6 Facilitating the Fungus: Insights from the Genome of the Rice Blast Fungus, *Magnaporthe Oryzae*

Nicole M. Donofrio, Jinnan Hu, Thomas K. Mitchell, and Richard A. Wilson

6.1 Introduction: The Past, Present, and Future Importance of the Rice Blast Fungus

Magnaporthe oryzae is the causal agent of rice blast disease and one of the most devastating plant pathogenic fungi, worldwide. Rice blast has caused and continues to cause epidemics wherever rice is grown. A few examples of its sordid history on rice include a 1953 epidemic in Japan, with a 700,000 million ton yield loss, a panicle blast epidemic in Korea in 1978, resulting in 40 % yield loss, a 2006 epidemic in China where in one province alone, 1.3 million metric tons of yield was destroyed, and in 2009 where Arkansas rice farmers lost up to 80 % of their yield due to a blast outbreak (McBeath and McBeath 2010; http://deltafarmpress.com/rice/rice-blast-increasing). History, therefore, dictates that the next epidemic is a matter of "where, when, and how bad", rather than "if". Since 1979, when *M. oryzae* (then *Magnaporthe grisea*) was first defined as a genetically useful fungal "model" by Barbara Valent et al. , research has been intensively focused on characterizing fungal pathogenicity mechanisms, and plant host resistance mechanisms. The last decade of rice blast research has been defined by the age of "omics", or the ability to look at large sets of genes and biological processes simultaneously. Our review herein attempts to summarize these "omics-age" results and to highlight their importance in the elucidation of fungal virulence. We conclude our review with future perspectives on the importance of pushing this research forward, as the threat of a new wheat-infecting strain of *M. oryzae* looms menacingly for U.S. wheat varieties.

Over a decade ago, Couch and Kohn (2002) used molecular taxonomic markers to split what was once known as *M. grisea* into two species; *M. grisea* isolates grouped together into one clade associated with infection of grass (*Digitaria*) species, while *M. oryzae* isolates grouped into another clade associated with rice infection. Currently, there is an ongoing discussion about nomenclature for the rice blast pathogen, *M. oryzae* or *Pyricularia oryzae*, the teleomorph and anamorph, respectively. *Magnaporthe oryzae* has been widely adopted by the community and appears in numerous published studies; while the anamorph (asexual) form is commonly found in the field and associated with infection, *M. oryzae* is used by a large proportion of the scientists who study rice blast and is the term recognized most

N. M. Donofrio (✉)
Plant and Soil Sciences Department, University of Delaware, Newark, DE 19716, USA
e-mail: ndonof@udel.edu

J. Hu · T. K. Mitchell
Department of Plant Pathology, The Ohio State University, Columbus, OH 43210, USA
e-mail: jinnan.hu@gmail.com

R. A. Wilson
Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, NE 68583, USA

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widely by those outside the rice blast research community. As we move toward a "one fungus one name" system, phylogenetic studies like the one from Luo and Zhang 2013, may help clarify taxonomic distinction of current and future isolates, and together with input from the rice blast research community, will ultimately determine the most appropriate name for the rice blast fungus. For the purposes of this chapter, we will utilize the widely recognizable *M. oryzae*.

Magnaporthe oryzae subdivides further into a strain isolate that infects (wheat) *Triticum* species, which was first described in Brazil in 1985 (Igarashi et al. 1986). Since then, wheat blast has caused major epidemics in South America; should it spread into the United States, an epidemic will likely occur as current U.S. wheat cultivars are susceptible to the disease (Marangoni et al. 2013). We are just beginning to scratch the surface in terms of understanding this particular isolate and how to control it. In the next section, we describe current comparative genomics projects of numerous isolates and what this information can tell us about virulence mechanisms and host adaptation.

Apart from its past, present, and potentially strong future concerns as a major pathogen of small grain crops, *M. oryzae* is also a genetically tractable organism with a well-defined and fascinating infective life cycle. Valent et al. began defining this fungus' utility as a model for genetics with their studies on the vegetative diploid phase, and development of genetic mutants to examine specific traits, such as melanin production (Crawford et al. 1986; Chumley and Valent 1990). Since then numerous studies and reviews have chronicled the *M. oryzae* infective life cycle, hence we need not provide substantial detail here (reviewed in Li et al. 2012). In brief, conidiospores, the asexual and arguably the most important stage of the fungus for disease, will germinate on a hydrophobic leaf surface. Given sufficient moisture levels, the spore will undergo autophagy to allow proper formation of the penetration structure called the appressorium. This dome-shaped, melanized structure utilizes mechanical pressure to breach the leaf surface, growing invasively into the first epidermal cells by means of invasive hyphae. This brief phase is rapidly followed by a lengthier biotrophic phase whereby bulbous hyphae grow within epidermal cells, producing "biotrophy-interfacial complexes" or "BICs" (Kankanala et al. 2007; Mosquera et al. 2009; Fig. 6.1). BICs are, in effect, factories for the production and release of fungal effectors into plant cells, as beautifully detailed by Khang et al. (2010). By 48 h post-inoculation, the fungus has become necrotrophic, producing thin, invasive hyphae followed by eventual development of lesions and production of more conidiophores (Fig. 6.1).

The vast majority of recent studies on *M. oryzae*'s infective life cycle were greatly facilitated through the completion of genome sequencing in 2005 (Dean et al. 2005), funded by both USDA and NSF via the jointly administered USDA/NSF Microbial Sequencing Program. This is evidenced by the fact that to date, this seminal paper has been cited 741 times. Part of the Broad Institute's Fungal Genome Initiative, the *M. oryzae* genome has been publicly housed on its website for the last 8 years. The genome is robust, having been through eight iterations of annotation, and within the last year alone was visited by 18,000 individuals (personal communication from web developers at Genome Sequencing and Analysis Program, Broad Institute), indicating an extremely high impact. In the next section, we explore how, as more isolates are sequenced, comparative genomics helps us unravel how particular isolates came to infect important small grain crops such as rice and wheat. There are more than 200 rice blast researchers world-wide, and while we attempted to be comprehensive, due to space considerations, it is not possible to include all recent published works enabled by the genome sequences.

6.2 The Genomic Era: Genome Structure and Variation Informs Host Adaptation

Over the past decade, the collective rice blast research community has been developing extensive resources for whole genome studies.

