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Unterschrift des Betreuers

TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

Diplomarbeit

Functional characterization of a fungal gene family encoding plant defense response-eliciting proteins in *Trichoderma* biocontrol species

ausgeführt am

Institut für Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften

der Technischen Universität Wien

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Unterschrift

Deutschsprachige Zusammenfassung

Spezies des filamentösen Pilzes *Trichoderma* (Teleomorph: *Hypocrea*) sind in der Natur weit verbreitet und sind in allen Klimazonen von der Tundra bis zu den Tropen zu finden. Aufgrund ihrer vielseitigen Eigenschaften finden *Trichoderma/ Hypocrea* Spezies auch große Bedeutung in industriellen Anwendungen. *Trichoderma atroviride* und *Trichoderma virens* werden in der Landwirtschaft als biologische Pflanzenschutzmittel verwendet und stellen daher eine Alternative zu chemischen Pestiziden dar. Der Einsatz von *Trichoderma spp.* beruht auf der mykoparasitischen Wirkung gegen pflanzenpathogene Pilze und auf einer direkten Interaktion mit den Pflanzen und daraus resultierenden positiven Effekten auf das Pflanzenwachstum.

Cerato-Platanine sind kleine, sezernierte Proteine die vier konservierte Cysteine besitzen. Diese Proteine kommen nur in filamentösen Pilzen vor und werden mit der Induktion von Abwehrreaktionen in Pflanzen und allergischen Reaktionen bei Menschen assoziiert. Die primäre Funktion von Cerato-Plataninen im Wachstum und Entwicklung von Pilzen ist bisher jedoch nicht bekannt. Das Ziel dieser Arbeit war es herauszufinden ob Cerato-Platanine in den Pilzen *T. atroviride* und *T. virens* eine wichtige Funktion während des Wachstums erfüllen und wie die entsprechenden Gene reguliert sind.

Cerato-Platanine in *T.atroviride* heißen EPLs (eliciting plant-response-like proteins) und in *T. virens* SMs (small proteins). Um die biologischen Funktion von Cerato-Plataninen zu erforschen, wurden in dieser Arbeit *epl1, epl2, epl1epl2* und *sm1, sm2* Gen-Knockout Stämme untersucht. Die Morphologie und das Wachstum der Stämme wurden unter verschiedensten Bedingungen analysiert. Es wurden phänotypische Untersuchungen auf folgende Eigenschaften hin durchgeführt: Wachstum auf Agar-Platten mit verschiedenen Wachstumsbedingungen, Bildung der Lufthyphen, Konidienbildung, Biomasseausbildung, Chlamydosporen-Bildung, osmotischer Stress, Zellwand Stress und Mykoparasitismus. In diesen Analysen wurden jedoch weder in *T. atroviride* noch in *T. virens* Unterschiede

zwischen dem Wildtyp/ Mutterstamm und den Gen-Knockout-Stämmen gefunden. Diese Ergebnisse legen daher nahe, dass Cerato-Platanine in *T. atroviride* und *T. virens* keine essentielle Funktion im Wachstum der Pilze haben.

In der Literatur wurde berichtet, dass die Chlamydosporenbildung mit der Genexpression des Cerato-Platanins von *Ceratocystis platani* in Verbindung steht. Aufgrund dessen wurde dies für *T. atroviride* und *T. virens* überprüft und mikroskopische Analysen durchgeführt. Die Resultate lassen darauf schließen, dass in *T. atroviride* und in *T. virens* die Chlamydosporenbildung nicht mit der Genexpression der *epls/ sms* zusammenhängt. Chlamydosporen wurden in Submerskulturen sowohl im Wildtyp als auch in den Gen-Knockout-Stämmen von *T.atroviride* gefunden. Dies wurde auch für *T. virens* beobachtet, bei dieser Spezies war die Chlamydosporenbildung sogar viel stärker ausgeprägt und auch schon zu früheren Probennahme-Zeitpunkten zu erkennen.

Zusätzlich wurden die drei Gene, die für die Cerato-Platanine in *T. atroviride* (*epl1, epl2, epl3*) und *T. virens* (*sm1, sm2, sm3*) kodieren, untersucht. Experimente bezüglich der Genexpression von *epl1, epl2, epl3* in *T. atroviride* und von *sm1, sm2, sm3* in *T. virens* wurden durchgeführt, um festzustellen ob diese Gene co-reguliert sind und/ oder diese Gene in verschiedenen Entwicklungsstadien exprimiert werden. Während *epl1/ sm1* vorwiegend während des Hyphenwachtsums exprimiert sind, waren *epl2/ sm2* nur während der Sporenbildung exprimiert und e*pl3/ sm3* waren unter diesen Bedingungen so gut wie gar nicht exprimiert. Diese Ergebnisse zeigten, dass die drei Gene die für die Cerato-Platanine kodieren weder in *T. atroviride* noch in *T. virens* co-reguliert sind und diese Gene in unterschiedlichen Entwicklungsstadien exprimiert werden.

Summary

Species of the filamentous fungus *Trichoderma* (teleomorph: *Hypocrea*) are commonly found in nature and occur in climates ranging from the tundra to the tropics. Due to the versatile properties of *Trichoderma/ Hypocrea* species they have great importance in industrial applications. *T. atroviride* and *T. virens* are used in agriculture as biological pesticides and are of increasing interest as alternatives to chemical pesticides. The use of *Trichoderma spp*. is based on the mycoparasitic activity against plant pathogenic fungi and beneficial effects on plant growth.

Cerato-platanins are small, secreted proteins with four conserved cysteine residues. They are commonly found in filamentous fungi and are associated with the induction of defense responses in plant and allergic reactions in humans. The primary function of cerato-platanins in fungal growth and development is not known yet. The aim of this thesis was to investigate the potential involvement of cerato-platanins in hyphal growth and development in *T. atroviride* and *T. virens* and to analyze their gene regulation.

In *T.atroviride* cerato-platanins are called EPLs (eliciting plant-response-like proteins) and the orthologues in *T. virens* are called SMs (small proteins). In this study, in order to analyse their biological functions, gene knockout strains of *epl1, epl2* from *T. atroviride* and *sm1, sm2* from *T. virens* were analysed. Several morphological parameters were assessed, including growth on agar plates, formation of aerial hyphae, conidiation, formation of biomass, cell wall and osmotic stress, chlamydospore formation and mycoparasitic potential. The findings that knockout strains of *ep1* and *epl2* and as well as *sm1* and *sm2* showed no detectable phenotype in any of this analyses suggests that these proteins are not essential for fungal growth and development of *T.atroviride* and *T. virens.*

A connection between cerato-platanin gene expression and the formation of chlamydospores in *C. platani* had been reported in literature previously. Based on these findings this was

tested for *T. atroviride* and *T. virens*. The results showed that there is no connection between chlamydospore formation and *epl/ sm* gene expression in *T. atroviride* and *T. virens*. Microscopic analyses showed that chlamydospores were formed in the wild-type and in the gene knockout strains of *T. atroviride.* In *T. virens* chlamydospore formation was even more pronounced than in *T. atroviride* and was also observed in the knockout strains.

Furthermore, transcriptional profiles of the three cerato-platanin encoding genes of *T. atroviride* (*epl1, epl2, epl3*) and of *T. virens* (*sm1, sm2, sm3*) were generated in order to determine whether these genes are co-regulated or rather expressed during different growth stages. The results showed that *epl1/ sm1* are predominantly expressed during hyphal growth, whereas *epl2/ sm2* were only expressed during spore formation. For *epl3/ sm3* hardly any expression was observed. These findings showed that in *T. atroviride* and in *T. virens* the three genes encoding cerato-platanins are not co-regulated, but are expressed during different growth stages.

Inhalt

Deutschsprachige Zusammenfassung…….. I

Summary.. III

1. Introduction 1

1.1 Fungi 1

1.2. The genus *Trichoderma*/ *Hypocrea*.. 2

1.2.1 Classification of *Trichoderma*/ *Hypocrea* 2

1.2.2. Features and applications of *Trichoderma* 3

1.2.3. Biocontrol by *Trichoderma* 4

1.2.4. Mycoparasitism 6

1.3. The species *T. atroviride* and *T. virens*.. 8

1.4. Cerato-platanin proteins 9

1.4.1. Eliciting plant-response-like proteins (EPLs) and small proteins (SMs)..................10

1.5. Aims of this thesis 14

2. Materials and methods...15

2.1. Instruments, chemicals and devices 15

2.1.1. Instruments 15

2.1.2. Chemicals 16

2.1.3. Media..18

2.2. Fungal strains...19

2.3. Growth tests of fungal strains on agar plates 19

2.3.1. Mycoparasitism assays with T. atroviride/ T. virens against R. solani and B. cinerea 19

2.3.2. Determination of the spore concentration 20

2.3.3. Shake flask cultivation of fungi for gene expression and biomass-tests 20

2.3.4. Biomass tests 21

2.4. Analysis of morphological features of fungal strains...21

2.5. RNA Isolation and cDNA synthesis..21

2.6. Reverse transcription polymerase chain reaction (RT-PCR) 23

2.7. Agarose gel electrophoresis 25

3. Results...27

3.1. Overview of cerato-platanin genes in *T. atroviride* and *T. virens* and generation of gene knockout strains 27

3.2. Phenotype analysis...30

3.2.1. Morphological analysis of the gene knockout strains from *T. atroviride* and *T. virens* 31
3.2.2. Stress tests 38
3.2.3. Biomass formation....39
3.2.4. Mycoparasitism assays....41
3.3. Gene expression analysis 46
4. Discussion....55
5. References....60
6. Danksagung....66

1. Introduction

1.1 Fungi

Living organisms are subdivided into five major kingdoms: the Chromista (algae), the Protozoa (phagotrophic unicellular organism), the Plantae (plants), the Animalia (animals) and the **Fungi.** Fungi are heterotrophic organisms, and the kingdom of fungi includes filamentous fungi with mycelial growth and yeasts with unicellular growth (Watkinson 2001).
Fungi are divided into genera, families, orders, classes, phyla and species (Dingley 1957). The kingdom of fungi contains seven major phyla including Basidiomycota, Ascomycota, Glomeromycota, Zygomycota, Neocallimastigomycota, Blastocladiomycota and Chytridiomycota. However, the classification is continuously undergoing changes (Hibbett, Binder et al. 2007).
Fungi are able to proliferate sexually and asexually, but for several species, known as Fungi Imperfecti, only asexual reproduction structures have been observed so far. More recently several of these asexual species could be attributed to their sexual based on DNA sequence comparisons in phylogenetic studies. The sexual state of a fungus is called teleomorph and the asexual state anamorph. Currently, the nomenclature of fungi is based on their sexual state (holomorph concept) (Carlile, Watkinson et al. 2005).

Fungi have a strong impact on human life and on the ecosystem. They recycle nutrients in the environment and some of them enter symbioses with plants or can be used as biocontrol agents in agricultural applications (Druzhinina and Kubicek 2005). Others are infectious agents and can be dangerous for plants, animals and humans by causing diseases (Idnurm and Heitman 2005).

1.2. The genus *Trichoderma/ Hypocrea*

1.2.1 Classification of *Trichoderma/ Hypocrea*

The anamorphic fungal genus *Trichoderma (teleomorph Hypocrea)* was first recognized by Persoon in 1794 (Persoon 1794). It took, however, 200 years from the discovery of the genus *Trichoderma* until it was taxonomically classified. The problems of the identification were mis- and reidentifications of several strains. In history nearly all strains of *Trichoderma* were identified as *T. viride* (Bisby 1939). In 1969 Rifai described nine different aggregates, some of which comprised two or more morphologically indistinguishable species (Rifai 1969). The results of the research of Rifai and Bisset (Rifai 1969; Bissett 1984) showed five sections of the genus *Trichoderm: Longibrachiatum*, *Pachybasium*, *Trichoderma*, *S*aturnisporum and *Hypocreanum* (Bissett 1991; Bissett 1991; Bissett 1991; Bissett 1992). Due to the fact that the identification of species was based mainly on morphological characteristics, the classification of *Trichoderma* was extremely difficult. Further development in DNA based phylogenetic analysis in the past few years enabled re-evalution of the genus *Trichoderma.* These new techniques have shown several aspects of the genus *Trichoderma* and its subdivision that could not be resolved based on morphological characterization (Rehner 1995). Nowadays ca. 150 different *Trichoderma* species have already been identified and new species are still being described each year. DNA-based phylogenetic analysis of *Trichoderma* species were developed into online identification tools: a DNA barcoding system (TrichoKEY) and a customized similarity search tool (TrichoBLAST), provided by the website www.isth.info. Sequences of internal transcribed spacer regions of rDNA (ITS1-5.8S-ITS2 complex), translation-elongation factor (EF-1α), the D1 and D2 region of the 28S rDNA, the small subunit of the mitochondrial rDNA (ssu-mDNA) and a fragment of *each42* (chi18-5) are most frequently used to differentiate between *Trichoderma* strains and it was possible to get detailed information of the biodiversity of *Trichoderma* (Kubicek, Baker

et al. 2008). The first sequenced *Trichoderma* genome was from *Trichoderma reesei* (Martinez, Berka et al. 2008) followed by the genomes of *Trichoderma atroviride* IMI206040 and *Trichoderma virens* Gv29-8 (Kubicek, Herrera-Estrella et al. 2011). The research of the large number of *Trichoderma/ Hypocrea* species is still ongoing (Druzhinina and Kubicek 2005).

1.2.2. Features and applications of *Trichoderma*

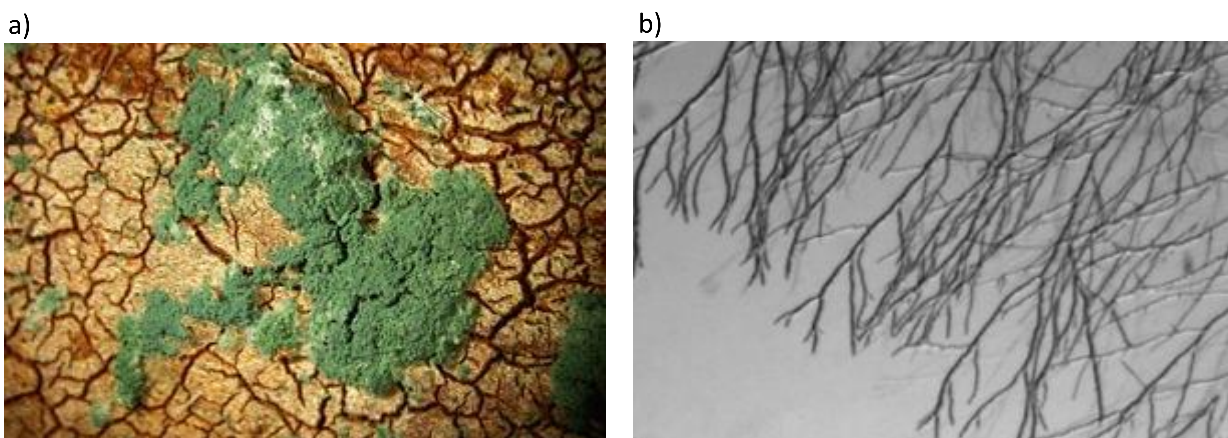


Fig.1: *Trichoderma harzianum* colony in nature (a) taken from Walter M. Jaklitsch (TU Wien) and microscopic picture from *Trichoderma atroviride* (b) taken from V. Seidl-Seiboth (unpublished).

Trichoderma spp. are filamentous, cosmopolitan fungi and occur in climates ranging from the tundra to the tropics. They are normally found in root, soil and foliar environments (Gams and Bissett 1998). They are among the most widely distributed and common fungi in nature, probably due to their diverse metabolic capability and aggressively competitive nature (Kubicek, Herrera-Estrella et al. 2011). Species of *Trichoderma/ Hypocrea* can grow saprotrophically on decaying wood, but many *Trichoderma* spp. are also opportunistic, necrotrophic mycoparasites (Druzhinina, Seidl-Seiboth et al. 2011). They have often high growth rates and minimal nutritional requirements (Samuels 1996; Klein and Eveleigh 1998). Many species of the genus are being used for the production of secondary metabolites or as biocontrol agents against plant pathogenic fungi for a wide variety of crops and climates (*H.*

atroviridis/*T. atroviride*, *H. lixii*/*T. harzianum*, *H. virens*/*T. virens, T. asperellum*) (Harman, Howell et al. 2004). Others are industrial producers of enzymes such as cellulases and hemicellulases (*H. jecorina*/*T. reesei*) (Seiboth, Herold et al. 2012). However, there are also some negative aspects of *Trichoderma* spp. like the degradation of cotton fabrics due to their high cellulolytic potential. *T. aggressivum* is pathogenic on commercial mushrooms species like *Agaricus* and *Pleurotus*and, it was reported that *T. longibrachiatum* is an opportunistic pathogen of immunocomprised mammals including humans (Seaby 1998; Kredics, Antal et al. 2003).

1.2.3. Biocontrol by *Trichoderma*

In agriculture serious crop losses occur every season due to plant diseases caused by fungal and bacterial pathogens. The plants are damaged by the pathogens, leading to a reduction in harvesting yields and in addition the crops may be contaminated with toxins that are produced by the plant pathogens. Prevention of this damage has so far been mainly achieved through the use of chemical pesticides. However, the number of resistant pathogens against chemical plant protectants increases constantly. In addition, awareness of the consumers about chemical pollution of the environment and pesticide residues in food is increasing. Therefore, there is a growing interest in sustainable agriculture and biological control as a good alternative to chemical pesticides (Cook 1983). The biocontrol process by *Trichoderma* comprises three different mechanisms:

- competition for nutrients and living space with plant pathogenic organisms
- direct attack and destruction of the pathogens (mycoparasitism)
- promotion of plant beneficial processes such enhancement of plant growth and induction of systemic and localized resistance.

(Cortés, Gutiérrez et al. 1998; Hjeljord and Tronsmo 1998; Zeilinger, Galhaup et al. 1999; Harman, Howell et al. 2004).

However, the exact process of the *Trichoderma spp.*-plant interaction and communication is not clear yet. *Trichoderma spp.* are qualified as biocontrol agents due to their high reproductive capacity, efficiency in the utilization of nutrients, ability to survive under unfavourable conditions, strong aggressiveness against phytopathogenic fungi and stimulation of plant growth and plant-defence responses (Chet and J.Inbar 1997).

Some *Trichoderma* strains are able to colonize plant roots and enter the epidermal layers of the roots without being pathogenic for the plant. This leads to changes in gene expression, induction of local and systemic resistance in plants, alterations in plant physiology and therefore may result in the improvement of abiotic stress resistance, nitrogen fertilizer uptake and resistance to pathogens. The result of all these effects is an increase in productivity and plant growth (Hermosa, Viterbo et al. 2012).

