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ORIGINAL ARTICLE

Reference genes for RT-qPCR analysis in *Citrus* and *Poncirus* infected by zoospores of *Phytophthora parasitica*

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Abstract *Phytophthora* species are highly destructive phytopathogens, associated with massive damage in natural ecosystems and agriculture. Citrus production is also affected, mainly by the hemibiothrophic oomycete *Phytophthora parasitica*, which causes root rot and gummosis. *Poncirus trifoliata* and *Citrus sunki* (two rootstocks widely used in citrus orchards) pose a resistance and a susceptible interaction with *P. parasitica,* respectively, which makes them suitable models to study plant defense mechanisms. Gene expression analysis is a very important tool in this type of study, in particular PCR (RT-qPCR). Hence, it is crucial to use appropriate reference genes for expression normalization. Our aim was to evaluate the stability of several candidate reference genes to determine which set is best suited for normalization in citrus infected with *P. parasitica*. We evaluated five candidate reference genes selected from the database CitEST. GeNorm and NormFinder algorithms were used to assess the best reference genes. We found that the more stable genes to be used for RT-qPCR analysis in *P. trifoliata* plants were *GAPC2* and *F-BOX,* while *EGIDH* and *GAPC2* were more suitable to *C. sunki*. These four genes were found to be excellent normalizers, being stable throughout the infection regardless of pathogen attack or symptom development.

Keywords Oomycetes · Gene expression · Housekeeping genes · Molecular biology

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Introduction

The world's citrus production has been widely damaged by several diseases caused by viruses, bacteria, fungi, oomycetes, and nematodes. *Phytophthora parasitica* (Dastur) (syn. *P. nicotianae*) is a destructive oomycete capable of infecting more than 250 plant families (Kamoun et al. 2015. In citrus, it is associated with root rot and gummosis, which affects the productivity but can also lead to the declining of the tree (Panabieres et al. 2016). It is estimated that in average 10 to 30% of the total production of citrus orchards is affected due to *P. parasitica*-associated diseases (Timmer et al. 2000).

During infection, pathogens secrete hundreds of effector proteins, some of which are recognized by plant proteins activating defense systems, while others are fundamental to the disease establishment and pathogen virulence (Osswald et al. 2014). In any case, there are changes in the transcription program of the plants, which could lead to susceptibility or resistance. These alterations in the genetic expression profile of plants are still obscure, especially in citrus.

In the interaction between *P. parasitica* and citrus, two genotypes, widely used as rootstocks, stand out because of their opposite outcomes regarding development of symptoms. While *Poncirus trifoliata* (L.) Raf. presents a clearly resistant interaction, Sunki mandarin (*Citrus sunki* (Hayata) hort. ex. Tanaka) is highly susceptible to the pathogen. These distinct responses to infection make the two species very convenient to study mechanisms of attack and defense, particularly at the molecular level. Gene expression analysis has been a very important tool in this type of study. One of the techniques with great accuracy, high sensitivity and specificity for this type of

work is quantitative reverse transcription-PCR (RT-qPCR) (Gachon et al. 2004; Nicot et al. 2005). However, for improved efficiency and accuracy it is necessary to use appropriate reference genes to normalize the expression levels of target genes (Nicot et al. 2005; Jain et al. 2006; Czechowski et al. 2005; Vandesompele 2002; Radonic et al. 2004; Brunner et al. 2004; Iskandar et al. 2004).

Genes are considered good normalizers when their expression does not change under different conditions or treatments, different tissues or cell types, developmental stages or stresses (biotic or abiotic) (Wong and Medrano 2005; Nolan et al. 2006; Borges et al. 2012). Many housekeeper genes encoding proteins involved in basic cellular processes, such as *ACTIN* (Zheng et al. 2011), *EF1* (Sharifi-Sirchi et al. 2011), *TUBULIN* (Tan and Swain 2007), *GAPDH* (Fan et al. 2010) and *18 s-RNA* (Albrecht and Bowman 2008) have been used as normalizers to study gene expression in citrus under different conditions and stresses without first testing them for their stability (Mafra et al. 2012). (Volkov et al. 2003) and (Matta et al. 2011) have demonstrated that several genes involved in basic cellular processes are not always stable under different conditions, which could lead to deviated results in gene expression analyses (Czechowski et al. 2005; Nicot et al. 2005; Remans et al. 2008). Nolan et al. (2006) published a protocol for reliable RT-qPCR and concluded that for each different experimental set-up, internal reference genes should be validated. To our knowledge, in the citrus-*P. parasitica* interaction, the stability of candidate reference genes was never tested in experiments in which the infection was performed via inoculation of zoospores in roots. The inoculation of zoospores in roots would be the experimental condition more related to what happens in nature, and therefore, the experimental set-up of choice in future studies on this pathosystem in detriment of stem or leaf wounding.

