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# Evaluation of Potential Reference Genes for Quantitative RT-PCR Analysis in *Fusarium graminearum* under Different Culture Conditions

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**The filamentous fungus *Fusarium graminearum* is an important cereal pathogen. Although quantitative real-time PCR (qRT-PCR) is commonly used to analyze the expression of important fungal genes, no detailed validation of reference genes for the normalization of qRT-PCR data has been performed in this fungus. Here, we evaluated 15 candidate genes as references, including those previously described as housekeeping genes and those selected from the whole transcriptome sequencing data. By a combination of three statistical algorithms (BestKeeper, geNorm, and NormFinder), the variation in the expression of these genes was assessed under different culture conditions that favored mycelial growth, sexual development, and trichothecene mycotoxin production. When favoring mycelial growth, *GzFLO* and *GzUBH* expression were most stable in complete medium. Both *EF1A* and *GzRPS16* expression were relatively stable under all conditions on carrot agar, including mycelial growth and the subsequent perithecial induction stage. These two genes were also most stable during trichothecene production. For the combined data set, *GzUBH* and *EF1A* were selected as the most stable. Thus, these genes are suitable reference genes for accurate normalization of qRT-PCR data for gene expression analyses of *F. graminearum* and other related fungi.**

***Keywords*** **:** *Fusarium graminearum*, gene expression, quantitative real-time PCR, reference genes

Quantitative real-time PCR (qRT-PCR) is the most sensitive and specific method for quantifying mRNA expression levels of individual target genes of interest. qRT-PCR expression analysis has several advantages over other conventional methods, but requires data normalization with appropriate reference gene(s) whose expression should be unaffected throughout the study conditions (Anderson et al., 2004; Bustin et al., 2004; Gutierrez et al., 2008). Several housekeeping genes involved in basic cellular functions, such as ribosomal genes, and those encoding actin, beta-tubulin (BTUB), translation elongation factor (EF1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been used as reference genes for qRT-PCR in humans and other model organisms. However, several studies have demonstrated that some of these traditional housekeeping genes are unsuitable references because their expression levels vary under certain conditions (Czechowski et al., 2005; Dheda et al., 2004; Lee et al., 2002; Suzuki et al., 2000). Therefore, it is necessary to evaluate candidate reference genes for qRT-PCR expression analysis under specific experimental conditions in the organism to be studied.

The filamentous fungus *Fusarium graminearum* (telomorph: *Gibberella zeae*) is an important cereal pathogen, and produces mycotoxins that are harmful to humans and animals (Desjardins and Proctor, 2007; McMullen et al., 1997). Complete genome sequencing of *F. graminearum* (Cuomo et al., 2007) has allowed for genome-wide expression profiling using DNA microarrays under various developmental and physiological conditions, such as mycelial growth (Güldener et al., 2006) sexual development (Hallen et al., 2007), germination (Seong et al., 2008) and pathogenesis (Güldener et al., 2006; Stephens et al., 2008). Although the microarray data in these studies were not validated by qRT-PCR, it has become the most common method to obtain specific expression patterns of genes identified from gene expression profiles (Chen et al., 2011; Liu et al., 2011; Pandolfi et al., 2010) or functional studies (Lee et al., 2009; Lysøe et al., 2009). However, the expression stabilities of the housekeeping genes used as references for qRT-PCR analysis in even these studies have not been properly validated.

The objective of this study was to identify *F. graminearum* reference genes suitable for normalization of qRT-PCR data under different conditions such as mycelial growth, sexual development, and mycotoxin production. We selected 15 candidate references, including the common housekeeping genes used in previous studies (Chen et al., 2011; Lee et al., 2009; Liu et al., 2011; Lysøe et al., 2009; Pandolfi et al., 2010) and a new set of genes selected from

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the whole transcriptome sequencing (RNA-seq) analysis of a *F. graminearum* strain (unpublished results). By comparing the variation in the expression of these genes under various conditions using three different algorithms [geNorm (Vandesompele et al., 2002), NormFinder (Anderson et al., 2004), and BestKeeper (Pfaffl et al., 2004)] we identified suitable reference genes that can be used in subsequent expression studies.

