Chapter 15
Methods in Fungal Genetics

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15.1 Introduction

15.1.1 Evolution of Modern Gene Manipulations

Whether in saving starter for sourdough bread or making koji mold for Sake fermentation, humans have been manipulating fungi for their own purposes for millennia. The Tyrolean iceman, Ötzi, had fungi among his personal items although whether for medicinal or practical use is still debated (Rollo et al. 1995). With the development of modern germ theory and the exposition of Koch's postulates, practical microbiology included saving and characterizing pure cultures of microorganisms. Even before Fleming discovered his *Penicillium* strain (Bennett and Chung 2001) that led to modern pharmaceutics, culinary strains were selected for high spore production, lack of toxic metabolites, flavor profiles, purity, and stability (Machida et al. 2008). It can be argued that once strain improvement for higher antibiotic production began, modern biotechnological manipulation was on a natural continuum, and while the specific techniques have changed, the general aims remain the same. These aims include high productivity, lack of contaminating side products, purity, and stability.

15.1.2 Research Versus Commercial Applications

As biological sciences went from being purely descriptive, and experimental biology became practical, fungi were brought into the laboratory for characterization and as model systems for larger biological inquiry. Among the foundational

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discoveries made using model fungal organisms are the one gene-one enzyme hypothesis (Beadle and Tatum 1941), the existence of suppressor mutations (Tatum and Perkins 1950), multifunctional gene clusters, and regulatory mutations (Horowitz et al. 1961). In recent years, fungi continue to be valuable models for population genetics, genome organization, epigenetic gene modification, circadian rhythms, and host-pathogen interactions.

Commercial use of characterized and improved fungal material touches on many aspects of agriculture, medicine, food and fiber processing, and increasingly in the day-to-day activities of a modern lifestyle (Bohlin et al. 2010). Whether it be biocontrol of insect pests or invasive non-crop plants, the manipulation of field populations of toxin-producing or pathogenic organisms, the production of industrial chemicals, bio-processing, or the production of enzymes or pharmaceuticals, genetically characterized and manipulated strains represent the foundation and reference against which all progress is measured (OECD 2001).

Many of the research uses of fungi are removed from practical applications, although both approaches increasingly rely on the use of genetically manipulated strains. It is important in this context to emphasize that genetically manipulated strains may be generated by meiotic genetics, mitotic recombination, and by DNA-based genetic transformation using genetic constructs produced in vitro.

15.1.3 GMO Organisms and Regulatory Status

Materials collected in the environment, whether from intact wilderness, from agricultural, or even urban settings, are considered to be genetically wild type, and strains that have been derived from them by classical mutagenesis or by Mendelian or even mitotic recombination are evaluated and regulated according to the same criteria. Materials that have been generated by genetic manipulation using engineered or synthetic molecules are subject to regulation as hazardous biological materials. Moreover, shipment of GMO materials is governed by the same guidelines as dangerous goods. At the time of the writing of this review, this circumstance is in a state of flu15. The Cartagena Protocol on Biosafety to the Convention on Biological Diversity (http://bch.cbd.int/protocol/) is an international treaty negotiation with purview over the safe handling, transport, and use of GMO organisms, which they describe as “living modified organisms.” This protocol further describes its purpose as the protection of biodiversity from the potential risks of living modified organisms. It entered into force in September 2003, having been ratified by 50 countries. Regardless of the purpose, this protocol will have implications for the use of modified organisms in research, agriculture, and in the areas of pharmaceutical and industrial research and production.

15.1.4 Scope

The present chapter will describe the development of tools for manipulation of filamentous fungi with emphasis on tools for research application. It will describe the evolution of modern molecular genetic tools and include circumspection on their characteristics. Emphasis will be placed on the use of materials in the collection of the Fungal Genetics Stock Center, and description of these materials will be elaborated, to the extent that it provides insight into directions of research over many years.

15.2 Classical Genetic Manipulation

Once fungi had been domesticated and put to practical uses, and simultaneous with the rediscovery and validation of Mendel's work on pea genetics by de Vries and others in the late nineteenth century (Hurst 2009), fungi were subject to investigations of their genetics both to explore the nature of genes and as a practical means of strain improvement. While some organisms were studied just to identify principles of genetics (Shear and Dodge 1927; Blakeslee 1904), others were studied primarily because of their practical application or impact (Takamine 1914; deBary 1853).

