Disruption of the *Coniothyrium minitans* PIF1 DNA helicase gene impairs growth and capacity for sclerotial mycoparasitism

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A non-mycoparasitic restriction enzyme-mediated DNA integration (REMI) mutant of *Coniothyrium minitans* (R2427) contains two tandem plasmid copies integrated towards the 3' end of an ORF. The predicted polypeptide (845 aa) exhibits high similarity with DNA-helicase proteins from other filamentous fungi and yeasts that play a role in mitochondrial DNA maintenance and repair. Disruption of the *C. minitans* PIF1 DNA helicase gene results in altered morphology, reduced growth rates and a concomitant loss in ability to mycoparasitize sclerotia of *Sclerotinia sclerotiorum*. In infection bioassays, R2427 exhibited sparse mycelial growth on the surface of live sclerotia, but no mycelia were detected inside the sclerotia. Conversely, R2427 readily colonized autoclaved sclerotia. Complementation of the mutant with wild-type PIF1 restored normal mycelial growth and mycoparasitic capability, confirming a functional role in the host–pathogen interaction. The *C. minitans* PIF1 DNA helicase may maintain mitochondrial stability in response to reactive oxygen species, either produced endogenously within the mycoparasite, or exogenously from the sclerotial host.

INTRODUCTION

*Coniothyrium minitans* is a sclerotial mycoparasite of the plant-pathogenic fungus *Sclerotinia sclerotiorum*, which can infect over 400 plant species and causes severe economic loss in a wide variety of crops (Boland & Hall, 1994; Budge *et al.*, 1995; Escande *et al.*, 2002; Huang *et al.*, 2000; Willetts & Wong, 1980). *C. minitans* is an effective biocontrol agent against *Sclerotinia*, in both field and glasshouse (Budge & Whipps, 2001; Li *et al.*, 2003), that can achieve levels of disease control comparable to fungicide treatments (Budge & Whipps, 2001; Gerlagh *et al.*, 1996). However, when disease levels are high, *C. minitans* fails to provide adequate control (Budge *et al.*, 1995; Jones *et al.*, 1999). To improve the efficacy of *C. minitans* as a biocontrol agent, better understanding of the molecular mechanisms involved in the fungal–fungal interaction and sclerotial mycoparasitism is required.

Infection of sclerotia by *C. minitans* does not involve specialized infection structures such as appressoria (Huang & Hoes, 1976), but begins when the highly melanized sclerotial rind is penetrated by hyphal tips intracellularly or through intercellular gaps (Whipps & Gerlagh, 1992). *C. minitans* hyphae penetrate host cell walls through a combination of mechanical pressure and degradation by extracellular enzymes, such as β-glucanases and chitinases (Huang & Kokko, 1987; Jones & Watson, 1969; Jones *et al.*, 1974). Hyphal growth through infected tissues is followed by lysis and almost complete disintegration of the host cell walls accompanied by production of numerous pycnidia of *C. minitans* both internally and on the surface of the sclerotia.

Molecular characterization of *C. minitans* mycoparasitism is limited. The *cmg1* exo-β-1,3-glucanase gene was shown to be upregulated when the fungus was grown on media containing sclerotia as the sole carbon source (Giczey *et al.*, 2001). Recently we have identified 251 unique transcripts of *C. minitans* upregulated during growth on sclerotia, and representing genes involved in diverse processes including signalling, detoxification and stress responses (Muthumeenakshi *et al.*, 2007). The development of efficient and reliable transformation and insertional mutagenesis systems for *C. minitans*, both protoplast-mediated and *Agrobacterium*-mediated (ATMT), are enabling functional characterization of key genes (Jones *et al.*, 1999; Rogers *et al.*, 2004). Similar approaches have been used to identify pathogenicity genes in other plant-pathogenic filamentous fungi, including *Magnaporthe*.
grisea and Fusarium graminearum (Balhadère et al., 1999; Kahmann & Basse, 1999; Seong et al., 2005).

