

REFERENCE GENE SELECTION IN *ASPERGILLUS*

INVESTIGATION OF CANDIDATE REFERENCE GENES FOR REVERSE-TRANSCRIPTION  
QUANTITATIVE POLYMERASE CHAIN REACTION OF *ASPERGILLUS*

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## **Lay Abstract**

*Aspergillus* is a globally distributed genus of fungi, with some species threatening opportunistic human infection. To combat infection with the opportunistic species, *Aspergillus fumigatus*, antifungal drugs including: itraconazole, voriconazole and amphotericin B are used. Recent years have seen a rise in antifungal resistance in *A. fumigatus*. To understand this and other mechanisms in *Aspergillus*, changes in gene expression must be examined. My thesis aimed to determine how reference genes are selected for reverse-transcription quantitative polymerase chain reaction, a method applied to measure gene expression changes in *Aspergillus*. It was discovered that very few studies between the years 2001 and 2020 experimentally validated that the reference genes used were stably expressed, with only 17 out of 90 studies providing validation. In part two of my thesis, genes overexpressed in *A. fumigatus* when exposed to antifungal drugs, from formerly published articles, were summarized to better understand the role of gene expression in antifungal drug resistance.

## **Abstract**

The genus *Aspergillus* possesses broad functionality and occupation of ecological niches. Underpinning this are changes in the transcriptome of these species. Transcriptional changes are clinically relevant with respect to understanding triazole resistant isolates of *Aspergillus fumigatus*. Reverse-transcription quantitative Polymerase Chain Reaction (RT-qPCR) is a highly specific means of measuring changes in gene expression. The most common method of which requires normalization to experimentally validated, stably expressed reference genes. Ideal reference genes are unaffected by differences in the experimental conditions or strains/isolates and are expressed at levels near the target gene(s). The first study reviewed current practices for reference gene selection and validation for RT-qPCR gene expression analysis of the genus, *Aspergillus*. Information on the species examined, experimental conditions, sample type, normalization strategy, reference gene(s) and their state of validation was obtained from 90 primary studies. Twenty reference genes were used, with the most popular reference genes used encoding beta-tubulin, actin, 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase. Seventeen of the 90 studies experimentally validated the expression stability of the reference genes used, out of which eight used more than one reference gene. The results of three studies conflicted with others described in the literature, with no experimental validation of the reference genes available to aid in interpreting the conflicting findings. In the Genome-Wide Association Study, genes noted to increase in expression in response to itraconazole and/or voriconazole treatment of *A. fumigatus* were extracted from published RNA-sequencing or RT-qPCR studies. Ten ATP-binding cassette transporters, four major facilitator superfamily transporters and 16 transcription factors were identified. Collectively, the findings of this thesis show a large disparity in experimentally validated reference genes as well as future targets of gene expression analysis in triazole resistant isolates of *A. fumigatus*.

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Thank you to my supervisor, Dr. Jianping Xu, for allowing me to transfer into his lab to work on this project and for supporting me during my M.Sc. work. I would also like to thank him for encouraging me to get involved in paper publications, conference presentations and to apply for scholarships early in my research career. It is with Dr. Xu's guidance that I have been able to accomplish as much as I have during the last year of the Covid-19 pandemic and I am very grateful to him for his help. Thank you as well to my supervisory committee members, Dr. Marie Elliot and Dr. Ben Evans, for providing feedback on my experimental design throughout the course of my project. I would also like to thank the members of the Xu Lab for welcoming me into their group, just before the Covid-19 pandemic hit. Thank you as well to my roommate, Rhys, for their constant support, coffee presents and musical movie night breaks. Finally, I would like to thank my parents, both McMaster University Biology alumni, for being my inspiration for going into Biology at McMaster University. In my B.Sc. thesis, I thanked my father for being my scientific role model and I attribute my interest in learning more about bioinformatics to him and his love of computer science. However, in my M.Sc. thesis I would also like to thank my mother because she has been my number one cheerleader from day one and I would not be the person that I am today without her. I am forever grateful for the support and for my time in the Department of Biology. Thank you to everyone who has made my time at McMaster University special.

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## List of Abbreviations

<b>AMM</b>	<i>Aspergillus</i> minimal medium
<b>ABC</b>	ATP-binding cassette
<b>AmB</b>	Amphotericin B
<b>CAP</b>	Citric acid production
<b>CD</b>	Czapek–Dox
<b>cDNA</b>	Complementary DNA
<b>CM</b>	Complete medium
<b>Ct or Cq</b>	Cycle threshold
<b>CYA</b>	Czapek-Dox modified yeast agar
<b>GAPDH</b>	Glyceraldehyde-3-phosphate dehydrogenase
<b>GMM</b>	Glucose minimal medium
<b>GMPY</b>	Glucose maltose polypeptone yeast extract
<b>GWAS</b>	Genome-wide association study
<b>IA</b>	Invasive aspergillosis
<b>ISP2</b>	International Streptomyces project-2
<b>MEA</b>	Malt extract agar
<b>MEB</b>	Malt extract broth
<b>MEM</b>	Minimal essential medium
<b>MFS</b>	Major facilitator superfamily
<b>MIQE</b>	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
<b>MM</b>	Minimal medium

<b>MYA</b>	Malt yeast agar
<b>OTA</b>	Ochratoxin A
<b>PBS</b>	Phosphate-buffered saline
<b>PDA</b>	Potato dextrose agar
<b>PDB</b>	Potato dextrose broth
<b>PEG</b>	Polyethylene glycol
<b>PVDF</b>	Polyvinylidene fluoride
<b>RPMI</b>	Roswell Park Memorial Institute
<b>RNA-seq</b>	RNA-sequencing
<b>RT-qPCR</b>	Reverse-transcription quantitative Polymerase Chain Reaction
<b>SMKY</b>	Sucrose magnesium sulphate potassium nitrate yeast
<b>SNP</b>	Single-nucleotide polymorphism
<b>YAG</b>	Yeast extract-agar-glucose
<b>YE</b>	Yeast extract
<b>YEM</b>	Yeast extract medium
<b>YEP</b>	Yeast extract peptone
<b>YEPD</b>	Yeast extract-peptone-dextrose
<b>YES</b>	Yeast extract sucrose
<b>YESP</b>	Yeast extract sucrose peptone
<b>YESP-N</b>	YESP medium modified with 1% sodium nitrate
<b>YESP-T</b>	YESP medium modified with 1% tryptophan
<b>YG</b>	Yeast glucose
<b>YPD</b>	Yeast peptone dextrose

**YUU**

YAG supplemented with 5 mM uridine and 10 mM uracil

## **Declaration of Academic Achievement**

I am the first author of the paper included as Chapter 2, “Current Practices for Reference Gene Selection in RT-qPCR of *Aspergillus*: Outlook and Recommendations for the Future”. For this paper I conducted the literature search, extracted, summarized, and generated graphics to visualize the information, wrote the manuscript, and assisted in editing the final draft. I am the fourth author of the excerpt from the paper included as Chapter 3, “Genome-Wide Association Analysis for Triazole Resistance in *Aspergillus fumigatus*”. As, the fourth author, I have only included my contributions to the “Results” section of the paper in this thesis as section 3.3. “Results”. For this section, I extracted and summarized the genes known to be overexpressed during triazole exposure in *Aspergillus fumigatus*, which were later compared to the single-nucleotide polymorphisms in triazole-resistant *A. fumigatus*, by my colleagues and co-first authors of the article: YuYing Fan and Yue Wang. Since my co-author, Greg Kofanty was the author of the “Introduction” for our paper, I have written a separate one for inclusion in my thesis, as section, 3.2. “Introduction”. As the information in Chapter 3 is my contribution to my paper, with a unique “Introduction”, “Discussion”, and “References” section written by myself, I believe that it’s inclusion as part of my M.Sc. thesis is justified.

## **Impact of Covid-19 on Research Plan**

The first Covid-19 lockdown occurred shortly after my transfer from my previous lab to the Xu lab and this greatly limited my interactions with my peers as well as my ability to familiarize myself with the lab. Fortunately, my supervisor Dr. Xu suggested that I began my M.Sc. work with a literature review examining the way that reference genes were currently selected and validated for reverse-transcription quantitative Polymerase Chain Reaction gene expression analysis for the genus of my research organism, *Aspergillus*. This review paper was published on June 24, 2021, and is my biggest contribution to my research project, and proudest achievement as a graduate student. While I was writing this paper, it was very challenging for me to go into the lab, as we had a large lab and we could only go in for three-person shifts, it was difficult to be trained on new protocols. Additionally, due to the mandate from McMaster University and the Government of Ontario, stating that all individuals capable of working from home should work from home, during the time that writing was my primary focus, I did not go to the lab. This impacted my time in the lab significantly. For this reason, my final project includes my work from my review paper and as the fourth author on our Genome-wide Association Study, which was published on June 4, 2021.

## Chapter 1

### General Introduction

#### 1.1. The Genus *Aspergillus* and it's Diverse Implications

*Aspergillus* is a genus of saprophytic, filamentous fungi with more than 300 species capable of living in many different ecological niches around the world (Bennett, 2010). These species possess unique functionality, with some posing a threat to human health as opportunistic human fungal pathogens, including *Aspergillus fumigatus*, *Aspergillus terreus* and *Aspergillus flavus* (Guarro, Xavier, & Severo, 2009), and others aiding the food industry through the production of soy sauce, sake, such as through *Aspergillus oryzae*, and citric acid, such as through *Aspergillus niger* (F. J. Jin, Hu, Wang, & Jin, 2021; Yu, Liu, Wang, Wang, & Zhang, 2021). Interestingly, *A. fumigatus*, the primary causative of invasive aspergillosis (IA) in humans, can also cause infection in dogs, cats, honeybees, horses, birds, and monkeys (Seyedmousavi et al., 2015). In addition, many species of *Aspergillus* are also important to agriculture, with some such as *A. niger*, possessing potential biocontrol properties (N. Jin et al., 2019), and others, including *Aspergillus flavus* and *Aspergillus parasiticus*, posing mycotoxin crop contamination risk (Caceres et al., 2020). An essential component to understanding the mechanisms underlying the diverse capabilities of these species and their capacity to adapt to environmental conditions, is understanding their corresponding gene expression dynamics.

#### 1.2. Reverse-transcription Quantitative Polymerase Chain Reaction and Selection of Candidate Reference Genes

Reverse-transcription quantitative Polymerase Chain Reaction (RT-qPCR) is a cost-effective and simple way to examine gene expression (Huggett, Dheda, Bustin, & Zumla, 2005), and can be



performed in one or two steps (Adams, 2020). If completed in two steps, the first step is reverse-transcription, during which the enzyme, reverse transcriptase, converts the mRNA from the sample into complementary DNA (cDNA) (Adams, 2020). In the second step, the cDNA is amplified through PCR to produce more of the target gene (Adams, 2020). The more cDNA that is amplified, the greater the amount of fluorescence that is produced by the associated fluorescent probe or intercalating dye (Adams, 2020). The point at which the fluorescence crosses the fluorescence threshold (ie. is significantly greater than the background fluorescence at baseline) is called the cycle threshold (Ct) value and this is used as the proxy for gene expression. The more product, the earlier the threshold is crossed and the lower the Ct value, indicating higher the expression (Adams, 2020). Some of the information described below overlaps with the section 2.2. of Chapter 2, “Introduction”.

The standard method for measuring changes in gene expression using RT-qPCR is the relative quantification method, which requires the use of stably expressed reference genes to normalize the expression of the target gene (Adams, 2020). A stably expressed reference gene is unaffected by the experimental conditions used in the study or by the strains/biological material used. As noted in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE), the traditional method for comparing the expression of the target gene to that of the reference gene between samples, is through using one reference gene for comparison in the  $\Delta\Delta C_t$  calculation (Bustin et al., 2009). However, it is recommended that multiple reference genes be used to normalize RT-qPCR expression data (Bustin et al., 2009), because it helps mitigate the effects of reference genes being at different abundances (Vandesompele et al., 2002). Therefore, alternative methods for calculating relative changes in gene expression using multiple reference genes, such as the qBase approach (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007) and BestKeeper (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004), have been developed. To aid in the selection of multiple

stably expressed reference genes, several software tools have been developed (Andersen, Jensen, & Orntoft, 2004; Pfaffl et al., 2004; Vandesompele et al., 2002; Xie, Xiao, Chen, Xu, & Zhang, 2012).

Table 1.1 below summarizes the function of four reference gene evaluation algorithms, geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004) and RefFinder (Xie et al., 2012), and describes their metric for evaluating reference gene stability and indication of greater reference gene stability.

**Table 1.1.** Summary of reference gene ranking algorithms and their metric for stability ranking.

<b>Algorithm</b>				
	<b>geNorm</b>	<b>NormFinder</b>	<b>BestKeeper</b>	<b>RefFinder</b>
<b>Summary of Algorithm</b>	Ranks the stability of all reference genes and provides a recommendation for the number of reference genes to use.	Calculates the associated <i>stability value</i> for all candidate reference genes.	Computes the inter-gene variation and compares the associated correlation coefficients to the <i>BestKeeper index</i> .	Combines the output from the three previous programs and the $2^{-\Delta\Delta CT}$ calculates a final stability rank.
<b>Metric for Stability Rank</b>	<i>M</i> – the average pairwise variation of the reference gene compared to all other candidate reference genes.	<b>Stability value</b> – the combined estimates of the intra- and inter-group variation for each gene	<b>BestKeeper Index</b> – the geometric mean of all the highly correlated reference gene’s Ct values	<b>Final Ranking</b> – Assigns a weight to each reference and calculates the final ranking based on the geometric mean of these weights.
<b>Indicator of Greater Stability</b>	Lower <i>M</i> value	Lower <i>stability value</i>	Lower standard deviation in the Ct value of the <i>BestKeeper index</i>	Lower geometric mean weights
<b>Ref.</b>	(Vandesompele et al., 2002)	(Andersen et al., 2004)	(Pfaffl et al., 2004)	(Xie et al., 2012)

In the past, many studies have used genes that are classically considered to be “housekeeping” genes, named such because they are involved in important metabolic or physiological processes. However, the instability of “housekeeping” gene stability has been documented since the early 2000s (Vandesompele et al., 2002). For instance, Vandesompele et al. demonstrated that the expression of *actB*, encoding beta-actin, varies in expression in human heart compared to fibroblast tissue (Vandesompele et al., 2002). The researchers note that this is likely because the heart is mostly made of muscle, whereas *actB* is a non-muscular cytoskeletal actin (Vandesompele et al., 2002). This further highlights the necessity of experimental validation of reference gene expression stability, at the onset of the study.

### **1.3. Triazole-Resistance in *Aspergillus fumigatus***

Please note that some of the information presented below overlaps with section 3.2., “Introduction”, of Chapter 3. As mentioned in section 1.1. above, several species of *Aspergillus* can cause infection in humans. In the most severe form of infection, IA (Jenks & Hoenigl, 2020), it is recommended that the patient is treated first with the triazole, voriconazole, followed by the polyene, amphotericin B, should treatment be unsuccessful (Misch & Safdar, 2016). Triazole drugs are recommended first because they have positive patient outcomes and low host toxicity (Misch & Safdar, 2016). These drugs, including voriconazole and itraconazole, inhibit ergosterol biosynthesis through the binding of their fourth nitrogen atom to the ferric iron moiety in the active site of lanosterol 14 $\alpha$ -demethylase, also called Cyp51A, an important enzyme in the ergosterol biosynthesis pathway of *Aspergillus* and other molds (Cowen, Sanglard, Howard, Rogers, & Perlin, 2014; Rybak, Fortwendel, & Rogers, 2019). Ergosterol is an integral part of fungal membranes and contributes to membrane fluidity and helps regulate the cell cycle (Alcazar-Fuoli & Mellado, 2012). When the active site of Cyp51A is blocked,

the methyl group of lanosterol can no longer be removed by Cyp51A, which prevents the production of ergosterol precursors and leads to accumulation of toxic methylated 14 $\alpha$ -lanosterol and eventually cell death (Rybak et al., 2019). In contrast, amphotericin B binds directly to ergosterol once it has been integrated into the fungal membrane, and this binding is theorized to disrupt the membrane, either due to the formation of pores, adsorption of ergosterol to the phospholipid membrane, sequestering of ergosterol or oxidative damage (Carolus, Pierson, Lagrou, & Van Dijck, 2020). At the present, there is no consensus as to which of the four models correctly outlines the mode of action for amphotericin B.

In addition to their clinical applications, azole antifungals are also applied extensively in agriculture and are estimated to persist in the environment for months (Chen et al., 2013) to years (Kahle, Buerge, Hauser, Muller, & Poiger, 2008). *A. fumigatus* resistance to itraconazole was first reported in 1997 (Denning et al., 1997). Due to their high combined uses, *A. fumigatus* resistance to triazole drugs is on the rise (Ashu et al., 2018; Chowdhary, Kathuria, Xu, & Meis, 2013). Interestingly, *A. fumigatus* isolates that are resistant to five agriculturally applied azoles also exhibit cross-resistance to the medical azoles, itraconazole and voriconazole (Snelders et al., 2012). Structural similarities between agricultural and medical azoles are likely the reason for the observed cross-resistance (Snelders et al., 2012). Mutations in *cyp51A*, the gene encoding the azole target, Cyp51A, are commonly associated with azole resistance, and may be acquired during patient treatment (Howard et al., 2009) or from the environment (Snelders et al., 2008). However, there is mounting evidence that alternative mechanisms of non-*cyp51A* mutations contribute to triazole resistance in *A. fumigatus* (Sharma, Nelson-Sathi, Singh, Radhakrishna Pillai, & Chowdhary, 2019). To date, little is known about the mechanism(s) behind amphotericin B resistance in *A. fumigatus*. Altogether, understanding

how gene expression is altered in the presence of triazole drugs and how differential expression may influence resistance to triazoles outside of *cyp51A* mutations warrants further study.

#### **1.4. Objectives and Hypothesis**

The objectives of this thesis project are to: (1) determine what reference genes are used for RT-qPCR of *Aspergillus* species and how they are selected, (2) determine the frequency of experimental validation of reference gene stability and (3) identify candidate reference genes for RT-qPCR of *A. fumigatus*. The hypothesis is that the expression stability of candidate reference genes is dependent on the experimental conditions used.

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## **Chapter 2**

### **Current Practices for Reference Gene Selection and Validation in RT-qPCR of *Aspergillus*: Outlook and Recommendations for the Future**

#### **2.1. Preface**

This literature review was published in the journal “Genes” on June 24, 2021. The authors of this paper are Meagan Archer and Dr. Jianping Xu. I am the first author on this publication. I conducted the literature search using PubMed, and manually curated the 575 publications returned by the query to extract 90 primary articles and one reference gene validation study for the literature review. I also

conducted the second complementary literature search through Google Scholar. I wrote the manuscript and prepared all in-text and supplementary tables and figures. Dr. Jianping Xu conceptualized and outlined the review paper, supervised the work, and edited the manuscript. Please note that minor formatting changes to the published work have been made to conform with the thesis submission requirements established by the School of Graduate Studies at McMaster University, and to consider thesis committee member recommendations.

The citation for this review article is provided here: Archer, M., & Xu, J. (2021). Current Practices for Reference Gene Selection in RT-qPCR of *Aspergillus*: Outlook and Recommendations for the Future. *Genes (Basel)*, 12(7). doi:10.3390/genes12070960.

The journal link to the supplementary files for Chapter 2 can be accessed and downloaded using the following link: “<https://www.mdpi.com/2073-4425/12/7/960>”.

## **2.2. Abstract**

*Aspergillus* is a genus of filamentous fungi with vast geographic and ecological distributions. Species within this genus are clinically, agriculturally and biotechnologically relevant, leading to increasing interest in elucidating gene expression dynamics of key metabolic and physiological processes.

Reverse-transcription quantitative Polymerase Chain Reaction (RT-qPCR) is a sensitive and specific method of quantifying gene expression. A crucial step for comparing RT-qPCR results between strains and experimental conditions is normalisation to experimentally validated reference gene(s). In this review, we provide a critical analysis of current reference gene selection and validation practices for RT-qPCR gene expression analyses of *Aspergillus*. Of 90 primary research articles obtained through our PubMed query, 17 experimentally validated the reference gene(s) used. Twenty reference genes were used across the 90 studies, with those encoding beta-tubulin being the most used reference

gene, followed by actin, 18S rRNA and glyceraldehyde 3-phosphate dehydrogenase encoding genes. Sixteen of the 90 studies used multiple reference genes for normalisation. Failing to experimentally validate the stability of reference genes can lead to conflicting results, as was the case for four studies. Overall, our review highlights the need to experimentally validate reference genes in RT-qPCR studies of *Aspergillus*.

### **2.3. Introduction**

The ascomycete genus *Aspergillus* is among the first described filamentous fungi, dating back to 1729, as recorded by Pier Antonio Micheli, an Italian biologist and priest. Under a microscope, the asexual spore-forming structure of these fungi looks like an aspergillum, a “holy water sprinkler”, and Micheli named these fungi in the genus *Aspergillus* [1,2]. Since then, over 300 species have been described and recognised in this genus [3]. These species differ in their morphological, physiological, and phylogenetic characteristics. *Aspergillus* fungi are broadly distributed across the globe and are found in diverse ecological niches such as soil, composts, water, buildings, air, and in or on plants [4]. Species in this genus have significant impacts on many fields, including biotechnology (e.g., antibiotics production) [5], fermented food production [6], food safety (e.g., mycotoxin production and food contamination) [7] and human health [2]. In addition, several *Aspergillus* species have been model organisms for understanding the fundamental biology, including physiology and genetics, of fungi and eukaryotes [8,9].

An emerging theme on the studies of these organisms is the regulations of gene expression and metabolic pathways, and how regulated expression is related to their beneficial and detrimental effects to human welfare. For example, several *Aspergillus* species are employed frequently in the food and beverage industry as fermenters of soy, to make sake, miso and soy sauce [6]. Understanding how the

genes are involved in producing these products could help develop strategies to control their expression for optimal commercial production. On the other hand, several other *Aspergillus* species are opportunistic human fungal pathogens (HFPs), including *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus nidulans* [10,11]. Globally, each year there are approximately 4.8 million cases of allergic bronchopulmonary aspergillosis (including asthma), 3 million cases of chronic pulmonary aspergillosis and 250,000 cases of IA [12]. The dominant cause of aspergillosis in humans is *A. fumigatus* [10]. Furthermore, *A. flavus* can not only cause human infections, but along with *Aspergillus parasiticus*, can also produce aflatoxins that contaminate foods and severely impact human health, with long-term exposure leading to infertility and endocrine disorders [7].

