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Leptosphaeria maculans, a fungal pathogen of *Brassica napus*, secretes a subtilisin-like serine protease

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Abstract

Leptosphaeria maculans, a fungal pathogen of *Brassica napus*, secretes large amounts of a 28 kDa protein (SP2) in liquid culture. This protein shows high sequence similarity to secreted serine proteases from other ascomycetes and is the major component of culture filtrate with protease activity, as analysed on casein zymogels. The *sp2* gene is expressed during infection of *B. napus* cotyledons when *L. maculans* hyphae are growing between mesophyll cells, as well as at later stages when the fungus invades the vascular tissue.

Introduction

Blackleg disease, caused by the dothideomycete *Leptosphaeria maculans*, is the most serious disease of canola (*Brassica napus*) worldwide (Howlett, 2004). Ascospores or pycnidiospores germinate on the leaf surface and the resultant hyphae enter the leaf tissue via stomata or wounds. The fungus initially colonises cotyledon or leaf tissue as a biotroph, growing intercellularly between the mesophyll cells, but behind the hyphal front it becomes necrotrophic. Hyphae grow down the petiole and invade and kill cells of the stem cortex resulting in a blackened canker at the crown (Hammond et al., 1985; Hammond and Lewis, 1987; Chen and Howlett, 1996).

Infection and colonization processes of phytopathogenic fungi often involve a number of secreted proteins; for example, cell wall-degrading enzymes (Ten Have et al., 2002) and the avirulence gene products of *Cladosporium fulvum*, *Rhynchosporium secalis*, *Pyrenophora tritici-repentis* and *Magnaporthe grisea* (Laugé and De Wit, 1998). Few *L. maculans* proteins or genes have been characterised. We previously described a gene, *sp1*, expressed during infection of canola that encoded a 12.3 kDa protein with a predicted secretion signal and four cysteine residues (Wilson et al., 2002). In this paper we report the characterisation of a *L. maculans* secreted protein (SP2) with serine protease activity.

Materials and methods

Preparation of SP2 from L. maculans *culture filtrate*

L. maculans isolate IBCN 18 (4×10^6 pycnidiospores) was inoculated in 200 ml of Czapek-Dox broth (17.5 g l^{-1}, Difco, USA) and yeast extract (0.005%) and grown for 10 days on an orbital shaker (80 rpm). Mycelia were harvested on Miracloth (Calbiochem, USA), and the culture filtrate was lyophilised and resuspended in water before being dialysed (CelluSepT1 MW cut-off 3.5 kDa; Membrane Filtration Products Inc., USA) against milli-Q water. Dialysed samples were subjected to successive steps of ethanol precipitation and centrifugation, and then analysed on precast 4–20% SDS polyacrylamide gels (Gradipore, USA) for the presence of protein bands. Gels were blotted onto PVDF-plus transfer membrane (0.45 μm;

Osmonics Inc., USA), stained in 0.25% Coomassie brilliant blue R250 and destained. N-terminal amino acid sequencing was carried out by automated Edman degradation (Matsudaira, 1990) on a Beckman gas-phase sequencer (model LF 3400; USA). The ethanol-precipitated protein fractions were also applied to a Sephadex G50 column (Amersham Pharmacia Biotech) pre-equilibrated in 20 mM HEPES 150 mM NaCl pH 7.4. Fast performance liquid chromatography (FPLC) was conducted at room temperature at a flow rate of 3 ml min^{-1}. Fractions containing the 28 kDa protein were concentrated using Amicon Centricon 3 (Millipore), electrophoresed on SDS PAGE gels and stained with 'Plus One' silver stain (Amersham Pharmacia Biotech, UK).

Gene isolation and characterisation

The gene encoding the SP2 protein was isolated by a strategy based on knowledge of its N-terminal sequence to which a degenerate primer (5′-CGI ATRCANTGIGTYCGNWG-3′) was designed and used in 5′ Rapid Amplification of cDNA Ends (RACE) with the GeneRacer kit (Invitrogen, USA). The cDNA template was reverse-transcribed from total RNA prepared from mycelia of isolate IBCN 18 grown in 10% Campbells V8 juice (Sexton and Howlett, 2000). The resultant product was purified using the Perfectprep Gel Cleanup kit (Eppendorf, USA), cloned into the pGEM-T Easy vector (Promega, USA) and sequenced. A gene specific primer 5′-GTCAAGATGAAGAACGAG AACC-3′ was designed from the 5′ cDNA sequence and 3′ RACE was conducted with the GeneRacer kit and the product cloned and sequenced as described above. Primers (5′-ATC AAAGCAGCCCTCAGTC-3′ and 5′-ACTCCACT CGATGGCTTTC-3′) were used to amplify a 1586 bp PCR fragment from genomic DNA of isolate IBCN 18 that was then sequenced. Genomic DNA and cDNA sequences were compared to identify intron positions, which confirmed those predicted by FGENESH gene prediction software (www.softberry.com). The presence of a secretion signal was sought using SignalP software (www.cbs.dtu.dk/services/SignalP/) and the cDNA sequence compared to the GenBank database using BLAST (Altschul et al., 1997).

