THE ROLE OF AUTOPHAGY IN *ARABIDOPSIS THALIANA* DURING BIOTROPHIC AND HEMIBIOTROPHIC FUNGAL INFECTIONS

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ABSTRACT

A plants response to pathogen infection is tailored dependent on infection strategy. Successful plant pathogens employ various infection strategies to avoid or reduce plant defense responses for the establishment of host compatibility. Autophagy is a non-selective degradation pathway conserved in eukaryotic organisms, which has been implicated in the regulation of cell survival or cell death, depending on cell type and stimulus. In *Arabidopsis thaliana*, an autophagic response has been reported to be activated during nutrient deprivation. Cellular contents, such as cytoplasm and organelles, are sequestered into double-membraned autophagosomes and delivered to the vacuole for degradation; degradative products, such as amino acids, are released back into the cell and reutilized to maintain cellular function. In this study, the response of the autophagy pathway was investigated in *A. thaliana* leaf tissues upon biotrophic *Erysiphe cichoracearum* and hemibiotrophic *Colletotrichum higginsianum* infections. Expression of some autophagy genes was induced in *A. thaliana* at 9 days post infection with *E. cichoracearum* and, 3 and 5 days post infection with *C. higginsianum*. Using a transgenic *A. thaliana* plant line over expressing autophagosome associated protein autophagy-8e (*ATG8e)* conjugated to green fluorescent protein (GFP) (*ATG8e-GFP*), confocal analysis revealed that autophagosomes specifically accumulated at the infection sites during *E. cichoracearum* and *C. higginsianum* invasions. These results indicate that the plant autophagic pathway responds to an interaction between *A. thaliana* and fungal pathogens. None of the defense signaling molecules including salicylic acid, jasmonic acid, ethylene, hydrogen peroxide and nitric oxide consistently triggered expression of autophagy genes. The insensitivity to defense signaling molecules and the delayed induction of autophagy genes compared to expression of pathogenesis-related genes suggest that the activation of this pathway does not contribute to host resistance responses during the infection process. In *A. thaliana* mutants, *atg4a/b, atg5-1, atg9-1* and *atg9-6* deficient for the autophagic response, virulence of *E. cichoracearum* was retarded whereas pathogenesis of *C. higginsianum* was accelerated. Taken together, these data suggest that the autophagy pathway is a potential host susceptibility factor for pathogen infection, possibly involved in establishing/facilitating biotrophy in *A. thaliana*.

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LIST OF ABBREVIATIONS

ATG: Autophagy
atg: Autophagy mutant
Ams1: α- mannosidase
Avr: *Avirulence*
Bgh: *Blumeria graminis hordei*
cDNA: Complimentary DNA
CK: Control
Con A: Concanomycin A
CVT: Cytoplasm to vacuole targeting pathway
CWA: Cell wall appositions
DNA: Deoxyribonucleic acid
Dpi: Days post infection
EHM: Extrahaustorial membrane
EHMAT: Extrahaustorial matrix
ERF: Ethylene responsive factor
ET: Ethylene
GFP: Green fluorescent protein
H_2O_2: Hydrogen peroxide
Hpi: Hours post infection
Hpt: Hours post treatment
HR: Hypersensitive response
HST: Host specific toxins
HXT1: Hexose transport 1
IN: Inoculated
ISR: Induced systemic resistance
JA: Jasmonic acid
LRR: Leucine rich repeat
MAPK: Mitogen activated protein kinase
MDC: Monodansylcadaverine
MLO: Mildew locus O
MPa: Megapascals
NBS: Nucleotide binding site
NO: Nitric oxide
NSF: N-ethylmalemide-sensitive
OH-: Hydroxyl oxide
PAMPs: Pathogen Associated Molecular Patterns
PAS: Preautophagosomal structure
PCD: Programmed cell death
PCR: Polymerase chain reaction
PDF: Plant defensin
PE: Phosphatidylethanolamine
PEN: Penetration
PH: Primary hyphae
PR: Pathogenesis related gene
PrApe1: Pre-Aminopeptidase 1

R: Resistance
RNA: Ribonucleic acid
ROS: Reactive oxygen species
SA: Salicylic acid
SAR: Systemic acquired resistance
SH: Secondary hyphae
SNARE: Soluble NSF attachment protein receptor
SNP: Sodium nitroprusside
SOD: Superoxide dismutase
T-DNA: Transferred DNA
TLR: Toll-like receptor
TMV: Tobacco mosaic virus
UTR: Untranslated region

CHAPTER 1
GENERAL INTRODUCTION

1.1 Introduction to plant-pathogen interactions

1.1.1 Types of interactions

Plants are non-motile eukaryotic organisms. As do animals, plants can suffer from infections and stress. Plant illness can result from either biotic or abiotic factors. Plant pathology is the study of a plant's interaction with its parasitic biotic or abiotic environment. Plant-microbe interactions range from beneficial symbiotic interactions to deadly necrotrophic interactions. Infectious microbes range from the simplest viroids to higher parasitic plants. Table 1.1 illustrates the range of microbes that are infectious to plants and their various modes of infection. More than 10,000 species of fungi have been identified as parasites of plants, compared to a mere 50 to humans (Agrios 1999) and fungal parasitism contributes to an annual 10% crop yield reduction globally (Strange and Scott 2005). To date, plant diseases have been predominately controlled by the use of toxic chemicals or pesticides (Agrios 1999). The challenge for plant pathologists now is to employ advancing technologies in order to understand the molecular mechanisms governing these interactions such that more environmentally friendly means of controlling plant diseases can be identified. Plants and fungal pathogens have coevolved specialized and sophisticated strategies for establishing resistance and infection, respectively. Virulent (disease causing) fungal pathogens employ one of three infection strategies: 1) biotrophy, where the fungus lives on or in the plant and receives nutrients from living tissue, 2) necrotrophy, where the pathogen kills the host tissue taking nutrients from the dead or dying tissue or, 3) hemibiotrophy, where the pathogen lives as a biotroph initially and then switches to a necrotrophic lifestyle. A given plant species is either considered a host to members of a pathogen species, that is, they are susceptible to those members, or they are considered a nonhost, unable to support infection from a particular species or members of a species. The modes of fungal infections and host versus nonhost will be discussed in more detail in the following sections.

Table 1.1 Types of plant microbe interactions

	Examples				
Parasitic Microbes and Higher Plants	Symbiotic	Biotrophic / obligate	Hemibiotrophic	Necrotrophic	Saprophytic
Viruses / Viroids	*n/a	*Tobacco mosaic virus*	n/a	n/a	n/a
Prokaryotes	*Rhizobium* spp.	n/a	*Agrobacterium* spp.	*Erwinia* spp. *Pseudomonas* spp	n/n
Protozoa	n/a	*Plasmodiophora* spp., *Spongospora* spp.	n/a	n/a	*Physarum* spp.
Oomycetes	n/a	*Albugo* spp.	*Phytophthora* spp	*Pythium* spp.	n/a
Fungi	Mycorrhizae Lichens	*Blumeria* spp. *Erysiphe* spp. Rusts	*Colletotrichum* spp.	*Fusarium* spp.	Various species
Nematodes	n/a	*Meloidogne* spp.	n/a	n/a	Various ectoparasites
Parasitic Higher Plants	n/a	Dodders Mistletoes	n/a	n/a	n/a

*n/a: not applicable

1.1.1.1 Biotrophy

Biotrophic fungal pathogens require a living host on or in which to survive and reproduce. Since their goal is to colonize and "feed" off their living host they must do so inconspicuously in order to avoid host recognition and a resistance response. Biotrophic pathogens are more highly specialized than hemibiotrophic or necrotrophic pathogens and as a result have a very narrow host range. To date, most biotrophic fungi cannot be grown axenically and are not amenable to molecular transformations.

Biotrophic fungi were described by Mendgen and Hahn (2002) to possess five characteristic properties: "1) highly developed infection structures; 2) limited secretory activity, especially of lytic enzymes; 3) carbohydrate-rich and protein-containing interfacial layers that separate fungal and plant plasma membranes; 4) long-term suppression of host defenses; 5) haustoria, specialized hyphae for nutrient absorption and metabolism". These characteristics allow the pathogen to have high efficiency nutrient uptake while bypassing their host's surveillance system. Figure 1.1 depicts the typical infection strategy and infection structures of biotrophic pathogens.

Conidia, the asexual fungal spore of the biotrophic fungi *Erysiphe*, land on a suitable host surface and almost immediately begin to germinate. Conidia of *Erysiphe* spp., the focus of a large part of this thesis, germinate to produce a single germ tube within 2-4 hours of landing on the host surface. A septum is formed near the tip of the germ tube which then swells and differentiates into the appressorium (Kunoh et al., 1979; Singh and Singh 1983; Carver et al., 1996). The appressorium is a lobed or tubular infection structure that facilitates attachment to the host surface and penetration (Agrios 1999). An amorphous extracellular material is visibly secreted under the appressorium, and it has been suggested that this substance aids in attachment and penetration since it is observed to be selectively secreted based on stimuli from hydrophobicity, cellulose and cutin monomers. However, the exact mechanisms of adhesion and penetration are still being debated (Kobayashi et al., 1991; Carver et al., 1996; Francis et al., 1996). The mechanical pressure exerted by the appressorium on the plant surface forces a penetration peg through the cuticle and cell wall of the plant cell (Agrios 1999). It has been proposed that an increased concentration of glycerol in the appressorium combined with a semipermiable appressorial membrane triggers uptake of water from the host tissue surface and an increased turgor pressure is created within the appressorium (Green et al., 2003). In *Magnaporthe grisea*, a melanized appressorium-producing hemibiotrophic pathogen, the pressure exerted by the appressorium has been measured to be up to 8 megapascals (MPa) (Howard et al., 1991). To successfully infect the host, the penetration peg must breach the cuticle, the cell wall and the papilla, a cell wall apposition produced by the plant in response to recognition of a pathogen.

It has long been debated whether biotrophic pathogens use only mechanical pressure to breach the plant cuticle, cell wall and papilla or whether they also employ enzymes to aid in

penetration. In 1998, Suzuki et al. identified cellulase activity in powdery mildew conidial exudant as well as an increase in pectinase gene expression in the conidia. As early as 1970, electron micrographs taken at infection sites revealed that cellulose microfibrils did not disassemble during penetration suggesting that they were degraded by enzyme activity (Edwards and Allen 1970). It was also noted that microfibril degradation was restricted to an area 0.1 mm in diameter to the infection site. These experiments also revealed stress fractures in the papillae near the tip of the infection peg suggesting mechanical force was also used to breach this barrier (Edwards and Allen 1970). The question arises as to the ability of the pathogen to remain inconspicuous when it employs such destructive means for penetration. More recently, data from Liu et al. (2007a) presents a new approach to penetration.

Since *Erysiphe* and *Blumeria*, two powdery mildew species, lack melanized appressoria and turgor pressure has been observed to be only 4 MPa (Pryce-Jones et al., 1999), it is safe to assume the penetration is not solely by mechanical means. During *Blumeria* infection, Liu et al. (2007a) noticed iron accumulation under appressoria and penetration pegs in plant cells. Iron is found in its ferric (Fe_2O_3) state within the plant cell. A conversion to ferrous iron (Fe^{2+}), induced by the pathogen, signals an accumulation of hydrogen peroxide (H_2O_2) and the formation of hydroxyl oxide (OH^-) radicals. This reaction could stimulate the degradation of the surrounding environment including the plant cell wall and papilla facilitating penetration. It is proposed that the reductase involved in the conversion of Fe_2O_3 to Fe^{2+} is secreted by the pathogen, or more specifically, the emerging penetration peg, into the plant apoplast (between cell wall and membrane). This conversion and reaction, as of yet, is unable to elicit a complete resistance response (Liu et al., 2007a).

Once the penetration peg has breached the cuticle, cell wall and papilla, a specialized feeding structure called the haustorium is formed between the plant cell wall and plasma membrane and is comprised of the haustorial cytoplasm, membrane and wall (Gil and Gay 1977). It was previously assumed that the plant plasma membrane simply invaginated around the haustorium. This membrane is referred to as the extrahaustorial membrane (EHM). However, recently Koh et al. (2005) identified that the EHM must be synthesized de-novo since they were unable to identify any of eight plasma membrane markers in this membrane. These data support the findings that the plant *MLO* (*Mildew Locus O*) gene, required for powdery mildew infection in barley among other monocots and dicots, codes for a membrane-localized syntaxin that has a

role in the delivery of plasma membrane materials to the plasma membrane allowing for the development of the haustorium (Panstruga 2005). However, the membrane rearrangement and synthesis in the plant cell during haustorium development is still being elucidated.

The fungal membrane and EHM are separated by the extrahaustorial matrix (EHMAT). This gel-like amorphous layer produced by both the plant pathogen and the infected plant cell is enriched in carbohydrates (Perfect and Green 2001; Szabo and Bushnell 2001). The EHMAT is a sealed compartment between the fungal haustorium, EHM and the callose of the papillum that is deposited into a neckband surrounding the penetration site (Szabo and Bushnell 2001). It is the intermediate site of nutrient transfer between the plant cell and fungal haustorium and is thought to facilitate nutrient uptake by the fungus by creating a sealed-off environment for the creation of a proton gradient required for the production of energy needed for the uptake of nutrients (Szabo and Bushnell 2001). The presence of the EHMAT is also speculated to hinder contact between the fungal haustorium and the EHM of the plant as part of an attempt to remain hidden by the pathogen (Mendgen and Hahn 2002). The haustorial cell wall is considered similar to a typical fungal cell wall comprised of polysaccharides and chitin (Chard and Gay 1984). The haustorial membrane, however, is distinct from the membrane of the appressorium and surface hyphae.

A gene involved in sugar transport was identified to be expressed in the haustorium of *Uromyces fabae* (rust of bean) (Voegele et al., 2001). These authors later identified that the encoded sugar transporter, hexose transport 1 (HXT1), was localized in the haustorial membrane and that it is specific for D-glucose and D-fructose. Sucrose is the major form of translocated carbon in plant cells, yet it is speculated that glucose is the major form of transferred carbon between plants and biotrophic fungal pathogens. Phosphatase activity has been implicated in nutrient uptake through the EHM, the EHMAT and the fungal haustorial wall/membrane into the haustorium. ATPase activity has been speculated to be the active import mechanism driving this nutrient uptake by the haustorium (Spencer-Phillips and Gay 1981). Since the EHMAT is completely sealed off, it is plausible that H+-ATPases could generate a proton gradient across the haustorial membrane that would provide the energy needed for active transport and indeed, in 1998, Struck et al. identified PMA1, a haustorial plasma membrane H+-ATPase. As for the export of sugars from the plant cell, it is currently accepted that passive transport is due to its leaky structure since the EHM was observed to lack ATPase activity, however, this is still under debate (Manners and Gay 1983; Manners 1989).

Along with sugars, amino acids and lipids have also been suggested to be taken up by the haustorium. Three genes (*AAT1, AAT2* and *AAT3*) encoding amino acid transporters have been shown to be expressed throughout parasitic hyphae, but their transcripts have been found to accumulate in haustoria (Hahn and Mendgen 2001; Wirsel et al., 2001; Struck et al., 2002). Amino acid transport is suggested to involve a proton-symport mechanism (Voegele et al., 2001; Struck et al., 2002). Taken together, these data demonstrate that the bulk of nutrient uptake is accomplished by the haustorium. A successful transfer of nutrients allows the growth of the surface hyphae and eventually sporulation (the production of conidia) in powdery mildew.

1.1.1.2 Hemibiotrophy

Hemibiotrophic fungal pathogens employ a biotrophic mode of infection initially and later switch to a destructive necrotrophic mode of infection. Hemibiotrophic pathogens have a wider host range than biotrophic pathogens and do not rely on living material for survival. *Colletotrichum* species, a hemibiotrophic group of pathogens and the focus of a large part of this thesis, cause the agronomically important disease anthracnose world-wide on a range of crops. *Colletotrichum* spp. cause two distinct types of diseases: post-harvest (latent infection of maturing fruits and vegetables in storage) and pre-harvest (infection of plant tissues in the field) (O'Connell et al., 2000). Their infection strategies can also be divided into two distinct classes: intracellular hemibiotrophy and subcuticular intramural necrotrophy (O'Connell et al., 2000). Figure 1.1 depicts the intracellular infection strategy and infection structures of typical *Colletotrichum* species.

Conidia of *Colletotrichum* spp. land on a suitable host surface and adhere to the cuticle (regardless of the infection strategy). *Colletotrichum* conidia possess a unique conidial coat that is a spongy, porous structure composed largely of glycoproteins versus characteristic hydrophobic layers of other fungi (Bobichon et al., 1994; Kershaw and Talbot 1998; O'Connell et al., 1996). This type of surface layer has been found in *Candida albicans* (yeast) and been implicated in the regulation of cell surface hydrophobicity and adhesion to host cells (Hazen and Hazen 1992). Removal of the spore coat abolished the ability for the conidium of *C. musae* and *C. graminicola* to adhere to polystyrene (a hydrophobic surface, Styrofoam) (Hazen and Hazen

1992). Taken together this evidence suggests that adhesion to the host surface by *Colletotrichum* conidia is due largely to hydrophobic interactions.

A short germ tube germinates from the conidium and at its tip differentiates into an appressorium. Appressoria of *Colletotrichum* species are distinct from those of *Erysiphe* or *Blumeria* in that they are melanized. Melanin is deposited in a layer of the appressorial wall close to the plasma membrane (Kubo and Furusawa 1986). A mature appressorium is asymmetrical, domed at the upper region and flattened at the basal region. The appressorium adheres to the plant epicuticular surface via hydrophobins, which are fungal proteins that connect hydrophobic and hydrophilic interfaces (Beckerman and Ebbole, 1996). Turgor pressure is built up in the appressorium and with the combination of extracellular cuticular and cell wall degrading enzymes the penetration peg moves through the cuticle and cell wall of the plant cell (Karr and Albersheim 1970; English et al., 1971 and 1972; Anderson 1978; Anderson and Nicholson 1996).

Intracellular hemibiotrophic species of *Colletotrichum* produce a biotrophic feeding structure very similar to the haustorium of biotrophic fungi, called the primary hypha. Primary hyphae are observed to be separated from the host plasma membrane by an interfacial matrix supposedly to avoid detection or suppress host defense responses (Green et al., 1995). Biotrophic hyphae are observed to be larger than haustoria and can become branched and septate. Knowledge regarding the exact mode of nutrient uptake by the primary hyphae is vague. It is assumed that primary hyphae actively uptake nutrients from the host as do haustoria, however, the exact mechanisms of nutrient transfer and the genes involved remain to be elucidated.

For whatever reason, the availability of nutrients or recognition by the host and therein recognition by the pathogen that a resistance response is looming, hemibiotrophic pathogens switch to a necrotrophic mode of infection. They develop small secondary hyphae from the tips of the biotrophic primary hyphae that are destructive in nature. Theses hyphae grow through and between plant cells. Metabolites released by the fungus either elicit a resistance response by the plant in the form of cell death, and lesions begin to appear on the infected surface, or are potent enough to kill the invaded cells (Bailey et al., 1992). The tip of the secondary hypha remains ahead of the host defense response, and the dead tissue left behind becomes heavily colonized by secondary necrotrophic hyphae (Rodriguez and Redman 2000). Little is known about the specificities of the necrotrophic stage of *Colletotrichum* species, however, pathogenesis of

necrotrophic pathogens has been well studied and the following discussion can lend itself to this stage of hemibiotrophic infection.

1.1.1.3 Necrotrophy

Unlike biotrophic and hemibiotrophic pathogens, necrotrophic pathogens survive and reproduce on dead or dying plant material. The typical defense reaction elicited by a plant to a biotrophic or hemibiotrophic pathogen is programmed cell death or apoptosis (Mayer et al., 2001). Necrotrophic pathogens benefit from this reaction since they can colonize dead material and therefore they generally have a very wide host range (Farr et al., 1989; Govrin and Levine, 2000). Necrotrophs are considered to be less specialized than biotrophs and hemibiotrophs since their form of attack is to destroy and kill, however they actually posses unique features that allow them to colonize plant tissues; they subdue their host plants using very subtle mechanisms.

The infection process of necrotrophic parasites (fungi) is well studied, as several necrotrophic pathogen genomes have been sequenced (for example, *Botrytis cinerea,* a grey mould to grapes and *Fusarium graminearum* causing head blight to wheat) (Narusaka et al., 2004; O'Connell et al., 2004). Necrotrophic pathogens secrete toxins and lytic enzymes that degrade plant tissues; some toxins are only functional on certain plant species and are referred to as host specific toxins (HST). Pathogens that use HST have a narrow host range compared to the broad-host range necrotrophic pathogens.

Although necrotrophic pathogens have a wider host range, their interactions are still specific. Necrotrophic spore germination is selective and is determined by the chemical and physical properties of the plant surface (Doelemann et al., 2005 and 2006). Some necrotrophs produce appressoria on the host surface; however, penetration into host tissues is primarily accomplished by the secretion of digestive enzymes such as cutinase and lipase rather than mechanical penetration (Van Kan et. al., 1997; Reis 2005). Evidence for this lies in the fact that there is no observable septum between the appressorium and germ tube (observed in both *Erysiphe* and *Colletotrichum*) and that the deposition of melanin into the walls of the appressorium is scant (de Jong et al., 1997; Gourgues et al., 2004). Breaching of the cuticle by the penetration peg triggers the production of reactive oxygen species (ROS) by the plant (Tenberge et al., 2002; Tenberge 2004). ROS generation elicits a cell death response and a

suitable feeding environment for the pathogen. Thin mycelia grow within plant tissues secreting digestive enzymes and macerating surrounding cells. Figure 1.1 depicts the intracellular infection strategy and infection structures of a typical necrotrophic pathogen *Fusarium graminiarum*.

An interesting characteristic of necrotrophic pathogens is their ability to withstand the accumulation of ROS. The production of ROS leading to oxidative stress and the hypersensitive response causes a localized plant cell death response (Agrios 1999). ROS such as the superoxide ion O_2^- and the hydroxyl radical OH^- are very reactive molecules that accumulate at infection sites and cause damage to all cellular components including DNA and proteins (Schafer and Buettner 2001). Yet necrotrophic mycelia can tolerate these substances because they are well equipped with enzymes such as superoxide dismutase (SOD), peroxidase and catalase that protect them from ROS (Mayer et al., 2001). SOD catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide (H_2O_2), still considered a ROS although less reactive (Mayer et al., 2001).

If invasion does not elicit ROS production, necrotrophic pathogens produce and secrete toxins that can kill host tissues. Two well-studied toxins are vomitoxin produced by *F. graminearum* and tentoxin produced by *Alternaria alternata* (Proctor et al., 1997; Colmenares et al., 2002; Lemmens et al., 2005). Host specific toxins, like the two previously mentioned, are required for pathogenicity while non-specific toxins are not essential for pathogenesis but do increase pathogen virulence. Vomitoxin inhibits cellular protein synthesis whereas tentoxin inhibits photosynthesis rendering the plant defenseless (Agrios, 1999).

