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Functional characterization of *Magnaporthe oryzae* (anamorph. *Pyricularia oryzae*) effector during infection

Samuel de Paula

Thesis presented to obtain the degree of Doctor in Science. Area: Plant Pathology

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Samuel de Paula
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I dedicate this work to

my parents João and Néva,

my brother Kaciano, my spouse Aline, and my daughter Annelise,

who have all provided unwavering support throughout my journey.

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“Today is difficult,
tomorrow is much more difficult,
but the day after tomorrow is beautiful.
Most people die tomorrow evening”.

- *Jack Ma*

CONTENTS

RESUMO

Caracterização funcional de efetor de *Magnaporthe oryzae* (anamorfo: *Pyricularia oryzae*) durante a infecção

O fungo ascomiceto filamentoso *Magnaporthe oryzae* (sin. *Pyricularia oryzae*), agente causal da brusone do arroz, representa um desafio crítico à segurança alimentar, por se tratar de um patógeno com grandes desafios de manejo. Essa doença é responsável por perdas de produção estimadas entre 10–30% do total da produção mundial de arroz. *M. oryzae* utiliza um arsenal sofisticado de efetores para contornar os mecanismos de defesa do hospedeiro e invadir com sucesso as células de arroz. Esses efetores são proteínas especializadas que interagem com os mecanismos celulares do hospedeiro, manipulando-os para facilitar a infecção, colonização e reprodução do patógeno. O objetivo principal deste estudo foi fornecer uma revisão abrangente sobre efetores de *M. oryzae* e ampliar a caracterização do efetor Bas83, avaliando seu impacto na sintomatologia da brusone em arroz. Para elucidar o papel do efetor Bas83 na dinâmica de infecção e progresso da doença, a pesquisa utilizou a cepa tipo selvagem (WT) *M. oryzae* Guy11. Este estudo empregou uma abordagem dupla em manipulação genética, incorporando técnicas de superexpressão e silenciamento gênico mediado por RNA de interferência (RNAi). Essas metodologias foram escolhidas estrategicamente para proporcionar um entendimento abrangente dos papéis funcionais do Bas83 no processo de infecção, oferecendo *insights* sobre como sua modulação afeta a manifestação da doença no arroz. Além disso, o estudo investigou o papel de domínios proteicos específicos no Bas83 em ditar a localização e a dinâmica de secreção do efetor dentro das células hospedeiras. Isso foi alcançado através de uma abordagem de deleção de domínio proteico direcionado, permitindo uma avaliação detalhada de como domínios específicos influenciam a distribuição espacial e a atividade funcional do Bas83 no processo de interação hospedeiro-patógeno. Este estudo não conseguiu produzir cepas de superexpressão Bas83:mRFP, mas gerou com sucesso três cepas com silenciamento notável de *BAS83* (68%, 69% e 79%). Essas cepas mostraram um impacto direto nos estágios de infecção em comparação com a Guy11 WT. Ensaios *in planta* indicaram que as cepas RNAi*Bas83* apresentaram menores intensidades de doença (2-3) em comparação com a Guy11 WT (4-5), confirmando a influência significativa do efetor Bas83 no desenvolvimento da brusone. Além disso, os resultados mostram que deleções de domínios na proteína Bas83 alteram de forma marcante sua localização e secreção no complexo interfacial de biotrofia (BIC) de *M. oryzae*. Enquanto o domínio intacto da proteína Bas83 marcado com mRFP acumula-se na região do BIC, deleções como o sinal de secreção (SS) e sequências de aminoácidos 22-61, 62-120 e 121-173 bloquearam a secreção no BIC, levando ao acúmulo da hifa invasiva. Portanto, o silenciamento do Bas83 afeta significativamente os estágios de infecção de *M. oryzae* e o progresso da doença. Além disso, a deleção de domínios proteicos específicos no Bas83 influencia a localização e secreção do efetor através dos BICs.

Palavras-chave: *Magnaporthe oryzae*, Efetor Bas83, Proteína, Brusone do arroz

ABSTRACT

Functional characterization of *Magnaporthe oryzae* (anamorph. *Pyricularia oryzae*) effector during infection

The filamentous ascomycete fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*) represents a critical challenge to global food security due to its role in causing devastating blast diseases in rice. These diseases account for yield losses estimated at 10–30% of total global rice production. *M. oryzae* utilizes a sophisticated arsenal of effectors to circumvent the innate immune defenses of the host and successfully invade plant cells. These effectors are specialized proteins that interact with the host's cellular mechanisms, manipulating them to facilitate infection and proliferation of the pathogen. The primary objective of this study was to provide a compreensive review on *M. oryzae* and to augment the characterization of the *M. oryzae* effector Bas83 and to assess its impact on the symptomatology of rice blast disease. In order to elucidate the role of the Bas83 effector in the infection dynamics and progression of the blast fungus, the research utilized the *M. oryzae* Guy11 wild-type (WT) strain. This study employed a dual approach in genetic manipulation, incorporating both overexpression and RNA interference (RNAi)-mediated gene silencing techniques. These methodologies were strategically chosen to provide a comprehensive understanding of Bas83's functional roles within the infection process, offering insights into how its modulation affects disease manifestation in rice. Additionally, the study investigated the role of specific protein domains in Bas83 in dictating the effector's localization and secretion dynamics within host cells. This was achieved through a targeted protein domain deletion approach, allowing for a detailed assessment of how particular domains influence the spatial distribution and functional activity of Bas83 in the host-pathogen interaction process. This study failed to produce Bas83:mRFP overexpression strains but successfully generated three strains with notable *Bas83* silencing (68%, 69%, and 79%). These strains showed a direct impact on infection stages compared to the Guy11 WT. *In planta* assays indicated that RNAi*Bas83* strains had lower disease scores (2-3) versus the Guy11 WT (4-5), confirming Bas83's significant influence on blast disease development. Also, the findings show that domain deletions in the Bas83 protein markedly alter its localization and secretion into the biotrophic interfacial complex (BIC) of *M. oryzae.* While the intact Bas83 protein tagged with mRFP accumulates into the outlayers of BICs, the deletions of the secretion signal (SS) and amino acid sequences 22-61, 62-120, and 121-173 blocked their secretion into BICs, leading to their accumulation into fungal invasive hyphae. In conclusion, the silencing of Bas83 significantly impacts the infection stages of *M. oryzae* and the progression of blast disease. Additionally, the deletion of putative protein domains in Bas83 influences the effector's localization and secretion through the BICs.

