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MINI-REVIEW

Extracellular enzymes and the pathogenesis of nematophagous fungi

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Abstract Nematophagous fungi are an important group of soil microorganisms that can suppress the populations of plant-parasitic nematodes. The pathogenic mechanisms of nematophagous fungi are diverse: They can be parasitical–mechanical through producing specialized capturing devices, or toxin-dependent. During infections, a variety of virulence factors may be involved against nematodes by nematophagous fungi. In this review, we present up-to-date information on the modes of infection by nematophagous fungi. The roles of extracellular hydrolytic enzymes and other virulence factors involved in infection against nematodes were summarized. The biochemical properties and peptide sequences of a special group of enzymes, the serine proteases, were compared, and their implications in infections were discussed. We also discussed the impact of emerging new techniques on our understanding of this unique group of fungi.

Introduction

Plant-parasitic nematodes cause severe damages to world agriculture (Siddiqui and Mahmood 1996) every year. In recent years, nematophagous fungi, one of the natural enemies of nematodes, have been proposed as biological agents to control the harmful nematodes because of their unique ability to infect and kill the nematodes (Siddiqui and Mahmood 1996; Nordbring-Hertz et al. 2000). This strategy is attracting increasing attention because the traditional method using chemical nematicides has resulted in significant environmental pollutions. In addition, the development of resistance among nematodes to these chemical nematocides has emerged and is beginning to decrease their effectiveness (Kerry 2000; Larsen 2000).

Nematophagous fungi include a wide and diverse range of fungi that can antagonize nematodes. They can be grouped into three categories according to their different pathogenic mechanisms: nematode-trapping fungi, parasitic fungi, and toxic fungi (Siddiqui and Mahmood 1996; Li et al. 2000; Nordbring-Hertz et al. 2000). At present, the detailed molecular pathogenic mechanisms against nematodes by nematophagous fungi have not yet been fully elucidated. However, increasing evidences show that extracellular hydrolytic enzymes including proteases, collagenase, and chitinase may be involved in nematode-cuticle penetration and host-cell digestion (e.g., Åhman et al. 2002; Huang et al. 2004; Morton et al. 2004). Ultrastructural and histochemical studies supported the hypothesis that the penetration of nematode cuticles by fungi involved the activities of hydrolytic enzymes (Lopez-Llorca and Robertson 1992; Lopez-Llorca et al. 2002). Various aspects of the biological control of nematodes using fungi have been reviewed by Jaffee (1992), Siddiqui and Mahmood (1996), and Kerry (2000). Nordbring-Hertz et al. (2000) reviewed the infection process and the evolution of nematophagous fungi. In this paper, we present up-to-date information of pathogenesis by nematophagous fungi with a focus on the analysis and comparison of serine proteases at the molecular level.

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Nematophagous fungi and their infection models against nematodes

Nematode-trapping fungi

Nematode-trapping fungi were traditionally classified into three genera based on the morphological characters of their conidia: *Arthrobotrys* Corda, *Dactylella* Grove, and *Monacrosporium* Oudem (Subramanian 1963). Recent studies with internal transcribed spacer (ITS) and 18S ribosomal DNA (rDNA) sequences indicated that trapping devices are more informative than other morphological structures in delimiting genera (e.g., Liou and Tzean 1997; Ahrén et al. 1998; Scholler et al. 1999; Li et al. 2005). Nematode-trapping fungi form different nematode-trapping devices that include adhesive hyphae, adhesive networks, adhesive knobs or branches, and non-adhesive rings (Fig. 1; Nordbring-Hertz et al. 2000; Zhang and Mo 2006). The ultrastructures of the nematode-trapping devices have been extensively studied (e.g., Heintz and Pramer 1972; Nordbring-Hertz and Stalhammar-Carlemalm 1978; Dijksterhuis et al. 1994). Although there is variation in morphology, different types of adhesive traps (branches, nets, and knobs) share some common features that clearly distinguish them from normal vegetative hyphae (Heintz and Pramer 1972; Dijksterhuis et al. 1994). One shared feature is the presence of numerous cytosolic organelles (dense bodies) within the trapping hyphal cells (Heintz and Pramer 1972; Nordbring-Hertz and Stalhammar-Carlemalm 1978). Another feature is the presence of extensive layers of extracellular polymers. These polymers have been considered important for the attachment of the traps to nematode surfaces (Tunlid et al. 1991).

During the past 20 years, Tunlid et al. have studied extensively the interaction between nematophagous fungi and their hosts (nematodes), using the soil-living fungus *Arthrobotrys oligospora* as their model species (e.g., Tunlid and Jansson 1991; Tunlid et al. 1994, 1999; Åhman et al. 1996, 2002). They identified that nematode-trapping fungi infect their hosts through a sequence of events. Recognition and adhesion were the first steps in the infection. However, little is known about the molecular mechanisms of recognition and adhesion. To date, only lectin has been reported to be involved in the recognition process. The interests of studying lectins in nematode-trapping fungi