## Materials and Methods

**Fungal strain and growth conditions.** The *F. graminearum* PH-1 strain used in this study belongs to lineage 7 of the *F. graminearum* species complex (O'Donnell et al., 2000), and its genome has been sequenced (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/MultiHome.html). It was retrieved from 25% glycerol stock cultures stored at −80 °C and maintained on potato dextrose agar (PDA; Difco Laboratories, USA). We investigated four different culture conditions. Fungal growth in complete medium (CM) liquid culture, performed as previously described (Leslie and Summerell, 2006), represents mycelial (vegetative) growth under nutrient-rich conditions. Carrot agar (Leslie and Summerell, 2006) represents sexual development in *F. graminearum*, and favors two different developmental stages. The first 6-day growth on carrot agar represents the formation of aerial mycelia under nitrogen starvation conditions, which is a prerequisite for sexual development. The subsequent 6-day growth on the same medium, as previously described (Lee et al., 2006), represents the formation of sexual fruiting bodies (perithecia). Mycelial growth in defined liquid medium amended with agmatine (AG), performed as previously described (Gardiner et al., 2009), represents trichothecene production; toxin accumulation peaks at days 6–7 (Gardiner et al., 2009). The time points for RNA extraction under each growth condition were as follows: days 2, 4, and 6 in CM; days 2, 4, 6 on carrot agar for aerial mycelia; days 2, 4, 6 on carrot agar after removing aerial mycelia grown for 6 days on carrot agar; days 3 and 6 in AG liquid medium.

**RNA extraction.** The fungal mycelia or perithecia were harvested from each culture at the chosen time points and ground to fine powder in liquid nitrogen (Lee et al., 2006). Total RNA from the samples was extracted using the Total RNA extraction kit (iNtRON Biotechnolgy, Korea) according to the manufacturer's protocol. All RNA samples were assessed for quality by agarose gel electrophoresis and quantified using ultraviolet spectrophotometry at a 260 nm/280 nm absorbance ratio (between 1.8 and 2.0). The first-strand cDNA was synthesized from total RNA using PrimeScript RT reagent kit (TaKaRa, Japan).

**Reference genes, primer design, and PCR efficiency test.** Fifteen candidate reference genes were selected (Table 1). Seven were *F. graminearum* orthologs of common housekeeping genes that are frequently used as references for data normalization. The remaining eight were selected from the *F. graminearum* PH-1 genome based on the read frequencies of individual mRNAs by RNA-seq analysis, each of which was expressed constitutively at similar levels under the different conditions described above (unpublished results). Primers for the newly selected candidate genes were designed using PrimerSelect program (DNASTAR, USA), and those for the commonly used housekeeping genes were obtained directly from the published literature (Table 1). The amplification efficiencies of all genes were determined using RNA samples from a single time point in each condition followed by analysis of the slope of a serial 10-fold cDNA dilution curve (Pfaffl et al., 2001) (Table 1).

**Quantitative real-time PCR and data analysis.** qRT-PCR was performed with the SYBR Green Super Mix (Bio-Rad, USA) and a real-time PCR system (Eppendorf Mastercycler ep realplex4, Germany). The changes in fluorescence of the SYBR Green dye in each cycle were monitored by the system software, and the threshold cycle (Ct) above the background for each reaction was calculated. The expression stabilities of the candidate reference genes were evaluated using three Microsoft Excel-based statistical tools, geNorm (Vandesompele et al., 2002), NormFinder (Anderson et al., 2004) and BestKeeper (Pfaffl et al., 2004). Gene expression was measured in two separate samples from each time point in each condition (biological replicates). Each individual reaction was run in triplicate (technical replicates). The average Ct value of each triplicate reaction was converted into linear expression values for subsequent analysis with geNorm and NormFinder.

## Results

**Expression analysis of candidate reference genes.** qRT-PCR primers generated amplicons ranging from 91 to 250 bp (Table 1). The average PCR efficiency of the primer sets (except *BTUB*) ranged from 1.95 to 1.99. Although the standard deviations for three genes (*EF1B*, *UBC*, and *CYP2*) were slightly higher than 0.05, all of the average E values indicated that the amplification efficiencies were near the theoretical optimum level of 2. We also observed a single peak in real-time melt-curve analysis of all the genes (data not shown). Thus, gene-specific amplification by all primers was confirmed. In all subsequent data, *BTUB* was not considered reliable because it displayed an unexpect-

