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CHAPTER 23

SEX AND VIRULENCE IN BASIDIOMYCETE PATHOGENS

GUUS BAKKEREN, EMILIA K. KRUZEL, and CHRISTINA M. HULL

INTRODUCTION TO THE BASIDIOMYCOTA

Among the fungi, the largest and most explored groups are the Ascomycota and the Basidiomycota, which comprise the Dikarya. There are over 40,000 described species of Ascomycota, including the model yeast *Saccharomyces cerevisiae*, many filamentous fungi, and most of the human fungal pathogens (Hawksworth, 2001; Mitchell, 2005; Morrow and Fraser, 2009). The Basidiomycota contains approximately 30,000 described species, including mushrooms, yeasts, rust, and smut fungi. These fungi are known for their ubiquitous nature in the environment; they are found in nearly all terrestrial ecosystems and are the major wood-degrading organisms on earth, essential for maintaining the earth's carbon cycle (Hibbett, 2006; Kirk et al., 2001; Mitchell, 2005). Some, such as certain mycorrhizal symbionts, are universal in these ecosystems where they are essential for forest health and may have been fundamental to land colonization by plants (Martin et al., 2008). While no single morphological feature or property defines all basidiomycetes, the production of several specialized cell types occurs in the majority of the basidiomycetes described thus far. Stable dikaryons (the occurrence of two haploid nuclei in the same cell), clamp cells, and basidia all characterize these fungi and play important roles in sexual development (Casselton and Olesnicky, 1998; Raper, 1983). In turn, sexual development is closely linked with the success of the basidiomycetes, particularly those that cause disease in plants and animals. Approximately 8000 rust fungi and smut species are plant pathogens, and at least 40 basidiomycetes cause disease in mammals (Blackwell, 2011; Kirk et al., 2001; Mitchell, 2005). Two genera represent the models most utilized for studying the pathogenesis of the basidiomycetes. Smut fungi of the genus *Ustilago* have been studied extensively for their plant pathogenic properties, and members of the *Cryptococcus* species complex are among the leading causes of fungal meningitis in humans (Banuett, 1995; Park et al., 2009). In this chapter, we discuss the roles of sexual development in virulence of these two representative pathogen-containing groups. We also

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present information emerging from recent studies of the cereal rust fungi, which are among the most devastating crop pathogens (Bolton et al., 2008; Brown and Hovmøller, 2002; Goellner et al., 2010; Singh et al., 2011).

Mating-Type Loci

Sexual development of fungi is controlled by a specialized region of the genome known as a mating-type (*MAT*) locus (Fraser and Heitman, 2003). Akin to the sex chromosomes of larger eukaryotes, this region of the genome houses the genes required to establish differences between sex cells (aka mating types in fungi). Thus, mating-type determination by *MAT* is critical for establishing which cells will engage in productive fusion events. There is striking variation among fungal *MAT* loci; however, ascomycetes generally have a single *MAT* locus that houses transcription factors that govern cell type. This results in two mating types (a bipolar mating system) (Herskowitz, 1988; Kronstad and Staben, 1997). Basidiomycetes are more flexible in their *MAT* configurations. For these fungi, it is common to have two *MAT* loci: one encoding homeodomain transcription factors and one encoding pheromones and pheromone receptors (Casselton and Olesnicky, 1998; Raper, 1966). In principle, this allows for four mating types (a tetrapolar mating system). However, many basidiomycetes have multiple alleles of the genes housed in these loci, increasing the potential production of many more mating types in a population—up to tens of thousands of potential mating types for higher mushrooms. To undergo sexual reproduction, it is critical that distinct cell types be established, and the *MAT* locus is the lynchpin in this process. Although the specific nature of contributions of *MAT* loci to sexual development varies among species, in all cases described, developmental cell types are controlled by the expression of different pheromones and pheromone receptors and the function of a heterodimeric transcription factor complex composed of a pair of *MAT*-encoded homeodomain proteins with one member contributed by each parent (Casselton and Olesnicky, 1998; Hsueh and Heitman, 2008; Kronstad and Staben, 1997). This paradigm governs *MAT* control of sexual development and provides a common platform from which to explore the diversity of basidiomycete *MAT* loci in greater detail.

Sexual Development

Consistent with the diversity of *MAT* loci, sexual development among basidiomycetes varies in its details. A relative constant, however, is that sexual development is initiated when cells of compatible mating types fuse with one another, and this fusion eventually results in the formation of a fruiting body known as a basidium (after which this group is named) on which sexual spores are produced (Casselton and Olesnicky, 1998). A second characteristic feature of basidiomycetes is the production of a prolonged binucleate cell known as a dikaryon, which results from the fusion of compatible cells containing genetically distinct nuclei. Although cell fusion and cytoplasmic mixing occur, the nuclei do not undergo immediate karyogamy and remain distinct. The nuclei are replicated relatively synchronously but are maintained as single entities throughout this growth phase (Iwasa et al., 1998; Raper, 1983). In some basidiomycetes, the dikaryon can grow indefinitely, but in others, the dikaryotic state is transient. In both cases, the process of dikaryon replication and maintenance is coordinated to ensure that each cell contains two nuclei, one from each parent. A cell type critical for this process is the clamp cell, a specialized protrusion that must fuse with the filament to maintain two nuclei in each filament cell aligned at the cell midline (Inada et al., 2001; Scherer et al., 2006). The selective pressures leading to the maintenance of dikaryons are essentially unknown. The complex nuclear migration events that occur require significant input of energy, and the outcome is eventually the same: To complete sexual development, the two nuclei in the terminal filament cell eventually fuse with one another and undergo meiosis. Studies of mushrooms indicate that dikaryons diverge phenotypically to a much greater extent than monokaryons do, suggesting that dikaryotic growth could provide an increased potential for genetic and phenotypic variation, resulting in a fitness advantage (Clark and Anderson, 2004).