15.2.1 Mendelian Genetics

The development of model systems for filamentous fungi was based on the ability to conduct Mendelian genetics. The demonstration of segregation of mating type was made in a number of fungal systems including *Neurospora* (Lindegren 1932), *Schizophyllum*, *Ustilago*, *Sordaria*, and yeast (Kronstad and Staben 1997). Similarly, mating type was characterized in a number of water molds, some of which are no longer considered fungi. These included *Allomyces* (Emerson and Wilson 1949), *Achlya* (Raper 1939), and *Phytophthora* (Judelson et al. 1995). In parallel, mating and other characteristics were described for slime molds, and these had significant parallels to fungal systems (Bonner 1944). Classical genetics has been foundational for the understanding of fungal biology and for many years was the best way to characterize interesting traits. This also included extensive study of the mechanisms of genetics, the nature of intra- and intergenic recombination, as well as population and quantitative genetics. In the most well-characterized systems, such as *Neurospora*, *Aspergillus*, yeast, and numerous plant-pathogenic fungi, genetic maps were constructed demonstrating the number of linkage groups and the relationship of markers on these linkage groups. This was corroborated by cytogenetic analysis of stained chromosomes, (McClintock 1945) although for many fungi, the number

of chromosomes is underestimated because most of the chromosomes are too small for classical microscopic analysis as demonstrated by electrophoretic karyotype analysis (Mills and McCluskey 1990). For example, *Neurospora* has seven linkage groups and also seven microscopically visible chromosomes, while *Ustilago* has as many as 20 electrophoretically resolvable chromosomes (Kinscherf and Leong 1988) but only 4 that are visible in cytological preparations (Lindegren 1948). Moreover, while many years of mutagenesis and characterization have yielded as many as 1,200 markers on the *Neurospora* genetic map (Galagan et al. 2003), whole-genome sequence has identified closer to 10,000 genes in this fungus. The systematic gene deletion program for *N. crassa* (Colot et al. 2006) has produced gene deletion mutants for over 8,000 unique ORFs, and of these, 1,500 are only available as heterodikaryons, suggesting that these ORFs are essential. This suggests that the saturation mutagenesis of many years was successful in mutating most ORFs for which a phenotype could be recovered. Moreover, these KO strains effectively carry an antibiotic resistance marker at every locus, and this can be used for fine structure mapping (Hammond et al. 2012).

15.2.2 Non-Mendelian Genetics

Because the demonstration of Mendelian genetics requires appropriate mating pairs or specific conditions to produce a teleomorph, some fungi were considered to be asexual, although mating-type alleles and even sexual reproduction have been identified for some species (Arie et al. 1997; O'Gorman et al. 2008). For these, the ability to carry out mitotic recombination was a valuable tool for pseudo-genetic manipulation. For example, the first markers mapped in *Aspergillus nidulans* were characterized by mitotic mapping (Pontecorvo 1956). Other approaches to non-Mendelian genetics included protoplast fusion between noncompatible strains and species. This leads to the production of potentially aneuploid strains, and the reduction of this aneuploidy can generate stable strains with traits not available through natural processes (Peberdy 1979). In a similar vein, interspecies crosses can be carried out for some organisms. The spore-killer element from *Neurospora intermedia* was introgressed into *N. crassa* (Turner and Perkins 1979) to allow better characterization. This element is carried on a 1.9 Mb region on the left arm of contig 3 presumably including the entire meiotic drive element along with other sequences (McCluskey et al. 2011).

Similarly, the characterization of strains carrying aneuploid or rearranged chromosomes has been useful both with regard to the insight it provides into fungal chromosome biology and also as a tool for characterization of gene location and epigenetics (Perkins 1997). Supernumerary chromosomes, sometimes referred to as B chromosomes (Hurst and Werren 2001) or, more recently, lineage-specific chromosomes (Ma et al. 2010), have been characterized in a number of fungi (Covert 1998). These chromosomes do not follow typical Mendelian genetics and are historically considered to be segregation distorters (Jones et al. 2008). The

distribution of these elements is not uniform, and while some fungi, such as *Neurospora*, do not tolerate foreign DNA (Windhofer et al. 2000), others, such as *Nectria haematococca* (Coleman et al. 2009) or *Fusarium oxysporum* (Ma et al. 2010), have a significant portion of their genomes on these dispensable chromosomes (Croll and McDonald 2012).

Now characterized in many organisms, epigenetic phenomena were characterized in filamentous fungi beginning with the demonstration of the mutagenic phenomenon induced by duplicated genes and known as RIP (Cambareri et al. 1989). Similarly, vegetative silencing in fungi was demonstrated by silencing of genes for carotenoid biosynthesis (Cogoni et al. 1996). Epigenetic phenomena are now known to be present in myriad fungi (Selker 1997).

15.2.3 Mutagenesis

Early research with fungi leading to such hallmark discoveries as the one gene–one enzyme hypothesis and intragenic recombination was carried out with filamentous fungi. Among these were the demonstration of spore color mutants in the homothallic species *Sordaria* (Sang and Whitehouse 1983), light sensing in *Phycomyces* (Delbruck and Meissner 1968), and auxotrophy and morphological anomalies in *Neurospora* (Beadle and Tatum 1941). For many fungi, mating type was the most reliable character subject to Mendelian analysis in fungi, and this one area was disturbingly not amenable to mutational analysis.