**Table 1.** Selected candidate reference genes evaluated in this study

| Gene | Locus[a] | Gene description | Primer sequence[b] (5'-3') | Amplicon size (bp) | Tm (°C) | E[c] | Reference |
|---|---|---|---|---|---|---|---|
| *EF1B* | FGSG_01008.3 | eukaryotic translation elongation factor 1 beta | GAGTACCGCAAGAAGAAGGAGAA-CAAG/ | 176 | 60.8 | 1.95 ± 0.06 | Lee et al., 2009 |
| | | | ACCAAAACCAACGGGGACGAG/ | | 61.6 | | |
| *GAPDH* | FGSG_06257.3 | glyceraldehyde-3-phos-phate dehydrogenase | ACCACCGTCCACTCCTACAC/ GGCGAACAGTCAAGTCAACA | 219 | 60.0 70.0 | 1.97 ± 0.02 | Pandolfi et al., 2010 |
| *UBC* | FGSG_10805.3 | ubiquitin conjugating enzyme | TCCCCTTACTCTGGCGGTGTC/ TTGGGGTGGTAGATGCGTGTAGT | 103 | 59.8 59.2 | 1.97 ± 0.05 | Lysøe et al., 2009 |
| *EF1A* | FGSG_08811.3 | eukaryotic translation elongation factor 1 alpha | GGCTTTCACCGACTACCCTCCTCT/ ACTTCTCGACGGCCTTGATGACAC | 91 | 61.7 62.3 | 1.94 ± 0.01 | Lysøe et al., 2009 |
| *BTUB* | FGSG_09530.3 | beta-tubulin | GGTCTCGACAGCAATGGTGTT/ | 110 | 56.1 | 2.92 ± 0.52 | Lysøe et al., 2009 |
| | | | GCTTGTGTTTTTCGTGGCAGT | | 56.4 | | |
| *CYP1* | FGSG_7439.3 | cyclophilin 1 | TCAAGCTCAAGCACACCAAGAAGG/ | 191 | 61.4 | 1.93 ± 0.02 | |
| | | | GGTCCGCCGCTCCAGTCT | | 59.6 | | |
| *CYP2* | FGSG_00777.3 | cyclophilin 2 | CTACGGTGAGAAGTTCGCTGACG/ | 250 | 59.8 | 1.95 ± 0.05 | |
| | | | TCTTGGGCTTCTGGGTGTACTTGATGG | | 65.7 | | |
| *GzHSP70* | FGSG_00838.3 | heat shock protein 70 | TCAACGGAAAGGAGCCCAACAAGT/ | 136 | 63.6 | 1.95 ± 0.04 | this study |
| | | | GGGGGCGACGTCGAGGAGCAGAAT | | 70.5 | | |
| *GzUBH* | FGSG_01231.3 | ubiquitin C-teminal hydrolase | GTTCTCGAGGCCAGCAAAAAGTCA/ CGAATCGCCGTTAGGGGTGTCTG | 168 | 62.4 65.2 | 1.94 ± 0.05 | this study |
| *GzRBP1* | FGSG_02497.3 | RING-box protein 1 | ATAACTGCGCCATCTGCCGTAAT/ | 165 | 60.5 | 1.92 ± 0.02 | this study |
| | | | GGACAAACAGATCGAGCCTTCAAC | | 59.8 | | |
| *GzFLO* | FGSG_05633.3 | putative cell surface flocculin | GTTGAGAAGCCCGCACCTACGAC/ CTCCTGGCGCTGGGGCTCCTTTGT | 179 | 62.7 70.5 | 1.98 ± 0.03 | this study |
| *GzDGA* | FGSG_06688.3 | diacylglycerol acyltransferase | TGGGCCCATCTCTTCAACATTA/ GCTCGCGCTCTCCTTCTACAG | 195 | 57.9 58.0 | 1.94 ± 0.04 | this study |
| *GzC2H044* | FGSG_06701.3 | C2H2 finger protein | GGCCCTTTCCCCTCTTGATGA/ | 181 | 60.9 | 1.99 ± 0.03 | this study |
| | | | GCTTTTCGGCTCGGTGGTTGC | | 64.5 | | |
| *GzSNC1* | FGSG_08537.3 | synaptobrevin | CCGTGGGTGTGATGCGTGAC/ | 114 | 61.6 | 1.98 ± 0.04 | this study |
| | | | CCTCGGCGGAAACCTTGTGCTGAA | | 68.5 | | |
| *GzRPS16* | FGSG_09438.3 | mitochondrial ribosomal protein S16 | GACCGATCCTTACGACGACTCTG/ ATCCTTCTGCACAACACCCTTTAT | 192 | 58.6 56.8 | 1.99 ± 0.01 | this study |

[a]from the *F. graminearum* genome database (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/MultiHome.html).
[b]forward/reverse primer.
[c]average E value ± standard deviation.

edly high E value (2.92). This may be attributable to the position of the reverse primer, spanning the third intron region in the *BTUB* nucleotide sequence. The Ct values determined from the amplification curves of the samples are shown in Tables S1-3. The average Ct value ranged from 17 (for *CYP2*) to 25 (for *GzC2H044*). The variation in the expression of the candidate reference genes under the various conditions were assessed by BestKeeper, geNorm, and NormFinder.

