Oxidative stress and the presence of bacteria increase gene expression of the antimicrobial peptide aclasin, a fungal CSαβ defensin in Aspergillus clavatus

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ABSTRACT

Background: Antimicrobial peptides (AMPs) represent a broad class of naturally occurring antimicrobial compounds. Plants, invertebrates and fungi produce various AMPs as, for example, defensins. Most of these defensins are characterised by the presence of a cysteine-stabilised α-helical and β-sheet (CSαβ) motif. The changes in gene expression of a fungal CSαβ defensin by stress conditions were investigated in Aspergillus clavatus. A. clavatus produces the CSαβ defensin Aclasin, which is encoded by the aclasin gene.

Methods: Aclasin expression was evaluated in submerged mycelium cultures under heat shock, osmotic stress, oxidative stress and the presence of bacteria by quantitative real-time PCR.

Results: Aclasin expression increased two fold under oxidative stress conditions and in the presence of viable and heat-killed Bacillus megaterium. Under heat shock and osmotic stress, aclasin expression decreased.

Discussion: The results suggest that oxidative stress and the presence of bacteria might regulate fungal defensin expression. Moreover, fungi might recognise microorganisms as plants and animals do.

INTRODUCTION

Antimicrobial peptides (AMPs) are a diverse group of naturally occurring molecules that are produced by a wide range of organisms, both prokaryotes and eukaryotes. AMPs are short peptides (consisting of 12–50 amino acids), commonly cationic and amphipathic. AMPs show a broad-spectrum activity against bacteria and other organisms, including gram-positive and gram-negative bacteria, parasites, fungi, viruses and even cancer cells (Mahlapuu et al., 2016). Additionally, AMPs are part of the innate defence mechanism in animals and plants (Heine, 2008). The role of AMPs is less understood in prokaryotes and lower eukaryotes. It has been suggested that AMPs might help them to compete for nutrients with other microorganisms (Ageitos et al., 2017).
The largest group of AMPs are defensins. Defensins are cysteine-rich peptides found in many organisms throughout the eukaryotic kingdom. Vertebrate defensins contain a three-stranded antiparallel $\beta$-structure. Defensins produced by invertebrates, plants and fungi mostly contain a common structure composed of an $\alpha$-helix linked to a $\beta$-sheet by three to four disulphide bridges. This structure is called cysteine-stabilised $\alpha$-helical and $\beta$-sheet (CS$\alpha\beta$) motif (Silva, Gonçalves & Santos, 2014).

Defensin genes can be differentially or constitutively expressed in plants, vertebrates and invertebrates. In these three groups of organisms, defensins are produced as host defence response to bacteria and fungi (Harder et al., 1997; Irving et al., 2001; Penninckx et al., 1996). Signalling pathways of host defence response share similarities among plants, invertebrates and vertebrates (Han et al., 1998; Ip et al., 1993; Ryals et al., 1997). For example, the receptors, which recognise pathogens, are highly conserved among these organisms (Medzhitov, Preston-Hurlburt & Janeway, 1997; Whitham et al., 1994).

In plants, presence of pathogens and abiotic stress (salt, drought and cold) activate the plant immune system signalling cascade whose defence response includes the expression of defensins as well as other AMPs (Campos et al., 2018; Taji et al., 2004). It has been suggested that defensins are involved in stress adaptation in addition to the antimicrobial activity (Do et al., 2004; Koike et al., 2002).

Fungi are attractive sources of antimicrobial compounds. However, only few fungal CS$\alpha\beta$ defensins have been characterised in terms of their antimicrobial activity. Some of these defensins are plectasin, copsin and eurosin, which are synthesised by Pseudoplectania nigrella, Coprinopsis cinerea and Eurotium amstelodami, respectively (Essig et al., 2014; Mygind et al., 2005; Oeemig et al., 2012). These fungal defensins have antimicrobial activity mainly against gram-positive bacteria.

Another class of defensins found in fungi is defensin with antifungal activity, also called defensin-like antifungal. The structure of defensin-like antifungals consists of five antiparallel $\beta$-sheets forming a $\beta$-barrel. The gene expression of defensin-like antifungals from Aspergillus giganteus and A. niger is altered by environmental stress conditions (Meyer & Stahl, 2002; Meyer, Wedde & Stahl, 2002; Paeghe et al., 2016). For example, carbon starvation increases the expression of these defensin-like antifungals (Meyer, Wedde & Stahl, 2002; Paeghe et al., 2016). In A. giganteus, osmotic stress, heat shock and the presence of other fungi enhance the gene expression of antifungals (Meyer & Stahl, 2003; Meyer, Wedde & Stahl, 2002). In Penicillium chrysogenum, limited glucose induces the expression of antifungals (Marx et al., 1995). In both cases, defensin-like antifungal genes contain putative stress response element (STRE) sequences in their promoters (Marx, 2004; Meyer, Wedde & Stahl, 2002). STRE is a consensus sequence (5'-AGGGG-3' and 5'-CCCCT-3') and is present in the promoters of genes that are regulated by stress.

