



Published in final edited form as:

Trends Microbiol. 2018 July ; 26(7): 582–597. doi:10.1016/j.tim.2017.12.007.

Rise of a cereal killer: The biology of *Magnaporthe oryzae* biotrophic growth

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Abstract

The rice blast fungus, *Magnaporthe oryzae*, causes one of the most destructive diseases of cultivated rice in the world. Infections caused by this recalcitrant pathogen leads to the annual destruction of approximately 10–30% of the rice harvested globally. The fungus undergoes extensive developmental changes to be able to break into plant cells, build elaborate infection structures, and proliferate inside host cells without causing visible disease symptoms. From a molecular standpoint, we are still in the infancy of understanding how *M. oryzae* manipulates the host during this complex multifaceted infection. Here, we describe recent advances in our understanding of the cell biology of *M. oryzae* biotrophic interaction and key molecular factors required for the disease establishment in rice cells.

Keywords

Rice blast; appressorium; cytoplasmic effectors; apoplastic effectors

The impact of rice blast disease on rice production

Rice (*Oryza sativa*) is by far the most important staple food for more than half of the human population, providing approximately 19% of the daily calories consumed worldwide [1]. By 2050, the human world population is predicted to increase from nearly 7.6 to 9.8 billionⁱ. Thus, to keep up with future rice demands, rice producers must increase global rice yields by 25% before 2030ⁱⁱ. To support this increase, rice crops will face several future challenges that will seriously jeopardize its annual production. Among these challenges are fungal diseases threatening rice production that cause decreasing annual yields and increasing cultivation costs [2]. Rice blast disease represents a significant threat to rice production worldwide. The causal agent of this disease is the **hemibiotroph** (see **Glossary**) filamentous

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ⁱhttps://esa.un.org/unpd/wpp/Publications/Files/WPP2017_KeyFindings.pdf

ⁱⁱ<http://ricetoday.irri.org/the-importance-of-sustainable-rice-supply-systems/>

fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*), which annually contributes to the loss of enough rice to feed 60 million people [3]. Unfortunately, neither traditional breeding nor chemical approaches have been able to contain this disease, due to the fact that the fungus can rapidly adapt and mutate to evolve resistance to multiple rice varieties [3]. Despite outstanding efforts to understand *M. oryzae* biology and its interactions with the host, this pathogen continues to be a major threat to global food security [3]. Additionally, rice is not the only plant susceptible to this pathogen. Several *M. oryzae* **pathotypes** are also able to infect a wide variety of annual and perennial grass species including economically important cereal crops, such as wheat (Box 1), barley, and millet [2]. In this review, we focus on recent advances in our understanding of how *M. oryzae* undergoes different morphological changes in order to penetrate and invade rice cells. In particular, to gain a better understanding of how the fungus colonizes rice tissues, we highlight several aspects of the *Magnaporthe* life cycle and major key factors required for the establishment of rice blast disease. We do not discuss the biology of **avirulent** effector proteins in detail, because these have been reviewed recently [4].

Infection Stage, Part I: Host surface recognition and appressorium formation

Host cell contact

To be a successful pathogen, *M. oryzae* faces extensive developmental changes to colonize the plant tissues and complete its life cycle. Unlike other plant pathogens, *M. oryzae* has a hemibiotrophic life style, in which the fungus undergoes an initial **biotrophic** stage during which the plant immune system is suppressed, and then switches to a **necrotrophic** stage that promotes plant cell death. Once the three-celled asexual spores (known as conidia) settle on the leaf cuticle, the conidium releases an adhesive substance called spore tip mucilage from the conidial apex and attaches itself tightly to the hydrophobic plant surface (Figure 1) [5]. Furthermore, upon attachment, the conidium germinates and forms a polarized germ tube at one of the apical cells, and grows across the leaf surface (Figure 1). When the germ tube perceives physical cues, such as surface hardness and hydrophobic surface, the tip of the germ tube differentiates into a dome-shaped infection structure called the **appressorium** (Figure 1) [6–8]. As the appressorium matures, a thick layer of melanin deposition forms on the inner side of the appressorium cell wall providing an impermeable barrier to prevent the efflux of comparable solutes. At the same time, the conidium contents are recycled into the appressorium contributing to the next stages of appressorium development. Subsequently, a substantial internal **turgor pressure** of up to 8.0 MPa is generated in the appressorium, which is translated into a physical force acting at the base of the appressorium forming a **penetration peg** to rupture the rice cell cuticle (Figure 1) [9, 10]. During appressorial morphogenesis, *M. oryzae* accumulates high levels of endogenous reactive oxygen species (ROS) hence strengthening the appressorium cell wall [11]. This ROS accumulation is regulated by two *M. oryzae* NADPH oxidases, Nox1 and Nox2. Mutation on any of these Nox genes result defects in appressorium-mediated cuticle penetration. Once the appressorium matures, it remodels its actin cytoskeleton to form a toroidal F-actin network at the base of the cell, scaffolded by septins GTPases to promote the penetration peg emergence to invade plant tissue [12]. Remodeling of the F-actin cytoskeleton also requires

the action of Nox2 NADPH oxidase complex [13]. Following penetration of the leaf cuticle, the fungus rapidly colonizes and destroys the plant cells, generating necrotic lesions filled with spores to spread the diseases. To achieve this, *M. oryzae* utilizes different mechanisms to rapidly response to nutrient fluctuations and redox conditions inside the plant cell during infection (Box 2).

Translating the environmental cues via cyclic AMP and Pmk1 MAP kinase signaling pathways

The formation of a functional appressorium is an essential step in the infection cycle of *M. oryzae*. The infection begins when the conidium germinates on the leaf surface and the germ tube recognizes physical cues leading the fungus to develop an appressorium. The recognition of the physical cues is primarily regulated by highly conserved G-protein/cAMP-dependent signaling and the Pmk1 MAP kinase pathways (Figure 2) [8, 14, 15]. Upon conidia germination, Pth11 a bona fide G-protein-coupled receptor senses a hydrophobic surface and interacts with the G-proteins complex to mediate the activation of the cAMP signaling pathway (Figure 2) [20]. The Pth11 protein contains seven transmembrane regions and a CFEM (Common in several Fungal Extracellular Membrane protein) domain [21, 22]. Pth11 functions upstream of the cAMP signaling pathway in *M. oryzae* [20]. Interestingly, the Pth11 CFEM domain is required for the formation of appressorium and pathogenicity, implying that CFEM is the putative surface sensing function of Pth11 [23]. Moreover, ROS accumulation during appressorium formation is impaired in the *pth11* mutant strain. However, exposure to antioxidants induces appressorium formation in the *pth11* mutant strain on hydrophobic and *in planta* surface. *M. oryzae* contains three distinct G- α subunits: MagA, MagB and MagC as well as an adenylate cyclase, Mac1 [14, 16, 17]. Genetically, Rgs1 (Regulator of G-protein Signaling) modulates the G- α subunit MagA to enable *M. oryzae* to perceive and response to physical cues during appressorium formation [18]. PKA activity also is essential for vegetative growth and appressorium formation in *M. oryzae*. Two forms of PKA, cPKA and cPK2, have overlapping functions in cAMP cascade [14]. The generation of *cpkA cpk2* double mutants showed severe defects in growth and sporulation and failed to develop infection-related structures in *M. oryzae* [19]. Moreover, spontaneous suppressor of *cpkA cpk2* restores hyphal growth and appressorium formation on hydrophobic surface but not pathogenesis in *M. oryzae*. Interestingly, loss of function mutations in the transcription factor, MoSfl1, can bypass PKA activity to restores growth and appressorium formation in the *cpkA cpk2* mutant [19]. MoSfl1 interacts with the conserved Cyc8-Tup1 transcriptional co-repressor to block the transcription of target genes required for growth [19]. In general, phosphorylation of MoSfl1 by PKA disrupts MoSfl1-Cyc8-Tup1 interaction to regulate the expression of growth and infection-related genes [19].

