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Phytophthora nicotianae transformants lacking dynein light chain 1 produce non-flagellate zoospores

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ABSTRACT

Biflagellate zoospores of the highly destructive plant pathogens in the genus *Phytophthora* are responsible for the initiation of infection of host plants. Zoospore motility is a critical component of the infection process because it allows zoospores to actively target suitable infection sites on potential hosts. Flagellar assembly and function in eukaryotes depends on a number of dynein-based molecular motors that facilitate retrograde intraflagellar transport and sliding of adjacent microtubule doublets in the flagellar axonemes. Dynein light chain 1 (DLC1) is one of a number of proteins in the dynein outer arm multiprotein complex. It is a 22 kDa leucine-rich repeat protein that binds to the catalytic motor domain of the dynein γ heavy chain. We report the cloning and characterization of DLC1 homologues in *Phytophthora cinnamomi* and *Phytophthora nicotianae* (*PcDLC1* and *PnDLC1*). *PcDLC1* and *PnDLC1* are single copy genes that are more highly expressed in sporulating hyphae than in vegetative hyphae, zoospores or germinated cysts. Polyclonal antibodies raised against PnDLC1 locallized PnDLC1 along the length of the flagella of *P. nicotianae* zoospores. RNAi-mediated silencing of *PnDLC1* expression yielded transformants that released non-flagellate, non-motile zoospores from their sporangia. Our observations indicate that zoospore motility is not required for zoospore release from *P. nicotianae* sporangia or for breakage of the evanescent vesicle into which zoospores are initially discharged.

1. Introduction

Phytophthora is a genus of over 60 species, many of which are destructive plant pathogens. The genus belongs to the class Oomycetes and occurs within the Stramenopile assemblage of protists that share the characteristic of having tripartite tubular hairs (mastigonemes) adorning the anterior flagellum of the heterokont zoospores formed during asexual reproduction (Adl et al., 2005; Patterson, 1989). For most *Phytophthora* species, the motile biflagellate zoospores are the main infective agent that initiates plant disease. Zoospores are able to detect gradients of a variety of compounds including ions, amino acids and sugars and are chemotactically and electrotactically attracted to suitable infection sites (Appiah et al., 2005; Carlile, 1983; Van West et al., 2002). Having reached a potential host, the zoospores encyst during which process the flagella are typically detached, the spores become bonded to the host surface through secretion of adhesive material and a cellulosic cell wall is rapidly deposited (Hardham, 2001, 2007). The important contribution of zoospore motility to pathogen virulence and successful infection has been demonstrated through disruption of normal flagellar function through RNAi silencing of genes encoding G-α-subunit and bZip transcription factor proteins (Blanco and Judelson, 2005; Latijnhouwers et al., 2004).

Phytophthora and other Oomycete zoospores are formed within multinucleate sporangia through the polarisation of organelles and subdivision of the cytoplasm into uninucleate domains (Hardham, 2009; Hyde et al., 1991). In most *Phytophthora* species, once cleavage is complete, the zoospores are released through an exit pore at the site of an apical papilla, the wall of which becomes weakened and distended to form an evanescent vesicle into which the zoospores are emitted. Zoospores swim within the vesicle for a short time until it breaks and they are able to swim away.

Flagella of *Phytophthora* and other Oomycete species have the typical 9 + 2 axonemal structure found in eukaryotic flagella (Ginger et al., 2008; Hardham, 1987; Nicastro et al., 2005). While the proteins in a number of zoospore secretory vesicles and flagellar surface components have been shown to be synthesized during sporangium formation (sporangiogenesis), the flagella themselves are assembled during sporangial cleavage (zoosporogenesis) from two basal bodies that lie adjacent to the pointed end of the pear-shaped nuclei (Cope and Hardham, 1994; Hardham and Hyde, 1997). Studies of the transcriptomes of *Phytophthora infestans*, the cause of late blight of potato, have shown that the expression of specific sets of genes occurs during sporangiogenesis or zoosporogenesis (Judelson et al., 2008, 2009).

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In our studies of the sporulation transcriptomes of two broad host-range *Phytophthora* species, namely *Phytophthora cinnamomi* and *Phytophthora nicotianae*, we identified a gene that showed strong homology to the 22 kDa dynein light chain 1 (DLC1) protein of *Chlamydomonas reinhardtii*, LC1 (Benashski et al., 1999). In *C. reinhardtii*, LC1 is one of 13 proteins that comprise the large (~2 MDa) dynein outer arm attached to the A-tubule of the outer doublet microtubules of the flagellar axoneme (King, 2003; Pfister and Witman, 1982). The outer arm dynein is composed of one to three dynein heavy chains (α, ß, γ) responsible for ATPase/motor activity, and a variable number of intermediate chains and light chains (Wickstead and Gull, 2007). *C. reinhardtii* LC1 is a member of the SDS22+ subclass of the leucine-rich repeat protein family (Kobe and Kajava, 2001) and binds directly to the motor domain of the γ dynein heavy chain and to another uncharacterized 45 kDa axonemal protein (p45) (Benashski et al., 1999; King, 2003). Conformational changes in dynein structure lead to sliding of adjacent microtubule doublets relative to one another to generate flagellar bending or to transport of attached cargo towards the minus end of the microtubules. In the case of the flagellar axoneme, the minus end is that emerging from the basal body.

In the present paper, we describe the structure of the DLC1 genes in *P. cinnamomi* and *P. nicotianae*. We also report a phylogenetic comparison of the DLC1 proteins in *Phytophthora* and other protists, an analysis of the expression of the DLC1 gene during the asexual lifecycle in *P. cinnamomi* and *P. nicotianae* and immunolocalization of the DLC1 protein within *P. nicotianae* flagella. In studies of PnDLC1 function, we show that RNAi disruption of PnDLC1 synthesis in *P. nicotianae* completely inhibits flagella formation and zoospore motility. However, sporangial cleavage occurs as normal and the uninucleate, non-flagellate zoospores are still released from the sporangia, demonstrating unambiguously for the first time that zoospore motility is not required for zoospore release from *P. nicotianae* sporangia or for breakage of the evanescent vesicle into which they are initially released.

2. Materials and methods

2.1. Phytophthora strains and culture conditions

P. cinnamomi (H1000, ATCC 200982) and *P. nicotianae* (H1111, ATCC MYA 141) were grown on V8 nutrient medium as described previously (Hardham et al., 1991; Marshall et al., 2001a). Asexual sporulating mycelium of *P. nicotianae* was obtained by growing cultures on miracloth on 10% V8 agar for 5 days at 25 °C in the dark and then transferring the miracloth disks to 5% V8 broth in 20 mm deep Petri dishes. To induce sporulation, after 12 days growth in V8 broth in the light, fresh V8 broth was added for 24 h before the cultures were transferred to mineral salts solution. The appearance of sporangia was monitored and, in some cases, scored and mycelia harvested at selected times over the next 5 days.

2.2. Screening of cDNA and genomic libraries

A cDNA clone containing the partial sequence of the *PcDLC1* gene was identified by differential screening of an oligo(dT)-primed *P. cinnamomi* cDNA library made from mRNA isolated from hyphae harvested 4 h after induction of sporulation (Weerakoon et al., 1998). Replicates of the arrayed cDNA clones on HyBond N + nylon membranes (GE Healthcare) were hybridized with ^{32}P-labelled cDNA probes synthesized from mRNA isolated from hyphae harvested 4 h after induction of sporulation or from vegetative hyphae. Genomic clones of *PcDLC1* were obtained from a *P. cinnamomi* genomic library constructed in EMBL3 (Weerakoon et al., 1998) by screening under high stringency conditions as described previously (Marshall et al., 2001b) with a ^{32}P-labelled *PcDLC1* cDNA. After three rounds of screening, DNA was isolated from three putative positive *PcDLC1* genomic clones. Restriction digests and hybridization results showed that the three *PcDLC1* genomic clones were identical. One *PcDLC1* genomic clone was selected and digested with *Sac*I and *Eco*RI for subcloning into pBluescript (pBS) and sequencing.

The *PnDLC1* genomic clone was isolated from a *P. nicotianae* BAC genomic library (Shan and Hardham, 2004) by screening seven filters representing the whole library with the ^{32}P-labelled *PcDLC1* cDNA clone according to standard protocols. Genomic DNA from two of three putative positive clones was isolated and restriction digests and hybridization with the *PcDLC1* cDNA probe showed that the clones were identical. DNA from one clone was digested with *Eco*RI and *Bam*HI for subcloning into pBS and sequencing. GenBank accession numbers for *P. cinnamomi* and *P. nicotianae* DLC1 cDNA and genomic clones are pending.