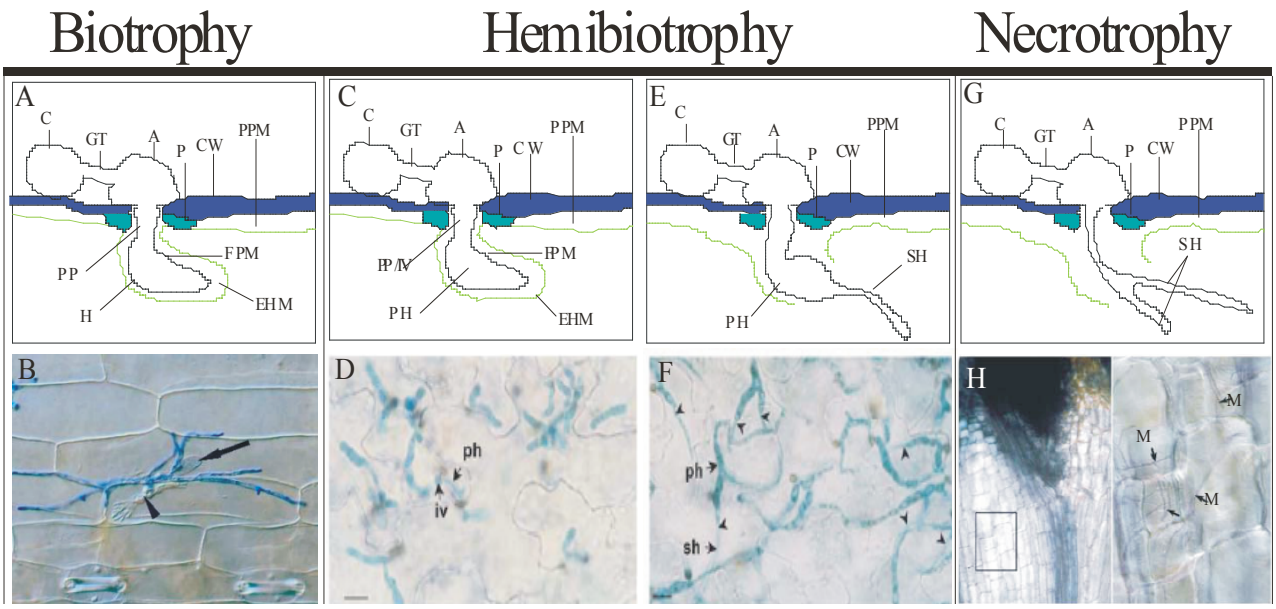

Figure 1.1: Types of fungal infection strategies. Images A and B represent biotrophic infection strategy/structure development. A conidium (*C*) germinates to produce a germ tube (*GT*) and from the *GT* an appressorium (*A*) is produced. The penetration peg (*PP*) pierces the plant cell wall (*CW*). The nutrient acquiring haustorium (*H*) is produced between the *CW* and plant plasma membrane (*PPM*). In a penetration attempt, callose (referred to as a papilla) is deposited beneath the penetration site (*P*). Nutrients are moved from the plant cell, across the *PPM* through the appoplast, the extrahaustorial matrix (*EHM*), and taken into the *H*. Image B was borrowed from Huckelhoven et al., 2005 and represents the haustorium and surface hyphae of a *Blumeria graminis* f.sp. *hordei* (*Bgh*) in a barley epidermal cell. Arrowheads indicate the conidium (on the surface of the epidermis) and the branched haustorium (within the epidermal cell and characteristic of *Bgh*). Images C-F represent hemibiotrophic infection strategy/structure development. A *C* germinates on the host surface to produce a short *GT*. From the *GT* a melanized *A* is produced. A *PP* punctures the host *CW* and *P*. A lobed or branched and often septate primary hyphae (*PH*) is produced between the *CW* and *PPM*. The second stage of hemibiotrophic infection (images E-F) begins with the production of secondary hyphae (*SH*) from the tip of the *PH*. The *SH* are destructive and pierce or degrade the *PPM*. Images D and F were taken by the Wei lab and represent infection structures of *Colletotrichum higginsianum*. Images G-H represent a necrotrophic infection strategy/structure development. A *C* lands on the host surface, germinates to produce a *GT* and from that an *A* is produced. Necrotrophic hyphae referred to as *SH* pierce the *CW* and *PPM* and this is usually accompanied by the production of degredative enzymes. Image H was borrowed from David Greenshields (Greenshields et al., 2004) and represents the necrotrophic infection of *Fusarium graminearum* in wheat.

1.1.1.4 Host versus nonhost

Regardless of the infection strategy used by a pathogen, not all pathogens can infect all plants. Logically, plants that can support the infection process of a pathogen are referred to as hosts and the pathogen is referred to as adaptive. To differentiate a host from a nonhost, the varying levels of resistance need to be examined and they include: basal resistance, pre-penetration resistance and post-penetration resistance and resistance-gene (R-gene) resistance. If a pathogen can overcome all four levels of resistance the plant is considered a compatible host.

Basal resistance is a level of resistance that all plants show to all pathogens, whether successful or not. A classic example of basal resistance is the formation of cell wall appositions (CWAs) localized at fungal penetration sites. These localized CWAs, also referred to as papillae, fortify the cell wall under attack making penetration difficult or impossible for non-host pathogens. This basal resistance reaction is so ubiquitous that it has been observed to occur in response to a pin-prick (Hardham et al., 2007). If the pathogen is unable to overcome this defense mechanism, it is considered to be non-pathogenic or a non-adapted pathogen on a non-host plant (Thordal-Christensen, 2003; Nurnberger and Lipka, 2005; Ellis, 2006).

Pre-penetration and post-penetration resistance are components of non-host resistance, where all members of a plant species are resistant to all members of a pathogen species. Pre-penetration resistance overlaps basal resistance and is characterized by the recognition of pathogen elicitors such as bacterial flagellin or fungal chitin by the plant's surveillance system resulting in a resistance response of CWA formation (Asai et al., 2002; Thordal-Christensen 2003; Ellis 2006; Kaku et al., 2006). In the non-adaptive *Blumeria graminis* f. sp. *hordei (Bgh)-A. thaliana* pathosystem, three genes have been identified that confer resistance via cell wall depositions at infection sites: *Penetration 1* (*PEN1)*, *PEN2* and *PEN3* (Thordal-Christensen 2003; Ellis 2006). *PEN1* was identified as a SNARE (soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor) that is recruited to the plasma membrane during attempted *Bgh* penetration, suggesting a role for vesicle trafficking in this resistance response (Koh et al., 2005). *pen* mutants and vesicle trafficking will be discussed further in the following section and in section 1.1.3.1.

Post-penetration resistance overlaps host resistance or *Resistance* (*R*)-gene mediated immunity (Lipka et al., 2005). All three *pen* mutants (*pen1*, *pen2*, *pen3*) as well as the *pen1 pen2*

double mutant are susceptible to invasion of an otherwise non-adapted pathogen yet are capable of mounting a post invasion resistance response, therefore distinguishing pre-penetration resistance from post-penetration resistance (Lipka et al., 2005). Near-adaptive pathogens, those that can surpass basal and pre-penetration resistance, are unable to successfully colonize plant tissue due to post-penetration resistance. This response is generally characterized by the hypersensitive response (HR) or localized cell death (Feys et al., 2005).

Some hosts may be resistant to certain subspecies or races of an otherwise infectious pathogen species; this resistance is referred to as host resistance. Host resistance is the final layer of possible immunity from infection. Members of a pathogen species that are defeated by host resistance are referred to as avirulent, meaning not-virulent or incapable of causing disease. Avirulent pathogens produce certain elicitors encoded by *Avirulence* genes (*Avr*) that can be recognized by *R*-gene products (receptors) possessed by the plant (Ellis et al., 2000). Identifying *R* and *Avr* genes has been the primary focus of many molecular plant pathologists since their identification can drive plant breeding programs. *Avr* gene product recognition by *R* gene products initiates a cascade of events leading to the eventual inhibition of pathogen development. Host resistance is most commonly carried out by HR and localized programmed cell death (PCD) (Gilchrist, 1998; Heath, 1999; Greenberg and Yao, 2004). HR is characterized by the production of ROS, ion fluxes, DNA fragmentation, mitochondrial and plastid leakage and ultimately cell death (Mittler et al., 1997; Morel and Dangl, 1997). Three plant hormones, salicylic acid, jasmonic acid and ethylene, have been identified to play major roles in the cascade leading to HR. Their role in defense will be discussed in more detail in section 1.1.2.3. A single *R*-gene locus can often confer resistance, therefore *R*-gene mediated resistance breeding has been widely used by plant breeders. However, often, selective plant breeding causes uniformity in crops that then make them vulnerable to other diseases.

1.1.2 Perception of infection

For an interaction between a plant and pathogen to occur, the pathogen must sense that it has encountered a susceptible host plant. For a plant to elicit a resistance response it must sense the threat of a pathogen. Disease development begins with the pathogen when it recognizes a suitable host. The pathogen's development can then trigger the surveillance system of the plant

through several mechanisms: gene-for-gene interactions and recognition of pathogen associated molecular patterns (PAMPs). Elicitation of a response by the plant then depends on its ability to recognize elicitors from the pathogen. Such a defense response is considered to be inducible, while others like CWA and the presence of pre-existing antimicrobial compounds are considered preformed (Broekaert et al., 2006). The perception of infection will be discussed further in the following sections emphasizing *Colletotrichum, Blumeria* and *Erysiphe* species.

1.1.2.1 Pathogen and plant sensing

The establishment of disease in a plant-pathogen interaction is generally governed by the pathogen. Compounds or the physical topography of the host signal conidium germination. The appressoria of *Colletotrichum gloeosporioides*, a pathogen of avocado, selectively develop on avocado fruits due to the chemical nature of the epicuticular wax (Podila et al., 1993). Conidium germination and appressorium formation in *Colletotrichum* species have also been induced by a combination of hard surface contact, or hydrophobicity, and the release of ethylene, a plant hormone (Kolattukudy et al., 1995). This combination is particularly observed during post harvest disease development. In the *Bgh*-barley pathosystem, the conidia secrete an extracellular proteinaceous matrix upon contact with the hydrophobic leaf surface. This matrix is suggested to break down the epicuticular wax (Wright et al., 2002). Epicuticular wax components are then absorbed by the conidium; the uptake of these compounds is suggested to trigger conidium germination (Nielsen et al., 2000). The aeciospores and urediospores of the biotrophic rust fungi *Uromyces* germinate to produce germ tubes. These germ tubes develop as a result of thigmotropism. The ridges of stomata signal the production or development of an appressorium from the germ tube. The penetration peg then enters the plant tissue directly through the stomatal opening (Wynn, 1976). *Colletotrichum* species that employ the use of cuticle and cell wall digestive enzymes, to aid their penetration into the host cell, display selective expression of endopolygalacturonase in response to pectin, a plant cell wall component. *Clpg2*, the gene encoding endopolygalacturonase, is expressed in the germinating conidium, appressorium and penetration peg of *Colletotrichum* species (Centis et al., 1997; Dumas et al., 1999). The interesting feature in all of these examples is that the germination and initiation of penetration or infection is specific and in most cases does not occur on non-host tissues.

Plant sensing is crucial to defense. Plants have evolved and continue to evolve the ability to recognize pathogen elicitors due to gene-for-gene interactions. Plant innate immunity is triggered by the perception of both avirulence gene products and PAMPs (Flor, 1942 and 1947; Parker, 2003; Gomez-Gomez, 2004; Desender et al., 2007). Avirulence (*Avr*) elicitors are proteins excreted by the pathogen that are recognized by the corresponding host resistance (*R*) protein (Catanzariti et al., 2006). A molecule secreted by a pathogen that is non-recognizable by the host is considered a virulence effector; when this molecule is recognizable it is considered an avirulence effector. There is constant evolution in resistance genes and virulence genes to confer resistance and overcome recognition, respectively. PAMPs are molecular motifs found on or produced by pathogens that are considered non-self motifs to plants. These non-self motifs are recognized by toll-like receptors (toll-like receptors are membrane bound receptors that are involved in innate immunity by recognizing non-self molecules derived by other organisms such as microbes); this mechanism for recognition of infection is found both in plant and animal systems (Parker, 2003).

Certain races of the rust, *Melampsora lini,* are avirulent to flax varieties due to avirulence gene product recognition by resistance gene products. These resistance gene products are generally located on the EHM and contain a nucleotide binding site (NBS) and leucine rich repeat (LRR) domains (Luderer and Joosten, 2001). This has been a model system for the study of haustorially expressed and secreted proteins (Anderson et al., 1997; Ayliffe et al., 2001; Dodds et al., 2001a, 2001b and 2004). Dodds et al. (2004) identified the Avr protein AvrL567, secreted by *M. lini* and the corresponding *R* gene alleles in flax that encode NBS-LRR intracellular receptor proteins; the interaction of these products triggered the HR. Avr proteins of biotrophic and hemibiotrophic oomycetes (ATR13 and ATR1 from *Hyaloperonospora parasitica*; Avr1b from *Phytophthora sojae* and Avr3a from *Phytophthora infestans* and AvrL567 from *M. lini*) all share a common conserved signal peptide with effector proteins from *Plasmodium falciparum*, the malaria parasite, required for translocation across the erythrocyte (red blood cell) membrane (Doddes et al., 2004; Hiller et al., 2004; Marti et al., 2004; Catanzariti et al., 2006). Although membrane bound proteins involved in translocating effector proteins in flax have yet to be identified, these data support intercellular gene-for-gene interactions of *Avr* and *R* gene products that initiate a cascade of events to confer resistance via the HR.

The classic, and most well studied PAMP is bacterial flagellin (Gomez-Gomez and Boller, 2000; Samatey et al., 2001). Flagellin is a protein building block of the flagella of eubacteria that is highly conserved at the N- and C-terminus ends (Samatey et al., 2001). Leaky flagellin, flagellin monomers that have escaped into the extracellular environment of the host cell, are recognized by extracellular receptors (Shimizu et al., 2003). Flagellin sensitive2 (FLS2), a flagellin receptor, was identified through map based cloning in *A. thaliana,* and possesses a predicted signal peptide, an extracellular leucine rich repeat domain, a transmembrane domain and an intercellular serine/threonine kinase domain representing the hallmarks of Toll-like receptors (TLR) in mammalian immune systems (Gomez-Gomez and Boller, 2000). FLS2 shares identity to the TLR of mammals and TLR5 specifically in humans (Gomez-Gomez and Boller, 2000; Hayashi et al., 2001). FLS2 kinase activity, due to the recognition of flagellin, was responsible for the activation of a MAP kinase cascade which further led to the activation of WRKY transcription factors, known to regulate the expression of defense-related genes in plants (Bauer et al., 2001; Gomez-Gomez et al., 2001).

1.1.2.2 Signal relay *in planta* in response to PAMPs and avirulence gene products

Once a plant cell has sensed a foreign invader a cascade of events relays that information to initiate a resistance response. In contrast, if a plant cell is unequipped to recognize such an invasion, there is minimal or no response to the attack. The relay of information occurs within the infected cell and throughout the plant. Until recently, little was known regarding the transmission of such a signal. In the animal systems, many TLRs recognize PAMPs and resistance is initiated via a common conserved signal transmittance pathway (Aderem and Ulevitch, 2000; Khush and Lemaitre, 2000). It was speculated that such a mechanism was acting in plants to confer resistance. In response to flagellin, the *A. thaliana* FLG2 LRR receptor kinase was found to activate a mitogen-activated protein kinase (MAPK) cascade. All of the MAPKs in this cascade were identified: MEKK1, MKK4/MKK5 and MPK3/MPK6; downstream to this cascade WRKY transcription factors, WRKY22/WRKY29, were observed to be phosphorylated and therefore inactivated which leads to the expression of genes involved in initiating a resistance response mediated by cell death (Asai et al., 2002).

Cell death, in response to infection is well known to be associated with a cellular increase of ROS (Kotchoni and Gachomo 2006). ROS also play a role in signal relay. In almost every situation involving defense, ROS have been observed to accumulate (Bolwell et al., 2001; Mellersh et al., 2002; Kotchoni and Gachomo 2006). ROS mediate the activation of MAPK pathways (Moon et al., 2003), play a role in cross-linking metabolites at infection sites aiding in penetration resistance (Thordal-Christensen et al., 1997), activate defense related genes such as those encoding pathogenesis-related proteins or fungal inhibiting compounds (Aist and Brushnell 1991; Kotchoni and Gachomo 2006) and cross-talk with the three major plant hormones involved in plant disease, salicylic acid, jasmonic acid and ethylene (discussed in section 1.1.2.3) (Shirasu et al., 1997). Although ROS can be detected during separate disease sensing and signal relay events in plant cells, the exact role in each situation is unclear. What is clear, however, is that ROS have a significant role in disease perception and signal relay, and that both positive and negative ROS feedback systems are at play (Kotchoni and Gachomo 2006).

1.1.2.3 Defense signaling molecules involved in disease perception and signal relay: salicylic acid, jasmonic acid and ethylene

Defense against biotrophic pathogens is generally mediated by programmed cell death. Programmed cell death associated with biotrophic infection is regulated by salicylic acid-dependent pathways (SA) (Glazebrook et al., 2005). Since necrotrophic pathogens benefit from this type of resistance response an alternate defense strategy involving ethylene (ET) and jasmonic acid (JA) confers resistance to these pathogens (Glazebrook 2005). There are however exceptions to every rule. For example, while ET plays crucial roles in plant development, such as senescence, it confers resistance during some plant-pathogen interactions and triggers conidium germination in some *Colletotrichum* species (Agrios 1999). Although the SA and JA/ET pathways interact extensively, SA and JA appear to be mutually inhibitory to the expression of several resistance genes under some circumstances. Induction of defense genes often requires both JA and ET but in other cases one hormone is sufficient to induce gene expression (Glazebrook 2005).

The term systemic acquired resistance (SAR) and the plant hormone SA are mutually inclusive. SAR is defined as acquired systemic immunity to secondary infection (Grant and

Lamb 2006). Simply, primary infection promotes a signal transduction throughout the plant conferring resistance. SA and several pathogenesis-related gene products play a major part in this resistance, however, as was previously thought, SA is not the mobile signal, even though it is observed to accumulate at the infection site and in tissues distal to infection (Vernooij et al., 1994; Durrant and Wong 2004; Bostock 2005). In the case of SAR, nitric oxide (NO), SA, JA and ET have all been implicated as possible signaling molecules (Grant and Lamb 2006).

Both pathogen attack and accumulation of ROS (or HR) trigger SA-dependent signaling (Glazebrook 2005). SA-dependent signaling involves SA accumulation. This has been directly associated with the expression of the pathogenesis related protein 1 (PR-1), among others (Van Loon and Van Strien 1999). PRs, in general, are proteins in plants that have the capacity to confer resistance. The exact role of PR-1 is unknown, however it is widely recognized as a marker for SA accumulation in plants, specifically in *A. thaliana* (Van Loon and Van Strien 1999). SA accumulation in *A. thaliana* involves the gene *SID2*, a gene encoding an isochorismate synthase, suggesting SA production during infection is largely from isochorismate rather than phenylalanine (Wildermuth et al., 2001). SA levels in the plant cell affect the gene *NPR1* (Nonexpresser of PR gene 1), an oligomeric protein found in the cytoplasm (Despres et al., 2003). Increased SA promotes disassociation of the oligomers into monomers that then enter the nucleus where they interact with TGA transcription factors (Fan and Dong 2002; Despres et al., 2003). Although the role of SA appears to be quite simple in the previously explained circumstance, there are other defense related pathways that operate independently of NPR1 but involve PR1 (Uquillas et al., 2004). The role SA plays during plant pathogen interactions cannot be simply described. It seems to be the modulator of resistance and interacts with genes involved in resistance, but this is unique to every plant-pathogen system. Unlike ET, there is no evidence that SA accumulation supports or encourages pathogen development; however, defense strategies mounted under SA control can be overcome by the pathogen.

Since programmed cell death is, in most cases, beneficial to necrotrophs, an alternate resistance response must be mounted for plant protection; this response is under the control of ET and JA (Broekaert et al., 2006). ET biosynthesis increases in response to recognition of certain avirulence elicitors (Cohn and Martin 2005). Accumulation of ET induces the expression of several defense related genes; genes involved in cell wall fortification, in the biosynthesis of antimicrobial compounds and PR's (Broekaert et al., 2006). Activation of *PR* genes other than

PR1 (which is induced in response to SA) such as *PR3* and *PR4* is a cooperative effort involving both ET and JA (Penninckx et al., 1998). Like the SA-dependent defense pathway, *PR* genes induced by ET and/or JA are induced both at the infection site and systemically (Broekaert et al., 2000).

The genes encoding plant defensins (PDFs), small peptides that inhibit the growth of a wide range of pathogenic fungi, contain unique GCC box promoter elements that are activated by ethylene responsive factor (ERF) transcription factors downstream to ET and JA accumulation (Penninckx et al., 1996; Lorenzo et al., 2003). PDFs specifically bind to target fungal membranes where they pierce the membrane creating a pore resulting in efflux of cellular components (Thomma et al., 2002 Thomma et al., 2003; Thevissen et al., 2004). The use of defensins in resistance is conserved among eukaryotes (Kim et al., 2005). In *A. thaliana,* defensin-like genes have been identified and they show differential expression in response to ET, JA and even SA (although some *PDF's* are induced in response to SA, the HR is the predominant form of defense to biotrophic infection). Interestingly, those PDFs that are responsive to SA lack the ET-inducible promoter GCC elements (Broekaert et al., 2006). Antimicrobial molecules are also crucial for defense to necrotrophs. An example of this is GLIP1, a secreted lipase under the control of ET accumulation, that confers resistance to *Alternaria brassicicola* in *A. thaliana* (Oh et al., 2005). The secretion of GLIP1 is ET-responsive based on recognition of effector molecules from the pathogen. It possesses lipase and antimicrobial activity that directly disrupt *A. brassicicola* spore integrity.

Since ET is involved in senescence and fruit ripening its production naturally can affect pathogen development and resistance or susceptibility (Agrios 1999). *Colletotrichum* species that cause post harvest diseases of fruits and vegetables have conidia that lay dormant on plant tissues and germinate in response to ethylene production in the ripening tissues. Ripening or senescing tissues are presumably easier to infect as: *i)* ripening breaks down sugars in the tissue which makes them more accessible for uptake by the pathogen and, *ii)* senescence is a slow form of cell death, a weaker cell is more easily colonized by opportunistic fungi/pathogens. Interestingly, upregulation of genes involved in ET biosynthesis is also observed during susceptible interactions, presumably to accelerate senescence (Liu et al., 2007b).

Environmental conditions need to be considered when associating ET with defense. Liu et al. (2007b) identified discrepancies between resistant-host *A. thaliana* Eil-0 and the pathogen

C. higginsianum. Upon leaf detachment, *A. thaliana* Eil-0 (an ecotype carrying an R-locus to *C. higginsianum* in an ET-dependent manner) became susceptible to *C. higginsianum.* It was concluded that detachment of leaves uncouples ET/JA defense pathways. These assays contribute to the knowledge of the already complex defense pathways governed by plant hormones.

JA is a member of the jasmonate class of plant hormones that are involved in the regulation of plant growth and development such as: growth inhibition, senescence and leaf abscission. Thus JA and ET are usually considered together (Xu et al., 1994; Creelman and Mullet 1997). However, JA is quite distinct from ET and SA. It is required to confer resistance to the biotrophic fungus *E. cichoracearum* in *A. thaliana*, some necrotrophic fungal pathogens, non-pathogenic fungi and a strain of the bacterium *Erwinia carotovora*. Members of the jasmonate family (oxylipins) are also anti-herbivory molecules to some arachnids and insects (Farmer et al., 2003).

