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Research review

Effector-triggered susceptibility by the rice blast fungus *Magnaporthe oryzae*

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Summary

Rice blast, the most destructive disease of cultivated rice world-wide, is caused by the filamentous fungus *Magnaporthe oryzae*. To cause disease in plants, *M. oryzae* secretes a diverse range of effector proteins to suppress plant defense responses, modulate cellular processes, and support pathogen growth. Some effectors can be secreted by appressoria even before host penetration, while others accumulate in the apoplast, or enter living plant cells where they target specific plant subcellular compartments. During plant infection, the blast fungus induces the formation of a specialized plant structure known as the biotrophic interfacial complex (BIC), which appears to be crucial for effector delivery into plant cells. Here, we review recent advances in the cell biology of *M. oryzae*–host interactions and show how new breakthroughs in disease control have stemmed from an increased understanding of effector proteins of *M. oryzae* are deployed and delivered into plant cells to enable pathogen invasion and host susceptibility.

I. Introduction

The filamentous ascomycete fungus *Magnaporthe oryzae* (synonym of *Pyricularia oryzae*) threatens global food security by causing devastating blast diseases on rice, millets, and most recently wheat (Gladieux *et al.*, 2018; Asibi *et al.*, 2019; Valent *et al.*, 2020). In rice alone, the fungus causes losses of *c.* 10–30% of the global rice production (Nalley *et al.*, 2016). The amount of rice destroyed by this disease is sufficient to feed 60 million people annually, at a cost of $66 billion (Pennisi, 2010; Nalley *et al.*, 2016). To durably control blast disease, the mechanisms by which the blast fungus colonizes host plants need to be understood. To colonize a host plant, *M. oryzae* must overcome two major layers of plant immunity (Oliveira-Garcia & Valent, 2015). The first layer involves cell surface pattern recognition receptors (PRRs) that detect conserved pathogen-associated molecular patterns (PAMPs) to trigger PAMP-triggered immunity (PTI; Jones & Dangl, 2006), while the second layer involves intracellular host resistance (R) immune proteins that recognize pathogen AVR (avirulence) effectors directly or indirectly, to trigger effector-triggered immunity (ETI; Jones & Dangl, 2006; Saur *et al.*, 2019). AVR-effector recognition in blast disease occurs through cytoplasmic nucleotide-binding domain leucine-rich repeat (NLR)-type immune receptors leading to effector-triggered immunity in a classical gene-for-gene interaction (Zipfel, 2014; Boutrot & Zipfel, 2017; Roudaire *et al.*, 2020). To overcome these two layers of immunity, *M. oryzae* secretes a battery of effector proteins that elude or suppress pathogen surveillance systems and hijack cellular processes (Yan *et al.*, 2023). Effectors typically exhibit a high level of genetic diversity due to an 'arms race'-like evolutionary dynamic between host and pathogen (Kanzaki *et al.*, 2012; Oliveira-Garcia *et al.*, 2021).

A recent study of gene expression of the blast fungus during rice infection using high-resolution transcriptional profiling provided an indication of the extent of differential fungal gene expression necessary to cause blast disease (Yan *et al.*, 2023). The pathogen transcriptome changes significantly during infection with major shifts in expression of genes involved in both primary and secondary metabolism, cell signaling, and transcriptional regulation. Fungal co-expression modules identified during plant infection revealed co-regulation of genes that encode effector proteins. These temporally co-regulated effectors possess structural similarity, even though sequence unrelated (Yan *et al.*, 2023). How effectors are temporally co-regulated is likely to be critical for

disease progression and a recent study reported a forward-genetic screen, which identified mutants showing constitutive effector gene expression. This screen identified *RGS1* as a key regulator of effector gene expression (Tang *et al.*, 2023). Rgs1 is a regulator of appressorium development, but also represses effector gene expression before plant infection. This genetic screen could lead to the discovery of further novel regulators of effector gene expression and reveal the hierarchical regulatory networks that control effector expression. The blast fungus shows orchestrated control of numerous effector-encoding genes during fungal biotrophic invasion, but the function of the overwhelming majority of effectors remains unknown (Giraldo *et al.*, 2013; Oliveira-Garcia & Valent, 2015). Large-scale screening to identify effector targets in the host plant will therefore be necessary to further reveal precisely how they suppress immunity so effectively during rapid tissue colonization (Oliveira-Garcia & Valent, 2015).

Much of our current knowledge of effector function comes from targeted mutagenesis and live cell imaging (Redkar *et al.*, 2015; Park *et al.*, 2016; Sakulkoo *et al.*, 2018). Live-cell imaging has demonstrated that fungal effectors are delivered to the apoplast, the gap between the fungal cell wall and host plasma membrane, or targeted to the host cell cytoplasm during infection (Mosquera *et al.*, 2009). Apoplastic effectors protect pathogens from recognition by inhibiting enzymes or scavenging molecules that trigger extracellular immune receptors (Hückelhoven & Panstruga, 2011; Sperschneider *et al.*, 2018). Cytoplasmic effectors, meanwhile, are translocated into host cytoplasm where they target cellular compartments to suppress immunity and manipulate host metabolism and signaling to facilitate fungal proliferation (Robin *et al.*, 2018). In this review, we focus on new insights into the mechanism of effector delivery and effector-triggered susceptibility during biotrophic invasion by *M. oryzae*. We emphasize how characterization of the repertoire of fungal effectors will be vital to develop new disease control strategies.

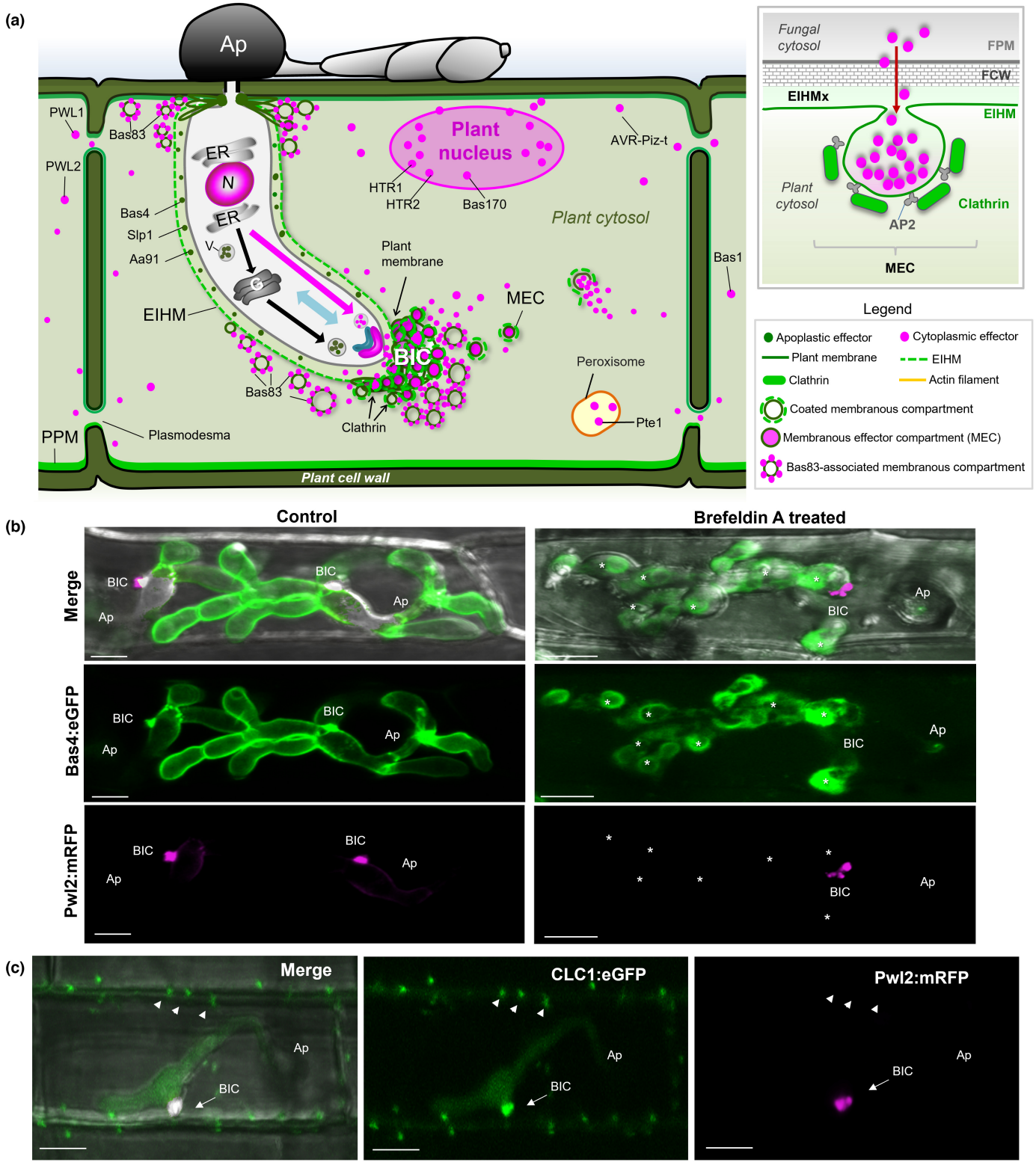
II. How does *M. oryzae* secrete effectors *in planta*?