Keywords: *Magnaporte oryzae*, Bas83 effector, Protein domain, Rice blast

1. GENERAL INTRODUCTION

Rice (*Oryza sativa*) is a staple food that is highly important for more than half of the world's population, as it is responsible for approximately 19% of the calories consumed daily worldwide (Elert, 2014). A meta-analysis projection suggests that the global food demand will increase from 35% to 56% between 2010 and 2050, meanwhile, the population at hunger risk will dramatically increase in the same period not only because of population growth but also because of the undergoing climate changes (van Dijk et al., 2021). Since forecasts indicate that the world population and food demand will continue to grow in the upcoming years, rice yields should increase by at least 28% in the next decades (Zhu et al., 2022). However, the yield rise is threatened by the climate changes such as extreme temperatures reported in several countries like Thailand, India, China, and the United States (Arunrat et al., 2020; Lafferty et al., 2021).

On the world stage, outside Asia, Brazil is the largest rice producer, with important regions for rice cultivation, such as the states of Maranhão in the northeast, Mato Grosso in the west, Minas Gerais in the east, and Santa Catarina and Rio Grande do Sul in southern Brazil (Sharma, 2010; Schwanck et al., 2015). These last two states are responsible for about 66% of rice production in Brazil under irrigated areas, reaching the highest yields in the country (Singh et al., 2017). Rice yield can be affected by several factors, such as water availability, soil fertility, climate, insects, and diseases (Jiang et al., 2020). The crop is mainly affected by several diseases caused by bacteria, viruses, and fungi (Dai et al., 2007).

Among the fungi pathogenic to rice, the causal agent of the blast is the first ranked within this group of phytopathogens (Dean et al., 2012). The ascomycete *Magnaporthe oryzae* (anamorphic *Pyricularia oryzae*) is responsible for causing rice blast. This pathogen is an excellent model organism for the investigation of molecular mechanisms of plant-pathogen interactions (Pennisi, 2010; Jacob et al., 2017). All plant tissues are subject to infection, but the rice panicle is the part that, when attacked, can lead to complete grain loss (Dean et al., 2012). The most typical yield losses are about 10 - 30%, although epidemics can lead to higher impacts (Dean et al., 2012).

During the interaction with the plant, *M. oryzae* uses several mechanisms to overcome the host barriers. These mechanisms can affect plant tissues to favor infection and colonization by the pathogen. The *M. oryzae* is a pathogen widely studied and known for the large number of secondary metabolites it produces, many of which are involved in important processes, including the phytotoxin pyriculol, which is linked to the induction of necrotic

lesions (Motoyama et al., 2021). Another secondary metabolite produced is melanin, which is crucial for the infection process, as it is directly involved with the appressoria melanization and it helps standing the high turgor pressure generated by the structure, processes that are fundamental for the success of *M. oryzae* in the interaction with the plant tissue (Motoyama et al., 2021).

In the same way that pathogens have mechanisms to facilitate their infectious process in plant tissue and succeed in infection, plants also have mechanisms to recognize pathogens and trigger their mechanisms to defend themselves against attacks (Jones and Dangl, 2006). Plants have an innate immune system that allow the recognition of molecules released by pathogens and enables the battle between plant and pathogen during the interaction (Jones and Dangl, 2006). In this battle, the pathogen uses various tools to overcome plant defense, which includes the secretion of a large repertory of effector proteins. Effectors are important features required for pathogenesis, mainly due to their ability to modulate plant immunity and facilitate infection (Macho and Zipfel, 2015).

The confrontation between rice and *M. oryzae* commences with the adhesion of the three-celled asexual *M. oryzae* spore (conidia) to the leaf cuticle facilitated by an adhesive substance known as spore tip mucilage (Figure 1) (Hamer et al. 1988). Subsequent to adhesion, germination occurs through the emergence of a polarized germ tube originating from one of the apical cells (Hamer et al. 1988). This germ tube extends across the leaf surface until it undergoes differentiation into an appressorium, a dome-shaped infection structure (Ryder and Talbot, 2015). The formation of an appressorium is essential for initiating plant infection, as it empowers the fungus to physically breach the cuticle of the plant host (Cruz-Mireles at al., 2021). Later, a high turgor pressure (reaching up to 8.0 MPa) is generetad within the appressorium, facilitating the penetration peg to rupture the host cell cuticle (de Jong et al., 1997). This marks the onset of primary hyphae development within the initially invaded cell, establishing an intimate biotrophic association (Fernandez and Orth, 2018). Then, the primary hyphae differentiates into invasive hyphae (IH) being surrounded by the extra-invasive hyphal membrane (EIHM) that separetes IH from the rice cytoplasm (Kankanala et al., 2007; Wilson, 2021). The extra-invasive hyphal membrane matrix (EIHM matrix) forms between the EIHM and the *M. oryzae* cell wall (Kankanala et al., 2007; Wilson, 2021). During the colonisation, the IH continues to grow and once it fills the first invaded cell, the IH moves to the adjacent uninfected cells through plasmodesmata (Kankanala et al., 2007; Martin-Urdiroz et al. 2016; Wilson, 2021). Rice blast lesions become visible 96-144 hours after infection, and optimal humidity conditions are essential for sporulation (Talbot,

