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Site-Directed and Random Insertional Mutagenesis in Medically Important Fungi

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1. Introduction

Site-directed and random mutagenesis have been useful tools in molecular biology. The application of directed mutagenesis in medically important fungi has been limited by the availability of molecular genetic techniques. Even species in which efficient genetic transformation methodologies exist, mutagenesis approaches were sparsely used due to diploidism. Lack of genetic tools hindered understanding of virulence mechanisms of medically important fungi. With the arrival of whole-genome sequencing, as well as improved techniques of genetic manipulation, the ability to address these questions is improving. A comprehensive review of mutagenesis in pathogenic fungi is outside the scope of this review, so not all studies were included. The intent of this review is to educate the reader on applications of site-directed and random insertional mutagenesis in medically important fungi in order to provide ideas for novel approaches to address major issues in pathogenic fungal research.

2. Site-directed mutagenesis

Site-directed mutagenesis has been exploited to understand signaling pathways, mechanisms of drug resistance, and identification of promoter DNA binding sites. Applications used less frequently have included protein localization and function of specific genes. In most instances, the site of the mutation was selected due to homology to model species or mammalian genes.

2.1 Signaling pathways

The most commonly reported application of site-directed mutants is the construction of dominant-negative and dominant-active alleles. The ability to make dominant-active alleles is particularly useful in diploid strains, since both endogenous alleles do not have to be disrupted. The amino acids chosen for mutation are often based on homology to *Saccharomyces cerevisiae* or *Aspergillus nidulans*. Although the roles of genes in signaling pathways were identified in model fungi, the regulation and downstream effects of these pathways are often very different in medically important fungi.

2.1.1 Phosphomimetics

The introduction of an amino acid substitution so the residue acts as constitutively phosphorylated or non-phosphorylated is a common technique to study cellular processes. Phosphomimetics have been used to study MAPK, cAMP-PKA, calcineurin, and two–component signaling, as well as cytokinesis and the heat shock response, in medically important fungi (Bockmühl and Ernst, 2001; Fox and Heitman, 2005; Hicks et al., 2005; Li et al., 2008; Menon et al., 2006; Nicholls et al., 2011).

The cAMP-PKA pathway regulates multiple cellular processes in eukaryotes. cAMP levels are regulated by phosphodiesterases (*PDE1*, *PDE2*), which in turn are regulated by protein kinase A (*PKA*) in some species. In *Cryptococcus neoformans*, the cAMP pathway is involved in multiple cellular processes, including virulence factor expression (melanin and capsule formation) (Hicks et al., 2005). Since the role in cAMP degradation and regulation by *PKA* of *PDE1* and *PDE2* differ among species, the goal of this study was to learn the functions of the PDEs in *C. neoformans*. In order to identify if *PDE1* was regulated by *PKA* through phosphorylation, a site-directed mutation in the *PDE1* at a putative *PKA* phosphorylation site was introduced based on work in *Saccharomyces*. Site-directed mutagenesis was performed by overlap PCR (Section 2.7), and the product was ligated into a *C. neoformans* transformation vector. The predicted outcome was an inactive *PDE1* and, thus, increased activation of the PKA pathway. In this way, the use of site-directed mutagenesis validated that PKA directly regulated the activation of *PDE1* in *C. neoformans* (Hicks et al., 2005).

Putative phosphorylated residues have not always been identified previously in model fungi. Consequently, *in silico* analysis can be utilized to identify putative phosphorylation sites (Bockmühl and Ernst, 2001; Li et al., 2008). *In silico* analysis predicted certain threonine residues as phosphorylation sites in the *Candida albicans* APSES protein Efg1p. These sites were mutated. Phenotypic analysis demonstrated that the mutations differentially affected morphogenesis, an important virulence attribute of *C. albicans* (Bockmühl and Ernst, 2001*).*

Unlike previous studies, target residues in the two-component response regulator, Ssk1p, were identified by sequence comparison to a bacterial response regulator (Menon et al., 2006). Invariant aspartic acid residues were substituted using site-directed mutagenesis. This study demonstrated that phosphorylation of two different residues affects regulation of different cellular processes involved in virulence (Menon et al., 2006).

2.1.2 G protein signaling

Another common application for site-directed mutagenesis has been G protein signaling. In *Aspergillus fumigatus,* asexual sporulation results in release of spores that are inhaled by man, which can lead to serious manifestations. In the non-pathogen, *Aspergillus nidulans*, it was known that G protein signaling pathways were responsible for both vegetative growth and conidiation. Activation of *flbA* is required for conidiation, and this was probably through the activation of the GTPase activity of the G alpha protein FadA. Mah et al. (2006) used this framework to determine if similar regulation occurred in the pathogen *A. fumigatus* (Mah and Yu, 2006). They were able to confirm that Af*flb* regulated the G protein signaling through GpaA (homolog of FadA). Gene disruption and random chemical mutagenesis confirmed the role of Af*flb* in conidiation. Dominant-active and dominant-negative mutant alleles of *gpaA* (made by overlap PCR) demonstrated that it is a

downstream target in this pathway. Interestingly conidiation appeared even in the absence of Af*flb* (Mah and Yu, 2006), which may aid in dissemination.

As in *Aspergillus*, G protein signaling is also responsible for cellular differentiation in *C. albicans*. Much of the initial molecular dissection of the signaling pathways involved in morphogenesis was deciphered by constructing dominant-active and dominant-negative alleles of the G signaling proteins. Site-specific mutations were introduced in *CDC42, RAC1, GPA2, RAS1* and *RAS2* based on homology to *Saccharomyces* and mammalian G proteins (Bassilana and Arkowitz, 2006; Feng et al., 1999; Sanchez-Martinez and Perez-Martin, 2002; vandenBerg et al., 2004). The mutant alleles were introduced into exogenous loci under the expression of constitutive or regulatable promoters (Bassilana and Arkowitz, 2006; Feng et al., 1999; Sanchez-Martinez and Perez-Martin, 2002). However, since *CDC42* is essential, the mutated alleles were introduced at the endogenous locus in a *CDC42/cdc42* heterozygote (VandenBerg et al., 2004). These studies demonstrated the existence of different hyphal induction pathways, cross-talk between the MAPK and cAMP pathways, and distinction between growth and morphogenesis.