Pathogens of Plants and Animals

Most human fungal pathogens are ascomycetes; however, basidiomycete pathogens of humans are a growing group, in large part because of increasing numbers of immunocompromised individuals due to other diseases, malnutrition, and complex medical procedures and drug regimes (Böhme and Karthaus, 1999; Brown, 2004; Mitchell and Perfect, 1995; Park et al., 2009). Some of these species may have adapted to life in human/animal hosts via the acquisition of tolerance mechanisms that provide resistance to various hostile environmental conditions (higher temperatures, pH, iron deficiency, etc.) (Casadevall et al., 2003; Roetzer et al., 2011a,b). The best-studied of these "new" human basidiomycete pathogens is the *Cryptococcus* species complex consisting of *Cryptococcus neoformans*, *Cryptococcus gattii*, *Cryptococcus albidus*, and *Cryptococcus laurentus* (Casadevall and Perfect, 1998). *C. neoformans* is the model for this group as well as for basidiomycete human pathogens overall. Very abundant, but not immediately life threatening, is a basidiomycete causing skin disease, *Malassezia globosa*, implicated in human dandruff and atopic eczema. The genome of *M. globosa* has recently been sequenced, and although the sexual cycle has not yet been observed in nature or under laboratory conditions, the genome sequence revealed conserved genes involved in mating and meiosis in other fungi, and what may represent a bipolar *MAT* locus, suggesting that this pathogen may be capable of sex (Xu et al., 2007). Other basidiomycete species implicated in opportunistic invasive diseases belong to the *Trichosporon*, *Rhodotorula*, and *Sporobolomyces* genera.

Among fungal species adapted to life in plants or trees, basidiomycetes account for several pathogen groups that cause major destruction worldwide. These include many rust fungi (Pucciniales) on many plant and tree hosts, and smut fungi (Ustilaginaceae) and bunts (Tilletiaceae) on grasses, including cereal crops (Bolton et al., 2008; Brown and Hovmøller, 2002; Goellner et al., 2010; Mitchell, 2005). Many of these fungi have come to prominence because they attack important agriculture and forestry crops; for instance, the wheat stem rust fungus has been observed since biblical times (Chester, 1946). Surveys of cereal rust fungi and targeted breeding of plants for resistance have contributed greatly over the last 60 years to efforts to maintain production levels in the presence of this unabating disease pressure (Li and Wang, 2009; Singh et al., 2011). However, targeted breeding has lead to rounds of introductions of disease resistance genes, monoculture production, and, consequently, the evolution of new strains of fungi overcoming introduced resistance properties. The emergence of novel, very virulent wheat stem rust isolates in Uganda in East Africa in the late 1990s (Ug99 and derivatives), likely through sexual recombination, has proven to be a severe threat to wheat production worldwide (Singh et al., 2011). In addition, large amounts of fungicides are applied annually to control smuts, bunts, and rusts, a costly necessity often not available in many parts of the world and often devastating to the environment.

SMUT FUNGI

In smut fungi, pathogenicity is tightly associated with sex because in these plant pathogenic fungi, mating between haploid partners of different mating types is necessary to form the pathogenic dikaryotic cell type. Diploid teliospores that are the dispersal and survival propagules of these plant pathogens germinate to produce the basidium in which meiosis occurs and four haploid basidiospores are produced. This cell type is nonpathogenic and can be easily germinated and maintained as budding cells and also manipulated in the laboratory. Mating type has segregated in these progenies, and only basidiospores having different mating types initiate the formation of a conjugation tube in response to each other, which can fuse. This then brings two parental haploid nuclei together in the same cell, forming the dikaryotic state, which is maintained throughout the rest of the life cycle up to spore formation and which is characteristic for many basidiomycetes. Combining nuclei with mating-type genes of different allelic specificities causes a major change in the regulation of many genes, resulting in a switch to filamentous growth now capable of infecting plant host tissue. This cell type represents the obligate parasitic stage of the life cycle since it cannot easily be maintained *in vitro* and needs the host for completion of its life cycle to produce the teliospores.

Mating-Type Loci

The complete genomes of three smut species have been sequenced and their mating-type loci analyzed. The corn smut fungus *Ustilago maydis* remains the model organism for this group and for basidiomycete plant pathogens in general (Brefort et al., 2009; Kamper et al., 2006). It is closely related to the genome of the corn and sorghum head smut fungus *Sporisorium reilianum* (Schirawski et al., 2010). The third sequenced smut genome is that of *Ustilago hordei*, which causes covered smut on barley and oats (Bakkeren et al., 2006; J. Laurie, S. Ali, G. Bakkeren, J. Schirawski, R. Kahmann, unpublished data). These fungi had been known for a long time to represent two different groups with respect to mating types: ones that possess a bipolar mating system and ones that are governed by a tetrapolar mating system. These designations were based on assessment of mating types of numerous progenies generated from many different natural smut fungal isolates that had been paired in various combinations. In species such as *U. hordei*, only one genetic locus determined mating type, and two allelic specificities seemed to segregate, designating it as a bipolar mating system. In other species such as *U. maydis*, mating type was governed by two unlinked genetic loci, designating it as tetrapolar (Fisher and Holton, 1957; Raper, 1966). In the latter mating system, one locus, termed the *a* locus, seemed to have two specificities (Bolker et al., 1992), although three specificities have been described in *S. reilianum* (Schirawski et al., 2005). For the other locus, termed the *b* locus, many allelic specificities have been found in nature.

Molecular analysis of *U. maydis* has elucidated functions underlying these genetic findings. The *a* locus spans no more than 10 kb and encodes two main components: a pheromone mating factor a (Mfa) and pheromone receptor A (PRA) (Fig. 23.1). The *a1* locus encodes Mfa1 and PRA1, and the *a2* locus encodes Mfa2 and PRA2. Mfa1 stimulates cells having PRA2 (and the *a2* specificity) and vice versa. Limited allelic variation has been found, although more than two alleles appear to be present in *S. reilianum* (Schirawski et al., 2005). The *b* locus has two divergently transcribed genes spanning no more than 4 kb and codes for heterodimeric homeodomain-containing transcription regulators, bE and bW. Component bE1 from mating