In the present study we aimed to evaluate the stability of candidate reference genes and determine which set of these genes is best suited for transcript normalization in resistant and susceptible citrus genotypes during infection with zoospores of *P. parasitica*. We evaluated five candidate reference genes (*CYP, DIM1, EGIDH, FBOX, GAPC2*), which were selected from the database CitEST. GeNorm and NormFinder algorithms were used to find the best reference genes in all the conditions evaluated.

Material and methods

Zoospore production and infection of plants

P. parasitica isolate IAC 01–95 was maintained in Petri dishes with carrot-agar medium, in the dark, at 20 °C. After the colony reached up to 80% of the plates, sporangia development was induced by pouring autoclaved water on the plate. The water was replaced daily for 1 week. After 7 days, zoospore release was induced by placing the Petri dishes at 4 °C for 1 h. The amount of zoospores released was recorded using a Neubauer chamber. The zoospore suspension was set to 1×10^5 zoospores/mL.

Seeds of *C. sunki* and *P. trifoliata* were germinated and grown in root containers with sterile substrate for 3 months in the greenhouse. Three days before initiating the experiment, the seedlings were carefully removed from the containers. The roots were rinsed off the substrate and placed in 50 mL Falcon tubes containing 50 mL of distilled water and sealed with parafilm. Roots were inoculated as follows: zoospores (1×10^5 zoospores per plant) were carefully pipetted from the zoospore suspension and transferred to the water surrounding the roots inside the Falcon tubes. Plants were maintained in growth chambers at 20 °C, light conditions of 250 μmol/m^2/s photosynthetic photon flux density (PPFD) and photoperiod of 12 h. Six plants were used for each treatment. The experiment was repeated twice.

Disease assessment

The plants were monitored daily for root necrosis, growth of visible mycelia on the root surface, wilting of leaves and mortality.

Genomic DNA was extracted from 100 mg frozen root material using the DNeasy Plant Mini kit (Qiagen) and was further purified using the Wizard DNA Clean Up System (Promega) according to the manufacturers' protocols. The DNA was diluted 1:10 in water to prevent the inhibition of the PCR. The amount of *P. parasitica* DNA in 5 μL of root extract was determined by Sybr Green quantitative PCR using an SDS7700 sequence detection system (Applied Biosystems), with the primer pair PN5b/PN6 (Ippolito et al. 2002). All analyses were performed in three technical replicates using Promega Sybr Green chemicals and performing 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 62 °C for 60 s. The Ct values of the samples were compared with a standard curve that was generated for pure *P. parasitica* genomic DNA extracted from mycelia grown in liquid culture (mineral medium M1) supplemented with 10 g/L glucose and 2 g/L L-asparagine). The standard curve concentrations ranged from 1 pg DNA/mL to 10 ng DNA/mL in five steps.

For the extraction of total RNA, 50 mg of roots were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the MasterPure Plant RNA Purification kit (Epicentre Biotechnologies) according to the manufacturer's protocol, including a DNAse I treatment. The concentration and quality of extracted RNA was measured using a nanodrop (NanoDrop 8000; Thermo Scientific) in which the samples were analyzed at 260 and 280 nm wavelengths. cDNA was reverse

transcribed using 1 μg of total RNA with oligo-dT primers and the MMLV Reverse Transcriptase First Strand cDNA Synthesis kit (Epicentre Biotechnologies). The transcript levels of the genes were analysed by using 0.05 μg of cDNA (analyzed by NanoDrop 8000) by qRT-PCR in three technical replicates using the ABsolute SYBRGreen ROX chemicals (ABgene) and performing 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30s. For all amplifications performed in RT-qPCR, we produced dissociation curves to check for nonspecific amplification resulting from possible contamination.

Light microscopy analysis

Root tip samples were taken after 96 h post infection and fixed in Karnovsky solution (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4). After 3 days, samples were washed in phosphate buffer (pH 7.4) and embedded in resin (Leica embedding kit) following the manufacturer's protocol. The cuttings were processed in a microtome (longitudinal cuts, 1 nm), colored with Toluidine blue (0.05% solution, pH 6.0, for eight minutes) and analyzed by light microscopy.

Reference genes selection

The five candidate genes evaluated in this experiment were selected from the CitEST (http://limonia.centrodecitricultura.br/blast/blast.html) and HarvEST (http://www.harvest-web.org/) citrus databases according to one or more of the following criteria: (1) reference genes traditionally used in citrus for transcript normalization; (2) citrus homologues of reference genes tested for transcript level normalization and quantification in *Arabidopsis*. The information regarding all the genes used in this work is summarized in Table 1.