Among human hosts infected with pathogenic *Aspergillus*, effective treatment often requires antifungal drugs such as itraconazole, voriconazole and amphotericin B [13]. Currently, voriconazole is the recommended first-line of treatment and prophylactic agent against IA [14]. However, drug-resistant strains are increasing in both environmental and clinical populations of *A. fumigatus* and other opportunistic species [2]. Infections by drug-resistant fungal pathogens are associated with elevated length of hospitalisation and higher mortality [15]. Understanding the mechanism(s) of drug resistance in *Aspergillus* pathogens, including *A. fumigatus*, could help with monitoring infection progression and resistance, and improve treatment options. In addition, there is increasing evidence that secreted enzymes in *A. fumigatus* play an important role in pathogen colonisation and host tissue damage. Intriguingly, strain Z5 of *A. fumigatus* has multiple xylanases [16], which can break down xylan into its constituents of xylose, arabinose and glucuronic acid, all of which can then be used in the production of biofuels [17]. Better control of the specific pathways involved in producing these beneficial enzymes could generate significant economic benefits. Over the years, several approaches

and techniques have been developed to monitor and quantify gene expression. These techniques include northern blotting, microarray hybridisation, high throughput transcriptome sequencing and reverse-transcription quantitative polymerase chain reaction (RT-qPCR). RT-qPCR quantifies the amount of mRNA in a biological sample and takes this as a measurement of gene expression [18,19]. RT-qPCR possesses several advantages over other methods: it is quick, capable of high-throughput processing, and is highly sensitive and specific [18,19]. Additionally, RT-qPCR is useful for detecting low-abundance transcripts, as the high annealing temperature used during RT-qPCR allows for highly specific primer binding to the target gene [20]. Indeed, RT-qPCR is often used to confirm the results obtained using other approaches [21–32]. However, to accurately quantify gene expression using RT-qPCR, the normalisation of mRNA levels to validated reference genes is required [18]. Through normalisation using appropriate reference genes, the impact of differences in RNA yield (due to variation in extraction), cDNA yield (due to variation in reverse-transcription and RNA template quality) and amplification efficiency on gene expression levels can be minimised [19]. Thus, by controlling for these differences, normalisation allows for the comparison of mRNA levels across different experimental treatments [19].

A good reference gene is one that is stably expressed under the experimental conditions being tested [19]. The Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines recommend using two or more validated reference genes to normalise gene expression data [19]. Using the geometric mean of multiple reference genes achieves high accuracy of normalisation, and is recommended over the arithmetic mean, as it better controls for differences in the amount of mRNA and outliers between genes [33]. When using multiple reference genes, it is also important to select genes that are not co-regulated, as co-regulated genes may lead to false positives as they lead to stable expression ratios [33]. Overall, inappropriate and/or insufficient reference genes can lead to the

wrong interpretation of results, and reduce the reliability of experimental data [18,19]. Therefore, careful consideration should go into the selection of reference genes for RT-qPCR analysis.

In this review, we describe the reference genes that have been used for normalising gene expression in RT-qPCR analyses for species in the genus *Aspergillus*. We review how the reference genes were selected in these studies, and whether they were validated under the specific experimental conditions.

In addition, we briefly summarise how these reference genes were used in RT-qPCR to help understand the important biological processes in these fungi. Towards the end, we discuss the potential areas of improvements for selecting robust reference genes.

## **2.4 Reference Genes for Gene Expression Analyses of *Aspergillus***

To assess the state of reference genes in RT-qPCR studies of *Aspergillus*, in this review, we searched PubMed using the search query: “*Aspergillus* qPCR”. The loose search criteria were used to ensure that we cast a wide net for any relevant studies available for our review. In total, this query returned 575 results which were manually curated to exclude those used to quantify fungal load, or those that did not specifically examine gene expression in *Aspergillus*, but that mentioned members of the genus in the abstract and with RT-qPCR studies in organisms other than *Aspergillus*. This allowed us to obtain information from 90 primary studies (Table 2.1., Table S2.1.)

[16,21,22,23,24,25,26,27,28,29,30,31,32,34,35,36,37,38,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53,54,55,56,57,58,59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74,75,76,77,78,79,80,81,82,83,84,85,86,87,88,89,90,91,92,93,94,95,96,97,98,99,100,101,102,103,104,105,106,107,108,109,110] and one reference gene validation study [111], from 2001 to 2020. For each of these 90 studies, we manually extracted information on the species, genes, experimental conditions, purpose of the study and analytical methods used.

**Table 2.1.** Species, the encoded reference gene(s) product(s) and experimental conditions used in 90 RT-qPCR studies of *Aspergillus*.

Species	Reference		Experimental Conditions	Ref.
	Gene-Encoded Product	Validation (Y/N)		
<i>A. aculeatus</i>	Actin	N	Minimal medium (MM) for 3, 5 and 7 days	[34]
	Histone 3			
	GAPDH	Y	MM with 1% (w/v) polypeptone, 1% (w/v) glucose, 1% (w/v) avicel, 1% (w/v) xylose, or 1% (w/v) arabinose for 3 h or 6 h	[35]
	18S rRNA	N	Czapek-Dox Modified Yeast Agar (CYA) medium in the dark at 30 °C for 2 days	[36]
<i>A. carbonarius</i>	Calmodulin	N	Co-culture with the actinobacterial strain, SN7, on International Streptomyces Project-2 (ISP2) medium at 28 °C for 4 days	[37]
	Ubiquitin-conjugating Enzyme	Y	MM at 25 °C, without shaking (ochratoxin A (OTA)-inducing conditions) for 4, 6 and 8 days in the dark	[32]
<i>A. cristatus</i>	Actin	N	Cellulose membrane on malt yeast agar (MYA) and on 17% NaCl MYA media for 5 days at 28 °C in the dark, after which mycelia were fixed (extraction from 7-day mycelia)	[24]
<i>A. fischeri</i>	Beta-tubulin	Y	Growth on a hydrophobic polyvinylidene fluoride (PVDF) membrane on top of oatmeal agar for 3, 6 or 30 days (wildtype only)	[38]
	Histone 3			
<i>A. flavus</i>	18S rRNA	N	Glucose minimal medium (GMM) containing 1% starch or 24 g/400 mL ground corn seed at 30 °C for 24, 48 and 72 h with shaking at 250 rpm	[39]
	18S rRNA	N	Yeast extract sucrose (YES) medium at 37 °C for 1.5 days and at 28 °C for 3 days, in the dark	[28]

18S rRNA	N	YES medium supplemented with 0.40 mmol/L of cinnamaldehyde, 0.56 mmol/L of citral, and 0.80 mmol/L of eugenol for 7 days	[40]
18S rRNA			
Beta-tubulin	Y	Co-culture with <i>Listeria monocytogenes</i> in malt extract broth (MEB) at 25 °C and 30 °C for 7 days	[41]
Calmodulin			
Actin	N	Co-incubation with soil isolates of <i>Streptomyces</i> on ISP2 medium at 28 °C	[42]
Beta-tubulin			
Actin	N	(1) Potato dextrose agar (PDA) or GMM supplemented with NH <sub>4</sub> <sup>+</sup> for 48 h (2) GMM supplemented with 50 mM ammonium or NaNO <sub>2</sub> for 30 h	[43]
Actin	N	YES medium containing 1.5 mg/100 mL silver nanoparticles at 28 °C for 14 days	[44]
Beta-tubulin			
Actin	N	YES or yeast peptone dextrose (YPD) medium for 5 and 7 days, respectively, at 37 °C in the dark	[45]
Beta-tubulin	Y	Inoculated onto 25 g of wheat and grown at 30 °C in open petri dishes with wetted filter paper for 9 days	[46]
Beta-tubulin	N	Peanut samples for 6 weeks at 25 °C in polyethylene sandwich boxes containing glycerol/water solutions to maintain the equilibrium relative humidity conditions	[47]
Beta-tubulin	N	YES or yeast extract peptone (YEP) medium at 28 °C for 4 days	[48]
*			
Beta-tubulin	N	Sucrose magnesium sulphate potassium nitrate yeast (SMKY) liquid medium 25 °C for 7 days	[49]
Beta-tubulin	N	Treatment or no treatment with the subinhibitory concentrations of carvacrol or trans-cinnamaldehyde in potato dextrose broth (PDB) at 25 °C for 5 days	[50]
*			
Beta-tubulin	N	Liquid culture at 30 °C with constant shaking at 120 rpm for 20 to 24 h	[51]



Beta-tubulin	Y	Malt extract agar (MEA) supplemented with 0.5 mM eugenol	[52]	
GAPDH				for 4 days at 27 °C in the dark
Beta-tubulin	N	Coconut agar at 25 °C for 2 or 7 days	[53]	
Calmodulin				
Beta-tubulin	Y	Co-incubation with <i>Streptomyces roseolus</i> on ISP2 medium at 30 °C for 4 days at a relative humidity of 80% in a Vötsch chamber	[54]	
GAPDH				
Beta-tubulin	Y	YES medium containing one of four antifungal peptides: PPD1, 66-10, 77-3, or D4E1	[55]	
*				
Beta-tubulin	N	PDB containing 30% (v/v) of <i>Eurotium cristatum</i> culture filtrate for 3 days in the dark at 28 ± 2 °C with shaking at 120 r/min	[56]	
<i>ITS1</i>	N	Cultures of differing water potential were supplemented with different antioxidant concentrations at 20 °C and 28 °C for 72 h	[57]	
<i>ITS4</i>				
18S rRNA	Y	Treatment with 125 µg/mL artemisinin or solvent for 3 h in Roswell Park Memorial Institute (RPMI) 1640 medium at 37 °C	[27]	
18S rRNA	N	PDA with different carbon dioxide concentrations and on Czapek medium with different C: N ratios for 48 h and 5 days	[58]	
18S rRNA	N	Liquid <i>Aspergillus</i> minimal medium (AMM) at 37 °C for 18 h with shaking at 200 rpm	[59]	
<i>A. fumigatus</i>	Actin	N	(1) GMM at 25 °C for 60 days with shaking at 280 rpm (2) High iron liquid media or low iron liquid media at 37 °C for 24 h with shaking at 280 rpm	[23]
Actin	N	Biofilm growth at 37 °C in minimal essential medium (MEM) supplemented with 5% v/v fetal calf serum (FCS) or 5% v/v phosphate-buffered saline (PBS) for 24 h and 48 h	[22]	
Actin	N	(1) CBS 144.89 and <i>ΔsrbA</i> : Treatment with MIC <sub>50</sub> of fluconazole or voriconazole in liquid GMM at 37 °C with shaking at 300 rpm	[60]	
GAPDH				
<i>tefA</i>				

CBS 144.89, $\Delta$ <i>srbA</i> and <i>pniiA-erg11A-<math>\Delta</math>srbA</i> : Liquid GMM plus 20 mM NO <sub>3</sub> for 12 h and liquid GMM plus 20 mM NH <sub>4</sub> at 37 °C with shaking at 300 rpm			
Actin	Y	Mandels' salt solution with 1% oat spelts xylan for 0, 2, 4, 6 and 17 h	[16]
Histone H4			
Actin	N	RPMI medium at 37 °C for 0, 2, 4, 6, and 8 h	[21]
Actin	N	Treatment with 24,700, 12,300 and 6170 µg/mL kombucha during growth in RPMI medium at 35 °C for 48 h	[61]
Beta-tubulin	N	Yeast extract-peptone-dextrose (YEPD) medium with 10 µg/mL (H11-20) or 100 µg/mL of itraconazole for 8 h at 37 °C	[62]
Beta-tubulin	N	Treatment with 0.5 µg of voriconazole or without voriconazole in yeast glucose (YG) medium for 30, 60, 120, and 240 min	[63]
Beta-tubulin	N	AMM containing 0, 1, 10, 100, 1000 µM of FeSO <sub>4</sub> at 37 °C for 24 h with 150 rpm shaking	[64]
Beta-tubulin	N	MEM supplemented with 10% (v/v) human serum (male) and 50 µM FeCl <sub>3</sub> at 37 °C for 6 h	[65]
Beta-tubulin	N	Growth in mouse lung alveoli for 4 h or 14 h	[66]
Beta-tubulin	N	Four formed fungi balls or 2 mL of fungal suspension in NaCl incubated for 3 h at 25, 30, 35 and 40 °C	[67]
Beta-tubulin	N	Treatment with sub-lethal and lethal amphotericin B (AmB) concentrations in Sabouraud medium for 24 h at 37 °C with shaking	[68]
Beta-tubulin	N	Treatment with 4 mg/L itraconazole or dimethyl sulfoxide in Vogel's medium at 37 °C with shaking at 250 rpm for 14 to 16 h	[69]
Beta-tubulin	N	Growth in mouse lungs subcutaneously injected with 40 mg/kg Kenalog 1 day, or intraperitoneally with 175 mg/kg of cyclophosphamide 2 days prior to inoculation	[70]
<i>tefA</i>			
GAPDH	N	Exposure of <i>A. fumigatus</i> to human airway epithelial cells or human bronchial epithelial cells	[31]

	GAPDH	N	Treatment with 10 ng/mL of itraconazole (CEA17) or 5 µg/mL of itraconazole (mutant strains) in YG medium supplemented with 1.2 g/L of uracil and uridine for 24 h at 37 °C	[71]
	Putative 1,3-beta- glucan synthase catalytic subunit	N	Barrat's minimal nitrate medium in the presence or absence of oxidative stress and/or iron-limitation for 33 h and 50 h	[72]
	<i>TEF1</i>	N	<i>in vitro</i> : (1) YPD medium for 4 h, 8 h and 1 to 7 days at 37 °C, (2) YPD medium for 24 h at 37 °C (3) YPD or RPMI medium for 24 h and 72 h at 37 °C, (4) YPD medium for 5 to 8 days at 37 °C <i>in vivo</i> : (1) Mouse lung incubation for 4 h, 8 h, and 1 to 7 days, (2) mouse lung incubation for 24 h and 72 h, (3) mouse lung incubation for 5 to 8 days	[73]
	<i>TEF1</i>	N	Growth in a glucose (3%)-yeast extract (YE; 1%) liquid medium at 37 °C for 16 h	[74]
<i>A. luchuensis</i>	Actin	N	Growth on rice as rice koji and sampling at the stages in the process of shimaishigoto (40 °C, 30 h) and dekoji (40 h)	[26]
	Actin	N	Citric acid production (CAP) medium at 30 °C for 0 h or 12 h with shaking at 163 rpm	[75]
	Actin <u>Beta-tubulin</u>	N	Low or high inorganic phosphate yeast extract medium (YEM) and MM at 37 °C for 17 h with shaking at 200 rpm	[76]
<i>A. nidulans</i>	Actin	Y	Yeast extract-agar-glucose (YAG) supplemented with 15% polyethylene glycol (PEG) (w/v) compared to YAG without supplementation for 2 days	[77]
	Actin	N	Complete medium (CM) or MM with inducing or non-inducing supplements at 28 °C for 16 h with 250 rpm shaking	[78]

Actin	N	YAG supplemented with 5 mM uridine and 10 mM uracil (YUU) at 37 °C for 24 h	[79]
Actin GAPDH	Y	MM supplemented with 0.1% fructose and 5 mM urea at 30 °C for 15 h with shaking at 150 rpm	[30]
Beta-tubulin	N	YG medium in the presence or absence of drugs (camptothecin, imazalil, itraconazole, hygromycin, and 4-nitroquinoline oxide) for 8 h	[80]
Beta-tubulin	N	3% lactose MM for 18 h, after which a source of induction and/or repression was added, and cultures incubated for 4 h	[81]
Beta-tubulin Histone 2B	N	MM with exposure to light or darkness at 23 °C for 24 h (spores) or 30 °C for 40 h in distilled water (conidia)	[82]
Beta-tubulin *	N	MM supplemented with 50 mM ethyl methyl ketone or 1% glucose at 37 °C for 16 h to 18 h with shaking at 150 rpm	[83]
EEF-3 Elongation Factor	N	Glucose-free minimal nitrate medium or minimal nitrate medium at 37 °C for 4 h or 24 h	[84]
NAPDH	N	Modified MMPKRUU medium under K sufficient or deficient conditions at 37 °C for 24 h with shaking at 220 rpm	[85]
Putative ribosomal protein L37	Y	Liquid MM under standard conditions	[86]
Putative ribosomal protein L3			
18S rRNA	N	PDA supplemented with different carbon and nitrogen sources	[87]
18S rRNA *	N	Subculturing of transformants in the presence of 1.25 mg/mL of 5-fluoroorotic acid with uridine and uracil	[88]
<i>A. niger</i> Actin	N	Liquid AMM for 0, 3, 6, 12 and 72 h and on AMM plates for 5 days	[89]
Actin	N		[90]

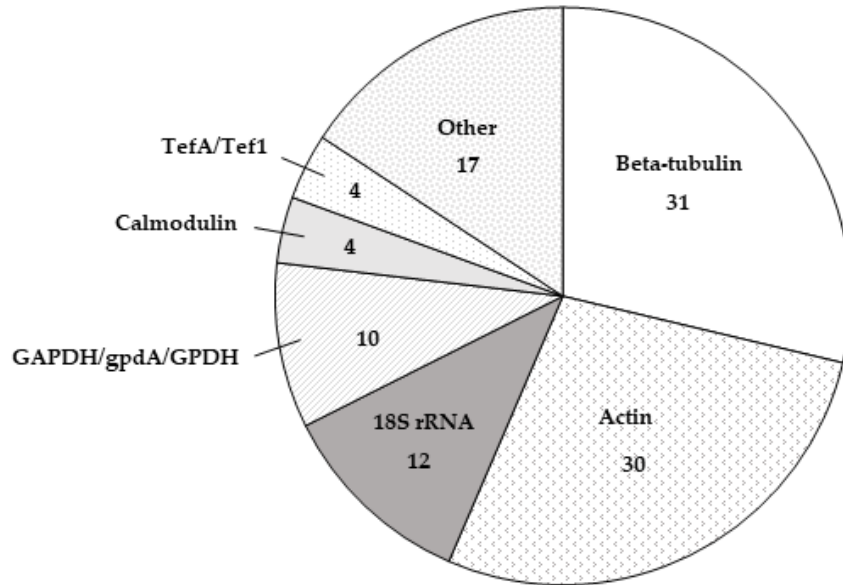
	GAPDH		Liquefied corn starch medium at 35 °C for 72 h with shaking at 330 rpm	
	Actin	N	CM for 25 h	[91]
	GAPDH	N	Glucose maltose polypeptone yeast extract (GMPY) medium at 30 °C and 250 rpm for 2 days	[92]
	Histone H2B	N	MM supplemented with L-rhamnose or L-rhamnonate at 30 °C with shaking at 250 rpm	[93]
	Histone- encoding Gene	Y	Growth in bioreactors on sorbitol as the carbon source with 1 mM D-xylose or 50 mM D-xylose	[94]
<i>A. nomius</i>	Calmodulin	N	Coconut agar at 25, 30 and 35 °C for 7 days	[95]
	18S rRNA *	N	Subculturing of transformants in the presence of 1.25 mg/mL of 5-fluoroorotic acid with uridine and uracil	[88]
	Actin	N	MM whole culture broth grown on a cellulose nitrate filter for 42 to 48 h until pigmented conidiospores present	[96]
	Actin	N	DPY agar medium for 48 h	[25]
	Actin	N	PDA medium at 30 °C for 7 days	[97]
	Beta-tubulin	N	Modified Czapek–Dox (CD) minimal agar at 30 °C for 48 h with 200 rpm shaking	[98]
<i>A. oryzae</i>	Beta-tubulin *	N	(1) $\alpha$ -amylase transcripts: Inducing (1% sorbitol plus 1% starch), non-inducing (1% sorbitol), and repressing (1% sorbitol plus 1% starch plus 2% sucrose) conditions for 48 h (2) glucoamylase A transcripts: Inducing conditions (in 1% starch) or repressing conditions (in 2% sucrose) at 30 °C for 24 h	[83]
	Histone H1	N	Growth on 5 g of wheat bran containing 2, 3, 4, 6 or 8 mL of water at 30 °C for 48 h	[99]
	Histone H1	N	MM at 30 °C for 5 days	[100]
	Histone H2A	N	DPY liquid medium at 30 °C for 2 days	[101]

	18S rRNA *	N	Adye and Mateles (1964) medium for 48 h and 72 h	[102]
	18S rRNA	N	Stationary phase culture growth in PDB 30 °C for 4 days in the dark	[29]
	Actin	Y	Treatment with 0, 0.25, 0.5 or 1.0 µg/mL aflastatin A for 1.5 to 3.5 days in PDB at 27 °C	[103]
	Actin	N	RPMI medium containing 25 or 50 mg/mL of vitamin C for 3 days at 28 °C	[104]
	Actin	Y	Co-incubation with <i>Kluyveromyces lactis</i> at 30 °C for 48 h	[105]
<i>A. parasiticus</i>	Beta-tubulin *	N	YES or YEP medium at 28 °C for 4 days	[48]
	Beta-tubulin	N	Yeast extract sucrose peptone (YESP) medium modified with 1% sodium nitrate (YESP-N) or 1% tryptophan (YESP-T) at 10, 15, 25, 30 and 37 °C for 96 h with shaking at 100 rpm	[106]
	Beta-tubulin *	N	Treatment or no treatment with the subinhibitory concentrations of carvacrol or trans-cinnamaldehyde in PDB at 25 °C for 5 days	[50]
	Beta-tubulin *	Y	YES medium containing one of four antifungal peptides: PPD1, 66-10, 77-3, or D4E1	[55]
<i>A. sojae</i>	18S rRNA *	N	Adye and Mateles (1964) medium for 48 h and 72 h	[102]
	Actin	Y	Lovastatin production medium at 27 °C for 10 days with shaking at 220 rpm	[107]
<i>A. terreus</i>	Actin	N	MID medium for 10 days after which 0.5, 1.0, 3.0 and 5.0% (w/v) of surface sterilised <i>Podocarpus. gracilior</i> leaves were added and incubated together for 20 days	[108]
	Beta-tubulin *	N	Four formed fungi balls or 2 mL of fungal suspension in NaCl incubated for 3 h at 25, 30, 35 and 40 °C	[67]
	Beta-tubulin *	N	Treatment with sublethal and lethal concentrations of AmB in Sabouraud medium at 37 °C for 24 h with slight shaking	[68]
<i>A. westerdijkiae</i>	Beta-tubulin	N	CYA in the presence or absence of <i>Debaryomyces hanseni</i> at 28 °C for 3 and 7 days	[109]

GAPDH	N	YES medium at 25 °C for 96 h	[110]
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Y, validation was provided; \*, analysed more than one species of *Aspergillus* and appear twice in the table.

Overall, 15 species of *Aspergillus* were examined: *A. aculeatus* [34,35]; *A. carbonarius* [32,36,37]; *A. cristatus* [24]; *A. fischeri* [38]; *A. flavus* [28,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53,54,55,56,57]; *A. fumigatus* [16,21,22,23,27,31,58,59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74]; *A. luchuensis* [26,75]; *A. nidulans* [30,76,77,78,79,80,81,82,83,84,85,86]; *A. niger* [87,88,89,90,91,92,93,94]; *A. nomius* [95]; *A. oryzae* [25,83,88,96,97,98,99,100,101]; *A. parasiticus* [29,48,50,55,102,103,104,105,106]; *A. sojae* [102]; *A. terreus* [67,68,107,108]; and *A. westerdijkiae* [109,110] (Table 2.1., Table S2.1.). The most common reference genes used in these studies were those encoding beta-tubulin, which were used 31 times in the literature [38,41,42,46,47,48,49,50,51,52,53,54,55,56,62,63,64,65,66,67,68,69,70,76,80,81,82,83,98,106,109], followed by those encoding actin (30 times) [16,21,22,23,24,25,26,30,34,42,43,44,45,60,61,75,76,77,78,79,89,90,91,96,97,103,104,105,107,108], the 18S ribosomal RNA gene (18S rRNA; 12 times) [27,28,29,36,39,40,41,58,59,87,88,102] and glyceraldehyde-3-phosphate (GAPDH, *gpdA* and *gpdh*; 10 times) [30,31,35,52,54,60,71,90,92,110] (Table 2.1., Figure 2.1., Table S2.1.). Reference genes that were used four or fewer times [16,27,34,37,38,41,53,57,60,70,72,73,74,82,84,85,86,93,94,95,99,100,101] are grouped under Section 2.4.5. of this review. Please note that reference genes are have been grouped together according to the common name for their corresponding end product (either protein or rRNA) and will be referred to by their corresponding end product name throughout the remainder of this Chapter and thesis.