**BestKeeper analysis** (http://www.gene-quantification.de/bestkeeper.html)**:** The BestKeeper tool expresses variation in gene expression as the standard deviation (SD) and coefficient of variance (CV) of the derived Ct values from each reference candidate gene. Descriptive statistics of the candidate genes under four different culture conditions are provided in Table S4–9. A low SD should be expected for most stably expressed genes under the various conditions. The SD values of all of the genes (except *EF1B* and *GAPDH*) were not greater than 1.0 (i.e., two-fold change) during the 6-day hyphal growth stage in CM liquid medium. The gene with the lowest expression levels under these conditions was *GzC2H044* with an SD of 0.24, which represents an acceptable 1.18-fold change in expression. The expressions of *GzUBH* and *GzFLO* also varied similarly (Table 2). In the mycelia growth condition on carrot agar for 6 days, *EF1A* was the most stable gene with an SD of 0.16, followed by *GzRPS16*, *EF1B*, *GzSNC1*,

**Table 2.** Ranking of the candidate reference genes for normalization under mycelial growth in CM and AG by three statistical algorithms

| Rank | CM | | | | | | AG | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BestKeeper | | geNorm | | NormFinder | | BestKeeper | | geNorm | | NormFinder | |
| | Gene | SD [±Ct] | Gene | M value | Gene | Value | Gene | SD [±Ct] | Gene | M value | Gene | Value |
| 1 | *GzC2H044* | 0.24 | *GzUBH* | 0.118 | *GzFLO* | 0.011 | *GzRBP1* | 0.32 | *GzRPS16* | 0.123 | *GzC2H044* | 0.144 |
| 2 | *GzUBH* | 0.27 | *GzFLO* | 0.133 | *GzRPS16* | 0.013 | *GzRPS16* | 0.33 | *GzRBP1* | 0.132 | *EF1A* | 0.196 |
| 3 | *GzFLO* | 0.29 | *UBC* | 0.138 | *GzRBP1* | 0.023 | *EF1A* | 0.38 | *EF1A* | 0.162 | *CYP1* | 0.219 |
| 4 | *GzSNC1* | 0.41 | *BTUB* | 0.156 | *EF1A* | 0.049 | *EF1B* | 0.42 | *GzC2H044* | 0.188 | *GzRPS16* | 0.230 |
| 5 | *GzRPS16* | 0.42 | *GzC2H044* | 0.168 | *GzUBH* | 0.070 | *CYP2* | 0.43 | *GzSNC1* | 0.36 | *GzRBP1* | 0.272 |
| 6 | *UBC* | 0.45 | *GzRBP1* | 0.182 | *GzC2H044* | 0.078 | *BTUB* | 0.45 | *CYP2* | 0.361 | *GzHSP70* | 0.281 |
| 7 | *BTUB* | 0.48 | *GzRPS16* | 0.195 | *BTUB* | 0.089 | *GzC2H044* | 0.47 | *EF1B* | 0.412 | *UBC* | 0.288 |
| 8 | *GzDGA* | 0.55 | *EF1A* | 0.205 | *CYP2* | 0.090 | *CYP1* | 0.50 | *GzHSP70* | 0.419 | *GzUBH* | 0.297 |
| 9 | *GzRBP1* | 0.61 | *GzSNC1* | 0.227 | *UBC* | 0.156 | *GzSNC1* | 0.77 | *GzUBH* | 0.443 | *BTUB* | 0.354 |
| 10 | *GzHSP70* | 0.72 | *GzDGA* | 0.24 | *GzSNC1* | 0.159 | *GzHSP70* | 0.77 | *UBC* | 0.456 | *GzSNC1* | 0.381 |
| 11 | | 0.86 | *GzHSP70* | 0.276 | *GzDGA* | 0.203 | *UBC* | 0.84 | *BTUB* | 0.491 | *GzDGA* | 0.437 |
| 12 | *EF1A* | 0.92 | *GAPDH* | 0.309 | *CYP1* | 0.255 | *GzUBH* | 0.85 | *GzDGA* | 0.55 | *EF1B* | 0.542 |
| 13 | *CYP1* | 0.98 | *CYP2* | 0.361 | *GAPDH* | 0.300 | *GzDGA* | 1.12 | *CYP1* | 0.664 | *GzFLO* | 0.561 |
| 14 | *EF1B* | 1.08 | *EF1B* | 0.412 | *GzHSP70* | 0.324 | *GzFLO* | 1.21 | *GzFLO* | 0.729 | *CYP2* | 0.594 |
| 15 | *GAPDH* | 1.32 | *CYP1* | 0.459 | *EF1B* | 0.385 | *GAPDH* | 1.33 | *GAPDH* | 0.781 | *GAPDH* | 0.616 |
| | | | *GzFLO* & *GzUBH*[a] (V2/3=0.047) | | *GzFLO* & *GzRPS16*[b] | 0.009 | | | *GzRBP1* & *GzRPS16*[a] (V2/3=0.074) | | *EF1A* & *GzUBH*[b] | 0.078 |

[a]Optimum pair of reference genes based on the average pairwise variation V.
[b]Optimum pair of reference genes.

