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Validation of reference genes for quantitative real-time PCR in Périgord black truffle (*Tuber melanosporum*) developmental stages

Osvaldo Zarivi *, Patrizia Cesare, Anna Maria Ragnelli, Pierpaolo Aimola, Marco Leonardi, Antonella Bonfigli, Sabrina Colafarina, Anna Maria Poma, Michele Miranda, Giovanni Pacioni

Department of Life, Health and Environmental Sciences, University of L'Aquila, 67100 L'Aquila, Italy

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ABSTRACT

The symbiotic fungus *Tuber melanosporum* Vittad. (Périgord black truffle) belongs to the Ascomycota and forms mutualistic symbiosis with tree and shrub roots. This truffle has a high value in a global market and is cultivated in many countries of both hemispheres. The publication of the *T. melanosporum* genome has given researchers unique opportunities to learn more about the biology of the fungus. Real-time quantitative PCR (qRT-PCR) is a definitive technique for quantitating differences in transcriptional gene expression levels between samples. To facilitate gene expression studies and obtain more accurate qRT-PCR data, normalization relative to stable housekeeping genes is required. These housekeeping genes must show stable expression under given experimental conditions for the qRT-PCR results to be accurate. Unfortunately, there are no studies on the stability of housekeeping genes used in *T. melanosporum* development. In this study, we present a morphological and microscopical classification of the developmental stages of *T. melanosporum* fruit body, and investigate the expression levels of 12 candidate reference genes (18S rRNA; 5.8S rRNA; Elongation factor 1-alpha; Elongation factor 1-beta; α-tubulin; 60S ribosomal protein L29; β-tubulin; 40S ribosomal protein S1; 40S ribosomal protein S3; Glucose-6-phosphate dehydrogenase; β-actin; Ubiquitin-conjugating enzyme). To evaluate the suitability of these genes as endogenous controls, five software-based approaches and one web-based comprehensive tool (RefFinder) were used to analyze and rank the tested genes. We demonstrate here that the 18S rRNA gene shows the most stable expression during *T. melanosporum* development and that a set of three genes, 18S rRNA, Elongation factor 1-alpha and 40S ribosomal protein S3, is the most suitable to normalize qRT-PCR data from all the analyzed developmental stages; conversely, 18S rRNA, Glucose-6-phosphate dehydrogenase and Elongation factor 1-alpha are the most suitable genes for fruiting body developmental stages.

1. Introduction

The symbiotic fungus *Tuber melanosporum* Vittad. (Périgord black truffle) belongs to the Ascomycota (Pezizomycota, Pezizomycetes, Pezizales, Tuberaceae) and forms mutualistic symbiosis, called ectomycorrhiza, with roots of trees and shrubs such as oaks, pine and hazelnut trees. The ectomycorrhizal symbiosis is indispensable to the truffle life cycle (Mello et al., 2006; Trappe et al., 2009), that results in the fruit bodies ripening in autumn and winter. Several members of the truffle genus *Tuber* are highly praised and priced gourmet food, and their aroma and taste are known worldwide (Rubini et al., 2007). Some of the most precious edible fungi are found amongst scented truffles: *Tuber magnatum* Pico – the Italian white truffle and *T. melanosporum* – the Périgord black truffle. The publication of the *T. melanosporum* genome (Martin et al., 2010) has given researchers a unique opportunity to learn more about the biology of the fungus. It is the first genome of a symbiontic ascomycetous edible fungus to be sequenced. Transcriptome analyses (microarray, RNAseq, qRT-PCR) have paralleled the sequencing project by studying the transcriptional gene expression in the free-living mycelium (FLM), ectomycorrhizal root tips and hypogeous fruiting bodies (FBs) developmental stages and the changes of gene expression due to symbiosis have been monitored by laser microdissection and microarray (Balestrini et al., 2012; Hacquard et al., 2013; Martin et al., 2010; Sillo et al., 2013; Tisserant et al., 2011; Zarivi et al., 2013). The identification of the genes differentially expressed from the FLM to the FB stages during truffle development may contribute to a better understanding of truffle morphogenesis and the factors affecting reproductive differentiation. One study has functionally annotated the truffle proteome from the 2010

* Corresponding author. Tel.: +39 862 433270; fax: +39 862 433273.
E-mail address: osvaldo.zarivi@univaq.it (O. Zarivi).

