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# Identification of Virulence Factors in Nematode-Trapping Fungi

Insights from Genomics, Transcriptomics and Proteomics

Karl-Magnus Andersson



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DOCTORAL DISSERTATION

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To be defended in public at the Ecology building, Blue Hall  
on Friday, October 25<sup>th</sup>, 2013 at 10.00 am.

*Faculty opponent*

Assoc. Prof. Peter Solomon  
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Abstract <p>Nematode-trapping fungi are soil-living organisms with the unique ability to capture and infect free-living nematodes. The interest in studying these fungi arises from their potential use as biological control agents for plant- and animal-parasitic nematodes. To enter the parasitic stage, nematode-trapping fungi develop different kinds of trapping structures. In order to understand more about the evolution of parasitism in the nematode-trapping fungi and to identify virulence factors in these fungi genomic, transcriptomic and proteomic studies were conducted.</p> <p>First, the genome of <i>Monacrosporium haptotylum</i> were sequenced and compared to the genome of the closely related <i>Arthrobotrys oligospora</i> and also to genomes of other ascomycetes. Two genomic mechanisms were identified that likely have been important for the adaptation to parasitism in these two nematode-trapping fungi. Firstly, the expansion of certain protein-domain families and a large number of species-specific genes indicated that gene duplications followed by functional diversification have played a major role in the evolution of the nematode-trapping fungi. Gene expression analyses indicated that many of these genes are important for pathogenicity. Secondly, comparisons of gene expression of orthologs between the two fungi during infection indicated that differential regulation was an important mechanism for the evolution of parasitism in nematode-trapping fungi.</p> <p>Second, the proteome of the trapping structure in <i>M. haptotylum</i> were characterized using mass spectrometry. The trapping structure in this fungus is called knob and is a single cell that can be separated from the vegetative mycelia. The results showed that there was a large difference in the protein content of the knob and that of the mycelium. The knob proteome was overrepresented in secreted proteins, including small secreted proteins, peptidases and proteins containing the carbohydrate-binding domain WSC. Transcripts encoding such proteins were also highly upregulated in <i>M. haptotylum</i> during infection. We suggest that the knob contains many of the proteins needed in the early stages of infection.</p> <p>Finally, to gain further insight about what genes that are generally regulated during infection we conducted a comparative transcriptome analysis of three nematode-trapping fungi infecting two nematode species. The analysis showed that the divergence in fungal interspecific gene expression was significantly larger than that related to the nematode host. We identified a core set of genes being expressed by all three fungi, and a more variable set being regulated depending on the fungal species or nematode host, respectively. The core set included several peptidases such as subtilisins and aspartic proteases but also ribosome-inactivating Ricin-B lectins. The variable set depending on the fungal species included fungal fruit-body lectins and D-mannose binding lectins. The host specific genes included glucosidases and genes encoding small secreted proteins.</p>	
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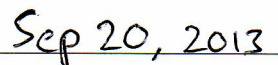
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# Identification of Virulence Factors in Nematode-Trapping Fungi

Insights from Genomics, Transcriptomics and Proteomics

Karl-Magnus Andersson



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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 summarizes the accompanying papers. These have either been published or are in manuscripts in various stages (in press, submitted or manuscript).

Front page: Adhesive knobs from *Monacrosporium haptotylum*. The knob is a single cell formed at the apex of a hypha. The micrograph is reproduced from Nordbring-Hertz et al., 1995, Courtesy of Institut für den Wissenschaftlichen Film, Göttingen.

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## List of papers

I. Meerupati, T., Andersson, K-M., Friman, E., Kumar, D., Tunlid, A., Ahrén, D.  
Genomic mechanisms accounting for the adaptation to parasitism in nematode-trapping fungi.  
(Accepted, *PLOS Genetics*).

II. Andersson, K-M., Meerupati, T., Levander, F., Friman, E., Ahrén, D., Tunlid, A. (2013).  
Proteome of the nematode-trapping cells of the fungus *Monacrosporium haptotylum*. *Applied and Environmental Microbiology*, 16: 4993-5004.

III. Andersson, K-M., Kumar, D., Bentzer, J., Friman, E., Ahrén, D., Tunlid, A. Interspecific  
and host-related gene expression patterns in nematode-trapping fungi.  
(Manuscript).

*All authors in the list of papers have given their consent for the use of their work in the thesis.*

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## **Author contributions**

**I.** Conceived and designed the experiments: DA, AT. Performed the experiments: TM, K-MA, EF, DK. Analyzed the data: TM, DA, AT. Wrote the paper: DA, TM, AT.

**II.** Conceived and designed the experiments: K-MA, DA, AT. Performed the experiments: K-MA, FL, EF. Analyzed the data: K-MA, TM, DA, AT. Wrote the paper: K-MA, FL, DA, AT.

**III.** Conceived and designed the experiments: DK, AT. Performed the experiments: DK, EF. Analyzed the data: K-MA, JB, DA, AT. Wrote the paper: K-MA, JB, DK, DA, AT





## Abstract

Nematode-trapping fungi are soil-living organisms with the unique ability to capture and infect free-living nematodes. The interest in studying these fungi arises from their potential use as biological control agents for plant- and animal-parasitic nematodes. To enter the parasitic stage, nematode-trapping fungi develop different kinds of trapping structures. In order to understand more about the evolution of parasitism in the nematode-trapping fungi and to identify virulence factors in these fungi genomic, transcriptomic and proteomic studies were conducted.

First, the genome of *Monacrosporium haptotylum* was sequenced and compared to the genome of the closely related *Arthrobotrys oligospora* and also to genomes of other ascomycetes. Two genomic mechanisms were identified that likely have been important for the adaptation to parasitism in these two nematode-trapping fungi. Firstly, the expansion of certain protein-domain families and a large number of species-specific genes indicated that gene duplications followed by functional diversification have played a major role in the evolution of the nematode-trapping fungi. Gene expression analyses indicated that many of these genes are important for pathogenicity. Secondly, comparisons of gene expression of orthologs between the two fungi during infection indicated that differential regulation was an important mechanism for the evolution of parasitism in nematode-trapping fungi.

Second, the proteome of the trapping structure in *M. haptotylum* was characterized using mass spectrometry. The trapping structure in this fungus is called knob and is a single cell that can be separated from the vegetative mycelia. The results showed that there was a large difference in the protein content of the knob and that of the mycelium. The knob proteome was overrepresented in secreted proteins, including small secreted proteins, peptidases and proteins containing the carbohydrate-binding domain WSC. Transcripts encoding such proteins were also highly upregulated in *M. haptotylum* during infection. We suggest that the knob contains many of the proteins needed in the early stages of infection.

Finally, to gain further insight about what genes that are generally regulated during infection we conducted a comparative transcriptome analysis of three nematode-trapping fungi infecting two nematode species. The analysis showed that the divergence in fungal interspecific gene expression was significantly larger than that related to the nematode host. We identified a core set of genes being expressed by all three fungi, and a more variable set being regulated depending on the fungal species or nematode host, respectively. The core set included several peptidases such as subtilisins and aspartic proteases but also ribosome-inactivating Ricin-B lectins. The variable set depending on the fungal species included fungal fruit-body lectins and D-mannose binding lectins. The host specific genes included glucosidases and genes encoding small secreted proteins.

## 1. Introduction

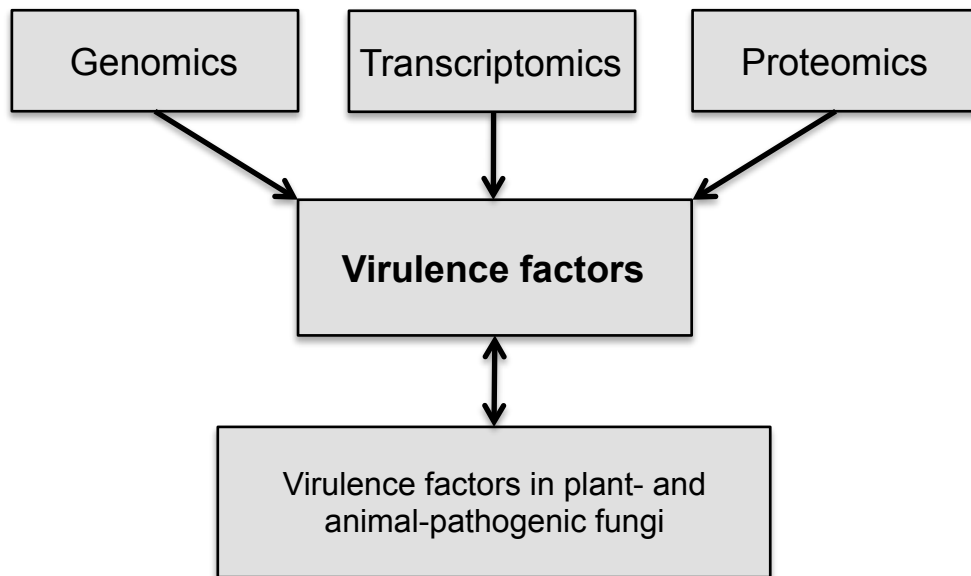
Helminths (parasitic worms) are the most common infection agents of humans in developing countries. Today, it is estimated that approximately two billion people around the world are infected (World Health Organization, Fact sheet N°366, June 2013). People living in developing regions of sub-Saharan Africa, East Africa, Asia and America are especially afflicted. Despite the large number of people infected, helminth infections are a neglected disease in terms of public recognition and funding for medical research. This could be due to that most infections with helminths are asymptomatic (Rana and Misra-Bhattacharya, 2013;Hotez et al., 2008;Stepek et al., 2006). However, helminth infection is a serious condition. Children tend to harbor the greatest number of intestinal worms leading to reduced growth, impaired memory and cognition and reduced school attendance (Crompton and Nesheim, 2002;Miguel and Kremer, 2004;Bundy et al., 2013). This reduces their wage-earning capacity later on in life (Bleakley, 2007). Furthermore, helminth infection during pregnancy is one of the major causes of anemia in poor communities causing neonatal prematurity, low birth weight and infant mortality (Brooker et al., 2008). In addition to human infections, infection by helminths causes major economic losses in livestock and crop industries. In the USA alone, it has been estimated that helminth infections cost the livestock industry \$2 billion dollars yearly in lost productivity and increased operating expenses (Rana and Misra-Bhattacharya, 2013). Helminths infecting plants causes annual crop damages exceeding \$100 billion worldwide (Bird and Kaloshian, 2003).

