

Translocation of the fungal effector AvrM into host cells

BY

PAMELA HUI PENG GAN

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Declaration

All research reported in this thesis is original and my own, except where acknowledgement is given, and has not been submitted for any other degree.

A handwritten signature in black ink, appearing to read 'Pamela Hui Peng Gan', with a stylized, cursive script.

Pamela Hui Peng Gan

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Abstract

Rust fungi are obligate biotrophic pathogens that are responsible for some of the most devastating diseases of economically important plants. It is thought that these pathogens are able to evade host defense responses and obtain nutrients from living host tissue through the actions of small, secreted proteins known as effectors, which alter the structure and function of plant cells. Therefore, elucidating the fate of these proteins during infection is important for understanding the molecular mechanisms underlying infection and for development of better disease control measures. The experiments described in this thesis aim to address this issue by examining the localisation of the flax rust effector AvrM during infection by the fungus of the host plant and by exploring molecular mechanisms potentially responsible for AvrM uptake into plant cells.

Two approaches were taken in the localisation studies: (1) immunolocalisation of AvrM and (2) the generation of transgenic flax rust fungi expressing AvrM-fluorescent protein fusions. Although flax rust transformants expressing AvrM fusion proteins were successfully isolated and fusion proteins could be detected on immunoblots, no fluorescent signal was observed. Thus, AvrM distribution was studied primarily by immunolocalisation. For this purpose, polyclonal antibodies against AvrM were generated and used in immunofluorescence and immunogold labelling experiments. While the antibodies did not detect the presence of AvrM on spores or *in vitro* germinated spores, labelling was observed *in planta* at the periphery of fungal hyphae and specialised fungal feeding structures known as haustoria. Strong cytoplasmic labelling was also detected within the cytoplasm of infected plant cells providing direct evidence that AvrM enters the host cell during infection. Examination at the electron microscope level indicated the presence of the protein in vesicles close to the host-derived extrahaustorial membrane at the plant-pathogen interface. Labelling was also associated with putative endomembrane structures within host cells.

Having confirmed the transfer of AvrM into the host during infection, the mechanism of effector uptake into host cells was investigated. This involved using viral-induced gene silencing of plant genes implicated in clathrin-mediated endocytosis and clathrin-independent endocytosis as well as the expression of dominant negative AP180 homologs, which are predicted to affect clathrin-mediated endocytosis. The results of these experiments did not provide conclusive

evidence for the involvement of any of the tested pathways in AvrM uptake. Finally, in light of evidence that lipid binding may be required for effector uptake into host cells, the lipid binding activities of AvrM and AvrL567, another flax rust effector, were tested using various deletion and truncation mutants of both proteins. This showed that while AvrL567 did not possess significant lipid binding activity, AvrM bound strongly to phosphatidylinositol, phosphatidylinositol monophosphates and phosphatidyl serine. However, two AvrM truncations that were able to enter host cells *in planta* displayed loss of lipid binding activity, suggesting that lipid binding is not required for uptake. The significance of the results obtained in the present study as well as future directions for research are discussed.

Abbreviations

ANTH	AP180 N-terminal homology
ATPase	Adenosine triphosphatase
Avr	Avirulence
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BIC	Biotrophic interfacial complex
Bp	Basepair
BSA	Bovine serum albumin
c/v	Column volumes
cDNA	Complementary DNA
Cds	coding sequence
CPP	Cell penetrating peptides
CTxB	Cholera toxin subunit B
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4', 6-diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
Dpi	Days post infiltration
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
EHD	Epsin homology domain
EM	Electron microscopy
ENTH	Epsin N-terminal homology
ER	Endoplasmic reticulum
ERAD	ER associated degradation
EST	Expressed Sequence Tags
ETI	Effector-triggered immunity
EtOH	Ethanol
FITC	Fluorescein isothiocyanate
G protein	Guanine nucleotide binding protein
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
GST	Glutathione S-transferase
GTPase	Guanosine triphosphatase
GUS	β -glucuronidase
H	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPF	High pressure frozen
Hpi	Hours post infection
HR	Hypersensitive response
IPTG	Isopropyl thio- β -D-galactoside

Kb	Kilo basepair
kDa	kiloDalton
L6-tobacco	<i>N. tabacum</i> transgenic for the <i>L6</i> resistance gene
LIC	Ligation-independent cloning
m/v	Medium volumes
MAMP	Microbe-associated molecular pattern
MES	2-(N-morpholino)ethanesulfonic acid
Min	Minutes
M-tobacco	<i>N. tabacum</i> transgenic for the <i>M</i> resistance gene
NB	Nucleotide binding
NBT	Nitroblue tetrazolium
Nt	Nucleotide
O.D. ₆₀₀	Optical density at 600 nm
O.D. ₂₆₀	Optical density at 260 nm
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PI3K	Phosphatidylinositol 3-kinase
PIPES	1,4-piperazine-diethanesulfonic acid
PMSF	Phenylmethylsulfonyl fluoride
PPD	<i>p</i> -phenylenediamine
PtdInsP	Phosphatidylinositol monophosphate
PTI	PAMP-triggered immunity
R protein	Resistance protein
RNA	Ribonucleic acid
ROP	Rho-related GTPase from plants
ROS	Reactive oxygen species
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
Sec	Seconds
STMV	Satellite tobacco mosaic virus
SVISS	Satellite virus-induced silencing system
TMV	Tobacco mosaic virus
TRITC	Tetramethylrhodamino isothiocyanate
TRV	Tobacco rattle virus
UV	Ultraviolet
v/v	Volume to volume ratio
Vol	Volumes
w/v	Weight to volume ratio
WGA	Wheat germ agglutinin

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CHAPTER 1

1 Biotrophic fungal infection of plants

1.1 Introduction

Plants have co-evolved with a variety of microbes and plant-associated microorganisms range from the beneficial to the pathogenic. Among plant pathogenic microbes, there are two main modes of nutrient acquisition, necrotrophy and biotrophy. Necrotrophic pathogens cause rapid cell death and feed off dead tissues whereas biotrophs feed on host plants without killing them. Biotrophs may be facultative, capable of host-independent growth such as *Ustilago maydis*, or may be obligate, only growing and reproducing in association with a host, such as in the case of the rust fungi and powdery mildews. In addition, many plant pathogens also have an intermediate lifestyle known as hemibiotrophy, where the pathogen initially propagates in living host tissue before making a switch to a necrotrophic phase.

Two major classes of eukaryotic plant pathogens are the fungi and the oomycetes. Comparisons between these two groups reveal that they share many morphological similarities which are likely to have arisen through convergent evolution as efficient mechanisms for plant infection, including dispersal through spores and growth as filamentous hyphae (Latijnhouwers et al. 2003). In fact, early plant pathologists considered oomycetes to be a class of fungi. However, structural and biochemical differences and comparisons at the level of nucleotide sequences have provided evidence that the two groups are phylogenetically separate (Hardham 2005). Indeed, oomycetes actually belong to the Kingdom Chromalveolata and are more closely related to alveolates, which include apicomplexan parasites such as *Plasmodium falciparum*, the causal agent of malaria (Baldauf et al. 2000). On the other hand, fungal plant pathogens are from the Kingdom Fungi with most fungal plant pathogens falling into the subkingdom dikarya, which consists of the basidiomycetes and the ascomycetes.

Some of the most successful pathogenic obligate biotrophs are the rust fungi, which are basidiomycetes in the class Urediniomycetes of the order Uredinales (Lawrence et al. 2007). This order includes two main families. In the Melampsoraceae, *Melampsora lini* (flax rust) has been extensively studied at a genetic level revealing many insights into plant pathogen interactions and

was the basis of the gene-for-gene hypothesis (Flor 1955 and see below), while *Melampsora larici-populini* (poplar rust) has attracted much recent attention at a molecular level and its genome has recently been sequenced (<http://genome.jgi-psf.org/Mellp1/Mellp1.home.html>). The other major family, the Pucciniaceae, includes rusts from the *Puccinia* and *Uromyces* genera (Lawrence et al. 2007). *Puccinia* species are the major cause of rust on cereal crops and are a major threat to world food supplies, while *Uromyces fabae* (broad bean rust) has served as a model for understanding molecular aspects of the biotrophic lifestyle of rusts. Rust fungi have been associated with the devastation of crops since the Bronze Age and indeed the Romans were documented to make sacrifices to appease a god of rust and mildews as far back as 700 BC (Voegelé et al. 2009). Rust diseases continue to plague human agriculture even today with significant economic and environmental impacts. In terms of their economic impact, although individual host ranges may be limited, collectively this group of pathogens infect a wide group of crop plants including flax, wheat, white pine, soybean and coffee (Staples 2000; Voegelé et al. 2009). In recent years, an extremely aggressive strain of wheat rust fungus known as Ug99 has caused much alarm as it spread from Uganda, where the strain was first discovered, through to Kenya, Ethiopia, Sudan, Yemen and Iran (Ehrenberg 2010). With 90% of 12000 varieties of wheat vulnerable to this fungus, Ug99 poses a real threat to the world's wheat supply (Stokstad 2007). Rust can also impose significant ecological threats. *Puccinia psidii* (guava rust) was first described infecting guava plants in Brazil, but is also able to infect introduced eucalyptus species in South America and has recently been detected in New South Wales, Australia, where spread of the disease could have potentially devastating consequences on native forests (Carnegie et al. 2010). Despite advances in pesticide application, the most successful and environmentally friendly strategy of rust control in agriculture has been the breeding and use of genetically rust-resistant crop varieties (Ehrenberg 2010). This genetic resistance is based on recognition of specific rust effector proteins by host immune receptors (see Section 1.3) and often breaks down when rust strains evolve to escape recognition. Thus it is important to develop a fuller understanding of the molecular mechanisms behind disease establishment in order to develop more robust rust control measures.

However, rust fungi are a difficult group of organisms to study owing to their obligate biotrophic nature that prevents culturing of the biotrophic phase *in vitro*, which can complicate analyses. The flax rust fungus *M. lini* infects flax and is a particularly useful model for studying

plant-pathogen interactions since the interaction between it and its host plant (*Linum usitatissimum*) has been well characterised genetically (Flor 1956; Lawrence 1988; Islam et al. 1989; Lawrence et al. 1995; Dodds et al. 2004; Catanzariti et al. 2006; Lawrence et al. 2007) as well as cytologically (Coffey 1976; Coffey and Allan 1983; Kobayashi et al. 1994). In fact, one of the earliest insights into the evolution of resistance underlying plant-pathogen immunity, known as the gene-for-gene theory, which states that every resistance gene in the plant has a corresponding avirulence gene in the pathogen, was obtained through studies utilising the flax-flax rust pathosystem (Flor 1955). Part of the reason this system has been used in so many studies as a model is that owing to the rust's sexual cycle, it is amenable to genetic crosses (Lawrence 1988). Further, dikaryotic uredospores of the flax rust build up rapidly on the host and can be stored for many years under vacuum facilitating stocking of various strains (Lawrence 1988). In addition, unlike many other rusts which are heteroecious, the fungus is autoecious, meaning that it is able to carry out both its asexual and sexual lifecycle stages on a single host (Lawrence 1988; Lawrence et al. 2007).

1.2 Flax rust lifecycle

The lifecycle of the rust consists of five separate spore stages: pycniospores, aeciospores, uredospores, teliospores and basidiospores as shown in Figure 1-1 (Lawrence et al. 2007). As part of the sexual cycle, dikaryotic mycelia are able to produce black, rod-shaped teliospores which are resistant to adverse environmental conditions, allowing the pathogen to persist over winter when there is no green host tissue (Lawrence et al. 2007). These spores germinate to form metabasidia on the surface of the host (Lawrence et al. 2007). Haploid basidiospores are ejected from conical sterigma which develop from metabasidia and are generally only dispersed locally since they desiccate and lose viability rapidly (Voegelé et al. 2009; Lawrence et al. 2007). Upon basidiospore germination on a leaf, a monokaryotic mycelium is formed that develops after 8 to 10 days into a pycnium of a specific mating type (Lawrence et al. 2007). Pycnia produce pycniospores, also known as spermatia, in liquid exudates or nectar (Voegelé et al. 2009). Since *M. lini* is heterothallic, pycniospores must be fertilised by spores of a different mating type with transfers being mediated by insects and surface water (Lawrence et al. 2007). Upon mating, dikaryotic aeciospores are produced in an aecium and the resultant aeciospores infect new hosts and develop uredospores after 9 to 10 days (Lawrence et al. 2007).

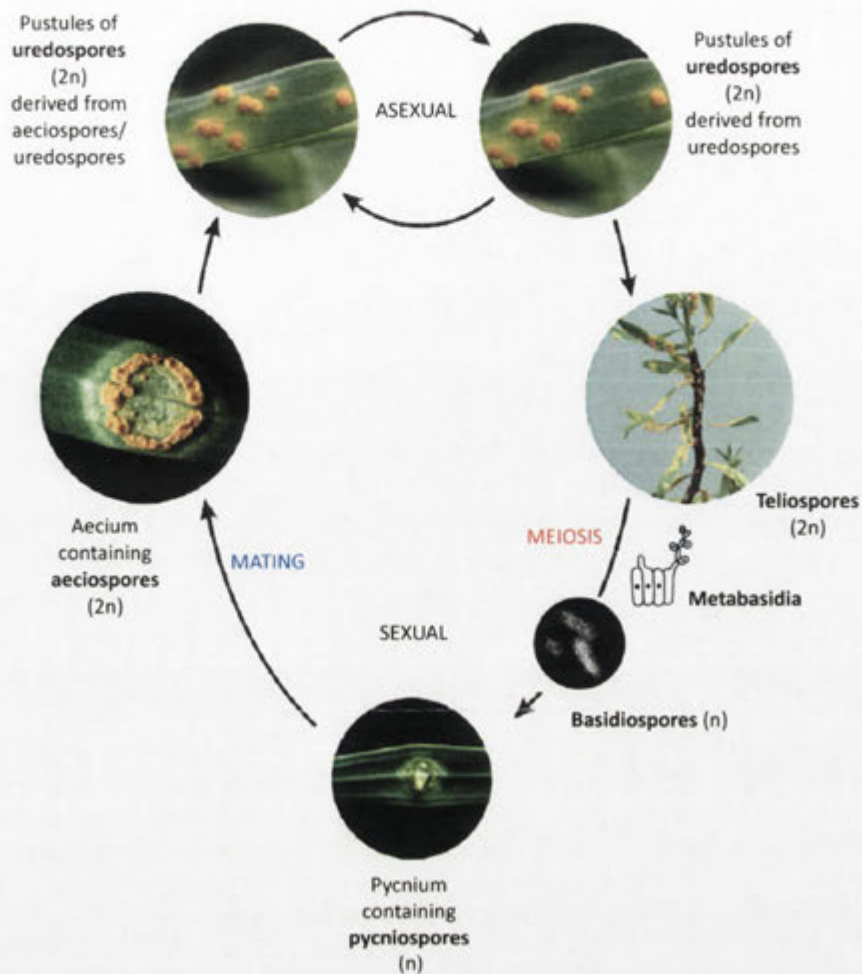


Figure 1-1 Lifecycle of the flax rust *Melampsora lini*. Diagram modified from Lawrence et al. (2007).

The asexual stage of the rust life cycle is perpetuated by uredospores. This asexual stage occurs as a repeating cycle of infection and spore production that builds up the number of spores very rapidly. In the case of the wheat stem rust *Puccinia graminis*, a single uredium may give rise to up to 20000 spores on plants in the heading growth stage although this number is lower in mature plants (Eversmeyer and Kramer 2000). Up to five or six infection cycles can occur within a single growing season and uredospores are easily disseminated widely by the wind and deposited on leaves by rain, so devastating disease epidemics can occur across wide regions (Voegelé et al. 2009; Li et al. 2009). Thus, although aeciospores and basidiospores are also able to act as infectious agents, infection is spread mainly through short- and long-range distribution of dikaryotic uredospores (Schumann 1991; Lawrence 1988; Lawrence et al. 2007).

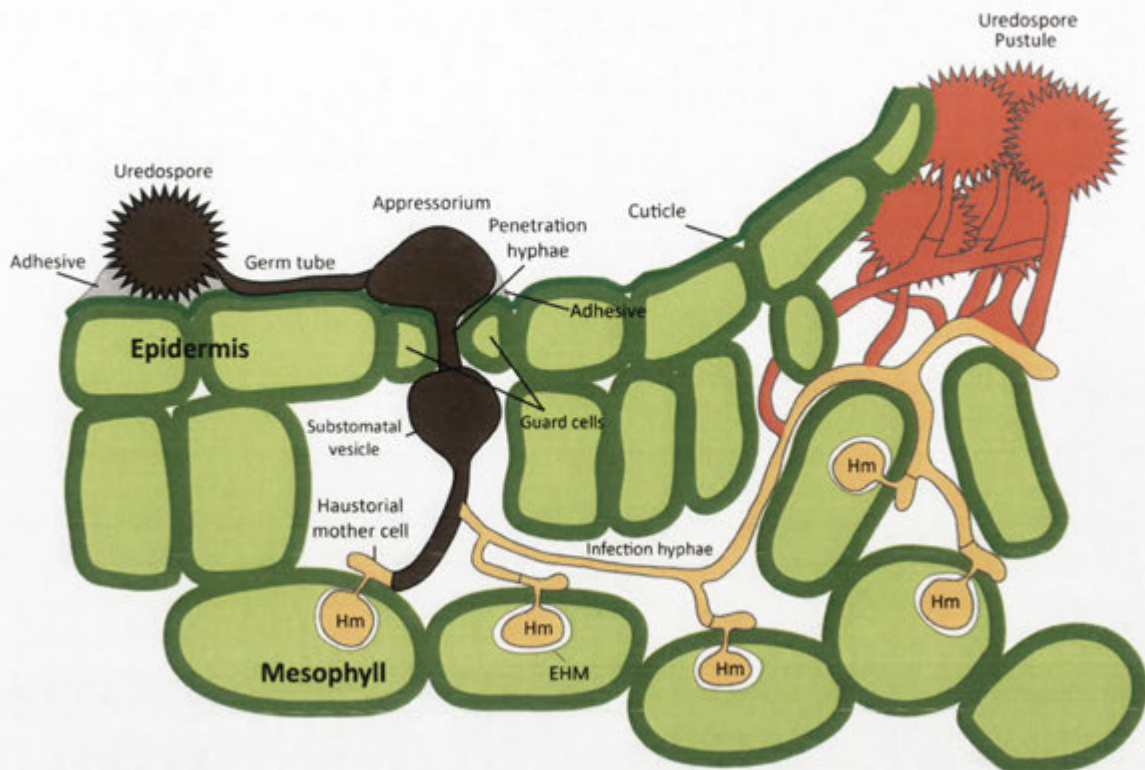


Figure 1-2 Schematic representation of the flax rust uredospore infection on a compatible host. Early infection structures from the 'penetration stage' including uredospore germination and entry into stomata are represented in dark brown, structures from the 'parasitic/ biotrophic stage' are represented in yellow and the structures from the 'sporulation stage' are represented in red. Image design taken from Voegelé et al. (2006). Hm: haustoria

Due to its contribution to disease and the ability to continually propagate, the rust uredospore infection process, represented in greater detail in Figure 1-2, is the most studied lifecycle stage of *M. lini*. When an uredospore lands on the surface of a plant, it adheres to the surface initially through hydrophobic interactions and subsequently forms an adhesive pad consisting of glycoproteins and carbohydrates (Clement et al. 1993; Deising et al. 1996). In spores of *U. fabae*, it was found that adhesive pad formation was largely a passive process, occurring also in dead, autoclaved spores, although full adhesion required the action of esterases and cutinases that are not present in autoclaved spores (Deising et al. 1991). Upon germination, a germ tube extends across the leaf surface until it reaches a stomate, where the tip swells to form an appressorium directly over the stomatal pore. Entry of the fungus into the tissue of the host then occurs through the stomatal cavity via a structure known as a penetration peg. Although some other pathogenic fungi, such as *Cladosporium fulvum*, also enter leaves through stomates, others

such as *Magnaporthe* and *Colletotrichum* penetrate the host cuticle and cell wall of epidermal cells directly (Willmer and Fricker 1996; Mendgen and Deising 1993).

Upon entry into the leaf tissue, the penetration peg expands to form a substomatal vesicle in the substomatal cavity. From this vesicle, one or more primary infection hyphae grow until contact with a host mesophyll cell whereupon hyphae differentiate to form haustorial mother cells (Heath and Skalamera 1997). Then, a penetration peg extends from the haustorial mother cell breaking through the cell wall of the host cell and a haustorial body forms within the cell wall and invaginates the host plasma membrane (Harder and Chong 1991). Upon establishment of haustoria, the fungus is no longer metabolically independent and enters a 'parasitic/biotrophic phase' during which it obtains nutrients from its host (Deising et al. 1996; Voegelé 2006).

In flax rust, young haustoria appear spherical but may develop up to five lobes as they mature, which presumably serves to maximise the surface area of the host-pathogen interface (Littlefield 1972). The haustorial cell wall was demonstrated to be differentiated from the rest of the fungal cell wall since monoclonal antibodies that are specific to a haustorial wall-specific epitope of flax rust have been raised (Murdoch and Hardham 1998). Interestingly, the same monoclonal antibodies did not recognise haustoria from maize rust (*Puccinia sorghi*) or wheat leaf rust (*Puccinia recondita*) indicating that the haustorial-specific component may also be species-specific (Murdoch and Hardham 1998).

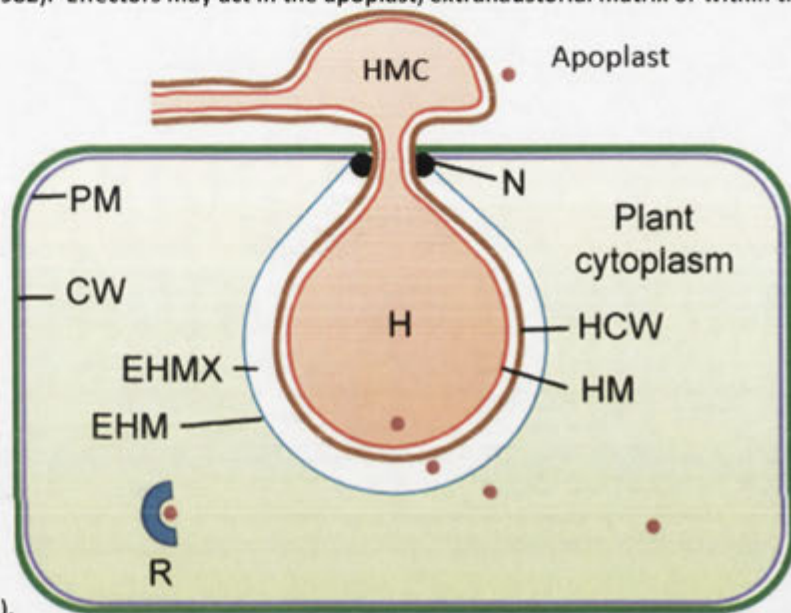
Cytological observations indicate that haustoria are separated from the host cytoplasm by an amorphous, gel-like compartment known as the extrahaustorial matrix as shown in Figure 1-3 (O'Connell 1987; Hahn and Mendgen 1992). As the extrahaustorial matrix lies between the host and the pathogen, it is believed to serve as a hub for the exchange of pathogen and plant proteins and signalling factors (Mendgen and Hahn 2002). The matrix consists of a mixture of components, primarily carbohydrates and glycoproteins, that are thought to be derived from both the fungus and the plant (Harder and Chong 1991). In rust infections, the extrahaustorial matrix is delimited on the pathogen side by the haustorial membrane and cell wall and on the host side by an extension of the host plasma membrane known as the extrahaustorial membrane (Harder and Chong 1991). The extrahaustorial matrix is sealed off from the apoplast by a structure known as the neckband which is thought to act as a selectively permeable barrier, like the Casparian strip at the endodermis (Heath 1976; Harder and Chong 1991).

Although contiguous with the plant plasma membrane, from which it is thought to be derived, the extrahaustorial membrane exhibits distinct molecular properties. Differences include a reduced glycolipid content and a reduced level of ATPase activity compared to the rest of the host plasma membrane (Baka et al. 1995; Perfect and Green 2001). In infections by some rust fungi, such as *Puccinia hemerocallidis* and *Puccinia striiformis*, tubular beaded elements contiguous with the extrahaustorial matrix have also been observed extending from the extrahaustorial membrane into the host cytoplasm and may represent infection-specific structures since they are not observed at other plant membranes (Mendgen et al. 1991; Mims et al. 2002; Mims et al. 2003). However, these structures were not reported at the host-haustorial interface during *P. recondita* or *Uromyces appendiculatus* rust-infection (Mendgen et al. 1991).

Haustoria have proved difficult to analyse at a molecular level as they are only produced *in planta*, requiring specific host factors for their development. In order to overcome this limitation, methods to isolate haustoria either by differential centrifugation or based on affinity purification with the lectin concanavalin A have been developed and employed (Gil and Gay 1977; Tiburzy et al. 1992; Hahn and Mendgen 1992; Voegelé and Mendgen 2003; Murdoch and Hardham 1998; Catanzariti et al. 2006). Due to the intimate connection with the host, haustoria were proposed to have a role in nutrient acquisition as specialised fungal feeding structures. This was supported by a study analysing genes that are preferentially expressed in isolated *U. fabae* haustoria, termed *In Planta* Induced Genes (PIGs), which resulted in the identification of two sequences with homology to genes involved in nutrient acquisition (Hahn and Mendgen 1997). These sequences, renamed AAT1 and AAT2, were found to encode amino-acid transporters (Hahn and Mendgen 1997). Further experiments demonstrated that AAT1 was a broad-specificity amino acid transporter (Struck et al. 2002). In addition, AAT2 was shown by immunolocalisation to occur specifically in the haustorial membrane, although no amino acid transport activity has yet been described for this protein (Mendgen et al. 2000). Further evidence for a role in nutrient acquisition came from studies of the *U. fabae* HXT1p protein, a haustorially-expressed sugar uptake transporter, which localises to the periphery of dikaryotic haustoria but not to intercellular hyphae (Voegelé et al. 2001; Voegelé and Mendgen 2003). HXT1p was shown to transport monosaccharides through a proton symport process when expressed in *Xenopus* oocytes demonstrating a potential nutrient uptake mechanism (Voegelé et al. 2001). Given that ATPases, which are required for the pumping of protons across membranes, are reduced on the extrahaustorial membrane but enriched on the

fungal haustorial membrane (Baka et al. 1995; Struck et al. 1998; Struck et al. 1996), this finding suggests that during infection, a proton gradient is generated between the extrahaustorial matrix and the haustorium that is used to drive active transport of nutrients towards the pathogen (Szabo and Bushnell 2001; Baka et al. 1995; Aist and Bushnell 1991).

Apart from this role in nutrient acquisition, it is also emerging that the secretion of pathogen proteins, known as effectors (described in greater detail below), from haustoria is important in establishing biotrophy by allowing manipulation of host metabolism and defense responses. In agreement with this scenario, compatibility between the host and the pathogen appears to be determined at the onset of haustorial development since spore germination, germ tube growth, appressorium formation and growth of primary infection hyphae develop on a similar time scale in both susceptible and resistant host plants (Dickinson and Lucas 1982). Effectors may act in the apoplast, extrahaustorial matrix or within the plant



cell (Figure 1-3).

Figure 1-3 The host-haustorium interface. In haustoria-forming biotrophic fungi, effectors (red dots) are secreted from haustoria (H) into the extrahaustorial matrix (EHMX), which is a discrete compartment sealed by a neckband (N). Effectors may cross the extrahaustorial membrane (EHM), a modified host plasma membrane that invaginates around the haustorium, into the host cytoplasm, where they may interact with target proteins, thus leading to host manipulation or resulting in recognition by cognate resistance (R) genes and the mounting of a defense response. Effectors may also be secreted into the apoplast. PM: plant plasma membrane; CW: plant cell wall; HM: haustorial membrane; HMC: haustorial mother cell; HCW: haustorial cell wall.

Other eukaryotic plant pathogens from distant phylogenetic groups, such as the obligate biotrophic powdery mildews (ascomycetes) as well as some hemibiotrophic fungi and oomycetes also produce haustoria, indicating that similar infection structures have evolved independently multiple times. Although powdery mildew haustoria differ in morphology from rust haustoria as they are often extensively branched, with multiple finger-like projections from a main body, they

share structural similarities to those of the rust fungi including the presence of a neckband and differentiation of the extrahaustorial membrane compared to the host plasma membrane, as indicated by the detection of a large (200 kDa) glycoprotein specifically in the extrahaustorial membrane formed during pea powdery mildew (*Erysiphe pisi*) infection (Micali et al. 2008; Roberts et al. 1993). Additional evidence for the specialisation of the extrahaustorial membrane comes from analysis of powdery mildew (*Erysiphe cichoracearum*) infections of *Arabidopsis*, where host plasma membrane proteins were shown to be excluded from extrahaustorial membranes, instead accumulating at the necks of haustoria (Koh et al. 2005). On the other hand, haustoria of hemibiotrophic fungi and oomycetes appear to be less differentiated compared to intercellular hyphae and generally lack neckbands, although some exceptions exist, as in the case of the oomycete *Albugo candida* which possesses a simple neckband and an extrahaustorial membrane that is differentially stained by phosphotungstic acid compared to the host membrane (Soylu 2004; Perfect and Green 2001). In addition, tubular extensions into the cytoplasm from the extrahaustorial membrane were detected during infection of the oomycete *A. candida*, although again, this is not a feature that has been reported in other oomycete phytopathogens (Baka 2008). The haustorial membranes of hemibiotrophs can also be differentiated from hyphal membranes since the membrane protein *Pihmp1* was shown to localise to and to be required for the formation of haustoria in the oomycete *Phytophthora infestans*, indicating that the haustorial membranes of oomycetes have specific components that distinguish them from the membranes of intercellular hyphae (Avrova et al. 2008). Underscoring the importance of haustoria as centers of pathogen protein secretion, specific secretion of proteins from oomycete haustoria has also been demonstrated (Whisson et al. 2007).

Other hemibiotrophic and biotrophic fungal pathogens, such as *Magnaporthe oryzae*, *U. maydis* and *Colletotrichum* spp., do not produce haustoria, but nevertheless form intracellular hyphae (Mendgen and Hahn 2002; O'Connell and Panstruga 2006). Interestingly, in these cases, a zone of separation still exists between the pathogen and the host cytoplasm. For example, intracellular hyphae of *M. oryzae* are also surrounded by a host-derived plasma membrane, termed the extra-invasive hyphal membrane, although no neckband structure was detected which might seal the space between the fungus and the host membrane (Kankanala et al. 2007). However, the extra-invasive hyphal matrix surrounding *M. oryzae* invasive hyphae appears to be distinct from the plant apoplast since addition of the lipid-binding dye FM4-64 to the apoplast

leads to the incorporation of the dye into the plasma membrane of plant cells but not intracellular hyphae (Kankanala et al. 2007). Fascinatingly, it has recently come to light that *M. oryzae* primary invasive hyphae have a specialised region of protein secretion termed the biotrophic interfacial complex (BIC) (Figure 1-4; Khang et al. 2010). It is of interest to note that in hemibiotrophic pathogens, the separation between the host and the pathogen is only maintained during the biotrophic stage. Upon the switch to necrotrophy, the host plasma membrane disintegrates around intracellular fungal structures suggesting the zone of separation between host and pathogen is important to maintaining a biotrophic relationship (Voegelé et al. 2009).

1.3 Host-pathogen specificity

As described in the previous section, rust fungi as well as other biotrophic and hemibiotrophic fungi are able to grow within living host cells and tissues. In order to maintain this intimate association, the pathogen alters and manipulates the host metabolism extensively. Among the changes that have been documented in host plants during rust infection are invagination of the host plasma membrane to accommodate the invading fungal structure; an increase in chlorophyll and photosynthetic activity, leading to the “green island effect”; redistribution of carbohydrates into infected leaves and movement of the host nucleus and endomembrane system to surround the extrahaustorial membrane (Scholes and Farrar 1985; Coffey 1972; Leckie et al. 1995; Voegelé et al. 2009).

On the other hand, plant cells are able to sense pathogen invasion by perception of mechanical wounding (Hardham et al. 2008) as well as by recognition of conserved pathogen-derived molecules, such as chitin fragments from fungal cell walls and GP42 proteins from oomycetes, which are collectively referred to as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs). Indeed, pattern recognition receptors that recognise PAMPs have been identified, such as the rice CEBiP receptor that recognises fungal chitin (Kaku et al. 2006). In interactions between a would-be pathogen and a non-host plant, detection of invasion by the plant results in a defense response known as PAMP-triggered immunity (PTI), which can be quite localised. Electron microscopy of host cells during infection by the barley powdery mildew *Blumeria graminis*, for example, showed the presence of vesicle-like bodies and pouch-like structures, including multivesicular bodies and vesicles with hydrogen peroxide and phenolics,

accumulating in the host cell at the site of penetration (An et al. 2006b). Apart from the generation of reactive oxygen species (ROS), PTI-associated defense responses include reinforcing the cell wall with cell wall appositions (papillae) and the induction of defense-related genes which inhibit further growth of the invader (Aist 1976; Torres 2010; Schwessinger and Zipfel 2008). As demonstrated during the interaction of *Uromyces vignae* with the non-host broad bean plant, clathrin-mediated endocytosis in the plant is active at this early stage of infection and may function to recycle activated defense-related receptors from the plant surface (Xu and Mendgen 1994; Leborgne-Castel et al. 2010).

It is the current paradigm that PTI, also known as basal resistance or innate immunity, is subverted by adapted pathogens through the deployment of pathogen-encoded effectors (Jones and Dangl 2006). Indeed, pre-infection by an adapted pathogen may permit subsequent growth by non-host pathogens, indicating that a general suppression of the host defense response occurs (Fernandez and Heath 1991).

In turn, plants have evolved resistance (R) proteins with the ability to recognise some of these effectors, termed avirulence (Avr) factors. Identified plant R proteins have a nucleotide binding (NB) domain and a C-terminal leucine-rich repeat (LRR) region (Dodds and Rathjen 2010). In addition, many R proteins also have an N-terminal Toll, interleukin-1 receptor (TIR) domain or a coiled-coil (CC) domain (Dodds and Rathjen 2010). These proteins have structural similarity to proteins that have been found to be important for immunity in animal systems, namely the Toll receptors in *Drosophila* and Toll-like receptors in mammals (Staskawicz et al. 2001). Collectively NB-LRR proteins confer resistance against various pathogens including fungi, oomycetes, bacteria, viruses and insects (Dodds and Rathjen 2010). Recognition of an Avr factor by a corresponding R protein results in effector-triggered immunity (ETI), which has been described as an accelerated and amplified form of PTI and which normally culminates in a form of programmed cell death known as the hypersensitive response (HR) (Dodds and Rathjen 2010; Jones and Dangl 2006). In flax plants, the M and L6 NB-LRR resistance proteins and their corresponding Avr proteins in the flax rust, AvrM and AvrL567 respectively, have been cloned (Lawrence et al. 1995; Anderson et al. 1997; Dodds et al. 2004; Catanzariti et al. 2006).

1.4 Effectors

Due to the importance of effectors in the infection process, these proteins have drawn considerable interest in the last few years. In recent literature, effectors have been defined as small molecules, including peptides, which alter the structure and function of the host, whether by increasing pathogen virulence or by triggering a defense response (Hogenhout et al. 2009; Kamoun 2009).

In the past, sequences encoding effectors such as AvrL567 from *M. lini*, AvrPita, PWL1 and PWL2 from *M. oryzae*, Avra10 and AvrK1 from the powdery mildew *B. graminis* and AvrLm1 and AvrLm6 from *Leptosphaeria maculans* were identified by positional cloning (Dodds et al. 2004; Valent et al. 1986; Sweigard et al. 1995; Orbach et al. 2000; Ridout et al. 2006; Gout et al. 2006; Fudal et al. 2007). However, advancements in DNA sequencing technologies have enabled the sequencing of entire phytopathogen genomes. Combining this with bioinformatic analysis, it is now possible to predict suites of candidate effectors based on the general criteria of identifying sequences encoding small proteins under diversifying selection with a signal peptide from pathogen genomes (Mueller et al. 2008; Dean et al. 2005; Kamper et al. 2006; Haas et al. 2009). Similarly, these search criteria have also been extended to cDNA libraries and EST (Expressed Sequence Tags) databases enriched with sequences from the biotrophic phase of pathogenic biotrophs or from the host-pathogen interface. Such a strategy resulted in the identification of AvrM, AvrP4 and AvrP123 from *M. lini*, UfRTP1 from the broad bean rust *U. fabae* and the candidate effectors BAS1-4 from *M. oryzae* (Catanzariti et al. 2006; Hahn and Mendgen 1997; Mosquera et al. 2009). In order to facilitate this process, programs to analyse genomes and EST library sequences encoding small secreted proteins have been designed (Torto et al. 2003; Choi et al. 2010). It is noted however that bioinformatic screens have their limitations. For instance, such programs would miss effectors without conventional signal peptides such as the *B. graminis* effectors Avra10 (Ridout et al. 2006).

Many important insights into effector biology have been acquired by studying the sequences of predicted effectors. One of the best examples of this is that sequence comparisons between known *Phytophthora* effectors have detected a conserved N-terminal RXLR dEER motif (RXLR-X₅₋₂₁-dEER; where upper case letters represent consensus amino acids in 10 out of 11 sequences and lowercase letters represent consensus amino acids in more than half of the genes) (Rehmany et al. 2005; Birch et al. 2006). Using this as an additional search criterion on the

sequenced genomes of *Phytophthora*, approximately 350 RXLR proteins were identified from the genome sequences of *Phytophthora sojae* and *Phytophthora ramorum*, respectively (Tyler et al. 2006). However, it is noted that other estimates range from 158 to more than 600 candidates in *P. sojae* and approximately 200 to more than 500 in *P. ramorum* based on different stringencies of the search settings, such as whether or not the dEER motif is also included and if hidden Markov Model statistics are applied (Win et al. 2007). Other motifs that have been identified by sequence comparisons of potential *Phytophthora* effectors include the W-, L- and Y-motifs in the C-terminal portions of some RXLR effectors, which have been hypothesised to be required for effector function (Jiang et al. 2008). The W-motif was found to be required for the HR mediated by the interaction between the Rpi-blb1 resistance protein and ipiO effectors (Champouret et al. 2009). Further, the W- and Y-motifs were shown to be required for the ability of Avr1b to suppress programmed cell death (Dou et al. 2008).

An additional family of *Phytophthora* secreted proteins known as Crinklers, which have a potential necrosis-inducing function in host plants, were also identified by analysis of ESTs encoding secreted sequences (Torto et al. 2003). Subsequent analysis of the genomes of *P. infestans*, *P. sojae* and *P. ramorum* later revealed that members of this family have a conserved LXLFLAK motif (Haas et al. 2009; Win et al. 2007). Similar analyses of EST sequences isolated from barley epidermal cells containing *B. graminis* powdery mildew haustoria identified the Y/F/WxC-motif in small secreted proteins encoded by this fungus (Godfrey et al. 2010). It is noted that not all pathogen effectors may have such conserved amino acid sequence motifs, thus restricting the amount of information that can be obtained by sequence comparisons. This appears to be the case with rust fungi, since no such motifs have yet been identified from ESTs isolated from poplar rust haustoria (Joly et al. 2010). In addition to this, it is important to keep in mind that whether or not all predicted proteins have an effector function is unknown and experimental characterisation of these proteins remains an important area of research to verify the biological significance of these findings. To this end, several high throughput techniques for functional screening of potential effectors have been developed, including screening for phenotypes from expression of agroinfiltrated proteins on potential host plants (Torto et al. 2003; Oh et al. 2009).

1.5 Importance of effectors during infection

The large number of effectors predicted from sequence analysis hints at the complex nature of pathogen host interactions. While it is possible that not all of the predicted proteins function as

effectors, one potential explanation for the large number of effectors is that different subsets of these proteins may be required at different stages of infection. For example, the deletion of a potential virulence cluster from the genome of *U. maydis* encoding several small secreted proteins which are up-regulated during infection led to defects in tumour and spore formation, but did not affect penetration or hyphal growth of the fungus within plant tissue (Kamper et al. 2006). In addition, expression of effectors may differ according to the type of host tissue infected, as indicated in *U. maydis* (Skibbe et al. 2010). Apart from this, it is noted that it seems common that multiple variants of an effector may exist in pathogens, as observed with the flax rust effector AvrM, which has six paralogs (Catanzariti et al. 2006). This is perhaps unsurprising given that effectors are under selective pressure to evolve to evade recognition by host R proteins and to retain virulence. Interestingly, it was discovered recently that in the case of the multigene *P. infestans* ipiO family, normal recognition of the IPI-O1 variant by the RB-resistance protein is impaired in the presence of the IPI-O4 variant, which is not recognised by the RB-resistance protein (Haltermann et al. 2010). Although this is not the case with all effectors, the protective effect conferred by sequence variants may be another possible explanation for the pathogen to encode or express a large number of effectors.

For the most part, efforts to elucidate the functions of effectors and to understand how they contribute to pathogenicity are complicated by the fact that many of these proteins do not have sequence homologies with other known proteins. Despite this limitation, a number of effectors have been shown to interfere with host basal defense responses. For instance *C. fulvum* seems to use a dual strategy to avoid triggering PTI. The Avr4 effector protein binds to chitin in the fungal cell wall and may protect it from the action of host chitinases (van den Burg et al. 2006). These enzymes target chitin for degradation, possibly reducing the fungal cell wall integrity, but importantly also releasing chitin oligomers which act as PAMPs and trigger PTI. A second *C. fulvum* effector, Ecp6, binds to digested chitin fragments and effectively competes with host PAMP recognition receptors for chitin binding, thereby preventing PTI induction by any residual degradation activity (de Jonge et al. 2010). Other effectors are predicted to target host enzymes that may be involved in plant defense including the EPI1 Kazal-like protease inhibitor from *P. infestans*, which inhibits the protease P69B, the EPIC2B protein which inhibits host papain proteases PIP1 and RCR3, the *C. fulvum* Avr2 effector which also inhibits RCR3, and the *P. infestans* glucanase inhibitors GIP1 and GIP2 (Tian et al. 2005; Tian et al. 2007; Song et al. 2009; Rooney et

al. 2005; van Esse et al. 2008; Shabab et al. 2008; Rose et al. 2002; Damasceno et al. 2008; Hein et al. 2009). Similarly, among the flax rust effectors that have been identified, AvrP123 has been shown to have homology to Kazal-like protease inhibitors although the biological significance of this has not yet been demonstrated (Catanzariti et al. 2006). Some effectors also appear to inhibit downstream PTI signalling processes. Indeed, the *P. infestans* Avr3a effector suppresses cell death induced by the elicitor INF1 in plants that lack the R3a resistance protein (Bos et al. 2009; Bos et al. 2006). Further, in *Fusarium oxysporum* infection of tomato, the effector Avr1 inhibits HR induced by Avr2 and Avr3 (Houterman et al. 2008).

The functions of many other effectors have yet to be demonstrated although many identified effectors are Avr proteins, and thus are known to trigger the resistance response when detected by a corresponding R protein. Interestingly, mutational analysis of Avr3a indicated that the avirulence and virulence functions of the effectors can be separated, since mutants that were recognised by the R3a resistance protein but which did not suppress INF1-induced cell death could be isolated (Bos et al. 2009). This suppression of INF1-induced cell death has recently been shown to be related to the interaction of Avr3a with the U-box E3 ligase CMPG1, where paralogs that interact with and stabilise CMPG1 prevent HR, and paralogs that do not are unable to do so (Bos et al. 2010). In the flax-flax rust system, it was demonstrated in yeast two-hybrid assays that avirulent but not virulent forms of the AvrL567 and AvrM effectors are able to interact directly with the L6 and M resistance proteins respectively, indicating that R protein recognition for these proteins at least may be mediated by direct interaction (Dodds et al. 2006; Catanzariti et al. 2010).

1.6 Delivery of effectors into host cells

An interesting observation made from studies analysing the functions of fungal effectors is that known targets of effectors can be extracellular (in the apoplast) or in the host cytoplasm. Early evidence for the delivery of eukaryotic phytopathogen proteins into host cells came from immunolocalisation of UfRTP1, a haustorially-expressed, secreted glycoprotein of unknown function originating from the broad bean rust (Kemen et al. 2005). From this study, it was shown that UfRTP1 was localised to the extrahaustorial matrix and within the host cell during infection (Kemen et al. 2005). Further, the protein was found to have a functional nuclear localisation signal and accumulated in the host nucleus during infection, although a closely related homolog from *Uromyces striatus*, UsRTP1, was not shown to possess this host nuclear localisation (Kemen et al. 2005).

In the case of the flax rust effectors, host cytoplasmic localisation was initially inferred from the discovery that transient expression of AvrL567 and AvrM in the host cytoplasm by *Agrobacterium*-mediated transformation was able to trigger HR in plants with the corresponding R proteins suggesting that the protein is targeted to the cytoplasm prior to HR during infection (Dodds et al. 2004; Catanzariti et al. 2006). In fact these host R proteins, L6 and M, are cytosolic (D. Takemoto et al., unpublished observations) and have been shown to interact directly with AvrL567 and AvrM respectively in yeast two-hybrid assays (Dodds et al. 2006; Catanzariti et al. 2010). Similar results were also obtained when transiently expressing cytoplasmic AvrP123 and AvrP4 from *M. lini* and Avra10 and AvrK1 from *B. graminis* in plants with the respective R genes indicating that delivery of effectors in the cytoplasm is common in fungal pathogens with haustoria (Catanzariti et al. 2006; Ridout et al. 2006; Barrett et al. 2009). Other experiments showed that recognition of some oomycete effectors is also intracellular. Interestingly, in haustoria-forming oomycetes the RXLR motif conserved in the N-terminus of many effectors has been shown to mediate uptake into plant cells (Whisson et al. 2007; Dou et al. 2008). Further, the RXLR motif is not the only host cell cytoplasm targeting motif in *Phytophthora* effectors. Members of the Crinkler family appear modular with a signal peptide followed by a conserved N-terminal motif (LXLFLAK) before a divergent C-terminal effector region (Haas et al. 2009; Win et al. 2007; Schornack et al. 2010). This class of protein was first identified as secreted proteins that induce necrosis when expressed in plant cells and further studies have shown that the C-terminal effector region is localised to the host nucleus during infection and is able to trigger cell death (Torto et al. 2003; Schornack et al. 2010). Similar to the RXLR motif, it was recently demonstrated that the LXLFLAK motif was required for targeting of Crinkler proteins into host cells since alanine substitution of the motif resulted in loss of protein uptake, although a recent proteomics study also identified a potentially cell wall associated Crinkler protein, indicating that these proteins may have different sub-cellular localisations during infection (Schornack et al. 2010; Grenville-Briggs et al. 2010). Interestingly, Crinkler effectors are more widely conserved among oomycetes compared to the RXLR effectors and some plant parasitic oomycetes, such as the legume rot pathogen *Aphanomyces euteiches*, that lack haustoria possess Crinklers but not RXLR effectors (Schornack et al. 2010).

In *M. oryzae*, which lacks haustoria but forms intracellular hyphae, secreted effectors have also been demonstrated to enter the host cytoplasm during infection. This includes the AvrPita

effector which was shown to bind to the host cytoplasmic Pi-ta protein from rice *in vitro* by yeast two-hybrid analysis (Jia et al. 2000). Expression of AvrPita by particle bombardment in the cytoplasm of plants expressing the Pi-ta resistance protein was also shown to trigger the HR response indicating that AvrPita avirulence function may also occur in the cytoplasm *in planta* (Jia et al. 2000). Intriguingly, recent, more direct analysis tracking the delivery of *M. oryzae* effectors into host cells revealed that many host cytoplasmic effectors, including AvrPita, PWL1, PWL2 and BAS1 appear to be secreted from the BIC (Figure 1-4; Khang et al. 2010). Secretion into the BIC appears to be directed by the signal peptide and/or part of the promoter encoding sequence since these regions of AvrPita, PWL1 and PWL2 are sufficient to direct fluorescent protein localisation to the BIC characteristic of the full-length protein fluorescent protein fusions (Khang et al. 2010). This regulated export of proteins is especially interesting since it appears that secretion of AvrPita occurs through the regular ER-mediated secretory pathway (Yi et al. 2009). At present, the role of the BIC and the mechanism behind effector uptake across the host membrane has yet to be characterised.

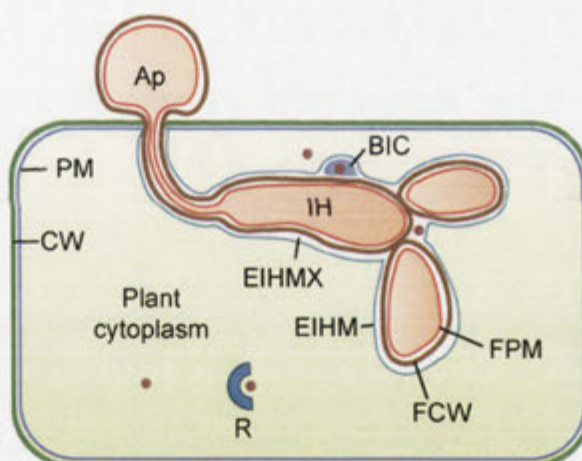


Figure 1-4 The host-pathogen interface of *M. oryzae*. During infection, *M. oryzae* grows within host cells in the form of invasive hyphae (IH), which are surrounded by the extra-invasive hyphal membrane (EIHM). A subset of effectors (red dots) are secreted at the tip of primary hyphae in a region that develops into the biotrophic interfacial complex (BIC), which may mediate effector entry across the EIHM into the host cell. Other effectors may be secreted into the space between the EIHM and the fungus known as the extra-invasive hyphal matrix (EIHMx). Entry of effectors into host cells may be detected by cognate resistance (R) genes which triggers a defense response. CW: plant cell wall; PM: plant plasma membrane; FCW: fungal cell wall; FPM: fungal plasma membrane; Ap: appressorium.

Given the large number of pathogen effectors that are predicted to enter the host cell, elucidating the mechanism of host cell entry is clearly an important step in understanding the infection process. In terms of plant pathogens, the best known system of effector translocation

into the host is that of phytopathogenic bacteria. In this case, effector delivery from the bacterial cell into the host occurs via a complex nanomachine, known as the Type III secretion system, that is conserved in Gram-negative bacteria (Cornelis 2006; Alfano and Collmer 2004; Galan and Wolf-Watz 2006). In general, these complexes consist of a basal structure that resembles a flagellum, with a needle- or pilus-like appendage (Cornelis 2006). In plant pathogenic bacteria, Hrc (HR and conserved) proteins are responsible for translocation of bacterial effector proteins across the bacterial membrane, while Hrp (HR and pathogenicity) proteins are required to translocate effectors into the host (Alfano and Collmer 2004). In this system, effectors are injected directly from bacteria into the host cytoplasm without passing through the apoplast as shown in Figure 1-5.

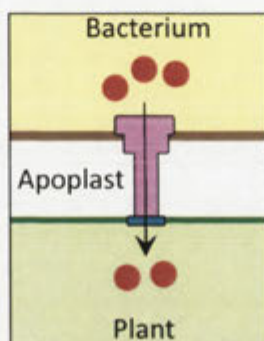


Figure 1-5 Schematic representation of the bacterial Type III secretion system, required for delivery of bacterial effectors into the plant cytoplasm. Red dots: effectors

Infection-specific tubular vesicular structures extending from the extrahaustorial membranes of some rust haustoria were also proposed as another potential route to mediate effector transfer into host cells (Voegelé et al. 2009). However, UfRTP1, which was demonstrated to enter the nucleus and cytoplasm of infected cells, was not documented to be localised to such structures in analyses of the *U. fabae* pathogen-host interface (Kemen et al. 2005). Further, the uptake of *M. lini* effectors into host cells appears to be independent of disease development since expression of secreted AvrM and AvrL567 by uninfected plant cells can still trigger HR in plants with the corresponding R protein in the absence of the pathogen (Catanzariti et al. 2006; Rafiqi et al. 2010). In addition, deletion analysis of AvrM and AvrL567 identified short regions of less than 31 amino acids in the N-terminal region of each mature peptide that were required for uptake into

host cells (Rafiqi et al. 2010). Unlike the RXLR and Crinkler effectors, no conserved motif was identified between the minimum uptake regions of the two fungal effectors (Rafiqi et al. 2010).

Like the flax rust effectors, the mechanism of RXLR protein uptake into host cells appears to be pathogen-independent (Dou et al 2008). This was demonstrated in experiments where GFP fluorescence was detected in the cytoplasm and nucleus of root cells incubated in purified Avr1b-GFP protein (Dou et al. 2008). This uptake was shown to be dependent on the RXLR-dEER motif since alanine substitution of this motif abolished the accumulation of GFP in the cytoplasm of root cells (Dou et al. 2008). The requirement for the RXLR-dEER motif for effector entry into host cells was also demonstrated in Avr3a-GUS proteins expressed from the pathogen *P. infestans* during infection (Whisson et al. 2007). Interestingly, SpHTP1, an RXLR protein from the fish pathogenic oomycete *Saprolegnia parasitica* was also shown to enter host cells in fish, indicating that the mechanism of RXLR entry is not only pathogen-independent but exploits an uptake pathway that is widely conserved among eukaryotic cells (van West et al. 2010).

Intriguingly, the RXLR motif is very similar to the host targeting (HT) or protein export element (PEXEL) of effectors from malarial pathogens from the genus *Plasmodium*. During infection, the malarial parasite is engulfed by erythrocyte cells but is separated from the host cytoplasm by the parasitophorous vacuole and surrounded by the parasitophorous vacuolar membrane (Goldberg and Cowman 2010). As such, the parasitophorous vacuole is similar to the extrahaustorial matrix or extra-invasive hyphal matrix that forms during infection by eukaryotic plant pathogens. The PEXEL motif [RXLX (E/Q/D)] is, like the RXLR motif, found at the N-terminal portion of proteins downstream from the signal peptide cleavage site and was found to be associated with the export of proteins from the parasite into the cytoplasm of the host (Hiller et al. 2004; Marti et al. 2004). Further experiments demonstrated that the PEXEL motif could functionally substitute the RXLR motif and vice versa (Bhattacharjee et al. 2006; Grouffaud et al. 2008; Dou et al. 2008). Recent work on the malarial pathogen showed that the PEXEL motif is actually a protease recognition site that is cleaved at the conserved leucine before subsequent acetylation at the N-terminus and that while the arginine and leucine are important for cleavage, it is the final residue (glutamic acid, glutamine or aspartic acid) that is required for export into the host (Chang et al. 2008; Boddey et al. 2009). Subsequent studies revealed that several pathogen factors are required for PEXEL export, including the PEXEL-processing protease Plasmepsin V, localised in the ER of the pathogen, and a multimeric complex consisting of parasite-encoded

proteins, termed the *Plasmodium* translocon of exported proteins (PTEX) that is localised to the parasitophorous vacuolar membrane during infection as shown in Figure 1-6 (Boddey et al. 2010; de Koning-Ward et al. 2009). Since these findings conflict with evidence that RXLR proteins are able to enter cells in a pathogen-independent manner, it appears that the mechanism of RXLR entry into host cells differs from that of the PEXEL proteins.

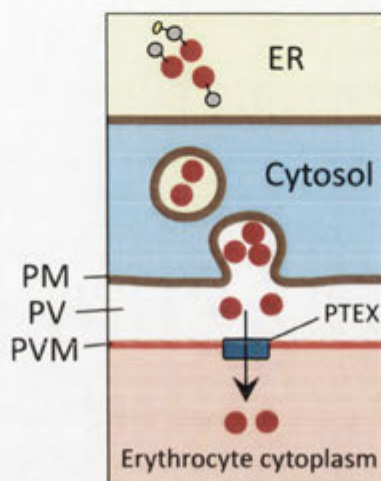


Figure 1-6 Model of the translocation of *Plasmodium* effector proteins with the PEXEL motif into the host erythrocyte cytoplasm. In the ER of the parasite, the signal peptide (yellow dot) and the majority of the PEXEL motif (RXL, grey dot) is recognised and cleaved sequentially from proteins targeted for export into the erythrocyte cytoplasm (red dots). Proteins are secreted from the parasite into the parasitophorous vacuole (PV) where the *Plasmodium* translocon of exported proteins (PTEX) translocates proteins across the parasitophorous vacuolar membrane (PVM) into the host. PM: *Plasmodium* plasma membrane.

