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A putative DEAD-box RNA-helicase is required for normal zoospore development in the late blight pathogen *Phytophthora infestans*

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ABSTRACT

The asexual multinucleated sporangia of *Phytophthora infestans* can germinate directly through a germ tube or indirectly by releasing zoospores. The molecular mechanisms controlling sporangial cytokinesis or sporangial cleavage, and zoospore release are largely unknown. Sporangial cleavage is initiated by a cold shock that eventually compartmentalizes single nuclei within each zoospore. Comparison of EST representation in different cDNA libraries revealed a putative ATP-dependent DEAD-box RNA-helicase gene in *P. infestans*, *Pi-RNH1*, which has a 140-fold increased expression level in young zoospores compared to uncleaved sporangia. RNA interference was employed to determine the role of *Pi-RNH1* in zoospore development. Silencing efficiencies of up to 99% were achieved in some transiently-silenced lines. These *Pi-RNH1*-silenced lines produced large aberrant zoospores that had undergone partial cleavage and often had multiple flagella on their surface. Transmission electron microscopy revealed that cytoplasmic vesicles fused in the silenced lines, resulting in the formation of large vesicles. The *Pi-RNH1*-silenced zoospores were also sensitive to osmotic pressure and often ruptured upon release from the sporangia. These findings indicate that *Pi-RNH1* has a major function in zoospore development and its potential role in cytokinesis is discussed.

1. Introduction

Oomycetes cause destructive diseases of plants, insects, crustaceans, fish and vertebrate animals. Among the oomycetes, *Phytophthora* spp. are arguably the most economically significant pathogens of dicotyledenous plants. The most notorious is *Phytophthora infestans* (Mont.) de Bary, which causes late blight on potatoes and blight on tomatoes, resulting in global losses exceeding US$ 5 billion per year (Duncan, 1999). Moreover, the genus includes devastating wide host-range pathogens that threaten natural vegetation, such as *P. ramorum* that has decimated oak forests in California (Appiah et al., 2004). Although they posses a filamentous growth habit, they are distantly related to fungi and instead are more closely related to brown algae in the group of Stramenopiles. Oomycetes have thus evolved distinct genetic and biochemical mechanisms for infection (Kamoun, 2003).

Infection by *Phytophthora* spp. typically initiates when sporangia release motile, biflagellate zoospores. Zoospores are essential for the disease cycles of many oomycete pathogens and are often the first point-of-contact with the host. Currently, little is known about the molecular biology of zoospore development or the regulation of its various stages. On short exposure to low temperature (cold shock) multinucleated sporangia rapidly differentiate by cytoplasmic cleavage to form several zoospores, which are released from the sporangial apex and exhibit an α-helical swimming pattern. Understanding the mechanisms underlying the rapidity by which this process occurs is a major goal of our research.

Zoospores serve as infectious agents and can swim for hours in the presence of an endogenous food reserve (Carlile, 1986). They remain motile until encystment and can display several tactic behaviors (Deacon and Donaldson, 1993; Hill et al., 1998; Griffith et al., 1988; van West et al., 2002). It is thought that two separate processes, zoospore taxis and zoospore immobilization, define the targeting of host tissue by zoospores. Only directional swimming of zoospores towards chemical, nutrient, ionic or electrical gradients constitutes a genuine tactical response (van West et al., 2002; Appiah et al., 2005).

On reaching a host, zoospores encyst. Encystment is a very fast process involving flagella detachment and primary cell wall formation (Griffith et al., 1988). Cysts form a germ tube, which usually differentiates into an appressorium (Grenville-Briggs et al., 2008). The appressorium forms a penetration peg that penetrates the epidermal cell layer. Subsequently, during *P. infestans* leaf infection,

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for example, an infection vesicle is usually formed in the epidermal cell and hyphae grow into the mesophyll cell layers both intra- and intercellularly. In most cases, many intracellular haustorial feeding structures are formed. After three-to-four days, *P. infestans* grows necrotrophically in the center of the growing lesion. Hyphae emerge through the stomata and sporangiophores are formed which produce numerous new sporangia on the underside of the leaf (van West and Vleeshouwers, 2004).

In our laboratories we are interested in genes that are up-regulated during zoospore development and zoospore release in *P. infestans*. Proteomic studies, cDNA-AFLP, suppression subtractive hybridization as well as microarray approaches have been employed to discover genes that may be implicated in these developmental processes (Avrova et al., 2003; Grenville-Briggs et al., 2005; Shepherd et al., 2003; Torto et al., 2003). In addition, comparison of expressed sequence tag (EST)-libraries revealed a putative DEAD-box RNA helicase that was over-represented in cDNA libraries derived from cleaving sporangia, zoospores and cysts compared to ESTs derived from mycelial and other stage-specific cDNA libraries. The objective of the research described here was to clone and functionally characterize the RNA helicase; experiments to investigate the role of the RNA helicase in zoospore development are described in this report.

2. Results

2.1. Identification of a putative DEAD-box RNA helicase gene in *P. infestans*

A large-scale gene discovery project generated 75,757 expressed sequence tags (ESTs) from *P. infestans* (Randall et al., 2005). The data provided an 18,256 unigene set for Pfam searches. Comparison of EST representation in different stage-specific cDNA libraries to identify ESTs that are specific for zoospore stages, revealed the presence of 10 partial cDNA clones, all of which showed similarity to DEAD box RNA helicase sequences from various organisms. The corresponding gene was tentatively named *Pi-RNH1*. Interestingly the DEAD/DEAH box motif was found to be one of the 40 most represented domains in *P. infestans* (Randall et al., 2005), suggesting that this motif may be important for numerous processes in *P. infestans*. *Pi-RNH1* was also described by Tani et al. (2004), who discovered, by employing cDNA array hybridization that *Pi-RNH1* is at least 18-fold up-regulated in cleaving sporangia compared to uncleaved sporangia. To delimit the full open reading frame (ORF) of *Pi-RNH1*, further ESTs were identified that corresponded to the predicted ORF of *Pi-RNH1*. From the existing EST datasets, only sequences that aligned to the 5′ end of the ORF were found. A BLASTN search of the *Phytophthora sojae* genome database (http://genome.jgi-psf.org/Physo1_1/Physo1_1. home.html) with the 5'end of *Pi-RNH1* revealed a close homologue (119997). Subsequently, this *P. sojae* sequence was used to search the *P. infestans* EST database for sequences that would be situated further towards the 3′ end. Four clones that corresponded to the 3' end of *Pi-RNH1* were found. However, sequences matching the central portion of the *P. sojae* open reading frame were absent. Primers RH-intron-F and RH-intron-R (Table 1) were designed to amplify the equivalent region from both *P. infestans* genomic DNA and cDNA. This resulted in the discovery of a 605 bp intron in the *Pi-RNH1* sequence; the largest intron identified in a gene from *P. infestans* to date. The *Pi-RNH1* sequence was submitted to Genbank (Accession No. EU195919).