The transcription of *sp2* was examined *in planta*. Ten days after seed germination, cotyledons of *B. napus* cv. Westar were inoculated with pycnidiospores of isolate IBCN 18 as previously described (Purwantara et al., 1998). Immediately, then 2, 5, 7, 9, 11 and 14 days later cotyledons were harvested. RNA was extracted from freeze-dried mycelia or cotyledons (Sexton and Howlett, 2000). Qualitative Reverse Transcriptase (RT) PCR with gene-specific primers (*sp2*; 5′-CCACATTCCTTGCCAACTAC-3′ and 5′-GATGGAATTTGTGCCACCG-3′, β-tubulin; 5′-CCGTATGATGGCCACCTTCTC-3′ and 5′-CTCCTGAATGGAGTCGAGTT-3′) was performed with the ThermoScript RT-PCR system (Invitrogen, USA). This experiment was carried out twice using independently prepared RNA samples.

Expression of recombinant SP2

To prepare large amounts of protein the *sp2* gene was expressed in *Escherichia coli*. Primers 5′-CATGAAGCTTTCAATCCTCC-3′ and 5′-GATTCAACTGCTGGGGTTGC-3′ were designed to amplify a 1349 bp protein-coding fragment from *sp2* cDNA. This fragment was cloned into pPROEX HT (Gibco-BRL), the resultant plasmid was transformed into *E. coli* BL21/03 and expression was induced. The resultant recombinant protein, which contained a histidine tag at its N-terminus was sized 30 kDa and was purified by affinity chromatography using Ni-NTA resin (Qiagen).

Zymogram protease assays

Zymogram analysis was conducted in 12% polyacrylamide gels containing casein as a co-polymerised substrate (BioRad, USA). Secreted protein samples and Proteinase K (Sigma, USA) were incubated for 20 min in 0.02 M Tris pH 8 with or without the protease inhibitors, 1 mM phenyl methyl sulfonyl fluoride (PMSF) or 1 μM pepstatin, at room temperature before incubation in SDS sample buffer at 40 °C for 1 h. Heat-inactivated samples were incubated in SDS sample buffer at 70 °C for 1 h. Gels were run at 4 °C at 20 mA, incubated in two 30 min washes of 2.5% Triton X-100 and 16 h in incubation buffer (0.05 M Tris–HCl, 200 mM NaCl, 6 mM $CaCl_2$, pH 7.5). All washes were at room temperature with gentle rocking. Gels were then stained in 0.25% Coomassie brilliant blue R250 and destained.

Results

A fraction enriched for the SP2 protein was obtained from gel filtration of the ethanol-precipitated culture filtrate fraction. Silver staining of SDS PAGE gels showed the protein composition of both ethanol-precipitated culture filtrate fractions and purified SP2 fractions. Proteinase K is loaded for comparison (Figure 1). An abundant 28 kDa protein (Secreted Protein 2, SP2) was isolated by electroblotting the protein and excising the band. Fifteen amino acids were sequenced (AYVTQASSTWGLARI) from the N-terminus of the protein and used to design degenerate primers for reverse transcriptase PCR. Complete cDNA sequence (1507 bp) for SP2 was obtained using 5′ and 3′ rapid amplification of cDNA Ends (RACE). Subsequently a 1586 bp PCR fragment from genomic DNA, which included *sp2,* was sequenced (GenBank AY422213).

