times, with at least three plants each time.

2.7. High-Throughput RNA-Sequencing and Analysis of the Sequence Data

Total RNA was isolated and purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the RNA amount and purity of each sample were quantified using NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). The RNA integrity was assessed by Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) with RIN number >7.0, and confirmed by electrophoresis with denaturing agarose gel. Poly (A) RNA was purified from 50 µg total RNA with Dynabeads Oligo (dT) 25-61005 (Thermo fisher scientific, Waltham, MA, USA), using two rounds of purification. Finally, we performed the \( 2 \times 150 \) bp paired-end sequencing (PE150) on an Illumina Novaseq™ 6000 platform (Illumina, Inc., San Diego, CA, USA). Raw reads used fastp software (https://github.com/OpenGene/fastp, accessed on 11 June 2021) to remove the reads that contained adaptor contamination, low-quality bases, and undetermined bases with default parameter. The mapping of clean reads onto the C. sativus reference genome was conducted using HISAT2 (http://daehwankimlab.github.io/hisat2, accessed on 12 June 2021). Next, StringTie (https://ccb.jhu.edu/software/stringtie, accessed on 12 June 2021) was used to perform expression level for all mRNAs from input libraries by calculating FPKM (total exon fragments /mapped reads (millions) \times \text{exon length} (kB)). RNA-seq data with details of datasets are available on the NCBI Sequence Read Archive BioProject-PRJNA837466 (https://www.ncbi.nlm.nih.gov/sra/PRJNA837466, accessed on 17 May 2022).

3. Results

3.1. Assessment of Primer Specificity and Amplification Efficiency

Eleven candidate reference genes were selected from among previously used genes for normalization in cucumber or other species. The sequences of the primers and their associated parameters are listed in Table 1. To identify the primer specificity, the amplified products were analyzed using 2% agarose gel electrophoresis. Only one clear band of the expected size appeared in each lane, and no primer dimers or non-specific amplification could be detected (Figure 2). The specific bands were cut off and sent to Sanger-sequencing. Blastn verified that the amplicons were from the targeted genes (data not shown).
To further assess the primer specificity, RT-qPCR was conducted. A single peak in the melting curve was obtained after the amplification of all 11 genes (Figure 3). The corresponding PCR amplification efficiencies (E) and the linear relationships between the Ct values and the log-transformed copies indicated by correlation coefficients ($R^2$) for all the tested reference genes were calculated from the RT-qPCR data (Table 1). The E values ranged from 91.99% to 96.24%, which were all within the acceptable range of 90–105%. Furthermore, the $R^2$ values ranging from 0.9924 to 0.9990 confirmed the specificity of the primer pairs.

3.2. Expression Levels and Variation in Candidate Reference Genes

In order to show the different transcriptional levels among the 11 candidate genes, the average Ct values were determined using all the experimental samples. The Ct values of
the candidate genes in all the samples are illustrated as a box plot in Figure 4. The average Ct values of the 11 genes ranged from 18.272 to 26.678. The GAPDH gene showed the highest expressive abundance, with the lowest Ct value of 17.727, while the F-BOX showed the lowest abundance with the highest Ct value of 27.548 (Figure 4). The Ct values for L23 (20.107–23.001) and TUA (20.796–25.152) showed the largest variation for one gene, whereas those for CYP (17.821–19.435) and GAPDH (17.727–19.489) showed the smallest variation.

Figure 4. Expression levels of the eleven candidate reference genes evaluated in prunus necrotic ringspot virus (PNRSV)-infected C. sativus. Values are given as the cycle threshold (Ct, mean of triplicate samples) and are inversely proportional to the amount of template. The three lines of the box represent the 25th quartiles, median, and 75th quartiles. The whiskers represent minimum and maximum values.

To further evaluate the expression stability of the candidate reference genes, four methods based on different algorithms were used to calculate the expression stability: geNorm, NormFinder, ∆Ct method, and BestKeeper.