2.2. Features of the predicted protein sequence of Pi-RNH1

The corresponding protein sequence, *Pi-RNH1*, was analyzed using conserved domain searches (CD-search) at http://www.ncbi.nlm.nih.gov/Structure/cdd (Marchler-Bauer and Bryant, 2004). *Pi-RNH1* belongs to the DEAD box family of RNA-helicases. Nine conserved motifs of DEAD box RNA-helicases, which have been named *Q*, *I*, *Ia*, *Ib*, *II*, *III*, *IV*, *V*, *VI* (Cordin et al., 2006), were all found in *Pi-RNH1* (Fig. 1A). These motifs are required for RNA binding, ATPase and helicase activities and regulation. The N- and C-termini of *Pi-RNH1* are glycine-rich. There are 56 glycine residues within the first 81 amino acids and 15 within the last 23 amino acids of the C-terminus.

The phylogenetic relatedness of *Pi-RNH1* was compared to other RNA helicases by neighbor-joining analysis (Fig. 1B). *Pi-RNH1* clusters with *P. sojae* and *P. ramorum* homologues sharing 97% and 95% identity, respectively, over a region of 440 amino acids. The oomycete *RNH1* sequences belong to a larger cluster containing putative RNA helicases from *Aspergillus*, *Oryza* and *Dictyostelium*, along with DBP2 from *Kluyveromyces*. DBP2 in yeast is involved in pre-mRNA splicing and cell cycle progression (Imamura et al., 1998) and, interestingly, was shown to be induced in response to cold shock (Schade et al., 2004). DDX4 and DDX3Y from humans and mouse are involved in germ-line development (Castrillon et al. 2000; Ditton et al. 2004). DDX53 from human is probably involved in cellular proliferation (Cho et al., 2002). In the second cluster are RNA helicases with various functions, including RHLB, which is involved in RNA decay (Py et al., 1996), and Xp54, which is involved in (suppression of) translation (Minshall and Standart, 2004).

2.3. Expression analysis of Pi-RNH1

Detailed investigation of *Pi-RNH1* expression during the life-cycle of *P. infestans* was required to confirm that *Pi-RNH1* was indeed up-regulated in the early stages of zoospore development

Table 1
Primer sequences

Gene	Primer name	Primer sequence	Application	Amplicon size (bp)
rnh1	RH-F	ATGGACGACCACCAGAAGTA	dsRNA synthesis	196
	T7F-RH	GTAATACGACTCACTATAGGGATGGACGACCACCAGAAGTA		
	T7F-RH	GTAATACGACTCACTATAGGGATGGACGACCACCAGAAGTA	dsRNA synthesis	196
	RH-R	ACTCACGCAGCACATAGTCA		
	RNAHEL-F	CGCAGGAGGAGCGTGACTAT	Real-time RT-PCR	76
	RNAHEL-R	GCCACATCAGTAGCGACTAGG		
	RH-intron-F	GACGAGACATGGTCGGTA	Intron detection	183/788
	RH-intron-R	CGCCGAACTTGTTACACT		
gfp	GFPF	GCTGGAGTACAACTACAACT	dsRNA synthesis	199
	GFPRT7	GTAATACGACTCACTATAGGGGGCAGATTGCGTGGACAGGT		
	GFPFT7	GTAATACGACTCACTATAGGGGCTGGAGTACAACTACAACT	dsRNA synthesis	199
	GFPR	GGCAGATTGCGTGGACAGGT		
actA	ActAF2	CATCAAGGAGAAGCTGACGTACA	Real-time RT-PCR	69
	ActAR2	GACGACTCGGCGGCAG		

A

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MYSSGGDRYGDSSSRRDYGGGGSGGYGGGGYGGGSRGGGGGGYGGGGGGY   50
SGGGGGYGGGGGGYSGGGRGGGGGGGFGGGGDLGSNLDTHIQWDLSKLPV  100
FEKNFYYEHPDVTKRSEDEYEKWKRDNQIIVSGKGVPKCVLSFEEASFPE  150
          Q-motif                  motif I
YVLEEVVRLGFDKPTPIQCQGWPMALSGRDMVGISATGSGKTLAFLLPAI  200
                    motif Ia
VHINAQPYLQPGDGPIVLMIAPTRELAVQIQAECNKFGASSKIKNTCVYG  250
                  motif Ib              motif II
GVPKGGQIADLRRGVEICICTPGRMIDMLSMGKTNLRRVTYLVLDEADRM  300
                        motif III
LDMGFEPQLRKIVSQIRPDRQTLMWSATWPKEIVALANDFLTDFIQVTVG  350
                                          motif IV
SLDLTANKRIKQIVEVMDDHQKYSSLQDHLRDIYEGGRIIIFCETKRGAD  400
                                            motif V
ELSRNLRNTRYICKAIHGNKSQEERDYVLREFKDGRTQILVATDVASRGL  450
                    motif VI
DIKDIRYVVNFDMPKNIEDYIHRIGRTARAGNKGTSISFFTASNNGRLAG  500
PLVKIMEEAEQEVPRELRDLVGRGGGGGRGRGGFGGGRGGRGRW*       544
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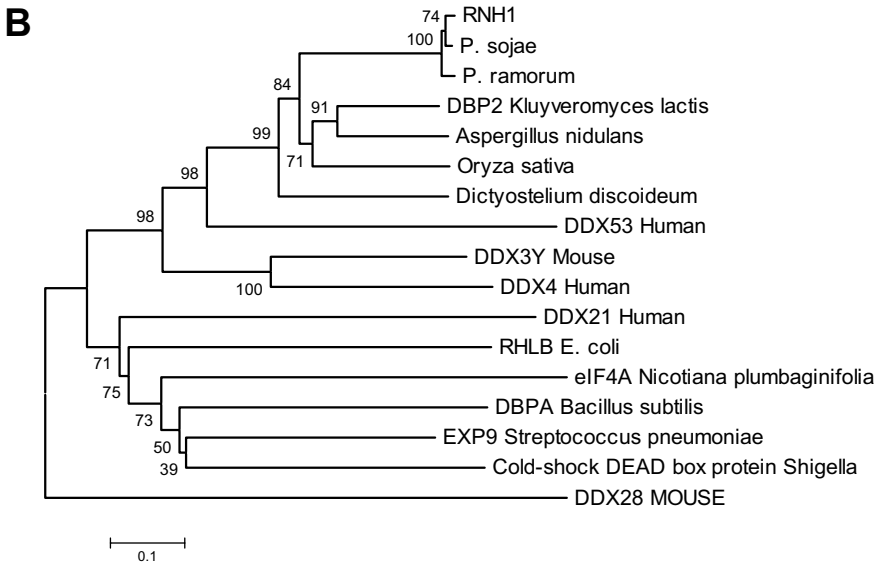

