Adaptation strategies of *Aspergillus* species to environmental challenges

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Summary

Aspergilli are ubiquitous fungi which can be found in various natural environments. Evolution shaped their adaptability to diverse stress conditions, such as low temperature or nutrition limitations that they also encounter during host infection. We applied different assays and multi-omics approaches to gain deeper understanding about these strategies.

The determination of intracellular ATP levels over the time of the freezing and defrosting processes revealed a high resistance of *Aspergillus nidulans* against cold- and cryostress. This suggests that *A. nidulans* is able to induce protective mechanisms to withstand low temperature and cryostress and to determine its lowest growth temperature at 10°C. Large-scale proteomics, transcriptomics and metabolomics allowed us to gain insights into the low temperature response of this fungus at 10°C. These analyses revealed an induction of cold protection mechanisms and a change in cell development and natural product biosynthesis. The induced cold protection mechanisms were known from other organisms and ranged from oxidative to osmotic stress responses and the induction of chaperones and glycine-rich RNA binding proteins. Further on, sexual development was triggered. This finding indicated the existence of a light-independent mechanism of activation of cell developmental signalling and regulation by cold. The low temperature response also led to a distinct biosynthesis of secondary metabolites (SMs), including the production of so-far uncharacterised compounds with inhibitory effects against fungi and Gram-positive bacteria. The insights of this study may help to improve the cryo-conservation of filamentous fungi. Our data set is also highly relevant for the optimisation of fermentation processes under low temperature conditions. Further on, SMs produced at low temperature stress may represent a valuable source for therapeutic compounds. They may also represent important mediators of microbial communication.

Another stress is caused by human cells. As a first step to characterise the adaptation, the regulation of extracellular protease secretion in the human-pathogenic fungus *Aspergillus fumigatus* was investigated by an LC-MS/MS based proteomic approach. The gene regulatory networks of the transcription factors (TFs) XprG and PrtT required for protease secretion were studied on the protein level. Deletion of these TFs resulted in a nearly absent degradation of substrate proteins. However, the double deletion strain Δ*XprG/*Δ*PrtT* did not show an attenuated virulence in a murine infection model of invasive aspergillosis. We concluded a genetic redundancy in proteolytic function and a putative induction of alternative virulence factors in the double mutant Δ*XprG/*Δ*PrtT*. Furthermore, XprG and PrtT influenced additional processes, such as cell wall modifications and allergen

production. Thus, the mutant strain Δ*XprG/*Δ*PrtT* may be useful for investigating the allergic response to fungal protein antigens.

In summary, Aspergilli used in this study showed strong adaptability to the applied stress conditions and a valuable multi-omics data set for future studies was generated.

Zusammenfassung

Aspergilli sind ubiquitäre Pilze, die an den verschiedensten Standorten in der Umwelt gefunden werden können. Sie haben sich im Laufe der Evolution an diverse Stressbedingungen wie niedrige Temperaturen oder eine durch den Wirt verursachte Limitierung der Nahrungsquelle angepasst. Um tiefere Einblicke in die Anpassungsstrategien zu erlangen, haben wir einen Multi-Omics Ansatz und weitere unterschiedliche Untersuchungsmethoden angewandt.

Durch Messungen der intrazellulären ATP Werte während des Einfrier- und Auftauvorgangs stellte sich heraus, dass *Aspergillus nidulans* eine hohe Resistenz gegenüber Kälte und Kryostress aufweist und 10°C die niedrigste Temperatur im Wachstum von *A. nidulans* darstellt. Der Pilz ist somit in der Lage, Schutzmechanismen gegen niedrige Temperaturen und Kryostress einzuleiten. Umfangreiche Proteomik-, Transkriptomik- und Metabolomik-Analysen erlaubten Einblicke in die Kältestress-Antwort des Pilzes bei 10°C. Dadurch konnte eine Induktion von Kälteschutzmechanismen, eine Veränderung der Zellentwicklung sowie eine Biosynthese von Naturstoffen nachgewiesen werden. Bei den induzierten Kälteschutzmechanismen handelt es sich im Allgemeinen um von anderen Organismen bekannte Mechanismen, die von oxidativen und osmotischen Stressreaktionen bis hin zu der Induktion von Chaperonen und Glycin-reichen RNA-Bindungsproteinen reichen. Weiter wurde die sexuelle Entwicklung nach Kältestress induziert, und es gab Hinweise auf einen lichtunabhängigen, kälteregulierten Mechanismus zur Aktivierung der Signalübertragung und Regulierung der Zellentwicklung. Außerdem führte die Kältestressantwort zur Biosynthese von diversen Sekundärmetaboliten (SMen). Dabei wurden bisher noch nicht charakterisierte, chemische Verbindungen produziert, die eine inhibitorische Wirkung gegen Pilze und Gram-positive Bakterien aufwiesen. Die Erkenntnisse dieser Studie können zu einer optimierten Kryokonservierung filamentöser Pilze beitragen. Weiterhin sind unsere Daten für die Optimierung von Fermentationsprozessen unter niedrigen Temperaturbedingungen von großer Bedeutung. Darüber hinaus können die kältestress-induzierten SMe eine wertvolle Quelle für therapeutische Verbindungen darstellen. Zudem ist es möglich, dass die SMe wichtige Signale der mikrobiellen Kommunikation übermitteln.

Eine weitere Stress-Art wird durch Immun-Zellen hervorgerufen. Als erster Schritt wurde zur Charakterisierung der Stress-Adaption mittels eines LC-MS/MS basierten proteomischen Ansatzes die Regulation der extrazellulären Protease-Sekretion im humanpathogenen Pilz *Aspergillus fumigatus* untersucht. Dazu wurde das genregulatorische Netzwerk der Transkriptionsfaktoren (TFen) XprG und PrtT auf Proteinebene erforscht. Die Deletion dieser TFen führte zu einem nahezu komplett zum Erliegen kommenden Abbau von Proteinsubstrat. Der Deletions-Stamm *ΔXprG/ΔPrtT* zeigte allerdings keine abgeschwächte Virulenz in einem murinen Infektionsmodell der invasiven Aspergillose. Entsprechend haben wir auf eine genetische Redundanz proteolytischer Funktion geschlussfolgert, bei der außerdem eine mutmaßliche Induktion alternativer Virulenzfaktoren in der Doppelmutante *ΔXprG/ΔPrtT* eine Rolle spielen könnte. Darüber hinaus konnten wir nachweisen, dass XprG und PrtT weitere Prozesse wie die Zellwandmodifikationen und die Produktion von Allergenen beeinflussen. Somit kann der Deletions-Stamm *ΔXprG*/*ΔPrtT* auch in der Erforschung der allergischen Reaktion auf Pilzproteinantigene verwendet werden.

Zusammenfassend weisen die in dieser Studie verwendeten *Aspergilli* eine starke Anpassungsfähigkeit an die angewandten Stressbedingungen auf, und es wurde ein Multi-Omics-Datensatz für zukünftige Studien erzeugt.

1 Introduction

This work focuses on the ability of *Aspergillus* species to adjust to various growth challenges and withstand harsh conditions in the natural environments or in the human body. For investigating the behaviour of the fungus in the human body or in particular studying the ability to degrade structural barriers of the host, the human pathogenic fungus *Aspergillus fumigatus* was chosen. The regulation of extracellular proteases that may participate in host tissue invasion and destruction were investigated in more detail. As a harsh environmental condition, low temperature stress was chosen, and the adaptation ability of *Aspergillus nidulans* was examined in more detail. In the following sections, the characteristics of *A. fumigatus* in terms of pathogenicity as well as *A. nidulans* in terms of secondary metabolism, cell development, and cold protection will be introduced.

1.1 The genus *Aspergillus*

Fungi represent one of the largest eukaryotic kingdoms (1), in which the phylum Ascomycota forms the biggest faction with over 64,000 described species belonging to approximately 6,400 genera (2). The filamentous fungi of the genus *Aspergilli* cover 350 different species (3). *Aspergilli* are moulds that are widespread in nature. As ubiquitous microbes with a large pool of enzymes, *Aspergilli* can degrade both plant and animal material (4,5), which is characteristic of a saprophytic life style (6). The different *Aspergillus* species can be distinguished by the morphology of their conidiophores (specific asexual fruiting structure) (6,7). With regard to their impact on humans, *Aspergilli* play an ambivalent role; some are beneficial while others can be harmful.

Aspergilli can exhibit toxic activity and cause life-threatening disease, *e.g.* in susceptible individuals. Among those, the most well-known species is *Aspergillus fumigatus* (8). *A. fumigatus* can invade the lungs and cause invasive aspergillosis (IA) in immunocompromised patients (9). It is found in about 90% of all clinical cases of IA (10) most probably due to its high adaptability (9,11). Although we inhale about 100 *A. fumigatus* conidia per day (12), healthy individuals are hardly affected due to efficient clearance by the immune system (13).

On the other side, *Aspergilli* are beneficial for mankind. In biotechnology, *Aspergillus* species are of industrial importance for food fermentations and the production of enzyme or organic acids (14–17). For example, *Aspergillus oryzae* is used for soybean fermentation (18), while *Aspergillus niger* produces citric acid in large quantities for industrial use (19). On the other hand, *A. niger* can also cause diseases in humans (20), which further demonstrates the ambivalent role of *Aspergilli*. Moreover, *Aspergillus* species are also interesting for the

pharmaceutical industry due to their ability to produce biologically active natural products (NPs) (21). Examples for compounds of medical significance are antimicrobial agents such as echinocandins or cholesterol-lowering drugs such as statins (22). To illustrate the relationships between the aforementioned *Aspergilli*, a phylogenetic tree of a regulatory gene in the aflatoxin biosynthesis gene cluster in a conserved region is shown (Figure 1).

Figure 1: Phylogenetic tree without distance corrections for *A. niger*, *A. oryzae*, *A. nidulans,* and *A. fumigatus* for a regulatory gene (An01g15010 in *A. niger*, *aflJ* in *A. oryzae*, *mdpA* in *A. nidulans,* and *tpcD* in *A. fumigatus*) of the aflatoxin biosynthesis gene cluster. For the investigated genetic region, *A. nidulans* is closer related to *A. oryzae*, which have both the same distance to *A. fumigatus* and *A. niger*.

1.1.1. Pathogenicity of *A. fumigatus*

A. fumigatus is an opportunistic airborne pathogen that can infect humans. With a size of approximately 2-3 µm in diameter, the spores are small enough to reach the lung alveoli of humans. Depending on the state of the immune system, spores are either killed or germinate and establish an infection in immunocompromised hosts. IA infections are accompanied by tissue damage. IA is associated with mortality rates between 30 to 90% (9,23).

The diagnosis of IA is difficult and therapeutic options are rather limited due to the similarity of the biology of eukaryotic host cells and the fungal pathogen. Infections are usually microbiologically diagnosed by the detection of cell wall components like galactomannan or β-1,3-glucan in serum (24), with the risk of false positives and negatives (25). Alternatively, *Aspergilli* can be diagnosed *via* a PCR reaction which has similar sensitivity and specificity compared to the aforementioned biomarker assays (26). *A. fumigatus* infections can be treated with three different groups of antifungal agents: azoles, polyenes and echinocandins (27,28). Azoles such as voriconazole and polyenes such as the polyene-macrolide amphotericin B interact with the fungal cell membrane component ergosterol and interfere with cell membrane integrity. Echinocandins have a completely different mechanism of action, they inhibit the synthesis of the cell wall polymer ß-1,3-glucan in a non-competitive manner, and therefore perturb cell wall functions (29). The increasing prevalence of antifungal drug resistance in *A. fumigatus* strains (30,31), *e.g.* against voriconazole or other

triazole-derivates, has become an emerging problem. Hence, searching for other agents active against fungi is crucial.

In the immunocompetent host, the immune system has an armoury of weapons to clear an *A. fumigatus* infection. After conidia are inhaled, *A. fumigatus* is confronted by the pulmonary innate and adaptive immune system. The innate immune system in the human lungs consists of physiological barriers like epithelium and endothelium, and cellular components like macrophages, neutrophils, natural killer cells, monocytes, and dendritic cells.

Upon reaching the bronchial system, the recognition of *A. fumigatus* takes place by pathogen-associated molecular patterns (PAMPs), which are detected by pattern-recognition receptors (PRRs) located on the surface of innate immune effector cells. Toll-like receptors TLR-2 and TLR-4, and the c-type-lectin-receptor dectin-1 support recognition of the ß-1-3-glucan polymer in the fungal cell wall (32,33). Once, conidia are internalised by epithelial, endothelial, or professional phagocytic cells (macrophages, neutrophils) they are killed by acidification (34,35) inside phagolysosomes (36). However, this process can be disturbed by the fungal conidial pigment melanin. Alveolar macrophages represent the largest number of immune cells that reside in the lung (37). They trigger the release of a multitude of proinflammatory cytokines and chemokines. This leads to the recruitment and activation of neutrophil granulocytes (38), which represent an essential line of defence of the immune system against *A. fumigatus*. They can degranulate and form so called neutrophil extracellular traps (NETs) (39), facilitating further recruitment of immune cells (36) and prevent spreading of the pathogen (40). It is also known that neutrophils can guide and differentiate dendritic cells (41), which can phagocytose and eliminate conidia and hyphae. After internalising conidia, dendritic cells secrete interleukin 12, inducing the protective T helper (Th-)1-cell immune response (42,43). In contrast, the phagocytosis of hyphae triggers the secretion of anti-inflammatory IL-10 and IL-4. The latter one activates a Th2-cell response (44).

Another important part of the innate immune system is the complement system, which consists of a number of proteins and acts as a cascade to initiate an immune defence reaction (45). Some complement proteins have a direct antimicrobial effect, while others lead to the formation of the membrane attack complex (MAC) that causes cell lysis. Their main function is to opsonize pathogens and attract immune cells (46). Since this process cannot discriminate between exogenic and endogenic, healthy or damaged cells, the activity of this system needs to be tightly regulated, *e.g.* with membrane integrated factors and/or with regulators in the serum. Therefore, a common evasion strategy of pathogens is to bind immune regulators that are present in the serum. *In vitro* studies showed that conidia from *A. fumigatus* are able to bind regulatory factors like factor H, FHL-1, plasminogen, and the

C4-binding protein C4bp, which is a regulator of the lectin pathway in order to avoid immune recognition (47). Hyphae can also evade the complement system by secreting the serine protease Alp1, which inactivates unspecific complement proteins by proteolytic degradation (48).

During the infection process, *A. fumigatus* secretes secondary metabolites like gliotoxin, helvolic acid and fumagillin (23) and proteins like mitogillin, which inhibit cell activity, damages the respiratory epithelial tissue (49) or may have a cytotoxic or hemolytic activity against macrophages, epithelial cells and erythrocytes (50,51). These secretions represent one of the strategies that allow *A. fumigatus* to successfully invade and thrive in the human body (11,52).

Beside challenges like the host immune response, the fungus also needs to adapt to a higher temperature, lower levels of oxygen, and nutrient limitations in the human host (11,23). An available nutrient source is the host tissue, which needs to be enzymatically degraded to low molecular mass carbon sources before uptake. It was shown in other microorganisms that the ability to utilize specific horst carbon sources is a requirement for invasive growth. For example, in *Candida albicans* (53) and *Mycobacterium tuberculosis* (54,55) the lipid metabolism is essential during infection. However, a study from Schöbel *et al.* (56) showed that *A. fumigatus* does not strongly depend on the ability to use lipids as a carbon source during infection (57). *A. fumigatus* developed different strategies to compensate nutrient deficiency. Amino acid starvation for instance results in accumulation of uncharged tRNA molecules, which activate the sensor kinase of the cross-pathway control system. This leads to the secretion of corresponding enzymes for amino acids uptake, which are essential for this fungus (58).

1.1.2. Secretion of proteins by *A. fumigatus*

It is well known that *A. fumigatus* secretes enzymes, like proteases, during lung invasion (59). After degradation of the host tissue, various nutrient sources are available for the fungus. Depending on the carbon source present, the enzyme methylcitrate synthase (MscA) is crucial for the survival of *A. fumigatus* in the host. When growing on carbon compounds such as propionate, isoleucine, valine, or methionine, the fungus accumulates the toxic metabolite propionyl-CoA, which is usually metabolised further by McsA and subsequent enzymes of the methylcitrate cycle. When McsA is lacking, the growth of *A. fumigatus* is strongly reduced on propionyl-CoA-generating carbon sources, including growth on protein sources. A Δ*mcsA* mutant of *A. fumigatus* showed attenuation of virulence in different infection models. This suggests that proteins and amino acids serve as growth-supporting nutrients during infection (57).

To aid host tissue degradation, the protein secretion machinery is maintained by the unfolded protein response (UPR) and the endoplasmic-reticulum-associated protein degradation (ERAD) process. The involvement of secreted proteins in the infection process, in particular proteases and lipases, was shown for bacteria, protozoa, and pathogenic yeasts (60,61). In the case of the opportunistic human pathogen *Cryptococcus neoformans*, the secreted phospholipase B1 (Plb1) was shown to be an essential virulence factor for nutrition acquisition, tissue invasion and immune evasion (62,63). However, in case of proteases, these enzymes often substitute each other's function to a certain extent. Hence, the functional analysis of single proteases is often very difficult by genetic methods. In *Candida albicans*, proteases can partially complement each other's functions (64). It was shown that the aspartyl proteases SAP 1, 2, and 3 are responsible for the proteolysis of complement proteins (65) and thus represent virulence determinants. In *A. fumigatus*, protease-deficient strains did not show reduced virulence in mouse infection models (66–68). However, PrtT, a transcriptional regulator of several secreted proteases, has been shown to play a role in damaging epithelial cells and erythrocytes (59,68). Further nutritional profiling studies with regard to extracellular proteolysis and uptake of oligopeptides were carried out (69). Only a limited number of transporters, mainly members of the oligopeptide transporter family (OPT) and to a lesser extent amino acid permeases and peptide transporters, are responsible for the uptake of peptides in fungi (70,71). A strain lacking the entire set of *opt* family genes and the extracellular protease regulator *prtT* revealed growth defects on porcine lung tissue agar, but showed no attenuated virulence in a mouse infection model (69,72). This underlines the robustness and the high degree of redundancy encoded by the *A. fumigatus* genome to ensure nutrient supply and growth in a complex and hostile environment.

1.2 The filamentous fungus *A. nidulans*

A. nidulans is a ubiquitous filamentous fungus that belongs to the class of ascomycetes in the order of Eurotiales. It represents a well-established model organism for cell biology and gene regulation (73). This mould is fast growing and can be easily cultivated. Moreover, the genome of *A. nidulans* has been sequenced (74), which allows whole-genome, transcriptome, and proteome studies (75,76). *A. nidulans* has a sexual cycle and can be easily crossed. Numerous basic studies on signal transduction (77), gene regulation (78–80) and cell biology and development (81–83) illustrate the high relevance of *A. nidulans* for various scientific fields including medicine and biotechnology (17,21,22).

Like many other moulds, the fungus has the ability to produce secondary metabolites (SMs) (84,85), secrete enzymes (86) and organic acids (87). Although *A. niger* is the most commonly utilized *Aspergilli* species for biotechnological use, *A. nidulans* has some

importance in applied biology and biotechnology as a source of biologically active natural products (NPs) and enzymes. The biosynthesis of SMs often coincides with development (sexual/asexual) in *A. nidulans* (80,88). The association between the SM production and cell differentiation or development has been reported for a long time (89,90). An interesting question is which environmental and genetic factors link these two processes with each other.

1.2.1. Cell development of *A. nidulans*

The cell development of *A. nidulans* depends on many different environmental signals such as nutrition (*e.g.* nitrogen sources, which can repress or activate certain cell developmental pathways (80)), partial oxygen pressure, pH, light, and further external stress factors. *A. nidulans* is homothallic (91) and can cross with a sexual partner in a heterothallic manner. It can reproduce via a parasexual, asexual, and sexual cycle (92,93). The parasexual cycle is an uncoordinated process, in which heterokaryons are formed. Hyphae with two homokaryons fuse, subsequently one cell possesses genetically different nuclei and other cytoplasmic components (93). After combination, the nuclei continue to divide mitotically (94). In the asexual cycle (Figure 2), a single haploid spore produces many identical asexual spores: Beginning with the germination and formation of hyphae, cells undergo vegetative growth. At a certain time-point, hyphae pause growth and enter the phase of asexual development. A specialized stalk, the conidiophore, is formed. On the head of the conidiophore, two layers of the uninucleate reproductive cells, the metulae and phialides, are formed (95), which produce the so-called conidia or conidiospores (95). For the sexual cycle (Figure 2), a fruiting body evolves in which a pair of nuclei undergoes meiosis. In the first step, ascogenous hyphae are formed. The hyphal tips develop into an ascus, where the fusion of two haploid nuclei occurs. The meiosis is followed by a post-meiotic mitosis. Here, eight haploid ascospores are generated (96). Thousands of ascospores are located in the fruiting body with a size of 125–200 µm in diameter. From the so-called cleistothecium, the ascospores are released after bursting (97). During the initial stages of cleistothecium ontogeny, the cleistothecia are surrounded by a nondeciduous chlamydospore-like structure called Hülle cells (98,99). These cells surround the dikaryotic hyphae and form spherical structures with 5-7µm thick cell walls, which can expand to a full size of up to 5 µm in diameter (92,96,100).

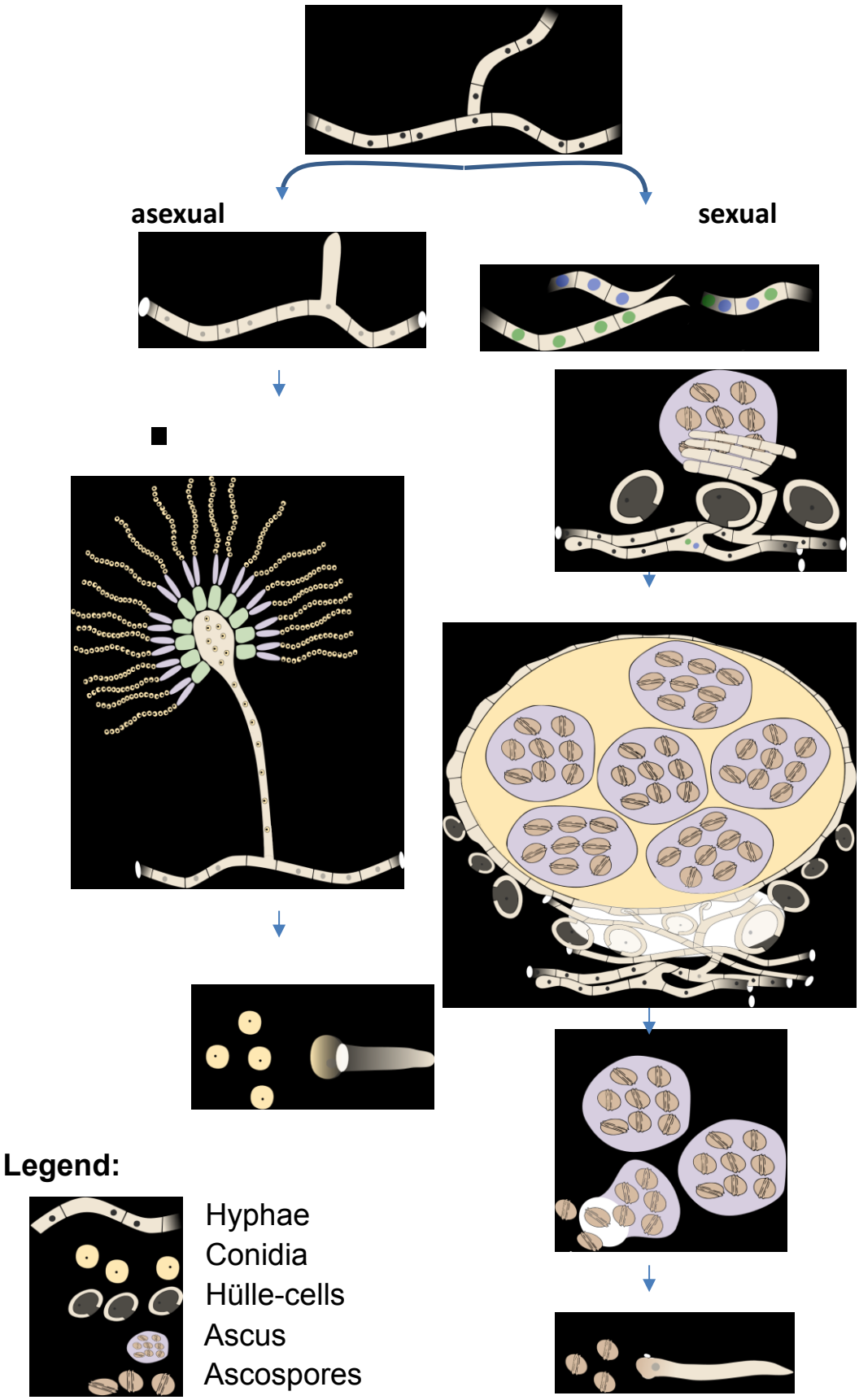

Figure 2: Developmental stages for asexual and sexual development of *A. nidulans*. (a) A vegetative hypha of *A. nidulans* is shown. The hyphae differentiate and can undergo asexual or sexual development. Asexual cycle (b-d): After formation of the footcell (b), the stalk

emerges and elongates. It takes about 6 hours until vesicle formation begins and metulae start to form at the stalk tip. Subsequently, phialides develop which give rise to long chains of conidia (c). Each conidium can germinate (d) and form vegetative hyphae. The sexual cycle is depicted in (e) – (i): In the beginning, ascogenous hyphae are formed (e). The fruiting body is produced (f) and surrounded by the Hülle cells. The thick-walled globose Hülle cells form a nest-like structure (g) (101) and the fruiting body formation is completed. The fruiting body is called a cleistothecium and is filled with thousands of ascospores. The asci release the ascospores (h), which can germinate again and form vegetative hyphae (i) (modified after Noble *et al.* (102)).

1.2.2. Asexual development

How asexual development in *A. nidulans* is initially triggered is not fully understood. Different ambient stimuli like variation in carbon levels, light intensity and wave lengths, or osmotic and oxidative stressors can influence the regulation of cell development in *A. nidulans* (103). It is known that the interplay between the trimeric G-protein signalling with two different heterotrimeric G-protein complexes and the cytoplasmic protein FluG together promotes vegetative growth and asexual development (104,105). FluG is suggested to be a central regulator for asexual sporulation (106) and is responsible for conidiophore formation and germination (107). Further downstream, FluG activates *brlA* (108), which induces the formation of conidiophore vesicles. The activation of *brlA* is sufficient for the induction of the asexual spore formation (108). This is accompanied by the activation of a cascade, including AbaA, WetA, AtuA, MedA and VosA (109). StuA and MedA are developmental modifiers, whereas StuA enables proper spatial expression and MedA proper temporal expression of *brlA* and *abaA* (95,109). The nuclear protein VosA controls the activation of asexual development (105), but is also important for sexual development (110).

1.2.3. Sexual development

It is believed that all *Aspergilli* can reproduce by sexual means (111), although some species such as *A. nidulans* (112), *A. flavus* (113) *A. parasiticus* (114) or *A. nomius* (115) are more prone to switch to the sexual development cycle than other species like *A. fumigatus* (116). The initiation of sexual development is triggered by environmental and intrinsic signals, which induce the mating type *(MAT*) genes (91). It is known that in *A. nidulans*, the loci *MAT1-1* and *MAT1-2* need to be induced to initiate sexual reproduction. These loci have several functions in filamentous ascomycetes. They determine cell type identity and are important for nuclear recognition, proliferation and pheromone signalling (117). Although no interaction between pheromones and their receptors is yet known in *A. nidulans*, psi factors (precocious sexual inducer) may interact with these receptors (118). A study on the identified pheromone

receptors GprA and GprB unveils their involvement in the homothallic sexual reproduction cycle (118). These receptors activate a G protein-coupled receptor protein (GPCR) which is essential for cleistothecia formation during the homothallic sexual cycle and fruiting body development (118–120). The G-protein α-subunit FadA activates *via* a serine/threonine kinase a mitogen-activated protein kinase (MAPK) pathway, which is involved in cleistothecia and heterokaryon formation (109,121).

1.2.4. Regulation of cell development

Early studies (122) revealed hundreds of genes which are putatively involved in cell development. Different regulators contribute to a complex network which modulates timing and balance of the cell development in *A. nidulans* (89).

One of these regulatory factors is the *psi* factor (precocious sexual inducer). Psi-factors are hormone-like molecules which are composed of hydroxylated analogues of linoleic (psi*α*) or oleic acid (psi*β*): PsiC is hydroxylated on position 5',8' (dihydroxy-acid), the psiB on position 8' (hydroxy-acid) and psiA is a lactonized ester of psiC (92,123). Champe *et al.* (124) described that psiAα alone stimulates asexual sporulation while psiBα and psiCα favour sexual and inhibit asexual development. Hence, the ratios of these psi-factors, and the activity of the *ppo* genes *ppoA*, *ppoB* and *ppoC* (125) that encode fatty acid oxygenases required for the production of *psi* factors, determine whether sexual or asexual sporulation is promoted (124,125). The transcript level of the *ppo* genes changes during sexual and asexual development and influences the timing of developmental events (125,126).

It is also known that *A. nidulans* prefers asexual development in light and sexual development during darkness (127,128). *A. nidulans* has several receptors for sensing light of different wave length. Red light is sensed by phytochrome FphA, blue light by the white collar complex LreA (WC-1) and LreB (WC-2) and UV light as well as blue light by cytochrome CryA (127). Together, these photoreceptors regulate development and induce or repress the asexual and sexual reproduction (129,130). FphA has an N-terminal photosensory module (GAF and PHY domain) and a C-terminal histidine kinase-related domain. It acts as an inducer of the asexual cycle and as a repressor of the sexual cycle. LreA contains a light-, oxygen-, and voltage-sensitive domain, protein-protein interaction domains and a GATA-type zinc-finger DNA binding domain. LreA and LreB are activators of sexual development. CryA is a combined cryptochrome/photolyase that is involved in repairing UV induced DNA damage. CryA is a repressor of sexual development and takes part in the regulation of fruiting body formation under both UV and blue light conditions (129,130).

Furthermore, some genes are known to be involved in modulating the sexual and asexual cycle, such as *LaeA* and *VeA* (131). *LaeA* is a global regulator which impacts the Hülle cell and cleistothecia formation (131) and the conidia production (132). *VeA* is a well-studied light dependent gene; its expression influences sexual development positively and down-regulates asexual reproduction (133). When the fungus is exposed to white or blue light, the protein VeA is localised in the cytoplasm, while in the dark or during red light it is predominantly found in the nucleus (81,131). Furthermore, the VeA protein does not only influence cell development, but also plays a role in regulation of secondary metabolism (134).

Hence, the production of SMs is strongly connected with sexual development in *A. nidulans* (83,129). For instance, deletion of *velA* led to inhibited sterigmatocystin and penicillin production in *A. nidulans* (132). The relevance of VelA for secondary metabolism has been shown for several filamentous fungi over the years (135–138).

1.3 Natural Products

Natural products (NPs) are chemical substances produced by a living organism (139). NPs are known to be extracted from all kinds of organisms, such as animals, plants, fungi, and bacteria (140,141). They are produced by pathways of the primary or secondary metabolism. The term secondary metabolites (SMs) for NPs indicates that these compounds are not essential for growth and survival of an organism. The biological role of the majority of SMs is not understood, but it is likely that these compounds often contribute to an advantage in the organism-specific ecological niche (142,143). Fungal SMs can function as anti-predator components to protect against natural enemies (144). For example, the fungivorous springtail *Folsomia candida* developed a preference for feeding on an SM-deficient *A. nidulans* strain over an SM-producing wild type (145). Another example is the production of siderophores like fusarinine C, triacetylfusarinine C and ferricrocin in *A. nidulans*. The siderophores allow the fungus to survive under iron-limiting conditions and provide advantages over competing microorganisms (146). Since siderophores are essential for the organisms during iron depletion, it can be debated whether siderophores should be regarded as typical NPs. Since the fungal siderophores in *Aspergilli* are produced by typical SM clusters, several publications assign siderophores as SMs (147,148).

NPs can also play a role as signalling molecules. As discussed above, oxylipins regulate asexual and sexual spore production in *A. nidulans* (92,126). Apart from the importance for the microorganism itself, NPs can be either beneficial or harmful to humans. Fungal species are a valuable source of NPs. These active compounds (ACs) are widely used as therapeutics, such as antibiotics, immunosuppressants, antiparasitics, cholesterol-lowering,

and antitumoral agents (149). In particular, penicillin (150,151), griseofulvin (152) and lovastatin (153) are known as ACs advantageous to humans. However, fungal-derived NPs can also threat human health. For instance, the mycotoxins gliotoxin (154), aflatoxin B1 (155), and ochratoxin A (156) exhibit strong carcinogenic and toxic activity.

1.3.1. Biosynthesis of secondary metabolites

In *Aspergilli*, the genes which code for enzymes involved in the production of SMs are organised in clusters (21,154). These gene clusters vary in size between 30 – 80 kb (157) and contain one or multiple central biosynthesis genes (158,159). The exceptions include oxylipins (125) and kojic acid (160). Although these metabolites are lacking a central biosynthesis gene and are derived from fatty acids (161) or glucose (160), they are still regarded as SMs (162) due to their similar biosyntheses and properties to other NPs (161). The main groups for assembling the SM backbone are multidomain enzymes like polyketide synthases (PKSs) (163) or non-ribosomal peptide synthetases (NRPSs) (164). PKS/NRPS hybrid, PKS- and NRPS-like enzymes or prenyltransferases (dimethylallyl tryptophan synthase (DMATS)) and terpene cyclases (DTS) are also participating in producing SM backbones.

