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Plant-induced cell death in the oomycete pathogen *Phytophthora parasitica*

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Summary

The activation of programmed cell death in the host during plant–pathogen interactions is an important component of the plant disease resistance mechanism. In this study we show that activation of programmed cell death in microorganisms also regulates plant–pathogen interactions. We found that a form of vacuolar cell death is induced in the oomycete *Phytophthora parasitica* – the agent that causes black shank disease in *Nicotiana tabacum* – by extracellular stimuli from resistant tobacco. The single-celled zoospores underwent cell death characterized by dynamic membrane rearrangements, cell shrinkage, formation of numerous large vacuoles in the cytoplasm and degradation of cytoplasmic components before plasma membrane disruption. *Phytophthora* cell death required protein synthesis but not caspase activation, and was associated with the production of intracellular reactive oxygen species. This characterization of plant-mediated cell death signalling in pathogens will enhance our understanding of the biological processes regulating plant–pathogen interactions, and improve our ability to control crop diseases.

Introduction

Programmed cell death (PCD) as a regulated and active mechanism has been described in eukaryotes at all stages of the evolutionary process: from amoebae to plants, nematodes, insects and mammals (Ameisen, 2002; Golstein *et al*., 2003). PCD can take many different forms including apoptosis, necrosis and autophagic vacuolar cell death (Kerr *et al*., 1972; Clarke, 1990; Golstein *et al*., 2003). In vertebrates and invertebrates, the apoptotic pathway is probably the most well-defined cell death program. Several biochemical and genetic approaches have been used to demonstrate that caspase family proteinases play a major role in apoptosis (Ellis and Horvitz, 1986; Nicholson and Thornberry, 1997). In higher plants, a form of PCD is associated with the hypersensitive response (HR), an induced defence strategy against pathogen infection that causes the death of host cells and restricts pathogen growth (Morel and Dangl, 1997; Pennell and Lamb, 1997; Lam, 2004). The virus-induced hypersensitive cell death is mediated by the vacuolar protease VPE in tobacco (Hatsugai *et al*., 2004). PCD pathways have also been shown to exist in unicellular or multicellular microorganisms (Cornillon *et al*., 1994; Christensen *et al*., 1998; Madeo *et al*., 1999; Vardi *et al*., 1999; Lee *et al*., 2002; Pinan-Lucarre *et al*., 2003) including kinetoplastid human parasites, such as *Trypanosoma cruzi* and *Leishmania amazonensis* (Ameisen, 2002). In these parasites the regulation of PCD ensures that cell differentiation is tightly coupled to cell survival (Ameisen *et al*., 1995; Moreira *et al*., 1996). Few studies have investigated the activation of cell death in plant pathogens or analysed the cell death pathways operating in these microorganisms. In addition, few studies have attempted to evaluate the role played by plant pathogen cell death in plant–pathogen interactions. Narasimhan *et al*. (2001) have shown that the tobacco defence protein osmotin activates apoptotic cell death in *Saccharomyces cerevisiae*, suggesting that plant stimuli can induce PCD in pathogens.

A major class of plant pathogens is found in the group Oomycetes. These eukaryotic microorganisms undergo filamentous growth and have sexual/asexual cycles. They are classified with chrysophytes, diatoms and brown algae within the taxon of Stramenopiles (Kumar and Rzhetsky, 1996; Paquin *et al*., 1997; Van de Peer and De Watchter, 1997). In this group, *Phytophthora* is an important genus of plant pathogens and most species cause devastating diseases in numerous economically important plant species (Erwin and Ribeiro, 1996; Kamoun *et al*., 1999; Judelson and Blanco, 2004). *Phytophthora* infection begins

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when the sporangia release unicellular, biflagellated zoospores on plant tissue. The zoospores are initially only surrounded by a cell membrane. After a motile period they encyst and develop a primary cell wall. These cysts then produce a germ tube. Plant colonization occurs when the oomycetes penetrate directly into the intercellular spaces or after formation of an appresorium. As the interaction proceeds, new sporangia are formed on the plant surface. The success of infection depends initially on the survival capacity of the zoospores at landing site on host tissue. This site may be the root surface, the upper plant where zoospores can be spread by splashing, or artificial and natural wounds (caused by cuttings or induced by wind or rain). At this site, plants may emit attractants forming concentration gradients from both root tips and wound sites, which attract zoospores and presumably represent optimal sites for infection (Tyler, 2002; Judelson and Blanco, 2004).

Here we describe the cell death induction in zoospores of *Phytophthora parasitica* at the infection site on wounded leaves of *Nicotiana tabacum*. As in most plant–pathogen interactions, the HR induced by *Phytophthora* infection is crucial for the ability of hosts to control disease (Kamoun *et al*., 1994; Ricci, 1997). Plant resistance to *Phytophthora* may also depend on the viability of the zoospores, a property regulated by host molecules. During the early stages of plant development, when the viability and germination of zoospores is promoted, *N. tabacum* is highly susceptible to leaf infection by *P. parasitica.* An HR-independent resistance pathway, involving zoospore-specific cytotoxic activity, develops during the later stages of *N. tabacum* development (Hugot *et al*., 1999). Thus, the commitment of pathogenic cells to germination or to death is dependent on stimuli from susceptible or resistant plants. We show that in resistant plant, zoospore death is triggered at the infection site, a property which can be regulated by apoplastic host molecules. To characterize zoospore death and to evaluate its role in pathogenic interactions, we developed a plant-free model system to study pathogen responses to cell death-inducing plant stimuli. We show that extracellular signals from a resistant plant can activate PCD in a plant pathogen. We demonstrate that tobacco-induced cell death in *P. parasitica* exhibits characteristics of vacuolar cell death and that *Phytophthora* cell death is associated with the production of reactive oxygen species. We propose that the regulation of both plant and pathogen PCD plays a central role in determining the outcome of plant–pathogen interactions.

Results

Induction of cell death in Phytophthora parasitica

For different soil-borne *Phytophthora* species penetration into host tissues occurs preferentially by a wound (Kaiser and Melendez, 1978; Biles *et al*., 1993; Adorada *et al*., 2000; Salas *et al*., 2000; Thomidis, 2003). In the case of *P. parasitica*, infection does not occur without wounding of *N. tabacum* leaves (Fig. 1A). The aerial contamination