In *Neurospora*, and other fungi, filtration enrichment was developed as a technique to increase the likelihood of identifying appropriate mutant strains (Woodward et al. 1954). In this ingenious approach, heavily mutagenized spores are allowed to germinate in liquid culture and then subject to multiple cycles of filtration over loosely woven cotton cloth or other coarse matrix. Conidia that germinated either in minimal medium, or in special cases under selective pressure such as elevated temperature, were trapped in the filter, and conidia with a nutritional or other requirement passed through the filter. Subsequent culture of these defective conidia in complete medium or at reduced selection pressure identified mutants with a frequency that is much higher than simple mutagenesis.

Because of the frequency of inducing secondary mutations, it has always been best practiced to backcross mutants to a well-known wild type. Estimates on the number of crosses suggest that as many as ten backcrosses may be necessary to purify a unique mutation (Leslie 1981), although in practice this large number of backcrosses was associated with a 20-map unit recombination block around mating type.

Whole-genome analysis of classical mutant strains of *N. crassa* has shown the impact of backcrossing into the reference genome (Table 15.1). Where the number of backcrosses was known for classical mutant strains, the lowest divergence from the reference genotype was seen in strains that had been backcrossed multiple times into the reference genotype. This analysis rests on the fact that most SNPs were not

Table 15.1 Impact of backcrossing on frequency of polymorphisms detected in whole genome sequence from classical mutant strains of *N. crassa*

Strain	Marker	Mutagen	Backcrosses	Total SNPs	% max SNPs	# nonsense mutations	Reference
106	*com*	UV	3	23,579	12.5	18	Perkins and Ishitani (1959)
305	*amyc*	–	3	90,195	47.9	67	Atwood and Mukai (1954)
309	*ti*	X-rays	3	13,274	7.0	11	Perkins (1959)
322	*ty-1*	Spontaneous	–	142,489	75.7	95	Horowitz et al. (1961)
821	*ts*	Spontaneous	–	188,346	100.0	122	Nakamura and Egashira (1961)
1211	*dot*	Spontaneous	3	20,493	10.9	19	Perkins et al. (1962)
1303	*fi*	Spontaneous	–	59,356	31.5	35	Perkins et al. (1962)
1363	*smco-1*	Mustard	–	146,641	77.9	137	Garnjobst and Tatum (1967)
2261	*do*	UV	3	44,839	23.8	37	Perkins et al. (1962)
3114	*Sk-2*	Introgression	1	41,085	21.8	31	Turner and Perkins (1979)
3246	*fs-n*	Spontaneous	–	21,533	11.4	14	Mylyk and Threlkeld (1974)
3562	*mb-1*	UV	–	106,533	56.6	78	Weijer and Vigfusson (1972)
3564	*mb-2*	UV	–	47,981	25.5	36	Weijer and Vigfusson (1972)
3566	*mb-3*	UV	–	37,516	19.9	27	Weijer and Vigfusson (1972)
3831	*ff-1*	Spontaneous	–	22,961	12.2	15	Tan and Ho (1970)
3921	*tng*	Spontaneous	2	80,311	42.6	84	Springer and Yanofsky (1989), Howe and Benson (1974)
7022	*fld*	Spontaneous	–	78,991	41.9	45	Perkins et al. (1962)
7035	*per-1*	UV	3	18,487	9.8	13	Howe and Benson (1974)

unique and the result of the particular mutagenesis. Rather, they were found in multiple strains and seem to be associated with the multiple lineages used in the *Neurospora* research community. Many of the same polymorphisms seen in these classical mutant strains were also found in an independent analysis of a separate classical mutant strain (Pomraning et al. 2011).

Interestingly, nonsense mutations were common in classic mutant strains. The distribution of nonsense mutations was correlated with the lineage of the strain and inversely related to the number of backcrosses. The strains with the fewest numbers of nonsense mutations had all been backcrossed three times, while the strains with the most polymorphisms had not been backcrossed into the reference genome lineage (Table 15.1), emphasizing the importance of multiple generations of backcrossing in strain construction.

For many years, strain improvement included mutagenesis. Penicillin-producing strains were a key example of the impact of strain improvement (Sermonti 1959). Useful yields of antibiotic increased by several orders of magnitude during classical strain improvement. Subsequently, whole-genome analysis of *A. niger* strains subject to classical strain improvement showed the impact of strain improvement on the genome (Andersen et al. 2011). This study revealed many differences between the improved and reference genome strains including a high level of nucleotide polymorphisms, significant genome rearrangements, deletions, and even sequence unrelated to the reference genome. Transcriptome analysis also indicated that significant changes in regulation of gene expression had occurred.

Numerous recent efforts have characterized classical mutant strains and among them are the studies of nine *Neurospora* strains carrying classical mutations in genes responsible for pigment biosynthesis. These strains were characterized by direct sequence analysis of the *albino-2* gene (Diaz-Sanchez et al. 2011). One of the strains produced an aberrant pigment while others were albino. The red pigment-producing strain had multiple non-synonymous mutations as well as other synonymous ones. Several of the mutants produced truncated or shortened polypeptides and these mutants were variously completely albino or leaky. Only some polymorphisms at *al-2* were shared among multiple strains suggesting that these mutant strains were not all generated in the identical genetic background, similarly to that which was seen among the classical mutant strains subject to whole-genome analysis. Similarly, well-mapped mutations at the *ad-8* locus in *Neurospora* were used to establish that intragenic recombination was responsible for some instances of allele complementation (Ishikawa 1962). A subset of these mutations were characterized by targeted sequence analysis (Wiest et al. 2012a), and unlike the mutants at *al-2*, the *ad-8* mutants did not contain multiple changes, nor did they show any influence of founder effects.