We previously used both REMI (restriction enzyme-mediated DNA integration) and ATMT insertional mutagenesis approaches to generate a library of over 4000 C. minitans transfectants, from which nine 'pathogenicity mutants', unable to colonize sclerotia, were identified (Rogers et al., 2004). By analysing REMI mutant R2427, we have identified a disrupted gene that shows high homology to the PIFI DNA helicase gene of Saccharomyces cerevisiae (Fourny & Lahaye, 1987). Functional complementation of R2427 has been achieved using the wild-type C. minitans gene. In other fungi, PIFI is involved in mitochondrial DNA (mtDNA) repair and recombination and has a role in the maintenance of mitochondria in response to oxidative stress (Doudican et al., 2005; Fourny & Lahaye, 1987). This paper describes molecular characterization of the C. minitans PIFI gene and demonstrates its concomitant role in mycelial growth and sclerotal mycoparasitism.

METHODS

Strains, plasmids and culture maintenance. The wild-type C. minitans strain Conio (IMI 134523), C. minitans REMI mutant R2427 (Rogers et al., 2004) and the host Scl. sclerotiorum isolate SB (IMI 390053) were maintained on potato dextrose agar (PDA, Oxoid) at 20°C unless otherwise stated, and where appropriate with the addition of hygromycin (Rogers et al., 2004). Sclerotia of Scl. sclerotiorum were produced from PDA cultures. The plasmid FHJS3 (a gift from F. H. J. Schuren, University of Groningen, The Netherlands) contains the ble gene, encoding a phleomycin-binding protein, from Streptalloteichus hindustanus (Schuren & Wessels, 1994) coupled with the Agaricus bisporus gpd III promoter (Harmsen et al., 1992) and Schizophyllum commune gpd terminator (Schuren & Wessels, 1998).

Molecular analysis of transformants. C. minitans genomic DNA was extracted from powdered freeze-dried mycelia, grown statically in potato dextrose broth (PDB, Oxoid) for 10–14 days, using the DNeasy Plant Mini kit (Qiagen). Southern blot analysis was performed according to standard protocols (Sambrook et al., 1989). Genomic DNA for PCR screening was isolated from C. minitans mycelia scraped from the surface of 4-day-old PDA cultures using a Chelex mini-prep method (Challen et al., 2003).

Total RNA was extracted from powdered freeze-dried mycelia grown for 10 days in PDB using the RNagents Total RNA Isolation System (Promega). Samples were DNase treated (Promega) and reverse-transcriptase PCR (RT-PCR) analysis was performed using the Eurogentec RT-PCR core kit with the following primers: X4prb (CGA ATG AAC CAG CTC CAA GG), X4prbc (GCG CTT GTA GTT CTA TC), PIFendC (CCC TTC TCC GTT TTG CTC TC), β-tubulin-1 (ACT TTC TCC GTC CCT TC), β-tubulin-3 (GCT GGT GAG AGC GAA TC).

Recovery of disrupted sequences from R2427. The REMI mutagenesis site was identified using plasmid rescue. Genomic DNA (approx. 3 μg) was restricted with Xbal, which cuts once within the transforming PANT-1 sequence (Punt et al., 1987), purified using a QiAquick PCR purification column (Qiagen), self-ligated and transformed into Escherichia coli DH5α competent cells (Invitrogen). Selection of ampicillin-resistant colonies allowed recovery of the recombinants containing the REMI-disrupted genomic DNA. A macro-arrayed cosmids-based genomic library of C. minitans (Muthumeenakshi et al., 2007) was probing using a radioactively labelled 396 bp portion of the recovered Xbal fragment. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit and a 373 sequencer (Applied Biosystems) at the Warwick HRI Genomics Resource Centre.

Sequence analysis. Homologous sequences were identified using online tools Blast (NCBI) or Fasta (EBI). The SE Central software package (Clone Manager Professional Suite v.8, Scientific & Educational Software) and GENSCAN (http://genes.mit.edu/) were used to identify restriction sites, ORFs and introns, and to translate putative genes. Predicted protein sequences were analysed for specific motifs using Promsite (ExPaSy) and the CBS Prediction Server (http://www.cbs.dtu.dk). The CLUSTAL W algorithm in the Megalign module (Lasergene v.7, DNASTAR) was used to create multiple protein sequence alignments, and presented using GeneDoc (v.2.6.002) (Nicholas et al., 1997). Dendrograms were created based on protein sequences using Megalign and the ProtDist algorithm from PHYLIP (version 3.5c) (Felsenstein, 1993), with bootstrap sampling (n=1000).