In general, PKSs contain a set of modules and domains which carry out three different reaction steps. The typical domains are the ketosynthase (KS), the acyltransferase (AT), and the acyl carrier protein (ACP). These domains are responsible for the starting, elongation, and termination stage. Dependent on the nature of the PKS (modular or iterative), these steps can be repeated multiple times. The iterative PKS allows use of modules in an iterative way, whereas the modular PKS needs a new module for each molecule that is connected or modified. A prominent example of a compound derived from an iterative PKS is lovastatin from *Aspergillus terreus* (165), and for a modular PKS erythromycin A from the bacterium *Saccharopolyspora erythraea* (166). A further classification refers to the fatty acid synthase nomenclature. Type I PKSs consist of large multifunctional enzymes, which are linearly arranged and have covalently fused catalytic domains, while type II PKSs have a dissociable complex and usually consist of monofunctional enzymes. PKSs of type III are described as chalcone synthases, which are multifunctional in selecting the starter unit (163).

NRPSs also use multidomain enzymes with different modules and domains to synthesize the SM backbones. Apart from proteinogenic amino acids, also non-proteinogenic amino acids, fatty acids and α-hydroxy acids can be incorporated (167). Similar to the PKSs, NRPSs are also composed of three main domains for the starting module, elongation, and termination. Each NRPS gene cluster consists of multiple elongation modules. Often this fully elongated molecule forms cyclic amides (lactams) or cyclic esters (lactones) (168).

Structural prediction of PKSs is very challenging due to the possibility of tailoring and iterative or modular processing of the PKSs. For NRPSs, the substrate specificity of the A-domain can be predicted with computational approaches such as NPRSpredictor2 (169). Bioinformatic tools, based on algorithms such as SMURF (170), CASSIS (171) and antiSMASH (172), predict that *A. nidulans* produces a wide diversity of SMs with up to 70 biosynthetic gene clusters (157,173) that are often silenced under standard growth conditions (174–176) and controlled by a complex regulatory network (142).

1.3.2. Regulation of secondary metabolite gene clusters

Fungi naturally produce a diverse array of SMs which are often regulated by cluster-specific transcription factors (177,178). For *A. nidulans*, a few products (about 20) of the 70 predicted putative SMs have been chemically identified so far. The fact that the majority of gene clusters remain uncharacterised illustrates the importance of finding methods to induce silent SM gene clusters.

Different strategies have been developed to study gene clusters and their regulation. The induction of certain stress factors, coincubation with different organisms (179), or alteration of growth conditions (180) represent strategies to identify new SMs and unveil the regulation of their production. Coincubations are often established with organisms that naturally share a common habitat, like the interaction between *A. nidulans* and the soil bacterium *Streptomyces rapamycinicus*, which led to the activation of the orsellinic acid gene cluster (*ors*) and the production of the polyketide orsellinic acid and its derivatives (181). It was shown that secreted bacterial NPs, like rapamycin (182) or trichostatin A (183,184), alone were not sufficient to induce the *ors* gene cluster. A physical contact between the two organisms is indispensable. This suggests that a bacterial protein or signal molecule needs to penetrate the fungus in order to induce the *ors* gene cluster (185).

Apart from triggering the production of SMs by different stresses and growth conditions, molecular biological methods can be used as well. Genome mining approaches use target gene inactivation and can unveil new NPs (186). Often transcription factors of corresponding SM gene clusters (187,188) or global regulators (178,189) are overexpressed to induce the production of SMs. This strategy led to a successful identification of formerly unknown metabolites like aspyridone A and B (178). Transcriptional regulators can be triggered constitutively or with an inducible promoter. Examples for common inducible promoters in *A. nidulans* are derived from the gene alcohol dehydrogenase (*alcA)* or glyceraldehyde-3-phosphate dehydrogenase (*gpdA)* (178,190). Due to the need of precursors for SM production, growth rate is often impeded when a constitutive promoter is used. It was also found that SM production can be increased with manipulation of chromatin modifying

enzymes or chemicals (191). Another possibility of genetical manipulation is the transfer of the whole gene cluster to another production organism. Heterologous gene expression is useful to elucidate the SM biosynthetic pathway when the homologous host is difficult to genetically manipulate, has a low production yield, or a low generation time (192,193).

Regulation of SM cluster expression is often closely connected to the developmental stage or the primary metabolism of a fungus (194,195). The methyltransferase-domain protein LaeA (196), which triggers the production of several secondary metabolites also contributes to developmental processes (131,197). It has been shown in several studies (83,129,132,133) that the central regulator VeA, a light-dependent regulator of developmental processes, regulates sterigmatocystin and penicillin production in *A. nidulans* (129,198). Lind *et al.* described an increased expression level and therefore a temperature-dependent regulation of *VeA*-controlled SM gene clusters of several SMs at lower temperatures (30°C versus 37°C) (199). How environmental factors influence the fungal SM production is not well studied. To elucidate the variety of SMs produced by *A. nidulans*, environmental variations like adaptation to low temperatures can contribute to a better understanding of fungal SM production and to the ecological meaning of *A. nidulans*.

1.4 Adaptation to low temperature stress

Since fungi are exposed to constant environmental changes, responding to these variations is a critical challenge and part of their lifestyle. During the seasonal cycle, fungi and many other microorganisms need to adapt to drastic changes in their environment, such as temperature shifts (200,201). An adaptation is challenging when the temperature drops below the temperature limit for growth of an organism or below the freezing point of water. Under these conditions, organisms have to deal with drastically reduced metabolic processes, because enzymes need to operate below their temperature optimum (202). Moreover, cold stresses are associated with the alteration of the pH of biological buffers (200) and decreased membrane fluidity (203,204). The main causes of cell damages during low temperature stress below 0°C results from the formation of intracellular ice crystals and dehydration (205). Often the cell wall, membrane, and cellular organelles are affected. Hence, the organisms need to develop effective strategies to adapt to these stresses (Figure 3).

To prevent or control ice crystal formation (206), many organisms that have to endure temperatures near the freezing point, can produce anti-freezing-proteins (AFPs) (207,208). Examples can be found in many different species like various fish (209), insects (210) and plant species (211). Similar to the AFPs, cryoprotectants are largely used to improve survival of organisms during low temperatures. They can be produced by organisms, or artificially

added, for example during cryo-conservation. Cryo-conservation is a process in which biological material such as cells or tissues are preserved at very low temperatures. Cryoprotectants lead to the excretion of water so that ice crystals cannot be formed intracellularly. Common cryoprotectants are sugars such as trehalose or glycerol, which are just a few examples of compounds produced by cold-adapted organisms (212) as a strategy to increase stress resistance against freezing (213).

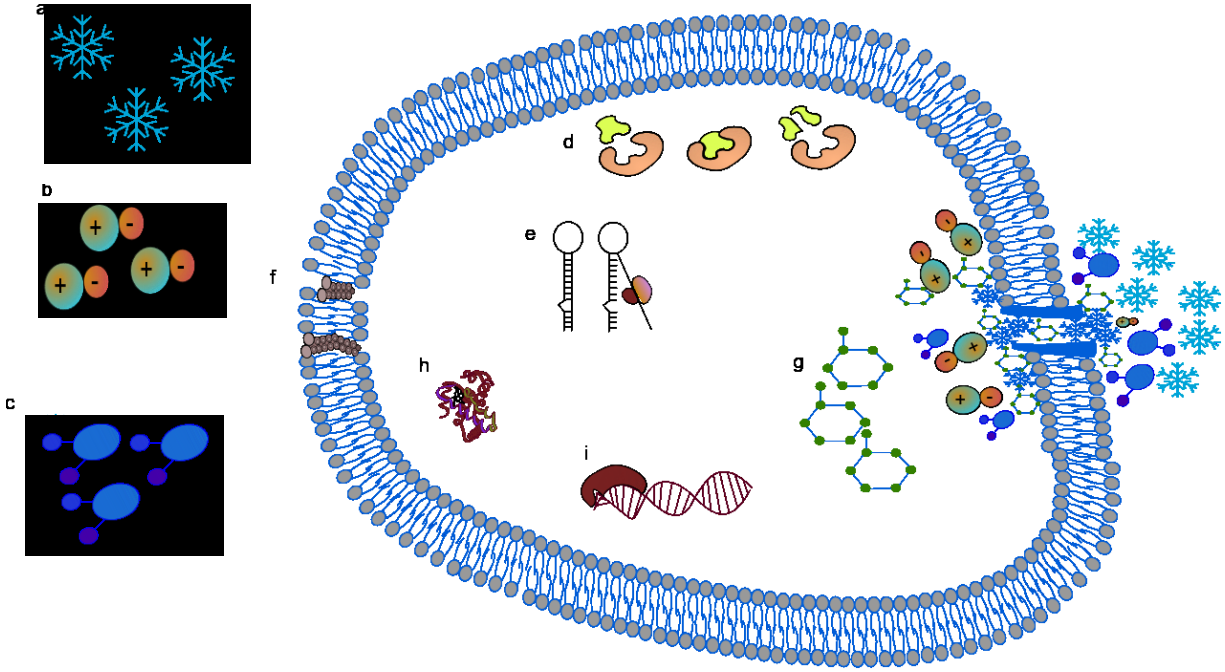

Figure 3: Schematic illustration of low temperature stress adaptation in a fungal cell. In (a), the ice crystal formation is depicted. Formation of ice crystals is accompanied by increased osmolarity and alteration of pH in biological buffer systems (b) due to an enrichment of solved salts in the residual water. Usually, the surrounding medium of the fungal cell cools down faster, hence the medium freezes first. Consequently, a water efflux from the cell can be measured, which leads to desiccation (c). The decreased temperature results in a lower rate of biological and chemical processes. In order to adapt to these effects, the fungus can develop different strategies. Enzymes can adapt to the lower temperatures with a decreased temperature optimum to ensure maintenance of metabolic processes (d). For sensing the temperature and facilitating transcription at a certain temperature the organism can use RNA thermometers, *e.g* as shown for bacteria (e). Furthermore, functioning membranes are essential for survival. In order to preserve membrane fluidity, the average chain length of fatty acids is reduced at lower temperatures (f). Membranes can also be stabilised with sugar molecules. Moreover, sugars can act as cryoprotectants and can induce a controlled freezing process (g). Therefore, the fungus can produce or incorporate sugar entities. Some organisms are able to produce AFPs, which have a similar function to cryoprotectants. Among others, they can increase the protein stability (h) and chaperones can assist in

maintaining protein functions by refolding misfolded proteins. Similarly, cold-adapted proteins facilitated transcription of DNA and translation of RNA at low temperatures. Glycine-rich RNA binding proteins (GRPs) destabilize the secondary structure of RNA and allow translation at low temperature stress (i) (modified after Robinson (214)).

1.4.1. Fungal strategies to adapt to low temperatures

Little is known about cryo-stress adaptation in filamentous fungi, but they are found in harsh environments like the Antarctic where average temperatures are around the freezing point (215). A common strategy of lower temperature adaptation is the accumulation of sugars and sugar alcohols (216,217) intracellularly (218,219) or extracellularly (205,220,221). The most prominent cryo-protective sugars or sugar alcohols are glycerol, arabitol, trehalose, and mannitol or sugar derivatives like polyols (222). These compounds stabilise membranes or maintain their integrity and function (223–226). Most of the molecular changes required for adaptation to low temperatures can be achieved under conditions above the freezing point, allowing an enhanced resistance to freezing (227).

Another strategy to gain a higher tolerance against freezing is to stabilize the cellular membranes. This can be achieved by the global reduction in the average chain length of fatty acids (204). The abundances of triacylglycerides with medium-chain fatty acids, triterpenes like squalene and sterol esters are increased, whereas that of phosphatidic acid and the ratio of phosphatidylcholine to phosphatidylethanolamine is decreased (228–230). These membrane changes lead to enhanced membrane fluidity at lower temperatures and prevents cell damages. Additionally, low-temperature adapted organisms like snow moulds (231) can produce anti-freezing proteins, which help to prevent or control the freezing process by modifying the growth of ice crystals (206).

However, the formation of ice crystals and consequent damages on the molecular or cellular level are not the only factors that have an impact survival of an organism. Dropping temperature can damage the organism in multiple ways. Ice crystal formation is accompanied by an increasing salt concentration in the surrounding medium, biological processes are slowed down, and molecules or cell structures can be damaged. The cell can react with a general protective stress response. Hence the survival under low temperature stress is affected by the capacity to detoxify reactive oxygen species (232,233) and the activity of heat shock proteins (234).

Further on, maintenance of metabolism at cold temperatures is of great importance. In order to enable translation at low temperatures, glycine-rich RNA binding proteins (GRPs) destabilize the secondary structure of RNA (235). GRPs may substitute for the function of cold shock domain proteins (or RNA chaperones) in fungi, facilitating to trigger numerous

biochemical, physiological, and metabolic changes needed for the acquisition of freezing-thawing tolerance (236). On the protein level, the activity of enzymes at low temperatures is important. Furthermore, the cold tolerance of fungi is increased by cold-adapted enzymes with high catalytic activities at low temperatures (237,238).

Finally, adaptation to the specific ecological niche is critical. Usually, the germination of fungal spores occurs in the warmer months and fungi can switch to an inactive, dormant state during the colder times of the year to avoid freezing stress (239,240). This flexible adaptation also enables fungi to undergo one or more growing seasons, alternating with the dormant state. In summary, evolution generated diverse stress responses in different organisms. What they all have in common is that fungi require the ability to sense temperature changes for each of those adaptations.

1.4.2. Temperature sensing

Sensing temperature is a critical parameter that needs to be monitored by organisms to efficiently adapt to changes in the environment (241). In order to sense temperature, a direct measurement is not necessarily needed. A highly conserved response to stress caused by an increased temperature is the unfolded-protein response (242,243). After a temperature-induced stress, many proteins misfold and the production of heat-shock proteins (Hsp) is initiated. Different Hsp chaperones can be produced in order to refold misfolded proteins (244). Hsp70 for example is a well-studied protein in the cytosol of *Saccharomyces cerevisiae* as a response to high temperatures (245). At low temperatures, a similar strategy exists (246,247). The so-called cold shock proteins are induced at low temperatures (248). Their main task is not the refolding of proteins, but RNA unwinding like the CsdA ribosomal associated protein (249) or facilitating the translation at low temperatures as the ribosomal binding factor RbfA (250).

Changes in membrane fluidity and membrane dynamics can act as a temperature sensor in cells. A rise in temperature results in an increase of fluidity due to a decrease of the molecular order of cellular membranes. In *Histoplasma capsulatum*, genes can be triggered by a change of saturated fatty acids as a result of a high temperature (251). In *S. cerevisiae, C. albicans,* and *C. neoformans*, transcriptional profiling revealed transcriptional activators (252–254), which may respond to temperature changes. Nevertheless, changes in membrane fluidity are slow processes. Hence, an existence of other primary temperature sensors is likely.

RNA thermometers (255) are able to react much faster to temperature changes and have the advantage of directly measuring temperature changes (256). RNA thermometers are *cis*-active regulatory elements which can switch between two different conformations (257), often

the stability of cold-shock mRNAs is increased at low temperatures, which may occur due to changes in the RNA structure (258). They are localised on the mRNA and regulate the access to mRNA binding sites, which can be blocked or released for translation (255,259). Up to date, nothing is known about RNA thermometers in fungi.

The adaptation of fungi to low temperature stress is only partially studied. Tsuji *et al.* (229) for example tested for the antifreeze activity of different fungi obtained from Antarctica and found secretion of extracellular polysaccharides or a higher amount of unsaturated fatty acids as an adaptation to cold. In their global study on *S. cerevisiae* to low temperature stress, Aquilera *et al.* (260) observed a cold-induced accumulation of trehalose, glycerol, and heat-shock proteins. In a chemostat-based transcriptome study on *S. cerevisiae*, the authors concluded that genes involved in lipid metabolism and ribosomal protein–encoding genes were overrepresented at low temperature and may be part of a compensation strategy for the underlying low-temperature–induced problems (261). In another global transcriptional analysis in *S. cerevisiae*, the authors compared the cold response of the yeast to other stress stimuli (262) and found that heat-shock proteins and the alteration of the membrane fluidity are crucial. Another proteome study on the macro-fungus *Flammulina velutipes* investigated the cold and light stress response mechanism underlying the fruiting processes.

In total, these and other studies addressed either very specific topics such as the phosphoproteome dynamics in *S. cerevisiae* (263) or only used very simple techniques (264,265) and therefore delivered restricted insights.

2 Aim of this study and motivation

Fungi are widespread in diverse natural environments and their exposure to a plethora of stress conditions shaped their evolution and adaptation to harsh conditions. This study investigated the adaptation strategies of both *A. nidulans* to low temperature stress and *A. fumigatus* to host-imposed nutrient limitations. Proteomics, transcriptomics, and metabolomics were applied to deepen our understanding of cold adaptation in a filamentous fungus. The goal of this study was not only to reveal cold protection mechanisms in *A. nidulans*, but also to follow other aspects of cold adaptation including cell development and natural product biosynthesis. A better understanding of the adaptation mechanisms to low temperature stress may also help to improve cryo-conservation and fermentation of filamentous fungi in cold conditions.

The second project aimed at exploring the role of the *A. fumigatus* transcription factors XprG and PrtT in controlling the secretion of extracellular proteins, in particular proteases, by means of LC-MS/MS based proteomics. Such a proteomic data set can contribute to our

understanding of the role of proteases secreted by *A. fumigatus* in establishing an infection in the immunocompromised host.

3 Manuscripts

3.1 ATP content and cell viability as indicators for cryostress across the diversity of life

Felizitas Bajerski, Johanna Stock, **Benjamin Hanf**, Tatyana Darienko, Elke Heine-Dobbernack, Maike Lorenz, Lisa Naujox, E. R. J. Keller, H. M. Schumacher, Thomas Friedl, Sonja Eberth, Hans-Peter Mock, Olaf Kniemeyer and Jörg Overmann

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Summary

In natural environments, organisms undergo temperature shifts within the seasonal cycle. During cold seasons or in standard preservation protocols, involving ultradeep freezing, organisms need to deal with temperatures below their optimum and are exposed to low temperature stress. Here, we use the intracellular ATP content as an indicator of the physiological state of cells in a comparable approach with different cell types such as bacteria, fungi, algae, plant tissue, as well as plant and human cell lines. In general, we observed a decrease of the intracellular ATP content along with the temperature drop in most of the organisms. Afterwards, the initial ATP level could be restored or exceeded subsequently to a regeneration phase. In parallel, cell viability was monitored and unveiled a positive correlation between ATP content and viability for cryosensitive algae *Chlamydomonas reinhardtii* SAG 11-32b and *Chlorella variabilis* NC64A, and in plant cell lines of *Solanum tuberosum*. Further on, it was noticeable that psychrophilic and cryotolerant bacteria or algae exhibited elevated ATP level during growth phases in comparison to mesophilic or cryosensitive representatives and achieved a better culturability after the process of cryo-conservation.

Contribution to the manuscript

Benjamin Hanf contributed to the manuscript by conducting the experiments for fungi and partially for algae and also wrote the section "*The Filamentous Fungus Aspergillus nidulans*"

and in part the introduction, results and discussion. He was involved in editing the manuscript.

Estimated contribution in percentage

Felizitas Bajerski	30 %
Johanna Stock and **Benjamin Hanf**	**30 %**
Tatyana Darienko, Elke Heine-Dobbernack, Maike Lorenz, Lisa Naujox, E. R. J. Keller, H. M. Schumacher, Thomas Friedl, Sonja Eberth and Hans-Peter Mock	25 %
Olaf Kniemeyer and Jörg Overmann	15 %

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ATP Content and Cell Viability as Indicators for Cryostress Across the Diversity of Life

Felizitas Bajerski[1], Johanna Stock[2], Benjamin Hanf[3,4], Tatyana Darienko[5], Elke Heine-Dobbernack[1], Maike Lorenz[5], Lisa Naujox[1], E. R. J. Keller[2], H. M. Schumacher[1], Thomas Friedl[5], Sonja Eberth[1], Hans-Peter Mock[2], Olaf Kniemeyer[3,4] and Jörg Overmann[1]*

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In many natural environments, organisms get exposed to low temperature and/or to strong temperature shifts. Also, standard preservation protocols for live cells or tissues involve ultradeep freezing in or above liquid nitrogen (−196°C or −150°C, respectively). To which extent these conditions cause cold- or cryostress has rarely been investigated systematically. Using ATP content as an indicator of the physiological state of cells, we found that representatives of bacteria, fungi, algae, plant tissue, as well as plant and human cell lines exhibited similar responses during freezing and thawing. Compared to optimum growth conditions, the cellular ATP content of most model organisms decreased significantly upon treatment with cryoprotectant and cooling to up to −196°C. After thawing and a longer period of regeneration, the initial ATP content was restored or even exceeded the initial ATP levels. To assess the implications of cellular ATP concentration for the physiology of cryostress, cell viability was determined in parallel using independent approaches. A significantly positive correlation of ATP content and viability was detected only in the cryosensitive algae *Chlamydomonas reinhardtii* SAG 11-32b and *Chlorella variabilis* NC64A, and in plant cell lines of *Solanum tuberosum*. When comparing mesophilic with psychrophilic bacteria of the same genera, and cryosensitive with cryotolerant algae, ATP levels of actively growing cells were generally higher in the psychrophilic and cryotolerant representatives. During exposure to ultralow temperatures, however, psychrophilic and cryotolerant species showed a decline in ATP content similar to their mesophilic or cryosensitive counterparts. Nevertheless, psychrophilic and cryotolerant species attained better culturability after freezing. Cellular ATP concentrations and viability measurements thus monitor different features of live cells during their exposure to ultralow temperatures and cryostress.

Keywords: cryopreservation, viability tests, ultradeep freezing, ATP level, adaptation, physiological, cold stress, cold temperature

INTRODUCTION

Over 80% of terrestrial and marine habitats are considered as cold, with temperatures residing permanently ≤15°C (Kirby et al., 2012). Cold stress of an organism occurs when outside temperatures fall below the optimum temperature range for growth and involves a slow-down of biochemical reactions, a decrease in pH of biological buffers, reduced membrane fluidity, cold denaturation of proteins, and a hydration of non-polar protein groups, affecting protein solubility and stability (Deming, 2002; Georlette et al., 2004). At temperatures below the freezing point, such as in arctic and antarctic environments, glacial and lake ice, high-altitude alpine sites, but temporarily also occurring in temperate regions (Georlette et al., 2004; Layborn-Parry et al., 2012; De Maayer et al., 2014), cells undergo cryostress, elicited by the formation of ice crystals. At slow cooling rates, ice formation often starts in the extracellular space whereas rapid cooling promotes intracellular crystallization (Mazur, 1969; Wisniewski and Fuller, 1999; Mazur, 2004). The resulting extracellular or intracellular decrease in water activity causes osmotic water efflux or influx, respectively, that effects the survival of the cells (Dumont et al., 2004). In addition, thawing or warming of cells may lead to recrystallization and the growth of larger ice crystals at the expense of smaller ones, which can cause significant cellular damage (Mazur, 1977).

Cellular adaptations to temperature decline involve the differentiation of permanent cell forms such as spores or akinetes, changing the phospholipid fatty acid inventory to maintain the functionality of cellular membranes (Bajerski et al., 2017), increasing the concentrations of enzymes (Willem et al., 1999), expressing cold-adapted isoenzymes (Hoyoux et al., 2001) or cold shock proteins stabilizing different cell constituents or changing gene expression (Phadtare et al., 1999; Weber and Marahiel, 2003; Wilson and Nierhaus, 2004), or preventing intracellular ice nucleation through the accumulation of sugars, or cryoprotectants like polyols (glycerol, arabitol, trehalose, and mannitol), secondary metabolites, or anti-freezing proteins (Duman and Olsen, 1993; Davies and Sykes, 1997; Sdebottom et al., 1997; Grant, 2004; Agrawal, 2009; De Maayer et al., 2014). Ice crystal formation can be inhibited and cellular proteins and membranes maintained in their native structure through the addition of glycerol or artificial cryoprotectants, particularly dimethyl sulfoxide (DMSO; Mazur, 1984). However, the physiological details of responses to rapid temperature decrease have mostly been studied at non-freezing temperatures [(cold stress; Graumann and Marahiel, 1996; Phadtare, 2004; Hannah et al., 2005; Cavicchioli, 2006), among many others] rather than under conditions of freezing (cryostress; Dumont et al., 2004; Miladi et al., 2013; Mykytczuk et al., 2013).

The different responses of living cells to cryostress are not only of fundamental scientific interest, but have direct practical significance for cryopreservation. Various bacteria and algae do not survive freezing under standard laboratory conditions (Smith, 2001; Day and Stacey, 2007). Standard preservation protocols for live cells or tissues involve ultradeep freezing in or above liquid nitrogen (−196°C or −150°C, respectively; Jacobsen and Stewart, 1973; Talwar, 2012). Therefore, an efficient cryopreservation is of increasing importance to safeguard biomaterials for follow-up scientific investigations (Schüngel et al., 2014), for subsequent medical applications, and for the maintenance of genetic resources in the agricultural sector, e.g., of crop plants which cannot be stored as seeds.

In order to monitor the physiological consequences of cryostress and to establish cryopreservation conditions, a suitable indicator for the physiological state of cells is needed. Across different cell types and tissues, the cellular content of ATP of growing cells is tightly regulated and maintained within a narrow concentration range (Holm-Hansen, 1970; Atkinson, 1977). Total cellular ATP content was suggested as a viability marker for cryopreserved cells, tissues and organs (Pegg, 1989) and cell cultures in general (Crouch et al., 1993; Niles et al., 2009). Cellular ATP concentration has been used in the past to monitor the physiological state of diverse prokaryotic and eukaryotic cells under starvation and (cold) stresses (e.g., Sobczyk et al., 1985; De Baulny et al., 1999; Napolitano and Shain, 2005; Amato and Christner, 2009; Tsai et al., 2010; Lin et al., 2011). Enzymes which are directly (F1-ATPase, V-ATPase) or indirectly related to adenylate anabolism might not work efficiently at subzero temperature. For example, a V-type ATPase is inhibited under cold stress in plants (Dietz et al., 2001). As a result the ATP level decreases (Napolitano and Shain, 2005). However, these enzymes differ across the diversity of life. Moreover, the kinases and phosphatases involved in the signal transduction during stress response affect intracellular adenylate levels (Corton et al., 1994; Huang et al., 2012). Therefore, ATP is a suitable biomarker to address cellular stress response. Indeed, cell damage induced by low temperatures has been linked to the shortage of cellular ATP (Amato and Christner, 2009).

In the present comparative study, a wide range of different cell types and tissues were subjected to cryostress to determine which parameters are suitable for monitoring the individual responses during freezing at ultralow temperatures as it occurs during cryopreservation. We show that cells and organisms across the diversity of life forms display changes of their cellular energy metabolism and concomitant changes in viability, but that both parameters in most cases are not tightly correlated.

MATERIALS AND METHODS

A series of cryostress experiments was conducted using bacteria, fungi, algae, plant tissues and cell lines, as well as human cell lines, as detailed in the following paragraphs. As higher multicellular organisms cannot be cryopreserved as a whole, spores, seeds, meristematic tissue or suspended cells (cell lines) are used. Accordingly, we chose a human cancer cell line (JURL-MK1) relevant for medical research, a cell culture of the major crop plant *Solanum tuberosum* cv. Desiree, and shoot tips of the species *Arabidopsis thaliana*, which is the bona fide model organism used in plant research. We focused on the effect of established and optimized cryopreservation conditions that are

currently in use for the different cells types (Supplementary Figure S1).

Bacterial Strains

Two different bacterial genera, the Gram-positive *Planococcus* (*Pla.*) and Gram-negative *Psychrobacter* (*Psy.*) were selected for the current study. Of the genus *Planococcus*, the mesophilic species *Pla. plakortidis* DSM 23997^T (Kaur et al., 2012), and the psychrophilic *Pla. donghaensis* DSM 22276^T (Choi et al., 2007) and *Pla. halocryophilus* DSM 24743^T (Mykytczuk et al., 2011) were analyzed. Similarly, the mesophilic *Psy. marincola* DSM 14160^T (Romanenko et al., 2002) was compared to the two psychrophilic species *Psy. aquaticus* DSM 15339^T (Shivaji et al., 2005) and *Psy. cryohalolentis* DSM 17306^T (Bakermans et al., 2006). Detailed growth experiments demonstrated that all psychrophilic species could grow at subzero temperatures in contrast to their mesophilic relatives (data not shown).

Planococcus strains were grown in Tryptic Soy Broth (Merck) supplemented with 0.3% yeast extract (w/v, TSY), *Psy. aquaticus* in Lysogeny Broth (LB; (Bertani, 1951) and the other two *Psychrobacter* strains in Marine Broth (MB, Merck). The mesophilic strains were routinely grown at 28°C and the psychophilic strains at 20°C. Cells were harvested at the end of the exponential growth phase. Cryostress experiments were conducted in three biological replicates in a final volume of 200 µl each using 500 µl 96-deep well plates, adding 10% dimethylsulfoxide (v/v, DMSO) as a cryoprotectant to the above described media. The 96 well plates were directly frozen in the gas phase of a liquid nitrogen tank and thawed after 24 h in a 30°C water bath. ATP content, OD_{600} and colony forming units (CFUs) were determined before freezing (BF), after adding the cryoprotectant (BF_treat), directly after thawing (AF) and after regrowth under optimum conditions at the end of the exponential growth phase (RG) (Supplementary Figure S1). Total cell numbers (TCN) were calculated from OD_{600} values based on calibration factors determined for each strain. CFUs were determined by plating 25 µl of a 10^{-6}-fold diluted culture suspension on the appropriate growth medium solidified with agar. Culturability values were calculated by dividing CFUs by TCN.

Algal Strains

Five strains of green microalgae were selected based on their different sensitivity to ultralow temperatures. The genera *Chlorella* and *Chlamydomonas* occur ubiquitously, serve as model systems in algae research and are of biotechnological and industrial relevance. The cryosensitive *Chlamydomonas reinhardtii* (SAG 11-32b) and *Chlorella variabilis* (strains ATCC 30562 and NC64A) were compared to the cryotolerant *Chlorella vulgaris* (SAG 211-11b) and *Micractinium conductrix* (SAG 241.80).

Chlorella and *Micractinium* strains were cultivated in basal medium with beef extract ("Erddekokt+Salze+Fleisch," ESFl, medium 1a; Schlösser, 1994) and the *Chlamydomonas* strain on Tris-Acetate-Phosphate (TAP) medium (Gorman and Levine, 1965). Axenic growth was tested in ESFl, basal medium with peptone (ESP, medium 1b; Schlösser, 1994) and in modified Bold's Basal Medium with 1.5% w/v glucose and 2% w/v proteose peptone (TOM; Nichols and Bold, 1965). All strains were grown at a temperature of 20°C using a 12 h/12 h dark/light regime of white fluorescent light (50 µE m^{-2} s^{-1}). After 2 weeks of growth, cultures in the exponential growth phase were harvested for cryostress assays. *Chlorella* and *Micractinium* strains were treated with 5% DSMO (v/v) according to the protocol introduced for *Chlorella* vulgaris using a controlled rate freezer (Day et al., 2007). For *Chlamydomonas* a protocol employing 3% (v/v) methanol as cryoprotectant was used (Crutchfield et al., 1999) since DMSO destroys the delicate cell envelope of *Chlamydomonas*. All cryopreserved strains were stored in the vapor phase of liquid nitrogen for 24 h and subsequently thawed as described previously (Day et al., 2007).

ATP content was measured at the start of the cryostress experiment (BF), after addition of cryoprotectants (BF_treat), in cultures after 24 h of cryostress [washed in culture medium and kept 24 h in the dark and 24 h under standard conditions (AF)] and after thawed cultures had been incubated for 2 weeks under standard growth conditions, transferred into fresh culture medium, and grown for two additional weeks (RG). The viability of the algae cells was determined at each sampling time through live/dead staining with fluorescein diacetate (FDA).