2003, Wilson, 2021). Subsequently, *M. oryzae* spores disseminate to healthy plants, completing the rice blast fungus life cycle (Talbot, 2003).

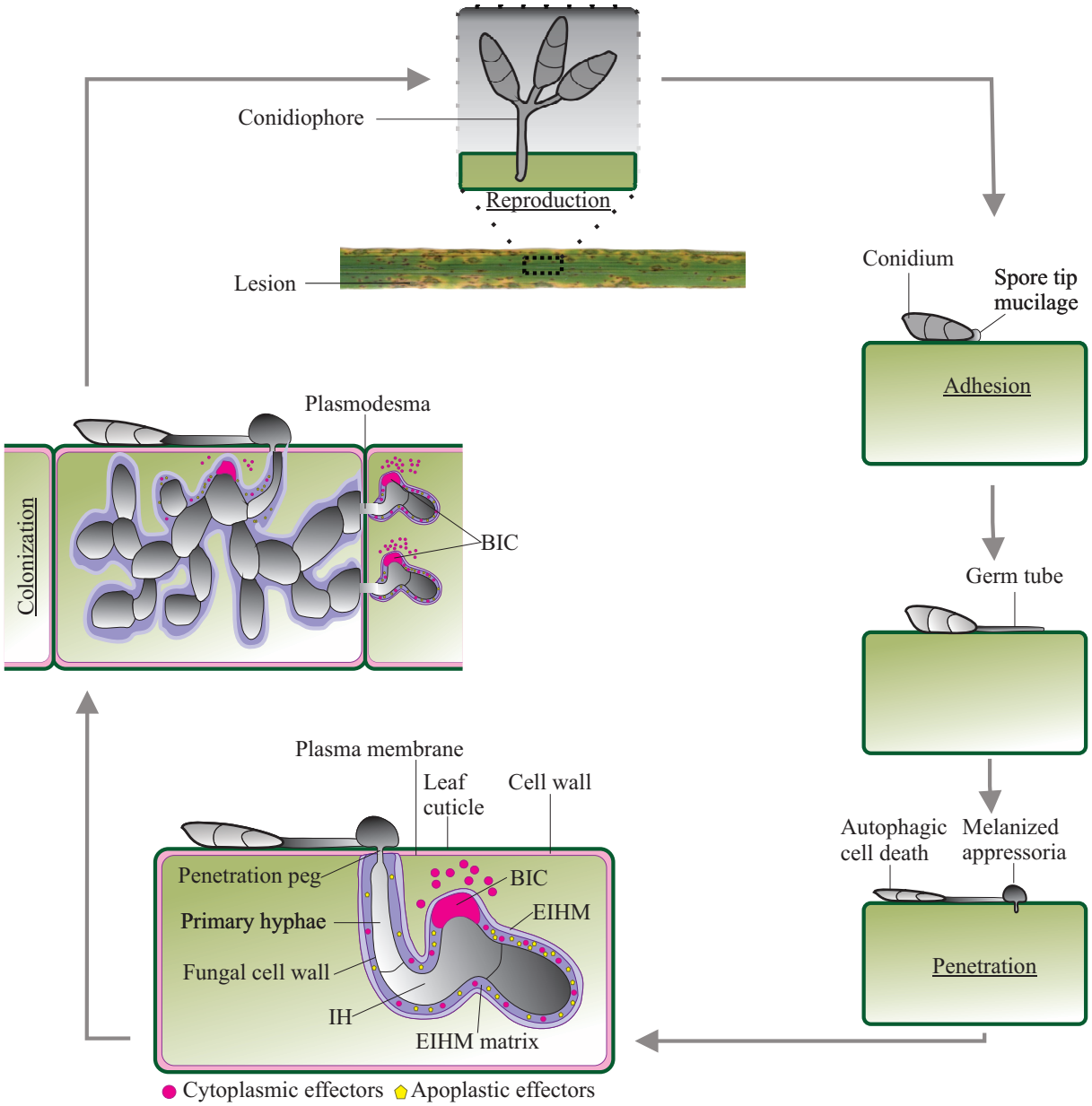

Figure 1. **Life cycle of the rice blast fungus *Magnaporthe oryzae***. The cycle begins with the conidium surface attachment facilitated by the spore tip mucilage (adhesion), progressing to germination after 2 hours. 4-6h hours post inoculation (hpi), the germ tube starts to swell and develops a melanized appressorium. By 24-28 hpi, the spore undergoes autophagy, and the penetration peg breaches the leaf cuticle to reach the underlying epidermal cells (penetration). Then, penetration peg differentiates into a filamentous primary hyphae, subsequently transitioning into an invasive hyphae (IH). The extra-invasive hyphal membrane (EIHM) serves to separe the IH from the rice cytoplasm, creating the EIHM matrix between the *M. oryzae* cell wall and EIHM. Apoplastic effectors (♦) can be find in the EIHM matrix, while the cytoplasmic effectors (●) are secreted through the biotrophy interfacial complex (BIC). IH moves to the adjacent cells 48 hpi and news BICs are generated, continuing the infection process (colonisation). Between 96-144 hpi, rice blast lesions become visible on the leaf surface, and the dispersal of new conidia concludes the life cycle (reproduction).