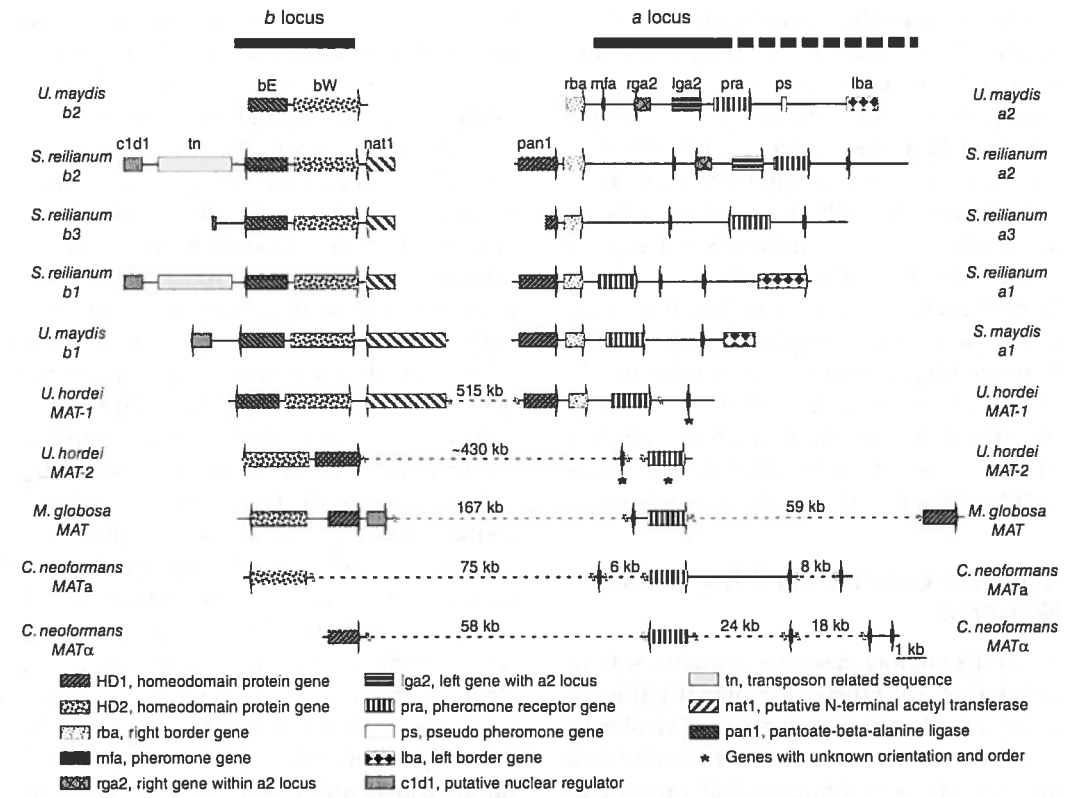

Figure 23.1 Genetic organization of the mating-type loci of selected basidiomycetes. Genes are indicated by arrows with the arrow denoting direction of transcription. Related genes are denoted by the same shading and respective gene functions are explained in the lower part of the figure. * indicates that the relative order and orientation of these genes have not been determined. In the tetrapolar species *Ustilago maydis* and *Sporisorium reilianum*, the *a*- and *b*-specific sequences reside on different chromosomes while they are linked by spacer regions (which are not drawn to scale and whose length is indicated) in the bipolar species *Ustilago hordei* and *Cryptococcus neoformans*, as well as in *Malassezia globosa*. The black bars on top of the figure indicate the regions of the *b* locus, which covers the two homeodomain protein genes *bE* and *bW*, and the *a* locus (which expands to different lengths in the different fungi, indicated by a broken line) from the *lba* gene to the *rba* gene. Sequence information was obtained from the following GenBank accessions: AF043940, AM118080, AACP01000083, AACP01000013, AJ884588, AJ884583, AJ884590, AJ884585, AJ884589, AJ884584, U37796, M84182, AF184070, AF184069, Z18531, AAYY01000003, AF542530, and AF542531. Reprinted from Bakkeren et al. (2008), with permission from Elsevier.

specificity 1 interacts, after cell fusion, with bWx (not 1) from cells having a different allelic specificity. Many different allelic variants have been found, and amino acid changes are responsible for altered interaction and subsequent downstream gene activation (Yee and Kronstad, 1998; reviewed in Brefort et al., 2009).

In the bipolar smut fungus *U. hordei*, the same *a* and *b* mating-type components are present, but they are physically linked or reside on the genetically described single *MAT* locus (Bakkeren and Kronstad, 1994). This linkage seems to be the defining difference between bipolar and tetrapolar mating systems. Astoundingly, the *a* and *b* mating-type complexes are separated by 527 kb at the *MAT-1* locus (Fig. 23.1). This region is suppressed for recombination with the *MAT-2* region in cells from the opposite mating type and has inversions (at least at the *b* gene complex), deletions (the

region between the *a* and *b* gene complexes at the *MAT-2* locus is 70 kb shorter), and over 50% of coding capacity is taken up by transposable elements and long terminal repeat (LTR)-like sequences. All these features may account for the lack of recombination and possibly for the genesis of specialized sex chromosomes (Bakkeren et al., 2006; Lee et al., 1999). A translocation of a chromosome segment harboring the *a* mating-type complex to link it to the *b* mating-type complex to constitute the large *MAT* locus appears to have occurred, possibly in a progenitor, and thus resulting in a new evolutionary clade that includes small grain-infecting bipolar smut fungi.

Signaling Cascades and Regulatory Networks

Several signaling cascades regulate sets of genes that are crucial for fungal pathogenicity or play a major role in virulence. Many components seem to be conserved also among nonpathogens, but their deletion does not always affect an *in vitro*/saprobic lifestyle. It is therefore likely that as species evolved to become pathogenic, they found novel ways of relaying and responding to or taking advantage of (danger) signals from host environments during infections.

As mentioned, in the smut fungi, the binding of mating pheromone Mfa to the correct PRA initiates conjugation tube formation and cell fusion (mating) between partners of different allelic specificities, *a1* and *a2*. Many components of two major signal transduction pathways involved, a G-protein-ras-adenylate cyclase cascade acting through cyclic AMP (cAMP), and a mitogen-activated protein kinase (MAPK) module of hierarchical kinases, have been identified (reviewed in Brefort et al., 2009; García-Pedrajas et al., 2008; Lee et al., 2003). Mating and pathogenic filamentation are initiated upon signaling by both the cAMP and pheromone MAPK cascades that converge on the Prf1 transcription factor (Hartmann et al., 1999). The PRA receptor, active in response to pheromone binding, signals through sequential MAP kinases, and results in the activating phosphorylation of Prf1. Signaling via the cAMP pathway activates a second kinase whose additional phosphorylation of Prf1 is required for complete activity (Hartmann et al., 1999). Prf1 integrates nutritional and mating signals, thus governing the initiation of sexual development and pathogenic growth. When active, Prf1 induces the expression of genes that promote haploid cell fusion and simultaneously activates the expression of the bE and bW homeodomain-containing transcription factors that heterodimerize upon cellular fusion. At this stage, bE/bW regulates the expression of 347 genes, inducing 212 and repressing 135 (Brachmann et al., 2001; Hartmann et al., 1996; Kahmann and Schirawski, 2007; Urban et al., 1996). Downstream of bE/bW lies a regulatory network composed of cascading transcription factors contributing to differential aspects of pathogenesis and biotrophic growth. Of the direct targets of bE/bW, Rbf1, a zinc finger transcription factor, is responsible for most *b*-dependent transcriptional and morphological effects. Rbf1 controls the expression of secondary transcription factors Hdp1 and Biz1, which contribute to propagation *in planta* and cell cycle regulation of dikaryotic growth (Flor-Parra et al., 2006; Heimel et al., 2010b). Among other genes regulated by bE/bW is MAPK *kpp6* involved in invasive growth of the newly formed dikaryotic filament (Brachmann et al., 2003).

