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„Utilization of the fungal surface-active protein
hydrophobin as a tag for monitoring physiological
responses of *Trichoderma* to biotic and abiotic
influences“

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1 Abstract

Trichoderma is a genus of over 300 species with versatile physiologies widespread across the globe and habitats. Several *Trichoderma* species are important in industry and agriculture, but some can also pose a threat to human health and applications. Understanding *Trichoderma* physiology and fungal-fungal interactions is vital for perfecting its application and understanding its challenges. The *Trichoderma* genome is enriched in small, secreted, amphiphilic, surface-active fungal proteins called hydrophobins, which are thought to play an integral part in the environmental opportunism of this genus, as it facilitates spontaneous hydrophobic switching of the fungal mycelial surface. In this thesis *Trichoderma guizhouense* overexpressing a hydrophobin gene encoding the hydrophobin 8 protein (HFB8) with an mRFP tag (Tgui_{OEhfb8}) was used to investigate *Trichoderma* fungal-fungal interactions. The heterologous *hfb8::mrfp* construct was controlled by the P_{cdna1} promoter from *T. reesei*, which is widely used in heterologous enzyme production in *Trichoderma*. However, the function of the native gene – *cdna1*, has so far not been studied thoroughly. To test the influence of ambient stresses on P_{cdna1} regulation, Tgui_{OEhfb8} was exposed to several environmental factors. P_{cdna1} activation was measured by extracting HFB8_{mRFP} and measuring mRFP intensity, as well as measuring transcription levels of the *cdna1* gene in three *Trichoderma* species by qPCR. The analysis of hyphal interactions between *T. guizhouense* and *Fusarium oxysporum* (FOC4) did not show any penetration or hyphal degradation, but revealed close hyphal contact, which was initiated by FOC4. P_{cdna1} was found to be ambiguously influenced by several ambient factors and is believed to be regulated by a complex interplay of different stress factors and cellular pathways. Its regulation further appears to be strongly strain-dependent. The overexpression of HFB8_{mRFP} under P_{cdna1} resulted in a strong phenotype including slower growth rate and reduced surface hydrophobicity (hydrophilic) of *T. guizhouense*. This is most likely the result of the downregulation of other regulatory pathways. HFB8_{mRFP} is heterogeneously distributed across the *T. guizhouense* mycelial surface and accumulates in vacuoles, which might hint at a specialized secretory pathway for hydrophobins. As HFB8_{mRFP} does not stay attached to the cell wall and was found to accumulate at the surface of the fungal interaction partner, it is not suitable for the monitoring of the fungal-fungal interactions. This thesis gave an insight into the interaction of *T. guizhouense* with FOC4 that was previously known as mycoparasitic with FOC4 being a host. Further, the accumulation of HFB8_{mRFP} gave a potential hint at a specialized secretory pathway of hydrophobins, which could result in the discovery of important properties of these essential proteins. And lastly, the study of the P_{cdna1} promoter resulted in strong evidence that it is not constitutive and that its underlying gene *cdna1* might play a part in *Trichoderma* stress response.

2 Kurzfassung

Trichoderma enthält über 300 Arten vielseitiger Physiologien, weltweit verbreitet über diverse Lebensräume. Etliche *Trichoderma* Arten sind wichtig für Industrie und Landwirtschaft, manche können jedoch eine Gefahr für menschliche Gesundheit und Anwendungen darstellen. Das Verständnis der Physiologie von *Trichoderma* und seiner Interaktionen mit anderen Pilzen ist für dessen Anwendung und das Überwinden seiner Herausforderungen von entscheidender Bedeutung. Das *Trichoderma* Genom ist angereichert mit kleinen, sezernierten, amphiphilen, oberflächenaktiven Proteinen genannt Hydrophobine, von denen angenommen wird, dass sie einen wesentlichen Beitrag zum Umwelt-Opportunismus dieser Gattung leisten, da sie das spontane hydrophobe Schalten der Pilzoberfläche ermöglichen. Zur Untersuchung von Pilz-Pilz-Interaktionen wurde In dieser Arbeit *Trichoderma guizhouense* verwendet, welches ein Hydrophobin-Gen, das das Hydrophobin 8 Protein (HFB8) kodiert, mit einem mRFP-Tag überexprimiert (TguiOE_{Hfb8}). Das heterologe *hfb8::mrfp* Konstrukt enthält den P_{cdna1} Promotor von *T. reesei*, der in der Enzymproduktion in *Trichoderma* weit verbreitet ist. Die Funktion seines nativen Gens *cdna1* wurde jedoch bisher nicht untersucht. Um den Einfluss von Stressfaktoren auf die P_{cdna1} Regulierung zu testen, wurde TguiOE_{Hfb8} mehreren Faktoren ausgesetzt. Die Aktivierung von P_{cdna1} wurde durch Extraktion von HFB8_{mRFP} und Messen der mRFP-Intensität sowie durch die Bestimmung des Transkriptionslevels von *cdna1* in drei *Trichoderma* Arten durch qPCR gemessen. Die Interaktion zwischen *T. guizhouense* und *Fusarium oxysporum* (FOC4) zeigte keine Penetration oder Abbau von Hyphen, jedoch engen Kontakt, der durch FOC4 initiiert wurde. Es wurde festgestellt, dass P_{cdna1} durch Umweltfaktoren beeinflusst wird. Es wird angenommen, dass der Promoter durch ein komplexes Zusammenspiel verschiedener Stressfaktoren und zellulärer Mechanismen reguliert wird, was auch stark vom *Trichoderma* Stamm abhängig zu sein scheint. Die Überexpression von HFB8_{mRFP} unter P_{cdna1} führte zu einem starken Phänotyp, einschließlich einer langsameren Wachstumsrate und verringerter Hydrophobie (hydrophil) des *T. guizhouense* Mycel. Dies ist vermutlich das Ergebnis der Herunterregulierung anderer regulatorischer Mechanismen. HFB8_{mRFP} ist heterogen über die Myzeloberfläche von *T. guizhouense* verteilt und reichert sich in Vakuolen an, was auf einen speziellen sekretorischen Pfad für Hydrophobine hindeuten könnte. Da HFB8_{mRFP} nicht in der Zellwand verankert ist und sich an der Oberfläche des Pilz-Interaktionspartners ansammelt, ist dieses Konstrukt nicht für die Untersuchung von Pilz-Pilz-Interaktionen geeignet. Diese Arbeit gab einen Einblick in die bisher als mykoparasitisch beschriebenen Interaktionen von *T. guizhouense* mit FOC4, wobei FOC4 bisher als Wirt galt. Darüber hinaus gab die Akkumulation von HFB8_{mRFP} in Vakuolen einen potenziellen Hinweis auf einen spezialisierten sekretorischen Weg von Hydrophobinen, was wichtige Eigenschaften dieser essentiellen Proteine aufdecken könnte. Und schließlich ergab die Studie des P_{cdna1} Promotors starke Beweise dafür, dass er nicht konstitutiv ist und dass sein natives Gen *cdna1* eine Rolle in der *Trichoderma* Stressreaktion spielen könnte.

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3 Introduction

Fungi are ubiquitously found in all environments. They can live in soils, on dead organic matter, in aquatic environments and the phyllosphere. They are heterotrophic organisms that feed by absorbing nutrients through their cell wall. This is typically facilitated by the secretion of metabolic enzymes into their environment, that break down macronutrients into small, soluble molecules. Fungi are important decomposers in many ecosystems, as they can live saprotrophically, feeding on dead organic material. However, they can also feed on living biomass, which leads to frequent biotrophic interactions with other organisms [1].

Fungi are well known for their preference to form symbiotic relationships with other microorganisms, plants (mycorrhizae) or algae (lichens) [1]. The exposure to a diversity of different environments and interactions with other organisms harbours a great source of stress for fungi. The ability to survive- or even thrive in- such changing conditions greatly depends on the adaptability to these external influences. Often, these adaptations can result in drastic changes in fungal physiology.

Due to their versatile physiology fungi are important for human applications. They are widely used in agriculture (biofertilizers, and biopesticides and bioremediators) [2]–[4] and food industry (food production)[5], [6] and have proven vital to improving world health as producers of bioactive compounds such as immunomodulators or antibiotics [7]–[9]. Beside their generally beneficial properties for the environment, some biotrophic fungi can also cause diseases in plants [10] and animals, including humans [11].

Unravelling fungal responses to biotic and abiotic environmental factors is essential not only for the understanding of basic fungal physiology, but it will also improve the efficiency of fungi-based technologies in modern industry and pharma.

3.1 Fungi and environmental stress

Stress signals, or stressors, can indicate unfavourable conditions or potential threats to a cell. A cell under stress can undergo different physiological and molecular changes in response to a stressor. These changes can be aimed at preventing or minimizing damage to the cell upon exposure to a stressor or can aim at adapting to changed environmental conditions [12].

Fungi show an extensive capability to adapt to various environmental stresses. They do so by increasingly adapting to mildly unfavourable conditions, and thus acquiring resistance to a wider range of potentially forthcoming stresses [13], [14]. These acquired “immunities” can even be transferred to progenies, thus driving evolutionary development and increasing competitive strength [15].

Environmental stresses do not only drive evolutionary development in fungi, they can also be responsible for the development of novel biotic interactions. On the one hand, environmental stress

can force fungi to develop new ways of energy acquisition or nutrient utilization. Engaging in a symbiotic relationship might facilitate access to a diverse set of enzymes for breakdown of complex nutrient sources, without the need to invest excessive energy. On the other hand, the fungi's adaptability to stresses enables close contact with interaction partners, which might produce stressors in the form of toxins for self-defence [16].

Stress response is not only important for the understanding of interactions and reactions of fungi living in natural environments. Fungi used for industrial production of enzymes or other biotechnological products are cultivated in "unnatural", and therefore highly stressful, conditions. This is important to keep in mind, as stress response might result in the unwanted production of secondary metabolites or negatively affect yield [17]–[19].

3.1.1 Toxicity of reactive oxygen species is concentration dependent

Cellular stress can impact the levels of reactive oxygen species (ROS) that are naturally produced by cells. These are highly reactive chemical compounds produced for different purposes in varying concentrations as by-products of energy metabolism, mostly in aerobic respiration, where oxygen serves as a terminal acceptor for oxidative phosphorylation, which is necessary for ATP production [20], [21]. ROS are important for many cellular processes, such as signalling, proliferation and differentiation [20]. Under stressful conditions, ROS levels can dramatically increase and become toxic to the cell, potentially damaging DNA, proteins, lipids and other cellular structures [22].

To prevent cellular damage by ROS, many organisms have developed antioxidant systems that enable the efflux or detoxification of radical compounds [20]. These systems include ABC efflux transporters [23] as well as many reducing enzymes and redox pathways [20].

Many environmental factors can act as stressors. However, these external influences don't always have to induce a state of distress by elevating the levels of free radicals to toxic concentrations. External factors can simply act as signals for differential activation of specific cellular pathways. Chemical agents (e.g. toxins), osmotic stress, climate or the reduced availability of energy sources can, among others, all cause specific cellular responses. In the following a few of the most important and common environmental signals and stressors are described.

3.1.2 Oxygen- vital for survival but highly toxic

Depending on its concentration, oxygen can be vital for cellular survival or cause severe damage. Different microorganisms have evolved to tolerate (or need) different oxygen conditions [21]. As previously described, oxygen serves as a terminal electron acceptor in aerobic respiration, resulting in the formation of toxic superoxide [20]. Exposure to high concentrations of oxygen can therefore have toxic effects on cells. Besides its importance in respiration, oxygen can also serve as a signalling factor

for orientation of microorganisms growing in several substrates, as increased oxygen levels can indicate surface exposure [24].

3.1.3 Light- a powerful energy source

All life on earth depends- directly or indirectly- on light. While only photosynthetic organisms rely on sunlight for the acquisition of organic matter and energy, light can play an important part in the regulation of many cellular processes in various organisms (e.g. circadian rhythm, differentiation, orientation) [24]–[26].

Sunlight corresponds to a relatively narrow fraction of the electromagnetic spectrum that consists of ultraviolet, infrared and visible light. Thus, it possesses wavelengths of different energy levels. Short wavelength radiation is known as ultraviolet (UV) light, which lies in a range of 10-400nm. It is a powerful energy source known to have potentially deteriorating effects on DNA [27]–[29]. UV radiation can further initiate uncontrolled free radical reactions, causing formation of ROS [29]. Visible light lies in the spectrum of ~ 390 to 700nm, which results in a lower energy level, and therefore less potentially damaging effects, than UV light. However, visible light can indirectly cause increased ROS production through energy transfer from light activated molecules, or chromophores, such as flavins [30]. Chromophores are organic molecules essential for the function of light receptors [24].

In fungi light is almost exclusively known for stimulation of development such as sexual and asexual reproduction [31]–[34]. Fungi usually grow inside substrates, hidden from harsh outside conditions such as high oxygen levels and illumination. Light might serve as a signal indicating exposure of hyphae to air on the surface, and thus triggering morphological changes to adapt to new conditions [24].

Filamentous fungi express several receptors sensitive to light of different wavelengths [24].

3.1.4 Temperature stress can induce production of ROS

Many saprotrophic filamentous fungi are mesophilic (live in mild temperatures), as they grow in soils or organic matter that shield them from extreme heat or cold. Seasonal or global climate changes can have high impacts on these temperature conditions. Temperature stress influences enzymatic pathways and consequently can also have other effects on cellular functions [35]. Changes in environmental temperature can have effects on activity levels, chemical bond formation and protein folding. Temperatures below optimal growth conditions cause many fungi to enter dormant stages, to preserve energy for basic survival. Heat stress can cause protein denaturation and cellular damage. Heat can also induce the production of ROS [36], [37]. The relationship between temperature and oxidative stress in filamentous fungi is however not yet well understood.

3.1.5 Nutrient sources as inducers for cellular responses to stress

Starvation conditions can have different effects on microbial cells. Mostly proliferation and differentiation are deprived or stagnate in scarce nutritional conditions. Reduced amounts of easily digestible energy sources can also lead to differential gene expression when more complex- and thus harder to metabolise- nutrient sources are present. As different enzymes are required for the digestion of complex carbohydrates, different cellular pathways must be activated [38]. Thus, carbon sources could potentially influence fungal stress reaction, by differential activation of pathways that control the production of co-regulators for this response.

3.1.6 Biotic interactions of fungi are frequently associated with stress

As previously mentioned, fungi show a preference to form symbiotic relationships with other organisms. Intercellular communication between interacting partners is often facilitated by signalling molecules [39]–[41]. Fungi and bacteria often produce radical oxygen species and secondary metabolites upon contact with other organisms [42]. As fungi might pose a threat to these organisms, may it be as a nutrient competitor or as a predator, this can often be a defence mechanism.

3.2 *Trichoderma*- an ecologically versatile genus that contains highly opportunistic species

Trichoderma (Hypocreales, Ascomycota, Dikarya) is a mycotrophic genus that includes at least 300 species [43]–[45]. Some members of the genus are present in all soils and rhizosphere, where they are one of the most prevalent isolated fungi [44]. According to the opinion of I. Druzhinina (personal communication), the ability to establish in soil of some *Trichoderma* species (max 15% of total diversity) can be considered as a marker of their remarkable nutritional versatility. Usually these species are adapted to grow in a diversity of habitats such as dead wood, other fungi, plants, soil, and the rhizosphere, thus demonstrating their high environmentally opportunistic potential [46]–[49]. Members of the genus are both saprotrophs and biotrophs, which means they rely on dead or decaying organic matter as well as other living organisms as nutrient sources, respectively [44], [50].

Trichoderma shows a multitude of human-beneficial applications. The fungi are of great importance to the biotechnology industry, where *T. reesei* is the primary source of cellulolytic enzymes and biofuel production [43], [51]. In agriculture, the fungus also has an important status, as some *Trichoderma* spp. show mycoparasitic properties (explained further in section 3.3), which means they attack other fungi, but do not harm the plants [52], [53]. This can be exploited for protection of crops by providing a natural control of plant-pathogenic fungi without harming the plants. Biocontrol formulations based on *Trichoderma* allow the reduced use of chemical fungicides and thus improve the agricultural practice. Soil-dwelling *Trichoderma* species are also used for the production of bioorganic fertilizers as

their presence in soil results in significant promotion of plant growth and induces resistance to pathogens [3], [23], [44], [54], [55].

The genus *Trichoderma* consists of at least 300 molecularly defined species. Most of these species are rare, while several are cosmopolitan and have strong opportunistic potential [43], [44]. Among them *T. reesei* is the prominent industrial species with the longest application history and the best available molecular toolkit. However, the most common (most opportunistic and dominant) infrageneric group of *Trichoderma* is a so-called *Harzianum* species complex that includes *T. harzianum sensu stricto*, *T. guizhouense* and several other recently distinguished species [56]. The establishment of an effective genetic transformation protocol for the strains from the *Harzianum* complex, and the availability of several whole genome sequences, put these species in the front line of *Trichoderma* research ([57], [58], Cai, Druzhinina, personal communication).

3.3 Fungal-fungal interaction of *Trichoderma*

Fungi of the *Trichoderma* genus form symbiotic relationships with many different organisms [44]. The symbiosis with other fungi is of constantly increasing interest, especially in agriculture, as *Trichoderma* shows strong antagonism against many known plant-pathogenic fungi [59]. As mentioned before, *Trichoderma* spp. follow both saprotrophic and biotrophic nutritional strategies. It has been widely observed that many *Trichoderma* spp. are mycotrophic, which means they use other fungi for nutrition (reviewed by Druzhinina et. al 2011 [44]).

The most widely observed mycotrophic strategy in *Trichoderma* is mycoparasitism [3], [46]. True symbiosis, including parasitism, is based on close and long-term biological interaction between two organisms. In a survey of >1,000 tested *Trichoderma* strains all showed mycoparasitic properties against three fungal species [44]. This widespread ability of *Trichoderma* to antagonize, and even kill, other fungi sparked the investigation of *Trichoderma* spp. as natural biocontrol agents for the fight against plant- pathogenic fungi [52], [53].

Some of the well-known plant-pathogenic fungi include *Fusarium oxysporum* f. sp *cubense* 4 (Hypocreales, Ascomycota, Dikarya) and *Pestalotiopsis fici* (Xylariales, Ascomycota, Dikarya).

Fusarium oxysporum f. sp *cubense*, or FOC, is believed to have originated in South-East Asia [60] and is the causative agent of banana wilt disease. It was found that *T. guizhouense* is a strong antagonist of *Fusarium oxysporum* f.sp *cubense* 4 [57], [58]. Under certain conditions, facultative mycoparasitic properties have also been observed in *Fusarium* spp. [61].

Pestalotiopsis fici is a known endophyte, but also plant-pathogen. The fungus is of great interest for medical applications as it is a prolific producer of bioactive secondary metabolites [62].

Both FOC and *P. fici* have been shown to be affected by *Trichoderma harzianum*, which has been observed to promote their recruitment in the rhizosphere of black pepper [50], [63]. As these fungi belong to the same order as *Trichoderma* (Hypocreales), these interactions are important observations for the study of closely related fungi, which might provide greater understanding of their common ancestral background and evolutionary development [50].

3.3.1 *Trichoderma* as a threat to agriculture

Because of its mycoparasitic properties against plant-pathogens *Trichoderma* is highly praised for its positive impact on human application, and symbioses with these fungi are well studied (*vide supra*). However, the targets of the genus's symbiotic relationships and strong fungal antagonism extend far beyond its close plant-pathogenic relatives. One of these targets is *Pleurotus ostreatus* (Agaricales, Basidiomycota, Dikarya), which is more commonly known as the oyster mushroom. Some *Trichoderma* spp. can attack this edible mushroom, causing the well-known green mold disease on mushroom farms [64], which makes this interaction an important research target for the food industry. Besides its importance for human application, this contact between *Trichoderma* spp. and *Pleurotus ostreatus* is also an excellent model for studying the relationships of more distantly related fungi, as *P. ostreatus* belongs to the Basidiomycota.

3.4 Hydrophobins- small, amphiphilic surface proteins enriched in *Trichoderma*

Among other factors, the environmental opportunism of some *Trichoderma* spp. is hypothesized to be linked to their high number and diversity of class II hydrophobins [65], [66]. Hydrophobins are small, amphiphilic, secreted proteins that are uniquely produced by filamentous or dimorphic fungi [67]–[70]. They can self-assemble into amphiphilic monolayers at hydrophobic/hydrophilic interfaces [71]. This property facilitates the attachment of fungal hyphae to a large variety of different substrates and living surfaces [65]. Hydrophobins are further needed for fungal development and differentiation. Here, the breach of the water-air interface between moist substrates and surface growth is facilitated by the amphiphilic properties of hydrophobin monolayers, as they decrease water surface tension [70]. Hydrophobins are also required for formation and stability of spores for dispersion by air, and the formation of fruiting bodies by stabilizing air channels [70], [72], [73].

The hyphal surface of fungi is a vital interface, as it facilitates growth, nutrition, defence and communication [1]. Filamentous fungi feed by secreting digestive enzymes and subsequently absorbing the digested nutrients through their cell wall. To increase the efficiency of this way of nutrition, hyphae grow in a tubular structure to create a high surface- volume ratio [1]. This large surface, however, may also be a weak point. The amphiphilic properties of hydrophobins may help *Trichoderma* balance between nutrition (hydrophilic), spore development (hydrophobic) and protection against external forces (hydrophobic).

The *Trichoderma* genus is highly enriched in hydrophobins, especially the *Virens* and *Harzianum* clades, with *T. virens*, *T. harzianum* and *T. guizhouense* expressing 12, 12 and 10 hydrophobins, respectively (Chenthamara, unpublished).

3.5 Protein tags and their different applications

Protein or amino acid tags are used for monitoring or extraction of proteins of interest. They can consist of short peptide sequences or larger, protein coding peptides, grafted onto genes of interest for the heterologous production of recombinant proteins. Protein tags have many different applications.

Affinity tags are used for purification of proteins after their biological production. This purification process, called affinity chromatography, is based on the binding affinity of specific molecular structures to a stationary phase, allowing the separation of the bound molecule from its undesired surrounding material [74].

Epitope tags are targets for antibodies. They consist of short peptide sequences and elicit high immunoreactivity. Antibody binding is highly specific, which makes epitope tags a perfect target for protein identification. The used antibodies are usually combined with some sort of reporter protein or enzyme, that enable visualization of the protein target.

Fluorescent tags are used for visualization of target proteins *in vivo*. This can be used to visualize protein location, activity or interactions with other cellular molecules. Fluorescent proteins such as the green fluorescent protein (GFP) from *Aequorea Victoria* (Leptothecata, Hydrozoa, Cnidaria) are the most commonly used fluorescent tags [75].

3.5.1 Fluorescent proteins- visualizing cellular physiology

Fluorescent proteins (FPs) can be used to visualize entire cells, cellular compartments or single proteins *in vivo* by creating recombinant proteins using transgenic approaches. The fusion of an FP to a protein target of interest enables quantitative analysis of the target protein by monitoring fluorescence intensity. Fluorescent protein tags eradicate the need for exogenous fluorophores, which often require cellular fixation and permeabilization, and thus killing of the cell. Among the most commonly used FPs are the green, the red and the yellow fluorescent protein. All FPs are ~25 kDa in size [75], [76].

3.5.2 Hydrophobin as a secretion vector for fluorescent tags

Although fluorophores are easily detectable by epifluorescence microscopy they can be technically challenging to quantify inside tissue. The addition of a secretion signal to a fluorophore is an efficient way to enable easy quantification, as secreted proteins are easily extractable from cultivation media. Hydrophobins show high surface activity, as they are secreted at high rates [77]. This property makes hydrophobins, together with a fluorescent tag, easily quantifiable and therefore easy to monitor. The

fluorophore chosen in this thesis is mRFP (mCherry), as many fungi show strong autofluorescence in the emission spectrum around 500nm [78], while RFP has an emission maximum of 583nm. This makes the mRFP signal less likely to interfere with the auto fluorescent signal and the distinction between actual signal and background fluorescence easier.

3.6 Regulation of fungal gene expression

Cellular physiology and metabolism are the result of the interplay of complex signalling pathways and gene expression systems that lead to the production of vital proteins and other cellular structures.

Environmental factors and stresses can induce differential gene expression for the cellular adjustment to changing conditions. Extracellular signals are translated to specific internal responses by intrinsic signalling cascades. This adaptation is important for the maintenance of homeostasis and the avoidance of cellular damage or even cell death [79].

3.6.1.1 Gene expression is regulated by promoters

Gene expression is controlled by promoters (and other regulatory elements). Promoters are DNA sequences of ~100-1000bp that are an essential part of a gene, which are located upstream of the gene's coding region, close to the transcription start site. Transcription initiation happens through the binding of the RNA polymerase to the promoter DNA. In eukaryotes, this process is regulated by several different transcription factors that facilitate the binding of the RNA polymerase to the promoter.

Promoters research is an important area in molecular biology. It is also investigated for biotechnological applications of gene expression. There are different types of promoters with distinct functionalities available, each serving different purposes.

Constitutive promoters control vital genes and are therefore active in all tissues and structures (if applicable) and largely independent of external influences. These promoters are usually very strong promoters and result in high production rates, which is often desirable for research of industrial applications.

Inducible promoters are highly dependent on environmental influences and can therefore be activated artificially by abiotic factors or specific nutrients. This enables precise control of the promoters in a controlled environment and therefore serves as a powerful tool for genetic engineering.

3.7 P_{cdna1} , a strong promoter from *Trichoderma reesei*

The P_{cdna1} promoter was originally discovered in *T. reesei* by Nakari-Setälä et al. [80]. As previously mentioned, *T. reesei* is an important industrial enzyme producer, best known for its high production rate of cellulases. The strong inducible promoter of the cellulase-encoding gene *cbh1* (= *cel7A*) had previously been used for heterologous enzyme production in *T. reesei* on cellulase containing media [81]–[85]. As these natural media promote protease production, and therefore negatively influence

protein yield, the use of a synthetic glucose medium was striven for. The *cbh1* promoter, however, is inhibited by glucose [86]. In the endeavour to find a strong promoter for glucose containing media, the P_{cdna1} promoter controlling the *cdna1* gene of still unknown function was discovered, showing the highest activity of all candidates [80].

The P_{cdna1} promoter has been proposed as constitutive and has been used frequently for recombinant protein production [87]–[92]. However, not much is known about its specific function or its underlying gene *cdna1*.

3.8 Hypothesis and Aims of the Thesis

In this thesis I hypothesise that a heterologous protein construct consisting of a native hydrophobin combined with mRFP is an appropriate tag for visualizing fungal ecophysiology. The environmentally opportunistic fungus *Trichoderma guizhouense* that follows versatile nutritional strategies, is highly resistant to abiotic stresses and interacts with a plethora of other fungi, plants and animal, appears to be an appropriate model organism for the experimental testing of this hypothesis.

To test this hypothesis the species *T. guizhouense* (NJAU TUCIM 4742) was genetically modified to overexpress its native hydrophobin 8 (*hfb8*) tagged with mRFP under the exogenous promoter P_{cdna1} from *T. reesei*.

This thesis aims at the following tasks:

- To study the relationships between a species of the genus *Trichoderma* and its fungal interaction partners of different phylogenetic proximity.
- To investigate the response of the promoter P_{cdna1} from *Trichoderma reesei* to a variety of biotic and abiotic factors. This analysis should lead to the further hypothesis on the function of the *cdna1* gene in *Trichoderma*.

Fungal-fungal interactions will be monitored in dual confrontation assays on agar plates and microscopy slides and will be monitored by photography and epifluorescence microscopy. The external regulation of P_{cdna1} will be investigated by exposing the HFB8_{mRFP} overexpressing *T. guizhouense* strains to several environmental stresses, which will be monitored by the quantification of mRFP and *cdna1* expression.

4 Material and Methods

4.1 Media and Chemicals

All Chemicals used were supplied by Roth (Germany), if not specifically stated otherwise.

4.2 *Trichoderma* strains

All strains used in this thesis (Table 1) were obtained from the Technical University of Vienna Collection of Industrial Microorganisms (TUCIM). Strains are stored in sterile glycerol stocks as mycelial plugs or spore suspensions at -80°C.

Name	Wild type strain	TUCIM	Description
Tgui	<i>Trichoderma guizhouense</i> NJAU 4742	4742	Wild type strain
Tgui ^{OE_{hfb8}} 7582	<i>Trichoderma guizhouense</i> NJAU 4742	7582	<i>hfb8</i> -overexpressing 4742 with mRFP and 6xHis tag at the C terminus under the control of P _{cdna1} promoter with Hygromycin resistance
Tgui ^{OE_{hfb8}} 7583	<i>Trichoderma guizhouense</i> NJAU 4742	7583	<i>hfb8</i> -overexpressing 4742 with mRFP and 6xHis tag at the C terminus under the control of P _{cdna1} promoter with Hygromycin resistance
Tgui ^{Δ_{hfb8}}	<i>Trichoderma guizhouense</i> NJAU 4742	7441	<i>hfb8</i> -deletion 4742 with Hygromycin resistance
<i>T. harzianum</i>	<i>Trichoderma harzianum</i> CBS 226.95	916	Wild type strain
<i>T. reesei</i>	<i>Trichoderma reesei</i> QM6a	917	Wild type strain
<i>P. ostreatus</i>	<i>Pleurotus ostreatus</i>	No number	Wild type strain
<i>P. fici</i>	<i>Pestalotopsis fici</i>	5788	Wild type strain
FOC	<i>Fusarium oxysporum</i> f.sp. <i>cubense</i>	4301	Wild type strain
FOC4	<i>Fusarium oxysporum</i> f.sp. <i>cubense</i> 4	4848	Wild type strain

Table 1: All fungal strains used in this thesis including their Technical University of Vienna Collection of Industrial Microorganisms (TUCIM) numbers

The Tgui mutant strains (TUCIM 7582, 7583 and 7441) were originally provided by Dr Feng Cai (TU Wien/ NAU).

4.3 Fermentation and protein biochemistry

HFB8_{mRFP} integrity and surface accumulation was tested by SDS-PAGE and Western Blot of flask fermentation samples of *Trichoderma guizhouense* wild type (TUCIM 4742) and hydrophobin 8 overexpressing strains (TUCIM 7582 and TUCIM 7583). No biological replicates were created.

4.3.1 Spore harvesting

Spores were harvested from equally old cultures grown on 6cm PDA Petri plates in an alternating rhythm of 12 hours illumination and 12 hours darkness (12 hours illumination) at 25°C (10-14 days). Spores were harvested with autoclaved physiological salt solution (0.9% NaCl, 0.05% Tween 20)

applied onto the cultures on PDA and distributed with a sterile spreader. This pore wash was filtered through sterilised glass wool to separate spores from the mycelium. Spore concentration was adjusted with a BIOLOG turbidimeter (Biolog Inc., USA) by light absorption at 590nm.

4.3.2 Shaking flask fermentation

For fermentation, 50ml of minimal medium (Table 2) were inoculated with 4×10^6 spores/ml.

Minimal medium			
	concentration (%)		concentration (%)
Glycerol	4	*	
Peptone	0,4	CaCl ₂	5
KH ₂ PO ₄	0,5	dissolved in dH ₂ O, filter sterilised (0,2um)	
(NH ₄) ₂ SO ₄	0,28	**	
MgSO ₄ ×7H ₂ O	0,05	FeSO ₄ ×7H ₂ O	2,5
Tween 20	0,2	MnSO ₄ ×H ₂ O	0,8
Dissolved CaCl ₂ *	10	ZnSO ₄ ×7H ₂ O	0,7
Trace elements**	10	CoCl ₂ ×2H ₂ O	0,1
		dissolved in dH ₂ O, filter sterilised (0,2um)	

Table 2: Composition of minimal medium used for submerged cultivation

Fermentation was carried out in 300ml shaking flasks at 28°C and 200rpm for 72 hours. After fermentation, the biomass was harvested for analysing the fermentation broth and a mycelial wash. For analysing the mycelium wash, fermentation product (except 1.5ml) was transferred to a 50ml Flacon tube, centrifuged for 5 minutes at 13krpm and the supernatant was discarded. 10ml wash buffer (0.1M Tris-HCl (pH9), 1%SDS) were used to wash ~1g of biomass pellet by vortexing and subsequent gentle shaking at 100rpm at room temperature for 10 minutes. The sample was centrifuged again, and the supernatant used for further analysis. For analysing the fermentation broth, 1.5ml fermentation product were transferred to a 1.5ml Eppendorf tube and centrifuged for 3 minutes at 13krpm. The supernatant was used for further analysis.

4.3.3 Protein concentration measurement

Protein concentrations of the samples were measured with a bicinchoninic acid (BCA) assay kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, USA) using a bovine serum albumin (BSA) standard curve (Figure 1).

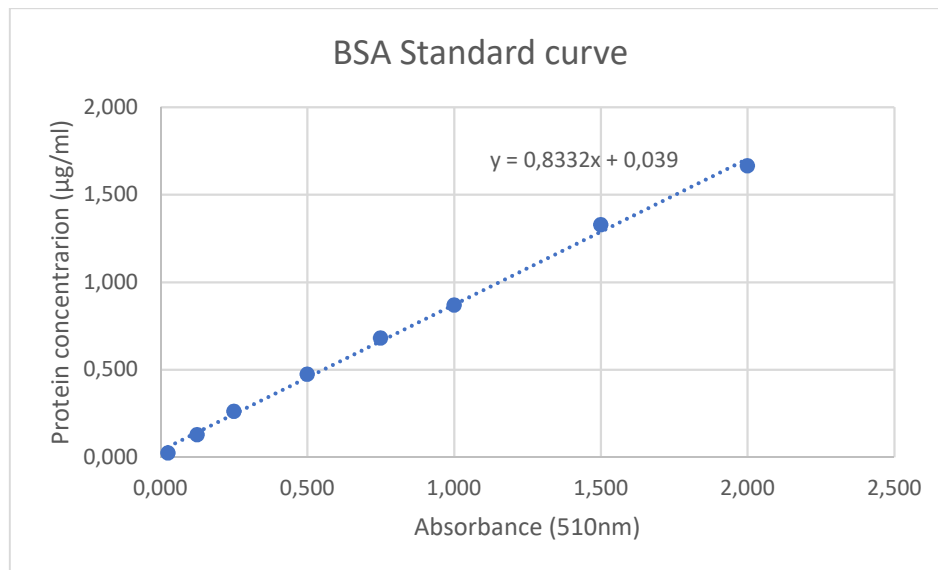


Figure 1: BSA standard curve for determining protein concentration

Mycelium wash samples were all diluted to 1.5mg/ml. Fermentation broth samples were all between 0.3-0.7 mg/ml and were not diluted.

1.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Leammli buffer (5x) was added to both the mycelial wash and fermentation broth samples in a 1:5 ratio and incubated at 98°C for 10 minutes. Denatured samples were applied to freshly poured sodium dodecyl polyacrylamide gels and run in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Biorad, USA) filled with running buffer (5mM Tris, 50mM glycine, 0.02% SDS) at 100V for ~1.5 hours. The protein ladder used was PageRuler™ Unstained Broad Range Protein Ladder (Thermo Fisher Scientific, USA). After electrophoresis, gels were developed by silver staining using the SilverQuest™ Silver Staining Kit (Thermo Fisher Scientific, USA) according to the manufacturer.

4.3.4 Immunoblotting Assay

For identification of HFB8_{mRFP}, and clarification that it is secreted intact and not cleaved, SDS-PAGE was carried out using PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo Fisher Scientific, USA) as a protein ladder, for subsequent immunoblotting. After SDS-PAGE, the gels were transferred in transfer buffer (20% MeOH, 190mM glycine, 25mM Tris) to nitrocellulose membranes by horizontal electrophoresis using the Mini Trans-Blot® Module (Thermo Fisher Scientific, USA) according to the manufacturer at 40mA for 14 hours. After transfer, the membranes were rinsed with TBST (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% Tween 20) and then blocked (5% milk powder in TBST) for 30 minutes. After blocking, membranes were washed three times with TBST for 5 minutes each. HFB8_{mRFP} contains a C-terminal 6x His-Tag. For detection of HFB8_{mRFP} by immunoblotting, a mouse-anti-His antibody (Thermo Fisher Scientific, USA) with a C-terminal alkaline phosphatase (AP) residue diluted 1:2000 in TBST was applied to the membranes for 1 hour. The membranes were washed again three times with TBST and treated with nitro-blue tetrazolium chloride/ 5-bromo-4-chloro-3'-indolylphosphate p-

toluidine salt (NBT/BCIP, Thermo Fisher Scientific, USA), which reacts with the AP, resulting in a dark purple coloration of the membrane.

4.4 Cultivation conditions

If not specifically stated otherwise, all fungal strains used in this thesis were cultivated on potato dextrose agar (PDA, Roth) at 25°C in darkness. PDA plates were inoculated either with mycelium samples on small agar plug (~5mm diameter) or with spore suspensions.

For investigating Tgui, Tgui_{OE_{Ehfb8}} and Tgui_{Δ_{hfb8}} phenotypes, the following strategies were used. Each setup was carried out in triplicates.

4.4.1 Growth monitoring

For growth monitoring strains were cultivated on 6cm Petri plates containing PDA in either darkness or in an alternating rhythm of 12 hours illumination and 12 hours darkness (12 hours illumination) for 72 hours. Growth was monitored by visual inspection and photography.

4.4.2 Sporulation

For monitoring of sporulation cultivation was carried out on 6cm Petri plates containing PDA under 12 hours illumination for 16 days. Sporulation was monitored by visual inspection and photography.

4.4.3 Aerial growth

Aerial growth was monitored in 2 ml liquid cultures in minimal medium (Table 2) grown in darkness. Aerial growth was monitored by visual inspection and photography.

4.4.4 Physical injury

The response to physical injury was monitored by the formation of spores at the sites of damage. Strains were cultivated on 6cm Petri plates containing PDA in darkness for 72 hours, cut in an orthogonal pattern with a sterile scalpel and incubated another 72 hours under 12 hours illumination. Sporulation was monitored by visual inspection and photography.

4.5 Hydrophobicity of mycelium and spores

The hydrophobicity of hyphae was determined by measuring the water contact angle (WCA) of a droplet applied to the mycelial surface of cultures grown on PDA for 72 hours in darkness. Spore hydrophobicity was measured on spore covered cultures grown on PDA for 10 days under 12 hours illumination. To measure the contact angle, a water droplet of 8μl was applied to mycelium or spore carpet and recorded after 2 seconds with a DSA100 contact angle measuring device (Krüss, Germany). The drop shape was analysed using the ImageJ [93] plugin LB-ADSA [94], based on the fitting of the Young-Laplace equation to the image data.

4.6 Fungal-fungal interactions

The fungal species used for the monitoring of *Trichoderma* fungal-fungal interactions are summarized in Table 3.

Name	Wild type strain	TUCIM	Description
<i>P. ostreatus</i>	<i>Pleurotus ostreatus</i>	No number	Wild type strain
<i>P. fici</i>	<i>Pestalotopsis fici</i>	5788	Wild type strain
FOC	<i>Fusarium oxysporum</i> f.sp. <i>cubense</i>	4301	Wild type strain
FOC4	<i>Fusarium oxysporum</i> f.sp. <i>cubense</i> 4	4848	Wild type strain

Table 3: Fungal species used for investigation of *Trichoderma* fungal-fungal interactions

4.6.1 Dual confrontation assays on PDA

To study the macromorphology of fungal-fungal interactions, dual confrontation assays were carried out. 9cm Petri plates containing PDA were inoculated on opposite sides, ~1cm from the edge of the plate, and incubated at 25°C in darkness for up to 3 weeks. Interactions were monitored by visual inspection and photography.

4.6.2 Microscopy cultivation setup

For investigation of the micromorphology of Tgui_{OE_{hfb8}} and its interaction with other fungi, cultivation was carried out directly on microscopy slides, to preserve structural integrity, which would be lost when taking samples from PDA plate cultures. Thin pieces of PDA were used as nutrient sources for the fungi.

For dual confrontation assays, the agar plugs were placed on the microscopy slides 5-10 mm apart. Each plug was incubated with either Tgui_{OE_{hfb8}} or one of the fungal strains summarized in Table 3. The setup was covered with a cover glass, placed in a Petri plate, sealed with Parafilm, and incubated at 25°C in darkness for up to 48 hours. Growth was checked every ~12 hours. Microscopic analysis was started upon growth overlap of ~1-2mm of the interacting colonies.

Epifluorescence microscopy was carried out with the Leica fluorescent DMI8 Microscope (Leica Microsystems, Germany) and images were created using the LAS X Software (Leica Microsystems, Germany) and ImageJ [93]. The red fluorescent signal of HFB8_{mRFP} was detected with a green excitation range filter (BP 545/30, Leica Microsystems, Germany). Fungal autofluorescence was detected with a red excitation filter (BP 610/75, Leica Microsystems, Germany).

4.7 External influence factors monitored by fluorescence quantification of extracted HFB8_{mRFP}

The *hfb8::mrfp* construct in Tgui_{OE_{hfb8}} is controlled by the promoter P_{cdna1} from *T. reesei* [80]. To test if and how this promoter is regulated by environmental stresses, Tgui_{OE_{hfb8}} cultures were exposed to different ambient influence factors. Cultivations were carried out on agar plates (PDA, unless specifically stated otherwise) or in liquid submerged cultures (minimal medium, unless specifically stated otherwise) in triplicates.

4.7.1 Illumination

White light exposure was carried out with a Philips Master 840 diode (15 Watts, Philips, Netherlands), blue light exposure with an OSRAM FLUORA 77 diode (15 Watts, OSRAM, Germany). Continued light exposure was carried out in an alternating rhythm of 12 hours illumination and 12 hours darkness (12 hours illumination) inside the incubator. Short light exposure was carried out for 10 minutes, outside of the incubator, in a self-made illumination chamber (Figure 2) to ensure a regulated light environment. Lux was measured with an Android App (Light Meter, My Mobile Tools Dev) on a Sony smartphone (Xperia Z3 compact, Sony, Japan) placed inside the chamber.