**Figure 2.1. Distribution of reference gene usage across 90 RT-qPCR studies of *Aspergillus*.** Note that in the 90 studies examined, reference genes were used a total of 108 times, as some studies used multiple reference genes. Therefore, 108 was used as the denominator when computing the frequency of reference gene usage. The group “other” is composed of reference genes that were used fewer than four times. Beta-tubulin was the most frequently used reference genes, at 31 times in the literature.

In Section 2.4.1., Section 2.4.2., Section 2.4.3., Section 2.4.4., and Section 2.4.5. below, we briefly describe the history of use for each reference gene, as well as the relevant research results of these gene expression studies in *Aspergillus*. We also provide an analysis of the consistency or inconsistency of the studies regarding the validation of reference genes. Validation of specific reference genes for use under the specific experimental conditions tested was provided in only 17 of the 90 studies (Table 2.1., Table S2.1.) [16,27,30,32,35,38,41,46,52,54,55,77,86,94,103,105,107]. Table 2.1. is organised alphabetically first by species and second by reference gene, followed by the date of publication, and includes the species examined, encoded product of the reference gene used, whether the reference gene was validated and the experimental conditions for each of the 90 papers



returned by our PubMed search. A brief explanation of how the reference genes were validated, the associated gene symbol for each reference gene, strains used, type of sample taken, number of biological replicates and normalisation strategy are described in supplementary Table S2.1. Together, our review highlights the need for more frequent reference gene validation and standardising normalisation practices in RT-qPCR gene expression studies of *Aspergillus*.

### **2.4.1. Beta-Tubulin**

Beta-tubulin is a subunit of a universal eukaryotic protein called tubulin, the basic structural unit of microtubule. Tubulin is a heterodimer, consisting of equal numbers of alpha-tubulin and beta-tubulin subunits [112]. Microtubules are part of the cytoskeleton and provide structure and shape to eukaryotic cells. Because of the essential role microtubules play, both the alpha- and beta- tubulin encoding genes are highly conserved; as a result, they are commonly used for evolutionary studies. For example, the beta-tubulin gene is commonly used in phylogenetic [113] and taxonomic [114] studies of fungi. However, the copy number of beta-tubulin-encoding genes in *Aspergillus* genomes can vary [115]. For example, there are two copies of beta-tubulin-encoding genes in *A. nidulans*, while there is only one copy in *A. niger* [115]. In *A. nidulans*, each beta-tubulin performs a different function. The *benA*-encoded beta-tubulin is involved in nuclear movement and vegetative growth during mitosis [112,116], while the *tubC*-encoded beta-tubulin is involved in conidiation [112]. Beta-tubulin has been used as reference gene in gene expression studies since 1988 [117], and in RT-qPCR studies as early as 2000 [118]. In this review, beta-tubulin was used as a reference gene 31 times in studies examining gene expression in *A. nidulans* (5) [76,80,81,82,83], *A. fumigatus* (9) [62,63,64,65,66,67,68,69,70], *A. flavus* (13) [41,42,46,47,48,49,50,51,52,53,54,55,56], *A. parasiticus* (4) [48,50,55,106], *A. oryzae* (2) [83,98], *A. terreus* (2) [67,68], *A. westerdijkiae* (1) [109] and *A.*

*fischeri* (1) [38] (Table 2.1., Table S2.1.). Of these 31 studies, nine provided a rationale for choosing beta-tubulin as a reference gene [38,41,46,47,52,55,62,98,109], though only six provided proper validated justification [38,41,46,52,54,55]. Many of these studies examined the impact of abiotic factors, such as the wavelength of light [82], nutrient availability [64,76,106], water activity [47], temperature [67,106] and pH [76] on the expression of metabolic and biosynthesis genes. A select few examined the effects of biotic factors, such as the impact of co-incubation with *Streptomyces* spp. [42,54], *Eurotium cristatum* [56], *Debaryomyces hansenii* CYC 1244 [109] and *Listeria monocytogenes* [41] on the expression of genes associated with aflatoxin biosynthesis. Several studies examined the effects of antifungal treatment on the expression of antifungal resistance-associated genes [62,68,80], while others explored alternative treatments for minimising aflatoxin production [42,50,52,54,55,56]. Targeted and spontaneous mutations were also heavily examined in the literature, characterising the function of putative genes [65], through molecular cloning, or the specific effects on metabolic [38,66,81,83] pathways, through comparing expression in mutant to wildtype strains, and antifungal resistance [69] pathways, through comparing expression in antifungal susceptible to resistant strains. The following two sections will discuss the experimental conditions and findings of the studies that validated beta-tubulin as a stable reference gene for use under the specific conditions of the study, and those that did not provide justification for its use, respectively. Studies that specifically aimed to validate reference genes under a specific set of conditions will be discussed below in Section 2.5.

#### **2.4.1.1. Studies That Validated Beta-Tubulin Expression Stability under the Experimental Conditions Tested**

Of the 31 studies that used beta-tubulin as a reference gene for RT-qPCR, only six validated the reference gene for use under the specific experimental conditions being studied [38,41,46,52,54,55]. Cacaes et al. 2016 and Lappa et al. 2019 followed the “gold standard” for reference gene selection by using NormFinder [119] to evaluate the stability of multiple reference genes (of which beta-tubulin was one of them) in their studies examining the expression of genes related to aflatoxin biosynthesis [41,52]. In their analyses, beta-tubulin was demonstrated to be stably expressed during *A. flavus* growth under specific experimental conditions: at 27 °C on malt extract agar (MEA) supplemented with 0.5 mM eugenol [52]; and during growth in malt extract broth (MEB) at 25 °C and 30 °C in the presence or absence of *L. monocytogenes* [41]. In addition to validating stability, both research groups also used multiple reference genes for normalisation [41,52], thus applying the “gold standard” for RT-qPCR data normalisation [19].

Previous studies have identified that 30 genes are involved in aflatoxin biosynthesis in *A. flavus* [7]. These genes include the specific regulators *aflR* and *aflS*, and several early (*aflA*, *aflB*, and *aflC* or *pksA*) and later (*aflD* or *nor-1*, *aflM* or *ver-1*, *aflN*, *aflO*, *aflP* or *omtA*, and *aflQ*) pathway genes as well as those not shown to be directly associated with the pathway (*aflT*) [7]. Here we will use the naming conventions of the aflatoxin biosynthesis genes as recommended by Yu et al. [120]. Aside from these genes, several global regulatory genes were also identified to be associated with aflatoxin biosynthesis, including *veA* [121], *mtfA* [122] and *msnA* [123]. With this background information, here we briefly review the studies that investigated the potential influences of environmental factors on aflatoxin production through RT-qPCR.

In their 2016 study, Carces et al. found that 26 out of the 27 aflatoxin biosynthesis genes that they examined showed decreased expression following treatment with 0.5 mM eugenol, where the only gene that did not decrease in expression was *aflT* [52]. They also demonstrated that the genes involved in the later steps of the AFB1 biosynthesis pathway, such as *aflQ*, were more affected by eugenol treatment than early pathway genes, such as *aflA* and *aflB* [52]. Moreover, they found that global regulators *veA*, *mtfA* and *msnA* were upregulated [52].

In another study published by the same group two years later, Caceres et al. assessed the efficacy of *S. roseolus* as a biocontrol agent against AFB1 aflatoxin production in *A. flavus* [54]. The group used the same method of quantification for RT-qPCR as they did previously, and the same reference genes, including beta-tubulin [54]. They examined the expression of 27 genes in the aflatoxin biosynthesis pathway, as well as the impact of *S. roseolus* co-incubation on the expression of genes involved in fungal development, response to external stimuli and oxidative stress [54]. As was observed in their study investigating the effects of 0.5 mM eugenol treatment, 26 of the 27 genes in the AFB1 aflatoxin biosynthesis pathway showed significantly decreased expression after four days of co-incubation with *S. roseolus*, where again, only *aflT* expression was not significantly reduced [54]. Moreover, the expression of the two AFB1 transcriptional regulators, *aflR* and *aflS*, was significantly reduced 6-fold, and the genes involved in early aflatoxin biosynthesis were less affected than those involved in the intermediate and later stages [54]. This suggests that reduction in the expression of aflatoxin biosynthesis pathway genes may be mediated by a reduction in *aflR* and *aflS*, where the few AFLR-AFLS complexes translated were used up for the expression of the early pathway genes, leading to fewer complexes available for the expression of the late pathway genes, and consequently a greater reduction in their expression [52]. Since *aflT* does not possess the binding site for the AflR transcription factor, and is therefore not regulated by the AFLR-AFLS complex [124], this may

explain why *aflT* expression is unaffected following either treatment with 0.5 mM eugenol [52], in their previous study, or by co-incubation with *S. roseoulus* [54]. Given that the results of the two studies are the same with regards to the expression pattern of the aflatoxin biosynthesis genes, the use of beta-tubulin (and GAPDH discussed below) is reasonable, despite the experimental conditions being different (treatment with 0.5 mM eugenol [52] versus co-incubation with *S. roseoulus*) [54]. Additionally, following co-incubation with *S. roseoulus*, several of the genes involved in fungal development increased in expression, with *mtfA* being the most affected global regulator, exhibiting a significant 3.5-fold increase in expression [54]. Of those involved in the fungal response to external stimuli, *gprA* was the most affected, with its expression significantly increasing 5.5-fold [54]. Of the 10 oxidative stress genes investigated, the two most affected genes were *atfB* and *cat2*, which decreased 149.2-fold and increased 3.7-fold, respectively [54]. Altogether, co-incubation with *S. roseoulus* was effective at reducing AFB1 biosynthesis and protein concentration, making it an attractive alternative to phytopharmaceuticals used in agriculture [54].

Lappa et al. demonstrated that there was a temperature threshold for aflatoxin expression, and that *L. monocytogenes* decreased aflatoxin production at the protein level [41]. Mayer et al. also provided experimental evidence for stable beta-tubulin expression in their study monitoring *aflD* expression in *A. flavus* during growth on wheat in a petri dish for nine days [46]. They found that *aflD* mRNA levels were highest on the fourth day of incubation, and decreased after day six, the day at which AFB1 protein levels first became detectable [46]. These results demonstrated that there was a transient induction of *aflD* expression in *A. flavus* before translation of AFB1 begins [46]. Similarly, Devi and Sashidhar stated that the antimicrobial peptides (AMPs) used in their study did not affect the expression of the reference gene, beta-tubulin, although they found that each of the four AMPs tested (PPD1 (FRLHF); 66-10 (FRLKFH); 77-3 (FRLKFHF); and D4E1- 12 (FKLRAKIKVRLRAKIKL))

significantly reduced the expression of the aflatoxin biosynthesis genes *aflR*, *alfC*, *aflD*, *aflM* and *aflP* in *A. flavus* and *A. parasiticus* [55]. Moreover, of the four AMPs tested, two (77-3 and D4E1) were the most effective inhibitors of expression [55]. Together, these RT-qPCR studies using beta-tubulin as a reference gene helped reveal the general conditions associated with increases (e.g., two days of incubation with wheat), and decreases (e.g., eugenol and AMP treatments and co-incubation with *L. monocytogenes*) in aflatoxin production by *A. flavus* and *A. parasiticus*.

Aside from being used as a validated reference for monitoring the expression of genes involved in mycotoxin production in *A. flavus*, beta-tubulin has also been used for studying the regulation of gene expression related to other phenotypes. Specifically, in their study examining the role of mannitol in conidia stress resistance and ascospore development, Wyatt and colleagues provided experimental evidence for the stability of beta-tubulin expression during *A. fischeri* growth on oatmeal agar for up to six days [38]. However, they found that the mRNA levels of beta-tubulin were low in 30-day ascospores, and thus chose to normalise beta-tubulin and target gene expression to an additional reference gene, experimentally validated histone 3 (*his-H3*), for 30-day culture data [38]. They measured the expression of *mpdA*, *mtdA*, *mtdB* and *esdC* in wildtype and in three *mpdA* deletion mutants [38]. *mpdA* was maximally expressed in 6-day ascospore-forming wildtype cultures, at which time *mtdA* and *mtdB* were also highly expressed, indicating that these genes may play a role in mannitol metabolism during early ascospore development [38].

#### **2.4.1.2. Studies Missing Proper Beta-Tubulin Expression Stability Validation**

Among the studies that used beta-tubulin as a reference gene, 25 did not provide experimental validation for its use under the specific conditions tested [42,47,48,49,50,51,53,56,62,63,64,65,66,67,68,69,70,76,80,81,82,83,98,106,109]. These studies

included those examining the expression of clinically relevant genes, such as those contributing to antifungal resistance [62,63,68,69,80], mycotoxin production [109], aflatoxin biosynthesis [42,47,48,49,50,53,56,106] and infection in steroid and chemotherapeutic mouse models [70]. They also included studies investigating changes in the expression of genes associated with nutrient-sensing [76], calcium transport [66], iron acquisition [64], germination and conidiation [82], as well as those with potential industrial application [83,98]. Other studies focussed on characterising proteins, such as the heat shock protein, Hsp60 [67] and sialidase enzyme [65]. One study also looked at how targeted mutations to the Spt-Ada-Gcn5-acetyltransferase components of *A. nidulans* affected the expression of an aldehyde dehydrogenase and three alcohol dehydrogenases [81].

When reference gene expression stability is not validated, it can be difficult to interpret conflicting RT-qPCR results. For example, Abdel-Hadi et al. reported that 90W water activity delayed the expression of the aflatoxin biosynthesis activator, *aflR*, and that the structural genes, *aflD*, *aflM* and *aflP*, were expressed prior to the detection of *aflR* mRNA [47]. Here, the water activity of 0.9 (90W) means that the vapour pressure within the food (peanuts) is 90% that of pure water. Moreover, at 85W, only *aflD* and *aflM* were expressed [47]. This indicates that structural gene expression may be independent of *aflR* under these conditions [47], contrary to the findings of Degola et al., who found that expression of the structural genes *aflD*, *aflO* and *aflQ*, was not detectable until 48 h of incubation in yeast extract sucrose (YES) agar, indicating reliance on prior *aflR* expression [125]. Interestingly, both research groups used beta-tubulin as a reference gene [47,125], demonstrating that their experimental conditions might have had a profound effect on gene expression of similar target genes. However, of greater interest and concern are the apparent differences in beta-tubulin expression in Abdel-Hadi et al.'s paper, as indicated by the changes in band intensity in Figure 4 of their study [47]. It seems that the expression of the reference gene changed substantially from week two to three at

85W [47], further suggesting that beta-tubulin expression stability was not validated by the authors, and that its expression was not stable under their experimental conditions. In this study's conditions, beta-tubulin was unlikely to be an appropriate reference gene.

Similarly, using beta-tubulin as a reference for normalisation, Fattahi et al. found that there was a significant increase in *cyp51A* expression in natural voriconazole-resistant *A. flavus* isolates, following growth in liquid medium at 30 °C [51]. Their results were consistent with studies examining mutations in *cyp51A*, contributing to voriconazole resistance in *A. fumigatus* [126] and *A. lentulus* [127], as well as those examining laboratory-induced resistance in *A. flavus* [128]. However, Liu et al. found that *cyp51A* did not contribute to voriconazole resistance in *A. flavus* BMU29791, a strain isolated from a patient with IA [129]. In their study, Liu et al. treated *A. flavus* with 0.25 µg/mL of voriconazole in minimal medium, and used actin as a reference gene, though they also did not provide experimental evidence for validation of its stability [129]. Since the reference genes were not experimentally demonstrated to be stable under the tested conditions in either case [51,129], instead of different mutations causing the drug resistances among strains, alternative explanations such as differences in stability of reference genes in the experimental conditions cannot be ruled out. If the beta-tubulin and actin genes were validated under the experimental conditions used in these studies, it would be easier to determine whether the differences observed were caused by genetic differences among the strains and species.



## 2.4.2. Actin

Actin forms microfilaments and is among the most abundant proteins in eukaryotic cells [130]. Actin was first used as a reference gene in gene expression studies as early as 1985 [131], and similar to beta-tubulin, actin was first used as a reference gene in RT-qPCR studies as early as 2000 [132].

In our literature search, we found 17 reported usages of actin

[16,21,23,25,26,34,42,43,60,75,77,78,79,89,91,96,108], 12 usages of beta-actin

[22,24,44,45,61,76,90,97,103,104,105,107] and one reported usage of gamma actin [30] as reference

genes. Unlike in the human genome, which contains about 20 copies of actin genes that make up six different isoforms of actin proteins [133], most fungi are known to contain only one actin gene

[134,135]. Thus, we were curious as to how similar the actin and beta-actin genes were in *Aspergillus*

fungi. We first checked the specificity of the primers used in these studies using Primer-Blast [136]

and found that several of the primer sequences listed for beta-actin matched an actin gene instead, and

one study matched both actin and beta-actin genes. We then conducted a BLASTn of the actin gene

(*Afu6g04740*) of *A. fumigatus* Af293 in FungiDB. The sequence matched the gamma-actin gene

(*P168DRAFT\_280232*) in *A. campestris* with 87% identity, the actin gene (*ACLA\_095800*) in *A.*

*clavatus* with 94% identity and the actin gene (*NFIA\_051290*) in *A. fischeri* with 99% identity. The

sequence also matched the gamma actin gene, *ANID\_06542*, for *A. nidulans* with 92% identity. The

gamma actin gene in *A. nidulans* is the only actin gene in the fungus [135], and we will therefore

include this reference gene with the counts for the actin reference gene in this review. We further ran

a second BLASTn using the beta-actin sequence (accession number: AF276240) that was previously

retrieved for *A. terreus* through Primer-Blast [136] (primers from Sorrentino and colleagues [107]),

and compared the results to the BLASTn results for *Afu6g04740*. Similarly, the beta-actin sequence

matched the gamma actin gene (*P168DRAFT\_280232*) in *A. campestris* with 87% identity, the actin

gene (*ACLA\_095800*) in *A. clavatus* with 94% identity and the actin gene (*NFIA\_051290*) in *A. fischeri* with 99% identity. Although the results from the FungiDB BLASTn did not contain an exhaustive list of all *Aspergillus* species, given that the two sequences BLASTed yield the same results in these three species, this demonstrates that actin and beta-actin are most likely the same gene. Here we follow the naming convention of *A. fumigatus* Af293 and call the gene actin. We have made this change in Table 2.1., reporting both actin- and beta-actin-encoding genes as actin (Table 2.1.). We have kept the original gene symbols in Table S2.1. for the reader's reference (Table S2.1.). Thus in total, actin was used in 30 studies examining gene expression in *A. niger* (3) [89,90,91], *A. nidulans* (4) [30,76,77,78,79], *A. fumigatus* (6) [16,21,22,23,60,61], *A. flavus* (4) [42,43,44,45], *A. parasiticus* (3) [103,104,105], *A. cristatus* (1) [24], *A. oryzae* (3) [25,96,97], *A. luchuensis* (2) [26,75], *A. terreus* (2) [107,108] and *A. aculeatus* (1) [34] (Table 2.1.). Of these 30 studies, seven studies provided an explanation for using actin under the conditions tested [16,45,75,78,89,103,105], although only six of these studies provided experimental validation of its expression stability under the specific conditions [16,30,77,103,105,107] (Table 2.1., Table S2.1.). Like those using beta-tubulin as a reference, many studies used RT-qPCR to investigate aflatoxin biosynthesis [42,44,45,103,104,105]. A few studies also focused on characterising putative biosynthesis pathway genes, such as those involved in trehalose synthesis [89], citric acid production [90] and galactofuranosylation [91], as well as the function of homologous genes in different species of *Aspergillus* [43,75]. Others looked to identify additional pathways contributing to antifungal resistance and potential antifungal drug targets [21,60,78,79], or tested the efficacy of natural antifungals [23,61]. Below in Section 2.4.2.1, we briefly describe the specific studies and experimental conditions that validated actin gene as an appropriate reference gene. Studies that did not provide validations are summarised in Section 2.4.2.2.

#### **2.4.2.1. Studies Validating Actin Expression Stability under the Experimental Conditions Tested**

Of the 30 studies using actin as a reference gene, only six studies verified its stability under the experimental conditions tested [16,30,77,103,105,107] (Table 2.1., Table S2.1.). Miao et al. followed the “gold standard” for reference gene selection by verifying the expression stability of two reference genes, actin, and the histone-encoding gene of *A. fumigatus* Z5 under the experimental conditions used in their study [16]. They used geNorm [33] to validate the two reference genes [16]. As such, actin has been demonstrated to be stably expressed during *A. fumigatus* Z5 growth in Mandels’ salt solution, supplemented with 1% oat speltis xylan, as the xylanase inducer [16]. Using RT-qPCR, Miao and colleagues found that 11 of the 13 xylanase-encoding genes in *A. fumigatus* Z5 are expressed in response to xylan, in addition to eight xylan-induced secreted proteins [16]. They identified four endoxylanases, two xylosidases, one  $\alpha$ -L-arabinofuranosidase and one acetyl xylan esterase as important xylan-degraders, and thus promising agents for future biofuel generation [16].

Gao et al. also followed the “gold standard” for reference gene selection by validating the stability of actin in *A. nidulans* during its growth in the two media conditions, yeast extract-agar-glucose (YAG) with and without 15% (w/v) polyethylene glycol (PEG), employed in their study [77]. RT-qPCR analysis revealed that the expression of four members of the calcium signalling pathway—*mida*, *crzA*, *pkaA* and *pmrA*—significantly increased under osmotic stress in the presence of 15% PEG in *mobB/cotA* mutants of *A. nidulans* [77].

The gold standard was also applied by Deloménie et al., who validated the expression stability of two reference genes, actin and GAPDH [30], using geNorm [33]. They then used these genes to normalise gene expression in their custom Agilent microarray for *A. nidulans* [30]. They used RT-qPCR to verify the accuracy of the microarray data, by checking the expression of five genes: two that were

stably expressed according to microarray analysis, *ANID\_08764* and *ANID\_05831*; and three that increased in expression, *ANID\_00858*, *v00296*, and *ANID\_02343* [30]. RT-qPCR analysis confirmed that *ANID\_08764* and *ANID\_05831* were stably expressed, and that *ANID\_00858*, *v00296* and *ANID\_02343* increased in expression, although the fold change was greater for RT-qPCR results [30]. As articulated by the researchers [30], this increase in fold change can be explained by the compression effect often observed for microarrays, and more severely in Agilent microarrays, when comparing RT-qPCR data [137]. The compression effect is attributed to technical limitations of microarrays, such as the limited dynamic range of signal intensities and cross-hybridisation among paralogous sequences [137].