Effector secretion is key to understanding biotrophic fungal invasion (Giraldo & Valent, 2013; Oliveira-Garcia *et al.*, 2023). *M. oryzae* possesses at least 548 genes predicted to encode effectors (Yan *et al.*, 2023). The presence of an N-terminal signal peptide remains a crucial criterion for effector identification (Mosquera *et al.*, 2009; Yan *et al.*, 2023), but algorithms such as EffectorP and deepredeff have been developed to synthesize characteristics of known effectors, such that more accurate predictions can be made (Kristianingsih & MacLean, 2021; Sperschneider & Dodds, 2022). When considered with other diagnostic traits, such as differential expression in plant tissue and localization to the host-derived biotrophic interfacial complex (BIC), the confidence in effector prediction in *M. oryzae* rises further, although experimental validation has been carried out in very few cases (Yan *et al.*, 2023).

M. oryzae employs two distinct secretion systems to deliver cytoplasmic and apoplastic effectors (Giraldo *et al.*, 2013). Secretion of apoplastic effectors is mediated through the endoplasmic reticulum (ER)–Golgi-dependent secretory pathway (Fig. 1a), which has long been considered the conventional secretion pathway in filamentous fungi. Cytoplasmic effectors, however, are secreted from invasive hyphae and accumulate in the BIC, a membrane-rich structure derived from the extrainvasive hyphal membrane (EIHM) that surrounds the invading fungus (Oliveira-Garcia & Valent, 2015). Secretion of cytoplasmic effectors is insensitive to Brefeldin A (BFA), which blocks Golgi-dependent secretion of apoplastic effectors (Fig. 1a,b; Giraldo *et al.*, 2013; Oliveira-Garcia & Valent, 2021).

Fluorescence recovery after photobleaching (FRAP) analysis has demonstrated that cytoplasmic effectors are continuously secreted into BICs even in the presence of BFA, providing evidence for Golgi-independent secretion of cytoplasmic effectors (Giraldo *et al.*, 2013; Oliveira-Garcia & Valent, 2021). Cytoplasmic effectors are therefore secreted by a nonconventional secretion pathway (Fig. 1b), but this involves exocyst components, such as Exo70 and Sec5, as well as SNARE proteins. Exo70, Sec5, and Sso1 deletion mutants of *M. oryzae* show abnormal accumulation of cytoplasmic effectors in biotrophic hyphae and reduced virulence on rice, highlighting the importance of the effector repertoire in fungal virulence (Giraldo *et al.*, 2013). Interestingly, oomycete effectors destined for delivery into the cytoplasm are also secreted

Fig. 1 Secretion and translocation of *Magnaporthe oryzae* effectors into rice cells. (a) This illustration represents the biotrophic interfacial complex (BIC) at the tip of primary hyphae (PH) at 22–24 h postinoculation (h.p.i.) delivering effectors into rice cell cytoplasm. Apoplastic effectors (green circles), including Bas4, Slp1, and Aa91, are secreted from the PH via Golgi-dependent secretion, and accumulate in the apoplast enclosed by the EIHM (green dashed line around the PH). By contrast, cytoplasmic effectors (magenta circles), including Pwl2, Pwl1, and Bas1, are secreted into BICs by a nonconventional secretion system involving the exocyst and SNARE protein Sso1 (magenta and blue curved structures). Cytoplasmic effectors are packaged in vesicles inside BICs and translocated via clathrin-mediated endocytosis (CME). The effector Bas83 binds to vesicles, possibly recruiting more host membrane to the BIC. Many cytoplasmic effectors, such as Bas1, Pwl2, and Pwl1, move through plasmodesmata into surrounding host cells to prepare them for invasion. Bas170, HTR1, and HTR2 are translocated from cytoplasm to nuclei and Pte1 to peroxisomes, where they reprogram specific cell processes associated with suppression of immunity. Key: Ap, appressorium; ER, endoplasmic reticulum; G, Golgi apparatus; N, fungal nucleus; PPM, plant plasma membrane; V, transport vesicle. Right insert shows a working model for *M. oryzae* effector internalization via CME. AP2, Adaptor Protein-2 complex; EIHMx, extrainvasive hyphal matrix; FCW, fungal cell wall; FPM, fungal plasma membrane. (b) Brefeldin A (BFA) blocks secretion of apoplastic effectors but not of cytoplasmic effectors. The cytoplasmic effector Pwl2:mRFP (magenta) shows BIC localization and Bas4:eGFP (green) shows apoplastic localization. In the presence of BFA, Bas4:eGFP (green) is retained in the fungal ER (asterisks), but Pwl2:mRFP remains BIC-localized, imaged with the same transformant 3 h after BFA treatment. Control: infected cell treated with 0.1% DMSO. (c) CME marker OsCLC1:eGFP (green) co-localizes with the cytoplasmic effector Pwl2:mRFP (magenta) in BICs in rice cells. (b, c) All images (bright field merged with mRFP and eGFP and mRFP and eGFP alone) are projections of confocal optical sections. Bar, 10 μm.



by an unconventional BFA-insensitive mechanism (Wang *et al.*, 2017).

A recent report has demonstrated that the unconventional secretion of cytoplasmic effector in *M. oryzae* depends on tRNA modification and codon usage patterns (Li *et al.*, 2023a). Functional characterization of the Uba4–Urm1 sulfur relay system mediating tRNA anticodon wobble uridine 2-thiolation (s^2U_{34}) demonstrated that loss of s^2U_{34} eliminates the translation of

AA-ending codon-rich mRNAs encoding cytoplasmic effectors, but mRNAs encoding apoplastic effectors are unaffected (Li *et al.*, 2023a). U_{34} thiolation and codon usage therefore modulate pathogen secretion of cytoplasmic effector in host rice cells (Li *et al.*, 2023a).

III. How does *M. oryzae* internalize effectors into the host cell cytoplasm?

The mechanisms by which fungal plant pathogens deliver effectors across the host plasma membrane into living host cells have remained largely unknown despite extensive study (Panstruga & Dodds, 2009; Giraldo & Valent, 2013; Lo Presti & Kahmann, 2017). Translocation motifs, such as the RXLR motif, have been identified for oomycete effectors, but cell entry motifs for fungal effectors have not been identified. The RXLR motif was originally shown to bind phosphatidylinositol-3-phosphate (PI3P) in the host membrane (Kale *et al.*, 2010). This assay is controversial, however, and could not be repeated by others (Yaeno *et al.*, 2011; Wawra *et al.*, 2013). Moreover, the RXLR motif from *P. infestans* effector AVR3a is cleaved before secretion (Wawra *et al.*, 2013), and hence, the function of the RXLR motif in cell entry remains unclear (Trusch *et al.*, 2018). Effectors might be translocated into plant cells via specialized translocon complexes in the plasma membrane. Recently, for example, a stable protein complex comprised of five unrelated fungal effectors and two fungal membrane proteins has been implicated in effector translocation by the corn smut fungus *Ustilago maydis* (Ludwig *et al.*, 2021). Despite these findings, the question of how effectors are taken up into plant cells remains largely unresolved in eukaryotic plant pathogens (Panstruga & Dodds, 2009; Giraldo & Valent, 2013; Oliveira-Garcia & Valent, 2015). In fact, visualization of effector translocation through the plant plasma membrane has been an enormous technical challenge in all fungal and oomycete systems studied to date.