2.2 Mechanisms of drug resistance

Many fungal species present antifungal drug resistance *in vivo*. Studies have enhanced our understanding of this resistance. In most species, drug resistance is due to increased expression of export channels and/or mutations in target genes of the antifungal agent. Further studies confirmed the importance of the mutations.

The azoles interfere with ergosterol biosynthesis by targeting lanosterol 14 alpha-demethylase (*ERG11, CYP51*). Mutations in the gene resulted in reduced binding by the azole compound. Mutational hotspots were identified by sequencing the gene of interest from fungal strains isolated from patients or strains that have been passaged in the presence of the drug *in vitro*. It was then necessary to confirm that the mutation correlated with reduced susceptibility to the drug. Site-directed mutagenesis is an ideal method for validation. Due to homology of the *ERG11* gene among fungal species, many studies were performed in the more genetically malleable yeasts, *S. cerevisiae* or *Pichea pastoris* (Alvarez-Rueda et al., 2011). These studies confirmed that the mutated expressed protein is more or less susceptible to drug, but they do not definitively prove that the mutation was the reason for the clinical resistance. With the advent of genetic transformation techniques in medically important fungi, it is now possible to perform these experiments in the appropriate fungal host.

The most studied gene is *ERG11* in *C. albicans*, reviewed in Morio et al. (2010). Over 144 amino acid substitutions have been identified. It is less clear how many of these contribute to *in vivo* resistance. Additionally, some mutations may result from *in vitro* manipulations. A recent screen of azole-susceptible and resistant clinical isolates demonstrated that mutations are associated with both susceptibility and reduced susceptibility. Only 18% of isolates had no polymorphisms (Morio et al., 2010). These results highlighted the need to confirm that a specific mutation correlated with acquisition of resistance. In *C. albicans,* this has been approached by site-directed mutagenesis. Initial studies cloned the *ERG11* open reading frame (ORF) in a plasmid and then used PCR to introduce site-directed mutations. The PCR products were ligated into a *S. cerevisiae* expression vector. *S. cerevisiae* (azole

susceptible strain) was transformed with plasmids containing the mutated *C. albicans ERG11* gene and tested in a series of assays for reduced azole susceptibility. In this manner, azole resistance was correlated with specific amino acid substitutions (Kakeya et al., 2000; Lamb et al., 2000; Lamb et al., 1997; Sanglard et al., 1998; Sheng et al., 2010). Direct mutagenesis of the *ERG11* gene in *C. albicans* has not been reported. However, direct mutagenesis of the azole-target gene *cyp51A*, was performed in *A. fumigatus* (Mellado et al., 2007; Snelders et al., 2011). Two approaches were used. In the first study, mutated sequences were amplified by PCR from clinical isolates that demonstrated reduced susceptibility to itraconazole (Mellado et al., 2007). Previously it was known that itraconazole resistance correlated with specific amino acid mutations at G54 and M220 (See Table 1 in the chapter by Figurski et al. for the amino acid codes). However, this study identified a new mutation site (L98H) in conjunction with a duplication in the promoter sequence. In order to confirm the importance of these mutations, an azole-susceptible strain was transformed with the mutated allele; and transformants were plated on itraconazole (Mellado et al., 2007). Although the importance of the mutation sites were confirmed, the transformation selection criteria were not efficient. A second study expanded upon this approach and used 3-D modeling to determine a mechanistic reason for the azole resistance conferred by the mutations (Snelders et al., 2011). Specific amino acids were substituted in the *cyp51A* using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene) (Section 2.7). The appropriate PCR products were cloned into a vector that contained a hygromycin resistance marker and flanking sequences for introduction into the endogenous *cyp51A* site. Therefore, positive transformants were selected on hygromycin and further tested for azole resistance/susceptibility phenotypes. Consequently, the inclusion of a dominant selective marker improved the efficiency of screening transformants (Snelders et al., 2011). To further expand identification of potential mutation sites, the same experimental approach could be used by subjecting the *cyp51A* gene to random PCR-directed mutagenesis (Palmer and Sturtevant, 2004) and thereby identify new mutation sites that confer altered susceptibility.

2.3 Promoter response elements

Mutagenesis is a common approach to identify DNA binding sites in promoters. Nested deletions are probably the most commonly reported method used in the medically important fungi. Site-directed mutagenesis has been used to introduce point mutations in the *hapB* promoter in *A. nidulans*; so this approach may be used in *A. fumigatus* in the future (Brakhage and Langfelder, 2002). Site-directed mutagenesis has been used to identify putative promoter elements in chitin synthases (*CHS2, CHS8*) in *C. albicans* (Lenardon et al., 2009). The significance of this study is that chitin synthases are up-regulated in response to cell wall stress and thus are important for fungal survival. In this study, the promoters of *CHS2* and *CHS8* were mutated by site-directed mutagenesis or nested deletions. The selected sites were chosen due to previous studies or by *in silico* analysis. The mutated *Candida* promoter sequences were ligated upstream of the *Streptococcus thremophilus lacZ* gene. If the mutated site in the promoter element were important for a specific cell wall stress, *lacZ* would not be induced; and colonies would be white instead of blue on X-gal-containing medium. (X-gal is 5-bromo-4-chloro-indolyl-β-D-galactopyranoside.) The chosen potential sites reflected induction of several pathways and included known binding motifs. Mutations of individual promoter elements selected by *in silico* analysis had no effect on expression. Consequently, they performed nested deletions using exonuclease III digestion