Similarly, Sorrentino and colleagues validated the use of actin as a reference gene under the conditions used in their work, examining the addition of linoleic acid to enhance lovastatin production in *A. terreus* [107]. *A. terreus* is the main producer of lovastatin, a drug used to lower cholesterol [138]. Using RT-qPCR, Sorrentino and colleagues found that addition of linoleic acid increased the expression of two lovastatin biosynthetic genes, *lovB* and *lovF* [139], when compared to control cultures, while noting that the expression of actin remained consistent [107]. However, the group did not provide the data demonstrating stable actin expression [107]. Thus, fatty acids such as linoleic acid enhance lovastatin production, and this knowledge can be applied to industrial fungal fermentation as a cost-effective method for increasing lovastatin yield [107].

Ghanbari et al. also validated the stability of actin expression in their study, examining the efficacy of *Kluyveromyces lactis* as a biocontrol agent against aflatoxin production and *aflR* expression in *A. parasiticus* [105]. Consequently, actin expression has been shown to be stable during co-incubation with  $1.5 \times 10^5$  CFU/mL of *K. lactis* at 30 °C for 48 h [105]. They found that there was a significant decrease in *aflR* expression in cells treated with *K. lactis* compared to untreated cells, and that this

corresponded with a decrease in aflatoxin production [105]. This indicates that *K. lactis* may decrease aflatoxin production through the downregulation of *aflR* [105].

Finally, Kondo et al. used actin as a reference gene to examine the effects of aflastatin A on aflatoxin expression in *A. parasiticus* [103]. The authors cite their previous work with the same species during exposure to blasticidin and its derivatives as justification for using actin as a reference gene [140].

Since the structures of aflastatin A and blasticidin are similar [141], hypothesising that actin expression would exhibit the same stability when exposed to aflastatin A is reasonable, and therefore the authors provide good justification for its use in their study. The group found that the expression of the aflatoxin biosynthesis genes *aflC*, *aflM*, *aflP* and *aflR* was significantly diminished following treatment with aflastatin A [103]. Moreover, they found that aflastatin A also decreased the expression of *aldA* and *facA* [103], which encode aldehyde dehydrogenase [142] and acetyl-CoA synthetase [143], respectively.

#### **2.4.2.2. Studies Missing Proper Actin Expression Stability Validation**

Twenty-four of the 30 studies did not include proper expression stability validation of actin as a reference gene [21,22,23,24,25,26,34,42,43,44,45,60,61,75,76,78,79,89,90,91,96,97,104,108].

Trevisan et al. and Verheecke et al., who used RT-qPCR to examine the expression of genes involved in nutrient-sensing [76] and aflatoxin biosynthesis [42] (as discussed above), respectively, also used actin as an unvalidated reference gene. As with beta-tubulin, several studies investigated the expression of genes facilitating antifungal resistance [60,61,79] and aflatoxin biosynthesis [42,44,104]. Additionally, two studies used RT-qPCR to confirm RNA-sequencing (RNA-seq) data, including the expression of the growth phase-associated [21] and amylolytic enzyme [26] genes. Similarly, several studies used RT-qPCR to confirm microarray expression data, including the expression of members of the gliotoxin gene cluster [22], non-ribosomal peptide synthetases [23],

sporulation-related genes [24] and highly expressed mating-type associated genes [25]. Others studied the expression of genes associated with Galf biosynthesis [78], nitrogen regulation and catabolism [43], citric acid production [75,90], spore color development [96] and conidiation [43,97], as well as putative trehalose synthesis genes [89] and one gene encoding alpha-glucan synthase [91].

Additionally, one study used RT-qPCR to verify increased transcription of genes associated with the industrially relevant product, taxol [108], while another study used RT-qPCR to characterise the *acr* biosynthetic cluster of a novel secondary metabolite, Acurin A, in *A. aculeatus* [34].

As mentioned previously, failure to properly validate reference genes can have negative consequences on the interpretation of results. Bruns et al. used actin as a reference gene in their RT-qPCR experiment, aimed at verifying the expression of four gliotoxin cluster genes obtained using microarray [22]. However, the group did not experimentally validate the expression of actin prior to conducting RT-qPCR [22]. Bruns et al. noted that there was a discrepancy in the expression levels of *gliN*, *gliP*, *gliG* and *gliT* when comparing the data obtained using microarray to that obtained using RT-qPCR [22]. They noted that the limited dynamic range of detection for microarrays and differences in probe design might have contributed to the differences observed in gene expression between methods [22]. However, as RT-qPCR was used by the research group to verify the results obtained using microarray [22], it would have been prudent for the researchers to ensure that the reference gene used for RT-qPCR exhibited stable expression under the conditions being tested. Similarly, Perrin et al. used RT-qPCR to confirm the microarray expression data for seven non-ribosomal peptide synthetases (not associated with siderophore biosynthesis) and an additional four ribosomal peptide synthetases associated with siderophore biosynthesis, specifically [23]. They also used actin as a reference gene, but did not validate its expression stability under the conditions tested [23]. They report that the siderophore gene, *sidE*, reaches maximum expression during growth under

low iron availability [23], conflicting with Reiber et al.'s finding that *sidE* is insensitive to changes in iron concentrations and is constitutively expressed, except under iron replete conditions (300  $\mu\text{M}$ ) at 24 h of growth [144]. Interestingly, Reiber et al. used calmodulin as the reference gene in their study, and demonstrated that calmodulin is constantly expressed in *A. fumigatus* under the conditions tested (mineral salt medium (pH 6.8), supplemented with 0, 20 and 300  $\mu\text{M}$ ) [144]. Curiously, Perrin et al. note that the differences in expression patterns observed between their study and Reiber's group are likely due to subtle differences in the growth conditions, yet the media composition and iron concentrations used were the same in both studies. The only difference between the two studies was the shaking speed used, which was 230 rpm for Perrin et al. [23] and 280 rpm for Reiber et al. [144]. Therefore, the differences in observed *sidE* expression may be due to differences in reference gene expression, where actin expression differed under the experimental conditions used by Perrin et al. [23]. In fact, Perrin et al. note that actin expression decreased in the *laeA* mutant examined during growth under low iron availability, indicating that actin was likely not an appropriate reference gene for their study [23]. To ascertain the cause for the observed differential expression, the actin reference gene must be validated under the experimental conditions used by Perrin's group. This case clearly illustrates the importance of reference gene validation in RT-qPCR analyses under the specific conditions being studied, and the consequences associated with improper reference gene use.

### **2.4.3. 18S rRNA**

The 18S rRNA gene encodes the small ribosomal RNA subunit of the translation apparatus in all eukaryotes. The rDNA copy number of the 18S rRNA gene in *A. fumigatus* is strain-dependent, ranging from 38 copies in the Af293 strain to up to 91 copies in select strains [145]. Unlike single-copy genes, this variable nature in copy number among strains represents a potential drawback of

using 18S rRNA as a reference in comparative analyses among strains. However, 18S rRNA is a highly conserved gene at the DNA sequence level, and has been used as a reference gene in gene expression analyses as early as 1990 [146]. Similar to the two previously discussed reference genes, beta-tubulin and actin, the 18S rRNA gene was first used in RT-qPCR analyses as early as 2000 [147]. Additionally, the product of the 18S rRNA gene is commonly used to confirm the overall integrity of the isolated RNA used in RT-qPCR [63,66].

In this review, we found that the 18S rRNA gene was used as a reference gene 12 times in studies examining gene expression in *A. niger* (2) [87,88], *A. fumigatus* (3) [27,58,59], *A. flavus* (4) [28,39,40,41], *A. parasiticus* (2) [29,102], *A. sojae* (1) [102], *A. oryzae* (1) [88] and *A. carbonarius* (1) [36] (Table 2.1., Table S2.1.). Only two of the twelve studies validated the use of the 18S rRNA gene under the conditions being tested [27,41], with the remaining ten studies offering no explanation for its use as a reference gene [28,29,36,39,40,58,59,87,88,102] (Table 2.1., Table S2.1.). As discussed above, several of these studies examined the efficacy of potential antifungals [27] and aflatoxin biosynthesis antagonists [40,41], as well as the influence of abiotic factors, such as temperature and water activity [28], on gene expression. Genetic alterations were also examined, including deletion mutants, to assess the importance of genes involved in aflatoxin biosynthesis [29], conidiation [29] and production of extracellular proteins [39], as well as overexpression strains, to assess the role of cofilin during oxidative stress and pathogenesis [59]. Two groups also examined the influence of carbon and nitrogen sources on the expression of genes in two relevant, yet different pathways: those involved in production of industrially useful xylanases [87]; and harmful allergen-encoding genes [58].



### **2.4.3.1. Studies Validating 18S rRNA Expression Stability under the Experimental Conditions Tested**

The 18S rRNA gene was only validated in two of the 12 studies examined in this review [27,41]. As mentioned in Section 2.4.1.1, Lappa et al. employed the “gold standard” for reference gene selection by validating the use of the three reference genes (beta-tubulin, 18S rRNA and calmodulin) under the experimental conditions used in their study examining aflatoxin biosynthesis [41]. Gautam et al. also used the “gold standard” for reference gene selection, by testing the stability of three candidate reference genes (actin, GAPDH and 18S rRNA) in preliminary experiments for their study investigating the efficacy of the antimalarial drug artemisinin against *A. fumigatus* [27]. Of the three candidate reference genes tested, the 18S rRNA gene exhibited the greatest stability [27]. Using RT-qPCR, seven of 745 genes displaying an altered expression following exposure to 125 µg/mL artemisinin, including the oxidative phosphorylation pathway-specific 64 kDa mitochondrial NADH dehydrogenase, were confirmed to exhibit a similar change in expression [27]. Interestingly, while the 64 kDa mitochondrial NADH dehydrogenase was downregulated upon artemisinin exposure, other oxidative phosphorylation genes were upregulated [27]. One explanation for this is that the 64 kDa mitochondrial NADH dehydrogenase is a specific target of artemisinin, and due to its downregulation, *A. fumigatus* overexpresses additional oxidative phosphorylation genes to equilibrate the membrane potential of the fungus [27]. Additionally, co-incubation with artemisinin and itraconazole produced a synergistic effect, suggesting that artemisinin may be useful in combination infection treatments with other azole antifungals [27].

#### **2.4.3.2. Studies Missing Proper 18S rRNA Expression Stability Validation**

Ten of the twelve studies that used the 18S rRNA reference gene did not validate its stability under the experimental conditions of their study [28,29,36,39,40,58,59,87,88,102]. Like studies described above in Section 2.4.2., Chang et al. used RT-qPCR to confirm microarray expression data for genes associated with oxidative stress [29]. Similarly, the expression of aflatoxin biosynthesis genes was also examined in studies using the 18S rRNA gene as a reference [40,102]. These studies also included those investigating the expression of miRNA-like genes [28] and xylanases [87], as well as the expression of genes associated with allergies [58], oxidative stress and cell wall polysaccharide biosynthesis [59]. Other studies looked at the expression of differentially secreted proteins, such as alpha amylase, in response to the deletion of VeA [39] and putative ochratoxin A (OTA) biosynthesis genes [36]. In addition, one study evaluated the expression of single-guide RNA for use in CRISPR-Cas9 editing of *A. niger* [88].

#### **2.4.4. GAPDH**

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, also called *gpdA* and *gpdh*) plays a vital role in glycolysis by catalysing the conversion of glyceraldehyde-3-phosphate (G3P) to 1,3-biphosphoglycerate in the presence of NAD<sup>+</sup> and inorganic phosphate [148]. The role of GAPDH extends to additional cellular processes, acting as a potential transcription factor (as demonstrated by binding to RNA polymerase II in *Schizosaccharomyces pombe*) [149], glucose availability sensor and corresponding regulator of cell growth [150], as well as apoptosis inducer [151].

GAPDH has been used in gene expression analyses as early as 1991 [152]. It was first used as a reference gene in RT-qPCR analyses as early as 1992 [153], making it the earliest reference gene used of four reference genes discussed in detail in this review. GAPDH was used as a reference gene in ten

studies examining gene expression in *A. niger* (2) [90,92], *A. nidulans* (1) [30], *A. fumigatus* (3) [31,60,71], *A. flavus* (2) [52,54], *A. westerdijkiae* (1) [110] and *A. aculeatus* (1) [35] (Table 2.1., Table S2.1.). These studies included those that investigated the function of proteins, including the type III polyketide synthase [92] and alternative oxidase [90] genes, using deletion and overexpression mutants. Several studies also used RT-qPCR to confirm the results from other gene expression analyses, including microarrays [30,31] and cDNA RDA [110]. Another study by Blosser and Cramer (also discussed above) examined the mechanism of triazole resistance in *A. fumigatus* mediated by SbrA [60]. Only four of the 10 studies using GAPDH as a reference gene experimentally validated its stability [30,35,52,54] (Table 2.1., Table S2.1.).

#### **2.4.4.1. Studies That Validated GAPDH Expression Stability under the Experimental**

##### **Conditions Tested**

Of the 10 studies that used GAPDH as a reference gene, four studies experimentally validated its expression stability under the conditions being tested [30,35,52,54]. As mentioned above, in Section 2.4.1.1, Caceres et al. employed the “gold standard” for selecting reference genes when selecting beta-tubulin and GAPDH in their 2016 study, examining the effects of 0.5 mM eugenol on expression of aflatoxin biosynthesis genes [52]. These same reference genes were also used in their latter study examining the impact of *S. roseoulus* on aflatoxin biosynthesis gene expression [54]. Similarly, Deloménie et al., also mentioned above in Section 2.4.1.1, employed the “gold standard” for reference gene selection by first validating the expression stability of actin and GAPDH [30].

Tani et al. also validated the expression stability of GAPDH as a reference gene using NormFinder [119], in their study investigating the role of the XlnR signalling pathway in *A. aculeatus* [35].

Through examining the expression of cellulase and hemicellulase genes (*bgl1*, *cbh1*, *cmc1*, *cmc2*,

*xyn1a*, and *xyn1b*) in *A. aculeatus* wildtype and  $\Delta xlnr$  strains, during growth in the presence of various carbon sources (1% (w/v) polypeptone, 1% (w/v) glucose, 1% (w/v) avicel, 1% (w/v) xylose, or 1% (w/v) arabinose), the researchers demonstrated that there are two different pathways mediating cellulase and hemicellulase expression [35]. Specifically, there is one pathway that mediates the expression of *cbh1*, *cmc2* and *xyn1a* in minimal medium containing 1% (w/v) avicel, which is independent of XlnR signalling [35]. Moreover, expression of *cbh1*, *cmc2* and *xyn1a* was induced by 30 mM cellobiose in the wildtype strain, whereas *xyn1b* and *cmc1* expression was not, suggesting that cellobiose is an exclusive inducer of the XlnR-independent pathway [35]. This study was the first to report diverging routes of gene induction via cellulose, to XlnR-dependent and -independent pathways, and makes a valuable contribution to our understanding of transcriptional inducers in filamentous fungi [35].

#### **2.4.4.2. Studies Missing Proper GAPDH Expression Stability Validation**

The remaining six studies using GAPDH as a reference gene did not validate its stability under the conditions tested [31,60,71,90,92,110]. Blosser and Cramer and Hou et al., who investigated the expression of genes involved in antifungal resistance [60] and citric acid production [90] using RT-qPCR and actin, as an unvalidated reference gene, respectively, also used GAPDH as a reference gene. Blosser and Cramer also used a third reference gene in their study, *tefA*, although this gene was not experimentally validated either [60]. As with the sections above, the expression of genes contributing to antifungal resistance [60,71] was examined. Again, RT-qPCR was used to verify the results of other methods of gene expression analysis, including the change in expression in eight genes in *A. fumigatus* conidia exposed to human airway epithelial cells [31] and three putative

oxidoreductases in *A. westerdijkiae* [110]. Additionally, one study investigated the expression of the type III polyketide synthase-encoding gene, *AnPKSIII* [92].

Oosthuizen et al. investigated dual organism transcriptomics, looking at the transcriptome of *A. fumigatus* conidia and *A. fumigatus*-infected human airway epithelial cells (AECs) using microarrays [31]. They used RT-qPCR to examine a representative group of genes for both *A. fumigatus* conidia incubated with AECs or human bronchial epithelial cells (16HBE14o-) and a representative group of genes expressed in AECs and 16HBE14o-[31]. For analysis of *A. fumigatus* conidia, this representative group of eight genes included those involved in vacuolar acidification and those that possess metallopeptidase activity [31]. Three of the eight *A. fumigatus* genes encoding—vacuolar ATPase 98 kDA subunit, MAP-1 and SkpA—were significantly upregulated following incubation with either AECs or 16HBE14o- [31]. In contrast, the tubulin-specific chaperone C and  $\beta$ -glucosidase significantly increased in expression, but only when incubated with AECs [31]. Similarly, *sidA* and *fdh* significantly increased in expression when incubated with 16HBE14o- cells [31]. Overall, microarray and RT-qPCR analysis elucidated similar trends in expression [31]. Through looking at the expression of genes in human epithelial cells and *A. fumigatus* conidia simultaneously, the researchers provided a better representation of the changing expression dynamics as host and pathogen interact [31].

Hou et al. characterised the role of the mitochondrial alternative oxidase gene, *aox1*, in citric acid production in *A. niger* [90]. Interestingly, the researchers did not validate the expression stability of either of their two reference genes, actin and GAPDH, under the experimental conditions used [90]. They compared the expression of six metabolic genes—*aox1*, *cox*, *cat*, *hk*, *pfk* and *cs*—in an *aox1* overexpression strain (102) and an  $\Delta$ *aox1* strain (3–4), to each other, as well as the parental strain, CGMCC 10142 [90]. In strain 3–4, *cox* expression increased compared to the parental strain, with a

coinciding increase in ATP concentration for all sampled time points, except for 36 h and 48 h [90]. In strain 102, the expression of *cox* decreased compared to the parental strain [90]. In the absence of *aox1*, *cat* expression was more constant and relatively higher during aerobic treatment, in relation to the parental strain [90]. Correspondingly, a negative correlation between *aox1* and *cat* expression was observed in strain 102, compared to the parental strain [90]. The expression of *hk*, *pfk* and *cs* genes was higher in strain 102 compared to strain 3–4 [90]. Citric acid was also produced at higher quantities in strain 102 [90]. This work establishes the linkage between *aox1* expression, genes involved in citric acid production, and the amount of citric acid produced, using methodology that can be applied to other industrially relevant biological mechanisms [90].

#### **2.4.5. Others**

Other reference genes that have been used over the last two decades to study gene expression in *Aspergillus* include those that encode: histones [16,34,38,82,93,94,99,100,101]; nicotinamide adenine dinucleotide phosphate+ hydrogenase [85]; translation elongation factors [84] and elongation factor subunits [60,70,73,74]; ribosomal proteins [86]; putative 1,3-beta-glucan synthase catalytic subunit [72]; calmodulin [37,41,53,95]; internal transcribed spacer regions [57]; and ubiquitin-conjugating enzyme [32] (Table 2.1., Table S2.1.).

Of those using the above-mentioned reference genes, four studies used multiple reference genes to normalise their expression data [16,41,60,82]. Six different studies experimentally validated the expression stability of the reference genes used under the conditions tested [16,32,38,41,86,94]. Several of the studies have already been discussed above [16,38,41], and we will therefore focus here on three studies that validated the expression of the reference genes used in their work identifying and characterising the conserved WDR gene (FPWDR) in *A. nidulans* [86], validating RNA-seq data

examining OTA biosynthesis in *A. carbonarius* [32] and modelling the expression dynamics of the XlnR regulon in *A. niger* [94].

Prior to assessing the impact of deleting FPWDR and the surrounding locus, on the expression of cell wall-associated genes in *A. nidulans*, Guerriero et al. used geNormPlus [33] to rank the expression stability of five candidate reference genes—*rpl37*, *rpl3*, actin, *CRP2* and *TEF1*—during growth in standard minimal medium [86]. As *rpl37* and *rpl3* were the most stable, they were chosen as reference genes for normalisation [86]. Following genomic analysis of FPDWR, Guerriero et al. deleted FPWDR and the surrounding locus (including *bf*), generating a heterokaryon transformant (hkΔAN1556) [86]. Since hkΔAN1556 possessed deformities in cell wall morphology [86], the group decided to look at the effect of the deletion on the expression of cell wall-associated genes, including members of the *chs* family, *csmA*, *csmB*, *celA*, *fksA*, *rhoA* [154], *pkcA* [155], *wscA*, *wscB* [156], *CPSI* and *bf*. Generally, the expression of cell wall-associated genes decreased in hkΔAN1556, with a significant reduction in *chsED*, *csmA*, *csmB*, *CPSI*, *fksA* and *wscB* expression [86]. Surprisingly, FPWDR and *bf* expression was similar in hkΔAN1556 compared to the control strain, which suggests that there are nuclei within hkΔAN1556 where FPWDR and the surrounding locus were not deleted, and thus their expression is retained [86]. Taken together with its proximity to the *chsD* gene and the presence of cell wall deformities, the alterations in gene expression observed for hkΔAN1556 indicate that FPWDR plays a role in the cell wall [86].

Gerin et al. used RT-qPCR to confirm their RNA-seq data, demonstrating differential expression of five polyketide synthases, four non-ribosomal peptide synthetases and one chloroperoxidase in *A. carbonarius* during OTA-inducing conditions [32]. To determine the best reference gene for normalisation, the group assessed the expression stability of beta-tubulin, calmodulin, and ubiquitin-conjugating enzyme during *A. carbonarius* growth in minimal medium at 25 °C in the dark without

shaking, using BestKeeper [157]. Of the three candidate reference genes tested, the ubiquitin-conjugating enzyme was the most stable, and therefore was used to normalise their RT-qPCR data [32]. The RT-qPCR analysis demonstrated similar patterns of gene expression as observed for RNA-seq, after *A. carbonarius* growth in minimal medium in the dark at 25 °C without shaking for four, six and eight days [32]. Overall, their results provide a comprehensive view of the different expression networks that may be connected to OTA production [32].

Omony et al. used the histone-encoding gene, *hist*, in their work building off their previous studies [158,159], and modelling the transcription dynamics of 23 genes in the XlnR regulon using time-course RT-qPCR expression data of *A. niger* [94]. The group previously experimentally validated the expression of the histone-encoding gene under the same experimental conditions, treatment with 1 mM or 50 mM D-xylose, and in the same strains used in their study, *A. niger* N400 (wildtype) and NW28 (a derepressed CreA mutant) [158]. As in their study four years earlier [158], Omony et al. found that the expression of XlnR regulon was higher for the 1 mM D-xylose treatment in both the wildtype and mutant strains [94]. They also found that the induction of hemicellulose genes was higher for the 1 mM D-xylose condition, and that *xlnR* expression was constant, regardless of functional CreA or D-xylose concentration [94]. The group also presents an updated kinetic differential equation model for the transcription of the XlnR regulon [94]. As the general results of their two studies were consistent, this is excellent support for the use of the histone-encoding gene for examining XlnR regulon transcription in *A. niger*, using 1 mM and 50 mM D-xylose for transcriptional induction [94,158].



## **2.5. Validation of Candidate Reference Genes in *Aspergillus***

The studies highlighted above use RT-qPCR to answer a diversity of research questions about gene expression in various species of *Aspergillus* under specific experimental conditions. In this section, we will highlight the need for studies validating the expression stability of candidate reference genes under standard laboratory conditions by discussing two large-scale validation studies in *A. flavus* and *A. niger*. We will also summarise all reference genes experimentally validated to date with their associated species and experimental conditions and highlight the traditional reference genes that were shown to be less stably expressed than the selected reference gene under the specific tested experimental conditions.