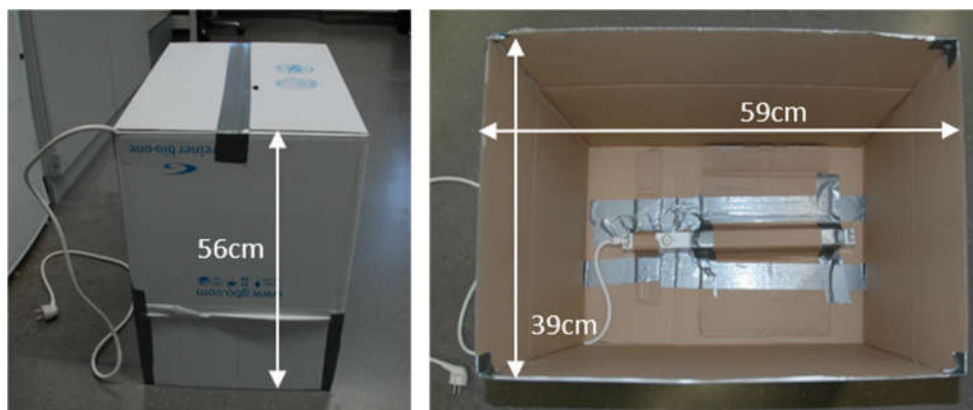


Figure 2: Self-made illumination chamber for short illumination exposure of fungal cultures

4.7.2 Aeration

Aeration of plates was carried out in a sterile environment (clean bench) by opening the sealed plates for 5 seconds under a Bunsen burner and resealing them with Parafilm for continued cultivation.

4.7.3 Temperature decrease

After 72 hours of growth on PDA at 25°C in darkness, cultures were transferred to 4°C in darkness and incubation was continued for 18 days.

4.7.4 Different carbon sources

The influence of nutrition on P_{cdna1} activation was tested on agar plates containing a complex carbon (1% rice flour, 1.5% agar agar) and using BIOLOG FF Microplates (Biolog Inc., USA), a high throughput assay involving 95 different carbon sources.

4.7.5 BIOLOG

BIOLOG FF Microplates (Biolog Inc., USA) contain 95 wells with different carbon sources (Supplementary Figure 5) and one well with water, designed for high throughput phenotypic analysis of fungi.

For inoculation of the microplates, spore suspensions of 4×10^6 spores/ml were created as previously described (section 4.3.1). The microplates were incubated at 25°C in either darkness, with addition of

0.05 mM menadione (added to the stock spore suspension) or under 12 hours illumination for seven days. Growth was measured every 24 hours by light absorption at 750nm.

4.7.6 Menadione

The influence of oxidative stress on P_{cdna1} was measured by inducing the production of high levels of reactive oxygen species by $Tgui_{OEhfb8}$ by addition of menadione. Menadione was dissolved in 96% pure EtOH, filter sterilized (0.2 μ m) and added either to the spore suspension (BIOLOG) or to the growth medium (cultivation in 2 ml minimal medium) to a final concentration of 0.05 mM.

4.7.7 Fluorescence measurements

The influence of environmental factors on P_{cdna1} activation as measured by HFB8_{mRFP} expression was quantified using the mRFP fluorescent signal. For this measurement, HFB8_{mRFP} was extracted from $Tgui_{OEhfb8}$ with a mild wash buffer (0.1M Tris-HCl pH9, 1% SDS) and quantified in a GloMax®-Multi Detection System (Promega, USA) using a green filter (520 nm excitation maximum).

For extraction from cultures grown on agar Petri plates, the wash buffer was applied directly onto the plate (in equal amounts per plate) and the mycelium was washed by homogenization with a sterile spreader and subsequent incubation for 10 minutes at room temperature. After incubation, the liquid was collected in 15ml Falcon tubes, centrifuged for 5 minutes and the supernatant was transferred to a microplate for fluorescence detection. Each measurement was carried out in triplicates.

For extraction of HFB8_{mRFP} from submerged 2 ml cultures, biomass was transferred to an Eppendorf (Germany) tube, which was filled up with wash buffer. The samples were vortexed for 30-60 seconds each and centrifuged for 5 minutes. The supernatant was transferred to a microplate for fluorescence detection. Each measurement was carried out in triplicates.

Cultures grown on BIOLOG FF Microplates (Biolog Inc., USA) were directly transferred to 96-well microplates for fluorescence detection, with cut sterile pipette tips. Each measurement was carried out in triplicates.

4.8 External influence factors monitored by *cdna1* mRNA quantification

For more precise analysis of P_{cdna1} regulation, mRNA quantification of its underlying gene *cdna1* was carried out by real-time quantitative polymerase chain reactions (qPCR).

Three *Trichoderma* species were chosen for this analysis (Table 4).

Strain	TUCIM	cDNA1 Accession number (NCBI)
<i>Trichoderma guizhouense</i> NJAU 4742	4742	OPB44063.1
<i>Trichoderma harzianum</i> CBS 226.95	916	XP_024774428.1
<i>Trichoderma reesei</i> QM6a	917	XP_006968441.1

Table 4: *Trichoderma* strains used for the investigation of *cdna1* expression

The sequence for the predicted protein cDNA1, discovered in *T. reesei* [80], was retrieved from Uzbass et. al, 2012 [88]. Homologous proteins in *T. guizhouense* and *T. harzianum* were retrieved from the National Center for Biotechnology Information (NCBI, USA) using BLAST [95], summarized in Supplementary Figure 8.

qPCR primers for *cdna1* were designed using Primer Premier 5.0 [96] and are summarized in Table 5.

Strain	Forward primer (5'→3')	Reverse primer (3'→5')
<i>Trichoderma guizhouense</i> NJAU 4742	CAGCACTGTTCGCAAGCCTA	TCATGGTGGTTGCGAATCTC
<i>Trichoderma harzianum</i> CBS 226.95	GCACTGTTCGCAAGCCTGTT	TTGCAGGCTTCATGGTGGTT
<i>Trichoderma reesei</i> QM6a	GGCTTCTTCATGCAAGTCCG	TAGCATAGTCGGGTCGTGCA

Table 5: Primer pairs used for amplification of *cdna1* and homologous genes

For normalization of technical sample to sample differences, *tef1* was used as a reference gene.

4.8.1 Conditions

The three *Trichoderma* species (Table 4) were cultivated on 6cm Petri plates containing PDA, covered in sterilised cellophane disks, for easy biomass extraction. PDA plates were each inoculated with 5μl of spore suspension (4×10^6 spores/ml). Cultures were subjected to ambient stress factors summarized in Table 6.

Environmental factors (predictors)	Condition 1 (approaching the environmental optimum of <i>T. guizhouense</i> in vitro)	Condition 2 (imitating environments stress conditions)
Illumination	Darkness	12 hours illumination (continuous) Short illumination (white light)
Temperature	Optimal condition (25°C)	Short cold stress (4°C) Short heat stress (37°C)

Table 6: Cultivation conditions for the investigation of environmental influences on P_{cdna1} regulation

All cultures were grown at 25°C in darkness (except for 12 hours illumination culture) until short exposure to an environmental stressor, after 48 hours of growth. Short illumination was carried out for 10 minutes, as described previously (section 4.7.1). Short temperature stress was carried out for 30 minutes. After the short stress exposure, cultures were returned to 25°C in darkness for 30 minutes (timepoint 1) or three hours (timepoint 2) before RNA extraction.

Additional to stress exposure, the activity of P_{cdna1} was measured in 10ml submerged cultures in potato dextrose broth (PDB) inoculated with 10μl spore suspension (4×10^6 spores/ml) to a final concentration of 4×10^3 spores/ml. Cultivation was carried out in 100ml flasks at 28°C in darkness, either standing or shaking at 250rpm. Each of these setups was done in triplicates.

4.8.2 mRNA extraction

After the final incubation, mRNA was extracted from the agar plate cultures by scraping off the biomass from the cellophane disks into a mortar with liquid nitrogen and thoroughly grinding the tissue to a

fine powder. Biomass from the submerged cultures was harvested by filtration through filter paper, using the drip-dried biomass to continue as before. The fine tissue powder was immediately decanted into 1.5ml Eppendorf tubes and mRNA extraction was carried out with the RNeasy® Plant Mini Kit (Qiagen, Germany) according to the manufacturer. mRNA was eluted in 35µl RNase free water.

4.8.3 cDNA synthesis

For cDNA synthesis the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was used. cDNA synthesis and prior removal of residual genomic DNA by treatment with DNase were carried out according to the manufacturer. cDNA concentration was measured with NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA).

4.8.4 qPCR

Reaction samples for real-time polymerase chain reaction (qPCR) were prepared with Luna® Universal qPCR Master Mix (NEB, USA), with final concentrations of 1x Luna® Universal qPCR Master Mix, 0.25µM forward primer, 0.25µM reverse primer and 400ng cDNA template in a final volume of 20µl (bioscience grade water (Roth, Germany) added to final volume). qPCR was carried out in a qTOWER 2.0 (Analytik Jena, Germany). The thermal program was set as 1 min at 95 °C initial denaturation, 40 cycles of denaturation for 15 sec at 95 °C and annealing for 30 sec at 60 °C. The melting curve was set from 60°C~95°C.

4.8.5 Relative gene expression calculation

Relative *cdna1* expression, or expression fold change, was calculated by the $2^{-\Delta\Delta C_t}$ method [97] normalized to a housekeeping gene (*tef1*) and to the C_t values of cultures not exposed to any environmental stresses (grown on PDA at 25°C in darkness).

4.9 Statistics

Statistical analyses, including graphical displays, were done with RStudio [98]. All obtained data was descriptively analysed (mean, minimum, maximum and standard deviation values) and checked for possible outliers. The significance of the influence of environmental stress factors on P_{cdna1} activation was tested through comparison of sample means by analysis of variance (ANOVA). To identify statistical relevance between all possible pairs of means, post-hoc comparison was done using Tukey's honest significant difference and Duncan's new multiple range test.

5 Results

The aim of this thesis was to investigate biotic and abiotic influences on fungal physiology. For this purpose, mutants of the filamentous fungus *Trichoderma guizhouense* NJAU 4742 were used, overexpressing its native hydrophobin 8 (*hfb8*) tagged with mRFP under the strong promoter P_{cdna1} from *Trichoderma reesei*. The *hfb8::mrfp* construct contains an N-terminal *hph* selection cassette and a C-terminal HIS6-tag for purification and detection, followed by the hydrophobin 8 terminator sequence (Figure 3). The predicted size of the expressed HFB8_{mRFP} is ~37kDa (mRFP: ~27kDa, HFB8: ~10kDa).

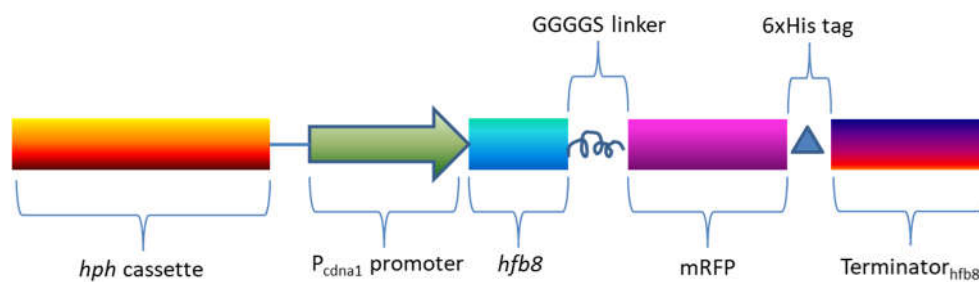


Figure 3: Schematic overview of the *hfb8::mrfp* gene construct

The *hfb8::mrfp* gene construct used for transformation of *T. guizhouense* contains an *hph* cassette for purification selection with hygromycin. The P_{cdna1} promoter controls *hfb8* with an mRFP tag, connected by a ggggs linker. The construct contains a C-terminal 6xHis tag for detection and purification, followed by the hydrophobin 8 terminator sequence. The whole construct has a size of ~5.8kb. (Image provided by Feng Cai)

The strains used in this thesis are described in detail in Table 7. Two *hfb8* overexpressing strains were used, namely TUCIM 5782 and TUCIM 5783. They are two different transformants containing the same *hfb8::mrfp* construct in their genome.

Name	TUCIM	Description
Tgui wt	4742	<i>Trichoderma guizhouense</i> NJAU 4742 wild type
Tgui _{O_Ehfb8}	5782	<i>hfb8</i> -overexpressing 4742 with mRFP and 6xHis tag at the C terminus under the control of P_{cdna1} promoter with Hygromycin resistance
Tgui _{O_Ehfb8}	5783	<i>hfb8</i> -overexpressing 4742 with mRFP and 6xHis tag at the C terminus under the control of P_{cdna1} promoter with Hygromycin resistance
Tgui _{Δhfb8}	7441	<i>hfb8</i> -deletion 4742 with Hygromycin resistance

Table 7: Description of *T. guizhouense* strains used in this thesis.

5.1 Applicability of HFB8_{mRFP} as a tag for the study of fungal physiology

5.1.1 HFB8_{mRFP} has an affinity for interface accumulation

To utilize HFB8_{mRFP} as an efficient tag for the visualization of fungal hyphal interactions with various surfaces, the fluorescently labelled protein must be secreted. The secretion into the cultivation broth was confirmed by SDS-PAGE and immunoblotting (Western blot) analysis of the two Tgui_{OEhfb8} mutants, namely TUCIM 5782 and TUCIM 5783 (Figure 4) grown in submerged condition. Both the fermentation broth and a mycelial debris, washed with a mild buffer (0.1M Tris-HCl pH 9, 1% SDS) that presumably does not destroy the integrity of the surface proteome, were analysed. Figure 4 A+B shows that HFB8_{mRFP} is present in the fermentation broth and in the mycelium wash of both strains, which confirms that it is secreted. The band intensity is stronger in TUCIM 5783 than in TUCIM 5782, which suggests that the TUCIM 5783 genome may contain more copies of the recombinant gene. If not specified otherwise, Tgui_{OEhfb8} will refer to TUCIM 5782 from now on.

The western blot in Figure 4 C confirms that the bands are indeed HFB8_{mRFP} and that it is secreted intact and not degraded. No bands are detectable by immunoblot in the fermentation broth (Figure 4D). This is most likely because HFB8_{mRFP} preferably attaches to surfaces and can therefore not be detected easily in the supernatant.

The florescent signal of HFB8_{mRFP} is not homogenously distributed across the mycelium

Utilizing the mRFP tag, the preference of the recombinant protein to attach to surfaces and the morphology of the fluorescent signal can also be observed by epifluorescence microscopy. Several different red fluorescent signal morphotypes have been observed.

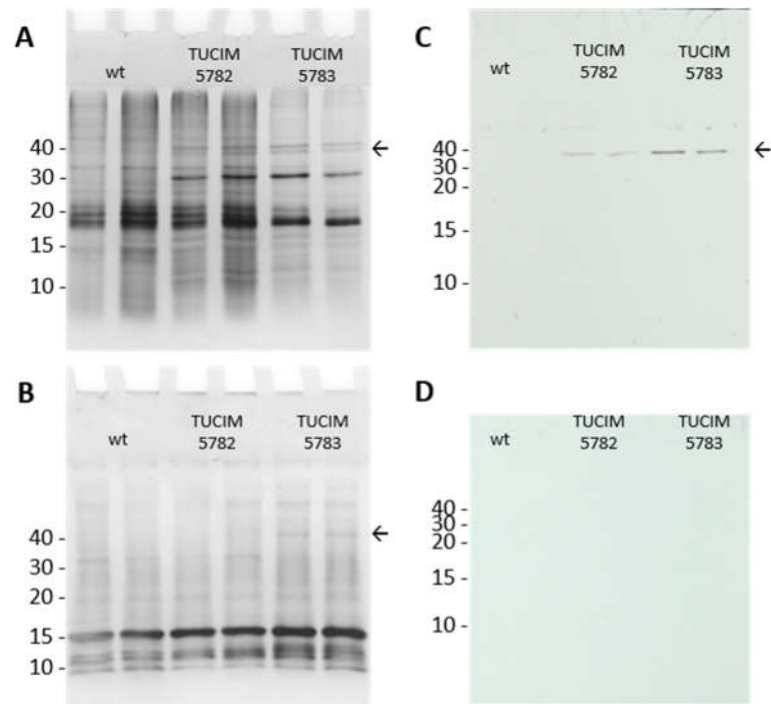


Figure 4: HFB8_{mRFP} is secreted and accumulates at the hyphae-water interface

SDS-PAGE and Western Blot of fermentation broth samples and mycelial wash samples from shaking flask fermentations of *Tgui* wild type (wt, TUCIM 4742), and two *Tgui^{OEhfb8}* strains (TUCIM 5782 and TUCIM 5783) cultivated in minimal medium. A) Silver stained SDS-PAGE gel of the mycelium wash. The arrow indicates the expected molecular weight of HF8_{mRFP} (~37kDa), which is present in both TUCIM 5782 and TUCIM 5783. B) Western Blot of the mycelial wash detected with mouse-anti-His Ab. TUCIM 5782 and TUCIM 5783 both show bands at the expected molecular weight of HFB8_{mRFP}, suggesting that the construct is intact and mRFP is not cleaved off. C) Silver stained SDS-PAGE gel of the fermentation broth. The arrow indicates the expected molecular weight of HF8_{mRFP} (~37kDa), which is faintly visible in TUCIM 5783. D) Western Blot of the mycelial wash detected with mouse-anti-His Ab. No bands are visible in any samples. This is most likely due to low amounts of soluble HF8_{mRFP} in the medium, due to its preference to accumulate at hyphal-water interfaces.

5.1.1.1 HFB8_{mRFP} accumulates at the hyphae – glass – air interface

Figure 5 shows TUCIM 5782 grown on hydrophilic glass microscopy slides. The fluorescent signal is accumulated at the hyphal surface, specifically at the triple interface between the hyphae, the air and the glass surface. This can be observed clearly in Figure 5 B, which shows that there is no intense signal in the contact area between the two interacting hyphae, only on the edges around the interaction. This might be another indication that the HFB8_{mRFP} protein is secreted and accumulates at interfaces, rather than staying attached to the hyphal wall.

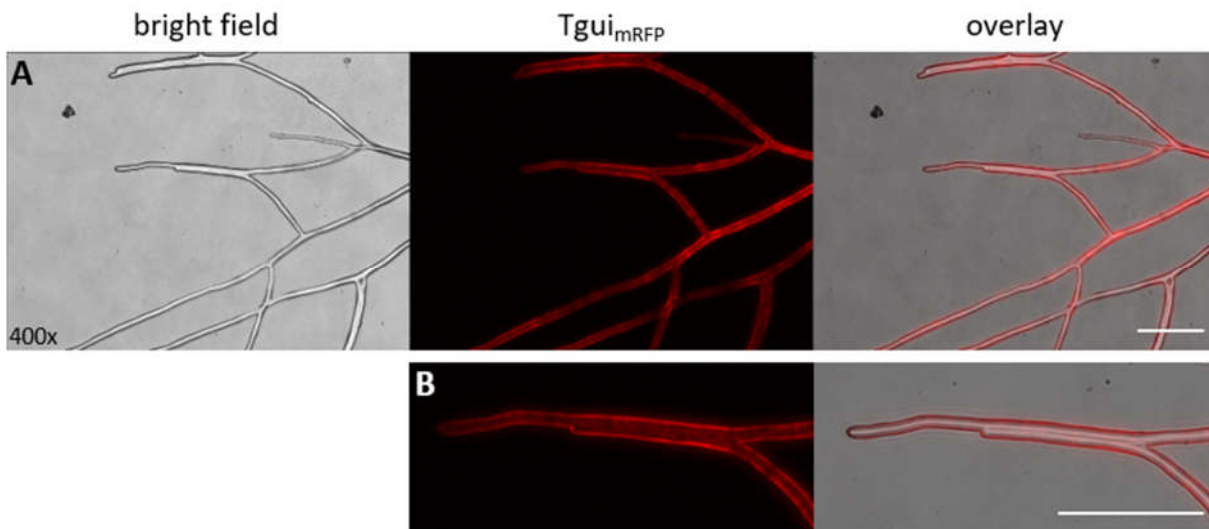


Figure 5: Water-air-hyphae interface signal of HFB8_{mRFP} in *Tgui^{OEhfb8}*

Microscopic image of the non-trophic hyphae of *Tgui^{OEhfb8}* on a microscopy glass slide surface recorded with Leica fluorescent DMI8 Microscope (Leica Microsystems, Germany). A) Image recorded at x 400 magnification. B) Enlarged section of A. HFB8_{mRFP} (shown in red) accumulates at the hyphae-water-air interface around the interacting hyphae, but not in their contact zone.

The size bars correspond to a length of 50µm.

5.1.1.2 Heterogeneous distribution of HFB8_{mRFP} on hyphal surface

Figure 6 (A+B) shows a heterogeneous (granular) structure of the fluorescent signal on the hyphal surface. This morphotype was observed in many different cultivation setups independent of the surface properties of the substrate offered to the non-trophic hyphae (glass, plastic) or the cultivation medium for the trophic mycelium (nutritional agar or submerged growth). All studied hyphae had otherwise healthy phenotype. These granular structures vary in size and distribution across the mycelium. The granular structures are believed to be HFB8_{mRFP} accumulated in vacuoles (Figure 6 B). Due to the observation that conidia do not appear fluorescent (shown below) and the visual confirmation that the signal appears inside the hyphae, it was ruled out that the granules might be spores attached to the hyphal surface.

5.1.1.3 HFB8_{mRFP} fluorescence intensity is often strongest at septae

When grown on glass or plastic surfaces, *Tgui^{OEhfb8}* hyphae often show a “segmented” morphology (Figure 6 C+D). The hyphae do not appear round and firm, but “deflated” with much more intensely visible segments corresponding to hyphal compartments (cells). The mRFP signal observed was very intense at the septae and weaker in the hyphae-surface-air interface.

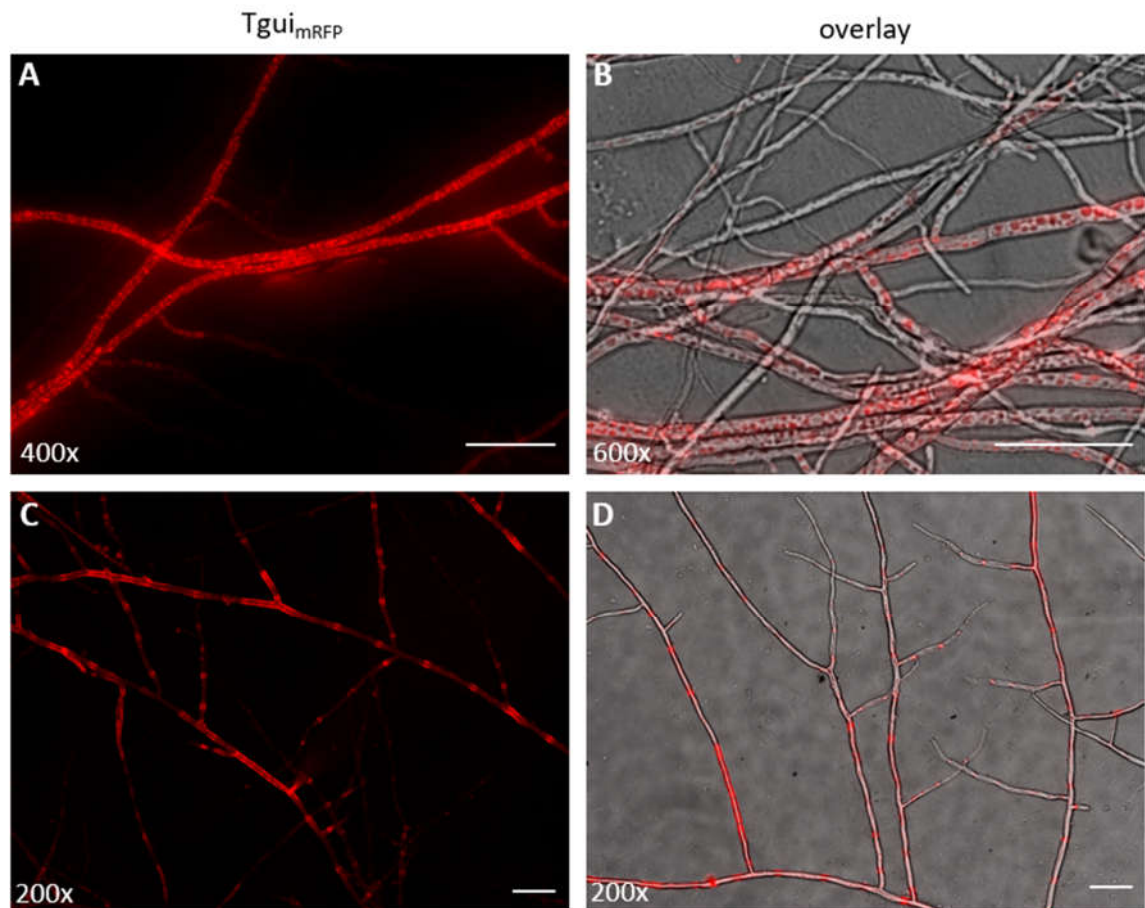


Figure 6: HFB8_{mRFP} heterologous fluorescence signal morphology

Investigation of HFB8_{mRFP} signal morphology on the non-trophic hyphae of Tgui_{OEhfb8} on a microscopy glass slide surface. A) Granular structure observed at x 400 magnification. B) Granular structure observed at x 600 magnification, overlain with the bright field image. C) Segmented HFB8_{mRF} signal morphology observed at x 200 magnification. D) Segmented HFB8_{mRF} signal morphology observed at x 200 magnification, overlain with the bright field image.

Images were recorded with Leica fluorescent DMI8 Microscope (Leica Microsystems, Germany).

The size bars correspond to a length of 50µm.

5.1.1.4 The mRFP signal morphology is setup dependent

Generally, the red fluorescent signal is not homogeneously distributed across Tgui_{OEhfb8} on either trophic (feeding) or non-trophic (aerial hyphae) hyphae in any experimental setup. In submerged liquid culture and on PDA, the granular structures are visible in most of the mycelium, but intensity and size of granules vary (Figure 7 A), and not all hyphae show a fluorescent signal (Figure 7 B). Investigation of samples of submerged cultures under the epifluorescence microscope showed no accumulation of HFB8_{mRFP} in the dual hyphae-liquid interface.

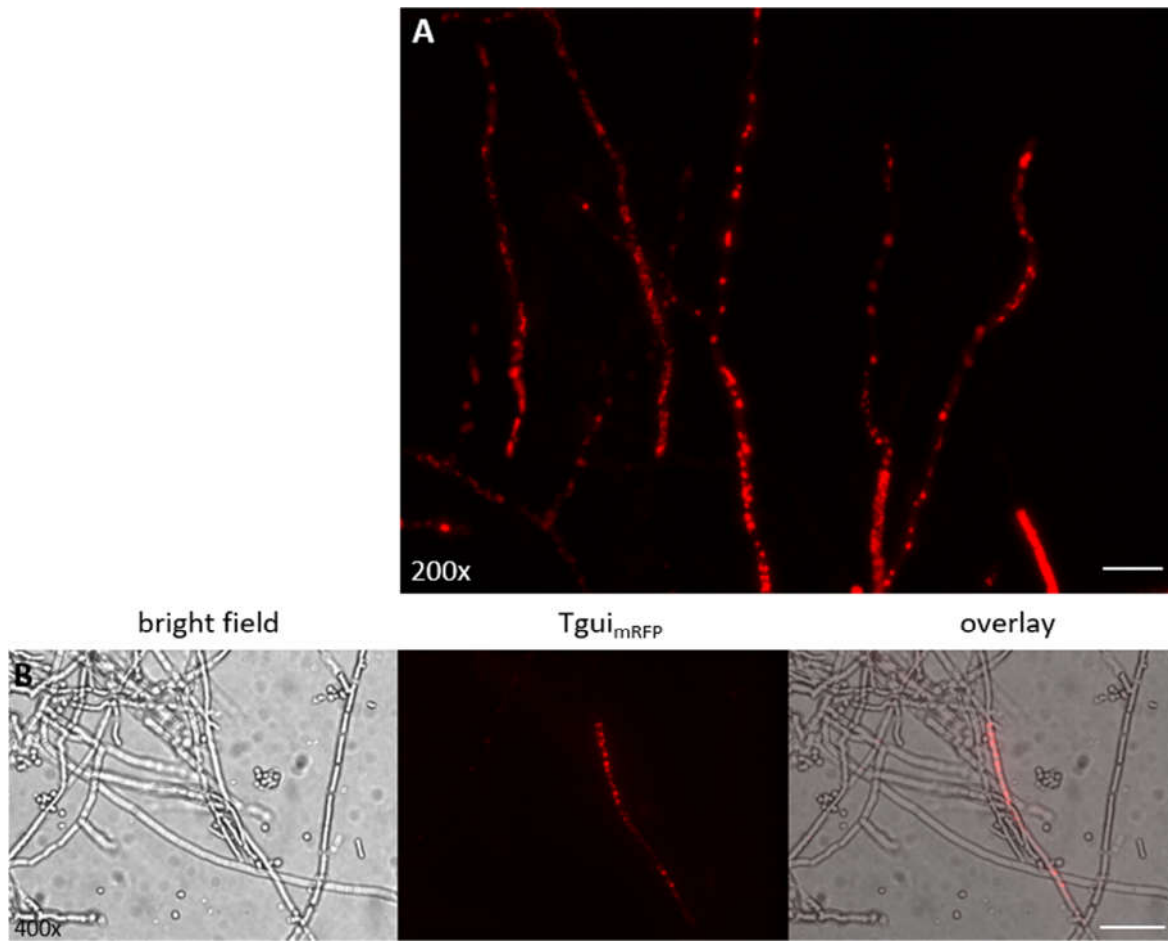


Figure 7: Samples of submerged liquid cultures of *Tgui^{OEhfb8}*

Samples of submerged liquid cultures of *Tgui^{OEhfb8}* observed on glass microscopy slides. A) The granular HFB8_{mRFP} signal (shown in red) is not homogeneously distributed across the mycelium, size and frequency of the granules varies (x 200 magnification). B) Not all hyphae show the red fluorescent signal of HFB8_{mRFP} (x 400 magnification).

Images were recorded with Leica fluorescent DMI8 Microscope (Leica Microsystems, Germany).

The size bars correspond to a length of 50µm.

Grown on glass slides or plastic petri dish surfaces, the interface signal is predominant. In this setup the previously observed granular structure is most intense near the source of nutrition, while young hyphae further away from the nutrition source do not show granular structures (Figure 8 A). While many hyphae show both the granular structure and the interface signal simultaneously (Figure 8 B), mostly these two signal types are mutually exclusive. Since the granular structures, which are believed to be vacuoles with accumulated HFB8_{mRFP}, only occur close to the nutrient source, this accumulation might be nutrient dependant.

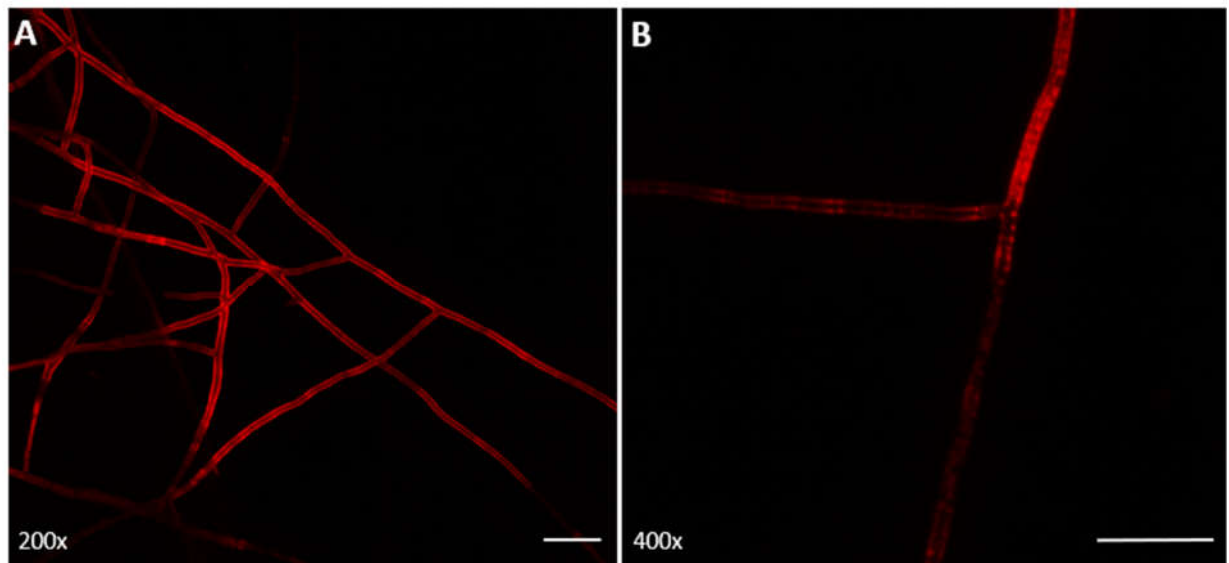


Figure 8: HFB8_{mRFP} signal morphology depends on proximity of non-trophic *TguiOE_{hfb8}* hyphae to nutrient source

Microscopic images of the non-trophic hyphae of *TguiOE_{hfb8}* on a microscopy glass slide surface. A) Young hyphae far away from the PDA plug nutrient source (x 200). The granular HFB8_{mRFP} signal is lost, the hyphae-water-air signal is predominant (shown in red). B) Older hyphae, closer to the nutrient source (x 400). Many hyphae show both the granular and the hyphae-water-air signal simultaneously (shown in red), but mostly those two signal types are mutually exclusive.

Images were recorded with Leica fluorescent DMI8 Microscope (Leica Microsystems, Germany).

The size bars correspond to a length of 50μm.

5.1.1.5 Conidiophores are brightly fluorescent

When observed under 400x magnification in the epifluorescence microscope, conidiophores of *TguiOE_{hfb8}* appear brightly fluorescent (Figure 9 A). However dispersed spores do not show any red fluorescent signal (Figure 7 B). When recorded in a fluorescent imager (Chemidoc, BioRad, USA), areas covered in green spores also do not show any fluorescent signal (Figure 9 B).

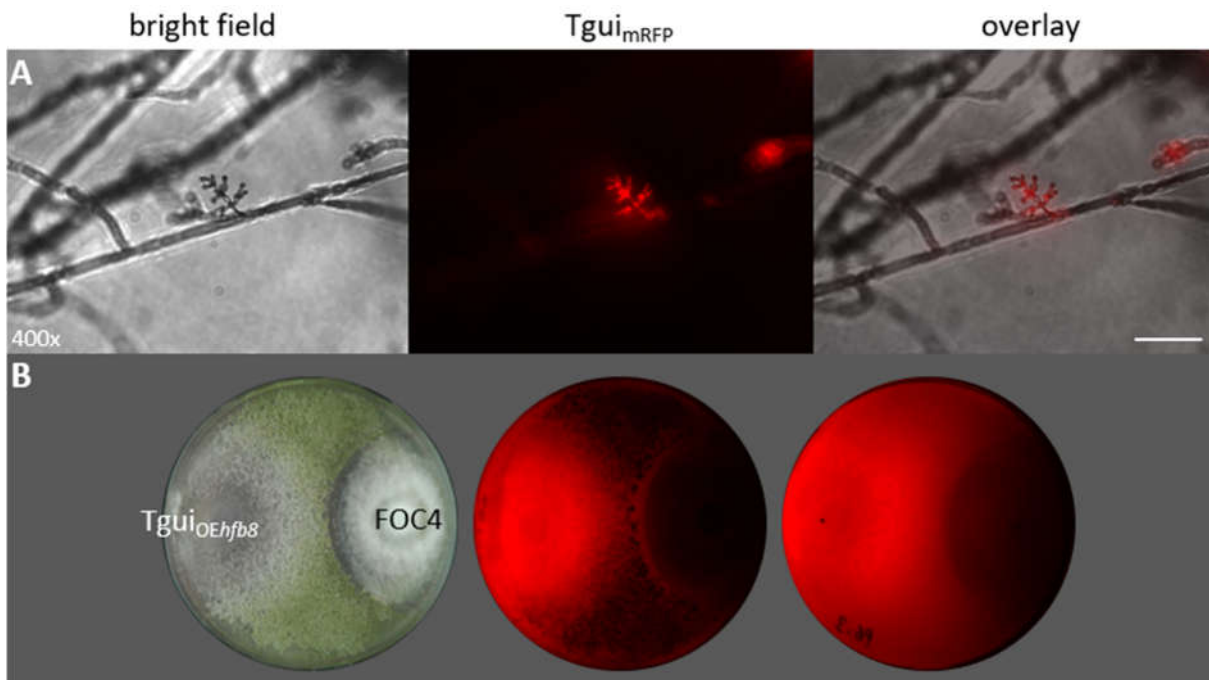


Figure 9: HFB8_{mRFP} fluorescence is present on conidiophores, but not on conidia.

A) Microscopic image of a conidiophore on a non-trophic hypha of Tgui_{OEhfb8} on a microscopy glass slide surface (x 400). Conidiophores appear brightly red fluorescent in every investigated setup. B) Dual confrontation of Tgui_{OEhfb8} against FOC4 on PDA, grown for 16 days at 25°C in darkness in a 9cm Petri plate. The left image shows the agar plate covered in green spores from Tgui_{OEhfb8}, recorded by photography. The other two images show the same plate recorded in ChemiDoc (Biorad, USA) from above (middle) and below (right). The area covered in spores does not appear red fluorescent, while the agar underneath does.

The size bar corresponds to a length of 50µm.

5.1.2 Influence of HFB8_{mRFP} overexpression on *T. guizhouense* vitality

To test possible implication of utilizing HFB8_{mRFP} as a tag, the characteristics of Tgui_{OEhfb8} were tested in a diversity of *in vitro* conditions and compared to the wild type and the Tgui_{Δhfb8} deletion mutant.

5.1.2.1 HFB8_{mRFP} overexpression is associated with a reduced growth rate

The impact of HFB8_{mRFP} overexpression on the surface growth rate was monitored on potato dextrose agar (PDA) plates incubated in darkness or in an alternating rhythm of 12 hours illumination and 12 hours darkness (12 hours illumination). Figure 10 shows the *T. guizhouense* parental strain, Tgui_{OEhfb8} and deletion mutants grown on PDA for three days in darkness. Deletion of *hfb8* does not affect the growth rate, while the HFB8_{mRFP} overexpression mutant grows slower (a non-quantitative visual observation).

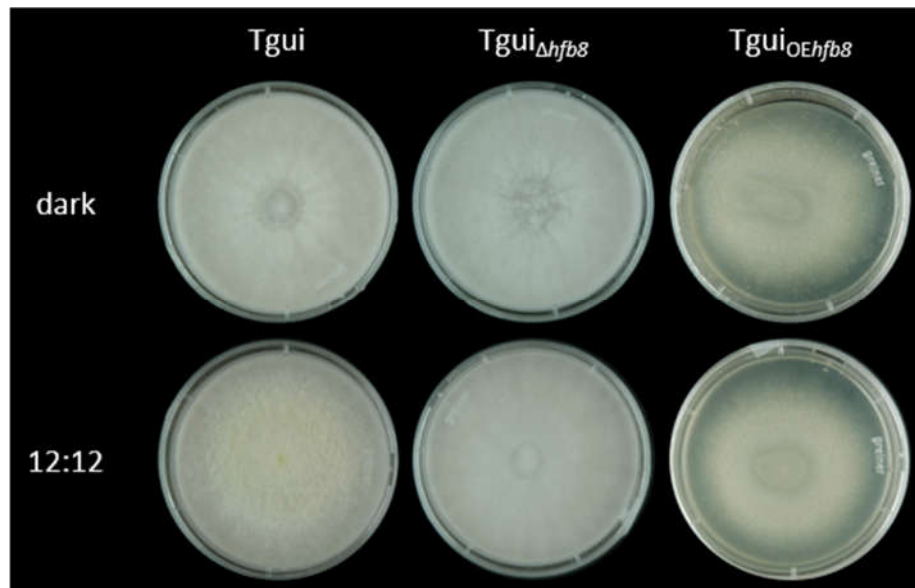


Figure 10: Influence of HFB8 mutations on growth rate of Tgui

T.guizhouense wild type (Tgui) and the HFB8 overexpressing (Tgui^{OEhfb8}) and deletion (Tgui^{Δhfb8}) strains grown on PDA in 6cm Petri plates at 25°C for three days. The cultures were grown in darkness or under 12 hours illumination. In darkness, Tgui^{Δhfb8} does not show any difference to Tgui, while Tgui^{OEhfb8} shows reduced growth. Under illumination the cultures appear similar to the ones in darkness, except Tgui is starting to sporulate, as indicated by the lightly green ring.

5.1.2.2 Sporulation is drastically reduced in Tgui^{OEhfb8}

When faced with unfavourable conditions fungi usually produce spores for aerial or aquatic dispersal to ensure survival. To monitor sporulation, Tgui, Tgui^{OEhfb8} and Tgui^{Δhfb8} were cultivated on PDA plates under 12 hours illumination or in darkness. The overexpression of HFB8_{mRFP} shows a drastic delay in sporulation compared to the parental strains, whereas the deletion of *hfb8* does not appear to have any implications on the amount of spores produced, although the sporulating areas seem to slightly differ from Tgui (Figure 11).

