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Molecular cytology of *Phytophthora*–plant interactions

Adrienne R. Hardham[A,B] *and Leila M. Blackman*[A]

[A]Plant Cell Biology Group, Research School of Biology, The Australian National University, Canberra, ACT 2601, Australia.
[B]Corresponding author. Email: Adrienne.Hardham@anu.edu.au

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Abstract. *Phytophthora* diseases cause widespread economic and environmental losses worldwide. Thousands of plant species are susceptible. Disease is typically initiated through the activity of motile, biflagellate zoospores. Plant penetration and colonisation are achieved through the secretion of a diverse range of cell wall-degrading enzymes and effector proteins. Effector proteins are especially important during biotrophic growth; they function to suppress host defence and regulate host metabolism to favour pathogen growth. Plants can detect the presence of *Phytophthora* cells and rapidly mount a basal defence response that often successfully inhibits disease development. A key aspect of basal defence is the formation of wall appositions that constitute a physical and chemical barrier to pathogen growth. Components of basal defence can be triggered by chemical and physical signals produced by invading *Phytophthora* cells. If basal defence fails to inhibit pathogen ingress, the plant cell under attack can undergo hypersensitive cell death. In *Phytophthora*–plant interactions, hypersensitive cell death can be triggered by elicitors or effectors. In the continuing arms race between pathogen and plant host, *Phytophthora* species have evolved a range of counter-defence mechanisms that include suppression of hypersensitive cell death, inhibition of plant degradative enzymes and protection against reactive oxygen species. This brief article provides an overview of the contribution of modern molecular cytology to our understanding of *Phytophthora*–plant interactions.

Additional keywords: elictins, flagellar structure and function, Oomycetes, plant cytoskeleton, plant secretion, spore adhesives.

Introduction

Many of the more than 80 species of *Phytophthora* are aggressive plant pathogens that cause extensive losses in agricultural crops, horticultural plants and natural ecosystems. Some *Phytophthora* species infect only a small number of plant species whereas others have extremely broad host ranges. *Phytophthora cinnamomi*, for example, is now known to infect over 3500 plant species, many of them native to Australia. *Phytophthora* and other members of the class Oomycetes form fungus-like hyphae and conidia-like asexual sporangia, but they are not fungi. The Oomycetes cluster with a diversity of other protists such as diatoms and coloured algae within the Stramenopiles, an assemblage whose taxon-defining characteristics include possession of tubular flagellar hairs.

Species of *Phytophthora* produce motile, biflagellate zoospores that play a key role in the initiation of plant disease. Zoospores target, adhere and encyst at favourable infection sites. Emergent hyphae penetrate the plant epidermis and colonise underlying plant tissues. Some *Phytophthora* species are hemibiotrophs which initially obtain nutrients through the development of haustoria within living plant cells before adopting a necrotrophic life style. During necrotrophic growth, nutrients are derived from dead or dying cells.

Plants are able to recognise the presence of *Phytophthora* cells on their surface and mount a rapid basal defence response that is often sufficient to inhibit disease either before or soon after penetration of the epidermis. This basal, or non-host, resistance involves structural reorganisation of the plant cell and the secretion of cell wall material and toxins to form a physical and chemical barrier to impede *Phytophthora* ingress. When basal defence fails to stop infection, a second level of defence may be invoked with the infected cell undergoing programmed cell death.

In this article, we review current understanding of cellular and molecular aspects of the interactions between plants and *Phytophthora* pathogens. In so doing, we highlight how modern molecular cytology is revolutionising our ability to elucidate the roles of selected proteins and cell components in *Phytophthora* pathogenicity and plant defence.