Data analysis and statistics

Data analysis was performed similarly to Mafra et al. 2012. Primer efficiency for each experimental set was estimated using an algorithm in real-time PCR Miner software (http://www.miner.ewindup.info/) that calculates primer efficiency and quantification cycle (Cq) values based on the kinetics of individual reactions without the need for a standard curve. Cq values, determined by the second derivative maximum for each biological sample, were converted into non-normalized relative quantities using the formula $Q = E^{\Delta Cq}$, where *E* represents the arithmetic mean of efficiency of all samples for each gene, and ΔCq represents the difference between the arithmetic mean Cq value across all samples for this gene

Table 1 Genes used in this work

Gene abbreviation	AGI*	Annotation	Primer sequences	Primer concentration (nM)
DIM1	At5g08290	*DIM1* homolog / *YLS8*	F: CGA AAC CTG TAT GCA GAT GG R: ACG GTT GAG GGA TCG TAA AG	120
FBOX	At5g15710	*FBOX* family protein	F: TTG GAA ACT CTT TCG CCA CT R: CAG CAA CAA AAT ACC CGT CT	120
GAPC2	At1g13440	Glyceraldehyde-3-phosphate dehydrigenase C2	F: TCT TGC CTG CTT TGA ATG GA R: TGT GAG GTC AAC CAC TGC GAC AT	150
CYP (Cyclo)	At2G36130	Cyclophilin	F: AGA GTA TGC AGA GGA ATG G R: GTC CTT AAC AGA AGT CCG T	107
EGIDH	At4G35260	NADP-isocitrate dehydrogenase	F: CAT TGA ACA TGC AGT TGA GG R: ATT CTC ATG ACG TGT CGG	91
CC-NBS	At4G27190	NBS-LRR protein	F: GTG GTT TGC CTC TTG CAC TTA TT R: AGC TCC ATT CCT CCT GTG ACT TC	150

AGI* Arabidopsis Gene Initiative (AGI) locus identifier number

and the Cq value of the sample in question, as recommended by Hellemans et al. 2007. These quantities were imported into geNorm v3.5 (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) for reference gene selection. First, we performed a global analysis composed of all biological samples in geNorm. Then, we analyzed each experimental condition individually in an attempt to identify specific reference genes according to the two citrus species. The geNorm software calculated the average of expression stability yielding an M value and the variation of the stability of the better gene pairs (V). This allowed us to exclude the less suitable genes, i.e., genes with the lowest M value (Vandesompele *et al.* 2002). Finally, NormFinder calculated both inter- and intragroup variation in the expression stability, thus identifying the best combination of reference genes. This software is based on the Ct method, in which data are linearized through transformation of the Ct values in relative quantity (Q), fixing the highest Ct value and using the amplicon average efficiency in the treatment (E). To do that, we have applied the following equation, called delta-Ct (Andersen *et al.* 2004): $Q = (E_{gene})^{\Delta Ct(lowest-highest)}$

To test the sets of optimal reference genes we have selected as target the gene *CC-NBS*, which is a coile-coil resistance-type gene, known to display differences in expression when comparing *P. trifoliata* and *C. sunki*. To calculate the expression of the *CC-NBS* gene we have used the method of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). We also applied the Kruskal-Wallis non-parametric test to check the significance among the treatments and time-points (Kruskall and Wallis 1952).

Results and discussion

In this work we present two sets of candidate reference genes suitable to analyze gene expression of *P. trifoliata* and *C. sunki* upon root infection with zoospores of *P. parasitica*.

Successful infection by *P. parasitica* in both species after inoculation via zoospores was confirmed by microscopy and/or pathogen DNA found in roots (Figs. 1 and 2). At 48 h post-inoculation (hpi) in *C. sunki*, the pathogen colonized the root tissues damaging the cortex and central cylinder. It is possible to visualize hyphae and sporangia near the central cylinder (Fig. 1c). Figure 2a shows *P. parasitica* colonizing the cortex of *C. sunki*. The infection is also confirmed by *P. parasitica* DNA quantification in roots, with the highest value at 48 hpi (Fig. 1d).

