The Zinc cluster transcription factor ZtfA is an activator of asexual development and secondary metabolism and regulates the oxidative stress response in the filamentous fungus *Aspergillus nidulans*

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Declaration of independence

Herewith I declare that the dissertation entitled "**The Zinc cluster transcription factor ZtfA is an activator of asexual development and secondary metabolism and regulates the oxidative stress response in the filamentous fungus *Aspergillus nidulans***" was written on my own and independently without any other aids and sources than indicated.

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This work was conducted in the group of Prof. Dr. Gerhard H. Braus at the Department of Molecular Microbiology and Genetics, Institute of Microbiology and Genetics, Georg-August-Universität Göttingen.

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Summary

The interconnection of developmental programs and secondary metabolism is regulated by the velvet domain proteins in numerous filamentous fungi. Velvet domain proteins constitute a family of fungal specific transcription factors with structural similarities in the DNA binding and dimerization domain of mammalian Rel-domains, including NF-κB as regulator for inflammation and infection. Velvet factors bind to promoters of thousands of genes and a large amount of their downstream targets remains to be analyzed. This study focuses on the <u>Z</u>inc cluster <u>t</u>ranscription <u>f</u>actor <u>A</u> (ZtfA) as repression target of the velvet factor VosA in the filamentous fungi *Aspergillus nidulans* and *A. fumigatus*. The *A. nidulans* Δ*ztfA* strain forms diminished numbers of conidiophores with conidiospores of short-term viability compared to the wild type. A *ztfA* overexpression strain forms conidiophores in conditions when the wildtype grows with vegetative hyphae. The *ztfA* overexpressing strain increases conidiophore formation during sexual development in the dark, where conidiation normally is repressed. The conidiation pathway proceeds in a strictly time-tuned manner and several regulators are involved in its temporal control. The *ztfA* gene product was exclusively found in nuclei of hyphae, conidiophores and germinating spores. ZtfA activates the conidiation pathway through the major regulatory gene *brlA* and the conidiation activator-encoding genes *flbC* and *flbD*. ZtfA represents a novel component of the timely adjusted choreography of conidiation. ZtfA controls expression of several secondary metabolite genes, including austinol or dehydroaustinol biosynthesis. It forms a complex with the transcription repressor RcoA and might execute parts of its regulatory functions as a heterodimer. The phosphorylation status of ZtfA is presumably part of its control function. ZtfA regulates genes of the oxidative stress response system in the presence of hydrogen peroxide. ZtfA is conserved among Aspergilli as exemplified by the characterization of the *A. fumigatus* counterpart. AfZtfA is part of the fungal adhesion, but dispensable for conidiation. In summary, ZtfA regulates asexual development, secondary metabolite expression and oxidative stress response downstream of the velvet factor VosA in the filamentous fungus *A. nidulans* and is involved in the regulation of adhesion factors in *A. fumigatus*.

Zusammenfassung

Velvet-Domänen-Proteine verknüpfen Entwicklungsprogramme und Sekundärmetabolismus in zahlreichen filamentösen Pilzen. Velvet-Domänen-Proteine stellen eine Familie von Pilz-spezifischen Transkriptionsfaktoren dar, welche in ihrer DNA-Binde- und Dimerisierungsdomäne strukturelle Gemeinsamkeiten mit Rel-Domänen, einschließlich der NF-κB Faktoren von Säugetieren aufweist. Velvet-Faktoren binden Promotoren tausender Gene und das Gros ihrer nachgeschalteten Ziele ist noch unbekannt. Die vorliegende Studie konzentriert sich auf den Zink Cluster Transkriptionsfaktor ZtfA als Repressions-Ziel des Velvet-Faktors VosA in den filamentösen Pilzen *Aspergillus nidulans* und *A. fumigatus*. Im Vergleich zum Wildtyp produziert der *A. nidulans ztfA* Deletionsstamm eine stark verminderte Zahl an Konidiophoren, welche Konidiosporen mit verkürzter Lebensfähigkeit hervorbringen. Eine *ztfA* Überexpression produziert Konidiophore sogar unter Bedingungen, unter denen der Wildtyp nur vegetative Hyphen bildet. Die *ztfA* Überexpression produziert eine erhöhte Anzahl an Konidiophoren während des sexuellen Wachstums im Dunkeln, in welchem normalerweise die Konidiosporulation reprimiert ist. Der Signalweg der Konidiosporulation läuft in einer strikten Zeitfolge ab und mehrere Regulatoren sind an seiner zeitlichen Kontrolle beteiligt. ZtfA aktiviert den Signalweg der Konidiosporulation über dessen Hauptregulator, kodiert durch das *brlA* Gen und über die Konidiations-Aktivatoren, kodiert durch *flbC* und *flbD* und stellt eine neue Komponente des zeitabhängigen Ablaufs der Konidiosporulation dar. ZtfA kontrolliert die Expression mehrere Sekundärmetabolit-Gene, einschließlich der Biosynthese von Austinol und Dehydroaustinol. Es bildet einen Proteinkomplex mit dem Transkriptionsrepressor RcoA und übt seine regulatorischen Funktionen vermutlich teilweise als Heterodimer aus. Der Phosphorylierungszustand von ZtfA ist vermutlich Teil seiner Funktionskontrolle. ZtfA reguliert Gene der oxidativen Stress-Antwort in der Gegenwart von Wasserstoffperoxid. ZtfA ist konserviert in Aspergillen, wie beispielhaft durch die Charakterisierung seines Gegenstücks in *A. fumigatus* gezeigt wird. AfZtfA ist Teil der Regulation der pilzlichen Adhäsion, jedoch entbehrlich für die Bildung von Konidiophoren. Zusammenfassend reguliert ZtfA in *A. nidulans* asexuelle Entwicklung, Sekundärmetabolit-Expression und die Antwort auf oxidativen Stress, nachgeschaltet zu dem Velvet-Faktor VosA. In *A. fumigatus* ist es wichtig für die Adhäsion.

1. Introduction

1.1 *Aspergillus nidulans* – a genetic model for filamentous fungi

Aspergillus nidulans, member of the most common fungal genus *Aspergillus*, is a well-established genetic model for filamentous fungi (de Vries *et al.*, 2017). It is able to produce asexual conidiophores and sexual cleistothecia in response to environmental conditions, such as pH, temperature and light. The soil borne mold is a member of the phylum Ascomycota, which comprises the mostly unicellular saccharomycetes and mycelial ascomycetes with a short dikaryotic phase in their life cycle. The latter group propagates vegetatively via formation of indefinite hyphae and forms complicated multicellular structures upon establishment of suitable internal and external conditions. A characteristic of all members of the phylum Ascomycota is the formation of the name-giving multicellular structure: the ascus. This sac-like structure represents a compartment within the fruit body, which produces sexual meiospores, called ascospores (Braus *et al.*, 2002; Pöggeler *et al.*, 2006) (FIGURE 1). The ascus is protected by the ascocarp (Greek: *askos* = sac, *karpos* = fruit), the visible fruit body. The ascocarp is called cleistothecium in *A. nidulans*, due to its closed form (Greek: *kleistos* = closed, *theke* = case) and is surrounded and nursed by multi-nucleated Hülle cells, which differentiate from hyphae forming nest-like structures around the developing cleistothecium (Latin: *nidulans* = nest). Fruit bodies are highly complex structures of the sexual life cycle of fungi and represent overwintering structures.

Aspergillus comprises a large genus with significant impact on humankind, since most Aspergilli are secondary metabolite producers. Fungal secondary metabolites can be useful or deleterious. Hence, several representatives of *Aspergillus* spp. are of medical or economic importance, such as *A. niger*, the main source for citric acid production, *A. oryzae*, indispensable for Asian cuisine, *A. flavus*, a wide-spread crop contaminant or *A. fumigatus*, a serious health threat in immunocompromised patients (Bhatnagar-Mathur *et al.*, 2015; Chen *et al.*, 2016; Despot *et al.*, 2016; Jöhnk *et al.*, 2016; Kobayashi *et al.*, 2007; Wang *et al.*, 2017). The large enzymatic variety of Aspergilli renders the genus a large source of industrially and medically important fungi.

A. nidulans is a homothallic fungus and as such is able to undergo sexual development without the presence of a partner with a different mating type (FIGURE 1). Mitotic division during asexual development yields conidiophores, which produce haploid mitotic conidiospores in two to three days after germination whereas sexual cleistothecia form

binucleate ascospores, which mature after approximately seven days (Braus *et al.*, 2002; Pöggeler *et al.*, 2006). In contrast to homothallic fungi, heterothallic fungi like *A. fumigatus* need a partner of opposite mating type to undergo sexual differentiation.
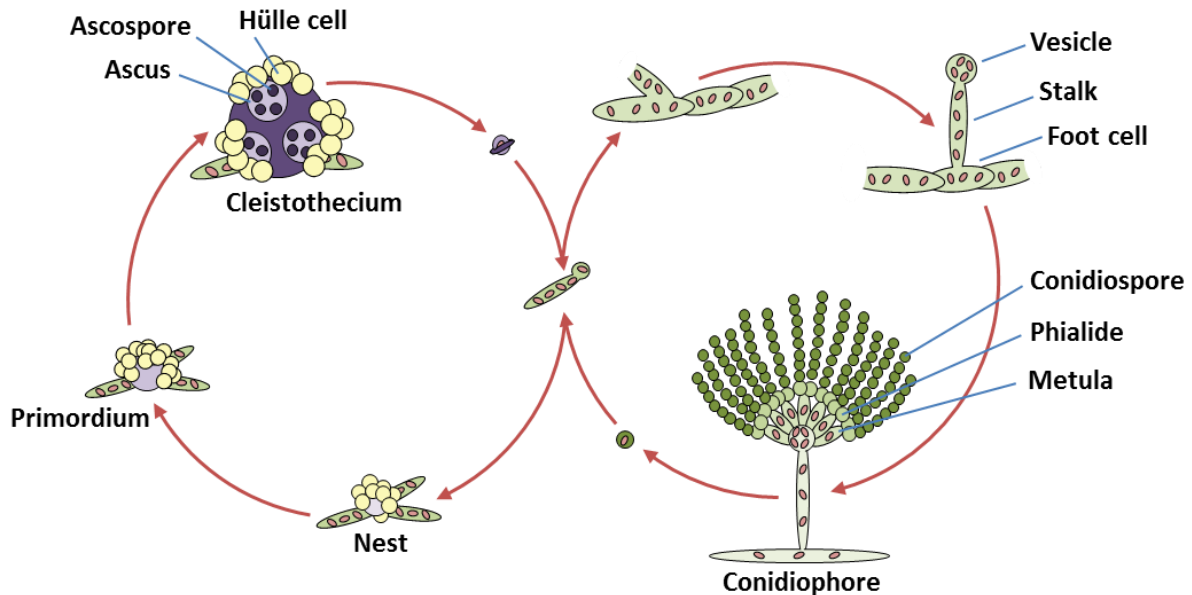
FIGURE 1: Developmental programs of *A. nidulans*.
A. nidulans can undergo sexual (left hand side) as well as asexual (right hand side) development leading to the formation of sexual cleistothecia (predominantly formed in the dark under low oxygen supply) or asexual conidiophores (formed in light when oxygen is present) as spore forming units. Adapted from Bayram *et al.*, 2010.

Since the genome of *A. nidulans* is completely sequenced (Galagan *et al.*, 2005) and an increasingly large number of phenotypical, transcriptomic and proteomic approaches have been carried out, it is one of the most feasible genetic models for haploid filamentous fungi.

1.2   The velvet regulators

Important regulators of developmental programs in filamentous fungi are the velvet proteins, which form complex regulatory networks (Bayram *et al.*, 2008a, 2008b; Käfer, 1965; Kato *et al.*, 2003; Kim *et al.*, 2002; Park *et al.*, 2012a; Satterlee *et al.*, 2016). Velvet proteins constitute a family of fungal specific regulatory proteins, which mostly comprises four members. The founding member of this family, velvet A (VeA), was identified more than half a century ago as a developmental regulator with a central role in transduction of the development inducing light signal (Käfer, 1965). The velvet family further comprises the factors VelB, VelC (velvet-like B and C) and VosA (viability of spores A). Velvet proteins share the name-giving velvet domain and are highly conserved among filamentous fungi

(Ahmed *et al.*, 2013; Bayram *et al.*, 2008a; Ni and Yu, 2007). The velvet domain does not exhibit sequence similarities with known protein domains, but structural similarities to the Rel homology domain of NF-κBs were found recently (Ahmed *et al.*, 2013). NF-κBs constitute a family of mammalian transcription factors. They are involved in apoptosis and inflammatory response but also in broad metabolic processes and cell proliferation (Engelmann and Haenold, 2016; Sun and Andersson, 2002). Velvet factors contain a DNA-binding and dimerization domain and act as transcription factors in *A. nidulans* and *Penicillium chrysogenum* (Ahmed *et al.*, 2013; Becker *et al.*, 2016).

VeA is involved in the coordination of sexual development and secondary metabolism and is part of the light control of fungal development (Alkahyyat *et al.*, 2015; Bayram *et al.*, 2008a; Calvo, 2008; Kim *et al.*, 2002; Mooney and Yager, 1990; Stinnett *et al.*, 2007). VeA is necessary for cleistothecia formation (Kim *et al.*, 2002). Involvement of VeA and other velvet proteins in virulence has been shown in several fungi, such as *A. flavus*, several *Fusarium* spp. and others (Duran *et al.*, 2009; Merhej *et al.*, 2012; Myung *et al.*, 2012; Wang *et al.*, 2016; Wiemann *et al.*, 2010). VeA interacts with several proteins. It forms a protein complex with the white-collar (WC) proteins LreA and LreB and the phytochrome FphA, which fulfils light-triggered regulatory functions (Hedtke *et al.*, 2015; Purschwitz *et al.*, 2008; Ruger-Herreros *et al.*, 2011). WC proteins are involved in light regulation in fungi and activate expression of the major conidiation regulator-encoding *bristle* gene (*brlA*) in response to light (Chen *et al.*, 2009; Froehlich *et al.*, 2002; He and Liu, 2005; Ruger-Herreros *et al.*, 2011; Smith *et al.*, 2010). VeA forms a heterotrimeric complex with VelB and the methyltransferase LaeA (lack of *aflR* expression A) in the nucleus, known as the velvet complex, which acts as a major regulator of secondary metabolism (Bayram *et al.*, 2008a; Estiarte *et al.*, 2016; Lind *et al.*, 2016; Sarikaya-Bayram *et al.*, 2010; Schumacher *et al.*, 2015; Wang *et al.*, 2016) (FIGURE 2) (see CHAPTER 1.3). The VeA-VelB heterodimer, which forms in the cytoplasm prior to velvet complex formation, is presumably the main mechanism for VelB to enter the nucleus as VelB does not exhibit a conserved nuclear localization sequence (NLS) (Bayram *et al.*, 2008a; Bayram and Braus, 2012; Sarikaya-Bayram *et al.*, 2010). Nuclear import of the VeA-VelB heterodimer is controlled by the methyltransferases VipC (VeA interacting protein C) and the VipC associated protein VapB (Sarikaya-Bayram *et al.*, 2014). Both methyltransferases are recruited by VapA to the plasma membrane and released upon environmental triggers (Sarikaya-Bayram *et al.*, 2014). The VipC-VapB heterodimer negatively influences VeA-VelB nuclear entrance after release from the plasma membrane. It also forms heterotrimeric complexes with VeA in the nucleus. Either VipC-VapB or the

heterotrimer acts positively on asexual and negatively on sexual development and influences histone posttranslational modifications (Sarikaya-Bayram *et al.*, 2014, 2015) (FIGURE 2).

VelB was proposed to be an activator of conidiation since a loss of *velB* results in diminished conidiophores, whereas an overexpression (OE) leads to increased conidiation (Park *et al.*, 2012b). VelB exhibits a positive regulation on the biosynthesis of sterigmatocystin, a potent mycotoxin (Bayram *et al.*, 2008a; Bayram and Braus, 2012; Bryant *et al.*, 2016; Gruber-Dorninger *et al.*, 2016).
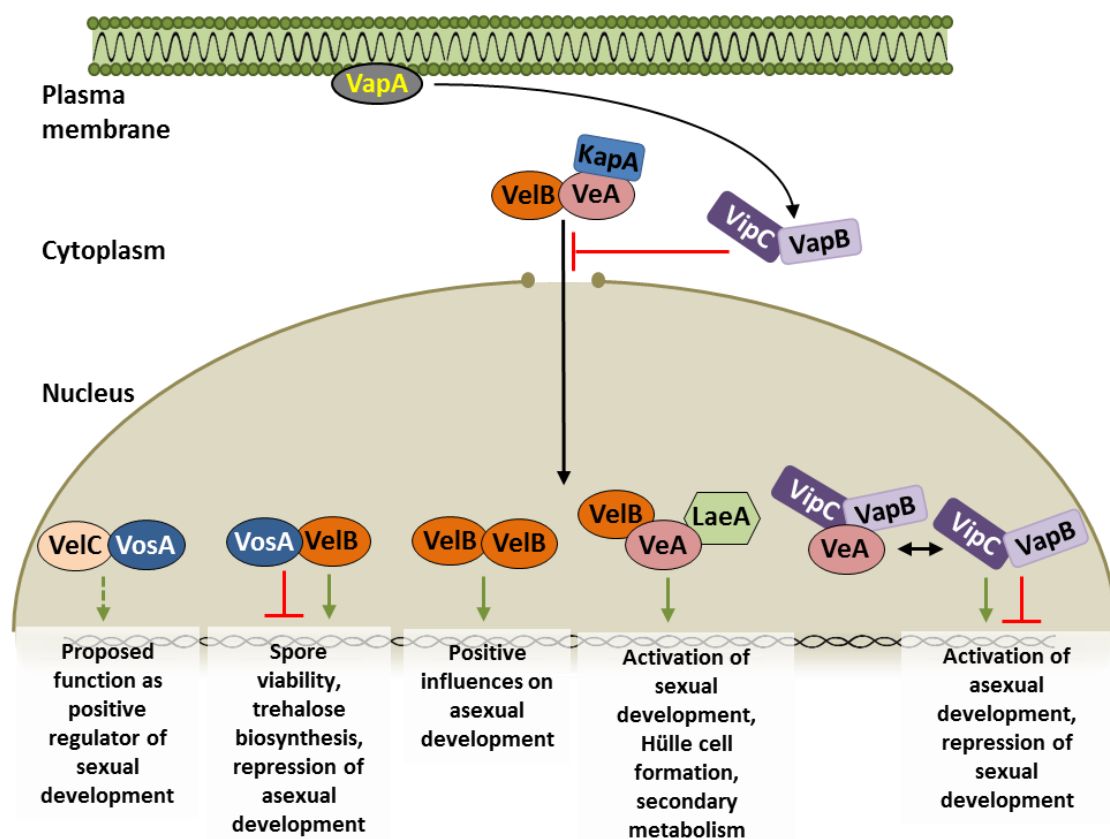
FIGURE 2: The velvet regulatory network.
The depicted schema summarizes the velvet protein network of *A. nidulans*. The α-importin KapA shuttles VeA-VelB into the nucleus. VipC-VapB is released from VapA at the plasma membrane and negatively regulates VeA-VelB nuclear entry. Both velvet proteins form several complexes in the nucleus. VeA-VelB recruits LaeA to form the velvet complex, which activates sexual development and secondary metabolism. VeA forms a heterotrimeric complex with VipC-VapB. Either this heterotrimer or VipC-VapB act as activator of asexual and repressor of sexual development and influence histone posttranslational modifications. VelB forms homodimers and presumably acts positively on asexual development. The VelB-VosA heterodimer is important for spore viability and trehalose biosynthesis and acts as a repressor of early asexual development. The function of the VosA-VelC heterodimer is not clear, but it is proposed to positively regulate sexual development. Positive regulatory influences are shown in green, negative regulatory influences in red. Adapted from Sarikaya-Bayram *et al.*, 2014, 2015.

VelB forms an alternative heterodimer with VosA in the nucleus (Sarikaya-Bayram *et al.*, 2010) (FIGURE 2). The VelB-VosA heterodimer exhibits a time dependent dual function: it represses *brlA* expression during vegetative growth but regulates conidiospore viability and maturation by activation of *wetA* (*wet*-*white* *A*) and other genes, which products are important for conidiospore maturation and trehalose biosynthesis during late asexual growth (Bayram *et al.*, 2008a; Lee *et al.*, 2016; Ni and Yu, 2007; Park *et al.*, 2012b; Sarikaya-Bayram *et al.*, 2010) (see CHAPTER 1.5.3).

VelB and VosA, and their homologs, are inter-dependent in promoting spore maturation and viability (Sarikaya-Bayram *et al.*, 2010; Wang *et al.*, 2014; Webster and Sil, 2008). VosA is involved in conidiospore quality and virulence of several pathogenic fungi as well (Li *et al.*, 2015; Wang *et al.*, 2015). VeA and VosA seem to be exchanged as VelB binding partners in VelB heterodimers, since a deletion of *laeA* leads to increased VosA-VelB heterodimer formation (Sarikaya-Bayram *et al.*, 2010).

The role of the fourth velvet protein VelC is a matter of ongoing investigation up to date. *In vitro* analyses suggest the formation of a VosA-VelC heterodimer, which was proposed to positively regulate sexual development (Park *et al.*, 2012a, 2014).

1.3   Secondary metabolism

Filamentous fungi, and especially the Aspergilli, are a reservoir for yet undescribed secondary metabolites (SMs) (Alberti *et al.*, 2017; Brakhage, 2013; Chen *et al.*, 2016; Despot *et al.*, 2016). *A. nidulans* produces several SMs, such as penicillins, sterigmatocystin, benzaldehydes, emericellamides, orsellinic acid, orcinol and orcinol-related compounds, diindoles, austinol and dehydroaustinol (Brakhage, 2013; Gerke *et al.*, 2012b; Giles *et al.*, 2011; Lo *et al.*, 2012; Nahlik *et al.*, 2010; Schroeckh *et al.*, 2009). SM genes often are organized in clusters in fungal genomes and are controlled by cluster-specific transcription factors and master regulators, which interconnect developmental programs with SM biosynthesis (Bok and Keller, 2004; Calvo *et al.*, 2002; Keller *et al.*, 2005). Therefore, SM biosynthesis is activated by environmental triggers such as light, temperature, pH, nutrient availability and presence of other organisms and connected to developmental programs (Bayram *et al.*, 2008a; Brakhage, 2013). Fruit body formation is genetically linked to secondary metabolism in response to illumination (Busch *et al.*, 2003; Kato *et al.*, 2003; Kim *et al.*, 2002). Expression of secondary metabolite genes is reoriented during onset of conidiation (Garzia *et al.*, 2013). SM gene clusters are often silent during laboratory growth (Gerke and Braus, 2014).

Bridging factors between developmental programs and secondary metabolism are the velvet factors. VeA regulates developmental programs together with secondary metabolism in response to environmental cues (Bayram *et al.*, 2008a, 2008b; Calvo *et al.*, 2004; Duran *et al.*, 2007; Kato *et al.*, 2003; Li *et al.*, 2006; Myung *et al.*, 2012; Sarikaya-Bayram *et al.*, 2010) (see CHAPTER 1.2). Deletion of *veA* leads to a change in secondary metabolite production and VeA is necessary for sterigmatocystin production (Dreyer *et al.*, 2007; Estiarte *et al.*, 2016; Kato *et al.*, 2003; Myung *et al.*, 2009). Sterigmatocystin is a potent toxin and in several Aspergilli the penultimate precursor of aflatoxin, which is related to apoptosis and disequilibrium between reactive oxygen species (ROS) and ROS defense mechanisms in host cells of pathogens (Chen *et al.*, 2013; Mughal *et al.*, 2017). This is reflected on transcriptional level: VeA is necessary for *aflR* expression, which encodes the major regulator of sterigmatocystin and penicillin biosynthesis in *A. nidulans* and is the ortholog of the <u>af</u>latoxin <u>r</u>egulator from *A. flavus* and *A. parasiticus* (Woloshuk *et al.*, 1994; Yu *et al.*, 1996a). A key element of the VeA-mediated regulation of secondary metabolism and developmental programs is the velvet complex (VelB-VeA-LaeA) (Bayram *et al.*, 2008a; Bok and Keller, 2004; Cohrs *et al.*, 2016; Martín, 2016; Schumacher *et al.*, 2015) (CHAPTER 1.2). LaeA is a master regulator of secondary metabolism in fungi and one of the very few SM regulators, which is conserved (Bok and Keller, 2004; Butchko *et al.*, 2012; Jiang *et al.*, 2016; Liu *et al.*, 2016; Reyes-Dominguez *et al.*, 2010; Wang *et al.*, 2016; Wu *et al.*, 2012). Secondary metabolism is silenced in the absence of *laeA*, whereas its overexpression results in increased production of several secondary metabolites (Amaike and Keller, 2009; Bok *et al.*, 2006b; Bok and Keller, 2004; Chettri and Bradshaw, 2016; Jiang *et al.*, 2016; Martín, 2016; Shaaban *et al.*, 2010). Its role in SM regulation, however, is species specific (Chettri and Bradshaw, 2016; Linde *et al.*, 2016; Liu *et al.*, 2016). Involvement of LaeA and the other velvet complex members in virulence, probably via activation of mycotoxin production, has been demonstrated for several pathogenic fungi (Estiarte *et al.*, 2016; Kumar *et al.*, 2016; López-Díaz *et al.*, 2017) (see CHAPTER 1.2). Further SM master regulators have been identified, such as the <u>m</u>ulti<u>c</u>luster <u>r</u>egulator <u>A</u> (McrA), which represses a number of SM genes, or the <u>r</u>emediator of <u>s</u>econdary <u>m</u>etabolism (RsmA) (Oakley *et al.*, 2016; Yin *et al.*, 2013). Other developmental regulators are involved in SM regulation as well, such as the Flb (<u>fl</u>uffy <u>l</u>ow <u>br</u>l<u>A</u>) factor FlbA or the <u>m</u>aster <u>t</u>ranscription <u>f</u>actor <u>A</u> (MtfA) (Hicks *et al.*, 1997; Keller *et al.*, 1994; Lind *et al.*, 2015; Yu *et al.*, 1996a).

1.4   Vegetative polar growth in *A. nidulans*

Initially, filamentous fungi form vegetative hyphae, also called mycelia. The filamentous growth mode allows fungi to adhere to substrates and invade them. Undifferentiated hyphae elongate by extending their plasma membrane and cell wall with new material from distal areas in a polarized manner until internal and external stimuli induce developmental programs (Adams *et al.*, 1998; Herrero-Garcia *et al.*, 2015; Riquelme, 2013). These hyphae form branched two dimensional networks by fusion via anastomosis tubes (Gabriela Roca *et al.*, 2005). A prerequisite for polarized hyphal growth is the Spitzenkörper, located at the center of the hyphal tip (Fajardo-Somera *et al.*, 2015; Harris, 2009; Schultzhaus *et al.*, 2017; Virag and Harris, 2006). The Spitzenkörper is a dynamic center for vesicle organization and supply, which is required for cell wall and plasma membrane component transport (Schultzhaus *et al.*, 2017; Virag and Harris, 2006). An important function of the hyphal tip is sensing and adaption to new environmental stimuli and the Spitzenkörper might serve as a signaling hub and protein-recycling center in this context (Harris, 2009; Schultzhaus *et al.*, 2015; Schultzhaus and Shaw, 2016).

An essential aspect of the vegetative life style is polarity of growth. FlbB and FlbE are transcription factors that are necessary for hyphal polarized growth and FlbB accumulates at the hyphal tip (Etxebeste *et al.*, 2008; Garzia *et al.*, 2009, 2010; Herrero-Garcia *et al.*, 2015) (FIGURE 3).
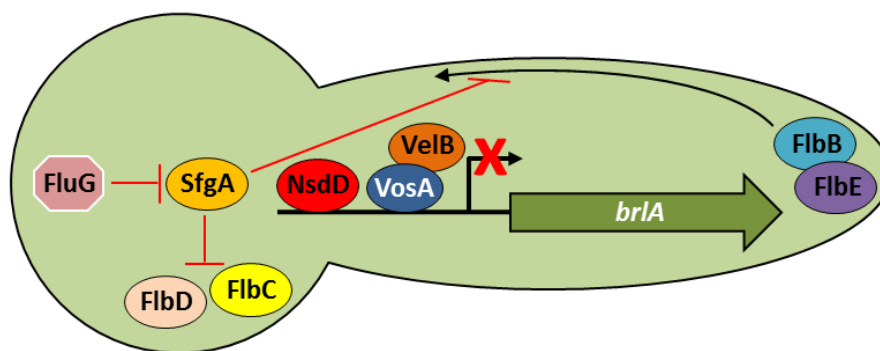
FIGURE 3: Repressors block conidiation during hyphal growth of *A. nidulans*.
VosA-VelB heterodimers and NsdD block *brlA* expression during vegetative growth. SfgA restricts the FlbB-FlbE heterodimer to apical localizations (apical nuclei) and negatively influences FlbD and FlbC. FluG starts to accumulate what removes the repressing effect of SfgA upon the Flb factors. Adapted from Lee *et al.*, 2016.

FlbE forms heterodimers with FlbB and is necessary for the apical FlbB accumulation (Herrero-Garcia *et al.*, 2015). Subsequently, FlbB migrates to the most apical nucleus, enters and accumulates in this and subsequent nuclei (Etxebeste *et al.*, 2008, 2009; Garzia *et al.*,

2009; Herrero-Garcia *et al.*, 2015) (FIGURE 3). This migration depends on an intact actin skeleton and is crucial for the gain of competence to induce asexual development (Garzia *et al.*, 2009; Herrero-Garcia *et al.*, 2015). In the nucleus, FlbB binds to the *brlA* promoter (Kwon *et al.*, 2010a) (see CHAPTER 1.5). *brlA* encodes the master regulator of conidiation and is repressed by VosA-VelB and NsdD (Adams *et al.*, 1988; Lee *et al.*, 2014, 2016) (FIGURE 3). The nuclear localization of FlbB is dependent on the conidiation repressor SfgA (<u>s</u>uppressor of <u>fluG</u> <u>A</u>), which restricts FlbB localization to the most apical nuclei in newly formed hyphal branches (Etxebeste *et al.*, 2008, 2009) (FIGURE 3).

1.5 Asexual development

1.5.1 The conidiophore

Conidiophores are the asexual spore-producing structures in *A. nidulans*, which consist of four cell types (the stalk with a vesicle, metulae, phialides and airborne conidiospores) and are produced after achievement of developmental competence approximately 18 to 20 h post germination (Axelrod *et al.*, 1973; Mims *et al.*, 1988; Yager *et al.*, 1982) (FIGURE 4). Conidiophores arise from a thick-walled hyphal foot cell, on which a stalk with a terminal multinucleated vesicle is produced (Mims *et al.*, 1988). On top of this vesicle, a layer of uninucleated metulae is formed, which produce two to three spore forming phialides per metula in a budding-like process (Adams *et al.*, 1998; Fischer, 2002; Garzia *et al.*, 2013; Mims *et al.*, 1988; Yu, 2010). Repeated mitoses of the phialides ultimately lead to formation of haploid airborne conidiospores, which are isogenic to the parental organism. Mature conidia are formed after approximately 15 to 24 h post induction of asexual development in light (Fischer, 2002; Mooney and Yager, 1990; Yu, 2010) (FIGURE 4).
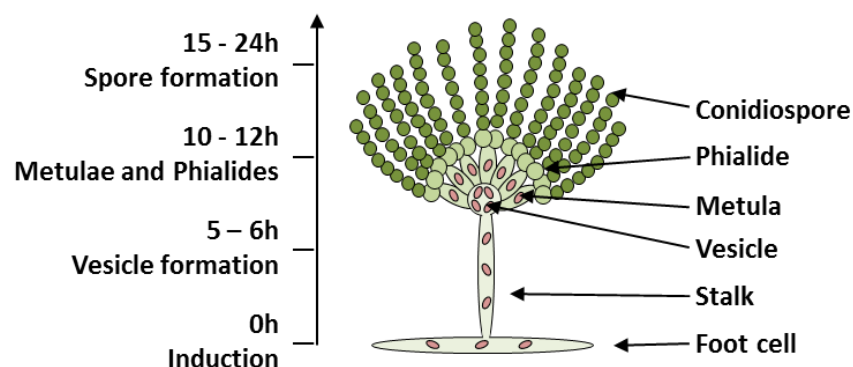
FIGURE 4: The conidiophore of *A. nidulans*.
The formation of a conidiophore over time is shown. Cell nuclei are given in red. Note that conidiospores and phialides are mononucleated (not shown). Adapted from Yu, 2010.

Attributes of mature conidiospores are four-layered cell walls, covered by a rodlet layer of hydrophobins and accumulate trehalose, which is proposed to promote long-time viability (Bayry *et al.*, 2012; Beever and Dempsey, 1978; d'Enfert and Fontaine, 1997; Ni and Yu, 2007; Sewall *et al.*, 1990a).

Asexual development is induced in response to external (e.g. light, temperature, pH, nutrient availability) and internal (e.g. metabolites) signals (Oiartzabal-Arano *et al.*, 2016; Rodríguez-Urra *et al.*, 2012). Transcriptional changes during ongoing development in response to illumination conditions affect approximately 19% of genes in *A. nidulans* (Bayram *et al.*, 2016). Developmentally competent mycelia differentially regulate approximately 5% of their genes when grown in light for only 30 min, the minimum time required for initiation of conidiation in *A. nidulans,* compared to growth in the dark (Mooney and Yager, 1990; Ruger-Herreros *et al.*, 2011). Several sexual development-related genes are downregulated during the transition from vegetative to asexual growth. *brlA* and genes, which products are involved in conidium differentiation, pigmentation and integrity are upregulated during this process (Garzia *et al.*, 2013). Around 7% of the genes are differentially expressed after 24 h of growth in light, compared to vegetative growth (Bayram *et al.*, 2016). Conidiation can be separated into two phases of genetic differentiation: the early phase where initiation of conidiophore development occurs due to <u>u</u>pstream <u>d</u>evelopmental <u>a</u>ctivators (UDAs) of *brlA*, and the late phase, characterized by the <u>c</u>entral <u>d</u>evelopmental <u>p</u>athway (CDP), which leads to spore formation and maturation (Etxebeste *et al.*, 2010a; Garzia *et al.*, 2013; Herrero-Garcia *et al.*, 2015; Oiartzabal-Arano *et al.*, 2015).

1.5.2 Upstream developmental activators induce conidiation after de-repression of *brlA* occurred

Premature asexual development is hindered during vegetative growth in *A. nidulans* by repression of the major conidiation activator BrlA. This repression is accomplished indirectly by SfgA and directly by VosA and NsdD (<u>n</u>ever in <u>s</u>exual <u>d</u>evelopment <u>D</u>) (Han *et al.*, 2001; Lee *et al.*, 2014, 2016; Ni and Yu, 2007; Seo *et al.*, 2006). De-repression occurs in a time dependent manner. Firstly, FluG (<u>flu</u>ffy <u>G</u>) removes the repressive effects of SfgA on conidiation by accumulation of a small molecular weight marker (Lee and Adams, 1994a; Lee *et al.*, 2014; Seo *et al.*, 2003, 2006). Secondly, the repressors NsdD and VosA dissociate from the *brlA* promoter, allowing the Flb factors to activate *brlA* expression (Garzia *et al.*, 2010; Lee *et al.*, 2014, 2016) (FIGURE 5).

VosA is proposed to exhibit this repression as homodimer or heterodimer with VelB and DNA-binding of VosA as well as the VosA-VelB heterodimer to the *brlA* promoter was shown (Ahmed *et al.*, 2013; Park *et al.*, 2012b; Sarikaya-Bayram *et al.*, 2010). Recently, a direct action upon the *brlA* promoter has been shown for NsdD as well (Lee *et al.*, 2016). Lee and co-workers found that a Δ*nsdD*Δ*vosA* double mutant produces conidiophores as early as 12 h in submerged cultures whereas the WT hardly forms conidiophores under these culture conditions (Lee *et al.*, 2016). The repression of conidiophore development by NsdD was observed in *A. flavus* and *A. fumigatus*, indicating a similar regulation of conidiation repression in Aspergilli in general (Lee *et al.*, 2016). Binding of NsdD and VosA to the *brlA* promoter together determines full suppression of conidiation (Lee *et al.*, 2016). After removal of, probably multiple, NsdD(s) and VosA from the *brlA* promoter (after approximately 18 h of vegetative growth), the Flb factors FlbB, FlbD and FlbC bind to the *brlA* promoter and activate *brlA* expression (Etxebeste *et al.*, 2008, 2009; Garzia *et al.*, 2010; Kwon *et al.*, 2010a; Wieser and Adams, 1995).

The conidiation cascade, or <u>c</u>entral <u>d</u>evelopmental <u>p</u>athway (CDP), BrlA→AbaA→WetA is activated by the <u>u</u>pstream <u>d</u>evelopmental <u>a</u>ctivators (UDAs) network (Adams *et al.*, 1988, 1998; Yu *et al.*, 2006). These are the products of the *flb* genes (Adams *et al.*, 1992; Wieser *et al.*, 1994). The *flb* genes *flbB*, *flbC*, *flbD* and *flbE* encode transcription factors, which activate *brlA* expression (Etxebeste *et al.*, 2008, 2009, Garzia *et al.*, 2009, 2010, Kwon *et al.*, 2010a, 2010b; Wieser and Adams, 1995) (FIGURE 5). Conservation of function has been shown for several of these genes in other fungi as well (Kwon *et al.*, 2010b; Malapi-Wight *et al.*, 2014; Matheis *et al.*, 2017; Son *et al.*, 2014; Yao *et al.*, 2016).
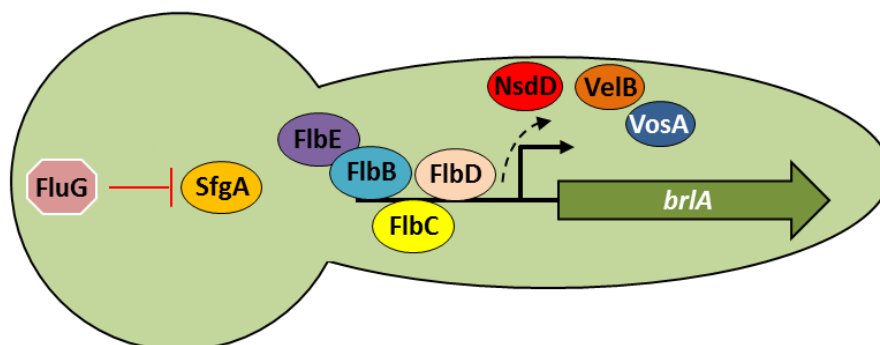
FIGURE 5: The upstream developmental activators induce *brlA* expression of *A. nidulans*. The accumulating FluG signal represses SfgA. NsdD and VosA-VelB dissociate from the *brlA* promoter and allow the Flb factors to activate *brlA*, which initiates conidiophore formation. Adapted from Lee *et al.*, 2016.

The UDA genes are already expressed during vegetative growth (Garzia *et al.*, 2013) (see CHAPTER 1.4). The *flb* gene products activate *brlA* in two cascades: the basic-leucine-zipper

(bZIP) transcription factor FlbB activates the cMyb transcription factor FlbD and, together with FlbE, which is important for proper FlbB activation (see CHAPTER 1.4), both transcription factors subsequently induce *brlA* expression (Etxebeste *et al.*, 2008, 2009, Garzia *et al.*, 2009, 2010; Herrero-Garcia *et al.*, 2015; Wieser and Adams, 1995). A prerequisite for this activation is the absence of repressive effects of SfgA upon nuclear localization of the Flb factors (Etxebeste *et al.*, 2009). Deletion of *flb* genes lead to the production of increased amounts of aerial hyphae and decreased and delayed conidiation due to impaired *brlA* activation, which leads to a fluffy cotton-like phenotype (Adams *et al.*, 1998; Wieser *et al.*, 1994; Yu *et al.*, 2006). Besides the FlbB/FlbE→FlbD→BrlA cascade, a second cascade exists: FlbC→BrlA (Kwon *et al.*, 2010a). *flbC* encodes a C2H2 transcription factor that binds to promoter regions of *brlA*, *abaA* (*abacus A*) and *vosA*, but not of *wetA in vitro* (Kwon *et al.*, 2010a; Sewall *et al.*, 1990b). *flbC* OE is sufficient to induce *brlA*, *abaA* and *vosA* expression (but not *wetA*) independently of FlbB and FlbE (Kwon *et al.*, 2010a).

The RGS (regulator of G-protein signaling) domain protein FlbA functions in an indirect activation of conidiation. Together with the other Flb factors, FlbA is required for light dependent activation of *brlA* and required for response of *fluG* and *flbB* to light (Ruger-Herreros *et al.*, 2011). However, FlbA is involved in normal asexual growth but not absolutely required for conidiation (Adams *et al.*, 1998; Lee and Adams, 1994b). FlbA regulates a heterotrimeric G-protein signaling pathway by inactivating FadA (Gα-subunit) (Yu *et al.*, 1996b). FadA is in its active form guanosine triphosphate (GTP)-bound and inhibits asexual development and production of the secondary metabolite sterigmatocystin by transmitting a proliferation supporting signal to its downstream factor PkaA (Hicks *et al.*, 1997; Kato *et al.*, 2003; Shimizu *et al.*, 2003; Shimizu and Keller, 2001; Yu *et al.*, 1996b). PkaA inhibits *brlA* and *aflR* expression (Shimizu and Keller, 2001; Yu *et al.*, 1996a) (FIGURE 6).
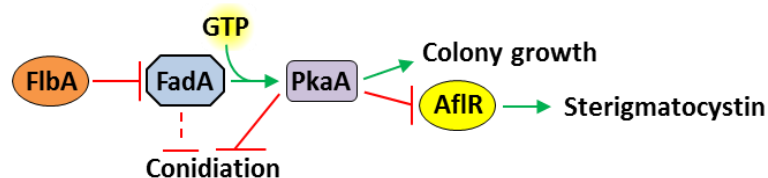
FIGURE 6: FlbA indirectly regulates conidiation of *A. nidulans*.
FlbA indirectly activates conidiation, proposedly by repression of a G-protein signaling pathway, which represses development via FadA. Activating effects are shown in green, repressing effects in red. Adapted from Shimizu *et al.*, 2003.

1.5.3 The central developmental pathway leads to conidiophore formation

Activation of *brlA* expression is the key step in the pathway leading to the formation of conidiospores (Adams *et al.*, 1988). *brlA* mRNA starts to accumulate during late vegetative growth at the onset of development after 24 h post germination, peaks at 6 h post asexual induction and is detectable until 48 h when asexual development is completed (Etxebeste *et al.*, 2008; Garzia *et al.*, 2009). *brlA* mRNA accumulation, as well as accumulation of *abaA*, *flb* gene, and *fluG* transcripts, is light dependent (Mooney and Yager, 1990; Ruger-Herreros *et al.*, 2011). *brlA* consists of two overlapping transcripts, *brlA*α and *brlA*β (Prade and Timberlake, 1993). *brlA*β regulates *brlA*α expression (Barton and Prade, 2008; Han *et al.*, 1993). *brlA* OE leads to spore formation from hyphae in vegetative cultures whereas strains lacking *brlA* form aerial hyphae but vesicles, metulae or further cell types are absent (Adams *et al.*, 1988; Boylan *et al.*, 1987).

The C2H2 zinc finger transcription factor BrlA activates *abaA* in the mid phase of conidiation (Adams *et al.*, 1988, 1990; Andrianopoulos and Timberlake, 1994; Boylan *et al.*, 1987). AbaA is necessary for phialide differentiation (Sewall *et al.*, 1990b). *abaA* mutants produce cells with intermittent tumefactions and non-separated conidiospores instead of conidia-chains (Clutterbuck, 1969). Phialides are absent in these mutants and metula-like cells are formed instead (Sewall *et al.*, 1990b). *brlA* as well as the AbaA downstream target *wetA* are differentially expressed in Δ*abaA*, indicating feedback regulation of the CDP (Boylan *et al.*, 1987) (FIGURE 7).
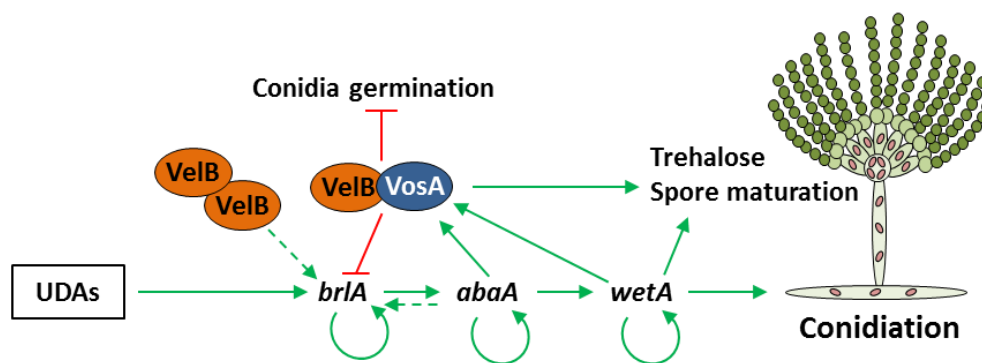
FIGURE 7: The central developmental pathway exhibits autoregulatory feedback loops in *A. nidulans*.
A model of the genetic interactions of the central developmental pathway (CDP) is shown. The CDP comprises BrlA→AbaA→WetA and exhibits several autoregulatory feedback loops. VelB-VosA represses *brlA* expression during vegetative growth and is necessary for spore viability and trehalose biogenesis during ongoing conidiospore formation and maturation. Green arrows indicate activating and red lines repressing effects. Dotted lines indicate proposed interactions. Adapted from Park *et al.*, 2012a; Yu, 2010.

AbaA, in contrast to BrlA, is not sufficient to induce conidiation as *abaA* OE does not produce conidiophores under non-inducing conditions (Mirabito *et al.*, 1989). In both, *abaA* OE as well as in Δ*abaA*, *brlA* expression is upregulated (Aguirre, 1993; Kwon *et al.*, 2010a; Ni and Yu, 2007; Tao and Yu, 2011). *wetA*, which gene product has self-regulating abilities, is activated by AbaA in the late phase of conidiation (Adams *et al.*, 1998; Boylan *et al.*, 1987; Mirabito *et al.*, 1989). WetA is necessary for the synthesis of conidiospore wall components and stability of mature conidia (Boylan *et al.*, 1987; Clutterbuck, 1969; Marshall and Timberlake, 1991; Mirabito *et al.*, 1989; Sewall *et al.*, 1990a). *wetA* mutants form colorless autolyzing conidia and accumulation of sporulation-specific mRNAs is absent (Boylan *et al.*, 1987; Marshall and Timberlake, 1991; Sewall *et al.*, 1990a). *wetA* OE is not sufficient to induce *brlA* or *abaA* expression but spore-specific mRNAs accumulate (Marshall and Timberlake, 1991).

Important factors for conidiospore maturation and viability are the velvet proteins VosA and VelB (Ni and Yu, 2007; Sarikaya-Bayram *et al.*, 2010) (see CHAPTER 1.2). Both are required for trehalose biogenesis and the activation of genes involved in spore maturation (Ni and Yu, 2007; Sarikaya-Bayram *et al.*, 2010; Wang *et al.*, 2015, 2014). Trehalose is a storage component and an important factor for conidiospore viability and rapid loss of trehalose is accompanied by viability loss in conidiospores (Fillinger *et al.*, 2001; Nguyen Van Long *et al.*, 2017; Ni and Yu, 2007; Novodvorska *et al.*, 2016; Sarikaya-Bayram *et al.*, 2010). Expression of both, *vosA* and *velB*, is activated by AbaA in the late phase of conidiation (Garzia *et al.*, 2013; Park *et al.*, 2012b) (FIGURE 7). VosA activates *wetA* and other genes, which products are important for the formation of cell wall components and for trehalose biogenesis, during spore maturation in *A. nidulans* (Al-Bader *et al.*, 2010; Borgia *et al.*, 1996; Fillinger *et al.*, 2001; Ni and Yu, 2007). VosA is abundant in phialides, metulae and conidiospores, but mRNA and protein levels decrease rapidly during vegetative growth after spore germination (re-establishment of a new colony) (Ni and Yu, 2007). Low levels of VosA are present in vegetative growth where it represses *brlA* expression (Ni and Yu, 2007) (see CHAPTER 1.2).

1.6   Sexual development

A. nidulans forms cleistothecia as closed sexual fruit bodies (FIGURE 1 and 8). The formation of fruit bodies is coupled to a plethora of environmental and endogenous factors like nutrient availability, surface contact, oxygen availability, illumination, pheromones or the cellular redox status (Busch and Braus, 2007; Ugalde and Rodríguez-Urra, 2016). It is an important

process because it opens up the possibility of genetic rearrangement. Two haploid nuclei from compatible mating partners are fused to a diploid (karyogamy). Since *A. nidulans* is a homothallic fungus, this can happen within the same individual (selfing) or involve two individuals (mating) (see CHAPTER 1.1). Selfing results in offspring genetically identical to the parent whereas mating results in genetic recombination events (Busch *et al.*, 2007). Karyogamy is followed by meiosis during which the genome is reduced to a haploid. The newly formed haploid nuclei are enclosed in new cells during ascosporogenesis, resulting in the formation of ascospores. The asci are protected by the cleistothecium (ascocarb), which is surrounded by multi-nucleated Hülle cells. Hülle cells differentiate from hyphae forming nest-like structures around the developing cleistothecium (FIGURE 8). The proposed function of the Hülle cells is the protection and nourishment of the maturating nests (Braus *et al.*, 2002; Sarikaya-Bayram *et al.*, 2010). Sexual ascospores are released from the cleistothecium after maturation and are easily transported by water and resistant to it.
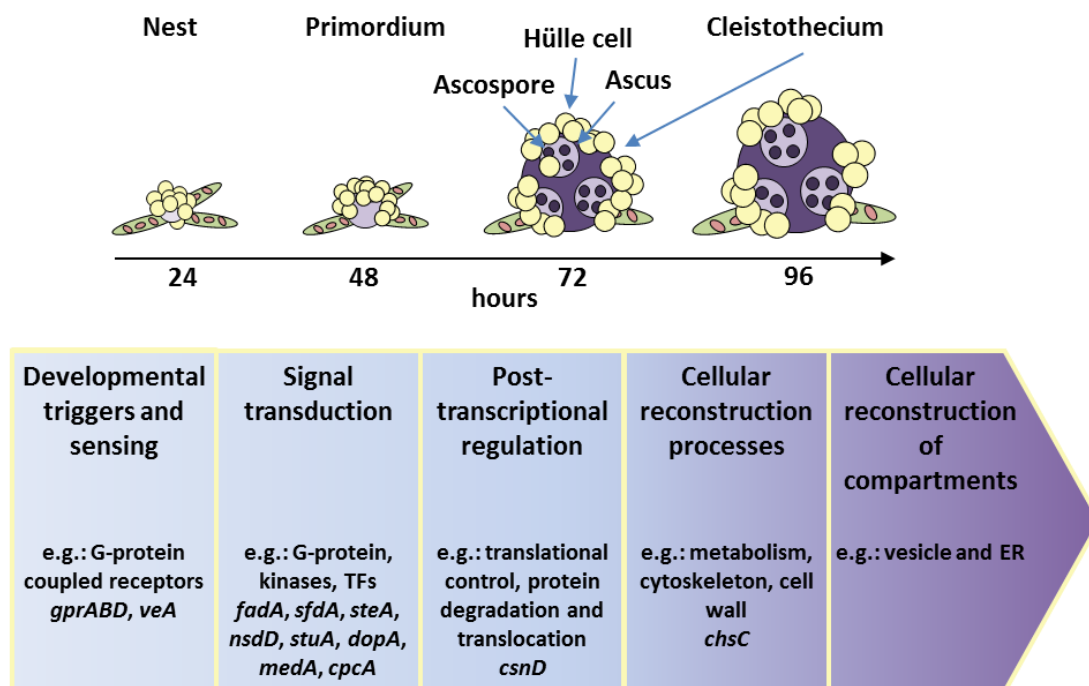
FIGURE 8: Cleistothecium development in *A. nidulans*.
Development of cleistothecia from nests is schematically depicted (upper part). Ascospores (dark violet) are formed within asci (closed structures, light violet). The cleistothecium (violet) is surrounded by Hülle cells (yellow). Schematic presentation of important steps in the transition from vegetative hyphae to cleistothecia and involved genes (lower part). Adapted from Busch and Braus, 2007.

Cleistothecia represent overwintering structures, which are formed in the soil. Darkness and limited oxygen supply, and thus increase in carbon dioxide pressure, are two major triggers of sexual development in *A. nidulans* (Busch and Braus, 2007; Champe *et al.*, 1994; Pöggeler *et*

al., 2006). A number of G-proteins, such as FadA (Gα-subunit), SfdA (Gβ-subunit) and GpgA (Gγ-subunit) as well as the mitogen-activated protein kinase SakA/HogA, are crucial for transduction of development inducing signals (Busch and Braus, 2007; Pöggeler *et al.*, 2006). Several transcription factors are involved in cellular rearrangement processes and cleistothecia formation, such as SteA, NsdD, StuA, DopA, MedA and CpcA (Busch and Braus, 2007; Han *et al.*, 2001; Pöggeler *et al.*, 2006; Vallim *et al.*, 2000). Except for SteA, the aforementioned transcription factors are involved in asexual development in *A. nidulans* as well (Busby *et al.*, 1996; Dutton *et al.*, 1997; Pascon and Miller, 2000). Phosphorylation events triggered by the kinase SakA (e.g. of the stress regulator AtfA) are common regulation mechanisms of growth states, cell cycle arrest and spore dormancy in fungi (Lara-Rojas *et al.*, 2011).