of restriction-digested plasmids containing the *CHS2* and *CHS8* genes. These mutated promoters were introduced into *Candida* by transformation, and induction of the *lacZ* reporter was assayed after stresses. In this manner they were able to identify regions, but not specific regulatory elements, in the *CHS2* and *CHS8* promoters that responded to cell wall stressors (Lenardon et al., 2009). In order to determine which signaling pathways acted upon these promoters, the mutated *CHS2* and *CHS8* constructs were introduced into *C. albicans* signaling pathway deletion mutants. These studies demonstrated that the cell wall integrity, calcineurin, and HOG (osmotic sensing) pathways mediated expression through the *CHS2* promoter; only the cell wall integrity pathway affected *CHS8*-mediated expression (Lenardon et al., 2009). A pitfall of the deletion method is that it does not identify exact residues. It is possible to lose structural consistency, and it may delete other regulatory elements. In order to identify appropriate binding sites, random mutagenesis of *CHS2* and *CHS8* promoters by XL1-Red (a mutator strain of *Escherichia coli* useful for mutating cloned fragments) could have been performed (Palmer and Sturtevant, 2004). The ensuing transformants could be quickly screened on cell wall stressor media.

In another study, the authors wanted to identify the promoter binding sites in the pH responsive gene, *PHR1*, in *C. albicans* (Ramon and Fonzi, 2003). The pH response pathway had been well researched in *A. nidulans*. However, the promoter binding elements in the *Aspergillus* pH responsive gene (*pacC)* could not be translated to *PHR1*. Therefore, regions of DNA binding were identified *in vitro* by ChIP (Chromatin Immunoprecipitation Assay) and then confirmed by site-directed mutagenesis (Ramon and Fonzi, 2003).

Site-directed mutagenesis and ChIP have also been used to identify the genes that a specific transcription factor binds. A good illustration is the gain-of-function allele of the transcription factor *CAP1* that was constructed by site-directed mutagenesis and then analyzed in ChIP assays (Znaidi et al., 2009).

2.4 Gene function-essential genes

Surprisingly, site-directed mutagenesis has not been used extensively to determine the function of a gene. Gene disruption is routinely the method of choice to study gene function. However, this is not possible when studying the function of essential genes. The use of conditional promoters is often used. Results can sometimes be misleading, since phenotypic testing is performed under suboptimal growth conditions due to promoter-dependent nutritional constraints. Even so, expression of an essential gene under a conditional reporter does not allow complete analysis of multifunctional genes. Very few studies have taken advantage of directed mutagenesis of a specific gene.

The first report of the use of site-directed mutagenesis of an essential and multifunctional gene was the signaling regulatory gene, *BMH1* (14-3-3 gene). There is only one 14-3-3 protein (Bmh1p) in *C. albicans*, and it is essential (Cognetti et al., 2002). Multiple approaches were attempted to express the gene under a regulatable promoter, but they were unsuccessful (Palmer et al., 2004). This may be because *BMH1* regulates multiple cellular processes involved in growth, and the phenotypic studies were performed under suboptimal growth conditions due to promoter-dependent nutritional constraints. Therefore, *BMH1* appeared to be an excellent candidate to test the feasibility of both site-directed and random mutagenesis. Amino acid residues in the 14-3-3 allele required for

ligand binding, dimerization, and growth were reported for other eukaryotic species. Due to the high degree of conservation between 14-3-3 proteins, the same residues were selected for substitution in the *C. albicans BMH1* allele. Six sites were chosen. Transformants were screened for filamentation and growth defects (Palmer et al., 2004). Two approaches tested the applicability of random mutagenesis of the *BMH1* allele (Palmer and Sturtevant, 2004). A plasmid containing the *BMH1* allele was propagated in the *E. coli* XL-1 Red strain (Stratagene), which is deficient in multiple primary DNA repair pathways and thus introduces random mutations in the plasmid. Mutagenized plasmids were isolated after 11 to 44 divisions and introduced into the remaining *BMH1* locus in a *BMH1* heterozygote strain. The second random mutagenesis approach was PCR-mediated. (DNA polymerases used for PCR can be mutagenic under certain conditions.) The *BMH1* allele was subject to PCR amplification with an unbalanced nucleoside pool. The PCR products were ligated into a *Candida* transformation vector, and pools were introduced into the *BMH1* heterozygote by transformation, as above. The *E. coli*-mediated mutagenesis resulted in a higher efficiency of correct integration of the mutated allele than did the PCR-mediated method. Around 1400 (1000 – *E. coli*-mediated; 368 – PCR-mediated) *C. albicans* transformants containing randomly mutagenized *BMH1* alleles were screened under a variety of phenotypic stresses. These tests were rapid and easily visible; thus, they translated easily into a screen. Mutant alleles were isolated from transformants that demonstrated altered phenotypes and were sequenced. In the end, from 1000 *E. coli*- and 368 PCR-mutated colonies, 2 and 4 alleles, respectively (0.4%), were identified with altered coding sequences. That these mutations were responsible for the altered phenotypes was validated by constructing *C. albicans* strains isogenic for the site-directed mutations, as described previously. While the efficiency of the random mutagenesis methods was lower than reported for bacteria, non-lethal mutants were identified. Thus, this is a valid approach to study gene function in fungi (Palmer and Sturtevant, 2004). The outcome of the site-directed and random mutagenesis approaches was a set of isogenic strains in which *BMH1* or a mutant *BMH1* allele was expressed under its own promoter at an exogenous locus. These strains were analyzed under a variety of environmental conditions reflecting stresses in the host. It was possible to discriminate between separate pathways involved in filamentation, growth, and survival in the host (Kelly et al., 2009; Palmer et al., 2004; Palmer and Sturtevant, 2004). Additional mutants may have been identified if transformants were screened in additional tests or in *in vivo* models. On the other hand, since *BMH1* is an essential gene, there may be a limited number of amino acids that can be mutated and still result in a non-lethal allele.