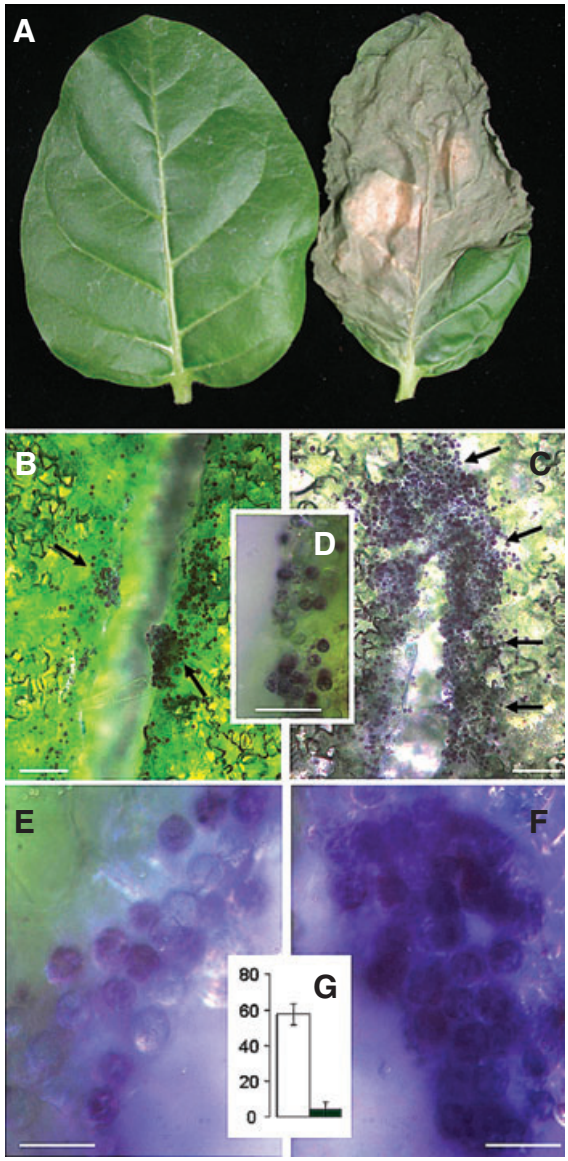

Fig. 1. Influence of host wounding on landing and viability of *P. parasitica* zoospores at leaf surface.
A. Tobacco leaves inoculated twice with 100 zoospores by the lower epidermis, immediately after wounding with a scalpel (right) or without wounding (left), 7 days after inoculation.
B–D. Micrographs of wounded tobacco leaves infected by immersion with zoospores (500 cells μl^{-1}) showing highly localized accumulation of cysts at the wound site (black arrows) after 45 min.
E–G. (E and F) Trypan blue staining and (G) percentage of living cysts at wound sites on leaf tissues from young susceptible plants (E and white bar in G) and from old resistant plants (F and black bar in G), 1 h after inoculation.
Bars: 100 μm in (B) and (C), 50 μm in (D), 20 μm in (E) and (F).

occurs during projection of water by zoospores splashing (Robin and Guest, 1994; Blancard, 1998), precisely at the level of wounded tissues. This phenomenon was visualized after immersion of tobacco wounded leaf tissues (Xanthi-nc) in a suspension of swimming zoospores of *P. parasitica*. In these conditions, the zoospores migrated and landed specifically at wounded sites before encystment and germination. This phenomenon was so spectacular that after 45 min of incubation wounds were decorated with aggregates of *Phytophthora* cells (Fig. 1B and C) in which most of the cells were ungerminated (Fig. 1D). A trypan blue test indicated that at this infection site the survival of *Phytophthora* cells before germination is crucial for regulation of *N. tabacum–P. parasitica* interactions. Indeed, viability of landing zoospores and then cysts was drastically lower on wounded tissues from old resistant plants (Fig. 1F and black bar in Fig. 1G) than on tissues from young susceptible plants (Fig. 1E and white bar in Fig. 1G). In this environment, these cells are in contact with all cellular compartments, and by incubation of zoospores with intercellular fluids, we reproduced, *in vitro*, the conditions of regulation of *Phytophthora* cells *in planta* (Hugot *et al*., 1999). Cell death was not induced when zoospores were incubated in a cleared crude extract prepared from resistant leaves after obtaining the intercellular fluid or in boiled intercellular fluid. These results indicate that, at sites for infection, heat-labile apoplastic content influences the viability of zoospores

To characterize *Phytophthora* cell death, we used the *in vitro* model system to reproduce the conditions present during the *Nicotiana–Phytophthora* interaction. We isolated *P. parasitica* zoospores from sporangia and incubated them with water, with the intercellular fluids from young susceptible (IFS) or old resistant (IFR) tobacco plants of the same line, Xanthi-nc (Hugot *et al*., 1999). In water, zoospores were motile for between 3 and 5 h. Incubation with IFS and with IFR induced synchronized germination or cell death respectively. Zoospores incubated with IFS lost their flagella after 10–20 min of treatment. In these conditions, the cysts displayed circular cytoplasmic movements (Movie S1 in *Supplementary material*). An average of 60 ± 10% of cysts germinated within 2 h of incubation and then produced elongated hyphae.

Induction of cell death in IFR-treated zoospores led to the loss of flagella within 1 or 2 min. In contrast to germinating cells, the cytoplasmic contents of cells undergoing cell death were static, whereas intense activity took place at the membrane. These dynamic membrane rearrangements were associated with cell shrinkage: a 20% decrease in cell size was observed after 90 min of treatment (Movie S2 in *Supplementary material*). The majority of cells lost their integrity after 2 h of incubation (95 ± 2%). The results of the trypan blue exclusion test showed that this loss of integrity could be considered as cell death. These cells were unable to germinate, even after an additional 24 h incubation with IFS or with a malt medium that supports *Phytophthora* growth. Cells incubated with IFR also formed cell aggregates. Most of the cells in these aggregates died but a few finally germinated. Thus, only a minority of the cells (4 ± 2%) were able to germinate in the presence of IFR.

Organelles and cell death

We investigated *Phytophthora* cell death by staining the organelles in dying cells and comparing these staining patterns with those in healthy zoospores, in cysts before the emergence of a germ tube and in germinated cysts. No nuclear fragmentation was detected in the dying cells with the karyophilic dye, Hoechst 33258. In a TUNEL (terminal deoxy uridine triphosphate nick end labelling) staining assay, 72 ± 12% of the dying cells were labelled after 2 h of incubation, whereas only a small number of germinating cells (6 ± 4%) were labelled (data not shown).

Mitochondrial modifications in the IFR-treated cells were revealed by staining with MitoTracker Red CM-H2XROS. This cellular probe only becomes fluorescent when it oxidized after entering actively respiring cells. We found that mitochondrial staining was very strong in IFR-treated cells undergoing cell death, indicating that this process was associated with high levels of respiratory activity. Round-shaped structures that were heterogeneous in size were detected in the dying cells 2 h after the beginning of the incubation with IFR. The width of these structures (2.2 ± 0.8 μm; Fig. 2G) was greater than that measured for any of the internal structures detected in zoospores (1.0 ± 0.2 μm; Fig. 2A), in cysts (1.0 ± 0.3 μm; Fig. 2B) or in germinated cysts (1.2 ± 0.3 μm; Fig. 2C). These observations are indicative of mitochondrial swelling or sequestration of content of these organelles in vacuoles. We observed similar staining patterns using rhodamine 123 as mitochondrial probe (compare Fig. 2H with D–F).

Induction of cell death also led to the formation of structures that were stained with monodansylcadaverine (MDC), a marker for lysosomal/autophagic vacuoles (Biederbick *et al*., 1995). Before incubation with IFR or IFS, only 2 ± 0.3% of the cells were stained with MDC. Incubation of cells in water for 2 h had no effect on the level of MDC staining (Fig. 3A). Treatment with IFS led to a small increase in the number of MDC-labelled cells: 12 ± 5% of the cysts (Fig. 3B) and 17 ± 11% of the geminated cysts (Fig. 3C) were stained respectively. The induction of cell death led to a large increase in the number of MDC-labelled vacuoles: 85 ± 10% of these cells were stained with MDC (Fig. 3D). In addition, the staining pattern observed in the IFR-treated cells was strikingly