The ubiquitin-dependent proteasomal protein degradation machinery is necessary for fruit body formation. Defects in the COP9 signalosome, which acts as a negative regulator for ubiquitin ligases that mark specific proteins for proteasomal degradation, lead to a block of sexual development at the stage of primordia (Beckmann *et al.*, 2015; Busch *et al.*, 2007; Busch and Braus, 2007; Meister *et al.*, 2016). Also deletions of particular F-box proteins, which act as substrate-specifying subunits of cullin ring ubiquitin ligases (CRL), such as Fbx15, Fbx23 and GrrA result in either blocked (Fbx15, GrrA) or constitutive (Fbx23) sexual development (Krappmann *et al.*, 2006a; von Zeska Kress *et al.*, 2012).

Significant transcriptomic changes occur during sexual development in *A. nidulans* (Bayram *et al.*, 2016). Genes involved in cell wall biogenesis, like the chitin synthase encoding *chsC*, are differentially regulated during these processes (Busch and Braus, 2007). Most specifically upregulated genes during sexual development are found after 72 h in contrast to asexual development, where induction of genes peaked at late developmental stage after 48 h (Bayram *et al.*, 2016). At this time point, sexual development is not yet finished and ascosporogenesis is in progress (FIGURE 8). These changes do not only comprise cell structure specific gene expressions. Also primary metabolism is altered to efficiently utilize accumulated nutrients to nurture developing cleistothecia (Bayram *et al.*, 2016; Busch and Braus, 2007).

1.7   Oxidative stress defense: survival mechanism in fungi

1.7.1   Enzymatic response to oxidative stress

Organisms produce reactive oxygen species (ROS) as by-products of aerobic respiration and other metabolic functions due to oxygen excitation, partial reduction and radical and peroxide

formation (Aguirre *et al.*, 2005). ROS are used as intracellular signaling molecules as well as for inter-species communication, for example in symbioses and in pathogenic processes (Marschall and Tudzynski, 2016; Nath *et al.*, 2016; Zhang *et al.*, 2016). The production of ROS, and thus oxidative stress during development can actively be regulated in fungi (Pöggeler *et al.*, 2006). ROS are produced as defense mechanism by host immune systems of animals and their counterparts in plants (Camejo *et al.*, 2016; Moye-Rowley, 2003). ROS can damage all kinds of biomolecules like nucleotides, proteins and lipids (Breitenbach *et al.*, 2015; Sato *et al.*, 2009). Therefore, fast and potent mechanisms to counteract ROS stress are crucial for fungal fitness and success.

ROS are detoxified by enzymatic mechanisms and redox systems, which provide reducing power (Aguirre *et al.*, 2005; Matsuzawa, 2017). Several enzymes, such as superoxide dismutases and catalases are involved in the oxidative stress response (OSR). At least five catalases exist in *A. nidulans*: catalases A-D and the uncharacterized *AN8553* gene product (Bayram *et al.*, 2016; Kawasaki *et al.*, 1997; Kawasaki and Aguirre, 2001; Navarro *et al.*, 1996). Deletion of *catA*, *catB* and *catC*, as well as double and triple deletions did not have developmental influences in *A. nidulans* (Kawasaki *et al.*, 1997; Kawasaki and Aguirre, 2001). CatA is preferentially found in conidiospores, whereas CatB is a hyphal catalase and both protect against external as well as internal H_2O_2 (Kawasaki *et al.*, 1997; Navarro *et al.*, 1996). CatC is proposed to act on very specific stress situations since expression of *catC* is not induced during oxidative or osmotic stress and only slightly upregulated by heat shock stress (Kawasaki and Aguirre, 2001). The catalase-peroxidase CatD functions as a H_2O_2 scavenger and during heat stress only in old mycelia (24 h and thereafter) (Kawasaki and Aguirre, 2001).

1.7.2   Thioredoxin and glutathione system

Besides the enzymatic OSR proteins, several oxidative stress defense systems have evolved. Key mechanisms in the OSR are the thioredoxin and the glutathione system (Aguirre *et al.*, 2005; Bakti *et al.*, 2017; Carmel-Harel and Storz, 2000; Sato *et al.*, 2009; Thön *et al.*, 2007).

The main cellular oxidative stress defense system is the glutathione system, a redox-buffer system (Bakti *et al.*, 2017; Breitenbach *et al.*, 2015). Glutathione peroxidase, for which glutathione functions as electron donor, specifically reduces H_2O_2 to H_2O (Breitenbach *et al.*, 2015; Meister and Anderson, 1983; Sato *et al.*, 2009). The glutathione system exhibits interplay with a second redox system, the thioredoxin system (Sato *et al.*, 2009; Thön *et al.*, 2007). Thioredoxins are small, omnipresent proteins of 12 to 13 kDa, which function as

oxidoreductases. They act as electron donors for thioredoxin peroxidases, similarly to glutathione (Sato *et al.*, 2009; Thön *et al.*, 2007). Glutathione and thioredoxin are reduced by their specific reductases, which use NADPH as electron donor, after the oxidation processes (Breitenbach *et al.*, 2015; Sato *et al.*, 2009; Thön *et al.*, 2007)

1.7.3 Transcription factors involved in the oxidative stress response

Rapid transcriptional regulation events are important for the fungal defense against ROS stress. The OSR is mainly regulated by nuclear localization control of specific transcription factors and their protein phosphorylation (Moye-Rowley, 2003). Several examples of fungal transcription factors are known, where nuclear localization is regulated by oxidative stress (da Silva Dantas *et al.*, 2015; Glover-Cutter *et al.*, 2014; Jin *et al.*, 2015; Morano *et al.*, 2012; Moye-Rowley, 2003). Yap1 from *Saccharomyces cerevisiae*, which corresponds to NapA of *A. nidulans*, is required for expression of thioredoxin *TRX2* and involved in the regulation of the glutathione biosynthesis (Asano *et al.*, 2007; Kuge and Jones, 1994; Moye-Rowley, 2003; Wu and Moye-Rowley, 1994). Transcriptional regulation by Yap1 in the OSR is regulated via an exportin: Yap1 enters the nucleus in unstressed situation but is rapidly shuttled out again, whereas oxidative stress leads to a nuclear Yap1 accumulation and subsequent transcriptional regulation of target genes (Isoyama *et al.*, 2001). Localization of its homologs from other yeasts is controlled in an oxidant-responsive manner as well (Moye-Rowley, 2003). Skn7 is, together with Yap1, required for oxidative stress tolerance (Moye-Rowley, 2003). Yap1 and Skn7 are interdependent and likely function in the same OSR pathway via activation of *TRX2* (thioredoxin) expression (Krems *et al.*, 1996; Morgan *et al.*, 1997). NapA (*A. nidulans* AP-1 homolog A) is the Yap1 ortholog in *A. nidulans.* It is important for the stress-mediated activation of several genes of the OSR, such as *catB*, *trxR* and *trxA* in *A. nidulans*. Strains, which lost *napA* are not able to grow on medium supplemented with oxidative stress inducers (Asano *et al.*, 2007). Several transcription factors are activated by mitogen-activated protein kinase (MAPK) phosphorylation cascades upon oxidative stress, such as Atf1 of *Schizosaccharomyces pombe* or its homolog in *A. nidulans*, AtfA (Hagiwara *et al.*, 2008; Lara-Rojas *et al.*, 2011; Shiozaki and Russelp, 1996). Deletion of the gene encoding the MAPK SakA, which interacts with AtfA, leads to increased sensitivity of conidiospores to oxidative stress and decreased spore viability in *A. nidulans* (Kawasaki *et al.*, 2002; Lara-Rojas *et al.*, 2011).

Another fungal mechanism in response to stresses is the adjustment of the cellular protein composition. Target proteins are labeled for degradation by multi-subunit SCF Cullin RING

ligases, which employ F-box proteins as substrate specific adaptors (Jöhnk *et al.*, 2016; Yu, 2010). The F-box protein Fbx15 in *A. fumigatus* is necessary to shuttle SsnF into the nucleus in response to oxidative stress (Jöhnk *et al.*, 2016). SsnF is a subunit of the transcriptional co-repressor complex RcoA-SsnF and mislocalization of SsnF in Δ*fbx15* correlates with an upregulation of *catB* (Jöhnk *et al.*, 2016). In *S. cerevisiae*, the corresponding Ssn6-Tup1 co-repressor complex coordinates the expression of three to five percent of the whole genome and is involved in mating, nutrient sensing, DNA-damage repair and stress response (Derisi *et al.*, 1997; Parnell and Stillman, 2011).

1.8   Zinc cluster proteins: a fungal specific type of transcription factors

Transcriptional control of genes is of importance for organisms in order to cope with changing environments and internal constitution. Cells possess a variety of regulatory proteins for these purposes. Amongst them the group of zinc binding proteins represents the largest group of transcription factors in eukaryotes. This group is divided into three subgroups: Cys_2His_2 (C2H2), Cys_4 (C4) and Cys_6 (C6). The last one, also called zinc cluster proteins, is primarily present in fungi (with few exceptions in other microorganisms) and is not found in bacteria, plants or animals (MacPherson *et al.*, 2006; Scazzocchio, 2014; Schjerling and Holmberg, 1996). C6 proteins are mainly involved in the regulation of either i) carbon and nitrogen metabolism, ii) secondary metabolism or iii) asexual or sexual development (Chang and Ehrlich, 2013). A number of C6 proteins are involved in virulence and pathogenicity in several fungi, such as *A. fumigatus* and *Candida albicans* (Bok *et al.*, 2006a; Boyce *et al.*, 2015; Dufresne *et al.*, 2000; Issi *et al.*, 2017; Lu *et al.*, 2014; Rybak *et al.*, 2017; Vandeputte *et al.*, 2011). DNA-target sequence specificity of C6 proteins is given by a unique linker region between the zinc fingers and, in many cases, a dimerization domain, which together constitute the DNA binding domain (DBD) (Johnston and Dover, 1987; MacPherson *et al.*, 2006; Mamane *et al.*, 1998; Reece and Ptashne, 1993). The DBD (C6) domain is often located near the N-terminus of proteins, but C-terminal localization has been shown as well (MacPherson *et al.*, 2006). C6 proteins contain in many cases a region of weak similarity that follows the C6 domain. This region was designated the middle homology region (MHR) since it is framed by the C6 domain and the regulation domain at the C-terminus of the protein, which is in most cases an acidic activation domain forming a negatively charged area (Schjerling and Holmberg, 1996) (FIGURE 9). C6 proteins are known to be able to form homodimers and heterodimers with other zinc cluster proteins, as well as with non-zinc cluster proteins (Akache *et al.*, 2004; Amar *et al.*, 2000; Karpichev *et al.*, 1997; Karpichev

and Small, 1998; Mamnun *et al.*, 2002; Rottensteiner *et al.*, 1997) (FIGURE 9). These monomers or dimers bind short DNA recognition sequences of inverted, everted or direct repeats (MacPherson *et al.*, 2006). Self-regulation via feedback loops was found for several C6 proteins (Delahodde *et al.*, 1995; Hiesinger *et al.*, 2001; Hon *et al.*, 2005; Larochelle *et al.*, 2006; Rottensteiner *et al.*, 1997; Zhang *et al.*, 2001).
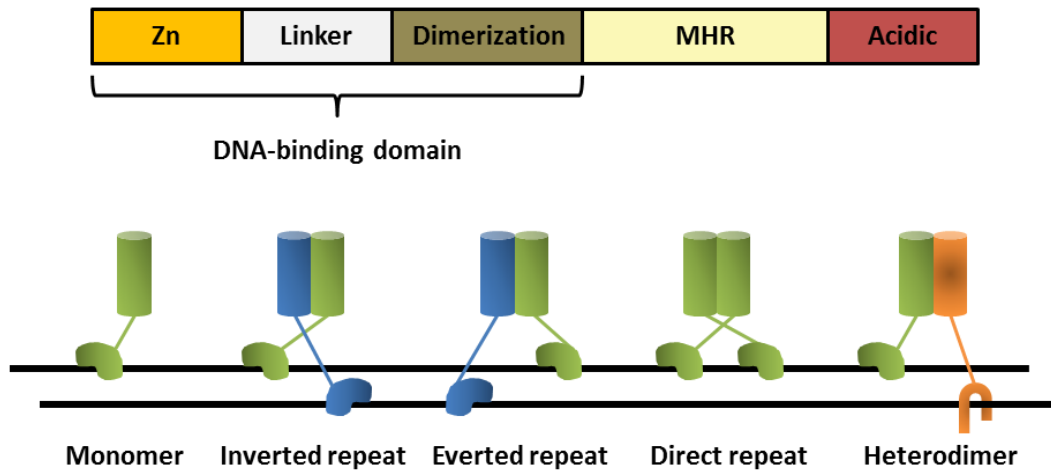
FIGURE 9: C6 proteins are fugal-specific DNA-binding proteins.
The upper part depicts a model of the functional parts of a typical C6 protein. The DNA-binding domain (DBD) comprises the zinc cluster (Zn), a linker region and the dimerization domain. The middle homology region (MHR) is framed by the DBD and the acidic activation region. A model of protein-DNA interactions exhibited by C6 proteins is depicted in the lower part. C6 proteins typically bind short DNA sequences with inverted, everted or repeated orientation as monomers, homo- and heterodimers. Adapted from MacPherson *et al.*, 2006.

Transcription factors need to be localized in the nucleus in order to fulfill their task of regulating gene expression. C6 proteins can be divided into two groups with respect to their localization: i) proteins, which are permanently localized in the nucleus and ii) proteins, which are localized in the cytoplasm and shuttled into the nucleus upon their activation signal (MacPherson *et al.*, 2006). For the former group it is postulated that its members are activated by target molecules or metabolic intermediates and constitutive promoter binding has been shown for a number of C6 proteins (Flynn and Reece, 1999; Harbison *et al.*, 2004; Kirkpatrick and Schimmel, 1995; MacPherson *et al.*, 2006; Sellick and Reece, 2003, 2005).

The second group needs to be transported into the nucleus with the help of importers. Several different and redundant import strategies have been shown for C6 proteins (Hasper *et al.*, 2004; MacPherson *et al.*, 2006; Nikolaev *et al.*, 2003). A general import strategy is not known for C6 proteins (MacPherson *et al.*, 2006). Protein transport into the nucleus takes place by the binding of the α/ß importin heterodimer to nuclear localization sequences (NLS) of the respective cargo protein and the import of the complex into the nucleus through nuclear pores

(Beck and Hurt, 2017; Garcia *et al.*, 2016; Görlich *et al.*, 1995; Köhler *et al.*, 1999; Lim *et al.*, 2015; Miyamoto *et al.*, 2016; Nakielny and Dreyfuss, 1999; Schwartz, 2016). The NLS in C6 proteins can be localized within as well as outside of the C6 domain.

1.9   *Aspergillus fumigatus* – a pathogenic mold

1.9.1   *A. fumigatus* as soil borne fungus with pathogenic potential

A. fumigatus represents an opportunistic pathogenic member of the genus *Aspergillus*. Like *A. nidulans*, it is a soil borne fungus, which lives on decaying biological material and plays an important role in the natural recycling of carbon and nitrogen sources (Adav *et al.*, 2015; Brakhage and Langfelder, 2002; Flipphi *et al.*, 2009; Wang *et al.*, 2012). Its versatile metabolism is one factor, besides other factors, like high thermotolerance, oxidative stress resistance, mycotoxin production, that renders *A. fumigatus* a potent pathogen (Krappmann and Braus, 2005). The saprophytic mold exhibits high genetic diversity, accompanied by high adaptability to changing environmental conditions (Debeaupuis *et al.*, 1997; Verweij *et al.*, 2016b). Its increasing resistance towards widely used fungicides renders it a serious health treat in industrialized countries (Verweij *et al.*, 2016a, 2016b). *A. fumigatus* is the second most common agent of fungal infections in humans after *Candida albicans* (Kaur and Singh, 2013). Immunocompetent individuals rarely encounter problems by inhaling of spores due to an efficient innate immune response. In immunocompromised patients, however, *A. fumigatus* spores can evoke life threatening infections with high mortality rates of up to 90% (Dagenais and Keller, 2009; Denning, 1998; Kousha *et al.*, 2011; Lamoth *et al.*, 2016; Latgé, 1999; Wasylnka and Moore, 2003).

1.9.2   Developmental programs of *A. fumigatus*

Asexual development in *A. fumigatus* is similar to *A. nidulans*. The conidiophore in *A. fumigatus* resembles the characteristics of the *A. nidulans* conidiophore in organization, except for the missing layer of metulae (Brakhage and Langfelder, 2002; Tao and Yu, 2011; Yu, 2010) (FIGURE 10). Conidiospores of this opportunistic pathogenic mold are considerably smaller compared to *A. nidulans*, what contributes to their virulence since they easily reach the lung alveoli after inhalation (Dagenais and Keller, 2009; Kaur and Singh, 2013; Yu, 2010). They are resistant to various stresses and stress defense mechanisms are extensively studied (Hagiwara *et al.*, 2008; Jöhnk *et al.*, 2016; Kwon-Chung and Sugui, 2009; Muszkieta *et al.*, 2016).
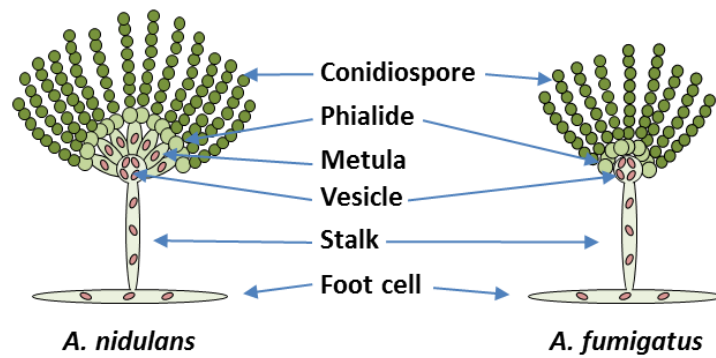
FIGURE 10: Comparison of conidiophores of *A. nidulans* and *A. fumigatus*.
Schematic comparison of conidiophores from *A. nidulans* and *A. fumigatus* is shown. The organization is similar in both fungi, but the conidiophores of *A. fumigatus* lack metulae. Cell nuclei are shown in red. Note that nuclei of the mononucleated conidiospores and phialides are not shown. Adapted from Yu, 2010.

A. fumigatus is, in contrast to *A. nidulans*, a heterothallic fungus. For long, *A. fumigatus* was designated a member of the *fungi imperfecti* (Deuteromycota), which do not possess a sexual reproductive cycle or for which such a cycle has not been discovered (Geiser *et al.*, 1996). The fungus possess all genetic requirements for a sexual life cycle (Galagan *et al.*, 2005; Nierman *et al.*, 2005). In 2009, sexual development could be shown for *A. fumigatus* (O'Gorman *et al.*, 2009). The process of sexual propagation requires specific temperature, media and extended periods of time (up to six months) (O'Gorman *et al.*, 2009). As for many other pathogenic fungi, the sexual life cycle remains a rare event in *A. fumigatus* and asexual propagation is favored (Dyer and O'Gorman, 2012; Ene and Bennett, 2014).

Corresponding OSR genes from *A. nidulans* are present in *A. fumigatus* as well, such as *trxR* (*trr1* in *A. fumigatus*), *catA, catB* and *catD* (*catA*, *1*, *2*) to name but a few (Abadio *et al.*, 2011; Calera *et al.*, 1997; Paris *et al.*, 2003). Moreover, conidiospores and hyphae of *A. fumigatus* are resistant to high temperatures of up to 75°C and 55°C, respectively (McCormick *et al.*, 2010; Perez-Nadales *et al.*, 2014; Sueiro-Olivares *et al.*, 2015).

1.9.3 The central developmental pathway is conserved in *A. fumigatus*

Similarly to *A. nidulans*, BrlA of *A. fumigatus* is a necessary developmental activator, which function is conserved among both species (Mah and Yu, 2006; Tao and Yu, 2011). *wetA* and *abaA* expression are dependent on BrlA in this fungus as well (Tao and Yu, 2011). AbaA function is conserved among *A. nidulans* and *A. fumigatus* as AbaA is involved in phialide differentiation and activation of *wetA* expression in both fungi (Tao and Yu, 2011). WetA is involved in cell wall biogenesis of conidiospores and is necessary for trehalose biogenesis, spore viability and integrity and stress tolerance (Tao and Yu, 2011). An *A. fumigatus* Δ*wetA*

mutant produces colorless conidia with imperfect separation and drastically reduced viability (Tao and Yu, 2011). During vegetative growth, hyphal branching is reduced in Δ*wetA*, comparable to the situation in *A. nidulans* (Tao and Yu, 2011). In both, Δ*abaA* and Δ*wetA*, *brlA* expression is upregulated, indicating negative feedback regulatory circuits (Tao and Yu, 2011). This shows that the conidiation cascade BrlA→AbaA→WetA is conserved between *A. nidulans* and *A. fumigatus* and plays a key role in both fungi. Nevertheless, regulatory details are distinctly different, especially with respect to autoregulation and feedback loops (Park *et al.*, 2012a; Shin *et al.*, 2015; Tao and Yu, 2011; Yu, 2010).

1.9.4 Differences of upstream developmental activator functions between *A. nidulans* and *A. fumigatus*

FluG is necessary for conidiation in *A. nidulans* (see CHAPTER 1.5). In contrast, FluG is not required for the activation of conidiation in *A. fumigatus*, as Δ*fluG* mutants conidiate like the WT during asexual development (Mah and Yu, 2006). *A. fumigatus* is able to sporulate in liquid cultures, where development is blocked in *A. nidulans* and only vegetative growth occurs. Though, sporulation under submerged conditions in *A. fumigatus* is dependent on FluG (Mah and Yu, 2006). Hence, existence of more than one conidiation pathway has been hypothesized in this fungus (Mah and Yu, 2006; Yu, 2010). FlbE is proposedly conserved among Aspergilli, as *flbE* of *A. fumigatus* fully complements the loss of *flbE* in *A. nidulans* (Kwon *et al.*, 2010b). A loss of *flbB* in *A. fumigatus* leads to decreased conidiation and delayed expression of *brlA* and *abaA*, but not of *wetA* and *vosA* (Xiao *et al.*, 2010). FlbB functions in the early phase of conidiation, comparable to the situation in *A. nidulans* (see CHAPTER 1.5). Production of the SM gliotoxin, a potent mycotoxin with antioxidant function, is absent in Δ*flbB* (Choi *et al.*, 2007; Owens *et al.*, 2014; Xiao *et al.*, 2010). This phenotype could not be fully restored by introduction of *flbB* from *A. nidulans* into *A. fumigatus* Δ*flbB* (Xiao *et al.*, 2010). This is due to the fact that *A. nidulans flbB* encodes one transcript, whereas *flbB* produces two transcripts in *A. fumigatus* (Etxebeste *et al.*, 2008; Xiao *et al.*, 2010). Two FlbB polypeptides are present in *A. fumigatus*, which are both important for gliotoxin biosynthesis and morphological development (Xiao *et al.*, 2010). Furthermore, *flbE* and *brlA* are involved in the regulation of gliotoxin production (Xiao *et al.*, 2010). *flbD* mRNA only accumulates if products of both, *flbB* and *flbE* are present, indicating a similar genetic dependency as found in *A. nidulans* (Garzia *et al.*, 2009, 2010; Xiao *et al.*, 2010) (see CHAPTER 1.5). In contrast to *A. nidulans*, where FlbB and FlbE are interdependent and form a

complex, both factors are independently expressed in *A. fumigatus* (Garzia *et al.*, 2009; Herrero-Garcia *et al.*, 2015; Mah and Yu, 2006).

1.9.5   Velvet factor control of secondary metabolism and development

The four velvet proteins, crucial developmental and SM regulators (see CHAPTER 1.2) are conserved in *A. fumigatus* (Park *et al.*, 2012a). Genomic and metabolomic analyses have revealed that *A. fumigatus* supposedly is able to produce several hundred secondary metabolites (Dolan *et al.*, 2015; Frisvad *et al.*, 2009; Frisvad and Larsen, 2016; Lind *et al.*, 2015). VeA and LaeA play important roles in the regulation of gliotoxin production, a potent mycotoxin, and couple SM production to virulence (Dagenais *et al.*, 2010; Dhingra *et al.*, 2013; Perrin *et al.*, 2007). Similar to the situation in *A. nidulans*, VeA represses *brlA* expression in *A. fumigatus* (Park *et al.*, 2012a). VelB represses *brlA* in *A. fumigatus* as well, which is in contrast to the situation in *A. nidulans*, where VelB is hypothesized to positively regulate *brlA* expression (Park *et al.*, 2012a). A loss of *veA* and *velB*, but not of *velC*, leads to conidiophore formation in submerged cultures in conidiation-suppressing media, whereas Δ*vosA* forms vesicle-like structures under these conditions and accumulates *brlA* mRNA after 24 h of vegetative growth (Park *et al.*, 2012a). Moreover, VosA and VelB are involved in trehalose biogenesis, as respective mutants showed decreased trehalose content of spores and decreased spore viability, indicating conservation of their general necessity for trehalose biogenesis (Park *et al.*, 2012a). In contrast, trehalose is abolished in corresponding mutants in *A. nidulans* (Ni and Yu, 2007; Sarikaya-Bayram *et al.*, 2010). VeA is besides VelB and VosA an important regulators of conidiosporogenesis and involved in the regulation of stress resistance of conidiospores in *A. fumigatus* (Shin *et al.*, 2016). These findings show that several developmental genes from *A. nidulans* are involved in virulence and secondary metabolite regulation in *A. fumigatus* and distinct reprogramming of the genetic machinery occurred after species differentiation.

1.10   Aim of this study

The aim of this study was to analyze the regulatory level downstream of the VosA control of transcription. Therefore, a VosA controlled transcription factor (ZtfA) was analyzed in more detail. Velvet regulators bridge developmental programs and secondary metabolism in filamentous fungi. VosA binds to promoter sequences of approximately 1500 genes but the vast majority of these targets is uncharacterized, amongst them the *ztfA* gene (Ahmed *et al.*,

2013). *ztfA* corresponds to *A. niger scl-2* (*sclerotia-like 2*) (A.F.J. Ram, personal communication). *scl-2* was originally described by Jørgensen and collaborators, who aimed at increasing secondary metabolite production in the industrial fungus *A. niger* (Jørgensen *et al.*, 2011). The *A. niger scl-2* mutant exhibits diminished asexual sporulation and the formation of sclerotia-like structures, which are thought to correspond to cleistothecia in *A. nidulans* and are rarely produced in the laboratory (Frisvad *et al.*, 2014; Jørgensen *et al.*, 2011). Besides its developmental phenotype, the *scl-2* mutant displays severe impairment in SM production. However, no further genetic analyses of this mutant or any putative ortholog among other fungi have been conducted so far.

The *ztfA* gene product exhibits a C6 domain as the only obvious protein domain and was therefore denominated Zinc cluster transcription factor A (ZtfA). Genetic relationships between the velvet proteins and ZtfA are investigated in the present study. The regulatory influences of ZtfA upon the conidiation pathways are examined through phenotypical and transcriptional analyses in detail in *A. nidulans*. Involvement of ZtfA in the regulation of secondary metabolite biosynthesis is analyzed as well. An investigation of SM production and its transcriptional regulation reveals further connections between developmental programs and secondary metabolism in *A. nidulans*. Oxidative stress response is often coupled to secondary metabolism and hence is investigated in addition.

A functional conversion of developmental to virulence regulators between non-pathogenic and pathogenic fungi is common and a possible role of the respective ortholog in pathogenicity of *A. fumigatus* is investigated. The examination of its involvement in important determinants for pathogenicity as well as virulence in an invertebrate model gives further insights into the aforementioned functional conversion of regulators between genetic and pathogenic model fungi.

2. Materials and methods

2.1 Chemicals and materials

Buffers, solutions and media were prepared with chemicals purchased from the companies APPLICHEM GMBH (Darmstadt, Germany), BD BIOSCIENCES (Heidelberg, Germany), CARL ROTH GMBH & CO. KG (Karlsruhe, Germany), FLUKA (Neu-Ulm, Germany), INVITROGEN (Carlsbad, CA, USA), MERCK KGAA (Darmstadt, Germany), BIOZYME SCIENTIFIC GMBH (Hessisch Oldendorf, Germany), ROCHE DIAGNOSTICS GMBH (Mannheim, Germany), SIGMA-ALDRICH CHEMIE GMBH (Munich, Germany), SERVA ELECTROPHORESIS GMBH (Heidelberg, Germany), OXOID DEUTSCHLAND GMBH (Wesel, Germany).

Plastic consumables, such as pipet tips, reaction tubes, inoculation loops etc., were purchased from SARSTEDT AG & CO. (Nümbrecht, Germany), STARLAB GMBH (Hamburg, Germany) and NERBE PLUS GMBH (Winsen/Luhe, Germany).

Polymerases and restriction enzymes were obtained from THERMO FISHER SCIENTIFIC (Schwerte, Germany), trypsin was purchased from SERVA ELECTROPHORESIS GMBH (Heidelberg, Germany). Primers were obtained from EUROFINS GENOMICS GMBH (Ebersberg, Germany). The GeneRuler 1kb DNA ladder and the PageRuler^{TM} Prestained Protein Ladder (THERMO FISHER SCIENTIFIC) were used for DNA and protein on-gel band size determination. Filtropur filters with a pore size of 0.2 and 0.45 µm for small-scale sterile filtration of chemicals were purchased from SARSTEDT. Ampicillin (ROTH), pyrithiamine hydrobromide (SIGMA-ALDRICH), clonNAT nourseothricin dihydrogen sulfate from WERNER BIOAGENTS (Jena, Germany) and phleomycin (INVIVOGEN) were used for selection of microorganisms.

DNA- and protein amounts were measured with a NanoDrop ND-1000 photospectrometer from PEQLAB BIOTECHNOLOGIE GMBH (Erlangen, Germany). Agarose gel electrophoresis was performed with Mini-Sub$^{®}$ Cell GT chambers and the PowerPac^{TM} 300 power supply and SDS-polyacrylamide gel electrophoresis and EMSAs were performed with the Mini-Protean$^{®}$ Tetra Cell, Mini Trans-Blot$^{®}$ Electrophoretic Cell and powered with the PowerPac^{TM} 3000 from BIO-RAD LABORATORIES (Hercules, CA, USA). Proteins were transferred from SDS-polyacrylamide gels onto Amersham^{TM} Protran^{TM} 0.45 µm NC nitrocellulose blotting membranes and DNA was blotted to Amersham^{TM} Hybond-N^{TM} nylon membranes from GE HEALTHCARE (Little Chalfont, United Kingdom). Chemiluminescence was detected either by utilization of Amersham^{TM} Hyperfilm^{TM}-ECL from GE Healthcare, which were exposed with the Optimax X-ray Film Processor from PROTEC GMBH & CO. KG (Oberstenfeld, Germany), or by exposure of the membranes with the Fusion SL chemiluminescence detector from

PEQLAB. For centrifugation of 1.5 and 2 ml reaction tubes, Biofuge fresco (cooled) and Biofuge pico centrifuges from HERAEUS INSTRUMENTS GMBH (Hanau, Germany) were used. For centrifugation of 10, 15 and 50 ml centrifuge tubes Rotixa/RP from ANDREAS HETTICH GMBH & CO. KG (Tuttlingen, Germany), 5804R from EPPENDORF AG (Hamburg, Germany) and 4K15C from SIGMA LABORZENTRIFUGEN GMBH (Osterode am Harz, Germany) were used. For pH determination a WTW bench pH/mV Routine meter pH 526 (SIGMA-ALDRICH) was used.

Further materials, instrumentations and suppliers are indicated in the subsequent chapters.

2.2   Media and growth conditions

Chemicals for media preparation were dissolved in dH_2O and sterilized by autoclavation at 121°C for 20 min at two bar. Thermally unstable supplementations were dissolved in dH_2O and sterile filtered.

2.2.1   Bacterial growth

Escherichia coli strains DH5α[TM] (Hanahan, 1985), DH10B[TM] (Lorow and Jessee, 1990) and One Shot[®] TOP10 (INVITROGEN) were used for construction and amplification of recombinant plasmids. Genotypes of these strains are given in TABLE 1. *E. coli* strains were cultivated in lysogeny broth (LB) (Bertani, 1951) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) on a rotary shaker at 37°C. Ampicillin was used as selective agent in a concentration of 100 mg/ml. Solid medium was prepared by supplementation with 2% (w/v) agar.

TABLE 1: *E. coli* **strains used in this study.**

Strain name	Genotype
DH5α[TM]	F-, Δ(*arg*F-lac)169, φ80d*lac*ZΔM15, Δ*pho*A8, *gln*X44(AS), λ-, *deo*R481, *rfb*C1, *gyr*A96(NalR), *rec*A1, *end*A1, *thi*E1, *sd*R17
DH10B[TM]	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC), φ80d*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara, leu*)7697, *gal*U, *gal*K, *rps*L, *end*A1, *nup*G
One Shot[®] TOP10	F- *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), φ80d*lac*ZΔM15, Δ*lac*X74, *rec*A1, *ara*D139, Δ(*araleu*)7697, *gal*U, *gal*K, *rps*L (StrR), *nd*A1 *nup*G

2.2.2   Fungal growth

The *A. nidulans veA*[+] strain AGB 551 (Bayram *et al.*, 2012) was used as wildtype (WT) host for *A. nidulans* strains constructed in this study. *A. fumigatus* strain AfS35 (Krappmann *et al.*, 2006b) was used as host for *A. fumigatus* strains constructed in this study.

A. nidulans and *A. fumigatus* strains were grown in <u>m</u>inimal <u>m</u>edium (MM) (1% (w/v) glucose, 7 mM KCl, 2 mM MgSO$_4$, 70 mM NaNO$_3$, 11.2 mM KH$_2$PO$_4$, 0.1% (v/v) trace element solution pH 5.5 (Käfer, 1977)). <u>L</u>ondon <u>m</u>edium (LM) (1% (w/v) glucose, 7 mM KCl, 2 mM MgSO$_4$, 5 mM (NH$_4$)$_2$C$_4$H$_4$O$_6$, 11.2 mM KH$_2$PO$_4$, 0.1% (v/v) trace element solution pH 5.5 (Käfer, 1977)) was used to prevent expression of *ztfA* OE driven by ^{P}*niaD*. 2% (w/v) agar was added for solid MM plates. Aspergilli were grown at 37°C in baffled flasks under shaking conditions on a rotary shaker for vegetative growth for 24 h, or for shorter time periods when indicated. *A. nidulans* strains were grown on solid MM plates under constant white light for two to four days (d), or longer where indicated, to induce asexual development. Sexual development was induced by growing fungal strains in the dark for up to 7 d on solid MM plates, which were tightly sealed with Parafilm$^®$ M (MERCK) to prevent oxygen supply.

A. fumigatus strains were grown in the presence of oxygen but without illumination. Strains were grown for 24 h in submerged cultures for synchronized growth. After this, mycelia were harvested and washed through sterile Calbiochem Miracloth filters (MERCK) and subsequently shifted onto solid MM plates (Fischer, 2002). Tests including the *ztfA* OE were carried out on MM supplemented with 10 µg/ml doxycycline to induce *ztfA* expression (Helmschrott *et al.*, 2013).

Conidiospores were harvested in 0.96% (w/v) NaCl with 0.002% (v/v) Tween-80 (SIGMA-ALDRICH) and stored at 4°C.

TABLE 2: Fungal strains used in this study.
^{P} = promoter, *phleo*^{R} = phleomycin resistance (non-recyclable), p.c. = personal communication. *A. nidulans* strains are denominated AGB or FGSC, *A. fumigatus* strains are denominated ACS, AfS or AfGB.

Strain name	Genotype	Reference
FGSC A4	*veA*$^+$	McCluskey *et al.*, 2010
AGB551	Δ*nkuA*::*argB*, *pyrG89*, *pyroA4, veA*$^+$	Bayram *et al.*, 2012
AGB596	^{P}*gpdA*::*sgfp-phleo*^{R}; *pabaA1, yA2, veA*$^+$	Bayram *et al.*, 2012
AGB1007	Δ*nkuA*::*argB*, *pyrG89*, *pyroA4*, *veA*$^+$, Δ*ztfA*::*six*	This study
AGB1008	Δ*nkuA*::*argB*, *pyrG89*, *pyroA4*, *veA*$^+$, *six*::^{P}*niaD*::*ztfA*	This study
AGB1009	Δ*nkuA*::*argB*, *pyrG89*, *pyroA4*, *veA*$^+$, *ztfA*::*sgfp*::*six*	This study
AGB1010	Δ*nkuA*::*argB*, *pyrG89*, *pyroA4*, *veA*$^+$, *sgfp*::*ztfA*::*six*	This study
AGB1011	Δ*nkuA*::*argB*, *pyrG89*, *pyroA4*, *veA*$^+$, *ztfA*::*six*	This study
AGB1012	AGB1009, transformed with pME3173	This study
AGB1013	AGB1010, transformed with pME3173	This study
AGB1014	AGB551, transformed with pME3173	This study
AGB1015	Δ*nkuA*::*argB*, *pyrG89*, *pyroA4*, *veA*$^+$,	This study

	$^P ztfA::ztfA^{S327A,T464A,S504-506A}::six$	
AGB1016	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta fluG::six$	This study
AGB1017	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta fluG::six$, $\Delta ztfA::six$	This study
AGB1018	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta fluG::six$, $six::^P niaD::ztfA$	This study
AGB1019	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $six::^P niiA::fluG$	This study
AGB1020	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta ztfA::six$, $six::^P niiA::fluG$	This study
AGB1022	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta aflR::six$	This study
AGB1023	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta aflR::six$, $\Delta ztfA::six$	This study
AGB1024	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta aflR::six$, $six::^P niaD::ztfA$	This study
AGB1025	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $six::^P niiA::aflR$	This study
AGB1026	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta ztfA::six$, $six::^P niiA::aflR$	This study
AGB1028	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta abaA::six$	This study
AGB1029	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta abaA::six$, $\Delta ztfA::six$	This study
AGB1031	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta brlA::six$	This study
AGB1032	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta brlA::six$, $\Delta ztfA::six$	This study
AGB1033	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta brlA::six$, $six::^P niaD::ztfA$	This study
AGB1035	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbB::six$	This study
AGB1036	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbB::six$, $\Delta ztfA::six$	This study
AGB1037	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbB::six$, $six::^P niaD::ztfA$	This study
AGB1039	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbC::six$	This study
AGB1040	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbC::six$, $\Delta ztfA::six$	This study
AGB1041	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbC::six$, $six::^P niaD::ztfA$	This study
AGB1043	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbD::six$	This study
AGB1044	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbD::six$, $\Delta ztfA::six$	This study
AGB1045	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbD::six$, $six::^P niaD::ztfA$	This study
AGB1047	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbE::six$	This study
AGB1048	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbE::six$, $\Delta ztfA::six$	This study
AGB1049	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta flbE::six$, $six::^P niaD::ztfA$	This study
AGB1051	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $phleo^R$, $^P niaD::ztfA::eyfp-C$, $^P niiA::rcoA::eyfp-N$	This study
AGB1052	AGB1051, transformed with pME3173	This study
AGB1053	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $phleo^R$, $^P niaD::ztfA::eyfp-C$, $^P niiA::eyfp-N$	This study
AGB1054	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $phleo^R$, $^P niaD::eyfp-C$, $^P niiA::rcoA::eyfp-N$	This study
AGB1055	AGB1053, transformed with pME3173	This study
AGB1056	AGB1054, transformed with pME3173	This study
AGB1057	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta vosA::six$	S. Thieme, p.c.
AGB1058	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta vosA::six$, $\Delta ztfA::six$	This study
AGB1059	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta vosA::six$, $six::^P niaD::ztfA$	This study
AGB1062	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta velC::six$	This study
AGB1063	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta velC::six$, $\Delta ztfA::six$	This study
AGB1064	$\Delta nkuA::argB$, $pyrG89$, $pyroA4$, veA^+, $\Delta velB::six$	S. Thieme, p.c.

AGB1065	Δ*nkuA*::*argB*, *pyrG89*, *pyroA4*, *veA⁺*, Δ*velB*::*six*, Δ*ztfA*::*six*	This study
AGB1066	Δ*nkuA*::*argB*, *pyrG89*, *pyroA4*, *veA⁺*, Δ*veA*::*six*	J. Gerke, p.c.
AGB1067	Δ*nkuA*::*argB*, *pyrG89*, *pyroA4*, *veA⁺*, Δ*veA*::*six*, Δ*ztfA*::*six*	This study
ACS39	Δ*akuA*::*loxP*, Δ*pyroA*::*pyroA-TetOn-ztfA*	C. Sasse, p.c.
AfS35	Δ*akuA*::*loxP*	Krappmann *et al.*, 2006b
AfGB129	Δ*akuA*::*loxP*, Δ*ztfA*::*six*	This study

2.3 Morphological methods: conidiospore and cleistothecia quantification

Determination of conidiospore numbers was performed by utilization of a Coulter Z2 particle counter (BECKMAN COULTER GMBH, Krefeld, Germany) or spore numbers were determined with a Thoma cell counting chamber (hemocytometer) (PAUL MARIENFELD GMBH AND CO. KG, Lauda-Königshofen, Germany). Total numbers of conidiospores were determined by complete harvesting of all conidiospores from plates in 0.96% (w/v) NaCl with 0.002% (v/v) Tween-80 (SIGMA-ALDRICH). Conidiospores per 5 mm^2 solid medium were determined by excising plugs of agar from point inoculated cultures with the larger end of a 200 µm pipette tip. Spores were harvested from these plugs and counted with a hemocytometer.

For quantifying cleistothecia, 2000 spores of indicated strains were point inoculated and grown for 8 d in light or dark to induce asexual or sexual development. Agar plugs of 5 mm^2 were cut out using the larger side of a 200 µl pipette tip and cleistothecia were individualized on a new agar plate and counted with help of a SZX12-ILLB2-200 binocular microscope (OLYMPUS DEUTSCHLAND GMBH, Hamburg, Germany). The quantification was tested with plated cultures (1*10^7 spores per strain) with similar results.

2.4 Nucleic acid methods

2.4.1 Isolation and purification of fungal genomic DNA

Strains were grown <u>o</u>ver<u>n</u>ight (o/n) in liquid cultures for extraction of genomic DNA. Mycelia were harvested through Miracloth filters, frozen in liquid nitrogen and ground with a MM400 table mill from RETSCH TECHNOLOGY GMBH (Haan, Germany). Ground mycelia were mixed with 500 µl genomic DNA lysis buffer (Lee and Taylor, 1990) (50 mM Tris-HCl ph 7.2, 50 mM EDTA, 3% (w/v) SDS, 1% (v/v) β-mercaptoethanol) and incubated 15 min at 65°C. Subsequently mycelia solution were mixed with 100 µl 8 M potassium acetate and centrifuged for 15 min at 13000 rpm at <u>r</u>oom <u>t</u>emperature (rt). Supernatant was mixed with 100 µl 8 M potassium acetate and centrifuged for 15 min at 13000 rpm at rt. Supernatant was transferred into new test tubes and mixed with 300 µl isopropanol and centrifuged 10 min at

13000 rpm at rt. DNA pellets were washed twice with 70% (v/v) ethanol and dried at 42°C before resolving in H_2O at 65°C.

2.4.2 Isolation and purification of fungal RNA

Strains were grown vegetatively or asexually for RNA isolation. Mycelia were harvested through sterile Miracloth filters (MERCK) and immediately frozen in liquid nitrogen. Frozen mycelia were ground with a table mill (RETSCH) directly before RNA extraction. RNA from approximately 200 µl of ground mycelia was isolated with the RNeasy® Plant Miniprep Kit from QIAGEN (Hilden, Germany) according to manufacturer's instructions without addition of β-mercaptoethanol. Concentrations were measured with a Nanodrop ND-1000 (PEQLAB). cDNA was transcribed from 0.8 µg RNA with the QuantiTect® Reverse Transcription Kit (QIAGEN) according to manufacturer's conditions.

2.4.3 Isolation and purification of plasmid-DNA and linearized DNA fragments

Plasmid DNA was extracted from *E. coli* cultures by utilization of the QIAprep® Spin Miniprep Kit (QIAGEN) or the NucleoSpin® Plasmid Kit (MACHEREY-NAGEL) according to manufacturer's specifications. Plasmid DNA was eluted from spin columns with dH_2O and stored at -20°C.

Linearized DNA fragments from PCR amplification or enzymatic digests for plasmid linearization and construct excision were mixed with 10x DNA loading dye (10% (v/v) Ficoll 400, 200 mM EDTA pH 8.0, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol FF), separated by agarose gel electrophoresis and gel pieces with respective DNA bands were cut out of the gel. DNA was purified from agarose gels by employing the QIAquick® Gel Extraction Kit (QIAGEN) or the NucleoSpin® Gel and PCR Clean-up Kit from MACHEREY-NAGEL GMBH & CO. KG (Düren, Germany).

2.4.4 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) (Saiki *et al.*, 1988) was employed to amplify DNA fragments for plasmid construction and to determine the presence of desired plasmids in *E. coli* after plasmid construction via seamless cloning or ligation (colony PCR) (Bergkessel and Guthrie, 2013; Hofmann and Brian, 1991). PCRs were performed in T Professional Standard 96, T Professional Trio 48 and T Professional Standard 96 Gradient thermocyclers from BIOMETRA GMBH (Göttingen, Germany) and in Primus 96 Thermal Cyclers from MWG BIOTECH AG (Ebersberg, Germany). Phusion® High-Fidelity DNA Polymerase (THERMO

FISHER SCIENTIFIC) was used for DNA amplification via PCR and PCR programs were designed after manufacturer's instructions and according to calculated melting temperatures (T_M) of utilized primers. The OligoCalc program was used for calculation of primer T_MS and salt adjusted temperatures were regarded as best assumption (Kibbe, 2007).

2.4.5 Agarose gel electrophoresis

Agarose gel electrophoresis was employed for separation of DNA fragments according to size (Lee *et al.*, 2012a). 1% (w/v) agarose gels containing 0.001 mg/ml ethidium bromide were used in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). DNA was applied in 10x DNA loading dye and visualized in-gel by exposure to UV light ($\lambda = 254$ nm) in a Gel iX20 Imager Windows Version and the Intas GDS gel documentation software from INTAS SCIENCE IMAGING INSTRUMENTS GMBH (Göttingen, Germany) or on a TFX-20 MX Vilber Lourmat Super Bright transilluminator (SIGMA-ALDRICH).

2.4.6 Quantitative real-time polymerase chain reaction

Gene expression was measured by quantitative real-time PCR (qRT-PCR) utilizing MESA GREEN qPCR MasterMix Plus for SYBR® Assay purchased from EUROGENTEC (Lüttich, Belgium) in a CFX Connect™ Real-Time System (BIORAD). The utilized qRT-PCR protocol is given in TABLE 3. Primers for qRT-PCR were designed utilizing the Primer3 software (Koressaar and Remm, 2007; Untergasser *et al.*, 2012) and are given in TABLE 4. Gene expression was measured from 1:5 dilutions of respective cDNA. Obtained qRT-PCR data was analyzed with the CFX Manager™ 3.1 software package (BIO-RAD) using the 2-$\Delta\Delta C_T$ method for relative quantification of gene expression (Schmittgen and Livak, 2008). Expression of *gpdA*, *h2A* (*AN3468*) and *15S rRNA* were used for *A. nidulans* qRT-PCR and *h2A* (*Afu3g05360*) and *gpdA* expression were used for *A. fumigatus* as references for relative quantification.

For measurement of the expression of oxidative-stress related genes, strains were grown in submerged cultures at 37°C on a rotary shaker for 24 h. Subsequently, 5 mM H_2O_2 was added. Control strains were left untreated. Incubation was prolonged for another 30 min shaking on the rotary shaker and mycelia were harvested as described above.

qRT-PCR measurements were conducted in several biological replicates as indicated. Each biological replicate was measured in three technical replicates.

TABLE 3: qRT-PCR program used in this study.
Steps 2 to 4 were repeated for 36 times.

Step	Temperature [°C]	Duration [min]	
1	95	5:00	
2	95	0:15	
3	60	0:22	repeated 36 times
4	72	0:40	
5	95	0:10	
6	Melt curve: 65 to 95, increment 0.5	0:05 per step	

TABLE 4: Primers for qRT-PCR used in this study.
Primers for qRT-PCR with *A. fumigatus* cDNA are marked with [af]. All other primers are for *A. nidulans* cDNA.

Designation	Gene	5' – sequence – 3'	Size
HO277	*Afu3g13110* [af] A	CCT GCC GTA ACA TTG CTT CTT G	22mer
HO278	*Afu3g13110* [af] B	CAC AGT CAT CAT CCT CCG ATC C	22mer
HO660	*Afu3g00880* [af] A	GCT CTG ACT CTC ACT GCC TTC G	22mer
HO661	*Afu3g00880* [af] B	AAG CTT GTT GAC GGG AGG GTA G	22mer
HO788	*uge3* [af] A	CCT ATG GCC GTA CCA AAT GGA T	22mer
HO789	*uge3* [af] B	GTG GGA GTC TGT CTG GGG TCT T	22mer
jg787	*flbA* A	CCC TTC TTC TTC TTC CCC TCC T	22mer
jg788	*flbA* B	AAA ACT GGG TGT GGT TGT GGT G	22mer
jg793	*aflR* A	GAA GGC AGG ACC ACC AGT TAC A	22mer
jg794	*aflR* B	CCC TCA AGA AGC GAA GGA GAA A	22mer
jg814	*veA* A	CAA CGA GCA TCA GCA CAA ACA T	22mer
jg815	*veA* B	AGC AGG AAT CGG CGT AGA AGA T	22mer
jg816	*velB* A	CCC CTC CGT GTA TCC GTC TAA T	22mer
jg817	*velB* B	AGC CGA GTG CTT CAC AAG ATT T	22mer
jg818	*vosA* A	CTT CCA TTC CAC CGT CTA CTG C	22mer
jg819	*vosA* B	CGT CCG TCT TTC GCA TTT CA	20mer
jg824	*stcU* A	TTG AGC ACT TCG GAT ACC TGG A	22mer
jg825	*stcU* B	TTG GAA CTT GTG AGG ATG ATG C	22mer
jg1482	*easA* A	ATC ACC AGC GAA CCT CTC TTA G	22mer
jg1483	*easA* B	AGG CTT TCA ATC ACC AGA CTC C	22mer
jg1484	*easB* A	TTC GTC AAG TTT AGT GGC GTT A	22mer
jg1485	*easB* B	CGT TGT GGG TCA AGA AGT AGG T	22mer
jg1486	*easC* A	ACC TTC ATT GGA AAC ATC AAC G	22mer
jg1487	*easC* B	TAG GGT CGT CAG GGA TTC TG	20mer
jg1488	*easD* A	AGC GAC TTC CAC CAT TAC AGT G	22mer
jg1489	*easD* B	AGT TTC TGC TTC CCT GAT GTT C	22mer
kt272	*ztfA* (*AN0585*) A	TTC AGT CTC ACC AAC GGG ACA T	22mer
kt273	*ztfA* (*AN0585*) B	GAT ACG CGA GTT TGG GTT TTC C	22mer
kt274	*brlA* A	CAG GAT CAC TCC CCA ACA ACA C	22mer
kt275	*brlA* B	GTA AGC GAG TCC TTG AGC GAC A	22mer

kt278	*15S rRNA* A	GAT CCG CGA AAA ACC TTA CCA C	22mer
kt279	*15S rRNA* B	TGG CAC GTC TAT AGC CCA CAG T	22mer
kt308	*gpdA* A	AAC GCT TCT TGC ACC ACC AA	20mer
kt309	*gpdA* B	ACC AGT GGA GGA GGG GAT GA	20mer
kt310	*velC* A	CCA ATC GAC TCC GCT CCT CT	20mer
kt311	*velC* B	AGA AGC ATG CCG GTG GTT TT	20mer
kt312	*h2A.X* A	TCT CGA GCT TGC TGG AAA CG	20mer
kt313	*h2A.X* B	CAC CCT GGG CAA TAG TGA CG	20mer
kt316	*h2A.X* ^{af} A	TGG AGT ATC TCG CTG CTG AA	20mer
kt317	*h2A.X* ^{af} B	GGA GAT GGC GAG GAA TGA TA	20mer
kt320	*ztfA (Afu6g11110)* ^{af} A	CAG CAG CAG ACT AGG GGT TC	20mer
kt321	*ztfA (Afu6g11110)* ^{af} B	TGA TGC GGA GCT ACT TCT CC	20mer
kt332	*brlA* ^{af} A	TCA TCA AGC AGG TGC AGT TC	20mer
kt333	*brlA* ^{af} B	TTG GAG TGG CTC TTC ATG TG	20mer
kt397a	*flbB* A	AGT TCG ACT TCT CGT CAG TTC C	22mer
kt398a	*flbB* B	TGG GGA TTG TCT TCA AAT ATC C	22mer
kt399a	*flbC* A	ATC TCA TCT GCA GGC TCT TAC C	22mer
kt400a	*flbC* B	GTT GTT GAG CTG TAA TCG GTG A	22mer
kt401a	*flbD* A	CAA CAA AGC ATC AAC AGC TCT C	22mer
kt402a	*flbD* B	GGT CCA TGA GGT ATA GGG TCT G	22mer
kt404	*fluG* A	GAC ATC AAT CTG CTG AAA TCC A	22mer
kt405	*fluG* B	TCG CGT GTA TAT GGG TAA GAT G	22mer
kt436	*ausA* A	AGG TGG AGA ACT GCT CAG GA	20mer
kt437	*ausA* B	CGA AGG AAA CGG ACT GAG AG	20mer
kt438	*ausF* A	TGT CCA CCA CAC GAG AAA AG	20mer
kt439	*ausF* B	TGC GAA TGG AGA GAA TTT CC	20mer
kt440	*ausH* A	GGA CTT CCA AGG GCT AAA GG	20mer
kt441	*ausH* B	ACT CGG TCT CAA ATC GAC CA	20mer
kt491	*nsdD* A	TCA TCT CAC CAG CCA CAA TTA C	22mer
kt492	*nsdD* B	CAG AGG TCA TAA CAG TGC TTG C	22mer
kt531	*catB* A	TTA ATC GAA TCT CGA ACG ACC T	22mer
kt532	*catB* B	GGT CGT GTT GTC GTG GTA GTA A	22mer
kt533	*trxR* A	CCC TAG AGG CTA ACG GTC TTT T	22mer
kt534	*trxR* B	ATG TAT CCG TCC TCA TCG AGT T	22mer
kt548	*flbE* A	TGA CGA AGA TGA GGA TGG TAT G	22mer
kt549	*flbE* B	TGT TAC TAG ACG ACC CAT CAC G	22mer
kt550	*sfgA* A	ACT TTT AGC GCT CTT CGA GAT G	22mer
kt551	*sfgA* B	AGG GTG ATT CAT TTC AGC AAC T	22mer
kt578	*catA* A	AGG AAG TTC TGG GCA ATG TG	20mer
kt579	*catA* B	GTC CTT GAG CAC CTT GAA GC	20mer
kt584	*napA (AN7513)* A	CCG GCA TCT TAC GAC ATT CT	20mer
kt585	*napA (AN7513)* B	ACT TTG TGG CAG GGT TGT TC	20mer
kt586	*rsmA* A	ATC GCT GGC AGT CAT TAT CC	20mer
kt587	*rsmA* B	TAA TTC CGA TTC CGT CCT TG	20mer
kt588	*glrA* A	CCG AAG TTG AGG ATT TGC AT	20mer

kt589	*glrA* B	TCG ACG TTG GTG TTT TGG TA	20mer
kt590	*trxA* A	GAA ATT CGC CCA GAC CTA CA	20mer
kt591	*trxA* B	CCA ACC ACA TCG CTA ACC TT	20mer
RH382	*orsA* A	GAT GAT GAC GCA GAG GAG GAG A	22mer
RH383	*orsA* B	AGG GCT TTC AGG TGG ATG TAG G	22mer

2.5 Plasmid construction for genetic manipulation of fungi

2.5.1 Cloning strategies

For the fusion of DNA sequences for plasmid construction fusion-PCR (Szewczyk *et al.*, 2006) and the GeneArt® Seamless Cloning and Assembly Kit (INVITROGEN) or the GeneArt® Seamless Cloning and Assembly Enzyme Mix (INVITROGEN) was used. As templates for PCR amplified DNA fragments genomic DNA from *A. nidulans* FGSC A4 and *A. fumigatus* AfS35 was used. All constructs harboring a recyclable marker cassette were cloned into the *EcoR*V multiple cloning site of pBluescript SK+. For excising of all genetic cassettes constructed this way outermost primers of each construct introduce *Mss*I restriction sites. Linearization of constructs with this strategy results in on-locus integration into the genome of transformed fungi.