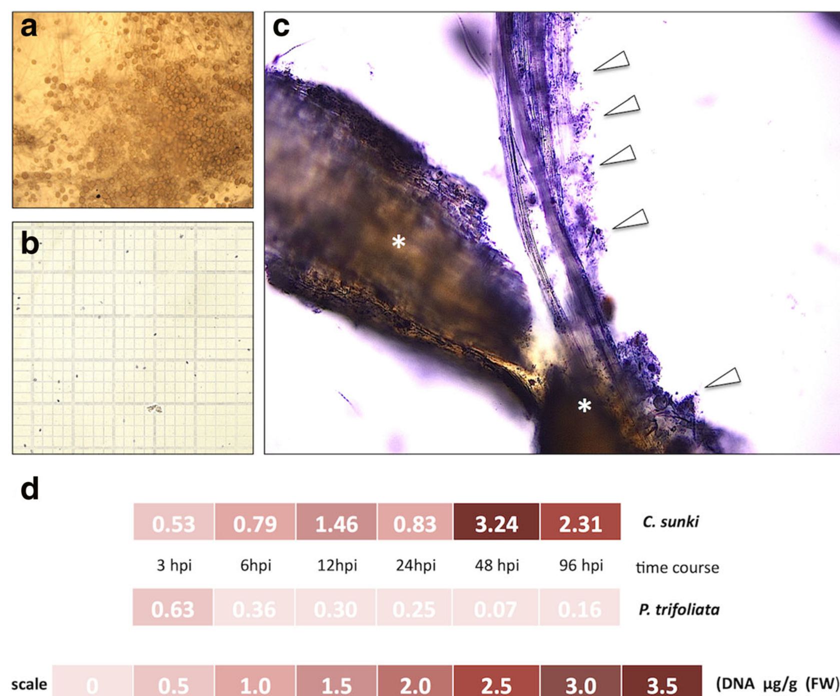

Fig. 1 **a** Light microscopy view of mycelium in Petri dish evidencing clusters of sporangia of *P. parasitica* cultivated in carrot agar. **b** Neubauer chamber picture showing zoospores of *P. parasitica* (1×10^5 zoospores/mL). **c** Light microscopy of *C. sunki* roots infected with *P. parasitica*. The *arrows* show *P. parasitica* colonization in the central cylinder of the damaged root and the *asterisks* show the tissue necrosis process, characterizing root rot in citrus (48 h post inoculation). Staining: toluidine *blue*. **d** Heat map showing *P. parasitica* genomic DNA values along the colonization of *C. sunki* and *P. trifoliata* seedlings (DNA expressed in µg/g FW), monitored by RT-qPCR over 3, 6, 12, 24, 48 and 96 h after inoculation

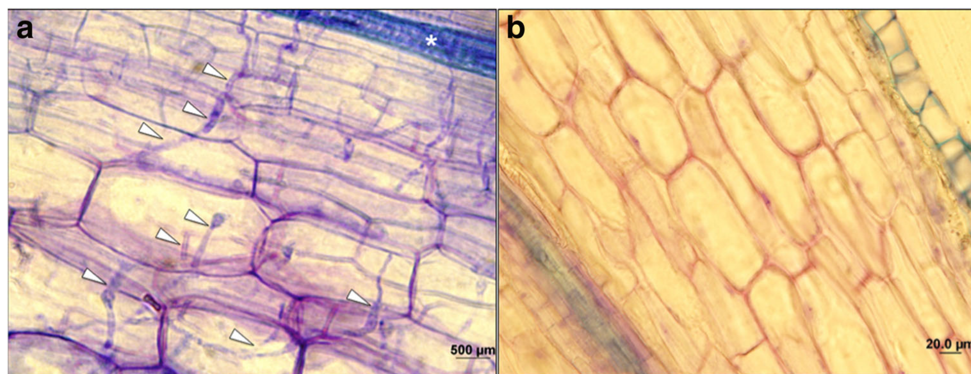

Fig. 2 Longitudinal section of *C. sunki* **(a)** and *P. trifoliata* **(b)** roots infected with 1×10^5 zoospores of *P. parasitica*. **a** The *arrows* point mycelia growing in root tissue of *C. sunki*. **b** Mycelia of *P. parasitica* was not found in *P. trifoliata* root tissues, despite qPCR confirmation of roots infection. *Asterisk* shows the central cylinder of *C. sunki* seedlings. Staining: toluidine *blue*

P. trifoliata was also infected by *P. parasitica* but to a lower degree compared to *C. sunki*. It was not possible to find hyphae in *P. trifoliata* root cuttings (Fig. 2b) but the infection was confirmed by the DNA analysis (Fig. 1d). This pattern was expected since *P. trifoliata* shows resistance/tolerance against *P. parasitica*, while *C. sunki* is highly susceptible. Similar results were reported by Boava et al. 2011.

Internal reference genes have been extensively analyzed over their capability to normalize transcripts in gene expression in citrus under different treatments and pathogen attack. The most used genes have been *EF-1α* (Endo *et al*. 2006; Cernadas *et al*. 2008; Nishikawa *et al*. 2009; Sharifi-Sirchi et al. 2011; Martinelli *et al*. 2012), *ACTIN* (Liu *et al*. 2007; Huerta *et al*. 2008; Liu *et al*. 2009; Chai *et al*. 2011; Zheng *et al*. 2011; Miao *et al*. 2011; Zhong