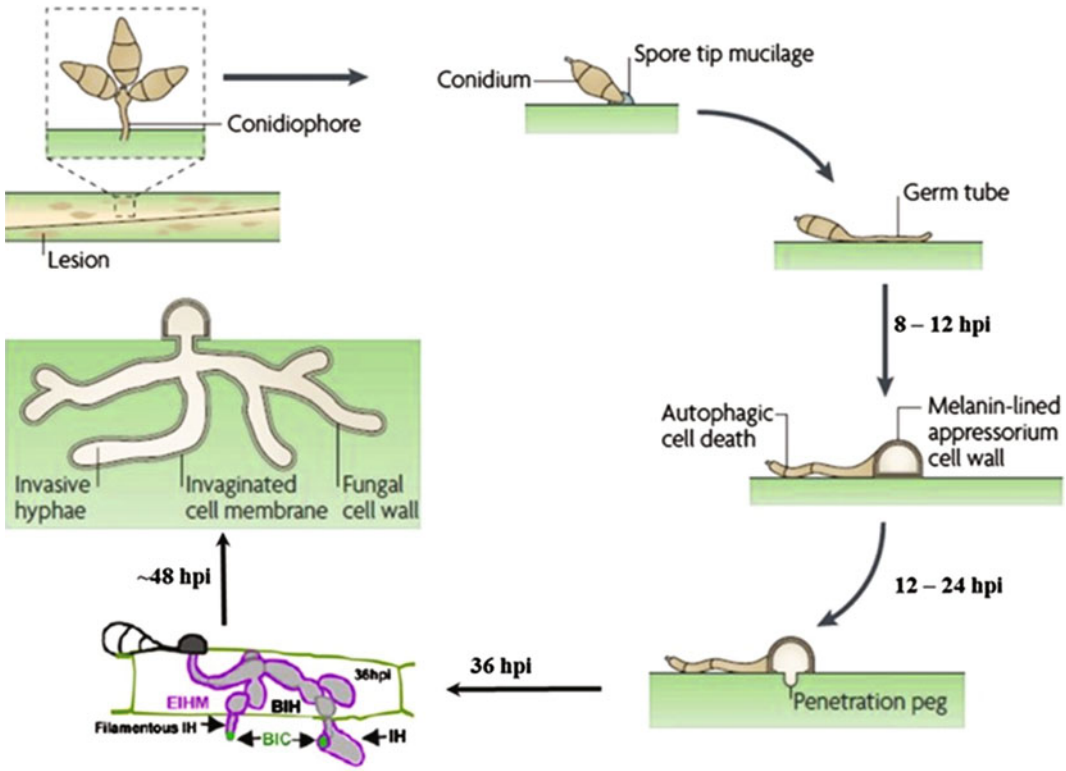

Fig. 6.1 Infective life cycle of *M. oryzae* adapted from Wilson and Talbot (2009) and Mosquera et al. (2009). The figure has been adjusted to include the biotrophic portion of the life cycle, which includes production of bulbous invasive hyphae (*IH*), biotrophic-interfacial complexes (*BICs*) and an extra-invasive hyphal membrane (*EIHM*)

The first funded effort to perform a whole genome sequence assembly was initiated by R. Dean's group in 2000 and took several years to complete with via a coordinated collaborative effort betweenacademic institutes and the Broad Institute using first generation sequencing technologies (Dean et al. 2005). During that time, a parallel effort to generate a complete assembly of the smallest chromosome, chromosome 7, was completed using a BAC-by-BAC approach (Thon et al. 2006). Since the initial release of the 70–15 *M. oryzae* genome in 2002, several annotated versions of the genome have been completed, with the most recent being V8 (Broad Institute 2010) released in April, 2011. 70–15 is not a naturally occurring isolate; it was derived from a fertile cross between isolate GUY11 (rice-infecting isolate from the French Guyana) and an isolate from weeping lovegrass. Progeny from that cross were backcrossed to GUY11 for several generations to produce 70–15 (Leung et al. 1988). This isolate was chosen as the model to be sequenced as it was the most widely used laboratory strain at the time, and its host range, physiology, and gene content were better understood than in other isolates.

6.2.1 The Flagship Sequence Strain 70–15 Launches Additional Efforts

Sequencing of 70–15 was a major accomplishment. It was the first (publically available) filamentous fungal pathogen sequenced, paving the way for others to come. The genome assembly became a powerful tool and a launching pad for the development of a wealth of genome-based

tools providing new insights into the evolution of fungal pathogenesis of plants. Notable, initial discoveries from studies of 70–15 include: (1) identification of a large suite of secreted proteins, many of which likely function as effectors; (2) dramatic invasion of active transposable elements; (3) expanded families of PKS and NRPS genes that function in secondary metabolism; (4) sets of predicted virulence associated genes; (5) localization of known avirulence (*Avr*) genes to telomeric regions and (6) evidence for ancient RIP (repeat-induced point mutation) mechanisms suggesting sexual recombination existed in nature at one point (Ikeda et al. 2002; Dean et al. 2005; Farman et al. 2007). These discoveries fueled an explosion of research, particularly in the area of functional genomics.

With the advent of next generation sequencing, researchers across the globe initiated projects to generate genome assemblies of other lab strains, authentic field isolates, and mapping lines for either interspecific or intraspecific crosses. Additional efforts resulted in sequences being generated and made available for related species including *M. grisea*, *Magnaporthe poae* (summer patch) and *Gaeumannomyces graminis var. tritici* (*G.g.t.*; causal agent of take all disease) (http://www.broadinstitute.org). A survey of the community reveals that a minimum of over 40 sequenced genomes have been generated for fungi in the Magnaporthaceae. These include isolates representing *M. oryzae* (>22 genomes), wheat blast (4 genomes), foxtail millet blast (2 genomes), finger millet blast (2 genomes), *Digitaria* blast (1 genome), *G.g.t.* (1 genome), *M. poae* (1 genome), and perennial ryegrass blast (1 genome). The only other fungal genome with more sequenced isolates is *Saccharomyces cerevisiae*, which has genomes available for as many as 52 isolates. The power of having numerous genomes of different isolates is just being realized with multiple laboratories worldwide currently initiating projects to sequence the genomes of hundreds of isolates from a large diversity of rice growing regions. These genomes represent one of the richest collections of genome sequences for any specific lineage of fungi. However, the current paradigm is that most of these are sequestered in the databases of individual researchers. As comparative genome studies are completed and published, many are expected to be released and the possibilities for comparative genomic queries, including adaptation strategies to new hosts, will be endless.

