

ORIGINAL RESEARCH

Identification of suitable reference genes during the formation of chlamyospores in *Clonostachys rosea* 67-1

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Funding information

National Natural Science Foundation of China, Grant/Award Number: 31471815; the Program of Modern Agricultural Industry Technology System, Grant/Award Number: CARS-25-B-02; the Ministry of Agriculture Special Scientific Research Task, Grant/Award Number: 201503112-2

Abstract

Clonostachys rosea is a potential biocontrol fungus that can produce highly resistant chlamyospores under specific conditions. To investigate the genes related to chlamyospore formation, we identified reliable reference genes for quantification of gene expression in *C. rosea* 67-1 during sporulation. In this study, nine reference genes, actin (*ACT*), elongation factor 1 (*EF1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), histone (*HIS*), RNA polymerase II CTD phosphatase Fcp1 (*RPP*), succinate-semialdehyde dehydrogenase (*SSD*), TATA-binding protein (*TBP*), ubiquitin (*UBQ*), and ubiquitin-conjugating enzyme (*UCE*), were selected and cloned from 67-1, and their expression stability during chlamyospore formation was determined using reverse transcription quantitative PCR and assessed using the software geNorm, NormFinder and BestKeeper. The Ct values of the candidates ranged from 19.9 to 29.7, among which *HIS*, *ACT* and *SSD* exhibited high expression levels. The statistical analysis showed that *ACT* and *SSD* were most stably expressed, while *UBQ* and *GAPDH* showed relatively large variations under different culture conditions. Calculation of pairwise variation value indicated that two reference genes were required for precise quantification. Finally, *ACT* and *SSD* were selected to normalize gene expression during chlamyospore production in *C. rosea* 67-1. To the best of our knowledge, this is the first report of *SSD* as a reference gene. This study will facilitate the accurate quantification of differentially expressed genes during the generation of chlamyospores and contribute to the investigation of the molecular mechanism underlying chlamyospore formation in *C. rosea*.

KEYWORDS

actin, chlamyospore, *Clonostachys rosea*, reference gene, reverse transcription quantitative PCR, succinate-semialdehyde dehydrogenase

1 | INTRODUCTION

Clonostachys rosea (syn. *Gliocladium roseum*) is a widely distributed mycoparasite, and it has shown great potential against various plant fungal pathogens through multiple mechanisms, such as mycoparasitism,

competition, antagonism, and induction of plant resistance (Morandi, Maffia, Mizubuti, Alfenas, & Barbosa, 2003; Mouekouba et al., 2014; Ortiz & Orduz, 2001; Papavizas, 1985; Rodríguez, Cabrera, Gozzo, Eberlin, & Godeas, 2011). The conidia of *C. rosea*, which are easily obtained by mass production, are commonly used as the main components of biocontrol agents. However, the relatively low adversity

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resistance of the conidiospores may result in instability and a short shelf life of fungal biopesticides, limiting their large-scale production and application. The chlamyospore is a type of vital resistant spore, and it is able to survive under unfavorable conditions (Armengol, Sales, & Garcia-Jimenez, 1999; Beagle-Ristaino & Papavizas, 1985; Caldwell, 1958; Eyal, Baker, Reeder, Devane, & Lumsden, 1997). Compared with conidia, chlamyospores are much larger and have a thicker cell wall, higher resistance to harsh environments, and equal biocontrol ability (Dong, Sun, Li, Peng, & Luo, 2014). Chlamyospores are of great significance in fungal survival as well as in the commercialization of fungal biopesticides. Several studies have revealed the nutrient and environmental conditions that influence chlamyospore formation in *C. rosea* (Li, Qu, Tian, & Zhang, 2005; Sun, Chen, Liu, Li, & Ma, 2014; Zhang, Sun, Zhang, Xie, & Li, 2010); however, the mechanism underlying chlamyospore formation has not yet been elucidated. Information on chlamyospore production-related genes will facilitate the development and wide applications of *C. rosea*.

For functional characterization of genes, reverse transcription quantitative PCR (RT-qPCR) is commonly used to measure the expression levels of candidate genes because of its high sensitivity and throughput, specificity, and accuracy. However, the results are affected by the sample amount, RNA quality and quantity, and efficiencies of cDNA synthesis and PCR amplification (Fleige & Pfaffl, 2006; Udvardi, Czechowski, & Scheible, 2008). Appropriate reference genes are available to eliminate systematic errors and precisely normalize gene expression during RT-qPCR (Guénin et al., 2009).

However, no reference gene is absolutely consistent in different organisms and their developmental stages; instead, the expression level of a reference gene is stable only under specific conditions. β -*tubulin* was used to normalize N-acetyl- β -D-glucosaminidase gene in *C. rosea* when confronting with *Fusarium culmorum* (Mamarabadi, Jensen, & Lübeck, 2009), however, it was not available in mycoparasitic process (Sun, Li, & Sun, 2015; Sun, Sun, & Li, 2015). In *Neurospora crassa*, the expression of *act* was not affected by temperature, but it was inhibited under all-dark conditions (Cusick et al., 2014). Therefore, selection of suitable reference genes under a specific condition is crucial for accurate gene normalization and function certification.

