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Transcriptome Analyses of the Nematode-trapping Fungus *Monacrosporium haptotylum*

Margareta Tholander



LUND
UNIVERSITY

**Doctoral thesis
2007**

En doktorsavhandling vid ett svenskt universitet består antingen av en monografi eller en samling artiklar. I det senare fallet utgörs avhandlingen formellt av introduktionen, vilken sammanfattar ingående artiklar. Dessa har antingen redan publicerats eller är i manuskriptform i olika stadier (i tryck, insänt eller utkast).

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarises the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in ms).

Front page: Adhesive knobs from *Monacrosporium haptotylum*. The knob cell is a spherical cell formed on the apex of a hyphal stalk. The micrograph is reproduced from Nordbring-Hertz *et al.*, (1995), courtesy of Birgit Nordbring-Hertz and IWF, Göttingen.

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1. List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I. Ahrén, D*, Tholander, M*, Fekete, C*, Rajashekar, B., Friman, E., Johansson, T. and Tunlid, A. (2005). Comparison of gene expression in trap cells and vegetative hyphae of the nematophagous fungus *Monacrosporium haptotylum*. *Microbiology*. 151: 789-803.
*These authors contributed equally to this work.

- II. Tholander, M., Kiel, J.A.K.W., van der Klei, I.J., Veenhuis. M. and Tunlid, A. Characterization of a *PEX11* homolog in the nematophagous fungus *Monacrosporium haptotylum*. (Manuscript).

- III. Fekete, C*, Tholander, M*, Rajashekar, B., Ahrén, D., Friman, E., Johansson, T. and Tunlid, A. Paralysis of nematodes: shifts in the transcriptome of the nematode-trapping fungus *Monacrosporium haptotylum* during infection of *Caenorhabditis elegans* (Manuscript).
*These authors contributed equally to this work.

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2. Abstract

Nematode-trapping fungi are soil-living organisms with the ability to infect and kill nematodes. These fungi have developed specialized infection structures, traps for the capture of nematodes. To be able to identify genes in nematode-trapping fungi that are involved in the infection of nematodes complementary DNA (cDNA) microarrays were constructed. The microarrays were printed with cDNA reporters obtained from expressed sequence tag (EST) sequencing of four different cDNA libraries representing the mycelium and traps (knobs) of the nematode-trapping fungus *Monacrosporium haptotylum*, as well as the fungus infecting the nematode *Caenorhabditis elegans* for 4 h and 24 h, respectively. In total, 8,466 EST sequences were generated from these libraries. Following an assembly of these sequences, 3,518 clones were amplified and printed on the array, 2,822 of fungal, 540 of worm, and 156 of unknown origin. In order to identify genes that are expressed and regulated during the development of traps, total RNA was isolated from knobs and mycelium and used in the hybridization of the cDNA array. In spite of the fact that knobs and mycelium were grown in the same medium, a total of 23.3% (657 of 2,822) of the gene representatives were differentially expressed in knobs as compared to mycelium. Several of the genes that were regulated in knobs showed sequence similarities to genes involved in regulating morphogenesis and cell polarity in fungi and to genes involved in development of plant-pathogenic infection structures (appressoria). Typical for trap cells in nematode-trapping fungi is the presence of numerous dense bodies which are organelles related to peroxisomes. The transcriptional profiling of *M. haptotylum* traps identified one gene representative with homology to the peroxisomal membrane protein Pex11p from *S. cerevisiae*. This homolog was significantly up-regulated in knobs as compared to mycelium. The Pex11p protein is known to have a role in peroxisome proliferation in yeast. In order to further characterize the *M. haptotylum* PEX11 homolog, the full length cDNA was cloned and expressed in the yeast *Hansenula polymorpha*. The result showed that the *M. haptotylum* Pex11p could not functionally complement the *Apex11* mutant of *H. polymorpha*. In the third study, the changes in the transcriptome of *M. haptotylum* were followed during the various stages of the infection. Isolated knobs from *M. haptotylum* were used in the infection of the nematode *C. elegans*. RNA was extracted from the infection samples at four different time-points corresponding to the various stages of the infection; adhesion and penetration (1 h and 4 h) and digestion (16 h and 24 h) and was subsequently used for hybridization to the cDNA array. RNA isolated from axenic knobs and non-infected nematodes were used as a reference sample in the hybridization of the cDNA array. In total, 58% (1,562) of the fungal genes represented on the array, were regulated in at least one of the stages of the infection. The most dramatic shift in the fungal transcriptome occurred after 4 hours, a time-point associated with the penetration of the nematode.

3. Introduction

As parasites, nematodes have a substantial impact on human welfare and economy. Plant-parasitic nematodes cause significant damage to a wide variety of crops. The economical losses due to plant-parasitic nematodes have been estimated to billions of US\$ every year (Dong and Zhang, 2006). Chemical nematicides are widely used for the control of plant-parasitic nematodes because of their non-selective nature and effectiveness in controlling nematodes. However, chemical nematicides are associated with environmental hazard, high costs, limited availability in many developing countries and diminished effectiveness following repeated applications (Dong and Zhang, 2006). Since these chemicals have a broad biocidal activity they will have harmful effects also on beneficial nematodes, insects and other soil animals. In addition nematicides such as methyl bromide, dibromochloropropane (DBCP) and ethylene dibromide (EDB) have recently been withdrawn from the market in most countries as a result of their harmful effects on humans and the environment (Oka *et al.*, 2000).

The animal-parasitic nematodes are causing severe infections of grazing domestic animals resulting in reduced efficiency of livestock production (Stear *et al.*, 2007). Control of animal-parasitic nematodes relies upon treatment with drugs called anthelmintics. This type of control is threatened by the widespread evolution of drug resistance in nematode populations (Bartley *et al.*, 2004). Several methods have been used in combination with chemicals to control the parasitic nematodes. These include grazing management and vaccination to control animal-parasitic nematodes and crop rotation and the use of resistant crops to control plant-parasitic nematodes. However, the continuing environmental problems associated with the use of nematicides and the resistance development against anthelmintics have introduced a sense of urgency into the search for alternative methods of nematode control (Kerry, 2000). In this aspect, the nematophagous fungi are attracting major interest, since they have the potential to be used as biological control agents against plant- and animal-parasitic nematodes (Larsen, 2000; Jansson and Lopez-Llorca, 2004).

The principle of biological control is based on the fact that a natural enemy is able to reduce the population number of the host. In nature, the population densities of nematophagous fungi are most often not high enough for successful control of parasitic nematodes. There are two

general ways of increasing the population of nematophagous fungi in soils: addition of large amounts of fungal inoculates to the soil; or stimulation of the activity of existing fungi using different amendments or treatments of the soils. There have been many studies, both in the laboratory and in the field, demonstrating the feasibility of using nematophagous fungi to control plant- and animal-parasitic nematodes (Atkins *et al.*, 2003; Mendoza-DE Gives *et al.*, 2006; Stromberg and Gasbarre, 2006). However, the results vary and there are few commercial formulations available on the market (Brand *et al.*, 2004). The nematode-trapping fungus *Peacilomyces lilacinus* has been used in several formulated biological nematicides. One such commercial agent is Bioact®WG (<http://www.prophyta.com>) which contains spores of *Peacilomyces lilacinus* in the form of water dispersible granules. Bioact®WG controls common plant-parasitic nematodes and does not affect beneficial insect pathogenic nematodes.