2.3. DNA blots

For DNA blots, 10 μg of genomic DNA was isolated from *P. cinnamomi* and *P. nicotianae* hyphae (Dudler, 1990), digested with *Bam*HI, *Hin*dIII, *Pst*I and *Sac*I (New England Biolabs), electrophoresed on a 1.0% agarose gel and transferred to Hybond-N+ membranes. The blots were probed with the *PcDLC1* cDNA clone labelled with ^{32}P-dCTP using the Megaprime kit (GE Amersham). Signals were detected by exposing the filters to a PhosphoImager screen (Molecular Dynamics, CA).

2.4. DNA sequencing and data analysis

DNA sequencing was done at the Australian Genome Research Facility (Brisbane, Qld). DNA and protein sequence searches were conducted against the non-redundant, expressed sequence tag (EST) and selected genome sequence databases using BLAST programs through the websites of the National Centre for Biological Information (NCBI, http://www.ncbi.nih.gov/BLAST), the *Phytophthora* Functional Genomics Database (http://www.pfgd.org/) and the Joint Genome Initiative (JGI; http://genome.jgi-psf.org/Physo1_1/Physo1_1.home.html). DNA sequences were searched for introns using FGENESH (http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind). For phylogenetic analysis, homologues of DLC1 were retrieved from NCBI and JGI databases. Protein sequence alignments were performed using the Clustal X 1.81 algorithm (Thompson et al., 1997) and BioEdit sequence editor (Hall, 1999). Phylogenetic analyses were carried out with Phylo-win Version 2 (Galtier et al., 1996). A phylogenetic tree was obtained with the maximum parsimony method (Felsenstein, 1985). Structural homology between PcDLC1, PnDLC1 and the *C. reinhardtii* LC1 protein (PDB accession 1DS9) was determined using Deep View/Swiss-PDB Viewer (http://www.expasy.org/spdbv/).

2.5. Quantitative real-time PCR (qPCR)

RNA was isolated from *P. cinnamomi* and *P. nicotianae* zoospores, 3-h germinated cysts and hyphae isolated before and after the induction of sporulation using TRIZOL® reagent (Invitrogen) according to the manufacturer's instructions. RNA was treated with RQ1 DNAse (Promega, Madison, WI, USA) and reverse transcribed using SuperscriptII reverse transcriptase (Invitrogen. Carlsbad, CA, USA) with oligo(dT) primers to produce cDNA according to the manufacturer's instructions. Minus RT controls to check for genomic DNA contamination were conducted by PCR and gel electrophoresis. DNAse-treated *P. cinnamomi* RNA was further purified with an RNeasyMini Kit (Qiagen Pty., Ltd., Doncaster, Victoria, Australia) prior to cDNA production. Levels of *PnDLC1* and

PcDLC1 transcripts were quantified using a Rotor-Gene 3000 real-time cycler (Corbett Life Science, Mortlake, NSW) and FastStart SYBR Green Master (Roche Applied Science, Mannheim, Germany), as described by the manufacturer using the conditions described in Blackman and Hardham (2008). *PnDLC1* transcripts were detected with the primer pair TCGCTCAACACGCTCAAG–GCCACAGTTCCTCCAACG and *PcDLC1* detected with the primers CCTGTCACTTGGTCGGAA–CTCGTAGATGGGGTTGCC. *WS041* (GenBank accession number CF891677), a gene shown to be constitutively expressed throughout *P. nicotianae* and *P. cinnamomi* lifecycle stages (Shan et al., 2004; Yan and Liou, 2006; Blackman and Hardham unpublished results), was used as a normalizing reference gene (*PnWS041* primers: CACGT ACACATGCCCGAGAC–TTCCCATGTAGGCCGAGTATTC; *PcWS041* primers: GCTGACCAACAACGGCACG–GATCACCTCCGTACCTGCG). Amounts of *DLC1* and *WS041* transcripts were calculated using the Comparative Quantification function (qPCR Analysis Software, version 6.1.81, Corbett Research) relative to the transcript levels in a single vegetative hyphae sample and the ratio between the two genes determined as described in Finnegan et al. (2005). A melt curve was examined after each qPCR run to check that primer dimers had not formed.

2.6. Anti-PnDLC1 antibody production and immunofluorescence microscopy

Two New Zealand White rabbits were inoculated with a synthetic peptide with the sequence AKIDGDMVKQTERD conjugated to keyhole limpet haemocyanin (KLH). The 14 amino acid peptide corresponded to the predicted C-terminus of PnDLC1 and PcDLC1 proteins. Pre-immune sera were collected before commencing inoculations. Immune sera were collected after third and subsequent inoculations and tested in enzyme-linked immunosorbent assays. Mouse monoclonal antibodies directed towards PnLpv, PnCpa and PnVsv proteins in zoospore peripheral vesicles (Gautam et al., 1999; Hardham et al., 1986; Hardham and Gubler, 1990) were also used. Immunofluorescence assays were conducted using *Phytophthora* zoospores and mycelia fixed in 4% formaldehyde in 50 mM Pipes buffer as described previously (Cope et al., 1996).

2.7. Vectors for RNAi silencing of PnDLC1

A 343-nucleotide *PnDLC1* coding region (Supplementary Fig. S1) was amplified using forward (PnDLC1dsRNAF1: GGACTAG TCCCCCGGGATGAAGAAGCTGCGG) and reverse (PnDLC1dsRNAR1: CCATCGATGGGCTTGACCATGTCACCGTCGA) primers, digested with *Spe*I and *Cla*I and ligated to a *Cla*I-digested kanamycin linker to produce a hairpin RNAi construct that was ligated into *Spe*I-digested pBS. The *PnDLC1*-kanamycin-*PnDLC1* hairpin fragment was removed from pBS using *Sma*I digestion and ligated into the expression plasmid, pTH210 (Judelson et al., 1993) that had been digested with *Sma*I. In this reaction, the *PnDLC1* RNAi cassette replaces the hygromycin-resistance gene in pTH210 and is under the control of the *B. lactucae* Hsp70 promoter and Ham34 terminator. Inserts were verified by sequencing. BLAST searches of the NCBI, JGI or Broad Institute databases did not retrieve any sequences other than those encoding DLC1 genes.

2.8. Transformation of P. nicotianae and analysis of PnDLC1 RNAi transformants

Protoplasts were generated from *P. nicotianae* encysted zoospores using 20 mg/mL driselase (Sigma) and 0.6 mg/mL cellulase (Sigma) as described previously (Bottin et al., 1999). Protoplasts were co-transformed with the linear blunt-ended *Sca*I-digested *PnDLC1* RNAi construct and the PCR product of pTH210. A polyethylene glycol–$CaCl_2$ treatment regime was used to introduce foreign DNA into *P. nicotianae* protoplasts, and transformants were recovered 5 days after transformation on 10% V8 agar + 100 μg/ml hygromycin. To obtain sporulating mycelium for phenotypic analysis, transformants were grown on miracloth on V8 agar containing 100 μg/ml hygromycin for 7 days, in V8 broth containing 50 μg/ml hygromycin for 14 days and then transferred to mineral salts solution. Sporangial cleavage was induced by washing the cultures in cold distilled water and incubating the plates on a light box at 18 °C. Zoospore release and motility were examined microscopically during the ensuing hour. *PnDLC1* transcript levels were determined in sporulating hyphae of five transformants generated in the presence of the hygromycin-resistance gene and the *DLC1* RNAi constructs, in one transformant generated in the presence of the hygromycin-resistance gene only and in wild-type *P. nicotianae* by qPCR as described earlier. Sporangia numbers were monitored between 0 and 7 days after transfer to mineral salts solution and samples taken at similar stages of sporulation. Between five and eight biological replicates were analysed for each isolate.