*GzUBH*, and *GzRBP1*; the remaining genes (except *BTUB*) still expressed within a two-fold range (SD < 1.0) (Table 3). The variation in the expression of the genes under the perithecial induction stage on carrot agar was approximately two-fold higher than under mycelial growth conditions on carrot agar. The most stable gene under this condition was *GzRPS16* (SD=0.46), followed by *EF1A* and *GzFLO*. Four genes (*GzDGA*, *GzC2H044*, *GAPDH*, and *GzRBP1*) displayed SD values greater than 1.0 (Table 3). When assessed over the entire sexual development stage on carrot agar, *GzRPS16* was the most stable (SD=0.37), followed by *GzFLO* and *GzSNC1* (Table 3). Under trichothecene production conditions for 6 days, *GzRBP1* was the most stable (SD=0.32). *GzRPS16*, *EF1A*, and *GzRBP1* also showed SD values of less than 0.4 (Table 2). When all the conditions were combined, the most stable gene was *EF1A* (SD=0.64), although three other genes (*GzUBH*, *GzRBP1*, and *UBC*) had similar SD levels (Table 4).

**geNorm analysis:** This algorithm defines the reference gene stability measure (M value) by determining the pairwise variation (V) in a reference gene compared to all other reference genes, and enables selection of the optimal reference genes for normalization. Genes with the lowest M values have the most stable expression. Ranking of candidate reference genes and the optimum pair of reference genes under each condition are shown in Tables 2–4 and Table S10. Highly ranked genes displayed very high or high reference target stabilities (average M ≤ 0.2 and ≤ 0.5, respectively), which is typically seen when reference targets are stably expressed or when evaluating candidate reference targets in a homogeneous set of samples (Hellemans et al., 2007). In CM, all genes had high expression stability with low M-values (below the default limit of M=0.5) and the most stable gene was *GzUBH* (M=0.12), followed by *GzFLO* and *UBC*. A low V value of 0.047 between *GzFLO* and *GzUBH*, which is much lower than the common cut-off threshold of 0.15, indicated that these genes can be used as references for normalization (Table 2). During the sexual developmental process on carrot agar, *EF1A* and *GzRPS16*, and *CYP1* and *GzRPS16* were the most stable pairs over the 6-day vegetative growth and subsequent perithecial production stages, respectively. When the two stages were combined, *GzRPS16* and *EF1A* were the best combination of reference genes (Table 3). Under trichothecene production conditions, *GzRBP1* and *EF1A* were highly stable (Table 2). Only three genes (*UBC, GzHSP70*, and *GzUBH*) displayed M values lower than 0.5, all of which were identified as the best combination of reference genes under combined conditions (Table 4). For the combined data set, the pairwise variation in *UBC* and *GzHSP70* was higher than 0.15, so a third gene (*GzRPS16*) was added to

**Table 3.** Ranking of the candidate reference genes for normalization under the sexual development by three statistical algorithms