Helminths are divided into two major phyla: the nematodes and the platyhelminths (Hotez et al., 2008). The nematodes, also known as roundworms, include the major intestinal worms and the filarial worms that cause lymphatic filariasis and onchocerciasis. The platyhelminths, also known as flatworms, include the trematodes and the cestodes that cause cysticercosis (Hotez et al., 2008). The drugs used to control the helminth infections are called anthelmintics. The most commonly used is ivermectin that was released 1981 (Geary, 2005). However, there are few types of anthelmintics out on the market and there is an increased drug resistance in nematodes. The resistance was first seen in nematode populations infecting livestock in the 1970s but also emerged in nematodes infecting humans during the late 1990s (Sutherland and Leathwick, 2011;Geerts and Gryseels, 2001). To control plant-parasitic nematodes chemical nematicides are widely used (Davies, 2005). However, chemical nematicides are associated with environmental hazards. One of the previously most used is methyl bromide, but it is now forbidden in the developed world and from year 2015 also in the developing part of the world because of its role in ozone depletion (Davies, 2005). The increased resistance development against anthelmintics and the environmental problems associated with chemical nematicides makes it necessary to develop alternative methods to control nematode infections. In this aspect, natural enemies of nematodes, such as the nematophagous fungi could be of major interest.

Nematophagous fungi have been seen as potential biological control agents against nematodes for a long time. First attempts were made in the 1930s to reduce plant-parasitic nematodes in soils (Linford, 1937). During the following 50 years, the research using nematophagous fungi to control nematode infections were only sporadic. In the 1980s, the research was reinforced and trials begun feeding live stock with spores of nematophagous fungi (Gronvold et al., 1987). It was found that especially the nematode-trapping fungus *Duddingtonia flagrans* was effective in reducing the worm burden (Larsen, 2000). However, despite many successful field studies, *D. flagrans* is not yet used as a commercial biological control agent against nematodes (Torres-Acosta et al., 2012). It has been suggested that a better knowledge about the infection process in

nematophagous fungi will improve the development of using these fungi to control nematode infections (Morton et al., 2004). Furthermore, knowledge about the genes being regulated during pathogenicity could lead to the development of new control agents (Davies, 2005).

During my PhD studies I have used different “omic” approaches to identify candidate virulence factors in the nematode-trapping fungi (Figure 1). Since very few virulence factors are known in these fungi, sequence similarity and protein domain comparison of known virulence factors in plant- and animal-pathogenic fungi were conducted.



**Figure 1.** Main studies conducted in this thesis leading to the identification of candidate virulence factors in nematode-trapping fungi. Comparisons to known virulence factors in plant- and animal-pathogenic fungi contributed to the identification of candidate virulence factors in the nematode-trapping fungi.

**The aims of this thesis are:**

- To identify genomic mechanisms important for the evolution of parasitism in the nematode-trapping fungi.
- To identify virulence factors in the nematode-trapping fungi.
- To assign putative functions of these virulence factors during pathogenicity.

## 2. Background of nematode-trapping fungi

### 2.1 Biology of nematophagous fungi

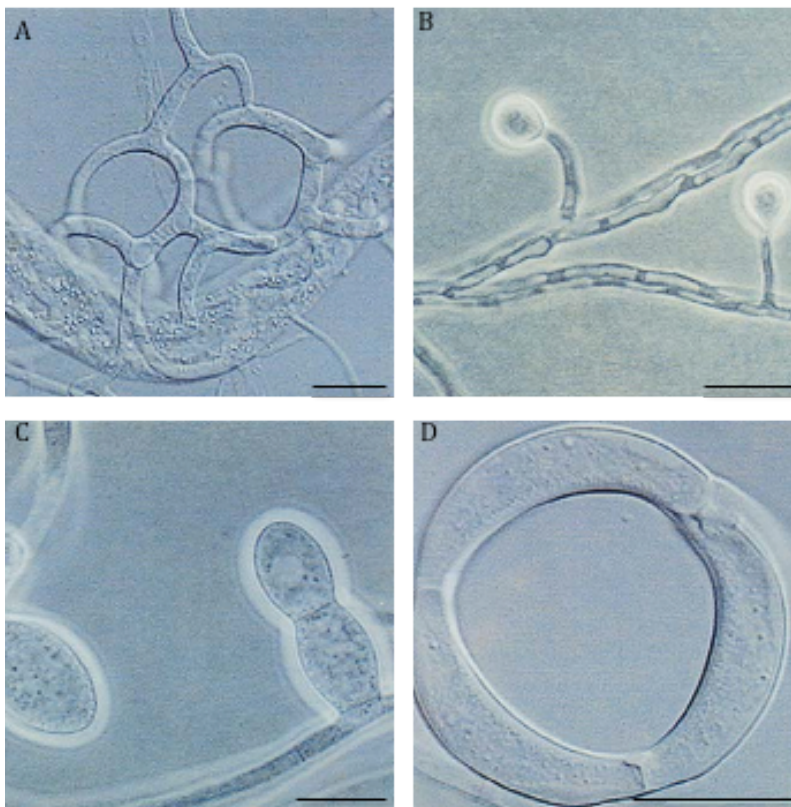
Nematophagous fungi comprise more than 200 species and exist in all regions of the world, from the tropics to the Antarctica (Barron, 1977). They are commonly found in soils that are rich in organic material. The density of nematophagous fungi is highest in the upper 20 cm of the soil horizon and very few are found below 40 cm of depth (Persmark et al., 1996). Most nematophagous fungi can grow both as saprophytes, decaying organic matters as substrates, and as parasites, using nematodes as nutrients (Barron, 1992). It has been proposed that the nematophagous fungi living in nitrogen-limiting habitats have an advantage over other fungal saprophytes, because nematodes can serve as an important source of nitrogen (Barron, 1992). Nematophagous fungi can be divided into three major groups: the nematode-trapping fungi, the endoparasitic fungi and the egg- and cyst-parasitic fungi (Nordbring-Hertz et al., 2006). The nematode-trapping fungi develop hyphal structures, such as nets, knobs, branches or rings to capture nematodes by adhesion or by mechanical means. The endoparasitic fungi are often obligate parasites and infect nematodes using spores, which either are swallowed by the nematode or that adhere to their surface. The egg- and cyst-parasitic fungi use hyphal tips to infect non-motile stages of nematodes (Nordbring-Hertz et al., 2006). A fourth group of nematophagous fungi are suggested to be the toxin-producing fungi, which immobilize nematodes with toxic droplets from its mycelium (Jansson et al., 1997). *Stropharia rugosoannulata* could represent a fifth group, using finger-like projections called acanthocytes to infect nematodes (Luo et al., 2006).

Many nematode-trapping fungi do not form traps spontaneously; instead they are dependent of external stimuli like living nematodes (Dijksterhuis et al., 1994). Already in the 1950s, a substance called nemin that could induce trap formation was extracted from culture filtrates of nematodes (Pramer and Stoll, 1959). Nemin was suggested to be a peptide of low molecular weight (Pramer and Kuyama, 1963). Later, short peptides were shown to induce traps in *Arthrobotrys oligospora* (Nordbring-Hertz, 1973). Recently, it was shown that ascarosides, which are constitutively secreted by nematodes, trigger trap formation in nematode-trapping fungi (Hsueh et al., 2013). Ascarosides are composed of the sugar ascarylose linked to a fatty-acid chain. More than 100 different ascarosides have been identified from various nematodes (von Reuss et al., 2012).

Among the nematophagous fungi, the nematode-trapping fungi are the most studied. Probably because they are easy to grow in the laboratory in contrast to the endoparasitic fungi (Dijksterhuis et al., 1994; Lohmann and Sikora, 1989). In several species of nematode-trapping fungi it is possible to control the development of traps by modifying the growth condition and the medium composition. Growing *A. oligospora* in aerated liquid cultures in a low-nutrient media containing soya peptone supplied with small amounts of the amino acids phenylalanine and valine allows significant trap formation (Friman et al., 1985). Growing *Monacrosporium haptotylum* in this system makes the knobs detach from the mycelium due to the air bubbling from the bottom (Friman, 1993b). The knobs can be separated from the mycelium by filtration and gives an unique opportunity to compare the isolated infection structure with the vegetative mycelium (Friman, 1993b).