2.5.2 Primer and plasmid design

Plasmids generated and used in this study are listed in TABLE 5 and the primers used for their construction are listed in TABLE 6. Primers and genetic construct maps were designed with the Lasergene software package from DNA STAR INC. (Madison, WI, USA). Genetic information was obtained from AspGD (Cerqueira *et al.*, 2014) and CADRE (Mabey Gilsenan *et al.*, 2012). Construction strategies are described in detail in the next chapter. For all plasmids containing constructs, which were excised with *Mss*I (in GTTT/AAAC motives) for transformation into Aspergilli, a naturally occurring AAAC or GTTT quadruplet was used as terminal sequence towards the plasmid backbone. Terminal primers of these constructs introduced the respective second half of the *Mss*I restriction site. This strategy leads to the absence of additional base pairs in the genomes of fungi transformed with respective plasmids and genes potentially lying in respective regions were not interrupted (FIGURE 11). Primers used for seamless cloning reactions were designed to introduce 15 base pairs (bp) complementary to adjacent sequences in the way that two adjacent sequences share a 15 bp homology region.
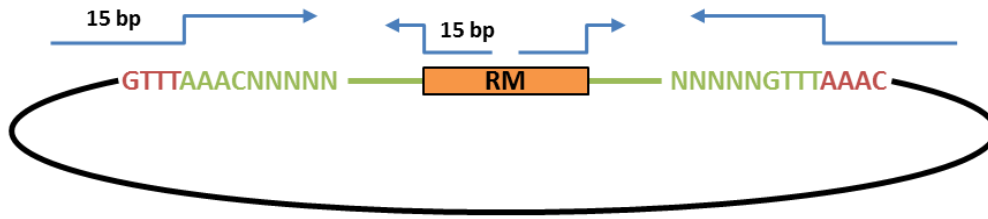
FIGURE 11: Cloning strategy employed for the generation of constructs for genetic manipulation of Aspergilli.
Schematic depiction indicates primer design and general architecture of plasmids constructed in this study. Primers comprise regions with 15 bp end homology to the adjacent DNA-fragment. Outermost nucleotide sequences naturally containing one half of the *Mss*I restriction site were chosen as terminal sequences for PCR-amplified constructs. The fragment amplified from Aspergillus genomic DNA is shown in green, the second half of the *Mss*I restriction site, introduced by the respective primer, is given in red, the plasmid backbone is given in black, RM = recyclable marker cassette. Note that after recycling of the marker cassette, only the mutated genomic region (green) and a *six* site as substitute for the recyclable marker cassette is present in the fungal genome.

TABLE 5: Plasmid constructed and used in this study.
A. nidulans genes are denoted with AN put in front, *A. fumigatus* genes are marked with Afu. All plasmids constructed in this study use pBluescript SK+ as backbone, if not stated otherwise. ^{P} = promoter, ^{t} = terminator, ^{R} = resistance, natRM = recyclable *nat*^{R} resistance cassette from pME4304, phleoRM = recyclable phleoRM resistance cassette from pME4305, ptrARM = recyclable *ptrA* resistance cassette from pSK485, p.c. = personal communication.

Plasmid	Description	Reference
pBluescript SK+	Cloning vector, *amp*^{R}	FERMENTAS GMBH
pJG137	Plasmid for BiFC containing *niaD*^{t}-*Swa*I-^{P}*niaD*/^{P}*niiA*-*Pme*I-*niiA*^{t}	J. Gerke, p.c.
pJG158	^{P}*veA*::ANΔ*veA*::natRM	J. Gerke, p.c.
pME3173	^{P}*gpdA*::intron::*mrfp*::*h2A* cDNA in *EcoR*V and ^{P}*gpdA*::*natR* in *Sma*I of pBluescript II KS	Bayram *et al.*, 2008a
pME3741	BiFC vector; ^{P}*niiA*::n*eyfp*::*culA*::*niiA*^{t} and ^{P}*niaD*::*candA-N*::C::*ceyfp*::*niaD*^{t}::*AfpyrG*::*wA*, *bla*	Helmstaedt *et al.*, 2011
pME4292	Plasmid contains *sgfp*	B. Jöhnk, p.c.
pME4304	*six*-^{P}*xylP*::β-*rec*::*trpC*^{t}-*nat*^{R}-*six*	J. Gerke, p.c.
pME4305	*six*-^{P}*xylP*::β-*rec*::*trpC*^{t}-*phleo*^{R}-*six*	J. Gerke, p.c.
pME4575	ANΔ*ztfA*::natRM	This study
pME4576	^{P}*ztfa*::AN*ztfA*::*sgfp*::natRM	This study
pME4577	AN*ztfA*::phleoRM	This study
pME4578	natRM::^{P}*niaD*::AN*ztfA*	This study
pME4579	^{P}*ztfA*::*sgfp*::AN*ztfA*::phleoRM	This study
pME4580	^{P}*ztfA*::AN*ztfA*$^{S327A,T464A,S504-506A}$::phleoRM	This study
pME4581	ANΔ*fluG*::phleoRM	This study
pME4582	phleoRM::^{P}*niiA*::AN*fluG*	This study
pME4584	ANΔ*aflR*::phleoRM	This study
pME4585	phleoRM::^{P}*niiA*::AN*aflR*	This study
pME4587	ANΔ*abaA*::phleoRM	This study

pME4589	ANΔ*brlA*::phleoRM	This study
pME4591	ANΔ*flbB*::phleoRM	This study
pME4593	ANΔ*flbC*::phleoRM	This study
pME4595	ANΔ*flbD*::phleoRM	This study
pME4597	ANΔ*flbE*::phleoRM	This study
pME4599	^P*niaD*::AN*ztfA*::*ceyfp* in *Swa*I restriction site, ^P*niiA*::AN*rcoA*::*neyfp* in *Pme*I restriction site of pJG137	This study
pME4600	^P*niaD*::AN*ztfA*::*ceyfp*, ^P*niiA*::*neyfp*	This study
pME4601	^P*niaD*::*ceyfp*, ^P*niiA*::AN*rcoA*::*neyfp*	This study
pME4602	ANΔ*velC*::phleoRM	This study
pME4603	ANΔ*vosA*::natRM	S. Thieme, p.c.
pME4605	ANΔ*velB*::natRM	S. Thieme, p.c.
pME4606	AfuΔ*ztfA*::ptrARM	This study
pSK485	*six*-p*xylP*::β-*rec*::*trpCt-ptrA-six*	Hartmann *et al.*, 2010

TABLE 6: Oligonucleotides used for sequence amplification and plasmid construction.

Name	5' – sequence – 3'	Size
kt145	CTA TAG GCC TGA GTG TAA GAA GTC AAG AAG CGG TCA ATG	39mer
kt146	ATA ATA TGG CCA TCT ATG TTT TGA GGG ACT CCA ACT C	37mer
kt203	CTG CAG GAA TTC GAT GTT TAA ACG CTG AAG TTT GTG GGA G	40mer
kt204	ATC GAT AAG CTT GAT GTT TAA ACG CTG TAA GTT GGA TCA GC	41mer
kt208b	CTG CAG GAA TTC GAT GTT TAA ACC TGG TAT GAA CGA CTT TCC	42mer
kt209	CTG CAG GAA TTC GAT GTT TAA ACC ATC GCT CTG GTA GCT TC	41mer
kt211	ATA ATA TGG CCA TCT CTC AAC CGC CTA TCA CTC TAG	36mer
kt214	CTA TAG GCC TGA GTG CTG GTA GTC TTA CGG TGA GTT G	37mer
kt215	CTG CAG GAA TTC GAT GTT TAA ACA GGA TTC GGT GAT TTC TTT C	43mer
kt218	ATA ATA TGG CCA TCT TCG TCT CCT ACA GCA GGA C	34mer
kt221	CTA TAG GCC TGA GTG TTG GTA GGT TGA GGG TCC C	34mer
kt224	ATC GAT AAG CTT GAT GTT TAA ACC CTA CTT TCA CAA CGA GG	41mer
kt225	ATC GAT AAG CTT GAT GTT TAA ACC GGA GCG TAT CAC CTA TC	41mer
kt226	ATC GAT AAG CTT GAT GTT TAA ACC AAA GAC CCA GCT AAA AAC	42mer
kt228	ACC ACC GCT ACC ACC GTC GTT GAC CAT ATC ATC AA C	37mer
kt229	CTA TAG GCC TGA GTG TTA CTT GTA CAG TTC GTC CAT G	37mer
kt230	CTA CTT GTA CAG TTC GTC CAT GC	23mer
kt231	CTA TAG GCC TGA GTG TTA GTC GTT GAC CAT ATC ATC C	37mer
kt234	ACC ACC GCT ACC ACC GTC GTT CAC CAT ATC ATC AA G	37mer
kt241	ATG CAA TCA CTC GTC CTC CC	20mer
kt251	ATA ATA TGG CCA TCT GAT GGC GGG CGC GGT GAT T	34mer
kt252	GAC GAG TGA TTG CAT GTG AGA GTA TGG GAT AGG AAA ATA AT	41mer
kt253	ATA AGC TTG ATG TTT AAA CAT TAA GTA TCC AGT ATG ATC AG	41mer
kt300	GAT GGC GGG CGC GGT GAT	18mer
kt302	GTG AGA GTA TGG GAT AGG AAA ATA	24mer
kt307	GCC CTT GCT CAC CAT CTG GTA GTC TTA CGG TGA GTT G	37mer
kt337	ATA ATA TGG CCA TCT GTG AGA GTA TGG GAT AGG AAA ATA	39mer

kt341	CTG CAG GAA TTC GAT GTT TAA ACA GAC ATC TCC ATG CCG GTT ATG	45mer
kt342	CTA TAG GCC TGA GTG GGC GAT GAA CCA GCA AAC TAA AGG AC	41mer
kt343	ATA ATA TGG CCA TCT GTC TAA TCT TTC TCC TGA GCG TAT TCA C	43mer
kt345	ACC GCG CCC GCC ATC ATG GCC ACT CTC TCT TCA CTC CG	38mer
kt348	CTG CAG GAA TTC GAT GTT TAA ACT GGC GTT GCA CCT TGG GTT G	43mer
kt349	CTA TAG GCC TGA GTG GAT ATT TGC ATA TGA TAC AGG CCC GCA TTG	45mer
kt350	ATA ATA TGG CCA TCT GGT TGA ATA ATC TGG AAT GAT ATT TAT GCG ATC	48mer
kt353	ACC GCG CCC GCC ATC ATG GAG CCC CCA GCG ATC AG	35mer
kt354	CTG CAG GAA TTC GAT GTT TAA ACC CTG GTC AGA CAC TGA GCA TG	44mer
kt355	CTA TAG GCC TGA GTG GGA GCA GAC CCC AAG ATT CGC TC	38mer
kt356	ATA ATA TGG CCA TCT CCT CCT TTA CCA TGT CTA TGA ACA GAC G	43mer
kt361	ATC GAT AAG CTT GAT GTT TAA ACA AAA TAT GAT CGT GCT TCG GCA CTT GG	50mer
kt362	ATC GAT AAGC TTG ATG TTT AAA CAC CAA CTG CAG GCC TCG G	41mer
kt363	ATC GAT AAG CTT GAT GTT TAA ACG ACG AGT ACG CTG TAA CAG CAA TTC	48mer
kt364	ATC GAT AAG CTT GAT GTT TAA ACA CGC CGC CGC TAA GCG	39mer
kt365	ATC GAT AAG CTT GAT GTT TAA ACT CGT CTG CGG CTG AGT CG	41mer
kt407	CAT ACT CTC ACA TTT ATG CAA TCA CTC GTC CTC CC	35mer
kt409	CGC CCG CCA TCG TTT ATG CGC AGC ATT GAC CAA CC	35mer
kt415	TCT TGC AGG CCG GGC GGT CGT TGA CCA TAT CAT CCA AC	38mer
kt416	TGC GAA CCC GTA TTT TCA CTT GTA CAG CTC GTC CAT	36mer
kt417	CGC CCG GCC TGC AAG ATC	18mer
kt418	CGT GGC GAT GGA GCG CCGT CCA GTG TAC GCG GAG	34mer
kt421	CGC TCC ATC GCC ACG GTG AGC AAG GGC GAG GAG	33mer
kt422	TAT CCT CGT CAG TTT TCA CAT GAT ATA GAC GTT GTG GCT	39mer
kt430	GTG CGA CTG AAG TCA TTG AC	20mer
kt431	TGA CTT CAG TCG CAC CAA CGG GAC ATG TCC CAA TC	35mer
kt432	CTC TGG AGC TTG CTG CTG C	19mer
kt433	CAG CAA GCT CCA GAG CCC CGC CAG GGC TTC ATG	33mer
kt434	GGC GGC CAA GCC TAG TTG GCT TTG GCT TTG GCG C	34mer
kt442	CCA ACT AGG CTT GGC CGC CGC G	22mer
kt487	CTG CAG GAA TTC GAT GTT TAA ACG CCC AAC CCC ACA CTG	39mer
kt488	CTA TAG GCC TGA GTG GTC TTC GAG CGA CGG GGC	33mer
kt489	ATA ATA TGG CCA TCT AAC AGA AAC AAA GAG GGC TGA TC	38mer
kt490	ATA AGC TTG ATG TTT AAA CAA GAA CGT AAC CTA CCG TAA G	40mer
kt515	CTG CAG GAA TTC GAT GTT TAA ACG CTC CTT CTT CCA CTT C	40mer
kt516	CTA TAG GCC TGA GTG GGT GGT CGA GCT GTG AAT AG	35mer
kt517	ATA ATA TGG CCA TCT CCT GAC AGC TCG CTT TTT TTC	36mer
kt518	ATC GAT AAG CTT GAT GTT TAA ACA TAG TGT ATG ACA CGC CC	41mer
kt519	CTG CAG GAA TTC GAT GTT TAA ACC CAC TGC TCA AGC TCA G	40mer
kt520	CTA TAG GCC TGA GTG TGA GGA TAG TCG TTT TGA AAG AG	38mer
kt521	ATA ATA TGG CCA TCT TCG TTT CAA TCG ACC TGC CC	35mer
kt522	ATC GAT AAG CTT GAT GTT TAA ACG GCG TCG AGA AGG C	37mer
kt523	CTG CAG GAA TTC GAT GTT TAA ACG AAC AAG TGC CGA CTC	39mer
kt524	CTA TAG GCC TGA GTG TTG CGA AAC TGT GTT GGT GAT G	37mer

kt525	ATA ATA TGG CCA TCT ACG ATC ACA CGA CTC TCT TC	35mer
kt526	ATC GAT AAG CTT GAT GTT TAA ACA CCG TAG ACT TGT CCA G	40mer
kt527	CTG CAG GAA TTC GAT GTT TAA ACG ACT TGT TTG CTC GTC TC	41mer
kt528	CTA TAG GCC TGA GTG GGT AAG GCG ACG ACG GC	32mer
kt529	ATA ATA TGG CCA TCT TTG CTG TAC GAG TTA TAT TAC GAC	39mer
kt530	ATC GAT AAG CTT GAT GTT TAA ACT AGT GAG ACC TAC CAG C	40mer
kt539	CTA TAG GCC TGA GTG TCA TGA ATA CAT CGT CTC ATC AG	38mer
kt540	CTA TAG GCC TGA GTG TTA CTC TTC GTC ATC GCC TG	35mer
kt541	CTA TAG GCC TGA GTG TCA GTT CAA GAG GTT GTC GAG	36mer
kt542	CTA TAG GCC TGA GTG TCA CGA AAA CGT TTT GTT GAA GAA TC	41mer
kt543	CTA TAG GCC TGA GTG TCA TTC ATC CCA GCC GTC C	34mer
kt544	CTA TAG GCC TGA GTG TCA ATA CCT CTC CAC AAG CC	35mer
kt545	CTA TAG GCC TGA GTG TCA GGC GTG GCG GAG GAT	33mer
kt546	CTA TAG GCC TGA GTG CTA GAC AGC CTC AAC CGC	33mer
SR120	ATG GTG AGC AAG GGC GAG GAG	21mer
SR121	ACC ACC GCT ACC ACC CTT GTA CAG TTC GTC CAT GCC	36mer
SR18	GGT GGT AGC GGT GGT GTG AGC AAG GGC GAG GAG	33mer
SR193	CGC CCG CCA TCG TTT ATG GTG AGC AAG GGC GAG	33mer
SR195	ACT CTC ACA TTT ATG GCC GAC AAG CAG AAG AAC G	34mer
kt182	CAT CAG TGC CAG CTG TCT TCG	21mer
kt183	GAT GTG CTG CAA GGC GAT TAA GTT G	25mer
kt184	GGC TTT ACA CTT TAT GCT TCC G	22mer
kt266	GAG AAG CGC GAT CAC ATG G	19mer
kt267	GCA TGG CGG ACT TGA AGA AG	20mer
kt268	GAA CCC GTG CCC TAT ACT ATC	21mer
kt290	CTT TTT GTG GCC CTT CCT CC	20mer
kt291	AAA CCA TCG CTC TGG TAG CTT C	22mer
kt338	CGT GGA ACA GCT GAA GTC AC	20mer
kt339	CTC TGA TAT CTA TAG GTC AAT AGA G	25mer
kt372	CAT AGA TAG AGA TAG GGC TTG	21mer
kt373	CAT AAT ATG GCC ATC TGT GAG	21mer
kt374	GAA TCC TGT TAA AAT CAG TAT ATC ATG	27mer
kt375	GGA ATG ATT CTT CTT TTG TTG AAG G	25mer
kt424	CAA CTA GGC TTG AGC TCC TC	20mer
kt425	GAA CAA TCT TCG AGA TTC TGC TC	23mer
kt426	CCA ATC AGA GCC TCG GAA TC	20mer
kt427	GTT CTA TGG ACT GTT ACC GAT TC	23mer

2.5.3 Recyclable marker cassettes as selection markers

All plasmids constructed during this study, which were used for homologues recombination, harbor recyclable marker cassettes based on the bacterial recombination system, which employs a prokaryotic small β-serine recombinase and its *six* recognition sequences (Canosa *et al.*, 1996; Hartmann *et al.*, 2010; Rojo *et al.*, 1993; Rojo and Alonso, 1994). This system

allows the excision of the respective marker cassette off the fungal genome after successful transformation (FIGURE 12). This procedure allows a marker-free mutation and only leaves a relatively small *six* recognition site of 100 nucleotide base pairs (bp). After excision of the marker cassette, the same selection marker can be used again in the same host strain. This system prevents large resistance cassettes to interfere with the genetic equipment of the host. Three different recyclable marker cassettes were used, allowing utilization of three different selective agents. pSK485 harbors the *A. oryzae ptrA* gene, which confers resistance against pyrithiamine (Hartmann *et al.*, 2010). The pyrithiamine resistance marker cassette from pSK485 is termed ptrARM (*ptrA* recyclable marker) in the following. pME4304 (J. Gerke, p.c.) harbors the *nat1* gene from *Streptomyces noursei*, which grants resistance against nourseothricin (Kück and Hoff, 2006). The nourseothricin resistance marker cassette from pME4304 is termed natRM in the following. pME4305 (J. Gerke, p.c.) harbors the *ble* gene from *Streptoalloteichus hindustanus*, which confers resistance to phleomycin (Drocourt *et al.*, 1990). The phleomycin resistance marker cassette is termed phleoRM in the following.
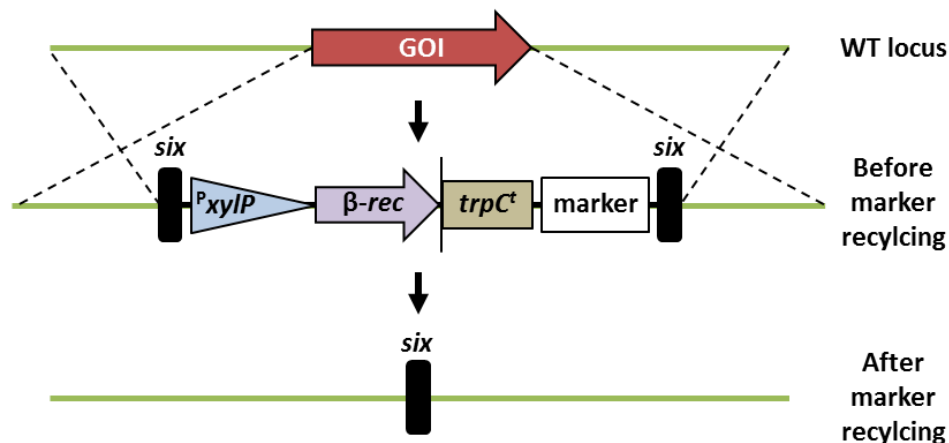
FIGURE 12: Schematic depiction of integration and recycling of a recyclable marker cassette.
A gene deletion is given as example. The ORF (red) of the gene of interest (GOI) is replaced by a recyclable marker cassette, comprising the β-*serine recombinase* gene (β-*rec*), driven by the xylose-inducible promoter (^P*xylP*) and employing the *trpC* terminator (*trpC*^t). Expression of ^P*xylP*::β-*rec* is induced by supplementation of the medium with xylose. This leads to an excision of the whole cassette from the fungal genome, only leaving a small *six* site as scar. Dotted lines indicate 5' and 3' region (green) adjacent to the GOI ORF, which are used for homologous recombination. This promotes on-locus integration of the respective construct.

2.5.4 Sequencing of plasmids

Plasmids constructed in this study were sequenced by SEQLAB SEQUENCE LABORATORIES GMBH (Göttingen, Germany). Obtained sequences were analyzed with the Lasergene software package (DNA STAR INC.). All plasmids constructed in this study and cloned in

pBluescript SK+ were sequenced with the primers kt182, kt183, kt184 and kt339, which bind near both *six* sites of the recyclable marker cassettes and near the *EcoR*V cloning site of pBluescript SK+. Further primers are indicated in the respective sub-chapters (see CHAPTER 2.5.5).

2.5.5 Plasmid and strain construction of *A. nidulans* mutant strains

All DNA fragment sizes given in the upcoming sections are rounded. Genomic DNA of FGSC A4 (*A. nidulans* WT, *veA*⁺) and AfS35 (*A. fumigatus* WT, Δ*akuA*), respectively, was used as template, if not stated otherwise.

2.5.5.1 Construction of the Δ*ztfA* cassette and Δ*ztfA* strain in *A. nidulans*

For construction of an *A. nidulans* Δ*ztfA* strain the 2.7 kb long 5' region of the *ztfA* (*AN0585*) gene was amplified from *A. nidulans* FGSC A4 genomic DNA with primers kt208b/214 introducing overhangs of 15 bp homolog to pBluescript SK+ and the *six* site of the recyclable marker cassette, respectively. The respective 2.2 kb long 3' region was amplified with primers kt211/224 likewise introducing overhangs of 15 bp homolog to the *six* site of the recyclable marker cassette and pBluescript SK+. Both sequences and the natRM cassette were cloned into the *EcoR*V multiple cloning site of pBluescript SK+ in a seamless cloning reaction according to manufacturer's conditions, resulting in pME4575. The deletion cassette was subsequently excised with *Mss*I and integrated into AGB551, resulting in the strain AGB1007. The correct replacement of the original gene with the deletion construct was verified by Southern hybridization before as well as after marker recycling.

2.5.5.2 Construction of plasmid pME4578 and *ztfA* OE strain in *A. nidulans*

For the overexpression of *ztfA* the 1.3 kb nitrate-inducible promoter (ᴾ*niaD)* was amplified with primers kt251/252, which introduce overhangs of 15 bp complementary to the *six* site of the recyclable marker cassette and the *ztfA* gene, respectively. The *ztfA* open reading frame (ORF) itself and a small part of the 3' region (1.8 kb) were amplified with primers kt241/253. The 5' region was amplified with primers kt208b/214. The 5' region, natRM cassette, ᴾ*niaD* and the *ztfA* gene were fused and cloned into pBluescript SK+ in a seamless cloning reaction, resulting in plasmid pME4578. The plasmid was sequenced with additional primers kt290, kt291, kt338, kt372 and kt373. The *ztfA* OE construct was subsequently excised with *Mss*I and integrated into AGB551, resulting in AGB1008. Homologous recombination was verified by Southern hybridization.

2.5.5.3   **Plasmid and strain construction of GFP-fusions of ZtfA in *A. nidulans***

sgfp was amplified from pME4292 using primers, which introduce a 15 bp linker region between the *ztfA* and the *sgfp* gene. For a *ztfA*::*sgfp* construct *sgfp* was amplified using primers SR18, introducing the 15 bp linker and kt229 introducing 15 bp overhang to *six*. The *ztfA* ORF and its 5' flanking region (4.4 kb) was amplified using primers kt208b/228. The latter one introduces the deletion of the stop codon of *ztfA* and 15 bp homolog to the linker of *sgfp*. The *ztfA* 3' region was amplified using primers kt211/224. The three sequences (5' UTR and *ztfA*, *sgfp,* 3' UTR) together with the natRM cassette were cloned into pBluescript SK+ resulting in pME4576. The plasmid was sequenced with additional primers kt266, kt267, kt290, kt291 and kt338. Subsequently, the *ztfA*::*sgfp* construct was excised off the plasmid and integrated into AGB1007 resulting in AGB1009. Successful transformation at the correct locus was verified by Southern hybridization.

An N-terminally tagged fusion construct (*sgfp*::*ztfA*) was obtained by a seamless cloning reaction cloning the 5' flanking region of *ztfA*, *sgfp*, *ztfA* ORF, the phleoRM cassette and the *ztfA* 3' flanking region in pBluescript SK+, resulting in pME4579. Therefore, the 1.9 kb *ztfA* 5' flanking region was amplified with primers kt209/307. kt307 introduces a 15 bp overhang to *sgfp* and a start codon. *sgfp* was amplified with primers SR120/121. The *ztfA* ORF was amplified with primers kt230, introducing an overhang for the linker of *sgfp* and a deletion of the start codon, and kt231. The 1.3 kb 3' flanking region was amplified using primers kt211/225. The plasmid was sequenced with additional primers kt266, kt267, kt290, kt291 and kt338. Subsequently, the *sgfp*::*ztfA* construct was excised from pME4579 with *Mss*I and integrated into AGB1007, obtaining AGB1010. Homologous integration of the construct was verified by Southern hybridization. The plasmid pME3173 containing $^{P}gpdA$::*rfp*::*h2A* was integrated into AGB1009 and AGB1010, resulting in AGB1012 and AGB1013, respectively, for a better visualization of nuclei and ectopic integration was verified by microscopy. To obtain a negative control for microscopy, pME3173 was integrated into AGB551 resulting in AGB1014.

2.5.5.4   ***ztfA* complementation in *A. nidulans***

The *ztfA* ORF and its 5' UTR (4.4 kb) was amplified with primers kt208b/231. The *ztfA* 3' UTR was amplified with primers kt211/224 and both fragments together with the phleoRM cassette were cloned into pBluescript SK+, giving rise to pME4577. The plasmid was sequenced with additional primers kt290 and kt338. The *ztfA* complementation cassette was excised and cloned into AGB1007, resulting in AGB1011.

2.5.5.5   Construction of plasmid and strain: *ztfA*^S327A,T464A,S504-506A^ in *A. nidulans*

A strain expressing a permanently dephosphorylated ZtfA protein was constructed. Therefore plasmid pME4580 was constructed as follows: 1.9 kb of the 5' region adjacent to the *ztfA* ORF and first 1 kb part of the *ztfA* ORF was amplified with primers kt209/430. Thymine at position 1038 is exchanged with guanine using primer kt430, therefore introducing the first mutation in the gene product of the serine residue at amino acid position 327 to alanine. The next 431 bp of the *ztfA* ORF were amplified with primers kt431/432. kt432 introduces a mutation of arginine to guanine at bp position 1449, which leads to an exchange of threonine at amino acid residue 464 to alanine. Adjacent 135 bp were amplified with the primer pair kt433/434, which introduce a mutation of arginine and guanine to guanine and cytosine at bp position 1569 and 1570 and thymine to guanine at position 1572. The last 172 bp of the *ztfA* ORF were amplified with the primer pairs 442/231. kt442 introduces a mutation of thymine to guanine at bp position 1575. These alterations lead to an exchange of the serine stretch at amino acid position 504 to 506 with an alanine stretch. The four fragments together constituting the mutated *ztfA* ORF and its 5' adjacent region were fused in a series of fusion PCRs (Szewczyk *et al.*, 2006), resulting in one fragment. The 3' adjacent region to the *ztfA* ORF was amplified with the primer pair kt211/225. Both fragments and the phleoRM cassette were cloned into pBluescript SK+ in a seamless cloning reaction. The plasmid was sequenced with additional primers kt290, kt291 and kt338. The *ztfA*^S327A,T464A,S504-506A^ cassette was excised and integrated into AGB1007, resulting in AGB1015.

2.5.5.6   Construction of plasmids and strains: Δ*fluG*, *fluG* OE and the *fluG/ztfA* double mutants in *A. nidulans*

1 kb of the 5' flanking region of *fluG* was amplified with primers kt341/342, which introduce 15 bp overhangs to the *EcoR*V restriction site of pBluescript SK+ and the *six* site, respectively. 1 kb of the 3' flanking region was amplified with the primer pair 343/364 and both fragments together with the phleoRM cassette were cloned into the *EcoR*V restriction site of pBluescript SK+ in a seamless cloning reaction, giving rise to pME4581. The *fluG* deletion cassette was excised from pME4581 and integrated into the genome of AGB551, AGB1007 and AGB1008, resulting in AGB1016, AGB1017 and AGB1018, respectively. The same 5' flanking region, the *fluG* gene with a short part of its 3' region (3 kb), amplified with kt345/365, the ^P^*niiA*, amplified with primers kt337/300 and the phleoRM cassette were cloned into pBluescript SK+ in a seamless cloning reaction, resulting in pME4582. The plasmid was sequenced with additional primers kt372, kt373 and kt375. The ^P^*niiA*::*fluG* cassette was excised and integrated into AGB551 and AGB1007, resulting in AGB1019 and AGB1020.

2.5.5.7   Construction of plasmids and strains: Δ*aflR*, *aflR* OE and the *aflR*/*ztfA* double mutants in *A. nidulans*

1 kb of the 5' flanking region adjacent to *aflR* was amplified with kt348/349. 1.5 kb of the 3' region adjacent to *aflR* was amplified with kt350/361. Both fragments together with the phleoRM cassette were cloned into pBluescript SK+ in a seamless cloning reaction, resulting in pME4584. The Δ*aflR* cassette was excised from pME4584 and integrated into AGB551, AGB1007 and AGB1008, resulting in AGB1022, AGB1023 and AGB1024, respectively.

The *aflR* OE construct was accomplished by cloning the same 5' region together with the phleoRM cassette, $^P niiA$, amplified with kt337/300, and the *aflR* ORF and a short 3' flanking region, together spanning 2.5 kb (primer kt353/362), into pBluescript SK+, resulting in pME4585. The plasmid was sequenced with additional primers kt372, kt373 and kt374. The $^P niiA$::*aflR* cassette was excised and integrated into AGB551 and AGB1007, giving rise to AGB1025 and AGB1026, respectively.

2.5.5.8   Construction of plasmids and strains: Δ*abaA* and the *abaA*/*ztfA* double mutants in *A. nidulans*

1.5 kb of the 5' region adjacent to the *abaA* ORF were amplified with kt354/355 and together with the phleoRM cassette and 1.4 kb of the 3' region adjacent to *abaA* (primers kt356/363) cloned into pBluescript SK+, resulting in pME4587. The Δ*abaA* cassette was excised and integrated into AGB551 and AGB1007, resulting in AGB1028 and AGB1029, respectively.

2.5.5.9   Construction of plasmids and strains: Δ*brlA* and the *brlA*/*ztfA* double mutants in *A. nidulans*

1.7 kb of the 5' region adjacent to the *brlA* ORF was amplified with kt487/488. 1.2 kb of the *brlA* 3' region was amplified with kt489/490. Both fragments and the phleoRM cassette were cloned into pBluescript SK+ in a seamless cloning reaction, resulting in pME4589. The Δ*brlA* cassette was excised and integrated into AGB551, AGB1007 and AGB1008, resulting in AGB1031, AGB1032 and AGB1033, respectively.

2.5.5.10   Construction of plasmids and strains: Δ*flbB* and the *flbB*/*ztfA* double mutants in *A. nidulans*

1.2 kb of the 5' region adjacent to the *flbB* ORF was amplified with kt515/516. 1 kb of the respective 3' region was amplified with kt517/518. Both fragments together with phleoRM cassette were cloned into pBluescript SK+ in a seamless cloning reaction, resulting in pME4591. The Δ*flbB* cassette was excised from the plasmid and integrated into AGB551, AGB1007 and AGB1008, resulting in AGB1035, AGB1036 and AGB1037, respectively.

2.5.5.11 Construction of plasmids and strains: Δ*flbC* and the *flbC*/*ztfA* double mutants in *A. nidulans*

1.2 kb of the 5' region adjacent to the *flbC* ORF was amplified with kt519/520. 1 kb of the respective 3' region was amplified with kt521/522. Both fragments together with the phleoRM cassette were cloned into pBluescript SK+ in a seamless cloning reaction, resulting in pME4593. The Δ*flbC* cassette was excised from the plasmid and integrated into AGB551, AGB1007 and AGB1008, resulting in AGB1039, AGB1040 and AGB1041.

2.5.5.12 Construction of plasmids and strains: Δ*flbD* and the *flbD*/*ztfA* double mutants in *A. nidulans*

1.1 kb of the 5' region adjacent to the *flbD* ORF was amplified with kt523/524. 1.2 kb of the respective 3' region was amplified with kt525/526. Both fragments together with the phleoRM cassette were cloned into pBluescript SK+, resulting in pME4595. The Δ*flbD* cassette was excised from the plasmid and integrated into AGB551, AGB1007 and AGB1008, resulting in AGB1043, AGB1044 and AGB1045, respectively.

2.5.5.13 Construction of plasmids and strains: Δ*flbE* and the *flbE*/*ztfA* double mutants in *A. nidulans*

1.3 kb of the 5' region adjacent to the *flbE* ORF was amplified with kt527/528. 1.1 kb of the respective 3' region was amplified with kt529/530. Both fragments together with the phleoRM cassette were cloned into pBluescript SK+, resulting in pME4597. The Δ*flbE* cassette was excised from the plasmid and integrated into AGB551, AGB1007 and AGB1008, resulting in AGB1047, AGB1048 and AGB1049.

2.5.5.14 BiFC plasmid and strain construction for interaction studies of ZtfA with RcoA in *A. nidulans*

For BiFC studies one half of a split *yfp* was fused to *ztfA*, resulting in *ztfA*::*ceyfp*, and the other half was fused to *rcoA*, leading to *rcoA*::*neyfp*. Both gene fusions were set under the control of the bidirectional nitrate-inducible ^{P}*niiA*/^{P}*niaD* promoter and terminators and integrated ectopically in respective mutants. All BiFC plasmids constructed in this study were sequenced with additional primers kt268, kt372, kt373, kt424, kt425, kt426 and kt427.

For these constructs, *ztfA* was amplified from cDNA from vegetatively grown WT cultures (instead of genomic DNA) with primers kt407/415. *ceyfp* was amplified from pME3741 with primers kt416/417 and *neyfp* with primers kt421/422. *rcoA* was amplified from cDNA with primers kt409/418. pJG137 was utilized as backbone vector, which was digested in a two-step digestion with *Mss*I and *Smi*I to excise the bidirectional nitrate-inducible promoter, which was

reintroduced as fragment amplified with the primers kt300/302. Prior to utilization of a seamless cloning reaction to clone the fragments together, resulting in pME4599, *ztfA* and *rcoA* were fused to their respective *eyfp* parts by fusion PCR (Szewczyk *et al.*, 2006). pME4599 was ectopically integrated into AGB1007 resulting in AGB1051 and AGB1014, resulting in AGB1052. As controls for BiFC experiments both genes fused to the same half of the split *eyfp* used for pPME4599 were cloned into pJG137 as backbone together with the nitrate-inducible promoter and the respective other half of the split *eyfp* without a gene connected to it. For the free half of the *eyfp* primers introduced a start codon to allow for free eYFP expression. Free *ceyfp* was amplified with primers kt416/SR195 and cloned in a seamless cloning reaction with *rcoA*::*neyfp* and the bidirectional nitrate-inducible promoter into pJG137, resulting in pME4601. pME4601 was introduced into AGB551 and AGB1014, resulting in AGB1054 and AGB1056, respectively. Free *neyfp* was amplified with primers kt422/SR193 and cloned in a seamless cloning reaction into pJG137 together with *ztfA*::*ceyfp* and the nitrate-inducible promoter, resulting in pME4600. pME4600 was introduced into AGB551 and AGB1014, resulting in AGB1053 and AGB1055 respectively.

2.5.5.15 Construction of plasmids and strains for *velvet*/*ztfA* double mutant strains of *A. nidulans*

The 2.4 kb 5' region of *velC* was amplified with primers kt203/145 and the 2.1 kb 3' region was amplified with primers kt146/204. Both sequences together with the phleoRM cassette were cloned into pBluescript SK+, resulting in pME4602. The deletion construct was excised from the plasmid and integrated into AGB551 resulting in AGB1062. To obtain a double deletion of *velC* and *ztfA*, the *ztfA* deletion cassette from pME4575 was integrated into AGB1062, resulting in AGB1063.

pME4603 (Δ*vosA*, harbors the natRM marker; S. Thieme, p.c.) was integrated into AGB1007 and AGB1008, resulting in AGB1058 and AGB1059, respectively.

The *ztfA* deletion cassette from pME4575 was integrated into AGB1066 (Δ*veA*::*six*; J. Gerke, p.c.) to obtain the Δ*veA*Δ*ztfA* strains (AGB1067) and into AGB1064 (Δ*velB*::*six*; S. Thieme, p.c.) to obtain the Δ*velB*Δ*ztfA* strain (AGB1065).

2.5.5.16 Plasmid for Δ*ztfA* and strain construction in *A. fumigatus*

2 kb of the *ztfA* 5' flanking region from *A. fumigatus* were amplified with the primer pair kt215/221 and 2 kb from the respective 3' flanking region were amplified with the primer pair kt218/226. Both fragments and the ptrARM were cloned into pBluescript SK+, resulting in

pME4607. The Δ*ztfA* cassette was excised from the plasmid and integrated into Afs35, resulting in AfGB129.

2.6 Genetic manipulation of microorganisms

2.6.1 Transformation of fungi

A. nidulans and *A. fumigatus* were transformed by polyethylene glycol-mediated protoplast fusion as described before (Punt and van den Hondel, 1992). For all genetic modifications in *A. nidulans*, AGB551 (Bayram *et al.*, 2012) or AGB551 derived strains were used as transformation hosts and AGB551 was used as WT. AfS35 (Krappmann *et al.*, 2006b) was used as WT and transformation host of the *A. fumigatus* Δ*ztfA* strain. All transformation hosts used in this study (AGB551 and AfS35 and their derivatives) harbor the Δ*nkuA* and Δ*akuA* mutation, respectively. Loss of these orthologous genes remarkably increases homologous recombination during transformation and results in on-locus integration of linearized genetic constructs (Krappmann *et al.*, 2006b; Nayak, 2005).

Host strains were grown o/n in submerged cultures on a rotary shaker at 37°C. Mycelia were harvested through sterile Miracloth filters (MERCK) and washed with sterile citrate buffer (150 mM KCl, 580 mM NaCl, 50 mM Na-citrate pH 5.5). Mycelia were transferred into sterile filtered protoplastation solution (30mg/ml Vinoflow® Max or Vinotaste® Pro from NOVOZYMES (Bagsvaerd, Denmark) and 15 mg/ml lysozyme (SERVA), dissolved in citrate buffer and sterile filtered through 0.2 µm filters (SARSTEDT)) and incubated for 100 min at 30°C under constant agitation to allow for protoplastation. Formation of protoplasts was monitored by microscopy. Protoplasts were filtered through sterile Miracloth filters and collected in pre-cooled sterile 50 ml centrifuge tubes (SARSTEDT), filled up to 50 ml with ice cold STC 1700 buffer (1.2 M sorbitol, 10 mM Tris pH 5.5, 50 mM CaCl$_2$, 35 mM NaCl) and chilled on ice for 10 min. Subsequently, protoplasts were centrifuged at 2600 rpm at 4°C for 12 min and washed with ice cold STC1700 and this step was repeated. Protoplasts were incubated with approximately 10 µg of respective DNA constructs (either linearized by excision from respective plasmids, or in circular form in case of pME3173, pME4599, pME4600 and pME4601) for 30 min on ice. 1.35 ml sterile PEG solution (10 mM Tris pH 7.5, 50 mM CaCl$_2$, 60% (v/v) PEG4000) was added successively in three steps to increase DNA uptake of protoplasts and they were incubated for another 40 min over the ice. Subsequently, protoplasts were centrifuged at 2600 rpm for 12 min and distributed on freshly prepared solid MM plates, supplemented with 1.2 M sorbitol and respective selecting agents

(pyrithiamine 1:1000, nourseothricin 0.7:1000, phleomycin 1:1000). Transformed clones were picked after three to seven days and individualized on selective MM plates. Successful transformation of constructs into *A. nidulans* and *A. fumigatus* hosts was verified by Southern hybridization. Recyclable marker cassettes were eliminated from the genome of respective mutants by singularizing clones on MM/xylose plates (0.5% (w/v) glucose, 0.5% (w/v) xylose, 7 mM KCl, 2 mM MgSO$_4$, 70 mM NaNO$_3$, 11.2 mM KH$_2$PO$_4$, 0.1% (v/v) trace element solution pH 5.5) (Hartmann *et al.*, 2010). Successful marker recycling was monitored by Southern hybridization.

2.6.2 Transformation of bacteria

E. coli transformations were carried out as described in Hanahan *et al.*, 1991; Inoue *et al.*, 1990. Briefly, chemi-competent *E. coli* cells were incubated with plasmid DNA for 30 min on ice and subsequently heat shocked at 42°C to allow plasmid uptake. Heat shocked *E. coli* cells were cooled on ice for one to two minutes, 600 µl LB was added and cultures were shaken for 30 to 60 min at 37°C on a rotary shaker. That followed, *E. coli* cells were harvested by centrifugation and inoculated on solid LB plates supplemented with 1:1000 ampicillin to prevent plasmid loss and allow for selection of clones, which successfully took up the plasmid, and grown o/n at 37°C. *E. coli* clones were screened for successful uptake of constructs via PCR amplification of fragments specific to respective plasmids (colony PCR).

2.7 Southern hybridization

Southern hybridization was employed to confirm successful mutagenesis of genetic loci (Southern, 1975). Restriction enzymes (THERMO FISHER SCIENTIFIC) were utilized according to manufacturer's instructions. Genomic DNA of respective fungal mutant strains was digested with restriction enzymes o/n, which were chosen the way that resulting DNA fragments span parts of the respective mutated locus and at least one restriction site was outside of the integrated construct to confirm on-locus integration. Moreover, restriction enzymes were chosen according to the premise that respective DNA fragments show clear size differences between mutants and the WT when separated by agarose gel electrophoresis. After separation of DNA fragments according to size by agarose gel electrophoresis, gels were washed for 10 min in wash buffer 1 (0.25 M HCl), followed by washing with buffer 2 for denaturation (0.5 M NaOH, 1.5 M NaCl) for 25 min and 30 min in buffer 3 (0.5 M Tris, 1.5 M NaCl, pH 7.4) for neutralizing. All washing steps were performed under constant

agitation at room temperature (rt). Subsequently, DNA was transferred onto Amersham^{TM} Hybond^{TM}-N nylonmembranes (GE HEALTHCARE) by dry blotting for 2 h at rt. Membranes were subsequently dried at 75°C for 10 min and DNA was cross-linked to the membrane by UV light exposure (λ = 254 nm) for 3 min per side. Membranes were pre-hybridized in hybridization solution from the Amersham^{TM} Gene Images AlkPhos Direct Labelling and Detection Sytem (GE HEALTHCARE, prepared after manufacturer's instructions) for 30 min at 55°C in a HERA hybrid R hybridization oven (HERAEUS INSTRUMENTS) prior to application of the DNA probe. DNA probes were prepared with the aforementioned kit according to manufacturer's instructions (GE HEALTHCARE). Hybridization of the membranes with the respective DNA probes was performed o/n at 55°C. Subsequently, membranes were washed twice in post-hybridization buffer I (1 mM MgCl2, 3.5 mM SDS, 50 mM sodium phosphate buffer, 150 mM NaCl, 2 M Urea, 0.2% (w/v) blocking reagents) for 10 min at 55°C and twice in post-hybridization buffer II (2 mM $MgCl_2$, 50 mM Tris, 100 mM NaCl, pH 10) for 5 min at rt under constant agitation. For detection of DNA bands CDP-Star (GE HEALTHCARE) was applied and membranes were exposed to Amersham^{TM} Hyperfilm^{TM} ECL (GE HEALTHCARE).

2.8   Secondary metabolite extraction

2.8.1   Sterigmatocystin isolation

The isolation of sterigmatocystin and thin layer chromatography (see next chapter) was performed as described (Bayram *et al.*, 2008a). $1*10^5$ spores were point inoculated on solid MM and grown for three to seven days at 37°C in light or dark. Colonies or colony centers were cut out with a 50 ml centrifuge tube (SARSTEDT) and the resulting agar plug was cut into small pieces. Agar pieces were shaken in 50 ml Falcon tubes and six small glass bullets in 3 ml H_2O for 30 min at rt. Subsequently, 3 ml chloroform was added and samples were shaken for another 30 min at rt. After this, tubes were centrifuged 10 min at 1000 rpm to separate phases and the lower chloroform phase was transferred into glass tubes and evaporated o/n at rt under the hood.

2.8.2   Thin layer chromatography

Sterigmatocystin samples were resuspended in 50 µl methanol and 15 µl of isolated sterigmatocystin per sample was applied to pre-coated SIL G/UV254 Polygram$^®$ DC-foil TLC-sheets (MACHEREY-NAGEL) (thin layer chromatography plates) in three steps of 5 µl to prevent distribution over the TLC plate. TLC plates were run in 1:4 (v/v) acetone:chloroform

for 40-50 min and photographed at 366 and 254 nm with a Camag TLC Visualizer 2 system from CAMAG (Muttenz, Switzerland) after 5 min drying at rt. That followed, TLC plates were sprayed with 20% (v/v) aluminum chloride in 95% (v/v) ethanol and baked at 70°C for 10 min. Developed plates were photographed again at 366 and 254 nm with a Camag TLC Visualizer 2 system and processed with the winCATS 1.4.4 software (CAMAG).

2.8.3 Secondary metabolite isolation for HPLC measurements

Procedure was followed as described by Gerke and collaborators for the extraction of SMs from vegetatively grown cultures (Gerke *et al.*, 2012b). $1*10^7$ spores were grown vegetatively for 48 h at 37°C on a rotary shaker and mycelia were removed. Remaining media were adjusted to pH 5 with HCl, equal amounts of ethyl acetate were added and media and ethyl acetate were mixed in a shaking flask to extract secondary metabolites. The formed water phase was discarded and the ethyl acetate was transferred into round bottom flasks and evaporated in a Hei-VAP-Advantage rotary evaporator from HEIDOLPH INSTRUMENTS GMBH & CO. KG (Schwabach, Germany) with a MWG Lauda RM6 from LAUDA-BRINKMANN LP (Delran, NJ USA) and a Laboxact KNF vacuum system (SIGMA-ALDRICH) at 37°C under constant gyration.

For extraction of secondary metabolites from asexually and sexually grown cultures $1*10^6$ spores were plated and grown for three or seven days under asexual or sexual development promoting conditions. Subsequently, fungal cells were washed off with cotton swabs and 0.96% (w/v) NaCl solution, containing 0.0002% (v/v) Tween. The agar was cut into small pieces and transferred into flasks, covered with 300 ml ethyl acetate and shaken at 160 rpm at 30°C for 30 min followed by 15 min ultra-sonication in a Bandelin Sonorex™ Digital 10P ultrasonic bath from BANDELIN ELECTRONIC GMBH & CO.KG (Berlin, Germany) at highest level. Ethyl acetate was transferred to round bottom flasks and evaporated in a rotary evaporator at 37°C under constant gyration.

Secondary metabolites were resolved in 3 ml methanol by swirling and transferred into small glass tubes. Methanol was evaporated in a rotary evaporator at 37°C under constant gyration and samples were stored at -20°C. Samples were resolved in 500 µl methanol, centrifuged and 250 µl taken for measurements with high-performance liquid chromatography (HPLC).

2.8.4 Secondary metabolite analysis by high-performance liquid chromatography (HPLC) coupled with a UV diode array detector (UV-DAD)

HPLC measurements were executed by Dr. Jennifer Gerke (Department of Molecular Microbiology and Genetics, Georg-August University Göttingen, Germany).

Analytical HPLC/UV-DAD measurements were performed using the following system: HPLC pump 420, SA 360 autosampler, Celeno UV-DAD HPLC detector, ELSD-Sedex 85 evaporative light-scattering detector (ERC)) with a Nucleodur 100-5 C18 end-capped (ec) column (250 mm x 3 mm) and the solvent system: A = H_2O + 0.1% (v/v) trifluoroacetic acid (TFA), B = acetonitrile + 0.1% (v/v) TFA (from GOEBEL INSTRUMENTELLE ANALYTIK GMBH, Au/Hallertau, Germany). Secondary metabolite extracts were dissolved in 500 µl methanol and an injection volume of 20 µl was analyzed under gradient conditions (20% B to 100% B in 20 minutes) with a flow rate of 0.5 ml/min.

HPLC data was analyzed with the Geminyx III software from GOEBEL INSTRUMENTELLE ANALYTIK GMBH (Au/Hallertau, Germany).

2.9 Protein methods

2.9.1 Protein isolation

Strains were grown under vegetative conditions. For protein isolation from asexually or sexually grown cultures, cultures were grown vegetatively for 22 h and subsequently shifted onto solid MM plates and grown asexually or sexually for 12 h. Mycelia were harvested through sterile filter (MIRACLOTH), washed with 0.96% (v/v) sterile NaCl supplemented with 1 mM PMSF and 1% (v/v) DMSO and subsequently mycelia were frozen in liquid nitrogen. Frozen mycelia were ground in liquid nitrogen with a MM400 table mill (RETSCH) and approximately 200 mg was mixed with 300 µl B^+ buffer (300 mM NaCl, 100 mM Tris pH 7.5, 10% (v/v) glycerol, 1 mM EDTA, 0.1% (v/v) NP-40) supplemented with 1.5 mM DTT, 1 tablet/50 ml complete EDTA-free protease inhibitor cocktail (ROCHE), 1 mM PMSF, phosphatase inhibitor mix (1 mM NaF, 0.5 mM sodium-orthovanadate, 8 mM ß-glycerolphosphate disodium pentahydrate and 1.5 mM benzamidine) and centrifuged for 15 min at 13000 rpm at 4°C. Supernatant was transferred into fresh test tubes and protein concentration was measured with a NanoDrop ND-1000 spectrophotometer (PEQLAB). Concentration of samples were adjusted to same values with B^+ buffer and samples were mixed with 3x SDS sample buffer (250 mM Tris-HCl pH 6.8, 15% (v/v) β-mercaptoethanol, 30% (v/v) glycerol, 7% (v/v) SDS, 0.3% (w/v) bromphenol blue) and boiled at 95°C for 5 min

followed by 5 min incubation on ice. Samples were either used directly for further experiments or stored at -20°C.