3.3. Analyses of Candidate Reference Gene Stability Using Four Different Types of Software

3.3.1. geNorm Analysis

The raw Ct values from RT-qPCR were transformed into quantities for the geNorm analysis. The average gene-expression stability measurement (M-value) of the 11 candidate genes were obtained through the program, and the genes were ranked based on the M-value, from highest to lowest. The genes with the highest M-values showed lower stability, while the genes with the lowest M-values indicated higher stability. All of the 11 selected genes showed an acceptable level of expression stability with M < 0.702, which was below the cut-off value of 1.5 suggested by geNorm (Figure 5a). F-BOX and UBI-1 were the most stable genes, followed by EF1α, and the TUA and TIP41 genes showed the least stability according to geNorm. In addition, the optimal number of reference genes required for reliable normalization was also determined by geNorm. The pairwise value for two genes (V2/3) was 0.107, which was lower than the threshold of 0.15 (Figure 5b). This result indicated that two reference genes were sufficient for accurate analyses of the gene expression in the context of PNRSV infection.
Figure 5. Values of gene-expression stability of the eleven candidate genes. (a) geNorm-expression stability measurement (M-value). A lower M-value indicates more stable gene expression. (b) The optimal number of reference genes required for effective qRT-PCR data normalization by geNorm. Pairwise variation ($V_n/V_{n+1}$) between the normalization factors NF$_n$ and NF$_{n+1}$ (NF$_n$ is the expression values for the $n$ first-ranked candidate reference genes). (c) Expression stability of the candidate reference genes analyzed by NormFinder. A lower stability value indicates more stable gene expression. (d) The STDEV values analyzed by ΔCt method. A lower value indicates more stable gene expression.

3.3.2. NormFinder Analysis

NormFinder was used to evaluate the stability of the reference genes based on the SV parameter. Lower SV values meant higher stability. The SVs of the 11 candidate genes are shown in Table 2 and ranked in Figure 5c. The results of the NormFinder analysis illustrated that the two most stable genes were $EF1\alpha$ and $F-BOX$, and the two least stable candidate genes were $TUA$ and TIP41.

Table 2. Expression stability ranking of the nine candidate reference genes calculated by four different types of software. M-value: stability measurement; r: coefficient of correlation.

| Gene Name | geNorm | NormFinder | Delt Ct | BestKeeper | RefFinder |
|-----------|--------|------------|---------|------------|-----------|
|           | M-Value| Ranking | Stability | Ranking | Stability | Ranking | r Value | Ranking | Stability | Ranking |
| EF1\alpha | 0.310  | 3       | 0.213    | 1       | 0.56      | 1       | 0.873   | 1       | 1.73      | 1       |
| F-BOX     | 0.244  | 1       | 0.273    | 2       | 0.58      | 2       | 0.826   | 4       | 2.00      | 2       |
| UBI-1     | 0.244  | 1       | 0.412    | 4       | 0.64      | 4       | 0.765   | 5       | 3.46      | 3       |
| CYP       | 0.519  | 7       | 0.487    | 6       | 0.70      | 6       | 0.327   | 7       | 3.98      | 4       |
| PP2A      | 0.328  | 4       | 0.315    | 3       | 0.60      | 3       | 0.845   | 3       | 4.12      | 5       |
| GAPDH     | 0.459  | 6       | 0.523    | 7       | 0.73      | 7       | 0.387   | 8       | 4.92      | 6       |
| L23       | 0.361  | 5       | 0.471    | 5       | 0.68      | 5       | 0.761   | 6       | 5.44      | 7       |
| UBI-ep    | 0.584  | 8       | 0.586    | 8       | 0.73      | 7       | 0.356   | 9       | 7.11      | 8       |
| CACS      | 0.613  | 9       | 0.596    | 9       | 0.73      | 7       | 0.413   | 10      | 8.13      | 9       |
| TIP41     | 0.633  | 10      | 0.648    | 10      | 0.78      | 10      | 0.421   | 11      | 10.00     | 10      |
| TUA       | 0.702  | 11      | 0.930    | 11      | 1.01      | 11      | 0.856   | 2       | 11.00     | 11      |
3.3.3. ΔCt Analysis

The comparative ΔCt method identified that EF1α and F-BOX were the most two suitable genes, and TUA and TIP41 were the least suitable (Figure 5d). The ranking order of the 11 candidate genes was similar to that obtained by NormFinder analysis.

3.3.4. Bestkeeper Analysis

The three variables, SD, coefficient of correlation (r), and CV play key roles in gene-expression stability analyses using the Bestkeeper program. Candidate genes with SD values > 1 should be excluded, since they are considered inconsistent. In the study, the analysis of the data from all the candidate genes using Bestkeeper showed that all the candidate reference genes had SD values < 1. Across all the candidate genes, TUA had relatively high SD and CV values (SD TUA = 0.81 and CV TUA = 3.66), which can be considered to have been eliminated in the subsequent analysis (Table 3). In addition, the Bestkeeper analysis showed that the p-values of the genes CYP, GAPDH, UBI-ep, CACS, and TIP41 were over 0.05 (0.105 < p-value < 0.216), which meant they could also be excluded from the subsequent ranking. The remaining candidate genes were ranked from the most stable, with the highest coefficient of correlation, to the least stable, with the lowest value. EF1α (r = 0.873; p-value = 0.001) was the most stable gene, followed by PP2A (r = 0.845; p-value = 0.001) and F-BOX (r = 0.826; p-value = 0.001) (Table 2). These three genes (EF1α, F-BOX, and PP2A) were also the most stable genes identified by NormFinder analysis and the ΔCt method.