6.2.2 Location, Location, Location: Genome-Assisted Identification of Avirulence Genes

While many Resistance (R) genes in rice have been identified and cloned, the identification of *Avr* genes in *M. oryzae* has been limited. There are three *Avr* genes involved in host species specificity: PWL1 (Kang et al. 1995), PWL2 (Sweigard et al. 1995), and Avr-CO39 (Farman and Leong 1998), and at least six involved in rice cultivar specificity, including Avr-Pita (Orbach et al. 2000), ACE1 (Bohnert et al. 2004), AvrPiz-t (Li et al. 2009), AvrPia (Miki et al. 2009; Yoshida et al. 2009), Avr-Pii (Yoshida et al. 2009), and Avr-Pik/km/kp have been cloned to date (Yoshida et al. 2009). While R proteins are reported to contain some of the canonical, conserved functional domains—such as NBS-LRR—no obvious sequence or structural patterns can be assigned to *Avr* genes, which make their identification difficult. However, "genome-wide association analysis" (GWAS) can now help in identifying novel *Avr* genes based on the association between *Avr* genes and the cultivar specific virulence. In one study focusing on Avr-Pita family including AvrPita1, AvrPita2, and Avr-Pita3, it was found that AvrPita1 and AvrPita2 were associated with transposon elements and that they localized to different chromosomes in various field isolates (Chuma et al. 2011). This location variability, as well as high diversifying selection, makes it almost impossible to identify them without genome sequencing and association analysis.

The first identification of novel *Avr* genes resulting from a whole genome comparison was

reported in 2009 (Yoshida et al. 2009). An attempt to associate polymorphisms in secreted proteins in 70–15 and different field isolates with pathogenicity resulted in little success. However, comparison of the sequence of a field isolate "Ina168", which is known to contain nine *Avr* genes, with 70–15 identified a 1.68 Mb unique region in 70–15. Through an association study for genes located on this isolate-specific region, three new *Avr* genes were identified. The project demonstrated the value of the 70–15 reference genome for identifying novel *Avr* genes in field isolates.

One of the first published whole genome comparison studies of *M. oryzae* isolates was published in 2012 (Xue et al. 2012). Two field isolates of *M. oryzae*—isolate Y34 from China and isolate P131 from Japan were sequenced and their genomes compared with the reference strain 70–15 (version 6). Sequencing was performed using a mix of traditional Sanger technology and next-generation 454 sequencing. The de novo assembly of the two field isolates showed a slightly smaller genome size than 70–15. One significant finding was the large amount of isolate-specific sequence; 1.69 Mb identified in P131 and 2.56 Mb in Y34. Confirmation of isolate-specific sequences was completed by CHEF gel and Southern hybridization using unique sequences as the probes. Genes were predicted de novo in Y34 and P131, with the result showing a similar total number of genes as compared to 70–15. While all three genomes shared a "core" gene set, comparisons among the three genomes showed that there are about 300 field isolate-specific genes absent from the 70–15 genome, with most of them annotated with an "unknown" function. To determine the biological function of these, nine Y34-specific genes and three P131-specific genes were selected for functional characterization. While most of these deletion mutants produced no obvious phenotypic changes, four mutants showed altered virulence. Multiple duplicated genomic sequences were also identified in the analysis, which were enriched in chromosome II, IV, V, and VII. The non-synonymous to synonymous nucleotide substitution rate (Ka/Ks) analysis showed 697 genes with only non-synonymous nucleotide substitutions and six genes with a Ka/Ks >1. Six known virulence factors were among these. With regard to repetitive sequences, a similar percentage ($\sim$10 %) was identified in all three genomes, while the two field isolates showed them to have a more similar distribution pattern. A total of roughly 200 genes were disrupted in the three genomes by transposable elements, with many associating with isolate-specific or duplicated sequences. This study revealed that gain or loss of unique genes, DNA duplication, gene family expansion, and frequent translocation of transposon-like elements are all important factors contributing to genome variation of *M. oryzae*.

It is interesting to note that these first two studies using whole genome sequencing analysis confirmed by CHEF gel analysis that *M. oryzae* contains supernumerary chromosomes that vary in size, and that *Avr* genes may localize to them. Supernumerary chromosomes, sometimes called "conditionally dispensable chromosomes", were first discovered and gained attention in *Alternaria* species (Johnson et al. 2001; Hatta et al. 2002); however, through whole genome sequencing studies they were reported in different fungi including *Fusarium oxysporum* (Ma et al. 2010), *Nectria haematococca* (Han et al. 2001; Coleman et al. 2009), *Mycosphaerella graminicola* (Stukenbrock et al. 2010), *Cochliobolus heterostrophus* (Tzeng et al. 1992), and *Leptosphaeria maculans* (Leclair et al. 1996).

6.2.3 Is Bigger Always Better? Genome Size Comparisons Across Fungi

Since before the application of next generation sequencing, it was widely known that fungal genomes are variable with regard to size, but the degree of variability is only now being confirmed. Fungal phytopathogens and oomycetes

have extreme variability in genome size. For example, the genome size difference between smut fungi *Ustilago maydis* (~19–21 Mb) and *Phytophthora infestans* (~220–280 Mb) is nearly 15-fold (Kamper et al. 2006; Haas et al. 2009; Raffaele et al. 2010; Schirawski et al. 2010). Generally, filamentous Ascomycete phytopathogens have larger genomes than their yeast type relatives (Haas et al. 2009; Spanu et al. 2010; Duplessis et al. 2011). For example, the powdery mildew *Golovinomyces orontii* has the largest sequenced genome in that phylum at approximately 160 Mb (Spanu et al. 2010). For Basidiomycetes, the rust fungus *Melampsora larci-populina* has the largest genome at over 89 Mb, although the coffee rust fungus is predicted to be close to 500 Mb (Duplessis et al. 2011; personal communication, N. Donofrio). Compared with pathogens, genomes of non-pathogenic fungi sequenced so far are typically ~40 Mb, such as *Aspergillus oryzae* at 37 Mb (Machida et al. 2005), *Neurospora crassa* at 41 Mb (Galagan et al. 2003) and *Schizophyllum commune* at 39 Mb (Ohm et al. 2010). However, the trend of fungal phytopathogens toward larger genomes is not absolute, and some filamentous pathogens actually have relatively small genomes, possibly due to gene loss such as in *Albugo laibachii* (Kemen et al. 2011), intron loss as in *U. maydis* (Kamper et al. 2006), or reduced transposon content in *Sclerotinia sclerotiorum* (Amselem et al. 2011). Given the large variability and plasticity in the size of fungal genomes, it is perhaps not surprising the published and unpublished results of sequencing additional *M. oryzae* isolates shows the genome size to vary dramatically with different isolates having up to 2 MB differences in genome size.

Unpublished reports on single nucleotide polymorphism (SNP) variation show that about 10,000–15,000 SNPs and 3,000–10,000 Indels exist between three field isolates and the lab strain, 70–15. Among the SNPs, 355 were expected to introduce premature stop codons, 752 were expected to alter initiation methionine residues, and 132 were expected to disrupt splicing donor or acceptor sites. Large effect insertion/deletion polymorphisms (IDPs) were identified as the cause of frame shifts. There were 47,083 IDPs identified for all strains and 910 among them were large effect IDPs. It was reported in previous studies that SNPs and IDPs were not distributed evenly along the genome, but were in fact found enriched in some regions and thus make sequences in the regions highly variable (Wei et al. 2007).