M. oryzae effectors are secreted via two mechanisms. Apoplastic effectors are released into the EIHM matrix via the conventional endoplasmic reticulum (ER)-Golgi pathway, while cytoplasmic effectors follow an unconventional secretion pathway utilizing the biotrophy interfacial complex (BIC) (Giraldo et al., 2013). For *M. oryzae*, especially, there are numerous examples of effectors already studied. Mentlak et al (2012) demonstrated that *M. oryzae* was able to overcome the first line of defense in rice plants by secreting an effector called LysM Protein1 (Slp1), which accumulates at the interface between the fungal cell wall and the plant's plasma membrane, suppressing the response chitin-induced immunity. Chen et al (2013) demonstrated that five effectors (MoCDIP1-MoCDIP5) from *M. oryzae* were able to induce cell death. On the other hand, effectors have been showed to be capable of suppressing host cell death and possibly involved with the biotrophic phase of the pathogen (Dong et al., 2015). During the biotrophic phase of *M. oryzae*, various effector proteins can be secreted to helping to suppress the immune system, which can alter the host's physiology (Were, 2018). Also, *M. oryzae* proteins were identified as biotrophy-associated secreted (BAS) proteins, which are up-regulated and secreted in the earlier moments of infection in the biotrophic phase. Some BAS proteins remain outside the host cells in the apoplastic space, while others can be translocated within the cytoplasm of the host's living cells and move simplastic in the near cells (Mosquera et al., 2009; Khang et al., 2010). However, the way in which BAS proteins act in mediating the virulence of *M. oryzae* remains unknown in many cases. Recently, a new membrane-associated candidate effector, named Bas83, which localizes at BIC, EIHM, and plasma membrane of vesicles, has been under characterization (Oliveira-Garcia et al., 2023). The evidence suggests that the rice plasma membrane may be a target to *M. oryzae* effectors.

Numerous studies have shown the *M. oryzae* effectors repertoire used to manipulate the host defense system and succesfully invade the plant tissue. Recently, Yan et al. (2023) demonstrated major changes in fungal gene expression during host infection, reporting 546 *M. oryzae* protein genes, of which 32 use the BIC to target the cytoplasm of rice cells. Also, Li et al. (2023) identified 16 *M. oryzae* genes that are up- or down-regulated during early infection processes and cause cell death BAX-mediated supression, indicating its potential association to pathogenicity. Given all the scientific advancements in this field and the information available thus far, the importance of effectors for pathogens is undeniable, particularly as a mechanism to overcome plant defense barriers (Zhang et al., 2022). Despite the progress in research, *M. oryzae* remains one of the most significant pathogens affecting rice cultivation, causing substantial losses in global rice production (Savary et al., 2019). The ongoing

research efforts, coupled with the pursuit of practical applications based on the acquired information, have the potential to mitigate the losses caused by this pathogen. In an effort to enhance comprehension of the rice blast disease and its effectors, this work aimed to:

- Dissect the role of *M. oryzae* efectors and their relation to plant susceptibility, providing a compreensive review to guide future studies on rice blast disease (**Chapter 2**);
- Characterize a plasma membrane-associated effector Bas83 during *M. oryzae* infection through overexpression and RNAi silencing approaches (**Chapter 3**);
- Describe how the deletion of putative protein domains impact *M. oryzae* Bas83 effector secretion (**Chapter 4**).

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2. *Magnaporthe oryzae* EFFECTORS AND PLANT SUSCEPTIBILITY

Abstract

The filamentous fungus *Magnaporthe oryzae* is responsible for rice blast, the most devastating disease affecting cultivated rice globally. In its quest to infect plants, *M. oryzae* employs a diverse array of effector proteins. These effector molecules function to inhibit the defensive responses of plants, modulate various cellular mechanisms, and promote the proliferation of pathogens. Certain effectors are secreted by appressoria before the penetration of the host occurs, whereas others amass in the apoplast or infiltrate living plant cells, where they are localized in particular subcellular structures. During the progression of plant infection, the blast fungus develops a specialized interfacial structure known as the biotrophic interfacial complex (BIC). This complex is believed to be instrumental in the translocation of effector proteins into plant cells, underscoring its vital role in the infection process. This review delves into recent advancements in the cell biology of *M. oryzae*-host interactions, demonstrating how a deeper understanding of the deployment and delivery of *M. oryzae* effector proteins into plant cells has led to breakthroughs in disease control, contributing to the comprehension of pathogen invasion and host susceptibility.

Keywords: host susceptibility, effector proteins, *Pyricularia oryzae*, biotrophic phase, biotrophic interfacial complex, effector secretion, rice blast.