The Filamentous Fungus *Aspergillus nidulans*

The ubiquitous filamentous fungus *Aspergillus nidulans* is saprophytic and exhibits cold-, heat- and osmo-tolerance. It represents an established model organism in eukaryotic cell biology and was therefore chosen for the present investigation. Cultures were produced in 100 ml *Aspergillus* minimal medium (AMM; Barratt et al., 1965), inoculated with 10^6 *Aspergillus nidulans* spores per ml and incubated for 12 h to allow for the germination of spores and formation of sufficient biomass. The resulting mycelia were frozen at −80°C without cryoprotectant at a rate of 1°C min^{-1} using Mr. Frosty™ (Nalgene®) and samples were then stored frozen for 4 h. Since physiological activity of microorganisms has been found to cease ≤−70°C (Christner, 2002), the results obtained could be compared to those of the other organisms. Afterwards, cells were thawed in a water bath at 37°C for 150 s (i.e., at a rate of 46.8°C min^{-1}). For recultivation, mycelia were transferred into fresh AMM. For determination of ATP concentrations, mycelia were harvested at four different time points: after initial incubation under optimal growth condition (37°C for 12 h) (BF), after freezing (BF_treat), after thawing (AF) and after the recultivation of the cells for 12 h in AMM (RG). Oxygen consumption was monitored as an indicator of cell viability since viability measurements for cells in complex mycelia via colony forming units is not feasible. Mycelia were harvested at the beginning (BF) and after the cryostress and cell recovery (RG). The mycelium was transferred to fresh medium, overlaid with paraffin oil to prevent oxygen diffusion from air, and incubated at 37°C with gentle shaking

(80 rpm). Oxygen concentrations were measured in time intervals of 15 min with an oxygen sensor (Oxygen Sensor Spot, SP-PSt3-NAU, PreSens Precision Sensing, Regensburg, Germany). Afterwards, dry cell masses were determined after separation of mycelia from the supernatant by filtering with Miracloth (Merck Millipore) and 5 days of drying at 60°C.

Arabidopsis thaliana Tissues

For cryostress experiments of *Arabidopsis thaliana*, the established cryopreservation protocol was employed (Stock et al., 2017). Briefly, excised shoot tips were immersed overnight in liquid Murashige and Skoog medium (MS; Murashige and Skoog, 1962) containing 0.1 M sucrose, and the tips subsequently dehydrated for 20 min in MS medium containing 2 M glycerol and 0.4 M sucrose. This solution was then replaced by plant vitrification solution (PVS2; consisting of 30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v dimethyl sulfoxide, 0.4 M sucrose in MS, pH 5.8) for 1 h at 4°C in the dark. After droplet vitrification by LN_2 shock freezing, shoot tips were heated to 22°C and placed onto recovery medium [0.1 M sucrose, 0.5 mg L^{-1} zeatin riboside, 0.2 mg L^{-1} gibberellic acid (GA_3), 0.5 mg L^{-1} indole-3-acetic acid, 1% agar in MS medium, pH 5.8]. For regeneration, the explants were maintained for 3 days in the dark at 22°C, then for 4 days under low light, long day conditions (16 h-photoperiod, irradiance 20–30 μmol m^{-2} s^{-1} at 22°C, 8 h in the dark at 20°C), and finally for additional 18 days under normal light conditions (16 h-photoperiod, irradiance 150 μmol m^{-2} s^{-1} at 22°C, 8 h in the dark at 20°C). During regeneration, the cells of plant shoot tips die and a new plant develops from meristem cells. After this recovery period, all explants lacking signs of development were considered as dead, while those which formed incomplete structures were classified as surviving; the third category (recovered) represented those tips which developed into normal plantlets. Only recovered plantlets were included in the statistical analyses. All values reported represent the mean of three replicates, each of which comprised a group of 30 shoot tips.

Solanum tuberosum Cell Line

The wild type of *Solanum tuberosum* cv. Désiree (DSMZ No. PC-1182) was subcultured in 300 ml Erlenmeyer flasks with 100 ml cell suspension at 23°C and harvested as previously described (El-Banna et al., 2010). For cryostress experiments suspended cells were taken from the logarithmic growth phase 3 days after the last transfer. The controlled-rate cooling approach based on the method of Withers and King (Withers and King, 1980) was applied using the minitest system (Heine-Dobbernack et al., 2008) and the protocol described by Vaas et al. (2012). Cell suspensions were pretreated for 48 h with sorbitol (final concentrations, 0, 0.3, 0.6, and 1.2 M) then incubated in 5% (v/v) DMSO for 1.5 h, cooled at rate of −0.25°C min^{-1} to −40°C, and finally immersed in liquid nitrogen. After storage in liquid nitrogen for 1 day, samples were rapidly thawed at 40°C in a water bath. All individual experiments were conducted in six biological replicates.

Cellular ATP content and viability of the cells were determined after sorbitol and DMSO pretreatment (BF_treat), directly after thawing (AF), as well as after 1 week (RG1) and 5 weeks (RG5) of regeneration in 4X medium (Gamborg et al., 1968) containing 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 0.5 mg L^{-1} indole-3-acetic acid, 0.5 mg L^{-1} 1-naphtylacetic acid and 0.4 mg L^{-1} kinetin. Untreated inoculum cells from the logarithmic growth phase were used as reference (BF). Viability was determined by Evans Blue staining modified after Suzuki et al. (1999). Plant cells (150–300 mg fresh weight) were stained for 5 min with 0.5% Evans Blue in 4X medium. Afterwards cells were washed four times with 4X medium (BF, RG1, and RG5 cells) or with 4X medium supplemented with the respective sorbitol concentration (BF treat and AF cells). For Evans Blue staining of regrown cells the content of the cryotubes was poured onto 4X agar plates covered with three sterile filter paper disks. After 2 h of incubation the uppermost filter was transferred to fresh 4X agar. All agar plates were incubated in the dark at 23°C and the percentage of viable cells was determined after 1 and 5 weeks, respectively.

Human Cell Line JURL-MK1

The human chronic myeloid leukemia cell line JURL-MK1 was obtained from the repository of the Leibniz Institute DSMZ, and cultured in humidified air at 5% CO_2 and 37°C in 80% RPMI 1640 medium (Life Technologies) supplemented with 20% (v/v) fetal bovine serum (FBS) (Sigma). Cell cultures were maintained at 0.5 to 2.0 × 10^6 cells ml^{-1}. For cryostress experiments, four million cells of an exponentially growing culture (sample BF) were harvested and resuspended in 1 ml of freezing medium (70% RPMI 1640, 20% FBS, 10% v/v DMSO) per cryotube (sample BF_treat). These cell suspensions were frozen at a rate of −1°C min^{-1} until −80°C using a freezing container (Mr. Frosty™, Nalgene®) and subsequently transferred to the vapor phase of liquid nitrogen for storage. After 4–6 days, cryotubes were rapidly thawed in a water bath at 37°C and cell suspensions immediately diluted 10-fold with complete culture medium (sample AF). Thawed cells were then sedimented, the supernatant containing freezing medium discarded, and cells subcultivated at a density of 0.8 × 10^6 cells ml^{-1} as described above (sample AF). Finally, survival and regrowth were determined 3 days post thawing (sample RG). At each sampling point, aliquots were subjected to vital staining using trypan blue (Sigma) to determine cell density and viability using a Neubauer chamber (Brand GmbH & Co Kg).

ATP Assay

ATP was measured using the Bac- or CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the instructions of the manufacturer. Bacterial cultures were diluted 10-fold and human cell suspensions adjusted to 0.8 × 10^6 viable cells ml^{-1} and 50 µl of the reagent were added to 50 µl of cell suspension in white opaque 96-well plates (Greiner). For algae, fungi, plant cell lines and plant tissue, specifically adapted ATP extraction protocols had to be applied prior to ATP measurement.

Algal and fungal cells (500 µl re-suspended pellet of 50 ml algae cultures corresponding to 3–5 × 10^7 cells ml^{-1}) and fungal mycelia were mechanically disrupted in liquid nitrogen. ATP was extracted from 100 to 200 mg cell mass by mixing with 400 µl Tris/EDTA-buffer (0.1 M Tris-Acetate-Buffer, 2 mM EDTA, pH 7.75) and 500 µl cold (4°C) TCA/EDTA-solution (10% w/v trichloroacetic acid, 4 mM EDTA). The emulsion was centrifuged (10 min at 4°C, 20.000 × *g*) and 20 µl of the supernatant were transferred into a 96 well plate and four 10-fold serial dilutions were prepared in Tris/EDTA-buffer. 100 µl Tris/EDTA and 100 µL BacTiter-Glo reagent buffer were added to each sample.

For plant cell lines and tissue, ATP extraction was performed as described in (Smyth and Black, 1984) with small adaptations. 150 to 300 mg (fresh weight) plant cells were supplemented with 0.3 ml cold perchloric acid (0.83 M) and homogenized for 30 s. Another 0.7 ml cold perchloric acid were added and samples centrifuged for 15 min at 4°C and 10.000 × *g*. Then 225 µl Bicine (1 M, pH 7.75) were added to 900 µl of the supernatant, the sample was immediately placed on ice and the pH adjusted to 7.6–8.0 by addition of 4 M KOH. The white precipitate was centrifuged for 10 min at 4°C. 100 µl BacTiter-Glo® reactant was added to 100 µl of extract.

Luminescence of all samples was measured with an integration time of 0.5 s and automatic attenuation using a luminescence plate reader (Tecan Infinite M200). Samples were measured in technical duplicates (or triplicates for human cells) and ATP-standard curves with ATP diluted in culture medium or the respective extraction buffer included in each measurement. The amount of cellular ATP was calculated in moles per biomass. To calculate the ATP amount per mg cell material, the cell mass (dry weight), the protein concentration (Bradford, 1976) or the whole protein amount per biomass (dry weight) were determined depending on the organisms.

Statistics

For the measured parameters (cellular ATP and viability), the means of three replicates (4–6 parallels for the plant cell line) and 95% family wise confidence levels were calculated with R version 3.4.3 (R Core Team, 2017). Significant differences between consecutive sampling time points were calculated by one-way-ANOVA with multiple comparisons of means using Tukey Contrasts (package multcomp; Hothorn et al., 2008) and shown as compact letter display (cld; Piepho, 2004). Correlations for the association between paired samples were tested (R, corr.test) using two-sided Spearman's rank correlation rho. Distinct patterns in ATP content across the model organisms under cryostress, were evaluated employing shape-based time-series clustering (R, dtwclust; Sardá-Espinosa, 2017; Sardá-Espinosa et al., 2017) that refers to the Dynamic Time Warping distance (DTW; Keogh and Ratanamahatana, 2005; Lemire, 2009). Five different clustering algorithms [DTW basic, DTW with window of 1 and shape as centroid, cluster κ-shape, Time-series Anytime Density Peaks Clustering and a distance based on Global Alignment Kernels (GAK) with shape as centroid] using two to six clusters were evaluated using internal cluster validity indices. A particular algorithm was identified as optimum, if at least three of the indices yielded the best results.

RESULTS

Cellular ATP as an Indicator for Cryostress of Different Groups of Organisms

Cellular ATP concentrations were used to probe the physiological responses of the different cell types to optimum growth conditions (BF), to cryoprotectant (BF_treat), deep freezing (AF), and after regeneration of the cells from cryostress (RG). Methods of cryopreservation that had previously been established for each group of organisms were applied (Supplementary Figure S1) in order to compare the cryostress response across all cell types under their specific, currently optimized conditions.

For all groups of organisms, a decrease in the specific ATP content was observed during cryostress experiments. Minimum values were determined upon treatment with cryoprotectant (BF_treat) and/or after the freezing period (AF) (**Figure 1** and Supplementary Table S1). When data were aggregated groupwise, this decline in cellular ATP content was significant for all groups of organisms except for the combined six bacterial strains and for the human cell line (**Figure 1**, asterisks). After the phase of regrowth, bacterial cells and plant tissue even reached higher cellular ATP levels than the initial cultures (**Figure 1** and Supplementary Table S1). Thus, the initial ATP was exceeded up to four times in bacteria (*Psy. marincola*), up to three times in *Arabidopsis thaliana* shoot tips and 1.5 times in *Solanum tuberosum* cell lines treated with 1.2 M sorbitol (Supplementary Table S1). Based on these groupwise data, deep freezing was the single step during the cryostress experiment that affected the cellular ATP content most pronouncedly across almost all groups of organisms.

Changes in the Cellular ATP Content and in Viability of Different Microbial Strains

During the cryostress experiment all six bacterial strains studied exhibited similar patterns of changes in their cellular ATP content (**Figure 2A** and Supplementary Table S1). This is also corroborated by 12 out of the 15 possible pairwise correlations of ATP contents that proved to be highly significant (Supplementary Table S2). The addition of DMSO before freezing (step BF_treat) led to a significant decrease of ATP levels in *Pla. halocryophilus, Psy. cryohalolentis,* and *Psy. marincola.* Directly after thawing (step AF), ATP levels were significantly elevated in *Pla. plakortidis, Psy. marincola,* and *Psy. aquaticus* (**Figure 2A** and Supplementary Table S1).

In order to verify and classify the distinct patterns in ATP content across the different model organisms under cryostress, the pattern determined for each strains or organism was fitted to different shapes employing the R package dtwclust. The GAK algorithm performed best to describe the observed trends in cellular ATP. This analysis resulted in clusters of strains or organisms that could be

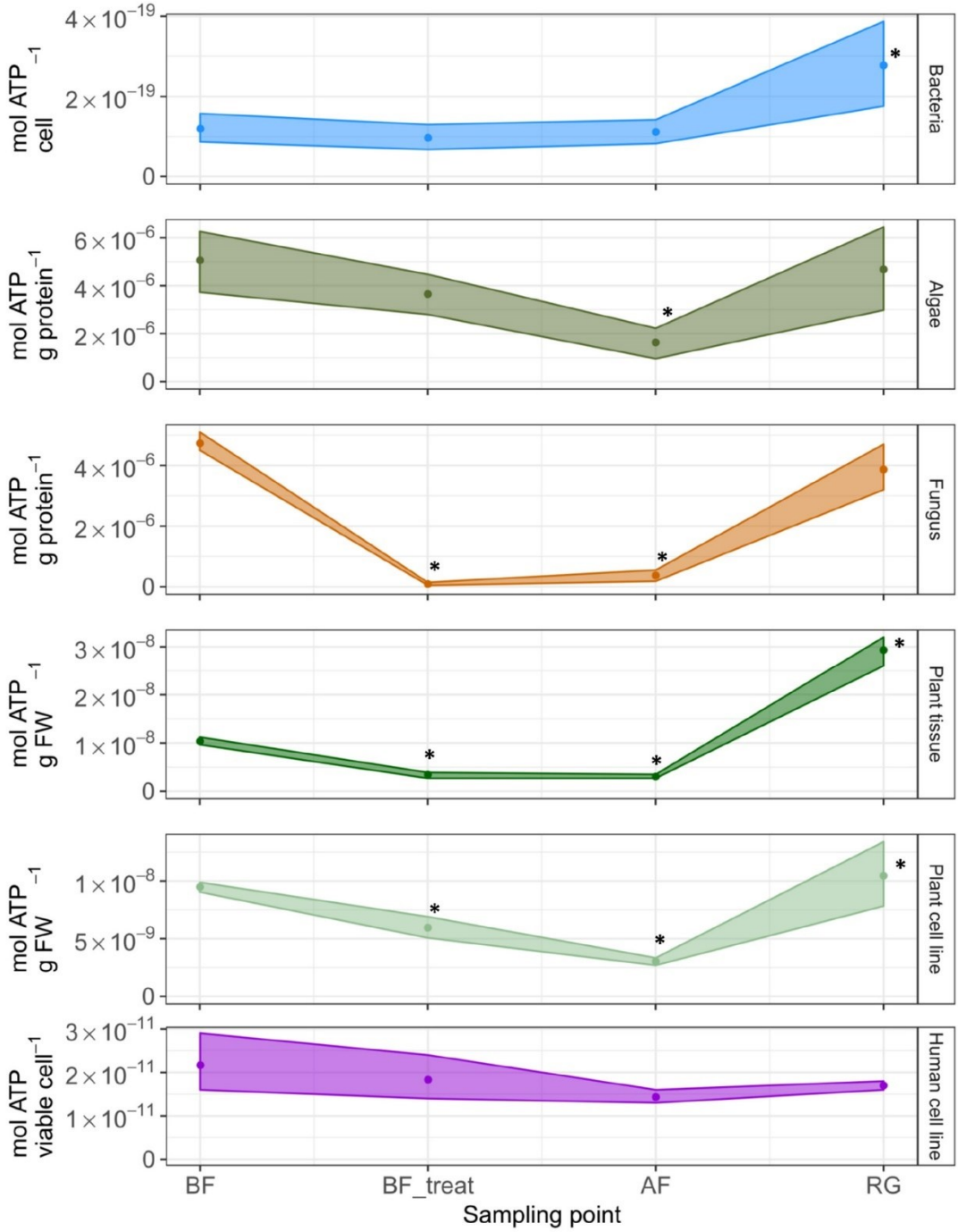

FIGURE 1 | Changes in intracellular ATP levels observed for the different model organisms during cryostress experiments. Points denote the mean ATP content of all representatives of a group of organisms at the individual sampling points. 95% confidence intervals are displayed as ribbons. Values that were significantly different ($p < 0.05$) from the BF value within a group of organisms are marked with *. FW, fresh weight; BF, before freezing; BF_treat, after treatment with cryoprotectant; AF, after freezing; RG, after the regrowth phase. Compare Supplementary Figure S1 for details on individual sampling points.

assigned to four different shapes (Supplementary Figure S2). Most bacterial strains fell into a cluster characterized by a pattern of limited decrease in ATP concentrations upon DMSO addition and a pronounced increase in ATP content after regeneration (cluster 4; **Figure 3** and Supplementary Table S1). Only *Pla. plakortidis* did not exhibit this pronounced final increase in ATP and hence fell into cluster one (**Figure 3**).

Notably, culturability and ATP contents were not correlated in cryostress experiments with bacterial strains (Supplementary Table S2 and **Figure 4A**). After regeneration, the initial culturability was reached in the psychrotolerant species *Pla.*

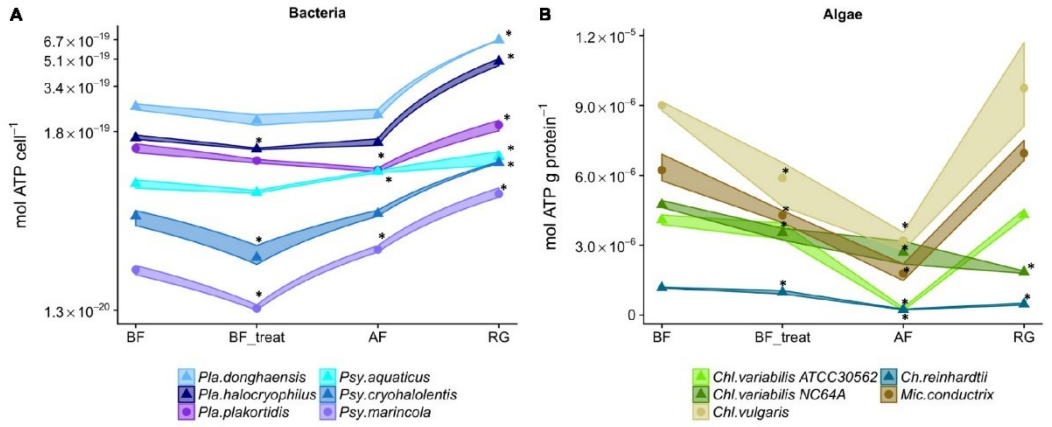

FIGURE 2 | Strain-specific alterations in intracellular ATP levels during cryostress experiments of bacteria and green algae. ATP content of cells of individual strains before freezing (BF), after the addition of a cryoprotectant (BF_treat), after freezing and thawing (AF) and after a regrowth phase (RG). Depicted are the means of three biological replicates with 95% confidence intervals presented as ribbons. **(A)** Response of the six different bacterial strains. **(B)** Response of the five different strains of green algae. ▲ psychrotolerant bacteria/cryotolerant algae, ● mesophilic bacteria/cryosensitive algae. * denotes values that were significantly different ($p < 0.05$) from the BF value of the respective strain. *Pla.*, *Planococcus*; *Psy.*, *Psychrobacter*; *Ch.*, *Chlamydomonas*; *Chl.*, *Chlorella*; *Mic.*, *Micractinium*.

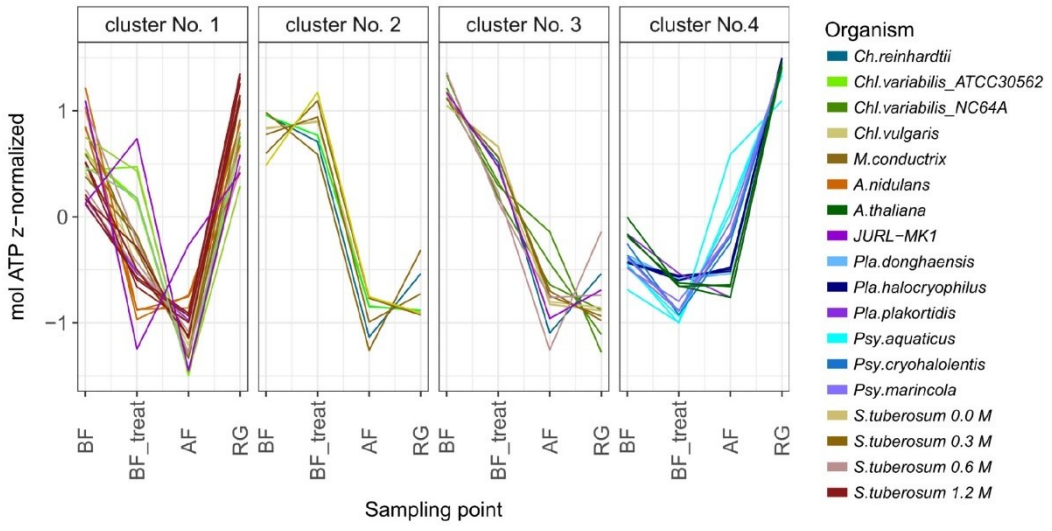

FIGURE 3 | Patterns of changes in intracellular ATP content observed for different organisms during cryostress. The pattern of ATP content observed under cryrostress for each strain or organism was fitted to different shapes employing the R package dtwclust. This analysis resulted in clusters of strains or organisms that could be assigned to four different shapes (Supplementary Figure S2). BF, before freezing; BF_treat, after treatment with cryoprotectant; AF, after freezing; RG, after the regrowth phase. *Pla.*, *Planococcus*; *Psy.*, *Psychrobacter*; *Ch.*, *Chlamydomonas*; *Chl.*, *Chlorella*; *Mic.*, *Micractinium*; *A. nidulans*, *Aspergillus nidulans*; *A. thaliana*, *Arabidopsis thaliana* Col-0 shoot tips; *S. tuberosum*, *Solanum tuberosum* cv. Desiree suspension cells after pretreatment with different sorbitol concentrations.

donghaensis and *Pla. halocryophilus*, but this did not mirror the distinctively elevated ATP contents. For the mesophilic *Pla. plakortidis*, both, culturability and ATP content, declined after freezing and for the mesophilic *Psy. marincola*, both, culturability and ATP content, were elevated after freezing, but the ATP content was not commensurate with culturability after regeneration. While the ATP content was increased after regeneration, culturability dropped below the initial point. Overall, the culturability of mesophilic bacteria in this study was found to be lower after the final regeneration than for psychrophilic.

The cellular ATP concentrations of green algae followed two principles and distinct patterns during cryostress experiments. One cluster of strains (No. 1, **Figure 3**) exhibited a strong ATP decline after freezing and thawing, followed by an increase in ATP content after regrowth which recovered fully back to initial levels. This cluster comprised the cryotolerant *Chl. vulgaris*, *Chl. variabilis* A, and *Mic. conductrix* (**Figures 2B**, **3**). Interestingly, cellular ATP content of the cryosensitive strains *Ch. reinhardtii* SAG 11-32b and *Chl. variabilis* NC64A did not return to the high initial values even after regrowth but instead reached only 40% of these levels (**Figure 2B**); these strains thus fell into another cluster of strains with a different pattern (no. 3, **Figure 3**). Thus, the two *Chl. variabilis* strains showed similar ATP contents before freezing (4.08×10^{-06} and 4.74×10^{-06} mol ATP g protein^{-1} in ATCC30562 and NC64A, respectively) and also after cryoprotectant treatment, but differed in later stages of the cryostress experiments. The extent of ATP decrease during freezing and thawing was more pronounced in the cryotolerant ATCC strain but cellular ATP levels were completely restored after regrowth, whereas the ATP levels of NC64A remained below the values of starting cultures. Cell size and ATP content per g protein were negatively correlated within the different algae species ($p = 0.038$, $R^2 = 0.742$, estimate = −0.9, linear regression calculated with Pearson's product-moment correlation), due to the relatively higher protein amount of the motile *Chlamydomonas* algae (Ø 4.6 µg ml^{-1}), compared to *Chl. vulgaris* (Ø 1.4 µg ml^{-1}) or *Chl. variabilis* (Ø 3.6 µg ml^{-1}). Therefore viability and ATP content had to be compared for each strain separately. The ATP content was significantly correlated to viability in the cryosensitive *Ch. reinhardtii* and *Chl. variabilis* NC64A (**Figure 4B** and Supplementary Table S1), whereas viability does not reflect the ATP content of the cells in *Chl. variabilis* ATCC 30562, *Chl. vulgaris* and *Mic. conductrix*.

The cellular ATP content of the fungus *Aspergillus nidulans* exhibited a pattern similar to that of cryotolerant green algae (cluster with pattern No. 1; **Figure 3** and Supplementary Table S1). After a pronounced decline of the cellular ATP content during freezing and thawing, ATP levels were fully restored after the regeneration period of 12 h and no significant differences to the values of the first sampling time point were observed (**Figure 2**). At optimum growth, the oxygen consumption rate per dry weight was significantly lower ($p < 0.01$) in comparison to the cells regenerated after thawing. Since the decrease of the oxygen consumption (i.e., the respiration rate) stayed constant over the whole course of the cryostress experiment, it has to be concluded that no significant cell death occurred. The oxygen consumption

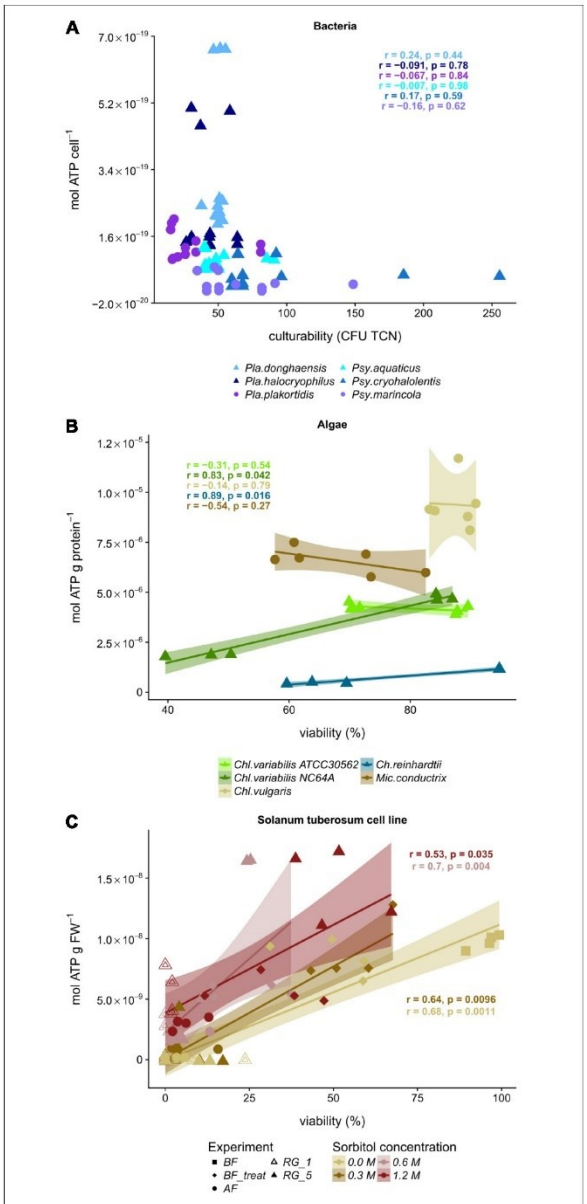

FIGURE 4 | Correlation of ATP levels and viability during freezing of bacteria, green algae and a plant cell line. **(A)** Depicted is the ATP content vs. culturability (CFU, colony forming units per TCN, total cell numbers) of the different bacterial strains (*Pla.*, *Planococcus*, *Psy.*, *Psychrobacter*). **(B)** ATP content vs. viability with 95% confidence interval and linear regression of the green algae *Ch.*, *Chlamydomonas*; *Chl.*, *Chlorella*, and *Mic.*, *Micractinium*. ▲ psychrotolerant bacteria/cryotolerant algae, • mesophilic bacteria/cryosensitive algae. **(C)** ATP content vs. viability with 95% confidence interval and linear regression of suspension cultures of *Solanum tuberosum* cv. Desiree after pretreatment with different sorbitol concentrations before freezing (BF), after the addition of a cryoprotectant (BF_treat), after freezing and thawing (AF) and after a regrowth phase (RG). r, Spearman's rank correlation, estimated measure of the association; p, *p*-value.

rate did not correlate with the ATP content (Supplementary Table S2).

Taken together, the results obtained for the 12 different types of microorganisms clearly demonstrate that the cellular ATP contents do not mirror the cell viability (Supplementary Table S2). In most cases, bacteria and algae showed high ATP levels after regrowth regardless of a lower viability. A significant positive correlation of ATP content and viability was determined only under optimal conditions (BF) and for the cryosensitive algae *Chlamydomonas reinhardtii* and *Chl. variabilis* NC64A as well across experiments.

Cryostress Response of Cells From Multicellular Eukaryotes

The ATP concentration of *Arabidopsis thaliana* in wild-type seedlings (1.09×10^{-8} mol ATP g FW^{-1}) and freshly excised shoot tips (9.89×10^{-8} mol ATP g FW^{-1}) did not vary significantly (Supplementary Table S2). The ATP level during cryopreservation was characterized by an ATP decline after cryoprotectant treatment and after freezing and thawing and this was similar to the patterns observed for bacteria or the fungus (**Figures 3**, **5A**). Within the first 3 days of regeneration (RG_3), the ATP level increased steadily to concentrations comparable to that of the seedling stage (1.15×10^{-8} mol ATP g FW^{-1}) (compare Supplementary Figure S3A) but continued to increase until day 5 when even higher levels were attained (**Figure 5A**) accompanied by a rapid development of the seedlings (Supplementary Figure S3B). The viability after a regeneration of 25 days was close to 100% (Supplementary Table S1).

Plant cell lines are especially vulnerable to cryostress due to their high water content and the presence of water in large vacuoles. Since cryopreservation of plant cell lines involves specific pretreatment with sorbitol, the effect of the resulting osmotic stress was investigated systematically, using cell suspensions of *Solanum tuberosum* cv. Désiree. Before freezing and under optimal growth conditions, the ATP content was 9.6 nmol ATP $(g\ FW)^{-1}$ and 96% of the cells were viable (Supplementary Table S1). Already in the absence of sorbitol, addition of DMSO decreased the viability significantly to 50% (Supplementary Figure S3C). Pretreatment of the cells with 0.6 or 1.2 M sorbitol reduced the ATP content significantly to about 60% (**Figure 5B**), after pretreatment of the cells without or 0.3 M sorbitol the decrease was not significant based on multiple comparisons by ANOVA-testing. The lower viability of cells pretreated with these higher sorbitol concentrations was not significant as compared to cells exposed to no or lower concentrations of sorbitol (Supplementary Table S1, cld "c"). Interestingly, the decreases in cellular ATP content during deep freezing and after regeneration were inversely correlated with the concentrations of sorbitol during pretreatment (**Figure 5B** and Supplementary Table S1). Thus, the ATP contents of cells without sorbitol or pretreated with 0.3 M sorbitol did not recover even in later stages of regeneration (i.e., clustered with patterns 2 and 3, **Figure 3**) whereas the higher sorbitol concentrations resulted in elevated cellular ATP content at the end of the cryostress experiment (pattern 1, **Figure 3**). Viability values showed a similar massive decline and dropped to $\leq$6% during deep freezing but unlike the ATP content remained low also during the subsequent 1st week of regeneration (Supplementary Table S1). After 5 weeks of regeneration, viability values had recovered to

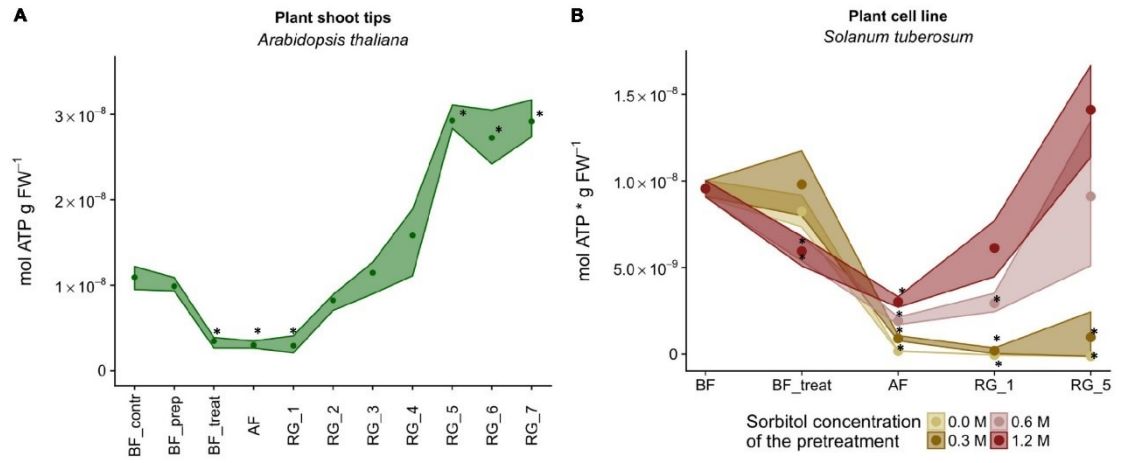

FIGURE 5 | Strain-specific responses in intracellular ATP concentrations during cryostress experiments of plant shoot tips and a plant cell line. ATP content of cells from the individual strains before freezing (BF), after the addition of a cryoprotectant (BF_treat), after freezing and thawing (AF) and after a regrowth phase (RG). Depicted are the means of the biological replicates with 95% confidence intervals represented as ribbons. **(A)** Response of *Arabidopsis thaliana* Col-0 shoot tips. BF_contr, control (wild-type seedlings); BF_prep, prepared (freshly excised shoot tips); RG_1 – RG_7, regrowth after 1–7 days. **(B)** Response of *Solanum tuberosum* cv. Desiree suspension cells after pretreatment with different sorbitol concentrations. RG_1 and RG_5, regrowth after 1 and 5 week(s). * denotes values that were significantly different ($p < 0.05$) from the BF value of the respective strain. FW, fresh weight.