Another interesting feature of JA during plant pathogen interactions is that unlike SA and ET there is little up regulation of genes involved in JA biosynthesis distal to infection sites suggesting that if JA is involved in the signal relay aspect of defense that it could be synthesized at the point of infection and then travels distally (Li et al., 2002; Strassner et al., 2002). JA has, to date, been predominately associated with ET during disease resistance. ET and JA together are required to induce gene expression of *PDF1.2* in *A. thaliana* in response to *A. brassicicola*. Neither the ET-insensitive mutant (*ein2*) nor the JA-insensitive mutant (*coi1*) could mount a defense to *A. brassicicola* (Penninckx et al., 1996). There are no clear examples of JA operating alone in plant defense or susceptibility to pathogens; however, it is clear that it is highly active alongside ET. It acts antagonistically with SA (in some cases), but cross-talk between all three plant hormones is characteristic to many plant-pathogen interactions.

1.1.3 Plant cellular responses to pathogen attack

Not only are the HR and antimicrobial compounds responsible for resistance, but the cellular components of infected cells and cells distal to infection react in an effort to stop an invading pathogen. Cytoskeleton, membrane and cytoplasmic reorganization and extensive vesicle trafficking occur in plant cells in response to infection, typically in an effort to stop penetration

through localized fortification of the cell wall, or the production of CWAs (De Bary 1863). Interestingly, these responses (cellular remobilization) occur during resistance and susceptibility. The following section will discuss cellular responses to pathogen attack during both resistant interactions and susceptible interactions, with the focus on susceptible interactions since this thesis focuses on plant cell response to virulent fungi.

1.1.3.1 Cellular response during non-host infections

In *A. thaliana*, a non-adapted (non-pathogenic) *Colletotrichum* species can germinate on the leaf surface to produce a melanized appressorium. Penetration events of this non-adapted species trigger actin microtubule reorganization towards the infection site (Opalski et al., 2005; Shimada et al., 2006). Treatment of *A. thaliana* with cytochalasin, an actin depolymerization inhibitor, compromises resistance to non-adapted *Colletotrichum* species, supporting the importance of this cytoskeletal reorganization in resistance (Kobayashi and Hakuno 2003; Yun et al., 2003). Due to cytoskeletal rearrangement, cytoplasmic streaming is redirected to the infection site (Koh et al., 2005). Following cytoplasmic streaming, endoplasmic reticulum (ER), mitochondria and the nucleus accumulate beneath the infection site (Skalamera and Heath 1998; Koh et al., 2005). The plant cell is now poised for defense.

Small vesicles, presumably containing contents necessary for the formation of CWAs accumulate around infection sites (Koh et al., 2005; Liu et al., 2007a). Vesicle trafficking during non-host resistance has only recently been dissected. It is assumed, though not clarified, that the vesicles accumulating under the infection sites during non-host penetration contain precursors for CWA formation. The material identified with CWAs includes callose, pectic substances, phenolic compounds, suberin and some metal ions (Smart et al., 1991). Recently, three loci in *A. thaliana* have been identified to confer penetration resistance to a non-host powdery mildew (*Bgh*), *PEN1*, *PEN2* and *PEN3* (Collins et al., 2003). *PEN1* encodes a Soluble NSF (Assaad et al., 2004; Bhat et al., 2005). Simply, PENs play a role in vesicle fusion once the vesicle has arrived at its target membrane. Since PEN1 contains a plasma membrane syntaxin, it is fitting that a mutation in this locus would promote penetration and therefore susceptibility to an otherwise avirulent fungal strain. If a vesicle containing precursors cannot dock and fuse to the target membrane, those precursors cannot be used in the formation of a CWA. Interestingly, even

though a mutation at any of the three *PEN* loci allows efficient *Bgh* penetration, *A. thaliana* is still capable of mounting a resistance response in the form of cell death and further fungal growth is stopped (Collins et al., 2003). Thus vesicle trafficking in response to non-pathogenic fungi appears to be required at the level of basal resistance.

It should be noted that the deposition of CWAs at infection sites requires ROS (Thordal-Christensen et al., 1997). Localized release of ROS is visible and presumably required for oxidative coupling, used in building or cross linking several CWA components, such as cell wall phenolics (Fry 1986; Wallace and Fry 1999). Liu et al. (2007b) demonstrated that ROS could be detected under penetration sites as early as 3 hours post inoculation. ROS have also been detected within vesicles traveling to infection sites, supporting their requirement/role in cross-linking of CWA materials for resistance (Hückelhoven et al., 1999; Collins et al., 2003).

1.1.3.2 Cellular response during susceptible infections

During susceptible plant-pathogen interactions, cytoskeletal rearrangement, membrane and cytoplasmic reorganization and vesicle trafficking are all observed to occur in infected cells (Koh et al., 2005; Opalski et al., 2005; Robatzek 2007). This remobilization may be in response to pathogen recognition and be an attempt to resist their invasion; however, accumulation and reorganization during virulent infections has unique roles to those in resistance. The following discussion will focus on interactions between plants and biotrophic pathogens since during biotrophic infection the plant cell remains intact.

The formation of CWAs at penetration sites is observed during both resistant and susceptible penetration attempts. Those fungi that make it through the barrier then continue to develop their infection structures. Cytoskeletal reorganization in a barley line susceptible to *Bgh* was more subtle than in the barley line resistant to *Bgh* (Opalski et al., 2005). This lack of reorganization is under the control of the barley *MLO* locus. The *mlo* mutant line is highly resistant to *Bgh* infection and this mutation has been bred into commercial barley lines for the past two decades (Lyngkjaer et al., 2000). Polarized deposition of CWA constituents and defense related compounds are found to be lacking in susceptible barley cells and the few actin filaments that did reorient towards an infection site were observed to depolymerize after the initiation of haustorium formation (Opalski et al., 2005). Since actin depolymerization is not observed in

resistant barley lines, the authors suggest that these fungi may have the capacity to actively counteract actin polymerization during the initiation of haustorium formation (Opalski et al., 2005). Interestingly, actin filaments were observed to surround a fully formed haustorium that had invaginated the host plasma membrane. This phenomenon is also observed in symbiotic mycorrhizal interactions (Genre and Bonfante 1998). Since actin filaments are linked via actin-binding proteins to the plasma membrane, it is suggested that the actin remodeling around the haustorium is involved in plasma membrane invagination (Baluska et al., 2003). Therefore, it is proposed that powdery mildew fungi have evolved the ability to use the host papilla response (redirection of actin and microtubules) for the establishment of infection (reorganization of plasma membrane) (Vorwerk et al., 2004).

Cytoplasm is observed to accumulate at the infection site as early as 1-2 hours post infection (Koh et al., 2005). With the redirected cytoplasmic streaming the nucleus, ER, mitochondria, Golgi bodies and peroxisomes accumulate around the developing and developed haustoria. This redirection is presumably facilitated by actin microfilaments, the mode of transportation for these organelles, which are also redirected to the infection site. The nucleus is twice as large in an infected cell versus an uninfected cell (Baluska et al., 1995; Koh et al., 2005). The oversized nucleus is proposed to be a result of active transcription (Baluska et al., 1995). The ER, peroxisomes, mitochondria and Golgi bodies were all aggregated beneath and around haustoria (Koh et al., 2005). Unidentified double membrane vesicles have also been closely associated with haustoria in infected cells (Coffee et al., 1972). The significance of organelle localization to the infection site is poorly understood, however, it had been suggested that the fungus itself is able to manipulate a host resistance response. Perhaps aggregation of organelles to the haustorium is another example of the pathogens ability to utilize plant cell substances/pathways/machinery for infection establishment and nutrient acquisition.

The question of nutrient acquisition by the pathogen has long baffled plant pathologists. As discussed previously, nutrients must be transferred from the host through the EHM, the EHMAT and the fungal plasma membrane. Recently, Koh et al. (2005) labeled eight plasma membrane proteins with green fluorescent protein (GFP) markers, all of which were visualized in the plasma membrane of uninfected cells and in the plasma membrane of infected cells excluding the membrane that surrounded the haustorium. Interestingly, these results suggest *de novo* synthesis of the EHM, but for what purpose? Is the synthesis of the specialized membrane

initiated (controlled) by the pathogen? Does a newly synthesized plant membrane facilitate nutrient acquisition? All of the plant cell machinery is in place to deliver nutrients to the fungi, but the mechanisms governing the operation remain to be elucidated.

1.2 Autophagy

Autophagy (ATG), literal meaning "self-eating", is a degradation and recycling system in eukaryotic cells (van Doorn and Woltering 2005). Unlike selective protein degradation via the ubiquitin 26S proteasome pathway, autophagy is thought to be an indiscriminant bulk degradation pathway that involves sequestration of cellular components into double membrane vesicles, autophagosomes, and subsequent delivery to the vacuole or lysosome for breakdown (van Doorn and Woltering 2005). This pathway has, until recently, been studied predominately in yeast; genetic screening has helped to elucidate the molecular mechanisms involved in this adaptive response (Abeliovich and Klionsky 2001; Stromhaug and Klionsky 2001; Wang and Klionsky 2003; Tsujimoto and Shimizu 2005).

1.2. Autophagy in yeast

The autophagosome was first reported by Clark in 1957 when he observed via electron microscopy a unique double membraned organelle inside the degradative vacuole of mice kidney cells (Clark 1957). This observation prompted several studies in mammalian cells that led to the identification of a new trafficking pathway and vesicle termed macroautophagy and autophagosome respectively (Deter et al., 1967a and b). Due to the lack of technology at the time of discovery, scientists had limited resources for further genetic dissection of this pathway until 1992 when Ohsumi and colleagues discovered macroautophagy in the yeast *Saccharomyces cerevisiae.*

Vesicular trafficking in eukaryotic cells had long been accepted as the main mode of transportation of materials between membrane-bound compartments or organelles (Palade 1975). Since vesicular trafficking aids in the maintenance of cellular homeostasis, it is considered constitutive. Newly identified autophagy, on the other hand, appeared to be a vesicular-trafficking pathway that was inducible (Abeliovich and Klionsky 2001). In yeast, the

discriminate cytoplasm to vacuole targeting pathway (CVT) was observed to share many functional proteins with the autophagy pathway (Baba et al., 1997). This pathway specifically targets cytosolic pre-aminopeptidase1 (PrApe1) and α-mannosidase (Ams1) for acquisition and delivery to the vacuole via a double membraned transport vesicle where they are cleaved to become functional vacuolar hydrolases (Baba et al, 1997). The CVT vesicles were observed constitutively; however, during nutrient stress (carbon and nitrogen specifically) a second double membraned transport vesicle, the autophagosome, was observed to accumulate within yeast cells (Baba et al., 1997). Although one pathway is constitutive and one is inducible, CTV and autophagy share physical attributes, require several of the same proteins for function and share mechanistic themes. This allowed researchers to dissect the autophagy pathway in yeast using already established assays (Kim et al., 1997).

The autophagic process has been divided into seven distinct steps: induction, cargo selection and packaging, vesicle nucleation, vesicle expansion and completion, vesicle targeting docking and fusion and vesicle breakdown (refer to Figure 1.2 during the following discussion) (Levine and Klionsky 2004). Induction of autophagy has been identified to be negatively regulated by a protein kinase TOR (Target of Rapamycin) (Schmelzie and Hall 2000). TOR is thought to play two roles in autophagy inhibition: i) it may, control translation or transcription of autophagy genes via activation of downstream effectors or, ii) it is directly or indirectly responsible for hyperphosphorylation of ATG13, a subunit of ATG1 protein kinase (Abeliovich and Klionsky 2001). A hyperphosphorylated ATG13 has low affinity for ATG1; the formation of the ATG13/ATG1 complex is required for the induction of autophagy (Levine and Klionsky 2004). To date, the target of the ATG13/ATG1 complex has not been identified and neither have other proteins required for autophagy induction. TOR kinase activity on ATG13, however, is dependent on the nutritional status of the cell. During starvation conditions it is inactive on ATG13 allowing ATG13/ATG1 complex formation and the induction of autophagy (Levine and Klionsky 2004).

Cargo selection and packaging have been predominately characterized in yeast for the CVT pathway rather than autophagy, although the two pathways share several components (Stromhaug and Klionsky 2004). The specificity of the CVT pathway is an example of how a non-selective autophagy can become a selective process, with the addition of components,. The recognition of the PrApe1 complex and Ams1 is governed by Cvt19 (ATG19) of the CVT

pathway. Cvt19 is not a conventional receptor as it is recruited by the formation of the PrApe1 complex and not visa versa. It then recruits Ams1. Cvt9 is then recruited by the Cvt19-complex and is involved in mediating the delivery of the cargo to the preautophagosomal structure (PAS) (Wang and Klionsky 2003). The PAS is a center that recruits cargo for packaging into the double membraned vesicle, the autophagosome or the CVT vesicle (Wang and Klionsky 2003). Once cargo has reached the PAS vesicle expansion can begin. The origins of proteins involved in the PAS are still poorly understood.

The initiation of vesicle formation at the PAS, also referred to as vesicle nucleation, is associated with the phosphatidylinositol (PtdIns) 3-kinase complex I, which contains the PtdIns 3-kinases Vps34, Vps15, Vps30/ATG6 and ATG14 (Kihara et al., 2001). ATG9, a transmembrane protein, is also thought to be associated with early vesicle formation (Levine and Klionsky 2004). The exact role of this complex and ATG9 are unknown; however, synthesis of the CVT vesicle and the autophagosome are considered to be a *de novo* process unlike membrane recycling of the endomembrane system. The membrane required for the formation of these vesicles is speculated to arise from the ER although the exact mechanisms that divert membrane to CVT vesicle or autophagosome formation remain to be elucidated (Fengsrud et al., 2004).

Vesicle expansion and completion is probably the most characterized step in autophagy. It involves two ubiquitin-like conjugation steps. A cysteine protease, ATG4, cleaves the C-terminal end of ATG8 exposing a glycine residue. The E1-like enzyme ATG7 then activates and transfers ATG8 to an E2-like enzyme ATG3. ATG8 is then conjugated to phosphatidylethanolamine (PE). The second conjugation step results in the formation of an isopeptide bond between ATG12 and ATG5. This conjugation again involves the E1-like enzyme ATG7 and E2-like enzyme ATG10. ATG16 is recruited to the ATG12/ATG5 complex and the resulting complex is recruited to the PAS where it is thought to play a role in vesicle formation, necessary for the generation of a spherical vesicle. None of the proteins involved in the second conjugation step are retained within the mature autophagosome, leading to the assumption that the complex functions similar to coat proteins: in the curvature generation process (Wang and Klionsky 2003).

Vesicle targeting was originally thought to require ATG8, which was characterized as a microtubule-associated protein thought to function with ATG4 in transporting the mature

autophagosome to the vacuole; however, later it was identified as a component of the conjugation step in vesicle formation (Lang et al., 1998). Levine and Klionsky (2004) discovered that the mechanisms involved in vesicle trafficking of the endomembrane system were also responsible for targeting and docking of autophagosomes and CVT vesicles. The SNARE proteins Vam3, Vam7, Vit1 and Ykt6; the NSF, Sec17; the SNAP, Sec18 and the GDI, Sec19 as well as the Rab protein Ypt7 have all been identified in both the CVT vesicle and autophagosome and found to be involved in targeting, tethering and fusion of these structures to the vacuole (Levine and Klionsky 2004).

Upon fusion to the vacuole, the outer autophagic membrane becomes associated with the vacuolar membrane and the inner autophagic membrane containing the cargo is digested, which is dependent on the acidity (pH) of the vacuolar lumen. The PAS is associated with the vacuole and it is speculated that there could be some recycling of the autophagosomal membrane in the formation of the PAS post vesicle fusion. During starvation conditions the breakdown of cytoplasmic contents and organelles facilitates cell survival via the release of building blocks for the maintenance of cellular homeostasis (Levine and Klionsky 2004).

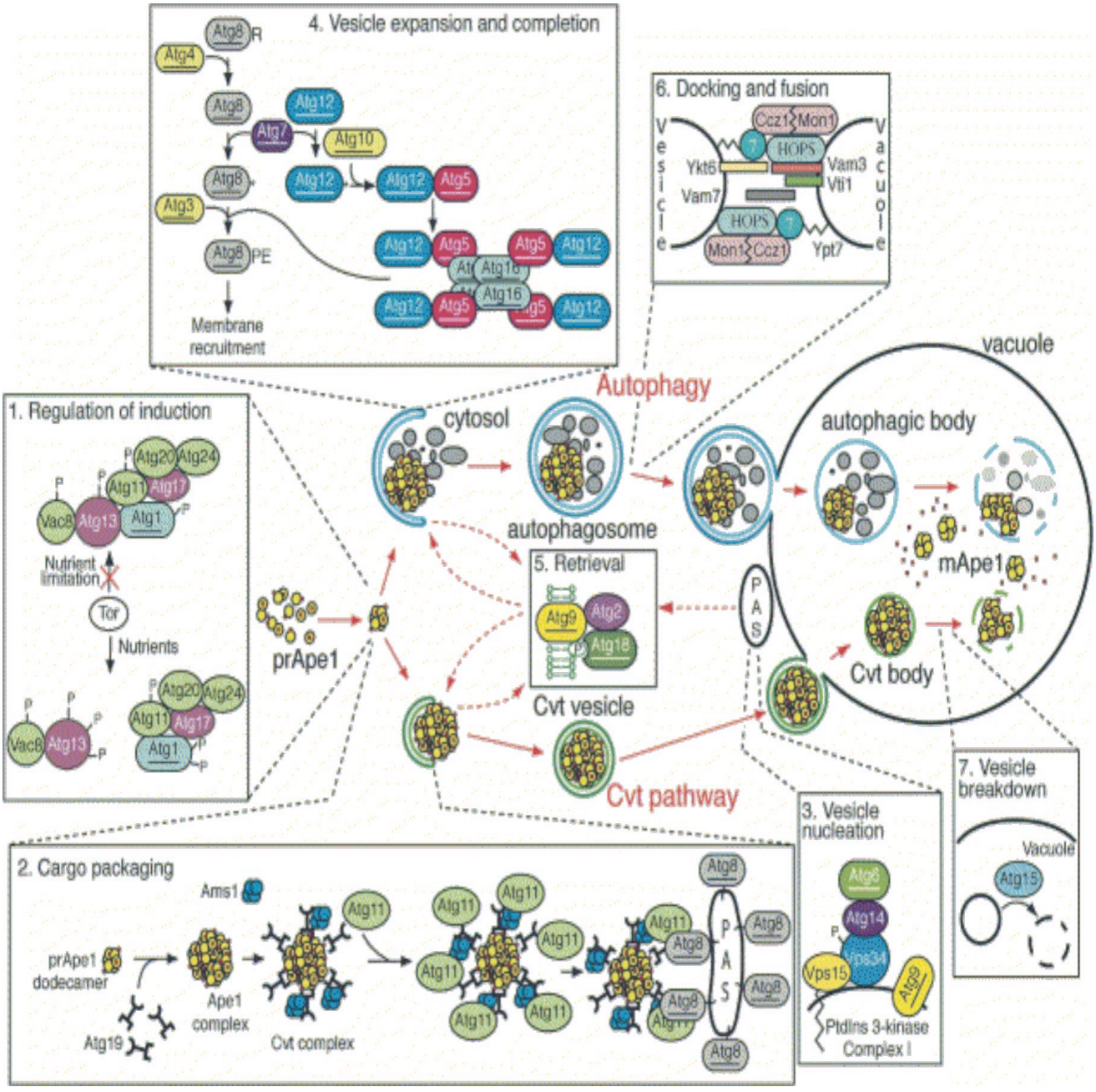

Figure 1.2 Representation of the cytoplasm to vacuole targeting and autophagy pathways in yeast. With emphasis on the autophagy pathway, the process, characterized predominately in yeast, can be divided into seven steps: Induction (1), cargo packaging (2), vesicle nucleation (3), vesicle expansion and completion (4), retrieval (5), docking and fusion (6) and vesicle breakdown (7). 1) Vesicle induction is negatively regulated by TOR kinase; it phosphorylates ATG13 which is then unable to form a complex with ATG1. During starvation conditions, TOR is inactive on ATG13, the ATG13/ATG1 complex is formed and through as of yet unknown down stream effectors autophagosome formation is initiated. 2) In the CVT pathway a PrApe1 complex recruits ATG19 and Ams1. ATG11 is then recruited by ATG19 and the entire complex is recruited to the PAS. 3) Vesicle nucleation is observed to require lipid kinase complexes I and II. Vsp34, a requirement to both complexes is involved in generating PtdIns (3)phosphate at the

PAS. 4) Vesicle expansion and completion involves two ubiquitin-like reactions. ATG8 is post-translationally processed by ATG4 for subsequent activation by the E1-like ATG7. An E2-like enzyme, ATG3, conjugates ATG8 to PE. The ATG8-PE complex is then recruited to the PAS. ATG12 is also activated by ATG7 and then conjugated to ATG5 via ATG10. The ATG12/ATG5 complex recruits ATG16 and that complex is recruited to the PAS where it is thought to play a similar role as do coat proteins of the endomembrane system. 5) Retrieval is poorly characterized as ATG9, the transmembrane protein, has been associated with a developing autophagosome, which is recycled by ATG2 and ATG18. 6) Vesicle targeting, docking and fusion involves the SNARES: Vam3, Vam7, Vit1 and Ykt6 (docking proteins) and the Rab: Ypt7 (targeting protein) thought to target the autophagosome to the vacuole. 7) The degradation of the autophagic body (inner autophagic membrane and cargo) is observed to be dependent on vacuolar hydrolase activity and ATG15 a lipase within the vacuolar lumen. This figure was borrowed from Levine and Klionsky 2004.

1.2.2 Autophagy in *A. thaliana*

With the complete *A. thaliana* genome sequence available, orthologous autophagy genes could then be identified in this higher eukaryote. In 2002, Hanaoka et al. conducted a genome wide search of the *A. thaliana* genome for genes orthologous to autophagy genes in yeast. They identified 25 *A. thaliana* autophagy genes orthologous to 12 yeast autophagy genes based on sequence similarity, all essential to autophagy in yeast. Yeast *APG1* has three orthologs in *A. thaliana*, *ATG1a-c*; *APG4* has two, *ATG4a-b*; *APG5, APG6, APG7* and *APG9* have only one, *ATG5, ATG6, ATG7* and *ATG9* respectively; *APG8* has nine, *ATG8a-i* and *APG12* has two *ATG12a-b* (Hanaoka et al., 2002). The conservation of autophagy genes in *A. thaliana* suggests that plants may use this pathway in a similar way to yeast, however, the observation that *A. thaliana* possessed multiple orthologs suggests that this process may be more complex in this higher eukaryote (Doelling et al., 2002; Hanaoka et al., 2002).