2.1. Introduction

The ascomycete fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*) represents a significant threat to global food security due to its role in causing devastating rice blast disease (Savary et al. 2019). In rice, it accounts for approximately 10–30% of worldwide yield losses (Dean et al., 2012). Understanding the fungal strategies for host colonization is paramount for effective blast disease management. *M. oryzae* navigates through two primary layers of plant immunity for successful infection (Oliveira-Garcia and Valent, 2015). The first layer involves the plant's pattern recognition receptors (PRRs) on the cell surface, which detect pathogen-associated molecular patterns (PAMPs), triggering PAMP-triggered immunity (PTI) (Jones & Dangl, 2006). The second layer comprises host resistance (R) proteins that recognize specific pathogen AVR (avirulence)-effectors, leading to effector-triggered immunity (ETI) (Jones & Dangl, 2006). To overcome these defenses, *M. oryzae* deploys a suite of effectors that circumvent or suppress these immune systems and manipulate host cellular processes to its benefit (Yan et al., 2023). These effectors demonstrate substantial genetic diversity, indicative of an ongoing evolutionary arms race characterized by continual adaptation and counter-adaptation between the host and the pathogen (Anderson et al., 2010).

Recent high-resolution transcriptional profiling studies have revealed extensive changes in the gene expression of the rice blast fungus *M. oryzae* during plant infection, highlighting a larger-than-anticipated repertoire of at least 548 effector-encoding genes crucial for blast disease development (Yan et al., 2023). This comprehensive analysis identified significant shifts in genes related to metabolism, cell signaling, and transcriptional regulation, with a focus on the co-regulated expression of effector genes. These effectors, showing temporal expression patterns and structural similarities without sequence homology, play a vital role in disease progression. A novel forward-genetic screen has further pinpointed RGS1 as a key modulator of effector gene expression, suggesting its dual role in appressorium development and pre-infection effector repression (Tang et al., 2023). Despite advances in understanding the regulation of effector genes, the functions of most effectors remain elusive. Future large-scale screenings are essential to identify effector targets within the host, providing insights into their role in suppressing host immunity and facilitating rapid pathogen colonization (Oliveira-Garcia and Valent, 2015).

A substantial portion of our existing knowledge regarding fungal effector function is derived from targeted mutagenesis and live cell imaging techniques (Jones and Khang, 2018; Sakulkoo et al., 2018). Live-cell imaging investigations have revealed that fungal effectors are either dispatched to the apoplast—the interstitial space between the fungal cell wall and the host plasma membrane—or directed to the host cell cytoplasm during infection (Mosquera et al., 2009). Apoplastic effectors fulfill a protective function by concealing pathogens from host detection, neutralizing host enzymes, or intercepting molecules poised to activate extracellular immune receptors (Zhang and Zu, 2014; Lo Presti et al., 2015). In contrast, cytoplasmic effectors, once secreted into the plant cell cytoplasm, target specific intracellular compartments to undermine host immunity (Zhang and Xu, 2014). This review delves into the latest understanding of the pathways and mechanisms governing the delivery of effectors and their role in inducing susceptibility during the biotrophic phase of *M. oryzae* infection. It underscores the critical importance of comprehensive effector characterization, highlighting its indispensable role in the development of innovative and effective strategies for the management and control of blast disease.

2.2. Conclusion and Outlook

The persistent threat of rice blast disease to global rice agriculture, now exacerbated by the emerging and global spread of wheat blast disease, underscores the urgent need for

novel control methodologies (Latorre et al., 2023). This urgency is heightened by the genomic dynamism and rapid evolutionary adaptation of *M. oryzae* in natural environments. An in-depth understanding of the processes involved in the production, deployment, and action of effectors is crucial for the development of rice varieties that are resistant to blast disease over the long term (Oliveira-Garcia and Valent, 2015). Although a vast array of putative effectors has been identified in *M. oryzae* (Mosquera et al., 2009; Yan et al., 2023), detailed molecular characterization of the interactions between these effectors and their host targets is still sparse. This gap highlights the imperative to identify and understand effector targets to fully elucidate the strategies employed by *M. oryzae* to undermine plant immune systems. Key to this understanding is the elucidation of the mechanisms underlying the translocation and release of cytoplasmic effectors into host cells. For example, deciphering how the Bas83 effector interacts with the EIHM may provide significant insights into the function of the BIC. The recent application of gene-editing technology to discover rice mutants that confer blast resistance is particularly significant (Sha et al., 2023). Taken together, these recent advancements highlight the importance of comprehensively understanding the effector biology in the *M. oryzae*-rice interaction to develop innovative and sustainable strategies for managing this fungal pathogen.

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3. FUNCTIONAL CHARACTERIZATION OF THE PLASMA MEMBRANE-ASSOCIATED EFFECTOR BAS83 OF THE RICE BLAST PATHOGEN *Magnaporthe oryzae*

Abstract

Rice (*Oryza sativa*) serves as a fundamental dietary component for more than half the global population, supplying crucial nutrients and energy. The rice fields are at risk from a fungal disease caused by the fungus *Magnaporthe oryzae*. The blast pathogen can cause up to 30% in yield losses annually. Plants have evolved complex defense mechanisms to protect themselves against pathogens. Conversely, *M. oryzae* secretes effectors to circumvent plant immunity and successfully invade rice cells. Then, this study aimed to enhance the characterization of the *M. oryzae* effector Bas83 and its influence on rice blast symptoms. To elucidate the function of the Bas83 effector in infection dynamics and disease progression of blast fungus, the study utilized the *M. oryzae* Guy11 wild-type (WT) strain and employed dual genetic manipulation techniques: overexpression and RNA interference (RNAi)-based gene silencing. In this study we were unable to generate Bas83:mRFP overexpression strains. However, we successfully generated three strains with substantial *Bas83* silencing (68%, 69%, and 79%) and demonstrated that this effector directly impacts the infection stages of the RNAi*Bas83* strains compared to the Guy11 WT. Additionally, in planta assays revealed that the RNAi*Bas83* strains exhibited lower disease scores (2-3) compared to the Guy11 WT (4-5), confirming that the Bas83 effector significantly impacts the development of blast disease. In conclusion, Bas83 plays a significant role in influencing the infection process and progression of blast disease in rice. Future studies, particularly those achieving success in *Bas83* knockout or gene deletion, could offer a more in-depth understanding of Bas83's role in these infection processes.