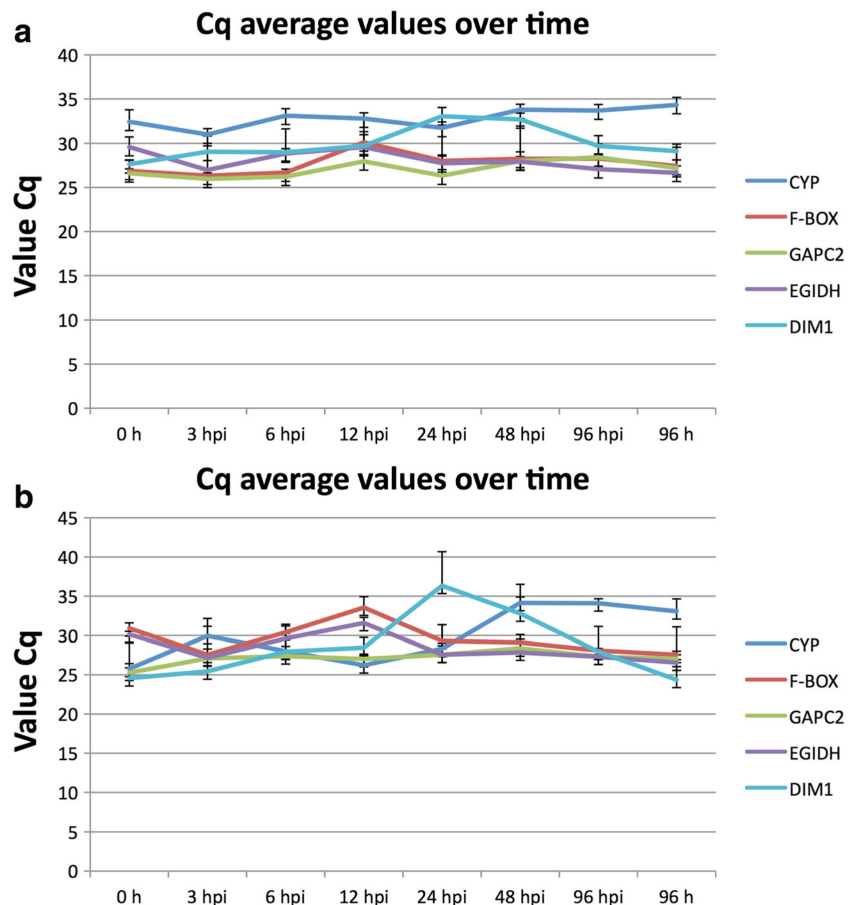

Fig. 3 Cq values of endogenous candidate genes. The arithmetic mean ($n = 6$) of Cq values of the genes of plants infected by *P. parasitica* over time were plotted in the graphs. **a** *P. trifoliata* values. **b** *C. sunki* values. Time points: 0, 3, 6, 12, 24, 48, 96 h (96 hpi: infected plants at 96 h; 96 h: control plants at 96 h). Bars represent standard deviation

Table 2 Citrus candidate genes ranked according to their expression stability as determined by NormFinder

Gene	Stability value
C. sunki	
GAPC2	0.146
EGIDH	0.286
DIM1	0.287
FBOX	0.300
CYP	0.477
P. trifoliata	
FBOX	0.092
CYP	0.096
GAPC2	0.140
DIM1	0.169
EGIDH	0.225

et al. 2015), *18 s rRNA* (Albrecht and Bowman 2008; Aritua *et al.* 2013), *UBQ* and *CYP* (Boava *et al.* 2011; Mafra *et al.* 2012; Rodrigues *et al.* 2013). Specifically for the interaction between *P. parasitica* and citrus, the genes already evaluated are *ETEF2*, *EGIDH*, *CYP*, *UBQ* and *TUB* (Boava et al. 2011) and *FBOX, GAPC2, SAND, UPL7, EF1, ADP, PTB1, TUB, UBC21, UBC9, ACT2, DIM1, CtP, CYP* (Mafra et al. 2012). For this study we have selected the following genes: *CYP, DIM1, EGIDH, FBOX, GAPC2*. All genes selected were proven to be stable in the citrus-*P. parasitica* interaction, however, the experimental set up, citrus genotypes and *P. parasitica* inoculation were different in those previous works.

In order to check the stability of the selected candidate reference genes, we have performed RT-qPCR to obtain Cq

