scientific reports

Check for updates

OPEN

Selection of stable reference genes in *Prunus persica* fruit infected with *Monilinia laxa* for normalisation of RT-qPCR gene expression data

Agata Lizzio[1,2,3]✉, Valerio Battaglia[2], Ernesto Lahoz[2], Massimo Reverberi[3] & Milena Petriccione[1]

Reverse transcription-quantitative PCR (RT-qPCR) is a powerful tool for quantifying gene expression. However, reference genes (RGs) for gene expression analysis in peach (*Prunus persica*) during interactions with *Monilinia laxa*, a major fungal pathogen that causes brown rot, have not been established. In this study, we analysed 12 candidate RGs in this pathosystem by analysing samples from 12 to 144 HAI. The stability of the RGs was evaluated using the ΔCq method and BestKeeper, NormFinder, and geNorm algorithms. Our results identified AKT3, RNA pol II (RPII) and SNARE (using geNorm), RPII, AKT3 and TEF2 (using NormFinder), AKT3, SNARE and RPII (using BestKeeper) and RPII, MUB6 and AKT3 (using the ΔCq method) as the most stable RGs for mRNA normalisation in this pathosystem across all tested samples. The geNorm algorithm was used to determine the optimal number of suitable RGs required for proper normalisation under these experimental conditions, indicating that the three RGs were sufficient for normalisation. Analysis of the results obtained using different algorithms showed that AKT3, RPII, and SNARE were the three most stable RGs. Furthermore, to confirm the validity of the reference genes, the expression levels of six genes of interest, involved in different metabolic pathways, were normalized in inoculated and uninoculated peach fruit. These findings provide a set of RGs for accurate RT-qPCR analysis in studies involving peach and *M. laxa* interactions, facilitating deeper insights into the molecular mechanisms underlying this important plant–pathogen relationship.

Keywords Peach, Pathosystem, Brown rot, RGs selection and normalisation, Algorithms

Monilinia laxa (Aderh. & Ruhland) Honey is an important fungal pathogen responsible for brown rot in stone fruits, including peaches (*Prunus persica* (L.) Batsch). This disease is a major threat to peach production worldwide, causing significant pre- and post-harvest economic losses that can exceed 50%, even reaching 100%, when environmental conditions are favourable for its development[1]. Recent studies have focused on elucidating the pathogenic mechanisms of *M. laxa*, identifying host resistance factors, and developing effective management strategies[2]. Endo- and exo-polygalacturonases play crucial roles in degrading plant cell walls, enabling the pathogen to penetrate and spread within the host tissue[2,3]. In addition, the production of reactive oxygen species (ROS) by *M. laxa* contributes to its virulence and pathogenicity by inducing oxidative stress in host plants[3]. Understanding host resistance mechanisms against *M. laxa* is essential for developing resistant peach cultivars. Recent studies have identified several peach genes associated with brown rot resistance. For example, the genes involved in the phenylpropanoid pathway, which is responsible for synthesising lignin and other phenolic compounds, have been linked to enhanced resistance[4]. Additionally, genes encoding pathogenesis-related (PR) proteins, such as PR-1 and PR-5, are upregulated in resistant peach cultivars in response to *M. laxa* infection[5].

Advances in omics technologies such as genomics, transcriptomics, and metabolomics offer powerful tools for identifying key genes and metabolic pathways involved in resistance and susceptibility[6]. Gene expression analysis is crucial for understanding the intricate regulatory networks of genetic, signalling, and metabolic pathways involved in plant–pathogen interactions. While gene expression microarrays offer a comprehensive

[1]CREA Council for Agricultural Research and Economics, Fruit and Citrus Crops (CREA-OFA), Olive, Caserta, Italy. [2]CREA Council for Agricultural Research and Economics, Cereal and Industrial Crops (CREA-CI), Caserta, Italy. [3]Department of Environmental Biology, "Sapienza" University of Rome, Rome, RM, Italy. ✉email: agata.lizzio@crea.gov.it

overview of global gene transcriptional activity, RT-qPCR assays are typically used to validate these results on a smaller scale owing to their high sensitivity, specificity, and reproducibility[7]. mRNA transcription is highly sensitive to external factors and requires a well-defined experimental design[8]. Several critical steps, including accurate RNA extraction, high-quality RNA to ensure integrity and purity, efficient reverse transcription to cDNA, careful primer design, thorough RT-qPCR validation, and appropriate selection of RGs, should support a robust experimental design[8]. According to the Minimum Information for Publication of Real-Time Quantitative PCR Experiments (MIQE) guidelines, normalisation of gene expression levels using a set of stable reference genes (RGs) is essential to minimise variability in gene expression quantification, thereby increasing the accuracy and reliability of the results[8]. Several studies have examined the stability of candidate RGs in different pathosystems and have revealed that some RGs exhibit a degree of variability. Plant pathogens, including viruses, bacteria, and fungi, can induce metabolic changes and reprogram gene expression in different organs of the host plant, thereby affecting the stability of the RGs[9,10]. The proteomic responses to infection by *M. fructicola* and *M. laxa* differ among peach cultivars with different levels of resistance against brown rot. Multiple classes of proteins are differentially expressed between resistant and susceptible cultivars[1].

Different RGs have been used for the expression normalisation of target genes in *P. persica* under abiotic and biotic stress such as tubulin alpha-5 (TUA5), tubulin beta-1 (TUB1), ubiquitin 10 (UBQ10), actin (ACT), cyclophilin 2 (CYP2), translation elongation factor 2 (TEF2), initiation factor 1 A (IF-1 A), RPII and small subunit 18 S rRNA (18 S)[11–16]. In addition, the stability of some RGs, as well as RNA polymerase II (RP II), has been evaluated in different tissues, genotypes, regulator treatments, and developmental stages of peaches[14].

The aim of this study was to select 12 RGs as candidates and evaluate the most stable ones in the peach/*M. laxa* pathosystem. Four statistical algorithms, i.e., geNorm[17] NormFinder[18] delta Ct[19] and BestKeeper[20] were used to determine RGs expression stabilities at six time points after fungal infection in peach fruit.

Results

Selection of RGs, amplification specificity and efficiency test

Twelve candidate RGs were chosen as internal controls for the expression studies in peach fruits inoculated with *M. laxa*. Notably, eight of these RGs had been previously tested in peach under different experimental conditions, as detailed in Table 1. The specificity of each primer pair was verified by the presence of a single band of expected size on a 2% agarose gel (Fig. S1 and S2). Furthermore, the melting curve of each primer pair was characterised by a single peak, confirming the absence of dimers (Fig S). No signal was detected in the melt-curve analysis of the negative controls for any of the candidate RGs. Analysing the standard curves of all primer pairs, the PCR efficiency (E) varied from 100.9 to 109.8%, and the regression coefficient (R^2) ranged from 0.989 to 1.00 (Table 1), confirming that the selected RGs were appropriate for RT-qPCR analysis.