3. Results

3.1. Identification of a P. cinnamomi cDNA clone encoding dynein light chain 1

Differential screening of a *P. cinnamomi* cDNA library constructed from mRNA isolated from hyphae harvested 4 h after induction of asexual sporulation led to the identification of a number of cDNA clones that were more highly expressed during early sporulation than in vegetative hyphae. When the ESTs were compared with sequences in GenBank, one showed homology to a gene encoding the flagella outer arm dynein light chain 1 (DLC1). The gene corresponding to this cDNA was named *PcDLC1* for *P. cinnamomi* Dynein Light Chain 1. The *PcDLC1* cDNA produced a full length transcript of ~622 bp, which included a 37-bp untranslated 3′ region upstream of the poly (A+) tail, and encoded a polypeptide with a predicted molecular mass of 20.2 kDa. In GenBank database comparisons, PcDLC1 has the highest similarity to the DLC1 from *C. reinhardtii*, LC1 (GenBank accession number AAD41040) with which it has 52% amino acid identity. Alignment of PcDLC1 with DLC1 homologues from *C. reinhardtii* and other species demonstrates strong sequence preservation especially in the highly conserved leucine-rich repeat regions (Supplementary Fig. S2). Residues thought to be involved in binding to the dynein γ heavy chain in the outer arm complex and to p45, the only other protein known to interact with DLC1 (Horvath et al., 2005; Sakato and King, 2004), are also conserved in PcDLC1. Protein modelling of PcDLC1 based on the *C. reinhardtii* LC1 structure (Wu et al., 1999, 2003; Wu et al., 2000) revealed a similar topology, including the locations of the dynein γ heavy chain-interacting sites within the β-sheet face of the protein and the α-helical regions that interact with p45 (Supplementary Fig. S3).

3.2. DLC1 is a single copy gene in P. cinnamomi and P. nicotianae

Blots of genomic DNA from *P. cinnamomi* and *P. nicotianae* were hybridized with a radiolabeled *PcDLC1* cDNA probe (Fig. 1). There is a restriction site for *Sac*I in *PcDLC1*. The hybridization patterns in both cases are consistent with there being a single copy of the *DLC1* in both *P. cinnamomi* and *P. nicotianae* genomes. Comparison of *PcDLC1* and *PnDLC1* with *P. infestans*, *Phytophthora ramorum*, *Phytophthora sojae* and *Phytophthora capsici* genomic sequence databases revealed that each of these species also contains a single copy of the DLC1 gene.

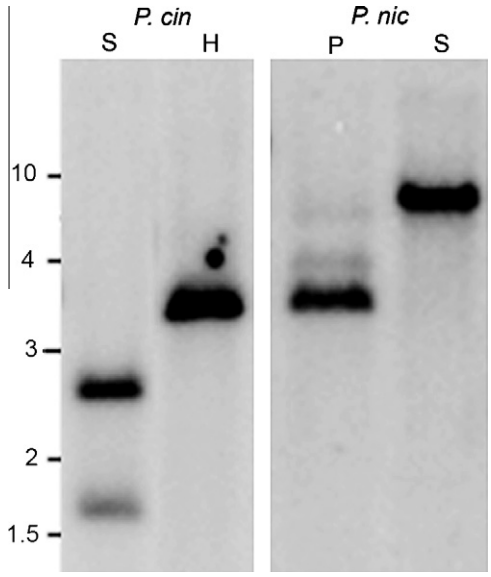

Fig. 1. DNA blot analysis of *PcDLC1* and *PnDLC1*. Genomic DNA from *P. cinnamomi* (*P. cin*) and *P. nicotianae* (*P. nic*) was digested with *Sac*I (S), *Hin*cIII (H) and *Pst*I (P). The filters were hybridized with a ^{32}P labelled probe made using *PcDLC1* cDNA clone. The sizes of the marker bands are indicated at the left in kbp.

3.3. Sequence analysis of PcDLC1 and PnDLC1 genomic clones

The *PcDLC1* genomic clone was isolated from a *P. cinnamomi* EMBL3 genomic library and sequenced. Comparison with the cDNA sequence indicated that the *PcDLC1* genomic clone contained two introns, 240 bp and 64 bp in length (Supplementary Fig. S2), and a 17-bp CT-element 105–122 bp upstream of the ATG. CT-elements are frequently seen in the promoter region of *Phytophthora* genes (McLeod et al., 2004). At the 5′-end of the CT-element is a CTCCTTCT sequence that, apart from one base pair, conforms with the consensus initiator (Inr) sequence, CTCANTCT (Smale and Baltimore, 1989). The initiator site overlaps the transcription start site and is capable of acting alone as a minimal transcription control element in directing transcription at a low level in most eukaryotic genes, including species of *Phytophthora* (Chen and Roxby, 1997). A consensus eukaryotic polyadenylation signal (AATGAA) was observed in the 3′-untranslated regions of the *PcDLC1* genomic clone.

The *PnDLC1* genomic clone was isolated from a *P. nicotianae* BAC genomic library (Shan and Hardham, 2004) by screening with a radiolabelled *PcDLC1* cDNA probe. Sequencing of the *PnDLC1* genomic clone and analysis using FGENESH indicated that this gene also contained two introns, 74 bp and 69 bp in length, and a 17-bp CT-element 86–103 bp upstream of the ATG. *PnDLC1* did not have a consensus eukaryotic polyadenylation signal (AA[TG/A]AA) but did have an AT-rich (TTTTTGTAAA) sequence in the 3′-untranslated region similar to the polyadenylation signal commonly found downstream of the open reading frames of Oomycete genes. Analysis of partial EST and gDNA sequence data from *P. capcisi* indicate that the position but not the length of the second intron is conserved in different *Phytophthora* species.

3.4. Phylogenetic analysis

A maximum parsimony tree was obtained after alignment of PcDLC1 and PnDLC1 amino acid sequences with 19 homologues of DLC1 from a range of eukaryotic protists and representative animal species (Supplementary Fig. S4). The cladogram revealed that DLC1 sequences from *Phytophthora* species clustered with those from the two diatoms, *Aureococcus anophagefferens* and *Thalassiosira pseudonana*. The *Phytophthora* species (Class Oomycetes) and the diatoms belong to the Stramenopile protist taxon (Van de Peer et al., 1996). DLC1 proteins in the Stramenopile species are most closely related to those in the two Alveolate groups, the apicomplexans and the ciliates. DLC1 sequences in the green algae, kinetoplastids and animals are more distantly related to *Phytophthora* DLC1.

3.5. Analysis of PcDLC1 and PnDLC1 expression

Patterns of expression of the *DLC1* gene in *P. cinnamomi* and *P. nicotianae* were analysed using quantitative real-time RT-PCR (qPCR) (Fig. 2). RNA was isolated from vegetative hyphae, sporulating hyphae, zoospores and germinated cysts.

In *P. cinnamomi*, sporulation can be synchronously induced by transferring non-sporulating mycelium (growing in fresh V8 broth) to mineral salts solution (Chen and Zentmyer, 1970). Analysis of the time-course of sporulation revealed that *PcDLC1* expression begins to be up-regulated 6–12 h after induction of sporulation and maximum transcript levels are reached between 12 and 24 h after transfer to mineral salts solution (Fig. 2a). In *P. cinnamomi*, sporangia typically first appear about 8 h after the induction of sporulation (Dearnaley et al., 1996). Levels of *PcDLC1* mRNA were a little higher in zoospores and 3-h germinated cysts (i.e. cysts sampled 3 h after germination) than they were in vegetative hyphae but significantly lower than the levels reached during sporulation.

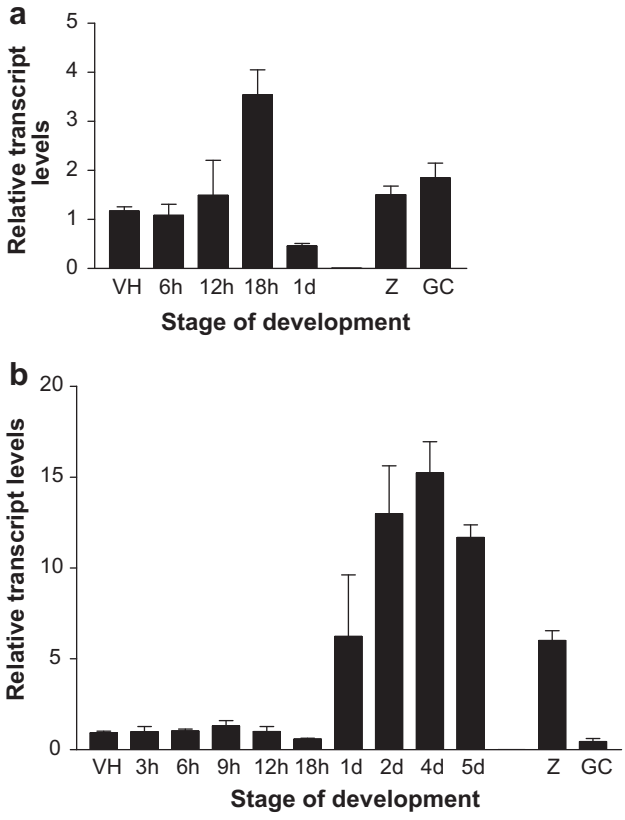

Fig. 2. qPCR analysis of *DLC1* expression in *P. cinnamomi* and *P. nicotianae*. (a) Relative *PcDLC1* transcript levels in *P. cinnamomi* vegetative hyphae (VH), sporulating hyphae sampled at 6 h, 12 h, 18 h and 1 day after transfer to mineral salts solution, zoospores (Z) or cysts sampled 3 h after germination (GC). (b) Relative *PnDLC1* transcript levels in *P. nicotianae* vegetative hyphae (VH), sporulating hyphae sampled at 3 h, 6 h, 9 h, 12 h, 18 h, 1 day, 2 days, 4 days and 5 days after transfer to mineral salts solution, zoospores (Z) or cysts sampled 3 h after germination (GC). DLC1 transcript levels are relative to those of the constitutively expressed *P. nicotianae* gene, *WS041* (Shan et al., 2004; Yan and Liou, 2006). Bars indicate the positive value of the standard error of the mean of three biological replicates.