A recent study identify endosomal compartments to function as signaling scaffolds that anchor the components of the G-protein/cAMP signaling in *M. oryzae* [22]. Upon surface sensing, the key activators and regulators, including Pth11, MagA, Mac1 and Rgs1, localized in the dynamic tubulo-vesicular endosomal compartments (Figure 2) [22]. Vps39 is a key member of the homotypic fusion and vacuole protein-sorting complex, essential for the conversion of early endosome into late endosomal compartments. Loss on *MoVPS39*

function disrupts the endosomal localization of the Pth11, MagA, Mac1, and Rgs1, cAMP signaling and inhibit appressorium development (Figure 2) [22]. Exogenous cAMP treatments restore appressorium formation on *vps39* mutant strain suggesting that functional Vps39 is essential for proper cAMP signaling and pathogenesis. Late endosomal scaffolding and trafficking of G-protein signaling components are vital for robust cAMP signaling leading to appressorium development in *M. oryzae* (Figure 2).

Mass spectrometry analysis identified a *M. oryzae* Ark1 (actin regulating kinase) interacting protein, MoEnd3, that is required for endocytosis transport and F-actin assembly (Figure 2). MoEnd3 function is linked to the internalization of Pth11 and the membrane sensor MoSho1 to the endosomal compartments [24]. Deletion of *MoEnd3* gene resulted in delays in endocytic transport and appressorium development [24]. Moreover, the authors demonstrate how MoEnd3 is involved in multiple cellular processes including autophagy, MAP kinase pathway and might has additional role in effector secretion during plant infection [24].

The Pmk1 MAP kinase pathway regulates late stages of appressorium formation, penetration and invasive growth (Figure 2) [14, 25]. Pmk1 is orthologous to the terminal kinase in the MAP kinase cascade, Fus3/Kss1 MAP kinases (MAPKs), found in the budding yeast *Saccharomyces cerevisiae* [26]. Loss in Pmk1 function results in an appressorium defective mutant that is non-pathogenic. Upstream to Pmk1, MAPK kinase (MEK) Mst7, MEK kinase (MEKK) Mst11 and an adaptor protein Mst50 have been identified [14, 27, 28]. Mst7 and Mst11 interact with Mst50, to activate the Pmk1 MAPK cascade. As expected, deletion of any of these genes upstream of Pmk1 result in impairments of appressorium formation and plant infection [27–29].

New evidence has revealed two cell-surface signaling mucins, Msb2 and Cbp1, promote the activation of Pmk1 MAPK pathway through the GTP-binding proteins Ras2 interaction [30]. The extracellular mucin domain region of Msb2 is required for appressorial formation whereas the cytoplasmic domain is essential for *M. oryzae* penetration and invasive growth [30]. Previously that it was demonstrated two Ras GTP-binding proteins, Ras1 and Ras2, play an essential role in Pmk1 activation by interacting with Mst11 and Mst50 (Figure 2) [28]. Contrary to Ras1 that displays no obvious phenotype, Ras2 has been proven essential for *M. oryzae* appressorium morphogenesis [29]. Interestingly, the generation of dominant active *RAS2* protein in *M. oryzae* resulted in improper activation of both Pmk1 MAPK and cAMP signaling pathways, and the formation of appressoria on non-inductive surfaces [29]. Therefore, constitutive expression of Ras2 enables the fungus to bypass the physical cues and attachment required for initial appressoria development. Moreover, the interaction of MEKK Mst11 with activated Ras2 and two phosphorylation events releases Mst11 from its self-inhibitory binding and activates the Pmk1 pathway [31].

Additionally, two other *M. oryzae* thioredoxin genes, *TRX1* and *TRX2*, have been found to play an important role in pathogenesis [32]. *TRX2* interacts with the Mst7 kinase and regulates the activation of the Pmk1 MAPK pathway for appressorium formation (Figure 2) [32]. Interestingly, both *TRX1* and *TRX2* are implicated in another signaling pathway that involves intra-cellular reactive oxygen species (ROS) signaling [32].

Both cAMP PKA and Pmk1 MAPK signaling pathways positively regulate appressorial morphogenesis and maturation. However, recent evidence has revealed a negative regulatory pathway of cAMP PKA and Pmk1 MAPK signaling that prevents appressorium formation in *M. oryzae* [33]. Marroquin and Wilson have demonstrated that the activation of **Target of Rapamycin (TOR)** signaling pathway in response to the accumulation of intracellular glutamine trigger the inhibition of appressorium development (Figure 2) [33]. The GATA transcription factor, Asd4, regulates intracellular glutamine levels and promotes appressorium formation. *asd4* mutant strains are unable to develop an appressorium on inductive surface, and contain high levels of glutamine [33]. Interestingly, the inhibition of TOR pathway by rapamycin restores appressorium formation in both *asd4*, *cPKA* mutants but not in the MAP Kinase *Pmk1* mutant [33]. Therefore, TOR inactivation requires a functional Asd4 to regulate intracellular glutamine levels and develop an appressorium in *M. oryzae*.