Phytophthora pathogenicity

Zoospores locate favourable infection sites

The two flagella that emerge from the centre of the groove that runs along the ventral surface of *Phytophthora* zoospores have an internal structure typical of eukaryotic flagella, with nine

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microtubule doublets surrounding a central pair of microtubules (Fig. 1*a*, *b*), stabilised by nexin links between doublets and radial spokes between the doublets and central microtubules (Hardham 1987; Ginger *et al*. 2008). The two flagella differ in length and surface ornamentation, with the shorter, anterior flagellum possessing two rows of tubular, tripartite hairs termed mastigonemes, the common structure giving rise to the name of the Stramenopile taxon (= straw hair). In eukaryotic flagella, sliding of adjacent microtubule doublets relative to one another is achieved through the action of two rows of dynein arms attached to the A-tubule of each doublet. In *Phytophthora* this generates a sinusoidal wave that propagates from the flagellar base to its tip. This should, by rights, propel the zoospores backwards but the movement of the mastigonemes during wave propagation reverses the thrust of flagellar beat and pulls the zoospores forward (Jahn *et al*. 1964; Cahill *et al.* 1996). The shaft of the mastigoneme is composed of a 40-kDa protein (Robold and Hardham 1998). The gene encoding the mastigoneme protein in *P. nicotianae* has now been cloned revealing possession of an N-terminal secretion signal sequence and three conserved epidermal growth factor signature-like domains typically involved in protein–protein interactions in mammalian extracellular matrix proteins (L. M. Blackman, M. Arikawa, S. Yamada, T. Suzaki and A. R. Hardham, pers. obs.). The longer, posterior flagellum trails behind, occasionally flexing to create a change in swimming direction.

Inner and outer dynein arms are large (~2 MDa), multiprotein complexes possessing ATPase motor activity. One of the 13 proteins in the complex is a 22-kDa leucine-rich repeat protein called dynein light chain 1, DLC1 (Benashski *et al*. 1999; King 2003). In *Trypanosoma brucei* silencing of the DLC1 gene results in loss of the tip-to-base beat typical of trypanosome flagellar motility and reversal of swimming direction (Baron *et al*. 2007). In *Phytophthora nicotianae*, silencing of *PnDLC1* leads to inhibition of flagellar formation during zoosporogenesis and loss of zoospore motility (R. Narayan, L. M. Blackman and A. R. Hardham, pers. obs.). Loss of flagella does not, however, inhibit zoospore discharge from sporangia, giving further evidence that zoospore release is driven by generation of high hydrostatic pressure within the sporangium (Money and Webster 1989).

Phytophthora zoospores are chemotactically and electrotactically attracted to the surface of potential host plants through detection of chemical or electrical gradients emanating from the plant (Tyler 2002). This directed zoospore movement allows the spores to target particular regions of the plant that are favourable for infection such as the root elongation zone (Fig. 1*c*) (Gow 2004). *Phytophthora* zoospores show an even finer level of specificity in that they may be preferentially attracted to the grooves between adjacent epidermal cells (Fig. 1*d*) (Hardham 2001). Chemoreceptors are likely to be located on the zoospore surface (Sakihama *et al*. 2004) and there is evidence that signal transduction during spore motility may involve a trimeric G-protein and a bZIP transcription factor (Latijnhouwers *et al*. 2004; Blanco and Judelson 2005).