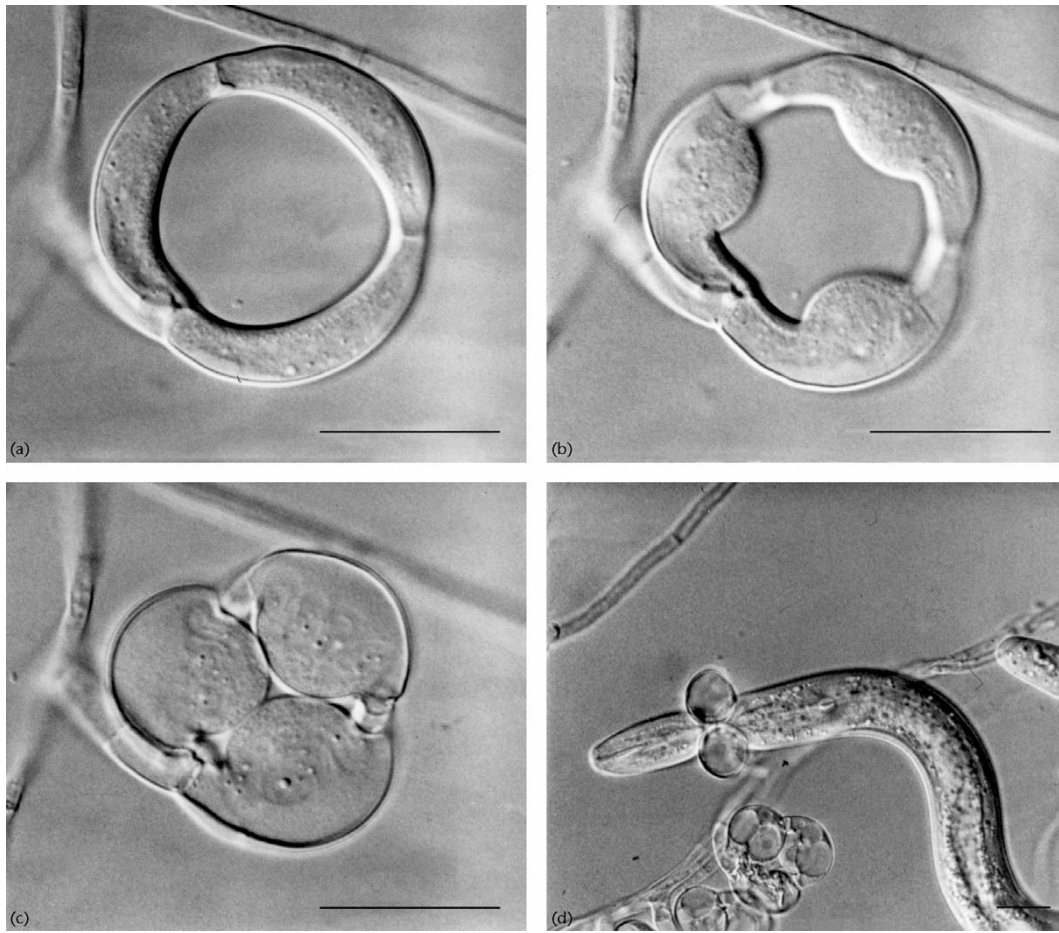
## 2.2 Diversity of infection structures in nematode-trapping fungi

The nematode-trapping fungi use different kinds of traps to capture and infect nematodes. In this thesis, fungi having all the four major types of traps have been studied (Figure 2). *A. oligospora* traps nematodes by an adhesive three-dimensional net (Nordbring-Hertz et al., 2006; Nordbring-Hertz et al., 1995). *M. haptotylum* develops at the apices of hyphal branches a structure called knobs. The knob is an adhesive single cell that can detach from the mycelia, travel along with the nematode and subsequently penetrate the nematode cuticle and infect the nematode. *Monacrosporium cionopagum* has adhesive hyphal branches that consist of one or more cells to infect nematodes. *Arthrobotrys dactyloides* uses a mechanical trap called constricting ring to trap nematodes (Nordbring-Hertz et al., 2006; Dijksterhuis et al., 1994). This is a fascinating structure that consists of three cells (Higgins and Pramer, 1967). When a nematode enters the ring, the three cells inflate and capture the nematode (Figure 3). The closure of the trap is very rapid (0.1 s) and is triggered by physical contact between the nematode and the constricting ring cells (Higgins and Pramer, 1967). The closure is also stimulated by heat or by touching the luminal side of the ring with a needle (Nordbring-Hertz et al., 2006). A fifth type of trapping structure not studied in this thesis is the sessile adhesive knobs (Yang et al., 2012). A sixth type is the non-constricting ring (Liu et al., 2009). The non-constricting ring is formed when an erect lateral branch of the vegetative hyphae thickens and curves to form a three-cells ring that fuses with the stalk. The non-constricting rings are always accompanied with adhesive knobs (Liu et al., 2009). In addition, a few nematode-trapping fungi such as *A. oligospora* and *A. dactyloides* form conidial traps (Nordbring-Hertz, 2004). The conidial traps develop directly along the germination of the conidia, without an intermediate hyphal phase (Nordbring-Hertz, 2004).



**Figure 2.** Major types of traps in nematode-trapping fungi.

**A**, adhesive net of *Arthrobotrys oligospora* (bar 20  $\mu\text{m}$ ); **B**, adhesive knobs of *Monacrosporium haptotylum* (bar 10  $\mu\text{m}$ ); **C**, adhesive branches of *Monacrosporium gephyropagum* (bar 10  $\mu\text{m}$ ); **D**, constricting ring of *Arthrobotrys brochopaga* (bar 5  $\mu\text{m}$ ). The micrographs are reproduced from Nordbring-Hertz et al., 1995, Courtesy of Institut für den Wissenschaftlichen Film, Göttingen.

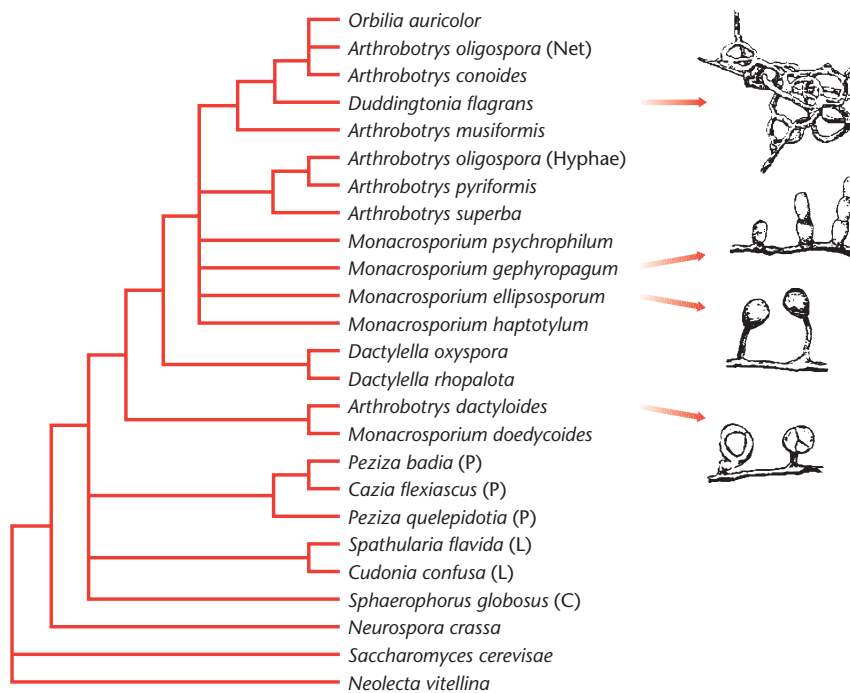


**Figure 3.** Trapping mechanism of the constricting ring of *Arthrobotrys brochopaga*. **a-c**, closure of the ring was triggered by heat (bars 5  $\mu\text{m}$ ). The closure that is irreversible is accompanied by a large increase in cell volume. The three cells building up the constricting ring are clearly visible. **d**, A nematode firmly trapped in a constricting ring (bar 10  $\mu\text{m}$ ). The micrographs are reproduced from Nordbring-Hertz et al., 1995, Courtesy of Institut für den Wissenschaftlichen Film, Göttingen.

### 2.3 Phylogeny

Phylogenetic analyses using DNA sequence information from the small subunit ribosomal genes (18S rDNA) revealed that a majority of nematode-trapping fungi belong to a monophyletic clade within the phylum of ascomycetes (Figure 4) (Ahrén et al., 1998). The phylogenetic patterns within this clade were concordant with the morphology of the infection structures rather than with the morphology of the conidia or conidiophores that traditionally have been used characters in taxonomy. This indicates that these fungi have a common ancestor and that the ability to capture nematodes has been an important trait during the evolution (Ahrén et al., 1998; Ahrén and Tunlid, 2003). A more recent phylogenetic study using five nuclear genes shows that the trapping structures have evolved along two major lineages; one leading into constricting rings and one leading into the adhesive traps (Yang et al., 2012). Furthermore, among the species forming adhesive traps, the adhesive nets and the adhesive knobs species are sister clades and are separated from species forming adhesive branches (Yang et al., 2012).