51% only in cultures pretreated with 1.2 M sorbitol, reached lower values (mean, 16%) after pretreatment with 0.6 M sorbitol but remained at low levels for the other cultures. These significant differences indicate a major effect of sorbitol pretreatment on the survival of plant cell during cryostress (Supplementary Table S1). Due to the different kinetics of ATP concentrations and viability of the cells, both parameters were not significantly correlated for cells treated with 0.6 and 1.2 M sorbitol (**Figure 4C**).

In the cancer cell line JURL-MK1, cellular ATP levels remained stable during freezing and thawing and the observed differences were not statistically significant (**Figure 1** and Supplementary Table S1). Also, the freezing and thawing steps had no considerable effect on cell viability which remained constantly high >95% (Supplementary Table S1).

DISCUSSION

So far, the physiological responses to temperature decrease have mostly been studied under cold stress rather than for ultradeep freezing conditions. Few individual organisms have been tested at subzero temperatures (Christner, 2002; Junge et al., 2006; Morrison and Shain, 2008; Amato and Christner, 2009), but in these cases changes in ATP concentrations and viability have not been determined together. In particular, the effects of cryopreservation on the physiological state of the cells have not been assessed systematically. Our comparative cryostress experiments employed established cryopreservation procedures and monitored the resulting changes in cellular ATP and their relation to cell viability across a wide range of different types of organisms.

The intracellular ATP concentrations determined for growing cells in the present study are comparable to those in the literature [10^{-19} mol ATP cell^{-1} for *Psy. cryohalolentis* (Amato and Christner, 2009); 37.6–161.4 μmol ATP mg chlorophyll a^{-1} h^{-1} for algae (Sawa et al., 1982), 1–17 μmol ATP mg chlorophyll a^{-1} in our study; 6×10^{-6} mol ATP g dry weight^{-1} for *Saccharomyces cerevisiae* (Thomsson et al., 2003); 30–80 nmol g FW^{-1} for maize cell cultures (Sowa et al., 1998); 50 nmol g FW^{-1} in *Arabidopsis* seedlings (Zhu et al., 2012); 3.95×10^{-11} mol ATP cell^{-1} for a chronic myelogenous leukemia cell line (Mikirova, 2015)]. Considerable differences in the cellular ATP contents were observed between the six bacterial strains investigated in this study. Since the cellular ATP concentrations were correlated significantly ($p = 0.044$, $R^2 = 0.596$, estimate = 0.82, linear regression calculated with Pearson's product-moment correlation) with the respective cell volumes of the strains (0.39–1.02 μm^3 × cell^{-1}), the differences in cellular ATP content can be explained by the different size of the bacteria. Cell size and ATP content (determined per g protein) were negatively correlated for the different algae species, which most likely is due to the motility and the higher relative protein content of the larger *Chlamydomonas* algae.

Cold shock (i.e., a decrease of incubation temperature by 10–20°C below temperature for growth optimum, for 120 min) has been demonstrated to result in a rapid loss of ATP and total adenylate nucleotides in mesophilic bacteria, fungi and protists, but on the opposite leads to increased intracellular concentrations in psychrophilic representatives, including *Psy. cryohalolentis* which was investigated also in the present study (Napolitano and Shain, 2005; Amato and Christner, 2009). It has therefore been suggested that the mode of regulation of the adenylate pool differs in psychrophiles, and enables maintenance of elevated ATP concentrations that offset reductions in molecular motion and Gibb's free energy of ATP hydrolysis (Morrison and Shain, 2008). In plant cells, the physiological adaptations during cold acclimation (so-called "hardening") require several weeks of incubation at non-freezing temperatures (Sobczyk and Kacperska-Palacz, 1978; Uemura and Steponkus, 1994). Winter rape (*Brassica napus* L. var. *oleifera* L.) plants maintain intracellular ATP concentrations in the dark upon cooling to 0°C. After cold acclimation, freezing results in increased ATP concentrations in leaves but decreased concentrations in roots (Sobczyk and Kacperska-Palacz, 1978).

In contrast to these previously published results, increases of cellular ATP concentrations at ultradeep temperatures were never observed for any of the cell types tested. Instead, representatives of bacteria, fungi, algae, plant tissue, and plant cell lines directly subjected to ultradeep freezing all showed significant declines in cellular ATP levels which in most cases recovered to the initial or even higher values upon regeneration. In bacteria and plant (tissue) ATP levels during regeneration exceeded those before freezing by upto four times. This increase can be attributed to a higher energy demand during exponential growth phase and the development of new tissue (Morita and Morita, 1997). The plant shoot tip cells die during regeneration and a new plant develops from the meristem cells. This cell differentiation and active metabolism are typically characterized by elevated ATP levels (Rolletschek et al., 2004).

Most biochemical processes cease when ambient temperatures fall below −70 to −80°C (Christner, 2002; Junge et al., 2006). At −40°C, bacterial metabolism is very low and appears to be limited to the repair of macromolecular damage of the largely dormant cells (Price and Sowers, 2004). Previously, increases in cellular ATP concentrations of psychrophilic bacteria upon exposure to subzero temperatures were observed when employing slow cooling rates [0.32–1.91°C min^{-1}; (Amato and Christner, 2009)] that may elicit a physiological response of the bacterial cells while still in liquid cultures. Similarly, the time course of cooling used in established cryopreservation protocols (Supplementary Figure S1) is much too short to allow for cold-adaptation of plant cells and tissues. Reduced ATP values as a consequence of cryoprotectant exposure have been reported in catfish spermatozoa (De Baulny et al., 1999), gorgonian coral (Tsai et al., 2010, 2014a,b, Lin et al., 2011) and porcine oocytes (Tsuzuki et al., 2009), but did not seem to occur in the human cell line when the established cryopreservation procedures were employed. Furthermore, cryoprotectant treatment was avoided in starved psychrophilic bacteria after incubation at 22°C for 88 h before cryostress (Amato and Christner, 2009). In addition, cryoprotectants and plant vitrification solutions have been reported to induce hypoxic stress in plant tissues, such as garlic shoot tips (Subbarayan et al., 2015). Similarly, an ATP decrease has been related to hypoxia during barley seed development

(Rolletschek et al., 2004). Mitochondrial dysfunction resulting in hypoxia and apoptosis of retinal ganglion cells could be prevented by taurine treatment of the cells, accompanied by cellular ATP loss (Chen et al., 2009). Furthermore, mitochondria of maize seedlings have shown to be a target for chilling-induced oxidative stress that finally impairs cellular respiration and ATP formation (Prasad et al., 1994). Thus, cryopreservation related stressors, such as oxidative stress or cryoprotectants, can cause mitochondrial dysfunction leading to hypoxia, accompanied by ATP depletion.

Different biochemical processes are known to affect cellular ATP content upon decreasing temperatures. In bacteria, futile cycling of protons or other ions (K^+, NH_4^+) occurs if ATP-consuming active transport is neutralized by reverse transmembrane movements due to turgor-activated membrane channels or changes in membrane resistance, thereby causing a pronounced decrease in cellular ATP on a short-term scale (Russell and Cook, 1995). In leaves of winter rape, the decrease in intracellular ATP concentrations is associated with changes in membrane permeability, initially through the inactivation of ion-dependent ATPases. Subsequent irreversible membrane damages result in degradation of ATP (Sobczyk et al., 1985). No significant changes in cellular ATP content could be observed for the continuous human JURL-MK1 cell line. In contrast, primary human peripheral blood leukocytes have been shown to be compromised in mitochondrial function and ATP production during cryopreservation (Keane et al., 2015). Hence, in eukaryotic cells, mitochondrial function may be affected by cryostress, while the plasma membrane remains intact. Slow cooling of human umbilical vein endothelial cells resulted in depolarization and dysfunction of mitochondria immediately after thawing (Reardon et al., 2015), whereby the detailed mechanisms of the indirect effects of cooling on cell organelles need further investigations. However, the observation of a consistent decline in intracellular ATP concentrations observed across almost all other organisms, independent of their psychrophilic, psychrotolerant, or mesophilic character, together with the speed of temperature decline, indicate that compensatory physiological responses to cold stress did not occur at the rapid freezing rates used in the present study. Rather, constitutive biochemical processes dominated the energetic state of the cells during cryopreservation as well as the impairment of cellular respiration.

The goal of the established, often complex cryopreservation techniques is to maintain as many cells as possible in a viable state that ensures growth upon thawing. In order to investigate whether ATP is a major determinant of cell survival (Pegg, 1989), we systematically tested the correlation between cellular ATP content and viability during different stages of cryopreservation and across different types of organisms. Such a correlation is suggested by some previously published observations. Elevated ATP concentrations induced in an *Escherichia coli* knockout mutant resulted in a significantly increased survival after exposure to 0°C compared to the wild type (Morrison and Shain, 2008). Other stresses [such as anaerobic carbon starvation in the yeast *Saccharomyces cerevisiae*; (Thomsson et al., 2003) which result in energy deprivation and drastically reduced intracellular ATP-concentrations] also cause a major loss in physiological activity and viability upon subsequent substrate addition. Yet, the correlation between ATP content and biomass formation in bacteria is often poor due to maintenance energy requirements and other ways of non-growth energy dissipation, the latter can exceed the former by an order of magnitude (Russell and Cook, 1995).

The lack of correlation between cellular ATP content and viability after ultradeep freezing was observed across almost all types of organisms covered in the present study. A link between cellular ATP content and viability was only observed for two cryosensitive alga and plant cell cultures kept at low osmotic pressures, in which ATP concentrations did not return to pre-cryostress levels, suggesting irreversible damage of the cells under the conditions applied. Our results support the conclusion that factors other than ATP content determine changes in cell viability as long as cryopreservation does not irreversibly affect the cells. This is also commensurate with the observation that an increased respiration rate occurred during the regeneration of *Aspergillus nidulans*, enabling the return to initial values of cellular ATP values. Based on our results, adaptations to cold stress do not occur during cryopreservation. Instead, the physiological response and survival of almost all cell types is determined by other, constitutive processes such as changes in membrane permeability and ion transport. The cold stress response may provide additional opportunities for improving cryopreservation techniques but to our knowledge, this has not been studied systematically to date. From a practical point of view, our results clearly indicate that intracellular ATP content cannot be used as a reliable predictor of cell viability for further optimization of cryopreservation for the organisms tested in the present study.

AUTHOR CONTRIBUTIONS

JO, OK, H-PM, SE, TF, HS, EK, ML, and FB designed the study. FB, JS, BH, TD, EH-D, and LN performed parts of the lab work and mainly analyzed and interpreted the data. JO, OK, H-PM, SE, TF, HS, EK, and ML substantial contributed to the interpretation of the results and valuable discussion. FB was drafting the work. JS, BH, TD, EH-D, and SE contributed the drafting. JO, OK, H-PM, TF, HS, EK, and ML revised it critically for important intellectual content. All authors finally approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00921/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2 Transcriptomic and proteomic profiling of the *Aspergillus nidulans* response to low temperature stress revealed a distinct profile of secondary metabolites and the induction of sexual development

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Summary

Fungi are exposed to regular temperature changes due to the seasonal cycles and need to adapt to harsh environmental conditions such as low temperatures. This study investigates the global adaptation of filamentous fungi to low temperature stress for the first time. Different omics approaches (transcriptomics, proteomics and metabolomics) were applied to investigate the adaptation of *A. nidulans* to 10°C and 37°C.

An enrichment analysis revealed differentially regulated proteins and transcripts at 10°C, in the categories of cold stress protection, cell development and biosynthesis of natural products. This data showed that several protective mechanisms were induced, which are among others commonly known from oxidative, osmotic and cold stress response in different organisms. Further on, an altered secondary metabolite (SM) expression profile was found at low temperature stress, with cold-induced SMs that show activity against Gram-positive bacteria and fungi. Additionally, sexual development was induced, suggesting an alternative, light-independent activation for an altered cell development.

Understanding low temperature stress adaptation strategies of *A. nidulans* give new insights in the regulation of cell development and is highly relevant for natural product research as SMs can be used as valuable sources for therapeutic compounds or exert important tasks as mediators of biological communication.

Contribution to the manuscript

Benjamin Hanf contributed to the manuscript by conducting all the experiments and also writing and editing the whole manuscript.

Estimated contribution in percentage

Benjamin Hanf	**60 %**

Thomas Krüger, Derek Mattern, Maria Stroe, Theresia Conrad, Boyke Bunk, Gerhard Braus and Jörg Overmann	20 %
Axel Brakhage and Olaf Kniemeyer	20 %

Transcriptomic and proteomic profiling of the *Aspergillus nidulans* response to low temperature stress revealed a distinct profile of secondary metabolites and the induction of sexual development

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Keywords: low temperature stress, cold tolerance, cell development, sexual development, secondary metabolites

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Summary

Fungi are found in nearly every terrain on earth. The seasonal cycle exposes fungi to regular temperature changes. For tolerating harsh environmental conditions such as low temperatures, adaptation strategies are essential.

To gain detailed insights into the low temperature adaptation of the important model fungus *Aspergillus nidulans*, we analysed the dynamics of the transcriptome, proteome, and secondary metabolome during low temperatures that still allow growth. The low temperature stress response was investigated *via* comparative gel-based (Difference in gel electrophoresis, DIGE) and gel-free (Liquid chromatography–tandem mass spectrometry, LC-MS/MS) proteomic approaches. Our multi-omics analysis revealed that genes and proteins involved in the categories of cold stress protection, cell development and biosynthesis of natural products were enriched at 10°C. Common cold protective mechanisms such as osmotic stress response proteins were induced. In addition, an altered secondary metabolite (SM) production profile was confirmed at the metabolite level by LC-MS/MS analysis. Sharp temperature shifts triggered among others the expression of silent gene clusters such as the anthraquinone pigment asperthecin. Some of the known SMs, like asperfuranone, have been reported to exhibit biological activity such as anti-proliferative effects in human cancer cells. Likewise, the cold-induced SMs inhibited growth of Gram-positive bacteria and fungi. On the transcript and protein levels, our analyses showed the induction of factors, which regulate the sexual cycle in *A. nidulans.* A shift towards sexual development was confirmed by the observation that Hülle cells (a nondeciduous chlamydospore-like structure that surrounds cleistothecia) were formed at 10°C.

Collectively, this study demonstrates that low temperature stress triggers sexual development, activates common cold protective mechanisms and induces the production of a variety of SMs in *A. nidulans*.

1 Introduction

With the seasonal cycle, microorganisms including fungi are exposed to temperature changes (1). Hence, many microorganisms experience drops of ambient temperature in natural environments and need to adapt continuously in order to survive. Low temperatures do not exclusively occur in natural environments but are also used for strain maintenance and storage (2). Some microorganisms survive or even grow under these low temperatures while others perish (3,4). Therefore, it is important to understand the principles of low temperature adaptation. Some aspects have already been investigated in fungi, but many details remain obscure. Several studies focused on the temperature adaptation of mycorrhizal fungi and their adaptation to temperature changes in the soil (5). Cucumber plants benefit from their symbiosis with arbuscular mycorrhizal fungi that lower the hydrogen peroxide accumulation in the plant's roots and therefore increase their chilling stress (chilling stress is a defined range from 6 - 15°C) tolerance (6). For pathogenic fungi, the ability to adapt to temperature shifts is crucial. Studies on human and plant pathogenic fungi revealed that temperature greatly influences their latent period (generation time) and snow moulds have established their niche towards plant pathogens that prevail when covered in snow (7). Further on, cultivation of fungal production strains at low temperatures such as cold fermentation is used in the food industry because it leads to reduced evaporation of volatile flavourings (8). Cold-adapted Arctic and Antarctic fungi are mostly psychrotrophic, i.e., they make use of mechanisms to endure temperatures close to the freezing point (9). These mechanisms include the increase of intracellular osmolytes like trehalose and polyols, of unsaturated membrane fatty acids, the secretion of antifreeze proteins, and the production of enzymes that are active at low temperatures (9). During prolonged periods of extreme low temperatures, many fungi survive through the formation of dormant spores, thus avoiding adverse environmental conditions (10).

Until now, little is known about the cold adaptation of moulds belonging to the medically and biotechnologically important genus *Aspergillus*. Therefore, here, we have studied the species *Aspergillus nidulans* (11), which is a model organism in cell and developmental biology and biotechnology (12,13). It has been shown that *A. nidulans* mycelium is relatively robust against

cryostress (14). Moreover, temperature, light and nutrients, influence fruiting body formation (15). Also the production of secondary metabolites (SMs) is affected by differences in temperature in several *Aspergillus* species (13,16). Typically, the genes involved in the production of SMs are organised in clusters. Bioinformatic analyses predict up to 70 SM gene clusters (17–19) in the genome of *A. nidulans*. However, the true ecological role of most of them is unknown (20). In general, the ecological function of SMs can range from chelation of essential trace elements over outcompeting rivals *e.g.* by growth inhibition (21–24) to cross-species communication (25). Most SM gene clusters are silent under standard growth conditions in the laboratory (26). The question of whether low temperature stress activates specific SM clusters and whether the produced compounds play a role under these conditions remains to be addressed.

Here, we provide insight into the adaptive response of *A. nidulans* to low temperature stress by determining changes on the transcript, protein, and SM level using RNA-seq, LC-MS/MS-based proteomics and metabolomics. By applying this integrative, multi-omics approach, this study provides a global overview of the *A. nidulans* stress response to low temperature adaptation. Further, our data reveal new biologically active SMs and demonstrate a connection between the cold shock response and the induction of sexual development in *A. nidulans*.

2 Material and Methods

2.1 *A. nidulans* strain and culture conditions

A. nidulans strain R21 (*yA2*, *pabaA1*, *veA*) (27) was cultivated in 100 ml *Aspergillus* minimal medium (AMM; (28)). For production of conidia, the strain was incubated for 3 days at 37°C on AMM agar (1.5% (w/v) agar). The conidia were harvested in Tween-saline solution (NaCl 0.8% (w/v), 0.025%(v/v) Tween80). Experimental cultures were inoculated with 10^6 conidia/ml and incubated for approximately 18-24h at 37°C or 3-4 weeks at 10°C. At a residual sugar amount of 10 – 20 nmol/l glucose in the growth medium, mycelia were harvested in order to ensure the formation of sufficient biomass and similar growth conditions under the two different temperatures.

2.2 Determination of glucose concentration

The glucose concentration of the culture supernatant was measured with the BIOSEN C-Line analyzer (EKF Diagnostic, Germany) according to the manufacturer's instructions.

2.3 Protein extraction for proteome analysis

Protein extracts of *A. nidulans* mycelium were generated after grinding in liquid nitrogen by trichloroacetic acid (TCA)/acetone precipitation as previously described (29). Protein concentration was determined with the Bradford assay as described in (30). For gel-free proteomics we followed our previously established protocol (31) with slight modifications: After isolating the proteins, SDS and contaminants were removed by chloroform-methanol purification after Wessel-Flügge (32) and water-saturated ethyl acetate clean-up after Yeung and Stanley (33). After that, the proteins were solubilized in 50 mM TEAB and the protein concentration was determination by Direct Detect System (Merck Millipore, Germany) according to the manufacturer's instructions.

2.4 Difference in gel electrophoresis (DIGE) two-dimensional gel electrophoresis

DIGE analysis was carried out as previously described (34) with slight modifications: At least 6 biological replicates were used. The generated gel images were analysed with the software Delta2D 4.6 (Decodon, Germany) to quantify relative changes in protein spot abundances (37°C *versus* 10°C). Only proteins with a *p*-value below 0.01 using Wilcoxon, Mann-Whitney-Test with a false discovery rate of <0.01 and a ratio greater or smaller than 2 were considered as significantly different.

2.5 Mass spectrometry analyses

2.5.1. MALDI-TOF/TOF:

The MS (35) data were collected with the software flexControl 3.3 and further analysed with flexAnalysis 3.3 (Bruker Daltonics, Germany). The peptide mass fingerprint (PMF) and peptide fragmentation fingerprint (PFF) data were submitted to an MASCOTserver (MASCOT 2.3,

Matrix Science, U.K.), using the NCBI database restricted to the taxon fungi. The database searching parameters were the following: a peptide mass tolerance of 100 ppm, oxidation of Met (variable) and carbamidomethylation of Cys (fixed). Results with a *p*-value ≤ 0.05 according to the MASCOT score (> 54) were regarded as statistically significant.

2.5.2. Tandem mass tag (TMT) labelling:

The TMT labelling was conducted as described previously (31) with slight modifications: Protein digestion was performed using 4 µg trypsin+LysC mix (Promega Cat. # V5072) followed by an incubation for 18 h at 37°C. Afterwards the peptides were labelled with the TMTsixplex™ Isobaric Label Reagent (ThermoFisher Scientific, Dreieich, Germany) according to the manufacturer's instructions. The three biological replicates of the 37°C control samples were labelled with TMT-126, TMT-128 and TMT-130 reagents. The 10°C samples were accordingly labelled with TMT-127, TMT-129, and TMT-131. After the reaction had been stopped (8 µL 5 % (v/v) hydroxylamine, 15 min) all 6plex reactions were combined and mixed.

2.5.3. Strong cation exchange (SCX) fractionation:

Following SpeedVac evaporation of the multiplex samples the labelled peptides were resolubilised in 800 µL F0 solution (25% ACN, 0.05% HCOOH) for SCX fractionation on Hyper Sep SCX (50 mg, 1 mL) SPE cartridges (Thermo Scientific, Waltham, MA, USA). After reconstitution of the SCX resin with 1 mL ACN and 2 × 1 mL F0, the sample was loaded and subsequently fractionated using NH_4HCO_3 at concentrations of 25 mM (F1), 50 mM (F2), 100 mM (F3), and 400 mM (F4). The flow-through fraction (F0) and the stepped elution fractions (F1-4) were evaporated using a SpeedVac, resolubilized in 25 µL 0.05% TFA and 2% ACN, sonicated in a water bath for 15 min, filtered through 10 kDa MWCO spin filters (VWR) at 16,500 × *g* for 15 min and stored at -80°C until LC-MS/MS analysis.

2.5.4. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis

LC-MS/MS analysis and protein database search, including reporter ion quantification, was carried out as described previously (31) with slight modifications.

Tandem mass spectra were searched against the AspGD protein database of *A. nidulans* FGSC A4 (http://www.aspergillusgenome.org/download/sequence/A_nidulans_FGSC_A4/current/A_nidulans_FGSC_A4_current_orf_trans_all.fasta.gz; status: 2015/02/19). TMT reporter ion ratios were calculated by comparison of all biological replicates of 10°C cultures against the 37°C cultures.

2.6 Microscopic imaging

After culturing mycelia at 37°C or 10°C for up to four weeks, aliquots of 10 µl were taken and transferred on microscope slides and were covered with cover slips. Microscope images were recorded by using a light microscope (Leica DM 4500B, Leica Microsystems, Germany).

2.7 Statistical analyses

If not otherwise stated, Student's *t*-test was used, with at least three biological replicates. Differences between two groups were considered as significant, if the $P \leq 0.05$ or $P \leq 0.01$ were calculated. One or two asterisks, respectively, indicate the confidence intervals for the *p*-values. Standard deviations of means are indicated by error bars. Each experiment was conducted with at least three biological replicates.

2.8 Molecular biological methods

RNA preparation was essentially carried out as previously described (36), with the use of the RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). For spectrophotometrical determination of the RNA amount, the NanoDrop 1000 (Thermo Fisher Scientific) was used. RNA quality was calculated with the Bioanalyzer 2100 (Agilent Technologies, USA, RNA 6000 Nano LabChip® kit). DNA contaminations were eliminated with the RNase-Free DNase Set (Quiagen, Venlo, Netherlands) and reverse transcribed with MyTaq™ One-Step RT-PCR Kit (Bioline, London, UK).

For transcriptome analysis, a total of 1µg mRNA was purified and polyA selection was performed using the Illumina TruSeq RNA Sample Preparation v2 (Illumina, New York, USA). A library was prepared using total RNA from the samples with ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre, Madison, Wisconsin, USA). For quality control, libraries were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Cluster generation of the prepared libraries was performed using the cBot (Illumina) using TruSeq SR Cluster Kit v3–cBot-HS (Illumina, New York, USA). The concentration of cDNA libraries loaded in the flow-cells was 12 pM. Sequencing of the clustered flow-cell was done on the HiSeq 2500 instrument (Genome Analytics at the Leibniz Institute DSMZ-German collection of Microorganisms and Cell Cultures) using TruSeq SBS Kit v3 - HS (Illumina, New York, USA) for 50 cycles. Image analysis and base calling were performed using the Illumina pipeline v 1.8. For data evaluation, overrepresented and underrepresented genes were defined as genes that showed significantly different expression in the three biological replicates employing the statistical test DEseq for differential expression based on a model using the negative binomial distribution (37); adjusted *p*-value < 0.01 and a transcriptional difference which was at least two-fold: $\log_2$FC>1 for overrepresented genes and $\log_2$FC<−1 for underrepresented genes, fold-change (FC) stands for 10°C sample / 37°C sample.

The qRT-PCR analysis was carried out as described before (38) on BioRad MyIQ real-time PCR detection system in 96-well plates (AB-1900/KI, ThermoFisher Scientific, Dreieich, Germany). Primers are shown in the supplement, tableS1.

2.9 Extraction and analysis of secondary metabolites

Secondary metabolites were extracted as described in Valiante *et al.* (39) with slight modifications. SMs were extracted from the growth medium with ethyl acetate containing 5% (v/v) MeOH. The dried extract was resuspended in 1ml of LC-MS grade methanol or in water, respectively, and filtered through a 0.2 µm PTFE (Carl Roth, Karlsruhe, Germany) (for methanol-extract) or nylon (Whatman Puradisc Syringe Filters, Nylon, 25 mm, 0.2 µm GE Healthcare, Illinoin, US) (for H_2O extract) filter. Then, 20 µl samples were analysed by LC-

MS/MS using the LC-system UltiMate 3000 binary RSLC with photo diode array detector (Thermo Fisher Scientific, Dreieich, Germany) combined with an LTQ XL Linear Ion Trap mass spectrometer (ThermoFisher Scientific, Dreieich, Germany) as described before (40). For high-resolution mass spectrometry data, a Q-Exactive Plus Orbitrap high-performance Benchtop LC-MS/MS with an electrospray ion source and an Accela HPLC system (Thermo Fisher Scientific, Bremen, Germany) was used, equipped with a C18 column (Betasil C18 3 µm 150 × 2.1 mm).

3 Results

3.1 Temperature limit of growth of *A. nidulans*

For defining low temperature stress conditions, we characterised the temperature limits of growth of *A. nidulans*. Previous studies showed that *A. nidulans* is able to grow in a wide temperature range from 6°C to 47°C, with an optimum between 35°C to 37°C (41,42). Since the measurement of optical density does not allow accurate monitoring of mycelial growth, we used glucose consumption as an indirect indicator for mycelial growth. Figure 1 shows the glucose consumption rate at four different temperatures. 37°C reflects the optimal growth temperature and 12, 11 or 10°C are close to the described minimal temperature allowing growth. At 10°C the slowest glucose consumption was observed in comparison to 11 and 12°C. Thus, a temperature of 10°C was used to induce low temperature stress in *A. nidulans*. At 10°C, *A. nidulans* requires three to four weeks to generate biomass in sufficient amounts for the following large-scale analysis of the low temperature stress response. During the incubation time, about 30 mmol/l glucose were consumed. Lower temperatures than 10°C were not applied due to extremely long incubation times and the resulting issue of an increased risk of microbial contaminations of the cultures.

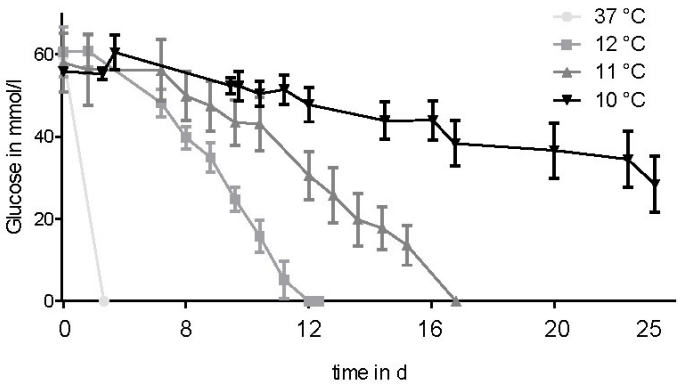

Figure 1: Glucose consumption of *A. nidulans* at 37°C (light grey circles), 12°C (grey squares s), 11°C (dark grey triangles) and 10°C (black, down-pointing triangles). The glucose concentration is given in mmol/l and was determined in the supernatant of *A. nidulans* cultures.

3.2 Global analysis of the adaptation of *A. nidulans* to low temperature stress by transcriptomics and proteomics

In order to investigate the molecular changes at 10°C, omics analysis were conducted. In the following, the transcripts or proteins found in the analysis are designated as identifiers (IDs). To calculate the statistical significance of the results, DEseq analysis was employed for the transcriptome analysis, and Wilcoxon/-Mann-Whitney analysis for the gel-based proteome analysis. The fold changes between the conditions 10°C and 37°C were calculated as a ratio of the abundance of proteins or transcripts ($abundance_{10°C}$ / $abundance_{37°C}$). For the transcriptome and gel-based proteome analysis, a threshold of 2-fold change ($log_2FC > 1$ = overrepresented, $log_2FC < -1$ = underrepresented) and for the gel-free proteomic approach a threshold of 1.5-fold change ($log_2FC > 0.585$ = overrepresented, $log_2FC < -0.585$ = underrepresented) was applied. The transcriptome analysis based on RNA-seq revealed 1715 statistically significant differentially abundant transcripts at 10°C in comparison to 37°C (supplementary Table S2). 651 transcripts were underrepresented, while 1064 were overrepresented. The proteome analysis included a gel-free and a 2D gel-based approach. In the gel-free approach, in total, 3896 proteins were detected (supplementary Table S3). 539 proteins were underrepresented and 691 overrepresented at 10°C. In the gel-based approach

(Figure 2), a total of 671 significantly differentially regulated protein spots were detected (supplementary Table S4), with 132 over- and 91 underrepresented proteins, and 256 different proteins identified by MALDI-TOF/TOF and LC-MS/MS (one protein spot can contain more than one protein). The overlap between the proteome and transcriptome data was 12.8% (351 IDs). 144 IDs were found to be overrepresented both on the proteome and transcript level, while 152 IDs were underrepresented in both analyses (Figure 3).

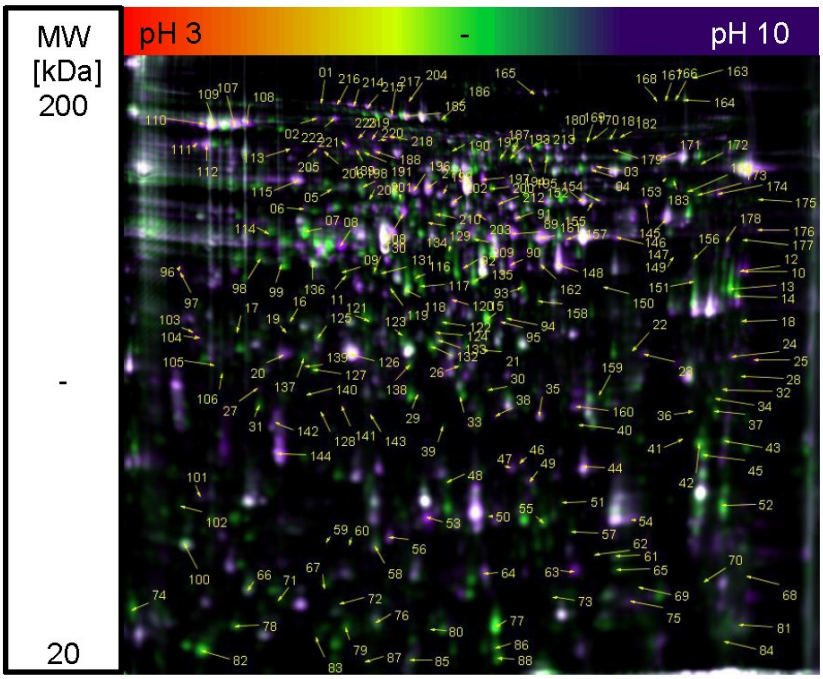

Figure 2: Representative overlay image of the 2D-DIGE analysis of the proteome of *A. nidulans* at 10°C (pink) *vs.* 37°C (green) incubation temperature of *A. nidulans*. The proteins were separated in two dimensions: on the x-axis according to their molecular weight and on the y-axis according to their isoelectric point (pI) (left: acidic proteins, low pI and right: basic proteins, high pI). Pink-coloured spots indicate overrepresented proteins at 10°C, green-coloured spots are underrepresented proteins at 10°C. The yellow labels indicate protein spots with a different ratio of abundance (normalised spot-intensity$_{10°C}$/ normalised spot-intensity$_{37°C}$) of at least 2-fold.