Fig. 1. (A) Amino acid sequence of *Pi-RNH1*. Indicated are the conserved motifs (*Q*, *I*, *Ia*, *Ib*, *II*, *III*, *IV*, *V*, and *VI*), which are found in DEAD-box RNA helicases (Cordin et al., 2006). Underlined in the sequence is the 605 bp intron. Note the large number of glycine residues at the N-, and C-terminus of the protein sequence. Numbers on the right indicate number of amino acid residues. (B) A neighbor-joining distance tree constructed from the conserved region of selected helicases corresponding to position 143–518 of *Pi-RNH1*. Percentage bootstrap values are reported from 1000 replicates. The scale bar indicates the number of substitutions per site for a unit branch length.

and zoospore release. Real-time RT-PCR analysis was used to characterize expression of *Pi-RNH1* in uncleaved sporangia, cleaving sporangia, zoospores, germinated cysts, and germinated cysts with appressorium-like structures and during the infection of susceptible potato cv. Bintje with a compatible race of *P. infestans* at 24, 48 and 72 h post-inoculation (hpi). Primers were designed to anneal to *Pi-RNH1* (Table 1). The *actA* gene from *P. infestans* was used as a constitutively-expressed endogenous control and the expression of *Pi-RNH1* was determined relative to *actA* expression. Expression of *Pi-RNH1* in different samples was compared to the level of its expression in a calibrator sample, which was cDNA from uncleaved sporangia. The expression of *Pi-RNH1* in the uncleaved sporangia cDNA sample was assigned the value of 1.0. *Pi-RNH1* showed approximately 140-fold higher expression in zoospores compared to uncleaved sporangia (Fig. 2A) confirming the cDNA array hybridization results described by Tani et al. (2004). PiRNH1 was not significantly upregulated in any other lifecycle stage or during infection of potato.

2.4. Transient silencing of Pi-RNH1 expression

A transient gene silencing strategy, based on RNA interference (RNAi), was employed to determine the role of *Pi-RNH1* in zoospore development. This method was recently developed and validated by Whisson et al. (2005). We have also used the RNAi technique to investigate the role of four cellulose synthases in the development of appressoria in *P. infestans* (Grenville-Briggs et al., 2008). The RNAi-method results in very fast scoring of any potential phenotypes after transient silencing of the gene of interest. *P. infestans* protoplasts were incubated overnight with *in vitro* synthesized dsRNA of *Pi-RNH1*, and the regenerating protoplasts were subsequently cultured in liquid pea broth in microtitre wells for 3 days. As a control, dsRNA was made from a gene encoding green fluorescent protein (GFP) that is not present in the wild type strains CY29 or 88069. Single colonies were picked from the *Pi-RNH1* or *gfp*-treated protoplasts and transferred to solid medium. Twelve days later, these colonies were flooded with cold water to induce zoospore formation. After 2 h, the suspension was collected and an ali-

