The role of the toxin sirodesmin in virulence of the blackleg fungus

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Abstract

Sirodesmin PL is a secondary metabolite toxin produced by *Leptosphaeria maculans*. Its biosynthesis involves a cluster of 18 co-regulated genes. A mutant that does not produce sirodesmin PL forms similar sized lesions on cotyledons of *B. napus* to those formed by the wild type. However, it is half as effective in colonising stems, as indicated by reduced lesion size and reduced fungal biomass. This implicates sirodesmin PL as a virulence factor in *B. napus* stems. Expression patterns of two of the cluster genes is consistent with the distribution of sirodesmin PL *in planta*, as revealed by mass spectrometry experiments. The sirodesmin-deficient mutant has less antibacterial and antifungal activity than the wild type, suggesting that sirodesmin PL may play a role in protecting *L.maculans* against other micro-organisms, particularly in the soil where Brassica residues host the fungus.

Introduction

Sirodesmin PL is a non-host specific toxin produced by *Leptosphaeria maculans*, which causes blackleg (phoma stem canker) of oilseed rape (*Brassica napus)*. As well as causing chlorotic (yellow) lesions on leaves, sirodesmin PL has antibacterial and antiviral properties (Rouxel et al. 1988). Sirodesmin PL is a member of the epipolythiodioxopiperazine (ETP) class of fungal secondary metabolites, which are characterised by a sulphur-bridged dioxopiperazine ring synthesised from two amino acids (Figure 1). The disulphide bridge confers toxicity by enabling ETPs to cross-link proteins via cysteine residues, and to generate reactive oxygen species through redox cycling.

At least 14 different ETPs are known, and all are produced by ascomycetes (for review see Gardiner et al. 2005). The best-characterised ETP, gliotoxin (Figure 1C), is produced by the opportunistic human pathogen *Aspergillus fumigatus,* as well as *A. terreus, A. flavus, A.niger, Penicillium terlikowskii* and *Trichoderma virens,* a fungus that controls root diseases of plants. Other ETPs with roles in animal diseases include sporidesmins, produced by *Pithomyces chartarum* which infects grasses and causes facial eczema and liver diseases of grazing animals. Chaetomin and chaetocin are produced by *Chaetomium globosum,* a systemic pathogen of immunocompromised humans. The cytotoxicity of some ETPs has made them attractive as potential anticancer agents and accordingly interest in these molecules is increasing.

Sirodesmin PL is derived from serine and tyrosine. A prenyl transferase is predicted to catalyse the addition of a dimethylallyl group to either the dipeptide cyclo-L-tyrosyl-L-serine or free tyrosine, before condensation with serine, to produce the intermediate cyclic dipeptide, phomamide. The genes responsible for the biosynthesis of sirodesmin PL in *L. maculans* were identified six years ago by an approach that took advantage of the clustering of genes encoding biosynthetic enzymes for secondary metabolites in fungal genomes (Gardiner et al. 2004). A homologue of prenyl transferase from the endophyte *Neotyphodium coenphialum* was identified from an *L. maculans* Expressed Sequence Tag (EST) library. This EST was used to probe an *L. maculans* cosmid DNA library and a cosmid (35 kb) containing the prenyl transferase was sequenced. Regions flanking this prenyl transferase gene (named *sirD*) contained a cluster of 18 genes which, based on best matches to genes in databases, could be assigned roles in sirodesmin PL biosynthesis. These included a two module non-ribosomal peptide synthetase (*sirP),* acetyl transferase (*sirH),* methyl transferases (*sirM* and *sirN),* an ATP-binding cassette (ABC) type transporter (*sirA),* responsible for toxin efflux, and a member of the zinc binuclear cluster ($Zn(II)_2Cys_6$) family (*sirZ)* (Figure 2A). Of the nine cluster genes tested, all were co-regulated and timing of expression was consistent with the production of sirodesmin PL in culture, suggesting the gene cluster is involved in sirodesmin PL biosynthesis. Furthermore, the disruption of the peptide synthetase, *sirP,* resulted in a mutant isolate unable to produce sirodesmin PL, confirming that this gene is essential for

the synthesis of this toxin, and that the biosynthetic gene cluster was correctly identified (Fox and Howlett 2008). *Aspergillus fumigatus* has a similar cluster of genes for gliotoxin biosynthesis (Figure 2B).