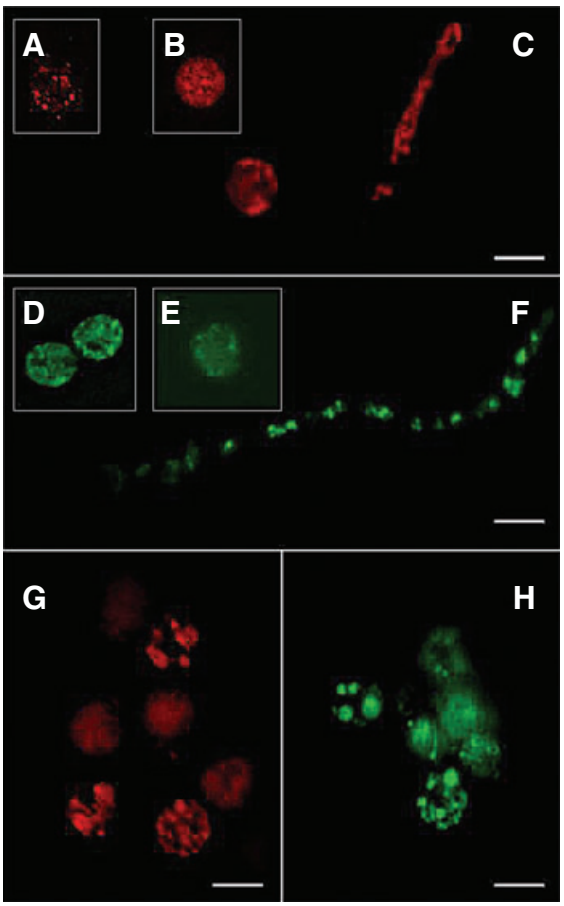

Fig. 2. Staining of *Phytophthora* cells with mitochondrial probes. MitoTracker Red CM-H2XROS (A–C and G) and rhodamine 123 (D–F and H) staining were monitored at 2 h in the water-treated zoospores (A and D), at 45 min in the IFS-treated cysts (B and E), at 2 h in the IFS-treated germinated cysts (C and F), at 2 h in the IFR-treated dying cells (G and H). Dying cells contain large and strongly stained structures (G and H). These structures are smaller in the zoospores (A and D), in cysts (B and E) and in germinated cysts (C and F). Bars, 10 μm.

different from that observed in the IFS-treated cells. Cysts contained small (1.1 ± 0.2 μm) weakly stained structures, whereas the dying cells contained intensely stained, large (2.52 ± 1.27 μm) spherical structures. Colocalization of MDC and MitoTracker Red CM-H2XROS was not observed in zoospores (Fig. 3A, E and I), in cysts (Fig. 3B, F and J) and in germinated cysts (Fig. 3C, G and K) whereas it was common in dying cells (Fig. 3D, H and L): the co-staining reached 72 ± 2% in dying cells, 1 ± 0.2% in zoospores, 3 ± 1% in cysts and 7 ± 4% in germinated cysts. Co-localization of MDC and Nile red, a probe for lipid droplets, was also observed in dying cells but not in zoospores or cysts (data not shown). The colocalization of MDC and stains that label cytoplasmic organelles suggests that cellular material was sequestered in membrane vacuoles during the cell death process. However, we cannot rule out the possibility that the various cellular probes had lost their specificity.

Ultrastructural characterization

To further investigate the *Phytophthora* cell death process, we examined the cellular ultrastructure of cells by transmission electron microscopy. Our analysis revealed that a cell wall surrounds the cysts by the early stages of germination and that these germinating cells contain fully developed organelles, which migrate towards the growing germ tube (Fig. 4).

Zoospores that had only just been released from sporangia had a central nucleus containing a highly visible nucleolus. All the cytoplasmic components were present in these cells together with rod-shaped or globular mitochondria surrounded by an outer membrane and with conspicuous cristae (Fig. 5A). A similar intracellular architecture was observed in zoospores that had been incubated for 1 h with water (Figs 5B and 6A) or in ungerminated cysts (Fig. 5C).

We observed gradual degradation of cellular components and concomitant vacuolization in zoospores undergoing cell death (Fig. 5D–F). The cytoplamic content was progressively constricted to thin layers between the plasma membrane and the large vacuoles. These vacuoles gradually increased in size. The mitochondria, like most other cellular structures, were still intact after 30 min of treatment with IFR. The electron density of the plasma membrane revealed its structural integrity at this time point. Similar observations concerned the mitochondrial cristae (Fig. 6B). These results, together with those obtained using the MitoTracker Red CM-H2XROS stain, show that mitochondria remain structurally and functionally intact during the early stages of the cell death process. However, the mitochondria began to degenerate as length of the incubation increased (60–90 min after the start of the treatment). The total number of mitochondria per cell was substantially lower for the dying cells than for the zoospores or the cysts. There was also an overall decrease in the number of cristae, accompanied by a vesiculation after 90 min of incubation (Fig. 6C). At this time point, vacuolization was nearly complete: enlarged vacuoles occupied up to 50–60% of the cellular volume (Figs 5F and 6C). Some cells contained intravacuolar vesicles (400–600 nm) or vesicles predominately located adjacent to the vacuoles. These vesicles contained membrane-derived components and granular masses of cytoplasm (Fig. 7). The cells did not have a well-defined cell wall at any of the time points analysed. They were surrounded by a plasma membrane, which progressively lost its electron density (Fig. 6C). Thus, we found that the prominent features of cell death were the degradation of

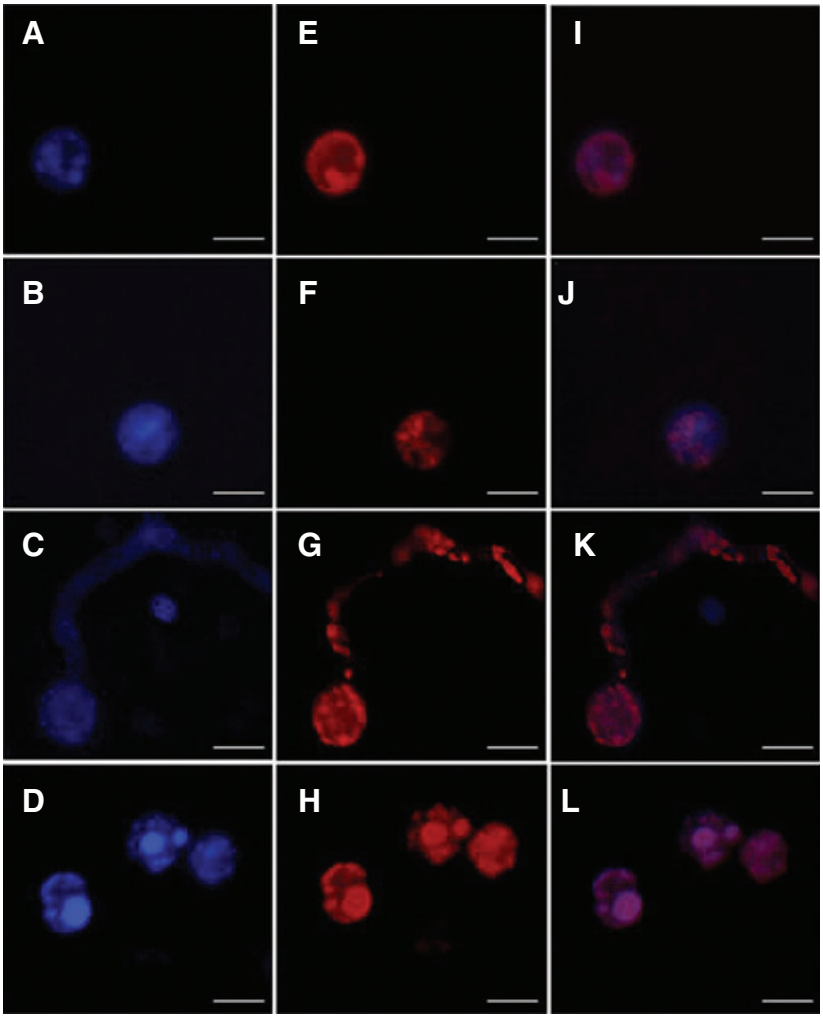

Fig. 3. Co-staining of *Phytophthora* cells with monodansylcadaverine (MDC) and MitoTracker Red CM-H2XROS. MDC (A–D) and MitoTracker (E–H) staining were monitored at 2 h in the water-treated zoospores (A, E and I), at 45 min (B, F and J) and at 2 h (C, G and K) in the IFS-treated cysts and at 45 min the IFR-treated dying cells (D, H and I). MDC-labelled structures are specifically present in the dying cells (compare D with A–C). Co-staining with MDC and MitoTracker Red was also frequently observed in the dying cells (D and H). No colocalization of MDC and MitoTracker Red was observed in zoospores (A and E), in cysts (B and F) or in germinated cysts (C and G). (I)–(L) are merged images showing MDC and MitoTracker Red double labelling. Bars, 10 μm.

cytoplasmic components, extensive vacuolization and then disruption of the plasma membrane.