In *P. nicotianae*, induction of synchronous sporulation is difficult, however, we have found that after obtaining sufficient biomass in nutrient medium, most sporangia will disappear within 24 h when the cultures are transferred to fresh V8 broth (L.M. Blackman and A.R. Hardham, unpublished observations). If the cultures are then washed with mineral salts solution, sporulation will occur in the subsequent 2–5 days. Sporangial counts can be used as an indicator of the progress of sporulation of the culture.

RNA was isolated from *P. nicotianae* mycelia in a time-course after transfer to mineral salts solution. The number of sporangia in the cultures was counted daily. Sporangia first appeared during the first 24 h after transfer to mineral salts solution, increased gradually over the next 2 days and then more rapidly during days 4 and 5. Analysis using qPCR revealed that *PnDLC1* transcript levels in the sporulating cultures were similar to those in vegetative hyphae until about 1 day after transfer to mineral salts solution (Fig. 2b). Thereafter, *PnDLC1* transcript levels increased to reach a maximum between 2 and 5 days after transfer to mineral salts solution. *PnDLC1* transcript levels in zoospores were significantly higher than in vegetative hyphae but the level in 3-h germinated cysts was similar to that in vegetative hyphae.

3.6. Immunolocalization of PnDLC1 in zoospores of P. nicotianae

Polyclonal antisera from two rabbits inoculated with a DLC1-KLH synthetic peptide were tested in immunoblots of *P. nicotianae* zoospore proteins but neither showed a specific reaction with a 20 kDa polypeptide (data not shown). In immunofluorescence assays with *P. cinnamomi* and *P. nicotianae* zoospores, sera from both rabbits labelled the zoospore flagella brightly (Fig. 3a). There was some labelling of the cell body but as polyclonal antisera tend to give relatively high levels of non-specific cytoplasmic labelling in the zoospores, the significance of this labelling is not clear. There was either no or weak background labelling by the pre-immune sera (Fig. 3b).

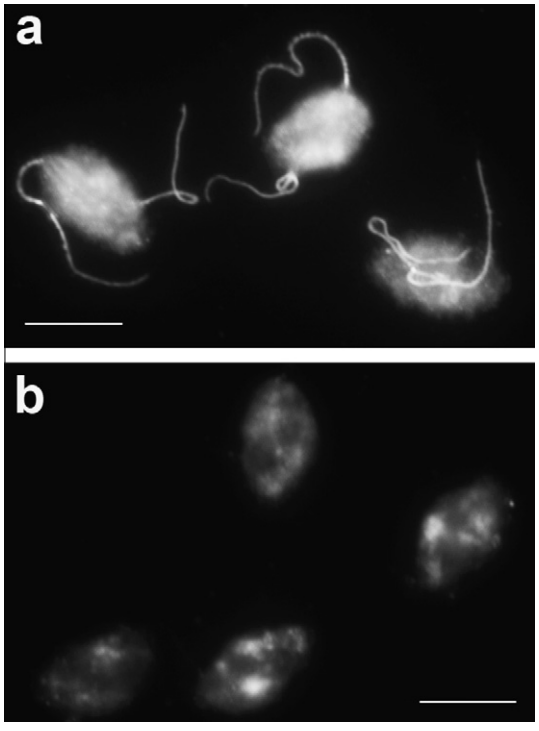

Fig. 3. (a) Zoospores incubated with polyclonal antiserum raised against an 18-amino acid synthetic peptide from the C-terminus of PcDLC1 and PnDLC1. The antibody labels the two flagella of each zoospore. (b) Zoospores incubated with pre-immune antiserum. Zoospore flagella are not labelled. Concentrations of the antisera, image exposure times and digital enhancement were all the same for both images. Bar = 10 μm.

3.7. Analysis of PnDLC1 RNAi transformants

In order to investigate the function of the DLC1 protein, the expression of *PnDLC1* was inhibited through RNAi silencing of the *PnDLC1* gene. In seven co-transformation experiments in which constructs encoding the hygromycin-resistance gene (pTH210) and a *PnDLC1* RNAi silencing cassette were transformed into *P. nicotianae* protoplasts, a total of 331 hygromycin-resistant transformants were isolated. Control experiments in which only the hygromycin-resistance gene construct, pTH210, was present were run in parallel. None of the drug-resistant transformants showed any abnormal phenotypes when grown on V8 agar or V8 broth.

Because expression of *PnDLC1* is up-regulated during sporulation and PnDLC1 forms part of the flagellar axoneme in zoospores, assessment of possible phenotypic changes in the transformants was conducted using sporulating hyphae and zoospores. *P. nicotianae* transformants sporulate poorly when 100 μg/ml hygromycin is included in the V8 broth, however, mycelia will sporulate if grown in the presence of 50 μg/ml of hygromycin and then transferred to mineral salts solution. All drug-resistant transformants were grown in this way before inducing zoospore release from sporangia by rinsing in cold distilled water.

Zoospore release from sporangia was monitored in each transformant from about 30 min after the induction of cleavage. In most drug-resistant transformants, sporangial cleavage and zoospore release and motility were normal but in four transformants, designated PnDLC1#31, PnDLC1#50, PnDLC1#66 and PnDLC1#68, only non-motile zoospores were released from the sporangia. As in wild-type *Phytophthora* cultures (Fig. 4a–c; Supplementary Video 1), in the four *PnDLC1* transformants, zoospores were initially released into an evanescent vesicle derived from the apical sporangial papilla (Fig. 4d–f, Supplementary Video 2). This vesicle subsequently broke but instead of swimming away the zoospores remained immobile. None of the zoospores produced by the PnDLC1#31, PnDLC1#50, PnDLC1#66 or PnDLC1#68 transformants possessed flagella.

Other aspects of zoospore formation, structure and function in the *PnDLC1*-silenced transformants appeared to be normal. Immunolabelling of three categories of zoospore peripheral vesicles in sporulating hyphae, cleaving sporangia, zoospores and cysts, for example, showed that these vesicles were synthesized during sporulation, segregated during zoosporogenesis and secreted during encystment as normal [compare immunolocalisations in Fig. 5 to those in wild-type cells in Cope et al. (1996), Hardham (2005), Hyde and Hardham (1992)]. The water expulsion vacuole in the anterior end of the cells also dilated and contracted normally, with a mean cycle time of 5.02 ± 0.46 s. This compares to a cycle time of 5.7 + 0.71 s in wild-type zoospores (Mitchell and Hardham, 1999).

Zoospores or the cysts derived from them were isolated and used to establish 20 single-zoospore lines from three of the four primary transformants. Analysis of sporangial release and zoospore motility showed that all the single-zoospore lines displayed the same phenotype of non-flagellate, non-motile zoospores. Silencing of the *PnDLC1* gene in these transformants is stable as the lines showed the same phenotype of non-motile zoospores when examined over 2 years after their initial generation.