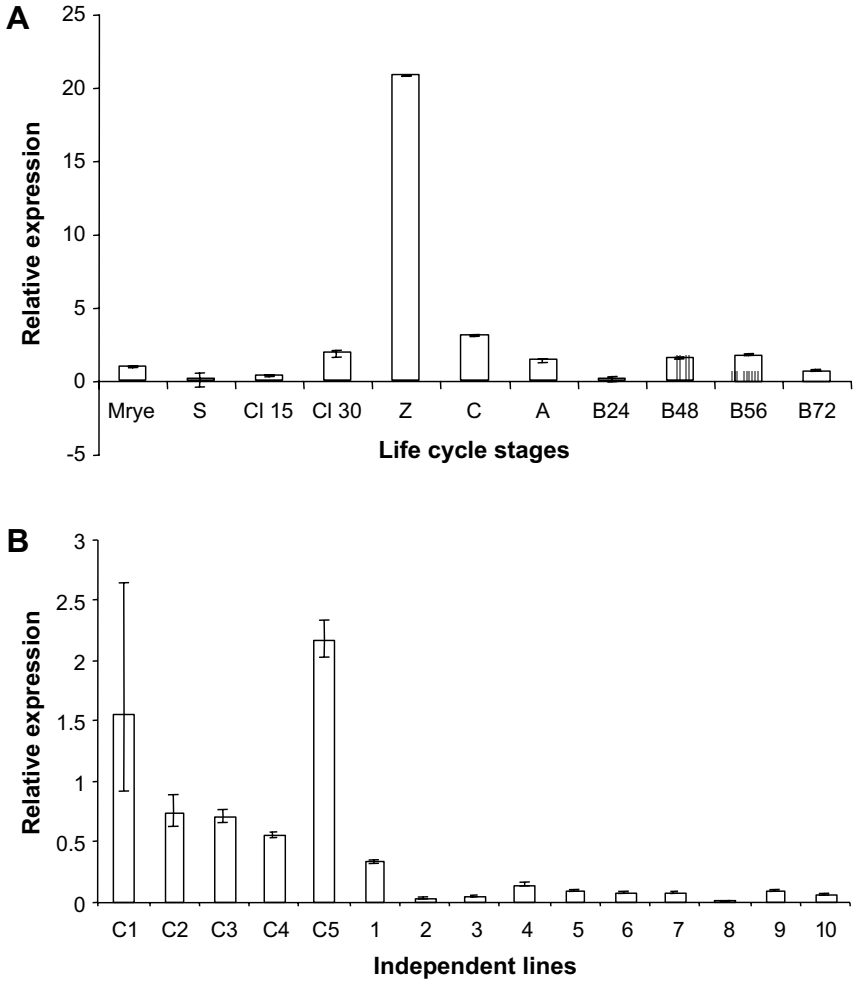

Fig. 2. Real-time RT-PCR expression profiles of *Pi-RNH1*. (A) Real-time RT-PCR expression profile of *Pi-RNH1* in pre-infection stages (mycelium, M; sporangia, S; cleaving sporangia, CS (15 min and 30 min); zoospores, Z; germinating cyst, C; and germinating cysts with appressoria), and *in planta* (24 (B24), 48 (B48) and 72 (B72) hours post-inoculation of potato cultivar Bintje with a compatible race of *P. infestans*, relative to their expression in non-cleaving sporangia (S). Two biological replicates were used for each sample, yielding the same expression profiles. Error bars represent 95% confidence intervals calculated using standard deviation of three technical replicates for each sample within the RT-PCR assay. (B) Relative expression levels of *Pi-RNH1* in individual RNAi lines from two independent experiments measured 15 days after exposure to *Pi-RNH1* dsRNA or *gfp* dsRNA (controls—C). Variation in expression levels in individual control lines and lines exposed to *Pi-RNH1* dsRNA is shown as 95% confidence intervals calculated using standard deviation of three technical replicates in the real-time RT-PCR assays.

quot of it was analyzed by microscopy (see below). The remaining zoospore suspension was centrifuged and the pellet was frozen for RNA isolation. Individual lines were obtained from two independent experiments (five controls and ten *Pi-RNH1*-dsRNA treated lines, Fig. 2B). Subsequently, quantitative real-time RT-PCR was performed with the extracted RNA to determine whether the dsRNA-treated lines were silenced for *Pi-RNH1* expression. Relative expression values were assessed 15 days after exposure to *Pi-RNH1* dsRNA or *gfp* dsRNA (control). Results from two independent experiments are presented in Fig. 2B. The *Pi-RNH1*-silenced lines showed a reduction in *Pi-RNH1* mRNA abundance, with mRNA levels ranging from 1 to 34% of the *gfp* dsRNA treated controls. Therefore, successful silencing of *Pi-RNH1* was achieved using RNAi allowing any resulting phenotypes to be studied.

2.5. Phenotypic characterization of Pi-RNH1-silenced P. infestans lines

Both the *Pi-RNH1*-dsRNA treated (silenced) and the *gfp*-dsRNA-treated (control) lines grew normally on rye medium and produced similar numbers of sporangia (data not shown). Wild type sporangia usually release about 6 biflagellated uninucleate zoospores (length 17.5 μm, width 11.4 μm) that have a swimming speed of up to 100 μm/s (Appiah et al., 2005). The control line zoospores behaved normally, with similar swimming speeds. Moreover, zoospore morphology, size, and numbers of zoospores released per sporangium in control lines were similar to the wild-type (Figs. 3A and 4A). However, zoospores released from the *Pi-RNH1*-silenced lines had undergone various phenotypic changes. Most sporangia of *Pi-RNH1*-silenced lines released one-to-three large zoospores per sporangium (Figs. 3B, 3C and 4B). The size of these aberrant zoospores had often doubled in length from 17.5 μm for the control zoospores to 35 μm in several zoospores from the *Pi-RNH1*-silenced lines. In some silenced lines this phenomenon was found in up to 70% of all harvested sporangia. In addition, several aberrantly shaped zoospores, presumably resulted from release of partially cleaved sporangia, were found in the *Pi-RNH1*-silenced lines (Fig. 4B). The large aberrant zoospores were rarely seen in the control lines (<0.1%). In some silenced lines, aberrant zoospores were found less frequently (approximately 10% of cases). However, it should be noted that in these silenced lines the *Pi-RNH1*-expression levels were higher than those found in lines that had more than 50% of large aberrant zoospores. Thus, the level of gene silencing was closely associated with the severity of the phenotype.

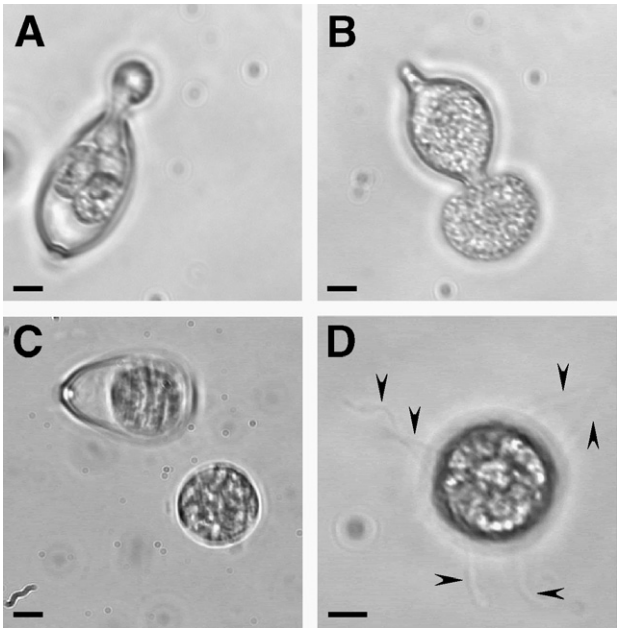

Fig. 3. Zoospore release and sporangial cytoplasmic cleavage in *Pi-RNH1* silenced lines derived from wild type strain CY29. (A) Zoospore release of a *gfp*-dsRNA-treated line showing normal zoospore morphology and release. (B) Large aberrant zoospore produced from undifferentiated sporangium of a *Pi-RNH1*-dsRNA treated line. (C) Large aberrant zoospores of a *Pi-RNH1*-dsRNA treated line. One zoospore was unable to release itself from the sporangium. The other has moved slowly away from the sporangium. (D) Large aberrant zoospores of a *Pi-RNH1*-dsRNA treated line. Indicated, with small arrows, are 6 flagella on the surface of the zoospore. Scale bars represent 10 μm.