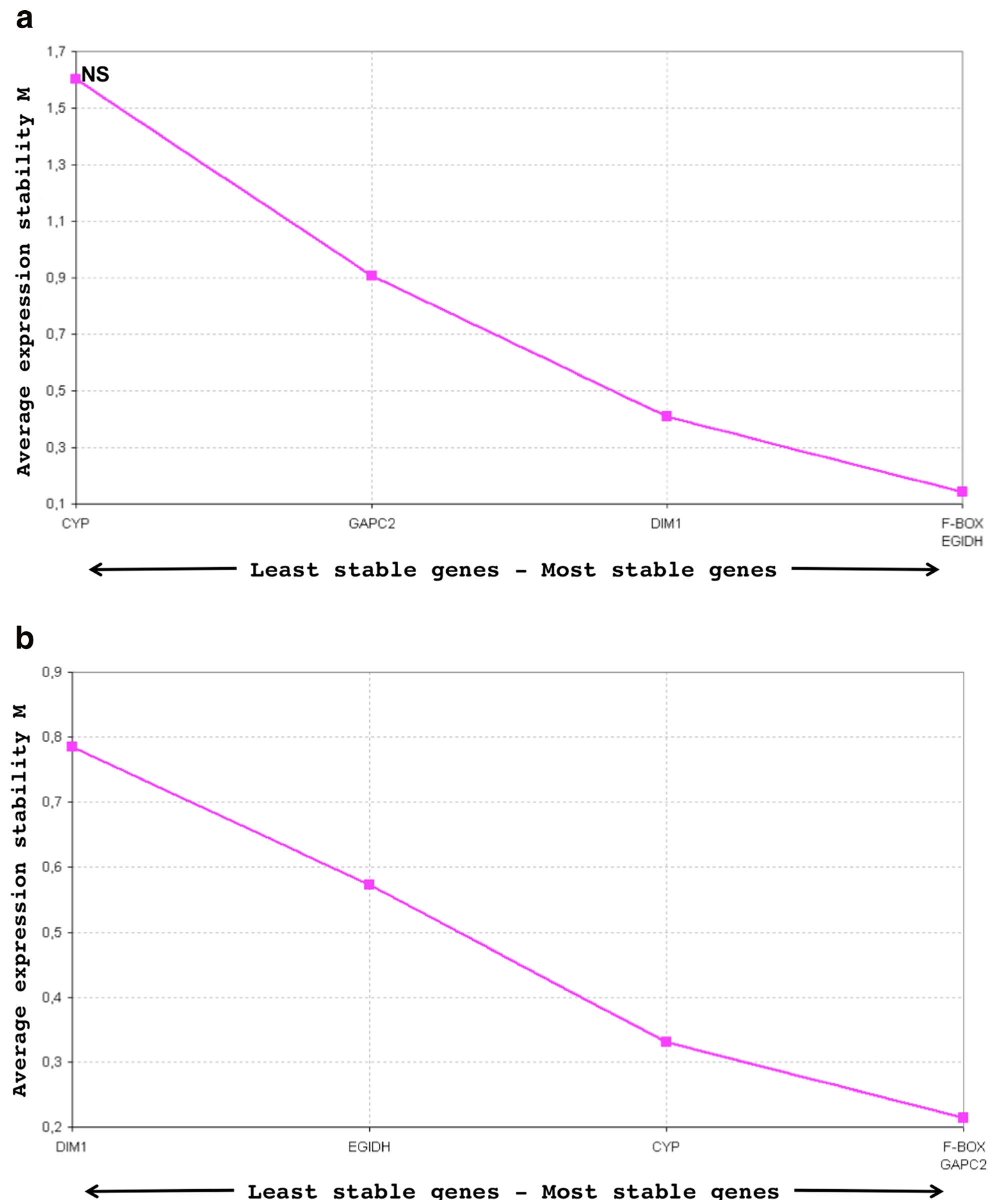

Fig. 4 Stability values of the genes tested in the geNorm software for *C. sunki* **(a)** and *P. trifoliata* **(b)**. Efficiency values plotted for the five genes evaluated as best normalizers. *CYP* gene showed a M value greater than 1.5, therefore was not ranked as a stable gene. NS, not stable

Table 3 *C. sunki* candidate endogenous genes ranked according to their expression stability as determined by geNorm

Time	1° *FBOX*	2° *EGIDH*	3° *DIM1*	4° *GAPC2*	5° *CYP*	Normalisation fator
0 h*	0.17334	0.17303	0.22032	1.00E+00	1.00E+00	0.2851
96 h	0.21080	0.22939	0.20007	0.75755	0.57867	0.2926
3 hpi	0.25984	0.30717	0.17812	0.59467	0.32146	0.3032
6 hpi	0.31705	0.40349	0.15485	0.46449	0.16985	0.3097
12 hpi	0.43169	0.49768	0.29646	0.42093	0.09119	0.4046
24 hpi	0.56692	0.61429	0.48228	0.40202	0.05168	0.5098
48 hpi	0.76953	0.73482	1.00E+00	0.37933	0.02669	0.6805
96 hpi	1.00E+00	1.00E+00	0.70812	0.34163	0.01685	0.7013
M	1.262	1.275	1.374	1.456	2.642	

*h: hour(s); hpi: hours post infection

M values are ranked from the most stable pair of genes to the least stable gene

values of these genes following the inoculation of *P. trifoliata* and *C. sunki*. For most of the genes, the Cq values were maintained with low variability when compared to Cq values of control plants at 0 and 96 h for both genotypes. Even at the latest time points (48 and 96 hpi), when the infection was fully established in *C. sunki*, imposing a great stress to the plant, the Cq values of the genes were comparable to the Cq values of control plants (Fig. 3), indicating that the majority of the genes were stable throughout the experiment.

