Chapter 4:

Discussion and Conclusion

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The cell wall-degrading enzymes (CWDEs) produced by microbial pathogens, are of great importance due to their significant roles in pathogenicity and also industrial applications. Foliar plant pathogens encounter the host leaf cuticle and cell wall as initial barriers to infection. A CWDE thus may be a crucial virulence factor, as it may aid the pathogen in successful host invasion. For instance, cellulases are involved in host penetration and invasion in *M. oryzae* (Vu et al., 2012) and act as major virulence determinants in a tomato wilt bacterial pathogen *Clavibacter michiganensis* (Lorrai and Ferrari, 2021). Cellulases are also found to be involved in root penetration by a yellow potato cyst nematode, *Globodera rostochiensis* (Lorrai and Ferrari, 2021). Cutinase 2, a cutin-degrading enzyme that acts on leaf cuticle, is involved in host-surface sensing required for proper appressorium differentiation and formation of penetration peg in *M. oryzae* (Skamnioti and Gurr, 2007). Further, pectinases such as polygalacturonases (PG) are found to be involved in pathogenicity of a variety of saprotrophic fungal plant-pathogens such as 1) *Aspergillus flavus*, known to infect variety of cereals, legumes and cotton 2) *Alternaria citri*, a causal agent of black rot in citrus plants and 3) *Claviceps purpurea*, which infects rye and other related cereals. A necrotrophic pathogen such as *Botrytis cinerea* also produces a variety of pectinases, including PGs that can induce tissue collapse and necrosis in several important plants such as tomato, apple, broad-beans and *Arabidopsis* (Lorrai and Ferrari, 2021). In addition to its role in infection, PG activity, particularly that of endo-PG, is important in providing a source of host-derived nutrients in phytopathogenic fungi such as *C. purpurea*, *A. citri* and *B. cinerea*. Although a putative endo-PG (encoded by MGG_08938.6) is not important for pathogenesis, it is found to be involved in proper conidial germination in *M.*

oryzae (Quoc and Chau, 2017). Whereas, *M. oryzae* pectate lyase encoded by *MoPL1* is involved in tissue colonization during necrotrophic phase of the fungal infection cycle (Wegner et al., 2021). Interestingly, phytopathogens such as *Proteobacterium caratovorum* and *B. cinerea* can induce the expression of host pectinases (e.g., *Arabidopsis thaliana*-pectin methylesterase3) to enable tissue-colonization by the pathogens and make the plant more susceptible. Similarly, extracellular xylanase activity is required for virulence of *M. oryzae* and in tissue necrosis by *B. cinerea*. A functional xylanase has also been identified in a cotton root-knot nematode *Meloidogyne incognita*, suggesting its possible role as virulence factor in nematodes as well (Lorrai and Ferrari, 2021). In addition to the main groups of CWDEs, accessory enzymes such as feruloyl esterases also play an important role in plant cell wall degradation. In apple tree canker pathogen *Valsa mali*, feruloyl esterases are required for full virulence of the fungus (Xu et al., 2018). Here, considering the involvement of various CWDEs in pathogenesis, role of feruloyl esterases - a relatively under-explored group of CWDEs - has been studied in pathogenicity of an economically-important and a model plant-pathogen *M. oryzae*.

Feruloyl esterases (Fae) are known to hydrolyse the ester bonds between hydroxycinnamic acid and hemicellulose or certain pectins. A previous study, attempted to decipher the role of a predicted homolog of *Penicillium funiculosum* Fae in *M. oryzae* (MGG_01403.5) that was found to be upregulated during necrotrophic phase (72 hpi onwards) of the fungal infection cycle, but not required for pathogenicity (Zheng et al., 2009). In the present study, at least twelve putative Fae sequences containing a tannase or esterase domain (both characteristic features of Fae) have been identified in *M. oryzae*. Nine out of these twelve sequences belonged to the type B category with homology to well-characterized type B Fae sequences of *Aspergillus oryzae* (AoFaeB; PDB: 3WMT_B) and *Neurospora crassa*

(NcFaeB; GenBank: AJ293029). Surprisingly, the hmm-based search for the Tannase or Esterase domain containing Fae sequences could not find the previously studied Fae (MGG_01403; Zheng et al., 2009). This could be partly due to the difference in the search methodology followed and/or use of different versions of the annotated *M. oryzae* reference genome sequence. Here, a relatively recent version of the *M. oryzae* 70-15 reference genome was used for the *in silico* analyses. Multiple sequence alignment with these nine Fae in *M. oryzae* showed the presence of a conserved GXSXG motif and were found to belong to α/β-hydrolase-fold superfamily (Chapter 3, Section 3.1.1).