Table 3. Descriptive statistics of the eleven candidate reference genes by Bestkeeper analysis. n: number of samples; Geo Mean (Ct): geometric mean of Ct value; AR mean (Ct): arithmetic mean of Ct value; Min and Max (Ct): extreme values of Ct; SD: standard deviation; CV: coefficient of variation; Min and Max (x-fold): extreme values of expression levels; SD (±x-fold): standard deviation of the absolute regulation coefficients; r: coefficient of correlation.

| Ranking | EF1α | TUA | PP2A | F-BOX | UBI-1 | L23 | CYP | GAPDH | UBI-ep | CACS | TIP41 |
|---------|------|-----|------|-------|-------|-----|-----|-------|-------|------|-------|
| n       | 16   | 16  | 16   | 16    | 16    | 16  | 16  | 16    | 16    | 16   | 16    |
| Geo Mean (Ct) | 20.21 | 22.19 | 24.13 | 26.67 | 24.24 | 21.48 | 18.62 | 18.27 | 20.04 | 24.99 | 26.10 |
| AR Mean (Ct) | 20.22 | 22.22 | 24.13 | 26.68 | 24.25 | 21.49 | 18.62 | 18.27 | 20.05 | 24.99 | 26.11 |
| Min (Ct) | 19.43 | 20.80 | 23.11 | 25.74 | 23.22 | 20.11 | 17.82 | 17.73 | 19.09 | 23.82 | 24.75 |
| Max (Ct) | 21.24 | 25.15 | 24.97 | 27.55 | 25.31 | 23.00 | 19.44 | 19.49 | 21.25 | 26.12 | 26.89 |
| SD (±Ct) | 0.40 | 0.81 | 0.48 | 0.41 | 0.53 | 0.47 | 0.31 | 0.36 | 0.41 | 0.43 | 0.54 |
| Min (x-fold) | −1.72 | −2.63 | −2.02 | −1.91 | −2.03 | −2.59 | −1.74 | −1.45 | −1.94 | −2.24 | −2.55 |
| Max (x-fold) | 2.04 | 7.79 | 1.79 | 1.83 | 2.09 | 2.87 | 1.76 | 2.33 | 2.31 | 2.19 | 1.73 |
| SD (±x-fold) | 1.32 | 1.76 | 1.39 | 1.33 | 1.44 | 1.28 | 1.24 | 1.29 | 1.33 | 1.35 | 1.46 |
| coeff. of corr. (r) | 0.99 | 3.66 | 1.97 | 1.54 | 2.17 | 2.38 | 1.65 | 1.98 | 2.07 | 1.71 | 2.09 |
| p-value | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.216 | 0.138 | 0.176 | 0.112 | 0.413 | 0.421 |

3.3.5. RefFinder Analysis

To obtain a final overall ranking, RefFinder, a web-based comprehensive evaluation platform, was performed, based on the results of the above four programs. According to the RefFinder analysis, EF1α and F-BOX were the top two suitable reference genes for normalizing the transcripts in PNRSV-infected C. sativus, whereas TIP41 and TUA were the least stable genes (Table 2). According to the optimal number of reference genes evaluated by geNorm, EF1α and F-BOX were selected as the optimal multiple reference genes for normalization in PNRSV-infected C. sativus at 12 dpi.

3.4. Influence of Different Selection of Reference Genes on the Relative Expression of Target Genes

To investigate the influences of different reference genes on the expression of the target mRNA, we selected AGO1, AGO4, and RDR6, which are the important genes in RNA interference (RNAi)-based antiviral immunity (Figure 6), for additional RT-qPCR analyses. To identify the primer specificity, the amplified products were analyzed using
2% agarose gel electrophoresis and the melting curve (Figures S1 and S2). The most stable genes, EF1α and F-BOX, were used in combination for normalization, while the less stable genes, TIP41 and TUA, were also used as comparison. The relative expression levels of AGO1 and AGO4 showed significant upregulation, and the relative expression level of RDR6 showed no significant difference after PNRSV infection when we normalized using EF1α and F-BOX in combination. The AGO1, AGO4, and RDR6 genes exhibited similar expression trends when using TIP41 as the reference gene, while the upregulated folds were much larger than when using EF1α and F-BOX in combination, which indicated that the relative expressions were obviously overestimated (Figure 6a–c). By contrast, the relative expression of AGO1 and AGO4 in the PNRSV-inoculated C. sativus showed quite different trends when TUA was used for normalization, and did not show any differences compared with mock-inoculated plants.