Site-directed mutagenesis was also used to decipher the role of the hemoglobin response gene (*HBR1*) in vegetative growth. It was known that *HBR1* induced mating type genes, but mating is not an essential process in *C. albicans* (Peterson et al., 2011). Sites required for optimal growth and the oxidative stress response in the homologous gene in *Saccharomyces* were targeted in the *C. albicans* gene. The mutant alleles were introduced into a *HBR1* heterozygote and were regulated by the *MET3* promoter. This study identified amino acid residues important for mating locus regulation, but not for vegetative growth. Thus, amino acids identified to be important in model fungal species do not always translate to related pathogenic fungi (Peterson et al., 2011).

Essential genes are often prospective drug targets. One such gene is *MET6*. In *C. albicans*, Prasannan et al. (2009) constructed GST fusions of mutated *C. albicans MET6* and expressed the fusion protein in a *MET6 Saccharomyces* mutant. A 3-D model that was modified from

the known crystallized structure of the *Arabidopsis* enzyme was used to select sites for mutation in *MET6*. Eight residues were chosen based on conservation across species and probability of being catalytic sites. Site-directed mutagenesis was introduced by the Quikchange kit from Stratagene. The mutant GST-Met6p fusion proteins demonstrated varied enzymatic activity validating the use of this approach in the design of new antifungal drugs (Prasannan et al., 2009).

2.5 Gene function – genes with multiple functions

The transcriptional regulator, *EFG1*, regulates multiple cellular processes in *C. albicans*. *EFG1* is a member of the APSES protein family. Although the domain that defines this family is known, the actual structure-function relationships were not understood. Thus, defined regions within and flanking the APSES domain were deleted. This was mediated by PCR. Instead of amino acid substitution, 15 – 103 nucleotides were deleted from within the *EFG1* gene, similar to what is done for promoter bashing (mutating promoters) (Noffz et al., 2008). A disadvantage to this approach is that it is not possible to discriminate if an altered phenotype is due to the compromise of protein structure or to the absence of protein expression. However, immunoblotting confirmed that the mutated protein was expressed in all mutants. Two mutants did express lower levels of Efg1p that could account for altered phenotypes (Noffz et al., 2008). Thus, it is important to confirm protein expression of mutants. The authors were able to associate specific regions of Efg1p with distinct cellular processes that it regulates. The deletion alleles were also used in over-expression and one-hybrid (gene-fusion technology to identify a DNA-binding domain) experiments. Thus, this approach was successful in determining structure – function relationships of an APSES protein (Noffz et al., 2008).

2.6 Other applications

Site-directed mutagenesis has been used to determine how GPI-tagged proteins discriminate between localization to the plasma membrane and cell wall (Mao et al., 2008). N and C termini of cell wall or plasma proteins were fused to GFP. The termini were subjected to truncation and mutagenesis. Localization of mutant alleles was examined by microscopy. One potential pitfall, however, is that the GFP tag itself can cause protein mislocalization. These experiments identified the omega cleavage site. Further domain exchange and mutagenesis studies identified which residues dictated cell wall or plasma membrane localization (Mao et al., 2008).

2.7 Methodology

Site-directed mutagenesis (*i.e.*, targeted substitution of one or more nucleotides) in a gene was normally performed via overlap PCR and/or the QuikChange Site-Directed Mutagenesis Kit (Stratagene/Agilent Technologies). The principle of these methods is the same. Complementary primers are designed with the nucleotide substitution at the desired site of the mutation. The primers are complementary to the region of the template with the wild-type residue. The template is a double-stranded DNA vector (usually a plasmid) containing a DNA clone of the region of interest. PCR with a high fidelity polymerase results in a plasmid with the mutation of the primer. The product is digested with *Dpn*I,

which cleaves only the parental plasmid (template) because *Dpn*I requires fully or hemi-methylated DNA. (The parental plasmid is methylated by the *E. coli* host; DNA amplified by PCR is unmethylated.) The resulting DNA is then introduced into competent cells by transformation. Resulting plasmids are sequenced to confirm the mutation. It is also important to confirm that the mutation does not affect gene expression. Single-site mutagenesis has also been used to introduce silent mutations that result in construction of a restriction enzyme site in order to facilitate genetic manipulation (Cognetti et al., 2002; Schmalhorst et al., 2008).

3. Insertional mutagenesis

Insertional mutagenesis methods are commonly used in model fungi species. Although genomes are similar between model and medically important fungal species, there are still significant differences. Forward screens (screens for new genes that are involved in a phenotype, often using homologs) in model fungi will not identify genes important for pathogenesis, since these species are usually attenuated in virulence or are avirulent. Signaling pathways are shared among fungi, but downstream targets and regulation vary. It is estimated that only 61% of the essential genes in *S. cerevisiae* are also essential in *C. albicans*. There may be even more differences in filamentous fungi (Carr et al., 2010). The advent of improved genetic techniques and whole-genome sequencing has dramatically improved the ability to perform forward screens in the medically important fungi. One major drawback has been diploidism. Ways to circumvent the problem of diploidy have included parasexual genetics (non-meiotic conversion of a diploid to a haploid) (Carr et al., 2010; Firon et al., 2003) and haploid insufficiency (a phenotype resulting from the loss of one allele in a diploid) (Uhl et al., 2003). Additional requirements that are species–specific include a 'mutagen' and an appropriate screen/phenotype. Insertional mutagenesis is normally now facilitated by transposons, but it is still necessary to identify transposons that work efficiently in the fungal species of choice. Much of the initial work demonstrated a bias for insertions, including a bias of non-coding regions. A recent analysis of three transposons has identified Tn*7* as having the least insertion bias in *Candid*a *glabrata* (Green et al., 2012). This would probably translate to other fungal species whose genomes are also rich in A/T sequences. Certainly, in the post-genomics era, utilization of forward genetics approaches have increased due to the improved ability to identify the site of insertion.