Sequences for C. minitans PIFI are deposited in the EMBL nucleotide sequence database under accession numbers AM778530 and AM778832.

Complementation of R2427. The 4597 bp genomic DNA region encompassing the C. minitans PIFI gene was amplified from a cosmid template, using the Expand High Fidelity system (Roche) with primers PIFamplC (GCC GGT GTA GAT GTT GGA GA), cloned using pGEM T Easy (Promega) and sequenced to check for amplification errors. The resulting pGEM_PIFI plasmid and pFHJS3 were co-transformed into mutant R2427 using protoplast-mediated transformation (Rogers et al., 2004). Phleomycin-resistant colonies were selected and tested for their ability to parasitize Scl. sclerotiorum sclerotia as described previously (Rogers et al., 2004). Transformants were screened by PCR and Southern hybridization to confirm the presence of wild-type PIFI.

Scanning electron microscopy (SEM). Sclerotia were inoculated with wild-type C. minitans or R2427 using a conidial suspension and placed on water agar plates at 20°C for 0–30 days. Sclerotia were attached to the sample holder using Leit-C (Neubauer) investigative carbon cement before transfer to the cold stage of the cryo-preparation chamber (−160 to −180°C). Samples were sputter-coated with gold for 5 min (1.5 kV and 1.5–2 mA) and transferred to the SEM cold stage. Examination of the samples was performed at approx. −180°C using a 10 kV accelerating voltage in the presence of an anti-contaminator.

RESULTS

Phenotype of REMI mutant R2427

Previous work had shown that the growth rate of R2427 on PDA (1.13±0.16 mm per day) was substantially reduced compared to wild-type (3.77±0.2 mm per day) (P<0.001) and that R2427 does not infect live sclerotia of Scl. sclerotiorum (Rogers et al., 2004). The mycelial colony morphology of R2427 was also markedly altered (Fig. 1), although R2427 was still able to sporulate normally. SEM revealed that spores of R2427 could germinate and form sparse mycelial growth on the surface of live sclerotia (Fig. 1). However, no growth of within the sclerotia was detected during three replicated mycoparasitism tests.
of 20 sclerotia. R2427 readily colonized autoclaved sclerotia (Fig. 1), although pycnidial development occurred later than with wild-type *C. minitans*.

**R2427 contains a disrupted PIF1 gene**

Southern blot analysis of R2427 genomic DNA revealed tandem copies of pAN7-1 as a single integration at a *Hind*III recognition site, shown by a single hybridizing band in *Hind*III-digested R2427 DNA at approx. 6.8 kb, the same size as the linear plasmid (Fig. 2).

Sequences upstream of the integration site were recovered by plasmid rescue using *Xba*I. A 2540 bp fragment of *C. minitans* DNA was sequenced and an internal 396 bp PCR product was used to detect the wild-type sequences. Comprehensive sequencing of the downstream flank of the integration site in R2427 was not performed, but PCR analysis showed that significant deletions had not occurred (data not shown) and that other ORFs were not disrupted by the REMI event.

Sequencing of a cosmid clone from the genomic library revealed a putative ORF of 2586 bp with a single 48 bp intron between positions 1148 and 1195 inclusive. Intron location was confirmed by sequencing two separate clones of the cDNA transcript (data not shown). The predicted 845 amino acid polypeptide (Fig. 3) had high similarity to the *PIF1* genes of *Aspergillus fumigatus* (55% identity),

![Fig. 1](image-url). Growth of *C. minitans* wild-type (WT) and the mutant R2427 on PDA and sclerotia of *Scl. sclerotiorum*. (A) 21 days’ growth on PDA, single inoculation. R2427 has a reduced growth rate. (B) 18 days’ growth on live sterile sclerotia, wild-type pycnidial initials visible, but no apparent growth of R2427. (C) 8 days’ growth on autoclaved sclerotia. (D) SEM images of the sclerotial surface after 9 days’ growth; sparse mycelial growth of R2427 compared to wild-type (bars, 50 μm).

