



Note

Evaluation of quantitative PCR reference genes for gene expression studies in *Tribolium castaneum* after fungal challenge

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ABSTRACT

To investigate gene expression in *Tribolium castaneum* exposed to *Beauveria bassiana*, reference genes for qPCR were evaluated. Of these, β -actin, α -tubulin, and RPS6 were not stable. The most stable were ribosomal protein genes, RPS3, RPS18, and RPL13a. Syntaxin1, syntaxin6, and E-cadherin may be appropriate for some experimental systems.

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Quantitative real-time PCR (qPCR) is one of the most accurate methods to measure small changes in mRNA levels for individual genes. However, the quality of the results is directly related to normalization with reference genes whose expression is stable with experimental procedures. Much work has been published on normalization using housekeeping genes with presumed stability of expression but without validation under the specific experimental conditions. According to Vandesompele et al. (2002), errors of up to 20-fold can result from using only one reference gene, and at least two are recommended. Furthermore, the expression of reference genes can vary greatly among cell types and experimental systems, and many of the "classical" reference genes are unsuitable for general use (Radonić et al., 2004). Accordingly, potential reference genes should be validated to assure stability of expression under specific experimental conditions.

The honey bee, *Apis mellifera* is currently the only insect for which reference genes have been validated and published (Lourenço et al., 2009; Scharlaken et al., 2008). The red flour beetle, *Tribolium castaneum*, is a model organism and is the only beetle for which there is a published genome sequence. The tolerance of *T. castaneum* to fungi and other pathogens makes it an especially good model for studies of insect immune defenses. With the exception of Altincicek et al. (2007), who used the ribosomal protein RPS18 and α -tubulin

for injected adult *T. castaneum*, single genes have been used as sole normalizers for *T. castaneum* qPCR including RPS3 (Mahroof et al., 2005; Zou et al., 2007), RPS6 (Arakane et al., 2009; Morris et al., 2009), polyubiquitin (Arakane et al., 2005), and RPS18 (Knorr et al., 2009). Zou et al. (2007) used RPS3 as the sole normalizer for *T. castaneum* after injection with bacteria or non-entomogenous fungi. It should be noted that injection with fungi induces gene expression in response to wound and sepsis in addition to response to the fungus.

We have identified several suitable reference genes for gene expression studies using larvae of *T. castaneum* strain GA2, the sequenced strain, at four time points after treatment with commercially produced conidia of the entomopathogenic fungus *Beauveria bassiana* GHA (Laverlam, Butte, MT, USA).

T. castaneum larvae 11 days post-oviposition were exposed by contact for 24 h to a maximum challenge dose of 1 mg of *B. bassiana* conidia/g of crimped wheat. At 24, 48, 72, and 96 h from initial exposure, treated and control larvae were flash frozen in liquid nitrogen, and 20 mg of whole larvae from each replicate was used for RNA collection. RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions including an optional on-column DNase treatment. A second DNase treatment was performed in 2.25 mM Tris buffer (0.17U DNase/ μ l) and cleaned using Absolutely RNA miniprep kit (Stratagene, LaJolla, CA, USA). First strand cDNA was synthesized from 1 μ g of total RNA using SuperScript[®] III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Concentration and RNA purity were estimated by a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA).

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Table 1

Nucleotide sequences of primers for real-time PCR reactions (forward primers are listed first).

Symbol	Name	GenBank accession	Primer sequence (5' to 3')
ACTB	β -actin	XM_970977	TCCATCATGAAGTGGCATGT CCACATCTGTGGAAATGTCG
RPS3	Ribosomal protein S3	XM_965494	ACCTCGATACACCATAGCAAGC ACCGTCGTATTCTGTAATTGAC
RPS6	Ribosomal protein S6	XM_963302	AGATATATGGAAGCATCATGAAGC CGTCGTCTCTTTGCTCAAATTG
RPS18	Ribosomal protein S18	XM_968539	CGAAGAGTTCGAGAAAATCG CGTGGTCTTGGTGTGTTGAC
RPL13a	Ribosomal protein 13a	XM_969211	ACCATATGACCGCAGGAAAC GGTGAATGGAGCCACTTGTT
CAD	E-cadherin	XM_961215	AACGAGCCAAGGACAGCTAA TAGATTGAGCGGTGCTCT
SYN1	Syntaxin1	XM_965112	GGCTTCATGGATGCATTTTT TTAAGCTTGGCAGGACTTT
SYN6	Syntaxin6	XM_962400	CAGAGATCGTGATCGTACCG GGAATCACCGATAGCTTCCA