There are many factors that could presumably affect the effectiveness by which nematophagous fungi could control parasitic nematodes in soils. Due to the fact that many nematophagous fungi can grow both as saprophytes and parasites, the population sizes of these fungi depend not only on the population densities of nematodes, but also on the competition for nutrients with other saprotrophs present in soils. One way to improve the competitive potential of nematophagous fungi in soils is to develop better formulations for adding the biological control agent (Jaffee and Zasoski, 2001). Another possibility is to use genetic engineering to improve the pathogenicity and survival of the introduced fungus. Until recently, however, such a strategy has been precluded due to the lack of information about the molecular background of fungal infection of nematodes. The first, and so far only report in this area, is a study showing that strains with improved pathogenicity of a nematophagous fungus could be formed by adding additional copies of a gene encoding a subtilisin with nematotoxic activity (Ahman *et al.*, 2002). Mutants containing additional copies of the subtilisin gene possessed a higher number of traps and had an increased speed of capturing and killing of nematodes (Ahman *et al.*, 2002).

The aims of this thesis are:

- To identify groups of fungal and nematode genes that are expressed during the infection process.
- To assign putative functions of these groups of genes (fungal and nematode) using bioinformatics tools.
- To identify components of the defence system of *C. elegans* that are expressed during the infection.

4. Nematode-trapping fungi

Nematophagous fungi consist of a taxonomically diverse range of fungi all sharing the ability to infect and digest nematodes. More than 200 species of nematophagous fungi have been described so far. Based on their mechanisms for infecting nematodes, the nematophagous fungi can be divided into three main groups (Barron, 1977):

Nematode-trapping fungi

Endoparasitic fungi

Egg and cyst parasitic fungi

The three categories of fungi differ in their dependency on nematodes for growth and survival (Dijksterhuis *et al.*, 1994). Nematode-trapping fungi can grow as saprophytes in soils using complex organic matters as substrates. They enter the parasitic stage by developing special hyphal structures called traps, such as nets, knobs, branches or rings, in which nematodes are captured mechanically or by adhesion. The killed nematodes provide the fungi with an additional nutrient source that is rich in nitrogen. Accordingly, it has been proposed that the parasitic habit give the fungi a competitive advantage over many other fungal saprotrophs growing in soil environments that are characterized by low levels of nitrogen (Barron, 1992). The endoparasitic fungi are often obligate parasites and are dependent on nematodes for their survival. They infect nematodes with adhesive or non-adhesive spores which are swallowed

by the nematode or adhere to the nematode surface. The third category is the egg and cyst parasitic fungi that parasitize these non-motile stages of nematodes with their hyphal tips (Barron, 1977). The egg and cyst parasitic fungi can also survive as saprophytes in soils.

The nematode-trapping fungi are the best known group among the nematophagous fungi probably due to their remarkable morphological adaptations and their effective infection of nematodes. This group includes the species of nematophagous fungi where the mechanisms of infection have been most extensively investigated (Dijksterhuis *et al.*, 1994).

Diversity of trapping structures

The traps of nematode-trapping fungi are formed on vegetative hyphae and can be produced either spontaneously or be induced in response to signals from the environment, including peptides and other compounds secreted by the host nematode (Dijksterhuis *et al.*, 1994). One inducing factor is living nematodes, which triggers formation of traps by touching the mycelium. In addition the nutritional levels in the environment are important, especially the ratio of carbon/nitrogen (Dijksterhuis *et al.*, 1994). One of the most common nematode-trapping fungi in soil is *Arthrobotrys oligospora* which forms three dimensional nets (Figure 1). The genus *Monacrosporium* possesses adhesive branches or adhesive knobs. *Monacrosporium haptotylum* produces both adhesive knobs and non-constricting rings (Nordbring-Hertz *et al.*, 1997). A number of species of nematode-trapping fungi e.g. *Arthrobotrys brochophaga* and *Arthrobotrys dactyloides* produce a type of mechanical trap called the constricting ring. These rings appear to be the most highly developed and most remarkable trapping structures. They are an active form of trap; when a nematode enters, the ring cells swell and the ring closes the opening so that the nematode can not escape. The closing takes only 0.1 second and is triggered by the touch of the nematode (Pramer, 1964). Many of the *Arthrobotrys* species do not form traps spontaneously whereas the trapping structures of other fungi, such as branches, knobs and constricting rings are often formed spontaneously (Nordbring-Hertz *et al.*, 1997).

Phylogeny of nematode-trapping fungi

Phylogenetic analyses of 18S ribosomal DNA (rDNA) sequences have shown that a majority of the identified species of nematode-trapping fungi belong to a monophyletic clade among

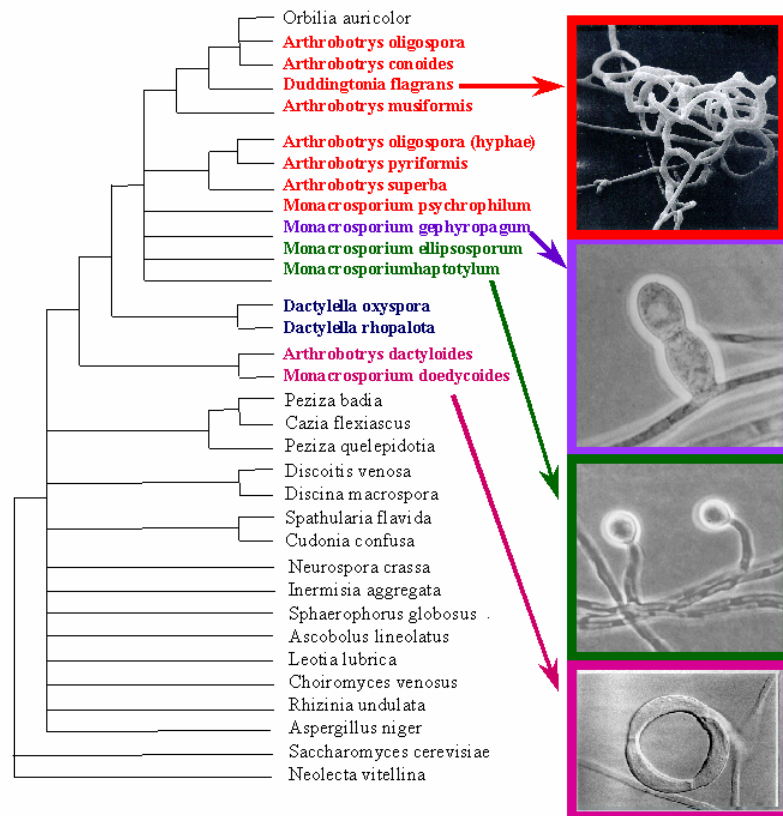


Figure 1. A cladistic tree showing the relationship between various nematode-trapping fungi and other ascomycetes based on 18S rDNA sequences. Only branches with bootstrap support values above 50 are shown. Note that the nematode-trapping fungi form a monophyletic clade among an unresolved cluster of apothecial ascomycetes. The phylogenetic pattern within the clade of nematode-trapping fungi is concordant with the morphology of the traps. The tree is redrawn from Ahren *et al.*, (1998). Shown on the micrographs from top to bottom are adhesive net (*Arthrobotrys oligospora*), adhesive branch (*Monacrosporium gephyropagum*), adhesive knob (*Monacrosporium haptotylum*) and constricting ring (*Arthrobotrys brochophaga*). The micrographs are reproduced from Nordbring-Hertz *et al.*, (1995), courtesy of Birgit Nordbring-Hertz and IWF, Göttingen.

the apothecial ascomycetes (Figure 1) (Liou and Tzean, 1997; Ahren *et al.*, 1998; Hagedorn and Scholler, 1999). As can be seen from Figure 1, the phylogenetic pattern of the nematode-trapping fungi is linked to the morphology of the trapping structures. Three lineages of nematode-trapping fungi are supported from the tree; the species with constricting rings, the non-parasitic species and the species having various adhesive structures (nets, hyphae, knobs and non-constricting rings) (Ahren *et al.*, 1998). It has been proposed that the ability to capture nematodes has arisen at least twice, once in a lineage leading to species forming constricting rings and once in a lineage leading to the formation of adhesive infection structures (Ahren *et al.*, 1998). Since there is a close genetic relationship among the nematode-trapping fungi it can be expected that many of the molecular mechanisms for trapping and killing nematodes are shared among these fungi. Accordingly, information from

the studies of *M. haptotylum* in this thesis can also apply to other species of nematode-trapping fungi.