C. rosea 67-1 is an efficient biocontrol fungus active against various plant fungal pathogens (Ma et al., 2011; Zhang, Gao, Ma, & Li, 2004). In previous study, we developed a technique that was able to promote chlamyospore formation in 67-1 and constructed the transcriptome of chlamyospore formation of the fungus. In this study, nine candidate reference genes—actin (*ACT*), elongation factor 1 (*EF1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), histone (*HIS*), RNA polymerase II CTD phosphatase Fcp1 (*RPP*), succinate-semialdehyde dehydrogenase (*SSD*), TATA-binding protein (*TBP*), ubiquitin (*UBQ*), and ubiquitin-conjugating enzyme (*UCE*) were isolated from *C. rosea* 67-1 and screened for precise normalization of the differentially expressed genes during the generation of chlamyospores. To our knowledge, this is the first report of reference genes for chlamyospore formation. Our study will provide a basis for further investigation of the genes related to chlamyospore

formation and the molecular mechanism underlying chlamyospore formation.

2 | MATERIALS AND METHODS

2.1 | Strain

The strain *C. rosea* 67-1 was originally isolated from a vegetable yard in Hainan Province, China, using the sclerotium baiting method (Zhang et al., 2004). It is preserved in the Biocontrol of Soilborne Diseases Lab of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences.

2.2 | Production of chlamyospores

C. rosea 67-1 was cultured on potato dextrose agar (PDA) plates at 26°C for 10 days, and the spores were eluted using 10 ml of sterile distilled water and a sterile glass spatula. One milliliter of the spore suspension was inoculated into 50 ml of PD broth in a 250 ml conical flask and incubated at 27°C on a rotary shaker at a speed of 180 r·min⁻¹. After 36 hr, the seed liquid was transferred into medium (25 g of glucose, 7 g of soybean cake powder, 0.35 g of urea, 1.0 g of K₂HPO₄·3H₂O, 0.5 g of MgSO₄, and 50 mg ZnSO₄·7H₂O in 1 L water) to produce chlamyospores. The conidiation of *C. rosea* in the medium of corn flour in which no chlamyospores were detected during the whole sampling time was taken as the control. The fungus was incubated at 27°C on a rotary shaker at 180 r·min⁻¹, and the thallus were harvested at 36 hr (initial stage of chlamyospore formation) and 72 hr (stage of chlamyospore mass production) after inoculation by using a centrifuge (Sigma 3-15, Osterode, Germany) at 8000g for 15 min and washed three times with sterile distilled water. The residual water was removed using sterile filter paper, and the fresh samples of the fungus were frozen in liquid nitrogen immediately and stored at -80°C. Three completely independent replications were conducted.

2.3 | RNA extraction and cDNA synthesis

Total RNA of each sample was extracted using the TRIzol reagent (Invitrogen, CA), according to the manufacturer's instructions; RNase-free DNase I (TaKaRa, Dalian, China) was used to eliminate DNA contamination. RNA integrity was verified using agarose gel electrophoresis, and RNA concentration and quality were detected using a micro-spectrophotometer (SimpLiNano, Cambridge, UK).

One microgram of the RNA sample was reverse-transcribed to cDNA in a 20 μ l reaction system by using the cDNA FastQuant RT Kit (Tiangen, Beijing, China), and the first-strand cDNA was maintained at -20°C for real time PCR.

2.4 | Primer design for the reference genes

On the basis of the transcriptome data for *C. rosea* 67-1 chlamyospore formation, a total of nine candidates were selected and cloned

from the genome of *C. rosea* (Sun, Li et al., 2015; Sun, Sun et al., 2015), namely, *ACT*, *EF1*, *GAPDH*, *HIS*, *RPP*, *SSD*, *TBP*, *UBQ*, and *UCE* (GenBank accession numbers: KP274072, KP274074-KP274077, KX686112-KX686115). The primers for the reference genes used for RT-qPCR were designed using Primer Premier 6 (Table 1), and their specificity was assessed with the following PCR program: 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 20 s; final extension at 72°C for 10 min. The amplification efficiency (*E*) of each primer pair was assayed by amplifying twofold serially diluted cDNA templates; the following equation was used: $E = (10^{[-1/\text{slope}] - 1}) \times 100$.

2.5 | Reverse transcription quantitative PCR

RT-qPCR was performed in a 96 well plate with the IQ 5 Multicolor Real-time PCR Detection System (Bio-Rad, CA). A 25 µl reaction system was used, and it contained 1 µl of the cDNA template, 1 µl of each forward and reverse primer with the concentration of 10 µmol/L, 9.5 µl of RNase-free water, and 12.5 µl of 2× SYBR Premix Ex Taq II (TaKaRa, Dalian, China). RT-qPCR was conducted as follows: denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 55°C for 30 s. The melting curve was generated from 55°C to 95°C every 0.5°C to check the specificity of the primers. Each reaction was performed three times.