The contribution of sirodesmin PL to virulence of *L. maculans* on oilseed rape (*Brassica napus*) has been determined unequivocally, by exploitation of a defined sirodesmin-deficient *L. maculans* mutant, that had a DNA insertion in the non-ribosomal peptide synthetase gene (*sirP*). When the *sirP* mutant was inoculated onto cotyledons of *B. napus*, it caused similar-sized lesions on cotyledons as the wild type isolate, indicating that sirodesmin PL was not a virulence factor at this stage of infection. Subsequently the mutant caused fewer stem lesions and was half as effective as wild type in colonising stems, as shown by quantitative PCR analyses (Elliott et al. 2007). Thus sirodesmin PL contributes to virulence in *B. napus* stems. The expression of two cluster genes, the peptide synthetase, *sirP* and an ABC transporter, *sirA*, was also studied during infection. Fungal isolates containing fusions of the green fluorescent protein gene (GFP) with the promoters of these genes fluoresced after ten days post-inoculation (dpi). This expression pattern was consistent with the distribution of sirodesmin PL in both cotyledons and stems, as revealed by mass spectrometry experiments (Elliott et al. 2007).

As to why sirodesmin PL contributes to colonisation of stems but not necrotic symptoms of lesions in the cotyledons is unknown. Sirodesmin PL may suppress plant defences during biotrophic growth down the stem, or it might play a role in competition between *L. maculans* and other microorganisms *in planta*, or even on stubble during the saprophytic stage of its life cycle. Germination of several fungal species including *Fusarium graminaerum* and *L. biglobosa* 'brassicae' was inhibited in the presence of a 13 day old colony of the wild type sirodesmin PL-producing isolate when grown *in vitro,* illustrating the antifungal activity of this molecule (Elliott et al. 2007).

The role that secondary metabolites play in the biology of fungi is elusive. Fungi that produce toxins often do not rely on growth on a host to complete their life cycle. Organisms that produce such molecules may have enhanced survival. Many such organisms live saprophytically in the soil exposed to a harsh environment with many

competing organisms. Fungal virulence has been proposed to have evolved to protect fungi in such an environment against amoebae, nematodes or other invertebrates that can feed on fungi (Mylonakis et al. 2007). Secondary metabolite toxins could play a role in such behaviour. The recent availability of defined mutants in the biosynthesis of secondary metabolites will enable this hypothesis to be tested for some molecules and their producing-organisms.

Acknowledgements

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References

Elliott CE, Gardiner DM, Thomas G, Cozijnsen AJ, Van De Wouw A, Howlett BJ, 2007. Production of the toxin sirodesmin PL by *Leptosphaeria maculans* during infection of *Brassica napus.* *Molecular Plant Pathology* 8, 791-802

Fox EM, Howlett BJ, 2008. Biosynthetic gene clusters for epipolythiodioxopiperazines in filamentous fungi. *Mycological Research* 112, 162-169

Gardiner DM, Cozijnsen AJ, Wilson L, Pedras MSC, Howlett BJ, 2004. The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. *Molecular Microbiology* 53, 1307-1318.

Gardiner DM, Howlett BJ, 2005. Bioinformatic and expression analysis of the putative gliotoxin gene cluster of *Aspergillus fumigatus*. *FEMS Microbiology Letters* 248, 241-248.

Gardiner DM, Waring P, Howlett BJ, 2005. The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions and biosynthesis. *Microbiology* 151, 1021-1032.

