ELSEVIER

The oomycete *Pythium oligandrum* expresses putative effectors during mycoparasitism of *Phytophthora infestans* and is amenable to transformation

Neil R. HORNER[1,2], Laura J. GRENVILLE-BRIGGS[2], Pieter VAN WEST*

Aberdeen Oomycete Laboratory, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK

ABSTRACT

The oomycete *Pythium oligandrum* is a mycoparasitic biocontrol agent that is able to antagonise several plant pathogens, and can promote plant growth. In order to test the potential usefulness of *P. oligandrum* as a biocontrol agent against late blight disease caused by the oomycete *Phytophthora infestans*, we investigated the interaction between *P. oligandrum* and *Ph. infestans* using the green fluorescent protein (GFP) as a reporter gene. A $CaCl_2$ and polyethylene-glycol-based DNA transformation protocol was developed for *P. oligandrum* and transformants constitutively expressing GFP were produced. Up to 56 % of *P. oligandrum* transformants showed both antibiotic resistance and fluorescence. Mycoparasitic interactions, including coiling of *P. oligandrum* hyphae around *Ph. infestans* hyphae, were observed with fluorescent microscopy. To gain further insights into the nature of *P. oligandrum* mycoparasitism, we sequenced 2376 clones from cDNA libraries of *P. oligandrum* mycelium grown *in vitro*, or on heat-killed *Ph. infestans* mycelium as the sole nutrient source. 1219 consensus sequences were obtained including transcripts encoding glucanases, proteases, protease inhibitors, putative effectors and elicitors, which may play a role in mycoparasitism. This represents the first published expressed sequence tag (EST) resource for *P. oligandrum* and provides a platform for further molecular studies and comparative analysis in the Pythiales.

© 2011 British Mycological Society. Published by Elsevier Ltd.

Introduction

CORE
Provided by Elsevier - Publisher Connector

The which display fungal-like morphology. Current molecular research and genomics have focused predominantly on Peronosporales oomycetes. *Phytophthora* species are particularly associated with lethal and devastating diseases, such as late blight caused by *Phytophthora infestans*, sudden oak death, caused by the highly destructive *Phytophthora ramorum* and the equally destructive dieback caused by *Phytophthora*

cinnamomi. These diseases have significantly impacted farm-

The 100 or so recognised species within the *Pythium* genus also include many important plant pathogens that cause post and preemergence damping-off of seedlings or seeds as well as fruit, stem, and root rots (van West *et al.* 2003). Several other species have evolved to infect animals, including insects (*Pythium carolinianum*) (Chen *et al.* 2005), fish (*Pythium undulatum*) (Sati 1991), and mammals, including humans (*Pythium*

insidiosum) (De Cock *et al.* 1987); the latter a significant and sometimes fatal problem in tropical countries. A number of *Pythium* species are able to antagonise plant pathogenic fungi and oomycetes, including *Pythium acanthicum*, *Pythium periplocum* (Ribeiro & Butler 1995), *Pythium nunn* (Laing & Deacon 1991), *Pythium lycopersicum* (Karaca *et al.* 2008), and *Pythium oligandrum* (Deacon 1976).

Of these, *P. oligandrum* is an effective parasite of economically important soil-borne pathogens such as *Rhizoctonia solani*, *Botrytis cinerea* (Laing & Deacon 1991), *Fusarium solani* (Bradshaw-Smith *et al.* 1991), *Pythium ultimum* (Berry *et al.* 1993), and *Phytophthora parasitica* (Picard *et al.* 2000b). *Pythium oligandrum* was commercialised as a biocontrol agent for the protection of a wide variety of plant species under license as Polygardron in the Slovak Republic (Brozova 2002). It is also an endophyte capable of colonising the root rhizosphere of many crop plants (Martin & Hancock 1987). Beneficial effects of this association include induction of a variety of plant defence responses. It has been reported to protect sugar beet, wheat, grapevine, and tomato plants from colonisation by many different fungal pathogens (Benhamou *et al.* 1997; Picard *et al.* 2000a; Le Floch *et al.* 2003; Hase *et al.* 2006; Mohamed *et al.* 2007; Takenaka *et al.* 2008). Induction of plant defences by *P. oligandrum* is mediated by extracellular or cell wall bound elicitins (Picard *et al.* 2000a; Hase *et al.* 2006; Hondo *et al.* 2007; Takenaka & Tamagake 2009; Masunaka *et al.* 2010). *Pythium oligandrum* may also promote plant growth, *via* production of tryptamine, an auxin precursor (Le Floch *et al.* 2003).

It seems likely that *P. oligandrum* reduces pathogen load by occupying the available space on the root surface and by consuming free nutrients (Takenaka *et al.* 2008). A direct antagonistic mycoparasitic interaction, as demonstrated extensively *in vitro* (Deacon 1976; Laing & Deacon 1991) is also likely to play a role. Mycoparasitism occurs when *P. oligandrum* hyphae come into close physical contact with host hyphae or resting structures. The interaction events are varied and host-specific, including coiling of *P. oligandrum* around host hyphae, penetration of host hyphae, host cytoplasmic disorganisation, and finally lysis (Deacon 1976; Davanlou *et al.* 1999). Secretion of antimicrobials, such as volatile chemicals (Bradshaw-Smith *et al.* 1991), other antibiotics (Lewis *et al.* 1989; Benhamou *et al.* 1999) and cellulases (Picard *et al.* 2000b), also contribute to the success of this organism as a mycoparasite. With the exception of cell wall degrading enzymes, mycoparasitic effectors have, so far, not been identified in *P. oligandrum*.

Despite the beneficial effects of *P. oligandrum* towards crop plants and its potential as an effective biocontrol agent, very little is known about the molecular mechanisms involved in the interactions with its hosts. We are interested in the molecular, cellular, and physiological processes underlying *P. oligandrum* mycoparasitism. We therefore wanted to develop a molecular tool kit to aid in examining the interaction between *P. oligandrum* and plant–pathogenic microbes. Here we analyse the suitability of green fluorescent protein (GFP) as a reporter gene for the analysis of interactions between the mycoparasitic oomycete *P. oligandrum* and the destructive plant pathogenic oomycete *Ph. infestans*. Stable DNA transformation was first described in the oomycetes, in *Ph. infestans* (Judelson *et al.* 1991). Since then, multiple species have been stably transformed with a variety of methods, including *Phytophthora*

capsici (Huitema *et al.* 2011), *Phytophthora sojae* (Judelson *et al.* 1993), *Saprolegnia monoica* (Mort-Bontemps & Fevre 1997), *Phytophthora nicotianae* (Bottin *et al.* 1999), *Phytophthora palmivora* (van West *et al.* 1999a), *Phytophthora brassicae* (Si-Ammour *et al.* 2003), *Pythium aphanidermatum* (Weiland 2003), *P. ultimum* (Vijn & Govers 2003), and *Ph. ramorum* (Riedel *et al.* 2009). Reporter genes such as β-glucuronidase (GUS) or GFP have proved useful in advancing our understanding oomycete growth and development, by enabling detailed analyses both *in vitro* and *in planta* (van West *et al.* 1998). For example, reporter genes have been used to study growth of *Ph. brassica* in *Arabidopsis*, *Ph. infestans* in potato, *Ph. nicotianae* in tobacco and tomato and *P. aphanidermatum* in sugar beet. Measurements of reporter gene expression allowed host resistance and effector secretion to be assayed (Kamoun *et al.* 1998a, 1998b; Bottin *et al.* 1999; Si-Ammour *et al.* 2003; Weiland 2003; Whisson *et al.* 2007; Le Berre *et al.* 2008). A $CaCl_2$ and polyethylene-glycol-based DNA transformation protocol was developed in *P. oligandrum* and transformants expressing GFP were produced. The use of these reporter genes to study mycoparasitic interactions between the two oomycetes is discussed.

To further illuminate the mycoparasitic interaction we employed a small-scale pilot expressed sequence tag (EST) sequencing approach, to gain an initial perspective on gene diversity in *P. oligandrum*. Characterisation of *P. oligandrum* genes could facilitate the development of more effective biocontrol strategies, as has been possible with mycoparasitic *Trichoderma* species (Flores *et al.* 1997; Lorito *et al.* 1998). Accordingly, here, we also present the main findings from the analysis of two *P. oligandrum* cDNA libraries, from which 2376 clones were sequenced. We highlight transcripts that code for proteins that may be involved in the interaction with its hosts. The ESTs have been deposited into the public NCBI dbEST database GenBank: EV243424 to EV248081.

Materials and methods

In vitro *growth of* Pythium oligandrum *and* Phytophthora infestans

Pythium oligandrum strain CBS 530.74 and CBS 200.184 were obtained from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). *Pythium oligandrum* strain 7 was a gift from Franck Panabieres, INRA France. *Pythium oligandrum* was maintained on V8 medium (1.5 g of $CaCO_3$, 100 ml of V8 Vegetable Juice) at 24 °C as described by Rahimian & Banihashemi (1979). *Phytophthora infestans* strain 88069 was maintained on Rye medium supplemented with 2 % sucrose at 18 °C as described by Caten & Jinks (1968). Nonsporulating *Ph. infestans* mycelium was grown in submerged liquid V8 broth for 5 d. All strains used were grown under the Scottish Executive Environment and Rural Affairs licence number PH/37/2010.

Growth of Pythium oligandrum *on* Phytophthora infestans

Nonsporulating *Ph. infestans* mycelium was grown in V8 broth for 5 d, after which time the medium was replaced with sterile dH_2O. Mycelial plugs of *P. oligandrum* were inoculated and both

organisms were cocultured for 2 d before visualising with fluorescent microscopy.

Plasmids for transformation of Pythium oligandrum

Vectors used for cotransformation were pVW2 (van West *et al.* 1999a), containing a mammalian enhanced GFP open reading frame fused to the *ham34* promoter and terminator from *Bremia lactucae*, used in conjunction with either pTH209 or pHAMT34H, kindly provided by H. Judelson. Plasmid pTH209 consists of the *hsp70* promoter of *B. lactucae* fused to the coding sequence of the neomycin phosphotransferase gene (*nptII*), which confers resistance to the aminoglycoside G418, and the *ham34* terminator. pHAMT34H contains the *ham34* promoter and terminator, fused to the coding sequence of the hygromycin phosphotransferase gene (*hpt*) (Judelson *et al.* 1991).

Transformation of Pythium oligandrum

Stable transformation of *P. oligandrum* was carried out according to the protocols of Judelson *et al.* (1991) and van West *et al.* (1998, 1999a, b) with the following minor modifications. Forty-eight-h-old mycelium from 8 to 10 plates of V8-liquid grown cultures was washed three times with KC osmoticum (0.6 M KCl, 0.2 M CaCl$_2$) and incubated with 125 mg cellulase (5 mg ml^{-1}) (Sigma C8546) and 75 mg lysing enzymes from *Trichoderma harzianum* (3 mg ml^{-1}) (Sigma L1412) at room temperature with 30 rpm shaking for 90 min. Protoplasts were filtered through a 70 μM mesh, pelleted at 700 g, washed once in KC osmoticum, once in KC/MT (0.6 M KCl, 0.2 M CaCl$_2$/0.8 M Mannitol, 10 mM Tris−HCl) and resuspended in MT buffer (0.8 M Mannitol, 10 mM Tris−HCl) pH7.5 containing 25 mM CaCl$_2$ at a concentration of 0.1−1 × 10^7 protoplasts per ml. A mixture containing 30 μg vector DNA (linearised) plus 60 μg Lipofectin reagent was preincubated at room temperature and added to 1 ml of protoplast solution. A 1 ml solution of polyethylene-glycol (PEG)-Ca (50 % w/v PEG 3350, 25 mM CaCl$_2$, 10 mM Tris−HCl pH7.5) was slowly added to the protoplast mixture and incubated at room temperature for 4 min. A solution of 2 ml of clarified V8 broth containing 0.8 M Mannitol (V8M) was added to the DNA/protoplast mixture, inverted and incubated for 1 min. A further 1 ml of V8M was added to the mixture, which was then transferred into a solution of 25 ml V8M with the addition of antibiotics (100 μg ml^{-1} ampicillin, 50 μg ml^{-1} vancomycin). *In vitro* growth of *P. oligandrum* strain CBS 530.74 on V8 agar amended with hygromycin or geneticin (G418) determined the minimum inhibitory concentration (MIC) at 30 μg ml^{-1} for both antibiotics. Protoplasts were allowed to regenerate at 24 °C for between 15 and 18 h, after which time they were pelleted at 700 g, resuspended in 3 ml V8M and spread onto V8 agar supplemented with 30 μg ml^{-1} G418 (pTH209) or hygromycin (pHAMT34H) antibiotic. Colonies appeared within 2−3 d and were subsequently propagated on V8 agar with the addition of either G418 or hygromycin at 30 μg ml^{-1}.

Molecular analysis of gene integration

Polymerase chain reaction (PCR) was used to check the integration of the *gfp* gene into *Pythium oligandrum*. DNA was extracted from liquid grown mycelium of *P. oligandrum* using a phenol/chloroform extraction method as described in Raeder & Broda (1985). Integrity of the DNA was tested by agarose gel electrophoresis. Thirty-five cycles of PCR were carried out, annealing at 60 °C. Primers targeting the GFP gene were GFP-F (ATGGGCAAGGGCGAGGAACTGTTCAC) and GFP-R (TCACTTGTAGAGTTCATCCATGCCATGCG).

