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Comprehensive Identification of Reliable Reference Genes for qRT-PCR Normalization of *Fusarium oxysporum*-Resistant Genes' Expressions in *Lilium sargentiae* Wilson

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Abstract: *Fusarium* wilt (caused by *Fusarium oxysporum* f. sp. *Lilii*) is one of the most damaging diseases in lily (*Lilium sargentiae* Wilson). Although some *F. oxysporum*-resistant lily varieties have been identified and are being utilized in resistant breeding, the regulation network of the resistance-associated mechanisms is yet to be studied due to the lack of reliable reference genes for qRT-PCR (quantitative reverse transcription PCR) normalization. The reliability of results by qRT-PCR relies mainly on the stability of the reference genes. This study investigated the reliability of nine candidate reference genes (*CYP*, *EF1-α*, *GAPDH*, *TUB*, *UBQ*, *AQP*, *HIS*, *PGK*, and *RPL13*) for qRT-PCR analysis of *F. oxysporum*-resistant genes. Expression stability analysis via common programs GeNorm, BestKeeper, and NormFinder, at different time points post-inoculation of *F. oxysporum,* revealed that all nine genes met the basic requirements of reference genes. Amongst them, *HIS* and *GAPDH* displayed the highest and the lowest expression stability, respectively. The reliability of *HIS* was further validated by analyzing the expression levels of four resistance-related candidate genes. The expression patterns of the four target genes were consistent with their responses to pathogenetic fungi in other plants. Our results show that *HIS* is the most suitable reference gene for accurately normalizing *F. oxysporum*-resistant genes' expressions in *L. sargentiae*.

Keywords: *Lilium sargentiae* Wilson; *F. oxysporum* inoculation; gene expression; stability

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1. Introduction

Gene expression analysis is crucial in dissecting the regulation network of physiological and metabolic processes in plants [1–3]. At present, transcriptome sequencing is the most widespread method to explore transcriptional changes during diverse biological processes in various species [4–6]. However, transcriptome data are not always reliable, and qRT-PCR analysis is the most used approach to validate transcriptome data [6–8]. It is highly sensitive, specific, accurate, and reproducible [9–11]. Nevertheless, its accuracy is influenced by diverse factors, including the quality of RNA, initial sample capacity, primer specificity, reverse transcription efficiency, and amplification efficiency [11,12]. It is necessary to utilize one or more stable reference genes as the calibration standard to normalize the relative expression levels of target genes [2,13]. In plant science, reliable reference genes are commonly chosen from the stably expressed housekeeping genes, including polyubiquitin (*UBQ*), actin (*ACT*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 18S ribosomal RNA (*18S*), elongation factor (*EF*), Tubulin (*TUB*), and so on [2,3,10,14,15]. These genes are associated with fundamental cellular functions and processes, maintenance of cell structure and properties, and primary metabolism [12]. A reliable reference gene should exhibit stable expression in all plant organs at different physiological and developmental stages and under various experimental conditions [10,16]. However, some studies have

shown that reference genes' expression levels are not always stable under changing growth conditions in many species [15,17–20]. Thus, an improper selection of reference genes may lead to erroneous results and conclusions by qRT-PCR analysis [21–23]. Accordingly, it is essential to identify suitable and stable reference genes for specific experimental conditions and in each species [10].

Lily (*Lilium* spp.) is an important ornamental plant that contributes considerably to the global cut flower market economy. Yunnan, China, is one of the main producers of lily cut flowers in the world. With the expansion of lily planting areas, lily diseases have become more serious in recent years. *Fusarium* wilt disease (also labeled as basal rot, stem rot, or root rot) is one of the most limiting threats to bulbs and flower production in lilies, specifically under special growing conditions in greenhouses [24–26]. The soilborne fungus *Fusarium oxysporum* f. sp. *Lilii* is the main pathogen that causes lily *Fusarium* wilt. It can survive for a long time as mycelium, chlamydospore, or sclerotium with diseased residue in the soil or as mycelium in the lily bulbs [27]. *F. oxysporum* is difficult to control effectively. Therefore, the most effective and safe approach for controlling this disease is to understand resistance-related molecular mechanisms and identify and exploit key genome resources in breeding novel *F. oxysporum*-resistant lily varieties. A few lily breed cultivars (Asiatic hybrids, LA hybrids) and some wild species (*L. sargentiae* Wilson, *L. regale* Wilson, and *L. pumilum* DC) have shown resistance to *F. oxysporum* [24,27–30].

L. sargentiae is endemic to China and is naturally distributed in the southwest regions [31]. Due to its resistance to *F. oxysporum* and ornamental characters, it represents one of the important parents used in lily cross-breeding [32,33]. However, the regulation of the resistance to *F. oxysporum* in *L. sargentiae* is largely unknown. In a recent study (unpublished data), we applied high-throughput transcriptome sequencing technology and identified potential candidate genes for *F. oxysporum* resistance in *L. sargentiae*. To enable in-depth studies of these genes, reliable reference genes for qRT-PCR normalization are required. Herein, we selected nine candidate reference genes based on the RNA-seq data and evaluated their expression stability at different time points following *F. oxysporum* inoculation in *L. sargentiae*. Three available statistical programs, i.e., GeNorm, BestKeeper, and NormFinder, were used, and their respective results were fitted to unveil the most suitable reference gene for qRT-PCR normalization in *L. sargentiae* during infection with *F. oxysporum*. Our findings will facilitate the molecular dissection of *Fusarium* wilt disease resistance in *L. sargentiae*.