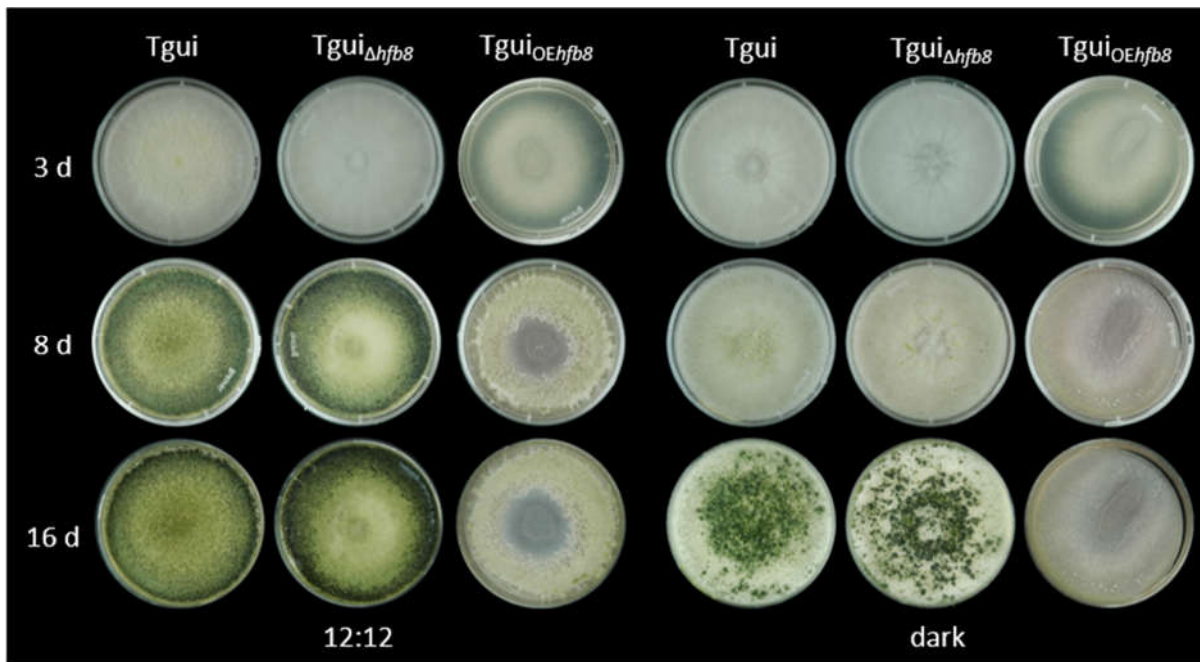


Figure 11: Influence of HFB8 mutations on sporulation of Tgui

T. guizhouense wild type (Tgui) and the HFB8 overexpressing (Tgui^{OEhfb8}) and deletion (Tgui^{Δhfb8}) strains grown on PDA in 6cm Petri plates at 25°C under 12 hours illumination. After eight days under illumination, the Tgui cultures are completely covered in spores. Sporulation of Tgui^{OEhfb8} is strongly reduced, compared to Tgui. The deletion of *hfb8* (Tgui^{Δhfb8}) does not appear to influence the amount of spores produced but does seem to slightly affect which areas show sporulation.

5.1.2.3 The overexpression of HFB8_{mRFP} alters response to mechanical injury

A known fungal stressor is mechanical injury to hyphae, which induces the release of reactive oxygen species (ROS) [99]. Tgui and the HFB8 mutants (Tgui^{OEhfb8} and Tgui^{Δhfb8}) were grown on PDA in the dark for 72 hours, cut with a sterile cold scalpel and incubated for another 72 hours. The parental strain and Tgui^{Δhfb8} show strong sporulation at the site of injury, while Tgui^{OEhfb8} does not show the same response to physical damage (Figure 12).

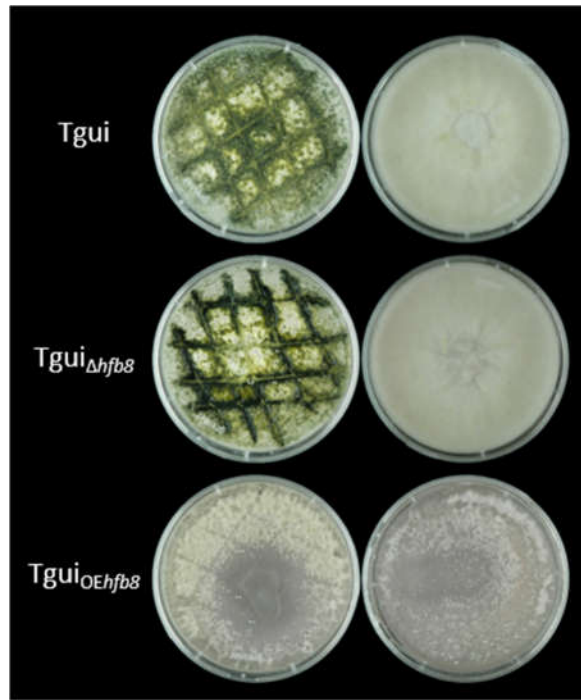


Figure 12: Influence of HFB8 mutations on response to physical damage of Tgui

T. guizhouense wild type (Tgui) and the HFB8 overexpressing (Tgui^{OEhfb8}) and deletion (Tgui^{Δhfb8}) strains grown on PDA in 6cm Petri plates at 25°C in darkness for three days, physically damaged by a sterile scalpel, and incubated for another three days under 12 hours illumination. Tgui responds to this injury by strong sporulation at the affected area, as does Tgui^{Δhfb8}. Tgui^{OEhfb8} does not show any response by sporulation. The cultures on the right are negative controls, not subjected to physical damage.

5.1.2.4 Formation of aerial hyphae in liquid culture is lost in Tgui^{OEhfb8}

The formation of aerial hyphae plays an important role in the reproduction and distribution of filamentous fungi. Since fungi often colonize moist environments (tissues, soils and other substrates), they must breach the water-air interface for the formation of aerial mycelium. Due to high water surface tension, breach by pure force is difficult or not possible for filamentous fungi. Hydrophobins play a vital role in this breach, as they spontaneously form amphiphilic monolayers at the water-air interface, which reduces surface tension and thus facilitates aerial growth [100].

To investigate the importance of HFB8_{mRFP} in the formation of aerial hyphae, a standing liquid cultivation of Tgui and Tgui^{OEhfb8} in minimal medium was performed in the dark for five days. Figure 13 shows the formation of fluffy aerial mycelium in the parental culture, while no aerial hyphae were formed in the Tgui^{OEhfb8} mutant culture. This suggests that the overexpression of HFB8_{mRFP} alters the regulation of the *T. guizhouense* hydrophobome, preventing the formation of an amphiphilic monolayer and consequently the development of aerial hyphae.



Figure 13: Influence of HFB8_{mRFP} overexpression on the formation of aerial hyphae

T. guizhouense wild type (Tgui) and Tgui^{OEhfb8} grown in 2 ml minimal medium at 25°C in darkness for five days. Tgui shows fluffy, aerial mycelium covering the entire well, while no hyphae have penetrated the water-air interface in Tgui^{OEhfb8}.

5.1.2.5 Surface hydrophobicity is reduced in Tgui^{OEhfb8}

To quantify the role of HFB8_{mRFP} in hyphal hydrophobicity the water contact angle (WCA) of a water droplet applied to the fungal mycelium was measured (Figure 14). The red line in Figure 14 A marks the 90° contact angle between the droplet and the surface, the hydrophobicity threshold. The parental strain shows high contact angles (94.58 ± 16.25), indicating that its aerial mycelium is hydrophobic. While very low angles (30.80 ± 24.05) were measured on the mycelium of the Tgui^{OEhfb8} strain, the hyphae of the deletion mutant Tgui^{Δhfb8} displayed contact angles (108.11 ± 4.60) even higher than the parental strain. Analysis by ANOVA shows that the overexpression of HFB8_{mRFP} significantly reduces hydrophobicity ($p < 0.001$) of aerial hyphae, while the deletion of *hfb8* (Tgui^{Δhfb8}) results in a slight, but not statistically significant ($p = 0.54$), increase in hydrophobicity.

The same measurements were carried out on older mycelium covered in spores. The contact angles on spores were higher overall, the biggest difference between mycelium (30.80 ± 24.05) and spores ($73.99 \pm 30.80 \pm 35.03$) was measured on Tgui^{OEhfb8} ($p = 0.008$). Almost no difference could be measured between parental spores ($114.93 \pm 30.80 \pm 99.45$) and Tgui^{Δhfb8} spores ($115.70 \pm 30.80 \pm 5.94$). The contact angles measured on Tgui^{OEhfb8} spores were significantly lower than the angles of the other strains ($p < 0.001$). However, Tgui^{OEhfb8} mycelium was not completely covered in spores (Figure 14 B), which most likely influenced the measurement. This alone leaves inconclusive results to whether HFB8 significantly influences spore hydrophobicity. But, as it has been previously shown, HFB8_{mRFP} most likely does not cover the spore surface, as no red fluorescent signal was observed on spores. Together these findings suggest that HFB8, at least when expressed with an mRFP tag under the *P_{cdna1}* promoter, does not have any influence on *T. guizhouense* spore hydrophobicity.

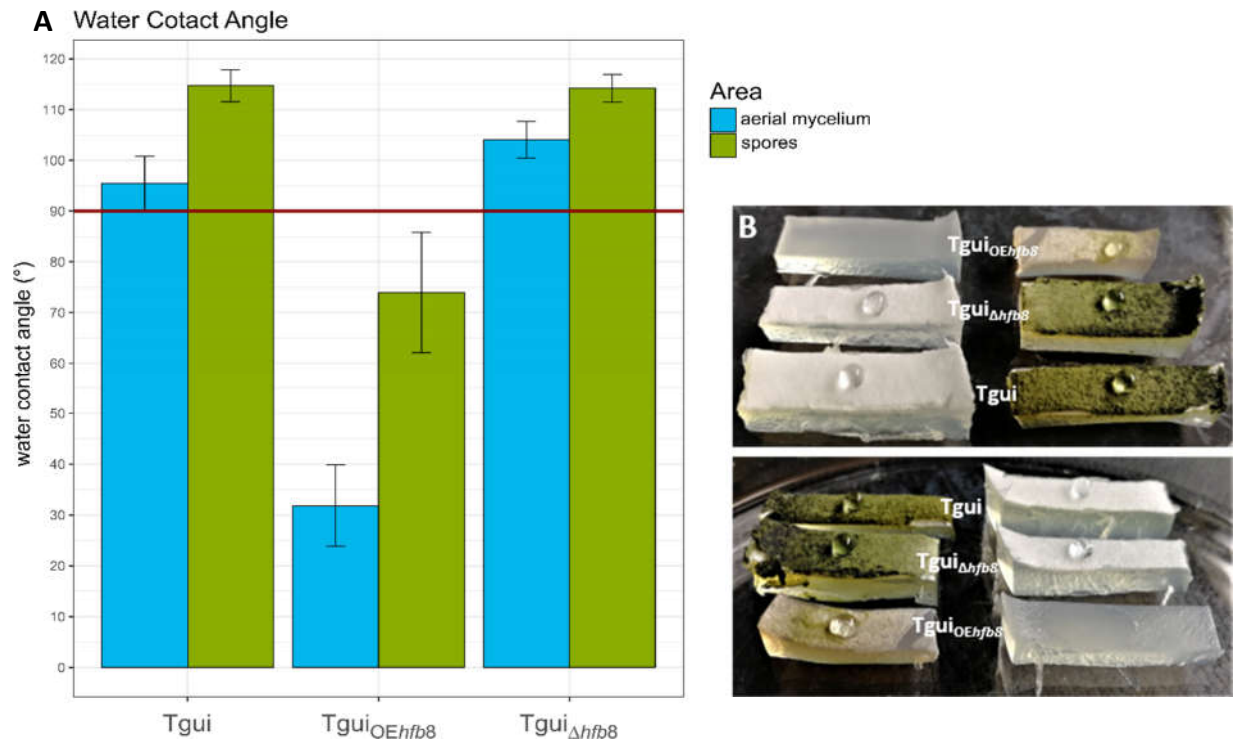


Figure 14: Influence of HFB8 mutations on hydrophobicity of Tgui hyphae and spores

A) The hydrophobicity of hyphae and spores of Tgui and HFB8 mutant strains, recorded by the contact angle of an 8μl water droplet. The red line at 90° indicates the hydrophobic-hydrophilic threshold. The Tgui mycelium is hydrophobic. The Tgui^{Δhfb8} mycelium shows slightly increased hydrophobicity compared to Tgui, while the Tgui^{OEhfb8} mycelium is strongly hydrophilic. Tgui spores are more hydrophilic than its mycelium. There is no difference between Tgui and Tgui^{Δhfb8} mycelium, Tgui^{OEhfb8} spore hydrophobicity seems strongly reduced, but the mycelium was not completely covered by spores which most likely influenced the results. B) Water droplets on Tgui, Tgui^{OEhfb8} and Tgui^{Δhfb8} mycelium after three days incubation in darkness and on spores after 10 days under 12 hours illumination.

5.1.2.6 HFB8_{mRFP} overexpression influences macromorphology of the colony

The influence of HFB8 mutation on the morphology of fungal mycelium was investigated on PDA plates. Figure 10 shows that the overexpression of HFB8_{mRFP} drastically reduces the formation of fluffy mycelium, whereas *hfb8* deletion does not seem to have a strong influence on fluffiness.

The overexpression of HFB8_{mRFP} does not only influence the volume of aerial mycelium, but also its morphology. Figure 15 shows the parental strain on the left, which grows in a directed, “streaky” manner. The fluffy mycelium covers the whole plate homogeneously. The right image in Figure 15 shows the mycelium of Tgui^{OEhfb8}. The growth does not look directed and not homogeneous. Younger hyphae (right side) look less fluffy and a hydrophilic circle is visible around the inoculation site (aged hyphae).

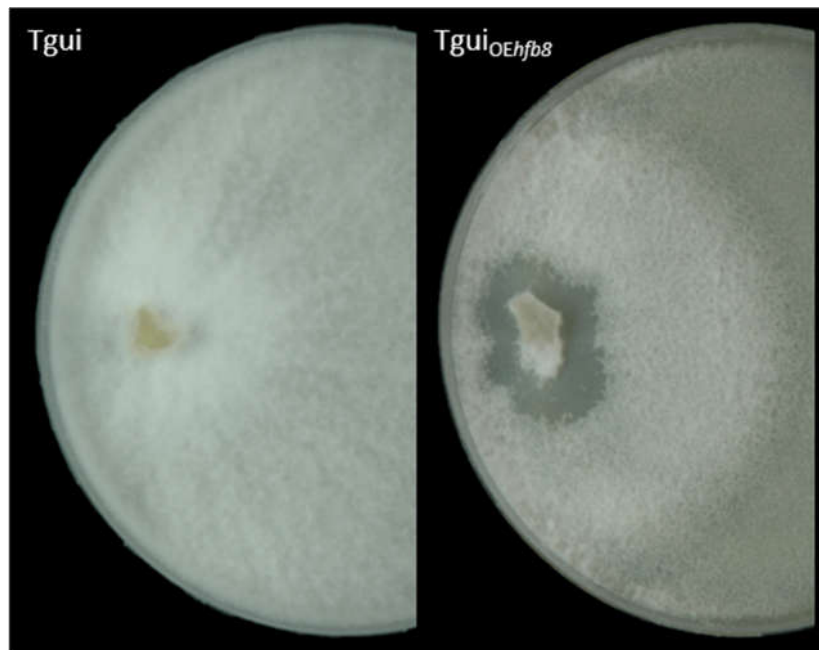


Figure 15: Influence of HFB8_{mRFP} overexpression on mycelial macromorphology

T. guizhouense wild type (Tgui) and Tgui_{OEhfb8} grown on 9cm PDA Petri plates at 25°C in darkness for five days. Tgui shows fluffy mycelium, growing in a directed, “streaky” morphology. Tgui_{OEhfb8} growth is less directed, the aerial mycelium is less voluminous and fluffy. A hydrophobic patch is forming around the inoculation site (aged hyphae).

5.1.2.7 Tgui_{OEhfb8} shows altered morphology of non-trophic hyphae

The heterogeneous morphology of Tgui_{OEhfb8} hyphae is even more apparent on a microscopic scale. As shown previously (section 5.1.1.2), Tgui_{OEhfb8} is able to produce strong, healthy-looking hyphae. However, it also seems more vulnerable and prone to irregularities and formation of unhealthy hyphae than the parental strain.

Figure 16 A shows a self-confrontation of Tgui_{OEhfb8} on a glass microscopy slide. Hyphae are weak-looking, stunted and not growing straight. As mentioned before (Figure 6 C+D), hyphae often show a “segmented” morphology when grown on glass or plastic (Figure 16 B). Another observed form of this segmentation is shown in Figure 16 C. The bulb-like structures in this figure are most likely vacuoles or single segments that are inflated due to liquid aggregation. This morphology occurs along very thin and weak-looking hyphae that appear to be dying or are starting to degrade. Another frequently observed morphology is hyphae which clump together, which might be due to their altered surface hydrophobicity (Figure 16 D).

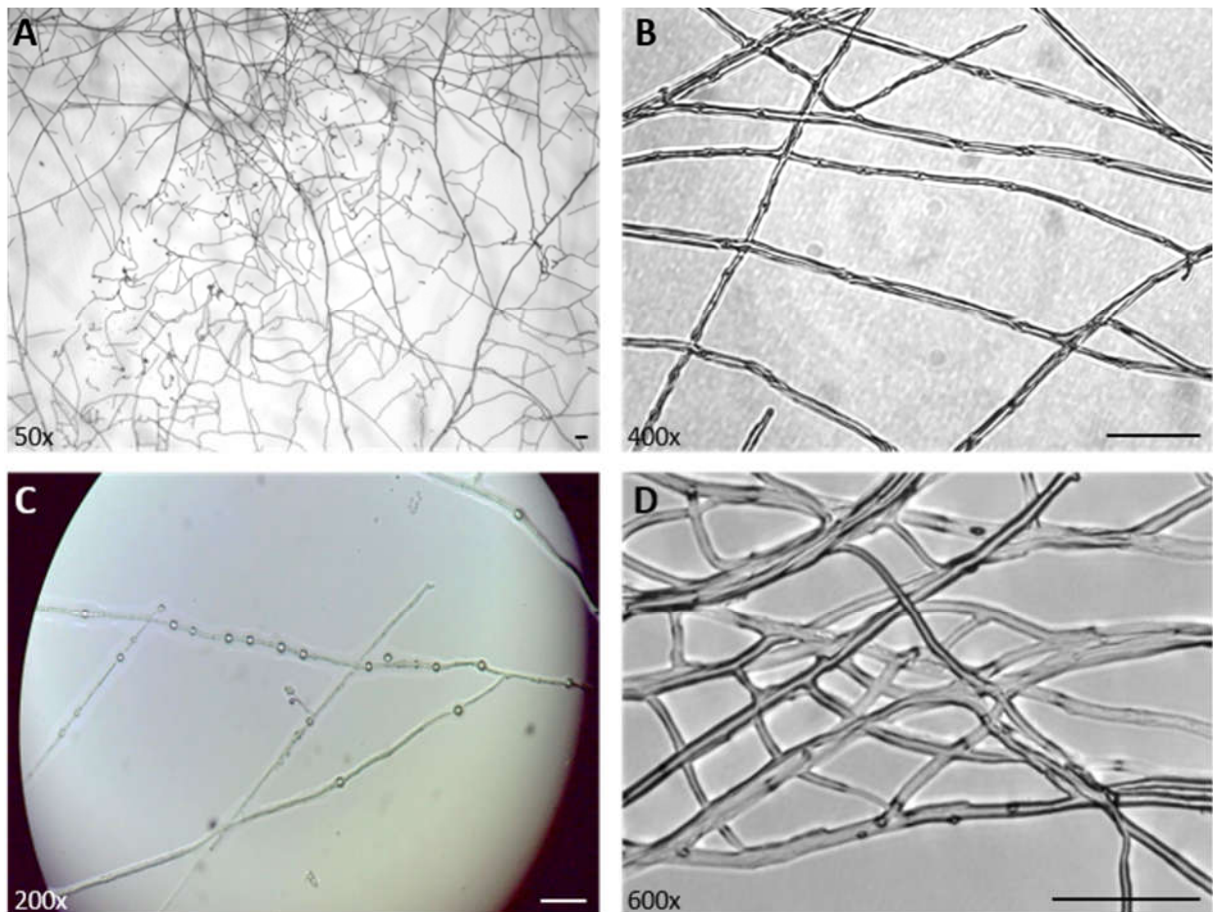


Figure 16: Influence of HFB8_{mRFP} overexpression on morphology of non-trophic hyphae on microscopy slides

Microscopic images of the non-trophic hyphae of *Tgui^{OEhfb8}* on microscopy glass slide surface. A) Self confrontation of *Tgui* and *Tgui^{OEhfb8}* (x 50). Hyphae are stumpy and unhealthy. B) “Segmented” hyphal morphology (x 400). Cellular compartments appear flat, except at septae. C) Bulb-like, inflated segments (x 200). Hyphae are degrading, some cells are inflating, which is most likely due to liquid aggregation. D) Hyphae are clumping together (x 600).

Images were recorded with Leica fluorescent DMI8 Microscope (Leica Microsystems, Germany).

The size bars correspond to a length of 50µm.

The previous observations were all recorded on glass or plastic surfaces, and therefore in an unfavourable and nutrient-low environment. This might lead to the conclusion that these morphotypes occur only due to starvation or surface properties. However, these abnormalities occurred with much lower frequency in the same setups with the parental strain. Additionally, unhealthy morphology of *Tgui^{OEhfb8}* was also recorded in a more favourable setup. As Figure 17 shows, young hyphae in agar plugs look unhealthy and stumpy. They appear weak and are not growing in a smooth, straight morphology, although they do not appear as curled as hyphae growing on glass surfaces shown in Figure 16. Since the agar plug used in this setup does not contain PDB, this unhealthy morphotype might be caused by nutrient deprivation.

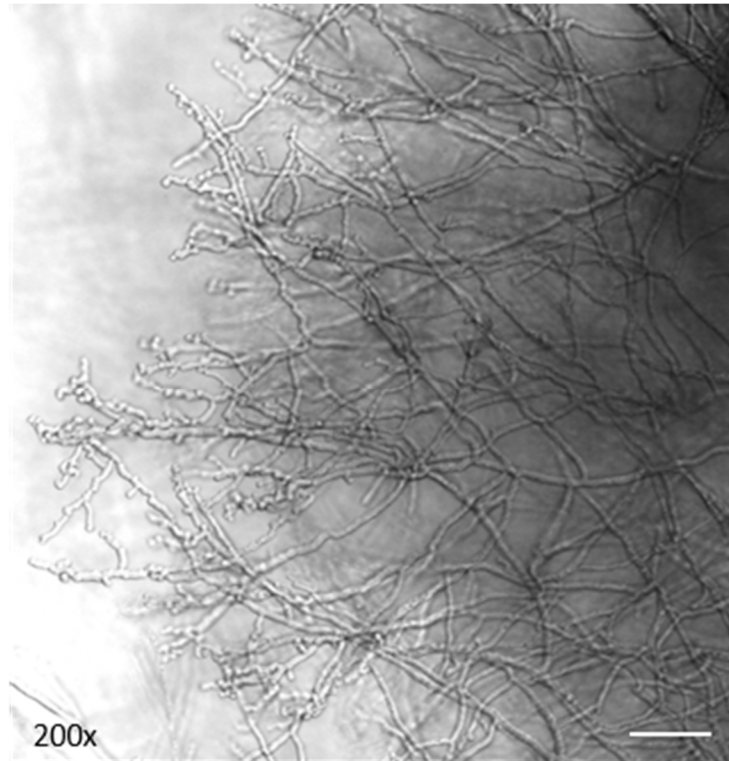


Figure 17: Influence of HFB8_{mRFP} overexpression on morphology of non-trophic in agar plugs

Microscopic images of Tgui_{OEhfb8} hyphae on microscopy glass slides inside agar agar plugs (x 200) recorded with Leica fluorescent DMi8 Microscope (Leica Microsystems, Germany). Young hyphae appear stumpy and weak. This morphology is most likely caused by nutrient deprivation, as agar agar does not provide enough nutrients for Tgui.

The size bar corresponds to a length of 50μm.

5.1.3 Challenges of HFB8_{mRFP} as a tag for fungal interactions and gene expression

The overexpression of HFB8_{mRFP} has an influence on growth, sporulation, stress response and hydrophobicity, summarized in

Table 8. Hyphae are more vulnerable, but the formation of healthy mycelium is not prevented. The mRFP signal observed under the epifluorescence microscope is not homogeneous throughout the entire mycelium and dependent on different setups. This must be taken into consideration when using the construct as a tag for *T. guizhouense* fungal- fungal interactions and gene expression.

Phenotype	Tgui _{OEhfb8}	Tgui _{Δhfb8}
Growth rate	Reduced	No change
Sporulation	Delayed	No change
Response to mechanical injury	No sporulation	No change
Aerial hyphae formation	Delayed/Lost	No change
Hyphal hydrophobicity	Strongly reduced	Slightly increased
Spore hydrophobicity	Inconclusive	No change

Table 8: Summary of the recoded phenotypes of HFB8 mutants

5.2 Macromorphology of the interactions between *Trichoderma guizhouense* and other fungi

Fungal - fungal interactions play an important role in both natural ecosystems and manmade environments (e.g. in agriculture). To investigate its interaction with other fungi, *T. guizhouense* (wt and Tgui_{OEhfb8}) was cultivated in frontal confrontation setups with different fungal partners.

5.2.1 Dual confrontation assays on agar plates

The four fungal interaction partners selected in this study are *Pleurotus ostreatus*, an edible mushroom, *Pestalotiopsis fici*, a prominent producer of antimicrobial substances, and two members of *Fusarium oxysporum*, which are known plant pathogens and mycotoxin producers. These four fungi were selected to study *T. guizhouense* interactions with partners of different taxonomic proximity.

A common setup for investigating fungal- fungal interactions is as dual confrontations on agar plates (Figure 18). Two different fungal cultures are inoculated at opposite sides of an agar plate and incubated together. In these conditions, fungi are forced to a combative interaction because the resources in the Petri plate are limited. They may either prevent growth of each other prior to contact through the secretion of volatile antifungal metabolites, or combat in a direct physical contact. In the latter case, fungi may stay in a dead lock reaction (growth of both colonies stops after contact) or one fungus can overgrow the other one in either agonistic or mycoparasitic manner [50]. In this study, PDA plates were used, and cultures were inoculated either as mycelium plugs or spore suspensions.



Figure 18: Dual confrontation setup on PDA Petri plates

Dual fungal confrontation assays on PDA on 9cm Petri plates. Colonies are inoculated at opposite sides on the agar plate, ~1cm from the edge of the plate. Fungal cultures are inoculated as either spore suspensions (left) or as mycelium plugs extracted from monocultures on PDA (right).

Figure 19 shows *T. guizhouense* confrontations with each of the previously mentioned fungi. The first apparent observation is that *T. guizhouense* grows a lot faster in this setup than its opponents. Even though Tgui^{OE_{hfb8}} grows slower than the parental strain, there seems to be no difference in growth radius of the interaction partners in Tgui^{OE_{hfb8}} confrontations compared to wt confrontations.

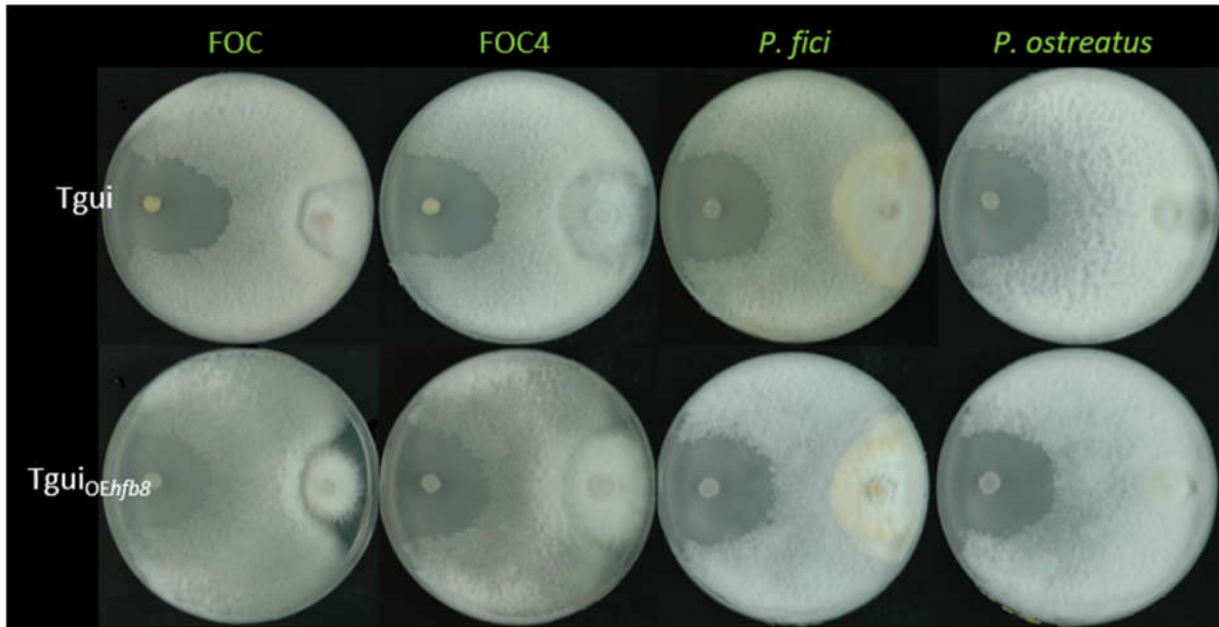


Figure 19: Dual confrontation assays of Tgui and Tgui^{OE_{hfb8}} against 4 different fungi

Dual fungal confrontation assays of Tgui and Tgui^{OE_{hfb8}} against *P. ostreatus*, *P. fici*, FOC and FOC4 grown on PDA on 9cm Petri plates at 25°C in the darkness for five days. Tgui and Tgui^{OE_{hfb8}} are growing a lot faster than the other strains in this setup, covering the entire plate (until contact with the other colony).

5.2.1.1 *Trichoderma guizhouense* overgrows *Pleurotus ostreatus*

Pleurotus ostreatus (Agaricales) is an edible mushroom, which can be cultivated in farms. *Trichoderma* frequently attacks these mushroom farms, causing the green mold disease [64]. *P. ostreatus* belongs to the Basidiomycota, other than *T. guizhouense*, which belongs to the Ascomycota. Therefore *P. ostreatus* is an interesting partner for the investigation of interactions of *Trichoderma* to distantly related fungi. Figure 20 shows a confrontation setup on PDA plates. *Trichoderma guizhouense* quickly overgrows the entire plate, including the *Pleurotus ostreatus* mycelium. There is no pigmentation that could suggest the formation of secondary metabolites observed. Tgui^{OE_{hfb8}} behaves the same as the parental strain regarding this interaction. Investigation of this setup under the stereomicroscope does not show any interaction of the two partners, and no penetration or degradation of hyphae (not shown).

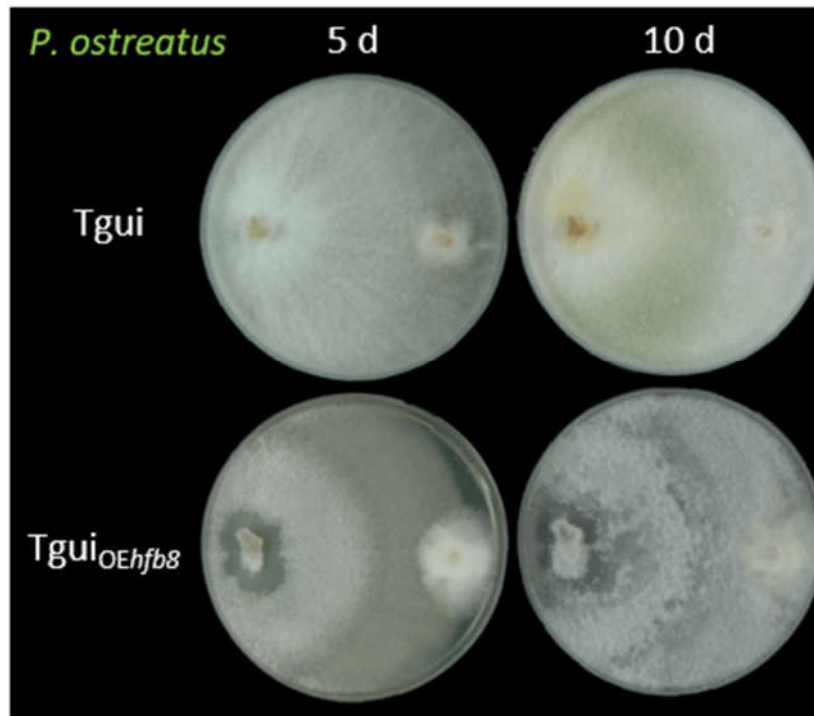


Figure 20: Dual confrontation assays of Tgui and Tgui^{OEhfb8} against *P. ostreatus*

Dual fungal confrontation assays of Tgui and Tgui^{OEhfb8} against *P. ostreatus* grown on PDA on 9cm Petri plates at 25°C in the darkness for 10 days. Tgui^{OEhfb8} growth is delayed upon contact with *P.ostreatus*, but both Tgui strains eventually completely overgrow *P.ostreatus*.

5.2.1.2 *Pestalotiopsis fici* produces secondary metabolites when confronted with *Trichoderma guizhouense*

Pestalotiopsis fici (Xylariales) is a plant endophyte and known producer of various secondary metabolites [62]. It occupies the same ecological niche as *Trichoderma* and endoparasitism of *T. reesei* on *P. fici* has been suggested *in vitro* [50]. *P. fici* belongs to the same class as *Trichoderma*, Sordariomycetes, which makes it an interesting candidate for studying fungal-fungal interactions on this taxonomic level. A confrontation of these two fungal species on PDA shows that *P. fici* produces secondary metabolites- as shown by yellow pigmentation- upon contact with *T. guizhouense* mycelium (Figure 21). The metabolites do not seem to have a strong inhibitory effect on *T. guizhouense* growth, as no clear area is forming on the agar. The *T. guizhouense* parental strain is able to grow over the *P. fici* culture. This ability is reduced in Tgui^{OEhfb8}, which only partly covers the *P. fici* colony.

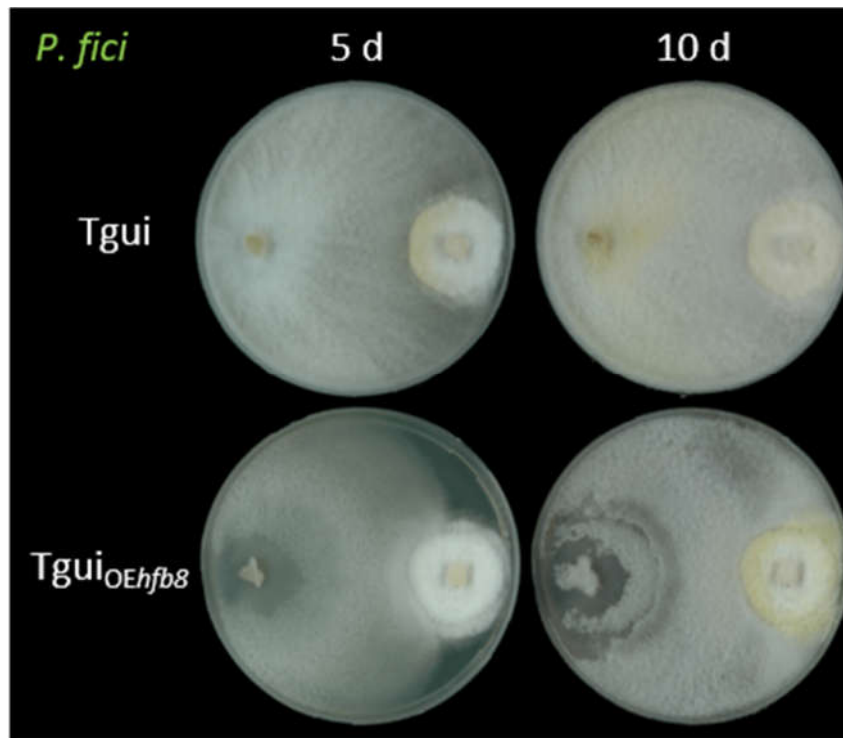


Figure 21: Dual confrontation assays of Tgui and Tgui^{OEhfb8} against *P. fici*

Dual fungal confrontation assays of Tgui and Tgui^{OEhfb8} against *P. fici* grown on PDA on 9cm Petri plates at 25°C in the darkness for 10 days. *P. fici* produces secondary metabolites (as indicated by yellow pigmentation) upon contact with Tgui and Tgui^{OEhfb8}. Tgui is able to overgrow *P. fici* completely. Tgui^{OEhfb8} growth is delayed upon contact with *P. fici* but is eventually able to partly overgrow *P. fici*.

5.2.1.3 *Fusarium oxysporum* f. sp. *cubense* 4 (FOC4) shows strong defense against *Trichoderma guizhouense*

Fusarium spp. (Hypocreales) are mycotoxin-producing plant-pathogenic fungi that may inhabit the same ecosystem with *Trichoderma*. They belong to the same orders as *Trichoderma*, which makes these fungi excellent candidates for studying interactions of *Trichoderma* with closely related fungi (adelphoparasitism) [50]. Here, *Fusarium oxysporum* f. sp. *cubense* (FOC) and *Fusarium oxysporum* f. sp. *cubense* 4 (FOC4) were used to investigate this interaction.

Figure 22 shows a confrontation setup of Tgui and Tgui^{OEhfb8} against FOC and FOC4 on PDA plates. As previously mentioned, *T. guizhouense* has a faster growth rate than its interaction partners. Upon contact with FOC4, Tgui growth is inhibited. This inhibition is even stronger against Tgui^{OEhfb8}. FOC does not seem to inhibit Tgui growth as strongly as FOC4 but shows the same inhibition against Tgui^{OEhfb8} as FOC4.

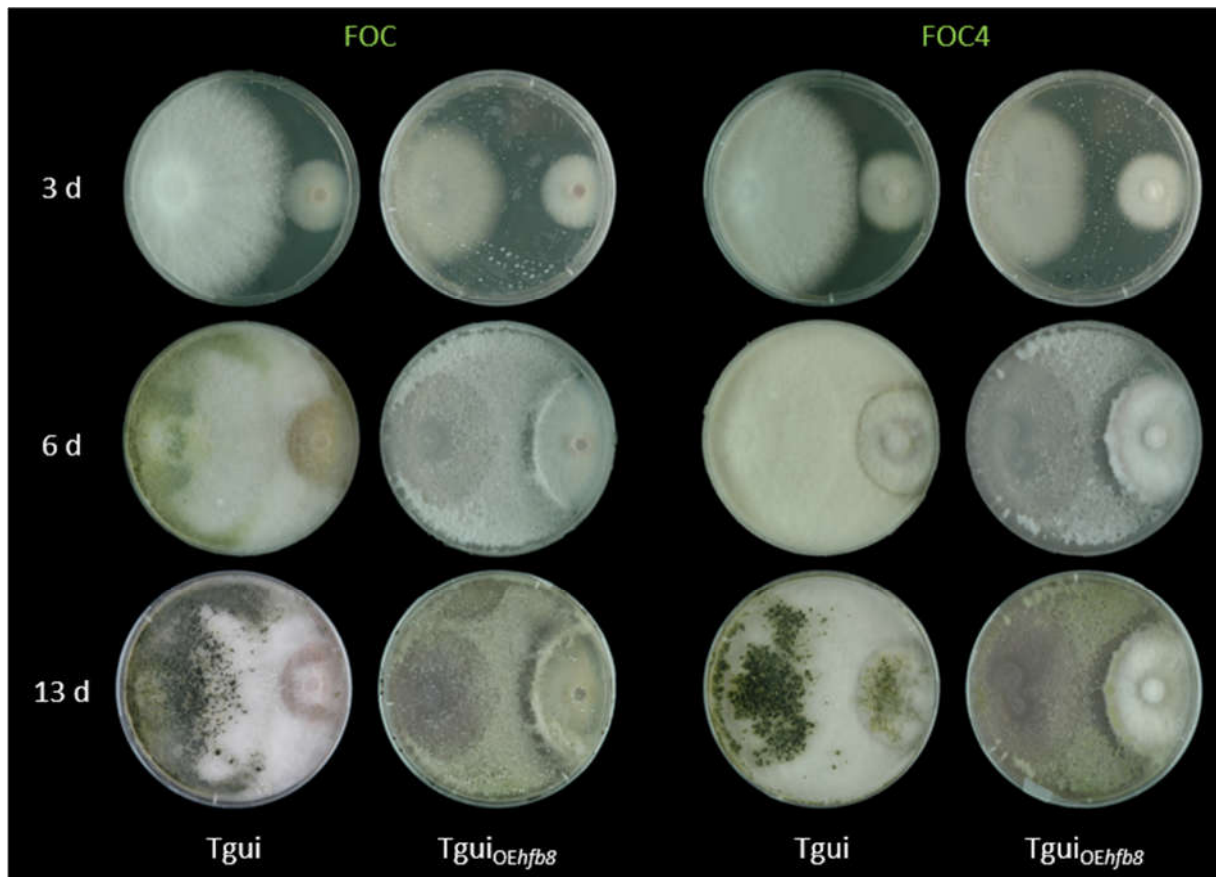


Figure 22: Dual confrontation assays of Tgui and Tgui^{OEhfb8} against FOC and FOC4

Dual fungal confrontation assays of Tgui and Tgui^{OEhfb8} against FOC and FOC4 grown on PDA on 9cm Petri plates at 25°C in the darkness. Tgui growth is strongly inhibited upon contact with FOC4, but only lightly by FOC. Tgui^{OEhfb8} growth is strongly inhibited by both FOC and FOC4, stronger than Tgui.

In the contact zone between FOC4 and Tgui a clear circle free of mycelium (antibiosis zone) is forming as indicated by the red arrows in Figure 23. This clear area does not form in the interaction with FOC. In the confrontation of both FOC and FOC4 with Tgui^{OEhfb8}, the clear zone is more prominent. This zone is most likely enriched in secondary metabolites (mycotoxins) secreted by FOC(4), toxic to *T. guizhouense* and inhibiting its growth. To overcome this barrier, *T. guizhouense* can form mycelium “bridges”, to avoid contact with the toxic agar, and continue growth onto the FOC(4) mycelium, which can be seen in Figure 23 B (indicated by red arrows). The putative molecular mechanisms of this interaction are described in Zhang et al. [58].

The overexpression of HFB8_{mRFP} seems to strongly reduce the combative strength of Tgui against FOC(4).