2.6 | Analysis of the stability of gene expression

Three software tools, geNorm (Vandesompele et al., 2002), NormFinder (Andersen, Jensen, & Ørntoft, 2004), and BestKeeper

(Pfaffl, Tichopad, Prgomet, & Neuvians, 2004), were used to evaluate the expression stability of the reference genes during chlamydospore generation of *C. rosea* 67-1. For the geNorm algorithm, the expression stability value *M* was calculated; the gene with the lowest *M* value was regarded to be the most stable, while a higher *M* value indicated lower stability. The pairwise variation value *V* was computed to determine the optimum numbers of reference genes for reliably normalizing gene expressions during 67-1 chlamydospore formation; the criterion $V_{n/n+1} < 0.15$ was considered as the minimum number of reference genes. Expression stability of the candidate reference genes was estimated based on the analysis of intra- and intergroup variation by using NormFinder software; a lower *M* value meant higher stability in gene expression. The BestKeeper software provided standard deviation (*SD*) and coefficient of variance (*CV*) values calculated from Ct values of the reference genes; lower *CV* and *SD* values indicated higher stability.

3 | RESULTS

3.1 | RNA quality and primer specificity

All RNA samples exhibited two obvious bands of 28S and 18S when agarose gel electrophoresis was performed, and their A_{260}/A_{230} and A_{260}/A_{280} values ranged from 1.9 to 2.2, suggesting high quality for cDNA synthesis.

Assessment of the primers showed a single band with an expected size for each candidate in agarose gel electrophoresis (Figure 1) and a single peak in the melting curve, indicating that the primers of the nine reference genes had high specificity and were suitable for the analysis

TABLE 1 Primers used in this study

| Reference gene | Primer sequence (5'-3') | Fragment length (bp) | Tm value (°C) | E-value (%) |
|----------------|-------------------------|----------------------|---------------|-------------|
| <i>ACT</i> F | CACTCCCGTGACCATTTC | 138 | 55.6 | 101.1 |
| <i>ACT</i> R | AACGAACCAATCCACCCT | | 54.4 | |
| <i>EF1</i> F | TCGATGTCGCTCCTGACT | 169 | 53.5 | 104.6 |
| <i>EF1</i> R | AGCGTGACCGTTTATTGTA | | 54.0 | |
| <i>GAPDH</i> F | GGTGCTCGACAGAGTTGC | 109 | 53.7 | 109.8 |
| <i>GAPDH</i> R | ATCCATTACCCTACATTCTCT | | 50.4 | |
| <i>HIS</i> F | CGCAAGGAGACCTACAGC | 102 | 53.4 | 99.3 |
| <i>HIS</i> R | GACGAAAGAGTTCAGGATGG | | 54.3 | |
| <i>RPP</i> F | GCCGTGACGAGAATGGAA | 122 | 57.0 | 104.9 |
| <i>RPP</i> R | CAGGTTTGGGCGGTTTCAT | | 58.7 | |
| <i>SSD</i> F | TTTCTTCGCCTTTGACACG | 100 | 57.0 | 100.1 |
| <i>SSD</i> R | GCACCTGCTTGCACTTCTTG | | 57.3 | |
| <i>TBP</i> F | ATCCGTGAACCCAAGACA | 108 | 53.2 | 105.5 |
| <i>TBP</i> R | GCGAGCATATTTGCGTGA | | 56.0 | |
| <i>UBQ</i> F | TTACCATCCGCCGACCTG | 187 | 59.7 | 102.7 |
| <i>UBQ</i> R | GATCAAACGCACCACCAA | | 55.0 | |
| <i>UCE</i> F | CTCCTATCAGCCACCAA | 160 | 54.3 | 100.8 |
| <i>UCE</i> R | TTCTGTCCATCTGCTCCA | | 51.4 | |

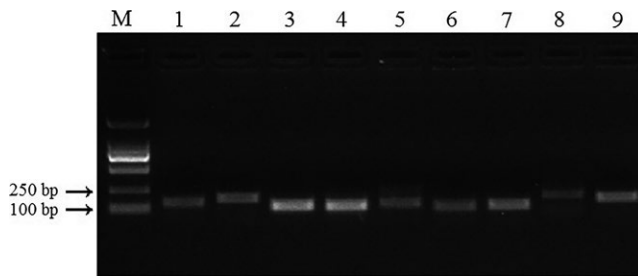


FIGURE 1 Verification of primer specificity of nine candidate reference genes from *Clonostachys rosea* 67-1. M: Marker; 1: Actin; 2: Elongation factor 1; 3: Glyceraldehyde-3-phosphate dehydrogenase; 4: Histone; 5: RNA polymerase II CTD phosphatase; 6: Succinate-semialdehyde dehydrogenase; 7: TATA-binding protein; 8: Ubiquitin; 9: Ubiquitin-conjugating enzyme