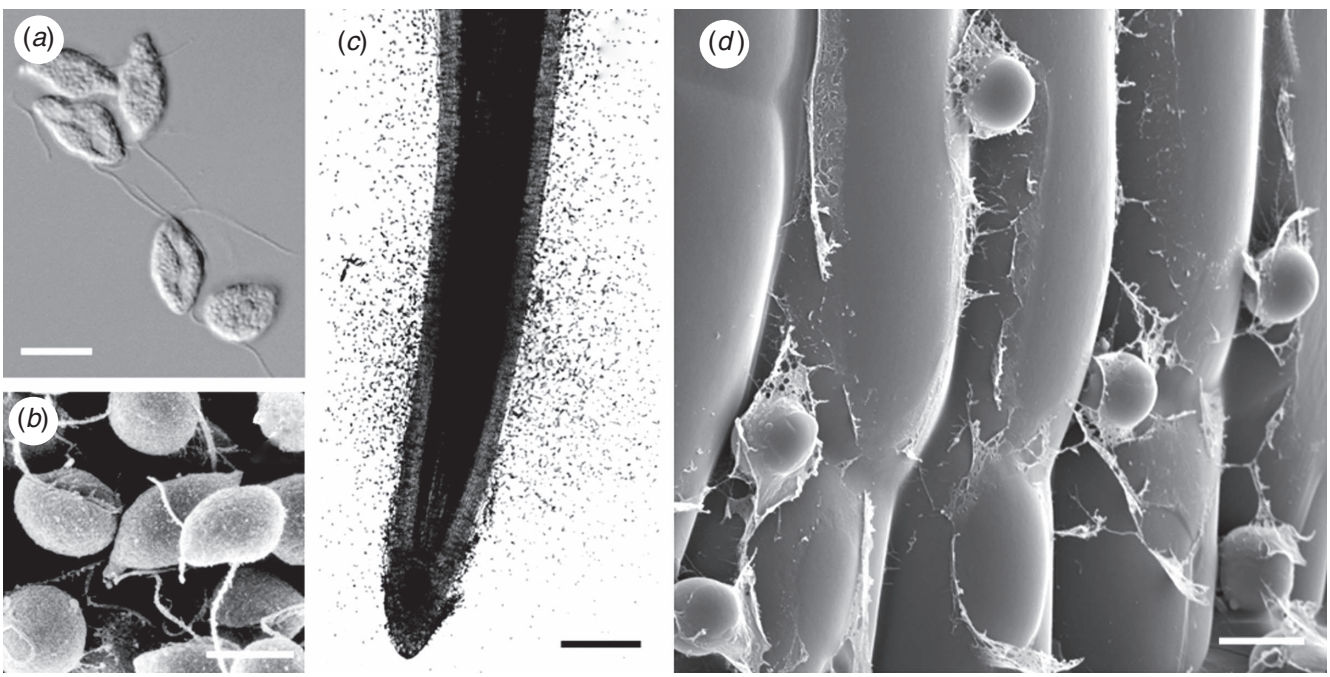

Fig. 1. *Phytophthora cinnamomi* zoospores and cysts. (*a*) Light micrograph of biflagellate zoospores showing emergence of the two flagella from the centre of the ventral groove. Bar = 10 µm. (*b*) Scanning electron micrograph of zoospores and cysts on a root surface. Bar = 10 µm. (*c*) Light micrograph of zoospores that have been chemotactically attracted to the elongation zone of an onion root. Bar = 0.5 mm. (*d*) Cryoscanning electron micrograph of spores that have targeting and settled in the grooves on a root surface. Reproduced with permission from Hardham (2005). Bar = 10 µm.

Rapid secretion of adhesins immobilises spores at the infection site

At the plant surface, *Phytophthora* zoospores manoeuvre so that their ventral surface faces the plant before, quite abruptly, the flagella are detached and proteins from three different cortical vesicles are secreted onto the zoospore surface (Hardham and Gubler 1990; Škalamera and Hardham 2006). High molecular weight glycoproteins from one category of vesicle below the dorsal surface may form mucilage that serves a protective role (Fig. 1*d*). Proteins released from ventrally located vesicles are adhesins that bond the spores to the plant surface. In *P. cinnamomi*, this adhesin, PcVsv1, is a 200-kDa protein containing 47 copies of the thrombospondin type I repeat motif, a conserved 50-amino acid sequence found in a range of extracellular adhesive molecules secreted by mammalian cells and malarial parasites (Robold and Hardham 2005). Homologues of PcVsv1 occur throughout *Phytophthora* and other Oomycete genera. Another 20-kDa putative adhesin is also secreted from large cortical vesicles beneath the zoospore surface (Škalamera and Hardham 2006).

Secretion of enzymes and effectors facilitates penetration and colonisation of the plant

Encysted *Phytophthora* spores germinate from the centre of the ventral surface and the hyphae typically grow along the anticlinal walls between epidermal cells. Penetration sometimes involves formation of appressoria-like structures and is facilitated by the secretion of enzymes that degrade components of the plant cell wall. *Phytophthora* genomes contain large multigene families encoding cell wall-degrading enzymes. Some species have more than 20 genes encoding endopolygalacturonases or β-glucanases that degrade pectin and β-glucans, respectively (Götesson *et al*. 2002; McLeod *et al*. 2003; Wu *et al*. 2008). Cell wall-degrading enzymes are released as a temporal cascade, with pectinases often being the first to be secreted. Digestion of pectins leads to plant cell separation, tissue maceration and exposure of other cell wall components for degradation.