Using qPCR, levels of *PnDLC1* transcripts were measured in PnDLC1#50, PnDLC1#66 and PnDLC1#68 transformants and compared to levels in wild-type *P. nicotianae* and in a number of other control lines (Fig. 6). These latter control isolates included a drug resistant line that had been transformed with the cassette containing the hygromycin-resistance gene (pTH210) only, and two drug-resistant transformants that had been transformed in the presence

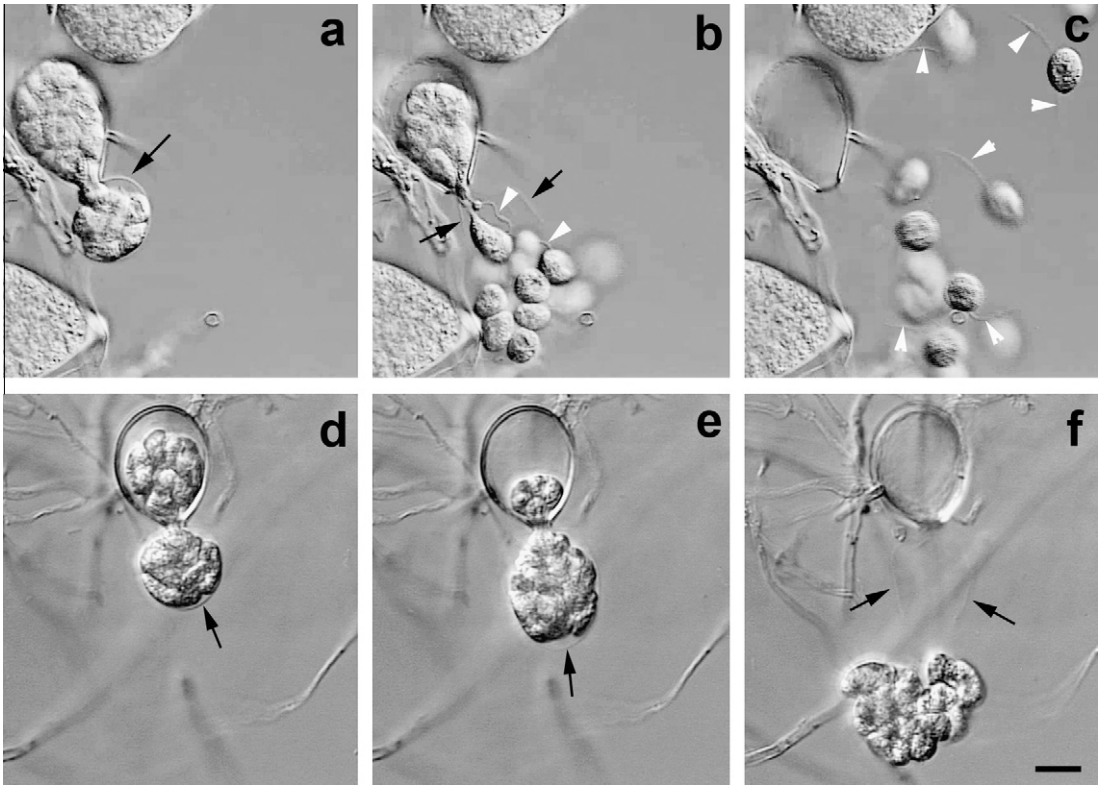

Fig. 4. Zoospore release from *P. nicotianae* sporangia. (a–c) In wild-type *P. nicotianae*, biflagellate zoospores quickly swim away after breakage of the evanescent vesicle (black arrows) into which they have been released following rupture of the apical papilla. (d–f) In the *PnDLC1#68* silenced line, zoospores were released from the sporangia with the same speed as in wild-type cultures. However, as the zoospores lack flagella and are non-motile they do not swim away and the cluster of released zoospores slowly floats away. White arrowheads: flagella. Bar = 10 μm.

of the *PnDLC1* RNAi silencing construct but which did not show the non-motile zoospore phenotype (designated #6 and #41). The results showed that levels of *PnDLC1* mRNA were significantly lower in the non-motile zoospore transformants, PnDLC1#50, PnDLC1#66 and PnDLC1#68, than in wild-type, pTH210, #6 and #41 controls. These three lines showed *PnDLC1* mRNA levels 3%, 8% and 22% of those in wild-type *P. nicotianae*, respectively.

4. Discussion

4.1. The importance of zoospore motility for Phytophthora pathogenicity

Zoospore motility plays an important role in spore dissemination and disease initiation in *Phytophthora*. Zoospores can swim for hours at speeds of up to about 200 μm/s (Cahill et al., 1996), thus, potentially covering distances of several metres over their lifetime. Zoospores are negatively geotropic, swimming towards the surface of their liquid environment and they autoaggregate (Carlile, 1983; Reid et al., 1995). They are also chemotactically and electrotactically attracted to the surface of potential host plants (Gow, 2004; Tyler, 2002) and can even target specific regions on the host surface such as the root elongation zone (Van West et al., 2002) or the grooves between adjacent epidermal cells (Hardham, 2005). Prior to zoospore encystment, motility is used to orient the ventral surface of the zoospore so that adhesive material is released from ventral vesicles onto the plant surface in order to attach the spore to the plant (Hardham and Gubler, 1990).

4.2. Synthesis of flagella and other zoospores components during asexual sporulation

Many of the proteins and sub-cellular components (e.g. secretory vesicles) destined for *Phytophthora* zoospores are synthesized during sporangium formation (sporangiogenesis), including a number of flagellar surface components (Cope and Hardham, 1994; Dearnaley et al., 1996; Hardham and Hyde, 1997). The expression of genes encoding proteins stored or functioning in zoospores has been shown to occur after the induction of sporulation (Judelson et al., 2008, 2009; Marshall et al., 2001b; Robold and Hardham, 2005). Our demonstration of the up-regulation of expression of the DLC1 gene in *P. cinnamomi* and *P. nicotianae* during sporulation is in accord with the results of a recent study of the *P. infestans* sporulation transcriptome (Judelson et al., 2009). While the increase in *P. cinnamomi* appears to be more modest, in *P. nicotianae*, qPCR measurements indicated an approximately 18-fold increase in *PnDLC1* transcript levels coincident with the development of sporangia in the cultures. In *P. infestans*, genes encoding a number of flagellar proteins are up-regulated during sporangiogenesis, including a 50-fold increase in transcripts of one (Pi015853) encoding a homologue of a dynein light chain in *Paramecium tetraurelia* (CAK88700) (Judelson et al., 2009).

In contrast to the secretory vesicles which are made during sporangiogenesis, the flagella are not formed until the partitioning of the multinucleate sporangia into uninucleate zoospores (zoosporogenesis) (Cope and Hardham, 1994). During this process, the microtubular flagellar axonemes are assembled from the pair of basal bodies located at the narrow end of each nucleus and extend within so-called cleavage vacuoles which become the plasma membrane covering the flagella (Hyde et al., 1991). Components destined for the flagellar surface become clustered at the base of the flagellum during its assembly and are then moved along the flagellar membrane (Cope and Hardham, 1994). However, by analogy with other systems, it seems likely that the dynein outer and inner arms become distributed along the microtubule doublets as the flagellar axonemes are assembled.