An alternative mechanism that has recently been proposed for RXLR effector entry into host cells is uptake mediated by binding of effectors to the lipid phosphatidylinositol-3-phosphate (PtdIns(3)P) on the extracellular leaflet of the lipid bilayer of the host plasma membrane (Kale et al. 2010). In the study, it was reported that RXLR effectors bound to PtdIns(3)P and that lipid binding was abolished by substitution of the motif with alanines (Kale et al. 2010). Further, uptake was inhibited by co-incubation of effectors with PtdIns(3)P-binding proteins, presumably due to competition for PtdIns(3)P-binding sites on the host plasma membrane (Kale et al. 2010). RXLR effector uptake was also shown to be reduced by treatment with wortmannin which inhibits the kinase activity of phosphatidylinositol-3 kinase, required for generation of PtdIns(3)P in cells (Kale

et al. 2010). Further experiments by Kale et al. (2010) provided evidence that fungal effectors including AvrL567 from *M. lini* as well as AvrLm6 and AvrLm2 from *L. maculans* may also enter host cells by PtdIns(3)P-binding.

Another fungal protein that is able to internalise into host plant cells in the absence of the pathogen is the PtrToxA toxin from the causal agent of wheat tan spot, *Pyrenophora tritici-repentis* (Manning and Ciuffetti 2005). Host cell entry of this toxin is dependent on the presence of an RGD vitronectin-like motif (Manning et al. 2008). This motif has been demonstrated to be involved in the binding of members of the integrin protein family, which associate actin within cells with the extracellular matrix and are known to be targets of mammalian pathogens which internalise into host cells via a form of clathrin-mediated uptake (Isberg and Tran Van Nhieu 1994). Interestingly, the uptake of this protein occurred in ToxA-sensitive but not ToxA-insensitive host mesophyll cells and was concentrated close to the infiltration site, indicating possible interaction with a high-affinity receptor on the surface of the host (Manning and Ciuffetti 2005). Together these results were taken to indicate that the ToxA fungal protein may be internalised by a form of receptor-mediated endocytosis. The RGD motif is also present in the *P. infestans* IPI-O effector (which also contains an RXLR motif) and IPI-O has been shown to compete with other RGD peptides in binding to the plasma membrane (Senchou et al. 2004). However, this motif is absent from the AvrM and AvrL567 minimum uptake regions identified by Rafiqi et al. (2010) indicating that binding to integrins may not be the mechanism of host cell entry for these flax rust effectors.

In addition, another group of proteins that is able to enter cells without requiring translocation machinery are proteins containing cell penetrating peptides (CPPs). The mechanism of CPP entry is not widely understood, although it is thought that these peptides of less than 30 amino acids in length are able to internalise into animal and plant cells by virtue of secondary structure and a net positive charge (Drin et al. 2003; Drin et al. 2001). Intriguingly, there is evidence that CPP internalisation may occur by different mechanisms including by direct membrane penetration through pore formation as well as various forms of endocytosis (Drin et al. 2003; Richard et al. 2005; Wadia et al. 2004; Ter-Avetisyan et al. 2009; Joliot and Prochiantz 2004).

Endocytosis is the process by which cells internalise a variety of molecules at the cell surface, such as nutrients, ligands and transmembrane receptors, as well as the plasma membrane. Currently, the many endocytic internalisation pathways that have been identified are broadly categorised into two major groups: the clathrin-dependent and the clathrin-independent

pathways (Mayor and Pagano 2007; Doherty and McMahon 2009). Clathrin-dependent endocytosis, a major endocytic pathway of animal cells, was only recently shown to be an important internalisation mechanism in plant cells (Dhonukshe et al. 2007). In this form of endocytosis, internalisation occurs at coated pits consisting of polymerised clathrin and several other accessory factors, which are required for cargo recognition, clathrin recruitment, membrane association, membrane budding and scission (Schmid and McMahon 2007). It is emerging that clathrin-independent endocytosis pathways are also important in contributing to uptake and membrane turnover. Examples of clathrin-independent uptake that have been identified in mammalian cells are phagocytosis, macropinocytosis, caveolin-dependent endocytosis, flotillin-dependent endocytosis, the clathrin-independent carrier/GPI-AP enriched early endosomal compartment (CLIC/GEEC) pathway, the IL2 receptor β (IL2R β) pathway and the Arf6 dependent pathway (Doherty and McMahon 2009).

1.7 Objectives and experimental approaches

In order to achieve a better understanding of the mechanism of fungal disease establishment in plants, it is important to establish the fate of secreted fungal effectors during infection. In an effort to address this issue, one of the objectives of the work presented in this thesis is to directly examine the localisation of the flax rust effector AvrM in host plant cells during flax rust infection. This was done, as described in Chapter 2, by raising antibodies against AvrM and using these antibodies to perform immunofluorescence and immunogold localisation assays of infected material. This objective was further addressed, as described in Chapter 3, by generation of transgenic flax rust fungi expressing fluorescent protein fusions of AvrM. The results presented provide direct evidence for the translocation of AvrM into the cytoplasm of host cells. The mechanism of AvrM uptake into plant cells was further investigated in a number of ways (Chapters 2, 4 and 5). The intracellular localisation of AvrM within infected host cells was determined at the ultrastructural level (Chapter 2). Different methods to assay the uptake of AvrM into host cells were tested (Chapter 4). Further, the effects of inhibiting various host endocytic components by viral-induced gene silencing and dominant negative expression of endocytic proteins on AvrM uptake into plant cells were examined. Chapter 5 reports the results of testing the lipid binding requirement of AvrM and AvrL567 effector proteins in the context of effector uptake into host

cells by expressing various deletion and truncation mutants of each effector with different abilities to enter host cells and testing the lipid binding activity of these proteins on lipid blots.

CHAPTER 2

2 Immunolocalisation of AvrM

2.1 Introduction

The avirulence gene *AvrM* was identified by screening for highly expressed sequences in flax rust haustoria encoding secreted proteins (Catanzariti et al. 2006). Expression analysis of *AvrM* transcripts showed that the gene is expressed in *in vitro* germinated spores and that transcript levels in total extracts of infected plant tissue increase over time during infection (Catanzariti et al. 2006). Homologs of *AvrM* have also been identified in poplar rust during analysis of the fungal *ex planta* secretome (Joly et al. 2010). In the flax rust strain used for this study, CH5, a total of six paralogs of *AvrM* have been identified (Table 2-1). The virulence locus encodes a single paralog *avrM*, while the avirulence locus encodes five paralogs (*AvrM-A*, *-B*, *-C*, *-D* and *-E*) (Catanzariti et al. 2006).

Table 2-1. Predicted sizes of *AvrM* paralogs in CH5 rust (Catanzariti et al. 2006).

Protein	Number of amino acid residues	Size (kDa)		Similarity to <i>AvrM-A</i> *	Necrotic response in plants with the M resistance protein expressing secreted effector
		With signal peptide	Without signal peptide		
<i>AvrM-A</i>	343 (C-terminal truncation)	38.5	35.3	-	Strong necrotic response
<i>AvrM-B</i>	377	42.4	39.2	90.7 %	Weak necrotic response
<i>AvrM-C</i>	377	42.4	39.2	91.0 %	Weak necrotic response
<i>AvrM-D</i>	350 (C-terminal truncation and 7 amino acid insertion)	39.3	36.1	98.0 %	Strong necrotic response
<i>AvrM-E</i>	212 (7 amino acid insertion and premature STOP codon)	23.3	20.1	59.8 %	No response
<i>avrM</i>	314 (2 deletions)	36.0	32.9	80.2 %	No response

*Similarity calculated using the BLOSUM50 substitution matrix on the Matrix Global Alignment Tool (MatGAT) v 2.02 (Campanella et al. 2003).

Of these, AvrM-A elicits the strongest and quickest hypersensitive response (HR) when expressed transiently via *Agrobacterium* in flax and tobacco plants expressing the M resistance protein (Catanzariti et al. 2006). Catanzariti et al. (2006) demonstrated that there was no difference between the HR response elicited by AvrM-A and -D proteins expressed by plant cells with and without the signal peptide. This indicated that AvrM is recognised inside plant cells, suggesting that it is delivered into host cells during infection. However no direct evidence of this occurring during infection has been available.

Two approaches commonly used to localise plant pathogen effectors during infection are: (1) transformation of pathogens to express effectors fused to fluorescent proteins, as in the case of *Ustilago maydis* Pep1 and *Phytophthora infestans* Avr3a (Doehlemann et al. 2009; Whisson et al. 2007) or (2) immunolocalisation using antibodies raised specifically against the effector protein of interest, such as in the case of the *Uromyces fabae* UfRTP1 protein (Kemen et al. 2005). For obligate biotrophs, which are more difficult to transform, the second method is the more technically feasible option. Both approaches were followed in the present study of the localisation of AvrM. Experiments to generate transgenic flax rust expressing AvrM fused to a fluorescent protein are discussed in the next chapter. In this chapter, experiments to generate antibodies against AvrM and immunolocalisation assays to confirm if the protein enters the host cytoplasm during infection are described.

2.2 Materials and Methods

2.2.1 Transformation of *E. coli*

2.2.1.1 Preparation of calcium-manganese based (CCMB) competent cells

Competent cells were routinely prepared using the CCMB method (Hanahan et al. 1991). *E. coli* cells were streaked from glycerol stocks onto an LB agar plate (Appendix), which was then incubated overnight at 37°C. A single colony was selected and used to inoculate 5 mL of LB broth (Appendix). This was incubated overnight at 37°C on a shaker. An aliquot of 400 µL of the overnight culture was used to inoculate 5 mL of LB broth. The culture was incubated on a 37°C shaker and harvested at the exponential phase of growth when O.D.₆₀₀ 0.4-0.5. Cells were harvested by centrifuging at 4000 × *g* for 10 min at 4°C. Pellets were gently resuspended in 1/5 of the original culture volume in CCMB buffer [10% (v/v) glycerol, 80 mM CaCl₂, 20 mM MnCl₂, 10 mM MgCl₂, 10 mM CH₃CO₂K] and then centrifuged at 15800 × *g* for 30 sec at 4°C. Resuspension and centrifugation of the pellet was repeated once and the resulting pellet was resuspended in 1/10 of the original culture volume in CCMB buffer. Aliquots of 0.1 mL were either used directly or frozen in liquid nitrogen and stored at -80°C.

2.2.1.2 Transformation

Competent *E. coli* cells prepared as described in Section 2.2.1.1 were thawed in an ice bath and mixed by gently flicking the tubes. Fifty nanograms of plasmid was mixed with 100 µL of cells and then incubated for 10 min on ice. Cells were heat shocked for 60 to 90 sec in a 42°C water bath. Tubes were immediately returned to the ice bath for 5 min. This was followed by the addition of 900 µL of room temperature SOC medium (Appendix). Cells were then incubated at 37°C for 1 h with shaking. An aliquot of the transformation mix was plated onto LB agar plates with the appropriate antibiotics to allow selection of transformants. Plates were incubated at 37°C overnight. The following day, colonies were selected with sterile tips and patched on to fresh selective agar plates. These plates were incubated overnight at 37°C.

2.2.1.3 Plasmid DNA isolation from bacterial clones

Plasmids were isolated from *E. coli* cultures grown in LB broth with appropriate antibiotics using the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA quality and concentration were determined by agarose gel electrophoresis and by spectrophotometry on a BioPhotometer (Eppendorf) assuming that 50 µg of DNA per mL has an O.D.₂₆₀ of 1.0.

2.2.1.4 DNA sequencing

Plasmid DNA at 50-125 ng/ μ L and gene-specific primers at 0.8 μ M were submitted to the Australian Genome Research Facility, Brisbane for sequencing.

2.2.2 Bacterial expression of His-tagged AvrM

The AvrM-A coding sequence lacking the signal peptide was cloned into the pET15b.ep plasmid (Novagen) to generate expression vectors encoding His-tagged AvrM (His-AvrM) fusion protein by Dr A-M Catanzariti (CSIRO, Plant Industry) and transformed into BL21 (DE3) pLysS *E. coli* cells by Dr S. Williams (Flinders University). Cultures were grown in LB with 0.1 % (w/v) glucose, 100 μ g/mL ampicillin and 50 μ g/mL chloramphenicol to an O.D.₆₀₀ of 0.6-0.8. Then, cultures were chilled before inducing protein expression by addition of isopropyl thio- β -D-galactoside (IPTG) to 1 mM and incubating overnight at 16°C with shaking.

2.2.3 Extraction and purification of AvrM proteins

Cells were harvested by centrifugation at 8217 $\times g$ for 10 min at 4°C in a JA10 rotor and then resuspended in Lysis buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.0, 300 mM NaCl, 10 mM imidazole]. Protease inhibitors (Sigma) compatible with nickel affinity chromatography were added to the cell suspension. Cells were lysed either by pressure lysis by performing two passes at 20000 pounds per square inch using a chilled pressure cell or by three successive freezing (-80°C) and thawing cycles. Cells were incubated for 10 min at 4°C with 0.5 mg/mL lysozyme, to increase lysis efficiency, and 50 μ g/mL DNaseI, to reduce the viscosity of the suspension.

His-AvrM protein was purified from the crude lysate using the protocol obtained from Dr S. Williams (personal communication). The lysate was cleared by centrifugation at 10000 $\times g$ for 30 min at 4°C, filtered through a 0.45 μ m filter (Nalgene) and loaded on a 1 mL Ni Sepharose 6 Fast Flow (GE Healthcare) column pre-equilibrated with protein lysis buffer. After loading protein samples, a further 5 column volumes (c/v) of lysis buffer were run through the column. This was followed by 25 c/v of Wash buffer A [20 mM HEPES pH 8, 300 mM NaCl, 65 mM imidazole]. The His-tagged protein was then eluted with approximately 4 c/v of Elution buffer A [20 mM HEPES pH 8, 300 mM NaCl, 250 mM imidazole]. Proteins were solvent exchanged into phosphate buffered saline pH7 [PBS pH7; 2.7 mM KCl, 138 mM NaCl, 1.5 mM KH₂PO₄, 20.4 mM NaH₂PO₄, pH 7] using an Amicon Ultra-15 centrifugal device with a molecular weight cut off point of 30 kDa (Millipore Corporation). Samples were then frozen in liquid nitrogen and stored at -80°C. Protein

concentrations were determined using the Bradford assay (Bradford 1976) with bovine serum albumin (BSA) diluted in protein extraction buffer as a standard.

2.2.4 Expression and purification of His-tagged, Ubiquitin-tagged AvrL567

The pHUE10-1 plasmid, for expression of AvrL567-A with a hexahistidine-tag and a Ubiquitin-tag (His-Ubi-AvrL567; Dodds et al. 2006) was transformed into BL21 (DE3) *E. coli*. The expression of His-Ubi-AvrL567 protein was induced using IPTG as described for AvrM. Bacterial cultures were lysed and the recombinant protein purified on a Ni-sepharose column as described for AvrM with the following modifications: After loading the crude lysate onto the column, the column was washed with 10 c/v of Wash buffer B [20 mM HEPES pH 8, 300 mM NaCl, 50 mM imidazole] and eluted with 4 c/v of Elution buffer B [20 mM HEPES pH 8, 300 mM NaCl, 150 mM imidazole].

2.2.5 Antibody production

2.2.5.1 Immunisation of rabbits

The pre-immune sera of New Zealand white rabbits were tested for immune reactions on sections of butyl-methylmethacrylate (BMM) embedded infected flax leaves (CH5 *Melampsora lini* on *Linum ussittassimum*) harvested 5 days post-infection (5 dpi) as well as on uninfected flax leaves by immunofluorescence and immunoblotting assays. Animals with pre-immune sera that did not produce a reaction were used to produce antibodies. Two rabbits were given three intraperitoneal injections at 3-week intervals, of 0.4 to 0.5 mg of antigen in PBS pH7 mixed with an equal volume of Freund's adjuvant. The rabbits were bled at 8 weeks after the first immunisation.

2.2.5.2 Purification of antibodies

Several protocols based on the antibody purification method described by Robinson et al. (1988) were tested.

2.2.5.2.1 Two-step affinity purification by positive and negative adsorption

A total of 1.5 mg semi-purified His-tagged AvrM protein (purified with 20 c/v of Wash Buffer A instead of 25 c/v) was loaded onto two 12% acrylamide gels and transferred to two Hybond-C extra nitrocellulose membranes (GE Healthcare). Blots were stained with Ponceau S [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] and the band corresponding to the His-AvrM protein was excised (Blot A) for the positive adsorption step and the remainder of the blot, containing contaminating bacterial proteins (Blot B), was kept for the negative adsorption step. The bands were destained with PBS pH7, rinsed in deionised water and dried. Poorly bound protein was removed by soaking

the membrane in 100 mM glycine, pH 2.5 for 5 min. Membranes were then rinsed twice for 5 min in Tris-buffered saline [TBS; 20 mM Tris-Cl, pH 7.4, 500 mM NaCl] and then blocked in 3% (w/v) BSA in TBS for 1 h at room temperature. Blot B was cut to 5 mm × 5 mm pieces and incubated overnight at 4°C with 10 mL of a 1:5 dilution of serum in TBST [TBS and 0.05% (v/v) Tween-20] (negative adsorption). Then, unbound antibodies were incubated with Blot A (containing the AvrM protein) cut to 5 mm × 5 mm pieces overnight at 4°C (positive adsorption). Membranes were then rinsed twice for 5 min in TBS, followed by a further two rinses in PBS pH 7 for 5 min each. Bound antibodies were eluted by vortexing the membranes at room temperature in 1 mL of 100 mM glycine, pH 2.5. After 10 min of vortexing, 70 µL of 1 M Tris-Cl, pH 8, was added to the eluate to neutralise the pH. This elution and neutralisation step was repeated once more and the eluates were pooled together. Finally, the antibodies were stabilised by addition of sodium azide to 5 mM and BSA to 1 mg/mL. Aliquots of the antibodies were frozen at -20°C.

2.2.5.2.2 Three-step affinity purification by positive and negative adsorption

The antibodies were purified as described in Section 2.2.5.2.1 with the following modifications: After positive adsorption, 1 mL volumes of the purified antibodies at concentrations of 1:100, 1:250, 1:500, 1:1000 and 1:5000 were incubated overnight at 4°C with 0.5 mg of protein extracted from uninfected flax plants (Section 2.2.5.3) bound to Hybond-C extra membranes (negative adsorption). Proteins were bound by directly applying the plant protein extracts onto the membranes and allowing the membranes to dry. Membranes were checked for the presence of protein by staining with Ponceau S and rinsed three times for 5 min in TBS. Poorly bound protein was removed by washing with 200 mM glycine, pH 2.5. This was followed by rinsing the membranes three times for 5 min with TBST and blocking with 1% BSA (w/v) and 2% (w/v) non-fat milk for 1 h at room temperature. Membranes were rinsed in TBST, cut to 5 mm × 5 mm and finally incubated overnight at 4°C with diluted serum so that antibodies which recognised plant protein were bound to the membrane. The diluted antibodies were used directly for immunolabelling experiments.

2.2.5.2.3 One-step affinity purification by negative adsorption

Sera were purified by negative adsorption against proteins from uninfected flax plants bound to Hybond-C extra nitrocellulose membranes as follows: A total of 1 mg of proteins extracted from uninfected flax plants (Section 2.2.5.3) was spotted onto a Hybond-C extra membrane, stained with Ponceau S and rinsed three times for 5 min in TBS. Poorly bound protein was removed by

washing with 200 mM glycine, pH 2.5. This was followed by rinsing the membranes three times for 5 min with TBST and blocking with 1% BSA (w/v) and 2% (w/v) non-fat milk for 1 h at room temperature. Membranes were rinsed in TBST, cut to 5 mm × 5 mm and finally incubated overnight at 4°C with 500 µL of 1:100 diluted sera so that antibodies which recognised plant protein were bound to the membrane. The diluted serum was then used on immunoblots at a concentration corresponding to 1:1000 of the original antiserum (1:10 of the diluted serum).

2.2.5.3 Immunoblot

Total proteins were extracted by grinding samples in a mortar and pestle in liquid nitrogen. Samples were resuspended in either Extraction Buffer A [50 mM Tris-Cl pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 10% (v/v) glycerol] or Extraction Buffer B [150 mM NaCl, 20 mM Tris-Cl pH 6.5, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (v/v) β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Both extraction buffers were supplemented with Roche Complete protease inhibitor cocktail (Roche Diagnostics Ltd). Samples were centrifuged to remove cellular debris before boiling for 5 min in the presence of 1 × Laemmli buffer with β-mercaptoethanol [60 mM Tris-Cl pH 6.8, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 0.1% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol]. Fifty micrograms of total protein extracted from plants were loaded per lane onto 12% acrylamide gels (Laemmli 1970) using a mini ProteanII gel apparatus (Bio-Rad), and run for approximately 1 h at 180 V. Proteins were transferred onto a Hybond-C extra membrane overnight at 4°C at 20 V in Hybond-C transfer buffer [25 mM Tris pH8.3, 150 mM glycine, 20% methanol]. Transfer of 5 µL Kaleidoscope™ Precision Plus pre-stained molecular weight standards (Bio-Rad), added to one lane, and staining in Ponceau S, was routinely used as an indicator for protein transfer from the gel to the membrane.

Blots were blocked to reduce non-specific binding of antibodies for 2 h with 1% (w/v) BSA, 2% (w/v) non-fat milk in PBS pH7, washed three times for 5 min each in PBT [PBS pH7 with 0.2% (v/v) Tween-20] and incubated for 45 min at room temperature in primary antibody. Then, unbound primary antibody was removed by washing the blot three times for 5 min in PBT. This was followed by incubating the blot for 1 h at room temperature in the secondary antibody, a 1:1000 dilution of anti-rabbit sheep antibody conjugated to alkaline phosphatase (Chemicon International). Finally, membranes were washed three times in PBT and then three times for 5 min in PBS to remove unbound secondary antibody. Membranes were equilibrated in alkaline phosphatase buffer [0.1 M Na₂CO₃, 1 mM MgCl₂·6H₂O, pH 9.8] for 2 min. Colour was developed in

a 0.3 mg/mL nitroblue tetrazolium (NBT) and 0.15 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution in alkaline phosphatase buffer. Once the colour had developed, the membrane was rinsed in distilled water for 10 min. All antibodies were diluted in 1% (w/v) BSA in PBS pH 7.

For dotblots, different amounts of purified His-AvrM or His-Ubi-AvrL567 were spotted onto a Hybond-C extra membrane and allowed to dry for 1 h at room temperature. Proteins were detected using chemiluminescence detection. In brief, blots were blocked in 4% (w/v) non-fat milk in PBS pH 7.4 [0.01 M phosphate buffer, 2.7 mM KCl and 0.137 M NaCl, pH 7.4] before incubation with the primary antibody for 1 h. Blots were rinsed three times for 10 min each in PBST [PBS pH 7.4 with 1% (v/v) Tween-20]. Primary antibodies were detected with a goat anti-rabbit antibody conjugated to horse radish peroxidase (Sigma) at a 1:10000 dilution. Antibodies were then detected using the Supersignal West Pico chemiluminescent Substrate kit (Pierce Chemicals) according to the manufacturer's instructions.

Gels, immunoblots and dotblots were scanned using an hpScanJet 4570c scanner (Hewlett-Packard), a perfection 4990 photo scanner (Epson), a Lumix DMC-FX7 camera (Panasonic) or a Lumix TZ7 camera (Panasonic).

2.2.6 Immunolocalisation

2.2.6.1 Immunofluorescence microscopy

2.2.6.1.1 Tissue fixation and embedding

Leaves from flax plants were sampled at 5 dpi (or 120 hours post infection (hpi)). The lower epidermis was peeled off and leaves were cut into 3 mm × 3 mm pieces and fixed under vacuum in 4% (v/v) formaldehyde with 0.2% (v/v) glutaraldehyde in 50 mM 1,4-piperazine-diethanesulfonic acid (PIPES) buffer, pH 7.0 and 0.02% (v/v) Triton X-100 in a vacuum for 2 h and then dehydrated and embedded in BMM as described by Kobayashi et al. (1994). Infected leaf material at other time points specified were also collected and treated similarly.

In vitro germinated spores were prepared by Dr G. Lawrence (CSIRO, Plant Industry) by incubating a one-cell thick layer of spores on water overnight. Spores and *in vitro* germinated spores were fixed and embedded in BMM as described for *Phytophthora* zoospores by Hardham (2001). Cells were fixed for 30 min at room temperature by the addition of one volume of triple-strength fixative to two volumes of cell suspension. Cells were rinsed three times in 100 mM PIPES

buffer, pH7, by repeated centrifugation and resuspension, and finally resuspended in 2% (w/v) low melting point agarose dissolved in 100 mM PIPES buffer which was then solidified in an ice bath for 20 min. The agarose was cut into blocks and then dehydrated and embedded as described for flax leaves.

BMM-embedded samples were cut at 2 to 6 μm using an Ultracut microtome (Reichert-Jung) and air-dried onto uncoated, multiwelled slides on a heating block. Sections were etched in acetone for 45 min before immunolabelling experiments.

For cryosectioning, samples were fixed according to the same protocol as for BMM-embedded sections, rinsed three times in 100mM PIPES buffer, pH 6.8, placed in Tissue Tek cryomolds (Miles Inc.), and then snap frozen in a drop of OTC compound embedding medium (ProSciTech) and stored at -20°C . Sections were cut at 12 μm using a Cryostat 2800 Frigocut-E (Reichert-Jung) and placed onto polyethylenimine (PEI)-coated slides and kept in a moist chamber. PEI-coated slides were prepared by adding drops of 0.1% (v/v) PEI on clean slides for 1 to 2 min and rinsing the slides in distilled water and drying before use.

For vibratome-cut sections, infected leaves were cut into 5 mm $\times$ 5 mm segments and were either fixed as for BMM-embedded samples or left untreated. Leaves then were embedded in 3% (w/v) low melting point DNA-grade agarose. Samples were cross-sectioned at 150 μm thickness on a vibratome (1000 plus; Vibratome Company). Fixed sections were transferred into PBS pH 7 and stored at 4°C . Unfixed sections were immediately fixed as described for BMM-embedded samples and stored at 4°C in PBS pH 7. Samples were labelled with antibodies in multiwelled slides.

2.2.6.1.2 Immunofluorescence labelling

Sections fixed as described in Section 2.2.6.1.1 as well as BMM-embedded samples from Kobayashi et al. (1994) of CH5-Hosh flax plants at 12, 16, 24, 64 and 120 hpi were blocked for 1 h in 1% (w/v) BSA and 0.1% (v/v) fish scale gelatin in PBS pH 7. Blocking solutions were removed and sections were incubated in primary anti-AvrM antibodies (prepared as described in Section 2.2.5.2.3) for 2 h at 37°C , rinsed three times and then incubated in 1:1000 goat anti-rabbit antibodies conjugated to Alexa A488 (Molecular Probes) for 2 h at 37°C . Slides were rinsed and mounted in a glycerol-based mounting medium, Mowiol, with *p*-phenylenediamine (PPD) or 1,4-diazabicyclo[2.2.2]octane (DABCO) antifade agent. For double-labelling studies, sections were co-

incubated with rabbit anti-AvrM antibodies and mouse monoclonal anti-haustorial antibody ML1 (Murdoch and Hardham 1998) followed by co-incubation with 1:1000 goat anti-rabbit antibodies conjugated to Alexa A488 and 1:75 sheep anti-mouse antibodies conjugated to rhodamine (Silenus Laboratories) or 1:800 goat anti-mouse conjugated to Dyelight (Sigma) for 2 h at 37°C. In other experiments, double-labelling was performed by co-incubation of AvrM antibodies with wheat germ agglutinin (WGA), which binds to sialic acid and N-acetylglucosaminyl sugar residues in fungal cell walls, conjugated to tetramethylrhodamino isothiocyanate (TRITC) (Sigma) at a concentration of 50 µg/mL. All antibody dilutions were made up in 1% (w/v) BSA and 0.1% (v/v) fish scale gelatin in PBS pH7. Some sections were also stained with 1 µg/mL 4', 6-diamidino-2-phenylindole (DAPI) (Sigma), which binds to nucleic acids, for 10 min after secondary antibody incubation. Following incubation with secondary antibodies, slides were rinsed and stored in the dark at 4°C. Sections were observed with an Axioplan microscope (Zeiss) equipped with a digital camera (Princeton Instruments Inc.) using differential interference contrast (DIC) optics and fluorescence microscopy or on a Leica SP2 confocal microscope (Leica).

2.2.6.2 Electron microscopy (EM)

2.2.6.2.1 Chemical fixation

The lower epidermis was removed from infected leaves and leaves were fixed in a vacuum for 2 h in 2% (v/v) glutaraldehyde in 50 mM PIPES buffer, pH 6.9 and embedded in Lowicryl K4M (Chemische Werke Lowi) as previously described (Hardham 2001).

2.2.6.2.2 High pressure fixation

High pressure fixation was performed by Dr Rick Webb (Centre for Microscopy and Microanalysis, University of Queensland). Flax leaves were couriered at room temperature to Queensland and were infiltrated with hexadecane and a leaf disc was punched out with a biopsy punch or cut with a sapphire scalpel before freezing in a Leica EMPACT2 high pressure freezer (Leica). For immunogold labelling, samples were freeze substituted in 0.2% uranyl acetate, 5% (v/v) water in acetone and held at -85°C for 6.9 h and then brought up to -50°C over 11.4 h, dehydrated in acetone twice for 20 min at -50°C in and infiltrated in acetone at -50°C with increasing concentrations of Lowicryl K4M resin (20%, 40%, 60%, 80%) followed by three changes in 100 % Lowicryl K4M for 24 h. The embedded samples were then polymerised at -50°C for 48 h and 20°C for 48 h.

2.2.6.2.3 Immunogold labelling

Lowicryl ultrathin sections cut on an Ultracut microtome (Reichert-Jung) were floated onto formvar coated gold grids and blocked with 1% (w/v) BSA, 0.1% (v/v) fish scale gelatin in PBS for 60 min and then incubated with primary antibody diluted in 1% (w/v) BSA, 0.1% (v/v) fish scale gelatin in PBS for 1 h at room temperature. Sections were rinsed in PBT and incubated with 1:5 dilution of goat anti-rabbit conjugated to 10 nm gold (GE-Healthcare) for 1 h at room temperature. Sections were counterstained with 6% uranyl acetate for 2 to 4 min and lead citrate for 1 min and examined with a H7100FA transmission electron microscope (Hitachi) equipped with a digital camera.

2.2.6.3 Immunolocalisation of clathrin heavy chain

Labelling of clathrin heavy chain was performed using a mouse antibody against human clathrin heavy chain (BD Biosciences) as a primary antibody at 1:25 dilution with 1:800 goat anti-mouse antibodies conjugated to Dyelight (Sigma) as a secondary antibody for immunofluorescence microscopy and at 1:5 dilution with a 1:5 dilution of goat anti-mouse antibody conjugated to 10 nm gold (Amersham) as a secondary antibody for immunogold labelling. This primary antibody was selected as previous studies had shown that it is able to recognise plant clathrin in tobacco and Arabidopsis (Lam et al. 2007, Hinz et al. 2007). In addition, labelling was also performed using a goat antibody raised against bovine clathrin heavy chain (Sigma) tested previously by Dr. David Collings for plant clathrin labeling (personal communication) at a 1:200 dilution with a rabbit anti-goat-conjugated to fluorescein isothiocyanate (FITC)(Cappel) as a secondary antibody for immunofluorescence labelling and a 1:400 dilution with a 1:30 rabbit anti-goat antibody conjugated to 10 nm gold (BioCell) for immunogold labelling.

2.2.6.4 Immunolocalisation of AvrL567

Labelling of AvrL567 proteins was performed as described for AvrM using rabbit anti-AvrL567 antibodies (Catanzariti 2006, Thesis).

2.2.7 Image processing

Image processing was performed using ImageJ (National Institutes of Health), Adobe Photoshop CS2 (Adobe Systems) and Adobe Illustrator CS2 (Adobe Systems).

2.3 Results

2.3.1 Polyclonal antibody production

His-AvrM protein, consisting of AvrM-A lacking the signal peptide fused to an N-terminal hexahistidine tag, was expressed by IPTG induction and purified from BL21 (DE3) pLysS *E. coli* by immobile metal affinity chromatography using the protocol developed by Dr S. Williams (personal communication)(Figure 2-1).

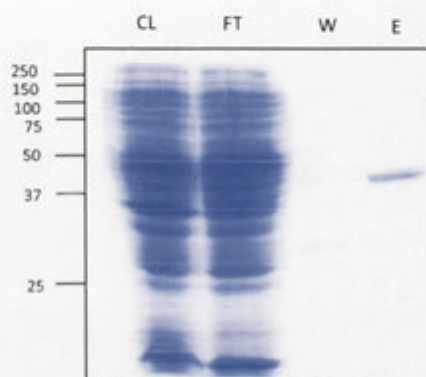


Figure 2-1 Coomassie stained SDS-PAGE gel showing purification of His-AvrM. CL: Crude lysate; FT: Flowthrough off nickel column; W: 65 mM imidazole wash and E: Eluate

Pre-immune sera from seven rabbits were screened by immunoblots and by immunofluorescence microscopy on 4 μ m BMM sections of uninfected and infected flax leaf tissue attached to multiwelled slides. From these, two rabbits with pre-immune sera that did not label any of these samples were selected for further injections with purified AvrM. Screening of sera from the immunised rabbits (S1 and S2) by immunofluorescence microscopy showed that they did not react with sections of uninfected plant tissue embedded in BMM. However, on immunoblots, there was a weak labelling of proteins in uninfected plants (Figure 2-2). This included several distinct bands smaller than the expected size of AvrM in the case of S1 and two bands in the case of S2, one that was approximately the same as the expected size of AvrM, and a second that was between 50 and 70 kDa.

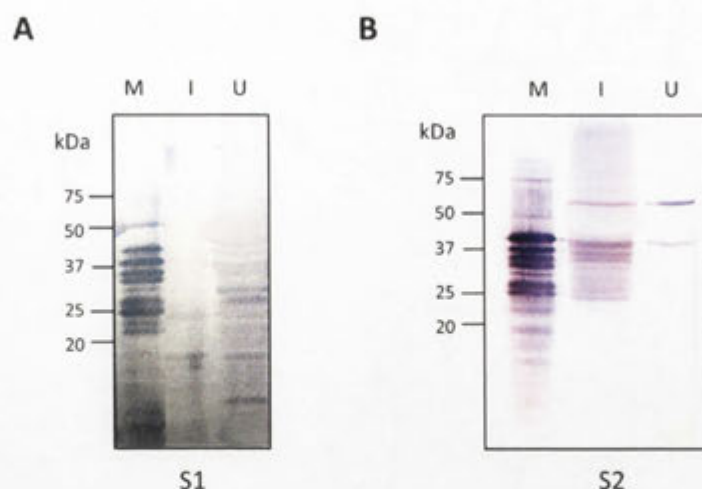


Figure 2-2 Immunoblots using sera (A) S1 and (B) S2 raised against AvrM from rabbits. M: His-AvrM expressed by *E. coli*; Total protein extracted from I: infected flax leaves and U: uninfected flax leaves.

2.3.1.1 Affinity purification of antibodies

To reduce the non-specific reaction with plant proteins, several affinity purification protocols were tested based on methods by Robinson et al. (1988). In the first approach, antibodies were purified by positive adsorption to His-AvrM bound to a nitrocellulose membrane. This was followed by negative adsorption to other bacterial proteins. The affinity purified antibody (S1-1) detected His-AvrM but not His-Ubi-AvrL567 expressed from *E.coli* when diluted to a concentration of 1:5000 and above (data not shown). S1-1 was then tested at a range of dilutions against immunoblots of proteins extracted from uninfected and infected flax plants (Figure 2-3). At dilutions of 1:1000 and below, the S1-1 antibodies detected a protein of approximately 40 kDa in protein extracts of uninfected flax plants, indicating that the antibodies retained some cross-reaction to plant protein. However, an additional protein band of about 35 kDa was detected in infected plants under the same conditions, which may correspond to the AvrM protein. At higher dilutions (1:2500 and 1:5000), the antibody did not detect any bands in either infected or uninfected plants.

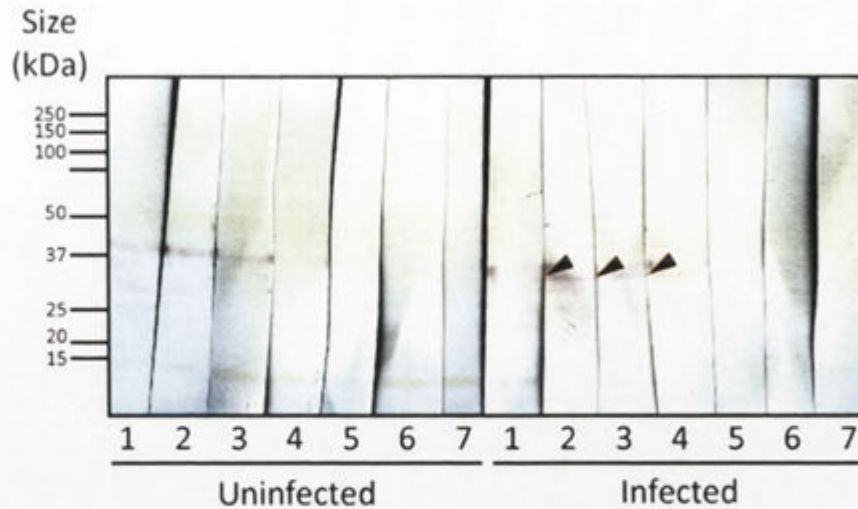


Figure 2-3 Immunoblot of uninfected and infected flax leaves using S1-1. Detection with S1-1 antibodies diluted to (1) 1:100, (2) 1:250, (3) 1:500, (4) 1: 1000, (5) 1: 2500, (6) 1: 5000 and (7) pre-immune sera at 1:100. Arrowheads: Bands detected in infected plants only.

In order to overcome this non-specific labelling of flax protein, the S1-1 antibody was further purified by negative adsorption against protein from uninfected plants. The resulting anti-AvrM antibody preparation (S1-2) detected bands in protein extracts of infected flax plants but did not label any proteins in uninfected flax plants (Figure 2-4).

Interestingly, no signal was detected in lanes of plants infected with flax rust homozygous for the *avrM* virulence locus, potentially indicating differential labelling of the homologs. However, labelling was weak with the antibody showing much reduced labelling of His-AvrM (data not shown) compared to the unpurified antiserum suggesting loss of binding specificity. Further, immunofluorescence screening of sections of infected flax plants detected only weak labelling of fungal structures (data not shown).

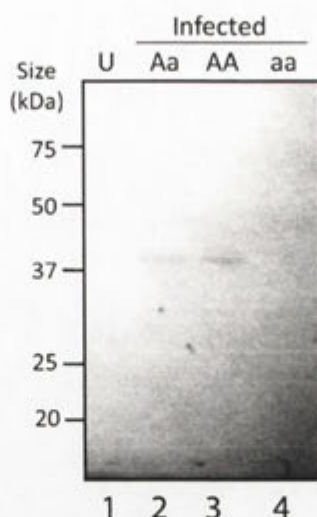


Figure 2-4 Immunoblot labelled with anti-AvrM (S1-2) antibody. Anti-AvrM labelling against total protein extracts from 1: uninfected plant cells (U), 2: plants infected with flax rust heterozygous for the AvrM avirulence and virulence alleles (Aa), 3: plants infected with flax rust homozygous for the avirulence allele of AvrM (AA) and 4: plants infected with flax rust homozygous for the virulence allele of AvrM (aa).

Based on the experiments described, it was hypothesised that negative adsorption against uninfected plant protein may be sufficient to reduce reaction with uninfected plant protein extracts in immunoblots. Thus, a new anti-AvrM antiserum preparation, S1-3, was purified by negative absorption of the primary anti-AvrM (S1) antibody against uninfected plant proteins. Figure 2-5 A shows immunoblot analysis of S1-3 against various protein extracts. This preparation retained strong labelling of the 39 kDa purified His-AvrM protein, but not purified His-Ubi-AvrL567. In addition, it did not bind to any contaminating proteins in uninfected plants, but did strongly detect protein of approximately 35 kDa in extracts from flax leaves infected with rust strains expressing AvrM-A. However, no band was detected in extracts from flax plants infected by a rust strain homozygous for the *avrM* virulence allele of this locus, indicating that the major band detected corresponded to the gene products of the avirulence paralogs. The S1-3 antibody also recognised AvrM fused to the cerulean fluorescent protein transiently expressed in tobacco plants, but did not label protein from untreated tobacco.

After longer colour development, the S1-3 antisera also detected smaller bands in immunoblots of rust-infected flax leaves, which were not present in protein extracts of uninfected flax plants (Figure 2-5 B), suggesting the presence of some processed forms of the AvrM protein.

When tested on a dotblot loaded with different concentrations of *E. coli* expressed His-AvrM, the antibody was found to detect as low as 0.1 ng of His-AvrM protein when used at a concentration of 1:10, corresponding to 1:1000 of the S1 antiserum (Figure 2-5 C). This antibody preparation also retained the ability to label sections of infected plant tissue as assessed by immunofluorescence (data not shown).

The S2 anti-AvrM antiserum was also purified by negative adsorption against uninfected plant protein to produce the S2-3 preparation. Immunoblot analysis (Figure 2-5 D) yielded similar results to S1-3, except that S2-3 also detected a band in protein extracts of untreated tobacco leaves. This corresponded to the size of a background band detected by the unpurified sera in flax plant extracts (Figure 2-2). However, background bands were negligible in uninfected flax plants.

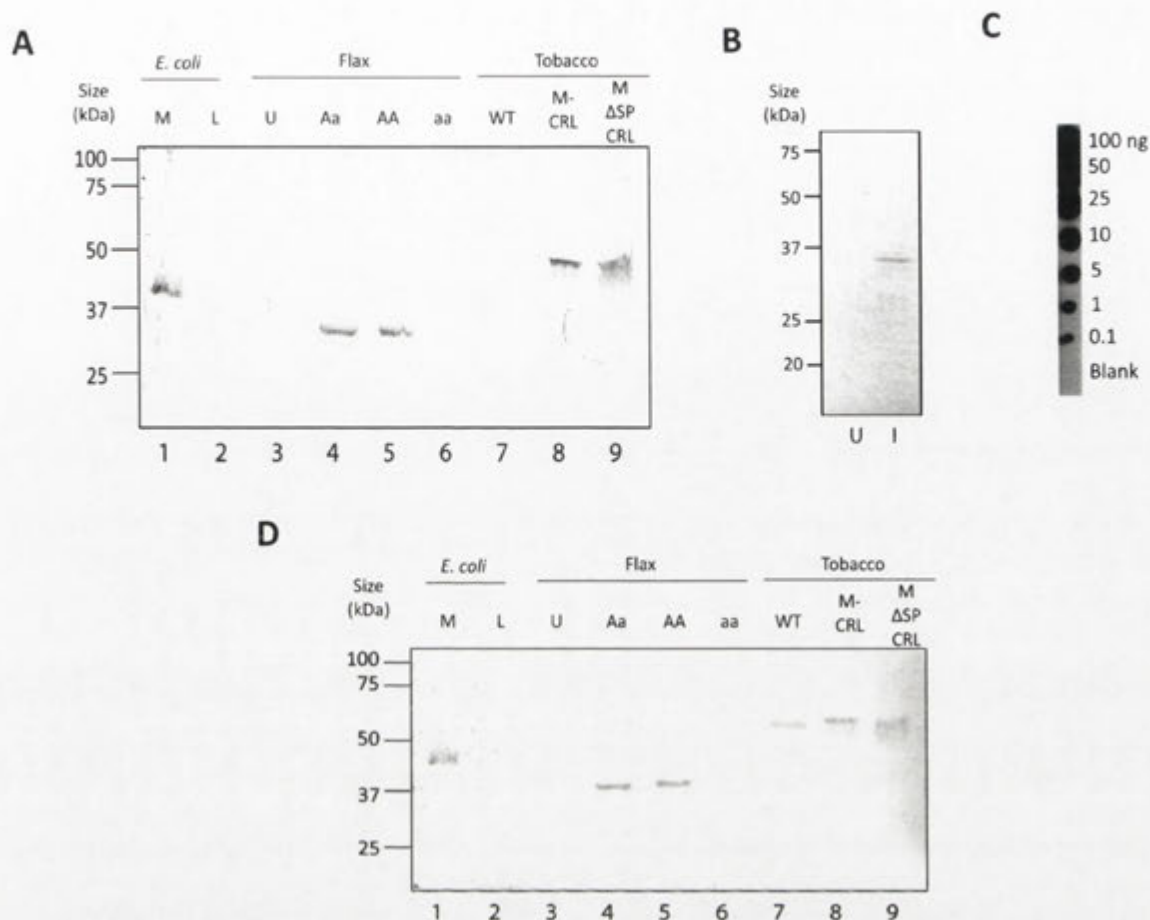


Figure 2-5 Immunoblots labelled with anti-AvrM antibodies. **A.** Immunoblot labelled with anti-AvrM (S1-3) antibody. Anti-AvrM labelling against 1: His-AvrM (M; 39 kDa), 2: His-Ubi-AvrL567 (L; 25 kDa) and total protein extracts from 3: uninfected flax plants (U), 4: plants infected with flax rust heterozygous for the AvrM avirulence and virulence alleles (Aa), 5: plants infected with flax rust homozygous for the avirulence allele of AvrM (AA), 6: plants infected with flax rust homozygous for the virulence allele of AvrM (aa); and tobacco plants 7: wildtype (WT), 8: agroinfiltrated with AvrM-Cerulean (M-CRL; 65 kDa) and 9: agroinfiltrated with AvrM ΔSP-Cerulean (MΔSP CRL; 62 kDa). **B.** Anti-AvrM labelling against uninfected (U) and infected (I) flax plants. **C.** Dotblot showing detection of His-AvrM at concentrations of as low as 0.1 ng. **D.** Immunoblot labelled with anti-AvrM (S2-3). The order of protein loading is the same as for the immunoblot in **A**.

Thus, the affinity purified anti-AvrM antisera S1-3 and S2-3 both showed specific detection of AvrM with no cross-reaction to flax proteins and the immunolocalisation experiments described in the rest of this chapter, as well as immunoblots described elsewhere in this thesis, were all carried out with either of these antiserum preparations.

2.3.2 Immunolocalisation of AvrM

2.3.2.1 Immunofluorescence localisation of AvrM

The localisation of AvrM was studied by immunofluorescence labelling of BMM-embedded sections that were prepared from either isolated spores, spores germinated overnight (16 h) *in vitro*, or infected plant tissues at various time points after infection (Figure 2-6 and -7; Table 2-2). No signal was detected in the isolated spores or *in vitro* germinated spores beyond the level of autofluorescence (Figure 2-6 A-D).

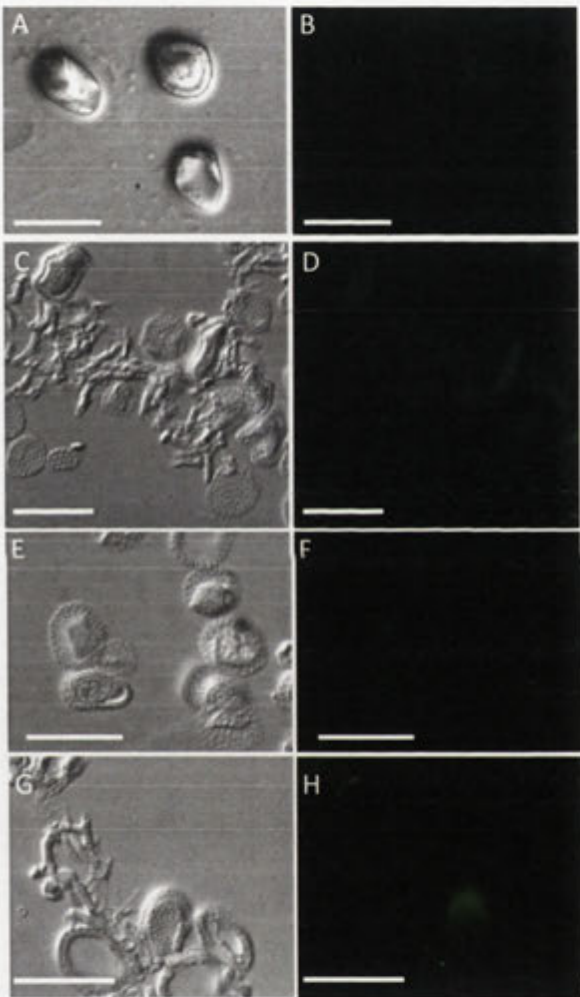


Figure 2-6 Immunolocalisation of AvrM on spores (A and B), overnight *in vitro* germinated spores (C and D). Unlabelled spores (E and F) and overnight *in vitro* germinated spores (G and H) demonstrate the level of background fluorescence.. Panels A, C, E and G are DIC images of panels B, D, F and H. Scale bars = 100 μ m

Infected material sampled at 12, 16, 24, 64 and 120 h after inoculation were labelled with anti-AvrM antibodies. No signal was detected at 12 hpi, at which stage rust hyphae were observed

growing within host tissue but no haustoria were present (Figure 2-7 A and B). At 16 hpi, young haustoria were observed and were strongly labelled by the antibody (Figure 2-7 C and D; Table 2-2). Haustorial labelling was detected at all subsequent time points tested (Figure 2-7 E and F; Table 2-2) suggesting that AvrM is present in very young haustoria and is expressed throughout the life of haustoria. Other than haustorial labelling, the antibody also labelled the tips of infection hyphae from 64 hpi onwards (Figure 2-7 E and F; Table 2-2).

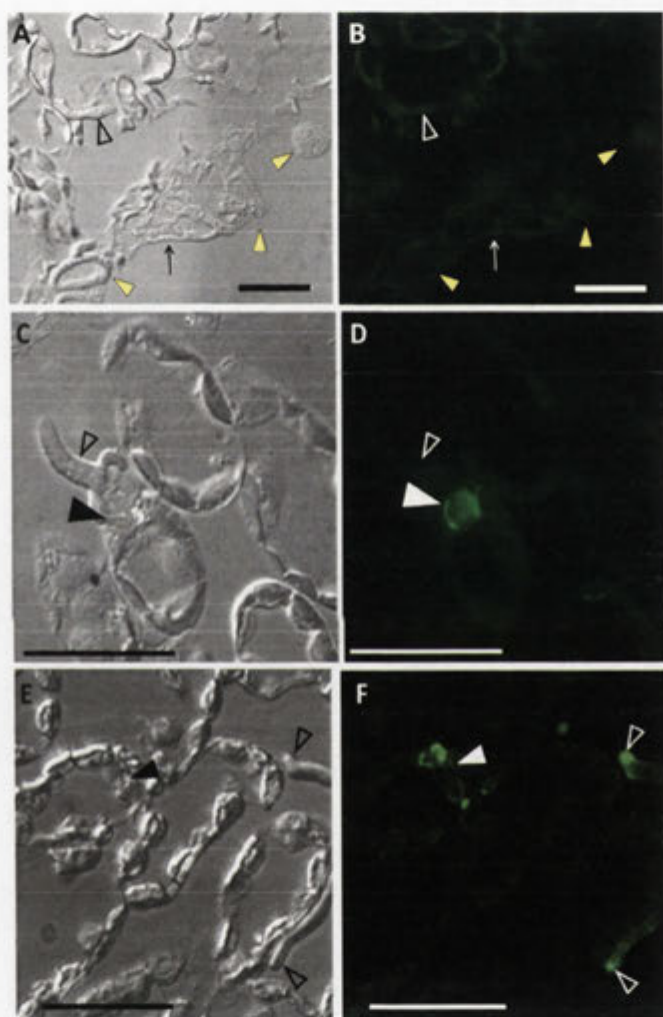


Figure 2-7 Immunolocalisation of AvrM in rust-infected leaf material at 12 hpi (A and B), 16 hpi (C and D) and 120 hpi (E and F). Unfilled arrowheads: hyphae; yellow arrowheads: uredospores; open arrow: germ tube; white/black arrowheads: haustoria; scale bars = 100 μ m.

In addition, closer examination by confocal microscopy revealed that both hyphal and haustorial peripheral labelling was punctate, with some labelling detected outside the haustorial

cell wall, defined by WGA-labelling, potentially indicating secretion from the fungus (Figure 2-8 A-D). In addition, at 120 hpi anti-AvrM was observed to strongly label regions around the chloroplasts, corresponding to the plant cytoplasm, in approximately 13% of host cells containing haustoria (n=87) (Figure 2-8 E-G; Table 2-2). It is noted that in the case of cytoplasmically labelled cells with haustoria, labelling was not detected in neighbouring, uninfected plant cells. In plant cells with cytoplasmic anti-AvrM labelling, AvrM could be observed to be excluded from host nuclei (Figure 2-9).

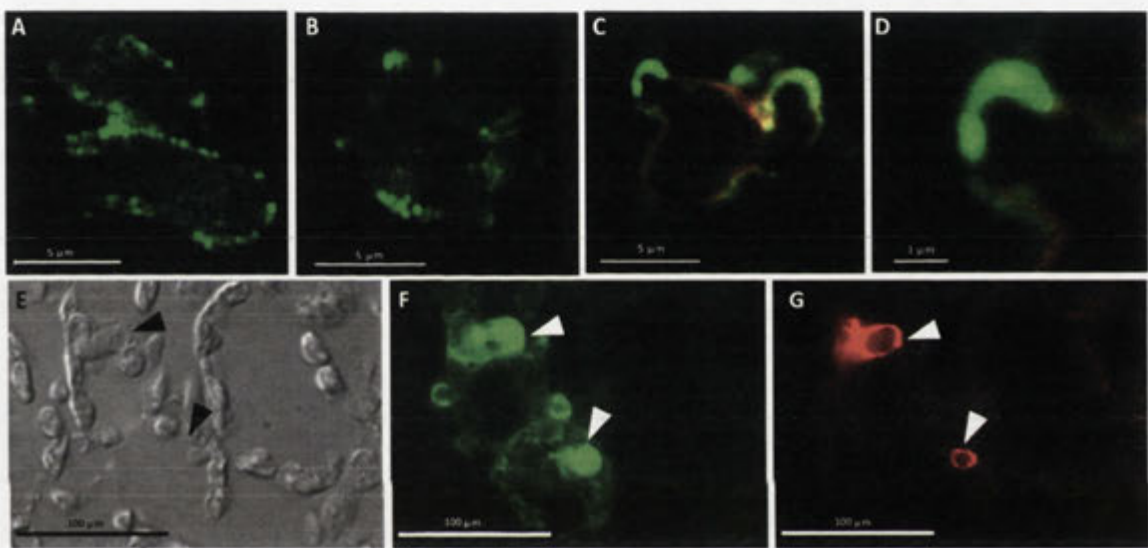


Figure 2-8 Immunolocalisation of AvrM in rust-infected leaf material at 120 hpi (5 dpi). **A**. Confocal image of hyphal tips showing punctate labelling of AvrM (green) at the periphery of hyphae. **B-D**: Confocal images of haustoria showing punctate labelling of AvrM (green) at the periphery of haustoria. In **G** the haustorial cell wall is also labelled by WGA (red) and AvrM could also be detected external to the WGA-labelled structure Panel **D** shows a close up of a region from **C**. **E-G**. Haustorial and plant cytoplasmic labelling of AvrM. Panels **E**: DIC image; **F**: anti-AvrM labelling (green) and **G**: labelling with the monoclonal ML1 anti-haustorial antibody (red). Filled arrowheads: haustoria; scale bars as indicated.

Table 2-2 Localisation of AvrM at 12, 16, 24, 64 and 120 h after inoculation. The labelling pattern of anti-AvrM antibody was observed in sections from at least three different leaves infected with CH5 flax rust per time point. No haustoria were observed at 12 hpi. N/A: not applicable; +: observed; -: not observed.

	Time post inoculation				
	12 h	16 h	24 h	64 h	120 h

Perimeter of haustoria	N/A	+	+	+	+
Perimeter of hyphae, especially apex	-	-	-	+	+
Plant cytoplasm	-	-	-	-	+

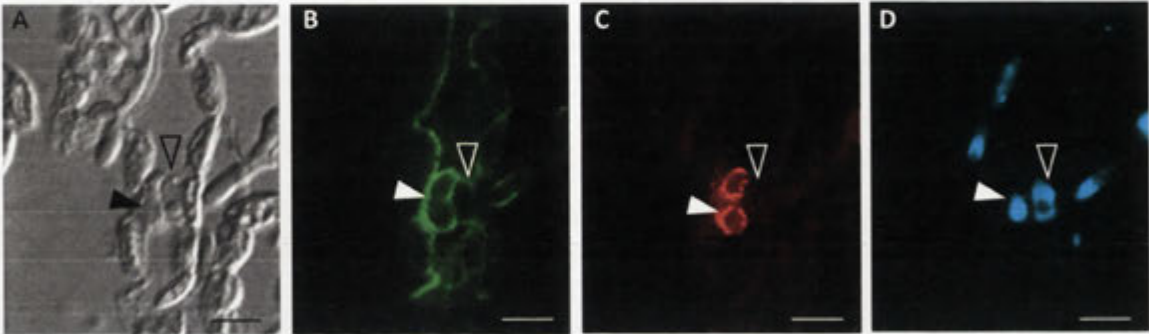


Figure 2-9 Immunolocalisation of AvrM in rust-infected leaf material showing cytoplasmic localisation of AvrM but exclusion from the plant nucleus. Panels **A**: DIC; **B**: labelling with anti-AvrM (green); **C**: labelling with anti-haustorial antibody (red) and **D**: labelling with DAPI (blue). Filled arrowheads: haustoria; unfilled arrowheads: host nucleus; scale bars = 50 μm.