### **2.5.1. Validation of *hisH4* and *cox5* for Studying Aflatoxin Biosynthesis in *A. flavus***

From our PubMed search, only one study was returned that assessed the expression of multiple candidate reference genes under a specific set of conditions [111]. To find reference genes suitable for RT-qPCR analysis of aflatoxin biosynthesis genes in *A. flavus*, Suleman and Somai tested the expression stability of four reference genes (*actA* (actin), *sarA* (secretion associated binding protein), *hisH4* (histone H4) and *cox5* (cytochrome C oxidase subunit V)) under aflatoxin-inducing conditions, growth in sucrose low salts (SLS) or SLS supplemented with 117 mM ammonium sulphate (SLS + NH<sub>4</sub>), as well as non-inducing growth in lactose low salts (LLS), conditions at acidic (pH = 4.0) or alkaline (pH = 8.5) pH [111]. They chose actin, *sarA* and *cox5* for testing, as their expression stability was previously demonstrated in *A. niger* [160], and *hisH4*, as it had been previously used to normalise expression in *A. oryzae* [161].

Prior to assessing the stability of the candidate reference genes, Suleman and Somai assessed the primer pairs for each gene [111]. An ideal primer pair should: (1) produce an amplification efficiency

of ~100% (indicating a doubling of product per cycle); (2) produce a standard curve with a slope of  $-3.32$ , y-intercept less than 40 and correlation coefficient of  $>0.990$ ; and (3) amplify one product (as indicated by a single peak in melt-curve analysis) [19,162]. The amplification efficiencies of *cox5* and *hisH4* were ~100%, while the amplification efficiencies of *sarA* and actin were less than 80%, despite attempts to redesign and optimise the primers [111]. Therefore, only *cox5* and *hisH4* were assessed further [111]. As the standard deviations of the Cq values were less than one for both *cox5* and *hisH4* when comparing different treatment conditions, BestKeeper and REST2009 analyses demonstrated that there was no significant change in expression for either gene [111].

As mentioned in the introduction above, using multiple, validated reference genes is the MIQE recommended practice for normalising gene expression data [19]. Thus, to assess whether normalisation with one reference gene (*cox5* or *hisH4*) or both reference genes together yielded more robust results, the researchers normalised the expression of a “dummy” reference gene, with a set Cq value of 15, to the experimentally obtained Cq values of each reference gene, as well as to the sum of their Cq values, using REST2009 [111]. Using this method, the “dummy” gene should not exhibit a change in expression (expression ratio of ~1.0 with a p-value  $> 0.055$ ) [163]. When comparing normalisation with *hisH4* or *cox5* alone, to normalisation with both *cox5* and *hisH4* together, a better overall expression ratio was observed when using both reference genes for normalising the data from acidic and alkaline conditions [111]. When assessing the expression data under specific conditions, they found that normalisation with both reference genes also produced the best expression ratio for SLS + NH<sub>4</sub> [111]. However, under three sets of conditions (when normalising expression data following growth on SLS under acidic and alkaline conditions; when comparing expression during growth on SLS + NH<sub>4</sub> to SLS under acidic and alkaline conditions; and when comparing expression during growth on LLS to SLS under acidic conditions), normalisation with *hisH4* yielded the best

expression ratio [111]. Normalisation with *cox5* only yielded a better expression ratio for data obtained following growth on LLS, as well as when comparing expression during growth on LLS to SLS under alkaline conditions [111]. Collectively, these results show that the use of multiple reference genes is not always optimal for the conditions being tested [111]. Thus, this study highlights the need to validate the stability and the utility of reference genes under the specific conditions of each experiment.

### **2.5.2. Validation of *actA*, *sarA* and *cox5* for Studying *glaA* Expression in *A. niger***

Bohle et al. experimentally validated the expression stability of ten candidate reference genes (*act*, *sarA*, *cox5*, *apsC* (aminopeptidase C), *gpd* (GAPDH), *glkA* (glucokinase), *g6pdh* (glucose-6-phosphate dehydrogenase), *icdA* (isocitrate dehydrogenase precursor), *pfkA* (phosphofructokinase) and *pgiA* (phosphoglucose isomerase) in *A. niger* during growth in batch and continuous cultures, with *glaA*-inducing and -non-inducing conditions [160]. They first examined the expression stability of each gene in fedbatch cultures of *A. niger*, with glucose as the carbon source and continuous glucoamylase (*glaA*)-induction, for nine time points using geNorm [160]. Interestingly, one of the most used reference genes, GAPDH (used in ten studies highlighted in this review [30,31,35,52,54,60,71,90,92,110], two of which examined expression in *A. niger* [90,92] specifically) was the least stable under these conditions [160]. This further illustrates the importance of experimentally validating commonly used reference genes for use in study-specific conditions. Since the researchers were interested in examining expression stability for a diverse range of experimental conditions, they decided to examine the six most stably expressed genes, *act/sarA*, *g6pdh*, *cox5*, *apsC* and *pgiA* (most to least stable), under *glaA*-inducing (glucose as the carbon source) and -non-inducing (xylose or maltose as the carbon source) conditions in modified Vogel-Medium with varying stir speeds (400–1000 per min) and pHs (3.0 to 5.5) [160]. They found *act* and *sarA* to

be the most stably expressed, followed by *cox5* [160]. Since their dataset contained more samples for *glaA*-inducing conditions, they repeated their analysis with an equally represented dataset to ensure that the same three genes were consistently the most stable, irrespective of induction [160]. They found that the order of stability differed following the second analysis, where *act* and *cox5* exhibited the greatest stability, followed by *sarA* again, demonstrating the influence of experimental conditions on reference gene stability [160].

To further investigate the potential advantage of normalisation using three validated reference genes, they compared the correlation coefficients computed following regression with the normalisation factor for *act*, *sarA* and *cox5* ( $N_{act,sarA,cox5}$ ), the unvalidated reference gene, GAPDH ( $N_{GAPDH}$ ) and total RNA [160].  $N_{act,sarA,cox5}$  resulted in the highest correlation coefficient, demonstrating that the combination of the three validated reference genes was the best approach under the experimental conditions [160].

An important consideration noted by the authors is that these genes are all from different functional classes, and thus co-regulation of these genes is highly unlikely [160]. The absence of co-regulation is critical when using geNorm, as co-regulated genes can lead to high stability ranking and the inclusion of false positives [33]. Therefore, this work presents three experimentally validated reference genes that are suitable for studying *glaA* expression, while continuing to highlight the crucial first step of experimentally validating reference genes.

### **2.5.3. Reference Genes Currently Validated for Use in *Aspergillus***

Based on our review of the literature in Section 2.4., Section 2.5.1., and Section 2.5.2. above, the genes shown in Table 2.2. are recommended as reference genes for the specified experimental conditions. In this table, we also provide the species that these genes have been validated for, as well as the primers for each species and each recommended reference gene (Table 2.2.). Table 2.2. is

organised alphabetically by species first and reference gene second, followed by date. The information in Table 2.2. was extracted from 21 publications [16,27,30,32,35,38,41,46,52,54,55,77,86,94,103,105,107,111,141,144,160].

**Table 2.2.** Recommended reference genes for specific species and experimental conditions based on experimental validation.

Species	Reference		Reverse Primer (5'-3')	Experimental Conditions	Ref.
	Gene- Encoded Product	Forward Primer (5'-3')			
<i>A. aculeatus</i>	GAPDH	TACCGCTGCCCA GAACATCA	GGAGTGGCTGTC ACCGTTCA	Minimal medium (MM) with 1% (w/v) polypeptone, 1% (w/v) glucose, 1% (w/v) avicel, 1% (w/v) xylose, or 1% (w/v) arabinose for 3 h or 6 h	[35]
<i>A. carbonarius</i>	Ubiquitin- conjugating Enzyme	CCGAAGGTCAA CTTCACCAC	GGCATATTTGCG AGTCCATT	MM at 25 °C, without shaking (ochratoxin A (OTA)-inducing) conditions, for 4, 6 and 8 days in the dark	[32]
<i>A. fischeri</i>	Beta-tubulin  Histone 3	GCTCTTCCGTCC CGATAACTT CAAGAAGCCTC ACCGCTACAAG	CCTTGGCCCAGT TGTTACCA GACTTCTGGTAG CGACGGATTT	Growth on a hydrophobic polyvinylidene fluoride (PVDF) membrane on top of oatmeal agar for 3, 6 or 30 days (wildtype only)	[38]
<i>A. flavus</i>	18S rRNA  Beta-tubulin  Calmodulin	GCAAATTACCCA ATCCCGACAC CGCATGAACGTC TACTTCAACGAG CTTCCCCGAATT CCTTACC	GAATTACCGCG GCTGCTG AGTTGTTACCAG CAGCGGACT TCACGGATCATC TCATCGAC	Co-culture with <i>Listeria monocytogenes</i> in malt extract broth (MEB) at 25 °C and 30 °C for 7 days	[41]

	Beta-tubulin	CTTGTTGACCAG GTTGTCGAT *	GTCGCAGCCCTC AGCCT*	Inoculated onto 25 g of wheat and grown at 30 °C in open petri dishes with wetted filter paper for 9 days	[46]
	Beta-tubulin	AACGTCTACTTC AACGAGGCCA	GTACCAGGCTCA AGATCAACGAG	Malt extract agar (MEA) supplemented with 0.5 mM eugenol for 4 days at 27 °C	[52]
	GAPDH	CGTGTTGTTGAC CTCATTGCCT	GGTGACCTGATA ATCCGGGAAC	in the dark	
	Beta-tubulin	AACGTCTACTTC AACGAGGCCA	GTACCAGGCTCA AGATCAACGAG	Co-incubation with <i>Streptomyces roseolus</i> on International <i>Streptomyces</i>	
	GAPDH	CGTGTTGTTGAC CTCATTGCCT	GGTGACCTGATA ATCCGGGAAC	Project-2 (ISP2) medium at 30 °C for 4 days at a relative humidity of 80% in a Vötsch chamber	[54]
	Beta-tubulin	TCTCCAAGATCC GTGAGGAG	TTCAGGTCACCG TAAGAGGG	Yeast extract sucrose (YES) medium containing one of four antifungal peptides: PPD1, 66-10, 77-3, or D4E1	[55]
	Cytochrome C oxidase Subunit V	CGTCATTCACCT GTTGCGCTAAG	CCTTGGCATACT CGTTGGAAG	Sucrose low salts (SLS), SLS supplemented with 117 mM ammonium sulphate, and lactose low salts, at acidic	[111]
	Histone H4	TCGTCGTGGTGG TGTC AAG	TTGGCGTGCTCA GTGTAGG	or alkaline pH	
	18S rRNA	TCTTGTTAAACC CTGTCGTGCTGG	GTGTACAAAGG GCAGGGACGTA AT	Treatment with 125 µg/mL artemisinin or solvent for 3 h in Roswell Park Memorial Institute (RPMI) 1640 medium at 37 °C	[27]
<i>A. fumigatus</i>	Actin	TGCCCTTGCTCC CTCGTCTA	ACCGCTCTCGTC GTACTCCT	Mandels' salt solution with 1% oat spelts xylan for 0, 2, 4, 6 and 17 h	[16]
	Histone H4	GCTCGTCGTGGT GGTG TCAA	TGGCGTGCTCAG TG TAGGTG		
<i>A. nidulans</i>	Actin	TCAATCCCAAGT CCAACC (Tm 57 °C)	TACGACCGGAA GCATACA (Tm 57 °C)	Yeast extract-agar-glucose (YAG) supplemented with 15% polyethylene	[77]

	AATGGTTCGGGT	ACGCTTGGACTG	glycol (PEG) ( <i>w/v</i> ) compared to YAG	
	ATGTGC (T <sub>m</sub> 60 °C)	TGCCTC (T <sub>m</sub> 60 °C)	without supplementation for 2 days	
Actin-like protein	GTACGATGAGA GCGGTCCTT	CAGAAAATACG CGACAACGA	MM supplemented with 0.1% fructose and 5 mM urea at 30 °C for 15 h with shaking at 150 rpm	[30]
GAPDH	CGACAACGAGT GGGGTTACT	GGCATCAACCTT GGAGATGT		
Calmodulin	CCGAGTACAAG GAAGCTTTCTC	GAATCATCTCGT CGACTTCGTCGT CAGT	WB/MM supplemented with high, low or no iron for 24, 48 and 72 h	[144]
Putative ribosomal protein L3	TTCCTCGCAAGA CTCACAAG	TTGTGGTTGCAA GAGGTACG	Liquid MM under standard conditions	[86]
Putative ribosomal protein L37	CGCCACAACAA AACTCACAC	TCTCGCTCCAGT TGTACTIONG		
Actin	GGTCTGGAGAG CGGTGGTAT	cactgcGAAGAAG GAGCAAGAGCA GtG		
Cytochrome C oxidase Subunit V	GACCAAGGAGT GGCAGGAG	gaactgGGTGGGA GGCAGCAGtTC	<i>glaA</i> -inducing and -non-inducing conditions in modified Vogel-Medium with stir speeds and pH from 400–1000 per min and 3.0 to 5.5, respectively	[160]
Secretion Associated Binding Protein	gaacctACGGGTAA GGGCAAGGtTC	TCGCAACAATA AAGTCAACAGC		
Histone-encoding Gene	ATCTTGCGTGAC AACATCCA	CACCCTCAAGG AAGGTCTTG	Growth in bioreactors on sorbitol as the carbon source with 1 mM D-xylose or 50 mM D-xylose	[94]

*A. niger*

<i>A. parasiticus</i>	Actin	CTTGACTTCGAG CAGGAGAT	TCTGGATACGGT CGGAGATA	Treatment with or without 1.0 µM of blasticidin A in potato dextrose broth (PDB) at 27 °C for 2 days	[140]
	Actin	CGCGGATACAC CTTCTCCACTA *	ACGTAGCAGAG CTTCTCCTTGA *	Treatment with 0, 0.25, 0.5 or 1.0 µg/mL aflastatin A for 1.5 to 3.5 days in PDB at 27 °C	[103]
	Actin	NA	NA	Co-incubation with <i>Kluyveromyces lactis</i> at 30 °C for 48 h	[105]
	Beta-tubulin	TCTCCAAGATCC GTGAGGAG	TTCAGGTCACCG TAAGAGGG	YES medium containing one of four antifungal peptides: PPD1, 66-10, 77-3, or D4E1	[55]
<i>A. terreus</i>	Actin	TCGTGACTTGAC CGACTACC	TGATGTCACGGA CGATTCA	Lovastatin production medium at 27 °C for 10 days with shaking at 220 rpm	[107]

\*, used TaqMan system and therefore these primers have an associated probe, NA, not available.

While the reference genes described above are validated for use under the specific conditions described in Table 2.2. above, three papers that tested the expression stability of multiple candidate reference genes demonstrated that several traditionally used reference genes were less stably expressed under the experimental conditions tested than the reference genes that they chose [27,32,86]. In their preliminary experiments, Gautam et al. found that actin and GAPDH were less optimal reference genes than the 18S rRNA gene for RT-qPCR gene expression analysis of *A. fumigatus* during exposure to 125 µg/mL artemisinin or solvent control for 3 h at 37 °C [27]. Similarly, Gerin et al. found that beta-tubulin and calmodulin were less suitable reference genes than the ubiquitin-conjugating enzyme for RT-qPCR gene expression analysis of *A. carbonarius* during growth in minimal medium under OTA-inducing conditions for four, six and eight days in the dark at 25 °C [32]. Guerriero et al. also demonstrated that actin was less optimal for assessing gene expression in *A. nidulans* during growth in liquid minimal medium under standard conditions, than



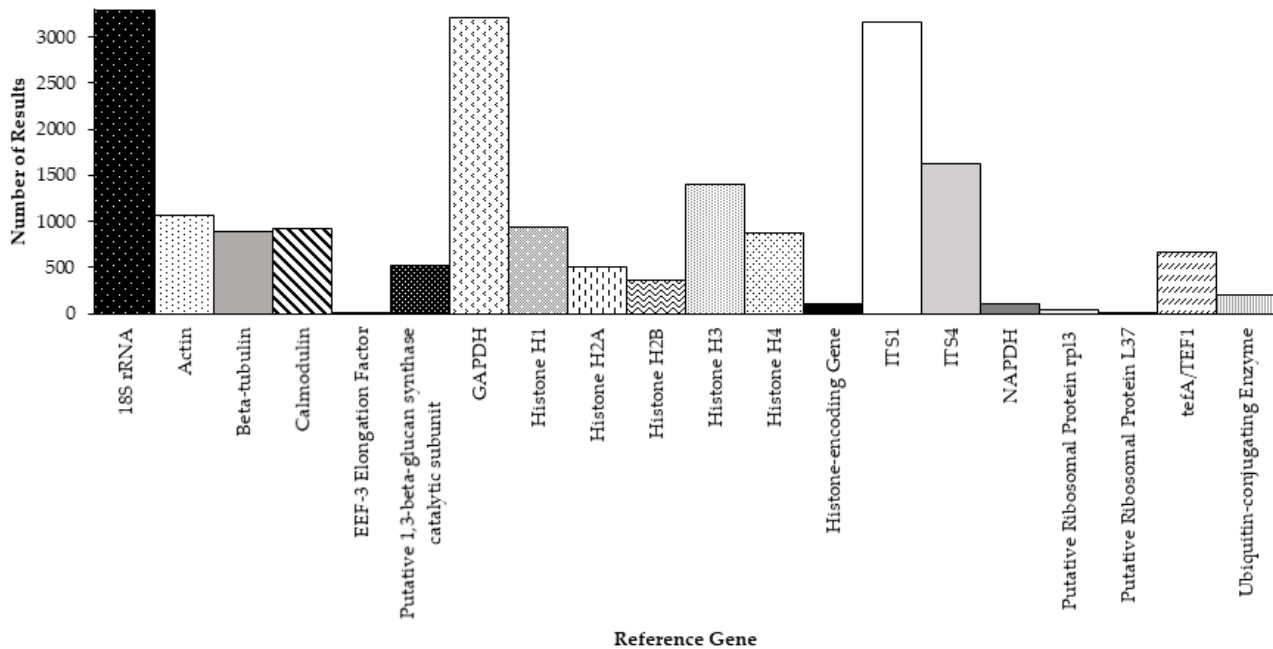
two putative ribosomal protein-encoding genes, *L37* and *L3* [86]. These two putative ribosomal genes were also found to be more suitable reference genes than *CRP2* and *TEF1* under these experimental conditions [86].

Additionally, as highlighted in the candidate reference gene validation study by Bohle et al. and discussed in Section 2.5.2 above, a traditionally used reference gene, GAPDH, was found to be the least stable candidate reference gene in feed-batch cultures of *A. niger*, with glucose and continuous *glaA*-induction [160]. Additionally, the candidate reference genes, *icdA*, *glkA* and *pfkA* (most to least stable), demonstrated low stability, and are therefore not the most suitable reference genes for use under these conditions [160]. Under *glaA*-inducing and non-inducing conditions in modified Vogel-Medium with a pH range of 3.0–5.5 and stir speed of 400–1000 per minute, the candidate reference genes *pgiA*, *apsC* and *g6pdh* (most to least stable) were demonstrated to be less stable than actin, *sarA* and *cox5* [160].

## 2.6. Reference Gene-Specific Google Scholar Queries

In this study, we focused our analyses on PubMed search results. However, though PubMed is a major database for the biomedical literature, there are other databases. As a broader search for RT-qPCR studies of *Aspergillus* fungi, we conducted an additional set of queries using Google Scholar, with the following structure: “*Aspergillus* “reference gene” qPCR”, where “reference gene” corresponds to the gene symbol associated with the reference gene of interest. We added quotation marks around the reference gene symbol to specifically search for the reference gene symbol as an entire string and ensure that the substrings comprising the reference gene symbol would not be searched separately. For example, the 18S rRNA gene was queried as “*Aspergillus* “18S rRNA” qPCR” and not “*Aspergillus* 18S rRNA qPCR”. The dates of the returned results of the queries ranged

from 1983 to 2021. Figure 2.2. summarises the search results for all reference genes reviewed in our initial PubMed searches. The Google Scholar query that returned the most results corresponded to the 18S rRNA gene, returning 3290 results total (Figure 2.2.). This was followed by the query for GAPDH with 3209 results, ITS1 with 3170 results and ITS4 with 1630 results (Figure 2.2.).



**Figure 2.2.** Number of search results for each reference gene based on their corresponding Google Scholar query. The 18S rRNA reference gene returned the most results, with 3290 results returned.

It is important to note that the search queries described in this section have several limitations. One limitation is that because qPCR is also used as a diagnostic tool for the detection and quantification of *Aspergillus* species [164], the results of each query likely contain these studies, as well as relevant gene expression studies. Additionally, any papers that contain “*Aspergillus*” in the body of their report, such as the introduction, but do not specifically examine gene expression in *Aspergillus*, may

also be returned by the queries. Therefore, while the total results returned by each query may be used as a proxy for reference gene use frequency in qPCR gene expression analysis, the Google Scholar search results require manual curation as was done during the original PubMed search described in Section 2.4. above, in order to determine the relevance of the Google Scholar search results literature to our current study.

## **2.7. Concluding Remarks and Recommendations**

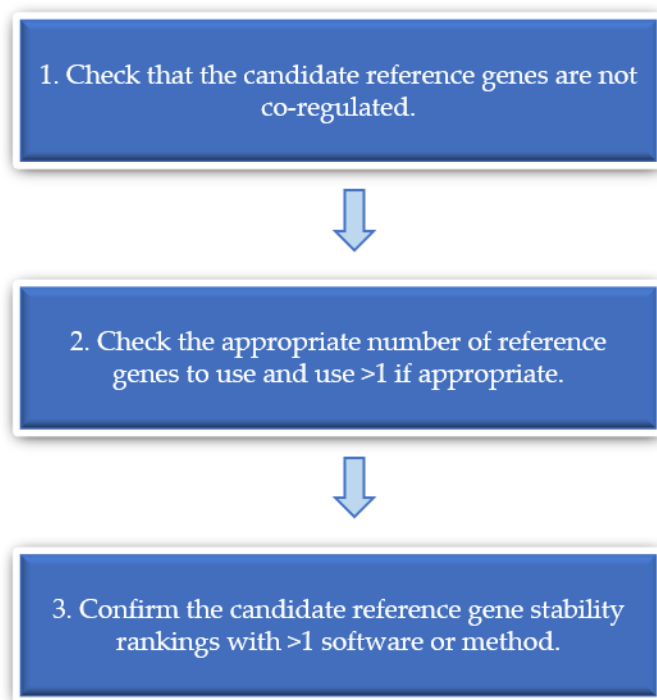
In a recent 2018 review of reference gene validation practices for RT-qPCR of insects, Shakeel and colleagues discuss the validation of reference genes thus far for select insect species, while emphasising the need for a comprehensive group of studies to be conducted under diverse experimental conditions for all species of insects [165]. They note that several studies of classical housekeeping genes show varying expression under different experimental conditions, and indicate ribosomal genes as a promising new set of genes for further stability analysis in insect-specific studies [165].