2.9.2 SDS-PAGE and western hybridization

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to separate proteins according to size for western hybridization (Laemmli, 1970; Schinke *et al.*, 2016). Equal amounts of protein, which were determined with a NanoDrop ND-1000 photospectrometer (Thermo Fisher Scientific), were loaded on 10% SDS gels (separation gel: 2.8 ml H_2O, 3.75 ml 1 M Tris pH 8.8, 100 µl 10% (w/v) SDS, 3.3 ml 30% (v/v) acrylamide, 10 µl TEMED, 50 µl 10% (w/v) APS; stacking gel: 3.67 ml H_2O, 625 µl 1 M Tris pH 6.8, 30 µl 10% (w/v) SDS, 650 µl 30% (v/v) acrylamide, 5 µl TEMED, 25 µl 10% (w/v) APS) and separated according to size at 200 V in running buffer (25 mM Tris, 0.25 M glycine, 0.1% (w/v) SDS). Proteins from SDS gels were blotted for 1 h at 100 V on Amersham^{TM} Protran^{TM} 0.45 µm NC nitrocellulose membranes (GE Healthcare) in ice cooled transfer buffer (25 mM Tris, 192 mM glycine, 0.02% (w/v) SDS) or at 35 V o/n at rt in transfer buffer (Towbin *et al.*, 1979). Membranes were blocked with 5% (w/v) skim milk powder dissolved in TBST buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween 20) for 1 h at rt and subsequently probed with 1:250 mouse α-GFP antibody (sc-9996, SANTA CRUZ BIOTECHNOLOGY, Dallas, TX, USA) in TBST-M (TBST buffer, supplemented with 5% (w/v) skim milk powder) and incubated o/n at 4°C. That followed, membranes were washed three times in TBST for 10 min under constant agitation at rt and incubated for 1 h with 1:1000 horseradish peroxidase coupled goat α-mouse antibody (115-035-003, JACKSON IMMUNO RESEARCH, West Grove, CA, USA) as secondary antibody in TBST-M. Subsequently, membranes were washed for three times 10 min with TBST under constant agitation at rt. That followed, membranes were covered with a 1:1 (v/v) mixture of solution A (2.5 µM luminol, 400 µM paracoumarat, 100 mM Tris-HCl pH 8.5) and solution B (5.4 mM H_2O_2, 100 mM Tris-HCl pH 8.5) and incubated for 5 min under constant agitation at rt in the dark (Suck and Krupinska, 1996). Chemiluminescent signals were detected with a Fusion-SL7 chemiluminescence detection system (PEQLAB) and pictures were recorded with the Fusion 15.15 software from VILBER LOURMAT (Marne-la-Vallée cedex 3, France). As loading control membranes were stained with Ponceau staining (Romero-Calvo *et al.*, 2010).

2.9.3   GFP-trap pull-downs

Protein pull-downs employing GFP-Trap®_A beads from CHROMOTEK (Planegg-Martinsried, Germany) were conducted as described earlier (Jöhnk *et al.*, 2016). *A. nidulans* strains were inoculated in a concentration of $5*10^8$ spores in 500 ml MM and grown vegetatively for 24 h (vegetative samples), or for 22 h vegetatively and subsequently mycelia were shifted onto solid agar plates and grown for 12 h in light (asexual samples) or in the dark and sealed with Parafilm® to induce sexual development (sexual samples). Mycelia were harvested and immediately frozen in liquid nitrogen. Frozen mycelia were ground with a MM400 table mill (RETSCH) in liquid nitrogen. 5 ml ground mycelia were mixed with B^+ buffer in a relation of 1:1 (v/v) and centrifuged twice for 20 min at 4000 rpm at 4°C. Supernatant was filtered through 0.2 µm sterile filters (SARSTEDT) and mixed 1:100 with GFP-Trap®_A beads (CHROMOTEK) and incubated o/n rotating at 4°C. Subsequently, GFP-Trap®_A beads were washed twice with freshly prepared B^+ buffer and transferred into 1.5 ml reaction tubes. GFP-Trap®_A beads were centrifuged at 3000 rpm at rt for 1 min and subsequently boiled in 50 µl 3x SDS sample buffer at 95°C for 10 min. Protein extracts were stored at -20°C until further processing.

2.9.4   Bioinformatic analyses of protein features

The InterPro database (Finn *et al.*, 2016) was searched using InterProScan (https://www.ebi.ac.uk/interpro/search/sequence-search) (Jones *et al.*, 2014) to predict protein domains. The presence of putative orthologs of ZtfA in other fungi was analyzed *in silico* with the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1990). Orthologs were investigated in pairwise sequence alignments using EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) (Li *et al.*, 2015; McWilliam *et al.*, 2013; Rice *et al.*, 2000). Putative nuclear localization sequences were searched with cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) (Kosugi *et al.*, 2009) and NucPred (http://www.sbc.su.se/~maccallr/nucpred/cgi-bin/single.cgi) (Brameier *et al.*, 2007). LocNES (http://prodata.swmed.edu/LocNES/LocNES.php) (Xu *et al.*, 2015) and NetNES 1.1 (http://www.cbs.dtu.dk/services/NetNES/) (La Cour *et al.*, 2004) were employed to identify nuclear export signals *in silico*. Phosphorylation sites were determined *in silico* with NetPhos 3.1 (http://www.cbs.dtu.dk/services/NetPhos/) (Blom *et al.*, 1999). The cutoff was set to 0.7 (score values from 0 to 1).

2.10 Identification of proteins from GFP pull-downs with mass spectrometry

2.10.1 Tryptic protein digestion

Protein LoBind Tubes PCR Clean from EPPENDORF AG (Hamburg, Germany) were used for the whole procedure.

Tryptic digestion of proteins was performed as published by Shevchenko and collaborators using Sequencing Grade Modified Trypsin (PROMEGA) (Shevchenko *et al.*, 1996). Briefly, protein samples were separated according to size on a 10% SDS gel (see CHAPTER 2.9.2). Complete lanes were excised and cut into small pieces of approximately 2 mm. Gel pieces were shaken in acetonitrile for 10 min at rt and dried in a SpeedVac Concentrator (THERMO FISHER SCIENTIFIC). That followed proteins were reduced in-gel by incubating the gel pieces in 10 mM DTT in 100 mM NH_4HCO_3 at 56°C for 1 h. Subsequently, the DTT solution was exchanged with 55 mM iodoacetamid in 100 mM NH_4HCO_3 to allow alkylation of reduced cysteine residues, and the samples were incubated for 45 min in the dark. Afterwards, the gel pieces were washed with 100 mM NH_4HCO_3 for 10 min and dehydrated in acetonitrile for 10 min. This procedure was repeated and the gel pieces were dried in a SpeedVac Concentrator (THERMO FISHER SCIENTIFIC) at 50°C. That followed, the gel pieces were covered with trypsin-digestion buffer (PROMEGA; prepared according to manufacturer's specifications) and incubated on ice for 45 min, followed by an incubation in 25 mM NH_4HCO_3 o/n at 37°C. Following, supernatants were collected into new reaction tubes and the gel pieces were covered with 20 mM NH_4HCO_3 and incubated for 10 min at rt for the extraction of acidic peptides. Supernatants were collected and the gel pieces were incubated in 50% (v/v) acetonitrile and 5% (v/v) formic acid and incubated for 20 min at rt. Subsequently, supernatants were collected. This procedure was repeated three times to extract remaining peptides. The combined supernatants were dried completely in a SpeedVac Concentrator (THERMO FISHER SCIENTIFIC). Peptides were resolved in 20 µl resuspension buffer (98% H_2O, 2% (v/v) acetonitrile, 0.1% (v/v) formic acid) and incubated in an ultrasonic bath at 35°C for 3 min at maximum power.

2.10.2 C18 StageTip purification of trypsin-digested samples

Protein LoBind Tubes PCR Clean from EPPENDORF AG (Hamburg, Germany) were used for the whole procedure.

Prior to measurement with LC-MS/MS, peptides were purified from salts and other contaminations, using the StageTip purification method (Rappsilber *et al.*, 2003, 2007). For

this, C18 (reversed-phase material) stage tips were prepared by introducing C18 plugs into 200 µl pipet tips. StageTips were equilibrated with 100 µl of 0.1% (v/v) formic acid in HPLC grade methanol, followed by 100 µl of 0.1% (v/v) formic acid in 70% (v/v) acetonitrile and 100 µl of 0.1% (v/v) formic acid in dH_2O. The last step was repeated. Peptides resolve in resuspension buffer were loaded onto the StageTips and centrifuged 5 min at 3500 rpm. This was repeated. Subsequently, the StageTips were washed twice with 100 µl of 0.1% (v/v) formic acid in dH_2O and peptides were eluted with 60 µl of 70% (v/v) acetonitrile containing 0.1% (v/v) formic acid after incubation for 5 min. Peptides were dried completely in a SpeedVac Concentrator (THERMO FISHER SCIENTIFIC) at 50°C.

For mass spectrometry, peptides were resolved in 20 µl resuspension buffer (98% H_2O, 2% (v/v) acetonitrile, 0.1% (v/v) formic acid) and incubated in an ultrasonic bath at 35°C for 3 min at maximum power.

2.10.3 LC-MS/MS identification of proteins and protein phosphorylation

Mass spectrometry was performed by Dr. Oliver Valerius (Department of Molecular Microbiology and Genetics, Georg-August University Göttingen, Germany) utilizing an Orbitrap Velos Pro (THERMO FISHER SCIENTIFIC) as described (Jöhnk *et al.*, 2016; Kleinknecht *et al.*, 2016; Lin *et al.*, 2015; Schinke *et al.*, 2016).

Liquid chromatography-coupled mass spectrometry was done using the Orbitrap Velos Pro mass spectrometer and the RSLCnano Ultimate 3000 chromatography system (THERMO FISHER SCIENTIFIC). Peptides of proteins hydrolyzed by trypsin and purified with C18 stage tips were separated at nano-flow with Acclaim PepMap RSLC columns (THERMO FISHER SCIENTIFIC) through a water-acetonitrile gradient. Online ionization of eluting peptides through nano-electrospray was achieved by the use of the Nanospray Flex Ion Source (THERMO FISHER SCIENTIFIC). Full scans within the mass range of 300-1850 were recorded with the Orbitrap-FT analyzer at a resolution of 30.000. In parallel data-dependent top ten collision-induced dissociation (CID) in the LTQ Velos Pro linear ion trap took place. For phosphopeptide analyses precursor peptides were either CID fragmented in the multi-stage activation mode in the linear trap (MSA) or with higher-energy collisional dissociation (HCD) within the C-trap. The XCalibur^{TM} 2.2 software (THERMO FISHER SCIENTIFIC) was used for LC-MS method programming and the mass spectra acquisition. MS/MS2 data processing for peptide analysis and protein identification was performed either with the MaxQuant 1.5.1.0 and Perseus 1.5.3 or the Proteome Discoverer 1.4 software (THERMO SCIENTIFIC) using the SequestHT and the Mascot search engines. As protein database an *A. nidulans* specific

database with common contaminants was used. For further details see the LC-MS analysis section of Materials and Methods in the recent publication from Schmitt and co-workers (Schmitt *et al.*, 2017).

2.11 Spore viability assay

Viability of spores over time was analyzed as described (Ni and Yu, 2007; Sarikaya-Bayram *et al.*, 2010). Conidia were harvested after two days and counted with a Coulter Z2 particle counter (BECKMAN). $1*10^5$ spores were plated on solid MM plates, supplemented with 1.2 M sorbitol. Conidia were harvested after two, five and ten days and 200 spores were spread on solid MM plates and grown at 37°C in light. This test was performed in triplicates per experimental day. Colony formation was monitored after two days and calculated as ratio of the number of growing colonies to the number of inoculated spores.

2.12 Trehalose assay

Trehalose assays were performed as described (d'Enfert and Fontaine, 1997; Ni and Yu, 2007; Sarikaya-Bayram *et al.*, 2010). $1*10^7$ spores per strain were grown for two days on solid MM at 37°C in light. $1*10^7$ conidiospores were harvested and washed in dH$_2$O. Subsequently, $1*10^8$ conidiospores per strain were resuspended in 200 µl dH$_2$O and boiled at 95°C for 20 min to allow for trehalose extraction from spores. Spores were collected by centrifugation, supernatants were transferred into fresh reaction tubes and mixed with equal amounts of 0.2 M sodium citrate pH 5.5 and incubated for exactly 8 h at 37°C with and without 3 mU trehalase (SIGMA-ALDRICH). Glucose amounts generated by the trehalase from trehalose were assayed by employing the Glucose (GO) Assay Kit (SIGMA-ALDRICH) according to manufacturer's instructions. Glucose amounts from untreated samples were deducted from the amounts from trehalase treated samples.

2.13 Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described (Ahmed *et al.*, 2013). DNA probes were generated by annealing the reverse-complementary oligonucleotide pair kt379/380 with the sequences `GACTTTCCTCCGCGGACGCCGCGTCGATTTTAG/CTAAAATCGACGCGGCGTCCGCGGAGGAAAGTC`. GST-VosA purified protein (Ahmed *et al.*, 2013) in indicated concentrations was mixed with

1200 ng DNA and incubated for 15 min at rt in 2 µl HEPES/NaCl buffer (10 mM HEPES pH 7.4, 150 mM NaCl). The protein-DNA mix was mixed with 4x sample buffer (40% (w/v) glycerol in 0.25 M Tris-HCl, pH 6.8) and dispersed according to size on a native 6% (v/v) polyacrylamide gel in TBE running buffer (45 mM Tris-borate, 1 mM EDTA) prior to staining with ethidium bromide. As control the gel was stained with Coomassie brilliant Blue G-colloidal staining (SIGMA-ALDRICH). Prior to the staining, gels were incubated for 1 h under constant agitation in fixation solution (40% (v/v) ethanol, 10% (v/v) acetic acid) (Schinke *et al.*, 2016), followed by rinsing with dH$_2$O for several times. Fixation solution was subsequently utilized for destaining.

2.14 Microscopy

Photomicrographic images were obtained by utilization of an Axiolab microscope (CARL ZEISS MICROSCOPY GMBH, Jena, Germany) and a SZX12-ILLB2-200 binocular microscope (OLYMPUS). Both systems were equipped with a SC30 digital camera (OLYMPUS). Pictures were processed with the cellSens Dimension 1.4 software (OLYMPUS).

For monitoring conidiophore development in liquid cultures, strains were grown in submerged cultures for 18 h and mycelial balls were transferred onto microscopic slides. For analyses of premature conidiophore development, strains were grown for 14 h in LM and transferred into liquid MM via filtration trough sterile Miracloth filters (MERCK) and grown for 9 h in liquid MM under vegetative conditions (see Chapter 2.2.2).

Fluorescence microscopy was performed with a Zeiss AxioObserver Z.1 inverted confocal microscope, equipped with Plan-Neofluar 63x/0.75 (air), Plan-Apochromat 63x/1.4 oil and a Plan-Apochromat 100x/1.4 oil objectives (ZEISS) and a QuantEM:512SC camera (PHOTOMETRICS, Tucson, AZ, USA). Pictures were processed with the SlideBook 6.0 software package (INTELLIGENT IMAGING INNOVATIONS GMBH, Göttingen, Germany).

For fluorescence microscopy 2000 spores per strain were inoculated in 8-well borosilicate cover glass system (THERMO FISHER SCIENTIFIC) in 400 µl liquid MM for vegetative growth or on glass slides for microscopy, covered with 1 ml solid MM for asexual and sexual growth at 37°C or 30°C. Cultures were incubated for 24 to 36 h. Fluorescence values of WT background fluorescence were subtracted from strains expressing GFP-fusion proteins to allow for normalization of fungal auto fluorescence. Nuclei were visualized by ectopic integration of ^{P}*gpdA::rfp::h2A* (pME3173) into respective strains or via staining with 0.1%

(w/v) 4',6'-diamidino-2-phenylindole (DAPI) (ROTH) and incubation for 20 min at 37°C prior to microscopy.

2.15   Isolation of polysaccharides of *A. fumigatus*

Polysaccharides were extracted as described (Fontaine *et al.*, 2011; Gravelat *et al.*, 2013). $5*10^7$ conidiospores per strain were inoculated in 100 ml modified Brian-medium (20 g/L asparagine, 2.4 g/L NH_4HCO_3, 10 g/L KH_2PO_4, 2g/L $MgSO_4*7\ H_2O$, 26 mg/L $ZnSO_4*7\ H_2O$, 2.6mg/L $CuSO_4*5\ H_2O$, 1.3 mg/L $CoCl_2*6\ H_2O$, 65 mg/L $CaCl_2$, pH5.4, supplemented with 5% (w/v) glucose after sterilization) and grown for 24 h at 37°C on a rotary shaker. Mycelia were removed by filtration trough Miracloth filters (MERCK) and flow through was transferred into 500 ml bottles and mixed with 250 ml 70% (v/v) ethanol to precipitate polysaccharides. To increase precipitation yields, bottles were shaken o/n at 4°C. The liquid was transferred into centrifugation tubes and centrifuged in a Sorvall RC-3B Plus Refrigerated Centrifuge (THERMO FISHER SCIENTIFIC), supernatant was discarded and precipitated polysaccharides were dried o/n at rt. Weight of the centrifugation tubes was determined prior and after polysaccharide precipitation to calculate weight of total precipitated polysaccharides.

2.16   Fungal stress tests

Stress tests with 1% (v/v) H_2O_2 or 1% (w/v) sodium dodecyl sulfate (SDS) dissolved in dH_2O were carried out as described (Lessing *et al.*, 2007). $1*10^7$ spores were mixed with 25 ml MM with 2% (w/v) agar and supplemented with 10 µg/ml doxycycline to induce *ztfA* expression, when needed, shortly before solidification (Helmschrott *et al.*, 2013). This allows pouring plates, which incorporate evenly distributed spores in the agar. After solidification, an agar plug was removed by excision with a 15 ml centrifugation tube and the hole was filled with 150 µl of the respective stress inducing agent in indicated concentrations. Strains were grown for 2 d at 37°C in light (*A. nidulans*) or dark (*A. fumigatus*) and inhibitions zones were measured.

2.17   *A. fumigatus* infection assay with *Galleria mellonella* larvae

G. mellonella larvae were infected as described (Renwick *et al.*, 2006). Larvae were obtained from FAUNA TOPICS GMBH (Marbach am Neckar, Germany) and directly employed upon arrival to ensure maximal initial health. Larvae were infected in groups of 12 individuals with

$8*10^6$ spores in 20 µl sterile 0.96% (w/v) NaCl, supplemented with 0.002% (v/v) Tween-80 (SIGMA-ALDRICH) per strain. 10 µg/ml rifampicin was added to prevent infection with other microorganisms. Per experimental repetition, 12 larvae were mock infected with NaCl Tween solution and six larvae were left untreated to monitor general health of the animals and to ensure that neither the infection procedure nor the storage conditions were responsible for observed mortality. Micro-Fine^{TM}+ 0.3 ml insulin syringes (BD BIOSCIENCES) were utilized for inoculation and sterilized with 100% ethanol after each treatment and discarded after infection of three individuals to decrease contamination risk. Larvae were kept at 30°C in petri dishes, separated according to the fungal strain they were infected with, and with litter they came in. Survival was monitored at least daily. Moribund larvae were suspended when no movement was observable upon contact and dark discoloration was observable. Suspended larvae were sacrificed at -20°C prior to autoclave sterilization.

3. Results

3.1 *AN0585/ztfA* encodes the putative Zinc cluster transcription factor ZtfA

3.1.1 The *AN0585* gene product is a $Zn(II)_2Cys_6$ fungal transcription factor

The velvet domain transcription factor VosA binds to promoters of approximately 1500 genes (Ahmed *et al.*, 2013). Amongst these putative VosA targets are several so far uncharacterized genes. By employment of UV-mediated random DNA damage Jørgensen and collaborators generated a mutant strain (*scl-2*), which showed reduced asexual sporulation and the formation of sclerotic-like structures in *Aspergillus niger* that correspond to cleistothecia in *A. nidulans* (Jørgensen *et al.*, 2011). Velvet proteins are fungal master regulators of developmental programs and secondary metabolism and VosA specifically negatively regulates the major conidiation activator-encoding *brlA* gene. The gene corresponding to the *scl-2* mutant phenotype could therefore be a downstream factor of one of the velvet proteins. The *A. niger* gene later was identified as *An08g07710* but so far, no further research has been conducted to characterize it (A.F.J. Ram, personal communication). The putative ortholog in *A. nidulans*, *AN0585*, is among the genes regulated by the velvet factor VosA (Ahmed *et al.*, 2013).

The *AN0585* open reading frame (ORF) comprises 1730 nucleotides with one intron of 59 nucleotides (FIGURE 13). The gene product is a protein of 556 amino acids with a predicted molecular mass of 60.3 kDa. The AN0585 protein shows an amino acid sequence similarity of 65.2% to An08g07710 of *A. niger*, 55.4% to Afu6g11110 of *A. fumigatus* and 63.2% to AO090023000506 of *A. oryzae* in pairwise sequence alignments carried out with EMBOSS Needle (Li *et al.*, 2015; McWilliam *et al.*, 2013; Rice *et al.*, 2000). Sequence similarity indicates percentage of matches between two sequences. Further putative orthologs among Aspergilli were found in searches employing the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) for *A. flavus*, *A. terreus*, *A. acidus*, *A. aculeatus*, *A. carbonarius*, *A. brasiliensis*, *A. glaucus*, *A. tubingensis*, *A. wentii*, *A. zonatus*, *A. kawachii*. All orthologs from these Aspergilli are C6 proteins with Aacu16872_038210 being the only exception: *in silico* screens could not identify any conserved domains in the ortholog of *A. aculeatus* (FIGURE 13). Amino acid sequence based searches using the AN0585 sequence as query reveal putative orthologs in other Ascomycota as well. Several *Penicillium* spp. harbor proteins with high query coverages of 70 to 100% and sequence identities around 40% to *A. nidulans* ZtfA. A number of *Talaromyces* spp. might harbor orthologs as well (query

coverage of 54 to 91% and sequence identities of 30 to 40%). Query coverage indicates the percentage of the alignment that covers the primary amino acid sequence of *A. nidulans* ZtfA.
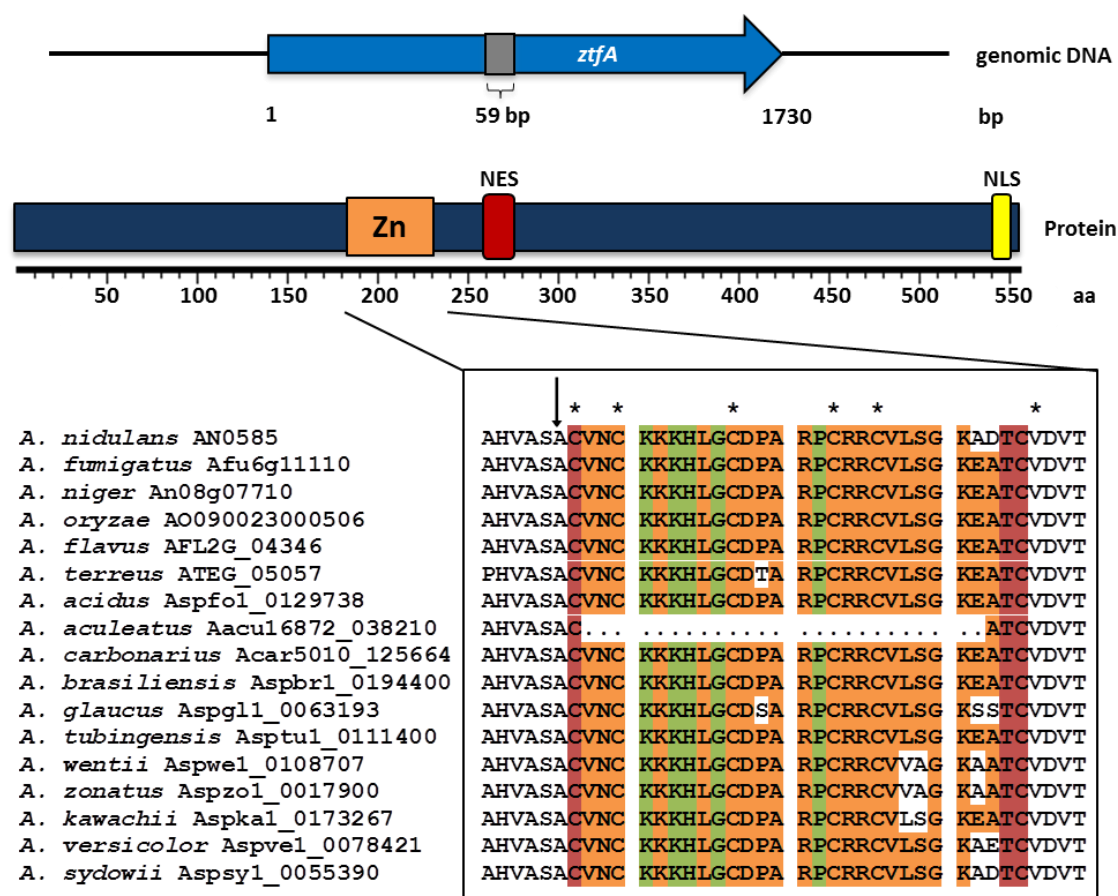
FIGURE 13: *ztfA* (*AN0585*) **encodes a C6 transcription factor.**
Graphical representation of *ztfA* (zinc cluster transcription factor A; *AN0585*) and its gene product (upper part). The grey box represents an intron, bp = base pairs, Zn = Zn(II)$_2$-Cys$_6$ fungal-type DNA-binding domain, NLS = nuclear localization sequence, NES = nuclear export signal, aa = amino acids. Multiple amino acid sequence alignments of the C6 domain (colored) of ZtfA orthologs from Aspergilli (lower part). Red = absolutely conserved, orange = conserved in ≥ ½ of indicated sequences. Residues presumably involved in DNA-binding are given in green. Asterisks mark the cysteine residues of the C6 domain. The small arrow indicates the absolutely conserved antecedent alanine residue.

AN0585 orthologs have not yet been characterized up to date. Query coverages for putative orthologs from most other Ascomycota identified in BLAST searches are below 30% and in almost all of these cases the region harboring the C6 domain is the only region with similarities to AN0585. BLAST analyses using the AN0585 amino acid sequence as query against Basidiomycota, Zygomycota, Glomeromycota and Chytridiomycota revealed query coverages in single-digit or low double-digit percental range (< 20% query cover, with exception of two putative proteins from *Rhizophagus irregularis* with 21% and 22% query

cover, respectively). Therefore, orthologs of AN0585 are abundant in Aspergilli and supposedly present in other Ascomycota, but seem to be absent in other fungal taxa.

A search of the InterPro database (Finn *et al.*, 2016) using InterProScan (Jones *et al.*, 2014) for conserved domains revealed a $Zn(II)_2$-Cys_6 (C6) fungal-type DNA-binding domain as the only conserved domain. Therefore, the protein was given the name ZtfA (Zinc cluster transcription factor A) and, consequently, its gene was named *ztfA*.

NucPred (Brameier *et al.*, 2007) and cNLS Mapper (Kosugi *et al.*, 2009) both predict a nuclear localization sequence (NLS) with high probabilities, starting at amino acid position 541 or 543 to position 548 or 550, respectively. LocNES (Xu *et al.*, 2015) and NetNES 1.1 (La Cour *et al.*, 2004) conformably predict a nuclear export signals (NES) starting at position 259 (LocNES) or 265 (NetNES) to 273. The predicted score, indicating the probability of the actual existence of the NES, was relatively low (score value between 0 and 1, predict score for NES in ZtfA around 0.6).

3.1.2 The C6 domain architecture of ZtfA is found in 5.7% of all *A. nidulans* C6 proteins

Typical DNA-binding sites of C6 proteins consist of terminal trinucleotides of direct or inverted repeats separated by six to eleven residues. Whereas this structure is conserved, the consensus sequence of the trinucleotides of targets differ greatly (Todd and Andrianopoulos, 1997). Gal4 of *Saccharomyces cerevisiae* is the founding member of the group of C6 proteins and one of the best studied examples for this protein group. The zinc cluster DNA-binding domain (DBD) of Gal4 has a $CX_2CX_6CX_6CX_2CX_6C$ architecture. Concurrently, this is the most common C6 architecture in *A. flavus* and *A. nidulans* (Chang and Ehrlich, 2013). In general, the cysteines within the first part of this motif are absolutely conserved, whereas the second part varies and forms $CX_2CX_6CX_{5-16}CX_2CX_{6-8}C$ (Todd and Andrianopoulos, 1997). A previous study found 330 C6 proteins in *A. nidulans* (Wortman *et al.*, 2009). An up-to-date *in silico* analysis conducted in the present study under employment of the AspGD and FungiDB databases (Cerqueira *et al.*, 2014; Stajich *et al.*, 2012) reveals two additional C6 proteins. This increases the number of C6 proteins to 332 in *A. nidulans*. ZtfA shows a $CX_2CX_6CX_5CX_2CX_8C$ architecture, which is found in 19 out of 332 C6 proteins in *A. nidulans* (approximately 5.7%). The amino acid residues within the first CX_2CX_6C motif are conserved to a certain extent among all C6 proteins known up to date, and mutagenesis studies showed their importance for DNA binding (Johnston and Dover, 1987; Todd and Andrianopoulos, 1997; Yuan *et al.*, 1991). The first, third, fourth and sixth residue between

the second and third cysteine of the C6 domain are in most cases basic residues and mutations of these residues have been shown to abolish DNA-binding in several C6 proteins (Todd and Andrianopoulos, 1997). For ZtfA, these four residues are lysine, lysine, histidine and glycine in orthologs of all Aspergilli (except *A. aculeatus*) (shown in green in FIGURE 13). A conserved proline at position C4 – X$_{1-2}$ functions in DNA binding in several known C6 proteins and was shown to prevent twist in the loop formed between the cysteines (Johnston and Dover, 1987; Marmorstein *et al.*, 1992; Todd and Andrianopoulos, 1997; Turcotte and Guarente, 1992; Yuan *et al.*, 1991). This proline is conserved in all putative Aspergilli ZtfA orthologs (shown in green in FIGURE 13). The entire C6 domain is strongly conserved among ZtfA orthologs in Aspergilli. The antecedent residue of the C6 domains is in most cases a small amino acid (Todd and Andrianopoulos, 1997) and for all ZtfA orthologs it is alanine. In conclusion, ZtfA has a quite uncommon C6 domain architecture and its orthologs in Aspergilli share high conservation of their amino acid sequences.

3.2   ZtfA is necessary for conidiation of *A. nidulans*

A mutant of the *ztfA* ortholog in *A. niger* (*scl-2*) produces drastically diminished numbers of conidiophores but is able to form sclerotia-like structures (Jørgensen *et al.*, 2011). Consequently, an *A. nidulans* knock out strain was created to analyze the influence of *ztfA* on developmental programs. The absence of *ztfA* leads to a phenotype with drastically diminished conidiophore numbers under asexual growth promoting conditions (FIGURE 14).
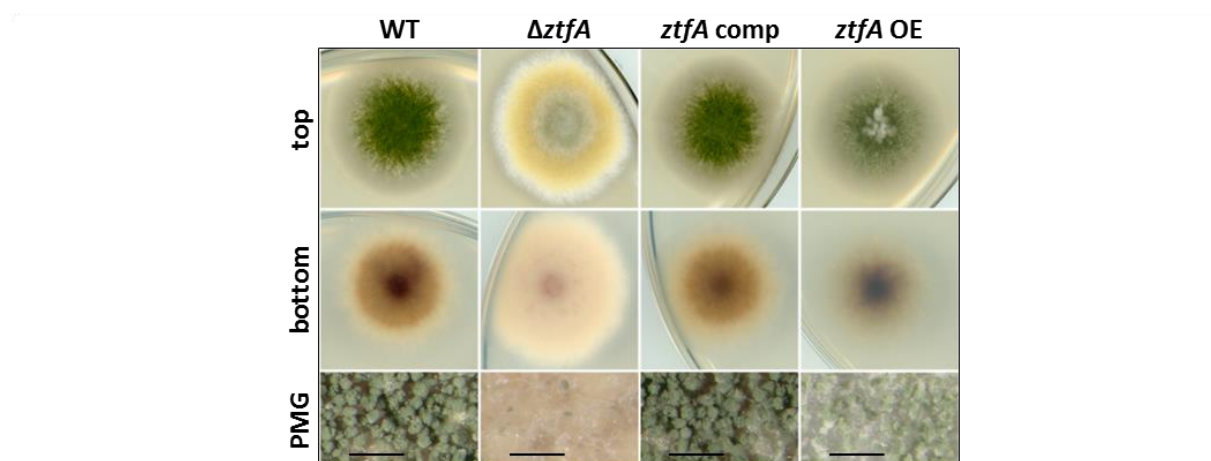
FIGURE 14: ZtfA is necessary for conidiation.
The absence of *ztfA* leads to diminished conidiophore formation. 2000 spores of WT, Δ*ztfA*, *ztfA* comp and *ztfA* OE (^P*niaD::ztfA*) were point inoculated on solid minimal medium (MM) and incubated for 3 d at 37°C in light. Cultures were photographed from above (top) and below (bottom). Photomicrographs (PMG) show that Δ*ztfA* forms reduced numbers of conidiophores (green). Black bars = 200 μm.

A. nidulans produces high numbers of conidiophores during asexual development in light and reduces conidiophore formation during sexual growth (in the dark with limited oxygen supply). A strain that overexpresses *ztfA* (*ztfA* OE) under a nitrate-inducible promoter (^P*niaD*::*ztfA*) produces increased numbers of conidiophores under sexual inducing conditions (FIGURE 15A). The *ztfA* OE phenotype is especially intense when the strains are plated instead of point inoculated on <u>mi</u>nimal <u>me</u>dium (MM). This leads to a greenish appearance of the *ztfA* OE strain in comparison to WT, Δ*ztfA* and the complemented strain (*ztfA* comp) when grown in the dark (FIGURE 15A). Point inoculation leads to simultaneous germination and circular growth of the whole colony from the same location. Such radial colonies exhibit zones of different age due to ongoing growth: the center comprises the oldest parts of the colony whereas structures at the periphery are the youngest (vegetative zone) (Etxebeste *et al.*, 2010b). In contrast, plated strains comprise colonies emerging from single germinating spores, which form a coherent mycelium due to hyphal fusion via anastomosis tubes, and are of same age at every spot (Etxebeste and Espeso, 2016; Gabriela Roca *et al.*, 2005). This leads to simultaneous developmental progression of the whole culture.

Quantification of conidiospores produced by *ztfA* mutants confirms that Δ*ztfA* forms drastically diminished numbers of conidiospores compared to WT, *ztfA* OE and the complemented strain (FIGURE 15B). *ztfA* OE produces one order of magnitude more conidiospores in the dark, compared to WT and the complemented strain (FIGURE 15B).

The *A. niger scl-2* mutant strain produces sclerotia-like structures, which are rarely formed by *A. niger* WT under laboratory conditions, indicating a repressing effect of *scl-2* upon sclerotia formation (Frisvad *et al.*, 2014; Jørgensen *et al.*, 2011). Hence, cleistothecia formation, which presumably corresponds to sclerotia, was monitored in *A. nidulans*. It is noteworthy, that the WT (AGB551) used in this study produces increased amounts of cleistothecia compared to the commonly used FGSC A4 WT even in light (Bayram *et al.*, 2012; McCluskey *et al.*, 2010). The Δ*ztfA* mutant produces similar amounts of cleistothecia compared to WT when grown in light (FIGURE 15C). In contrast, *ztfA* OE produces slightly more cleistothecia when grown in the dark under tested conditions. Differences between *ztfA* mutants and the WT are relatively small in both asexual and sexual development, indicating, that ZtfA is not a major regulator of cleistothecia formation in *A. nidulans*. Nests and cleistothecia in the Δ*ztfA* strain are more apparent compared to WT when grown in light, where these structures are covered by green layers of conidiophores (FIGURE 16). Ascospores of Δ*ztfA* are viable. These findings indicate that the *ztfA* gene product is not required for ascospore formation.
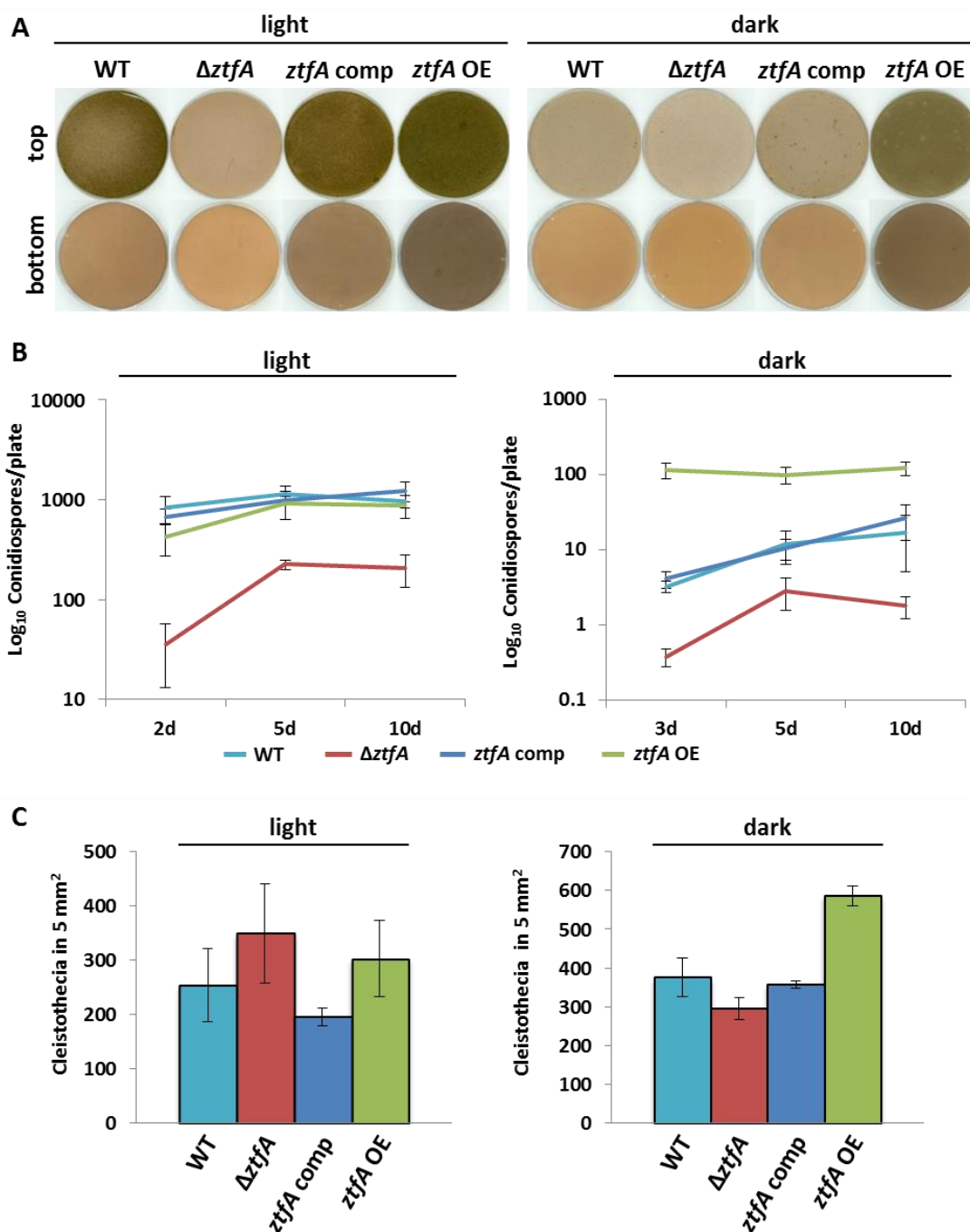
FIGURE 15: ZtfA is important for asexual development.
A) Colony morphology of plated cultures of WT, Δ*ztfA*, *ztfA* comp and *ztfA* OE strains. Solid MM plates were inoculated with $1*10^7$ spores and incubated for 7 d in light or dark at 37°C. B) Amount of conidiospores produced by indicated strains during a 10 days' time course during asexual development (light) and sexual development (dark), given in a logarithmic scale (Log_{10}). Plates were inoculated with $1*10^5$ spores of WT, Δ*ztfA*, *ztfA* comp or *ztfA* OE and spores were counted after 2, 5 and 10 days grown in light or dark at 37°C. C) Amounts of cleistothecia produced by WT and the *ztfA* mutant strains after 8 d grown in light or dark.
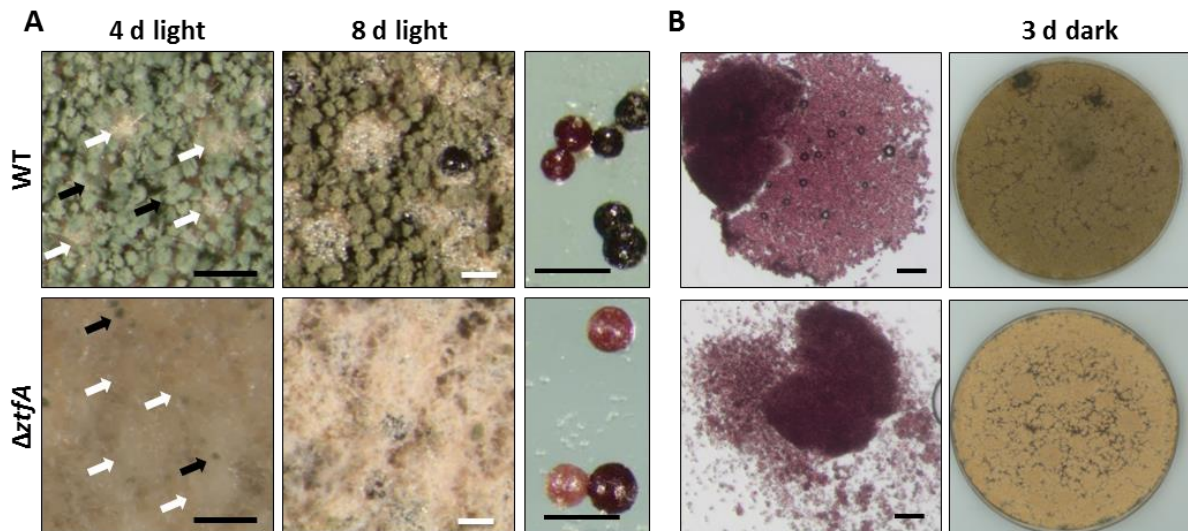
FIGURE 16: *ztfA* **is dispensable for cleistothecia formation and ascospore viability.**
A) Δ*ztfA* forms only few conidiophores (black arrows) and mostly nest like structures (white arrows), which produce mature cleistothecia. Black bars = 200 µm, white bars = 100 µm. B) Crushed cleistothecia (violet) of 7 d old cultures from WT and Δ*ztfA*. Black bars = 50 µm. Ascospores of WT and Δ*ztfA* were plated on solid MM and incubated for 3 d in dark.

3.3 The velvet protein VosA is a repressor of *ztfA* gene expression

3.3.1 VosA is a negative regulator of *ztfA*

VosA binds a palindromic CCGCGG recognition sequence upstream of approximately 1500 target ORFs (Ahmed *et al.*, 2013). *ztfA* is among these genes putatively regulated by VosA. The region containing the CCGCGG motif was identified upstream of the *ztfA* ORF. Electrophoretic mobility shift assays (EMSAs) with a synthesized DNA probe of this region show that GST-VosA (Ahmed *et al.*, 2013) binds this region *in vitro* and dosage-dependently (FIGURE 17A). This shows a specific binding of VosA to this motif upstream of *ztfA* and confirms the ChIP-on-Chip results from Ahmed and collaborators.

The molecular mechanism of regulation is unclear for most of the putative VosA targets. Hence, VosA's regulatory role upon *ztfA* expression was analyzed. cDNA was transcribed from RNA isolated from asexually grown WT, Δ*ztfA* and Δ*vosA* (S. Thieme, p.c.) cultures and quantitative real-time polymerase chain reaction (qRT-PCR) analyses were conducted to analyze whether VosA regulates *ztfA* expression (FIGURE 17B). *ztfA* transcription is upregulated in the absence of *vosA* in asexually grown cultures after 24 h but not during vegetative growth. This shows that VosA represses *ztfA* expression during the late phase of conidiation.
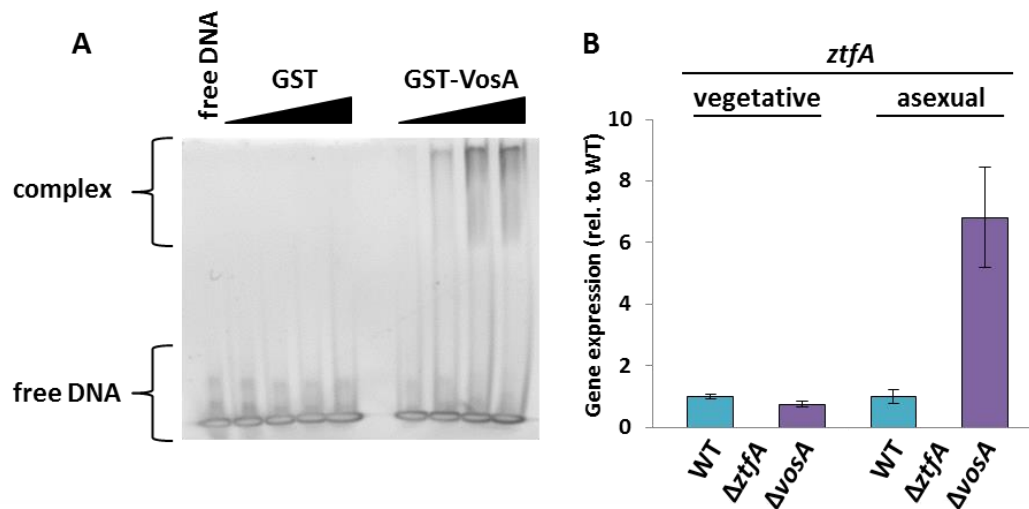
FIGURE 17: VosA binds upstream of the *ztfA* open reading frame and represses *ztfA* gene expression.
A) Electrophoretic mobility shift assay (EMSA) using serial diluted GST-VosA protein and a 33 bp DNA probe of the *vosA* recognition site upstream of *ztfA*. DNA and protein were used in molar ratios of 1:0.3, 1:1, 1:3 and 1:4. Free GST is shown as negative control. B) *ztfA* expression is upregulated in the absence of *vosA* during asexual growth, as indicated by qRT-PCR analyses. Strains were grown for 24 h in submerged cultures and mycelia were harvested (vegetative), or shifted onto solid MM plates and grown for 24 h in light (asexual).
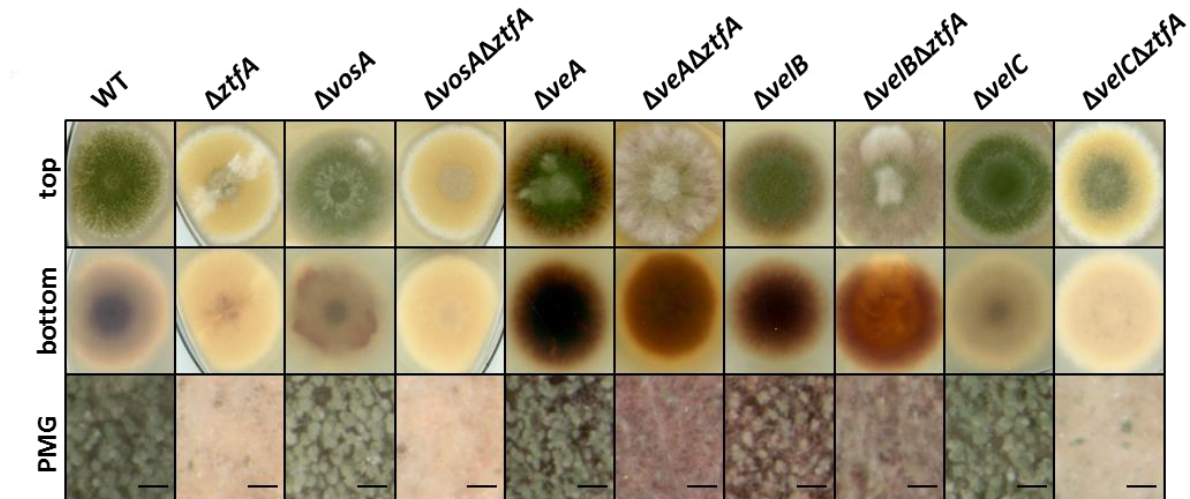
3.3.2 *ztfA* is epistatic towards *vosA*

VosA forms heterodimers with VelB and VelC (Park *et al.*, 2012, 2014), which fulfill different functions in fungal development and interconnected secondary metabolism. Single and double knock out mutants of the velvet factors and *ztfA* were constructed to investigate whether ZtfA functions up- or downstream of VosA and to analyze possible genetic relations with other velvet factors.

The Δ*vosA* strain forms grey-greenish conidiophores (Ni and Yu, 2007). The Δ*ztfA* phenotype predominates in a Δ*ztfA*Δ*vosA* double mutant strain (FIGURE 18). This indicates that *ztfA* is epistatic towards *vosA*. The Δ*veA* and Δ*velB* single mutants both show drastic phenotypes on solid MM without cleistothecia formation and with production of dark reddish pigments (Bayram *et al.*, 2008a; Palmer *et al.*, 2013; Park *et al.*, 2012b) (FIGURE 18). Both Δ*ztfA*Δ*veA* and Δ*ztfA*Δ*velB* double mutants show additive phenotypes. The dominant phenotype is similar to the Δ*veA* and Δ*velB* single mutant, respectively, but with increased amounts of aerial hyphae and drastically reduced greenish colony centers (FIGURE 18). These additive phenotypes indicate an action of ZtfA independently of VeA or VelB, or their heterodimers. The Δ*velC* single mutant shows an almost WT-like phenotype on solid MM but with increased amounts of conidiophores (Park *et al.*, 2014) (FIGURE 18). The Δ*ztfA*Δ*velC* double deletion strain shows an additive phenotype with the Δ*ztfA* phenotype as the predominant

phenotype but an increased greenish colony center. Taken together, ZtfA functions specifically downstream of VosA, because it does not function in VeA, VelB or VelC governed pathways.

FIGURE 18: Phenotypes of *ztfA* and velvet mutants.
Phenotypic analyses indicate that *ztfA* is epistatic towards *vosA*. Double mutants with Δ*ztfA* and either Δ*veA*, Δ*velB* or Δ*velC* show additive phenotypes. Strains were point inoculated on solid MM and grown for 4 d in light at 37°C. PMG = photomicrograph, black bars = 200 μm.

3.3.3 ZtfA is necessary for spore viability

VosA and VelB are crucial for trehalose biogenesis (Ni and Yu, 2007; Sarikaya-Bayram *et al.*, 2010). Conidiospore viability assays were conducted to test whether ZtfA is involved in spore viability as well. These tests were carried out on solid MM supplemented with 1.2 M sorbitol to decrease Hülle cell contaminations in conidiospore solutions (Han *et al.*, 2003). Conidiospores of Δ*ztfA* show a rapid loss in spore viability (FIGURE 19A). This can be complemented by reintroducing the *ztfA* gene into the *ztfA* deletion background (*ztfA* comp). The assay was performed several times with similar results without sorbitol to exclude an effect of sorbitol on spore viability. Consequently, trehalose amounts were analyzed in spores of WT and the *ztfA* mutants. However, no difference in trehalose amounts in spores of Δ*ztfA* or *ztfA* OE in comparison to WT could be found (FIGURE 19B). This finding shows that ZtfA specifically supports spore viability without affecting trehalose biogenesis, which might require a second VosA controlled regulatory gene.
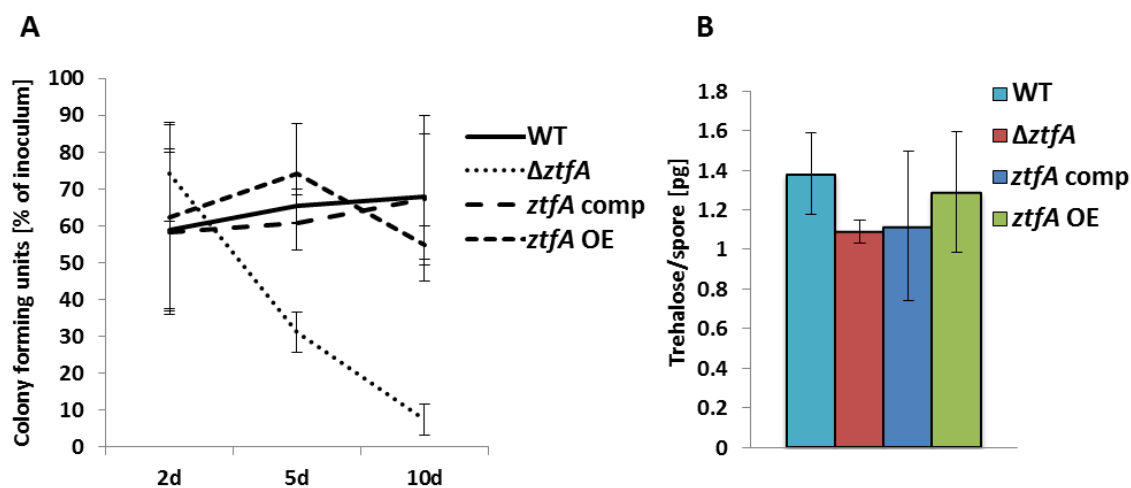
FIGURE 19: ZtfA supports spore viability.
A) Conidiospores show a rapid loss in viability in the absence of *ztfA*. $1*10^5$ spores were plated and grown for up to 10 d in light at 37°C. Spores were harvested at indicated time points and 200 spores per strain were plated. Emerging colonies were counted after two days. Numbers of emerging colonies are given as percent of inoculate. This assay was performed in three biological replicates on MM supplemented with sorbitol and repeated several times without sorbitol with similar results. B) Trehalose amounts do not differ between WT and the *ztfA* mutant strains. Trehalose amounts of WT and the *ztfA* mutant strains were analyzed in 2 d old conidiospores from asexually grown cultures. Amounts are given in pg (picogram) per spores.