15.3 Transformation

Originally demonstrated in *Streptococcus* in response to fundamental questions of the nature of the material of inheritance (Alloway 1932), the ability to transfer traits by introducing foreign DNA, either natural or from synthetic origins, among strains is foundational to every modern research system. While the techniques for accomplishing this transformation vary, all rely on the ability to introduce foreign DNA into a strain and have that strain express that DNA as though it were part of the natural genome. For some organisms, this is relatively straightforward, and the introduced DNA can become part of the nuclear genome. For other organisms, gene silencing and even mutation can limit the ability to mobilize and express foreign genes at high levels. Also, and in a manner similar to the requirement for backcrossing strains arising from explicit mutagenesis, transformants often need to be purified. This is especially important for organisms where the transforming cells are multinucleate, and nuclei that are not transformed can persist in hyphae

that are shared with a nucleus expressing the selectable marker gene. Moreover, transformation itself is mutagenic, and this has been shown both for insertion-associated mutations (Perkins et al. 1993) and also for second-site mutations (Keller et al. 1990).

15.3.1 Protoplast/Polyethylene Glycol

Protoplast fusion, whether in the presence of synthetic DNA or between cells carrying unique traits, has been used for strain manipulation and improvement for many years (Turgeon et al. 2010). The best results are available when high-quality protoplasts are available (Daboussi et al. 1989). The distinction between spheroplasts and regenerable protoplasts lies in the fact that protoplasts contain all the requirements to form a new colony while spheroplasts are merely osmotically sensitive liquid-filled micelles (Peberdy 1987).

The production of protoplasts requires enzyme cocktails to remove the cell wall polysaccharides and to make the cell permeable to DNA in the presence of polyethylene glycol (PEG) or similar polymer (Peberdy 1987). While generic enzyme cocktails may work for some species, other species require custom enzymes or other pretreatment. *Neurospora*, *Saccharomyces*, *Ustilago maydis*, and *A. nidulans* are generally amenable to protoplasting with generic enzyme cocktails, although even these amenable species are vulnerable to inconsistencies in enzyme specificity (de Bekker et al. 2009). Some organisms, such as *U. hordei*, *A. fumigatus*, or *Schizophyllum*, require specific enzyme cocktails. While this is sometimes accomplished by the addition of specific purified enzymes, the best protoplasting cocktails are generated by growing the enzyme-producing fungus, typically *Trichoderma* or *Aspergillus niger*, on purified cell walls from the target organism (Peberdy 1987). For example, to produce cell wall-degrading enzymes to make protoplasts from *S. commune*, one would first grow a large batch of *S. commune* mycelia and then use the purified cell walls of the *S. commune* strain as substrate to culture the *Trichoderma* or *A. niger* strain from which the cell wall-degrading enzymes are to be extracted.

Another approach is to use a strain that either constitutively or conditionally fails to produce a cell wall. In *N. crassa*, a strain known as slime contains three genetic lesions that combine to produce a wall-less strain (Selitrennikoff 1979). Regardless of how the protoplasts are produced, all transformation techniques rely on the use of PEG. The role played by the PEG in transformation was analyzed and compared to other molecules, and it was considered that the PEG functioned in concentrating the DNA and that it does not directly cause protoplast fusion (Kuwano et al. 2008).

15.3.2 Electroporation

This technique takes advantage of the ability of a pulse of electrical energy to create pores in biological membranes which allows DNA to enter a cell (Dower et al. 1988). While the exact mechanism by which electroporation facilitates DNA uptake and entry into the nucleus is unknown, alternate theories include the passage of DNA through pores (Levine and Vernier 2010), the formation and uptake of vesicles (Kawai et al. 2010), or even the electrophoretic acceleration of DNA in an electric field.

While the characteristics of the electrical discharge are subject to discussion (Weaver 1995) and the ideal system for electroporation should have a square discharge wave, practical issues mean that a logarithmic discharge is more readily achieved and produces adequate transformation efficiencies (Chen et al. 2006).

15.3.3 Chemical

Typically involving lithium acetate and modeled after bacterial transformation (Chung et al. 1989), some species can be readily transformed by preparation of competent cells using salt treatment. This has been shown not only for yeast-like fungi including Saccharomyces but also in *Microbotryum violaceum* (as *U. violacea*) (Bej and Perlin 1989). The role of lithium, the preference for different lithium salts, and the ability of different cations to replace lithium have been exhaustively studied (Kawai et al. 2010).