Keywords: effector protein, plant immunity, infection, *Oryza sativa*, *Pyricularia oryzae*

3.1. Introduction

Rice (*Oryza sativa*) is a staple food for over half of the world's population, providing essential nutrients and calories (Fukagawa and Ziska, 2019). Its global significance in agriculture and food security makes understanding and addressing threats to its cultivation vital (Rezvi et al., 2023). Like other crops, rice suffers from diseases caused by fungi, viruses, nematodes, and bacteria, resulting in significant annual yield losses worldwide (Savary et al., 2019). Rice blast, a fungal disease caused by the hemibiotrophic ascomycete *Magnaporthe oryzae*, is one of the most significant threats to rice crop producers, resulting in yearly yield losses of up to 30% (Talbot, 2003). Symptoms caused by *M. oryzae* can be found on the leaf, panicle, and other parts of the plants (Ashkani et al., 2015). Blast symptoms on leaves typically present as extended, diamond-shaped lesions, featuring a brown or reddish-brown border with a gray or white center (Ashkani et al., 2015).

Plants have evolved intricate defense mechanisms to protect against pathogens (Kaur et al., 2022). Plant immunity involves both constitutive defenses and inducible responses triggered by the recognition of pathogen-associated molecular patterns (PAMPs) (Jones and Dangl, 2006). These defense mechanisms are crucial for maintaining crop health. Fungal effectors play a pivotal role in the interaction between pathogens and plants. These molecules enable fungi like *M. oryzae* to manipulate host cellular processes, subverting plant defenses and facilitating successful infection (Stergiopoulos and de Wit, 2009).

Despite advancements in live-cell imaging, molecular biology, and effector investigation, the functional characterization of most reported *M. oryzae* effectors remains incomplete (Liu et al., 2023). A variety of effectors from *M. oryzae* have been delineated and studied., such as avirulent effectors (AVR-Pita, AVR-Piz-t, AVR-CO39AVR-Pia, AVR-Pik/km/kp, AVR-Pib, AvrPi9, Pwl1, Pwl2, ACE1 and AVRPi54), secreted proteins (Slp1, MC69 and Msp1) and biotrophy-associated secreted proteins (BAS1, BAS2, BAS3, BAS4, BAS83 and BAS107) (Fernandez and Orth, 2018; Oliveira-Garcia et al., 2023). *BAS83*, for example, was identified as a unique *M. oryzae* efector gene upregulated 36-fold during biotrophic invasion (Mosquera et al., 2009).

M. oryzae effectors are secreted using two distinct protein secretion pathways (Giraldo etal., 2013). Live-cell imaging of fluorescently-labelled effectors has shown that apoplastic effectors, such as Bas4, Bas113, and Slp1, are held within the extra-invasive hyphal matrix (EIHMx) (Kankanala et al., 2007; Masquera et al., 2009; Mentlak et al., 2012). This matrix constitutes the enclosed apoplastic compartment formed between the EIHM and the cell wall of the invasive hyphae (IH). On the other hand, cytoplasmic effectors, including Pwl2, Bas1, and Bas107, primarily accumulate within a plant-derived structure surrounded by the extra-invasive hyphal membrane (EIHM), known as the biotrophic interfacial complex (BIC), and subsequently translocate through the EIHM into the rice cytoplasm (Khang et al., 2010; Park et al., 2012; Giraldo et al., 2013; Giraldo and Valent, 2013).

The first two IH cells are linked to the translocation of cytoplasmic effectors (Kankanala et al., 2007; Khang et al., 2010). These effectors are secreted through a specialized Golgi-independent secretion system that exhibits insensitivity to brefeldin A (Giraldo et al., 2013). Recently, a live-cell imaging study demonstrated the colocalization of a *M. oryzae* effector (Bas83) with the rice plasma membrane (Oliveira-Garcia et al., 2023). This study provide evidence that the translocation of cytoplasmic effectors is mediated clathrin-mediated encocytosis in BICs (Oliveira-Garcia et al., 2023). Bas83 effector is suggested to

recruit plant membrane promoting the dynamic formation of vesicle-like membranous effector compartments that package effector proteins (Oliveira-Garcia et al., 2023).

Understanding how fungal effectors specifically impact the defense mechanisms of rice is crucial for developing effective strategies against rice blast disease. Fungal effectors can interfere with plant signaling pathways, suppressing immune responses, and promoting pathogenicity (Koeck et al., 2011). This study aimed to enhance the characterization of the *M. oryzae* effector Bas83 and its influence on rice blast symptoms.