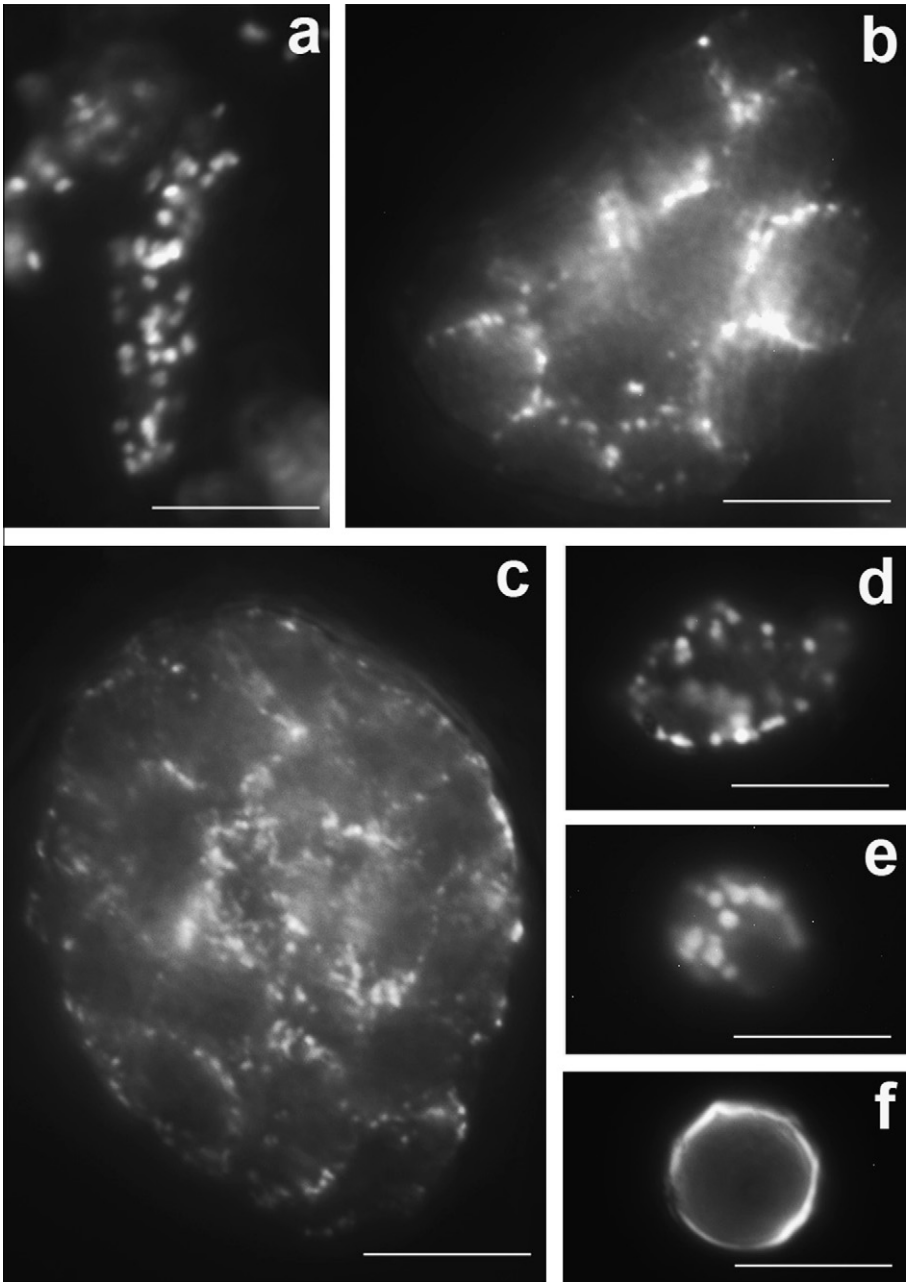

Fig. 5. Immunofluorescence micrographs of silenced line *PnDLC1#68* hyphae from sporulating mycelium (a), cleaving sporangia (b, c), zoospores (d, e) and cyst (f) immunolabelled with monoclonal antibodies specific for PnLpv in large peripheral vesicles (a, d), PnCpa in dorsal vesicles and the cyst surface (b, e, f) and PnVsv in ventral vesicles (c). The immunolabelling indicates that these three categories of zoospore peripheral vesicles are synthesized during sporulation (a) and segregated during sporangial cleavage (zoosporogenesis). In (b), PnCpa-containing dorsal vesicles do not occur along the future zoospore ventral surface which lies adjacent to the sporangial wall, giving a scalloped pattern to the labelling. In (c), PnVsv-containing vesicles cluster in regions along the ventral surface. In zoospores, large peripheral vesicles (d) and dorsal vesicles (e) occur over most of the cell surface but avoid the groove along the ventral surface. PcCpa is secreted as usual during encystment (f). These vesicle distributions are the same as those seen in wild-type *P. cinnamomi* and *P. nicotianae* cultures (Cope et al., 1996; Hardham, 2005; Hyde and Hardham, 1992). (Bars = 10 μm).

4.3. The role of DLC1 in flagella

Within the flagellar axoneme, each microtubule doublet is linked to adjacent doublets by nexin links and to the central pair of single microtubules by radial spokes. Flagella function is achieved through the sliding of adjacent microtubule doublets and is powered by the ATPase activity of inner and outer dynein arms that are distributed along the A-tubule of each doublet (Gibbons, 1982). Although, it has been known for some time that DLC1 binds directly to the catalytic domain of the γ dynein heavy chain and to p45, a putative microtubule-binding protein (Benashski et al., 1999), until recently, there has been little information on the function of DLC1. However, epitope tagging and RNAi silencing have been used to study the function of the DLC1 homologue from *Trypanosoma brucei* (Baron et al., 2007). Silencing of the expression of the *TbLC1* gene resulted in disruption of the dynein outer arm and the complete loss of the tip-to-base beat typical of trypanosome flagellar motility. Instead, the flagella propagate a base-to-tip beat which propels the cells backwards. To our knowledge, our silencing of *PnDLC1* in *P. nicotianae* is only the second example of DLC1 disruption and provides a contrasting result to that obtained in *T. brucei*.

RNAi silencing of the expression of *PnDLC1* in *P. nicotianae* led to complete inhibition of flagellar formation during zoosporogenesis in four independent transformants. This phenotype has not been

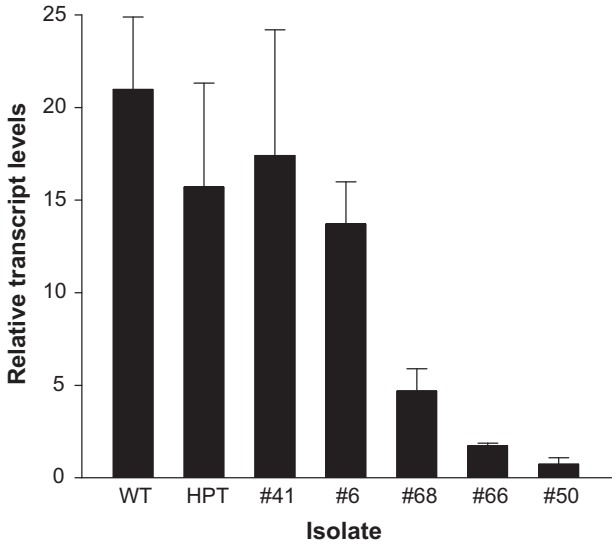

Fig. 6. qPCR analysis of *PnDLC1* expression in *P. nicotianae* wild-type (WT) and transformant lines. Transformant lines which did not show the non-motile zoospore phenotype include an isolate transformed with the hygromycin-resistance gene construct only (HPT) and two isolates transformed in the presence of the hygromycin-resistance gene construct and the DLC1 RNAi construct (#6, #41). Three transformants that did show the non-motile zoospore phenotype are PnDLC1#50, (#50), PnDLC1#66 (#66) and PnDLC1#68 (#68). PnDLC1 transcript levels are relative to those of the constitutively expressed *P. nicotianae* gene, *WS041* (Shan et al., 2004; Yan and Liou, 2006). Bars indicate the positive value of the standard error of the mean of five to seven biological replicates.

observed in any other drug-resistant transformants we have generated including control transformants expressing the hygromycin-resistance gene only or transformants expressing six other RNAi silencing constructs. qPCR analysis of three of the four transformants that produced non-motile zoospores showed that two (PnDLC1#50, PnDLC1#66) had *PnDLC1* transcript levels less than 10% of that in wild-type cells. Although, PnDLC1#68 had *PnDLC1* transcript levels about 22% of those in wild-type cells, 100% of the zoospores that were produced were non-flagellate and non-motile. It may be that this level of *PnDLC1* mRNA is too low to support adequate PnDLC1 synthesis for normal flagellar formation. It has been shown that degradation of mRNAs via microRNAs (miRNAs) and small interfering RNAs (siRNAs) is also associated with translational inhibition of the targeted mRNA (Brodersen et al., 2008). Thus, the level of functional PnDLC1 protein may be very low in all transformants showing the non-motile zoospore phenotype.

In *Chlamydomonas*, mutations in individual dynein outer arm proteins typically lead to instability of the outer arm dynein and impairment of function (King, 2003; Liu et al., 2008; Pazour and Witman, 2001; Sakato and King, 2004), however, they do not inhibit flagellar formation as observed in this study of *P. nicotianae*. In *T. brucei* and *C. reinhardtii*, interference with flagellar assembly does, however, result from silencing or deletion of genes encoding proteins responsible for intraflagellar transport (Absalon et al., 2008; Pazour and Witman, 2001). Homologues of proteins typically involved in retrograde intraflagellar transport in other organisms have been shown to be absent from some flagellate protists (Ginger et al., 2008). Thus, it is possible that in *Phytophthora*, DLC1 might be in some way involved in retrograde intraflagellar transport and hence its silencing inhibits flagellar assembly.