In silico analysis indicated the identification of eight families of putative CS$\alpha\beta$ defensins from published fungal genomes (Zhu, 2008; Zhu et al., 2012). This prediction was based on the sequence similarity and the presence of the CS$\alpha\beta$ motif. Among the putative defensins described is Acasin (encoded by acasin gene), which is produced by the filamentous fungus A. clavatus (Zhu, 2008).
As mentioned previously, environmental conditions can alter the expression of plant defensins and some defensin-like antifungals from fungi. However, the transcriptional regulation of CSαβ defensins is not well-understood. Stress conditions might also change the gene expression of fungal CSαβ defensins, such as aclasin. Aclasin expression might be a defence response to bacterial presence. The present work aimed to investigate aclasin expression under stress conditions (heat shock, osmotic, oxidative and presence of bacteria) in submerged mycelium of *A. clavatus*. The present investigation demonstrates that oxidative stress and the presence of bacteria increased two-fold aclasin expression after 30 min of exposure. Our findings suggest that filamentous fungi would produce CSαβ defensins as defence response. Moreover *A. clavatus*, as other fungi, might possess specific mechanisms of bacterial recognition.

**MATERIALS AND METHODS**

**Strains and conditions of culture**

*Aspergillus clavatus* DSM 3410 and *Bacillus megaterium* DSM 32 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *A. clavatus* was grown on malt extract broth (MEB; 2% malt extract, 2% glucose, 0.1% peptone) at 28 °C. *B. megaterium* was cultured on Luria-Bertani (LB) medium (0.5% yeast extract, 1% tryptone, 1% NaCl) at 37 °C.

For conidia production, *A. clavatus* was grown on MEB at 28 °C for 5 days. Conidia were harvested by washing MEB agar plates with five ml of phosphate buffered saline containing 0.01% Tween 80 and filtered through VWR 413 filter paper (VWR International BVBA, Leuven, Belgium). For submerged cultures of *A. clavatus*, a conidia suspension was inoculated in MEB to reach a final concentration of 10⁵ conidia/ml.

**Biomass and glucose determination**

The biomass was determined from the vegetative mycelium of submerged *A. clavatus* after 12, 24, 36, 48, 72, 96 and 120 h of cultivation. Mycelia were filtered through VWR 413 filter paper (VWR International BVBA, Leuven, Belgium). The filter papers were washed with water and dried at 80 °C for 24 h.

Glucose in the medium was measured after 12, 36, 48, 72, 96 and 120 h of cultivation. Glucose was quantified based on the combined action of glucose oxidase (GOD) and peroxidase (POD) (*Barham & Trinder, 1972*). Briefly, two μl of each culture was incubated with 200 μl of GOD-POD reagent, which consists of 100 mM potassium phosphate buffer, pH 7.40, 10 mM phenol, 0.3 mM 4-aminoantipyrine (Fluka Chemie, Buchs, Switzerland), 10 kU/l GOD (Sigma-Aldrich, Saint Louis, MO, USA) and 700 U/l peroxidase (Sigma-Aldrich, Saint Louis, MO, USA). Samples were incubated for 30 min at 37 °C. Then, the absorbance was read at 505 nm using a TECAN spectrophotometer (Infinite 200 PRO NanoQuant, Grödig, Austria).

**Heat shock, osmotic and oxidative stress conditions**

For heat shock conditions, submerged cultures of *A. clavatus* were incubated in MEB for 17 h at 28 °C and 150 rpm. Then, cultures were transferred to 37 or 47 °C. Aliquots
were taken after 30 and 60 min of incubation for subsequent RNA expression analysis. For osmotic stress conditions, submerged cultures were treated with one M NaCl final concentration. Samples were taken after 30 min of incubation. For oxidative stress, submerged cultures were treated with 2, 5 or 10 mM hydrogen peroxide (H₂O₂) based on previous studies in Aspergillus ssp. (Fountain et al., 2015; Reverberi et al., 2008). Samples were taken after 30 and 60 min of incubation with H₂O₂.

**Co-incubation with B. megaterium**

*Bacillus megaterium* was grown in LB medium at 37 °C to an optical density at 600 nm of two exponential phase. The bacterial suspension was concentrated and stored at 4 °C for 30 min. For heat-killed culture, it was treated at 60 °C for 30 min. Colony-forming units (CFUs) were counted for each bacterial suspension after 16 h of incubation on LB agar plates at 37 °C.

*Aspergillus clavatus* was grown 17 h at 28 °C, 150 rpm. Viable (10⁷ CFU/ml final concentration) and heat-killed bacteria were inoculated in submerged culture of *A. clavatus*. Samples were taken at 15 min after inoculation for later RNA expression analysis.