The *M. oryzae*-rice pathosystem possesses advantages for the study of effector cell biology due to the specific localization pattern of cytoplasmic effectors within BICs, which are easily visible in infected cells (Khang *et al.*, 2010; Giraldo *et al.*, 2013; Oliveira-Garcia & Valent, 2015, 2021). To date, all tested cytoplasmic fluorescently labelled effectors of *M. oryzae* localize in the outer layers of BICs and apoplastic effectors at the periphery of invasive hyphae (Khang *et al.*, 2010; Park *et al.*, 2012; Giraldo & Valent, 2013; Oliveira-Garcia *et al.*, 2023; Yan *et al.*, 2023). The distinct localization patterns of cytoplasmic and apoplastic effectors raise questions regarding the mechanism by which effectors are translocated by IH within living plant cells. High-resolution laser confocal images demonstrate that cytoplasmic effectors of *M. oryzae* are packed into vesicle-like structures inside BICs (Oliveira-Garcia *et al.*, 2023). These vesicle-like structures are termed membranous effector compartments or MECs (Oliveira-Garcia *et al.*, 2023). Colocalization assays of the plant plasma membrane marker LTi6b-GFP and *M. oryzae* effectors clearly show that BICs are plant membrane-rich structures (Giraldo *et al.*, 2013; Oliveira-Garcia *et al.*, 2023) that accumulate fungal effectors. By contrast, colocalization between the fungal H+ATPase Pma1 and the cytoplasmic effector Pwl2 confirms that the BIC structure does not contain fungal plasma membrane, which is inconsistent with a mechanism by which *M. oryzae* effectors are translocated within fungal exosomes (e.g. Giraldo *et al.*, 2013). Moreover, the discovery of a novel plasma membrane-associated effector, Bas83, which localizes to MECs associated with BICs, suggests it may be involved in recruiting plant plasma membrane to support rapid membrane turnover within BICs (Oliveira-Garcia *et al.*, 2023). This is consistent with the idea that *M. oryzae* controls plant membrane dynamics actively, perhaps via the action of secreted effectors.

Regulation of plant membrane dynamics is therefore a necessary prerequisite to fungal infection by the blast fungus due to invagination of invasive hyphae by the EIHM and is also critical for effector delivery. Consistent with this, genes involved in host endocytosis are upregulated during early stages of infection of the Japanese apple rust fungus *Gymnosporangium yamadae* in host tissue (Tao *et al.*, 2020). The main mechanism by which plant cells internalize extracellular or membrane-bound cargoes is clathrin-mediated endocytosis (CME; Dejonghe *et al.*, 2019; Narasimhan *et al.*, 2020). CME involves invagination of the host plasma membrane which is induced by clathrin upon binding of a ligand to a receptor, resulting in clathrin-coated vesicle formation (Chen *et al.*, 2011; Dragwidge *et al.*, 2023). CME is initiated by association of adaptor protein complex 2 (AP2) with the plasma membrane via binding to phosphatidylinositol-4,5- bisphosphate [PI(4,5)P2]. Membrane-associated AP2 recruits clathrin and accessory proteins. Then, AP2 binds cargo proteins and continues to recruit clathrin, which polymerizes into a coat as the membrane invaginates (Narasimhan *et al.*, 2020). The GTPase dynamin-related protein functions in detachment of the vesicle from the plasma membrane. The vesicles lose their clathrin coat when released to the cytoplasm and proceed through early and late endosomes to their ultimate destinations (Narasimhan *et al.*, 2020). To test the importance of CME in effector uptake from *M. oryzae* at the BIC, colocalization assays using clathrin light chain 1 (CLC1), a CME marker, showed that plant clathrin focally accumulates in BICs and co-localizes with fungal effectors (Fig. 1a, c). Silencing rice *ADAPTOR PROTEIN COMPLEX 2 subunit 2α* (*OsAP2α*) and *CLATHRIN HEAVY CHAIN 1* (*CHC1*)-encoding genes led to abnormal accumulation of cytoplasmic effectors into BICs and reduced virulence on rice, providing genetic evidence that CME is required for effector translocation. Similarly, chemical inhibition of CME using the clathrin heavy chain inhibitor Endosidin 9-17 (ES9-17; Dejonghe *et al.*, 2019) also inhibited effector uptake. Moreover, both silencing and chemical inhibition of CME led to the accumulation of cytoplasmic effectors under the appressorium pore, suggesting that some effector translocation begins during appressorium-mediated penetration (Oliveira-Garcia *et al.*, 2023). By contrast, inhibition of clathrin-independent endocytosis, which also occurs in plants and involves lipid raft formation, did not affect effector uptake (Mayor & Pagano, 2007; Ewers & Helenius, 2011; Oliveira-Garcia *et al.*, 2023). When considered together, these experiments suggest that clathrin-mediated endocytosis is necessary for fungal effector delivery into the cytoplasm (Oliveira-Garcia *et al.*, 2023).

Internalization of effectors into host cell cytoplasm via clathrin-mediated endocytosis is not, however, unique to *M. oryzae* or indeed to fungi. Recently, for instance, it has been reported that the oomycete *Phytophthora infestans*, which causes late blight of potato, also utilizes clathrin-mediated endocytosis to internalize effectors into host cells (Wang *et al.*, 2023). Transient silencing of NbCHC, which encodes clathrin heavy chain in *Nicotiana benthamiana*, attenuated *P. infestans* infection and reduced translocation of RXLR effectors into host cells. The mechanisms involved in cytoplasmic effector translocation may therefore be conserved across some fungal and oomycete pathogens. Determining the extent of this conservation is an important next step, to define whether effector uptake is an ancient, conserved process likely predating the divergence of oomycete and fungal pathogens, or whether it has evolved more recently and on more than one occasion within diverse pathogen lineages.

IV. How do *M. oryzae* effectors trigger susceptibility in rice?

The ability to manipulate and reprogram host plant cell signaling and metabolism is a hallmark of biotrophic pathogens (Oliveira-Garcia & Valent, 2015; Presti *et al.*, 2015; Uhse & Djamei, 2018). Effectors of *M. oryzae* can act in specific host cell compartments, such as chloroplasts, nuclei, or ER, to reprogram host metabolism and promote biotrophic growth (Khang *et al.*, 2010; Kim *et al.*, 2020; Liu *et al.*, 2022; Ning *et al.*, 2022). This is the case, for example, with the peroxisome-targeted effector protein Ptep1, which localizes to plant peroxisomes and is required for infection (Ning *et al.*, 2022). Several *M. oryzae* effectors are also mobile, being translocated initially into the cytoplasm of rice cells and then moving to noninfected neighboring cells to prepare them for subsequent infection (Khang *et al.*, 2010; Kim *et al.*, 2020). As with candidate cytoplasmic effectors from diverse pathogens, putative effectors from the crucifer anthracnose pathogen, *Colletotrichum higginsianum*, have been shown to accumulate in distinct plant cell compartments, where they have been proposed to affect immune responses in host organelles (Robin *et al.*, 2018).

A subset of *M. oryzae* cytoplasmic effectors are recognized by intracellular immune receptors, which are the products of dominant disease resistance (R) loci and trigger ETI (Park *et al.*, 2016; Ortiz *et al.*, 2017). Recent evidence suggests that some effectors are recognized directly by binding to the NLR receptor, or may interact with other proteins that function as guardees or decoys to trigger NLR activation (Takken & Goverse, 2012; Ortiz *et al.*, 2017). Although rare, direct effector recognition can be mediated by noncanonical domains incorporated into NLR receptors (Maqbool *et al.*, 2015; Sarris *et al.*, 2015). These integrated domains are structural mimics of proteins normally targeted by effectors for immune suppression. Effectors likely evolved as virulence factors providing benefits to the pathogen during host colonization (Giraldo & Valent, 2013; Kotsaridis *et al.*, 2022), and recent evidence suggests that even though individual effector mutants do not display significantly reduced virulence in spray infections, when analyzed in competition assays with isogenic strains, a fitness penalty can be observed (Yan *et al.*, 2023). *M. oryzae* effectors have been classified as virulence factors (Table 1) and host specificity factors, depending on whether they are known to confer a general benefit or to promote infection on specific host plants (Giraldo & Valent, 2013). Here,

Table 1 Selection of well-characterized *Magnaporthe oryzae* effectors triggering susceptibility.