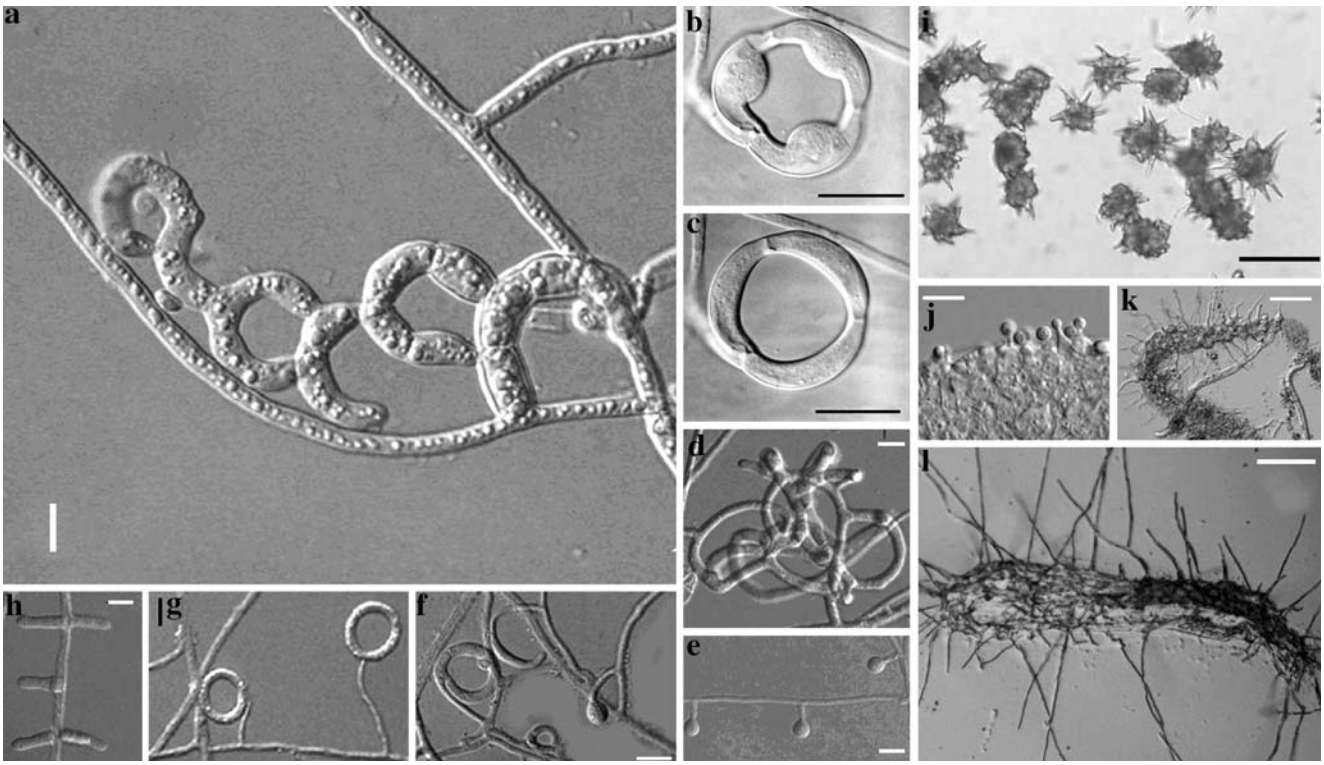

Fig. 1 Diversity of trapping structures and infection model of nematophagous fungi. **a** Adhesive network of *Dactylella dianchiensis* (bar = 10 μm). Reproduced from Zhang and Mo (2006). **b** and **c** Constricting ring of *Arthrobotrys brochopaga* (bar = 5 mm). Reproduced from Nordbring-Hertz et al. (2000). **d** Adhesive network of *A. oligospora* (bar = 10 μm). **e** Adhesive knobs of *Monacrosporium ellipososporum* (bar = 10 μm). **f** Adhesive knob and non-constricting rings of *M. candidum* (bar = 10 μm). **g** Non-constricting rings of *M. candidum* (bar = 10 μm). **h** Adhesive branches of *M. cionopagum* (bar = 10 μm). **d**–**h** Reproduced from Zhang and Mo (2006). **i** Acanthocytes of *Stropharia rugosoannulata* (bar = 20 μm). Reproduced from Luo et al. (2006). **j** *Panagrellus redivivus* infected by *Plesiospora polyspora* (bar = 10 μm). **k** *P. redivivus* infected by *Catenaria anguillulae* (bar = 10 μm). **j** and **k** Reproduced from Li (2005). **l** *P. redivivus* infected by *L. psalliotae* (bar = 100 μm). Reproduced from Yang et al. (2005a)

came from an observation that the interaction between *A. oligospora* and nematodes were mediated by a GalNAc-(*N*-acetyl-D-galactosamine) specific fungal lectin binding to receptors present on the nematode surface (Nordbring-Hertz and Mattiasson 1979). Similar experiments have indicated that lectins likely play a role in the adhesion to host surfaces by a number of parasitic and symbiotic fungi (Nordbring-Hertz and Chet 1986). Recently, a gene encoding such a lectin (*A. oligospora* lectin, AOL) was deleted in *A. oligospora* by homologous recombination (Balogh et al. 2003). However, the deletion mutant showed little decrease in spore (conidia) germination, saprophytic growth, and pathogenicity. This result suggested that the fungus might be capable of compensating the absence of the lectin by expressing other proteins with similar function(s) as AOL.

Nematode-trapping fungi capture nematodes by their particular hyphal structures (Fig. 1; Nordbring-Hertz et al. 2000; Zhang and Mo 2006). The nematode cuticle is a complex structure important for motility, for maintaining their morphological integrity, and for providing protection against the environment stresses and potential pathogens (Cox et al. 1981). The structure and physical properties of nematode cuticles vary with life stage, as reflected by the transient expression of certain collagen genes during different life stages (Abrantes and Curtis 2002). As with other pathogens, the nematode-trapping fungi enter into the host through both enzyme degradation and mechanical pressure. Several extracellular hydrolytic enzymes including serine proteases and collagenases have been detected and partly identified from different nematode-trapping fungi (e.g., Schenck et al. 1980; Tunlid et al. 1994; Tosi et al. 2001; Wang et al. 2006b). These studies suggested that extracellular hydrolytic enzymes are key virulence factors involved in the penetration process. After penetration, the hosts will be eventually degraded by the invading fungi. These fungi obtain nutrients from the nematodes for their growth and reproduction.

