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# The emerging role of autophagy in plant pathogen attack and host defence

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Autophagy is emerging as an important process in plant infection by pathogenic fungi, which develop differentiated infection cells to breach the plant cuticle. Conversely, autophagic processes are also important in the defence responses of plants that are able to perceive and react to invading pathogens. The pivotal role of autophagy in both fungal pathogenesis and disease resistance is linked to its function in the regulation of programmed cell death which is a key component of plant immunity responses and fungal infection-related development.

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## Introduction

Autophagy is widely conserved among eukaryotes and is responsible for the degradation and recycling of proteins, organelles and cytoplasm [1,2,3]. Autophagic recycling can occur as a cell survival mechanism under the conditions of severe starvation or environmental stress and also as a precursor, or mechanism of programmed cell death [4]. Autophagy can be broadly divided into two processes, which share some common components, but have quite distinct cellular functions. The first, macroautophagy, describes the bulk recycling of cellular materials, which is initiated by the formation of a cup-shaped single membrane structure called a phagophore, which engulfs and encapsulates organelles and/or cytoplasm that are/is destined for recycling [1,2]. Membranes of the phagophore elongate and fuse to form a double membrane-bound spherical autophagosome, which enlarges before fusion with lysosomes in animal cells, or the vacuole in plants and fungi. The outer autophagosome membrane fuses with the vacuolar membrane, releasing a vesicle into the vacuole lumen that is bounded

by the inner membrane, called an autophagic body [1,2,3,4]. Vacuolar hydrolases degrade the autophagic body making its contents available for re-use in the cytoplasm. By contrast, the second major form of autophagic recycling, selective autophagy is used to degrade peroxisomes (pexophagy), mitochondria (mitophagy) or endoplasmic reticulum (reticulophagy) in an organelle-specific process that requires a distinct set of proteins, necessary for cargo selection, some of which also participate in the cytoplasm to vacuole targeting (Cvt) pathway that has been described in yeast [5–8]. In addition to these main forms of autophagy, plants undergo developmentally regulated microautophagy by which they deposit storage proteins in seeds and mobile starch granules during seed germination (for review, see [9]). Other forms of autophagy have also been reported, such as aggrephagy which describes the selective degradation of protein aggregates, crinophagy to describe fusion of secretory vesicles with lysosomes and xenophagy to describe the selective degradation of microbial pathogens [5]. When considered together, a wealth of recent studies provides increasing evidence that autophagy is a fundamental process in eukaryotic organisms, necessary for the cellular response to stress, for cellular differentiation and tissue remodelling during embryonic development, host defence against pathogens, and as a key regulatory mechanism in metabolic control [1,2,3,4]. Many excellent reviews have appeared recently describing the process of autophagy and these provide a comprehensive overview of its mechanism and diverse roles [1,2,3,4,5]. In this review, we have focussed on the role of autophagy in plant–microbe interactions and, specifically, the recent evidence that autophagy is necessary for both fungal pathogenicity and host plant immunity responses.

## The molecular mechanism of autophagy

The molecular basis of autophagy has been most intensively studied in *Saccharomyces cerevisiae* where 31 proteins have been described that play a role in autophagy [1]. In yeast, autophagy is initiated under the conditions of starvation stress by the Target of Rapamycin (TOR) kinase, which acts as a negative regulator of autophagy. TOR appears to act, directly or indirectly, to hyperphosphorylate Atg13, which prevents its interaction with the Atg1 kinase inhibiting autophagy [10]. Induction of autophagy therefore involves a protein complex including Atg13 and Atg1, which may be tethered to the pre-autophagosomal structure (PAS) by the scaffold protein Atg17 [11]. Induction of autophagy requires the action of the Atg1 kinase, although the direct targets of Atg1 are not yet