Commercial usage of various *Trichoderma* species for the protection of plants has increased in the last few years. Available products are for example RootShield^{TM}, BioTrek 22G^{TM}, T-22HB^{TM} (Bio-Works, USA), Supresivit^{TM} (BorregaardBioPlant, Denmark), Binab^{TM} (Bio-Innovation, Sweden),Trichopel^{TM} and Promot^{TM} (J.H.Biotech, USA) (Paulitz and Bélanger 2001).

1.2.4. Mycoparasitism

Mycoparasitism is the phenomenon where one fungus is parasitic on another fungus (Fig.2). This is already a very old phenomenon, as was shown by 400 million years old fossil evidence (Taylor, Hass et al. 2005).

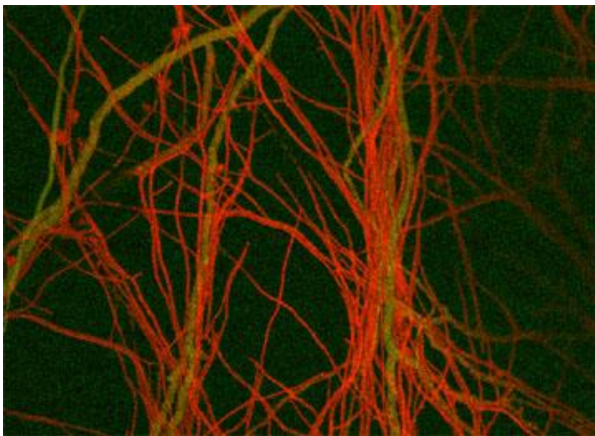


Fig.2 : *T. atroviride* (red) growing along the hyphae of a host fungus (green).
Photo by V. Seidl-Seiboth, TU Vienna and N. Read, Univ. of Edinburgh.

As mentioned above, this lifestyle has special pertinence when the prey is a plant pathogen, because these strategies for biological control can be used for protection of pests in plants (Vincent, Goettel et al. 2007). A potential mycoparasitic role for *Trichoderma* was first proposed by Weindling 1934. He described the parasitic activity of *Trichoderma spp.* against the plant pathogenic fungus *Rhizoctonia solani*. Since then, a large number of studies approved several *Trichoderma* species as a potent mycoparasite (Weindling 1934). Species of the genus *Trichoderma,* especially *T. atroviride and T. virens,* are not only used in agriculture as biocontrol agents, but are also model organisms for mycoparasitism research (Harman, Howell et al. 2004). They are usually used against plant-pathogenic fungi such as *Rhizoctonia solani, Botrytis cinerea, Sclerotium rolfsii, Sclerotinia sclerotiorum*, *Pythium* spp., and *Fusarium* spp., as a good alternative to chemical pesticides (Barak and Chet 1986; Chet 1987; Harman and Björkman 1998; Howell 2003). The mycoparasitic process is the result of different mechanisms and metabolic actions. There are a large number of proteins from

different families and other metabolites known so far, which are directly involved in the interaction of *Trichoderma* and its host (Kubicek, Herrera-Estrella et al. 2011).

The mycoparasitic attack includes the recognition of the host, the attachment to the prey hyphae, the secretion of secondary metabolites and the lysis of the host cell wall. First *Trichoderma* recognizes the host and attaches to the host hyphae via coiling (Fig.3) and helix- shaped hyphae.

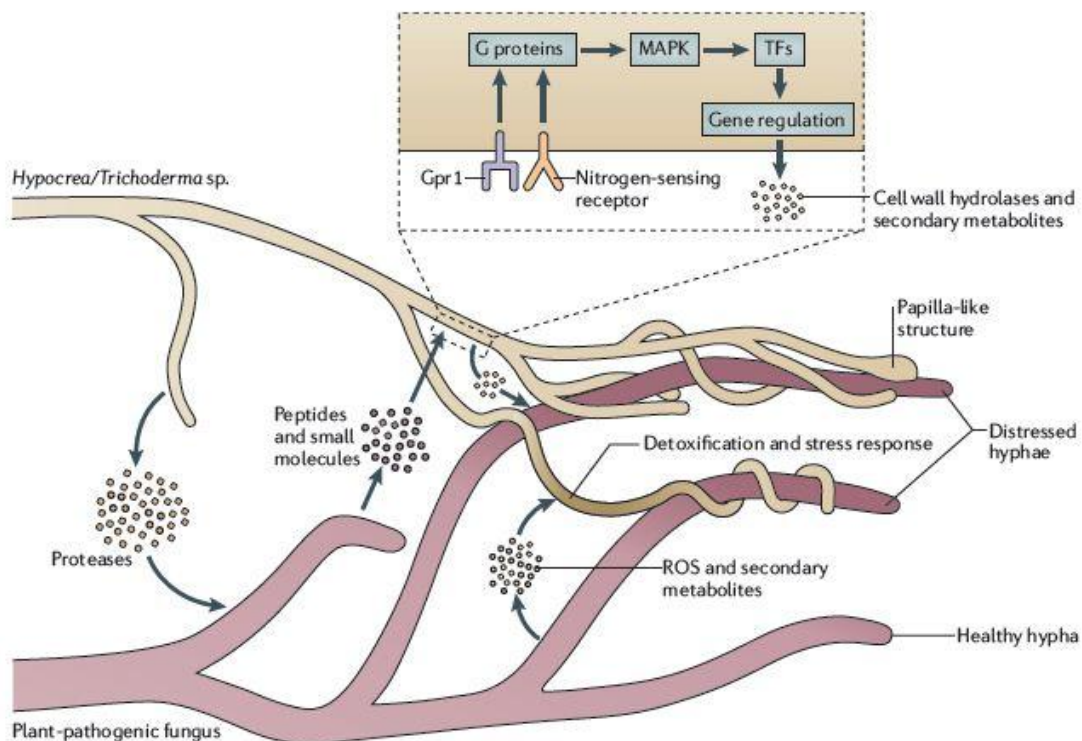


Fig.3 : Mycoparasitism of *Trichoderma* spp.. The attachment of *Trichoderma* spp.via coiling to the host hyphae (Druzhinina, Seidl-Seiboth et al. 2011).

It has been suggested that the attachment is controlled by the binding of *Trichoderma* cell wall carbohydrates to lectins on the host hyphae (Elad, Barak et al. 1983; Druzhinina, Seidl-Seiboth et al. 2011). After the attachment it secretes an array of lytic enzymes and secondary metabolites, which weaken and lyse the host cell wall. Hydrolytic enzymes such as chitinases, glucanases and proteases are involved in the degradation of the host cell wall, as these protein families are expanded in mycoparasites and/or have been shown to be upregulated during the mycoparasitic attack (Gruber and Seidl-Seiboth 2012). *Trichoderma* species are a rich source of secondary metabolites. They additionally produces polyketides,

isoprenoid-derived metabolites, pyrones and a variety of different peptaibols, a special class of non-ribosomally synthesized peptides, which can self-assemble, form pores in liquid membranes and act as ion-channels and leads to the loss of osmotic balance and finally to cell death of the host. Recently sequenced genomes of *T. atroviride* and *T. virens* are enriched in secondary metabolism-related genes and gene clusters (Druzhinina, Seidl-Seiboth et al. 2011; Mukherjee, Horwitz et al. 2012). The lytic enzymes are responsible for degrading the cell wall of the host, which act synergistically with peptaibols like Alamethicin (from H. virens/ T. virens), Trichokonone (from H. koningii/ T. koningii) and Trichorzianine (from H. lixit// T. harzianum), which affect the capacity of the hyphae to repair the damage of the cell wall degrading by inhibiting the synthesis of chitin and β-glucan (Schirmbock, Lorito et al. 1994; Lorito, Farkas et al. 1996; Komon-Zelazowska, Neuhof et al. 2007).

1.3. The species *T. atroviride* and *T. virens*

T. atroviride and *T. virens* are filamentous, mycoparasitic fungi that occur in soil, on decaying wood and on plant debris (Kubicek, Komon-Zelazowska et al. 2008). *T. atroviride* was first described in 1892 by Karsten, but this species has been frequently mistaken in literature with the superficially similar species *T. harzianum,* which also has smooth, globose to subglobose conidia (Karsten 1892). Bissett and Samuel uncovered the mistake and described the differences between the two species (Bissett 1991; Samuels, Dodd et al. 2002). The genome of both, *T.atroviride* and *T.virens,* is already sequenced and the genome size is 36.1 Mbp for *T.atroviride,* comprising 11865 gene models and for *T.virens* 38.8Mbp, comprising 12428 gene models. The two *Trichoderma* species share 1273 orthologues that are not found in the genome of the saprotrophic species *T. reesei*. Therefore, this gene pool might be associated with the strong mycoparasitic potential of *T. atroviride* and *T. virens* (Kubicek, Herrera-Estrella et al. 2011).

In the 1980ies and 1990ies the mycoparasitic potential of both *Trichoderma* species, *T.atroviride* and *T.virens*, was discovered and their important role in biological control

recognized. Today both strains are commonly applied in the mycoparasitism research (Lorito, Woo et al. 2010).

1.4. Cerato-platanin proteins

The name-giving protein for the cerato-platanin family was the protein CP (cerato-platanin) from the plant pathogen *Ceratocystis platani,* a fungus causing canker stain on plane trees (Pazzagli, Cappugi et al. 1999). Members of the cerato-platanin family are small, secreted cysteine-rich proteins that are uniquely found in filamentous fungi and are readily recognized by other organisms. They are associated with the induction of defense responses in plants and allergic reaction in humans (Seidl, Marchetti et al. 2006). Orthologues of the cerato-platanins are strongly conserved through the fungal kingdom. They can be found in filamentous fungi with different lifestyles such as human and plant pathogens, medically and industrially relevant, biocontrol and saprotroph fungi. Fungal genomes analysis showed that filamentous fungi have typically two or three gene encoding cerato-platanin (Frischmann, Neudl et al. 2012). In previous studies it had been suggested that cerato-platanins might have similar functions as hydrophobins, which are amphiphilic fungal proteins that self-assemble at hydrophobic-hydrophilic interfaces and invert the polarity of surfaces (Boddi, Comparini et al. 2004; Seidl, Marchetti et al. 2006) and the NMR-structure showed similarities to plant expansins, which are involved in cell wall expansion (de Oliveira, Gallo et al. 2011). Recently published data show that cerato-platanins increases the polarity of solutions and surfaces, this is the opposite effect of what is observed for hydrophobins, due to this effect cerato-platanins are not hydrophobin-like proteins. In *C. platani* it was reported that the expression levels of *cp* are connected with the chlamydospore formation (Baccelli, Comparini et al. 2012). However, the primary function of cerato-platanins is not known yet (Frischmann, Neudl et al. 2012).

1.4.1. Eliciting plant-response-like proteins (EPLs) and small proteins (SMs)

T. atroviride secretes three proteins called EPL1, EPL2 and EPL3 (eliciting plant-response-like proteins) and the orthologues in *T.virens* are called small proteins SM1, SM2 and SM3. These proteins are members of the cerato-platanin family (Vargas, Djonovic et al. 2008).

Cerato-Platanin proteins are strongly conserved according to their aa-sequence and have 4 cysteine residues (Pazzagli, Cappugi et al. 1999). The sequences of the three cerato-platanins from *T. atroviride* EPL1, EPL2, EPL3 and the orthologues from *T. virens* SM1, SM2, SM3 are shown in Fig.4. The spacing of the cysteine residues (colored in red in Fig.4) is highly conserved among the sequences of the cerato-platanin proteins.
An alignment of the cerato-platanin proteins from *T. atroviride* and *T. virens* is shown Fig.5.
So far only EPL1 and SM1 have been studied. The size of these proteins is 12.6 kDa. EPL1 and SM1 are detected in their monomeric and dimeric forms (Seidl, Marchetti et al. 2006).

EPL1 and SM1 have been shown to be elicitors of plant defense-responses. However, only the monomeric forms are able to induce such responses (Vargas, Djonovic et al. 2008). Dimer formation was found to be dependent on double oxidized tryptophan residues (Seidl, Marchetti et al. 2006) and also on a single glycosylation site that is present in SM1 but not in EPL1. Therefore, SM1 is mainly found in its monomeric form, whereas EPL1 has a high tendency to form dimers. Thus SM1 was found to have a higher potential to induce plant defense responses than EPL1 (Vargas, Djonovic et al. 2008). The capability of SM1 to provide protection to ward fungal pathogen and to act as an elicitor of plant defense reactions was suggested to play a key role in the induction of systemic resistance in plants by *T. virens* (Djonovic, Pozo et al. 2006).

>EPL1

MQFSSLFKLALFTAAVSADTVSYDTGYDDASRSLTVVSCSDGANGLITRYHWQTQGQIPRFPYIGGVQ
AVAGWNSPSCGTCWKLTYSGKTIYVLAVDHSAAGFNIGLDAMNALTNGNAVQYGRVDATASQVAVSN
CGL

>EPL2

MQLSNIFTVATLVTAITATYVSFDPGYDDASRSLRDVSCSDGLNGLITKYHWETQGQISRFPYIGGVQG
TTWNSTLCGACYKLEYADRSIHVLGIDAVYNGGLNIGLHALNDLTNGNAVAWGHVDATVTQVSGRGC
GLPNAHKAN

>EPL3

MVPLGICQVAAIISLASAETVSVTFNSLYDDPSRSLSEVACWRKGTGFMPNLDWKIQKDALDFIGIDAIR
GSSDAQCFSCWKLEYGDEHVSLFAIDGADSGFVLSLNAMQSLTGGQARELVRIDVEATQVDVSNCGV
SAAELHKYDF

>SM1

MQLSNIFTLALFTAAVSADTVSYDTGYDNGSRSLNDVSCSDGPNGLETRYHWSTQGQIPRFPYIGGAA
AVAGWNSASCGTCWKLQYSGHTIYVLAVDHAASGFNIALDAMNALTGGQAVQLGRVSATATQVPVKN
CGL

>SM2

MQLGSLFNAATLVAAATATYVSFDTGYDDPSRSMTQVACSDGVNGLITKYHWQTQGEVSNFPYIGGV
QGIQWNSTQCGTCHRLEYGGRSIHILAVDAAYNGGYNIALKALDTLTDGHAVEWGHVDAVATQVSVN
ECGLFTVY*

Fig.4: Sequences of EPL1, EPL2, EPL3 from *T. atroviride* and SM1, SM2, SM3 from T*. virens* searched in the Gji data bank. The cysteine residues are shown in red.

```
                *        20         *        40         *        60         *        80
SM1  : MQLSNIFTLALFTAAVSADTVSYDTGYDNGSRSLNDVSCSDGPNGLETRYHWSTQGQIPRFPYIGGAAAVAGWNSASCGTCWKLQYSGH :  89
EPL1 : MQFSSLFKLALFTAAVSADTVSYDTGYDDASRSLTVVSCSDGANGLITRYHWQTQGQIPRFPYIGGVQAVAGWNSPSCGTCWKLTYSGK :  89
SM2  : MQLGSLFNAATLVAAATATYVSFDTGYDDPSRSMTQVACSDGVNGLITKYHWQTQGEVSNFPYIGGVQGIQWNSTQCGTCHRLEYGGRS :  89
EPL2 : MQLSNIFTVATLVTAITATYVSFDPGYDDASRSLRDVSCSDGLNGLITKYHWETQGQISRFPYIGGVQGTTWNSTLCGACYKLEYADRS :  89
SM3  : MLASRLFQIAAIVAPVSADIVSATFDTIYDDPSRSLSEVACWRKPTGFMPNLDWKLQQDAVGFIGVDSITRLKSSKCFSCWTLEYNDKS :  89
EPL3 : MVPLGICQVAAIISLASAETVSVTFNSLYDDPSRSLSEVACWRKGTGFMPNLDWKIQKDALDFIGIDAIRGSSDAQCFSCWKLEYGDEH :  89
       M    6f A      3A VS           S          g          2    5IG

       *       100        *       120        *       140
SM1  : TIYVLAVDHAASGFNIALDAMNALTGGQAVQLGRVSATATQVPVKNCGL---------- : 138
EPL1 : TIYVLAVDHSAAGFNIGLDAMNALTNGNAVQYGRVDATASQVAVSNCGL---------- : 138
SM2  : IHILAVDAAYNGGYNIALKALDTLTDGHAVEWGHVDAVATQVSVNECGLFTVY*----- : 142
EPL2 : IHVLGIDAVYNGGLNIGLHALNDLTNGNAVAWGHVDATVTQVSGRGCGLPNAHKAN--- : 145
SM3  : ISLIALDGADSGIVMSLSALQFLTDGRAHELGRVDIHATEVDTSKCGLPAKILHAYDF* : 147
EPL3 : VSLFAIDGADSGFVLSLNAMQSLTGGQARELVRIDVEATQVDVSNCGVSAAELHKYDF- : 147
```

Fig.5: Alignment of the three cerato-platanins from *T. atroviride* EPL1, EPL2, EPL3 and their orthologues from *T. virens* SM1, SM2, SM3

Recently the biochemical properties of EPL1 were studied in detail. It was shown that EPL1 readily self-assembles at air/water interfaces and forms protein layers (Fig.6), but no ordered, rodlet-like structures, as have been reported for hydrophobins, were detected in these proteins layers.