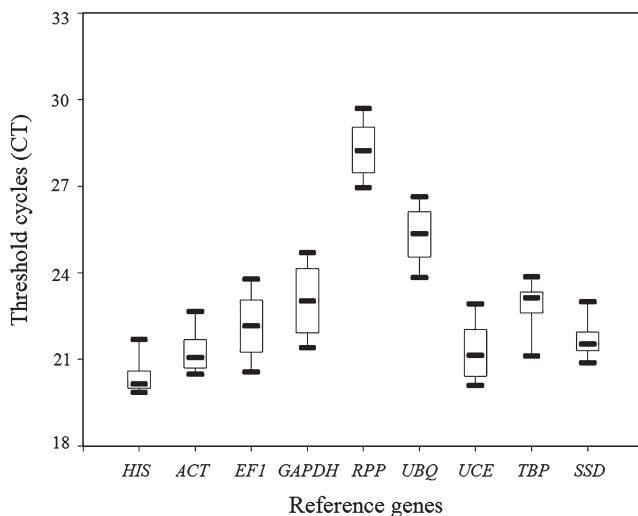


FIGURE 2 Expression abundance of nine candidate reference genes in *C. rosea* 67-1 during chlamydospore production. The boxes corresponding to the reference genes indicate the 75th and 25th percentiles, and the horizontal lines in the box-plots represent the median of three replicates. The bars indicate the maximum and minimum values. HIS, Histone; ACT, Actin; EF1, Elongation factor 1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; RPP, RNA polymerase II CTD phosphatase; UBQ, Ubiquitin; UCE, Ubiquitin-conjugating enzyme; TBP, TATA-binding protein; SSD, Succinate-semialdehyde dehydrogenase

of gene expression. PCR amplification efficiencies of the primers were calculated, and they were in the range of 99%–110% (Table 1).

3.2 | Expression of the reference genes

The expression levels of the nine reference genes were reflected by Ct values; *HIS* had the lowest Ct value of 19.9, indicating that the expression levels of the gene were the highest in different sporulation approaches and different stages. *ACT*, *UCE*, and *SSD* exhibited high expressions during *C. rosea* sporulation. *RPP*, with the highest Ct value of 29.7, showed the lowest expression abundance (Figure 2).

3.3 | Stability of gene expression

3.3.1 | geNorm analysis

The average *M* value of each candidate reference gene was less than 1.5, revealing high expression stability. *ACT* and *HIS* were considered to be the most stable with the lowest *M* value of 0.301, followed by *SSD* with an *M* value of 0.327. *UBQ*, which had a high *M* value of 1.071, was not suitable for gene quantification during *C. rosea* chlamydospore formation (Figure 3). Analysis of pairwise variation yielded $V_{2/3} = 0.102$, indicating that two reference genes should be used to accurately and reliably normalize gene expression during chlamydospore production in *C. rosea* 67-1 (Figure 4).

3.3.2 | NormFinder analysis

Calculation of expression stability values showed that the *M* value of *SSD* was the lowest (0.243), followed by *ACT* (0.406) and *HIS* (0.535); this demonstrated that *SSD*, *ACT*, and *HIS* were stably expressed during chlamydospore production of *C. rosea*. In contrast, *UBQ*, with an *M* value of 0.835, showed a large variation (Table 2).

3.3.3 | BestKeeper analysis

The expressions of *HIS* and *SSD* were consistent with low *SD* values of 0.616 and 0.629, respectively, while *GAPDH*, with the highest *SD* value of 1.288, showed relatively low stability during the production of chlamydospores in *C. rosea*.

Based on the results of the three software tools, and according to a comprehensive ranking method described by Wang et al. (2012), the most stable reference gene was *SSD*, followed by *ACT*, *HIS*, *TBP*, *RPP*, *UCE*, *EF1*, *UBQ*, and *GAPDH*. Therefore, *SSD* and *ACT* were finally selected as the optimal reference genes for normalizing gene expression during the generation of chlamydospore in *C. rosea* 67-1.

4 | DISCUSSION

RT-qPCR is an important and widely used method for the quantitative analysis of gene expression in various organisms, and its accuracy and reliable normalization depends on suitable reference genes. Generally, the expression of a reference gene needs to be consistent in all tissues and developmental stages of a species. Therefore, traditional housekeeping genes such as *ACT*, *EF1*, *GAPDH*, *UBQ*, and *UCE* are frequently used as reference genes for RT-qPCR. However, these genes are not always expressed stably at different periods and treatment conditions. For example, *ACT1* exhibited a significantly lower expression level than the other candidates in *Saccharomyces cerevisiae* under sulfite stress (Nadai, Campanaro, Giacomini, & Corich, 2015), and the variation of *act* expressions in *Trichoderma reesei* was detected when incubated under different conditions (Steiger, Mach, & Mach-Aigner, 2010). *GAPDH* was also found to be unstable during azole treatment in *Candida*

FIGURE 3 geNorm analysis of expression stability (M) of 9 candidate reference genes in chlamyospore production in *C. rosea* 67-1. The gene with the lowest M value is the most stable, while the gene with the highest M value is the least stably expressed. UBQ, Ubiquitin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; RPP, RNA polymerase II CTD phosphatase; TBP, TATA-binding protein; EF1, Elongation factor 1; UCE, Ubiquitin-conjugating enzyme; SSD, Succinate-semialdehyde dehydrogenase; ACT, Actin; HIS, Histone