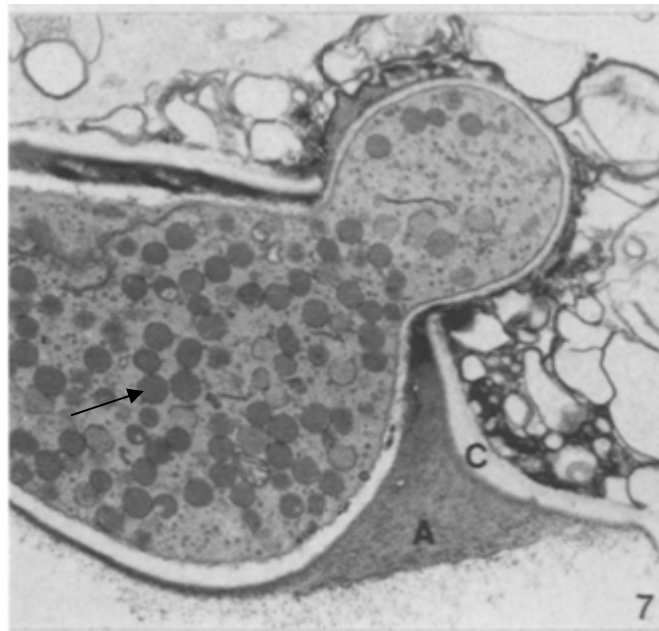


Figure 2. Electron micrograph of a thin section of *Arthrobotrys oligospora* demonstrating the presence of electron-dense microbodies (arrow) in the penetration hyphae, shortly after penetration of the nematode cuticle. (4h after capture; 10,000 x), 7; this is micrograph number 7 in a series originally published in Veenhuis *et al.*, (1985). A, adhesive coating; C, cuticle of the nematode.

Ultrastructure of trap cells

The ultrastructure of trap cells have been extensively studied in several nematode-trapping fungi, particularly in *A. oligospora* (Veenhuis *et al.*, 1985; Veenhuis *et al.*, 1989a). These studies have shown that the trap cells are highly specialized cells with a unique ultrastructure that clearly distinguishes them from vegetative hyphae. One feature of trap cells is that they contain numerous electron dense microbodies (Figure 2), which are having properties common to peroxisomes (Veenhuis *et al.*, 1984). Microbodies appear to be present in all types of trap cells (Dijksterhuis *et al.*, 1994). Microscope pictures of trap cells in *A. oligospora* show these dense bodies as dark structures with a dense core surrounded by a single membrane (Figure 2) and being tightly packed in the cytoplasm. It has been shown that these organelles contain catalase and D-amino acid oxidase, which are peroxisomal enzymes (Veenhuis *et al.*, 1984). In *A. oligospora*, dense bodies develop from specialized regions of the endoplasmatic reticulum (ER) (Veenhuis *et al.*, 1984). The dense bodies are only present

in the trap cells and are completely absent in normal vegetative cells. It has also been shown that the dense bodies disappear from the invading trap cell at the moment of nematode penetration. This suggests that they have a specific function during nematode penetration possibly in the formation of the penetration tube. Dense bodies are also believed to be involved during the digestion of nematodes (Veenhuis *et al.*, 1985; Veenhuis *et al.*, 1989a).

Another structure that appears to be common to the adhesive type of traps (nets, knobs, branches and non-constricting rings) is the presence of an extensive layer of extracellular polymers, which are thought to be important for attachment of traps to the surface of the nematode (Tunlid *et al.*, 1991).

The infection process

Nematode-trapping fungi infect their hosts in a sequence of events including adhesion to the host surface, followed by penetration, digestion and growth into the host tissue (Dijksterhuis *et al.*, 1994). The mechanism of the attachment between the surfaces of the trap cells and nematode is not yet known in detail (Tunlid *et al.*, 1992). The adhesive polymers in *A. oligospora* can be visualized as an electron-dense fibrillar layer and chemical analyses have indicated that the layer contains neutral sugars, uronic acids and proteins (Tunlid *et al.*, 1991). After contact with the nematode, the fibrils become directed perpendicularly to the nematode surface, probably to facilitate the anchoring (Nordbring-Hertz *et al.*, 1997). Studies of the adhesion mechanism in *A. oligospora* have also suggested the involvement of a lectin (carbohydrate binding protein) -carbohydrate interaction (Nordbring-Hertz and Mattiasson, 1979).

Following adhesion, the nematode-trapping fungi form a penetration tube which pierces the nematode cuticle (Dijksterhuis *et al.*, 1994). In *A. oligospora*, the penetration process is a combination of the activity of hydrolytic enzymes, such as proteases, and a mechanical pressure generated by the penetrating growing fungus. The nematode cuticle consists mainly of proteins including collagen (Cox *et al.*, 1981) and several proteases have been isolated from nematophagous fungi that can hydrolyse proteins of the nematode cuticle (see further below). Once inside the nematode, the penetration tube swells to form a large infection bulb. The mature bulb contains the common cell organelles but with the endoplasmatic reticulum (ER) especially well developed. In *A. oligospora* it has been shown using enhanced contrast

microscopy that the numbers of dense bodies present in the trap cell forming the penetration tube, rapidly decreased. During subsequent penetration and development of the infection bulb the decrease continues. Electron microscopy revealed that the dense bodies were degraded by means of an autophagic process. The degradation process was initiated early in the infection process and after 1-2 hours no dense bodies were present (Veenhuis *et al.*, 1985; Veenhuis *et al.*, 1989a). From the mature infection bulb, trophic hyphae are developing allowing the fungus to grow saprophytically and utilize the nutrients of the nematode. At this stage the original trap cell, characterized by numerous dense bodies, is transformed into an active vegetative hyphal cell, containing typical cell organelles such as nuclei, mitochondria, ER, vacuoles and normal microbodies but no dense bodies (Veenhuis *et al.*, 1984). When the infection has proceeded for 24 h, numerous lipid droplets accumulate inside the hyphal cells and the development of a fungal mycelium outside the nematode is initiated. The lipid droplets is a means of storing nutrients derived from the nematode. Later these lipid droplets will disappear and the hyphae will become vacuolated. The infection process is usually completed within 48-60 h (Veenhuis *et al.*, 1989b).

Molecular mechanisms

Until recently, the knowledge about the molecular mechanisms behind the infection process of nematode-trapping fungi has been limited. Much of the knowledge is originating from research on the nematode-trapping fungus *A. oligospora*. Two putative pathogenicity factors have been identified in *A. oligospora*; an extracellular subtilisin-like serine protease (designated PII) and a lectin (designated AOL) (Rosen *et al.*, 1992; Ahman *et al.*, 1996). These proteins have been partly sequenced, and based on this information, the corresponding genes have been cloned and characterized. Following the development of a transformation system for *A. oligospora* (Tunlid *et al.*, 1999) the functions of these proteins have been further studied by generating deletion and over-expressing mutants.