3.4 ZtfA activates the conidiation pathway

3.4.1 *ztfA* overexpression results in conidiophore formation during vegetative growth

ztfA OE forms increased numbers of conidiospores during sexual development (FIGURE 16). Phenotypes of WT and the *ztfA* mutants were monitored in submerged cultures to investigate whether *ztfA* OE is sufficient to induce conidiophore formation even during vegetative growth. Submerged culture conditions completely suppress developmental programs in *A. nidulans* and result in solely vegetative hyphal growth. Strains were grown in liquid cultures for 18 h and mycelia were investigated under the microscope. *ztfA* OE forms conidiophores in submerged cultures (FIGURE 20), whereas no conidiophores are found in cultures of WT, Δ*ztfA* or *ztfA* comp. This shows that *ztfA* OE is able to undergo asexual development even under development suppressing conditions.

A. nidulans achieves developmental competence after approximately 18 h of vegetative growth (Axelrod *et al.*, 1973; Lee *et al.*, 2016). Experiments were conducted to investigate if an overexpression of *ztfA* is sufficient to induce conidiophore development earlier than 18 h post germination. *ztfA* OE and the WT were grown for 14 h in submerged London medium

(LM), which represses the expression of *ztfA* driven by the nitrate-inducible promoter (^P*niaD::ztfA*) in the *ztfA* OE strain. Subsequently, cultures were shifted into liquid MM, which contains nitrate as sole N-source and therefore induces expression of ^P*niaD::ztfA* in the *ztfA* OE strain. *ztfA* OE forms conidiophores as early as 9 h post induction, whereas the WT does not form conidiophores under described culture conditions. This underlines an activating effect of ZtfA towards conidiation.
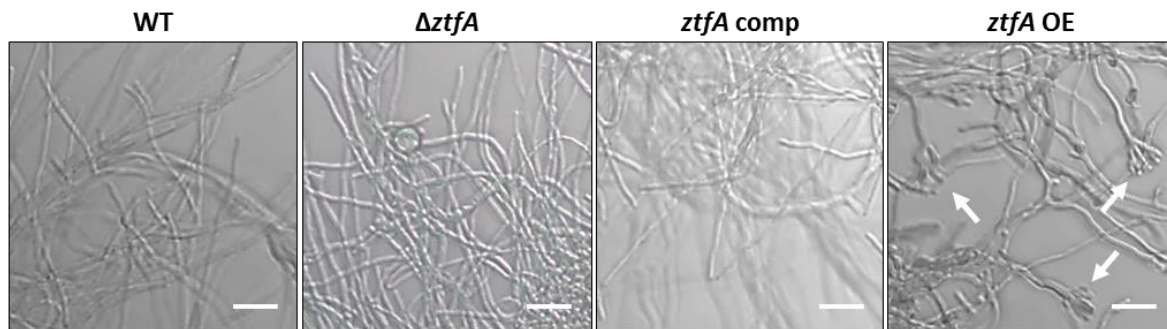
FIGURE 20: *ztfA* OE induces *A. nidulans* conidiophore formation in submerged cultures. Photomicrographs of strains grown for 18 h in submerged cultures in liquid MM. White arrows indicate conidiophores. White bars = 20 µm.

3.4.2 ZtfA functions downstream of the conidiation-pathway activator FluG

Phenotypes of the Δ*ztfA* and *ztfA* OE strains suggest that ZtfA is a conidiation activator. FluG is a key upstream activator of the conidiation pathway (Lee and Adams, 1994b). A *fluG* deletion cassette was constructed and integrated into the WT and the Δ*ztfA* strain, as well as the *ztfA* OE strain. The deletion of *fluG* leads to a drastically reduced conidiation and a fluffy phenotype (Lee and Adams, 1994a) (FIGURE 21A). The back of the colony shows a light orange color indicating an alteration in secondary metabolite production. The Δ*fluG*Δ*ztfA* double mutant shows an additive phenotype to the Δ*fluG* single mutant and completely failed to produce conidiophores (FIGURE 21A). The orange color of metabolites released by this mutant is less bright compared to the single Δ*fluG* mutant.

The Δ*fluG* phenotype is not rescued by an overexpression of *ztfA*. However, an overexpression of *fluG* does not rescue the Δ*ztfA* deletion phenotype. This indicates an action of the ZtfA protein downstream, or independently of the FluG pathway.
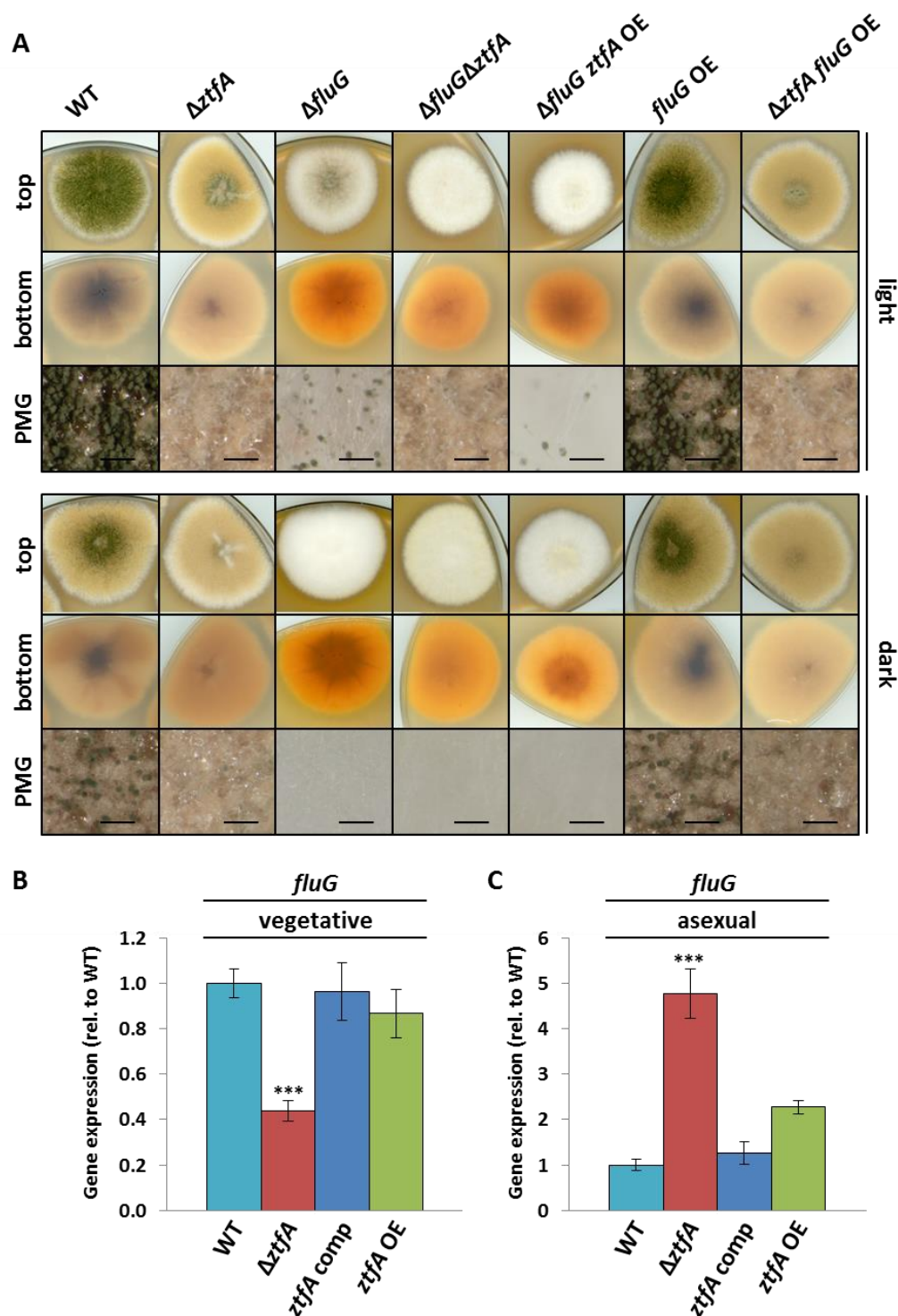
FIGURE 21: ZtfA regulates *fluG* expression.
A) Phenotypic analyses of *ztfA* and *fluG* mutants. 2000 conidiospores of each strain were point inoculated and grown for 4 d in light (upper panel) and dark (lower panel) at 37°C. PMG = photomicrographs, black bars = 200 µm. B) and C) qRT-PCR shows that *fluG* gene expression during vegetative growth after 24 h is downregulated B) but upregulated during late asexual phase (24 h post induction) in the absence of *ztfA* (***$P < 0.005$). $1*10^7$ spores were inoculated in MM and grown for 24 h vegetatively and harvested B) or transferred to plates and incubated for 24 h in light at 37°C to promote asexual development C). Gene expression is given relative to WT from three biological replicates.

In qRT-PCR analyses, *fluG* is not found to be upregulated during vegetative growth in *ztfA* OE, as would be expected if *ztfA* would be an activator of *fluG* (FIGURE 21B). In the absence of *ztfA*, however, a slight downregulation of *fluG* of about two fold is found. In contrast, *fluG* is upregulated fivefold in the absence of *ztfA* during asexual growth after 24 h in comparison to WT (FIGURE 21C). The differential expression in Δ*ztfA* is completely restored in *ztfA* comp. This indicates that ZtfA has different influences on *fluG* transcription during vegetative growth and during late asexual development.

3.4.3   ZtfA regulates upstream activators of conidiation

Several <u>u</u>pstream <u>d</u>evelopmental <u>a</u>ctivators (UDAs) are involved in the activation of *brlA* gene expression, downstream of FluG. These UDAs are the *flb* (*fluffy low <u>b</u>rlA expression*) genes, which gene products activate *brlA* expression in two cascades: FlbB/FlbE→FlbD→BrlA and FlbC→BrlA (see CHAPTER 1.5) (Seo *et al.*, 2003). Expression of the *flb* genes was analyzed in vegetative and asexual growth to investigate whether ZtfA regulates the UDA pathway. qRT-PCR analyses show that several *flb*s are differentially regulated in the absence of *ztfA* or in a *ztfA* OE background during vegetative growth (FIGURE 22). *flbB* and *flbE*, which encode co-activators of *brlA* via FlbD, were not found to be significantly differentially regulated in the *ztfA* mutants compared to WT during late vegetative growth. Strikingly, *flbD* is found to be downregulated about 11 times compared to WT in the absence of *ztfA* (FIGURE 22). *flbC* is downregulated about 2.5 fold in Δ*ztfA* compared to WT but upregulated in *ztfA* OE. This suggests an activating role of ZtfA towards the UDA cascade during vegetative growth and especially towards *flbC* and *flbD* gene expression.

During asexual growth *flbB*, *flbC* and *flbD* are upregulated about three fold in the absence of *ztfA* (FIGURE 22). These findings indicate that ZtfA coordinates expression of genes of the conidiation pathway differentially at different developmental stages. In vegetative growth, ZtfA activates the UDA pathway, which members then bind to the *brlA* promoter (Garzia *et al.*, 2010; Kwon *et al.*, 2010a). ZtfA represses expression of the UDA genes during late asexual growth after 24 h post induction, when conidiophores are present and conidiospores mature.

The fifth *flb* gene is *flbA* and codes for a RGS domain protein, which antagonizes the action of a G-protein mediated pathway through FadA. This pathway represses conidiation and sterigmatocystin biosynthesis (see CHAPTER 1.5.2) (Hicks *et al.*, 1997). *flbA* gene expression was monitored during vegetative and during asexual growth as well. Expression levels of *flbA*

were not altered during vegetative growth in *ztfA* mutant strains in comparison to WT (FIGURE 22). However, ZtfA has a repressing effect on *flbA* expression during asexual growth since *flbA* is upregulated in the absence of *ztfA* (FIGURE 22). As *flbA* expression was not affected during asexual growth in *ztfA* OE, this regulation might be indirect.
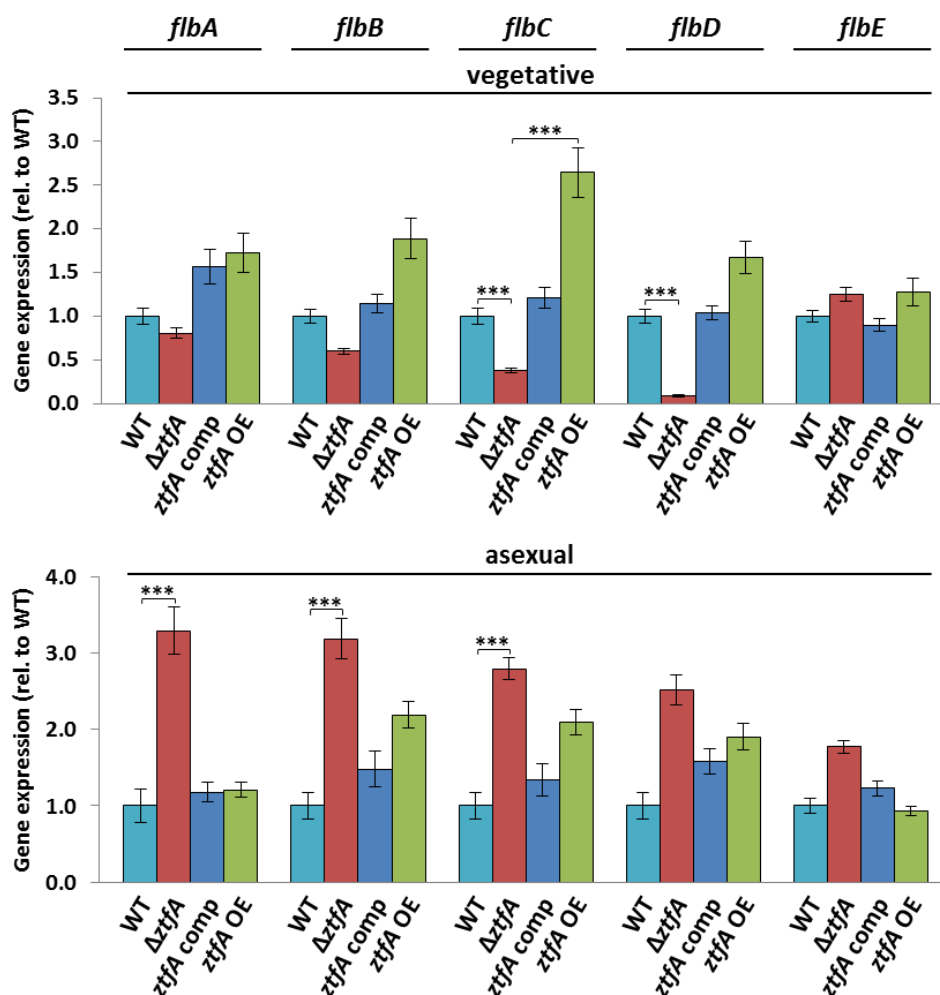
FIGURE 22: ZtfA regulates *flb* genes in a time dependent manner.
Expression of the *flb* genes in WT and the *ztfA* mutants determined by qRT-PCR. Gene expression of *flbC* and *flbD* is downregulated in Δ*ztfA* compared to WT during vegetative growth (left hand side). *flbC* expression is upregulated under same growth conditions in *ztfA* OE. Gene expression of *flbA*, *flbB*, *flbC* and *flbD* is upregulated in Δ*ztfA* during asexual development after 24 h (right hand side). $1*10^7$ spores were grown in submerged cultures for 24 h and mycelia were harvested (vegetative) or transferred onto solid agar plates and grown for 24 h in light (asexual). Gene expression relative to WT is given from three (asexual) and four (vegetative) biological replicates with three technical replicates (***P<0.005).

Single and double knock out strains were created to investigate genetic relations between *ztfA* and the *flb* genes. All single deletions of the *flb* genes show fluffy phenotypes (Wieser *et al.*, 1994) (FIGURE 23). Δ*flbC* shows a phenotype very similar to Δ*ztfA*. Double deletions of *ztfA* and each of the *flb* genes show distinct phenotypes with additional phenotypical effects.

Conidiophore development in the double deletions is completely absent in all mutant combinations (FIGURE 23). Notably, $\Delta flbC\Delta ztfA$ resembles the $\Delta flbB$ and $\Delta flbE$ phenotype as well as all $\Delta flb\Delta ztfA$ double mutant phenotypes. In contrast to the single $\Delta ztfA$ mutant, all double deletion strains produce increased numbers of aerial hyphae, which leads to a fluffy phenotype. ZtfA is a positive regulator of $flbC$ and $flbD$ gene expression (FIGURE 22). The fact that $\Delta flbB\Delta ztfA$ and $\Delta flbE\Delta ztfA$ show a similar phenotype to $\Delta flbD\Delta ztfA$ supports this, since FlbB and FlbE function upstream of FlbD. Furthermore, this is supported by the observation that $\Delta flbC\Delta ztfA$ resembles the other $\Delta flb\Delta ztfA$ phenotypes. Taken together, this indicates that ZtfA functions upstream of both, FlbC and FlbD. This shows that ZtfA activates both cascades of the Flb pathway through FlbD and FlbC. These findings suggest a necessity for both, ZtfA and the Flb factors for conidiation.
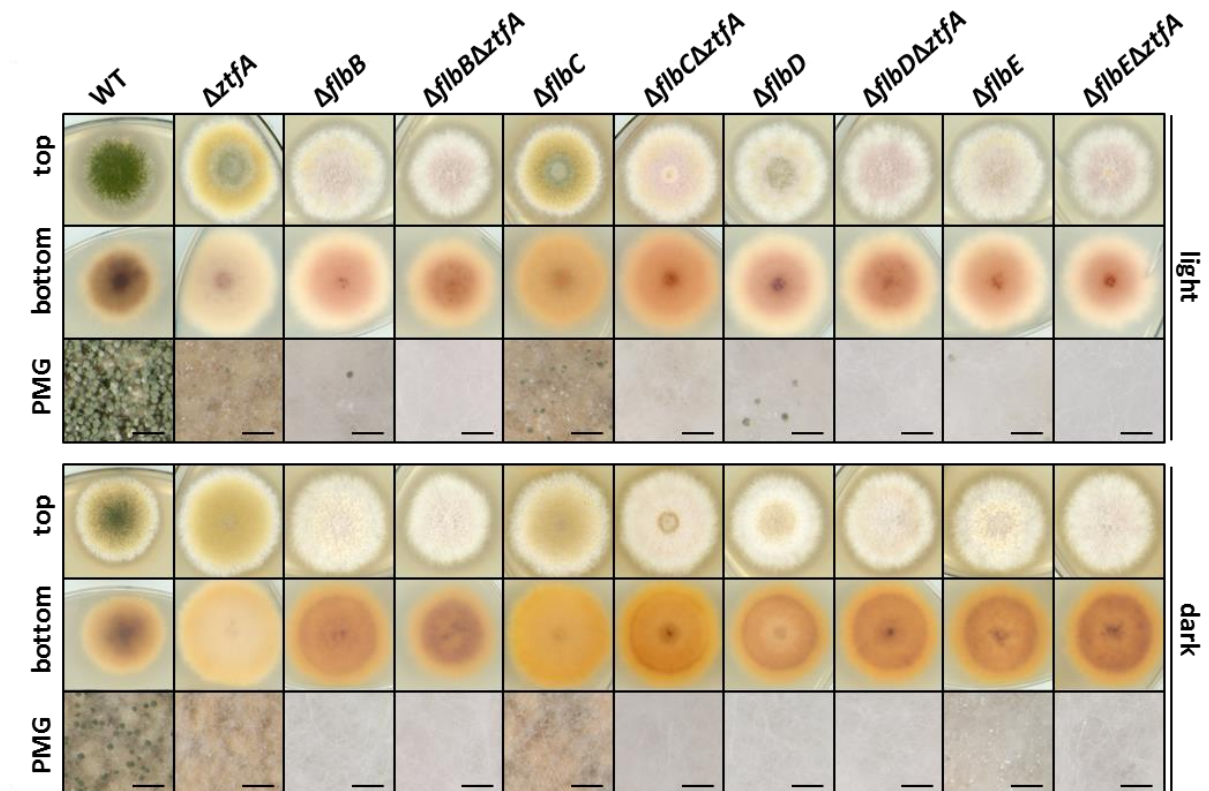
FIGURE 23: *ztfA* and the *flb* genes are necessary for conidiation of *A. nidulans*.
Loss of *ztfA* has additional effects in Δflb mutants. 2000 conidiospores per strain were point inoculated on solid MM and grown for 3 d in light (upper panel) or dark (lower panel) at 37°C. PMG = photomicrograph, black bars = 200 µm.

flb genes were knocked out in *ztfA* OE background to test whether *ztfA* OE is sufficient to rescue the *flb* phenotypes. *ztfA* OE is not sufficient to complement *flb* knock out phenotypes in any case (FIGURE 24). This clearly shows that ZtfA does not act downstream of the Flb factors. Taken together, these epistasis analyses show a necessity of both, *ztfA* and the *flb*

genes for asexual development in an interdependent manner. These findings indicate that ZtfA functions upstream of the UDA pathway and is important for conidiation, specifically through activation of *flbC* and *flbD* gene expression.
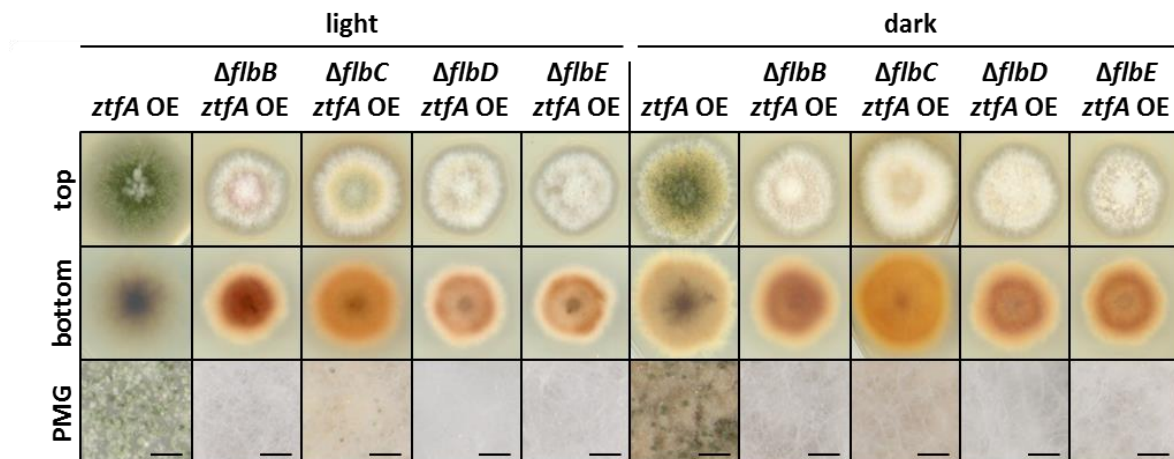
FIGURE 24: *ztfA* OE is not sufficient to rescue fungal Δ*flb* phenotypes.
2000 conidiospores per strain were point inoculated on solid MM and grown for 3 d in light and dark at 37°C. PMB = photomicrograph, black bars = 200 μm.

3.4.4 ZtfA is an activator of *brlA* gene expression

BrlA is the major regulator of conidiophore development (Adams *et al.*, 1988). Since *ztfA* OE forms conidiophores under development repressing conditions and ZtfA together with the Flb factors are necessary for conidiation, the question arises, whether ZtfA activates *brlA* expression. *brlA* gene expression in WT and *ztfA* mutant strains was analyzed by qRT-PCR. The WT only expresses basal levels of *brlA* when grown in liquid cultures. In contrast, transcript levels of *brlA* are highly upregulated in *ztfA* OE (FIGURE 25A).

brlA is also upregulated during vegetative growth in the absence of *vosA* (Lee *et al.*, 2016; Ni and Yu, 2007) (FIGURE 25A). This opens the question whether ZtfA activates *brlA* expression. Expression of *brlA* in a Δ*vosA* mutant in *ztfA* OE background was tested to investigate this possibility. *brlA* gene expression is already upregulated about 40 times in *ztfA* OE compared to WT under submerged culture conditions. The Δ*vosA ztfA* OE mutant shows a more than 10 times higher upregulation of *brlA* compared to the *ztfA* OE single mutant and over 400 times more compared to WT (FIGURE 25A). This additional upregulation indicates that *ztfA* OE is sufficient to activate *brlA* expression in the absence of *vosA*.

Phenotypical analyses were conducted to confirm an epistasis of *ztfA* towards *brlA*. The Δ*brlA* mutant strain shows a phenotype with drastically diminished conidia and increased numbers of aerial hyphae, leading to a fluffy phenotype when grown in light (FIGURE 25B). It

resembles the Δ*ztfA* strain when grown in the dark, where no aerial hyphae are formed (FIGURE 25B).
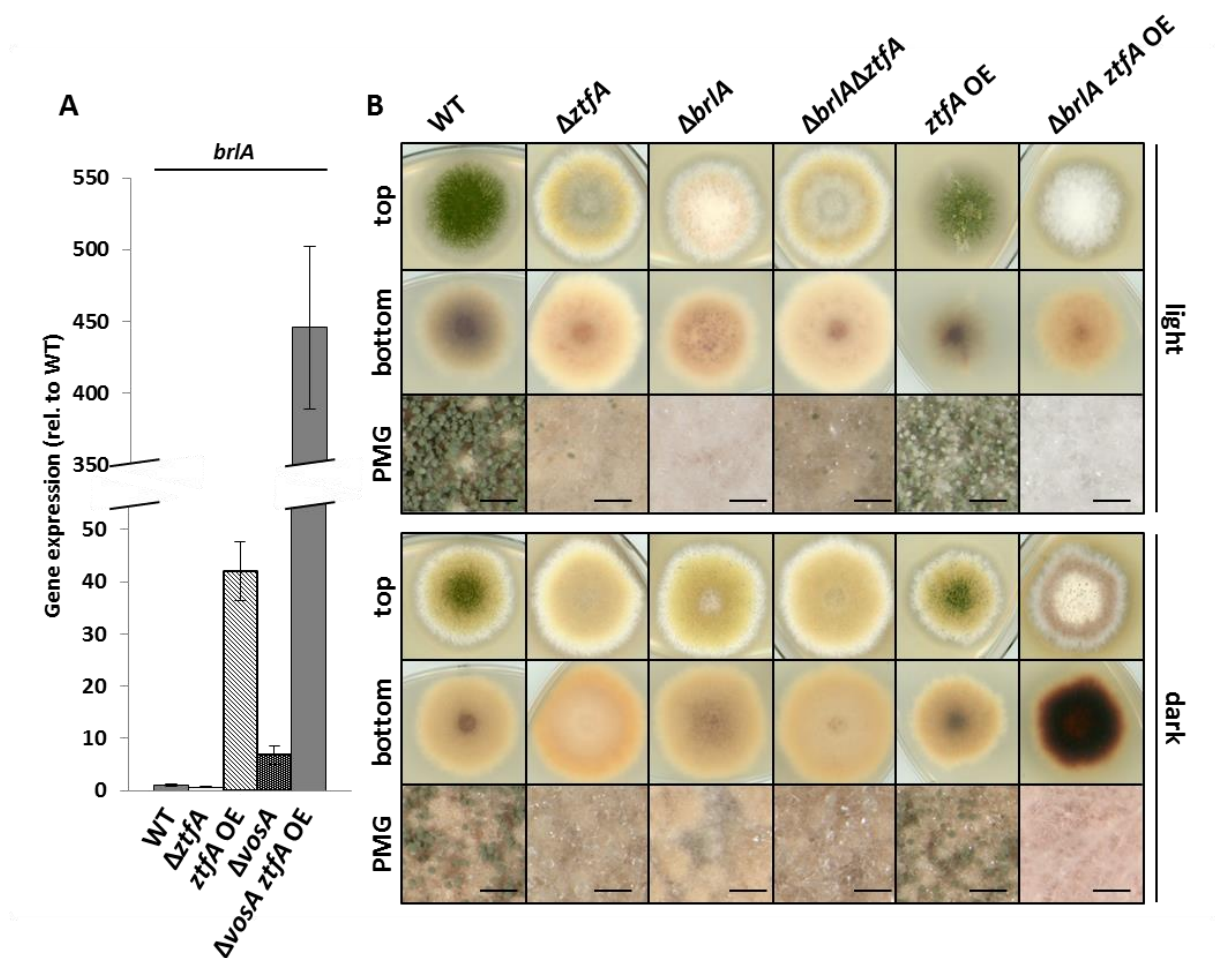
FIGURE 25: ZtfA activates *brlA* gene expression.
A) qRT-PCR shows that *ztfA* OE induces *brlA* gene expression in vegetatively grown cultures. This is intensified in the absence of *vosA*. Note that the axis of ordinates is interrupted to allow a better visibility of the expression values below 50 relative to WT. This was repeated with three biological and three technical replicates. B) *ztfA* is epistatic towards *brlA*. Strains were point inoculated on solid MM and grown for 3 d in light or dark at 37°C. PMG = photomicrograph, black bars = 200 μm.

The Δ*brlA*Δ*ztfA* double mutant shows the Δ*ztfA* single mutant phenotype in both light and dark. This underlines an action of ZtfA upstream of BrlA in developmental programs. A Δ*brlA ztfA* OE mutant shows a fluffy phenotype due to increased amounts of aerial hyphae. This shows that ZtfA alone is not able to induce conidiation but ZtfA-mediated conidiation induction relies on *brlA*. AbaA is a direct downstream factor of BrlA (Andrianopoulos and Timberlake, 1994). Hence, the genetic relations of *abaA* and *ztfA* were analyzed as well. A loss of *abaA* leads to the formation of brownish conidiophores, which are impaired in correct conidiospore separation and distinctly decreased in number (Sewall *et al.*, 1990b) (FIGURE

26). The Δ*ztfA*Δ*abaA* mutant shows the Δ*ztfA* single mutant phenotype but lost the greenish colony center when grown in light (FIGURE 26). The double mutant is indistinguishable from the Δ*ztfA* single mutant, but distinctly different to the Δ*abaA* single mutant when grown in the dark. This confirms an action of ZtfA upstream of BrlA and AbaA.
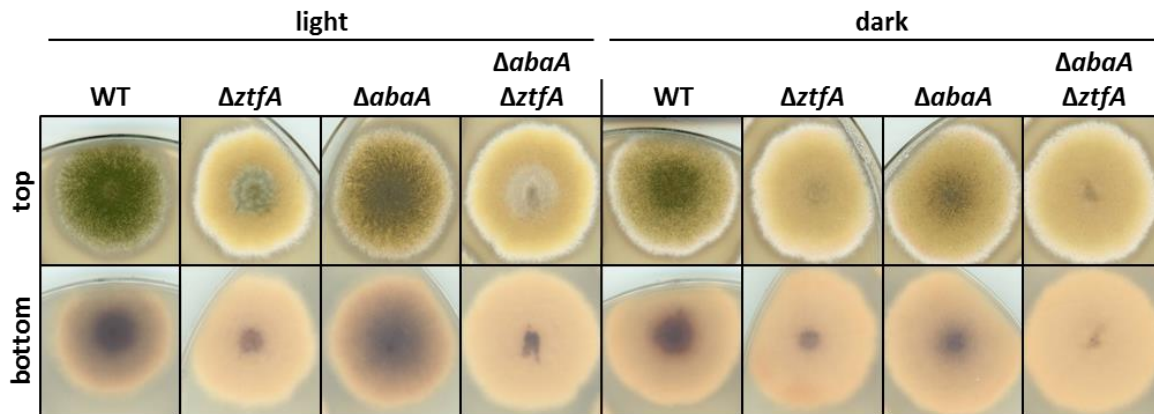
FIGURE 26: *ztfA* is epistatic towards *abaA*.
ztfA and *abaA* mutants, grown in light (left hand side) or dark (right hand side), are shown. 2000 conidiospores per strain were point inoculated on solid MM and grown for 4 d in light or dark at 37°C.

3.4.5 ZtfA regulates conidiation independently of developmental repressors

Activation of the conidiation pathway is hindered by the repressors NsdD and VosA during vegetative growth, which are released from the *brlA* promoter when the fungus becomes developmentally competent (Lee *et al.*, 2014; Ni and Yu, 2007). SfgA represses conidiation through negative regulation of the Flb factors (Seo *et al.*, 2003, 2006). Expression of *sfgA*, *nsdD* and *vosA* genes during vegetative growth and late asexual development was analyzed via qRT-PCR to exclude the possibility that ZtfA influences the conidiation pathway via downregulation of these repressors. None of the three genes is differentially regulated neither during vegetative nor during asexual growth in *ztfA* mutants (FIGURE 27). This clearly shows that *ztfA* does not negatively regulate gene expression of the conidiation repressors and it supports the hypothesis that ZtfA is a direct activator of *brlA* expression.
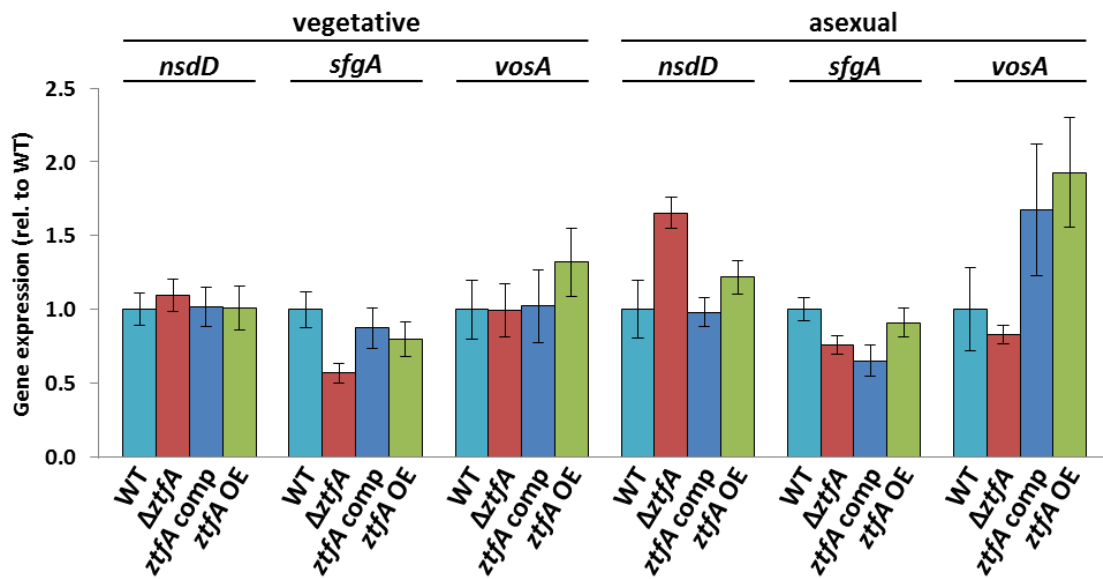
FIGURE 27: Gene expression of various regulatory genes of fungal development is independent of cellular ZtfA protein levels.
qRT-PCR shows that gene expression of repressors of asexual development is not significantly altered in absence or overexpression of *ztfA*. $1*10^7$ spores were grown in submerged cultures for 24 h and mycelia were harvested (left hand side) or transferred onto solid agar plates and grown for 24 h in light (right hand side). This was repeated with three biological and three technical replicates.

3.5   ZtfA supports expression of several secondary metabolite genes

3.5.1   ZtfA regulates gene expression of *aflR* and sterigmatocystin biosynthesis

Secondary metabolite (SM) production is tightly interconnected with developmental programs in filamentous fungi (Brakhage, 2013). The velvet regulatory networks play key roles in this interconnection (Bayram *et al.*, 2008a). It was examined whether ZtfA, as a downstream factor of VosA, is involved in the regulation of secondary metabolism. Sterigmatocystin production was compared in different *ztfA* mutant strains. Cells were grown for three days under asexual or sexual conditions. Sterigmatocystin samples were extracted and analyzed using thin layer chromatography (TLC). Sterigmatocystin production is increased during asexual as well as sexual development in both, the Δ*ztfA* strain as well as in the *ztfA* OE strain (FIGURE 28A). AflR is the major sterigmatocystin regulator in Aspergilli (Yu *et al.*, 1996a). It was examined whether the deletion or overexpression of *ztfA* is sufficient to restore sterigmatocystin production in Δ*aflR*. *aflR* was knocked out in Δ*ztfA* or *ztfA* OE background (FIGURE 28B). Neither a loss nor an overexpression of *ztfA* restored ST production in the absence of *aflR* (FIGURE 28B).

qRT-PCR analyses were carried out to examine whether this increase in sterigmatocystin production can be retraced to changes in gene expression of *aflR* and *stcU* in asexually grown cultures. *stcU* encodes a ketoreductase and transcript levels are commonly used as indicator for sterigmatocystin cluster activation (Hicks *et al.*, 1997; Kato *et al.*, 2003). In accordance with the TLC results, *aflR* expression is upregulated about three fold in *ztfA* OE and about eight fold in Δ*ztfA* (FIGURE 28C). Confirmatively, *stcU* is highly upregulated in both *ztfA* OE and Δ*ztfA* (FIGURE 28C).
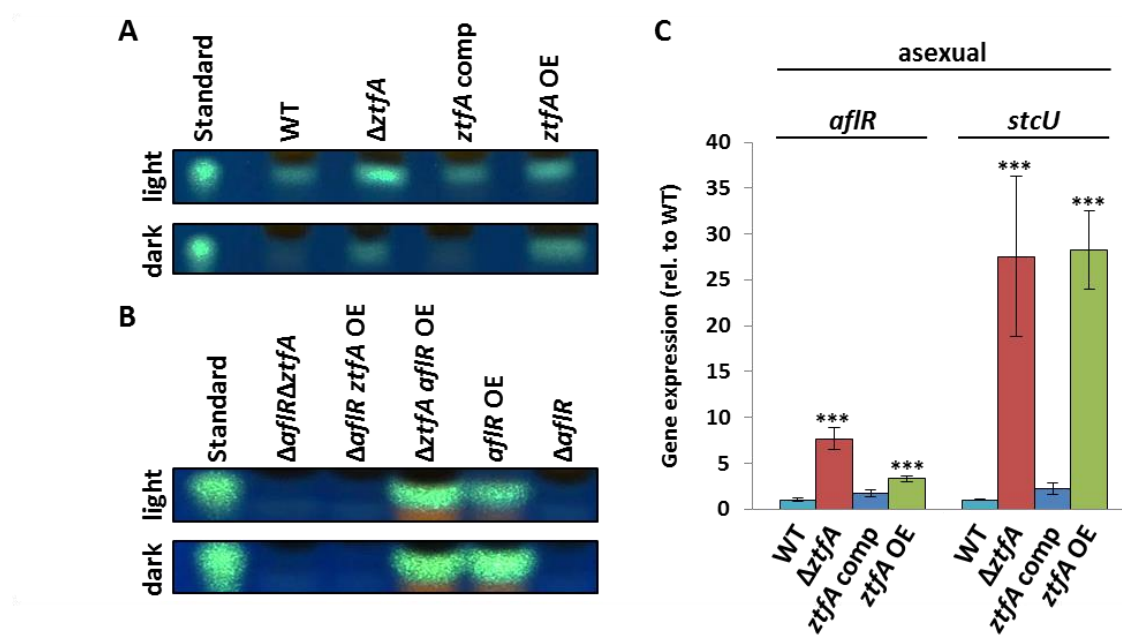
FIGURE 28: ZtfA is involved in the regulation of sterigmatocystin production and *aflR* and *stcU* gene expression.
A) Absence and overexpression of *ztfA* lead to increased sterigmatocystin production. B) *ztfA* OE is not sufficient to induce ST production in Δ*aflR*. A) and B) ST was isolated from strains grown for 3 d in light or dark. Thin layer chromatography was performed and TLC plates were sprayed with aluminum chloride to allow ST analysis. C) qRT-PCR shows that loss and overexpression of *ztfA* result in increased transcription of *aflR* and *stcU* during asexual growth (***$P < 0.005$, **$P < 0.01$).

The relation between *aflR* and *ztfA* on developmental levels was analyzed. Δ*aflR*, Δ*ztfA* and the double mutants, together with the *ztfA* OE and *aflR* OE mutants, were point inoculated on solid MM and grown for three days under asexual or sexual conditions (FIGURE 29). The Δ*aflR* single mutant resembles in its colony morphology the WT (Wilkinson *et al.*, 2004) (FIGURE 29). The Δ*aflR*Δ*ztfA* double mutant exhibits the Δ*ztfA* phenotype, indicating an epistasis of *ztfA* towards *aflR* in developmental programs. Consistently, the phenotype of a strain, which lacks the *aflR* gene, but overexpresses *ztfA* shows the *ztfA* OE phenotype. The *aflR* OE mutant shows a phenotype with decreased conidiation and dispensing of a red

pigment into the surrounding medium (FIGURE 29). The Δ*ztfA* in *aflR* OE background shows further diminished conidiophores (FIGURE 29). This confirms an epistasis of *ztfA* towards *aflR* in developmental programs.
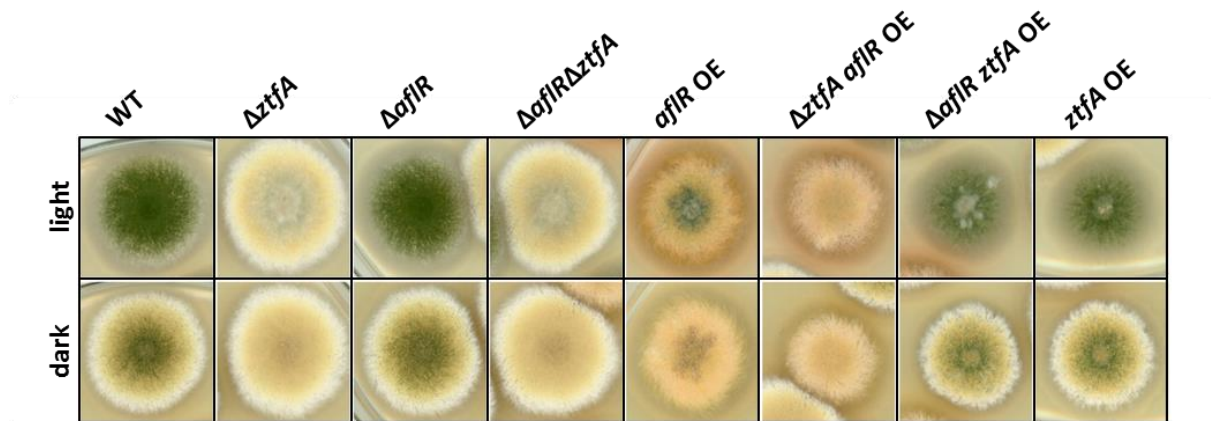
FIGURE 29: *ztfA* **is epistatic towards** *aflR*.
Point inoculated colonies of *ztfA* and *aflR* mutants grown in light (upper part) or dark (lower part) are shown. *ztfA* mutant phenotypes predominate the Δ*aflR* phenotype grown in light (asexual development) and dark (sexual development). 2000 spores per strain were point inoculated on solid MM and grown for 3 d in light at 37°C.

3.5.2 ZtfA is a positive regulator of austinol cluster genes and is required for austinol and dehydroaustinol biosynthesis

High-performance liquid chromatography (HPLC) was employed to analyze production of further secondary metabolites in *ztfA* mutants. Secondary metabolites were extracted with ethyl acetate from cultures grown for three and seven days in light or dark. Austinol and dehydroaustinol were identified according to their masses and UV/VIS absorption maxima (Szewczyk *et al.*, 2008) in SM samples extracted from asexually grown WT and *ztfA* OE cultures after both, three and seven days of asexual growth (light) (FIGURE 30A), but not during sexual growth (dark) (FIGURE 30B). Both compounds were absent in HPLC measurements from SM samples extracted from Δ*ztfA* cultures (FIGURE 30A and B). The production of austinol and dehydroaustinol during asexual growth is restored by reintroduction of the *ztfA* gene (*ztfA* comp) into the knock out mutant (FIGURE 30A).

Transcriptional analyses were conducted to investigate whether the HPLC measurements correlate to transcriptional changes of genes of the austinol cluster in the *ztfA* mutants. Expression of three genes, which products are involved in the austinol and dehydroaustinol synthesis pathway, was analyzed in vegetatively grown cultures: *ausA*, coding for a polyketide synthase producing 3,5-dimethyl orsellinic acid, the first intermediate in the austinol and dehydroaustinol biosynthesis pathway, as well as *ausH*, whose gene product is

required for production of both secondary metabolites (Lo *et al.*, 2012; Nielsen *et al.*, 2011). The third tested gene, *ausF*, codes for a protein required for austinol and dehydroaustinol production at WT levels (Lo *et al.*, 2012). The *ausA* and *ausF* transcripts were not expressed during vegetative growth, neither in WT nor in the Δ*ztfA* or the *ztfA* comp strain. *ausH* gene expression could be detected in WT and *ztfA* comp, but not in Δ*ztfA*. *ztfA* OE is sufficient to induce gene expression of all three genes (FIGURE 31). This shows that ZtfA activates *aus* (austinol cluster) gene expression.
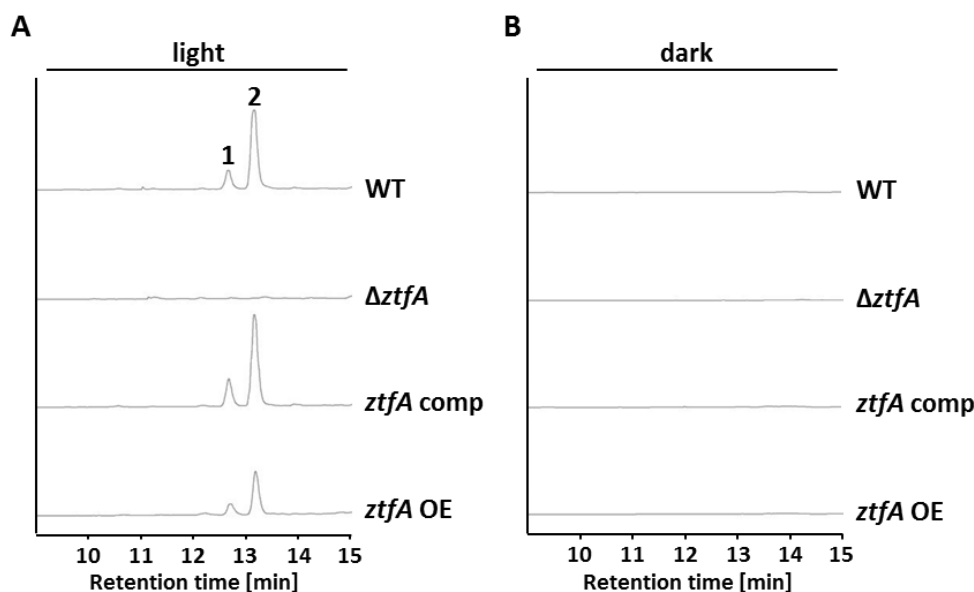
FIGURE 30: *ztfA* **is necessary for austinol and dehydroaustinol production.**
Secondary metabolites were extracted from cultures grown for 3 d in light or dark on solid MM plates at 37°C. SM profiles shown resemble these after 7 d. A) Austinol (1) and dehydroaustinol (2) are not produced in the absence of *ztfA*, but in WT, *ztfA* comp and *ztfA* OE during asexual growth (light). B) Austinol and dehydroaustinol are absent in SM samples extracted from sexually grown cultures (dark).

Neither austinol nor dehydroaustinol were identified in SM samples extracted from *ztfA* OE grown in liquid submerged cultures for 48 h (FIGURE 32). This indicates that the upregulation of austinol cluster genes in the *ztfA* OE strain is not sufficient to induce austinol and dehydroaustinol production during vegetative growth. The Δ*ztfA* strain produces compounds during vegetative growth, which were identified as peaks at 21 and 21.5 min retention time and thereafter with HPLC (FIGURE 32, in red). These compounds were absent in WT, as well as in *ztfA* comp and *ztfA* OE strains. These peaks might represent SMs, which are not produced in WT under these conditions or SM intermediates, where formation of the final product is blocked in the absence of *ztfA*. This finding underlines the importance of ZtfA for secondary metabolite production in *A. nidulans*.

Taken together, ZtfA acts as activator of genes of the austinol and dehydroaustinol cluster and is important for regulation of further, yet unidentified, SMs.
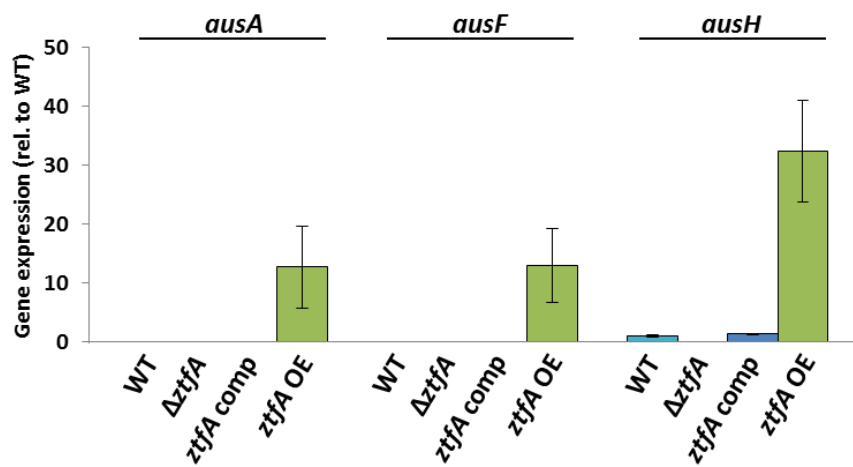
FIGURE 31: Overexpression of *ztfA* leads to an upregulation of *aus* genes during vegetative growth.
Gene expression of selected *aus* genes measured with qRT-PCR. Note that values with a quantification cycle (Cq) > 30 were considered as not expressed and are therefore not shown. $1*10^7$ spores were grown for 24 h under submerged culture conditions. Data from three biological and three technical replicates is shown.
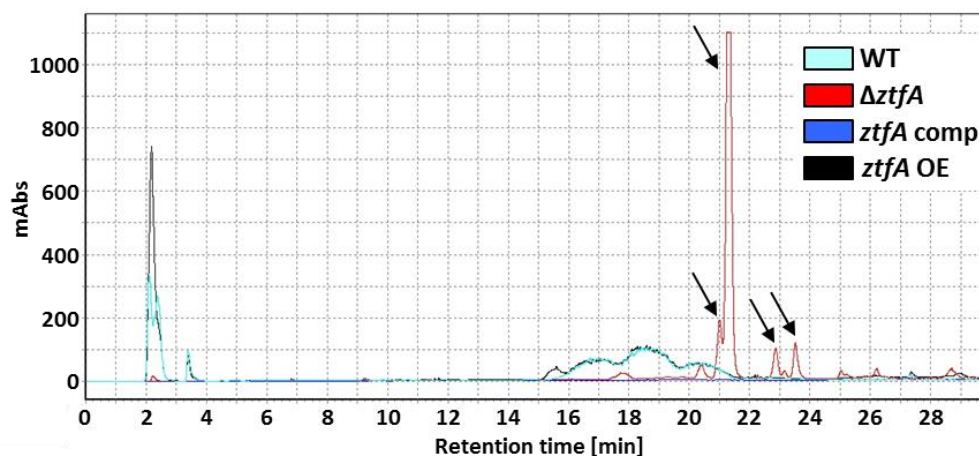
FIGURE 32: Further secondary metabolites are produced in the absence of *ztfA* during vegetative growth.
Strains were grown vegetatively for 48 h and subsequently secondary metabolites were extracted with ethyl acetate and analyzed with HPLC. Δ*ztfA* (red) produces several unidentified compounds, which are not found in WT (light blue), *ztfA* comp (dark blue) or *ztfA* OE (black). Examples of these compounds are indicated by arrows. mAbs = milli absorbance units.

3.5.3 ZtfA activates expression of emericellamide and orsellinic acid cluster genes

Gene expression of novel ZtfA controlled secondary metabolite genes was tested in an attempt to better understand the regulatory effects of ZtfA upon SM expression in *A. nidulans*.

orsA encodes the polyketide synthase (PKS) of the F9775 secondary metabolite gene cluster and is required for biosynthesis of orsellinic acid and its derivatives F9775A, F9775B and lecanoric acid (Bok *et al.*, 2009; Gressler *et al.*, 2015; Sanchez *et al.*, 2010; Schroeckh *et al.*, 2009). *orsA* is basally expressed in WT and *ztfA* comp but significantly upregulated in *ztfA* OE during vegetative growth (FIGURE 33). In contrast, *orsA* is not expressed in the absence of *ztfA*. This shows that *ztfA* is required for *orsA* gene expression during vegetative growth.