| Rank | carrot-vegetative | | | | | | carrot-perithecial | | | | | | combined on carrot | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BestKeeper | | geNorm | | NormFinder | | BestKeeper | | geNorm | | NormFinder | | BestKeeper | | geNorm | | NormFinder | |
| | Gene | SD [±Ct] | Gene | M value | Gene | Value | Gene | SD [±Ct] | Gene | M value | Gene | Value | Gene | SD [±Ct] | Gene | M value | Gene | Value |
| 1 | *EF1A* | 0.16 | *EF1A* | 0.118 | *GzSNC1* | 0.113 | *BTUB* | 0.40 | *GzRPS16* | 0.168 | *UBC* | 0.147 | *GzRPS16* | 0.37 | *GzRPS16* | 0.367 | *UBC* | 0.182 |
| 2 | *GzRPS16* | 0.20 | *GzRPS16* | 0.126 | *EF1A* | 0.149 | *GzRPS16* | 0.46 | *CYP1* | 0.197 | *GzUBH* | 0.202 | *GzFLO* | 0.44 | *EF1A* | 0.377 | *EF1A* | 0.256 |
| 3 | *EF1B* | 0.21 | *GzUBH* | 0.132 | *GzRPS16* | 0.182 | *EF1A* | 0.48 | *EF1A* | 0.213 | *EF1B* | 0.242 | *GzSNC1* | 0.49 | *UBC* | 0.385 | *GzUBH* | 0.288 |
| 4 | *GzSNC1* | 0.24 | *GzSNC1* | 0.179 | *UBC* | 0.206 | *GzFLO* | 0.49 | *UBC* | 0.311 | *EF1A* | 0.261 | *GzUBH* | 0.57 | *EF1B* | 0.427 | *GzRPS16* | 0.299 |
| 5 | *GzUBH* | 0.30 | *GzRBP1* | 0.212 | *GzRBP1* | 0.224 | *CYP1* | 0.55 | *EF1B* | 0.358 | *GzSNC1* | 0.265 | *EF1A* | 0.58 | *CYP2* | 0.443 | *GzSNC1* | 0.314 |
| 6 | *GzRBP1* | 0.30 | *CYP2* | 0.241 | *GzUBH* | 0.229 | *EF1B* | 0.64 | *GzDGA* | 0.367 | *CYP2* | 0.322 | *CYP1* | 0.59 | *GzUBH* | 0.479 | *GzDGA* | 0.374 |
| 7 | *GzC2H044* | 0.38 | *UBC* | 0.276 | *GzFLO* | 0.251 | *CYP2* | 0.64 | *CYP2* | 0.376 | *GAPDH* | 0.339 | *UBC* | 0.65 | *CYP1* | 0.508 | *CYP2* | 0.396 |
| 8 | *GzFLO* | 0.38 | *EF1B* | 0.3 | *EF1B* | 0.283 | *GzSNC1* | 0.75 | *GzUBH* | 0.415 | *GzRPS16* | 0.380 | *GzDGA* | 0.71 | *GzFLO* | 0.533 | *EF1B* | 0.397 |
| 9 | *CYP2* | 0.43 | *GzFLO* | 0.335 | *CYP2* | 0.294 | *GzHSP70* | 0.75 | *GzFLO* | 0.471 | *CYP1* | 0.388 | *GzHSP70* | 0.71 | *GzDGA* | 0.552 | *GzHSP70* | 0.420 |
| 10 | *GzDGA* | 0.50 | *GzDGA* | 0.367 | *GzDGA* | 0.310 | *UBC* | 0.76 | *GzSNC1* | 0.514 | *GzHSP70* | 0.400 | *CYP2* | 0.71 | *GzHSP70* | 0.58 | *GzFLO* | 0.429 |
| 11 | *UBC* | 0.52 | *GAPDH* | 0.409 | *GAPDH* | 0.398 | *GzUBH* | 0.83 | *GzHSP70* | 0.549 | *GzDGA* | 0.431 | *EF1B* | 0.76 | *GzSNC1* | 0.605 | *GzRBP1* | 0.436 |
| 12 | *GzHSP70* | 0.53 | *CYP1* | 0.439 | *GzC2H044* | 0.401 | *GzDGA* | 1.00 | *GAPDH* | 0.621 | *BTUB* | 0.528 | *GzRBP1* | 0.77 | *GzRBP1* | 0.649 | *CYP1* | 0.445 |
| 13 | *GAPDH* | 0.57 | *GzHSP70* | 0.47 | *GzHSP70* | 0.403 | *GzC2H044* | 1.03 | *BTUB* | 0.661 | *GzFLO* | 0.552 | *BTUB* | 0.80 | *GzC2H044* | 0.693 | *GAPDH* | 0.484 |
| 14 | *CYP1* | 0.66 | *GzC2H044* | 0.503 | *CYP1* | 0.466 | *GAPDH* | 1.04 | *GzRBP1* | 0.706 | *GzRBP1* | 0.566 | *GzC2H044* | 0.87 | *GAPDH* | 0.731 | *GzC2H044* | 0.496 |
| 15 | *BTUB* | 1.04 | *BTUB* | 0.668 | *BTUB* | 0.803 | *GzRBP1* | 1.26 | *GzC2H044* | 0.758 | *GzC2H044* | 0.594 | *GAPDH* | 1.07 | *BTUB* | 0.771 | *BTUB* | 0.745 |
| | | | *GzRPS16* & *EF1A*[a] (V2/3=0.035) | | *GzSNC1* & *EF1A*[b] | 0.072 | | | *CYP1* & *GzRPS16*[a] (V2/3=0.08) | | *UBC* & *GzUBH*[b] | 0.104 | | | *EF1A* & *GzRPS16*[a] (V2/3=0.08) | | *EF1A* & *GzUBH*[b] | 0.148 |

[a]Optimum pair of reference genes based on the average pairwise variation V.
[b]Optimum pair of reference genes.