![Fig. 2](image-url). Molecular analysis of the *C. minitans PIF1* gene and the R2427 mutant. (A) Southern blot of DNA from wild-type and R2427. *Hind*III- or *Xba*I-restricted DNA was probed with a 2401 bp DIG-labelled pAN7-1 *Bgl*II-*Eco*RI fragment. Lanes: M, DIG-labelled DNA marker (Roche); 1, *Hind*III-restricted wild-type DNA; 2, *Xba*I-restricted R2427 DNA; 3, *Hind*III-restricted R2427 DNA. The smaller hybridizing band in lanes 2 and 3 corresponds to DNA fragment containing *C. minitans* disrupted sequence. (B) Schematic of *C. minitans PIF1* gene region (not to scale); 2540 bp fragment cloned via plasmid rescue.
Neurospora crassa (48% identity) and Schizosaccharomyces pombe (47% identity). The C. minitans PIF1 ORF has two putative start codon methionine residues (Fig. 3; residues 1 and 53). The C. minitans PIF1 protein contains features consistent with DNA helicases: an ATPase domain (GSAGTGKS) at residues 380–387 and a putative DNA-binding domain (KGQAYVAL) at residues 780–787. Both (GSAGTGKS) at residues 380–387 and a putative DNA-replication/recombination and repair.

Analysis of the C. minitans PIF1 sequence showed a signal peptide predicted to target the protein to the mitochondria, with a cleavage site between residues 19 and 20. A nuclear exportation signal (NES) was predicted at position residue 759. Consistent with localization to the mitochondria, a prokaryotic membrane lipoprotein lipid attachment site was identified at residues 409–419. The REMI site in C. minitans contains helices involved in DNA replication, recombination and repair.

PIF1 disruption affects growth and sclerotial mycoparasitism

The pGEM_PIF1 plasmid encompasses wild-type C. minitans PIF1, flanked by ≥1 kb of 5′ and 3′ sequences. Co-transformation of R2427 yielded 21 phleomycin-resistant (Ph^KR^) transformants from six independent transformation experiments, 16 of which contained wild-type PIF1 (Fig. 5). Restoration of wild-type colony growth rate and full sclerotial pathogenicity was observed in all 16 transformants containing wild-type PIF1 (type 1 and 3 transformants; Fig. 5). The five transformants that did not contain the wild-type gene were indistinguishable from R2427 in terms of growth and sclerotial pathogenicity. In six of the 16 complemented transformants the hph transgene could not be detected, suggesting that a homologous gene-replacement event had occurred at the PIF1 locus (type 3 transformants).

Hybridization of the 396 bp PIF1 probe to wild-type, R2427 and 14 randomly selected Ph^KR^ R2427 transformants indicated that wild-type C. minitans contains a single copy of the PIF1 gene (data not shown). In R2427, the XbaI-restricted hybridizing band was larger (approx. 9.3 kb) than in the wild-type (approx. 5.7 kb), due to disruption of the ORF by the integrating plasmid. Complemented transformants (type 1 and 3) always contained the 5.7 kb band. Type 2 (non-complemented) transformants contained the 9.2 kb band but not the 5.7 kb band (Fig. 5B).

PIF1 transcript analysis

PIF1 transcripts were detectable in both wild-type and R2427 RNA samples from mycelia grown in PDB for 10 days (Fig. 6). Although 5′ regions could be amplified using appropriate oligonucleotide primers, full-length PIF1 transcripts (3′ regions) could not be amplified from R2427 using RT-PCR (Fig. 6), suggesting that the transcript was truncated at the 3′ end. These observations were consistent with the integration of pAN7-1 after nucleotide 2394 in the PIF1 gene (Fig. 2B).

Virtual Northern analysis using randomly primed cDNAs indicated that levels of wild-type PIF1 expression were similar during both mycoparasitism and growth in PDB, and that transcript levels in R2427 grown in PDB were not altered (data not shown).

DISCUSSION

We have identified a putative DNA-helicase PIF1 gene from C. minitans that when disrupted results in reduced mycelial growth and an inability to mycoparasitize Scl. sclerotiorum sclerotia. Functional complementation of the PIF1 REMI mutant (R2427) and restoration of wild-type growth and mycoparasitic phenotypes was achieved using wild-type PIF1. We believe this is the first C. minitans gene shown to have a functional role in growth with concomitant effect on sclerotial mycoparasitism.