2. Materials and Methods

2.1. Plant and Fungal Materials

A total of 20 lily bulbs of wild *L. sargentiae* Wilson plants were collected from a locality of Weixi county (altitude, 1906 m; 27°20′ N, 99°39′ E) of Yunnan province, China, and were grown in a greenhouse of Flower Research Institute, Yunnan Academy of Agricultural Sciences. *F. oxysporum* f. sp. *Lilii* was isolated from the infected oriental hybrid lily plants with standard *Fusarium* wilt symptoms. The pathogen was isolated and purified by conventional tissue separation and agar plate dilution and then was identified according to the morphology of spore and mycelium and molecular fingerprints [34]. The molecular fingerprint is a ribosomal DNA ITS (internal transcribed spacer) sequence (GenBank accession no. GU371875) from *F. oxysporum* strain (No. Fol-2) [34]. The detailed procedures for identifying pathogen by molecular fingerprint were described in reference [34]. The highly aggressive *F. oxysporum* strain (No. Fol-2) was preserved and offered by the Yunnan Flower Breeding Key Laboratory.

2.2. Cultivation and Inoculation

The tissue culture seedlings of wild *L. sargentiae* Wilson were used in this study. The tissue culture seedlings were cultivated under controlled conditions (growth chamber: temperature, 25 °C; humidity, 30%–40%; photoperiod, 14 h light/10 h dark). The tissue culture seedlings with a bulb diameter from 1 to 1.5 cm and strong root tissue were screened

out and separated into two groups (treatment and control) for inoculation [30]. Each group comprised three bottles with ten plants in each bottle. The *F. oxysporum* isolates were cultivated in potato sucrose liquid medium (potato, 20 g · L^{-1}; sucrose, 20 g · L^{-1}; RO water, 1 L) on a shaker (100 rpm/min) for 15 days. Next, the spore suspension of *F. oxysporum* was subsequently filtrated through a wire gauze of 200 mesh and diluted to a final concentration of approximately 1.0×10^6 spores/mL by microscopic count using the Bürker-Türk hemocytometer [26,28,30].

To induce the *Fusarium* wilt disease, the root tips of seedlings (treatment group) were cut off to create a wound and then inoculated with the spore suspension using the root dip inoculation method. In parallel, a mock inoculation was performed in seedlings within the control group with sterile distilled water [30]. The tissue culture seedlings were collected at 0, 6, 12, 24, 48, and 72 h after inoculation with *F. oxysporum* (treatment) and sterile water (control), respectively. Ten inoculated tissue culture seedlings with the same conditions (same time and same treatment) and same growth potential were sampled and mixed equally to constitute one experimental sample. All samples were immediately frozen directly in liquid nitrogen and kept in a freezer at −80 °C until use.

2.3. Selection of Candidate Reference Genes

We have performed high-throughput comparative transcriptome of *L. sargentiae* tissue culture seedlings at 24 h post-inoculation with *F. oxysporum* and sterile water, respectively (data unpublished). By examining the expression levels of genes, nine reference genes, including cyclophilin (*CYP*), elongation factor 1-α (*EF1-α)*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)*, tubulin (*TUB)*, ubiquitin (*UBQ)*, aquaporin (*AQP*), histone (*HIS*), phosphoglycerate kinase (*PGK*), and 60S ribosomal protein L13-1(*RPL13*), were chosen as potential candidate reference genes. These genes exhibited relatively (similar or indistinguishable) stable expression levels in all six samples based on (fragments per kb per million reads) FPKM values (Table S1). Meanwhile, based on the RNA-Seq result, four potential *Fusarium* wilt resistance candidate genes that showed differential expression patterns were also selected to verify the efficiency of candidate reference genes for qRT-PCR validation (Table S1). They included genes for peroxidase (*POD*), phenylalanine ammonia-lyase (*PAL*), chitinase (*CHI*), and dirigent-like protein (*DIR*). Specific primers of each gene were designed in Primer Premier 5.0 software and are listed in Table 1.

Table 1. Primers of the candidate reference genes and target genes for qRT-PCR analysis.