One important aspect of the life cycle is cell cycle control in relation to morphogenesis and, hence, infection and pathogenicity. Growth by budding of the basidiospores, switching to the formation of conjugation tubes, mating, filamentous growth, and the production of teliospores are momentous changes. Initiation of mating requires cells to be arrested in G2 (Garcia-Muse et al., 2003), which rearranges the cytoskeleton and prepares the cell for (fast) polar growth; such cycling between cell proliferation and G2 arrest to allow filamentous growth might be a hallmark of hyphae penetrating host cells during infection (Brefort et al., 2009; Flor-Parra et al., 2006, 2007; Perez-Martin et al., 2006). The dimorphic switch to hyphal growth is essential for pathogenicity in the smut fungi, and the disruption of many genes regulating or involved in cell polarity, such as cyclin-dependent kinases and microtubule kinesin, myosin, and dynein motor proteins, affects pathogenicity or virulence (Castillo-Lluva et al., 2007; Steinberg, 2007). Interestingly, DNA damage checkpoint kinases Chk1 and Atr1, involved in a widely conserved signaling cascade, may have additional roles regulating cell type by controlling cell cycle arrest and maintenance of the dikaryotic state during infection (de Sena-Tomás et al., 2011).

Another signaling pathway responding to environmental signals such as sensing pH (H^+) and other ions, high temperature, Ca^{2+} levels (through Ca^{2+}-binding calmodulin), cell wall stress, and so on, acts through calcineurin, a calcium/calmodulin-regulated serine/threonine phosphatase consisting of a catalytic subunit A (CNA) and regulatory subunit B (CNB). In pathogenic fungi, including *Ustilago* species, disrupting this central regulator affects virulence (Cervantes-Chávez et al., 2011; Egan et al., 2009; Fox et al., 2001). A comprehensive comparative genomic study of MAPK and calcium–calcineurin signaling components in plant and human pathogenic fungi was published recently (Rispail et al., 2009).

Pathogenesis and Virulence

To identify pathogenicity and virulence genes, functional assays and pathogenicity tests, based on mating tests and host interaction and disease ratings, have been employed to screen mutants generated by chemical mutagens or radiation, random insertional mutagenesis, or by reverse genetic approaches through gene deletion of candidate genes. Many genes have been found to impact the ability of the smut fungi to cause disease (pathogenicity factors) or the degree of disease and symptom formation (virulence factors). Studies, mainly in *U. maydis*, are too numerous to list here and the reader is referred to the reviews listed below. As mentioned, the haploid basidiospores can be easily manipulated experimentally, but upon mating, which in nature usually occurs on the leaf or coleoptile (emerging seedling) surface, the resulting dikaryotic hyphae in the smut fungi represent biotrophic cell types that cannot be cultured easily and need the host for completion of the life cycle, that is, the sporulation process. Therefore, in mutational screens, extensive use has also been made of pathogenic haploid strains to circumvent the need for mating (see reviews Brefort et al., 2009; Kahmann and Kamper, 2004). Distinguishing between virulence factors *sensu stricto*, that is, contributing to the disease process such as penetration and suppression of the host defense responses, and genes merely involved in fitness and/or general metabolism, is extremely difficult in the biotrophs and is often a matter of definition and interpretation. However, the acquisition of such factors led saprobic fungi to evolve pathogenic lifestyles. In general, the environment in which fungi can thrive and reproduce is likely to be unimportant to them, but the emergence of pathogenicity during evolution of the smut fungi needing host infection to produce progeny, or the rust fungi that have become strict biotrophs (see further), must have provided selective advantages such as a guaranteed source of nutrients, a superior method of producing progeny, and a dispersal mechanism.

Virulence factors include secreted effectors, which are part of the so-called secretome, and proteins predicted to be secreted,

often having an N-terminal signal peptide. Effectors in oomycetes and fungi are relatively small (<350 amino acids and can be smaller than 80 aa), and most do not match sequences with annotated functions in databases. Effectors can be conserved among related fungi, but subsets are more genus or even species specific. Some have multiple cysteine residues in a conserved spacing pattern among homologs, possibly allowing for disulfide bridge formation and characteristic folding for function and/or protection against proteases encountered in the host environment. Their highly variable nature and the fact that many often reside in series of paralogous family members, often in physical clusters, suggest that at least a subset is under diversifying selection, likely under selection pressure from interactions with host components.

Large sets of small secreted proteins (SSPs) have been identified in basidiomycete pathogen genomes, and numbers range from 350 to 400 in *U. maydis* (Mueller et al., 2008), *S. reilianum* (Schirawski et al., 2010), and *U. hordei* (J. Laurie, S. Ali, R. Kahmann, J. Schirawski, and G. Bakkeren, unpublished data), and as many as 1184 in the poplar leaf rust fungus *Melampsora larici-populina* (*Mlp*) and 1386 in the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici* (*Pgt*) (Duplessis et al., 2011; Joly et al., 2010). In smut fungi, several clusters of SSP genes are highly upregulated once the fungus is inside the host and are needed for full virulence (Kamper et al., 2006; Schirawski et al., 2010). Individual effectors, such as PEP1, PIT2, and UhAVR1, suppress host defense responses (Doehlemann et al., 2009, 2011; X. Song, S. Ali, J. Laurie, and G. Bakkeren, unpublished data), and PEP1 is also essential for penetration. In some *U. hordei* species, UhAVR1 triggers resistance in barley host cultivars having the resistance gene allele that recognizes this effector.