It is clear that the genomes of filamentous fungi are plastic and continue to evolve rapidly. Ongoing re-sequencing projects for *M. oryzae* from around the globe support the fact that the genome of this fungus is highly variable between field populations. The potential consequence of this level of variability is the fungus being able to rapidly adapt to changing environmental conditions and new hosts as well as the ability to overcome host resistance mechanisms.

6.3 Transcriptomics: How Genomics Has Enabled Progress from Microarray to RNA-Seq

Since at least 2003, researchers have been using large-scale approaches to study gene expression in the rice blast fungus; topics explored have ranged from gene expression during different developmental stages, to gene expression during various abiotic stresses, to examining the impact on gene expression of losing one specific gene. Pre-genome the methodology of cDNA library sequencing was employed (Takano et al. 2003), but after its public release researchers were able to capitalize on genome-wide approaches. The first whole genome microarray chip was built in 2004 as a coordinated effort among public universities, the Broad Institute, and Agilent Technologies.

6.3.1 Coordinated Global Gene Regulation of Developmental Programs

Given the importance of the specialized infection structure for rice blast disease, much research has focused on characterizing genes

thought to be involved in appressorial development. However, pre-genomics, this had to be performed gene-by-gene. The year 2005 saw the publication of the rice blast genome, along with the first global gene study on appressorial development using the first generation of the Agilent microarray (Dean et al. 2005). This experiment yielded families of genes, including membrane-anchored CFEM domain proteins that play a role in appressorial formation, supporting the genetic CFEM mutant *pth11* identified in 1996 by Sweigard et al. (1998). The genome has enabled studies on global expression during certain developmental regimens, or in response to stress. In 2008, researchers again used the Agilent microarrays to perform a more in depth analysis of appressorial development, and how this process is affected by addition of cyclic AMP (Oh et al. 2008). A comparison of *M. oryzae* spores germinating on hydrophobic (appressoria-inducing), hydrophilic (appressoria-inhibiting), and hydrophilic plus cAMP (appressoria-inducing) yielded the previously unknown finding that appressorium formation requires coordinated down-regulation of genes involved in protein synthesis and associated translational machinery, and up-regulation of genes involved in protein degradation and lipid metabolism (Oh et al. 2008). Putatively secreted genes were also found to be enriched in the upregulated group. In 2012, a microarray-based study examined gene expression during the important process of conidiation, the formation of asexual spores (Kim and Lee 2012). In the rice blast fungus, these are the most important spores in terms of the fungus' ability to infect. Prior to this study, conidiation in fungi had been studied in depth in the model ascomycetes *Aspergillus nidulans* and *N. crassa*, but only a small handful of conidiation-related genes were known in *M. oryzae*. Their study found 603 and 557 genes to be induced and repressed during conidiation, respectively, with the majority of them being involved in catalytic activity.

6.3.2 May-Day! Gene Expression Under Stress

There is a rich history of studies on the relationship between nitrogen stress and fungal infection, from plant to human pathogens (reviewed in Solomon et al. 2003; Hartmann et al. 2011). In the 1990s, studies revealed that nitrogen limitation (NL) in the rice blast fungus was a cue for induction of pathogenicity genes such as the hydrophobin *MPG1* and the regulatory loci *NPR1* and *NPR2*, supporting the idea that NL conditions prevail within the host (Talbot et al. 1997). This pivotal research laid the ground-work for examinations into whether in vitro NL mimicked host colonization. Again, the *M. oryzae* microarray was put to work examining global gene expression during NL (Donofrio et al. 2006). Five known pathogenicity genes, including *MPG1*, were upregulated in response to a shift to nitrogen limitation, and genes for nitrogen catabolite repression were also induced. This study also revealed a new role for a serine protease in both nitrogen utilization and virulence. In 2011, global gene expression during nitrogen stress was re-visited in a larger experiment; gene expression profiles of *M. oryzae* undergoing various types of in vitro and in planta stresses revealed that overall, fungal gene expression in planta most closely resembled that of fungal gene expression during NL (Mathioni et al. 2011). Stresses including carbon and nitrogen, oxidative-inducing paraquat, temperature upshift and invasive growth in rice and barley were then investigated. Results exposed a group of 55 and 129 "stress consensus" genes that were increased or decreased in expression during every condition, respectively. The elevated genes appeared to be involved in carbon metabolism, membrane function or oxidoreduction reactions, while the down-regulated genes were largely involved in molecular transport, signal transduction, and nitrogen metabolism. The ability to analyze how groups of genes behave in concert provides the evidence that

acquisition and/or breakdown of nutrient sources is an important function for the rice blast fungus during substantially different stresses.

Most recently, a 2013 study by Park et al. (2013) used bioinformatics approaches to identify 495 predicted transcription factor genes in *M. oryzae*, 206 of which were examined for expression changes during different stress conditions. Stress conditions studied included several in planta conditions, and 26 in vitro stresses; the magnitude of this study, and the new discoveries on how this fungus regulates stress responses, could only be made manifest by a robust genome sequence.

6.3.3 Global Gene Expression Analyses of Mutants: One Gene Can Impact Many

Global gene expression has allowed us to characterize genetic fungal mutants in new ways; not only can we collect physiologic and phenotypic data, but also obtain a molecular profile of a how fungal genes collectively behave in a single-gene deletion mutant. Table 6.1 provides examples of single-mutant microarray studies that have been performed in *M. oryzae* to date. Some single gene deletions, like the high-affinity phosphodiesterase *PDEH*, impact both pathogenicity and expression of virulence-related genes (Zhang et al. 2011). Others, like the class VII chitinase, *Con7*, and the conidiation regulator, *MoHOX2*, affect cell wall-associated genes and conidial regulation (Odenbach et al. 2007; Kim and Lee 2012). *Con7* was identified in an earlier study, and the mutant was found to be completely defective in its ability to cause disease (Shi and Leung 1995). Global gene expression studies on the *Δcon7* mutant revealed a suite of cell wall-related genes whose expression was dependent upon this putative transcription factor (Odenbach et al. 2007). One in particular, the *CHS7* gene encoding a class VII chitin synthase, was also defective in its ability to cause disease via misshapen appressoria.