![Figure 6](image)

**Figure 6.** Relative expression of (a) AGO1, (b) AGO4, and (c) RDR6 in PNRSV-infected C. sativus with different reference genes. EF1α and F-BOX were the most stable genes, while TIP41 and TUA were the least stable genes. (d) The relative expression levels of AGO1, AGO4, and RDR6 were calculated by high-throughput RNA sequencing data. * means p < 0.05, ** means p < 0.01, and *** means p < 0.0001 (based on t-test analysis).

Moreover, the relative expression levels of AGO1, AGO4, and RDR6 were calculated by high-throughput RNA sequencing data, which indicated that the expression of the AGO1 in the PNRSV-inoculated C. sativus was 2.28 times that in healthy plants, the expression of AGO4 in PNRSV-inoculated C. sativus was 1.61 times that in healthy plants, and the expression level of RDR6 showed no significant difference after PNRSV infection (Figure 6d). The relative expression levels of AGO1, AGO4, and RDR6 by transcriptome analyses were similar to the RT-qPCR results, which validated the use of EF1α and F-BOX as normalization genes.
4. Discussion

RT-qPCR is one of the most widely used methods for quantifying gene expression changes [5]. Relative changes in gene expression can be determined without knowing the absolute quantity of the reference genes, while the accuracy and repeatability of RT-qPCR analysis highly depends on many factors, such as sample quality, RNA quality and integrity, primer specificity, PCR conditions and amplification efficiency, etc. Based on these factors, the selection of a suitable reference gene is of primary importance [18,47]. In this study, we proposed a systematic process through which to identify the optimal reference genes in the leaf tissues of C. sativus under PNRSV infection. Firstly, the primer specificity of the candidate reference genes was experimentally validated by PCR amplification, Sanger sequencing, and melting curves. Secondly, the Ct value, E value, and R² were achieved for the tested reference genes. The suitable Ct values, 95% < E < 105%, and R² > 99%, are the prerequisite for using the 2^ΔΔCt method for data analysis [48]. Subsequently, four methods based on different algorithms and one platform for ranking order were used to evaluate the expression stability of the candidate reference genes [48,49]. geNorm is considered one of the best methods to determine the most stable genes and the optimal number of genes [16]. F-BOX, UBI-1, and EF1α were the most stable genes and TUA and TIP41 showed the least stability according to geNorm. At the same time, geNorm indicated that two reference genes were sufficient in our search. NormFinder uses the variation among candidate genes to rank, taking into account intra-group and inter-group variation, and reduces bias through co-regulation [17]. When we used NormFinder, EF1α and F-BOX were the most stable genes. The ranking results showed almost no differences between the NormFinder and ΔCt methods. BestKeeper is a useful approach for calculating the coefficients of correlation, SD and CV [18]. The EF1α was the most stable gene and TUA had a high r value, but its high SD and CV values suggested instability. Combining these four methods, EF1α and F-BOX were the top-ranked reference genes, while TUA and TIP41 were the lowest-ranked, according to the Reffinder analysis. Finally, three virus-immunity-associated genes were selected to validate the importance of the selection of suitable reference genes.