Parasitic fungi

Parasitic fungi infect nematodes mainly by ingestive spores (*Harposporium* spp.) (Shimazu and Glockling 1997) or adhesive spores (*Drechmeria coniospora*; Jansson et al. 1987). Some endoparasites, e.g., *Catenaria anguillulae* (Fig. 1), can produce zoospores that are attracted to nematodes before adhesion. The attachment is followed by encystment on the cuticle surface (Deacon and Saxena 1997).

Parasitic fungi cannot form trapping devices, and eggshells might be the main barriers to their infections against nematode eggs. Eggshells of root-knot and cyst nematodes are composed of three layers: the outer vitelline, the middle chitin, and the inner lipo-protein layers (Khan et al. 2004). The thickness of these layers varies considerably among nematode species (Blaxter and Robertson 1998). Before penetration, spores and penetration structures such as appressoria must first adhere to the host surface (Jansson and Lopez-Llorca 2001). Appressoria formed by the nematode egg parasite *Verticillium suchlasporium* (syn. *Pochonia rubescens*) have been studied using scanning electron microscopy (SEM; Lopez-Llorca and Claugher 1990). Mucilaginous material between the surface of the appressoria and the eggshell was observed. This material could function as an adhesive to assist in eggshell penetration by the fungus (Lopez-Llorca and Claugher 1990). A similar material was found in *Dactylella oviparasitica* appressoria infecting the *Meloidogyne* spp. eggs (Stirling and Mankau 1979). These infectious structures were formed likely as an adaptation to concentrate the mechanical forces and enzymatic degradation in a small area to facilitate host penetration (St Leger 1993; Lopez-Llorca et al. 2002).

Extracellular hydrolytic enzymes also play important roles in the infection process of these parasitic fungi. In 1990, Lopez-Llorca isolated the first pathogenic serine protease P32 from *V. suchlasporium*. Soon afterwards, Lopez-Llorca and Robertson (1992) confirmed the role of P32 in the pathogenicity of this fungus to nematode eggs by immunocytochemical localization studies. Recently, *Lecanicillium psalliotae* (syn. *V. psalliotae*), an opportunistic fungus, was reported to parasitize the free-living nematode *Panagrellus redivivus* (Fig. 1). *L. psalliotae* produces an alkaline serine protease that can immobilize the nematode *P. redivivus* and degrade the nematode cuticle within hours (Yang et al. 2005a).

Toxic fungi and other nematophagous fungi

Many microorganisms produce toxic metabolites, such as antibiotics, to prevent other microorganisms from competing for nutrients. Similarly, toxin-producing fungi can attack plant-parasitic nematodes by the production of nematicidal toxins (Dong et al. 2006; Stadler et al. 2006). The modes of action of these compounds against nematodes are diverse and complex. Recently, a novel nematicidal mode was reported during the study of the basidiomycetous fungi *Coprinus comatus* and *Stropharia rugosoannulata* (Luo et al. 2004, 2006). These two species produce a special nematode-attacking device: acanthocyte (Fig. 1). The microscopical observations showed that some acanthae resembled a sharp sword that could cause damage to the nematode cuticle, resulting in leakage of nematode inner materials. The results suggested that mechanical force is an important virulence factor in these fungi (Luo et al. 2006).

Extracellular enzymes involved in infection against nematodes

Serine proteases

Serine proteases are a family of enzymes that utilize a uniquely activated serine residue in the substrate-binding pocket to catalytically hydrolyze peptide bonds (Schultz and Liebman 1997; Siezen and Leunissen 1997). Serine proteases carry out a diverse array of physiological functions (Yousef et al. 2003) and have been reported as pathogenic factors found in bacterial or fungal pathogens against insects, nematodes, and even humans (e.g., Tunlid et al. 1994; Joshi et al. 1995; Tian et al. 2006). Serine proteases are major extracellular enzymes produced in large amounts by virulent isolates of *V. lecanii* (Jackson et al. 1985) and *Metarhizium anisopliae* (St Leger et al. 1992). The first pathogenicity-related serine protease P32 was identified from *P. rubescens* (Lopez-Llorca 1990). Subsequently, similar proteases were also found in other nematophagous fungi. In 1994, other two pathogenic proteases (PII and VCP1) were identified from *A. oligospora* (Tunlid et al. 1994) and *Pochonia chlamydosporia* (syn. *V. chlamydosporium*; Segers et al. 1994), respectively. Recently, more pathogenic serine proteases including pSP-3, Aoz1, Ver112, Mlx, PrC, and Ds1 were identified from nematophagous fungi *Paecilomyces lilacinus* (Bonants et al. 1995), *A. oligospora* (Zhao et al. 2004), *L. psalliotae* (Yang et al. 2005a), *Monacrosporium microscaphoides* (Wang et al. 2006a), *Clonostachys rosea* (syn. *Gliocladium rosea*; Li et al. 2006), and *Dactylella shizishanna* (Wang et al. 2006b), respectively. The partial biochemical properties of these serine proteases were summed in Table 1.