To ensure the stability of the genes, all Cq values of all target candidate reference genes were used as input in the NormFinder algorithm. After NormFinder analysis, the most stable genes were (in order): *GAPC2* (0.146), *EGIDH* (0.286), *DIM1* (0.287), *F-BOX* (0.300) and *CYP* (0.477), for *C. sunki*, and *F-BOX* (0.092), *CYP* (0.096), *GAPC2* (0.140), *DIM1* (0.169) and *EGIDH* (0.225) for *P. trifoliata* (Table 2).

The geNorm analysis showed that all five genes showed stable values ($M < 1.5$) for both species, except for *CYP* in *C. sunki* with an M value of 2.642 (Table 3). For *P. trifoliata*, the software ranked *F-BOX* ($M < 0.60$) and *GAPC2* ($M < 0.56$) as the more stable genes, while the genes *F-BOX* ($M < 1.26$) and *EGIDH* ($M < 1.27$) were ranked for *C. sunki*. The stability values of the genes tested in the geNorm software are shown in Fig. 4.

Mafra et al. (2012) analyzed endogenous genes in citrus plants infected with several pathogens, including *P. parasitica*. However, the experiment, despite having the same objective, was different from ours. The authors used older plants, different citrus genotypes in other rootstocks and they infected the stem using mycelia discs in wounded tissues. Furthermore, the infected plants were compared with wounded control plants. The infection in roots via zoospores, as in our work, reflects better what happens in nature and does not impose further stress to the plant by artificially wounding its tissues. Therefore, we expected differences in the ranking of the normalizing genes, which was indeed the case for both *C. sunki* and *P. trifoliata*. However, some similarities were also found, such as the *F-BOX* gene. We have found that the *F-BOX* gene is stable under all conditions for *C. sunki* as well as *P. trifoliata,* and was high-ranked by geNorm and ranked fourth by Normfinder (despite having a high stability value) (Fig. 4; Tables 2, 3 and 4). The *F-BOX* genes are grouped in a very large multigenic superfamily controlling several important biological functions in plants. At least 694 and 687 *F-BOX* genes were found in the genome of *Arabidopsis thaliana* and *Oryza sativa*, respectively (Gagne et al. 2002; Jain et al. 2007). In *Citrus sinensis* (L.) Osbeck, 363 *F-BOX*

Table 4 *P. trifoliata* candidate endogenous-genes ranked according to their expression stability as determined by geNorm

Time	1° *GAPC2*	2° *FBOX*	3° *CYP*	4° *EGIDH*	5° *DIM1*	Normalisation factor
0 h*	1.00E+00	0.82477	1.00E+00	0.23851	1.00E+00	0.7224
96 h	0.97496	0.77310	0.97329	0.29749	0.69213	0.6852
3 hpi	0.95097	0.78501	0.82678	0.35825	0.47993	0.6385
6 hpi	0.93880	0.81659	0.66189	0.41959	0.32118	0.5848
12 hpi	0.92303	0.86429	0.58759	0.50234	0.19178	0.5382
24 hpi	0.92790	0.92708	0.62055	0.62118	0.16968	0.5624
48 hpi	0.90681	1.00E+00	0.58553	0.75233	0.15644	0.5743
96 hpi	0.77758	0.92573	0.48325	1.00E+00	0.20330	0.5887
M	0.562	0.602	0.629	1.028	1.102	

h*: hour(s); hpi: hours post infection

M values are ranked from the most stable pair of genes to the least stable gene

Table 5 The more suitable pairs of endogenous genes for gene expression normalization in citrus

Species	geNorm	NormFinder
P. trifoliata	*F-BOX* and *GAPC2*	*CYP* and *F-BOX*
C. sunki	*F-BOX* and *EGIDH*	*EGIDH* and *GAPC2*

domain ontologies were found (phytozome.org). The main functions of F-BOX proteins are related to ubiquitination and protein degradation, embryogenesis, hormone responses, seedling development, senescence and floral ontology. All these fundamental processes render this gene a good reference gene status (Lechner et al. 2006).

The *CYP* (Cyclophilin) gene was also found to be a suitable normalizer and was well-ranked by geNorm and Normfinder for *P. trifoliata* analysis. However, for *C. sunki*, *CYP* received the lowest M score in geNorm and the least stable value by Normfinder (Fig. 4; Tables 2, 3 and 4). In the study of Mafra et al. (2012), the *CYP* gene was not found as a stable normalizer in any condition or genotype. The *CYP* gene encodes a molecular chaperone protein which is involved in protein folding by peptidyl prolil isomerization in the plant cell (Maruyama et al. 2004). Since these functions are essential for the physiology of the plant, this gene can also be considered a good candidate reference gene.