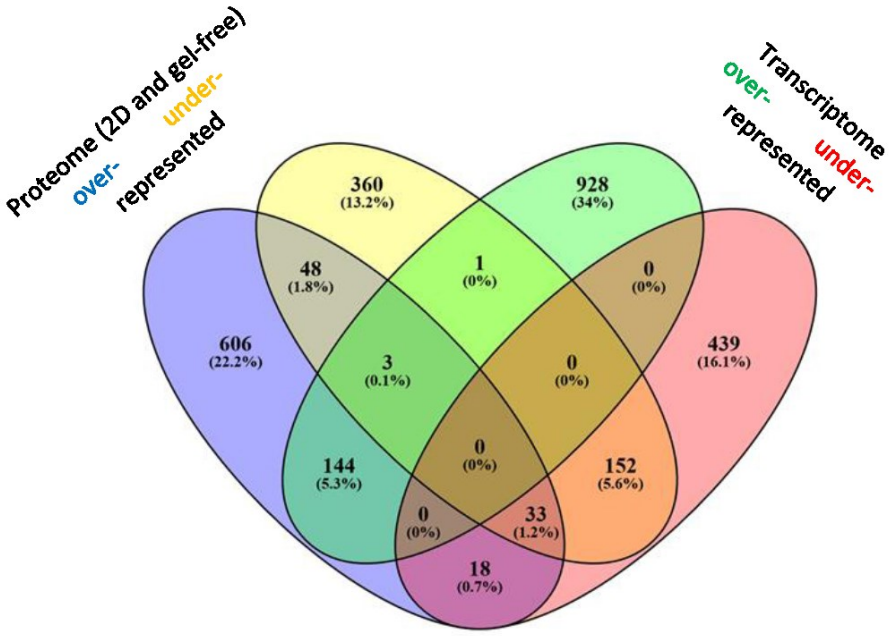

Figure 3: Venn-Diagram (43) showing the overlap of proteome- and transcriptome data. The blue and yellow colour indicate the over- and underrepresented proteins, the light green and light red colour the over- and underrepresented transcripts, respectively. 606 proteins were overrepresented in the proteome and 928 transcripts in the transcriptome. In contrast, 360 proteins were underrepresented in the proteome and 439 transcripts were underrepresented in the transcriptome at low temperature stress. The total overlap of proteome and transcriptome data was 12.8% which included 351 different IDs.

The 10 strongest up- and down-regulated IDs on the transcript and protein level (gel-free and gel-based) are listed in Table 1. Among others, IDs with functions in cell development (*e.g.* the kinase Stk19 with a role in ascospore formation and sexual reproduction (44), table 1b), biosynthesis of SMs (*e.g.* the transcripts AN1029, AN1031 and AN1032 of the asperfuranone biosynthesis gene cluster, Table 1a) or stress responses (*e.g.* peroxiredoxin AN10223 and co-chaperone AN5602) showed increased abundance at 10°C.

Table 1: Top 10 proteins and transcripts with the highest and lowest ratio (abundance$_{10°C}$ / abundance$_{37°C}$) of the transcriptome, gel-free proteome or gel-based (2D-DIGE) proteome analysis. Log2FC indicates log_2-fold changes (10°C/37°C).

a) Transcriptome analysis top 10 over- and underrepresented IDs

log_2FC 10°C/37°C	Accession number	ID Description
12.96	AN1031	Putative efflux pump; involved in asperfuranone biosynthesis
12.49	AN1029	Protein with homology to CtnR, citrinin biosynthesis transcriptional activator
12.2	AN3574	Protein of unknown function
12.04	AN2471	Ortholog of *A. niger* CBS 513.88
11.86	AN9002	Predicted monooxygenase; predicted SM gene cluster member
11.61	AN11157	Ortholog of *A. oryzae* RIB40: AO090026000764
11.49	AN8060	Has domain(s) with predicted NAD binding, oxidoreductase activity
11.19	AN5409	Has domain(s) with predicted catalytic activity
10.96	AN3388	Putative α-amylase with a predicted role in starch metabolism
10.77	AN1032	Putative oxidoreductase; required for asperfuranone biosynthesis
-8.01	AN3482	Protein of unknown function
-8.04	AN12330	Putative polyketide synthase (PKS)-like enzyme
-8.08	AN8135	Ortholog of *Aspergillus* versicolor: Aspve1_0089071
-8.17	AN1549	Ortholog of *A. fumigatus* Af293: Afu8g05600
-8.42	AN4148	Putative xylose transporter; transcriptionally induced by growth on xylose
-8.49	AN7053	Ortholog of *Aspergillus* versicolor: Aspve1_0054863
-8.82	AN10296	Ortholog(s) have fumarate reductase (NADH) activity
-9.02	AN7214	Ortholog of *A. nidulans* FGSC A4: AN8162
-9.13	AN4127	Ortholog(s) have intracellular localization
-9.37	AN3481	Ortholog(s) have carbohydrate binding activity

b) Gel-free proteome analysis top 10 over- and underrepresented IDs

log_2FC 10°C/37°C	Accession number	ID Description
3.59	AN2122	Has domain(s) with predicted DNA binding
2.76	AN5759	Ortholog(s) have role in ascospore formation
2.68	AN8339	Transcript induced by light in in developmentally competent mycelia
2.55	AN11721	Ortholog(s) have 5'-flap endonuclease activity
2.52	AN3123	Ortholog(s) have role in chromatin silencing at centromere
2.51	AN5090	Predicted ADP ribosylation factor (Arf) GTPase
2.44	AN11062	Ortholog(s) have role in mitochondrial genome maintenance
2.43	AN9002	Predicted monooxygenase; predicted SM gene cluster member
2.38	AN3386	Putative polyketide synthase; involved in SM production
2.37	AN6353	Ortholog of *A. fumigatus* Af293: Afu3g07750
-1.72	AN7208	SET domain protein
-1.72	AN6631	Putative F1F0-ATPase complex subunit

-1.73	AN1549	Ortholog of *A. fumigatus* Af293: Afu8g05600
-1.75	AN7169	fhbA: NirA-dependent flavohemoprotein
-1.78	AN5719	Ortholog(s) have structural constituent of ribosome activity
-1.82	AN5226	acpA; Acetate permease, involved in acetate uptake
-1.82	AN1074	Ortholog(s) have glycine dehydrogenase (decarboxylating) activity
-1.89	AN7444	Ortholog(s) have role in cellular response to cadmium ion detoxification
-1.93	AN1152	Protein expressed at decreased levels in a *hapX* mutant vs wild-type
-2.15	AN2702	Protein of unknown function

c) 2D-DIGE proteome analysis top 10 over- and underrepresented IDs

log_2FC 10°C/37°C	**Accession number**	**ID Description**
10.04	AN8216	Nucleoside diphosphate kinase
8.86	AN10223	TPA: putative 1-Cys peroxiredoxin
8.86	AN10223	TPA: putative 1-Cys peroxiredoxin
8.26	AN4159	Glutamine synthetase
7	AN0240	Protein of unknown function
6.18	AN2875	Protein of unknown function
5.78	AN5602	TPA: co-chaperone
5.51	AN8009	NMT1_ASPPA NMT1 protein homolog
5.51	AN6232	Vacuolar ATP synthase subunit B
4.82	AN7708	Ortholog(s) have alditol: $NADP^+$ 1-oxidoreductase activity
-2.69	AN0183	Putative molybdopterin binding domain protein
-2.69	AN0687	spdA: Putative spermine or spermidine synthase
-2.69	AN0870	Putative transporter with a predicted role in small molecule transport
-2.69	AN1162	Ortholog(s) have guanyl-nucleotide exchange factor activity
-2.69	AN2968	Putative inorganic diphosphatase with a predicted role in energy metabolism
-2.81	AN3172	Protein of unknown function
-2.81	AN8674	Protein of unknown function
-2.91	AN8041	Glyceraldehyde-3-phosphate dehydrogenase
-2.91	AN10170	TPA: GTP-binding protein YchF
-3.2	AN10079	ureB: Urease, involved in utilization of urea as a nitrogen source

51 transcription factors (TFs) were found to be differentially regulated at 10°C. An extract of selected examples is mentioned in the following (table S5). Many of the differentially regulated TFs are involved in stress response regulation, SM biosynthesis or cell development and are discussed in more detail below.

IDs from the proteome and transcriptome approach were combined for further data analysis. According to the FunCat classification implemented in FungiFun web tool (https://elbe.hki-jena.de/fungi-fun/fungifun.php), the enrichment analysis of transcripts and proteins with significant changes in abundance identified 14 major categories, including 207 sub-categories. The major categories are listed in Figure 4a. The category "metabolism" represented the highest number of over- and underrepresented IDs suggesting metabolic adaptation during low temperature stress. The second largest group was the category "Protein with binding function or cofactor requirement".

For a more detailed insight, we also considered subcategories shown in Figure 4b; the complete table can be found in the supplement (Table S6). The subcategories contained several IDs (in total 269) with a function in "cell development". Many differentially regulated transcripts at 10°C were assigned to the induction of the sexual cycle in *A. nidulans* as reported from previous studies, such as the gene *bxlC* (overrepresented in the transcriptome with a 4.88 $\log_2$FC), which encodes a putative β-1,4-xylosidase; *plyH* (overrepresented in the transcriptome with a 5.08 $\log_2$FC), which encodes a protein with pectate lyase activity; and *mutA* (overrepresented in the transcriptome with a 3.63 $\log_2$FC), an α-1,3-glucanase gene that is specifically expressed in Hülle cells (a nondeciduous chlamydospore-like structure that surrounds cleistothecia) (45,46).

Of the 3432 IDs with differential abundance, 1988 IDs (58%) were annotated, while 1444 IDs (42%) represented hypothetical genes/proteins. In order to obtain some information about non-annotated IDs, further bioinformatic analyses were conducted by a functional enrichment analysis of all IDs based on the GO terms (Figure 4c), KEGG pathways (Figure 4d) and functional domain analysis based on the Pfam database (Figure 4e). The GO classification resulted in 34 enriched categories sorted by biological activity, the KEGG enrichment resulted in 16 enriched KEGG pathways and the Pfam domain search in 56 enriched Pfam domains (Table S5).

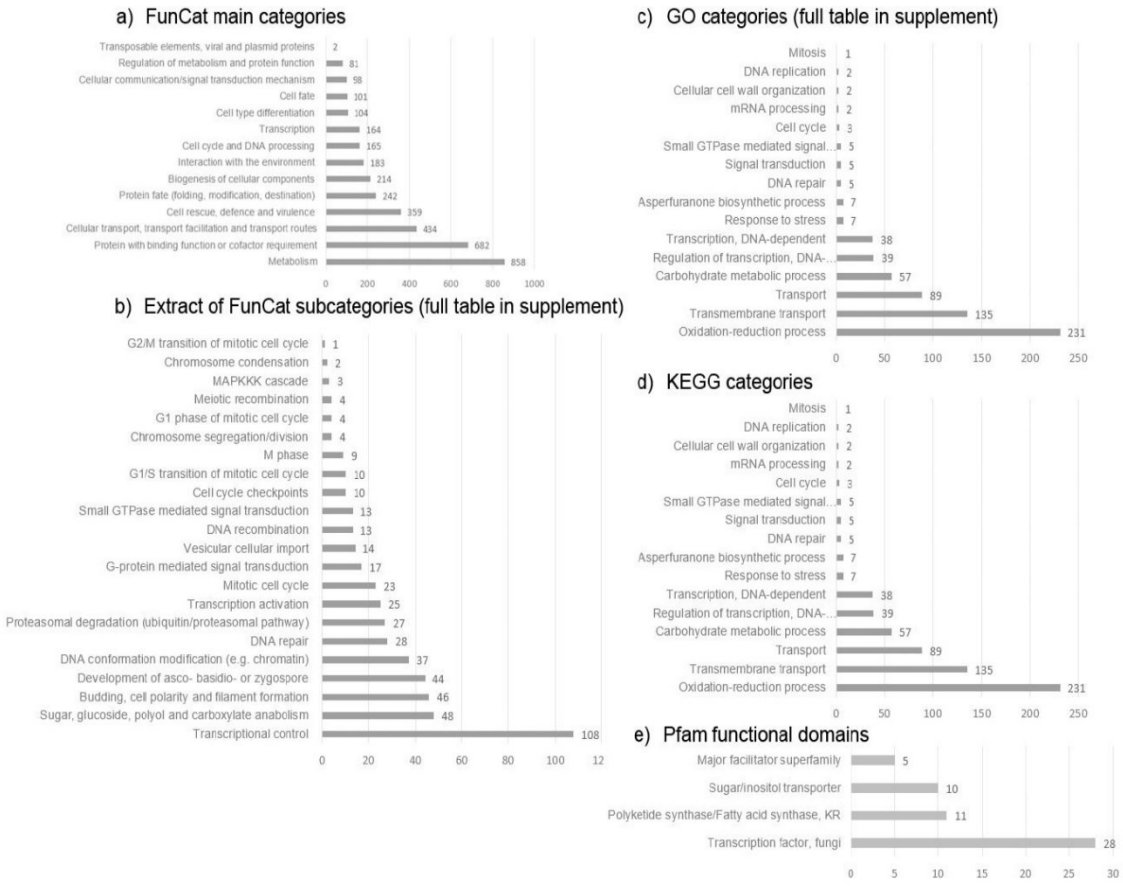

Figure 4: Functional category analysis of the transcriptomic and proteomic analysis with the FungiFun package (https://elbe.hki-jena.de/FungiFun/FungiFun.cgi, default settings: Species *A. nidulans* FGSC A4, classification ontology FunCat or GO as indicated in the figure, *p*-value ≤ 0.05), over- and underrepresented IDs were combined.

In general, the gene set enrichment analysis results were consistent with the FungiFun data, IDs in the categories “cell development” (GO: “Cell cycle”, “DNA replication”, “Mitosis”), “biosynthesis of SMs” (GO: “Asperfuranone and aflatoxin biosynthetic process”, KEGG: “Biosynthesis of SMs”, Pfam: “Polyketide synthase/Fatty acid synthase, KR”) and stress response (GO: “Oxidation-reduction process”, “Response to stress”, “DNA repair”) were enriched.

Since quite similar categories showed up in all enrichments analyses, three main categories most highly relevant for low temperature stress adaptation were defined as “cold protection”, “biosynthesis of SMs”, and “cell development.” Subcategories of these main categories are listed in the supplement Table S7. The category “cold protection” contained many IDs, which may directly contribute to a protection of *A. nidulans* against low temperatures. The category “biosynthesis of SMs” indicates that low temperature influences SM production. The enriched number of IDs regulating cell development implies that cold temperature exposure has an effect on the asexual and sexual development of *A. nidulans*. Protein and genes of these three categories are described below in more detail.

In the following, we selectively discuss responses which were found at all investigated levels, the transcriptome, proteome, and secondary metabolome.

3.3 Altered proteins and transcripts involved in cold protection of *A. nidulans*

The category “temperature reception and response” was overrepresented at 10°C as well as IDs in the categories of “DNA repair” or “DNA damage response”. IDs such as AN6114 (overrepresented in the transcriptome with a 4.34 log_2FC) were enriched, which have a predicted DNA-directed DNA polymerase activity. Along with this, genes/proteins of the category “protein fate (including protein folding and stabilization, modification and destination)” and RNA binding proteins were overrepresented at 10°C. These categories consist of various (19 in total) heat-shock proteins (HSPs) (Hsp20, Hsp30, Hsp70, Hsp78, Hsp90 and Hsp104), chaperones, and glycine-rich RNA binding proteins. Hsp30 and Hsp90 are regulated in response to pH sensing and AN5602 is presumably a co-chaperone, which has a chaperone binding activity in response to heat. In addition, IDs which support the oxidation-reduction process, oxidoreductase activity, cell rescue, defence and virulence, detoxification, cold shock response, and ER quality control were more abundant at low temperature stress. Stress response (GO: “Response to stress”) IDs such as the transcription factor *CrzA*, which is involved in cation homeostasis and the osmotic stress response (in the proteome with a 0.62 log_2FC) were found. Further IDs such as AN5602 (underrepresented in the transcriptome with

a -3.85 log_2FC and overrepresented in the proteome with a 54.88 log_2FC), and AN3508 or AN3509 (both overrepresented in the transcriptome with a >12 log_2FC) were enriched. AN3508 or AN3509 have similarities to RTA1 like proteins with a predicted role in response to stress and putative function as a fungal lipid-translocating exporter.

After the induction of low temperature stress, several cellular transporters including sugar transporters and IDs of the trehalose pathway were overrepresented: Trehalose functions as a storage carbohydrate, but also as stress metabolite. Levels of the putative trehalose-6-phosphate synthase AN8639, which is predicted to play a role in trehalose biosynthesis and the putative trehalose transmembrane transporter AN3515 increased at 10°C on transcript level. A complete list of the sugar metabolism-related IDs including the ratios can be found in Table S8. Further on, the bZIP transcriptional regulators AN6849 (overrepresented in the transcriptome with a 4.91 log_2F) and AN8643 (overrepresented in the transcriptome with a 5.80 log_2F) show both sequence similarities to the ATF/CREB family regulator AtfB of *A. oryzae* and are therefore putatively involved in the cellular response to heat and hydrogen peroxide (47).

Apart from stabilising the membrane with natural cryoprotectants such as trehalose, the increase in proportion of unsaturated to saturated fatty acids in the cellular membrane is a wide-spread adaptive response observed among microorganisms (48). Thus a re-organisation of the cell membrane often takes place to maintain fluidity of the membrane (49). In our samples, this is reflected by the increased production of IDs involved in unsaturated fatty acid biosynthesis. Apart from biosynthetic process, fatty acid biosynthetic and metabolic process at low temperature stress like the enzymes SdeA or SdeB, which are both putative desaturases with essential function, were enriched (50). Interestingly, desaturases found in these analyses like *sdeA, sdeB,* and further putative desaturases as well as IDs with a predicted role in sterol metabolism were underrepresented at 10°C. Further examples of the category “fatty acid biosynthesis” are the IDs AN6450 (involvement in melanin biosynthesis) and *basA* (AN0640). *basA* plays a role in sphingolipid metabolism, altering *psi* factors. Together with *ppoC* and *ppoB,* the regulation of asexual and sexual development is influenced. A complete list of

enriched IDs in the category of fatty acid biosynthesis can be found in the supplement in Table S8.

3.4 Low temperature stress triggers changes of proteins and transcripts in cell development

At 10°C, many IDs linked to cell development were overrepresented. Categories directly linked to cell development like e.g. “fruiting body development” and also categories without a direct connection such as stress response (GO: “Response to stress”) or the “fatty acid biosynthesis” (for instance the ID *basA*) include IDs concerning cell development. Example IDs in the category of stress response are *ppoC* (underrepresented in the transcriptome with a -4.82 log_2FC, and overrepresented in the proteome with a 4.85 log_2FC) and *ppoB* (underrepresented in the transcriptome with a -3.85 log_2FC). *ppoC* is a fatty acid oxygenase which catalyses the formation of the psi-factor component psiB-*β* and *ppoB* is involved in oxylipin and SM biosynthesis and favours conidiation. A representing example in the category “fruiting body development” is the cold-enriched protein CsnB (overrepresented in the proteome with a 0.63 log_2FC), which is a subunit of the COP9 signalosome and required for cleistothecia formation. Moreover, the kinase Stk19 (AN5759) (overrepresented in the proteome with a 2.76 log_2FC) that has a putative role in ascospore formation was found to be induced. Further categories connected to cell type differentiation were also enriched. Dyer *et al.* published a list of known genes which are involved in sexual reproduction in *Aspergilli* (51). After comparing our datasets with the sexual activators and repressors in this review, we found that following activators were overrepresented in our transcriptome or proteome data: AN2290, *steA*, a transcription factor involved in the mating processes and signal transduction and AN4783, *csnB*, COP9 signalosome subunit which is a regulatory protein of sexual development. AN5674, *STE20*, a MAP kinase kinase kinase kinase (MAPKKKK) which is part of the mating processes and signal transduction in which a gene deletion would lead to an incomplete sexual development were also overrepresented. The repressor AN7169, *fhbA* (*fhbB*) was underrepresented, a nitrate response gene, involved in perception of environmental signals. Further on, many transcription

factors were enriched, belonging to the supercategory of cell development. Orthologues of AN0917 (underrepresented in the proteome with a -1.79 log_2FC) play a role in ascospore formation. *FlbD* (underrepresented in the transcriptome with a -2.49 log_2FC) is a putative TF which is involved in the asexual development (asexual spore production), sexual development (development of a peridium, a protective layer that surrounds the ascogenous system) and in the nitrogen response. The central regulators of asexual sporulation BlrA (regulates the conidiophore development), AbaA (required for phialide differentiation and therefore is essential for asexual development), and WetA (a regulatory protein involved in conidial development, which activates conidium-specific gene expression) were not significantly regulated at 10°C in comparison to 37°C. Several IDs in all three supercategories contain IDs which are positively involved in sexual reproduction and show connections especially to ascospore formation and Hülle cell production.

Besides changes on the transcript and protein level, the fungal cultures at 10°C showed morphological differences in comparison to the 37°C cultures. Fungal cells cultivated at 37°C solely consisted of hyphae that formed the hyphal mass, while the 10°C cultures showed both hyphal and spherical structures (Figure 5). The spherical structures resembled Hülle cells, which are specific for fruiting body development (52,53). We found some IDs involved in the regulation of Hülle cell production (46). The peroxidase CpeA (overrepresented in the transcriptome with a 3.03 log_2FC and proteome with a 1.12 log_2FC), which is expressed during sexual development and the mutanase MutA (overrepresented in the transcriptome with a 3.63 log_2FC), being specifically expressed in Hülle cells. In order to confirm the induction of the sexual development in *A. nidulans* at 10°C, selected genes indicative for the sexual and asexual development were investigated by qRT-PCR. The regulators *brlA* (AN0973), *abaA* (AN0422), *wetA* (AN1937), and the putative glucanase AN3883 were chosen as genes specific for asexual development (conidiation), while the putative β-1,4-xylosidase *bxlC* (AN1477), the pectate lyase *plyH* (AN8453) and the α-1,3-glucanase *mutA* (AN7349) represented genes specific for sexual development (51). Moreover, the expression of *flbD* (AN0279) was

determined. FldB is a putative transcription factor involved in the regulation of both the asexual and sexual development in response to nitrogen starvation and is also overrepresented during conidiation. AN3883 is a putative glucanase, which might be involved in cell wall hydrolysis. It is formed during the early stages of asexual reproduction, but not during sexual reproduction. BxlC is involved in the cell wall degradation in the later stages of sexual development. PlyH is a pectin hydrolysing enzyme, which is specific for sexual development and already produced in the early stage of sexual development. MutA is specifically formed in Hülle cells and its gene is highly expressed during sexual development (54).

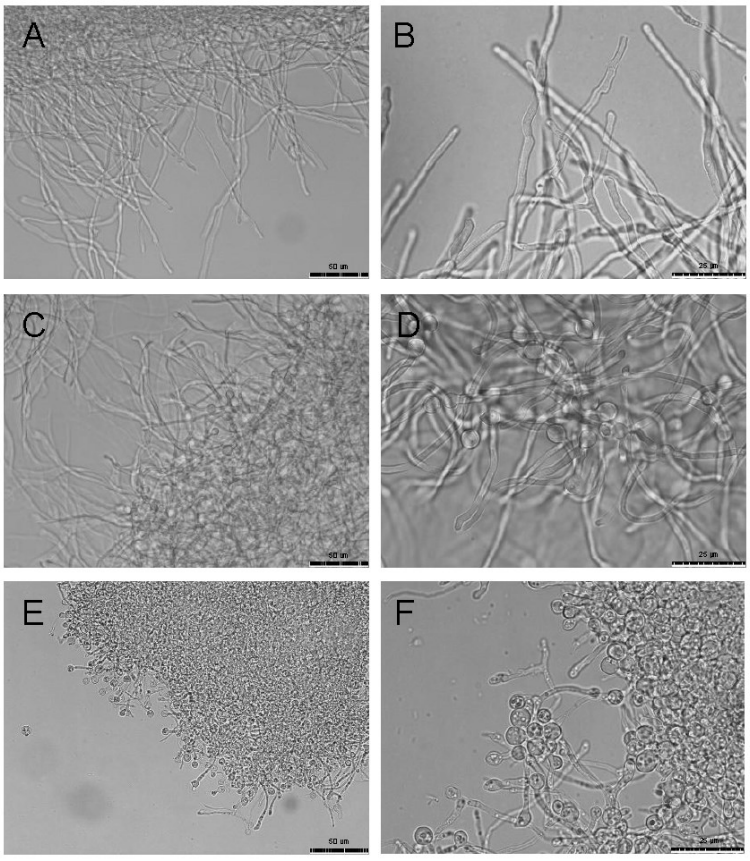

Figure 5: Microscopic image of *A. nidulans* with a 400x magnification (left) and 1000x magnification (right). The mycelia in A and B were incubated at 37°C for 18h, C and D for 8h at 37°C and switched to 10°C for 12h, E and F for 8h at 37°C and subsequently at 10°C for 21 days. The bar in the bottom right corner indicates 50 µm for the 400x magnification and 25µm for the 1000× magnification. In A and B, long hyphal filaments are shown, in C – F spherical Hülle became apparent.

In the qRT-PCR analysis, the measurement of the asexual production-specific genes did not lead to an inconclusive result. *BrlA* was significantly overrepresented at 37°C and underrepresented at 10°C, while *abaA* was slightly overrepresented at 10°C in comparison to 37°C. The expression of *wetA* showed no significant difference between the 10°C and 37°C samples. The glucanase AN3883 was similarly highly expressed in comparison to the actin control gene at 37°C and underrepresented at 10°C. In contrast, the sexual cycle-specific genes showed a clear profile for an induction of the sexual development at 10°C. *FlbD* was underrepresented in the 10°C sample and reached transcript level similar to the control gene actin at 37°C. *bxlC* and *plyH* were highly overrepresented at 10°C. The expression of the *mutA* gene was highly overrepresented at 10°C, but not at 37°C (Figure 6 a and b). In summary, qRT-PCR confirms change in the expression of genes associated with sexual development in *A. nidulans*.

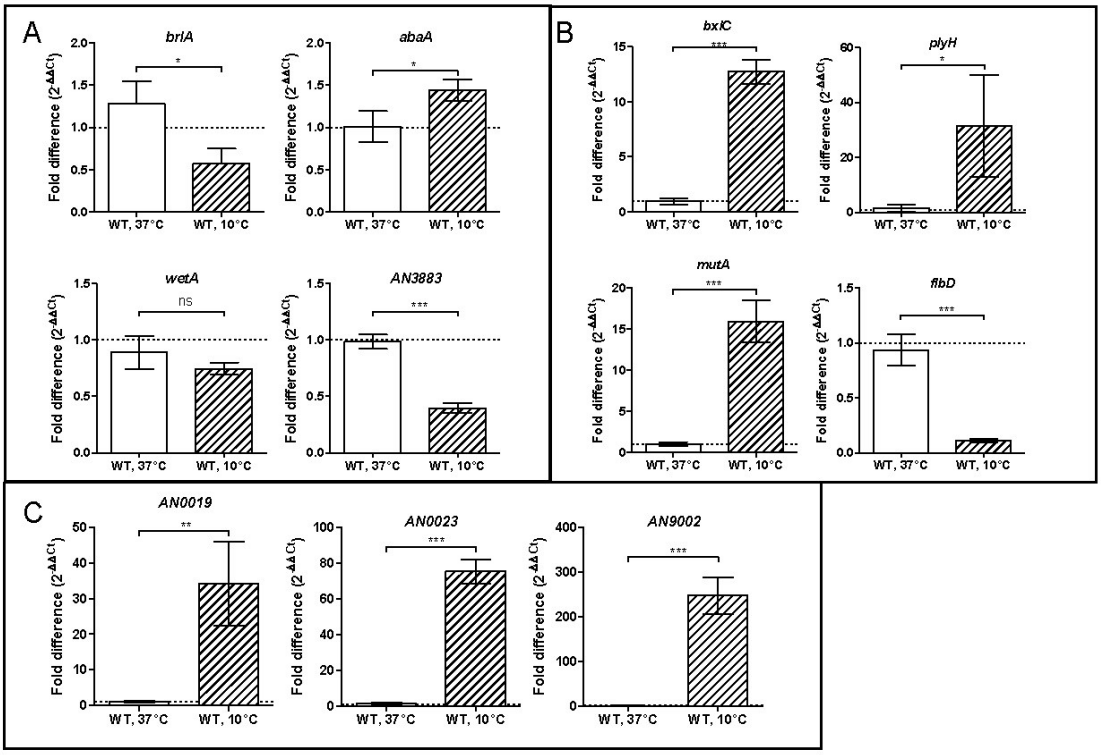

Figure 6: qRT-PCR-analysis of genes involved in asexual development (A), sexual development (B) and genes specific for the biosynthesis of SMs (C). In each graph, gene expression of *A. nidulans* at 37°C and 10°C is plotted. The y-axis shows the fold difference of

the expressed mRNA level, normalised to the *actA* gene. According to Student's t-test, 0.01< p <0.05 is indicated with *, 0.001<p<0.01 with **, p<0.001 with *** and p>0.05 with ns. *BrlA* is significantly overrepresented at 37°C, *abaA* is significantly underrepresented at 37°C, *wetA* shows no significant difference between 10°C and 37°C and AN3883 is significantly overrepresented at 37°C. *BxlC*, *plyH*, and *mutA* are significantly underrepresented at 37°C, *flbD* is significantly overrepresented at 37°C. AN0019, AN0023, and AN9002 are significantly underrepresented at 37°C.

3.5 Biosynthesis of secondary metabolites upon low temperature induction

Upon low temperature stress, the activity of SM genes and proteins changed. IDs in several secondary metabolite biosynthetic processes were overrepresented at 10°C. In total, 230 IDs of predicted or known SM gene clusters were significantly differentially regulated, of which 174 IDs were overrepresented. A full list of differentially regulated SM IDs can be found in table S9. In the asperfuranone cluster, most IDs were found to be differentially regulated at 10°C (8 IDs upregulated). Furthermore, a representative example for overrepresented, enriched IDs is the gene *afoA* (overrepresented in the transcriptome with a 12.49 $\log_2$F) which encodes a known regulator of the asperfuranone biosynthesis. Additionally, the putative efflux pump AfoB (overrepresented in the proteome with a 1.22 $\log_2$FC and transcriptome with a 12.96 $\log_2$FC), which is putatively involved in asperfuranone biosynthesis, was enriched. Moreover, asperthecin (5 IDs), emericellamide (4 IDs), *o*-orsellenic acid (3 IDs), and further predicted biosynthetic processes were detected in the supernatant of the 10°C samples. Genes or proteins of the aspyridone (2IDs), austinol (2 IDs), monodictyhphenone (6 IDs), microperfuranone (2 IDs), nidulanin A (3 IDs), penicillin (2 IDs), sterigmatocystin (7 IDs) and terriquinone (3 IDs) biosynthetic processes were also differentially regulated at 10°C but not detected in the supernatant.

The categories "generation of precursor metabolites and energy" and "regulation of secondary metabolite biosynthetic process" were overrepresented after the induction of low temperature stress. This data was confirmed for the uncharacterised generic non-ribosomal peptide

synthetase (NRPS) and polyketide synthase (PKS) IDs AN0019, AN0023, and AN9002 by qRT-PCR (figure 6c). For the cluster with the predicted central biosynthesis gene AN0016, 9 of 16 IDs were differentially regulated and for the predicted central biosynthesis gene AN9005, 5 out of 12 IDs. IDs with predicted regulatory functions of the gene clusters pkdA, pkg, and the AN3252 cluster were also enriched. IDs AN0533 (overrepresented in the transcriptome with a 7.05 log_2FC), AN7073 (overrepresented in the transcriptome with >20 log_2FC), *sndA* (underrepresented in the proteome with a 0.83 log_2FC), AN3255 (overrepresented in the transcriptome with a 3.45 log_2FC), AN2025 (overrepresented in the transcriptome with an 8.65 log_2F) have a predicted role in SM production and the putative TF AN8645 (overrepresented in the transcriptome with a 3.20 log_2FC), whose orthologues have a predicted role in aflatoxin biosynthesis.

Table 2: Identified overexpressed SM genes and proteins with positive detection of respective SMs in the supernatant of *A. nidulans* at 10°C. The names of the SM biosynthetic gene clusters are given. The middle column indicates the number of proteins detected for a respective gene cluster, and in the last row, the respective genes from the transcriptomics data. In the last line, a total of 29 predicted SM gene clusters contained 42 overrepresented proteins and 57 transcripts were overrepresented.