Effector	*In planta* localization	Function	Reference
Bas3	Cytoplasmic/cell wall crossing points	Potential role in cell-to-cell movement	Mosquera *et al*. (2009)
Bas4	Apoplastic	Major component of the EIHMx	Mosquera *et al*. (2009)
Bas83	Cytoplasmic	Plant plasma membrane-associated effector	Oliveira-Garcia *et al*. (2023)
Bas113	Apoplastic	Major component of the EIHMx	Giraldo *et al*. (2013)
Bas170	Cytoplasmic/nuclear	Potential role in host transcription reprogramming	Oliveira-Garcia *et al*. (2023)
AVR-Piz-t	Cytoplasmic	Targets host ubiquitination; AVR effector for rice R gene Piz-t	Park *et al*. (2016)
AVR-Pii	Cytoplasmic	Targets rice exocyst complex; AVR effector for rice R gene Pii	De la Concepcion *et al*. (2022)
AVR-Pik	Cytoplasmic	Targets rice heavy metal-associated (HMA) proteins; AVR effector for rice R gene Pii	Oikawa *et al*. (2020)
AVR-Pita	Cytoplasmic	Increasing COX activity in rice mitochondria and reduce ROS production	Han *et al*. (2021)
MoIug4	Cytoplasmic	Targets the rice ethylene pathway	Liu *et al*. (2022)
Iug6	Putative cytoplasmic	Targets both salicylic acid and ethylene pathways	Dong *et al*. (2015)
Iug9	Putative cytoplasmic	Targets both salicylic acid and ethylene pathways	Dong *et al*. (2015)
MoHTR1	Cytoplasmic/nuclear	Host transcription reprogramming	Kim *et al*. (2020)
MoHTR2	Cytoplasmic/nuclear	Host transcription reprogramming	Kim *et al*. (2020)
MoPte1	Cytoplasmic/peroxisomes	Targets peroxisomes and suppress immunity	Ning *et al*. (2022)
Slp1	Apoplastic	Chitin-binding effector; suppress chitin-triggered immunity	Mentlak *et al*. (2012)
MoPMO9A*	Apoplastic	CEBiP-binding effector; suppress chitin-triggered immunity	Li *et al*. (2020) and Martinez-D'Alto *et al*. (2023)

Bas, biotrophy-associated secreted; HTR, host transcription reprogramming; Iug, isolate-unique gene; PMO, polysaccharide monooxygenase; Pte1, peroxisomes-targeted effector protein; Slp1, secreted LysM protein1.
*Alternatively named MoAA9A or MoAA91 (auxiliary activity protein 9A or 91).

we divide the effectors of *M. oryzae* into major classes, according to the functions they perturb in the host plant during fungal infection. However, we recognize that these classes are not exclusive because effectors can have multiple functions that may overlap.

1. Effectors evading recognition of PAMPs by host PRRs

To successfully colonize host plants, *M. oryzae* must evade the recognition of its PAMPs (e.g., chitin, glucan, and ergosterol) by plant PRRs. In rice, two important chitin receptors have been identified, the LysM receptors CEBiP and OsCERK1, which collectively mediate chitin elicitor signaling and immunity (Desaki *et al.*, 2018). To suppress chitin-triggered immunity, pathogens secrete extracellular effectors containing LysM amino acid domains (carbohydrate-binding modules that bind GlcNAc into the plant cell apoplast). In the apoplast, LysM effectors can prevent the release of chitin oligosaccharides from fungal cell walls by plant chitinases and sequester released oligosaccharides to prevent their recognition by CEBiP and OsCERK1 (Fig. 2). Chitin-binding effectors have been reported to be important for virulence in a range of fungal pathogens, including Avr4 and Ecp6 effectors of the extracellular tomato leaf mold pathogen *Cladosporium fulvum* and Mg1LysM and Mg3LysM of *Zymoseptoria tritici* (Kombrink & Thomma, 2013). In *M. oryzae*, the LysM effector Slp1 (Secreted LysM Protein1) binds chitin oligosaccharides and suppresses chitin-induced immunity mediated by the CEBiP PRR protein (Mentlak *et al.*, 2012; Fig. 2). N-glycosylation of Slp1 is a post-translational modification critical for effector function to suppress host immunity (Chen *et al.*, 2014). Most recently, a novel apoplastic effector protein MoAa91 (*Magnaporthe oryzae* auxiliary activity protein, alternatively named MoPMO9A) was shown compete with the immune receptor chitin elicitor-binding protein precursor (CEBiP) for chitin binding (Fig. 2), suppressing chitin-triggered plant immunity (Li *et al.*, 2020; Martinez-D'Alto *et al.*, 2023). MoPMO9A contains a catalytic domain predicted to act on cellulose and a carbohydrate-binding domain that binds chitin (Martinez-D'Alto *et al.*, 2023). Interestingly, MoPMO9A is not active on cellulose but shows activity on cereal-derived mixed (1 → 3, 1 → 4)-β-D-glucans (MBG), which suggests it has a role in MBG degradation during rice blast infection (Martinez-D'Alto *et al.*, 2023). MoPMO9A is secreted extracellularly and may also play a role in appressorium-mediated plant infection by *M. oryzae*. MoPMO9A deletion mutants show reduced virulence which may be due to CEBiP activation by fungal GlcNAc that triggers immune responses (Li *et al.*, 2020) and/or its function in plant cell wall disruption (Martinez-D'Alto *et al.*, 2023).

2. Effectors targeting immune response pathways

Many effectors appear to target components of immune signaling pathways (Bos *et al.*, 2010; Park *et al.*, 2012). Defense responses may entail changes in plant hormone levels, which can be mediated by ubiquitin-dependent proteolysis (Trujillo & Shirasu, 2010). The AVR-effector *Avr3a* from *P. infestans* was, for example, characterized as targeting host ubiquitin proteolysis. Avr3a binds and stabilizes the potato U-box E3 ubiquitin ligase CMPG1, thereby blocking Inf1-induced mediated cell death (Bos *et al.*, 2010). In *M. oryzae*, the cytoplasmic effector AvrPiz-t targets proteasome activity through interaction with RING E3 ubiquitin ligases APIP6 and APIP10. This leads to their mutual degradation and suppression of PAMP-triggered immunity in rice (Park *et al.*, 2012, 2016; Fig. 3). Ectopic expression of AVR-Piz-t in transgenic rice suppresses flg22- and chitin-triggered immunity, induces reactive oxygen species (ROS) generation and enhances susceptibility to *M. oryzae*, indicating that AVR-Piz-t functions to suppress PTI in rice (Park *et al.*, 2012). Recently, Shi *et al.* (2018) reported that AVR-Piz-t can also, however, target potassium (K^+) uptake in rice cells (Fig. 3). Potassium (K^+) is essential for plant growth and development and required for immunity against pathogens (Williams & Smith, 2001; Shi *et al.*, 2018). AVR-Piz-t interacts with the plasma membrane-localized K^+ channel protein OsAKT1 and suppresses OsAKT1-mediated K^+ currents, thereby disrupting K^+ signal transduction. Remarkably, AVR-Piz-t interferes with the association of OsAKT1 and its upstream regulator, the cytoplasmic kinase OsCIPK23 (Fig. 3), which leads to decreased K^+ content in rice cells and suppression of plant immunity (Shi *et al.*, 2018). How AVR-Piz-t evolved to recognize such different protein targets is, however, not clear and the relative affinities of each target await further investigation.