In the literature reviewed above, we discussed several methods employed by those who validated the reference genes for normalisation in their studies, including geNorm [33], BestKeeper [157] and NormFinder [119]. Shakeel and colleagues discussed the benefits of these and other methods, RefFinder and  $\Delta C_t$ , for assessing reference gene stability, and noted how each method may lead to slight differences in reference gene stability rankings [165]. As stated by the group, both geNorm and NormFinder are excellent programs for the initial assessment of candidate reference gene stability, each with their own advantage, with geNorm capable of determining the number of reference genes to use [33], and NormFinder computing the stability of each reference gene separately to avoid the consequences associated with co-regulation [119]. Given that different programs for determining

reference gene stability may yield different stability rankings, we recommend that researchers use more than one program to validate the stability of the reference genes used under the experimental conditions being tested. In agreement with Shakeel and colleagues, because the results of some programs, such as geNorm, may be biased due to co-regulation, care should be taken to select candidate reference genes that are not co-regulated.

Interestingly, Shakeel and colleagues noted nearly the same four reference genes as those most used in RT-qPCR studies in general across organisms: beta-actin, GAPDH, beta-tubulin and 18S rRNA, citing papers as early as 2004 [165]. Our examination of 90 RT-qPCR studies, spanning 2001 to 2020, further demonstrates this to be the case for *Aspergillus*. The authors note that as of 2000, beta-actin and GAPDH were used 90% of the time without proper validation [165]. Out of the 30 usages of actin and 10 usages of GAPDH across the 90 studies we examined, actin was used without validation approximately 83% of the time, and GAPDH 60%. Similarly, of the 31 usages of beta-tubulin and 12 usages of 18S rRNA across the 90 studies we examined, beta-tubulin was used without validation approximately 81% of the time, and 18S rRNA, approximately 83%.

Since its publication in 2018, this article by Shakeel and colleagues on insects has been cited 22 times by articles in PubMed. Fourteen of these citing articles are those evaluating candidate reference genes [166,167,168,169,170,171,172,173,174,175,176,177,178,179]. We hope that our critical review will similarly stimulate future research on experimentally validating reference genes for gene expression studies in *Aspergillus* (and in fungi in general) using RT-qPCR. Without experimental validation of reference genes, it can be difficult to interpret the potential contributors to expression differences among strains, genes and treatments. Figure 2.3. below summarises our recommended practice for reference gene selection.



**Figure 2.3.** Recommended checkpoints to use when selecting reference genes.

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## 2.6. Supplementary Material

**Table S2.1.** Summary of 90 RT-qPCR studies.

Genus	Species	Strain	Sample	Biological Replication	Gene Symbol	Associated Product	Justification /Validation? (Yes/No)	Conditions	Normalization Strategy for Assessing Changes in Gene Expression	Ref .
<i>Aspergillus</i>	<i>niger</i>	A09	Mycelia	3	<i>18S</i>	18S rRNA	No	Growth in potato dextrose agar (PDA) media supplemented with different carbon sources (derived from xylan, xylose, glucose, maltose, cellobiose, arabinose, or lactose) and nitrogen sources (derived from yeast extract, NH <sub>4</sub> NO <sub>3</sub> , (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , peptone, or urea, and NaNO <sub>3</sub> , besides ammonium oxalate).	2 <sup>-</sup> ΔACT	[87]
		N402	Conidia, germinating spores and mycelia	4	<i>act</i>	Actin	Yes- Cite previous use by their research group, one of which cites Bohle et al. 2007 who validated the use of actin as a reference gene for <i>A. niger</i> [159]. However, the media conditions were not the same.	Growth in liquid <i>Aspergillus</i> Minimal Medium (AMM) for 0, 3, 6, 12 and 72h as well as on AMM plates for 5 days.	Pfaffl method	[89]
		J693 ( <i>tppB</i> +)								
		J685 ( <i>ΔtppB</i> )								
		MA169.4	Mycelia	NA	<i>gpdA</i>	Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	No	Growth in glucose maltose polypeptone yeast extract (GMPY) medium at 30°C, 250 rpm for 2 days.	NA	[92]
		MA169.4delAnPKSIII								
		MA169.4oeAnPKSIII								
		CBS 141257	Mycelia	2	<i>H2B</i>	Histone H2B	No	Mycelia transfer from complete medium (CM) with 2% D-fructose to 10 mL of minimal medium (MM) supplemented with 25 mM carbon source L-rhamnose or L-rhamnonate and grown at 30°C for 2 hours with 250 rpm shaking.	2 <sup>-</sup> ΔACT	[93]
		<i>AlraA</i>								
		<i>AlraB</i>								
		<i>AlraC</i>								
		<i>ArhaR</i>								

		N400 (CBS120.49)					Yes- Cite the methods in one of their other published papers, Mach-Aigner et al. 2012, which demonstrated the histone-encoding gene to be stably expressed under the same growth conditions, and with the same strains used [157].				
		NW283	Mycelia	2	<i>hist</i>	Histone-encoding Gene			Growth in bioreactors on sorbitol as the carbon source with 1 mM D-xylose or 50 mM D-xylose. The wildtype was sampled every 20 minutes over 5 hours. The mutant was sampled hourly over 5 hours.	Pfaffl method	[94]
		BCC4603	NA	NA	<i>18S</i>	18S rRNA *	No	Growth on PDA at 25°C under normal light (WT containing genomic DNA). The resulting transformants were selected on and subcultured three times in the presence of 1.25 mg/mL of 5-fluoroorotic acid with uridine and uracil to ensure the stability of transgene.	2 <sup>-ΔΔCT</sup>	[88]	
		Anig1_1									
		Anig1_2									
		Anig2_1									
		Anig2_2									
		CGMCC 10142	Spores	3	<i>β-actin</i>	Actin	No	Growth in liquefied corn starch medium at 35°C for 72 h with shaking at 330 rpm (sampling at 10, 16, 24, 36, 48, 60, 72 h).	2 <sup>-ΔΔCT</sup>	[90]	
		3-4			<i>GADPH</i>	GAPDH					
		102									
		<i>AgfsA</i>	Mycelia	NA	<i>actA</i>	Actin	No	Growth in complete medium (CM) for 25h.	ΔΔCq	[91]	
		<i>AgfsB</i>									
		<i>AgfsC</i>									
		<i>AgfsAC</i>									
		<i>AgfsAB</i>									
		<i>AgfsBC</i>									
		<i>AgfsABC</i>									
		<i>AugmA</i>									
		<i>wt</i>									
	<i>nidulans</i>	TN02A7	Frozen mycelia	3	NAPDH	Nicotinamide adenine dinucleotide phosphate + hydrogen	No	Growth in 100 mL of modified MMPKRUU medium in K sufficient (supplemented with 0.52 g/L KCl) or deficient (supplemented with 5.2 g K-feldspar powders) at 37°C for 24h with shaking at 220 rpm.	ΔCt	[85]	
			FGSCA4	Conidia and spores	3	<i>benA</i>	Beta-tubulin	No	Growth in minimal medium containing 2% glycerol, supplemented with arginine,	NA	[82]

			<i>H2B</i>	Histone 2B		uracil, uridine and vitamins during exposure to white-light or darkness at 23°C for 24h (spores) or 30°C for 40h in distilled water (conidia).		
FGSC26	Lyophilized mycelia	4	<i>eeF-3</i>	EEF-3 Elongation Factor	No	Growth in 100 mL of glucose-free minimal nitrate medium (carbon-starved media) or minimal nitrate medium containing 10 g/L of glucose at 37°C for 4h or 24h.	ΔCP	[84]
A1149	Lyophilized mycelia	3	<i>actA</i>	Actin	Yes- Cite previous literature in which actin was found to be a stable reference gene for <i>A. niger</i> . However, the media conditions differ.	Growth in CM with glucose or threonine or on MM with ammonium or nitrate, depending on the strain. Incubated at 28°C for 16h with shaking at 250 rpm.	2 <sup>-ΔΔCT</sup>	[78]
AAE1								
AAE2								
AAE5								
ASA1								
AAE12								
AAE13								
ASA4								
AKA10								
AKA11								
AKA12								
AF293								
pabaA1	Frozen mycelia	3	<i>β-actin</i>	Actin	No	Growth in low or high inorganic phosphate yeast extract medium (YEM) and MM at 37°C for 17h with shaking at 200 rpm.	ΔΔCt	[76]
pabaA1 palB7			<i>β-tubulin</i>	Beta-tubulin				
biaA1; riboB2; niiA4	Mycelia	3	<i>β-tubulin</i>	Beta-tubulin *	No	Growth in MM supplemented with 50 mM ethyl methyl ketone (EMK) or 1% glucose at 37°C for 16h to 18h with shaking at 150 rpm.	NA	[83]
creB1936								
hkΔAN1556	Hyphae	NA	<i>rpl37</i>	Putative ribosomal protein L37	Yes- Analyzed 5 candidate reference genes and ranked their stability using geNormPLUS S [33] and used the two most stable genes.	Growth in liquid MM under standard conditions.	qBasePLUS version 2.5	[86]
SAA.111			<i>rpl3</i>	Putative ribosomal protein L3				
TN02A7	Mycelia	3	NA	Actin	No	Growth in yeast extract-agar-glucose (YAG) supplemented with 5 mM uridine and 10 mM uracil (YUU) at 37°C for 24h.	2 <sup>-ΔΔCT</sup>	[79]
WJA01	Mycelia	3	<i>actA</i>	Actin	Yes - Noted the expression stability of actin under the conditions being tested in their experiments.	Growth in YAG supplemented with 15% polyethylene glycol (PEG) (W/V) compared to YAG without supplementation for 2 days.	2 <sup>-ΔΔCT</sup>	[77]
<i>cnaA</i> mutant								
<i>mobB/cotA</i>								
R21	Frozen mycelia	NA	<i>tubC</i>	Beta-tubulin	No	Growth yeast glucose (YG)	Standard Curve Method (Cycle	[80]

		ima532						medium in the presence or absence of drugs (25 µM camptothecin, 0.3 µg/mL imazalil, 0.2 µg/mL itraconazole, 100 µg/mL hygromycin, and 2.5 µg/mL 4-nitroquinoline oxide (4-NQO)) for 8h.	threshold (CT) values plotted against a logarithm of the DNA copy number	
		ima533								
		ima534								
		ima535								
		ima537								
		<i>areA217</i>						Growth in 3% lactose for 18h, after which a source of induction and/or repression was added and the cultures were grown for 4h. For <i>alcA</i> and <i>aldA</i> expression, EMK was used as the inducer and glucose or acetate was used as the repressor. For <i>alcB</i> expression analysis the strain of interest was grown under carbon-limiting conditions.	Standard Curve Method (CT values plotted against a logarithm of the DNA copy number)	[81]
		<i>acdXAareA217</i>	Mycelia	3	<i>tubC</i>	Beta-tubulin	No			
		<i>sptCAareA217</i>								
		CV125				<i>actA</i>	Actin	Yes- Tested the stability of the chosen reference genes using GeNorm in qBase PLUS tool [33].	Pfaffl method	[30]
		NA1363	Mycelia	4	<i>gpdA</i>	GAPDH				
		GFP-expressing strain derived from ATCC 13073	Conidia	NA	<i>gpdA</i>	GAPDH	No	Exposure of <i>A. fumigatus</i> to human airway epithelial cells (AECs) or human bronchial epithelial cells (16HBE14o-).	$2^{-\Delta\Delta CT}$	[31]
		Af293	Lyophilized mycelia	3	<i>fs1</i>	Putative 1,3-beta-glucan Synthase Catalytic Subunit	No	Growth in Barrat's minimal nitrate medium in the presence (H <sub>2</sub> O <sub>2</sub> at 3 mM final concentration, sampling at 1 hour) or absence of oxidative stress and/or iron-limitation (30 µM FeCl <sub>3</sub> for iron replete cultures and 0 µM FeCl <sub>3</sub> for iron deficient cultures) for 33h and 50h.	$\Delta\Delta CP$	[72]
	<i>fumigatus</i>	Af293	Frozen fungal culture containing mycelia	2	<i>18S</i>	18S rRNA	Yes- Performed preliminary experiments to assess the expression stability of GAPDH, actin and 18S rRNA genes under the conditions described. The 18S gene exhibited the greatest	Growth in 100 mL of Roswell Park Memorial Institute (RPMI) 1640 medium (with L-glutamine and sodium bicarbonate) with treatment with artemisinin (125 µg/ml) or solvent control at 37°C for 3h.	$\Delta\Delta CT$	[27]

						expression stability.			
	CM237	Mycelia	3	<i>βact</i>	Actin	No	Treatment with 24,700 , 12,300 and 6,170 µg/mL kombucha during growth in RPMI medium at 35°C for 48h.	REST software (2009) (uses 2 <sup>-ΔΔCT</sup> )	[61]
	CM2627								
	10 azole-susceptible								
	5 azole-resistant								
	AfIR974	Conidia	4	<i>actin</i>	Actin	No	Growth on sabouraud agar slants for 5 days at 30° C followed by dilution in RPMI medium and growth at 37°C for 0, 2, 4, 6, and 8h.	ΔΔCT-method	[21]
	AfIR964								
	F17727	Mycelia	3	<i>tub</i>	Beta-tubulin	No	Growth in Vogel's medium supplemented with 1% glucose at 37°C with shaking at 250 rpm for 14–16h, with added 4 mg/L itraconazole or DMSO.	2 <sup>-ΔΔCT</sup>	[69]
	F18149								
	F19980								
	F17999								
	F18085								
	F20140								
	F18304								
	F20063								
	F20451								
	F18454								
	F18329								
	F15483								
	Af293 (CBS 101355)								
	Af210								
	CEA10 (CBS 144.89)								
	Z5	Frozen mycelia	NA	NA	Actin	Yes- were determined to be the most stable genes out of five potential reference genes tested using geNorm [33].	Growth in Mandels' salt solution with 2% sucrose for 20h at 50°C with shaking at 150 rpm. Following 20h incubation, the mycelia were washed with distilled water and added to Mandels' salt solution with 1% oat spelt xylan and incubated for 0, 2, 4, 6 and 17h.	Pfaffl method	[16]
					Histone H4				
	CEA17 <i>Aku80</i>	Frozen mycelia	3	<i>18S</i>	18S rRNA	No	Growth in liquid AMM and cultured for 18 h at 37°C with shaking at 200 rpm.	2 <sup>-ΔΔCT</sup>	[59]
	cofilin OE								



		ATCC, #34506	Mycelia	NA	<i>18S</i>	18S rRNA	No	Growth on PDA plates in the presence of different carbon dioxide concentrations (280, 325, 360, 392, 450, 560 ppm), and on Czapek media, with different C : N ratios (5:1, 8:1, 10:1, 12:1 15:1, reverse for changes to the nitrogen concentrations) for 48h and 5 days.	2 <sup>-AACT</sup>	[58]
		Amphotericin B-susceptible - was used as a control for <i>A. terreus</i> expression	Cells	2	<i>BTU</i>	Beta-tubulin *	No	Growth in Sabouraud medium for 24h at 37°C with slight shaking and treatment with sub-lethal (0.25 mg/mL) and lethal Amphotericin B (AmB) (1 mg/mL) concentrations.	Pfaffl method	[68]
		ATCC 46645	Biofilm and planktonic mycelia	3	<i>act1</i>	Actin	No	Biofilm growth at 37°C in minimal essential medium (MEM) supplemented with 5% v/v fetal calf serum (FCS) or 5% v/v phosphate buffered saline (PBS). Exposure to 5% CO <sub>2</sub> for 2h was used to promote fungal adherence to the surface of the flask. Unattached conidia were washed with MEM (supplemented with either FCS or PBS) and the remaining were left to grow for 24 and 48h.	NA	[22]
		CBS 144.89	Lyophilized mycelia	2-3	<i>GAPDH</i> - only used for the inducible mutant	GAPDH	No	For gene expression analysis of CBS 144.89 and <i>AsrbA</i> only; treatment with MIC <sub>50</sub> of fluconazole (CBS 144.89, 128 µg/mL; <i>AsrbA</i> strain, 0.50 µg/mL) or voriconazole (CBS 144.89, 0.0625 µg/mL; <i>AsrbA</i> strain, 0.006 µg/mL) and grown in liquid glucose minimal medium (GMM) at 37°C with shaking at 300 rpm. For gene expression analysis including <i>pniiA-erg11A-AsrbA</i> : All three strains were grown in liquid GMM plus 20 mM NO <sub>3</sub> for 12h and liquid GMM plus 20 mM NH <sub>4</sub> at 37°C with shaking at 300 rpm.	iQ5 optical system software package (uses 2 <sup>-AACT</sup> )	[60]
		<i>AsrbA</i>			<i>actin</i>	Actin				
		<i>pniiA-erg11A-AsrbA</i>			<i>tefA</i>	NA				

		ATCC 13073	Mycelia	NA	NA	Beta-tubulin	No	Growth in MEM supplemented with 10% (v/v) human serum (male) and 50 µM FeCl <sub>3</sub> at 37°C for 6h.	2 <sup>-</sup> ΔΔCT	[65]
		Af293	Mycelia	≥2	TEF1	Putative Translation Elongation Factor EF-1 Alpha Subunit	No	<i>in vitro</i> : (1) growth in yeast peptone dextrose (YPD) medium for 4h, 8h, and 1 to 7 days at 37°C, (2) growth in YPD medium for 24 h at 37°C, (3) growth in YPD or RPMI medium for 24h and 72h at 37°C, (4) growth in YPD medium for 5 to 8 days at 37°C; <i>in vivo</i> : (1) growth in mice lungs for 4h, 8h, and 1 to 7 days, (2) growth in mice lungs for 24h and 72h, (3) growth in mice lungs 5 to 8 days.	Pfaffl method	[73]
		complimented <i>stuA</i>								
		<i>AstuA</i>								
		CEA17/ <i>ΔakuB</i> <sup>KU80</sup>	Mycelia	3	TEF1	Putative Translation Elongation Factor EF-1 Alpha Subunit	No	Growth in a glucose (3%)-yeast extract (YE; 1%) liquid medium for 16 h at 37°C.	2 <sup>-</sup> ΔΔCT	[74]
		<i>ΔcsmA</i>								
		<i>ΔcsmB</i>								
		<i>ΔcsmA/ΔcsmB</i>								
		ATCC 204305	Homegenised cells	NA	<i>β-tubulin</i>	Beta-tubulin *	No	Four formed fungi balls or 2 mL of fungal suspension in NaCl (corresponding to a McFarland 1 standard) were then incubated for 3h at 25, 30, 35 and 40°C.	The comparative SYBR Green/ΔCt method	[67]
		Af293	Frozen mycelia	5	<i>β-tubulin</i>	Beta-tubulin	No	Growth in mouse lung alveoli for 4 or 14h.	Standard Curve Method (CT values plotted against a logarithm of the DNA copy number)	[66]
		<i>ΔCaIA</i>								
		<i>ΔcrzA</i>								
		<i>ΔpmcA</i>								
		<i>ΔpmcB</i>								
		CEA17 (pyrG), ATCC 46645	Frozen mycelia	NA	<i>tubC</i>	Beta-tubulin	No	Growth in YG medium containing or without 0.5 µg of voriconazole for 30, 60, 120, and 240 min	Standard Curve Method (CT values plotted against a logarithm of the DNA copy number)	[63]
		<i>ΔpkaC1</i>								
		<i>ΔacyA</i>								
		CEA10	Lyophilized mouse lung tissue	NA	TefA	NA	No	Growth in mouse lung subcutaneously injected with 40 mg/kg Kenalog (a steroid) 1 day, or intraperitoneally with 175 mg/kg of cyclophosphamide 2 days prior to inoculation.	ΔΔCT method	[70]
					<i>β-tubulin</i>	Beta-tubulin				

		AF293						(1) Growth in GMM at 25°C with shaking at 280 rpm for 60 days and (2) Growth in high iron liquid media (25 g/L glucose, 3.5 g/L (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 2.0 g/L KH <sub>2</sub> PO <sub>4</sub> , 0.5 g/L MgSO <sub>4</sub> (heptahydrate), and 8 mg/L ZnSO <sub>4</sub> (heptahydrate) + 300 µM Fe(III)Cl <sub>3</sub> (pH 6.3)) or low iron media (same as high iron, but without the Fe(III)Cl <sub>3</sub> at 37°C with shaking at 280 rpm for 24h.		
		TW54.2 ( <i>DlaeA</i> )	Fungal mats	2 or 3	NA	Actin	No		2- <sup>Δ</sup> ACT	[23]
		TW68.6 ( <i>DlaeA</i> + <i>laeA</i> )								
		H11-20								
		RIT1								
		RIT2								
		RIT3								
		RIT4								
		RIT5								
		RIT6								
		RIT7								
		RIT8								
		RIT9								
		RIT10								
		RIT11								
		RIT12								
		RIT13	Mycelia	3 to 5	β-tubulin	Beta-tubulin	Yes- Note that its mRNA levels are constitutively expressed at a high level under various environmental conditions. However, did not validate themselves for their specific experimental conditions.	Growth in 15ml of yeast extract-peptone-dextrose (YEPD) medium supplemented with 10 µg/mL (H11-20) or 100 µg/mL of itraconazole for 8h at 37°C.	Standard Curve Method (CT values plotted against a logarithm of the DNA copy number)	[62]
		RIT14								
		RIT15								
		RIT16								
		RIT17								
		RIT18								
		RIT19								
		RIT20								
		RIT21								
		RIT22								
		RIT23								
		RIT24								
		RIT25								
		RIT26								
		CEA17								
		G10-1-50A	NA	NA	<i>GPDH</i>	GAPDH	No	Treatment with 10 ng/mL of itraconazole (CEA17) or 5 µg/mL of itraconazole	NA	[71]
		G10-1-50B								



	KSU116								
	KSU117								
	KSU118								
	KSU119								
	KSU120								
	KSU121								
	KSU122								
	KSU001								
	KSU002								
	KSU003								
	KSU004								
	ATCC28542	NA	NA	<i>β-actin</i>	Actin	No	Growth in YES medium containing 1.5 mg /100mL silver nanoparticles (AgNps HA1N, AgNps EH, and AgNps HA2) at 28°C for 14 days.	2 <sup>-ΔΔCT</sup>	[44]
	ICB-1	NA	3	<i>β-tubulin</i>	Beta-tubulin	No	Growth on coconut agar at 25°C for 2 or 7 days.	Pfaffl method	[53]
	ICB-12								
	ICB-54								
	ICB-141								
	ICB-161								
	ICB-198			<i>cal</i>	Calmodulin				
	YC-15	Mycelia	NA	<i>18S</i>	18S rRNA	No	Growth in YES medium supplemented with 0.40 mmol/L of cinnamaldehyde, 0.56 mmol/L of citral, and 0.80 mmol/L of eugenol for 7 days.	2 <sup>-ΔΔCT</sup>	[40]
	NRRL3357	Mycelia	3	NA	Actin	No	(1) Growth on PDA or GMM supplemented with NH <sub>4</sub> <sup>+</sup> for 48h and (2) growth on GMM supplemented with 50 mM ammonium or NaNO <sub>2</sub> (as the only nitrogen source) for 30h.	2 <sup>-ΔΔCT</sup>	[43]
	<i>ΔnmrA</i>								
	<i>ΔnmrA::nmrA</i>								
	NRRL 62477	Frozen mycelia	≥3	<i>β-tub</i>	Beta-tubulin	Yes- Evaluated the stability of several potential reference genes using NormFinder [119] and used the most stable reference genes for normalization.	Growth on malt extract agar (MEA) supplemented with 0.5mM eugenol for 4 days at 27°C in the dark.	2 <sup>-ΔΔCT</sup>	[52]
			<i>gpdA</i>	GAPDH					