PII belongs to the subtilisin family of serine proteases. These proteases have been suggested to have a role during the fungal penetration of the nematode cuticle and/or during the digestion of the internal tissues of the nematode (Ahman *et al.*, 2002). *PII* deletion mutants produced lower number of infection structures (traps) and caused a limited effect on virulence (Ahman *et al.*, 2002). Mutants overexpressing *PII* developed an increased number of infection structures and captured and killed nematodes more rapidly than wild type (Ahman *et al.*,

2002). The toxic activity of PII was verified by demonstrating that a heterologously produced PII in *Aspergillus niger* had a nematocidal activity when added to free living nematodes (Ahman *et al.*, 2002). AOL belongs to a family of low molecular weight, saline-soluble lectins that appear to be unique to filamentous fungi (Rosen *et al.*, 1996). It has been suggested that these lectins could be involved in recognition, storage of nutrients and fruit body development (Giollant *et al.*, 1993; Oguri *et al.*, 1996; Inbar and Chet, 1997; Rosen *et al.*, 1997; Boulianne *et al.*, 2000; Elifio *et al.*, 2000). Deletion of the AOL gene did not affect the phenotype of *A. oligospora* (Balogh *et al.*, 2003). However, expression studies suggest that one of the functions of AOL is to store the nitrogen obtained from infected nematodes. During the infection of nematodes large amount of AOL is synthesized in *A. oligospora*. Later, the lectin is transported from the infected nematode to other parts of the mycelium, where it can be degraded and support the growth of the fungus (Rosen *et al.*, 1997).

5. Transcriptome analyses using microarrays

Based on the analysis of fungal genome sequences, it can be predicted that a “typical” ascomycete genome contains between 7,000-15,000 genes (Xu *et al.*, 2006). Genes being active are transcribed into mRNA, and the term transcriptome is used to describe the set of all mRNA molecules, or transcripts, produced in one or a population of cells. Recent experiments have shown that a large fraction of the genes in fungal genomes can be actively expressed at any given time, but the transcriptome vary with external environmental conditions, and the tissues being analyzed (Breakspear and Momany, 2007). In view of these observations, we could expect that a large number of genes, probably several thousands, are expressed and regulated during the development of traps and during the infection process of nematode-trapping fungi.

Since their introduction in the mid-1990s, DNA microarrays have become one of the major tools for exploring the genome-wide patterns of gene expression, i.e. the transcriptome (Brown and Botstein, 1999). The DNA microarray technique is in principle very simple. The microarray is fabricated as a ‘chip’ containing thousands of DNA sequences (typically pre-synthesized oligonucleotides or inserts from cDNA clones) printed and immobilized onto a glass slide or other substrate. To compare the abundance of transcripts in a sample, the mRNA

is extracted (the “hybridization extract”), reverse transcribed to cDNA, labelled, and hybridized to the arrayed DNA (the “reporter”). After washing, the amount of bound hybridization extract is estimated by fluorescence scanning or phosphorous imaging. The power of DNA microarrays as experimental tools derives from the exact specificity and affinity of complementary base-pairing between the DNA sequences of the “reporter” and the “hybridization extract”. Due to binding between complementary sequences, the level of specific transcripts can be estimated even in an extremely complex mixture (Brown and Botstein, 1999).

In the absence of a complete genome sequence, information from partly sequenced complementary DNA (cDNA) clones is well suited for the construction of DNA microarrays (Brown and Botstein, 1999). Briefly, cDNA libraries are constructed from mRNAs extracted from various tissues and growth conditions of the organism being studied. Random clones from the cDNA libraries are collected and partly sequenced. The obtained sequences (expressed sequence tags or ESTs) are assembled into clusters; each cluster is presumably corresponding to one, unique gene. Clones representing a uniset of genes are amplified and spotted onto glass slides using a robotic arrayer. The mRNA to be used for hybridization, is extracted from the tissues being analyzed, reverse transcribed into cDNA and a fluorescent dye label is incorporated. One sample of cDNA is labelled with a ‘green’ dye and the other with ‘red’. The samples are then mixed and applied onto the array, where the dye-labelled cDNA strands can hybridize to their complementary sequences on the array. Un-hybridized cDNA is washed off, and the green and red fluorescence are measured from each spot on the array.

To date, the global gene expression in over 20 species of filamentous fungi has been examined by microarrays. The research areas covered include metabolism, development, pathogenesis, symbiosis and industrial applications. The majority of studies have concerned metabolism and pathogenicity. The first 50 microarray studies in filamentous fungi were recently reviewed by Breakspear and Momany (2007). It should be remembered that microarray analyses are measuring the relative amounts of mRNAs in the cells and this is not directly proportional to the amount of proteins expressed. The number of protein molecules that is synthesized from a given mRNA template varies depending on the translational regulation such as translation initiation factors and also the ability of the promoter element of the specific mRNA molecule in recruiting ribosomes for protein translation. One example that

the translational regulation of specific mRNAs is important for controlling gene expression is the binding of trans-acting factors to the 5'- and 3'-untranslated regions (UTRs) of the mRNA molecule. Further, the 5'-cap and 3'-poly(A)-tail are important determinants of translational efficiency (Wilkie *et al.*, 2003).

Table 1. Genes characterized in *M. haptotylum* in this thesis.

Gene name	cDNA ID ^a	EST clone ID ^b	GenBank Homologue*	Paper
<i>prf1</i>	AY635995	CN795458	Profilin (<i>Candida albicans</i>), (Acc.No. P53696)	Paper I
<i>cof1</i>	AY635996	CN795276	Cofilin Cof1 (<i>Saccharomyces cerevisiae</i>), (Acc.No. NP_013050)	Paper I
<i>rho1</i>	AY635989	CN800249	GTPase Rho1 (<i>Neosartorya fischeri</i>), (Acc.No. XP_001257807.1)	Paper I
<i>rdi1</i>	AY635991	CN794767	Rho GDP dissociation inhibitor Rdi1p (<i>Aspergillus clavatus</i>), (Acc.No. XP_001274435)	Paper I
<i>rac1</i>	AY635990	CN795728	GTPase Rac1 (<i>Schizophyllum commune</i>), (Acc.No. AAQ88447)	Paper I
<i>ras1</i>	AY635992	CN799032	Ras homologue (<i>Colletotrichum trifolii</i>), (Acc.No. O42785)	Paper I
<i>gks1</i>	AY635993	CN800713	GAS1 (MAS3) protein (<i>Magnaporthe grisea</i>), (Acc.No. AAK52794.1)	Paper I
<i>gph1</i>	AY635994	CN795977	Glycogen phosphorylase GPH1 (<i>Neosartorya fischeri</i>), (Acc.No. XP_001264471)	Paper I
<i>PEX11</i>	EF419889	CN796915	Peroxisomal membrane protein Pex11 (<i>Aspergillus fumigatus</i>), (Acc.No. XM_745572)	Paper II
<i>spr1</i>	-	CN799628	Serine protease Spr1 (<i>Monacrosporium megalosporum</i>), (Acc.No. AB120125)	Paper III
<i>spr2</i>	-	CN800950	Protease B PRB1 (<i>Saccharomyces cerevisiae</i>), (Acc.No. M18097.1)	Paper III
<i>CFEM1</i>	-	CN794863	CFEM domain protein, (<i>Neosartorya fischeri</i>), (Acc.No. XM_001258531)	Paper III
<i>CFEM2</i>	-	CN794582	CFEM domain protein, (<i>Neosartorya fischeri</i>), (Acc.No. XM_001258531)	Paper III

^aNCBI/GenBank accession number

^bNCBI/dbEST accession number

*Closest homologue in GenBank (not *M. haptotylum*), (Acc.No. is the NCBI accession number)

6. Summary of thesis

The major goal of the studies described in this thesis has been to identify and characterize genes in nematode-trapping fungi that are involved in the infection process. In 2002, when I started my PhD work, only two such pathogenicity factors had been characterized, *PII* and *AOL* of *A. oligospora*. Both genes were identified based on the observation that the infection process could be at least partially inhibited by treating the fungus with various chemicals including enzyme-inhibitors and hapten-sugars, followed by protein isolation, peptide sequencing, and cloning of the corresponding genes. The functions of these proteins were further examined by studying the patterns of expression and by mutant analyses (Ahman *et al.*, 2002; Balogh *et al.*, 2003). In this thesis, putative pathogenicity factors have been identified by following the changes in the transcriptome of a nematode-trapping fungus during the various stages of the infection using microarray analyses. Several shifts in the transcriptome were observed that were associated with the differentiation of trap cells, as well as with the adhesion, penetration and digestion of the captured nematodes. Based on their pattern of expression, several genes were selected for further characterization (Table 1). Among these genes, was a homolog to *PEX11* of *S. cerevisiae*, encoding a peroxisomal membrane protein, known to have a role in peroxisome proliferation (Erdmann and Blobel, 1995; Marshall *et al.*, 1995). The full length cDNA was cloned and expressed in a Δ *pex11* strain of *Hansenula polymorpha*. Furthermore, among the infection-regulated transcripts, we identified a cohort of genes with disabled open reading frames (i.e. containing stop codons and frame-shifts). The function of these non-coding RNAs is not yet known.