known. In selective autophagy, the selection of cargo for degradation and their packaging involves Atg11, which encodes a peripheral membrane protein that is the adaptor required for cargo loading in pexophagy and delivery of aminopeptidase I to the vacuole in the Cvt pathway, and Atg19 which is involved in cargo selection at the PAS [1]. Vesicle nucleation at the PAS proceeds through *de novo* generation of the phagophore, although the membrane required may be supplied by the endoplasmic reticulum [3\*,5]. There is evidence that the phosphatidylinositol 3-kinase (PI3K) complex I is involved in vesicle nucleation, including the *VPS34*-encoded PI3K, Atg6 (Beclin1/VPS30), Vps15 and Atg14 [12]. Phagophore and autophagosome expansion involves the activity of a large number of proteins including Atg3, Atg4, Atg5, Atg7, Atg8, Atg10, Atg12 and Atg16 [13\*\*] (Figure 1). The novel ubiquitin-like protein Atg8 is modified at its C-terminus via conjugation to phosphatidylethanolamine (PE) [14]. Atg8 is first processed by the cysteine protease Atg4, exposing a C-terminal glycine residue that is conjugated to phosphatidylethanolamine by a ubiquitin E1-like activating enzyme, Atg7 and the E2-like conjugating enzyme Atg3 [15]. A protein complex composed of Atg5, Atg12 and Atg16 brings about the conjugation of PE, perhaps acting in a similar manner to a ubiquitin E3 ligase [16] during proteasome-mediated protein destruction. Atg8 is present at the PAS and its localisation there requires Atg9, a putative membrane carrier protein, and the PI3K complex. Recent evidence has shown that Atg8 controls the expansion of the phagophore during the formation of autophagosomes, and the size of autophagosomes is dependent on the amount of Atg8 that is present [15]. The association of Atg8 with mature autophagosomes has made this a very widely utilized marker for autophagy [17]. Generation and expression of a GFP–Atg8 fusion protein provides a means of determining the spatial regulation of autophagy by measuring the distribution of punctate GFP–Atg8 autophagosome-associated signal within cells, whilst western blotting can be used to determine the proportion of conjugated Atg8–PE and free Atg8 protein in order to investigate the temporal dynamics of autophagy and to investigate the role of individual protein components [17]. Recycling and retrieval of autophagy proteins is mediated by Atg2, Atg9 and Atg18 [1,2,3\*,4\*]. Autophagosome docking and membrane fusion involves the SNARE proteins Vam3, Vam7, Ykt6 and Vti1, in addition to the Rab protein Ypt7 and Sec17, Sec18 and Sec19, and the homotypic fusion and vacuole protein sorting (HOPS) complex [18]. Autophagic bodies are then degraded via the Atg15 lipase and vacuolar hydrolases degrade their contents.

### The role of autophagy in plant pathogenic fungi

Many plant pathogenic fungi elaborate specialised infection structures, which are used to bring about cuticle penetration and entry to plant tissue. These structures, called appressoria, can be unicellular, dome-shaped cells,

or occasionally more elaborate multicellular structures [19]. In the rice blast fungus *Magnaporthe oryzae* (formerly *M. grisea*), appressoria are melanin pigmented unicellular structures that generate enormous turgor in order to breach the rice leaf cuticle [20,21]. A narrow penetration hypha is produced at the base of the appressorium and enters the underlying epidermis, rupturing the cell wall and invaginating the plasma membrane. The fungus is therefore able to occupy living rice cells, which remain intact and viable throughout the early stages of infection [21]. Appressorium turgor is generated by the accumulation of osmotically active solutes, most notably glycerol, which are formed from the contents of the three-celled conidium from which the appressorium forms, shortly after germination and extension of a short germ tube [22]. Appressorium formation by *M. oryzae* occurs only on hard, hydrophobic surfaces and appears to be genetically controlled by the cell cycle. During germination of the three-celled conidium, a nucleus enters the germ tube and undergoes mitosis within four to six hours. After nuclear division, one daughter nucleus moves into the developing appressorium, whilst the other nucleus migrates back into the original conidial cell [23]. Addition of hydroxyurea to conidia prevents nuclear division within the germ tube and also inhibits appressorium development, indicating that entry into mitosis is necessary for the initiation of appressorium formation. Consistent with this idea, a thermo-sensitive *MonimA<sup>E.37G</sup>* mutant of *M. oryzae* which was unable to undergo mitosis at the non-permissive temperature, was unable to form mature appressoria [23]. Interestingly, appressorium development in *M. oryzae* is also always accompanied by collapse and programmed cell death of the fungal conidium, which requires autophagy. Mutants lacking the *MoATG8* gene, a functional homologue of the yeast Atg8-encoding gene, were unable to undergo conidial collapse and cell death [23]. As a consequence,  $\Delta Moatg8$  mutants were non-pathogenic because they produced appressoria that were not able to form penetration hyphae and breach the host plant cuticle. During a differential cDNA screen for appressorium-expressed genes, a homologue of the Atg1 kinase-encoding gene was identified and targeted gene deletion showed that this too is essential for plant infection by *M. oryzae* [24]. It has recently been shown that MoAtg8 plays a role in the regulation of glycogen metabolism during conidiation [25].