**RNA purification, cDNA synthesis and qPCR**

Total RNA of three biological replicates was extracted. Mycelia were harvested by vacuum filtration using a VWR 413 filter paper (20 μm pore size; VWR International BVBA, Leuven, Belgium). Then, mycelia were washed with water and immediately frozen in liquid nitrogen. Samples were kept at –80 °C until RNA purification. Mycelia were disrupted by grinding with a mortar and pestle using liquid nitrogen. RNA was purified by FastGene RNA Premium Purification Kit (Nippon Genetics, Tokyo, Japan) according to the manufacturer’s instructions. RNA was treated with DNase I (Nippon Genetics, Tokyo, Japan). The integrity of RNA checked by agarose gel electrophoresis. RNA was quantified spectrophotometrically at 260 nm. The synthesis of cDNA was performed using FastGene Scriptase II cDNA Kit (Nippon Genetics, Tokyo, Japan), two μg RNA and oligo (dT) according to the manufacturer’s protocol.

Quantitative real-time PCR (qPCR) was carried out in triplicate in a LightCycler 96 instrument (Roche, Mannheim, Germany) using 100 ng cDNA, 0.5 μM each primer (Table S1) and qPCR BIO SyGreen Mix Lo-ROX (PCR Biosystem, London, UK) in 10 μl of total volume. The act1 gene (ACLA_095800) was used as reference gene (Bohle et al., 2007; Verheecke et al., 2015). Threshold cycle (C<sub>t</sub>) determination was performed using LightCycler 96 software (Roche, Mannheim, Germany). Expression levels were calculated according to the 2<sup>ΔΔCt</sup> method (Schmittgen & Livak, 2008). Results are presented as the mean of three independent data. Statistical analyses were performed in GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

**In silico analysis for transcription factor binding sites**

The 5′ untranslated region (UTR) of eight genes of fungal CSβ-defensins were analysed to identify putative transcription factor binding sites (TFBS) (Table S2). 1,500 bp upstream of the translation start site were analysed by the *Saccharomyces cerevisiae* promoter.
database of the Cold Springs Harbor Laboratory (http://rulai.cshl.edu/SCPD/) (Zhu & Zhang, 1999) and the TRANSFAC 6.0 database using the Patch 1.0 program (http://gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi) (Matys et al., 2006). The actl gene (ACLA_095800) was included as negative control (GenBank accession number: NW_001517095.1; Region: 176194-177694).

RESULTS

Aclasin expression during vegetative mycelium growth

The aclasin gene (ACLA_006820) encodes Aclasin, a putative CSβ defensin produced by A. clavatus (Zhu, 2008). Aclasin expression was detected by qPCR in different stages of the vegetative mycelium growth in submerged cultures: during exponential (24 h), late exponential (36 h), early stationary (48 h) and stationary growth phase (96 h). Simultaneously, the glucose content of the culture was determined during mycelium growth. The results of the aclasin mRNA expression are shown in Fig. 1. Aclasin was expressed in vegetative mycelium in these four stages, and no significant differences among them were observed. Aclasin expression did not change when glucose in the medium was depleted (96 h). These results indicate that steady-state level of aclasin does not change under glucose depletion.

Aclasin expression under stress conditions

Submerged cultures of A. clavatus were subjected to heat shock, osmotic and oxidative stress. The aclasin mRNA expression was assessed by qPCR through the change in the mRNA level compared to the untreated culture.
**Heat shock and osmotic stress decreased aclasin expression**

For heat shock conditions, *A. clavatus* was grown in liquid MEB medium for 17 h and transferred to 37 or 47 °C. Changes in the aclasin gene expression were evaluated after 30 and 60 min; the results are presented in Fig. 2A. The aclasin gene expression showed a significant decrease compared to the untreated control after 60 min: 10 and 2.8 fold at 37 and 47 °C, respectively. In addition, hsp30 (ACLA_088240) expression analysis was included as a control because hsp30 is upregulated by heat shock (*Seymour & Piper, 1999*). Hsp30 encodes to a heat shock-like protein (HSP-30) that is responsible for the heat shock response in fungi (*Tiwari, Thakur & Shankar, 2015*). Hsp30 expression was enhanced both at 37 and 47 °C, corroborating a heat shock response at transcriptional level. The lowest expression of aclasin was observed at 37 °C and 60 min of incubation. On the contrary, the highest expression of hsp30 was observed in the same conditions.

Aclasin relative expression decreased eight and three fold at 37 °C and 47 °C, respectively. An increase of nine fold in hsp30 relative expression was observed at 37 °C which suggests a stronger heat shock response at 37 than 47 °C in *A. clavatus*.

To investigate the expression of aclasin under osmotic stress conditions, submerged cultures of *A. clavatus* were treated with one M NaCl. Aclasin expression decreased four fold in comparison to the untreated control after 1 h of incubation, as shown in Fig. 2B.