The predicted PIF1 protein (845 aa) has strong similarity with DNA-helicases from several other fungi and yeasts, including A. fumigatus, N. crassa and S. pombe. Interestingly, there was a high level of conservation in the C-terminal half of the proteins, in contrast to the N-termini where the similarity was low between all species. Non-conserved N-terminal regions within PIF1 helicases may reflect specific properties such as differences in protein interactions, subcellular localization or additional enzymic activities (Bessler et al., 2001).

The role of PIF1 has been well characterized in Sac. cerevisiae and S. pombe, where it has been shown to be involved in repair and recombination of mitochondrial DNA (mtDNA) (Foury & Lahaye, 1987), chromosomal DNA replication (Ivessa et al., 2000; Zhou et al., 2002), and maintenance of telomeres and rDNA (Ivessa et al., 2000; Schulz & Zakian, 1994). PIF1 is not essential during the vegetative growth of Sac. cerevisiae, while the PIF1 homologue (Pfi1) of Sch. pombe is essential, indicating different roles for PIF1 between species. The C. minitans PIF1 ORF has two putative start methionine codons separated by approx. 50 aa; similar architecture is observed in the yeast, human, A. fumigatus and N. crassa genes. The Sac. cerevisiae PIF1 is localized to both the nucleus and the mitochondria (Schulz & Zakian, 1994; Zhou et al., 2000).
**Fig. 3.** Multiple sequence alignment of the PIF1 predicted proteins. Cm, *Coniothyrium minitans*; Af, *Aspergillus fumigatus* (accession no. XP_753317); Nc, *Neurospora crassa* (accession no. CAF05978); Sp, *Schizosaccharomyces pombe* (accession no. Q9UUA2). Shading represents 100% conserved residues. The predicted signal peptide is boxed; the ATP/GTP-binding site motif A (P-loop) is underlined by a cross-hatched bar; the putative DNA-binding site is underlined with a solid bar. The diagonal striped bar denotes the location of the prokaryotic membrane lipoprotein lipid attachment site. Arrows indicate positions of the nuclear export signal (NES) and site of REMI integration (REMI).
The nuclear and mitochondrial forms of PIF1 result from two length-disparate forms of the protein produced from different start codons (Schulz & Zakian, 1994). In *Sac. cerevisiae*, mutation of the first methionine (*PIF1-M1*) resulted in mitochondrial instability, but telomere length and production remained as for wild-type. Conversely, mutation of the second methionine (*PIF1-M2*) affected telomere maintenance but not mitochondria (Schulz & Zakian, 1994). Work with yeast and human PIF1 has also demonstrated that the ATP-binding domain plays an essential role in the function of the nuclear form, particularly in contributing to the maintenance of telomeres (Zhang *et al.*, 2006; Zhou *et al.*, 2000).

In R2427 the site of *PIF1* disruption is located in the highly conserved C-terminal putative DNA-binding domain. Functional complementation of R2427 with wild-type PIF1 indicates that the DNA-binding domain and/or downstream sequences are responsible for the observed phenotype. It is noteworthy that while *Sac. cerevisiae* PIF1-
M2 mutant cells could grow as well as wild-type cells, PIF1-M1 mutants (mitochondrial form) exhibited significantly higher proportions of petite cells (Schulz & Zakian, 1994). The C. minitans R2427 mutant exhibits significantly reduced mycelial growth rates (Rogers et al., 2004). Aside from a possible nuclear role for the C. minitans PIF1, the above observations, coupled with the predicted N-terminal signal peptide for mitochondrial export, suggest that mitochondrial instability could result in the reduced growth and the loss of mycoparasitic capacity seen in R2427.

Mycoparasitism of C. minitans involves colonization of sclerotia (rather than antibiotics or competition for nutrients) and it would not be unexpected that inactivation of a gene critically impairing growth would also prejudice its mycoparasitic abilities. However, the loss of mycoparasitism in R2427 does not simply appear to be due to a reduction in growth rate. During insertional mutagenesis of C. minitans, several other transformants with reduced growth rates were found able to infect sclerotia over the same timeframe (Rogers, 2004). Our SEM studies showed that R2427 was able to germinate and grow on the sclerotial surface. The mutant was also able to colonize autoclaved sclerotia without difficulty and therefore was not impaired in its ability to mechanically degrade and use dead sclerotial material as a nutrient source. Disruption of PIF1 therefore prevents C. minitans from penetrating and colonizing live sclerotia. However, PIF1 expression levels were not influenced by interaction with the host.