Cycloheximide inhibits cell death

To evaluate whether cellular metabolism participates in the cell death process and its associated phenomena, we analysed zoospores treated with cycloheximide. First, we examined the effect of cycloheximide on the germination of IFS-treated *Phytophthora* to determine the concentration required for complete inhibition of protein synthesis. Freshly released zoospores were pre-incubated with various concentrations of cycloheximide (in water) for 15 min before being exposed to IFS. Pre-incubation with 100 ng ml^{-1} cycloheximide inhibited germination for 2 h, whereas a concentration of 1 μg ml^{-1} cycloheximide inhibited germination for 24 h (Fig. 8A). Analysis of cells stained with trypan blue showed that treatment of the cells with 1 μg ml^{-1} cycloheximide altered moderately cell viability. Thus, we used a concentration of 1 μg ml^{-1} cycloheximide to investigate the effect of inhibiting cellular metabolism on cell death in IFR-treated cells. We found that cycloheximide had a less dramatic effect on cell survival than on germination. However, after 2 h of incubation with IFR, cell viability was significantly higher for cells pretreated with 1 μg ml^{-1} cycloheximide (IFR-C) than for water-pretreated controls (IFR). Percentage cell viability was 15 ± 1.4% for IFR-C-treated cells, whereas it was 34 ± 1.2% for IFS-C-treated cells (Fig. 8B). When cell death was inhibited by cycloheximide, dynamic membrane rearrangements and cell shrinkage were also prevented (Movie S3 in *Supplementary material*). These results indicate that *Phytophthora* cell death and cellular deformation require protein synthesis or *de novo* translation.

The production of reactive oxygen species in zoospores

One of the events taking place during PCD in plants and animals is an oxidative burst and the production of reactive oxygen species. To determine whether induction of

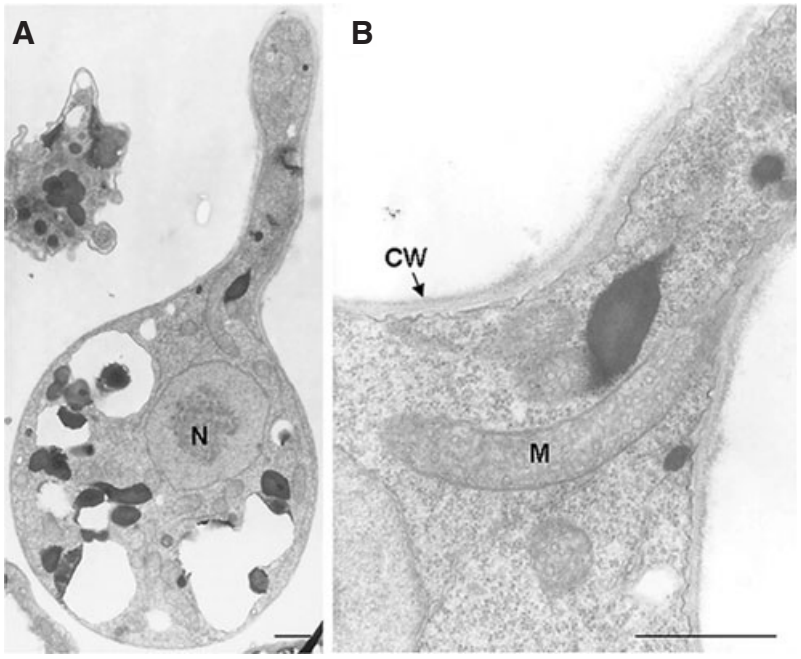

Fig. 4. Ultrastructure of germinating cysts.
A. The germinating cysts develop a germ tube and a cell wall.
B. The cytoplasm migrates in a rostro-caudal pattern.
CW, cell wall; M, mitochondrion. Bars, 1 μm.

cell death in *Phytophthora* was also associated with an oxidative burst, we stained zoospores with uncharged non-fluorescent dihydrorhodamine 123 to detect intracellular free radicals. Dihydrorhodamine 123 diffuses passively across cell membranes and is then oxidized to cationic rhodamine 123, a fluorescent dye that is readily sequestered by active mitochondria. We found that the dramatic loss of viability in IFR-treated cells was associated with strong rhodamine 123 staining (Fig. 9), indicating that the induction of cell death leads to the accumulation of intracellular reactive oxygen species. In IFS, 1.1 ± 1% and 12.4 ± 7% of cysts were stained with rhodamine 123, respectively, after 45 min (Fig. 9A) and 90 min (Fig. 9C) of incubation. None of these cells were germinated. At the same time points, around 35 ± 5% and then 71 ± 10% of IFR-treated zoopores were stained with rhodamine 123 and again we found that most of these cells had not germinated (Fig. 9B and D). The few cells that did germinate were not, or were only weakly, stained with rhodamine 123. Thus, our results show that cell death in *Phytophthora* is associated with the production of reactive oxygen species.

Caspase inhibitors do not block Phytophthora cell death

To investigate whether caspase activity was required for cell death in *Phytophthora*, we treated cells with the cell-permeable fluoromethylketone protease inhibitors benzyloxycarbonyl-Val–Ala–Asp-fluoromethylketone (z-VAD-FMK), benzyloxycarbonyl-Asp–Glu–Val–Asp-fluoromethylketone (z-DEVD-FMK) and benzyloxycarbonyl-Phe–Ala-fluoromethylketone (z-FA-FMK). No toxic effects were detected in zoospores cultured for 30 min in water in the presence of 10 μM and 50 μM concentrations of these inhibitors. After a 2 h incubation with IFR, we detected no significant differences in cell viability between cells treated with the three inhibitors and those treated with water or dimethylsulphoxide (DMSO) (Fig. 10A). These results show that these inhibitors do not block *Phytophthora* cell death. Analysis of zoospores stained before and after incubation in IFR with FITC-VAD-FMK, a conjugate of the cell permeable caspase inhibitor z-VAD-FMK, showed that this inhibitor was internalized (Fig. 10B and C). These data were confirmed by *in silico* analysis. Human caspases sequences (Accession No. P55211 and P42574) were used to query databases and subjected to TBLASTN analysis (expect level: 10) against 8000 *P. parasitica* expressed sequence tags (ESTs) (Panabières *et al*., 2005), 75 000 *Phytophthora infestans* ESTs (Randall *et al*., 2005), 23 000 *Phytophthora sojae* ESTs (http://www.pfgd.org/, Phytophthora Functional Genomics Database) and the genome sequences of *P. sojae* and *Phytophthora ramorum* (http://www.jgi.doe.gov). No sequences related to the caspase family were found. These results indicate the absence of structural and functional orthologues for these proteins in *Phytophthora*.