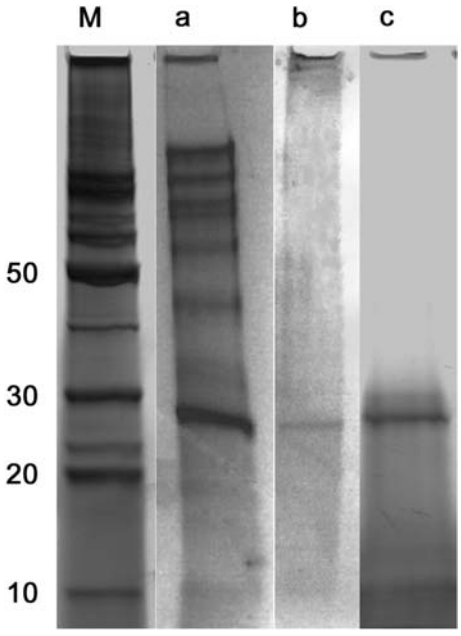

Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) of secreted proteins from *Leptosphaeria maculans*. Samples: (a) ethanol-precipitated protein fraction (1 mg), (b) SP2-enriched fraction (1 μg) and (c) proteinase K (100 μg) were electrophoresed on 4–20% polyacrylamide gels and protein was visualized by colloidal silver staining. Molecular weight markers (M, Benchmark, Invitrogen, USA) are shown in kDa.

Comparison of genomic DNA and cDNA sequences using FGENESH gene prediction software (*www.softberry.com*) identified three introns. Southern blot analysis confirmed that *sp2* is single copy and high stringency blots using DNA from other *L. maculans* isolates and two other dothideomycetes, *Alternaria alternata* and *Cochliobolus heterostrophus* also had a single copy (data not shown). An *sp2* homologue was identified in *M. grisea*, *Aspergillus nidulans*, *Fusarium graminearum*, *Ustilago maydis* and *Neurospora crassa* genomes (*www-genome.wi.mit.edu*).

The predicted protein sequence for *L. maculans* SP2 included a 15 amino acid (aa) secretion signal, 93 aa propeptide and a 285 aa mature protein (28.5 kDa) whose first 15 aa correspond to the N-terminal sequence obtained from the 28 kDa secreted protein (Figure 2). The *sp2* gene showed a high degree of sequence similarity to a group of subtilisin-like serine proteases secreted by pathogenic fungi including Pr1, the cuticle-degrading protease of *Metarhizium anisopliae* (St-Leger et al., 1992), Prt1, a subtilase from *Fusarium oxysporum* f. sp. *lycopersici* (Di Pietro et al., 2001), an alkaline serine protease from *Verticillium chlamydosporium* var. *chlamydosporium* (GenBank AJ427454) and a cuticle-degrading protease from *Beauveria bassiana* (GenBank AF104385). These proteins are very similar in amino acid sequence to the universal endoprotease proteinase K from the ascomycete *Tritirachium album* (GenBank X14689) and to the bacterial endoprotease subtilisin from *Bacillus licheniformis* (GenBank X03341). The three amino acids (asparagine, histidine and serine) characteristic of a serine protease active site, are conserved within all these proteases. With the exception of the *L. maculans* SP2 which contains two cysteine residues, the fungal proteases all contain four conserved cysteine residues and the bacterial subtilisin contains two. Comparison of SP2 and its fungal homologues shows a high degree of conservation between the secretion signals.

The transcript levels of *β*-tubulin and *sp2* during *L. maculans* infection of cotyledons of the susceptible *B. napus* cultivar Westar were examined by reverse-transcriptase PCR (Figure 3). *L. maculans β*-tubulin, which is believed to be constitutively transcribed and reflects fungal biomass in the plant, was detected 2 days after inoculation. A band for *sp2* was detected at all times after 2 days post-inoculation.