Expression profiling of RGs

The expression levels of candidate RGs were assessed using raw quantification cycle (Cq) values, where RGs with high Cq values require a high number of cycles to reach threshold fluorescence and have the lowest expression abundance. (Fig. 1, Table S1). In this experimental condition the samples displayed average Cq values ranged from 23.01 ± 1.51 to 29.24 ± 1.22, with values between 21.27 and 32.96. IF1A exhibited the highest expression level among RGs, characterised by the lowest mean Cq value. In contrast, CYP2 had the highest average Cq values and the lowest expression levels. As shown in the Fig. 1, SNARE, AKT3, and 18 S showed the least variation in gene expression, and their Cq values ranged from 28.06 to 29.78, 24.94 to 26.82 and 28.17 to 30.36, respectively (< 2.2 cycles). Therefore, these genes might be the most stable. In contrast, TUB1 exhibited the highest variation with Cq values from 25.76 to 32.96.

Expression stability of the RGs

Stability analysis of the 12 candidate RGs was carried out using four statistical applets: BestKeeper, geNorm, NormFinder, and deltaCt, and the results are shown in Table 2. The results of BestKeeper analysis highlighted that seven RGs had the least overall variation ($SD < 1$; $p < 0.001$), whereas five RGs showed unstable expression under *M. laxa* infection in peaches (Table 2). AKT3 and SNARE were the most stable RGs with SD values of 0.39 and 0.41, while ACT and TUB 1 were identified as the least reliable RGs. geNorm analysis revealed that all tested RGs exhibited minimal variance, with M-values below the default threshold of 1.5 ($M \leq 1.5$), indicating high stability under our experimental conditions. The most stable RGs were AKT3 ($M = 0.52$) and RPII ($M = 0.52$). TUB1 was the least stable candidate RG ($M = 1.30$) (Table 2). Furthermore, the geNorm algorithm was used to determine the optimal number of suitable RGs required for proper normalisation under these experimental conditions. geNorm analysis revealed that the stepwise calculation of the pairwise variation value $V_{3/4}$ was below the threshold of 0.15, suggesting that the three RGs could be used for normalisation (Fig. 2). This indicates that the optimal number of RGs for normalisation was three, and adding a fourth RG did not significantly affect the normalisation of gene expression. The three most stable RGs in all the tested samples were AKT3, RPII, and SNARE.

NormFinder analysis of the 12 candidate RGs revealed that RPII was the most stable, with an SV of 0.21. In contrast to other software packages, NormFinder identified UBQ10 ($SV = 0.67$), rather than TUB1, as the least stable gene.

The results obtained from the deltaCt method agreed with those of NormFinder, indicating that RPII was the most stable RG candidate. TUB1 was considered the worst RG candidate by BestKeeper and geNorm.

Gene name (Gene symbol)	Accession number	Primer sequence (5′-3′)	Amplicon length (bp)	Efficiency (%)	R^2	Reference
Tublin alpha-5 (TUA5)	XM_020565543.1	TTCTCTCTACTCATTCCCTCCTTGGATTGGTGTATGTTGGT CTCTCG	117	109.8	0.997	11
Tubulin beta-1 (TUB1)	XM_007222035.2	CCGAGAATTGTGACTGCCTTCAAGAGCATCATCCTGTCTG GGTATTCC	124	107.4	0.999	11
Ubiquitin 10 (UBQ10)	XM_007211368.2	CGGGAGGACTCTTGCAGATTACTTCTCACTGCTCCGACAA	127	107.3	0.996	In this study
Actin (ACT)	XM_007211382.2	ATGTTCCCCGGTATTGCAGACCCTCCAATCCAGACACTGT	117	104.5	0.999	In this study
Membrane-anchored ubiquitin-fold protein 6 precursor (MUB6)	XM_007225786.2	GTTCATCAATGGCAGCAGGTTGTACAGTGGTAGATGGGCT	103	110	1	In this study
Soluble-N-ethylmaleimide-sensitive-factor accessory-protein receptor (SNARE)	XM_007205173.2	TGCGCAAAGCGTTCTCAAGCTTGGCTGCAACACCCATTATT	101	103.8	0.999	12
AKT Serine/Threonine Kinase 3 (AKT3)	XM_007209248.2	CAGTCCGTCCTCGCATTGATTCATTTCATACTTCCTTTCC TTCCA	100	109.5	0.994	12
Cyclophilin 2 (CYP2)	XM_007199662.2	ACTCCAAAGCGTGTTAGAAAAGGGTCTCTTCCACCATAAC GATAGG	120	108.9	0.998	12
Initiation factor 1 A (IF-1 A)	XM_020558850.1	GCCCAAGTGCTTCGTATGCTATCACCGGCTGCAATCCA	120	104.4	0.997	12
Translation elongation factor 2 (TEF2)	XM_020562217.1	GGTGTGACGATGAAGAGTGATGTGAAGGAGAGGGAAGGT GAAAG	129	100.9	0.989	14
RNA pol II (RPII)	XM_020569750.1	TGAAGCATACACCTATGATGATGAAGCTTTGACAGCACCA GTAGATTCC	128	108.1	0.996	14
Small subunit 18 S rRNA (18 S)	XM_007211699.2	TTGTTCCGCCAGACACAAAGGCAGAGCCTACATAAACCGC	124	107.8	0.999	In this study
Target genes						
WRKY transcription factor (WRKY)	XM_007215847.2	ACCAAGCCTCTCCGACTTTT CCCGTTGCAATTCCTCCAAT	130	97	0.995	In this study
Peroxidase (POX 73)	XM_007205465.2	TGGCCACCAGAGATGTCATT AATTGAAGGTTGGCTTGGGG	126	106.8	0.991	In this study
Calcium-dependent phospholipid-binding Copine family protein (BON1)	XM_007220862.2	ATCAGAAACCACGTCCCTGTTGAGAAATAGGGTGGTGGGG	92	104	0.992	In this study
Aminocyclopropane carboxylate oxidase (ACC oxidase)	XM_007218994.2	ACATGCATCTCCGAGGTCAT TGAGTAACCTATGCCGACCC	117	96.1	0.997	In this study
Phenylalanine ammonia-lyase (PAL)	XM_007220568.2	GCAGAGCAACACAACCAAGACAGATCAACAGCTTGGCACA	129	100.6	0.984	In this study
Thaumatin-like protein 1 (Thau1)	XM_007215802.2	AAGGGATCTGATGGAAGCGTATGTCTCCGGCTTGTCGTTA	100	98.5	0.999	In this study

Table 1. Descriptions of 12 candidate reference and six target genes in *Prunus persica*, primer sequences, amplicon traits and parameters derived from RT-qPCR analysis.

Comprehensive analysis of candidate RGs stability

Analysis of the 12 candidate RGs showed different stability rankings according to the algorithms used by the four tools (BestKeeper, geNorm, NormFinder, and deltaCt), and the geometric mean was used to obtain the final ranking (Table S2). RPII, AKT3, and SNARE were the most stable genes, whereas TUA5, UBQ10, and TUB1 were the least stable.

Target gene expression analysis for RG validation

Expression studies of six target genes or genes of interest (GOIs) were performed to validate the most and least stable selected RGs (Table S3). The selected GOIs are involved in different metabolic pathways. The results obtained with the most stable RGs showed upregulation of the WRKY gene after 24, 96, 120, and 144 h after inoculation (HAI), with 3.99-, 1.22-, 8.11 and 4.45-fold changes, respectively. POX73 was upregulated 24, 72, 120, and 144 HAI, with 1.99-, 0.91-, 4.31 and 6.09-fold changes, respectively. BON1 was overexpressed after 24, 120, and 144 HAI, and the same trend was observed for ACC and THAU1. PAL transcript levels gradually increased from 72 to 144 HAI ranging from 0.73 to 2.73-fold changes, respectively (Fig. 3).