Discussion

Characteristics of plant-induced cell death in Phytophthora

We investigated the characteristics of plant-induced cell death in a phytopathogenic organism, *P. parasitica*. We

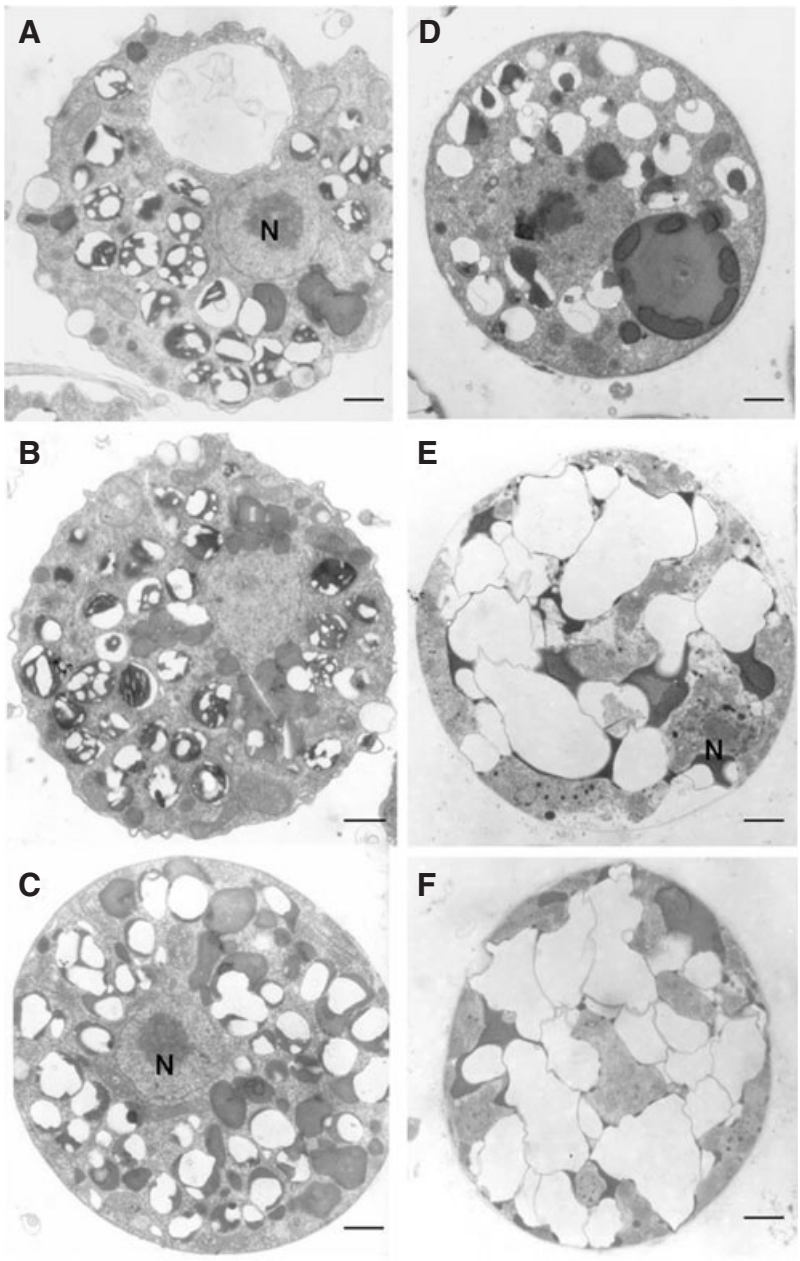

Fig. 5. Ultrastructure of *Phytophthora* cells. Ultrastructure of a representative zoospore freshly released from sporangia (A), of a zoospore incubated 60 min with water (B) and of a cyst (C). Ultrastructure of dying cells after 30 (D), 60 (E) and 90 (F) min incubations with IFR. Progressive vacuolization of the cytoplasm without collapse of the plasma membrane is specifically observed in almost all cells undergoing plant-mediated cell death (D–F). N, nucleus. Bars, 1 μm.

found a very clear correlation between tobacco resistance at late developmental stage and *in planta* cell death of *P. parasitica* cells before germination at wounded sites. We used a model system to reproduce the conditions present during the *Nicotiana–Phytophthora* interaction (Hugot *et al*., 1999). This *in vitro* model system allowed us to analyse this interaction without the constraints of *in planta* analyses. We used synchronized cellular populations to demonstrate that cell death in this plant pathogen is activated by plant stimuli. Two important traits of *Phytophthora* cell death were the rapid appearance in the cytoplasm of large vacuoles and the degradation of cytoplasmic content before plasma membrane disruption. It should be noted that the presence of vacuoles in *Phytophthora* is not exclusive to dying cells. Vacuoles may contribute to maintain turgor in zoospore (Fig. 5A) or accompany organelles transfer from the cyst to the germ tube (Fig. 4). In IFR-treated cells, the vacuolization and the extensive degradation of cytoplasmic components occurred concurrently and neither this process was observed in untreated zoospores or in cysts. Thus, it was not the presence of the vacuoles that signalled the induction of vacuolar cell death in *Phytophthora*, but rather the kinetics of vacuoles growth, the extent of vacuolization and the concomitant

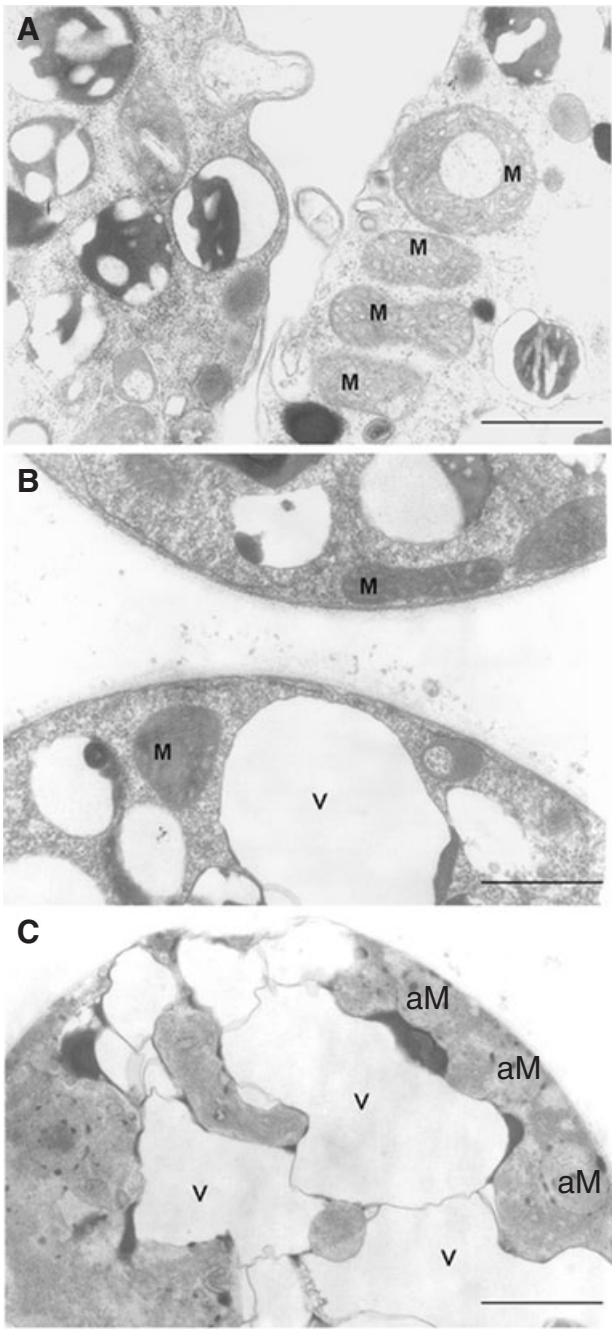

Fig. 6. Morphological changes in the ultrastructure of dying zoospores.
A. After an incubation of zoospores in water, cells display hemispherical projections filled with cytoplasm or water. These projections are caused by a reduction in intracellular osmotic pressure during incubation of the zoospores in water. The mitochondria in these zoospores have a mainly subplasma membrane localization.
B. At an early step of incubation with IFR (30 min) the vacuolization of the cytoplasm is observed with structurally well-defined mitochondria in almost all cells.
C. At late step of incubation with IFR (90 min) extensive vacuolization is associated with loss of plasma membrane electron density, with degradation of cytoplasmic components, and with altered mitochondrial morphology (absence of functional cristae).
N, nucleus; M, mitochondrion; aM, altered mitochondrion; V, vacuole. Bars, 1 μm.