Microscopic analysis of expression of GFP

Phase contrast and fluorescence microscopy were performed using an Axioplan 2 microscope (Zeiss) with standard rhodamine, ultra violet (UV), and GFP filter sets. Images were captured using a Hamamatsu CCD camera and analysed using Openlab 5.0.3 (Improvision).

cDNA library construction

A first cDNA library was created from mRNA isolated from vegetative mycelia of *Pythium oligandrum* (Library L1). Agar blocks containing hyphae of approximately 1 cm^2 of 7-d-old *P. oligandrum* cultures were inoculated in 10 % V8 broth and incubated at 25 °C in the dark and grown for 4 d. Mycelia was briefly washed in water and isolated on Whatman filter paper and then placed in 1.7 ml microcentrifuge tubes and frozen in liquid nitrogen, and stored at −80 °C.

A second cDNA library (L2) was created from RNA isolated from *P. oligandrum* interacting with *Phytophthora infestans*: Rye Sucrose Agar (RSA) plugs, approximately 1 cm^2, containing 11-d-old *Ph. infestans* mycelia were inoculated into 10 ml of 5 % V8 juice containing 100 mg ml^{-1} vancomycin and 100 mg ml^{-1} ampicillin before being incubated at 18 °C for 5 d until radial growth of around 20 mm had occurred. The cultures were incubated at 60 °C for 90 min to kill the mycelia. The V8 medium was removed from the Petri dishes by pipetting and replaced with distilled water. Two 1 cm^2 agar plugs of *P. oligandrum* were inoculated into the Petri dishes containing the killed *Ph. infestans* mycelia at opposite sides of the dish. The cultures were incubated at 25 °C to allow growth of *P. oligandrum* towards the killed *Ph. infestans* mycelia. After 3 d, the cultures were microscopically analysed to check for interaction signs, which were coiling of *P. oligandrum* hyphae around *Ph. infestans* hyphae, as well as lysis of some hyphae of *Ph. infestans*. Mycelia that appeared to be interacting with the killed *Ph. infestans* mycelia were isolated on Whatman filter paper and washed with water and placed into 1.7 ml microcentrifuge tubes before immediate freezing in liquid nitrogen. Mycelial samples were stored at −80 °C.

The following steps were carried out at Vertis Biotechnologie (Lise-Meitner-Str. 30, Freising, Germany): RNA was extracted and mRNA was isolated using oligo(dT) chromatography, cDNA synthesis was performed on mRNA primed with an oligo(dT) primer using Vertis Biotechnologie's full-length enriched technology. Size fractionation of the cDNA was performed using a cut-off of 500 bp. Cloning occurred into *Eco*R I and *Not* I sites of plasmid vector pBS II sk+. Plasmid libraries were electroporated into T1 phage resistant ElectroTen-Blue *Escherichia coli*.

Cloning of Pythium oligandrum *necrosis and ethylene inducing 1-like proteins (NLP's)*

Primers were designed to amplify the full predicted open reading frame (ORF) of (GenBank: EV243877), including the region downstream of the frame-shift (nlp-F: ATGCATGGACTCTATGC-GAGTCT; nlp-R: TCATTTCGACATGGACATGCTC). PCR products, amplified using KOD Hot Start DNA Polymerase (Novagen), were cloned into the *Sma*I site of plasmid pUC19 using standard techniques.

RT-PCR

Total RNA was extracted from approximately 100 mg of mycelia using the RNeasy Plant Mini Kit (Qiagen). Total RNA was checked on an agarose gel for integrity. cDNA was created from total RNA using the First-Strand cDNA Synthesis Kit (Amersham) according to the manufacturers instructions. Approximately 1 μg of RNA was used per reaction. Reverse transcriptase was primed by the addition of 1:25 dilution of 5 μg μl^{-1} *Not*I-d(T)$_{18}$ primer. cDNA was used in subsequent PCR reactions at 0.5 μl per 50 μl reaction with 300 nM of forward and reverse primers.

Sequence analysis

The L1 clones were sequenced by GATC Biotech (Germany). The L2 clones were sequenced by The Broad Institute, MIT (USA). The sequences were clustered into contigs that represent putative independent transcripts using the cap3 DNA assembly program (Huang & Madan 1999). Predicted protein sequences were obtained from the ESTs using the online programme, OrfPredictor (Min *et al.* 2005). Similarity searches were performed using either the BLASTALL or NetBLAST programmes (Altschul *et al.* 1997). Secreted proteins were predicted using SignalP 3.0 (Bendtsen *et al.* 2004). Conserved protein domains were predicted using a locally installed Inter-ProScan server (Zdobnov & Apweiler 2001). Motif scanning and miscellaneous sequence manipulation were performed with the EMBOSS software package (Rice *et al.* 2000) and the GP package (http://www.bioinformatics.org/genpak/). Protein alignments were performed using TCOFFEE software (Notredame *et al.* 2000). Neighbour joining phylogenetic trees were made using MEGA3.1 (Kumar *et al.* 2004). Phylogenetic trees were constructed using Poisson distance correction and 1000 bootstrap replications, and were rooted at midpoint. BLAST searches were performed against the NCBI nonredundant database and the oomycete genomic databases of *Pythium ultimum* (Levesque *et al.* 2010), *Phytophthora sojae*, *Phytophthora ramorum* (Tyler *et al.* 2006), *Phytophthora infestans* (Haas *et al.* 2009), as well as on a draft genome of the model biocontrol fungus *Trichoderma virens* (available at http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html).

Results

Transformation of Pythium oligandrum *with a* gfp *gene construct*

A construct containing the *ham34* promoter of the oomycete *Bremia lactucae* fused to the coding sequence of the mammalian enhanced GFP gene, pVW2 (van West *et al.* 1999a), was introduced into *P. oligandrum* strain CBS 530.74 by cotransformation of protoplasts with either pTH209, containing the Geneticin resistance gene (*nptII*), or with pHAMT34H, containing the Hygromycin resistance gene (*hpt*) (Judelson *et al.* 1991). In total 93 putative *ham34-gfp* transformants were obtained. Sixty of these were Geneticin resistant (G1-60), and 33 Hygromycin resistant (H1-33). Antibiotic resistance was maintained after multiple rounds of culturing in most transformed lines.

PCR analysis was used to check integration of the constructs and expression of *gfp* in *P. oligandrum* (Supplemental Fig S1). Strains PoG16 and PoH30 showed the brightest PCR product after gel electrophoresis.

Detailed microscopic analysis of expression of GFP

Fluorescence microscopy was used to observe the expression of GFP in *Pythium oligandrum* grown *in vitro*. GFP produced in transgenic strains fluoresces bright-green upon exposure to UV light with an excitation maximum of 488 nm. Twenty-three of the 60 Geneticin resistant *P. oligandrum* strains (38 %) analysed were fluorescent. Eighteen of the 33 Hygromycin resistant *P. oligandrum* strains (54 %) analysed were fluorescent. Growth rates of all *ham34-gfp* expressing strains were the same as wild-type strains on antibiotic free V8 agar. Among the transformants variation in the level of fluorescence was noted. This has previously been reported in other oomycetes transformed with the pVW2 *ham34-gfp* construct (van West *et al.* 1999a). Strains PoH30 and PoG16 were observed to be the most highly fluorescent, and so were used for further studies. *Pythium oligandrum* is a homothallic species, that produces abundant spiny oogonia in single culture (Deacon 1976). In those strains that were fluorescent, expression of *gfp* was observed in both *in vitro* grown mycelia and oogonia (as demonstrated by PoG16 in Fig 1A–C).

Infection of Phytophthora infestans *by* Pythium oligandrum

To investigate the nature of the parasitic interaction between *P. oligandrum* and *Ph. infestans*, 4-d old *Ph. infestans* mycelium, grown in liquid V8 medium, was inoculated with *P. oligandrum* hyphal plugs. The consistently highly fluorescent *P. oligandrum* strain PoG16 expressing GFP was chosen for this study, to enable easy distinction of host and parasite hyphae.

Forty-eight hours post-inoculation (hpi), small green fluorescent *P. oligandrum* hyphae were observed that coiled around the larger *Ph. infestans* hyphae (Fig 1D–F, *Ph. infestans* hyphae indicated by the arrow in Fig 1D). After 72 hpi, *Ph. infestans* hyphae became vacuolated and the cytoplasm became granulated in some areas. Abundant green fluorescent *P. oligandrum* oospores were also visible throughout the culture (Fig 1G–I). By the fourth day, debris from lysed hyphae became visible. These results indicate that *P. oligandrum* is able to parasitise *Ph. infestans*, and for this reason we used *Ph. infestans* as a host for a *P. oligandrum* interaction cDNA library. The use of strains of *P. oligandrum* expressing GFP allowed the clear distinction of host and parasite hyphae, and shows the close association of both hyphae during the interaction (Fig 1).
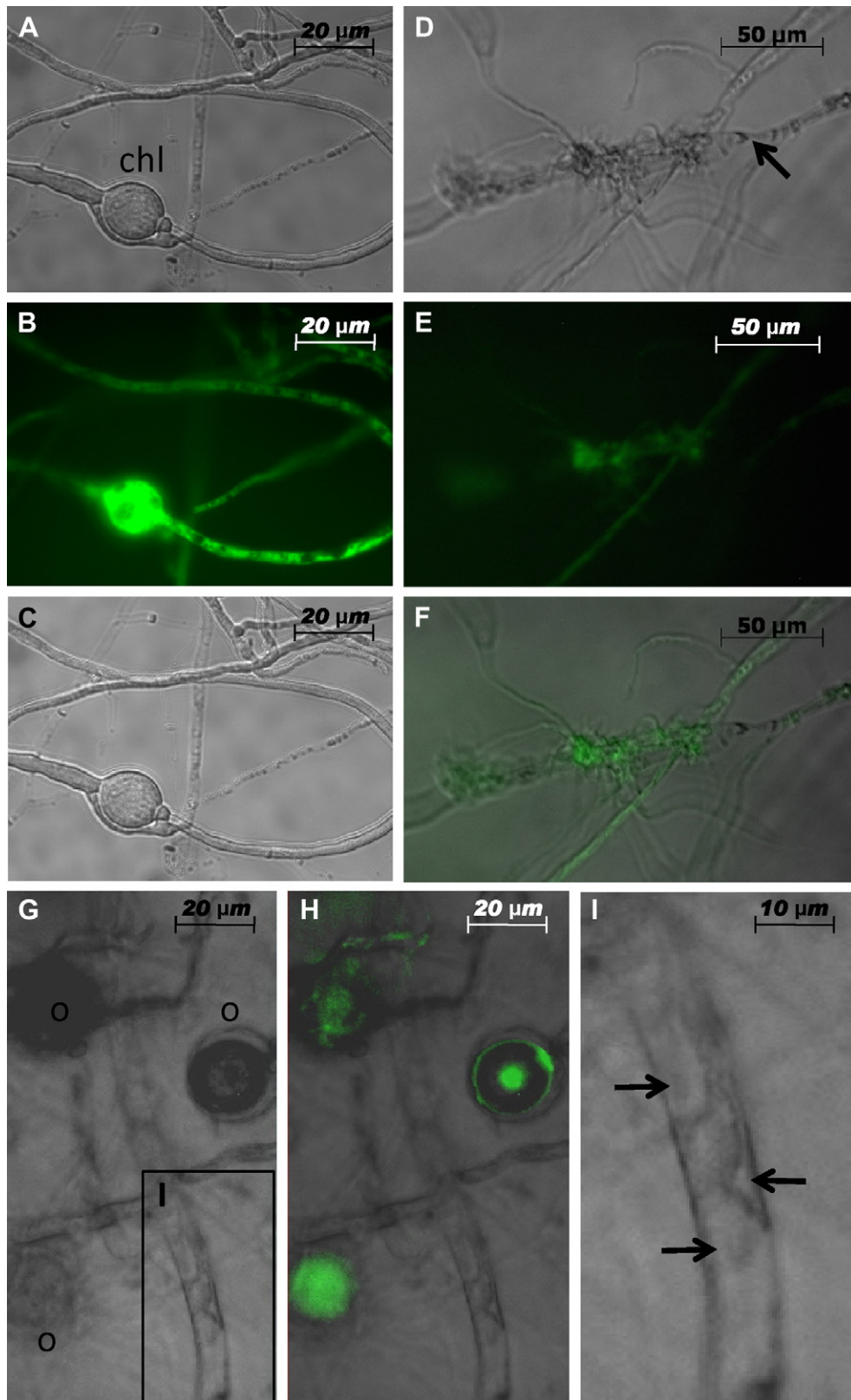
Fig 1 − **Microscopic analysis of a *Pythium oligandrum* transformant expressing *gfp* and the interaction with *Phytophthora infestans*. (A) Bright field image and (B) fluorescent image of *P. oligandrum* PoG16 hyphae and a chlamydospore (chl). (C) Merged image. (D) Bright field, (E) fluorescent image and (F) merged image of *P. oligandrum* PoG16 infecting *Ph. infestans* strain 88069, 48 hpi. *Ph. infestans* hypha (non fluorescent) is indicated by the arrow. (G) Bright field, (H) fluorescent and bright field merged image, and (I) zoomed in area of *P. oligandrum* PoG16 oospores (○) and hyphae of *Ph. infestans* strain 88069 72 h after infection. Vacuolated and granular *Ph. infestans* hypha indicated with arrows in (I).**

Table 1 — Summary of *P. oligandrum* ESTs.			
	Vegetative library L1	Interaction library L2	Total
Total reads	952	3724	4676
Average read length	668 bp	777 bp	
GC content (%)	55.21	55.43	
Singlets	417	246	
Contigs	119	562	
Unigenes	536	819	1219
Predicted to be secreted	48	74	
Similarity to nr database $< 1e^{-5}$	380	574	
Similarity to *P. ultimum* $< 1e^{-5}$	450	195	
Similarity to *T. virens* $< 1e^{-5}$	300	113	

Creation of two cDNA libraries and sequencing

Two cDNA libraries were made from *Pythium oligandrum* mRNA. The first library (L1) was produced from 4-d-old sporulating mycelia. From this library, 951 good quality 5′ sequences reads were obtained. The second library (L2) was obtained from a *P. oligandrum—Phytophthora infestans* interaction. To ensure that this library contained only *P. oligandrum*-derived transcripts, *Ph. infestans* mycelium was heat-killed (60 °C) before inoculating with *P. oligandrum*. *Pythium oligandrum* interaction with the dead mycelium continued for 4 d. In parallel, a heat-killed *Ph. infestans* control plate was left uninoculated to check for RNA degradation. An RNA preparation was performed on this *Ph. infestans* control material, and a sample was run on an ethidium-stained agarose gel. No RNA was visible on this gel (Supplemental Fig S2), showing that *Ph. infestans* RNA had been degraded, and that most, if not all, cDNA clones produced from this RNA would be of *P. oligandrum* origin. Four 384 well plates containing L2 clones were sequenced. Each clone from L2 was sequenced from both the 5′ and 3′ ends producing 3780 good quality reads. All sequences were

submitted to dbEST with GenBank accessions GenBank: EV243424—GenBank: EV248081.