The immunolocalisation pattern was similar for both polyclonal antibodies generated and was not observed in sections labelled with rabbit polyclonal antibodies raised against other antigens, such as SCAMP1, methyl transferase or ManI, provided by Dr Liwen Jiang (Hong Kong University) (data not shown), or in sections labelled with pre-immune sera indicating that the localisation pattern observed is specific for AvrM.

2.3.2.2 Immunogold labelling of AvrM

2.3.2.2.1 Chemically-fixed sections

The localisation of AvrM was analysed at the EM level in chemically-fixed flax rust-infected flax leaves at 120 hpi (5 dpi). In agreement with results from the immunofluorescence localisation experiments, the anti-AvrM antibody labelled putative secretory vesicles within hyphae as well as the periphery of all fungal hyphae observed (Figure 2-10 A). Haustorial labelling was found to be limited to the periphery of the haustorial body, with labelling predominantly in the extrahaustorial

matrix although some signal was associated with the haustorial wall or membrane (Figure 2-10 B). A small proportion of label was also found associated with the extrahaustorial membrane (Figure 2-10 B). A low level of labelling could also be detected in the host cytoplasm of some sections (Figure 2-10 C).

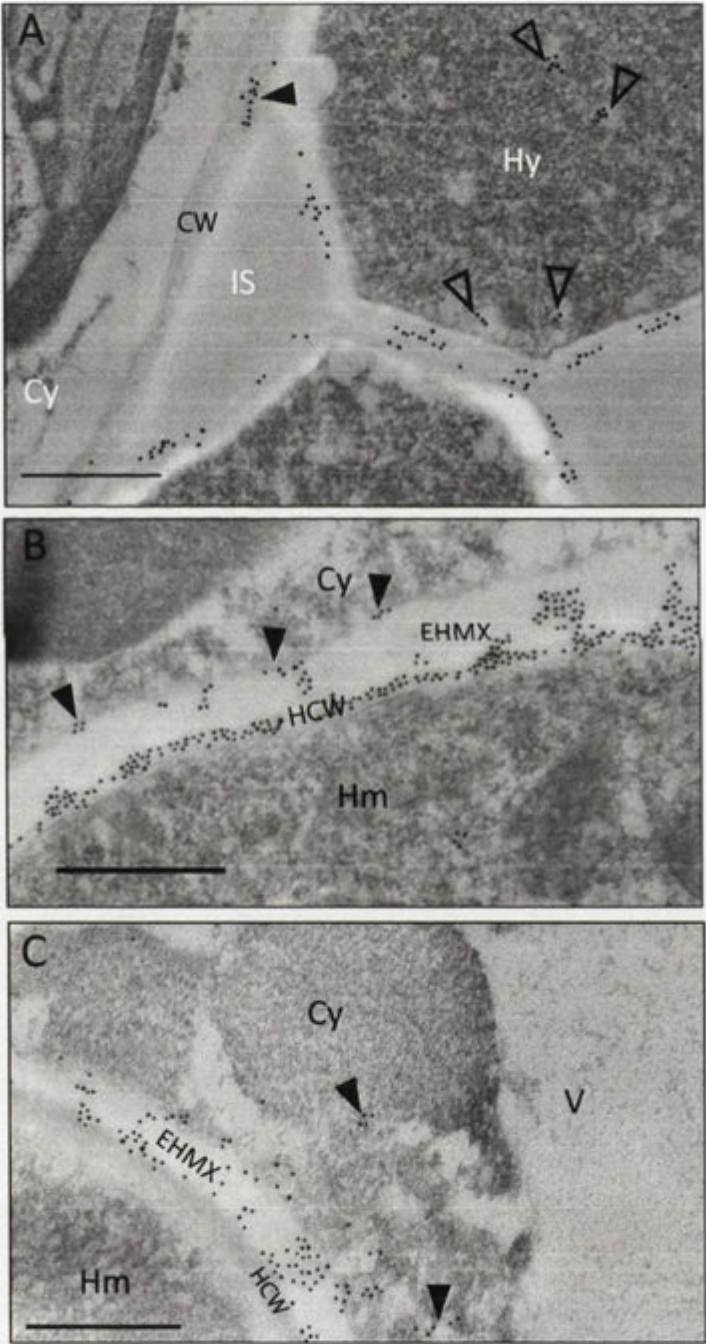


Figure 2-10 Immunogold localisation of AvrM on chemically-fixed rust-infected flax leaves at 5 dpi. **A.** Putative secretory vesicles (unfilled arrowheads) at the periphery of intracellular hyphae. Labelling of AvrM along a plant cell wall is indicated (filled arrowhead). **B.** Section through a flax rust haustorium with AvrM at the haustorial cell wall, in the extrahaustorial matrix and along the extrahaustorial membrane (filled arrowheads). **C.** Section through a flax rust haustorium showing AvrM in the extrahaustorial matrix and in the plant cytoplasm (filled arrowheads). Hm: haustorium; Hy: fungal hyphae; IS: intercellular space CW: plant cell wall, HCW: haustorial cell wall, Cy: plant cytosol, EHM: extrahaustorial matrix, V: plant vacuole; scale bars = 500 nm

One possible explanation for the lower level of host cytoplasmic labelling observed compared to the immunofluorescence experiments is that for samples processed for immunofluorescence microscopy embedded in BMM resin, the resin is removed by etching in acetone prior to antibody labelling, allowing access to antigens throughout the depth of the cut section. On the other hand, in samples processed for EM, embedded in Lowicryl resin, only antigens on the cut surface are accessible to antibody labelling. To test this hypothesis, etched and unetched BMM-embedded sections were labelled with the anti-AvrM antibody used for detection by immunofluorescence (Figure 2-11 A-D). The results from serial sections showed that while cytoplasmic labelling was not detected in unetched samples (Figure 2-11 A and B), labelling was detected in etched sections (Figure 2-11 C and D). In addition, flax leaves from 5 dpi susceptible plants were prepared in three different ways without any resin infiltration, to allow access to antigens throughout the cut sections, and then probed with the anti-AvrM antiserum. In the first approach, leaves were fixed and embedded in 3% agarose before sectioning with a vibratome (Figure 2-11E), while in a second approach the leaves were embedded in 3% agarose and cut on a vibratome before fixation (Figure 2-11F). In both cases strong labelling of haustoria was observed, and cytoplasmic labelling of plant cells was detected in the pre-fixed vibratome-cut sections, supporting the hypothesis that accessibility to the antigen may be a factor contributing to the lower level of antibody detection in the plant cytoplasm. In a third approach the tissues were fixed before embedding in OTC compound embedding medium and cryosectioning, but in this case no labelling was observed (Figure 2-11G).

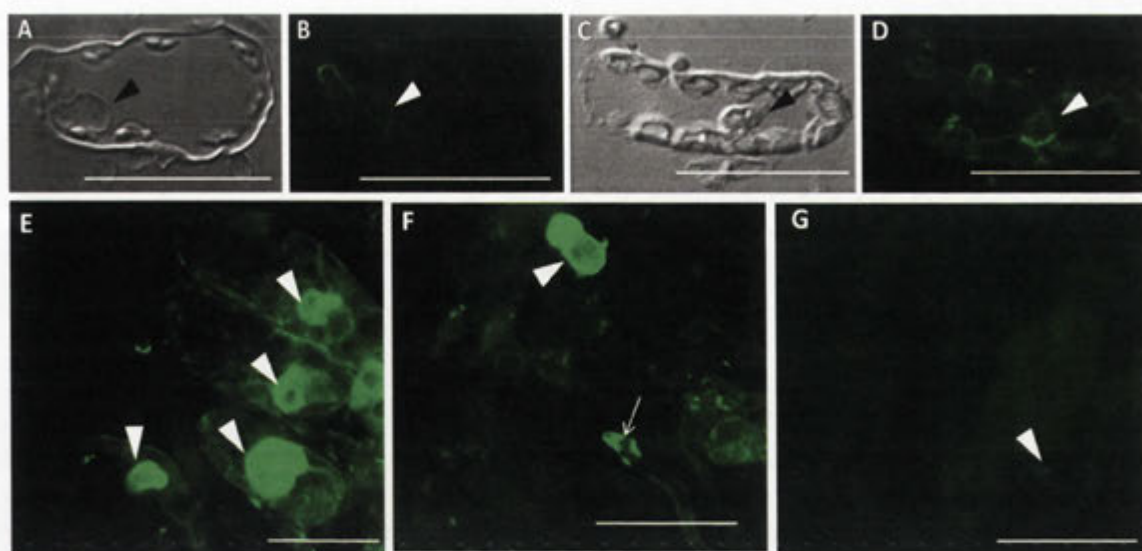


Figure 2-11 Unetched (**A** and **B**) BMM sections compared to etched (**C** and **D**) BMM serial sections. The intensity of anti-AvrM labelling (green) was reduced in the unetched sections. **A** and **C** represent the DIC images of **B** and **D** respectively. Arrowheads: haustoria; scale bar = 50 μ m. Panels **E-G** show labelling of AvrM in 5 dpi flax rust-infected leaves that were (**E**) pre-fixed, embedded in agarose and sectioned on a vibratome, (**F**) embedded in agarose, sectioned on a vibratome and then fixed and (**G**) pre-fixed, embedded in OTC and cryosectioned. Filled arrowheads: haustoria; arrow: hyphae; scale bars = 100 μ m

2.3.2.2.2 High pressure freezing (HPF)

Another factor that may contribute to the low level of cytoplasmic labelling observed at the EM level was the lack of preservation of the plant cytoplasm. In order to achieve better preservation, high pressure freezing (HPF) of infected flax leaves was tested. HPF was performed by Dr R. Webb (University of Queensland) on a Leica EMPACT2 machine available at the University of Queensland followed by freeze-substitution and embedding in Lowicryl HM20 resin.

Prepared samples were sectioned, labelled with anti-AvrM antibody and observed using EM. Although there was considerable variation in the quality of preservation even within the same specimen, the cytoplasm and membranes from plant and fungal cells were generally better preserved in HPF-treated material as compared to chemically-fixed material (Figure 2-12). For example, in the HPF material there was often good structural preservation and enhanced visualisation of mitochondria and membranous structures in fungal haustoria and plant cytoplasm.

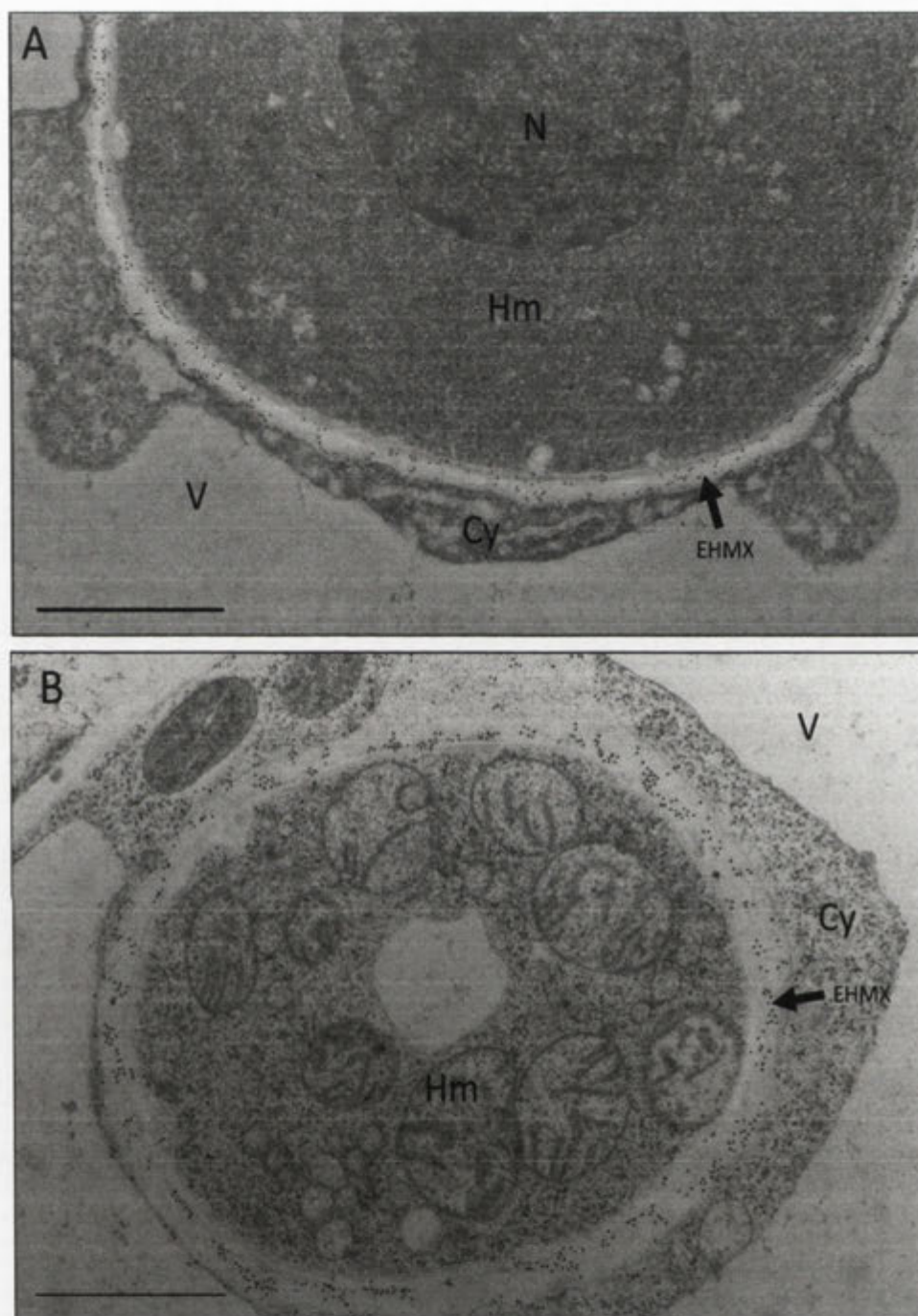


Figure 2-12 Comparison between the preservation of a chemically-fixed flax leaf infected with the flax rust fungus at 5 dpi (A) and a HPF-preserved leaf at 6 dpi (B). Both samples have been labelled with the anti-AvrM antibody. N: haustorial nucleus; Cy: plant cytosol; V: plant vacuole; Hm: haustorium; EHMx: extrahaustorial matrix; scale bars = 1 μ m.

As in sections of the chemically-fixed samples, the anti-AvrM antibody labelled the periphery of hyphae and the extrahaustorial matrix (Figure 2-12 and Figure 2-13 A-F). In addition, anti-AvrM labelled vesicular complexes within the cytoplasm of haustoria (Figure 2-13 B) and putative secretory vesicles within the haustorial cytoplasm (Figure 2-13 C).

While the majority of gold label was detected in the extrahaustorial matrix, in many cases labelling was also detected at the surface of the extrahaustorial membrane (Figure 2-13 D-F). At the extrahaustorial membrane, gold particles were observed in regions where the membrane appeared invaginated or in vesicles of approximately 40 nm in diameter in regions that were more electron dense than the surrounding plant cytoplasm (Figure 2-13 D-E). In addition, gold label could also be observed in larger vesicular structures within the plant cytoplasm close to the extrahaustorial membrane (Figure 2-13 F). Interestingly, within the plant cytoplasm, a low level of antibody labelling was detected at endomembrane structures, including multivesicular bodies (Figure 2-13 H) and the trans-Golgi network (Figure 2-13 G). The pre-immune serum did not show labelling to material in infected flax plants.

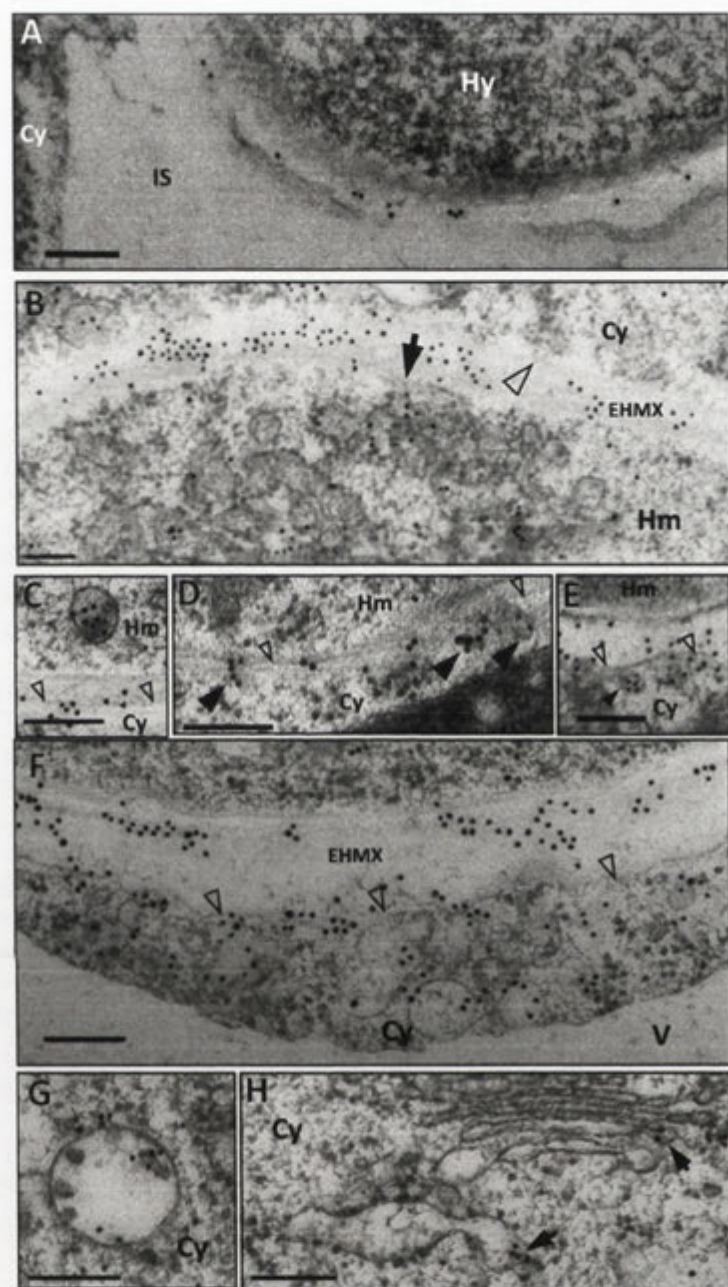


Figure 2-13 Immunogold localisation of AvrM in HPF preserved flax leaves infected with the rust fungus at 6 dpi. **A.** AvrM on the surface of hyphae. **B.** AvrM at tubular vesicular structures (arrow) within a haustorium and in the extrahaustorial matrix. **C.** Localisation of AvrM at a putative secretory vesicle within a flax rust haustorium. **D.** AvrM detection at the extrahaustorial membrane in regions where the membrane is invaginated (filled arrowheads). **E.** Labelling of a small vesicle (filled arrowhead) at the extrahaustorial membrane within the plant cytoplasm. **F.** AvrM in larger vesicles within the cortical cytoplasm of the infected plant cell. **G.** Labelling of a multivesicular body in the cytoplasm of a host cell containing a haustorium. **H.** Labelling of endomembrane structures (arrows) including the trans-Golgi network in the cytoplasm of a host cell within a haustorium. Hy: hyphae; IS: intercellular space; Cy: plant cytosol; Hm: haustorium; V: plant vacuole; unfilled arrowheads: extrahaustorial membrane; scale bars = 200 nm.

2.3.3 Test of antibodies against clathrin and other vesicle components

In order to further characterise the nature of the vesicles observed with anti-AvrM labeling at the extrahaustorial membrane, two antibodies against clathrin, one a mouse antibody, and the second, a goat antibody, were tested on infected plant sections with the aim of performing double-labelling experiments with anti-AvrM. The mouse anti-clathrin antibody had previously been shown to be able to detect clathrin heavy chain from tobacco and Arabidopsis cells (Lam et al. 2007; Hinz et al. 2007). Similarly, the goat anti-clathrin antibody had been used to label clathrin in plants (D. Collings, personal communication). In immunofluorescence experiments both antibodies were observed to have weak punctate labelling in the cytoplasm of plant cells (Figure 2-14) indicating that they were able to recognise plant proteins in the BMM sections. On the other hand, preliminary experiments showed that under conditions for labelling of sections for EM, no labelling was detected on sections of HPF-preserved infected leaves, possibly due in part to the low antibody sensitivity, indicating that further experiments to optimise labelling conditions are required. However, these experiments were not possible in the timeframe of this project.

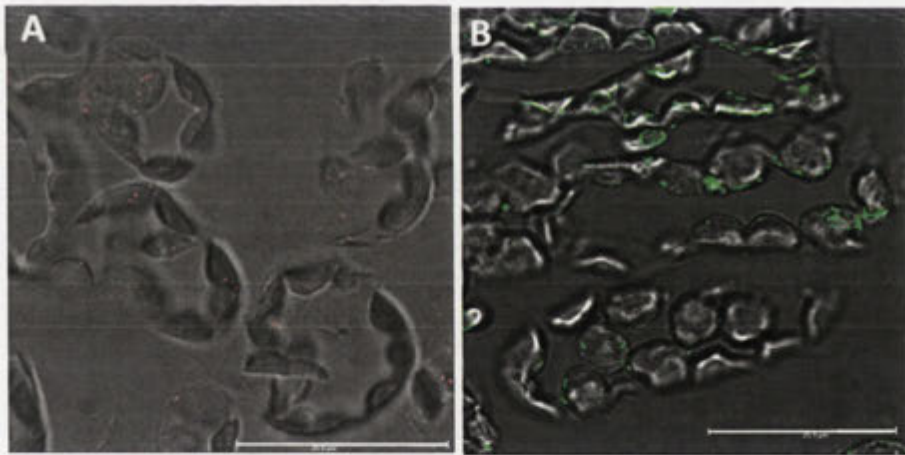


Figure 2-14 Immunofluorescence localisation of clathrin heavy chain on 5 dpi rust-infected leaves. Overlay of DIC images with **A.** a mouse anti-clathrin heavy chain antibody (red) and **B.** a goat anti-clathrin heavy chain antibody (green). Scale bars = 25 μm .

2.3.4 Immunolocalisation of AvrL567

In order to test if the localisation of AvrM is similar to that of other flax rust effectors, immunolocalisation of AvrL567 was performed. Previously, a polyclonal antibody had been raised against AvrL567 (Catanzariti 2006, Thesis). In immunoblots, this antibody was shown to label AvrL567 expressed from *E. coli*. However, despite attempts to do so, the antibody was not demonstrated to label AvrL567 expressed transiently in plants by *Agrobacterium*, AvrL567 expressed in transgenic tobacco and flax plants, or AvrL567 from flax rust-infected plants (Catanzariti 2006, Thesis). Labelling of the antibody on BMM-embedded sections indicated that the antibody labelled fungal mycelia at 5 dpi but was not reported to be associated with haustoria (Catanzariti 2006, Thesis).

In the present study, anti-AvrL567 was purified by negative adsorption against uninfected flax protein as described for AvrM. This antibody preparation was tested in immunoblot labelling of flax rust-infected plants as for the anti-AvrM immunoblots. Similar to the results from the earlier study, no band of the expected size was labelled in these blots (data not shown). However, the antibody was able to detect His-Ubi-AvrL567 expressed from *E. coli* (Figure 2-15 A). Testing of the antibody on a dotblot with different concentrations of His-Ubi-AvrL567 expressed from *E. coli* revealed that the anti-AvrL567 antibody is not as sensitive as the anti-AvrM antibody, only detecting as low as 5 ng of the *E. coli* expressed protein, when used at the same concentration corresponding to 1:1000 of the original antibody preparation (Figure 2-15 B).

Despite the lack of bands detected on immunoblots, the antibody was found to label fungal structures in infected flax leaves (Figure 2-15 C-J). In immunofluorescence microscopy anti-AvrL567 labelled the periphery of hyphae and haustoria. However, unlike AvrM, haustorial labelling was sometimes weak and only restricted labelling of host cells was detected (Figure 2-15 C). However, such labelling was not detected on controls consisting of uninfected flax plants (data not shown). Closer examination of fungal haustoria with high levels of labelling showed that the signal was punctate within haustoria, with low levels of signal found outside the haustorial cell wall, labelled by WGA (Figure 2-15 D-F).

At the EM level, low levels of anti-AvrL567 labelling were observed at tubular vesicular complexes within the haustorium (Figure 2-156 A), the extrahaustorial matrix and the cytoplasm of some plant cells containing haustoria (Figure 2-156 B). In agreement with immunofluorescence localisation, labelling of hyphae was detected on HPF-treated infected flax leaves (Figure 2-156 C).

Intriguingly, in one instance, low levels of AvrL567 appeared to be present in the plant cytoplasm in an endomembrane compartment near to a potential haustorial mother cell indicating that transfer may occur from an external structure (6 D). However, this experiment should be repeated to confirm these results.

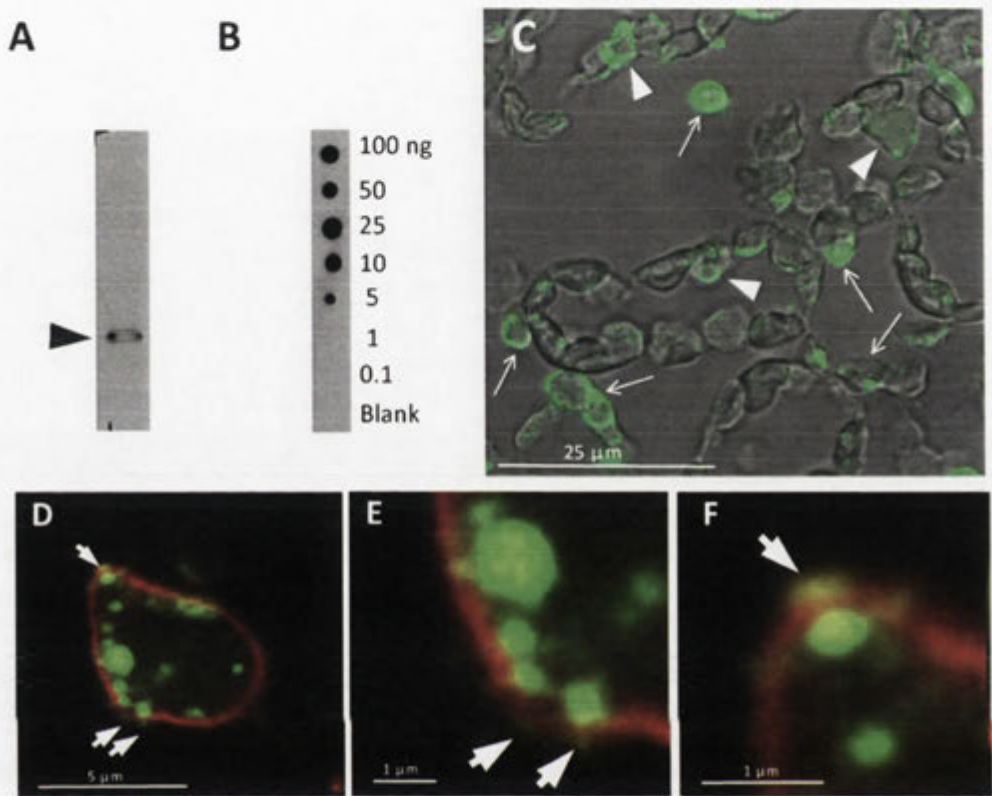


Figure 2-15 Labelling by the affinity purified anti-AvrL567 antibody. **A.** Detection of *E. coli*-expressed His-Ubi-AvrL567 (arrowhead). **B.** Dotblot showing that the anti-AvrL567 antibody detects as little as 5 ng of *E. coli*-expressed His-Ubi-AvrL567 protein. **C.** Overlay of DIC image and anti-AvrL567 labelling (green) on flax rust-infected leaves at 5 dpi. Anti-AvrL567 labelling was detected at the periphery of haustoria (filled arrowheads) and at hyphae (arrows). In addition, a low level of labelling was detected within host cells. **D.** Globular and punctate labelling of haustoria by anti-AvrL567. While the majority of labelling occurs within the haustorium, the arrows indicate regions where labelling was detected outside the haustorial cell wall (labelled red by WGA). Labelling at these regions is shown at higher magnification in **E** and **F**. Scale bars as indicated.

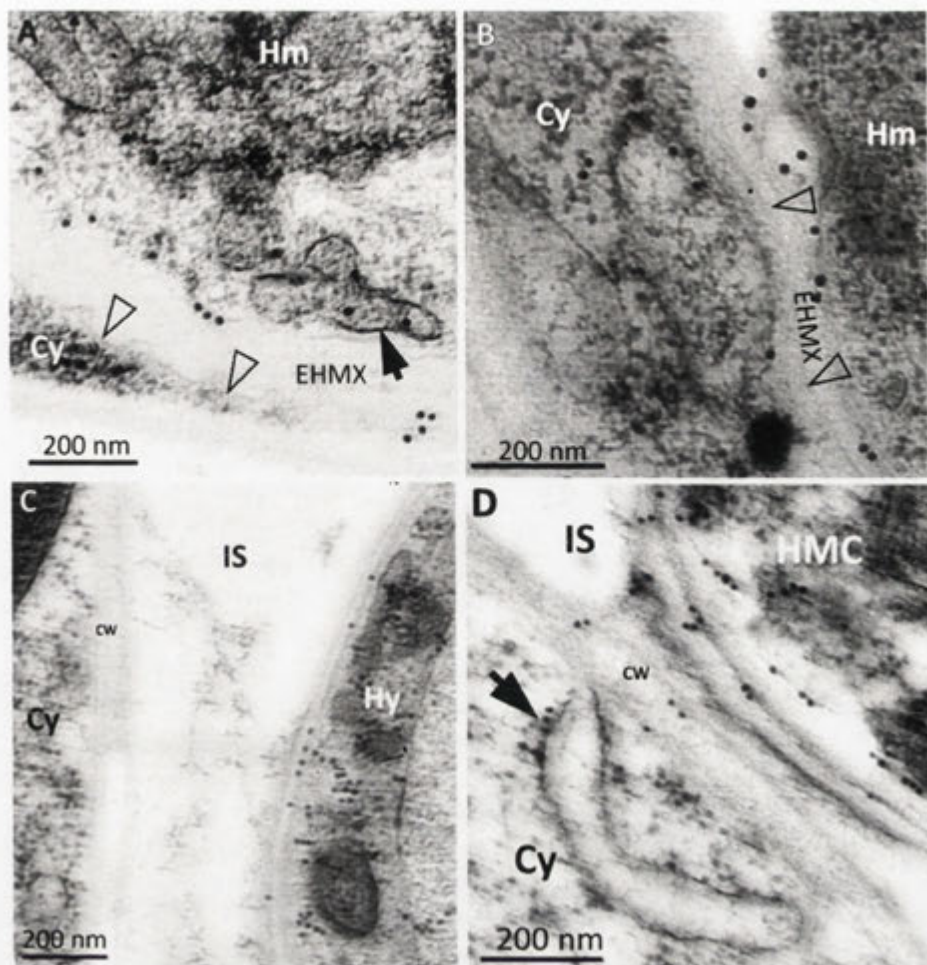


Figure 2-16. Immunogold labelling of HPF-preserved infected flax leaf sections was also performed . **A.** Labelling at part of a tubular vesicular complex in the haustorium (arrow). **B.** Detection of AvrL567 in the extrahaustorial matrix and within the host cytosol. **C.** Anti-AvrL567 labelling of a fungal hypha. **D.** Detection of anti-AvrL567 labelling at a haustorial mother cell and in a membranous structure within the host cytoplasm (arrow). Unfilled arrowheads: extrahaustorial membrane; Hm: haustorium; Cy: plant cytosol; EHMx: extrahaustorial matrix; IS: intercellular space; CW: plant cell wall; HMC: haustorial mother cell; scale bars as indicated.

2.4 Discussion

Previous work had provided indirect evidence for the delivery of AvrM into host cells during infection, based on the ability of cytoplasmic versions of this protein to induce cell death dependent on the cytoplasmic M resistance protein (Catanzariti et al. 2006). Subsequently, the AvrM and M proteins have been shown to interact directly by yeast two-hybrid assays (Catanzariti et al. 2010) and transiently expressed fluorescent fusions of AvrM secreted from tobacco and flax cells were shown to accumulate intracellularly (Rafiqi et al. 2010). The results of this chapter provide direct evidence that AvrM is delivered into host cells during infection and provide interesting insights into the fate of AvrM in flax plants during rust infection.

2.4.1 AvrM antibodies

Two polyclonal antisera were raised independently against His-AvrM. Both antisera reacted strongly with a band of approximately 35 kDa in infected but not in uninfected flax plants. A band of this size might correspond to the AvrM-A, -D and/or avrM proteins lacking the signal peptide (Table 2-1). Due to the lack of labelling in protein extracts of flax plants infected with rust homozygous for the virulence allele, the labelled protein is unlikely to be avrM. It has been reported that the AvrM-B, -C, -E and avrM proteins are also not recognised by the M resistance protein in yeast two-hybrid assays (Catanzariti et al. 2010), indicating that the structures of these proteins differ somewhat from AvrM-A and -D. However, the different paralogs are more than 95% identical at the amino acid sequence level, so it is likely that the antisera would detect these proteins, given that polyclonal antibodies recognise different epitopes of a full-length protein. In this case, it is possible that the non-detected gene products may not be expressed or are present at lower levels than AvrM-A and/or -D during infection. However, transcripts of the virulence allele and the avirulence paralogs have been detected during infection by RT-PCR (Catanzariti et al. 2006) indicating that other factors, such as lower protein stability, may also contribute to the lack of labelling.

Upon further colour development, additional bands of less than 35 kDa were also detected in protein extracts of infected plants labelled with anti-AvrM antibodies. While it is possible that these bands correspond to the products of other AvrM paralogs, it is likely that some of these additional bands represent N-terminal cleavage products of AvrM-A or -D since the unstructured N-terminal portion of AvrM is susceptible to cleavage in the absence of added proteases, even at

4°C (Catanzariti et al. 2010). In agreement with this possibility, an additional band of 25 kDa was labelled by the anti-AvrM antibody.

2.4.2 AvrM localisation

By immunofluorescence microscopy, AvrM was not detected on uredospores or *in vitro* germinated spores. Although northern blots have shown that AvrM is not expressed in spores, AvrM transcripts have been detected from RNA extracted from *in vitro* germinated spores (Catanzariti et al. 2006). It is possible that the protein is expressed and secreted from *in vitro* germinated spores but does not remain attached to germ tubes and is lost in the subsequent processing for immunolocalisation. Another possibility is that transcripts are present but not translated until haustorial formation.

In the experiments described here, AvrM was first detected by immunofluorescence at 16 hpi at the periphery of young haustoria and was observed at haustoria at all subsequent time points tested. Punctate labelling was detected at the edge of the cortical cytoplasm of some haustoria, possibly indicating secretory vesicles, while in most haustoria, the AvrM signal was found outside of the haustorial cell wall, indicating secretion from the fungus.

In addition, the results of this study provided evidence of AvrM entry into the plant cell during rust infection. Strong plant cytoplasmic labelling was only detected by immunofluorescence at 5 dpi and only in approximately 13% of infected cells. The detection of strong host cytoplasmic labelling at 5 dpi and not at earlier time points is contrary to expectations since if M and AvrM interaction in the host cytoplasm is required for HR induction, the protein would be expected to localise to the host cytoplasm early during infection for efficient attenuation of fungal growth. Indeed, in an incompatible interaction, with flax plants expressing the L6 resistance protein, which recognises the effector AvrL567, hypersensitive cell death was observed from 24 to 36 hpi (Kobayashi et al. 1994). This indicates that possibly only low amounts of AvrM, below the threshold of detection by fluorescence microscopy, enter the host cytoplasm at time points before 120 hpi and that this is sufficient to trigger HR. In a compatible interaction, AvrM may accumulate to high levels in the cytoplasm of host cells where infection has been established for a longer period of time, reaching levels detectable by the immunofluorescence assays.

In sections prepared for EM by chemical-fixation, immunogold labelling by anti-AvrM within the host cytoplasm was limited compared to the immunofluorescence experiments, even in

5 dpi plant tissue. Although this was partly due to the fact that the sections for EM had lower antigen accessibility compared to the sections prepared for immunofluorescence (surface labelling as opposed to labelling throughout the section) the limited labelling within the host cytoplasm may also be attributed to differences in preservation. Sections that were chemically fixed for EM were fixed using 2% glutaraldehyde alone whereas sections prepared for immunofluorescence microscopy were fixed using a combination of 4% formaldehyde with 0.2% glutaraldehyde. While glutaraldehyde cross-links more effectively than formaldehyde thus making it more widely used for EM work, its relatively larger sized molecules means that it is not able to penetrate thick samples as quickly as formaldehyde fixatives (Berod et al. 1981). Indeed, this problem is common in fixing leaf samples for EM as leaves are surrounded by a waxy cuticle and thick cell walls, making penetration of fixatives difficult to achieve (Ripper et al. 2008).

HPF is a technique that utilises liquid nitrogen at high pressures (2100 bar) to achieve preservation of specimens of up to 0.5 mm in thickness (Studer et al. 2001). Since its introduction in the late 1980s, it has been employed to study the host-pathogen interface in fungal diseases of plants (Welter et al. 1988; Knauf et al. 1989; Mendgen et al. 1991). In principle, the advantage of HPF is that antigens are immobilised immediately, avoiding artefacts caused by osmotic differences and changes in ionic concentrations encountered during chemical fixation with aldehydes in various buffers (Studer et al. 2001). However, as described in other studies (Mims et al. 2002; Mims et al. 2003), there was considerable variation in the quality of ultrastructural preservation and only small groups of well-preserved cells were obtained, possibly reflecting differing levels of natural cryoprotectants between different cells (Mims et al. 2003).

At the EM level, AvrM labelling was observed predominantly in the extrahaustorial matrix. Unlike the *Magnaporthe oryzae* effectors PWL2 and BAS1, which are localised to a specialised region of the extrainvasive hyphal matrix known as the biotrophic interfacial complex (Khang et al. 2010), AvrM was generally found throughout the extrahaustorial matrix. However, in some instances, clusters of gold labelling were observed. One possible explanation of this is that the clustering of anti-AvrM in the extrahaustorial matrix may occur as contents of secretory vesicles containing AvrM proteins are released into the matrix, resulting in regions with higher signal densities. Over time, these proteins may diffuse leading to a more even distribution around haustoria.

Anti-AvrM labelling of haustorial tubular vesicular complexes in the cortical cytoplasm of haustoria was also observed. These complexes appear morphologically similar to 'interesting' tubular vesicular complexes detected by Mims et al. (2002) in *Puccinia hemerocallidis* rust haustoria. As in *P. hemerocallidis*, three to four of these complexes could be observed in cross-sections of a haustorium. In plants, smaller complexes of tubular vesicular structures have been found to correspond to the early endosome sorting compartment (Lam et al. 2007). However, in the violet root rot fungus, *Helicobasidium mompa* which, like the flax rust fungus is also a basidiomycete, such structures were proposed to be a form of Golgi apparatus (Bourett et al. 2007). Further, in haustoria of the ascomycete *B. graminis*, similar structures were interpreted as blebbing of the ER (Hippe-Sanwald et al. 1992). The labelling of AvrM at these structures may indicate proteins being synthesised and sorted for secretion into the extrahaustorial matrix and may represent localised regions of high secretory activity distributed at the edge of the haustoria.

It was of particular interest to note that AvrM labelling was also detected within the plant cytoplasm at the extrahaustorial membrane in regions that were invaginated or in vesicles of approximately 40 nm in diameter that were slightly more electron dense than the surrounding host cytoplasm. In animal systems, different forms of endocytosis may be differentiated based on the morphology of the endocytic portal. For example, macropinocytosis is characterised by highly ruffled membranes, phagocytosis is marked by cargo shaped engulfments and clathrin-independent carrier/GPI-AP enriched early endosomal compartment-mediated endocytosis is defined by tubular/ring like invaginations (Doherty and McMahon 2009). The observation of labelled vesicles at the extrahaustorial membrane supports the scenario of AvrM entry into host cells by a form of vesicle-mediated endocytosis as opposed to the other pathways described or by direct membrane penetration. Several pathways that result in vesicular endocytic carriers include clathrin-mediated endocytosis, flotillin-mediated endocytosis, Arf6-dependent endocytosis and the IL2R β endocytic pathway (Doherty and McMahon 2009). While homologs of caveolins have not been identified in searches of plant gene sequence databases (Aniento and Robinson 2005), clathrin-coated pits of 50 to 70 nm in diameter have been detected at the extrahaustorial membrane during infection of *Vigna sinensis* by *Uromyces vignae* (Stark-Urnau and Mendgen 1995). However, the results reported in this chapter do not exclude the possibility of AvrM entry by a clathrin-independent vesicular endocytic pathway. Antibodies against coat components of various vesicular uptake pathways, such as clathrin heavy chain or flotillin, could be used to

further characterise the nature of the observed vesicles. With this in mind, preliminary experiments to test the labelling of two anti-clathrin heavy chain antibodies were performed. In immunofluorescence experiments, the antibodies were observed to have a punctate pattern of labelling, consistent with the localisation of clathrin heavy chain in tobacco BY2 cells (Lam et al. 2007), indicating that they may be useful for co-localisation studies in flax plants. However, further experiments to optimise labelling conditions for EM are required since no labelling was detected at the EM level under the conditions tested.

It is noted that tubular elements continuous with the extrahaustorial membrane observed by Mims et al. (2002) and Harder and Chong (1991) in the host cytoplasm during *P. hemerocallidis* and *Puccinia graminis* infections, respectively, were not observed in the present study. This may be due to poor host cytoplasmic preservation or may reflect biological differences in the different host-pathogen interactions.

Within the host cytoplasm, immunolocalisations indicated that AvrM within the host cell may be excluded from the host nucleus. This contrasts with studies which show a nucleocytoplasmic pattern of fluorescent protein fusions of AvrM when expressed transiently in tobacco and flax plants by *Agrobacterium*-mediated transformation (Rafiqi et al. 2010). This is interesting given that the approximately 35 kDa protein is below the size exclusion limit of the nuclear pore complex (Wang and Brattain 2007), and should theoretically be able to freely diffuse into the nucleus. One possibility is that the level of AvrM proteins entering the host cytoplasm is lower during infection compared to when expressed by agroinfiltration and that the protein is localised to the cytoplasm by specific interaction with cytoplasmic host proteins. During transient expression of AvrM *in planta* by agroinfiltration, binding sites to these host proteins may be saturated, thus allowing the protein to diffuse throughout the host cytoplasm.

In the host cytoplasm, antibody labelling was detected in vesicular structures that resembled tubular complexes observed during infection by *P. graminis* which were proposed to be derived from reorganisation of host endomembranes (Harder and Chong 1991). AvrM was also localised to other host cell endomembrane structures including multivesicular bodies and the trans-Golgi network. Localisation of AvrM at these structures could indicate its interaction with targets at host endomembranes. It is particularly interesting to note AvrM labelling at multivesicular bodies since these structures have been reported to fuse with the tonoplast, where the M resistance protein is purportedly localised (Dr M. Rafiqi, personal communication), and to

undergo vacuole-mediated autophagy during powdery mildew infection (An et al. 2006a; An et al. 2006b). However, no labelling was detected specifically at the tonoplast.

Another intriguing possibility is that AvrM occurrence at the trans-Golgi network could be indicative of the route by which these proteins escape from endosomes after entry into host cells. The shiga and ricin toxins, which enter cells when supplied exogenously through clathrin-dependent and -independent forms of endocytosis, are transported from early endosomes via the Golgi apparatus to the ER, where a small portion of these proteins exit to the cytosol in a process called retrotranslocation that normally functions in cells to transport misfolded proteins to the cytosol for proteosomal degradation (Frigerio et al. 1998; Di Cola et al. 2001; Johannes and Römer 2010). It is possible that AvrM also enters the cytosol via a similar pathway.

It is noted that cytoplasmic labelling was detected in infected cells but not in adjacent, uninfected, neighbouring cells. This differs from the *M. oryzae* PWL2 effector which translocates into neighbouring cells ahead of infection (Khang et al. 2010). The lack of transfer to neighbouring cells potentially reflects differences between *M. lini* and *M. oryzae*, where the former moves between cells as intercellular hyphae, whereas the latter moves from cell-to-cell in the form of intracellular hyphae through the plasmodesmata of infected cells (Kankanala et al. 2007).

In addition to the localisation of AvrM within host cells, AvrM was also detected at the periphery of fungal hyphae, especially at hyphal apices, although this signal was weaker than in the extrahaustorial matrix. This localisation is not surprising given that northern blot analysis of AvrM transcripts has shown that the gene is expressed in *in vitro* germinated spores (Catanzariti et al. 2006) and transcripts of an AvrM homolog have been detected in *ex planta* samples of poplar rust (Joly et al. 2010), in the absence of haustoria formation. Furthermore, the apices of fungal hyphae are known to be sites of active secretion (Bourett et al. 2007). Hyphae were not shown to be labelled before 64 hpi, at which time most appressoria penetration events would have resulted in two or more haustoria (Kobayashi et al. 1994), possibly indicating that AvrM is expressed in haustoria and in secondary hyphae, although the biological significance of this is unclear. One possibility is that the AvrM protein is not produced during the penetration stage of infection, when nutrients are still obtained from reserves in the spore, but is expressed when biotrophy is established (after the formation of the first haustorium), when nutrients are acquired from the host plant. Alternatively, the protein may be localised to the tips of secondary hyphae where it may be required from the early stages of contact with mesophyll cells for haustorial formation.

Another fungal protein that has been shown to enter plant cells, the *U. fabae* protein UfRTP1, which has a nuclear localisation signal, was shown to be localised to the periphery of haustoria as well as the cytoplasm and nucleus of plant cells, but was not reported to be present in hyphae (Kemen et al. 2005). Further, the *P. infestans* Avr3a effector that enters into the host cytoplasm has been shown to be secreted from haustoria and also does not appear to be expressed in hyphae (Whisson et al. 2007). There are several possible explanations for the localisation of AvrM in the intercellular space as well as in the host cytoplasm. It is possible that the primary function of AvrM is outside of host cells, such as in the case of the Avr2 apoplastic effector of *Cladosporium fulvum*, the EPIC1 and EPIC2B proteins from *P. infestans* and the Pep1 effector from *U. maydis* (Song et al. 2009; Doehlemann et al. 2009). In this scenario, internalisation into the host may not serve a virulence function but may be part of a pathogen detection mechanism. However, the conservation of the region required for uptake identified by Rafiqi et al. (2010) among the different AvrM alleles does not appear to support this hypothesis. Another possibility is that the protein serves an intracellular as well as an extracellular function. It is possible that the host target of AvrM has a dual localisation. Examples of host proteins that have multiple localisations and which are up-regulated during infection include some pathogenesis-related (PR) proteins (Edreva 2005). For instance, the barley PR1 protein is found not only in chloroplasts and mesophyll cell walls but also on the outer cell wall and cytoplasm of the invading fungus *Bipolaris sorokiniana* (Santén et al. 2005). Other groups of PR proteins with closely related structures have been shown to accumulate in the apoplast or in plant vacuoles as well as in the cell walls of invading pathogens (Bol et al. 1990; Edreva 2005). It is also conceivable that AvrM targets some intercellular plant defense signal that needs to be internalised into cells to serve its function.

2.4.3 Comparison between AvrM and AvrL567 immunolabelling

Similar to AvrM, several studies have indicated that the AvrL567 protein acts within host cells. AvrL567 transcripts are present during infection from the earliest time point tested up until 8 dpi but are not present in *in vitro* germinated spores (Dodds et al. 2004). Like AvrM, the protein interacts with a cytoplasmic host resistance protein (the L6 protein) in yeast two-hybrid assays and has been shown to internalise into plant cells when expressed as a secreted protein from tobacco and flax plant cells (Dodds et al. 2006; Rafiqi et al. 2010). The N-terminal portion of AvrL567 was also reported recently to enter soybean root cells by a lipid-dependent endocytic pathway when supplied exogenously (Kale et al. 2010). Despite a lack of primary sequence conservation between

the minimum uptake regions of AvrM and AvrL567 (Rafiqi et al. 2010), it is possible that the AvrL567 protein enters the host cell by the same pathway as AvrM.

In order to analyse the localisation of AvrL567, a polyclonal antibody generated against *E. coli*-expressed His-Ubi-AvrL567 (Catanzariti 2006, Thesis) was used. As in previous experiments, the antibody was shown to detect *E. coli* expressed His-Ubi-AvrL567 but did not label proteins in immunoblots of extracts from flax rust-infected leaf, which could be labelled by the anti-AvrM antibody. This may partly be due to a lower sensitivity of the antibody compared to anti-AvrM, as shown by dotblot analysis. Indeed, while the antibody did not detect AvrL567 in total protein extracts of flax or tobacco plants transiently expressing AvrL567 (Catanzariti 2006, Thesis), an anti-GFP antibody did detect transiently expressed AvrL567 fluorescent protein fusions (Rafiqi et al. 2010). However, this does not exclude the possibility that addition of the fluorescent protein tag may increase the stability of the protein and that AvrL567 itself is not stable and may be degraded during the process of protein extraction. Yet another explanation for the lack of immunoblot labelling is that the protein is present in a compartment that is not easily solubilised. Indeed, the extrahaustorial membranes of powdery mildew haustoria have been described as exceptionally stable and durable even when treated with acids, detergents and hydrostatic and mechanical stresses that would normally dissolve lipid membranes (Gil and Gay 1977).

Like the AvrM protein, AvrL567 was detected at fungal hyphae as well as at the periphery of haustoria. However, haustorial labelling was weaker in comparison to AvrM, possibly indicating a lower level of expression or the lower sensitivity of the antibody as discussed above. The majority of AvrL567 labelling at haustoria was detected in globular and punctate structures within haustoria, with only low levels of labelling outside the haustorial cell wall in regions of punctate peripheral labelling possibly indicating a more regulated secretion of AvrL567. The punctate labelling at the periphery of haustorial cortical cytoplasm could correspond to the sorting and secretion of these proteins at the tubular vesicular complexes observed. However, only a low level of labelling at these structures was observed at the EM level. In addition, labelling within the extrahaustorial matrix was also lower compared to AvrM. Further, unlike AvrM, anti-AvrL567-labelled vesicles at the extrahaustorial membrane were not observed in the sections examined. This may be due to a lower level of protein transfer into plant cells compared to AvrM, or could be caused by modification of the protein before translocation or after host cell entry. Indeed, the anti-AvrL567 antibody only detected weak cytoplasmic host labelling even at 5 to 6 dpi.

Unfortunately, given the low level of labelling observed in this experiment, it was not possible to determine whether if AvrL567 enters host cells by a similar pathway to that of AvrM. Potentially the use of a more sensitive anti-AvrL567 antibody, or a technique that allows for detection of weaker signals, such as the coupling of HPF with cryo-electron microscopy, which permits labelling of sections for EM without resin-embedment, may provide a better indication as to whether different flax rust effectors enter via a similar vesicle-mediated pathway.

CHAPTER 3

3 Expression of AvrM-Cerulean by *Melampsora lini*

3.1 Introduction

The transformation of fungi has proved to be an important tool in studying the expression pattern, localisation and function of fungal genes, whether by knocking out genes, or introducing reporter coding sequences (Mullins and Kang 2001; Olmedo-Monfil et al. 2004; Lorang et al. 2001). In general, transformation requires (1) a method of introducing foreign DNA into host cells and (2) a method of selecting transformants in which the DNA is stably integrated.

Various fungal transformation techniques have been reported (Table 3-1). Early protocols were based on the introduction of DNA into protoplasts in the presence of various chemicals, such as polyethylene glycol/CaCl₂ and a buffering agent (Hinnen et al. 1978). However, since it can be difficult to obtain or regenerate protoplasts, the use of other techniques which avoid this such as electroporation, biolistics and *Agrobacterium tumefaciens*-mediated transformation, have also been adopted (Olmedo-Monfil et al. 2004).

These techniques are more readily applied on fungi that can be cultured axenically, and transformation of obligate biotrophs that can only grow within host tissue has been much more difficult to achieve. Chaure et al. (2000) reported stable transformation of barley powdery mildew *Blumeria graminis* (formerly known as *Erysiphe graminis*) by biolistic bombardment of infected leaves using benomyl or bialaphos resistance as selectable markers. However, this result has not been reproduced in the subsequent 10 years despite significant interest within the powdery mildew research community. Recently, a transformation technique was developed for *Melampsora lini* which involves *Agrobacterium*-mediated introduction of transgenes to dikaryotic mycelium growing within the stem of a susceptible host (Lawrence et al. 2010). In this system, transformants are then selected for the silencing of the AvrL567 effector on the basis of their ability to grow on host plants containing the L6 resistance gene (Lawrence et al. 2010).

Table 3-1 Brief summary of fungal transformation techniques and systems to which they have been applied.

Method	System	Advantages	Disadvantages
Protoplasts with PEG/CaCl ₂	<i>Magnaporthe grisea</i> (Parson et al. 1987), <i>Ustilago maydis</i> (Wang et al. 1988), <i>Aspergillus nidulans</i> (Punt et al. 1991)	• No special equipment required • Relatively fast time to generate and isolate transformants	• Difficult to optimise • Lengthy preparation of cells for transformation • Some protoplasts are hard to obtain/regenerate • Transient
Electroporation	<i>Aspergillus niger</i> (Ozeki et al. 1994), <i>Colletotrichum gloeosporioides</i> (Robinson and Sharon 1999)	• Efficient • Short protocol	• Special equipment required
Biolistic transformation	<i>Erysiphe graminis</i> (Chaure et al. 2000), <i>Puccinia graminis</i> (Schillberg et al. 2000)	• Efficient	• Special equipment required • Preparation of projectiles required • Transient
Agrobacterium-mediated transformation	<i>Melampsora lini</i> (Lawrence et al. 2010), <i>Magnaporthe grisea</i> (Rho et al. 2002), <i>Aspergillus niger</i> , <i>Fusarium venenatum</i> , <i>Colletotrichum gloeosporioides</i> (De Groot et al. 1998)	• Efficient • No special equipment required	• Requires preparation of bacterial cells • Longer protocol

In this chapter, the technique developed by Lawrence et al. (2010) was utilised to transform *M. lini* to express the AvrM gene fused to the Cerulean fluorescent protein with the aim of complementing the immunolocalisation data in Chapter 2 by allowing live visualisation of AvrM during infection. The transformation work presented in this section was performed under supervision of Dr Greg Lawrence from the CSIRO Plant Industry.

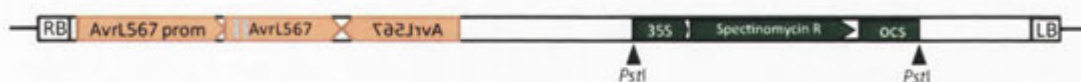
3.2 Materials and Methods

3.2.1 Cloning of rust transformation constructs

PCR products were amplified using the proof-reading Phusion DNA polymerase (Finnzymes) and cloned into pBluescript KS+ (Stratagene) and then into the *Pst*I site of the siAvrL567 T-DNA construct (Lawrence et al. 2010) by restriction enzyme digestion (Figure 3-1). In brief, approximately 1.1 kb of sequence immediately downstream of the AvrM coding region, corresponding to the genomic terminator of AvrM, was PCR amplified and cloned into the pBluescript plasmid with *Xba*I-*Nco*I linkers on the 5' end and *Sbf*I-*Sal*I linkers on the 3' end. The coding sequence of the Cerulean fluorescent protein (Rizzo et al. 2004), a modified form of the cyan fluorescent protein, was amplified from the plasmid pCerulean and cloned into *Xba*I-*Nco*I restriction sites. The entire coding region of AvrM including 1.4 kb of sequence immediately upstream of the AvrM coding region corresponding to the genomic promoter of AvrM was then cloned into the plasmid with *Sac*I-*Sbf*I linkers on the 5' end and the *Xba*I restriction site on the 3' end. Alternatively, the coding region comprising the genomic promoter of AvrM alone was cloned with the *Sac*I-*Sbf*I linkers on the 5' end and the *Xba*I restriction site on the 3' end. Entire constructs consisting of the genomic promoter, coding region and terminator, were then digested from the pBluescript backbone using the *Sbf*I restriction enzyme, which creates *Pst*I compatible ends, before cloning into the *Pst*I site of siAvrL567 T-DNA construct. Restriction enzyme digests were performed using the conditions specified by the manufacturer (New England Biolabs) for 2 h to overnight at 37°C. For some digests, additional units of enzymes were added to the reaction partway through the digestion.