The role of autophagy has also been studied in the appressorium-forming cucumber anthracnose fungus *Colletotrichum orbiculare* and has confirmed the significance of macroautophagy to appressorium-mediated plant infection by the deletion of *CoATG8* [26\*\*]. The resulting  $\Delta Coatg8$  mutants were impaired in appressorium development and pathogenicity [26\*\*]. However, analysis of the pexophagy-specific gene *CoATG26*, which putatively encodes a sterol glucosyltransferase previously studied in the methylotrophic yeast *Pichia*

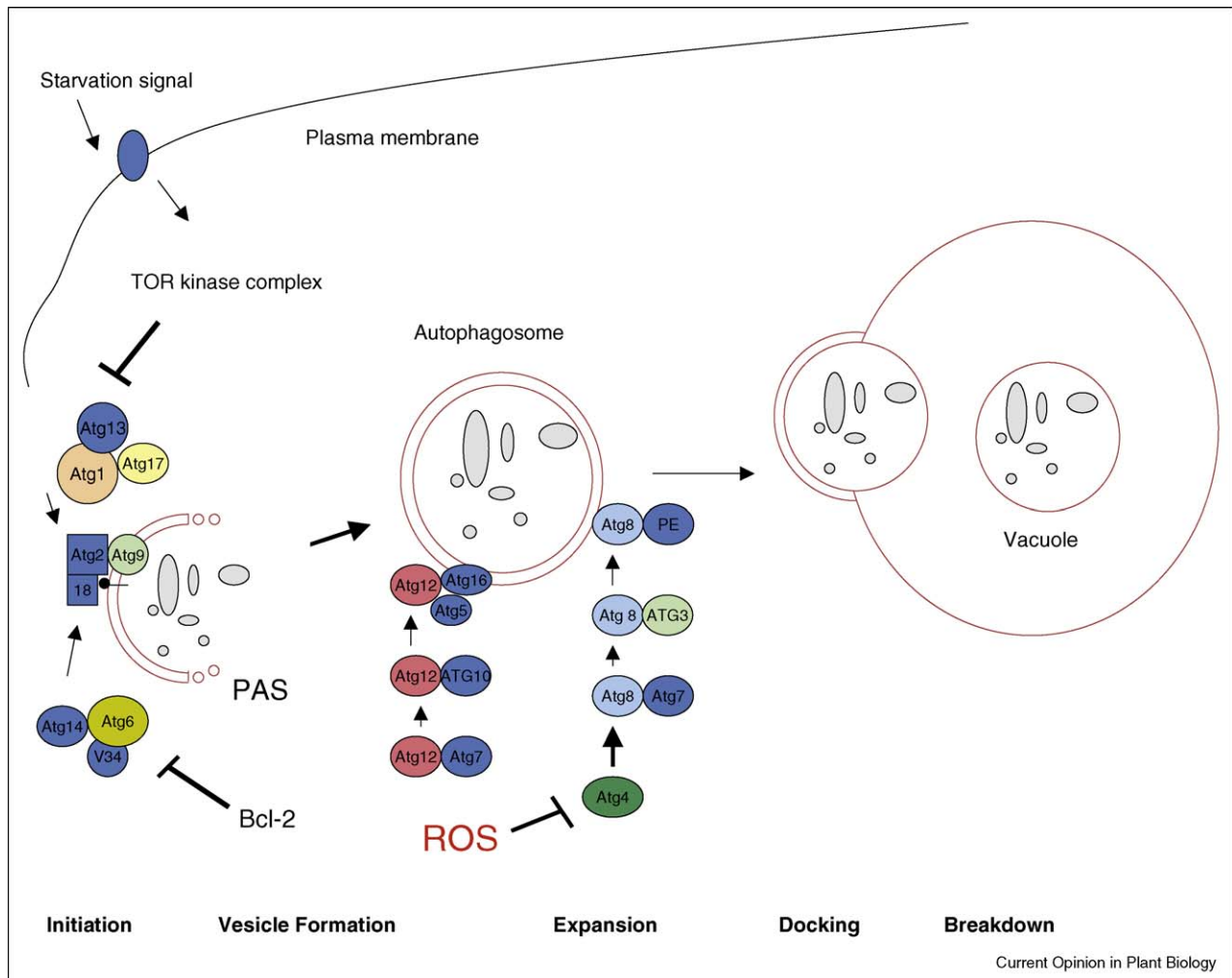
*pastoris*, showed a specific requirement for pexophagy during tissue colonisation following appressorium-mediated penetration. Peroxisome degradation within appressoria was observed in *C. orbiculare* and was significantly delayed in *Coatg26* mutants. In *M. oryzae*, turgor generation involves mobilisation of lipid bodies to developing appressoria, followed by their rapid lipolysis, a process that involves a large number of triacylglycerol lipases [27]. Peroxisomal fatty acid  $\beta$ -oxidation is also significant during appressorium in *M. oryzae* protein, because mutation of *MgPEX6*, a gene encoding a peroxin protein required for peroxisome biogenesis, or *MFPI*, which encodes the multi-functional  $\beta$ -oxidation enzyme, both impair appressorium function in *M. oryzae* [27–29]. Similarly, in *C. lagenarium* peroxisome biogenesis is essential for appressorium function [30]. The role of peroxisomal fatty acid  $\beta$ -oxidation in both fungi is likely to be associated with the importance of acetyl CoA as substrate for a large number of biosynthetic pathways, including perhaps most significantly the dihydroxynaphthalene melanin biosynthetic pathway. Cell wall-associated melanin is necessary for appressorium turgor generation in both *Magnaporthe* and *Colletotrichum* species [31,32], and its synthesis is clearly impaired in  $\Delta mfp1$ ,  $\Delta pex6$  and  $\Delta pth2$  (carnitine acetyl transferase) mutants [26<sup>••</sup>,27–29]. Recent evidence in mammals has demonstrated that autophagy may be necessary for lipid breakdown and inhibition of autophagy affects lipid mobilisation from lipid droplets, indicating that their normal degradation may be via a process that has been termed macrolipophagy [33<sup>••</sup>]. This is consistent with the responses observed in pathogenic fungi and it may be worth speculating that autophagy and lipid breakdown are intimately connected in pathogenic fungi, perhaps even at the level of triacylglycerol lipase activation. The importance of lipid mobilisation and peroxisomal fatty acid  $\beta$ -oxidation in pathogenic fungi, during appressorium maturation also indicates that selective forms of autophagy, such as pexophagy may be important in the initial stages of plant colonisation following appressorium-mediated plant infection. This appears to be the case in *C. orbiculare* because *Atg26* mutants are impaired in host invasion, but only following appressorium-mediated penetration and not after wounding plants [26<sup>••</sup>]. This role for autophagy was not, however, observed in experiments carried out in *M. oryzae* (MJ Kershaw and NJ Talbot, unpublished data), again highlighting the specific differences that occur between appressorium-forming fungi. However, when all of these studies are considered together the role of non-selective macroautophagy in fungal infection-related development appears to be significant. As fungal infection-related morphogenesis is a starvation-induced process, it appears likely that cellular recycling via autophagy will prove to be a pivotal process in the pre-penetration stages of many fungal pathogens. This may also be true for mutualistic symbiotic fungi, because autophagy

genes are induced during arbuscule formation by the mycorrhizal fungus *Glomus intraradices* [34<sup>•</sup>]. Selective autophagy, meanwhile, may be involved in fungal developmental processes that are preceded by intense peroxisomal activity or other organellar activities.