Clustering of the sequences resulted in 536 and 819 consensus sequences from L1 and L2 respectively. Clustering the consensus sequences derived from both libraries gave a total of 1219 unigenes (Table 1).

To rule out the possibility of *Ph. infestans* contamination in L2, ESTs were queried against the *Ph. infestans* genome database (Haas *et al.* 2009) using the BLASTN program. None of the ESTs had more than 90 % similarity to any *Ph. infestans* sequence, (data not shown) demonstrating that no *Ph. infestans* sequences were found in the EST libraries.

Highly represented sequences

Contigs representing the most highly abundant ESTs from L1 and L2 are listed in Table 2 — by far the most abundant sequence in L1 is L1C92 (GenBank: EV244088), a polyadenylated transcript with no similarity to sequences in the public databases and no significant similarity to *Pythium ultimum* genome sequences. L1C92 appears to have very low coding potential as the sequence contains only one ATG codon in the forward orientation, which at position 426 is relatively far from the 5′ of the sequence compared to other ESTs. Coding potential of L1C92 was determined using the Testcode software (Fickett 1992), which returned a value of 0.645, which suggests a noncoding sequence. The L1C92 ESTs represented 7.7 % of L1 transcripts. It is possible that the abundance of transcripts that represent L1C92 is due to bias introduced during the library construction. It is also notable that ESTs similar to L1C92 were absent in L2.

L1C113 (GenBank: EV244338) was represented by 11 transcripts but shows no similarity to known sequences in the public databases or to the *P. ultimum* genome. L1C37 (GenBank: EV243752) was predicted to encode a signal peptide-containing protein, and was represented by ten

Table 2 — Most highly represent ESTs.					
Representative EST	No. in contig	Top hit description	E value	Species	Library source
EV245172	96	Glycine-rich cell wall protein	1.6E-19	*P. ultimum*	L2
EV244402	95	Ribosomal protein L29e	6E-13	*M. truncatula*	L2
EV245173	94	Ribosomal protein S27a	8E-48	*C. familiaris*	L2
EV244382	83	Ribosomal protein S33	1E-12	*K. marxianus*	L2
EV244421	81	Ribosomal protein S14	5E-57	*B. napus*	L2
EV244743	77	HAF1, TFIID	2.9E-52	*P. ultimum*	L2
EV244088	74	No hits (possible noncoding sequence)	—	—	L1
EV244690	74	Ribosomal protein S17	4E-49	*Petunia x hybrida*	L2
EV244408	72	Ribosomal protein S12	2E-68	*Ph. infestans*	L2
EV244729	65	Ribosomal protein L12	3E-57	*C. elegans*	L2
EV244671	62	Ribosomal protein L39	5E-31	*C. hominis*	L2
EV243499	31	Elongation factor 1 alpha	4E-137	*C. incerta*	L1
EV244052	15	Putative S-phase specific ribosomal protein	1E-87	*L. edodes*	L1
EV243732	13	Protein kinase, TKL group	4E-32	*D. discoideum*	L1
EV243713	12	Gag-Pol	1E-123	*L. batatas*	L1
EV244338	11	No hits	—		L1
EV243561	11	ADP/ATP translocase	1E-143	*Ph. infestans*	L1
EV243474	10	S14 Ribosomal protein	4E-57	*B. napus*	L1
EV243752	10	Conserved hypothetical secreted protein	3E-11	*Ph. infestans*	L1
EV243463	9	Ubiquitin-conjugating enzyme E2	3E-28	*C. reinhardtii*	L1

sequences. This sequence has at least three significant hits in the *P. ultimum* genome, all to proteins of unknown function. The predicted gene product of L1C37 contains 19 % proline residues, a common feature of extracellular matrix and cell wall proteins (Williamson 1994). The other most abundant sequences from L1 are mostly similarity to housekeeping genes, apart from L1C11 (GenBank: EV243732), which was similar to a TKL group protein kinase, and L1C32 (GenBank: EV243713) that was similar to retrovirus-related sequences. Eight of the ten most abundant sequences from L2 are predicted to encode ribosomal proteins. L2C153 (GenBank: EV245172) was predicted to encode a small tyrosine-rich protein with a predicted signal peptide and shows similarity to a glycine-rich cell wall protein in *P. ultimum* (PYU1_T000208; 1 e -12). The other unigene, contig L2C365 (GenBank: EV244743), is similar to a HAF1 transcription initiation factor-like protein from *P. ultimum*, (PYU1_T001058; 2 e -04) (Table 2).

Sequence annotation

The consensus sequences from each library were annotated by searching the NCBI nonredundant protein database, the *Pythium ultimum* genome database (Levesque *et al.* 2010), the *Phytophthora infestans* genome database (Haas *et al.* 2009), the *Phytophthora sojae* and *Phytophthora ramorum* genome databases (Tyler *et al.* 2006) and a draft genome of the model mycoparasitic fungus *Trichoderma virens* (available at http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html). The percentage of sequences that had significant hits (defined here as an E value $< 10^{-5}$) to the NCBI nr database were 73 % and 68 % for L1 and L2 respectively. Eighty-four percent of L1 and 24 % L2 sequences had significant hits ($E < 10^{-5}$) to the *P. ultimum* genome. Fifty-six percent of L1 and 14 % of L2 sequences had significant hits ($E < 10^{-5}$) to the *T. virens* genome. ESTs that were assigned potential roles in mycoparasitism, based on similarity to known genes, are listed in Table 3. The contigs referred to herein are described by their library name followed by the contig id (e.g. L1C46), with a representative dbEST accession in parenthesis. Singlet sequences are referred to using just the dbEST accession. Sequences that were found in both libraries are named according to our contig id (e.g. Contig1).

Putative secreted proteins

The 1219 unigenes were processed using the ORFpredictor web tool (Min *et al.* 2005), to produce a set of predicted protein sequences. Signal peptides were predicted using the SignalP V3.0 Web server (Bendtsen *et al.* 2004). Of the predicted proteins, 47 and 68 from L1 and L2 respectively were predicted to contain N-terminal signal peptides after removal of probable false positives such as ribosomal proteins. In addition, 11 sequences from the L1 predicted secreted dataset and 12 sequences from the L2 predicted secreted dataset were predicted membrane proteins. Within the total putative secreted protein set, only two had representative ESTs from both libraries, GenBank: EV246257 and GenBank: EV244643. These were both similar to elicitin-like sequences. This suggests that the secretomes from the two different conditions used to make the libraries are significantly different, although this variation

could also be due to the limited sampling size of this pilot project. The predicted secreted protein dataset includes extracellular surface proteins such as mucins, surface glycoproteins, cell wall structural proteins, and adhesion proteins. Proteins such as these may be important for establishing contact with suitable host surfaces, or for evading host defence systems. Several other sequences potentially involved in mycoparasitism and pathogenicity were also identified. These are described in more detail below.

Transcripts putatively involved in host interaction

Cell wall degrading enzymes

There were a total of 16 distinct sequences that showed significant similarity to enzymes possibly involved in the degradation of carbohydrates and which could play an important role in mycoparasitic interactions, seven originating from L1 and nine from L2. There was no overlap of these sequences between the libraries (Table 3). Four contigs were similar to Cell 5A endo-1,4-beta-glucanase from *Phytophthora* spp. One of these, GenBank: EV243914, had a predicted N-terminal transmembrane span, as did the other 21 *Phytophthora* spp. sequences annotated as Cell 5A endo-1,4-beta-glucanase in the NCBI nr protein database. None of the other three Cell 5A-like sequences found in the *Pythium oligandrum* libraries have predicted transmembrane regions, possibly because the majority are nonfull-length open reading frames. Two sequences from L2 were similar to pectate lyases from *Aspergillus fumigatus* (GenBank: EV244943, E value $= 5e^{-5}$8), and *Neosartorya fischeri* (GenBank: EV246008, E value $= 2e^{-5}$3). No sequences similar to pectin/pectate-degrading enzymes were found in L1.

Transcripts possibly involved in obtaining nutrients from the host

Three unigenes, with predicted signal peptides, were annotated as having a role in the breakdown and utilisation of lipids. The L2 transcript Genbank: EV244973 was similar to a lipin acyltransferase from *Pythium ultimum* (PYU1_T012544; 3 e^{-52}). Contig26 (Genbank: EV244589) was similar to a choloylglycine hydrolase from *P. ultimum* (PYU1_T014908; 1 e^{-66}). L1C75 (Genbank: EV244043) was similar to a triacylglycerol lipase from *P. ultimum* (PYU1_T008733; 1 $^{e-173}$). L1C5 (Genbank: EV243484) was annotated as a metabolite and sugar transporter, with similarity to a putative metabolite transporter from *P. ultimum* (PYU1_T014850; 2.33 e^{-156}). The predicted protein contains a signal peptide, and ten transmembrane spanning regions. It also contains two major facilitator superfamily (MFS) domains (cd06174) and a sugar (or other molecule) transport domain (pfam:00083), these domains are found in a large family of diverse transport proteins, including those that function primarily in nutrient uptake.

Proteases

Ten unigenes similar to proteases were annotated as extracellular, by the presence of a predicted signal peptide, or in the case of missing 5′ sequence information, were similar to known extracellular proteases (Table 3). Three were identified from L2 and seven from L1. L2C361 (GenBank: EV245020) was similar to a trypsin protease from *Phytophthora infestans*

Table 3 – *P. oligandrum* sequences with potential mycoparasitic roles. BLASTX searches were performed with EST sequences against the NCBI non-redundant protein database, and the predicted proteomes of *P. ultimum* and *T. virens* to assign tentative biological roles.