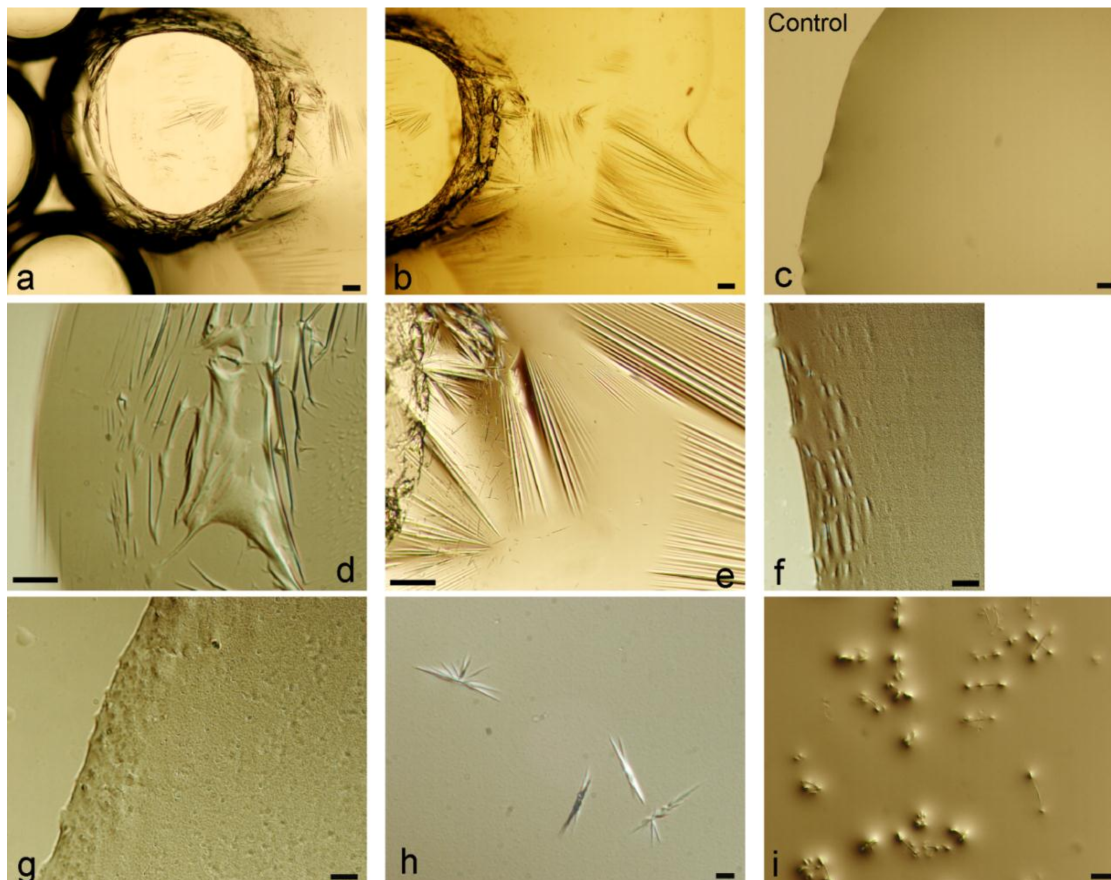


Fig.6 : EPL1 forms protein layers on the surface of water droplets, which also stabilize small air bubbles. Sodium acetate buffer as control (c). Scale bars: a, b: 100 µm, d, e, i: 50 µm, c, f, g, h: 10 µm; taken from Frischmann, Neudl et al. 2012.

Further, EPL1 increases the polarity of aqueous solutions and surfaces, which is the opposite as has been reported for hydrophobins, these data therefore indicated that the cerato-platanin protein is not a hydrophobin-like protein (Frischmann, Neudl et al 2012). In addition, EPL1 was found to bind to various forms of polymeric chitin, which suggests that cerato-platanins are carbohydrate-binding proteins and could indicate a localization of these protein in the fungal cell wall (Frischmann, Neudl et al. 2012).

1.5. Aims of this thesis

Cerato-platanins are small, secreted proteins with four conserved cysteine residues. They are commonly found in filamentous fungi and are associated with the induction of defense responses in plant and allergic reactions in humans (Seidl, Marchetti et al. 2006). The primary function of cerato-platanins in fungal growth and development is not known yet. The aim of this thesis was to investigate the potential involvement of cerato-platanins in hyphal growth and development in *T. atroviride* and *T. virens* and to analyze their gene regulation.

2. Materials and methods

2.1. Instruments, chemicals and devices

2.1.1. Instruments

Table 1: Instruments

Instrument:	Producer:
Centrifuge 5415R	Eppendorf AG, Hamburg, Germany
PCR Cycler, Thermocycler T3000	Biometra GmbH, Germany
Nikon T300 microscope	Nikon, Tokyo, Japan
Nanodrop^{R} ND$_{-1000}$ Spectrophotometer	Nanotrop Technologies, USA
Gel Doc Universal HoogII	Bio-Rad, California, USA
PowerPac Basis	Bio-Rad, California, USA
Gel chamber wide mini-sub cell gt	Bio-Rad, California, USA
Gel chamber mini-sub cell gt	Bio-Rad, California, USA
Waterbath	Fischer Scientific Inc., England
PHM 82 Standard pH Meter	Radiometer Copenhagen
CERTOclav Type CVII/1600	KELOMAT Haushaltsgeräte Traun, Austria
Sanyo Incubator MIR-153	Sanyo Electric Co., Ltd. Japan
Incubator Infors HT Multitron	Infors HT, Switzerland
Incubator B5060E	Heraeus, Germany
IKA^{R} Vortex	IKA, Germany
Thermomixer compact 5350	Eppendorf AG, Hamburg, Germany
Magnetic stirrer Hei Standard	Heidolph Instruments, Germany

Miracloth 475855	Calbiochem, Austria
Whatman Glass Microfibre filters, 47mm	Whatman International Ltd., England
Pipetman Neo P10N, 1-10µl	Gilson, Wisconsin, USA
Pipetman Neo P20N, 2-20µl	Gilson, Wisconsin, USA
Pipetman Neo P200N, 20-200µl	Gilson, Wisconsin, USA
Pipetman Neo P1000N, 100-1000µl	Gilson, Wisconsin, USA

2.1.2. Chemicals

Tabel 2: Chemicals

Chemicals	Producer	Article No.
Water for Molecular Biology	Merck GmbH, Germany	T143.4
Ethanol	Merck GmbH, Germany	1009712500
Methanol	Merck GmbH, Germany	1060092500
Sulfuric acid	Merck GmbH, Germany	1007312500
Tween 80	Merck GmbH, Germany	8221870500
Phosphoric aci	Merck GmbH, Germany	8150580010
Isoamyl alcohol	Merck GmbH, Germany	100979
Sodium hydroxide	Carl Roth GmbH, Germany	6771.2
Acetic Acid	Carl Roth GmbH, Germany	3738.1
Tris(hydroxymethyl)aminomethane	Carl Roth GmbH, Germany	4855,3
2-propanol	Sigma-Aldrich Chemie GmbH, Germany	650447
Trichloromethane/ Chloroform	Carl Roth GmbH, Germany	L1412
Potato Dextrose Agar	Difco, USA	213400
Phenol	Calbiochem, Austria	A1578.0500

Ribonuclease A from bovine pancreas	SERVA	34388.02
Glycerol	Carl Roth GmbH, Germany	3783.1
Sodium chloride	Carl Roth GmbH, Germany	3957.2
Malt extract	Merck GmbH, Germany	1.05391.0500
Agar-agar	Merck GmbH, Germany	1.01614.1000
K_2HPO_4	Merck GmbH, Germany	1.04873.1000
KH_2PO_4	Merck GmbH, Germany	C221563
$(NH_4)_2SO_4$	Carl Roth GmbH, Germany	3746.3
$CaCl_2.2H_2O$	Sigma-Aldrich Chemie GmbH, Germany	111K067
KCl	Merck GmbH, Germany	1.04936.1000
$MgSO_4.7H_2O$	Carl Roth GmbH, Germany	T888.2
$MgCl_2$	Carl Roth GmbH, Germany	2189.1
$FeSO_4.7H_2O$	Merck GmbH, Germany	3965
$ZnSO_4.7H_2O$	Merck GmbH, Germany	2761
$MnSO_4.H_2O$	Merck GmbH, Germany	4986
Peptone	Merck GmbH, Germany	1.02239.0500
Glucose	Carl Roth GmbH, Germany	6780.2
Congo Red	Sigma-Aldrich Chemie GmbH, Germany	C6767
Calcofluor White	Sigma-Aldrich Chemie GmbH, Germany	1000957490
Agarose	Starlab GmbH, Germany	LF45110033
Potato dextrose agar (PDA)	Becton, Dickinson and Company, USA	2087392
$SYBR^R$ Safe DNA gelstain	Invitrogen, USA	S33102

Chitin	Sigma-Aldrich Chemie GmbH, Germany	C7170

2.1.3. Media

Table 3: Media

	ISM	**SM**
KH_2PO_4	0.68 g/l	2 g/l
K_2HPO_4	0.87 g/l	____
$(NH_4)_2SO_4$	1.7 g/l	1.4 g/l
$CaCl_2.2H_2O$	0.2 g/l	0.3 g/l
KCl	0.2 g/l	____
$MgSO_4.7H_2O$	0.2 g/l	0.3 g/l
Trace elements 100x		
$FeSO_4.7H_2O$	0.5 g/l	0.5 g/l
$ZnSO_4.7H_2O$	0.2 g/l	0.2 g/l
$MnSO_4.H_2O$	0.2 g/l	0.2 g/l
Peptone	0.05 %	0.05 %
Carbon source (glucose)	1 %	1%

MEX	
Malt extract	3 %
Agar	2 %
PDA (Becton, Dickinson and Company)	39 g/l

2.2. Fungal strains

In this work *T. atroviride* P1 (ATCC 74058) and *T. virens* I10 (CBS 116947) were used and kept on potato dextrose agar (PDA) plates. For genetic manipulations the *T. atroviride* wild-type strain (WT) and a *T. virens* Δ*ku70* strain (Catalano, Vergara et al. 2011) were used. The advantage of Δ*ku70* strains is that they are deficient in non-homologous end-joining during DNA recombination, which results a higher rate of targeted DNA recombination events during fungal transformations in comparison to a wild-type strain.

2.3. Growth tests of fungal strains on agar plates

For comparison of different gene knockout strains that were analysed in this study (see results) the following growth tests were carried out: Growth of the *T. atroviride* wild-type/*T. virens* parental strain and knockout strains was compared on PDA plates with 12h/12h light/ darkness cycles at 28°C. Further, growth during osmotic stress was investigated on agar plates containing 2% (w/v) malt extract and 10% (w/v) glycerol or 1M NaCl. For cell wall stress tests growth was assayed on agar plates with SM medium containing 10µg/ml Calcofluor White or Congo Red. Additionally the growth on plates with SM medium containing 1% (w/v) colloidal chitin was tested.

2.3.1. Mycoparasitism assays with T. atroviride/ T. virens against R. solani and B. cinerea

All fungal strains were pre-grown on PDA plates at 28°C. For the confrontation assays pieces of well sporulated plates were cut out from the *T.atroviride* and *T.virens* wild-type/ parental strain and gene knockout strains (P1, *Δepl1, Δepl2, Δepl1Δepl2*, *Δku70*, *Δsm1, Δsm2*) and from the host fungi *Rhizoctonia solani* and *Botrytis cinerea*, respectively. The experiment was

performed on PDA plates incubated at 28°C with a 12 h/12 h light/dark cycle. In each case a piece of *Trichoderma* and a piece of the host fungus *R. solani* or *B. cinerea* were placed on opposite sides of the plate. Images were taken every 24 h to record the mycoparasitic potential of the *Trichoderma* wild-type/ parental strain and knockout strains.

2.3.2. Determination of the spore concentration

Spores from PDA plates were harvested with a NaCl/ Tween solution (0.9%NaCl, 0.05% Tween 80) and were filtered through a glass wool tube. 10 ml of a Biolog inoculation solution (0.25% Phytagel, 0.03% Tween 80) were added to a Biolog tube. The tube was put in the machine and turned until the transmission was on zero. Afterwards 10-50µl of the spore solution were added and the transmission measured until the transmission achieved 75 %. The added spore solution contained a concentration of $4x10^7$ spores. From this value the volume of the spore stock solutions was calculated that was needed to inoculate liquid cultivations (see 2.3.3).

2.3.3. Shake flask cultivation of fungi for gene expression and biomass-tests

The following experiments were carried out in 500ml Erlenmeyer flasks which contained 250ml of SM or ISM media. Media were inoculated with $1x10^6$spores/ ml and incubated on a shaker at 200rpm and 25°C for gene expression and 28°C for biomass tests. For the biomass experiment the shake flasks were emptied by filtering through glass microfiber filter (Whatman). For the gene expression assay mycelia was harvested by filtering through Miracloth (VWR), frozen in liquid nitrogen and stored at -80°C.

2.3.4. Biomass tests

Glass microfiber filters (Whatman) were weighed before use. The media containing the biomass were poured on these filters. Afterwards filters were washed with tap water and then dried at 80°C over night. On the next day the filters with the biomass were weighed again and biomass concentrations were calculated from these values.

2.4. Analysis of morphological features of fungal strains

Macroscopic and microscopic analyses of hyphal growth and aerial hypae were carried out. Photographic images were taken from agar plates and an inverted Nikon T300 microscope (Nikon, Tokyo, Japan) with differential interference contrast optics and a DXM1200F digital camera (Nikon) was used for microscopic imaging.

2.5. RNA Isolation and cDNA synthesis

All bottles and pipette tips were treated with 1% DEPC solution by immersing them in the DEPC solution over night, and afterwards autoclaved and dried at 80°C.

For RNA isolation 700 µl Chirgwin reagents and 6 µl β-mercaptoethanol were pipetted into a 2 ml Eppendorf tube and put on ice. The frozen mycelia were grinded in liquid nitrogen and the powder transferred in the tube and mixed by vortexing. Afterwards 70 µl 2M NaAc, 700 µl RNA-phenol and 400 µl chloroform/ isoamylalcohol were added and after each step the suspension was mixed by vortexing. Then the tube was put on ice for 15 minutes and subsequently centrifuged for 15 min at 13 000 *g* and 4°C. The upper phase was transfered into a new 2 ml tube containing 800 µl isopropanol, the solution was mixed by vortexing and the tube was incubated at -20°C for at least 60 min for RNA precipitation. Then the tube was centrifuged at 13 000 *g* and 4°C for 20 min and the supernatant was discarded. The pellet

was washed with 500 µl 70% ethanol, then dried at room temperature and finally dissolved in 30 µl DEPC-H_2O and stored at -80°C. Isolated RNAs were treated with DNAse I, according to the following protocol (Fermentas, St Leon-Rot, Germany):

Table 4: DNAse I digest composition.

RNA	5 µg
10xbuffer	1 µl
DNAse	3 µl
DEPC-H_2O	to 10 µl

Incubation for 45 min at 37°C, afterwards addition of 1 µl EDTA and incubation for another 10 min at 65°C.

cDNAs were generated with the Revert Aid H-minus cDNA synthesis kit (Fermentas, Germany).

Table 5: cDNA synthesis composition.

Template RNA	5 µg
Oligo(dT)$_{18}$ primer Random hexamer primer	0.5 µl 0.5 µl
DEPC ddH_2O	to 12.5 µl
5x reaction buffer	4 µl
RiboLock^{TM}RNase Inhibitor	0.5 µl
dNTP Mix 10mM	2 µl
REvertAid^{TM}H Minus Reverse Transcriptase	1 µl
Total volume	20 µl

The mixture was gently mixed and incubated for 10 min at 25°C followed by 60 min at 42°C and the reaction was terminated by heating at 70°C for 10 min. cDNAs were stored at -80°C.

2.6. Reverse transcription polymerase chain reaction (RT-PCR)

For the analysis of the gene expression of:

- control: *gpdh*- protein ID: 297741
- *epl1*- protein ID: 30292
- *epl2*- protein ID: 88590
- *epl3*- protein ID: 48225

Protein ID in the JGI *T.atroviride* genome database.

- control*: tef*- protein ID: 83874
- *sm1*- protein ID: 110852
- *sm2*- protein ID: 111830
- *sm3*- protein ID: 32154

Protein ID in the JGI *T.virens* genome database.

reverse transcription PCR (RT-PCR) was used.

Table 6: Components which were used for the setup of the PCR reaction with GoTaq^R DNA polymerase (Promega) in a volume of 25 µl.

cDNA	1 µl
5x Green GoTaq^R Flexi Buffer	5 µl
$MgCl_2$, 25 mM	2.5 µl
Primer	0.5 µl from 1:10 dilution
dNTP, 10mM	0.5 µl
GoTaq^R Flexi DNA Polymerase (Promega)	0.1 µl
ddH_2O	14.9 µl

Table 7: Thermal cycling conditions for GoTaq[R] DNA ploymerase

Hot start	95°C		
Denaturation	95°C	2 min	
Denaturation	95°C	30 sec	25-30 cycles
Annealing*		30 sec	
Extension	72°C	1 min/ kb	
Final Extension	72°C	7 min	
End	16°C		

*Annealing temperature depends on used primers, see table 7.

Table 8: Primers which were used for RT-PCR.

Primer	Sequence	Annealing temperature [C°]	Fragment length [bp]
gdph fw gdph rv	gatggaagagttgttgttgccgag ccagaacatcatccccagcagc	59°C	331
epl1 fw epl2 rv	cttgctctcttcaccgccgc ccgttggtcagggcattcat	59°C	220
epl3 fw epl3 rv	tgccaagttgctgctattatca cagatacgccgcagttagac	59°C	450
TV-tef1 fw TV-tef1 rv	gtcgttaccttcgctccttccaa cggacttgatgaacttgggggc	62°C	360
TV sm1 fw TV sm1 rv	ccaacatcttcactctcgctctc aatgttgaagccagaagcagcgtg	62°C	370
TV sm2 fw TV sm2 rv	cctcttcaatgctgctaccctc gccatcagtaagagtgtccagtg	62°C	390
TV sm3 fw TV sm3 rv	gctgccattgtcgccccg caccaatgaacccaactgcgtc	62°C	170

2.7. Agarose gel electrophoresis

0.8 %-2 % (w/v) agarose were suspended in a TAE buffer. Afterwards the suspension was heated in the microwave until the agarose was molten and then tempered in a 50°C water bath. Subsequently SYBR^{R} Safe DNA gel stain (intvitrogen) 2-4 µl were added to the mixture and the agarose solution was poured into a gel tray containing a comb. After solidification the gel was put into an electrophoresis chamber which was filled with 1xTAE buffer. Loading

buffer was added to the DNA samples before they were pipetted into the gel pockets. To analyse the DNA dimension 5 µl of a molecular weight standard (GeneRuler 1kb DNA ladder, Fermentas) was placed into the pockets. Gels were run at 90 V..