### The role of autophagy in plant defence

Autophagy has recently been identified as a significant component of the plant defence mechanism deployed by plants. The majority of plant autophagy-associated ATG genes are, for instance, expressed preferentially upon challenge with the aphid *Myzus persicae* or the bacterium *Pseudomonas syringae* [35]. Plant defence against potential pathogens involves the recognition of microbial proteins at the cell surface — often termed pathogen-associated molecular patterns (PAMPs) — that trigger defence signalling and responses such as secondary cell wall thickening and production of anti-microbial compounds [36]. Pathogenic micro-organisms, however, target plant defence signalling directly using secreted effector proteins and thereby suppress plant defence, facilitating their entry and colonization of plant tissue. As a response to the action of microbial effectors, plants have developed immunity from disease by the recognition of specific pathogen effectors [36]. This triggers a hypersensitive response (HR) in which plant cells at the site of infection are killed by a form of programmed cell death, thereby preventing the invading pathogen from further spread. How plants are able to restrict the HR to cells in the immediate area surrounding an infection has, until recently, been unclear, but autophagy appears to be necessary for the spatial restriction of programmed cell death [37]. An orthologue of the ATG6 (Beclin1) gene was studied in *Nicotiana benthamiana*. Silencing of the *NbBECLIN1* gene by RNA interference in plants that contained the *N* resistance gene resulted in reduced autophagy and uncontrolled HR upon infection with tobacco mosaic virus [38]. *N* belongs to the family of TIR–NBS–LRR immune receptor proteins (Toll/interleukin-1 receptor/nucleotide binding site/leucine-rich repeat protein) and recognises the helicase domain of the TMV replicase protein, which triggers HR and disease resistance [37,38]. The uncontrolled HR observed in *NbBECLIN1*-silenced plants was completely dependent on the action of *N*-mediated immunity, because no cell death was observed when the silenced plants were challenged with TMV in the absence in lines not containing the *N* gene. The role of ATG6/Beclin1 in HR regulation has also been confirmed in *Arabidopsis thaliana* where *AtATG6* antisense plants were found to show a spreading HR phenotype when infected with an incompatible *P. syringae* DC3000 bacterial pathogen expressing the AvrRpm1 effector protein in plants carrying the corresponding *RPM1* resistance gene [39<sup>••</sup>]. *AtATG6* furthermore showed enhanced expression upon infection with either virulent, or avirulent *P. syringae* bacteria and plant cell death caused by virulent bacteria was also affected by the reduced expression of *AtATG6* in antisense lines. These results indicate that autophagy is vital to

Figure 1



The process of macroautophagy based on studies carried out primarily in *Saccharomyces cerevisiae*. A single membrane phagophore forms and envelops organelles or cytoplasm that is targeted for recycling. The phagophore elongates and folds to form a spherical double membrane autophagosome, which fuses with the vacuole, releasing an autophagic body for processing by vacuolar hydrolases. Atg proteins facilitate vesicle nucleation, phagophore formation, autophagosome expansion, docking and recycling. See text for details. Filamentous ascomycete fungi contain a similar repertoire of proteins to *S. cerevisiae*, whereas plants contain multiple forms of most Atg proteins. In *Arabidopsis* there are, for example, nine putative orthologues of ATG8, suggesting that cell or tissue-specific forms of autophagy may operate.

restrict programmed cell death to localized sites of infection site during the HR and may also play a role in host cell death during plant disease. These studies do, however, leave many unanswered questions regarding the precise role of autophagy in plant defence [37]. Is autophagy, for instance, necessary only for the prevention of cell death in healthy tissue adjacent to sites of HR, or is it also a component of the mechanism of programmed cell death in cells undergoing HR as well? Autophagy is induced during *N*-mediated resistance to TMV infection within sites of HR induction, as well as in the surrounding tissue and the production of autophagosomes was impaired in *NbBECLIN1* RNAi plants [38]. Therefore, although it seems likely that autophagy plays a key role in restricting