An HMM-based phylogenetic analysis across different fungal species showed a significant genetic diversity in the Fae sequences therein. Intriguingly, non-phytopathogenic filamentous fungi such as *Aspergillus spp.* and *N. crassa* showed presence of multiple Fae sequences. A further detailed phylogenetic analysis is required to understand any evolutionary aspect of it. Importantly, *M. oryzae* Fae1 (encoded by MGG_08737) showed a significant divergence, likely in a host-specific manner, as compared to its paralogs and orthologs across different fungi. A careful examination of the Fae1 sequences from different host-specific isolates revealed a few amino acid substitutions among them (Table 4.1). Although minor, these differences in the Fae1 among host-specific isolates could be interesting and worth studying further with a site-directed mutagenesis approach. Although, *in silico* analysis (as shown earlier in Chapter 3, section 3.1.3) suggested that the Fae function could be attributed to specific hosts, the defect in the *fae1*Δ mutant could not be correlated to any of the host species used in this study. However, it remains to be studied whether *fae1*Δ has a differential phenotype with any other cereal crops.

Host-specific *M. oryzae* isolates	Sites in multiple sequence alignment for Fae1		
	122	275	460
70-15, GY11, PH14, P131, Y34	V	D	S
BR32	I	D	A
CD156, MZ5-1-6	V	D	A
US71	V	N	S

Table 4.1. Fae1 sequence comparison between different host-specific *M. oryzae* isolates. Amino acid substitutions at specific positions during multiple sequence alignment are highlighted with red font.

CWDEs often occur as gene family and are generally under tight transcriptional regulation in phytopathogenic fungi. In *Alternaria alternata*, a necrotrophic fungal pathogen causing brown spot disease in citrus plants, although a host-specific mycotoxin (ACT toxin) is primarily involved in pathogenicity, CWDEs/CAZymes such as cutinases (AaCut3 and AaCut7) are likely required to breach the plant physical barriers for successful penetration and colonization. Interestingly, *Alternaria*-CWDEs including cutinases are regulated by Ste12 transcription factor, and Ste12-deficient mutant shows downregulation of various CWDE-encoding genes (Ma et al., 2019). In filamentous fungi, expression of genes encoding xylanases, cellulase and pectinases are regulated by transcriptional activators (e.g., XlnR and AceII) and repressors (e.g., AceI and Cre) and play differential roles during growth and pathogenesis (Quoc and Chau, 2017). *M. oryzae* genome contains Xlr1, a homolog of transcription factor XlnR (xylanase regulator), which is shown to be involved in regulation of pentose catabolic pathway but not in that of hemi-cellulolytic enzymes. Moreover, *xlr1*Δ mutant did not show any significant reduction in fungal infection, suggesting that D-xylose catabolism is not required during *M. oryzae* infection (Battaglia et al., 2013). On the other hand, MoSET1, a *S. cerevisiae SET1* ortholog that encodes a histone

H3K4 methyltransferase, is involved in regulating genes coding for a wide range of CWDE including various glycosyl hydrolases during infection-related morphogenesis in *M. oryzae* (Vu et al., 2012 and Pham et al., 2015).