15.3.4 Biolistic

In some cases, DNA can be directly introduced to cells using microscopic pellets coated with the DNA one wishes to introduce. Because of the complexity of this protocol and because of the relatively low yield, it is preferential to use another approach where available. In some cases, biolistic transformation is the only option, and in these cases, it can be extremely useful (Olmedo-Monfil et al. 2004).

15.3.5 Agrobacterium

Many species of fungi, even filamentous fungi, can be transformed by conjugation of special plasmids. This is accomplished by co-culturing the target organism with *A. tumefaciens* cells carrying a plasmid mobilized by the presence of so-called T-DNA regions (Betts et al. 2007). The DNA that is transferred into the fungal cell

integrates randomly into the genome and generates a tagged integrant where the presence of the T-DNA marks the insertion (de Groot et al. 1998). Strains transformed with this method require careful evaluation to assure the presence of a unique insertion. Moreover, the stability of the inserted DNA has not been evaluated in all target species. This may be especially relevant for species where meiotic gene silencing or mutation depends on the presence of repeated DNA. The ability to transfer DNA via Agrobacterium was exploited in the generation of tagged-integrant mutants of *Magnaporthe grisea*, and 48,000 such strains were generated and phenotyped (Tucker and Orbach 2007; Soderlund et al. 2006).

15.4 Selectable Markers

Transformation by foreign DNA is relatively inefficient (Fincham 1989) with 1–100 transformants per microgram of transforming DNA and per 10^7 recipient cells being not uncommon. Because of this, it is not practical to screen for cells that have foreign DNA, and rather selectable markers, which allow only the cells carrying and expressing foreign DNA to grow, are employed. These markers vary in their characteristics, but all allow the growth of only cells expressing the marker gene. In addition to a robust selectable marker, appropriate fungal promoters and terminators are required for stable transformation. For many markers, the *Aspergillus nidulans* TrpC promoter and terminator were used (Yelton et al. 1984).

15.4.1 Auxotrophic Complementation

The first demonstration of transformation by complementation of auxotrophy was made by culturing cells of a Neurospora strain that required inositol with DNA from an inositol prototrophic strain (Mishra and Tatum 1973). Practical use of auxotrophic markers, however, awaited the demonstration of transformation of protoplasts of a leucine-requiring Saccharomyces strain with the DNA from a leucine prototroph (Hinnen et al. 1978) and soon thereafter with the LEU2 gene on an autonomously replicating shuttle plasmid (Beggs 1978). Among the most commonly used selectable markers are complementation of nutrient requirements, such as histidine (Case et al. 1979) and pyrimidines (Ballance et al. 1983). Other auxotrophies have been employed, such as tryptophan (Yelton et al. 1984), adenine (Kurtz et al. 1986), quinic acid (Case 1982), and nitrate reductase (Campbell et al. 1989).

15.4.2 Drug Resistance

Because complementation of auxotrophic strains has specific problems, the use of dominant markers such as drug resistance is highly desirable. For fungi this is complicated by the relatively few drugs available as drug/selectable marker systems. The most commonly used drug for selection in filamentous fungi is hygromycin, and many vectors are derived from the pAN7-1 plasmid first used to confer hygromycin resistance in *Aspergillus* (Punt et al. 1987). Other markers include bialaphos resistance (Avalos et al. 1989) and then less commonly used markers like sulfonylurea (Sweigard et al. 1997), phleomycin (Mattern et al. 1988), and nourseothricin (Kück and Hoff 2006).

15.4.3 Recyclable Markers

By careful expression of splicing sequences and the genes for their recognition, some markers can be evicted once transformation has been accomplished. These commonly use the cre-lox system and require induction of the genes for splicing. The end product is a transformed strain that no longer carries the selectable marker. These strains can then be transformed with the same marker increasing the value of such systems (Krappmann et al. 2005). In a similar vein, the FLP/FRT technique has also been adapted from *Saccharomyces cerevisiae* to recycle markers in *P. chrysogenum*, and other filamentous fungi, by codon optimization of the recombinase gene (KOPKE et al. 2010).

While not in the same category as markers that integrate into the genome and require subsequent excision, autonomously replicating plasmids are important for manipulation of some species. The AMA series of plasmids (Osherov et al. 2000) offer the benefit of autonomous replication for *Aspergillus* and have been widely used both as a genome library and as individual vectors for other uses. While there was some suggestion that autonomous replication could be observed in *Neurospora* (Grant et al. 1984), this has never been used to develop vectors for gene manipulation.

15.4.4 Novel Methods

Complementation of temperature-sensitive (TS) mutations was used as a means to identify the gene carrying the TS lesion. It was not until identification of the ribosomal S9 protein that this was proposed as a deliberate selectable marker. Subsequently this has been used to disrupt the *albino-1* gene in Neurospora in a strain carrying the TS allele. Complementation by the *Magnaporthe* ribosomal S9

gene allowed targeting without interference by homology with the native *Neurospora* locus (Wiest et al. 2012c).