As stated, smut fungi have various mating-type architectures (reviewed in Bakkeren et al., 2008), and these probably have affected the evolution of virulence. For example, unlinked *a* and *b* loci might have led to diversification of allelic specificities of the *b* genes, resulting in the many mating types observed for *U. maydis*. This is thought to favor outcrossing and hence increases possible recombination and diversification of virulence factors such as predicted secreted effectors, among mates. As mentioned, in smut fungi, many effectors are in clusters of related paralogous family members. In contrast, in the bipolar smut fungus *U. hordei*, the single *MAT* locus has been hypothesized to favor inbreeding, possibly stimulating other means of diversifying virulence components. Indeed, early analysis of an effector cluster in this fungus revealed fewer family members (S. Ali, J. Laurie, and G. Bakkeren, unpublished data).

CRYPTOCOCCUS SPECIES

C. neoformans is a budding yeast saprophyte that grows clonally via budding or undergoes a filamentous phase during sexual development (Casadevall and Perfect, 1998; Kwon-Chung, 1976). Unlike the plant pathogenic basidiomycetes, pathogenesis is not tightly associated with sex because full sexual development can take place outside the host, the yeast form is infectious in animal models, and there is no evidence that sexual development occurs in the host environment under any conditions. The link between sexual development and pathogenesis among *Cryptococcus* species is through the role that sex plays in the development of spores and in the generation of genetic diversity (Giles et al., 2009; Velagapudi et al., 2009).

C. neoformans is a bipolar fungus with two stable mating types (**a** and α) that can be grown and manipulated in the laboratory (Hull and Heitman, 2002; Kwon-Chung, 1976). When *C. neoformans* cells of opposite mating type encounter one another under appropriate nutrient conditions, they can signal via pheromones and pheromone receptors and fuse with one another (Davidson et al., 2000; McClelland et al., 2004; Shen et al., 2002; Stanton et al., 2010). After cell fusion, filamentous dikaryotic growth ensues and continues until the formation of a basidium in which nuclear fusion and meiosis occur. The meiotic products are then repeatedly replicated mitotically, and the resulting products are packaged into spores. These basidiospores are then budded onto the surface of the basidium in four long chains (Alspaugh et al., 2000; Idnurm, 2010).

Another form of sexual development in *C. neoformans* is known as haploid fruiting (Lin et al., 2005, 2006). During this process, α strains, in response to severe desiccation and nutrient limitation, undergo monokaryotic filament formation, basidium formation, and sporulation. The resultant spores are recombinant, arising from an early endoduplication or same-sex fusion event (Bui et al., 2008; Lin et al., 2005). The capacity to carry out a sexual cycle in the absence of a mating partner may confer a fitness advantage because strains could respond to undesirable environmental conditions by forming spores for dispersal in the absence of a suitable mating partner.

Mating-Type Loci

To date, five *Cryptococcus* genomes have been sequenced to completion and analyzed, including those of *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii*, and *C. gattii* (D'Souza et al., 2011; Loftus et al., 2005). In each case, the genome contains a single, unusually large *MAT* locus over 100 kb in size that harbors upward of 20 genes (Lengeler et al., 2002). One end of the locus encodes pheromones and pheromone receptors responsible for specifying haploid cell identity. These factors mediate mate recognition and cell fusion and show no allelic variation; that is, **a** cells encode Ste3**a** (the functional homolog of *Ustilago* PRA) and MF**a**, and α cells encode Ste3α and MFα, and the specificities of the pheromones and receptors do not vary (Fig. 23.1) (Chung et al., 2002; Davidson et al., 2000; McClelland et al., 2002; Moore and Edman, 1993). This limited allelic repertoire is also seen at the opposite end of the locus that encodes the homeodomain transcription factors Sxi1α and Sxi2**a** (the functional homologs of *Ustilago* bE and bW proteins, respectively; Fig. 23.1) (Hull et al., 2004, 2005). These transcriptional regulators are responsible for specifying the dikaryotic and diploid cell types, and unlike in many other basidiomycetes, they do not vary (Casselton and Olesnicky, 1998). As a result, *C. neoformans* is a bipolar fungus with only two mating types. The midsection of the *C. neoformans MAT* locus contains ~15 genes of varying types, including genes of unknown function and highly conserved housekeeping genes. The roles of these genes in sexual development (if any) are unknown, and most appear to have been captured via a series of transposon-mediated rearrangements (Fraser et al., 2004; Lengeler et al., 2002; Loftus et al., 2005). These rearrangements have led to a region suppressed in recombination, facilitating additional divergence between the *MAT* alleles. The resulting locus thus appears to represent an evolutionary intermediate in a tetrapolar to bipolar evolutionary transition, akin to the locus of *U. hordei* in which a tetrapolar mating system has been reduced to a bipolar arrangement via the linking of two *MAT* loci (Hsueh et al., 2008; Lee et al., 1999).

Signaling Cascades and Regulatory Networks

Many genes and signaling networks that contribute to development have been identified in *C. neoformans*. Interestingly, numerous signaling pathways contribute to both the ability to undergo sexual

development and survival within the host (Kozubowski et al., 2009). The evolutionary linkage between development and pathogenesis is not understood as *C. neoformans* exhibits a saprobic lifestyle in the environment and is not communicable host to host. Virulence traits are therefore unlikely to be under selection of the host environment. Despite the distinct natures of these two processes (sexual development and virulence) in the lifestyle of *C. neoformans*, a number of important signaling pathways and regulatory networks are in common, with many components contributing to both sexual morphogenesis and pathogenesis.

In *C. neoformans*, the protein kinase A (PKA)/cAMP signaling cascade contributes to mating, sexual differentiation, and the production of the virulence factors melanin and capsule. The cAMP pathway operates via a network of signaling proteins that regulate the activity of Cac1, adenylyl cyclase, which converts ATP to the signaling molecule cAMP. Mutants unable to produce cAMP (*gpa1*, *cac1*) show defects in melanin production, sexual filamentation, and capsule formation (Alspaugh et al., 1997, 2002). Specifically, cAMP levels regulate the expression of the laccase genes *LAC1* and *LAC2*, both critical for melanin production (Pukkila-Worley et al., 2005). cAMP levels influence these downstream events presumably via cAMP-dependent kinases such as Pka1. Interestingly, *pka1* mutant phenotypes are less severe than those of *gpa1* or *cac1*, suggesting a more redundant signaling network downstream of cAMP levels (D'Souza et al., 2001; Hu et al., 2007b). The only known Pka1 phosphorylation target is the transcription factor Nrg1, which is required for sexual development and influences the expression of *UGD1*, a gene involved in capsule production (Cramer et al., 2006). Additional targets of Pka1 phosphorylation are unknown, and much remains to be described regarding the mechanisms of cAMP signaling influencing downstream effectors that are important for morphogenesis and pathogenesis.