Organisms used

To be able to identify genes that are uniquely expressed in the infection structures and not in the mycelium of a nematophagous fungus, there must be a good method for the physical separation of the infection structures from the mycelium. For that reason, I have in my thesis used the nematode-trapping fungus *M. haptotylum* (*syn. Dactylaria candida*) (Figure 3). *M. haptotylum* infects nematodes by using adhesive knobs, which are developed on branches of vegetative mycelium. During growth in liquid cultures with heavy aeration the connections between the traps (knobs) and mycelium can be easily broken, and the knobs can be separated from the mycelium by filtration (Friman, 1993). The isolated knobs are still functional and can infect nematodes even if they are separated from the mycelium.

The nematode *Caenorhabditis elegans*, which is a bacteria-feeding nematode living in soils, was used as a host in the infection experiments. For many years, the nematode *C. elegans* has been a model organism for studying development, neurobiology, and apoptosis. More recently, it has become a model for the study of innate immunity and host-pathogen interactions (Millet and Ewbank, 2004; Gravato-Nobre and Hodgkin, 2005; Mylonakis and Aballay, 2005). Furthermore, the complete genome of *C. elegans* has been sequenced (The *C. elegans* sequencing consortium, 1998).

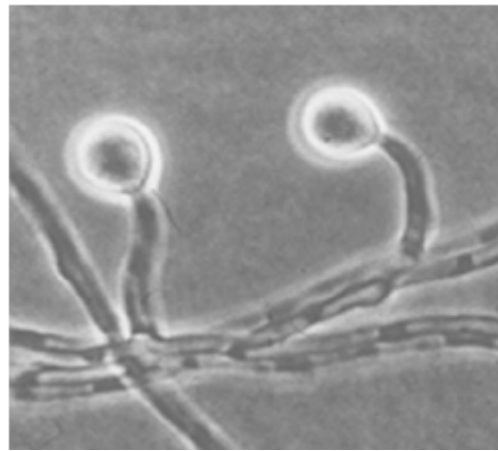


Figure 3. Trapping structures of *Monacrosporium haptotylum*. The knob cell is a spherical cell formed on the apex of a hyphal branch. The micrograph is reproduced from Nordbring-Hertz *et al.*, (1995), courtesy of Birgit Nordbring-Hertz and IWF, Göttingen.

Construction of cDNA microarrays

The cDNA microarrays used in my thesis were printed with cDNA reporters obtained from EST sequencing of four different cDNA libraries representing the mycelium and traps of *M. haptotylum*, as well as the fungus infecting *C. elegans* for 4 h and 24 h, respectively. In total, 8,466 EST sequences have been generated from these libraries. Following an assembly of these sequences, a uniset of 3,518 gene representatives were amplified, 2,822 of which are of fungal origin, 540 of worm origin, and 156 of unknown origin. Each reporter was printed at least in quadruplicate together with a number of control reporters. Constructions of the arrays are described in more detail in Ahren *et al.*, (2005).

Statistical analysis

For the statistical analysis, the microarray slides were scanned and visually inspected to exclude low-quality spots. For the spots remaining, the raw fluorescence intensities for each channel on each slide were collected. After local background correction for each spot, the reporters yielding intensities below twice the average background were excluded. In the following statistical analyses the mixed-model analysis of variance (ANOVA) (Wolfinger *et al.*, 2001) was used.

The statistical filtering of the microarray raw data was performed to serve two purposes; firstly, normalization of the data to remove systemic biases that may have affected all genes simultaneously, such as differences in the amount of RNA that was labelled for a particular replicate of a treatment, and secondly, assessment of the contribution of biological and experimental sources of error to the variation in the expression of each individual gene (Wolfinger *et al.*, 2001).

Validation of microarray data

To validate the microarray data, the identities of selected regulated genes were confirmed by re-sequencing of the cDNA clones. The expression levels of selected fungal and nematode EST cDNA clones were further analysed by quantitative real-time PCR. The real-time reverse transcription polymerase chain reaction (RT-PCR) uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction. The principle of quantification is straightforward: the more copies there are at the beginning of the assay, the fewer cycles of amplification are required to generate the number of amplicons that can be detected reliably. Consequently, fewer amplification cycles are required for the fluorescence to reach the threshold level of detection of the real-time detection instrument (Bustin *et al.*, 2005).

The real-time RT-PCR data was analyzed using relative quantification according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) (Stratagene software for the Mx3000P Real-Time PCR instrument). Relative quantification relates the PCR signal of the target transcript to that of another sample, such as an untreated control, which is used as a reference sample. The relative quantity was calculated as a fold change of the normalized expression of the target transcript relative to normalized expression levels in the reference sample. The gene expression values were normalized to an internal control gene.

Paper I : Comparison of the transcriptome in vegetative hyphae and trap cells

In this study the global pattern of gene expression was compared in knobs and mycelium of *M. haptotylum*. Total RNA was isolated from mycelium and knobs which were grown in the same medium. The microarray analyses were designed as two-sample comparisons, i.e. knobs versus mycelium. The RNA was reversed transcribed to cDNA, labelled and used for hybridization to the constructed cDNA microarray. There were substantial differences in the sets of genes expressed in knobs as compared to mycelium. In total, 23.3% (657 of 2,822) of the cDNA reporters showed differential regulation in knobs as compared to mycelium. Based on sequence homologies, the differentially expressed genes were annotated into different functional categories. Several of them showed significant sequence similarities to genes known to be involved in regulating morphogenesis and cell polarity in fungi. Furthermore, a number of genes were identified which appear to be commonly regulated in the infection structures of nematode-trapping fungi (trap cells) and plant-pathogenic fungi (appressoria).

Morphogenesis

The trap cell of *M. haptotylum*, the adhesive knob, is a spherical cell located at the tip of a slender hyphal stalk. Formation of the knob represents a shift from polar growth of the hyphal stalk to isotropic (unpolar) growth of the spherical knob cell. This shift can be expected to involve the regulation of genes important for morphogenesis and cell polarity. In the hyphae, cell polarity is established by a polarized actin cytoskeleton. Actin cables are assembled into cortical patches at the tip, guided by a cap of regulatory and cytoskeletal proteins. Actin cables then guide secretory vesicles to the cap, where they accumulate and fuse, thus polarizing growth. During isotropic growth, the proteins of the cap are more diffusely distributed, cortical patches are isotropically distributed, and actin cables form a meshwork (Pruyne and Bretscher, 2000a; Pruyn and Bretscher, 2000b).