Although, at this time, autophagy genes were identified in *A. thaliana* there had been no experimentation examining the role/function of these genes. Hanaoka et al. (2002) was the first group to characterize an *A. thaliana* autophagy gene. They identified a single ortholog in *A. thaliana* (*ATG9*) homologous to the yeast *APG9*. A T-DNA insertion mutation at this locus (*atatg9-1*) resulted in accelerated senescence during nitrogen and carbon starvation conditions and a reduction in seed set. *A. thaliana ATG9* could compliment a yeast *apg9* mutant. This group concluded that autophagy, in *A. thaliana*, is involved in maintenance of cellular viability during starvation conditions (Hanaoka et al., 2002). In 2002, Doelling et al. identified gene families

encoding proteins involved in the vesicle expansion and completion step of autophagy (the ATG8 and ATG12 conjugation step). They found a single *APG7* locus in *A. thaliana* (*ATG7*) encoding the E1-like activating enzyme required for both conjugation pathways during vesicle expansion and completion. A mutation at this locus did not disrupt normal growth and development; however, *atg7* plants were hypersensitive to nutrient limited conditions, exhibiting accelerated senescence. The accumulation of both *ATG7* and *ATG8* mRNA during senescence was observed suggesting that autophagy is induced during senescence. With the identification of orthologs in *A. thaliana*, conservation of autophagy in plants suggests its importance in carbon and nitrogen remobilization during starvation and senescence (Doelling et al., 2002; Hanaoka et al., 2002).

These early reports initiated a stream of research focusing on autophagy and cellular viability in *A. thaliana*. Although several orthologs were identified in *A. thaliana* it was not clear if they were all required for autophagy. For example, yeast *APG8* has nine orthologs in *A. thaliana* (*ATG8a-i*) and *APG4* has two (*ATG4a-b*). Yoshimoto et al. (2004) further characterized some of these orthologs during the autophagic process in *A. thaliana*. APG8 is cleaved by APG4 prior to being conjugated to PE in yeast. All nine orthologs of *APG8* in *A. thaliana*, *ATG8a-i*, as well as the two *APG4* orthologs, *ATG4a-b*, were found to be ubiquitously expressed at low levels in all *A. thaliana* organs, and induced upon nitrogen starvation (Yoshimoto et al., 2004). Interestingly this group found that only seven of the nine *ATG8* orthologs had the characteristic extension of the C-terminal end after the glycine residue (cleaved by ATG4 prior to conjugation to PE). In *A. thaliana* ATG4a and ATG4b are responsible for the cleavage of the C-terminal region exposing the glycine residue of ATG8a-g (Yoshimoto et al., 2004). Using subcellular fractionation, this group found that, ATG8 proteins are associated with membranes, as in yeast. Using an *atg4a/b* double mutant, Yoshimoto et al. (2004) demonstrated that ATG8 proteins did not conjugate into membranes and that ATG4a and ATG4b were responsible for cleaving the C-terminal end of ATG8 proteins essential for autophagosome formation. Another assay conducted by Yoshimoto et al, was to conjugate ATG8 to GFP which enabled them to visualize autophagosomes in the cytoplasm of both root cells and protoplasts of *A. thaliana* (Yoshimoto et al., 2004). With the availability of an autophagy marker, ATG8-GFP, cellular visualization of this pathway was facilitated. Slavikova et al. (2005) used concanamycin A (conA) to inhibit vacuolar ATPases and observed an accumulation of fluorescing structures, presumably

autophagosomes, within the vacuolar lumen. Thompson et al. (2005) demonstrated that an *A. thaliana atg5* mutant was susceptible to early senescence and hypersensitive to both carbon and nitrogen starvation. In both *atg5* and *atg7* mutants they were unable to visualize ATG8e-GFP accumulation in the vacuole after conA treatment supporting the important role of ATG5 and ATG7 in the conjugation pathways essential for autophagosome formation. In 2005, a new technique was developed that facilitated autophagosome visualization (Contento et al., 2005). Monodansylcadaverine (MDC) is a fluorescent probe that stains lytic compartments, therefore vacuoles. In yeast, the autophagosome itself was capable of degrading its contents prior to arrival at the vacuole; therefore, they were considered to be lytic. Using the MDC technique, Contento et al. (2005) were able to co-localize MDC and ATG8e-GFP autophagosomes in *A. thaliana* protoplasts.

In 2006, Rose et al. monitored the expression of *ATG3, ATG4a, ATG4b, ATG7* and *ATG8a-i*. They were curious if the expression would mimic the expected sequence of events, that is, expression of *ATG4a* and *ATG4b* first since they are required to cleave ATG8s; *ATG8a-i* second, followed by *ATG7* and *ATG3* (Levine and Klionsky 2004). Such a trend was observed when they subjected *A. thaliana* protoplasts to a sucrose free environment. Their results, along with others, confirmed that the processing of autophagy proteins and role of autophagy in plants appears to be similar to that identified in yeast (Rose et al., 2006).

1.2.3 Physiological roles of autophagy in mammals, plants and fungi

To date, autophagy has been implicated in processes such as programmed cell death, nutrient recycling during starvation conditions, nutrient remobilization during senescence and pollen germination, aging, cellular homeostasis, and immunity in eukaryotic cells (Doelling et al., 2002; Hanaoka et al., 2002; Levine and Klionsky 2004; Colombo 2005; Contento et al., 2005; Liu et al., 2005; Schmid and Munz 2005; Terman and Brunk 2005; Thompson et al., 2005; Bassham et al., 2006; Rose et al., 2006; Seay et al., 2006; Veneault-Fourrey et al., 2006). In mammalian cells, autophagy appears to play contradictory roles, dependent on the situation or cell type. It has been associated with cell death and so a third type of cell death termed 'autophagic cell death' has been introduced. In mammalian cardiovascular cells, autophagy has been implicated as the mechanism of cell death contributing to several diseases such as heart failure, aortic valve

degeneration and heart infractions (Mistiaen et al., 2006). Understanding autophagic cell death has been the goal of medical researchers in the hope of autophagy targeted drug therapies (Mistiaen et al., 2006).

In several cases autophagy has been observed to aid in cellular homeostasis, especially in aging cells and specialized cells (Terman and Brunk 2005). Neurons and cardiac cells are considered specialty cells since they are not easily renewable even with stem cell technology; therefore, the longer these cells can stay alive the better their corresponding systems are operating (Terman and Brunk 2005). In both of these types of cells autophagy is involved in turning-over damaged cell components, such as mitochondria, to renew biological materials within the cell. As mammalian cells age the process of autophagy is retarded. Cell aging in neurons is accompanied by an increase of events such as the misfolding of proteins (He and Klionsky 2006). This is especially harmful in postmitotic cells such as neurons because misfolded proteins cannot be diluted with cell divisions (Ross and Poirier 2004; He and Klionsky 2006). The accumulation of misfolded proteins in these cells contributes to such neurodegenerative diseases like Alzheimers and Parkinsons. Autophagy plays a considerable role in the degradation of misfolded proteins, also considered proteinopathies, since they are pathogenic to a cell (Williams et al., 2006).

Oncologists are interested in autophagy since this pathway is induced in cancer cells treated with anti-cancer therapies (Kondo and Kondo 2006). The visualization of autophagosomes within the cytoplasm of treated cells has oncologists wondering if autophagy is induced to help maintain cancer cell homeostasis or if it is induced to initiate cancer cell death (Kondo and Kondo 2006). The mechanisms of autophagy are under investigation, and targeted manipulation of this pathway is considered a new strategy in the fight against cancer.

There has been one example of the necessity of autophagic cell death in fungi for plant disease development and ultimately infection (Veneault-Fourrey et al., 2006). *Magnaporthe grisea*, the pathogen causing rice blast disease, geminates on the host surface to produce a melanized appressorium from which a penetration peg pierces the epidermal cell of rice leaves or stems (Veneault-Fourrey et al., 2006). This group noticed appressorium morphogenesis was always accompanied by spore collapse and tested whether autophagic cell death was responsible for spore collapse. Using *Mgatg8* knockout line they confirmed that autophagy was responsible for cell death of the spore as the mutant lines were unable to produce a penetration peg and

appressorium morphogenesis was not accompanied by spore cell death, and they could not cause infection in rice (Veneault-Fourrey et al., 2006).

In plants, morphological studies revealed that autophagy is induced in rice, maize, tobacco and *A. thaliana* in response to sucrose and nitrogen starvation (Chen et al., 1994; Aubert et al., 1996; Moriyasu and Ohsumi 1996). In these assays, double membraned autophagosomes accumulated in the cytoplasm and then are deposited into the vacuolar lumen. In intact maize plants, carbon starvation resulted in net protein reductions, which is associated with an autophagic process (Brouquisse et al., 2007). As early as 1978 autophagosomes were identified in root meristematic cells and implicated in vacuole formation (Marty 1978). In plants, autophagy has also been associated with senescence (Wittenbach et al., 1982; Doelling et al., 2002; Hanaoka et al., 2002; Slavikova et al., 2005). Protein degradation during leaf senescence is accomplished via chloroplast proteases; however, the degradation of the chloroplast is associated with autophagy (Wittenbach et al., 1982). More recently autophagosome accumulation and autophagy gene transcript accumulation have been identified in both *A. thaliana* and tobacco in response to carbon and nitrogen starvation (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Contento et al., 2005; Slavikova et al., 2005; Thompson et al., 2005; Rose et al., 2006). In *A. thaliana,* autophagy has been associated with controlling programmed cell death in response to infection, this role for autophagy will be discussed further in the following section.

1.2.4 Autophagy and immunity

A role for autophagy in immunity has been identified in both mammalian and plant systems. Generally, autophagy has been categorized into the innate immune response category, a non-specific generic recognition and response to pathogens that does not confer long-lasting immunity (Alberts et al., 2002). Admittedly, since autophagy is proposed to be non-selective, researchers consider it an understudied form of defense. It should be noted again that autophagy has been implicated in maintaining cellular viability against such things as aging organelles and proteinopathies in mammalian systems, and this type of maintenance could be considered a form of cellular immunity.

In mammalian systems, autophagy eliminates infectious agents that have accessed the cytoplasm (Rich et al., 2003). A classic example of this was demonstrated by Gutierrez et al. (2004) in macrophages infected by *Mycobacterium tuberculosis*. Interferon- γ (INF- γ) induces autophagy in macrophages, which arrests *M. tuberculosis* intracellular survival. Autophagic cell death has also been speculated to be a means of defense to intracellular pathogens, bacteria and viruses, but there are no clear mechanisms supporting this idea.

More intensively studied in mammalian systems is the ability of pathogens to use autophagy to avoid host defense responses intracellularly (Dorn et al., 2002; Swanson et al., 2002). Pathogenic microorganisms possess type IV secretory systems involved in secreting molecules that allow the pathogen to subvert host defenses (Segal et al., 2005). Interestingly, autophagy is activated by the *Legionella pneumophila* and *Coxiella burnetii* type IV secretory systems (Beron et al., 2002). It is suggested that these bacteria interact with the autophagy pathway to facilitate their replication (Colombo 2005). *Coxiella burnetii* replicates in a compartment labeled by ATG8 (Gutierrez et al., 2005). Cells treated with autophagic inhibitors had a dramatic reduction in bacterial persistence (Dorn et al., 2001). The common *Porphyromonas gingivalis* replicates in autophagosomes of endothelial cells (Dorn et al., 2002). The autophagosome is assumed to be a protective niche for replication that contains nutrients such as small peptides and amino acids that can be taken up by bacterial transporters (Colombo 2005).

To date, there has been only one example correlating autophagy and plant innate immunity. The innate immune response includes the HR, a localized form of PCD involving the accumulation of ROS. Localization of the HR is crucial to the survival of the infected plant. Liu et al. (2005) demonstrated in tobacco infected with an incompatible strain of Tobacco Mosaic Virus (TMV) that the induction of the HR was responsible for conferring resistance. They noticed the accumulation of ROS in infected cells and in cells distal to infection. Cells distal to infection survived and did not initiate PCD, even though they were experiencing an increased production of ROS (which in most cases initiates PCD). Plant BECLIN 1, an ortholog to yeast and mammalian ATG6/Vsp30/BECLIN1 (involved in vesicle nucleation step), ATG3 and ATG7 were involved in regulating unrestricted HR/PCD in cells distal to the infection and observed autophagic vesicles in both infected cells and cells distal to infection. Therefore autophagy was induced during infection, and plant BECLIN 1 was required for its induction. Plants with a

beclin1 mutation exhibited unrestricted cell death distal to the primary infection site compared to wild type plants. They concluded that autophagy negatively regulates HR/PCD during this plant innate immune response.

1.3 Themes and Research Questions

This thesis is focused on elucidating the role of autophagy in resistance or susceptibility during plant and fungal pathogen interactions. Since autophagy is induced as an intracellular coping mechanism during carbon and nitrogen starvation, and is a negative regulator of senescence, two *A. thaliana* pathosystems were employed to examine both functions of autophagy. The *A. thaliana-Erysiphe cichoracearum* pathosystem facilitates the exploration of the involvement of autophagy in maintaining cellular viability during susceptible infection. Using this pathosystem it is also possible to examine the likelihood of autophagy having a role in attempted resistance. The *A. thaliana-Colletotrichum higginsianum* pathosystem facilitates an exploration of the effects of the process of senescence on pathogenicity, and thereby the importance of autophagy, the negative regulator of senescence, in immunity. This system could also be used in an attempt to duplicate the observed results of *A. thaliana-E. cichoracearum* pathosystem since both systems share several developmental similarities in the earlier stages of infection. Autophagy gene expression in *A. thaliana* during infection with *E. cichoracearum* and *C. higginsianum* was investigated to determine if this pathway was induced during infection. Using four *A. thaliana* lines defective in autophagosome formation, the importance of this pathway for susceptibility was investigated. Using an *A. thaliana* transgenic line, *AtATG8e*-GPF, autophagosomes were visualized in real time during infection by *Erysiphe* and *Colletotrichum.*

The objectives of this thesis are to:

- Investigate expression of autophagy genes in *A. thaliana* in response to infection by *E. cichoracearum* and *C. higginsianum.*
- Examine the formation and reorganization of autophagosomes as a cellular response to infection of *A. thaliana* by *E. cichoracearum* and *C. higginsianum.*
- Determine the role of the autophagy pathway in *A. thaliana* immunity against *E. cichoracearum* and *C. higginsianum*

CHAPTER 2

AUTOPHAGY IN *ARABIDOPSIS THALIANA* IN RESPONSE TO BIOTROPHIC AND HEMIBIOTROPHIC FUNGAL INFECTION

2.1 Introduction

Autophagy, an indiscriminant-recycling pathway, has recently been identified in *Arabidopsis thaliana* (Doelling et al., 2002; Hanaoka et al., 2002). Autophagic trafficking vesicles, autophagosomes, are unique to the trafficking vesicle of the endomembrane system in that they are double membraned (Clark 1957; Marty 1978). ATG8 is the only known autophagy protein associated with the inner autophagic membrane and the green fluorescence labeled ATG8e is used extensively *in vivo* (*At*ATG8e-GFP) as a marker for autophagy (Contento et al., 2005). A second *in vivo* marker for autophagosomes is the stain monodansylcadaverine (MDC). MDC is an autofluorescent amine that reacts with lipids within autophagosomes. This property of MDC is based on two independent effects: i) ion trapping in acidic compartments draws this stain because of the presence of an amino group and ii) the dansyl moiety of the molecule emits a fluorescent signal upon interaction with lipid molecules in a hydrophobic environment (Biederbick et al., 1999; Niemann et al., 2001). This stain stains lytic compartments and has been used to stain autophagosomes in the cytosol and vacuole of *A. thaliana* protoplasts (Contento et al., 2005).

Autophagosome accumulation within the cell is observed during carbon and nitrogen stress (deficiency), resistance response to TMV in *Nicotiana* and senescence in *A. thaliana*. Although autophagy is not required for senescence it is assumed to play a role in nutrient remobilization during the senescence process (chloroplast degradation) (Doelling et al., 2002; Hanaoka et al., 2002). The process of autophagy during stress conditions involves the acquisition of cytosolic components (organelles and cytoplasm) which are encapsulated into autophagosomes and transferred to the vacuole for degradation; this results in a release of building blocks such as amino acids and carbon back into the cytosol where they can be used to maintain cellular viability rather than *de novo* synthesis (Wang and Klionsky 2003).

Previous studies using *A. thaliana* protoplasts revealed that several autophagy genes are induced during carbon and nitrogen starvation (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Contento et al., 2005; Liu et al., 2005; Slavikova et al., 2005; Thompson et al., 2005; Rose et al., 2006). Responsive genes include *ATG1, ATG3, ATG4a-b, ATG5, ATG7, ATG8a-i, ATG9, ATG12, BECLIN1* and *Vsp34*. Autophagy mutants (*atg4a/b, atg5-1, atg7, atg9-1*) display an accelerated senescence phenotype and are more susceptible to carbon or nitrogen deficiency (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005). An *AtATG8e*-GFP fusion construct has been used to transiently transform *A. thaliana* protoplasts enabling visualization of accumulated autophagosomes in both the cytosol and vacuolar lumen during starvation events. These data support the role of autophagy in maintaining cellular viability (Contento et al., 2005).

The establishment of biotrophic parasitism by a single *Erysiphe* conidium occurs within a single plant epidermal cell. Nutrients, primarily glucose, are taken up by the fungal biotroph *E. cichoracearum* through a feeding structure called the haustorium (Panstruga 2003). Though, exact mechanisms of nutrient transfer are unknown. Hemibiotrophic pathogens, such as *Colletotrichum higginsianum* establish infection initially via biotrophy, producing haustorium-like primary hyphae, then switch to necrotrophy (O'Connell et al., 2004). The duration of biotrophy is variable between *Colletotrichum* species and the factors that affect it are the age of the infected plant, biochemical reactions of the infected plant (such as the increase in ethylene production) and the density of infection (Wei et al., unpublished data). It appears that *Colletotrichum* species are sensitive biotrophs, switching to necrotrophy when nutrient conditions become limiting.

Autophagy has a role in limiting programmed cell death in the resistance response of tobacco to TMV (Liu et al., 2005). To date, in plants, no other pathosystem has been reported to involve autophagy. It is therefore justified to examine a possible role for this pathway in the susceptible *A. thaliana-E. cichoracearum* and -*C. higginsianum* pathosystems. It is unknown whether the stress of biotrophic parasitism is substantial enough to elicit a starvation response, such as autophagy *in planta*, or whether autophagy is involved as an attempted resistance response to these pathogens.

2.2 Materials and Methods

2.2.1 Plant and pathogen materials

A. thaliana ecotypes Col-0 and Ws-0 were obtained from the Arabidopsis Biological Resource Center. The *At*ATG8e-GFP transgenic line was received from Contento et al. (2005) (D.C. Bassham, Department of Genetics, Development and Cell Biology, Plant Sciences Institute and Interdepartmental Plant Physiology, Iowa State University). All *A. thaliana* seeds were sown on soil, cold-treated prior to germination at 4^{o}C for 2 days and then transferred to either the greenhouse (plants to be inoculated with *E. cichoracearum*) with a natural photoperiod and at temperature constant at 25^{o}C or to a growth chamber (plants to be inoculated with *C. higginsianum*) with a cycle of 16 h light 8 h dark at 22^{o}C day and 18^{o}C night. *C. higginsianum* conidia were propagated at room temperature on V8 or potato dextrose agar (PDA) medium as described previously by Wei et al. (2004). *E. cichoracearum* was maintained on a susceptible cucumber cultivar (National Picking) in the greenhouse at 25^{o}C.

2.2.2 Inoculation procedure

E. cichoracearum conidia from 9 to 14-day old colonies were used in each experiment. Intact *A. thaliana* plants, approximately 3 to 4 weeks old, to be inoculated with *E. cichoracearum,* were placed in a box. Cucumber leaves with sporulating powdery mildew were detached from the plant and conidia were brushed onto *A. thaliana* ecotypes Col-0 and *AtATG8e*-GFP. Inoculated *A. thaliana* plants were removed from the box approximately 1 hour after inoculation and exposed to regular greenhouse conditions. Conidia from *C. higginsianum* were collected from 7 to 9-day old plates and spore suspensions were created (1x10^{6} spores ml^{-1} in distilled water). Spray and droplet inoculations (5-10 µl droplets on either side of the leaf mid-vein) were employed depending on the assay. Intact plants that were inoculated with *C. higginsianum* were kept at 100% humidity in a growth chamber immediately after inoculation (Narusaka et al., 2004; O'Connell et al., 2004). Detached leaves from 3 to 4-week old plants were inoculated using either the spray or droplet method and incubated in Petri dishes (with wet filter paper) and

maintained under laboratory conditions (room temperature) with a 12 h photoperiod at a light intensity of 30 µE $m^{-2}sec^{-1}$ (Liu et al., 2007b).

2.2.3 RNA isolation and cDNA preparation

A. thaliana leaves with and without infection were collected at varying time intervals (3, 6 and 9 days post infection (dpi) with *E. cichoracearum* and 1, 3 and 5 dpi with *C. higginsianum*) and stored at -80°C. Total RNA was extracted using a phenol/chloroform method as described by Liu et al. (2005). cDNA was generated using the SuperScript™ II Reverse Transcriptase kit (Invitrogen) following the manufacturer's instructions.

2.2.4 Reverse Transcription-Polymerase Chain Reaction analysis

Each reverse transcription PCR (RT-PCR) was performed using 1 µl of cDNA, 0.1 µl Platinum®Taq DNA Polymerase (Invitrogen), 1 µl dNTP (2.5 mM each dATP, dTTP, dCTP, dGTP), 2.5 µl 10x buffer and water for a final volume of 25 µl. All reactions were carried out using an Eppendorf Mastercycler®. Gene-specific primers used in each reaction are listed in Table 2.1. The PCR reactions for *ATG1, ATG3, ATG4a, ATG4b, ATG5, ATG7, ATG8b, ATG8c, ATG8d, ATG8f, ATG8g, ATG9, PR1* and *PDF1.2* were run at 94°C for 3 min; 94°C for 50 s; 55°C for 30 s; 72°C for 1 min at 32 cycles; *ATG8a* and *ATG8i* ran under the same conditions for 34 cycles. The reactions for *ATG8h*, *ATG8e* and *Telf1-2* and were run at 94°C for 3 min; 94°C for 50 s; 50°C for 30 s; 72°C for 1 min at 32 cycles.

2.2.5 Concanomycin A (conA) and monodansylcadaverine (MDC) treatments for confocal microscopy

Mock and *Colletotrichum*- and *Erysiphe*-inoculated leaves of *AtAtg8e*-GFP plants were collected at varying time points post-inoculation and immediately placed in 300 µl of 1 µM conA followed by vacuum infiltration for approximately 30 sec each. Infiltrated tissue was kept at 4°C for 12 to16 h (Contento et al., 2005; Thompson et al., 2005). For MDC staining, conA treated tissue was placed in 0.1 mM MDC for 2 h and kept on ice at 4°C followed by 2 to 3 vacuum

infiltrations for approximately 30 sec each immediately prior to microscopy as modified from Contento et al. (2005). The tissue was then rinsed twice in PBS to remove excess MDC.

Table 2.1 List of primers used for RT-PCR analysis of *A. thaliana* leaves.