degradation of cytoplasmic content. Vesicles containing cytoplasmic components were detected in dying cells. These vesicles were closely associated with vacuoles suggesting that they may function in processes occurring within these organelles during cell death (Fig. 7). Another trait of cell death was the staining with MDC. This staining was also observed *in planta* for landed and ungerminated cysts at wounded sites from resistant plants indicating that, *in planta*, cell death phenotype is similar to that observed *in vitro* (see Fig. S1 in *Supplementary material*). *In vitro*, the observation of specific co-staining with MDC and with the MitoTracker Red demonstrates the perturba-

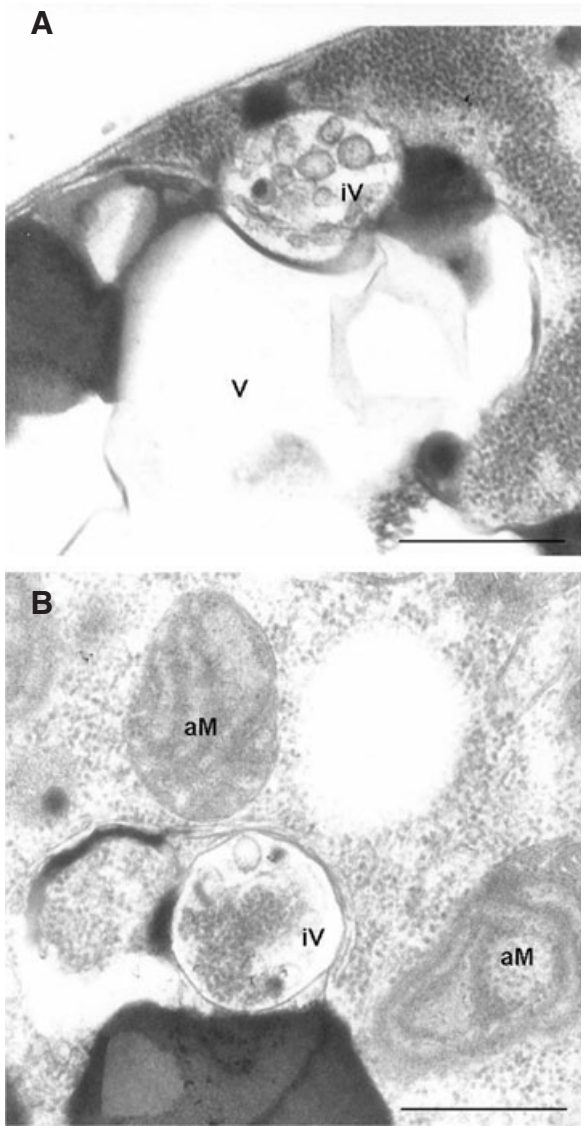

Fig. 7. Intravacuolar vesicles in IFR-treated cells. Vesicles containing partially degraded or electron-dense cytoplasmic material were observed after 60 (A) and 90 (B) min incubations with IFR. The size of the vesicles varies from 400 to 600 nm. aM, altered mitochondrion; iV, intravacuolar vesicle; V, vacuole. Bars, 500 nm.

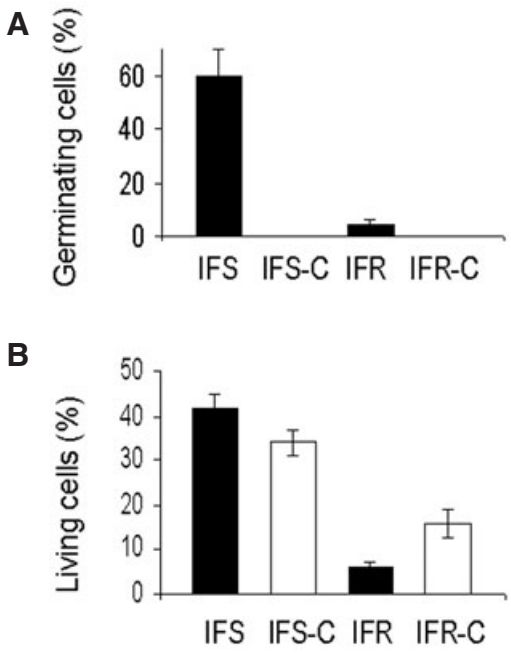

Fig. 8. Effects of cycloheximide on zoospore germination and cell death.
A. Percentage germination of IFS- and IFR-treated cells pre-incubated in the presence (IFS-C, IFR-C) and absence (IFS, IFR) of 1 μg ml^{-1} cycloheximide.
B. Percentage viability of IFS- and IFR-treated cells pre-incubated in the presence (IFS-C, IFR-C) and absence (IFS, IFR) of 1 μg ml^{-1} cycloheximide.
Data were analysed using the Student's *t*-test. The differences between IFR and IFR-C are statistically significant ($P < 0.0005$, $n = 4$).

tion of intracellular compartmentalization during cell death. The labelling of IFR-induced cytoplasmic vacuoles by MDC and the extensive vacuolization were suggestive of autophagic degeneration. However, autophagic vacuoles were not observed by transmission electron microscopy. The additional characteristic features of cell death were translation requirement and production of reactive oxygen species. Taken together, these traits indicate that a form of vacuolar PCD takes place in *P. parasitica* zoospores in response to stimuli from resistant tobacco.

Programmed cell death involving vacuolization has been observed in several biological contexts in unicellular and multicellular eukaryotes from different branches of the phylogenetic tree (Cornillon *et al*., 1994; Sperandio *et al*., 2000; Lee and Baehrecke, 2001; Levraud *et al*., 2003; Pinan-Lucarre *et al*., 2003). Our study on *Phytophthora* indicates that a related form of vacuolar cell death exists in a supplementary class of organisms, the oomycetes. The phylogenetic position of the oomycetes within the Stramenopiles group should make it be extremely interesting to compare the cell death pathways in different taxons (Wyllie and Golstein, 2001; Golstein *et al*., 2003).

Activation and execution of Phytophthora *cell death*

The mechanisms involved in the activation and execution phases of PCD in *P. parasitica* are not known. The characterization of the events that activate cell death in zoospores will be an important step in increasing our understanding of the *Phytophthora* PCD pathway. PCD may be induced in response to a lack of essential growth nutrients in the intercellular fluid of resistant plants. Starvation provokes PCD in several organisms (Golstein *et al*., 2003). Thus, it is possible that the PCD pathway plays an important role in controlling the intercellular growth of *Phytophthora in planta* and that this pathway is triggered when the resources from the host plant become limited. However, this hypothesis is not consistent with observation that zoospores are able to swim for several hours on water, an activity that relies on endogenous food reserves (Skalamera *et al*., 2004) and does not result in extensive activation of the cell death pathway. These observations suggest that *Phytophthora* zoospores are not particularly sensitive to starvation. Alternatively, the PCD pathway may be activated in response to signals from the host. The finding that the phytuberin, a phytoalexin isolated from infected potato tubers, provokes highly vacuolization of intact *P. infestans* zoospores supports this hypothesis (Harris and Dennis, 1977). The only thing known about the putative stimuli from plants inducing *P. parasitica* cell death is that boiling abolishes their activity. The PR-5 protein of tobacco induces apoptotic cell death in *S. cerevisiae* (Narasimhan *et al*., 2001); however, this compound is not present in the intercellular fluids of old resistant plants (Hugot *et al*., 2004), suggesting that it is not involved the activation of *Phytophthora* cell death. The PR proteins of class 1 and 2 are good candidates for activators of *Phytophthora* cell death (van Loon and Strien, 1999). These proteins are secreted into the apoplasm of resistant plants (Hugot *et al*., 2004) and exhibit antimicrobial activity against oomycetes (Mauch *et al*., 1988; Alexander *et al*., 1993; Niderman *et al*., 1995). However, the involvement of these proteins in the induction of cell death in *Phytophthora* has not yet been analysed. Identification of molecules with antimicrobial activity from tobacco will help to characterize the *Phytophthora* PCD pathway.