These pathogenic proteases from nematophagous fungi are highly sensitive to the inhibitor phenylmethyl sulfony fluoride (PMSF; Table 1), indicating that they belong to the subtilin-like serine protease family (Siezen and Leunissen 1997). They have similar molecular weights ranging from 32 to 39 kDa and share a broad range of protein substrates including casein, gelatin, nematode cuticle, eggshells, etc. Moreover, the biochemical properties of proteases PII (*A. oligospora*), Aoz1 (*A. oligospora*), Mlx (*M. microscaphoides*), and Ds1 (*D. shizishanna*; Tunlid et al. 1994; Zhao et al. 2004; Wang et al. 2006a,b) are similar and share lower pI, all of which were isolated from nematode-trapping fungi. Interestingly, the biochemical properties of proteases P32 (*P. rubescens*), VCP1 (*P. chlamysporia*), pSP-3 (*P. lilacinus*), Ver112 (*L. psalliotae*), and PrC (*C. rosea*; Lopez-Llorca 1990; Segers et al. 1994; Bonants et al. 1995; Yang et al. 2005a; Li et al. 2006) are also similar to each other except that they share a higher pI. These enzymes were isolated from parasitic fungi. Therefore, these pathogenicity-related proteases from nematophagous fungi can be divided into two categories according to the differences of their biochemical properties: Class I is composed of proteases from nematode-trapping fungi and class II consists of proteases from parasitic fungi (Yang et al. 2005b).

Table 1 Biochemical characterization of serine proteases isolated from nematophagous fungi

Nematophagous fungi	Group	Protease	MW (kDa)	Inhibitors	pI	Optimum pH	Substrates	References
A. oligospora	Trapping fungi	PII	35	PMSF, anti-pain pCMB, chymostatin	4.6	7–9	Casein, bovine serum albumin (BSA), gelatin, denatured collagen, and nematode cuticle	Tunlid et al. (1994)
A. oligospora	Trapping fungi	Aoz1	38	PMSF, SSI	4.9	6–8	Casein, gelatin, azocoll, and nematode cuticle	Zhao et al. (2004)
M. microscaphoides	Trapping fungi	Mlx	39	PMSF	6.8	9	Casein, BSA, gelatin, skimmed milk, collagen, and nematode cuticle	Wang et al. (2006a)
D. shizishanna	Trapping fungi	Ds1	32	PMSF	–[a]	10	Casein, skimmed milk, BSA, gelatin, collagen, and nematode cuticle	Wang et al. (2006b)
P. suchlasporia	Parasitic fungi	P32	32	PMSF, pCMB	–	8.5	Proteins from mature nematode eggs	Lopez-Llorca (1990)
P. chlamydosporia	Parasitic fungi	VCP1	33	PMSF	10.2	–	Casein, BSA, azocoll, eggshells of *Meloidogyne incognita*	Segers et al. (1994)
P. lilacinus	Parasitic fungi	pSP-3	33.5	PMSF	10.2	10.3	Colloidal chitin, vitellin and intact eggs of Meloidogyne hapla	Bonants et al. (1995)
L. psalliotae	Parasitic fungi	Ver112	32	PMSF	–	10	Casein, skimmed milk, BSA, gelatin, collagen, and nematode cuticle	Yang et al. (2005b)
C. rosea	Parasitic fungi	PrC	33	PMSF	10	9–10	Casein, skimmed milk, BSA, gelatin, collagen, and nematode cuticle	Li et al. (2006)

[a] No data in references

Previous results indicated that the higher pI value was important for the hydrolytic activity and for the binding of the enzyme to fragments of insect cuticle (St Leger et al. 1986). Moreover, these serine proteases also showed different nematicidal activity to different nematodes (Wang et al. 2006b), a result suggesting that substrate recognition sites of these proteases might differ among fungi with different host preferences. These observations suggest potentially effective methods for selecting nematophagous fungi for the biocontrol of different nematode diseases.

Chitinases

Chitin is an important structural polymer found in the cell walls of fungi and in the exoskeletons of invertebrates. It is an important component of the middle layer of nematode eggshells (Wharton 1980; Bird and Self 1995). Egg-parasitic fungi, such as *P. rubescens* and *P. chlamydosporia*, must first penetrate the nematode eggshell to cause infection (Lysek and Krajci 1987). There is extensive evidence for the production of chitinases by fungal parasites during infection based on ultrastructural studies (e.g., Lopez-Llorca and Robertson 1992; Tikhonov et al. 2002; Khan et al. 2004).

Recently, an extracellular chitinase CHI43 was identified from *P. chlamydosporia* and *P. rubescens*, and this enzyme was found to serve as a nematicidal factor in infecting nematode eggs (Tikhonov et al. 2002). Chitinase activity was also observed in the culture supernatant of the nematophagous fungus *P. lilacinus* strain 251 in a minimal medium containing chitin, and after separation by isoelectric focusing, six proteins were detected that showed chitinolytic activity (Khan et al. 2003). Chitinase activity was further confirmed on nondenaturing 1D and 2D gels using a sandwich assay with glycol chitin as a substrate. Studies on the extracellualr enzymes of *P. lilacinus* revealed that the application of the protease and chitinase drastically altered the eggshell structures when applied individually or in combination (Khan et al. 2004).

Collagenases and other hydrolytic enzymes

Collagenases are enzymes that can catalyze the hydrolysis of collagen and gelatin rather than other proteinaceous substrates (Maclennan et al. 1953). Collagen is the main constitutive component of the nematode cuticle (Blaxter and Robertson 1998). Therefore, collagenase from these fungi may play an important role in infection against nematodes. However, reports on collagenase production by nematophagous fungi are relatively rare (Schenck et al. 1980; Tosi et al. 2001). Recently, collagenases were identified in *Arthrobotrys* spp. (Tosi et al. 2001), and all of the *Arthrobotrys* species examined produced collagenase when they were grown in liquid medium free of proteose-peptone (proteosepeptone induces collagenase production). This result shows that collagenase is a constitutive enzyme in these fungi.