The *C. neoformans* MAPK cascades are also critical for pathogenesis and sexual morphogenesis. The high osmolarity glycerol (HOG) MAPK pathway is central to the resistance of *C. neoformans* to host stress conditions but contributes minimally to sexual development (Bahn et al., 2005, 2007). The pheromone-activated MAPK cascade influences phenotypes relating to mating, but certain components affect both morphogenic and pathogenic phenotypes, suggesting an interesting link between these two growth stages. During sexual development, pheromone binding to its complementary receptor activates its associated G protein; this activation then triggers an intracellular MAPK cascade (Davidson et al., 2003). The pheromone signal transduction pathway requires the sequential activity of the kinases Ste20**a**/α (PAK kinase), Ste11**a**/α (MAPK kinase kinase), Ste7 (MAPK kinase), and Cpk1 (MAPK) (Clarke et al., 2001; Davidson et al., 2003; Nichols et al., 2004; Wang et al., 2002). A number of these components are required for sexual development, and deletion mutants of *gpb1*, *ste11*, *ste7*, and *cpk1* exhibit dominant sterile phenotypes during development and cannot undergo cellular fusion with a wild-type mating partner. When assessed for their capacity to cause disease in murine models, the same mutations have little to no effect on virulence (Clarke et al., 2001; Davidson et al., 2003; Wang et al., 2000). However, Ste20 is required for full virulence in *C. neoformans* var. *grubii* (Nichols et al., 2004; Wang et al., 2002).

Downstream of the pheromone MAPK signaling cascade, several transcription factors mediate the cellular response to a mating partner. The recently identified Mat2 regulator appears to play a central role during morphogenesis and is a potential direct target of Cpk1 phosphorylation, but when Mat2 is deleted, virulence is unaffected (Lin et al., 2010). Downstream of Mat2 regulation are the mating-type specific regulators Ste12**a** and Ste12α, named for their sequence similarity to the pheromone response and pseudohyphal growth regulator Ste12 of *S. cerevisiae* (Chang et al., 2001; Fields and Herskowitz, 1985; Wickes et al., 1997; Yue et al., 1999). In *C. neoformans*, they contribute to cellular fusion and hyphal growth but are not required, and comprise a branch of the MAPK regulatory network that functions in parallel to currently unknown components (Davidson et al., 2003). Interestingly, Ste12**a** and Ste12α are important for pathogenesis and have been linked to the full expression of certain virulence factors (melanin, capsule). They influence expression of the virulence genes *LAC1* and *CAP59* (Chang et al., 2000; Clarke et al., 2001; Wickes et al., 1997).

The Mat2 transcription factor also activates the expression of the downstream master regulatory genes *SXI1*α and *SXI2***a** (Lin et al., 2010). Despite their induction during early development, they are dispensable for mating and cellular fusion (Hull et al., 2005). Current models suggest that the *SXI* genes are induced prefusion so that their gene products can function immediately postfusion, when they heterodimerize to form an active complex. The Sxi1α–Sxi2**a** heterodimer then acts to initiate the fusant to filament transition via the transcriptional regulation of target effector genes (Stanton et al., 2009). The downstream effectors of Sxi1α-Sxi2**a** activity are unknown, aside from the likely direct target *CLP1* (Ekena et al., 2008). Microarray studies suggest that the Sxi1α–Sxi2**a** complex influences the expression of hundreds of genes during sexual development. Studies suggest that many of these changes may be indirect, and that a number of transcription factors may be among the direct Sxi-regulon (M.E. Mead and C.M. Hull, unpublished data). Further studies should elucidate whether Sxi1α–Sxi2**a** fits the model established in *U. maydis*, where the bE/bW heterodimer acts to initiate a tiered transcription factor cascade controlling dikaryotic growth and the full sexual/pathogenic cycle leading to spore production (Heimel et al., 2010a,b).

Pathogenesis and Virulence

Humans and other animals acquire *Cryptococcus* from the environment via a respiratory route of infection. *C. neoformans* cells, either desiccated yeast or spores, are inhaled into the lung alveoli where they generally cause little or no respiratory disease but can disseminate to other tissues (Casadevall and Perfect, 1998; Garcia-Hermoso et al., 1999; Goldman et al., 2001). Dissemination to the brain in humans results in the development of meningoencephalitis, which is fatal without treatment (Casadevall and Perfect, 1998; Hull and Heitman, 2002). Most cryptococcal diseases occur in immunocompromised people; approximately 1 million cases and over 600,000 deaths are estimated to occur worldwide each year (Park et al., 2009). Numerous virulence properties have been identified in *C. neoformans*, including growth at high temperature, the α mating type, the ability to produce a polysaccharide capsule, and the ability to produce melanin (Chang and Kwon-Chung, 1994; Kwon-Chung et al., 1992a,b). Furthermore, specific genes associated with these pathways have also been identified and tested in a mouse model of cryptococcosis.

Interestingly, over 95% of isolates of *Cryptococcus* isolated from both patients and the environment are of the α mating type (Erke, 1976; Lin et al., 2005, 2006; Wickes et al., 1996). This bias suggests that sexual development itself or genes associated with sexual identity or development somehow influence fitness, persistence, and/or pathogenesis. In fact, some α strains of *C. neoformans* var. *neoformans* have been shown to be more virulent than the **a** counterparts in a mouse model of infection

(Kwon-Chung et al., 1992a). One hypothesis is that haploid fruiting (which occurs preferentially in α cells) could lead to better dispersal of α strains, thus leading to more opportunities to persist in the environment and to infect hosts (Lin et al., 2005, 2006). A caveat to this proposal is that α fruiting has only been detected in *C. neoformans* var. *neoformans*, and the vast majority of isolates in the world are *C. neoformans* var. *grubii*, in which α fruiting has not been detected (Hull and Heitman, 2002; Wickes et al., 1996). Thus, it remains to be determined what parameters of *C. neoformans* growth lead to differences in virulence and how sexual reproduction contributes to this. Among the human fungal pathogens, *Cryptococcus* is the only one with a sexual cycle that can be readily manipulated in the laboratory. The ability to induce sexual development and link genotype to phenotype through classical genetic studies is a hallmark of the system, resulting in a relatively large number of known virulence genes.