Reagents:

TAE buffer (50x):	
Tris	242 g/l (2 M)
Acetic acid	57.1 ml (1 M)
EDTA	100 ml (50 mM)
Add ddH_2O to 1 liter	
pH 8.0 with HCl	

Loading buffer (6x):	
Ficoll 400	15 % (w/v)
Bromphenolblue	0.25% (w/v)
EDTA	60 mM
pH 8.0	

3. Results

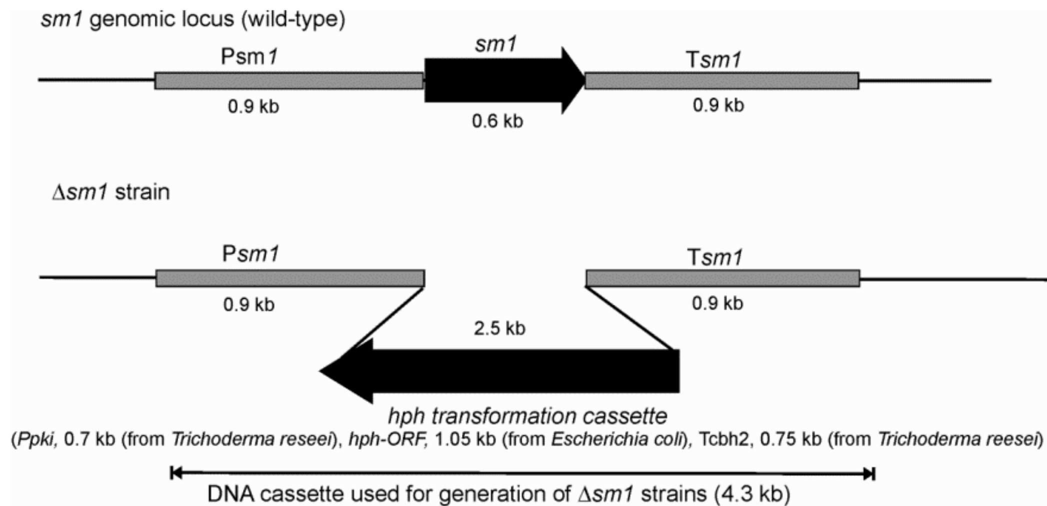
3.1. Overview of cerato-platanin genes in *T. atroviride* and *T. virens* and generation of gene knockout strains

Members of the cerato-platanin family are small, secreted cysteine-rich proteins that are uniquely found in filamentous fungi. They are associated with the induction of defense responses in plants and allergic reaction in humans (Kubicek, Herrera-Estrella et al. 2011). Orthologues of the cerato-platanins are strongly conserved throughout the fungal kingdom. Fungal genomes analysis showed that filamentous fungi have typically two or three gene encoding cerato-platanin (Frischmann, Neudl et al. 2012). Cerato-platanins in *T. atroviride* are called EPLs (eliciting plant response-like proteins) and the ortholouges in *T. virens* are the SM proteins (small proteins) (Vargas, Djonovic et al. 2008). However, the primary function of these proteins in fungal development is not known yet. In order to analyse their biological functions, gene knockout strains were generated previously in our research group. In this thesis the strains were characterized. For clarity reasons the generation of the gene knockout strains is described here briefly and maps of the genomic loci are shown in Fig. 7, 8. In *T. atroviride* single and double gene knockout strains of *epl1* and *epl2* were generated and in *T. virens* gene knockout strains of *sm1* and *sm2* were created.

To generate these knockout strains, the respective genes were deleted by replacement with dominant marker cassettes. For *epl1* deletion the selection marker *amdS* (encoding an acetamidase) (Penttila, Nevalainen et al. 1987) for *epl2* and *sm1* deletion the selection marker *hph* (encoding hygromycin phosphotransferase) (Mach, Schindler et al. 1994) were used, and for *sm2* the selection marker *ptr* (encoding a pyrithiamine resistance gene) (Kubodera et al. 2002). The selection marker cassettes contained the selection marker genes with a promoter and a terminator as outlined in the respective references. These marker cassettes were flanked by the up- and downstream regions of the target genes in

order to achieve gene replacements through homologous recombination by a double cross over mechanism.

a)



b)

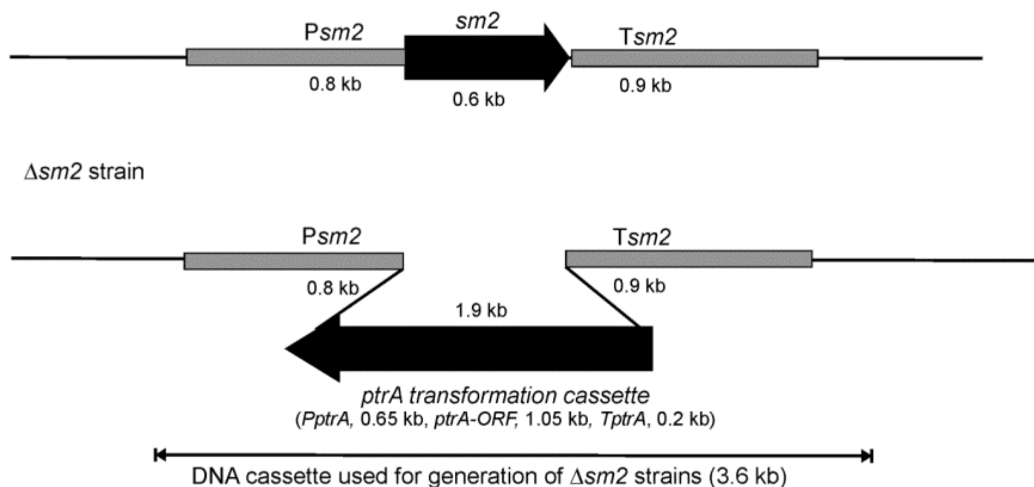


Fig.7: Schematic illustration of double cross over mechanism to create the *sm1* a) and *sm2* b) deletion cassettes. Taken from V. Seidl-Seiboth.

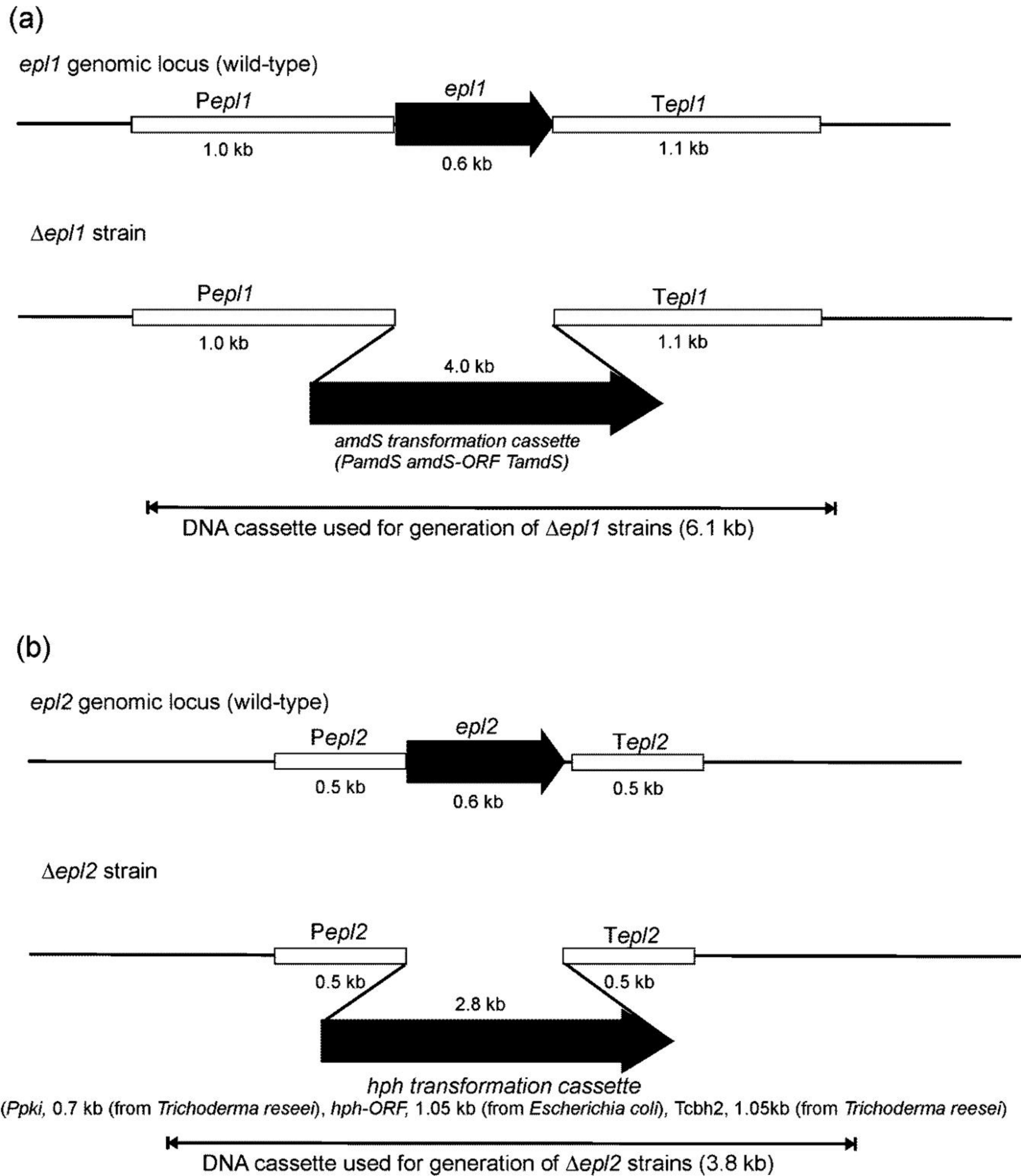


Fig.8: Schematic illustration of double cross over mechanism to create the *epl1* a) and *epl2* b) deletion cassettes. Taken from J Biol Chem, revised manuscript submitted.

3.2. Phenotype analysis

In this study, the following knockout strains were phenotypically characterized in order to elucidate the potential functions of *epl/ sm* genes:

- *T. atroviride* Δ*epl1*
- *T. atroviride* Δ*epl2*
- *T. atroviride* Δ*epl1*Δ*epl2*
- *T. virens* Δ*sm1*
- *T. virens* Δ*sm2*

As reference strains *T. atroviride* P1 (wild-type) and *T. virens* I10 Δ*ku70* (parental strain) were used.

The following parameters were assessed:

- morphological analysis:
  - ➢ growth on agar plates
  - ➢ sporulation
  - ➢ growth on cultivation with colloidal chitin as carbon-source
  - ➢ growth in liquid cultivations
  - ➢ hyphal morphology
  - ➢ formation of aerial hyphae
  - ➢ hydrophobicity of the mycelium
- osmotic stress (NaCl and glycerol), cell wall stress (Calcofluor White and Congo Red) and desiccation stress
- biomass formation in shake flask cultivations
- mycoparasitic potential

3.2.1. Morphological analysis of the gene knockout strains from *T. atroviride* and *T. virens*

Growth on agar plates and sporulation patterns

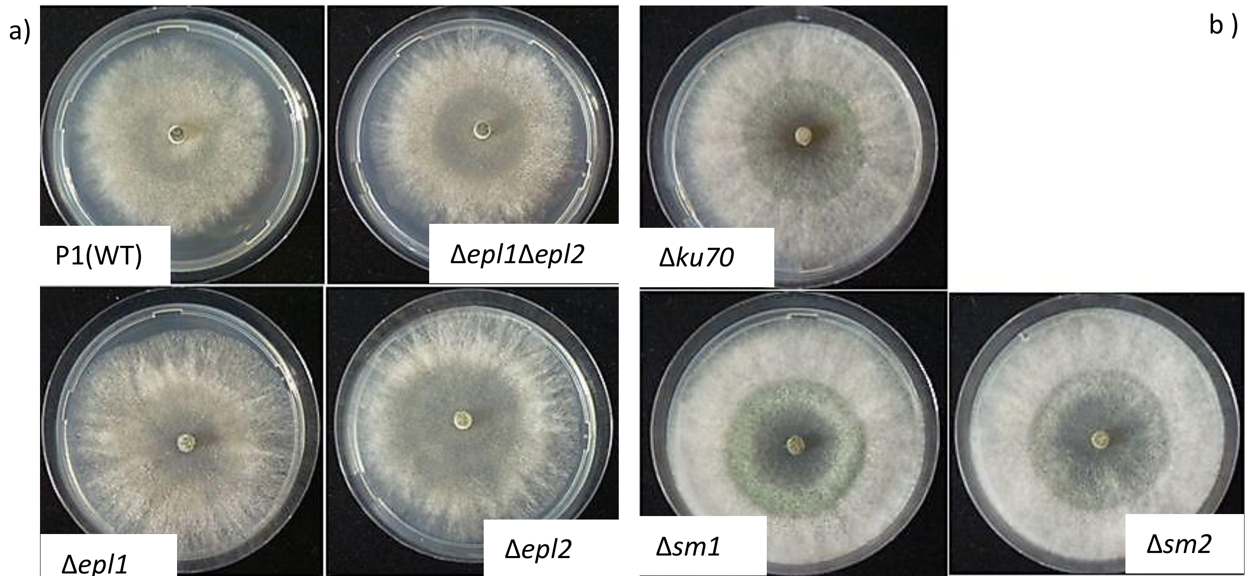


Fig.9: Growth on agar plates of the wild-type/ parental strain and the gene knockout strains from *T. atroviride* (a) and from *T. virens* (b) after three days.

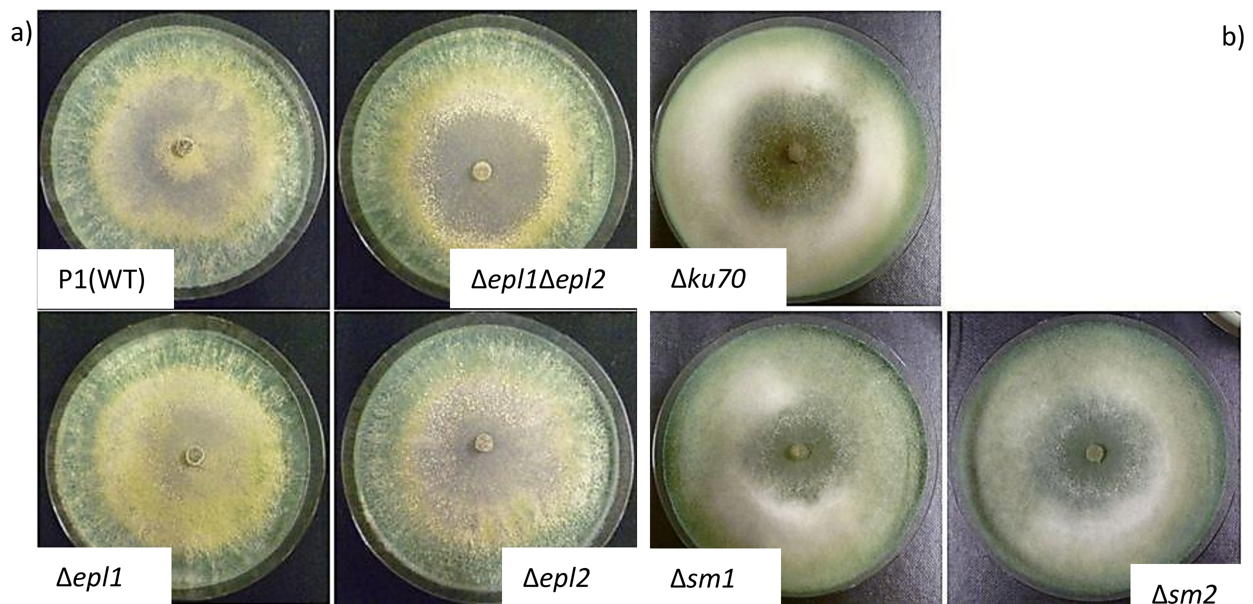


Fig.10: Sporulation pattern on agar plates of the wild-type/ parental strain and gene knockout strains from *T. atroviride* (a) and *T.virens* (b).

In Fig. 9 and 10 the growth and sporulation pattern of *T. atroviride* and *T. virens* on agar plates grown at 28°C with a 12h/ 12h light-darkness cycle are shown. Different light/ darkness cycle conditions were tested in order to evaluate growth and sporulation patterns of the strains. However, in all these analyses there was no difference in growth between the wild-type and the gene knockout strains in *T. atroviride* (Fig. 9a), and the same was observed for *T. virens* (Fig. 9b). The sporulation pattern (Fig.10) also showed no difference in the wild-type/ parental strain and gene knockout strains.

Growth with chitin as carbon source

A recently conducted study shows that EPL1 binds to various forms of chitin, which is a component of the fungal cell wall. Therefore growth tests of the wild-type/ parental strain and gene knockout strains from *T. atroviride* containing 1% (w/v) chitin were carried out. The analysis was performed in liquid standing culture with SM medium as well as on plates with SM medium containing 1,5% agar, with and without 0,1% peptone. All plates were incubated at 28°C with a 12h/ 12h light-darkness cycle. In Fig. 11 a the growth on agar plates containing chitin and peptone is shown, the black mark indicates the extent of the growth after 3 days and the red mark shows growth development after 4 days. Growth in liquid standing cultivations is shown in Fig. 11 b. In all these analyses no difference in growth was observed between the wild-type and the gene knockout strains of *T. atroviride* (Fig.11a, b).

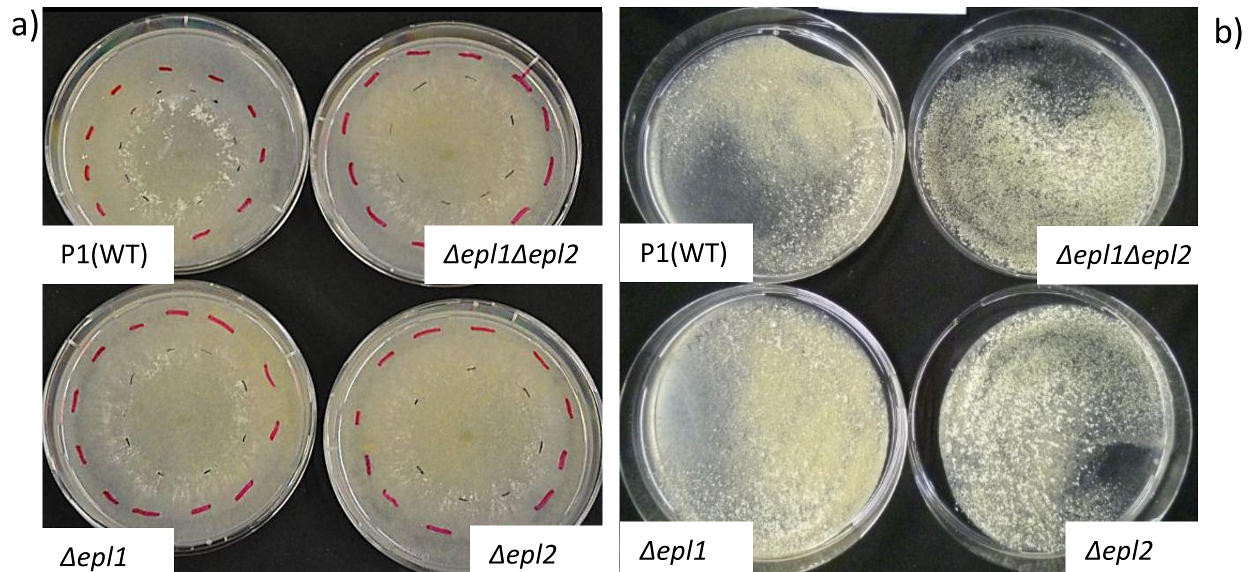


Fig.11: The growth on SM plates containing 1% (w/v) colloidal chitin of *T. atroviride* wild-type and gene knockout strains. Plates containing 1,5% agar are shown in a), black mark shows the growth after 3 days and red mark after 4 days. Growth on liquid standing culture is shown in b).