**Figure 4.** Phylogenetic tree based on 18S rDNA sequence information showing the relationships between various nematode-trapping fungi and the position of this clade among species from the Pezizales (P), Leotiales (L) and Calciales (C). Only branches with bootstrap values above 50 are shown. The phylogenetic pattern within the clade of nematode-trapping fungi is concordant with the morphology of their infection structures. The tree is redrawn from Ahrén et al., 1998. Drawings of nematode-trapping structures are from Nordbring-Hertz et al., 2006 and display from top to bottom; adhesive nets, adhesive branches, adhesive knobs and constricting rings.

## 2.4 Infection mechanism

### 2.4.1 Attraction

Despite morphological differences in the trapping structures, the infection mechanism is similar between different nematode-trapping fungi (Dijksterhuis et al., 1994). The infection mechanism starts with attraction. Since the fungi are non-motile in comparison to nematodes they need to attract the nematodes in some way. The mycelia and traps release compounds that attract the nematodes. The traps have a greater attractiveness than the vegetative mycelium (Field and Webster, 1977; Jansson, 1982). Furthermore, fungal species being relatively more parasitic show an increased attractiveness as compared to more saprophytic types (Nordbring-Hertz et al., 2006). A volatile or a small and rapidly diffusing compound that is continuously produced by the fungus has been suggested to be responsible for the attraction (Jansson and Nordbring-Hertz, 1980). Until recently, the exact components of these compounds have been unknown. However, chemical studies on the culture medium of the knob-producing *Arthrobotrys entomopaga* led to the isolation of two compounds that showed strong nematode-attracting abilities (Wu et al., 2013). The first compound, paganin A, was isolated as colourless oil with the molecular formula



C<sub>9</sub>H<sub>11</sub>O<sub>2</sub>. The second compound was blumenol A with the molecular formula C<sub>13</sub>H<sub>20</sub>O<sub>3</sub> (Wu et al., 2013).

#### **2.4.2 Adhesion**

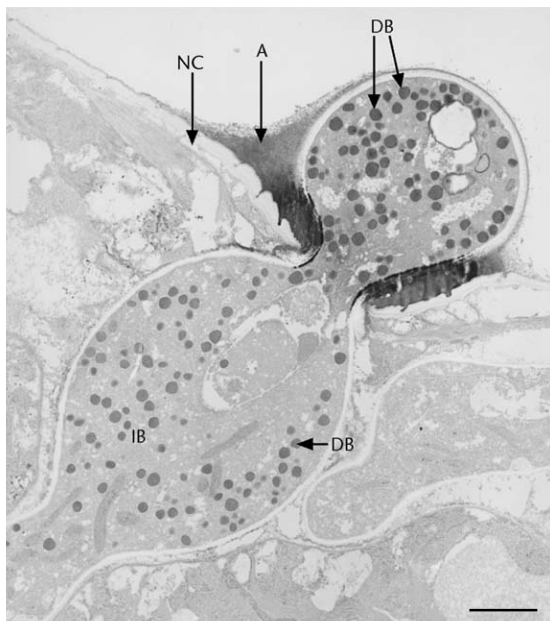
The adhesion between the fungi and the nematode has been extensively studied during the years. Electron-microscopically analysis has shown that the traps of *A. oligospora* have a layer of polymers on their surface even before contact with the nematode (Veenhuis et al., 1985). After contact there is an increased secretion of surface polymers and the fibrillar layer becomes oriented in one direction, perpendicularly to the orientation of the nematode (Veenhuis et al., 1985). Gel chromatography showed that the surface polymers contain neutral sugars, uronic acids, and proteins (Tunlid et al., 1991). In 1979, based on sugar inhibition experiment, it was suggested the infection process in *A. oligospora* was initiated by binding between a lectin present on the trap and a carbohydrate ligand found on the nematode surface (Nordbring-Hertz and Mattiasson, 1979). Subsequently, a N-acetylgalactosmine (GalNac)-specific lectin was isolated from *A. oligospora* (Borrebaeck et al., 1984). Pre-treatment of nematodes with the lectin reduced the adhesion, which further strengthen a lectin-mediated interaction (Borrebaeck et al., 1984; Premachandran and Pramer, 1984). Later, a lectin named AOL, probably identical to the previously identified lectin, was purified and characterized from *A. oligospora* (Rosén et al., 1992). However, observations showed that AOL could also function as a storage protein during both parasitic and saprophytic growth (Rosén et al., 1997). Further studies also showed that deletion of the AOL gene did not affect the mutants ability to infect nematodes (Balogh et al., 2003). Recently, using mass spectrometry 26 cell-wall proteins were identified being upregulated in traps as compared to vegetative mycelium in *A. oligospora* (Liang et al., 2013), and included glycosyl hydrolases, oxidases, pectin lyases and proteases. The authors suggested that the adhesive coating, besides its role in adhesion, also serves as a matrix, harboring many extracellular virulent proteins (Liang et al., 2013). In conclusion, the molecular mechanisms of the attachment of the traps to the nematode cuticle are not yet known.

#### **2.4.3 Penetration**

Following adhesion, the nematode-trapping fungi form a penetration tube that pierces the nematode cuticle (Veenhuis et al., 1985). Likely, this step includes both enzyme activities and mechanical pressure. The penetration site is effectively sealed, which prevent leakage of nematode contents out into the environment (Veenhuis et al., 1985). The fact that the nematode cuticle mainly consists of proteins including collagens (Cox et al., 1981) makes it likely that the nematode-trapping fungi penetrate the cuticle with aid of proteases. Indeed, many proteases have been isolated and characterized during the years. PII is a serine protease purified from *A. oligospora* that could digest proteins present on the nematode cuticle (Tunlid et al., 1994). Additional copies of *PII* in a genetically modified strain of *A. oligospora* even increased the speed of capturing and killing of the nematode as compared to the wild type. However, *PII*-deleted mutants did not show any significant change in pathogenicity (Åhman et al., 2002). A newly published paper analyzing the genome and proteome of *A. oligospora* showed that the expression of *PII* did not change when the fungus was exposed to nematode extracts (Yang et al., 2011). However, another serine protease, named *P186* was highly expressed. Mutants with *P186* being genetically disrupted showed 24 h after infection of nematodes a decreased fatality rate of 24-32%. PII and P186 belong to a group of subtilisin-like serine proteases (Åhman et al., 1996; Yang

et al., 2011). In *M. haptotylum*, a microarray analysis showed that two genes encoding subtilisins, *spr1* and *spr2*, were upregulated during infection of the nematode *Caenorhabditis elegans* (Fekete et al., 2008). The two subtilisins were highly upregulated after 1 h of infection, then downregulated after 4 and 16 h, and finally upregulated at end of the experiment (24 h) (Fekete et al., 2008). Several other extracellular subtilisins have been characterized from various nematophagous fungi (reviewed in Yang et al 2007). Chitinases, collagenases and other hydrolytic enzymes have also been shown to have nematicidal activities (Yang et al., 2007).

A common feature for all nematode-trapping fungi is the presence of dense bodies inside the trap (Figure 5). Dense bodies are cytosolic and contain catalase and amino-acid oxidase activity which indicates that they are peroxisomal. Dense bodies are rapidly degraded after the formation of the infection bulb and it has been suggested that they contain material to facilitate the penetration and the initial development of trophic hyphae (Veenhuis et al., 1989;Dijksterhuis et al., 1994).



**Figure 5.** Transmission electron micrograph of the penetration of the nematode cuticle by the nematode-trapping fungus *Arthrobotrys oligospora*. Dense bodies (DB) are visible in the trap and in the infection bulb (IB). The adhesive coating (A) is shown between the penetration tube and the nematode cuticle (NC). The micrograph is reproduced from Nordbring-Hertz et al., 2006 and originally published in Veenhuis et al., 1985. (bar 1  $\mu\text{m}$ )

#### 2.4.4 Degradation

Following penetration, the infection tube swells inside the nematode and form an infection bulb (Dijksterhuis et al., 1994). Trophic hyphae develop from the infection bulb and the infecting fungus digests the nematode. Morphologically, both the infection bulb and the following trophic hyphae show the same characteristics as normal vegetative mycelium. However, the endoplasmic reticulum is highly proliferated in both structures (Veenhuis et al., 1989). The time course for the infection process varies between different nematode-trapping fungi, and also depending on the nematode species being infected and even between different individuals of the same nematodes species. In *A. oligospora*, the process from adhesion until penetration and immobilization of the nematode usually takes 1-4 h (Nordbring-Hertz and Stålhammar-Carlemalm, 1978;Veenhuis et al., 1985). The infection bulb is formed and trophic hyphae are developed. After 12-24 h the growth rate of the trophic hyphae is retarded and growth of fungal mycelium outside the nematode is initiated. The infection process is usually completed within 48-60 h (Dijksterhuis et al., 1994).

