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Validation of RT-qPCR reference genes for *in planta* expression studies in *Hemileia vastatrix*, the causal agent of coffee leaf rust

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ABSTRACT

Hemileia vastatrix is a biotrophic fungus, causing coffee leaf rust in all coffee growing countries, leading to serious social and economic problems. Gene expression studies may have a key role unravelling the transcriptomics of this pathogen during interaction with the plant host. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is currently the golden standard for gene expression analysis, although an accurate normalisation is essential for adequate conclusions. Reference genes are often used for this purpose, but the stability of their expression levels requires validation under experimental conditions. Moreover, pathogenic fungi undergo important biomass variations along their infection process *in planta*, which raises the need for an adequate method to further normalise the proportion of fungal cDNA in the total plant and fungus cDNA pool. In this work, the expression profiles of seven reference genes [glyceraldehyde-3-phosphate dehydrogenase (*GADPH*), elongation factor (*EF-1*), Beta tubulin (*β-tubulin*), cytochrome c oxidase subunit III (*Cyt III*), cytochrome b (*Cyt b*), *Hv00099*, and 40S ribosomal protein (*40S_Rib*)] were analysed across 28 samples, obtained *in vitro* (germinated uredospores and appressoria) and *in planta* (post-penetration fungal growth phases). Gene stability was assessed using the statistical algorithms incorporated in geNorm and NormFinder tools. *Cyt b*, *40S_Rib*, and *Hv00099* were the most stable genes for the *in vitro* dataset, while *40S_Rib*, *GADPH*, and *Cyt III* were the most stable *in planta*. For the combined datasets (*in vitro* and *in planta*), *40S_Rib*, *GADPH*, and *Hv00099* were selected as the most stable. Subsequent expression analysis for a gene encoding an alpha subunit of a heterotrimeric G-protein showed that the reference genes selected for the combined dataset do not differ significantly from those selected specifically for the *in vitro* and *in planta* datasets. Our study provides tools for correct validation of reference genes in obligate biotrophic plant pathogens, as well as the basis for RT-qPCR studies in *H. vastatrix*.

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Introduction

Gene expression analysis is crucial for enhancing our understanding of the signalling and metabolic pathways which underlie cellular and developmental processes. Although several methods have been used to quantify gene expression, including Northern blotting, RNase protection assay, *in situ* hybridisation, and cDNA microarray technology, Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is considered the gold standard for quantifying gene expression, thanks to its sensitivity, specificity, dynamic range, and high throughput capacity (Freeman *et al.* 1999; Bustin *et al.* 2005; Udvardi *et al.* 2008). This technique can detect very low quantities of a target transcript even if only a few copies are present in the sample (Wong & Medrano 2005). However an accurate normalisation is required to obtain a reliable quantification of the transcript (Huggett *et al.* 2005; Derveaux *et al.* 2010).

Different procedures to normalise RT-qPCR data have been proposed over the years, evolving from techniques such as sample size, total RNA or genomic DNA quantification, and artificial molecules controls, to techniques that take into account sample variations, such as differences in the quantity and quality of RNA, and efficiencies of reverse transcription or PCR (Huggett *et al.* 2005; Cruz *et al.* 2009; Teste *et al.* 2009; Walker *et al.* 2009). In this perspective, internal control genes (references genes) are the most commonly used to normalise RT-qPCR data, though the success of this procedure relies on the appropriate choice of control genes (Pfaffl *et al.* 2004; Dheda *et al.* 2005). Typically, reference genes should exhibit two major properties: (i) they should be essential for the maintenance of cellular function and viability, and therefore should be constitutively expressed in all tissues; (ii) their transcription should not be affected by the conditions under study (Derveaux *et al.* 2010). However, some evidences show that almost all genes seem to be regulated under some conditions and there are always some variations in transcript levels, so that none of the commonly exploited genes can be viewed as a universal reference gene (Thellin *et al.* 1999; Stürzenbaum & Kille 2001; Dheda *et al.* 2005). In many cases, the use of, a single reference gene is inadequate, and can produce wrong biological conclusions (Stürzenbaum & Kille 2001; Hu *et al.* 2009; Teste *et al.* 2009). Additionally, gene expression studies as well as reference gene validations are mainly limited to human and other well-established model organisms, while non-model species often suffer from lack of background information available (Axtner & Sommer 2009). For all these reasons, a careful evaluation of one or even more suitable reference genes is essential prior to any gene expression profiling study. Currently, several methods such as geNorm (Vandesompele *et al.* 2002) and NormFinder (Andersen *et al.* 2004) based on different statistical algorithms have been developed to select the most stable reference genes to appropriately normalise RT-qPCR results (Vandesompele *et al.* 2002; Andersen *et al.* 2004; Pfaffl *et al.* 2004).