Gene expression of four representatives of the emericellamide synthesis cluster was analyzed as well (*easA*, *easB*, *easC* and *easD*). *easA* and *easB* code for the non-ribosomal peptide synthase and the PKS of the emericellamide cluster, respectively (Chiang *et al.*, 2008). *easC* and *easD* code for an acyltransferase and an acyl-CoA ligase, respectively (Chiang *et al.*, 2008). *easA* and *easD* are expressed in WT and *ztfA* comp during vegetative growth (FIGURE 33). *easA*, but not *easB*, *easC* or *easD*, is expressed under same conditions in Δ*ztfA*. In contrast, *easA*, *easB*, *easC* and *easD* are highly upregulated in *ztfA* OE. This shows that ZtfA activates the emericellamide gene cluster.
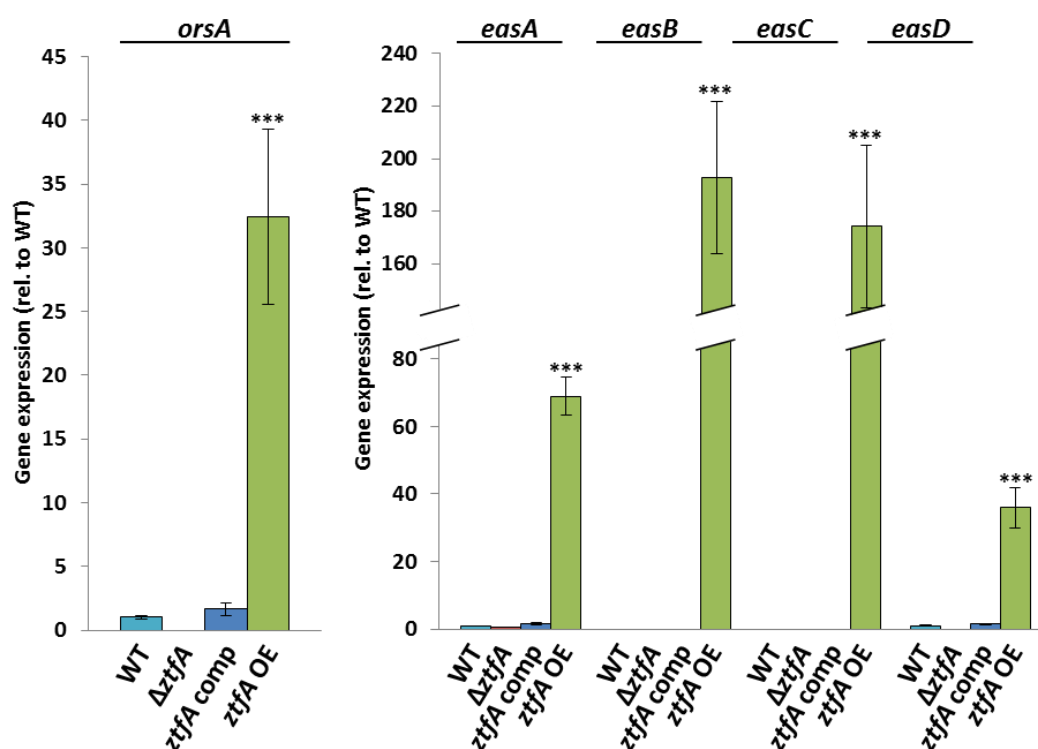
FIGURE 33: ZtfA is an activator of *eas* and *orsA* gene expression.
Gene expression of *orsA*, *easA*, *easB*, *easC* and *easD* was measured with qRT-PCR in vegetatively grown cultures. *orsA*, *easB*, *easC* and *easD* are not expressed in the absence of *ztfA*. Note that values with a quantification cycle (Cq) > 30 were considered as not expressed and are therefore not shown. Data from three biological and three technical replicates is shown (***$P < 0.005$).

3.6 ZtfA is involved in the regulation of an appropriate oxidative stress response in *A. nidulans*

3.6.1 ZtfA reduces the cellular response to hydrogen peroxide induced stress

The adaptive response to oxidative stress is crucial for developmental programs in fungi, since reactive oxygen species (ROS), which are by-products of metabolic functions, can damage all kinds of biomolecules. Tests with H_2O_2 as stressor were executed to analyze a possible influence of ZtfA upon cellular response to oxidative stress. The loss of *ztfA* leads to an increased tolerance towards oxidative stress (FIGURE 34). This suggests a repressive role of ZtfA in the regulation of the oxidative stress response (OSR) in *A. nidulans*.
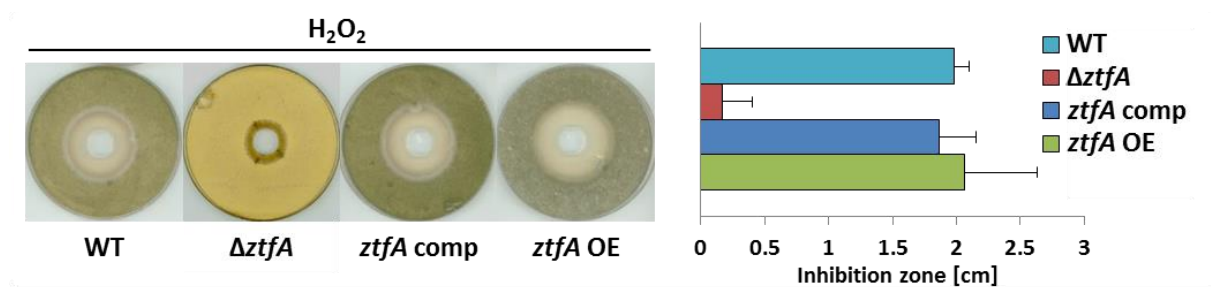
FIGURE 34: ZtfA is involved in the oxidative stress response.
Loss of *ztfA* leads to an increased tolerance towards H_2O_2. Spores were plated on solid MM and plugs of agar were cut out and filled with 150 μl 1% (v/v) H_2O_2. Inhibition zones were measured after 2 d growth in light at 37°C.

3.6.2 ZtfA regulates redox systems in *A. nidulans*

Transcriptional analyses were carried out to investigate regulatory influences of ZtfA upon genes, which products are involved in the OSR. The glutathione and the thioredoxin systems are core elements of the fungal OSR (Moye-Rowley, 2003). Consequently, expression of genes encoding parts of these redox systems was monitored by qRT-PCR during vegetative growth in unstressed cultures in comparison with cultures which were treated with 5 mM H_2O_2 for 30 min.

glrA encoding a glutathione reductase (Bakti *et al.*, 2017; Sato *et al.*, 2009) is upregulated in WT, *ztfA* comp and *ztfA* OE in response to H_2O_2. In Δ*ztfA*, only a very slight induction below threshold of two fold is found (FIGURE 35). The thioredoxin system-encoding genes *trxA* (thioredoxin) and *trxR* (thioredoxin reductase) were tested as well. *trxA* is induced in WT, *ztfA* comp and *ztfA* OE about two to four times upon H_2O_2 treatment compared to unstressed situation. In Δ*ztfA* induction upon treatment with H_2O_2 is below threshold (FIGURE 35). In addition, *trxR*, which is induced about three fold in WT in the presence of H_2O_2, is not

induced in Δ*ztfA* (FIGURE 35). *ztfA* OE induces *trxR* expression above six fold in the presence of H_2O_2. These findings indicate that ZtfA is necessary to induce *trxR* expression upon oxidative stress.
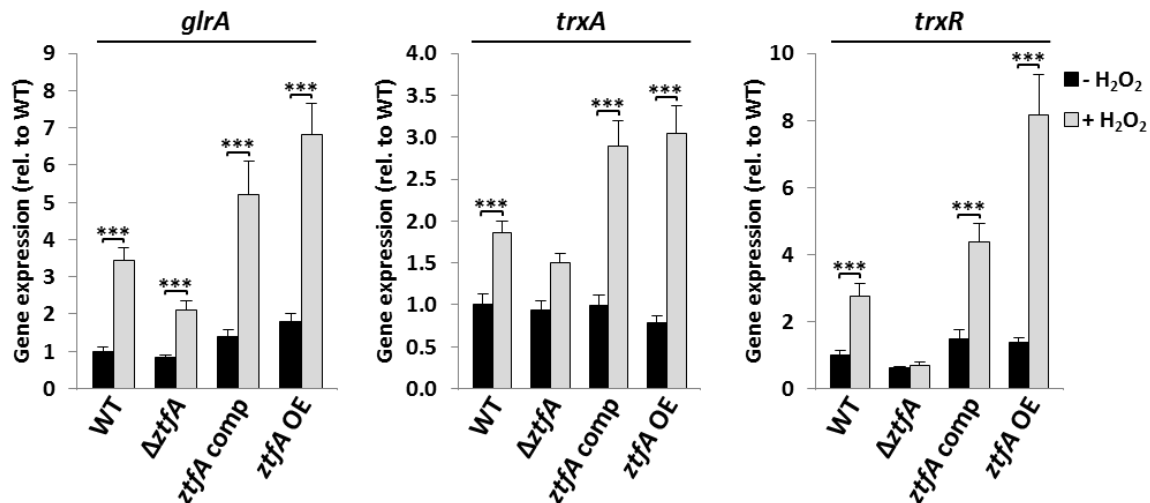
FIGURE 35: ZtfA regulates gene expression of the fungal redox systems.
qRT-PCR analyses show that ZtfA regulates parts of the thioredoxin systems in response to H_2O_2. Submerged cultures were incubated with $1*10^7$ spores per strain and grown for 24 h at 37°C on a rotary shaker. Subsequently, cultures were supplemented with (grey) or without (black) 5 mM hydrogen peroxide and incubated for 30 min at 37°C on a rotary shaker (***$P < 0.005$).

3.6.3   ZtfA activates *catA* gene expression for catalase A in response to H_2O_2

Gene expression of *catA* and *catB* was analyzed to test whether ZtfA is involved in the regulation of catalase expressing genes as well. The expression of *catA*, encoding the spore-specific catalase A, is four fold upregulated in WT and *ztfA* comp, but the gene is not induced in Δ*ztfA* in presence of H_2O_2 in comparison to unstressed situation (FIGURE 36).

Expression of *catA* in *ztfA* OE, where the gene was already upregulated about eight fold in unstressed situation compared to WT, is induced about six fold in the presence of H_2O_2 compared to unstressed growth (about 38 fold compared to WT unstressed growth). *catB*, which codes for the hyphal catalase B, is not significantly upregulated in WT, *ztfA* comp or Δ*ztfA*. In *ztfA* OE, however, *catB* expression is four fold induced in the presence of H_2O_2 in contrast to the non-stressed situation. These findings indicate a necessity of ZtfA for appropriate activation of the enzymatic OSR. Furthermore, an upregulation of genes of redox systems or catalase encoding genes is not sufficient to induce an appropriate OSR. Other factors seem to be more important for the fungal OSR, which were not tested. These might be other enzymes, such as further catalases or catalase-peroxidases.
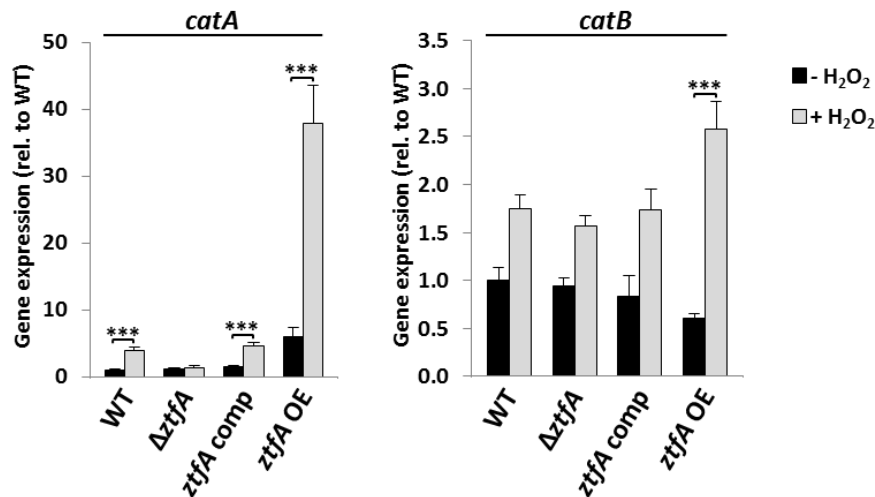
FIGURE 36: ZtfA regulates expression of *catA* and *catB* in response to oxidative stress.
qRT-PCR indicates that ZtfA is necessary for the upregulation of *catA* gene expression and sufficient to induce *catB* expression in the presence of H_2O_2. Submerged cultures were incubated with $1*10^7$ spores per strain and grown for 24 h at 37°C on a rotary shaker. Subsequently, cultures were supplemented with (grey) or without (black) 5 mM hydrogen peroxide and incubated for 30 min at 37°C on a rotary shaker (***$P < 0.005$).

3.6.4 ZtfA regulates gene expression for transcription factors during oxidative stress

RsmA (remediator of secondary metabolism A) is a transcription factor, which is involved in the regulation of both, secondary metabolism and OSR (Emri *et al.*, 2015; Yin *et al.*, 2013). *rsmA* gene expression is induced in WT when H_2O_2 stress is applied (FIGURE 37). In *ztfA* OE *rsmA* expression is strongly upregulated in the presence of H_2O_2. In Δ*ztfA*, *rsmA* expression is not induced during H_2O_2 stress. This shows that ZtfA is involved in upregulation of *rsmA* gene expression during oxidative stress. *ztfA* itself is upregulated in WT, *ztfA* comp and in *ztfA* OE upon addition of H_2O_2 in comparison to an unstressed situation (FIGURE 37).

This activation of *ztfA* gene expression by oxidative stress underlines its involvement in the oxidative stress response regulation. In contrast to these findings, the most prominent oxidative stress regulator in *A. nidulans*, encoded by *napA* (*A. nidulans* AP-1 homolog A), was not found to be upregulated under conditions applied in this study (FIGURE 37).

Taken together, ZtfA is involved in the regulation of the oxidative stress response via expression regulation of redox, catalase and transcription factor encoding genes. However, expression of the genes tested in this study is not sufficient to induce an appropriate OSR as they were not upregulated in the absence of *ztfA* and still, the strain is more tolerant towards H_2O_2. This shows that additional ZtfA controlled OSR genes are important for the cellular response to oxidative stress, which have not been identified as ZtfA targets yet. The increased tolerance of the Δ*ztfA* strain suggests that these are repression targets of ZtfA.
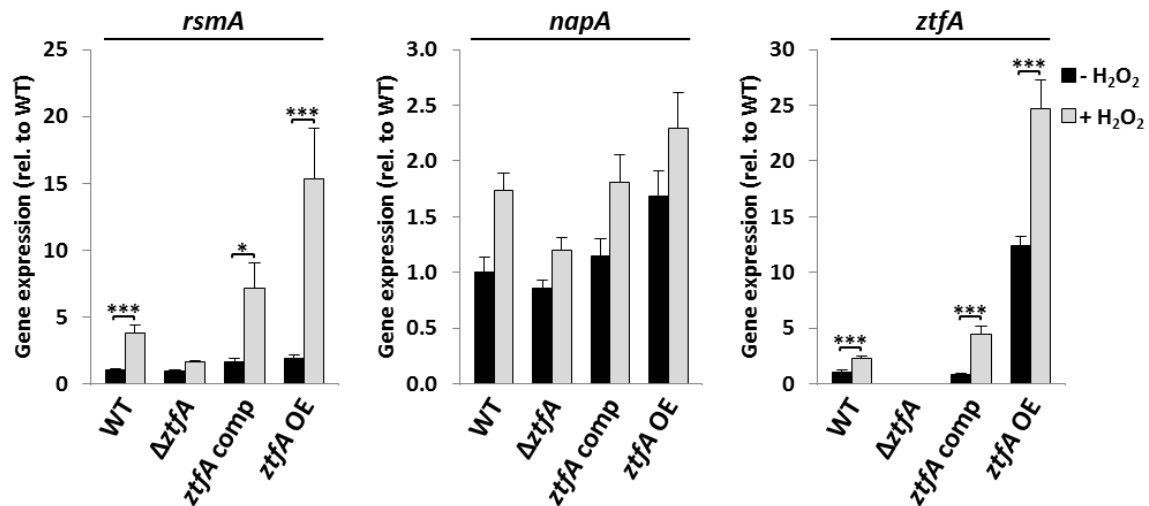
FIGURE 37: ZtfA regulates other transcription factor-encoding genes in response to oxidative stress.
rsmA upregulation in response to H_2O_2 is ZtfA-dependent. *napA* is not regulated by ZtfA under these conditions. *ztfA* expression is upregulated in response to H_2O_2. Submerged cultures were incubated with $1*10^7$ spores per strain and grown for 24 h at 37°C on a rotary shaker. Subsequently, cultures were supplemented with (grey) or without (black) 5 mM hydrogen peroxide and incubated for 30 min at 37°C on a rotary shaker (*$P < 0.05$, ***$P < 0.005$).

3.7 ZtfA forms protein-complexes in nuclei

3.7.1 ZtfA is localized in the nucleus

Transcription factors need to be localized in the nucleus in order to execute DNA-binding and regulation of gene expression. In accordance with the assumption that ZtfA is a nuclear protein, CELLO (Yu *et al.*, 2004) and WoLF PSORT (Horton *et al.*, 2007) predict ZtfA to be localized exclusively in the nucleus with almost 100% probability. ZtfA was fused to sGFP to examine subcellular localization *in vivo*. An N- and C-terminally tagged version (sGFP-ZtfA and ZtfA-sGFP) was constructed and expressed in the Δ*ztfA* strain to analyze, whether both versions are functional. Both fusions were expressed under control of the native *ztfA* promoter and complemented the loss of *ztfA* (FIGURE 38A). Whereas ZtfA-sGFP produces slightly less conidiospores compared to WT, sGFP-ZtfA produces slightly more spores (FIGURE 38B). Expression of both *sgfp*-tagged versions of *ztfA* is slightly upregulated during vegetative growth in both GFP strains (FIGURE 38C). Taken together, both GFP-tagged versions of ZtfA are functional and mostly complement the loss of *ztfA*.

The predicted molecular masses of the fusion proteins are 87.46 kDa in both cases. Western hybridization experiments show slightly higher molecular masses (FIGURE 39A). This indicates possible post-translational modifications of ZtfA. Fluorescence microscopy reveals a

subcellular localization of both versions of the ZtfA fusion protein in nuclei of hyphae during all growth conditions tested (vegetatively, asexually and sexually grown) as well as in conidiospores (FIGURE 39B) and germlings (FIGURE 39C).
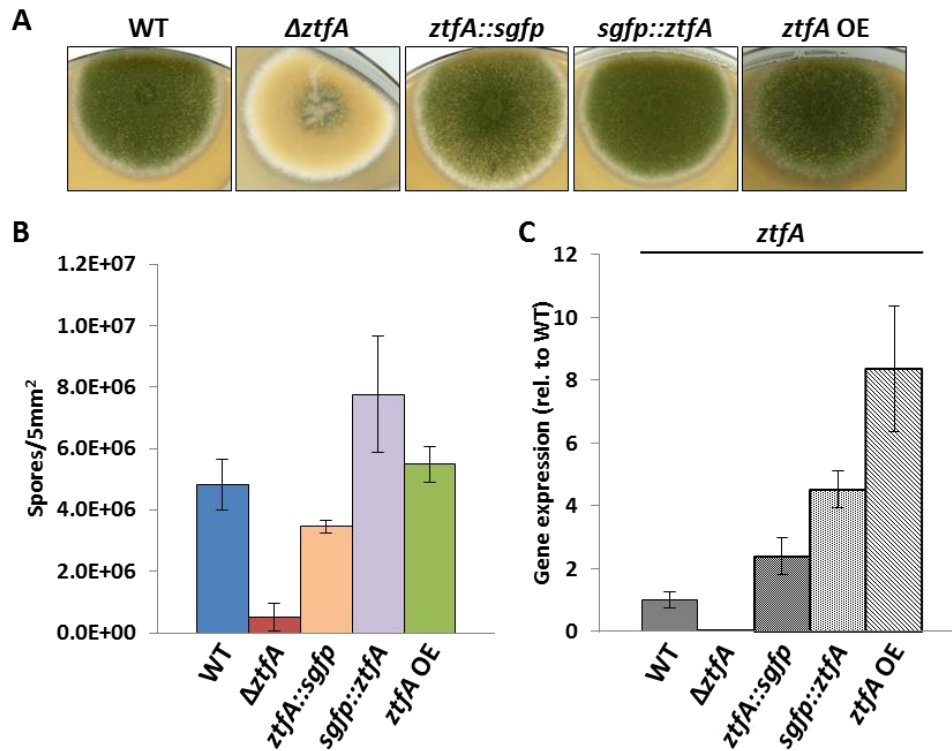
FIGURE 38: GFP-fusions of ZtfA are functional.
A) Strains expressing an N- or C-terminally tagged version of ZtfA (*sgfp::ztfA* and *ztfA::sgfp*) in the Δ*ztfA* background are shown. Expression of both GFP-tagged versions of ZtfA complements the Δ*ztfA* phenotype. Strains were point inoculated on solid MM and grown for 6 d in light. B) Strains were point inoculated for 6 d in light. Spores were counted from agar plugs with a diameter of 5 mm^2. Four plugs were cut out per plate and the average was calculated. Error bars indicate standard deviation from three biological repetitions. C) qRT-PCR shows that *ztfA* is slightly overexpressed in GFP fusions compared to WT. RNA was extracted from strains grown in submerged cultures for 24 h (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).
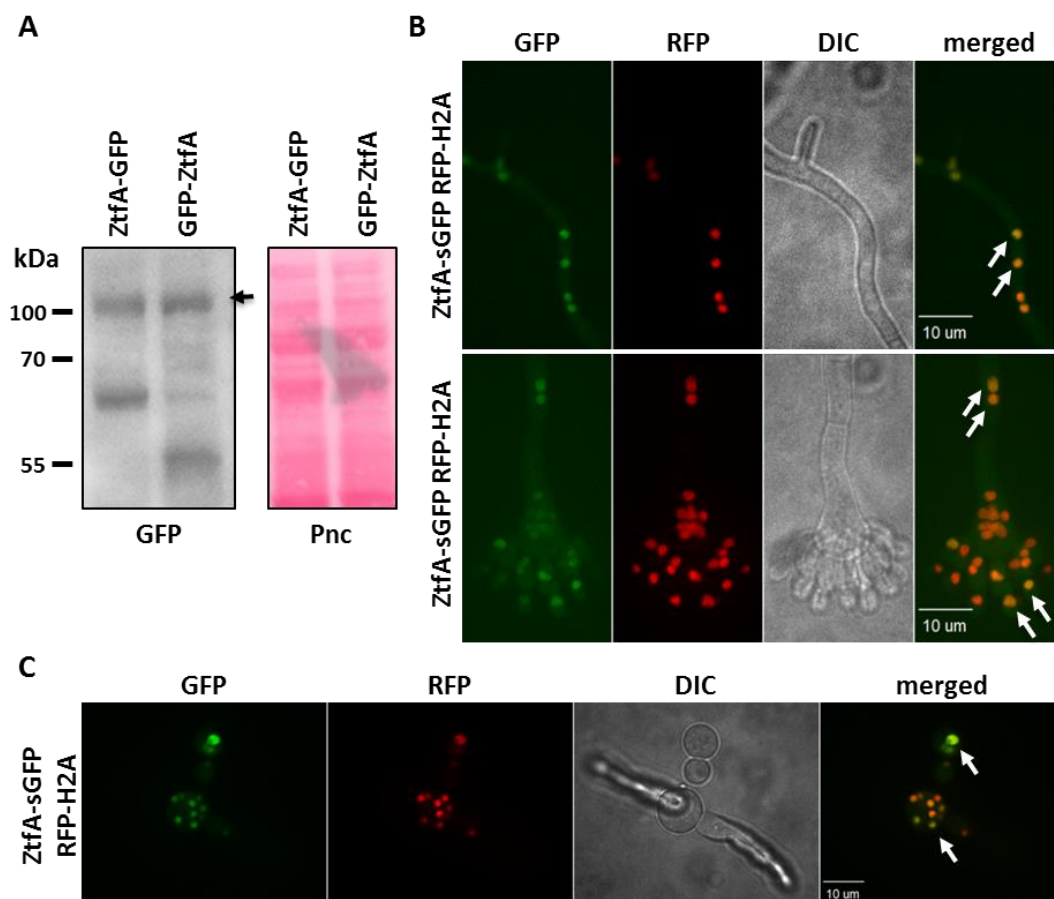
FIGURE 39: GFP-fusions of ZtfA are localized in the nucleus of hyphae, conidiospores and germlings.
A) Western hybridization shows expression of ZtfA-sGFP and sGFP-ZtfA under native promoter. Ponceau stained membrane (Pnc) is shown as loading control. The black arrow indicates the protein band representing the full-length fusion protein. B) and C) Fluorescence microscopic photos of ZtfA-sGFP (green) in ΔztfA background. RFP-H2A was introduced into this strain to allow identification of nuclei (red). White arrows show co-localization of GFP and RFP signals. Strains were grown for 24 h in submerged culture (upper panel) or for 36 h on solid MM to induce asexual differentiation (lower panel and C).

3.7.2 ZtfA is phosphorylated at S327, T464 and S506, respectively

Western hybridization show higher molecular masses for the ZtfA GFP-fusion proteins and therefore indicates post-translational modifications (FIGURE 39). To investigate this possibility, the phosphorylation status of ZtfA was monitored. The *in silico* prediction of phosphorylation sites in the ZtfA amino acid sequence with NetPhos 3.1 (Blom *et al.*, 1999) predicted 24 serine, ten threonine and four tyrosine residues to be phosphorylated (score value between 0 and 1, cutoff >0.7) (FIGURE 40A). LC-MS/MS analyses were conducted to investigate these putative phosphorylation sites in more detail. The peptide coverage of the whole ZtfA protein in LC-MS/MS analyses was around 40%.
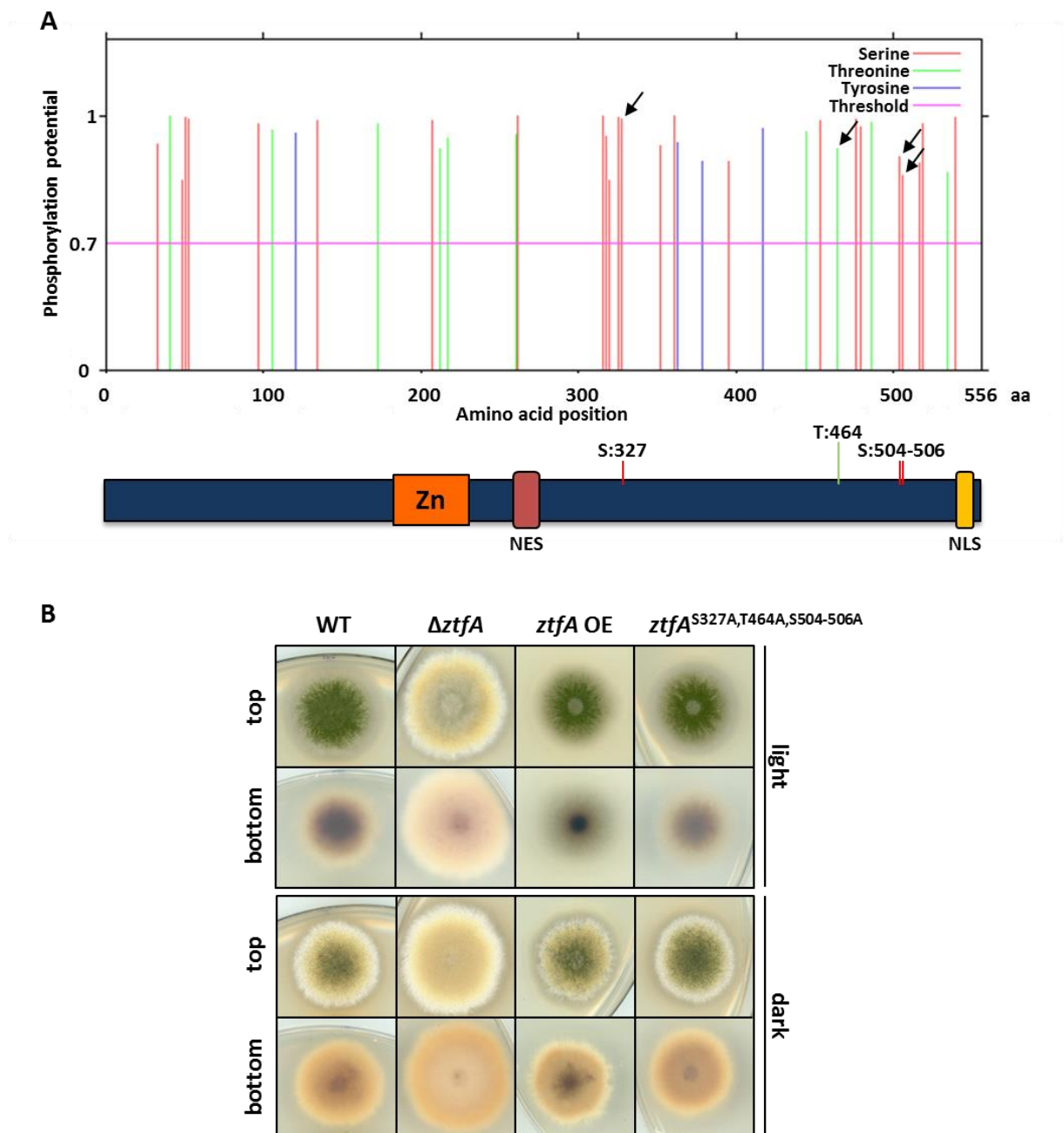
FIGURE 40: Phosphorylation of ZtfA has regulatory effects.
A) Putative phosphorylation sites in the ZtfA amino acid sequence, determined with NetPhos 3.1 (Blom *et al.*, 1999). The cutoff was set to 0.7 (score values from 0 to 1). Arrows indicate phosphorylation sites, which were mutated in a strain that expresses a permanently dephosphorylated ZtfA (*ztfA*$^{S327A,T464A,S504-506A}$). A model of the ZtfA protein is shown (dark blue). Mutated amino acid (aa) residues and their position within the amino acid sequence are indicated. S = serine, T = threonine, NLS = nuclear localization sequence, NES = nuclear export signal, Zn = zinc cluster. B) Colony morphology of the *ztfA*$^{S327A,T464A,S504-506A}$ strain in comparison to WT, Δ*ztfA* and *ztfA* OE strains. Permanently dephosphorylated version of ZtfA (*ztfA*$^{S327A,T464A,S504-506A}$) shows a lacerated phenotype when point inoculated and grown in light (left side) and might produce more conidiophores in the dark (right side). Strains were point inoculated and grown for 3 d in light and dark at 37°C.

Three phosphorylation sites for ZtfA were found in vegetatively grown cultures: a serine residue at position 327 (S327), a threonine residue at position 464 (T464) and a serine residue at position 506 (S506). At the latter position the protein exhibits a short stretch of three serines, spanning from position 504 to 506. All of these identified positions (S327, T464 and S506) were predicted by NetPhos 3.1 (FIGURE 40A). A mutant strain was constructed, in which all three serines at position 504 to 506, the serine at position 327 and the threonine at position 464 were replaced by alanine to mimic permanent dephosphorylation ($ztfA^{S327A,T464A,S504-506A}$). The respective genetic construct was integrated in $\Delta ztfA$ background and rescues conidiophore formation (FIGURE 40B). The $ztfA^{S327A,T464A,S504-506A}$ strain shows lacerated colony morphology comparable to the $ztfA$ OE phenotype when point inoculated and grown in light. These findings suggest that the $ztfA$ OE strain might produce more insufficiently phosphorylated ZtfA protein and that correct ZtfA phosphorylation is important for WT colony morphology.

3.7.3   ZtfA pulls down several proteins in GFP-trap experiments

LC-MS/MS showed phosphorylated amino acid residues in ZtfA. GFP-trap pull-downs, followed by LC-MS/MS analyses, were executed with both versions of GFP-fusion proteins of ZtfA (sGFP-ZtfA and ZtfA-sGFP) to analyze, if ZtfA interacts with phosphatases or kinases and other proteins. Several proteins were identified in these experiments (FIGURE 41). GFP-trap pull-downs were conducted with cultures grown vegetatively, asexually and sexually.
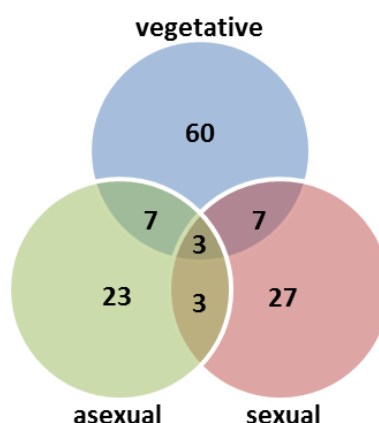
FIGURE 41: ZtfA pulls down proteins as putative interactions partners.
GFP-trap pull-downs with ZtfA followed by LC-MS/MS revealed several proteins, which might be putative interactions partners. Protein compositions differ in vegetative (blue), asexual (green) and sexual (red) growth. Numbers represent proteins identified in at least two biological repetitions with unique peptides ≥ 3 and MS/MS counts ≥ 3 and which were absent in the negative control.

Proteins with three or more unique peptides (Zhao and Lin, 2010) and three or more MS/MS counts identified in at least two out of three biological repetitions were considered for further analyses. Proteins for which also unique peptides or MS/MS counts were found in the negative control ($^{P}gpdA$::*gfp*) were excluded from further analyses. In vegetative cultures GFP-tagged ZtfA pulled 77 and in asexually grown cultures 36 proteins, respectively (FIGURE 41). 40 proteins were identified from sexually grown cultures. Out of these, seven proteins were identified in both, vegetative and asexual samples and seven proteins were identified in both, vegetative and sexual samples. Three proteins were identified in samples from asexually and sexually grown strains and three (including ZtfA bait) were identified in samples of all three growth states. Identified candidates were scanned for kinases, phosphatases, potential transcription factors, DNA binding proteins, nuclear transporters and proteins involved in chromatin remodeling. These criteria were chosen to investigate, whether the group of identified proteins comprises candidates, which possibly might support ZtfA in its regulatory roles. Identified proteins with these functions, are given in TABLE 7. Proteins with these roles, which were identified in two or more biological replicates below threshold, are given in TABLE 8. A comprehensive list of all proteins identified with the above mentioned threshold (three or more unique peptides and three or more MS/MS counts) is given in TABLE 9.

TABLE 7: Selection of proteins identified in GFP pull-downs with ZtfA, followed by LC-MS/MS.
For pull-downs ZtfA-sGFP and sGFP-ZtfA were used as bait. Descriptions from AspGD (Cerqueira *et al.*, 2014) are given. Sys. name = systematic name, Std. name = standard name, Ident. in = identified in (refers to the growth state of the sample): v = vegetative, a = asexual, s = sexual. Unchar. = uncharacterized protein. * = RcoA was identified in two biological replicates in asexual growth, but below threshold in one of these two biological replicates.

Sys. name	Std.name	Description	Ident. in
Nuclear transport			
AN6734	KapF	Essential karyopherin (importin)	v
AN2120	KapJ	Karyopherin (importin)	v, a
AN0906	KapB	Essential karyopherin (importin)	a, s
AN5717	KapI	Non-essential karyopherin family protein; required for normal hyphal growth and conidial development	a, s
Chromatin remodeling			
AN6705	unchar.	Ortholog of *S. pombe* Ssr2 (subunit of chromatin remodeling complex)	v
AN5102	unchar.	Putative ortholog of *S. pombe* Spt16 (FACT complex subunit)	s
AN6687	unchar.	Putative ortholog of *S. pombe* Pob3 (FACT complex subunit)	s

Transcription factors

AN2012	RfeF	Putative transcription factor	v
AN6505	RcoA	Tup1 homolog of *S. cerevisiae* repressor domain, WD40 repeat protein; required for sexual development and for sterigmatocystin production, RcoA-SsnF repressor complex member	v, s, (a)*

Kinases

AN2943	RfeA	Putative regulatory role in secondary metabolism, protein kinase domain	a

TABLE 8: Proteins identified in at least two out of three biological repetitions, but below threshold in GFP pull-downs with ZtfA.

For pull-downs both ZtfA-sGFP and sGFP-ZtfA were used as bait. Descriptions from AspGD (Cerqueira *et al.*, 2014) are given. Sys. name = systematic name, Std. name = standard name, Ident. in = identified in (refers to the growth state of the sample): v = vegetative, a = asexual, s = sexual. Unchar. = uncharacterized protein. All putative interactions partners given here were identified in two or more biological replicates, but identification was below threshold.

Sys. name	Std. name	Description	Ident. in
RNA maturation and processing			
AN0327	unchar.	Putative RuvB-like helicase 2	a, s
AN1971	unchar.	Putative RuvB-like helicase 1	v, s
AN10944	unchar.	Putative ortholog Cdc5 (*S. pombe*) is involved in mRNA splicing	s
DNA-binding			
AN0242	unchar.	Putative transcription factor (putative ortholog of *S. pombe* Snd1)	s
AN0809	unchar.	DNA binding activity	s
Signaling			
AN3719	MpkB	MAP kinase, involved in regulation of secondary metabolism and developmental programs	v

The majority of the identified proteins (85 out of 129) pulled down with ZtfA are uncharacterized proteins. Two protein kinases were identified: the mitogen-activated protein (MAP) kinase MpkB and the uncharacterized RfeA protein. MpkB might be important for phosphorylation of ZtfA. RfeA is uncharacterized in *A. nidulans* but *in silico* analyses indicate that it is a protein kinase with a predicted role in secondary metabolism. Interactions with kinases are of short duration what explains the relatively low MS/MS counts and unique peptides identified for MpkB.

Four karyopherins (importins) were identified in total. This indicates a strong nuclear import for ZtfA. The putative, so far uncharacterized, transcription factor RfeF was identified solely in vegetative samples. Notably, the transcription repressor RcoA was found in samples of all three developmental stages, even though in asexual samples, RcoA was found slightly below

the above mentioned threshold (it was identified above threshold in one, but below threshold in a second biological replicate). RcoA is involved in asexual development and secondary metabolism in *A. nidulans* (Hicks *et al.*, 2001). This finding indicates that RcoA and ZtfA might form complexes during all parts of the *A. nidulans* life cycle.

TABLE 9: Comprehensive list of proteins identified in GFP-trap pull-downs with sGFP-tagged ZtfA (sGFP-ZtfA and ZtfA-sGFP) as bait.
Proteins were identified in at least two out of three biological replicates with a threshold of $3 \geq$ MS/MS counts and $3 \geq$ unique peptides, according to functional groups. Descriptions from AspGD (Cerqueira *et al.*, 2014) are given. Proteins identified solely in vegetative samples are highlighted in blue, proteins identified solely in developmental samples are given in green, proteins identified in vegetative and developmental samples are given in orange. Sys. Name = systematic name, std. name = standard name, ident. in = identified in, v = vegetative, a = asexual, s = sexual, [1] = ZtfA was used as bait, * = RcoA was identified in two biological replicates in asexual growth, but below threshold in one of these two biological replicates.

Sys. name	Std.name	Description	Ident. in
Bait			
AN0585	ZtfA[1]	Activator of asexual development and secondary metabolism	v, a, s
Nuclear transport			
AN6734	KapF	Essential karyopherin (importin)	v
AN2120	KapJ	Karyopherin (importin)	v, a
AN5717	KapI	Non-essential karyopherin family protein; required for normal hyphal growth and conidial development	a, s
AN0906	KapB	Essential karyopherin (importin)	a, s
Transcription/chromatin			
AN2012	RfeF	Putative transcription factor	v
AN6505	RcoA	Tup1 homolog of *S. cerevisiae* repressor domain, WD40 repeat protein; required for sexual development and for sterigmatocystin production, RcoA-SsnF repressor complex member	v, s, (a)*
AN6705	unchar.	Ortholog of *S. pombe* Ssr2 (subunit of chromatin remodeling complex)	v
AN5102	unchar.	Putative ortholog of *S. pombe* Spt16 (FACT complex subunit)	s
AN6687	unchar.	Putative ortholog of *S. pombe* Pob3 (FACT complex subunit)	s
RNA processing			
AN10557	unchar.	Putative ATP-dependent RNA helicase, putative *A. fumigatus* ortholog Ded1	v
AN5931	unchar.	Putative ATP-dependent RNA helicase, putative *S. cerevisiae* ortholog of Dbp2	v
AN7659	unchar.	Putative ortholog of *S. pombe* Dbp5 (RNA helicase)	v
AN0646	unchar.	Putative ortholog of *S. pombe* Upf1 (ATP-dependent RNA helicase)	s
AN6004	unchar.	Protein with an RNA recognition motif, putative ortholog of *S. pombe* Vip1	v

AN10257	unchar.	Putative ortholog of *C. albicans* Pbp2 (putative RNA binding protein)	v
AN2068	unchar.	Putative ortholog of *S. pombe* Vgl1 (RNA binding protein)	v, a
AN1408	unchar.	Putative U5 snRNP-specific protein, putative ortholog of *S. pombe* Cwf1	s
AN0111	unchar.	Putative ortholog of *S. pombe* and *S. cerevisiae* Syf1 (pre-mRNA splicing factor)	s
AN0289	unchar.	Putative ortholog of *S. pombe* Cwf22 (pre-mRNA splicing factor)	s
AN1208	unchar.	Putative ortholog of *S. pombe* Prp5 (pre-mRNA splicing factor)	s
AN4523	Prp8	Putative mRNA-splicing protein	s
AN0310	unchar.	Putative ortholog of *S. pombe* Pwp1 (RNA processing protein)	s
AN6906	unchar.	Putative ortholog of *S. pombe* Prp19 (pre-mRNA processing factor)	s
AN11052	unchar.	Putative ortholog of *S. pombe* Exo2 (exonuclease)	v

Translation

AN3413	unchar.	Putative ribosomal protein S2 and S5, putative ortholog of *S. cerevisiae* Rps2	a
AN0074	unchar.	Putative ortholog of *S. cerevisiae* Ebp2 (required for 25S rRNA maturation and 60S ribosomal subunit assembly)	s
AN0247	unchar.	Putative ortholog of *S. pombe* Nat10 (ribosome biogenesis ATPase)	s
AN10740	unchar.	Ortholog of Afu2g07970 (60S ribosomal protein L19), putative ortholog of *S. pombe* Rpl1902	s
AN1095	unchar.	Putative ortholog of *S. pombe* Mrpl10 (predicted ribosomal protein subunit L15)	s
AN3167	Nop58	Putative ribosome biogenesis protein	s
AN6902	unchar.	Putative ribosomal protein, putative ortholog of *S. cerevisiae* Mrt4	s
AN10182	unchar.	Putative translation initiation factor 3, subunit f (eIF-3f)	v
AN4038	unchar.	Putative translation initiation factor eIF5B	v
AN7540	unchar.	Putative ortholog of *S. pombe* Moe1 (translation initiation factor eIF3d)	v
AN6060	unchar.	Ortholog of *A. fumigatus* eukaryotic translation initiation factor subunit eIF-4F	v
AN7350	unchar.	Ortholog of *A. fumigatus* translation initiation factor 4B	v
AN1158	unchar.	Putative ortholog of *S. cerevisiae* Ssd1 (translational repressor)	v, s
AN10475	unchar.	Putative ortholog of *S. pombe* Wrs1 (tryptophan-tRNA ligase)	v
AN4086	unchar.	Putative ortholog of *S. pombe* Frs1 (phenylalanine-tRNA ligase)	v
AN8867	unchar.	Putative ortholog of *S. pombe* Srs1 (serine-tRNA ligase)	v
AN1913	unchar.	Putative lysyl-tRNA synthetase, putative ortholog of *S. pombe* Krs1	a
AN8224	unchar.	Putative ortholog of *S. pombe* cytoplasmic glutamate-tRNA ligase Gus1 (predicted)	a
AN3702	unchar.	Putative ortholog of *S. pombe* Lrs1 (leucine-tRNA ligase)	s
AN0705	unchar.	Putative ortholog of *S. pombe* Irs1 (isoleucine-tRNA ligase)	v, s
AN10474	unchar.	Has domain(s) with predicted tRNA binding activity, putative ortholog of *S. cerevisiae* Arc1	v, s

Protein folding/chaperons

AN0858	Hsp104	Putative chaperone	v
AN5713	Cct7/CctA	Putative chaperonin complex component, TCP-1 eta subunit; ortholog of *S. cerevisiae* Cct7p	a
AN2149	Cct1	Putative chaperonin complex component, TCP-1 alpha subunit; ortholog of *S. cerevisiae* Tcp1p	s

AN10351	unchar.	Putative ortholog of *S. pombe* Aap1 (aspartyl metalloaminopeptidase, chaperone-mediated protein folding)	v
AN3592	ClxA	Putative calnexin with a predicted role in protein folding and protein quality control on the ER membrane	v
AN4583	Cyp7/Cpr6	Putative peptidyl-prolyl cis-trans isomerase D	v
AN8605	Cyp1	Peptidyl-prolyl cis-trans isomerase (PPIase); cyclophilin	v

Protein degradation

AN1700	unchar.	Putative 26S proteasome regulatory subunit (*S. pombe* Rpn2 ortholog)	v
AN1922	unchar.	Putative ortholog of *A. niger* RpnG (proteasome regulatory subunit)	v
AN2213	unchar.	Putative ortholog of *S. pombe* Rpt2 (proteasome regulatory subunit)	v
AN4236	unchar.	Putative 26S proteasome subunit (*S. pombe* Rpt5 ortholog)	v
AN4282	unchar.	Putative ortholog of *A. oryzae* AspB (lysine aminopeptidase)	v

Primary metabolism

AN10901	unchar.	Putative ortholog of *S. pombe* Gcv2 (glycine cleavage complex subunit), one-carbon metabolic process	v
AN2873	LysA	Saccharopine dehydrogenase (NAD$^+$, L-lysine-forming)	v
AN3031	unchar.	Putative threonine synthase, predicted role in glycine, serine, and threonine metabolism, putative ortholog of *S. cerevisiae* Thr4	v
AN5610	unchar.	Putative L-aminoadipate-semialdehyde dehydrogenase, predicted role in lysine metabolism, putative ortholog of *S. pombe* Lys1	v
AN6639	McdB	Putative 2-methylcitrate dehydratase, predicted role in lysine metabolism	v
AN0708	AromA	Putative pentafunctional AROM polypeptide with 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate 5-dehydrogenase, shikimate kinase, and EPSP synthase activities, predicted role in aromatic amino acid biosynthesis	a
AN7451	GdhB	Putative NAD-glutamate dehydrogenase, predicted role in glutamate and glutamine metabolism	a
AN2964	PdhX	Pyruvate dehydrogenase complex component	v
AN3829	unchar.	Putative succinate-semialdehyde dehydrogenase [NAD(P)$^+$], putative ortholog of *A. fumigatus* Uga2	v
AN3894	unchar.	Putative aconitate hydratase, predicted role in the TCA cycle, putative ortholog of *S. cerevisiae* Aco2	v
AN3901	unchar.	Putative lactic acid dehydrogenase, predicted role in energy metabolism, putative ortholog of *S. cerevisiae* Cyb2	v
AN6525	AciA	Formate dehydrogenase, predicted role in oxalic acid metabolism	v
AN0034	unchar.	Putative glycerone kinase, predicted role in glycerol metabolism, putative ortholog of *N. crassa* Dak1	a
AN0565	PyrABCN	Multifunctional enzyme with carbamoyl-phosphate synthase and aspartate carbamoyltransferase activities	a
AN0567	unchar.	Putative alcohol oxidase, predicted role in glycerol metabolism	a
AN5162	PdhB	Putative pyruvate dehydrogenase (lipoamide), predicted role in pyruvate metabolism	a
AN7895	CipB	Oxidoreductase; contains Zn-dependent alcohol dehydrogenase domain	v, a, s
AN5716	unchar.	Putative inosine-5'-monophosphate dehydrogenase, predicted role in purine metabolism, putative ortholog of *S. pombe* Mug70	v
AN6541	AdF/Ad9	Putative ligase, predicted role in purine metabolism	v
AN1015	unchar.	Putative phosphorylase, predicted role in glycogen degradation, putative ortholog of *S. cerevisiae* Gph1	v, a
AN8010	unchar.	Putative glycogen (starch) synthase, predicted role in glycogen biosynthesis, putative ortholog of *N. crassa* Gsy-1	v, a, s

Secondary metabolism

AN5130	unchar.	Putative ortholog of *A. niger* HemF (coproporphyrinogen III oxidase)	v
AN11008	ErgA	Putative ortholog of *S. pombe* Erg1 (squalene monooxygenase)	v
AN2943	RfeA	Putative reguatory role in secondary metabolism, protein kinase domain	a
AN8435	unchar.	Putative ortholog of *A. oryzae* tyrosinase MelB	a
AN7897	DbaB	FAD-binding monooxygenase with a role in secondary metabolism; member of the dba gene cluster	s
AN7902	DbaH	FAD-binding monooxygenase with a role in secondary metabolism; member of the dba gene cluster	s

Cell compartments/cytoskeleton/septa

AN9149	unchar.	Putative ortholog of *S. pombe* Tcb3 (ER-plasma membrane tethering protein)	v
AN0261	Sec23	COPII coat component; considered a prototypic marker of transitional ER (endoplasmic reticulum)	v, a
AN8233	unchar.	Putative ortholog of *S. cerevisiae* Sfh5 (phosphatidylinositol transfer protein)	v, a
AN3720	unchar.	Putative ortholog of *S. pombe* Sec24 (COP II cargo receptor)	v
AN6257	unchar.	Putative ortholog of *A. oryzae* Sec31 (subunit of vesicle coat complex COPII, ER to Golgi transport)	a
AN1177	unchar.	Putative ortholog of *S. pombe* Sec26 (coatomer subunit, ER-Golgi transport)	s
AN4547	unchar.	Putative ortholog of *S. pombe* Sec21 (coatomer subunit, ER-Golgi transport)	s
AN8023	VpsA	Putative ortholog of *S. pombe* Vps1 (dynamin), required for vacuole biogenesis	v
AN7687	unchar.	Putative ortholog of *S. pombe* Tom70 (translocase receptor)	v
AN3843	unchar.	Putative ortholog of *S. cerevisiae* Mic60 (mitochondrial complex member)	v
AN4064	unchar.	Putative ADP/ATP carrier protein	a
AN4402	unchar.	Putative ortholog of *S. cerevisiae* Por1 mitochondrial porin	a
AN5803	FimA	Predicted fimbrin protein	v
AN8862	MyoV	Myosin V	v
AN6838	TubC	Beta-tubulin	a
AN6341	unchar.	Protein with similarity to *S. cerevisiae* Crn1p; predicted role in actin patch assembly	v, a
AN4667	AspA	Septin, involved in development; prevents formation of inappropriate germ tubes and branches; required for formation of normal conidiophores	a
AN6688	AspB	Putative septin B; localizes to septa during early septum formation and to branch points during vegetative growth; localizes to the vesicle/metula, the metula/phialide and the phialide/conidium interfaces during conidiophore development	a
AN4695	HexA	Putative Woronin body protein; HapX-regulated gene	v, s
AN3026	CopA	Alpha-COP coatamer-related protein involved in the establishment and maintenance of polarized growth	v, s
AN7111	FoxA	Peroxisomal multifunctional enzyme involved in fatty acid beta-oxidation	a

Membranes/cell wall

AN0595	unchar.	Putative NADPH-cytochrome P450 reductase with a predicted role in energy metabolism, putative ortholog of *S. pombe* Ccr1	v
AN2210	unchar.	Probable ABC-transporter, putative ortholog of *S. cerevisiae* Arb1	v

AN3163	StoA	Putative stomatin ortholog, predicted to have scaffolding functions in maintenance of lipid microdomains in membranes	v
AN6287	unchar.	Putative F1F0-ATPase complex subunit, predicted role in energy metabolism, putative ortholog of *A. niger* Atp5	v
AN0317	unchar.	Putative ortholog of *S. cerevisiae* Ede1 (scaffold protein involved in endocytosis)	v
AN0870	unchar.	Putative transmembrane transporter with a predicted role in small molecule transport, putative ortholog of *S. cerevisiae* Mir1	a
AN6232	VmaB	Putative F1F0-ATPase complex subunit, nitrogen and amino acid metabolism	s
AN12492	unchar.	Putative dynamin	s
AN2532	unchar.	Putative ortholog of *A. oryzae* AoxA (amine oxidase)	v
AN1911	unchar.	Putative mannose-1-phosphate guanyltransferase, putative ortholog of *S. pombe* Mpg2	v
AN2314	unchar.	Putative 1,4-alpha-glucan branching enzyme, predicted role in starch metabolism, putative ortholog of *A. niger* GbeA	a
AN4727	UgeA	UDP-glucose 4-epimerase, involved in galactose metabolism	a
AN7657	GelA	Putative 1,3-beta-transglycosidase with a predicted role in glucan processing; predicted glycosyl phosphatidylinositol (GPI)-anchor	v, a

Oxidative stress response			
AN9339	CatB	Hyphal catalase	v

Unknown function			
AN0753	unchar.	Protein of unknown function	v
AN1378	unchar.	Protein of unknown function	v
AN5141	unchar.	Protein of unknown function	v
AN7710	unchar.	Protein of unknown function	v
AN5421	unchar.	Protein of unknown function	a
AN5741	unchar.	Protein of unknown function	s
AN7014	unchar.	Protein of unknown function	s
AN7836	unchar.	Protein of unknown function	s
AN5446	unchar.	Protein of unknown function	v, s
AN2954	unchar.	Protein of unknown function	a, s

3.7.4 ZtfA interacts with the repressor RcoA in hyphal nuclei *in vivo*

RcoA was identified in pull-down experiments conducted with vegetatively, asexually and sexually grown samples. Bimolecular fluorescence (BiFC) experiments (Kerppola, 2008) were performed to verify direct interaction of ZtfA and RcoA *in vivo*. For BiFC analyses, a strain was constructed, which expresses fusion proteins of ZtfA and one half of a split YFP (cYFP) and RcoA fused to the other half (nYFP). A signal of the joint YFPs is only emitted, if both YFP halves are in close proximity due to a direct interaction of ZtfA and RcoA. This strain was grown vegetatively and investigated under a fluorescence microscope. A signal of the joint YFP halves could be identified in nuclei of hyphae. This indicates a direct interaction of ZtfA with RcoA *in vivo* (FIGURE 42A).