Other hydrolytic enzymes are also reported to be involved in the infection of nematodes by fungi. For example, eggs of *Heterodera schachtii* infected by fungi appeared to have their inner lipid layers degraded in comparison to uninfected eggs, and this was attributed to the lipolytic activity of the fungus (Perry and Trett 1986).

Phylogenetic analysis of serine proteases

So far, six pathogenicity-related serine proteases (PII, Azo1, Mlx, pSP-3, VCP1, and Ver112; Tables 1 and 2) have been cloned from different nematophagous fungi (Bonants et al. 1995; Åhman et al. 1996; Morton et al. 2003; Zhao et al. 2004; Yang et al. 2005b; Wang et al.

Table 2 Molecular characterization of serine proteases from nematophagous and entomophagous fungi

Gene	Species	GenBank accession no.	Intron	PP/MP	Cysteine	Oxyanion hole	Catalytic triad in mature peptide	References
PII	*A. oligospora*	X94121	1	408:286	$Cys_{81,\ 233}$	Asn_{165}	$Asp_{41}His_{77}Ser_{230}$	Åhman et al. (1996)
Aoz1	*A. oligospora*	AF516146	1	426:303	$Cys_{81,\ 151,\ 233}$	Asn_{165}	$Asp_{41}His_{77}Ser_{230}$	Zhao et al. (2004)
Mlx	*M. microscaphoides*	AY841167	1	405:285	$Cys_{81,\ 233}$	Asn_{165}	$Asp_{41}His_{77}Ser_{230}$	Wang et al. (2006b)
Prot K	*T. album*	X14689	1	384:279	$Cys_{34,\ 73,\ 123,\ 178,\ 249}$	Asn_{161}	$Asp_{39}His_{69}Ser_{224}$	Gunkel and Gassen (1989)
Alp	*F. oxysporium*	AB110909	2	397:289	$Cys_{36,\ 126,\ 181,\ 251}$	Asn_{164}	$Asp_{41}His_{72}Ser_{227}$	Morita et al. (1994)
pSP-3	*P. lilacinus*	L29262	2[a]	367:284	$Cys_{36,\ 76,\ 126,\ 181,\ 253}$	Asn_{164}	$Asp_{41}His_{72}Ser_{227}$	Bonants et al. (1995)
PrA	*M. anisopliae*	AJ251972	3	388:284	$Cys_{36,\ 126,\ 181,\ 253}$	Asn_{164}	$Asp_{41}His_{72}Ser_{227}$	St Leger et al. (1992)
Pr1	*B. bassiana*	AF154118	3	379:280	$Cys_{36,\ 75,\ 125,\ 180,\ 251}$	Asn_{163}	$Asp_{41}His_{69}Ser_{226}$	Joshi et al. (1995)
VCP1	*P. chlamydosporia*	AJ427454	3	387:281	$Cys_{36,\ 76,\ 126,\ 181,\ 253}$	Asn_{164}	$Asp_{41}His_{70}Ser_{227}$	Morton et al. (2003)
Ver112	*L. psalliotae*	AY692148	3	382:280	$Cys_{36,\ 75,\ 125,\ 180,\ 251}$	Asn_{163}	$Asp_{41}His_{69}Ser_{226}$	Yang et al. (2005b)
pepD	*A. niger*	L19059	3	416:295	Cys_{158}	Asn_{163}	$Asp_{41}His_{72}Ser_{228}$	Jarai et al. (1994)

PP Polypeptide, *MP* mature peptide
[a] Unpublished data from our laboratory

2006a). Sequence analyses and comparisons showed that serine proteases from nematophagous fungi shared extensive similarities to the subtilisin family of serine proteases from non-nematophagous fungi (Fig. 2). The comparison of these deduced peptide sequences to proteinase K (prot K) from *Tritirachium album* (Gunkel and Gassen 1989) revealed that they were typical fungal serine proteases possessing a pre-pro-peptide structure (Fig. 2; Siezen and Leunissen 1997). The deduced peptide sequence of PII (*A. oligospora*) showed 84.7, 41.3, 43.5, 39, 40, 41.7, and 38.8% identity, respectively, to Mlx (*M. microscaphoides*), pSP-3 (*P. lilacinus*), VCP1 (*P. chlamydosporia*), Ver112 (*L. psalliotae*), prot K (*T. album*), Pr1 (*Beauveria bassiana*; Joshi et al. 1995), and PrA (*M. anisopliae*; St Leger et al. 1992). Their signal peptide cleavage sites were very conserved; the signal peptides consisted of 15–21 amino acid (aa) residues, and the last aa was alanine (Ala) except in prot K (Ala replaced by glycine). The pro-peptides