Rep. dbEST	Lib	Best informative hit description	Species	GenBank AC	E value
Cell wall degrading enzymes					
EV243721	L1	Cell 5A endo-1,4-beta-glucanase	*P. ramorum*	ABL75348	4.00E-32
EV243914	L1	Cell 5A endo-1,4-beta-glucanase	*Ph. infestans*	ABG91063	2.00E-59
EV243844	L1	Cell 5A endo-1,4-beta-glucanase	*Ph. infestans*	ABL75352	1.00E-50
EV244394	L2	Cell 5A endo-1,4-beta-glucanase	*Ph. infestans*	AF494015	3.00E-18
EV245189	L2	Putative endo-1,3-beta-glucanase	*Ph. infestans*	AAM18482	3.00E-63
EV243780	L1	Beta-glucosidase	*Fervidobacterium*	AAN60220	2.00E-46
EV244528	L2	Beta-glucosidase	*Fervidobacterium*	AAN60220	6.00E-38
EV247541	L2	Endo-1,3; 1,4-beta-glucanase	*Ph. infestans*	AAM18486	3.00E-63
EV244829	L2	Endo-1,3-beta-glucanase	*S. pombe*	NP_594547	6.00E-35
EV243491	L1	Thermostable beta-glucosidase	*S. aurantiaca*	ZP_01465947	7.00E-11
EV243787	L1	Putative glycosyl hydrolase	*Proteobacterium*	ZP_01223540	2.00E-09
EV245605	L2	TonB-like (glycoside hydrolase 1)	*S. degradans*	YP_529070	3.00E-66
EV247393	L2	TonB-like (glycoside hyrolase 1)	*S. degradans*	YP_529070	4.00E-39
EV244254	L1	Beta-glucan-binding protein 2	*M. truncatula*	ABB69782	8.00E-39
EV244943	L2	Pectate lyase	*A. fumigatus*	XP_747393	5.00E-58
EV246008	L2	Pectate lyase, putative	*N. fischeri*	XP_001262134	2.00E-53
Proteases/other degradative enzymes					
EV244520	L2	Aspartic protease	*Ph. infestans*	AAY43365	7.00E-59
EV245020	L2	Trypsin protease GIP-like	*Ph. infestans*	AAY43395	1.00E-39
EV245351	L2	Zinc metalloproteinase	*A. aegypti*	EAT47551	8.00E-11
EV244200	L1	Aspartic protease	*S. parasitica*	AAY53768	6.00E-28
EV244235	L1	Papain family cysteine protease	*T. thermophila*	EAR83820	2.00E-25
EV243881	L1	Subtilisin-like serine proteinase	*A. astaci*	AAK39096	5.00E-27
EV243518	L1	Cysteine proteinase.	*M. crystallinum*	AAA74430	4.00E-08
EV243709	L1	Serine carboxypeptidase precursor	*T. cruzi*	EAN95917	7.00E-17
EV243996	L1	Cathepsin-like cysteine protease	*Ph. infestans*	AAY43370	7.00E-63
EV244317	L1	Papain family cysteine protease	*T. thermophila*	EAR92683	4.00E-16
EV244462	L2	Zn2+ dependent hydrolase,	*A. fumigatus*	XP_001267519	8.00E-78
EV244532	L2	Hydrolase or acyltransferase	*L. interrogans*	AAN50345	1.00E-07
Uptake and utilisation of host nutrients					
EV244973	L2	Lipin-acyltransferase	*P. ultimum*	PYU1_T012544	3.00E-52
EV244589	L2	Choloylglycine hydrolase	*P. ultimum*	PYU1_T014908	1.00E-66
EV244043	L1	Triacylglycerol lipase	*P. ultiumum*	PYU1_T008733	1.00E-173
EV243484	L1	Metabolite transporter	*P. ultimum*	PYU1_T014850	2.33E-156
Cell wall/extracellular matrix					
EV244122	L1	Mucin-like protein	*H. glycines*	AAC62109	8.00E-34
EV245072	L2	Mucin-like protein	*Ph. infestans*	AAC72308	4.00E-14
EV244009	L1	Mucin-like protein	*Ph. infestans*	AAC72308	2.00E-14
EV244143	L1	Mucin-like protein	*P. ultimum*	PYU1_T011067	5.60E-33
EV244632	L2	Glycine-rich cell wall structural protein	*P. ultimum*	PYU1_T000208	3.70E-20
EV244654	L2	Glycine-rich cell wall structural protein	*P. ultimum*	PYU1_T000200	6.00E-13
EV245146	L2	Glycine-rich cell wall structural protein	*P. ultimum*	PYU1_T000208	1.00E-17
EV246106	L2	Glycine-rich cell wall structural protein	*P. ultimum*	PYU1_T000200	1.60E-26
EV245340	L2	P48 eggshell protein precursor*	*S. mansoni*	M74170	2.00E-19
EV245847	L2	P48 eggshell protein precursor*	*S. mansoni*	M74170	6.00E-13
EV245907	L2	Glycine-rich cell wall protein*	*O. sativa*	P10496	1.00E-32
EV244139	L1	Cell surface glycoprotein putative*	*H. walsbyi*	AM180088	3.00E-15
EV244304	L1	Extensin-like protein*	*Bacillus* sp.	ZP_01183899	1.00E-07
EV243691	L1	Vegetative cell wall protein GPL-like	*P. ultimum*	PYU1_T008449	8.80E-44
Defence/counter-defence					
EV245133	L2	Agrin-like protein	*P. ultimum*	PYU1_T000142	5.00E-51
EV245779	L2	Four domain protease inhibitor	*P. ultimum*	PYU1_T005024	5.00e-36
EV243682	L1	Agrin-like protein	*P. ultimum*	PYU1_T000142	8.00E-47
EV244785	L2	Mini-agrin	*Mus musculus*	AAX09643	4.00E-6
EV244419	L2	Cystatin-like protease inhibitor	*Ph. infestans*	AAY21183	1.00E-17
EV243901	L1	MDR-like ABC transporter	*O. sativa*	CAD59587	3.00E-35
EV243484	L1	MFS	*T. carboxydivorans*	ZP_01666400	3.00E-74
EV244332	L1	PDR-type ABC transporter 2	*N. tabacum*	BAD07484	3.00E-83
EV243602	L1	Multidrug resistance protein	*F. rubripes*	XP_788510	2.00E-26

Table 3 (continued)

Rep. dbEST	Lib	Best informative hit description	Species	GenBank AC	E value
EV243680	L1	ABC transporter AbcB3	*D. discoideum*	XP_629966	3.00E-60
EV243803	L1	ABC Protein	*P. chrysosporium*	CAD98883	4.00E-43
EV244280	L1	ATP-binding cassette	*H. sapiens*	XP_542642	6.00E-20
EV244758	L2	Multidrug resistance protein	*O. sativa*	NP_001046148	8.00E-45
EV243825	L1	Glutathione peroxidase	*A. thaliana*	NP_180715	3.00E-41
EV243847	L1	Glutathione s-transferase	*X. laevis*	AAM82563	5.00E-17
EV245748	L2	Hydroperoxide glutathione peroxidase	*Ph. sojae*	ABA29804	2.00E-07
EV243948	L1	Xenobiotic reductase	*B. marina*	ZP_01093518	1.00E-59
EV244311	L1	Xenobiotic reductase	*S. elongatus*	ZP_01620253	1.00E-54
EV243726	L1	Thioredoxin peroxidase	*Ph. infestans*	AAN31487	5.00E-76
EV244540	L2	Thioredoxin h2	*M. truncatula*	AAZ98843	6.00E-22
EV244261	L1	Callose synthase catalytic subunit	*G. hirsutum*	AAD25952	5.00E-18
EV244453	L2	Beta-lactamase	*S. usitatus*	YP_823764	4.00E-12
Putative effectors/elicitors					
EV244702	L2	CRN family protein	*Ph. infestans*	XP_002895699	6.00E-17
EV244880	L2	CRN-like CRN5	*Ph. infestans*	AAY43399	2.00E-70
EV244643	L2	Elicitin-like protein 1 precursor	*P. oligandrum*	Q1ESR5	9.00E-55
EV244523	L1	Elicitin-like protein SOL13A	*Ph. sojae*	ABB56009Q	0.017
EV244529, EV244523	L2L1	Putative elicitin Elicitin-like protein SOL13A	*Ph. infestans Ph. sojae*	XP_002897306ABB56009	1.00E-270.017
EV246257, EV244529	L2L2	Elicitin-like protein1putative elicitin	*P. oligandrum Ph. infestans*	Q1ESR4XP_002897306	1.00E-22, 1.00E-27
EV244386, EV246257	L2L2	Elicitin-like protein RAL13D Elicitin-like protein 1	*P. ramorum P. oligandrum*	ABB55953Q1ESR4	1.00E-6, 1.00E-22
EV243877, EV244386	L1L2	NPP1-containing protein Elicitin-like protein RAL13D	*Pe. atrosepticum P. ramorum*	YP_051177ABB55953	4.00E-42, 1.00E-6
EV245688, EV243877	L2L1	Small cysteine rich − SCR122NPP 1-containing protein	*Ph. infestans Pe. atrosepticum*	AF424683YP_051177	4.00E-8, 4.00E-42
EV245366, EV245688	L2L2	Small cysteine rich − SCR76Small cysteine rich − SCR122	*Ph. infestans Ph. infestans*	AF424670AF424683	8.00E-12, 4.00E-8
EV245712, EV245366	L2L2	CBEL Small cysteine rich − SCR76	*Ph. parasitica Ph. infestans*	CAA65843AF424670	6.00E-78, 8.00E-12
EV243525, EV245712	L2L2	Transglutaminase elicitor family M81BCBEL	*Ph. infestans Ph. parasitica*	AAP74660CAA65843	3.00E-46, 6.00E-78
EV245749, EV243525	L2L2	FK506-binding protein 2 precursor transglutaminase elcitor family M81B	*C. neoformans Ph. infestans*	AE017345AAP74660	2.00E-41, 3.00E-46
EV243988, EV245749	L1L2	ROC7 PPIaseFK506-binding protein 2 precursor	*A. thaliana C. neoformans*	AAF05760AE017345	9.00E-82, 2.00E-41
EV243988	L1	ROC7 PPIase	*A. thaliana*	AAF05760	9.00E-82

* indicates that the low-complexity filter was switched off.

(GenBank: AAY43395.1, E value = 1e^{-39}), and was also similar to a glucanase inhibitor protein (GIP) from *Phytophthora sojae* (GenBank: AAL11721, E = 8e^{-35}). GIPs are related to trypsin proteases, but posses mutated catalytic triads, which abolish proteolytic activity, whilst retaining the protein interaction capacity that confers glucanase inhibiting properties (Rose *et al.* 2002). An alignment of L2C361 with GIPs and GIP-like trypsin proteases showed that the H-D-S catalytic triad, which is required for protease activity, was intact in L2C361, suggesting that it retains protease functionality and is not a GIP (Data not shown).

Putative effectors

Genome sequencing of several oomycete pathogens has driven forward the search for oomycete effector molecules; molecules that manipulate the host to facilitate infection and/or trigger defence responses. We were interested in whether such molecules were also present in *Pythium oligandrum* and expressed during mycoparasitism. We,

therefore, mined our pilot dataset for potential effector sequences.

Crinkling and necrosis-inducing like (CRN) effectors

In *Phytophthora* spp. the Crinkler (*Crn*) genes encode a large family of secreted effector proteins with a conserved motif, LxLFLAK, in the amino terminal domain, (Haas *et al.* 2009). Several proteins with similar N-terminal domains were also discovered in *Aphanomyces euteiches* (Gaulin *et al.* 2008) and *Hyaloperonospora parasitica* (Win *et al.* 2007). Recent evidence suggests that the N-terminal sequence motif mediates translocation of these proteins into plant cells and in some cases into the host plant nucleus, to trigger plant cell death (Schornack *et al.* 2010). Two L2 sequences, with significant hits to uncharacterised proteins in *Pythium ultimum* were similar to *Phytophthora infestans* CRN-like proteins. L2C566 GenBank: EV245135 was predicted to encode a full open reading frame that was similar to CRN-like 5 (GenBank: AAY43399, E value = 2e^{-70}), and which had a predicted signal peptide. Another EST, GenBank: EV244702 was similar to a

CRN family protein (GenBank: XP002895699, E value $= 6e^{-17}$), but was lacking the 5′ and 3′ regions encoding the start and stop of the putative ORF. The CRN protein family is highly conserved in all oomycete pathogens sequenced to date (Levesque *et al.* 2010) and includes 196 members in *Ph. infestans*, 100 members in *Phytophthora sojae*, 19 members in *Phytophthora ramorum* and 26 members in *P. ultimum* (Haas *et al.* 2009; Levesque *et al.* 2010). The *P. ultimum* CRN proteins all share a modified version of the motif seen in *Phytophthora* CRN proteins, LXLYLA[RK]. Phylogenetic analysis of the *P. ultimum* CRN proteins reveals that they are more divergent and predominantly basal to the more *Phytophthora* CRN proteins (Levesque *et al.* 2010). L2C566 (GenBank: EV245135) also contains the LXLYLA[RK] motif identified in *P. ultimum*, (Fig 2) indicating, that this may be a *Pythium* version of the CRN motif, from which the *Phytophthora* motif later diverged. Library L1 was devoid of CRN-like sequences.

RXLR effectors
The genomes of the fully sequenced members of the *Phytophthora* genus, *Phytophthora infestans*, *Phytophthora sojae* and *Phytophthora ramorum* contain large numbers of predicted effector molecules with the amino terminal domain containing the RXLR and dEER motifs that are thought to mediate entry into host cells (Whisson *et al.* 2007; Grouffaud *et al.* 2008; 2010; Haas *et al.* 2009). We were interested to see if putative RXLR effectors were present amongst the *Pythium oligandrum* ESTs. Initial searches of the *P. oligandrum* ESTs identified three potential RXLR effectors. Two of these were predicted to have N-terminal signal anchor sequences, suggesting they are not secreted into the extracellular milieu. A nonanchored candidate, L2C559

GenBank: EV246291, displayed similarity (E value $= 2e^{-80}$) to a predicted protein from the *Ph. infestans* genome database (protein ID PITG_09585), which was annotated as a putative secreted RXLR effector peptide (Haas *et al.* 2009). L2C559 and PITG_09585 were predicted to be tetratricopeptide-containing proteins, a motif involved in protein—protein interactions. PITG_09585 contained a KDEL ER retention motif at the C-terminus, although it is possible that this motif could be masked and remains nonfunctional. The top BLAST hits to L2C559 in the *Ph. sojae* ad *Ph. ramorum* databases also have C-terminal KDEL motifs thereby suggesting these proteins are all retained in the ER. The remaining two potential RXLR effectors, L1C102 GenBank: EV244253 and L1C28 GenBank: EV243691 had close homologues in the public oomycete databases, but none of these had an RXLR motif in the expected region.