Sexual development of *Cryptococcus* is additionally important because spores are suspected infectious particles in human disease. Recently, spores were purified for the first time in numbers sufficient for biochemical, immunological, and virulence studies (Botts et al., 2009). (All previous studies of *Cryptococcus* had been carried out with the yeast form of the organism.) Spores have now been shown to be resistant to many environmental stresses and to cause disease in the mouse inhalation model of cryptococcosis (Botts et al., 2009; Giles et al., 2009; Velagapudi et al., 2009). Furthermore, spores interact with the mammalian immune system in a fundamentally different manner than the genetically identical yeast form does (Giles et al., 2009). These intriguing findings imply that *Cryptococcus* could infect mammals as spores, as yeast, or as a mixture, ultimately leading to differences in disease profile and outcome. As more fertile strains are recovered from patients and the environment, the full spectrum of spore production and virulence can be determined (Litvintseva et al., 2003). One area of great interest is in the role that sexual development and resulting spore production are playing in human infections in sub-Saharan Africa. The largest concentration of people with AIDS is in this area, in which there is strong evidence for *Cryptococcus* sexual development and resulting strain diversity (Litvintseva et al., 2003; Park et al., 2009). Because sexual development creates genetic diversity, the opportunity for more virulent strains to arise in susceptible hosts may be imminent. Clearly, sexual development and pathogenesis are linked in *Cryptococcus* in ways that have not been fully recognized previously. Future studies promise to elucidate these links and to provide insights into how the relationship evolved in this and other basidiomycetes.

RUST FUNGI

Sex in Relation to Virulence

In contrast with smut fungi, the cereal rust fungi have a complex life cycle including five different spore types and two hosts (macrocyclic). For example, the wheat leaf rust fungus *Puccinia triticina* (*Pt*), formerly called *Puccinia recondita* f. sp. *tritici*, produces brown-colored urediniospores from which the rust obtained its name, on wheat (*Triticum aestivum* L.). They are the asexual dikaryotic, infectious propagules, which are easily carried long distances by prevailing winds and spread on wheat through reinfection, which can lead to epidemics. On senescing wheat plants, teliospores can be produced, which are primarily two celled, with each cell containing two haploid nuclei that have paired, if not fused, to form the diploid state (Mendgen, 1984). Similar to smuts, in rust fungi, teliospores are the survival structures and produce haploid basidiospores. A third mitotic division can result

in basidiospores having two nuclei while being genetically monokaryons, containing two nuclei of the same type (Anikster, 1983). These spore types are ephemeral and infect meadow rue (*Thalictrum speciosissimum* L.), the so-called alternate host on which the fungus completes its sexual stage. Upon infection through direct penetration of the leaf surface, monokaryotic hyphae produce specialized pycnia, which generate pycniospores embedded in nectar. Because they originated from haploid meiotic products, the pycniospores in a specific pycnium represent one mating type and need to cross-fertilize (heterothallism), often through the action of insects attracted by the nectar. During fertilization, one pycniospore fuses with a receptive hyphum in the pycnium of a different mating type. This cellular fusion is followed by nuclear transfer, and the newly formed dikaryon undergoes developmental reprogramming. The resultant mycelium traverses the leaf and forms aecia on the underside, in which dikaryotic aeciospores develop. Aeciospores are dispersal propagules that will infect the primary wheat host (Bolton et al., 2008; Horton et al., 2005; Samborski, 1985). Very similar life cycles can be described for wheat stem rust, *Pgt*, and wheat stripe rust, *Puccinia striiformis* f. sp *tritici* (*Pst*), although these have their sexual cycles on *Berberis* species; this was very recently discovered for *Pst* (Jin et al., 2010).

Unlike the smuts, the rust fungi do not require sex for infection (of wheat) and the asexual cycle can produce such enormous populations that changes in virulence are thought to occur through mutation or parasexual interactions. The latter hypothesis was recently substantiated when vegetative or parasexual recombination was shown to occur between hyphae of germinated *Pt* urediniospores (Wang and McCallum, 2009). Large reductions in *Pt* populations due to the planting of resistant wheat cultivars and control of the disease through the use of large amounts of fungicides have correlated with reductions in the variability of virulence. The *Thalictrum* species that serve as good hosts for the sexual reproduction of *Pt* are mainly found in the Mediterranean basin and in the Middle East, the regions where wheat and progenitors coevolved with their rust pathogens. *Thalictrum* species indigenous in North America are widespread and can support sexual reproduction but to limited degrees (Saari et al., 1968). A striking correlation exists between the availability of the alternate host for *Pgt* and *Pst*, *Berberis* species that can support sexual reproduction, and the occurrence of isolates (races) with a high genetic variability and diverse virulence spectrum (Jin et al., 2010). Indeed, barberry bush eradication campaigns in the Great Plains in the United States since the 1930s coincided with a steady decline of race variation, probably because the populations became asexual. It seems therefore that virulence increases through sexual recombination in the grass-infecting rust fungi and as such is dependent on the action of the mating-type genes, but not in the same sense as in the smuts where mating is a prerequisite for pathogenicity. In these rusts, reshuffling genes likely leads to new combinations of effector repertoires, which can overcome defenses put up by the grass hosts.

Whether these correlations and findings extend to the rusts infecting dicots has not been researched to the same extent as for the cereal rusts, where large collections of genetically typed pathogens and hosts are available. In the poplar leaf rust *Melampsora medusae*, more genetic variabilities seem to be present in populations where hosts for both the telial (*Populus* spp.) and the aecial (*Larix* spp.) are present, although this does not correlate with virulence profiles (Bourassa et al., 2007). In *M. larici-populina*, increased variability in virulence is indicated in populations where the sexual host is also present (Gerard et al., 2006). Various populations of *Melampsora lini*,

which causes rust of flax (*Linum* species), have different levels of genetic variability and virulence, but it is unclear whether this is solely correlated with the presence of sexual reproduction. In addition, like the cereal rust fungi, this fungus is macrocyclic, but in contrast to the cereal rust fungi, all stages occur on the same host, flax (autoecious rust). It is currently unknown whether this particular property affects possible correlations of sex and the evolution of virulence compared to the cereal rust fungi (reviewed in Lawrence et al., 2007). In the bean rust fungus *Uromyces appendiculatus*, there is no clear indication of fewer genetic polymorphisms in asexual populations, based on a limited set of virulence and isoenzyme markers (Groth et al., 1995). Economically important legume-infecting *Phakopsora* species, such as the soybean rust pathogen *Phakopsora pachyrhizi*, have not been observed to produce teliospores (and therefore to go through a sexual cycle) in North America. A teliospore stage has been found in Asia, but germination has not been reported in nature and it is not known whether this fungus is autoecious or heteroecious (reviewed in Goellner et al., 2010). Analyses of Nigerian asexual populations of *P. pachyrhizi* revealed low genetic differentiation and a limited variability in pathotypes (Twizeyimana et al., 2011).