**Oxidative stress increased aclasin expression**

Oxidative stress conditions were generated by H$_2$O$_2$. The aclasin gene expression was evaluated at 2 mM H$_2$O$_2$ after 30 and 60 min (Fig. S1). In response to oxidising conditions, fungi produce different scavengers against H$_2$O$_2$, as the mycelial bifunctional catalase-peroxidase (*Paris et al., 2003*) that is encoded by the cat2 gene (ACLA_044200). The cat2 expression was assessed as a control to confirm the response to oxidative stress at transcriptional level (*Paris et al., 2003*). A significant increase in aclasin expression was observed after 30 min of incubation. No difference in aclasin expression was observed between cultures treated with 2 mM H$_2$O$_2$ and non-treated ones after 60 min.

Submerged cultures of *A. clavatus* were treated with higher concentrations of H$_2$O$_2$, 5 or 10 mM, and aclasin expression was evaluated (Fig. 2C). Aclasin expression increased two fold after 30 min of incubation (*P* < 0.05). Higher expression of the cat2 expression was observed both at 5 and 10 mM H$_2$O$_2$, as expected.

**Aclasin expression under co-incubation with B. megaterium**

To determine whether the presence of microorganisms can alter aclasin expression, *A. clavatus* was co-incubated with the gram-positive bacterium, *B. megaterium*. *A. clavatus*, after 17 h of incubation, was inoculated with a viable (10$^7$ CFU/ml) or a heat-killed *B. megaterium* suspension. Bacterial suspension was heat-inactivated to prevent nutrient depletion and basification of the medium due to bacterial growth (*Farrell & Finkel, 2003*). The results are shown in Fig. 3. The aclasin gene expression significantly increased two fold (*P* < 0.01 and *P* < 0.05, respectively) both in the presence of viable and heat-killed *B. megaterium* cultures.
Identification of putative regulatory elements in the 5′ untranslated region

The 5′ untranslated region of aclasin was analysed to identify putative regulatory elements. Several putative TFBSs were found, including TATA box, STRE, Yap1 response element.

Figure 2 Relative aclasin gene (ACLA_006820) expression under heat shock, osmotic, and oxidative stress conditions. Submerged cultures of A. clavatus were incubated for 17 h in MEB at 28 ºC and 150 rpm. (A) For heat shock stress, cultures were transferred to 37 or 47 ºC. Aliquots were taken at 30 and 60 min. (B) Hsp30 (ACLA_088240) was included as a positive control. (C) For osmotic stress, cultures were treated with 1 M NaCl for 1 h. (D) For oxidative stress, cultures were treated with 5 or 10 mM H₂O₂ for 30 min. (E) Cat2 gene (ACLA_044200), which encodes a mycelial bifunctional catalase-peroxidase, was used as a positive control for a gene upregulated by oxidative stress. Relative aclasin mRNA expression was determined by qPCR according to the 2^ΔΔCt method. The act1 gene (ACLA_095800) was used as reference gene and the untreated condition as control. Controls have a value of 1. Data represent the mean (± standard deviations) of three biological replicates. Relative expressions were analysed by ANOVA followed by Bonferroni post-tests in comparison to the control for heat shock and oxidative stress, and student t-test for the osmotic assay (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

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Yap1 is a transcriptional activator of associated genes to oxidative stress (Hong, Roze & Linz, 2013). CRE is recognised by transcriptional factors relating to oxidative and osmotic stress responses in Aspergillus ssp. (Balázs et al., 2010; Hong, Roze & Linz, 2013). In addition, the 5’ UTR of the other seven CSβ defensins (Zhu, 2008; Zhu et al., 2012) were analysed to identify STRE, CRE and YRE sequences. Putative STRE sequences were found in all of the 5’ UTR analysed (Fig. S3). Nevertheless, STRE is a short sequence, and it was also found in the 5’ UTR of the housekeeping act1 gene that was included as a negative control.

**DISCUSSION**

The transcriptional regulation of fungal CSβ defensins was an open question. Understanding their transcriptional regulation could help us to comprehend the biological functions of AMPs and the interactions between microorganisms. Considering that the structure of CSβ defensins is highly conserved, understanding their transcriptional regulation might help us to understand these functions in higher eukaryotes. Furthermore, A. clavatus (as other Aspergillus sp.) is a good model system for molecular biology studies because it grows on inexpensive, defined media and can be stored for long periods (Golduran & Morris, 1995).

In this study, aclasin expression increased about two fold after treatment with H2O2. Oxidative stress could directly or indirectly regulate aclasin expression. Previous studies have shown that a CSβ defensin is involved in oxidative stress signalling (Eigentler, Pócsi & Marx, 2012).