For qPCR, primers were designed using Primer3 software (Rozen and Skaletsky, 2000) and sequences were validated against the *T. castaneum* genome. Primers were obtained from IDT Technologies (Coralville, IA). Takara SYBR Premix Ex Taq II (Takara Bio, Madison, WI, USA) reaction mix was used for optimization of concentrations and quantification according to the manufacturer's recommendations with 10 ng of cDNA. Real-time PCR reactions were performed with a MX3000P thermocycler (Stratagene) with the following thermocycler conditions: initial denaturation 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 55 °C for 20 s, and 72 °C for 15 s. The primer concentrations (F/R) were RPS18, 900/900nM; RPL13a, 300/300 nM; E-cadherin, 900/300 nM; syntaxin6, 900/900 nM; syntaxin1, 300/900 nM; RPS3, 300/900 nM; β -actin, 900/50 nM; and RPS6, 50/300 nM. We evaluated nine biological replicates using averages from three technical replicates.

Two Microsoft Excel-based tools that gave different results were used for comparison of candidate gene expression stability: geNorm (Vandesompele et al., 2002), which bases a gene expression stability measure (*M*) on the pair-wise comparisons of geometric means to minimize outlier effects, and NormFinder (Andersen et al., 2004). Raw Ct values were converted by the comparative Ct method for both algorithms. The data for each time post-exposure were analyzed separately.

Eight reference genes were selected (Table 1). Also tested but not included in the group is α -tubulin (NCBI XM_968998), which was not stable in *T. castaneum* under our conditions and did not provide consistent Ct values. β -actin, which has been reported to vary considerably in mammals (Selvey et al., 2001) but was identified as among the most stably expressed genes in the heads of bacteria-challenged bees (Scharlaken et al., 2008), was not stable in fungus-

Table 3

Expression stability calculated by NormFinder of candidate reference genes for normalization.

Gene name	Stability value			
	24 h	48 h	72 h	96 h
B-actin	0.207	0.490	2.102	–
RPL13a	0.143	0.085	0.051	0.048
E-cadherin	0.193	0.249	0.051	0.058
Syntaxin6	0.179	0.128	0.217	0.565
Syntaxin1	0.100	0.094	0.082	0.061
RPS18	0.128	0.029	0.106	0.048
RPS3	0.071	0.115	0.210	0.064
RPS6	0.127	0.586	0.469	1.808
Best gene	RPS3	RPS18	RPL13a/E-cadherin	RPL13a/S18

infected *T. castaneum*. The ribosomal protein gene, RPS6, another commonly used reference gene, also was not stable under our experimental conditions.

The genes that were most stable according to both assessment programs and across incubation periods were for ribosomal proteins RPS3, RPS18, and RPL13a (Tables 2 and 3). In addition, three genes in this study not commonly used as references were evaluated. Syntaxins 1 and 6 are reportedly stable in qPCR with all life stages of *T. castaneum* (Arakane, unpublished). Cadherins have been used as reference genes in human tissue (Eisenberg and Levanon, 2003), and E-cadherin was found to be stably expressed in larval *T. castaneum* heads and gut (Morris et al., 2009). In our analyses, all three were deemed moderately stable and may be appropriate for some experimental systems.

It should also be noted that many of the housekeeping genes that are commonly used as references for qPCR, including those for ribosomal proteins, contain multiple subunits. In some cases, individual subunits may be differentially expressed in various tissues. Since qPCR primer design amplifies such a small region of the target gene (typically 50–100 bp), it is critical to design and validate primers so that they amplify only the specific target gene. Normalization with carefully validated genes is especially important for late stage disease development when multiple homeostatic processes may be compromised, perhaps at different rates. This is especially true in the case of fungal infections wherein a substantial portion of the host tissue is replaced by pathogen. We have identified three genes that are stable for use as qPCR normalizers in *T. castaneum* larvae, whether healthy or in early to late stages of fungal infection.

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Table 2

Expression stability (*M* value) of candidate reference genes for normalization calculated by geNorm. Genes with *M* values <0.15 are considered best.

Gene name	Stability (<i>M</i>) value						
	24 h		48 h		72 h		96 h
RPL13a/S18	0.10	RPL13a/S18	0.09	Syntaxin6/S18	0.12	RPL13a/S18	0.14
RPS3	0.129	RPS3	0.11	RPS3	0.17	E-cadherin	0.17
RPS6	0.147	Syntaxin1	0.16	RPL13a	0.19	Syntaxin1	0.18
Syntaxin1	0.189	Syntaxin6	0.17	Cadherin	0.21	RPS3	0.20
E-cadherin	0.238	E-cadherin	0.24	Syntaxin1	0.24	Syntaxin6	0.32
Syntaxin6	0.270	β -actin	0.38	RPS6	0.33	RPS6	0.98
β -actin	0.290	RPS6	0.51	β -actin	1.01		

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