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T. melanosporum genome, and the results clearly indicate that the black truffle's unique biology, in the context of its evolution, shows only a distant relationship to any other commonly studied fungus (such as yeast) (Islam et al., 2013). The use of reference gene(s) as internal control for the measurement of target gene expression variation is the currently preferred method for normalizing qRT-PCR data because reference genes can capture all non-biological variations (Dheda et al., 2004; Vandesompele et al., 2009). The lack of an exact qRT-PCR normalization may affect the results and bias conclusions. Ideally, the internal control expression level should be constant in all samples (Kozera and Rapacz, 2013). The housekeeping genes, that are constitutively expressed to maintain basal cellular functions, are the least affected by experimental conditions and the most used by the investigators, often without any previous control. In reality, a universal housekeeping gene does not exist (Butte et al., 2001). Although no gene exhibits constant expression under all experimental conditions, studies validating reference genes have been driven by several algorithms and freely available software packages, e.g., geNorm (Andersen et al., 2004; Pfaffl et al., 2004; Vandesompele et al., 2002) and RefFinder, which can analyze the stability of gene expression. The expression of reference genes can differ widely over developmental stages, in different tissues, and under different environmental conditions. Therefore, a rational optimization approach to select and standardize reference genes is now an important requirement in qRT-PCR-based transcriptome studies (Radonić et al., 2004). There is no validated reference gene(s) for *T. melanosporum* development. In previous RT-qPCR studies on *T. melanosporum*, reference genes such as 18S rRNA (Ceccaroli et al., 2011) and Elongation factor 1-beta (Sillo et al., 2013) were selected based on consensus or results from other species. The aim of this work is to identify suitable reference gene(s) and minimize the risk of coregulation artefacts in *T. melanosporun*. Expressions of 12 candidate reference genes (18S rRNA, *18S*; 5.8S rRNA, *5.8S*; Elongation factor 1-alpha, *EF1A*; Elongation factor 1-beta, *EF1B*; α-tubulin, *TUBA*; 60S ribosomal protein L29, *RPL29*; β-tubulin, *TUBB*; 40S ribosomal protein S1, *RPS1*; 40S ribosomal protein S3, *RPS3*; Glucose-6-phosphate dehydrogenase, *G6PD*; β-actin, *ACTB*; Ubiquitin-conjugating enzyme, *UBC*) were examined in seven *T. melanosporum* developmental stages (FLM and FB 1, 2, 3, 4, 5, 6 stages). Furthermore, the consistency of the best-scoring reference gene was tested by different statistical approaches (geNorm, BestKeeper, NormFinder, GenEx, Delta Ct method and RefFinder). In this work we refer to free-living mycelium and six developmental stages of *T. melanosporum* FB assessed by morphological and cytological features, providing a new definitive classification relating to the ontogeny of *T. melanosporum* FB surpassing the previous proposals (Kulifaj, 1984; Montant et al., 1983; Pacioni et al., 1995). As the advanced FB developmental stages are mainly investigated in the literature (Harki et al., 2006; Le Tacon et al., 2013), due to the hypogeous truffle habitat that makes the sampling of the early developmental stages difficult, we present here, as a further contribution, a detailed morphological and microscopical description of *T. melanosporum* FB 1, 2, 3, 4, 5, 6 developmental stages to allow a precise identification.

2. Results and discussion

2.1. Morphological and microscopic staging of T. melanosporum development

On the basis of the structural organization and morphological features, six different stages can be distinguished during the development of *T. melanosporum* fruiting bodies, as reported in Pacioni et al., 1995. Representative pictures are reported in Fig. 1 and a brief description of each stage is given below, while further details can be found in Table 1, where sub-stages are also defined. Stage 1 (''hyphal stage'') fruiting bodies are undifferentiated mycelial pellets, yellowish[1] in color and with dimensions ranging from about 50 to 300–550 μm (Fig. 1A). At stage 2 (''peridial stage''), fruiting bodies appear as larger pellets, up to 2 mm, initially depressed-excavate from the top, with a finely warty surface and yellowish or yellow-reddish in color. In this stage hyphae of the outer layer transform into roundish cells that will originate the external structures of the fruiting body (hypothecium and peridium), enveloping the internal gleba (Fig. 1B). The subsequent stage 3 (''veined stage'') is characterized by a reddish peridium with flattened and radially fissured warts, while the whitish-marbled gleba is formed by increasingly complex and compact veins and paraphyses begin to appear at the interface between developing sterile and fertile veins (Fig. 1C). Dimensions range from around 0.8 to 3 cm. At stage 4 (''ascal stage'') sterile and fertile veins are clearly distinguishable in the gleba; fertile veins contain developing asci (Fig. 1D). Dimensions range from 2 to 7 cm. In the subsequent stage 5 (''sporal stage'') ascospores are produced within the asci; their wall, which is smooth at first, undergoes a process of formation of ornamentation (Fig. 1E). Peridium appears reddish-brown and dimensions range from 3 to 10 cm or more. Eventually, ascospores are fully ripe at stage 6 (''pigmented stage''), when they exhibit completely developed ornamentation and dark-brown pigmentation; in this stage the gleba appears brown-black with widely developed fertile veins, while sterile veins are reduced to thin bundles (Fig. 1F). Peridium is dark brown-black and definitive size of the fruiting body is reached.

2.2. Total RNA quality and PCR amplification efficiencies

The integrity of all total RNA samples was confirmed using 1.2% agarose and imaging analysis (Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software Bio RAD) (Fig. S2). All PCR assays produced a single amplicon of the expected size, as shown by the absence of nonspecific bands in electrophoresis analysis and the presence of a single sharp peak in melting curve analysis, and temperatures were in accordance with those calculated (Fig. S1; Table 2). The gene-specific amplification efficiency was calculated by linear regression analysis of the standard curve and ranged between 95.87% (*RPS3*) and 99.01% (*UBC*) (File S1). The coefficient of correlation (r^2) of the linear regression analysis was always greater than 0.992, indicating a linear relationship between Ct values and log-transformed transcript quantities in the range of the standard curve.

2.3. Expression stability of candidate reference genes

The transcripts of all the 12 candidate genes were found from all the stages investigated. Global variations on expression profiles of the candidate reference genes can be observed in (Fig. 2), while the detailed expression of each gene in the different developmental stages is shown in (Fig. S3). The means of the Ct ± SEM spanned from 12.96 ± 0.06 (*18S* gene) to 27.14 ± 0.09 (*G6PD* gene) in group A and from 12.92 ± 0.06 (*18S* gene) to 26.91 ± 0.09 (*G6PD* gene) in group B (Table S1). The fluorescence peak after about 12.96 cycles showed that *18S* was the most abundantly transcribed, whereas *G6PD* was the least abundant transcript with a Ct value of 28 or higher. Expression of *EF1B* showed the least variation across samples, in contrast to *5.8S*, *EF1A* and *RPS1*. Whatever the stage was, the genes appeared organized in the same order according to their level of expression.

[1] For interpretation of color in Fig. 1, the reader is referred to the web version of this article.

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