All 5’ UTR of analysed defensin genes contained STREs sequences; however, it was also found in 5’ UTR of act1. Act1 is considered a housekeeping gene, and its gene expression is stable under stressful conditions in fungi (Jacob et al., 2012). STRE is a short sequence, and it was also found in the 5’ UTR of the housekeeping act1 gene that was included as a negative control.
sequence (5 bp) and can be found randomly in 5′ UTR of genes. Presence of putative STREs has been linked to gene regulation by stress in fungi without experimental data. Nevertheless, the implication of regulatory elements requires experimental analysis.

A significantly lower expression of aclasin was found at high temperatures and elevated osmolarity. In plants, an indirect down-regulation of defensin by heat shock factors has been observed (Kumar et al., 2009). This phenomenon also might occur in fungi. A possible explanation for this response is that many bacteria are not capable of surviving under high temperature and high osmolarity conditions. Filamentous fungi would not require the defensins against bacteria in these conditions.

As mentioned earlier, defensin-like antifungals are induced by glucose limitation (Marx, 2004). Moreover, the gene expression of a defensin-like antifungal, AFP, decreased under oxidative stress (Meyer, Wedde & Stahl, 2002). In contrast, the aclasin expression did not change under glucose limitation (stationary phase) in this study. Furthermore, aclasin expression was enhanced in the presence of H₂O₂.

Although defensin-like antifungals and CSαβ defensins are AMPs, their gene expression patterns are not comparable.

The gene expression of a fungal CSαβ defensin produced by A. nidulans (anisin1) was associated with conidiation (asexual development) (Eigentler, Pócs & Marx, 2012). The expression of defensin-like antifungals from fungi also relates to asexual development (Meyer & Jung, 2018). The correlation between aclasin expression and conidiation was not the focus of this work.

Invertebrate and fungal Csαβ defensins share structures (CSαβ motifs), mechanisms of action (Schmitt et al., 2010; Schneider et al., 2010) and have been proposed to have a common origin (Zhu, 2008). Their induction might also be similar. In this work, the presence of both viable and heat-killed B. megaterium increased aclasin expression two fold. In invertebrates, induction of defensins is triggered by bacterial recognition. Specifically, plants and animals recognise microbe-associated molecular patterns (MAMPs) (Nürnberger et al., 2004). Aspergillus and other fungi might detect MAMPs through physical interaction (Schroeckh et al., 2009; Svahn et al., 2014). Nevertheless, this detection mechanism has not been found in fungi.

Future research could be addressed to study aclasin expression after addition of peptidoglycan, lipoteichoic acid or other MAMPs to identify what trigger aclasin expression. Moreover, it would be attractive to study the aclasin expression under presence of fungi and gram-negative bacteria. Because, both fungi and gram-negative bacteria upregulate the gene expression of defensins in the red flour beetle, although these defensins are not active against these microorganisms (Tonk et al., 2015).

Previous studies have reported that in A. giganteus, co-cultivation with fungi induced expression of a defensin-like antifungal (Meyer & Stahl, 2003). This is the first report in which the presence of bacteria can induce a fungal CSαβ defensin. This would suggest a prominent role as antibacterial agent. We have shown previously that recombinant AfusinC (a CSαβ defensin from A. fumigatus), as well as other CSαβ defensins, exhibit prominent antibacterial activity against gram-positive bacteria (Contreras et al., 2018; Essig et al., 2014; Mygind et al., 2005).
CONCLUSIONS
In this work, we demonstrated that aclasin was expressed in submerged mycelia of A. clavatus. The gene expression did not significantly change between 24 and 96 h of mycelial growth, which suggests that it is constitutively expressed in mycelia. Moreover, oxidative stress and the presence of bacteria slightly enhanced the aclasin mRNA expression. It would be interesting to investigate the corresponding protein levels of Aclasin under these conditions. Our results suggest that oxidative stress and the presence of bacteria could, directly or indirectly, regulate aclasin expression. These stress conditions could also induce the gene expression of other CSαβ defensins.

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Competing Interests
Michael Wink is an Academic and Section Editor for PeerJ.

Author Contributions
- Gabriela Contreras conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Nessa Wang conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Holger Schäfer authored or reviewed drafts of the paper, approved the final draft.
- Michael Wink conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
Data Availability
The following information was supplied regarding data availability:

The raw data are available as a Supplemental File. The raw data shows the qPCR Cycle Threshold (Ct) mean values of the housekeeping gene (act1) and the genes tested (aclasin, cat2, hsp30).

Ct mean was calculated using three technical replicates.

Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.6290#supplemental-information.

REFERENCES
Ageitos JM, Sánchez-Pérez A, Calo-Mata P, Villa TG. 2017. Antimicrobial peptides (AMPs): Ancient compounds that represent novel weapons in the fight against bacteria. Biochemical Pharmacology 133:117–138 DOI 10.1016/j.bcp.2016.09.018.