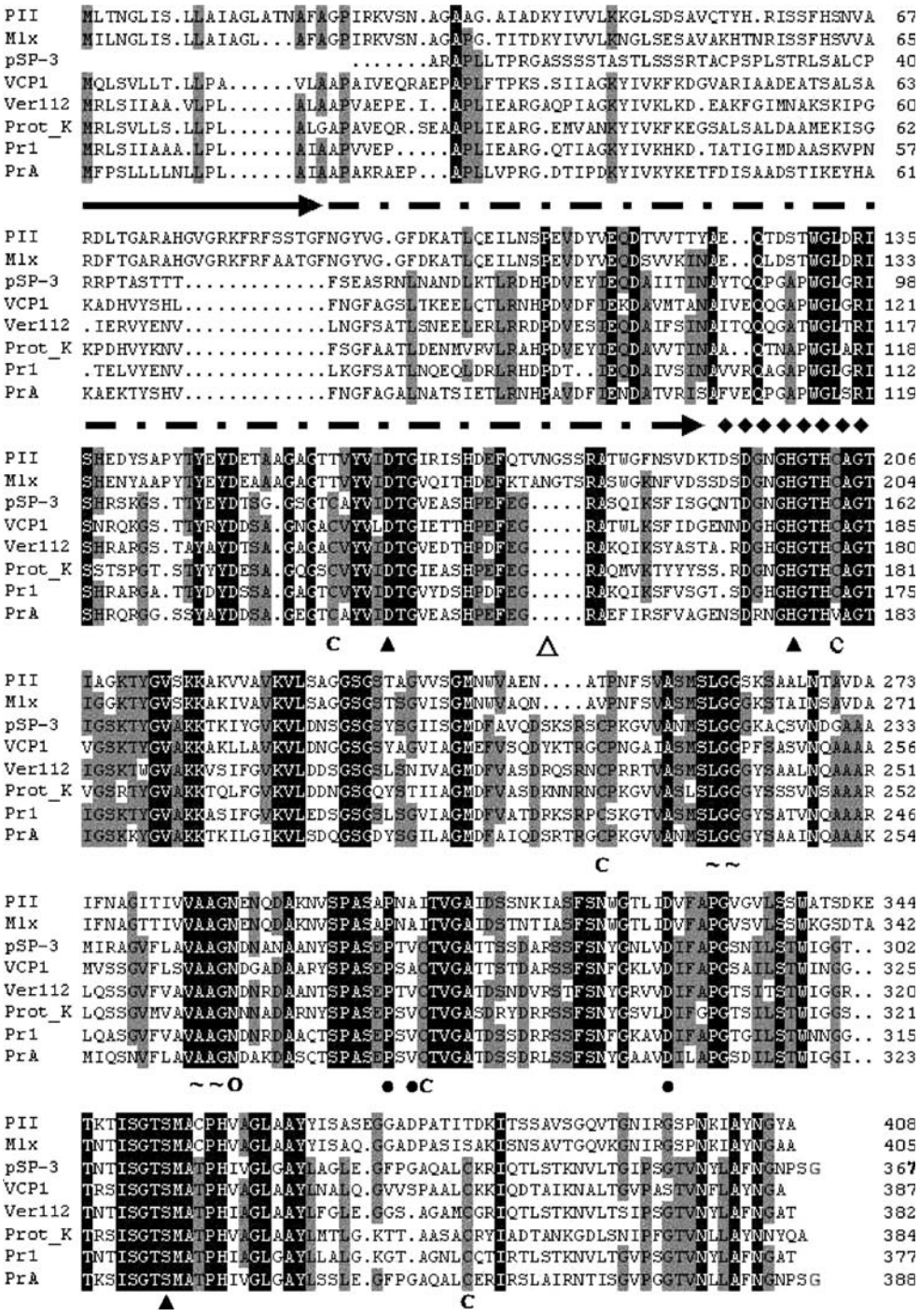

Fig. 2 Alignment of deduced peptide sequences from different fungi. The GenBank accession numbers of proteases PII, Mlx, pSP-3, VCP1, Ver112, Prot K, Pr1, and PrA are CAA63841, AAW21809, AAA91584, CAD20584, AAU01968, CAA32820, AAK70804, and CAB64346, respectively. Areas *shaded in black* are conserved regions (100% similarity), areas shaded in *gray* have high degrees of homology (more than 75% similarity), and *unshaded* areas are regions of variability between these proteases. Signal peptide sequences are marked on the *arrow*, and pro-peptides are marked on the *discontinuous arrow*. The *diamonds* indicate the N-terminal sequences of mature peptides. The *tildes* indicate the substrate-binding S1 pocket in subtilisin. The *filled triangles* indicate the aspartate (Asp_{39})-histidine (His_{69})-serine (Ser_{224}; in prot K) catalytic triad. The *open circles* indicate the oxyanion hole residue Asn_{161} (in prot K). *C* indicates the cysteine. The *open triangle* indicates the potential N-linked glycosylation sites in PII and Mlx. The *filled circles* indicate the conserved Ca^{2+}-binding sites

consist of 82–102 aa, which are removed before the enzyme is secreted into the extracellular environment. The active site of the prot K consists of the catalytic triad aspartate (Asp_{39})-histidine (His_{69})-serine (Ser_{224}), as well as the oxyanion hole residue asparagine (Asn_{161}) that are absolutely conserved among these enzymes (Fig. 2, Table 2). The functions of the catalytic triad and of the oxyanion hole in catalysis have been clearly established (Betzel et al. 1988, 2001). In this mechanism, Ser functions as the primary nucleophile and His plays dual roles both as proton acceptor and donor at different steps in the reaction. The role of Asp is thought to bring the His residue in the correct orientation to facilitate nucleophilic attack by Ser, and the role of the oxyanion hole is to stabilize the developing negative charge on the oxygen atom of substrate during the formation of the tetrahedral intermediate (Kraut 1977; Betzel et al. 2001). The two blocks of side-chains that form the sides of the substrate-binding S_1 pocket in subtilisin occur in regions of high conservation and consist of $Ser_{132}Leu_{133}Gly_{134}Gly_{135}$ and $Ala_{158}Ala_{159}Gly_{160}$, respectively, in prot K (Kraut 1977).