The RNA-binding protein RBP35 is fascinating, as its deletion impacts both full virulence and preprocessing of mRNAs via polyadenylation (Franceschetti et al. 2011). Preprocessing of messages, especially at the 3' end, is essential for proper expression, as well as regulation (Millevoi and Vagner 2010). Hence, it is no surprise that the targets of RBP35 would be subjects of interest. Microarray results identified 159 genes with differential expression. A subset of five were down-regulated only in the 3' UTRs and not in their coding sequences. Furthermore, these genes showed alternative splicing, and several additional experiments revealed that *RBP35* was likely involved in processing them with longer 3' UTRs. Interestingly, one of these genes was 14-3-3, a family of proteins that integrates signals through regulation of signaling cascades (Morrison 2009). *MoHOX2* is a homeobox transcription factor; when deleted, fungal mutants produce the spore-bearing stalks, but cannot produce conidia and hence are deficient in disease (Kim et al. 2009). In 2012, microarrays were employed to obtain the fungal profile of this mutant (Kim and Lee 2012). The authors compared their whole-genome expression results with microarray results from an earlier conidiation study in wild type, and determined a "core set" of genes involved in this important developmental process. They identified 137 significantly down-regulated genes that were increased during conidiation in the wild type fungus, and repressed in the *ΔMohox2* mutant. Among this gene set were those involved in regulation, such as transcription factors and kinases, metabolic genes like peptidases and synthases, and cell wall associated genes like chitin-binding proteins. Here, the ability to access the entire genome coupled with genetic mutation, provided much-needed insight into the processes regulating conidiation. Understanding what the rice blast fungus requires in order to form asexual spores provides opportunities to block these pathways, thus control the disease.

Table 6.1 Single mutant whole-genome expression studies

Mutant	Function	Genome Impacts	References
Microarray approach			
Δcon7	Transcription factor	Cell wall-associated proteins	Odenbach et al. (2007)
Δmgwc-1	Blue light receptor	Melanin biosynthetic genes	Kim et al. (2011)
ΔpdeL	Low-affinity phosphodiesterase	Altered expression of many genes	Zhang et al. (2011)
ΔpdeH	High-affinity phosphodiesterase	Required for expression of path genes	Zhang et al. (2011)
Δrbp35	Gene-specific polyadenylation factor	Alternative splicing in pre-mRNA	Franceschetti et al. (2011)
Next-gen. approach			
Δdcl1	Dicer 1	NDE[a]	Raman et al. (2013)
Δdcl2	Dicer 2	Specific size classes of sRNAs	Raman et al. (2013)
Δdcl1/ Δdcl2	Double knock-out	Specific size classes of sRNAs	Raman et al. (2013)
Δdcl2/ Δdcl1	Double knock-out	Specific size classes of sRNAs	Raman et al. (2013)
Δago1	Argonaute	In progress[b]	Raman et al. (2013)
Δago2	Argonaute	In progress	Raman et al. (2013)
Δago3	Argonaute	Conidiation	Raman et al. (2013)
Δrdrp1	RNA-dependent RNA polymerase	Conidiation	Raman et al. (2013)
Δrdrp2	RNA-dependent RNA polymerase	In progress	Raman et al. (2013)
Δrdrp3	RNA-dependent RNA polymerase	Specific size classes of sRNAs	Raman et al. (2013)

[a] *NDE* no discernable effect

[b] In progress indicates that the mutants were used as a tool for the first publication (Raman et al. 2013) and are being more thoroughly examined for additional work

6.4 Turned Down and Turned Off: Genome-Enabled, High-Throughput, Single Gene Analyses

Analyses of the genome sequence resulted in a plethora of data on predicted genes and their function. However, predictions, at some point, must be elevated to the status of "confirmed" in order to truly understand the function of the roughly 11,000 genes in *M. oryzae*. To this end, several high-throughput mutant collections were generated to allow for forward genetic phenotypic screens. In 2007, Betts et al. and Meng et al. detailed the generation and utilization of about 60,000 randomly tagged insertion lines. The latter study in part provided important information on the distribution of T-DNA insertions with respect to gene locations, while the former in part examined phenotypic assays as well as recovery of flanking sequences (Betts et al. 2007; Meng et al. 2007). In the same year, Lee et al. published on their collection of approximately 21,000 random insertion lines, generated exclusively through Agrobacterium-mediated transformation (Choi et al. 2007). While development of constructs for these random insertions studies could not have easily been performed without genome knowledge, the abundant information about virulence mechanisms in this fungus gleaned from reverse genetics, would not exist without the genome sequence.

6.4.1 Turned Down: RNA Interference Goes to Work in Magnaporthe oryzae

RNA interference (RNAi) is a powerful genetic tool, especially for organisms recalcitrant to other gene targeting approaches. Although the homology-based recombination approaches in the rice blast fungus show acceptable efficiency, RNAi provides value especially for essential genes that cannot be fully deleted and for gene families where members have redundant function (Fire et al. 1998). The very first evaluation of RNAi applied in *M. oryzae* as a genetic modification tool, was reported in 2003 (Kadotani et al. 2003), where authors first tested the ability of three different forms of RNA to induce RNA silencing by introducing plasmid constructs expressing sense, antisense, and hairpin RNAs. Similar to the results found in other organisms, hairpin RNAs were found to have the highest efficiency. Additionally, this study showed at least three different sizes of small interfering RNAs (siRNAs) with function. Although being demonstrated as an acceptable method for knocking down the expression of genes, the construction of a hairpin RNA vector requires two steps of oriented cloning, and thus, the application was limited to a small or moderate scale. In 2008, the same research group reported an improved method: a dual promoter system of RNA silencing in filamentous fungi, allowing a high-throughput application (Nguyen et al. 2008). The silencing vector, pSilent-Dual (pSD1) carries two convergent opposing RNA polymerase II promoters and multicloning sites between them. Using this novel system, 37 calcium signaling-related genes were targeted and silenced, with the resultant 26 (70.2 %) genes examined being involved in hyphal growth, 35 (94.6 %) involved in sporulation, and 15 (40.5 %) involved in pathogenicity. Another RNAi study focusing on the function of xylanase enzymes was reported in 2011, where a combination of 40 bp sequences from each of the 10 endoxylanase genes were used to make an artificial RNAi trigger, which was coined as the "building blocks method" (Nguyen et al. 2011). After the functionally redundant xylanase genes were significantly reduced in mutants, their pathogenicity was reduced resulting from a lowered number of lesions, rate of penetration, and extent of infected cells. The level of pathogenicity reduction was associated with the degree of silencing of xylanase mRNA. Overall, the RNAi approach has been demonstrated to operate in *M. oryzae* and has been adapted for high-throughput functional genomics projects.