Plasmids are naturally found in mitochondria of several fungi (Griffiths 1995), and while these suggest that it should offer a route for manipulation of fungal physiology, this has not proven to be useful in practice.

15.5 Plasmids/Strains

For fungi where dominant selectable markers are available, they are widely used, and a series of vectors based on the same genes are often available for use across multiple target species. With the demonstration that targeting was enhanced in strains deficient in nonhomologous end joining, many such strains were developed to allow modern gene targeting. Among these strains are representatives from most of the commonly used model species (Table 15.2).

15.5.1 Neurospora

Most strains of *Neurospora* are sensitive to hygromycin and so are readily transformed with the most commonly available vectors. Additional strains are used for special purpose transformation including auxotrophic strains, temperature-sensitive strains, and strains defective in nonhomologous end joining (Table 15.2). Among the auxotrophic complementation systems, the most widely used was the *his-3* complementation which allowed targeting to the *his-3* locus in *Neurospora*. This robust system generated significant numbers of homologous integrants, but because of the variability at *his-3* among laboratory strains (Yeadon et al. 1998), most researchers use strains carrying the 1-234-723 allele of *his-3* (Margolin et al. 1997).

15.5.2 Aspergillus

Some species of *Aspergillus* are naturally resistant to hygromycin, and so complementation of auxotrophies is the most common method for selecting for transformants. The most commonly used marker is pyrG (Oakley et al. 1987a), and several vectors exist for selecting transformants in the most commonly studied species of Aspergillus (Table 15.3). Similarly, complementation of mutations at the riboB locus (Oakley et al. 1987b) has been used for selecting transformants in *A. nidulans* and *A. fumigatus* (Nayak et al. 2006). One of the commonly used transformation systems for *Aspergillus* uses the *amdS* gene on the plasmid p3SR2 (Tilburn et al. 1983) to confer the ability to grow on acetamide as the sole nitrogen

Table 15.2 Strains engineered for targeted gene disruption

FGSC #	Species	Characteristics	Reference
8071	*N. crassa*	am target strain TEC39	Cambareri and Kinsey (1994)
8072	*N. crassa*	am target strain TEC41	Cambareri and Kinsey (1994)
9538	*N. crassa*	mus-51 delta::Bar; his-3	Ishibashi et al. (2006)
9539	*N. crassa*	mus-52 delta::Bar; his-3	Ishibashi et al. (2006)
9567	*N. crassa*	mus-52::Hyg^r	Ishibashi et al. (2006)
9568	*N. crassa*	mus-52::Hyg^r	Ishibashi et al. (2006)
9595	*N. crassa*	mus-51::Hyg^r	Ishibashi et al. (2006)
9717	*N. crassa*	delta mus-51::bar+; his-3	Ishibashi et al. (2006)
9719	*N. crassa*	delta mus-52::bar+	Ishibashi et al. (2006)
9720	*N. crassa*	delta mus-52::bar+; his-3	Ishibashi et al. (2006)
9718	*N. crassa*	delta mus-51::bar+	Ishibashi et al. (2006)
20277	*N. crassa*	NCU08290.2 (mus-51)	Colot et al. (2006)
20278	*N. crassa*	NCU08290.2 (mus-51)	Colot et al. (2006)
10216	*N. crassa*	un-16, mus-52	McCluskey et al. (2007)
10217	*N. crassa*	un-16, mus-52	McCluskey et al. (2007)
10218	*N. crassa*	un-16, mus-51	McCluskey et al. (2007)
10219	*N. crassa*	un-16, mus-51	McCluskey et al. (2007)
A1421	*A. flavus*	CA14 deltaKu70 delta PyrG	Chang et al. (2009)
A1181	*A. niger*	Δ kusA pyrG-	Meyer et al. (2007)
A1279	*A. niger*	KusA::amdS; pyrG	Carvalho et al. (2010)
A1180	*A. niger*	Delta kusA	Meyer et al. (2007)
A1515	*A. niger*	pyrG-, KusA::AfpyrG	Chiang et al. (2011)
A1182	*A. niger*	Delta kusA::AmdS	Meyer et al. (2007)
A1179	*A. niger*	Delta kusA pyrG-	Meyer et al. (2007)
A1421	*A. flavus*	CA14 deltaKu70 delta PyrG	Chang et al. (2009)
A1280	*A. fumigatus*	akuA::loxP	Hartmann et al. (2010)
A1160	*A. fumigatus*	DeltaKU80 pyrG-	Krappmann et al. (2006), Krappmann (2006)
A1159	*A. fumigatus*	akuA::loxP	Krappmann et al. (2006), Krappmann (2006)
A1158	*A. fumigatus*	akuA::loxP-hygro^R/tk	Krappmann et al. (2006), Krappmann (2006)
A1157	*A. fumigatus*	akuA::ptrA	Krappmann et al. (2006), Krappmann (2006)
A1151	*A. fumigatus*	pyrG^AF::Delta KU80	Krappmann et al. (2006), Krappmann (2006)
10386	*M. grisea*	P1.2-deltaKU80	Villalba et al. (2008)
10385	*M. grisea*	Guy11-deltaKU80	Villalba et al. (2008)
A1190	*A. parasiticus*	ordA, Ku70	Chang (2008)
A1243	*A. parasiticus*	Delta ku70	Ehrlich et al. (2008)
A1244	*A. parasiticus*	Delta ku70 Delta pyrG	Chang et al. (2009)