Pythium ultimum predicted YXSL[KR] effectors
In the absence of candidate RXLR effectors in the *P. ultimum* genome, Levesque *et al.* (2010) identified a group of secreted proteins with an amino terminal YXSL[KR] motif as a new class of candidate oomycete effector proteins. Searches of our dataset revealed the presence of an amino terminal YXSL[KR] in two sequences, Contig113 Genbank: EV244817 and L2C381 Genbank: EV246952. However, Genbank: EV244817 was not predicted to be secreted. L2C381 Genbank: EV246952 had significant similarity to the C-terminal domain of an E3 Ubiquitin ligase in *P. ultimum* (PYU1_T000045; 4 e -09). However, L2C381 Genbank: EV246952 was lacking the 5′ region encoding the start of the predicted ORF, and therefore it was not possible to accurately predict the presence of a signal peptide for secretion.
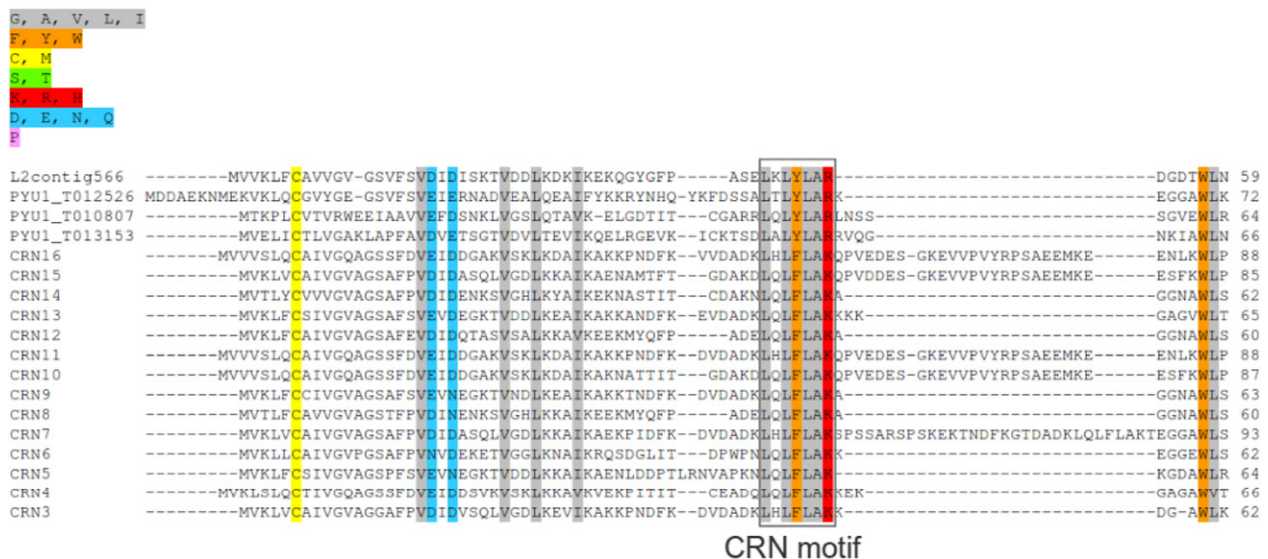

Fig 2 − Multiple alignment of L2C566 with *Phytophthora infestans* CRN-like sequences. The N-terminus of the conceptual translation of *P. oligandrum* L2C566 (GenBank: EV245135) was aligned with *Ph. infestans* and *P. ultimum* CRN-like sequences to show conservation of the signal peptide sequence and the 'LFLAK' or 'LYLAR/K' motif. Multiple alignment was carried out using ClustalW. GenBank accessions for *Ph. infestans* sequences: CRN3 GenBank: AAY43397, CRN4 GenBank: AAY43398, CRN5 GenBank: AAY43399, CRN6 GenBank: AAY43400, CRN7 GenBank: AAY43401, CRN8 GenBank: AAY43402, CRN9 GenBank: AAY43403, CRN10 GenBank: AAY43404, CRN11 GenBank: AAY43405, CRN12 GenBank: AAY43406, CRN13 GenBank: AAY43407, CRN14 GenBank: AAY43408, CRN15 GenBank: AAY43409, CRN16 GenBank: AAY43410. *P. ultimum* sequences were downloaded from the genome website at http://pythium.plantbiology.msu.edu/.

NLP's

A single EST from L1 (GenBank: EV243877), appeared to be a member of the family of plant elicitors, the NLPs . The NLPs are defined by an NPP1 domain (IPR008701). NPP1 domain-containing proteins are thought to behave as virulence factors. NLPs may also act as Microbial Associated Molecular Patterns (MAMPs), inducing innate immune responses in dicotyledonous plants (Qutob *et al.* 2006). GenBank: EV243877 had no significant similarity to sequences within the *Pythium ultimum* genome, but was similar to an NLP protein from *Pectobacterium atrosepticum* (GenBank: YP_051177.1, E value = $4e^{-42}$). According to Gijzen & Nurnberger's classification (Gijzen & Nürnberger 2006), GenBank: EV243877 is a type II NLP due to the presence of four conserved cysteine residues (Fig 3.) All other NLPs found within the Oomycetes to date are type I, which are characterised by the presence of just two conserved cysteines. A frame-shift in GenBank: EV243877 at position 452 was predicted by the Genio online server (http://www.biogenio.com/frame/), and resulted in a predicted truncated protein product, relative to characterised NLPs, of 139 amino acids. This frame-shift was caused by an apparent deletion. The translational frame was restored *in silico* by the addition of two random bases. This resulted in a predicted protein of 247 residues, which aligned well with other NLPs, apart from an in frame deletion found only in GenBank: EV243877 (Fig 3). Primers were designed to amplify the whole ORF, including the predicted stop downstream of the frame-shift, from genomic DNA. After cloning and sequencing of GenBank: EV243877 from gDNA and cDNA, it was found that this deletion is indeed present in the genome, and that this gene is intronless (data not shown).

The same primers were used in PCR reactions using cDNA from other *Pythium oligandrum* strains to identify homologues. PCR products of about 800 bp from *P. oligandrum* strains 7 and CBS 200.184 were cloned into the *Sma*I site of the pUC19 plasmid.

The clones were sequenced and aligned with GenBank: EV243877. The alignment showed that GenBank: EV243877 had a 44 bp deletion relative to strain 7 (Supplemental Fig S3) and the same deletion was present relative to a sequence amplified from *P. oligandrum* CBS 200.184. These data show that several *P. oligandrum* strains possess type II NLPs. The deletion that was observed in GenBank: EV243877 may be unique to *P. oligandrum* CBS 530.74. To our knowledge, this is the first report of a type II NLP found within the Oomycetes.

Elicitins

Elicitins are a family of structurally related extracellular proteins that induce a hypersensitive cell death (HR) in *Nicotiana* spp. (Kamoun *et al.* 1997, 1998a, 1998b) and may also have virulence functions (Kamoun 2006). We identified five putatively secreted unigenes that were highly similar to elicitins and elicitin-like proteins from *Pythium ultimum* and *Phytophthora* spp. Contig2 (GenBank: EV244643) shared 99 % identity with *Pythium oligandrum* elicitin POD-1 (GenBank: Q1ESR5), Contig15 GenBank: EV246257 was identical to oligandrin-D1 previously identified from *P. oligandrum* strain MMR2 (Masunaka *et al.* 2010).

POD-1 is present in *P. oligandrum* cell walls, and induces defence responses, but not necrosis, in sugar beet (Takenaka *et al.* 2006). L2C1 GenBank: EV244523 was similar to *Phytophthora sojae* elicitin SOL13D (GenBank: ABB56009, E value = 0.017). L2C67 GenBank: EV244529 was similar to putative elicitin from *Phytophthora infestans* (GenBank: XP_002897306, E value $1e^{-27}$). In total, seven elicitin-like proteins have now been identified from *P. oligandrum*, including those from previous studies. A phylogenetic reconstruction of representative members of the elicitin (ELI), and elicitin-like (ELL) protein family, taken from Jiang *et al.* (2006), was carried out along with the seven *P. oligandrum* members (Fig 4.). L2C67 (GenBank: EV244529) clusters with the ELL2 group. L2C1 (GenBank: EV244386) is the most divergent
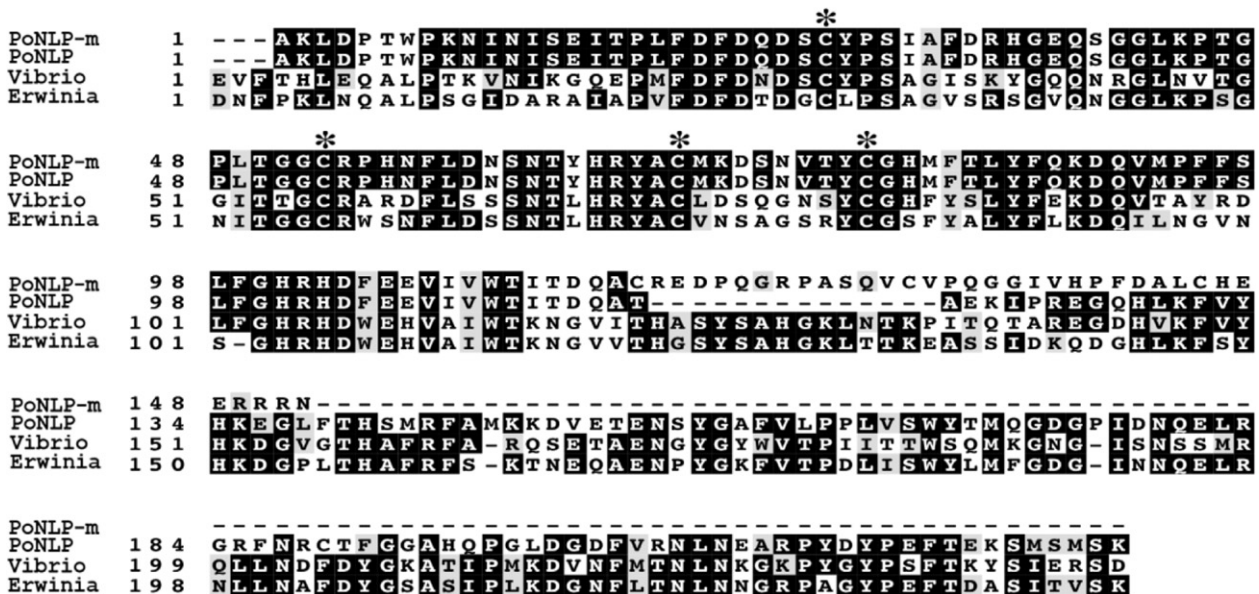

Fig 3 – Alignment of *Pythium oligandrum* NLP and bacterial homologues. Protein alignment of GenBank: EV243877 (PoNLP) and PoNLP-m, which shows the sequence with the frame-shift removed by the addition of two extra bases at the site of a predicted frame-shift mutation. Also in the alignment are *Vibrio* (GenBank: Q93IK1 and *Erwinia* GenBank: Q2XT34) homologues. Asterisks represent the positions of conserved cysteine residues. Alignment was performed with TCOFFEE.
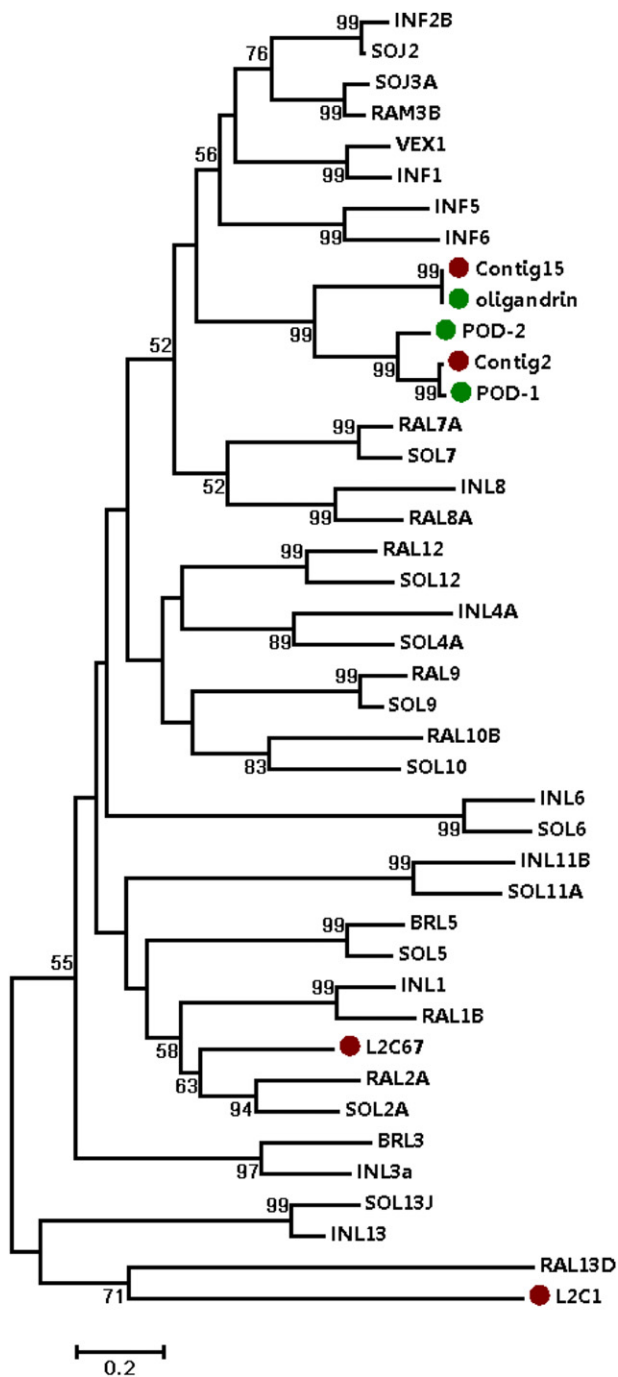
and clusters with the ELL13 group, and is predicted to contain a C-terminal GPI anchor sequence by big-PI Predictor (Eisenhaber *et al.* 1999) and GPI-SOM (Fankhauser & Maser 2005) Contig15 and Contig2 cluster very closely with, and may be orthologous to, oligandrin and POD-1 respectively.

Other putative elicitors

An L2 Contig, L2C437 (Genbank: EV245712) was similar to CBEL, (Genbank: CAA65843; 6e -78) a cellulose binding (CB), elicitor of defence in plants (E) and lectin-like (L) protein from *Phytophthora parasitica*, which is involved in cell wall deposition and adhesion to cellulose (Gaulin *et al.* 2002). L2C437 (Genbank: EV245712) contains an Apple_Factor_XI_like domain (cd01100) a cellulose binding domain (smart00236) and PAN domain (pfam00024). The L2 sequence L2C100 (Genbank: EV243525) was similar to a transglutaminase elicitor family M81B from *Phytophthora infestans* (Genbank: AAP74660; 3 e^{-46}). Other sequences with putative effector or elicitor roles included transcripts similar to cyclophilins (Table 3).