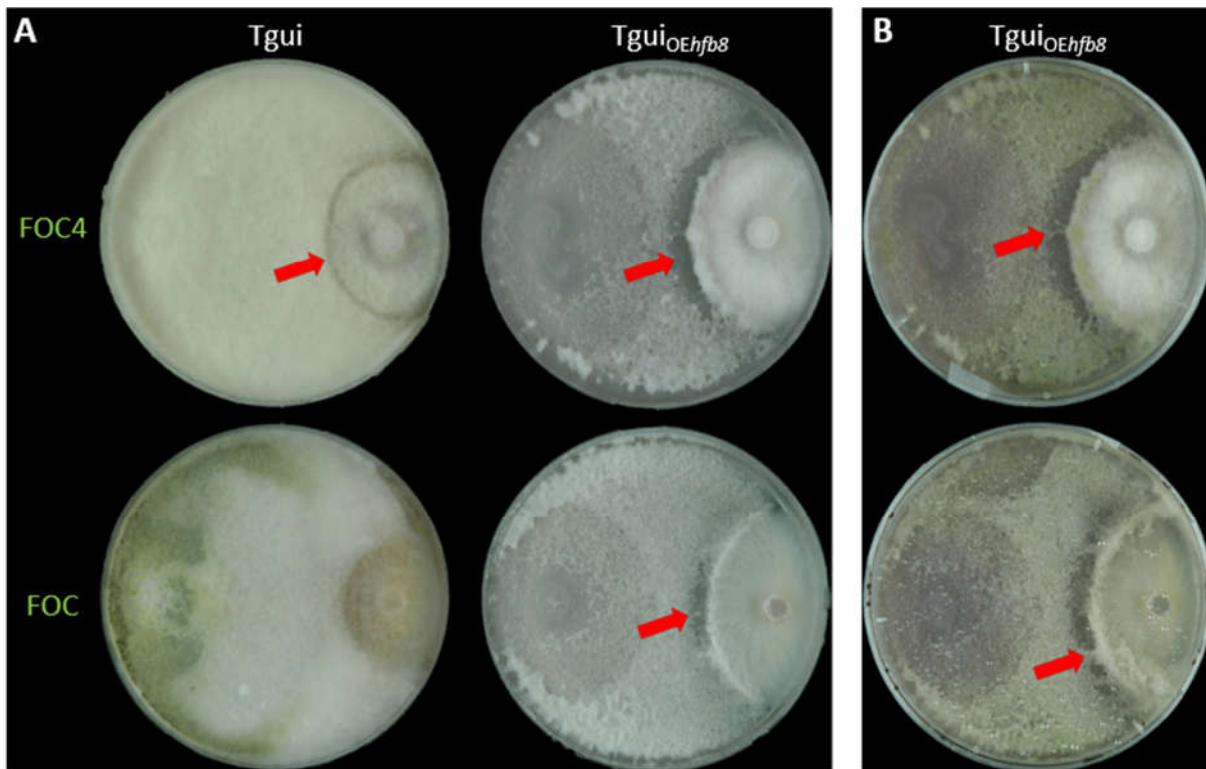


Figure 23: Antibiosis zone between FOC(4) and Tgui/ Tgui_{OEhfb8}

Dual fungal confrontation assays of Tgui and Tgui_{OEhfb8} against FOC and FOC4 grown on PDA on 9cm Petri plates at 25°C in the darkness. A) An antibiosis zone (indicated by red arrows) is forming between FOC4 and Tgui. The zone is larger between FOC4 and Tgui_{OEhfb8}. There is no antibiosis zone visible between FOC and Tgui, but the zone is forming between FOC and Tgui_{OEhfb8}. B) Tgui_{OEhfb8} is able to form mycelium “bridges” onto FOC(4) mycelium to avoid the toxic agar (indicated by red arrows). These bridges can be observed more clearly in confrontations of Tgui_{OEhfb8} compared to Tgui (not shown here), as the antibiosis zone is wider.

5.2.2 Interactions on microscopy slides

The confrontation setup on agar plates allows insights into the behaviour of fungal partners upon contact but not the detailed monitoring of fungal-fungal interactions. To visualize these details, light microscopy was used. To prevent disruption of the mycelium, and therefore the contact zone, when investigating fungal interactions, the fungi were directly confronted on microscopy slides incubated in sterile petri dishes (Figure 24). PDA plugs serve as nutrition source and inoculation sites. This setup poses challenges, as hyphae are growing on glass or plastic, which potentially influences their structural integrity (see section 5.1.2.7). The microscopic morphology of the hyphae of filamentous fungi cultivated in a confrontational setup is challenging to distinguish correctly, especially when hyphal structure might be altered due to setup conditions. To counteract this problem, fluorescent tags are commonly used to label microorganisms or cellular compartments for their identification by

epifluorescence microscopy. Here, HFB8_{mRFP} is used as such a tag. The red fluorescent protein was chosen due to strong autofluorescence of some fungal mycelium in the green emission spectrum.

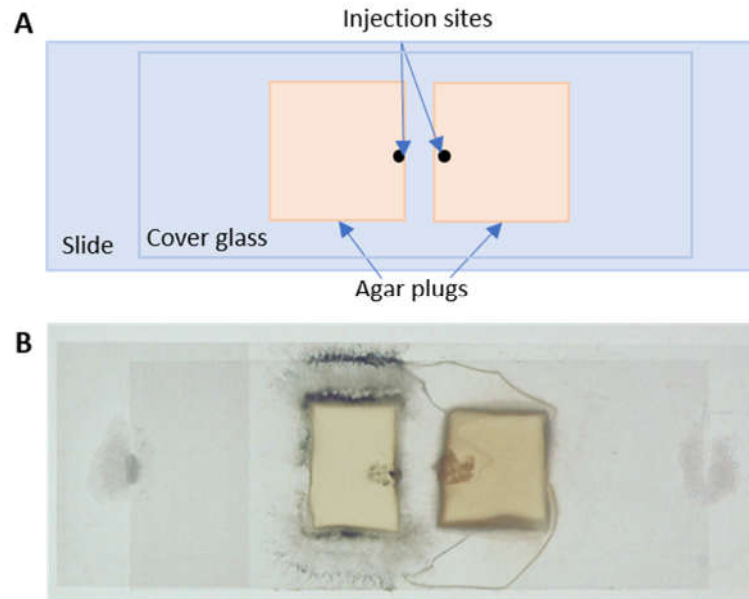


Figure 24: Setup of dual confrontation assays on microscopy slides

For microscopic analysis of dual fungal confrontations, fungal cultures were inoculated on PDA plugs on microscopy slides. The plugs were placed about 5-10 mm apart. After inoculation the slides were covered with a cover glass. These slides were then cultivated inside of sterile plastic Petri plates, sealed with Parafilm, at 25°C in darkness. A) Schematic image of the microscopy slide setup. B) Photograph of fungal confrontation on microscopy slide after several days of incubation.

5.2.2.1 HFB8_{mRFP} accumulates on all hyphae on glass slides

As previously mentioned, HFB8_{mRFP} has an affinity to attach to surfaces, especially interfaces. This causes problems for the identification of Tgui_{OEhfb8} in the confrontation, as the mRFP signal has the potential to accumulate to hyphae of the confrontation partner. This accumulation can be observed in Figure 25. Figure 25 A shows the (assumed) interaction of Tgui_{OEhfb8} (indicated by white arrow) with FOC4. The Tgui_{OEhfb8} hypha shows the previously observed granular mRFP signal, while the surrounding hyphae show the water-air-hyphae interface signal. Figure 25 B shows FOC4 mycelium close to its inoculation site, farther away from Tgui_{OEhfb8} mycelium, identified by its rod-shaped spores. Again, hyphae show the water-air-hyphae interface signal. This was observed in setups on glass slides. These setups were prone to accumulate a lot of condensation water, which facilitates the distribution of the secreted construct throughout the slide and subsequent accumulation on the confrontation partner's hyphae. Due to this observation, the setup was changed to cultivation directly on plastic petri dishes (still using PDA agar slices as nutrient sources), which decreased the accumulation of condensation water.

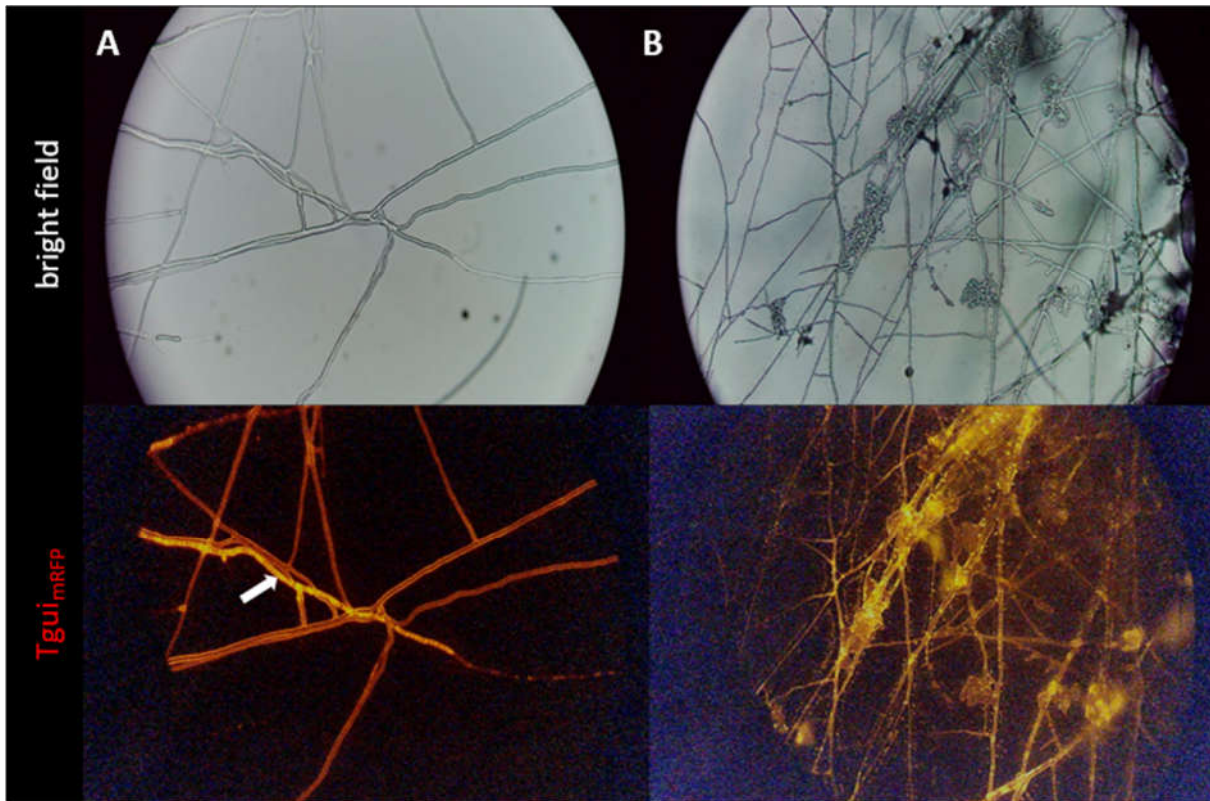


Figure 25: HFB8_{mRFP} accumulation on FOC4 mycelium

Microscopy investigation of non-trophic hyphae of a dual confrontation of Tgui_{OEhfb8} with FOC4 on microscopy glass slides. A) Accumulation of HF8_{mRFP} red fluorescence signal on hyphae-water-air interface of FOC4 interacting with Tgui_{OEhfb8} (white arrow). B) HF8_{mRFP} signal accumulated on FOC4 colony close to its inoculation site. The colony can be identified by its typical rod-shaped spores.

Images were recorded with Leica fluorescent DMI8 Microscope (Leica Microsystems, Germany).

Images are unscaled.

5.2.2.2 Restrictions linked to the use of HFB8_{mRFP}

As mentioned in section 5.1.1.2, the mRFP signal is not homogeneous throughout the Tgui_{OEhfb8} mycelium. Some hyphae do not show any red fluorescence (Figure 7 B). This prevents the identification of the fungal interaction partners, as Tgui_{OEhfb8} shows self-interaction (Supplementary Figure 1) and non-fluorescent hyphae. We noted that the diameter of the non-trophic hyphae (the aerial hyphae growing on the solid surface) are similar for all studied fungi. Therefore, the confrontation with *P. fici* and FOC could not be resolved in the epifluorescence microscope.

When analysing the green autofluorescence of the investigated fungi it was found that FOC4 shows a strong signal compared to Tgui_{OEhfb8} (Supplementary Figure 2). This enabled the clear distinction between Tgui_{OEhfb8} and FOC4 and therefore microscopic analysis was continued with Tgui_{OEhfb8} and FOC4 only.

5.2.2.3 Dual confrontation of Tgui_{OEhfb8} and FOC4 on plastic Petri plates show no penetration or degradation, but close hyphal interactions

Figure 26 and Figure 27 show the confrontation of Tgui_{OEhfb8} (appears red) with FOC4 (appears green) under the epifluorescence microscope. Tgui_{OEhfb8} and FOC4 are both seeking proximity, but FOC4 preferentially initiates hyphal contact (Figure 26).

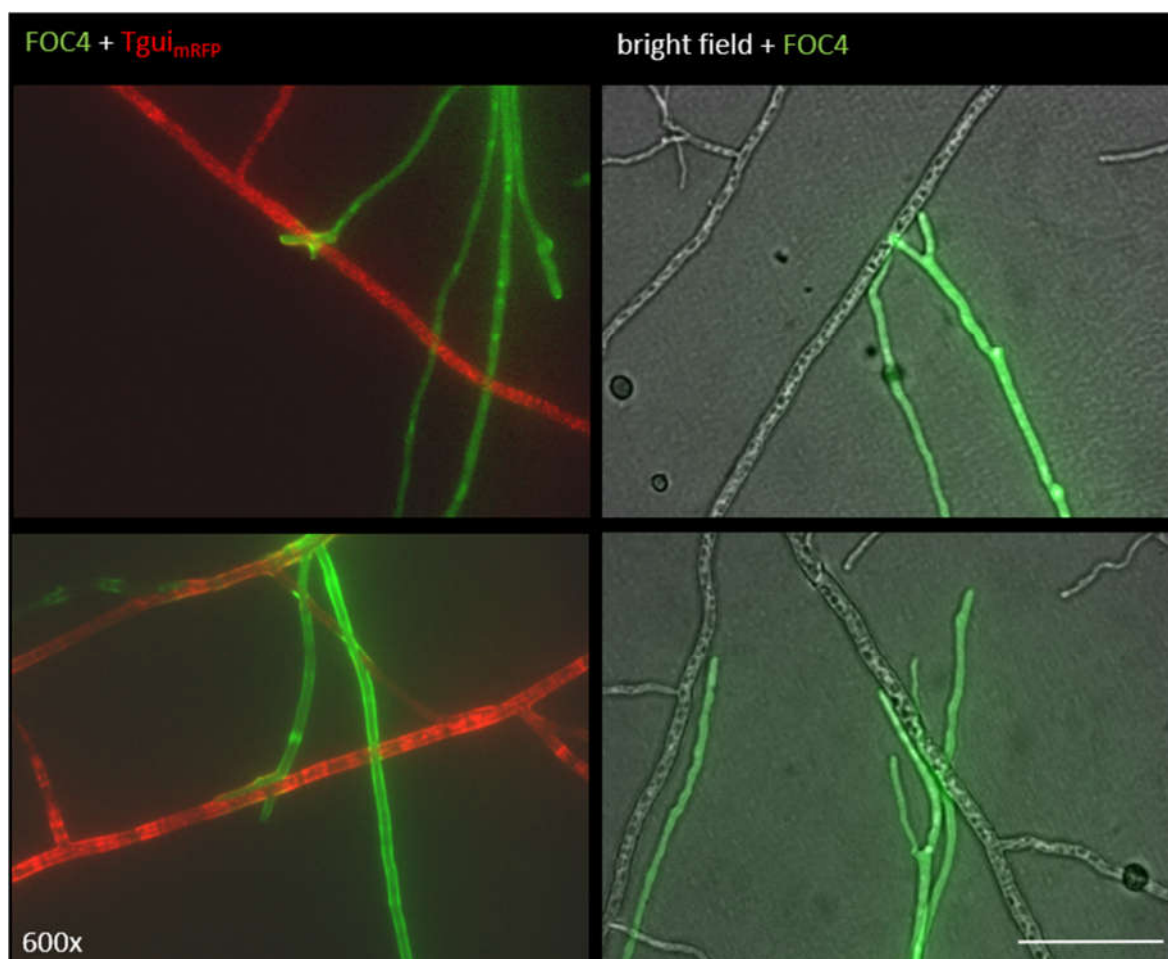


Figure 26: Contact initiation of FOC4 confronted with Tgui_{OEhfb8} on plastic Petri plate

Microscopy investigation of non-trophic hyphae of a dual confrontation of Tgui_{OEhfb8} (shown in red) with FOC4 (shown in green) on microscopy glass slides (x600). FOC4 preferentially initiates the contact to Tgui_{OEhfb8}, as can be seen by the tips of young hyphae reaching out to Tgui_{OEhfb8} hyphae. After this initiation, FOC4 starts to grow along the hyphae of Tgui_{OEhfb8}.

Images were recorded with Leica fluorescent DMI8 Microscope (Leica Microsystems, Germany).

The size bar corresponds to a length of 50µm.

After this initiation, the hyphae of the both interacting partners grow alongside each other in close contact (Figure 27). This preference of FOC4 to initiate hyphal contact is in contrast to the hypothesis that *T. guizhouense* is the aggressor in this interaction [57], [58], which was also previously described in dual confrontation assays on PDA, where *T. guizhouense* builds bridges onto the FOC4 mycelium.

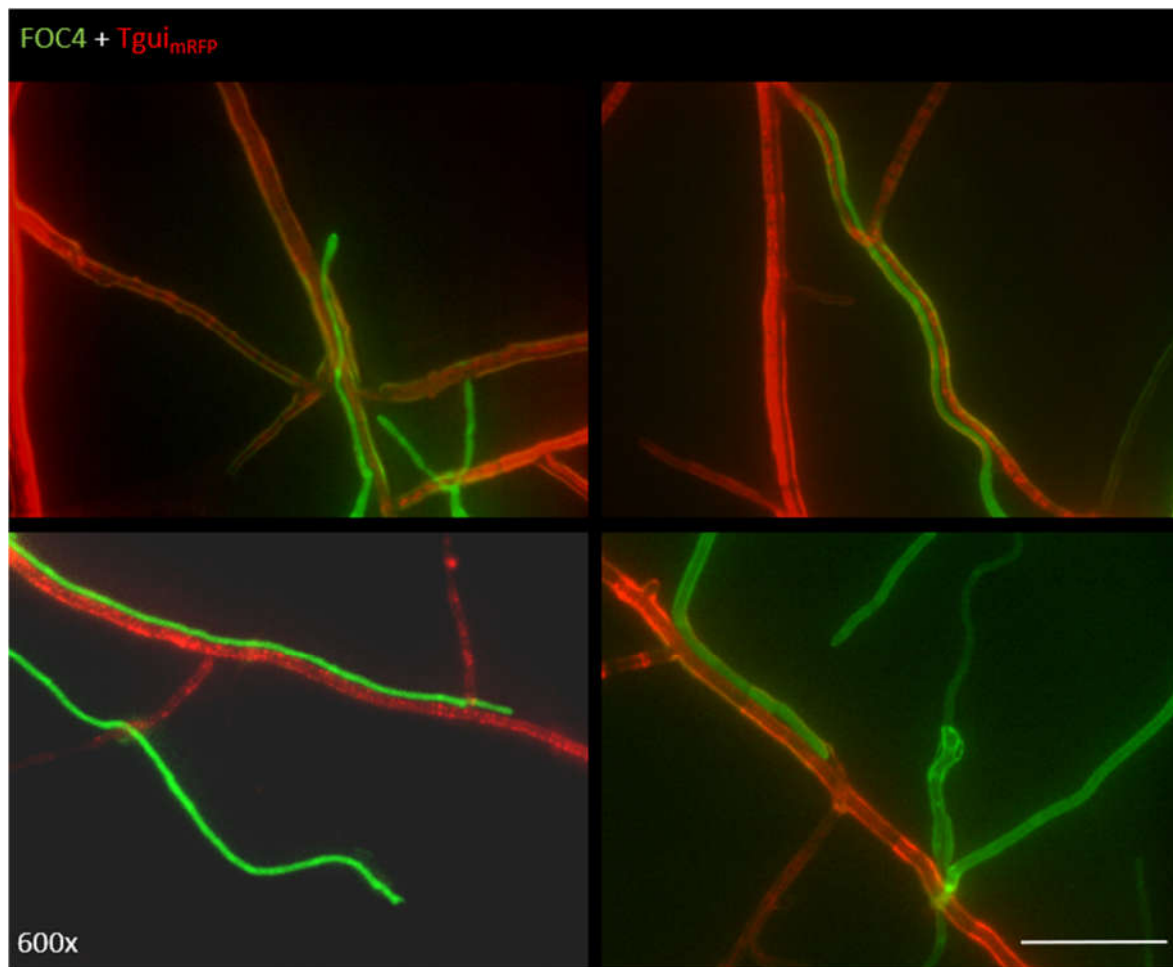


Figure 27: FOC4 and Tgui^{OEhfb8} grow in close hyphal contact alongside each other

Microscopy investigation of non-trophic hyphae of a dual confrontation of Tgui^{OEhfb8} (shown in red) with FOC4 (shown in green) on microscopy glass slides (x 600). After contact initiation by FOC4, hyphae start to grow along the hyphae of Tgui^{OEhfb8} and the two fungi continue to grow in close hyphal contact with each other.

Images were recorded with Leica fluorescent DMi8 Microscope (Leica Microsystems, Germany).

The size bar corresponds to a length of 50µm.

No penetration was observed in any setup between these two partners, neither was degradation of hyphae upon contact. This observation leads to the question whether this interaction is truly mycoparasitic. The exchange of nutrients between these two fungal partners was not recorded.

As previously shown, the overexpression of HFB8_{mRFP} influences *T. guizhouense* antagonism against FOC4 (section 5.2.1.3). This alteration could be- at least partly- responsible for this unexpected behaviour, along with the non-trophic growth on an unnatural, hydrophilic surface. A dual confrontation of FOC4 against a *T. guizhouense hfb8* deletion strain (Tgui^{Δhfb8}) showed no clear differences to the confrontations against the wild type (Supplementary Figure 3).

5.3 Utilizing HFB8_{mRFP} as a tag for monitoring gene expression

For the overexpression of HFB8_{mRFP}, the fungal promoter P_{cdna1}, a promoter for a gene of unknown function from *Trichoderma reesei*, was used. This promoter is commonly used in the overexpression of lignocellulolytic enzymes in *T. reesei*, as it was found to be a strong promoter in respective conditions and was proposed as constitutive [80].

Due to the high expression levels of the mRFP containing protein construct, achieved by the P_{cdna1} promoter, PDA plates cultivated with both Tgui_{OEhfb8} strains (TUCIM 5782 and TUCIM 5783) turned pink after several days of incubation, due to secretion and accumulation of the protein construct in the agar. This allowed the unique possibility of the visual monitoring of P_{cdna1} activity. During routine cultivation of the Tgui_{OEhfb8} strains in different ambient conditions several observations were made regarding the level of pinkness of the PDA plates.

Generally, all plates were cultivated at 25°C in darkness. For specific purposes, e.g. for the induction of conidiation, fungi were cultivated under 12 hours illumination. Figure 28 shows cultures after several days of cultivation in either darkness or under 12 hours illumination. Both TUCIM 5782 and TUCIM 5783 show pink coloration after seven days in light, while no coloration appears on the plates cultivated in darkness. After 10 days, more intense (than in illumination) coloration appears in TUCIM 5782 cultivated in darkness, while still no coloration is observed in the TUCIM 5783 culture. After another five days of cultivation at 28°C, the TUCIM 5783 culture incubated in darkness turned brightly pink, with much higher intensity than the other plates.

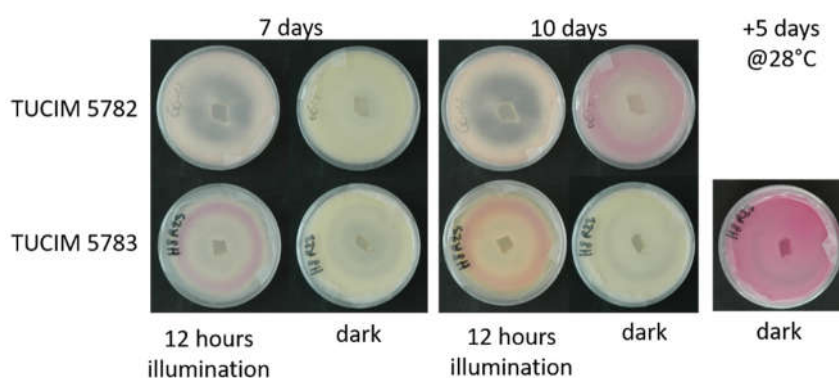


Figure 28: Pink coloration of Tgui_{OEhfb8} strains TUCIM 5782 and TUCIM 5783 under illumination and in darkness

Tgui_{OEhfb8} strains TUCIM 5782 and TUCIM 5783 grown on 6cm PDA Petri plates at 25°C in either darkness or under 12 hours illumination for 10 days, and additional growth at 28°C for five days. Under illumination, pink coloration occurs faster than in darkness (after seven days) but pink coloration of plates grown in darkness eventually show more intense coloration. TUCIM 5782 shows earlier coloration in darkness than TUCIM 5783, but the latter shows stronger coloration overall.

Influence of temperature on pinkness intensity was observed during storage. Fungal cultures are stored at 4°C short term. When Tgui_{OE_{hfb8}} overexpressing strains on PDA were stored at 4°C the plates increased colour intensity within days (not shown).

Fungal cultures are generally cultivated in large incubators often containing many different species and strains. To prevent contamination and desiccation, agar plates are sealed with Parafilm. Mycelial samples are regularly extracted from growing PDA cultures for re-plating or sampling. For this purpose, the plates must be opened (in a sterile environment), which ultimately allows gas exchange. Cultures of Tgui_{OE_{hfb8}}, which were opened for sampling during cultivation, showed a much stronger level of pinkness several hours after the sampling (data not shown).

These observations lead to the hypothesis that the P_{cdna1} promoter used in the *hfb8::mrfp* construct is not constitutive, but influenced by many different environmental factors. Utilizing the mRFP tag in the construct, environmental influences on the promoter can be monitored and quantified.

In a first investigation four different abiotic ambient factors were tested, namely illumination, temperature, aeration and carbon source (full setup Table 9). TUCIM 5782 and TUCIM 5783 were grown on PDA plates for several days and the appearance of the pink coloration was monitored.

Environmental factors (predictors)	Condition 1 (approaching the environmental optimum of <i>T. guizhouense</i> in vitro)	Condition 2 (imitating environments stress conditions)
Illumination	Darkness	12 hours illumination
Temperature	Optimal condition (25°C)	Cold stress (4°C)
Carbon nutrition	Complex medium (PDA)	- Cellulose enhanced substrate or rich medium (x2 PDA) - Starvation (agar agar) - Complex medium (rice flour)
Aeration	Limited (Parafilm sealed plates)	- Unlimited - Unlimited coupled with 12 hours illumination

Table 9: Experimental setup for investigation of the influence of several environmental factors on P_{cdna1} regulation as measured by HFB8_{mRFP} expression

To estimate the influence of these factors on the expression of the P_{cdna1} controlled construct, a mycelium wash was extracted after 21 days of incubation and RFP intensity was measured. The results of the measurements are depicted in Figure 29. The control samples show that grown on PDA in darkness, TUCIM 5782 shows slightly higher mRFP intensity than TUCIM 5783. Growth under 12 hours illumination seems to increase HFB8_{mRFP} expression almost 4-fold in both strains. The influence of exposure to oxygen was also tested under 12 hours illumination (plates in the dark not usable due to technical issues). The obtained values are almost the same as plates grown in light for TUCIM 5783, TUCIM 5782 values, however, are essentially lower. A temperature change to 4°C does not seem to have any influence on HFB8_{mRFP} expression in either strain. The temperature change occurred three

days after inoculation. As *T. guizhouense* becomes (nearly) inactive at 4°C, a reduction in the expression of HFB8_{mRFP} would be expected. The absence of such a decrease might suggest that the expression of HFB8_{mRFP} is highest in the first days of growth. Since hyphal growth is exponential and inhibited by nutrient availability a limitation in gene expression is expected in cultivation on a limited nutrient source.

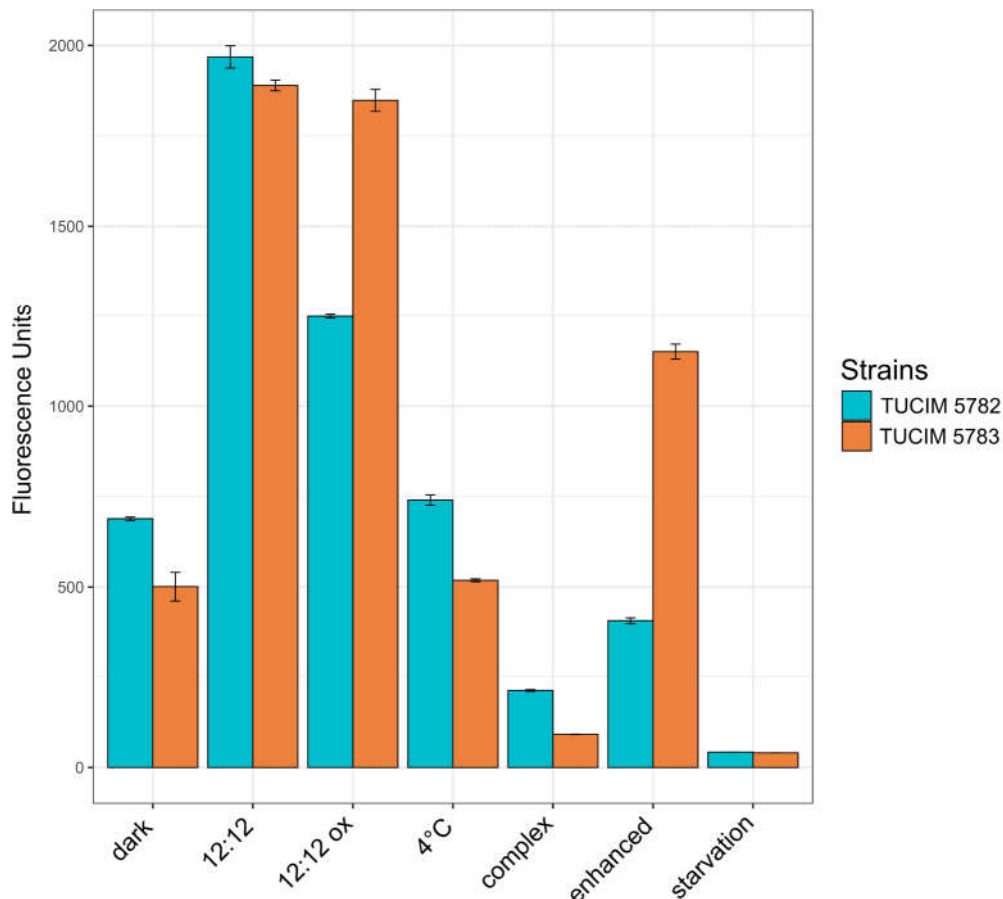


Figure 29: Environmental influences on P_{cdna1} regulation as measured by HFB8_{mRFP} expression of *TguioEhfb8* cultivated on PDA

Fluorescence intensity of HFB8_{mRFP} extracted from the two *TguioEhfb8* mutants TUCIM 5782 (in orange) and TUCIM 5783 (in blue) cultivated on PDA under seven different environmental conditions for 21 days. 12 hours illumination strongly enhances HFB8_{mRFP} expression, while growth on complex carbon and starvation strongly reduce HFB8_{mRFP} expression. A temperature-decrease to 4°C does not seem to have any influence. Growth under the 12:12 cycle in combination with aeration and growth on enhanced PDA show strain dependant, ambiguous influences.

12:12- 12 hours illumination; 12:12 ox- unsealed under 12 hours illumination; complex- rice flour agar; enhanced- double concentrated PDB agar; starvation- agar agar;

Error bars indicate the standard error (SE) of the means.

The role of nutrition in the expression of the construct was tested with three setups. The growth on the complex carbon source (rice flour agar) was associated with a significantly reduced expression of

HFB8_{mRFP} in both strains. This is most likely due to decreased energy-availability for the fungus. As complex carbohydrates require more energy and a larger set of genes for nutrition, it might be possible that the genes that are not involved in energy acquisition and associated processes are downregulated under these conditions. Double concentrated PDA decreased mRFP intensity in TUCIM 5782, while it more than doubled mRFP intensity in TUCIM 5783. Oversaturation of easily available nutrient sources can also cause downregulation of certain genes, as no stored energy must be recycled for survival. Starvation (only agar) resulted in the dropped HFB8_{mRFP} expression to background fluorescence level in both strains. This is most likely linked to no or very low growth and a low energy state.

As growth rates were not recorded in this experiment mRFP levels were not normalized.

A two-way ANOVA showed significant influence on HFB8_{mRFP} expression of almost all factors (except temperature) and of the strains used in this experiment (Supplementary Table 1).

Based on these findings, a more detailed investigation of the different influences was carried out.

5.3.1 Tgui^{OEhfb8} response to different illumination conditions is ambiguous

Light is an important stress factor for fungi which generally grow in dark conditions. They have several illumination receptors specific to light signals of different wavelengths [24].

To test the influence of different illumination sources and light intensities on P_{cdna1} activation, two Tgui^{OEhfb8} strains (TUCIM 5782 and TUCIM 5783) were cultivated on agar plates in varied illumination conditions (Table 10).

Environmental factors (predictors)	Condition 1 (approaching the environmental optimum of <i>T. guizhouense</i> in vitro)	Condition 2 (imitating environments stress conditions)
Illumination	Darkness	12 hours illumination (continuous) Short white illumination (10 min) Short blue illumination (10 min)
Carbon nutrition	Complex medium (PDA)	Simple medium (2% glucose)

Table 10: Experimental setup for investigation of the influence of different illumination conditions on P_{cdna1} regulation as measured by HFB8_{mRFP} expression

The cultures were grown on PDA and 2% glucose agar. The results showed that the medium strongly influenced the fungal growth and therefore most likely gene expression levels (PDA: 863 mean FU, glucose: 44 mean FU). Consequently, mRFP levels were analysed separately for PDA and glucose agar cultivated samples. Figure 30 shows the influence of the different light conditions on TUCIM 5782 and TUCIM 5783 grown on PDA. Due to varying replicates the standard error (SE) of means is high, as indicated by the error bars. The different light conditions showed no statistically significant influence on HFB8_{mRFP} expression on PDA ($p = 0.895$). When grown on 2% glucose agar medium, both fungal strains showed strongly reduced growth (data not shown) and low mRFP intensity (43.38 mean FU). As the measured mRFP values all lay near zero, statistical significance was not considered.

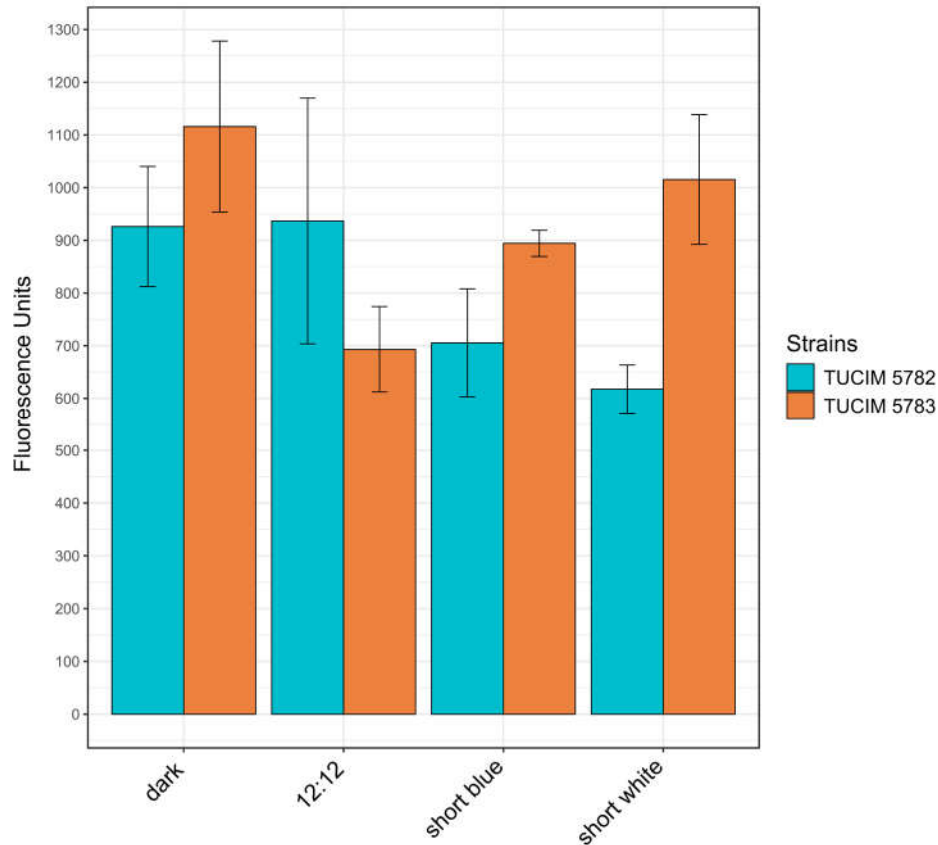


Figure 30: Influence of different illumination conditions on P_{cdna1} regulation as measured by HFB8_{mRFP} expression of Tg_{uiOEhfb8} cultivated on PDA

Fluorescence intensity of HFB8_{mRFP} extracted from the two Tg_{uiOEhfb8} mutants TUCIM 5782 (in orange) and TUCIM 5783 (in blue) cultivated on PDA under different illumination conditions for 14 days. Short white light illumination and continuous 12 hours illumination (12:12) was done with Philips Master 840 diode, short blue illumination with Osram FLUORA 77 diode. Short illumination was carried out once for 10 minutes (~400 Lux). The different illumination conditions show no statistically significant influence on fluorescence intensity ($p=0.895$), due to a high variety between replicates and thus high error. Error bars indicate the standard error (SE) of the means.

As measuring the biomass on agar plates is difficult, the data in the previous experiment is not normalized by it. This makes it impossible to determine whether the different illumination conditions influence P_{cdna1} activation or fungal growth and activity in general. For easy growth measurement (by light absorbance at 750nm), the same setup (as shown in Table 10) was cultivated in 2 ml liquid standing cultures (minimal medium).

As illumination can induce stress in the form of reactive oxygen species (ROS) [29], a setup with 0.05 mM menadione was added to test whether the influence of light might be indirectly inducing

expression by formation of ROS rather than directly affecting light activated signalling pathways for P_{cdna1} regulation.

Figure 31 shows the mRFP intensity of TUCIM 5782 and TUCIM 5783 on the last day of incubation (day seven) normalized by the measured fungal biomass. Plates incubated under 12 hours illumination showed half of the mRFP intensity of plates grown in darkness ($p<0.001$). The addition of menadione significantly reduced HFB8_{mRFP} expression compared to growth in darkness ($p<0.001$). When comparing the influence of the 12 hours illumination and menadione samples, TUCIM 5782 and TUCIM 5783 were affected differently. TUCIM 5783 showed no significant difference between light and menadione influence ($p=0.97$), while TUCIM 5782 did ($p=0.023$) suggesting that light does not affect P_{cdna1} the same way as the formation of reactive oxidants.

To compare the influence of different wavelengths of light on P_{cdna1} activation, cultures were exposed once to blue or white light for 10 minutes. TUCIM 5782 and TUCIM 5783 were highly differently affected by short blue illumination. In TUCIM 5782 short blue illumination did not show significant difference to 12 hours illumination ($p=0.64$) but was significantly reduced compared to growth in darkness ($p<0.001$). Short blue exposed TUCIM 5783 showed no influence on mRFP levels compared to TUCIM 5783 grown in darkness ($p=0.99$), but significantly higher mRFP levels than cultures exposed to 12 hours illumination ($p<0.001$). Cultures under short white illumination showed reduced mRFP intensity compared to the ones in darkness ($p=0.012$), while mRFP values were higher in cultures exposed to 12 hours illumination ($p=0.031$). TUCIM 5782 showed no significant difference between short white and short blue illumination treatments ($p=0.064$), but TUCIM 5783 was significantly affected by different wavelengths ($p<0.001$).

These results show that TUCIM 5782 and TUCIM 5783 grown in 2 ml liquid standing cultures were differently affected by various illumination conditions.

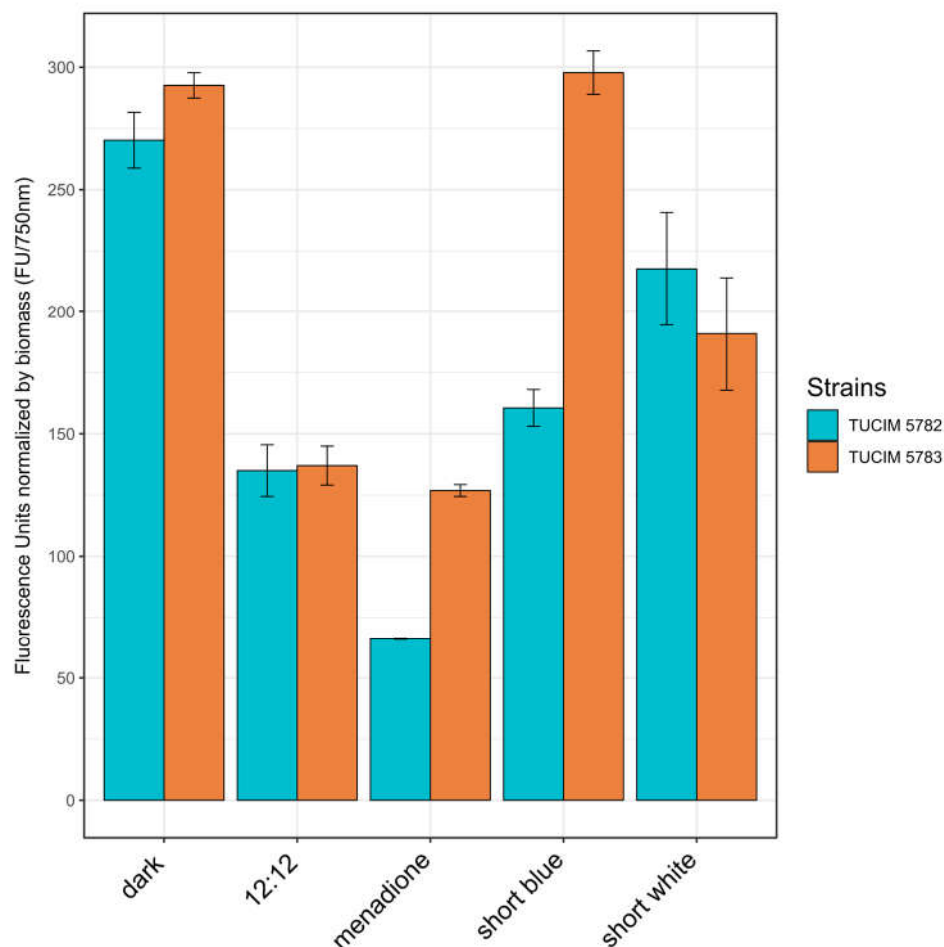


Figure 31: Influence of menadione and different illumination conditions on P_{cdna1} regulation as measured by HFB8_{mRFP} expression of Tg_{uiOEhfb8} cultivated in liquid submerged culture

Fluorescence intensity of HFB8_{mRFP} extracted from the two Tg_{uiOEhfb8} mutants TUCIM 5782 (in orange) and TUCIM 5783 (in blue) cultivated in 2 ml minimal medium under different illumination conditions or with addition of 0.05 mM menadione for 7 days. White light illumination (12 hours continuous and short white) was done with Philips Master 840 diode, short blue illumination with Osram FLUORA 77 diode. Short illumination was carried out once for 10 minutes (~400 Lux). Illumination under 12 hours illumination (12:12) strongly reduced HFB8_{mRFP} expression, as did the addition of menadione, but only for TUCIM 5782. Short white illumination slightly increased HFB8_{mRFP} expression, short blue illumination was ambiguous and highly strain dependent.