Cell cycle progression during appressorium formation

In addition to physical cue responses by cAMP and MAPK signaling pathways, *M. oryzae* utilizes essential cellular processes to ensure functional appressorial formation and maturation. *M. oryzae* conidia undergo a sequential cell cycle progression and autophagic cell death for appressoria development [34, 35]. The conidium has three nuclei, one of which migrates into the developing appressorium where it undergoes a single mitotic division (Figure 1). This process generates two daughter nuclei, of which one nucleus migrates into the developing appressorium and the other one migrates back to the conidium. Once the daughter nucleus translocates to the appressorium, a septum is formed between the germ tube and the appressorium structure [36]. Therefore, this daughter nucleus is the source of all genetic material during *in planta* infection. The initial appressorium formation is controlled by a cell cycle progression at the germ tube, in which DNA replication in the S-phase is required to initiate swelling at the germ tube tip [35]. Inhibition of DNA replication by either hydroxyurea (HU) or generation of a temperature sensitive *nim1* mutant prevent the germ tube tip from differentiating into appressorium [35]. It is now known that *M. oryzae* requires two independent S-phase checkpoints that ensure proper timing of initial appressorium morphogenesis and appressorium repolarization [37]. The first Sphase checkpoint is regulated by the protein kinase Cds1 involving the DNA damage response (DDR) pathway. HU treatments on *cds1* mutant result in defects on conidium cell death, and the formation of unmelanized appressorium. The second S-phase checkpoint take place at the appressorium, where it is vital for penetration peg formation and plant infection. This novel S-phase checkpoint is independent of DDR pathway and is regulated by turgor sensing and linked to melanin biosynthesis. Following S-phase, G2/M cell cycle checkpoints are required for maturation of the appressorium and subsequent plant cell infection [35]. Indeed, an inactivated TOR pathway is required to arrest the cell cycle at G2 and initiate both autophagy and appressorial development. Nutrients released from autophagy re-activate TOR signaling to reinitiate cell cycle progression through mitosis. Then the appressorial nuclei arrest at G1 because TOR signaling is inactivated again [38]. After appressorium formation, the three remaining nuclei in the conidium degrade, and the conidium dies via an autophagy process [34]. During this nuclear degradation, *M. oryzae* employs a nonselective macroautophagy mechanism to successfully infect the plant cell [39]. Subsequently, the

conidium contents are recycled into the appressorium contributing to the next stages of appressorium development.

In a recent report, a transcription factor for polarity control, TPC1, was identified and shown to be a key regulator of vegetative growth and appressorium-mediated plant infection processes in *M. oryzae* [40]. Tpc1 function is associated with the activation of the Pmk1 MAP kinase and Atg1 kinase signaling pathways. Interestingly, Tpc1 activation leads to the transcription of target genes required for autophagy, glycogen/lipid degradation and septin-mediated asymmetric reorganization of F-actin cytoskeleton to facilitate plant cell invasion [40].

Infection stage, part II: Under the radar and inside the leaf

Biology of the invasive growth

Once the melanized appressorium matures, the fungus breaches the host surface and develops a narrow penetration peg to enter into the first host cell (Figure 3, Key Figure). After penetration, the peg differentiates into thin filamentous primary **invasive hyphae (IH)** and grows inside the live rice cell. At this stage of infection, *M. oryzae* and the rice cell establish an intimate biotrophic association, in which the fungus switches from filamentous primary IH to bulbous intracellular IH that are surrounded by a plant-derived **extrinsic invasive hyphae-membrane (EIHM)** (Figure 3) [41]. This biotrophic association creates an enclosed **apoplastic** compartment between the intracellular IH and the rice cell membrane [41]. The bulbous IH grows inside the first cell and switches back to primary IH to move into uninfected adjacent cells, potentially through the host's **plasmodesmata** [41].

In planta live-cell imaging with fluorescently labeled proteins have become a valuable tool to track and visualize *M. oryzae* biotrophic invasion and the host cellular events occurring during pathogen colonization. For instance, high spatio-temporal resolution imaging studies demonstrate how *M. oryzae* appressorium remains mitotically active post-penetration and during IH proliferation [42]. Once the appressorial mitosis occur, a one new nucleus migrates into the swollen apex of the first bulbous IH, while the other nucleus remains in the appressorium (Figure 1) [43]. This cellular event leads to a septum formation in the filamentous region of the first IH [43]. The mitotic nucleus displays a remarkable constriction and elongation during migration through penetration peg [42]. Furthermore, a second round of appressorial mitosis was observed during second IH formation implying that the appressorium viability contributes the genetic material for IH proliferation. However, it remains to be elucidated whether the appressorial mitotic activity persists throughout the IH proliferation in the first infected cell and the adjacent cells. Another study developed a live cell imaging method using two fluorescent vital dyes, fluorescein diacetate (FDA, indicating green viable cells) and propidium iodide (PI, indicating dead red cells), in rice cells to gain new insights into the dynamics of host cell death during rice blast infection [44]. The authors demonstrated how the first infected rice cell loses viability once *M. oryzae* advances into adjacent cells [44]. Additionally, during biotrophic growth *M. oryzae* IH invaginates the host vacuole membrane causing vacuolar shrinkage [45]. However, early collapse of the host vacuole during early infection with *M. oryzae* has an effect on hyphal

growth. This study elegantly demonstrates that the host vacuolar integrity in the first infected rice cell is important for *M. oryzae* early stages of infection.

As the primary IH develops, a plant membrane-rich structure known as **biotrophic interfacial complex (BIC)** appears at the tip of the hyphae, where BIC is positioned at the differentiated bulbous IH (Figure 3A,B). As the fungus proliferates inside the first rice cell, BIC remains behind the bulbous IH and then appears again at the tip of the filamentous IH that will move into adjacent cells (Figure 3B, C) [46, 47]. At this biotrophic stage of infection, *M. oryzae* secretes a repertoire of effector proteins into rice cells to suppress plant immunity and manipulate host cell physiology for the next stages of infection (Figure 3) [48]. To date, many *M. oryzae* effectors have been identified, although the precise function of most of these effectors has not yet been determined (Table 1). Most fungal effectors are small-secreted proteins without known conserved domains or motifs and presumably have functional redundancy due to the lack of visible virulence phenotypes in single deletion strains [49]. Therefore, it remains as a challenge to predict their function inside the host cell. Despite the lack of sequence commonalities among fungal effectors, recent efforts to understand fungal effector function has revealed that 50% of the *M. oryzae* avirulence effectors and other fungal effectors belong to a new family of structurally conserved MAX effectors (*Magnaporthe* Avr and ToxB like) [50]. Interestingly, the vast majority of the *M. oryzae* MAX effectors are expressed during the biotrophic stage of infection.

All localization matter: cytoplasmic versus apoplastic effectors

Live-cell imaging of fluorescently labeled effector proteins has proven to be an outstanding tool for monitoring secreted proteins in living rice cells during early *M. oryzae* infections with minimal perturbation of their normal function [47, 51]. Indeed, fluorescently labeled blast effectors reveal two groups of effectors based on their localization *in planta*. Some effectors such as the known avirulent (Avr) effectors Avr-Pita, Avr-Pizt, Pwl1, Pwl2 as well as the secreted protein MC69 and the biotrophic-associated secreted (BAS) protein Bas1, are first accumulated in the BIC structure at the primary IH before their translocation into the rice cytoplasm (Figure 3) [52–54]. The native promoter region of these cytoplasmic effectors is responsible for BIC localization, but this activity is not linked to any obvious sequence motifs. Interestingly, once cytoplasmic effectors are translocated into the rice cells, some effectors accumulate around the host cell wall crossing points or they move into uninfected neighbor cells, presumably preparing the adjacent uninvaded host cell for further infection (Figure 3A, B). Fluorescence recovery after photobleaching has revealed that fluorescent cytoplasmic effectors such as Pwl2 and Bas1 are continuously secreted into the BIC while the bulbous IH grows elsewhere in the rice cell [52]. Intriguingly, BIC structures appear in every newly infected cell and accumulate cytoplasmic effectors. How this accumulation at the BIC mediates the delivery of *M. oryzae* effectors into the rice cytoplasm remains to be elucidated.