3. Effectors targeting transcriptional regulation

Plant nuclei are the control center for the immune system against pathogens (Jagodzik *et al.*, 2018). MAP (mitogen-activated protein) kinase-dependent signaling cascades are activated upon PAMP recognition to transfer signals to plant nuclei and promote changes in gene regulatory networks required for host immunity (Jagodzik *et al.*, 2018; Kim *et al.*, 2020). Genome-wide transcriptome analysis of host plants undergoing infection has demonstrated, for example, that host metabolism and immunity are reprogrammed in order to enable proliferation of the pathogen (Schaker *et al.*, 2016; Schurack *et al.*, 2021; Yang *et al.*, 2021).

A series of nuclear effectors associated with host immunity suppression have been identified in diverse fungal pathogens, including *Ustilago maydis* See1, *Puccinia striiformis* f.sp. *tritici* PstGSRE1, *Colletotrichum graminicola* CgEP1, *Ascochyta rabiei* ArPEC25, and VdSSR1 (secretory silencing repressor 1) from *Verticillium dahliae* (Redkar *et al.*, 2015; Vargas *et al.*, 2016; Qi *et al.*, 2019; Kim *et al.*, 2020; Zhu *et al.*, 2022; Singh *et al.*, 2023). In *M. oryzae*, two nuclear effectors, MoHTR1 and MoHTR2 (*M. oryzae* Host Transcription Reprogramming Proteins 1 and 2) are delivered into the rice cell cytoplasm via the BIC, translocated into nuclei, and appear to move from cell-to-cell (Kim *et al.*, 2020). Both *MoHTR1* and *MoHTR2* affect the expression of immunity-associated genes such as PR protein-encoding genes and phytohormone signaling genes. Expression of *MoHTR1* and *MoHTR2* in transgenic rice increases susceptibility to hemibiotrophs such as *M. oryzae* and *Xanthomonas oryzae* pv *oryzae*. Knockout and overexpression of *MoHTR1* and *MoHTR2* impact the virulence of *M. oryzae* on rice plants, providing evidence that these two nuclear effectors may affect the regulation of plant immunity (Kim *et al.*, 2020).

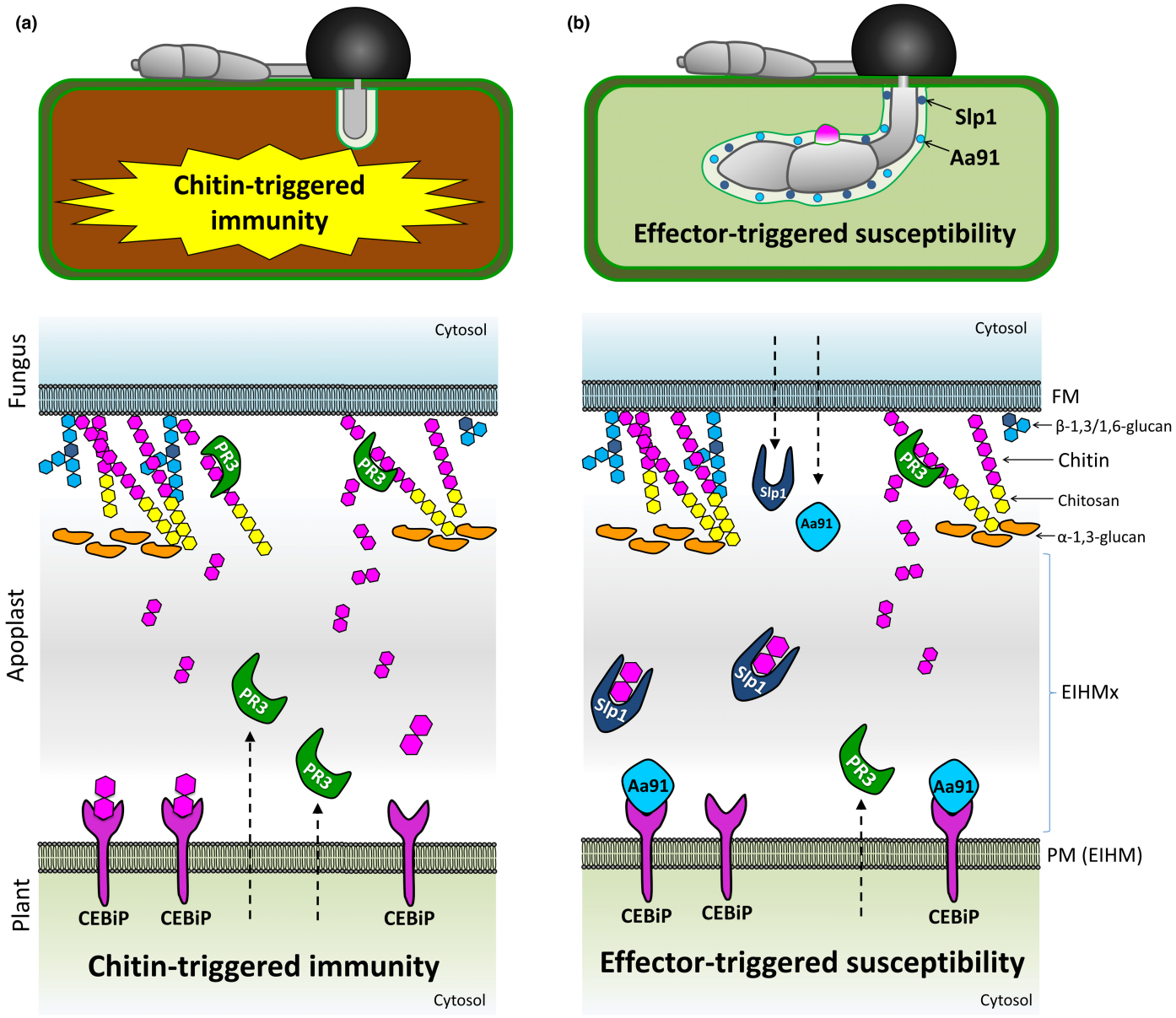


Fig. 2 Secretion of the *Magnaporthe oryzae* effectors Slp1 and Aa91 helps the fungus evade chitin-triggered immunity. (a) Pattern recognition receptors (PRRs; e.g., CEBiP, chitin receptor) at the rice plasma membrane restrict the spectra of pathogenic microorganisms. Rice plants secrete lytic enzymes into the apoplast, such as the chitinase PR3, which releases chitin oligomers from the fungal cell wall that are recognized by CEBiP receptor triggering host immunity. (b) The EIHMx contains apoplastic effectors, such as the chitin-binding effector Slp1 and the CEBiP-binding effector MoPMO9A. Slp1 recognizes and sequesters these chitin oligomers, evading recognition by host chitin receptors. CEBiP-binding effector MoAa91 (alternatively named MoPMO9A) binds to CEBiP chitin receptor blocking the chitin-binding domain, thereby leading to loss of CEBiP function and effector-triggered susceptibility. EIHM, extrainvasive hyphal membrane; EIHMx, extrainvasive hyphal matrix; FPM, fungal plasma membrane; MoPMO9A, CEBiP-binding effector; PPM, plant plasma membrane; PR3, pathogenesis-related protein 3 (plant chitinase); Slp1, Chitin-binding-like effector.

4. Effectors targeting the ethylene biosynthesis pathway

Ethylene (ET) plays a major role in host responses to biotic and abiotic stresses in several plant systems (Binder, 2020). In rice, genes associated with the ethylene biosynthesis pathway enhance resistance to rice blast disease (Liu *et al.*, 2022). For instance, transgenic rice lines expressing OsEIN2 and OsEIL1, two genes involved in ET biosynthesis, showed activation of ET signaling and enhanced resistance to *M. oryzae* (Liu *et al.*, 2021). OsEIN1 and OsEIL2 function as transcription factors that activate expression of ERF1 regulate many ET response genes (Liu *et al.*, 2022).