Growth in submerged cultivations and analysis of hyphal morphology

Strains were grown in shake flask cultivations and samples taken after 24h, 30h, 48h and 72h. Macroscopic and microscopic analyses of the wild-type/ parental strain and all gene knockout strains from *T. atroviride* and *T. virens* were carried out. Macroscopically there was no difference between the wild-type and the gene knockout strains from *T.atroviride* or *T. virens* observed at any time point with respect to color of the medium, foaming, biomass appearance, etc. Samples from these cultivations were also microscopically investigated. In Fig.12 the hyphal morphology of *T. atroviride* wild-type on ISM and SM medium after 24h (Fig.12 a, b), 30h (Fig.12 c, d) and 48h (Fig.12 e, f) is shown, representative for all *T. atroviride* strains. Additionally, microscopic pictures of Δ*epl1* strain (Fig.13 a, b), Δ*epl2* strain (Fig.13 c, d) and Δ*epl1*Δ*epl2* knock out strain (Fig.13 e, f) on ISM and SM medium at 48 hours are shown. Fig.14 demonstrates the hyphal morphology of the *T. virens* parental strain (Fig.14 a, b, c), *sm1* (Fig.14 d, e, f) and *sm2* knockout strains (Fig.14 g, h, i) on ISM medium at 24h, 30h and 48h. It was conspicuous that the growth morphology between *T. atroviride* and *T. virens* was strongly different. While *T. atroviride* formed a rather homogenous

meshwork of hyphae, *T. virens* grew mainly in form of pellets. However, no morphological differences were observed between the wild-type and the gene knockout strains in *T. atroviride* at any time point and also not for the *T. virens* parental strain and knockout strains. Therefore, it can be concluded from these experiments that growth, hyphal morphology, the formation of hyphal networks and branching was not altered upon deletion of *epl1/sm1* and/or *epl2/sm2*.

T. atroviride P1 (WT)

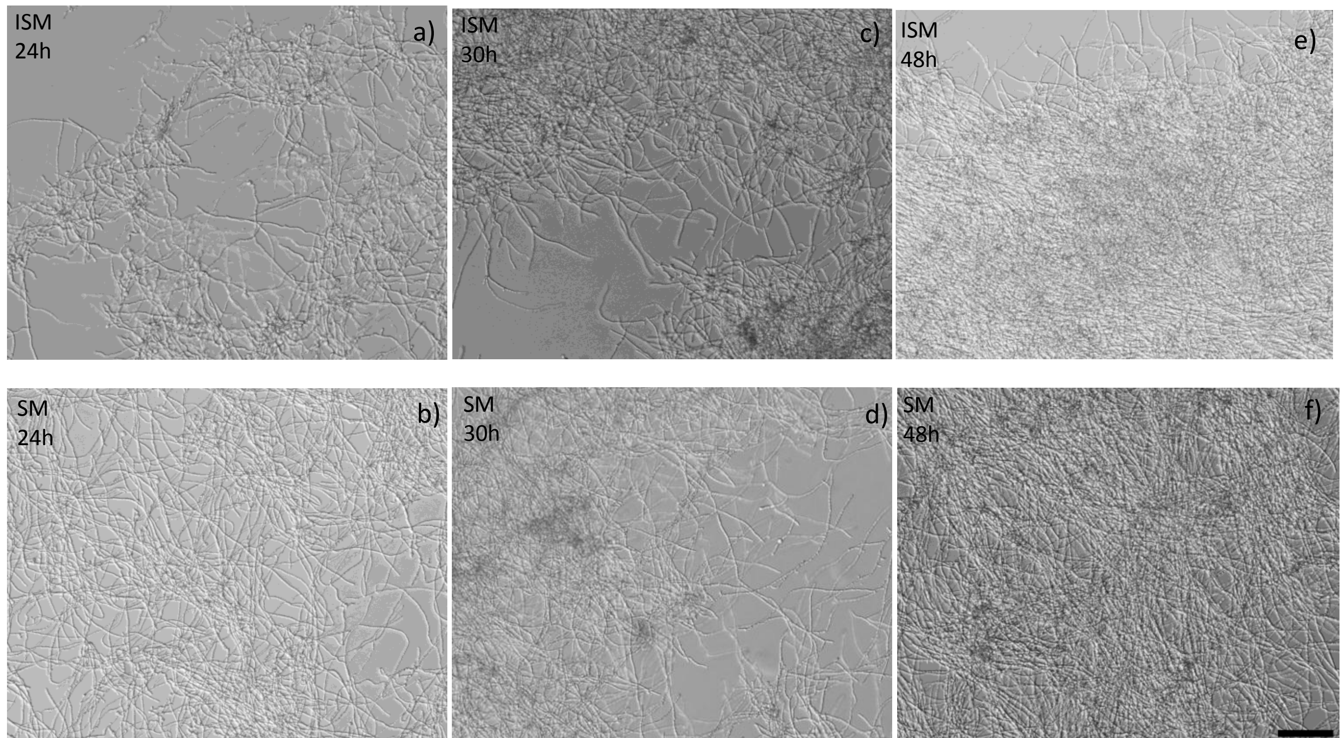


Fig 12: Microscopic analysis of the hyphae formation from *T. atroviride* P1 wild-type on ISM and SM media after 24h, 30h, 48h. Scale bars=100µm.

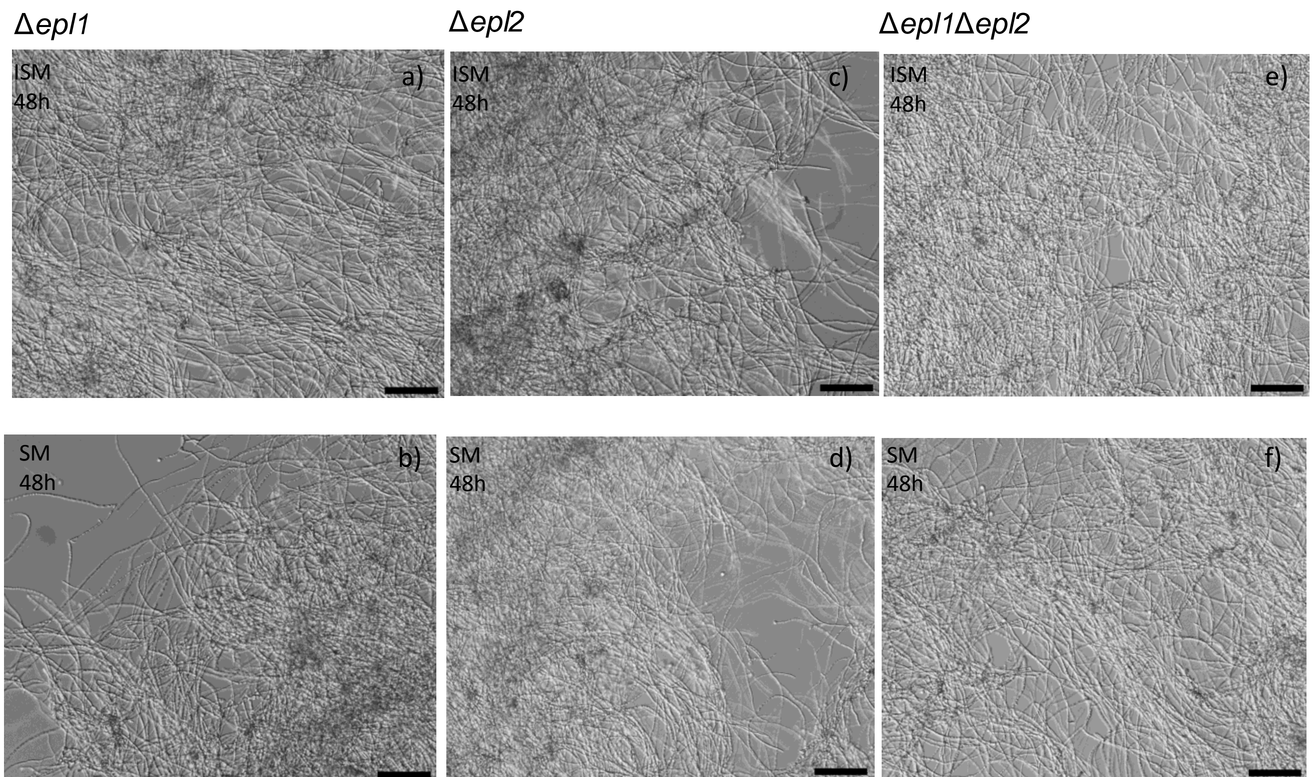


Fig.13: Microscopic analysis of the hyphae formation from *T. atroviride*: *Δepl1* knockout strain (a, b), *Δepl2* knockout strain (c, d) and *Δepl1Δepl2* knockout strain (e, f) on ISM and SM after 48h. Scale bars=100µm.

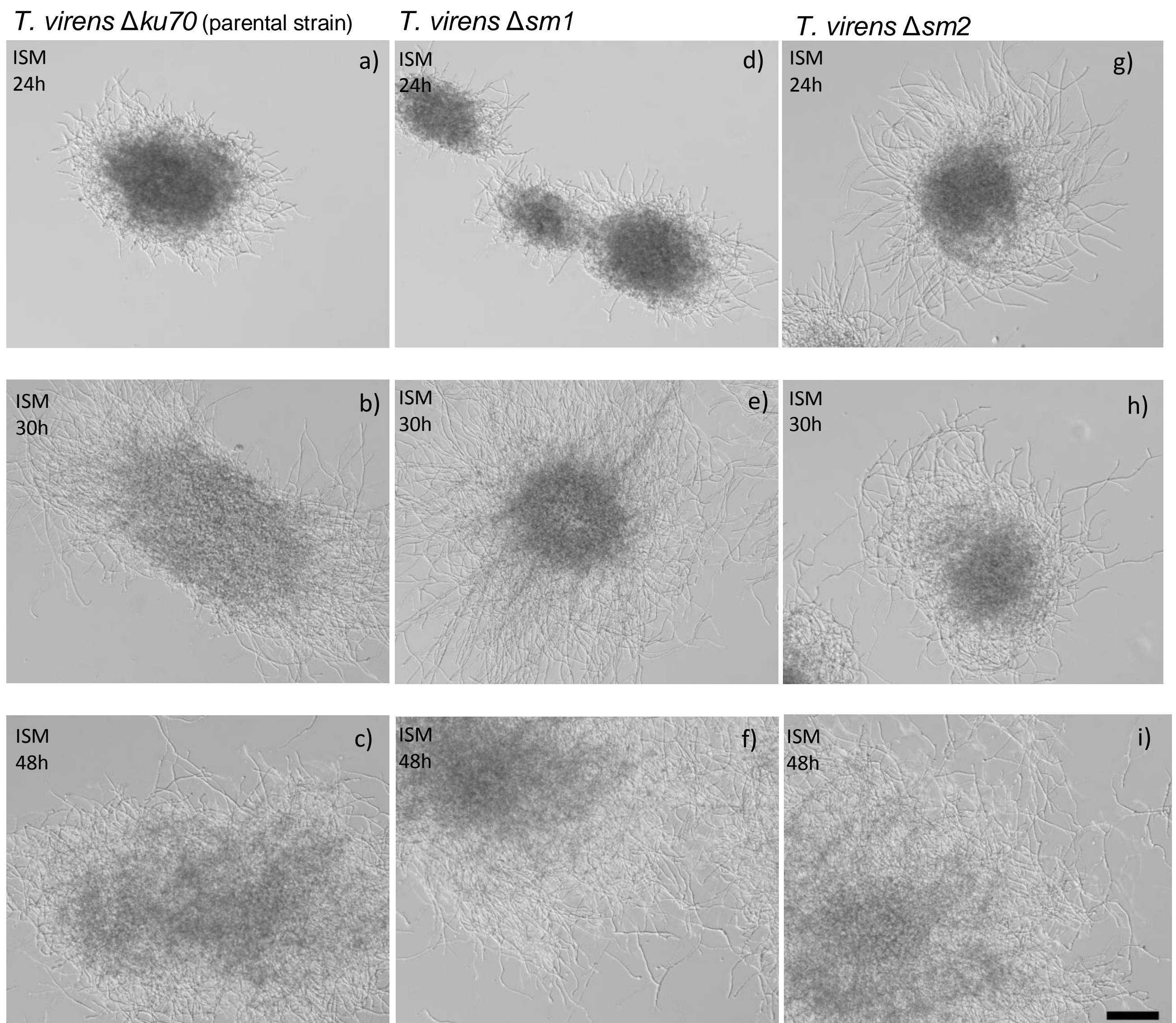


Fig.14: Microscopic analysis of the hyphae formation from *T. virens Δku70* parental strain (a, b, c), *Δsm1* knockout strain (d, e, f) and *Δsm2* knockout strain (g, h, i) on ISM medium after 24h, 30h and 48h. Scale bars=100µm.

Formation of aerial hyphae

Special attention was paid to the formation of aerial hyphae in different experimental setups. Beside the observation of aerial hyphae on normal agar plates, the bridging of gaps of 2 – 10 mm between agar pieces was investigated, either by leaving those gaps dry or by wetting them with medium in order to facilitate hyphal growth. Further, agar plates were inverted and the lids (then at the bottom) were filled with medium and hyphal growth was observed. No

peculiarities in the formation of the aerial hyphae were observed in any of the strains. Thus, in all of these tests no differences between the wild-type/ parental strain and the gene knockout strains from *T. atroviride* and *T. virens* were found.

Hydrophobicity of the mycelium

Furthermore the hydrophobicity of the mycelium of the wild-type/ parental strain and gene knockout strains from *T. atroviride* and *T. virens* was investigated. In Fig. 15 the phenomenon of the hydrophobicity in the parental strain of *T. virens* (Fig. 15 a) and the wild-type of *T. atroviride* (Fig. 15 b) is shown. Due to the hydrophobic properties of the aerial hyphae and spores on agar plates, water-droplets form round drops upon the mycelium instead of flowing apart and seeping into the mycelium. This behaviour was found to be the same in all strains and therefore no difference between the wild-type/ parental strain and gene knockout strains from *T. atroviride* and *T. virens* was found.

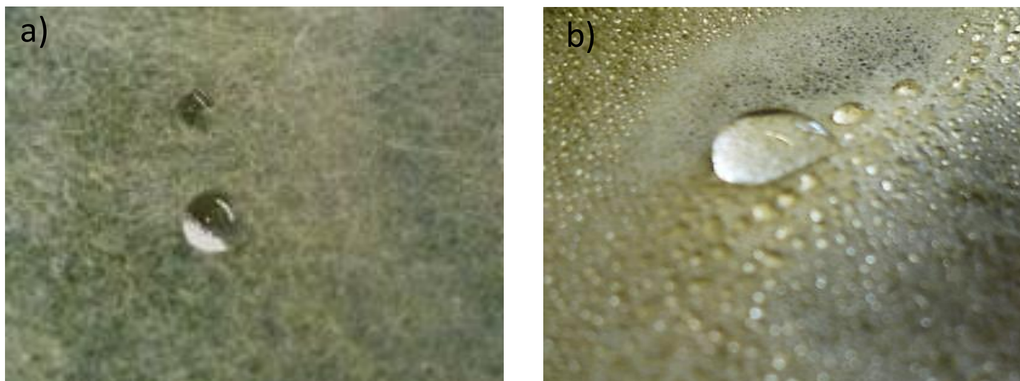


Fig.15: Due to the effect of the hydrophobicity of the mycelium water forms a round droplet upon the mycelium of *T. virens* parental strain a) and *T. atroviride* wild-type b).

In summary, no morphological differences were observed in any of the analyzed knockout strains. Therefore, the results from the morphological analyses showed that the *epl/ sm* knockout strains do not show any morphological defects with respect to hyphal growth on agar plates and in liquid cultivations, sporulation, formation of aerial hyphae and hydrophobicity of the mycelium.

3.2.2. Stress tests

In order to evaluate whether the knockout strains show a phenotype under different forms of stress, growth tests were made under cell wall stress, osmotic stress and desiccation stress conditions. For cell wall stress tests growth on agar plates with SM medium containing 10µl/ ml Calcofluor White or Congo Red was investigated. Calcofluor White binds preferentially to chitin in the fungal cell wall and Congo Red binds mainly to glucans. Both compounds interfere with the correct assembly of the fungal cell wall and abolish fungal growth at higher concentrations. In media supplemented with either Congo Red or Calcofluor White the knockout strains showed the same growth retardation as the *T. atroviride* wild-type and *T. virens* parental strain, respectively. Therefore, these results indicated that deletion of the *epl/ sm* genes does not lead to increased sensitivity towards cell wall stress.

To test the growth during osmotic stress analysis on agar plates containing 2% (w/v) malt extract and 10% (w/v) glycerol or 1M NaCl were made. However, also in these assays there was no difference bewteen the wild-type and gene knockout strains of *T. atroviride* and *T. virens*.

For desiccation stress tests pieces of well sporulated agar plates were cut out from *T.atroviride* and *T.virens* wild-type/ parental strains and gene knockout strains and placed on very thin PDA plates, which were incubated at 28°C with a 12 h/12 h light/dark cycle. These thin plates dry out within ca. three days. The results showed again no difference in growth or sporulation patterns between the wild-type and gene knockout strains from *T. atroviride* and the same results were observed for *T. virens*.

In conclusion, in none of these stress tests a difference between the wild-type/ parental strain and the gene knockout strains was found.

3.2.3. Biomass formation

Biomass measurements form shake flasks cultivations with ISM and SM medium (table 3) for *T. atroviride* and ISM medium for *T. virens* were carried out. Samples were taken after 24h and 48h. *T. atroviride* shows fast growth on SM medium, but rather slow growth on ISM medium, although these two media only slightly differ in their salt composition (table 3). In contrast to that, *T. virens* grows well on ISM medium, but shows hardly any growth on SM medium. Both media were used with 1 % glucose (w/v) as carbon source.

Fig. 16 shows the biomass formation of the wild-type and gene knockout strains from *T. atroviride* on SM (red) and ISM (blue) media at 24 and 48 hours. As expected, in ISM medium after 24h biomass formation of *T. atroviride* was reduced in comparison to SM medium. However, at the time point of 48h biomass formation was nearly identical in both media, indicating that the carbon source had been consumed completely and that maximal biomass formation had been reached. At all time points and on both media no significant differences in biomass content between the wild-type and the gene knockout strains were observed.

Biomass formation of the parental strain and gene knockout strain from *T. virens* is shown in Fig. 17. Again no significant differences were observed, although generation of parental strain biomass was slightly higher than in the gene knockout strains after 24h and at time point 48h biomass formation of the parental strain was slightly lower than in the gene knockout strains.