In contrast to cytoplasmic effectors, apoplastic effectors are not associated with the BIC structure. The apoplastic effectors such as the secreted LysM protein 1 (Slp1), Bas4 and Bas113 accumulate at the enclosed apoplastic matrix between the fungal cell wall and EIHM outlining the entire IH (Figure 3) [47, 51, 55]. For instance, Slp1 directly binds to chitin

oligosaccharides and competes with the plant receptor CEBiP to evade host recognition and subsequent chitin-triggered immune responses [55]. To achieve this, an α -1,3-mannosyltransferase, Alg3 mediates the N-glycosylation of the Slp1 effector, and this posttranslational modification is required to prevent host innate immunity [56]. In addition, the apoplastic effector Bas4 was observed to uniformly outline the bulbous IH, and also N-glycosylated by Alg3 [52, 56].

Using a transient expression assay, five apoplastic effectors in *M. oryzae*, named as MoCDIP1–5, were shown to induce plant cell death in rice protoplasts [57]. Applying similar transient expression approaches in *Nicotiana benthamiana* leaves, a recent study identified 11 suppressors of plant cell death (SPD) effectors from *M. oryzae* that were able to suppress plant cell death induced by necrosis-inducing protein 1 (Nep1) or the apoptosis regulator BAX protein [58]. The biological function of a *M. oryzae* specific gene, Required-for-Focal-BIC-Formation 1 (RBF1) was identified as a virulence gene essential for focal BIC formation during biotrophic growth [59]. Similar to cytoplasmic effectors, *RBF1* accumulates in the BIC structure and translocates into rice cytoplasm. However, loss of *RBF1* function resulted in dispersed BIC formation and low effector translocation efficiency [59]. In addition, the lack of *RBF1* caused not only a pathogenicity defect but also the inability to suppress host immune responses [59]. Notably, the focal BIC structure plays a critical role in the establishment of biotrophic growth to evade or suppress host defenses responses by secreting effectors into plant cell. However, understanding the molecular mechanism by which *M. oryzae* Rbf1 mediates the focal BIC formation will bring new clues on the mode of action of BIC and host immune suppression.

Secretion mechanisms during biotrophic stage

To understand effector secretion, a recent study demonstrated that *M. oryzae* has two distinct secretory pathways for effector proteins. In the first, cytoplasmic effectors preferentially accumulate at the BIC and are subsequently delivered into the plant cytoplasm (Figure 3) [51]. Targeted deletion of two genes in the exocyst complex, SEC5 and EXO70, in *M. oryzae* revealed significant loss of pathogenicity and inefficient secretion of these cytoplasmic effectors. In addition, a t-SNARE called Sso1 in *M. oryzae* is required for BIC development and pathogenesis [51]. This study provides evidence that cytoplasmic effectors require an unconventional pathway in the BIC for secretion that involve the exocyst complex and t-SNARE components. Moreover, live-cell imaging of cytoplasmic effectors fused to fluorescent proteins demonstrated that some effectors are not only translocated into the infected cell but also into neighboring uninfected cells [52].

Perturbations in the aforementioned pathway had no effect on the secretion of apoplastic effectors, indicating that these effectors use a second secretion pathway for delivery into the apoplastic matrix during biotrophic invasion. Indeed, treatments with brefeldin A, an inhibitor of conventional endoplasmic reticulum (ER)-Golgi secretion pathway, blocked apoplastic effector secretion into the apoplastic space without having an impact on BIC-mediated accumulation of cytoplasmic effectors [51]. These findings provide evidence that the fungus uses distinct pathways to secrete effector proteins during early stages of rice cell colonization.

Hormonal mimicking to modulate plant metabolism

Plant pathogens employ sophisticated strategies to disrupt hormonal homeostasis in order to create a suitable niche and facilitate nutrient uptake and colonization in plant tissues [60]. To achieve this many pathogens either target components of the phytohormone metabolism or synthesize compounds that can act as phytohormone mimics during infection to modulate plant physiology. For instance, *M. oryzae* secretes a monoxygenase, Abm, which hydroxylates endogenous free jasmonic acid (JA) into 12OH-JA to disable JA-based host innate immunity to facilitate pathogenesis [61, 62]. Moreover, the fungus also secretes the metabolite 12OH-JA during host penetration to subvert the plant immune responses [62]. In fact, it has been shown that loss of *ABM* function in the fungus induces host immune responses and inhibits plant colonization [62]. Fungi also produce hormonal compounds similar to plant hormones in order to hijack plant development and nutrient allocation process to promote sustained colonization and dissemination [60]. For example, a secreted cytokinin synthase 1, *Cks1*, has been shown to be essential for cytokinin (CK) biosynthesis and full virulence in *M. oryzae* [61]. Fungus-derived CKs likely contribute to nutrient fluxes and rice defense inhibition. The *cks1* mutants induce an early and strong transcriptional response of rice defense-related genes during infection [61]. Moreover, *M. oryzae* *Cks1* was clearly implicated with nutrient fluxes due to the inability of the *cks1* mutants to maintain key nutrient levels at the infection site [61]. Remarkably, in the presence of *cks1* mutant, the relative expression of rice CK-responsive genes were altered *in planta*, suggesting that *M. oryzae* CKs are likely perceived by the plant system during infection [61].

Concluding Remarks and Future Perspectives

Undoubtedly, rice blast disease remains a threat to global rice production and food security. With its remarkable biology, *M. oryzae* actively secretes a large repertoire of small-secreted proteins to suppress or evade host pathogen surveillance. Upon spore-leaf attachment, the fungus undergoes distinct morphological changes to develop an appressorium, generate IH and subsequently move the IH to adjacent cells. These changes are intrinsic to rice blast disease progression, but how the fungus maintains a biotrophic association and switches to a necrotrophic behavior remains unclear. Many questions remain open regarding *M. oryzae* biotrophic biology (see Outstanding Questions). Significant advances have been made in recent years to understand effector secretion during biotrophic interaction, revealing how *M. oryzae* deploys repertoires of effectors to suppress plant immunity and allow the fungus to propagate inside the host cell. Despite the outstanding discoveries on *M. oryzae* effector biology, our understanding on how these effectors are translocated into host cell is unknown. Elucidating the precise composition of apoplastic matrix between the IH and the plant membrane will improve our knowledge of the effector translocation mechanisms. Furthermore, the mechanism underlying effector-host defense suppression is not yet understood, and with this, the question of how *M. oryzae* biotrophic effectors suppress plant immune defenses to promote intracellular colonization is of particular interest. The use of biochemical approaches to detect protein-protein associations and protein complexes in living cells may facilitate the identification of host targets. Answers to these outstanding questions will provide us with clues to the general understanding of how pathogenic fungi successfully colonize plant tissues and subvert plant immunity.