4.4. Zoospore release from Phytophthora sporangia

In *Phytophthora*, zoospore flagella begin beating within the sporangium before expansion of the papilla and the initiation of zoospore discharge. Some observers have had the impression that flagellar beating might contribute to the passage of the zoospores through the exit pore and into the evanescent vesicle (Gisi and Zentmyer, 1980). However, while in some cases the last few zoospores may swim through the exit pore, there is good evidence to support the hypothesis that in *Phytophthora*, as in other Oomycetes, discharge of zoospores from sporangia is driven by hydrostatic pressure generated within the sporangium (Gisi, 1983; Money et al., 1988; Money, 2001; Money and Webster, 1989). Our observations of the *PnDLC1*-silenced lines confirm that flagellar function is not required for the release of all zoospores from the sporangium. Breakage of the evanescent vesicle into which the zoospores are initially discharged usually occurs when its volume is approximately the same as that of the sporangium (Gisi and Zentmyer, 1980). In wild-type cells, rapid motility of zoospores within the evanescent vesicle gives the impression that the motile spores may contribute to or be responsible for vesicle breakage. However, our studies of the non-flagellate *P. nicotianae* zoospores indicate that this is not the case and zoospore motility is not required for vesicle breakage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.04.008.

References

Absalon, S., Blisnick, T., Kohl, L., Toutirais, G., Doré, G., Julkowska, D., Tvenet, A., Bastin, P., 2008. Intraflagellar transport and functional analysis of genes required for flagellum formation in trypanosomes. Mol. Biol. Cell 19, 929–944.

Adl, S.M., Simpson, A.G.B., Farmer, M.A., Andersen, R.A., Anderson, O.R., Barta, J.R., Bowser, S.S., Brugerolle, G., Fensome, R.A., Fredericq, S., James, T.Y., Karpov, S., Kugrens, P., Krug, J., Lane, C.E., Lewis, L.A., Lodge, J., Lynn, D.H., Mann, D.G., McCourt, R.M., Mendoza, L., Moestrup, O., Mozley-Standridge, S.E., Nerad, T.A., Shearer, C.A., Smirnov, A.V., Spiegel, F.W., Taylor, M.F.J.R., 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J. Eukaryot. Microbiol. 52, 399–451.

Appiah, A.A., Van West, P., Osborne, M.C., Gow, N.A.R., 2005. Potassium homeostasis influences the locomotion and encystment of zoospores of plant pathogenic oomycetes. Fungal Genet. Biol. 42, 213–223.

Baron, D.M., Kabututu, Z.P., Hill, K.L., 2007. Stuck in reverse: loss of LC1 in *Trypanosoma brucei* disrupts outer dynein arms and leads to reverse flagellar beat and backward movement. J. Cell Sci. 120, 1513–1520.

Benashski, S.E., Patel-King, R.S., King, S.M., 1999. Light chain 1 from the *Chlamydomonas* outer dynein arm is a leucine-rich repeat protein associated with the motor domain of the γ heavy chain. Biochemistry 38, 7253–7264.

Blackman, L.M., Hardham, A.R., 2008. Regulation of catalase activity and gene expression during *Phytophthora nicotianae* development and infection of tobacco. Mol. Plant Pathol. 9, 495–510.

Blanco, F.A., Judelson, H.S., 2005. A bZIP transcription factor from *Phytophthora* interacts with a protein kinase and is required for zoospore motility and plant infection. Mol. Microbiol. 56, 638–648.

Bottin, A., Larche, L., Villalba, F., Gaulin, E., Esquerré-Tugayé, 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 Microbiol. Lett. 176, 51–56.

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L., Voinnet, O., 2008. Widespread translational inhibition by plant miRNAs and siRNAs. Science 320, 1185–1190.

Cahill, D.M., Cope, M., Hardham, A.R., 1996. Thrust reversal by tubular mastigonemes: immunological evidence for a role of mastigonemes in forward motion of zoospores of *Phytophthora cinnamomi*. Protoplasma 194, 18–28.

Carlile, M.J., 1983. Motility, taxis, and tropism in *Phytophthora*. In: Erwin, D.C., Bartnicki-Garcia, S., Tsao, P. (Eds.), Phytophthora. Its Biology, Taxonomy, Ecology, and Pathology. American Phytopathology Society, St. Paul, Minnesota, pp. 95–107.