**Table 4.** Ranking of the candidate reference genes for normalization across all conditions by three statistical algorithms

| Rank | BestKeeper | | geNorm | | NormFinder | |
|---|---|---|---|---|---|---|
| | *Gene* | SD[±Ct] | *Gene* | M value | *Gene* | Value |
| 1 | *EF1A* | 0.65 | *UBC* | 0.409 | *GzUBH* | 0.213 |
| 2 | *GzUBH* | 0.66 | *GzHSP70* | 0.445 | *EF1A* | 0.219 |
| 3 | *GzRBP1* | 0.66 | *GzUBH* | 0.467 | *CYP1* | 0.286 |
| 4 | *UBC* | 0.69 | *GzRBP1* | 0.557 | *GzRPS16* | 0.327 |
| 5 | *GzSNC1* | 0.70 | *EF1A* | 0.606 | *GzRBP1* | 0.335 |
| 6 | *GzRPS16* | 0.70 | *CYP1* | 0.67 | *GzHSP70* | 0.353 |
| 7 | *GzC2H044* | 0.75 | *GzDGA* | 0.704 | *UBC* | 0.419 |
| 8 | *GzHSP70* | 0.75 | *GzRPS16* | 0.726 | *GzDGA* | 0.424 |
| 9 | *BTUB* | 0.77 | *GzSNC1* | 0.745 | *EF1B* | 0.429 |
| 10 | *GzFLO* | 0.78 | *CYP2* | 0.773 | *GzC2H044* | 0.454 |
| 11 | *EF1B* | 0.80 | *EF1B* | 0.793 | *CYP2* | 0.492 |
| 12 | *CYP1* | 0.81 | *GzFLO* | 0.817 | *GzSNC1* | 0.494 |
| 13 | *CYP2* | 0.89 | *GzC2H044* | 0.843 | *GzFLO* | 0.495 |
| 14 | *GzDGA* | 0.90 | *GAPDH* | 0.932 | *BTUB* | 0.546 |
| 15 | *GAPDH* | 1.51 | *BTUB* | 1.023 | *GAPDH* | 0.936 |
| | | | *GzRPS16*, *GzHSP70*, & *UBC*[a] (V3/4=0.147) | | *GzUBH* & *EF1A*[b] | 0.106 |

[a]Optimum pair of reference genes based on the average pairwise variation V.
[b]Optimum pair of reference genes.

normalize gene expression (Table 4).

**NormFinder analysis.** Unlike the geNorm algorithm, NormFinder identifies the optimal reference genes among a set of candidate genes based on the stability of gene expression between the groups, resulting in an optimum pair of reference genes (Table S11). Under CM conditions, eight genes had expression stability values of less than 0.1. The most stable gene was *GzFLO*, followed by *GzRPS16* and *GzRBP1*. When two genes are used as references for normalization, *GzFLO* and *GzRPS16* would be the best combination because they had stability values (0.009) less than that of the single most stable gene (*GzFLO*, stability value of 0.011) (Table 2). During vegetative growth on carrot agar, four genes showed stability values less than 0.2. The three most stable genes were *GzSNC1*, *EF1A*, and *GzRPS16*; *GzSNC1* and *EF1A* were the best combination. During the perithecial induction stage, only *UBC* had a stability value of less than 0.2, followed by *GzUBH*; these genes achieved values of 0.104 (Table 3). For the combined data sets for sexual development, *UBC* was ranked as the most stably expressed gene (> 0.2), followed by *EF1A* and *GzUBH*, but the combination of second- and third-ranked genes (*EF1A* and *GzUBH*) achieved a lower stability value than that of *UBC* (Table 3). Under AG conditions, *GzC2H044* and *EF1A* were ranked as the best genes, but *EF1A* and the eight-ranked *GzUBH* gene (SV=0.297) were identified as the optimum reference pair (Table 2). When all conditions were combined, the reference gene rank was: *GzUBH* > *EF1A* > *CYP1* (Table 4).

**Identification of best candidate reference genes by combining three programs.** The output data from geNorm and NormFinder can be easily compared because they both use the same input data (linear expression values), whereas that of BestKeeper may be different as it uses Ct values. Nevertheless, it was possible to select the best reference genes from the top three most stable genes identified by each analysis. In CM, *GzFLO* was always identified as one of the best reference genes (third in BestKeeper, second in geNorm, and first in NormFinder). As an alternative, *GzUBH* ranked second in BestKeeper, first in geNorm, and fourth in NormFinder. Similarly, both *EF1A* and *GzRPS16* were stably expressed during both vegetative and perithecial induction conditions on carrot agar. These two genes were also identified as the best reference genes under the AG condition. When all conditions were combined, *GzUBH* (second in BestKeeper, third in geNorm, and first in NormFinder), and *EF1A* (first in BestKeeper, fifth in geNorm, and second in NormFinder) were very stable. The primers for *GzUBH*, *GzRPS16*, and *GzFLO* have been submitted to the RTPrimerDB (with ID 8322, 8323, and 8324, respectively). GAPDH was among the least stably expressed genes under most conditions.