Plasmids digested for cloning were routinely incubated at 80°C for 20 min to inactivate remaining enzymes and then treated with Antarctic phosphatase (New England Biolabs) or calf-intestinal phosphatase (New England Biolabs) according to the manufacturer's instructions. All digests were purified with the Wizard SV Gel and PCR Clean-Up Kit (Promega) according to the manufacturer's instructions before ligation with T4 DNA ligase (Promega). Plasmids were transformed into chemically competent XL1Blue or DH5α cells as described in Chapter 2. All constructs were fully sequenced at the Australian Genomic Resource Facility, Brisbane.

A



B

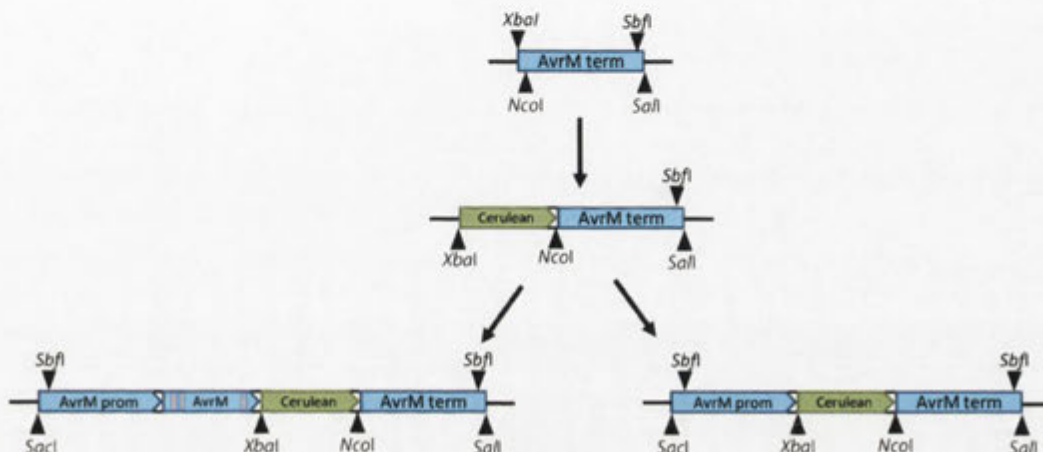


Figure 3-1 Cloning strategy for expression of AvrM fused to Cerulean and Cerulean alone. **A.** Schematic representation of the siAvrL567 T-DNA construct for transformation of rust from Lawrence et al. (2010). **B.** Schematic representation of cloning of AvrM-Cerulean or Cerulean for expression under control of the genomic promoter of AvrM into pBluescript. The SbfI gene fragments were inserted into the PstI site to replace the spectinomycin resistance gene. AvrM term: AvrM genomic terminator; Cerulean: cerulean coding sequence; AvrM prom: AvrM genomic promoter; AvrM: AvrM coding region; AvrL567 prom: AvrL567 promoter sequence; AvrL567: AvrL567 coding region; RB: T-DNA right border; LB: T-DNA left border; 35S: 35S promoter; Spectinomycin R: Spectinomycin resistance gene; ocs: OCS terminator. The presences of introns are indicated by grey boxes and introduced restriction sites are shown by arrowheads. Diagrams are not drawn to scale.

3.2.2 Rust transformation

Binary vectors were transformed into chemically competent *Agrobacterium* using the freeze-thaw method described by Annamalai and Rao (2006). Then, the transformation of flax rust was performed as described in Lawrence et al. (2010). Briefly, *A. tumefaciens* GV3101::pMP90 cultures containing the binary vector were grown overnight in LB media with 12.5 µg/mL tetracycline, 50 µg/mL gentamicin and 50 µg/mL rifampicin at 28°C and supplemented with 200 µM acetosyringone the following morning. After approximately 5 h growth at 28°C, cells were pelleted and resuspended in 4/3 volumes of original culture volume in 5% sucrose supplemented with

200 μ M acetosyringone. Following this, the stems of Hosh plants inoculated with wildtype CH5-89 rust 5 days beforehand were extensively punctured with a wire brush and immersed into the *Agrobacterium* suspension. A total of eight pots with five to six plants per pot were used in each experiment. The uredospores obtained from rust growing on these plants were subsequently screened by Dr Greg Lawrence for the ability to grow on H3 x Birio flax plants expressing the L6 resistance gene. Spores obtained from this screen were increased by two rounds of infection on Hosh plants, which do not undergo HR in response to AvrL567 or AvrM.

3.2.3 DNA extraction and PCR

Genomic DNA was extracted from infected leaves 7 days after infection using the protocol described in Young et al. (2001). In brief, approximately 100 mg of leaf was ground in a 1.5 mL microcentrifuge tube in 200 μ L DNA extraction buffer [0.2 M Tris-Cl pH 9.0, 0.4 M LiCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS)] using a plastic pestle. An additional 300 μ L extraction buffer was added to the tube and the leaf was ground further before centrifugation at $15800 \times g$ for 5 min at 4°C in a benchtop centrifuge. Then, 340 μ L of the supernatant was transferred into another microcentrifuge tube and DNA was precipitated by addition of 350 μ L isopropanol. DNA was pelleted by centrifugation at $15800 \times g$ for 10 min at 4°C. The supernatant was discarded and the pellet washed with 70% EtOH before air drying at room temperature. The pellet was then dissolved in 300 to 400 μ L MilliQ-filtered water (Millipore Corp.) for 30 min to 1 h at room temperature.

For PCR analysis, 2 μ L of sample was used as the template for each PCR reaction. Templates were placed in a 0.2 mL microcentrifuge tube with 1 $\times$ Promega PCR mix (Promega) and 0.5 μ M each of forward and reverse primers in a final volume of 20 μ L. PCR amplification was performed by denaturation at 95°C for 5 min followed by 40 cycles at 94°C for 30 sec, annealing at 50°C temperature for up to 1 min, and extension at 72°C for up to 90 sec with a final extension at 72°C for 5 min. For detection of the vector sequence, the primers 10-1.15 and 10-1.19, which uniquely amplify a product from the AvrL567 inverted repeat, were used (Lawrence et al. 2010). For detection of the Cerulean gene, the XbaI-Cerulean and NcoI-Cerulean rev primers were used (Appendix).

3.2.4 Reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from infected plants using the protocol developed by Oñate-Sánchez and Vicente-Carbajosa (2008). RNA was converted to cDNA using MMLV-reverse transcriptase according to the manufacturer's instructions (Promega). In brief, 1 µg of Promega RQ1 RNase-free DNaseI-treated RNA was incubated with 1 µg Oligo dT primer in a total volume of 14 µL at 72°C for 5 min before chilling at 4°C for 5 min to prevent secondary structure formation. Each reaction was then incubated with 1 × reaction buffer, 0.5 mM dNTPs, 25 units RNasin RNase inhibitor (Promega) and 200 units MMLV-Reverse transcriptase (Promega). Each tube was incubated at 40°C for 10 min, followed by extension for 50 min at 55°C and a final step at 72°C for 15 min. For RT-PCR reactions, 2 µL of cDNA was used as template. Reverse transcriptase was omitted from -RT controls.

For RT-PCR analysis, templates were placed in a 0.2 mL microcentrifuge tube with 1 × Promega PCR mix (Promega) and 0.5 µM each of forward and reverse primers in a final volume of 20 µL. PCR amplification was performed by incubating at 50°C for 2 min followed by denaturation at 95°C for 5 min followed by repeated cycles at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 15 sec with a final extension at 72°C for 10 min. Volumes of 5 µL of each reaction were run on agarose gels containing 0.2 µg/mL ethidium bromide. The intensities of bands run on the same gel and exposed under the same conditions were analysed using the ImageJ (National Institutes of Health) densitometry function. For PCR amplification of Cerulean-encoding transcripts, the primers Cerulean qF and NcoI-Cerulean rev were used. Transcript levels were assessed relative to the intensity of bands from the rust β -tubulin gene (AF317682) amplified using the TUB1 qF and TUB1 qR primers.

3.2.5 Immunoblots

Proteins from infected and non-infected plants were extracted in protein extraction Buffer B (Appendix) and immunoblots with alkaline phosphatase detection were performed as described in Chapter 2. For chemiluminescence detection, blots were blocked in 4% non-fat milk in PBS pH7.4 (Appendix) before incubation with the primary antibody anti-GFP (Roche Diagnostics Ltd) for 1 h. Blots were rinsed three times for 10 min each in PBST (Appendix). The anti-GFP antibodies were detected with goat anti-mouse conjugated to horse radish peroxidase (Sigma) at a 1:50000 dilution. Antibodies were then detected using the Supersignal West Pico chemiluminescent Substrate kit (Pierce Chemicals) according to the manufacturer's instructions.

3.2.6 Microscopy

Plant tissue samples were harvested from infected flax leaves and observed before the eruption of uredospores, between 4 to 7 days post inoculation, and tissue was directly imaged. Live tissue imaging was performed on a Leica SP2 confocal microscope (Leica), using 20× and 63× C-Apochromat water immersion objectives. The 458 nm or 405 nm laser lines with appropriate emission filters were used to image Cerulean fluorescent protein and chloroplast autofluorescence.

3.3 Results

3.3.1 Cloning of AvrM-Cerulean for expression in rust

A genomic fragment of 17 kb containing the sequence of AvrM-A, one of the five avirulence alleles of AvrM with the quickest necrotic response in plants with the M resistance protein, was previously isolated (Catanzariti et al. 2006) from a genomic DNA library prepared from rust strain CH5 (Ayliffe et al. 2001). Previously it was shown that upstream of the AvrM-A coding sequence is a sequence encoding a reverse transcriptase and downstream are four open reading frames, with no similarity to other database sequences (Catanzariti et al. 2006; Figure 3-2 A). Thus, this indicated that the two fragments chosen, which encompassed the genomic sequence between AvrM-A and the other open reading frames, were sufficient to cover the promoter and terminator regions of AvrM-A. As shown in Figure 3-2 B, approximately 1.1 kb of sequence immediately downstream of the AvrM-A coding region was amplified as the genomic terminator of AvrM. Further, the region approximately 1.4 kb upstream of the AvrM-A start codon was amplified as the genomic promoter of AvrM. These fragments were cloned into the pBluescript backbone in the correct orientation to express either AvrM-Cerulean or the Cerulean coding sequence alone. Constructs were then cloned into the *Pst*I site of the siAvrL567 T-DNA construct, which has previously been used for *M. lini* transformation (Lawrence et al. 2010). Constructs were checked by PCR for the same orientation relative to the AvrL567 promoter on the siAvrL567 T-DNA construct before transformation into *Agrobacterium*. This check was conducted because the reverse orientation might allow read-through from the AvrL567 transcript to generate antisense RNA of the AvrM construct. *Agrobacterium* cultures were then used to transform the *M. lini* strain CH5-89 growing on Hosh plants lacking the L6 or M resistance genes. Subsequently, spores were collected by Dr Greg Lawrence and used to infect H3 x Birio flax plants expressing the L6 resistance gene. From this experiment, five single pustules from transformation with the AvrM-Cerulean construct and eight pustules from transformation with the Cerulean only construct were isolated. Spores were increased through two rounds of infection on Hosh plants by Dr Lawrence.

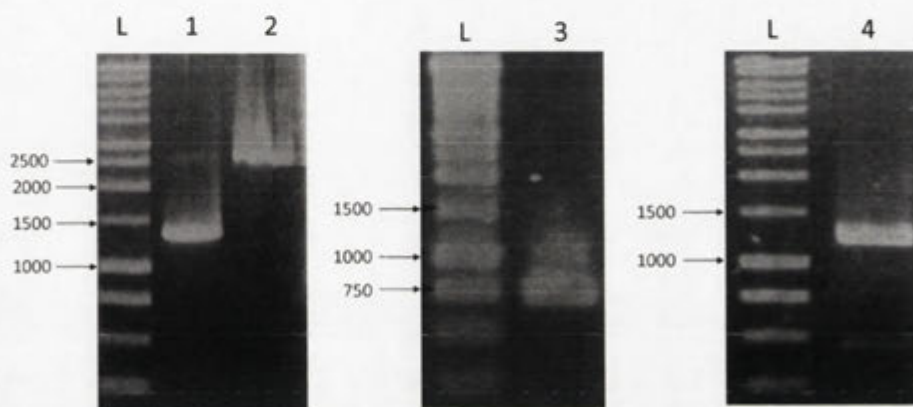
A**B**

Figure 3-2 Cloning of AvrM genomic promoter and terminator for expression of AvrM fused to Cerulean or Cerulean alone. **A.** Schematic representation of genomic region containing the AvrM-A avirulence locus (Catanzariti et al. 2006). RT: reverse transcriptase; UbiA: UbiA prenyltransferase; ORF: open reading frame. **B.** Restriction enzyme digests of fragments for cloning of constructs. L: 1 kb DNA ladder (Promega); 1: AvrM promoter; 2: AvrM promoter and AvrM coding region; 3: Cerulean and 4: AvrM terminator. Sizes of fragments are indicated on left in bp.

3.3.2 Screening of AvrM-Cerulean rust transformants and assessment of expression

Five AvrL567-silenced transformant pustules were isolated from a single transformation experiment using the AvrM-Cerulean construct. Genomic DNA samples were extracted from flax plants 5 days post inoculation (dpi) with spores from these pustules and used as the template for PCR amplification with primers specific for either the Cerulean coding sequence or the transformation vector. Using these primers, no bands were amplified from the negative control which was DNA extracted from plants infected by the parent wildtype strain (Figure 3-3). On the other hand, all five of the DNA samples extracted from plants infected with the transformants were found to be positive for the Cerulean sequence as well as the transformation vector sequence confirming that the AvrM-Cerulean construct had been integrated into the genome of the fungus (Figure 3-3).

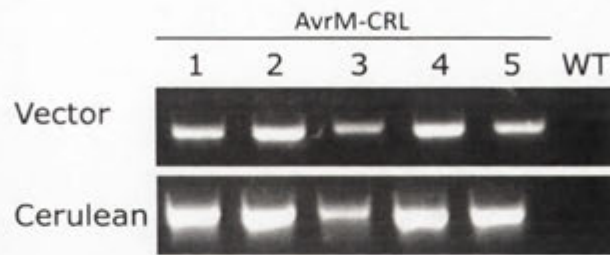


Figure 3-3 PCR of genomic DNA isolated from plants infected by AvrM-Cerulean (AvrM-CRL) rust transformants showing the detection of the Cerulean gene (717 bp) and transformation vector sequences (900 bp). PCR of plants infected by rust expressing the AvrM-Cerulean protein (1-5) and plants infected by wildtype rust (WT).

Apart from confirming the presence of the AvrM-Cerulean transgene, immunoblots were used to assess the translation of the full-length fusion protein (Figure 3-4). Proteins extracted from rust-infected plants were labelled with anti-GFP, which had previously been demonstrated to cross-react with the cerulean protein (Rafiqi et al. 2010) and anti-AvrM antibodies. Both antibodies detected proteins that corresponded to the expected size of full-length AvrM-Cerulean fusion proteins only in total protein extracts of plants infected with the AvrM-Cerulean rust transformants but not in uninfected plants or plants infected with the parental wildtype rust strain. The results of this experiment confirmed that the full-length fusion protein was being expressed.

In samples labelled with the anti-AvrM antibody, bands of 35 kDa or smaller were detected in proteins from plants infected with wildtype as well as AvrM-Cerulean transformed rust, but not in uninfected plants. These most likely correspond to wildtype AvrM-A and potentially other allelic versions of AvrM, and their degradation products, that are present in the wildtype parental rust strain, which is heterozygous for the AvrM avirulence and virulence alleles (see Chapter 2).

Additional, less intense bands, just beneath the band corresponding to the expected size of AvrM-Cerulean, were detected by anti-AvrM in protein extracts from plants infected with AvrM-Cerulean transformed rust but not wildtype rust. While it is possible that these bands are non-specific background labelling, it is likely that they indicate potential N-terminal degradation

products since two of these bands were also detected in the anti-GFP labelled immunoblot, a result which would occur if both antibodies recognise the same degradation products. In addition, in the anti-GFP antibody labelled blot, bands corresponding to the expected size of Cerulean alone were detected, indicating possible cleavage between AvrM and the Cerulean protein. However, the intensity of such bands was always much lower compared to the full-length fusion protein, suggesting that the majority of protein was intact. Further, in other immunoblots (Figure 3-7 B) the band corresponding to Cerulean alone was not detected, which supports the conclusion that cleavage was sample dependent and possibly occurs during protein extraction. It is noted that samples were boiled before electrophoresis on SDS polyacrylamide gel. Boiling has been shown to result in cleavage of the Cerulean fluorescent protein from AvrM-Cerulean (Rafiqi et al. 2010).

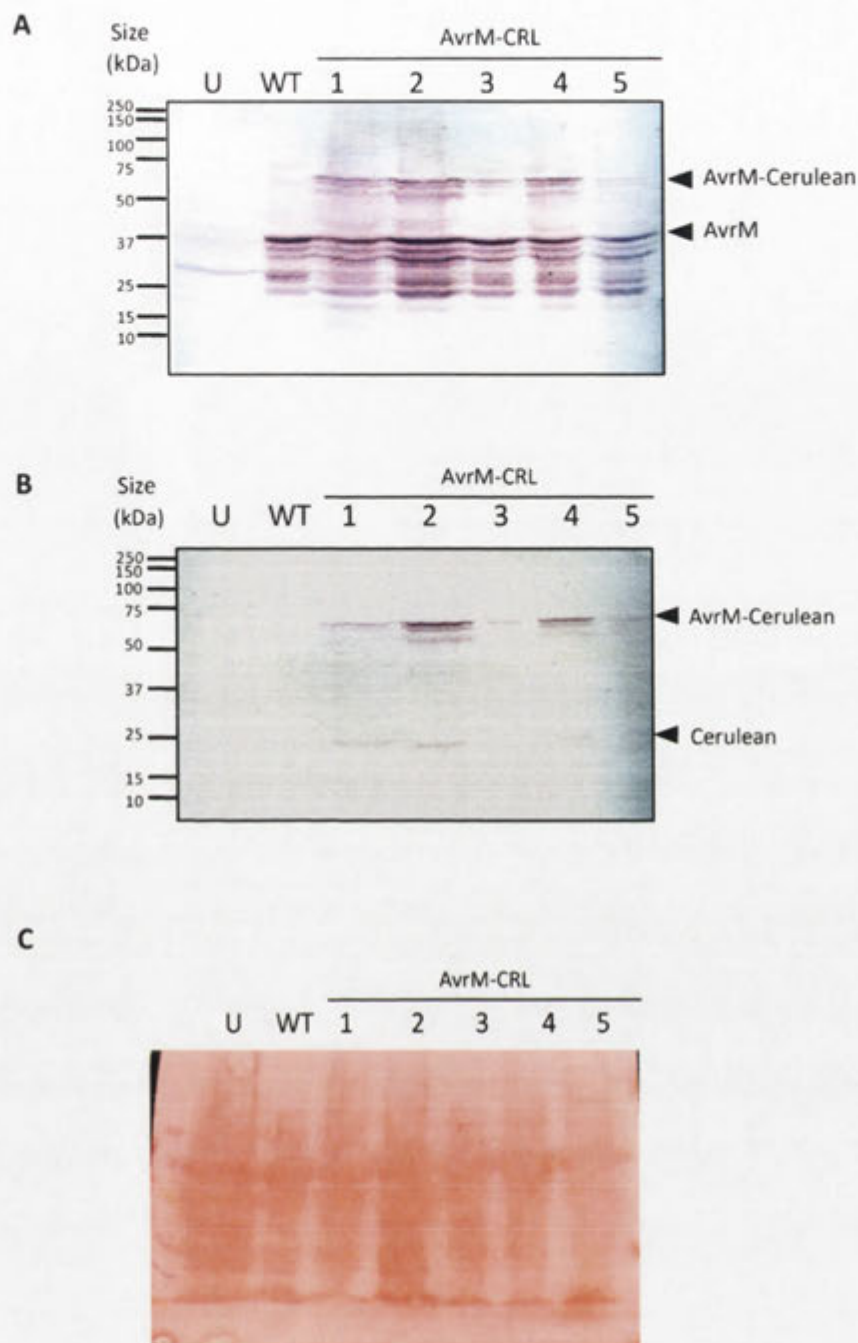


Figure 3-4 Immunoblots showing alkaline phosphatase colourimetric detection of AvrM-Cerulean (AvrM-CRL) by **A.** anti-AvrM and **B.** anti-GFP antibodies from total protein extracted from plants infected with AvrM-Cerulean rust transformants at 7 dpi (**1-5**). As controls, protein from plants infected by parent wildtype strain (**WT**) and uninfected plants (**U**) were included. The immunoblots showed that the major band detected by anti-GFP was that of the full-length AvrM-Cerulean fusion protein. Wildtype AvrM protein was detected in all rust-infected plants by anti-AvrM due to endogenous AvrM expression. **C.** Ponceau S stain to show proteins transferred onto the immunoblots. Sizes of proteins are indicated in kDa on left.

3.3.3 AvrM-Cerulean localisation

Having established that the AvrM-Cerulean gene transcripts were present and that the protein was synthesised in AvrM-Cerulean rust, the localisation of the fluorescent fusion protein was then assessed in rust transformants lines 1-4 growing within leaves between 4 and 7 dpi. However, the level of fluorescence in AvrM-Cerulean rust was similar to that in wildtype rust. Fluorescence was not observed even at hyphal tips (Figure 3-5) despite evidence from immunolocalisation data (Chapter 2) that the protein is present there. Further, no strongly fluorescent structures which might correspond to haustoria were detected within host mesophyll cells even from the rust lines 2 and 4, which showed high protein levels.

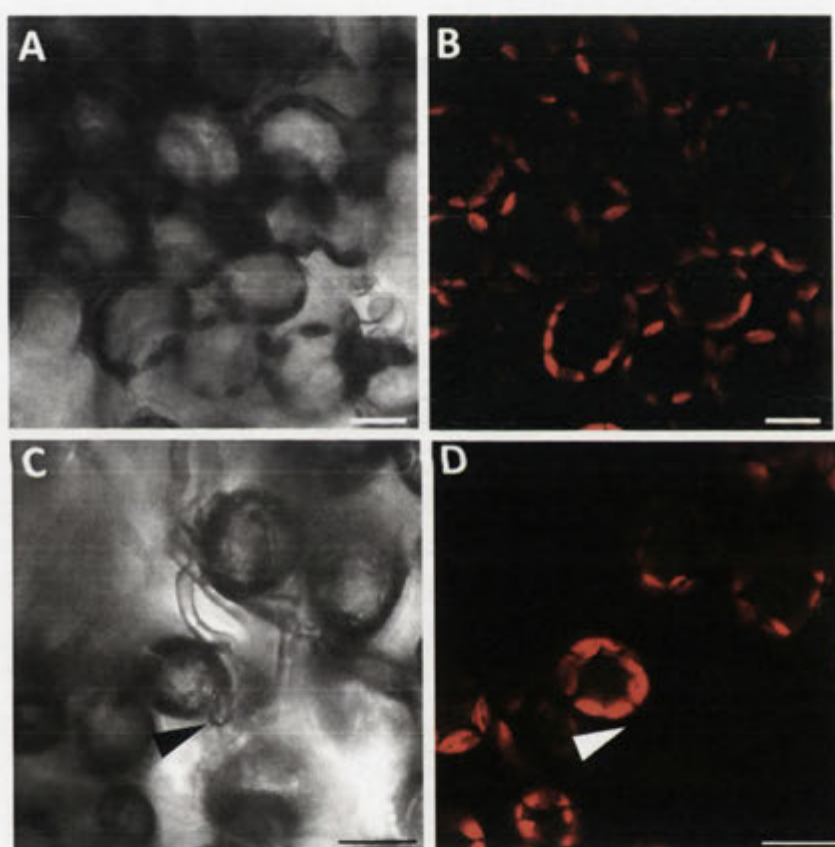


Figure 3-5 Confocal images of flax rust hyphae growing throughout a susceptible flax leaf at 6 dpi. **A.** Brightfield and **B.** fluorescence image of wildtype hyphae during infection. **C.** Brightfield and **D.** fluorescence image of transformed flax rust hyphae line 1 expressing AvrM-Cerulean during infection. Arrowheads indicate hyphal tip. Scale bars = 20 μ m. Similar results were observed for AvrM-Cerulean rust lines 2, 3 and 4.

Although immunoblot analysis showed accumulation of the AvrM-Cerulean protein, it was possible that the Cerulean domain failed to fold and mature properly into a fluorescent form.

Since Cerulean possesses the S72A mutation, which improves GFP protein folding at 37°C (Rizzo et al. 2004; Cubitt et al. 1999), infected plants were incubated at 37°C and then examined on the confocal microscope at various time points from 1 h to overnight. However, no increased fluorescence was observed after 1, 2, 3 or 5 h of incubation at 37°C. Furthermore, incubation at 37°C overnight resulted in autofluorescence of wildtype as well as AvrM-Cerulean fungi (Figure 3-6).

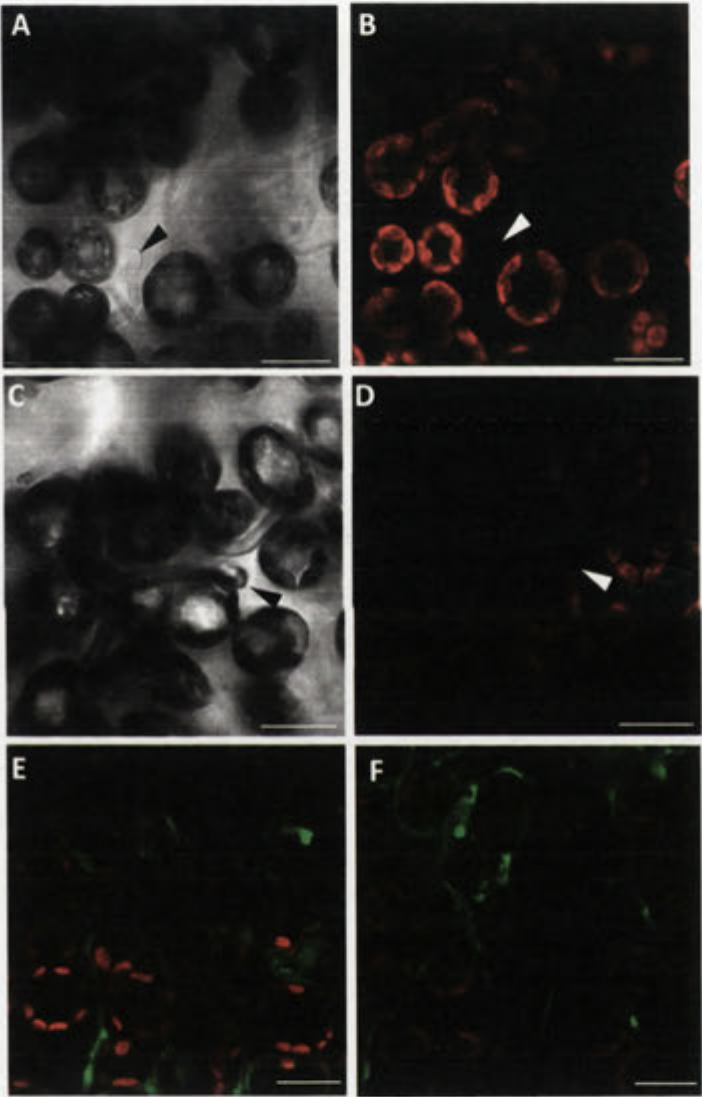


Figure 3-6 Brightfield (A and C) and fluorescence (B and D) images of wildtype (A and B) and AvrM-Cerulean flax rust line 3 (C and D) hyphae growing throughout a susceptible flax leaf 3 h after incubation at 37°C. The levels of fluorescence were similar to samples incubated for 1, 2 and 5 h at 37°C. Fluorescence was observed in plants infected by wildtype (E) and AvrM-Cerulean (F) hyphae after overnight (16 h) incubation at 37°C. Arrowheads indicate hyphal tips. Scale bar = 20 μm. Similar results were observed for AvrM-Cerulean rust lines 1, 2 and 4. Red: chloroplast autofluorescence.

3.3.4 Screening of cerulean rust transformants and assessment of gene expression

A total of eight AvrL567-silenced transformants were isolated after transformation with the Cerulean only construct and five of these were selected for further analysis. Cerulean protein expression in these strains was examined by immunoblot analysis of proteins extracted from infected leaves at 5 dpi with anti-GFP antibodies. Although a positive control consisting of proteins extracted from plants infected with an AvrM-Cerulean rust strain showed the presence of a band corresponding to full-length AvrM-Cerulean, no Cerulean protein was detected in any of the five Cerulean rust transformants (Figure 3-7A). Two strains (number 1 and 4) were further tested at 7 dpi and again no protein expression was observed (results not shown).

To determine if the lack of protein was due to a problem with transcript expression, RT-PCR was performed using primers specific to the Cerulean coding sequence (Figure 3-7). All five Cerulean transformants that were tested showed bands of the same size as that of the positive control, which was cDNA from plants infected by AvrM-Cerulean rust. The negative controls, from plants infected by untransformed rust or uninfected plants, showed no bands. No products were observed in a control sample from which the reverse transcriptase was omitted, indicating that the PCR products were derived from cDNA and not contaminating genomic DNA. This demonstrated that the expression construct was present in Cerulean rust and that the transcript was being expressed.

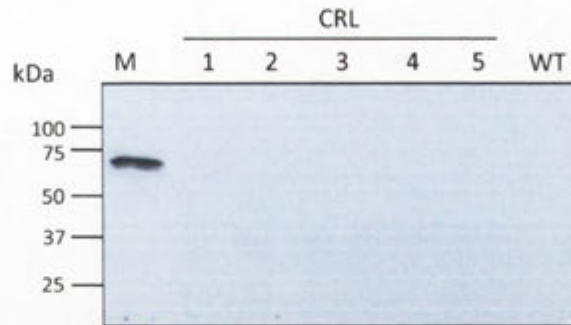
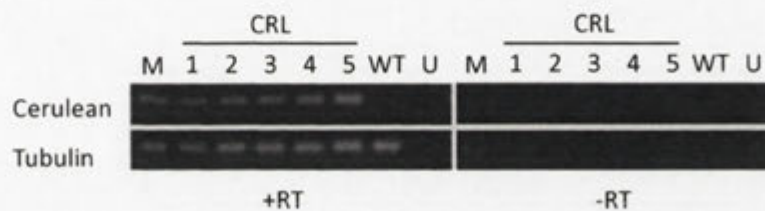
A**B**

Figure 3-7 Expression of Cerulean protein and transcripts of Cerulean (CRL) transformants at 5 dpi. **A.** Immunoblot showing chemiluminescent detection of Cerulean protein using anti-GFP antibodies from total protein extracted from infected plants. Cerulean protein was not detected in plants infected by Cerulean only transformants although Cerulean protein was detected in the AvrM-Cerulean transformant-infected plant. **B.** RT-PCR analysis (35 cycles) showing transcription of the Cerulean gene in plants infected by rust transformants. PCR was performed using primers specific for the Cerulean gene as well as for the flax rust β -tubulin gene. Material from plants infected by Cerulean rust transformants (CRL1-5), AvrM-Cerulean transformed rust (M), wildtype rust (WT) and uninfected plants (U) were included.

As this RT-PCR analysis had been performed with 35 cycles of amplification, when product amplification may have been saturated and transcript expression levels cannot be compared, semi-quantitative RT-PCR was used to determine if there was a difference in Cerulean transcript expression levels between the AvrM-Cerulean and Cerulean only transformants. First, a range of cycles was tested to determine a cycle number at which PCR product amplification is not saturated and where amplification is potentially exponential and transcript levels can be compared. This was found to occur after 28 cycles of amplification (Figure 3-8 A). The transcript levels of AvrM-Cerulean transformant lines 1, 4 and 5 and Cerulean transformant lines 1, 3 and 4 were assessed by ImageJ densitometry analysis and normalised to the rust tubulin transcript. This showed that

Cerulean encoding transcript levels did not differ significantly between the two types of transformants (Figure 3-8 B).

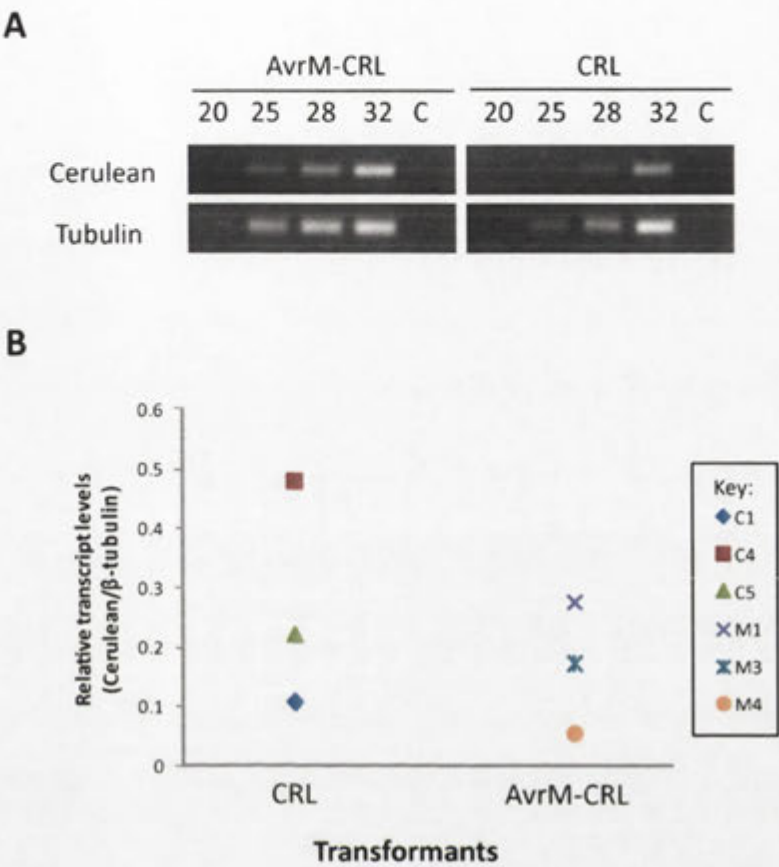


Figure 3-8 Semi-quantitative PCR showing expression levels of Cerulean transcripts in AvrM-Cerulean (AvrM-CRL) and Cerulean (CRL) transformants at 7 dpi relative to rust β -tubulin. **A.** A number of PCR cycles ranging from 20 to 32 were tested on cDNA from AvrM-Cerulean and Cerulean transformants to determine the range at which transcripts are undergoing exponential growth. Numbers on the top of each lane indicate number of PCR cycles. C is the no RT control, which was amplified with 32 cycles. **B.** Comparison of expression levels between a number of AvrM-Cerulean and Cerulean only transformants after 28 cycles by densitometry analysis. Values are shown relative to levels of the rust β -tubulin gene. Lines tested were Cerulean lines 1, 4 and 5 (C1, C4 and C5) and AvrM-Cerulean lines 1, 3 and 4 (M1, M3 and M4).

All Cerulean transformants were also screened using confocal microscopy and no Cerulean fluorescence was detected in any of the samples (data not shown).

3.4 Discussion

The aim of the work presented here was to generate flax rust expressing AvrM fused to a fluorescent protein marker. Such transformants would allow further analysis of the biology of the flax rust fungus as well as increase the understanding of the dynamics of AvrM uptake and its fate during and after entry into host cells. In order to achieve this, *Agrobacterium*-mediated transformation of *M. lini* which has previously been used to silence the AvrL567 gene was performed (Lawrence et al. 2010).

Transformation of *M. lini* is a new technique that is still being developed and optimised. In the experiments described here, five transformants were isolated in the case of AvrM-Cerulean and eight in the case of Cerulean. The results of PCR analysis indicate that all tested transformants were positive for the transgene. This showed that silencing of AvrL567 is a robust selectable marker and that the full construct including the gene of interest was stably inserted into the rust in all cases. As transformant pustules were increased twice on Hosh plants, which lack resistance against AvrL567, without loss of the transgene, the transformation appears to be stable as reported previously by Lawrence et al. (2010).

In this study, it was shown that the region 1.4 kb upstream of the AvrM-A transcriptional start site was sufficient to drive expression of transcripts to a level where protein accumulation of AvrM-Cerulean protein could be observed, although this appeared to be lower than wildtype AvrM, which served as an internal control in immunoblots labelled with anti-AvrM antibodies. A possible explanation for the observed difference between AvrM-Cerulean and wildtype AvrM expression is that there are five AvrM alleles at the avirulence locus of AvrM and that the AvrM antibody may not differentiate between the protein products of different alleles with approximately the same size. It is possible that as mentioned in Chapter 2, the products of more than one avirulence allele were being detected. Further, cleavage between AvrM and Cerulean in AvrM-Cerulean fusion proteins may also contribute to the intensity of the 35 kDa AvrM bands. In any case, it is noted that the results reported here do not indicate that AvrM is weakly expressed in haustoria since total protein extracts from infected plants were used and not protein from haustoria alone.

In the experiments described, it was found that transformation of the AvrM-Cerulean but not the Cerulean only transgene led to the expression of immunoblot-detectable Cerulean protein.

It is known that wild-type GFP is not efficiently translated in some fungi due to sub-optimal codon usage (Spellig et al. 1996; Cormack et al. 1997) and not in plants due to the presence of a cryptic intron splice site (Haseloff et al. 1997). However, although versions of GFP that have been codon optimised for use in plants, such as SGFP and EGFP, have been used successfully to achieve visualisation of fluorescent plant pathogenic fungi and oomycetes *in planta*, including *U. maydis*, *Colletotrichum trifolii* and *Phytophthora nicotianae* (Spellig et al. 1996; Lorang et al. 2001; Bottin et al. 1999), it is possible that the codon usage of the Cerulean coding sequence is not optimal for expression in *M. lini*. Cloning of AvrM fused to a *M. lini* codon-optimised fluorescent protein sequence may increase expression levels of the reporter gene to a level that can be detected by confocal microscopy. *M. lini* is a basidiomycete and studies on the model basidiomycete *Schizophyllum commune* have found that the expression of GFP may be inhibited by several factors, including AT-rich stretches (Scholtmeijer et al. 2001; Schuren and Wessels 1998). However, the Cerulean coding region has a GC content of 60.97%, indicating that the presence of the gene itself is unlikely to affect expression negatively.

Another factor that affects the expression of gene products in basidiomycetes is a lack of introns (Lugones et al. 1999; Scholtmeijer et al. 2001). The intron requirement for efficient expression of GFP was also found to apply to the basidiomycetes *Phanerochaete chrysosporium*, *Agaricus bisporus* and *Coprinus cinereus* (Ma et al. 2001; Burns et al. 2005). It has been hypothesised that introns may also be required for expression of genes in *M. lini* based on the finding that all known avirulence genes of the fungus contain two or more introns, despite encoding relatively small protein products (Lawrence et al. 2007). This includes the AvrM-A gene which possesses two introns towards the 5' end and one intron towards the 3' end of the coding region (Catanzariti et al. 2006). In this study, the fact that the genomic version of AvrM (including introns) fused to the Cerulean coding sequence was demonstrated to yield detectable amounts of protein on immunoblots but not the Cerulean coding sequence alone, indicates that introns may also be important for expression in *M. lini*. However, the detection of Cerulean-encoding transcripts by RT-PCR analysis in both AvrM-Cerulean and Cerulean only transformants indicates that the lack of detectable protein in Cerulean only transformants is most likely caused by a post-transcriptional process. One possibility is that, as in other higher eukaryotes, intron removal may influence the stability of the 3'-end of transcripts (Nesic and Maquat 1993) although the level of transcripts in Cerulean rust compared to AvrM-Cerulean transformants is not significantly different

in the transformants tested. It is possible that addition of just the first intron of AvrM, which occurs after the signal peptide, may be sufficient for efficient translation of transcripts, as in the case of *P. chrysosporium* where addition of the first intron flanked by 15 bp of the exons of the glyceraldehyde-*p*-dehydrogenase gene is enough to allow efficient expression of GFP protein (Ma et al. 2001). It is noted, however, that GFP gene expression without introns does occur efficiently in some other basidiomycetes including *Ustilago maydis* (Spellig et al. 1996).

Despite the detection of the AvrM-Cerulean protein on immunoblots by anti-GFP and anti-AvrM antibodies, no Cerulean fluorescence was detected under the conditions tested. The Cerulean fluorescent protein is a modified form of enhanced cyan fluorescent protein (ECFP) with the additional mutations of S72A, Y145A and H148D (Rizzo et al. 2004). These mutations collectively confer a brighter signal than ECFP, and have been used to express fluorescent proteins *in planta*, including in the apoplast of tobacco and flax plants indicating that it is stable under apoplastic conditions (Rizzo et al. 2004; Rafiqi et al. 2010; Martin et al. 2009). However, the major disadvantage of using this protein is its relatively weak fluorescence in comparison to other forms of GFP, with only approximately 82.5% of the signal intensity of EGFP (Rizzo et al. 2004). Given that a weak signal may be insufficient for visualisation of the fusion protein, several other strategies may be employed to detect these proteins *in planta*. Among these include the use of forms of fluorescent proteins with higher signal intensity such as EGFP and mCherry, which have been successfully employed to detect fluorescent effector proteins expressed and secreted from *Magnaporthe oryzae*, *U. maydis* and *P. infestans* (Mosquera et al. 2009; Khang et al. 2010; Doehlemann et al. 2009; Whisson et al. 2007). By using other fluorescent protein variants, the detection of fungal autofluorescence, which overlaps with the Cerulean emission spectrum, as seen when infected leaves were incubated at 37°C overnight, may also be avoided. Potentially misfolded Cerulean fusion proteins could also be detected by immunolocalisation with anti-GFP antibodies as described for anti-AvrM in Chapter 2, although this requires fixation of infected tissue.

In addition to this, strategies to detect weak signals may be employed such as the use of other reporters such as the GUS reporter protein, which was used to detect RxLR-mediated Avr3a within host cells (Whisson et al. 2007). However, one of the disadvantages of this latter approach is that it does not allow live visualisation of reporter activity. Another strategy to increase the sensitivity of protein detection is the attachment of nuclear localisation signals to fluorescent

fusion proteins of interest, since concentration of the protein in a confined compartment in the cell would enhance the local strength of fluorescence. This strategy was successfully employed recently to detect the transfer and subsequent cell-to-cell movement of the *M. oryzae* effectors PWL2 and BAS1 in infected plant cells (Khang et al. 2010). The ability of the AvrM-A protein to induce HR would also be a sensitive, indirect verification of AvrM-Cerulean expression and transfer *in planta*. In the experiments described in this chapter, however, this assay was not possible as the parent strain of rust used (CH5-89) is heterozygous for the avirulence and virulence alleles of AvrM. However, transforming a rust strain that is homozygous for the virulent form of AvrM would allow for such an assay to be conducted on plants expressing the M resistance protein. Recently this was performed using constructs derived from the AvrM-Cerulean transformation construct generated in this chapter, where the Cerulean coding sequence was replaced with the coding sequence for the Citrine fluorescent protein which has stronger signal strength relative to Cerulean. Transformation of flax rust homozygous for the virulence allele of AvrM resulted in the loss of virulence (or gain of avirulence) of transformants on flax with the M resistance protein (Dodds et al., personal communication). Studies to localise the fluorescent protein fusion are underway.

CHAPTER 4

4 Analysis of the role of host endocytic components in AvrM uptake

4.1 Introduction

In the study by Rafiqi et al. (2010) it was shown that AvrM expressed and secreted from tobacco and flax epidermal cells is able to re-enter these cells. This result provides evidence that AvrM entry into plant cells does not require pathogen-encoded machinery and that the mechanism of internalisation is host-dependent. Further, it also indicates that the host uptake pathway is widely conserved, at least between different plant species. Endocytosis, or the process of internalisation of small molecules at the cell surface, has typically been classified as either clathrin-dependent or clathrin-independent.

4.1.1 Clathrin-dependent endocytosis

In clathrin-dependent endocytosis in mammalian cells, clathrin associates with the AP2 adaptor complex which is exclusively involved with coated vesicle formation at the plasma membrane, as opposed to the AP1, AP3 and AP4 clathrin-adaptor complexes, which function in the processes of sorting at the endosomes and the trans-Golgi network (Boehm and Bonifacino 2001). The AP2 complex is a heterotetramer of α -, β 2-, μ - and σ -adaptins that is involved in internalisation of transmembrane proteins containing a cytoplasmic tyrosine sorting motif (YXX Φ). The YXX Φ motif, which is present on many plasma membrane and trans-Golgi network localised proteins, has been relatively well characterised and shown to associate by a direct interaction with the AP2 μ -subunit (Haucke and De Camilli 1999). Evidence that this mechanism is conserved in plants comes from protein pull down assays which demonstrated binding of a μ -adaptin protein to the YXX Φ motif of the vacuolar sorting receptor, VSR-PS1 (Happel et al. 2004). However, AP2 is by no means the only clathrin-cargo adaptor protein and redundancies in terms of adaptors have been postulated to give greater robustness to clathrin-mediated uptake (Doherty and McMahon 2009). An example of an alternative adaptor is the AP180 protein which also binds to clathrin and can stimulate clathrin cage assembly (Ford et al. 2001). In plants, the range of clathrin adaptors and other accessory factors has not been well characterised. An example of a potential clathrin accessory factor is the epsin homology domain (EHD) protein AtEHD2, which has been shown to increase the

uptake of FM4-64 and the clathrin-mediated endocytosis marker transferrin when silenced (Bar et al. 2008). Further, the related protein, AtEHD1, which was shown to reduce FM4-64 uptake when silenced, may also be a potential clathrin accessory factor in plants (Bar et al. 2008).

4.1.2 Clathrin-independent uptake pathways

Recently, it has been found that clathrin-independent endocytosis pathways are also important in cellular uptake and membrane turnover. Clathrin-independent uptake pathways include, macropinocytosis, caveolin-dependent endocytosis, flotillin-dependent endocytosis, the clathrin-independent carrier/GPI-AP enriched early endosomal compartment (CLIC/GEEC) pathway, the IL2 receptor β (IL2R β) pathway and the Arf6 dependent pathway (Doherty and McMahon 2009). These pathways differ in terms of the accessory factors or structural components required, although some overlap between the different pathways exists (Table 4-1).

In mammals, one of the better characterised of these mechanisms is that of caveolin-dependent endocytosis. Caveolins are transmembrane proteins that define specialised lipid rafts by which internalisation can occur. However, in plants, no homologs of caveolins have been identified in database searches (Aniento and Robinson 2005). Nevertheless, the presence of homologs of another protein associated with a separate class of lipid raft-mediated endocytosis, flotillin, has been demonstrated in detergent resistant membranes of *Arabidopsis* (Borner et al. 2005). In animal cells, flotillin-mediated endocytosis has been associated with the internalisation of the cholera toxin subunit B (CTxB) and glycosylphosphatidylinositol (GPI)-linked proteins (Glebov et al. 2006).

The other clathrin-independent internalisation pathways do not have characteristic structural components but may be defined morphologically (i.e. phagocytosis, which is characterised by the engulfment of large particles; macropinocytosis, which is characterised by membrane ruffling and CLIC/GEEC, which is defined by tubular/ring-like invaginations) and/or in terms of the requirement for specific proteins, usually small G-proteins for signalling (i.e. the Arf6 dependent pathway, which requires the G-protein Arf6 and the IL2R β pathway, which requires the G-proteins RhoA and Rac1 (Doherty and McMahon 2009). There is also evidence that the G-protein Rac1 is involved in uptake of the cell penetrating peptide MPG, which is able to enter cells autonomously through an undefined, non-endocytic pathway, by facilitating actin remodelling at entry sites (Gerbal-Chaloin et al. 2007). Many of these G-proteins are members of the Rho family of GTPases, which belong to the Ras superfamily of GTPases. Plants lack homologs of mammalian

Rho G-proteins but have a unique class of Rho family GTPases known as Rho-related GTPases from plants (ROPs) although they are sometimes referred to as RACs or Rac-like GTPases in the literature (Vernoud et al. 2003; Gu et al. 2004). Of these, some members have been associated with actin and microtubule reorganisation and may conceivably also function in membrane turnover (Chen et al. 2003; Opalski et al. 2005).

Table 4-1 Summary of major endocytic pathways in animal cells (modified from Doherty and McMahon (2009) and Mayor and Pagano (2007)).

Endocytic mechanism	Structural components and associated proteins	Examples of cargo
Clathrin-mediated endocytosis	Clathrin, epsin, actin, AP2 complex (α -adaptin, β 2-adaptin, μ 2-adaptin and σ 2 adaptin), AP180, dynamin, Rab5, and many other accessory factors, EHD proteins	Transferrin receptor, anthrax toxin, receptor tyrosine kinase
Caveolin-dependent	Caveolins*, actin, PKC, src, dynamin, Rab5	Cholera toxin B subunit (CTxB), GPI-linked proteins
Flotillin-dependent	Flotillin1, Flotillin 2	CTxB, proteoglycans
CLIC/GEEC	Actin, GRAFs, Cdc42, possibly phosphatidylinositol 3-kinase (PI3K)	Fluid phase markers, CTxB, GPI-linked proteins
IL2R β pathway	Rac1, RhoA, PAK1, PAK2, possibly PI3K	IL2R β , cytokine receptor
Phagocytosis	Rac1/cdc42/Arf6/rhoA, actin, amphiphysin1	Pathogens, apoptotic remnants
Macropinocytosis	Rac1, PI3K	Receptor tyrosine kinase, fluid phase markers

* No evidence of caveolin homologs in plants

After entry, proteins that enter via vesicular-mediated uptake pathways still require a pathway to escape from membrane-bound vesicles of the endosomal system into the cytoplasm. While some proteins disrupt endosomal membranes to be released, others are known to enter the cytoplasm from the endoplasmic reticulum via translocons in a process known as retrotranslocation (Sandvig and van deurs 2000).

The aim of the research described in this chapter was to assess the effect of inhibiting different host cell protein internalisation pathways on AvrM uptake. Uptake pathways could be disrupted by using chemical inhibitors, by silencing or mutation of genes encoding endocytic components or by expressing dominant negative forms of proteins involved in endocytosis. The

use of chemical inhibitors requires controlled external conditions and a short assay time. Hence, a rapid assay based on direct protein delivery was tested in protoplasts, epidermal peels and whole leaves. Silencing, on the other hand, is performed *in planta* and can be combined with assessment of hypersensitive cell death induced by recognition of the AvrM effector after its secretion and subsequent uptake. Hypersensitive cell death has been shown to correlate well with entry of secreted AvrM into host cells after agroinfiltration with effector-encoding constructs in *N. tabacum* leaves in plants stably expressing the M resistance protein (Rafiqi et al. 2010). In *N. tabacum*, host genes can be silenced by the satellite virus-induced silencing system (SVISS) (Gosselé et al. 2002). An alternative silencing system is that of tobacco rattle virus (TRV)-mediated silencing of *N. benthamiana* genes. The use of this latter system required generation of *N. benthamiana* plants stably expressing the M resistance protein and demonstration that AvrM-induced HR could be used as an assay for AvrM uptake in these plants.

In the research described in this chapter, clathrin-mediated endocytosis was targeted by knockdown of α -adaptin, AP180, EHD1 and EHD2 gene homologs. Flotillin-dependent endocytosis was investigated by targeting the sequence of flotillin. A homolog of the *NtRac1* GTPase that has been shown to regulate actin (Tao et al. 2002) was also targeted as a potential component of clathrin-independent pathways. In addition to potential components of uptake pathways, the *CDC48* gene, whose product may be involved in retrotranslocation transport of proteins from the ER to the cytosol (Marshall et al. 2008), was also targeted for silencing.

4.2 Materials and Methods

4.2.1 Protein infiltration assay

4.2.1.1 Ligation-independent cloning (LIC) plasmid construction for expression of GST-tagged proteins

The *ccdB* coding region, which is cytotoxic to *E. coli* with wildtype DNA gyrase, was amplified from the pYY13 plasmid using the primers KpnI-LIC adaptor 1-PstI-*ccdB*-F and XhoI-LIC adaptor 2-PstI-*ccdB*-R. These primers introduced a PstI digestion site on either side of the *ccdB* coding region and two ligation-independent cloning (LIC) adaptors on either side (Figure 4-1). The PCR product was then inserted into the pET41 vector (Novagen), used for expression of N-terminal His-tagged glutathione S-transferase (GST) fusion proteins, at the KpnI and XhoI site to yield pET41.1. As the region amplified was found to be insufficient for the inhibition of *E. coli* growth, the *ccdB* gene was amplified by Phusion polymerase (Finnzymes) from pYY13 using primers pYY13F and pYY13R according to the manufacturer's instructions. In brief, 1 pg to 10 ng of plasmid DNA was incubated with 20 μ M dNTP, 1x Phusion HF buffer, 0.5 μ M template-specific primers and 1 U of Phusion polymerase in a 50 μ L reaction. Templates were denatured at 98°C for 10s followed by 35 cycles of amplification with denaturing at 98°C for 10 sec, annealing at 70°C for 20 sec and extension at 72°C for 0.06 sec /bp. Cycling was followed by a final extension at 72°C for 10 min. PCR fragments were cloned using restriction enzyme digestion into pET41.1 at the PstI site to yield

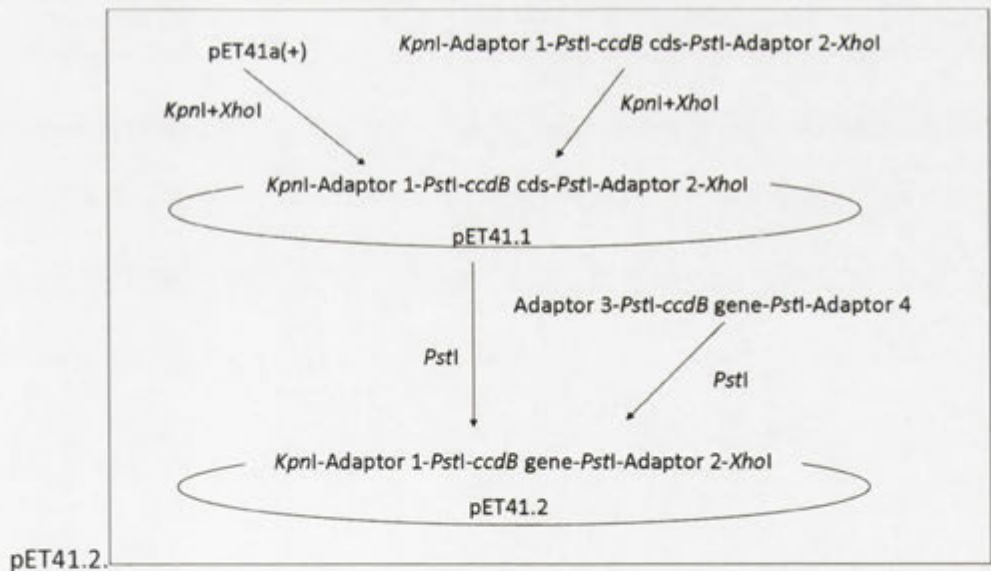


Figure 4-1 Construction of the pET41.2 plasmid. A cassette containing the *ccdB* coding region flanked by two PstI digestion sites and adaptors was inserted into the expression vector pET41. The pET41.2 vector was

then generated by replacing the *ccdB* coding sequence (cds) with the *ccdB* gene at the *PstI* site. Adaptor 3 and Adaptor 4 correspond to the primers pYY13F and pYY13R.

4.2.1.2 Expression and purification of proteins

Expression and purification of His-tagged AvrM lacking the N-terminal signal peptide (His-AvrM) was performed as specified in Chapter 2. For AvrM 156-Cerulean, PCR fragments encoding residues 28-156 of AvrM fused to the Cerulean fluorescent protein flanked by *Bam*HI were obtained from Dr M. Rafiqi. These fragments were cloned into the *Bam*HI site of the pET15b.ep vector (Novagen) using T4 DNA ligase (Promega) according to the manufacturer's instructions before transformation into the C41(DE3) overexpression *E. coli* strain (Lucigen Corp.). For growth of C41(DE3) cells on solid media, YT agar (Appendix) was used instead of LB agar. For N-terminal GST-tagged AvrM-A lacking the signal peptide (GST-AvrM) and Cerulean (GST-Cerulean) the AvrM and Cerulean protein coding sequences were amplified with LIC adaptor 1 and LIC adaptor 2 flanking the coding sequence and cloned into the pET41.2 plasmid by LIC as described by Dong et al. (2007).

