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Selection of reference genes for expression analysis in the entomophthoralean fungus *Pandora neoaphidis*

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ABSTRACT

The selection of suitable reference genes is crucial for accurate quantification of gene expression and can add to our understanding of host–pathogen interactions. To identify suitable reference genes in *Pandora neoaphidis*, an obligate aphid pathogenic fungus, the expression of three traditional candidate genes including 18S rRNA(*18S*), 28S rRNA(*28S*) and elongation factor 1 alpha-like protein (*EF1*), were measured by quantitative polymerase chain reaction at different developmental stages (conidia, conidia with germ tubes, short hyphae and elongated hyphae), and under different nutritional conditions. We calculated the expression stability of candidate reference genes using four algorithms including geNorm, NormFinder, BestKeeper and Delta Ct. The analysis results revealed that the comprehensive ranking of candidate reference genes from the most stable to the least stable was *18S* (1.189), *28S* (1.414) and *EF1* (3). The *18S* was, therefore, the most suitable reference gene for real-time RT-PCR analysis of gene expression under all conditions. These results will support further studies on gene expression in *P. neoaphidis*.

Introduction

Pandora neoaphidis is one of the most important fungal pathogens of aphids and has great potential for use in biocontrol.[1–4] Although the life cycle of *P. neoaphidis* is well known, a detailed molecular understanding of gene expression during the infection process is still lacking. A diversity of genes can be up- or down regulated during host–pathogen interactions,[5] however, little is known about the expression of reference genes in this particular entomophthoralean fungus.

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Due to its high throughput capacity, sensitivity and specificity, quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) represents a good method for the measurement of gene expression levels across different samples.[6,7] Indeed, it has been used for this purpose with several fungi.[8–11] However, a number of critical aspects must be optimized for effective qRT-PCR analysis; these include the efficiency of RNA extraction, the quality of the RNA, the presence of inhibitors, the efficiency of the reverse transcription and the selection of a suitable reference gene as an internal control.[12] While the majority of these potential sources of error can be avoided by adhering closely to standardized protocols, selection of appropriate reference genes is frequently the greatest challenge because it requires a species-specific solution.[13,14] An ideal reference gene should have constant expression across all samples to be investigated, regardless of biotype, developmental stage or any other biological or experimental variability.

Selection of an inappropriate reference gene with variable expression, leads to erroneous calculations of the expression of target genes and, therefore, incorrect assumptions about the function of those target genes.[15] Identification of suitable reference genes is essential for accurate transcript expression analysis. Several statistical algorithms have been developed to identify the most suitable internal controls with the least variability in expression; these algorithms are based on qRT-PCR data from a given set of candidate genes and rank putative reference genes according to their expression stability, thereby indicating the best reference gene or combination of reference genes for accurate normalization. The four most commonly used algorithms for assessing the appropriateness of reference genes are geNorm,[16] NormFinder,[17] Best-Keeper[18] and Delta Ct.[19] These software packages are freely available to download from the authors' websites and have been widely used to identify suitable reference genes.[20,21]

In this study, three putative housekeeping genes (*18S*, *28S* and *EF1*) were evaluated as potentially reliable reference genes in *P. neoaphidis* using qPCR. Fungal infection of the host usually involves multiple developmental stages.[22] Therefore, profiling gene expression in multiple development stages is important for understanding the mechanisms of pathogenesis. We tested four developmental stages including conidia, conidia with germ tubes, short hyphae and long hyphae. Profiling gene expression under various nutritional conditions is also a routine approach to study gene function. We evaluated the stability of gene expression in three different nutrient media. The expression stability of each gene in all samples was analyzed using the geNorm, NormFinder, Best-Keeper and Delta Ct programs, and the most suitable reference gene for accurate normalization was selected.

Material and methods

Isolate and culture conditions

P. neoaphidis isolate ARSEF 5403 was obtained from the USDA Collection of Entomopathogenic Fungal Cultures, Ithaca, NY. It was subcultured on SEMA (Sabouraud dextrose agar [SDA] supplemented with egg yolk and milk[23]; in 9 cm diameter Petri dishes for 10 d at 18 °C in a 12:12 light:dark regimen.

Sample preparations

Propagules at different stages of germination were prepared. Mycelial mats from liquid culture were produced using the method described by Xu and Feng.[24] Primary conidia actively discharged from the mycelial mat during the period of peak sporulation were harvested into 0.01 mol/L sterile phosphate buffered saline (PBS) solution (130 m mol/L NaCl, 7 m mol/L Na_2HPO_4, 3 m mol/L NaH_2PO_4, pH 7.3).[25] The conidial suspension was filtered through glass wool to remove any mycelia and the conidia centrifuged at 4500 rpm for 10 min before being inoculated into GLEN medium in flasks[26] at a concentration of 10^{10} conidia per milliliter followed by incubation at 20 °C and 150 rpm in a 12:12 light:dark regimen for different periods of time.[2] Samples were taken after ~0 h for conidia, after ~6 h for conidia with germ tubes, after ~12 h for early, short hyphae (i.e. the length of germ tube was a maximum of 200 μm), and after 24 h for elongated hyphae (i.e. hyphae that exceeded 200 μm). At each stage the fungal structures were observed microscopically to ensure they had attained the correct developmental stage (Fig. 1). Samples of each developmental stage were collected after vacuum filtration through Whatman 54 paper and stored at −80 °C until total RNA was isolated. There were three replicate flasks for each developmental stage, i.e. 12 flasks in total were set up.

To provide samples grown under different nutritional conditions, the mycelium from half a Petri dish (agar medium removed using a scalpel) was added to 100 mL flasks containing either 30 mL GLEN medium,[26] 30 mL of Grace's medium (Invitrogen, USA) or 30 mL of OS-SDB medium (Sabouraud Dextrose Broth [SDB; Difco, BD, USA] supplemented with 0.5% (v/v) sesame oil and 0.1% (w/v) sugar esters of fatty acids [Emulsifier E473, CAS No. 37318-31-3, Liuzhou Gaotong Food Chemicals Co. Ltd., China]). Flasks were incubated at 20 °C and 150 rpm in a 12:12 light:dark regimen for three days.[2] Mycelium samples grown under different nutritional conditions were collected by vacuum filtration through Whatman 54 paper and stored at −80 °C until total RNA was isolated. There were three replicate flasks for each nutritional condition i.e. nine flasks in total.

Candidate gene and primer design

Nucleotide sequences of the *18S* and *28S* genes (Genbank accession numbers HQ677591.1 and EF392405.1, respectively), were downloaded from Genbank (http://www.ncbi.nlm.nih.gov/genbank/), while the *EF1* gene sequence was acquired through degenerate PCR. Primer pairs for each gene were designed using the Primer Premier 5.0 program (Table 1). Specificity of primer pairs for each candidate gene was confirmed using melting curve analysis and agarose gel electrophoresis.

Total RNA isolation and cDNA synthesis

RNA was prepared for each sample using the RNeasy Mini Kit (QIAGEN, Cat. No. 74104, Mississauga, Canada), and genomic