The Search for Functional Homologs Related to Sex

Molecular genetic analysis of the rust fungi has only recently received a boost mainly due to several large-scale genome sequencing projects. Although molecular genetic research has been performed for the last 20 years by a few tenacious researchers, the fact that the rust fungi are obligate biotrophic pathogens has made analyses of gene function very difficult. The lack of transformation systems made using reverse genetic or mutation complementation techniques impossible. Studies were limited to the isolation of (conserved) candidate genes and expression analyses, and *in situ* localization of gene products and comparative analyses to genes of other pathogens.

Several studies have attempted to shed light on the mating-type system in rust fungi. Conclusions and speculations vary from rust fungi having a simple bipolar system in several *Puccinia* and *Uromyces* species (Anikster et al., 1999) to a more complicated tetrapolar system with multiple allelic specificities in *M. lini* (Lawrence, 1980) and related oat crown rust pathogen, *P. coronata* (Narisawa et al., 1994). Different rust fungi may have different mating systems and harbor similar gene complexes in various arrangements as in the smuts and mushrooms. Indeed, searches of the public *Pgt* genome identified smut *a*-like pheromone (*mfa*) and pheromone receptor (*Pra* or *STE3*) genes and one set of divergently transcribed, *b*-like homeodomain-containing genes. Examination of the partial *Pt* genome reveals homologs of PRA1 and PRA2, and of two *bE* and *bW* pairs likely representing the two allelic specificities in the two haploid nuclei, bE1/bW1 and bE2/bW2 (G. Bakkeren, L. Szabo, J. Fellers, C. Cuomo, et al., unpublished data).

The Search for Functional Homologs Related to Pathogenicity

The study of effectors with avirulence functions, that is, effectors that trigger resistance in hosts carrying resistance gene alleles that recognize them, has been a focus in plant pathology for the last 30 years. Despite genetic evidence of these in the rust fungi since 1942 (Flor, 1942), molecular work aimed at identifying and cloning them in the rust fungi is rather recent (Zambino et al., 2000). Candidate gene approaches, using specific cDNA libraries from haustoria (specialized feeding structures established after plant infection), has accelerated research into

such effectors from bean (Hahn and Mendgen, 1997; Link and Voegele, 2008; Puthoff et al., 2008) and flax rust fungi (Catanzariti et al., 2006; Dodds et al., 2004) and from *Mlp* (Joly et al., 2010), *Pst* (Yin et al., 2009), and *Pt* (Xu et al., 2011). Candidate effectors have also been revealed in haustoria using a proteomic approach (Song et al., 2011). As mentioned, many effectors are predicted in the *Mlp* and *Pgt* genomes (Duplessis et al., 2011).

It is still challenging to investigate the functionality of identified genes in rust fungi, although progress has been made in developing transformation systems (Lawrence et al., 2010; Webb et al., 2006). With regard to effectors, an RNA silencing approach was used in *M. lini* to demonstrate an avirulence function that at the same time served as selection for genetic transformation (Lawrence et al., 2010). Also in populations of this fungus, allelic variation at effector gene loci correlated with pathogenicity. This is a direct demonstration of how evolution of effector loci can change virulence profiles in natural populations that are likely under selection pressure of coevolving hosts (Barrett et al., 2009).

Having several complete rust fungal genome sequences and expressed sequence tag (EST) collections from several more species, many candidate pathogenicity and virulence genes other than effectors have been identified. These are often based on proven functionality in other pathosystems that are more amenable to molecular genetic manipulation. However, in a promising approach using two somewhat related basidiomycete pathogens, Hu et al. expressed a *Pt* MAP kinase in *U. maydis* strains in which the homologous MAPK genes, *Ubc3*/*Kpp2* and *Kpp6*, were deleted, and showed that heterologous expression can restore mating and pathogenicity defects (Hu et al., 2007a). This approach opens up avenues along which to functionally analyze genes from biotrophs. *U. maydis* seems particularly suited for exploitation because it is a well-researched model system for which numerous deletion mutants exist. In another approach, RNA silencing, when induced in the host plant but targeted to fungal genes, can suppress the expression of such genes, thus allowing their functional analysis. Initially demonstrated in the powdery mildew fungus–barley pathosystem (Nowara et al., 2010), this technique seems also functional in the *Pst*– and *Pgt*–wheat pathosystem, presumably by uptake of siRNA molecules via haustoria (Yin et al., 2011). More importantly, this approach can also be used to target *Pt*, *Pst*, and *Pgt* fungal genes such as the mentioned MAPK involved in pathogenicity in other systems, and in suppression of disease development in wheat (V. Panwar and G. Bakkeren, unpublished data).

SUMMARY

Basidiomycetes comprise an eclectic mixture of saprobic organisms and facultative pathogens able to live saprobic lifestyles but able to engage in opportunistic invasive growth on or within hosts. A subset has evolved to become highly specialized, true biotrophic pathogens that establish intercellular hyphae and haustoria while inflicting minimal damage to host cells and maintaining the integrity of the host tissue (Brefort et al., 2009; Hibbett, 2006; Kirk et al., 2001). The adaptation of saprobic fungi to pathogenic lifestyles, utilizing host resources and exhibiting varying degrees of virulence, requires coevolution with hosts on a population level and also requires the ability to subdue or subvert host defense responses. It is an open question of whether such adaptations have occurred or are occurring more quickly in organisms displaying sex (Heitman, 2011; Morrow and Fraser, 2009). From detailed studies in the individual model organisms and other systems, it is becoming apparent that molecular determinants involved in sex are

a part of gene networks impinging on and interacting with other networks involved in pathogenicity (Sahni et al., 2009, 2010). We are only beginning to understand the implications emanating from these discoveries, but, apart from satisfying academic interests and shedding light on evolutionary questions, knowledge about these gene networks and the molecular basis underpinning pathogenicity and virulence will allow for the design of novel strategies for disease control in humans, animals, and crops.

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