### 3. Summary of thesis

#### 3.1 Paper I: Genomic mechanisms accounting for the adaption to parasitism in nematode-trapping fungi

##### 3.1.1 Summary of paper I

In this study we sequenced the genome of *M. haptotylum* by 454 pyrosequencing. The genome size was estimated to 40.4 Mb and the number of predicted protein-encoding genes to 10,959. These numbers were similar to the genome of the net-forming *A. oligospora* (40.1 Mb genome and 11,479 protein-encoding genes) (Yang et al., 2011). Comparative genomics of these two genomes together with the genomes of other ascomycetes showed that the two nematode-trapping fungi contained a large number of lineage-specific and species-specific genes. In total ~62% of the genes in the genomes of *M. haptotylum* and *A. oligospora* were shared by other fungi; ~20% of the genes were shared between the two nematode-trapping fungi and up to 16% of the genes were unique for each genome. The lineage-specific and species-specific genes were shorter than the core genes and also enriched in secreted proteins including small secreted proteins (SSP). A Pfam analysis identified 25 expanded protein families in *M. haptotylum* compared to other ascomycetes. They included several peptidases, plant cell-wall degrading enzymes and virulence factors usually found in plant-pathogenic fungi. In addition to genome sequencing, the transcriptomes of *M. haptotylum* and *A. oligospora* during infection of the nematode *Caenorhabditis briggsae* were analyzed by sequencing. In *M. haptotylum*, 117 genes were identified that were among the 10% most expressed transcripts and also ten-fold upregulated in the infecting hyphae as compared to knobs. These 117 genes were enriched in species-specific genes, encoding secreted proteins, SSPs, and genes identified to belong to expanded gene families. A detailed comparative transcriptome analysis was made comparing the expression levels of ortholog pairs in *M. haptotylum* and *A. oligospora*. The orthologs displaying the largest differences in expression values were again enriched in lineage-specific genes, encoding secreted proteins, SSPs and genes identified to belong to expanded gene families. Conclusively, the comparison of the *M. haptotylum* and *A. oligospora* genomes to each other and to the genomes of other ascomycetes suggested two genomic mechanisms that likely have been important for the adaption to parasitism in the nematode-trapping fungi. Firstly, the expansion of gene families and the large number of species-specific genes indicated that gene duplication followed by functional diversification may have played a major role in the evolution of the nematode-trapping fungi. Many of these genes were also highly expressed and regulated during infection, indicating their importance for pathogenicity. Secondly, the differential gene expression of orthologs between the two fungi during early infection indicated that differential regulation have been an important mechanism for the evolution of parasitism in the nematode-trapping fungi in response to the nematode host.

##### 3.1.2 Similarity to plant-pathogenic fungi

During the course of my PhD education the first whole genome sequence of a nematode-trapping fungi was published (Yang et al., 2011). Yang and co-workers sequenced the well-studied *A. oligospora* using 454 pyrosequencing combined with Sanger sequencing. They showed by comparative genomics that *A. oligospora* shared many more genes with pathogenic fungi than with non-pathogenic fungi. The *A. oligospora* genome contained a larger number of genes

encoding pathogenicity-related enzyme families such as subtilisins, cellulases, pectinesterases and cellobiohydrolases as compared to the other analyzed fungi (Yang et al., 2011). In Paper I we show that the nematode-trapping fungi share more gene families being expanded in plant-pathogenic fungi as compared to insect- or animal-pathogenic fungi. Previously, it has been shown that both *M. haptotylum* and *A. oligospora* has the ability to colonize roots of tomato (Persson and Jansson, 1999). The adhesive traps of nematode-trapping fungi have several structural and functional similarities with appressoria formed by plant-pathogenic fungi (Ahrén et al., 2005). Both are specialized infection structures that develop from hyphae. The structures contain an adhesive layer that binds to the host surface. Following attachment, both traps and appressoria form a hypha that penetrates the host using a combination of physical force and extracellular enzyme activities (Ahrén et al., 2005). The physical force is in *Magnaporthe grisea* generated by a high turgor pressure (Tucker and Talbot, 2001). The turgor pressure is the result of a rapid influx of water into the appressoria against a concentration gradient of glycerol, maintained by a melanin layer in the cell wall (Tucker and Talbot, 2001). The high concentration of glycerol is built up by the breakdown of glycogen and lipids (Thines et al., 2000). In *A. oligospora* enzymes involved in the degradation of glycogen were up-regulated while enzymes involved in lipid degradation were down-regulated during trap formation (Yang et al., 2011). Interestingly, it was experimentally confirmed that glycerol accumulates during trap formation (Yang et al., 2011). This indicates that the high turgor pressure in *A. oligospora* is generated by glycerol accumulation, similarly to that of *M. grisea*. Whether or not this mechanism applies to other nematode-trapping fungi needs further studies.

### **3.1.3 Small secreted proteins**

In paper I, 36 SSPs were identified among the 117 genes that were 10% most expressed during infection and also ten-fold upregulated in the infecting hyphae compared to knobs.. Among these 36 SSPs, 17 were orphans, defined as lacking known homologs and not to contain any Pfam domains. Therefore, it is hard to predict their function. The importance of SSPs for successful infection of the host has been reported from both insect- and plant-pathogenic fungi (Xiao et al., 2012;Doehlemann et al., 2009). The expansion of SSPs related to pathogenicity appears to be an evolutionary strategy used by these fungi (Lowe and Howlett, 2012). Many of the SSPs involved in pathogenicity are effectors. Effectors are generally cysteine-rich, lack transmembrane domains and are often species specific (Lowe and Howlett, 2012). The function of effectors varies; they can manipulate the host cell structure and function and thereby facilitate the infection (Kimura et al., 2001). In *Cladosporium fulvum*, effectors target and inhibit plant proteases important for host defence (Rooney et al., 2005). In *Ustilago maydis*, the effector Cmu1, that is a chorismate mutase, changes the metabolic status of the plant cell, leading to a reduced plant defence (Djamei et al., 2011). Another type of SSP is the hydrophobins that are uniquely found in filamentous fungi (Wosten, 2001). Hydrophobins are short (about 100 amino-acid residues), characterized by high levels of hydrophobicity and contain eight conserved cysteine residues. Hydrophobins always seem to relate to interactions with interfaces or surfaces and they have functions in protection, adhesion and growth (Wosten, 2001;Bayry et al., 2012). In *M. grisea*, the hydrophobin Mpg1 is required for pathogenicity since it acts as a development sensor for appressorium formation (Talbot et al., 1996). In *Beauveria bassiana*, hydrophobins create the spore-coat rodlet layer and are required for attachment to the insect epicuticle and virulence (Zhang et al., 2011). However, only two genes were found containing the Pfam domain Hydrophobin (PF01185) among 10,959 gene models predicted in *M. haptotylum*. This indicates that very few of the SSPs identified in *M.*

*haptotylum* are hydrophobins. Further studies are needed to evaluate if the SSPs in *M. haptotylum* function as effectors or have other function(s). In Paper I we propose a model for the origin and diversification of SSPs in nematode-trapping fungi. Gene duplication through unequal crossing over has generated a large number of SSPs in *M. haptotylum*. Following such duplications, the SSP have undergone rapid diversification through the repeat-induced point mutations (RIP) mechanism. RIP is a fungus-specific mechanism that generates point mutations in repeated regions of a genome. RIP was discovered in *Neurospora crassa* and leads to the enrichment in TpA because of CpG to TpA transitions (Cambareri et al., 1989). In *Leptosphaeria maculans*, RIP has been described as an evolutionary mechanism leading to virulence since it creates rapid sequence diversification (Fudal et al., 2009).

## **3.2 Paper II: Proteome of the nematode-trapping cells of the fungus *Monacrosporium haptotylum***

### ***3.2.1 Summary of paper II***

In this study the proteome of the trapping structure of the nematode-trapping fungi *M. haptotylum* was characterized. *M. haptotylum* was grown in aerated liquid cultures. After 10 days of incubation, when sufficient numbers of traps (knobs) had formed and detached from the mycelia, the knobs were separated from mycelia by filtration. Proteins were extracted from both mycelia and knobs and analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography-tandem mass spectrometry (LC-MS-MS). Since the genome sequence of *M. haptotylum* was available (Paper I) the peptides generated from the mass-spectrometry runs could be assigned to gene models. In total, 336 proteins were identified, 95 of these proteins were uniquely found in knobs, 89 were uniquely found in mycelia and 152 proteins were found in both structures. Quantitative proteomics revealed that 54 proteins were upregulated at least 2-fold in knobs *versus* mycelia. The upregulated knob proteome was characterized by an overrepresentation of secreted proteins including SSPs. Other extracellular proteins such as peptidases and proteins containing the carbohydrate-binding domains WSC and GLEYA were also overrepresented in the knob. Furthermore, thioredoxin, glutathione S-transferase and HSP70 are all proteins involved in various stress responses, and were found more expressed in knobs than in mycelia. The upregulated knob proteome was also enriched in members of protein families that we in Paper I classified as gene families being expanded in *M. haptotylum*. They included proteins containing WSC, GLEYA, peptidase\_S8, aspartyl protease, tyrosinase and mucin domains. In addition to the proteomic analysis, a transcriptomic analysis comparing the transcriptional profiles of knobs and mycelia was conducted. The results showed that in total 851 gene models were more than 2-fold upregulated in the knobs as compared to mycelia. However, only six of those were found upregulated on the protein level. The knob transcriptome was as well as the knob proteome characterized by an overrepresentation of secreted proteins including SSPs. The knob proteome was also compared with the transcriptome of hyphae infecting the nematode *C. briggsae*. Eleven of the in total 842 gene models that were more than 2-fold upregulated in the penetrating hyphae (in a pairwise comparison with the knob transcriptome) were also upregulated at the protein level in the knob. Interestingly, five of those were proteins containing the WSC domain.