		RCP08108	Frozen mycelia	3	<i>ITS1</i>	Internal Transcribed Spacer Regions	Yes- Cite previous use in the literature.	First, the fungi were grown on peanut meal extract agar (PMEA) for 5 days at 28 °C to obtain heavily sporulating cultures. The water activity of cultures were modified using nonionic solute glycerol to -2.8, -7.1, -9.9 and -16.0 MPa to generate water activities of 0.98, 0.95, 0.93 and 0.89, respectively. Next, cultures of differing water activity were supplemented with 1+1 mM 2(3)-tert-butyl-4 hydroxyanisole-2,6-di (tert-butyl)-p-cresolor 5 + 5 mM 2(3)-tert-butyl-4 hydroxyanisole-2,6-di (tert-butyl)-p-cresol (i.e. differing antioxidant concentrations) at 20°C and 28°C for 72 hours.	NA	[57]
		PFCC 83	Frozen mycelia	NA	<i>tub1</i>	Beta-tubulin <sup>*</sup>	No	YES, aflatoxin-inducing, or yeast extract peptone (YEP) medium, non-inducing, at 28°C for 4 days.	NA	[48]
		PFCC 86								
		PFCC 101								
		PFCC 116								
		PFCC 137								
		PFCC 147								
		PFCC 149								
		PFCC 154								
		PFCC 165								
		PFCC 176								
		PFCC 186								
		PFCC 191								
		PFCC 193								
		PFCC 209								
		PFCC 213								
		PFCC 224								
		PFCC 244								
		PFCC 247								
		PFCC 267								
		PFCC 285								
		PFCC 288								
		PFCC 309								
		PFCC 313								
		PFCC 316								
		CMI 102566								

	CMI 93803								
	NRRL	Frozen mycelia	2	<i>act1</i>	Actin	No	Co-incubation with soil isolates of <i>Streptomyces</i> on ISP2 medium at 28°C.	qbase+ software (biogazelle)	[42]
				<i>βtub</i>	Beta-tubulin				
	NRRL3357	Fungal tissue	3	<i>18S</i>	18S rRNA	No	Growth on YES medium at 37°C for 1.5 days and 28°C for 3 days (to obtain the same amount of biomass at a lower temperature), in the dark.	ΔΔCt	[28]
	NRRL-6513	Mycelia	3	<i>β-tubulin</i>	Beta-tubulin *	Yes- The researchers note that the peptides created did not have an effect on β-tubulin expression - no data shown.	Growth in 20 mL of YES medium containing one of four antifungal peptides: PPD1 peptide (4 μg/mL), 66-10 peptide (3.5 μg/mL), 77-3 peptide (3 μg/mL), or D4E1 (12 μg/mL).	2 <sup>-ΔΔCT</sup>	[55]
	NRRL 3357	Cells	6	<i>TUB</i>	Beta-tubulin	No	Treatment or no treatment with the subinhibitory concentrations of carvacrol (0.02%) or trans-cinnamaldehyde (0.005%) in 10 mL of PDB at 25°C for 5 days.	Comparative Critical Threshold Method	[50]
	NRRL 4123								
parasiticus	ATCC #15517	Frozen mycelia	NA	<i>ACT1</i>	Actin	Yes- State that they demonstrated beta-actin to be stably expressed in the presence of <i>Kluyveromyces lactis</i> .	Treated or untreated with 1.5 × 10 <sup>5</sup> CFU/mL of <i>Kluyveromyces lactis</i> and incubated at 30°C for 48h.	REST, 2008 V2.0.2 software (ΔΔCt)	[10 5]
	BN9Δ <i>amsA</i>	Frozen mycelia	4	<i>18S</i>	18S rRNA	No	Growth of stationary phase cultures in PDB at 30°C for 4 days in the dark.	Relative Standard Curve Method (CT values plotted against a logarithm of the DNA copy number)	[29]
	NRRL-2999	Mycelia	3	<i>β-tubulin</i>	Beta-tubulin *	Yes - the researchers note that the peptides created did not have an effect on β-tubulin expression.	Growth in 20 mL of YES medium containing one of four antifungal peptides: PPD1 peptide (4 μg/mL), 66-10 peptide (3.5 μg/mL), 77-3 peptide (3 μg/mL), or D4E1 (12 μg/mL).	2 <sup>-ΔΔCT</sup>	[55]
	CECT 2688	Frozen mycelia	3	<i>TUB</i>	Beta-tubulin	No	Yeast extract sucrose peptone (YESP) medium modified with 1% sodium nitrate (YESP-N) or 1% tryptophan (YESP-T) at 10, 15, 25, 30 and 37°C for 96h with shaking at 100 rpm.	2 <sup>-ΔΔCT</sup>	[10 6]
	NRRL 2999	Frozen mycelia	NA	<i>tub1</i>	Beta-tubulin *	No	Growth in YES or YEP medium at 28°C for 4 days.	NA	[48]
	PFCC 14								



	NRRL 2999	Mycelia	NA	$\beta$ -actin	Actin	Yes- Used in a previous study with the same species conducted by the same authors.	Treatment with 0, 0.25, 0.5 or 1.0 $\mu$ g/mL aflastatin A for 1.5 to 3.5 days in PDB at 27°C.	NA	[10 3]
	ATCC 24690								
	ATCC15517	Mycelia	NA	$\beta$ -actin	Actin	No	Growth in RPMI medium containing 25 or 50 mg/mL of vitamin C for 3 days at 28°C.	Statistical Package for the Social Sciences (SPSS) software for Windows, using the t-test and Fisher's exact test, where a p-value less than 0.05 was considered to be statistically significant.	[10 4]
<i>sojae</i>	SRRC 2043	Spheroplasts	NA	18S	18S rRNA	No	Growth in Adye and Mateles (1964) medium for 48h and 72h.	2 <sup>-</sup> AACT	[10 2]
	299								
	1123								
	1126								
<i>cristatus</i>	E4 (CGMCC 7.193)	Mycelia	NA	$\beta$ -actin	Actin	No	Cellulose membrane on malt yeast agar (MYA) and on 17% NaCl MYA media for 5 days at 28°C in the dark, after which mycelia were fixed (extraction from 7 day mycelia).	2 <sup>-</sup> AACT	[24]
<i>oryzae</i>	RIB40 <i>creBA</i>	Mycelia	3	$\beta$ -tubulin	Beta-tubulin *	No	Growth conditions dependent on transcript of interest. (1) $\alpha$ -amylase transcripts: inducing (1% sorbitol plus 1% starch), non-inducing (1% sorbitol), and repressing (1% sorbitol plus 1% starch plus 2% sucrose) conditions for 48h. (2) glucoamylase A transcripts: inducing conditions (in 1% starch) or repressing conditions (in 2% sucrose) at 30°C for 24h.	NA	[47]
	DAR3699								
	Aory1_1	NA	NA	18S	18S rRNA *	No	Growth on PDA at 25°C under normal light (WT containing genomic DNA). The resulting transformants were selected on and subcultured three times in the presence of 1.25 mg/mL of 5-fluoroorotic acid with uridine and uracil to ensure the stability of transgene.	2 <sup>-</sup> AACT	[88]
	Aory1_2								
	Aory2_1								
	Aory2_2								
	$\Delta$ LaeA	NA	NA	HI	Histone 1	No	Growth in MMcontaining 0.6% NaNO <sub>3</sub> , 0.052% KCl, 0.052% MgSO <sub>4</sub> , 0.152% KH <sub>2</sub> PO <sub>4</sub> and 1% glucose at 30°C for 5 days.	2 <sup>-</sup> AACT	[10 0]
PyrG+									

	RkuN16ptr1(pyrG) ( <i>Δku70::ptrA</i> , <i>ΔpyrG.pyrG</i> +)						Growth in DPY (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH <sub>2</sub> PO <sub>4</sub> , and 0.05% MgSO <sub>4</sub> ·7H <sub>2</sub> O, pH 5.8) liquid medium at 30°C for 2 days.	Comparative Cycle Threshold (Ct) Method	[101]
	<i>ΔsclR</i> ( <i>Δku70::ptrA</i> , <i>ΔsclR::pyrG</i> )	NA	3	<i>H2A</i>	Histone H2A	No			
	<i>Δbr1A</i>	Frozen mycelia and conidia	2	<i>β-actin</i>	Actin	No	Growth on PDA medium at 30°C for 7 days.	NA	[97]
	NS4								
	MAT1-1	Mycelia	NA	NA	Actin	No	Growth (conidia) in DPY (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH <sub>2</sub> PO <sub>4</sub> , and 0.05% MgSO <sub>4</sub> ·7H <sub>2</sub> O) liquid medium supplemented with 0.5% uridine and 0.2% uracil. After 24h of growth, 200 mg of mycelia were transferred onto DPY agar medium and grown for an additional 48h.	NA	[25]
	MAT1-2								
	<i>ΔMAT</i>								
	JaL250	Conidiospores	NA	NA	Actin	No	Growth in MM supplemented with 10 mM uridine and 1% maltose at 34°C with gentle shaking for 22 to 24h, after which 25-75 mL was transferred to a cellulose nitrate filter and grown for 42-48h until pigmented conidiospores were present.	NA	[96]
	DLM2								
	DLM3								
	DLM17								
	DLM24								
	DLM29								
	NS4DLP and several transformants	Frozen hyphae	NA	AO090038000317	Beta-tubulin	Yes- previously exhibited stability in microarray work.	Growth in modified Czapek–Dox (CD) medium (CD minimal agar supplemented with 5 mM uridine, 10 mM uracil, 10 mM sodium glutamate, and/or 30 μg/ml methionine and an increased glucose concentration of 10%) at 30°C for 48h with 200 rpm shaking.	NA	[98]
	RIB40	Culture	NA	<i>HI</i>	Histone H1	No	Growth on 5 g of wheat bran containing 2, 3, 4, 6 or 8 mL of water at 30°C for 48 h.	2 <sup>-ΔΔCT</sup>	[99]
<i>lucluensis</i>	RIB2601	Rice	NA	NA	Actin	No	Growth on rice as rice koji and sampling at the stages in the process of shimaishigoto (30h, 40°C) and dekoji (40h).	NA	[26]
	U1								
	SO2	Mycelia	NA	<i>actA</i>	Actin	Yes- Cited the use of actin as a reference gene in the literature. However, the cited	Precultured in M medium for 36h at 30°C with shaking at 180 rpm, then transferred to Citric acid production (CAP) medium and	NA	[75]
	<i>ΔLaeA</i>								

	<i>ALaeA + LaeA</i>					study uses different media conditions.	grown for 0 or 12h at 30°C with shaking at 163 rpm.		
<i>terreus</i>	Clinical isolate, W.B. 016.02	Homogenised cells	NA	<i>β-tubulin</i>	Beta-tubulin *	No	Four formed fungi balls or 2 mL of fungal suspension in NaCl (corresponding to a McFarland 1 standard) incubated for 3h at 25, 30, 35 and 40°C, respectively.	The comparative SYBR Green/ΔCt method	[67]
	Amphotericin B-resistant	Cells	2	<i>BTU</i>	Beta-tubulin *	No	Treatment with sub-lethal (2 mg/mL for AMR; 0.1 mg/mL AMS) and lethal AmB (5 mg/L AMR; 1 mg/mL AMS) concentrations during growth in Sabouraud medium for 24h at 37°C with slight shaking.	Pfaffl method	[68]
	Amphotericin B-susceptible								
	MUCL 38669	Homogenised cells	NA	<i>β-actin</i>	Actin	Yes - Note that there was no change in the transcript levels under the conditions tested, however, they did not show their data.	Growth in lovastatin production medium at 27°C for 10 days with shaking at 220 rpm.	Light cycler SDS Software	[107]
	EFB108	Mycelia	NA	<i>actaA</i>	Actin	No	Growth on MID medium for 10 days, after which 0.5, 1.0, 3.0 and 5.0% (w/v) of surface sterilized <i>Podocarpus gracilior</i> leaves were added and incubated together for 20 days.	Standard Curve Method (CT values plotted against a logarithm of the DNA copy number)	[108]
<i>westerdijkiae</i>	CECT 2948	Frozen mycelia	2	<i>βtub</i>	Beta-tubulin	No	Growth on Czapek-Dox Modified Yeast Agar (CYA) in the presence or absence of <i>Debaryomyces hansenii</i> at 28°C for 3 and 7 days.	$2^{-\Delta\Delta CT}$	[109]
	AOPD16-1								
	UEL91	Mycelia	NA	<i>GAPDH</i>	GAPDH	No	Growth on YES medium at 25°C for 96h.	$2^{-\Delta\Delta CT}$	[104]
	ITAL163								
<i>carbonarius</i>	W04-40	Frozen mycelia	NA	<i>18S</i>	18S rRNA	No	CYA medium in the dark at 30°C for 2 days.	Relative Expression Software Tool (REST)	[36]
	W04-46								
	S402	Frozen mycelia	NA	<i>cal</i>	Calmodulin	No	Co-culture with the actinobacterial strain, SN7, incubated at 28°C for 4 days on solid ISP2 medium.	NA	[37]
	AC49	Frozen mycelia	2	<i>ubc</i>	Ubiquitin-conjugating Enzyme	Yes- Performed preliminary experiments to assess the expression stability of <i>β-tub</i> , <i>cal</i>	Growth in MM in the dark at 25°C, without shaking (ochratoxin A (OTA)-inducing conditions), for 4, 6 and 8 days.	$2^{-\Delta\Delta CT}$	[32]

		AC67					and <i>ubc</i> genes under the conditions described using BestKeeper [156]. The <i>ubc</i> gene exhibited the greatest expression stability and therefore was used as the reference gene.			
<i>aculeatus</i>	Aacbh589	Mycelia	3	<i>gpdA</i>	Glyceraldehyde-3-phosphate dehydrogenase A (GAPDH)	Yes- Tested the stability of the reference gene under the tested condition using NormFinder [119].	Growth in MM containing 1% (w/v) polypeptone, 1% (w/v) glucose, 1% (w/v) avicel, 1% (w/v) xylose, or 1% (w/v) arabinose for 3h or 6h.	Standard Curve Method (CT values plotted against a logarithm of the DNA copy number)	[35]	
	<i>ΔxlnR</i> mutants									
	ACU7	Cells	NA	<i>actA</i>	Actin	No	Growth on solid MM for 3, 5 and 7 days.	Fold change = 2x, x = (geneOE-housekeepingOE)-(generef.-housekeepingref.)	[34]	
	acrR-OE (ACU41)			<i>hhtA</i>	Histone 3					
<i>nomius</i>	INCQS 40010	Frozen mycelia	NA	<i>CAL</i>	Calmodulin	No	Growth on coconut agar at 25, 30 and 35°C for 7 days.	ΔΔCT	[95]	
	Seven strains isolated from Brazil nuts									
<i>fischeri</i>	CBS 317.89 CBS-KNAW (wildtype)	Frozen ascospores	NA	<i>his-H3</i>	Histone 3	Yes- Beta-tubulin was used for 3-day and/or 6-day-old, but because beta-tubulin expression was so low, they evaluated its expression in relation to the histone H3 gene and found that histone H3 was a better reference as it was expressed at a higher copy number.	Growth on a hydrophobic polyvinylidene fluoride (PVDF) membrane on top of oatmeal agar for 3, 6 or 30 days (wildtype only).	2 <sup>-ΔΔCT</sup>	[38]	
	<i>AmpdA</i>			<i>β-tub</i>	Beta-tubulin					

\*, analyzed more than one species of *Aspergillus* and appear twice in the table.

## Chapter 3

### Genome-wide association analysis for triazole resistance in *Aspergillus*

#### *fumigatus*

#### 3.1. Preface

This publication was done in collaboration with co-first authors, Yuying Fan and Yue Wang, Gregory

Korfanty and Dr. Jianping Xu and was published on June 4, 2021, in the journal “Pathogens”. For this paper, I was the fourth author. I have included my contribution to the “Results” section, ie. over-expressed genes that were previously shown to be associated with triazole resistance in *A. fumigatus* through RT-qPCR and/or RNA-sequencing. Here, I researched the overexpressed genes and prepared the written component and corresponding table for the manuscript. I also contributed to the “Discussion” section of this paper, by writing the section discussing the functional validation of non-*cyp51A* single-nucleotide polymorphisms (SNPs) in *Hmg1*, *AFUA\_7G01960* and *AFUA\_2G1060*. Finally, I helped to edit the manuscript. Since my contribution to the “Introduction” was through edits to the manuscript, I have not included it as part of my thesis, but I have included all relevant information to Chapter 3 within a separate introduction written as part of this thesis as section 3.2. Please note that the “Results” section is an embedded copy from the original publication and the corresponding references from the reference list follow this section. I have also written a separate “Discussion” section as 3.3. to discuss my contribution to the paper. In addition, Table S3.1. was modified from the supplementary table included in the manuscript to include superscripts to describe the method used to determine fold change, ex. fold change relative to the wildtype.

The full reference for the publication is provided here: Fan, Y., Wang, Y., Korfanty, G. A., Archer, M., & Xu, J. (2021). Genome-Wide Association Analysis for Triazole Resistance in *Aspergillus fumigatus*. *Pathogens*, 10(6). doi:10.3390/pathogens10060701

The journal link to access the supplementary files for Chapter 3 is provided here:

<https://www.mdpi.com/2076-0817/10/6/701>

## 3.2. Introduction

Due to the rise in triazole resistant isolates of *A. fumigatus*, there is increasing interest from the medical and scientific communities regarding the molecular mechanisms that enable resistance to occur. Many studies have focused on the contributions of mutations in the gene encoding the azole target protein, *cyp51A* [1,2], however, much of the observed *A. fumigatus* azole resistance is not due to mutations in this gene or alteration of its expression [3]. For instance, in a study of 230 isolates, 43% of the observed triazole resistance was unaccounted for by *cyp51A* mutations [3]. In section 3.3. below, from our Genome-wide Association Study (GWAS) of triazole resistance in *A. fumigatus*, we sought to curate a list of genes identified in previous gene expression analyses to be upregulated during exposure to one or both of the following triazoles, itraconazole or voriconazole.

## 3.3. Results

### 3.3.1. Genes Overexpressed with Triazole Exposure

Table 3.1. summarizes the genes that were overexpressed upon exposure to each antifungal. The overexpression of these genes under triazole stress were determined using RT-qPCR and RNA-seq information [4,5,6]. Supplementary Table S3.1. describes the details on the experimental conditions and setup associated with each gene listed in Table 3.1. Specifically, previous work demonstrated that 10 ATP-binding cassette (ABC) transporters (*abcA-1*, *abcA-2*, *abcB*, *abcC*, *abcD*, *abcE*, *atrF*, *mdr1*, *mdr4*, and *AFUA\_5G02260*), four major facilitator superfamily (MFS) transporters (*AFUA\_2G11580*, *mfs56*, *mfsA* and *mfsC*), the 14-alpha sterol demethylase *cyp51A*, and 16 transcription factors (*ace1*, *AFUA\_1G02870*, *AFUA\_1G04140*, *AFUA\_1G16460*, *AFUA\_2G01190*, *AFUA\_3G09130*, *AFUA\_4G06170*, *AFUA\_4G13600*, *AFUA\_5G02655*, *AFUA\_5G06350*, *AFUA\_5G07510*, *AFUA\_6G01960*, *AFUA\_6G03430*, *AFUA\_7G03910*, *AFUA\_8G07360*, and *fumR*) were

overexpressed following itraconazole exposure [4,5]. Similarly, five ABC transporters (*mdr1*, *abcB*, *abcC*, *abcD* and *abcE*), three MFS multidrug transporters (*mfsA*, *mfsB* and *mfsC*), a F-box domain protein (*fbpA*), an AAA-family ATPase (*aaaA*), a C6 zinc finger domain protein (*finA*), a BZIP transcription factor (*cpcA*), and a putative C2H2 zinc-finger transcription factor (*zfpA*) were overexpressed with voriconazole exposure [5].

**Table 3.1.** Overexpressed genes associated with triazole exposure in *A. fumigatus* from previous RT-qPCR and RNA-seq studies.

Overexpressed Gene Name	Encoded Protein	Fold Change When Exposed to Itraconazole	Fold Change When Exposed to Voriconazole	Ref.
<i>abcA-1</i> (AFUA_1G17440)		7.1	NA	[4]
<i>abcA-2</i> (AFUA_2G15130)		~6.50	NA	[4]
<i>abcB</i> (AFUA_1G10390)		~4.50	~5.00–13.00	[4,5]
<i>abcC</i> (AFUA_1G14330)	ABC multidrug	~5.50	~5.00–>20.00	[4,5]
<i>abcD</i> (AFUA_6G03470)	transporter	~4.50	~2.00–>20.00	[4,5]
<i>abcE</i> (AFUA_7G00480)		~1.00	~2.00–>20.00	[4,5]
<i>atrF</i> (AFUA_6G04360)		31.7	NA	[4]
<i>mdr1</i> (AFUA_5G06070)		~5.00	~2.00–5.00	[4,5]

<i>mdr4</i> (AFUA_1G12690)		~4.70	NA	[4]
<i>AFUA_5G02260</i>	ABC multidrug transporter, putative	~4.90	NA	[4]
<i>AFUA_2G11580</i>	MFS multidrug	14.2	NA	[4]
<i>mfs56</i> (AFUA_1G05010)	transporter, putative	~4.50–700.00	NA	[4]
<i>mfsA</i> (AFUA_8G05710)		~4.70	~1.50–11.00	[4,5]
<i>mfsB</i> (AFUA_1G15490)	MFS multidrug transporter	NA	~4.00–18.00	[5]
<i>mfsC</i> (AFUA_1G03200)		~7.90	~2.50–30.00	[4,5]
<i>cyp51A</i> (AFUA_4G06890)	14-alpha sterol demethylase	21.00–550.90	NA	[4]
<i>fbpA</i> (AFUA_1G14050)	F-box domain protein	NA	~ >50.00–600.00	[5]
<i>aaaA</i> (AFUA_7G06680)	AAA-family ATPase, putative	NA	~2.00–90.00	[5]
<i>finA</i> (AFUA_8G05800)	C6 zinc finger domain protein	NA	~4.00–40.00	[5]
<i>AFUA_1G02870</i>	Transcription factor involved in oxidative stress response, putative	2.48–2.61	NA	[6]
<i>AFUA_1G04140</i>		2.04–2.94	NA	[6]
<i>AFUA_6G01960</i>	C6 finger domain protein, putative	2.01–3.02	NA	[6]
<i>AFUA_6G03430</i>		2.78–2.93	NA	[6]



<i>fumR</i> (AFUA_8G00420)	C6 zinc finger transcription factor	4.00–4.70	NA	[6]
AFUA_5G07510	factor	2.39–3.50	NA	[6]
AFUA_3G09130	C6 transcription	1.73–2.22	NA	[6]
AFUA_8G07360	factor, putative	1.90–1.92	NA	[6]
<i>cpcA</i> (AFUA_4G12470)	BZIP transcription factor	NA	>1.50–~5.50	[5]
AFUA_1G16460	BZIP transcription factor (LziP), putative	1.75–2.12	NA	[6]
AFUA_7G03910	C2H2 zinc finger protein	2.50–2.86	NA	[6]
<i>ace1</i> (AFUA_3G08010)	C2H2 zinc-finger	1.66–2.32	NA	[6]
AFUA_4G13600	transcription	2.30–2.71	NA	[6]
<i>zfpA</i> (AFUA_8G05010)	factor, putative	NA	~1.50–60.00	[5]
AFUA_2G01190	Cu-dependent DNA-binding protein, putative	1.30–2.10	NA	[6]
AFUA_4G06170	Predicted DNA- binding	3.79–3.89	NA	[6]
AFUA_5G02655	transcription factor	2.75–3.84	NA	[6]
<i>ada</i> (AFUA_5G06350)	DNA repair and transcription factor, putative	1.23–2.05	NA	[6]

### 3.4. Discussion

As of 2005, it was estimated that *A. fumigatus* encoded 278 ABC and 49 MFS transporter genes [7]. As discussed in section 3.2., previous work identified 10 ABC transporters to be upregulated during exposure to itraconazole [4], five of which are also upregulated during exposure to voriconazole [5] (Table 3.1.). The role of ABC transporters in facilitating azole resistance in *A. fumigatus* has been documented since at least 1999 [8]. ABC transporters use the energy generated from hydrolyzing ATP to transport molecules, including antifungal drugs, across the cell membrane [8]. Upregulation of ABC transporters is also associated with azole resistance in other human fungal pathogens, including *Candida albicans* [9] and *Candida glabrata* [10], suggesting that their role in azole resistance is conserved. Recently, overexpression of the ABC transporter, *abcC* (also called *abcG1*), was shown to promote azole resistance in an *A. fumigatus* strain with a *cyp51A* promoter duplication and L98H amino acid change in the encoded protein [11]. This demonstrates the connectivity of the mechanisms underlying azole resistance in *A. fumigatus* and highlights the need to look for relationships between changes in the transcriptome and known single-nucleotide polymorphisms (SNPs).