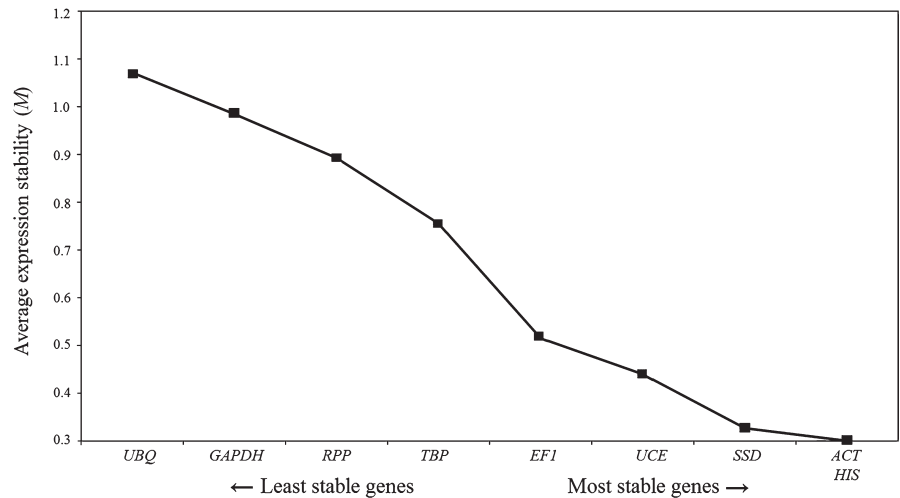


FIGURE 4 Determination of the optimal number of reference genes during *C. rosea* 67-1 sporulation by using geNorm analysis. The pairwise variation ($V_{n/n+1}$) value was used to estimate the optimal number of reference genes for normalizing gene expression; the value lower than 0.15 indicates that no additional reference gene is required for the normalization of gene expression

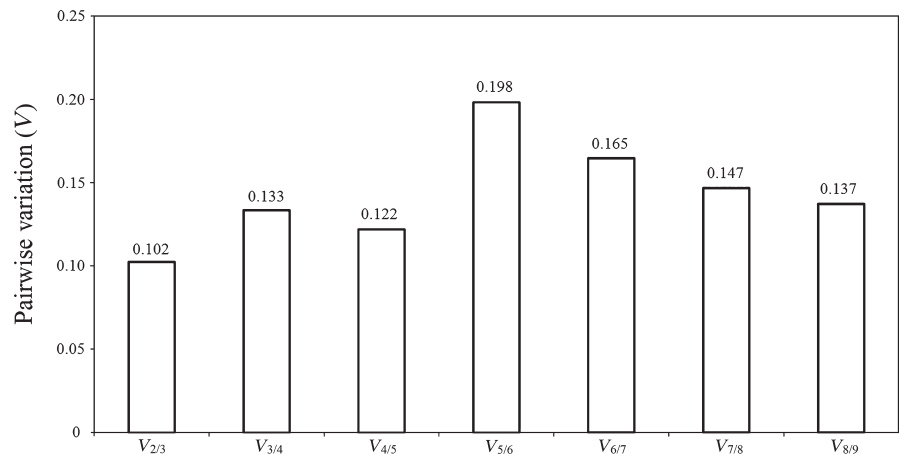


TABLE 2 Expression stability analysis of nine candidate reference genes by using BestKeeper and NormFinder programs

| Reference gene | Bestkeeper ^a | | NormFinder |
|----------------|-------------------------|-----------|------------|
| | SD [\pm Ct] | CV [% Ct] | Stability |
| ACT | 0.692 | 3.247 | 0.406 |
| EF1 | 1.135 | 5.122 | 0.601 |
| GAPDH | 1.288 | 5.593 | 0.739 |
| HIS | 0.616 | 3.011 | 0.535 |
| RPP | 0.984 | 3.479 | 0.566 |
| SSD | 0.629 | 2.896 | 0.243 |
| TBP | 0.849 | 3.722 | 0.454 |
| UBQ | 0.987 | 3.901 | 0.835 |
| UCE | 1.012 | 4.746 | 0.575 |

^aSD [\pm Ct] indicates standard deviation of the Ct value; CV [% Ct] represents coefficient of variance expressed as the percentage of the Ct value.

glabrata (Li, Skinner, & Bennett, 2012). Therefore, new reference genes that exhibit stable and high expression levels under specific conditions should be considered. In this study, new candidates such

as *RPP* and *SSD* were used to assess their expression stability during chlamyospore formation in *C. rosea* 67-1.

To explore functional genes in *C. rosea*, several reference genes have been adopted. e.g. *act* was used to normalize ABC transporter genes when suffering to secondary metabolites produced by biocontrol bacteria (Kamou et al., 2016). Zapparata (2014) found that *act* was most stable in different nutrient conditions and glyceraldehyde 3-phosphate dehydrogenase gene *gpd1* was available when analyzing the interaction with *F. graminearum*. The gene *tub* was also used in the quantification of gene expressions in *C. rosea* when confronting with pathogenic fungi and plant nematodes (Mamarabadi et al., 2009; Zou, Tu, Liu, Tao, & Zhang, 2010). In our previous study, we found that *EF1* was expressed most stably in *C. rosea* 67-1 during parasitism on *Sclerotinia sclerotiorum* sclerotia, and we obtained a reliable expression profile of mycoparasitism-related genes by using the reference gene *EF1* (Sun, Li et al., 2015; Sun, Sun et al., 2015). In this study, analyses using geNorm, BestKeeper, and NormFinder showed that the expression of *EF1* was not satisfactory during chlamyospore formation in *C. rosea*. Similar results were obtained during the selection of other reference genes. *MGG_Actin* was proved available to normalize gene expression during the infection of *Magnaporthe oryzae*, but not

suitable during fungal vegetative growth (Omar, Bentley, Morieri, & Preston, 2016). Zhou et al. (2012) found that *CrzA* was most stable during the developmental stages of *Beauveria bassiana*, but its stability varied sharply under different stress conditions. The stability of reference genes may vary in response to various environmental conditions, and they may change during the life cycle of an organism; therefore, it is essential to select suitable reference genes for specific conditions.