Defence and counter-defence transcripts

Library L2 contained three contigs that were predicted to encode extracellular kazal protease inhibitor domain-containing sequences. L2C265 (GenBank: EV245133) was predicted to contain three kazal_1 inhibitor domains (Interpro domain IPR002305) and was similar to a *Pythium ultimum* agrin-like protein PYU1_T000142 (5 e^{-51}). L2C453 (GenBank: EV245779) was predicted to encode two kazal_2 inhibitor domains (Interpro domain IPR011497), and was similar to the *P. ultimum* four domain protease inhibitor PYU1_T005024 (5.6e^{-36}). L1C16 (Genbank: EV243682) was composed of two putative kazal_1 inhibitor domains (Interpro domain IPR002305) and was similar to a *P. ultimum* agrin-like protein PYU1_T000142 (8.7e^{-47}). Searches of the NCBI non-redundant database revealed all three putative protease inhibitors were also similar to the haemocyte kazal-type protease inhibitor from the tiger shrimp *Penaeus monodon* (GenBank: AY267200). L2C283 (GenBank: EV244785) contained one predicted kazal_1 domain, but was shown by BLASTP to be weakly similar to the mini-agrin precursor from *Mus musculus* (GenBank: AAX09643, E value = 4e^{-06}). Another putative protease inhibitor, encoded by L2C337 (GenBank: EV244419) was identified from L2 with a predicted cystatin domain (Interpro domain IPR000010) and similarity to the cystatin-like cysteine protease inhibitor EPC2B from *Phytophthora infestans* (Genbank: AAY21183; 1e^{-17}).

ABC transporter-related homologues, which may be involved in actively transporting toxic compounds out of the cytoplasm or in the secretion of virulence factors, were identified in both libraries. Other sequences with possible defence-related functions include xenobiotic reductases, and proteins involved in the detoxification of reactive oxygen species, such as glutathione transferases, and thioredoxin peroxidase (Table 3).

Putative cell wall proteins

Tyrosine-rich proteins

We identified a group of very similar transcripts predicted to encode tyrosine-rich proteins that were between 86 and 110 amino acids in length, which we have called PoSTR1-5 (*Pythium*

Fig 4 – Phylogenetic analysis of *Pythium* and *Phytophthora* elicitin and elicitin-like sequences. Phylogenetic tree of *Phytophthora* and *Pythium* elicitin and elicitin-like sequences. The evolutionary history was inferred using the Neighbour-Joining method in MEGA3. Evolutionary distances were computed using the Poisson correction method (number of amino acid substitutions per site). Node values are bootstrap values from 1000 replicates. Root was placed at midpoint. Red and green dots mark *P. oligandrum* sequences identified in this or other studies, respectively. Bootstrap values lower than 50 % are not shown.

oligandrum <u>S</u>mall <u>T</u>yrosine <u>R</u>ich) PoSTR1 (GenBank: EV245847), PoSTR2 (GenBank: EV244654), PoSTR3 (GenBank: EV245146), PoSTR4 (GenBank: EV245340), PoSTR5 (GenBank: EV244632). The predicted proteins were highly rich in tyrosine and glycine residues, characteristics of cell wall and extracellular matrix proteins. The PoSTR family are similar to a group of glycine-rich proteins from *Pythium ultimum* (as well as to the *Phytophthora infestans* M96 family that is comprised of 22 highly similar proteins); PoSTR5 was 46 % similar to a region of M96-4 (GenBank: Q2Q570), although the M96 proteins are larger at approximately 300 amino acids. The M96 genes encode proteins rich in tyrosine, glycine, and serine, and most are induced during mating. It is thought that they may be constituents of the *Ph. infestans* oospore cell wall or act as an adhesive between the mating gametangia (Cvitanich *et al.* 2006). STR5 was also 48 % similar to sexually-induced P48 eggshell protein of *Schistosoma mansoni* (GenBank: AAA29908) (Chen *et al.* 1992).

Discussion

A protocol for the first successful genetic transformation of the oomycete *Pythium oligandrum* has been developed. Using a PEG/CaCl$_2$ and liposome-mediated cotransformation protocol, we were able to integrate a cassettes containing *gfp* as a visible marker expressed under the control of the *ham34* promoter and terminator sequences, and either *nptII* or *hpt* as selectable markers. The expression of the transformed *gfp* gene could be confirmed by direct observation with fluorescent microscopy. Green fluorescence was detected in living cells. Similar observations have been made with *Phytophthora palmivora* and *Phytophthora ramorum* transformed with *ham34* promoter regulated *gfp* (van West *et al.* 1999a; Riedel *et al.* 2009) and *Phytophthora parasitica* var. *nicotianae* transformed with an *hsp70* promoter regulating *gfp* expression (Bottin *et al.* 1999). Thirty-eight percent of geneticin resistant and 54 % of hygromycin resistant transformants showed *gfp* expression using fluorescent microscopy. Transformation of *Phytophthora* species with *gfp* resulted in similar (41 % van West *et al.* 1999a), lower (13 % Bottin *et al.* 1999; Riedel *et al.* 2009) or higher (85 % Si-Ammour *et al.* 2003) percentages of GFP fluorescent transformants. These differences in transformation efficiency may reflect genotypic differences that have been reported to influence oomycete transformation efficiencies (Si-Ammour *et al.* 2003).

Having produced *P. oligandrum* strains that express *gfp* we were able to visualise the interaction between the mycoparasitic *P. oligandrum* and *Phytophthora infestans* as a host. We were able to clearly distinguish the two different oomycete hyphae using fluorescent microscopy, providing evidence of a physical interaction between the two hyphae. *Gfp* expressing *P. oligandrum* strains represent a valuable resource for future microscopic studies of mycoparasitic oomycete interactions.

Similar to previous reports of *P. oligandrum* behaving as a mycoparasite towards various fungi and oomycetes (Deacon 1976; Berry *et al.* 1993; Picard *et al.* 2000b), we found that *Ph. infestans* is a host for *P. oligandrum*. The *gfp* expressing *P. oligandrum* strains allowed us to clearly observe coiling around *Ph. infestans* hyphae along with morphological changes and ultimately host lysis. These observations adds

another host to the large range of fungi and oomycetes that *P. oligandrum* is able to parasitise.

This study also represents the first pilot sequencing project published from the oomycete *P. oligandrum*. Two *P. oligandrum* cDNA libraries were made: one from mRNA isolated from vegetative mycelia of *P. oligandrum*, and the other from mRNA isolated from *P. oligandrum* interacting with heat-killed *Ph. infestans* hyphae. A total of 1219 unigenes were obtained. This initial survey of the *P. oligandrum* transcriptome, with a relatively limited amount of sequence data, has already uncovered a wealth of information. The generated sequence data will facilitate molecular biology studies of *Pythium* species and in particular mycoparasitic oomycetes.

The identities of the most abundant transcripts in both libraries were somewhat unexpected, being represented by previously unreported transcripts. Often the most highly represented sequences in cDNA sequencing projects derive from housekeeping genes such as those involved in protein expression (Pappas *et al.* 2005; Akao *et al.* 2007; Baker *et al.* 2007). The most abundant transcript from the L1 library is probably a noncoding transcript. Recently a large number of infection-specific noncoding transcripts were identified in a *Ph. infestans* suppression subtractive hybridisation (SSH) cDNA library (Avrova *et al.* 2007). It was suggested that such transcripts are produced by the action of enhancers recruiting the Polymerase II machinery, which then is delivered to downstream promoters forming noncoding transcripts in the process (Ling *et al.* 2005; Avrova *et al.* 2007).

The most abundant transcripts from L2 were similar to glycine-rich proteins from *Pythium ultimum* and sexual-specific transcripts from *Ph. infestans* that encode the M96 protein family (Cvitanich *et al.* 2006). They were also similar to the P48 eggshell protein from *Schistosoma mansoni*. Cvitanich *et al.* (2006) also noted similarity of M96 to P48, but they ascribed this to the low complexity of the proteins and not to shared homology. Based on expression profile and amino acid content, M96 was proposed to be either an adhesive between gametangia (sexual structures), or an oospore wall structural protein. Because of the high tyrosine content of the predicted M96 proteins Cvitanich *et al.* (2006) hypothesised that they could form higher order structures by forming intermolecular cross-links.

The oomycete cell wall consists predominantly of (1–3)-β-D-glucans, (1–6)-β-D-glucans, with small amounts of cellulose playing important structural roles (Grenville-Briggs *et al.* 2008). Proteins make up around 10 % of the oomycete cell wall (Meijer *et al.* 2006) and may be differentially expressed depending on developmental stage (Grenville-Briggs *et al.* 2010). However, oospore specific cell wall proteins have not yet been identified from oomycetes. Oospores may represent an important inoculum source for many oomycetes (Jeger *et al.* 1998; Schmitthenner 1999; Dyer & Windels 2003; Rossi *et al.* 2008; Brurberg & Nordskog 2009) and therefore future studies characterising these proteins may be useful in the development of novel control strategies.

The interaction library contained many *P. oligandrum* oospores, which is in line with these sequences being oospore specific. However, none of these sequences were present in the L1 library, which also contained oogonia. It is possible that the host-derived cues triggered an advanced stage of sporulation in the L2 cultures.

We were interested in identifying transcripts that encoded for secreted proteins that may function as extracellular effectors in mycoparasitic interactions. To increase the production of mycoparasitism-related transcripts, we created one library from *P. oligandrum* grown in the presence of dead *Ph. infestans* since the presence of host material, even when dead, can induce effector gene expression in mycoparasites, (Mach *et al.* 1999; McQuilken & Gemmell 2004). However the size of the current study does not allow us to make statistical comparisons between the two libraries. Further gene expression studies will be required to assign roles to these newly-discovered genes.

Over the past decade the number of effector proteins identified in plant-pathogenic oomycetes have steadily increased, and now includes a wide range of degradative enzymes, enzyme inhibitors, toxins, and plant defence elicitors, (reviewed by Oliva *et al.* 2010), many of which have potential representatives in the libraries described here.

Polysaccharide-degrading enzymes are deployed as effector molecules by both plant pathogens and mycoparasites (e.g. as reported in Klemsdal *et al.* 2006; Heller & Thines 2009; King *et al.* 2011) and have the potential for commercial exploitation. There were 16 contigs, based on BLAST results and SignalP analysis that probably encode secreted polysaccharide-degrading enzymes including various glucanases and pectinases (Table 3). Four sequences were similar to Cell 5A 1,4-beta-glucanase (cellulase) from *Phytophthora* species. One of these cellulases was predicted to contain a single transmembrane helix. This could indicate that this cellulase is involved in cell wall synthesis or restructuring as shown for membrane-bound cellulases in *Arabidopsis* (Nicol *et al.* 1998). Two sequences with similarity to pectate lyase were present in L2. To our knowledge, unlike plants *Ph. infestans* does not contain pectin in the cell wall. The presence of these transcripts in the interaction library may be a result of general effector gene upregulation in response to starvation or host presence and not in response to *Ph. infestans per se*. Alternatively, the sequence in question may only resemble a pectate-degrading enzyme, and could have some other role, may be as a degrader of other oomycete polysaccharides.

Some of predicted polysaccharide-degrading enzymes identified in these libraries could have biotechnological applications, as is the case with many enzymes from mycoparasitic *Trichoderma* spp. Several enzymes are produced in large scale by *Trichoderma* spp., including commercially available cellulases that are widely used to generate protoplasts. Also, genes encoding hydrolytic enzymes, such as chitinase, have been expressed in plants, resulting in protection from fungal parasites (Lorito *et al.* 1998). It would be interesting to see if the polysaccharide-degrading enzymes identified here have high enough activities and stability to be commercially exploitable.

Sequences similar to CRN-like proteins from *Ph. infestans* were identified in L2. It was suggested recently that the CRN-like N-terminal LXLFLAK motif may act as a host targeting signal (Haas *et al.* 2009). An alignment of L2C556 (GenBank: EV245135) with the *Ph. infestans* CRN-like sequence showed that L2C556 did not have the LXLFLAK motif, but instead contained the *P. ultimum* LXLYLA[RK] CRN-motif variant. CRN sequences have been identified in all plant pathogenic oomycetes sequenced to date and therefore may represent ancient and important pathogenicity determinants.

As previously described, the *P. oligandrum* elicitin-like protein, oligandrin, when applied exogenously to plants induces resistance to *P. parasitica* without inducing a hypersensitive response (Picard *et al.* 2000a). This type of response to a *P. oligandrum* elicitor may in part explain the reciprocal nature of *P. oligandrum*—plant interactions. Proteinaceous elicitors are used agriculturally to induce resistance in plants. Harpin proteins, for instance, produced by pathogenic gram-negative bacteria, are commercially available as a foliar treatment of plants, which induces disease resistance and increases crop yield (Wei *et al.* 1992). Elicitors derived from mutualists such as *P. oligandrum* could be of interest if they prove to be less phytotoxic than pathogen elicitors. Therefore it will be interesting to identify *P. oligandrum* homologues of pathogen elicitors and to characterise elicitor activity and stability of these proteins for potential commercial use.

A type II NLP similar to a sequence from *Erwinia carotovora* was present in L1. NLPs induce plant defence responses as well as acting as phytotoxins (Qutob *et al.* 2006). GenBank: EV243877 represents the first type II NLP identified in an oomycete, all others being of type I. GenBank: EV243877 was predicted to encode a truncated protein relative to its bacterial homologues. Sequencing of a GenBank: EV243877 homologues from two other *P. oligandrum* strains showed that this truncation was due to a deletion event, which resulted in a frameshift and the introduction of a premature stop codon. It would be interesting to see if other strains have this deletion, and whether the presence or absence of this deletion is associated with altered reactions of host plants.