Complementation was achieved by co-transformation of the PIF1 gene with FHJS3, a plasmid containing the Streptolactoleichis hindustanus ble gene under the control of an Agaricus bisporus promoter, resulting in stable phleomycin-resistant transformants. This is believed to be the first reported use of the phleomycin resistance gene as a selectable marker in C. minitans, providing a useful tool for future molecular investigations. In a high proportion of the complemented transformants, the hygromycin resistance gene was replaced with the wild-type PIF1 gene, demonstrating that homologous recombination can occur at high frequency. Targeted gene knockouts are thus feasible in C. minitans and we have recently exploited this technology to disrupt other genes implicated in sclerotial mycoparasitism (C. W. Rogers and others, unpublished data).

Maintenance of the mitochondrial genome by PIF1 and repair of mtDNA following oxidative damage have been directly linked in yeast (O’Rourke et al., 2002; Zhou et al., 2002). In Sac. cerevisiae PIF1 plays a major role in maintaining mitochondrial integrity in response to oxidative stress (Doudican et al., 2005) and mutants showed a 30-fold increase in mitochondrial DNA mutagenesis (O’Rourke et al., 2002). There is also evidence that mtDNA is more susceptible than nuclear DNA (Yakes & Van Houten, 1997) to oxidative damage by extracellular reactive oxygen species (ROS). Produced as unwanted byproducts of normal mitochondrial respiration during production of ATP through the oxidative phosphorylation pathway, ROS can damage cellular macromolecules, including mtDNA (Doudican et al., 2005). ROS can also originate exogenously, such as during the oxidative burst response to pathogen attack in plants (Apel & Hirt, 2004). Such responses have been recently observed in a fungal–fungal interaction between Ag. bisporus and Verticillium fungicola (Savoie & Largeteau, 2004).

During mycoparasitism, C. minitans mtDNA may be exposed to increases in endogenous and/or exogenous ROS. Mycoparasitic growth and pressure-driven penetration of host cells could lead to endogenous ROS increases in mitochondria in C. minitans, while Scl. sclerotiorum may generate exogenous ROS in response to the mycoparasite. C. minitans PIF1 would therefore protect mtDNA from oxidative damage (and maintain mitochondrial stability) when exposed to increased ROS levels. R2427 with a dysfunctional PIF protein would suffer oxidative damage through its weakened repair mechanisms. This has been observed in Sac. cerevisiae, where PIF1 mutants display mitochondrial instability (Bessler et al., 2001). In turn, this would prevent the mutant from performing energy-demanding processes, such as penetration and colonization of the heavily melanized sclerotia. This would explain our observations that R2427 is able to germinate and grow on the surface of the sclerotia but fails to penetrate and colonize internally.

The Scl. sclerotiorum host produces ROS as signalling molecules during sclerotial differentiation (Georgiou et al., 2001), but their presence in resting sclerotia or release during pathogen attack has not been reported. C. minitans expresses a wide range of genes related to ROS scavenging, oxidative stress response and DNA damage repair, including a histidine biosynthesis gene during sclerotial mycoparasitism (Muthumeenakshi et al., 2007). However, attempts to use histidine and proline as ROS scavengers (Chen & Dickman, 2005; Son et al., 2005) with R2427 have...

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Fig. 6. RT-PCR analysis of C. minitans PIF1 after growth for 10 days in PDB. M, DNA marker (Bioline). Primers used for second strand synthesis: lanes 1–3, β-tubulin-1 and β-tubulin-3; lanes 4–6, X4prb and X4prbC (PIF1 control region, ~400 bp); lanes 7–9, X4prb and PIFendC (spanning PIFI integration site in R2427). W, wild-type cDNA; R, R2427 cDNA; − , water control.
hitherto proved inconclusive. Further work is required to provide a mechanistic understanding of the role of PIF1 DNA helicase in C. minitans development and sclerotial mycoparasitism.

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