Cluster name	Proteins	Genes
Asperfuranone	7	8
Orsellinic acid	2	3
Emericellamide	4	0
Asperthecin	6	2
Triacetylfusarinine	1	0
Predicted SM clusters (29)	42	57

These data suggested that several SMs were only produced at 10°C but not at 37°C. Therefore, the SM profile of supernatants of the *A. nidulans* cultures grown at 10°C and 37°C was investigated (Table 2). The SM signals were compared by their retention time and identified with their exact *m*/*z* by mass spectrometry. The 10°C samples showed more SM signals than the 37°C samples. The resulting masses were matched to a library of already known SMs from *A. nidulans* (table 3). Beside already known SMs, further masses indicated the existence of unidentified SMs produced at 10°C. Metabolites extracted from the supernatants of these cultures (10°C) inhibited Gram-positive bacteria and fungi in agar diffusion assays (Figure 7). In contrast, the 37°C supernatants did not show similar inhibitory effects.

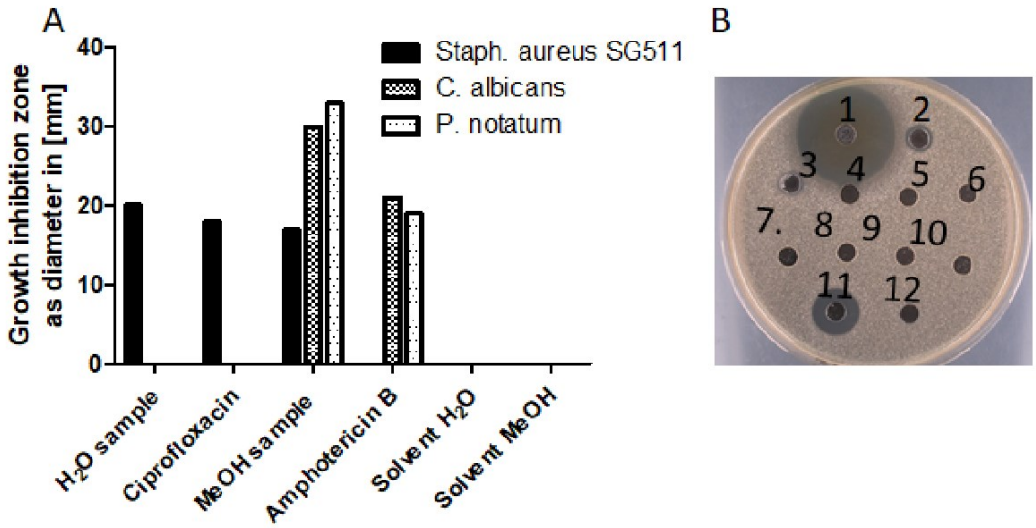

Figure 7: (A) Biological activity measurement based on agar diffusion assay. The different microorganisms which showed an inhibition in growth are shown on the x-axis (*Staphylococcus aureus SG511*, *Candida albicans* and *Penicillium notatum*). The growth inhibition zone is displayed as diameter in mm on the y-axis. (B) Agar diffusion assay with fractions of the culture supernatants of *A. nidulans* after incubation at 10°C. Numbers 1 to 10 indicate the different samples which were fractionated (Fraction A – J, respectively), number 11 represents the positive- (Amphotericin B) and number 12 the negative- (solvent MeOH) control.

4 Discussion

Although highly studied for their roles in the environment and the clinic, little is known about the response of filamentous fungi to low temperature stress. Different molecular mechanisms to survive low temperatures have been described, but how they contribute to the survival of filamentous fungi under low temperature stress are largely unknown (55). In a field study, soil fungi showed a better adaptation towards cold than soil bacteria (56). Understanding the underlying mechanisms of low temperature adaptation in *A. nidulans* and other fungi will contribute to explaining how the soil microbial community is shaped by temperature. Further, it will help to explore organisms adapted to low temperatures.

To gain detailed insights into the low temperature adaptation of *A. nidulans,* we analysed the changes of the transcriptome, proteome, and secondary metabolome during growth at 10 and 37°C. Interestingly, a temperature change of 1°C (from 11°C to 10°C) extended the incubation time needed to reach a similar amount of biomass by several days (Figure 1). This suggests that the fungus approaches its temperature limit at around 10°C. At this temperature, all cellular processes are far beyond their temperature optimum (9,57,58). Therefore, this condition resembles the transition to winter time in temperate regions (59). Previous studies showed that temperature drops were linked to decreased enzyme activity (60) as well as increased oxidative (61,62) and osmotic stress (63,64). Gocheva *et al.* observed typical signs of oxidative stress response in fungi belonging to the genus *Penicillium* after reducing the temperature from the optimal growth temperature to 15 or 6°C. Apart from a significant reduction of biomass production, increased levels of oxidative damage of proteins and an accumulation of storage carbohydrates were measured (carbohydrates such as trehalose can function as natural cryoprotectant) (61). In order to reduce oxidative damage of proteins, cells induce antioxidative enzymes for scavenging reactive oxygen species. Among others, we observed a simultaneous induction of these cellular responses to low temperature stress. The connection between oxidative and osmotic stress can be illustrated by the gene *prxA*. As a thioredoxin peroxidase, the gene was found to be induced by oxidative stress (36) and osmoadaptation (65).

Additionally, we found further effects apart from a general stress response to cold. Firstly, we identified several SMs which were only produced at 10°C. Secondly, we observed a morphological change from asexual to sexual development in *A. nidulans*.

The adaptation to low temperatures requires a sensing of the temperature change (66). There are many mechanisms known for temperature perception (66) such as the unfolded-protein response (UPR) mediated by ER stress sensor proteins (67,68) or the synthesis of cold shock proteins (69). The synthesis of RNA thermometers, predominantly found in prokaryotes, can regulate the access to mRNA binding sites and therefore facilitate the translation (70).

The omics analysis revealed a putative glycine-rich RNA-binding protein (GRP, AN2989, overrepresented with a 1.75 log_2FC in proteome data), which shows similarities to the cold induced RNA chaperone Crp2 of the entomopathogenic fungus *Metarhizium anisopliae* (60.7% of identical amino acids), facilitating the RNA translation at low temperatures (71).

Altogether, many cold-shock induced IDs represent heat-shock proteins, chaperones or GRPs (19), which were differentially regulated. However, *bipA*, a UPR target gene resident in the endoplasmic reticulum (ER), was not found to be overrepresented. Moreover, IDs localised in the ER were enriched, although their function does not suggest an involvement in the UPR. However, Hsp20 (AN10507), which exerts an UPR activity and is activated in response to heat stress (72), was found to be underrepresented at 10°C. This finding indicates that UPR as a temperature perception mechanism may rather be activated under heat stress than at low temperatures in *A. nidulans*. Different variants of Hsp90 (*e.g.* AN8269) and Hsp70 (*e.g. sgdE*, AN6010) were found to be enriched at low temperature stress. The Hsp90 chaperone is essential for growth at high temperatures (73) and Hsp70 is expressed at increased levels during osmoadaptation. Another highly conserved heat shock response gene is HSR1 (Heat Shock RNA 1, orthologue in *A. nidulans* is AN5003, overrepresented 2.60 log_2FC in transcriptome data, statistical significance based on DEseq2), which induces several cytoprotective genes as a response to elevated temperature (74). These examples suggest other cellular tasks than UPR-mediated temperature perception of HSPs under low

temperature stress and highlight an increased response to osmotic stress and further cryoprotective mechanisms at 10°C. In yeast, cold-shock induces several HSPs. The Hsp12 and Hsp26 are up-regulated at temperatures between 10 and 18°C, Hsp12, Hsp42 and Hsp104 at temperatures below 10°C (75). Interestingly, besides a trehalose response, we also found Hsp104 being induced at low temperatures in *A. nidulans*. This suggests a similar stress response mechanism like in yeasts, where it is described to be necessary to co-ordinated induction of trehalose biosynthesis and specific stress proteins for surviving near-freezing temperatures.

Cold stress-induced damages can range from desiccation to osmotic stress (9). In line, the activation of the osmotic stress response of *A. nidulans* found in this study confirms the alteration of the osmotic shock tolerance during cold adaptation (64). The fungus seems to react to cold shock by increasing the abundance of proteins involved in the high-osmolarity glycerol (HOG) signalling pathway, such as *YpdA, PbsA, TcsB or HogA,* which are all involved in the regulation of the osmotic stress response (76–79). Further, TFs such as CrzA, which is known to be up-regulated under osmotic stress, were overrepresented at 10°C, too.

Low temperature stress also interferes with protein biosynthesis and transcription due to a disturbance of the protein structures and energy depletion. (80,81). The expression of chaperones only may not be sufficient to protect against damages resulted by low temperature stress. Accordingly, DNA repair mechanisms need to be activated (71,82,83). Overrepresented IDs are involved in DNA repair or in the DNA damage response including proteins such as the family X DNA polymerase AN6114, which is most probably involved in gap filling during base excision repair. Furthermore, orthologues of the co-chaperone AN5602 are known to be activated in response to DNA replication stress (84), while the helicase AN5514 facilitates DNA or RNA unwinding to initiate transcription and translation at low temperature (69,71,85,86). DNA damages may also result from a low temperature-induced accumulation of reactive oxygen species (ROS) (87). Gocheva *et al.* (61) also described an increase in superoxide dismutases, catalases, and ROS after a temperature-induced

antioxidant response in fungi. Similarly, we found IDs characteristic of an increased antioxidant response overrepresented (61). Among others, we found several known and putative catalases (*catA*, *catB*, *catC*, *cpeA*, AN5918, AN7388, AN8637 and AN8553) and IDs with oxidoreductase activity such as AN5276, as a putative monoamine oxidase, which is suggested to be related to the formation of ROS (88,89) (tableS6). In yeast, low temperature stress as well as oxidative stress induce genes implicated in detoxification processes, such as glutathione transferase, glutathione peroxidase isoforms, glutaredoxin and thioredoxin peroxidase (90). In this study, we found induction of glutathione transferases (*gstA, gst3*), a glutathione peroxidase (*gpxA*) and thioredoxin peroxidases (putative, AN8218 such as AN3581, *trxR* and *prxA*, which is osmoadaptation-induced). Hence, the regulation of the low temperature response may be similarly regulated as in yeast, in which the transcriptional activators Msn2p and Msn4p are involved in the cold-specific response. However, the homologue to the yeast transcriptional activator Msn2p, *msnA* in *A. nidulans,* was not found to be significantly overrepresented in our omics analysis.

Upon cold stress, in many microorganisms cryoprotectants are able to preserve the plasma membrane integrity (91), to maintain enzyme activity and to prevent freezing damages caused by temperatures below 4°C. Examples for cryoprotectants are anti-freezing proteins or compatible osmolytes like trehalose (92), which *A. nidulans* is able to produce (75). The pathway of trehalose biosynthesis (*e.g. tpsA* and AN8639) was increased after low temperature stress induction. Further, sugar transporters such as predicted trehalose transmembrane transporter (AN3515) and glucose transporter of the major facilitator superfamily (*mstE*) were up-regulated as well (Table S8). Thus, this fungus may use sugar entities as cryoprotectants, for a better survival of freezing stress.

Apart from sugars supporting the membrane structure, maintenance of the membrane flexibility is essential for many low temperature-adapted organisms (49). Usually, unsaturated fatty acids ensure the membrane fluidity. However, some examples demonstrate that unsaturated fatty acids are not necessarily needed for maintenance of membrane flexibility. In

Bacillus megaterium, Suutari and Laasko (93) describe a decrease in the amount of unsaturated fatty acids when lowering the incubation temperature towards 10°C. Nevertheless, the overall melting temperature of fatty acids decreased in *B. megaterium* at low temperatures and this may also hold true for *A. nidulans*. Further examples of the category fatty acid biosynthesis show that enriched IDs in this category do not invariably contribute to the fitness of the cells under low temperature stress, but also accomplish other important functions such as the regulation of cell development.

Our omics data also suggest a switch to sexual reproduction at low temperatures. Pöggeler *et al.* (94) described a temperature-influenced fruiting body formation in ascomycetes. Our data confirms that besides light, nutrient supplementation or other factors temperature has the capability to alter the developmental cycle in *A. nidulans*. Switching to sexual development under harsh conditions, such as cold, may represent a survival strategy since ascospores exhibit a higher stress resistance than conidia (95). They have a thicker cell wall, two nuclei, increased HSP levels, and elevated concentrations of sugars, like trehalose (51,95,96). In addition, fruiting bodies also have a higher resistance against fungivores than vegetative mycelia or asexual-derived spores (97). These changes became apparent not only on the molecular, but also on the macroscopic and microscopic level. After three to four weeks of growth at 10°C, the development of Hülle cells was observed.

In contrast, at 37°C asexual development occurs, which is reflected by expression of the major regulatory gene of conidiophore development, *brlA*. However, *abaA* is slightly overrepresented at 10°C. In opposite to the clear activation of sexual development, asexual development is neither clearly triggered nor suppressed. It should be further considered that the expression of development-specific genes change dynamically over time, as shown for *wetA* (98,99). Another reason for the unclear observation of asexual development on molecular level may be that mycelial cultures do not consist of a homogenously growing hyphal mass.

The induction of sexual development in *A. nidulans* is known to be regulated *via* the velvet gene *veA*. The R21 strain used in this work is a *veA*$^{-}$ strain carrying a truncated *veA* gene that

is non-functional, hence, the light-induced sexual development conferred by this gene cannot be activated. Interestingly, the fungus is still able to induce the first steps of sexual development, which implies the existence of additional regulators initiating sexual development. Sexual development is known to be triggered by nitrogen starvation, darkness, and low oxygen levels. In this study, standard growth conditions (day-night-cycle, no starvation conditions, no oxygen limitation) were applied, which do not explicitly favour sexual development. Additionally, the omics data does not suggest any of the above-mentioned triggers. For instance, the expression profile of *flbD* indicates that no nitrogen starvation conditions occurred at 10°C (100). This suggests that low temperature stress contributes to the initiation of sexual development. Most likely, low temperature stress triggers sexual development as a strategy to survive winter cold in temperate zones through the production of stress-resistant ascospores (95). Further on, the sexual cycle allows the fungus to acquire new genes by recombination for greater survival in complex environments.

From many fungi it is known that sexual development is connected to secondary metabolism (101). The *A. nidulans* velvet protein VeA is the molecular link between secondary metabolism and development. The study of a Δ*veA* mutant, which could not produce any sexual fruiting bodies, showed the connection of ST and penicillin production and sexual development through the global regulator *velA* (102). This suggests that low temperature-induced SM production is linked to the switch to sexual development.

However, we could find both increased levels of SMs typically produced during asexual development, *e.g.* emericellamide and terrequinone A, and SMs synthesised during sexual development, such as asperthercin (98). This indicates that not only the induction of the sexual cycle, but also other factors, even perhaps the cold stress itself, influence the SM production at low temperatures. It is also possible that indirect parameters of the low temperature stress induce SM production, such as the increased osmolarity as shown in marine-derived fungi (103). Most probably, metabolic differentiation may increase the ecologic fitness in natural environments under harsh conditions including low temperature stress.

In line, *A. nidulans* produced so-far uncharacterised compounds during low temperature stress that exhibited growth inhibitory activity against Gram-positive bacteria and fungi. The SM profile at 10°C may serve as a chemical shield to protect against competitors (97,104) under extreme conditions such as low temperatures.

In summary, our study elucidates the global stress response of *A. nidulans* to low temperature. Different low temperature-induced effects such as activation of protective stress response mechanisms, increased SM production and altered cell development were identified in *A. nidulans* on the transcriptome, proteome, and metabolome level. Several known adaptation mechanisms to cold stress such as activation of RNA/protein chaperones, DNA repair proteins, osmotic stress response proteins and enzymes involved in trehalose product, were detected in *A. nidulans*.

A new finding has been the initiation of sexual development and SM production under cold stress. Earlier publications describe a close connection of cell developmental stage to SM production (105), whereas other studies focussed on the detection of global regulators which trigger SM production without influencing the fungal development (106). In particular, it would be interesting in further studies to uncover the regulatory mechanisms in place during low temperature stress. The ability of the fungus to induce sexual development at low temperatures, despite the truncated VeA protein is noteworthy. Which signalling pathways are involved and how sexual development is triggered in the absence of a functional *veA* gene remain open questions. Furthermore, the SM profile at low temperature requires further investigations.

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3.3 Phenotypic and proteomic analysis of the *Aspergillus fumigatus* Δ*PrtT*, Δ*XprG* and Δ*XprG*/Δ*PrtT* protease-deficient mutants

Einav Shemesh†, **Benjamin Hanf†**, Shelly Hagag, Shani Attias, Yana Shadkchan, Boris Fichtman, Amnon Harel, Thomas Krüger, Axel A. Brakhage, Olaf Kniemeyer and Nir Osherov

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Summary

Aspergillus fumigatus is an airborne opportunistic fungal pathogen which can be inhaled and can lead to life-threatening diseases in immunocompromised hosts. Once reaching the lung alveoli, the fungus can germinate and penetrate the pulmonary epithelia which is a crucial step of the infection process in humans. It is assumed that *A. fumigatus* degrades structural barriers with secreted proteases. In this study, we examine the role of the transcription factor XprG in regulating extracellular proteolysis, alone and in combination with PrtT, which is known from former studies to control secreted proteolytic activity. Deletion mutants of these regulators showed a reduced or nearly absent degradation activity of substrate proteins. Nevertheless, virulence in the murine systemic and pulmonary model of infection was unaffected. Proteomic analysis revealed the regulation of secreted proteases by XprG and PrtT and suggested an influence on additional cellular processes, such as cell wall modifications and allergens. This study elucidates the high adaptive potential of the fungus and demonstrates the gene redundancy of critical biologically functions for *A. fumigatus,* such as the ability to degrade organic matter for nutrition uptake.

Contribution to the manuscript

Benjamin Hanf contributed to the manuscript by conducting the proteomics experiments, the data analysis and interpretation. Further on, he contributed to writing and editing the introduction, method section, results and discussion and created figures.

Estimated contribution in percentage

Einav Shemesh and **Benjamin Hanf**	**50 %**

Shelly Hagag, Shani Attias, Yana Shadkchan, Boris Fichtman, Amnon Harel and Thomas Krüger	20 %
Axel A. Brakhage, Olaf Kniemeyer and Nir Osherov	30 %

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Phenotypic and Proteomic Analysis of the *Aspergillus fumigatus* Δ*PrtT*, Δ*XprG* and Δ*XprG*/Δ*PrtT* Protease-Deficient Mutants

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Aspergillus fumigatus is the most common mold species to cause disease in immunocompromised patients. Infection usually begins when its spores (conidia) are inhaled into the airways, where they germinate, forming hyphae that penetrate and destroy the lungs and disseminate to other organs, leading to high mortality. The ability of hyphae to penetrate the pulmonary epithelium is a key step in the infectious process. *A. fumigatus* produces extracellular proteases that are thought to enhance penetration by degrading host structural barriers. This study explores the role of the *A. fumigatus* transcription factor XprG in controlling secreted proteolytic activity and fungal virulence. We deleted *xprG*, alone and in combination with *prtT*, a transcription factor previously shown to regulate extracellular proteolysis. *xprG* deletion resulted in abnormal conidiogenesis and formation of lighter colored, more fragile conidia and a moderate reduction in the ability of culture filtrates (CFs) to degrade substrate proteins. Deletion of both *xprG* and *prtT* resulted in an additive reduction, generating a mutant strain producing CF with almost no ability to degrade substrate proteins. Detailed proteomic analysis identified numerous secreted proteases regulated by XprG and PrtT, alone and in combination. Interestingly, proteomics also identified reduced levels of secreted cell wall modifying enzymes (glucanases, chitinases) and allergens following deletion of these genes, suggesting they target additional cellular processes. Surprisingly, despite the major alteration in the secretome of the *xprG/prtT* null mutant, including two to fivefold reductions in the level of 24 proteases, 18 glucanases, 6 chitinases, and 19 allergens, it retained wild-type virulence in murine systemic and pulmonary models of infection. This study highlights the extreme adaptability of *A. fumigatus* during infection based on extensive gene redundancy.

Keywords: *Aspergillus fumigatus*, protease secretion, transcription factor, proteomics, virulence

INTRODUCTION

Aspergillus fumigatus is a common saprophytic mold which produces abundant microscopic conidia (2–4 micrometers) that can be inhaled into the pulmonary alveoli to cause a variety of pathological conditions (Kwon-Chung and Sugui, 2013). In the context of the immunocompromised patient, the infection, termed Invasive Pulmonary Aspergillosis (IPA), is life-threatening and severe (Kosmidis and Denning, 2015). The neutropenic status of these patients culminates in their inability to destroy inhaled conidia resulting in fungal growth and penetration through the pulmonary epithelium into the blood stream (Latge, 2001).

The success of *A. fumigatus* as a pathogen is not a result of direct adaptation to the host. Rather it should be viewed as an accidental interaction between a common and hardy environmental mold and a weakened host. *A. fumigatus* survives in the compromised host due to a chance combination of pre-existing capabilities. They include the abundant release of small conidia protected by a non-immunogenic layer of hydrophobins and oxygen-radical quenching pigments. Growing hyphae effectively endure oxidative stress and hypoxia, efficiently collect scarce iron and secrete toxins that further depress host immune function and proteases that degrade host tissue (Abad et al., 2010; Kwon-Chung and Sugui, 2013). In human A549 alveolar epithelial cells, culture filtrates (CFs) of *A. fumigatus* can disrupt the actin cytoskeleton, activate NFκB signaling and induce the production of proinflammatory cytokines. These cellular events can be prevented by addition of serine protease inhibitors to the secreted CF, implying that they are directly dependent on secreted fungal proteases (Kogan et al., 2004; Sharon et al., 2011). *A. fumigatus*-secreted proteases and gliotoxin also induce platelet activation that may serve as a mechanism for activating the immune defenses and inducing inflammation (Speth et al., 2013). Additionally, the secreted *A. fumigatus* alkaline protease Alp1 cleaves the complement components C3, C4, and C5 that contribute to evasion from the host immune response (Behnsen et al., 2010). Alp1 is also a major allergen (Aspf 13) and promotes airway hyper-responsiveness and bronchoconstriction in asthma (Balenga et al., 2015).

In previous work we have identified the transcription factor PrtT, a positive regulator of secreted proteases in *A. fumigatus*. Deletion of *prtT* results in greatly reduced secreted protease activity and a reduction in the transcription of secreted proteases. Δ*prtT* CF showed reduced killing of A549 lung alveolar cells and erythrocyte lysis (Bergmann et al., 2009; Sharon et al., 2009, 2011). However, the Δ*prtT* strain showed wild-type virulence in infected neutropenic mice suggesting that perhaps residual protease activity was sufficient to enable virulence in this setting (Bergmann et al., 2009; Sharon et al., 2009).

In the related mold *A. nidulans*, that lacks a *prtT* homolog, the transcription factor XprG regulates extracellular protease production in response to nutrient stress (Katz et al., 2013, 2015; Katz and Cooper, 2015). Deletion of *A. nidulans xprG* resulted in complete loss of halo formation on skimmed milk (SM) agar plates and an inability to grow on medium containing BSA as sole carbon or nitrogen source. XprG is a member of the p53-like transcription factors, also known as the *NDT80/PhoG*-like family. *NDT80* transcription factors are found in animals, fungi, and amoeba. Fungal *NDT80* genes were studied in detail in *Saccharomyces cerevisiae* (Pak and Segall, 2002), *Neurospora crassa* (Hutchison and Glass, 2010) *Candida albicans* (Chen et al., 2004; Sellam et al., 2009, 2010) and *A. nidulans* (Katz et al., 2013, 2015; Katz and Cooper, 2015). The consensus is that in response to nutrient stress and deprivation they activate specific target genes such as proteases, phosphatases, secondary metabolites, and genes involved in meiosis (itself a stress response initiated by starvation) and autolysis.

We hypothesized that XprG also co-regulates protease production in *A. fumigatus* and that deletion of both *prtT* and *xprG* would completely abolish secreted protease activity, reducing fungal virulence. To test this hypothesis we prepared *A. fumigatus* strains deleted in *prtT*, *xprG* alone and in combination. The effects of these mutations *in vitro* and during infection *in vivo* are described.

MATERIALS AND METHODS

Strains and Culture Conditions

The strains used in this study are detailed in **Table 1**. *A. fumigatus* conidia were harvested in 0.2% (*vol/vol*) Tween 20, resuspended in double-distilled water (DDW) and counted with a hemocytometer. For continuous growth, *A. fumigatus* strains were grown on YAG medium, that consists of 0.5% (*wt/vol*) yeast extract, 1% (*wt/vol*) glucose, and 10 mM $MgCl_2$, supplemented with trace elements, vitamins, and 1.5% (*wt/vol*) agar when needed (Bainbridge, 1971). SM medium consisted of 1% (*wt/vol*) glucose, 1% (*wt/vol*) SM (Difco, Livonia, MI, United States), 0.1% (*wt/vol*) Casamino Acids (Difco), 7 mM KCl, 2 mM $MgSO_4$ and 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 5.3), supplemented with vitamins, trace elements and 1.5% agar when needed. $NaNO_3$-depleted *Aspergillus* minimal medium (Weidner et al., 1998) was used for collagen medium with 0.1% (*wt/vol*) yeast extract, 0.5% (*wt/vol*) glucose and collagen as sole carbon and nitrogen source. Peptone medium contained 1% (*wt/vol*) glucose, 0.4% peptone (Difco), 7 mM KCl, 2 mM $MgSO_4$ and 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 5.3), supplemented with vitamins and trace elements. Genetically modified organisms and pathogens used in this study were maintained in accordance with TAU Institutional Policies.

Generation and Verification of *A. fumigatus* Mutant Strains

All strains were prepared in the *Ku80* null background, strain $AkuB^{KU80}$ (da Silva Ferreira et al., 2006). Full details of the construction and verification of the strains shown in **Table 1**, including a list of the primers used (Supplementary Table S1), are provided in the Supplementary Data Section.

TABLE 1 | Strains used in this study.

Strain	Genotype	Source
KU80 (ΔakuB)	CEA17, AFUA_2G02620::*pyrG*	da Silva Ferreira et al., 2006
ΔPrtT	AFUA_4G10120::*hph*	This work
ΔXprG	AFUA_8G04050:: *ptrA*	This work
ΔXprG/ΔPrtT	AFUA_8G04050:: *ptrA*; AFUA_4G10120::*hph*	This work
PrtT KI	AFUA_4G10120::*hph*; AFUA_4G10120-*phl*	This work
XprG KI	AFUA_8G04050:: *ptrA* AFUA_8G04050-hph	This work
ΔXprG/PrtT KI	AFUA_8G04050:: *ptrA*; AFUA_4G10120::*hph* AFUA_4G10120-*phl*	This work

Conidial Stability in Detergent Storage

Freshly harvested *A. fumigatus* conidia (10^7/ml) were suspended in DDW +0.5% (*vol/vol*) Tween 20 at 37°C. At different time-points, aliquots were diluted, plated on YAG plates and the number of colonies counted.

Conidial Disruption by Glass Beads

Freshly harvested *A. fumigatus* conidia (5×10^7/ml) were suspended in 0.5 ml DDW +0.1% Tween 20 and mixed with 0.5 ml (packed volume) of acid washed glass beads, 150–212 μm (Sigma–Aldrich Corp., St. Louis, MO, United States). They were then vortexed on medium strength for up to 10 min. At each time point a sample was taken, diluted and plated on YAG plates. The plates were incubated at 37°C for 24–36 h, colonies were counted and survival rates were calculated as the percentage of viable spores.

Analysis of Fungal Enzymatic Activity

Proteolytic activity on solid medium was assessed by spotting conidia on SM plates containing 0.1% Tween 20. The colonies were grown for 48 h at 37°C, then transferred to room temperature for another 48 h and subsequently photographed. Supernatants were collected from *Aspergillus* cultures grown in liquid SM for 48 h at 37°C. Azocasein (Sigma) was dissolved at a concentration of 5 mg/ml in assay buffer containing 50 mM Tris (pH 7.5), 0.2 M NaCl, 5 mM $CaCl_2$ and 0.05% Triton X-100 as previously described (Kogan et al., 2004). The azocasein solution (400 μl) was mixed with 100 μl portions of supernatants from *Aspergillus* cultures and incubated by shaking for 90 min at 37°C. The reactions were stopped by adding of 150 μl 12% (*vol/vol*) trichloroacetic acid, and the reaction mixtures were allowed to stand at room temperature for 30 min. Tubes were then centrifuged for 3 min at 8,000 *g*, and 100 μl of each supernatant was added to 100 μl of 1 M NaOH. The absorbance of released azo dye at 436 nm was determined with a spectrophotometer. Proteolytic activity on bovine serum albumin (BSA) was measured by growing *A. fumigatus* in 24-well plates with 1 ml/well liquid peptone medium containing 0.1% BSA, respectively, for 24–72 h at 37°C. Supernatants were boiled in sample buffer and run on an 8% SDS-PAGE gel followed by Coomassie staining to visualize the proteins.

Scanning Electron Microscopy (SEM) Analysis

Aspergillus fumigatus wild-type and mutant strains were grown for 72 h at 37°C on YAG agar plates. Fixation and processing of samples for SEM was performed as described earlier (Fichtman et al., 2014; Hover et al., 2016). Briefly, small areas of conidiating mycelium were carefully excised from the zone of interaction and vapor fixed with 8% (*vol/vol*) paraformaldehyde and 4% (*vol/vol*) glutaraldehyde dissolved in water for 1 h in a closed chamber. Secondary vapor fixation was then carried out in an aqueous solution of 2% (*wt/vol*) osmium tetroxide for 1 h. The samples were submerged for 10 min in DDW and then dehydrated (10 min, twice for each step) under a series of ethanol concentrations (7.5, 15, 30, 50, 70, 90, 95, and 100%). Next, samples underwent critical point drying (CPD) using a K850 CPD dryer (Quorum Technologies, United Kingdom). Coating was done with 3 nm iridium using a Q150T coater (Quorum Technologies, United Kingdom). Samples were imaged with a Merlin scanning electron microscope (Zeiss, Germany).

LC-MS/MS Analysis and Identification of Secreted Proteins

Aspergillus fumigatus strains at a concentration of 10^6 condia/ml were grown in liquid collagen medium for 72 h at 37°C. Total protein was TCA-precipitated from the CFs, digested with trypsin, iTRAQ-labeled and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as detailed in the Supplementary Data.

Murine Models for Invasive Aspergillosis

For the cyclophosphamide/cortisone acetate neutropenic model (Ejzykowicz et al., 2009), 6-week-old female ICR mice were injected intraperitoneally with cyclophosphamide (150 mg/kg in PBS) at 3 days prior to infection, on the day of infection and at 3 days post-infection. Cortisone acetate (150 mg/kg PBS with 0.1% Tween 20) was injected subcutaneously at 3 days prior to conidial infection.

For intranasal infection, mice were anesthetized by intraperitoneal injection of a solution of 250 μl xylazine (VMD, Arendonk, Belgium) and ketamine (Imalgene, Fort Dodge, IA, United States) at a concentration of 1.0 and 10 mg/ml, respectively (dissolved in PBS). Following anesthesia, the mice were inoculated intranasally with 2.5×10^5 (intranasal model) or intravenously (IV) through the tail vein (IV model) with freshly harvested conidia of $AkuB^{KU80}$, *ΔXprG*, or *ΔXprG/ΔPrtT* in PBS +0.1% (*vol/vol*) Tween 20. The inoculum was verified by quantitative culture. The animals were monitored for survival for up to 18 days. For infection of immunocompetent mice, 6-week-old female ICR mice were inoculated intranasally with 1×10^7 conidia and sacrificed 24 h or 48 h later. Excised lungs were ground and plated on YAG agar plates for CFU enumeration. Histological analysis was performed with Gomori methenamine

silver stain (GMS, stains fungal elements black) or haematoxylin and eosin stain (H&E, stains host-cell nuclei purple, cytosol pink). Statistical analysis of mouse survival was performed with GraphPad Prism 4 software (GraphPad Software, San Diego, CA, United States). Animal studies were authorized by the Tel Aviv University Animal Welfare Committee according to the Israel Ministry of Health guidelines and carried out in accordance with Tel Aviv University institutional policies.

RESULTS

Generation of *A. fumigatus prtT*, *xprG*, *xprG/prtT* Null Mutants and Reconstituted Strains

A single *A. fumigatus xprG* ortholog (AFUA_8G04050) was identified by amino-acid similarity to *A. nidulans* XprG (70% identity). It is predicted to be 1752 nucleotides long and to contain one intron. *A. fumigatus* XprG encodes a protein 583 amino acids in length, containing a predicted *NDT80/PhoG* like DNA-binding domain (amino-acid residues 156–327) and a MAP65/ASE1 domain (amino acid residues 336–505) shared by microtubule-binding proteins. The similarity to *NDT80* family genes *vib-1* from *N. crassa* and *S. cerevisiae ndt80* is limited to the *PhoG* like DNA-binding domain. To analyze the function of XprG and its interaction with PrtT in *A. fumigatus*, we prepared Δ*PrtT*, Δ*XprG*, Δ*XprG*/Δ*PrtT* null mutants and corresponding reconstituted strains *PrtT-KI*, *XprG-KI*, and *PrtT-KI*/Δ*XprG* by transformation with a circular plasmid containing the *PrtT* gene and the phleomycin resistance cassete for selection (see Supplementary Data for full details about strain construction and verification).