During the early biotrophic growth phase in hemibiotrophic species, nutrients are obtained from living plant cells. This is achieved by penetration of the plant cell wall, but not the plasma membrane, and formation of a haustorium that is specialised for nutrient uptake from the host cell. As in fungi, haustoria of *Phytophthora* and other Oomycetes secrete effector proteins that are transported across the plant plasma membrane into the host cytoplasm from where they orchestrate metabolic changes that favour pathogen development (Birch *et al*. 2009). During necrotrophic growth, nutrients required for pathogen growth and reproduction are obtained from dead and dying cells in the necrotic lesions that develop as the pathogen colonises the plant.

Plant defence

Basal resistance defeats most potential pathogens

Plants react rapidly to attempted infection by *Phytophthora*. Early responses include deposition of callose, synthesis of reactive oxygen species and cytoplasmic aggregation beneath the pathogen (Hinch *et al*. 1985; Gross *et al*. 1993; Able *et al*. 2003; Belhaj *et al*. 2009). Immunocytochemistry and green fluorescent protein (GFP)-tagging have revealed that cytoplasmic aggregation is accompanied by dynamic reorganisation of actin (Fig. 2*a*), microtubules (Fig. 2*b*), endoplasmic reticulum (ER; Fig. 2*c*), Golgi bodies (GA) and peroxisomes (Takemoto *et al*. 2003). Actin, ER, GA and peroxisomes become focussed on the infection site and are likely to be responsible for secretion of toxins and formation of wall appositions that inhibit hyphal penetration of the plant cell wall. Changes in microtubule arrays are more variable but include localised microtubule depolymerisation at the infection site (Gross *et al*. 1993; Takemoto *et al*. 2003). Subsequent defence responses include changes in gene expression, and production of phytoalexins and pathogenesis-related proteins (Schmelzer *et al*. 1989; Chirapongsatonkul *et al*. 2008; Schlink 2009).

Plants may detect both physical and chemical signals from the invading pathogen

Treatment of plant cells with purified *Phytophthora* elicitors can induce at least some components of the basal defence response seen during pathogen infection (Gus-Mayer *et al*. 1998; Blume *et al*. 2000; Braga *et al*. 2006; Chirapongsatonkul *et al*. 2008). Studies of *Phytophthora*–plant interactions have provided evidence that receptors responsible for detecting these chemicals are located on the plant plasma membrane (Diekmann *et al*. 1994; Fliegmann *et al*. 2004).

Plants can also respond to mechanical stimulation, and some of the changes that are induced by touching a plant with a fine microneedle are similar to those occurring during the defence response. Touch can lead to rapid changes in gene expression, including the upregulation of disease resistance genes (Wick *et al*. 2003; Braam 2005), cytoplasmic aggregation and movement of the plant cell nucleus to the contact site (Kennard and Cleary 1997; Gus-Mayer *et al*. 1998). Gentle mechanical stimulation applied to the outer epidermal wall of *Arabidopsis* plants expressing GFP-tagged cell components results in a reorganisation of subcellular components similar to that observed during attempted infection by *Phytophthora* and other fungal or Oomycete pathogens (Fig. 2*d–f*) (Hardham *et al*. 2008). Within 5 min of touching the cotyledon surface, actin microfilaments become focussed on the site of contact, and ER and peroxisomes accumulate beneath the needle tip. A subpopulation of the cortical microtubules surrounding the point of contact depolymerises, generating a microtubule-depleted zone around a patch of concentrated tubulin subunits. The focal patches remain beneath the needle tip when moved across the leaf surface and disperse within minutes of removal of the needle's pressure. These results suggest that plant cells may be able to detect the force exerted by fungal or Oomycete hyphae attempting to penetrate the plant epidermis, thus triggering at least some components of the basal defence response.