Balázs A, Pócsi I, Hamari Z, Leiter É, Emri T, Miskei M, Oláh J, Tóth V, Hagedűs N, Prade RA, Molnár M, Pócsi I. 2010. AtfA bZIP-type transcription factor regulates oxidative and osmotic stress responses in Aspergillus nidulans. Molecular Genetics and Genomics 283(3):289–303 DOI 10.1007/s00438-010-0513-z.

Barham D, Trinder P. 1972. An improved colour reagent for the determination of blood glucose by the oxidase system. Analyst 97(1151):142–145 DOI 10.1039/an9729700142.

Bohle K, Jungebloud A, Göcke Y, Dalpiaz A, Cordes C, Horn H, Hempel DC. 2007. Selection of reference genes for normalisation of specific gene quantification data of Aspergillus niger. Journal of Biotechnology 132(4):353–358 DOI 10.1016/j.jbiotec.2007.08.005.

Campos ML, de Souza CM, de Oliveira KBS, Dias SC, Franco OL. 2018. The role of antimicrobial peptides in plant immunity. Journal of Experimental Botany 69(21):4997–5011 DOI 10.1093/jxb/ery294.

Contreras G, Braun MS, Schäfer H, Wink M. 2018. Recombinant AfusinC, an anionic fungal CSβα defensin from Aspergillus fumigatus, exhibits antimicrobial activity against gram-positive bacteria. PLOS ONE 13(10):e0205509 DOI 10.1371/journal.pone.0205509.

Do HM, Lee SC, Jung HW, Sohn KH, Hwang BK. 2004. Differential expression and in situ localization of a pepper defensin (CADEFI) gene in response to pathogen infection, abiotic elicitors and environmental stresses in Capsicum annuum. Plant Science 166(5):1297–1305 DOI 10.1016/j.plantsci.2004.01.008.

Eigentler A, Pócsi I, Marx F. 2012. The anisin1 gene encodes a defensin-like protein and supports the fitness of Aspergillus nidulans. Archives of Microbiology 194(6):427–437 DOI 10.1007/s00203-011-0773-y.

Essig A, Hofmann D, Münk D, Gayathri S, Künzler M, Kallio PT, Sahl H-G, Wider G, Schneider T, Aebl M. 2014. Copsin, a novel peptide-based fungal antibiotic interfering with the peptidoglycan synthesis. Journal of Biological Chemistry 289(50):34953–34964 DOI 10.1074/jbc.M114.599878.

Farrell MJ, Finkel SE. 2003. The growth advantage in stationary-phase phenotype conferred by rpoS mutations is dependent on the pH and nutrient environment. Journal of Bacteriology 185(24):7044–7052 DOI 10.1128/JB.185.24.7044-7052.2003.
Fountain J, Scully B, Chen Z-Y, Gold S, Glenn A, Abbas H, Lee R, Kemerait R, Guo B. 2015. Effects of hydrogen peroxide on different toxigenic and atoxigenic isolates of *Aspergillus flavus*. Toxins 7(8):2985–2999 DOI 10.3390/toxins7082985.

Golduran GH, Morris NR. 1995. *Aspergillus nidulans* as a model system for cell and molecular biology studies. Methods in Molecular Genetics 6:48–65 DOI 10.1016/S1067-2389(06)80006-3.

Han ZS, Enslen Hé, Hu X, Meng X, Wu I-H, Barrett T, Davis RJ, Ip YT. 1998. A conserved p38 mitogen-activated protein kinase pathway regulates *Drosophila* immunity gene expression. Molecular and Cellular Biology 18(6):3527–3539 DOI 10.1128/MCB.18.6.3527.

Harder J, Bartels J, Christophers E, Schröder J-M. 1997. A peptide antibiotic from human skin. Nature 387(6636):861–861 DOI 10.1038/43088.

Heine H. 2008. *Innate immunity of plants, animals, and humans*. Heidelberg: Springer-Verlag.

Hong S-Y, Roze L, Linz J. 2013. Oxidative stress-related transcription factors in the regulation of secondary metabolism. Toxins 5(4):683–702 DOI 10.3390/toxins5040683.

Ip YT, Reach M, Engstrom Y, Kadalayil L, Cai H, González-Crespo S, Tatei K, Levine M. 1993. Dif, a dorsal-related gene that mediates an immune response in Drosophila. Cell 75(4):753–763 DOI 10.1016/0092-8674(93)90495-C.

Irving P, Troxler L, Heuer TS, Belvin M, Kopczynski C, Reichhart J-M, Hoffmann JA, Hetru C. 2001. A genome-wide analysis of immune responses in *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America 98(26):15119–15124 DOI 10.1073/pnas.261573998.

Jacob TR, Peres NTA, Persinoti GF, Silva LG, Mazucato M, Rossi A, Martinez-Rossi NM. 2012. rpfb2 is a reliable reference gene for quantitative gene expression analysis in the dermatophyte *Trichophyton rubrum*. Medical Mycology 50(4):368–377 DOI 10.3109/13693786.2011.616230.