Four major facilitator superfamily (MFS) transporters were expressed at higher levels during itraconazole exposure [4] and three MFS transporters were expressed at higher levels during voriconazole exposure [5] (Table 3.1.). One of these transporters, *mfsC*, which has been shown to be upregulated during exposure to both triazoles [4,5] (Table 3.1.), was recently shown to increase 5-fold in expression in a resistant environmental (agricultural field) isolate lacking any *cyp51A* mutations, and 3-5-fold in three other resistant isolates of *A. fumigatus*, relative to the standard Af293 strain [12]. Six to eight SNPs were identified in the *mfsC* gene of the four resistant strains, which may contribute to the higher expression and associated resistance [12].

The same study identified SNPs in the transcription factor, *ace1* and the AAA-ATPase family protein, *aaaA* [12], whose expression has been demonstrated to increase during itraconazole [4] and voriconazole [5] exposure, respectively. Interestingly, *ace1* is also upregulated during *A. fumigatus* conidia exposure to human airway epithelial cells [13]. One hypothesis pertaining to the *aaaA*-encoded enzyme is that it mediates the disassembly of protein aggregates/complexes or proteolysis, thereby leading to voriconazole resistance in *A. fumigatus* [5].

Surprisingly, Amarsaikhan and colleagues reported that the BZIP transcriptional regulator of cross-pathway control system of amino acid biosynthesis, *cpcA* [14], decreases during *A. fumigatus* exposure to voriconazole [15], which contradicts the findings of da Silva Ferreira *et al.* [5] (Table 3.1.). Moreover, genes involved in the amino acid biosynthesis pathway also follow the same pattern for their respective studies [5,15]. Amarsaikhan *et al.* also observed an increase in voriconazole resistance when *cpcA* was deleted [15]. Collectively, their results warrant future study into the specific role of CpcA in voriconazole resistance in *A. fumigatus*.

Altogether, the data presented here shows that additional pathways and transporters may contribute to non-*cyp51A*-mediated resistance and that there is still a lack of data regarding their differential gene expression in response to both triazole drugs.

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### 3.6. Supplemental Materials

**Table S3.1.** Additional data pertaining to the studies for which overexpressed genes in response to triazole exposure were identified in *Aspergillus fumigatus*.

Strain	Overexpressed Gene(s)	Encoded Protein(s)	Function and/or Associated Pathway	Fold Change	Experimental Conditions	Reference
Af293 (wildtype)	<i>AFUA_2G11580</i>	MFS Multidrug Transporter, putative	Membrane transport activity	14.20 <sup>A</sup>	Exposure to 1 mg/L itraconazole in Vogel's 1% glucose minimal medium for 4 hours.	[4]
	<i>abcA-1 (AFUA_1G17440)</i>	ABC Multidrug Transporter	Membrane transport activity	7.10 <sup>A</sup>		
	<i>mdr1 (AFUA_5G06070)</i>	ABC Multidrug Transporter	Membrane transport activity	~5.00 <sup>A</sup>		
	<i>abcB (AFUA_1G10390)</i>	ABC Multidrug Transporter	Membrane transport activity	~4.50 <sup>A</sup>		
	<i>abcC (AFUA_1G14330)</i>	ABC Multidrug Transporter	Membrane transport activity	~5.50 <sup>A</sup>		
	<i>abcD (AFUA_6G03470)</i>	ABC Multidrug Transporter	Membrane transport activity	~4.50 <sup>A</sup>		
	<i>abcE (AFUA_7G00480)</i>	ABC Multidrug Transporter	Membrane transport activity	~1.00 <sup>A</sup>		
	<i>mdr4 (AFUA_1G12690)</i>	ABC Multidrug Transporter	Membrane transport activity	~4.70 <sup>A</sup>		
	<i>mfsA (AFUA_8G05710)</i>	MFS Multidrug Transporter	Membrane transport activity	~4.70 <sup>A</sup>		
	<i>mfsC (AFUA_1G03200)</i>	MFS Multidrug Transporter	Membrane transport activity	~7.90 <sup>A</sup>		

	<i>AFUA_5G02260</i>	ABC multidrug transporter, putative	Membrane transport activity	~4.90 <sup>A</sup>	
	<i>abcA-2 (AFUA_2G15130)</i>	ABC Multidrug Transporter	Membrane transport activity	~6.50 <sup>A</sup>	
	<i>mfs56 (AFUA_1G05010)</i>	MFS Multidrug Transporter, putative	Membrane transport activity	~4.50 <sup>A</sup>	
F20140 (non- <i>cyp51A</i> mutant and itraconazole-resistant)	<i>abcC (AFUA_1G14330)</i>	ABC Multidrug Transporter	Membrane transport activity	>25.00 <sup>A</sup>	Growth in Vogel's 1% glucose minimal medium with DMSO added in place of itraconazole.
F18304 (non- <i>cyp51A</i> mutant and itraconazole-resistant)				>70.00 <sup>A</sup>	
F17727 (non- <i>cyp51A</i> mutant and posaconazole, voriconazole and itraconazole-resistant)				>25.00 <sup>A</sup>	
F19980 (non- <i>cyp51A</i> mutant and voriconazole)				7.20 <sup>A</sup>	

and itraconazole-resistant)					
F20063 (non- <i>cyp51A</i> mutant and itraconazole-resistant)				6.50 <sup>A</sup>	
F20451 (non- <i>cyp51A</i> mutant and itraconazole-resistant)				3.60 <sup>A</sup>	
F18454 (non- <i>cyp51A</i> mutant and voriconazole-resistant only)				5.10 <sup>A</sup>	
F15483 (azole-susceptible)				2.10 <sup>A</sup>	
F15483 (azole-susceptible)	<i>mfs56 (AFUA_1G05010)</i>	MFS Multidrug Transporter, putative	Membrane transport activity	~7.00 <sup>B</sup>	Exposure to 1 mg/L itraconazole in Vogel's 1% glucose minimal medium for 4 hours.
F18329 (non- <i>cyp51A</i> mutant and voriconazole-resistant)				~7.00 <sup>B</sup>	



F18304 (non- <i>cyp51A</i> mutant and itraconazole- resistant)			~5.00 <sup>B</sup>		
F18149 (non- <i>cyp51A</i> mutant and posaconazole, voriconazole and itraconazole- resistant)			~110.00 <sup>B</sup>		
F20063 (non- <i>cyp51A</i> mutant and itraconazole- resistant)			~550.00 <sup>B</sup>		
F18085 ( <i>cyp51A</i> mutation A248T and itraconazole- resistant)			~500.00 <sup>B</sup>		
F17999 ( <i>cyp51A</i> mutations G448S and H147Y and voriconazole			~700.00 <sup>B</sup>		

and itraconazole- resistant)					
F19980 (non- <i>cyp51A</i> voriconazole and itraconazole- resistant)				27.80 <sup>B</sup>	
F20140 (non- <i>cyp51A</i> itraconazole- resistant)				35.60 <sup>B</sup>	
F20451 (non- <i>cyp51A</i> itraconazole- resistant)				17.70 <sup>B</sup>	
F17999 ( <i>cyp51A</i> mutations G448S and H147Y and voriconazole and itraconazole- resistant)	<i>atrF</i> (AFUA_6G04360)	ABC Multidrug Transporter	Membrane transport activity	31.70 <sup>B</sup>	

F18304 (non- <i>cyp51A</i> mutant and itraconazole- resistant)				550.90 <sup>B</sup>		
F19980 (non- <i>cyp51A</i> mutant and voriconazole and itraconazole- resistant)	<i>cyp51A</i> (AFUA_4G06890)	Sterol 14-alpha demethylase	Ergosterol Biosynthesis	21.00 <sup>B</sup>		
ATCC 46645 (wildtype)	<i>mdr1</i> (AFUA_5G06070)	ABC Multidrug Transporter	Membrane transport activity	~2.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.	[5]
				~5.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.	
	<i>abcB</i> (AFUA_1G10390)	ABC Multidrug Transporter	Membrane transport activity	~7.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 30 minutes during growth at 37°C.	

			~7.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 60 minutes during growth at 37°C.
			~13.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.
			~5.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.
<i>abcC</i> (AFUA_1G14330)	ABC Multidrug Transporter	Membrane transport activity	~5.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 60 minutes during growth at 37°C.
			~10.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.

			>20.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.
<i>abcD</i> (AFUA_6G03470)	ABC Multidrug Transporter	Membrane transport activity	~2.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 60 minutes during growth at 37°C.
			>2.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.
			>20.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.
<i>abcE</i> (AFUA_7G00480)	ABC Multidrug Transporter	Membrane transport activity	~2.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 30 minutes during growth at 37°C.

			~7.5 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 60 minutes during growth at 37°C.
			>10.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.
			>20.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.
<i>mfsA</i> (AFUA_8G05710)	MFS Multidrug Transporter	Membrane transport activity	~11.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 30 minutes during growth at 37°C.
			~5.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 60 minutes during growth at 37°C.

				~1.50 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.
				~2.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.
				~4.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 60 minutes during growth at 37°C.
	<i>mfsB</i> (AFUA_1G15490)	MFS Multidrug Transporter	Membrane transport activity	~10.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.
				~18.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.

	<i>mfsC</i> (AFUA_1G03200)	MFS Multidrug Transporter	Membrane transport activity	~2.50 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 60 minutes during growth at 37°C.
				>15.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.
				~30.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.
	<i>fbpA</i> (AFUA_1G14050)	F-box domain protein	NA	>50.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.
				~600.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.



	<i>aaaA</i> (AFUA_7G06680)	AAA-family ATPase, putative	NA	~5.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 60 minutes during growth at 37°C.
				~2.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.
				~90.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.
	<i>finA</i> (AFUA_8G05800)	C6 zinc finger domain protein	NA	~4.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 30 minutes during growth at 37°C.
				~5.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 60 minutes during growth at 37°C.

				>5.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.
				~40.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.
<i>cpcA</i> (AFUA_4G12470)	BZIP transcription factor	NA	>1.50 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 30 minutes during growth at 37°C.	
			~3.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.	
			~5.50 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.	

				~2.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 30 minutes during growth at 37°C.	
				~1.50 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 60 minutes during growth at 37°C.	
	<i>zfpA</i> (AFUA_8G05010)	C2H2 zinc-finger transcription factor, putative	NA	~5.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 120 minutes during growth at 37°C.	
				~60.00 <sup>A</sup>	Exposure to 0.5 µg/mL voriconazole for 240 minutes during growth at 37°C.	
A1160C' (wildtype)	<i>fumR</i> (AFUB_086150) (Systematic name in other strains: AFUA_8G00420)	C6 zinc finger transcription factor	NA	4.70 with RNA-seq <sup>C</sup>	Exposure to 0.5 µg/mL itraconazole for 120 minutes during growth at 37°C.	[6]
				4.00 with qRT-PCR <sup>C</sup>		

<p><i>AFUB_063290</i> (Systematic name in other strains: <i>AFUA_4G06170</i>)</p>	<p>Predicted DNA-binding transcription factor</p>	<p>NA</p>	<p>3.89 with RNA-seq<sup>C</sup></p>
			<p>3.79 with qRT-PCR<sup>C</sup></p>
<p><i>AFUB_051190</i> (Systematic name in other strains: <i>AFUA_5G02655</i>)</p>	<p>Predicted DNA-binding transcription factor</p>	<p>NA</p>	<p>3.84 with RNA-seq<sup>C</sup></p>
			<p>2.75 with qRT-PCR<sup>C</sup></p>
<p><i>AFUB_055060</i> (Systematic name in other strains: <i>AFUA_5G07510</i>)</p>	<p>C6 transcription factor</p>	<p>NA</p>	<p>3.50 with RNA-seq<sup>C</sup></p>
			<p>2.39 with qRT-PCR<sup>C</sup></p>
<p><i>AFUB_004490</i> (Systematic name in other strains: <i>AFUA_1G04140</i>)</p>	<p>C6 finger domain protein, putative</p>	<p>NA</p>	<p>2.94 with RNA-seq<sup>C</sup></p>
			<p>2.04 with qRT-PCR<sup>C</sup></p>
<p><i>AFUB_089440</i> (Systematic name in other strains: <i>AFUA_7G03910</i>)</p>	<p>C2H2 zinc finger protein</p>	<p>NA</p>	<p>2.86 with RNA-seq<sup>C</sup></p>
			<p>2.50 with qRT-PCR</p>

<p><i>AFUB_094860</i> (Systematic name in other strains: <i>AFUA_6G03430</i>)</p>	<p>C6 finger domain protein, putative</p>	<p>NA</p>	<p>2.78 with RNA-seq<sup>C</sup></p>
			<p>2.93 with qRT-PCR</p>
<p><i>AFUB_003250</i> (Systematic name in other strains: <i>AFUA_1G02870</i>)</p>	<p>Transcription factor involved in oxidative stress response, putative</p>	<p>NA</p>	<p>2.48 with RNA-seq<sup>C</sup></p>
			<p>2.61 with qRT-PCR<sup>C</sup></p>
<p><i>AFUB_070520</i> (Systematic name in other strains: <i>AFUA_4G13600</i>)</p>	<p>C2H2 finger domain protein, putative</p>	<p>NA</p>	<p>2.30 with RNA-seq<sup>C</sup></p>
			<p>2.71 with qRT-PCR<sup>C</sup></p>
<p><i>AFUB_018270</i> (Systematic name in other strains: <i>AFUA_2G01190</i>)</p>	<p>Cu-dependent DNA-binding protein, putative</p>	<p>NA</p>	<p>2.10 with RNA-seq<sup>C</sup></p>
			<p>1.30 with qRT-PCR<sup>C</sup></p>
<p><i>ada</i> (<i>AFUB_053880</i>) (Systematic name in other strains: <i>AFUA_5G06350</i>)</p>	<p>DNA repair and transcription factor, putative</p>	<p>NA</p>	<p>2.05 with RNA-seq<sup>C</sup></p>
			<p>1.23 with qRT-PCR<sup>C</sup></p>

<p><i>AFUB_096380</i> (Systematic name in other strains: <i>AFUA_6G01960</i>)</p>	<p>C6 finger domain protein, putative</p>	<p>NA</p>	<p>2.01 with RNA-seq<sup>C</sup></p>
			<p>3.02 with qRT-PCR<sup>C</sup></p>
<p><i>AFUB_080380</i> (Systematic name in other strains: <i>AFUA_8G07360</i>)</p>	<p>C6 transcription factor, putative</p>	<p>NA</p>	<p>1.90 with RNA-seq<sup>C</sup></p>
			<p>1.92 with qRT-PCR<sup>C</sup></p>
<p><i>AFUB_015800</i> (Systematic name in other strains: <i>AFUA_1G16460</i>)</p>	<p>BZIP transcription factor (LziP), putative</p>	<p>NA</p>	<p>1.75 with RNA-seq<sup>C</sup></p>
			<p>2.12 with qRT-PCR<sup>C</sup></p>
<p><i>AFUB_040000</i> (Systematic name in other strains: <i>AFUA_3G09130</i>)</p>	<p>C6 transcription factor, putative</p>	<p>NA</p>	<p>1.73 with RNA-seq<sup>C</sup></p>
			<p>2.22 with qRT-PCR<sup>C</sup></p>
<p><i>ace1</i> (<i>AFUB_041100</i>) (Systematic name in other strains: <i>AFUA_3G08010</i>)</p>	<p>C2H2 transcription factor, putative</p>	<p>NA</p>	<p>1.66 with RNA-seq<sup>C</sup></p>
			<p>2.32 with qRT-PCR<sup>C</sup></p>

<sup>A</sup>Fold change relative to the wildtype (Af293) without itraconazole treatment; <sup>B</sup>Fold change relative to the wildtype (Af293) treated with 1 mg/mL itraconazole for four hours; <sup>C</sup>Fold change relative to the wildtype A1160C') without itraconazole treatment

## Chapter 4

### General Conclusion

#### 4.1 Concluding Remarks and Future Directions

In conclusion, this thesis work investigated gene expression in *Aspergillus*, with the major focus on understanding the reference gene selection practices for RT-qPCR analysis of this globally distributed genus and on the expression dynamics of triazole resistance genes in *A. fumigatus*. In Chapter 2, the literature was extensively reviewed to determine the status of the reference gene selection and validation processes in RT-qPCR of the genus *Aspergillus*. Information regarding the species of *Aspergillus* examined, sample type, experimental conditions, method of normalization, reference gene(s) used and whether they were experimentally validated was extracted from 90 primary articles from 2001 to 2021 (Archer & Xu, 2021). The key results of each article were also summarized (Archer & Xu, 2021). Of these studies, 17 experimentally validated the expression stability of the reference genes used and eight of these studies used more than one reference gene (Archer & Xu, 2021). In total 16 studies used more than one reference gene (Archer & Xu, 2021). Reference genes encoding beta-tubulin was the most used references gene out of a total of 20 unique reference gene-encoded products (Archer & Xu, 2021). Three of the 90 studies reported conflicting experimental results with other published studies, however, due to the missing experimental validation of reference gene stability, whether these differences are caused by differences in the experimental conditions, or the strains/isolates used is inconclusive (Archer & Xu, 2021) Twenty-three of the 90 studies focused

specifically on expression in *A. fumigatus*, of which only two experimentally validated the expression stability of the genes used (Archer & Xu, 2021). The expression of the 18S rRNA gene was experimentally demonstrated to be stable during exposure to 125 µg/mL of artemisinin in RPMI-1640 medium, and the expression of actin and histone H4-encoding genes were shown to be stable during growth in Mandels' salt solution with 1% oat spelts xylan (Archer & Xu, 2021). Due to the disparity in reference gene validation for this species, it is evident that further validation of candidate reference genes is required. Overall, the work presented in this review paper support the hypothesis that the expression stability of reference genes is unique to the experimental conditions employed in the study. The review paper outlined in this Chapter also allowed for the identification of seven candidate reference genes previously used in RT-qPCR analysis of gene expression in *A. fumigatus* for future research. These include the top four most frequently used reference genes for all species of *Aspergillus* examined in this review, those encoding beta-tubulin, actin, 18S rRNA and GAPDH, as well as those encoding putative 1,3-beta-glucan synthase catalytic subunit, translation elongation factor 1-alpha, and histone 4 (Archer & Xu, 2021). Of note, the 18S rRNA gene may be problematic for use as a reference gene for several reasons. The first is that the 18S rRNA gene may be expressed at higher levels, as observed for other organisms, leading to lower Ct values (Kohsler, Leitsch, Muller, & Walochnik, 2020) which is a problem because ideally reference genes are expressed at approximately the same level as target genes for normalization (Vandesompele et al., 2002). One way to circumvent this is to dilute the sample, however, this introduces the risk of dilution errors (Vandesompele et al., 2002). Additionally, 18S rRNA transcription is mediated by RNA polymerase I (Azouzi et al., 2021), while mRNA transcription is mediated by RNA polymerase II (Muniz, Nicolas, & Trouche, 2021). This is problematic because the different pathways for RNA synthesis may result in differences in RNA levels of expression, which would not allow for direct, accurate comparisons



between the two (Chapman & Waldenstrom, 2015). Therefore, careful experimental validation of 18S rRNA is critical to ensure that it is an appropriate reference gene for RT-qPCR analysis of *A.*

*fumigatus*.

In Chapter 3, previously identified triazole treatment-associated overexpressed genes were extracted from previous RNA-seq and RT-qPCR studies and summarized (Fan, Wang, Korfanty, Archer, & Xu, 2021). In summary, 10 ABC transporters, four MFS transporters and 16 transcription factors were identified as upregulated during triazole exposure (Fan, Wang, Korfanty, Archer, & Xu, 2021). As discussed in section 3.4, “Discussion”, Amarsaikhan, et al. reported an opposite transcriptional response to voriconazole exposure for *cpcA* to da Silva Ferreira et al., where Amarsaikhan, et al. reported a decrease in *cpcA* expression (Amarsaikhan et al., 2017) and da Silva Ferreira et al. reported an increase (da Silva Ferreira et al., 2006). As Amarsaikhan et al. noted, the differences in expression may be attributable to the use of different media (AMM compared to YG medium) and different strains/isolates in the different group’s experiments (Amarsaikhan et al., 2017). Both research groups used the same voriconazole treatment concentration of 0.5 µg/mL and included drug exposure times of two and four hours (Amarsaikhan et al., 2017; da Silva Ferreira et al., 2006). Although the two research teams used different methods to quantify gene expression, northern blotting (Amarsaikhan et al., 2017) versus RT-qPCR (da Silva Ferreira et al., 2006), it highlights the potential differences that changes to experimental conditions may have on the transcriptional outcome, as was reviewed in Chapter 2.

Future research should focus on the experimental validation of the expression stability of the seven candidate reference genes under clinically relevant experimental conditions. These conditions include oxidative, nitrosative, temperature and antifungal stress. Moreover, as the expression profiles of many transporters and transcription regulator genes has not been established for both itraconazole and

voriconazole yet, another extension of this work would be to use the validated reference genes from the antifungal exposure conditions to examine whether the expression of these genes' changes during exposure to the missing antifungal. Overall, this work serves as a steppingstone to improving the availability of experimentally validated, stable reference gene for future study of *Aspergillus* species.

## 4.2. References

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