Different trends and fold changes were observed when the normalisation method was performed using the least stable RGs (Fig. 4). POX73 showed lower relative expression level at 24, 72, 120, and 144 HAI, while BON1 was also upregulated 12 HAI. PAL was upregulated 12, 24 and 144 HAI whereas was downregulated 72, 96 and 120 HAI.

Discussion

Interactions between plants and pathogens are regulated by dynamic and complex molecular mechanisms. Understanding these interactions is essential for effective disease management and control[21]. Although there are different molecular techniques to study gene expression levels, such as RNA sequencing (RNA-Seq),

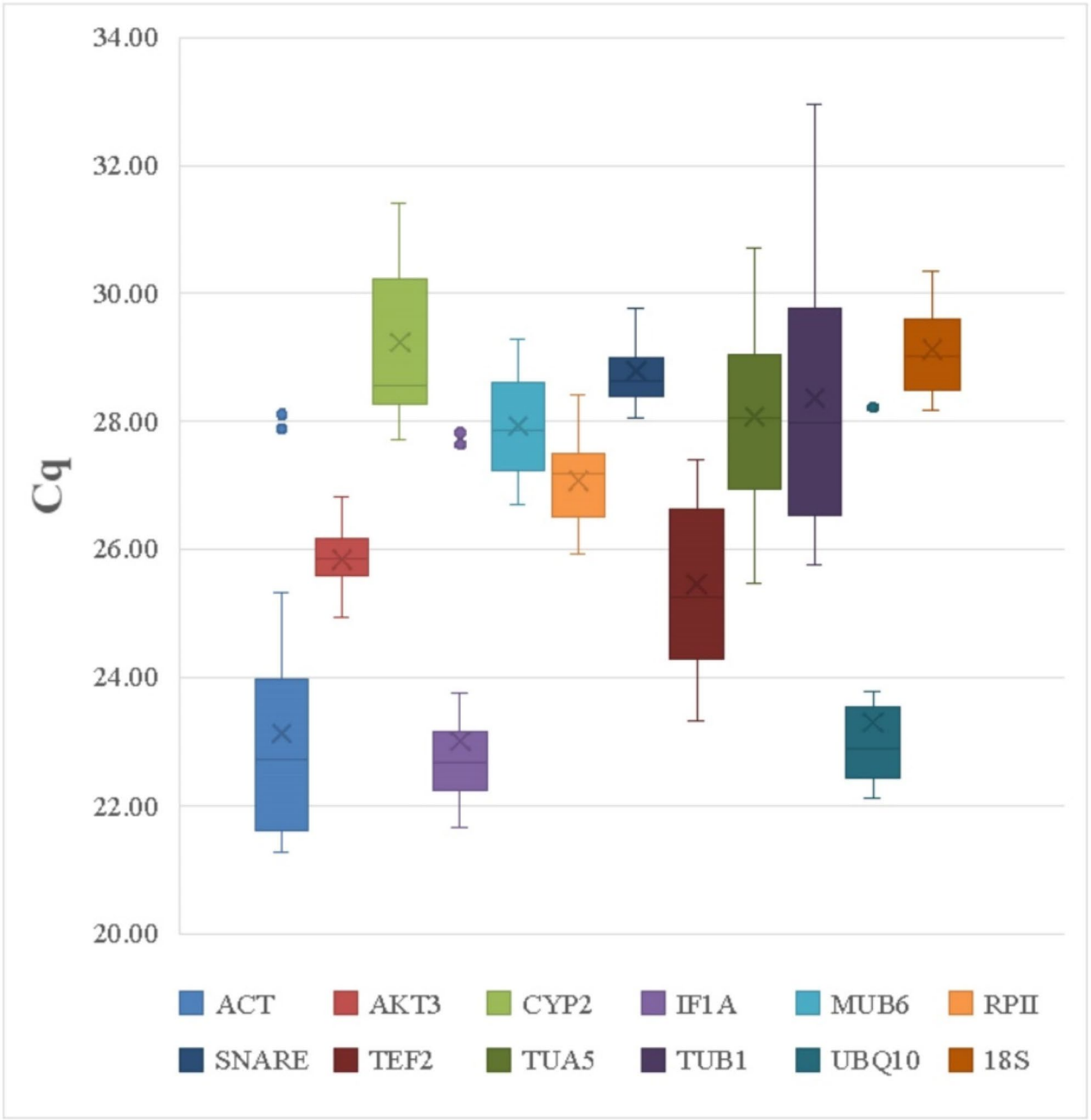

Fig. 1. Variation of Cq values for 12 candidate reference genes. Each box represents the 25th and the 75th percentiles. The line inside the box indicates the median and the "X" denotes the mean Cq value. The lines extending from the boxes (whiskers) show the range of the data and dots outside represent outliers.

microarrays, and northern blotting, RT-qPCR is widely used for studying gene expression changes under different experimental conditions, and RGs should be chosen carefully to compare mRNA levels between different test conditions in experimental design[8]. The use of internal RGs is the best normalisation method for obtaining accurate results, as suggested by the MIQE guidelines[8]. A comparison of the results obtained using the different algorithms of the four software types showed slight differences in the RGs rankings. Furthermore, there is no established number of RGs to validate GOI, but these are defined using the geNorm algorithm, and their numbers differ for each experimental condition[8,17]. Our results showed a pairwise variation $V_{3/4}$ value below 0.15, which indicates that combination of three-RGs was sufficient for optimal normalization of GOI.

Various studies have investigated RGs in *P. persica* under different biotic and abiotic stresses, and the most commonly used candidate RGs are actin, tubulin, elongation factor, ubiquitin, 18 S rRNA, and cyclophilin[11,14,16,22]. In this study, we examined a set of 12 RGs selected from literature data using the same primer sequences or redesigned according to MIQE guidelines[8,11,12,14,16,22] (Table 1).

	BestKeeper		geNorm		NormFinder		ΔCq	
Rank	Gene	SD	Gene	M	Gene	SV	Gene	SD
1	AKT3	0.386	AKT3	0.527	RPII	0.215	RPII	1.08
2	SNARE	0.409	RPII	0.527	AKT3	0.287	MUB6	1.1
3	RPII	0.540	SNARE	0.582	TEF2	0.288	AKT3	1.14
4	18 S	0.551	MUB6	0.605	SNARE	0.290	SNARE	1.16
5	MUB6	0.726	18 S	0.676	MUB6	0.339	TEF2	1.21
6	UBQ10	0.911	CYP2	0.777	ACT	0.367	CYP2	1.32
7	IF1a	0.924	TEF2	0.869	18 S	0.431	18 S	1.35
8	CYP2	1.095	TUA5	0.969	CYP2	0.462	IF1a	1.4
9	TEF2	1.177	IF1A	1,108	TUB1	0.519	TUA5	1.42
10	TUA5	1.285	UBQ10	1,197	IF1a	0.553	ACT	1.42
11	ACT	1.388	ACT	1.253	TUA5	0.575	UBQ10	1.48
12	TUB1	1.803	TUB1	1.306	UBQ10	0.670	TUB1	1.57

Table 2. The average stability ranking of the 12 candidate reference genes in *Prunus persica*. SD, standard deviation with BestKeeper. M, average of stability values calculated using geNorm. SV, stability value calculated by NormFinder. SD standard deviation of comparative ΔCq value.