### 3.2.2 Previously proteome studies in nematode-trapping fungi

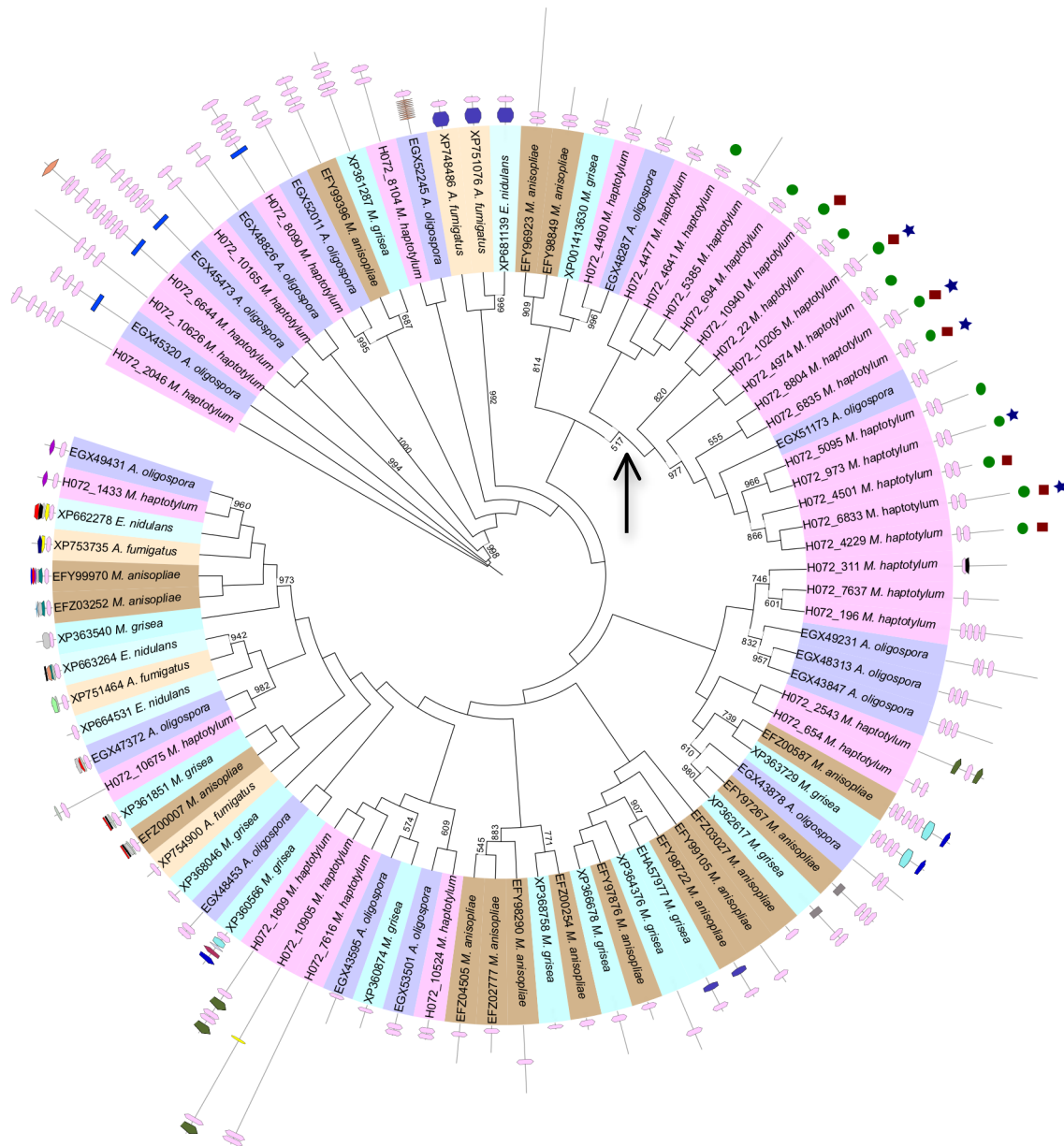
Our study was the first proteomic study where isolated trapping cells of a nematode-trapping fungus have been characterized in detail. Previously, the proteome in the knob-producing *Monacrosporium lysipagum* and the proteome in the net-producing *A. oligospora* have been analyzed (Khan et al., 2008; Yang et al., 2011). The study with *M. lysipagum*, analyzing mycelia containing knobs, identified 51 proteins encoded by 31 genes. The identified proteins included mainly house-keeping proteins, enzymes and membrane-associated proteins (Khan et al., 2008). In *A. oligospora* the proteome during trap-formation was characterized. In total, 90 out of 861 proteins were upregulated more than 1.5 times in samples 10 h after induction of traps as compared to vegetative mycelium. They included proteins involved in translation, posttranslational modification, amino-acid metabolism, carbohydrate metabolism, energy conversion and cell wall and membrane biogenesis (Yang et al., 2011). The uniqueness of our proteome analysis is that it is based on isolated trapping cells, which allowed the identification of proteins that were upregulated or uniquely expressed in these structures. We found that the knob is an active cell structure in which different kinds of metabolism were found upregulated as compared to the vegetative mycelia. When comparing the expression of the proteins with the expression of their transcripts the upregulated knob proteins showed a higher concordance with the transcript during infection ( $r=0,32$ ) than to the transcription of knobs that were not infecting nematodes ( $r=-0,20$ ). Furthermore, several proteins believed to be involved in pathogenicity were identified in knobs. These findings indicates that the knob contain, even before the adhesion to a nematode, many of the proteins needed in the early stages of infection. An earlier study showing that inhibition of translation does not affect the infection process supports this idea (Friman, 1993a).

### 3.2.3 WSC

In Paper I we identified 33 genes with the Pfam domain WSC in the genome of *M. haptotylum*, and 13 of those were found upregulated in the transcriptome during infection. In this study (Paper II) we identified five proteins upregulated in the knobs containing the WSC domain. All these five proteins were included among the 13 WSC proteins that were found upregulated during infection. A phylogenetic analysis revealed that there was a clade of 15 WSC paralogs (Figure 6). This clade only has one ortholog in *A. oligospora*, indicating that the expansion of the *M. haptotylum* paralogs within this clade has occurred after the speciation of *M. haptotylum* and *A. oligospora*. Interestingly, this clade contains all 13 genes that were upregulated during infection.

The WSC domain is putatively a carbohydrate-binding domain that is found in proteins with diverse functions (Ponting et al., 1999). In *Trichoderma harzianum* the WSC domain has been found in a  $\beta$ -1,3-exoglucanase (Cohen-Kupiec et al., 1999). However, the WSC proteins that have been most studied are those involved in the cell-wall integrity (CWI) pathway (Futagami et al., 2011). These so-called cell-surface sensors have been extensively characterized in yeast, where they monitor cell stress, including internal turgor pressure, through the CWI pathway (Verna et al., 1997; Lodder et al., 1999; Merchan et al., 2004).





**Figure 6.** Phylogeny of the WSC Pfam family in *Monacrosporium haptotylum*. Bootstrap values are marked in the tree, and branches with a lower bootstrap value than 50% are not shown. Arrow shows the clade of 15 rapidly expanded WSC proteins in *M. haptotylum*. This clade contains the five proteins that were more than 2-fold upregulated in the knob proteome as compared to the mycelium (blue star), the 13 proteins that were more than 2-fold upregulated during infection as compared to the knob transcriptome (green circles) and seven proteins that were highly upregulated during infection as compared to the knob transcriptome (more than 10-fold) and highly expressed during infection (10% of the most expressed transcripts) (red squares). For the full version of the figure see Paper II.

One out of in total 33 WSC-encoding genes in the genome of *M. haptotylum* displayed sequence similarity to WscB in *Aspergillus nidulans*, which is involved in the CWI pathway (Futagami et al., 2011). However, based on sequence similarity searches, the function of the other WSC proteins in *M. haptotylum* remains unclear. A majority of the WSC proteins have features commonly found in fungal adhesions, such as a signal peptide, tandem repeats and O-

glycosylation sites (Linder and Gustafsson, 2008). Therefore we suggest that a large proportion of the WSC proteins are extracellular and located on the surfaces of the knob cells. One question that needs attention is whether WSC proteins are long enough to reach the outermost cell wall so that an interaction with the nematode is possible. It has been shown that the cell surface sensor WSC1 in *Saccharomyces cerevisiae* behaves like a nanospring capable of resisting high mechanical force and of responding to cell-surface stress (Dupres et al., 2009). The full-length WSC1 protein consists of 378 amino-acid residues and it has been estimated that the extracellular part extend 86 nm from the plasma membrane. Since the approximate thickness of the yeast cell wall is 110 nm, WSC1 is too short to reach the outermost cell wall. The WSC domain in WSC1 is located in the N-terminal region, which is followed by a serine/threonine-rich region, a single transmembrane region and a short cytoplasmic tail. It is suggested that the cysteines of the WSC domain are connected to cell-wall glucans and that the serine/threonine regions in the WSC1 are glycosylated to give stiffness to the protein (Dupres et al., 2009). In *M. haptotylum*, the length of the 15 WSC proteins in the expanded clade of rapidly evolving WSC proteins varied between 223 – 1102 residues with the average length being 560 residues. The thickness of the knob cell wall in *M. haptotylum* appears to be 100-150 nm when examining electron micrographs (Saikawa and Kaneko, 1994). This indicates that a majority of the WSC proteins in the expanded clade in *M. haptotylum*, if they have the same properties as WSC1 in *S. cerevisiae*, are capable of reaching the outermost cell wall. However, further studies are needed to investigate whether these WSC proteins participate in the adhesion of nematodes, function as cell-surface sensors or have any other function.