Abnormal Conidiogenesis in the Δ*Xprg* and Δ*XprG*/Δ*PrtT A. fumigatus* Mutants

The aforementioned null and reconstituted strains were point inoculated on YAG agar plates and examined for differences in morphology after growth for 72 h at 37°C. Results show that the Δ*XprG*, Δ*XprG*/Δ*PrtT* null strains produced lighter colored conidia as compared to the $AkuB^{KU80}$, Δ*PrtT*, and *XprG-KI* strains (**Figure 1A**). Examination of the conidiophore structure by light microscopy and SEM revealed that the Δ*XprG* and Δ*XprG*/Δ*PrtT* strains produced significantly smaller, more compact conidiophores ($P < 0.02$) with shorter conidial chains (**Figures 1B,E**). The conidial hydrophobin outer rodlet layer remained intact and unchanged (**Figure 1C**) and the surface hydrophobicity of the mutant colonies, based on their ability to exclude water, was unchanged (not shown). Reflecting the shorter conidial chains, the number of conidia produced by the Δ*XprG* and Δ*XprG*/Δ*PrtT* strains was significantly reduced three–fourfold per plate ($P < 0.05$) compared to $AkuB^{KU80}$ and Δ*PrtT* (**Figure 1D**). Radial growth rates of the Δ*PrtT*, Δ*XprG*, Δ*XprG*/Δ*PrtT* strains were similar to that of $AkuB^{KU80}$ at both 37 and 48°C (Data not shown). The lighter conidial color of the *PrtT-KI* complemented strain compared to the deleted strain (**Figure 1A**) is unexpected and may be due to multiple integration of the complementing plasmid.

The Conidia Produced by the Δ*Xprg* and Δ*XprG*/Δ*PrtT A. fumigatus* Mutants Are More Fragile

We evaluated the ability of the mutant conidia to withstand osmotic or detergent disruption by measuring their survival in water or in water containing Tween 20 detergent. Conidia from the Δ*XprG* and Δ*XprG*/Δ*PrtT* strains exhibited similar increased susceptibility to the detergent, losing >90% viability after 4 h as compared to loss of only <20% viability in the $AkuB^{KU80}$ and Δ*PrtT* strains (**Figure 2A**). There were no differences in conidial stability during storage in DDW, suggesting the mutants are not osmotically sensitive (data not shown). The ability of the mutant conidia to withstand physical disruption was assessed by subjecting them to agitation in the presence of glass beads. Conidia from the Δ*XprG* and Δ*XprG*/Δ*PrtT* strains exhibited increased susceptibility to glass-bead agitation, losing all viability after 4 min agitation, compared to 10 min for the $AkuB^{KU80}$ and Δ*PrtT* strains (**Figure 2B**).

Taken together these results show that deletion of *xprG* in the $AkuB^{KU80}$ and Δ*PrtT* strains resulted in the production of smaller conidiophores containing less and more fragile spores.

Deletion of *xprG* and *prtT* in *A. fumigatus* Results in an Additive Reduction in the Ability to Degrade Casein and Albumin

To investigate the effect of *xprG* deletion on secreted protease activity, we point inoculated the strains on SM agar plates and visually determined the formation of a proteolytic halo due to casein degradation around the fungal colony. As we have previously shown (Sharon et al., 2009), deletion of *prtT* resulted in the formation of a very weak halo compared to the control strain $AkuB^{KU80}$, whereas deletion of *xprG* (Δ*XprG*) led to a partially reduced halo. Deletion of both *xprG* and *prtT* (Δ*XprG*/Δ*PrtT*) completely abolished the formation of a proteolytic halo (**Figure 3A**) and reduced azocasein degradation by over 90% (**Figure 3B**). Halo formation was restored in the reconstituted strain *XprG-KI* and slightly increased in the *PrtT-KI* and *PrtT-KI*/Δ*XprG* strains. This increase, however, was not seen in the azocasein or BSA degradation assays described below. We used a more sensitive SDS-PAGE-based BSA degradation assay to better differentiate between the proteolytic activities of the Δ*XprG*,Δ*PrtT* and Δ*XprG*/Δ*PrtT* mutants (**Figure 3C**). Strains were grown for 24–72 h on peptone medium containing 0.1% BSA. The supernatant was separated on an SDS-PAGE gel followed by Coomassie staining to visualize the degradation of the BSA protein. The control $AkuB^{KU80}$ and reconstituted strains substantially degraded the BSA substrate protein after 48 h of incubation, whereas the Δ*PrtT*, Δ*XprG*, and Δ*XprG*/Δ*PrtT* strains did not (**Figure 3C**). After 72 h of incubation, all strains except Δ*XprG*/Δ*PrtT* had completely degraded the BSA. Taken together, our findings indicate that deletion of both *prtT* and *xprG* results in an additive reduction in the

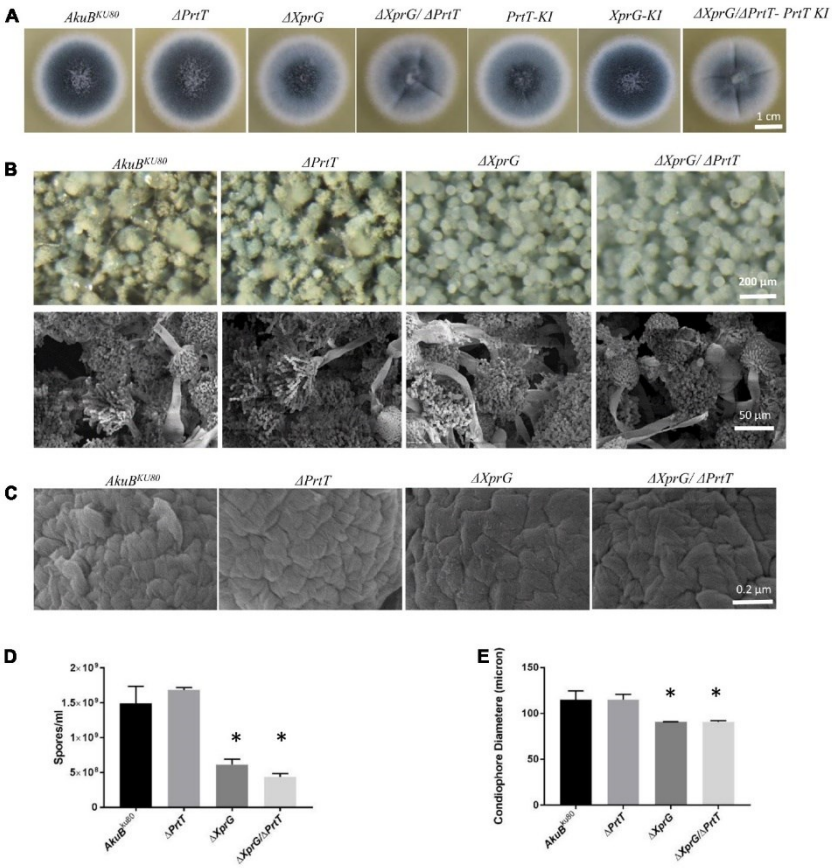

FIGURE 1 | Deletion of *xprG* and *xprG/prtT* leads to abnormal conidiogenesis. Phenotypic analysis of the Δ*XprG* and Δ*XprG*/Δ*PrtT* strains compared to the wild-type $AkuB^{KU80}$ and Δ*PrtT* strains shows that they produce **(A)** lighter colored conidia when growing on YAG agar plates **(B)** conidiophores with reduced diameter and shorter spore chains (Light microscopy and SEM) **(C)** conidia with a normal rodlet outer layer (SEM, high magnification), **(D)** a significantly reduced number of conidia ($^*P < 0.005$). **(E)** Quantification of reduction in conidiophore diameter (based on the diameter of 50 conidiophores per strain) ($^*P < 0.02$).

ability to degrade casein or albumin. Previous analyses using protease inhibitors and deletion mutants have demonstrated that secreted *A. fumigatus* serine proteases, and in particular Alp1, are primarily responsible for the degradation of these substrates (Jaton-Ogay et al., 1994; Tomee et al., 1997; Kogan et al., 2004).

Proteomic Analysis of the Δ*PrtT*, Δ*XprG*, and Δ*XprG*/Δ*PrtT* Secretomes

To identify the repertoire of proteins secreted by the Δ*PrtT*, Δ*XprG*, and Δ*XprG*/Δ*PrtT* mutants compared to the control $AkuB^{KU80}$ strain, conidia were cultured in liquid MM containing collagen for 72 h at 37°C with shaking. Supernatants were TCA-precipitated, treated after Yeung and Stanley (2009), followed by a Wessel-Flügge precipitation, labeled with iTRAQ 4-plex isobaric labeling for quantitative proteomics approach and analyzed by LC-MS/MS. A total of 938 proteins were identified in the fungal supernatants, 274 of which were predicted to be secreted by *in silico* analysis (secretion signal found by signalP for 236 proteins and targetP for 274 proteins) (Supplementary Table S2).

Cell lysis cannot be completely excluded during cultivation and sample preparation. It is therefore possible that a proportion of the secretome consisted of proteins without a secretion signal, which had been released by cell lysis. However, the comparison of the abundance (based on normalized PSM values) of typical intracellular, non-secreted proteins like actin (*Afu6g04740*) or tubulin (*Afu1g02550*) with the abundance of a secreted protein like lap2 (*Afu3g00650*) revealed a depletion by a factor between 12 and 106. In addition, fungi are able to release typical intracellular proteins via extracellular vesicles to the extracellular space (Joffe et al., 2016). It can be therefore assumed that the contamination of the secretome by cell lysis was relatively low.

Deletion of *prtT* resulted in strongly reduced secretion of 22 proteases, including Lap1, Lap2, Cp1, Cp3 Alp1, Alp2, and Mep

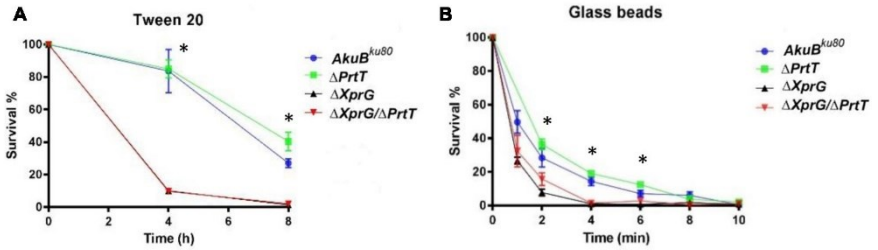

FIGURE 2 | The Δ*XprG* and Δ*XprG*/Δ*PrtT* mutants produce conidia that are more sensitive to chemical and mechanical stress. **(A)** 10^3 conidia/ml were suspended in 0.5% Tween 20 for the time indicated, plated and the number of colonies counted in comparison to controls suspended in DDW alone. $^*P < 0.001$ $AkuB^{ku80}$ vs. Δ*XprG* and Δ*XprG*/Δ*PrtT* mutants. **(B)** 5×10^7 conidia/ml were agitated in the presence of acid-washed glass beads for the time indicated, plated and the number of colonies counted in comparison to untreated controls. $^*P < 0.05$ $AkuB^{ku80}$ vs. Δ*XprG* and Δ*XprG*/Δ*PrtT* mutants.

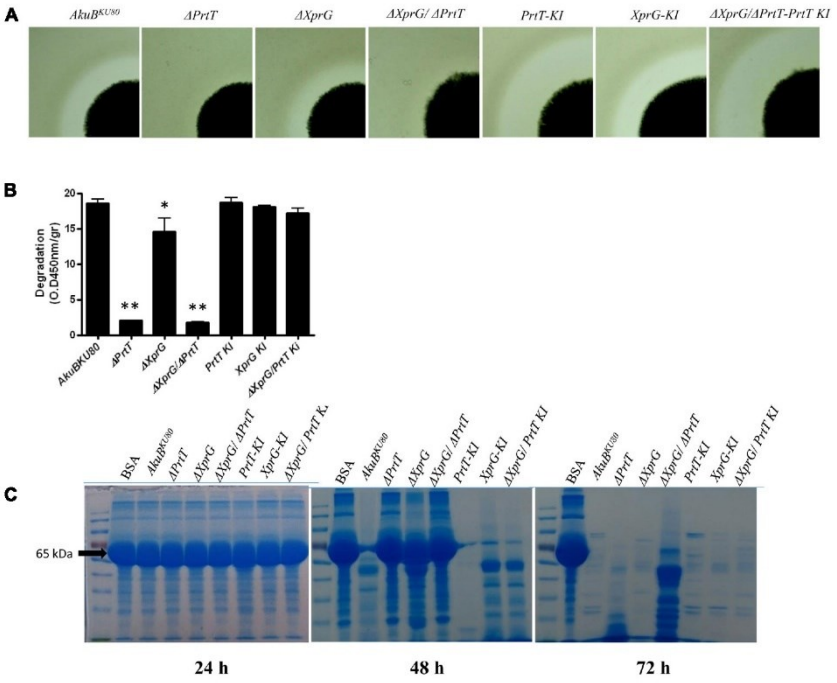

FIGURE 3 | Proteolytic activity following deletion of *prtT* and *xprG*. Proteolytic activity of the fungal strains was assessed by **(A)** Halo formation on SM agar plates containing 0.1% Tween 20 following 48 h of growth at 37°C followed by another 48 h at room temperature. **(B)** Degradation of azocasein by *A. fumigatus* culture filtrates (CFs). The absorbance of the released azo dye was measured spectrophotometrically at 450 nm. ($^*P < 0.05$, $^{**}P < 0.0001$). **(C)** BSA proteolysis by *A. fumigatus* CFs followed by SDS-PAGE analysis of the cleaved substrate. BSA (65 kDa, arrow) and other proteins were stained by Coomassie.

(**Figure 4** and Supplementary Table S3). Deletion of *xprG* resulted in reductions in the secretion of 19 proteases, including most strongly Lap1, Sxa2, DppV, DppIV, Mep, and SedC. Notably, several proteases that were strongly reduced in the Δ*PrtT* mutant were only slightly reduced (Pep2, DapB, Cps1, Cp1, Cp3) or not reduced (CtsD, Ape3, Lap2, Alp1, Alp2) in the Δ*XprG* strain (**Figure 4** and Supplementary Table S3). This provides a good explanation for the higher secreted protease activity of the Δ*XprG* vs. the Δ*PrtT* mutant, especially the lack of reduction in the major neutral serine protease Alp1 (Behnsen et al., 2010). Deletion of both *prtT* and *xprG* resulted in the strongest reductions in the secretion of 24 proteases comprising the entire joint dataset of the Δ*PrtT* and Δ*XprG* mutants, including 14 serine proteases, 7 metalloproteases and 3 aspartic proteases (**Figure 4** and Supplementary Table S3). Interestingly, in all three mutant strains, the secretion of numerous glucanases,

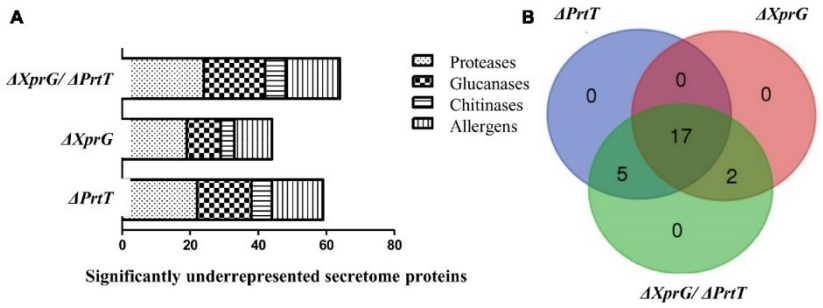

FIGURE 4 | Effect of the deletion of *prtT*, *xprG* and *xprG/prtT* on the secretome of *A. fumigatus*. Deletion of *prtT*, *xprG* and *xprG/prtT* **(A)** reduces the secretion not only of proteases but also of glucanases and chitinases participating in cell wall biosynthesis and of numerous allergenic proteins. **(B)** Venn diagram illustrating the degree of overlap in the secretome datasets of the mutant strains.

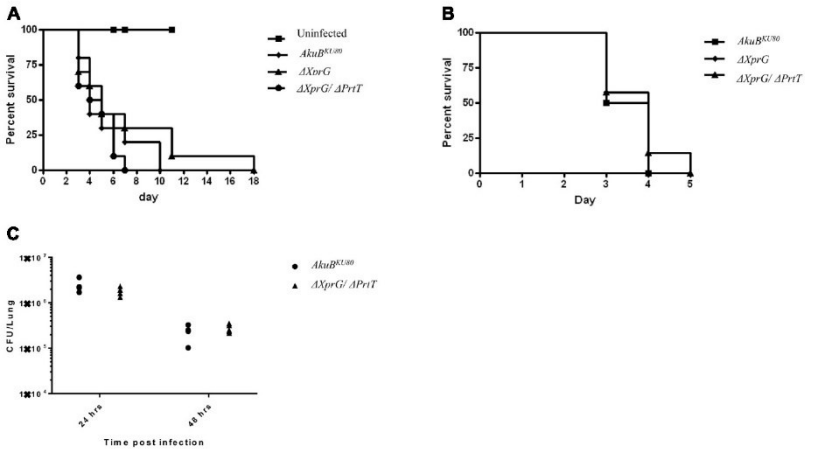

FIGURE 5 | Deletion of *xprG* and *xprG/prtT*, does not affect virulence in a neutropenic murine model of **(A)** pulmonary aspergillosis, **(B)** disseminated aspergillosis and **(C)** pulmonary fungal load in immunocompetent mice. Cortisone acetate plus cyclophosphamide (neutropenia model) were used to immunocompromise the infected mice **(A,B)**. Survival curves are shown for mice infected **(A)** intranasally with an inoculum of 2.5×10^5 conidia per mouse ($n = 10$ mice/group) and **(B)** intravenously via the lateral tail vein with an inoculum of 2.5×10^5 conidia per mouse ($n = 7$ mice/group). These experiments were repeated twice with similar results. **(C)** Immunocompetent mice were infected intranasally with an inoculum of 1×10^7 conidia per mouse ($n = 5$ mice/group) and total lung fungal load (CFU/lung) assessed 24 and 48 h post-infection. (Additional histological analysis is presented in the Supplements- Supplementary Figure S6).

chitinases, and allergens was also reduced, indicating that these transcription factors not only regulate the expression of secreted proteases but also of cell wall enzymes and antigenic proteins (Supplementary Table S3). The significance of these results will be explained in the discussion. No proteins showed increased secretion in the mutants vs. the control $AkuB^{KU80}$ strain.

The Δ*XprG* and Δ*XprG*/Δ*PrtT* Strains Exhibit Normal Virulence in Infected Mice

Previous studies have shown that deletion of *prtT* in *A. fumigatus* does not affect virulence in lung-infected neutropenic mice (Bergmann et al., 2009; Sharon et al., 2009). We hypothesized that the almost complete lack of secreted protease activity in the Δ*XprG*/Δ*PrtT* strain would result in a noticeable reduction in virulence. To test this, we compared the virulence of the control $AkuB^{KU80}$, Δ*XprG* and Δ*XprG*/Δ*PrtT* strains in two mouse models of invasive aspergillosis. Both models reproduce profound neutropenia (as found, for example following chemotherapy in leukemic patients), by immunosuppressing the mice with a combination of cortisone acetate and cyclophosphamide. In the first model, the mice were infected intranasally with freshly harvested conidia. In the second model reproducing disseminated infection, mice were infected IV. The number of live mice in each group was recorded daily throughout the experiment. The Δ*PrtT* strain was not included as we have previously shown it to be fully virulent in these models (Sharon et al., 2009). **Figure 5** shows survival curves obtained during the course

of the experiment for the lung (**Figure 5A**) and disseminated (**Figure 5B**) models. No significant differences in virulence were found for the Δ*XprG* and Δ*XprG*/Δ*PrtT* mutants compared to the control $AkuB^{KU80}$ strain (P-value > 0.3). Next, we analyzed the response of immunocompetent mice to intranasal pulmonary infection (10^7 conidia/mouse) with Δ*XprG*/Δ*PrtT* and control $AkuB^{KU80}$ strains. Outputs included lung CFU (**Figure 5C**) and histology (Supplementary Figure S6), 24 and 48 h following infection. There was no significant difference in lung fungal load (P-value > 0.2) between the Δ*XprG*/Δ*PrtT* and control $AkuB^{KU80}$ strains (**Figure 5C**). Histology revealed abundant conidia in the bronchi of both mutant and control strains 24 h after infection (GMS stain, right panel, arrows) accompanied by a considerable influx of red blood cells and lymphocytes (H&E stain, left panel) (Supplementary Figure S6). As expected in immunocompetent mice, the number of conidia strongly decreased 48 h after infection, as did the number of red blood cells and lymphocytes (Supplementary Figure S6). Taken together, these results suggest that immunocompetent mice mount a similar and effective immune response to both Δ*XprG*/Δ*PrtT* and control $AkuB^{KU80}$ infection.

DISCUSSION

This study describes the disruption of the putative transcriptional activator XprG in *A. fumigatus*, alone and in combination with the previously described transcription factor PrtT. We chose to study XprG in *A. fumigatus* because (i) it regulates protease expression in *A. nidulans* and we reasoned it may also do so, in combination with PrtT, in *A. fumigatus*, (ii) regulation of protease expression by combinations of transcription factors in pathogenic filamentous fungi is poorly understood and (iii) we hypothesized that deletion of two transcriptional regulators controlling the expression of multiple proteases could shed more light on the involvement of secreted proteases in the pathogenesis of *A. fumigatus*.

We gained several important insights from this study. First, we found considerable divergence in the role of XprG in *A. nidulans* and *A. fumigatus*. In *A. nidulans* that lacks a PrtT homolog XprG is the major activator of secreted protease activity under starvation. In *A. fumigatus,* this function is carried out by PrtT, with XprG performing a secondary role. Increasing functional divergence is found in the *N. crassa* XprG homolog vib-1 – in addition to controlling conidial pigmentation and increasing extracellular protease activity it also controls protoperithecial sexual development (Hutchison and Glass, 2010). Further functional separation has occurred in *S. cerevisiae* ndt80 (Pak and Segall, 2002) that controls meiosis and *C. albicans* CaNdt80 (Chen et al., 2004; Sellam et al., 2009, 2010), that regulates azole resistance, hyphal growth and virulence. The basis for this diversity likely stems from the extreme sequence divergence outside of the conserved *NDT80/PhoG* like DNA-binding domain.

We also found evidence for overlap in ndt80/vib-1/XprG function. As previously shown in *A. nidulans xprG* and *N. crassa vib-1* null mutants, deletion of *A. fumigatus xprG* resulted in the formation of pale conidia, suggesting a role in melanin biosynthesis. We further showed that in *A. fumigatus*, conidiogenesis is impaired and the resulting conidia are weaker and more prone to chemical and physical disruption. Most interestingly, we show here that *A. fumigatus* XprG and PrtT activate expression of glucanases, chitinases and numerous allergens. *C. albicans* CaNdt80 also activates genes encoding cell wall components including expression of chitinase Cht3p and cell wall glucosidase Sun41p that are essential for the completion of cell separation (Sellam et al., 2010). However, despite reduced levels of numerous secreted glucanases and chitinases in *A. fumigatus* Δ*XprG,* we found no obvious evidence for cell wall alterations by either SEM, transmission electron microscopy (TEM) or cell wall inhibitor analysis (data not shown). CaNdt80 also activates CDR1 efflux pump and ergosterol biosynthesis and its deletion results in azole sensitivity. Nevertheless, deletion of *A. fumigatus xprG* did not increase azole sensitivity (data not shown) suggesting that it does not affect these targets.

Our analysis of XprG and PrtT showed that both genes are not essential for maintaining virulence. Considering that the double-mutant has almost no detectable secreted protease activity *in vitro* and exhibits strong (two–fivefold) reductions in 24 secreted proteases [including all 5 proteases that are activated during *in vivo* infection (McDonagh et al., 2008)] as well as 18 glucanases and 6 chitinases, the unaffected virulence in the mouse model is unexpected. The *A. fumigatus* genome encodes approximately 50 putative secreted proteases, and some may be alternatively activated *in vivo*, compensating for the reductions seen in Δ*PrtT*/Δ*XprG*. Another possible explanation is that under *in vivo* stress, Δ*XprG*/Δ*PrtT* activates alternative virulence determinants that are not activated in the wild type. For example, we have previously shown that deletion of *prtT* resulted in the upregulation of four secondary metabolite clusters, including genes for the biosynthesis of toxic pseurotin A (Hagag et al., 2012). These factors could increase its virulence to wild type levels, despite its inability to produce secreted proteases. Functional gene redundancy could also account for our findings. Such redundancy has been repeatedly shown in *A. fumigatus*, following multiple deletions in large gene families encoding chitin synthases (Muszkieta et al., 2014), α-1,3-glucan synthases (Henry et al., 2012), and oligopeptide transporters (Hartmann et al., 2011).

Interestingly, the *A. fumigatus* strains generated here showed reductions in the expression of 21 of the 23 allergens identified in *A. fumigatus*, including those encoding the proteases Alp1/Aspf13, Alp2/Aspf18, Pep1/Aspf10, and Mep/Aspf5. Allergens encoded by fungal proteases activate an allergic Th-2 response *in vivo* by cleaving airway fibrinogen and generating fragments that act as TLR4 ligands on alveolar macrophages and airway epithelium (Millien et al., 2013). While secreted *A. fumigatus* proteases are not critical for infection of the immunocompromised host, our findings propose that they

could be highly important in determining the response in fungal allergies. This possibility can be further explored with the *A. fumigatus* strains generated in this study.

AUTHOR CONTRIBUTIONS

ES, BH, SH, SA, YS, BF, and TK performed the experiments. AH, AB, OK, and NO conceived and planned the project and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02490/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4 Additional materials and methods

For studying the global comparison of intracellular ATP content (manuscript “ATP Content and Cell Viability as Indicators for Cryostress Across the Diversity of Life”), it was necessary to find out which conditions *A. nidulans* would survive during cryo-conservation. Therefore, the viability of the fungus after cryo-conservation was measured. The results can be found in section 5.1 (Glucose consumption after freezing of *A. nidulans*).

Furthermore, we conducted surface proteomics approach on *Lichtheimia corymbifera* spores. *L. corymbifera* is one of the major causative agents of mucormycosis, a fatal disease especially in immunocompromised patients. Previous studies (266) suggested that the protein surface structures of *Lichtheimia* spores are interacting with immune cells, particularly with murine alveolar macrophages (MH-S). These studies demonstrated the *Lichtheimia corymbifera* strain FSU:09682 is virulent while the strain FSU:10164 is attenuated in a mouse infection model. Further proteomic studies of the spore surface could contribute to a deeper knowledge about the different virulence mechanisms of these two strains and gain insights into the infection mechanism in *Lichtheimia* species (Section 5.2, “Surface proteomics of *L. corymbifera”*).

4.1 Glucose consumption of *A. nidulans* after freezing

A. nidulans strain R21 (*yA2*, *pabaA1*, *veA*) (267) was cultivated in 100 ml *Aspergillus* minimal medium (AMM; (268)). The fungus was incubated for 18 hours at 37°C, inoculated with 10^6 spores/ml. Afterwards the sample was divided into two parts. One half was stopped from growing by incubation at 4°C – 8°C (control), the other half was frozen to -80°C with a controlled cooling rate of about 1°C per minute (sample). Both samples were incubated for 24h. The samples were defrosted over the time-course of 1 minute up to 37°C and transferred into new medium. At the time points of 0, 15, 18, 21, 23 and 38 hours, the glucose concentration of the culture supernatant was determined with the BIOSEN C-Line analyzer (EKF Diagnostic, Germany) according to the manufacturer’s instructions. The measurement was repeated in 3 biological replicates.

4.2 Proteomic analysis of *Lichtheimia corymbifera* spore surface

Freshly collected spores of the two strains *Lichtheimia corymbifera* strains FSU:09682 and FSU:10164 were re-suspended in ammonium bicarbonate buffer (25 mM, pH 8), 10 µg of trypsin (Sigma) was added to 10^8 spores/ml for 30 min at 37°C. The enzymatic reaction was stopped with 1% (v/v) formic acid. Supernatants were filtered (0.22 µm) and stored at -80°C

(269) for LC-MS/MS analysis. In parallel, the trypsin-treated spores were used for a phagocytosis assay.

For LC-MS/MS analysis, the protocol from Baldin *et al.* (270) was applied with the following modifications. After treating the protein samples with 200 mM tris (2-carboxyethyl) phosphine and Iodoacetamide (375 mM), samples were loaded on an Ultimate 3000 nano RSLC system coupled to a QExactive Plus mass spectrometer (both Thermo Scientific Fischer). The pre-concentrated sample was eluted on the analytical column (Acclaim PepMap RSLC, 15 cm x 75 µm, 2 µm). The binary mobile phase consisted of ((A) 0.1% (v/v) formic acid in H_2O and (B) 0.1% (v/v) formic acid in 90/10 ACN/H_2O) to separate the peptides with a 135 min gradient elution: 0-5 min at 4% B, 10-20 min at 4.5-6% B, 25-30 min at 6.6-7.2% B, 35-45 min at 7.9-9.3% B, 50 min at 10.2% B, 55 min at 11.4% B, 60 min at 12.9% B, 65 min at 15% B, 70 min at 17.1% B, 75 min at 20.4% B, 80 min at 24% B, 88 min at 30% B, 94 min at 38% B, 100 min at 55% B, 103 min at 66% B, 106 – 114 min at 96% B, and 115-135 min at 4% B. Positive charged ions were generated at the Nanospray Flex Ion Source (Thermo Fisher Scientific) as described in Baldin *et al.* (270), the mass range of the precursor ions was set to m/z 300 – 1800 at a resolution of 70k full width at half maximum (FWHM) with a maximum injection time of 120 ms and an automatic gain control target of 1 x 10^6 The quadrupole / orbitrap mass analyser worked in Full MS/ddMS^2 (TopN) mode. For data-dependent acquisition, up to eight most abundant precursor ions with an assigned charge of z = 2-6 were selected for further fragmentation by the quadrupole in each scan cycle with an isolation width of m/z 2.0. In the collision cell, the fragments were generated at normalised collision energy of 30 V with nitrogen gas. Precursor ions were excluded dynamically for 35 s. With a maximum injection time of 120 ms and an automated gain control target of 2 x 10^5, the fragment ion resolution was set to 17.5k FWHM. The software Thermo/Dionex Chromeleon Xpress v6.80 SR13 build 3818 and the Thermo QExactive Plus Tune/Xcalibur v3.0.63 2.3 build 1765 graphical interface software was used on for controlling the LC-MS/MS instrument and data acquisition.

The raw files generated by the LC-MS/MS were further processed by the software Proteome Discoverer v1.4.0.288 (Thermo). Tandem mass spectra were searched against the NCBI *Lichtheimia corymbifera* protein database with the algorithm of MASCOT v2.4.1 (Matrix Science, UK), SEQUEST HT, and MS Amanda for database analysis with a maximum of two missed cleavages. The mass tolerances were set to 10 ppm for the precursor ion and to 0.02 Da as a fragment mass tolerance. As modifications, oxidation of Met (variable) and carbamidomethylation of Cys (fixed) were considered. For validation (q value) of the peptide spectra matches, a reverse decoy database and a percolator node were used with a maximum ΔCn of 0.05 and a target false discovery rate of 0.01 (strict) and 0.05 (relaxed). A

minimum of 2 peptides per protein hit were required and only unique peptides were used for quantification. The reporter ions were normalised to the protein median. Spectral counting (normalized spectral abundance factor, NSAF) was performed according to (271). A change in abundance of ≥1.5-fold was considered as significant threshold.

5 Additional results

5.1 Glucose consumption after freezing of *A. nidulans*

In Figure 4, the glucose consumption of *A. nidulans* after freezing and the control were monitored. The control culture consumed glucose only slightly faster than the frozen culture.

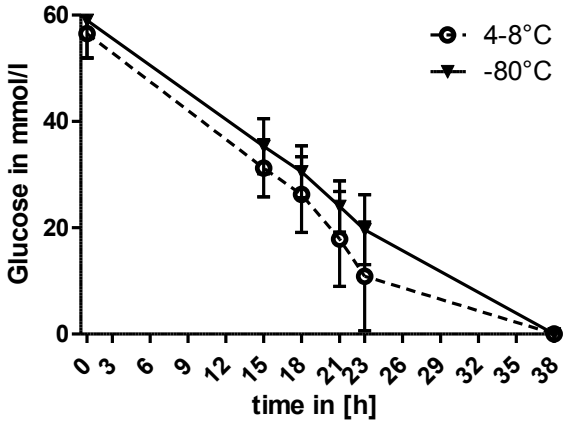

Figure 4: Glucose consumption after freezing of *A. nidulans*. On the x-axis, the time in hours is indicated, on the y-axis the glucose concentration in mmol/l.