The ET pathway is a key target for plant pathogen effectors to promote infection. This is the case of the *Xanthomonas* type III effector XopD (e.g. Kim *et al.*, 2013), PsAvh238 of *Phytophthora sojae* (Yang *et al.*, 2019), SsERP1 of *Sclerotinia sclerotiorum* (Fan *et al.*, 2021), and the Jsi1 effector of *Ustilago maydis* (Darino *et al.*, 2021). In *M. oryzae*, the cytoplasmic effector Iug4 (*M. oryzae*

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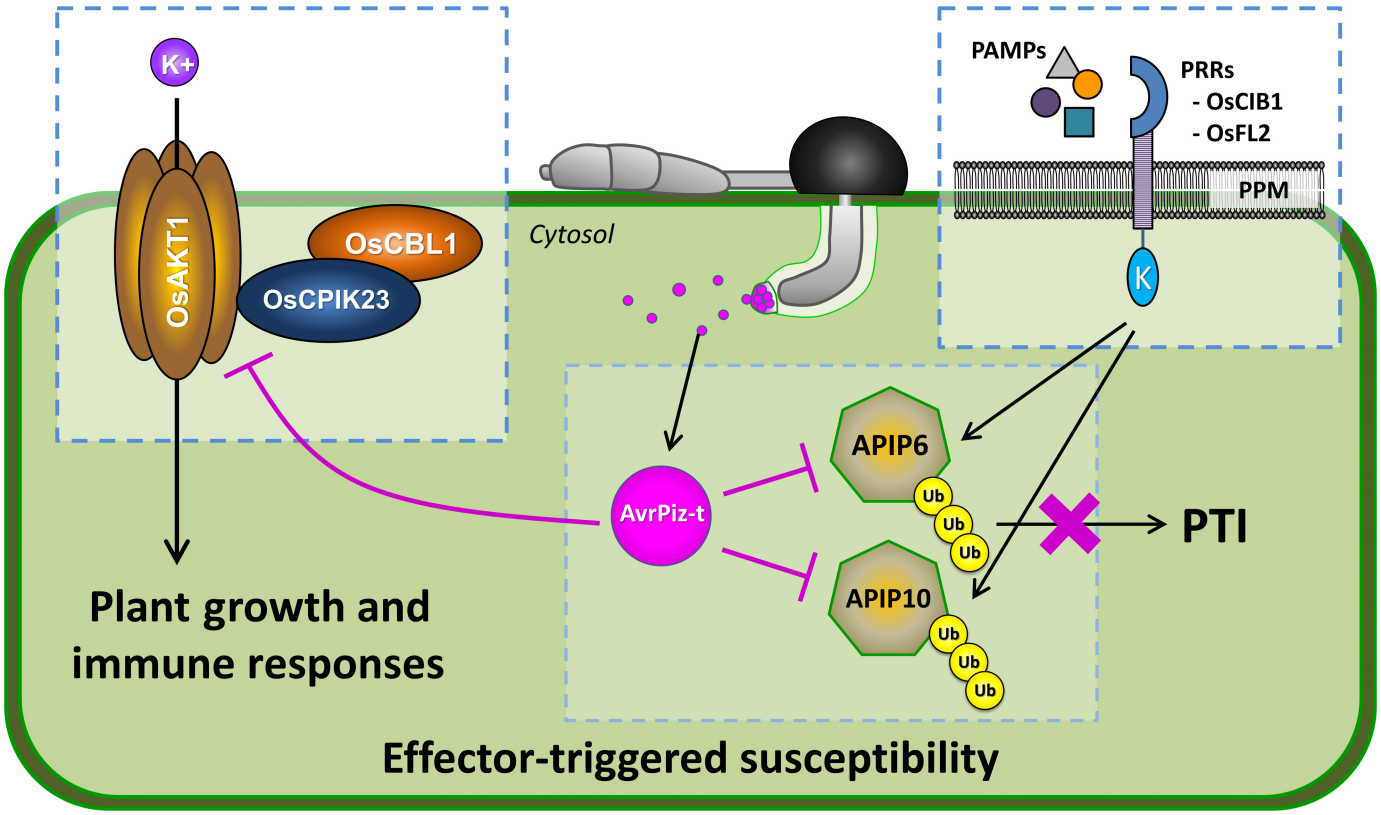

Fig. 3 *Magnaporthe oryzae* effector AVR-Piz-t triggers susceptibility in rice. PTI is initiated upon recognition of PAMPs by plasma membrane-localized PRRs (e.g., OsCIB1 and OsFL2), restricting fungal infection. Rice APIP6/10 are positive regulators of PTI. To suppress PTI, *M. oryzae* secretes effector proteins including AVR-Piz-t. AVR-Piz-t is translocated from the BIC to rice cell cytoplasm to promote the degradation of APIP6/10. The rice CBL1-CIPK23-AKT1 complex modulates K^+ signal transduction required for plant growth and disease resistance. AVR-Piz-t suppresses the activity of OsAKT1 and/or disrupts the OsAKT1-OsCIPK23 complex to suppress K^+ signal transduction, thereby triggering host susceptibility. PAMP, pathogen-associated molecular patterns; PPM, plant plasma membrane; PRRs, pattern recognition receptors; PTI, PAMP-triggered immunity; Ub, ubiquitin ligase.

isolate-unique gene 4) acts as a transcriptional repressor to target the ethylene pathway, via OsAHL1, to disrupt host immunity (Liu *et al.*, 2022; Fig. 4). Specifically, MoIug4 binds to the promoter of rice *OsEIN2* which encodes a central signal transducer in ethylene signaling. Moreover, MoIug4 interacting protein, OsAHL1, acts as an AT-hook motif-containing protein binding to the A/T-rich promoter regions and positively regulates plant immunity in response to *M. oryzae* infection (Liu *et al.*, 2022). Both MoIug4 and OsAHL1 possess transcriptional regulatory activities by binding the OsEIN2 promoter region (Fig. 4). MoIug4 binds to the promoter of *OsEIN2* with a higher affinity than OsAHL1 and functions as a transcription factor to repress *OsEIN2* expression *in planta*. *MoIug4* deletion mutants in *M. oryzae* showed reduced virulence, indicating that the effector plays a role in suppressing host immunity (Liu *et al.*, 2022).

5. Effectors targeting plant peroxisome-function

Peroxisomes are multifunctional eukaryotic organelles that play important roles in stress tolerance, metabolism, immune response signaling, and pathogen defense (Dixit *et al.*, 2010). In plants, peroxisomes are involved in ROS generation in response to pathogen infection (Pan *et al.*, 2020). The disruption of ROS production is a common strategy used by pathogens to cause successful infection, and therefore, peroxisomes are widely targeted by pathogen effectors. Fungal pathogens and even nematodes use effectors to impair peroxisome-mediated ROS production. For instance, the anthracnose pathogen *Colletotrichum higginsianum*, for example, has two effectors containing a tripeptide signal sequence required for typical PTS1 (peroxisomal targeting signal 1) pathway functioning in peroxisomes and, therefore, localize to the peroxisomal matrix of tobacco cells (Robin *et al.*, 2018).

The cytoplasmic effector MoPtep1 (peroxisomes-targeted effector protein 1), a small secreted protein (198 aa) containing a cupredoxin domain, for example, targets host peroxisome compartments during transient expression in *N. benthamiana* plants (Ning *et al.*, 2022). Moreover, MoPtep1 interacts with a rice thaumatin-like host protein (OsIP-4) involved in host defense (de Jesús-Pires *et al.*, 2020). *MoPTEP1* deletion mutants showed reduced virulence on rice (Ning *et al.*, 2022). MoPtep1 therefore plays an important role in suppressing host immunity during biotrophic invasion of *M. oryzae* via peroxisome targeting, but it is likely that other effectors target ROS production to facilitate fungal infection.