Mechanisms involved in the execution of PCD in *Phytophthora* also remain to be characterized. We found that cell death occurred concurrently with a rapid accumulation of intracellular reactive oxygen species, suggesting that cell death in *Phytophthora* is associated with an oxidative burst. Further investigations are necessary to establish whether these reactive oxygen species are involved in a signal transduction pathway or in the execution of cell death. Our results also indicate that caspases with high structural similarities to the metazoan homologues are unlikely to be required for cell death in *Phytophthora*.

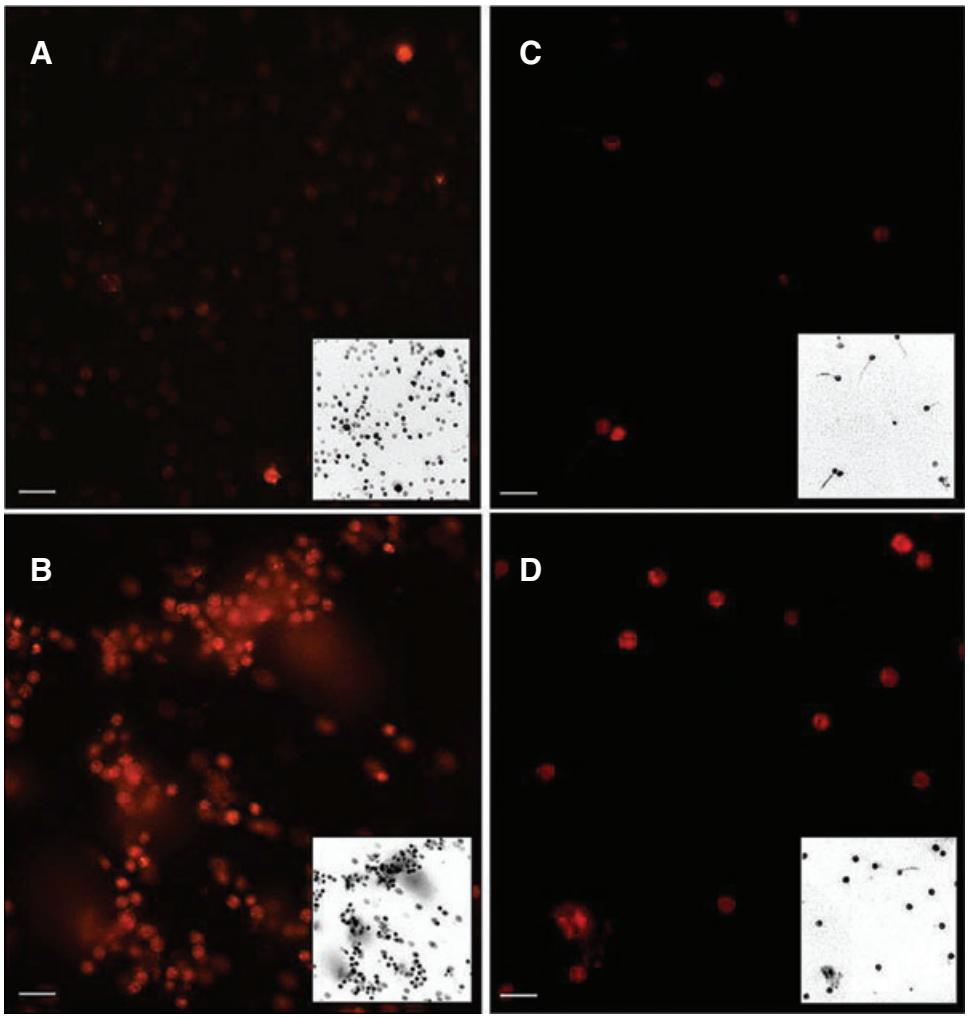

Fig. 9. Detection of intracellular free radicals. Freshly released zoospores were immediately exposed to dihydrorhodamine 123 (2 μM), incubated with IFS (A and C) or IFR (B and D) for 45 (A and B) and 90 (C and D) min, and then monitored through a rhodamine optical filter. Rhodamine 123-labelled cells are mainly present in IFR while only few sparse cysts were stained in IFS. Insets show the whole cellular population photographed. Bars, 30 μm.

Microorganism cell death during plant–pathogen interactions

Phytophthora parasitica is a plant pathogen and thus our finding that this microorganism undergoes PCD has several implications. First, our results raise the possibility that regulation of pathogen PCD is as crucial for the outcome of the plant–pathogen interaction as regulation of plant PCD. The activation of the HR is associated with the inhibition of pathogen growth and overall plant survival. We show here that there is a very clear relationship between the induction of vacuolar cell death in *Phytophthora* and *Phytophthora* resistance in tobacco.

Second, it is possible that the ability of crop pathogens to self-destruct is an element of regulation of the infectious cycle of these disease-causing agents. However, the biological significance for microbe growth of PCD activation in resistant plants remains obscure. At present it seems that *Phytophthora* PCD, like plant PCD, is only advantageous to the host. However, it is likely that the plant-driven balance between cell death and survival is critical for pathologic function. Arnoult *et al*. (2002) proposed that the ability of intracellular *Leishmania major* amastigotes to undergo PCD might reduce the host immune response and favour overall parasite survival. Similarly, PCD activation in *P. parasitica* may limit plant defence responses and facilitate the survival of some zoospores. PCD may also be beneficial to the pathogen: the self-destruction of most zoospores may help to ensure the propagation of at least some cells when conditions for zoospore development are unfavourable. In *Dictyostelium discoideum*, PCD is a part of the development of transient multicellular aggregated

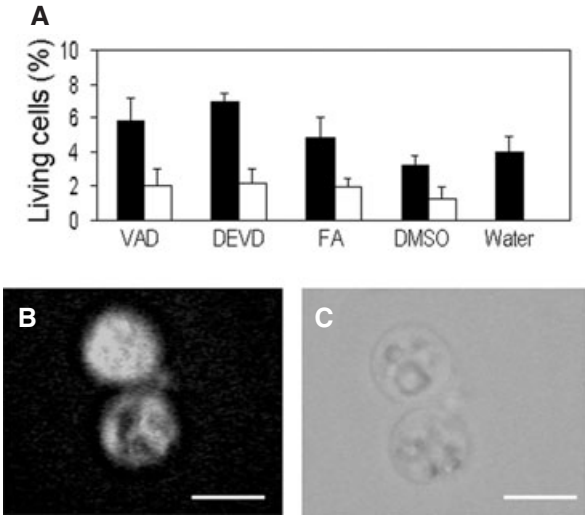

Fig. 10. Influence of protease inhibitors on zoospore death.
A. Influence of Z-VAD-FMK (VAD), Z-DEVD-FMK (DEVD), Z-FA-FMK (FA) and DMSO on cell death in *Phytophthora* zoospores. Bars represent percentage of survival of IFR-treated cells incubated with various inhibitors (10 μM, black bars, and 50 μM, white bars). For DMSO, the concentrations were 0.1% v/v (black bar) and 0.5% v/v (white bar). Values are the means ± SD of four replicates. As attested by a Student's *t*-test, the differences in percentage of cell survival between cells treated with the caspase or cathepsin inhibitors and those treated with DMSO or water are not statistically significant.
B. FITC-VAD-FMK treatment of zoospores incubated for 1 h in IFR.
C. Cellular morphology of cells stained with FITC-VAD-FMK.
Bars, 10 μm.

bodies that favour the overall survival of the spores (Cornillon *et al*., 1994). We found that *Phytophthora* zoospores aggregated upon induction of cell death. Most of these cells died; however, a minority germinated. The germination of this small number of cells may have been promoted by interactions between dying cells.