Therefore, the results of the biomass formation analysis showed no significant difference between wild-type/ parental strain and gene knockout strains from *T. atroviride* and *T. virens.*

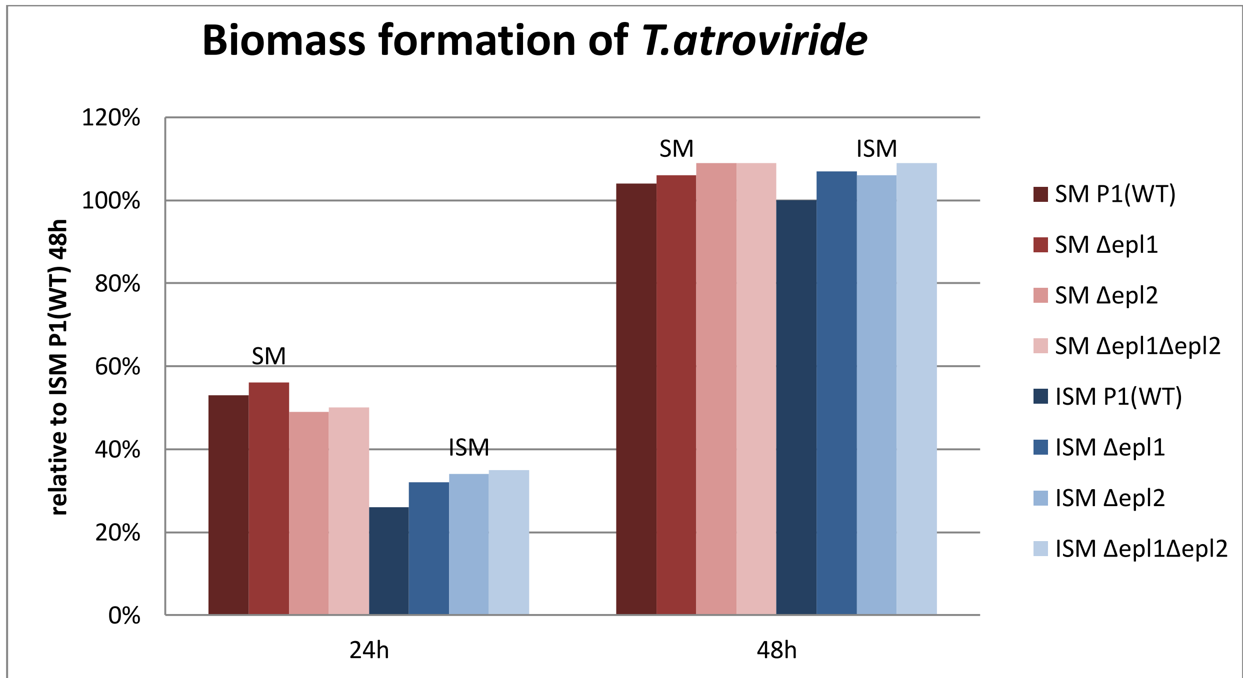


Fig 16: Biomass formation of wild-type and gene knockout strains from *T. atroviride* on SM (red) and ISM (blue) media at the time points 24h and 48h. Averaged values from three independent biological experiments are shown.

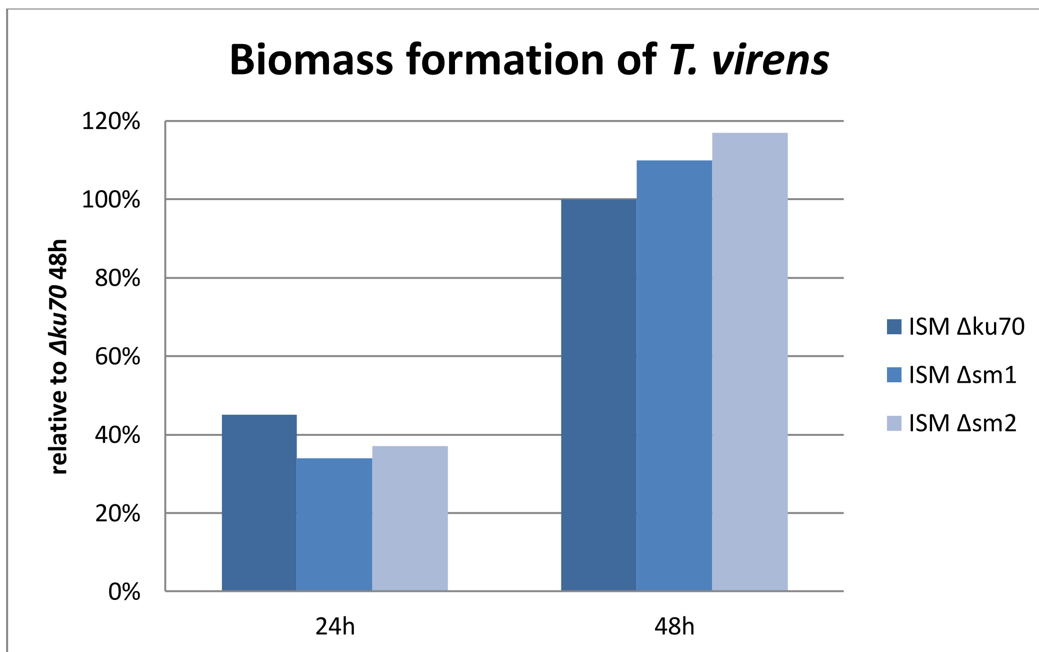


Fig. 17: Biomass formation of parental strain and gene knockout strains from *T. virens* on ISM medium at the time points 24h and 48h.

3.2.4. Mycoparasitism assays

The mycoparasitic potential of *T. atroviride* and *T. virens* against the plant pathogenic fungi *Rhizoctonia solani* and *Botrytis cinerea* was analyzed. These two fungi are commonly used hosts for mycoparasitism assays with *Trichoderma*. Pictures were taken every 24 hours in order to document the mycoparasitic attack and overgrowth of the host fungi by *T. atroviride* and *T. virens* (Fig. 18-21). On the second day the mycelia of *T. atroviride* and the host fungi *R. solani* (Fig. 18) and *B. cinerea* (Fig. 19) had come into physical contact. At this point the mycoparasites started to antagonize and overgrowth the host fungi, as can be seen by the delimitation of growth of the host fungi. The white edge of the mycelium of *R. solani* at the interaction zone with *T. atroviride* indicates the ongoing antagonism and mycoparasitic attack. After five days growth of the host fungi had clearly stopped and *T. atroviride* continued to overgrow the host fungi. Finally, after seven days *T. atroviride* had completely overgrown the host fungus and had sporulated on the complete agar plate, including the mycelium of the host fungus. The same phenomenon was observed for *T. virens* against *R. solani* (Fig. 20) and *B. cinerea* (Fig. 21). There was no difference in the mycoparasitic potential between the wild-type and the gene knockout strains in *T.atroviride* and the same results were found for *T.virens.* Based on these results it can be concluded that the mycoparasitic potential is not affected in any of the *epl/ sm* gene knockout strains. Therefore, the respective proteins do not appear to play a major role in mycoparasitism of *Trichoderma*.

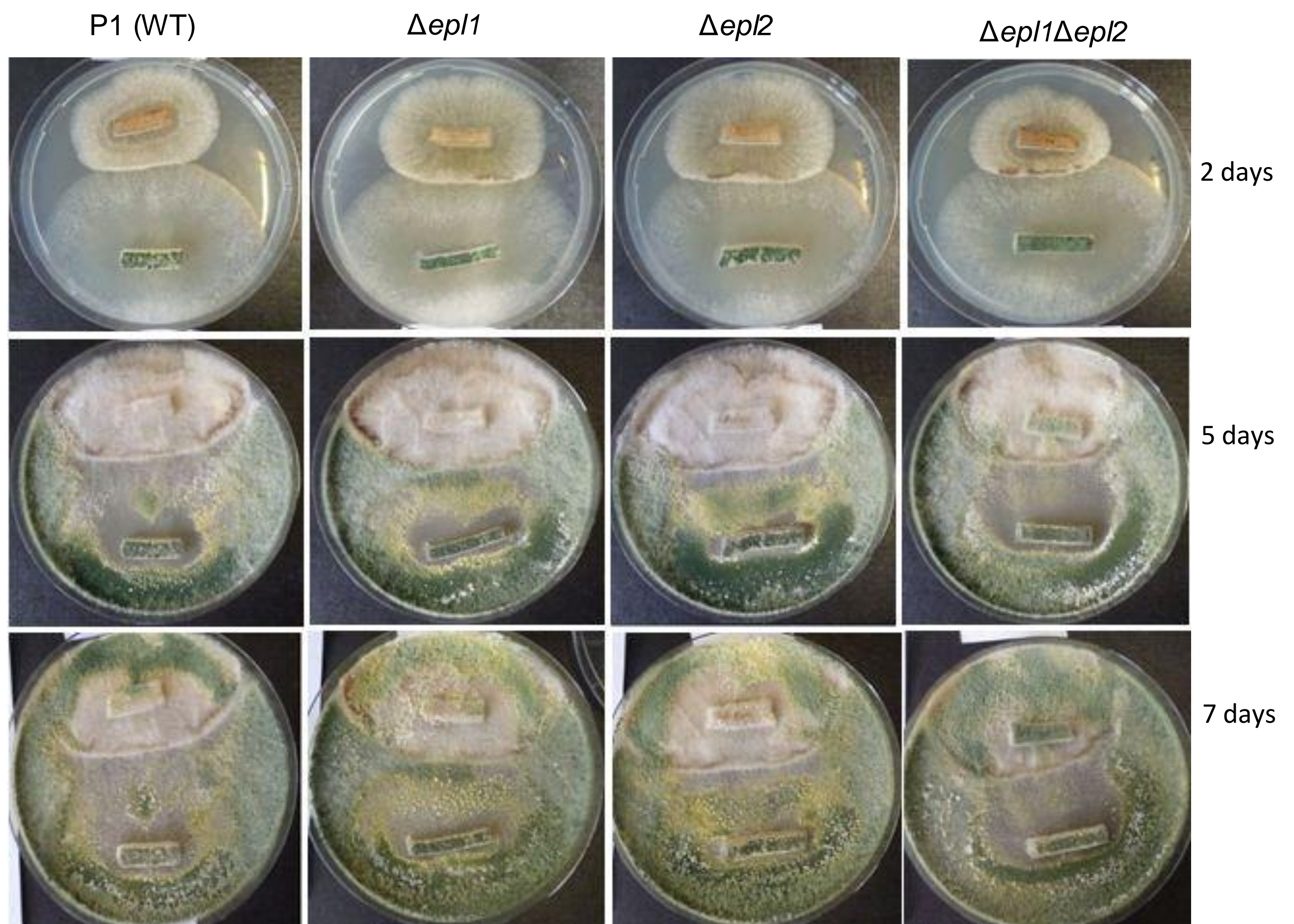


Fig. 18: Mycoparasitic potential from *T. atroviride* wild-type and gene knockout strains against *R. solani.*

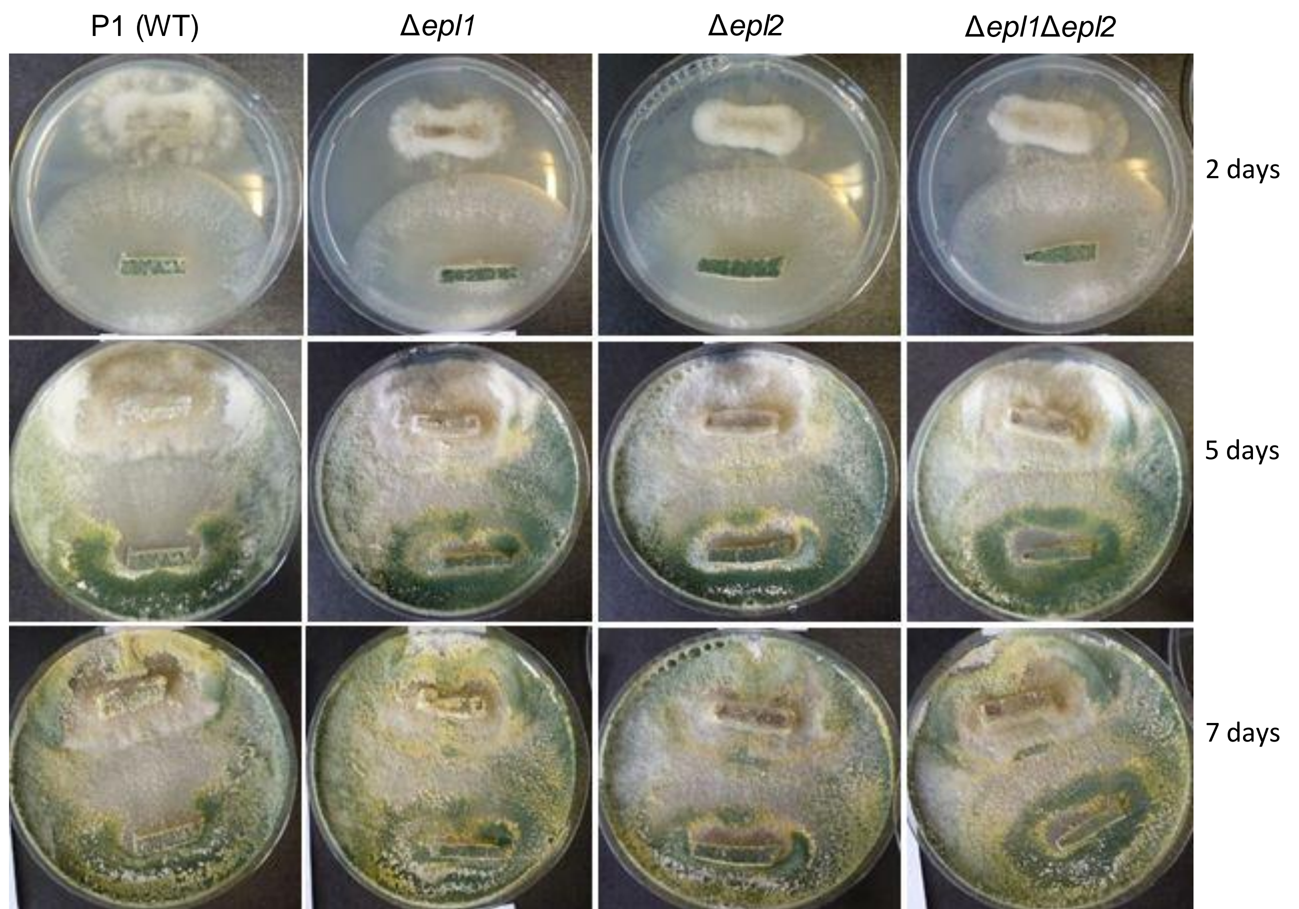


Fig.19: Mycoparasitic potential from *T. atroviride* wild-type and gene knockout strains against *B. cinerea.*

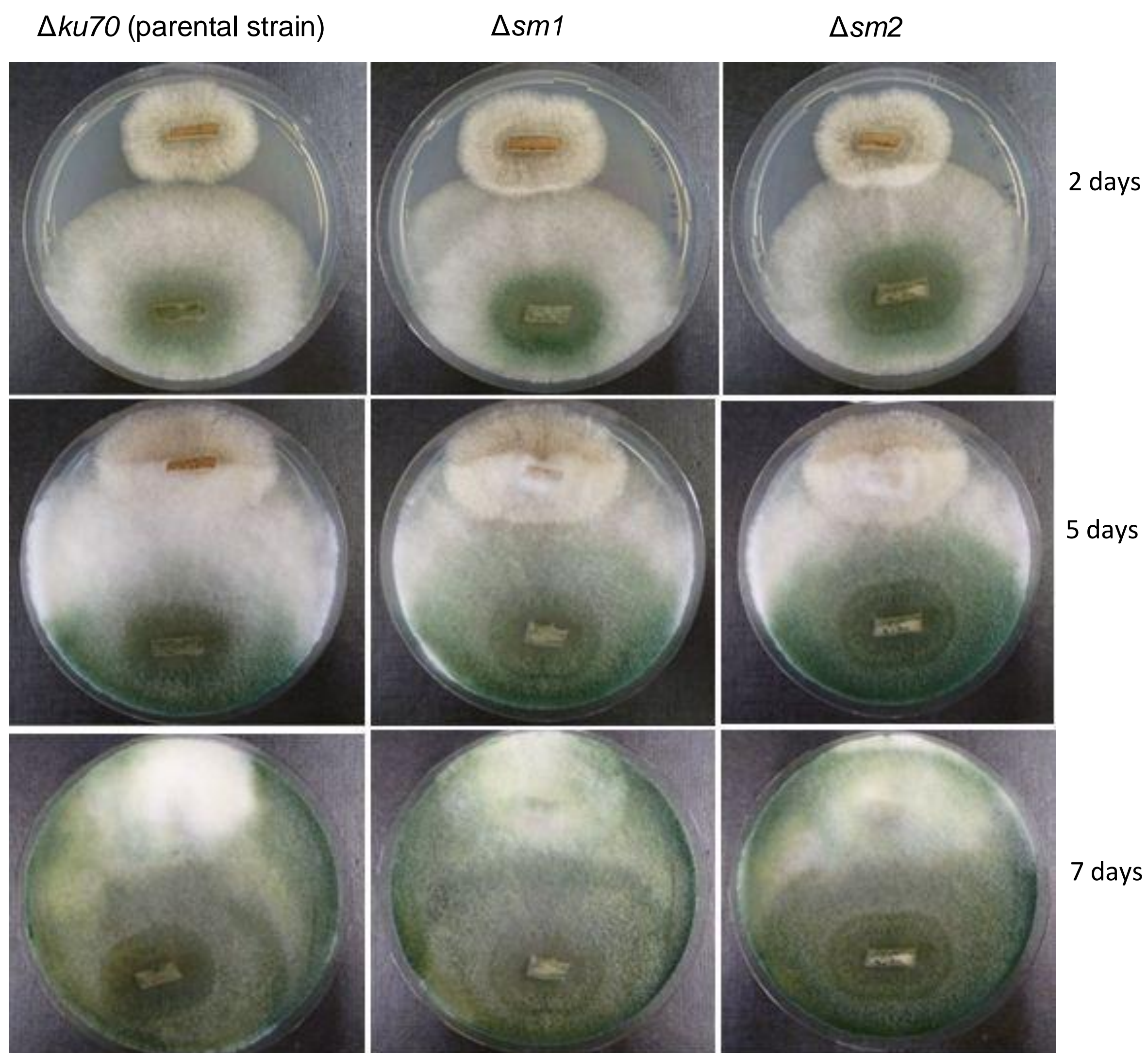


Fig.20: Mycoparasitic potential from *T. virens* parental strain and gene knockout strains against *R. solani*.