Several viruses infecting woody plants in the genus Ilarvirus cause serious damages in the fruit industry [39]. PNRSV induced significant losses in stone-fruit trees when it broke out in Turkey, India, etc. [50,51]. The ApMV and ApNMV viruses were identified as having induced apple mosaic disease, which is characterized by the symptoms of mosaic leaves and occurs widely in major apple-producing areas around the world [52,53]. PDV, another ilarvirus infecting stone-fruit species, caused deteriorated fruit marketability and reduced fruit yields [39]. The fact that the infectious clones of the above four viruses caused clear symptoms in cucumbers, the breakthrough of transgenic technology, and the establishment of VIGS systems on cucumbers, have prompted the application of cucumber as a useful and crucial experimental material to facilitate studies on the infection mechanism of ilarviruses. The study of pathogenic mechanisms is very important in the unravelling the viral infections and provide clues for the development of resistant materials [54]. Therefore, the validation of reference genes for gene expression studies using RT-qPCR after viral infection in C. sativus is highly necessary. Recently, some studies have identified a variety of reference genes in cucumbers at different developmental stages and under different biotic or abiotic stresses. TUA and UBI-ep were the most stably expressed genes when the cucumber seedlings at the second true leaf stage were treated with hormones, and EF1α showed a relatively stable expression level when the seedlings were treated with salt and drought stress [47,55]. The genes CACS, TIP41, F-BOX, and EF1α showed the highest expression stability under different nitrogen nutrition regimens and the combined use of three or four references helped to obtain reliable results [48]. In terms of biotic stress, EF1α and GAPDH were the most reliable reference genes for normalizing the miRNA expression after cucumber green mottle mosaic virus infection in leaf, root, and stem samples [56]. In our research, we identified EF1α and F-BOX as the most suitable reference genes among the eleven candidate genes for normalization in the PNRSV-infected leaf tissues of C. sativus at 12 dpi. By contrast, the validated reference genes of TUA, TIP41,
CACS, and UBI-ep in the previous experiments were relatively unstable and ranked lower in the stability ranking, which further indicated that there is no universal reference gene that can be stably expressed and used under all conditions for all kinds of tissue. The stability of reference genes depends on the experimental parameters, and the selection of reference genes should be studied in advance, case by case, to prevent subsequent false data interpretation and conclusions.

RNAi-mediated plant immunity is believed to be a universal pathway against viruses [57]. To further validate our conclusion, we investigated the relative expression of AGO1, AGO4, and RDR6 using the normalization factor generated with EF1α and F-BOX in combination, which showed that AGO1 expression was greatly and significantly upregulated in PNRSV-infected leaf tissues compared with the mock-inoculated control plants, and suggested that AGO1 may play an important role in PNRSV infection. Furthermore, when using TUA or TIP41 as reference genes, the differential expression of AGO1 and AGO4 was ignored or overemphasized. To support these conclusions, transcriptome-wide high-throughput RNA sequencing was conducted to provide a more accurate, unbiased estimate of the transcript abundance of AGO1, AGO4, and RDR6. The changes in the expression trends were similar to the RT-qPCR results. These findings further indicate that the use of appropriate reference genes is crucial to obtain accurate results.

In this study, we assessed eleven candidate reference genes in C. sativus in the context of PNRSV infection and determined that the combined use of EF1α and F-BOX is the best choice. This study represents the first attempt to select suitable reference genes in C. sativus in the context of ilarvirus infection, which will facilitate the functional study of genes related to viral infection.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14061269/s1, Figure S1: Amplification products of AGO1, AGO4 and RDR6 in Cucumis sativus. Lane M, Trans2K DNA marker; lane 1, AGO1; lane 2, AGO4; lane 3, RDR6. Figure S2: Dissociation curves of AGO1, AGO4, and RDR6 evaluated under experimental conditions.

Author Contributions: Conceptualization, B.Z. and S.L.; software, Z.D.; validation, Z.D.; formal analysis, Z.D. and B.Z.; investigation, Z.D.; writing—original draft preparation, Z.D. and B.Z.; writing—review and editing, B.Z. and S.L.; supervision, B.Z. and S.L.; project administration, S.L.; funding acquisition, B.Z. and S.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key R & D Program of China, grant number 2019YFD1001800.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Special thanks to Hongguang Cui (from Hainan University) for the gift of infectious clones.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Kozian, D.H.; Kirschbaum, B.J. Comparative gene-expression analysis. Trends Biotechnol. 1999, 17, 73–78. [CrossRef]
2. Robert, C. Microarray analysis of gene expression during early development: A cautionary overview. Reproduction 2010, 140, 787–801. [CrossRef] [PubMed]
3. De Klerk, E.; AC’t Hoen, P. Alternative mRNA transcription, processing, and translation: Insights from RNA sequencing. Trends Genet. 2015, 31, 128–139. [CrossRef] [PubMed]
4. Bustin, S.A. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 2000, 25, 169–193. [CrossRef]
5. Bustin, S.A.; Benes, V.; Nolan, T.; Pfaffl, M.W. Quantitative real-time RT-PCR—A perspective. J. Mol. Endocrinol. 2005, 34, 597–601. [CrossRef]
6. Allison, D.B.; Cui, X.; Page, G.P.; Sabirpouri, M. Microarray data analysis: From disarray to consolidation and consensus. Nat. Rev. Genet. 2006, 7, 55–65. [CrossRef]