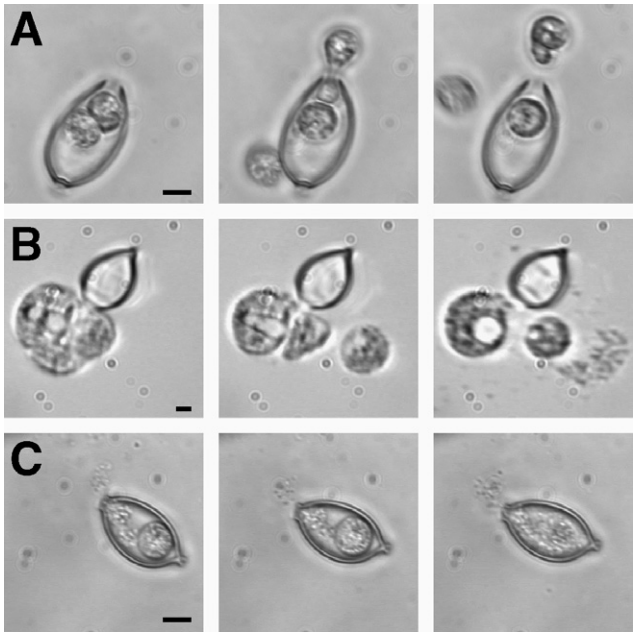

Fig. 4. Zoospore release and bursting in *Pi-RNH1* silenced lines derived from wild type strain CY29. (A) Zoospores released from a sporangium of a *gfp*-dsRNA-treated line. (B,C) Three large aberrant zoospores are released from a sporangium (B) and one remaining in the sporangium (C) of a *Pi-RNH1*-dsRNA treated line. The zoospores were unable to control their osmotic pressure and swelled and eventually burst. Pictures were taken with 20 s interval. Scale bars represent 10 μm.

It was frequently found that the *Pi-RNH1*-silenced lines were unable to release the aberrant large zoospore(s) (Fig. 3C). Remarkably, in the silenced lines the large aberrant zoospores that remained in the sporangium moved constantly towards the sporangial base rather than the sporangial opening, therefore failing to be released from sporangia (Figs. 3C and 4C). The large aberrant zoospores produced by the *Pi-RNH1*-silenced lines were observed to have multiple flagella. In some cases up to 6 flagella per zoospore were seen (Fig. 3D). The presence of multiple flagella resulted in the zoospore being pulled in several different directions simultaneously, causing a shaking movement and therefore exhibiting a greatly reduced mobility, ranging from motionless to only a few micrometers per second.

A large proportion of the aberrant zoospores from the *Pi-RNH1*-silenced lines showed low tolerance to osmotic pressure. Particularly, the larger aberrant zoospores had a tendency to swell rapidly and burst after a short period in distilled water (Fig. 4B and C). Furthermore, wild type-sized *Pi-RNH1*-silenced zoospores were also sensitive to osmotic pressure and showed the bursting phenotype. Therefore, further sporangial release experiments were performed in 1 M mannitol, which resulted in less than 5% of the zoospores being ruptured.

RNA-interference experiments were performed on ten independent occasions with isolate CY29 with over 100 silenced lines being analyzed. For each experiment, silenced lines exhibited similar phenotypes. Besides isolate CY29, *P. infestans* strain 88069 was also treated with dsRNA of *Pi-RNH1* and *gfp* and over 20 individual lines were tested. Comparable phenotypic changes in zoospore morphology, size and swimming pattern were found in the *Pi-RNH1*-silenced lines of strain 88069 as to those seen for isolate CY29, while the *gfp*-dsRNA treated lines of strain 88069 retained the wild-type phenotype (data not shown).

2.6. Transmission electron microscopy

Transmission electron microscopy (TEM) was used to study the effect of *Pi-RNH1*-silencing on the organization of internal organelles during sporangial cleavage and zoospore formation. Zoospore samples were taken from both *gfp*-dsRNA-treated lines (control) and *Pi-RNH1*-silenced lines at the induction of sporangial cleavage (t_0), 30 min (t_{30}), and 2 h (t_{2hr}) after induction. No significant difference in the cellular structures of control and *Pi-RNH1*-silenced sporangia were observed at induction of sporangial cleavage (t_0). In both control and silenced lines, sporangia were multinucleated with undifferentiated cytoplasm; the size, form and structure were also similar (data not shown). However, there were significant differences between sporangia from the control and *Pi-RNH1*-silenced lines 30 min after induction of sporangial cleavage. The cytoplasm of control sporangia had differentiated to form on average 6 fully developed zoospores (Fig. 5A). In contrast, the sporangial cytoplasm from *Pi-RNH1*-silenced lines remained undifferentiated and, notably, several vesicles (lipid bodies) had apparently fused (Fig. 5B). Two hours (t_{2h}) after induction of sporangial cleavage additional differences were seen in cytoplasm and organelle organization of the control and silenced lines. In fact, normal-sized zoospores from both the control and the silenced lines differed in cytoplasmic composition. Control zoospores contained several relatively small individual lipid bodies (Fig. 5C). However, as seen at t_{2h}, the *Pi-RNH1*-silenced zoospores contained several large vesicles. It appeared that smaller lipid bodies had fused to produce larger vesicles (Fig. 5D). Similar differences were seen in cysts from both lines at t_{2h}. Many cysts from *Pi-RNH1*-silenced lines were large and multinucleated, whereas control cysts were smaller and contained only one nucleus.

3. Discussion

A putative DEAD-box RNA-helicase gene, *Pi-RNH1*, from *P. infestans* was identified and shown to be significantly up-regulated in