Genes encoding actin, glyceraldehyde-3-phosphate dehydrogenase, elongation factor, ß-tubulin, and 28S and 18S ribosomal genes are frequently used in molecular studies as reference genes for RT-qPCR without appropriate validation, which may jeopardise the reliability of data (Kim *et al.* 2003; Nicot *et al.* 2005; Fang & Bidochka 2006; Bohle *et al.* 2007). In fungi and oomycetes, the determination of the most stable genes for expression studies was only carried out for a limited number of species, with different reference genes validated in each species. These include actin, secretion associated GTP-binding protein (*sarA*), and cytochrome c oxidase (*Cox5*) from *Aspergillus niger* (Bohle *et al.* 2007), elongation factor (*Mlp-ELF1a*) and α-tubulin (*Mlp-aTUB*) from *Melampsora larici-populina* (Hacquard *et al.* 2011), elongation factor (EF-1), a glyceraldehyde-3-phosphate dehydrogenase (*GADPH*), and tryptophan biosynthesis enzyme (*tryp*) from *Metarhizium anisopliae* (Fang & Bidochka 2006), ubiquitin-conjugating enzyme (*Ubc*), Beta tubulin (*Tub-b*), and 40S ribosomal protein (*WS21*) from *Phytophthora parasitica* (Yan & Liou 2006) and mannosyltransferase activity (*ALG9*), RNA Pol II transcription factor activity (*TAF10*), RNA Pol III transcription factor activity (*TFC1*), and ubiquitin-protein ligase activity (UBC6) from *Saccharomyces cerevisiae* (Teste *et al.* 2009). In this way, it is crucial to validate reference genes prior to expression studies, namely in non-model organisms such as *Hemileia vastatrix*.

Hemileia vastatrix is responsible for coffee leaf rust, a disease that can lead to yield losses of 30 % in *Coffea arabica* if no control measures are applied. This pathogen establishes a biotrophic interaction with its host and is completely dependent of plant living cells to grow and reproduce (Silva *et al.* 2006; Nunes *et al.* 2009; Ramiro *et al.* 2009; Azinheira *et al.* 2010; Talhinhas *et al.* 2010). In spite of its biotrophic lifestyle, *H. vastatrix* uredospores can germinate and differentiate appressoria *in vitro*, but further differentiation of infection structures (i.e. infection hyphae, haustoria, and sporulation structures) requires the presence of the host plant (Azinheira *et al.* 2001). In coffee leaves, appressoria formed over stomata differentiate an infection hypha which invades the substomatic cavity, from which the fungus grows colonising the leaf tissues inter- and intra-cellularly, feeding from living coffee cells by specialised structures named haustoria (Silva *et al.* 1999). Leaf tissues become heavily colonised by the fungus and the infection cycle is completed with the formation of sporogenic hyphae and the release of uredospores, which occurs from 21 d after the infection (Rodrigues *et al.* 1975; Silva *et al.* 1999). Therefore, the proportion of *H. vastatrix* biomass *in planta* increases along the infection process, which can be a problem for validation of reference genes. In this way, an additional normalisation step is required to successfully validate *H. vastatrix* reference genes for *in planta* studies. Although this is a normalisation in conceptual terms, we termed it 'correction' to avoid confusion with the common use of 'normalisation' in qPCR. It should be noted that, even though quantification of fungal pathogen DNA is frequently carried out by qPCR (Atallah *et al.* 2007; Mideros *et al.* 2009), including rust fungi (Boyle *et al.* 2005; Jackson *et al.* 2006; Acevedo *et al.* 2010), this strategy for the validation of RT-qPCR fungal reference genes 'in host' is still a novelty. To date, such validation of reference genes following a correction step has been only defined in the poplar rust fungus *M. larici-populina* (Rinaldi *et al.* 2007; Hacquard *et al.* 2011).

The main focus of this work was the identification of the best *H. vastatrix* reference genes suitable for the normalisation