Several of the gene representatives that were differentially expressed in knobs and mycelium are known to be involved in regulating morphogenesis and cell polarity. Examples are the actin-binding proteins profilin and cofilin. Three small GTPases, including the *Rho1*, *Rac* and *Ras* homologs were differentially expressed in knobs and mycelium. The *Rho* and *Ras* family of small GTPases, including Rho, Rac and Cdc42, play an important role in regulating the

actin part of the cytoskeleton (Pruyne and Bretscher, 2000a). Among the small GTPases, *Rac* has been identified only in filamentous fungi and there are no *Rac* homologs in the yeasts *Saccharomyces cerevisiae* or *Saccharomyces pombe*. *Rac* has been identified in the filamentous fungi *Penicillium marneffei*, *Wangiella dermatitidis*, and *Magnaporthe grisea* and appears to be especially important for polarized growth in filamentous fungi. In *P. marneffei*, strains deleted for the *Rac* homolog *cflB* make conidiophores and hyphae that do not polarize properly (Boyce et al., 2003).

Similarities to appressoria

The infection of a plant-pathogenic fungus starts when a spore (conidium) germinates on the surface of a leaf. A specialized hypha, called the germ tube, is growing out from the conidium. Eventually, the germ tube swells slightly and develops an appressorium at its end. Appressoria are swollen dome shaped cells specialized for plant infection (Tucker and Talbot, 2001). This process exhibits several similarities to the development of traps (knobs) of *M. haptotylum*. Like a knob, an appressorium is a specialized infection structure, which develops as a spherical cell at the tip of a hypha (germ tube) (Ahren et al., 2005). Both structures are covered by an adhesive layer which mediates the attachment to the host surface. In addition, both appressoria and knobs secrete extracellular enzymes and generate physical force to bring about cuticle penetration of the host.

Gene expression profiles have been published for the two appressoria forming fungi, *Blumeria graminis* and *Magnaporthe grisea* (Takano et al., 2004; Both et al., 2005a; Both et al., 2005b). Interestingly, a number of the genes that are specifically expressed and regulated in appressoria were also found to be regulated in knobs. Both et al., (2005a) identified a cluster of virulence related genes in the fungus *B. graminis* that were up-regulated during appressorium development. This cluster contained genes that are homologous to known virulence genes of plant-pathogenic fungi, such as *egh16*, *egh16H1*, *cPKA* and *clap1*. Another group of genes identified in the cluster were homologous to genes associated with pathogenic development or with establishment of the infection, including genes encoding for NADPH oxidase, serine protease, aspartic protease, cyclophilin and MAP kinase. The genes *egh16* and *egh16H1* in *B. graminis* are homologs of the *gas1/mas3* and *gas2/mas1* genes of *M. grisea*. In *M. grisea* these genes have been shown to contribute to the pathogenicity of the fungus and deletion of *gas1/mas3* and *gas2/mas1* caused reduced appressorial penetration and lesion development in plants infected by *M. grisea* (Xue et al., 2002). In the microarray analysis

made by Takano *et al.*, (2004), genes were identified that were specifically up- or down-regulated during appressorium formation in the fungus *M. grisea*. Among the genes being differentially expressed in appressoria as compared to vegetative mycelium in *M. grisea*, were homologs encoding for; metallothionein, GAS1/MAS3, serine protease and chitinase. Comparison of the gene expression profiles of knobs with that of appressoria in *M. grisea* and *B. graminis* reveals that there are similarities in the genes expressed in the infection structures of nematode-trapping and plant-pathogenic fungi. Commonly regulated in knobs of *M. haptotylum* and appressoria were homologs encoding; Egh16, chitinase, serine protease and cyclophilin in *B. graminis* (Both *et al.*, 2005a; Ahren *et al.*, 2005) and GAS1/MAS3, chitinase, serine protease, cyclophilin and metallothionein in *M. grisea* (Takano *et al.*, 2004; Ahren *et al.*, 2005).

Paper II : Characterization of a PEX11 homolog

Typical for trap cells in nematode-trapping fungi is the presence of numerous dense bodies which are organelles related to peroxisomes (Dijksterhuis *et al.*, 1994). The transcriptional profiling of *M. haptotylum* traps identified one gene representative with homology to the peroxisomal membrane protein Pex11p from *S. cerevisiae*. This Pex11p homolog was significantly up-regulated in traps (knobs) as compared to vegetative mycelium (Ahren *et al.*, 2005) and was also regulated in the gene expression analysis of *M. haptotylum* infecting the nematode *C. elegans* (Paper III). The expression of *PEX11* was significantly down-regulated at all the different stages of infection (Paper III). The Pex11p protein is known to have a role in peroxisome proliferation in yeast (Erdmann and Blobel, 1995; Marshall *et al.*, 1995). In order to further characterize the *M. haptotylum* *PEX11* homolog we cloned the full length cDNA of the *PEX11* gene (*MhPEX11*). The deduced amino acid sequence reveals a protein of 237 amino acids (MhPex11p) showing significant sequence similarity to Pex11p from *S. cerevisiae* (28% amino acid identity). To analyze whether the MhPex11p has a function in peroxisome proliferation we expressed the corresponding cDNA in a $\Delta pex11$ strain of the yeast *H. polymorpha*. The result showed that the *M. haptotylum* Pex11p could not functionally complement the $\Delta pex11$ mutant of *H. polymorpha*. There was a slight effect on the peroxisome numbers; however there was no full complementation. This implies that the *H. polymorpha* Pex11p protein contains an additional activity that is absent in MhPex11p.

Dense bodies

Characteristic of trap cells are that they contain numerous electron dense microbodies which have properties common to peroxisomes (Veenhuis *et al.*, 1984). These organelles are completely absent in normal vegetative cells (Veenhuis *et al.*, 1989a) and it has been proposed that they have a specific function during nematode penetration. By using enhanced contrast microscopy of *A. oligospora* it was shown that the number of dense bodies present in the trap cells rapidly decreased when the penetration tube was formed. During the subsequent penetration of the nematode and development of the infection bulb this decrease continued (Veenhuis *et al.*, 1989a). Trap cells must have access to endogenous storage material to bring about the initial penetration. In traps developing on low nutrient media, common storage materials like glycogen and lipid droplets are not observed (Veenhuis *et al.*, 1989a). The finding that the dense bodies disappear at the same time as the development of the penetration tube, suggests that they play a role in this process. Dense bodies may be degraded to provide building blocks for new cell material (building up the penetration tube) and hydrolytic enzymes which are excreted during penetration (Veenhuis *et al.*, 1985).

PEX genes in fungi

PEX genes encode proteins, termed peroxins that are required for the biogenesis and proliferation of microbodies (peroxisomes). The peroxisome biogenesis in yeast is well studied and there are 32 *PEX* genes identified (van der Klei and Veenhuis, 2006). In yeast Pex11p is a 24- to 30-kDa peroxisomal membrane protein, that regulates peroxisome number and size. Deletion of *PEX11* in yeast will give rise to cells containing few, large peroxisomes (Erdmann and Blobel, 1995). Conversely, overexpression of *PEX11* causes proliferation of many small peroxisomes in yeast (Marshall *et al.*, 1995; Sakai *et al.*, 1995). In the *S. cerevisiae* *pex11* deletion mutant, the large peroxisomes can not enter cell buds and growth is arrested (Erdmann and Blobel, 1995). Until now, in filamentous fungi, only a limited number of genes involved in peroxisome biogenesis and proliferation have been identified (Kiel *et al.*, 2006). *Penicillium chrysogenum* is the only filamentous fungus where Pex11p has been characterized (Kiel *et al.*, 2005). In *P. chrysogenum* peroxisomes are involved in the production of β -lactam antibiotics (Martin and Gutierrez, 1995). Overexpression of Pex11p in *P. chrysogenum* results in proliferation of tubular-shaped peroxisomes and an increase in the level of penicillin in the culture medium. Pex11p from the *P. chrysogenum* has been overexpressed in *H. polymorpha* resulting in overproliferation of peroxisomes (Kiel *et al.*, 2005). Mutational analyses of other *PEX* genes in filamentous fungi have generated

phenotypes different from those in yeast species (Kiel *et al.*, 2006). In the plant-pathogenic fungus *Colletotrichum lagenarium* the *pex6* mutant is unable to infect plant cells (Kimura *et al.*, 2001) and in *P. chrysogenum* the *pex5* mutant is affected in asexual spore formation (Kiel *et al.*, 2004). These unexpected phenotypes of filamentous fungal *pex* mutants may reflect the highly complex phenotypes of human peroxisome biogenesis disorders (PBDs) (Kiel *et al.*, 2006).