Acknowledgments

This work was supported by the Welch Foundation grant I-1561 and Once Upon a Time...Foundation. JF was funded by the Microbiology Training (grant T32 AI007520-17) and the Once Upon a Time... Foundation. K.O. is a Burroughs Wellcome Investigator in Pathogenesis of Infectious Disease, a Beckman Young Investigator, and a W. W. Caruth, Jr., Biomedical Scholar and has an Earl A. Forsythe Chair in Biomedical Science. We thank the members of the Orth lab for thoughtful discussion and revision of this manuscript. We are very thankful to Dor Salomon and Richard A. Wilson for their critical review of our manuscript.

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Glossary

Apoplastic (also known as apoplast)

Extracellular space outside of the plant cell membrane. It facilitates transport of water and solutes across the cell wall to different plant tissues.

Appressorium

A specialized infection structure formed at the tip of the germ tube that many plant pathogenic fungi use to penetrate the plant cuticle and colonize the host cell.

Avirulence

Effector proteins that are recognized by host cognate resistance (R) proteins

Biotrophic Interface Complex (BIC)

A membrane rich complex in which the fungus accumulates a specific group of secreted proteins.

Biotrophic

Refer to a fungal pathogen that has a close relationship with the host and requires living plant tissue to survive. Biotrophs suppress host immune systems and derive nutrients from living host cells.

Extrainterspersive hyphal membrane (EIHM)

A plant membrane that surrounds the fungal cell wall.

Hemibiotroph

A pathogenic organism that initially establishes a close biotrophic association with the host without causing any symptoms that later turns into necrotrophic association, resulting in death of the plant cells.

Invasive hyphae (IH)

Fungal cells that develops during host infection, and grows and proliferates inside the plant cell.

Necrotrophic

Refer to a plant pathogen that colonizes host tissues by secreting toxins and enzymes that kill the living plant cells, followed by acquisition of nutrients from the dead and dying host tissues.

Pathotypes

A fungal pathogen that is distinguished from others of the same species by its specificity for a specific host.

Penetration Peg

A specialized fungal hyphae that the fungus uses to pierce the host plant surface. It is formed at the base of the mature appressorium.

Plasmodesmata

Small channels that connect the plant cytoplasm of neighboring cells. These channels facilitate the movement of molecules and communication between the cells

Target of Rapamycin (TOR)

Signaling pathway responsible for cell metabolism, growth, proliferation and survival under favorable conditions.

Turgor pressure

A hydrostatic force generated by the accumulation of solutes in a cell.

Box 1: Wheat Blast - potential threat to global wheat production

The *Magnaporthe oryzae Triticum* pathotype (MoT) is the causal agent of wheat blast or 'brusome' disease. This disease was first identified infecting wheat plants in the Paraná State of Brazil in 1985 [63]. Soon after, the pathogen spread to other wheat-producing regions of Brazil, and neighboring countries such as Bolivia, Paraguay and Argentina (see review [64]). The first wheat blast outbreak outside of South America was detected in Bangladesh in February 2016 [65]. The disease originates from a *M. oryzae* subpopulation that infects *Triticum* species (wheat) but not rice plants. This fungus can infect all above-ground tissues of wheat plants. However, the most obvious and damaging symptom of wheat blast infections is the bleaching of the spike. It occurs when the fungus attacks rachis at the base of the wheat heads, thereby blocking the transport of nutrients to the upper head parts resulting in the bleached appearance and hindrance of seed development [66]. Moreover, MoT is recognized as a seedborne pathogen, meaning that it can spread through infected seeds [64]. Therefore, wheat blast disease is a major threat to wheat production because the pathogen can severely infect wheat heads and result in serious yield losses-up to 100% in susceptible varieties [67]. The continued spreading of MoT to new wheat-growing regions is particularly worrisome because this disease is a potential threat to global wheat production and there is lack of effective disease control. Therefore, the need for a better understanding of the wheat blast biology and epidemiology is a top priority for the blast research community. Recently, researchers sought to understand the origin of this aggressive pathogen in 11 districts in Bangladesh, confirming that the outbreak resembles that of the South American wheat blast isolates [68, 69]. Moreover, a recent study revealed the key reasons why the blast fungus jumped hosts, from oat or perennial ryegrass to wheat, in Brazil. Genetic studies identified two Avr effectors, PWT3 and PWT4, from ryegrass and oat fungal isolates, which elicit a rapid host response in wheat plants containing resistance genes *rwt3* and *rwt4* [70]. In the early 1980s, a new high-yield wheat cultivar lacking the cognate resistance gene to Avr effector PWT3 was introduced into Brazil, allowing certain fungal isolates to colonize wheat plants. Wheat cultivars still carrying the cognate resistance *pwt3* gene in nearby areas imposed selective pressure that shaped the current genetics of *M. oryzae* PWT3-containing isolate, resulting in a new nonfunctional PWT3 strain [70]. These events prompted the emergence of a wheat blast outbreak a few years thereafter in Brazil. Thus, the use of wheat cultivars possessing both the Rwt3/Rwt4 is essential for reoccurrence of host jumps or for wheat blast disease prevention.

Box 2. Metabolic strategies employed by *M. oryzae* in planta

Once *M. oryzae* spores germinate on the leaf cuticle, the germ tube constantly monitors its environment for physical cues to generate infection structures to colonize plant tissues. At this point of infection, the fungus actively moves from the nutrient-poor leaf surface conditions to the nutrient-rich conditions inside the plant cell. How does *M. oryzae* cope with nutrient fluctuations during plant infection? Sugar sensing may enable the fungus to respond rapidly to nutrient availability in the environment. Indeed, trehalose-6-phosphate synthase (TPS1) plays a pivotal role in glucose-6-phosphate (G6P) sensing. Tps1 is a biosynthetic enzyme required for the production of the non-reducing disaccharide trehalose from G6P and uridine diphosphate (UDP)-glucose [71–73]. Additionally, Tps1 regulates gene expression in response to G6P availability during *M. oryzae* infection [72, 74]. Previous studies demonstrated that Tps1 is required for *M. oryzae* pathogenesis due to G6P binding independent of its trehalose production [71, 75]. In response to G6P, Tps1 activates glucose-6-phosphate dehydrogenase (G6PDH) to generate high levels of NADPH in the oxidative stage of the pentose phosphate pathway (PPP). This in turn leads to the activation of different NADPH-requiring genes, some of which are involved in nitrogen metabolism, pathogenicity via NADPH metabolism, and repression of genes required for alternative carbon sources [72]. Furthermore, NADPH levels lead to the activation of several GATA factors including Nut1, a nitrogen regulator in *M. oryzae*, and to G6P displacement from the Tps1 active site [72]. Notably, Tps1 acts as a central regulator of G6P sensing to manipulate NADPH levels, leading to rapid genetic responses that will enable *M. oryzae* to adapt and grow in such a harsh environment like the host cell. A recent study provided evidence that G6P/Tps1 sensing and NADPH production are essential for both glutathione and thioredoxin antioxidant systems during *M. oryzae* biotrophic growth [76]. Another study demonstrated how glucose metabolism through the transketolase enzyme, encoded by *TKL1*, is essential for *M. oryzae* invasive growth and cell-to-cell movement during infection [77]. Loss of *TKL1* in *M. oryzae* has severe effects on ATP levels, IH growth and cell cycle progression *in planta* [77]. Addition of exogenous ATP restores IH growth and reverses mitotic delay in *tkl1* mutant strains during infection. This supports the notion that glucose metabolism through the activation of the non-oxidative PPP to generate ATP and NADPH is an important process for *M. oryzae* biotrophic growth and infection. Interestingly, connections have been made between primary metabolism and suppression of host plant defenses during rice blast infections [78]. The sugar sensor Tsp1 regulates the expression of nitronate monooxygenase 2, encoded by the *NMO2* gene, which catalyzes the oxidative denitrification of nitroalkanes. Loss of *NMO2* resulted in strains severely attenuated in IH growth, unable to grow under nitrate and nitrite-containing media, and highly susceptible to nitrooxidative stress conditions and plant immune responses [78]. In general, *M. oryzae* uses *NMO2* to protect itself against nitrooxidative stress conditions, and to maintain redox balance by suppressing the first line of plant defenses to avoid plant recognition during rice blast infection.