Gene	Primer Sequence (5′ to 3′)	Product Length (bp)
CYP	F:CGGTGGCGAGTCTATCTAT; R:TGGTATTAGGCCCAGCAT	104
EF1-α	F:TATTGACAAGCGTGTGATCG; R:TTATCAAGCACCCACGCA	87
GAPDH	F:ACATGGCTGCAACCAAGTA; R:CATGAAGCCGGCATTTATT	80
TUB	F:GCTGTTTCATCCCGAGCA; R:CAGGCACAGGTCAACAAT	103
UBQ	F:CACCCTTGCGGACTATAAC; R:TTCCGGTGAGCGTCTTGA	98
AQP	F:CGTTTCCTCTCTATCTCCAG;R:CCATGAGCAGATCCATGTATT	117
HIS	F:GATTCACAGGCAGTTGAAGT; R:TTCAGATCCTTGCTCGCAT	138
PGK	F:GGCTAAAGGCGTACGACTA; R:GCAGGTACAACCTTGCTC	87
RPL13	F:GCAGGTACAACCTTGCTC; R:CCTTGCTCCTCTTGATCTTTA	93
POD	F:CCGAGCCTCTTGAGTACAATTA; R:CCATTGACAAAGCAGTCGT	110
PAL	F:CTGATCAAGGTCATAGACAGG; R:TAGTGCGTGCTCCACCAATA	111
CHI	F:GCCGACAATAAGATGGCG; R:AGGTTAGCTCCATAGCTGAC	80
DIR	F:GAGCGGCCTATTCAGGCTA; R:CGTACTCGACGAGAGATG	89

The Tm of all primers in this table is 60 °C. F, forward primers; R, reverse primers.

2.4. RNA Extraction and cDNA Synthesis

The mirVana™ RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) was used for total RNA extraction from each sample, following the instructions by the manufacturer. The NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used

to evaluate the yield and quality of RNA. Finally, the RNA integrity was assessed via electrophoresis with 1% agarose gel.

cDNAs synthesis was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The RT (reverse transcription) reaction consisted of 0.5 μg RNA, 2 μL of 5 × TransScript All-in-one SuperMix (TransGen Biotech, Beijing, China), 0.5 μL of gDNA Remover, and a total volume of 10 μL with ddH_2O. The RT reaction conditions were 15 min at 42 °C and 5 s at 85 °C. Each RT product was diluted ten times with nuclease-free water and then stored at −20 °C.

2.5. qRT-PCR Analysis

The qRT-PCR was carried out on LightCycler®480 IIReal-time (Roche, Basel, Switzerland). The PCR reaction was composed of 1 μL cDNA, 5 μL 2 × PerfectStart™ Green qPCR Super Mix (TransGen Biotech, Beijing, China), 0.2 μL each primer (10 pmol/L), and 3.6 μL of nuclease-free water. The conditions were as follows: 94 °C for 30 s, 45 cycles of 94 °C for 5 s, and 60 °C for 30 s. At the end of the PCR cycles, we conducted melting curve analysis to evaluate the quality of each PCR product. Each gene was analyzed in three technical and biological replicates. The Ct (cycle threshold) values of each gene were computed automatically [23].

2.6. Expression Stability Analysis and Validation of Reference Genes

Based on standard curves obtained in PCR runs, we considered the Ct values as relative quantities and used them directly in performing gene expression stability analysis [35]. The expression stability of the candidate reference genes were assessed utilizing NormFinder [6,35], geNorm [1], and BestKeeper [36]. The results obtained from the three programs were fitted to generate a comprehensive stability ranking of each candidate reference gene via the GM (geometric mean) method [37]. Microsoft Excel 2016 and Data Processing System were used for data analysis and statistics [37,38].

To confirm the most reliable reference gene, the four selected resistance-related genes (*POD*, *PAL*, *CHI*, and *DIR*) were also analyzed by qRT-PCR. The PCR reaction systems and conditions were the same as those above. The most stable candidate reference gene served as the internal control for target genes' expression normalization. In theory, the gene expression level was the lowest at the 0 h time point with sterile water treatment (0 h W). So, 0 hW was used as the control, and other time points were compared with 0 h W. The relative expression levels of four target genes were normalized according to the $2^{-\Delta\Delta Ct}$ method [39]. One-way ANOVA with Tukey's HSD post-hoc test was used to compare the means of multiple samples at the 5% significance level [37].

3. Results

3.1. Verification of Primer Specificity and PCR Amplicons

To identify suitable reference genes for qRT-PCR validation analysis of potential disease-resistant candidate genes in *L. sargentiae*, nine candidate reference genes were selected from RNA-seq data of *L. sargentiae* for qRT-PCR analysis. The qRT-PCR melting curves of most candidate reference genes showed a single peak in all samples (the red color curves), indicating that the primers were highly specific (Figure 1), and the gene amplification curves for each sample had excellent repeatability, showing that the qRT-PCR results were accurate and reliable. However, in some samples (the green color curves), *GAPDH* showed a non-specific peak, indicating that its primer specificity was not high.