Role of peroxisomes in fungal pathogens

In yeast, microbodies are predominantly involved in primary metabolism of various unusual carbon and organic nitrogen sources for growth (Veenhuis and Harder, 1988). In filamentous fungi microbodies also play a role in the primary metabolism, but in addition they can perform biosynthetic and structural functions. In filamentous fungi four types of microbodies have been described; glyoxysomes, peroxisomes, Woronin bodies and hydrogenosomes (van der Klei and Veenhuis, 2006).

The role of peroxisomes in fungal pathogenesis is not well understood. However, there are examples of plant-pathogenic fungi where peroxisomes have been shown to contribute to the pathogenicity. In the plant-pathogenic fungus *Colletotrichum lagenarium* it has been demonstrated that peroxisomal function is required for appressorium-mediated plant infection (Kimura *et al.*, 2001; Asakura *et al.*, 2006). The *pex6* deletion mutants formed small appressoria with severe reduced melanization that failed to generate penetration hyphae (Kimura *et al.*, 2001). A *PEX6* homolog has also been identified in the plant-pathogen *M. grisea*, where *PEX6* is required for growth on fatty acids and is essential for pathogenicity (Ramos-Pamplona and Naqvi, 2006). Loss of *PEX6* function in *M. grisea* led to completely non-melanized appressoria, which were unable to elaborate penetration pegs and infection hyphae (Ramos-Pamplona and Naqvi, 2006).

Fatty-acid metabolism plays a crucial role during fungal pathogenesis (Kimura *et al.*, 2001; Both *et al.*, 2005b). Deletion of different glyoxylate cycle enzymes such as isocitrate lyase and malate synthetase have resulted in reduced pathogenicity in fungi (Lorenz and Fink, 2001; Idnurm and Howlett, 2002; Wang *et al.*, 2003; Solomon *et al.*, 2004). This indicates that fatty acid β -oxidation which occurs primarily within peroxisomes seems to be the predominant catabolic process during early pathogenesis in fungi (Ramos-Pamplona and Naqvi, 2006). In *M. grisea* the degradation of lipid reserves contributes to turgor generation in

the developing appressorium (Thines *et al.*, 2000). Furthermore, in *M. grisea*, the activity of triacylglycerol lipase, which generates glycerol and fatty acids from triacylglycerol, has been shown to be induced during appressorium formation and maturation (Kimura *et al.*, 2001). Conidia of fungal pathogens contain storage compounds that are used for infection-related morphological developments. Conidia of *C. lagenarium* contain large numbers of lipid bodies (Kimura *et al.*, 2001) and rapid degradation of lipid bodies occurs during appressorium formation and subsequent penetration of the host plant, implying that lipolysis provides the compounds required for appressorium formation and function (Yamauchi *et al.*, 2004).

Paper III : *Gene expression during invasion of the host*

In this study, the responses in the transcriptome of *M. haptotylum* during the infection of the nematode *C. elegans* were examined. Knobs were added to nematodes and samples were taken at four different time points (1, 4, 16 and 24 h) corresponding to the various stages of the infection; adhesion and penetration (1 h and 4 h) and digestion (16 h and 24 h). RNA was extracted, reverse transcribed to cDNA, labelled and hybridized to the cDNA array. For comparison, RNA was also isolated from reference materials consisting of axenic knobs and non-infected nematodes, respectively. Based on a statistical analysis, genes were identified that were significantly regulated during the infection as compared to the reference material. In total 58% (1,562 of 2,684) of the fungal genes represented on the array, were regulated in at least one of the stages of the infection. Bioinformatic analyses were used to identify putative secretory proteins and enzymes encoded by these gene representatives. The most dramatic shift in the fungal transcriptome occurred after 4 h, a time-point associated with the penetration of the nematode. At this time-point, a majority of the fungal gene representatives were down-regulated. However, based on clustering patterns, a set of genes (372) was identified to be transiently and specifically up-regulated after 1 h and 4 h, and not at the later time-points. These genes were defined as early expressed genes. A large fraction of these gene representatives (293 out of 372) were classified as orphans, displaying no significant similarity to proteins in other organisms. DNA sequencing of several of these transcripts showed that they included both those with full-length open reading frames (ORFs), but also transcripts with disabled ORFs (frameshifts and premature stop codons). The role of these early expressed genes and non-coding RNAs in the infection is not yet known. Finally, the

transcriptional responses in the defence system of *C. elegans* were analyzed during the infection process.

Proteases and secretory proteins

Surprisingly few hydrolytic enzymes were identified to be regulated during the fungal infection of *C. elegans*. The enzymes found to be regulated included the subtilisin-like serine proteases, otherwise few enzymes were found among the regulated genes.

The factors that contribute to the rapid killing and immobilization of captured nematodes have been the subject of research for a long time. Early studies in *A. oligospora* were trying to purify a nematotoxic substance, a fungal metabolite that is toxic to nematodes, and might be involved in the immobilization of nematodes. It was proposed that *A. oligospora* secretes a nematotoxic substance which paralyzes nematodes following the capture and penetration of them (Olthof and Estey, 1963). This enzyme was supposed to be secreted from the fungus upon contact with the nematode. Although many attempts, the only nematotoxic compounds identified from nematophagous fungi are fatty acids, including linoleic acid (Kwok *et al.*, 1992; Stadler *et al.*, 1993).

Recently, several studies of proteases from nematophagous fungi have been reported (Table 2). The first serine protease, P32 from a nematophagous fungus was isolated in 1992 from the endoparasite *Pochonia suchlasporium* (syn. *Verticillium suchlasporium*) and it was found to be involved in penetration of nematode eggs (Lopez-Llorca and Robertson, 1992). Following that, eight additional serine proteases from nematophagous fungi have been isolated and characterized as virulence factors, as listed in Table 2.

The serine proteases of nematophagous fungi show high similarity to Protease K of the subtilisin-like serine protease family, with the conservation of the aspartic acid - histidine - serine catalytic triad (Huang *et al.*, 2004). Serine protease inhibitors such as PMSF can significantly decrease their activities and the purified proteases can degrade the proteins from the nematode cuticle. Light microscope and scanning electron microscopy (SEM) showed that the outer layers of eggshells and cuticles of nematodes exfoliated; indeed large flaws even appeared with treatment of the purified protease. Serine proteases have also been identified in insect pathogenic fungi. A subtilisin-like serine protease PR1 has been isolated from the insect pathogenic fungus *Metarhizium anisopliae* (St Leger *et al.*, 1992). This protease is

highly active against cuticle proteins and is produced in large amounts by infection structures. A trypsin-like serine protease PR2 has also been cloned from *M. anisopliae* (Smithson *et al.*, 1995).