Outstanding questions

- What other processes are involved in conidia cell death and degradation during appressorial development?
- Once inside the plant cell, how does *M. oryzae* maintain host cell membrane integrity during early biotrophic infection stage?
- How do *M. oryzae* effectors translocate from the IH into the host cytoplasm?
- Does *M. oryzae* deliver the effector proteins by extracellular vesicles such as exosomes into apoplastic interface? How is this accomplished? How is effector secretion regulated?
- How does the fungus manipulate the plant plasmodesmata channels to move from cell-to-cell?
- How are cytoplasmic effectors delivered into the BIC structures? Is the translocation mechanism BIC-effector accumulation dependent?
- How do *M. oryzae* effectors suppress basal plant defenses during biotrophic growth?
- What are the main signaling pathways that effectors proteins target, and how does this contribute to the infection process?
- What are the intracellular cues that promote the fungus to switch from biotrophic growth to necrotrophic behavior

Highlights

- The internalization and transport of the G-protein signaling components to late endosomal compartments plays a critical role during appressoria development in rice blast disease.
- TOR pathway activation by intracellular glutamine levels negatively regulate appressorium development.
- Rice blast fungus has evolved two secretion mechanisms to deliver effector proteins into the host cell during biotrophic growth.
- *M. oryzae* disrupts hormonal homeostasis to evade or suppress host defenses during plant cell invasion.

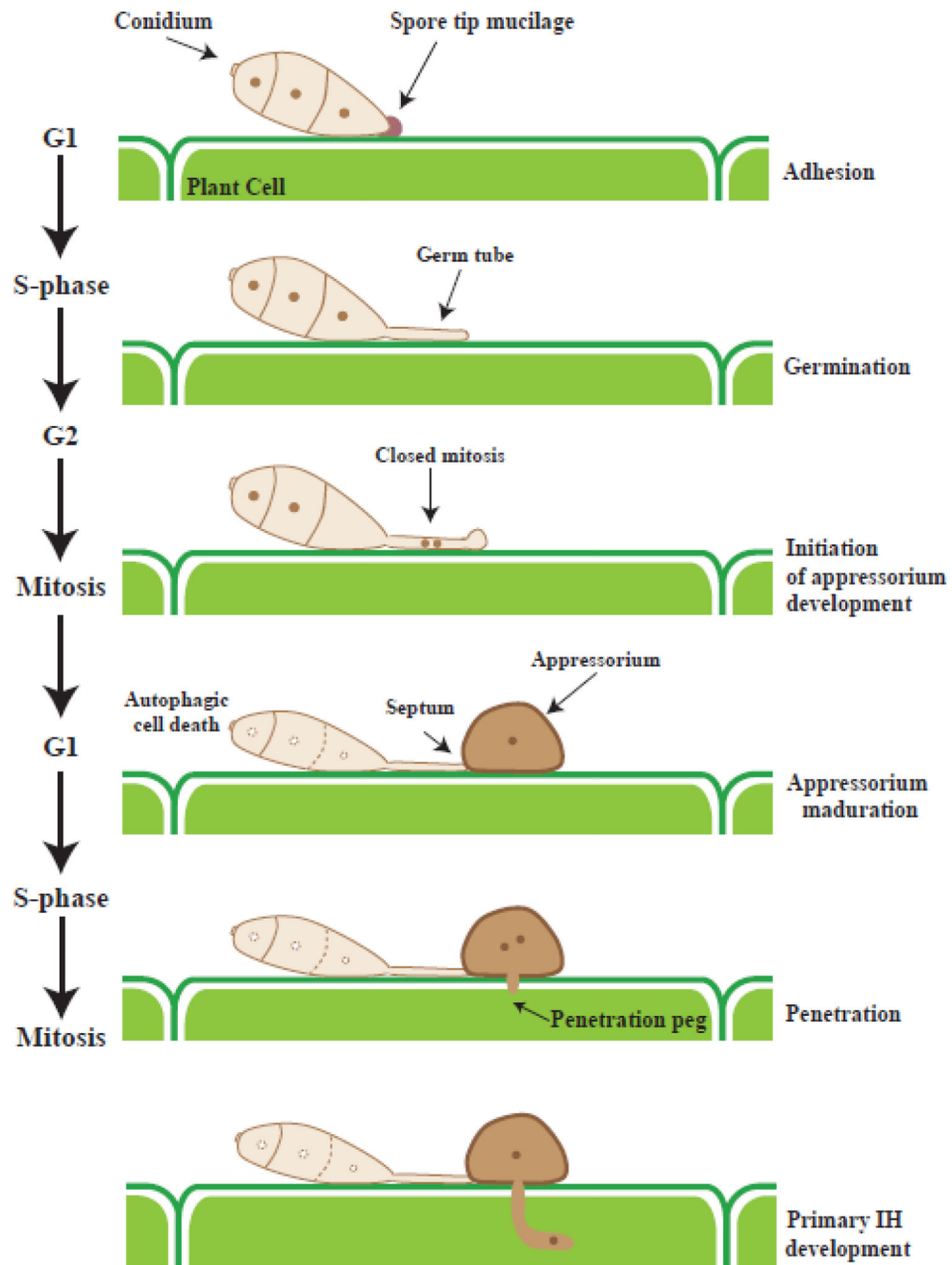


Figure 1. Schematic representation of cell cycle progression during appressorium development in the rice blast fungus *M. oryzae*. The fungus three-celled conidium adheres to the plant hydrophobic cuticle and germinates. Each cell contains one nucleus. One of the nuclei migrates to the germ tube and goes through a single mitotic event, in which two daughter nuclei are generated in a closed mitosis. On daughter nucleus migrates into the immature appressorium and a septum is formed, while the other nucleus migrates back to the conidium, which then goes through an autophagic cell death process. Appressorium remains

mitotically active after maturation. Brown circles: nucleus, dashlines: conidium degradation, HI: invasive hyphae.