Mylonakis E, Casadevall A, Ausubel FM, 2007. Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. *PLoS Pathogens* 3, e101.

Patron NJ, Waller RF, Cozijnsen AJ, Straney DC, Gardiner DM, Nierman WC, Howlett BJ, 2007. Origin and distribution of epipolythiodioxopiperazine (ETP) gene clusters in filamentous ascomycetes. *BMC Evolutionary Biology* 7, 174.

Rouxel T, Chupeau Y, Fritz R, Kollmann A, Bousquet J-F, 1988. Biological effects of sirodesmin PL, a phytotoxin produced by *Leptosphaeria maculans*. *Plant Science* 57, 45-53.

Legends

Figure 1: Structures of epithiodioxopiperazines

A. Core epithiodioxopiperazine (ETP) moiety; **B**. Sirodesmin PL **C.** Gliotoxin

Figure 2: The *Leptosphaeria maculans* sirodesmin PL (A) and *Aspergillus fumigatus* gliotoxin (B) biosynthetic gene clusters

Common ETP moiety genes (white text on black background) include those with best matches to non-ribosomal peptide synthetase (*P*), thioredoxin reductase (*T*), methyl transferases (*M* and *N*), glutathione *S*-transferase (*G*) and cytochrome P450 mono-oxygenase (*C*), amino cyclopropane carboxylate synthase (ACCS) (*I*), dipeptidase (*J*), as well as a transcriptional regulator (*Z*) and a transporter (*A*). Other genes (black text on white background) do not have obvious homologues in the other cluster and are thought to be involved in modification of the side chains of the core ETP moiety. These encode cytochrome P450 mono-oxygenases *(F, B* and *E)*, a prenyl transferase *(D)*, an acetyl transferase *(H)*, epimerases *(Q, S* and *R)*, an oxidoreductase *(O)* and a hypothetical protein *(K)* (Gardiner and Howlett 2005). Genes shaded in grey encode proteins with best matches to proteins with no potential roles in ETP biosynthesis. The forward slash marks represent a 17 kb region of repetitive DNA.

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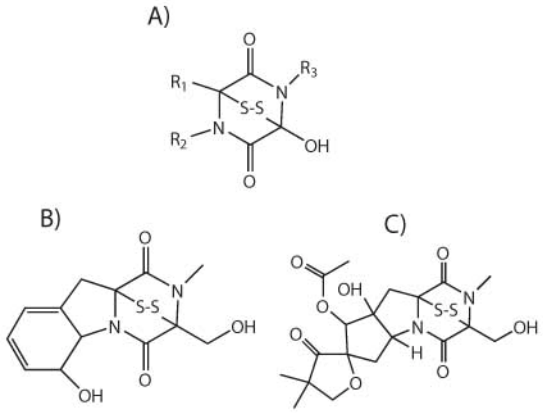

Figure. 1

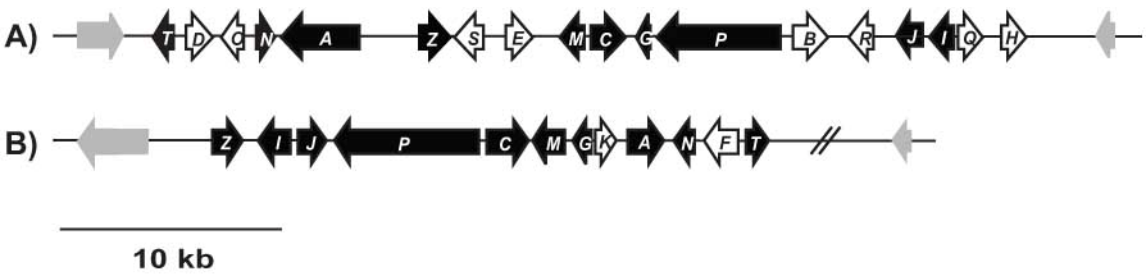

Figure 2