In brief, a total of 200 ng of purified PCR product, purified using the Wizard SV Gel and PCR Clean-up Kit (Promega) was treated with T4 DNA polymerase (New England Biolabs) in 1 × NEBuffer2 supplemented with 5 mM dATP and 5 mM dithiothreitol for 30 min at 22°C. The enzyme was then inactivated at 75°C for 20 min. The pET41.2 vector was prepared by digesting 3 µg plasmid with *PstI* enzyme in a total volume of 30 µL at 37°C for 3 h and then purifying the band containing the vector backbone using the Wizard SV Gel and PCR Clean-Up Kit (Promega) following the manufacturer's protocol for purification of PCR products. Then, 200 ng of the purified vector was treated with T4 DNA polymerase in the same way as the PCR product with the exception that dATP was substituted with dTTP. Treated vector and insert were then mixed together in equal amounts of up to 100 ng and allowed to anneal at room temperature or at 22°C for 30 min. Mixtures were then transformed into chemically competent XL1Blue *E. coli* cells as described in Chapter 2. Plasmid integrity was confirmed by sequencing before transformation into chemically competent C41(DE3) *E. coli*.

Expression of AvrM 156-Cerulean, GST-AvrM and GST-Cerulean was performed by autoinduction at 37°C overnight. For autoinduction, volumes of 250 mL of LB media supplemented with 1 × 5052 [50 × 5052: 0.5% glycerol, 0.05% glucose, 0.2% α-lactose] and 1 × PPS

[20 × PPS: 1 M Na₂HPO₄, 1 M KH₂PO₄] as well as 1 mM MgSO₄ were inoculated with 1/50 vol of overnight *E. coli* culture and incubated on a shaker overnight for 16 to 20 h at 37°C. Subsequently, cultures were harvested and lysed as described in Chapter 2 before batch purification using Ni-sepharose 6 fast flow resin (GE Healthcare).

For AvrM 156-Cerulean, crude lysate from 1 L of overnight culture was incubated with 800 µL Ni-sepharose 6 fast flow resin for 1 h at 4°C on a rotator. Then, the resin was washed by resuspending in 30 medium volumes (m/v) of Wash buffer C [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.0, 300 mM NaCl, 20 mM imidazole], centrifugation of the resin at 1000 × *g* for 3 min at 4°C and removing the buffer containing unbound protein. This was followed by two washes with 10 m/v each of Wash buffer A (Appendix) and a single wash of 5 m/v with Wash buffer A. Finally, protein was eluted by incubating the resin with 1.25 m/v of Elution buffer A (Appendix) for 5 min at 4°C before centrifugation of the resin at 1000 × *g* for 3 min at 4°C and collecting the supernatant. This elution step was repeated an additional three times.

For the purification of GST fusions, proteins were purified by incubating crude lysate from 250 mL of overnight culture with 250 µL Ni-sepharose Fast flow resin (GE Healthcare) for 1 h at 4°C on a rotator. This was followed by a wash with 30 m/v Wash buffer C, three washes with 10 m/v Wash buffer A and four elution steps with 2 m/v of Elution buffer B.

In addition, as described in Chapter 2, proteins were buffer exchanged and concentrated using an Amicon ultracentrifugation device with the appropriate molecular weight cut off (Millipore). Then, the concentration of proteins was determined using the Bradford assay and bovine serum albumin (BSA) as the standard (Bradford 1976).

4.2.1.3 Epidermal peels

Epidermal peels were prepared from young wildtype *N. benthamiana* leaves by peeling off the lower epidermis with a scalpel. Peels were incubated in 1 mg/mL protein diluted in distilled water for the indicated times and washed in distilled water for 15 min in the dark before mounting in distilled water or 100 µM propidium iodide for visualisation on an Axioplan epifluorescence microscope (Zeiss).

4.2.1.4 Protoplasts

Protoplasts of *N. benthamiana* mesophyll cells were prepared using a method modified from He et al. (2007). In brief, young leaves were cut into 1 mm strips and floated on the surface of

protoplasting buffer [1% cellulase, 0.5% BSA, 0.5% macerage, 1% driselase in CPW buffer (0.5 M mannitol pH 5.6, 0.2 mM KH_2PO_4 , 1 mM KNO_3 , 13.3 mM CaCl_2 , 2 mM MgSO_4)] and incubated for 3 to 4 h at 28°C. Protoplasts were released by gently swirling the solution and separated from cell wall material by pipetting through nylon gauze (70 μm mesh). Suspensions were centrifuged in a round bottom flask to pellet cells at approximately $1000 \times g$ on a swinging rotor clinical tabletop centrifuge for 2 min and the supernatant was removed. Cells were gently resuspended by swirling in 10 mL CPW buffer before repeating the centrifugation step. The washing step was repeated once more before resuspending the pellet in protein solution in CPW buffer or 0.5 M mannitol for the specified times. Cells were pelleted and resuspended in CPW buffer before visualisation on the epifluorescence microscope.

4.2.1.5 Protein infiltrations

Concentrated proteins were adjusted to a desired concentration in phosphate buffered saline pH 7 [PBS pH 7; 2.7 mM KCl, 138 mM NaCl, 1.5 mM KH_2PO_4 , 20.4 mM NaH_2PO_4 , pH 7] or MS media [4.4 g MS basal medium (Sigma), 0.5 g MES, 20 g Sucrose/L, pH 5.8] and infiltrated into leaves with a 1 mL needleless syringe. For Proteinase K treatment, aliquots of proteins were incubated with 1 unit of Proteinase K conjugated onto agarose beads (Sigma) at 37°C for 4 h and then centrifuged at $10000 \times g$ for 15 min at 4°C to pellet Proteinase K before infiltration of the supernatant into plants.

4.2.1.6 Growth of plants

Hosh flax plants, lacking the M resistance gene, and Dakota flax plants, containing the M resistance gene, were raised and maintained by Dr Greg Lawrence (CSIRO, Plant Industry).

Transgenic *N. benthamiana* and *N. tabacum* plants expressing the M resistance protein and *N. tabacum* plants expressing the L6 resistance protein were generated by Kim Newell (CSIRO, Plant Industry). Seeds from transgenic plants were surface sterilised by rinsing with 70% EtOH for 30 sec followed by rinsing in 2.5% sodium hypochlorite in water for 10 min in a 1.5 mL microcentrifuge tube. Seeds were rinsed three times for 10 min each in sterile distilled water before plating on an MS agar plate [MS media with 8 g/L Bactoagar (Difco)] plate supplemented with 50 $\mu\text{g}/\text{mL}$ spectinomycin. Plates were maintained in a 16 h light/ 8 h dark cycle. Under spectinomycin selection, non-transgenic seedlings have a photo-bleached phenotype. Transformant seedlings were transferred to soil after germination. Homozygous lines were selected based on segregation of spectinomycin resistance. The presence of the M resistance

gene was confirmed by agroinfiltration of leaves with AvrM, which induces the hypersensitive response (HR).

4.2.1.7 Agrobacterium-mediated transient transformation of AvrM, AvrM Δ SP or AvrM Δ 106-153 in plant leaves

Agrobacterium tumefaciens GV3101::pMP90 cultures containing binary plasmids for expression of AvrM, AvrM Δ SP or AvrM Δ 106-153 fused to Cerulean under the nopaline synthase promoter and terminator (Rafiqi et al. 2010) were routinely infiltrated into plants at O.D.₆₀₀ 0.2. In brief, *Agrobacterium* cultures grown on a plate were used to inoculate LB media supplemented with 50 μ g/mL kanamycin, 50 μ g/mL gentamicin and 50 μ g/mL rifampicin as well as MES and acetosyringone to final concentrations of 10 mM and 20 μ M, respectively. Cultures were grown overnight on a shaker incubator at 28°C and harvested the next day by centrifugation at 4000 $\times$ g for 10 min in an F13/14 rotor. Pellets were resuspended in agroinfiltration buffer [10 mM MES, 10 mM MgCl₂, 200 μ M acetosyringone] and allowed to stand at room temperature for 4 to 6 h before infiltration into plants with a 1 mL needleless syringe.

4.2.2 Satellite virus-induced silencing system (SVISS)

4.2.2.1 Preparation of satellite virus transcripts

Target sequences were amplified with the PDS-NotI-rev and PDS-SbfI-for primers (Appendix) which added SbfI and NotI restriction enzyme site adaptors at the 5' and 3' ends, respectively, by PCR with Phusion Polymerase according to the manufacturer's instructions using cDNA extracted from wildtype *N. benthamiana* plants as a template. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up Kit, digested with SbfI and NotI, repurified and ligated into SbfI/NotI-digested pVE350 vectors (Figure 4-2; Gosselé et al. 2002; Gosselé and Metzlauff, 2005).

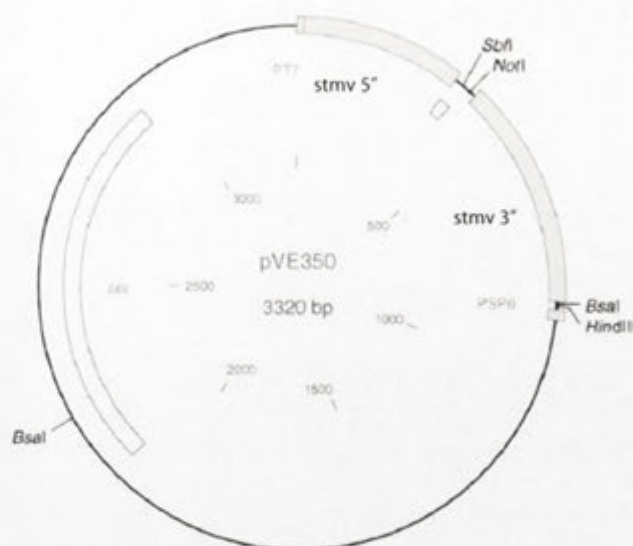


Figure 4-2 Physical map of the satellite tobacco mosaic virus (STMV) vector pVE350. Abbreviations *bla*: β -lactamase; PSP6: SP6 promoter sequence; PT7: T7 promoter sequence; stmv 5' and 3': STMV lacking 275 bp of the STMV coat protein, with a polylinker inserted (Modified from Gossele and Metzlauff (2005)).

Positive colonies were identified by colony PCR. In brief, 20 μ L PCR reactions were set up with 1x Promega PCR master mix (Promega), 0.5 μ M template-specific primers and bacterial cells from single colonies picked using sterile micropipette tips as templates. PCR amplification was performed with an initial denaturing/lysing step at 95° C for 5 min, followed by up to 39 cycles of denaturing at 94°C for 1 min, annealing at the melting temperature for 1 min and amplification at 72° C for 1 min/kb. This was followed by a final amplification step at 72° C for 5 min. Plasmids were isolated from positive colonies using the QIAgen Spin Miniprep Kit (QIAgen) and sequenced. Plasmids were digested with *HindIII* enzyme, treated with phosphatase and then purified using the Wizard SV Gel and PCR Clean-Up Kit. After verification of complete digestion by electrophoresis on an agarose gel, *in vitro* transcription was performed using the Megascript High-Yield T7 Transcription Kit (Ambion) according to the manufacturer's instructions. RNA quality was assessed using spectrophotometry and by electrophoresis on a denaturing agarose gel according to standard in-lab microbiology protocols (Appendix).

4.2.2.2 Plant inoculations

Four to six week old *N. tabacum* plants were inoculated with a tobacco mosaic virus (TMV) helper virus as described in Gossele and Metzlauff (2005). Plants were inoculated with 2.5 μ g *in vitro*

transcribed satellite virus transcripts and were co-inoculated with helper virus. As a control, buffer with helper virus alone was inoculated onto plants instead of the satellite virus transcript.

4.2.2.3 Northern blots

Inoculated and upper leaves were harvested 21 days post inoculation (dpi) and RNA was prepared as described in Oñate-Sánchez and Vicente-Carbajosa (2008). Five micrograms of RNA per sample was separated on a 1.8% denaturing gel and capillary-transferred overnight onto a nitrocellulose membrane (Biodyne) and fixed by exposure to UV light. DNA probes against the STMV genome (Figure 4-3) were amplified from the pVE350 plasmid containing the empty vector using the STMV for and STMV rev primers (Appendix). Prehybridisation and hybridisation with [³²P] dCTP-labeled DNA probes were performed in 7% (w/v) SDS, 1% (w/v) BSA, 0.5 M sodium phosphate, pH 7.2, and 1 mM EDTA at 65°C, and washing was in 2 × SSC [0.3 M NaCl and 0.03 M sodium citrate] and 0.1% SDS at 65°C.



Figure 4-3 Diagram depicting the genome of STMV showing cloning site of target gene and region as a STMV specific probe (Modified from Dodds 1998).

4.2.3 Tobacco rattle virus (TRV) silencing system

4.2.3.1 Cloning into pYY13 vector

As described by Dong et al. (2007), PCR primers were designed against target genes from *N. tabacum* and *N. benthamiana* to include LIC-linker specific sequences. The *EHD2* target sequence was amplified from *N. tabacum* cDNA as previously described (Bar and Avni 2009) and the *AP180* target sequence was amplified from *N. benthamiana* genomic DNA since the target sequence lacks introns. All other target sequences were amplified from *N. benthamiana* cDNA. PCR amplification was performed with Phusion Polymerase as described in Section 4.2.1.1 using primers: 5'-CGACGACAAGACCCT-target specific sequence-3' and 5'-GAGGAGAAGAGCCCT- target specific sequence-3' (Appendix). In these reactions, either 50 to 500 ng of genomic DNA or 50 to 100 ng of cDNA prepared as described in Chapter 3 were used as templates. PCR products were

inserted into the pYY13 plasmid by LIC as described in Section 4.2.1.2. Plasmids were then transformed into *Agrobacterium* using the freeze-thaw protocol (Annamalai and Rao 2006).

4.2.3.2 TRV inoculation onto plants

The protocol described in Section 4.2.1.7 was followed with the following modification: Pellets were resuspended in agroinfiltration buffer to a final O.D.₆₀₀ of 1.0. Cultures were allowed to stand at room temperature for 4 to 6 h before mixing cultures of *Agrobacterium* for expression of pTRV1 and pTRV2-TARGET encoded by pYL192 and derivatives of pYY13 (Figure 4-4; Dong et al. 2007) in a 1:1 ratio for inoculation. As an empty vector control, *Agrobacterium* with the pYL156 vector were used in place of *Agrobacterium* with the pYY13-derived plasmids (Figure 4-4). Plants were maintained under continuous light at 24 to 26°C and assessed from 3 weeks post inoculation. The fourth to sixth leaves above the infiltrated leaf were used for RT-PCR and phenotypic analyses.

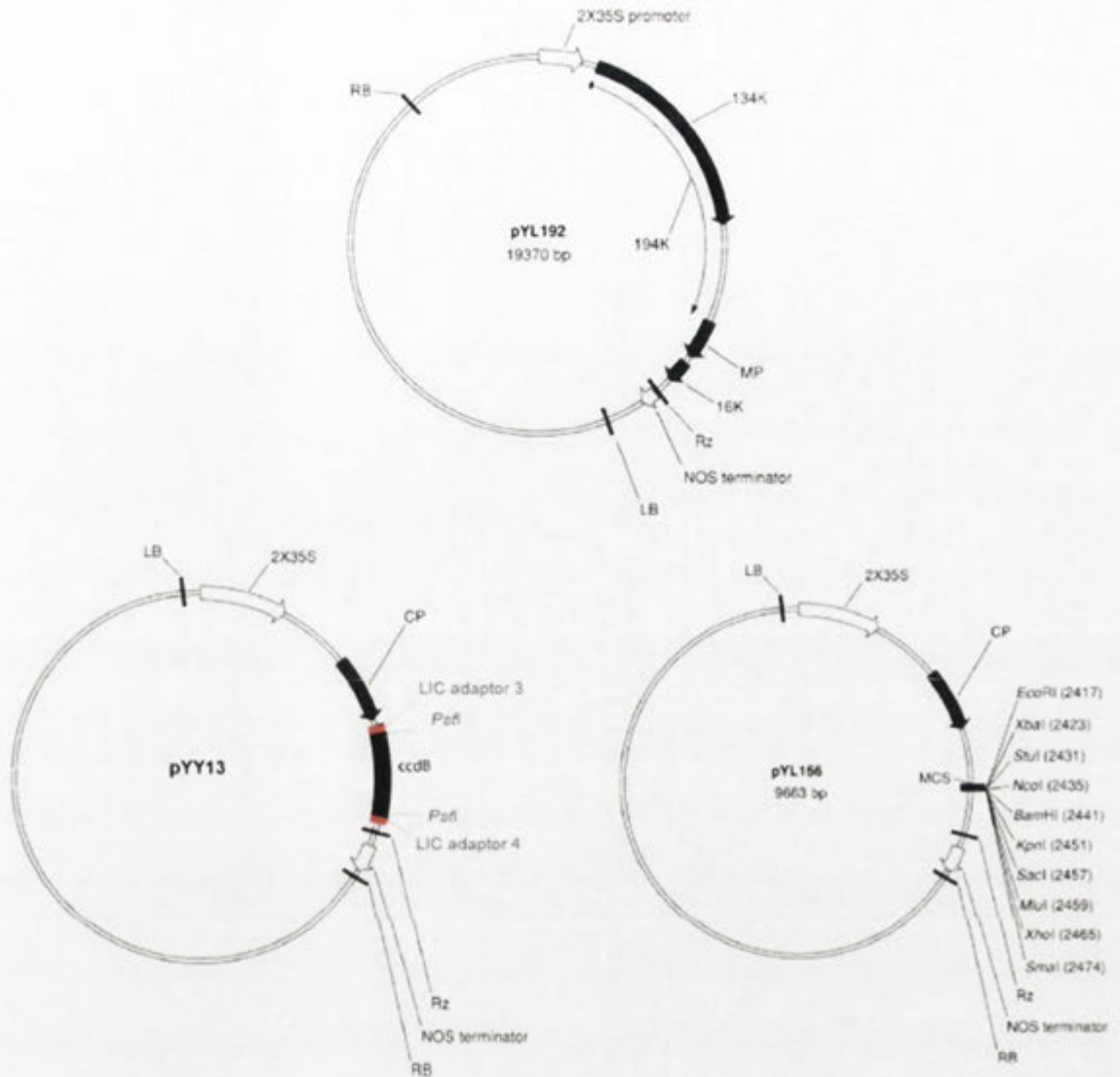


Figure 4-4 Physical map of vectors encoding RNA1 and RNA2 of the bipartite genome of TRV. The RNA1-encoding plasmid pYL192 and the RNA2-encoding plasmids pYL156 and pYY13. RB: right border; LB: left border; 2x35S: two copies of the cauliflower mosaic virus (CaMV) 35S promoter; CP: coat protein; MCS: multiple cloning site; Rz: ribozyme; NOS terminator: nopaline synthase terminator; ccdB: *E. coli* cytotoxic protein CcdB; LIC adaptor 3: pYY13F primer sequence; LIC adaptor 4: pYY13R primer sequence.

4.2.3.3 Neighbour-Joining tree analysis

Neighbour-Joining trees were calculated in Jalview (Waterhouse et al. 2009) using the BLOSUM62 matrix from sequences aligned by ClustalW (Larkin et al. 2007) .

4.2.3.4 Reverse transcriptase PCR (RT-PCR)

RNA samples from leaves collected prior to infiltration with AvrM were extracted using the RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. Alternatively, DNA-free RNA was extracted as described by Oñate-Sánchez and Vicente-Carbajosa (2008). RT-PCR analysis was carried out as described in Chapter 3.

4.2.3.5 Scoring of hypersensitive cell death phenotype

TRV-infected plants were agroinfiltrated to express AvrM, AvrM Δ SP or AvrM Δ 106-153 as described in Section 4.2.1.7. The infiltrated regions were subsequently scored 0, 1, 2 or 3 for cell death severity as shown in Figure 4-5 and the percentage of agroinfiltrated regions falling into each category was plotted.

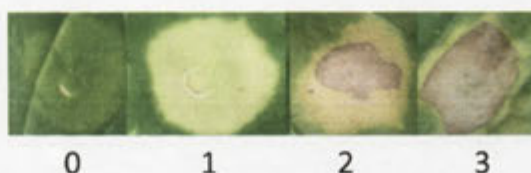


Figure 4-5 Appearance of infiltrated regions representative for the different scores used to classify cell death severities on *N. benthamiana* plants transgenic for the M resistance protein. Cell death severities were scored 0 for no cell death, 1 for yellowing, 2 for partial cell death and 3 for full cell death.

4.2.4 Dominant negative AP180

PCR using the Phusion polymerase with the primers *Clal*-AtAP180-336 F and *XbaI*-AtAP180 R was used to amplify the region encoding the C-terminus of the AtAP180 from genomic DNA of *Arabidopsis* Col-0 prepared according to the protocol described in Chapter 3. The full-length sequence of a homolog was not identified in *N. tabacum* genomic DNA. However, an *N. tabacum* EST encoding part of the C-terminal portion of a potential homolog was identified (AM82660) and amplified using the primers *Clal*-NtAP180-C-term F and *XbaI*-NtAP180 R. Similarly, the primers *Clal*-NtAP180-317 F and *XbaI*-NtAP180 R were used to amplify a homologous region of the predicted *N. tabacum* AP180 gene from genomic DNA of wildtype *N. tabacum* using Phusion polymerase as described in Section 4.2.1.1. PCR products were cloned using standard procedures into the pGREEN-derived in-house plasmid, pCBJ352, digested with *XbaI* and *Clal* for expression under the CaMV 35S promoter and nopaline synthase terminator. Plasmids were co-transformed with the pSOUP plasmid into *Agrobacterium* using the freeze-thaw protocol (Annamalai and Rao

2006). *Agrobacterium* strains carrying the plasmids were co-infiltrated with *Agrobacterium* strains carrying plasmids for expression of AvrM, AvrM Δ SP or AvrM Δ 106-153 fused to Cerulean as described in Section 4.2.1.7. For co-infiltrations, cultures carrying the pCBJ352-derived plasmids were prepared to O.D.₆₀₀ 1.5 and cultures carrying the Cerulean fusions of AvrM were diluted to O.D.₆₀₀ 0.2. After 4 h at room temperature, cultures were mixed 1:1 and infiltrated into plants with a 1 mL needleless syringe.

4.2.5 Image processing

Image processing was performed using Adobe Photoshop CS2 (Adobe Systems) and Adobe Illustrator CS2 (Adobe Systems).

4.3 Results

4.3.1 Development of assays of AvrM uptake

In order to experimentally study the mechanism of AvrM uptake, it was necessary to develop a method to assay the introduction of the protein into plant cells. This section describes tests of the following methods: (1) Incubation of purified protein from *E. coli* with epidermal leaf peels or protoplasts followed by assaying for uptake using fluorescence microscopy, (2) infiltration of purified protein from *E. coli* into leaves followed by assaying for uptake by observing HR, and (3) expression of secreted protein from plant cells via agroinfiltration followed by assessment of entry into plant cells by observing HR.

4.3.1.1 Incubation with epidermal peels and protoplasts

The uptake of proteins into cells may be followed by tracking the localisation of fluorescently-tagged proteins. This approach has previously been employed to study Avr1b uptake into soybean root cells and also the uptake of the ToxA toxin into wheat mesophyll cells (Dou et al. 2008; Manning and Ciuffetti 2005). Expression of AvrM Δ SP fused to Cerulean by *E. coli* did not yield highly soluble protein, as assessed by running crude lysates of induced *E. coli* cultures on a Coomassie gel (data not shown). However, a C-terminal fusion of Cerulean to residues 28-156 of AvrM (AvrM 156-Cerulean), which were shown to be sufficient for protein uptake *in planta* (Rafiqi et al. 2010), was found to be more soluble than even His- AvrM alone and was shown to retain the fluorescent properties of Cerulean (Figure 4-6).

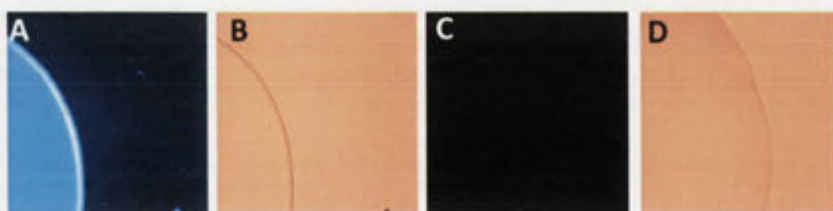
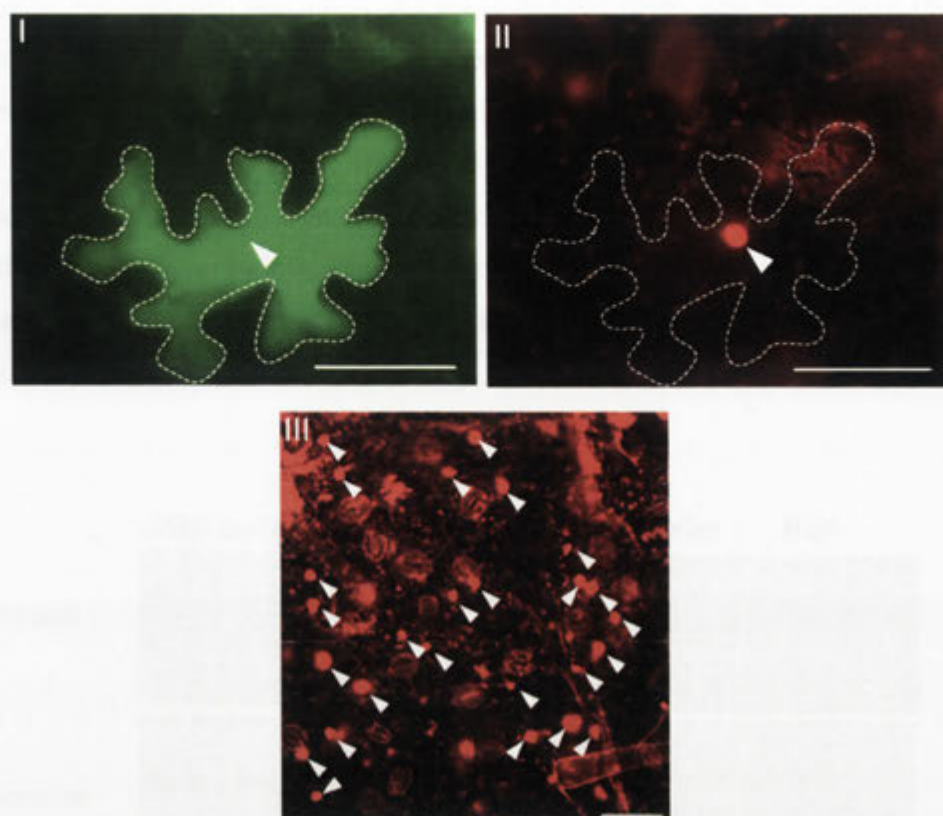


Figure 4-6 Fluorescence of a drop of AvrM 156-Cerulean diluted in distilled water (A) compared to water alone under UV light (C). B and D are DIC images of A and C respectively.

As for the assessment of the uptake of the elicitor cryptogein into tobacco BY2 cells (Leborgne-Castel et al. 2008) and the uptake of fungal toxin ToxA into mesophyll cells (Manning

and Ciuffetti 2005), AvrM 156-Cerulean protein was diluted in distilled water and its uptake was assayed. This involved assessing the uptake of the protein on epidermal leaf peels at concentrations of 1 mg/mL after incubation for 15 min. It was noticed that the space corresponding to the vacuole of many cells was completely filled with fluorescent signal possibly due to the hypotonic conditions used. Notably, a large proportion of cells were found to be permeable to propidium iodide even after only a 15 min incubation in protein solution, indicating the lack of membrane integrity characteristic of cell death, possibly as a consequence of cell damage during the peeling process (Figure 4-7). On the other hand, incubation of protoplasts with 1 mg/mL AvrM 156-Cerulean in CPW buffer or 0.5 M mannitol for 1 h, 3 h or even overnight did not result in higher levels of fluorescence compared to buffer only controls. Similar results were obtained from protoplasts incubated with GST-Cerulean (not shown).

A



B

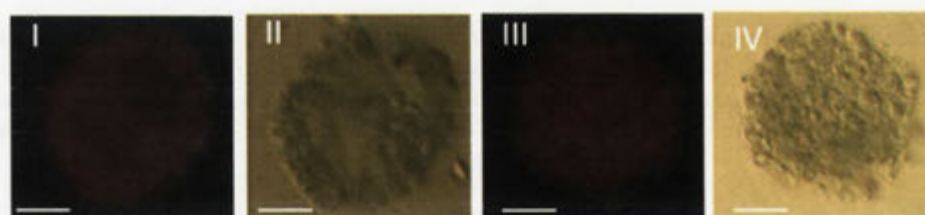


Figure 4-7 A. Incubation of *N. benthamiana* leaf epidermal peels with AvrM 156-Cerulean and propidium iodide. *N. benthamiana* leaf epidermal peel showing vacuolar localisation of AvrM 156-Cerulean (I; green) for 15 min. The same dead cell (dotted outline) with nuclear staining by propidium iodide is shown in panel II. Arrowheads indicate the location of the nucleus. The extent of cell death in the leaf is shown at low magnification in panel III. Nuclear staining of propidium iodide is indicated with arrowheads. Scale bars = 100 μ m. **B.** *N. benthamiana* mesophyll protoplasts incubated overnight with AvrM 156-Cerulean in 0.5 M mannitol (I) did not show more than background fluorescence as shown by cells incubated with buffer alone overnight at room temperature (III). The DIC images of I and III are shown in panels II and IV, respectively. Scale bars = 10 μ m.

4.3.1.2 Infiltration into host leaves

His-AvrM protein was infiltrated into leaves of tobacco and flax plants with and without the M-resistance protein with variable results. In initial experiments, infiltration of His-AvrM protein in PBS pH 7 into leaves of *N. tabacum* transgenic for the M resistance protein (M-tobacco) resulted in induction of cell death within 2 days on leaves of M-tobacco plants (Figure 4-8). HR was not induced by infiltration with the buffer alone or with BSA indicating that cell death is not caused by infiltration of the PBS buffer or by infiltration of proteins in general. In addition, HR was also not elicited in plants expressing the L6 resistance protein suggesting that this response was specifically mediated by the M resistance protein.

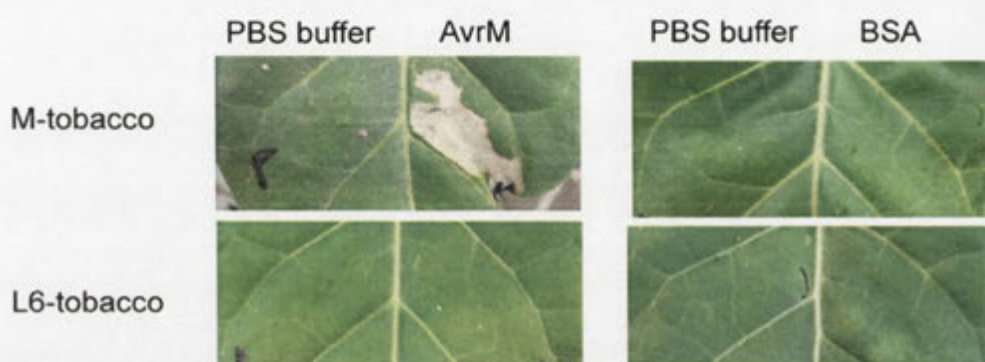


Figure 4-8 Infiltration of His- AvrM into tobacco transgenic for the M resistance protein (M-tobacco) or the L6 resistance protein (L6-tobacco). Individual leaf panels were infiltrated with 0.6 mg/mL AvrM in PBS buffer. As negative controls, panels were treated with PBS buffer alone or 0.6 mg/mL BSA. Leaves were photographed at 2 dpi.

This experiment was repeated in flax leaves, this time using MS media at an acidic pH (pH 5.8) rather than PBS at pH 7, as the infiltration buffer. AvrM Δ SP elicited cell death in flax plants with the M resistance protein at 11 dpi but not in plants lacking the resistance protein (Figure 4-9). Further, infiltration of flax leaves expressing the M resistance protein with Proteinase K-treated His-AvrM did not trigger cell death, confirming that the cell death response was caused by a proteinaceous agent in the infiltration solution.

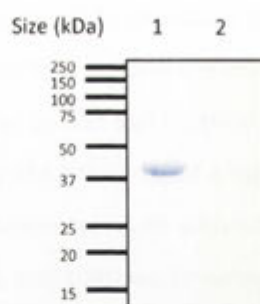
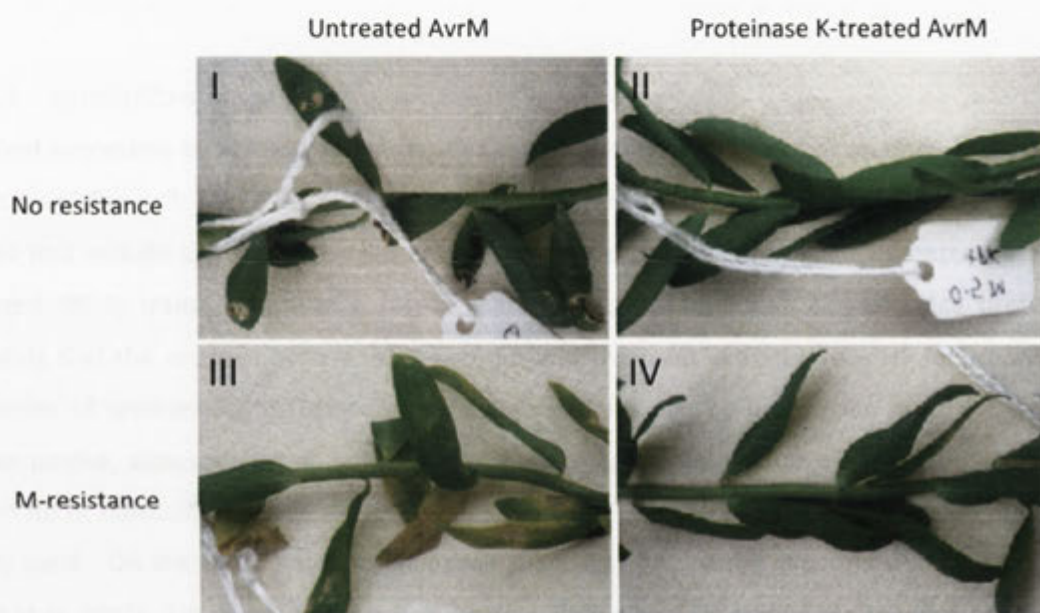
A**B**

Figure 4-9 Infiltration of Proteinase K-treated His-AvrM into flax plants. **A.** Coomassie gel of His-AvrM. Lane 1: Untreated His-AvrM and 2: His-AvrM incubated at 37°C for 4 h with Proteinase K. **B.** Untreated and Proteinase K-treated His-AvrM diluted in MS media at 0.5 mg/mL was infiltrated into flax plants lacking (I and II) or expressing (III and IV) the M resistance protein. Photographs were taken at 11 dpi.

However, despite the promising results obtained in these pre-liminary experiments, replicate experiments in flax and tobacco plants showed that the level of HR induced by infiltrated AvrM varied substantially between different batches of purified protein, with some samples not inducing cell death above that of the negative control, His-Ubiquitin-tagged AvrL567 lacking the signal peptide (His-Ubi-AvrL567)(data not shown). In addition, His-AvrM has low solubility and, therefore, low yields when expressed in *E. coli* (~0.5-0.8 mg/L induced culture, Chapter 2), which

limits the number of plants that can be treated from a single protein purification experiment. An approach to increase protein solubility in *E. coli* is to express a protein of interest as a fusion to another protein with higher solubility. Such tags include the maltose binding protein as well as GST (Sorensen and Mortensen 2005). Fusion to GST significantly increased the solubility of AvrM without the signal peptide as evidenced by the presence of a large band of the expected size in crude cell lysates of *E. coli* expressing GST-fusions of AvrM without the signal peptide (see Chapter 5) and increased the yield of protein from *E. coli* cultures. However, GST-tagged AvrM without the signal peptide failed to trigger cell death in flax plants even at 7.5 mg/mL (data not shown).

4.3.1.3 Agroinfiltration of AvrM

Transient expression by agroinfiltration has been used to assay the uptake of secreted AvrM into tobacco and flax cells (Rafiqi et al. 2010). This assay showed that expression of AvrM-Cerulean fusions that include the signal peptide nevertheless gave rise to intracellular fluorescence and triggered HR in transgenic tobacco plants expressing the cytoplasmic M resistance protein, indicating that the secreted protein was taken up into the plant cytosol. Thus HR induction by expression of secreted AvrM-Cerulean in M-tobacco plants can be used as an assay for AvrM protein uptake, allowing analysis of the role of host components by gene silencing. A silencing system for *N. tabacum* based on a satellite virus has been described (Gosselé et al. 2002), but not widely used. On the other hand, TRV-induced silencing in *N. benthamiana* is well established (Dong et al. 2007). Therefore, transgenic *N. benthamiana* plants expressing the M resistance gene were tested for recognition of transiently-expressed AvrM-Cerulean. As a positive control for HR signalling, a version of AvrM Δ SP was also infiltrated into leaves. In addition AvrM lacking the sequence required for uptake into host cells (AvrM Δ 106-153) was expressed as a negative control. Plants gave a consistent response to expression of the three constructs, with a strong HR in the region expressing AvrM Δ SP, a weaker HR in AvrM and an even weaker/ no reaction in AvrM Δ 106-153, although there was considerable variation as to when HR was detected, ranging from 5 to 19 dpi (Figure 4-10 A). However, nucleocytoplasmic fluorescence was detected in areas infiltrated with AvrM-Cerulean at 4 dpi indicating that the protein had been expressed and was localised into host cells by that time point (Figure 4-10 B).

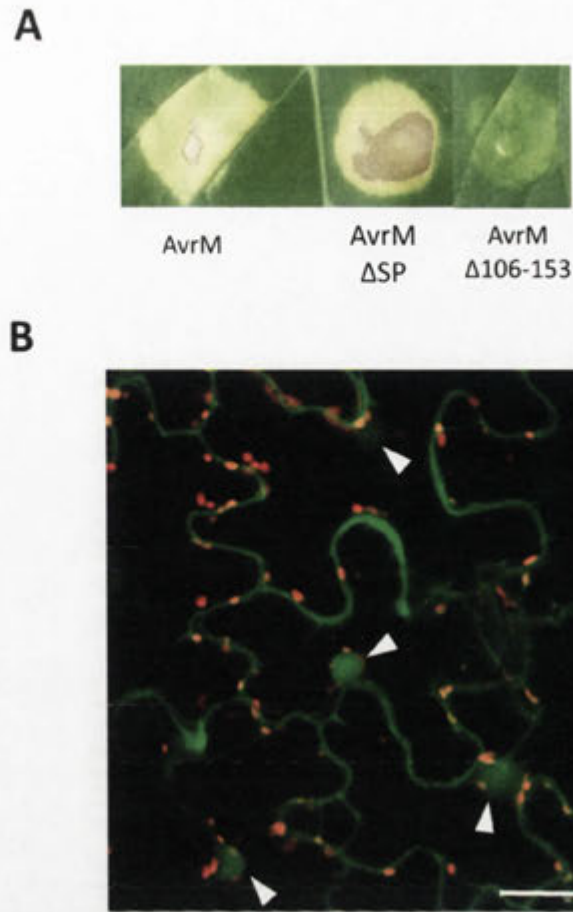


Figure 4-10 Internalisation of secreted AvrM-Cerulean in *N. benthamiana* leaf epidermal cells expressed by agroinfiltration. **A.** HR response to transient expression of AvrM in *N. benthamiana* plants transgenic for the M resistance protein. As controls, cytoplasmic AvrM lacking the signal peptide (AvrM Δ SP) and the AvrM Δ 106-153 deletion mutant with impaired uptake into plant cells were expressed. **B.** Confocal micrograph showing the nucleocytoplasmic localisation of AvrM (green) within wildtype *N. benthamiana* leaf epidermal cells at 4 dpi. Red: chloroplast autofluorescence; arrowheads: nuclei; scale bar = 50 μ m.

4.3.2 Inhibition of endocytosis components

The majority of studies reported in the literature in which endocytosis pathways are inhibited employ the following methods: (1) the use of chemical inhibitors, (2) silencing or knock down of specific components and (3) expression of dominant negative forms of endocytic components. In the research described in this chapter, the last two techniques were tested and their effect on AvrM-induced HR assessed.

4.3.2.1 *Satellite virus-induced gene silencing system in N. tabacum*

Since *N. tabacum* plants transgenic for the M resistance protein had been generated and the HR response of agroinfiltrated AvrM has been characterised (Rafiqi et al. 2010), silencing of endocytic components from *N. tabacum* was attempted with the satellite virus-induced silencing system (SVISS) which was developed specifically for *N. tabacum* (Gosselé et al. 2002). In this system, regions of the target gene of interest are cloned into the genome of the satellite tobacco mosaic virus (STMV). Chimeric transcripts are spread throughout the plant by a helper virus strain of the tobacco mosaic virus (TMV) and induce silencing as the virus replicates in the form of double-stranded RNA (Gosselé et al. 2002).

Two helper virus strains of TMV were tested: the U2 and the U2-like TMV viruses courtesy of Dr Paul Chu, CSIRO Plant Industry. Infection by U2 resulted in less severe leaf crinkling symptoms than the U2-like strain, and was therefore used for further experiments because subtle silencing phenotypes would be easier to distinguish. In order to test if silencing could be achieved, transcripts of chimeric STMV containing a fragment of the phytoene desaturase (PDS) gene, that was previously shown to be sufficient for PDS silencing, was used to infect *N. tabacum* plants (Gosselé et al. 2002). Despite the fact that SVISS is reported to achieve fully established gene-silencing for PDS within 21 days (Gosselé et al. 2002), no hint of the expected photobleaching phenotype was observed in plants even 30 days after inoculation (data not shown). Analysis of RNA extracts collected 19 days after inoculation showed that while TMV was present in inoculated and uninoculated leaves, indicating that TMV had spread systemically, STMV transcripts were restricted to inoculated leaves (Figure 4-11). The three bands detected in RNA of the inoculated leaf in Figure 4-11 most likely represent the detection of the full-length chimeric STMV-PDS virus genome, as well as transcripts encoding ORF1 and the STMV coat protein as these overlap in the region to which the probe was designed (Figure 4-3).

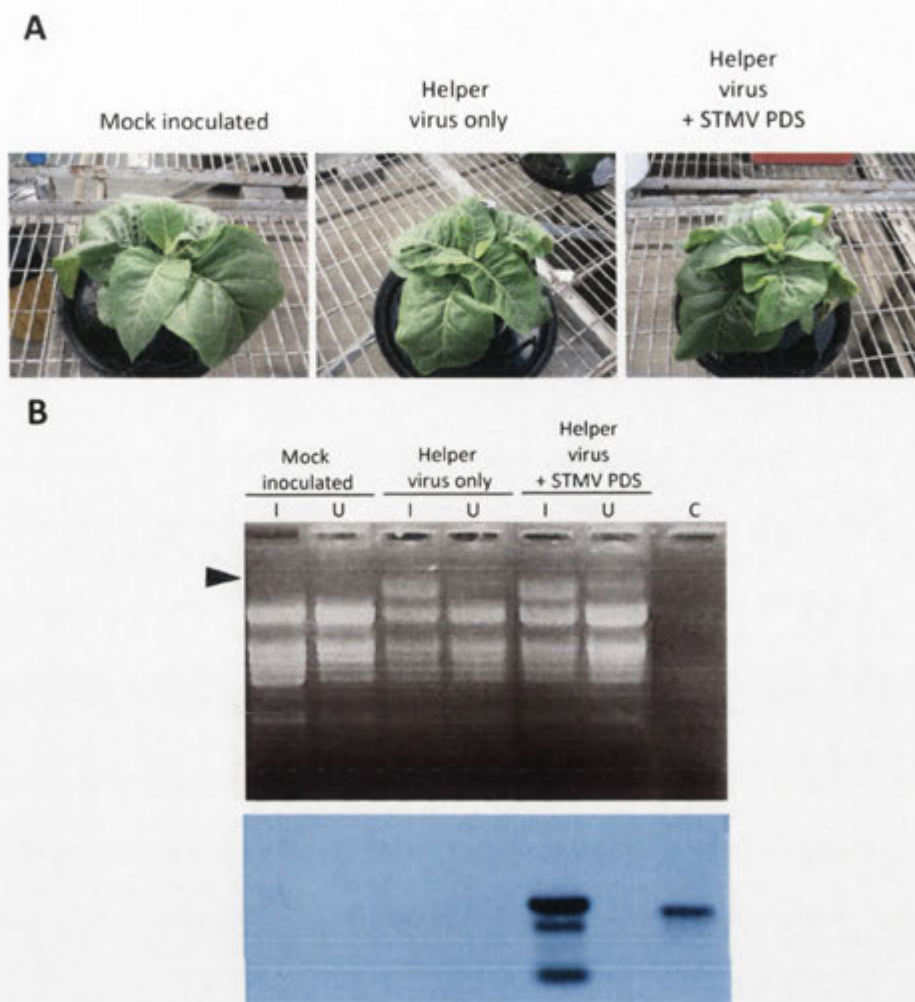


Figure 4-11 Infection of *N. tabacum* plants with STMV PDS chimeric virus and the U2 helper virus. **A.** Plants that were mock inoculated, inoculated with the helper virus only, or with the helper virus and the STMV PDS chimeric virus at 19 days post inoculation lack the photobleaching phenotype characteristic of PDS silencing. **B.** RNA gel (upper panel) of RNA extracted from plants that were mock inoculated, inoculated with the helper virus only, or with the helper virus and STMV at 19 days post inoculation. The arrowhead marks the size of the TMV helper virus. A northern blot (lower panel) with a probe specific for the STMV virus showed that the virus is present in an inoculated leaf (I) but not in an upper, uninoculated upper leaf (U). C: 3.4 ng of STMV PDS transcript used for plant inoculation.

4.3.2.2 Tobacco rattle virus (TRV) silencing

Given that SVISS did not work, TRV-mediated silencing was performed instead. However, TRV is more efficient at silencing genes in *N. benthamiana* than in *N. tabacum*. Thus, this experiment was conducted with *N. benthamiana* transgenic for the M resistance protein generated by Kim Newell (CSIRO, Plant Industry). TRV is a bipartite virus consisting of TRV-RNA1 and TRV-RNA2.

TRV-RNA1, encodes a viral replicase, a movement protein, a 16 kDa protein and a ribozyme, and TRV-RNA2, encodes a coat protein and a ribozyme (Caplan and Dinesh-Kumar 2006). As described by Dong et al. (2007), target gene sequences cloned into TRV-RNA2 were introduced into plants by *Agrobacterium*-mediated transformation along with TRV-RNA1. Silencing of the PDS gene resulted in photobleaching of upper, non-inoculated leaves as previously reported (Dong et al. 2007; Figure 4-12).

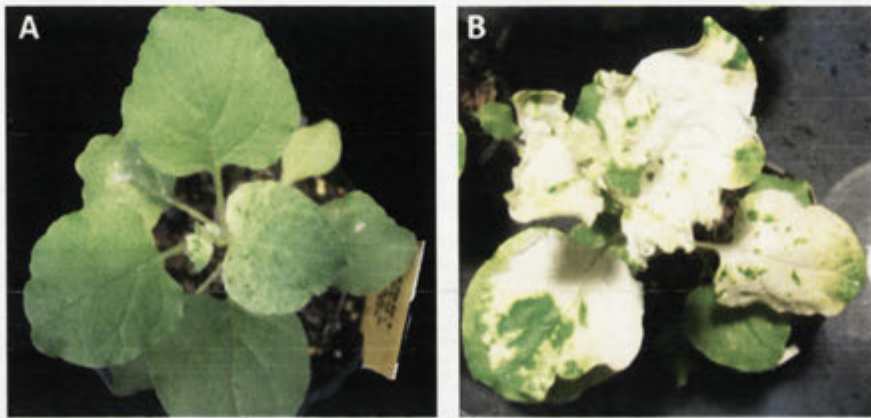


Figure 4-12 Silencing of the *PDS* gene results in photobleaching observable as early as 9 days after *Agrobacterium*-mediated transformation of *N. benthamiana* (A) and subsequently spreads systemically. The same plant is shown at 30 dpi (B).

In order to test if TRV silencing could knock down M-specific HR induced by AvrM, silencing of the *M* resistance gene was tested. Overall, silenced plants showed reduced HR in response to infiltration by AvrM and AvrM Δ SP, compared to plants inoculated with the empty vector control (Figure 4-13). The reduction in the HR response was observed for more than 19 days post agroinfiltration of plant cells to express AvrM and AvrM Δ SP.

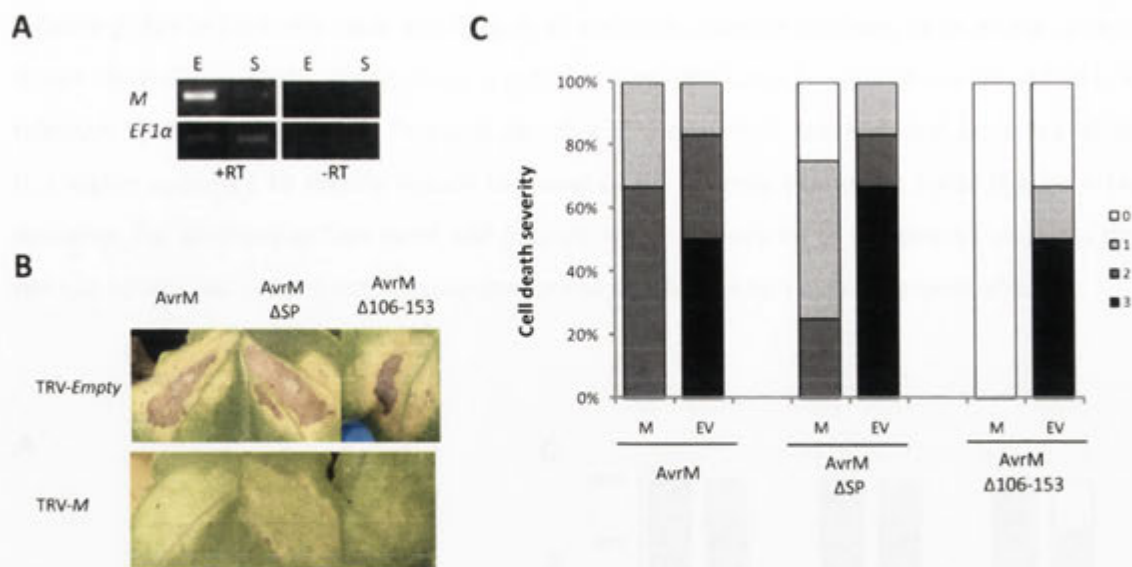


Figure 4-13 Silencing the *M* resistance gene in *N. benthamiana* transgenic for the *M* resistance protein. **A.** RT-PCR of *M* (35 cycles) showed that the *M* transcript was reduced in plants infected with the *M* silencing construct (S) compared to the empty vector controls (E). Amplification of *EF1α* (25 cycles) was used as a loading control. **B.** Representative photos of regions agroinfiltrated with AvrM, AvrM ΔSP or AvrM Δ106-153 were taken at 19 dpi. **C.** Percent of infiltrated regions exhibiting different severities of cell death 19 days after agroinfiltration with AvrM, AvrM ΔSP or AvrM Δ106-153. Two leaves were infiltrated on each of three independently silenced plants. Cell death was scored: 3 for full cell death, 2 for partial cell death, 1 for yellowing and 0 for no cell death. M: TRV-M and EV: TRV-empty vector. Each row shows infiltrated regions from the same leaf.

Once the system was established, the homologs of seven genes involved in various host uptake and trafficking pathways were selected as targets for silencing: α-adaptin, AP180, EHD1, EHD2, flotillin, Rac1 and CDC48.

4.3.2.2.1 Clathrin accessory proteins

As mentioned, α-adaptins are components of the heterotetrameric AP2 clathrin adaptor complex. In a previous study, two potential α-adaptin genes sharing 98.5% homology at the amino acid level (At5g22770 and At5g22780) were identified in Arabidopsis based on database searches (Barth and Holstein 2004). The coding regions for these two genes also showed significant homology (97% identical) to one another at the nucleotide level (Barth and Holstein 2004). In Arabidopsis, one of the two proteins, αC-adaptin has been shown through pull down assays to associate with clathrin,

indicating that this protein could also be part of a clathrin adaptor complex, as in animal systems (Barth and Holstein 2004). In this study, a putative α -adaptin coding sequence was identified in *N. tabacum* by BLAST (FS382929). Targeted silencing of a related *N. benthamiana* sequence within this region appeared to slightly reduce the level of HR in areas expressing AvrM (Figure 4-14). However, the observation that AvrM Δ SP-induced HR also appeared to be reduced indicates that not just uptake but either protein expression or cell death induction may have been affected.

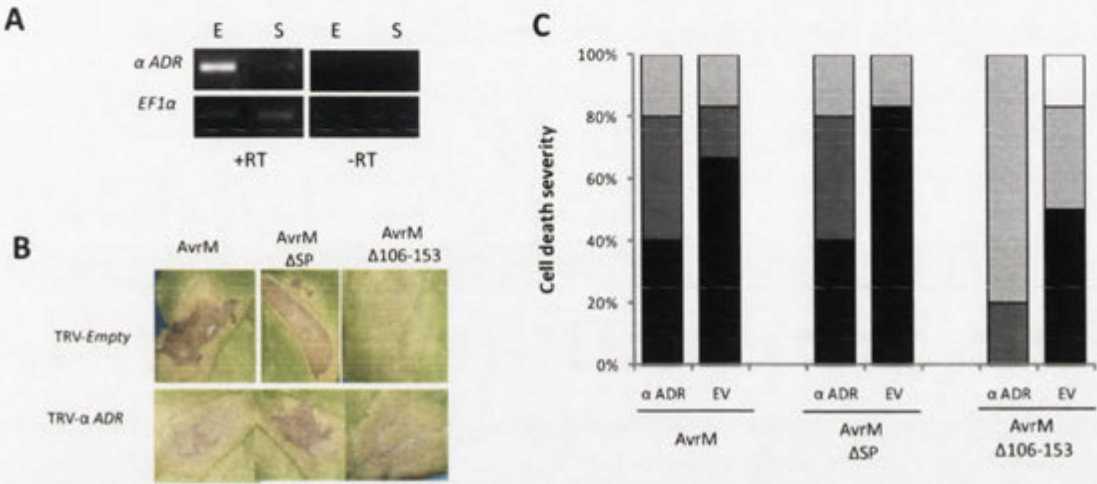


Figure 4-14 Silencing the α -adaptin (α -ADR) gene homolog in *N. benthamiana*. **A**. RT-PCR of α -ADR (33 cycles) showed that the α -ADR transcript was reduced in plants infected with the α -ADR silencing construct (S) compared to the empty vector controls (E). Amplification of *EF1 α* was used as a loading control. **B**. Representative photos of regions agroinfiltrated with constructs to express AvrM, AvrM Δ SP or AvrM Δ 106-153 were taken at 19 dpi. Each row shows infiltrated regions from the same leaf. **C**. Percent of infiltrated regions exhibiting different severities of cell death 19 days after agroinfiltration with AvrM, AvrM Δ SP or AvrM Δ 106-153. Two leaves were infiltrated on each of three independently silenced plants. Cell death was scored: 3 for full cell death, 2 for partial cell death, 1 for yellowing and 0 for no cell death. α -ADR: TRV- α -ADR and EV: TRV-empty vector. The results are representative of the trend from three independent experiments.

The monomeric clathrin adaptor protein, AP180, was also selected as a target for silencing. BLAST of tobacco genome survey sequences using the Arabidopsis homolog, AtAP180 (At1g05020), showed that only the N-terminal AP180 N-terminal homology (ANTH) domain was conserved in tobacco and a sequence within this region (ET929898) was used to design primers for amplification of the *N. benthamiana* homolog. Out of the eight ANTH-domain containing proteins in Arabidopsis, the translated *N. benthamiana* sequence identified showed highest homology to

AtAP180 (Figure 4-15A). Plants inoculated with the construct targeting the *AP180* gene amplified from *N. benthamiana* appeared to have a slightly higher level of HR in AvrM and AvrM Δ 106-153 infiltrated areas (Figure 4-15) but this effect was not observed in AvrM Δ SP infiltrated areas.