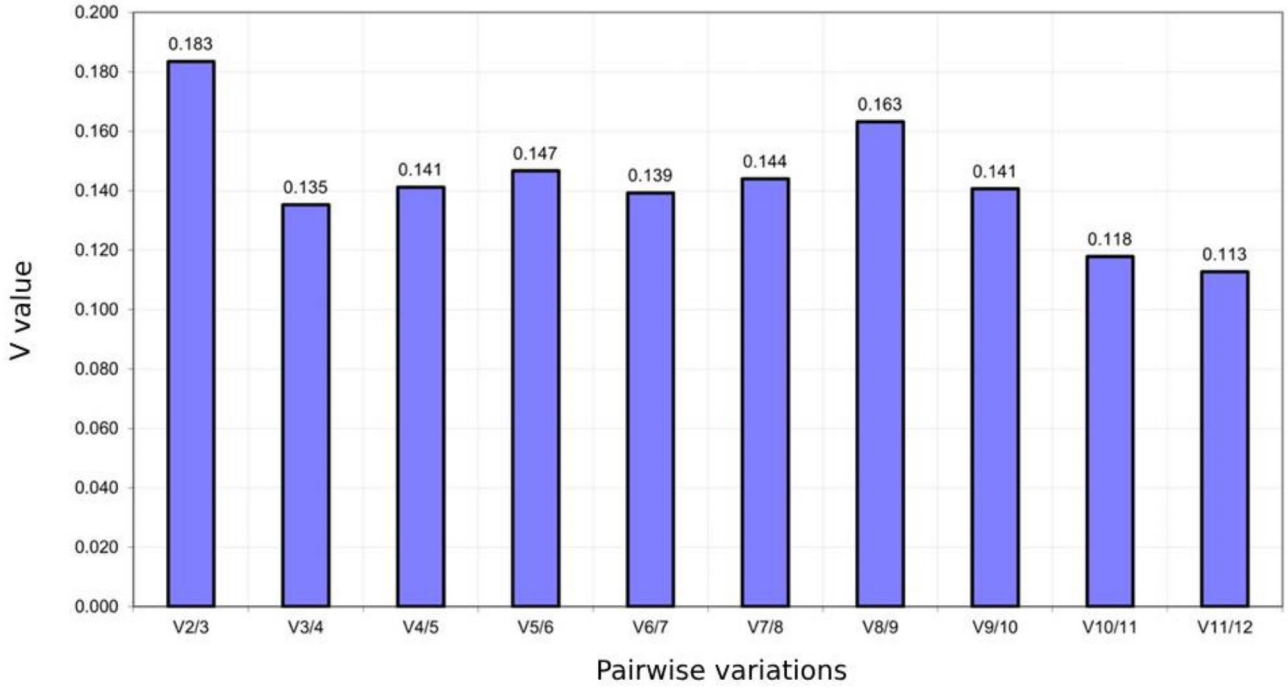

Fig. 2. Pairwise variation (V_n/V_{n+1}) was calculated between the normalization factors NF_n and NF_{n+1} using geNorm to establish the optimal number of reference genes for normalisation of all sample sets. Each pairwise variation value is compared with a recommended cut-off value 0.15, below which the inclusion of an additional reference gene is not required.

GeNorm, BestKeeper, and NormFinder alghorithms and deltaCt method were used to evaluate the stability of 12 candidate RGs in the pathosystem peach/*M. laxa*. The four tools showed different ranking orders, although the candidate RGs AKT3 and RPII were among the three most stable for all algorithms. Using geNorm and BestKeeper, the three most stable genes were AKT3, RPII, and SNARE. Instead of NormFinder, the best combination was obtained using RPII, AKT3, and TEF2, whereas the deltaCt method identified RPII, MUB6, and AKT3 as the most stable genes. According to NormFinder, BestKeeper, and delta Ct methods, ACT and TUB1 were the least stable genes. To address the differences observed between the four algorithms and to mitigate the individual limitations of each, the stability of the candidate reference genes was also evaluated using the geometric mean of the positions obtained from all four statistical algorithms. This approach ensured a more robust and comprehensive assessment of gene stability[23] (Table S2) The use of multiple RGs compensates for variability in the expression of individual genes, ensuring more reliable data normalization. The pairwise