Proteinase K has five cysteines (Cys) aside from a free Cys_{73} close to the active His; other Cys residues formed two disulfide bonds, Cys_{34}–Cys_{123} and Cys_{178}–Cys_{249}, that contribute to the stability of the tertiary structure consisting of an extended central parallel β-sheet decorated by six α-helices, three short anti-parallel β-sheets, 18 β-turns, and involving several internal, structurally important water molecules (Betzel et al. 1988). Similarly, proteases pSP-3, VCP1, Ver112, and Pr1 also have five cysteines each. However, PrA has four cysteines only, lacking the Cys_{180} that was replaced by valine. The two serine proteases, PII and Mlx, from the nematode-trapping fungi *A. oligospora* and *M. microscaphoides*, respectively, have only two cysteines (Cys_{81} and Cys_{233}) each. Proteinase K exhibits two Ca^{2+} -binding sites, one very strong and the other weak, which were the sites for the heavy atoms (Pb^{2+}, Sm^{3+}) used to solve the crystal structure. The weak binding site is ligated to the N and C termini, threonine (Thr_{16}) and Asp_{260}, and is only incompletely coordinated by oxygen ligands. The strong binding site is coordinated in the form of a pentagonal bipyramid with the side chain carboxylate from Asp_{200} and the C=0 of proline (Pro_{175}) as apex, and C=0 of valine (Val_{177}) and four water molecules in the equatorial plane (Betzel et al. 1988). However, only the strong Ca^{2+}-binding site is conserved in these serine proteases from nematophagous and entomopathogenic fungi. PII and Mlx each has a potential N-linked glycosylation site Asn_{55}, which follows the general rule of Asn–Xaa–Ser/Thr, where Xaa is any residue except, perhaps, aspartate, glutamic acid, and proline (Mononen and Karjalainen 1984). Prot K also contains a potential N-linked glycosylation site Asn_{99}. However, other proteases pSP-3, VCP1, Ver112, Pr1, and PrA lack corresponding sites for glycosylation, which may explain that the molecular mass of proteases from nematode-trapping fungi are larger than those from parasitic fungi.

Phylogenetic analyses based on the deduced peptide sequences of serine proteases from different fungi (Table 2) were performed with the PHYLIP software package (Fig. 3; Felsenstein 1991). This tree suggested that the serine proteases from the nematophagous and entomopathogenic fungi had one common ancestor (Fig. 3). From the tree, these proteases appeared to be divided into two subclades. One subclade consisted of three serine proteases from the nematode-trapping fungi *A. oligospora* (PII and Aoz1) and *M. microscaphoides* (Mlx), and two species of the nematode-trapping fungi *A. oligospora* and *M. microscaphoides*. Another subclade consisted of seven serine proteases from different fungi. The second subclade contained two additional clusters. One cluster consisted of three sub-clusters. Among them, pSP-3 (*P. lilacinus*) and PrA (*M. anisopliae*) formed one sub-cluster, Ver112 (*L. psalliotae*) and pr1 (*B. bassiana*) formed a second sub-

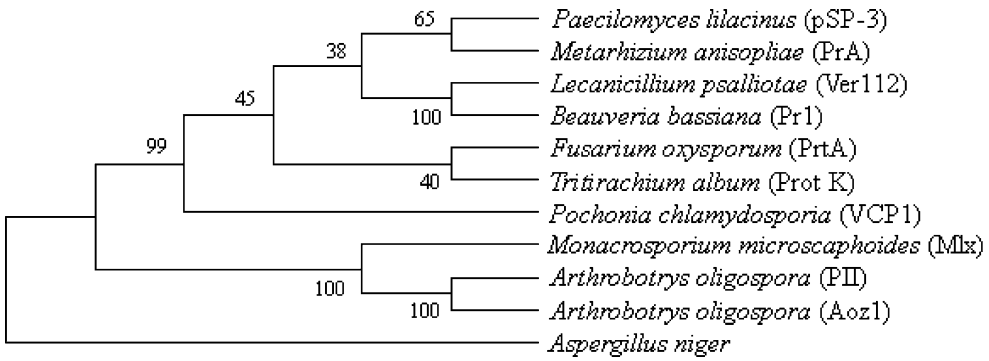

Fig. 3 Phylogenetic analyses based on the deduced peptide sequences of serine protease from different fungi. The GenBank accession number of protease from *Fusarium oxysporum* is BAD72940 and other proteases described in Fig. 2. *Aspergillus niger* (accession no. AAA32703) was used as the out-group. The phylogenetic tree was obtained by the neighbor-joining method using the PHYLIP software package. The numbers above the branches indicate the percentages with which a given branch was supported in 1,000 bootstrap replications

cluster, and two nonpathogenic proteases PrtA (*Fusarium oxysporium*; Morita et al. 1994) and Prot K (*T. album*) formed the third sub-cluster. Moreover, VCP1 (*P. chlamydosporia*) was distinct from others and formed another cluster (Fig. 3). From the above analyses, pathogenicity-related proteases (PII, Aoz1, and Mlx) from nematode-trapping fungi appeared to be distinct from those proteases from parasitic fungi. Moreover, proteases (Ver112, VCP1, pSP-3, Pr1, and PrA) from nematophagous and entomopathogenic fungi clustered together with the nonpathogenic proteases PrtA and Prot K. Therefore, these pathogenicity-related proteases evolved and developed to adapt to a different environment, which is important to the nematophagous and entomopathogenic fungi for parasitizing hosts.

New techniques used in nematophagous fungi

Recently, some new techniques including the green fluorescent protein (GFP) molecular marker, the over-expression systems of pathogenicity-related proteases, and the suppression subtractive hybridization (SSH) technique have been developed to study the interaction between the nematophagous fungi and their hosts (nematodes). The ultimate objective of which was to identify the developmental stage-specific genes for the targeted improvements of their biological control potential.