7. Bustin, S.A.; Benes, V.; Garson, J.A.; Helttemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 2009, 55, 611–622. [CrossRef]

8. Guerin, S.; Mauriat, M.; Pelloux, J.; Van Wuytswinkel, O.; Bellini, C.; Gutierrez, L. Normalization of qRT-PCR data: The necessity of adopting a systematic, experimental conditions-specific, validation of references. J. Exp. Bot. 2009, 60, 487–493. [CrossRef]

9. Czechowski, T.; Stitt, M.; Allmann, T.; Udvardi, M.K.; Scheible, W.-R. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 2005, 139, 5–17. [CrossRef]

10. Sundaram, V.K.; Sampathkumar, N.K.; Massaad, C.; Grenier, J. Optimal use of statistical methods to validate reference gene stability in longitudinal studies. PLoS ONE 2019, 14, e0219440. [CrossRef]

11. Lilly, S.T.; Drummond, R.S.; Pearson, M.N.; MacDiarmid, R.M. Identification and validation of reference genes for normalization of transcripts from virus-infected Arabidopsis thaliana. Mol. Plant-Microbe Interact. 2011, 24, 294–304. [CrossRef]

12. Liu, D.; Shi, L.; Han, C.; Yu, J.; Li, D.; Zhang, Y. Validation of reference genes for gene expression studies in virus-infected Nicotiana benthamiana using quantitative real-time PCR. PLoS ONE 2012, 7, e46451. [CrossRef]

13. Petit, C.; Pernin, F.; Heydel, J.-M.; Delye, C. Validation of a set of reference genes to study response to herbicide stress in grasses. BMC Res. Notes 2012, 5, 18. [CrossRef]

14. Baek, E.; Yoon, J.-Y.; Palukaitsis, P. Validation of reference genes for quantifying changes in gene expression in virus-infected tobacco. Virology 2017, 510, 29–39. [CrossRef]

15. Mascia, T.; Santovito, E.; Gallitelli, D.; Cillo, F. Evaluation of reference genes for quantitative reverse-transcription polymerase chain reaction normalization in infected tomato plants. Mol. Plant Pathol. 2010, 11, 805–816. [CrossRef]

16. Vandesompele, J.; De Preter, K.; Pattyn, F.; Poppe, B.; Van Roy, N.; De Paepe, A.; Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002, 3, 1–12. [CrossRef]

17. Andersen, C.L.; Jensen, J.L.; Ørntoft, T.F. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004, 64, 5254–5250. [CrossRef]

18. Pfaffl, M.W.; Tichopad, A.; Prgomet, C.; Neuvians, T.P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. Biotechnol. Lett. 2004, 26, 509–515. [CrossRef]

19. Xie, F.; Xiao, P.; Chen, D.; Xu, L.; Zhang, B. miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol. Biol. 2012, 80, 75–84. [CrossRef]

20. Pihur, V.; Datta, S.; Datta, S. RankAggreg, an R package for weighted rank aggregation. BMC Bioinf. 2009, 10, 62. [CrossRef]

21. Schmidt, G.W.; Delaney, S.K. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (Nicotiana tabacum) during development and abiotic stress. Mol. Genet. Genomics 2010, 283, 233–241. [CrossRef]

22. An, J.; Shen, X.; Ma, Q.; Yang, C.; Liu, S.; Chen, Y. Transcriptome profiling to discover putative genes associated with paraquat resistance in goosegrass (Eleusine indica L.). PLoS ONE 2014, 9, e99940. [CrossRef]

23. Moretti, M.L.; Alarcón-Reverte, R.; Pearce, S.; Morran, S.; Hanson, B.D. Transcription of putative tonoplast transporters in response to glyphosate and paraquat stress in Conyza bonariensis and Conyza canadensis and selection of reference genes for qRT-PCR. PLoS ONE 2017, 12, e0180794. [CrossRef]

24. Reddy, P.S.; Reddy, D.S.; Sivasakthi, K.; Bhatnagar-Mathur, P.; Vadez, V.; Sharma, K.K. Evaluation of sorghum [Sorghum bicolor (L.)] reference genes in various tissues and under abiotic stress conditions for quantitative real-time PCR data normalization. Front. Plant Sci. 2016, 7, 529. [CrossRef]

25. Zhu, H.; Ma, Y.; Guo, Q. Expression stability of internal reference gene in response to Trichoderma polysporum infection in Avena fatua L. Curr. Genet. 2021, 67, 909–918. [CrossRef]