6. Effectors targeting rice exocyst complex

The exocyst complex mediates tethering of secretory vesicles to the plasma membrane before SNARE-mediated membrane fusion, in

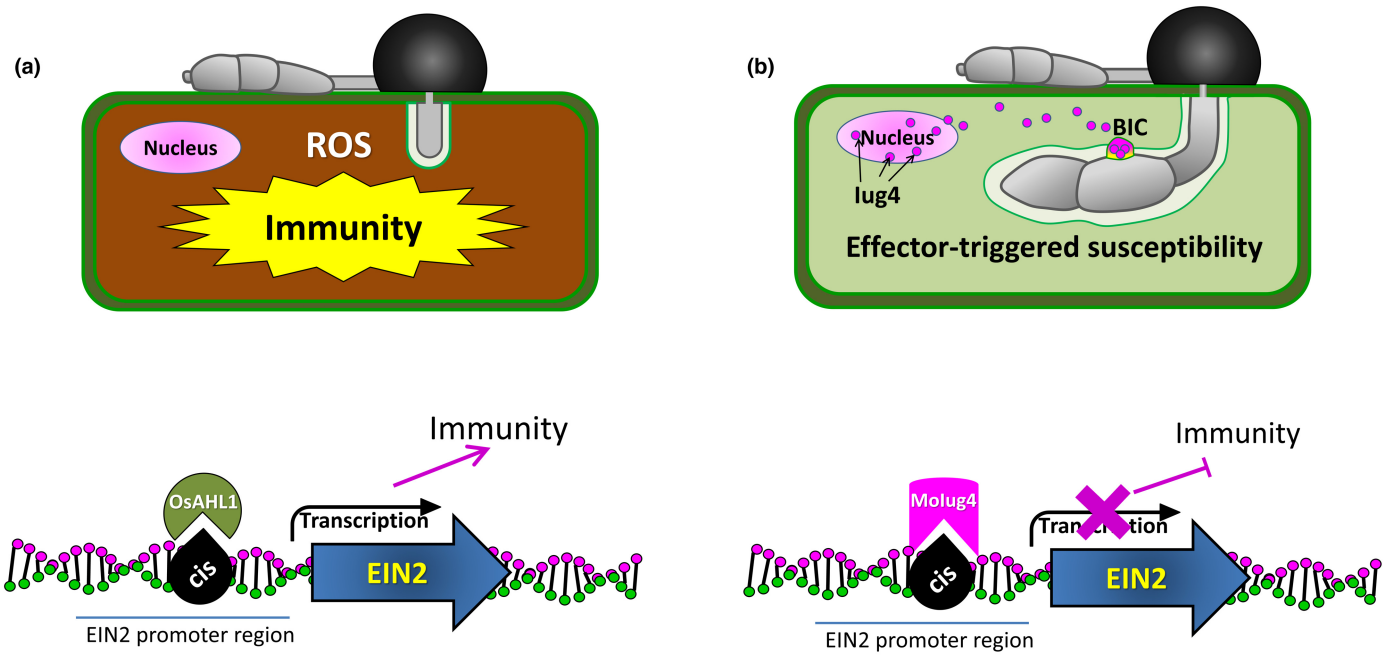

Fig. 4 *Magnaporthe oryzae* effector MoIug4 suppresses ethylene biosynthesis and *OsEIN2*-mediated immunity. (a) The rice OsEIN2 gene involved in the ethylene biosynthesis and plant immunity. OsEIN2 is positively regulated by AT-hook protein OsALHL1 transcription factor that enhances resistance to fungi. (b) Cytoplasmic effector MoIug4 is focally secreted into the biotrophic interfacial complex (BIC) and subsequently translocated into rice cell cytoplasm and rice nuclei during fungal infection. MoIug4 binds the promoter of *OsEIN2* with greater affinity than OsAHL1, thereby suppressing the expression of *OsEIN2* and *OsEIN2*-mediated immunity. *cis*, *cis* element; ROS, reactive oxygen species.

which the Exo70 subunit of the exocyst complex plays an essential role in plant immunity (Stegmann *et al.*, 2013). Components of the vesicle trafficking machinery are crucial to the immune response in plants and therefore are common targets for effectors, although the expansion of the Exo70 family in plants suggests alternative functions may also exist (Robatzek *et al.*, 2006; Smith *et al.*, 2014). In *Arabidopsis thaliana*, Exo70B2 and Exo70H1 orchestrate immune responses triggered by PAMPs (Stegmann *et al.*, 2012). Arabidopsis plants lacking Exo70B2, for instance, are more susceptible to the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Hpa) and the bacterial pathogen *Pseudomonas syringae* pv tomato (Stegmann *et al.*, 2013). Homologs of the Exo70 subunit and another tethering complex termed the conserved oligomeric Golgi complex (COG), Exo70F-like, and COG3 respectively, are required for penetration resistance of barley against *Blumeria graminis* f.sp. *hordei* (Ostertag *et al.*, 2013).

Some effectors of *M. oryzae* show a high degree of specificity toward host proteins of the exocyst system. This is the case for the effector AVR-Pii, for example, which targets two specific vesicle-tethering Exo70 subunits, OsExo70-F2 and OsExo70-F3 (De la Concepcion *et al.*, 2022). AVR-Pii exerts its activity via a zinc-finger effector fold (ZiF), which allows binding to a conserved interface of Exo70 (De la Concepcion *et al.*, 2022). Both *OsExo70-F2* and *OsExo70-F3* are involved in host immune responses and constitutively expressed in both healthy and *M. oryzae*-infected rice plants. This supports the idea that AVR-Pii, and other effectors associate with components of the exocyst complex (De la Concepcion *et al.*, 2022).

7. Effector targeting rice proteins containing heavy metal-associated-like domains

Proteins containing heavy metal-associated (HMA)-like domains have become massively expanded in plants (Barth *et al.*, 2009; de Abreu-Neto *et al.*, 2013) and play diverse roles in plant cellular processes. HMA proteins have been associated with plant defense and susceptibility toward pathogens. Effectors across different species have, for example, been shown to target HMA proteins. For instance, in *Arabidopsis thaliana*, the *AtHMAD1* enhances resistance against virulent *Pseudomonas syringae* DC3000 (Imran *et al.*, 2016) and *AtHIPP27* enhances resistance against beet cyst nematode (Radakovic *et al.*, 2018). Similarly, in monocots, the rice sHMA, Pi2, confers partial resistance against compatible isolates of *M. oryzae* (Fukuoka *et al.*, 2009) and wheat *TaHIPP1* enhanced resistance against stripe rust caused by *Puccinia striiformis* f. sp. *tritici* (Zhang *et al.*, 2015). The blast fungus also targets HMA domain-containing proteins to manipulate host immunity. For example, AVR-Pik binds a subset of related rice proteins containing a heavy metal-associated (HMA) domain (Fig. 5), included domains that have been integrated into plant intracellular NLR immune receptors (Oikawa *et al.*, 2020; Maidment *et al.*, 2021). Moreover, genetic data suggest that HMA proteins are susceptibility factors required for full virulence. Knockout of the OsHIPP20 gene, for example, reduces rice susceptibility toward *M. oryzae* (Oikawa *et al.*, 2020). Effectors targeting HMA proteins have been shown to contain a common structure and are termed MAX effectors (for

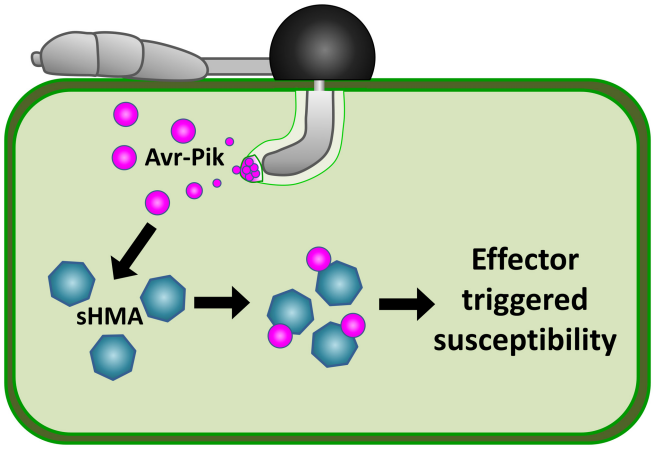

Fig. 5 *Magnaporthe oryzae* effector AVR-Pik targets rice heavy metal-associated proteins. In the compatible interaction, AVR-Pik binds rice heavy metal-associated proteins and heavy metal-associated isoprenylated proteins (also referred as small heavy metal-associated or sHMA proteins) and stabilizes them, possibly enhancing pathogen virulence.