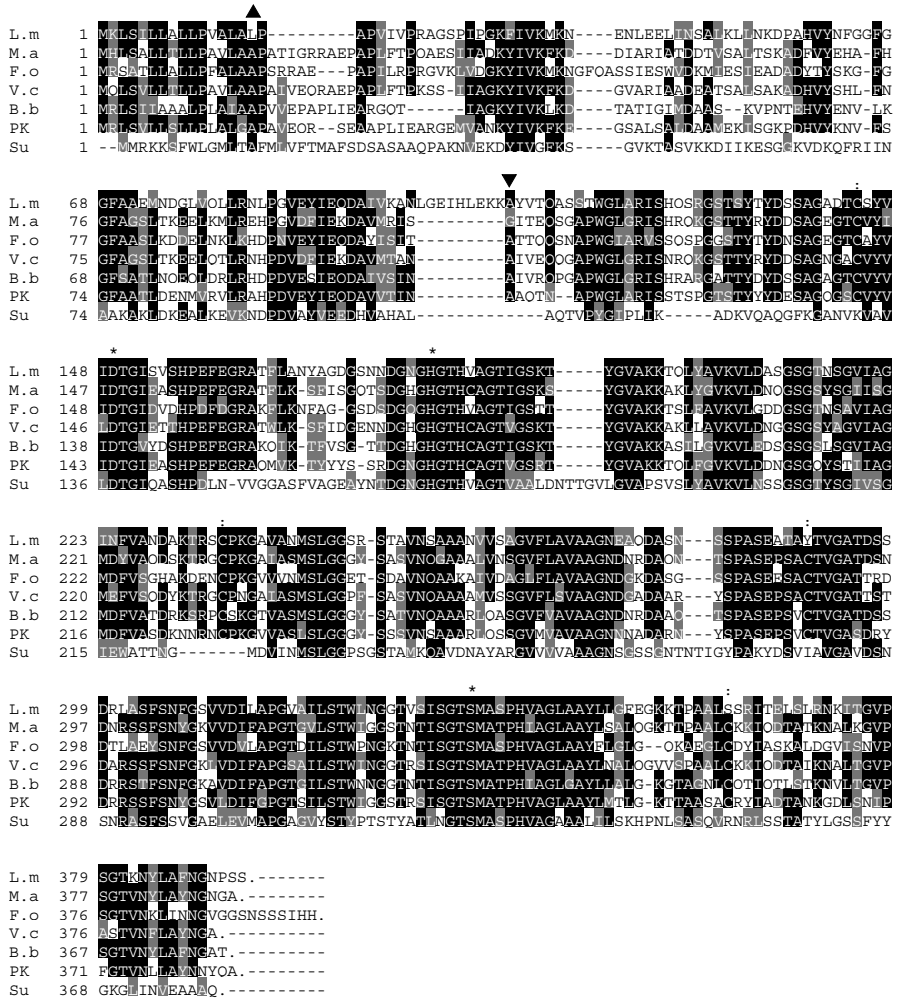

Figure 2. Alignment of the amino acid sequence of (L.m) *Leptosphaeria maculans* (GenBank AY422213) SP2 and its homologues (M.a *Metarhizium anisopliae* (GenBank AJ416688), F.o *Fusarium oxysporum* f. sp. *lycopersici* (GenBank AF074391), V.c *Verticillium chlamydosporium* var. *chlamydosporium* (GenBank AJ427454), B.b *Beauveria bassiana* (GenBank AF104385), PK proteinase K *Tritirachium album* (GenBank X14689), Su subtilisin bacterial endoprotease from *Bacillus licheniformis* (GenBank X03341). Signal peptide (▲) and propeptide cleavage (▼) sites, conserved aspartic acid, histidine and serine residues corresponding to the active site (*), conserved cysteine residues (:) and stop codons (.) are marked.

Zymogram analysis was conducted to detect protease activity in culture filtrate fractions. Casein zymography shows protease activity in the ethanol-precipitated protein fraction, the SP2 fraction and the proteinase K control (Figure 4). The SP2 fraction was enriched for protease activity in the purification process. The recombinant SP2 protein did not show protease activity suggesting that it is incorrectly folded or requires further modification for activity (data not shown). Proteinase activity in all samples was inhibited by PMSF and by incubation at 70 °C for 1 h, but not by pepstatin (data not shown). The main protease band in both the ethanol-precipitated and SP2-enriched fractions, presumed to be SP2, migrated a similar distance to the proteinase K band. Bands of proteolytic activity that migrated further may be of lower molecular weight and the product of autodigestion, or they may have different secondary structure. Variation in the profiles of the secondary enzymatic bands may be the result of differences in enzyme purity within the samples.

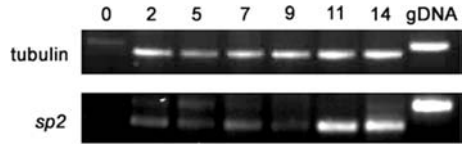

Figure 3. Transcription of *Leptosphaeria maculans* secreted protein 2 (*sp2*) and β-tubulin in infected cotyledons of *Brassica napus*, as shown by reverse transcriptase (RT) PCR. A 645 bp band amplified from the β-tubulin gene was used as a positive control for the detection of the fungus from 2 days post–inoculation. Primers specific to *sp2* amplify a 553 bp cDNA fragment from cotyledon tissue harvested at 0, 2, 5, 7, 9, 11 and 14 days post-inoculation. gDNA is genomic DNA. This experiment was repeated with independent RNA samples and similar results were found.

