

## Addendum

# Autophagic Cell Death and its Importance for Fungal Developmental Biology and Pathogenesis

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## KEY WORDS

*Magnaporthe grisea*, appressorium, autophagy, programmed cell death, host invasion

Addendum to:

*Autophagic Fungal Cell Death is Necessary for Infection by the Rice Blast Fungus*

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## ABSTRACT

In order to cause disease in plants, many fungal pathogens develop a specialized structure called an appressorium. We have recently shown that the rice blast fungus *Magnaporthe grisea* undergoes a regulated form of programmed cell death during appressorium development involving autophagy. Significantly, this form of cell death is a prerequisite for plant infection and fungal pathogenesis and part of a growing body of evidence implicating autophagy as a key process in fungal developmental biology.

*Magnaporthe grisea* is a filamentous fungus that is responsible for rice blast disease, the most devastating disease of cultivated rice.<sup>1</sup> The fungus is able to invade rice leaves directly by differentiating a specialized infection structure called an appressorium. The appressorium is a dome-shaped unicellular structure, which differentiates from the end of a fungal germ tube, shortly after spore germination on the leaf surface.<sup>2</sup> The appressorium develops substantial turgor that is translated into physical force to bring about rupture of the rice cuticle, allowing a narrow penetration hypha to invade the rice epidermis. From this initial site of infection the fungus proliferates rapidly in rice tissue bringing about a disease lesion within four days of its initial contact with the plant. Rice blast is responsible for serious harvest losses each year and the disease remains difficult to control effectively.<sup>1</sup> It occurs in all rice-growing regions of the world making the disease a significant threat to food security.

We recently investigated the development of appressoria by the rice blast fungus and carried out cytological analysis of nuclear division during formation of infection structures. Using a strain of the fungus in which a histone protein had been tagged with the green fluorescent protein we were able to show that appressorium formation was always associated with completion of mitosis in the germ tube.<sup>3</sup> Following mitosis, one daughter nucleus migrated to the nascent appressorium, while the other returned to the fungus spore. The fungal spore then underwent nuclear degeneration and cell death as the appressorium matured. We found that blocking mitosis, using either pharmacological agents such as hydroxyurea or benomyl, or genetically by generating a conditional NimA kinase mutation, prevented appressorium formation, indicating that completion of mitosis was a prerequisite for appressorium morphogenesis. Blocking mitosis also, however, prevented collapse and death of the fungal conidium, suggesting that appressorium morphogenesis and conidial cell death were linked developmental processes in *M. grisea*. To test this idea, we generated a mutant lacking the *M. grisea ATG8* gene, which is required for autophagy. Targeted deletion of *MgATG8* prevented starvation-induced autophagy from occurring in fungal hyphae but, importantly, also prevented conidial spore collapse during appressorium development. The resulting  $\Delta Mgatg8$  mutant was still able to produce appressoria, but was not able to cause rice blast disease because appressoria were nonfunctional and unable to produce penetration hyphae. When considered together, our data provide evidence that fungal spores of the rice blast fungus undergo autophagic cell death, which is necessary for the infection cell to function correctly and bring about rice blast disease.<sup>3</sup>

Appressorium development in *M. grisea* is an example of cellular differentiation that results from a set of external environmental cues including the presence of the hard, hydrophobic rice leaf surface and the absence of external nutrients.<sup>1,2</sup> There are now several examples in eukaryotic organisms, which show the involvement of autophagic type II programmed cell death in stress-induced cellular differentiation and development, such as ascospore differentiation in *Saccharomyces cerevisiae*, the production of a multicellular stage upon nutrient deprivation in the slime-mold *Dictyostelium discoideum* and the formation of the dauer larvae in the nematode *Caenorhabditis elegans*.<sup>4</sup> Involvement of the appressorial vacuole as a dynamic cellular compartment during infection-related development had in