the spread of HR-associated programmed cell death, it cannot be ruled out at this time, that it also plays a role in HR itself. Programmed cell death in plants does, for example, show morphological characteristic of autophagic cell death rather than apoptosis, such as uptake of organelles into the vacuole, an increase in vacuolar size, and ultimately vacuole lysis and cell death [9]. Furthermore, plants lack caspases that are critical to apoptosis in animal cells and the presence of a plant cell wall would prevent the take-up of the remains of dead cells by their neighbours, as occurs in animal apoptosis [7]. To test the hypothesis that autophagy is also essential for HR-mediated cell death, however, will require more systematic characterization of other more central components of macroautophagy, in

addition to Atg6/Beclin1 and the other autophagy proteins tested in *N. benthamiana*, *VPS34*, *ATG3* and *ATG7* [38]. In mammals, Beclin1 was actually identified because of its interaction with Bcl-2, an anti-apoptotic protein [40], highlighting its potentially wider role, although its main function is thought to be during autophagosome formation at the PAS, as discussed previously [1,4\*,9,40–43]. It will prove extremely interesting to carry out a systematic study of the spatial and temporal activity of the large family of plant ATG genes during HR and to study their biological functions via gene silencing in a range of host–pathogen interactions. When carried out in association with studies of lesion mimic mutants, which are also impaired in spatial regulation of HR [43], this may prove illuminating to dissecting the mode of programmed cell death operating during plant immunity [9,37].

## Conclusions

Recent studies have shown that autophagy is likely to be pivotal both to plant infection by pathogenic fungi and also to host defence responses. This highlights the importance of autophagic processes to developmental biology and extracellular responses in multi-cellular eukaryotes as a means of rapidly redeploying cellular resources under times of starvation stress or acute developmental need [1,2,3\*,4\*]. These studies must therefore be seen in the context of a very wide range of research that has shown autophagy to be involved in many diverse developmental processes in animals, as well as adaptive immunity, the response to oxidative or other abiotic stresses, tumorigenesis and its control, among many others [3\*,4\*,7,9]. However, clearly the study of autophagy in the context of plant–microbe interactions has already provided additional new insight into the mechanisms of both pathogen ingress and host defence, providing a platform for future investigation. Of particular interest will be to study the interplay between cell cycle control of appressorium development and the induction of autophagy within appressoria and conidia [23]. The spatial control of autophagy during *M. oryzae* will also be important, as it is not yet clear whether autophagy is spatially restricted to the conidium which undergoes cell death, or occurs in the appressorium, which undergoes growth of the penetration hyphae during plant infection. Similarly the regulation of lipolysis, and the potential that this is a predominantly autophagy-driven process in fungi as well as in mammals, requires further investigation in order to understand the physiology of fungal infection cells [26\*\*,27–30].

Perhaps most fundamental of all will be the necessity to define whether autophagy is primarily a cell survival response, that allows cells to survive the spread of other pro-death signals into healthy cells surrounding sites of HR in the plant immunity response, or whether autophagy is observed because it is a symptom of other forms of programmed cell death, perhaps resembling apoptosis [37]. Alternatively, autophagy may be the mechanism of pro-

grammed cell death in plant immunity, although this will need a more systematic functional study of autophagic components. Clearly in fungi, programmed cell death of conidia requires autophagy, which provides strong evidence that the process of cell death is primarily autophagic [23]. However, until apoptotic components such as the family of metacaspase genes that are present in filamentous ascomycetes such as *M. oryzae*, are functional analysed, it will not be possible to tell whether the process is exclusively autophagic, or merely requires autophagy as a component of an apoptotically triggered process [44]. There is now a growing interest in autophagy in fungal developmental biology [45\*\*], where it plays diverse role in vegetative incompatibility [46], for example, and the response to metal ion deficiency [47].

Finally, the control and manipulation of autophagy are clearly worth studying as a potential means of impairing plant infection by fungal pathogens, or manipulating host defence reactions, providing additional powerful incentives to understand the operation of autophagy during plant–pathogen interactions.

## Note Added in proof

A recent paper has appeared [Hofius et al., 2009 Autophagic components contribute to hypersensitive cell death in Arabidopsis. *Cell* 137; 773–778, 2009] which provides the first evidence that HR initiated by Toll/interleukin 1(TIR)-type immune receptors requires autophagy. This important paper therefore suggests that both the spatial restriction of HR lesions and the initiation of at least some forms of HR during plant defence, require autophagy.

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