## Discussion

This study evaluated numerous culture conditions, candidate genes, and algorithms to select the best reference genes for qRT-PCR, of which little is known in filamentous fungi. The conditions tested here can be divided into different categories based on fungal developmental or physiological status. The conditions in CM and AG, and on carrot agar favor only mycelial growth in *F. graminearum*; none of the other developmental processes such as asexual sporulation, sexual fruiting body formation, and sexual sporulation could be stimulated. However, the physiological or metabolic status is diverse among these conditions. In CM, metabolic pathways associated with high nutrient use are activated, whereas on carrot agar those for adapting to nitrogen starvation are activated, which is considered a prerequisite for switching to the sexual reproductive mode. The AG liquid culture specifically stimulates pathways producing several secondary metabolites, such as trichothecenes (Gardiner et al., 2009) In contrast, perithecial formation on carrot agar is heterogeneous in terms of fungal development. Even though this represents a typical sexual development process in *F. graminearum*, it also includes asexual spores as well as ground mycelia. In addition to the culture conditions, we assessed the time-course variation in gene expression by monitoring two or three time points under each condition. Because these diverse conditions represent primary developmental and/or physiological stages in *F. graminearum,* this increases the reliability of the reference genes selected in this study.

The candidate reference genes evaluated here are more diverse in terms of number and class than those in previous reports. Seven housekeeping genes, which have been commonly used as endogenous controls in various organisms, were assessed simultaneously under the various conditions using three algorithms. In most *F. graminearum* studies, only single housekeeping gene, such as actin, *BTUB*, *EF1A*, *EF1B*, or 18S rRNA, is used as an internal control without in-depth evaluation (Chen et al., 2011; Gardiner et al., 2009; Lee et al., 2009; Liu et al., 2011; Pandolfi et al., 2010). Lysøe et al. (Lysøe et al., 2009) evaluated three housekeeping genes (*BTUB*, *EF1A*, and *UBC*) using BestKeeper, but did not provide detailed data.

The results of the present study question the reliability of housekeeping genes as internal controls in qRT-PCR analysis. We found that *GAPDH* is an unsuitable reference gene, as previously demonstrated in animals (Barber et al., 2005; Olsvik et al., 2005). In addition, other housekeeping genes such as *EF1B*, *CYP1*, and *CYP2* were ranked among the least stable genes under certain growth conditions. Only two housekeeping genes (*EF1A* and *UBC*) displayed relatively stable expression patterns under all conditions.

Among the eight new candidate genes, three had expression stabilities similar to the housekeeping genes. Interestingly, two of the three genes exhibited the features similar to commonly used housekeeping genes, supporting the reliability of the procedure for selecting new reference genes using RNA-seq data. *GzUBH*, annotated as a hypothetical protein carrying an ubiquitin carboxyl-terminal hydrolase domain, may be involved in deubiquitination (the process of removing ubiquitin molecules from polyubiquitinated peptides) in *F. graminearum*. Because the *UBC* gene encoding ubiquitin-conjugating enzyme is frequently used as reference gene in various organisms including *F. graminearum*, it is possible that other genes, such as *GzUBH*, which is involved in the ubiquitin-mediated protein degradation pathway can be stably expressed under several conditions in *F. graminearum* and other organisms. *GzRPS16* has been annotated as a mitochondrial ribosomal protein S16 that characterized as a human housekeeping gene (Eisenberg and Levanon, 2003). In addition, ribosomal protein S4 has been used as a reference gene for qRT-PCR in a mycorrhizal fungus (Govindarajulu et al., 2005). *RPS3* and *RPS18* were the most stable in a fungus-infected red flour beetle (Lord et al., 2010). In contrast to these genes, *GzFLO*, annotated as a hypothetical protein similar to the putative cell surface flocculin, would be a good reference gene specific to only the CM condition because it was not stable under the other conditions.

The present study suggests that these genes can function as reference genes in other filamentous fungi as well as in *F. graminearum*. In addition, these genes can be applied to other conditions not examined in this study because the three conditions tested here represent the primary physiological processes in *F. graminearum*. However, evaluation of these genes *in planta* is required.

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## Supplementary data

Supplementary data (Tables S1-11) associated with this article can be found.

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