Discussion

Filamentous fungi secrete a range of proteolytic enzymes with a variety of functions. Fungal subtilases are typically extracellular and have a role in nutrition (Segers et al., 1999). Fungal proteases can degrade host proteins to provide nitrogen for plant pathogenic fungi (Sreedhar et al., 1999). In the interaction between the biotroph *C. fulvum* and its host tomato, nitrogen levels increase in the apoplast during invasion. One proposed source of this nitrogen is proteolysis and the proteolytic activity measured in the apoplast during this interaction is attributed to a serine protease (Solomon and Oliver, 2001). In *L. maculans* SP2 was the most abundant protein in ethanol-precipitated protein fractions, isolated from cultures grown with yeast extract as a supplemented nitrogen source.

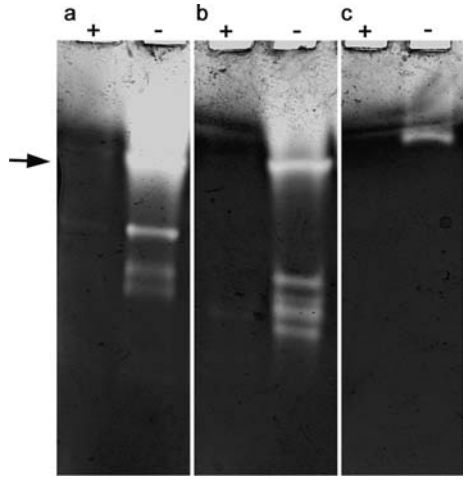

Figure 4. Casein zymography of *Leptosphaeria maculans* protein extracts in polyacrylamide gels (12%) stained with Coomassie blue; (a) ethanol-precipitated protein fraction (50 µg), (b) SP2-enriched fraction (100 ng), (c) proteinase K control (20 pg). Samples were incubated in the presence (+) and absence (−) of 1 mM phenyl methyl sulfonyl fluoride (PMSF). The band predicted to be SP2 is indicated (→). Bands of proteolytic activity that have migrated further may be of lower molecular weight and the product of autodigestion, or they may have different secondary structure. Variation in the profiles of the secondary enzymatic bands may be the result of differences in enzyme purity within the samples.

The *sp2* gene is also expressed during infection of *B. napus*. Transcripts were detected from 2 to 14 days post-inoculation. This suggests that the protease may play a role in the different infection stages of the fungus, including the early stages of infection, when the hyphae are growing intercellularly between the mesophyll cells and later when hyphae invade cells and the fungus undergoes a switch from biotrophic to necrotrophic growth (Hammond and Lewis, 1987; Chen and Howlett, 1996). A number of other genes encoding cell wall degrading enzymes, including a polygalacturonase and two cellobiohydrolases, have been identified in *L. maculans*. Transcription of one of the cellobiohydrolases, as well as cyanide hydratase, is also detected 14 days post inoculation of *B. napus* cotyledons (Sexton et al., 2000).

The role of fungal extracellular proteases in pathogenicity is controversial. Protease activity correlates with pathogenicity in some instances (Ball et al., 1991; St Leger, 1995; Sreedhar et al., 1999); however, in most cases no loss of pathogenicity is observed when such genes are mutated (Jaton-Ogay et al., 1994; Murphy and Walton, 1996; Ramesh and Kolattukudy, 1996; Di Pietro et al., 2001; Bindschedler et al., 2003). In cases where targeted gene disruption of a protease does not cause changes in pathogenicity, the fungus compensates for the loss of one enzyme activity by up-regulating the production of others (Ramesh and Kolattukudy, 1996; Bindschedler et al., 2003; Plummer et al., 2004). *Pyrenopeziza brassicae* also a pathogen of *B. napus*, produces an extracellular serine protease that is a pathogenicity determinant, but this protease is not a close homologue of SP2 (Batish et al., 2003). However, it is difficult to make comparisons between *P. brassicae* and *L. maculans* as their modes of infection are very different.

Successful invasion by plant pathogens depends on their ability to penetrate the host, to utilize nutrient sources provided by plants and to evade plant defence mechanisms. Since *L. maculans* invades leaves through stomatal apertures, rather than by degrading the host surface, it is unlikely that SP2 would be required for penetration. Whilst it is unlikely that SP2 is essential for pathogenicity, it may be involved in hydrolysing proteins in the apoplastic space, to provide a nutrient source for the fungus during the initial intercellular phase of infection, or it may be involved in the degradation of cell walls later in infection when the fungus begins to invade cells. Recently draft genome sequences of two filamentous fungal plant pathogens, *Magnaporthe grisea* and *Fusarium graminaerum,* have been released and sequencing of several more fungal genomes is underway. Bioinformatic analyses of these sequences are likely to find many open reading frames encoding secreted proteins. The combination of genome sequences and mutant banks will lead to an exponential amount of information about the roles of such secreted proteins in plant–pathogen interactions.

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