Chen, Y., Roxby, R., 1997. Identification of a functional CT-element in the *Phytophthora infestans piypt*1 gene promoter. Gene 198, 159–164.
Chen, D.-W., Zentmyer, G.A., 1970. Production of sporangia by *Phytophthora cinnamomi* in axenic culture. Mycologia 62, 397–402.
Cope, M., Hardham, A.R., 1994. Synthesis and assembly of flagellar surface antigens during zoosporogenesis in *Phytophthora cinnamomi*. Protoplasma 180, 158–168.
Cope, M., Webb, M.C., O'Gara, E.T., Philip, B.A., Hardham, A.R., 1996. Immunocytochemical comparison of peripheral vesicles in zoospores of *Phytophthora* and *Pythium* species. Mycologia 88, 523–532.
Dearnaley, J.D.W., Maleszka, J., Hardham, A.R., 1996. Synthesis of zoospore peripheral vesicles during sporulation of *Phytophthora cinnamomi*. Mycol. Res. 100, 39–48.
Dudler, D., 1990. The single-copy actin gene of *Phytophthora megasperma* encodes a protein considerably diverged from any other known actin. Plant Mol. Biol. 14, 415–422.
Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.
Finnegan, E.J., Kovac, K.A., Jaligot, E., Sheldon, C.C., Peacock, W.J., Dennis, E.S., 2005. The downregulation of FLOWERING LOCUS C (FLC) expression in plants with low levels of DNA methylation and by vernalization occurs by distinct mechanisms. Plant J. 44, 420–432.
Galtier, N., Gouy, M., Gautier, C., 1996. SeaView and Phylo_win, two graphic tools for sequence alignment and molecular phylogeny. Comput. Appl. Biotech. 12, 543–548.
Gautam, Y., Cahill, D.M., Hardham, A.R., 1999. Development of a quantitative immunodipstick assay for *Phytophthora nicotianae*. Food Agric. Immunol. 11, 229–242.
Gibbons, I.R., 1982. Sliding bending in sea urchin sperm flagella. In: Amos, W.B., Duckett, J.G. (Eds.), Prokaryotic and Eukaryotic Flagella. Cambridge University Press, Cambridge, pp. 225–287.
Ginger, M.L., Portman, N., McKean, P.G., 2008. Swimming with protists: perception, motility and flagellum assembly. Nat. Rev. Microbiol. 6, 838–850.
Gisi, U., 1983. Biophysical aspects of the development of *Phytophthora*. In: Erwin, D.C., Bartnicki-Garcia, S., Tsao, P.H. (Eds.), Phytophthora. Its Biology, Taxonomy, Ecology, and Pathology. American Phytopathological Society, St. Paul, Minnesota, pp. 109–119.
Gisi, U., Zentmyer, G.A., 1980. Mechanism of zoospore release in *Phytophthora* and *Pythium*. Exp. Mycol. 4, 362–377.
Gow, N.A.R., 2004. New angles in mycology: studies in directional growth and directional motility. Mycol. Res. 108, 5–13.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.
Hardham, A.R., 1987. Microtubules and the flagellar apparatus in zoospores and cysts of the fungus *Phytophthora cinnamomi*. Protoplasma 137, 109–124.
Hardham, A.R., 2001. The cell biology behind *Phytophthora* pathogenicity. Aust. Plant Pathol. 30, 91–98.
Hardham, A.R., 2005. *Phytophthora cinnamomi*. Mol. Plant Pathol. 6, 589–604.
Hardham, A.R., 2007. Cell biology of fungal and oomycete infection of plants. In: Howard, R.J., Gow, N.A.R. (Eds.), The Mycota, Biology of the Fungal Cell, vol. VII. Springer-Verlag, Heidelberg, pp. 251–289.
Hardham, A.R., 2009. The asexual lifecycle. In: Lamour, K., Kamoun, S. (Eds.), Oomycete Genetics and Genomics: Diversity, Interactions, and Research Tools. Wiley-Blackwell, pp. 93–119.
Hardham, A.R., Gubler, F., 1990. Polarity of attachment of zoospores of a root pathogen and pre-alignment of the emerging germ tube. Cell Biol. Int. Rep. 14, 947–956.
Hardham, A.R., Hyde, G.J., 1997. Asexual sporulation in the oomycetes. Adv. Bot. Res. 24, 353–398.
Hardham, A.R., Suzaki, E., Perkin, J.L., 1986. Monoclonal antibodies to isolate-, species- and genus-specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*. Can. J. Bot. 64, 311–321.
Hardham, A.R., Gubler, F., Duniec, J., Elliott, J., 1991. A review of methods for the production and use of monoclonal antibodies to study zoosporic plant pathogens. J. Microsc. 162, 305–318.
Horvath, J., Fliegauf, M., Olbrich, H., Kispert, A., King, S.M., Mitchison, H., Zariwala, M.A., Knowles, M.R.S., Fekete, G.E.A., 2005. Identification and analysis of axonemal dynein light chain 1 in primary ciliary dyskinesia patients. Am. J. Respir. Cell Mol. Biol. 33, 41–47.
Hyde, G.J., Hardham, A.R., 1992. Confocal microscopy of microtubule arrays in cryosectioned sporangia of *Phytophthora cinnamomi*. Exp. Mycol. 16, 207–218.
Hyde, G.J., Lancelle, S., Hepler, P.K., Hardham, A.R., 1991. Freeze substitution reveals a new model for sporangial cleavage in *Phytophthora*, a result with implications for cytokinesis in other eukaryotes. J. Cell Sci. 100, 735–746.
Judelson, H.S., Coffey, M.D., Arredondo, F.R., Tyler, B.M., 1993. Transformation of the oomycete pathogen *Phytophthora megasperma* f. Sp. *glycinea* occurs by DNA integration into single or multiple chromosomes. Curr. Genet. 23, 211–218.
Judelson, H.S., Ah-Fong, A.M.V., Aux, G., Avrova, A.O., Bruce, C., Calkir, C., da Cunha, L., Grenville-Briggs, L., Latijnhouwers, M., Ligterink, W., Meijer, H.J.G., Roberts, S., Thurber, C.S., Whisson, S.C., Birch, P.R.J., Govers, F., Kamoun, S., Van West, P., Windass, J., 2008. Gene expression profiling during asexual development of the late blight pathogen *Phytophthora infestans* reveals a highly dynamic transcriptome. Mol. Plant–Microbe Interact. 21, 433–447.
Judelson, H.S., Narayan, R., Ah-Fong, A.M.V., Kim, K.S., 2009. Gene expression changes during asexual sporulation by the late blight agent *Phytophthora infestans* occur in discrete temporal stages. Mol. Genet. Genom. 281, 193–206.
King, S.M., 2003. Organization and regulation of the dynein microtubule motor. Cell Biol. Int. 27, 213–215.
Kobe, B., Kajava, A.V., 2001. The leucine-rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. 11, 725–732.
Latijnhouwers, M., Ligterink, W., Vleeshouwers, V.G.A.A., Van West, P., Govers, F., 2004. A Gα subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*. Mol. Microbiol. 51, 925–936.
Liu, Z.M., Takazaki, H., Nakazawa, Y., Sakato, M., Yagi, T., Yasunaga, T., King, S.M., Kamiya, R., 2008. Partially functional outer-arm dynein in a novel *Chlamydomonas* mutant expressing a truncated gamma heavy chain. Eukaryot. Cell 7, 1136–1145.
Marshall, J.S., Ashton, A.R., Govers, F., Hardham, A.R., 2001a. Isolation and characterization of four genes encoding pyruvate, phosphate dikinase in the oomycete plant pathogen *Phytophthora cinnamomi*. Curr. Genet. 40, 73–81.
Marshall, J.S., Wilkinson, J.M., Moore, T., Hardham, A.R., 2001b. Structure and expression of the genes encoding proteins resident in large peripheral vesicles of *Phytophthora cinnamomi* zoospores. Protoplasma 215, 226–239.
McLeod, A., Smart, C.D., Fry, W.E., 2004. Core promoter structure in the oomycete *Phytophthora infestans*. Eukaryot. Cell 3, 91–99.
Mitchell, H.J., Hardham, A.R., 1999. Characterisation of the water expulsion vacuole in *Phytophthora nicotianae* zoospores. Protoplasma 206, 118–130.
Money, N.P., 2001. Biomechanics of invasive hyphal growth. In: Gow, N.A.R., Howard, R.J. (Eds.), The Mycota VIII. Springer-Verlag, Berlin, pp. 3–17.
Money, N.P., Webster, J., 1989. Mechanism of sporangial emptying in *Saprolegnia*. Mycol. Res. 92, 45–49.
Money, N.P., Webster, J., Ennos, R., 1988. Dynamics of sporangial empyting in *Achlya intricata*. Exp. Mycol. 12, 13–27.
Nicastro, D., McIntosh, J.R., Baumeister, W., 2005. 3D structure of eukaryotic flagella in a quiescent state revealed by cryo-electron tomography. Proc. Natl. Acad. Sci. USA 102, 15889–15894.
Patterson, D.J., 1989. Stramenoplies: chromophytes from a protistan perspective. In: Green, J.C., Leadbeater, B.S.C., Diver, W.L. (Eds.), The Chromophyte Algae: Problems and Perspectives. Clarendon Press, Oxford, pp. 357–379.
Pazour, G.J., Witman, G.B., 2001. Forward and reverse genetic analysis of microtubule motors in *Chlamydomonas*. Methods 22, 285–298.
Pfister, K.K., Witman, G.B., 1982. Purification and polypeptide composition of dynein ATPases from *Chlamydomonas* flagella. Cell Motil. 2, 525–547.
Reid, B., Morris, B.M., Gow, N.A.R., 1995. Calcium-dependent, genus-specific, autoaggregation of zoospores of phytopathogenic fungi. Exp. Mycol. 19, 202–213.
Robold, A.V., Hardham, A.R., 2005. During attachment *Phytophthora* spores secrete proteins containing thrombospondin type 1 repeats. Curr. Genet. 47, 307–315.
Sakato, M., King, S.M., 2004. Design and regulation of the AAA+ microtubule motor dynein. J. Struct. Biol. 146, 58–71.
Shan, W., Hardham, A.R., 2004. Construction of a bacterial artificial chromosome library, determination of genome size, and characterization of an *Hsp70* gene family in *Phytophthora nicotianae*. Fungal Genet. Biol. 41, 369–380.
Shan, W., Marshall, J.S., Hardham, A.R., 2004. Stage-specific expression of genes in germinated cysts of *Phytophthora nicotianae*. Molec. Plant Pathol. 5, 317–330.
Smale, S.T., Baltimore, D., 1989. The 'initiator' as a transcription control element. Cell 57, 103–113.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Tyler, B.M., 2002. Molecular basis of recognition between *Phytophthora* pathogens and their hosts. Annu. Rev. Phytopathol. 40, 137–167.
Van de Peer, Y., Van der Auwera, G., De Wachter, R., 1996. The evolution of stramenopiles and alveolates as derived by "substitution rate calibration" of small ribosomal subunit RNA. J. Mol. Evol. 42, 201–210.
Van West, P., Morris, B.M., Reid, B., Appiah, A.A., Osborne, M.C., Campbell, T.A., Shepherd, S.J., Gow, N.A.R., 2002. Oomycete plant pathogens use electric fields to target roots. Mol. Plant–Microbe Interact. 15, 790–798.
Weerakoon, N.D., Roberts, J.K., Lehnen, L.P., Wilkinson, J.M., Marshall, J.S., Hardham, A.R., 1998. Isolation and characterization of the single β-tubulin gene in *Phytophthora cinnamomi*. Mycologia 90, 85–95.
Wickstead, B., Gull, K., 2007. Dyneins across eukaryotes: a comparative genomic analysis. Traffic 8, 1708–1721.
Wu, H., Maciejewski, M.W., Benashski, S.E., Mullen, G.P., King, S.M., 1999. ^{1}H, ^{15}N and ^{13}C resonance assignments for the 22 kDa LC1 light chain from *Chlamydomonas* outer arm dynein. J. Biomol. NMR 13, 309–310.
Wu, H.W., Maciejewski, M.W., Marintchev, A., Benashski, S.E., Mullen, G.P., King, S.M., 2000. Solution structure of a dynein motor domain associated light chain. Nat. Struct. Biol. 7, 575–579.
Wu, H., Blackledge, M., Maciejewski, M.W., Mullen, G.P., King, S.M., 2003. Relaxation-based structure refinement and backbone molecular dynamics of the dynein motor domain-associated light chain. Biochemistry 42, 57–71.
Yan, H.-Z., Liou, R.-F., 2006. Selection of internal control genes for real-time quantitative RT-PCR assays in the oomycete plant pathogen *Phytophthora parasitica*. Fungal Genet. Biol. 43, 430–438.