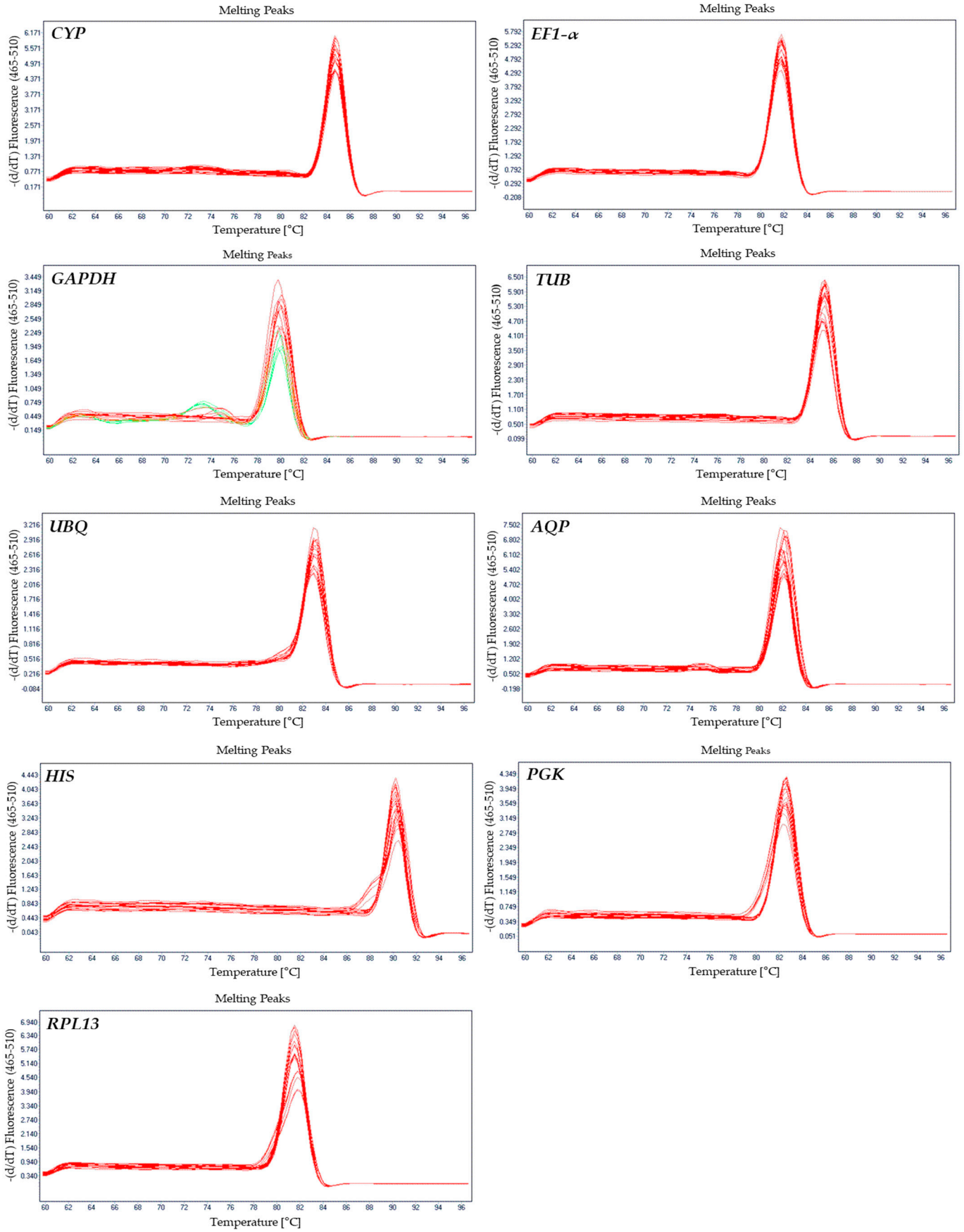

Figure 1. Melting curves of the nine selected potential reference genes.

3.2. Expression Profile Analysis of the Candidate Reference Genes

To explore the expression levels of the nine potential reference genes, we computed their respective Ct values from the qRT-PCR (Figure 2). The Ct values of the nine genes varied from 20.21 to 34.82. The gene with the highest transcripts was *EF-1α* (average Ct value 21.53), followed by *UBQ*, *CYP*, *AQP*, *RPL13*, *HIS*, *TUB*, *PGK*, and *GAPDH*. *GAPDH* exhibited the lowest relative expression levels (average Ct value 32.61) with the greatest variation, ranging from 30.45 to 34.82. *AQP* and *PGK* showed the least variation in gene expression levels, with Ct values varying from 25.30 to 27.62 and 27.03 to 29.07, respectively.