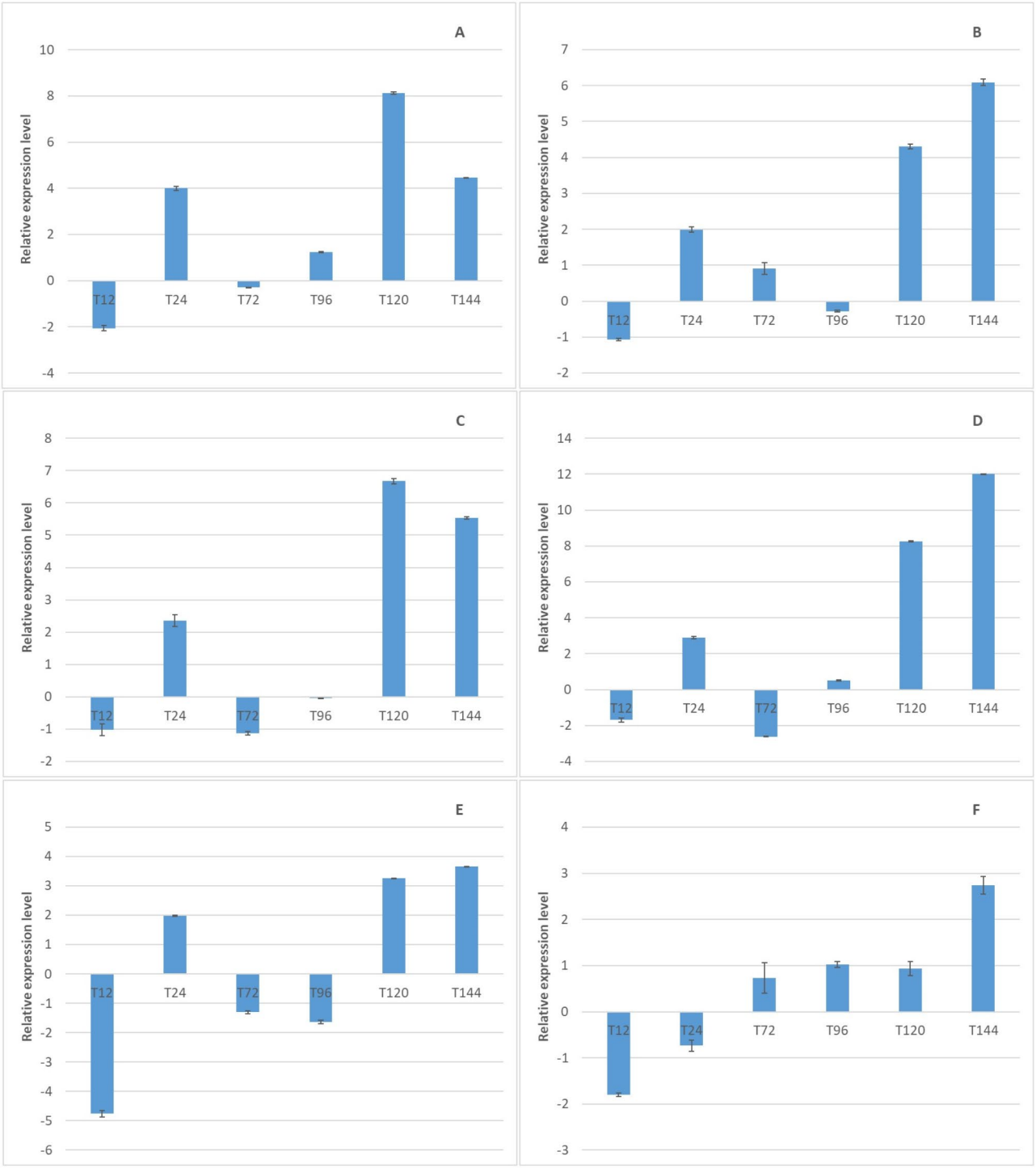

Fig. 3. Relative expression level of the WRKY transcription factor (A), peroxidase 73 (B), calcium-dependent phospholipid-binding copine family protein (C), thaumatin-like protein 1 (D), aminocyclopropane carboxylate oxidase (E), and phenylalanine ammonia-lyase (F) genes normalised with the three most stable genes (AKT serine/threonine kinase 3, RNA pol II, and soluble-N-ethylmaleimide-sensitive-factor accessory-protein receptor) in peach fruit inoculated with *M. laxa*. Data are expressed as mean ± standard deviation.

variation estimate $V_{3/4}$ in this experimental condition was below 1.5 (Fig. 2), indicating that three genes are adequate for normalizing qRT-PCR data. RPII, AKT3, and SNARE were the most stable genes, whereas TUA5, UBQ10, and TUB1 were the least stable. RPII was identified as the most stable gene in different vegetative tissues, fruits, and flowers of two different genotypes of *P. persica*[14], eight genotypes of *P. avium* L[24]. and in the leaves of six different *Citrus* genotypes[25]. Xu et al.[26] demonstrated that RPII was one of the most stable RGs in several *Raphanus sativus* L. species under different abiotic conditions. Contrary to the results obtained in our study, Kou

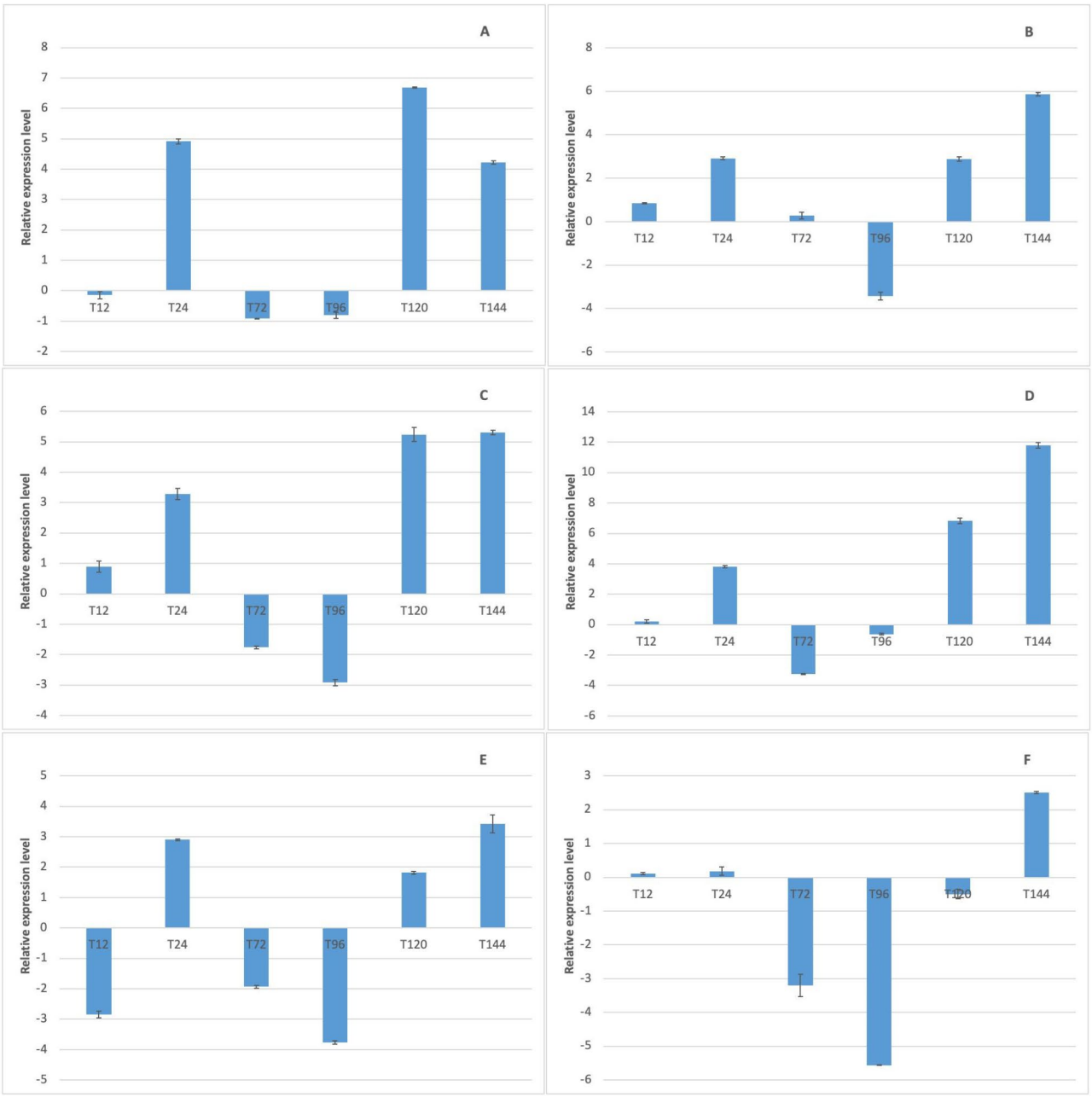

Fig. 4. Relative expression level of the WRKY transcription factor (A), peroxidase 73 (B), calcium-dependent phospholipid-binding copine family protein (C), thaumatin-like protein 1 (D), aminocyclopropane carboxylate oxidase (E), and phenylalanine ammonia-lyase (F) genes normalised with the three least stable genes (tubulin alpha-5, ubiquitin 10, and tubulin beta-1) in peach fruit inoculated with *M. laxa*. Data are expressed as mean ± standard deviation.

et al.[12] demonstrated that AKT3 was the least stable gene in peaches stored at different temperatures, owing to the different stress conditions and tested cultivars. The expression stability of the gene SNARE was reported by Kou et al.[12] in peach fruits stored at 15 °C and 25 °C and at different development stages. According to Marini et al.[13], TUA5 was the least stable RG in peach genotypes with different chilling requirements, whereas Xu et al.[27] showed that TUA5, together with CYP2, was the most stable RG in peach fruits of different genotypes infected with tobacco crackling virus. The gene UBQ10 showed high variation in expression under our experimental conditions. In contrast, it has been recognised as the most stable gene in peach fruits[14], *Brachypodium distachyon*[28] and *Oryza sativa* L. exposed to metal stresses[29]. Auler et al.[30] demonstrated that under water stress, UBQ10 was the most stable gene for the reproductive stages in one rice genotype, whereas it was the least stable gene in the vegetative stages. In the present study, TUB1 was identified as the least RG, as reported in different *Citrus* genotypes[25], but in different studies that investigated RG stability in several peach cultivars, TUB1 was identified as the most stable gene[14,27].