3.1 Selection of insertion mutants by complementation of auxotrophy

Initial studies used complementation of auxotrophy as a 'mutagen.' Auxotrophic strains were transformed with plasmids carrying an auxotrophic marker (*e.g*., *URA3/5*). For example, in *C. neoformans*, capsule formation is associated with virulence. Laccase is required for capsule formation. To identify the laccase gene, a *ura*-deficient mutant was transformed multiple times (to obtain independent mutants) with a *URA* construct that has an *E. coli*-specific replicon. When expressed in *C. neoformans,* the construct integrates randomly into the genome and complements the uracil auxotrophy. Transformants were selected for growth on medium lacking uracil. They were then screened on differential media that would identify strains with laccase deficiency due to a pigment change. Out of 1000 transformants, nine strains with an altered phenotype were identified. Plasmid rescue was performed to identify the insertion point. (Plasmid rescue results from cleaving

genomic DNA with the appropriate restriction enzyme. The inserted fragment, along with a piece of the interrupted gene, is released. The released DNA can circularize in the presence of ligase and form a plasmid that replicates in *E. coli.* Sequencing of the piece of interrupted gene is easily done and identifies the gene, which can then be cloned intact.). In this manner, a novel virulence attribute was identified, the vacuolar (H+) – ATPase subunit (*VPH1*) (Erickson et al., 2001). In general, the drawbacks to the auxotrophic approach were inefficient integration, integration via homologous rather than non-homologous recombination, and difficulty in identification of the insertion site.

3.2 Signature-tagged mutagenesis (STM)

Signature-tagged mutagenesis is a method originally designed to identify genes required for pathogenesis (Hensel et al., 1995). A large number of mutants were created by insertional mutagenesis. The inserted DNA includes a unique oligonucleotide tag that resembles a 'barcode.' In principle, up to 96 mutants can be inoculated into one host; strains not recovered are thought to harbor a mutation specific for *in vivo* growth (Hensel et al., 1995). This method was first used in *Salmonella* and was modified for *C. glabrata, A. fumigatus,* and *C. neoformans* (Brown et al., 2000; Cormack et al., 1999; Nelson et al., 2001). There were certain considerations in translating this approach to fungi, including larger genomes, non-coding DNA, inefficient methods for insertion, selection of the appropriate host environment (Brown et al., 2000), and inoculation parameters (Nelson et al., 2001). These issues were addressed in the studies below (Brown et al., 2000; Nelson et al., 2001).

The first studies were performed prior to the identification of useful transposons. In order to identify virulence factors in *A. fumigatus,* two approaches were used to address random insertion of signature tags (Brown et al., 2000). The first used restriction-mediated integration (REMI). Protoplasts of the recipient strain were transformed with clones with tags in the presence of the restriction enzyme *Kpn*I (96 transformations). The rationale was that these clones would integrate into *Kpn*I sites randomly situated in the genome. The construction of the second library relied on ectopic integration, and *Aspergillus* was transformed with linearized clones (84 transformations). The tags for the transformation constructs for both approaches were generated by PCR using templates developed for *Salmonella typhimurium* and cloned into a fungal transformation vector that carries a gene for hygromycin resistance (Brown et al., 2000). A similar approach was used for *Cryptococcus neoformans*, and the selection of insertions was based on ectopic integration of a linear plasmid conveying hygromycin resistance (Nelson et al., 2001). Further analysis demonstrated that integration was mostly random, except for one hotspot that was the actin/*RPN10* promoter. In both cases, integration efficiency was lower than reported for bacteria.

Many of the medically important fungi can cause different types of infections and/or colonize and infect multiple organs. Unlike bacteria, they do not have true 'virulence factors'; but they do have virulence "attributes." Since *in vivo* murine models are involved, it is important to limit the number of mice used; and thus it is necessary to predetermine the appropriate model, the time points and the organs to harvest. For *Aspergillus fumigatus*, the STM libraries were tested in an immunosuppressed murine inhalation model (Brown et al., 2000). For *C. neoformans*, Nelson et al. (2001) carefully determined the course of infection in a murine model and chose a time point that reflected attenuated or increased virulence based

on cfu (colony forming units) counts in the brain (Nelson et al., 2001). They also asked an interesting question: Would a virulent strain allow survival of an attenuated strain? For instance, if the virulent strain damaged endothelium, normally avirulent strains might theoretically have increased abilities to disseminate. They tested this by co-infecting with acapsular (avirulent) and capsular (virulent) strains. The avirulent acapsular strains were not recovered, and they concluded that virulent strains would not help avirulent strains (Nelson et al., 2001). However, this may not be true for all attenuated strains; and a strain's ability to piggyback upon another will depend on its defect. This is a general drawback of STM and confirms that virulence tests with single strains have to be performed.

Another important parameter is the number of strains that can be injected into a mouse and have an equal opportunity to survive. Nelson et al. (2001) did a prescreen with hygromycin and G418 resistant strains (100:1) and ascertained that it was possible to inoculate 100 strains. However, studies with hybridization signals showed that they could not reliably detect more than 80 strains. Experiments were performed with pools of 48 strains. Six hundred seventy-two mutants were screened, and 39 gave different output signals. Twenty-four of the mutants were tested singly in the mouse, and 6 of these had significant changes in virulence (Nelson et al., 2001). Brown et al. (2000) determined that subsequent hybridization efficiency was 80%, so, although they used pools of 96, they always inoculated 2 mice per pool (Brown et al., 2000). In total 4648 tagged strains were screened, and 35 strains (0.8%) gave weak signals in the output pool after two rounds of STM. These strains were tested in a competitive inhibition infection, in which the attenuated strain was present as 50% of the inoculum. Nine strains showed a competitive disadvantage, and two of these demonstrated significantly reduced virulence. The site of the mutation of one strain was not identifiable; the second mutation was upstream of the PABA synthetase gene. Further analysis confirmed that *pabaA* is required for virulence.