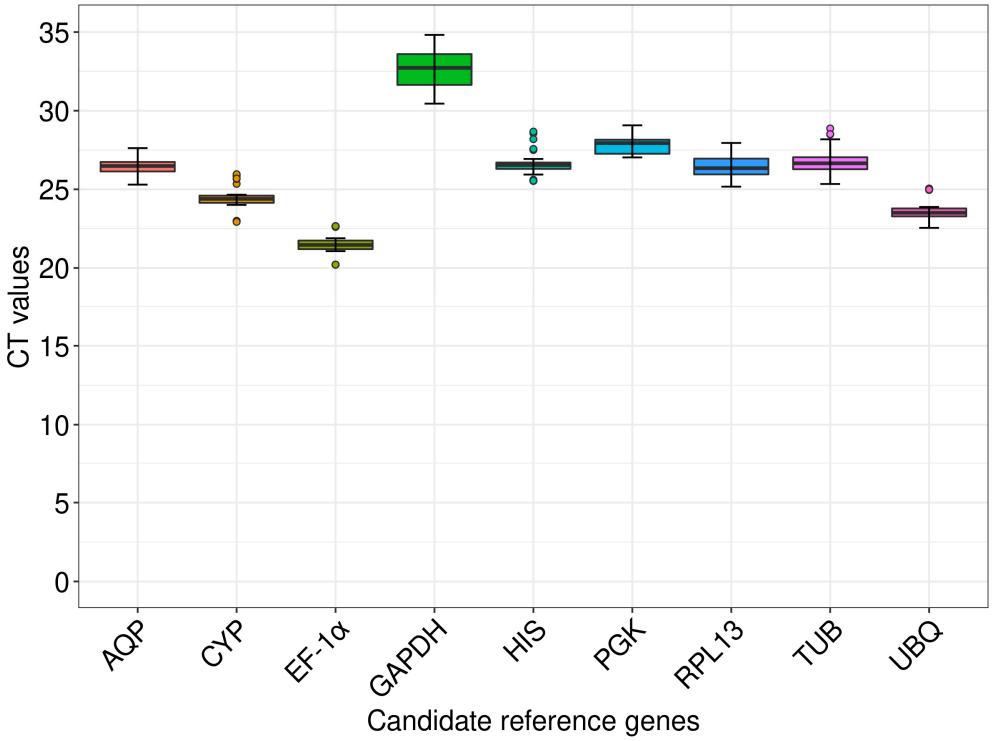

Figure 2. qRT-PCR Ct values for all candidate reference genes in all samples. The box graph represents the 75th to 25th percentiles and the line across the box represents the median. The whiskers on each box represent the minimum and maximum values and the circles represent outliers.

3.3. Expression Stability Analysis of the Candidate Reference Genes

Three programs (GeNorm, BestKeeper, and NormFinde) were used to determine the rankings of the nine potential reference genes separately. Thereafter, the GM method was applied to compute the comprehensive stability ranking.

By computing the gene expression stability measure (M) based on the average pairwise expression ratio, GeNorm was used to determine the most stable reference gene. The gene with the lowest M value is considered the most stable. A gene can be considered a reference gene if its M value is less than 1.5; lower M value indicates a more stable expression of the gene [3]. Eight of these reference genes had M values lower than 1.0, with the exception of *GAPDH*, which recorded an M value of 1.466. *HIS* and *EF-1α* were certified as the two most stable reference genes, with M values of 0.533 and 0.535, respectively (Table 2). In some gene expression analyses, a single reference gene may not be able to satisfy the experiment requirements if the precision requirements are high or the quantity change of gene expression has to be expressed quantitatively. It is therefore necessary to use two or more reference genes in order to obtain accurate and reliable normalization results. It is possible to determine the optimal number of reference genes for normalization based on the pairwise variation value (Vn/n + 1, V value). When Vn/n +1 is greater than or equal to 0.15, the optimal number of reference genes for accurate normalization should be n + 1. On

the contrary, if Vn/n + 1 is less than 0.15, n is the optimal number of reference genes [3,5]. GeNorm V value analysis showed that when the V value of the two to eight reference genes (V2/3 to V7/8) was less than 0.15 (Figure 3), the optimal number of reference genes is two. Based on the V values, the most appropriate combination of reference genes for qRT-PCR normalization of resistance-related genes' expression levels in *L. sargentiae* was *HIS* and *EF-1α*.

Table 2. Stability rank of candidate reference gene expression by geNorm, NormFinder, and BestKeeper analysis.

Stability Rank	geNorm		NormFinder		BestKeeper	
	Reference Genes	M	Reference Genes	SV	Reference Genes	SD
1	*HIS*	0.533	*HIS*	0.086	*CYP*	0.126
2	*EF-1α*	0.535	*PGK*	0.174	*AQP*	0.134
3	*CYP*	0.543	*CYP*	0.175	*EF-1α*	0.135
4	*UBQ*	0.598	*EF-1α*	0.182	*HIS*	0.146
5	*PGK*	0.611	*AQP*	0.211	*UBQ*	0.153
6	*TUB*	0.615	*TUB*	0.233	*TUB*	0.166
7	*AQP*	0.620	*UBQ*	0.280	*RPL13*	0.180
8	*RPL13*	0.717	*RPL13*	0.420	*PGK*	0.204
9	*GAPDH*	1.466	*GAPDH*	0.994	*GAPDH*	0.217