### 3.3 Paper III: Interspecific and host-related gene expression patterns in nematode-trapping fungi

#### 3.3.1 Summary of paper III

In this study the transcriptomes of three species of nematode-trapping fungi representing different trapping mechanisms infecting two species of plant-parasitic nematodes were characterized. Included were the nematode-trapping fungi *Arthrobotrys oligospora* (adhesive nets), *Monacrosporium cionopagum* (adhesive branches) and *Arthrobotrys dactyloides* (constricting rings) that were tested on the nematode species *Meloidogyne hapla* (root knot nematode) and *Heterodera schachtii* (sugar beet cyst nematode). These nematodes are both sedentary endoparasites belonging to the group of nematodes causing the most damages to crops (Williamson and Gleason, 2003). Specimens of *M. hapla* or *H. schachtii* were added to plates containing *A. oligospora*, *A. dactyloides* or *M. cionopagum* and the infection was followed under a light microscope. Materials were collected from all infection stages (trapped, paralyzed and infected nematodes). RNA was extracted, mRNA was purified and cDNA was synthesized and sequenced using 454 pyrosequencing. The raw reads from the two libraries from *A. oligospora* were mapped against its published genome (Yang et al., 2011). Since there were no genome sequences of *A. dactyloides* and *M. cionopagum*, the reads from these libraries had to be assembled prior mapping. To be able to compare this data with *A. oligospora*, the two libraries from *A. oligospora* were also assembled. The result showed that the divergence in interspecific gene expression was significantly larger than the divergence due to the nematode being infected. A core set of genes was identified that were highly expressed by all fungi independent of trapping mechanism. They included several peptidases such as subtilisins and aspartic proteases, cell surface proteins containing the carbohydrate-binding domain WSC and ribosome inactivating Ricin-B lectins. Many of the



genes identified within this core set, were found to encode protein families that were previously found expanded among nematode-trapping fungi (Paper I). One of these families was the Ricin-B lectins that are ribosome-inactivating proteins (Michiels et al., 2010). Other lectins such as the fungal fruit-body lectins and D-mannose binding lectins were only highly expressed in the fungus forming adhesive nets and not in the species forming adhesive branches or constricting rings. Host specific gene expression was analyzed using the mapped reads of *A. oligospora*. That is, genes that are differentially expressed dependent on which nematode *A. oligospora* infects. Genes encoding secreted proteins were enriched among the differentially expressed genes. They included peptidases, glucosidases and several gene families that were expanded in the genomes of nematode-trapping fungi such as genes encoding DUF3129, WSC and tyrosinase.

### ***3.3.2 Defense against reactive oxygen species***

In Paper III one group of genes that were highly expressed in all fungi independent of trapping structure and independent of nematode species being infected were genes encoding stress proteins. Several stress proteins were also upregulated in the proteome of the knob as compared to the vegetative mycelia in *M. haptotylum* (Paper II). Among these putative stress proteins many antioxidant enzymes were identified, such as thioredoxins, catalases and superoxide dismutases. Antioxidants are enzymes involved in the protection of the cell from oxidative damages induced by reactive oxygen species (ROS) (Morano et al., 2012). One question that needs answering is what role ROS have in the fungal-host interaction. ROS are continuously produced in the cell as byproducts from various metabolic pathways and have an important role(s) in signaling (Heller and Tudzynski, 2011). In *M. grisea*, ROS-generating NADPH oxidases (Nox1 and Nox2) are essential for pathogenicity (Egan et al., 2007). The authors suggest that the generated ROS accumulate in the appressorium to facilitate oxidative cross-linking of cell-wall proteins. This leads to a strengthening of the appressorium cell wall that will eventually resist high turgor pressure (Egan et al., 2007). Homologs to Nox1 and Nox2 proteins were regulated in the nematode-trapping fungi during infection (Paper III). ROS are also produced in plants as a defense strategy against invading pathogens (Wojtaszek, 1997). Interestingly, ROS are produced in *C. elegans* as a response to pathogenic bacteria (Chavez et al., 2007). Whether this also applies to nematodes infected by nematode-trapping fungi needs to be examined. Although, the identification of NADPH oxidases regulated in the nematode-trapping fungi during infection and the identification of antioxidant enzymes in the knob of *M. haptotylum* prior to infection makes it likely that the ROS derive from the fungus.

### ***3.3.3 Peptidase\_S8***

Serine proteases containing the Pfam domain peptidase\_S8 are important virulence factor in the nematode-trapping fungi (Tunlid and Ahrén, 2011; Yang et al., 2007). Peptidase\_S8 is an expanded gene family in *M. haptotylum* (59 genes) and in *A. oligospora* (52 genes) (Paper I). In Paper III, genes encoding proteins containing the peptidase\_S8 domain were found among the group of core genes, expressed by all fungi. However, among the 500 most expressed transcripts only one transcript was detected in each library containing the peptidase\_S8 domain. All these five peptidase\_S8 containing transcripts matched in BlastX searches either G1XLL2 in *A. oligospora* or H072\_8474 in *M. haptotylum*. Interestingly, these two proteins are orthologs (T. Meerupati, B. Canbäck, D. Ahrén, A. Tunlid, manuscript). Furthermore, G1XLL2 was the most

expressed peptidase\_S8-encoding gene in *A. oligospora* during early infection (6 and 10 hours) of *C. briggsae* and H072\_8474 was the second most expressed peptidase\_S8 gene in *M. haptotylum* during early infection (4 hours) of *C. briggsae* (Paper I). H072\_8474 was also identified in the proteome of both knobs and the mycelia (Paper II). The most expressed peptidase\_S8-encoding gene in *M. haptotylum* during infection of *C. briggsae* was H072\_5672 (Paper I) and is representing the gene *spr2* that was detected in a microarray analysis when *M. haptotylum* was infecting *C. elegans* (Fekete et al., 2008). This shows that despite the large number of peptidase\_S8 paralogs only a few of those are highly expressed during infection and furthermore of those being highly expressed in different nematode-trapping fungi, many are orthologs. However, to identify the most potent virulence factor among the peptidase\_S8 gene could be challenging. One virulence factor in *A. oligospora* that has been experimentally verified is the peptidase\_S8 gene *P186* (Yang et al., 2011). Mutants with a disrupted *P186* gene showed a decreased fatality rate of 24-32% 24 h after infection of *C. elegans* (Yang et al., 2011). In Paper III, no transcripts were found representing this gene and in Paper I 31 peptidase\_S8 genes showed higher expression levels than *P186* during infection of *C. briggsae*.

#### 4. Conclusions and future aspects

By sequencing the genome of *M. haptotylum* and by doing comparative genomics and transcriptomics to the closely related *A. oligospora* we could also identify following mechanisms that likely have been important for the adaptation to parasitism in these fungi:

- Gene duplication followed by functional diversification has played a major role in the evolution of the nematode-trapping fungi.
- Differential regulation of orthologous genes has been an important mechanism for the evolution of parasitism in the nematode-trapping fungi in response to the nematode host.
- Repeat-induced point mutations (RIPs) have caused rapid sequence diversification of small-secreted proteins (SSPs) of which many are regulated during infection.

In Table 1 I list candidate virulence factors with assigned functions that have been identified in this thesis. Some have been identified in earlier studies such as the peptidase\_S8, CFEM and DUF3129 (Fekete et al., 2008). Others are novel virulence factors previously not identified in nematode-trapping fungi such as WSC and RicinB lectins. The list is based on the genomic, transcriptomic and proteomic studies that were conducted. The genomic study revealed several gene families that were expanded in nematode-trapping fungi as compared to other ascomycetes (Paper I). Lineage-specific gene expansion has been shown to be one of the most important means of adaptation in eukaryotes (Lespinet et al., 2002). Therefore, it is likely that many of these expanded gene families are important for virulence in the nematode-trapping fungi. The transcriptional studies discovered a wide range of genes that were regulated in the nematode-

trapping fungi during infection of nematodes (Paper I and III). By using bioinformatics tools such as sequence-similarity searches putative function(s) of these genes were assigned. Comparison of these genes to known virulence factor in plant- and animal-pathogenic fungi shortened the list of candidate virulence factor in the nematode-trapping fungi. The proteomic study of *M. haptotylum* showed genes that truly were expressed in this fungus (Paper II). Even though only a small proportion of the proteome could be identified (in total 336 proteins), it is the most abundant proteins that will be detected. *M. haptotylum* offers unique opportunities since it is possible to separate the trapping structure from the vegetative mycelium and analyze these structures separately (Friman, 1993b). By doing quantitative proteomics we could identify 54 proteins that were significantly more highly expressed in the knobs than in the mycelia (Paper II). Among these, five proteins contained the carbohydrate-binding domain WSC. Interestingly, the transcripts representing all these five WSC proteins were more than 2-fold upregulated during infection (infected hyphae *versus* knob) (Paper I). To further investigate the WSC proteins a transformations protocol for *M. haptotylum* needs to be developed. It would then be possible to knock out one or several WSC genes and the adhesion ability of these mutants would be allowed to be compared to the wild type. Another possibility is to raise antibodies against the WSC protein(s) and follow their localization during infection.