fact already been suggested in the fungal pathogens *Colletotrichum gloeosporioides* and *M. grisea* previously,<sup>5,6</sup> but our study has provided evidence that autophagy in *M. grisea* has a causative role in spore collapse and cell death, and is a necessary prerequisite for successful infection of plant tissue through the differentiation of a fully functional appressorium. This result is in contrast with findings in the filamentous fungus *Podospora anserina* that have indicated that autophagy does not play a causal role in programmed cell death during fungal vegetative incompatibility,<sup>7</sup> but is consistent with studies in mammalian cells showing that *ATG* genes can promote programmed cell death by autophagy.<sup>8</sup> It appears that autophagic-programmed cell death may be a conserved process of ancient origin because it has been observed in such a highly diverse range of unicellular and multicellular eukaryotes including *Dictyostelium discoideum*, the nematode *C. elegans*, plants, oomycetes and filamentous fungi.<sup>4,9-12</sup> Autophagic cell death may therefore have developed as a developmental process prior to apoptosis.<sup>4</sup> It is becoming apparent that programmed cell death in fungi can, however, also occur through apoptosis as demonstrated in *Candida albicans*,<sup>13</sup> *A. nidulans*<sup>14</sup> and *Colletotrichum trifolii*.<sup>15</sup> However, as yet no direct link between these two types of cell death has been established in fungi. Evidence from other eukaryotic organisms suggests that type I (apoptosis) and type II (autophagic) cell death are not mutually exclusive phenomena.<sup>16,17</sup> For example, studies on mammalian cells have revealed that a mechanistic overlap can exist between the two-types of cell death via caspase activity, and in *C. elegans*, apoptosis is triggered when autophagy is genetically blocked.<sup>18</sup> The demonstration that autophagic cell death is a component of cellular differentiation during infection-related morphogenesis in a plant pathogenic fungus may provide a useful starting point for further studies to understand both the importance of autophagy in developmental biology in fungi and its interplay with apoptotic mechanisms that have yet to be studied in phytopathogenic species.

The principal challenge, however, will be to determine precisely why mutants of *M. grisea* that are impaired in autophagy are unable to cause disease. The differentiation of appressoria by the rice blast fungus and the development of high internal turgor within these cells<sup>19</sup> requires high metabolic demand and occurs in the absence of external nutrient sources. It is also clear that the entire contents of the conidium is mobilized to the appressorium following autophagic cell death. These requirements are consistent with the role of autophagy as a mechanism for turnover and recycling of proteins and organelles during nutrient stress and consequent tissue remodeling. Surprisingly, however, *M. grisea* mutants impaired in autophagy still elaborate appressoria that are able to support turgor, but which instead are completely blocked in their ability to repolarize and penetrate plant tissue. It is possible that the appressorium that is formed in the absence of autophagic recycling from the conidium is unable to synthesize the components necessary for penetration hypha formation and the metabolic transition necessary for plant tissue colonization. This highlights the potential role of autophagy as a molecular mechanism necessary for the adaptation of a fungus to growth in a new environment, which may be widespread among fungal species and a likely consequence of the osmotrophic, mycelial growth habit of filamentous fungi.

Another possibility, however, is that autophagy is essential for the actual process of repolarization by the appressorium. In cells undergoing apoptosis, actin, cytokeratins, lamins and other cytoskeletal proteins are depolymerized or cleaved, whereas the cytoskeleton in cells undergoing autophagic cell death has been found to be redistrib-

uted, but largely preserved.<sup>20,21</sup> A large-scale reorganization of the cytoskeleton is observed during penetration peg development at the base of an appressorium,<sup>2,22</sup> which suggests the need for preserved cytoskeletal components during penetration. Interestingly, it is also clear that polarity of the penetration peg is not established in the absence of autophagic cell death of the spore and the germ tube.<sup>3</sup> It is possible therefore that induction of autophagy is necessary as a checkpoint for organization of the penetration hypha and reestablishment of polarized hyphal growth.

In summary the demonstration that autophagy plays a significant role in the biology of plant infection by a pathogenic fungus is consistent with an emerging body of evidence highlighting the importance of autophagic processes to fungal growth and development. It also provides fundamental new information regarding the molecular basis of infection-related processes in pathogenic species, which may ultimately lead to more effective disease control strategies.

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