During transcriptome sequencing and analysis of chlamyospore formation in *C. rosea* 67-1, we found that some genes, including *SSD*, were consistently expressed at the early stage (36 hr) and stationary phase (72 hr) of sporulation (unpublished). *SSD* is a hydrolase that can catalyze the oxidation of succinic semialdehyde to succinic acid. It is a key enzyme in the catabolism of gamma-aminobutyric acid (GABA), and it plays a major role in anticonvulsant activity (Kumar, Kumar, & Puneekar, 2015; Piplani, Verma, & Kumar, 2016). Deficiency of *SSD* may lead to neurologic disorders (Gahr, Connemann, Schönfeldt-Lecuona, & Freudenmann, 2013; Lin, Weng, & Lee, 2015; Püttmann et al., 2013). Currently, researchers are focusing on the structure and dynamics of *SSD* (Jang, Park, Chi, & Lee, 2015; Park, Park, & Lee, 2014); however, there is limited information on its expression and variations in organisms. We selected *SSD* and the other candidates to decide whether they could be used as reference genes during fungal sporulation and found that *SSD* was the most stable in chlamyospore production in *C. rosea*, in accordance with a commonly used housekeeping gene *ACT*. To our knowledge, this is the first report of *SSD* as a reference gene for the normalization of gene expression.

In this study, three software tools, geNorm, NormFinder, and BestKeeper, were used to screen the reference genes for quantifying gene expressions during *C. rosea* sporulation. However, differences in the rankings of candidate genes are possible when different statistical algorithms are used (Bansal et al., 2015; Leal et al., 2015). In our study, some differences were observed during the software analyses. geNorm revealed that *ACT* and *HIS* were the most stable, followed by *SSD*. However, NormFinder showed that *SSD* exhibited the highest stability, followed by *ACT* and *TBP*. According to the results of both software tools, *UBQ* and *GAPDH* displayed the largest variation. According to BestKeeper software, *HIS*, *SSD*, and *ACT* were ranked as the top 3 candidate genes, while *GAPDH* and *EF1* expressed with the least stability. However, the trends in general were consistent. To minimize the impacts of the reference genes, a pairwise variation value was calculated based on the expressions of the nine candidate reference genes, and it confirmed that two reference genes were required for reliable and accurate normalization of gene expression. Based on the results, we recommend *ACT* and *SSD* as reference genes for the quantification of gene expression during chlamyospore formation in *C. rosea* 67-1.

Chlamyospore is of great importance in fungal growth and survival, and it is especially important in the commercialization of biocontrol fungi. To the best of our knowledge, this is the first report on the selection of suitable reference genes for normalizing gene expression during chlamyospore production in a biocontrol fungus. Identification of related genes will facilitate to understand the molecular mechanism underlying chlamyospore formation and promote large-scale applications of biocontrol agents.

5 | CONCLUSION

We analyzed the expression stability of nine reference genes during chlamyospore formation in *C. rosea* 67-1. The results indicated that two reference genes were required for precise normalization of gene expression. *SSD* and *ACT* were the most stable and were selected as reference genes for *C. rosea* sporulation. To the best of our knowledge, this is the first report of screening of sporulation-related reference genes of biocontrol fungi, and *SSD* was used for the first time as a reference gene. This study will contribute to further investigation of the genes related to chlamyospore production in *C. rosea*.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (31471815), the Program of Modern Agricultural Industry Technology System (CARS-25-B-02), and the Ministry of Agriculture Special Scientific Research Task (201503112-2).

CONFLICTS OF INTEREST

None declared.

REFERENCES

- Andersen, C. L., Jensen, J. L., & Ørntoft, T. F. (2004). 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 Research*, *64*, 5245–5250.
- Armengol, J., Sales, R., & Garcia-Jimenez, J. (1999). Effects of soil moisture and water on survival of *Acremonium cucurbitacearum*. *Journal of Phytopathology*, *147*, 737–741.
- Bansal, R., Mittapelly, P., Cassone, B. J., Mamidala, P., Redinbaugh, M. G., & Michel, A. (2015). Recommended reference genes for quantitative PCR analysis in soybean have variable stabilities during diverse biotic stresses. *PLoS ONE*, *10*, e0134890.
- Beagle-Ristaino, J. E., & Papavizas, G. C. (1985). Survival and proliferation of *Trichoderma* spp. and *Gliocladium virens* in soil and in plant rhizospheres. *Phytopathology*, *75*, 729–732.
- Caldwell, R. (1958). Fate of spores of *Trichoderma viride* Pers Ex Fr. introduced into soil. *Nature*, *181*, 1144–1145.
- Cusick, K. D., Fitzgerald, L. A., Pirlo, R. K., Cockrell, A. L., Petersen, E. R., & Biffinger, J. C. (2014). Selection and evaluation of reference genes for expression studies with quantitative PCR in the model fungus *Neurospora crassa* under different environmental conditions in continuous culture. *PLoS ONE*, *9*, e112706.
- Dong, P. P., Sun, M. H., Li, S. D., Peng, Y., & Luo, M. (2014). Biological characteristics of chlamyospores of *Clonostachys rosea* 67-1. *Mycosystema*, *33*, 1242–1252.
- Eyal, J., Baker, C. P., Reeder, J. D., Devane, W. E., & Lumsden, R. D. (1997). Large-scale production of chlamyospores of *Gliocladium virens* strain GL-21 in submerged culture. *Journal of Industrial Microbiology and Biotechnology*, *19*, 163–168.
- Fleige, S., & Pfaffl, M. W. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine*, *27*, 126–139.
- Gahr, M., Connemann, B. J., Schönfeldt-Lecuona, C. J., & Freudenmann, R. W. (2013). Succinic semialdehyde dehydrogenase deficiency:

- An inheritable neurometabolic disease. *Fortschritte der Neurologie Psychiatrie*, 81, 154–161.
- Guénin, S., Mauriat, M., Pelloux, J., Van Wuytswinkel, O., Bellini, C., & Gutierrez, L. (2009). Normalization of qRT-PCR data: The necessity of adopting a systematic, experimental conditions-specific, validation of references. *Journal of Experimental Botany*, 60, 487–493.
- Jang, E. H., Park, S. A., Chi, Y. M., & Lee, K. S. (2015). Structural insight into the substrate inhibition mechanism of NADP(+)-dependent succinic semialdehyde dehydrogenase from *Streptococcus pyogenes*. *Biochemical and Biophysical Research Communications*, 461, 487–493.
- Kamou, N. N., Dubey, M., Tzelepis, G., Menexes, G., Papadakis, E. N., Karlsson, M., ... Jensen, D. F. (2016). Investigating the compatibility of the biocontrol agent *Clonostachys rosea* IK726 with prodigiosin-producing *Serratia rubidua* S55 and phenazine-producing *Pseudomonas chlororaphis* ToZa7. *Archives of Microbiology*, 198, 369–377.
- Kumar, S., Kumar, S., & Puneekar, N. S. (2015). Characterization of succinic semialdehyde dehydrogenase from *Aspergillus niger*. *Indian Journal of Experimental Biology*, 53, 67–74.
- Leal, M. F., Belangero, P. S., Figueiredo, E. A., Cohen, C., Loyola, L. C., Andreoli, C. V., ... Cohen, M. (2015). Identification of suitable reference genes for gene expression studies in tendons from patients with rotator cuff tear. *PLoS ONE*, 10, e0118821.
- Li, L., Qu, Q., Tian, B., & Zhang, K. Q. (2005). Induction of chlamydospores in *Trichoderma harzianum* and *Gliocladium roseum* by antifungal compounds produced by *Bacillus subtilis* C2. *Journal of Phytopathology*, 153, 686–693.
- Li, Q. Q., Skinner, J., & Bennett, J. E. (2012). Evaluation of reference genes for real-time quantitative PCR studies in *Candida glabrata* following azole treatment. *BMC Molecular Biology*, 13, 22.
- Lin, C. Y., Weng, W. C., & Lee, W. T. (2015). A novel mutation of ALDH5A1 gene associated with succinic semialdehyde dehydrogenase deficiency. *Journal of Child Neurology*, 30, 486–489.
- Ma, G. Z., Wang, S. F., Bao, Z. H., Li, S. D., Sun, M. H., Fu, H. R., & Ge, P. H. (2011). Inhibition and protective activities of *Gliocladium roseum* 67-1 to *Rhizoctonia solanii*. *Crops*, 6, 77–79.
- Mamarabadi, M., Jensen, D. F., & Lübeck, M. (2009). An N-acetyl-beta-D-glucosaminidase gene, *cr-nag1*, from the biocontrol agent *Clonostachys rosea* is up-regulated in antagonistic interactions with *Fusarium culmorum*. *Mycological Research*, 113, 33–43.
- Morandi, M. A. B., Maffia, L. A., Mizubuti, E. S. G., Alfenas, A. C., & Barbosa, J. G. (2003). Suppression of *Botrytis cinerea* sporulation by *Clonostachys rosea* on rose debris: A valuable component in Botrytis blight management in commercial greenhouses. *Biological Control*, 26, 311–317.
- Mouekouba, L. D., Zhang, L., Guan, X., Chen, X., Chen, H., Zhang, J., ... Wang, A. (2014). Analysis of *Clonostachys rosea*-induced resistance to tomato gray mold disease in tomato leaves. *PLoS ONE*, 9, e102690.
- Nadai, C., Campanaro, S., Giacomini, A., & Corich, V. (2015). Selection and validation of reference genes for quantitative real-time PCR studies during *Saccharomyces cerevisiae* alcoholic fermentation in the presence of sulfite. *International Journal of Food Microbiology*, 215, 49–56.
- Omar, S. C., Bentley, M. A., Morieri, G., & Preston, G. M. (2016). Validation of reference genes for robust qRT-PCR gene expression analysis in the rice blast fungus *Magnaporthe oryzae*. *PLoS ONE*, 11, e0160637.
- Ortiz, A., & Orduz, S. (2001). *In vitro* evaluation of *Trichoderma* and *Gliocladium* antagonism against the symbiotic fungus of the leaf-cutting ant *Atta cephalotes*. *Mycopathologia*, 150, 53–60.
- Papavizas, G. C. (1985). *Trichoderma* and *Gliocladium*: Biology, ecology, and potential for biocontrol. *Annual Review of Phytopathology*, 23, 23–54.