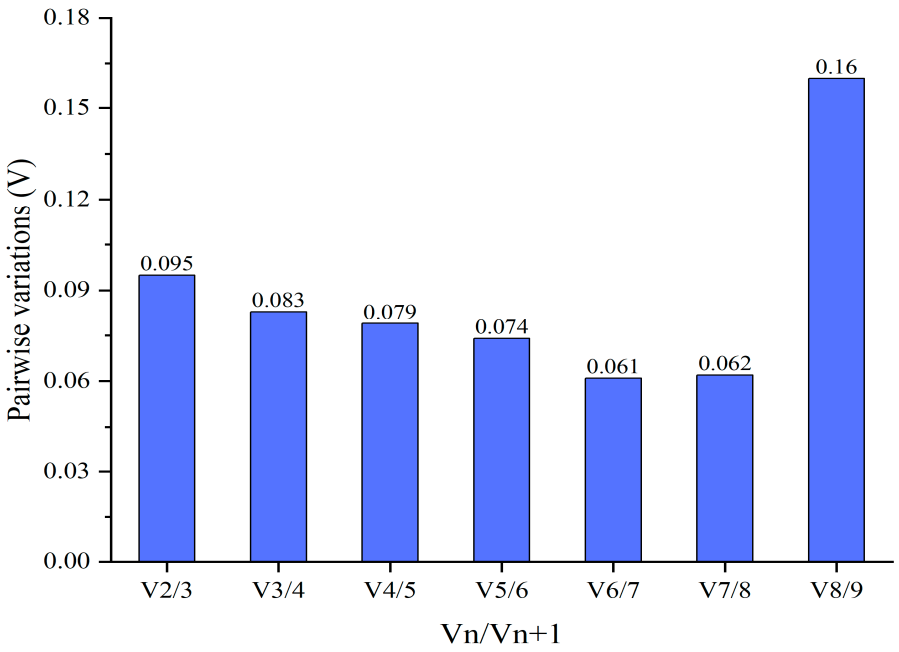

Figure 3. The pairwise variation values (Vn/Vn + 1, V) of candidate reference genes.

Unlike GeNorm analysis, the NormFinder is commonly used to evaluate the stability value (SV) of reference genes and to detect, via intra- and inter-group variations, the optimal number of reference genes for precise normalization. A gene is considered more stable if its expression level is lower than the average expression stability. The results of NormFinder analyses were the same as the GeNorm results in stability rankings of five reference genes including *HIS*, *CYP*, *TUB*, *RPL13*, and *GAPDH* (Table 2). Similarly, *HIS* (SV = 0.086) and *GAPDH* (SV = 0.994) were confirmed as the most stable and unstable genes, respectively.

BestKeeper is another tool to explore the stability index of potential reference genes through the evaluation of SD (standard deviation), CV (coefficient of variation), and *R* (correlation coefficient) [36]. If SD < 1.0, the gene is considered stable [1,4]. According to the BestKeeper results, all nine genes are suitable for use as reference genes (SD < 1.0). *CYP*

(SV = 0.126) and *GAPDH* (SV = 0.217) ranked first and last in terms of stability, respectively (Table 2). All three programs showed that *GAPDH* was the most unstable gene.

In order to determine the comprehensive stability ranking of the nine candidate reference genes, we integrated the results from the three programs based on GM value of ranking. GM value indicated that *HIS* was the most suitable reference gene, followed by *CYP*, *PGK*, *UBQ*, *AQP*, *EF-1α*, *TUB*, *RPL13*, and *GAPDH* (Table 3).

Table 3. The comprehensive ranking of candidate reference gene stability by GM method.

Comprehensive Ranking	Reference Genes	GM Values of Ranking
1	*HIS*	1.587
2	*CYP*	2.080
3	*PGK*	2.884
4	*UBQ*	4.121
5	*AQP*	4.309
6	*EF-1α*	5.192
7	*TUB*	6.000
8	*RPL13*	7.652
9	*GAPDH*	9.000

3.4. Expression Stability Validation of the Most Reliable Reference Gene

To verify the stability and reliability of *HIS* (the most stable reference gene) for accurate normalization of the expression levels of *Fusarium* wilt-related genes, we evaluated the expression of four potential resistance-related genes, including *POD*, *PAL*, *CHI*, and *DIR*, using qRT-PCR. As presented in Figure 4, the results showed that the expression patterns of the four target genes were the same during the first three time periods (0 h, 6 h, and 12 h) after inoculating; all genes showed the lowest expression at the 0 h time-point and began to significantly up-regulate in 6 h and then down-regulated again in 12 h. In the next three time periods (24 h, 48 h, and 72 h), they again showed a certain upregulation. The time period of their highest expression was not exactly the same. However, the general expression levels of the four target genes were stronger in most samples inoculated with *F. oxysporum* than that in control samples (Figure 4).