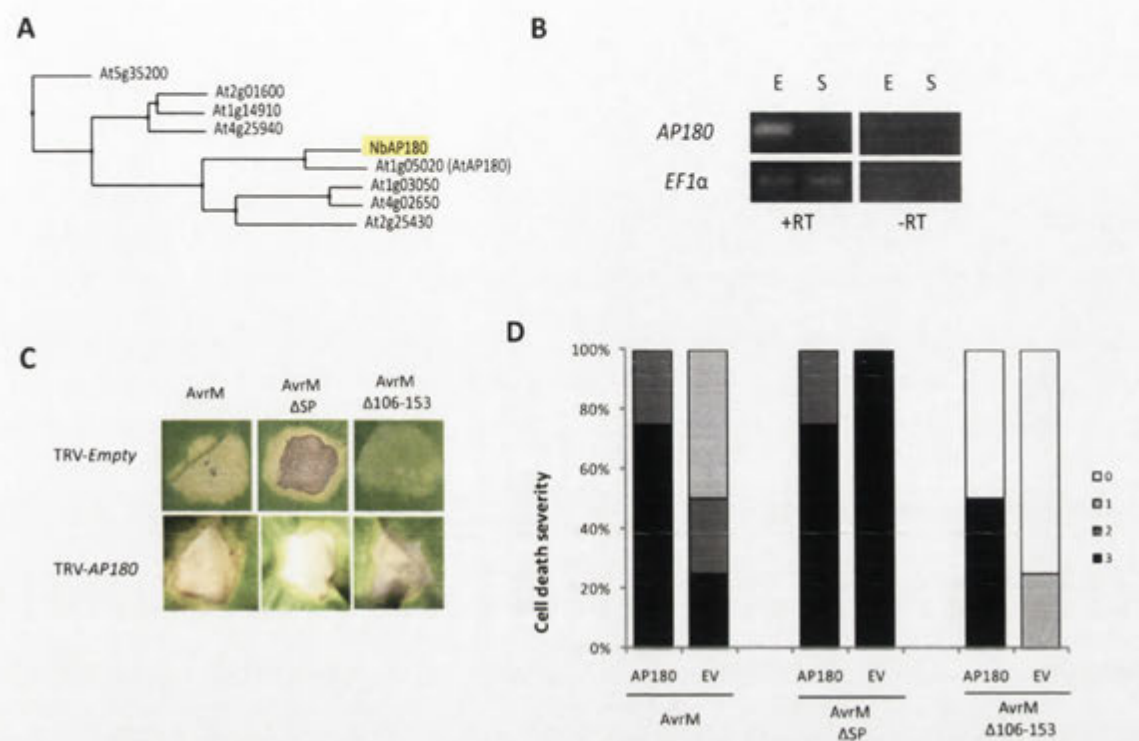


Figure 4-15 Silencing the *AP180* gene homolog in *N. benthamiana*. **A**. Neighbour-Joining tree of ANTH-domain containing sequences from Arabidopsis and the translated sequence used for silencing of the potential *N. benthamiana* *AP180* homolog. **B**. RT-PCR of *AP180* (40 cycles) showed that the *AP180* transcript was reduced in plants infected with the *AP180* silencing construct (S) compared to the empty vector controls (E). Amplification of *EF1α* was used as a loading control. **C**. Representative photos of regions agroinfiltrated with constructs to express AvrM, AvrM Δ SP or AvrM Δ 106-153 were taken at 19 dpi. Each row shows infiltrated regions from the same leaf. **D**. Percent of infiltrated regions exhibiting different severities of cell death 19 days after agroinfiltration with AvrM, AvrM Δ SP or AvrM Δ 106-153. Two leaves were infiltrated on each of three independently silenced plants. Cell death was scored: 3 for full cell death, 2 for partial cell death, 1 for yellowing and 0 for no cell death. AP180: TRV-AP180 and EV: TRV-empty vector. The results are representative of the trend from three independent experiments.

Other than the two potential clathrin adaptors, genes encoding homologs of the epsin homology domain (EHD) proteins, AtEHD1 and AtEHD2 were selected for analysis. Analysis of the protein sequences showed the presence of four clathrin-binding FS motifs on AtEHD1 and two clathrin-binding DLL motifs on AtEHD2 (data not shown) indicating potential clathrin association. An *N. benthamiana* sequence corresponding to the *N. tabacum* EHD1 homolog (FG164661) was

targeted for silencing. Further, the same sequence used for silencing of EHD2 in *N. benthamiana* by Bar and Avni (2009) was used to target EHD2 in this study (BP133939). Plants with either construct did not have an altered response to infiltration with AvrM, AvrM Δ SP and AvrM Δ 106-153 compared to empty vector controls (Figure 4-16 and Figure 4-17), suggesting that these proteins are not necessary for uptake or for HR signalling. It is noted however, that silencing was not as efficient for *EHD2* as *EHD1* despite the fact that efficient silencing had been demonstrated with the same target sequence (Bar and Avni 2009).

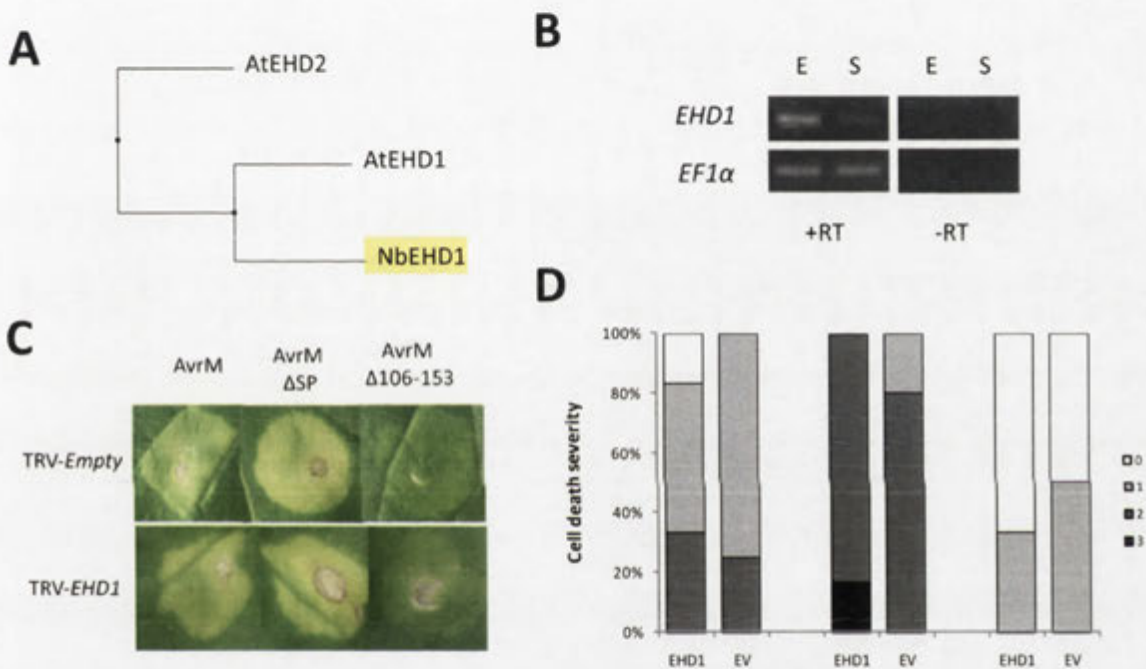


Figure 4-16 Silencing the *EHD1* gene homolog in *N. benthamiana*. **A**. Neighbour-Joining tree of EHD protein sequences from Arabidopsis and *N. benthamiana*. **B**. RT-PCR of *EHD1* (40 cycles) showed that the *EHD1* transcript was reduced in plants infected with the *EHD1* silencing construct (S) compared to the empty vector controls (E). Amplification of *EF1α* was used as a loading control. **C**. Representative photos of regions agroinfiltrated with constructs to express AvrM, AvrM Δ SP or AvrM Δ 106-153 were taken at 11 dpi. Each row shows infiltrated regions from the same leaf. **D**. Percent of infiltrated regions exhibiting different severities of cell death 11 days after agroinfiltration with AvrM, AvrM Δ SP or AvrM Δ 106-153. Two leaves were infiltrated on each of three independently silenced plants. Cell death was scored: 3 for full cell death, 2 for partial cell death, 1 for yellowing and 0 for no cell death. EHD1: TRV-EHD1 and EV: TRV-empty vector. The results are representative of the trend from three independent experiments.

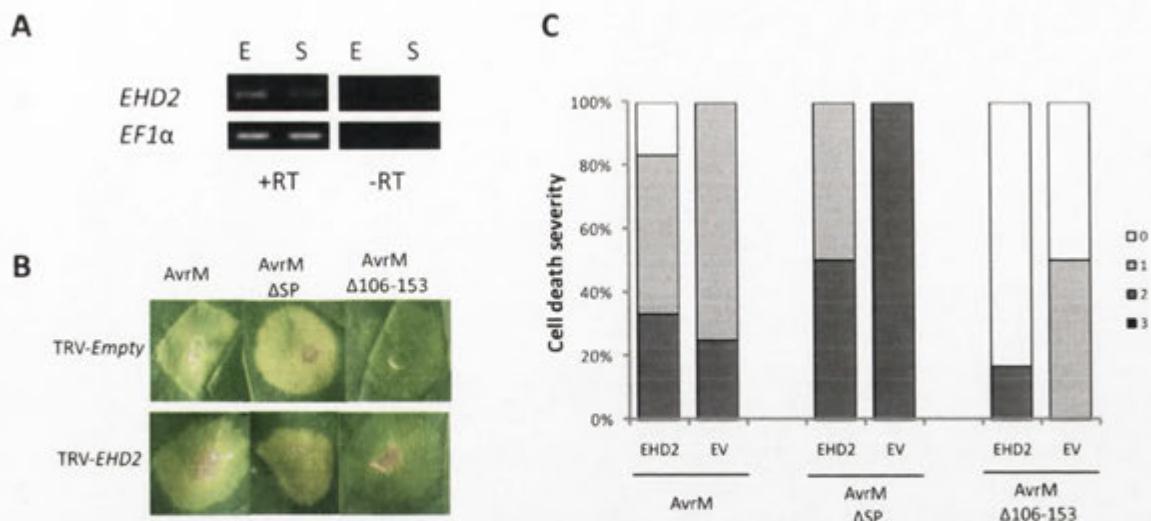


Figure 4-17 Silencing the *EHD2* gene homolog in *N. benthamiana*. **A**. RT-PCR of *EHD2* (33 cycles) showed that the *EHD2* transcript was reduced in plants infected with the *EHD2* silencing construct (S) compared to the empty vector controls (E). Amplification of *EF1α* was used as a loading control. **B**. Representative photos of regions agroinfiltrated with constructs to express AvrM, AvrM ΔSP or AvrM Δ106-153 were taken at 11 dpi. Each row shows infiltrated regions from the same leaf. **C**. Percent of infiltrated regions exhibiting different severities of cell death 11 days after agroinfiltration with AvrM, AvrM ΔSP or AvrM Δ106-153. Two leaves were infiltrated on each of three independently silenced plants. Cell death was scored: 3 for full cell death, 2 for partial cell death, 1 for yellowing and 0 for no cell death. EHD2: TRV-EHD2 and EV: TRV-empty vector. The results are representative of the trend from three independent experiments.

4.3.2.2.2 Flotillin

The structural component of flotillin-dependent endocytosis, flotillin, was selected as a target for silencing. Three potential flotillin-encoding genes in the Arabidopsis genome; At5g25250 (AtFlot1), At5g25260 and At5g64870 were identified by BLAST using AtFlot1 as a query sequence. Similarly, using the amino acid sequence of AtFlot1 as a query, three sequences were identified in the tomato genome, indicating that these genes are also conserved in solanaceous plants. An *N. tabacum* genome survey sequence fragment containing the partial 5' end of a flotillin-encoding sequence, which is conserved among all three tomato homologs (FH671323), was identified by BLAST. This was used to amplify a construct to target *N. benthamiana* flotillin. Neighbour-Joining analysis showed that this sequence did not cluster with the Arabidopsis flotillins (Appendix). However, this trait was also observed with *Medicago truncatula* flotillins possibly reflecting the diversification of flotillins following speciation. Silencing of this sequence did not significantly affect HR induced by AvrM (Figure 4-18).

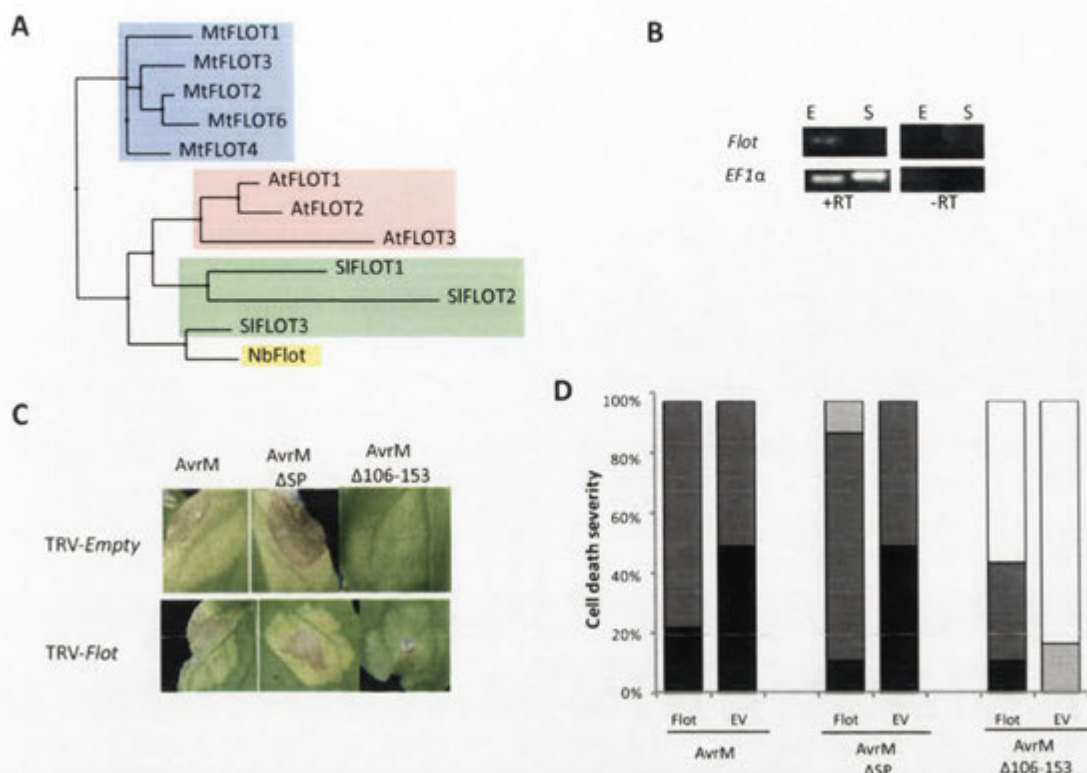


Figure 4-18 Silencing of flotillin in *N. benthamiana*. **A**. Neighbour-Joining tree of the NbFlot sequence as well as flotillins from tomato, *Arabidopsis* and *Medicago truncatula* (Haney and Long 2010) showing the clustering of the proteins according to species. Blue box: *M. truncatula* sequences; red box: *Arabidopsis* sequences. **B**. RT-PCR of *Flot* (33 cycles) showed that the *Flot* transcript was reduced in plants infected with the *Flot* silencing construct (S) compared to the empty vector controls (E). Amplification of *EF1α* was used as a loading control. **C**. Representative photos of regions agroinfiltrated with constructs to express AvrM, AvrM ΔSP or AvrM Δ106-153 were taken at 19 dpi. Each row shows infiltrated regions from the same leaf. **D**. Percent of infiltrated regions exhibiting different severities of cell death 19 days after agroinfiltration with AvrM, AvrM ΔSP or AvrM Δ106-153. Three leaves were infiltrated on each of three independently silenced plants. Cell death was scored: 3 for full cell death, 2 for partial cell death, 1 for yellowing and 0 for no cell death. Flot: TRV-Flot and EV: TRV-empty vector. The results are representative of the trend from three independent experiments.

4.3.2.2.3 Rac GTPase

Rac1 is a member of the Rho family of G-proteins and is associated with a number of uptake pathways that require actin such as the IL2Rβ pathway, phagocytosis and macropinocytosis (Doherty and McMahon 2009). Experiments have shown that like human Rac1, the *N. tabacum* NtRac1 gene may be involved in signalling to regulate actin binding and depolymerisation (Chen et

al. 2003) making it a potential functional homolog of human Rac1 although its direct effect on membrane trafficking has not been analysed. Neighbour-Joining analysis with the ROP family from rice showed that NtRac1 clusters more closely with OsRac5 and OsRac6 (Figure 4-19 A) compared to the other ROPs that have been identified in rice. Further, like OsRac5, OsRac6 and OsRac7, NtRac1 has a conserved plasma membrane binding motif (the CaaL motif) at its C-terminal end (Appendix). Using the sequence of NtRac1, primers were designed and used to amplify a homologous sequence from *N. benthamiana*. Interestingly, plants with the NtRac1 homolog silenced in *N. benthamiana* showed a more rapid HR response in AvrM-infiltrated areas compared to empty vector controls elicited by AvrM and AvrM Δ SP (Figure 4-19). In fact, at later time points it was noticed that HR was also observed in AvrM Δ 106-153 infiltrated areas (data not shown). This response was M-specific since wildtype plants infected with the silencing construct did not demonstrate increased cell death.

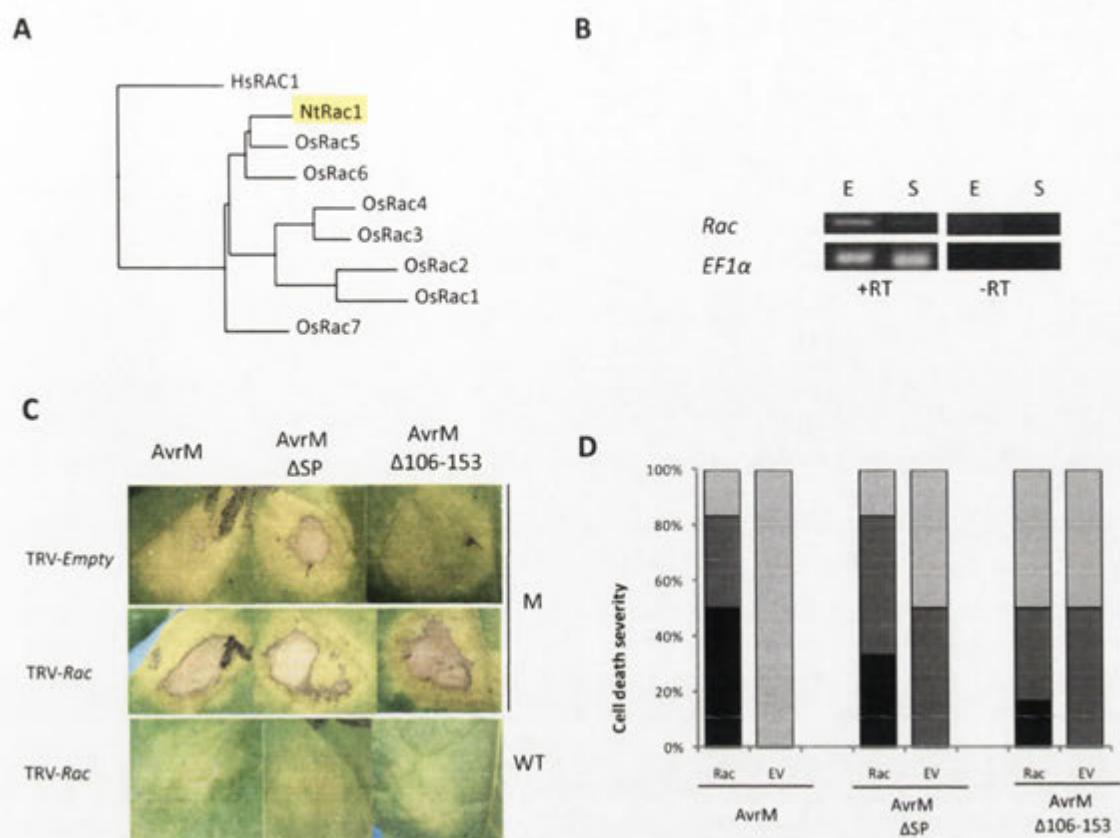


Figure 4-19 Silencing the *NtRac1* gene homolog in *N. benthamiana*. **A**. Neighbour-Joining tree of the *NtRac1* sequence as well as *Rac* proteins from rice (*OsRac1-7*; Chen et al. 2010). The human *Rac1* protein (*HsRac1*) was also included. **B**. RT-PCR of *Rac* (38 cycles) showed that the *Rac* transcript was reduced in plants infected with the *Rac* silencing construct (S) compared to the empty vector controls (E). Amplification of *EF1α* was used as a loading control. **C**. Representative photos of regions agroinfiltrated with constructs to express AvrM, AvrM ΔSP or AvrM Δ106-153 were taken at 7 dpi. Each row shows infiltrated regions from the same leaf. **D**. Percent of infiltrated regions exhibiting different severities of cell death 11 days after agroinfiltration with AvrM, AvrM ΔSP or AvrM Δ106-153. Two leaves were infiltrated on each of three independently silenced plants. Cell death was scored: 3 for full cell death, 2 for partial cell death, 1 for yellowing and 0 for no cell death. Rac: TRV-Rac and EV: TRV-empty vector. The results are representative of the trend from three independent experiments.

4.3.2.2.4 CDC48

Three CDC48 proteins - CDC48-A, CDC48-B and CDC48-C - have been identified in Arabidopsis, one of which (CDC48-A) has been shown to be involved in the retrotranslocation of toxins such as ricin from the ER to the cytosol (Marshall et al. 2008). The homolog of CDC48-A in *Nicotiana glutinosa* is a CDC48 ER-localised protein (Figure 4-20 A) that has previously been partially characterised and found to be important for growth (Bae et al. 2009). BLAST of the *N. benthamiana* EST database

using the *N. glutinosa* *CDC48* gene as a query sequence was used to identify a homolog in *N. benthamiana*. As previously reported for *N. glutinosa*, *NbCDC48*-silenced *N. benthamiana* plants (Bae et al. 2009) developed photobleached leaves (Figure 4-20 B and C) and stunted growth. *CDC48*-silenced plants also showed a more rapid cell death response (Figure 4-20 D) in all agroinfiltrated areas. However, the response was not M-specific since *N. benthamiana* plants lacking the M resistance protein also showed rapid cell death demonstrated by browning or blackening of infiltrated regions and reflected by the lack of *EF1α* housekeeping gene transcripts (Figure 4-20 B). Interestingly, cell death occurred in black rather than brown lesions characteristic of HR (Figure 4-20 C).

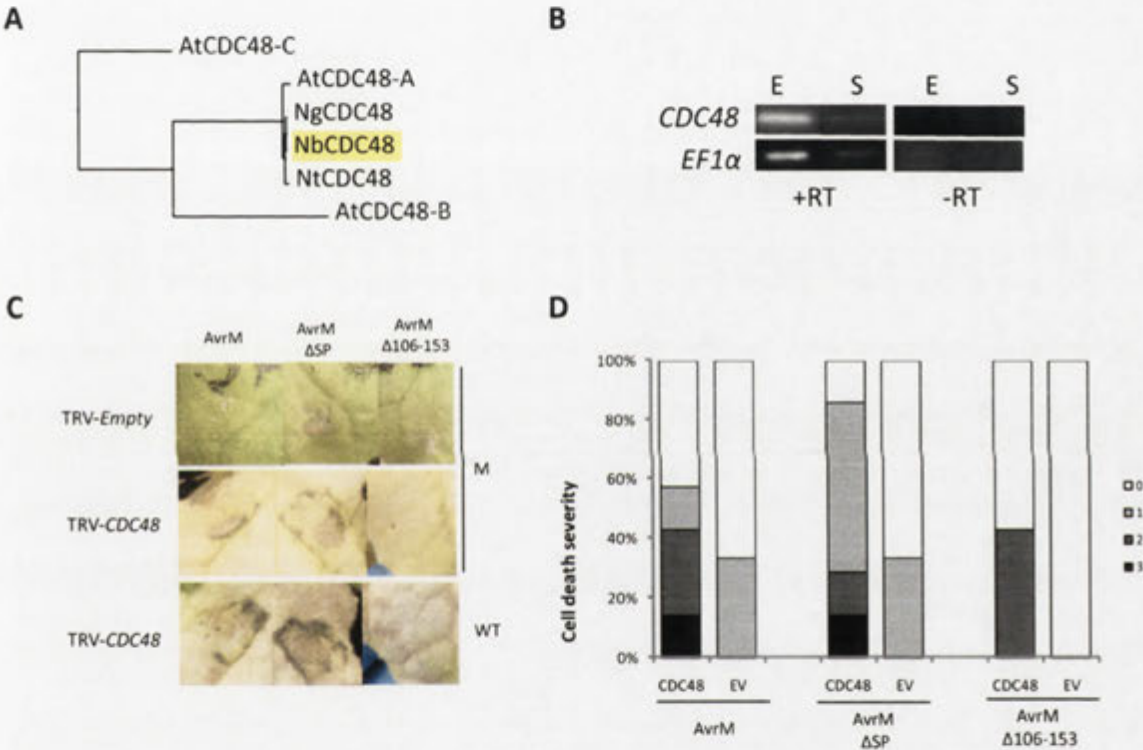


Figure 4-20 Silencing the *CDC48* gene homolog in *N. benthamiana*. **A**. Neighbour-Joining tree of *CDC48* sequences from *Arabidopsis*, *N. benthamiana*, *N. tabacum* and *N. glutinosa*. **B**. RT-PCR of *CDC48* (30 cycles) showed that the *CDC48* transcript was reduced in plants infected with the *CDC48* silencing construct (S) compared to the empty vector controls (E). Amplification of *EF1α* was used as a loading control. **C**. Representative photos of regions agroinfiltrated with constructs to express *AvrM*, *AvrM ΔSP* or *AvrM Δ106-153* were taken at 5 dpi. Each row shows infiltrated regions from the same leaf. **D**. Percent of infiltrated regions exhibiting different severities of cell death 11 days after agroinfiltration with *AvrM*, *AvrM ΔSP* or *AvrM Δ106-153*. Two leaves were infiltrated on each of three independently silenced plants. Cell death was scored: 3 for full cell death, 2 for partial cell death, 1 for yellowing and 0 for no cell death. *CDC48*: TRV-*CDC48* and EV: TRV-empty vector. The results are representative of the trend from three independent experiments.

4.3.3 Inhibition of AP180 by dominant negative AP180

Another means of inhibiting uptake is by over-expressing dominant negative forms of endocytic components. The expression of dominant negative endocytosis components has the added advantage in that it can potentially also be applied to flax plants during infection, since transient expression of proteins in flax plants can be achieved by agroinfiltration. In this section, the effect of co-expressing dominant negative AP180 and AvrM on M-mediated HR is analysed.

AP180 genes consist of an N-terminal lipid binding motif (the ANTH domain) and a C-terminal protein association domain that associates with clathrin and other accessory factors through motifs that are common among clathrin accessory proteins. Examples of these are the DLL and FS motifs, for binding to clathrin, DPF for interaction with α -adaptin, NPF for interaction with epsin-homology (EH) domains and DPW for interaction with ubiquitin (Holstein and Oliviusson 2005). Over-expression of the AP180 C-terminal region lacking the lipid binding domain has a dominant negative effect on clathrin-mediated endocytosis in mammalian cells, presumably through sequestration of available clathrin binding sites (Ford et al. 2001).

BLAST of the tomato genome using the AtAP180 identified three similar sequences above the threshold expect score ($E = 1e-50$). All three sequences showed higher homology at the N-terminus compared to the C-terminus of the protein and contained the ANTH signature lipid-binding motif [KAT(X)₅/P(X)₃K/RH/Y]. In fact, BLAST of the tomato genome with the C-terminus of AtAP180 alone did not result in any reported hits ($E = 1e-10$). The top hit, found on chromosome 12 of *Solanum lycopersicum* (nt 109825-10744 of AC209585) was intronless, similar to AtAP180, and was designated SIAP180-1.

AtAP180 possesses one each of the DLL, DPF, DPW, NPF and FS motifs (Holstein and Oliviusson 2005). In contrast, SIAP180-1 possesses an NPF motif, a DPF motif and five FS clathrin binding motifs while lacking DLL and DPW motifs (Figure 4-21). The presence of the DPF motif indicates that SIAP180-1 may associate with α -adaptin, as for AtAP180. Interestingly, loss of the DLL motif in AtAP180 reduces clathrin cage assembly activity, although this was not observed for mammalian AP180 (Barth and Holstein 2004; Morgan et al. 2000). The second closest AtAP180 homolog in tomato (SIAP180-2; nt 63245 – 64597 of AC232643) also lacks a DLL motif but possesses only a single FS motif along with a NPF and a DPF motif. No full-length gene sequences were found using BLAST against the tobacco genome survey and EST sequences using SIAP180-1 as a query sequence. However, a fragment encoding a region homologous to the C-terminus

excluding two of the FS domains was identified (AM82660) and cloned (NtAP180-C(EST)). By analysing matching regions in *N. tabacum* genome survey sequences, primers were designed to amplify a greater portion of the C-terminus. Interestingly, this sequence (NtAP180-C) was not identical to the NtAP180-C(EST) sequence. There were several polymorphisms including an additional FS clathrin-binding motif in the region of overlap (Figure 4-21; Appendix), indicating that the two are potential homologs.



Figure 4-21 Diagrammatic representation of protein domain organisation of AP180 homologs. Drawing not done to scale.

In contrast to the results from the silencing of putative AP180, expression of C-terminal NtAP180 in tobacco resulted in the delay of HR in panels that were co-infiltrated with AvrM-Cerulean (Figure 4-22). While this could reflect some uptake of AvrM into cells before full inhibition of clathrin-mediated endocytosis is achieved, the overall level of Cerulean fluorescence in areas co-infiltrated with NtAP180 C-terminal fragment (NtAP180-C (EST)) was lower compared to the empty vector controls suggesting that AvrM-Cerulean protein expression levels may also be reduced (Figure 4-22). Similar results were demonstrated with NtAP180-C (data not shown). The fact that AvrM Δ SP-mediated HR is also delayed in panels co-infiltrated with NtAP180 C-terminal sequences also agrees with the latter scenario. However, the assessment of Cerulean fluorescence under the microscope was only performed once and more repetitions are required to confirm these preliminary results.

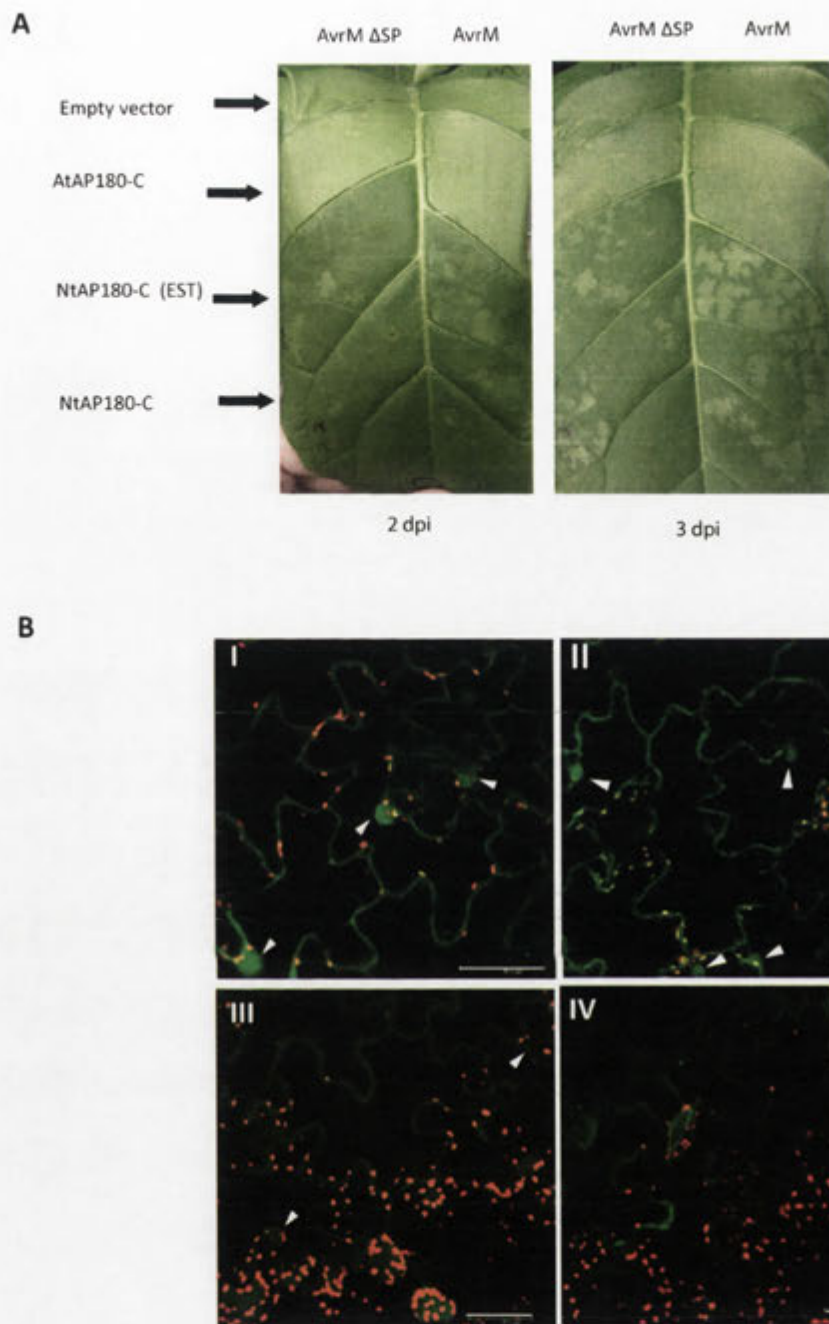


Figure 4-22 Co-expression of NtAP180 with AvrM and AvrM Δ SP in tobacco. **A.** Fusions of Cerulean to AvrM Δ SP (left panels) and AvrM (right panels) were co-expressed with Empty vector, AtAP180-C, NtAP180-C (EST) and NtAP180-C at 2 dpi and 3 dpi. **B.** Localisation of AvrM-Cerulean fusions at 3 dpi in panels co-infiltrated with the empty vector (I), AtAP180-C (II), or NtAP180-C (EST) (III). The level of Cerulean fluorescence was lowest in panel (III) where AvrM-Cerulean has been co-infiltrated with NtAP180-C (EST). The control, AvrM Δ SP co-infiltrated with NtAP180-C (IV) also showed lower levels of Cerulean fluorescence compared to panels I and II. Image exposure and processing are the same between the different panels. Arrowheads: nuclei, scale bar = 50 μ m.

4.4 Discussion

In this chapter, various methods to assay host cell internalisation of the effector AvrM were tested. Having developed a suitable assay system, components of host endocytic pathways were inhibited, with the aim of identifying potential pathways that might be required for AvrM uptake.

4.4.1 Assay for uptake of AvrM

Three methods for assaying AvrM uptake were tested. First, plant cells or tissue were incubated with a purified, fluorescent protein fusion, which was assessed for uptake directly by fluorescence microscopy. In addition, since there is a good correlation between entry of AvrM and the induction of HR (Rafiqi et al. 2010), HR was assayed as a measure of protein uptake for the other two methods tested. One of these methods was infiltration of purified AvrM protein into leaves containing the M resistance protein followed by assessment of HR. Finally, uptake was also assayed by expression of secreted AvrM *in planta* by Agrobacterium-mediated transformation followed by assessment of HR.

The main advantage of assessing protein uptake by directly observing the internalisation of fluorescent protein fusions into cultured cells or epidermal peels is that internalisation can be studied using chemical inhibitors of endocytosis since the external environment of target cells can be well controlled. This is an important consideration since some chemical inhibitors can affect cell viability at certain concentrations upon extended incubation times (Vercauteren et al. 2010). This method was used to measure the uptake of the RXLR effector Avr1b-GFP fusion into soybean root tips and, more recently, has been also applied to analyse the uptake of Avr1b-GFP and AvrL567-GFP into soybean root suspension cultures and mammalian cells (Dou et al. 2008; Kale et al. 2010). However, it has been shown previously that root cells take up proteins, including GFP even at low concentrations and over a short incubation time, into the cytoplasm as a nitrogen source for growth (Paungfoo-Lonhienne et al. 2008), raising doubts about the suitability of this assay system for effector delivery. Further, different cell types differ in terms of the relative proportions that various uptake mechanisms contribute to membrane turnover and caution should be employed in generalising results from one cell type to another (Doherty and McMahon 2009). Since the uptake of AvrM 156-Cerulean has been demonstrated to occur in agroinfiltrated tobacco leaf epidermal cells, the localisation of exogenously supplied AvrM156-Cerulean into epidermal peels, which lack a waxy cuticle to prevent protein contact with cells, was tested.

However, extensive cell death was observed on epidermal peels indicating that the peeling treatment itself may be damaging cells and compromising membrane integrity.

It is known that for the flax rust fungus, mesophyll cells are the main site of infection (Lawrence et al. 2007). While it is difficult to control conditions within the leaf, mesophyll protoplasts are readily prepared. The use of protoplasts and fluorescent-tagged proteins also lends itself to assays using fluorescence sorting methods such as fluorescence activated cell sorting (FACS), which would allow statistical analysis on uptake in a population of cells. Further, protoplasts are relatively easy to fix for immunolocalisation analyses since fixatives can achieve contact with the cells rapidly. Thus, mesophyll protoplasts were prepared and tested for uptake. However, even after extended incubations with AvrM-Cerulean protein, fluorescence levels were no higher compared to buffer only controls and GST-Cerulean controls. One possible explanation is that non-specific degradation of cell surface components occurred during digestion of cell wall material and that these components are required for uptake. Another possibility is that uptake mechanisms into protoplasts in general may be impaired. Indeed, it has been reported that the general marker of endocytosis, FM4-64, was not internalised into viable *Arabidopsis* mesophyll protoplasts even after an overnight incubation at room temperature (Aniento and Robinson 2005). In addition, inhibition of uptake may be caused by the hypertonic environment in which protoplasts are maintained, which is known to inhibit the interaction of clathrin with its adaptor proteins in mammalian cells (Oksche et al. 2000). The endocytosis of GABA receptors, which are internalised via a clathrin-independent pathway was also shown to be inhibited when incubated with high concentrations of sucrose (Cinar and Barnes 2001). However, uptake via other pathways does occur in protoplasts under hypertonic conditions. In fact, uptake of the cell penetrating peptides transportan, penetratin and pVEC was reported to occur in *N. tabacum* cv. SR-1 and triticale mesophyll protoplasts in a concentration dependent, non-saturable process that was not inhibited by incubation at 4°C or treatment with chemical inhibitors such as the general endocytic inhibitors sodium azide and nocodazole, and the macropinocytosis inhibitors, cytochalasin D or EIPA (Mäe et al. 2005; Chugh and Eudes 2007; Chugh and Eudes 2008). Given the results of this study, it appears that AvrM 156-Cerulean does not share the uptake properties of these proteins although a direct comparison to show that these cell penetrating peptides are able to internalise under the same conditions and that cell viability is not compromised, is needed to confirm this.

The infiltration of effector proteins into leaves has been employed to analyse the uptake of the RXLR oomycete effector Avr1b (Shan et al. 2004). In that study, crude extracts of proteins expressed in yeast were infiltrated into host plants and shown to induce HR in plants with the corresponding resistance protein (Shan et al. 2004). A more recent study employed bacterially expressed and purified Avr1b protein and infiltration into leaves (Kale et al. 2010). In the experiments described in this chapter, His-tagged AvrM lacking the signal peptide purified from *E. coli* was infiltrated directly into the intercellular space, to mimic the secretion of mature AvrM into the apoplast by the fungus. It was shown that this was able to induce HR in tobacco and flax plants in some experiments. Cell death was not elicited by infiltration of buffer alone or by non-specific proteins (BSA). Further, the HR-triggering agent was destroyed by incubation with Proteinase K, indicating that it is a protein. In addition, cell death was specific for plants expressing the M resistance protein. However, the HR response observed was weak and variable, in some cases not eliciting cell death above the level of the negative controls. Further, the yield of soluble AvrM Δ SP was low when expressed in *E. coli* and several independent batches of purified protein were used, limiting sample sizes and possibly contributing to the experimental variability. A GST-AvrM Δ SP fusion protein resulted in higher protein yields, but infiltration of this protein into flax leaves expressing the M resistance protein did not elicit the HR response. It is possible that the uptake motif is required at the N-terminus of the protein to direct efficient uptake. This hypothesis could be tested using the *Agrobacterium* transient expression assay, by expression of an N-terminal fusion of GFP to AvrM. One other possibility is that the GST fusion may interfere with the avirulence function of AvrM. Indeed, it has previously been reported that some GST proteins are able to protect against oxidative stress-induced cell death (Yin et al. 2000). The GST vector generated in this study, pET41.2, was designed to have an enterokinase cleavage site (DDDK) just before the start codon of the gene of interest. Thus, future experiments could trial expressing large quantities of GST fused to mature AvrM, digesting the GST tag and then infiltration of the protein, lacking a tag, into leaves.

The third AvrM uptake assay tested was expression of secreted forms of AvrM by *Agrobacterium*-mediated transformation. As mentioned, this method was successfully employed to analyse uptake of AvrM mutants in tobacco and flax plants and both full-length AvrM and AvrM lacking the signal peptide can trigger equivalent levels of cell death within 24 h (Rafiqi et al. 2010). In this chapter, the uptake of agroinfiltrated AvrM into *N. benthamiana* plants expressing the M

resistance protein was tested. Interestingly, AvrM-induced cell death in *N. benthamiana* was delayed relative to tobacco or flax plants and stronger in areas expressing the non-secreted form of AvrM compared to the secreted form of AvrM (Catanzariti et al. 2006; Rafiqi et al. 2010). One possible explanation for this is that M resistance protein signalling is weaker in *N. benthamiana* compared to tobacco and flax plants, and that the different reactions may reflect differences in host signalling requirements.

4.4.2 Satellite-virus induced silencing system (SVISS)

The satellite-virus induced silencing system (SVISS) is a bipartite viral-based silencing system developed by Gossele et al. (2002) which utilises STMV as a vector to introduce target genes for silencing into host plants. Unfortunately, in the experiments described here, inoculation of a chimeric PDS-STMV virus onto tobacco plants did not result in silencing of the PDS gene. Indeed, northern blot analysis showed that chimeric STMV transcripts were not being spread systemically and did not accumulate to as high a level compared to those observed by Gossele et al. (2002).

The natural helper virus strain of STMV is the TMV-U2 virus, also known as the tobacco mild green mosaic virus (Dodds 1998). However, mutations to the 5' end of the STMV genome were found to change the helper virus specificity of the satellite virus (Kurath et al. 1993), presumably due to its reliance on components supplied externally by the helper virus to allow systemic satellite virus replication and movement (Dodds 1998). Given that the TMV virus experiences an average mutation rate of 10^{-5} per base pair with a high rate of multiple mutations (Malpica et al. 2002) and that there is no selective pressure on TMV to retain compatibility to the STMV virus, it is possible that the TMV-U2 virus strain used in the experiments described here was incompatible for STMV propagation. Indeed, the different strains of TMV (U1, U2, U5 and so on) were classified in the 1950s based on physical, chemical and biological characteristics of infection (Rappaport et al. 1956) rather than nucleotide sequence and it is conceivable that an incompatible strain, classified as U2, may have been used as the helper virus strain. Using a helper virus from the same source as the original paper may be required to overcome this problem.

4.4.3 Inhibition of host endocytosis pathways in *N. benthamiana* by TRV silencing

Fortunately, knock down of *N. benthamiana* transcript accumulation levels could be achieved using TRV-induced silencing, which allowed analysis of the roles of several endocytic components.

Endocytosis pathways in plants have not been well characterised compared to those in animal systems and despite database analysis showing the conservation of animal homologs, there are differences between the two systems (Aniento and Robinson 2005). Notably, high turgor pressure exists in plant cells and database analyses indicate that plant genomes do not encode caveolin, the structural component of one of the known classes of lipid-raft mediated endocytosis in mammals (Aniento and Robinson 2005). Nevertheless, endocytosis has an important role in plant cells including in plant innate immunity and it has been shown that many defense-related signalling events may require internalisation of plant receptors from the plasma membrane (Geldner and Robatzek 2008; Leborgne-Castel et al. 2010). For example, the plasma membrane-localised Cf9 and Cf4 resistance proteins, which recognise the Avr9 and Avr4 effectors of the pathogen *Cladosporium fulvum*, respectively, have YXXΦ clathrin-mediated uptake motifs (Jones et al. 1994; Takken et al. 1998). Internalisation of receptors is also important in the perception of microbe-associated molecular patterns (MAMPs) (Geldner and Robatzek 2008). Notable examples include the uptake of the FLS2 receptor upon binding to the bacterial flagellin epitope flg22 (Robatzek et al. 2006) and endocytosis of the LeEix receptor, which also possesses a YXXΦ motif for clathrin-mediated uptake, upon exposure to the fungal elicitor, ethylene-inducing xylanase (Eix) (Bar and Avni 2009). Further, the oomycete elicitor cryptogein and bacterial lipopolysaccharide (LPS) were also shown to enter plant cells in processes that were inhibited by clathrin-mediated endocytosis inhibitors tyrphostin A23 and amantadine respectively (Leborgne-Castel et al. 2008; Gross et al. 2005). Nevertheless it is very likely that, as in animal cells, other uptake pathways also exist in plants and that these mechanisms are co-opted and exploited by pathogens.

A study has recently reported that fungal and oomycete effectors may be internalised by lipid-raft mediated endocytosis that is dependent on the presence of phosphatidylinositol-3-phosphate (PtdIns(3)P) phospholipid. In that study, uptake of Avr1b and AvrL567 was affected by treatment with the PI3K inhibitor wortmannin and the cholesterol sequestering chemicals, nystatin and filipin (Kale et al. 2010). However, further analysis is required since it was shown that treatment with filipin and nystatin did not influence the total amount of Avr1b protein taken up into cells over an extended period of time and that treatment with LY294002, which is also a PI3K inhibitor like wortmannin, did not affect uptake of Avr1b or AvrL567 (Kale et al. 2010). Further, PI3K is known to be required for certain forms of clathrin-independent uptake (Table 4-1; Abrami et al. 2010).

membrane. The Rac1 protein (not shown) may be involved in clathrin-independent uptake; arrows: trafficking pathways within plant cells

From the viral-induced silencing experiments, reducing the transcript levels of genes associated with clathrin-mediated endocytosis resulted in inconsistent effects on AvrM-induced HR. Specifically a slight decrease was detected in α -adaptin-silenced plants, a slight increase in AP180-silenced plants and almost no difference in EHD1- or EHD2-silenced plants. One possibility as to why only slight differences were observed might be that redundancies among the adaptors exist and possibly targeting more than one clathrin accessory factor is required to have an inhibitory effect. Interestingly, it was recently shown that clathrin-mediated uptake of the anthrax toxin was independent of the major clathrin accessory proteins including the AP2 complex and the EHD protein, epsin, due to the utilisation of less conventional, alternative adaptors (Abrami et al. 2010). Another possibility for the lack of effect of silencing various components of the clathrin-mediated uptake pathway on AvrM-induced HR is that the targeted proteins have low turnover rates and that even low levels of expression are sufficient for internalisation to occur. Certainly, clathrin and components of the AP2 complex are considered to be relatively stable and complete depletion of these proteins is not easily achieved (Neumann-Staubitz et al. 2010; Doherty and McMahon 2009). This has been known to cause complications since not all cargoes that enter by clathrin-mediated endocytosis are sensitive to partial depletion of these components (Doherty and McMahon 2009). In these cases, it has been shown that over-expression of the C-terminus of AP180 (AP180-C) has a dominant negative effect that can inhibit clathrin-mediated endocytosis (Ford et al. 2001).

In experiments to express dominant negative AP180, HR induced by secreted AvrM was shown to be reduced in panels expressing C-terminal sequences of putative *N. tabacum* AP180 proteins compared to panels co-infiltrated with AtAP180-C or an empty vector control. However, this difference was also noticed in panels infiltrated with the cytoplasmic form of AvrM (i.e. AvrM Δ SP). This implies that the effect of NtAP180-C and NtAP180-C(EST) protein expression may not have been caused by the suppression of AvrM uptake, but by more general suppression of HR, for instance by inhibition of signalling or protein expression. In Arabidopsis, there are eight genes with predicted ANTH domains, one of which has been characterised as AtAP180 (Holstein and Oliviusson 2005; Barth and Holstein 2004). Although tobacco sequences with the highest

homology to AtAP180 were chosen as targets for inhibition, it is possible that the sequences amplified are not functional homologs of AP180 in tobacco. Interestingly, expression of AtAP180-C did not inhibit uptake of AvrM-Cerulean, despite the fact that AtAP180-C has been shown to associate with Arabidopsis clathrin. This could reflect differences in clathrin between Arabidopsis and tobacco, although this is not highly likely since clathrin is a well conserved protein and indeed, monoclonal antibodies which recognise Arabidopsis clathrin also recognise tobacco clathrin (Hinz et al. 2007; Lam et al. 2007). Another possibility is that clathrin-mediated uptake is not required for AvrM internalisation. This would agree with the results from the TRV silencing experiments as well as the Kale et al. (2010) study which showed that tyrphostin A23 did not affect uptake of AvrL567 into soybean root suspension cultures.

Internalisation via mechanisms of clathrin-independent endocytosis is cholesterol-dependent and there are varying requirements for dynamin, actin and small G-proteins (Doherty and McMahon 2009). The major components of one potential cholesterol-dependent uptake pathway are the flotillins. In *M. truncatula*, flotillins have been found to localise to microdomains on the plasma membrane and two flotillins are required for establishment of nodules, possibly by trafficking or endocytosis of a nodulation signal, or by co-ordinating early symbiotic events (Haney and Long 2010). However, silencing of flotillin sequences only slightly reduced AvrM-induced HR. Based on the fact that there are three flotillin-like sequences in *Arabidopsis* and tomato, there are potentially three flotillin-encoding sequences in *N. benthamiana*. Since silencing was only assessed for one of these genes it is possible that the expression levels of other flotillin genes were not affected. It is noted that the results reported here do not necessarily conflict with the findings of Kale et al. (2010), since more than one form of lipid-raft mediated endocytosis may exist, as in the case of animal cells.

In mammalian systems, the Rac1 gene is associated with several clathrin-independent uptake pathways. The *N. tabacum* NtRac1 gene may be involved in signalling to regulate actin binding and depolymerisation (Chen et al. 2003) making it a potential functional homolog of human Rac1 although its direct effect on membrane trafficking has not been analysed. In this study, it was observed that inhibition of the NtRac1 homolog in *N. benthamiana* resulted in an increase in M resistance protein-mediated HR. Interestingly, expression of constitutively active human Rac1 has been shown to have the opposite effect and promotes the oxidative response in soybean cell cultures upon exposure to elicitors (Park et al. 2000). Seven members of the ROP

family have been identified in rice, some of which have potential functions in disease signalling (Chen et al. 2003). Neighbour-Joining analysis of the ROPs showed that NtRac1 clusters together with OsRac5, which is a negative regulator of rice blast resistance and OsRac6, which, although it was found to have a minor contribution in rice blast disease resistance, is the closest homolog of the barley HvRacB G-protein that is a negative regulator of barley powdery mildew (Chen et al. 2010; Schultheiss et al. 2003; Schultheiss et al. 2002). The increase in M-mediated HR indicates that the sequence selected for silencing is also a negative regulator of disease resistance. Constitutively active and dominant negative forms of Rac1 are known and expression of these mutants in tobacco or flax coupled with analysis by microscopy and/or tests of infectivity may expand our understanding of the role of Rac in infection.

The results of these experiments do not provide any strong evidence that AvrM uptake is mediated by any of the targeted uptake pathways. One possibility for these results are that although the sequences chosen were the most closely related to genes of known endocytic components in terms of BLAST homology, other related sequences may act as the functional homologs of these components. Thus, silencing of the targeted sequences may not have resulted in inhibition of the targeted pathways. In order to resolve this, silencing of other related sequences could be attempted.

Although it is possible that the targeted pathways were not completely inhibited, as discussed above, another potential explanation for the results described is that AvrM may enter cells by an alternative pathway, such as the PtdIns(3)P-mediated uptake model (explored further in Chapter 5) proposed by Kale et al. (2010). However, these results also do not exclude the possibility of AvrM entering plant cells by more than one pathway as in the case of the toxins of many pathogens during infection of mammalian cells including the CTxB toxin of the bacterium *Vibrio cholerae*, which can be internalised by clathrin-, flotillin- and caveolin-dependent uptake (Sotgia et al. 2002; Torgersen et al. 2001); the Shiga toxin of *Shigella dysenteriae*, which may be internalised by receptor-independent macropinocytosis but also receptor-dependent lipid raft uptake and clathrin-mediated uptake (Sandvig 2001; Malyukova et al. 2009; Torgersen et al. 2005) and the plant toxin ricin, which binds to glycolipids and glycoproteins on the cell surface and therefore may be internalised by any of the membrane turnover pathways (Sandvig and van Deurs 1996; Sandvig and van Deurs 2000). In this scenario, different uptake pathways may be co-regulated and inhibition of one pathway may up-regulate another, through which the AvrM

protein may enter. For example, fluid-phase endocytosis is up-regulated following the expression of a temperature-sensitive dynamin mutant that inhibits clathrin-dependent endocytosis (Damke et al. 1995). In addition, the depletion of caveolin has been shown to induce the activation of Cdc42, a Rho-GTPase that regulates CLIC/GEEC and phagocytosis (Nevins and Thurmond 2006). A hint that AvrM too may have multiple routes of entry comes from the fact that AvrM Δ 106-153 was also observed to induce cell death after extended periods (not shown), and therefore, may potentially enter cells by a pathway that does not require the uptake motif. However, such pathways may not be specific and may not be the main pathway through which the AvrM protein is normally internalised.

Finally, although trying to identify the internalisation mechanism of AvrM was the main focus of this chapter, another question of importance is how internalised protein from endocytosed vesicles ends up in the cytoplasm, where AvrM and the plant cytoplasmic M resistance protein have been predicted to interact (Catanzariti et al. 2010). Insights on how proteins can exit the endosomal system of animal cells has come from studies on toxins such as ricin and the shiga toxin (Sandvig and van Deurs 2000). In plant cells, some evidence exists for the exit of a small portion of the ricin toxin from the ER to the cytosol in a process called retrotranslocation that normally functions in ER associated degradation (ERAD), directing misfolded ER proteins to the cytosol for proteosomal degradation (Frigerio et al. 1998; Di Cola et al. 2001). More recently it was shown that retrotranslocation of ricin in tobacco protoplasts requires the AAA-ATPase CDC48 (Marshall et al. 2008). Unfortunately, silencing of CDC48 was lethal to *N. benthamiana*, possibly due to an accumulation of misfolded proteins, and the effect of CDC48 inhibition could not be assayed. However, dominant negative forms of CDC48 can be generated and could be expressed in *N. tabacum*, where the HR assay is faster and less variable, to test the effect of inhibiting retrotranslocation on AvrM entry into the cytoplasm.

CHAPTER 5

5 Lipid binding activity of flax rust effectors

5.1 Introduction

Recently, the entry of AvrM and AvrL567 into plant cells has been demonstrated to occur in the absence of the pathogen (Rafiqi et al. 2010). However, the ability of exogenous proteins to transverse plasma membranes is not unique. Other than oomycete effectors containing the RXLR motif, which are also able to enter plant cells in a pathogen-independent manner (Dou et al. 2008), an interesting example of independent uptake is a group of proteins with protein transduction domains whose members include the HIV Tat protein and the transcription factor Antennapedia (Frankel and Pabo 1988; Green and Loewenstein 1988; Joliot et al. 1991; Thorén et al. 2000). In these proteins, regions less than 30 amino acids in size, known as cell penetrating peptides (CPPs), were found to be sufficient to mediate uptake into cells (Joliot and Prochiantz 2004). These sequences are highly divergent, although many share the property of being amphipathic or cationic in nature (Drin et al. 2003). The mechanism of CPP uptake has been the subject of intense research since their discovery. Such studies have found that CPPs can enter different animal and plant cell types and can also mediate the uptake of attached cargoes, including low molecular weight drugs, proteins, nucleic acids and peptides (Rothbard et al. 2000; Nagahara et al. 1998; Simmons et al. 1997; Shibagaki and Udey 2002; Chugh and Eudes 2008). CPP internalization can occur by different mechanisms – possibly even in combination – including clathrin-dependent endocytosis, lipid raft-mediated macropinocytosis, clathrin- and caveolin-independent endocytosis and direct membrane penetration (Richard et al. 2005; Wadia et al. 2004; Ter-Avetisyan et al. 2009; Joliot and Prochiantz 2004). Interestingly, it was found that the CPP penetratin, from Antennapedia, may associate with negatively charged conserved membrane-associated components, such as the glycosaminoglycan heparan sulfate, prior to endocytic transport (Goncalves et al. 2005; Richard et al. 2005; Jiao et al. 2009). Recently, there has been a report that effectors of fungal pathogens and the RXLR uptake motif of oomycete effectors may also interact with conserved membrane components, specifically phosphoinositol-3-phosphate (PtdIns(3)P) (Kale et al. 2010).