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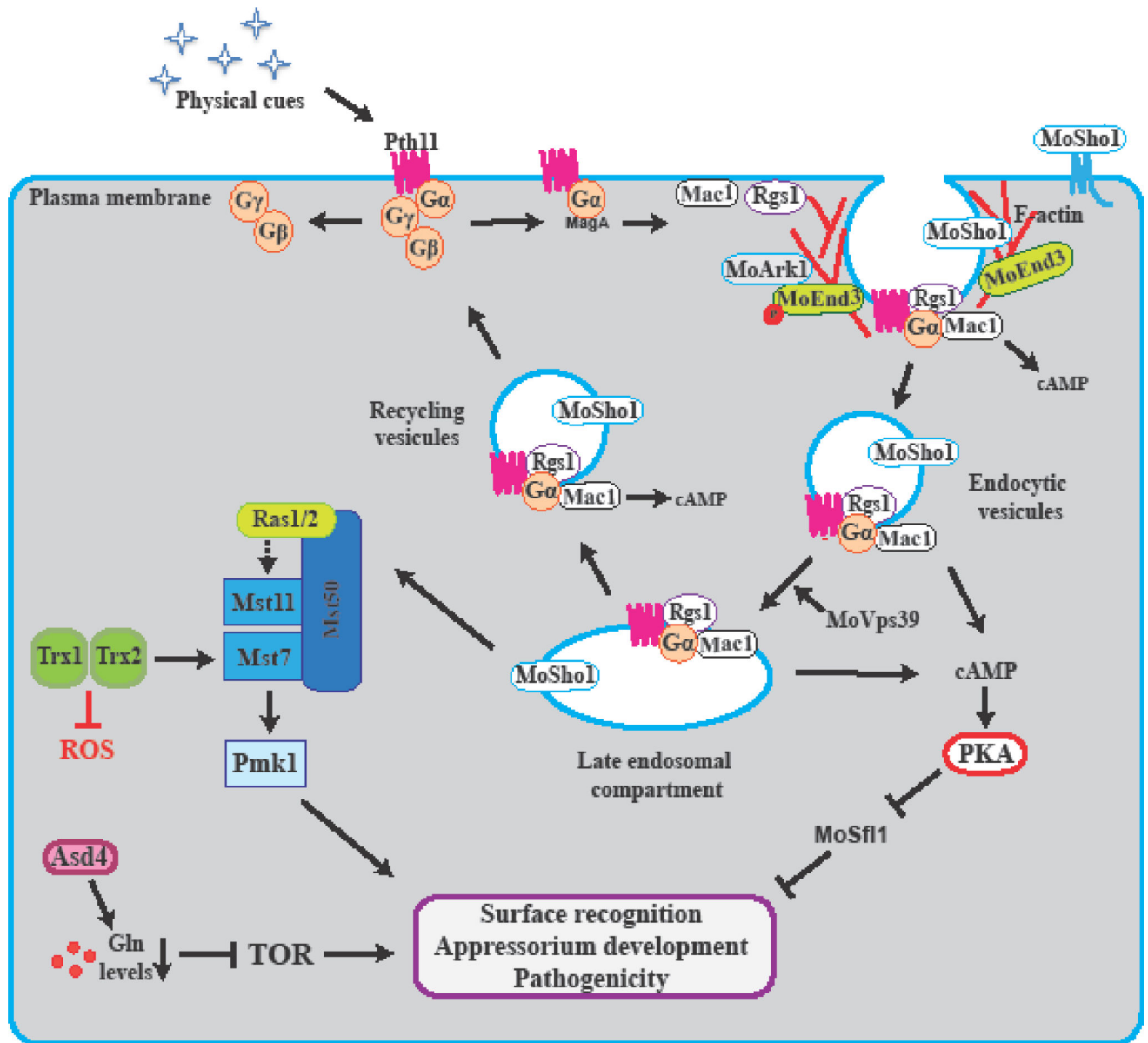


Figure 2. Schematic representation of the spatio-temporal cell dynamics of the G-protein/Pth11 and cAMP-signaling pathway during surface sensing and appressorium morphogenesis. Upon conidia germination, the key activators and regulators of G protein/cAMP (Pth11, membrane sensor MoSho1, G-proteins, Rgs1, and Mac1) are internalized to endosomal compartments leading to the accumulation of cAMP and subsequently PKA activity. MoEnd3 mediates the receptors Pth11 and MoSho1 endocytosis. MoArk1 negatively regulates MoEnd3 through protein phosphorylation. Mst50 interacts with Mst11/Mst7 and Ras1/2 proteins to activate the Pmk1 MAPK cascade and regulate appressorium development, penetration and pathogenicity. Asd4 acts downstream to cPKA, maintains intracellular glutamine levels in the cell, and promote appressorium formation. TOR signaling pathway inhibits the

appressorium formation under conditions of high glutamine levels or in the presence of glucose. Diagram modified from [24