Pythium oligandrum's ability to colonise plant tissues without inducing a hypersensitive response could be conferred by an ability to subvert normal cellular defence responses. This could be achieved by translocating proteins across the host plasma membrane to directly interfere with cellular processes in the same way plant pathogenic oomycetes and fungi are thought to do (Manning & Ciuffetti 2005; Kemen *et al.* 2005; Whisson *et al.* 2007). The RXLR motif of a large number of *Phytophthora* secreted effector proteins is thought to aid entry of the protein into host cells; and has been demonstrated for the RXLR-avirulence protein Avr3a (Whisson *et al.* 2007). Recently an RXLR protein, SpHtp1 was characterised from *Saprolegnia parasitica*, which is a fish pathogenic Oomycete. This putative effector protein is also able to translocate into fish cells (van West *et al.* 2010). We therefore searched for RXLR motifs in the predicted secreted proteins derived from the current ESTs. Only one sequence was classified as a possible RXLR-containing sequence. All others were excluded due to prediction of retention in the ER lumen or membrane, as well as alignments with *Phytophthora* spp. homologues. We were also unable to conclusively identify putative secreted proteins containing the *P. ultimum* YSXL[RK] motif in our limited dataset. Since RXLR proteins were not identified in the recently sequenced *P. ultimum*, it could be that *Pythium* species do not contain RXLR proteins. However, it is possible that the YXSL[Rk] motif identified in *P. ultimum* functions as a translocation motif in *Pythium* species. It would be informative to obtain transcripts from *P. oligandrum* interacting with plant hosts, as potential RXLR, or YXSL[RK]

encoding genes would most likely be expressed during these interactions.

Pythium oligandrum, along with a handful of other *Pythium* species, is unusual among the oomycetes in having multiple hosts that span several kingdoms of life. Whether the potential effector-encoding transcripts identified here are active in enabling the establishment of a parasitic interaction with fungi and oomycetes, or are deployed in order to establish mutualistic/commensal interactions with plants, or both, remains unknown. Future work to functionally characterise these gene products could shed light on these questions.

Acknowledgements

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.funbio.2011.09.004.

REFERENCES

Akao T, Sano M, Yamada O, Akeno T, Fujii K, Goto K, Ohashi-Kunihiro S, Takase K, Yasukawa-Watanabe M, Yamaguchi K, Kurihara Y, Maruyama J, Juvvadi PR, Tanaka A, Hata Y, Koyama Y, Yamaguchi S, Kitamoto N, Gomi K, Abe K, Takeuchi M, Kobayashi T, Horiuchi H, Kitamoto K, Kashiwagi Y, Machida M, Akita O, 2007. Analysis of expressed sequence tags from the fungus *Aspergillus oryzae* cultured under different conditions. *DNA Research* **14**: 47–57.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.

Avrova AO, Whisson SC, Pritchard L, Venter E, De Luca S, Hein I, Birch PRJ, 2007. A novel non-protein-coding infection-specific gene family is clustered throughout the genome of *Phytophthora infestans*. *Microbiology* **153**: 747–759.

Baker ML, Indiviglio S, Nyberg AM, Rosenberg GH, Lindblad-Toh K, Miller KD, Papenfuss AT, 2007. Analysis of a set of Australian northern brown bandicoot expressed sequence tags with comparison to the genome sequence of the South American grey short tailed opossum. *BMC Genomics* **8**: 50.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S, 2004. Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology* **340**: 783–795.

Benhamou N, Rey P, Cherif M, Hockenhull J, Tirilly Y, 1997. Treatment with the mycoparasite *Pythium oligandrum* triggers induction of defense-related reactions in tomato roots when challenged with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Phytopathology* **87**: 108–122.

Benhamou N, Rey P, Picard K, Tirilly Y, 1999. Ultrastructural and cytochemical aspects of the interaction between the mycoparasite *Pythium oligandrum* and soil-borne plant pathogens. *Phytopathology* **89**: 506–517.

Berry LA, Jones EE, Deacon JW, 1993. Interaction of the mycoparasite *Pythium oligandrum* with other *Pythium* species. *Biocontrol Science and Technology* **3**: 247–260.

Bottin A, Larche L, Villaba F, Gaulin E, Esquerre-Tugaye M-T, Rickauer M, 1999. Green fluorescent protein (GFP) as gene expression reporter and vital marker for studying development and microbe–plant interaction in the tobacco pathogen *Phytophthora parasitica* var. nicotianae. *FEMS Microbiology Letters* **176**: 51–56.

Bradshaw-Smith RP, Whalley WM, Craig GD, 1991. Interactions between *Pythium oligandrum* and the fungal footrot pathogens of peas. *Mycological Research* **95**: 861–865.

Brozova J, 2002. Exploitation of the mycoparasitic fungus *Pythium oligandrum* in plant protection. *Plant Protection Science* **38**: 29–35.

Brurberg MB, Nordskog B, 2009. The role of oospores in the epidemiology of potato late blight. *Acta Horticulturae* **834**: 61–68.

Caten CE, Jinks L, 1968. Spontaneous variability of single isolates of *Phytophthora infestans* I, cultural variation. *Canadian Journal of Botany* **46**: 329–347.

Chen LL, Rekosh DM, LoVerde PT, 1992. *Schistosoma mansoni* p48 eggshell protein gene: characterization, developmentally regulated expression and comparison to the p14 eggshell protein gene. *Molecular and Biochemical Parasitology* **52**: 39–52.

Chen CM, Hsieh H-J, Hu B-Y, Fu C-H, 2005. Mosquito-killing water molds isolated from soil samples collected in Taiwan. *Pedobiologia* **49**: 585–589.

Cvitanich C, Salcido M, Judelson HS, 2006. Concerted evolution of a tandemly arrayed family of mating-specific genes in *Phytophthora* analyzed through inter- and intraspecific comparisons. *Molecular Genetics and Genomics* **275**: 169–184.

Davanlou M, Madsen AM, Madsen CH, Hockenhull J, 1999. Parasitism of macroconidia, chlamydospores and hyphae of *Fusarium culmorum* by mycoparasitic *Pythium* species. *Plant Pathology* **48**: 352–359.

Deacon JW, 1976. Studies on *Pythium oligandrum*, an aggressive parasite of other fungi. *Transactions of the British Mycological Society* **66**: 383–391.

De Cock AW, Mendoza L, Padhye AA, Ajello L, Kaufman L, 1987. *Pythium insidiosum* sp. nov: the etiologic agent of pythiosis. *Journal of Clinical Microbiology* **25**: 344–349.

Dyer AT, Windels CE, 2003. Viability and maturation of *Aphanomyces cochlioides* oospores. *Mycologica* **95**: 321–326.

Eisenhaber B, Bork P, Eisenhaber F, 1999. Prediction of potential GPI-modification sites in proprotein sequences. *Journal of Molecular Biology* **292**: 741–758.

Fankhauser N, Maser P, 2005. Identification of GPI anchor attachment signals by a Kohonen self-organizing map. *Bioinformatics* **21**: 1846–1852.

Fickett JW, 1992. Recognition of protein coding regions in DNA sequences. *Nucleic Acids Research* **10**: 5303–5318.

Flores A, Chet I, Herrera-Estrella A, 1997. Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene prb1. *Current Genetics* **31**: 30–37.

Gaulin E, Janeau A, Vilalba F, Rickauer M, Esquerre-Tugaye M-T, Bottin A, 2002. The CBEL protein of *Phytophthora parasitica* var nicotianae is involved in cell wall deposition and adhesion to cellulosic substrates. *Journal of Cell Science* **115**: 4565–4575.

Gaulin E, Madoui M-A, Bottin A, Jacquet C, Mathé C, *et al.*, 2008. Transcriptome of *Aphanomyces euteiches*: new oomycete putative pathogenicity factors and metabolic pathways. *PLoS ONE* **3** (3): e1723. doi:10.1371/journal.pone.0001723.

Gijzen M, Nürnberger T, 2006. Nep1-like proteins from plant pathogens: recruitment and diversification of the NPP1 domain across taxa. *Phytochemistry* **67**: 1800–1807.

Grenville-Briggs LJ, Anderson VL, Fugelstad J, Avrova AO, Bouzenzana J, Williams A, Wawra S, Whisson SC, Birch PRJ, Bulone V, van West P, 2008. Cellulose synthesis in *Phytophthora infestans* is required for normal appressorium formation and successful infection of potato. *The Plant Cell* **20**: 720–738.

Grenville-Briggs LJ, Avrova A, Hay R, Bruce C, Whisson S, van West P, 2010. Identification of appressorial and mycelial cell wall proteins and a survey of the membrane proteome of *Phytophthora infestans*. *Fungal Biology* **114**: 702–723.

Grouffaud S, van West P, Avrova AO, Birch PRJ, Whisson SC, 2008. *Plasmodium falciparum* and *Hyaloperonospora parasitica* effector translocation motifs are functional in *Phytophthora infestans*. *Microbiology* **154**: 3743–3751.

Grouffaud S, Whisson SW, Birch PJR, van West P, 2010. Towards an understanding of how RxLR effector proteins are translocated from oomycetes into host cells. *Fungal Biology Reviews* **24**: 27–36.

Haas BJ, Kamoun S, Zody MC, Jiang RHY, Handsaker RE, Cano LM, Grabherr M, Kodira CD, Raffaele S, Torto-Alalibo T, Bozkurt TO, Ah-Fong AMV, Lucia Alvarado L, Anderson VL, Armstrong MR, Avrova A, Baxter L, Beynon J, Boevink PC, Bollmann SR, Bos JIB, Bulone V, Cai G, Cakir C, Carrington JC, Chawner M, Conti L, Costanzo S, Ewan R, Fahlgren N, Fischbach MA, Fugelstad J, Gilroy EM, Gnerre S, Green PJ, Grenville-Briggs LJ, Griffith J, Grunwalk NJ, Horn K, Horner NR, Hu C-H, Huitema E, Jeong D-H, Jones AME, Jones JDG, Jones RW, Karlsson EK, Kunjeti SG, Lamour K, Lui Z, Ma L, MacLean D, Chibucos MC, McDonald H, McWalters J, Meijer HJG, Morgan W, Morris PF, Munro CA, O'Neill K, Ospina-Giraldo M, Pinzon A, Pritchard L, Ramsahoye B, Ren Q, Restrepo S, Roy S, Sadanandom A, Savidor A, Schornack S, Schwartz DC, Schumann UD, Schwessinger B, Seyer L, Sharpe T, Silvor C, Song J, Studholme DJ, Sykes S, Thines M, van de Vondervoort PJI, Vipaporn Phuntumart V, Wawra S, Weide R, Win J, Young C, Zhou S, Fry W, Meyers BC, van West P, Ristanio J, Govers F, Birch PRJ, Whisson SC, Judelson HS, Nusbaum C, 2009. Genome sequence of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* **461**: 393–398.

Hase S, Shimizu A, Nakaho K, Takenaka S, Takahashi H, 2006. Induction of transient ethylene and reduction in severity of tomato bacterial wilt by *Pythium oligandrum*. *Plant Pathology* **55**: 537–543.

Heller A, Thines M, 2009. Evidence for the importance of enzymatic digestion of epidermal walls during subepidermal sporulation and pustule opening in white blister rusts (*Albuginaceae*). *Mycological Research* **113**: 657–667.

Hondo D, Hase S, Kanayama Y, Yoshikawa N, Takenaka S, Takahashi H, 2007. The LeATL6-associated ubiquitin/proteasome system may contribute to fungal elicitor-activated defense response via the jasmonic acid-dependent signaling pathway in tomato. *Molecular Plant–Microbe Interactions* **20**: 72–81.

Huang X, Madan A, 1999. CAP3: a DNA sequence assembly program. *Genome Research* **9**: 868–877.

Huitema E, Smoker M, Kamoun S, 2011. A straightforward protocol for electro-transformation of *Phytophthora capsici* zoospores. *Methods in Molecular Biology* **712**: 129–135.

Jeger MJ, Gilijamse E, Bock CH, Frinking HD, 1998. The epidemiology, variability and control of the downy mildews of pearl millet and sorghum, with particular reference to Africa. *Plant Pathology* **47**: 544–569.

Jiang RH, Tyler BM, Whisson SC, Hardham AR, Govers F, 2006. Ancient origin of elicitin gene clusters in *Phytophthora* genomes. *Molecular Biology and Evolution* **23**: 338–351.

Judelson HS, Coffey MD, Arredondo FR, Tyler BM, 1993. Transformation of the oomycete pathogen *Phytophthora megasperma* f. sp. *glycinea* occurs by DNA integration into single or multiple chromosomes. *Current Genetics* **23**: 211–218.

Judelson HS, Tyler BM, Michelmore RW, 1991. Transformation of the oomycete pathogen, *Phytophthora infestans*. *Molecular Plant–Microbe Interactions* **4**: 602–607.

Kamoun S, van West P, de Jong AJ, Vleeshouwers VGAA, Govers F, 1997. A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. *Molecular Plant–Microbe Interactions* **10**: 13–20.

Kamoun S, van West P, Vleeshouwers VGAA, de Groot KE, Govers F, 1998a. Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. *The Plant Cell* **10**: 1413–1426.

Kamoun S, van West P, Govers F, 1998b. Quantification of late blight resistance of potato using transgenic *Phytophthora infestans* expressing beta-glucuronidase. *European Journal of Plant Pathology* **104**: 521–525.

Kamoun S, 2006. A catalogue of the effector secretome of plant pathogenic oomycetes. *Annual Review of Phytopathology* **44**: 135–162.

Karaca G, Tepedelen G, Belghouthi A, Paul B, 2008. A new mycoparasite, *Pythium lycopersicum*, isolated in Isparta, Turkey: morphology, molecular characteristics, and its antagonism with phytopathogenic fungi. *FEMS Microbiology Letters* **288**: 163–170.

Kemen E, Kemen AC, Rafiqi M, Hempel U, Mendgen K, Hahn M, Voegele RT, 2005. Identification of a protein from rust fungi transferred from haustoria into infected plant cells. *Molecular Plant–Microbe Interactions* **18**: 1130–1139.

King BC, Waxman KD, Nenni NV, Walker LP, Bergstrom GC, Gibson DM, 2011. Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. *Biotechnol Biofuels* **4**: 4.