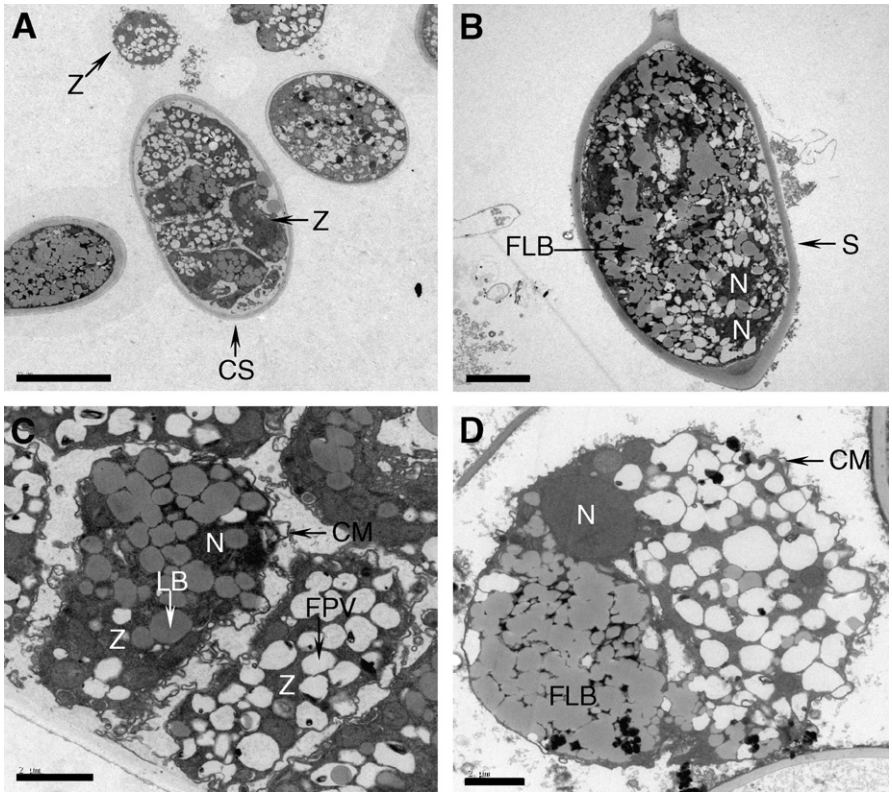

Fig. 5. Transmission electron microscopy studies of the dsRNA treated lines derived from wild-type strain CY29. (A) A cleaving sporangium (CS) of a *gfp*-dsRNA-treated line containing 6 zoospores (Z), 30 min after cold shock treatment. Scale bar represents 10 μm. (B) A sporangium of a *Pi-RNH1*-dsRNA-treated line two hours after cold shock treatment. The sporangium (S) is undifferentiated and several vesicles, lipid bodies, are fusing (FLB). Also indicated are some nuclei (N). Scale bar represents 5 μm. (C) Two zoospores (Z) in a cleaving sporangium of a *gfp*-dsRNA-treated line 30 min after cold shock. Indicated are the nucleus (N), the finger print vacuoles (FPV), lipid bodies (LB) and cellular membrane (CM). Scale bar represents 2 μm. (D) Large zoospore (Z) of a *Pi-RNH1*-dsRNA-treated line two hours after cold shock. Indicated are the nucleus (N), fusing lipid bodies (FLB), and the cellular membrane (CM). Scale bar represents 2 μm.

cleaving sporangia and zoospores. Functional studies demonstrated that *Pi-RNH1* is essential for zoospore development. Transmission electron microscopy revealed that *Pi-RNH1*-silenced lines produced large aberrant zoospores that had formed due to partial cleavage of sporangial cytoplasm and these zoospores often had multiple flagella on their surface. The *Pi-RNH1*-silenced zoospores were also sensitive to osmotic pressure with large zoospores often rupturing upon release from sporangia. We hypothesize that the RNA helicase-like protein is likely to play a role in activation of pre-mRNA molecules. This would explain the pleiotropic effects upon *Pi-RNH1*-silencing, since translation of intron-containing pre-mRNA's may be stalled.

RNAi, a transient gene silencing method triggered by sequence-specific dsRNA, has previously been demonstrated to occur in *P. infestans* (Whisson et al., 2005). At that time we demonstrated that two genes, *inf1* and *cdc14*, could successfully be silenced in *P. infestans* after bathing protoplasts in high concentrations of dsRNA that is complementary to these target genes. Silencing the genes by RNAi caused a significant reduction in respective mRNA expression levels, resulting in a change in phenotype (Whisson et al., 2005). Silencing efficiencies of up to 99% were achieved in some of the transiently silenced lines, which allowed functional characterization of *Pi-RNH1*. *Pi-RNH1* silencing was tested in two *P. infestans* strains, which resulted in similar phenotypes. Since the method is dependent on the efficiency of dsRNA uptake by the protoplasts, several lines were found that exhibited a range of both silencing efficiency and phenotypic changes. In general, lines that showed the most severe phenotypes also showed the highest levels of silencing. This is in line with previous observations in which we silenced four cellulose synthase genes by employing the same RNAi method. Again the strongest phenotypic changes were observed in the most silenced lines (Grenville-Briggs et al., 2008).

It should be noted that it is inevitable that individual sporangia within the silenced populations of individual lines will have different silencing efficiencies and thus show different severities of any potential phenotype. Nevertheless, we consider this technique a major breakthrough in functional characterization of genes in *P. infestans*, since it enables a more rapid identification of silenced phenotypes than conventional transformation methods. Furthermore, it could be used for high-throughput screening of target genes, and is potentially useful for functional characterization of essential genes.

Several studies have been conducted to investigate the role of calcium in sporangial cleavage and zoospore release and encystment (reviewed by van West et al., 2003). Zoosporogenesis requires a large increase in cytoplasmic Ca^{2+} concentration, thought to be triggered by phospholipid signals (Jackson and Hardham, 1996). A second, prolonged rise in Ca^{2+} is required during cytoplasmic cleavage to regulate cytokinesis. It was found that zoospore cleavage is blocked by phospholipase C (U-73122) and inositol trisphosphate (IP_3)-gated Ca^{2+} channel (2-APB) inhibitors (Tani et al., 2004). A model was proposed where immersion either releases a germination inhibitor or hydrates a receptor on the sporangial surface. The low temperature required for cold shock reduces plasma membrane fluidity, which stimulates membrane bound sensors such as phospholipase or G-proteins. Ca^{2+} enters the sporangial cytoplasm through activation of an IP_3 receptor-gated calcium channel, activating proteins involved in zoosporogensis (Tani et al., 2004; Judelson and Blanco, 2005). Previously,

Judelson and Roberts (2002) found that the calcium channel blocker verapamil and the calmodulin antagonist trifluoroperazine inhibit zoosporogenesis and encystment (Judelson and Roberts, 2002). A reduction in zoospore release was observed after treatment and in some cases the sporangial cytoplasm was released as an uncleaved, multiflagellated cytoplasmic mass, which burst shortly after release. Interestingly, a similar phenotype was observed in the *Pi-RNH1*-silenced zoospores, which suggests that silencing *Pi-RNH1* may have affected mRNA processing of one or more genes involved in Ca^{2+}-dependent release processes.