Koike M, Okamoto T, Tsuda S, Imai R. 2002. A novel plant defensin-like gene of winter wheat is specifically induced during cold acclimation. Biochemical and Biophysical Research Communications 298(1):46–53 DOI 10.1016/S0006-291X(02)02391-4.

Kumar M, Busch W, Birke H, Kemmerling B, Nürnberg T, Schöffl F. 2009. Heat shock factors HsfB1 and HsfB2b are involved in the regulation of *Pdf1.2* expression and pathogen resistance in *Arabidopsis*. Molecular Plant 2(1):152–165 DOI 10.1093/mp/ssn095.

Mahlapuu M, Håkansson J, Ringstad L, Björn C. 2016. Antimicrobial peptides: an emerging category of therapeutic agents. Frontiers in Cellular and Infection Microbiology 6:194 DOI 10.3389/fcimb.2016.00194.

Marx F. 2004. Small, basic antifungal proteins secreted from filamentous ascomycetes: a comparative study regarding expression, structure, function and potential application. Applied Microbiology and Biotechnology 65(2):133–142 DOI 10.1007/s00253-004-1600-z.

Marx F, Haas H, Reindl M, Stößler G, Lottspeich F, Redl B. 1995. Cloning, structural organization and regulation of expression of the *Penicillium chrysogenum* paf gene encoding an abundantly secreted protein with antifungal activity. Gene 167(1–2):167–171 DOI 10.1016/0378-1119(95)00701-6.

Matys V, Kel-Margoulis OV, Fricke E, Liebich I, Land S, Barre-Dirrie A, Reuter I, Chekmenev D, Krull M, Hornischer K. 2006. TRANSFAC® and its module TRANSCompe1®: transcriptional gene regulation in eukaryotes. Nucleic Acids Research 34(90001):D108–D110 DOI 10.1093/nar/gkj143.

Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature 388(6640):394–397 DOI 10.1038/41131.
Meyer V, Jung S. 2018. Antifungal peptides of the AFP family revisited: are these cannibal toxins? *Microorganisms* 6(2):50 DOI 10.3390/microorganisms6020050.

Meyer V, Stahl U. 2002. New insights in the regulation of the *afp* gene encoding the antifungal protein of *Aspergillus giganteus*. *Current Genetics* 42(1):36–42 DOI 10.1007/s00294-002-0324-9.

Meyer V, Stahl U. 2003. The influence of co-cultivation on expression of the antifungal protein in *Aspergillus giganteus*. *Journal of Basic Microbiology* 43(1):36–42 DOI 10.1002/jobm.200390007.

Meyer V, Wedde M, Stahl U. 2002. Transcriptional regulation of the antifungal protein in *Aspergillus giganteus*. *Molecular Genetics and Genomics* 266(5):747–757 DOI 10.1007/s00438-001-0609-6.

Mygind PH, Fischer RL, Schnorr KM, Hansen MT, Sönksen CP, Ludvigsen S, Raventós D, Buskov S, Christensen B, De Maria L, Taboureau O, Yaver D, Elvig-Jørgensen SG, Sorensen MV, Christensen BE, Kjærulff S, Zasloff M, Kristensen H-H. 2005. Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. *Nature* 437(7061):975–980 DOI 10.1038/nature04051.

Nürnberg T, Brunner F, Kemmerling B, Piater L. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological Reviews* 198(1):249–266 DOI 10.1111/j.0105-2896.2004.0119.x.

Oeemig JS, Lynggaard C, Knudsen DH, Hansen FT, Nørgaard KD, Schneider T, Vad BS, Sandvang DH, Nielsen LA, Neve S, Kristensen H-H, Sahl H-G, Otzen DE, Wimmer R. 2012. Eurocin, a new fungal defensin structure, lipid binding, and its mode of action. *Journal of Biological Chemistry* 287(50):42361–42372 DOI 10.1074/jbc.M112.382028.

Paege N, Jung S, Schäpe P, Müller-Hagen D, Ouedraogo J-P, Heiderich C, Jedamzick J, Nitsche BM, van den Hondel CA, Ram AF, Meyer V. 2016. A transcriptome meta-analysis proposes novel biological roles for the antifungal protein AnAFP in *Aspergillus niger*. *PLOS ONE* 11(11):e0165755 DOI 10.1371/journal.pone.0165755.

Paris S, Wysong D, Debeaupuis J-P, Shibuya K, Philippe B, Diamond RD, Latge J-P. 2003. Catalases of *Aspergillus fumigatus*. *Infection and Immunity* 71(6):3551–3562 DOI 10.1128/IAI.71.6.3551-3562.2003.

Penninckx IA, Eggermont K, Terras FR, Thomma B, De Samblanx GW, Buchala A, Métraux J-P, Manners JM, Broekaert WF. 1996. Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. *Plant Cell* 8(12):2309–2323 DOI 10.1105/tpc.8.12.2309.