6.4.2 Turned Off: Forward or Reverse, Genetic Analyses Have Provided Many Answers

In 2007, Lee et al. performed functional analysis of the *M. oryzae* genome by designing and executing an efficient screening process, looking for mutants defective in different life cycle stages (Choi et al. 2007). They utilized Agrobacterium-mediated transformation to generate and then screen approximately 21,000 mutants, from which they recovered about 200 mutants defective in various aspects of the fungus' infective life cycle. This screen proved bountiful, stimulating at least five in-depth studies of fungal genes involved in various aspects of pathogenicity, some of which, like *MoDES1*, would have likely never have been found via reverse genetic means. Among the more striking examples is the discovery of *MoMCK1*, a MAP kinase kinase kinase that when deleted, causes autolysis of mycelia and reduced production of aerial hyphae (Jeon et al. 2008). *MoDES1* is another excellent example; this serine-rich protein surprisingly has a role in tolerance to oxidative stress (Chi et al. 2009). When deleted, mutants inoculated onto plants elicit a stronger suite of defenses from the host, such as defense gene expression and production of reactive oxygen species. The *MoDES1* gene sequence was not reminiscent of ROS-scavenging genes, and without a forward genetic approach, its important function might not otherwise have been discovered. Additionally, the T-DNA

screen unearthed *MoSDR1*, a short chain dehydrogenase/reductase, which, to the best of our knowledge, had not yet been characterized in any way in this fungus. Upon deletion, the fungus is rendered substantially defective in many developmental aspects, including conidial formation and germination, appressorial formation, penetration, and invasive growth (Kwon et al. 2010).

Reverse genetic strategies have also contributed immensely to the determination of gene function, most of which could not be accomplished, without knowledge of the genome sequence. In 2009, Kershaw and Talbot used the genome sequence to design primers to amplify and fuse gene-of-interest flanking regions to selectable markers for high throughput deletion of all the autophagy genes (Kershaw and Talbot 2009). As mentioned previously, this family of genes is involved in development of a fully mature and functional appressorium. In 2010, Kim et al. compared fungal genomes for homeobox domain-containing proteins. They found eight such genes in the *M. oryzae* genome, two of which, *MoHOX2* (mentioned in the preceding section) and *MoHOX7* are required for conidiation and appressorial formation. The discovery of *MoHYR1*, likewise, was via reverse genetics, revealing a gene that, like the aforementioned *DES1*, is involved in regulating ROS scavenging in the fungus and required for successfully coping with the plant's defensive oxidative burst (Huang et al. 2011). In 2013, genome-wide comparisons and analyses allowed for the identification of ABC transporters in *M. oryzae*, known to have important roles in toxin tolerance and resistance. Fifty such genes were identified in *M. oryzae* from which three were selected for further examination. Upon deletion, *MoABC6* and *7* were reduced in conidiation but not virulence, while *MoABC5* was highly reduced in virulence (Kim et al. 2013a, b). Together, these studies based on genome sequence comparisons, set the stage for a better understanding of transcriptional control during pathogenesis, response to oxidative stress, and the role of transporters during invasive growth.

These genetic approaches provide the foundation for determining the mechanism behind how each of these genes, likely in conjunction with other genes, control stages of pathogenicity, and importantly, how the fungus adapts to changing conditions.

6.5 Genome to Protein to Pathway: The Importance of Biochemistry in Rice Blast Infection

Understanding the biochemical processes that allow *M. oryzae* to infect and destroy plants, and how these pathogenic pathways are controlled, is important in understanding the underlying biology of rice blast disease. Prior to the availability of the genome, several studies had success in identifying and characterizing biochemical pathways necessary for host infection. For example, Thompson et al. (2000) contributed to the elucidation of melanin biosynthesis by characterizing a second naphthol reductase gene cloned from *M. oryzae* by identification of cDNA fragments with weak homology to the cDNA of trihydroxynaphthalene reductase. Other biochemical pathways associated with infection that were characterized at this time include the glyoxylate cycle, where the gene encoding isocitrate lyase, *ICL1*, was isolated from an appressorial specific EST library (Wang et al. 2003). Subsequent disruption showed the glyoxylate cycle is required for full virulence; and glycogen and lipid turnover were shown to be pathways important for turgor generation (Thines et al. 2000). Several large-scale efforts to characterize biochemical pathways were also initiated. Hamer et al. (2001) coupled high throughput gene deletion strategies with extensive phenotyping to validate pathways, such as tryptophan and phenylalanine catabolism, which might be developed as new fungicide targets (Hamer et al. 2001). Tanzer et al. (2003) studied the response of *M. oryzae* (and other model and phytopathogenic fungi) to the toxic glutamine synthetase inhibitor glufosinate and the toxic

alcohol analogue allyl alcohol during growth on a wide range of nitrogen and carbon sources, respectively. This provided an outline of the likely metabolic pathways operating in these fungi, and how they could be controlled. Their results suggested that the regulation of nutrient utilization by *M. oryzae* might provide insights into the nutrients typically encountered by the fungus, i.e., during infection.

6.5.1 Genome-Enabled New Insights on Nutrient Requirements

Following the sequencing of the *M. oryzae* genome, progress in elucidating important biochemical pathways has accelerated. Availability of the genome has allowed biochemical pathways to be deduced using post-genomic, high-throughput technologies, such as transcriptomics, proteomics and metabolomics, which rely on an available genome for their utility. For example, in the aforementioned study by Mathioni et al. (2011), they compared transcript abundances under different stress conditions and in planta growth to determine that carbon metabolism and oxidoreduction reactions were likely important processes for infection. Soanes et al. (2012) recently used next generation sequencing to identify transcripts associated with appressorium development. By mapping the transcripts to the genome, and using the genomic sequence to reveal homologous genes with known functions, the authors were able to highlight the role of autophagy, melanin biosynthesis, and lipid metabolism in appressorium function. In addition, the observation that quinate permeases and the quinate utilization cluster are upregulated in appressoria, suggested that quinate produced in rice cells might be an important carbon source for *M. oryzae*. Proteomics studies have also started to reveal biochemical processes associated with infection. Gokce et al. (2012) and Franck et al. (2013) have studied the conidial and appressorium proteomes and identified proteins associated with melanin biosynthesis, lipid metabolism, glycogen metabolism, and anti-oxidation. Advances in metabolomics also promise to reveal how metabolite identity and amount changes during infection, thus giving a glimpse of the underlying biochemical pathways active during infection. Most success to date in this regard has been achieved by monitoring metabolite changes in the host cell following infection. Using metabolic fingerprinting, Parker et al. (2009) have demonstrated changes to metabolism in the cells of rice and *Brachypodium distachyon* following challenges with *M. oryzae*. They demonstrated that in infected cells, the generation of mannitol and glycerol appear to drive hyphal growth. Moreover, while some metabolite changes during infection might be consistent with ameliorating ROS activities, elevated levels of glutamate, aspartate, and GABA suggested a major source of nitrogen for protein synthesis in growing hyphae. Therefore, although metabolite measurements of infected leaves have so far been unable to ascertain changes occurring in the fungus, documenting metabolite changes in the host during infection generates information regarding which sources of nitrogen, for example, the fungus appears to manipulate the plant into producing, thus indicating which biochemical pathways are active to assimilate nutrients from the host.