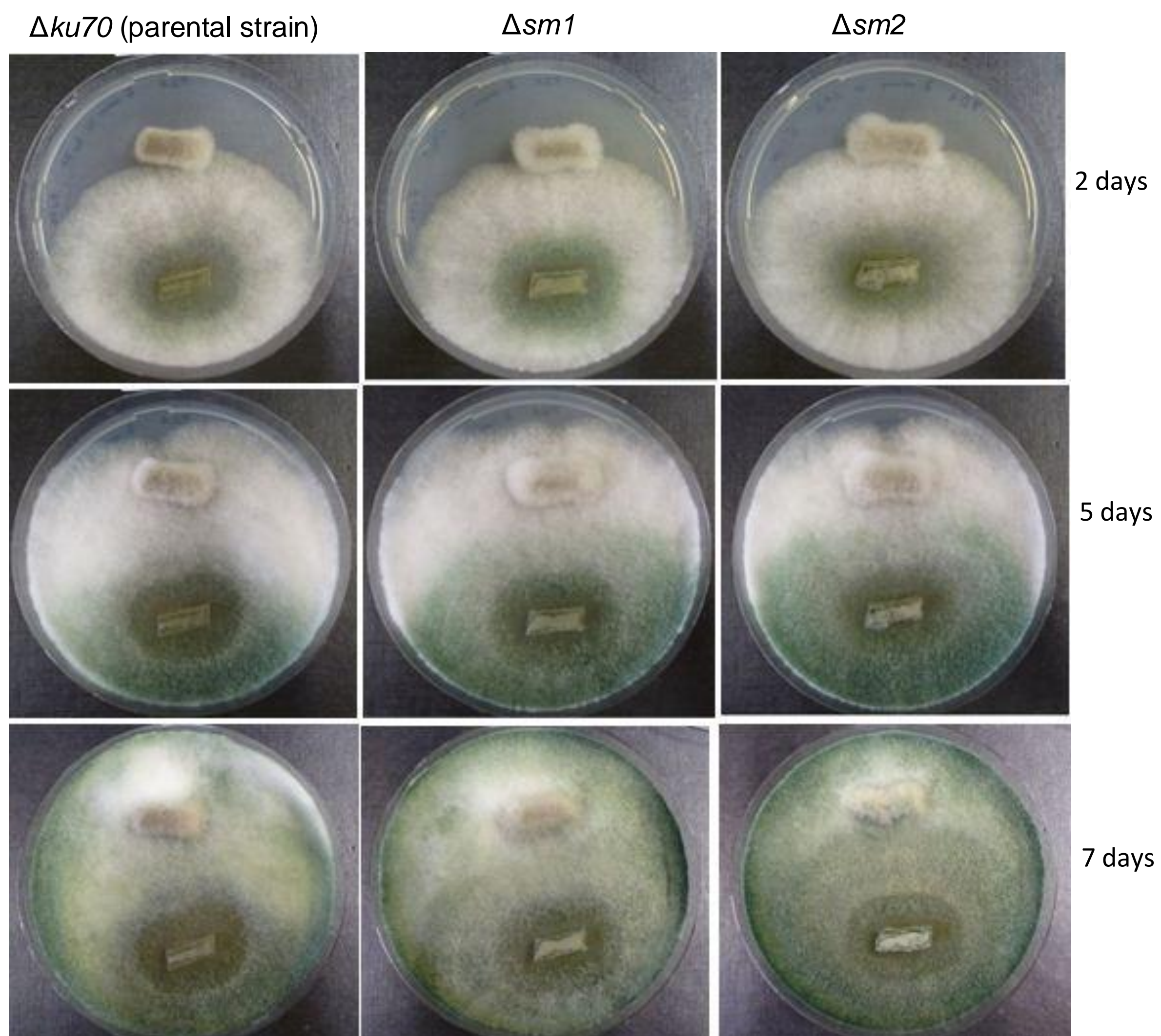


Fig.21: Mycoparasitic potential from *T. virens* parental strain and gene knockout strains against *B. cinerea*.

The finding that the knockout strains of *ep1* and *epl2*, as well as *sm1* and *sm2,* showed no detectable phenotype in any of this analysis suggests that these genes are not essential for fungal growth and development of *T.atroviride* and *T. virens*.

3.3. Gene expression analysis

Cerato-platanin proteins are strongly conserved throughout the fungal kingdom. They are conserved in fungi with different life styles such as pathogenic, parasitic and saprotrophic fungi (Frischmann, Neudl et al. 2012). Most fungal genomes contain two or three genes encoding cerato-platanin proteins.

Trichoderma species, including the mycoparasites *T. atroviride* and *T. virens* have three genes encoding cerato-platanin proteins: *epl1*, *epl2*, *epl3* in *T. atroviride* and *sm1*, *sm2*, *sm3* in *T. virens*. So far it was only known that *epl1* is expressed on different carbon sources, but it had not been studied yet if the expression of *epl1* is associated with certain growth stages and whether cerato-platanin genes are co-regulated. Therefore, in this thesis it was analyzed whether these genes are co-regulated or rather expressed during different growth stages.

Gene expression under different growth conditions was investigated. Shake flasks cultivations were made with two different media for *T. atroviride*, SM and ISM (table 3), and only with ISM for *T. virens* because this species hardly grows on SM medium. ISM and SM media differ only slightly in potassium and phosphate content and both media were used with 1% glucose as carbon source. Samples were taken at the time points of 24h, 30h, 48h and 72h. Additionally, spores were harvested at different maturation stages from sporulating mycelia from PDA plates: white spores, young light-green spores and mature dark-green spores. Afterwards transcriptional profiles were investigated with RT-PCR.

Epl1 was expressed during long periods of hyphal growth on SM medium (Fig.22 b; Fig.23 b). On ISM medium *epl1* was only expressed at later time points in the wild-type and *Δepl2* knockout strain (Fig. 22 a; Fig.23 a) and of course no expression was found in the *Δepl1* strain. In contrast to *epl1*, *epl2* was only found to be expressed during the maturation stages of conidia in the wild-type, whereas *epl1* expression was very low in these samples (Fig. 23 c). For *epl3* only low expression levels in the wild-type on SM medium after 24 and 48 hours were detected (Fig.22 b), during all other tested growth conditions there was no expression

for *epl3* observed. Therefore *epl1* gene is predominantly expressed during hyphal growth whereas *epl2* gene expression occurs only during spore formation and *epl3* is hardly expressed.

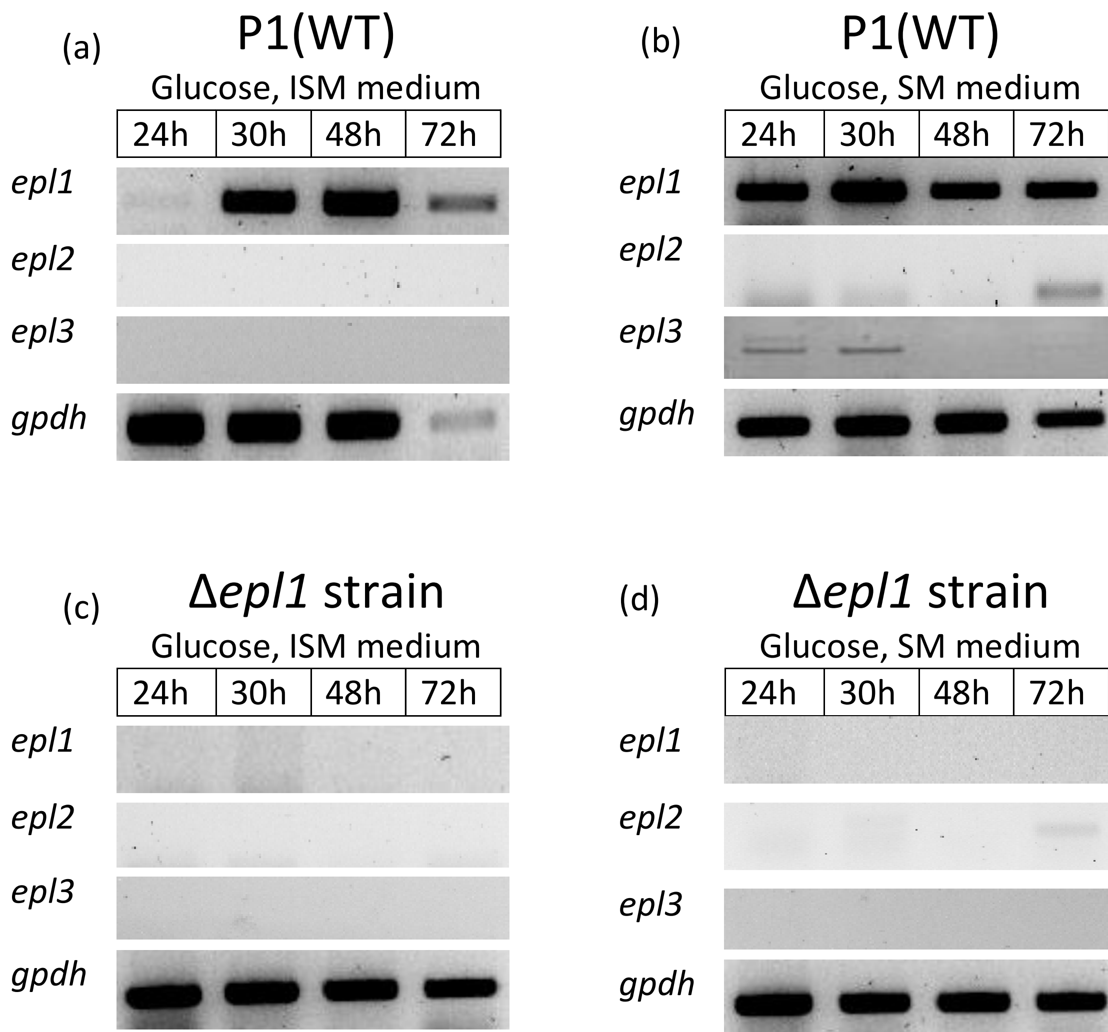


Fig.22: Fig. Gene expression of *epl1*, *epl2* and *epl3* in the P1 wild-type on ISM (a) SM medium (b) and *Δepl1* knockout strain on ISM(c) and SM medium (d) *Gpdh* was used as reference.

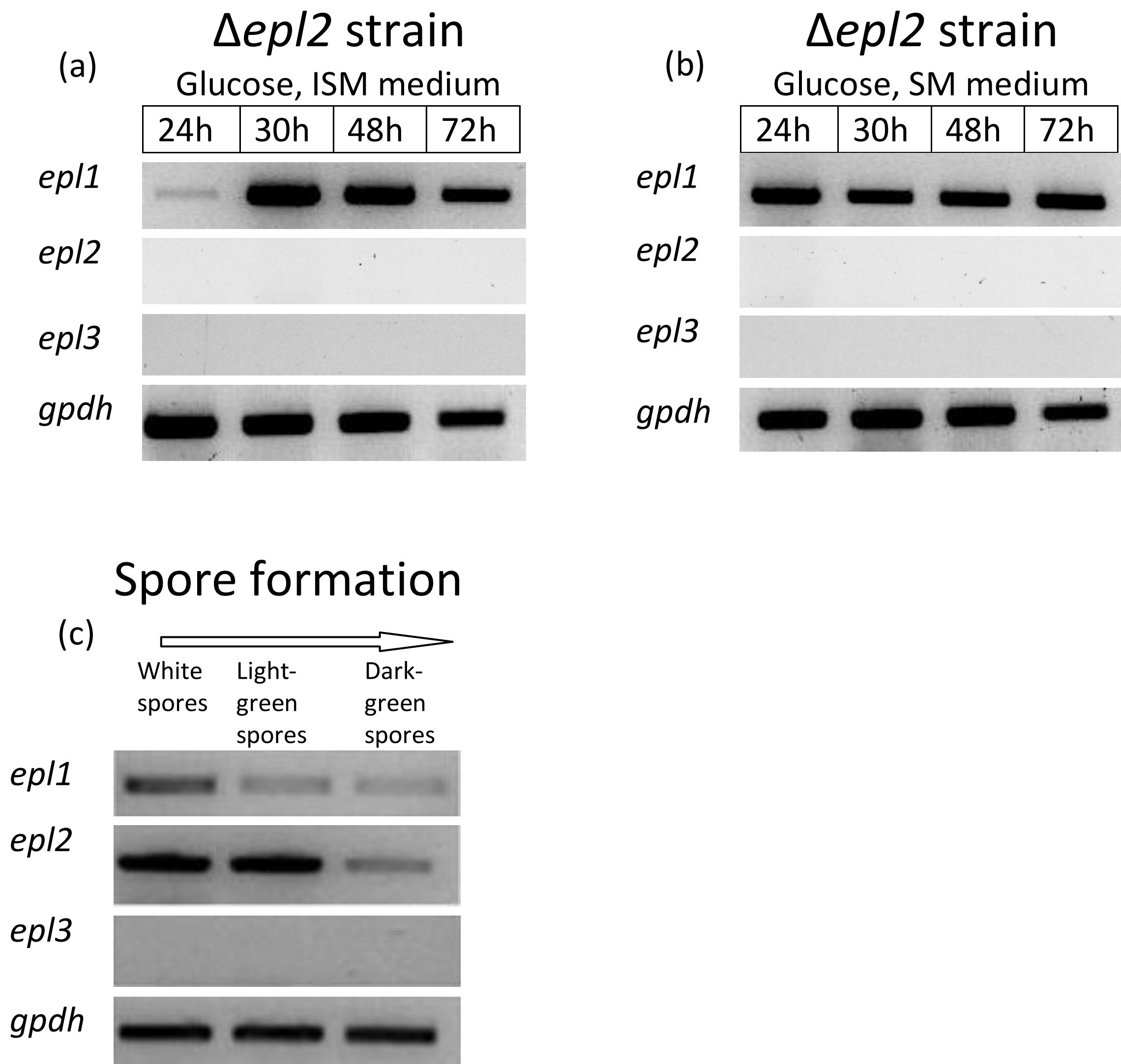


Fig.23: Gene expression of *epl1, epl2* and *epl3* in *Δep2* knockout strain on ISM (a) and SM medium (b). Gene expression of *epl1, epl2*, and *epl3* at different maturation stages in the wild-type is shown in (c). *Gpdh* was used as reference.

From all time points (24h, 30h, 48h, 72h) samples were taken for microscopic analysis. In Fig. 24 images of *T. atroviride* after 72 h on ISM and SM medium are shown. In a previous study with *C. platani* a connection between cerato-platanin (*cp*) gene expression and the formation of chlamydospores was reported (Baccelli, Comparini et al. 2012). Fig.24 b shows increasing numbers of chlamydospores on SM medium after 72 hours in the *T. atroviride* wild-type. However, this effect was also observed in all gene knock out strains (Fig.24 d, f, h). Further, at later time points in shake flask cultivations (48 and 72 h, see Fig. 22 a) *epl1* gene expression is rather decreasing and also *epl2* and *epl3* are not expressed. Therefore it can be concluded that in *T. atroviride* cerato-platanin gene expression is not associated with chlamydospore formation and that the cerato-platanin genes are not essential for chlamydospore formation.

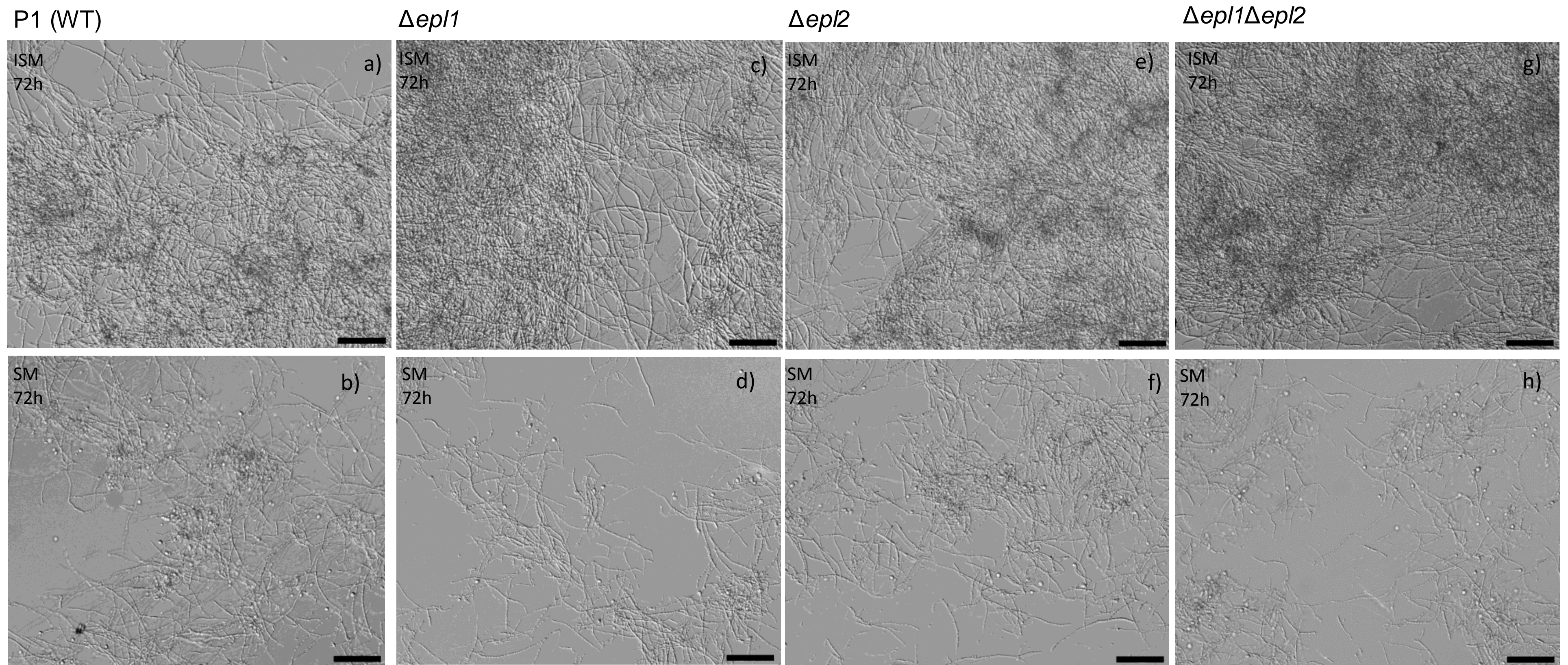


Fig.24: Microscopic analysis of hyphal growth and chlamydospore formation of *T. atroviride* wild-type (a, b), *Δepl1* (c, d), *Δepl2* (e, f) and *Δepl1Δepl2* (g, h) knockout strain on ISM and SM media after 72h. Scale bars=100µm.