Gene Name	Accession Number	Primer Sequence	Reference
AtATG1	*At*3g61960	5′-CCGAAAGTCTAACGGGTCAA-3′	This Study
		5′-ACCGGCACTCCATAAGTCAG-3′	
AtATG3	*At*5g61500	5′-TCATCCACACTTGCCTGGTA-3′	Rose *et al.*, 2006
		5′-CCGAGATCAAAGTCCATTGTG-3′	
AtATG4a	*At*2g44140	5′-GGCTGCATTGCAACTAGATTT-3′	Rose *et al.*, 2006
		5′-GAATCATGCAACCCCAGTTC-3′	
AtATG4b	*At*3g59950	5′-CTTTCACGTTCCCTCAAAGC-3'	Rose *et al.*, 2006
		5′-TTGCAATGGTAAGACGATGTG-3′	
AtATG5	*At*5g17290	5′-TCCTCCTCCTGCTCTTGTGT-3′	This study
		5′-CACCCACGAAAACGGTATCT-3′	
AtATG7	*At*5g45900	5′-CGTTCCCTGCGTTTGTACTT-3′	This study
		5′-GGTAATGCTCGCCATCTCAT-3′	
AtATG8a	*At*4g21980	5′-ACATTGACTTCTCTCTCTCGATT-3′	This study
		5′-ATCACGGGAATTCTGTCAGG-3′	
AtATG8b	*At*4g04620	5′-AGTTTCGTCTTTTGGTTTCGAG-3′	This study
		5′-AGATGGCTTTTTCAGCTCCA-3′	
AtATG8c	*At*1g62040	5′-ACACGAAACCCATCAAAATCGAAT-3′	This study
		5′-GATAGCCTTTTCGGCACTCA-3'	
AtATG8d	*At*2g05630	5′-TGGAGTTACCCAATTTCGACGTTG-3′	This study
		5′-TTCTCGGGACTGAGCTTGAT-3′	
AtATG8e	*At*2g45170	5′-ACCCTGATCGAATTCCTGTG-3′	This study
		5′-TTGAAGAAGCACCGAATGTG-3′	
AtATG8f	*At*4g16520	5′-TAGCGTTGTGCTCTGGAGAAGT-3′	This study
		5′-CCTGCTGGAGGAAGAACATT-3′	
AtATG8g	*At*3g60640	5′-GTCGAATCCAAAGTTTTTGATC-3′	This study
		5′-CGGTGGGAGGAAGAACATTAT-3′	
AtATG8h	*At*3g06420	5′-CATAAAATTGAACACATCCT-3′	This study
		5′-CATGTCTCGTGGGACCAAGT-3′	
AtATG8i	*At*3g15580	5′-ACTTCGCCGCTTGCTTTTGAGA-3′	This study
		5′-TCCATCAGAGCAGCAGTTTG-3′	
AtATG9	*At*2g31260	5′-TTGGATCTTTTTGTCGAAAGGCTCTAC-3'	Hanaoka *et al*., 2002
		5′-AAAGCTGCAAACATGGCCTACACC-3'	
PR1	*At*2g14610.1	5′-TATGTGAACGAGAAGCCATACTAA-3′	Liu *et al*., 2007b
		5′-ATGAATTTTACTGGCTATTCTCGA-3′	
PDF1.2	*At*2g26020.1	5′-TGTATCTGTTACGTCCCATGTTAA-3′	Liu *et al*., 2007b
		5′-ATGGCTAAGTTTGCTTCCATCATC-3′	
Telf1-2	*At*2g37370	5′-CGTGAGAGGGGTATCACCATTG-3′	Liu *et al*., 2007b
		5′-GAGTGAAAGCAAGGAGGGCGTGC-3′	

2.2.6 Confocal microscopy for GFP and MDC fluorescence

MDC and GFP were visualized *in vivo* in transgenic *A. thaliana* plants, *AtATG8e*-GFP, and in control wild-type ecotypes Col-0 and Ws leaves using a Zeiss Meta 510 Confocal laser-scanning microscope (Carl Zeiss, Gottingen, Germany). MDC fluorescence was measured with an excitation wavelength of 405 nm and a band-pass filter set at 505-530 nm. GFP was visualized with an excitation of 488 nm and a 505-530 nm band-pass filter. Prior to experimentation, *AtATG8e*-GFP seedlings were screened for GFP expression and selected for further use accordingly. *AtATG8e*-GFP seedlings were screened for optimal expression five to seven days post-germination.

2.3 Results

2.3.1 Autophagic response to *E. cichoracearum* and *C. higginsianum* infection in *A. thaliana* revealed by transcript profiling

To elucidate the involvement of autophagy in *A. thaliana* during biotrophic infection of *E. cichoracearum* and hemibiotrophic infection of *C. higginsianum* the expression of autophagy genes (*ATG1, ATG3, ATG4a, ATG4b, ATG5, ATG7, ATG8e, ATG9*) during infection was examined. Pathogenesis-related genes *PR1* and *PDF1.2* were also examined since they are molecular markers for plant defense activation. Transcription elongation factor 1-2 (*Telf1-2*) a constitutively expressed gene in all conditions, was included as an internal control for RNA levels. Intact *A. thaliana* Col-0 plants were inoculated with powdery mildew and *C. higginsianum* conidia and leaf tissue were collected for RNA extraction at 3, 6 and 9 dpi and 1, 3 and 5 dpi respectively. cDNA was prepared and subjected to RT-PCR using gene-specific primers. *ATG1, ATG3, ATG4a, ATG4b, ATG5, ATG7* and *ATG9* were ubiquitously expressed at low levels in control (CK) and *E. cichoracearum*-inoculated (IN) tissues (Figure 2.1 a). Autophagy genes *ATG5* and *ATG8e* appear to be slightly induced to infection at 9 dpi (Figure 2.1 a). *PR1*, a molecular marker for salicylic acid associated defense responses to biotrophic infection, was observed to be induced at 3, 6 and 9 dpi (Figure 2.1 a). *PDF1.2* a molecular marker associated with ethylene/jasmonic acid-dependent defenses against necrotrophic pathogen infection was also induced during the infection time course. Evaluation of autophagy genes during the *C. higginsianum* infection time course in *A. thaliana* revealed marginal induction of expression (Figure 2.1 b). *ATG1, ATG3, ATG4a, ATG4b, ATG7* and *ATG9* are constitutively expressed in control and inoculated tissues and appear not to respond to infection. *ATG5* and *ATG8e* however appear to be induced at 3 and 5 dpi respectively. *PR1* is induced during *C. higginsianum* infection time course, however, *PDF1.2* shows constitutive expression. The control tissue in the *C. higginsianum* inoculation assays was treated with water and kept at high humidity (see materials and methods); this treatment may explain the discrepancy observed in expression levels of autophagy genes between *E. cichoracearum* and *C. higginsianum* inoculation assays.

Since *ATG8e* was observed to be slightly induced during *C. higginsianum* infection in *A. thaliana,* I further investigated the response of all nine members of the *ATG8* gene family, *ATG8a-i*. As described previously, intact plant tissue inoculated with either *E. cichoracearum* or *C. higginsianum* were collected for RNA extraction at 3, 6 and 9 dpi and 1, 3, and 5 dpi respectively. cDNA was prepared and subjected to RT-PCR analysis using gene-specific primers (see materials and methods).

In leaf tissue infected with *E. cichoracearum*, *ATG8a*, *ATG8b*, *ATG8d*, *ATG8f*, *ATG8g*, *ATG8h* and *ATG8i* exhibit a constitutive expression pattern (Figure 2.2 a). *ATG8c* and *ATG8e* appear to be moderately induced in infected tissues 9 dpi. In tissue infected with *C. higginsianum ATG8a*, *ATG8c* and *ATG8g* exhibit a constitutive expression pattern (Figure 2.2.b). *ATG8d* and *ATG8f* appear to be moderately induced at 1 and 3 dpi, correlating with the biotrophic phase of infection. *ATG8b*, *ATG8e*, *ATG8h* and *ATG8i* are moderately induced at 5 dpi, correlating with the necrotrophic stage of infection. These data taken together suggest that the *ATG* gene family members are inconsistently responsive to both *E. cichoracearum* and *C. higginsianum* infection in *A. thaliana* and therefore do not appear to be transcriptionally regulated by either infection.

2.3.2 Visualization of GFP-tagged autophagosomes in *A. thaliana* in response to *E. cichoracearum* and *C. higginsianum* infection

Autophagosomes, the trafficking vesicles of autophagy, accumulate in the cytosol and vacuolar lumen of *A. thaliana* protoplasts and root cells during nitrogen and carbon starvation (Yoshimoto et al., 2004; Contento et al., 2005; Slavikova et al., 2005; Thompson et al., 2005). ATG8, the only protein associated with the mature autophagosome, was located in the inner autophagic membrane (Yoshimoto et al., 2004; Contento et al., 2005; Slavikova et al., 2005; Thompson et al., 2005). Transgenic *A. thaliana* plants *ATG8e*-GFP35S have recently been used to observe autophagosomes as markers for autophagy *in planta* using confocal microscopy during various stress imposed assays (Yoshimoto et al., 2004; Contento et al., 2005; Slavikova et al., 2005; Thompson et al., 2005).

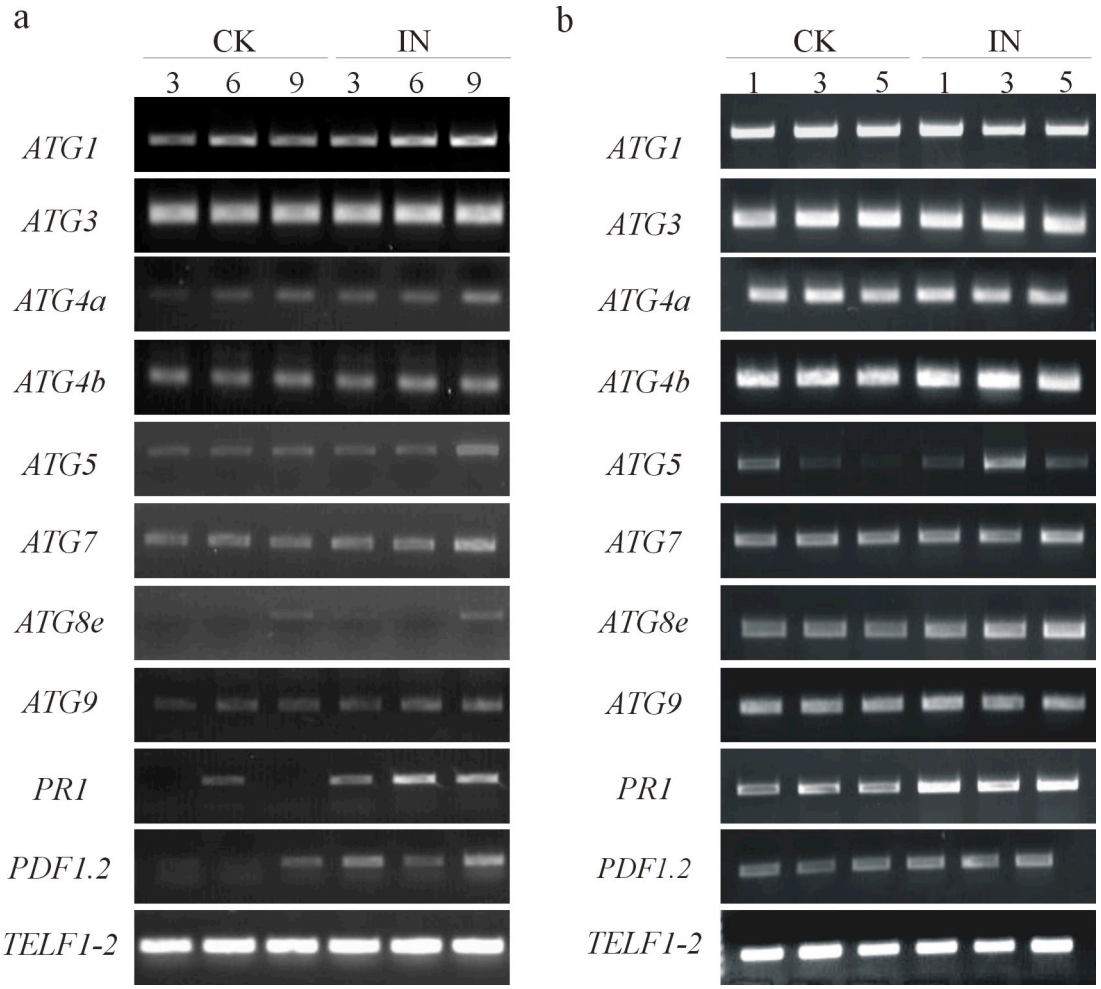

Figure 2.1 Expression of autophagy and pathogenesis-related genes in *A. thaliana* during *E. cichoracearum* and *C. higginsianum* infection time course. CK, control; IN, inoculated **(a and b)** RT-PCR analysis of autophagy and defense-related genes in *A. thaliana* during *E. cichoracearum* (a) and *C. higginsianum* (b) infection time courses. *Telf1-2* was included as a constitutive expressed loading control in both assays. This figure is representative of three biological replicates.

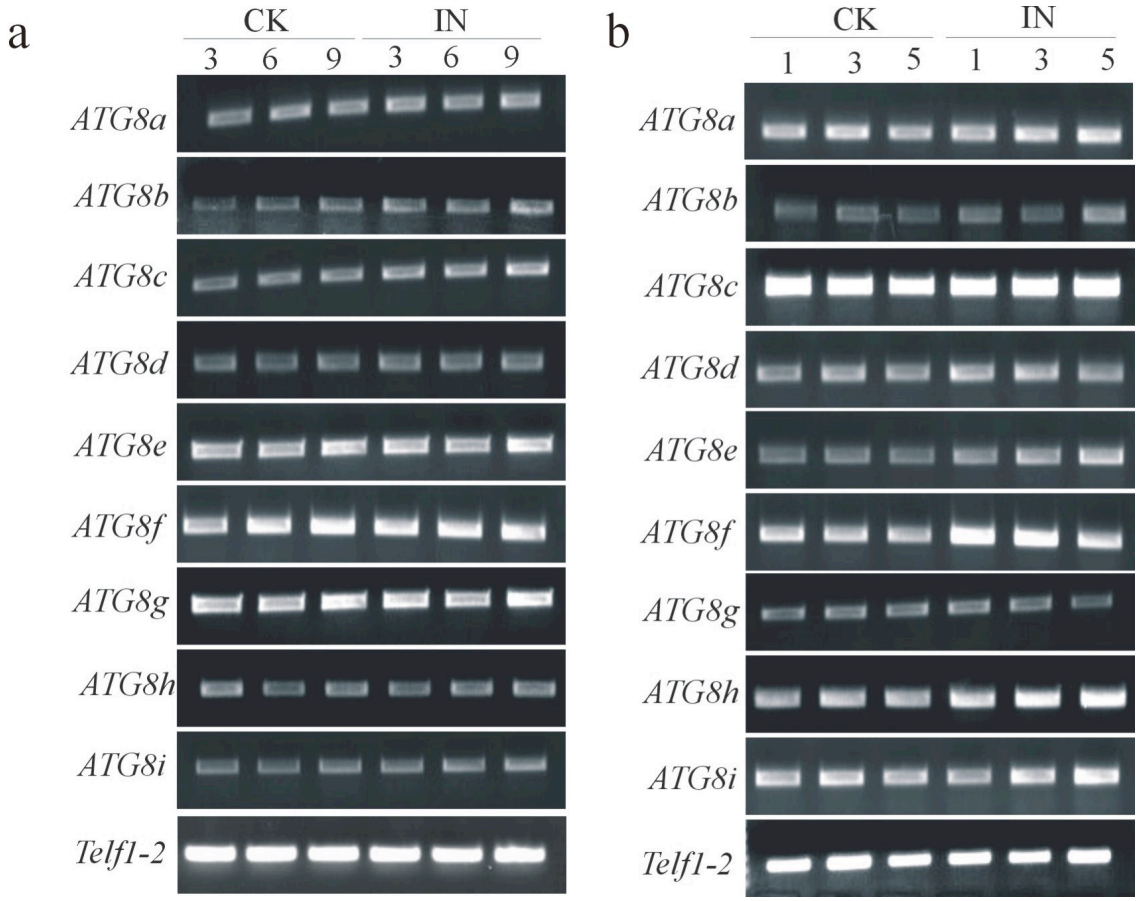

Figure 2.2 *ATG8* gene family expression in *A. thaliana* leaves during *E. cichoracearum* and *C. higginsianum* infection time courses. CK, control tissue; IN, inoculated tissue. **(a and b)** RT-PCR of *ATG8* gene family members in response to *E. cichoracearum* infection (a) and *C. higginsianum* infection (b). *Telf1-2* was included as a constitutive expressed loading control in both assays. This figure is representative of three biological replicates.

In combination with the transgenic *A. thaliana ATG8e*-GFP lines, conA, a vacuolar enzyme inhibitor, was applied to infected and control tissue 12 hours prior to observation in order to detect if the inner autophagic body had been deposited into the vacuolar lumen. Without this inhibitor, the autophagic body would be degraded and visualization of autophagosomes would be restricted to the cytosol. In an attempt to visualize an induction, accumulation or redirection response by autophagy (autophagosomes) to pathogen infection *AtATG8e*-GFP plants were inoculated with *E. cichoracearum* and *C. higginsianum* and visualized using confocal microscopy during infection. In these assays the autophagic response was investigated in control and inoculated cells as well as in cells distal to infection.

Since autophagy gene induction during *E. cichoracearum* infection was observed after 6 dpi, infected tissues of *AtATG8e*-GFP transgenic plants were collected at 7 dpi for microscopy. Tissues were pre-treated with conA for 12 hours (Contento et al., 2005) and then examined using confocal microscopy. Interestingly, autophagosomes did not increase in abundance in infected or adjacent non-infected cells at this time point. Autophagosomes were observed to aggregate to infection sites surrounding haustoria of *E. cichoracearum* (Figure 2.3 A-F). In non-infected *AtATG8e*-GFP transgenic plants autophagosomes were observed in the cytosol and vacuolar lumen of leaf epidermal cells, and appear not to be organized in any particular area of the cell (Figure 2.2 A-C). Aggregation of autophagosomes was also observed at infection sites at 3 dpi in *AtATG8e*-GFP transgenic tissues inoculated with *C. higginsianum* even though there was no significant induction of autophagy genes during this time period of the infection time course (Figure 2.3 G-I). Autophagosomes were observed to accumulate around the biotrophic primary hyphae of *C. higginsianum*. Using the identical confocal parameters as described above, no autophagosomes were observed in epidermal cells of wild-type plants, indicating the autophagosomes observed were not due to background autofluorescence (Figure 2.3 J-L).

2.3.3 Co-localization of GFP and monodansylcadaverine (MDC) labeled autophagosomes

An interesting feature of autophagosomes is that they contain an inner lytic compartment, and are therefore able to degrade their contents prior to arrival or deposition into the vacuolar lumen (Moriyasu et al., 1996). This characteristic has been employed to confirm the identity of autophagosomes by co-localizing the ATG8-GFP and monodansylcadaverine (MDC) signals in mammalian cells (Biederbick et al., 1995) and sucrose-starved *A. thaliana* suspension cells since MDC specifically stains lytic compartments in living cells (Contento et al., 2005). In an attempt to confirm my previous observations, I treated *E. cichoracearum*-infected and non-infected *AtATG8e*-GFP transgenic plants with 0.1 mM MDC stain and 1 μm conA and observed the co-localization of signals using confocal microscopy. Non-infected, non-transgenic *A. thaliana* plants (Ws) were also treated with MDC and conA and examined using confocal microscopy. In non-transgenic leaves, under no starvation conditions, MDC-stained spherical structures were randomly distributed in epidermal cells and aggregated around the nucleus (Figure 2.4 A and B). These structures were approximately 4 μm in diameter versus the characteristic ~2 μm diameter

of autophagosomes. In mock-inoculated *AtATG8e*-GFP transgenic plants treated with MDC and conA, small (~2 μm in diameter) spherical structures were observed to be randomly distributed throughout the epidermal cells (Figure 2.4 D-F) and MDC and GFP specific signals were co-localized to these structures (Figure 2.4 D-F). In *E. cichoracearum*-inoculated *AtATG8e*-GFP transgenic leaves spherical structures, co-labeled with MDC and GFP, appeared to aggregate around infection sites (Figure 2.4 G-I). Notably, in Figure 2.4 E and H, the larger central vacuole was stained with MDC emitting a signal, this signal was not observed using the GFP specific excitation wavelength suggesting that co-localization of signals is not due to a signal bleeding through wavelengths.

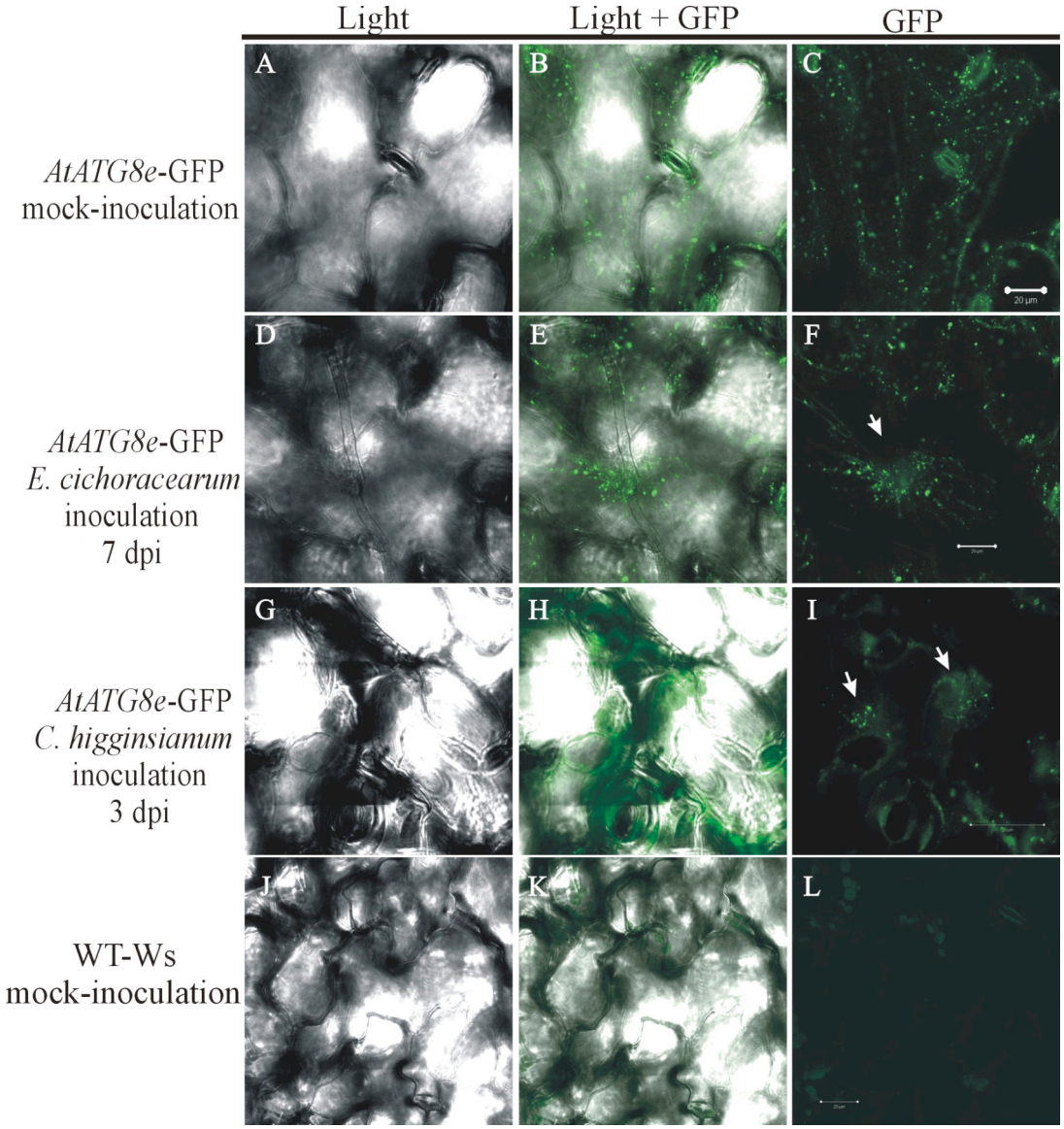

Figure 2.3 Detection of autophagosomes in *A. thaliana ATG8e*-GFP transgenic plants infected with *E. cichoracearum* and *C. higginsianum.* Light, image from light channel during confocal microscopy; Light + GFP, a merged image from the light and argon laser channels; GFP, image from argon channel set at GFP specific parameters (488 nm). **(A-C)** Non-infected transgenic *A. thaliana AtATG8e*-GFP leaves treated with 1 µM conA and examined using confocal microscopy. Autophagosomes were observed to randomly distribute throughout the epidermal cells. **(D-F)** *A. thaliana AtATG8e*-GFP transgenic leaves inoculated with *E. cichoracearum*, treated with 1 µM conA and examined using confocal microscopy at 7 dpi. Autophagosomes were observed to aggregate around haustoria (infection sites). **(G-I)** *A. thaliana AtATG8e*-GFP transgenic leaves inoculated with *C. higginsianum*, treated with 1 µM conA and examined using confocal microscopy at 3 dpi. Autophagosomes were observed to aggregate around primary hyphae (arrow heads in image I). **(J-L)** Non-transgenic *A. thaliana* (Ws) leaves approximately 30 days old were treated with 1 µM conA and examined using confocal microcopy for background autofluorescence.