A second layer of defence is triggered by recognition of Phytophthora *elicitins and effectors*

Basal defence mechanisms do not always successfully inhibit *Phytophthora* ingress but plants have a second system of

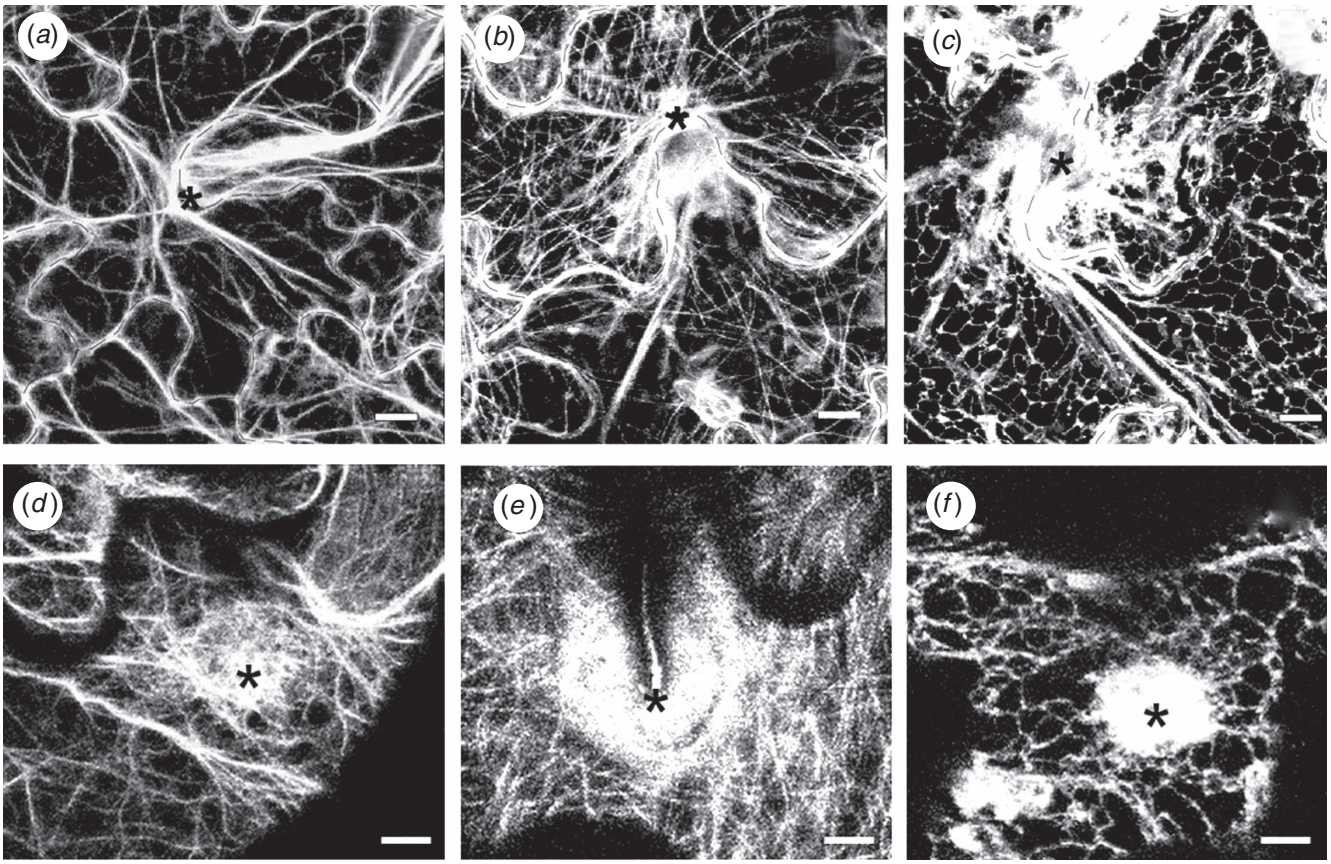

Fig. 2. Reorganisation of actin microfilaments (*a* and *d*), microtubules (*b* and *e*) and endoplasmic reticulum (*c* and *f*) tagged with green fluorescent protein (GFP) in transgenic *Arabidopsis* cotyledons. Asterisks in (*a–c*) show the site of attempted penetration by *Phytophthora sojae*. Asterisks in (*d–f*) show contact site of microneedle with cotyledon surface. (*a*) Actin cables focus on the penetration site. (*b*) Microtubules in cytoplasmic strands and a region of GFP-tubulin subunits surround the penetration site. (*c*) Endoplasmic reticulum has become concentrated at the penetration site. (*d*) A patch of actin microfilaments has formed beneath the microneedle. (*e*) A cloud of GFP-tubulin subunits has formed beneath the microneedle. (*f*) Endoplasmic reticulum has become concentrated beneath the microneedle. Bar = 10 μm. (*a–c*) Reproduced with permission from Takemoto *et al*. (2003). (*d–f*) Reproduced with permission from Hardham *et al*. (2008).

resistance that involves the recognition of specific pathogen molecules and the consequent induction of programmed cell death, also referred to as the hypersensitive response. As in fungal-plant interactions, hypersensitive cell death is a highly effective means of restricting pathogen growth and development. In *Phytophthora*–plant interactions there are two main classes of molecules that induce the hypersensitive response, elicitins and effectors.