Third, the existence of PCD in a plant pathogen may open up the possibility of developing new strategies for crop protection based on characterization of plant molecules that induce pathogen PCD and understanding of the biochemical changes leading to microbe PCD. Our results indicate that the regulation of PCD in both the plant and the pathogen is an integral part of the complex events involved in stable plant–pathogen interactions. The induction of pathogen cell death in response to plant signals supports the idea that PCD plays an essential role in the development, survival and evolution of most, if not all, organisms (Ameisen, 2002).

Experimental procedures

Reagents

MitoTracker Red CM-H2XROS was purchased from Molecular Probes (USA) and diluted in DMSO (final concentration: 1 mM). Protease inhibitors z-VAD-FMK, z-DEVD-FMK and z-FA-FMK were obtained from ICN-Enzyme-Systems Products and dissolved in DMSO (final concentration: 20 mM). All other reagents were purchased from Sigma Chemical (USA). Solutions of cycloheximide (10 mM), dihydrorhodamine 123 (5 mM) and MDC (2 mM) were made up in water, DMSO and acetic acid (0.5%) respectively. For each treatment, controls experiments were performed using the appropriate volume of solvent.

Plant material

Nicotiana tabacum cv. Xanthi-nc tobacco plants were grown in a growth chamber at 24°C with a 16 h photoperiod at a light intensity of 100 $\mu Em^{-2}\ s^{-1}$. Leaves were taken from 7- to 8-week-old *P. parasitica*-susceptible and 14- to 15-week-old *P. parasitica*-resistant tobacco plants (Hugot *et al*., 1999). Intercellular fluid was extracted as described by Hammond-Kosak (1992). Extracts were supplemented with 50 mM Tris-HCl buffer (pH 7) and filtered through a syringe filter (pore size: 0.22 μm, Labosi, France) and then used further for incubating *P. parasitica* zoospores.

Oomycete material and culture conditions

Phytophthora parasitica isolate 329 was obtained from the INRA (Sophia-Antipolis) *Phytophthora* collection. Cells were cultivated on malt-agar at 24°C in the dark (Lacourt *et al*., 1994). For the production of zoospores, mycelia were cultivated for 1 week in V8 liquid medium at 24°C under continuous light. This material was then macerated and incubated for a further 4 days on water supplemented with 2% agar. The zoospores were released by the following heat shock treatment: incubation at 4°C for 1 h followed by incubation at 37°C for 30 min. Water (10 ml) was added in between incubations. Germination or cell death was induced by incubating cell suspensions ($4–6 \times 10^5$ cells per millilitre of water) at 25°C with water containing intercellular fluids (50% v/v) from 7- to 8- or 14- to 15-week-old tobacco plant (Hugot *et al*., 1999). Cells were counted before and after incubation to determine the proportion of living and germinated cells. Cell viability was determined by a trypan blue exclusion test.

Digital image processing and animation

Cells were analysed using a microscope (Olympus CH40, Japan), with a CCD camera (Kappa, Germany). Fifteen minutes after the beginning of the incubation in intercellular fluids, images were captured every 30 s using the Archimed software (Microvision Instruments, France) for 90 min. Images were processed further using the Photoshop software (version 6.0, Adobe, USA). Time-lapse sequences were re-sampled to convert them from .tiff to .avi format and were then processed using the Adobe Premiere software (version 6.0, USA). Movies were produced in QuickTime format with a speed of 10 images per second. Cells stained with several probes were analysed using an Axioplan fluorescence microscope (Carl Zeiss MicroImaging, Germany) with appropriate filters. The size and number of stained organelles were determined using ImageJ version 1.29 (http://rsbweb.nih.gov/ij/download.html). Colour images were converted to greyscale, processed with an appropriate threshold (making only stained structures visible) and converted into binary images. The size and number of particles were then measured.

Mitochondrial staining with MitoTracker and Rhodamine 123

Zoospores were released into water supplemented with MitoTracker Red CM-H2XROS (0.5 μM) or dihydrorhodamine 123 (2 μM). Cells were then incubated for 2 h at 25°C with water or intercellular fluids in 12-well glass culture slides (VWR, France). Oxidative activity in cells was detected by monitoring the conversion of non-fluorescent dihydrorhodamine 123, to fluorescent rhodamine 123. Zoospores were incubated at 25°C for 60 min after the uptake of the probe before being observed.

Visualization of MDC-labelled vacuoles

Sequential double staining with MitoTracker Red CM-H2XROS and MDC was performed by a first incorporation of the MitoTracker Red CM-H2XROS (0.5 μM) when zoospores were released from sporangia in water. Incorporation of MDC was performed after incubation in the presence of intercellular fluids for 2 h at 25°C. Cells were incubated with PBS supplemented with 0.05 mM MDC for 10 min at 37°C. After incubation, cells were washed four times with PBS and immediately analysed by fluorescence microscopy.

Transmission electron microscopy

Following incubation in water or intercellular fluids, 2.5×10^5 zoospores were collected by centrifugation, fixed with 1.6% glutaraldehyde diluted in 0.1 M phosphate buffer (pH 7.4), post-fixed with 1% osmium tetroxide diluted in 0.1 M phosphate buffer (pH 7.4), dehydrated by incubation in a series of solutions containing increasing concentrations of ethanol and embedded in epoxy resin. Ultrathin sections (80 nm) were placed onto (150-mesh) copper grids, stained with uranyl acetate and lead citrate and examined with a CM12 Philips transmission electron microscope at 80 kV.

Protease inhibitor assays

We used the following caspase inhibitors: z-VAD-FMK, which is broad specificity caspase inhibitor, and z-DEVD-FMK, an inhibitor of caspases 3 and 7. We also used Z-FA-FMK, an inhibitor of cathepsins B and L but not of caspase activity. Zoospores were incubated for 30 min in water supplemented with the various inhibitors. Inhibitor concentrations ranged from 1 to 50 μM. Cell death was induced by adding intercellular fluids from resistant plants to the culture. Two hours later, cell viability was determined by a trypan blue exclusion test.

Statistical analysis

At least 200 cells were counted in 20 different images for each experiment. Data presented are the means ± standard deviation. A paired *t*-test was used to analyse differences between two groups. Each experiment was repeated at least three times.

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Supplementary material

The following supplementary material is available for this article online:

Movie S1. The germination of a *P. parasitica* cell in IFS.

Movie S2. The cell death in IFR.

Movie S3. Cell death inhibition with cycloheximide 1 μg ml^{-1}.

Figure S1. Staining with MDC of landed and ungerminated cysts of *P. parasitica* at wounded sites on leaves from a resistant plant which have been immersed in a suspension of swimming zoospores for 1 h.