Magnaporthe Avrs and ToxB like), even though they are sequence unrelated (de Guillen *et al.*, 2015), including AVR-Pik, AVR-Pia, and AVR1-Co39. Interestingly, MAX effectors are temporally co-regulated during plant infection (Yan *et al.*, 2023), suggesting that HMA targeting (and potentially that of other MAX targets) is critical during the early stages of plant tissue colonization (de Guillen *et al.*, 2015). A comprehensive survey of MAX effectors suggests that typically 58–78 MAX effector genes are present per genome in a set of 120 *M. oryzae* isolates representing seven host-associated lineages. Furthermore, MAX effectors display high rates of nonsynonymous substitutions suggesting they are under positive selection and may exhibit diversification in function (Le Naour-Vernet *et al.*, 2023).

8. Effectors targeting rice cytochrome c oxidase assembly protein in mitochondria

ROS plays many essential roles in plant defense signaling. Recognition of plant pathogens triggers rapid ROS bursts leading to the hypersensitive response and programmed cell death in host cells (Smirnoff & Arnaud, 2019). To suppress ROS formation in rice, *M. oryzae* secretes the zinc metalloprotease protein, AVR-Pita, which inhibits host mitochondrial ROS formation (Fig. 6). Inside rice mitochondria, AVR-Pita targets cytochrome c oxidase (COX) assembly protein OsCOX11, a key regulator of mitochondrial ROS metabolism in rice, thereby inhibiting ROS accumulation and suppressing immunity (Fig. 6). Ectopic expression of *AVR-PITA* in rice also enhances susceptibility to *M. oryzae* (Han *et al.*, 2021).

V. Conclusion and outlook

In this review, we have focused on new research findings that have begun to reveal the mechanism of effector uptake and the likely *in planta* functions of the diverse effector repertoire of *M. oryzae*. Rice blast disease is a constant threat to rice production world-wide, but when combined with the threat to wheat caused by emergence and intercontinental spread of wheat blast disease (Latorre *et al.*, 2023), the need for understanding effector biology and developing durably blast-resistant varieties is urgent (Oliveira-Garcia & Valent, 2015). Although large repertoires of putative effectors have been identified in *M. oryzae* (Mosquera *et al.*, 2009; Yan *et al.*, 2023), there are only

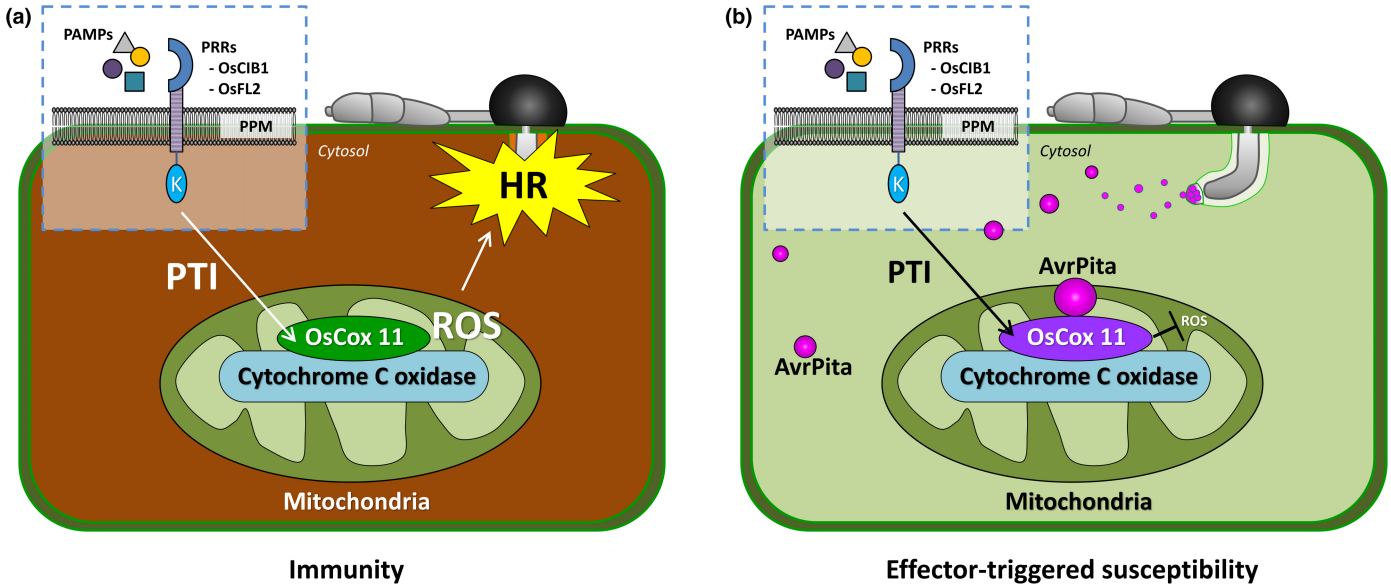

Fig. 6 *Magnaporthe oryzae* effector *AVR-Pita* inhibits mitochondrial ROS formation and triggers host susceptibility. (a) PRRs recognize PAMP molecules from *M. oryzae* and activate PTI leading to ROS production and HR to block pathogen invasion (left). (b) In *M. oryzae* isolates carrying the effector AVR-Pita (right) can suppress mitochondrial ROS production. AVR-Pita binds OsCOX11 in the host mitochondria to promote COX activity, thereby inhibiting ROS accumulation and triggering host susceptibility. HR, hypersensitive response; PAMP, pathogen-associated molecular pattern; PRRs, pattern recognition receptors; PTI, PAMP-triggered immunity; ROS, reactive oxygen species.

limited examples of effector-host target interactions having been defined at the molecular level, most of which we have described here. Our analysis clearly reveals the need to focus on the identification of effector targets in order to understand how *M. oryzae* can overwhelm the plant immune system so effectively. Strikingly, many targets of effectors are conserved across different pathogens, suggesting there are key processes that lead to PTI targeted by diverse fungi, including, for example, HMA proteins required for ROS generation. However, the challenge of identifying targets of > 546 blast effectors is a daunting one and will require high-throughput approaches to be developed. In this regard, the advent of structural prediction using Alphafold has already been hugely valuable in predicting effector functions (Seong & Krasileva, 2023). When coupled with the use of Alphafold Multimer (Evans *et al.*, 2022) to predict protein–protein interactions, this may lead to more rapid, global identification of putative effector targets which can then be experimentally validated more rapidly. Defining the relative fitness value of individual effectors in susceptible interactions is equally important (Yan *et al.*, 2023) and could be achieved at higher throughput by barcoding *M. oryzae* mutants, for instance, and then using sequence analysis of infected rice tissue to determine the relative proportion of isogenic strains of the fungus during successive infections. Such analyses are required if a holistic understanding of the effector complement of the pathogen is to be achieved.

The mechanism by which the cytoplasmic effectors are internalized and transported into plant cells is also beginning to be addressed. However, the precise mechanism of MEC uptake and effector release is not clear and requires functional studies of endocytic mutants of rice, coupled with more sensitive delivery assays. Internalization of effectors via clathrin-mediated endocytosis is a common feature in both *M. oryzae* and *P. infestans* and may therefore be applicable to other fungal and oomycete diseases. The precise roles of effectors in inducing endocytosis need to be addressed, as well as the recently identified role of fungal autophagy in modulating host plasma membrane dynamics at the BIC (Li *et al.*, 2023b). Elucidation of the mechanism by which the Bas83 effector binds to the EIHM, for instance, could provide new insight into BIC function and CME-dependent effector uptake. A gene editing approach to identify rice mutants conferring blast resistance and, significantly, recently identified a cytidine diphosphate diacylglycerol (CDP-DAG) synthase, that is required for phospholipid biosynthesis at the BIC, perhaps impairing effector delivery as a means of conferring disease resistance (Sha *et al.*, 2023). This demonstrates the utility of developing an understanding of effector delivery at the BIC and also in adopting large-scale genetic screens to look for novel mechanisms leading to disease resistance, distinct from NLR deployment. When considered together, these recent advances show how a knowledge of effector biology in blast disease may provide novel and durable methods to control this devastating pathogen.

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Competing interests

None declared.

Author contributions

EO-G designed the review article, performed the microscopy, created the figures, and wrote the manuscript. NJT, XY, MO-R and SdP wrote and edited the review article.

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