The appropriateness of the selected RGs was assessed using six genes of interest (WRKY, POX73, BON1, THAU1, ACC_OX, and PAL) involved in several metabolic pathways. The;

In this study, the ACC_OX gene responsible for ethylene production[31], showed a transient increase of up to two-fold after 24 HAI, was downregulated after 72 and 96 HAI, and was upregulated at 120 and 144 HAI. Barò-Montel et al.[32] reported that *PpACO* family showed a similar trend in peach fruits inoculated with different strains of *M. laxa* and *M. fructicola*, but the expression level of PpACO genes is strictly related with the chosen gene[33]. In addition, the overexpression of these genes could be linked to pathogen-induced senescence and an increase in the production of ethylene[32]. The WRKY transcription factor, which is a gene related to stress response, was always upregulated, except at 12 and 72 HAI. Upregulation of these genes has been reported in several studies on peaches after *M. fructicola* infection, particularly after 48 HAI[34–36]. WRKY is also involved in fruit ripening and senescence processes of the fruits[37]. Gou et al.[38] demonstrated the upregulation of this family of genes in peach fruits treated with 1-naphthaleneacetic acid, caused by higher ethylene production and softening. Ethylene is a hormone involved in both fruit ripening and interaction stages after necrotrophic fungal attack on fruits[39,40]. Fungal infections trigger a series of defence mechanisms in which PAL plays a crucial role in this process[41]. In this study, we observed the upregulation of PAL from 72 to 144 HAI. Tsalgatidou et al.[35] showed that after 48 h of interaction between *M. fructicola* and peaches, PAL was strongly overexpressed. A similar trend was observed in apple fruits inoculated with *Penicillium expansum* and *P. digitatum*, showing significant activation of the gene after 48 HAI[42].

The copine gene BON1 encodes a calcium-dependent phospholipid-binding protein that plays a crucial role in disease resistance and plant growth homeostasis[43]. Our results showed that this gene was upregulated 24, 120, and 144 h after HAI. Several studies have shown that the upregulation of this gene in *P. persica* fruits inoculated with *M. fructicola* occurs after 12 HAI[34–36].

Thaumatin-like proteins are involved in host defence systems after pathogen attack or other stress[44]. Tobias et al.[45] demonstrated that thaumatin-like proteins show antifungal activity via different mechanisms, such as spore lysis and reduced viability of germinated spores. In our study, THAU was upregulated 24, 120, and 144 HAI. In addition, in cherry fruits treated with methyl jasmonate and inoculated with *P. expansum*, THAU1 was overexpressed at 24, 48 and 120 HAI[46]. Tsalgatidou et al.[35] showed that the interaction between *M. fructicola*-peach fruit and *M. fructicola*-peach fruit-chitosan caused upregulation of the THAU gene after 48 HAI. Another study demonstrated that THAU1 was upregulated during *M. fructicola*-peach fruit interactions at 12 and 24 HAI[41]. This gene was upregulated in *P. persica* fruit after a week of cold storage at 5 °C[47].

Peroxidases are a family of genes that play crucial roles in several physiological processes, including defence against pathogens, growth and development of fruits, and protection against reactive oxygen species[48–50]. Our results showed the upregulation of POX73 after 24, 120, and 144 HAI, as reported by other studies that showed overexpression of this gene after 12, 24, and 48 HAI in the interaction between peach–chitosan–*M. fructicola*[35] and peach-*Bacillus amyloliquefaciens* QST 713-*M. fructicola*[36].

In conclusion, our study on the interaction between peaches and *M. laxa* identified RPII, AKT3, and SNARE as the most stable RGs for normalising RT-qPCR data. These genes exhibited consistent expression across various experimental conditions, ensuring reliable and accurate quantification of gene expression during peach-*Monilia* interactions. RPII, AKT3, and SNARE demonstrated superior stability compared to the other tested RGs, highlighting their suitability for normalisation in studies focusing on pathogen responses in peaches. The robust performance of these RGs will enable the precise measurement of gene expression changes, facilitating a deeper understanding of the molecular mechanisms underlying peach resistance or susceptibility to *M. laxa*.

Identification of these stable RGs is crucial for advancing research on peach pathology and improving the accuracy of gene expression analyses. These findings contribute to the specific study of peach-*M. laxa* interactions, but also offers valuable insights for broader applications in gene expression studies across various pathosystems.

Methods

Plant material and Monilia laxa inoculation

Peach fruits (*P. persica*) 'Fairtime' and *M. laxa* were used in this bioassay. The fungus was isolated from symptomatic fruits harvested in experimental orchard owned by CREA - Research Centre for Olive, Fruits and Citrus Crops (Caserta, Italy), and the identification was carried out with a multiplex PCR assay using the reverse primer MO368-5, and three species-specific forward primers: MO368-8R, MO368-10R and Laxa-R2 according to Côté et al.[51] 2004. Peach fruits ($n = 30$) were randomly harvested from the experimental orchard of the CREA, and no previous fungicide applications were received during the vegetative period. Fruits were washed in water, sterilised in sodium hypochlorite (0.3% v/v) for 10 min, rinsed twice with sterile distilled water, and dried in a laminar flow hood. For artificial inoculation, three small wounds (5 mm deep) were created in the equatorial position of each fruit using a sterile needle. *Monilinia laxa* inoculum was performed using conidia collected from a 2-week-old culture grown on potato dextrose agar (PDA, Oxoid) at 25 °C. The concentration of the conidial suspension was adjusted to 1×10^5 conidia mL^{-1} using a haemocytometer. Peach fruits were randomly divided into two batches (21 fruits in three biological replicates) and placed in sterilised plastic containers lined with wet paper towels. The first batch was inoculated by pipetting a drop of conidial suspension (60 µL) (treated samples) while 60 µL of sterile water was used in the second batch (control samples). Each sample was sealed airtight to ensure high relative humidity need for the infection, stored at 25 °C.

Experimental design and biological samples

Three biological replicates and three technical replicates were analysed for each sample. Sampling was carried out before inoculation and at 12, 24, 72, 96, 120, and 144 HAI (Fig. S5) and at the same timings in control ones. Samples were immediately frozen in liquid nitrogen and stored at − 80 °C until RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA from peaches inoculated with *M. laxa* and control samples was extracted according to Rubio-Piña and Zapata-Perez[52]. All samples were treated with RNase-free DNase (Invitrogen Life Technologies, Carlsbad, CA, USA) to eliminate genomic DNA contamination. The RNA concentration and purity were determined using a NanoDrop 2000/2000c spectrophotometer (Thermo Scientific, Milan, Italy). All the samples with $OD_{260/280}$ of 1.8–2.0 and $OD_{260/230} > 2$ were used for further analysis, while RNA structural integrity was verified using agarose gel electrophoresis (2%). RNA samples were used to synthesise single-stranded cDNA using iScript Select cDNA Synthesis Kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions.

Selection of candidate RGs and PCR primer design

Twelve candidate RGs (TUA5, TUB1, UBQ10, ACT, MUB6, SNARE, AKT3, CYP2, IF-1 A, TEF2, RPII, and 18 S) were selected to investigate their robustness as internal controls for RT-qPCR in *P. persica*. In addition, six genes were selected as genes of interest (GOIs) (WRKY, POX 73, BON1, ACC oxidase, PAL, and THAU1). Gene-specific primers, such as UBQ10, ACT, MUB6, 18 S, and all GOIs, were designed using Primer Expression Software version 3 (Table 1).