Phosphoinositides (PIs) constitute a small but important group of phospholipids within cells. They possess an inositol head-group which may be phosphorylated at hydroxyl groups at positions D-3, D-4 and D-5 in different combinations due to the combined actions of specific PI kinases and phosphatases (Figure 5-1). Plants possess three phosphatidylinositol monophosphate (PtdInsP) isomers [PtdIns(3)P, PtdIns(4)P and PtdIns(5)P] and three phosphatidylinositol biphosphate (PtdInsP₂) isomers [PtdIns(3,4)P₂, PtdIns(3,5)P₂ and PtdIns(4,5)P₂] but do not have phosphatidylinositol triphosphate (PtdInsP₃) [PtdIns(3,4,5)P₃], which is present in animal cells (reviewed by Heilmann 2009).

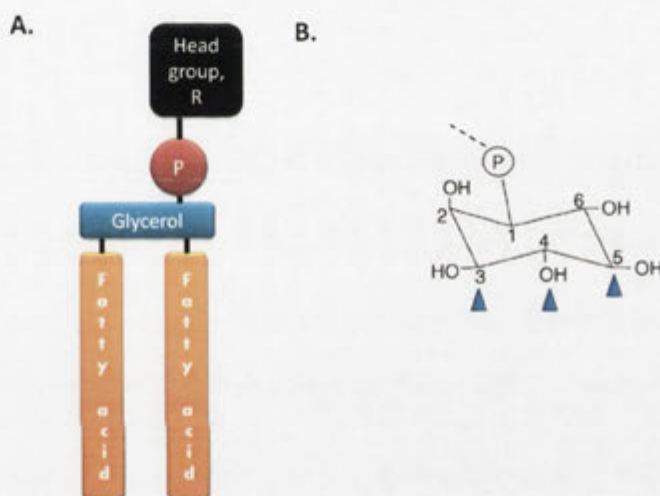


Figure 5-1 General structure of phosphoinositides. **A.** Phosphoinositides consist of a glycerol backbone linked to two fatty acyl chains and a phosphate group, to which the inositol head group is attached. **B.** The hydroxyl groups of the inositol head group at positions D-3, D-4 and D-5 (arrowheads) are differentially phosphorylated in different isomers (Modified from Heilmann 2009; Meijer and Munnik 2003).

In animal systems, PIs and their derivatives are associated with a variety of processes including signal transduction, vesicle trafficking and actin remodeling (reviewed in Martin 1998; Simonsen et al. 2001; Di Paolo and De Camilli 2006). In plants, PIs are also involved in signalling pathways in response to biotic and abiotic stress as well as in plant growth (reviewed in Laxalt and Munnik 2002; Meijer and Munnik 2003; Munnik and Testerink 2008; Heilmann 2009). In signal transduction pathways of both plants and animals, PIs are converted into secondary messages through the activation of PI metabolising enzymes. One of the best known examples is that of

phospholipase C (PLC) action on PtdIns(4,5)P₂, to form free cytosolic inositol-1,4,5-triphosphate (IP₃) and membrane bound diacylglycerol (DAG). In animals, IP₃ increases cytosolic calcium ion levels and DAG activates protein kinase C (PKC) members. In plants, which lack PKC kinases, DAG is rapidly converted into phosphatidic acid (PA) (Munnik et al. 1998; van der Luit et al. 2000). Levels of PA have been shown to increase within minutes in response to general elicitors such as xylanase, flagellin or chitinotetraose as well as the effector AVR4 (van der Luit et al. 2000; de Jong et al. 2004).

In addition to their role as messengers in signal transduction, PIs also provide a level of spatial regulation of cellular organisation because different subsets of PIs are enriched at specific organelle membranes and regions on membranes (Heilmann 2009). In fact, specific PI localisation is important in establishing the identity of compartments throughout the endomembrane system (Heilmann 2009; De Matteis and Godi 2004). This compartmentalisation may also contribute to signalling by concentrating signalling components at docking sites for PI-specific lipid binding domains (Laxalt and Munnik 2002; Furt et al. 2010).

Other than intracellular signaling, it has recently been uncovered that, like the lipid *N*-acylethanolamines (NAEs) and sphingosine-1-phosphate, PtdIns(4)P may also function in intercellular signalling in plants (Regente et al. 2008; Gonorazky et al. 2010). In sunflower seeds, PtdIns(4)P levels were found to increase after exposure to the hormone jasmonic acid while PA and phosphatidylglycerol levels increased in the presence of abscisic acid (Regente et al. 2008). In addition, exposure to the elicitor xylanase was followed by an increase in levels of extracellular PtdIns(4)P and application of PtdIns(4)P was shown to mimic the xylanase response in inducing reactive oxygen species production and cell death (Gonorazky et al. 2008). This is interesting because in eukaryotes, many studies have shown that under most conditions, the outer leaflet of the plasma membrane lipid bilayer is enriched with choline-containing phospholipids and glycosphingolipids while amine-containing phospholipids, such as phosphatidylethanolamine, phosphatidylserine (PS), as well as minor phospholipids like PA and PIs, are enriched within the inner leaflet of the plasma membrane bilayer (Daleke and Lyles 2000). This implies that extracellular PtdIns(4)P are either endocytosed into plant cells or insert into the extracellular leaflet of the plasma membrane and are flipped into the intracellular leaflet where enzymes are able to act on them and release cytosolic PA as a secondary messenger.

In terms of protein uptake, phospholipid binding has been associated with the translocation of the programmed cell death-detecting protein annexin A5 (Kenis et al. 2004). It is postulated that annexin A5 may elicit a form of pinocytosis by inducing membrane curvature upon binding to surface exposed PS, which can be found on the external leaflet of the plasma membrane as an intercellular signal under certain conditions including cell death (Kenis et al. 2004). Further, there is evidence that the CagA protein from *Helicobacter pylori*, also enters host cells by binding to PS lipids that are transiently exposed on the outer leaflet of the plasma membrane, although in this case, PS exposure on the external leaflet is induced during infection and is therefore pathogen-dependent to a certain degree (Murata-Kamiya et al. 2010).

A recent study suggests that uptake of oomycete and fungal effectors, including AvrL567, is mediated by binding to PtdIns(3)P present on the outer leaflet of plant cells (Kale et al. 2010). In this chapter, the phospholipid binding activities of AvrM and AvrL567 were assessed in the context of protein uptake into cells bioinformatically and experimentally. Full-length and truncated versions of both effectors with and without the ability to enter plant cells were tested for phospholipid binding.

5.2 Materials and Methods

5.2.1 Bioinformatic analysis

Full-length amino acid sequences of AvrM-A (ABB96258.1), AvrL567-A (AAS66948.1), AvrP123 (ACD49721.1), AvrP4 (ACD49710), Avr1b (AAM20937.1), myristoylated alanine-rich C kinase substrate (MARCKS) (P12624) and the CPP-containing proteins HIV Tat (BAF32556.1) and *Drosophila melanogaster* Antennapedia (AAS65114.1) were used as input sequences in the SVMProt program, which identifies potential lipid binding proteins with 94.1% overall accuracy on tested datasets (Cai et al. 2003; Lin et al. 2006). These sequences were aligned using ClustalW (Available at: <http://www.ebi.ac.uk/Tools/clustalw2/index.html>), and then subjected to Neighbour-Joining and principal component analysis clustering by Jalview (Clamp et al. 2004) using the BLOSUM62 matrix. The secondary structure of AvrM was predicted using the JPRED secondary structure prediction tool (Cole et al. 2008). Figures of alignments were prepared using Jalview (Clamp et al. 2004).

5.2.2 Cloning of glutathione S-transferase (GST) fusion proteins

AvrM and AvrL567 protein coding sequences were amplified using the forward primer, 5'-GACGACGACAAGACT-protein specific sequence-3', and the reverse primer, 5'-GAGGAGAAGCCCGGC-protein specific sequence-3' by Phusion polymerase as described in Chapter 4, and inserted into the ligation-independent cloning (LIC) cloning site of pET41.2 for cloning of N-terminal GST fusion proteins as described in Chapter 4. Mixtures were then transformed into chemically competent C41(DE3) *E. coli* cells using standard procedures as described in Chapter 2. Sequences of cloned genes were confirmed by sequencing at the Australian Genome Research Facility, Brisbane. The cloning of GST-Cerulean and AvrM 156-Cerulean is described in Chapter 4.

5.2.3 Expression and purification of proteins

Expression of the GST fusions was induced in transformed C41(DE3) *E. coli* cells by autoinduction overnight at 37°C using the protocol described in Chapter 4. Cells were harvested and lysed by freeze-thaw lysis as described in Chapter 1. Crude lysates of GST fusions were routinely incubated with 50 µl of Ni Sepharose 6 Fast Flow (GE Healthcare) pre-equilibrated with Lysis buffer (Appendix) for 1 h at 4°C. Then, the resin was centrifuged at 1000 × *g* for 3 min at 4°C. The resin was washed with 30 medium volumes (m/v) of Wash buffer C [20 mM HEPES pH 8.0, 300 mM NaCl, 20 mM imidazole]. This was followed by washing thrice with 10 m/v of Wash buffer

A (Appendix). Then, proteins were eluted thrice with 2 m/v of Elution buffer A (Appendix). Protein concentrations were determined using the Bradford assay (Bradford 1976) with bovine serum albumin (BSA) as a standard. The expression and purification of His-tagged AvrM (His-AvrM), His-Ubiquitin-tagged AvrL567 (His-Ubi-AvrL567) and AvrM 156-Cerulean protein was performed as described in Chapters 1 and 4. Purified GFP protein was a gift from Jan Elliot (Research School of Biology, Australian National University).

5.2.4 Lipid protein overlay

PIP strips (Figure 5-2; Echelon Biosciences) were blocked with 4% (w/v) non-fat milk in phosphate buffered saline pH 7.4 (PBS pH 7.4; Appendix) for 1 h at room temperature. This was followed by incubation of the blot with the protein of interest in 4% (w/v) non-fat milk in PBS pH 7.4 overnight at 4°C at a specific concentration. This was 6.45 nM for AvrM fusions or 174 nM for AvrL567 fusions. Membranes were rinsed three times for 10 min in PBST (Appendix), before incubation with primary antibodies to detect the proteins of interest for 1 h at room temperature. This was followed by three rinses for 10 min each in PBST and then incubation with the appropriate secondary antibody for 1 h at room temperature. The blots were then rinsed again in PBST three times for 10 min each before developing them in Supersignal West Pico chemiluminescent Substrate (Pierce Chemicals) according to the manufacturer's instructions. An IgG purified anti-GST antibody raised in rabbits (Upadhyaya et al. 1995; Li et al. 1996) was kindly provided by Dr Narayana Upadhyaya (CSIRO) and used at a concentration of 1 µg/mL and the anti-green fluorescent protein (GFP) antibody (Roche Diagnostics Ltd) was used at 1:12500 dilution. His-AvrM and His-Ubi-AvrL567 were detected using affinity purified anti-AvrM antibody (Chapter 2; Rafiqi et al. 2010) and anti-AvrL567 prepared by Dr Ann-Maree Catanzariti (CSIRO), respectively, at 1:1000 dilutions. The secondary antibodies, goat anti-rabbit conjugated to horse radish peroxidase (Sigma) was used at a 1:10000 dilution while goat anti-mouse conjugated to horse radish peroxidase (Sigma) was used at a 1:50000 dilution.

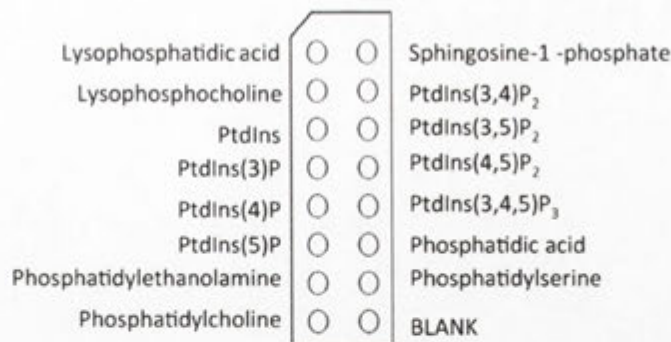


Figure 5-2 Schematic diagram of a PIP strip. Each spot has been pre-loaded with 100 pmol of the specified lipids.

5.2.5 Immunoblots

Proteins were run on 12% polyacrylamide sodium dodecyl sulfate (SDS) gels and transferred to Hybond-C extra membranes (GE Healthcare) as described in Chapter 2. Blots were blocked for 1 h with 4% (w/v) non-fat milk in PBS pH7.4 before protein detection by chemiluminescence with primary and secondary antibodies as described for the lipid blots in Section 5.2.4.

5.2.6 Preparation of lipid arrays

Lipid arrays were prepared as previously described (Dowler et al. 2002). Briefly, PtdIns(3)P or PtdIns(5)P lipids (Echelon Biosciences) were reconstituted in a 1:1 solution of methanol: chloroform to a final concentration of 1 mM. Solutions with different concentrations of each lipid were prepared by diluting in a 2:1:0.8 solution of methanol: chloroform: water. Aliquots of 1 μ L each were spotted onto a Hybond-C extra membrane. Membranes were allowed to dry in the dark at room temperature for 1 h before treatment as described for the lipid blots in Section 5.2.4. Unused lipid arrays were stored in the dark at 4°C and all lipid stocks were stored at -80°C.

5.3 Results

5.3.1 Bioinformatic prediction of lipid binding

As lipid binding proteins are diverse in sequence, form and structure, searches based on sequence similarity such as BLAST, can sometimes be inefficient in predicting lipid binding domains. In order to overcome this limitation, a program using the statistical learning method Support Vector Machines, SVMProt, was utilised to predict lipid binding (Table 5-1). Using AvrM-A and AvrL567-A as input sequences, SVMProt predicted that neither protein possessed lipid binding activity. This was also true for the HIV Tat protein, which contains the Tat CPP, and the Antennapedia protein, which contains the penetratin CPP. At the same time, the Avr1b effector from *Phytophthora infestans*, which has two RXLR motifs, was predicted to have lipid binding activity with 83.9% confidence. This level of confidence dropped upon substitution of one of the two RXLR motifs with alanines to either 73.8% (RSLR to AAAA) or 71.3% (RFLR to AAAA), or to 65.4% upon substitution of both RXLR motifs with alanines (RSLR and RFLR to AAAA and AAAA) indicating that each RXLR motif contributes to the predicted lipid binding of Avr1b. AvrP123 and AvrP4 were both predicted to be lipid binding proteins with low P-values of 58.6%.

Table 5-1 Results indicating the prediction of various effectors to be classified as lipid binding proteins according to SVMProt analysis. R-value represents a reliability score with a higher score representing a higher confidence. P-value indicates expected classification accuracy.

Sequence	R-value	P-value (%)
AvrM	-	-
AvrL567	-	-
AvrP123	1.0	58.6%
AvrP4	1.0	58.6%
Avr1b	2.0	83.9%
Avr1b (RSLR and RFLR to AAAA and AAAA)	1.2	65.4%
Avr1b (RSLR to AAAA)	1.5	73.8%
Avr1b (RFLR to AAAA)	1.4	71.3%
HIV Tat	-	-
Penetratin	-	-

Comparison of the AvrM and AvrL567 minimum uptake region with CPP sequences (Figure 5-3 A) showed that, like CPPs, the two minimum regions that have been demonstrated to mediate uptake *in planta* (AvrM 106-156 and AvrL567 25-50) are amphipathic in nature, with hydrophobic

residues interspersed with polar amino acids. Furthermore, both regions are predicted to have some secondary structure: alpha-helices in the case of AvrM according to JPRED prediction (Figure 5-3 D) and beta-sheets in the case of AvrL567, according to its crystal structure, although the N-terminal portion (residues 26-37) which is required for uptake (Rafiqi et al. 2010) was unstructured (Wang et al. 2007). Compared to the majority of CPPs, the minimum uptake regions of AvrM and AvrL567 show a larger proportion of negatively charged residues. A Neighbour-Joining tree based on the BLOSUM62 substitution matrix was drawn using the sequences used by Hansen et al. (2007) to cluster CPPs (Figure 5-3 B). However, in agreement with their findings, only the closely related Tat and Arg9 and TP10 and Transportan, were found to cluster together. The majority of CPPs as well as the AvrM and AvrL567 minimum uptake regions did not group together. As expected, these results were consistent with clustering based on the BLOSUM62 substitution matrix using principal component analysis (Figure 5-3 C).

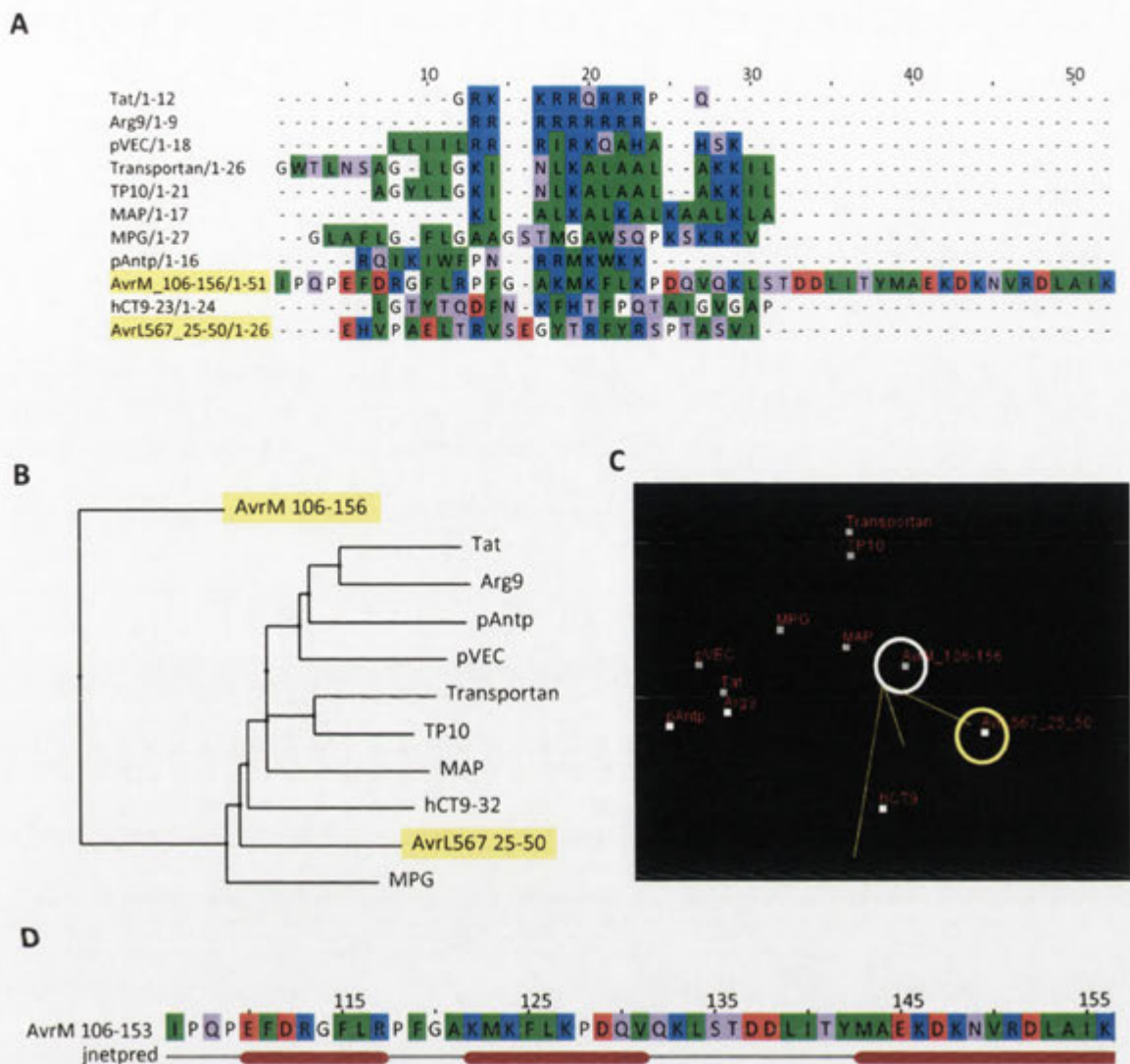


Figure 5-3 Comparison of AvrL567 and AvrM uptake regions against various CPP sequences. Sequences available from Hansen et al. (2007). **A.** ClustalW alignment. **B.** Neighbour-Joining tree. **C.** Principle component analysis. White circle: AvrM uptake region; yellow circle: AvrL567 uptake region. **D.** Consensus predicted secondary structure at AvrM minimum uptake region by JPRED. Green: hydrophobic amino acids; purple: polar uncharged amino acids; blue: positively-charged amino acids and red: negatively-charged amino acids; red tubes: alpha-helices

5.3.2 Lipid binding specificities of AvrM and AvrL567

Binding of *E. coli* expressed His-AvrM and His-Ubi-AvrL567 to phospholipids was tested by a lipid overlay assay using PIP strips, which contain an array of phospholipids attached to a membrane (Figure 5-4). The assay involves incubation of the membranes with the protein of interest followed

by immunodetection of bound protein after washing away unbound protein. In initial experiments, individual PIP strips were incubated with 0.5 $\mu\text{g/mL}$ of each protein which was detected using protein specific antibodies. As shown in Figure 5-4, under these conditions, the positive control, the PIP2 grip protein, consisting of the pleckstrin homology (PH) domain of phospholipase C fused to GST, showed specific binding to $\text{PtdIns}(4,5)\text{P}_2$, as expected. Further, the negative control, GFP alone, showed no lipid binding activity. In addition, the results showed that AvrM bound to PtdIns , $\text{PtdIns}(3)\text{P}$, $\text{PtdIns}(4)\text{P}$, $\text{PtdIns}(5)\text{P}$, $\text{PtdIns}(3,5)\text{P}_2$, PA and PS. On the other hand, AvrL567 was found not to bind to any of the lipids on the PIP strip at 0.5 $\mu\text{g/mL}$.

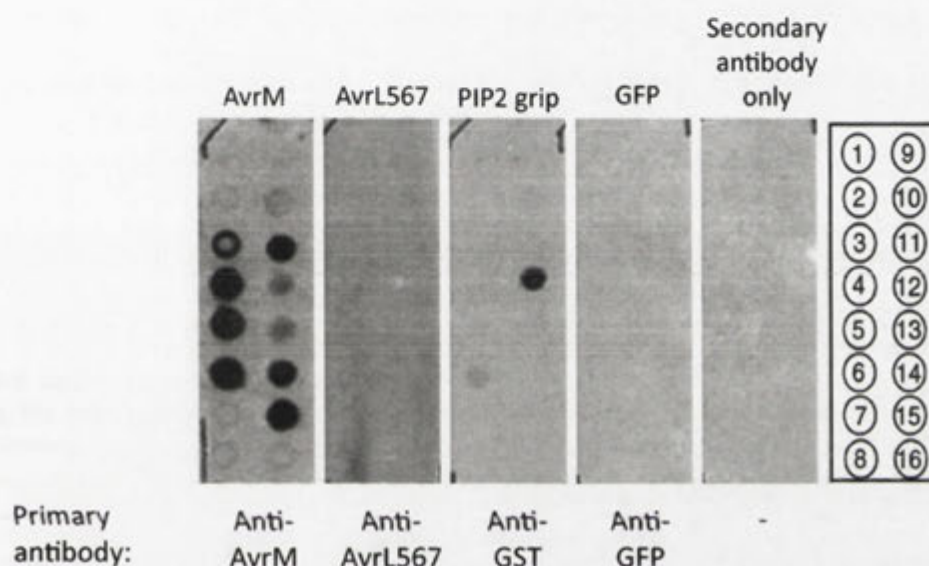


Figure 5-4 Binding of His-AvrM and His-Ubi-AvrL567 to lipids. His-AvrM and His-Ubi-AvrL567 were affinity purified and used to probe blots spotted with various lipids. PIP2 grip, a GST fusion which binds to $\text{PtdIns}(4,5)\text{P}_2$ was used as a positive control and GFP as well as the secondary antibody only were used as negative controls. Primary antibodies used to detect each protein are specified below each blot. Compounds spotted on the membrane: 1, lysophosphatidic acid; 2, lysophosphatidylcholine; 3, PtdIns ; 4, $\text{PtdIns}(3)\text{P}$; 5, $\text{PtdIns}(4)\text{P}$; 6, $\text{PtdIns}(5)\text{P}$; 7, phosphatidylethanolamine (PE); 8, phosphatidylcholine (PC); 9, sphingosine-1-phosphate; 10, $\text{PtdIns}(3,4)\text{P}_2$; 11, $\text{PtdIns}(3,5)\text{P}_2$; 12, $\text{PtdIns}(4,5)\text{P}_2$; 13, $\text{PtdIns}(3,4,5)\text{P}_3$; 14, phosphatidic acid (PA); 15, phosphatidylserine (PS); 16, blank. Each spot consisted of 100 pmol of lipid. All proteins were incubated with lipids at concentrations of 0.5 $\mu\text{g/mL}$.

Since the lack of AvrL567 binding might be attributed to a lower lipid binding affinity of the protein, another set of lipid blots was tested by increasing the concentration of AvrL567 protein to 5 $\mu\text{g/mL}$. Further, in order to distinguish lipids with the highest binding affinity to AvrM, blots were probed with 0.25 $\mu\text{g/mL}$ AvrM. The results in Figure 5-5 indicate that AvrM has the highest binding affinity to $\text{PtdIns}(3)\text{P}$ and $\text{PtdIns}(5)\text{P}$ and, to a lower extent, also to $\text{PtdIns}(4)\text{P}$. Longer exposures of

the film showed that AvrM had an even lower affinity to PtdIns and PS (data not shown). At the higher concentration (5 $\mu\text{g}/\text{mL}$), AvrL567 showed weak binding to PtdIns(3)P and PtdIns(5)P. Longer exposures for AvrL567-bound blots resulted in very high background preventing the detection of binding to other lipids.

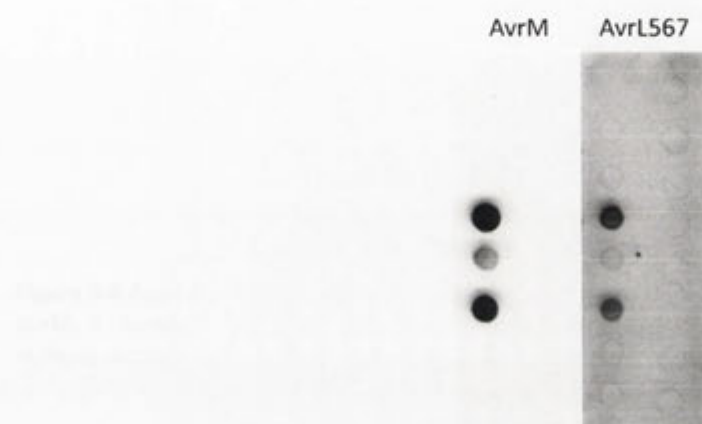


Figure 5-5 Lipid binding specificities of His-AvrM at 0.25 $\mu\text{g}/\text{mL}$ and His-Ubi-AvrL567 at 5 $\mu\text{g}/\text{mL}$ after adjusting the concentrations of protein used to probe blots spotted with various lipids. Both proteins showed binding to PtdIns(3)P and PtdIns(5)P and to a lesser extent, PtdIns(4)P.

5.3.3 Binding activities of AvrM and AvrL567 uptake mutants

To explore the possibility that AvrM and AvrL567 lipid binding is required for uptake of these proteins into host cells, AvrM and AvrL567 uptake mutants identified by Rafiqi *et al.* (2010) were tested for lipid binding activity. These mutants consisted of the minimum regions required for uptake (AvrM 106-153 and AvrL567 26-50) as well as peptides lacking the regions required for uptake (AvrM Δ 106-153 and AvrL567 Δ 26-37). In order to provide a common epitope for antibody recognition, an N-terminal GST fusion of each protein was cloned.

As described in Chapter 4, fusion to GST significantly increased the solubility of AvrM. The deletion mutants of AvrM and AvrL567 were also shown to be highly expressed, with strong bands corresponding to the expected sizes of the proteins detected in Coomassie stained protein gels of crude cell lysates (not shown). The exception to this was the AvrM Δ 106-153 GST fusion where there was no predominant band at the expected size in crude lysates. However, after purification on a nickel column, a band of the expected size was purified, although with lower yield compared

to other GST fusion proteins (Figure 5-6), which might indicate protein misfolding or a lack of stability of protein when expressed by *E. coli*.

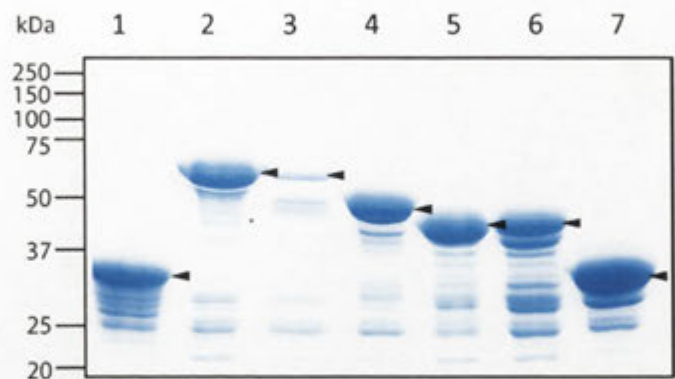


Figure 5-6 Expression of AvrM and AvrL567 GST fusion proteins. Semi-purified GST fusions of 1: GST only, 2: AvrM, 3: AvrM Δ 106-153, 4: AvrM 106-153, 5: AvrL567, 6: AvrL567 Δ 26-37 and 7: AvrL567 26-50. Arrows indicate masses of expected size for each target protein. Each lane was loaded with 15 μ L of protein eluate.

AvrL567 variants were adjusted to 174 nM, equivalent to the concentration of His-Ubi-AvrL567 protein at 5 μ g/mL, and incubated with lipid blots as described above. Bound protein was detected with an anti-GST antibody (Li et al. 1996). The results showed that the lipid binding activities of the AvrL567 Δ 26-37 and AvrL567 26-50 fusions to GST were not different from the binding activity of GST alone (Figure 5-7), indicating that the levels of lipid binding detected for these proteins were close to that of background. It is noted that the AvrL567 26-50 was smaller than the negative control, which was GST expressed from the empty pET41 vector, due to the expression of amino acids encoded by the multiple cloning site and an additional C-terminal hexahistidine tag in the pET41 empty vector control (Figure 5-7A). In effector GST fusions, a STOP codon was added before this additional His-tag.

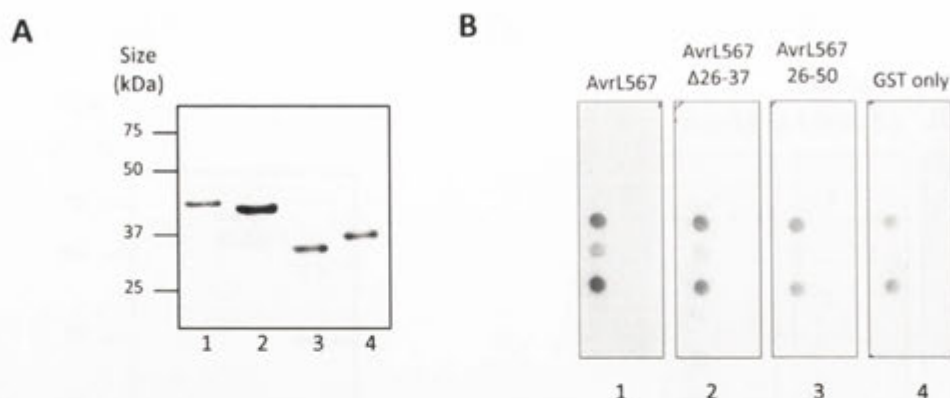


Figure 5-7 Lipid-binding specificities of GST-tagged AvrL567 variants at 174 nM. **A.** Immunoblot showing the detection of AvrL567 variants fused to GST. The amounts loaded per lane correspond to the amount of protein incubated with each lipid blot per 200 μ L. Lane 1: GST-AvrL567; 2: GST-AvrL567 Δ 26-37; 3: GST-AvrL567 26-50 and 4: GST only. Mass of protein standards shown on left. **B.** Lipid binding specificities of semi-purified GST fusions of AvrL567, AvrL567 Δ 26-37, AvrL567 26-50 and GST at 174 nM.

AvrM variants were incubated with lipid blots at a concentration of 6.45 nM, equivalent to the concentration of His-AvrM at 0.25 μ g/mL. Detection using anti-GST antibodies, showed that, like His-AvrM, the AvrM GST fusion binds to PtdIns, PtdIns(3)P, PtdIns(4)P, PtdIns(5)P and PS (Figure 5-8). After additional exposure, binding to PtdIns(3,5)P₂ was also detected, confirming the results obtained with the anti-AvrM antibody and suggesting that the N-terminal GST tag does not disrupt lipid binding. Further, the results showed that the AvrM variant lacking the minimum uptake region identified by Rafiqi et al. (2010) had a much reduced lipid binding activity compared to the full-length protein. However, a peptide corresponding to the proposed minimum region required for uptake (AvrM 106-153) did not bind to any lipids.

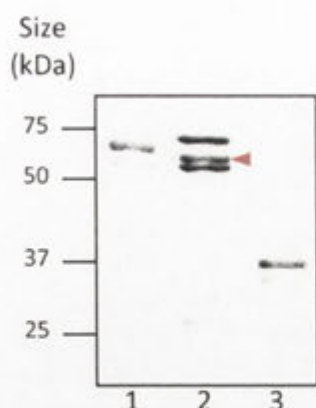
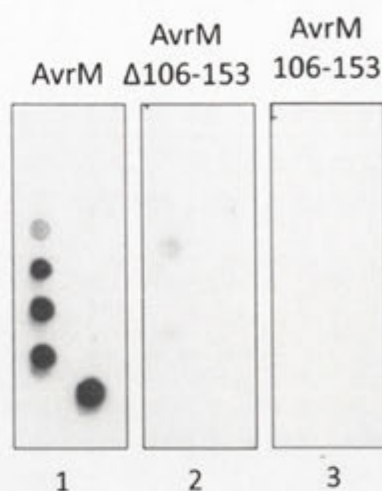
A.**B.**

Figure 5-8 Lipid-binding specificities of GST-tagged AvrM variants at 6.45 nM. **A.** Immunoblot showing the detection of AvrM variants fused to GST. The amounts loaded per lane correspond to the amount of protein incubated with each lipid blot per mL. Lane 1: GST-AvrM; 2: GST-AvrM Δ 106-153 and 3: GST-AvrM 106-153. Mass of protein standards shown on left. The expected size of AvrM Δ 106-153 is indicated by the arrowhead. **B.** Lipid binding specificities of semi-purified GST fusions of AvrM, AvrM Δ 106-153 and AvrM 106-153 at 6.45 nM.

As AvrM was shown to bind most strongly to PtdIns(3)P and PtdIns(5)P, the lipid binding affinities of the various forms of AvrM were assessed by binding to a dilution series of these lipids. The results of this (Figure 5-9) showed that the full-length AvrM protein had a high affinity for PtdIns(3)P and PtdIns(5)P. Under the same exposure conditions, the AvrM mutant lacking the minimal region required for uptake (AvrM Δ 106-153) had reduced affinity for the two lipids tested. However, the minimum uptake region by itself (AvrM 106-153) did not show any PtdIns(3)P or PtdIns(5)P binding under the same conditions. Constructs containing the N-terminal 153 or 156 amino acids of AvrM excluding the signal peptide (AvrM 153 and AvrM 156), which have been shown to internalise successfully into cells *in planta* (Rafiqi et al. 2010; Dr M Rafiqi, personal communication), also did not demonstrate binding to PtdIns(3)P or PtdIns(5)P (Figure 5-9 and Figure 5-10). The results of AvrM 156 binding could be replicated by replacing the N-terminal GST tag with a C-terminal fusion to the Cerulean fluorescent protein and detecting bound protein using anti-GFP antibodies. This result indicates that the lack of binding was not due to hindrance by the N-terminal GST domains (Figure 5-11). This suggests that for full phospholipid-specific binding, the uptake region, as well as the C-terminal domain of AvrM is required. This hypothesis is supported

by the fact that binding of AvrM lacking the first 106 amino acids (AvrM Δ 106) is equivalent to that of full-length mature AvrM under the same exposure conditions as shown in Figure 5-10.

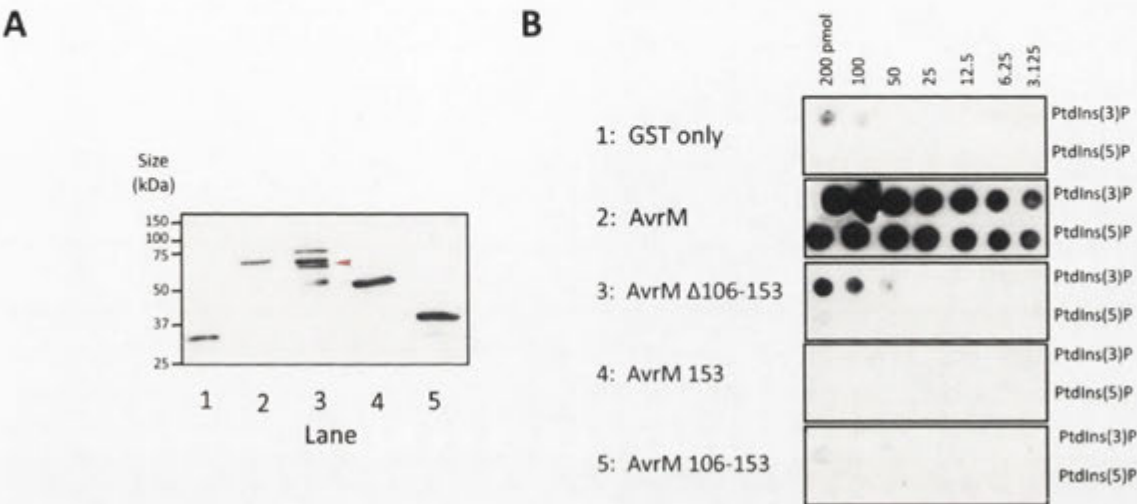


Figure 5-9 Lipid binding affinity of GST-tagged AvrM uptake variants. **A.** Immunoblot showing the detection of AvrM variants fused to GST. The amounts loaded per lane correspond to the amount of protein incubated with each lipid array per mL. Lane 1: GST alone; 2: GST-AvrM; 3: GST-AvrM Δ 106-153; 4: GST-AvrM 153 and 5: GST-AvrM 106-153. Mass of protein standards shown on left. The expected size of AvrM Δ 106-153 is indicated by the arrowhead. **B.** Lipid binding affinities of semi-purified GST fusions of GST alone, AvrM, AvrM Δ 106-153, AvrM 153 and AvrM 106-153 at 6.45 nM.

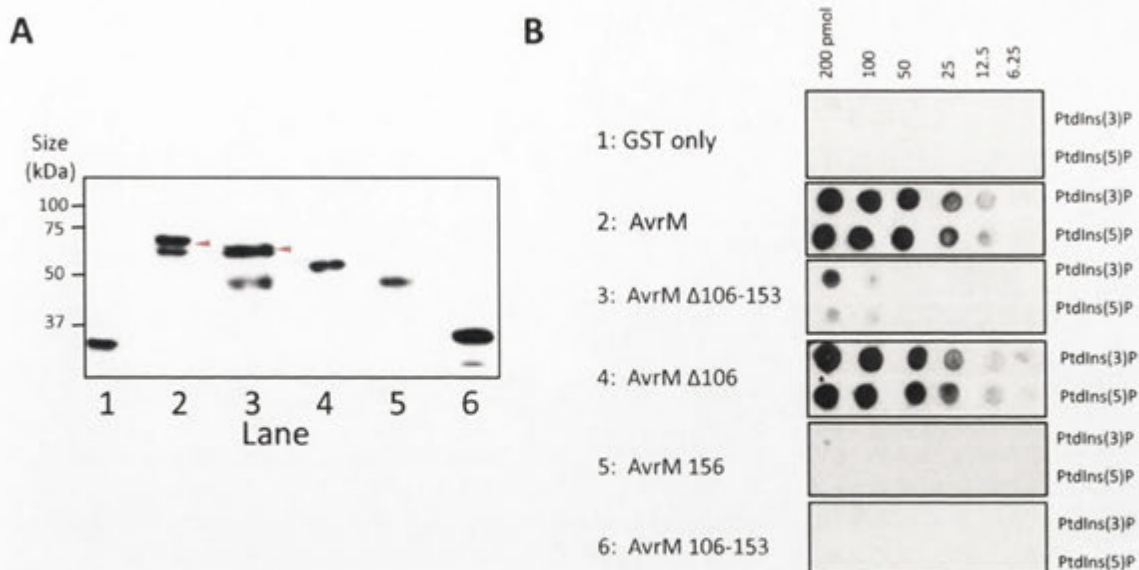


Figure 5-10 Lipid binding affinity of GST-tagged AvrM uptake variants. **A.** Immunoblot showing the detection of AvrM variants fused to GST. The amounts loaded per lane correspond to the amount of protein incubated with each lipid array per mL. Lane 1: GST alone; 2: GST-AvrM; 3: GST-AvrM Δ 106-153; 4: GST-AvrM Δ 106; 5: GST-AvrM 156 and 6: GST-AvrM 106-153. Mass of protein standards shown on left. The expected size of AvrM and AvrM Δ 106-153 GST fusions are indicated by arrowheads. **B.** Lipid binding affinities of semi-purified GST fusions of GST alone, AvrM, AvrM Δ 106-153, AvrM Δ 106, AvrM 156 and AvrM 106-153 at 6.45 nM.

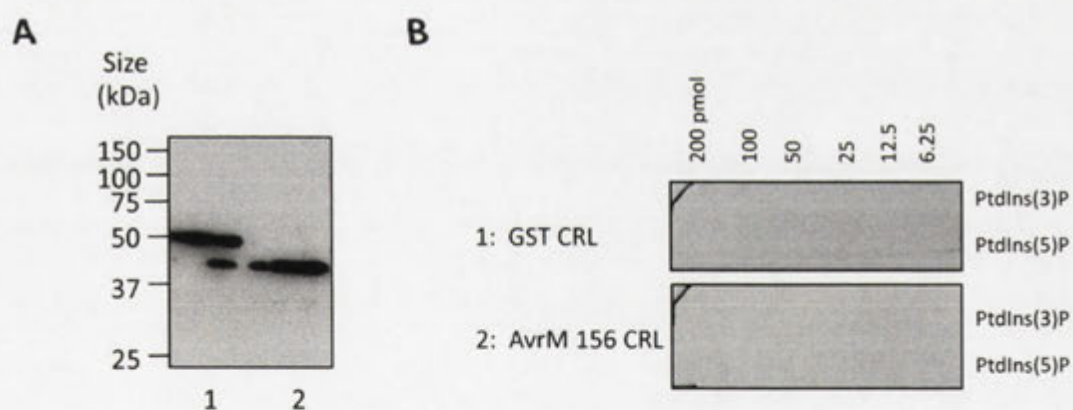


Figure 5-11 Lipid binding affinity of cerulean-tagged AvrM 156 and GST. **A.** Immunoblot showing the detection of AvrM 156-Cerulean and GST-Cerulean fusion proteins detected by anti-GFP antibodies. The amounts loaded per lane correspond to the amount of protein incubated with each lipid array per mL. Lane 1: AvrM 156 Cerulean; 2: GST Cerulean. Mass of protein standards shown on left. **B.** Lipid binding affinities of semi-purified GST-Cerulean (GST CRL) and AvrM 156-Cerulean (AvrM 156 CRL) at 6.45 nM.

5.4 Discussion

The flax rust effectors AvrM and AvrL567 have been shown to be able enter host cells in the absence of the pathogen (Rafiqi et al. 2010). Lipid binding activity is potentially required for uptake into host cells in different systems, such as in the case of the CPPs and potentially also some pathogen effectors (Kale et al. 2010). In this chapter, AvrM and AvrL567 variants with differing abilities to enter host cells were bioinformatically and experimentally assessed for their lipid binding activities.

5.4.1 Bioinformatic prediction of lipid binding

Several lipid binding domains have been identified and characterised in recent years. These can be divided into two classes: (1) those with stereo specific recognition of particular phospholipids and (2) those which associate with lipids less specifically based on the physical properties of the membrane, including charge, amphiphilicity and curvature (Lemmon 2008). Examples of domains that fall within the first class include the C1 domain, which recognises DAG (Colón-González and Kazanietz 2006), and the PH domain of phospholipase C δ_1 , which binds preferentially to the head group of PtdIns(4,5)P $_2$ (Kavran et al. 1998). Examples of protein sequences with less specific phospholipid binding include the AP180 N-terminal homology (ANTH) domain, which binds to PtdIns(4)P and PtdIns(3,5)P $_2$ although it has the highest affinity for PtdIns(4,5)P $_2$ (Ford et al. 2001), the Bin, amphiphysin, Rvs (BAR) domain, which binds to acidic phospholipids and may associate preferentially with curved membranes (Itoh and De Camilli 2006) and the MARCKS protein, which is a natively unfolded protein with a high proportion of charged residues and a few hydrophobic residues that binds to PtdInsP $_2$ in membranes by virtue of electrostatic interactions (McLaughlin and Murray 2005). Many of these lipid binding domains lack primary sequence similarity thus making it difficult to predict novel types of lipid binding domains by analysis of amino acid sequence features alone, although the majority of lipid-binding domains do contain cationic surfaces by which they interact with anionic lipids (Hansen et al. 2007; Stahelin 2009).

Bioinformatic prediction of lipid binding domains by support vector machine statistical analysis supposedly overcomes this by classifying proteins according to the physicochemical properties of functional classes of proteins, rather than by assessing changes in the amino acid sequence, as in the case of programs which utilise substitution matrices (Cai et al. 2003). The SVMProt program classifies Avr1b as a potential lipid binding protein and indeed, it has recently been reported that the full-length effector binds to PtdIns(3)P and PtdIns(4)P (Kale et al. 2010). In

contrast, the AvrM and AvrL567 proteins were not predicted to be lipid binding proteins (Table 5-1). However, the CPP-containing proteins HIV-Tat and *Drosophila* Antennapedia, which bind non-specifically to negatively charged lipid head groups in plasma membranes, were also not predicted to be lipid binding proteins (Ziegler et al. 2003; Drin et al. 2001). Nevertheless, SVMProt was able to predict the lipid binding activity of the MARCKS protein (Appendix), which shares similar charge properties with CPPs. It is noted that the SVMProt program has a false negative rate of approximately 10% for prediction of lipid binding proteins and has not been trained to recognise proteins with CPP sequences (Lin et al. 2006).

Several statistical methods based on physicochemical property prediction have been proposed to predict CPPs. However, the success rate has been low (~68%) although newer methods combining several statistical prediction approaches supposedly have higher (80-100%) predictive capabilities (Hällbrink et al. 2005; Dobchev et al. 2010). In general, CPPs have more positive charges than negative charges, with more arginines than lysines and an amphipathic, usually helical, structure, although certain CPPs, such as the polyarginines and the closely related Tat sequence, consist of stretches of positively charged residues (Hansen et al. 2007). In comparison to CPPs, the minimum uptake regions of AvrM and AvrL567 contain a greater proportion of negatively charged amino acid groups. Amphipathic structures are common features of lipid-associating proteins as hydrophobic residues are able to insert into membranes while charged residues bind to charged lipid head groups, leading to disruption of the lipid bilayer or induction of membrane curvature (Dom et al. 2003; Ford et al. 2001; Lemmon 2008). In fact, some amphipathic peptides are able to translocate across membranes by inducing pore formation (Nir and Nieva 2000; Bechinger 1997). Although there is no evidence of this occurring with the flax rust effector proteins, the minimum uptake regions of AvrM and AvrL567 are both amphipathic.

5.4.2 AvrL567 and AvrM lipid binding activity

A recently published study by Kale et al. (2010), showed through liposome binding and lipid protein overlays that the N-terminus of AvrL567, consisting of residues 24-67, fused to GFP binds to PtdIns(3)P and that this binding was abolished by mutation of the amino acids RFYR to alanines. However, the first three amino acids of the putative motif (RFY) are predicted to be part of the β 1-sheet of AvrL567 and it is known that while mutation of RFYR to AAAA prevents protein uptake into plant cells, it also abolishes recognition of AvrL567 by the L6 resistance protein, indicating that the overall structure of the protein may be affected (Wang et al. 2007; Rafiqi et al. 2010). In this

study, the lipid binding activities of full-length AvrL567 as well as AvrL567 26-50, which only lacks one amino acid from a region that is sufficient for uptake *in planta*, and AvrL567 Δ 26-37, which was found to be insufficient for uptake but is still recognised by the L6 resistance protein (Rafiqi et al. 2010), were tested.

In contrast to the results presented by Kale et al. (2010), under the conditions described in this study, weak binding was detected for AvrL567 to both PtdIns(3)P and PtdIns(5)P at a concentration of 174 nM but not at 17.4 nM. However, at a concentration of 174 nM, GST, a non-lipid binding protein, was shown to have the same lipid binding pattern suggesting that the binding detected for AvrL567 was close to background. Similarly, AvrL567 26-50 and AvrL567 Δ 26-37 both did not show differences in PI binding compared to full-length AvrL567 at 174 nM. One possible explanation for the differences between the Kale et al. (2010) study and the work presented here, aside from the differences in the constructs used, is that in the study by Kale and colleagues, binding was performed at a concentration of 1 μ g/mL (approximately 31 nM) using 3% BSA as a blocking buffer and it is possible that these differences in binding conditions resulted in the dissimilar results.

Nonetheless, it was shown that AvrM binds to phospholipids, especially PtdIns(3)P and PtdIns(5)P even at the low concentration of 6.25 nM. In addition, AvrM was also shown to bind with a lower affinity to other negatively charged phospholipids: PtdIns(4)P, PtdIns, PtdIns(3,5)P₂, PA and PS. As mentioned, many other phospholipid binding domains also demonstrate some target binding promiscuity. In these cases, specificity occurs by changes to lipid binding affinity in response to other factors such as cytosolic calcium levels, as in the case of some C2 domains, and membrane curvature in the case of the BAR and FCH-BAR (F-BAR) domains, which allows for exquisite spatial and temporal regulation of lipid binding events (Lemmon 2008).

As summarised in Figure 5-12, full-length mature AvrM has a high affinity for lipids. Deletion of the uptake region, corresponding to residues 106-153, substantially reduced lipid binding but also compromised protein expression levels and/or solubility in *E. coli*. A reduction in solubility sometimes reflects protein misfolding (Sorensen and Mortensen 2005), indicating that this deletion may negatively affect protein stability and/or folding which may explain its lack of binding. On the other hand, AvrM Δ 106 was found to be sufficient for recovery of lipid binding activity. Interestingly, AvrM Δ 107-225, which is a slightly larger deletion than AvrM Δ 106-153, was shown to be incapable of dimerisation, while AvrM Δ 107, which is almost equivalent to AvrM

Δ106, is able to self-dimerise (Catanzariti et al. 2010). Thus, one possible model for explaining the lipid binding activity of AvrM is that as a monomer it may have weak lipid binding affinity that is enhanced combinatorially by dimerisation. This has been shown to be the case for the PH domain of dynamin (Klein et al. 1998) and possibly also the Fab 1, YOTB, Vac 1 EEA1 (FYVE) domain of the Early Endosome Antigen 1 (EEA1) protein (Dumas et al. 2001). Such a model could be tested by determining the lipid binding activity of AvrM Δ107-225 or the avrM protein, which dimerises weakly (Catanzariti et al. 2010) or by testing the dimerisation ability of AvrM Δ106-153.

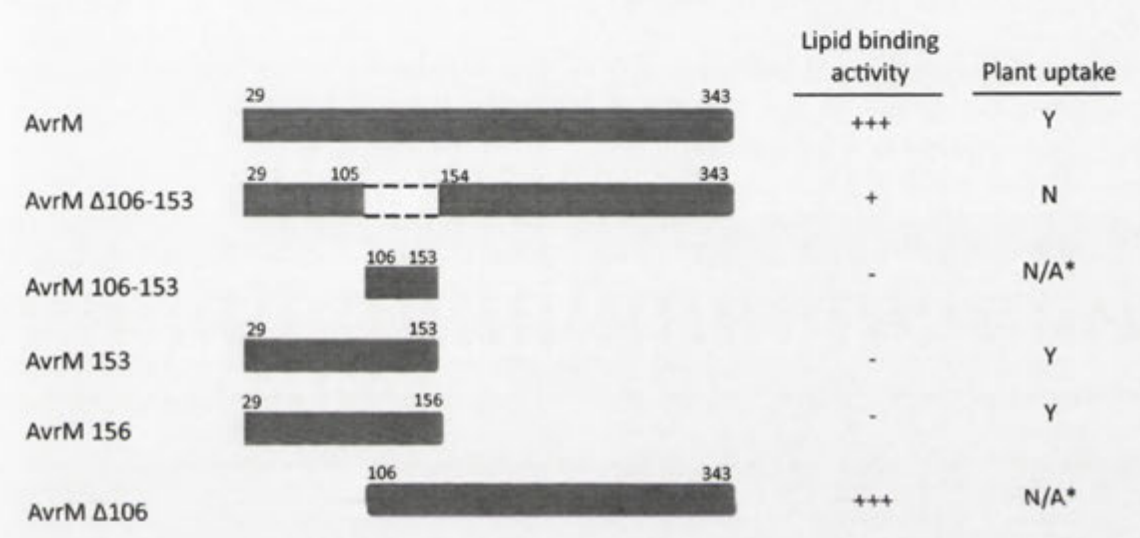


Figure 5-12. Summary of the lipid binding affinities of different AvrM uptake variants tested. +++: strong binding; +: weak binding; -: no binding; Y: uptake occurs; N: no uptake occurs; N/A: not assessed. The uptake of all assessed constructs into plant cells is described in (Rafiqi et al. 2010) with the exception of AvrM 153 (Dr M. Rafiqi, personal communication). *The similar constructs AvrM 106-156, AvrM 29-156 and AvrM Δ35-106 were found to internalise into plant cells.

Notably, AvrM 156 and AvrM 153, which are sufficient for translocation *in planta* (Rafiqi et al. 2010; Dr M. Rafiqi, personal communication), were found to be insufficient for PtdIns(3)P and PtdIns(5)P lipid binding activity. Thus, the results reported here do not support a role for specific phospholipid binding of AvrL567 or AvrM in protein uptake into cells. Nevertheless, they do not exclude a role of transient, non-specific lipid association by these proteins, such as that observed for the penetratin CPP of Antennapedia and HIV Tat (Drin et al. 2001; Ziegler et al. 2003).

Given that phospholipid binding may not be required for protein uptake, it is interesting to speculate on the potential role of AvrM phospholipid binding during infection. Of the two phospholipids to which AvrM has the highest affinity binding, PtdIns(3)P and PtdIns(5)P, more studies have been conducted on PtdIns(3)P. In plants, PtdIns(3)P constitutes approximately 5 to 15% of total PtdInsPs, making it the second most abundant PtdInsP in plant cells after PtdIns(4)P, which makes up to 80% of cellular PtdInsPs (Meijer et al. 2001). Localisation studies utilising a fluorescent protein fusion to two PtdIns(3)P-binding FYVE domains showed that PtdIns(3)P co-localises with Ara7, an endosomal marker in all tissue types tested (Vermeer et al. 2006). Further, PtdIns(3)P was shown to localise to the vacuolar membrane of leaf epidermal and guard cells and was found in vesicles at newly formed cell plates, although it was absent from newly formed membranes (Vermeer et al. 2006). PtdIns(3)P is believed to function in plants in a variety of processes including late endosomal or recycling endosome trafficking, and plants silenced for the phosphatidylinositol 3-kinase gene responsible for PtdIns(3)P synthesis (AtVPS34) are severely affected in growth and development (Vermeer et al. 2006; Welters et al. 1994; Lee et al. 2008). Kale et al. (2010) reported that PtdIns(3)P is also abundant on the outer leaflet of the plasma membrane, although at present it is unclear what its function on the extracellular leaflet *in planta* may be (Kale et al. 2010).