In the present study, *M. oryzae* culture treated with rice leaf extract showed significantly enhanced extracellular Fae-activity, suggesting that *FAE*s are likely induced by host-derived factor(s) (Chapter 3, Section 3.1.6). Transcript profile of nine putative *FAE*s in *M. oryzae* under host- or pathogenicity-mimic conditions showed a differential gene expression pattern, with majority of them accumulating >1.5 fold higher, in response to individual plant cell wall components (Chapter 3, Section 3.1.7.1). This observation is consistent with a previous report on *Fusarium graminearum* (a causative agent of blight disease in wheat and other cereals), wherein, two out of seven putative *FAE*s viz. *FAEB1* and *FAED1* are induced in the presence of aromatic compounds such as ferulic acid and caffeic acid. It remains to be tested whether a combination of more than one host cell wall component would cause further upregulation in *FAE* expression in *M. oryzae*. Interestingly, *F. graminearum FAED1* is also upregulated by xylose, but repressed by glucose or galactose (Balcerzak et al., 2012). Similarly, presence of glucose represses expression of various CWDEs including exoglucanase and feruloyl esterase (encoded by MGG_05529) via a sugar sensor Tps1-dependent regulation in *M. oryzae* (Fernandez et al., 2012), suggesting a possible upregulation of CWDEs under nutrient-limiting condition that often prevails at the host-pathogen interface. However, role of any transcriptional regulator(s) affecting the Fae1 gene expression in *M. oryzae* has not been established. While such studies could provide insight into nutrient acquisition strategies employed by the fungus during pathogenesis, knowledge of transcriptional regulators of CWDEs would also provide an alternative approach to characterize them by specifically inactivating the regulator gene. A secreted *M. oryzae*

chitinase (MoChia1), that binds to chitin to suppress plant immune response during infection (Yang et al., 2019), could likely digest free chitin to monomeric NAG, which in turn could be sensed by the blast fungus to express Fae. However, this hypothesis needs to be tested further.

As shown in Chapter 3 (Section 3.1.7.2), *FAE1* was specifically upregulated during both pre-invasive appressorial development (12 and 24 hpi) and post-penetration host colonisation stages (48 to 72 hpi) of the infection cycle. This observation is consistent with a recent transcriptome study (Jeon et al., 2020) showing that MGG_08737 (Fae1) is significantly upregulated at 18 hpi (161-fold), 27 hpi (82-fold), 36 hpi (130-fold), 45 hpi (147-fold) and 72 hpi (228-fold) during infection cycle of *M. oryzae*. Accordingly, while deletion of *FAE1* in *M. oryzae* did not affect the vegetative growth and asexual development, the *fae1*Δ mutant is specifically impaired in invading and colonizing rice, barley and wheat tissue (Chapter 3, Section 3.2.5.1). Thereby, *fae1*Δ mutant showed significantly reduced pathogenesis on rice or barley plants, mainly due to impaired host invasion and colonisation. Importantly, very few appressoria (<5%) that were able to form invasive hyphae in the mutant were defective in spreading to the adjacent host cells and were rather restricted to the first cell invaded, even after prolonged incubation (96 hpi; Chapter 3, Section 3.2.5.1 and 3.2.5.2). These observations are in line with the hypothesis that Fae, and CWDEs in general, likely play an important role in cell-to-cell spread of the fungus within the host tissue, and subsequent necrotrophic growth phase (Zheng et al., 2009). Its intriguing that deletion of just one *FAE* (i.e., *FAE1*) led to such a significant defect in host invasion and that other putative *FAE*s in *M. oryzae* did not compensate for the loss of Fae1 function. However, as shown earlier (Chapter 3, Section 3.1.2), *in silico* analysis of Fae sequences in *M. oryzae* showed that Fae1 has been diverged and no significant identity was observed

between any two Fae proteins in *M. oryzae*. This possibly suggests why the loss-of-function of Fae1 alone could have a significant defect in host invasion and thereby pathogenicity of *M. oryzae*. Further studies on localization of Fae1 *in planta* could highlight any stage-specific secretion/accumulation of Fae1 during infection.

Plant cell walls are often modulated and restructured to increase the resistance against the pathogenic invasion (Quoc and Chau, 2017). While fungal CWDE could act as elicitors by mediating release of plant cell wall monomers that could induce host defense response (Bellen et al., 2006), host plants produce a variety of inhibiting proteins against such microbial CWDEs and help trigger an immune function. A cell wall-associated polygalacturonase inhibiting protein (PGIPs), employed by plants, is involved in activation of plant immune response (Ferrari et al., 2012). Plants also produce other microbial CWDE-inhibiting proteins such as pectin methylesterase inhibiting protein, xylanase inhibitor protein and xyloglucan-endoglucanase inhibiting protein to protect from invading pathogen (Juge, 2006 and Lionetti et al., 2007). Further, plants secrete fungal cell wall-digestive enzymes such as chitinases and glucanases, which release chitin and glucans that act as PAMPs and elicit immune response. However, fungal pathogen, for instance *M. oryzae*, masks such immune elicitors by accumulating α-1,3-glucan on its cell surface, to mask β-1,3-glucan and chitin in the cell wall of the infection structures (Quoc and Chau, 2017). Importantly, a delay in fungal penetration, as observed due to knockdown of *M. oryzae*-xylanases or cellulases (Nguyen et al., 2011 and Vu et al., 2012), allows timely/successful activation of host defense response.