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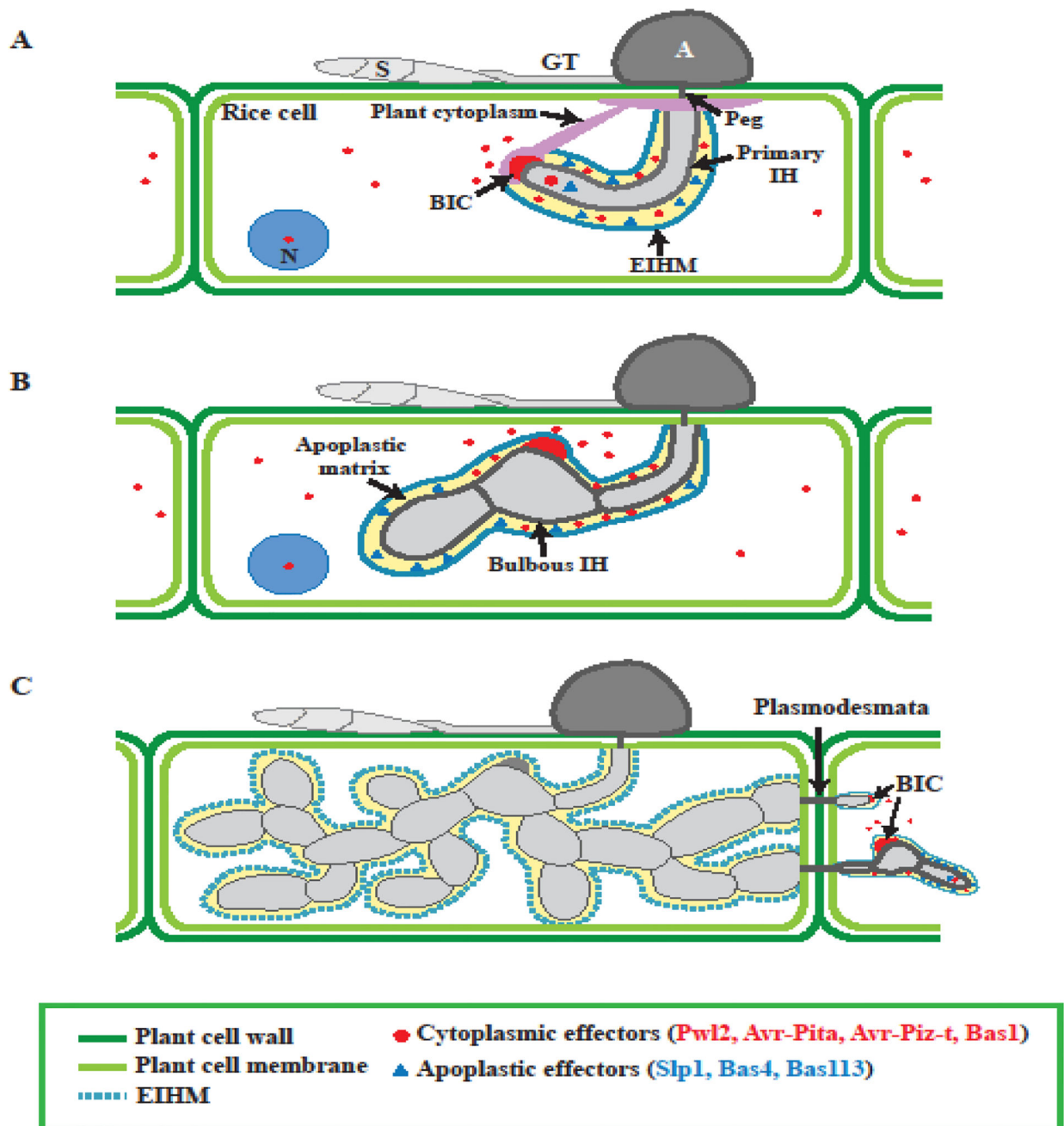


Figure 3.

BIC development and effector secretion during biotrophic growth. These illustrations represent the growth of the invasive hyphae (IH) from 22–40 hour post inoculation inside rice cells. (A) BIC develops at the tip of the primary filamentous invasive hyphae (IH). (B) BIC localizes at a subapical position in the first bulbous IH, and remains behind as the fungus grow in the rice cell. (C) New BICs are formed in the IH tip in the adjacent cells. Cytoplasmic effectors (red circles), including *Pwl2*, *Avr-Pita*, *Avr-Piz-t* and *Bas1*, accumulate at the BIC and are secreted into the host cytoplasm. In contrast, apoplastic effectors (blue triangles), including *Slp1*, *Bas4* and *Bas113* are secreted into the apoplastic

matrix between EIHM and fungal cell wall. Abbreviations: S, Spores; GT, Germ tube; A, Appressorium; N, Nucleus; BIC, Biotropic interface complex; IH, Invasive hyphae; EIHM, Extraintvasive invasive hyphae membrane

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Table 1

M. oryzae effector proteins identified to date.

Effector name	ID number	Size ^a	Function/Expression/Localization ^b	R ^c	References	
Avirulent effectors						
AVR-Pita	AF207841	224	Zinc metalloprotease/ <i>In planta</i> / BIC; Cytoplasm	Pita	[47,79–81]	
AVR-Piz-t	HE578813	108	Target rice U3 ubiquitin ligase; reduces Fig-22 and chitin induced ROS production/ <i>In planta</i> /Cytoplasm	Piz-t	[53]	
AVR-CO39	AF463528	89	Interact with RGA4/RGA5/ <i>In planta</i> /Cytoplasm	CO39	[82,83]	
AVR-Pia	AB498873	85	-/ <i>In planta</i> /Cytoplasm	Pia	[84]	
AVR-Pii	AB498874	70	C2H2 Zinc finger motif/ <i>in planta</i> /Cytoplasm	Pii	[84]	
AVR-Pik/km/xp	AB498875-AB498879	113	-/ <i>In planta</i> /Cytoplasm	Pik	[84]	
AVR-Pib	KM887844	74	-/ <i>Cytoplasm</i> predicted	Pib	[85]	
AvrP19	MGG_12655	91	-/ <i>In planta</i> /BIC; Cytoplasm	P19	[86]	
PWL1	AB480169	147	-/ <i>In planta</i> /BIC; Cytoplasm	-	[87]	
PWL2	MGG_04301	145	-/ <i>In planta</i> /BIC; Cytoplasm	-	[52,87–89]	
ACE1	AJ704622	4035	Polyketide-synthase-peptide synthetase/Appressorium penetration/Appressorium; Cytoplasm	Pi33	[90]	
AVR-Pi54	MGG_01947	153	-/ <i>In planta</i> /Appressorium	Pi54	[91]	
Secreted proteins						
SLP1	MGG_10097	162	LysM domains, binds to chitin oligosaccharides; suppresses chitin-induced immunity in rice/ <i>In planta</i> /Apoplast	-	[55]	
MC69	MGG_02848	54	-/ <i>in planta</i> /Apoplast	-	[54]	
MSPI	MGG_05344	137	Cerato-platanin family; triggers autophagic cell death and elicits host defense responses/ <i>In planta</i> /Apoplast	-	[92]	
Biotrophy-associated secreted proteins						
BAS1	MGG_04795	115	-/ <i>In planta</i> /BIC; Cytoplasm	-	[46,52]	
BAS2	MGG_09693	102	-/ <i>In planta</i> / BIC; Cell wall crossing points	-	[46,52]	
BAS3	MGG_11610	113	-/ <i>In planta</i> /BIC; Cell wall crossing points	-	[46,52]	
BAS4	MGG_10914	102	-/ <i>In planta</i> /EIHM	-	[46,52]	
BAS 107	MGG_10020	132	-/ <i>In planta</i> /BIC; Cytoplasm	-	[89]	
Group of predicted effector proteins						
IUGs	Isolate unique genes; Suppression of SA and ET signaling/ <i>In planta</i> /BIC; Cytoplasm					[93]
MoHEGs	<i>M. oryzae</i> hypothetical effector genes; suppress plant defenses; virulence function in Barley plants/ <i>In planta</i> /Appressorium					[94]

Effector name	ID number	Size ^a	Function/Expression/Localization ^b	R ^c	References
MoCDIPs			Induced plant cell death in rice protoplasts/ <i>In planta</i> /Apoplast		[57]
SDPs			Suppression of plant host defenses/ <i>In planta</i> /Apoplast and cytoplasm		[58]

^a Indicates the number of amino acids residues of the predicted protein.

^b Inside the host plant cell upon rice blast infection.

^c Cognate resistance protein in rice plants.

* *In planta*: expression during biotrophic growth; - unknown function or host interactor.