3.2. Conclusion

The present study encountered limitations in characterizing the role of *Bas83* overexpression in the infection process *of M. oryzae*. However, we successfully generated three strains exhibiting RNAi-mediated *Bas83* silencing (with a minimum silencing efficiency of 68%) and utilized these to elucidate the significant role of the Bas83 effector in pathogen infection. This study demonstrates the influence of RNAi*Bas83* silencing on the infection stages of *M. oryzae*. Additionally, it was observed that the RNAi*Bas83* strains deviated from the standard infection pattern exhibited by the *M. oryzae* Guy11 wild-type and resulted in less severe rice blast symptoms. Further insights into Bas83's role in infection could be gleaned from future studies, particularly if strategies to achieve *Bas83* knockout or gene deletion are successful.

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4. DELETION OF PROTEIN DOMAINS AFFECTS LOCALIZATION AND SECRETION OF THE *M. oryzae* BAS83 EFFECTOR VIA THE BIOTROPHIC INTERFACIAL COMPLEX

Abstract

This study delves into the pathogenicity mechanisms of the rice blast fungus *Magnaporthe oryzae*, a significant agricultural pathogen causing extensive yield losses. *M. oryzae* employs a suite of secreted effectors to suppress host defenses and facilitate infection. These include apoplastic and cytoplasmic effectors, with distinct localizations and functions. Notable among these are the biotrophy-associated secreted (BAS) proteins, with varying localizations such as the biotrophic interfacial complex (BIC), extracellular invasive hyphal membrane (EIHM), and cell wall crossing points, playing crucial roles in host-pathogen interactions. Recent discoveries in effector biology, such as the association of Bas83 to host membranes, highlight the complexity of *M. oryzae*'s invasion strategies. This study was conducted to elucidate the role of the Bas83 protein domain in determining the effector's localization and secretion dynamics within host cells. To achieve this, a protein domain deletion strategy was utilized, employing a double-joint PCR methodology to create various constructs. These constructs were designed to represent five distinct scenarios: the full-length protein domain, deletion of the secretion signal (SS), and deletions spanning amino acid residues 22 to 61, 62 to 120, and 121 to 173, respectively. This approach allowed for a detailed analysis of the functional contributions of specific amino acid sequences within the Bas83 protein domain. The Guy-11 wild-type (WT) strains of *M. oryzae* were genetically transformed with the various Bas83 domain deletion constructs. These modified strains were then inoculated into YT-16 rice leaf sheaths, a cultivar known for its susceptibility to rice blast. Post-inoculation, the inoculated leaf sections were examined using confocal microscopy. This allowed for detailed observations of the cellular localization and secretion patterns of the mRFP-tagged Bas83 protein within the rice tissue. The results indicate that deletions within the protein domain significantly affect the localization and secretion of Bas83 in *M. oryzae* BICs. The intact Bas83 protein domain, when tagged with mRFP, accumulates preferencialy inside and around BICs. In contrast, deletions such as the secretion signal (SS), amino acid sequences 22-61, 62-120, and 121-173 blocks of their secretion into BICs, leading to their accumulation in the cytoplasm of fungal invasive hyphae. This altered distribution underscores the essential role of the Bas83 protein's domain structure in directing its proper localization and subsequent functional activity in the pathogenesis process.

Keywords: *Magnaporthe oryzae*, rice blast, effector protein, secretion, Double-Joint PCR

4.1. Introduction

The rice blast fungus, *Magnaporthe oryzae* (syn. *Pyricularia oryzae*), is responsible for one of the most destructive diseases in rice fields worldwide (Wilson and Talbot, 2009). This hemibiotrophic ascomycete causes a 6% annual yield loss and up to 30% during outbreaks (Wilson and Talbot, 2009; Savary et al., 2019). *M. oryzae* can infect rice plants at all stages of development, spreading in the fields by its asexual conidia (Huang et al. 2022a).

Once the three-celled conidium lands on the leaf surface, it attaches via spore tip mucilage, germinates, and initiates the infection process (Martin-Urdiroz et al., 2016). This process starts with the formation of a melanized appressorium, which generates high turgor pressure (up to 8 MPa) (Howard et al., 1991; Howard and Valent, 1996). The rice epidermis is then penetrated using physical force by a penetration peg, a result of this high turgor pressure, leading to host invasion (Martin-Urdiroz et al., 2016). The primary hyphae differentiate into bulbous invasive hyphae that fills the first invaded cell and move cell to cell via plasmodesmata (Kankanala et al., 2007).

In order to successfully invade the host tissue, *M. oryzae* possesses a variety of effectors that help it enter and establish infection by suppressing defense mechanisms (Oliveira-Garcia and Valent, 2015). Effectors are described as small secreted proteins that target the host receptors or defense-signaling mechanisms to inhibit immune responses (Wei et al., 2023). *M. oryzae* uses two distinct effectors secretion system to mediate host invasion (Giraldo et al., 2013). Apoplastic effectors are known to remain outside the host cell, dispersed and retained in the Extracellular Invasive Hyphal Membrane (EIHM), which outlines the entire Invasive Hyphae (IH) (Giraldo et al., 2013). On the other hand, cytoplasmic effectors accumulate in the biotrophic interfacial complex (BIC) and translocate across the Extracellular Invasive Hyphal Membrane (EIHM) into the cytoplasm of living cells (Khang et al., 2010; Giraldo et al., 2013). Certain cytoplasmic effectors demonstrate the capability to migrate to adjacent host cells, a mechanism hypothesized to facilitate preparatory steps for subsequent colonization phases (Khang et al., 2010).