Since serine proteases in nematophagous fungi all have relatively broad substrate specificity they are believed to have two functions; one involves saprophytic growth and the other involves infection of nematodes (Tunlid *et al.*, 1994; Ahman *et al.*, 1996; Huang *et al.*, 2004). In *M. haptotylum*, two different serine proteases have been identified. One of them (*spr1*) displays significant sequence similarity to serine proteases of the subtilisin family including the cuticle degrading subtilisin PII of *A. oligospora* (Ahman *et al.*, 1996) and *spr1* of *Monacrosporium megalosporium* (Kanda *et al.*, 2006). The expression of the subtilisin-like serine protease (*spr1*) in *M. haptotylum* is in correlation with the proposed functions. It was up-regulated at 1 h, indicating a function during penetration of the nematode and also at 24 h demonstrating a role in the degradation of host tissue when the fungus has started to grow saprophytically. The other serine protease of *M. haptotylum* (*spr2*) which does not show significant homology to PII was slightly up-regulated during the 1h and 24 h time-points. This serine protease shows significant sequence similarity to a serine protease from the entomopathogenic fungus *Verticillium lecanii* (GenBank Acc.No. AAC99421). In contrast to the *spr1*, which encodes a secreted, extracellular serine protease, the *spr2* encodes an endocellular subtilisin protease.

Several global transcriptome analyses have been published of the insect pathogenic fungus *M. anisopliae* (Freimoser *et al.*, 2005; Wang *et al.*, 2005; Wang and St Leger, 2005). Freimoser *et al.*, (2005) analyzed the gene expression in *M. anisopliae* in response to diverse insect cuticles *in vitro* by using cDNA microarrays. Genes which were up-regulated in contact with an insect cuticle included those involved in cuticle-degradation (e.g. proteases), amino acid/peptide transport and transcription regulation. It was demonstrated that the transcriptional responses of *M. anisopliae* were specific to different cuticles. Like other ascomycete pathogens, *M. anisopliae* secretes a great variety of proteases (Hu and St Leger, 2004). Some of the proteases have been associated with virulence, because they mediate rapid penetration, solubilization and inactivation of antimicrobial peptides (St Leger *et al.*, 1996). The peptide/amino acid transport systems is supposed to function in transport of host degradation products.

Table 2. Subtilisin-like serine proteases characterized in Nematophagous fungi.

Identified and purified enzymes	Species of Nematophagous fungus	Reference
P32	<i>Pochonia suchlasporium</i>	(Lopez-Llorca and Robertson, 1992)
PII	<i>Arthrobotrys oligospora</i>	(Tunlid <i>et al.</i> , 1994)
Aoz	<i>Arthrobotrys oligospora</i>	(Zhao <i>et al.</i> , 2004)
pSP-3	<i>Paecilomyces lilacinus</i>	(Bonants <i>et al.</i> , 1995)
VCP1	<i>Pochonia chlamydosporia</i>	(Segers <i>et al.</i> , 1994)
Ver112	<i>Lecanicillium psalliotae</i>	(Yang <i>et al.</i> , 2005)
spr1	<i>Monacrosporium megalosporium</i>	(Kanda <i>et al.</i> , 2006)
Ds1	<i>Dactylella shizishanna</i>	(Wang <i>et al.</i> , 2006a)
Mlx	<i>Monacroporium microscaphoides</i>	(Wang <i>et al.</i> , 2006b)

Non-coding RNAs

Among the 372 genes which were specifically up-regulated in *M. haptotylum* at the early time-points of 1 h and 4 h, a large proportion was classified as orphans, showing no homology to the sequence information in various publicly available databases. When a subset of these orphans was re-sequenced it was clarified that some of them are potential non-coding RNAs. This was unexpected and interesting since it is becoming more and more evident that the non-coding RNAs can function in the regulation of other genes or may have other regulatory functions (Costa, 2007). Non-coding RNAs can originate from so-called pseudogenes. Two forms of pseudogenes have been described: “processed” pseudogenes, where an mRNA transcript is reversed transcribed and re-integrated into the genome; and “non-processed” pseudogenes, which arise from duplication of a gene in the genomic DNA with subsequent disablement of the copy which is not used (Benovoy *et al.*, 2005). Processed pseudogenes are characterized by an absence of promoters, a lack of introns, the presence of a poly(A)-tail at their 3’-end and small direct repeats at their 5’- and 3’-termini as well as frame shifts and/or premature stop codons (Benovoy *et al.*, 2005). In *Drosophila* and humans, the pseudogenes have features that would not be expected if pseudogenes were non-functional sequences of genomic DNA, so called “junk” DNA. Furthermore, changes in expression levels of pseudogenes have been associated with different kinds of cancer and neurological diseases

(Costa, 2007). There are indications that pseudogenes can be functional by being involved in regulation of gene expression and in generating genetic diversity (Balakirev and Ayala, 2003). Since pseudogenes have been linked to different processes they seem to be more important than previously thought. The non-coding RNAs identified in *M. haptotylum* were only expressed during infection and were not represented in the mycel or knob EST cDNA libraries.

Defence response in C. elegans

Since *C. elegans* lives in the soil, it can be expected to possess effective antimicrobial defence mechanisms against a great diversity of potential pathogens. A number of bacterial and fungal pathogens are able to infect *C. elegans*. Microarray analysis of *C. elegans* infected by the bacterial pathogen *Microbacterium nematophilum* identified genes encoding C-type lectin domains as the most abundant class of the regulated genes (O'Rourke *et al.*, 2006). This indicates that C-type lectins and other putative pathogen-recognition molecules are important for the innate immune defence in *C. elegans*, as previously suggested. In agreement with this several homologs to C-type lectins were also regulated when *C. elegans* was infected by the fungus *M. haptotylum* (Paper III).

Infection of *C. elegans* causes specific changes in gene regulation. The induced genes include those encoding antimicrobial peptides and proteins which can act directly against invading microorganisms (Millet and Ewbank, 2004). Among the genes known to be induced in *C. elegans* are those encoding lipases, lysozymes (*lys1-lys10*), the saposin-like protein family (*spp1-spp20*), the Thaumatin/PR-5 family and Metridin-like ShK toxin (Nicholas and Hodgkin, 2004). Lipases may act directly against invading microorganisms and lysozymes are antibacterial and act through cleavage of bacterial cell walls (Mallo *et al.*, 2002). The saposin-like proteins are a family of proteins showing similarities to amoebapores of *E. histolytica* which kill bacteria by forming ion-channels in the membranes (Banyai and Patthy, 1998). Saposin-like proteins are regulated by the transcription factor DAF16 (Murphy *et al.*, 2003) which is part of the DAF-2 pathway, a pathway that in *C. elegans* is believed to regulate the expression of a number of potentially antimicrobial gene products. Thaumatin is a member of the PR-5 family of plant pathogenesis-related proteins, which have antifungal activities (Murphy *et al.*, 2003). Antimicrobial factors with homology to Metridin-like ShK toxin are regulated by the DAF-16 transcription factor and are suggested to have antifungal activity (Murphy *et al.*, 2003).

Several gene representatives with similarities to these effectors of the *C. elegans* innate immune response were regulated during infection by the fungus *M. haptotylum*. These included a member of the saposin-like protein family (*spp5*) and sequences with homology to the Metridin-like ShK toxin (Paper III). Additionally factors corresponding to the innate immune response in *C. elegans* immunity and which was shown to be regulated in our study included homologs to genes encoding; asparatyl proteases, protease inhibitors, neuropeptide-like protein, chaperones, life span genes, and collagens.

7. Outlook and future studies

Mutational analyses of candidate genes

In the studies included in this thesis, a number of putative pathogenicity factors have been identified in the nematode-trapping fungus *M. haptotylum* by following the responses in the transcriptome during the infection of the nematode *C. elegans*.

The putative function of these genes is based on sequence similarities to genes with known functions in other fungi. The next step will be to investigate the function of these genes in *M. haptotylum*. For this a transformation system for *M. haptotylum* needs to be developed. Following that mutational analysis can be performed in *M. haptotylum* it will be possible to elucidate the function of the putative pathogenicity factors. Disruption of gene function by making deletion mutants will give information about gene function by studying the effect on the phenotype. By making mutants containing extra copies of a certain virulence gene it will be possible to increase the pathogenicity of the fungus. In the future it will be possible to use these genetically engineered strains for more effective biological control of parasitic nematodes.

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