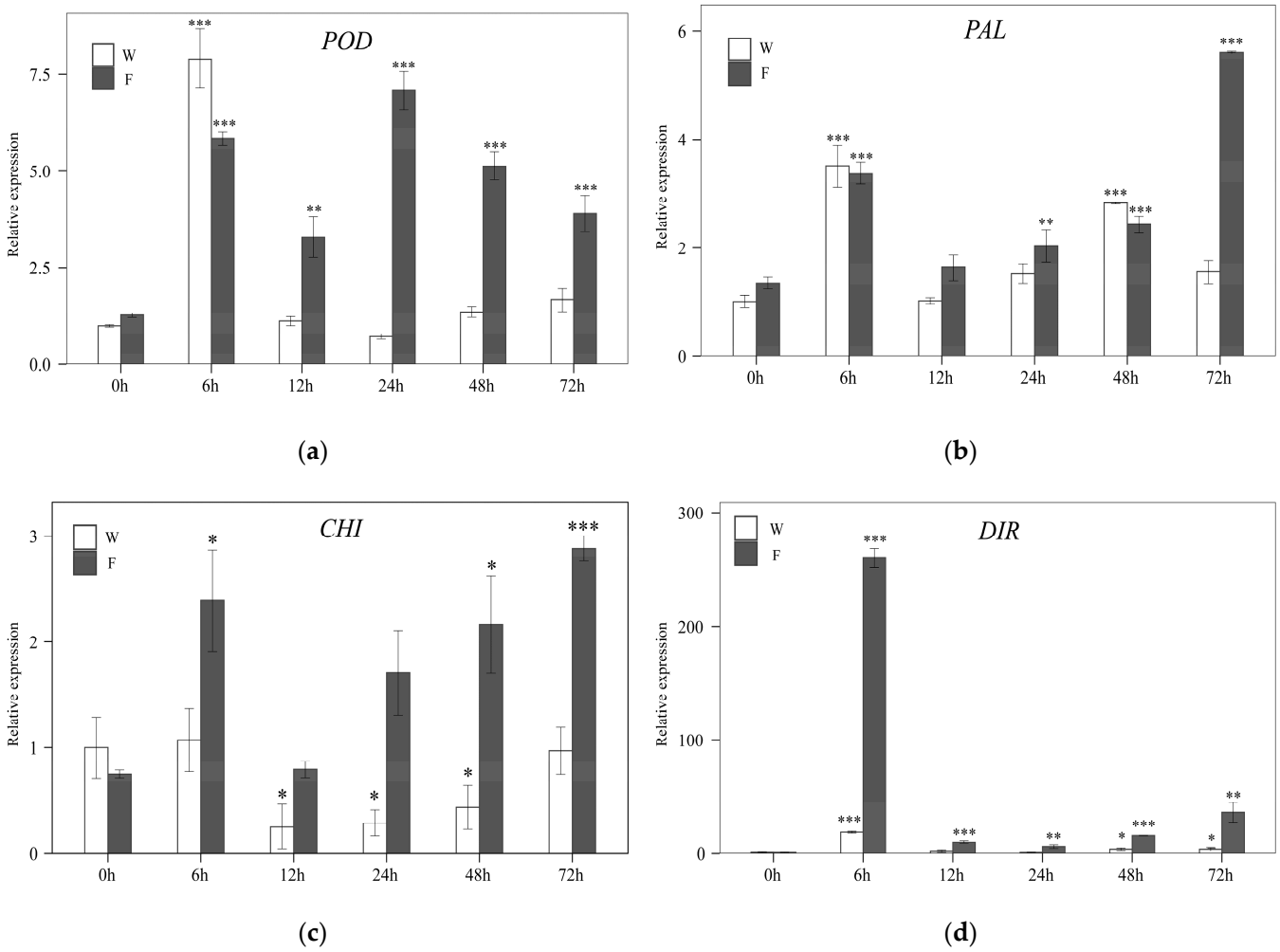

Figure 4. The relative expression levels of four resistance-related genes at different time points of *L. sargentiae.* The relative expression levels of *POD* (**a**), *PAL* (**b**), *CHI* (**c**), and *DIR* (**d**). F and W represent the different treatments with *F. oxysporum* and sterile water, respectively. The 0 h time point with sterile water treatment (0 hW) is the control. Each time period was compared with the control. *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. The expression of the four target genes was normalized according to the most suitable reference gene *HIS* using the $2^{-\Delta\Delta Ct}$ method. One-way ANOVA with Tukey's HSD post-hoc test was used to compare the means of multiple samples at the 5% significance level.

4. Discussion

Fusarium wilt disease is one of the biggest threats endangering lilies. It can cause huge economic losses through its adverse impacts on lily yield and quality [24–26]. Currently, chemical control is the primary method to control or prevent this disease. However, chemical pesticides have shown diminishing effects due to the soil-borne nature of *F. oxysporum* and the development of pathogenic resistance [27]. Moreover, chemicals are expensive and harm the environment [24]. Therefore, breeding novel resistant varieties is the most effective approach for managing *Fusarium* wilt diseases in lilies [28,40]. Some Chinese endemic wild lily species, including *L. sargentiae* Wilson, *L. regale* Wilson, and *L. pumilum* DC, have shown higher resistance to *F. oxysporum* and have been integrated into disease resistance breeding programs [30,33]. With the rapid developments of genomics-assisted breeding, it is essential to integrate quantitative genetics and functional genomics to decipher the regulation network of *Fusarium* wilt resistance in lilies. These studies require qRT-PCR analysis for precise and efficient quantification of expression levels of

target genes. Accordingly, the present study identified reliable reference genes for precise qRT-PCR normalization of candidate *Fusarium* wilt-related resistant genes' expressions to facilitate further molecular studies in *L. sargentiae*. Diverse molecular studies have been conducted in *L. regale* Wilson [25,28,29,41–43] and *L. pumilum* DC [30], but rarely in *L. sargentiae* Wilson.