Table 15.3 Selectable markers and plasmids for transforming filamentous fungi

Selection	Marker	Plasmids	Reference
Dominant selection			
Benomyl	BmlR/ben	pBT6, pBenA3	Orbach et al. (1986), Jung et al. (1992)
Hygromycin	Hph/ HygR	pES20, etc.	Staben et al. (1989)
Bialaphos	bar	pBARKS1, etc.	Pall and Brunelli (1993)
Phleomycin	phleo	pBC-phleo	Silar (1995)
Sulfonylurea	sur	pCB1528, etc.	Sweigard et al. (1997)
Nourseothricin	nat1	pD-Nat1	Kuck and Hoff (2006)
Recessive selection			
Histidine	*his-3*	pNH60, pRAUW122, pJHAM002	Legerton and Yanofsky (1985), Aramayo and Metzenberg (1996), Lee et al. (2003)
Acetamide	*amd-S*	p2SR2	Wernars et al. (1985)
Purine	*pyrG*	ppyrG, pPL6, Anep2, etc.	Oakley et al. (1987a), Storms et al. (2005)
Pyridoxine	*pyroA*	pTN1, pFB6	Nayak et al. (2006)
Inositol	*inl*	pINL, pOKE01, pRATT19	Akins and Lambowitz (1985)
Riboflavin	*riboB*	pLO1, pPL1	Oakley et al. (1987b)
TS lethal	*un-16*	pUN16-6	McCluskey et al. (2007)

source. This plasmid is one of the most highly distributed (Table 15.4) and has been used for the transformation of a variety of fungi including *A. niger* (Kelly and Hynes 1985), *Cochliobolus* (Turgeon et al. 1985), *Penicillium* (Beri and Turner 1987), and *Trichoderma* (Rahman et al. 2009, p. 6942.

15.5.3 Other Fungi

While not all of the tools developed for use in *Neurospora* or *Aspergillus* are directly applicable to use in other research systems, many of the vectors used in these systems can be engineered to function in unrelated systems (Meyer 2008). Similarly, many of the approaches used in *Neurospora* and *Aspergillus* are employed to generate specific characteristics, such as chlorate resistance which is useful for forcing anastomosis (Bowden and Leslie 1992) or for transformation directly (Daboussi et al. 1989).

Finally, interspecific transfer of genes is especially useful when one is doing gene targeting. For example, the *Aspergillus nidulans riboB* gene is useful for targeting in *A. fumigatus* (Nayak et al. 2006), and the *Magnaporthe* ribosomal S9 gene can be used to transform *un-16* TS-lethal mutant strains of *Neurospora* without interfering with targeting [e.g., to the *al-1* locus (Wiest et al. 2012c)].

Table 15.4 Most commonly requested plasmids from the FGSC collection from January 2000 to September 2012

Plasmid name	Number of distributions	Reference
pSilent-1	114	Nakayashiki et al. (2005)
pBC-phleo	86	Silar (1995)
pCSN44	78	Staben et al. (1989)
pBARGPE1	71	Pall and Brunelli (1993)
pMF272	67	Freitag et al. (2004)
pRS426	63	Christianson et al. (1992)
gGFP	56	Maor et al. (1998)
pCB1003	56	Caroll et al. (1994)
pCB1004	52	Caroll et al. (1994)
pPK2	48	Covert et al. (2001)
pCSN43	46	Staben et al. (1989)
pBARMTE1	42	Pall and Brunelli (1993)
p3SR2	42	Wernars et al. (1985)
pBC-hygro	41	Silar (1995)
pMYX10	37	Campbell et al. (1994)
pMYX2	35	Campbell et al. (1994)
pMT-mRFP1	35	Toews et al. (2004)
pBARKS1	34	Pall and Brunelli (1993)
ppyrG	34	Oakley et al. (1987a)
pMT-BFP	34	Toews et al. (2004)
pMF280	32	Freitag et al. (2004)
pFNO3	32	Yang et al. (2004)
pAO81	30	Yang et al. (2004)
pRG3-AMA1-NotI	29	Liu et al. (2004)
pMF334	29	Freitag and Selker (2005)
pXDRFP4	28	Yang et al. (2004)
pMF309	28	Freitag et al. (2004)
cosmid An26	28	Taylor and Borgmann (1996)
pMT-sGFP	27	Toews et al. (2004)
pSD1	26	Nguyen et al. (2008)
pD-Nat1	25	Kuck and Hoff (2006)
pCB1532	24	Sweigard et al. (1997)
pMOcosX	24	Orbach (1994)
pMF332	24	Freitag and Selker (2005)
pME2891	22	Krappmann et al. (2005)
pMF331	22	Freitag and Selker (2005)
p500	22	Vogt et al. (2005)
pMG2254	22	Gerami-Nejad et al. (2009)
pCCG::N-GFP	21	Honda and Selker (2009)
pA-HYG OSCAR	21	Paz et al. (2011)
pBARGEM7-2	21	Pall and Brunelli (1993)
pCCG::C-Gly::HAT::FLAG	21	Honda and Selker (2009)
pBT6	21	Orbach et al. (1986)
pYFP	20	Bardiya et al. (2008)
pOSCAR	20	Paz et al. (2011)