TEM revealed several phenotypic changes in the *Pi-RNH1*-silenced lines. After 30 min of induced zoospore release a large number of sporangia were found to have a less-differentiated cytoplasm with aberrant large lipid bodies, whereas, wild-type sporangia, zoospores, and cysts contained small round lipid bodies within their cytoplasm. The observation of vesicle fusion was also made in ageing sporangia of *P. palmivora* (Hemmes and Hohl, 1975). Similarly we noticed vesicle fusion to some extend also in the *gfp*-dsRNA and wild type lines. However in the *Pi-RNH1*-silenced sporangia this observation was much more pronounced and it appeared that lipid bodies had fused to produce several very large vesicles. This phenomenon was also seen in zoospores and cysts of *Pi-RNH1*-silenced lines. At present we do not have an explanation for why and how the *Pi-RNH1*-silenced sporangia seem to accelerate lipid body fusion in the cytoplasm. Vesicle fusion in cleaving sporangia has been reported before (reviewed by Hemmes, 1983) and it was proposed that this process is required to generate the new plasma membrane of the zoospore. It was argued that sporangial cleavage is a two-stage process in which specialized cleavage vesicles first become positioned at the boundaries of each future subdivision and secondly these vesicles fuse to compartmentalize the sporangium. However, rapid freezing and freeze substitution studies performed with cleaving sporangia of *P. cinnamomi* by Hyde et al. (1991), indicated that cleavage is more likely to result from the progressive extension of paired sheets of membrane along the future subdivision boundaries. It was found that these sheets finally interconnect and subdivide the sporangium. Hyde et al. (1991) only found cleavage vesicles in the preliminary stages of the cleavage process and the vesicles were never aligned along the future boundaries. Apparently the initial hypothesis as reviewed by Hemmes (1983) results from artifacts introduced due to chemical fixation (Hyde et al., 1991). Since we analyzed chemically fixed material we were not able to detect paired sheets of membranes in the cleaving sporangia.

A further phenotype observed in the silenced lines was greatly reduced zoospore mobility as a result of incomplete cleavage. Wild type zoospores have an α-helical swimming pattern brought about by two flagella. The anterior flagellum is responsible for pulling and the posterior for steering, enabling zoospores to successfully target host tissue. The large aberrant zoospores were unable to swim in the α-helical pattern probably due to the presence of multiple flagella, which causes the zoospore to be pulled in several different directions simultaneously, resulting in a 'rolling' movement. The rolling movement and spherical shape of the large aberrant zoospores resembled, to some extent, the characteristic rolling movement of the alga, Volvox (Kirk, 2004).

At present, only two *P. infestans* genes, a G-alpha gene (*Pigpa1*; Latijnhouwers et al. 2004) and a bZIP transcription factor (*Pibzp*; Blanco and Judelson 2005) have been characterized as having a role in zoospore motility. Silencing of each gene resulted in aberrant swimming patterns of the zoospores. However, the swimming phenotypes were different from the one we observed here with the silenced lines of the putative RNA-helicase. The *Pigpa1*-silenced mutants produced zoospores that appeared normal, but they turned six to eight times more frequently, while zoospores of the *Pibzp* silenced mutants spun in tight circles causing them to swim only short distances compared to wild-type zoospores.

At present, it is unclear whether sporangia are pre-programmed to undergo zoosporogenesis or if *de novo* transcription is required, since some evidence for both possibilities exist. For example, the transcription inhibitor actinomycin D does not block zoospore release in *Phytophthora* (Clark et al., 1978; Penington et al., 1989). Consequently it can be concluded that *de novo* transcription is not required for zoosporogenesis, which strongly argues in favor of pre-programming. It is interesting to note that Chytridiomycetes, zoosporic fungi that produce and release zoospores in a similar way to oomycetes, have mRNA stored in the nuclear cap prior to germination of the zoospore (Gong and Lovett, 1977; Smith and Burke, 1979; Silva et al., 1987). The same study also found that treatment with actinomycin-D had no effect on protein synthesis of germinating zoospores of *Blastocladiella emersonii* (Silva et al., 1987).

Following the experiments in *Phytophthora* performed by Clark et al. (1978), Penington et al. (1989) described above, Judelson and Roberts (2002) demonstrated that actinomycin D may not have entered into the sporangia rapidly enough to inhibit transcription. Their own work concludes that *de novo* RNA synthesis does take place in sporangia as transcription of a protein kinase gene is induced in sporangia within minutes of a cold shock both in the presence or absence of actinomycin D (Judelson and Roberts 2002). Further experiments demonstrated that this kinase interacts with a bZIP transcription factor that is required for normal zoospore swimming and infection of host tissue (Blanco and Judelson, 2005). Furthermore, Tani and Judelson (2006) described the discovery of a so-called cold-box-motif in the promoter region of a group of genes up-regulated during zoosporogenesis in *P. infestans*. Their data suggests that a pathway exists in which sporangia perceive cold temperatures through membrane rigidity, which in turn activates signals that drive both zoosporogenesis and cold-box-mediated transcription. This cold-box-motif was not found in the promoter region of *Pi-RNH1* (data not shown).

The discoveries made by these researchers strongly argue that *de novo* transcription takes place during zoospore development. However, their experiments do not demonstrate that *de novo* transcription is required for normal zoosporogenesis, nor do their experiments dispute earlier results obtained by Clark et al. (1978), Penington et al. (1989). Therefore, it remains possible that both *de novo* transcription and pre-programming could play a role in zoosporogenesis.

In many eukaryotic organisms pre-made or 'masked' RNAs have been found and they play an important role in several developmental processes. Sexual fate in *Caenorhabditis elegans* hermaphrodite germline and the specification of pattern along the anteroposterior body axis in *Drosophila* are regulated by maternal mRNAs (Wickens et al., 2000). This translational control of masked mRNAs regulates the formation of oocytes and the progression of cleavage in organisms such as Xenopus, Drosophila, mice and the surf clam *Spisula solidissima* (Schultz, 1993; Standart, 1992; Wickens et al., 2000; Wormington, 1993). Interestingly, oocyte experiments performed by Minshall et al. (2001) revealed that a DEAD-box RNA helicase, p54, is important for repression of translation of the stored pools of masked RNAs. This particular RNA-helicase belongs to the transcription initiation factor 4α (eIf4α) family. However, it is envisaged that other RNA-helicases such as members of the DBP2 family may be required to activate the pool of masked mRNAs, since these DBP2-like RNA helicases have been shown to be involved in pre-mRNA splicing and cell cycle progression (Ludgren et al., 1996; Imamura et al., 1998; Liu, Z., 2002; Will et al., 2002; Lin et al., 2005). Thus, it is tempting to speculate that *Pi-RNH1*, which shows high homology to the DBP2-like RNA-helicases, might play a role in activating pre-mRNAs and possibly masked mRNAs if these were to be present in *Phytophthora* sporangia.