Reverberi M, Zjalic S, Ricelli A, Punelli F, Camera E, Fabbri C, Picardo M, Fanelli C, Fabbri AA. 2008. Modulation of antioxidant defense in *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for the ApyapA gene. *Eukaryotic Cell* 7(6):988–1000 DOI 10.1128/EC.00228-07.

Ryals J, Weymann K, Lawton K, Friedrich L, Ellis D, Steiner HY, Johnson J, Delaney TP, Jesse T, Vos P. 1997. The Arabidopsis NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. *Plant Cell* 9(3):425–439 DOI 10.1105/tpc.9.3.425.

Schmitt P, Wilmes M, Puginie M, Aumelas A, Bachère E, Sahl H-G, Schneider T, Destoumieux-Garzón D. 2010. Insight into invertebrate defensin mechanism of action: oyster defensins inhibit peptidoglycan biosynthesis by binding to lipid II. *Journal of Biological Chemistry* 285(38):29208–29216 DOI 10.1074/jbc.M110.143338.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative C_{t} method. *Nature Protocols* 3(6):1101–1108 DOI 10.1038/nprot.2008.73.

Schneider T, Kruse T, Wimmer R, Wiedemann I, Sass V, Pag U, Jansen A, Nielsen AK, Mygind PH, Raventos DS, Neve S, Ravn B, Bonvin AMJJ, De Maria L, Andersen AS,
Gammelgaard LK, Sahl H-G, Kristensen H-H. 2010. Plectasin, a fungal defensin, targets the bacterial cell wall precursor Lipid II. Science 328(5982):1168–1172 DOI 10.1126/science.1185723.

Schroeckh V, Scherlach K, Nützmann H-W, Shelest E, Schmidt-Heck W, Schuemann J, Martin K, Hertweck C, Brakhage AA. 2009. Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. Proceedings of the National Academy of Sciences of the United States of America 106(34):14558–14563 DOI 10.1073/pnas.0901870106.

Seymour IJ, Piper PW. 1999. Stress induction of HSP30, the plasma membrane heat shock protein gene of Saccharomyces cerevisiae, appears not to use known stress-regulated transcription factors. Microbiology 145(1):231–239 DOI 10.1099/13500872-145-1-231.

Silva PM, Gonçalves S, Santos NC. 2014. Defensins: antifungal lessons from eukaryotes. Frontiers in Microbiology 5(47):97 DOI 10.3389/fmicb.2014.00097.

Svahn KS, Göransson U, Chryssanthou E, Olsen B, Sjölin J, Strömstedt AA. 2014. Induction of gliotoxin secretion in Aspergillus fumigatus by bacteria-associated molecules. PLOS ONE 9(4):e93685 DOI 10.1371/journal.pone.0093685.

Taji T, Seki M, Satou M, Sakurai T, Kobayashi M, Ishiyama K, Narusaka Y, Narusaka M, Zhu JK, Shinozaki K. 2004. Comparative genomics in salt tolerance between Arabidopsis and Arabidopsis-related halophyte salt cress using Arabidopsis microarray. Plant Physiology 135(3):1697–1709 DOI 10.1104/pp.104.039909.

Tiwari S, Thakur R, Shankar J. 2015. Role of heat-shock proteins in cellular function and in the biology of fungi. Biotechnology Research International 2015(2):1–11 DOI 10.1155/2015/132635.

Tonk M, Knorr E, Cabezas-Cruz A, Valdés JJ, Kollewe C, Vilcinskas A. 2015. Tribolium castaneum defensins are primarily active against Gram-positive bacteria. Journal of Invertebrate Pathology 132:208–215 DOI 10.1016/j.jip.2015.10.009.

Verheecke C, Liboz T, Anson P, Diaz R, Mathieu F. 2015. Reduction of aflatoxin production by Aspergillus flavus and Aspergillus parasiticus in interaction with Streptomyces. Microbiology 161(5):967–972 DOI 10.1002/mic.000070.

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B. 1994. The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. Cell 78(6):1101–1115 DOI 10.1016/0092-8674(94)90283-6.

Zhu S. 2008. Discovery of six families of fungal defensin-like peptides provides insights into origin and evolution of the CSaβ defensins. Molecular Immunology 45(3):828–838 DOI 10.1016/j.molimm.2007.06.354.

Zhu S, Gao B, Harvey PJ, Craik DJ. 2012. Dermatophytic defensin with antiinfective potential. Proceedings of the National Academy of Sciences of the United States of America 109(22):8495–8500 DOI 10.1073/pnas.1201263109.

Zhu J, Zhang MQ. 1999. SCPD: a promoter database of the yeast Saccharomyces cerevisiae. Bioinformatics 15(7):607–611 DOI 10.1093/bioinformatics/15.7.607.