Elicitins are 10-kDa proteins that are produced by *Phytophthora* and *Pythium* species and function in the uptake of sterols from host plant membranes (Osman *et al*. 2001). Elicitins are recognised by tobacco and some *Brassica* species and induce the hypersensitive response (Huitema *et al*. 2005). In *Nicotianae benthamiana*, the INF1 elicitin from *Phytophthora infestans* triggers hypersensitive cell death through interaction with the lectin-like receptor kinase, NbLRK1 (Kanzaki *et al*. 2008).

Effector-triggered hypersensitive resistance is a widespread response induced by race-specific proteins from bacterial, fungal or Oomycete pathogens (Boller 2009; Dodds *et al*. 2009; Ellis *et al*. 2009). In resistant plants, these effectors are avirulence proteins that are recognised either directly or indirectly in a gene-for-gene-specific manner by resistance proteins (Jones and Takemoto 2004). In *Phytophthora*, four avirulence effectors have been identified to date (Birch *et al*. 2009). In all cases, they are recognised by cytoplasmic plant resistance proteins of the NBS-LRR class that have a nucleotide binding site and a leucine-rich repeat domain (Tyler 2009).

Phytophthora counter-defence

The full range of functions of pathogen effectors are only beginning to be elucidated but there is evidence for roles in avoiding recognition, suppressing defence responses, protecting against chemical defences and orchestrating metabolic and structural changes in the plant cells. Several bacterial effectors are known to suppress components of basal defence including Ca^{2+} influx and callose deposition (Boller and Felix 2009). In fungi, the effector Avr1 of *Fusarium oxysporum* f. sp. *lycopersici* suppresses resistance triggered by specific recognition of other avirulence proteins (Houterman *et al*. 2008). In *Phytophthora*, the effector Avr3a from *P. infestans*

has been shown to suppress hypersensitive cell death triggered by the *P. infestans* elicitin, INF1 (Bos *et al*. 2006). The Avr1b effector from *P. sojae* also suppresses programmed cell death (Dou *et al*. 2008). Other *Phytophthora* effectors are inhibitors of plant pathogenesis-related proteases and glucanases (Rose *et al*. 2002; Tian *et al*. 2005).

It has been known for many years that *Phytophthora* spores and hyphae can produce water-soluble glucans or proteins that inhibit hypersensitive cell death and phytoalexin production in a range of plants (Doke *et al*. 1980; Ziegler and Pontzen 1982; Sanchez *et al*. 1994; Andreu *et al*. 1998). *Phytophthora* cells also synthesise metabolites and proteins that inhibit the production of, or provide defence against, reactive oxygen species produced during the plant's oxidative burst (Shiraishi *et al*. 1997). In a recent study, *P. nicotianae* expression of a gene encoding the hydrogen peroxide scavenging enzyme, catalase, was shown to be highly upregulated and *P. nicotianae* catalase activity increased during the infection of susceptible tobacco plants (Blackman and Hardham 2008).

Concluding remarks

The integration of cellular and molecular approaches has revolutionised studies of cell structure and function. When applied to investigations of *Phytophthora* pathogenicity, the production of antibodies and immunocytochemical labelling has facilitated the discovery and characterisation of a range of *Phytophthora* spore components that play key roles in the infection of host plants. Immunocytochemistry and GFP-tagging of plant proteins has allowed observations of dynamic changes occurring in living cells during *Phytophthora*–plant interactions. These studies have revealed the location of plant receptors involved in *Phytophthora* pathogen detection and mechanisms underlying basal defence. There is little doubt that molecular cytology will continue to be at the heart of cutting-edge research into the biology of *Phytophthora* effector proteins in *Phytophthora*–plant interactions.

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