5.2 Surface proteomics of *Lichtheimia corymbifera*

To explore which proteins are located on the spore surface of two *L. corymbifera* strains and to investigate whether a difference between the cell surface proteome of the two strains exists, spores were shortly treated by tryptic digestion to release accessible proteins from the cell surface of spores. These surface proteins were identified by liquid chromatography-mass spectrometry (LC-MS/MS). A total of 113 proteins were identified in both samples (Table S1 in attachment) of which 30 were predicted to be located on the surface based on the presence of a secretory signal peptide. Overall the sequence of 14 detected proteins could be annotated based on the presence of functional domains (Table S1 in attachment). The ratio of peptide-spectrum matches (PSM) score to the number of amino acids (AA) of a protein was used as an indicator for the relative abundance of a protein. These quantitative

data revealed differences in abundance of proteins between the two strains, which exhibit different virulence. Among these proteins, the spore coat protein CotH and the hydrophobic surface binding protein HsbA were ten-fold more abundant on the surface of the virulent strain FSU:09682 than on the attenuated strain FSU:10164 (Table S1). Of note, we also found proteins without a signal peptide for secretion which suggests a secretion by non-classical pathways. However, in this study, we focused on surface proteins with an N-terminal signal peptide for secretion. Three CotH proteins were identified on the spores of *L. corymbifera*; namely LCor12344, LCor04095 and LCor04208 (Table 1 in attachment). Based on PhylomeDB (phylomedb.org) data base analysis, these proteins are similar to CotH2/4 of *Rhizopus oryzae* which have been described as virulence factor during interaction with immune cells. For the HsbA protein, four different protein copies were detected. The proteins LCor05141, LCor05147, LCor05809 and LCor07988 were only found on the surface of the virulent strain FSU:09682 and were absent on the surface of the attenuated strain FSU:10164. The similarity between the various copies of the HsbA proteins does not exceed 40% on the protein sequence level as shown in table 2. Genome mining of mucoralean fungi showed that *Mucor circinelloides* and *Rhizopus delemar* have seven and four copies of HsbA proteins, respectively.

Table 2: Comparisons of the protein sequences of the four HsbA proteins that were detected on the surface of *L. croymbifera* spores by proteomic analyses. The highest sequence similarity was detected for the comparions of LCor05147 with LCor05141 (46% similarity). However, the study centred on the proteins LCor05809 and LCor07899 that showed a sequence similarity of 26%.

	LCor05141	LCor05147	LCor05809	LCor07899
LCor05141	100 %	41 %	31 %	39 %
LCor05147	46 %	100 %	28 %	38 %
LCor05809	34 %	38 %	100 %	26 %
LCor07988	36 %	28 %	26 %	100 %

6 Discussion

Fungi play an important role in terrestrial ecosystems. They account for a large proportion of decomposed organic matter as a part of energy cycling (272,273) and interact in a symbiotic manner with the majority of plants, throughout the world (274). A high stress tolerance is crucial to survive harsh environmental conditions such as extreme temperatures (275,276), high osmolarity (277), oxidative stress (278), desiccation (279), radiation (280) and nutrient limitation (281). Fungi have developed a plethora of survival strategies to efficiently adapt to natural stressors. This stress resistance may help fungi to occupy various ecological niches but also to persist and develop in the human host (243,282). Besides the great ecological importance, an exploration of fungal adaptation strategies to low temperatures is also of biotechnological benefit as documented in several studies (283,284). Therefore, a deeper understanding of these strategies is not only scientifically pertinent, but also of great practical relevance. This study investigated for the first time the adaptation of filamentous fungi to low temperature stress by using omics technologies.

6.1 Effect of low temperature stress on *A. nidulans*

In natural environments, *A. nidulans* is exposed to low temperatures in the seasonal cycle. This suggests the establishment of adaptation mechanisms to low temperature stress in *A. nidulans*. We could show that the fungus survives freezing stress at temperatures far below the freezing point of water, without the addition of any cryoprotectant (Figure 4). The study of the *A. nidulans* low temperature response, which results in high tolerance to cold- and cryostress, was the focus of this work.

In a genome-wide comparative approach with different cell types such as bacteria, fungi, algae, plant tissue, as well as plant and human cell lines, the intracellular ATP content was used as an indicator of the physiological state of the cells studied. In parallel, cell viability was monitored. A direct dependence of the intracellular ATP content on the temperature was observed for fungi and algae. Biochemical processes like the ATP-anabolic pathways are temperature-dependent and become less effective at lower temperatures or are even inoperative at freezing temperatures (285,286). Thus, intracellular ATP decreases by energy-consuming pathways, such as maintenance and repair processes that are activated by cold shock damage (287). Apart from reduced ATP levels due to impaired energy-generating metabolic pathways and ATP consumption by repair processes, further reasons are

discussed in the literature. Russell *et al.* (288) and Sobczyk *et al.* (289) described the connection between impaired cellular membrane transport processes and the decrease in intracellular ATP levels. In our study, the intracellular ATP content decreased in dependence of the temperature, but could be restored to initial levels after defrosting and a certain regeneration phase. Psychrophilic organisms such as *Psychrobacter cryohalolentis* and *Psychrobacter aquaticus* showed an already increased intracellular ATP level after defrosting, which indicates a fast initiation of ATP level regeneration and a high cyrostress tolerance. In our study, psychrophilic and cryotolerant bacteria or algae showed a better cultivability after cryo-conservation compared to mesophilic or cryo-sensitive microorganisms. The catabolic pathways in psychrophiles are better adapted to low temperatures due to a lower temperature optimum of metabolic enzymes. Thus, intracellular ATP levels can be rapidly regenerated after cold shock (290). In bacteria and plants, the initial intracellular ATP level even exceeded the initial levels during the regeneration phase. An increased ATP level in plants was found during starch storage and was coupled to an elevated metabolic activity (291). The higher metabolic activity was highlighted by our observation of a higher respiration rate in *A. nidulans* after freezing and subsequent defrosting. Probably, an elevated respiration rate is needed to recover the initial intracellular ATP levels (287). Hence, the intracellular ATP level may indicate an increased activity of catabolic activity, but does not necessarily correlate with the viability of cells after ultradeep freezing. Therefore ATP levels do not allow predicting survival of cells after the exposure to low temperature stress (292).

In summary, a correlation between intracellular ATP levels and decreasing temperatures was observed for *A. nidulans*. An elevated ATP level and increased metabolic activity after a regeneration phase may indicate a mechanism to return to initial values of the intracellular ATP content after cold stress. To further investigate the metabolic changes in *A. nidulans* after a freezing period, multi-omics analyses of the low temperature response were conducted.

6.2 Survival strategies of *A. nidulans* as a stress response to low temperature

The low temperature response of *A. nidulans* was investigated by analysing the transcriptome, proteome and secondary metabolome. Since intracellular ATP levels indicated nearly no metabolic activity at temperatures below the freezing point, a temperature was determined, where the fungus undergoes low temperature stress, but is still growing and metabolically active. The results indicate that the fungus reaches its growth limit at about 10°C, which corresponds to the average temperature in the moderate climate zone (293).

Acclimation strategies are already known from other organisms such as plants (294) or vesicular arbuscular mycorrhiza fungi (295), while strategies of saprophytic filamentous fungi are mainly unacknowledged (260). A common strategy of plants is a rise in the proportion of unsaturated phospholipids in the plasma membrane after 4 weeks of acclimatisation, which leads to higher freezing tolerance (289). Other typical strategies in low temperature-adapted microorganisms and plants are the production of cryo-protective agents (222), cold shock (236,296,297) and anti-freezing (206) proteins or the usage of cold-adapted enzymes (237,238,298,299).

The first step to initiate these strategies is the sensing of temperature changes (241). The multi-omics analysis revealed some possible sensing mechanisms for *A. nidulans*. We found an overrepresented putative glycine-rich RNA-binding protein (GRP) with high similarities (>50%) to orthologues of GRPs in other *Aspergilli*. RNA thermometers are predominantly found in prokaryotes. In eukaryotes, GRPs facilitate the translation of RNA at low temperatures by administering access to the binding site (255). Another common temperature sensing mechanism is the ER-mediated unfolded-protein response (UPR) (243,300) which results in upregulation of heat-shock proteins (HSPs). Most HSPs are hardly characterised in *A. nidulans* and their biological function can be only predicted based on sequence similarities (301). Nevertheless, the function of several enriched HSPs in this analysis could be assigned a putative function. Hsp20 (AN10507) was found to have an unfolded protein binding activity in response to heat shock and was underrepresented at 10°C. Other HSPs have a function in osmoadaptation (Hsp70) or in growth at high temperatures (Hsp90). These findings lead to the conclusion that a UPR-mediated temperature perception is rather unlikely at low temperatures and may be more important for a heat shock stress response. On the other side, *A. nidulans* induces heat shock genes at 10°C, which indicates the utilisation of heat shock response mechanisms also at low temperatures. Noteworthy, the heat shock response regulator HSF1 (AN8035), which triggers several cytoprotective genes at elevated temperatures (302), was overrepresented at low temperature.

The induction of cytoprotective response genes is necessary because temperature stress is usually accompanied by damages on the cellular and molecular level. At low temperatures, these damages can be caused by desiccation or osmotic stress for example (236). In *Cryptococcus flavescens*, cold adaptation leads to an increased liquid hyperosmotic shock tolerance and an improved desiccation tolerance (303). In our omics data, we also observed an increase in osmotic stress-related identifiers (genes and proteins, IDs) such as IDs from the high-osmolarity glycerol (HOG) signalling pathway and additional IDs connected to osmotic shock tolerance.

Another low temperature effect is that cellular processes operate below their temperature optimum (304,305), which promotes a decreased enzyme activity (306) because of the lower turnover rates (276). This consequently interferes with transcription and protein biosynthesis and causes a disturbance of the protein structure (307,308). Thus, cellular functions are compromised, and as a result increased oxidative stress can be detected (309,310) and repair mechanisms are induced (235,311,312). We observed an induction of several cellular repair systems represented by IDs in the categories DNA repair or DNA damage response. Damages also result from the formation of reactive oxygen species (ROS). Li *et al.* (313) reported on an accumulation of ROS after low temperature exposure in the plant *Jatropha curcas* and also described an increased chilling (5°C) tolerance of cells with elevated antioxidant enzyme activity levels. We determined an enrichment of IDs in the category of oxidative stress response at low temperature stress in *A. nidulans*. Moreover, IDs such as chaperones and RNA or DNA stabilizing IDs were overrepresented.

Damages may also occur from a reduced fluidity of the cellular membrane. Several examples found in the literature state that a decrease in temperatures leads to a re-organisation (203,314,315) or stabilisation (225) of the cell membrane to ensure flexibility *e.g.* by increasing the membrane fluidity at lower temperatures (203). Unsaturated fatty acids, a reduction of the average chain length of fatty acids and membrane lipid modification, *e.g.* mediated by desaturases, contribute to an increased membrane fluidity (316). It was demonstrated in *Synechocystis sp.* PCC 6803 that a decrease in temperature from 34°C to 22°C increased the level of desaturases by 10-fold (317,318) and conferred cold hardiness (319,320). Our results show a downregulation of IDs involved in sterol metabolism or desaturase activity at 10°C. Suutari and Laasko (321) made a similar observation after incubating *Bacillus megaterium* at 10°C and observed a lower amount of unsaturated fatty acids in comparison to incubation at higher temperatures. However, additional measurements revealed that the overall melting temperature of fatty acids decreased. Hence, the production of sterols, fatty acid desaturases and unsaturated fatty acids does not necessarily imply a higher membrane fluidity or *vice versa*. Accordingly, further experiments are required to elucidate the modification of the cytoplasmic membrane of *A. nidulans* at low temperature stress. Additionally, membrane stabilisation can be accomplished by incorporating cryoprotectants such as trehalose (212), which, in addition, can also prevent ice crystal formation by lowering the freezing point of intracellular fluids. Furthermore, anti-freezing proteins or osmolytes maintain enzyme activity and therefore reduce the low temperature stress. Our omics data-sets and results on freezing the fungus without addition of any cryoprotectant suggest that *A. nidulans* may use sugar entities as cryoprotectants and therefore benefits from an increased low temperature tolerance.

In summary, the analysis of the stress response to low temperatures in *A. nidulans* reveals several protective mechanisms. *A. nidulans* is able to restore the intracellular ATP content after being frozen and may accomplish the molecular requirements for the ability to sense temperature changes, *e.g.* by producing several HSPs or putative GRPs. Induced protective mechanisms include the activation of RNA/protein chaperones, an elevated antioxidant enzyme activity, DNA repair proteins, osmotic stress response proteins and enzymes of the trehalose biosynthesis pathway. In summary, the fungus prevents and repairs cellular damages that occur during low temperature stress. Furthermore, *A. nidulans* produces the cryoprotectant trehalose (75), which maintains membrane stability, enzyme activity, and prevents ice crystal formation.

6.3 Low temperature stress triggers a change in cell development of *A. nidulans*

Further strategies to survive temporal low temperature conditions include morphological changes or entry into dormancy. In our study, we observed a morphological change of the fungus at 10°C. On microscopic level, Hülle cell formation was observed, which is known to be accompanied by an increase in intracellular ROS (322). ROS levels can be regulated by catalases and peroxidases (323) by converting harmful H_2O_2 into oxygen and water. NADPH oxidases (Nox), which are also involved in ROS generation and play a role in cell differentiation, were found to be not significantly regulated in our omics data. We further assume that low temperature exposure promotes the production of ROS, resulting for instance from mitochondrial ROS which are not sufficiently detoxified due to a decreased biological activity at low temperatures. Consequently, the level of antioxidative enzymes is increased during low temperature stress (324). Our multi-omics data suggest that temperature stress triggers a switch from asexual to sexual development in *A. nidulans*. It is known in ascomycetes that factors such as nutrients, light, temperature, aeration, and pH commonly promote fruiting body development (96). In *A. nidulans*, nitrogen starvation, low oxygen levels, and a light-dependent activation of the velvet gene *veA,* are known mechanism for inducing sexual development. In this study, a *veA*$^{-}$ strain with a non-functional velvet gene is used and we showed that low temperature is an inducer for sexual development in *A. nidulans*. No nitrogen starvation or a low oxygen level were applied in this study, nevertheless we observed sexual development. This indicates that another, yet unknown mechanism for the regulation of sexual development exists. Therefore, low temperature stress is suggested to contribute to the initiation of sexual development in *A. nidulans*.

It is known that sexually derived ascospores show an elevated stress resistance compared to asexually derived conidia (325). The thicker cell wall, two nuclei, increased levels of HSPs, and an elevated level of sugars, such as trehalose, explain the higher stress resistance of ascospores compared to conidia (112,325,326). Additionally, fruiting bodies are more resistant against fungivores in comparison to conidia or vegetative mycelium. Therefore, the morphological change to fruiting bodies favours not only an avoidance of low temperature exposure in a sensitive growth phase (vegetative mycelium), but also provides further advantages for the fungus such as an elevated thermo-resistance or an increased ability to withstand desiccation (112,325). Hence, *A. nidulans* may benefit from a change in cell development at low temperature stress with an increased fitness and therefore a higher possibility to claim its ecological niche. The production of the stress-resistant ascospores (325) enables *A. nidulans* to withstand winter cold in temperate zones.

In summary, low temperature stress contributes to an induction of sexual development in *A. nidulans* and we suggest the presence of a new mechanism to trigger this development. The switch from asexual to sexual reproduction may improve fungal survival in harsh environmental conditions by changes in fungal morphology.

6.4 Low temperature stress induces distinct SM profile in *A. nidulans*

In fungi, secondary metabolism is known to be associated with cell development. Many coregulations of cell development and secondary metabolism by transcriptional regulators are described for *A. nidulans* (81,105,327). Bayram *et al.* (81) showed that the velvet complex consisting of VelB/VeA/LaeA is a light-responding regulator for cell development in *Aspergillus* and the regulation of the *velA* - *velC* genes is accompanied by a SM expression (105). The velvet gene *veA,* for instance, is involved in the regulation of sexual fruiting body formation, but also induces the production of sterigmatocystin and further SMs (132). This indicates a connection between low temperature-induced cell development and SM production.

In our study, we found SMs typically associated with asexual as well as sexual development at an incubation temperature of 10°C. Emericellamide and terrequinone A are commonly known for sexual development, and asperthercin can be found during asexual development (328). This indicates that the induction of sexual development may not be the only factor that affects secondary metabolism at low temperature stress. Low temperature itself may be a direct or indirect trigger of secondary metabolite production. Marine-derived fungi, for

instance, showed an induced SM production during increased osmotic stress (329), which may also trigger SM production at low temperatures.

The altered SM profile at 10°C may contribute to ecological fitness in natural environments (144,145). This is emphasized by the production of so-far uncharacterised SMs with an inhibitory effect on other microorganisms such as fungi and Gram-positive bacteria. Hence, metabolic differentiation may represent a defence mechanism against competitors under severe growth conditions. It has been hardly proven yet that SMs serve as defence mechanism against predators in the natural environment (330). Rohlfs *et al.* (331) studied the relationship of *A. nidulans* under the attack of the fungivorous springtail Folsomia candida. The authors found that the production of SMs grants a certain protection to the fungus and the arthropod prefers feeding on the mutant strain of *A. nidulans* that is deficient in the *laeA* gene. This ultimately regulates the cell development and expression of several SMs in this fungus (332). Döll *et al.* (333) investigated grazer-challenged *A. nidulans* cultures and observed a higher production of toxic SMs as an adjustment to the fungivore environmental challenge. Upon grazer challenge, a sexual reproduction was triggered, which led to an increased survival of the fungus. Therefore, it is possible that synthesis of SMs is induced as a defence and protective mechanism in *A. nidulans* after the ecological challenge of low temperature stress. Low temperatures can induce secretion of SMs which have the ability to inhibit the growth of natural enemies. Thus, the fungus can prevent to be overgrown and is able to defend its ecological niche. Other functions of SM differentiation may help to acquire nutrients (334,335) such as the secretion of siderophores for iron uptake. It is also possible that SMs serve as signal molecules, *e.g.* to regulate cell development in *A. nidulans* (327). Linoleic acid-derived compounds such as PpoA for instance coordinate the sporulation of *A. nidulans* (126,336). In summary, low temperature stress leads to an altered SM profile of *A. nidulans*. The SM production is possibly linked to cell development or is a direct or indirect effect of the low temperature stress response. Previously uncharacterised SMs with an inhibitory effect on other microorganisms are produced. Most likely, this leads to an increased ecological fitness under low temperature stress.

6.5 Regulation of extracellular proteases in *A. fumigatus*

The human body is a further habitat which can be colonised by *Aspergilli*. Since *Aspergilli* are ubiquitous fungi, the spores from *A. fumigatus* for instance are dispersed widespread in nature and frequently inhaled by humans (12). After inhalation, the spores start to germinate in the immunocompromised host, where they have to overcome different obstacles such as attack by the residual human immune system or nutrient limitations. This work explored the role of secreted proteases in the pathogenesis of *A. fumigatus*.

Extracellular protease activity is mainly controlled by global regulators. The *A. fumigatus* genome encodes the TF PrtT and XprG. PrtT is the major regulator for the secretion of extracellular protease activity, while XprG plays a minor role. However, this is in contrast to the situation in other *Aspergilli*. For instance, in *A. nidulans*, PrtT is not present and the secretion of proteolytic enzymes is mainly controlled by XprG. The double mutant Δ*XprG/*Δ*PrtT* in *A. fumigatus* has nearly no remaining proteolytic activity. Nevertheless, the fungus is still able to penetrate the epithelium and the transcriptional regulators PrtT and XprG are not essential for maintaining virulence in a mouse model of invasive aspergillosis. Of the 50 putative secreted proteases, 24 are underrepresented in the double mutant, including 5 proteases which are induced during *in vivo* infection (337). It is possible that the residual 26 putative proteases that are not underrepresented, and the reduced amount of the 24 underrepresented proteases, are sufficient for degrading the host tissue *in vivo*. This indicates the high functional gene redundancy in substrate-degrading proteases. The saprophytic life style of *Aspergilli* explains the importance for organic matter-degrading enzymes and emphasizes from an evolutionary point of view the priority for the high redundancy of these enzymes. Examples for functional gene redundancies are commonly found in large gene families such as chitin synthases (338), α-1,3-glucan synthases (339), and oligopeptide transporters (69). Alternatively, secretion of proteases may be induced *in vivo* by alternative mechanisms, which compensate the reduced secretory activity of the deleted genes *prtT* and *xprG*. Additionally, *in vivo* stress may activate alternative virulence determinants that are lacking in the wild type, such as the synthesis of toxic substances. Hagag *et al.* (340) described an upregulation of four SM clusters in the deletion mutant Δ*PrtT.* Independent of the reduced protease secretion in the double mutant, formation of toxic compounds such as pseurotin A and others could increase the virulence of this mutant strain.

As shown for the deletion mutant Δ*PrtT* (340)*,* the double mutant Δ*XprG/*Δ*PrtT* also shows side effects apart from the reduced extracellular protease activity. The double mutant shows a reduced expression of 21 of the 23 allergens identified in *A. fumigatus*. *In vivo*, cleavage of fibrinogen by fungal allergens (341) cause an allergic Th-2 response by acting as Toll-like receptor 4 ligands on alveolar macrophages and airway epithelium (342). Thus, our findings provide a good platform to determine the allergy response to fungi in more detail.

Briefly, secreted proteases regulated by the transcription factors XprG and PrtT in *A. fumigatus* are not critical for the infection of an immunocompromised host, although the double mutant Δ*XprG/*Δ*PrtT* has almost no detectable secreted protease activity. However, the fungus can still grow in the host, probably as a result of the high functional gene

redundancy in proteolysis. The observation of a reduced allergen expression may be important for investigating the fungal allergy response in future studies.

6.6 Surface protein HsbA as a virulence factor in *L. corymbifera*

Another fungus which can cause invasive infections in humans is the mucoralean fungus *Lichtheimia corymbifera*. It can cause mucormycosis with high rates of mortality, especially in patients suffering from immunodeficiency (343). The infection process of *L. corymbifera* is not fully understood yet. After entering the human body, the fungus can be recognised by the immune system by pattern recognition receptors. Some pathogens developed strategies to avoid recognition by the immune system by masking their surface, as shown for the fungal pathogens *Cryptococcus neoformans* (344), which uses galactoxylomannan and glucuronoxylomannan as a capsule. Another example is *A. fumigatus*, preventing the immune recognition of spores by various immune cells with the surface-hydrophobin RodA (345,346). However, it has not been elucidated yet, whether similar mechanisms exist in spores of *L. corymbifera*.

Therefore, we identified the surface proteins that vary in two *L. corymbifera* strains, which may explain their difference in virulence and recognition by the immune system. Annotation was carried out dependant on functional domains of the proteins. Proteins which showed differences in protein abundance between the two strains were three copies of the spore coat protein homologues CotH (LCor12344, LCor04095 and LCor04208) and four copies of hydrophobic surface binding protein A, HsbA (LCor05141, LCor05147, LCor05809, and LCor07988). CotH homologues are the most conserved proteins on the surface of mucoralean fungi and facilitate their invasion of host cells. Furthermore, CotH was reported to be recognised by the GRP78 receptor on the surface of endothelial cells (347). HsbA orthologues are antigenic galactomannoproteins that are exclusively detected in Mucormycotina and Eurotiales fungi and are highly distributed among various mucoralean species (348). HsbA proteins were identified as virulence factor for several fungal species during different interactions, including both fungal-plant and fungal-animal interactions. In the interaction of *Colletotrichum fructicola* with strawberry plants (*Fragaria* × ananassa Duch) (349) and *Magnaporthe oryzae* infection of rice (*Oryza sativa*) (350), HsbA may be involved in appressorium development. Moreover, HsbA played a role in the formation of fungal hyphae in the entomopathogenic fungus *Beauveria bassiana* during the infection of the host insect *Frankiniella occidentalis* (351). In *Talaromyces marneffei*, the HsbA proteins bind to lipid structures, such as arachidonic acid, and downregulate a cytokine production (352). Further studies indicated that HsbA has the capability to degrade hydrophobic structures (353) and plant biomass (354).

In summary, HsbA proteins are moonlighting proteins that have various functions in preventing immune recognition and mediating adhesion. However, the interaction of HsbA with immune cells has not been studied on the molecular level yet. Therefore, our findings form a good basis for investigating the contribution of the spore surface structure to the virulence of *L. corymbifera*.

On this basis, future experiments are planned to heterologously overexpress the LCor07988 HsbA protein in *Pichia pastoris* for further experiments. In addition, the interaction of the LCor07988 HsbA protein with immune cells such as the human monocytic cell line THP-1, human cell line Mono Mac 6 and murine alveolar macrophages (MH-S) will be investigated. These additional experiments should address the differences in the interaction of the immune system with the virulent (FSU:09682) and attenuated (FSU:10164) *L. corymbifera* strains (virulent in a mouse model). Our aim is to find out whether the HsbA protein can inhibit phagocytosis and contribute to the survival of *L. corymbifera* inside host cells. This may help to develop new therapeutic tools against mucoralean fungi.

In summary, our analysis revealed two differentially expressed proteins in the two *L. corymbifera* strains, which are assumed to contribute to the virulence of these strains. The protein CotH is known for its ability of masking the pathogen cell surface, facilitating an invasion to the host cells. The second protein of our interest, HsbA, exhibits properties that may contribute to the virulence of *L. corymbifera*.

7 Conclusion and future prospects

The main foci of this thesis were the investigation of the adaptation strategies of *A. nidulans* and *A. fumigatus* to harsh environmental conditions. In *A. nidulans*, I investigated the adaptation to low temperatures; in *A. fumigatus* I studied the regulation of protease secretion, which is essential for host tissue degradation.

First, I elucidated the viability of *A. nidulans* at temperatures down to -80 °C. Based on the findings, we hypothesised that the fungus utilises an elevated energy metabolism to provide ATP for repairing processes which are essential for the survival of low temperatures and cryostress. The physiological changes during low temperature adaptation were investigated further by multi-omics analyses of the low temperature response of *A. nidulans*.

The omics analysis revealed a plethora of protective mechanisms which were induced in response to low temperatures and which may help the fungus to survive these harsh conditions. The mechanisms include the activation of chaperones, an elevated abundance of antioxidant enzymes, DNA repair proteins, osmotic stress response proteins and elevated

trehalose levels in the cells. Other studies reported on the induction of osmotic stress response pathways and accordingly an increased osmotic stress tolerance after exposure to cold. In line with this observation, we found several osmotic stress-related proteins such as proteins involved in the HOG signalling pathway and in osmoadaptation. Additionally, our results proposed that the saturation status of membrane fatty acids may not be altered at low temperatures. The incorporation of sugars may be the major mechanism to maintain the membrane stability. We suggested the presence of mechanisms for sensing temperature changes which would be a good target for additional experiments. The analysis of the putative GRP AN2989 for instance could unveil the role of GRPs in *Aspergilli* and contribute to a better understanding of the temperature perception in filamentous fungi. In conclusion, the gained knowledge may also contribute to an optimised process of cryo-conservation in filamentous fungi.

Beside strategies which could directly be assigned to protection against damages occurring at low temperatures, decreased growth temperature triggered sexual development in *A. nidulans*. In the literature, an induction of a light-induced sexual development is described *via* the velvet gene *veA*. We used a *veA*$^{-}$ strain which does not possess a functioning velvet gene and therefore propose another, so-far uncharacterised low temperature-based induction of sexual development. In summary, this work delivers a good basis for further investigation to deepen the knowledge about the signalling pathways triggering cell development.

Another effect of applying low temperature stress to *A. nidulans* is an altered SM profile and an induced production of several SMs. We suggest that a distinct SM profile leads to an increased ecological fitness under harsh environmental conditions, which is emphasised by the inhibitory effect of SMs on other microorganisms. These SMs may protect against natural competitors in cold seasons and may prevent being overgrown in a phase where the fungus has slowed down its metabolism. Low temperature stress induction may represent a valuable strategy to exploit the potential of filamentous fungi as sources of novel SMs and the combined knowledge of the low temperature stress response and SM production may assist to improve fermentation of filamentous fungi at low temperatures.

The second focus of this work was the response of *A. fumigatus* to nutrient limitations inflicted by the host by investigating the transcriptional regulators XprG and PrtT. A detailed protein profile of each single mutant and the double mutant was created. The double mutant Δ*XprG*/Δ*PrtT* showed reduced conidiogenesis and an impaired ability to degrade substrate proteins but was still able to invade the immunocompromised host. We showed that proteases whose secretion was regulated by XprG and PrtT are not crucial for establishing an infection in the immunocompromised host. The consistent virulence in the mouse model

may result from the high gene redundancy in proteases. Further analysis of the role of remaining secreted proteases in destroying host tissue could contribute to evaluate the importance of extracellular proteins in the infection process of *A. fumigatus*. Further on, alternative virulence determinants triggered by the lack of the regulators XprG and PrtT, may lead to an increased production of SMs in the Δ*XprG/*Δ*PrtT* strain, which may compensate the depletion in protease activity. Although the double mutant did not lead to a reduced virulence in the mouse infection model, our proteomic data set demonstrated the importance of the regulators XprG and PrtT for determining the fungal allergy potential. 21 of 23 known fungal allergens were underrepresented in the double mutant Δ*XprG/*Δ*PrtT*. Hence, the *A. fumigatus* Δ*XprG/*Δ*PrtT* strain can be used in future studies of the allergic response to *A. fumigatus*.

Overall, the studies of *Aspergilli* in extreme environments provide insights into novel pathways and mechanisms for gene regulations and the application of adaptation strategies to different environmental triggers. These discoveries offer new possibilities for therapeutics, fermentation processes and basic research in the fields of developmental and immune biology.

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10 Eigenständigkeitserklärung

Die geltende Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena ist mir bekannt. Die vorliegende Dissertation habe ich selbständig verfasst und dabei weder Textabschnitte aus einer eigenen Prüfungsarbeit oder von dritten ohne Kennzeichnung übernommen. Es wurden keine anderen als die von mir angegebenen Quellen, persönliche Mitteilungen und Hilfsmittel verwendet. Ich versichere, dass die aufgelisteten Publikationen und Manuskripte, ausschließlich auf Ergebnissen beruhen, die während meiner Promotion generiert wurden.

Bei der Auswahl und Auswertung des Materials, wie auch bei der Herstellung der Manuskripte haben mich die in der Danksagung meiner Dissertation genannten Personen unterstützt.

Personen, die bei der Anfertigung der Publikationen und Manuskripte beteiligt waren, sowie deren Eigenanteil sind gekennzeichnet. Die Hilfe eines Promotionshelfers wurde nicht in Anspruch genommen. Auch haben Dritte weder unmittelbar noch mittelbar geldwerte Leistungen, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, erhalten.

Ich habe die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Ferner habe ich nicht versucht, diese Arbeit oder eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation einzureichen.

Jena, den 24.10.2018

Benjamin Hanf

11 List of publications

1. Lota F, Wegmüller S, Buer B, Sato S, Bräutigam A, **Hanf B**, Bucher M. 2013. The cis-acting CTTC-P1BS module is indicative for gene function of LjVTI12, a Qb-SNARE protein gene that is required for arbuscule formation in *Lotus japonicus*. Plant J 74:280–93.
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8. Bajerski F, Stock J, **Hanf B**, Darienko T, Heine-Dobbernack E, Lorenz M, Naujox L, Keller J, Schumacher HM, Friedl T, Eberth S, Mock HP, Kniemeyer O, Overmann J, The effect of cryostress on ATP content and cell viability across the diversity of life submitted in Frontiers in Physiology | Environmental, Aviation and Space Physiology

12 List of public presentations

1. **Hanf B**, Krüger T, Hagag S, Osherov N, Brakhage AA, Kniemeyer O. Role of the two transcriptional regulators *prtT* and *xprG* on the secretome of *Aspergillus fumigatus*. International Leibniz Research School (ILRS) Symposium (2014), Jena, Germany (Poster presentation).
2. **Hanf B**, Krüger T, Mattern D, Kniemeyer O, Brakhage AA. Adaptation of the filamentous fungus *Aspergillus nidulans* to low temperature stress. Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM) (2015), Marburg, Germany (Oral presentation).
3. **Hanf B**, Krüger T, Mattern D, Kniemeyer O, Brakhage AA. Low temperature stress activates proteins involved in the sexual development of the filamentous fungus *Aspergillus nidulans*. Proteomic Forum (2015), Berlin, Germany (Poster presentation).
4. **Hanf B**, Krüger T, Mattern D, Kniemeyer O, Brakhage AA. Adaptation of the filamentous fungus *Aspergillus nidulans* to low temperature stress. Vereinigung für Microbial Communication (MiCom) (2015), Jena, Germany (Oral presentation).
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6. **Hanf B**, Krüger T, Mattern D, Kniemeyer O, Brakhage AA. Adaptation of the filamentous fungus *Aspergillus nidulans* to low temperature stress. ILRS Symposium (2015), Jena, Germany (Oral presentation).

7. **Hanf B**, Krüger T, Mattern D, Kniemeyer O, Brakhage AA. Adaptation of the filamentous fungus *Aspergillus nidulans* to low temperature stress. Society for Cryobiology (Cryo) (2015), Ostrava, Czech Republic (Oral presentation).
8. **Hanf B**, Mattern D, Krüger T, Kniemeyer O, Brakhage AA. Activation of natural product gene clusters of *Aspergillus nidulans* at low temperature stress. DECHEMA - European Conference on Natural Products (2015), Frankfurt, Germany (Poster presentation).
9. **Hanf B**, Krüger T, Mattern D, Bunk B, Overmann J, Kniemeyer O, Brakhage AA. Adaptation of the filamentous fungus *Aspergillus nidulans* to low temperature stress. MiCom (2017), Jena, Germany (Poster presentation).
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15. **Hanf B**, Krüger T, Mattern D, Bunk B, Overmann J, Kniemeyer O, Brakhage AA. Adaptation of the filamentous fungus *Aspergillus nidulans* to low temperature stress. VAAM (2018), Wolfsburg, Germany (Oral presentation).