The investigation of biotrophy-associated secreted proteins (BAS) began over a decade ago (Mosquera et al., 2009). These proteins can be found in both the cytoplasm and the apoplast (Mosquera et al., 2009). Like other well-known avirulence effectors, Bas1 and Bas2 predominantly accumulate in the BIC (Mosquera et al., 2009). Bas4 and Bas113 are recognized as apoplastic effectors that localize at the EIHM matrix following their secretion onto the external surface of the *M. oryzae* hyphae (Mosquera et al., 2009; Giraldo et al., 2013). In contrast, Bas3 displays a unique localization pattern, predominantly situated near cell wall junctions, which implies its role in the fungal infection processes of adjacent cells (Mosquera et al., 2009). Additionally, Bas170, identified as a cytoplasmic effector, has been observed to accumulate beneath the appressoria and in rice cell nuclei prior to the penetration by *M. oryzae* (Oliveira-Garcia et al., 2023). This accumulation underscores the initiation of the plant-pathogen interaction before the host is penetrated. Recently, the Bas83 cytoplasmic effector has been discovered to recruit host membranes and localize at membranous

compartments (Oliveira-Garcia et al., 2023). It is hypothesized that this recruitment of plant membranes facilitates rapid membrane turnover in the BICs (Oliveira-Garcia et al., 2023). Despite recent advances, numerous questions remain unresolved and warrant further investigation.

Most effectors proteins possess functional domains, which can significantly influence their roles and functions in the processes of infection and colonization (Dean, 2011). Importantly, most of the fungal effectors do not contain conserved protein domains, which challenges effector function characterizations and protein domain studies (Huang et al., 2022b). Effector domains can influence gene repression or activation by interfering in the chromatin (Frietze and Farnham, 2011). In *M. oryzae*, the MIF-like domain has garnered attention for its potential impact on cellular differentiation and virulence, as investigated by Galli et al. (2023). This domain acts as a regulator of fungal virulence, modulating the balance between biotrophic and necrotrophic phases during host infection. Additionally, it plays a role in down-regulating the necrotrophic stage and inhibiting rice cell death (Galli et al., 2023). The MoMIF1 domain in *M. oryzae* is positioned towards the middle of the protein sequence, which spans a total length of 392 amino acids. Specifically, the MIF domain is located between positions 164 and 277 base pairs (Galli et al., 2023). Additionally, the deletion of MoAA91 in *M. oryzae* has been shown to result in delayed appressorium formation and reduced virulence, underscoring its role in suppressing host defense (Li et al., 2020). This suppression occurs through the inhibition of the rice receptor CEBiP, which is involved in chitin-triggered immunity. MoAA91, secreted during appressorium development, comprises a signal peptide (1-19 amino acids), a Glyco_hydro_61 domain (20-235 amino acids), and a C-terminal chitin-binding domain (504-556 amino acids) (Li et al., 2020). Recent deletion assays, as reported by Gao et al. (2019), have highlighted the significance of specific domains for the complete functionality of MoGrp1. These studies demonstrate that these domains influence both the nuclear localization and the biological functions of the protein.

In this study, we investigate the Bas83 cytoplasmic effector, which is hypothesized to recruit plant membranes, thereby facilitating the formation of membranous compartments. To understand the impact of its protein domain on localization and secretion into living rice cells, we employed a protein domain deletion strategy.

4.2. Conclusion

The deletion of specific domains within the Bas83 protein critically influences its localization and secretion. Specifically, the removal of the secretion signal or any segment of the amino acid sequence hinders the accumulation of the Bas83 effector in the biotrophic interfacial complex (BIC) of *M. oryzae*. As a result of such deletions, the effector protein remains distributed within other hyphal structures of the fungus but fails to localize in the BIC, which is the pivotal site for its secretion into the cytoplasm of rice cells. This altered distribution underscores the essential role of the Bas83 protein's domain structure in directing its proper localization and subsequent functional activity in the pathogenesis process.

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5. FINAL CONSIDERATIONS

The comprehensive study of *M. oryzae*, particularly focusing on the Bas83 effector, has significantly advanced our understanding of the molecular interactions underlying rice blast disease, a major threat to global rice agriculture. Our findings demonstrate that Bas83 plays a critical role in the infection process of *M. oryzae*, as evidenced by RNAi-mediated Bas83 silencing in three strains, which led to a deviation from the typical infection pattern and reduced severity of rice blast symptoms. This suggests that Bas83 is crucial to the pathogen's ability to infect host plants effectively. Moreover, the study has revealed that the specific domain structure of Bas83 is crucial for its correct localization to the biotrophic interfacial complex (BIC), a key site for effector delivery into host cells. Deletions within Bas83's domain structure hinder its accumulation at the BIC, thereby disrupting its functional role in the pathogenesis process. This finding underscores the importance of effector domain structures in the disease mechanism of rice blast.

The urgency of addressing rice blast disease is further highlighted by the dynamic genomic nature and rapid evolutionary adaptation of *M. oryzae*. In this context, the application of gene-editing technologies, such as the discovery of rice mutants conferring blast resistance, offers promising avenues for developing durable disease management strategies.

In summary, this research not only elucidates the fundamental role of the Bas83 effector in the pathogenesis of rice blast disease but also highlights the potential of targeted molecular interventions in developing long-term disease resistance strategies. This study sets a precedent for future research aimed at a deeper understanding of Bas83 effector biology and its application in managing significant plant pathogens like *M. oryzae*.

These insights, coupled with the application of gene-editing technologies, pave the way for developing novel, long-term control strategies against rice and wheat blast diseases, addressing a critical need in global food security. Future research should continue to focus on effector biology and host-pathogen interactions to fully exploit these strategies for sustainable disease management.