In summary, our experimental data indicate that *Pi-RNH1* is required for normal zoospore development and that silencing the gene resulted in pleiotropic phenotypic effects possibly due to incorrect processing of pre-mRNAs.

4. Experimental procedures

4.1. Growth of *P. infestans*, potato plants, plant inoculation

Growth of *P. infestans* strains 88069 and CY29, potato plants, and plant inoculation with *P. infestans* strain 88069 were carried out as described in Grenville-Briggs et al. (2005). Samples of mature leaves were taken before inoculation (B0), and at 24 h post inoculation (hpi) (B24), 48 hpi (B48) and 72 hpi (B72), and leaf material from each time point was combined prior to RNA extraction.

4.2. Cloning and sequence analysis of Pi-RNH1

Initially, several ESTs were identified in Genbank that correspond to the 5′ start region (e.g. CV950429) or the 3′ stop region (e.g. CV948652) of the putative open reading frame. Primers RH-intron-F and RH-intron-R (Table 1) were designed to amplify the missing part of *Pi-RNH1* from both genomic DNA and cDNA. The amplified fragments were cloned into pGemT-easy (Promega, USA) and sequenced in both directions, using AB BigDye Terminator cycle sequencing kit and ABI Model 377 DNA sequencer (Applied Biosystems, USA).

Pi-RNH1 homologs were found using BLAST searches of the Swissprot, genbank, and *P. sojae* and *P. ramorum* databases (http://genome.jgi-psf.org/). Multiple sequence alignment of representative homologues using the conserved region corresponding to amino acids 143–518 of *Pi-RNH1*, and phylogenetic analysis was carried out using the MEGA3 software (Kumar et al., 2004) Phylogenetic trees were constructed by Neighbor-Joining analysis, and the inferred phylogeny was tested by 1000 bootstrap replicates. Genbank Accessions are: *Oryza sativa* (NP_913140), *Aspergillus nidulans* (XP_663535), DBP2 *Kluyveromyces lactis* (Q6CIV2), *Dictyostelium discoideum* (XP_629279), DDX53 HUMAN (Q86TM3), DDX3Y MOUSE (Q62095), DDX4 HUMAN (Q9NQI0), DDX21 HUMAN (Q9NR30), RHLB *E. coli* (P0A8J8), eIF4A *Nicotiana plumbaginifolia* (P41380), DBPA *Bacillus subtilis* (P42305), EXP9 *Streptococcus pneumoniae* (P0A4D8), DEAD *Shigella flexneri* (P0A9P8), DDX28 MOUSE (Q9CWT6). JGI protein ids are: *P. sojae* (119997) and *P. ramorum* (71687).

4.3. RNA-interference

Oligionucleotide primers were designed to amplify a 196 bp fragment for the *P. infestans* gene *Pi-RNH1* and a 199 bp fragment of the non-endogenous control *gfp* (Table 1). RNA-interference was essentially carried out as described by Whisson et al. (2005). Transfection was allowed to proceed overnight at 20 °C. Protoplasts were transferred to pea broth and allowed to regenerate as single colonies for three days before transfer to solid agar plates. Fifteen days after transfection, agar plates were flooded with 10 ml of cold sterile distilled water and zoospores were harvested as described by van West et al. (1998). The *P. infestans* EST sequences deposited in Genbank were screened with the 196 bp fragment of *Pi-RNH1* sequence (using BLASTN) to investigate whether there are stretches of 20 nucleotides or more in other open reading frames that are completely identical. We envisage that theoretically, only a short 20–30 bp region of similarity is needed to potentially generate off-target effects with RNAi. However, we were unable to identify any other sequence in the EST database that had significant homology. Consequently we are confident that the observed phenotype in the *Pi-RNH1*-silenced lines was solely due to silencing of the *Pi-RNH1* gene.

4.4. SYBR green real-time RT-PCR assays

Template cDNA was derived from mycelium grown in rye broth, non-cleaving sporangia, cleaving sporangia, zoospores, germinating cysts and germinating cysts with appressoria, as well as potato leaves before inoculation (B0), and at 12 h post inoculation (hpi) (B12), 24 hpi (B24), 33 hpi (B33), 48 hpi (B48), 56 hpi (B56) and 72 hpi (B72). The *actA* gene from *P. infestans* was used as a constitutively expressed endogenous control and the expression of *Pi-RNH1* gene in sporangia was determined relative to *actA* using the $\Delta\Delta C_t$ method, as described in Avrova et al. (2003). Expression of *Pi-RNH1* in different life cycle stages was compared to the level of its expression in a calibrator sample, which was cDNA from non-cleaved sporangia. The expression of *Pi-RNH1* in the sporangia cDNA sample was assigned the value of 1.0. For expression of *Pi-RNH1* in potentially silenced lines, the calibrator sample was the mean expression value from all the control lines for each gene; this mean number was assigned the value of 1.0. RT-PCR assays for the expression of *Pi-RNH1* throughout the lifecycle were carried out using two biological replicates each containing three technical replicates. RT-PCR assays for the expression of genes in silenced lines were carried out using three technical replicates. It is not possible to use biological replicates of individual silenced lines, since each line was harvested on the day of maximum silencing (15 days after transfection) and only produced enough RNA for a single assay containing three replicates. RNAi is a transient phenomenon and by 20 days after transfection of *P. infestans* expression levels of silenced genes return to normal (Whisson et al., 2005; Grenville-Briggs et al., 2008), therefore each assay using silenced lines was performed using three technical replicates, with individual lines serving as biological replicates for the experiment as a whole.

4.5. Transmission electron and light microscopy

Samples for transmission electron microscopy (TEM) were harvested and fixed in 0.5 ml of 2.5% glutaraldehyde in 0.1 M PBS (pH 7.3) overnight. The following day, samples were washed in water, the cells collected by centrifugation and embedded in 3% low melting point agarose in distilled water. Thereafter, agarose embedded cells were post-fixed in osmium tetroxide and uranylacetate, dehydrated in ethanol, and embedded in Epon. Ultrathin sections (60–90 nm) were stained using uranylacetate and lead citrate and then examined using a Philips EM201 microscope. Light microscopy was carried out with an Axioplan 2 microscope (Carl Zeiss Ltd., UK). Microscopy images were captured using the Openlab system openlab v3.0 (Improvision, Coventry, UK) with a Hamamatsu C4742-95 digital camera (Hamamatsu Photonics, Hamamatsu, Japan).

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