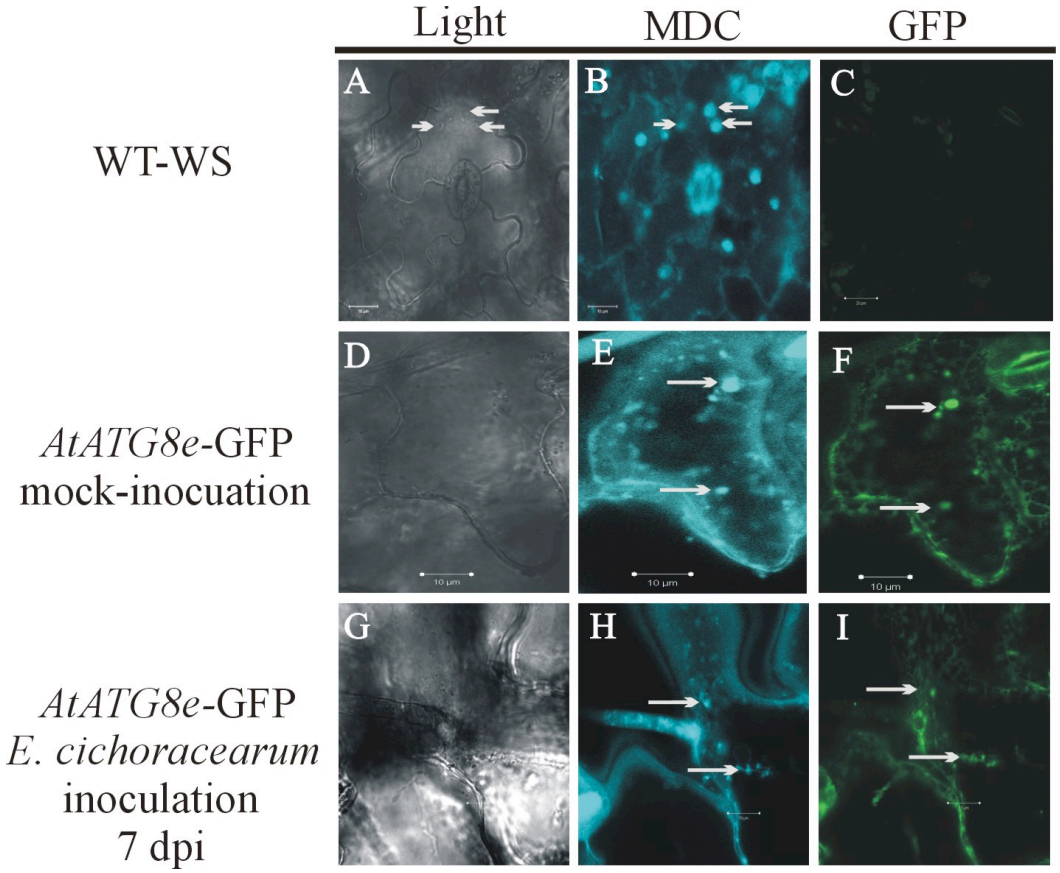

Figure 2.4 Co-localization of MDC staining and GFP signals in *A. thaliana ATG8e* transgenic plants. Light, image from light channel during confocal microscopy; MDC, image taken using diode laser specific for MDC excitation (405 nm); GFP, image from argon laser specific for GFP parameters (488 nm). **(A-C)** Epidermal cells of non-transgenic *A. thaliana* (Ws) stained with 0.1 mM MDC and treated with 1 µM conA and visualized using MDC and GFP specific excitation wavelengths. Arrowheads point out small spherical structures. **(D-F)** Epidermal cells of non-infected transgenic *A. thaliana AtATG8e*GFP stained with 0.1 mM MDC and treated with 1 µM conA and visualized using MDC and GFP specific excitation wavelengths. **(E and F)** Spherical structures are colocalized with the MDC (E) and GFP (F) signals and appear to be randomly distributed throughout the cell. **(G-I)** *E. cichoracearum* infected (7 dpi) transgenic *A. thaliana AtATG8e*GFP cells stained with 0.1 mM MDC and treated with 1µM conA and visualized using MDC and GFP specific excitation wavelengths. **(H and I)** Spherical structures are co-localized with the MDC (H) and GFP (I) signals and appear to accumulate around infection structures.

2.4 Discussion

2.4.1. Autophagy gene expression in response to pathogen attack

As a physiological process, autophagy has been implicated in playing a role in cellular differentiation and development, in the removal of organelles within active cells that are functioning abnormally or in senescing cells (see Moriyasu and Klionsky 2003), and in maintaining cellular homeostasis during stressful conditions (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Contento et al., 2005; Slavikova et al., 2005; Rose et al., 2006). The autophagic process produces amino acids, phospholipids and other elements required for basic metabolic activities. (Rose et al., 2006). The autophagy genes tested by Rose et al. (2006), required for the autophagic process, were expressed constitutively at basal levels in *A. thaliana* cells. An induction of autophagy genes (*ATG4a, ATG4b, ATG5, ATG7, ATG8a-i, ATG9, ATG12, BECLIN1*) has been observed in response to dark treatments and carbon or nitrogen starvations between 1 hour and 5 days of treatment (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Contento et al., 2005; Slavikova et al., 2005; Rose et al., 2006). All autophagy genes previously mentioned, except for *BECLIN1* and *ATG9,* are involved in the ATG8/ATG12 conjugation step essential for autophagosome formation (Moriyasu and Klionsky 2003; Thompson et al., 2005). When the ATG8/ATG12 conjugation step was blocked, *A. thaliana* plants were hypersensitive to chlorosis and cell death (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Contento et al., 2005; Slavikova et al., 2005; Rose et al., 2006).

During susceptible infection of *A. thaliana* by *E. cichoracearum* the processes of conidial germination, appressorium development and haustorium formation take approximately 24 hours (Panstruga 2003; Koh et al., 2005). Once the pathogen has established a successful infection, fungal surface hyphae develop and eventually penetrate cells distal to the primary infection sites and withdraw nutrients from newly infected cells (Panstruga 2003). My data shows that autophagy genes do not collectively respond at the transcriptional level in response to *E. cichoracearum* infection. There was marginal induction at 9 dpi by *ATG5* and *ATG8e*. This induction initially seemed to be delayed, since previous studies have detected autophagy gene induction as early as 1-2 hours post starvation treatment (Rose et al., 2006). However, at 9 dpi,

correlating with sporulation of the pathogen, a single conidium can have established up to 15 haustoria, each withdrawing nutrients from the host cells (Panstruga 2003). It is reasonable to assume that at this time point, although the host is capable of photosynthesis and obtaining nitrogen, that the withdrawal of host nutrients in support of the massive mildew growth exceeds production and therefore autophagy may be induced to help the infected cells relocate cell nutrients thereby maintaining cellular homeostasis, although, response by only two autophagy gene does not infer the pathway is actually induced. Interestingly, *ATG5* is a single copy gene within the *A. thaliana* genome (Doelling et al., 2002) and therefore, this gene could be a regulator of the autophagy pathway at the transcriptional level.

During hemibiotrophic parasitism, the timeframe of the biotrophic stage is shorter and variable compared to biotrophic pathogens. *C. higginsianum* conidia germinate to produce appressoria which penetrate the host cell surface and generate primary hyphae within the host cells. The primary mode of nutrient uptake is via primary biotrophic hyphae (O'Connell et al., 2004). The primary hyphae of *Colletotrichum* species are thought to be analogous to haustoria of biotrophic pathogens. Autophagy gene induction was assessed during the *C. higginsianum-A. thaliana* infection time course to elucidate whether induction was in response to biotrophy or was pathogen specific. *ATG5* and *ATG8e* are slightly induced in response to infection at 3 and 5 dpi respectively. *ATG5* and *ATG8e* are involved in the ATG8/ATG12 conjugation step required for autophagosome formation (Thompson et al., 2005). Previous studies indicate that *C. higginsianum* lives as a biotroph in *A. thaliana* from 2.5 to ~5 dpi before it switches to necrotrophy (Liu et al., 2007b). *C. higginsianum* produces large, branched primary biotrophic hyphae that can, at later stages of biotrophy, fill up to 50% of the cellular area (Liu et al., 2007b). The degree of nutrient stress by fungal pathogens on infected epidermal cells is unknown. It is possible that the observed transcriptional induction of some autophagy genes was an attempt by the host to maintain cellular homeostasis in response to infection. Since only two genes tested responded to infection their role remains inconclusive. However, the observation that *ATG5*, a single copy gene, is induced during infection could suggest that this gene is in fact a regulator for the autophagy pathway at the transcriptional level during infection.

During incompatible interactions, host plants generally recognize the pathogen and initiate an array of resistance responses to avoid infection. CWA, the hypersensitive response (HR) and the expression of pathogenesis-related proteins are examples of this type of defense

strategy to biotrophic pathogens (Agrios 1999). These defense strategies occur immediately in an attempt to block primary haustoria formation and colony development. Autophagosomes are small compartments containing intact and partially or completely degraded cytoplasm, cytoplasmic contents and organelles. An induction of autophagy at such a late stage of the *E. cichoracearum* infection could possibly be an attempt of resistance by the plant, as autophagosomes could be shuttling defense products to the infection sites. However, it is unlikely that this is their function. Given that autophagy is non-selective, it is unlikely that it is involved in trafficking defense-related products to infection sites.

2.4.2 Retargeting of autophagosomes in response to pathogen attack

Autophagosomes are the trafficking vesicles for autophagy. Sequestered cellular contents are delivered to the vacuole for degradation (Basham 2007). Delivery to the vacuole is thought to be accomplished via the same mechanisms used by the endomembrane system (Thompson et al., 2005). The capturing (involving coat proteins and clathrin-coated proteins), targeting (involving Rab proteins), docking (involving Rabs) and fusion (involving SNARE proteins) mechanisms of the endomembrane system have only recently been understood, and to date their specificity conveys the complexity of this process. Several targeting (Rabs: Ypt7) and fusion proteins (v-SNARES: Vam3, Vam7, Vti1, Ykt6 and Sec17-19) have been associated with autophagosomes (Surpin et al., 2003; Wang and Klionsky 2003; Liu et al., 2005), yet their roles have not been further characterized for autophagy in *A. thaliana*.

The retargeting of autophagosomes to infection sites could be a cellular mechanism of a resistance attempt or a susceptibility factor. The idea of autophagosomes as susceptibility factors is interesting, a double-membraned vesicle carrying degraded organelles and cytoplasm seems like a feast to an invading pathogen. However, how might an autophagosome be retargeted from the vacuole to the cell membrane and more specifically to an infection site? Interestingly, there is no evidence that trafficking vesicles of the endomembrane system are equipped for redirection which would require more than one set of targeting, docking and fusion proteins. However, material destined for degradation in mammalian cells has been identified to be packaged in early small endosomes containing Rab5; this type of endosome is then seen to mature into a lysosome or late endosome containing Rab7 (Rink et al., 2005). Rink et al. (2005) suggested that a Rab-

conversion mediated by the lysosomal HOPS complex resulting in the redirection of the vesicle. A second possibility is that autophagosomes do possess more than one set of targeting and docking proteins that are differentially turned on based on where the cell wants that autophagosome to go. The question remains then whether autophagosomes are equipped for redirection or whether Rab-conversions are taking place, and which factors are directing this re-localization.

If autophagosomes were equipped with more than one targeting mechanism it is possible that effector proteins originating from the pathogen enter into the plant cytoplasm and are responsible for the redirection of autophagosomes either directly or indirectly. Vesicles of the endomembrane system are not always ready for traffic. The process is actually well regulated, as Rab proteins require several other proteins to activate and deactivate them. There are several conceivable strategies for redirection and some evidence to support interaction between vesicles of the endomembrane system and pathogens. The bacterium *Pseudomonas syringae* secretes an effector protein that degrades an adenosine diphosphate ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) involved in Rab activation (Nomura et al., 2006). ARF-GEFs are major regulators of vesicle formation and intracellular trafficking in eukaryotic cells (Robatzek 2007). Nomura et al. (2006) suggested that this targeted vesicle formation inhibition retards antimicrobial compound delivery and therefore promotes infection. A similar scenario has also been observed in the *Alternaria carthami-A. thaliana* pathosystem (Driouich et al., 1997). Brefeldin A, a compound produced by the pathogen, enters the plant cell and inhibits Golgi-derived vesicle formation and promotes infection (Driouich et al., 1997). MLO, a plasma membrane-localized syntaxin, is required for successful powdery mildew infection in both monocots and dicots (Panstruga 2005). It is assumed that MLO plays a role in the delivery of plasma membrane materials to allow for the development of the haustorium and that a pathogen effector protein interacts with MLO (Panstruga 2005). Therefore, if autophagosomes are equipped with Rabs and SNARES specific for more than one membrane, targeted induction of plasma membrane specific Rabs and SNAREs, located on the autophagosome, by powdery mildew is conceivable since there is evidence showing that powdery mildews are capable of interfering with mechanisms of the endomembrane system (Jorgensen 1992; Driouich et al., 1997; Panstruga 2005; Consonni et al., 2006; Robatzek 2007). Why would powdery mildew want to retarget autophagosomes to the infection site? It has already been demonstrated that

autophagosomes in *A. thaliana* are lytic. It is possible that the redirection facilitates nutrient acquisition by the pathogen, from autophagosomes, at the infection site. Hypothetically, the outer membrane of the autophagosomes could fuse with the plasma membrane surrounding the haustorium depositing the inner autophagic body containing degraded cellular compounds into the EHMAT for uptake by the pathogen.

As there is evidence in support of a role for the endomembrane system, and mechanisms governing it, in disease establishment, so too is there for disease resistance. It is unusual to see a pathway involved in an attempted resistance response at later stages in the infection, if I could consider the slight induction of autophagy genes during powdery mildew infection at 9 dpi as such a response. However, the redirection of autophagosomes as a resistance response will be considered since it is possible that autophagosome redirection and gene induction are uncoupled, that is, the autophagosomes are already present in the cytosol and are redirected by the plant as a resistance response that is post-transcriptionally regulated. As discussed previously, both *Alternaria* and *Pseudomonas* secrete effector proteins that either directly or indirectly alter vesicle trafficking and presumably the deposition of antimicrobial molecules to infection sites (Driouich et al., 1997; Nomura et al., 2006). Hyphae of powdery mildews grow on the surface of the plant until they produce an infection structure in a cell distal to the primary infection. Autophagosome redirection was observed in cells distal to the primary infection 7 and 9 dpi. Logically, at this time point the haustoria could be less than 1, 2, 3 or more days old. The redirection of autophagosomes could therefore be a resistance response to those newly formed infection haustoria. It is reasonable to propose that the redirection involves the deposition of antimicrobial compounds; however, this is something normally taken care of by the endomembrane system, whereas autophagy is considered to be non-selective.

Expression of *ATG8e-GFP* in the transgenic *A. thaliana* line is under the control of a 35S promoter. This type of promoter assures constitutive expression of its accompanying gene. In previous assays investigating the roles of autophagy in *A. thaliana* as a physiological response, the fluorescing green spheres observed were confirmed to be autophagosomes since their presence in stressful conditions was confirmed using western-blot analysis for ATG8e and they were associated within a membrane (Doelling et al., 2002; Hanaoka et al., 2002; Levine and Klionsky 2004; Colombo 2005; Contento et al., 2005; Liu et al., 2005; Thompson et al., 2005; Bassham et al., 2006; Rose et al., 2006). With this being said, the number of autophagosomes per

cell during infection versus non-infected cells was not quantified. This leaves one other possibility for the retargeting of autophagosomes observed in these assays. Instead of autophagosomes being redirected to the infection sites, some unidentified vesicles of the endomembrane system could carry over-expressed ATG8e-GFP proteins. However, how these proteins would be tagged and packaged for traffic to the plasma membrane is unknown.

2.4.3 Co-localization of MDC and GFP

MDC had initially been observed to stain double membraned organelles that were abundant in both the cytosol and vacuole (peroxisomes) under nutrient limited conditions and the fluorescent signals were co-localized to the ATG8e-GFP marker in mammalian cells (Biederbick et al., 1995; Munafo and Colombo, 2001). Contento et al. (2005) were the first to demonstrate co-localization of MDC and ATG8e-GFP signals when applied in the *A. thaliana* system. Since the MDC signal co-localized with the ATG8e-GFP, MDC application would be a more convenient tool for autophagosome visualization annulling the need for a transgenic line. MDC application is also an effective tool to compliment genetic and biochemical approaches to the studies of autophagy as in the case of this study. In non-inoculated tissues of *AtATG8e*-GFP transgenic plants treated with MDC, autophagosomes were observed to be randomly distributed throughout the leaf epidermal cells. In *Erysiphe* infected *AtATG8e*-GFP transgenic tissue at 7 dpi, MDC-stained spherical structures accumulated at infection sites. In both assays, the GFP and MDC signals were co-localized using confocal microscopy at excitation wavelengths specific to each stain. To confirm the GFP signal was not bleeding due to the two-excitation wavelengths, wild type plants (Ws) infected with powdery mildew were treated with MDC and observed using confocal microscopy and indeed, spherical fluorescing structures were visible surrounding a powdery mildew infection structure.

Since the *AtATG8e*-GFP expression is operating under a 35S promoter, it is difficult to measure a temporal and spatial increase in autophagosome formation during infection. Surprisingly, it was a redirection of autophagosomes in these assays that became of interest. MDC therefore can be used both as a compliment to transgenic GFP studies and on its own to visualize autophagy *in vivo* during nutrient limited conditions.

CHAPTER 3

INTERPLAY BETWEEN THE AUTOPHAGIC RESPONSE AND DEFENSE SIGNALING PATHWAYS

3.1 Introduction

Three plant hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play major roles in disease perception and defense signaling. Defense against biotrophic pathogens is generally associated with SA-dependent pathways whereas defense against necrotrophic pathogens is associated with JA and ET-dependent pathways. Plant defense against hemibiotrophic pathogens is associated with the SA- and JA and ET-dependent pathways. Pathogen attack and recognition or the occurrence of the HR leads to SA production and increased levels of this plant hormone triggers defense gene activation and systemic acquired resistance (SAR). Pathogenesis-related (*PR*) genes are involved in conferring resistance in plants to pathogens. Members of the *PR* gene family are found to be differentially responsive depending on infection and the signaling pathway induced by the infection. The classic example of a SA-activated gene is *PR-1*. The exact role of *PR-1* in plant defense is unknown; however, *PR* genes in general have the ability to confer resistance (Glazebrook 2005). SAR is the acquired resistance that a plant attains after an initial avirulent pathogen attack (Glazebrook 2005). This resistance is associated with an accumulation of SA and *PR* gene expression. Along with conferring resistance in some instances, SA levels have also been observed to rise in response to cell death (HR) and believed to initiate cell death (Glazebrook 2005). Nonetheless, the SA pathway plays a major role in establishing resistance in plants to pathogens.

The defense signaling cascade initiated by JA and ET is unknown (Dong 1998); however, there are examples of induced systemic resistance (ISR) initiated by these signaling molecules that is completely uncoupled from the SA pathways and *PR* gene expression. *PDF1.2*, a gene

encoding a plant defensin, responds to increased cellular levels of JA and ET, which is induced in response to recognition of necrotrophic or hemibiotrophic fungal pathogen attack and is involved in conferring resistance (O'Connell et al., 2004). This second form of inducible defense, induced systemic defense has been shown to be effective to several pathogens with or without the expression of *PDF1.2* (O'Connell et al., 2004). Therefore, there seems to be several inducible pathways that confer resistance due to an accumulation of these two signaling molecules; however, the exact mechanisms are still under investigation.

Of the three hormones, only ET is associated with enhancing disease susceptibility. ET insensitive mutants (*ein*), those that do not perceive and respond to ET accumulation via senescence, are resistant to several pathogens. The natural production of ET by plants causes chlorosis, leaf abscission, increased permeability of membranes and fruit ripening, which facilitates infection of some opportunistic necrotrophic and hemibiotrophic fungal pathogens as well as some bacterial pathogens (Agrios 1999).

One of the earliest steps in defense response, before induction of SAR or ISR, is the production of reactive oxygen (ROS: H_2O_2 and O^2) and nitrogen species (NO) at the infection site (Broekaert et al., 2006). These signaling molecules were shown to induce accumulation of SA, JA and ET. Since the signaling molecules previously mentioned, SA, JA, ET, H_2O_2 and NO, were shown to play crucial roles in eliciting defense responses in plants to pathogens, this study investigated the response of autophagy genes in *A. thaliana* to these well-characterized signaling molecules.

A role for autophagy in plant innate immunity or defense has yet to be completely elucidated. In plants, autophagy is involved in sequestering ROS that are induced systemically in response to avirulent bacterial infection (Liu et al., 2005). Simply, it appears to have a role in maintaining cellular homeostasis due to an apparent systemic over reaction to infection, but could this role be under the control of any of these defense related molecules? Since autophagy responded post-transcriptionally to infection by both biotrophic and hemibiotrophic pathogens (see chapter 2), research was conducted into the possible induction mechanism; if autophagy is involved in resistance or susceptibility it is possible that it is under the control of SA, JA, ET, H_2O_2 or NO.

Interestingly, the post-translational autophagic response that was observed in chapter 2 appears to be independent from defense signaling molecules. Collectively, the group of

autophagy genes did not respond to any molecules tested. *ATG5* transcript levels were slightly induced by SA, JA, ET and sodium nitroprusside (SNP) (a representative reactive nitrogen molecule). *ATG9* was slightly induced by SA. *ATG8f* and *ATG8i* were slightly induced by H_2O_2.

3.2 Materials and Methods

3.2.1 Plant material

A. thaliana ecotypes Col-0 was obtained from the Arabidopsis Biological Resources Center. *A. thaliana* seeds were sown on soil and cold treated prior to germination at 4^{o}C for 2 days and then transferred to the a growth camber with a cycle of 16 h light 8 h dark at 22^{o}C day and 18^{o}C night. *A. thaliana* Col-0 plants were treated with signaling molecules at approximately 4 weeks.