Cormack et al. (1999) exploited STM to construct a mutant library in *C. glabrata* (Cormack et al., 1999). Each strain could be easily identified by a distinct tag. Ninety-six unique strains were generated by integrating 96 different tags, flanked by identical primer sites, into the already disrupted *URA3* locus. Since *C. glabrata* has an efficient system of non-homologous recombination, the *Saccharomyces URA3* gene was used for random mutagenesis. Transformants were selected on media minus uracil. Pools of the 96 tagged strains were screened for adherence to human cultured epithelial cells. Out of 4800 mutants (50 pools of 96), 31 mutants demonstrated aberrant adherence. Sixteen of these were non-adherent. Interestingly, 14/16 of these integrated into non-coding sequence upstream of the same gene, *EPA1*. This led to the identification of subtelomeric transcriptional silencing (Cormack et al., 1999). However, this method would not have identified *EPA1* by traditional STM, since *EPA1* null mutants are virulent *in vivo*.

3.3 Transposon-mediated insertional mutagenesis

Transposon technology has been used in pathogenic fungi to construct libraries, add epitope tags, and understand cellular processes. The technology has been adapted for diploid organisms using the parasexual cycle, haploid insufficiency, and homologous recombination (Carr et al., 2010; Davis et al., 2002; Firon et al., 2003; Juarez-Reyes et al., 2011; Spreghini et al., 2003; Uhl et al., 2003). The use of transposons has superseded auxotrophic and STM approaches.

Essential genes are often considered good drug targets. Firon et al. (2003) exploited the parasexual cycle to develop a transposon-mediated insertional mutagenesis protocol to identify essential genes in *A. fumigatus* (Firon et al., 2003). A diploid strain, homozygous auxotrophic for pyrimidines and heterozygous for a spore color marker, was randomly mutagenized with an *imp160*::*pyrG* transposon. The candidate mutant strains were induced to become haploid by the mitochondrial destabilizer, benomyl. The genotype of the parent strain allowed haploid progeny to be identified by pigmentation. Diploid strains were grey-green, but haploid progenies were white or reddish. Replica plating identified the haploid progeny that harbored transposons. If haploid strains carried a transposon-inactivated allele, they expressed pyrG and grew on both selective (without uridine/uracil) and non-selective media. Conversely, strains without a transposon grew only on non-selective media. If the transposon inactivated an essential gene, the haploid strain did not grow on either medium. With this approach, 3% of the haploid progeny of 2,386 diploid strains were found to be unable to grow on either medium and, therefore, possibly had mutations in essential genes. These strains were propagated further on selective media and haploid progeny could not be obtained from 1.2% of the resultant diploid revertants. The sites of insertion were determined by 2-step PCR using semi-random primers and 5′-end transposon-specific primers (see Section 3.5.1). Ninety percent of insertion sites were identified (Firon et al., 2003). Since the insertion rate of the transposon into essential loci was lower than expected, additional transposon insertion sites were analyzed. Although an insertion site did not depend on genome sequence or chromosomal location, there did appear to be a bias toward noncoding regions (34%) (Firon et al., 2003). Carr et al. (2010), who observed that transposon mobilization could be induced at 10 °C, improved upon this approach. Therefore, using the same screen, 96 additional essential loci were identified. They found no obvious bias of insertion in noncoding regions. Interestingly, only half of the genes had essential homologs in *Saccharomyces,* confirming the necessity for species-specific screening.

Uhl et al. (2003) developed a transposon mutant library in *C. albicans*. Restriction enzyme-digested *C. albicans* gDNA (genomic DNA) was mixed with a linearized donor transposon Tn*7*–containing plasmid. This plasmid harbored elements for replication in *E. coli*, for selection in both *E. coli* and *C. albicans* and a fungal *lacZ* reporter system. The fragments were ligated and introduced into *E. coli* by transformation. Plasmids were isolated from over 200,000 transformants and batch isolated. Transposon–gDNA junctions were sequenced in plasmids to confirm random integration. *C. albicans* was transformed with the Tn*7*-gDNA plasmids to give an 18,000-strain transposon mutant library. It was assumed that each strain had an independent insertion. That would mean there was a transposon approximately every 2.5 kb. However, only one allele of a gene was disrupted in these strains. (*C. albicans* is diploid, so one allele remains non-disrupted.) Uhl et al. (2003) exploited haploid insufficiency to screen for filamentation mutants, since heterozygote strains in genes involved in morphogenesis exhibit reduced filamentation. This screen was rapid and successful for identifying processes that required genes sensitive to dosage effects. However, this certainly will not be the case for all genes involved in pathogenesis.

Davis et al. (2002) constructed a transposon mutant library in *C. albicans*, but these strains harbored insertions in both alleles. This approach was based on a homologous recombination model that allowed the disruption of both alleles of *C. albicans* in one transformation step (Enloe et al., 2000). A cassette (UAU), which contains the *URA3* gene

disrupted with a functional *ARG4* gene, was inserted into transposon Tn*7*. the Tn*7*–UAU transposon was inserted randomly into a *C. albicans* library. Digestion with the appropriate restriction enzyme released DNA fragments that contained *C. albicans* DNA interrupted with the Tn*7*-UAU transposon. These fragments were used to transform *C. albicans*. Homology from the interrupted DNA allowed replacement of the chromosomal wild-type version by homologous recombination. The chromosomal version was then mutated because it carried the Tn*7*-UAU transposon. Recombinants could be selected because transformation into the recipient ura- arg- *Candida* strain will confer arginine prototrophy. Occasionally the other intact copy of the gene acquired the transposon. Thus, both copies of the gene were mutated. Using arginine selection, homozygous mutants could not be distinguished from the heterozygotes. However, in a small percentage of ARG^+ transformants, the *ARG4* gene is spontaneously looped out. If there were two copies of the transposon and if looping out occurred in one, it gave an ARG^+, URA^+ strain. Thus, both alleles were disrupted. This allowed for the construction of a large set of mutants, though it was still not as efficient as it would be for a haploid strain. This library is widely used by the *Candida* community (Davis et al., 2002; Norice et al., 2007; Park et al., 2009).