Evaluation of specificity and amplification efficiency

The amplification efficiency of the candidate RGs and GOIs was determined by RT-qPCR using a pool of all cDNA samples. A five-point standard curve was generated using a tenfold dilution series. The slope of the standard curve was used to calculate the correlation coefficient (R^2) and primer efficiency (E) according to the equation $E\ (\%) = (10^{(-1/\text{slope})} - 1) \times 100$ as reported by Radonić et al.[53].

Quantitative real-time PCR

Quantitative real-time-PCR was performed using a CFX Connect Real-time PCR Detection System (Bio-Rad, Milan, Italy). A total reaction mixture contained 10 µL SsoAdvanced SYBR Green Supermix (Bio-Rad, Milan, Italy), 1 µL of each PCR primer (500 nM), 2 µL of cDNA template (20 ng), and 6 µL DNase-free water. The RT-qPCR amplification protocol started with polymerase activation and DNA denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 30 s. A melting curve analysis was performed for each run to verify the specificity of amplification and the absence of primer dimers.

Stability analysis of RGs

The raw quantification cycle (Cq) and relative quantities were imported into the geNorm and NormFinder, Bestkeeper, and deltaCt methods. The geNorm algorithm calculates the expression stability value (M; cut-off value of 0.5) for all candidate RGs and then the pairwise variation (V) of each gene with the others. The most stable, RG, exhibited the lowest M value. GeNorm calculates the V value of $Vn/Vn+1$ between two sequential normalisation factors and defines the number of genes required for optimal normalisation ($Vn/Vn+1 < 0.15$)[17]. NormFinder calculates the expression stability value (SV) by analysing both the intra- and inter-group variations in gene expression for each RG (cut-off value of 0.15). The most stable genes showed lower SV values[18]. Bestkeeper is an Excel-based tool that selects the most stable RGs by applying a statistical analysis based on the Pearson correlation coefficient (r), coefficient of variance (CV), and standard deviation (SD). Subsequently, it combines the BestKeeper index (BKI) genes with a low SD and a high r value and calculates the correlation coefficient between each candidate RG and the BKI value, along with the associated *p*-value. An SD value greater than 1 suggests considerable variability in expression levels, indicating a lack of stability[20]. The delta Ct (dCt) method allows the identification of the most stable RGs by examining the relative expression of pairs of RGs across each sample[19].

Finally, the method by Velada et al.[23] was used for a comprehensive ranking of RG stability obtained using four algorithms across different experimental conditions. In this ranking, the most stable RG was assigned the number 1 and the least stable RG was assigned the number 12. By calculating the geometric mean of these values, a ranking of the RGs using the four algorithms together was obtained and referred to in this paper as the geometric mean. All statistical analyses were performed using the SPSS v. 20.0.

Validation of RGs

The relative expression levels ($\log_2$ fold changes) in GOIs (WRKY, POX 73, BON1, ACC oxidase, PAL and Thau1) were analyzed using stably expressed genes and unstable expressed genes according to the comparative $2^{-\Delta\Delta Ct}$ method[54] to verify the stability and reliability of the selected reference genes.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Received: 17 October 2024; Accepted: 13 February 2025
Published online: 25 February 2025