3.2.2 Signaling molecule treatment

Attached *A. thaliana* Col-0 leaves of four week-old plants were sprayed with water, methyl jasmonic acid (MeJA, 200 μM), Ethephon (200 μM, for releasing ET), SA (200 μM), SNP (for production of NO, 1 mM) or H_2O_2 (10 mM) and covered with a plastic lid and kept at greenhouse conditions for 24 hours. Three biological replicates were assayed and similar results were obtained. All chemicals were purchased from Sigma, except for MeJA (Bedoukian Research, Danbury, CT). Tissue was collected for RNA extraction 24 hours after treatment and stored at -80^{o}C.

3.2.3 RNA isolation and cDNA preparation

Total RNA was extracted by using a phenol/chloroform method as described by Liu et al. (2005). cDNA was generated using the SuperScript^{TM} II Reverse Transcriptase kit (Invitrogen) following the manufacturer's instructions. cDNA derived from 5 ug total RNA was used as a template for each RT- PCR.

3.2.4 RT-PCR Analysis

RT-PCR was performed using the gene specific primers listed in Table 2.1. RT-PCR was performed as per described in section 2.2.4.

3.3 Results

3.3.1. Expression of autophagy genes in response to signaling molecule treatments in *A. thaliana*

Autophagy genes responded slightly to infection by two different fungal pathogens, *E. cichoracearum*, a biotrophic pathogen and *C. higginsianum*, a hemibiotrophic pathogen. Therefore, an investigation was carried out into the control of this gene induction. *A. thaliana* plants were treated with defense signaling molecules in order to elucidate the mechanisms involved in autophagy gene induction and autophagosome redirection. There was no widespread induction of autophagy genes to any of the signaling molecule treatments (Figure 3.1). *PR1* responded to SA treatment and *PDF1.2* responded with a decrease in transcript production to SA and SNP (Figure 3.1). Transcripts of *ATG4a* were detectable at higher levels in the control tissues treated with water compared to any other treatment suggesting that this gene may be down regulated in response to signaling molecules. However, *ATG4b* did not exhibit a similar pattern of expression. *ATG5* and *ATG9* appeared to be slightly induced by SA at 24 hpt (Figure 3.1). This is interesting since the two genes are involved in two distinctly different steps during the autophagy process. A comparative analysis of *ATG5* and *ATG9* genomic DNA using ClustalW revealed that these two genes show only 41% sequence similarity (data not shown). *ATG5* gene expression also appeared to be significantly decreased in response to H_2O_2 (Figure 3.1). Interestingly, a W-box element (site of attachment of WRKY transcription factors) can be found in the 5'UTR (5' Un-Translated Region) of *ATG5*. WRKY transcription factors, involved in conferring resistance through resistance gene activation, are responsive to signaling molecules such as SA, JA, ET and SNP (Yang et al., 1999; Euglem et al., 2000; Euglem and Somssich 2007). This W-box sequence, TGAC, is not found in the 5'UTR of *ATG9*.

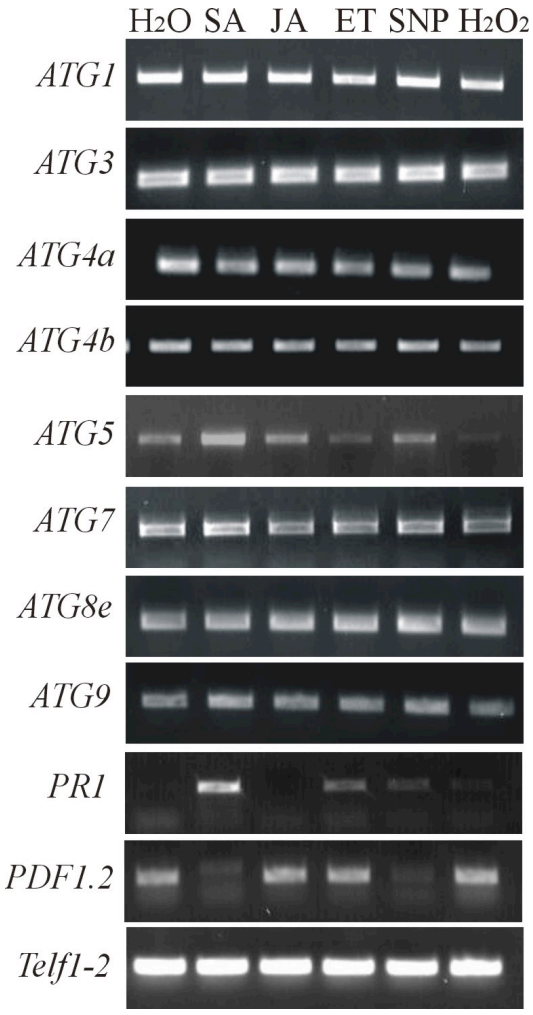

Figure 3.1 Expression patterns of autophagy and pathogenesis-related genes in response to signaling molecule treatments in *A. thaliana*. H_2O, water; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; SNP, sodium nitroprusside, H_2O_2, hydrogen peroxide. RT-PCR of autophagy genes: *ATG1, ATG3, ATG4a, ATG4b, ATG5, ATG7, ATG8e* and *ATG9* and pathogenesis related genes *PR1* and *PDF1.2* from *A. thaliana* leaves 24 hours post treatment with plant defense signaling molecules. *ATG4a* expression is negatively affected in response to all signal molecule treatments. *ATG5* and *ATG9* are slightly induced by SA. *ATG5* is down regulated by H_2O_2. *Telf1-2* was included as a constitutively expressed loading control in both assays. This figure is representative of three biological replicates.

3.3.2 Expression of the *ATG8* gene family in response to signaling molecule treatments in *A. thaliana*

Differences were observed in the expression of the *ATG8* gene family in response to *E. cichoracearum* and *C. higginsianum* infection; therefore the response of these genes to signaling molecule treatment was also investigated. As was observed in the previous assay, there was no

consistent induction of members of the *ATG8* gene family in response to signaling molecule treatment (Figure 3.2). *ATG8a* is constitutively expressed in all treatments. *ATG8b* is slightly induced by SA, ET and SNP treatments (Figure 3.2) *ATG8d* appears to be slightly down regulated by all treatments compared to water. *ATG8g* is down regulated by SNP treatment (Figure 3.2). *ATG8h* appears to be up regulated by SA and down regulated by H_2O_2 (Figure 3.2). *ATG8f* and *ATG8i* are slightly induced by H_2O_2 treatment. Based on ClustalW sequence alignment, *ATG8f* and *ATG8i* do not cluster in the same clade and share only 56% genomic DNA sequence similarity (data not shown). *ATG8f* lacks the W-Box sequence in the 5'UTR whereas a W-Box sequence is present in the 5'UTR of *ATG8i*.

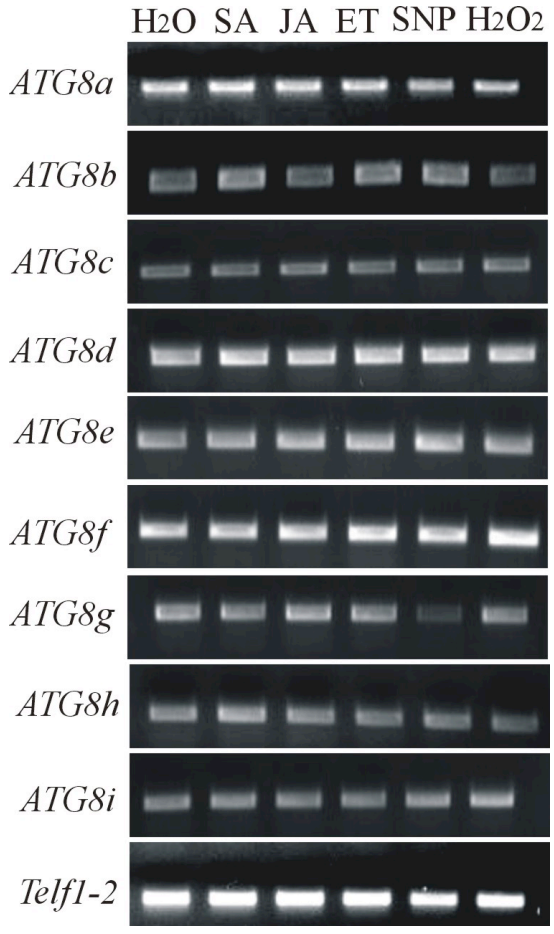

Figure 3.2 Expression pattern of *ATG8* gene family in response to plant signaling molecule treatments in *A. thaliana*. H_2O, water; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; SNP, sodium nitroprusside, H_2O_2, hydrogen peroxide. RT-PCR of *ATG8* family members: *ATG8a -i* from *A. thaliana* leaf tissue 24 hours post treatment with plant signaling molecules. This figure is representative of three biological replicates.

3.4 Discussion

In chapter 2, the autophagy pathway was assessed for its role in plant immunity. These assays revealed that autophagy genes did not respond collectively to either *C. higginsianum* or *E. cichoracearu*m infection. Since plant defense- and susceptibility-related cellular events are correlated to signaling molecule induction/accumulation, the autophagic response to these signaling molecules was investigated.

The slight responses of *ATG4a, ATG5* and *ATG9* to signaling molecule treatment are not enough to suggest that this pathway is in any way responsive to the signaling molecules that are classically responsible for eliciting defense responses in *A. thaliana*. Interestingly, *ATG5* was induced by SA and transcription was down-regulated by H_2O_2. The induction could be associated with the presence of a W-box element, a sequence TGAC found in the 5'UTR region of the gene. This is significant since WRKY transcription factors found to be involved in regulating defense related genes in plants bind to these W-box elements (Euglem et al., 2007). Since this W-box sequence is not found in the 5' UTR of *ATG4a* or *ATG9*, it could explain why *ATG5* is responsive to more than one of the signaling molecules (Rushton et al., 1996) even though the induction may be unrelated to defense. *PR* genes in parsley and tobacco have been found to possess the TGAC W-box sequence in their 5'UTR region and WRKY transcription factors are required to bind to these sequences to confer resistance to pathogens (Rushton et al., 1996; Yang et al., 1999).

ATG8i and *ATG8f* were slightly induced by H_2O_2 treatment after 24 hours in *A. thaliana*. *ATG8i* was observed to be the most dramatically induced member of the *ATG8* family after 12 hours of nitrogen starvation of 5 week old intact *A. thaliana* plants (Yoshimoto et al., 2004). This group concluded that nitrogen starvation induced senescence and therein induced autophagy genes. In this assay, the gene was responsive to H_2O_2 treatment, H_2O_2 being an ROS that can be responsible for directly or indirectly initiating cell death. The type of cell death associated with H_2O_2 is generally uncoupled from ET, or other molecules that would initiate senescence. The response of autophagy genes to signaling molecule suggests that the autophagic response to infection, observed in chapter 2, is not under the control of defense related signaling molecules; therefore, it is unlikely linked to resistance.

CHAPTER 4

VIRULENCE OF *ERYSIPHE CICHORACEARUM* AND *COLLETOTRICHUM HIGGINSIANUM* IS ALTERED IN *ARABIDOPSIS THALIANA* AUTOPHAGY MUTANTS

4.1 Introduction

A common assumption is that a change in animal behavior is the result of a change in its environment. The progression of disease development for both *E. cichoracearum* and *C. higginsianum* has been extensively documented. *E. cichoracearum* conidia land on the host surface and within approximately six hours germinate to form an appressorial germ tube (Koh et al., 2005). The tip of this germ tube swells to form an appressorium, which is essential for proper penetration of the host tissue. Via enzymatic and mechanical power a penetration peg penetrates the host epidermal cell wall (Green et al., 2002). Penetration of the host's surface occurs within 12 hours (Koh et al., 2005) and within 24 hours a fully functional feeding structure, the haustorium, will be produced between the epidermal cell wall and the plant plasma membrane. Surface hyphae germinate from the conidia on the surface of infected epidermal cells (Huckelhoven et al., 2005; Koh et al., 2005). As the disease progresses the surface hyphae will penetrate epidermal cells distal to the initially infected epidermal cell and secondary haustoria will be produced to help maintain the growing fungal colony.

C. higginsianum infects its host using an intracellular hemibiotrophy infection strategy (Shen et al., 2001). Conidia of *C. higginsianum* land on the host surface and germinate to produce an appressorial germ tube; this event takes approximately 24 hours (Shen et al., 2001). The tip of the appressorial germ tube swells and a melanized appressorium is produced. A penetration peg penetrates the host epidermal cell wall and a primary biotrophic infection hypha (equivalent to the haustorium) is formed between the plant cell wall and plasma membrane within 48 hours (Shen et al., 2001). The primary infection structures grow progressively longer and are observed to branch and develop septa; however, they remain within the initially infected epidermal cell. Between 3-5 days post infection (dpi) narrow necrotrophic secondary hyphae

emerge from the tips of the primary hyphae, radiate outwards and penetrate cells distal to the primarily infected cell. Since the pathogenesis of both *E. cichoracearum* and *C. higginsianum* is well documented one can observe their behavior in mutant plant lines compared to wild type lines in an attempt to understand if a pathway or protein has a role in disease susceptibility or resistance.

To date, several autophagy genes (*ATG4a/b, ATG5* and *ATG9*) that function in autophagosome formation and completion have been disrupted in *A. thaliana* (Doelling et al., 2002; Hanaoka et al., 2002; Levine and Klionsky 2004; Colombo 2005; Contento et al., 2005; Liu et al., 2005; Thompson et al., 2005; Bassham et al., 2006; Rose et al., 2006). The characteristics of these genes include a single copy within the *A. thaliana* genome (with the exception of *ATG4* which has two genes; however, a double knockout was created for these two gene loci (Rose et al., 2006)) and a non-lethal mutation at these loci in *A. thaliana*. The phenotypic effect of a mutation at either of these loci is the inability for autophagosomes to be formed, therefore the nutrient recycling autophagy pathway is blocked.

Pathogenesis of both pathogens was observed in these autophagy mutants to elucidate the role of this pathway in disease development. The autophagy pathway was not essential for either *E. cichoracearum* or *C. higginsianum* infection; however, the development of surface hyphae was compromised in *atg* mutant lines infected with *E. cichoracearum* at 2 dpi. The retardation of secondary hyphal production suggests that either penetration is hindered or haustorium formation/establishment is hindered for *E. cichoracearum* in autophagy mutants and therefore the autophagy pathway may play a role in susceptibility. *C. higginsianum* infection (virulence) was accelerated (very short biotrophic phase) in autophagy mutants compared to wild type suggesting a possible role for this pathway in the establishment of biotrophy.

4.2 Materials and Methods

4.2.1 Plant and pathogen materials

A. thaliana lines Col-0 and Ws were obtained from the Arabidopsis Biological Resource Center. Autophagy mutants (Table 4.1), *atg4a/b* and *atg9-1* were kindly received from Y. Ohsumi, (Department of Cell Biology, National Institute for Basic Biology, Okazaki, Japan). *atg5-1* was received from R. D. Viersta, (Department of Genetics, University of Wisconsin). *atg9-6* was created by G. Liu (Department of Biology, University of Saskatchewan). All *A. thaliana* seeds were sown on soil, cold-treated prior to germination at 4^{o}C for 2 days and then transferred to either the greenhouse (plants to be inoculated with *E. cichoracearum*) with a natural photoperiod and at temperature constant at 25^{o}C or to a growth camber (plants to be inoculated with *C. higginsianum*) with a cycle of 16 h light 8 h dark at 22^{o}C day and 18^{o}C night. *Colletotrichum* conidia were propagated at room temperature on V8 or potato dextrose agar (PDA) medium as described previously by Wei et al., (2004). *E. cichoracearum* was isolated from and maintained on a susceptible cucumber cultivar, national picking variety, in the greenhouse at 25^{o}C.

Table 4.1 The source and background of autophagy mutants and transgenic lines used in this study

Mutant	Type of mutation	Background	Source
Atatg4a/b	T-DNA insertion in first exon of *ATG4a* and T-DNA insertion in fifth intron in *ATG4b*	Ws	Yoshimoto *et al.*, 2004
Atatg5-1	T-DNA insertion in fifth intron	Col-0	Thompson *et al.*, 2005
Atatg9-1	T-DNA insertion in third intron	Ws	Yoshimoto *et al.*, 2004
Atatg9-6	T-DNA insertion in the fifth exon	Col-0	This study
AtATG8e-GFP	Transgenic lines constructed using a modified pJ4GFP-XB vector	Col-0	Contento *et al.*, 2005

4.2.2 Inoculation procedure

E. cichoracearum conidia from colonies 9-14 days old were used in each experiment. Intact plants, approximately 3-4 weeks old were placed in a box for inoculation with *E. cichoracearum.* Cucumber leaves with sporulating powdery mildew were detached from the plant and conidia were brushed onto *A. thaliana* lines: Col-0, WS, *atg4a/b*, *atg5-1*, *atg9-1* and *atg9-6.* Plants were removed from the box approximately 1 hour after inoculation and replaced to regular greenhouse conditions. Conidia from *C. higginsianum* were collected from 7-9 day old plates and spore suspensions were prepared ($1x10^6$ spores ml^{-1} in distilled water). Spray or droplet inoculation (5-10 µl droplets on the either side of the leaf mid-vein) was employed depending on assay. Intact plants that were inoculated with *C. higginsianum* were kept at 100% humidity in a growth chamber immediately after inoculation (Narusaka et al., 2004; O'Connell et al., 2004). Detached leaves removed from 3-4 week old plants were inoculated and incubated in Petri dishes (with wet filter paper) using either the spray or droplet method, depending on the assay, and maintained in laboratory conditions with a 12 hour photoperiod at a light intensity of 30 µE $m^{-2}sec^{-1}$ (Liu *et al.,* 2007b).

4.2.3 Histological staining and light microscopy

Infected *A. thaliana* leaves were collected at varying times post infection and fixed in a fixation solution (60% methanol: 30% chloroform: 10% acetic acid). Prior to staining, samples were rehydrated using an ethanol gradient (2 hours at each concentration: 100%, 80%, 60%, 40%, 20% and overnight in water). Samples were then stained overnight with trypan blue, then rinsed and mounted in glycerol for microscopy. Photographs were taken using a Zeiss Axioplan microscope equipped with a Zeiss Axiocan ICc 1 camera and Axiovision LE version 4.6.3.0 software. Statistical analysis (paired sample students t-test) were conducted using SPSS Statistics software version 17.0.

4.3. Results

4.3.1 Surface hyphal development of *E. cichoracearum* is compromised in *A. thaliana* autophagy mutants

To determine the effects of the host autophagy pathway on *E. cichoracearum* virulence, intact *A. thaliana* plants of wild type and *atg* mutants (*atg4a/b*, *atg5-1*, *atg9-1* and *atg9-6*) were inoculated at approximately 30 days post germination. *E. cichoracearum* conidia were observed to develop to the functional haustorium within host tissue and surface hyphae grow on the host surface by 2 dpi; therefore leaf tissue inoculated with *E. cichoracearum* at 2 dpi was examined using light microscopy to investigate altered virulence. Successful development of surface hyphae was counted versus unsuccessful development of surface hyphae over the entire leaf inoculated with *E. cichoracearum* conidia (three biological replicates). Figure 4.1b represents unsuccessful surface hyphal development (SH-) and successful surface hyphal development (SH+) at 2 dpi. Paired sample t-tests were conducted to access the statistical differences between the wild type and mutant plants on infection rates and secondary hyphal production, respectively. Infection rate of *E. cichoracearum* in wild type Col-0 and WS plants was not statistically different (Pair Col-0-Ws: t = 1.083, df = 2, p-value = 0.392) with a 68% infection rate compared to a 62% infection rate, respectively (Figure 4.1a). The *atg* mutants, *atg4ab* and *atg9-1*, having a Ws background, were both considered less-susceptible to *E. cichoracearum* at 2 dpi demonstrating 17% and 20% reduction of secondary hyphal development, respectively. This difference is statistically relevant at the 95% confidence level (Pair Ws-*atg4ab*: t = 5.575, df = 2, p-value = 0.031; Pair Ws-*atg9-1*: t = 4.674, df = 2, p-value = 0.043). At the 90% confidence level, *atg9-6* is significantly less susceptible to *E. cichoracearum* infection compared to wild type Col-0 with a 36% reduction in surface hyphal production at 2 dpi (Pair Col-0-*atg9-6*: t = 3.605, df = 2, p-value = 0.069) (Figure 4.1a). The surface hyphal production in *atg5-1* versus Col-0 (27 % less surface hyphal production in *atg5-1* versus Col-0) was observed not to be statistically different according to the paired sample t-test (Pair Col-0-*atg5-1*: t = 2.647, df = 2, p-value = 0.118). Together, the retardation of surface hyphal development in autophagy mutants suggests that this pathway is involved in facilitating either penetration or haustorium development, and therefore can possibly be considered a susceptibility factor.

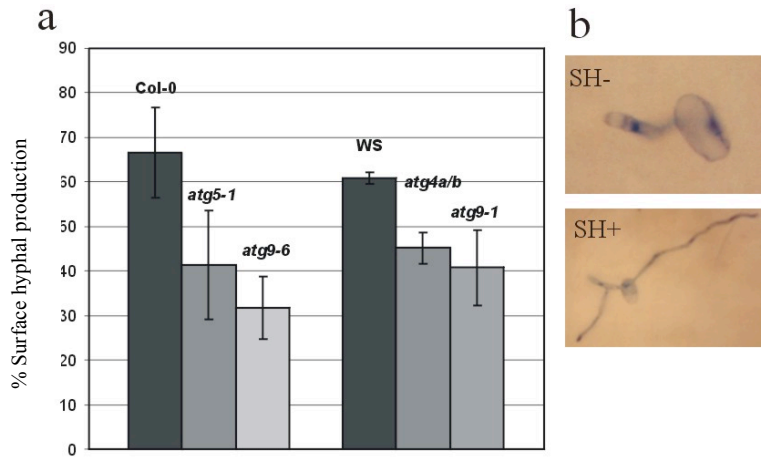

Figure 4.1 Surface hyphal development of *E. cichoracearum* infected *A. thaliana* autophagy mutants. SH-, unsuccessful surface hyphal development; SH+ successful surface hyphal development. **(a)** *E. cichoracearum* conidium was more successful at establishing infection in wild type *A. thaliana* (Col-0 and WS) compared to *atg* mutant (*atg4/ab*, *atg5-1, atg9-1* and *atg 9-6*) plants 2 dpi. **(b)** SH- image depicting failed surface hyphal development at 2 dpi on *A. thaliana* leaf tissue; SH+, image depicting successful surface hyphal development at 2 dpi on *A. thaliana* leaf tissue. This figure is representative of three biological replicate assays.