Error bars indicate the standard error (SE) of the means.

5.3.2 The influence of aeration on P_{cdna1} regulation as measured by HFB8_{mRFP} expression is not statistically significant

Most filamentous fungi live in the substrate. However, for reproduction in one or another form or, sometimes, for the colonization of new territory, they form aerial hyphae. Oxygen levels vary greatly between the substrate interior and the open air, as aeration of dense soils or tissues is limited.

Therefore, oxygen exposure can serve as a signal for changing conditions, e.g. surface exposure, which may trigger reproductive development (for example, sporulation for aerial dispersal [24]).

To test the influence of gas exchange on P_{cdna1} activation, TUCIM 5782 and TUCIM 5783 were grown on PDA plates in darkness or under 12 hours illumination and were differentially exposed to oxygen by opening the sealed plates in a sterile environment (Table 11).

Environmental factors (predictors)	Condition 1 (approaching the environmental optimum of <i>T. guizhouense</i> in vitro)	Condition 2 (imitating environments stress conditions)
Illumination	Darkness	12 hours illumination
Aeration	Limited (Parafilm sealed plates)	- One-time aeration - Several times aeration

Table 11: Experimental setup for investigation of the influence of illumination and aeration on P_{cdna1} regulation as measured by HFB8_{mRFP} expression

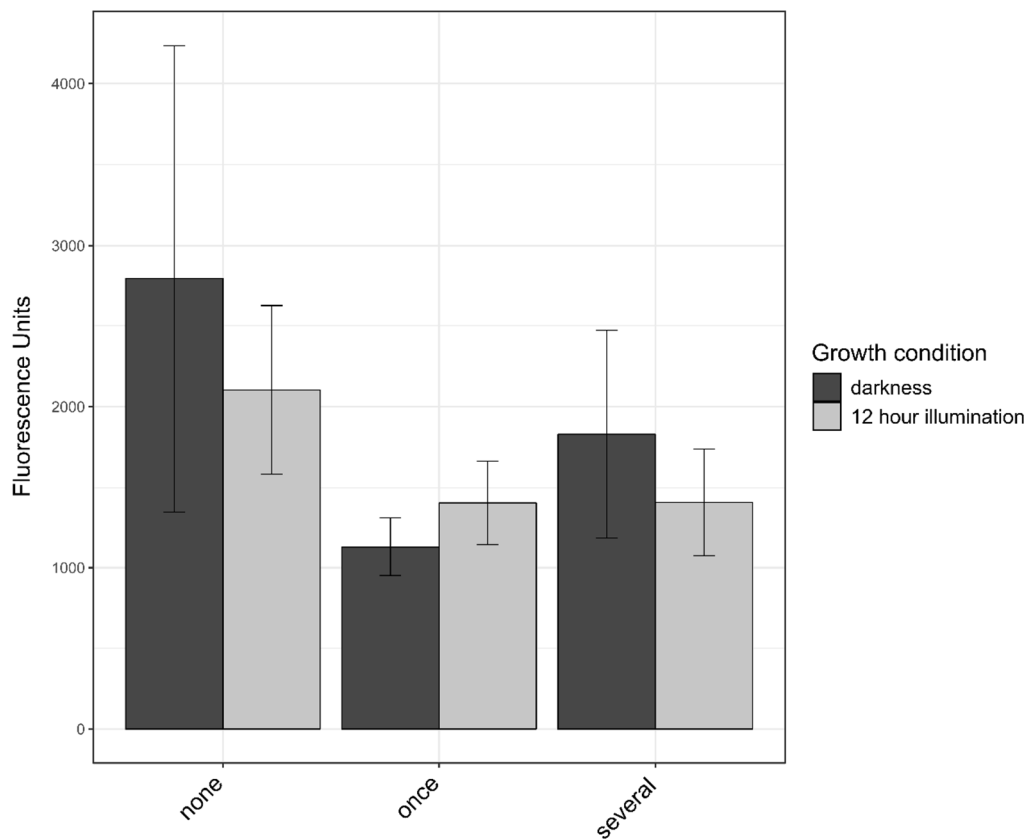


Figure 32: Influence of aeration on P_{cdna1} regulation as measured by HFB8_{mRFP} expression of *TguiOEhfb8* cultivated on PDA

Fluorescence intensity of HFB8_{mRFP} extracted from the two *TguiOEhfb8* mutants TUCIM 5782 and TUCIM 5783 on PDA under different aeration conditions in either darkness (dark grey) or under 12 hours illumination (light grey) for 14 days. Due to high error rates, no significant influence could be measured for any condition.

None- plates not opened; Once- plates opened once; Several- plates opened 3 times during cultivation; Error bars indicate the standard error (SE) of the means.

Figure 32 shows the influence of oxygen exposure on TUCIM 5782 and TUCIM 5783 together (separately in Supplementary Figure 4). The experiment was carried out in three repeats which all showed high variation, resulting in a high SE (as indicated by error bars). Due to this high error neither the setups nor the strains showed any statistically significant correlation. Due to growth on agar plates, no normalization of mRFP intensity with fungal growth was carried out.

To enable normalization by growth, the influence of oxygen exposure was tested in 2 ml liquid standing cultures. Oxygen exposure was limited to one timepoint. Cultures were incubated in darkness, under 12 hours illumination or with the additions of 0.05 mM menadione to test the influence of multiple stresses on P_{cdna1} regulation.

Figure 33 A shows the influence of oxygenation and cultivation condition on TUCIM 5782 and TUCIM 5783. No statistical correlation was found in a two-way ANOVA analysis between mRFP intensity and oxygenation or other conditions.

As a multiple ANOVA of the two strains showed a significant difference in fluorescence intensity ($p < 0.001$), impact of oxygenation and condition were tested on each strain, separately. TUCIM 5782 showed significant differences on mRFP intensity when cultivated under illumination and in darkness ($p = 0.011$), but the addition of menadione did not significantly influence mRFP values ($p = 0.96$), as is depicted in Figure 33 B. No statistical influence of cultivation conditions on TUCIM 5783 was found (Figure 33 C). Neither strain showed significant influence of oxygenation on mRFP intensity.

These findings suggest that oxygenation does not directly influence P_{cdna1} activation and that TUCIM 5782 and TUCIM 5783 are affected differently by illumination, as shown above.

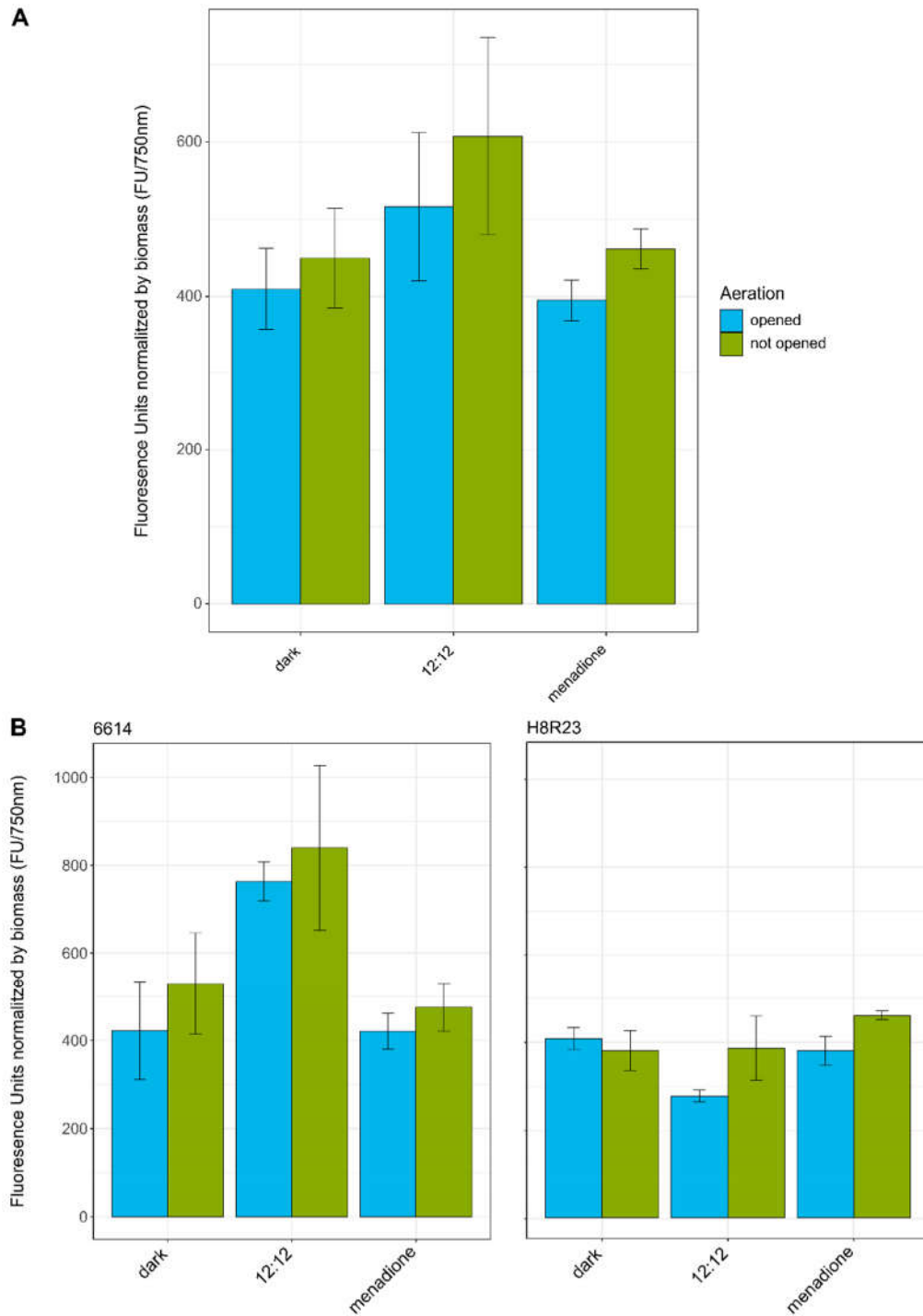


Figure 33: Influence of menadione and aeration on P_{cdna1} regulation as measured by HFB8_{mRFP} expression of TguioEhfb8 cultivated in liquid submerged culture

Fluorescence intensity of HFB8_{mRFP} extracted from the two TguioEhfb8 mutants TUCIM 5782 and TUCIM 5783 cultivated in 2 ml minimal medium in either darkness, in darkness with the addition of 0.05 mM menadione or under 12 hours illumination (12:12) for 12 days. The cultures were either exposed to oxygen by opening the plates (blue) or were kept sealed throughout the entire cultivation (green). A) TUCIM 5782 and TUCIM 5783 plotted together. Due to high error rates, no significant influence could be measured for any condition. B) TUCIM 5782 plotted alone. Illumination shows significant increase in HFB8_{mRFP} expression, but no other condition does. B) TUCIM 5783 plotted alone. No condition shows significant influence on HFB8_{mRFP} expression.

5.3.3 BIOLOG Phenotype MicroArrays show significant influence of carbon sources on P_{cdna1} regulation as measured by HFB8_{mRFP} expression

Trichoderma is ubiquitously found in several environments containing an abundant diversity of carbon sources. Nutrition is a key influence factor on growth and development. The following experiment aimed to test the potential influence of carbon sources on the sensitivity of P_{cdna1} to stress by illumination or exposure to menadione.

As the strain TUCIM 5782 has higher stability and more efficient spore production compared to the strain TUCIM 5783 (shown by the above results), TUCIM 5782 was selected to be performed in the next tests. It was cultivated in a high throughput setup on BIOLOG FF Microplates (Biolog Inc., USA) containing 95 different carbon sources (Supplementary Figure 5) in three conditions (Table 12).

Environmental factors (predictors)	Condition 1 (approaching the environmental optimum of <i>T. guizhouense</i> in vitro)	Condition 2 (imitating environments stress conditions)
Illumination	Darkness	12 hours illumination
Oxidative stress	-	0.05 mM menadione
Carbon nutrition	-	95 different carbon sources

Table 12: Experimental setup for investigation of the influence of different carbon sources in combination with illumination and menadione induced oxidative stress on P_{cdna1} regulation as measured by HFB8_{mRFP} expression

Fungal growth was measured by light absorbance at 750nm every 24 hours. The experiment was carried out for three and seven days (one replicate) and mRFP intensity was measured once on the last day of incubation. The mean mRFP intensity (normalized by biomass) measured under each condition is shown in Figure 34 A+C. mRFP intensity increased slightly from day three until day seven, which is most likely due to the accumulation of the secreted HFB8_{mRFP} in the well. In both measurements the addition of menadione seemed to slightly increase mRFP expression compared to the ones grown in darkness, while growth under illumination did not seem to influence mRFP levels. Figure 34 B+D show the mRFP intensity measured from each well in all three conditions plotted against the measured biomass. Both carbon sources and conditions seemed to differentially influence P_{cdna1} activation, as measured by mRFP intensity. For clearer visualisation the y-axis in Figure 34 was scaled to a maximum of 1000FU. Plots with the original scale can be seen in Supplementary Figure 6.

This was statistically tested by a two-way ANOVA of log₁₀ transformed fluorescence values. Measurements after three days show no significant correlation of mRFP intensity and carbon sources, but growth conditions significantly influence mRFP intensity ($p < 0.001$). mRFP intensity of cultures with added menadione significantly differed from the ones grown under illumination ($p < 0.001$) and in darkness ($p = 0.008$), but no statistical difference between the latter two is observed ($p = 0.716$). After seven days of incubation, both carbon sources and growth conditions showed highly significant

influence on mRFP intensity (both $p < 0.001$). Again, only menadione seemed to influence mRFP intensity ($p < 0.001$), but not illumination ($p = 0.238$).

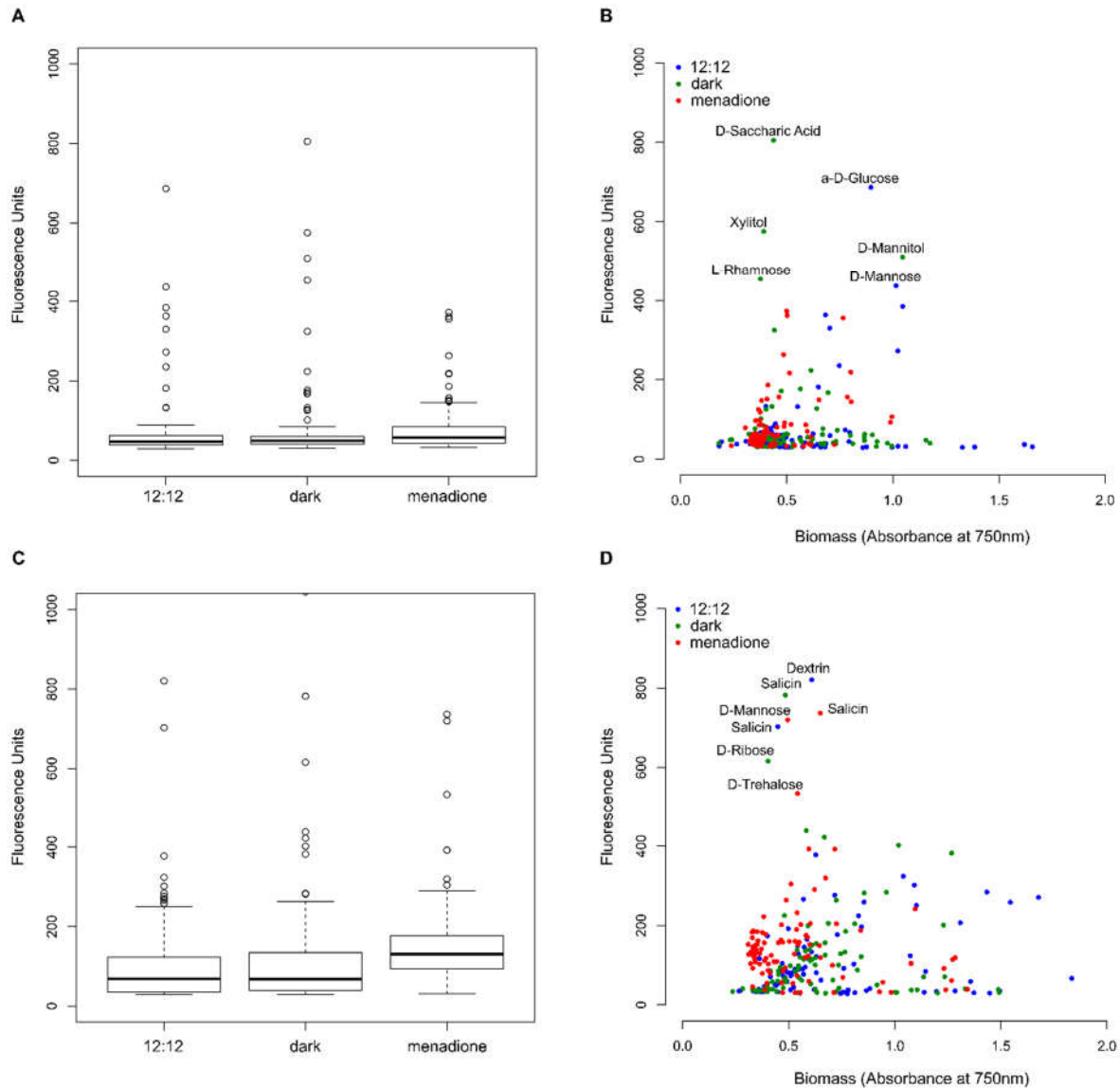


Figure 34: Influence of menadione and illumination on P_{cdna1} regulation as measured by HFB8_{mRFP} expression of TguioEhfb8 cultivated on 95 different carbon sources

Fluorescence intensity of HFB8_{mRFP} extracted from the two TguioEhfb8 mutants TUCIM 5782 and TUCIM 5783 cultivated in on BIOLOG FF Microplates containing 95 different carbon sources, in darkness with the addition of 0.05 mM menadione under 12 hours illumination for three or seven days. A) Mean fluorescence intensity across all substrates after three days of incubation. Fluorescence is slightly increased in cultures with added menadione. B) Fluorescence units (FU) of each well plotted against its measured biomass after three days. A few substrates show strongly increased fluorescence intensity, highly dependent on cultivation conditions. C) Mean fluorescence intensity across all substrates after seven days of incubation. Overall FU increased compared to three days incubation, menadione still shows slight positive influence on FU. D) Fluorescence units (FU) of each well plotted against its measured biomass after seven days.

These findings suggest that the previously observed influence of light on P_{cdna1} activation is most likely carbon dependant, as it does not seem to significantly affect overall mRFP expression for all substrates. The influence of ROS induced by menadione also differs from previous observations, seen by much higher mRFP intensity than in the setups without menadione.

Since no replicates were created, no growth curves are shown. However, an R script was written for easy plotting the growth curves measured in each of the 96 wells in the microplate in a PDF output. This script and a single well sample plot are shown in supplementary material 9.1.

5.3.4 Influence of environmental factor on *cdna1* relative expression in three *Trichoderma* species

The *hfb8::mrfp* construct is a useful tool to easily monitor mRFP expression in *TguioEhfb8* by pink coloration of the growth medium. However, as shown by the previous experiments, the extraction and quantification of the mRFP signal is error prone. Moreover, the measurement of an expressed protein is not an ideal way to study the control of a promoter on the transcription of its underlying gene in fungi, as proteins in eukaryotes can undergo several post-transcriptional modifications which might influence the observed expression levels.

For more accurate quantification of environmental influences on P_{cdna1} regulation, mRNA transcription quantification of its underlying gene *cdna1* was carried out using real-time polymerase chain reaction (qPCR) in three *Trichoderma* species, namely *T. reesei* QM6a (TUCIM 917), the original source of the P_{cdna1} promoter, *T. guizhouense* NJAU 4742 (TUCIM 4742) and *T. harzianum* CBS 226.95 (TUCIM 916), a close relative of *T. guizhouense* and an important industrial enzyme producer. The gene sequence for *cdna1* (from *T. reesei*) was retrieved from Uzbas et al. 2012 [88]. Its homologous genes in *T. guizhouense* and *T. harzianum* were retrieved by NCBI BLAST [95] search (Supplementary Figure 8).

These three strains were incubated under various conditions listed in Table 13.

Environmental factors (predictors)	Condition 1 (approaching the environmental optimum of <i>T. guizhouense</i> in vitro)	Condition 2 (imitating environments stress conditions)
Illumination	Darkness	12 hours illumination (continuous) Short illumination (white light)
Temperature	Optimal condition (25°C)	Short cold stress (4°C) Short heat stress (37°C)

Table 13: Experimental setup for investigation of the influence of several environmental factors on P_{cdna1} regulation as measured by *cdna1* expression

The cultures suspected to short exposure conditions were cultivated at baseline conditions (25°C, darkness) before and after the exposure. The exposure was carried out after 48 hours of cultivation, when the fungal mycelium covered the whole PDA plate. After exposure plates were returned to baseline conditions. Each of these short exposure setups was done in two sets, for which two different

timepoints of mRNA extraction were adopted (30 min and three hours after exposure). This was done to test how fast the P_{cdna1} promoter responds to these environmental influences. Further, a standing/shaking liquid fermentation in PDB was done, as the investigated fungal strains are important for industrial protein production. Fermentation was carried out for 72 hours.

The relative expression, or fold change, of the *cdna1* gene was calculated by the $2^{-\Delta\Delta C_t}$ method, normalized to a housekeeping gene (*tef1*) and to the cultures at baseline conditions (grown on PDA at 25°C in darkness). To correct for skewed distribution of frequencies, the fold change was log10 transformed (Supplementary Figure 9 and Supplementary Table 4).

Figure 35 shows *cdna1* expression fold change (log10 transformed) by condition. The highest fold changes were observed in the submerged shaking and standing cultures. The standing fermentation seems to increase fold change compared to growth on PDA in all three strain. *T. guizhouense* (TUCIM 4742) showed the strongest upregulation of *cdna1* expression levels in the liquid cultures, especially in the shaking fermentation.

To test statistical significance of the influence of different conditions on *cdna1* expression, a two-way ANOVA was calculated. Differences between means of groups were analysed by posthoc analysis using Duncan's new multiple range test (95% confidence interval). Statistical analysis of this data showed a significant difference ($p < 0.001$) of the influence on target gene expression between either standing or shaking fermentation and all other tested conditions across all strains, but not between the two different liquid fermentation samples ($p = 0.369$). Samples exposed to 37°C (extracted at timepoint 1) showed significant differences to samples exposed to 4°C (extracted at timepoint 1) or grown under 12 hours illumination ($p < 0.005$). Since significant differences of overall fold change between TUCIM 4742 and the other strains was calculated ($p < 0.001$), further analysis was carried out on each strain individually.

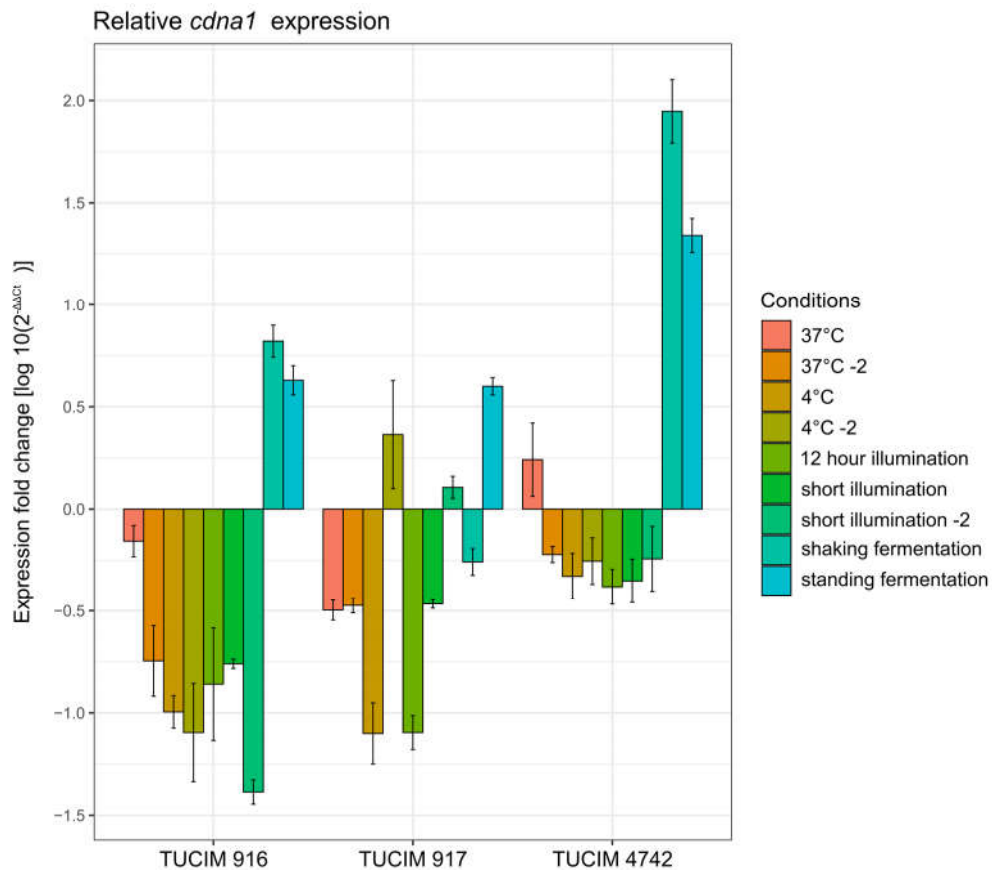


Figure 35: Influence of different environmental factors on *cdna1* expression

Relative expression of *cdna1* (log10 transformed) in *T.guizhouense* (TUCIM 4742), *T.harzianum* (TUCIM 916) and *T.reesei* (TUCIM 917) measured by real-time polymerase chain reaction (qPCR). Strains were cultivated either on PDA for 48 hours and exposed to different environmental stresses or in PDB liquid submerged culture for 72 hours under agitation (shaking) or standing.

The different environmental stresses were short exposure to temperature changes (4°C and 37°C) and exposure to illumination for 10 minutes (short) or under 12 hours illumination. Cultures exposed to short stress pulses were returned to baseline conditions and further incubated for 2 time periods (1: 30 minutes, 2: three hours). Illumination (12:12 and short) was done with a Philips Master 840 diode.

Shaking and standing fermentation show overall high upregulation of *cdna1* expression, while the other conditions show overall downregulation of *cdna1* expression.

Expression fold was calculated by the $2^{-\Delta\Delta Ct}$ method, normalized against the housekeeping gene *tef1* and values obtained from cultures grown at baseline conditions (on PDA at 25°C in darkness).

For individual analysis of each strain one-way ANOVA and Duncan's new multiple range test were done. Analysis of TUCIM 4742 (Figure 36) showed similar results for standing and shaking fermentations as before. Samples exposed to 37°C (extracted at timepoint 1) showed significant differences to all other conditions tested ($p < 0.01$), which all seem to contribute to the downregulation of *cdna1* expression. No other statistically relevant differences were observed.

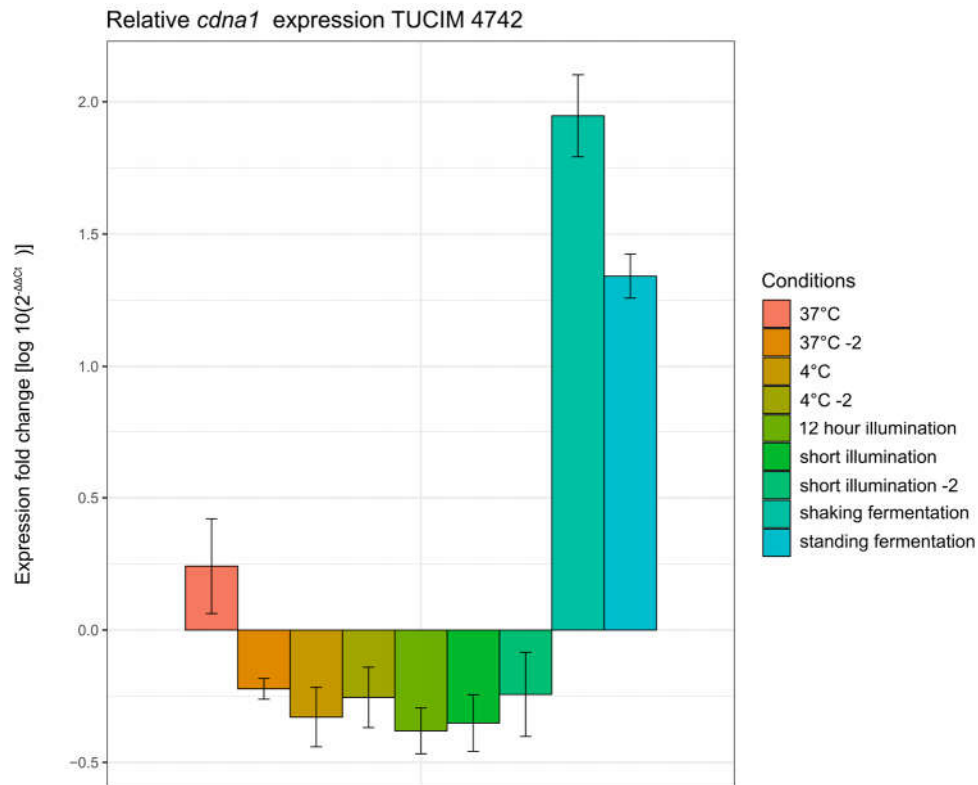


Figure 36: Influence of different environmental factors on *cdna1* expression on TUCIM 4742

Relative expression of *cdna1* (log10 transformed) in *T.guizhouense* (TUCIM 4742) measured by real-time polymerase chain reaction (qPCR). Strains were cultivated either on PDA for 48 hours and exposed to different environmental stresses or in PDB liquid submerged culture for 72 hours under agitation or standing.

The different environmental stresses were short exposure to temperature changes (4°C and 37°C) and exposure to illumination for 10 minutes (short) or under 12 hours illumination. Cultures exposed to short stress pulses were returned to baseline conditions and further incubated for 2 time periods (1: 30 minutes, 2: three hours). Illumination was done with a Philips Master 840 diode.

Shaking and standing fermentation show high upregulation of *cdna1* expression, exposure to 37°C and recovered for 30 minutes shows slight upregulation, while the other conditions show downregulation of *cdna1* expression.

Expression fold was calculated by the $2^{-\Delta\Delta C_t}$ method, normalized against the housekeeping gene *tef1* and values obtained from cultures grown at baseline conditions (on PDA at 25°C in darkness).

In TUCIM 916 *cdna1* expression is upregulated in liquid standing and shaking fermentation and downregulated under all other conditions (Figure 37). Similar to results in TUCIM 4742, significant differences ($p < 0.001$) between either standing or shaking fermentation and all other tested conditions were calculated, but not between the two different liquid fermentation samples ($p = 0.379$) and samples exposed to 37°C at timepoint 1 showed significant differences to all other conditions tested ($p < 0.01$). Extraction timepoints of cultures exposed to 37°C ($p = 0.012$) and short illumination ($p = 0.039$) seem to influence *cdna1* expression. Samples extracted after three hours show stronger

downregulation than samples extracted after 30 minutes at baseline conditions after short exposure. No highly significant difference between extraction timepoints was measured for cultures exposed to 4°C ($p=0.641$).

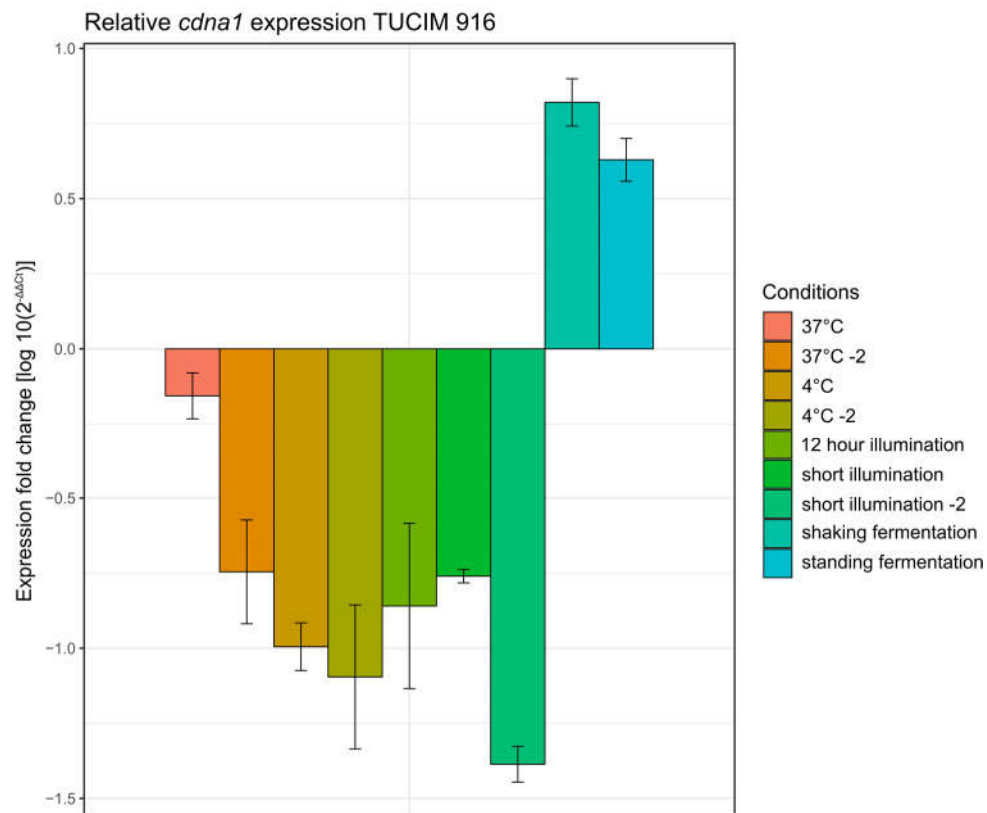


Figure 37: Influence of different environmental factors on *cdna1* expression on TUCIM 916

Relative expression of *cdna1* (log₁₀ transformed) in *T.harzianum* (TUCIM 916) measured by real-time polymerase chain reaction (qPCR). Strains were cultivated either on PDA for 48 hours and exposed to different environmental stresses or in PDB liquid submerged culture for 72 hours under agitation or standing.

The different environmental stresses were short exposure to temperature changes to (4°C and 37°C) and exposure to illumination for 10 minutes (short) or under 12 hours illumination. Cultures exposed to short stress pulses were returned to baseline conditions and further incubated for 2 time periods (1: 30 minutes, 2: three hours). Illumination was done with a Philips Master 840 diode.

Shaking and standing fermentation show high upregulation of *cdna1* expression, while the other conditions show downregulation of *cdna1* expression. Strong differences are observed between samples recovered for 30 minutes and three hours when exposed to 37°C or short illumination.

Expression fold was calculated by the $2^{-\Delta\Delta C_t}$ method, normalized against the housekeeping gene *tef1* and values obtained from cultures grown at baseline conditions (on PDA at 25°C in darkness).

TUCIM 917 shows a very different expression profile compared to the other two strains. *cdna1* expression seems to be downregulated in shaking fermentation but upregulated after short exposure to temperature decrease to 4°C or illumination, extracted after three hours of subsequent incubation

at baseline conditions (25°C, darkness). Extraction timepoints show significant influence on *cdna1* expression when exposed to 4°C ($p<0.001$) or short illumination ($p=0.0129$), but not when exposed to 37°C ($p=0.9$).

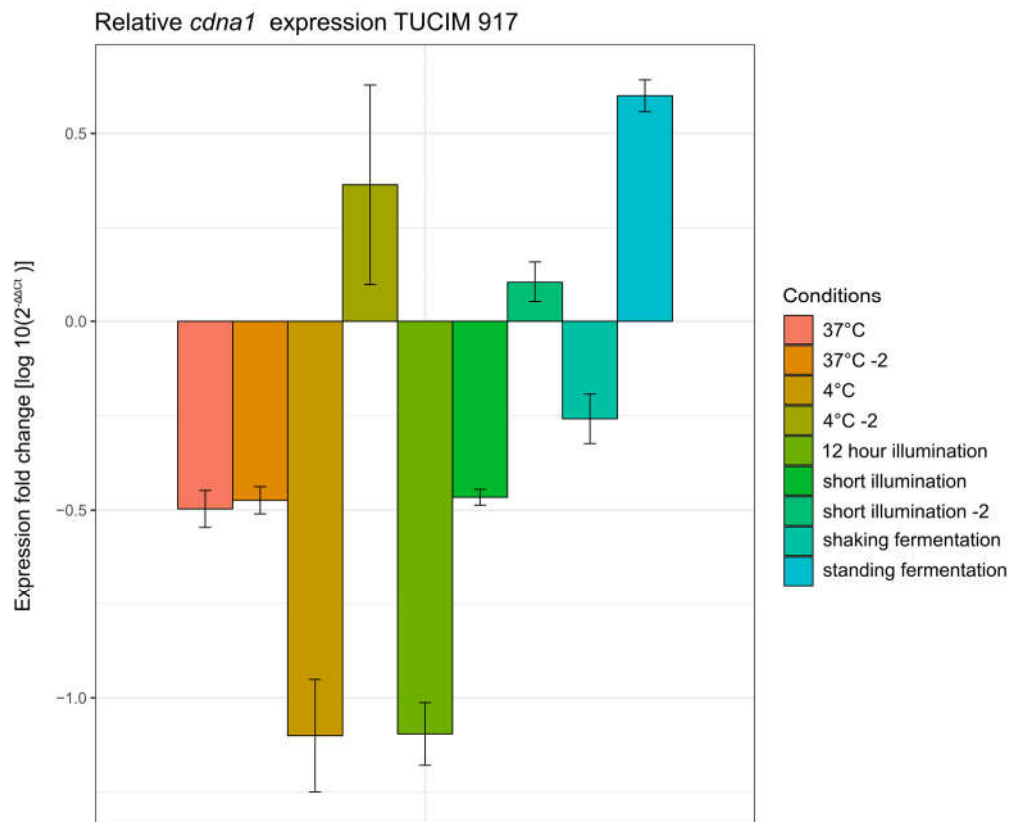


Figure 38: Influence of different environmental factors on *cdna1* expression on TUCIM 917

Relative expression of *cdna1* (log10 transformed) in *T.reesei* (TUCIM 917) measured by real-time polymerase chain reaction (qPCR). Strains were cultivated either on PDA for 48 hours and exposed to different environmental stresses or in PDB liquid submerged culture for 72 hours under agitation or standing.

The different environmental stresses were short exposure to temperature changes to (4°C and 37°C) and exposure to illumination for 10 minutes (short) or under a 12 hours cycle of alternating illumination and darkness. Cultures exposed to short stress pulses were returned to baseline conditions and further incubated for 2 time periods (1: 30 minutes, 2: three hours). Illumination was done with a Philips Master 840 diode.

Shaking fermentation and exposure to 4°C and short illumination recovered for 30 minutes show upregulation of *cdna1* expression, while the other conditions show downregulation of *cdna1* expression. Strong differences are observed between samples recovered for 30 minutes and three hours when exposed to 4°C or short illumination.

Expression fold was calculated by the $2^{-\Delta\Delta C_t}$ method, normalized against the housekeeping gene *tef1* and values obtained from cultures grown at baseline conditions (on PDA at 25°C in darkness).

A Principle Coordinates Analysis (PCoA) of the obtained data showed no obvious clustering by strain or condition (Supplementary Figure 10). A heatmap analysis showed strong differences of TUCIM 4742

cultivated as shaking and standing conditions, but otherwise no strong clustering (Supplementary Figure 11).

The P_{cdna1} promoter analysed in this thesis originates in *T. reesei* (TUCIM 917), which makes this strain the best model for comparing results obtained from HFB8_{mRFP} and *cdna1* mRNA quantification. The time of cultivation at baseline conditions seems to be highly significant for P_{cdna1} regulation. Interestingly, short recovery time at baseline conditions after stress exposure shows strong downregulation, while longer recovery time results in upregulation of *cdna1* expression. Response time of the promoter does not seem to be the direct cause for this phenomenon, as the strong downregulation would not be observed. It is possible that *cdna1* is downregulated upon exposure to stress to reduce energy expenditure. The strong upregulation, after the cell has returned to homeostasis, might be a way of compensating for low cDNA1 levels. The downregulation of *cdna1* expression in shaking fermentation might be an indication that TUCIM 917 is sensitive to agitation and is thus stressed by this condition. The strong differences between the three strains suggests that P_{cdna1} regulation is strongly strain dependant. Further, the promoter sequences of *cdna1* homologous in TUCIM 916 and TUCIM 4742 have not been analysed, so no details are known about their sequences. All statistical analyses are summarized in the supplementary material (Supplementary Table 5- Supplementary Table 8).

The overall results on P_{cdna1} regulation obtained from HFB8_{mRFP} quantification by its fluorescent signal and *cdna1* expression measured by qPCR show highly ambiguous results and a strong strain dependency. Exposure to illumination, oxygen and temperature decrease all seem to have an influence on P_{cdna1} activation, although these observations were not constant and high error rates between biological replicates were obtained.