Spreghini et al. (2003) exploited transposon mutagenesis to add an epitope to the putative cell wall protein, Dfg5p. Since conventional epitope tagging of amino and carboxyl termini was not an option, they wanted to identify an internal site which, when disrupted with a tag, did not compromise function. The Tn*7* transposon was used to mutagenize the *DFG5* insert in a plasmid and insertions within *DFG5* coding region were confirmed by sequencing. Then the mutagenized plasmid was redigested to get rid of the majority of Tn*7*, leaving only a 15-bp (base pair) insertion, which resulted in an insertion of 5 amino acids that did not disrupt function and could be recognized by an available antibody. The internally tagged *DFG5* insert was then ligated into *C. albicans* vectors for further study (Spreghini et al., 2003).

In the transposon examples above, mutagenesis was performed *in vitro*, and then mutagenized DNA was introduced into recipient strains. Magrini and Goldman (2001) took a different approach by directly mutagenizing *Histoplasma capsulatum in vivo*. The transformation cassette was a linear telomere vector (because the presence of a telomeric sequence is required for efficient homologous recombination in *Histoplasma*) containing the selection marker *URA5*, the *MOS1* transposase gene regulated by a strong promoter, and the hygromycin resistance gene flanked by *MOS1* terminal repeats to create a synthetic transposon. *Histoplasma* transformants were selected in presence of 5-FOA (5-Fluoroorotic acid, which selects against URA5) to select for loss of the donor plasmid and on hygromycin for the presence of the synthetic transposon, which encodes hygromycin resistance. It is not known if this library has been utilized because T-DNA appears to be more commonly used in *Histoplasma* (see below).

A novel use of random insertion was the analysis of subtelomeric silencing of *C. glabrata* adhesin genes. Learning where silencing occurred was accomplished by randomly placing a *URA3* reporter at different distances from a telomere and examining where *URA3* was silenced. The transposon Tn*7*–*URA3* was introduced into a subtelomeric sequence of *C. glabrata* cloned on an *E. coli* plasmid. Resulting constructs were integrated into subtelomeric regions of *C. glabrata* by homologous recombination. It was possible to select for 'silenced' *URA3* on 5-FOA media (Juarez-Reyes et al., 2011).

3.4 *Agrobacterium* T-DNA

Agrobacterium tumefaciens carries an approximately 200-kbp (kilobase pair) tumor-inducing (Ti) plasmid. A portion of this plasmid is called T-DNA. In plants, the T-DNA randomly inserts in the genome; and the outcome is a tumorous growth. This plasmid has been modified for genetic manipulation purposes to retain the insertional DNA (T-DNA). The plasmid vector can also replicate in *E. coli* and has cloning sites for additional DNA. T-DNA has been used to construct mutants with increased, reduced, or no expression of genes, depending on the plasmid used (Krysan et al., 1999). In the last decade, insertional mutagenesis via T-DNA has been successfully adapted for medically important fungi. In general, a fungal selectable marker is ligated into the *Agrobacterium* Ti plasmid within the T-DNA region and introduced into *A. tumefaciens* by electroporation. Equal concentrations of *A. tumefaciens* carrying the delivery plasmid and target fungal strain are incubated together for varying lengths of time under conditions that mimic plant wound conditions, which are accomplished by low pH and the addition of acetosyringone. The T-DNA is transferred to the target organism by a conjugation-like mechanism. A mutant that contains an insertion of T-DNA is selected with the appropriate fungal selective marker.

Prior to T-DNA mutagenesis, insertional mutagenesis was attempted by electroporation or biolistic transformation of naked DNA. Researchers have developed protocols that have improved the efficiency of transformation using T-DNA in *C. neoformans* (Idnurm et al., 2004), *Histoplasma* and *Blastomyces* (Brandhorst et al., 2002; Edwards et al., 2011; Gauthier et al., 2010; Laskowski and Smulian, 2010; Marion et al., 2006; Smulian et al., 2007; Sullivan et al., 2002). In addition, T-DNA mutagenesis protocols have been developed for *Coccidioides* (Abuodeh et al., 2000), *Trichoderma* spp. (Cardoza et al., 2006; Dobrowolska and Staczek, 2009; Yamada et al., 2009), and *Penicillium marneffei* (Kummasook et al., 2010; Zhang et al., 2008). In *C. neoformans*, the use of T-DNA improved both the efficiency and the stability of transformation events. The resulting transformants also demonstrated less complicated integrations and less additional gene rearrangements. There did seem, however, to be a bias for promoter sequences. In one study, some of the integration events were not linked to *NAT*, the gene for the Nourseothricin resistance marker on the inserted DNA (Idnurm et al., 2004).

Blastomyces, in particular, is a challenge to transform, since it is multinucleate. Transforming DNA often integrates at multiple sites (Brandhorst et al., 2002). This is usually bypassed by transforming conidia or performing multiple rounds of selection to enrich for homokaryons (all the nuclei are genetically identical). Sullivan et al. (2002) developed a protocol for both *Histoplasma* and *Blastomyces*. Many conditions were tested, including bacteria:yeast ratios, life stage of the recipient strain, and the choice of selectable marker. Interestingly, the efficiency of transformation was 5–10 times higher with uracil selection than with hygromycin selection. Southern analysis confirmed that integration was random, but there were often direct repeat concatemers in *Blastomyces.* There were clear improvements over electroporation, including increased efficiency, ability to use spores as the recipient, and single-site integrations (Sullivan et al., 2002). Additional studies in *Blastomyces* using T-DNA have identified genes involved in phase transition (Gauthier et al., 2010).