In analogy to the gene expression experiments and analysis of chlamydospore formation in *T. atroviride* described above, the same investigations were made for *sm1* and *sm2* gene expression and chlamydospore formation in the parental strain and knockout strains from *T. virens*. *Sm1* gene expression was detected during hyphal growth in the parental strain (Fig.25 a) and Δ*sm2* (Fig. 25 c) knockout strain. No expression was observed for *sm2* and *sm3* at these growth conditions and time points. Additionally, the expression of *sm1*, *sm2* and *sm3* was tested during different maturation stages of conidia in the parental strain. It should be noted that the mycelial mass on sporulating agar plates is much thicker than that from *T. atroviride*. While the respective samples from *T. atroviride* contain mainly conidia, those from *T. virens* are a mixture of hyphae and conidia. In these samples *sm1* was found to be highly expressed in biomass samples with white spores and expression levels got weaker in samples with light and dark green spores. In comparison to that, *sm2* gene expression was relatively low in samples with white spores and grew stronger in light and dark green spores (Fig.25 d). No expression for *sm3* was observed in any of the tested growth conditions.

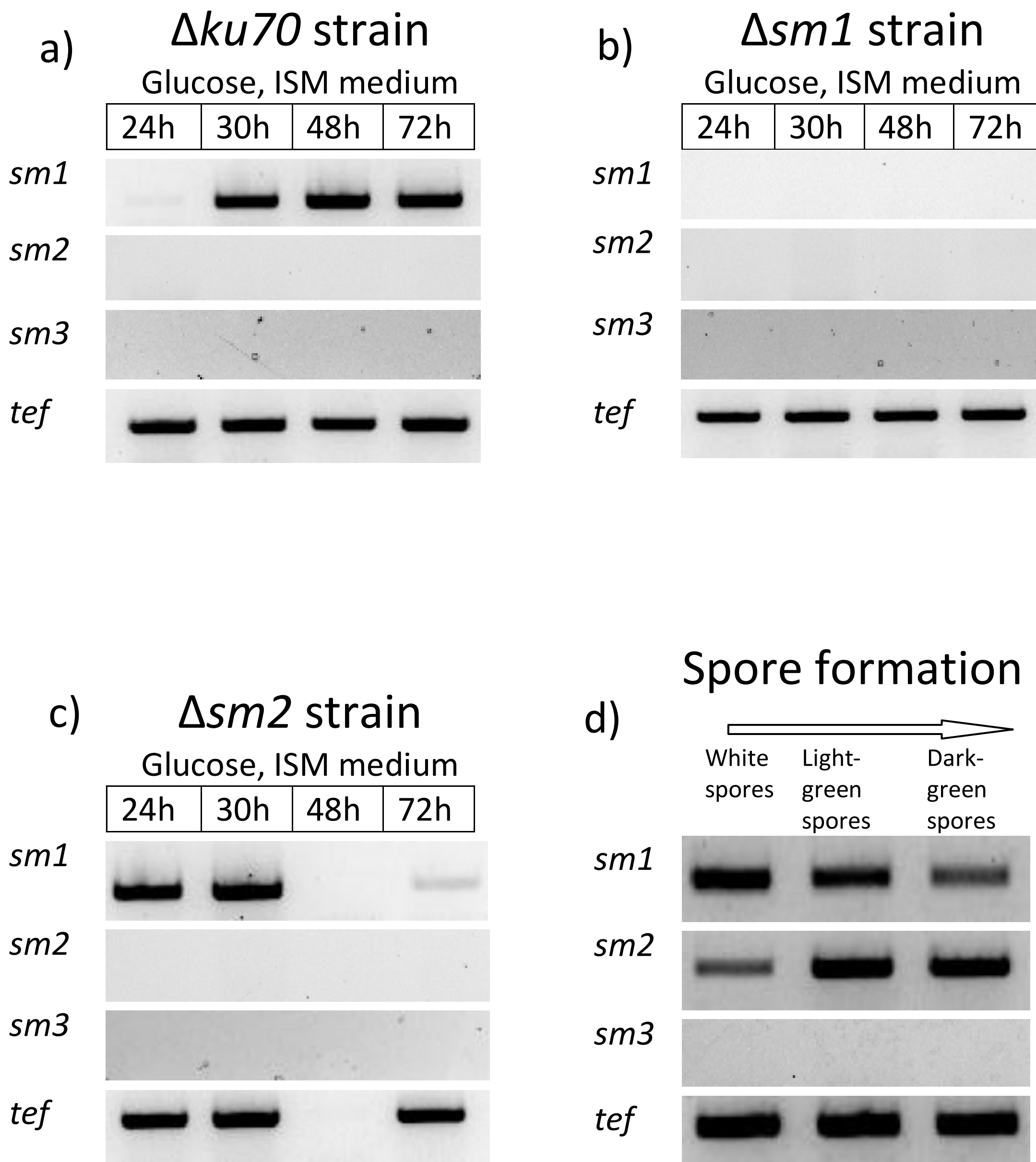


Fig.25: Gene expression of *sm1, sm2* and *sm3* in *Δku70* parental strain (a), Δ*sm1* knockout strain (b) and Δsm2 knockout strain (c).Gene expression of *sn1, sm2*, and *sm3* at different maturation stages in the parental stain is shown in (d). *Tef* was used as reference.

Microscopic analysis of the *T. virens* parental strain and gene knockout strains showed no different in hyphal growth and development at any of the investigatged time points. Interestingly, chlamydospore formation in *T. virens* was far more pronounced than in *T. atroviride*. In *T. virens* large numbers of chlamydospores are already visible in samples from

shake flask cultivations after 48 h (Fig.26 a, c, e) whereas there were only few chlamydospores visible in *T. atroviride* cultivations after 72 h. In general, growth of *T. virens* was more pellet-like than *T. atroviride* (Fig. 26), which could possibly contribute to enhance chlamydospore formation. However, no association of *sm1*, *sm2* or *sm3* gene expression and chlamydospore formation was observed in *T. virens* and there was also no difference between the parental strain and the gene knockout strains.

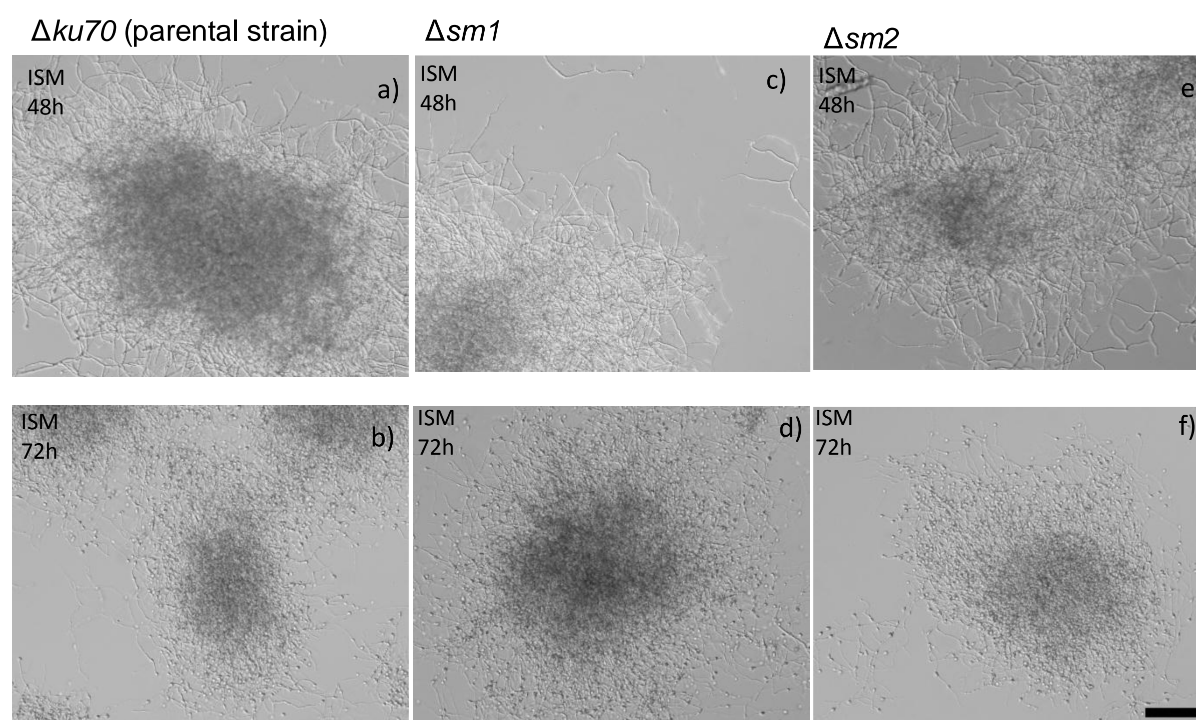


Fig.26: Microscopic analysis of hyphal growth and chlamydospore formation of *T. virens* parental strain (a, b), *Δsm1* (c, d) *and Δsm2* (e, f) knockout strain on ISM medium at the time points 48h and 72h.Scale bars=100µm.

Thus, from these data it can be concluded that chlamydospore formation and cerato-platanin gene expression are not associated in *Trichoderma* and that the cerato-platanins do not have any essential functions in hyphal growth and development in these fungi.

Gene expression of *epl1* and *epl2* was not altered in *Δepl1* and *Δepl2* gene knock out strains in *T. atroviride* and the same results apply in *T. virens* for *sm1* and *sm2*, indicating that the regulation of these genes is not connected in such a way that they compensate for each other in single knockout strains. Further, the cerato-platanin genes are not co-regulated but are expressed during different growth stages. While *epl1*/ *sm1* are predominantly expressed during hyphal growth, *epl2*/ *sm2* are expressed during the maturation of conidia and *epl3*/ *sm3* were hardly/ not expressed under the tested growth conditions. Therefore, these results indicate that the respective proteins are involved in different stages during fungal growth and development.

4. Discussion

The aim of this thesis was to investigate the potential involvement of cerato-platanins in hyphal growth and development in *T. atroviride* and *T. virens* and to analyze their gene regulation. Members of the cerato-platanin family are small, secreted proteins. They are strongly conserved throughout the fungal kingdom. Cerato-platanins are associated with the induction of defense responses in plants and allergic reaction in humans. However, the primary function of cerato-platanins in fungal growth and development is not known yet.

Phenotypic analysis of Δ*epl1,* Δ*epl2,* Δ*epl1*Δ*epl2* strains from *T. atroviride* and of Δ*sm1*, Δ*sm2* from *T. virens* were made in order to evaluate the functions of these genes. The following parameters were assessed: morphology and hyphal development, osmotic/ cell wall stress tests, biomass formation in shake flask cultivations and mycoparasitic assays.

In none of these investigations difference observed between the wild-type/ parental strain and the gene knockout strains from *T. atroviride* and *T. virens* were observed. The strains showed the same growth behaviour on agar plates under different light/darkness cycle conditions and during various stress conditions. There were also no morphological difference with respect to the formation of aerial hyphae and sporulation. Further, the mycoparasitic potential was not altered in the knockout strains.

In previous studies it had been suggested that cerato-platanins might have similar properties as hydrophobins, which are amphiphilic fungal proteins that self-assemble at hydrophobic-hydrophilic interfaces and invert the polarity of surfaces (Limon et al. 2004). However, a recently conducted study shows that EPL1 *in*creases the polarity of solutions and surfaces, which is the opposite effect of what is observed for hydrophobins and therefore cerato-platanins are not hydrophobin-like proteins (Frischmann et al. 2012). Further, analysis of the protein surface by structural modeling using *T. virens* SM1 (PDB 3M3G, unpublished) as template showed that the protein surface is mainly covered with

hydrophilic protein residues and no large hydrophobic areas were found (Frischmann et al. 2012).

The paper by Frischmann et al. contains also some of the results from this diploma thesis, namely the analysis of the *T. atroviride* gene knockout strains and the transcriptional profiling of *epl*-genes of *T. atroviride*. The findings that the hydrophobicity of the mycelium was not altered in the gene knockout strains of *T. atroviride* and *T. virens* is in agreement with the findings that cerato-platanin proteins do not behave like hydrophobins.

Based on its NMR-structure, the features of CP indicated similarities to Barwin-like endoglucanases and related types of plant expansins (de Oliveira, Gallo et al. 2011). Expansins unlock the network of cell wall polysaccharides, permitting turgor-driven cell enlargement (Cosgrove 2000). It is therefore tempting to speculate that cerato-platanins have functions that are related to plant expansins. The function of plant expansins has been elegantly studied with extensometer assays (Cosgrove 2000). Although it would be an interesting approach to study this aspect in fungi, such types of experiments have not been established for fungal cell walls yet. Cerato-platanin proteins from pathogenic fungi were previously reported to be human allergens and to induce plant defense responses. Interestingly, this is another analogy that can be detected to expansins, because the group of beta expansins also contains grass pollen allergens (Cosgrove 2000). The results that gene knockout strains of *epl1* and *epl2*, as well as *sm1* and *sm2,* showed no detectable phenotype suggests that these genes are not essential for fungal growth and development of *T.atroviride* and *T. virens*.

However, in view of the fact that cerato-platanins occur in all filamentous fungi and that orthologues of EPL1 are strongly conserved throughout the fungal kingdom and are highly expressed under different growth conditions, the question still remains what the function of these proteins is. It is possible that the loss of function of these genes in knockout strains is compensated by other mechanisms. Another possibility is that these

proteins are rather involved in interaction processes with other organisms than in growth of the development of the fungal mycelium which secretes them at a given time point. Such interactions could range from an effect on germination, growth or sporulation of other fungi (either of the same species or other species) to interactions with other organisms, e.g. plants. Although these aspects will need to be tested to further elucidate the functions of cerato-platanin proteins, it should be noted that the results from this thesis showed that the mycoparasitic potential was not affected in knockout strains. However, mycoparasitism is known to be a mixture of many different mechanisms (Kubicek, Herrera-Estrella et al. 2011) and it has so far also for other components that are involved in mycoparasitism, e.g. hydrolytic enzymes or secondary metabolites, been difficult to demonstrate essential roles. With respect to the involvement of cerato-platanins in fungal-plant interactions, it is known that these proteins from plant pathogenic fungi as well as plant beneficial fungi such as *Trichoderma* spp., induce plant defense responses. Although *T. virens* SM1 has been described to be a major determinant in this process, it is questionable whether this is a function of these proteins or rather a side-effect due to their abundant expression, which makes them easily recognizable targets for plants.

In *Trichoderma*, so far only SM1 and EPL1 have been analyzed in detail (Djonovic, Vargas et al. 2007; Vargas, Djonovic et al. 2008). In this study, gene expression of the cerato-platanin encoding genes *epl1, epl2, epl3* from *T. atroviride* and *sm1, sm2, sm3* from *T. virens* was investigated to find out whether these genes are co-regulated or rather expressed during different growth stages. The outcome that *epl1/ sm1* were predominantly expressed during hyphal growth shows that the expression is related to certain developmental phases of hyphal growth, although no significant differences in hyphal morphology could be detected under the microscope. In contrast to *epl1/ sm1*, high levels of *epl2/ sm2* expression were only observed during conidiation. When conidia from different maturation stages were harvested and analyzed, *epl2/ sm2* was expressed during the maturation stages of conidia, whereas *epl1/ sm1* expression was very weak in

these samples. For *epl3* weak expression was detected on SM medium after 24 and 48 h, but during all other tested growth conditions no expression of *epl3* was found and for *sm3* no gene expression was observed at all.

As mentioned above, for EPL1 and SM1 it is known that they induce plant defense responses. The respective genes, *epl1* and *sm1*, are expressed during long times of hyphal growth and they are produced in high quantities. The results from this thesis showed that gene expression of *epl2* is very weak during hyphal growth and therefore only small amounts of EPL2 are likely to be present. Therefore it can be suggested that, unlike EPL1, EPL2 is not available in large quantities and consequently the plant might not recognize this protein as efficiently as EPL1. The same applies for *epl3*, which is hardly expressed at all and also its aa-sequence differs strongly from EPL1 and EPL2. This could indicate that EPL2 and EPL3 might not play a major role in the induction of plant defense response.

The findings that the gene expression of *epl1/ sm1* and *epl2/ sm2* was not altered in *Δepl1/ Δsm1* and *Δepl2/ Δsm2* strains indicate that the regulation of these genes is not connected in such way that they compensate for each other in single knockout strains. Therefore these genes are clearly not co-regulated and these results indicate that the respective proteins are involved in different stages during fungal growth and development.

In *C. platani* a connection between cerato-platanin expression and chlamydospore formation was reported (Baccelli et al. 2012). In this thesis we re-investigated the gene expression of *epl1/ sm1* and *epl2/ sm2* under growth conditions related to the formation of chlamydospores. In *T. virens* chlamydospore formation was far more pronounced than in *T. atroviride*. Microscopic analysis of mycelia showed that in submerged cultivations of *T. atroviride* chlamydospores are formed at late time points. In *T. virens* chlamydospore formation was far more pronounced and started at earlier time points. However, the phenomenon was observed in the wild-type/ parental strain and all knockout strains.

Therefore it can be concluded that neither cerato-platanin gene expression levels correlate with chlamydospore formation in *Trichoderma*, nor are these genes essential for this process.

The findings from this thesis expand our knowledge about the fungal cerato-platanin protein family. Several aspects that had been previously suggested to be connected with the function and properties of cerato- platanin proteins were addressed. The results showed, somewhat surprisingly, that despite the abundant expression of EPL1/SM1, cerato-platanin proteins do not appear to have any major functions in these developmental stages and processes. Although further studies will be necessary to elucidate the functions of cerato-platanin proteins, the results from this thesis will contribute to advance our understanding of this protein family and their roles in fungal biology.

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6. Danksagung

Mein Dank gilt Dr. Bernhard Seiboth, der mir dieses interessante Thema zur Verfügung gestellt hat.

Besonders herzlich bedanke ich mich bei meiner Betreuerin Dr. Verena Seidl-Seiboth für die intensive Betreuung, die vielen Methoden die ich kennen lernen durfte, sowie die großartige Unterstützung in der praktischen Arbeit und beim Schreiben dieser Arbeit.

Ich möchte mich bei meinen Laborkollegen für den Spaß während meiner Arbeitszeit bedanken, besonders bei Alexa und Doris für die Unterstützung während dieser Zeit und die vielen lustigen Stunden.

Großes Dankeschön gilt auch meinem Freund Harald, der immer für mich da war.

Zum Schluss möchte ich mich bei meiner Familie für die Unterstützung bedanken.