6 Discussion

Filamentous fungi are ubiquitously present in all environments where they play a key role in carbon cycling [1]. In their habitat fungi are constantly exposed to various biotic and abiotic external influences. One of the most prevalent genera isolated from soil is *Trichoderma*. Members of *Trichoderma* are well known for their environmental opportunism and their preference to interact with other fungi [44]. Fungal-fungal interactions are believed to be the major nutritional strategy of *Trichoderma*, which ultimately influences its behaviour and physiology. Unravelling these interactions will help to better understand the genus's evolutionary development and its role in the environment.

The quantification and monitoring of the physiological response to these external stress factors and biological interactions can be precisely measured through gene transcription levels (mRNA) and with high definition microscopy such as electron microscopy. However, these methods can be expensive, time consuming and challenging and might therefore not be suitable for high throughput analysis of multiple factors.

In some circumstances, estimation of the expression levels of a gene can be done easier and faster by quantifying the produced protein. Labelling the protein with fluorescent tags as reporters is commonly applied as it facilitates visualization. If the labelled protein of interest is secreted, it can be extracted, and fluorescence can be quantified. This method is fast, but as nascent proteins can undergo several post-translational modifications in eukaryotes, it only gives an estimate of expression levels. Nonetheless, it is a suitable method to give an overview and first insights in a high throughput setup and can serve as a starting point for subsequent mRNA quantification or other high-precision analyses.

6.1 The overexpression of HFB8_{mRFP} indirectly influences *T. guizhouense* physiology

Hydrophobin 8 used in this study was discovered in *T. guizhouense* (Cai, Chethamara, Druzhinina, personal communication). It was observed that the overexpression of HFB8 tagged with mRFP under the P_{cdna1} promoter evokes strong physiological responses in *T. guizhouense*. The Tgui_{OEhfb8} strains (TUCIM 5782 and TUCIM 5783) showed reduced surface hydrophobicity (are hydrophilic), slower growth, delayed sporulation and reduced/lost antagonism against *F. oxysporum*. The Tgui_{Δhfb8} strain, however, showed no significant physiological implications or changes compared to the *T. guizhouense* parental strain for the tested parameters. This finding suggests that HFB8 on its own is not essential for the tested phenotypic characteristics and that its strong overexpression might not be an effect of HFB8 itself but of the downregulation of other hydrophobins or molecular pathways.

A phenotypical analysis of a *T. guizhouense* strain overexpressing hydrophobin 4 under the same P_{cdna1} promoter in *T. guizhouense* showed similar results to those of hydrophobin 8 (F. Cai, personal communication). Hydrophobicity, sporulation and antagonism against *F. oxysporum* were reduced.

The HFB4 deletion mutant, however, showed similar physiology to the overexpressing strain. This suggests that HFB4 is essential for the balance of the *T. guizhouense* hydrophobome and skewing its expression either way results in an imbalance of surface hydrophobicity. Since the deletion of HFB8 did not show any strong implications of *T. guizhouense* physiology it might be expressed at much lower levels than HFB4. This could be tested by expressing HFB4 and HFB8 tagged with antibody-binding epitopes under their native promoters to quantify their expression by western blot, or quantifying mRNA levels by quantitative polymerase chain reaction.

In 2017, another class II hydrophobin HFB7 -that may be a putative ortholog of HFB8- was overexpressed in a *T.virens* strain under the P_{cdna1} promoter [89]. The resulting phenotype differed from those of HFB4 and HFB8, growth was increased in submerged culture and there was no effect on hydrophobicity of hyphae observed. The growth medium chosen in this study was synthetic glucose medium, while the investigation of HFB8 was largely carried out on PDA. These strong differences between OE*hfb8* and OE*hfb7* suggest that the strong overexpression of any hydrophobin does not result in the same phenotype, so the effect might not simply be caused by a skewed balance in the hydrophobome. However, a different species was used in the HFB7 study, which makes it hard to compare it to the overexpression of HFB8 in *T. guizhouense*, as different *Trichoderma* species show different expression rates [80], [81].

Another aspect to consider is the copy number of the inserted *hfb8::mrfp* construct. TUCIM 5783 showed a stronger phenotype than TUCIM 5782, which suggests a higher copy number of the construct in the TUCIM 5783 genome. It remains unclear, however, whether the stronger expression of the construct causes the stronger phenotype due to a higher concentration of HFB8 or due to stronger downregulation of other hydrophobins. One aspect that underlines the assumption that the overexpression of the construct results in the downregulation of other hydrophobins is the response of the mutant strain to fungal confrontations. The HFB8_{mrFP} overexpressing strains shows less aggressive/ effective defence against FOC4 than the *T. guizhouense* parental strain. This could be a result of the downregulation of cellular pathways responsible for fungal antagonism and response to external influences, but not essential for basic survival. The deletion of *hfb8* showed no influence on fungal antagonism.

6.2 Fungal-fungal interactions

One aim of this study was to investigate fungal-fungal interactions between *T. guizhouense* and four fungal species, *Pleurotus ostreatus*, *Pestalotiopsis fici*, *Fusarium oxysporum* f.sp. *cubense* (FOC) and *Fusarium oxysporum* f.sp. *cubense* 4 (FOC4). These species were chosen for their different taxonomic proximity to *T. guizhouense* and their importance for human applications.

6.2.1 *Trichoderma guizhouense* overgrows *Pleurotus ostreatus*

Pleurotus ostreatus (Agaricales), is a cultivated fungus that is frequently attacked by *Trichoderma* spp. in mushroom farms. The interactions of two *Trichoderma* spp, namely *T. pleurotum* and *T. pleuroticola*, which both belong to the Harzianum clade, have been previously investigated in dual confrontation assays. It was observed that *T. pleurotum* caused a strong antagonistic response in *P. ostreatus*, which also implemented pigmentation of *P. ostreatus* mycelium in the contact zone. The confrontation with *T. pleuroticola* did not result in any visible antagonism against *P. ostreatus*. Both *Trichoderma* species were able to eventually overgrow *P. ostreatus* [64].

In dual confrontation assays on PDA, *T. guizhouense* wt and *P. ostreatus* showed no pigmentation or growth inhibition of *T. guizhouense*, which completely overgrew the *P. ostreatus* mycelium, similar to the observations of Komon-Zelazowska et al. 2007 on *T. pleuroticola* [64]. The Tgui_{OE_{hfb8}} strain showed inhibited growth upon contact with *P. ostreatus*, but eventually also overgrew it completely. Investigations under the light microscope gave no indication of hyphal penetration, degradation or any form of interaction between TUCIM 5782 and *P. ostreatus*. This suggests that *T. guizhouense* might not have true mycoparasitic properties against *P. ostreatus*, although this cannot be claimed undoubtably. *P. ostreatus* vitality during this confrontation was not investigated and might be tested in the future by live-dead staining.

6.2.2 *Pestalotiopsis fici* produces defensive secondary metabolites when confronted with *Trichoderma guizhouense*

Pestalotiopsis fici (Xylariales) occupies the same ecological niche as *Trichoderma* and is a known producer of many secondary metabolites [62]. Druzhinina et al. 2018 [50] showed endoparasitism of *T. reesei* on *P. fici* in vitro, demonstrating the mycoparasitic properties of *Trichoderma* against this species.

Upon interaction with *T. guizhouense* in dual confrontations on PDA, *P. fici* produced secondary metabolites, which was observed as yellow pigmentation of the mycelium. These secondary metabolites do not seem to have a strong inhibitory effect on *T. guizhouense*, which could still overgrow the *P. fici* mycelium. Due to technical problems, the interactions between these two fungal species could not be studied efficiently on a microscopic level.

The inhibitory effect on *T. guizhouense* of the metabolites produce by *P. fici* might be dependent on their concentration. This might be tested by exposing *T. guizhouense* to a pure ethanolic extract of the metabolites at different concentrations in the future.

6.2.3 FOC4 initiates interaction with Tgui_{OEhfb8} in non-trophic interaction on plastic Petri plates and not hyphal penetration or degradation was observed

Fusarium spp. (Hypocreales) are relatively closely related mycotoxin-producing plant-pathogenic fungi that may inhabit the same ecosystem as *Trichoderma*. *Fusarium oxysporum* f.sp. *cubense* 4 (FOC4) is the known causative agent of banana wilt disease, and therefore of great interest to human application.

It was previously discovered that *T. guizhouense* shows strong antagonism against FOC4 [57], [58] *in vitro*. Dual confrontations of these two species on PDA underlined these findings. FOC4 stagnated *T. guizhouense* growth by secreting secondary metabolites into the surrounding agar, creating an antibiosis zone toxic to *T. guizhouense*. After this initial growth inhibition, however, *T. guizhouense* formed hyphal bridges onto the FOC4 mycelium, evading contact with the toxic antibiosis zone, and continued growth on the FOC4 mycelium. Similar discoveries were made in the confrontation between *T. guizhouense* and FOC, although FOC showed a lot weaker antagonism against *T. guizhouense*, as the antibiosis zone is a lot smaller or not observable at all. FOC antagonism against TUCIM 5782, however, does result in a similarly pronounced antibiosis zone, which indicates that the overexpression of HFB8 under the P_{cdna1} promoter might either implicate *T. guizhouense* resistance to FOC metabolites or might alter *T. guizhouense* production of signalling molecules necessary for fungal-fungal communication, sensed by FOC, by downregulation of specific molecular pathways.

For a more detailed picture of these interactions HFB8_{mRFP} was utilized for observation of Tgui_{OEhfb8} interactions under the epifluorescence microscope. Due to the accumulation of HFB8_{mRFP} at the mycelial surface of both interaction partners, the tag is not optimal for the monitoring of these fungal-fungal relationships, which only allowed the thorough investigation of the interaction between *T. guizhouense* and FOC4, due to the strong autofluorescence of FOC4 (explained further below).

The previously described mycoparasitic attack of *T. guizhouense* on FOC4 is challenged by microscopic analysis, although close symbiotic interactions between *T. guizhouense* (TUCIM 5782) and FOC4 are still observed. Both interacting partners were seeking contact, while FOC4 preferentially initiated it, which is in strong contrast to the observed “attack” of *T. guizhouense* in dual confrontations on PDA. After this initiation, hyphae of the two fungal species grew in tight contact alongside each other. There was no penetration or degradation of hyphae of either fungus observed, which leads to the question whether this interaction is truly mycoparasitic. However, nutrient exchange was not monitored and could give more detailed insights into the nature of this fungal-fungal interaction. Investigations of TUCIM 5782 showed that the overexpression of the HFB8_{mRFP} protein construct influences *T. guizhouense* physiology. This, along with cultivation conditions such as nutrient source or growth

surface, might have drastic influences on the interaction between these two fungal species, which needs to be investigated further.

6.3 HFB8_{mRFP} as a tag for studying fungal-fungal interactions is associated with challenges

As previously described, the HFB8_{mRFP} tag used for the monitoring of *T. guizhouense* behaviour in dual confrontations with fungal partners, is not ideal for this task. The distinction between *T. guizhouense* and other fungal hyphae is often not trivial and using fluorescent tags for strain identification can help master this challenge.

As HFB8 is mostly secreted, it does not completely stay attached to the cell wall and, depending on the microscopy setup, e.g. how much water accumulates in the interaction zone, the protein construct was transferred across the whole microscopy slide and accumulates at hyphae-water-air interfaces, which include the cell surface of the interaction partner. This makes the use of this tag for the identification of *T. guizhouense* in a microscopy setup redundant, as it does not help with its identification. The use of plastic Petri dishes as growth surfaces for the confrontations strongly reduced the accumulation of condensation water in the interaction zone and thus reduced the spread of HFB8_{mRFP}. However, the only clearly distinguishable confrontation was with FOC4. As FOC4 showed high autofluorescence in the green emission spectrum, much higher than *T. guizhouense*, this aspect could be used for the identification of this strain, as this signal could be clearly distinguished from mRFP.

6.3.1 The HFB8_{mRFP} fluorescence signal is not homogeneously distributed across hyphae and is setup dependant

During epifluorescence microscopy analysis it was discovered that the red fluorescent signal is not homogeneously distributed across the surface of *T. guizhouense* mycelium. The fluorescent signal was observed in a granular structure in hyphae inside growth medium and on glass and plastic surfaces close to the nutrient source. Older hyphae of *T. guizhouense* growing on glass slides or plastic petri dishes, further away from the nutrient source, displayed red fluorescent signal only on the outer edges, at the hyphae-water-air interface. This signal did not seem to cover the entire hyphal surface, but only accumulated at the interface between hyphal cell wall and the growth surface (glass or plastic). As water accumulates around hyphae attached to these surfaces it is assumed that this fluorescent signal mirrors the preference of HFB8_{mRFP} to accumulate at water-air-hyphae interfaces.

The signal morphology is setup dependant, hyphae growing on glass slides or plastic petri dishes displayed very heterogenous signals, while hyphae growing in and on PDA or in submerged culture displayed mostly the granular signal. This could be a further indication of the importance of hydrophobins in the attachment to different surfaces. Hydrophobic surfaces might trigger the secretion of HFB8, enabling strong hyphal attachment to these otherwise unfavourable surfaces.

Another interesting observation made was that conidiophores of Tgui_{OE_{Hfb8}} are brightly red fluorescent, while spores did not show any fluorescent signal. At first look this might indicate that HFB8 is involved in conidiation and spore release but is not an integral part of the outer spore coat. However, HFB8_{mRFP} investigated in this thesis is not expressed under its native promoter, but under P_{cdna1}. Thus, the described observations are most likely rather an indication of the regulation of P_{cdna1} and the importance of this regulation in conidiation. It might further indicate that P_{cdna1} is repressed in spores, suggesting that its native underlying gene *cdna1* is not active in spores and therefore not essential for spore survival.

One more setup dependant observation was that the red fluorescent signal of Tgui_{OE_{Hfb8}} was strongly reduced in the confrontation zone against FOC4 compared to hyphae growing on the sides or away from FOC4 in the same setup. This observation might indicate that the contact with FOC4 triggers a reduction in the expression of HFB8_{mRFP} by influencing the regulation of the P_{cdna1} promoter.

6.4 HFB8_{mRFP} appears to strongly accumulate in vacuoles

The granular signal found in hyphae growing close to or inside of nutrient sources (PDA or liquid medium) are suggested to be excessive HFB8_{mRFP} accumulating in vacuoles inside the fungal hyphae, which is not expected for secreted proteins. The regular, or well described, secretory pathway of fungi is shortly described in the following. Nucleotide sequences of secreted proteins contain N-terminal signal peptide sequences, which direct the transcribed mRNA to the endoplasmic reticulum (ER). The nascent protein is then either cotranslationally or posttranslationally translocated into the ER lumen, where it undergoes modifications (usually N-glycosylation) and is checked for misfolding. Misfolded proteins are either sent to vacuoles (lysosomes) [101] or the cytoplasm, where they are degraded by proteasomes [102]. Correctly folded proteins are packaged into vesicles and transported to the Golgi apparatus for further processing and subsequent vesicular transportation to the plasma membrane for secretion or to vacuoles.

However, the accumulation of enzymes destined for secretion in vacuoles has been observed in filamentous fungi before [103], [104]. The question remains, why the observed accumulation is happening. As previously mentioned, misfolded proteins are translocated to vacuoles containing lytic enzymes for degradation. As HFB8_{mRFP} shows characteristic behaviour of hydrophobins (interface aggregation), and the predicted molecular weight, the protein appears to be folded correctly. It has been shown before that a vacuolar sorting receptor in *Aspergillus oryzae* (Eurotiales, Ascomycota, Dikarya) might be involved in the (partial) degradation of heterologous proteins [105], as its mutation resulted in increased secretion of these proteins. The addition of mRFP could thus lead to the degradation of the heterologous protein in vacuoles. Vacuoles are not only used for degradation, but also for storage [106] of proteins. The excessive expression of HFB8_{mRFP} might thus lead to the observed

accumulation in vacuoles. Upon nutrient deprivation, these storages might be recycled for energy acquisition to ensure survival. This might also explain why no granular mRFP signals were observed in young, non-trophic hyphae on glass or plastic surfaces, far away from the PDA nutrient source.

These mentioned strategies suggest that the observed aggregation of HFB8_{mRFP} in the vacuoles is merely a side effect of its heterologous nature or overexpression. However, this vacuolar accumulation might also be a hint at a specialised secretory pathway of hydrophobins. Vacuoles might provide a closed off compartment that allow specific conditions, such as low pH, which might be essential for the correct folding into the active form of hydrophobin, making this “detour” to vacuoles an essential step in hydrophobin secretion. However, it was observed that hydrophobins are stable over a wide pH range [107], [108]. Another theory would be that correctly folded and functional hydrophobins might “block” the regular secretory pathway, due to their high surface activity. By direct sorting to vacuoles (instead of the Golgi), and from there to the plasma membrane, this blockage could be avoided.

If hydrophobins are intentionally targeted to vacuoles, they must be specifically directed there. Vacuolar targeting in fungi is best studied in yeast. Vacuolar proteins are localized to the organelles by two pathways, one of which requires a sorting signal [109]. It was discovered that the residues “QRPL” are an integral part of these signals. In order to find out whether hydrophobins in *Trichoderma* are regularly transported to vacuoles as an integral step in their secretion pathway, vacuolar signal sequences, and essential motifs, need to be identified in this genus for a comparison to potential signal sequences found in hydrophobins. If hydrophobins contain a signalling sequence that likely directs them to vacuoles, then it is most likely that vacuoles play an integral role in the secretion of these proteins. If no such sequence is present, it is likely that the accumulation of HFB8_{mRFP} is only a side effect of its overexpression or heterologous nature.

It was further observed that the vacuolar signal only occurred close to the nutrient source in hyphae growing on glass microscopy slides or plastic Petri dishes. This suggests that the accumulation of HFB8_{mRFP} in vacuoles could be nutrient dependant. Starvation might lead to degradation of the accumulated proteins for energy acquisition or trigger release and secretion.

6.5 Regulation of P_{cdna1} by environmental influence factors shows ambiguous results

The P_{cdna1} promoter was discovered in *T. reesei* in 1995, in an endeavour to find a strong, constitutive promoter for cultivation in synthetic glucose containing medium, which provides optimal conditions for high enzyme yields, as the production of proteases is reduced [80]. The previously used promoter *cbh1* is an inducible promoter, which is inhibited by glucose [80], [110]. P_{cdna1} showed the highest transcription rate in the glucose containing medium [80]. Its underlying gene *cdna1* is of unknown function and has thus far not been studied thoroughly.

The *hfb8::mrfp* construct is regulated by P_{cdna1} . The strength of this promoter results in such high expression rates that the $\text{Tgui}_{\text{OEhfb8}}$ cultures grown on PDA cause pink coloration of the agar, due to the accumulation of high levels of secreted HFB8_{mRFP}. In the course of this study it was observed that the intensity of the pink coloration differs between plates cultivated in different conditions, such as in darkness or under illumination. This observation lead to the assumption that the expression of HFB8_{mRFP} might be influenced by external factors and, hence, P_{cdna1} is not constitutive.

This hypothesis was tested by exposing $\text{Tgui}_{\text{OEhfb8}}$ cultures to different environmental factors (illumination, temperature changes, aeration, carbon sources, menadione) followed by extraction of HFB8_{mRFP} as a mycelium wash and measuring of the mRFP signal in a fluorescence reader.

The results obtained from these measurements are ambiguous. Illumination resulted in both increase and decrease of fluorescent signal, or no significant influence on signal intensity. These fluctuations were observed in both cultures grown on agar plates and in submerged 2 ml cultures. The influence of illumination time and light source was tested by short time (10 min) exposure to white and blue light. Results showed significant differences between illumination times and light sources, but again, the results are not consistent. Several experiments were carried out for both $\text{Tgui}_{\text{OEhfb8}}$ strains (TUCIM 5782 and TUCIM 5783), which showed a significant influence on signal intensity. TUCIM 5783, which is believed to contain a higher copy number of the *hfb8::mrfp* gene construct, seemed to be more sensitive to the influence by external factors.

Light is also known to induce reactive oxygen species (ROS) in fungi [99]. To get a clearer picture whether the response of P_{cdna1} to light is simply a general stress response, cultures were exposed to menadione, which induces increased production of ROS, thereby creating an artificial stress state [111]. The influence of menadione on P_{cdna1} showed similarly ambiguous results as the influence by light, but the measured fluorescence signals never showed the same intensity between these two conditions. This points to the assumption that the response of P_{cdna1} to illumination is not a simple stress response, but most likely involves additional regulatory pathways.

The importance of oxygenation for P_{cdna1} regulation was tested by gas exchange (opening of sealed cultivation plates). No significant influence of oxygen exposure was recorded, the differences between biological replicates were too high. Here, again, the fluctuation was especially high for TUCIM 5783.

It has been shown previously that different carbon compounds in available nutrient sources have a strong influence on *Trichoderma* growth rates and gene expression in combination with photostimulation [112]–[114]. The cultivation on microplates containing 95 different carbon sources showed that P_{cdna1} regulation is highly dependent on nutrient sources. This cultivation was done in three setups, namely in darkness, under 12 hours illumination and with the addition of menadione. In this experiment, no significant difference could be detected between mean mRFP signal intensity of

plates cultivated in darkness and under illumination, while mean signal intensity was increased when menadione was added. As cultures with added menadione also showed lower endpoint biomass, but higher growth rates, this result might be because the cultures with added menadione were still more active than the ones under illumination, as exponential growth was delayed due to the prior need to degrade or neutralise menadione [23].

These findings suggest that the response of P_{cdna1} to external influences does not depend on one factor alone but might be dependent on an interplay of several regulatory pathways.

Fungal response to illumination has been widely studied before [24]. In *Trichoderma*, light is mainly known to stimulate asexual sporulation [115], but has also been shown to stimulate growth of *T. reesei* [116]. Both of these responses have been shown to be carbon source dependent [113], [114], [116], suggesting that this light stimulation process requires an additional inducer. The results obtained in this thesis underline these previous findings and highlight the complexity of *Trichoderma* response to environmental influences.

Trichoderma responses to blue light depend on the blue light receptors BLR-1 and BLR-2 [113], [117]. The BLR-1 receptor contains a LOV (light-oxygen-voltage) sensory domain and a PAS domain, which is suggested to act as a redox and oxygen sensor [114]. This link between light and oxygen sensing suggests the indication of surface growth by these signals, and therefore a stimulation of morphological changes to adapt to these changing conditions, e.g. conidiation, as surface growth indicates the potential for aerial dispersal of spores. It was observed that $Tg_{uiOEhfb8}$ shows brightly red fluorescent conidiophores, caused by strong expression of $HFB8_{mRFP}$. This suggests that P_{cdna1} is highly active in these structures and might thus be involved in conidiation, again highlighting the possible importance of light and oxygen in P_{cdna1} activation.

The mRFP extraction method used in these experiments is rather simple and therefore fast and cheap. But it is not automated and prone to technical errors. It cannot be clearly distinguished how P_{cdna1} influences the properties of HFB8 in the protein construct. The high surface activity of HFB8 might influence the accumulation and extraction rates of $HFB8_{mRFP}$, and therefore skew the fluorescence signal. Furthermore, in fungi, proteins can undergo several post translational modifications, which would be overlooked in this quantification method.

The analysis of gene expression at transcript level can be analysed highly accurately by real-time quantitative polymerase chain reaction (qPCR). The gene sequence for the CDNA1 protein of unknown function from *T. reesei* (TUCIM 916), recovered from its original publication [80], was used to find homologous genes in *T. guizhouense* NJAU (TUCIM 4742) and *T. harzianum* (TUCIM 917) using NCBI's BLAST [95]. The expression of *cdna1* was investigated in these three *Trichoderma* species. *T. harzianum*

was added to this investigation for its close taxonomic relation to *T. guizhouense* (both belong to the *Harzianum* clade) and its widespread use in biofertilizers [2]–[4].

As *cdna1* transcription is measured directly in this approach, there is no interference by mRFP, hydrophobin properties or post translational modifications, which improves its accuracy. However, this method also requires more preparation (BLAST, primer design, RNA extraction) and is therefore more time consuming and expensive.

qPCR still showed ambiguous results of illumination. There was no statistical significance of illumination on *cdna1* expression observable overall, but the results were strain dependant. Increased temperature showed slight upregulation of *cdna1* expression in *T. guizhouense*, but downregulation in *T. reesei* and *T. harzianum*. The strongest response was observed in liquid submerged culture. *cdna1* expression was strongly upregulated in both standing and shaking submerged culture in *T. harzianum* and *T. guizhouense*, the latter showing the strongest response. In *T. reesei*, gene expression was downregulated in shaking culture. This strong response in submerged growth could suggest that this cultivation setup provides more effective cellular regulation by providing more efficient nutrient uptake and therefore a more efficient metabolism. Since the measured Ct levels were normalized by a housekeeping gene, it is not likely that this strong upregulation is due to technical differences in mRNA extraction from submerged culture.

The qPCR results showed significant differences in *cdna1* regulation between the three species. Since the expressed genes are not identical, but only homologous, and the sequences of their individual promoter regions are not known, this is not entirely unexpected. These findings highlight the fact that gene regulation could be very species and strain dependant and that P_{cdna1} is influenced by many different cellular pathways. This could also explain the ambiguous response of *T. guizhouense* to individual environmental factors, which suggests that it is not one factor alone but a complex interplay of many external influences and their respective signalling pathways that elicit a molecular response by P_{cdna1} .

All these findings support the hypothesis that P_{cdna1} is not a constitutive promoter and that its regulation depends on the complex interplay of different cellular pathways, highly depending on the *Trichoderma* species.

6.6 Outlook

In the course of this thesis multiple external influences on *T. guizhouense* physiology were investigated and resulted in various insights into the fungus's response to its environment. In the end, there are still many unanswered questions and room for improvements and future investigations.

The Tui_{OE_{Hfb8}} strains are very sensitive to slight differences in cultivation conditions. Since perfectly regulated conditions are hard to maintain, the number of biological replicates should be increased to reduce the often-high standard errors.

The vitality of the fungi used in dual confrontation assays was monitored by light microscopy, but not directly on PDA plates. Live-dead staining could be carried out in the future to follow the progression of these interactions.

HFB8_{mRFP} was used as a tag for monitoring fungal-fungal interaction of TUCIM 5782 with four fungal interacting partners by epifluorescence microscopy. As HFB8 is secreted and preferentially accumulates at water-air interfaces, the red fluorescent signal created by the construct is, depending on the microscopy setup, detectable across the whole microscopy slide, including the hyphae of the interacting partners, as water accumulates around their hyphae. This makes the use of this construct for the identification of *T. guizhouense* redundant. For species identification a tag that stays anchored to the cell wall and is not secreted would be more appropriate (e.g. chitin or a cell wall protein).

HFB8_{mRFP} provides a fast and cheap way of monitoring external influences on its integrated promoter P_{cdna1}. HFB8 provides the signal for secretion of the protein construct which can therefore be extracted and quantified by measuring red fluorescence intensity. Due to its amphiphilic properties and spontaneous aggregation at water-air interfaces and the fungal cell wall, the extraction needs to be very precise and is prone to technical errors. The properties and possible posttranslational modifications of HFB8 might also have an influence on the excretion of the construct under different environmental conditions and might thus skew the significance of the results.

Thus, it might be feasible to use a protein that stays in solution after secretion, or simply add a secretion signal sequence to mRFP as a more appropriate and easier to handle tag, as interplay between mRFP and HFB8 would not be an issue.

The heterogeneous accumulation of HFB8_{mRFP} in vacuoles of different sizes was observed in Tgui_{OE_{Hfb8}}. It remains unclear, whether this accumulation is merely a side-effect of the heterologous nature of the protein or its strong overexpression, or if this discovery might be a hint at a specialized secretory pathway for hydrophobins in *Trichoderma*, needed due to their high surface activity and amphiphilic nature. A first step in unravelling this question would be to search for potential vacuolar targeting motifs in the signal peptide sequences of known vacuolar proteins in *Trichoderma*, using SignalP [118] or similar signal peptide predictors. These sequences might then be compared to known *Trichoderma* hydrophobin signal peptide sequences to discover potential vacuolar localization motifs, which would strongly suggest that HFB8_{mRFP} is intentionally sorted to the vacuoles and that hydrophobins use a special secretory pathway.

The investigation of the interaction between *T. guizhouense* and FOC4 gave new insights into this fungal relationship. This interaction has been previously described as mycoparasitic [57], [58], in which *T. guizhouense* attacks FOC4, which defends itself by the production of mycotoxic secondary metabolites. Although these observations were confirmed in this thesis in dual confrontation assays on PDA plates, microscopic analysis of this interaction showed, that *T. guizhouense* does not seem to penetrate or cause degradation of FOC4 hyphae. FOC4 initiates hyphal interaction and grows alongside *T. guizhouense* hyphae in close contact. To better understand this interaction, nutrient exchange could be monitored and visualized by mass spectrometric methods [119]–[121]. Dual confrontation assays could be done on different substrates and under different environmental influences. As the overexpression of HFB8_{mRFP} seems to influence *T. guizhouense* antagonism against FOC4, it is not clear how strongly these observations were influenced by this overexpression. The use of a different tag for fungal identification (as described previously) could help unravel this open question.

In the course of this thesis the hypothesis was made that the P_{cdna1} promoter from *T. reesei* is not constitutive and is controlled by an interplay of many different external influence factors and their respective regulatory signalling pathways. It remains unclear to what extent which external factor influences this response and which cellular pathways are responsible for the regulation of P_{cdna1}. To investigate this further particular signal receptors (e.g. BLR-1/2) or their respective response pathways could be silenced or knocked out by mutagenesis to empirically test the response of P_{cdna1} to single external stressors [29], [113], [117].

The promoter's underlying gene *cdna1* has not been studied and is of unknown function. To investigate how this gene might contribute to the cellular stress response, gene deletion and overexpressing mutants should be created for subsequent phenotypic analysis. The construction of a *cdna1* deletion mutant of *T. guizhouense* was attempted in the course of this thesis (Supplementary Material 9.2) but was not finished due to time limitations. A thorough phylogenetic analysis of this gene must be done to find homologs across *Trichoderma* and the fungal kingdom and find their putative common ancestor to give insights into how and where this gene originated and if and how it might have been horizontally transferred. This could give greater insights into the function of this gene and help find a starting point for the molecular investigation of its regulation.

6.7 Conclusion

HFB8_{mRFP} is not optimal for the microscopic analysis of fungal-fungal interactions. The overexpression of the construct influences *T. guizhouense* physiology and therefore might have an impact on the interaction with other fungi. The construct is further not fully suitable for the identification of Tgui_{O_EHfb8} under the epifluorescence microscope, as it is secreted and accumulates across the entire microscopy slide, including the mycelium of the interaction partner.

The previously as mycoparasitic described relationship between *T. guizhouense* and FOC4 was investigated by light microscopy and revealed no penetration or degradation of FOC4 hyphae by *T. guizhouense*, but the two fungi grow in close hyphal proximity with each other upon contact. It was further observed that FOC4 preferentially initiates this contact, which undermines the belief that *T. guizhouense* attacks FOC4 and is therefore the driver of this interaction. These observations were made between FOC4 and *T. guizhouense* overexpressing HFB8_{mRFP}, which might influence *T. guizhouense* physiology. Therefore, these findings need to be investigated further.

Another application of HFB8_{mRFP} utilized in this thesis was the easy and affordable way to monitor the regulation of its integrated promoter P_{cdna1} by external influences. As this protein construct is secreted and contains a fluorophore, it can be extracted and quantified. However, the overexpression of the construct has an impact on *T. guizhouense* physiology and might be subjected to posttranslational modifications, which needs to be considered for its application. Further, it was observed that HFB8_{mRFP} accumulated in what are believed to be intracellular vacuoles. Through this accumulation, a lot of HFB8_{mRFP} would not be directly secreted and therefore not detectable by the used method. It is not clear if and how specific parameters might influence this accumulation or the release of HFB8_{mRFP} from these vacuoles for secretion, thus creating more potential for skewed results.

This accumulation of HFB8_{mRFP} might give a hint at a specialised secretory pathway used by hydrophobins in *Trichoderma*, needed because of their high surface activity. However, it is not known whether this accumulation is intended or a simple by-product of heterologous overexpression. Further research in this area is needed to unravel these questions.

Findings of the study of the P_{cdna1} promoter strongly support the hypothesis that P_{cdna1} is not constitutive and influenced by external biotic and abiotic factors. This influence is most likely a complex interplay of different external factors, which are highly dependent on the *Trichoderma* species and the available nutrient source. The molecular regulation of this promoter is still not understood, as is the function of the promoter's underlying gene *cdna1*, both of which should be investigated in the future for their potential importance in *Trichoderma* stress response.

7 References

- [1] K. Esser and P. A. Lemke, *The mycota*. Springer, 1994.
- [2] G. E. Harman, "Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzianum* T-22," *Plant Dis.*, vol. 84, no. 4, pp. 377–393, 2000.
- [3] G. E. Harman, C. R. Howell, A. Viterbo, I. Chet, and M. Lorito, "Trichoderma species—opportunistic, avirulent plant symbionts," *Nat. Rev. Microbiol.*, vol. 2, no. 1, pp. 43–56, 2004.
- [4] M. Lorito, S. L. Woo, and F. Scala, "Le biotecnologie utili alla difesa sostenibile delle piante: i funghi," *Agroindustria*, vol. 3, pp. 181–195, 2004.
- [5] C. W. Hesseltine, "A millennium of fungi, food, and fermentation," *Mycologia*, vol. 57, no. 2, pp. 149–197, 1965.
- [6] E. Caplice and G. F. Fitzgerald, "Food fermentations: role of microorganisms in food production and preservation," *Int. J. Food Microbiol.*, vol. 50, no. 1–2, pp. 131–149, 1999.
- [7] E. L. Ghisalberti and K. Sivasithamparam, "Antifungal antibiotics produced by *Trichoderma* spp," *Soil Biol. Biochem.*, vol. 23, no. 11, pp. 1011–1020, 1991.
- [8] K. Spelman, J. J. Burns, D. Nichols, N. Winters, S. Ottersberg, and M. Tenborg, "Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators," *Altern. Med. Rev.*, vol. 11, no. 2, p. 128, 2006.
- [9] U. Kück, S. Bloemendal, and I. Teichert, "Putting fungi to work: harvesting a cornucopia of drugs, toxins, and antibiotics," *PLoS Pathog.*, vol. 10, no. 3, 2014.
- [10] R. Dean *et al.*, "The Top 10 fungal pathogens in molecular plant pathology," *Mol. Plant Pathol.*, vol. 13, no. 4, pp. 414–430, 2012.
- [11] G. D. Brown, D. W. Denning, N. A. R. Gow, S. M. Levitz, M. G. Netea, and T. C. White, "Hidden killers: human fungal infections," *Sci. Transl. Med.*, vol. 4, no. 165, p. 165rv13–165rv13, 2012.
- [12] W. J. Welch, "How cells respond to stress," *Sci. Am.*, vol. 268, no. 5, pp. 56–64, May 1993.
- [13] D. B. Berry and A. P. Gasch, "Stress-activated genomic expression changes serve a preparative role for impending stress in yeast," *Mol. Biol. Cell*, vol. 19, no. 11, pp. 4580–4587, Nov. 2008.
- [14] D. B. Berry *et al.*, "Multiple Means to the Same End: The Genetic Basis of Acquired Stress Resistance in Yeast," *PLOS Genet.*, vol. 7, no. 11, p. e1002353, Nov. 2011.
- [15] Q. Guan, S. Haroon, D. G. Bravo, J. L. Will, and A. P. Gasch, "Cellular memory of acquired stress resistance in *Saccharomyces cerevisiae*," *Genetics*, vol. 192, no. 2, pp. 495–505, Oct. 2012.
- [16] Z. Karányi, I. Holb, L. Hornok, I. Pócsi, and M. Miskei, "FSRD: Fungal stress response database," *Database*, vol. 2013, pp. 1–6, 2013.
- [17] K. J. Verstrepen *et al.*, "Glucose and sucrose: hazardous fast-food for industrial yeast?," *Trends Biotechnol.*, vol. 22, no. 10, pp. 531–537, Oct. 2004.
- [18] J. R. M. Almeida, D. Runquist, V. Sanchez i Nogue, G. Liden, and M. F. Gorwa-Grauslund, "Stress-related challenges in pentose fermentation to ethanol by the yeast *Saccharomyces cerevisiae*," *Biotechnol. J.*, vol. 6, no. 3, pp. 286–299, Mar. 2011.
- [19] Q. Li, Z. Bai, A. O'Donnell, L. M. Harvey, P. A. Hoskisson, and B. McNeil, "Oxidative stress in fungal fermentation processes: the roles of alternative respiration," *Biotechnol. Lett.*, vol. 33, no. 3, pp. 457–467, Mar. 2011.

- [20] J. Aguirre, M. Ríos-Momberg, D. Hewitt, and W. Hansberg, "Reactive oxygen species and development in microbial eukaryotes," *Trends Microbiol.*, vol. 13, no. 3, pp. 111–118, 2005.
- [21] B. L. Taylor and I. B. Zhulin, "PAS domains: internal sensors of oxygen, redox potential, and light.," *Microbiol. Mol. Biol. Rev.*, vol. 63, no. 2, pp. 479–506, 1999.
- [22] B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, 3rd ed. Oxford: Oxford University Press, 1999.
- [23] M. Lorito, S. L. Woo, G. E. Harman, and E. Monte, "Translational Research on *Trichoderma* : From 'Omics to the Field," *Annu. Rev. Phytopathol.*, vol. 48, no. 1, pp. 395–417, 2010.
- [24] J. Rodriguez-romero, M. Hedtke, C. Kastner, M. Sylvia, and R. Fischer, "Fungi , Hidden in Soil or Up in the Air : Light Makes a Difference," 2010.
- [25] P. L. Lakin-Thomas and S. Brody, "Circadian rhythms in microorganisms: new complexities," *Annu. Rev. Microbiol.*, vol. 58, pp. 489–519, 2004.
- [26] L. M. Corrochano and P. Galland, "Photomorphogenesis and gravitropism in fungi," in *Growth, differentiation and sexuality*, Springer, 2006, pp. 233–259.
- [27] C. Li and T. J. Schmidhauser, "Developmental and photoregulation of al-1 and al-2, structural genes for two enzymes essential for carotenoid biosynthesis in *Neurospora*," *Dev. Biol.*, vol. 169, no. 1, pp. 90–95, 1995.
- [28] N. Arrach, R. Fernandez-Martin, E. Cerda-Olmedo, and J. Avalos, "A single gene for lycopene cyclase, phytoene synthase, and regulation of carotene biosynthesis in *Phycomyces*," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 98, no. 4, pp. 1687–1692, Feb. 2001.
- [29] M. Schmoll, E. U. Esquivel-naranjo, and A. Herrera-estrella, "Trichoderma in the light of day – Physiology and development," *Fungal Genet. Biol.*, vol. 47, no. 11, pp. 909–916, 2010.
- [30] F. Lledias and W. B. T.-M. in E. Hansberg, "[11] Catalase modification as a marker for singlet oxygen," in *Singlet Oxygen, UV-A, and Ozone*, vol. 319, Academic Press, 2000, pp. 110–119.
- [31] K. K. Tan, "Light-induced fungal development," *The filamentous fungi*, vol. 3, pp. 334–353, 1978.
- [32] E. Cerdá-Olmedo and L. M. Corrochano, "Genetics of *Phycomyces* and its responses to light," in *Comprehensive Series in Photosciences*, vol. 1, Elsevier, 2001, pp. 589–620.
- [33] G. Berrocal-Tito, L. Sametz-Baron, K. Eichenberg, B. A. Horwitz, and A. Herrera-Estrella, "Rapid blue light regulation of a *Trichoderma harzianum* photolyase gene," *J. Biol. Chem.*, vol. 274, no. 20, pp. 14288–14294, 1999.
- [34] G. Nagahashi and D. D. Douds Jr, "Synergism between blue light and root exudate compounds and evidence for a second messenger in the hyphal branching response of *Gigaspora gigantea*," *Mycologia*, vol. 96, no. 5, pp. 948–954, 2004.
- [35] R. Abrashev *et al.*, "Temperature-stress tolerance of the fungal strain *Aspergillus niger* 26: physiological and ultrastructural changes.," *World J. Microbiol. Biotechnol.*, vol. 30, no. 5, pp. 1661–1668, May 2014.
- [36] M. A. Noventa-Jordão *et al.*, "Catalase activity is necessary for heat-shock recovery in *Aspergillus nidulans* germlings," *Microbiology*, vol. 145, no. 11, pp. 3229–3234, 1999.
- [37] Z. Bai, L. M. Harvey, and B. McNeil, "Oxidative stress in submerged cultures of fungi," *Crit. Rev. Biotechnol.*, vol. 23, no. 4, pp. 267–302, 2003.
- [38] A. Schuster and M. Schmoll, "Biology and biotechnology of *Trichoderma*," *Appl. Microbiol.*

- Biotechnol.*, vol. 87, no. 3, pp. 787–799, 2010.
- [39] C. Kistner and M. Parniske, “Evolution of signal transduction in intracellular symbiosis,” *Trends Plant Sci.*, vol. 7, no. 11, pp. 511–518, 2002.
 - [40] F. Maillet *et al.*, “Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza,” *Nature*, vol. 469, no. 7328, pp. 58–63, 2011.
 - [41] G. E. D. Oldroyd, “Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants,” *Nat. Rev. Microbiol.*, vol. 11, no. 4, pp. 252–263, 2013.
 - [42] K. A. Aliferis and S. Jabaji, “Metabolite composition and bioactivity of *Rhizoctonia solani* sclerotial exudates,” *J. Agric. Food Chem.*, vol. 58, no. 13, pp. 7604–7615, 2010.
 - [43] C. P. Kubicek *et al.*, “Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*,” *Genome Biol.*, vol. 12, no. 4, p. R40, 2011.
 - [44] I. S. Druzhinina, V. Seidl-seiboth, A. Herrera-estrella, S. Zeilinger, I. V. Grigoriev, and C. P. Kubicek, “*Trichoderma* : the genomics of opportunistic success,” *Nat. Publ. Gr.*, vol. 9, no. 10, pp. 749–759, 2011.
 - [45] J. Bissett, W. Gams, W. Jaklitsch, and G. J. Samuels, “Accepted *Trichoderma* names in the year 2015,” *IMA Fungus*, vol. 6, no. 2, pp. 263–295, 2015.
 - [46] D. Klein and D. E. Eveleigh, “Ecology of trichoderma,” *Trichoderma and gliocladium*, vol. 1, pp. 57–74, 1998.
 - [47] Y. Brotman, J. G. Kapuganti, and A. Viterbo, “*Trichoderma*,” *Curr. Biol.*, vol. 20, no. 9, pp. R390–R391, 2010.
 - [48] W. M. Jaklitsch, “European species of *Hypocrea* Part I. The green-spored species,” *Stud. Mycol.*, vol. 63, pp. 1–91, 2009.
 - [49] W. M. Jaklitsch, “European species of *Hypocrea* part II: species with hyaline ascospores,” *Fungal Divers.*, vol. 48, no. 1, pp. 1–250, 2011.
 - [50] I. S. Druzhinina *et al.*, *Massive lateral transfer of genes encoding plant cell wall-degrading enzymes to the mycoparasitic fungus Trichoderma from its plant-associated hosts*, vol. 14, no. 4. 2018.
 - [51] D. Martinez *et al.*, “Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*),” *Nat. Biotechnol.*, vol. 26, no. 5, p. 553, 2008.
 - [52] C. R. Howell, “Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts,” *Plant Dis.*, vol. 87, no. 1, pp. 4–10, 2003.
 - [53] T. Benítez, A. M. Rincón, M. C. Limón, and A. C. Codon, “Biocontrol mechanisms of *Trichoderma* strains,” *Int. Microbiol.*, vol. 7, no. 4, pp. 249–260, 2004.
 - [54] C. Altomare, W. A. Norvell, T. Björkman, and G. E. Harman, “Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22,” *Appl. Environ. Microbiol.*, vol. 65, no. 7, pp. 2926–2933, 1999.
 - [55] F. Vinale *et al.*, “A novel role for *Trichoderma* secondary metabolites in the interactions with plants,” *Physiol. Mol. Plant Pathol.*, vol. 72, no. 1, pp. 80–86, 2008.
 - [56] P. Chaverri, F. Branco-Rocha, W. Jaklitsch, R. Gazis, T. Degenkolb, and G. J. Samuels, “Systematics of the *Trichoderma harzianum* species complex and the re-identification of commercial biocontrol strains,” *Mycologia*, vol. 107, no. 3, pp. 558–590, 2015.