- Park, S. A., Park, Y. S., & Lee, K. S. (2014). Kinetic characterization and molecular modeling of NAD(P)(+)-dependent succinic semialdehyde dehydrogenase from *Bacillus subtilis* as an ortholog Ynel. *Journal of Microbiology and Biotechnology*, 24, 954–958.
- Pfaffl, M. W., Tichopad, A., Prgomet, C., & Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnology Letters*, 26, 509–515.
- Piplani, S., Verma, P. K., & Kumar, A. (2016). Neuroinformatics analyses reveal GABA and SSADH as major proteins involved in anticonvulsant activity of valproic acid. *Biomedicine & Pharmacotherapy*, 81, 402–410.
- Püttmann, L., Stehr, H., Garshasbi, M., Hu, H., Kahrizi, K., Lipkowitz, B., ... Kuss, A. W. (2013). A novel ALDH5A1 mutation is associated with succinic semialdehyde dehydrogenase deficiency and severe intellectual disability in an Iranian family. *American Journal of Medical Genetics part A*, 161, 1915–1922.
- Rodríguez, M. A., Cabrera, G., Gozzo, F. C., Eberlin, M. N., & Godeas, A. (2011). *Clonostachys rosea* BAF3874 as a *Sclerotinia sclerotiorum* antagonist: Mechanisms involved and potential as a biocontrol agent. *Journal of Applied Microbiology*, 110, 1177–1186.
- Steiger, M. G., Mach, R. L., & Mach-Aigner, A. R. (2010). An accurate normalization strategy for RT-qPCR in *Hypocrea jecorina* (*Trichoderma reesei*). *Journal of Biotechnology*, 145, 30–37.
- Sun, M. H., Chen, Y. M., Liu, J. F., Li, S. D., & Ma, G. Z. (2014). Effects of culture conditions on spore types of *Clonostachys rosea* 67-1 in submerged fermentation. *Letters in Applied Microbiology*, 58, 318–324.
- Sun, Z. B., Li, S. D., & Sun, M. H. (2015). Selection of reliable reference genes for gene expression studies in *Clonostachys rosea* 67-1 under sclerotial induction. *Journal of Microbiological Methods*, 114, 62–65.
- Sun, Z. B., Sun, M. H., & Li, S. D. (2015). Draft genome sequence of mycoparasite *Clonostachys rosea* strain 67-1. *Genome Announcements*, 3, e00546–15.
- Udvardi, M. K., Czechowski, T., & Scheible, W. R. (2008). Eleven golden rules of quantitative RT-PCR. *Plant Cell*, 20, 1736–1737.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, 3, RESEARCH0034.
- Wang, Q., Ishikawa, T., Michiue, T., Zhu, B. L., Guan, D. W., & Maeda, H. (2012). Stability of endogenous reference genes in postmortem human brains for normalization of quantitative real-time PCR data: Comprehensive evaluation using geNorm, NormFinder, and Bestkeeper. *International Journal of Legal Medicine*, 126, 943–952.
- Zapparata, A. (2014). Gene expression analysis of ABC-and MFS-transporters in the fungal biocontrol agent *Clonostachys rosea*. Pisa, Italy: MSc thesis, University of Pisa.
- Zhang, Y. H., Gao, H. L., Ma, G. Z., & Li, S. D. (2004). Mycoparasitism of *Gliocladium roseum* 67-1 on *Sclerotinia sclerotiorum*. *Acta Phytopathologica Sinica*, 34, 211–214.
- Zhang, B. Y., Sun, M. H., Zhang, Y. H., Xie, X. M., & Li, S. D. (2010). Sporulation of *Clonostachys rosea* strain 67-1 in submerge fermentation and its resistance to adversity. *Acta Phytopathologica Sinica*, 40, 103–105.
- Zhou, Y. H., Zhang, Y. J., Luo, Z. B., Fan, Y. H., Tang, G. R., Liu, L. J., & Pei, Y. (2012). Selection of optimal reference genes for expression analysis in the entomopathogenic fungus *Beauveria bassiana* during development, under changing nutrient conditions, and after exposure to abiotic stresses. *Applied Microbiology and Biotechnology*, 93, 679–685.
- Zou, C. G., Tu, H. H., Liu, X. Y., Tao, N., & Zhang, K. Q. (2010). PacC in the nematophagous fungus *Clonostachys rosea* controls virulence to nematodes. *Environmental Microbiology*, 12, 1868–1877.

How to cite this article: Zhang J, Sun Z, Li S, Sun M.

Identification of suitable reference genes during the formation of chlamydospores in *Clonostachys rosea* 67-1. *MicrobiologyOpen*.

2017;6:e505. <https://doi.org/10.1002/mbo3.505>