Transcriptome sequencing technology was applied to uncover the resistance-related genes of *F. oxysporum* and gain insight into the molecular mechanisms that govern disease resistance in *L. sargentiae*. Based on the transcriptome data, we selected nine candidate reference genes for qRT-PCR normalization of *F. oxysporum*-resistant candidate genes' expressions. The expression stabilities of these genes were assessed via three public programs (GeNorm, BestKeeper, and NormFinder). To our knowledge, this study represents the first comprehensive identification of reliable reference genes for molecular dissection of *F. oxysporum*-resistance mechanisms in *L. sargentiae*. Previous related studies were focused on internal reference genes for other phenotypic traits of lily and other close species. For example, the genes *TIP41* and *ACT* were identified as the best combination of reference genes for analyzing candidate genes associated with shading in the lily cultivar 'Tiny Padhye' [2]. *EF-1α* and 18S rRNA were unveiled as the most suitable reference genes for investigating the expression levels of abiotic stresses related candidate genes, including heat, cold, heavy metals, and drought, in *L. brownii* var. *Viridulum* [44]. In *L. davidii* var. *Unicolor*, *FP*, *ACT*, *GAPDH*, and *AP4* were detected as reference genes for studying abiotic stress and developmental process-related genes [45]. These results indicate that reference genes are specific to experimental conditions, and for the same trait, they may differ according to the species and studied organs.

In *L. regale* Wilson, *GAPDH* is the commonly used reference gene to normalize *Fusarium* wilt-related candidate genes' expression levels at different time points of post-*F. oxysporum* inoculation [28,29,41–43]. Nevertheless, in this study, we found that *HIS* was the most reliable reference gene for similar studies in *L. sargentiae*. Instead, *GAPDH* was found to be the least stable reference gene among all nine tested reference genes (Table 3). Consistently, in *L. pumilum* DC, *18S* is the suitable reference gene, instead of *GAPDH* for resistance to *F. oxysporum*-related studies [30]. These results show that *GAPDH* is unsuitable for accurate qRT-PCR normalizing the expression levels of *F.oxysporum*-resistant genes in *L. sargentiae* and *L. pumilum*.

To confirm the suitability of the identified most reliable reference gene (*HIS*), it was used to normalize the expression levels of four resistance-related genes at different times in two treatments in *L. sargentiae*. The selected target genes, including *POD*, *PAL*, *CHI,* and *DIR,* are well-known to be involved in defense mechanisms against various pathogens in plants. *POD*, *PAL,* and *CHI* were significantly up-regulated in *L. regale* under *F. oxysporum* treatment [25,29,43,46]. *DIR* may play an important role in responses to different pathogenetic fungi in many plants. In *Pisum sativum*, the expression of *DIR* was significantly induced after inoculating with *F. solani* f. sp. *Phaseoli* [47]; *Erysiphe necator* in *Vitis vinifera* [48]; *Colletotrichum gloeosporioides* in *Physcomitrella patens* [49]; and *Marssonina brunnea* in *Populus* [50]. In the present study, the four resistance-related genes expression patterns via *HIS* normalization were consistent with the above results. This result further demonstrated that *HIS* should represent the reference genes for qRT-PCR normalization of the expression levels of target genes related to *F. oxysporum* resistance in *L. sargentiae.* Functional characterization of *POD*, *PAL*, *CHI,* and *DIR* is needed to uncover the regulatory mechanisms associated with their respective expressions in responses to *F. oxysporum* in *L. sargentiae* and close species.

5. Conclusions

Overall, a total of nine candidate reference genes were selected from the RNA-seq data of *L.* sargentiae based on their FPKM values in this study. The expression stability of all reference genes was validated under *F. oxysporum* inoculation by three different statistical algorithms (geNorm, NormFinder, and BestKeeper). Among them, *HIS* exhibited

the highest level of stability. The expression patterns of four *F. oxysporum*-resistant genes normalized by HIS were consistent with their responses to pathogenetic fungi in other plants. It indicates that *HIS* can be used as a suitable reference gene for the standardized qRT-PCR analysis of *F. oxysporum*-resistant genes in *Lilium*. Our findings will help provide comprehensive guidelines for identifying suitable reference genes in other lily species and also contribute to further explorations of the molecular mechanisms in *Lilium* in resisting *F. oxysporum*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13063395/s1, Table S1: Candidate reference genes and target genes in RNA-seq libraries of *L. sargentiae* induced by *F. oxysporum* and sterile water, respectively.

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