6.5.2 Gene to Pathway: Comparative Biochemistry Reveals Infection-Related Pathways

Availability of the genome has also enabled genes encoding structural enzymes to be mapped by sequence homology to known biochemical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG). Comparing biochemical pathways for *M. oryzae* predicted in KEGG with those from either other plant pathogenic fungi, or nonplant pathogenic fungi, may indicate what pathways might be important for a pathogenic lifestyle. Also, KEGG facilitates the identification of genes involved in specific biosynthetic pathways, such that those pathways that can be dismantled using homologous gene replacement. One recent example is the disruption of

methionine biosynthesis in *M. oryzae*. Using KEGG, the methionine biosynthetic pathway was identified and the function of one member, cystathionine beta-lyase, was disrupted (Wilson et al. 2012). The resulting mutant strains could form appressoria and penetrate host cuticles, but failed to grow in the cell, suggesting the ability to make methionine is critical to in planta growth. Therefore, combining genomic and KEGG analyses can provide new insights into the biology of infection by *M. oryzae*.

Other biochemical pathways have been targeted since the publication of the genome. These include eight lipase genes and a gene encoding fatty acid beta-oxidation enzyme, identified from the genome, which were shown to have roles in plant infection via the metabolism of fatty acids and the subsequent generation of acetyl CoA (Wang et al. 2007). In addition, mitochondrial β-oxidation (Patkar et al. 2012), and lipid mobilization via the action of peroxisomal alanine: glyoxylate aminotransferase (Bhadauria et al. 2012) are biochemical processes recently demonstrated to be necessary for rice blast disease.

The availability of the *M. oryzae* genome has facilitated the further understanding of biochemical pathways important for disease. Two other studies of note, on a larger scale than those discussed earlier, have used the genome to facilitate biochemical understanding. First, Kim et al. (2010) utilized advanced ChIP-chip and microarray gene expression technologies to locate the genes that are regulated by the Ca^{2+}/calcineurin responsive transcription factor *MoCRZ1*. Increased intracellular Ca^{2+} and calmodulin levels leads to dephosphorylation of CRZ1 by calcineurin, resulting in its nuclear localization. Loss of MoCRZ1 abolished the ability of *M. oryzae* to cause disease (Choi et al. 2009). The study by Kim et al. (2010) aimed to identify the genes directly activated by MoCRZ1. Three hundred and forty-six genes under MoCRZ1 were identified by ChIP-chip and after validation by microarrays, 140 were found to be directly regulated by MoCRZ1. Not only did this study reveal some known virulence factors such as Apt2 to be under MoCRZ1 control, but also revealed feedback regulation of the calcium signaling network by MoCRZ1. Thus, this work provided new insights into calcium signaling, and its outcome, in filamentous fungi.

A second study of note used the available genome sequence to undertake a genome-wide characterization of the components of autophagy. Autophagy is a biochemical process that results in the turnover of bulk cell cytoplasm and cellular components during starvation. Autophagy was known to be necessary for appressorium function (Veneault-Fourrey et al. 2006), and here, Kershaw and Talbot (2009) identified all 22 genes with identities to known autophagy components and disrupted them in the *M. oryzae* genome. In this manner, they demonstrated that loss of any of the 16 genes necessary for non-selective macroautophagy abolished pathogenicity due to loss of appressorium maturation, while the six genes necessary for selective forms of autophagy, such as pexophagy and mitophagy, were dispensable for plant infection. This genome-wide analysis, therefore, characterized nonselective autophagy as being a biochemical process essential for the establishment of rice blast disease.

6.5.3 Proteomic-Based Insight Into Infection

Genome-wide proteomics studies, which are facilitated by the availability of the *M. oryzae* genome, have recently been employed to shed light on the cellular processes associated with infection-related development. Proteomic changes occurring during germination and appressorial formation in response to cAMP treatment were recently determined using nanoLC-MS/MS and spectral counting-based, label-free quantification (Frank et al. 2013). Altered protein abundances during appressorial development revealed the involvement of a wide range of cellular activities during infection-related

development. These included enzymes involved in cell wall biosynthesis and remodeling (including six of the seven *M. oryzae* chitin synthases and other proteins involved in chitin metabolism, and proteins involved in melanin biosynthesis); changes in putative transporter abundances (including altered abundances of ion transporting ATPases and the down-regulation of two putative maltose transporters following conidial germination); altered levels of ribosomal proteins following germination; changes in proteins associated with secondary metabolism; and up-regulation of secreted proteins during appressorium formation. Thus, this work provides important insights into the cellular processes involved in the early stages of rice infection (Frank et al. 2013).

Additional proteomic studies have leveraged the *M. oryzae* genome to analyze the identity of apoplastically secreted proteins during early infection (Kim et al. 2013a, b), and to compare the proteome of wild type conidia and those of a mutant defective in the conidial regulator *COM1* (Bhadauria et al. 2010). Moreover, proteomic studies contributed to the elucidation of metabolic processes altered in a glucose-sensing mutant compared to wild type (Fernandez et al. 2012), thereby providing insights into glucose metabolism in *M. oryzae*, and led to novel mechanistic insights into the processes governing appressorium formation through the identification of ubiquitination, mediated by polyubiquitin, as an essential process in the formation of appressoria (Oh et al. 2012).

Taken together, the work described here demonstrates how basic knowledge of biochemical pathways underpinning rice blast infection has improved with the advent of the rice blast genome. Future challenges remain, particularly in planta, where studies are starting to reveal what biotrophic-specific biochemical pathways might be involved in infection (Wilson et al. 2012; Fernandez et al. 2013). Both of these gene functional studies support earlier, genome-enabled microarray work of Donofrio et al. (2006), which indicated early infection occurred in a nitrogen-poor environment.