CWDE-mediated release of plant cell wall carbohydrates might act as an energy source for the fungus at the host-pathogen interface and facilitate its entry and/or elaboration into the host. In *Aspergillus niger*, Fae act synergistically, with major CWDEs like cellulases, xylanases and pectinases, to degrade the complex plant cell wall carbohydrates (Faulds and Williamson, 1995). Interestingly, exogenously added glucose, as a simple carbon source, or ferulic acid (FA), a product of the Fae activity, significantly supported the invasive growth of the *fae1*Δ mutant in a concentration-dependent manner (Chapter 3, Section 3.2.5.3). A higher concentration of either of these compounds could significantly support invasive growth of the *fae1*Δ when compared with the WT. Although a combination of these two molecules did not show any additive effect on the *fae1*Δ, a significant reversal of the mutant defect in the presence of these molecules individually or in combination highlights the key role for such compounds at the plant-fungus interface (Chapter 3, Section 3.2.5.3 and 3.2.5.4). This shows that the Fae1-based feruloyl esterase activity, by targeting the plant cell wall, plays an important role in accumulating ferulic acid and/or sugar molecules, as a likely energy source, to enable host invasion and colonisation by *M. oryzae*.

Feruloyl esterases have a wide range of applications in other areas such as biofuel industry, food, cosmetic and pharmaceutical industry and paper and pulp industry, all of which involve plant biomass degradation (Dilokpimol et al., 2016). It is often used in conjunction with other plant cell wall deconstructing enzymes. Most of the applied aspects of feruloyl esterases have been studied in *Aspergillus spp*. Although *M. oryzae* is a phytopathogenic fungus, one could explore the potential use of recombinantly expressed Fae1 in industrial applications, both in terms of enzyme activity and range of substrate specificity. Similarly, ferulic acid, the product of feruloyl esterase enzyme action, has a large application in food and pharmaceutical industry (Dilokpimol et al., 2016). Thus, use of an efficient feruloyl

esterase (*M. oryzae* Fae1) for production of ferulic acid could also be explored. It has been reported that Fae can also act on synthetic esterified substrates such as methyl ferulate, methyl sinapate, methyl *p*-coumarate and methyl caffeate (Crepin et al., 2004). Therefore, it would be worth exploring whether Fae1, or feruloyl esterases in general, can also act on rutin complexed with glucose, thereby releasing quercetin, a plant flavanol with medicinal properties. This might also implicate another potential commercial application of Fae. Lastly, given the involvement of Fae1 specifically in pathogenesis of *M. oryzae*, it could be considered as a potential target for developing an antifungal strategy.

Concluding Remarks

Feruloyl esterases (Fae) hydrolyse the ferulic acid-polysaccharide linkages, thus weakening the cross-linked plant cell wall structure. This study characterized the *FAE*-gene family in the rice blast pathogen and studied *FAE1*, which is upregulated during plant infection, using a reverse genetics approach. Here, the *M. oryzae* Fae1 was found to be crucial for pathogenesis, likely by releasing the plant cell wall monomers that the fungus can use as an energy source during colonization of the host-tissue.

Key highlights of this study are as follows:

1. Fae sequences, across fungal species, show a significant genetic diversity, with a likely association between the divergence and host specificity in a few instances.
2. Fae expression in *M. oryzae* is responsive to the host-derived molecule(s) and are secretory in nature.
3. Fae1, a key feruloyl esterase in our study, acts as a virulence factor given its specific requirement for host colonisation by *M. oryzae* during plant infection.
4. Fae1 is a potential target for a novel antifungal strategy, and a candidate for a biotechnological application involving biofuel production.

I believe that the findings presented in this thesis, using a model plant pathogen, are of interest to a large scientific community studying plant-microbe interactions. This study shall also be of interest to the researchers developing novel antifungal strategies or working on industrial applications of different plant cell wall degrading enzymes. This is the first study to my knowledge showing the role of a feruloyl esterase in pathogenicity of a filamentous phytopathogenic fungus dependent on a specialised (appressorium-mediated) host entry.