Klemsdal SS, Clarke JL, Hoell IA, Eijsink VG, Brurberg MB, 2006. Molecular cloning, characterization, and expression studies of a novel chitinase gene (ech30) from the mycoparasite *Trichoderma atroviride* strain P1. *FEMS Microbiology Letters* **256**: 282–289.

Kumar S, Tamura K, Nei M, 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**: 150–163.

Laing SAK, Deacon JW, 1991. Video Microscopic Comparison of Mycoparasitism by *Pythium Oligandrum*, *P. nunn* and an Unnamed *Pythium* Species. *Mycological Research* **95**: 469–479.

Le Berre JY, Engler G, Panabieres F, 2008. Exploration of the late stages of the tomato-*Phytophthora parasitica* interactions through histological analysis and generation of expressed sequence tags. *New Phytologist* **177**: 480–492.

Le Floch G, Rey P, Benizri E, Benhamou N, Tirilly Y, 2003. Impact of auxin-compounds produced by the antagonistic fungus *Pythium oligandrum* or the minor pathogen *Pythium* group F on plant growth. *Plant and Soil* **257**: 459–470.

Levesque CA, Brouwer H, Cano L, Hamilton JP, Holt C, Huitema E, Raffaele S, Robideau GP, Thines M, Win J, Zerillo MM, Beakes GW, Boore JL, Busam D, Dumas B, Ferriera S, Fuerstenberg SI, Gachon CM, Gaulin E, Govers F, Grenville-Briggs L, Horner N, Hostetler J, Jiang RH, Johnson J, Krajaejun T, Lin H, Meijer HJ, Moore B, Morris P, Phuntmart V, Puiu D, Shetty J, Stajich JE, Tripathy S, Wawra S, van West P, Whitty BR, Coutinho PM, Henrissat B, Martin F, Thomas PD, Tyler BM, DeVries RP, Kamoun S, Yandell M, Tisserat N, Buell CR, 2010. Genome sequence of the necrotrophic plant pathogen *Pythium ultimum* reveals original pathogenicity mechanisms and effector repertoire. *Genome Biology* **11**: R73.

Lewis K, Whipps JM, Cooke RC, 1989. Mechanisms of biological disease control with special reference to the case study of *Pythium oligandrum* as an antagonist. In: Whipps JM, Lumdsen RD (eds), *Biotechnology of Fungi for Improving Plant Growth*. Cambridge University Press, Cambridge, UK, pp. 191–217.

Ling J, Baibakov B, Wenhu P, Emerson BM, Tuan D, 2005. The HS2 enhancer of the beta-globin locus control region initiates synthesis of non-coding, polyadenylated RNAs independent of a cis-linked globin promoter. *Journal of Molecular Biology* **350**: 883–896.

Lorito M, Woo SL, Garcia Fernadez I, Colucci G, Herman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zonia A, 1998.

Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proceedings of the National Academy Sciences of the United States of America* **95**: 7860–7865.

Mach RL, Peterbauer CK, Payer K, Jaksits S, Woo SL, Zeilinger S, Kullrig CM, Lorito M, Kubicek CP, 1999. Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. *Applied and Environmental Microbiology* **65**: 1858–1863.

Manning VA, Ciuffetti LM, 2005. Localization of Ptr ToxA produced by *Pyrenophora tritici-repentis* reveals protein import into wheat mesophyll cells. *The Plant Cell* **17**: 3203–3212.

Martin FN, Hancock JG, 1987. The use of *Pythium oligandrum* for the biological control of damping-off caused by *P. ultimum*. *Phytopathology* **77**: 1013–1020.

Masunaka A, Sekiguchi H, Takahashi H, Takenaka S, 2010. Distribution and expression of elicitin-like protein genes of the biocontrol agent *Pythium oligandrum*. *Journal of Phytopathology* **158**: 417–426.

McQuilken MP, Gemmell J, 2004. Enzyme production by the mycoparasite *Verticillium biguttatum* against *Rhizoctonia solani*. *Mycopathologia* **157**: 201–205.

Meijer HJG, van de Vondervoort PJI, Yuan Yin Q, de Koster CG, Klis FM, Govers F, de Groot PWJ, 2006. Identification of cell wall-associated proteins from *Phytophthora ramorum*. *Molecular Plant–Microbe Interactions* **19**: 1348–1358.

Min XJ, Butler G, Storms R, Tsang A, 2005. OrfPredictor: predicting protein-coding regions in EST-derived sequences. *Nucleic Acids Research* W677–W680.

Mohamed N, Lherminier J, Farmer M-J, Fromentin J, Bećno N, Houot V, Milat M-L, Blein J-P, 2007. Defense responses in grapevine leaves against *Botrytis cinerea* induced by application of a *Pythium oligandrum* strain or its elicitin, oligandrin, to roots. *Phytopathology* **97**: 611–620.

Mort-Bontemps M, Fevre M, 1997. Transformation of the oomycete *Saprolegnia monoica* to hygromycin B resistance. *Current Genetics* **31**: 272–275.

Nicol F, His I, Jauneau A, Vernhettes S, Canut H, Hofte H, 1998. A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. *The EMBO Journal* **17**: 5563–5576.

Notredame C, Higgins DG, Heringa J, 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology* **302**: 205–217.

Oliva R, Win J, Raffaele S, Boutemy L, Bozkurt TO, Chaparro-Garcia A, Segretin ME, Stam R, Schornack S, Cano LM, van Damme M, Huitema E, Thines M, Banfield MJ, Kamoun S, 2010. Recent developments in effector biology of filamentous plant pathogens. *Cell Microbiology* **12**: 705–715.

Pappas Jr GJ, Benabdellan K, Zingales B, Gonzales A, 2005. Expressed sequence tags from the plant trypanosomatid *Phytomonas serpens*. *Molecular and Biochemical Parasitology* **142**: 149–157.

Picard K, Ponchet M, Belin J-P, Rey P, Tirily Y, Benhamou N, 2000a. Oligandrin. A proteinaceous molecule produced by the mycoparasite *Pythium oligandrum* induces resistance to *Phytophthora parasitica* infection in tomato plants. *Plant Physiology* **124**: 379–395.

Picard K, Tirilly Y, Benhamou N, 2000b. Cytological effects of cellulases in the parasitism of *Phytophthora parasitica* by *Pythium oligandrum*. *Applied and Environmental Microbiology* **66**: 4305–4314.

Qutob D, Kemmerling B, Brunner F, Kuefner I, Engelhardt S, Gust AA, Luberacki B, Seitz HU, Stahl D, Rauhut T, Glawischnig E, Schween G, Lacombe B, Watanabe N, Lam E, Schlichting R, Scheel D, Nau K, Dodt G, Hubert D, Gijzen M, Nuernberger T, 2006. Phytotoxicity and innate immune responses induced by Nep1-like proteins. *Plant Cell* **18**: 3721–3744.

Raeder U, Broda P, 1985. Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17–20.

Rahimian MK, Banihashemi Z, 1979. A method for obtaining zoospores of *Pythium aphanidermatum* and their use in determining cucurbit seedling resistance to damping-off. *Plant Disease Reporter* **63**: 658–661.

Ribeiro WRC, Butler EE, 1995. Comparison of the mycoparasites *Pythium periplocum*, *Pythium acanthicum* and *Pythium oligandrum*. *Mycological Research* **99**: 963–968.

Rice P, Longden I, Bleasby A, 2000. EMBOSS: the European Molecular Biology Open Software Suite. *Trends in Genetics* **16**: 276–277.

Riedel M, Calmin G, Belbahri L, Lefort F, Gotz M, Wagner S, Werres S, 2009. Green fluorescent protein (GFP) as a reporter gene for the plant pathogenic oomycete *Phytophthora ramorum*. *The Journal of Eukaryotic Microbiology* **56**: 130–135.

Rose JK, Hem K-S, Darvill AG, Albersheim P, 2002. Molecular cloning and characterization of glucanase inhibitor proteins: coevolution of a counterdefense mechanism by plant pathogens. *Plant Cell* **14**: 1329–1345.

Rossi V, Cafti T, Giosue S, Bugiani R, 2008. A mechanistic model simulating primary infections of downy mildew in grapevine. *Ecological Modelling* **212**: 480–491.

Sati SC, 1991. Aquatic fungi parasitic on temperate fishes of Kumaun Himalaya, India. *Mycoses* **34**: 437–441.

Schmitthenner AF, 1999. Phytophthora rot of soybean. In: Hartman GF, Sinclair JB, Rupe JC (eds), *Compendium of Soybean Diseases*, 4th edn. APS Press, St Paul, MN, pp. 39–42.

Schornack S, van Damme M, Bozkurt TO, Cano LM, Smoker M, Thines M, Gaulin E, Kamoun S, Huitema E, 2010. Ancient class of translocated oomycete effectors targets the host nucleus. *PNAS* **107**: 17421–17426.

Si-Ammour A, Mauch-Mani B, Mauch F, 2003. Quantification of induced resistance against *Phytophthora* species expressing GFP as a vital marker: beta-aminobutyric acid but not BTH protects potato and *Arabidopsis* from infection. *Molecular Plant Pathology* **4**: 237–248.

Takenaka S, Nakamura Y, Kono T, Sekiguchi H, Masunaka A, Takahashi H, 2006. Novel elicitin-like proteins isolated from the cell wall of the biocontrol agent *Pythium oligandrum* induce defence-related genes in sugar beet. *Molecular Plant Pathology* **7**: 325–339.

Takenaka S, Sekiguchi H, Nakaho K, Tojo M, Masunaka A, Takahashi H, 2008. Colonization of *Pythium oligandrum* in the tomato rhizosphere for biological control of bacterial wilt disease analyzed by real-time PCR and confocal laser-scanning microscopy. *Phytopathology* **98**: 187–195.

Takenaka S, Tamagake H, 2009. Foliar spray of a cell wall fraction from the biocontrol agent *Pythium oligandrum* induces defence-related genes and increases resistance against *Cercospora* leaf spot in sugar beet. *Journal of General Plant Pathology* **75**: 340–348.

Tyler BM, Tripathy S, Zhang X, Dehal P, Jiang RH, Aerts A, Arredondo FD, Baxter L, Bensasson D, Beynon JL, Chapman J, Damasceno CM, Dorrance AE, Dou D, Dickerman AW, Dubchak IL, Garbelotto M, Gijzen M, Gordon SG, Govers F, Grunwald NJ, Huang W, Ivors KL, Jones RW, Kamoun S, Krampis K, Lamour KH, Lee MK, McDonald WH, Medina M, Meijer HJ, Nordberg EK, Maclean DJ, Ospina-Giraldo MD, Morris PF, Phuntumart V, Putnam NH, Rash S, Rose JK, Sakihama Y, Salamov AA, Savidor A, Scheuring CF, Smith BM, Sobral BW, Terry A, Torto-Alalibo TA, Win J, Xu Z, Zhang H, Grigoriev IV, Rokhsar DS, Boore JL, 2006. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science* **313**: 1261–1266.

van West P, Appiah AA, Gow NAR, 2003. Advances in research on oomycete root pathogens. *Physiological and Molecular Plant Pathology* **62**: 99–113.

van West P, de Bruijn I, Minor KL, Phillips AJ, Robertson EJ, Wawra S, Bain J, Anderson VL, Secombes CJ, 2010. The putative RxLR effector protein SpHtp1 from the fish pathogenic oomycete *Saprolegnia parasitica* is translocated into fish cells. *FEMS Microbiology Letters* **310**: 127–137.

van West P, de Jong AJ, Judelson HS, Emons AMC, Govers F, 1998. The *ipiO* gene of *Phytophthora infestans* is highly expressed in invading hyphae during infection. *Fungal Genetics and Biology* **23**: 126–138.

van West P, Kamoun S, van't Klooster JW, Govers F, 1999b. Internuclear gene silencing in *Phytophthora infestans*. *Molecular Cell* **3**: 339–348.

van West P, Reid B, Campbell TA, Sandrock RW, Fry WE, Kamoun S, Gow NAR, 1999a. Green fluorescent protein (GFP) as a reporter gene for the plant pathogenic oomycete *Phytophthora palmivora*. *FEMS Microbiology Letters* **178**: 71–80.

van West P, Vleeshouwers VGAA, 2004. The *Phytophthora infestans*–host interaction. In: Talbot NJ (ed.), *Plant Pathogen Interactions. Annual Plant Reviews*, vol. 11. Blackwell Scientific Publishers, Book chapter. pp. 219–242.

Vijn I, Govers F, 2003. *Agrobacterium tumefaciens* mediated transformation of the oomycete plant pathogen *Phytophthora infestans*. *Molecular Plant Pathology* **4**: 456–467.

Wei ZM, Laby RJ, Zumoff CH, Bauer DW, He SY, Coller A, Beer SV, 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257**: 85–88.

Weiland JJ, 2003. Transformation of *Pythium aphanidermatum* to geneticin resistance. *Current Genetics* **42**: 344–352.

Whisson S, Boevink PC, Moleleki L, Avrova AO, Morales JG, Gilroy EM, Armstrong MR, Grouffaud S, van West P, Chapman S, Hein I, Toth IK, Pritchard L, Birch PRJ, 2007. A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* **450**: 115–118.

Williamson PR, 1994. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. *Journal of Bacteriology* **176**: 656–664.

Win J, Morgan W, Bos J, Krasileva KV, Cano LM, Chaparro-Garcia A, Ammar R, Staskawicz BJ, Kamoun S, 2007. Adaptive evolution has targeted the C-terminal domain of the RXLR effectors of plant pathogenic oomycetes. *The Plant Cell Online* **19** (8): 2349–2369 [cited 2011 Jul 28].

Zdobnov EM, Apweiler R, 2001. InterProScan-an integration platform for the signature-recognition methods in InterPro. *Bioinformatics* **17**: 847–848.