Fungal Genetics and Biology 47 (2010) 663–671

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

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

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ARTICLE INFO

Article history:
Received 28 July 2009
Accepted 28 April 2010
Available online 6 May 2010

Keywords:
Dynein light chain 1
Flagellar formation
Phytophthora
Sporangial discharge
Zoospore motility

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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doi:10.1016/j.fgb.2010.04.008

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