Nowadays there is much interest in small secreted proteins (SSPs). Especially effectors among the SSPs in plant-pathogenic fungi have been extensively studied (Rep, 2005; Hacquard et al., 2012). Many SSPs were also highly expressed in *M. haptotylum* during infection (Paper I). Among the 117 genes that were among the 10% most expressed during infection and also ten-fold upregulated in the infecting hyphae compared to knobs 36 were SSPs (Paper I). SSPs often show high sequence diversity and homologs are frequently hard to identify (Rafiqi et al., 2012). Indeed, 17 out of the 36 highly expressed SSPs found in *M. haptotylum* were orphans, since they are lacking known homologs and do not contain any Pfam domains (Paper I). Therefore, it is challenging to predict their function. To further investigate the role of SSPs in the nematode-trapping fungi it would be interesting to do localization studies to see if they are transported into the nematode during early infection. Another way is to do heterologous protein expression and examine their nematotoxic activity. Bioassays have been developed where expressed proteins are added in a range of concentrations to nematodes in microtiter wells (Åhman et al., 2002). The nematotoxic potency can then be compared to anthelmintics drugs such as ivermectin (Geary, 2005). If I had to choose only one protein for further studies it would be a protein among the Ricin-B lectins. They are encoded by an expanded gene family in nematode-trapping fungi (Paper I) and are highly expressed during infection (Paper I and III). Ricin-B lectins are proteins inhibiting protein synthesis (Endo and Tsurugi, 1987). A nematotoxic Ricin-B lectin has been identified in the basidiomycete *Marasmius oreades* (Wohlschlager et al., 2011) and an insecticidal Ricin-B lectin has been identified in the ascomycete *Sclerotinia sclerotiorum* (Hamshou et al., 2010). Further studies of the Ricin-B lectins and other candidate virulence factors in the nematode-trapping fungi will hopefully lead to the discovery of new anthelmintics that can treat nematode-infections.

**Table 1.** Candidate virulence factors in nematode-trapping fungi.

Pfam domain	Putative function	Nematode-trapping fungus <sup>a</sup>
<b>Peptidases</b>		
Peptidase_S8	Penetration	Mh <sup>b</sup> ,Ao,Ad,Mc
Peptidase_S10	Penetration	Mh <sup>b</sup> ,Ao,Ad,Mc
Asp	Penetration	Mh <sup>b</sup> ,Ao,Ad,Mc
<b>Cell wall proteins</b>		
WSC	Adhesion	Mh <sup>b</sup> ,Ao,Ad,Mc
PA14_2	Adhesion	Ao
CFEM	Matrix formation	Mh,Ao,Ad,Mc
<b>Lectins</b>		
RicinB_lectin_2	Ribosome-inactivating	Ao,Ad,Mc
RicinB_lectin	Ribosome-inactivating	Mh
<b>Stress proteins</b>		
Thioredoxin	Defence against ROS	Mh <sup>b</sup> ,Ao,Ad,Mc
Sod_Cu	Defence against ROS	Mh,Ao,Ad,Mc
Sod_Fe	Defence against ROS	Ao,Mc
Catalase	Defence against ROS	Mh,Ao,Ad,Mc
<b>Others</b>		
Tyrosinase	Melanin synthesis	Mh <sup>b</sup> ,Ao,Ad,Mc
DUF3129	Unknown	Mh,Ao,Mc

<sup>a</sup> Column presenting the fungus that has the specified Pfam domain among the 500 most expressed genes during infection by *M. haptotylum* (Mh) (Paper I) or among the 500 most expressed genes during infection by *A. oligospora* (Ao), *A. dactyloides* (Ad) or *M. cionopagum* (Mc) (Paper III)

<sup>b</sup> The Pfam domain was found in the 54 proteins that were more than 2-fold upregulated in knobs as compared to mycelia in *M. haptotylum* (Paper II)

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## Svensk sammanfattning

Nematod-fångande svampar är en grupp av filamentösa svampar som lever i jorden. De kan som deras namn antyder fånga och infektera nematoder. Varför är det då viktigt att studera dessa svampar? Svaret är just nematoder. Nematoder, även kända som rundmaskar, kan infektera både människor, djur och växter. Vi i Europa är relativt förskonade mot dessa infektioner om man jämför med människor som lever i Asien, delar av Afrika samt delar av Amerika. Infektioner i boskap och grödor orsakar skador för miljardtals kronor varje år för dessa näringar. Det finns läkemedel för att bota dessa infektioner, och det går att bespruta grödor för att minska antalet nematoder. Nematodpopulationer har blivit mer och mer motståndskraftiga mot dessa läkemedel och en ökad efterfrågan av mer ekologiskt jordbruk har ökat behovet av en mer biologisk kontroll av dessa nematoder. De nematod-fångande svamparna är därav av intresse eftersom de är deras naturliga fiender. Redan på 1930-talet såg man denna potential och under 1980-talet så gjordes lyckade fältförsök där man kunde se att antalet nematoder i boskap minskade när man matade djuren med sporer av den nematod-fångande svampen *Duddingtonia flagrans*. Istället för att använda den levande svampen som kontrollmedel så har vi i vår forskning försökt att ta reda på vad det är i svampen som är skadligt för nematoden. Det kan i förlängningen leda till utveckling av läkemedel med nya verkningsmekanismer, eller möjlighet att genetiskt modifiera en svamp så att den blir ännu bättre på att oskadliggöra nematoder.

Vi har tagit reda på vilka gener som den nematod-fångande svampen *Monacrosporium haptotylum* innehåller och gjort jämförelser med gen-innehållet hos andra svampar. Vi fann ett antal gen-familjer som har expanderat i *M. haptotylum*, som vi tror är viktiga för dess förmåga att infektera nematoder. När de expanderade gen-familjerna jämfördes med andra svampar var *M. haptotylum* mer lik växt-patogena svampar än djur-patogena svampar. *M. haptotylum* fångar nematoder med en knopp-liknande struktur. Den är en enkel cell som bildas från ett utskott av en hyf. Svampen utsöndrar ämnen så att nematoden lockas att komma nära, när knoppen får kontakt med nematoden klistrar den sig fast till nematoden via en okänd mekanism. Bindningen är väldigt stark och det kan hända att knoppen bryts loss från hyfen när nematoden kämpar för att komma därifrån. Knoppen kan då färdas med nematoden och sedan infektera den utan hjälp av mycelet. Via ett särskilt odlings sätt kan stora mängder av dessa fångstorgan produceras. Svampen får växa i en vätskekultur med luft som bubblar underifrån. Luftbubblorna gör att knopparna släpper och dessa kan sedan isoleras från mycelet genom filtrering. Det här ger en unik möjlighet att studera vilka proteiner som det finns mer av eller som kanske till och med är unika i knoppen jämfört med mycelet. Vi har undersökt både genuttryck och proteininnehåll i dessa knoppar, och jämfört med mycelet. Vi har även studerat genuttrycket specifikt under infektion och kommit fram till en lista med kandidater viktiga för denna svamps parasitära förmåga. Listan inkluderar proteiner som vi tror är viktiga för knoppens förmåga att kunna fästa till en nematod samt enzymer som behövs för att kunna penetrera nematodens yttre skikt.

En annan studie gjordes där genuttrycket hos tre andra nematod-fångande svampar undersöktes när de infekterade två olika arter av nematoder. Två av dessa svampar fångar nematoder på samma sätt som *M. haptotylum*, genom att klistra sig fast vid nematoden. Den tredje svampen har en spännande struktur som fångar nematoden mekaniskt. Dess fångstorgan består av tre celler som bildar en ring. När nematoden simmar in i ringen och nuddar cellerna så sväller cellerna på mindre än en sekund och nematoden fastnar. Ett antal gener identifierades i dessa studier som uttrycks oavsett typ av fångstorgan. Flera av dessa gener var liknande de gener som den knopp-bildande svampen uttryckte under infektion. En intressant grupp av gener som var högt uttryckt i

alla tre svampar var de Ricin-liknande generna. Ricin är ett toxiskt ämne som utsöndras från ricinväxtens frön. Den är toxisk genom att den inhiberar syntesen av nya proteiner. De flesta generna i denna studie reglerades på samma sätt oavsett vilken nematod som svamparna infekterade. Dock så kunde ett litet antal gener urskiljas som reglerades olika. De inkluderade enzymer och även proteiner som binder kolhydrater. En teori är att de svampar som bildar adhesiva fångstorgan fångar nematoden genom proteiner på svampens yta som binder till kolhydrater på nematodens hud.

Sammanfattningsvis så har vi i dessa studier undersökt de molekylära mekanismerna som ligger bakom de nematod-fångande svamparnas förmåga att infektera nematoder. Ett antal gener har identifierats som vi tror är viktiga för infektionen. För att kunna verifiera dessa som virulensfaktorer så måste ytterligare experiment göras. Ett sätt är att slå ut en gen i taget så att inte proteinet som genen kodas för bildas. Sedan får svampen infektera nematoder, och virulensen jämförs med en svamp med korrekt genuppsättning. Ett annat sätt är att studera lokaliseringen av proteiner under infektion. Genom att bilda antikroppar mot ett speciellt protein kan man med hjälp av fluorescensmikroskopi se var dessa proteiner är placerade under infektionen. Finner man en stark virulensfaktor i dessa experiment så kan det förhoppningsvis leda till ett första steg i utvecklingen av nya viktiga läkemedel.

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