Two control strains expressing either ZtfA-cYFP and free nYFP or RcoA-nYFP and free cYFP were constructed to verify that this signal is a result of the direct interaction of ZtfA and RcoA and not of YFP-tagged ZtfA or RcoA with the respective other YFP part cleaved off its carrier protein. No YFP signal could be identified in either control strain at any condition tested (FIGURE 42B). These findings support that ZtfA interacts with RcoA *in vivo* and that both proteins form heterodimers or multimeric complexes in nuclei.
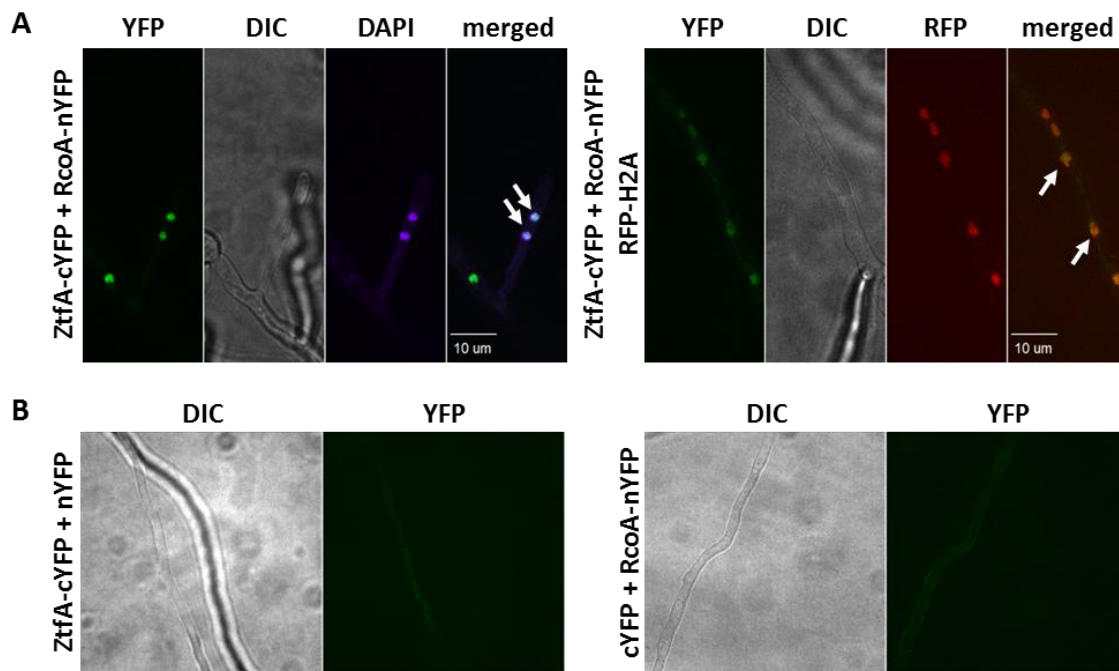
FIGURE 42: ZtfA interacts with RcoA *in vivo*.
A) Bimolecular fluorescence studies show interaction of ZtfA (*ztfA*::*cyfp*) and RcoA (*rcoA*::*nyfp*). Strains were grown for 36 h at 30°C in liquid cultures. Nuclei were stained with DAPI (left, blue) or by expression of RFP-H2A (red, right hand side). White arrows indicate double stained nuclei. B) Control strains expressing either ZtfA-cYFP and a free nYFP or RcoA-nYFP and a free cYFP do not show fluorescence signals.

3.8   ZtfA ortholog of *A. fumigatus*

3.8.1   *ztfA* encodes a C6 transcription factor in *A. fumigatus*

Bioinformatic analyses indicate that ZtfA is conserved among Aspergilli (see CHAPTER 3.1). The ZtfA ortholog of *A. fumigatus* was analyzed to examine conservation of ZtfA between the genetic model *A. nidulans* and its opportunistic counterpart. Blast analyses with the ZtfA amino acid sequence of *A. nidulans* as query reveal Afu6g11110 of *A. fumigatus* as putative ortholog (see CHAPTER 3.1 and FIGURE 43). The *ztfA* (*Afu6g11110*) ORF of *A. fumigatus* comprises 1724 nucleotides with one intron of 68 nucleotides (FIGURE 43). InterProScan

predicted a C6 domain as the only conserved protein domain in ZtfA of *A. fumigatus*, similar to ZtfA of *A. nidulans*. The ZtfA protein in *A. fumigatus* comprises 551 amino acids. *In silico* analyses revealed a putative NLS, starting at amino acid residue 535 to residue 546 for which cNLS mapper calculated a high score indicating high probability that this region really harbors a NLS (Kosugi *et al.*, 2009). Furthermore, LocNES (Xu *et al.*, 2015) predicts a putative NES (LocNES) with a relatively low score of approximately 0.4 (score value between 0 and 1), which spans from position 269 to residue 283. The C6 domain architecture of *A. fumigatus* ZtfA is similar to the one of *A. nidulans* (see CHAPTER 3.1) and completely conserved to ZtfA of *A. nidulans*, except the two amino acid residue antecedent to the last C-X_1 residue of the domain (FIGURE 13 and 43).
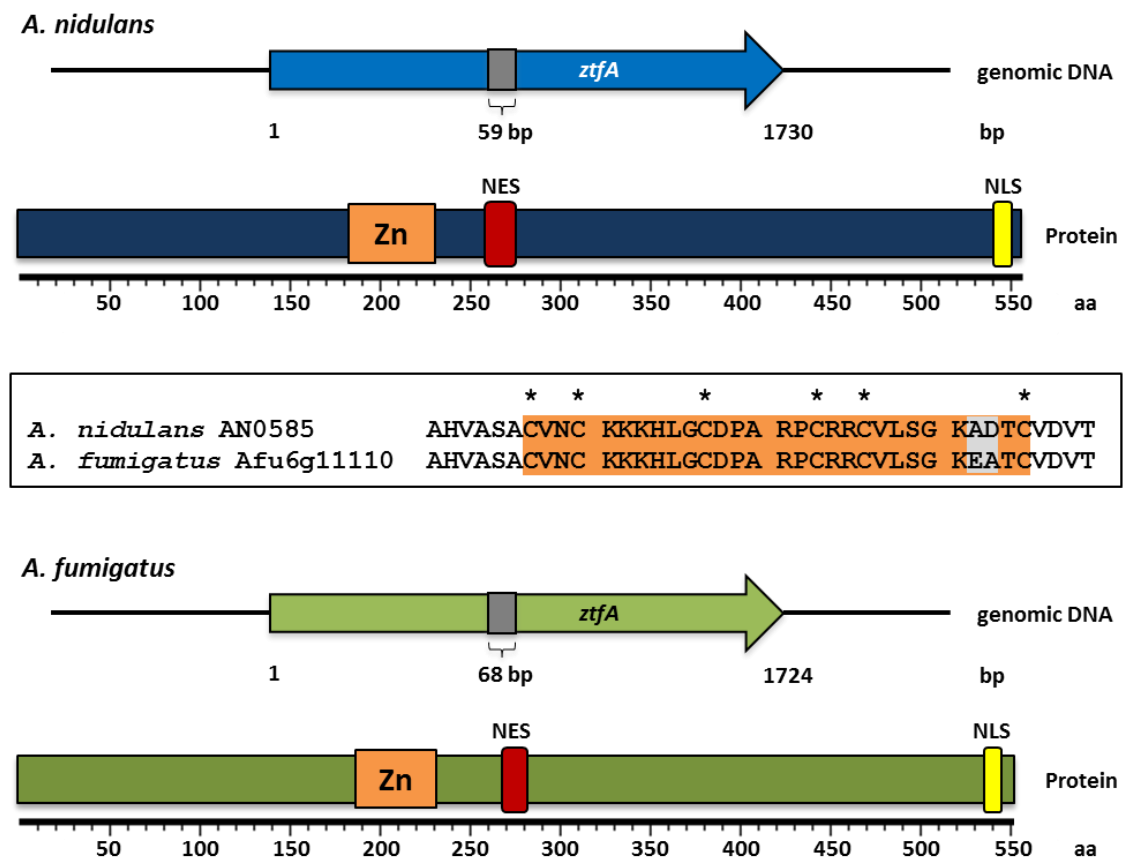
FIGURE 43: ZtfA of *A. nidulans* and *A. fumigatus*.
Comparison of *ztfA* and its gene products of *A. nidulans* (*AN0585*, upper part in blue) and *A. fumigatus* (*Afu6g11110*, lower part in green). The grey boxes represent introns, bp = base pairs, Zn = Zn(II)$_2$-Cys$_6$ fungal-type DNA-binding domain, NLS = nuclear localization sequence, NES = nuclear export signal, aa = amino acids. The amino acid sequences (aa) of the C6 domains (highlighted in orange) of both proteins is conserved with two exceptions (marked in grey, shown in the middle). Asterisks mark the six cysteines.

3.8.2 ZtfA is involved in regulation of *brlA* expression in *A. fumigatus*

ZtfA is a regulator of asexual development and activates *brlA* expression in *A. nidulans* (see CHAPTER 3.4). The BrlA→AbaA→WetA conidiation pathway is conserved between *A. nidulans* and *A. fumigatus*, but regulatory feedbacks and details of this cascade are different between both fungi (see CHAPTER 1.5 and 1.9).

The *ztfA* gene in *A. fumigatus* (*Afu6g11110*) was deleted and transcriptional analyses via qRT-PCR verify complete abolishment of *ztfA* transcription (FIGURE 44A). Deletion of the ortholog in *A. fumigatus* does not result in an obvious developmental phenotype, but the Δ*ztfA* mutant shows more diffuse colonies compared to WT, what can be best seen from below the colony (FIGURE 44A).
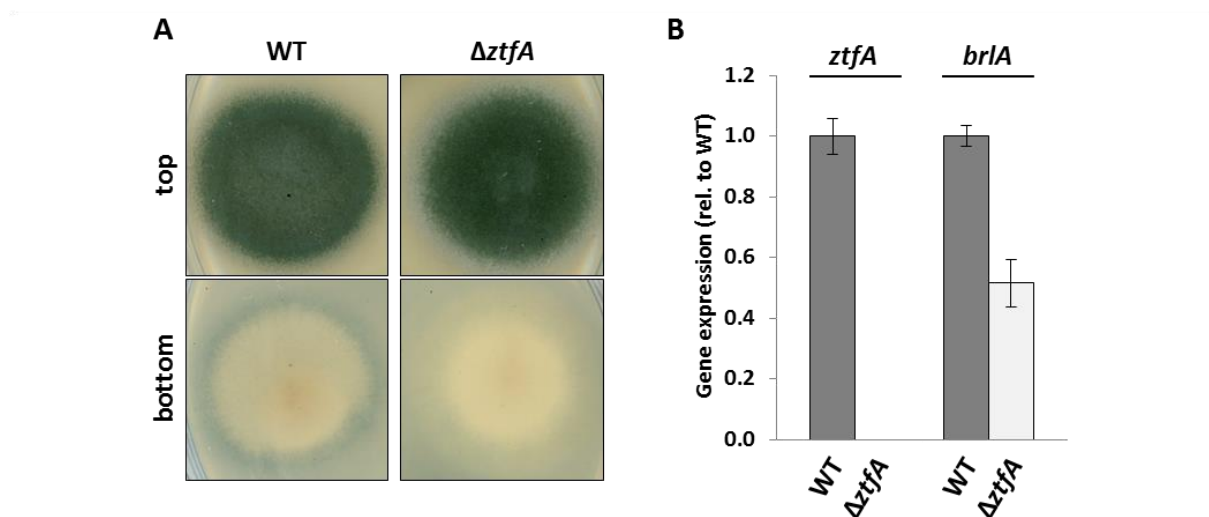
FIGURE 44: ZtfA is dispensable for conidiation in *A. fumigatus*.
A) The WT colony exhibits a defined morphology, while the Δ*ztfA* is more diffuse. B) qRT-PCR analyses indicate that ZtfA is involved in activation of *brlA* in *A. fumigatus*. Submerged cultures were inoculated with $1*10^7$ spores and grown for 24 h at 37°C. Subsequently mycelia was shifted onto solid agar plates and incubated for 24 h at 37°C in the dark. RNA from two independent clones of Δ*ztfA* was tested.

ZtfA activates *brlA* expression in *A. nidulans* (see CHAPTER 3.4.4). Consequently, *brlA* expression was tested in *A. fumigatus* Δ*ztfA* (FIGURE 44B). *brlA* expression is downregulated about two times in Δ*ztfA*.

One explanation for this finding might be the putative presence of a redundant transcription factor in *A. fumigatus*, which exhibits similar regulatory roles like ZtfA.

ztfA is a downstream target of, and negatively regulated by, VosA in *A. nidulans* (see CHAPTER 3.3). Hence, qRT-PCR experiments were carried out to analyze whether ZtfA regulates velvet gene expression in *A. fumigatus*. Expression of none of the velvet factors is

ZtfA dependent (FIGURE 45). This indicates that ZtfA is not a regulator of velvet gene expression and is in accordance with the finding that ZtfA in *A. nidulans* does not regulate *vosA* gene expression.
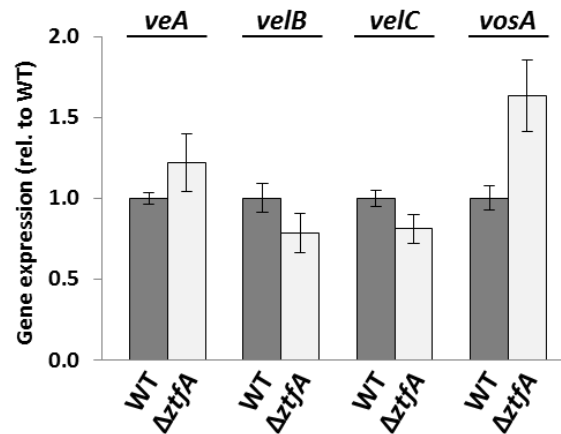
FIGURE 45: ZtfA is dispensable for expression of the velvet factors in *A. fumigatus*.
qRT-PCR analyses do not indicate regulatory effects of ZtfA upon the velvet-factor encoding genes as a loss of *ztfA* does not change their expression. Strains were grown vegetatively for 24 h and subsequently shifted onto solid MM and grown for 24 h in dark conditions at 37°C. Two independent clones of Δ*ztfA* were screened.

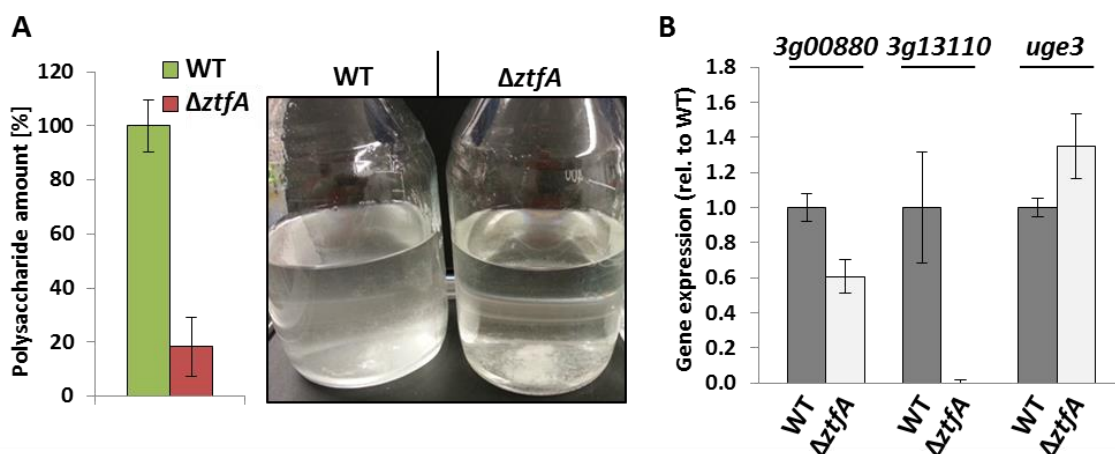### 3.8.3 ZtfA regulates polysaccharide production and biofilm formation in *A. fumigatus*

Polysaccharides, like the cell-wall component galactosaminogalactan, were shown to be involved in surface adhesion, a crucial step for host infection and virulence in pathogenic fungi (Gravelat *et al.*, 2013; Kaur and Singh, 2013; Lin *et al.*, 2015). Mutants, which exhibit developmental phenotypes in *A. nidulans* often have virulence phenotypes in *A. fumigatus* (examples are discussed in CHAPTER 4.8.1). Hence, polysaccharide production was analyzed in *A. fumigatus* Δ*ztfA*. The amount of polysaccharides produced by the mutant is only 20% of the quantity of WT (FIGURE 46A). This hints towards a putative decreased capability for surface adhesion. Consequently, expression of three genes associated with adherence was tested in WT and Δ*ztfA* to further elucidate a possible influence of ZtfA on surface adhesion capabilities: *Afu3g00880*, *Afu3g13110* and *uge3* (Chaudhuri *et al.*, 2011; Lin *et al.*, 2015). *Afu3g00880* and *Afu3g13110* encode putative adhesins (Chaudhuri *et al.*, 2011; Gravelat *et al.*, 2010). *Afu3g00880* is slightly downregulated in the absence of *ztfA* during asexual growth (FIGURE 46B). Importantly, *Afu3g13110* transcript is not detectable under these growth conditions in Δ*ztfA*, but expressed in WT (FIGURE 46B). Uge3 is a UDP-glucose 4-epimerase, which is involved in galactosaminogalactan synthesis (Gravelat *et al.*, 2013). *uge3* gene expression, however, was not significantly altered in Δ*ztfA* in comparison to WT.

FIGURE 46: ZtfA regulates polysaccharide formation and adhesin gene expression.
A) Polysaccharides were extracted from vegetatively grown cultures. Total amount of polysaccharides produced was measured and WT amounts were set to 100%. B) qRT-PCR indicates that *ztfA* regulates genes coding for putative adhesins. cDNA was transcribed from RNA extracted from cultures grown vegetatively for 24 h and subsequently 24 h on solid MM. Gene expression of two individual Δ*ztfA* clones was tested.

3.8.4 ZtfA is involved in H_2O_2 and cell wall stress response in *A. fumigatus*

Cell wall stress was tested since polysaccharide production was diminished in Δ*ztfA*. Stress tests were carried out with sodium dodecyl sulfate (SDS) as stressor. No significant difference between the size of the inhibition zones of Δ*ztfA* and the WT are found (FIGURE 47).
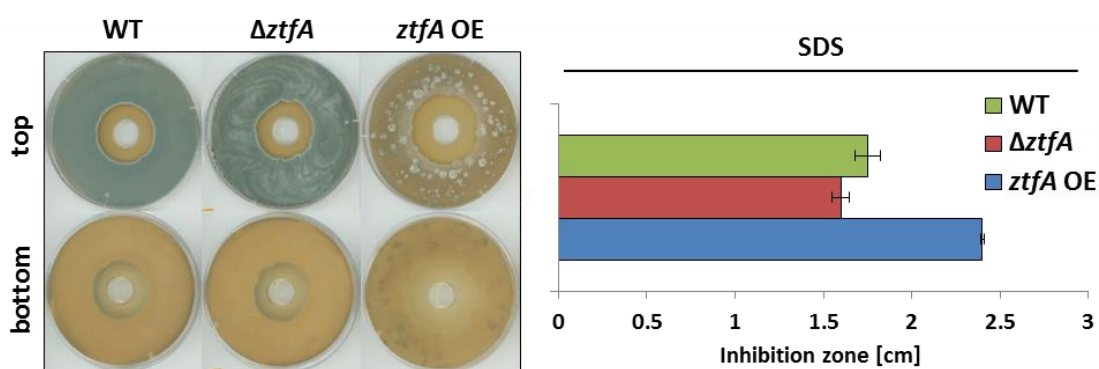
FIGURE 47: ZtfA is involved in SDS stress response in *A. fumigatus*.
A *ztfA* OE leads to decreased tolerance towards SDS stress. Strains were plated and agar plugs were cut out and filled with SDS. Formed inhibition zones were measured after 2 d incubation at 37°C in the dark. Three independent clones of Δ*ztfA* were tested. Media were supplemented with 10 µg/ml doxycycline to induce *TetOn-ztfA* (*ztfA* OE) expression.

The *ztfA* OE strain shows slightly larger inhibition zones of approximately 2.4 cm in contrast to WT (1.75 cm). This indicates that ZtfA might be involved in the cell wall stress response. It

has to be mentioned that the *ztfA* OE (*TetOn-ztfA*; C. Sasse, p.c.) shows impaired growth in comparison to WT, which might enhance the effect of SDS stress on this mutant strain.

ZtfA is an inhibitor of the OSR and regulates expression of redox and catalase genes in *A. nidulans* (see CHAPTER 3.6). Stress tests were carried out in *A. fumigatus* to examine whether ZtfA might be involved in stress responses in this fungus as well. A loss of *ztfA* increases the tolerance towards H_2O_2 stress in *A. fumigatus* only to a small extent (FIGURE 48). In comparison, loss of *ztfA* in *A. nidulans* leads to distinctly increased tolerance towards H_2O_2 compared to WT (see CHAPTER 3.6). The *ztfA* OE strain in *A. fumigatus* shows slightly larger inhibition zones in H_2O_2 tests. This indicates that ZtfA has negative influences upon the OSR in this fungus as well. As in both cases differences to WT were small, this suggests that redundant genetic systems exist in *A. fumigatus*, which can compensate for the loss of *ztfA*.
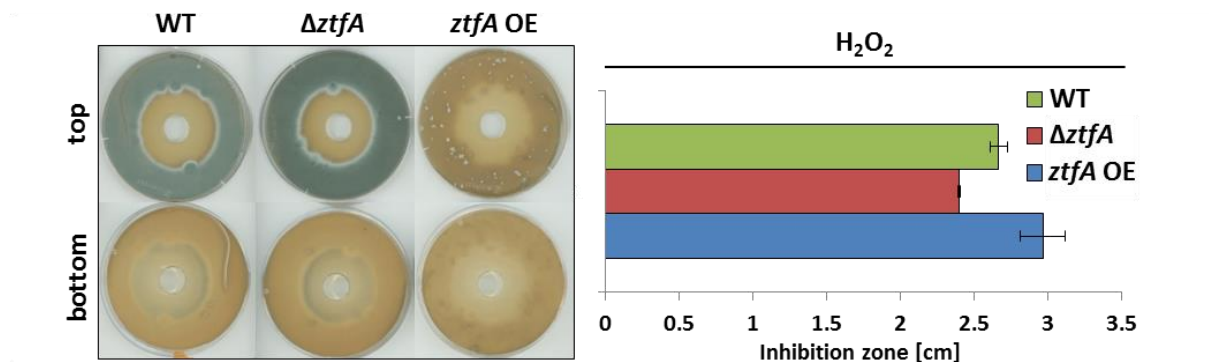
FIGURE 48: ZtfA negatively influences oxidative stress response in *A. fumigatus*.
Strains were plated and agar plugs were cut out and filled with 150 µl H_2O_2 1%. Strains were grown for 2 d at 37°C in the dark. Three independent clones of Δ*ztfA* were tested. Media were supplemented with 10 µg/ml doxycycline to induce *TetOn-ztfA* (*ztfA* OE) expression.

3.8.5 ZtfA is dispensable for virulence in *Galleria mellonella*

Larvae of the wax moth *Galleria mellonella* are frequently used as a non-vertebrate infection model (Brennan *et al.*, 2002; Reeves *et al.*, 2004; Renwick *et al.*, 2006; Smith and Calvo, 2014). Virulence studies often show correlations between infections in these larvae and *Mus musculus*, even though *G. mellonella* larvae do not exhibit pulmonary structures (Brennan *et al.*, 2002). *G. mellonella* larvae were infected with $8*10^6$ spores of WT and Δ*ztfA* and survival rates were monitored for several days. Survival rates of larvae infected with the Δ*ztfA* mutant were comparable to WT (FIGURE 49). This shows that *ztfA* is not required for virulence in an invertebrate model.
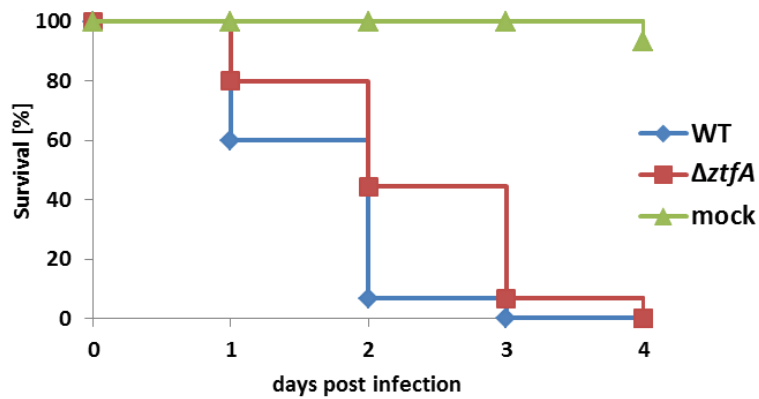
FIGURE 49: ZtfA is not involved in virulence of *A. fumigatus* in *G. mellonella*.
Larvae of the greater wax moth *G. mellonella* were infected with $8*10^6$ spores of WT and
Δ*ztfA* and incubated at 30°C for 4 d. 12 larvae were utilized per strain. This infection assay
was conducted with three independent clones of Δ*ztfA* and repeated with similar results.

In summary, the presence of *ztfA* and its gene product is conserved in *A. nidulans* and
A. fumigatus, but its regulatory role between these fungi differ. ZtfA is an important activator
of conidiation and secondary metabolism in *A. nidulans* but is dispensable for conidiation in
A. fumigatus. It is involved in adhesion in this pathogenic mold. The regulatory role of ZtfA
however is distinctly different in *A. nidulans* and *A. fumigatus*.

4 Discussion

4.1 ZtfA is a regulator of conidiation and secondary metabolism in *A. nidulans*

Members of the genus *Aspergillus* are among the most widespread fungi worldwide and colonize diverse ecological niches. Most of these filamentous fungi are saprophytic and important for biological substance cycles. A number of Aspergilli are secondary metabolite producers and several Aspergilli have deleterious as well as beneficial effects on humankind. Secondary metabolism is linked to developmental programs in filamentous fungi through the velvet proteins. This study characterizes a novel target of the velvet factor VosA: the transcription factor ZtfA. *ztfA* gene expression is repressed by VosA and both factors are necessary for spore viability. ZtfA activates conidiophore formation by expression regulation of the major conidiation activator-encoding *brlA* gene and its upstream activators, encoded by *flbC* and *flbD*. Moreover, ZtfA regulates biosynthesis of several secondary metabolites through activation of expression of their gene clusters. Besides, ZtfA is important for an appropriate response towards oxidative stress. It acts as activator of the thioredoxin system and *catA* gene expression, but represses other factors of the OSR. A summarizing model of the regulatory influences of ZtfA is shown in Figure 50.
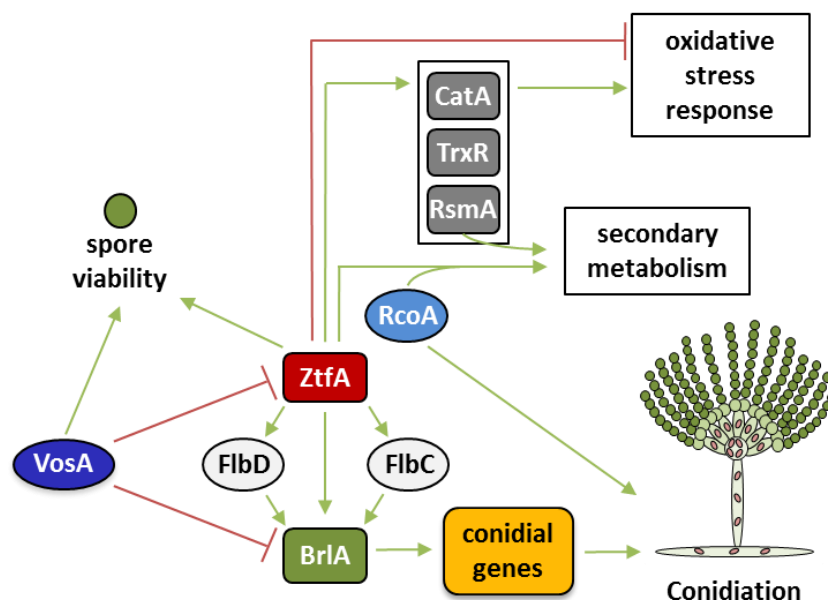
FIGURE 50: Comprehensive model of the regulatory role of ZtfA in *A. nidulans*.
The model describes the major regulatory roles of ZtfA as activator of conidiation and secondary metabolism and regulator of the oxidative stress response. Green arrows indicate positive and red lines negative influences on gene expression.

4.1.1   The C6 domain of ZtfA is highly conserved in Aspergilli

Proteins with a C6 domain constitute a group of fungal specific DNA binding proteins, which act as transcription factors (Chang and Ehrlich, 2013; MacPherson *et al.*, 2006; Schjerling and Holmberg, 1996). The C6 domain of ZtfA deviates from the domains of most common zinc cluster proteins in *A. nidulans*. Wortman and collaborators identified 330 C6 proteins in *A. nidulans* (Wortman *et al.*, 2009). An up-to-date *in silico* search, employing the fungal databases FungiDB and AspGD (Cerqueira *et al.*, 2014; Stajich *et al.*, 2012) reveals a recent number of 332 putative C6 proteins in *A. nidulans*. Less than six percent of all C6 proteins in *A. nidulans* exhibit a $CX_2CX_6CX_5CX_2CX_8C$ domain architecture, amongst them ZtfA. Only two other C6 proteins with this architecture have been characterized in *A. nidulans* so far: AcuM and ClrB (Coradetti *et al.*, 2012; Hynes *et al.*, 2007). Both are involved in primary metabolism: AcuM is involved in gluconeogenesis, whereas ClrB was identified as a regulator of cellulase gene expression. Its uncommon C6 architecture together with its broad regulatory influences renders ZtfA a very unique protein among C6 proteins in *A. nidulans*.

The C6 domain of the yeast DNA-binding protein Gal4, the best-studied C6 protein, has a $CX_2CX_6CX_6CX_2CX_6C$ architecture (Giniger *et al.*, 1985; Marmorstein *et al.*, 1992; Pan and Coleman, 1990; Rodgers and Coleman, 1994). This is the most common C6 architecture in *A. flavus* and *A. nidulans*, followed by $CX_2CX_6CX_5CX_2CX_6C$ in a ratio of 2:1 (Chang and Ehrlich, 2013). In general, the cysteines within the first part of this motif are conserved whereas the second part varies and forms different architectures: $CX_2CX_6CX_{5-16}CX_2CX_{6-8}C$ (Todd and Andrianopoulos, 1997). The databank searches conducted in this study as well as the data published by Wortman and collaborators reveal that the last part has to be extended for *A. nidulans* to $CX_2CX_6CX_{5-16}CX_2CX_{4-12}C$ and that few exceptions from this general architecture exist. An overview of the different architectures present in *A. nidulans* and characterized proteins exhibiting these architectures is given in TABLE 10.

The C6 domain is required for DNA binding (Bai and Kohlhaw, 1991; Burger *et al.*, 1991; Defranoux *et al.*, 1994; Johnston and Dover, 1987; Pfeifer *et al.*, 1989; Todd *et al.*, 1997; Todd and Andrianopoulos, 1997). Amino acids within the first CX_2CX_6C motif are conserved and mutagenesis studies showed their importance for DNA binding (Johnston and Dover, 1987; Todd and Andrianopoulos, 1997; Yuan *et al.*, 1991).

DNA recognition sites consisting of terminal trinucleotides of direct or inverted repeats, which are separated by six to eleven nucleotide residues, have been characterized for several C6 proteins. CCG triplets as everted or inverted repeats have been proposed to be typical C6 consensus sequences (MacPherson *et al.*, 2006; Marmorstein *et al.*, 1992). AcuM binds a

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CCGN$_7$CCG nucleotide consensus sequence, presumably as a heterodimer (Suzuki *et al.*, 2012). ClrB was shown to bind to CGGN$_8$CCG inverted repeats in a co-factor dependent manner, as well as to CGG/CCG single triplets in the absence of a co-factor (de Groot *et al.*, 2009; Li *et al.*, 2016; Yamakawa *et al.*, 2013). However, binding to triplets of different structure has been shown for other C6 proteins as well (Chang *et al.*, 1995; Todd *et al.*, 1998; Todd and Andrianopoulos, 1997).

TABLE 10: Comprehensive overview of C6 architectures present in *A. nidulans*.
The table provides an overview over the frequency of C6 architectures in *A. nidulans*, according to Wortman and collaborators, amended with the two additional proteins found in AspGD and FungiDB database searches (Cerqueira *et al.*, 2014; Stajich *et al.*, 2012; Wortman *et al.*, 2009). All characterized representatives of each group are indicated. ZtfA and its architectural group are given in bold.

C6 motif	Quantity	Metabolic processes	Development	Stress response
$CX_2CX_6CX_6CX_2CX_6C$	149	AflR, DbaA, MdpE, ArcA, ClrA, FacB, QutA, UaY	NosA, RosA, SfgA, OefC	PacX
$CX_2CX_6CX_5CX_2CX_6C$	73	AmyR, ApdR, GalR, GalX, InuR, PrnA, ScfA, XlnR		
$CX_2CX_6CX_8CX_2CX_6C$	27	FarA, FarB, TamA		
$CX_2CX_6CX_5CX_2CX_8C$	**19**	AcuM, ClrB, **ZtfA**	**ZtfA**	**ZtfA**
$CX_2CX_6CX_6CX_2CX_8C$	12	AcuK		
$CX_2CX_6CX_7CX_2CX_6C$	11			
$CX_2CX_6CX_9CX_2CX_6C$	11	LeuB		
$CX_2CX_6CX_{10}CX_2CX_6C$	4			
$CX_2CX_6CX_6CX_2CX_9C$	4			
$CX_2CX_6CX_{12}CX_2CX_6C$	2			
$CX_2CX_6CX_6CX_2CX_5C$	2			
$CX_2CX_6CX_7CX_2CX_7C$	2			
$CX_2CX_6CX_8CX_2CX_7C$	2	AmdR		
$CX_2CX_{10}CX_5CX_2CX_6C$	1			
$CX_2CX_{10}CX_6CX_2CX_6C$	1			
$CX_2CX_5CX_6CX_2CX_6C$	1			
$CX_2CX_6CX_{14}CX_2CX_6C$	1		SilA	
$CX_2CX_6CX_{15}CX_2CX_6C$	1			
$CX_2CX_6CX_{16}CX_2CX_6C$	1	AlcR		
$CX_2CX_6CX_5CX_2CX_{11}C$	1			
$CX_2CX_6CX_5CX_2CX_5C$	1	AraR		
$CX_2CX_6CX_5CX_2CX_7C$	1			
$CX_2CX_6CX_6CX_1CX_6C$	1			
$CX_2CX_6CX_6CX_2CX_{12}C$	1			SonC
$CX_2CX_6CX_6CX_2CX_7C$	1	NirA		
$CX_2CX_7CX_6CX_2CX_6C$	1			
$CX_2CX_9CX_{12}CX_2CX_6C$	1			

Several conserved amino acid residues are present in ZtfA giving a hint that ZtfA might recognize nucleotide triplet repeats as well. In Gal4, the lysine residues K17 and K18 were identified to be necessary for the specific base pair contact to the CGG triplet forming the DNA binding motif (Marmorstein *et al.*, 1992; Marmorstein and Harrison, 1994). RhaR is an example for a C6 protein in *A. nidulans*, which shows conservation of K18 but replacement of lysine K17 to arginine (R68) (Pardo and Orejas, 2014). RhaR binds to a $CGGN_{11}GGC$ DNA motif. ZtfA exhibits a stretch of three lysines at this position (K4-6). The relatively uncommon C6 architecture of ZtfA and indications for different interaction partners suggest that ZtfA is able to bind different DNA-binding sequences. Importantly, among ZtfA orthologs in Aspergilli the whole C6 domain of ZtfA is strongly conserved. These similarities in ZtfA orthologs imply that it might bind to similar binding motifs in different Aspergilli. It is therefore assumed, that ZtfA regulates the expression of orthologous genes and exhibits influences similar to the ones found for *A. nidulans* in other Aspergilli as well.

4.1.2   C6 proteins and their role in *A. nidulans*

The *A. nidulans* genome encodes 332 putative C6 proteins. In contrast to this high number, which makes up approximately three percent of all genes in *A. nidulans*, a relatively small number of only 33 C6 proteins (approximately 10%) has been characterized yet (TABLE 10). Several proteins of this group regulate primary or secondary metabolite gene clusters as cluster specific regulators in *A. nidulans*, such as AmdR, AlcR, QutA, NirA, PrnA, UaY, FacB, MdpE and AflR (Andrianopoulos and Hynes, 1990; Bergmann *et al.*, 2007; Beri *et al.*, 1987; Brown *et al.*, 1996; Burger *et al.*, 1991; Cazelle *et al.*, 1998; Chiang *et al.*, 2009, 2010; Felenbok *et al.*, 1988; Scazzocchio, 1994; Suarez *et al.*, 1995; Todd *et al.*, 1997). Two C6 proteins, SfgA and OefC, are involved in the regulation of asexual sporulation, and three C6 proteins, RosA, NosA and SilA, were found to be involved in sexual development (Han *et al.*, 2008; Lee *et al.*, 2005; Seo *et al.*, 2003, 2006; Vienken *et al.*, 2005; Vienken and Fischer, 2006). Employment of the AspGD Gene Onthology slim mapper (Cerqueira *et al.*, 2014) showed, that most manually annotated C6 proteins cluster with the terms *carbohydrate metabolic processes*, *developmental processes* and *response to chemical or stress*. Three are annotated to *secondary metabolic processes*, three for *sexual sporulation* and two for *asexual sporulation*. So far, most of the C6 proteins analyzed for *A. nidulans* regulate metabolic processes as cluster specific transcription factors or are involved in developmental programs. Regulation and interconnection of several processes and programs on a higher level, as this study shows for ZtfA, is uncommon for C6 proteins in *A. nidulans*. As only 10% of this

protein group has been investigated so far, it is likely to find more proteins which interconnect different pathways in this transcription factor group in future analyses.

In conclusion, ZtfA is one of the first C6 proteins that act as activator for asexual development and secondary metabolism.

4.2 ZtfA is a repression target of VosA and acts as a conidiation regulator in *A. nidulans*

4.2.1 The upstream developmental activator pathway is regulated by ZtfA

Velvet proteins are transcription factors, which interconnect developmental programs and secondary metabolism and regulate expression of thousands of genes (Ahmed *et al.*, 2013; Becker *et al.*, 2016). The velvet factor VosA represses premature conidiation and is important for spore viability (Ni and Yu, 2007). The present study shows that ZtfA is a downstream target of VosA. VosA is a repressor of *ztfA* gene expression and both factors together are important for viability of conidiospores.

VosA represses expression of the major conidiation regulator-encoding *brlA* gene during vegetative growth and therefore regulates developmental competence (Lee *et al.*, 2016; Ni and Yu, 2007). BrlA is the first factor in the central developmental pathway (CDP) (BrlA → AbaA → WetA) and is activated by the upstream developmental activators (UDAs) (Adams *et al.*, 1998; Lee and Adams, 1996; Lee *et al.*, 2016; Marshall and Timberlake, 1991; Mirabito *et al.*, 1989). The UDAs are repressed by SfgA and comprise the Flb pathway, which functions in two parts in parallel: FlbB/FlbE → FlbD → BrlA and FlbC → BrlA (Lee *et al.*, 2016; Ruger-Herreros *et al.*, 2011; Seo *et al.*, 2006; Yu *et al.*, 2010). FluG counteracts the repressing effect of SfgA, what allows activation of the Flbs (Garzia *et al.*, 2010; Kwon *et al.*, 2010a, 2010b; Ni and Yu, 2007; Seo *et al.*, 2006; Wieser and Adams, 1995).

It was previously hypothesized, that another yet unknown factor might exist that regulates conidiation together with the Flb cascades (Garzia *et al.*, 2010; Lee and Adams, 1996). Such a factor was anticipated to activate *brlA* gene expression or expression of either *flb* factors in the last steps of the UDA pathway, namely *flbC* and *flbD* (FIGURE 51). *flb* gene knock outs share the name-giving phenotype: a fluffy appearance of the colony due to increased aerial hyphae and decreased conidiophore production (Wieser *et al.*, 1994).

Phenotypical and transcriptional analyses show that ZtfA functions downstream of FluG and upstream of FlbC and FlbD. It is not involved in the regulation of *sfgA* expression but is an activator of *flbC* and *flbD* gene expression during late vegetative growth. Taken together,

ZtfA represents a new activator of conidiation, which functions upstream to the UDA pathway and is important for activation of both parts of the Flb cascade (FIGURE 51).
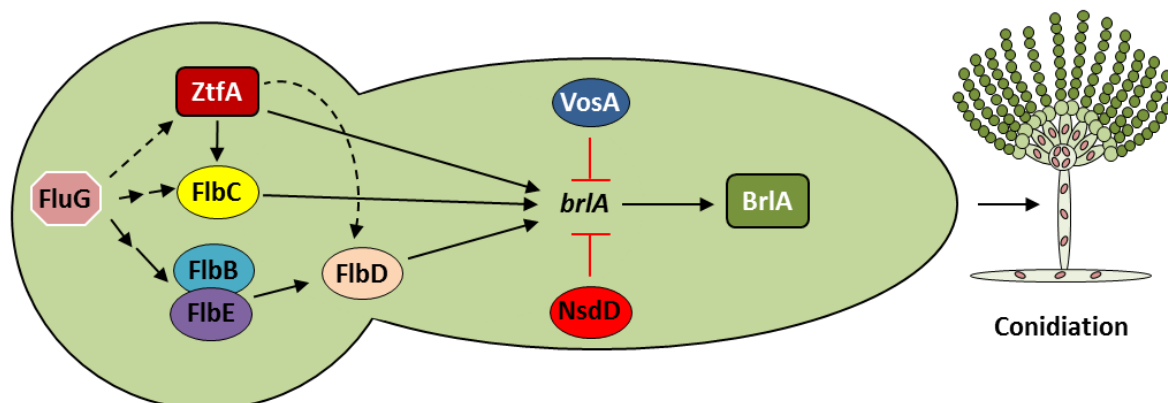
FIGURE 51: Gene expression of *flbC*, *flbD* and *brlA* is activated by ZtfA.
The model summarizes the current understanding of the regulatory role of ZtfA upon the UDA factors and *brlA* during late vegetative growth at the onset of conidiation. ZtfA activates *brlA* gene expression and is important for activation of FlbC and FlbD, downstream of FluG. Positive regulations are given in black, negative regulations (repression) are given in red.

4.2.2 ZtfA activates *brlA* expression in *A. nidulans*

ZtfA is sufficient to induce premature conidiophore development during vegetative growth and activates *brlA* expression under these conditions. This is further supported by the finding that this activation is significantly higher in the absence of *vosA*. VosA and NsdD repress *brlA* expression during vegetative growth (Lee *et al.*, 2016; Ni and Yu, 2007). *ztfA* OE produces normal conidiophores during these conditions, whereas the WT is not able to form conidiophores. Overexpression of *brlA* leads to conidiophore formation under vegetative conditions directly from vesicles formed at the hyphal tips (Adams *et al.*, 1988, 1998). Therefore, several aspects of the regulatory role of ZtfA are important for the formation of conidiophores, besides its activating role upon *brlA*. ZtfA regulates *flbD* expression and is important for *flbC* expression during vegetative growth. However, this is not sufficient to induce conidiophore development, since a *flbC* OE does not form conidiophores but vesicle-like structures at hyphal tips (Kwon *et al.*, 2010a). Therefore, induction of gene expression of *flbC*, *flbD* and *brlA* together is necessary for the conidiophore formation. BrlA activates *abaA* and *wetA*, which both encode transcription factors necessary for conidiation (Andrianopoulos and Timberlake, 1994; Lee *et al.*, 2016; Marshall and Timberlake, 1991; Ni and Yu, 2007; Sewall *et al.*, 1990a). In accordance with the finding that ZtfA activates *brlA*, phenotypical analyses show that *ztfA* is epistatic to both, *brlA* and *abaA*. Taken together, ZtfA activates both, the UDA factors through *flbC* and *flbD* and the CDP through *brlA*.

4.2.3 ZtfA and VosA function in achievement of developmental competence and spore maturation in *A. nidulans*

The onset of conidiation is genetically characterized by a time dependent de-repression of *brlA* expression (see CHAPTER 1.4 and 1.5) (Lee *et al.*, 2016). The conidiation cascade itself is also time dependent: *brlA* is activated by the UDA factors and ZtfA during the achievement of developmental competence (Etxebeste *et al.*, 2009, 2008; Garzia *et al.*, 2009, 2010; Kwon *et al.*, 2010a, 2010b; Wieser and Adams, 1995) (FIGURE 52).
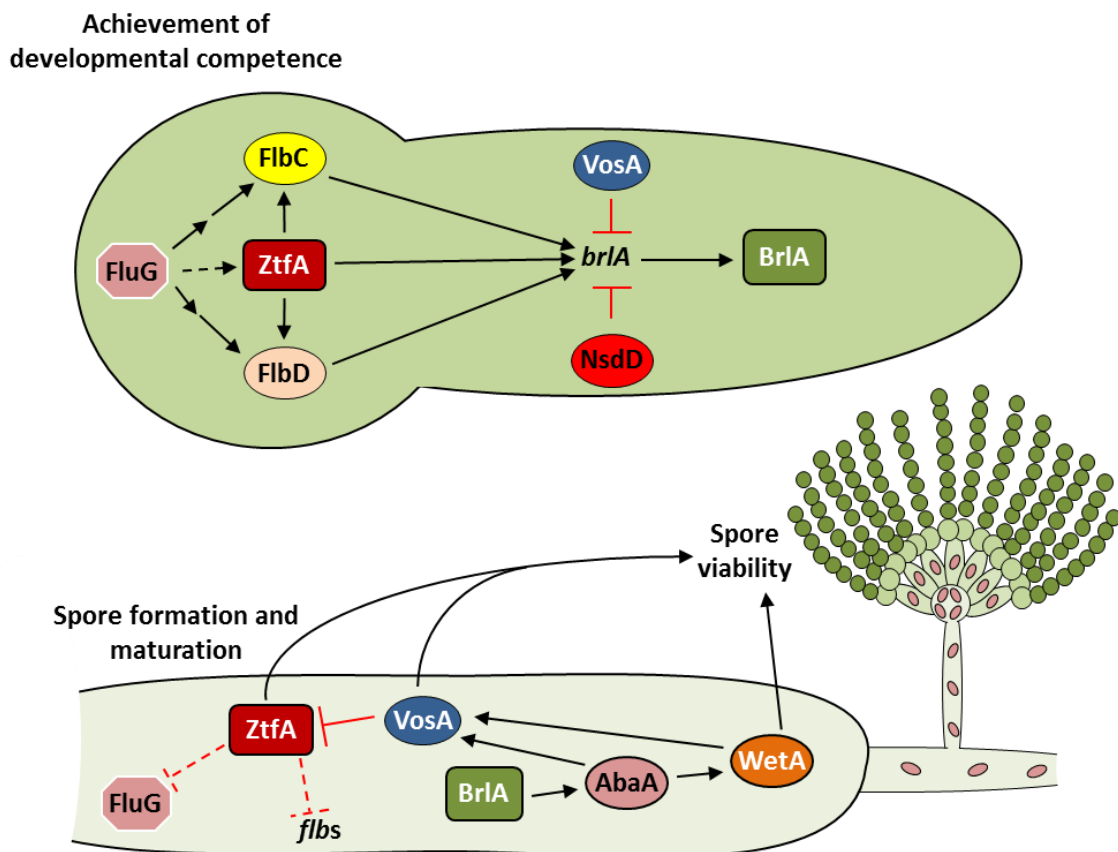
FIGURE 52: ZtfA and VosA regulate achievement of developmental competence and spore maturation.
The model depicts the regulatory roles of ZtfA and VosA upon conidiation during achievement of developmental competence (upper part) and ongoing spore formation and maturation (lower part). ZtfA activates gene expression of *brlA*, *flbC* and *flbD* during late vegetative growth and supports achievement of developmental competence. *brlA* expression is repressed by VosA and NsdD during this stage (upper part). ZtfA negatively regulates *fluG* and *flb* gene expression during ongoing development and supports spore maturation. *vosA* expression is activated by AbaA and WetA and VosA is necessary for trehalose biogenesis and spore maturation as well. VosA negatively regulates *ztfA* expression during asexual growth (lower part).

BrlA activates *abaA* expression during the mid-phase of conidiation (approximately 12 h post induction), whose product then activates *wetA* and *vosA* expression during the late phase of

conidiation (after 24 h post induction) (Adams *et al.*, 1988, 1990; Andrianopoulos and Timberlake, 1994; Boylan *et al.*, 1987; Marshall and Timberlake, 1991; Ni and Yu, 2007; Tao and Yu, 2011). VosA is involved in early time tuning of conidiation by repressing *brlA* expression until developmental competence is achieved (Lee *et al.*, 2016; Ni and Yu, 2007) (FIGURE 52). During late asexual growth, VosA negatively regulates *ztfA* expression, thereby exhibiting another level of regulation of the time adjusted choreography of conidiation. ZtfA is involved in late time tuning of the conidiation cascade as well: it negatively regulates *fluG* and the *flb* genes during late asexual growth after 24 h (FIGURE 52). This regulatory role confirms the model that ZtfA functions upstream of the UDA pathway as a rather global regulator of conidiation.

AbaA induces *vosA* expression during late asexual development (Park *et al.*, 2012b) (FIGURE 52). VosA is necessary for spore viability and trehalose biogenesis together with VelB (Ni and Yu, 2007; Sarikaya-Bayram *et al.*, 2010). Trehalose is a non-reducing disaccharide that is found in fungi, plants, bacteria and insects, where it functions as storage compound, but is also involved in stress resistance (Becker *et al.*, 1996; Elbein *et al.*, 2003; Jorge *et al.*, 1997; Ocón *et al.*, 2007). In germinating conidiospores, trehalose is rapidly degraded and deletion of the trehalose-6-phosphate synthase results in delayed germination (Al-Bader *et al.*, 2010; Fillinger *et al.*, 2001; Kane and Roth, 1974; Shin *et al.*, 2009).

Conidiospores are able to germinate in the absence of *ztfA*, but show a rapid loss in viability. Decreased spore viability has been shown before for *vosA* and *velB* mutants and for other *A. nidulans* mutant strains as well (Hagiwara *et al.*, 2008; Kawasaki *et al.*, 2002; Lara-Rojas *et al.*, 2011; Leiter *et al.*, 2016). Trehalose has been shown to be important for conidiospore viability: a loss in spore viability accompanies an insufficient trehalose concentration in conidiospores in Δ*vosA* and Δ*velB* strains (Ni and Yu, 2007; Sarikaya-Bayram *et al.*, 2010). Loss in spore viability in Δ*ztfA* is not coupled to trehalose biogenesis and ZtfA is not important for regulation of trehalose biosynthesis. Hence, ZtfA is not involved in the VosA-governed trehalose biogenesis regulation. Nevertheless, ZtfA probably is a factor of a VosA-governed spore viability regulation. ZtfA's role in spore viability, together with the activation of *brlA* and *flb* genes during late vegetative growth, supports the model that ZtfA is involved in the activation of conidiation and its regulation during ongoing spore formation and maturation. Such a regulation is probably not based upon VeA-VelB or VelB-VosA heterodimers since phenotypic analyses with Δ*veA*Δ*ztfA* and Δ*velB*Δ*ztfA* double mutants do not imply relations between these *velvet* genes and *ztfA*. A VosA-VelC heterodimer has been proposed but has not been proven *in vivo* up to date (Park *et al.*, 2014). Since little is known

about VelC at the moment, a regulatory effect upon spore viability cannot be excluded. A ΔvelCΔztfA double mutant does not show clear epistatic effects. Therefore, the regulatory effects of ZtfA are probably not dependent upon VelC.

4.3   Phosphorylation might represent an activity control of ZtfA

Three phosphorylated residues (S327, T464 and S506, respectively) were identified in ZtfA of *A. nidulans* in LC-MS/MS analyses and their importance could be demonstrated by construction of a strain that expresses a version of ZtfA, in which these residues were exchanged to alanine to mimic permanent dephosphorylation. Changes in the phosphorylation status have been shown to be important for function of several regulatory proteins (Bayram *et al.*, 2012; Jöhnk *et al.*, 2016; Rauscher *et al.*, 2016; Schinke *et al.*, 2016; Shimizu *et al.*, 2003). The phenotype of the dephosphorylated mutant (*ztfA*$^{S327A,T464A,S504-506A}$) does not resemble the Δ*ztfA* phenotype. This shows that ZtfA is still able to execute its activating effects on conidiation in the dephosphorylated mutant. Therefore, permanent dephosphorylation of identified residues does not hinder nuclear localization of ZtfA and does not lead to inactivation of the protein. The possibility exists that dephosphorylation of S327, T464 and S506 leads to increased ZtfA import into the nucleus. The dephosphorylated mutant strain resembles the *ztfA* OE strain in the scope of lacerated colony morphology. This suggests that the *ztfA* OE strain might produce increased numbers of incorrectly phosphorylated ZtfA protein and that this influences colony morphology. Several possible explanations for these findings exist. Phosphorylation might have a destabilizing effect and might represent an activity control for ZtfA (FIGURE 53).
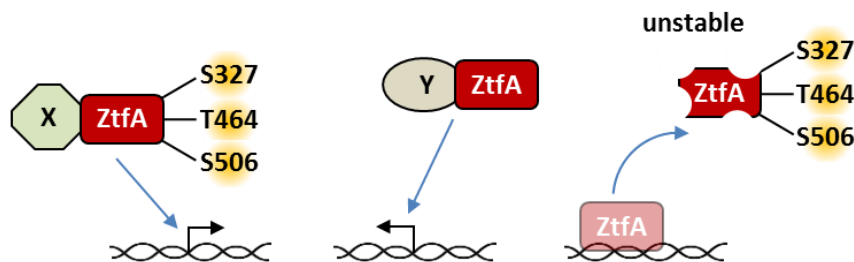
FIGURE 53: The phosphorylation status of ZtfA might influence DNA-binding specificity, protein-protein interaction and stability of the ZtfA protein.
The model depicts possible influences of the phosphorylation status of ZtfA on DNA-sequence specificity. The phosphorylation state of ZtfA might have influences on recruitment of different interaction partners and thus on DNA-binding specificity. It could also destabilize the ZtfA protein. Phosphorylation sites identified in this study are indicated. Note that blue arrows do not differ between activating and repressing effects.