- [57] J. Zhang *et al.*, "The neutral metallopeptidase NMP1 of *Trichoderma guizhouense* is required for mycotrophy and self-defence," *Environ. Microbiol.*, vol. 18, no. 2, pp. 580–597, 2015.
- [58] J. Zhang *et al.*, "Guttation capsules containing hydrogen peroxide: how an evolutionary conserved NADPH oxidase gains a role in wars between related fungi."
- [59] F. Vinale, K. Sivasithamparam, E. L. Ghisalberti, R. Marra, S. L. Woo, and M. Lorito, "Trichoderma – plant – pathogen interactions," vol. 40, pp. 1–10, 2008.
- [60] K. G. Pegg, N. Y. Moore, and S. Sorensen, "Fusarium wilt in the Asian Pacific region," in *International Symposium on recent developments in banana cultivation technology*. (Eds RV Valmayor, SC Hwang, RC Ploetz, SW Lee, VN Roa) pp, 1993, pp. 255–314.
- [61] P. Jeffries and T. W. K. Young, *Interfungal parasitic relationships*. Cab International, 1994.
- [62] L. Liu, "Bioactive metabolites from the plant endophyte *pestalotiopsis fici*," *Mycology*, vol. 2, no. 1, pp. 37–45, 2011.
- [63] P. Umadevi and M. Anandaraj, "Trichoderma harzianum MTCC 5179 impacts the population and functional dynamics of microbial community in the rhizosphere of black pepper (*Piper nigrum* L.)," *Brazilian J. Microbiol.*, vol. 49, no. 3, pp. 463–470, 2017.
- [64] M. Komoń-Zelazowska *et al.*, "Genetically closely related but phenotypically divergent *Trichoderma* species cause green mold disease in oyster mushroom farms worldwide," *Appl. Environ. Microbiol.*, vol. 73, no. 22, pp. 7415–7426, 2007.
- [65] C. P. Kubicek, S. Baker, C. Gamauf, C. M. Kenerley, and I. S. Druzhinina, "hydrophobin gene families of the ascomycete *Trichoderma* / *Hypocrea*," vol. 16, pp. 1–16, 2008.
- [66] L. Espino-Rammer *et al.*, "Two novel class ii hydrophobins from *Trichoderma* spp. Stimulate enzymatic hydrolysis of poly(ethylene terephthalate) when expressed as fusion proteins," *Appl. Environ. Microbiol.*, vol. 79, no. 14, pp. 4230–4238, 2013.
- [67] J. G. H. Wessels, "Fungi in their own right," *Fungal Genet. Biol.*, vol. 27, no. 2–3, pp. 134–145, 1999.
- [68] N. J. Talbot, "Fungal biology: Coming up for air and sporulation," *Nature*, vol. 398, no. 6725, pp. 295–296, 1999.
- [69] M. B. Linder, "Hydrophobins : the protein-amphiphiles of filamentous fungi," *FEMS Microbiol. Rev.*, vol. 29, no. 5, pp. 877–896, 2005.
- [70] H. A. B. Wösten, "Hydrophobins : Multipurpose Proteins," *Annu. Rev. Microbiol.*, vol. 55, no. 1, pp. 625–646, 2001.
- [71] H. A. B. Wösten and M. L. de Vocht, "Hydrophobins, the fungal coat unravelled," *Biochim. Biophys. Acta (BBA)-Reviews Biomembr.*, vol. 1469, no. 2, pp. 79–86, 2000.
- [72] H. A. Wösten, F. H. Schuren, and J. G. Wessels, "Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces.," *EMBO J.*, vol. 13, no. 24, pp. 5848–5854, 1994.
- [73] L. G. Lugones, H. A. B. Wösten, K. U. Birkenkamp, K. A. Sjollema, J. Zagers, and J. G. H. Wessels, "Hydrophobins line air channels in fruiting bodies of *Schizophyllum commune* and *Agaricus bisporus*," *Mycol. Res.*, vol. 103, no. 5, pp. 635–640, 1999.
- [74] A. J. Ninfa, D. P. Ballou, and M. Benore, *Fundamental laboratory approaches for biochemistry and biotechnology*. Fitzgerald Science Press Bethesda, MD, 1998.
- [75] R. Y. Tsien, "The green fluorescent protein," *Annu. Rev. Biochem.*, vol. 67, pp. 509–544, 1998.

- [76] N. C. Shaner, R. E. Campbell, P. A. Steinbach, B. N. G. Giepmans, A. E. Palmer, and R. Y. Tsien, "Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein," *Nat. Biotechnol.*, vol. 22, no. 12, p. 1567, 2004.
- [77] J. G. Wessels, O. M. de Vries, S. A. Asgeirsdottir, and J. Springer, "The thn mutation of *Schizophyllum commune*, which suppresses formation of aerial hyphae, affects expression of the Sc3 hydrophobin gene," *J. Gen. Microbiol.*, vol. 137, no. 10, pp. 2439–2445, Oct. 1991.
- [78] S. Sarrocco, L. Mikkelsen, M. Vergara, D. F. Jensen, M. Lübeck, and G. Vannacci, "Histopathological studies of sclerotia of phytopathogenic fungi parasitized by a GFP transformed *Trichoderma virens* antagonistic strain," *Mycol. Res.*, vol. 110, no. 2, pp. 179–187, 2006.
- [79] L. López-maury, S. Marguerat, and J. Bähler, "Tuning gene expression to changing to evolutionary adaptation," no. July, 2008.
- [80] T. Nakari-Setälä and M. Penttilä, "Production of *Trichoderma reesei* cellulases glucose-containing media," *Appl. Environ. Microbiol.*, vol. 61, no. 10, pp. 3650–3655, 1995.
- [81] A. Harkki, J. Uusitalo, M. Bailey, M. Penttilä, and J. K. C. Knowles, "A novel fungal expression system: secretion of active calf chymosin from the filamentous fungus *Trichoderma reesei*," *Nat. Biotechnol.*, vol. 7, no. 6, p. 596, 1989.
- [82] M. Saloheimo and M.-L. Niku-Paavola, "Heterologous production of a ligninolytic enzyme: expression of the *Phlebia radiata* laccase gene in *Trichoderma reesei*," *Nat. Biotechnol.*, vol. 9, no. 10, pp. 987–990, 1991.
- [83] J. M. Uusitalo, K. M. H. Nevalainen, A. M. Harkki, J. K. C. Knowles, and M. E. Penttilä, "Enzyme production by recombinant *Trichoderma reesei* strains," *J. Biotechnol.*, vol. 17, no. 1, pp. 35–49, 1991.
- [84] V. V. Joutsjoki, T. K. Torkkeli, and K. M. H. Nevalainen, "Transformation of *Trichoderma reesei* with the *Hormoconis resinae* glucoamylase P (gamP) gene: production of a heterologous glucoamylase by *Trichoderma reesei*," *Curr. Genet.*, vol. 24, no. 3, pp. 223–228, 1993.
- [85] E. Nyssönen, M. Penttilä, A. Harkki, A. Saloheimo, J. K. C. Knowles, and S. Keränen, "Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*," *Nat. Biotechnol.*, vol. 11, no. 5, pp. 591–595, 1993.
- [86] M. Penttilä, A. Saloheimo, M. Ilmen, and M.-L. Onnela, "Regulation of the expression of *Trichoderma* cellulases at mRNA and promoter level," in *The TRICEL93 Symposium*, 1993, pp. 189–197.
- [87] J. Li, J. Wang, S. Wang, M. Xing, S. Yu, and G. Liu, "Achieving efficient protein expression in *Trichoderma reesei* by using strong constitutive promoters," *Microb. Cell Fact.*, vol. 11, no. 1, p. 1, 2012.
- [88] F. Uzbaz, U. Sezerman, L. Hartl, C. P. Kubicek, and B. Seiboth, "A homologous production system for *Trichoderma reesei* secreted proteins in a cellulase-free background," pp. 1601–1608, 2012.
- [89] A. Przylucka *et al.*, "HFB7 – A novel orphan hydrophobin of the *Harzianum* and *Virens* clades of *Trichoderma*, is involved in response to biotic and abiotic stresses," *Fungal Genet. Biol.*, 2017.
- [90] T. Paasikallio, A. Huuskonen, and M. G. Wiebe, "Scaling up and scaling down the production of galactaric acid from pectin using *Trichoderma reesei*," *Microb. Cell Fact.*, vol. 16, p. 119, Jul. 2017.

- [91] Z.-B. Huang, X.-Z. Chen, L.-N. Qin, H.-Q. Wu, X.-Y. Su, and Z.-Y. Dong, "A novel major facilitator transporter TrSTR1 is essential for pentose utilization and involved in xylanase induction in *Trichoderma reesei*," *Biochem. Biophys. Res. Commun.*, vol. 460, no. 3, pp. 663–669, 2015.
- [92] N. Sun, Y. Qian, W. Wang, Y. Zhong, and M. Dai, "Heterologous expression of *Talaromyces emersonii* cellobiohydrolase Cel7A in *Trichoderma reesei* increases the efficiency of corncob residues saccharification," *Biotechnol. Lett.*, vol. 40, no. 7, pp. 1119–1126, 2018.
- [93] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, "NIH Image to ImageJ: 25 years of image analysis," *Nat. Methods*, vol. 9, no. 7, pp. 671–675, 2012.
- [94] A. F. Stalder, T. Melchior, M. Müller, D. Sage, T. Blu, and M. Unser, "Low-bond axisymmetric drop shape analysis for surface tension and contact angle measurements of sessile drops," *Colloids Surfaces A Physicochem. Eng. Asp.*, vol. 364, no. 1–3, pp. 72–81, 2010.
- [95] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, and D. J. Lipman, "Basic local alignment search tool.," *J. Mol. Biol.*, vol. 215, no. 3, pp. 403–410, Oct. 1990.
- [96] S. Lalitha, "Primer premier 5," *Biotech Softw. Internet Rep. Comput. Softw. J. Sci.*, vol. 1, no. 6, pp. 270–272, 2000.
- [97] K. J. Livak and T. D. Schmittgen, "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.
- [98] RStudio Team, "RStudio: Integrated Development for R." RStudio, Inc., Boston, MA, 2016.
- [99] H. Iigusa, Y. Yoshida, and K. Hasunuma, "Oxygen and hydrogen peroxide enhance light-induced carotenoid synthesis in *Neurospora crassa*," *FEBS Lett.*, vol. 579, no. 18, pp. 4012–4016, 2005.
- [100] J. G. H. Wessels, "Hydrophobins, unique fungal proteins," *Mycologist*, vol. 14, no. 4, pp. 153–159, 2000.
- [101] C. M. Coughlan, J. L. Walker, J. C. Cochran, K. D. Wittrup, and J. L. Brodsky, "Degradation of mutated bovine pancreatic trypsin inhibitor (BPTI) in the yeast vacuole suggests post-endoplasmic reticulum protein quality control," *J. Biol. Chem.*, 2004.
- [102] R. Y. Hampton, "ER-associated degradation in protein quality control and cellular regulation," *Curr. Opin. Cell Biol.*, vol. 14, no. 4, pp. 476–482, 2002.
- [103] W. Kurzatkowski *et al.*, "Glucose-induced secretion of *Trichoderma reesei* xylanases.," *Appl. Environ. Microbiol.*, vol. 62, no. 8, pp. 2859–2865, 1996.
- [104] I. Kuan and M. Tien, "Phosphorylation of lignin peroxidases from *Phanerochaete chrysosporium*. Identification of mannose 6-phosphate.," *J. Biol. Chem.*, vol. 264, no. 34, pp. 20350–20355, 1989.
- [105] J. Yoon, T. Aishan, J. Maruyama, and K. Kitamoto, "Enhanced production and secretion of heterologous proteins by the filamentous fungus *Aspergillus oryzae* via disruption of vacuolar protein sorting receptor gene *Aovps10*," *Appl. Environ. Microbiol.*, vol. 76, no. 17, pp. 5718–5727, 2010.
- [106] D. J. Klionsky, "Protein transport from the cytoplasm into the vacuole," *J. Membr. Biol.*, vol. 157, no. 2, pp. 105–115, 1997.
- [107] K. Kisko, G. R. Szilvay, U. Vainio, M. B. Linder, and R. Serimaa, "Interactions of hydrophobin proteins in solution studied by small-angle X-ray scattering," *Biophys. J.*, vol. 94, no. 1, pp. 198–206, 2008.

- [108] K. Meister *et al.*, "Observation of pH-Induced Protein Reorientation at the Water Surface," *J. Phys. Chem. Lett.*, vol. 8, no. 8, pp. 1772–1776, Apr. 2017.
- [109] E. Conibear and T. H. Stevens, "Multiple sorting pathways between the late Golgi and the vacuole in yeast," *Biochim. Biophys. Acta (BBA)-Molecular Cell Res.*, vol. 1404, no. 1, pp. 211–230, 1998.
- [110] S. Keränen and M. Penttilä, "Production of recombinant proteins in the filamentous fungus *Trichoderma reesei*," *Curr. Opin. Biotechnol.*, vol. 6, no. 5, pp. 534–537, 1995.
- [111] I. Pócsi *et al.*, "Comparison of gene expression signatures of diamide, H₂O₂ and menadione exposed *Aspergillus nidulans* cultures—linking genome-wide transcriptional changes to cellular physiology," *BMC Genomics*, vol. 6, no. 1, p. 182, 2005.
- [112] I. S. Druzhinina, M. Schmoll, B. Seiboth, and C. P. Kubicek, "Global Carbon Utilization Profiles of Wild-Type, Mutant, and Transformant Strains of *Hypocrea jecorina*," vol. 72, no. 3, pp. 2126–2133, 2006.
- [113] M. A. Friedl, M. Schmoll, C. P. Kubicek, and I. S. Druzhinina, "Photostimulation of *Hypocrea atroviridis* growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidative stress," pp. 1229–1241, 2008.
- [114] M. A. Friedl, C. P. Kubicek, and I. S. Druzhinina, "Carbon Source Dependence and Photostimulation of Conidiation in," vol. 74, no. 1, pp. 245–250, 2008.
- [115] V. Betina and V. Farkas, "Sporulation and light-induced development in *Trichoderma*," *Trichoderma Gliocladium Basic Biol. Taxon. Genet.*, vol. 2, pp. 131–151, 1998.
- [116] M. Schmoll, L. Franchi, and C. P. Kubicek, "Envoy, a PAS/LOV domain protein of *Hypocrea jecorina* (Anamorph *Trichoderma reesei*), modulates cellulase gene transcription in response to light," *Eukaryot. Cell*, vol. 4, no. 12, pp. 1998–2007, 2005.
- [117] S. Casas-Flores, M. Rios-Momberg, M. Bibbins, P. Ponce-Noyola, and A. Herrera-Estrella, "BLR-1 and BLR-2, key regulatory elements of photoconidiation and mycelial growth in *Trichoderma atroviride*," *Microbiology*, vol. 150, no. 11, pp. 3561–3569, 2004.
- [118] H. Nielsen, "Predicting secretory proteins with SignalP," *Protein Funct. Predict. Methods Protoc.*, pp. 59–73, 2017.
- [119] D. S. Cornett, M. L. Reyzer, P. Chaurand, and R. M. Caprioli, "MALDI imaging mass spectrometry: molecular snapshots of biochemical systems," *Nat. Methods*, vol. 4, no. 10, p. 828, 2007.
- [120] M. M. Smits *et al.*, "The fungal–mineral interface: challenges and considerations of micro-analytical developments," *Fungal Biol. Rev.*, vol. 23, no. 4, pp. 122–131, 2009.
- [121] N. Musat, F. Musat, P. K. Weber, and J. Pett-Ridge, "Tracking microbial interactions with NanoSIMS," *Curr. Opin. Biotechnol.*, vol. 41, pp. 114–121, 2016.
- [122] K. Sprouffske, "growthcurver: Simple Metrics to Summarize Growth Curves." 2016.
- [123] I. V Grigoriev *et al.*, "The genome portal of the department of energy joint genome institute," *Nucleic Acids Res.*, vol. 40, no. D1, pp. D26–D32, 2011.
- [124] F. Gruber, J. Visser, C. P. Kubicek, and L. H. de Graaff, "The development of a heterologous transformation system for the cellulolytic fungus *Trichoderma reesei* based on a pyrG-negative mutant strain," *Curr. Genet.*, vol. 18, no. 1, pp. 71–76, 1990.

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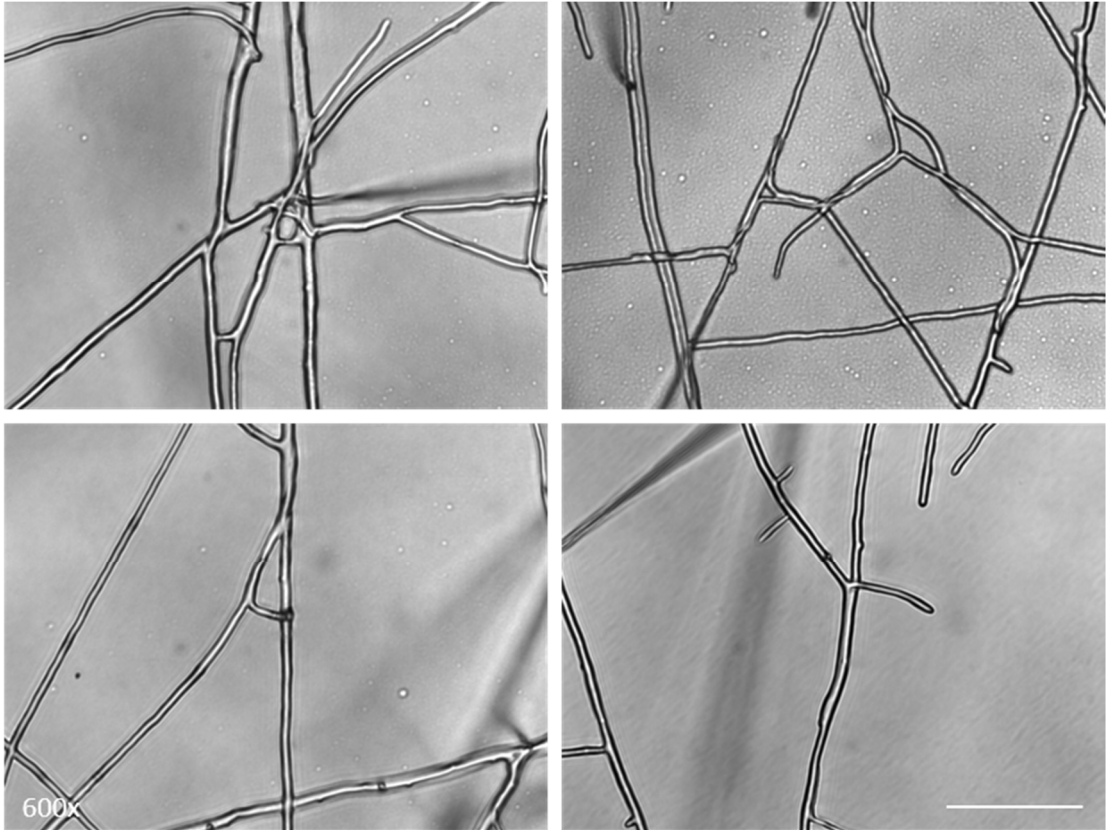
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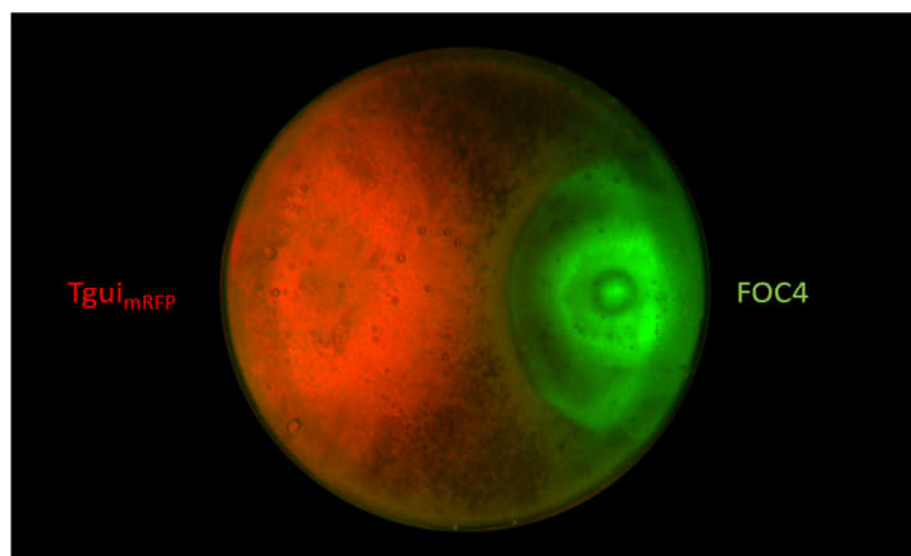
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9 Supplement

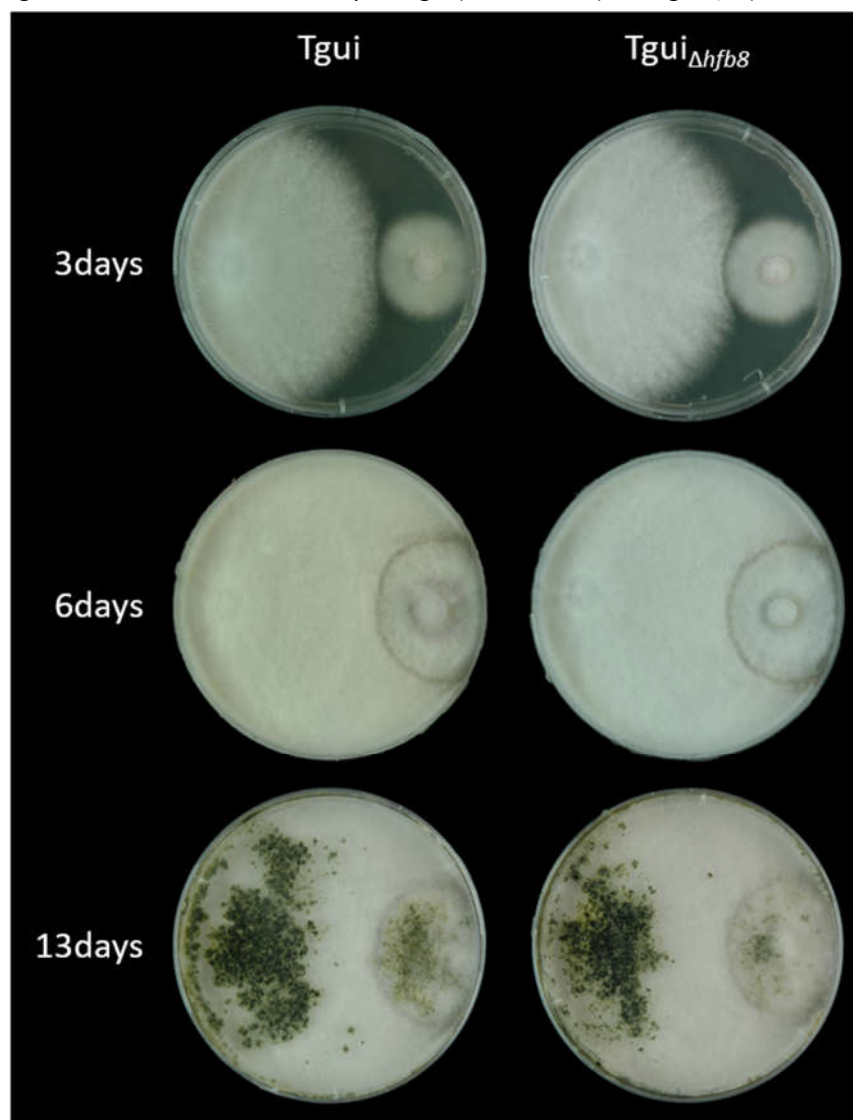
Supplementary Figure 1: *Trichoderma guizhouense* NJAU 4742 self-interactions on microscopy glass slides with 600x magnification



Supplementary Figure 2: Fluorescence signal of dual confrontation assay of Tgui_{OEhfb8} and FOC4. Strong autofluorescence of FOC (shown in green) overlain with red fluorescent signal of Tgui_{mRFP} (shown in red). The image was recorded with Chemidoc (Biorad, USA).



Supplementary Figure 3: Dual confrontation assays of Tgui (TUCIM 4742) and Tgui Δ_{hfb8} (TUCIM 7441) against FOC4



Supplementary Table 1: Statistical analysis of environmental influence factors on HFB8_{mRFP} expression as measured by red fluorescence signal

```
> res.aov2 <- aov(fluo ~ strain + cond, data = data)
> summary(res.aov2)
```

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
strain	1	154083	154083	3.809	0.0568 .
cond	6	23203395	3867232	95.610	<2e-16 ***
Residuals	48	1941500	40448		

```
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> library(multcomp)
> summary(glht(res.aov2, linfct = mcp(cond = "Tukey")))
```

Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts

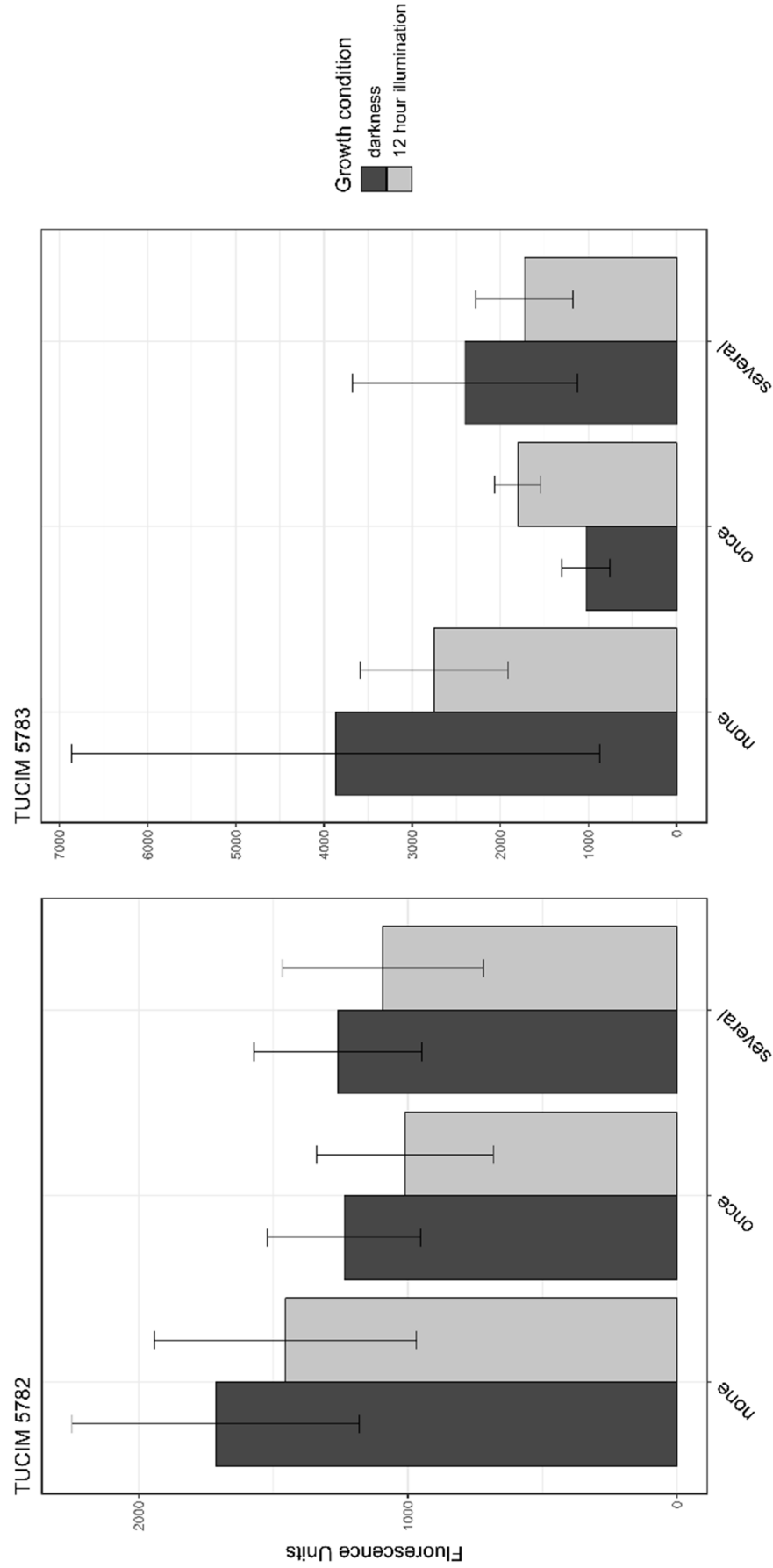
Fit: aov(formula = fluo ~ strain + cond, data = data)

Linear Hypotheses:

	Estimate	Std. Error	t value	Pr(> t)
light - control == 0	1333.58	100.56	13.262	< 0.001 ***
ox_light - control == 0	953.48	100.56	9.482	< 0.001 ***
4 - control == 0	34.59	100.56	0.344	0.99985
complex - control == 0	-443.22	100.56	-4.408	< 0.001 ***
enhanced - control == 0	182.45	100.56	1.814	0.54549
starvation - control == 0	-553.61	100.56	-5.505	< 0.001 ***
ox_light - light == 0	-380.10	100.56	-3.780	0.00754 **
4 - light == 0	-1298.99	100.56	-12.918	< 0.001 ***
complex - light == 0	-1776.80	100.56	-17.669	< 0.001 ***
enhanced - light == 0	-1151.13	100.56	-11.447	< 0.001 ***
starvation - light == 0	-1887.19	100.56	-18.767	< 0.001 ***
4 - ox_light == 0	-918.89	100.56	-9.138	< 0.001 ***
complex - ox_light == 0	-1396.70	100.56	-13.889	< 0.001 ***
enhanced - ox_light == 0	-771.03	100.56	-7.667	< 0.001 ***
starvation - ox_light == 0	-1507.09	100.56	-14.987	< 0.001 ***
complex - 4 == 0	-477.81	100.56	-4.752	< 0.001 ***
enhanced - 4 == 0	147.86	100.56	1.470	0.76047
starvation - 4 == 0	-588.20	100.56	-5.849	< 0.001 ***
enhanced - complex == 0	625.67	100.56	6.222	< 0.001 ***
starvation - complex == 0	-110.39	100.56	-1.098	0.92570
starvation - enhanced == 0	-736.06	100.56	-7.320	< 0.001 ***

```
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
(Adjusted p values reported -- single-step method)
```

Supplementary Figure 4: Influence of aeration on HFB8_{mRFP} expression of TUCIM 7582 and TUCIM 7583 cultivated on PDA

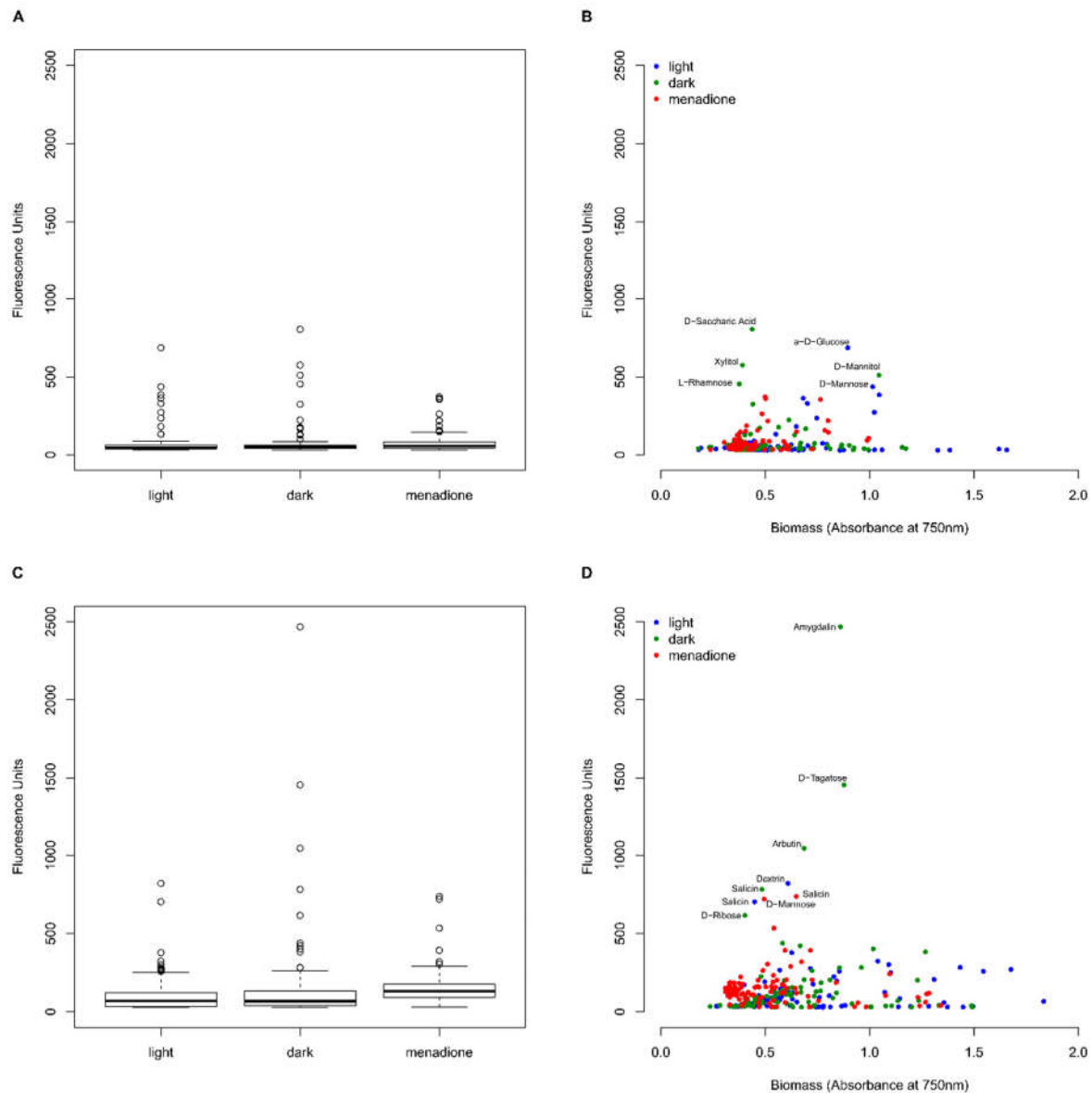


Supplementary Figure 5: Overview of 95 different carbon sources contained on BIOLOG FF Microplates (Biolog Inc.). Image obtained from <https://biolog.com/>.

FF MicroPlate™

A1 Water	A2 Tween 80	A3 N-Acetyl-D-Galactosamine	A4 N-Acetyl-D-Glucosamine	A5 N-Acetyl-D-Mannosamine	A6 Adonitol	A7 Amygdalin	A8 D-Arabinose	A9 L-Arabinose	A10 D-Arabitol	A11 Arbutin	A12 D-Cellobiose
B1 α -Cyclodextrin	B2 β -Cyclodextrin	B3 Dextrin	B4 D-Erythritol	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 D-Glucosamine	B12 α -D-Glucose
C1 Glucose-1-Phosphate	C2 Glucuronamide	C3 D-Glucuronic Acid	C4 Glycerol	C5 Glycogen	C6 m-Inositol	C7 2-Keto-D-Gluconic Acid	C8 α -D-Lactose	C9 Lactulose	C10 Malititol	C11 Maltose	C12 Maltotriose
D1 D-Mannitol	D2 D-Mannose	D3 D-Melezitose	D4 D-Melibiose	D5 α -Methyl-D-Galactoside	D6 β -Methyl-D-Galactoside	D7 α -Methyl-D-Glucoside	D8 β -Methyl-D-Glucoside	D9 Palatinose	D10 D- Psicose	D11 D-Raffinose	D12 L-Rhamnose
E1 D-Ribose	E2 Salicin	E3 Sedoheptulosan	E4 D-Sorbitol	E5 L-Sorbose	E6 Stachyose	E7 Sucrose	E8 D-Tagatose	E9 D-Trehalose	E10 Turannose	E11 Xylitol	E12 D-Xylose
F1 γ -Amino-butyric Acid	F2 Bromosuccinic Acid	F3 Fumaric Acid	F4 β -Hydroxy-butyric Acid	F5 γ -Hydroxy-butyric Acid	F6 p -Hydroxyphenyl-acetic Acid	F7 α -Keto-glutaric Acid	F8 D-Lactic Acid Methyl Ester	F9 L-Lactic Acid	F10 D-Malic Acid	F11 L-Malic Acid	F12 Quinic Acid
G1 D-Saccharic Acid	G2 Sebacic Acid	G3 Succinamic Acid	G4 Succinic Acid	G5 Succinic Acid Mono-Methyl Ester	G6 N-Acetyl-L-Glutamic Acid	G7 Alaninamide	G8 L-Alanine	G9 L-Alanyl-Glycine	G10 L-Asparagine	G11 L-Aspartic Acid	G12 L-Glutamic Acid
H1 Glycyl-L-Glutamic Acid	H2 L-Ornithine	H3 L-Phenylalanine	H4 L-Proline	H5 L-Pyrroglutamic Acid	H6 L-Serine	H7 L-Threonine	H8 2-Amino Ethanol	H9 Putrescine	H10 Adenosine	H11 Uridine	H12 Adenosine-5'-Monophosphate

Supplementary Figure 6: Influence of menadione and illumination on HFB8_{mRFP} expression of TguiO_{Ehfb8} cultivated on 95 different carbon sources (original scale)



9.1 Supplementary Material- Creating growth plots for BIOLOG data in R

Using the BIOLOG Microplate reader (Biolog Inc., USA), the growth rates of fungal cultures growing in each of the 96 different wells can be easily measured. Creating growth curves for different strains or conditions is usually not a big challenge and can be done with any graphical or statistical program (such as Excel), but creating 96 plots for easy side-by-side comparison can be difficult and result in large files. The following describes a pipeline for creating a PDF file of 96 growth plots RStudio [98]. Different conditions or strains investigated can be easily plotted together for direct comparison. The output file contains many details and is thus not meant for publication, as small font sizes would render the content unreadable. The 96-well PDF file is rather meant for investigation for subsequent plotting of individual wells. For publishable files containing 96 growth plots with little details the R package

“growthcurver” [122] can be used. The scripts necessary for creating the growth plots are described in Supplementary Table 2 (96-well plot) and Supplementary Table 3 (single well plot). The scripts do not include the plotting of error bars obtained from standard deviations of repeats. An example one-well plot is given in Supplementary Figure 7.

9.1.1 Input data

9.1.1.1 Growth data

The data obtained from BIOLOG measurements are saved in a long format, containing project names, dates of measurement, sample names and many other parameters, which can be (de-)selected in the BIOLOG program. For creating growth curves in R, only sample names, timepoints of measurement (in hours or days) and the obtained values for each well at the measured wavelength are used, no further columns should be present in the data table. An example is given below.

sample	time	A1	A2	A3	A4	A5	A6	...	H12
strain1_cond1	1	0.335	0.216	0.278	0.355	0.276	0.356	...	0.216
strain1_cond1	2	0.382	0.337	0.326	0.493	0.323	0.409	...	0.337
strain1_cond1	...	0.391	0.461	0.330	0.635	0.332	0.472	...	0.461
strain1_cond2	1	0.395	0.481	0.332	0.940	0.343	0.510	...	0.481
strain1_cond2	2	0.395	0.481	0.333	1.111	0.343	0.508	...	0.481
strain1_cond2	...	0.398	0.459	0.333	1.078	0.344	0.531	...	0.459
...									

9.1.1.2 Metadata (Substrates)

The substrates of each well are read into R in a metadata file. This file is in long format and contains the headers of each well (A1-H12) and the corresponding substrates in each well, respectively. An example is given below.