On the other hand, PtdIns(5)P was only recently discovered in plant and animal cells (Meijer et al. 2001) and much less is known about the function of PtdIns(5)P in eukaryotic cells. Indeed, PtdIns(5)P is relatively more abundant in plant cells than in animal cells (~2%) with up to 18% of the PtdInsP shown to be consisting of this isomer (Meijer et al. 2001). Since no enzymes to date have been found in plants to convert PtdIns to PtdIns(5)P, it is postulated that PtdIns(5)P forms through phosphatase action on PtdIns(3,5)P₂ (Heilmann 2009). In animal systems, this isomer has been indicated to be involved with stress signaling (Jones et al. 2006) and membrane trafficking from the late endosome to the plasma membrane (Lecompte et al. 2008). In plants, there is a possibility that PtdIns(5)P is induced by hyperosmotic stress (Meijer et al. 2001).

In animals, intracellular pathogens such as *Shigella flexnerii*, *Legionella pneumophila* and *Salmonella* spp. secrete proteins that interact with PIs to manipulate host trafficking processes (Weber et al. 2009; Drecktrah et al. 2004). As an example, *Mycobacterium tuberculosis* secretes PI phosphatases into host cells to deplete PtdIns(3)P levels on vacuoles containing the pathogen, which prevents phagosome maturation which would kill the bacterium (Vergne et al. 2005;

Beresford et al. 2007). Given that phospholipid binding is not required for uptake, sequestration of free, available PtdIns(3)P may be an effector function of AvrM. In plants, over-expression of the PtdIns(3)P-binding endosome binding domain (EBD) resulted in inhibition of vacuolar trafficking (Kim et al. 2001). However, cells that expressed the FYVE-binding PtdIns(3)P protein were shown to have two times the level of PtdIns(3)P compared to wildtype, indicating that PtdIns(3)P levels may also be up-regulated to compensate for the reduction in free PtdIns(3)P due to FYVE binding under certain circumstances (Vermeer et al. 2006).

Another potential function of PI binding may be to allow targeting of effectors to specific localisations where cellular targets of AvrM may reside. For example, in the case of *L. pneumophila*, PtdIns(3)P binding anchors the pathogen protein, LpnE, which is required for entry into host cells, to the site of pathogen entry (Weber et al. 2009). This recruits the host lipid metabolising phosphatase Oculocerebrorenal syndrome of Lowe 1 (OCRL1), which may be involved in membrane ruffling, to the site (Weber et al. 2009). While the lipid composition of the extrahaustorial membrane is unknown, in uninfected plant cells, PtdIns(3)P and PtdIns(5)P are potentially localised to endosomal compartments (Vermeer et al. 2006; Lecompte et al. 2008). Interestingly, the M resistance protein, which interacts directly with AvrM in yeast two-hybrid studies (Catanzariti et al. 2010), has been localised to the tonoplast of plant cells (Dr M. Rafiqi, personal communication). Trafficking studies of a GFP-EBD fusion indicate that while PtdIns(3)P is synthesised at the trans-Golgi network, it is transported to prevacuolar compartments before ending up at the tonoplast in vacuolar lumen vesicles (Kim et al. 2001) indicating that AvrM could co-localise with the M resistance protein at the tonoplast by association with PtdIns(3)P although as yet there is no evidence of this.

In summary, it has been shown that while AvrL567 does not appear to have significant PI lipid binding activity, AvrM binds with high affinity to PtdIns(3)P and PtdIns(5)P. Tests of the lipid binding activities of AvrM uptake mutants did not provide any evidence to indicate that lipid binding was required for entry into cells although it is possible that AvrM and AvrL567 transiently associate non-specifically with membranes by virtue of their charges. The tests also do not exclude the possibility of AvrM lipid binding as part of its effector function.

CHAPTER 6

6 Concluding remarks and future directions

Plant pathogenic biotrophs secrete small proteins known as effectors, which contribute to the establishment and maintenance of infection. Thus, in order to develop better disease control measures, elucidating the fate of these proteins during infection is important. With this in mind, the research in this thesis aimed to examine the localisation of the effector AvrM during infection of flax by the obligate fungal biotroph *Melampsora lini*. The work presented in this thesis provides direct evidence that the *M. lini* effector AvrM is secreted from haustoria and also that it is translocated into host cells during infection. These results are consistent with other studies on AvrM, which indicate that it is recognised by a cytoplasmic host resistance protein (Catanzariti et al. 2006; Catanzariti et al. 2010).

Several mechanisms have been proposed for translocation of extracellular proteins into host cells as shown in Figure 6-1. These can be broadly categorised into: (1) direct membrane penetration, (2) transport through a transmembrane transporter and (3) vesicle-mediated transport (Drin et al. 2003; Goldberg and Cowman 2010; Voegelé et al. 2009). For AvrM, the results of immunolocalisation experiments indicate that the effector may be endocytosed into host cells via vesicle-mediated uptake at the extrahaustorial membrane. This work highlights the value of combining high pressure freezing as a technique that allows better preservation of infected material compared to chemical fixatives, together with immunolocalisation at the electron microscope (EM) level which permits analysis of protein localisation at higher resolutions than fluorescence microscopy. Due to these technical advantages, employing these methods to examine the localisations of other host-targeted effectors is likely to be useful in understanding the delivery of other pathogen proteins. For instance, it would be interesting to compare the entry mechanisms of fungal effectors to those of oomycetes, given that the two groups are not closely related and that fungal effectors do not have a conserved uptake motif, whereas oomycetes have at least two known, conserved, uptake motifs (Rafiqi et al. 2010; Whisson et al. 2007; Schornack et al. 2010). Comparisons between the localisations of Crinkler effectors and RXLR effectors may also be noteworthy given that there is some evidence that Crinklers evolved in

oomycetes that do not form haustoria, whereas RXLR effectors are only present in oomycetes that do (Schornack et al. 2010).

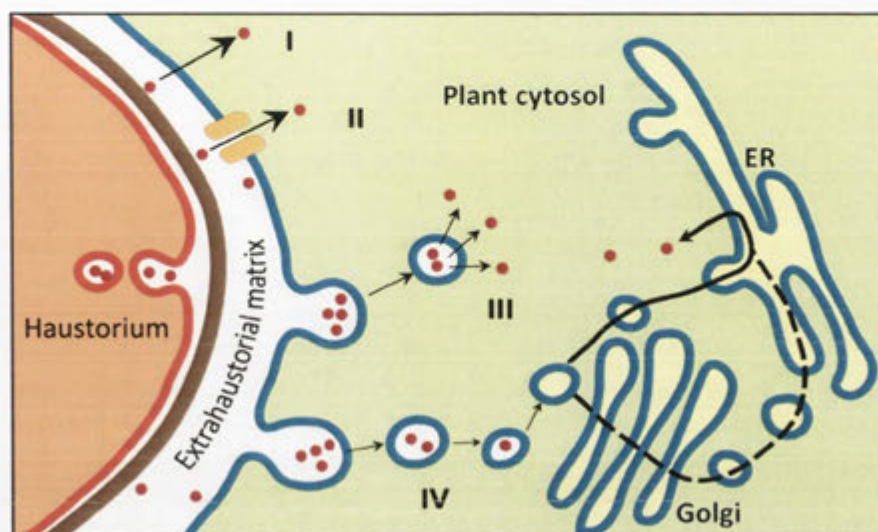


Figure 6-1 Possible routes for effector entry. Effectors (red dots) may enter plant cells in the absence of the pathogen via direct membrane penetration (I), transfer through a transmembrane transporter (II) and/or through vesicle-mediated uptake (III) and (IV). If entry through vesicle-mediated uptake occurs, then effectors may escape vesicles via endosomal escape (III) or via retrotranslocation through the Golgi apparatus (Golgi) and the endoplasmic reticulum (ER) (IV). Figure design taken from Voegelé et al. (2009).

In addition, the cryopreservation and immunogold labeling methods are also well suited to studying the trafficking and secretion of effectors from pathogens. Therefore, they might be useful in analysing the secretion of effectors from *Magnaporthe oryzae* during infection to elucidate the structure and function of the biotrophic interfacial complex, which may be involved in secretion of host-targeted effectors (Khang et al. 2010), or in studying the trafficking of effectors such as Avra10 and AvrK1 from the haustoria of *Blumeria graminis*, which do not possess identifiable signal peptides (Ridout et al. 2006). In the case of pathogens where transformation is possible, generating transformants expressing proteins of interest fused to epitope tags such as Myc or HA would allow the use of commercially available antibodies for immunolocalisation, avoiding the need to raise protein-specific antibodies and thereby potentially speeding up analyses.

Multiple forms of vesicle-mediated uptake have been documented in animal cells including clathrin-, caveolin- and flotillin-dependent uptake as well as forms that do not utilise any of these structural components (Doherty and McMahon 2009; Mayor and Pagano 2007). An important area of future research would be to determine which of these vesicles might be involved in uptake of AvrM. Such experiments would not only provide insights into uptake of effectors but also endocytosis in plants, of which very little is known. One approach to achieve this would be through co-localisation with known markers of the various pathways. As a first approach, pathways involving components with known homologs in plants according to database analysis, such as clathrin and flotillin could be tested. Commercially obtained antibodies against clathrin and flotillin raised against animal endocytic components may be useful in this case, provided that their homologs in flax are sufficiently well-conserved. Preliminary experiments described in Chapter 2 have identified clathrin heavy chain antibodies that are able to recognise proteins in flax plants that could be used for this purpose. Alternatively, co-localisation of proteins in transgenic plants expressing endocytic markers fused to a tag such as Myc, HA or GFP could be performed.

In different forms of vesicle-mediated uptake, escape into the cytoplasm may occur from the endosomes, as in the case of some cell penetrating peptides, or via retrotranslocation, where proteins are transported from the ER to the cytosol, as in the case of the Shiga and ricin toxins (Figure 6-1; Magzoub et al. 2005; Sandvig and van Deurs 1996; Sandvig and van Deurs 2000). Detection of AvrM at the trans-Golgi network of host plant cells is consistent with protein exit from vesicles into the cytosol via retrotranslocation rather than endosomal escape. Future work could aim to determine if this actually occurs by inhibiting retrotranslocation in the host and assessing its effect on AvrM uptake. Approaches to inhibit retrotranslocation include virus-induced gene silencing, the expression of dominant negative CDC48 or the use of chemical inhibitors such as wortmannin (Marshall et al. 2008; Utskarpen et al. 2007), although this last approach may not be suitable in the case of AvrM as it also inhibits PtdIns(3)P metabolism, which may be important for AvrM function given that it binds to PtdIns(3)P as shown in Chapter 5. Since the results in Chapter 4 demonstrate that agroinfiltrated regions undergo cell death when retrotranslocation is inhibited by virus-induced gene silencing, the uptake of proteins purified from a heterologous expression system such as *E. coli* may be tested rather than the uptake of proteins expressed *in planta* by agroinfiltration.

If retrotranslocation is required for AvrM uptake, then how AvrM avoids retrotranslocation from the pathogen ER must also be considered since escape of these proteins from the fungal ER into the cytosol may have unintentional effects. One possibility is that the conventional retrotranslocation process involved in ER associated degradation (ERAD) for quality control of proteins is not conserved in *M. lini*. Fungal retrotranslocation is not well characterised even in fungi that are used for heterologous protein expression such as *Aspergillus niger* (Nevalainen et al. 2005). A recent study generating *A. niger* mutants defective in the ERAD pathway showed that ERAD is not required for normal growth and that other alternative pathways may exist in fungi for clearing of misfolded proteins (Carvalho et al. 2010). One such alternative pathway that exists in mammalian cells is the degradation of proteins within the ER rather than retrotranslocation of proteins before degradation in the cytosol (Evnouchidou et al. 2009). Theoretically, the presence of such a pathway in fungal cells would circumvent the need for the retrotranslocation process. Bioinformatic analyses of the poplar rust and stem rust genomes may be helpful to indicate if key components required for retrotranslocation are conserved in rust fungi, although even if potential homologs of these genes are identified, functional analyses are still required to determine the relative contribution of such pathways. One way to test for the functionality of conventional retrotranslocation is to determine if the ricin toxin, which targets ribosomes, is able to inhibit fungal growth. However, only spores or *in vitro* germinated spores can be tested since the toxin is toxic to plants and as such, this assay cannot be performed *in planta*. . Since very little is known about the promoters of rust, inducible promoters from other basidiomycetes, such as the I-62 *lcc2* or *lcc3* promoters which are induced by alcohol, may be tested (Mansur et al. 1998).

On the other hand, it is possible that rather than a lack of a conserved retrotranslocation pathway, entry of proteins by retrotranslocation into the pathogen cytosol is avoided by post-translational modifications. This is the case with the ricin toxin which is synthesised by the castor bean plant. In this case, the protein is synthesised in the ER as a non-toxic form known as proricin, glycosylated and processed to its mature form - ricin toxins A and B - in the plant vacuole after exiting the ER. Accumulation of ricin in the vacuole does not affect the host cell producing it (Frigerio et al. 1998). However, no evidence of such modifications has emerged for AvrM as yet.

Localisation in the host endomembrane system could indicate that AvrM is interacting with target host proteins in these compartments. Indeed, tests of the lipid binding activity of

AvrM in Chapter 5 indicate that AvrM binds to PtdIns(3)P and PtdIns(5)P, two phospholipids that are associated with endosomes and endosomal trafficking, thus providing a possible mechanism for targeting to these compartments after entry into the cytoplasm. Such a scenario would also provide an explanation as to why AvrM may bind to lipids, given that lipid binding does not appear to be required for uptake, as suggested for other eukaryotic phytopathogenic effectors (Kale et al. 2010). In order to test if AvrM lipid binding is required for targeting within host cells, the region required for lipid binding could be identified through testing the lipid binding properties of further deletions and truncations of AvrM. Then, the localisation of deletion mutants could be analysed within host cells to determine if host endomembrane localisation is still observed.

Lipid binding could also indicate targeting of host lipid-based signalling, which could be a function of pathogen effectors that has not been previously reported. Taking this into consideration, advances in techniques to study lipid signalling and metabolism could be applied to infected tissue. For example, it is possible to isolate lipid binding proteins during infection by affinity purification of crude cell lysates using lipids immobilised to a solid support or using liposomes with an affinity tag such as biotin that can be isolated by affinity chromatography (Rusten and Stenmark 2006; Knodler and Mayinger 2005; Krugmann et al. 2002). Comparisons between proteins isolated from infected and uninfected tissue may identify differentially occurring lipid binding proteins originating from the pathogen that may interfere with lipid metabolism as an effector function.

In terms of the function of AvrM, it was interesting to observe that AvrM was not only found expressed in haustoria but also in secondary hyphae. This opens up the possibility that AvrM may have a role outside, as well as within, plant cells. As such, one area of future research would be to identify the targets of AvrM in plant tissue in order to determine if there is a biological significance to the dual localisation of the protein. Possible methods to do this include yeast two-hybrid screening and co-immunoprecipitation of tagged-proteins expressed from transgenic rust. The protocol to express introduced genes in rust is still being improved and the work described in Chapter 3 of this thesis has contributed to its development by demonstrating that introns may be required for efficient gene expression in *M. lini*. Further, using the flax rust transformation technique, AvrM deletion mutant strains of rust may be generated which may also provide clues about the function of AvrM during infection.

7 Appendix

7.1 Buffers and Media

Table 7-1 List of buffers and media used.

Buffer/Media	Recipe
50 × 5052	0.5% glycerol, 0.05% glucose, 0.2% α-lactose, autoclave
20 × PPS	1 M Na ₂ HPO ₄ , 1 M KH ₂ PO ₄ , autoclave
Lysis buffer	20 mM HEPES pH 8.0, 300 mM NaCl, 10 mM imidazole, filter sterilise
Wash buffer A	20 mM HEPES pH 8.0, 300 mM NaCl, 65 mM imidazole, filter sterilise
Wash buffer B	20 mM HEPES pH 8.0, 300 mM NaCl, 50 mM imidazole, filter sterilise
Wash buffer C	20 mM HEPES pH 8.0, 300 mM NaCl, 20 mM imidazole, filter sterilise
Elution buffer A	20 mM HEPES pH 8.0, 300 mM NaCl, 250 mM imidazole, filter sterilise
Elution buffer B	20 mM HEPES pH 8.0, 300 mM NaCl, 250 mM imidazole, filter sterilise
PBS pH7	2.7 mM KCl, 138 mM NaCl, 1.5 mM KH ₂ PO ₄ , 20.4 mM NaH ₂ PO ₄ , pH 7, autoclave
PBT	PBS pH7 with 0.2% (v/v) Tween-20, autoclave
PBS pH 7.4	0.01 M phosphate buffer, 2.7 mM KCl and 0.137 M NaCl, pH 7.4, autoclave
PBST	PBS pH7.4 with 1% (v/v) Tween-20
TBS	20 mM Tris-Cl, pH 7.4, 500 mM NaCl, autoclave
TBST	TBS and 0.05 % (v/v) Tween-20
LB broth	10 g NaCl, 10 g Bactotryptone (Difco) and 5 g Bacto yeast extract (Difco)/L, pH7, autoclave
LB agar	LB broth with 15 g Bactoagar (Difco), autoclave
SOC	2.0 g Bactotryptone, 0.5 g Bacto yeast extract, 1mL 1M NaCl, 0.25mL 1 M KCl, 1mL filter-sterilised 2M Mg ²⁺ stock [1M MgCl ₂ ·6H ₂ O, 1M MgSO ₄ ·7H ₂ O], 2 mL filter-sterilised 1M glucose, pH7, make up to 100 mL, filter sterilise
YT agar	16 g Bactotryptone, 10 g Bacto yeast extract, 5g NaCl/L, pH 7, autoclave
MS media	4.4 g Murashige and Skoog basal medium (Sigma), 0.5 g MES, 20 g Sucrose/L, pH 5.8, autoclave

MS agar	MS media with 8 g/L Bactoagar, autoclave
Extraction Buffer A	50 mM Tris-Cl pH 7.5, 5 mM EDTA pH 8.0, 10% (v/v) glycerol supplemented with Roche Complete Protease inhibitor
Extraction Buffer B	150 mM NaCl, 20 mM Tris-Cl pH 6.5, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (v/v) β -mercaptoethanol and 1 mM PMSF supplemented with Roche Complete Protease inhibitor
1 $\times$ Laemmli	60 mM Tris-Cl pH 6.8, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 0.1% (w/v) bromophenol blue and 5% (v/v) β -mercaptoethanol
Ponceau S	0.1% (w/v) Ponceau S in 5% (v/v) acetic acid
Hybond-C transfer	25 mM Tris pH8.3, 150 mM glycine, 20% methanol
Alkaline phosphatase buffer	0.1 M Na_2CO_3 , 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 9.8, autoclave
2 $\times$ SSC	0.3 M NaCl and 0.03 M sodium citrate
Protoplasting buffer	1% cellulose, 0.5% BSA, 0.5% macerage, 1% driselase in CPW buffer
CPW buffer	0.5 M mannitol pH 5.6, 0.2 mM KH_2PO_4 , 1 mM KNO_3 , 13.3 mM CaCl_2 , 2 mM MgSO_4
DNA extraction buffer	0.2 M Tris-Cl pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% SDS

Preparation of glycerol stocks

A 5 mL volume of LB broth with the appropriate antibiotics was inoculated with a single bacterial colony and incubated overnight at 37°C for *E. coli* or at 28°C for *Agrobacterium*. The following day, 0.9 mL of liquid culture was added to sterile 0.1 mL 80% glycerol in a microcentrifuge tube, mixed briefly and stored at -80°C.

Running of RNA gels

10 $\times$ MOPS running buffer: 0.2M 3-(N-morpholino)propane sulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, adjust to pH 7.0 with NaOH.

RNA loading buffer: 50% glycerol, 1 mM EDTA pH 8.0, 0.25% bromophenol blue, 50 mM MOPS pH 7.0

RNA loading mix: Two microlitres 10 × MOPS buffer, 10 µL formamide, 3.5 µL 37% formaldehyde, RNA and MilliQ-filtered water to a final volume of 20 µL. Heat in a 65° C water bath for 10 min. Add 2 µL of loading buffer to each tube.

RNA gel: Add 1.8 g agarose to 96 mL MilliQ-filtered water. Boil in microwave, cool to 50-60° C. Add 12 mL 10 × MOPS buffer and 12 mL 37% formaldehyde and pour into gel tank and allow to cool in fume cupboard.

RNA samples are loaded and run within a fume cupboard at approximately 5V/cm.

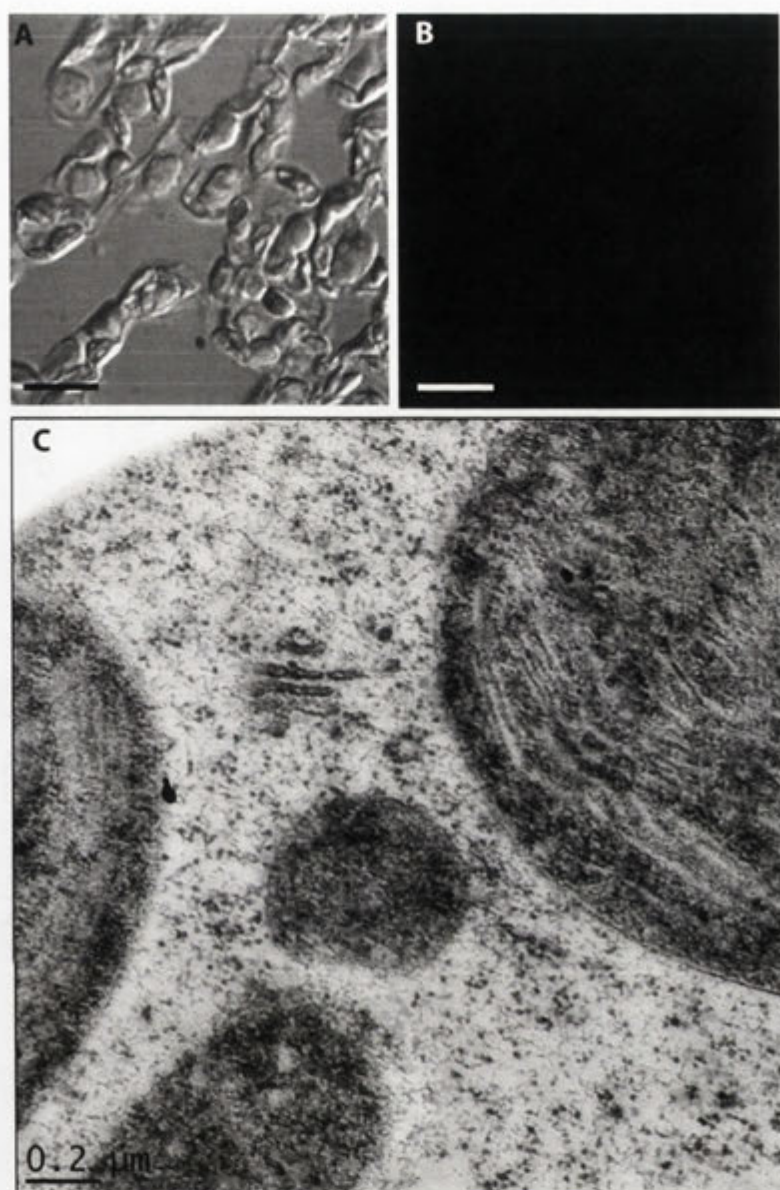


Figure 7-1. Labelling of uninfected plant cells by the anti-AvrM antibody. Labelling of sections preserved for immunofluorescence localisation (A and B). Labelling of a high-pressure frozen section (C).

7.2 Primer list

Table 7-2 List of primers used and sequences.

Name	Sequence
S-tag F	CGAACGCCAGCACATGGACAGC
T7 Rev	TGCTAGTTATTGCTCAGCGGT
AvrM-dSP-LIC-F	GACGACGACAAGACTCAC CCC ATGAACTCAGCAAAAC
AvrM-LIC-R	GAGGAGAAGCCCGGCTCACATGTCTGGAGATTTCATATC
AvrL567-dSP-LIC-F	GACGACGACAAGACTATGGAACATGTACCAGCAGAG
AvrL567-LIC-R	GAGGAGAAGCCCGGCTTAGGTCTTTGGAACCAACTTG
AvrM-106-LIC-F	GACGACGACAAGACTATCCCTCAACCAGAATTTGACAG
AvrM-153-LIC-R	GAGGAGAAGCCCGGCTTACAGGTCTCGTACATTTTTATC
AvrM-148-LIC-R	GAGGAGAAGCCCGGCTTATTTATCTTTTCTGCCATGTATGTG
AvrL567-26-LIC-F	GACGACGACAAGACTCATGTACCAGCAGAGTTGACC
AvrL567-50-R	GAGGAGAAGCCCGGCTTATATTACACTAGCCGTTGGGGACCG
AvrL567-d27-37-F	GACGACGACAAGACTATGGAATATACACGATTTTACC
SacI-SbfI-pAvrM-Fwd	TAGATGAGCTCCTGCAGGCAGTAACCTTTGTGAATTAAGTTGAC
AvrM-XbaI-Rev	TACTATCTAGACATGTCTTTATAATTTTAGGTC
XbaI-NcoI-tAvrM-Fwd	ATTAGTCTAGATAGTCCATGGTATAAGAGCGCTGGATTTCAGCC
Sall-SbfI-tAvrM-Rev	ATGTGGTCGACCTGCAGGGCCTGATTGACCCGTTTTCTC
pAvrM-XbaI-Rev	TAGTATCTAGAGAGTGAATCGATGCTGATTGGTC
NcoI-Cerulean rev	CATTCCATGGTTACTTGTACAGCTCGTCCATGC
XbaI-Cerulean	CTCTCTAGAATGGTGAGCAAGGGCGAGGAGC
TUB1 qF	TCCCTGGTCAACTCAACTCCG
TUB1 qR	GGTATTGTTGACTTCCACGAGCAG
Cerulean qF	GCTCGCCGACCACTACCAG
KpnI-LIC adaptor 1-PstI- ccdB-F	ATAGGTACCGATGACGACGACAAGACTGCAGGCTAAGGAAGCTA AACTTTTGCTGACG
XhoI-LIC adaptor 2-PstI- ccdB-R	TATCTCGAGTGAGGAGAAGCCCGGCTGCAGCAGACTGGCTGTGTA TAAGGGAGCCTG
pYY13F	CGACGACAAGACCCT
pYY13R	GAGGAGAAGAGCCCT
Clal-NtAP180-317 F	AAGATCGATGGATCAATCTTCATTTCCAG
XbaI-NtAP180 R	TGTTCTAGATTAAGACGTATGCTTAGCTATGATC
Clal-NtAP180-C-term F	AAGATCGATGCCTACTATTTCTAACAAC
LIC-AP180-for	CGACGACAAGACCCTGAGGAATTGGATCGTTGCC
LIC-AP180-rev	GAGGAGAAGAGCCCTGATGTCCTGCCAACTCAATAC
LIC-EHD1-for	CGACGACAAGACCCTCACGCCAGGATCTCAAGCAGG
LIC-EHD1-rev	GAGGAGAAGAGCCCTCCCCGGAATACTCCTTTTCATCAG
LIC-Flot1-for	CGACGACAAGACCCTGCTGCATCAATGACAATGGAGG

LIC-Flot1-rev	GAGGAGAAGAGCCCTCCACTTCCCTCTGCAATTCGG
LIC-NtRac1-for	CGACGACAAGACCCTCAATGGGAGTACTGTCAACCTAG
LIC-NtRac1-rev	GAGGAGAAGAGCCCTCTGCTTGGGTGGCTGGAGC
LIC-EHD2-for	CGACGACAAGACCCTTGTACATCAATAGTTGATGG
LIC-EHD2-rev	GAGGAGAAGAGCCCTAGTAAGCAACACTGAAGGTG
LIC-a-ADR-for	CGACGACAAGACCCTGGCTTTTCCAAATTTCTCAG
LIC-a-ADR-rev	GAGGAGAAGAGCCCTCATAAGGTGTCAACCCC
LIC-CDC48-for	CGACGACAAGACCCTGCTCTCAGAAGGTTTGG
LIC-CDC48-rev	GAGGAGAAGAGCCCTCCAAACCACATGG
LIC-M-for	CGACGACAAGACCCTCGAATTTGAAGTGG
LIC-M-rev	GAGGAGAAGAGCCCTGAAGTGGATAACTC
CRL-LIC-F	GACGACGACAAGACTATGGTGAGCAAGGGCGAGGAG
CRL-LIC-R	GAGGAGAAGCCCGGCTTACTTGTACAGCTCGTCC
STMV Rev	CGAAAGGTATGGCTCTGAC
STMV Fwd	CAATCAAAAGACCTAACCAACAG
PDS-NotI-rev	TATGCGGCCGAGCTTGAAGATATCCACT
PDS-SbfI-for	TCTCTGCAGGTTAGATGGTAACCCTCTGAGAGAC
RT-AP180F	CTCCTCCAACAAAGCCTATGC
RT-AP180R	ATCATCGCGGAAGCTAGAGA
RT-FLOT1F	GGAGAGGATGAATGCTTTGGC
RT-FLOT1R	GTAATCAATGATGTTGTCAAAGGC
RT- CDC48F	GATGTGGGAAAACCTTTCTTGC
RT-CDC48R	GCAGACTGTCTGGCCTTGTC
RT-A-ADRF	TCCAAAGTATCCCAGAGAAGC
RT-A-ADRR	ATATTTCCAACCAAAGTCAATGC
RT-M-F	AAGGTAGCGTGTGGTGGAAG
RT-M-R	TAGCCCATCCAAATCACCTC
RT-AP180F	CTCCTCCAACAAAGCCTATGC
RT-AP180R	ATCATCGCGGAAGCTAGAGA
RT-Flot1F	GGAGAGGATGAATGCTTTGGC
RT-Flot1R	GTAATCAATGATGTTGTCAAAGGC
RT-Rac1F	GTGCTCCAGCCACCCAAG
RT-Rac1R	CGAGACAAAGGAAAAACAAAGG
RT-EHD1F	ACATCCAAAACTAAAGCCTCTG
RT-EHD1R	CTGTCTGTGGTAGGCTCTGGC
RT-EHD2F	CAGAGCCTACCACCGACAG
RT-EHD2R	CAGTTCCAAAGTGTGACAGCGC

7.3 Alignments of sequences encoding host endocytic components

At- α -C-adr	MTGMRGLSVFISDVNRCNKEAERLRVDKELGNIRTCFKNEKVLTPYKKKKYVWKMLYIH	60
At- α -adr	MTGMRGLSVFISDVNRCNKEAERLRVDKELGNIRTCFKNEKVLTPYKKKKYVWKMLYIH	60
Nb- α -adr	---MRGLSVFISDIRNCONKEAERLRVDKELGNIRTRFKNEKGLTPYEKKKYVWKMLYIY	57
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	MLGYDVPFGHMEAVSLISAPKYPEKQVGYIVTSCLLNENHDFLKLAINTVRNDIIGRNET	120
At- α -adr	MLGYDVPFGHMEAVSLISAPKYPEKQVGYIVTSCLLNENHDFLKLAINTVRNDIIGRNET	120
Nb- α -adr	MLGYDVPFGHMEAVSLISAPKYPEKQVGYIVTSCLLNENHDFLRLAINTVRNDIIGRNET	117
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	FQCLALTIVGNIGGRDFAESLAPDVQKLLISSCRPLVRKKAALCLRLFRKNPDVAVNVD	180
At- α -adr	FQCLALTIVGNIGGRDFAESLAPDVQKLLISSCRPLVRKKAALCLRLFRKNPDVAVNVD	180
Nb- α -adr	FQCLALTIVGNIGGRDFAESLA-----	139
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	GWADRMQQLDERDLGVLTSSSTLLVALVSNNEAYSSCLPKCVKILERLARNQDVPQEY	240
At- α -adr	GWADRMQQLDERDLGVLTSSSTLLVALVSNNEAYSSCLPKCVKILERLARNQDVPQEY	240
Nb- α -adr	-----	
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	TYYGIPSPWLQVKAMRALQYFPTIEDPSTRKALFEVLQRILMGTDVVKVNNKNASHAVL	300
At- α -adr	TYYGIPSPWLQVKAMRALQYFPTIEDPSTRKALFEVLQRILMGTDVVKVNNKNASHAVL	300
Nb- α -adr	-----	
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	FEALSLVMHLDAEKEMMSQCVALLGKFISVREPNIIRYLGLNMTMLMVTVDQDIKKHQ	360
At- α -adr	FEALSLVMHLDAEKEMMSQCVALLGKFISVREPNIIRYLGLNMTMLMVTVDQDIKKHQ	360
Nb- α -adr	-----	
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	SQIITSLKDPDISIRRRALDLYGMCDSNAKDIVEELLQYLSTAEFMSREELSLSKAAIL	420
At- α -adr	SQIITSLKDPDISIRRRALDLYGMCDSNAKDIVEELLQYLSTAEFMSREELSLSKAAIL	420
Nb- α -adr	-----	
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	AEKFAPDLSWYVDVILQLIDKAGDFVSDDIWFRVVQFVTNNEDLQPYAASKAREYMDKIA	480
At- α -adr	AEKFAPDLSWYVDVILQLIDKAGDFVSDDIWFRVVQFVTNNEDLQPYAASKAREYMDKIA	480
Nb- α -adr	-----	
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	IHETMVKVSAYILGEYGHLLARQPGCSASELFSILHEKLPVSTPTIPILLSTYAKLLMH	540
At- α -adr	IHETMVKVSAYILGEYGHLLARQPGCSASELFSILHEKLPVSTPTIPILLSTYAKLLMH	540
Nb- α -adr	-----	
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	AQFPDPELQKKVNAVFKKYESCIDVEIQRAVEYFELSKKGPAFMDVLAEMPKFPERQSS	600
At- α -adr	AQFPDPELQKKVNAVFKKYESCIDVEIQRAVEYFELSKKGPAFMDVLAEMPKFPERQSS	600
Nb- α -adr	-----	
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	LIKKAENVEDTADQSAIKLRAQQQPSNAIVLADQPVNGAPPPLKVPILSGSTDPEVSAR	660
At- α -adr	LIKKAENVEDTADQSAIKLRAQQQPSNAMVLADQQPVNGAPPPLKVPILSGSTDPEVSAR	660
Nb- α -adr	-----	
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	SLSHPNGTSLNIDPQTSPDLLSDLLGLAIEAPPGAVSYEQHGPVGAEGVPDEIDGSAI	720
At- α -adr	SLSHPNGTSLNIDPQTSPDLLSDLLGLAIEAPPGAVSYEQHGPVGAEGVPDEIDGSAI	720
Nb- α -adr	-----	
*****;*****;*****;*****;*****;*****;		
At- α -C-adr	VPVEEQNTVELIGNIAERFHALCLKDSGVLYEDPHIQIGIKAEWRGHHGRVLVFMGNKN	780
At- α -adr	VPVEEQNTVELIGNIAERFHALCLKDSGVLYEDPHIQIGIKAEWRGHHGRVLVFMGNKN	780
Nb- α -adr	-----	

Figure 7-2 Alignment of the putative sequence of *Nicotiana benthamiana* α -adaptin to *Arabidopsis thaliana* α -adaptin sequences.

[illegible]

Figure 7-4 Alignment of predicted AP180 protein sequence from tomato (SIAP180-2) against Arabidopsis AtAP180 protein sequence identified by Barth and Holstein (2004). Black lines: ANTH-domain; Red boxes: putative ANTH lipid binding domain; blue boxes: endocytosis motifs on AtAP180; green boxes: endocytosis motifs on SIAP180-2.

NtAP180-C	RSSGTASSKPGSMKSSKSRHDSYGGQSEFSVATTEPYSERSTTTSGIGSPCSSLEELIRA	60
NtAP180-C_EST	-----	
NtAP180-C	TETGRKHPSISIDLEAYSIDIQFRKQCSEDVCDTGSARSLPVSMIDLVSNNWPGDDEYEN	120
NtAP180-C_EST	-----	
NtAP180-C	NEVKRQNPQPVVEKEREVNKDESKQEQPKKEKSLDSSSAKGWEAVLNEALTPSPSPSF	180
NtAP180-C_EST	-----	
NtAP180-C	GAFPEQQEPKQMSRTEANASSVEATSNNNGWDLGLFEATPQTKSEQPMPNTTANKVESFNT	240
NtAP180-C_EST	-----	
NtAP180-C	LPLPSFNAFQERQEQQLSRIGANVLSTEVSSCNNGWDLALFEATPQTKASQTLPTISNN	300
NtAP180-C_EST	-----AMPTISNN	8
	::* ***	
NtAP180-C	VNSFNSSTLDELYNKNPMSLFPNSDLSSLPIPNMNGNIYDQNPSTTFLPASN---NQIPV	357
NtAP180-C_EST	IN---SSTLDELNFQRSVSLFPNSGLSSLPMPTNTSSSYDQNPSTTFLPASNNAYNRIPV	65
	:* *****:~::~*****.*****:~*~ ***** ***** *:***	
NtAP180-C	MPQPHHYNPFQDTSTELPSNKFESTAAPTFQATPTFSAQDFSSAVVSDVNSDPEGTFS	417
NtAP180-C_EST	MAQANHYNPFQDTSTELPSNMLSTTAAPTFOATPTFSAQSSFSVQRDVNSDPEGTFS	125
	~~:*****~*~:*****~*~ ***** *****	
NtAP180-C	SEQMLNGAINQQNLLHEQQLWLQNQNKIIAKHTS	451
NtAP180-C_EST	SEQMLNGAINQQNLLHEQQLWLQNQNKIIAKHTS	159

Figure 7-5 Alignment of predicted C-terminal sequences of putative AP180 homologs from tobacco. Blue boxes: endocytosis motifs on NtAP180-C; red boxes: endocytosis motifs on NtAP180-C (EST).

AtEHD1	MEIESVAAGSCSKENQMIYKEWFEFSDSGDGRITGNDAIKFFTMSNLPPELKQIWAIA	60
NbEHD1	MEFDTSPISRCSKEHEKIYQOWFSLADSDGDGRITGGDAIKFFALSNLRSQDLKQVWAIA	60
	::: . . **: **::**::*****:*.*****:***. * :***:***	
AtEHD1	DSKRQGYLGFKEFIVAMQLVSLAQTGHEISHEVLISDVDFKNINPPTMEGLGVLMAKKKH	120
NbEHD1	DSKRQGFGLGFREFITSMQLVSLAQAGHPVTSDLLNAEVNFENLQPPPTLEGLDLLQAKKKR	120
	*****:***:***.*****:*** :: :* :*:***:***:***.*** :***:	
AtEHD1	SSKSSDPNMNGSPAADTSLTAHWFS--KSSKKISLSSVTSIVDGLKRLYIQKLKPLEVAY	179
NbEHD1	APKS--EPEQNGNPTVQSSVTANWFFSSTS VKKVSLSVTSIIDGLKKLYIQKLKPLEVAY	179
	::** :*: *.**:::***:***. * **:***:***:***:*****	
AtEHD1	RFNDFVSPLLTNSDFDAKPMVMMLGQYSTGKTTFIKHLKSTYPGAHIGPEPTTDRFVVV	239
NbEHD1	RFNDFVSPFLTNSDFDAKPMVMMLGQYSTGKTTFIKHLRSTYPGAHIGPEPTTDRFVVV	239
	*****:*****:*****:***:***:*****	
AtEHD1	MSGPDERSIPGNTVAVQADMPFSGLTTFGTAFLSKFECSQMPHPLLEHVTFFVDTPGVLSG	299
NbEHD1	MNGPDERSIPGN-----	251
	*.*****	
AtEHD1	EKQRTQRAYDFTGVTSWFASKCDLILLFDPHKLDVSDFKRVISSLRGHDDKIRVVLNK	359
NbEHD1	-----	
AtEHD1	ADQVDTQQLMRVYGALMWSLGKVLNTPEVSRVYIGSFSDKPINEAATGPIGRELFEKEQD	419
NbEHD1	-----	
AtEHD1	DLLADLKDI PKKACDRRINEFVKRARA AKIHAYII SHLKKEMPAIMGKAKAQKKLIDNLE	479
NbEHD1	-----	
AtEHD1	DEFGKVQREHHL PKGDFPNVDHFREVLSGYNIDKFEKLKPKMLQTVDMDLGYDIPPELLKN	539
NbEHD1	-----	
AtEHD1	FKNPYD 545	
NbEHD1	-----	

Figure 7-6 Alignment of the partial NbEHD1 sequence against AtEHD1 (Bar et al. 2008). The region encoding NbEHD1 residues 48-241 was targeted for silencing.


```

OsRac6      ----MSASRFIKCVTVGDGAVGKTCMLISYTSNTFPTDYVPTVFDNFSANVVVDGNTVNL 56
NtRac1      ----MSASRFIKCVTVGDGAVGKTCLLISYTSNTFPTDYVPTVFDNFSANVVVNGSTVNL 56
OsRac5      ----MSASRFIKCVTVGDGAVGKTCMLISYTSNTFPTDYVPTVFDNFSANVVVDGNTVNL 56
OsRac7      ----MSTARFIKCVTVGDGAVGKTCMLISYTSNTFPTDYVPTVFDNFSANVVVDGNTVNL 56
OsRac3      --MASSASRFIKCVTVGDGAVGKTCMLICYTSNKFPPTYIPTVFDNFSANVVVDSTTVNL 58
OsRac4      --MASSASRFIKCVTVGDGAVGKTCMLICYTSNKFPPTYIPTVFDNFSANVVVDGTTVNL 58
OsRac1      MSSAAAATRFIKCVTVGDGAVGKTCMLICYTCNKFPPTYIPTVFDNFSANVSVDGSSVNL 60
OsRac2      MS---GATKFIKCVTVGDGAVGKTCMLICYTSNKFPPTYIPTVFDNFSANVSVDGNI VNL 57
              .:::*****:*.:.:*****:*****:*.:.:***

OsRac6      GLWDTAGQEDYNRLRPLSYRGADVFLAFSLISKASYENVSKKWIPELKHYAPGVPIILV 116
NtRac1      GLWDTAGQEDYNRLRPLSYRGADVFLAFSLISKASYENVSKKWIPELKHYAPGVPIVLV 116
OsRac5      GLWDTAGQEDYNRLRPLSYRGADVFLAFSLISKASYENVSKKWIPELRHYAPGVPIILV 116
OsRac7      GLWDTAGQEDYNRLRPLSYRGADVFLAFSLISKASYENIHKKWIPELRHYAPNVPIVLV 116
OsRac3      GLWDTAGQEDYNRLRPLSYRGADVFLAFSLVSRASYENIMKKWIPELQHYAPGVPIVLV 118
OsRac4      GLWDTAGQEDYNRLRPLSYRGADVFLAFSLVSRASYENVMKKWLPELQHYAPGVPIVLV 118
OsRac1      GLWDTAGQEDYSRLRPLSYRGADVFLSFLISRASYENVQKKWMPELRRFAPGVPIVLV 120
OsRac2      GLWDTAGQEDYSRLRPLSYRGADIFVLAFSLISRASYENVLKKWMPELRRFAPNVPIVLV 117
              *****:*****:*****:*****:*****:*****:*****:*****:*****

OsRac6      GTKLDLRDDKQFFVDHPGAVPITTAQGEELRKQIGAPYYIECSSKTKQLNVKGVFDAAIKV 176
NtRac1      GTKLDLRDDKQFFIDHPGAVPITTAQGEELRKTIGAPAYIECSSKTKQNVKAVFDAAIKV 176
OsRac5      GTKLDLRDDKQFFVDHPGAVPISTAQGEELRKLIGAAAYIECSSKTKQNIKAVFDAAIKV 176
OsRac7      GTKLDLRDDKQFFLDHPGLAPISTAQGEELKRMIGAAAYIECSSKTKQNVKSVFDSAIV 176
OsRac3      GTKLDLRDDKHYLLDHPGMI PVTTAQGEELRKQIGAAAYIECSSKTKQNVKGVFDAAIKV 178
OsRac4      GTKLDLRDDKHYLLDHPSLVPVTTAQGEELRKHIGATCYIECSSKTKQNVKAVFDAAIKV 178
OsRac1      GTKLDLRDDRAYLADHPASSIITTEQGEELRKLIGAVAYIECSSKTKQNIKAVFDTAIKV 180
OsRac2      GTKLDLRDHRSYLADHPAASAITTAQGEELRKQIGAAAYIECSSKTKQNIKAVFDTAIKV 177
              *****:..::***:..::******:*** *****:*.:.:*****:*****

OsRac6      VLQPPKAK--KKKKAQKGACSII----- 197
NtRac1      VLQPPKQK--KKKGKAQKCSII----- 197
OsRac5      VLQPPKQK--KKKKKAQKCAII----- 197
OsRac7      VLCPPKPK--KKNTRKQSCWII----- 197
OsRac3      VIQPPTKQREKKKKKSRQGCSMMN--MFRGRKMSCFKS 214
OsRac4      VIKPPTKQDRKKKKKTRRGCSFFCKGVMSRRRLVCFK- 215
OsRac1      VLQPPRHKDVT--RKKLQSSSNRPVRRYFCGSACFA- 214
OsRac2      VLQPPRRRGETTMAKKTRRSTGCSLKNLMCGSACVV- 214
              *:  *  :

```

Type I

Type II

Figure 7-8 Alignment NtRac1 against Rac proteins from rice (Chen et al. 2010) showing the grouping of proteins into Type I Racs, with CaaL motifs (a, aliphatic amino acid) and Type II, which do not. Red boxes: CaaL motifs; blue line: polybasic C-terminal region; basic residues in the polybasic C-terminal region (R/K) are coloured red. The region encoding NtRac1 residues 50-183 were targeted for silencing.

NgCDC48	PALRRFGRFDREIDIGVPDEVGRLEVLRIHTKNMKLAEEVDLERISKDTHGYVGADLAAL	60
NbCDC48	PALRRFGRFDREIDIGVPDEVGRLEVLRIHTKNMKLAEEVDLERISKDTHGYVGADLAAL	60
NtCDC48	PALRRFGRFDREIDIGVPDEVGRLEVLRIHTKNMKLAEEVDLERISKDTHGYVGADLAAL	60
AtCDC48-A	PALRRFGRFDREIDIGVPDEIGRLEVLRIHTKNMKLAEDVDLERISKDTHGYVGADLAAL	60
AtCDC48-B	PALRRAGRFDALVEVSTNEEDRLKILQLYTKKVNLDPSVDLQAIASCNQYVGADLEAL	60
AtCDC48-C	PALRRSGRFETEIALTPADEDRAAEILSVVAQKLRLEGFPDKKRIARLTPGFVGADLESV	60
	***** ** : : . : * * : * : : : . * . * : * . * : ***** :	
NgCDC48	CTEAALQCIREKMDVIDLEDD-----SIDAEILNSMAVTNEHFQTALGTSNPS	108
NbCDC48	CTEAALQCIREKMDVIDLEDD-----SIDAEILNSMAVTNEHFQTALGTSNPS	108
NtCDC48	CTEAALQCIREKMDVIDLEDE-----TIDAEILNSMAVTNEHFQTALGTSNPS	108
AtCDC48-A	CTEAALQCIREKMDVIDLEDD-----SIDAEILNSMAVTNEHFHTALGNSNPS	108
AtCDC48-B	CREATISASKRSSD-----SLILTSQDFKIAKSVVGPS	93
AtCDC48-C	AYLAGRKAIKRILDSRKSEQSGDGEDDKSWLRMPWPEEELEKLFVKMSDFEEAVNLVQAS	120
	. * .. : . * : : : . . . * . . *	
NgCDC48	ALRETVEVPNVSWEDIGGLENVKRELQETVQYPVEHPEKFEKFGMSPSKGVLFYGPFGC	168
NbCDC48	ALRETVEVPNVSWEDIGGLENVKRELQETVQYPVEHPEKFEKFGMSPSKGVLFYGPFGC	168
NtCDC48	ALRETVEVPNVSWEDIGGLENVKRELQETVQYPVEHPEKFEKFGMSPSKGVLFYGPFGC	168
AtCDC48-A	ALRETVEVPNVSWNDIGGLENVKRELQETVQYPVEHPEKFEKFGMSPSKGVLFYGPFGC	168
AtCDC48-B	INRGITVEIPKVTWDDVGGLDKLKKLQQAVERNPIKHSAAAFVKMGISPMRGILLHGPPGC	153
AtCDC48-C	LTREGFSIVPDVKWDDVGGLDHLRLQFNRYIVRPIKKPDIYKAFGVDLETGFLLYGPFGC	180
	* : *. *. * : * : * : : : : : * : : : : * : * : * : * : * : *	
NgCDC48	GKTL LAKAIANECQANFISVKGPELLTMWFG	199
NbCDC48	GKTL LAKAIANECQANFISVKGPELLTMWFG	199
NtCDC48	GKTL LAKAIANECQANFISVKGPELLTMWFG	199
AtCDC48-A	GKTL LAKAIANECQANFISVKGPELLTMWFG	199
AtCDC48-B	SKTTLAKAAAANAQAASFFSLSCAELFSMYVG	184
AtCDC48-C	GKTLIAKAAAANAAGANFMHIKGAELLNKYVG	211
	. * : * * * * * : * : * : * : * : * : * : * : * : * : * : *	

Accession numbers of sequences of plant endocytic components

Table 7-3 NCBI GenBank accession numbers of plant endocytic components. Where possible, the nucleotide sequences of mRNA are provided. Amino acid sequences could be predicted from the coding regions of these sequences. Otherwise, protein coding sequences are provided.

Sequence	Accession number
Antennapedia	AAS65114.1
At α -Adaptin	AY099568
At α C-Adaptin	NM_122184
At1g03050	DQ446223.1
At1g14910	AY096496.1
At2g01600	AY133828.1
At2g25430	NM_128100
At4g02650	NM_116499.2
At4g25940	NM_118727.4
At5g35200	AY081284.1
AtAP180 (At1g05020)	BT005184.1
AtCDC48-A	P54609.1
AtCDC48-B	Q9ZPR1.1
AtCDC48-C	Q9SS94.2
AtEHD1	NM_112920
AtEHD2	NM_202788.1
AtFLOT1	Q501E6
AtFLOT2	AAY56411
AtFLOT3	ABJ17152
Avr1b	AAM20937.1
AvrL567-A	AAS66948.1
AvrM-A	ABB96258.1
AvrP123	ACD49721.1
AvrP4	ACD49710
HsRac1	CAB53579.5
MARCKS	P12624
MtFLOT1	ADA83094
MtFLOT2	ADA83095
MtFLOT3	ADA83096
MtFLOT4	ADA83097
MtFLOT6	ADA83098
NbCDC48	GO608110
NbEHD1	FG164661
NgCDC48	ACS28251.1
Nt α -Adr	FS382929
NtADR	FS382929
NtAP180-C(EST)	AM82660
NtAP180-N	ET929898
NtAP180-N	ET929898

NtCDC48	ABF59516
NtEHD2	BP133939
NtFLOT	FH671323
NtRac1	AY029330
OsRAC1	AB029508
OsRAC2	AB029509.1
OsRAC3	AB029510.1
OsRAC4	Q67VP4.1
OsRAC5	Q6EP31
OsRAC6	Q6ZHA3.1
SIAP180-1	AC209585 (nt 109825-10744)
SIAP180-2	AC232643 (nt 63245–64597)
SIFLOT1	AC240868 (nt 100953-99077)
SIFLOT2	AC23846 (nt 105031-107169)
SIFLOT3	AC209510 (nt 8255-6826)
Tat	BAF32556.1

TRV -targeted sequences

>adaptin

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```

>cdc48

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AAGTTTGAGAAATTTGGTATGTACCTTCAAAAGGTGTTCTTTTCTATGGTCCCCCAGGATGTGGGAAACTC
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>EHD1

```
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GATGAAAGGAGTATTCCGGGG
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>Rac1

```
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TTCGGGATGATAAGCAGTTCTTCATAGACCATCCTGGTGCTGTGCCAATTACTACTGCTCAGGCTGAGGAGCT
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GATGCTGCTATTAAGGTCGTGCTCCAGCCACCCAAGCAG
```

>Flot

```
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CAGAAACAAGGATTATATCGACACAAAGGGAAGGAGAAGGGAAGAAAGAGGAGATGAAGGTGAAGACTGATGT
GAAGGTTTATGAAAACCATAGAGAAGCAGAAGTTGCTGAAGCAAATGCAGAATTGGCAAAGAAGAAAGCAGGG
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GGGAAGTGG
```


>AP180

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ATTGGCAACGATTGATTGAGAGAGCAGTCGCTACAAGGCCAACAGGGGCAGCTAAGACGAATCGTCTTGTCCA
AATTGCTTTATATGCTGTTGTTCAAGAAAGTTTTGATCTTTACAAAGATATATCAGATGGGCTTGCTCTTGTT
CCTGATAGTTTTTTCCATTTACCATATCAATCATGTGTAAATGCATTCCAACTTGTGTCAAAGCAGCTAAAC
AATTTGAAGAAATTTGTGCTTTTTATTCTGTGTGTAAAAGTATTGGAGTTGGCAGGACATC

>EHD2

AGTAAGCAACACTGAAGGTGAAATGCACAACCTACCAACAATGGAGAGACAAAATCATTTAAAGTGATATGCGA
CTTCTAGCGGCTTTAGCTTTTGGATGTACAACCTCTTCAGTCCATCAACTATTGATGTGACA

7.4 SVMProt prediction

Table 7-4 Results indicating the predicted protein families of various effectors as predicted by SVMProt analysis. R-value represents a reliability score with a higher score representing a higher confidence. P-value indicates expected classification accuracy. Predictions of lipid binding activity are highlighted in yellow.

Sequence	Predicted protein family name	R-value	P-value (%)
AvrM	Outer membrane	1.0	58.6%
	Magnesium-binding	1.0	58.6%
AvrL567	Metal binding	1.0	58.6%
	DNA repair	1.0	58.6%
	Calcium-binding	1.0	58.6%
AvrP123	All lipid-binding proteins	1.0	58.6%
	Hormone	1.0	58.6%
	Metal-binding	1.0	58.6%
	Outer membrane protein secreting main terminal branch family	1.0	58.6%
	Transmembrane	1.0	58.6%
	Hydrolase	1.0	58.6%
	Amino acid-polyamine-organocation (APC) family	1.0	58.6%
AvrP4	All lipid-binding proteins	1.0	58.6%
	Outer membrane protein secreting main terminal branch family	1.0	58.6%
	Transmembrane	1.0	58.6%
	DNA replication	1.0	58.6%
Avr1b	All lipid-binding proteins	2.0	83.9%
	Hormone	1.0	58.6%
	Metal-binding	1.0	58.6%
	Copper-binding	1.0	58.6%
	Calcium-binding	1.0	58.6%
Avr1b (RXLR to AAAA)	All lipid-binding proteins	1.2	65.4%
	Hormone	1.0	58.6%
	Photosynthesis	1.0	58.6%
	Magnesium-binding	1.0	58.6%
Avr1b (RSLR to AAAA)	All lipid-binding proteins	1.5	73.8%
	Hormone	1.0	58.6%
	Metal-binding	1.0	58.6%
	Calcium-binding	1.0	58.6%
	Magnesium-binding	1.0	58.6%
Avr1b (RFLR to AAAA)	All lipid-binding proteins	1.4	71.3%
	Hormone	1.0	58.6%
	Metal-binding	1.0	58.6%
	Calcium-binding	1.0	58.6%
	Magnesium-binding	1.0	58.6%
Avr1b (dEER-)	All lipid-binding proteins	2.0	83.9%
	Hormone	1.0	58.6%
	Metal-binding	1.0	58.6%
	Copper-binding	1.0	58.6%
	Calcium-binding	1.0	58.6%
Avr1b (RFLR to QFLR)	All lipid-binding proteins	2.0	83.9%
	Hormone	1.0	58.6%
	Metal-binding	1.0	58.6%
	Copper-binding	1.0	58.6%
	Calcium-binding	1.0	58.6%

HIV Tat	Zinc-binding	1.2	65.4%
	Aptamer-binding protein	1.1	62.2%
	Metal-binding	1.0	58.6%
	RNA-binding	1.0	58.6%
Penetratin	Zinc-binding	1.5	73.8%
	Nuclear receptors	1.2	65.4%
	All DNA-binding	1.0	58.6%
	Metal-binding	1.0	58.6%
	Calcium-binding	1.0	58.6%
	Magnesium-binding	1.0	58.6%
MARCKS	All lipid binding proteins	3.5	96.4%
	Zinc-binding	1.0	58.6%
	Actin binding	1.0	58.6%
	DNA repair	1.0	58.6%

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