26. Zhang, Y.; Han, X.; Chen, S.; Zheng, L.; He, X.; Liu, M.; Qiao, G.; Wang, Y.; Zhuo, R. Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in Salix matsudana under different abiotic stresses. Sci. Rep. 2017, 7, 40290. [CrossRef]

27. Lee, J.M.; Roche, J.R.; Donaghy, D.J.; Thrush, A.; Sathish, P. Validation of reference genes for quantitative RT-PCR studies of gene expression in perennial ryegrass (Lolium perenne L.). BMC Mol. Biol. 2010, 11, 8. [CrossRef]

28. Yu, Y.; Zhao, Z.; Qin, L.; Zhou, Y.; Fan, H.; Zhang, Z.; Wu, Z.; Li, S. Incidence of major peach viruses and viroids in China. J. Plant Pathol. 2013, 95, 603–607. [CrossRef]

29. Manganaris, G.A.; Economou, A.S.; Boubourakas, I.N.; Katis, N.I. Elimination of PPV and PNRSV through thermotherapy and meristem-tip culture in nectarine. Plant Cell Rep. 2003, 22, 195–200. [CrossRef]

30. Mekuria, G.; Ramesh, S.A.; Alberts, E.; Bertozzi, T.; Wirthensohn, M.; Collins, G.; Sedgley, M. Comparison of ELISA and RT-PCR for the detection of prunus necrotic ring spot virus and prune dwarf virus in sweet cherry (Prunus avium) and Chinese cherry (Prunus pseudocerasus) in Shandong Province, China. In Proceedings of the VII International Cherry Symposium, Plasencia, Spain, 23 June 2013; pp. 469–474.
32. Oliver, J.E.; Freer, J.; Andersen, R.L.; Cox, K.D.; Robinson, T.L.; Fuchs, M. Genetic diversity of prunus necrotic ringspot virus isolates within a cherry orchard in New York. Plant Dis. 2009, 93, 599–606. [CrossRef] [PubMed]
33. Kinothi, W.M.; Nancarrow, N.; Dann, A.; Rodoni, B.C.; Constable, F.E. Updating the quarantine status of prunus infecting viruses in Australia. Viruses 2020, 12, 246. [CrossRef] [PubMed]
34. Amari, K.; Diaz-Vivancos, P.; Pallas, V.; Amelia Sanchez-Pina, M.; Antonio Hernandez, J. Oxidative stress induction by prunus necrotic ringspot virus infection in apricot seeds. Physiol. Plant. 2007, 131, 302–310. [CrossRef] [PubMed]
35. Hu, G.J.; Dong, Y.F.; Zhang, Z.P.; Fan, X.D.; Ren, F.; Li, Z.N.; Zhou, J. First report of prunus necrotic ringspot virus infection of apple in China. Plant Dis. 2016, 100, 1955–1956. [CrossRef]
36. Xing, F.; Gao, D.; Liu, H.; Wang, H.; Habihi, N.; Li, S. Molecular characterization and pathogenicity analysis of prunus necrotic ringspot virus isolates from China rose (Rosa chinensis Jacq.). Arch. Virol. 2020, 165, 2479–2486. [CrossRef]
37. Cui, H.; Hong, N.; Wang, G.; Wang, A. Genomic segments RNA1 and RNA2 of prunus necrotic ringspot virus codetermine viral pathogenicity to adapt to alternating natural prunus hosts. Mol. Plant-Microbe Interact. 2013, 26, 515–527. [CrossRef]
38. Pethybridge, S.J.; Wilson, C.R.; Ferrandino, F.J.; Leggett, G.W. Spatial analyses of viral epidemics in Australian hop gardens: Implications for mechanisms of spread. Plant Dis. 2000, 84, 513–515. [CrossRef]
39. Pallas, V.; Aparicio, F.; Herranz, M.C.; Amari, K.; Sanchez-Pina, M.A.; Myrta, A.; Sanchez-Navarro, J.A. Ilarviruses of Prunus spp.: A continued concern for fruit trees. Phytopathology 2012, 102, 1108–1120. [CrossRef]
40. Pappi, P.G.; Fotiou, I.; Ethimioni, K.E.; Katis, N.I.; Mallowka, V.I. Development of three duplex real-time RT-PCR assays for the sensitive and rapid detection of a phytoplasma and five viral pathogens affecting stone fruit trees. Mol. Cell. Probes 2020, 53, 101621. [CrossRef]
41. Sharma, A.; Ram, R.; Zaidi, A.A. Rubus ellipticus, a perennial weed host of prunus necrotic ring spot virus in India. Plant Dis. 1998, 82, 1283. [CrossRef]