References

1. Papavasileiou, A. et al. Proteomic analysis upon peach fruit infection with *Monilinia fructicola* and *M. Laxa* identify responses contributing to brown rot resistance. *Sci. Rep.* **10**, 7807 (2020).
2. Astacio, J. D., Melgarejo, P., De Cal, A. & Espeso, E. A. *Monilinia fructicola* genes involved in the cell wall-degrading process in early nectarine infection. *Int. J. Food Microbiol.*, **419**, 110750 (2024).
3. Balsells-Llauradó, M. et al. Depicting the battle between nectarine and *Monilinia laxa*: The fruit developmental stage dictates the effectiveness of the host defenses and the pathogen's infection strategies. *Hortic. Res.* **7**, 167 (2020).
4. Sharma, A. et al. Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. *Mol* **24**, 2452 (2019).
5. Islam, M. M. et al. Pathogenesis-related proteins (PRs) countering environmental stress in plants: a review. *S Afri J. Bot.* **160**, 414–427 (2023).
6. Murmu, S. et al. A review of artificial intelligence-assisted omics techniques in plant defense: current trends and future directions. *Front. Plant. Sci.* **15**, 1292054 (2024).
7. Derveaux, S., Vandesompele, J. & Hellemans, J. How to do successful gene expression analysis using real-time PCR. *Methods* **50**, 227–230 (2010).
8. Bustin, S. A. et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611–622 (2009).
9. Petriccione, M., Mastrobuoni, F., Zampella, L. & Scortichini, M. Reference gene selection for normalization of RT-qPCR gene expression data from *Actinidia deliciosa* leaves infected with *Pseudomonas syringae* Pv. *Actinidiae Sci. Rep.* **5**, 16961 (2015).
10. Rego, E. C. S. et al. Stable reference genes for RT-qPCR analysis of gene expression in the Musa acuminata-pseudocercospora musae interaction. *Sci. Rep.* **9**, 14592 (2019).
11. Xu, Z. et al. Selection and validation of reliable reference genes for gene expression studies in different genotypes and TRV-infected fruits of peach (*Prunus persica* L. Batsch) during ripening. *Genes* **13**, 160 (2022).
12. Kou, X., Zhang, L., Yang, S., Li, G. & Ye, J. Selection and validation of reference genes for quantitative RT-PCR analysis in peach fruit under different experimental conditions. *Sci. Hortic.* **225**, 195–203 (2017).
13. Marini, N., Bevilacqua, C. B., Büttow, M. V., Raseira, M. C. B. & Bonow, S. Identification of reference genes for RT-qPCR analysis in peach genotypes with contrasting chilling requirements. *Genet. Mol. Res.* **16**, 16029666 (2017).
14. Tong, Z., Gao, Z., Wang, F., Zhou, J. & Zhang, Z. Selection of reliable reference genes for gene expression studies in peach using real-time PCR. *BMC Mol. Biol.* **10**, 1–13 (2009).
15. Gu, R., Zhu, S., Zhou, J., Liu, N. & Shi, J. Inhibition on brown rot disease and induction of defence response in harvested peach fruit by nitric oxide solution. *Eur. J. Plant. Pathol.* **139**, 369–378 (2014).
16. You, S. et al. Selection and validation reference genes for qRT-PCR normalization in different cultivars during fruit ripening and softening of peach (*Prunus persica). Sci. Rep.* **11**, 7302 (2021).
17. Vandesompele, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biol.* **3**, 1–12 (2002).
18. 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.* **64**, 5245–5250 (2004).
19. Silver, N., Best, S., Jiang, J. & Thein, S. L. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol. Biol.* 7; (2006).
20. 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.* **26**, 509–515 (2004).
21. Vuong, U. T. et al. Engineering plant immune circuit: walking to the bright future with a novel toolbox. *Plant. Biotechnol. J.* **21**, 17–45 (2023).
22. Rickes, L. N., Klumb, E. K., Benitez, L. C., Braga, E. J. B. & Bianchi, V. J. Stable reference genes for studies of gene expression in '*Prunus persica*' under water stress. *Aust J. Crop Sci.* **10**, 1348–1356 (2016).
23. Velada, I., Ragonezi, C., Arnholdt-Schmitt, B. & Cardoso, H. Reference genes selection and normalization of oxidative stress responsive genes upon different temperature stress conditions in *Hypericum perforatum* L. *PLoS One*. 9; (2014).
24. Ye, X., Zhang, F., Tao, Y., Song, S. & Fang, J. Reference gene selection for quantitative real-time PCR normalization in different cherry genotypes, developmental stages and organs. *Sci. Hortic.* **181**, 182–188 (2015).
25. Yan, J., Yuan, F., Long, G., Qin, L. & Deng, Z. Selection of reference genes for quantitative real-time RT-PCR analysis in citrus. *Mol. Biol. Rep.* **39**, 1831–1838 (2012).
26. Xu, Y. et al. Evaluation of reference genes for gene expression studies in radish (*Raphanus sativus* L.) using quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* **424**, 398–403 (2012).
27. Xu, Z. et al. Selection and validation of reliable reference genes for gene expression studies in different genotypes and TRV-Infected fruits of peach (*Prunus persica* L. Batsch) during ripening. *Genes* **17**, 160 (2022).
28. Hong, S. Y., Seo, P. J., Yang, M. S., Xiang, F. & Park, C. M. Exploring valid reference genes for gene expression studies in *Brachypodium distachyon*by real-time PCR. *BMC Plant. Biol.* **8**, 112(2008).
29. Almas, D. E. & Kamrodi, A. R. Validation of appropriate reference genes for real-time quantitative pcr gene expression analysis in rice plants exposed to metal stresses. *Russ J. Plant. Physiol.* **65**, 890–897 (2018).
30. Auler, P. A. et al. Evaluation of stability and validation of reference genes for RT-qPCR expression studies in rice plants under water deficit. *J. Appl. Genet.* **58**, 163–177 (2017).
31. Houben, M. & Van de Poel, B. 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO): the enzyme that makes the plant hormone ethylene. *Front. Plant. Sci.* **10**, 695 (2019).
32. Baró-Montel, N. et al. Double-sided battle: the role of ethylene during *Monilinia* spp. infection in peach at different phenological stages. *Plant. Physiol. Biochem.* **144**, 324–333 (2019).
33. Tadiello, A. et al. On the role of ethylene, auxin and a GOLVEN-like peptide hormone in the regulation of peach ripening. *BMC Plant. Biol.* **16**, 1–17 (2016).
34. Cheng, C. et al. Metabolome and transcriptome analysis of postharvest peach fruit in response to fungal pathogen *Monilinia fructicola* infection. *LWT Food Sci. Technol.* **73**, 114301 (2023).
35. Tsalgatidou, P. C. et al. Global transcriptome analysis of the peach (*Prunus persica*) in the Interaction system of fruit–chitosan–*Monilinia fructicola*. *Plants* **13**, 567(2024).
36. Tsalgatidou, P. C. et al. Insights into the interaction between the biocontrol agent *Bacillus amyloliquefaciens* QST 713, the pathogen *Monilinia fructicola* and peach fruit. *Agron* **14**, 771 (2024).
37. Zhang, W. W. et al. FvWRKY48 binds to the pectate lyase FvPLA promoter to control fruit softening in Fragaria vesca. *Plant. Physiol.* **18**, 1037–1049 (2022).
38. Guo, S. et al. Transcriptomic Analysis Reveals Genes Associated with the Regulation of Peach Fruit Softening and Senescence during Storage. *Foods*. 12; (2023).
39. Glazebrook, J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**, 205–227 (2005).
40. Vall-llaura, N., Torres, R., Teixidó, N., Usall, J. & Giné-Bordonaba, J. Untangling the role of ethylene beyond fruit development and ripening: a physiological and molecular perspective focused on the *Monilinia*-Peach interaction. *Sci. Hortic.* **301**, 111123 (2022).

41. Ferreira, R. B. et al. The role of plant defence proteins in fungal pathogenesis. *Mol. Plant. Pathol.* **8**, 677–700 (2007).
42. Vilanova, L. et al. Transcriptomic profiling of apple in response to inoculation with a pathogen (*Penicillium Expansum*) and a non-pathogen (*Penicillium Digitatum*). *Plant. Mol. Biol. Rep.* **32**, 566–583 (2014).
43. Yang, D. L. et al. Calcium pumps and interacting BON1 protein modulate calcium signature, stomatal closure, and plant immunity. *Plant. Physiol.* **175**, 424–437 (2017).
44. Mahdavi, F., Sariah, M. & Maziah, M. Expression of rice thaumatin-like protein gene in transgenic banana plants enhances resistance to Fusarium wilt. *Appl. Biochem. Biotechnol.* **166**, 1008–1019 (2012).
45. Tobias, D. J., Manoharan, M., Pritsch, C. & Dahleen, L. S. Co-bombardment, integration and expression of rice chitinase and thaumatin-like protein genes in barley (*Hordeum vulgare* cv. Conlon). *Plant. Cell. Rep.* **26**, 631–639 (2007).
46. Wang, L. et al. Methyl jasmonate primed defense responses against *Penicillium Expansum* in sweet cherry fruit. *Plant. Mol. Biol. Rep.* **33**, 1464–1471 (2015).
47. Ogundiwin, E. A. et al. Development of Chill Peach genomic tools and identification of cold-responsive genes in peach fruit. *Plant. Mol. Biol.* **68**, 379–397 (2008).
48. Pandey, V. P., Awasthi, M., Singh, S. & Tiwari, S. Dwivedi U.N. A comprehensive review on function and application of plant peroxidases. *Biochem. Anal. Biochem.* **6**, 10000308 (2017).
49. Cosio, C. & Dunand, C. Specific functions of individual class III peroxidase genes. *J. Exp. Bot.* **60**, 391–408 (2009).
50. Bindschedler, L. V. et al. Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. *Plant. J.* **47**, 851–863 (2006).
51. Côté, M. J., Tardif, M. C. & Meldrum, A. J. Identification of *Monilinia Fructigena, M. Fructicola, M. Laxa, and Monilia polystroma* on inoculated and naturally infected fruit using multiplex PCR. *Plant. Dis.* **88**, 1219–1225 (2004).
52. Rubio-Piña, J. A. & Zapata-Pérez, O. Isolation of total RNA from tissues rich in polyphenols and polysaccharides of mangrove plants. *Electron. J. Biotechnol.* **14**, 1-8 (2011).
53. Radonić, A. et al. Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* **313**, 856–862 (2004).
54. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods* **25**, 402–408 (2001).

Acknowledgements

This work was supported by PNRR Project "National Research Centre for Agricultural Technologies (Agritech) – SPOKE 2 Crop Health: a multidisciplinary system approach to reduce the use of agrochemicals" CUP C23C22000450006 funded by the Italian Ministry for Universities and Research (MUR).

Author contributions

Conceptualization, AL and MP; formal analysis, AL and VB; funding acquisition MP, EL and MR; investigation AL and VB; methodology AL, VB and MP, writing original draft, AL and VB; writing, review and editing, MP, EL and MR. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-90506-1.

Correspondence and requests for materials should be addressed to A.L.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.