T-DNA was used to identify genes in *Histoplasma* involved in pathogenesis in a novel high-throughput macrophage-killing screen (Edwards et al., 2011). Transgenic (a novel gene was introduced) macrophage lines were constructed that constitutively expressed bacterial *lacZ*.

The activity of β- galactosidase, the product of *lacZ*, directly correlated to the number of macrophages. Thus, this line was used as a readout for macrophage killing. Over 2000 *Histoplasma* transformants made from *A. tumefaciens*-treated *Histoplasma* cells were incubated with macrophages and screened for killing activity after 7 days. Three strains were less efficient in killing, and one was significantly inefficient in killing both transgenic and primary macrophages. Flanking sequences were identified by PCR and sequencing. The authors identified a new virulence gene in *Histoplasma*, a homolog of Hsp82 (Edwards et al., 2011).

Marion et al. (2006) performed a more comprehensive analysis of insertional mutagenesis in *Histoplasma capsulatum* using *Agrobacterium*-mediated transformation. Optimal co-incubation times, bacteria:yeast ratios and temperature were determined. Southern hybridization analysis showed that approximately 90% of the insertions were random and at a single site. Inverse PCR and plasmid rescue were used to identify the flanking sequences. Their results indicated that mutagenesis by T-DNA resulted in the absence of chromosomal rearrangements and deletions. The biological relevance of the T-DNA mutants was approached by screening for genes involved in the biosynthesis of α-(1, 3)–glucan, which is posited to be a virulence attribute. The absence of α-(1, 3)–glucan was easily visualized since colonies have a smooth, rather than rough, morphology. Approximately 50,000 insertional mutants were screened, and 25 had smooth morphology. Eighty-eight percent had single insertions and reduced α-(1, 3)–glucan. Five of twenty-two had distinct insertions in the α-(1, 3)–glucan synthase gene (AGS1), which validated their screen. RNAi technology (synthetic inhibitory RNA) was used to confirm the insertion mutant phenotype with the wild-type allele. The phenotypes of the two other mutants were confirmed. One mutation was in *UGP1* (previously reported to play a role in glucan synthesis). The other mutation was in the amylase gene, which was previously unreported to play a role (Marion et al., 2006).

The use of T-DNA in *Histoplasma* has provided additional information. As with all genetic manipulations, it is important to confirm that the mutation is responsible for the ensuing phenotype. Smulian et al. (2007) wanted to make GFP-expressing strains and used hygromycin resistance as a marker and T-DNA as the tool for integration. It turned out that all the transformants were hypervirulent. Site-directed mutagenesis of the hygromycin resistant gene, *hph*, confirmed that the increased virulence was due to the acquisition of hygromycin resistance. One mutant actually gained the ability to form cleistothecia, a mating structure that was not present in the parent strain. This phenotypic trait was not due to the *hph* gene; and, thus, the strain may be used as a tool to study mating in *Histoplasma* (Laskowski and Smulian, 2010).

3.5 Methodologies to identify the site of insertion

3.5.1 Two-step PCR

In the first step of two-step PCR (Chun et al., 1997), sequence on one side of the insertion site is amplified with a degenerate primer and a primer homologous to the sequence in one of the ends of the inserted DNA. (There are two end-specific primers. A primer specific for only one end is used. Note that a tranposon can insert in either orientation.) The degenerate primer contains 20 nucleotides of defined sequence at the 5'-end, 10 nucleotides of

degenerate sequence (*i.e.*, all 4 nucleotides are used at each position for synthesis) + GATAT at the 3'-end. The sequence GATAT is predicted to occur every 600 bp in the yeast genome. The second step amplifies the first PCR product with two non-degenerate primers. The forward primer contains the 20 nt (nucleotides) of defined sequence in the degenerate primer. The reverse primer is immediately 3' (antisense strand) to the insertion-specific primer used in the first PCR reaction to guarantee that the desired DNA is amplified (Chun et al., 1997). This method was originally defined in *Saccharomyces* and was successfully used to identify transposon insertions in *A. fumigatus* (Carr et al., 2010; Firon et al., 2003).

3.5.2 Thermal asymmetric interlaced PCR (TAIL PCR)

TAIL PCR (Liu and Whittier, 1995) is another method to identify sequences flanking insertions. It is a modified version of hemispecific (one-sided) PCR. The purpose is to favor amplification of the desired product. It uses specific primers homologous to DNA in the integrating cassette or plasmid and a degenerate primer that can anneal to the gDNA flanking the insertion. The strategy is that the specific primers are long, nested, and have a high Tm; the degenerate primer is short and has a low Tm. The first five cycles are high stringency cycles to favor annealing to and linear amplification from the specific primer. Then there is one low stringency cycle to allow the degenerate primer to anneal. Because there are now several copies of the gDNA adjacent to the insertion, the chance of the degenerate primer annealing to the desired product is increased. However, other products might form from the primers finding additional annealing sites in the genome. Using a second and a third primer completely homologous to the inserted DNA will favor the desired product that is made from both the specific and degenerate primers instead of either one alone. This is accomplished by interlacing reduced stringency and high stringency cycles.

4. Closing remarks

Site-directed and insertional mutagenesis are techniques that can be used to advance our understanding of the pathogenesis of medically important fungi. The exploitation of these tools has resulted in a better understanding of drug-resistant mechanisms, transcription factors, signaling pathways and vital cellular processes. Site-directed mutagenesis could be better utilized to decipher the functions of essential and multi-functional genes. While all approaches cannot be used in the always-diploid strains, transposon-mediated insertional mutagenesis can be used to construct libraries. Additionally, T-DNA can be used to improve transformation efficiency in dimorphic fungi and in *C. neoformans.*

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