4.3.2 Virulence of *C. higginsianum* is accelerated in *A. thaliana* autophagy mutants

Since hemibiotrophic pathogens have the ability to switch their mode of nutrition from biotrophic parasitism to necrotrophic (destructive) parasitism, intact and detached *A. thaliana* leaves of wild type plants and autophagy mutants were inoculated with *C. higginsianum* and observed at the cellular level to identify any difference in the time period of biotrophic parasitism. It was expected that if *C. higginsianum* was in any way negatively affected by a lack of the autophagy response in the *atg* mutants, it would convert to a necrotrophic mode of nutrition sooner than it would in wild type plants. The result would then be an increase in virulence or disease severity in the *atg* mutants. Intact and detached wild type and *atg* mutants were inoculated via the spray inoculation method with *C. higginsianum* conidia. In both the intact plant and detached leaf assays disease symptoms in the form of chlorosis were observed (Figure 4.2 a and b). Small necrotic flecks were visible on wild type intact plant leaves at 5 dpi, whereas large necrotic lesions surrounded by chlorotic halos were observed in all *atg* mutants at this time point (Figure 4.2 a and b). In general, detached leaves were more susceptible to *C.*

higginsianum (Figure 4.2 b). In wild type detached leaves, a large necrotic lesion developed underneath the infection droplet and was surrounded by a thin chlorotic halo at 5 dpi (Figure 4.2 b). In all *atg* mutants, the necrotic lesions were surrounded by large chlorotic halos, in some cases extending to the leaf edge, suggesting extensive spread of this pathogen.

Fungal development was also observed at the cellular level in wild type Col-0 and *atg* mutant's *atg5-1* and *atg9-6*. To observe the biotrophic phase of infection, leaves infected with *C. higginsianum* at 3 dpi were collected and stained for microscopy. At this time point, biotrophic primary infection vesicles were visible between the plant cell wall and plasma membrane in wild type plants infected with *C. higginsianum* (Figure 4.2 c). Pathogen virulence was accelerated in *atg* mutants with secondary necrotrophic hyphae being developed from primary hyphae, growing intra- and intercellularly and spreading distally from the primary infection.

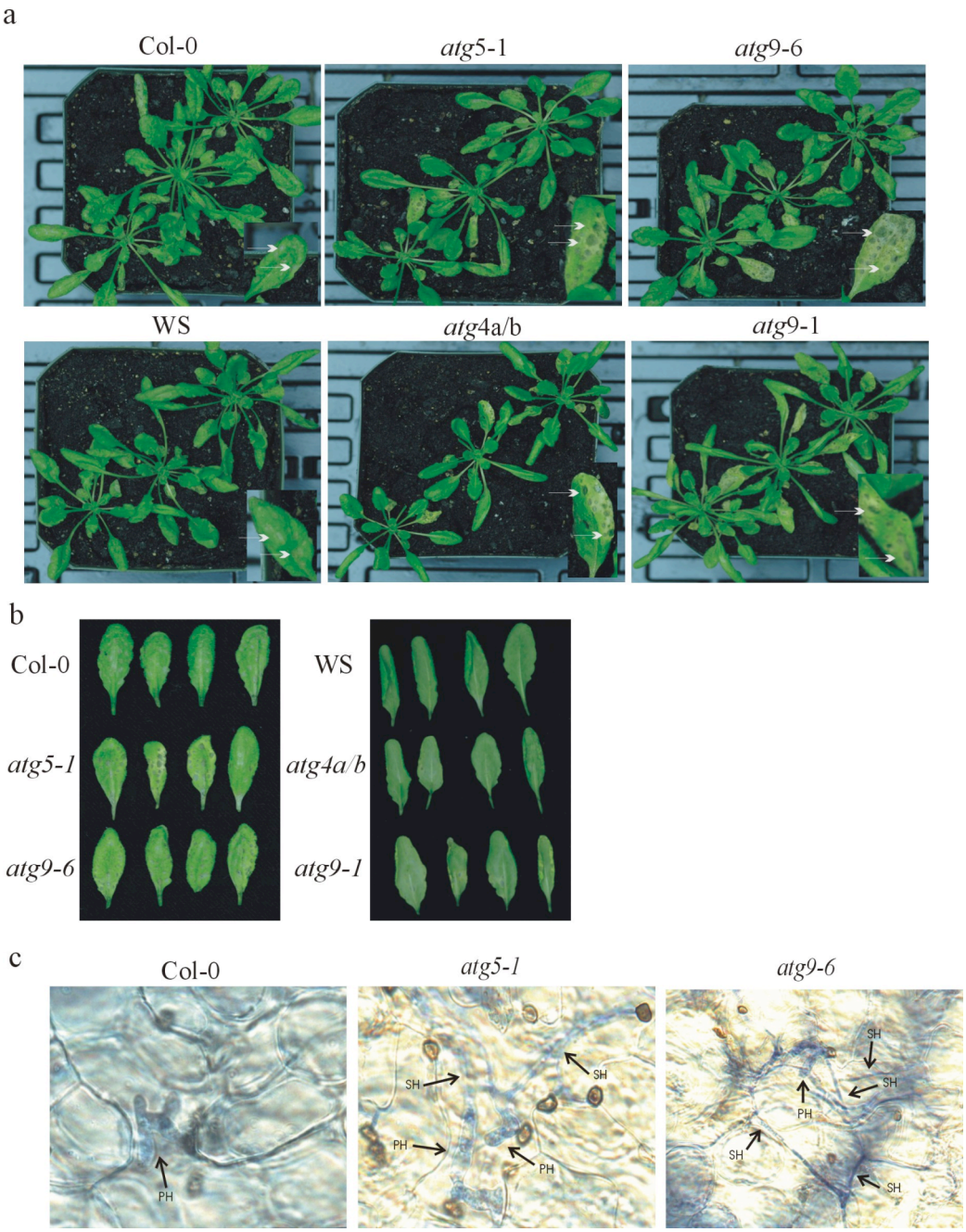

Figure 4.2 Virulence of *C. higginsianum* in *A. thaliana* intact and detached leaves of wild type plants and autophagy mutants. PH, primary biotrophic hyphae; SH, secondary necrotrophic hyphae **(a)** Intact wild type (Col-0 and Ws) and *atg* mutant (*atg4a/b, atg5-1, atg9-1* and *atg 9-6*) plants were inoculated with *C. higginsianum* and pathogenicity was accessed at 5 dpi. All *atg* mutants displayed an accelerated disease phenotype, as necrotic and chlorotic lesions (arrows) are more prominent at this time point. **(b)** Leaves of 35-day-old plants were detached and spray inoculated with *C. higginsianum*. Disease susceptibility was accessed at 5 dpi. All *atg* mutants displayed an accelerated disease phenotype, as necrotic and chlorotic lesions are more prominent at this time point. **(c)** Cytological examination of infected tissues at 3 dpi showing the accelerated transition to secondary hyphae in *atg* mutants compared to wild type.

4.4 Discussion

4.4.1 Autophagy as a susceptibility factor for *E. cichoracearum* infection

Since *E. cichoracearum* could successfully penetrate and cause infection in *atg* mutants one could infer that autophagy is not required for infection to take place. However, the retardation of pathogenesis in *atg* mutants suggests that rather than this pathway operating as a resistance response it could possibly be a susceptibility factor in establishing infection. The infection process of powdery mildews has been extensively studied. At 2 dpi, if a conidium has not produced surface hyphae one could assume that there is some limiting factor in the proper establishment of infection. All *atg* mutants displayed a similar pathogenic phenotype of retarded surface hyphal development at 2 dpi. Surface hyphae are important for two reasons; (i) they spread (relatively undetected) across the leaf surface and penetrate cells distal to primary infection in order to obtain nutrients for the growing colony and (ii) aerial conidiophores bearing the asexual conidia (spores) are born on these hyphae and are essential for the reproductive efficacy of the pathogen. One could deduce that with the retardation of surface hyphal development in *atg* mutants that the virulence of the pathogen is negatively affected by this mutation at either the penetration or haustorium formation stage. Therefore, autophagy could be considered a susceptibility factor for *E. cichoracearum* infection.

A well-documented susceptibility factor, MLO, is required for powdery mildew infection in barley (Panstruga 2005). MLO, the plasma membrane localized syntaxin is assumed to play a role in the delivery of materials required to re-build the plasma membrane around the infective haustorium (Panstruga 2005). This reorganization seems critical to proper infection establishment for powdery mildews since a mutation at this loci results in complete resistance. Another critical factor for infection establishment would be the availability of nutrients. These two requirements could be coupled and under the control of the same pathway or uncoupled using separate plant cellular machineries. As the endomembrane system is primarily responsible for the reorganization of membranes in plant cells, the question remains then what is the role of the autophagic vesicle in the proper establishment of infection?

There is a possible positive or negative mechanism for the involvement of autophagic vesicles in establishing biotrophic fungal parasitism. Although autophagy has been identified as

a non-specific pathway, it is possible that plasma membrane materials of host cells are reorganized and delivered to the infection site that are required for the proper establishment of the extrahaustorial membrane. Amino acid and sugar transporters have been localized to the haustorial membrane though little is known about the structure of the extrahaustorial membrane and the origin of materials used to synthesize it *de novo* (Hahn and Mendgen 2001; Mendgen and Hahn 2002; Struck et al., 2002). The second possibility is that autophagic vesicles, containing whole, partially or completely degraded organelles, proteins and cytoplasm are being redirected by the fungus in an attempt to acquire or high-jack nutrients from the plant cell. This idea is supported by two observations; (i) organisms need nutrients to live, and (ii) the outer autophagic membrane could be the mysterious new membrane surrounding the fungal haustorium.

Pathogens secrete effector proteins into plant cells to stop resistance responses. A well documented case is the effector protein secreted by *Pseudomonas syringae* that degrades a protein involved in Rab activation. This stops vesicles, presumably carrying antimicrobial compounds, from being delivered to the infection site (Nomura et al., 2006; Robatzek 2007). It is plausible then that effector proteins could be secreted into the plant cell apoplast that redirect the autophagic vesicle to the infection site. The fusion of the autophagic vesicle to the EHM would place a single-membraned vesicle into the EHMAT, available for uptake by the pathogen, and assist in the *de novo* synthesis of the EHM (membrane materials from the outer autophagic membrane). Interestingly, fluorescing spherical structures (possibly autophagosomes) were observed within the EHMAT of *A. thaliana* cells infected with *C. higginsianum* (data not shown).

In the previous discussion (2.4.2) it was suggested that the observed GFP-labeled structures are simply over-produced *At*ATG8e-GFP proteins being trafficked by the endomembrane system. However, the observations in this chapter support the idea that the structures being observed are true autophagosomes since for *E. cichoracearum* in their absence there is restricted pathogen development.

4.4.2 Autophagy and the maintenance of biotrophy for *C. higginsianum*

Colletotrichum is often used as a representative biotrophic pathogen in some pathology assays due to its unique biotrophic phase of life (Liu et al., 2007b). In previous assays, *C. higginsianum* persists as a biotroph, in wild type condition, for up to 4-5 days post infection. A rapid switch to necrotrophy could suggest several things; (i) a plant resistance response is threatening survival or (ii) the pathogen is not getting nutrients needed as a result of recognition and resistance by the plant. Virulence, and more specifically the duration of biotrophy, was assessed for *C. higginsianum* in *atg* mutants in order to clarify the role of autophagy in resistance or susceptibility. Since *C. higginsianum* is a virulent pathogen to *A. thaliana* Col-0 and Ws, a deviation in behavior (virulence) in *atg* mutants could aid in elucidating the role of this pathway in plant immunity.

In *atg* mutants, *C. higginsianum* switched to a necrotrophic mode of nutrition sooner than that observed in wild type conditions. The biotrophic phase was approximately 24 hours longer in wild type *A. thaliana* compared to *atg* mutants (Bailey et al., 1992; Shen et al., 2001). This disease phenotype implies one of two scenarios concerning the *A. thaliana*-*C. higginsianum* pathosystem; (i.) autophagy is involved as a defense response to *C. higginsianum* infection, since an accelerated infection was observed, or (ii.) autophagy is, as suggested for *E. cichoracearum*, involved in establishing biotrophy for *C. higginsianum*. The phenomenon of an accelerated switch to necrotrophy in *Colletotrichum* species has been observed in several assays. Liu et al. (2007b) observed this phenotype in intact *A. thaliana* plants impaired in the salicylic acid defense pathway (*NahG*, *sid2-1*, *pad4-1*) inoculated with *C. higginsianum*. They also noted that when leaves were detached from the plant and then inoculated, virulence was increased which suggested that the detachment uncoupled basal level resistance responses to infection (Liu et al., 2007b). The question then remains: Do *Colletotrichum* species live as biotrophs initially to avoid detection and resistance? If so, accelerated susceptibility in *atg* mutants suggests that in this pathosystem autophagy is involved in resistance. On the other hand, it is possible that autophagy is in some way involved in maintaining biotrophy, either in aiding the rearrangement of plasma membrane around the biotrophic hyphae or the delivery of cargo for nutrient uptake. Further investigations into the role of autophagy in this pathosystem are warranted.

CHAPTER 5

GENERAL DISCUSSION

The catabolic pathway autophagy has been predominately investigated for its ability to maintain cellular viability in times of stress (Doelling et al., 2002; Hanaoka et al., 2002; Levine and Klionsky 2004; Colombo 2005; Contento et al., 2005; Liu et al., 2005; Thompson et al., 2005; Bassham et al., 2006; Rose et al., 2006). Recent assays in plant and animal eukaryotic systems revealed that autophagy is not only involved in catabolic metabolism but also plays a role in cell death, cell differentiation and immunity (Doelling et al., 2002; Dorn et al., 2002; Hanaoka et al., 2002; Swanson et al., 2002; Rich et al., 2003; Gutierrez et al., 2004; Levine and Klionsky 2004; Colombo 2005; Contento et al., 2005; Liu et al., 2005; Thompson et al., 2005; Bassham et al., 2006; Rose et al., 2006). For its role in immunity, autophagy is involved in eliciting responses against several pathologies (infections). It is also associated with facilitating replication of viruses and bacteria, cancers, neurodegeneration, aging, and heart disease (Reviewed by Levine and Kroemer, 2008). Although this pathway seems to be predominately associated with cell rescue even its pro-survival functions can end up being deleterious. Due to the amenability of the *A. thaliana* plant system, the functions of autophagy in plants have confirmed the findings in other eukaryotic systems. In *A. thaliana*, autophagy plays a role in cellular differentiation during development and pollen formation, in maintaining homeostasis during carbon, nitrogen or light depletion and in immunity, having roles in both resistance and susceptibility (Doelling et al., 2002; Hanaoka et al., 2002; Levine and Klionsky 2004; Colombo 2005; Contento et al., 2005; Liu et al., 2005; Thompson et al., 2005; Bassham et al., 2006; Rose et al., 2006).

The role for autophagy in immunity is somewhat ambiguous in eukaryotic animal systems, as it is involved in resistance or susceptibility in a case specific manner. This pathway was found to be involved in maintaining cellular viability distal to the infection in response to the elicitation of the hypersensitive response (Liu et al., 2005). It was suggested that the response of autophagy was due to the HR and not the pathogen itself. Nonetheless, this response by autophagy was classified as a resistance response or to be involved in resistance. The work in this thesis provides the first evidence that autophagy is involved in susceptibility during pathogen infection in plants. It is not surprising that contradictory roles for this pathway have

been identified in plants as plant cell responses to pathogen attack vary widely between resistant and susceptible interactions.

Liu et al. (2005) suggested that the autophagy pathway acts as a scavenging mechanism for ROS in cells distal to infection. This would ensure that the infected cells die and the non-infected cells distal to infection live. It would be assumed then that the autophagy pathway could sequester ROS and deliver them to the vacuole for degradation. Prior to experimental execution and after considering the current functions identified for autophagy in plants, I considered the potential of autophagy as a rescue pathway for cells under biotrophic infection. If nutrient acquisition by the pathogen was substantial enough to stress the infected cell, autophagy may be involved in the recovery of the cell by sequestering cellular components and delivering them to the vacuole for breakdown and release. This too would be considered a resistance response, since it is benefiting the plant cell under pathogen infection conditions.

The pattern of autophagy gene expression that was observed during these assays did not validate my original hypothesis. The pathway was not dramatically responsive to infection or involved in resistance. Only after 6 dpi was there slight induction of several autophagy genes during *E. cichoracearum* infection and at 1 and 3 dpi during *C. higginsianum* infection in *A. thaliana*. Although I did not observe dramatic gene induction during infection, the results are significant. During *E. cichoracearum* infection a single conidium will produce several surface hyphae that eventually pierce cells distal to the originally infected cell and withdraw nutrients. When autophagy gene expression was induced it was at 9 dpi with *E. cichoracearum*. At this time point, the infection would be at the later stages of disease development and the leaf tissue heavily colonized by powdery mildew. Therefore, it is conceivable that the infection at this time point was heavy enough to elicit a stress response, such as autophagy, in *A. thaliana*. The autophagy gene induction during *C. higginsianum* infection correlated with the biotrophic stage of the pathogen life cycle. Interestingly, *C. higginsianum* primary biotrophic hyphae are much larger than those of *E. cichoracearum* and fill up to 50% of the plant apoplastic area. This is not to say that since they are larger they are more efficient at acquiring nutrients from the infected cell; however, it is possible that since they are so large, there is more biomass and this may be enough to elicit an autophagic response.

With the availability of an *ATG8e*-GFP line, the autophagic response was visually accessed in response to infection, assuming more autophagosomes within infected cells meant an

induction of the pathway. Images were taken at the same time points that the autophagy pathway was induced. Interestingly, autophagosomes were observed to be not only abundant in infected cells but to accumulate around infection hyphae. This observation introduced the possibility that autophagy was involved in host susceptibility.

In plants, the endomembrane system is tightly linked to resistance and susceptibility responses to pathogens. Cell wall fortification components are trafficked to the plasma membrane during penetration by a pathogen (Kabayashi and Hakuno 2003; Yun et al., 2003; Opalski et al., 2005; Shimada et al., 2006). To date, the proteins and enzymes required for proper targeting, delivery and docking of the endomembrane system have also been associated with mature autophagosomes. It is therefore conceivable that the redirection of autophagosomes to the infection site could be a resistance response; however, generally in plants, a resistance response would be elicited as soon as possible to stop the infection, not 6 to 9 days after pathogen infection. Redirection of autophagosomes then could be a susceptibility factor.

How could a pathway, assumed to be indiscriminant, be involved in susceptibility? There is evidence that effector proteins, secreted by the pathogen into the plant cell, have the ability to redirect plant cell machinery and gene expression, for their benefit (Koh et al., 2005; Opalski et al., 2005; Robatzek 2007). Interestingly, a mature autophagosome is assumed to possess the correct Rab and SNARE proteins for delivery to the vacuole; how then could it be directed to the plasma membrane? There is data that supports the possibility that in vesicles of the endomembrane system Rab conversions do take place. A Rab conversion involves the replacement of a Rab protein (possibly directing the vesicle to the vacuole) with a different Rab protein (possibly directing it to the nuclear membrane) (Rink et al., 2005). The redirection of autophagosomes could be triggered by the pathogen and carried out by Rab conversions using plant cellular machinery.

Biotrophic hyphae, more specifically haustoria, were assumed to simply invaginate the plant plasma membrane. Now it is recognized that at least in the *Erysiphe-A. thaliana* system, the plant membrane surrounding the haustorium is synthesized *de novo* (Koh et al., 2005). Interestingly, eight tested plasma membrane markers could not be identified in the membrane surrounding a haustorium in *A. thaliana* (Koh et al., 2005). It is therefore possible that the outer autophagic membrane is recruited to be used in the remodeling of the plant plasma membrane to facilitate hyphal growth and possible nutrient acquisition. In barley, a protein that is thought to

play a crucial role in plant plasma membrane remodeling during haustorium formation is the MLO protein (Lyngkjaer et al., 2000). Plants with a *mlo* mutation are resistant to infection and the pathogenesis is halted at the haustorium formation step. MLO is assumed to be associated with the endomembrane system and be involved in membrane material delivery to the plant plasma membrane (Lyngkjaer et al., 2000). MLO has not been associated with autophagy, but since these two pathways share other characteristics it is possible that the autophagy pathway assists in plant plasma membrane remodeling.

Another possible reason for the redirection of autophagosomes to the infection site is that in some way they are involved in nutrient transfer. Nutrients are assumed to leak through the plant plasma membrane into the extracellular matrix and be actively taken up by the haustorium. Autophagosomes in *A. thaliana* are lytic (Rose et al., 2006), that is the components they capture can be wholly or partially degraded prior to reaching the vacuole. The redirection of a vesicle carrying degraded compounds from the vacuole to the plasma membrane surrounding the infection site seems like it would be beneficial to a pathogen. Interestingly, GFP-tagged spheres were visible in the extracellular matrix of leaf cells of *A. thaliana* infected with primary hyphae of *Colletotrichum* (data not shown). ATG8s are associated with the inner autophagic membrane. It is therefore possible that the inner autophagic membrane is released into the extracellular matrix.

Traditionally, to elucidate the particular function of a pathway or a gene in host resistance or susceptibility, the target gene or a gene involved in the pathway would be disrupted in the plant and the behavior of the pathogen upon and during infection is assessed. This is a relatively easy way to determine the necessity of a pathway or gene involved in plant-pathogen interactions. In chapter 4, *A. thaliana* plants were blocked in the autophagy pathway at the autophagosome formation step at three locations (three different genes) in two wild type backgrounds. In all mutant lines, *E. cichoracearum* infection was retarded and *C. higginsianum* infection was accelerated. Interestingly, this simple assay differentiated the relocation of autophagosomes from being a resistance response to being a susceptibility factor. If infection was accelerated in *A. thaliana atg* mutant lines infected with *E. cichoracearum* then autophagy would be assumed to play a role in resistance. Since it was not completely halted, the pathway is considered not to be required for infection but since pathogenesis was negatively altered it can be considered as a susceptibility factor. The initial investigative theory was to elucidate a role for

autophagy during fungal biotrophy. *Colletotrichum* was included in these assays because it has recently become a model hemibiotrophic pathogen since its pathogenesis begins with the establishment of a biotrophic infection structure that feeds off living plant cells mimicking the infection strategy of biotrophic pathogens. *Colletotrichum* species are easily cultured anexically in laboratory conditions and have been successfully transformed with DNA constructs (O'Connell et al., 2004). Virulence of *C. higginsianum* was accelerated in *atg* mutants. This accelerated disease development has been observed previously and is associated with the concentration of inoculums (the higher the incolum concentration the faster the switch to necrotrophy), and the functionality of resistance genes and genes associated with resistance (Liu et al., 2007b). The rapid progression to necrotrophy suggests that this pathogen was not able to acquire what it needed and therefore had to turn to a more destructive mode of feeding. It does not necessarily mean that the autophagy pathway is involved in a resistance response to *C. higginsianum*. The fact that the autophagosomes were redirected to the infection sites during both types of infection (biotrophic and hemibiotrophic) suggests that this pathway could have a role in maintaining biotrophy during infection.

Finally, in order to elucidate the role of signaling in the induction of the autophagic response during infection, *A. thaliana* plants were treated with the common defense signaling molecules/hormones and autophagy gene expression was examined. SA, ET, JA, H_2O_2 and SNP are commonly observed to be, singly or in combination, involved in eliciting a resistance response during pathogen attack in plants. Expression of autophagy genes did not exhibit a coordinated response pattern upon treatment of these signaling molecules. The results of the signaling molecule treatments suggest that the autophagy pathway induction during infection is uncoupled from defense related signaling molecules. Taken together, the complex autophagy pathway appears to be involved in host susceptibility to infection. This thesis is the first example linking autophagy to susceptibility in *A. thaliana* during infection. Further investigations into autophagy and its diverse roles in immunity/susceptibility in plants are warranted.

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