The genes *DIM1* (18S rRNA dimethylase) and *EGIDH* (NADP-isocitrate dehydrogenase) were found to be suitable normalizers for *C. sunki* in both NormFinder and geNorm algorithms (Fig. 4; Tables 2, 3 and 4). However, while with acceptable normalizing values, the *DIM1* and *EGIDH* genes were low-ranked by both algorithms for *P. trifoliata* (Fig. 4; Tables 2, 3 and 4). Similar results were obtained by Boava et al. (2011). Mafra et al. (2012) found higher values for both genes in citrus plants. Several studies have been using *DIM1* and *EGIDH* as normalizers to study gene expression in plant-pathogen interactions or plant physiology (Mafra et al. 2012; Boava et al. 2011; Boava et al. 2010; Paux et al. 2004; Legay et al. 2007; Boiffin et al. 1998).

The *GAPC2* gene expresses a citosolic isoenzime GAPDH (gliceraldehide-3-fosfate Nad-dependent desidrogenase) which is involved in carbon metabolism in cells. It is frequently used as a normalizer gene. In our study, it was the most stable of all five genes tested by GeNorm and the third by NormFinder for *P. trifoliata* plants. In *C. sunki*, it was the best ranked gene in NormFinder and the fourth by geNorm (Fig. 4; Tables 2, 3 and 4). Mafra et al. (2012) also found *GAPC2* to be a suitable internal reference gene for citrus.

The best reference genes for expression normalization in *P. trifoliata* and *C. sunki*, based on geNorm and NormFinder are presented in the Table 5.

In order to check if the highly ranked genes are in fact good normalizers we performed a gene expression analysis in *P. trifoliata* and *C. sunki* infected with *P. parasitica*. The target gene was *CC-NBS* (NBS-LRR protein) (Rodrigues et al. 2013), known to be differentially expressed when comparing *P. trifoliata* and *C. sunki*. As normalizers, we used *F-BOX* and *GAPC2* as normalizers. The Kruskal-Wallis statistical test shows highly significant differences in gene expression levels for the *CC-NBS* in *P. trifoliata* seedlings in comparison with *C. sunki*

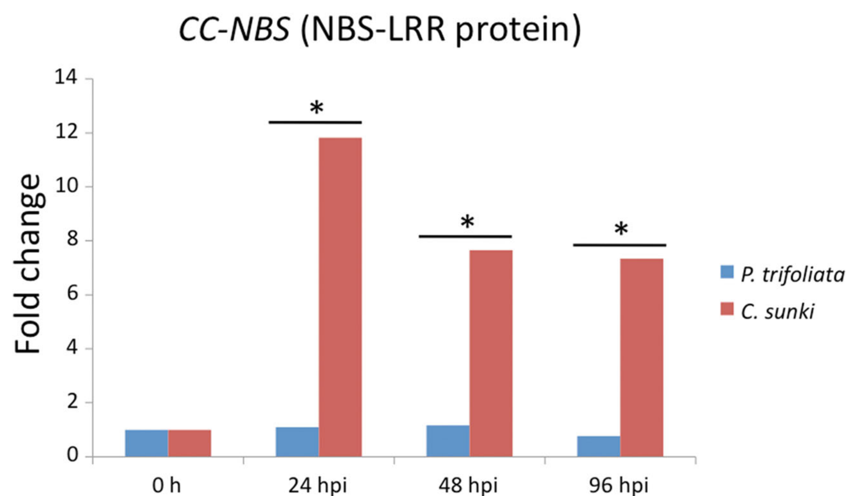

Fig. 5 *CC-NBS* (a resistance gene) differential expression in *P. trifoliata* and *C. sunki* seedlings infected with zoospores of *P. parasitica*. Genes *F-BOX, GAPC2* of *P. trifoliata* and *GAPC2, EGIDH* of *C. sunki* were used

for *P. trifoliata* and *GAPC2* and *EGIDH* for *C. sunki*., since those genes were highly ranked by both GeNorm and NormFinder (Table 5). The results are shown in Fig. 5.

As expected, the *CC-NBS* gene was differentially expressed in citrus roots comparing the two rootstocks at 24, 48 and 96 hpi. This result validates the selected reference genes for the study of gene expression in the citrus-*P. parasitica* interaction.

In summary, most of the genes studied were found to be suitable normalizers of citrus plants infected with *P. parasitica*. We recommend the use of *F-BOX* (best ranked in NormFinder) and *GAPC2* (best ranked in GeNorm) for *P. trifoliata*, and *EGIDH* (second best ranked in GeNorm; the first ranked, *F-BOX*, has a low score in NormFinder) and *GAPC2* (first ranked in NormFinder) for *C. sunki*. If only two genes were to be used for normalization of both *P. trifoliata* and *C. sunki* concomitantly, we suggest the use of *F-BOX* and *GAPC2* (overall best ranked genes).

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