A1	A2	A3	A4	A5	...	H12
Water	Tween 80	N-Acetyl- DGalactosamine	N-Acetyl- DGlucosamine	N-Acetyl- DMannosamine	...	Adenosine-5'- Monophosphate

Supplementary Table 2: R script for creating a PDF document containing growth curves for each well in BIOLOG FF microplates (Biolog Inc., USA)

```
#### set path to your file
setwd("../growth_table.txt") # put in path to your file

#### read in data table and meta data (substrate names)
growth_data<-read.table("growth_table.txt", header=T, sep="\t",dec=",")
substrates<-read.table("substrates_meta1.txt", header=T, sep="\t", quote = "",
                      check.names=FALSE, encoding = "UTF-8")

##### identify and count unique sample names
sample_names <- unique(growth_data$sample)
no_samples<-length(sample_names)

#### identify and count unique timepts of measurement
timepts<- unique(growth_data$time)
no_timepts<-length(timepts)

#### set color vector for unique samples
colors<-c(1:50) # can also be replaced by individual colors as c("col1","col2",...)

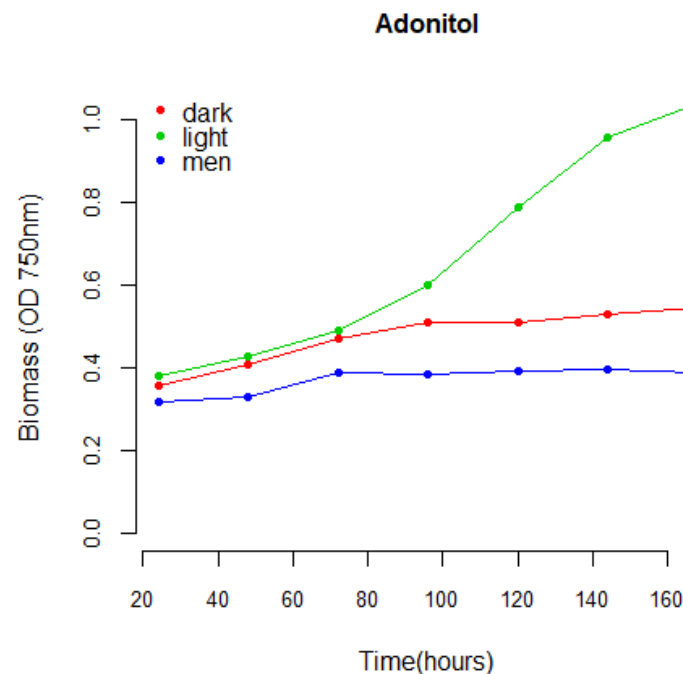
##### function that creates a pdf file of all 96 growth curves
max_biomass <- max(growth_data[3:98])# calculate max values to set margins of plots
pdf("growthcurves.pdf", height= 16.54, width = 23.39)#A2 format
par(mfrow=c(8,12),oma = c(2, 2, 0, 0)+ 1, mar = c(1, 1, 0, 0)+ 1,
    mgp = c(3, 0.5, 0), xpd = NA)#8 column, 12 rows
plotloop<- function(columnidx,growthdata,header,max_biom,
                    samples,no_samples,no_timepts,colors){
  plotvector <-growthdata[1:no_timepts, columnidx]
  plot(timepts,plotvector, ylim=c(0,max_biom), type="o", col=colors[1],
       main=header,pch = 20,ylab=NA, xlab=NA,
       bty='n',cex= 0.5, cex.axis= 0.5, cex.main= 1)
  for (i_plot in 2:no_strains){
    plotvector <- growthdata[((i_plot-1)*no_timepts+1):(i_plot*no_timepts),
                           columnidx]
    points(timepts,plotvector, col=colors[i_plot], type="o",pch = 20,
          bty='n', cex= 0.5)
  }
  legend("topleft", col=colors, legend = strain_names,pch = 20, bty='n', cex= 0.5)
}

#### run function through all 96 wells
for (index in 3:98){
  header <- substrates1[1,index-2]
  plotloop(index,growth_data,header,max_biomass,sample_names,no_samples,
          no_timepts,colors)
}
dev.off()
```

Supplementary Table 3: R script for creating a PDF document containing growth curves for one specific well in BIOLOG FF microplates (Biolog Inc., USA)

```
##### choose well you want to plot
well_no <- 6 # replace 6 with any well number you want to plot
input <- well_no +2
max_biomass <- max(growth_data[input])# calculate maximum values to set margins of plots
plotloop1<- function(columnidx,growthdata,header,max_biom,samples,no_strain,no_timepoints,colors){
  plotvector <-growthdata[1:no_timepoints, columnidx]
  plot(timepoints,plotvector, ylim=c(0,max_biom), type="o", col=colors[1], main=header,pch = 20,ylab="Biomass (OD
750nm)", xlab="Time(hours)", bty='n',cex= 1, cex.axis= 0.8, cex.main= 1)
  for (i_plot in 2:no_samples){
    plotvector <- growthdata[(((i_plot-1)*no_timepoints+1):(i_plot*no_timepoints), columnidx]
    points(timepoints,plotvector, col=colors[i_plot], type="o",pch = 20, bty='n', cex= 1)
  }
  legend("topleft", col=colors, legend = strain_names,pch = 20, bty='n', cex= 1)
}
for (index in input){
  header <- substrates1[1,index-2]
  plotloop1(index,growth_table1,header,max_biom,strain_names,no_samples,no_timepoints,colors)
}
#### make a pdf of the plot
pdf("growthcurve_ado.pdf")# give your output file a name, will overwrite existing files without asking
for (index in input){
  header <- substrates1[1,index-2]
  plotloop1(index,growth_table1,header,max_biom,strain_names,no_samples,no_timepoints,colors)
}
dev.off()
```

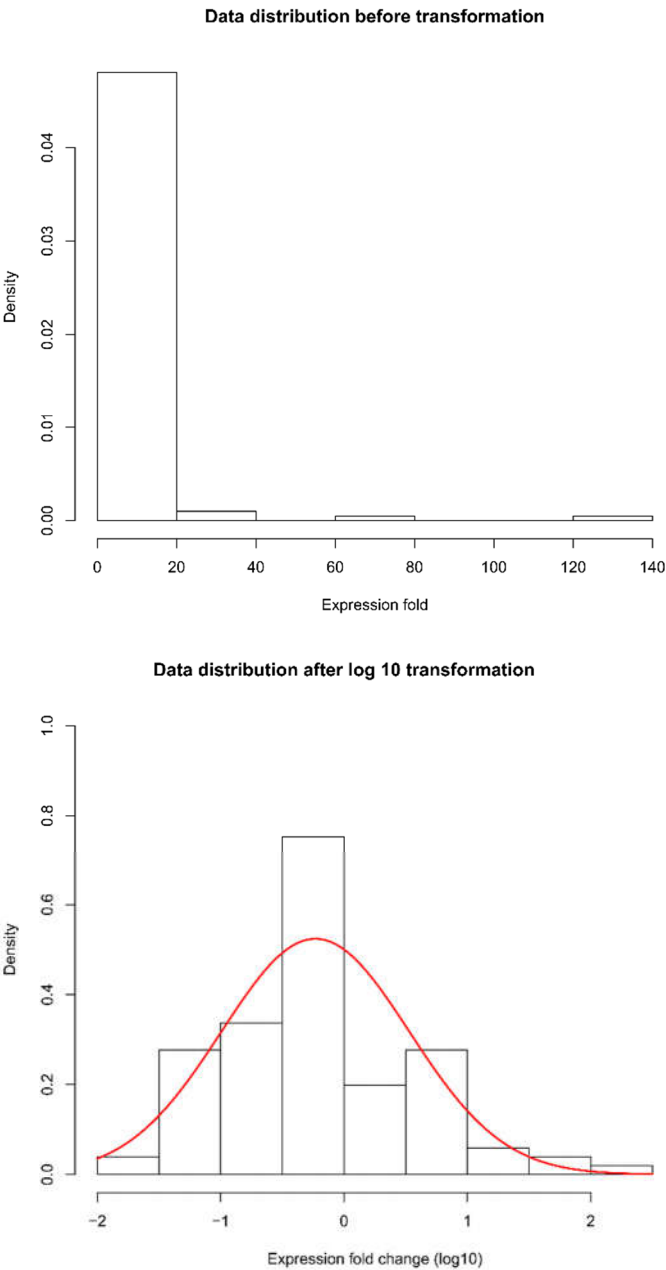
Supplementary Figure 7: Growth plot of fungal culture grown under three conditions in Adonitol.



Supplementary Figure 8: NCBI BLAST [95] search results for cDNA1 homologous proteins across *Trichoderma*

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☒	predicted protein [Trichoderma reesei QM6a]	179	179	100%	3e-60	100%	XP_006988441.1
☐	hypothetical protein A9Z42_0050230 [Trichoderma parareesei]	178	178	100%	1e-59	99%	QTA04450.1
☐	hypothetical protein M440DRAFT_1440150 [Trichoderma longibrachiatum ATCC 18648]	178	178	100%	2e-59	98%	PTB74729.1
☐	hypothetical protein BBK36DRAFT_1166516 [Trichoderma citrinoviride]	145	145	81%	1e-46	94%	XP_024751933.1
☒	hypothetical protein A0Q28_0023810 [Trichoderma guizhouense]	125	125	98%	1e-38	67%	QPB44063.1
☒	hypothetical protein M431DRAFT_86327 [Trichoderma harzianum CBS 226.95]	125	125	98%	1e-38	67%	XP_024774428.1

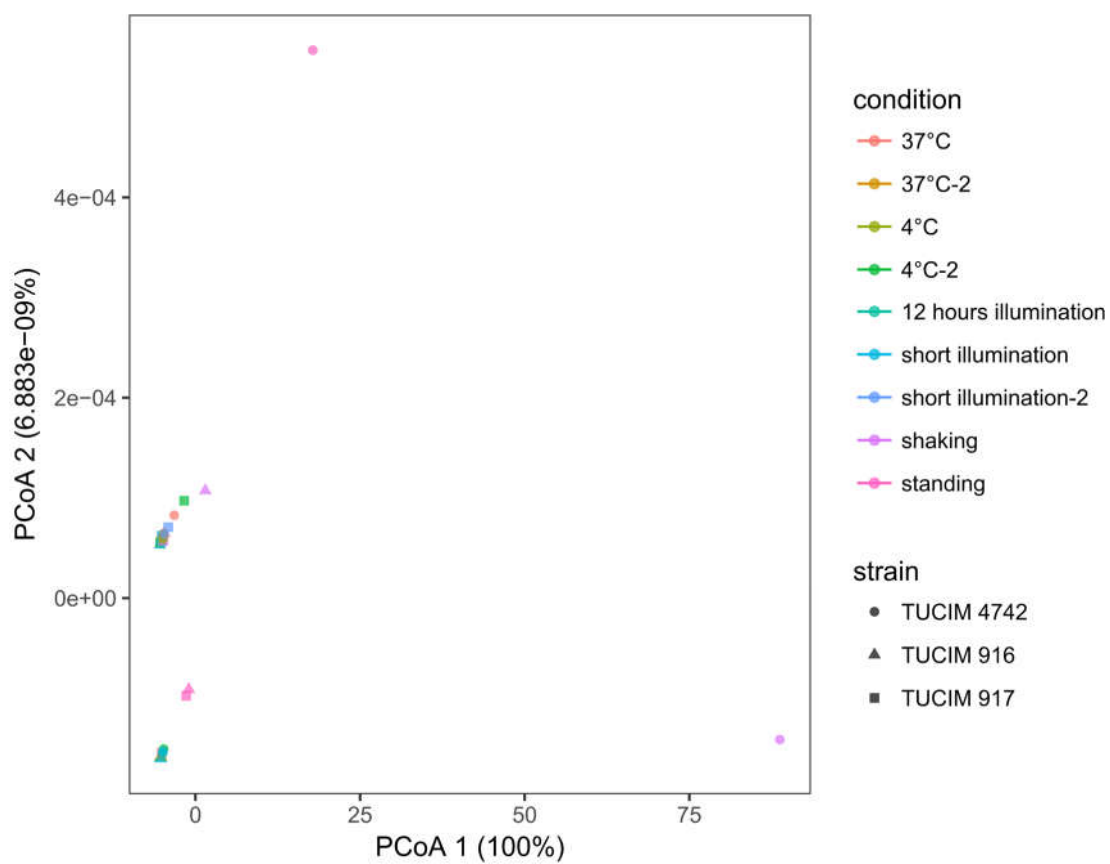
Supplementary Figure 9: Histogram of expression fold data before and after log10 transformation. Normal distribution curve is shown in red.



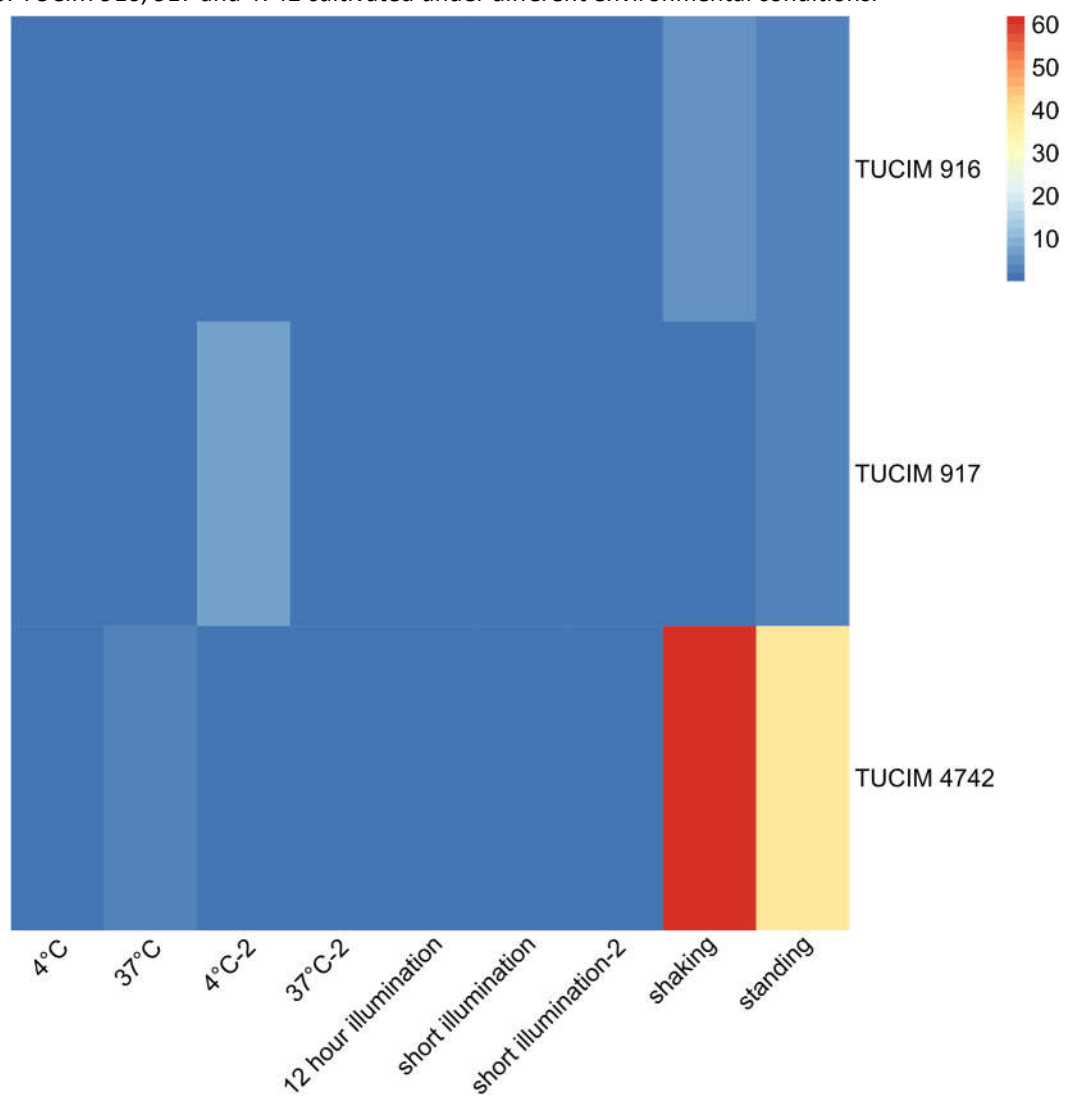
Supplementary Table 4: Shapiro-Wilk-normality test performed on log10 transformed *cdna1* expression fold. The alternative hypothesis that the data is not normally distributed cannot be rejected as the *p*-value < 0.05

```
> data$fold<-log10(data$fold)
> shapiro.test(data$fold)
      Shapiro-Wilk normality test
data:  data$fold
W = 0.96119, p-value = 0.004616
```

Supplementary Figure 10: Principal Coordinates Analysis (PCoA) of log10 transformed *cdna1* expression fold change levels as measured by qPCR of TUCIM 916, 917 and 4742 cultivated under different environmental conditions.



Supplementary Figure 11: Heatmap of log10 transformed *cdna1* expression fold change levels as measured by qPCR of TUCIM 916, 917 and 4742 cultivated under different environmental conditions.



Supplementary Table 5: Two-way ANOVA and Duncan's posthoc test of *cdna1* expression fold change (log10) in TUCIM 4742, TUCIM 916 and TUCIM 917

```
2-way ANOVA all strains
> res.aov2 <- aov(fold ~ strain + condition, data = data)
> summary(res.aov2)
      Df Sum Sq Mean Sq F value  Pr(>F)
strain    2   5.97   2.987  14.15 4.53e-06 ***
condition  8  32.96   4.119  19.52 < 2e-16 ***
Residuals 90  18.99   0.211
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> res.aov3 <- aov(fold ~ strain * condition, data = data)
> res.aov3 <- aov(fold ~ strain + condition + strain:condition, data = data)
> summary(res.aov3)
      Df Sum Sq Mean Sq F value  Pr(>F)
strain    2   5.97   2.987  44.35 2.18e-13 ***
```

```

condition      8 32.96  4.119 61.17 < 2e-16 ***
strain:condition 16 14.01  0.876 13.00 1.54e-15 ***
Residuals      74  4.98  0.067
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Duncan's test all strains
> PostHocTest(res.aov2, method= "duncan")

Posthoc multiple comparisons of means : Duncan's new multiple range test
95% family-wise confidence level

$strain
      diff   lwr.ci   upr.ci    pval
917-916  0.1912278 -0.03179901 0.4142547 0.09194 .
4742-916 0.5817405  0.34880787 0.8146731 1.7e-06 ***
4742-917 0.3905126  0.16748579 0.6135395 0.00078 ***

$condition
      diff   lwr.ci   upr.ci    pval
372-37  -0.34998851 -0.75088448 0.05090746 0.0874 .
4-37   -0.67079517 -1.09850036 -0.24308999 0.0016 **
42-37  -0.19140491 -0.56400292 0.18119311 0.3102
l-37   -0.64144475 -1.06324299 -0.21964651 0.0024 **
s-37   -0.42601401 -0.88940089 0.03737287 0.0724 .
s2-37  -0.37051775 -0.77552628 0.03449077 0.0736 .
sh-37  0.81628809  0.42550399 1.20707218 7.5e-05 ***
st-37  0.99390070  0.60181671 1.38598469 1.2e-06 ***
4-372  -0.32080666 -0.74458749 0.10297416 0.1411
42-372 0.15858360 -0.22238845 0.53955565 0.4104
l-372  -0.29145624 -0.70556722 0.12265474 0.1709
s-372  -0.07602550 -0.52228780 0.37023680 0.7402
s2-372 -0.02052924 -0.40150130 0.36044281 0.9150
sh-372 1.16627659  0.73281253 1.59974066 1.8e-07 ***
st-372 1.34388921  0.92010838 1.76767004 1.2e-09 ***
42-4   0.47939027  0.05759202 0.90118851 0.0251 *
l-4    0.02935042 -0.34324759 0.40194844 0.8760
s-4    0.24478116 -0.19358207 0.68314439 0.2759
s2-4   0.30027742 -0.10473111 0.70528595 0.1488
sh-4   1.48708326  1.03337350 1.94079302 1.9e-10 ***
st-4   1.66469588  1.22796886 2.10142289 5.5e-11 ***
l-42   -0.45003984 -0.86450567 -0.03557402 0.0328 *
s-42   -0.23460910 -0.68742240 0.21820420 0.3152
s2-42  -0.17911285 -0.57119684 0.21297114 0.3732
sh-42  1.00769299  0.59647183 1.41891415 2.5e-06 ***
st-42  1.18530561  0.78029708 1.59031414 2.0e-08 ***
s-l    0.21543074 -0.20114651 0.63200799 0.3070
s2-l   0.27092700 -0.12115700 0.66301099 0.1770

```

```

sh-l 1.45773284 1.00915185 1.90631382 3.0e-10 ***
st-l 1.63534545 1.20275017 2.06794073 6.3e-11 ***
s2-s 0.05549626 -0.36108099 0.47207350 0.7919
sh-s 1.24230210 0.75221683 1.73238737 4.4e-07 ***
st-s 1.41991471 0.94172578 1.89810365 4.5e-09 ***
sh-s2 1.18680584 0.75211041 1.62150127 8.7e-08 ***
st-s2 1.36441846 0.94262021 1.78621670 4.6e-10 ***
st-sh 0.17761262 -0.21317148 0.56839671 0.3690

---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

Supplementary Table 6: One-way ANOVA and Duncan's posthoc test of *cdna1* expression fold change (log10) in TUCIM 4742

```

2-way ANOVA 4742
> res.aov1 <- aov(fold ~ condition, data = data)
> summary(res.aov1)

      Df Sum Sq Mean Sq F value    Pr(>F)
condition  8 16.924  2.1156   38.56 3.52e-12 ***
Residuals 25  1.372  0.0549

---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> PostHocTest(res.aov1, method="duncan")

Posthoc multiple comparisons of means : Duncan's new multiple range test
95% family-wise confidence level

$condition
      diff   lwr.ci   upr.ci    pval
372-37 -0.46358619 -0.8047095 -0.1224629 0.0097 **
4-37   -0.57045184 -0.9476735 -0.1932302 0.0040 **
42-37  -0.49594692 -0.8653360 -0.1265578 0.0098 **
l-37   -0.62237952 -1.0100068 -0.2347522 0.0023 **
s-37   -0.59302909 -0.9761112 -0.2099470 0.0032 **
s2-37  -0.48465829 -0.8429821 -0.1263345 0.0096 **
sh-37   1.70533493  1.2664797  2.1441902 1.4e-08 ***
st-37   1.09951206  0.7583888  1.4406354 5.9e-07 ***
4-372  -0.10686565 -0.4762547  0.2625234 0.5624
42-372 -0.03236072 -0.3906845  0.3259631 0.8558
l-372  -0.15879332 -0.5418754  0.2242888 0.4069
s-372  -0.12944290 -0.5066646  0.2477788 0.4924
s2-372 -0.02107210 -0.3621954  0.3200512 0.8998
sh-372 2.16892112  1.7165137  2.6213285 1.6e-10 ***
st-372 1.56309825  1.2047744  1.9214221 1.5e-09 ***
42-4    0.07450492 -0.2666184  0.4156282 0.6567
l-4     -0.05192767 -0.4102515  0.3063961 0.7706
s-4     -0.02257725 -0.3637005  0.3185460 0.8927

```

```

s2-4  0.08579355 -0.2725303  0.4441174  0.6303
sh-4   2.27578677  1.8010423  2.7505313  1.0e-10 ***
st-4   1.66996390  1.2868818  2.0530460  7.8e-10 ***
l-42   -0.12643260 -0.4958217  0.2429565  0.4935
s-42   -0.09708217 -0.4554060  0.2612416  0.5862
s2-42  0.01128862 -0.3298347  0.3524119  0.9462
sh-42  2.20128184  1.7321040  2.6704597  1.7e-10 ***
st-42  1.59545898  1.2182373  1.9726806  1.6e-09 ***
s-l    0.02935042 -0.3117729  0.3704737  0.8608
s2-l   0.13772122 -0.2395004  0.5149429  0.4653
sh-l   2.32771444  1.8449640  2.8104649  7.8e-11 ***
st-l   1.72189158  1.3306510  2.1131322  5.4e-10 ***
s2-s   0.10837080 -0.2610183  0.4777599  0.5570
sh-s   2.29836402  1.8191941  2.7775339  9.2e-11 ***
st-s   1.69254115  1.3049139  2.0801684  6.8e-10 ***
sh-s2  2.18999322  1.7279929  2.6519935  1.6e-10 ***
st-s2  1.58417035  1.2147813  1.9535594  1.5e-09 ***
st-sh  -0.60582287 -1.0236119 -0.1880339  0.0062 **

---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

Supplementary Table 7: One-way ANOVA and Duncan's posthoc test of *cdna1* expression fold change (log10) in TUCIM 917

```

2-way ANOVA 917
> data<-subset(data, data$strain=="917") ####
> res.aov1 <- aov(fold ~ condition, data = data)
> summary(res.aov1)

      Df Sum Sq Mean Sq F value    Pr(>F)
condition  8 11.065  1.3832  24.93 7.83e-10 ***
Residuals 24  1.331  0.0555

---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> PostHocTest(res.aov1, method= "duncan")

Posthoc multiple comparisons of means : Duncan's new multiple range test
95% family-wise confidence level

$condition
      diff   lwr.ci   upr.ci    pval
372-37  0.022828108 -0.3484326  0.39408883 0.90007
4-37   -0.603565141 -0.9645752 -0.24255512 0.00187 **
42-37   0.860945788  0.4751418  1.24674976 7.0e-05 ***
l-37   -0.599049691 -0.9427703 -0.25532905 0.00145 **
s-37    0.030855574 -0.4112896  0.47300075 0.88806
s2-37   0.602812566  0.2228614  0.98276377 0.00273 **
sh-37   0.238566272 -0.1335443  0.61067686 0.20222

```

```

st-37  1.096501759  0.7061701  1.48683337  2.6e-06 ***
4-372  -0.626393249 -1.0283186 -0.22446788  0.00325 **
42-372  0.838117680  0.4277235  1.24851189  0.00021 ***
l-372  -0.621877799 -1.0118132 -0.23194241  0.00280 **
s-372   0.008027466 -0.4357140  0.45176890  0.97053
s2-372  0.579984458  0.1780591  0.98190983  0.00595 **
sh-372  0.215738164 -0.1741972  0.60567356  0.26858
st-372  1.073673651  0.6569577  1.49038957  9.9e-06 ***
42-4    1.464510928  1.0705899  1.85843191  2.1e-08 ***
l-4     0.004515449 -0.3392052  0.34823609  0.97859
s-4     0.634420715  0.1690774  1.09976401  0.00885 **
s2-4    1.206377707  0.8160461  1.59670932  5.5e-07 ***
sh-4    0.842131412  0.4563274  1.22793539  9.3e-05 ***
st-4    1.700066900  1.3032506  2.09688321  1.4e-09 ***
l-42    -1.459995479 -1.8503271 -1.06966387  1.9e-08 ***
s-42    -0.830090213 -1.2858307 -0.37434968  0.00078 ***
s2-42   -0.258133221 -0.6018539  0.08558742  0.13423
sh-42   -0.622379516 -0.9833895 -0.26136949  0.00142 **
st-42   0.235555972 -0.1081647  0.57927661  0.17008
s-l     0.629905266  0.1741647  1.08564580  0.00814 **
s2-l    1.201862258  0.8160583  1.58766624  5.1e-07 ***
sh-l    0.837615963  0.4576648  1.21756717  8.5e-05 ***
st-l    1.695551451  1.3016305  2.08947243  1.3e-09 ***
s2-s    0.571956992  0.1298118  1.01410217  0.01292 *
sh-s    0.207710697 -0.2132594  0.62868079  0.31867
st-s    1.065646185  0.6003029  1.53098948  5.3e-05 ***
sh-s2   -0.364246295 -0.7079669 -0.02052566  0.03871 *
st-s2   0.493689193  0.1326792  0.85469922  0.00898 **
st-sh   0.857935488  0.4858249  1.23004608  5.2e-05 ***

---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

Supplementary Table 8: One-way ANOVA and Duncan's posthoc test of *cdna1* expression fold change (log10) in TUCIM 916

```

2-way ANOVA 916
> data<-subset(data, data$strain=="916") #####
> res.aov1 <- aov(fold ~ condition, data = data)
> summary(res.aov1)

      Df Sum Sq Mean Sq F value    Pr(>F)
condition  8  18.98  2.3721   26.01 2.81e-10 ***
Residuals 25   2.28  0.0912

---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> PostHocTest(res.aov1, method="duncan")

Posthoc multiple comparisons of means : Duncan's new multiple range test
95% family-wise confidence level

```

```

$condition
      diff  lwr.ci  upr.ci  pval
372-37 -0.58926622 -1.0290917 -0.14944069 0.01068 *
4-37   -0.83836854 -1.3247373 -0.35199977 0.00124 **
42-37  -0.93921359 -1.4331385 -0.44528866 0.00044 ***
l-37   -0.70290504 -1.1791749 -0.22663515 0.00493 **
s-37   -0.60356514 -1.1694008 -0.03772943 0.03727 *
s2-37  -1.22970753 -1.7294927 -0.72992234 1.6e-05 ***
sh-37  0.97684234 0.5148394 1.43884526 0.00016 ***
st-37  0.78568829 0.3458628 1.22551382 0.00112 **
4-372  -0.24910232 -0.7253722 0.22716757 0.29723
42-372 -0.34994737 -0.8363161 0.13642139 0.15408
l-372  -0.11363882 -0.5756417 0.34836410 0.62102
s-372  -0.01429893 -0.5529730 0.52437514 0.95684
s2-372 -0.64044132 -1.1343662 -0.14651639 0.01225 *
sh-372 1.56610855 1.0898387 2.04237844 2.1e-07 ***
st-372 1.37495450 0.9129516 1.83695742 1.4e-06 ***
42-4   -0.10084505 -0.5406706 0.33898048 0.64087
l-4     0.13546350 -0.3043620 0.57528903 0.53163
s-4     0.23480340 -0.3310323 0.80063910 0.40561
s2-4   -0.39133900 -0.8533419 0.07066392 0.09433 .
sh-4    1.81521087 1.3154257 2.31499607 2.5e-08 ***
st-4    1.62405683 1.1301319 2.11798175 1.6e-07 ***
l-42    0.23630855 -0.2256944 0.69831147 0.30648
s-42    0.33564845 -0.2476607 0.91895755 0.25209
s2-42   -0.29049395 -0.7303195 0.14933158 0.18588
sh-42   1.91605592 1.4116119 2.42049991 1.0e-08 ***
st-42   1.72490188 1.2251167 2.22468707 6.4e-08 ***
s-l     0.09933990 -0.4393342 0.63801396 0.70729
s2-l    -0.52680249 -1.0030724 -0.05053260 0.03104 *
sh-l    1.67974738 1.1858224 2.17367230 9.0e-08 ***
st-l    1.48859333 1.0022246 1.97496209 6.2e-07 ***
s2-s    -0.62614239 -1.2218200 -0.03046474 0.03983 *
sh-s    1.58040748 0.9847298 2.17608513 6.0e-06 ***
st-s    1.38925343 0.8059443 1.97256254 3.1e-05 ***
sh-s2   2.20654987 1.6983364 2.71476331 6.9e-10 ***
st-s2   2.01539582 1.5109518 2.51983981 3.8e-09 ***
st-sh   -0.19115405 -0.6309796 0.24867148 0.37927

---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

9.2 Supplementary Material- *cdna1* deletion mutant construction

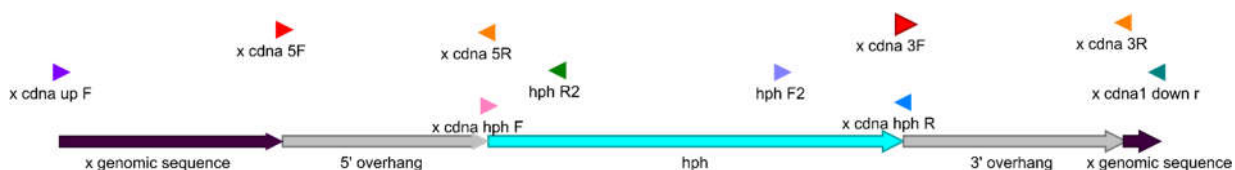
To gain insights into the function of the predicted protein cDNA1 from *Trichoderma reesei* QM6a (TUCIM 917) and its homologs in *Trichoderma harzianum* CBS 266.95 (TUCIM 916) and *Trichoderma*

guizhouense (TUCIM 4742), the construction of *cdna1* deletion mutants was attempted for phenotypic analysis. For *cdna1* deletion, replacement cassettes were created.

The three fungal genomes and the respective *cdna1* sequences were obtained from the MycoCosm portal of the Joint Genome Institute (JGI) [123]. The x in the following described primers refers to each of the used strains' TUCIM numbers, respectively.

A schematic overview of the deletion cassette and used primers is given in Supplementary Figure 12. A summary of all used primers is given in Supplementary Table 9.

Supplementary Figure 12: Schematic overview of *cdna1* replacement cassette and primer positions. The x refers to the used strains' TUCIM numbers.



Supplementary Table 9: All primers used for creation of *cdna1* replacement cassettes

Name	Orientation	Sequence	Length	Tm (°C)
916cdna 5F	5'→3'	CAGTCGATACACGCTTTCCAG	21	58.4
916cdna 5R	3'→5'	AGATGGTAGAGGCAATGGTATAAAG	25	58.7
916cdna 3F	5'→3'	GAGTTGCGATTGTCGTAAAGGT	22	58.9
916cdna 3R	3'→5'	CTCCGCTGCGTTCATGTATG	20	59.9
916cdna hph F	5'→3'	CTTTATACCATTGCCTCTACCATCTGGATCC GAGAGCTACCTTACATCAATATG	54	81.4
916cdna hph R	3'→5'	ACCTTTACGACAATCGCAACTCCTCGAGGG TACTATGGCTTAGATGGAATA	41	81.3
916cdna upF	5'→3'	ATACCAATATCCATTCTCTCGCG	23	60.4
916cdna downR	3'→5'	CTTGGGGCACAGTATGCTATTTAA	24	60.9
917cdna 5F	5'→3'	GACTTGATATGGCCTGATGGTC	22	58.5
917cdna 5R	3'→5'	AAAGTCTTTGGTTGTAGAGTTGGTG	25	59.2
917cdna 3F	5'→3'	TATATGCTATACTCTGGGGGCGTT	24	61.6
917cdna 3R	3'→5'	GCCAGTAACCAACATAGAGCTGC	23	61.0
917cdna hph F	5'→3'	CACCAACTCTACAACCAAAGACTTTGGATCC GAGAGCTACCTTACATCAATATG	54	81.4
917cdna hph R	3'→5'	AACGCCCCCAGAGTATAGCATATACTCGAG GGTACTATGGCTTAGATGGAATA	53	81.9
917cdna upF	5'→3'	CAGGTATAACTTGACGACGAGAAAT	25	59.3
917cdna downR	3'→5'	TGTTTCATCCCAAGTTGTGGACAC	23	62.0
4742cdna 5F	5'→3'	ACCGCAGATCCCCGCCATA	18	61.7
4742cdna 5R	3'→5'	ATTGAGAAGTGTTGAAGTTGAAGATA	27	58.5
4742cdna 3F	5'→3'	GAGGCAATGGTATAGAGTGGGAC	23	59.6
4742cdna 3R	3'→5'	ATTTAGCGCCAAAGCTCTCTTA	22	58.7
4742cdna hph F	5'→3'	TAATATCTTCAACTTCAAACACTTCTCAATG GATCCGAGAGCTACCTTACATCAATATG	59	79.8

4742cdna hph R	3'→5'	CACTCTATACCATTGCCTCTATCATCTCTCTC GAGGGTACTATGGCTTAGATGGAATA	58	82.4
4742cdna upF	5'→3'	AAGAGGTCAATAGCATCCGCG	21	61.9
4742cdna downR	3'→5'	CTCTGCCGGTTGTATAAAGAGC	22	58.9
hph F2	5'→3'	ATATGCTCCGCATTGGTCTTGA	22	62.1
hph R2	3'→5'	TTGGTCGTTTCTGACACAACG	21	59.3

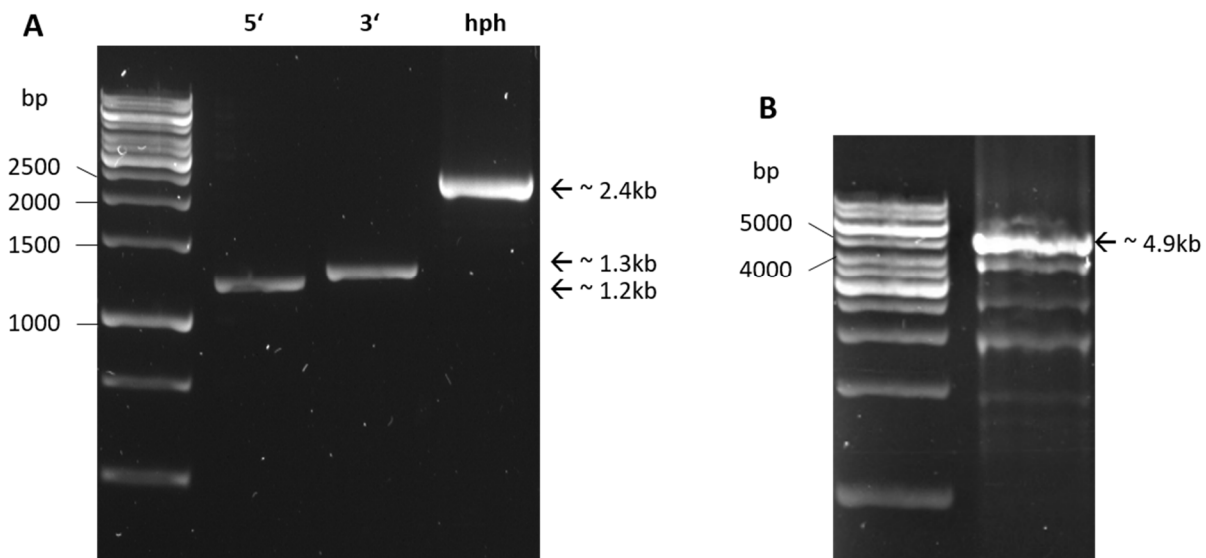
9.2.1 Construction of the deletion cassette

To construct the *cdna1* replacement fragment, a ~1.2 kb 5' upstream flanking fragment and a ~1.3 kb 3' downstream flanking fragment of the *cdna1* gene were amplified from genomic DNA of each of the used wild type strains by PCR using the primer pairs x_cdna_5F/x_cdna_5R and x_cdna_3F/x_cdna_3R. A ~2.4 kb fragment containing the hygromycin B expression cassette (*hph*) was obtained from the pPcdna1 plasmid [88] by PCR using the primer pair hph F2/hph R2. PCR was carried out using Q5® High-Fidelity DNA Polymerase (NEB, USA) with reaction samples containing final concentrations of 5x Q5 Reaction Buffer, 200 µM dNTPs, 0.5 µM forward and 0.5 µM reverse primer and 0.02 U/µl Q5 High-Fidelity DNA Polymerase added to a final volume of 25µl with HPCL grade water. Final concentration of DNA templates was 2-4 ng/ml. Polymerase chain reaction thermal program was set to 30 seconds initial denaturation at 98°C, 30 cycles of 10 seconds denaturation at 98°C, annealing for 20 seconds at 59°C and elongation for 60 seconds at 72°C, with a final elongation step of 2 minutes at 72°C. The obtained fragments were controlled on an agarose gel (Supplementary Figure 13 A).

The obtained fragments were joined by overlapping PCR using the primer pairs x_cdna_hphF/x_cdna_hphR. Overlapping PCR was carried out in two steps using CloneAmp™ HiFi PCR Premix (Takara, Japan) with reaction samples for the first step containing final concentrations of 1x CloneAmp HiFi PCR Premix and ~20 µM of each fragment obtained from the previous PCR added to a final volume of 25µl with HPCL grade water. The thermal cycling program was set to 30 seconds initial denaturation at 98°C, 15 cycles of annealing for 30 seconds at 60°C, elongation for 30 seconds at 72°C and a final elongation step of 2 minutes at 72°C. One µl of the first step PCR product was used in the second step reaction mix, which consisted of final concentrations of 1x CloneAmp HiFi PCR Premix, 0.5 µM forward and 0.5 µM reverse primer added to a final volume of 25µl with HPCL grade water. The thermal PCR program was set to 30 seconds initial denaturation at 98°C, 30 cycles of 10 seconds denaturation at 98°C, annealing for 30 seconds at 60°C and elongation for 2 minutes at 72°C, with a final elongation step of 2 minutes at 72°C.

The amplified fragment (~4.9 kb) was controlled on an agarose gel (Supplementary Figure 13 B) and purified using the mi-PCR Purification Kit (metabion, Germany) for transformation.

Supplementary Figure 13: Construction of *cdna1* deletion cassette fragments obtained by PCR



9.2.2 Creating protoplasts

Supplementary Table 10: Solutions used for creating protoplasts

Solutions for protoplasting	
Solution A	1.2M sorbitol, 0.1M KH ₂ PO ₄
Solution B	1M sorbitol, 50mM CaCl ₂ , 10mM Tris-HCl (pH 7.5)
Lysing solution	0.075% lysing enzymes from <i>Trichoderma harzianum</i> (Sigma, USA) in solution A, filter sterilized (0.2μm)

For transformation, protoplasts of were used. Spore suspensions (in sterile dH₂O) of freshly sporulated cultures (not older than 10 days) were used to inoculate 10 9cm PDA Petri plates covered in sterile cellophane discs (50-200 μl). Plates were incubated at 25°C for 8 hours, until spores started to germinate. After incubation, freshly prepared lysing solution was used to wash germinated spores off the cellophane discs by transferring one disc at a time to a sterile Petri plate containing 2-3ml lysing solution (per disc) and pulling the disc through the lysing solution using a sterile tweezer. The combined protoplasting suspension containing the spore washes from all discs was then incubated at 28°C for two hours under gentle agitation (90rpm). After incubation, the protoplasting suspension was filtered through glass wool into 50ml sterile Falcon tubes, using a few ml of solution A to wash the glass wool, on ice. Filtered solutions were centrifuged at 3200rpm for 10 minutes, at 4°C. The supernatant was discarded, and protoplasts resuspended in 1ml cold solution B, on ice.

9.2.3 Transformation

Transformation was performed according to standard poly-ethylene glycol (PEG)-mediated protoplast transformation for *Trichoderma* [124], using 2 μg of the donor DNA for transformation of 10⁷ protoplasts.