6.6 Creating a "Home": Fungal Effector Biology Gets a Boost from the Genome

The importance of pathogen produced effectors during the infection process has been well-studied in the infamous oomycete pathogen, *P. infestans* (reviewed in Kamoun 2006). However, effector biology has lagged behind in pathogenic filamentous fungi. In order to truly comprehend infection, we must learn how the fungus creates an appropriate environment for itself. In preceding sections, we have described genes contributing to its pre-penetration, penetration, and invasion stages. We are also beginning to understand the gene regulation and biochemistry underlying how *M. oryzae* adapts to a changing nutrient environment in the plant host. For the final section of this review, we delve into current efforts to understand how the rice blast fungus generates a hospitable environment for itself. Through the efforts of Valent, Talbot, and others, we begin to understand what these effectors are, where they are "built" and how they are delivered into the host cell.

6.6.1 Re-visiting Gene Expression Analyses: What Are the Effectors?

In 2009, Mosquera et al. developed a technique to enrich for *M. oryzae* effectors. A common limitation in examining fungal genes during invasive, in planta growth is a preponderance of plant RNA compared to fungal RNA, and more specifically, fungal RNA that is from noninvasive hyphae or spores. They overcame this limitation by combining knowledge of the timing of the biotrophic stage of *M. oryzae* (i.e., most hyphae are synchronously invading epidermal cells by 36 h post-inoculation; Fig. 6.1) and using an YFP-labeled fungal strain, in order to visualize groups of highly-infected cells. These areas were harvested for RNA preparation, thus highly enriching for RNAs potentially encoding effectors. Global gene expression patterns were

obtained using microarrays, comparing the enriched IH RNA, with plate-grown mycelial RNA. Overall, 1,120 genes were up-regulated in the IH RNA of which 262 were highly expressed and contained known *Avr* genes such as *Avr-PITA* (Jia et al. 2000). Four genes were characterized and defined as biotrophy-associated secreted (BAS) proteins. These are small, cysteine-rich proteins with secretion signals and no other discernible domains, based on homology searches. This study was seminal in opening the door into the world of pathogenic, filamentous fungal effectors. But the questions remained, how are they being delivered and how do they function?

6.6.2 How Are Effectors Moving, and Where Are They Going?

The Mosquera et al. (2009) study went on to determine that some of the BAS proteins, such as BAS1, are generated in the BICs, the aforementioned blast interfacial complexes that form when bulbous hyphae have invaded host cells. The BIC-generated BAS proteins were found to be cytoplasmically located in their plant hosts, while other small, secreted proteins found in the 2009 study were apoplastically localized (Khang et al. 2010). Research then turned to how effectors were being delivered into host cells. Part of the story comes from the deletion of an endoplasmic reticulum (ER) associated chaperone called LHS1, involved in proper protein import and folding in the ER. When this protein is deleted in *M. oryzae*, the fungus shows strongly attenuated virulence, specific to an inability to conidiate, penetrate, and grow biotrophically in a susceptible host (Yi et al. 2009). This study contributed to the effector story by revealing that without proper folding fungal proteins, among them effectors, lose the ability to function and can lead to impaired virulence.

Building upon this work, the labs of Valent and Talbot collaborated to determine how effectors were secreted into their plant hosts. Initially, they examined the *M. oryzae* genome for evidence of homologs to genes known for roles in polarization and secretion. Genome analysis identified six genes, including several involved in exocytosis, the polarisome and the Spitzenkörper functions (Giraldo et al. 2013). Using genetic deletions coupled with informed use of fluorescent marker constructs, they determined that each piece of the secretion machinery localized at growing hyphal tips. Importantly, they also demonstrated that effectors known to be secreted from BICs into host cells (Khang et al. 2010) were retained in the BICs in a Δ*exo7* mutant and a Δ*sec5* mutant. Importantly, both genes are part of the eight-protein exocyst, involved in docking vesicles to exocytosis sites. Both mutants were defective in secretion of particular effectors such as effector *Pwl2*, and both were reduced in pathogenicity, presumably as a result of inability to secrete specific effectors into host cell's, rendering a favorable environment for further infection.

6.7 Future Prospects and Potential Cures?

The previous section on effector biology in *M. oryzae* is the culmination, but not yet the finale, of where information from the genome can take us. Questions remain as to localization of the entire suite of fungal effectors, and what their host targets might be. Furthermore, it is now up to the research community at large to utilize knowledge gleaned from the genome-enabled comparative genomics, transcriptomics, proteomics, and genetics experiments (only some of which were described here), to find preventative therapies and/or cures for rice blast disease. For example, once we know host targets of these effectors, we might be able to block the interaction, or to manipulate the host target through transgenic approaches, making it unavailable to the fungal effector. Another strategy would be to utilize knowledge from the nutrient studies obtained both through biochemical and transcriptomic approaches, and again manipulate the host environment to reduce the amount of a

potential nutrient source, or sequester it to make it unavailable to the pathogen. This leads directly to an important area that we are just beginning to understand—the role of transporters in *M. oryzae*. Two recent studies suggest that transporters play important roles in pathogenicity, and may as yet represent another way the rice blast fungus, and perhaps fungi in general, manipulates or takes advantage of a host environment. One study comes from the anthracnose pathogen, *Colletotrichum gloeosporioides* and genetic deletion of its ammonia transporters (Shnaiderman et al. 2013). When the methyl ammonia permease *MEPB* was deleted, ammonium was not secreted. As a consequence, appressorial formation was reduced, as well as levels of cyclic AMP (cAMP), the secondary messenger required for proper appressorial formation. The aforementioned 2013 study on ABC transporters in *M. oryzae* (Kim et al. 2013a, b) revealed that three out of the 50 identified had roles in virulence, however the remaining 47 still need to be examined. Recently, Fernandez et al. (2012) identified a MATE transporter to play an important role in virulence. This citrate transporter is required for glucose assimilation, sporulation and pathogenicity and is the first study on this type of transporter in filamentous fungi. *M. oryzae* has two additional putative MATE transporters. Together, transporter studies in filamentous, pathogenic fungi are in an early discovery stage, and can provide valuable clues as to what the fungus needs to import and export in order for successful infection to occur. Transporters could indeed represent an excellent strategy for targeted inhibition.

The power of comparative genomics is just being realized. While we have learned a great deal about the plasticity of the *M. oryzae* genome from the few dozen genome sequences available for this fungus, what remains to be fully explored is the forces and mechanisms at work that drive these changes. Clearly, fungal genomes like that of *M. oryzae* are changing constantly, understanding rate, means, and type of changes will be critical for predicting disease control. For example, several scientists studying rice blast all over the globe are initiating associative genetic projects looking at the genomes of hundreds of *M. oryzae* field isolates within and across regions to develop markers associated with pathogenicity and virulence. These studies, when combined with whole genome-based genotyping of the rice host, will be powerful in predicting the most appropriate rice lines to cultivate, and will result in sustainable and ecofriendly approaches. These types of studies bring us closer to expertly deploying resistance genes and predicting when and how resistance will break down, so that major epidemics can be averted.

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