It is also possible that the phosphorylation status of ZtfA influences interaction with other proteins and thereby changes DNA-sequence specificity (FIGURE 53). It could also lead to hyperactivation of ZtfA. This would be an interesting task for further studies.

Two kinases, MpkB and RfeA, were identified as putative interaction partners. The three identified phosphorylation sites in ZtfA notably do not match the recognition site for MpkB (Thr-Glu-Tyr) (Kobayashi *et al.*, 2007). RfeA is not characterized yet and its recognition site is not known. The identified phosphorylation sites in ZtfA differ in their sequences. Therefore, RfeA might only be responsible for phosphorylation of one of these sites. RfeA is annotated as putatively involved in secondary metabolism. This might indicate that the phosphorylation status of ZtfA could be important for its regulatory roles in secondary metabolism. Not all peptides of the ZtfA protein were identified in LC-MS/MS analyses and bioinformatic analyses of the ZtfA amino acid sequence revealed 34 putative additional phosphorylation sites besides the ones investigated in this study. This implies that the constructed *ztfA*$^{S327A,T464A,S504-506A}$ strain might not produce a fully dephosphorylated ZtfA protein and that further phosphorylation sites with regulatory function exist. Moreover, the phosphorylation status of ZtfA might differ during different growth and developmental phases. This has to be analyzed in future studies.

4.4 Orthologs of ZtfA have regulatory roles in developmental programs in Aspergilli

Orthologs of ZtfA were found in Aspergilli as well as in non-Aspergilli during *in silico* analyses (see FIGURE 13 in CHAPTER 3.1).

The *scl-2* mutant of *A. niger* shows sporulation defects, similar to the situation in Δ*ztfA* of *A. nidulans* (Jørgensen *et al.*, 2011). The *scl-2* mutant has defects in the *ztfA* orthologous genetic locus (A.F.J. Ram, p.c.). This is in good agreement with the above proposed hypothesis, that ZtfA might show functional conservation among Aspergilli (see CHAPTER 4.1.2). The *scl-2* mutant forms sclerotia and the respective *A. niger* gene product might represent a repressor of sclerotia formation. Sclerotia are thought to correspond to cleistothecia in *A. nidulans*. ZtfA of *A. nidulans* is not a major repressor of sexual development since an absence of *ztfA* does not lead to a distinct increase in cleistothecia formation.

A loss of *ztfA* does not lead to an obvious conidiation phenotype in *A. fumigatus* (see CHAPTER 3.8). However, slight morphological effects on colony morphology are visible in *A. fumigatus* Δ*ztfA* as well. This discrepancy in conservation between *A. nidulans*, *A. niger*

and *A. fumigatus* is interesting as *A. niger* and *A. fumigatus* are more closely related to each other than the two species to *A. nidulans* (Peterson, 2008; Varga *et al.*, 2003).

The conidiation cascade of *A. fumigatus* exhibits some striking differences to the one in *A. nidulans* (see CHAPTER 1.9.3 to 1.9.4 and FIGURE 54). Factors, which regulate distinct events in one fungus, might exhibit a change in regulation in the other as has been shown for the velvet factors, but also for WetA and AbaA and several Flb factors (Mah and Yu, 2006; Park and Yu, 2012; Tao and Yu, 2011; Xiao *et al.*, 2010) (FIGURE 54).
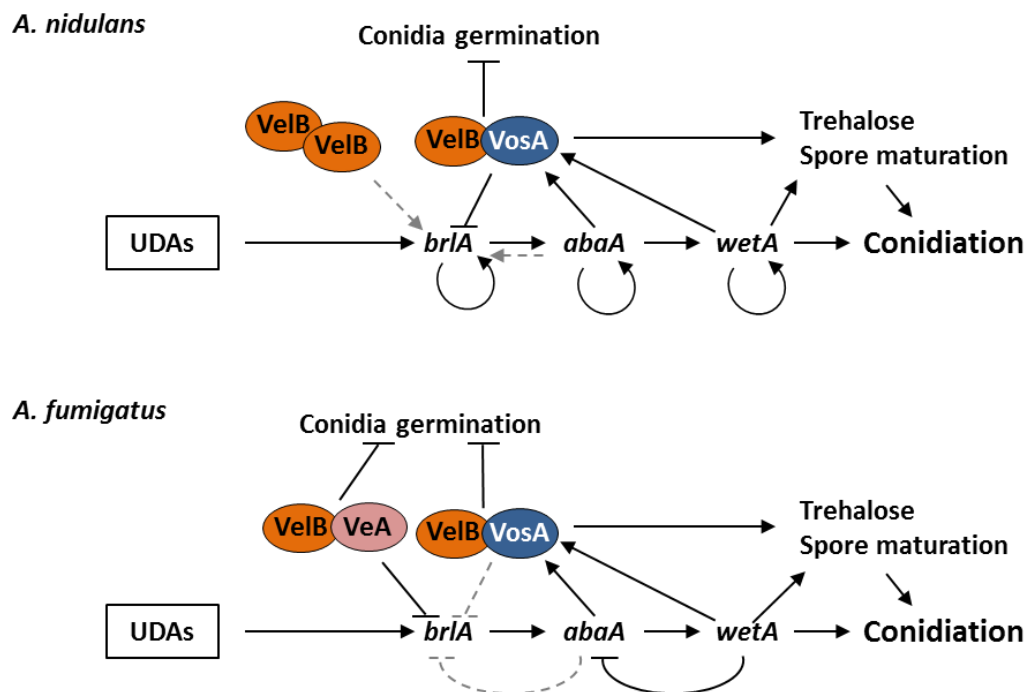
FIGURE 54: Differences in the regulation of conidiation between *A. nidulans* and *A. fumigatus*.
Regulation of the central developmental pathway for asexual propagation in *A. nidulans* (top) and *A. fumigatus* (bottom) reveals differences between both fungi. Black lines indicate trusted knowledge, grey dotted lines indicate proposed regulations. Modified from Park *et al.*, 2012a.

Notably, deletion of the upstream developmental factor-encoding *fluG* and *flbA* genes, which lead to fluffy phenotypes in *A. nidulans* due to decreased and delayed conidiation, do not result in fluffy phenotypes in *A. fumigatus* (Mah and Yu, 2006; Yu *et al.*, 2006). Levels of conidiation in Δ*flbA* are reduced but the colony appearance is WT-like. Loss of *fluG* does not result in a conidiation phenotype on solid medium when oxygen is present but hinders conidiophore development in submerged cultures, where the *A. fumigatus* WT is able to form conidiophores. ZtfA is a conidiation activator in *A. nidulans* and *A. niger*. However, this is not the case in *A. fumigatus*: a Δ*ztfA* mutant does not show an obvious sporulation defect. This is interesting as *A. niger* and *A. fumigatus* are more closely related to each other than the two

species to *A. nidulans* (Peterson, 2008; Varga *et al.*, 2003). Both species are proposed to be heterothallic, even though thallism is not completely clear for *A. niger* (Frisvad *et al.*, 2014; Varga *et al.*, 2003).

Loss of *ztfA* in *A. fumigatus* leads to a downregulation of *brlA* expression. This implies that ZtfA is involved in conidiation regulation in this fungus as well. More than one conidiation pathway have been hypothesized in other studies for *A. fumigatus* (Mah and Yu, 2006; Yu, 2010). Therefore, a redundancy in conidiation regulation is assumed, where another transcription factor would fulfill similar functions as ZtfA and might circumvent deleterious effects caused by *ztfA* deletion. Another possibility is that ZtfA might have undergone a functional conversion and might regulate different aspects of the *A. fumigatus* life cycle compared to *A. nidulans*.

In summary, *A. nidulans* ZtfA and its ortholog in *A. niger* share conserved functions, such as activation of conidiation. The *A. niger* ZtfA ortholog further functions as repressor of sclerotia formation. This function is probably not conserved in *A. nidulans*. The ZtfA ortholog of *A. fumigatus* is dispensable for conidiation and might have undergone a functional conversion. This is further discussed in CHAPTER 4.8.

4.5 ZtfA interconnects asexual development and secondary metabolism in *A. nidulans*

4.5.1 ZtfA is an activator of secondary metabolism

Several studies show a link between secondary metabolite (SM) production and developmental programs (Bayram *et al.*, 2008a; Bayram and Braus, 2012; Calvo, 2008; Calvo *et al.*, 2002; Estiarte *et al.*, 2016; Gerke *et al.*, 2012a; Lee *et al.*, 2012; Ramamoorthy *et al.*, 2012; Wang *et al.*, 2016; Yin *et al.*, 2013). ZtfA is a new transcription factor that interconnects asexual development and secondary metabolism in *A. nidulans*. This function is conserved to its ortholog in *A. niger*: the *scl-2* mutant exhibits diminished conidiophore formation and impaired secondary metabolite production (Jørgensen *et al.*, 2011).

ZtfA of *A. nidulans* is required for austinol and dehydroaustinol production, since both compounds are absent in the Δ*ztfA* mutant. Transcriptional analyses show that *ztfA* OE is sufficient to activate austinol-pathway gene expression during vegetative growth. Furthermore, *ztfA* OE is sufficient to upregulate expression of important genes of the F9775/orsellinic acid (*orsA*) and the emericellamide gene (*easA*, *easB*, *easC* and *easD*) cluster during vegetative growth. It was shown that expression of the *orsA* gene, encoding the PKS of the F9775 cluster, depends on environmental constituents: *orsA* expression is upregulated

when *A. nidulans* is co-cultivated with the soil bacterium *Streptomyces hygroscopius* but only basally expressed when the fungus is cultivated alone (Schroeckh *et al.*, 2009). An overexpression of *ztfA* is sufficient to induce *orsA* gene expression in the absence of another organism. ZtfA is a permanently nuclear localized transcription factor. Transcription factors with permanent nuclear localization are thought to be activated by environmental stimuli or secondary metabolite intermediates. It is therefore possible that intermediates produced by other organisms, such as *S. hygroscopius* activate ZtfA in the nature, where the fungus grows in diverse ecological environments. HPLC profiles of *ztfA* mutants suggest that further SMs are regulated by ZtfA as well. These might be SMs not produced in WT in the conditions tested and would indicate that ZtfA is not solely an activator but has also repressing capacities for specific SM gene clusters. On the other hand the possibility exists that these peaks in HPLC profiles represent SM intermediates in pathways where production of the final SM product is blocked in the absence of *ztfA* due to gene misregulation of crucial steps of the respective pathway.

Bridging factors in the interconnection of secondary metabolism and development are the velvet proteins, which form the velvet complex, a heterotrimer composed of VelB, VeA and the SM master regulator LaeA (Bayram *et al.*, 2008a; Bok and Keller, 2004; Sarikaya-Bayram *et al.*, 2010, 2015) (see CHAPTER 1.2 and 1.3). Phenotypic analyses show that ZtfA does not act in VeA- or VelB-governed pathways.

In conclusion, ZtfA interconnects asexual development and secondary metabolism and acts as activator of both programs.

4.5.2 Activation of conidiation is independent of ZtfA-mediated secondary metabolite regulation

As discussed above, ZtfA influences the conidiation pathway downstream of FluG and upstream of the Flb factors FlbC and FlbD. FluG is necessary for the synthesis of an unknown low molecular weight molecule, which is proposed to be important for conidiation activation (Lee and Adams, 1994a): Lee and Adams found that the conidiation defect in a Δ*fluG* strain can be rescued by a WT colony grown next to the Δ*fluG* colony and that a diffusible barrier does not hinder this rescue. Rodríguez-Urra and collaborators found that an adduct of dehydroaustinol and diorcinol is able to overcome the conidiation defect of the Δ*fluG* mutant. They hypothesized that one or both SMs, or dehydroaustinol- or diorcinol-like compounds are involved in the FluG signaling pathway (Rodríguez-Urra *et al.*, 2012). This would be an explanation for the developmental phenotype of Δ*ztfA*: the FluG signal cannot be formed

since austinol and dehydroaustinol are not synthesized and therefore, the conidiation cascade cannot be activated. In contrast to this hypothesis it was shown that both, austinol as well as dehydroaustinol, are not essential for conidiation: a Δ*ausA* strain is able to sporulate and the *ausA* gene is not necessary for asexual development (Nielsen *et al.*, 2011). FluG counteracts SfgA, which represses translocation and therefore conidiation activation of the FlbB-FlbE heterodimer (Etxebeste *et al.*, 2008, 2009; Herrero-Garcia *et al.*, 2015; Lee *et al.*, 2014; Seo *et al.*, 2006). Therefore, the phenotype of a *flbB* or *flbE* deficient mutant should resemble the Δ*ztfA* phenotype if ZtfA would be involved in the FluG signal upstream of the *flb* genes. Phenotypical analyses of the *flb* mutants in Δ*ztfA* contradict this (see CHAPTER 3.4.3). Deletion of *sfgA* completely bypasses *fluG* in conidiation and sterigmatocystin production (Seo *et al.*, 2006). This indicates that FluG mainly exhibits its activation towards the conidiation cascade by antagonizing negative effects of SfgA. Expression of *sfgA* is not regulated by ZtfA. A regulatory role of ZtfA upon *sfgA* expression would have been expected if ZtfA would be important for the activating effect of FluG on conidiation. Seo and co-workers proposed a model in which FluG activates FlbE, which then represses SfgA (Seo *et al.*, 2006). Neither phenotypical nor gene expression analyses support the possibility that ZtfA might act as a FluG-signal transducer in such a model.

4.5.3 Sterigmatocystin production is regulated by ZtfA

The SM sterigmatocystin is a potent mycotoxin and FluG is involved in its regulation (Hicks *et al.*, 1997; Seo *et al.*, 2003). This regulation is proposed to be accomplished via the activation of FlbA (Hicks *et al.*, 1997). FlbA is necessary to inhibit the FadA-mediated G-protein signaling pathway, which leads to a repression of conidiation and sterigmatocystin production via PkaA (Hicks *et al.*, 1997; Shimizu *et al.*, 2003) (FIGURE 55).

The deficiency in sterigmatocystin production in Δ*fluG* is a result of a lack of *flbA* activation (Hicks *et al.*, 1997). *aflR*, encoding the sterigmatocystin transcriptional regulator, and consequently *stc* gene (sterigmatocystin cluster) transcripts fail to accumulate in the absence of *flbA* (Hicks *et al.*, 1997). Likewise, a loss of *fluG* leads to a loss of *stc* transcript accumulation (Hicks *et al.*, 1997). An overexpression of *flbA* is able to induce premature *stc* transcript accumulation, followed by early sterigmatocystin production (Hicks *et al.*, 1997; Keller *et al.*, 1994). *flbA* expression is repressed by SfgA and absence of *sfgA* rescues sterigmatocystin production in Δ*fluG* (Seo *et al.*, 2006). Sterigmatocystin regulation is not a common function of conidiation regulators, as, besides FluG and FlbA, no other factor of the UDA or CDP pathway seems to be involved in sterigmatocystin regulation (Hicks *et al.*,

1997). ZtfA acts downstream of FluG and is involved in a feedback regulation of *fluG* expression during asexual growth. Nevertheless, ZtfA does probably not act in a sterigmatocystin regulation in between FluG and FlbA: ZtfA represses *flbA* expression during asexual growth. However, this might be indirect since a *ztfA* OE does not downregulate *flbA* expression.

Sterigmatocystin production is increased in both, the absence and the overexpression of *ztfA*. AflR is the major regulator of sterigmatocystin production in Aspergilli (Ehrlich *et al.*, 2003; Yu *et al.*, 1996a). Both, absence and overexpression of *ztfA* lead to upregulation of *aflR* and *stcU*, suggesting a tight balance of *ztfA* expression for maintenance of sterigmatocystin production at WT level. *stcU* transcript levels are used as indicator for *stc* cluster activation (Hicks *et al.*, 1997; Kato *et al.*, 2003). A similar regulation was found for the bZIP transcription factor RsmA, for which direct binding to the *aflR* promoter was shown (Yin *et al.*, 2012). Comparable to the regulation of sterigmatocystin via ZtfA, Δ*rsmA* as well as *rsmA* OE upregulate production of this SM (Shaaban *et al.*, 2010; Yin *et al.*, 2012). Since RsmA directly binds to the *aflR* promoter and *rsmA* OE upregulates *aflR*, Yin and collaborators proposed an induction of *aflR* through RsmA. A similar regulation could explain the observed sterigmatocystin increase in *ztfA* mutants even though direct binding of ZtfA to the *aflR* promoter is unclear at the moment (FIGURE 55).
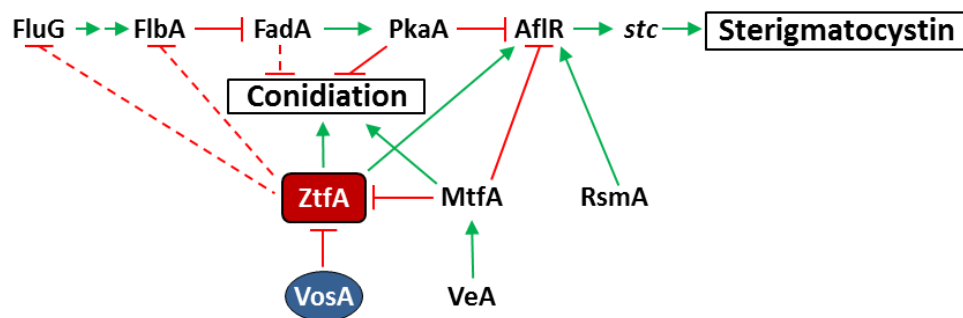
FIGURE 55: ZtfA is involved in sterigmatocystin biosynthesis regulation in *A. nidulans*. The depicted simplified model summarizes the regulatory role of ZtfA on sterigmatocystin production during asexual development. Red lines indicate negative regulations, green arrows show activating effects. Recently, MtfA was analyzed and might act as an antagonist of ZtfA in the regulation of sterigmatocystin biosynthesis; RsmA and ZtfA might have similar regulatory roles in sterigmatocystin regulation (Lind *et al.*, 2015; Ramamoorthy *et al.*, 2013; Yin *et al.*, 2012).

MtfA, downstream of VeA, regulates sterigmatocystin production in an inverted manner: loss as well as overexpression of *mtfA* resulted in reduced sterigmatocystin production and loss of *aflR* and *stcU* mRNA accumulation (Ramamoorthy *et al.*, 2013). Comparable SM regulation is known for VeA: a tight control of *veA* expression is necessary for penicillin biosynthesis, as

suggested by knock out and overexpression experiments (Kato *et al.*, 2003; Spröte and Brakhage, 2007). ZtfA and MtfA both are downstream factors of the velvet network. VosA and VeA both share VelB as binding partner (Bayram *et al.*, 2008a; Sarikaya-Bayram *et al.*, 2010). MtfA might therefore be an antagonist of ZtfA in sterigmatocystin production (FIGURE 55). This is further supported by the fact that transcriptomic analyses of a Δ*mtfA* strain showed an upregulation of *ztfA* gene expression, indicating a repressing effect of MtfA upon *ztfA* expression (Lind *et al.*, 2015).

4.5.4 Transcription factors with regulatory roles in secondary metabolism and oxidative stress response are regulated by ZtfA

Several transcription factors interconnect SM biosynthesis and the oxidative stress response (OSR) (Emri *et al.*, 2015; Hong *et al.*, 2013a, 2013b; Montibus *et al.*, 2015; Reverberi *et al.*, 2010; Roze *et al.*, 2011; Yin *et al.*, 2013, 2012). For instance, RsmA is involved in sterigmatocystin regulation and is important for the OSR (Emri *et al.*, 2015; Shaaban *et al.*, 2010). Hence, a putative regulatory role of ZtfA upon *rsmA* gene expression in response to H_2O_2 stress was investigated. *rsmA* expression is upregulated in the presence of hydrogen peroxide. This upregulation is ZtfA-dependent as it is intensified in *ztfA* OE upon H_2O_2 stress but no upregulation is found in response to H_2O_2 in Δ*ztfA*. A *rsmA* overexpression is able to overcome the block in sterigmatocystin production in Δ*laeA* (Shaaban *et al.*, 2010). ZtfA is necessary for *rsmA* activation during H_2O_2 stress but not for general expression of *rsmA* under non-stressed conditions. ZtfA therefore might be involved in stress-mediated SM regulation via *rsmA* gene-expression regulation.

In several cases, transcription factors, which couple SM and the OSR, are negative regulators of SM and positive regulators of the OSR. For instance, NapA negatively regulates emericellin, sterigmatocystin and other SMs as a *napA* OE strain produces lower amounts of these compounds compared to WT (Yin *et al.*, 2013). On the other hand, NapA is a positive regulator of the OSR as Δ*napA* shows decreased resistance against oxidative stress in *A. nidulans* and other fungi (Asano *et al.*, 2007; Thön *et al.*, 2010). NapA is the ortholog of the yeast Yap1 factor (Asano *et al.*, 2007; Toone *et al.*, 2001). Yap1 is important for expression of both, the thioredoxin and the glutathione system in *S. cerevisiae* (Kuge and Jones, 1994; Moye-Rowley, 2003; Wu and Moye-Rowley, 1994). *napA* expression was screened as well but was not influenced by the H_2O_2 stress conditions tested in this study. ZtfA seems to function in an inverted manner compared to NapA. It is an activator of secondary metabolism and a repressor of the OSR. Expression of *ztfA* itself is upregulated

upon oxidative stress as well. This stress-coupled induction emphasizes the finding that ZtfA is involved in regulation of the OSR in *A. nidulans*.

4.6   ZtfA is involved in the oxidative stress response of *A. nidulans* and *A. fumigatus*

4.6.1   ZtfA is an inhibitor of the oxidative stress response

Fungi have to cope with several stress factors. Amongst them, oxidative stress is one of the most prevalent stresses. It occurs due to reactive oxygen species (ROS) formed during aerobic respiration and other metabolic processes or derived from environmental sources (Aguirre *et al.*, 2005; Marschall and Tudzynski, 2016; Moye-Rowley, 2003; Nath *et al.*, 2016; Zhang *et al.*, 2016). Fungal pathogens have to face increased ROS production as defense mechanism of the innate immune system (Braem *et al.*, 2015; Leal *et al.*, 2012; Cramer *et al.*, 2013). ZtfA is an inhibitor of the OSR since loss of *ztfA* leads to distinctly increased tolerance towards H_2O_2 in *A. nidulans*, but only slightly increased tolerance to H_2O_2 in *A. fumigatus*. This difference can be explained with the sophisticated OSR system in *A. fumigatus*. Loss of either conidial or mycelial catalases can be circumvented due to a high quantity of OSR mechanisms in this pathogen (Brandon *et al.*, 2015; Dagenais and Keller, 2009; Paris *et al.*, 2003). Therefore, malfunction or loss of one element of the OSR can probably be compensated by factors with redundant function.

4.6.2   ZtfA activates the thioredoxin system during H_2O_2 stress in *A. nidulans*

The OSR in fungi comprises enzymes, such as catalases, as well as redox systems (Bayram *et al.*, 2016; Carmel-Harel and Storz, 2000; Kawasaki *et al.*, 1997; Kawasaki and Aguirre, 2001; Navarro *et al.*, 1996; Sato *et al.*, 2009; Thön *et al.*, 2007). The glutathione- and thioredoxin-dependent redox systems are important parts of the cellular oxidative stress defense mechanisms in fungi (Carmel-Harel and Storz, 2000; Emri *et al.*, 2015; Jamieson, 1998; Kawasaki *et al.*, 1997; Larochelle *et al.*, 2006; Thön *et al.*, 2007, 2010). ZtfA is necessary for induction of several stress response genes when H_2O_2 is present. The thioredoxin system comprises thioredoxin (TrxA) and its reductase (TrxR) (Holmgren, 1985, 2002). The small thioredoxins reduce disulfides in their targets and are re-reduced by their thioredoxin reductases, which use NADPH as electron donor and FAD as co-factor (Thön *et al.*, 2007) (FIGURE 56). ZtfA is necessary for induction of *trxR* gene expression in response to H_2O_2 but not involved in the general expression of the thioredoxin system during unstressed conditions.

The thioredoxin system possesses a key role in the redox regulation and is important for development in *A. nidulans* (Thön *et al.*, 2007, 2010). Hence, this regulation upon oxidative stress might represent a second layer of developmental regulation by ZtfA in response to intracellular redox homeostasis. The glutathione system functions similar to the thioredoxin system. Glutathione reduces ROS and is re-reduced by its glutathione reductase (GlrA) using NADPH as electron donor (Bakti *et al.*, 2017; Meister and Anderson, 1983). Influences of ZtfA upon the glutathione system were less pronounced and upregulation of *glrA* expression during H_2O_2 stress is not ZtfA dependent. The thioredoxin and the glutathione system interact in the redox regulation in fungi (Sato *et al.*, 2009; Song *et al.*, 2006; Thön *et al.*, 2007; Trotter and Grant, 2003). The weak regulatory effects upon the glutathione system observed in this study might be explainable by its interplay with the ZtfA-dependent thioredoxin system.
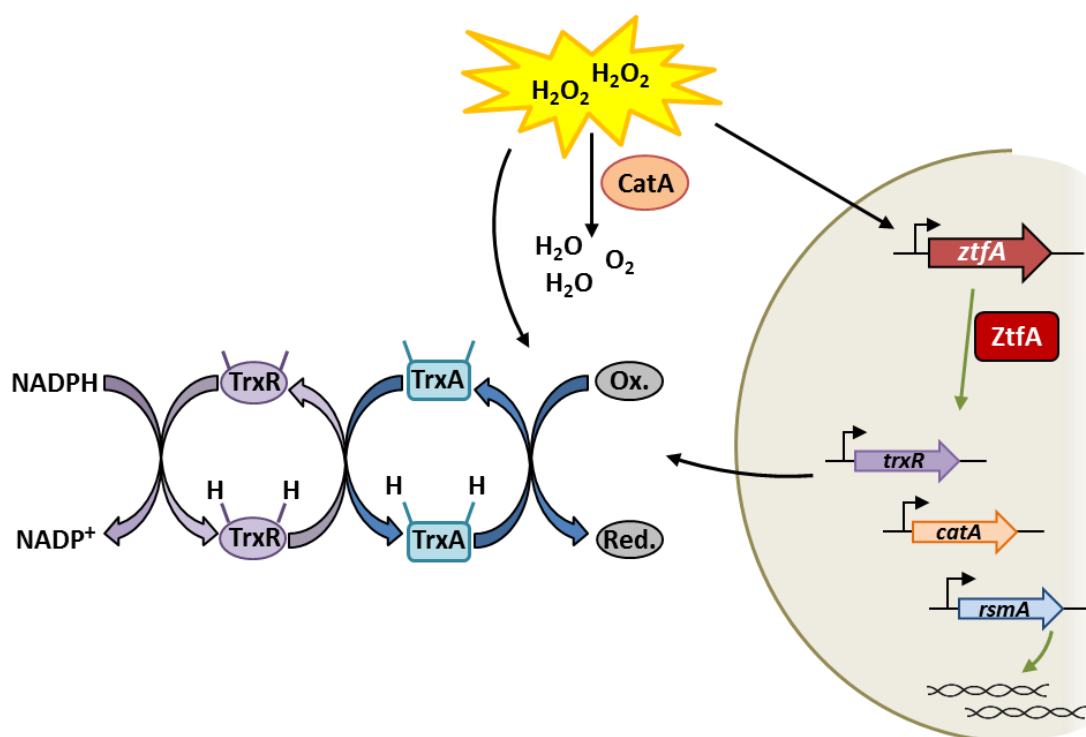
FIGURE 56: ZtfA regulates gene expression in response to hydrogen peroxide in *A. nidulans*.
A simplified model of ZtfA-dependent gene regulation in response to H_2O_2 in *A. nidulans* is shown. H_2O_2 stress (yellow) leads to oxidation of proteins (Ox., grey), which are reduced (Red., grey) by thioredoxin (TrxA). TrxA in turn is oxidized at its redox-active cysteine pair and subsequently is re-reduced in a NADPH-dependent reaction catalyzed by thioredoxin reductase (TrxR), which itself uses a redox-active cysteine pair. *ztfA* transcription is upregulated during H_2O_2 stress and increased ZtfA amounts result in upregulation of crucial parts of the OSR, such as *trxR*, *catA* and *rsmA*. The transcription factor RsmA then regulates further downstream genes.

4.6.3   ZtfA is important for catalase gene upregulation in response to H$_2$O$_2$ in A. nidulans

Antioxidant enzymes, such as catalases, are involved in the fungal OSR. At least five catalases exist and from these at least four are involved in the *A. nidulans* OSR (Bayram *et al.*, 2016; Kawasaki *et al.*, 1997; Kawasaki and Aguirre, 2001; Navarro *et al.*, 1996). CatA and CatB are involved in general OSR, whereas CatC and CatD activity was found in only certain stress situations or certain cellular structures (Kawasaki and Aguirre, 2001; Scherer *et al.*, 2002). Deletion of *catA*, *B* and *C*, as well as double and triple deletions did not have developmental influences in *A. nidulans* (Kawasaki *et al.*, 1997; Kawasaki and Aguirre, 2001). The hyphal catalase B (CatB) protects against external as well as internal H$_2$O$_2$ (Kawasaki *et al.*, 1997). *catC* is not induced during oxidative or osmotic stress and only slightly upregulated by heat shock stress whereas CatD activity was found to be even more specific (Kawasaki and Aguirre, 2001). *catA* and *catB* mRNA accumulate upon oxidative stress treatment in *A. nidulans* (Navarro and Aguirre, 1998; Noventa-Jordão *et al.*, 1999). In contrast to WT, *catA* expression is not induced in Δ*ztfA* upon H$_2$O$_2$ treatment but strongly induced in *ztfA* OE during vegetative growth. This might be due to the diminished conidiophores in Δ*ztfA*, since CatA is a spore specific catalase. However, it has been shown that spore formation is not a requirement for *catA* expression (Navarro *et al.*, 1996). Notably, in *ztfA* OE, *catA* is already upregulated in a non-stressed situation compared to WT. This might be due to the fact that *ztfA* OE already forms conidiophores during vegetative growth. Thereby, ROS are produced, which have to be detoxified. *catA* upregulation upon oxidative stress is strongly ZtfA dependent since a regulation upon hydrogen peroxide treatment was absent in Δ*ztfA*. *catB* expression is not upregulated in WT or Δ*ztfA* under tested conditions upon hydrogen peroxide treatment. This can be explained by the fact that different parts of the OSR react to different oxidative stressors (Emri *et al.*, 2015). Non-equivalent response to different oxidative stressors has been shown for OSR in other fungi as well (Moye-Rowley, 2003). Nevertheless, *ztfA* OE is sufficient to induce *catB* upregulation even under tested conditions. Since *ztfA* OE does not show increased tolerance towards H$_2$O$_2$, it is assumable that further mechanisms exist, which are more important in the OSR than the products of the genes tested in this study. It is likely that ZtfA regulates the OSR in general as the conditions tested here only show a snapshot of the total OSR in *A. nidulans*.

ZtfA is presumably not necessary for the general expression of OSR genes under non-stressed conditions since all tested genes are expressed, but several OSR genes are not regulated in the absence of *ztfA* upon hydrogen peroxide stress. A loss of *ztfA* does not result in increased

sensitivity but in a decreased sensitivity towards hydrogen peroxide stress. For several other mutants, where OSR genes are misregulated, increased sensitivity due to disturbed OSR was found. Deletion of *catA* and *catB* leads to decreased tolerance of conidiospores towards H_2O_2 and impaired colony growth in the presence of H_2O_2, respectively (Kawasaki *et al.*, 1997; Navarro *et al.*, 1996). Loss of *sskA* or *srrA* leads to hypersensitivity towards hydrogen peroxide and downregulation of *catA* and *catB* expression (Hagiwara *et al.*, 2007). Loss of *atfA* or *sakA* increases oxidative stress sensitivity as well (Emri *et al.*, 2015; Kawasaki *et al.*, 2002). ZtfA in general acts as repressor for the oxidative stress tolerance but positively regulates expression of redox system and catalase encoding genes in response to H_2O_2. ZtfA presumably regulates expression of further, yet unknown, factors of the OSR in *A. nidulans*, which are important for oxidative stress tolerance.

4.7 ZtfA is localized in nuclei of germlings, hyphae and conidiophores and interacts with RcoA in *A. nidulans*

4.7.1 Nuclear localization of ZtfA is important for *A. nidulans*

Transcription factors need to be localized in the nucleus in order to regulate gene expression. Zinc cluster proteins can be divided into two groups with respect to their localization: i) proteins, which are permanently localized in the nucleus and ii) proteins, which are localized in the cytoplasm and shuttled into the nucleus upon their activation signal (MacPherson *et al.*, 2006). For the first group it is postulated that its members are activated by target molecules or metabolic intermediates (Flynn and Reece, 1999; Harbison *et al.*, 2004; Kirkpatrick and Schimmel, 1995; Sellick and Reece, 2003, 2005). α/ß importin heterodimers bind to the NLS of cargo proteins and the complex shuttles into the nucleus through nuclear pores (Beck and Hurt, 2017; Garcia *et al.*, 2016; Lim *et al.*, 2015; Miyamoto *et al.*, 2016; Schwartz, 2016). *In silico* analyses predicted a NLS with high confidence for ZtfA and a NES with lower scores. Fluorescence microscopic analyses of GFP-tagged ZtfA proteins show a nuclear localization during all conditions tested. Four importins were identified in pull-down experiments as putative interaction partners of ZtfA. This suggests that a rapid nuclear localization of ZtfA is important for the fungus. This is supported by the nuclear localization of ZtfA in germlings, hyphae and conidiophores, which in conclusion, is important for *A. nidulans* during asexual growth and ongoing asexual development.

4.7.2 The RcoA-ZtfA complex might function in secondary metabolism and development

Several proteins were identified in pull-downs with GFP-tagged ZtfA. A number of these putative binding partners of ZtfA are uncharacterized proteins. Importantly, ZtfA pulled down different interaction partners during different developmental stages. This indicates that ZtfA might specifically interact with different proteins depending on developmental stages. RcoA was identified as putative interaction partner during vegetative, asexual and sexual growth. The interaction of RcoA and ZtfA was further verified *in vivo*, demonstrating the presence of a ZtfA-RcoA complex. RcoA is a VeA-dependent WD40 repeat protein, which regulates developmental programs, as well as sterigmatocystin production (Bayram and Braus, 2012; Hicks *et al.*, 2001; Todd *et al.*, 2003, 2006). RcoA fulfills its regulatory functions partly in a conserved co-repressor complex together with SsnF (García *et al.*, 2008; Hicks *et al.*, 2001; Jöhnk *et al.*, 2016; Todd *et al.*, 2003). SsnF was not found in GFP-trap experiments with ZtfA as bait. Therefore, it is not clear at the moment if ZtfA interacts with this co-repressor complex. The SsnF-RcoA co-repressor complex corresponds to the yeast Ssn6-Tup1 co-repressor, which binds different DNA-binding proteins as substrate linkers (Cupertino *et al.*, 2015; Hanlon *et al.*, 2011; Roy *et al.*, 2013). The Ssn6-Tup1 co-repressor is involved in hypoacetylation of H3 and H4 histones and the positioning of nucleosomes, thereby blocking DNA-accessibility for the transcription machinery (Church *et al.*, 2017; Davie *et al.*, 2003; Fleming *et al.*, 2014; Rizzo *et al.*, 2011; Watson, 2000). Furthermore, the co-repressor competes with transcription factors for promoter binding and can occlude promoters, thereby repressing gene transcription (Islam *et al.*, 2011; Merhej *et al.*, 2015) (FIGURE 57).
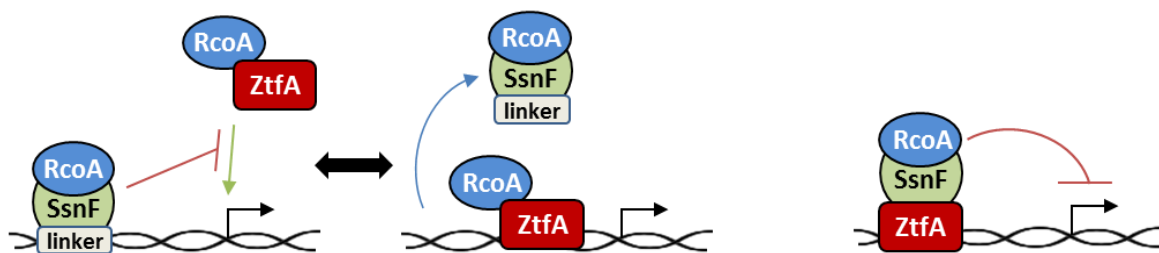
FIGURE 57: Regulatory roles of RcoA-ZtfA upon target genes.
The model summarizes two possibilities of gene expression regulation of RcoA-ZtfA. The complex could compete with the SsnF-RcoA co-repressor complex in both, special promoter binding and RcoA disposability (left hand side). ZtfA itself could also function as protein linker between SsnF-RcoA and DNA (right hand side). Both possibilities might coexist.

Transcriptional data gathered in this study suggest mainly activating effects of ZtfA towards downstream targets, but repressing effects upon *flb* genes were found during late asexual development as well. A switch from repression to activation of downstream targets in response to physiological conditions has been shown for RcoA (Hicks *et al.*, 2001). Accumulation of *brlA* mRNA is delayed in the absence of *rcoA* (Hicks *et al.*, 2001). Therefore it is possible, that an RcoA-ZtfA complex regulates *flbC*, *flbD* or *brlA* expression. In this scenario, RcoA-ZtfA could compete with the SsnF-RcoA co-repressor for promoter binding of target genes as well as for RcoA disposability (FIGURE 57). A second possibility emerges: ZtfA could also function as substrate linker between the SsnF-RcoA co-repressor and respective target promoters (FIGURE 57).

RcoA is involved in regulation of secondary metabolism in *A. nidulans*. *aflR* and *stcU* transcripts were not detected in the absence of *rcoA* and sterigmatocystin is not produced (Hicks *et al.*, 2001). Therefore, it is tempting to speculate that ZtfA regulates sterigmatocystin production as an RcoA-ZtfA complex. ZtfA or RcoA could further act as monomers or heteromeric complexes with other proteins in such a scenario. This would explain why a loss as well as an overexpression of *ztfA* upregulates sterigmatocystin biosynthesis: both situations would change the equilibrium of possible monomers or complexes. An interaction of RcoA and ZtfA is also interesting since HPLC data suggest that ZtfA regulates the production of further SMs, which could not be clarified in this study. Most SM gene clusters are silent under laboratory conditions in fungi and the proportion of unknown SMs is presumably significantly larger than the proportion of already known SMs (Gerke *et al.*, 2012b; Gerke and Braus, 2014; Hoffmeister and Keller, 2007; Khaldi *et al.*, 2010). Therefore, a better understanding of this RcoA-ZtfA protein complex, especially with respect to putative regulation of SM gene clusters, is important.

4.8   ZtfA regulates adhesion in *A. fumigatus*

4.8.1   Functional conversion of transcription factors between *A. nidulans* and *A. fumigatus*

Functional conversion of transcription factors and other regulatory proteins, which act as developmental regulators in *A. nidulans*, to SM or virulence factors in *A. fumigatus* has been shown in several studies. VeA is the most prominent example for such a functional conversion: VeA is a negative regulator of asexual development in response to light in *A. nidulans* (Bayram *et al.*, 2008a, 2008b; Käfer, 1965; Terfrüchte *et al.*, 2014). In contrast,

loss of *veA* in *A. fumigatus* does not result in an obvious phenotype under normal growth conditions, but VeA is rather a positive SM regulator in this fungus (Dhingra *et al.*, 2012, 2013; Krappmann *et al.*, 2005). MtfA is another example for a transcription factor, which is required for development in *A. nidulans*, but underwent a functional conversion in *A. fumigatus* (Ramamoorthy *et al.*, 2013; Smith and Calvo, 2014). *aflR* gene expression and sterigmatocystin production are decreased in the absence of *mtfA* and conidiation and Hülle cell numbers are diminished in *A. nidulans* (Ramamoorthy *et al.*, 2013). Deletion of *mtfA* in *A. fumigatus* leads only to a minor developmental phenotype, but virulence in *G. mellonella* infection is decreased (Smith and Calvo, 2014). Functional conversions of proteins, which do not act as transcription factors, have been shown as well. F-box proteins are substrate linkers for SCF complexes which, together with the COP9 signalosome (CSN), function in the conserved ubiquitin proteasome pathway (UPP) as target carriers for protein degradation via the ubiquitin 26S proteasome system (Braus *et al.*, 2010; Ciechanover, 1998; Meister *et al.*, 2016; von Zeska Kress *et al.*, 2012). Deletion of the F-box protein Fbx15 in *A. nidulans* results in drastically reduced sexual and asexual development (von Zeska Kress *et al.*, 2012). In contrast, the ortholog in *A. fumigatus* is not involved in asexual development, but essential for general stress response and virulence (Jöhnk *et al.*, 2016).

4.8.2 ZtfA is involved in the regulation of polysaccharide production, gene expression of adhesion factors and the response to cell wall stress in *A. fumigatus*

Polysaccharides are an important virulence determinant and crucial for surface adhesion, an important step during host invasion of pathogenic fungi (Gravelat *et al.*, 2013; Kaur and Singh, 2013; Lin *et al.*, 2015; Sheppard, 2011). ZtfA is involved in regulation of polysaccharide production in *A. fumigatus*. The genes *Afu3g13110* and *Afu3g00880* encode putative adhesins and *Afu3g00880* is regulated by MedA (Chaudhuri *et al.*, 2011; Gravelat *et al.*, 2010; Lin *et al.*, 2015). MedA is regulated by SomA and has been shown to regulate conidiation and adhesion in *A. fumigatus* (Gravelat *et al.*, 2010, 2013; Lin *et al.*, 2015). The function of MedA is conserved in *A. nidulans* as it is involved in conidiophore development via regulation of temporal *brlA* expression (Busby *et al.*, 1996; Clutterbuck, 1969). ZtfA induces *Afu3g00880* expression and is essential for *Afu3g13110* expression. This indicates that ZtfA regulates adhesion in *A. fumigatus* and might render ZtfA a possible virulence factor (FIGURE 58).

The fungal cell wall is an important interface between host and fungal cells and contains adhesins and other molecules necessary for host invasion (Bruneau *et al.*, 2001; Karkowska-

Kuleta *et al.*, 2009; Latgé *et al.*, 1993). At least two pigments are present in the *A. fumigatus* cell wall, which have protective functions against environmental stresses, such as UV-radiation and ROS (Heinekamp *et al.*, 2012; Rambach *et al.*, 2015; Schmaler-Ripcke *et al.*, 2009; Sugareva *et al.*, 2006). Tolerance to SDS was reduced in *ztfA* OE in *A. fumigatus*. SDS induces cell wall stress by disrupting the cell membrane (Fortwendel *et al.*, 2008; Ram *et al.*, 2004). Involvement of ZtfA in the regulation of defense against cell wall stress might represent another layer of a possible involvement in virulence.
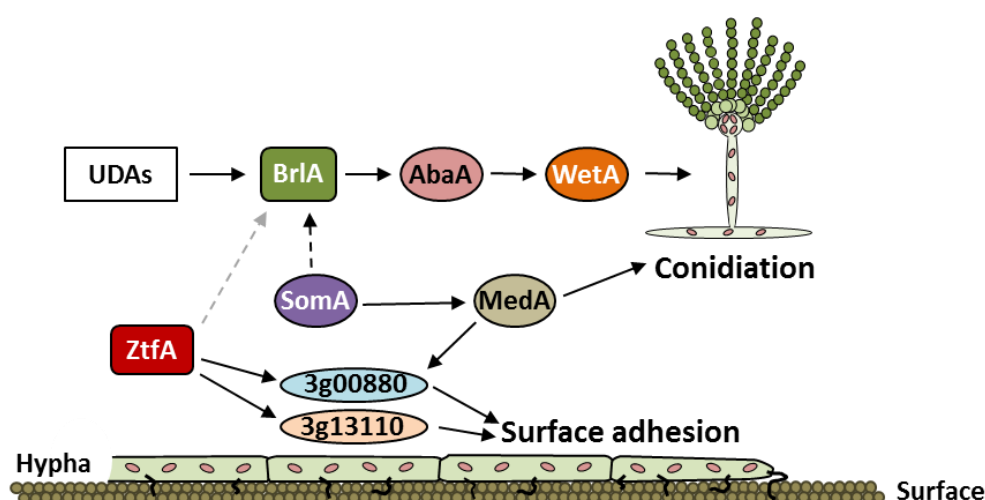
FIGURE 58: ZtfA regulates surface adhesion in *A. fumigatus*.
ZtfA regulates adhesion through regulation of expression of adhesin-encoding *Afu3g00880* (3g00880) and *Afu3g13110* (3g13110) and regulation of polysaccharide production in *A. fumigatus*. It might be involved in *brlA* gene expression regulation, but is dispensable for conidiation.

The *A. fumigatus* Δ*ztfA* strain did not show an obvious virulence phenotype in a *G. mellonella* infection assay. *G. mellonella* has been shown to serve as a suitable virulence model for developmental- and adhesion-defective *A. fumigatus* mutants and virulence data obtained in several studies are congruent with data from mice infections (Brennan *et al.*, 2002; Lin *et al.*, 2015; Renwick *et al.*, 2006; Slater *et al.*, 2011; Smith and Calvo, 2014). Similarly, *G. mellonella* has successfully been established as virulence model for other fungal pathogens, such as *C. albicans* strains defective in development and hyphal growth (Brennan *et al.*, 2002). The conformity in virulence between WT and Δ*ztfA* might be due to the fact that loss of *ztfA* did not lead to an obvious developmental defect in *A. fumigatus*. Utilization of insects as infection models emerged due to the fact that the innate immune response of insects and mammals, which is an important defense against fungal infections, is highly similar even though their immune systems differ greatly (Brennan *et al.*, 2002; Cohn *et al.*, 2001; Ratcliffe,

1985; Romani, 1999; Salzet, 2001). However, striking differences in virulence between *G. mellonella* and mice infections have been reported previously (Loh *et al.*, 2013; Olsen *et al.*, 2011). Cook and McArthur pointed out that, since genomic information are limited and standardized sources for *G. mellonella* larvae are missing, genetic variability might influence virulence tests, as previously shown for *Drosophila melanogaster* (Cook and McArthur, 2013; Tinsley *et al.*, 2006). Moreover, propagation conditions of *G. mellonella* larvae might differ greatly between suppliers and might influence associated microbiota, which may impact virulence assays as well (Cook and McArthur, 2013). Therefore, an involvement of ZtfA in other infection models cannot be excluded.

Taken together, ZtfA of *A. fumigatus* is involved in the regulation of fungal adhesion. ZtfA might be involved in virulence, since adhesion is an important virulence determinant, but is dispensable for *G. mellonella* infection.

4.9   Conclusion and outlook

This study shows that the transcription factor ZtfA is a novel activator of conidiation and secondary metabolism in *A. nidulans*. Its presence is conserved in Aspergilli. ZtfA is dispensable for conidiation in *A. fumigatus*, but regulates adhesion in this opportunistic pathogenic mold. ZtfA acts downstream of the velvet factor VosA in *A. nidulans*, which represses *ztfA* gene expression during late asexual growth.

Velvet factors constitute a family of transcription factors, which are important for the interconnection of developmental programs and secondary metabolism in fungi. This study underlines the importance of the genetic networks regulated by the velvet factors. Even though velvet factors are fungal specific proteins, these findings are of importance for the understanding of genetic networks in general, since fungal growth has striking similarities in common with neuronal development (Etxebeste and Espeso, 2016). The fungal-specific velvet proteins share structural similarities with NF-κBs of animals (Ahmed *et al.*, 2013), indicating a certain conservation of defense mechanisms and growth of cellular networks. NF-κBs can function as activators as well as repressors of target gene expression and possess auto-regulatory features (Snow and Albensi, 2016). NF-κBs are involved in regulation of neurogenesis and neuronal differentiation, cell viability, neuronal network formation, neuronal and synaptic plasticity and synaptic transmission in humans (Engelmann and Haenold, 2016; Snow and Albensi, 2016). Both, the fungal velvet genetic network as well as the NF-κB network exhibit nuclear import control mechanisms and execute their regulatory roles as homo- and heterodimers (Bayram *et al.*, 2008a; Sarikaya-Bayram *et al.*, 2015; Zabel

et al., 1993). The present study suggests further similarities between both regulatory networks, especially with respect to functions of their downstream factors (fungal growth/neuronal development). In conclusion, genetic networks of organisms, evolutionary as far away from each other as mammalians and fungi, share similarities on regulatory levels.

ZtfA is a new activator for *brlA* gene expression, which encodes the master conidiation regulator and its activators, encoded by *flbC* and *flbD*. EMSA or ChIP experiments are interesting to proof direct promoter binding of ZtfA to *brlA*, *flbC* and *flbD* and to further ZtfA targets. This is also interesting regarding ZtfA's regulation of the fungal OSR.

The ZtfA protein is phosphorylated at at least three amino acid residues and it will be interesting to learn more about its regulatory mechanisms. Further analyses of the *ztfA*$^{S327A,T464A,S504-506A}$ strain and investigations of possible further phosphorylation sites are attractive. Construction and analysis of a permanently phosphorylated ZtfA-expressing strain is of interest as well. Further studies of ZtfA in *A. fumigatus* are promising to increase knowledge about the mechanisms underlying functional conversions of regulators between genetic models and pathogenic fungi. Construction and analyses of mutants of *ztfA* and velvet or other developmental genes are promising in this respect.

ZtfA is an activator of several SM cluster genes in *A. nidulans* and represents a promising candidate for the identification of new SMs through further analyses of the *ztfA* mutant strains and the RcoA-ZtfA complex. A better understanding of SM regulation is important, as a vast amount of bioactive natural products is still unknown, which might have deleterious as well as beneficial potential to humankind (Gerke *et al.*, 2012b; Gerke and Braus, 2014; Keller *et al.*, 2005; Soukup *et al.*, 2016). The group of unknown SMs, which are not produced under laboratory conditions, is presumably significantly larger than the proportion of already known SMs (Hoffmeister and Keller, 2007; Khaldi *et al.*, 2010). Broadened knowledge of appropriate strategies against fungal food contaminants and health treats is another crucial outcome of future studies. Analyses of ZtfA's regulatory influences upon SM production in *A. fumigatus* are appealing in this respect as well.

Taken together, ZtfA is an important activator of conidiation and SM gene expression in *A. nidulans*. Parts of its functions are conserved in other Aspergilli, but functional conversion was observed as well. ZtfA represents a promising candidate to identify new SMs. Future studies will broaden our knowledge about interconnection of SM production and asexual development, as well as functional conversion of important regulators between *A. nidulans* and *A. fumigatus*.

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List of abbreviations

%	Percent
°C	Degree Celsius
Δ	Deletion
μg	Microgram
μl	Microliter
μM	Micromolar
aa	Amino acid(s)
ADP	Adenosine diphosphate
AspGD	*Aspergillus* genome database
ATP	Adenosine triphosphate
BiFC	Bimolecular fluorescence
BLAST	Basic local alignment search tool
bp	Base pairs
bZIP	Leucine zipper domain
C2H2	Cys_2His_2 zinc finger DNA binding domain
C4	Cys_4 zinc finger DNA binding domain
C6	$Zn(II)_2Cys_6$ zinc cluster fungal type DNA binding domain
CADRE	Central *Aspergillus* data repository
cDNA	Complementary DNA
CDP	Central developmental pathway
cm	Centimeter
cMyb	C-terminal myeloblastosis transcription factor domain
COP	Coat protein
COP9	Constitutive photomorphogenesis 9
CRL	Cullin-RING ligase
CSN	COP9 signalosome
C-terminus	Carboxy terminus
d	Day(s)
DBD	DNA binding domain
DIC	Differential intereference contrast
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia* = for example
ER	Endoplasmatic reticulum
FACT	Facilitates chromatin transcription
g	Gram
GFP	Green fluorescent protein
Glu	Glutamic acid
GTP	Guanosin triphosphate
h	Hour(s)
H2A	Histone
H_2O_2	Hydrogen peroxide

HPLC	High performance liquid chromatography
K	Lysine
kb	Kilo bases
kDa	Kilo Dalton
l	Liter
LB	Lysogeny broth
LC	Liquid chromatography
LM	London medium
M	Molar (mol/l)
mAbs	Milli absorbance units
MAP	Mitogen-activated (kinase)
mg	Milligram
min	Minute(s)
ml	Milliliter
MM	Minimal medium
mM	Millimolar
mm	Millimeter
mRNA	Messenger RNA
MS/MS	Tandem mass spectrometry
mU	Milli-units
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
natR	Nourseothricin resistance marker
natRM	Nourseothricin recyclable resistance marker
NES	Nuclear export signal
NLS	Nuclear localization sequence
N-source	Nitrogen source
N-terminal	Amino-terminal
o/n	Over night
OE	Overexpression
ORF	Open reading frame
OSR	Oxidative stress response
P	Promoter
p.c.	Personal communication
PCR	Polymerase chain reaction
pg	Pico gram
phleoRM	Phleomycin recyclable resistance marker
PKS	Polyketide synthase
ptrARM	Pyrithiamin recyclable resistance marker
qRT-PCR	Quantitative real-time PCR
R	Resistance
R	Arginine
RGS	Regulator of G-protein signaling
RING	Really interesting new gene
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species

rpm	Rounds per minute
rt	Room temperature
S	Serine
SDS	Sodium dodecyl sulfate
SM(s)	Secondary metabolite(s)
spp.	Species
t	Terminator
TCA	Tricarboxylic acid
Thr / T	Threonine
tRNA	Transfer RNA
Tyr / Y	Tyrosine
UDA	Upstream developmental activator
UDP	Uridine diphosphate
v/v	Volume per volume
w/v	Weight per volume
WT	Wildtype
YFP	Yellow fluorescent protein
Zn	Zinc

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