42. Akbas, B.; Ilhan, H.D. Widespread distribution of apple mosaic virus on apple in Turkey. Plant Dis. 2005, 89, 1010. [CrossRef]
43. Kinoti, W.; Plummer, K.; Constable, F.E.; Nancarrow, N.; Rodoni, B. The partial characterization of ilarviruses infecting prunus species in Australia. In Proceedings of the XXIX International Horticultural Congress on Horticulture: Sustaining Lives, Livelihoods and Landscapes (IHC2014): International Symposium on Nut Crops, Brisbane, QL, Australia, 23 February 2016; pp. 243–247.
44. Liu, M.; Liang, Z.; Aranda, M.A.; Hong, N.; Liu, L.; Kang, B.; Gu, Q. A cucumber green mottle mosaic virus vector for virus-induced gene silencing in cucurbit plants. Plant Methods 2020, 16, 9. [CrossRef]
45. Fang, L.; Wei, X.; Liu, L.; Zhou, L.; Tian, Y.; Geng, C.; Li, X. A tobacco ringspot virus-based vector system for gene and microRNA function studies in cucurbits. Plant Physiol. 2021, 186, 853–864. [CrossRef]
46. Wang, S.; Wang, R.; Liang, D.; Ma, F.; Shu, H. Molecular characterization and expression analysis of a glycine-rich RNA-binding protein gene from Malus hupehensis Rehd. Mol. Biol. Rep. 2012, 39, 4145–4153. [CrossRef]
47. Wan, H.; Zhao, Z.; Qian, C.; Sui, Y.; Malik, A.A.; Chen, J. Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. Anal. Biochem. 2010, 399, 257–261. [CrossRef]
48. Warzybok, A.; Migocka, M. Reliable reference genes for normalization of gene expression in cucumber grown under different nitrogen nutrition. PLoS ONE 2013, 8, e72887. [CrossRef]
49. Holmes, A.; Birse, L.; Jackson, R.W.; Holden, N.J. An optimized method for the extraction of bacterial mRNA from plant roots infected with Escherichia coli O157:H7. Front. Microbiol. 2014, 5, 286. [CrossRef]
50. Chandel, V.; Rana, T.; Hallan, V.; Zaidi, A.A. Evidence for the occurrence of prunus necrotic ringspot virus on peach in India by serological and molecular methods. Can. J. Plant Pathol. 2007, 29, 311–316. [CrossRef]
51. Sipahioglu, H.M.; Baloglu, S. The incidence of prunus necrotic ring spot (PNRSV) and apple chlorotic leaf spot (ACLSV) viruses on stone fruits grown in East Anatolia region of Turkey. In Proceedings of the XII ISHS Symposium on Apricot Culture and Decline, Stone Fruits, Can. J. Plant Pathol. 2012, 34, 343–357. [CrossRef]
52. Noda, H.; Yamaishi, N.; Yaegashi, H.; Xing, F.; Xie, J.; Li, S.; Zhou, T.; Ito, T.; Yoshikawa, N. Apple necrotic mosaic virus, a novel ilarvirus from miosis-diseased apple trees in Japan and China. J. Gen. Plant Pathol. 2017, 83, 83–90. [CrossRef]
53. Xing, F.; Robe, B.L.; Zhang, Z.; Wang, H.; Li, S. Genomic analysis, sequence diversity, and occurrence of apple necrotic mosaiv virus, a novel ilarvirus associated with miosis disease of apple trees in China. Plant Dis. 2018, 102, 1841–1847. [CrossRef]
54. Kovalskaya, N.; Hammond, R.W. Molecular biology of viroid–host interactions and disease control strategies. Plant Sci. 2014, 228, 48–60. [CrossRef]
55. Migocka, M.; Papierniak, A. Identification of suitable reference genes for studying gene expression in cucumber plants subjected to abiotic stress and growth regulators. Mol. Breed. 2011, 28, 343–357. [CrossRef]
56. Liang, C.; Hao, J.; Meng, Y.; Luo, L.; Li, J. Identifying optimal reference genes for the normalization of microRNA expression in cucumber under viral stress. PLoS ONE 2018, 13, e0194436. [CrossRef]
57. Ding, S.-W. RNA-based antiviral immunity. Nat. Rev. Immunol. 2010, 10, 632–644. [CrossRef]