Since Prasher et al. (1992) cloned a complementary DNA (cDNA) for the *gfp* gene from the jellyfish *Aequorea victoria* in 1992, the *gfp* gene as a molecular marker has been used widely in biocontrol studies. Recently, Cantone and Vandenberg (1999) expressed *gfp* in the entomopathogenic fungus *Paecilomyces fumosoroseus* through co-transformation with a vector that confers resistance to glufosinate ammonium, and Lu et al. (2004) used constitutive and inducible GFP reporter systems to study the interactions between the *Trichoderma* pathogen and its host plant in vivo. Lately, Atkins et al. (2004) developed a transformation system for the nematophagous fungus *P. chlamydosporia* by co-transformation with vectors pDH33 and pTEFEGFP, or transformed with pCT74 alone. Therefore, GFP is a useful marker to study the interaction between pathogenic fungi and their hosts and will help to elucidate the infection mechanism of pathogenic fungi (Maor et al. 1998).

One way to improve the biocontrol potential of nematophagous fungi would be to increase the expression of these pathogenicity-related proteases (Åhman et al. 2002). The development of a transformation system made it possible to examine the function of virulence factors in more detail, e.g., by constructing over expressing strains and knock-out mutants (Tunlid et al. 1999). Åhman et al. (2002) constructed Δ*PII* mutant in *A. oligospora* by homologous recombination. However, the pathogenicity of the mutant was reduced only slightly. It was suggested that there might be a significant residual proteolytic activity in the Δ*PII* mutant. Subsequently, a neutral serine protease Aozl was identified from *A. oligospora* and provided support for the important roles of one or more proteases in the pathogenicity of *A. oligospora* toward nematodes (Zhao et al. 2004). Moreover, a mutant containing additional copies of the *PII* gene developed a higher number of infection structures (capturing devices) and had an increased speed of capturing and killing nematodes compared to the wild type. This was the first report demonstrating that genetic engineering could be used to improve the virulence of a nematophagous fungus (Åhman et al. 2002). There are more successful cases using mycoparasitic and entomopathogenic fungi than with nematophagous fungi. The biocontrol efficiencies of *T. harzianum* and *B. bassiana* were improved by increasing the copy number of the basic proteinase gene *prb1* (Flores et al. 1997) and the endochitinase encoding gene *Bbchitl* (Fang et al. 2005), respectively.

SSH is a widely used method for separating DNA sequences that distinguish two closely related genomic DNA (gDNA) libraries. Specific amplification of genes with SSH has allowed identification of minute genomic differences between closely related microbial strains and have revealed many strain-specific, functionally important genes (Harakava and Gabriel 2003; Dai et al. 2004). Furthermore, it has also enabled the profiling of genetic diversity in an environmental metagenome (Galbraith et al. 2004).

The nematode-trapping fungi enter the parasitic stage by developing specific morphological structures called traps. Recently, Ahrén et al. (2005) compared the gene expression patterns in traps and in the mycelium of the nematode-trapping fungus *Monacrosporium haptotylum* by microarray analysis. Despite the fact that the knobs and mycelium were grown in the same medium, there were substantial differences in the patterns of genes expressed in the two cell types. In total, 23.3% (657 of 2,822) of the putative genes were differentially expressed in knobs versus mycelium. A number of the genes that were differentially expressed in trap cells were also known to be regulated during the development of infection structures in plant-pathogenic fungi. Therefore, SSH is a useful tool to study stage-specific functional genes, for example, the genes involved in the formation of traps, appressoria, chlamydospore, mycelia development, etc. in nematophagous fungi.

Conclusions and perspectives

The nematophagous fungi comprise more than 200 species of taxonomically diverse fungi that all share the ability to infect and kill living nematodes (Barron 1977). The interest

in studying these fungi arises from their potential use as biological control agents (Larsen 2000). Recently, increasing attention has been paid to understanding the molecular aspects of the infection process and identifying the potential virulence factors. Among them, extracellular enzymes including serine proteases, collagenases, and chitinases have been found to play important roles in the pathogenesis of nematophagous fungi. The identification of pathogenicity-related enzymes from different nematophagous fungi will help the selection of substrates, host preference, and targeted genetic engineering of the enzymes at the molecular level.

However, the development of fungal biological control agents for practical use may be limited by several factors. An effective biological control agent must be able grow well in the field to control the plant-parasitic nematodes. It is known that chemical, physical, and other biological factors in the soil can influence the growth of fungi (Mo et al. 2005), and some of the fungi can be inhibited by soil fungistatic compounds (Xu et al. 2004). Moreover, these factors may be unpredictable in the soil due to crop rotations. Therefore, the effect of fungal biological control agents may be unpredictable. One way to resolve these issues is to improve the expression of virulence factors by genetic engineering. However, releasing modified microorganisms in the field is a hot-button issue and might not be a good option in some parts of the world. Another way to enhance the biological control effect is to improve the growth and adaptability of nematophagous fungi in field soils by using appropriate agents (e.g., chlamydospore; Mo et al. 2005). Moreover, integrated pest management (IPM) may be a good alternative for pest management in sustainable agriculture system (Akhtar 1997).

The crystal and molecular structure of proteinase K from *T. album* Limber was determined by X-ray diffraction (Betzel et al. 1988, 2001). However, no crystal data on serine proteases from nematophagous fungi have been reported. Such information will be important for explaining the interaction between proteases and proteinous components of nematode cuticle and for understanding host preferences of nematophagous fungi. Moreover, novel techniques such as genomics, crystallography, and molecular biological methods including SSH should help us obtain important information about the encoding genes of traps, the signaling pathways that control the switch from saprotrophy to parasitism, and the molecular mechanism of the infection process. Such information should provide novel approaches to improve the efficacy of nematophagous fungi for biological control applications.

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