15.6 Fluorescent Proteins

15.6.1 Neurospora

GFP constructs were available for other organisms (Niedenthal et al. 1996; Zeilinger 1999, p. 68) before Freitag developed a codon-optimized version of GFP for *Neurospora* (Freitag et al. 2004). After this marker was introduced into the research community, numerous related fluorescent-tagged proteins were developed for use in *Neurospora* (Table 15.5). These include a split yellow fluorescent protein marker useful for the study of protein interactions (Hammond et al. 2011), as well as many fluorescent proteins targeted for specific organelles (Bowman et al. 2009). In addition to the fluorescent proteins described above, several vectors are available which will allow visualization of protein levels or location. The *lux* gene, derived from the firefly luciferase gene, has been engineered for use in *Neurospora* (Morgan et al. 2003) and ultimately employed as a signal of the levels of expression of the product of the *frq* locus (Gooch et al. 2008).

15.6.2 Aspergillus

With myriad fluorescent-tagged proteins available on convenient plasmids (Table 15.5), the growth in distribution of these plasmids reflects their value to the community (Table 15.4). The series developed in the laboratory of R. Fischer (Toews et al. 2004) includes green, red, and blue fluorescent proteins; these are all available from the FGSC and have been widely used in developing tools within the genus *Aspergillus* (Lubertozzi and Keasling 2009), as well as for cell biology studies in *Aspergillus* (Arratia-Quijada et al. 2012) and in unrelated fungi (Helber and Requena 2008). This reiterates the situation with selectable markers and tags; many of the tools developed for *Aspergillus* find direct utility in other systems underscoring the value of tool development in model systems.

15.6.3 Other Fungi

The most commonly used fluorescent protein used in a variety of systems is encoded by the gGFP vector developed by A. Sharon (Maor et al. 1998). This vector includes a hygromycin resistance cassette driven by the *A. nidulans* glyceraldehyde 3-phosphate dehydrogenase promoter and the terminator from the *A. nidulans trpC* gene. The GFP gene is also driven by the *gpd* promoter and uses the GFP gene and terminator from the plasmid pHSP70-SG (Spellig et al. 1996) which was originally generated for use in *U. maydis*. The gGFP plasmid was optimized for use in *Cochliobolus*, but has been requested for use in a broad variety of fungal systems including *Colletotrichum* (Horowitz 2002), *Verticillium* (Eynck et al. 2007), and others (Lorang et al. 2001).

Table 15.5 Plasmids carrying visualization tags

Name	Tag	Organism	Reference
pFNO3	GFP	Aspergillus	Yang et al. (2004)
pHL86	GA5-chRFP, riboB	Aspergillus	Liu et al. (2009)
pHL85	chRFP, pyroA	Aspergillus	Liu et al. (2009)
pHL84	GA5-GFP, pyroA	Aspergillus	Liu et al. (2009)
pHL83	GA5-GFP, loxP, pyrG	Aspergillus	Liu et al. (2009)
pHL82	GA5-GFP, riboB	Aspergillus	Liu et al. (2009)
pXDRFP4	RFP	Aspergillus	Yang et al. (2004)
pSK800	mRFP1	Aspergillus	Toews et al. (2004)
pSK494	GFP2-5	Aspergillus	Szewczyk and Krappmann (2010)
pSK495	yfp	Aspergillus	Szewczyk and Krappmann (2010)
pSK496	mCherry	Aspergillus	Szewczyk and Krappmann (2010)
pJH19	DsRedT4	Aspergillus	Toews et al. (2004)
pRF281	GFP	Aspergillus	Toews et al. (2004)
pDV2	sGFP	Aspergillus	Toews et al. (2004)
pRS54	GFP	Aspergillus	Suelmann and Fischer (2000)
pSK700	DsRedT4	Aspergillus	Toews et al. (2004)
pMT-sGFP	sGFP	Aspergillus	Toews et al. (2004)
pMT-BFP	BFP	Aspergillus	Toews et al. (2004)
pMT-mRFP1	mRFP1	Aspergillus	Toews et al. (2004)
pPND1	mRFP1	Aspergillus	Rischitor et al. (2004)
pRF280	GFP	Aspergillus	Toews et al. (2004)
pOT-eGFP	eGFP	Botrytis	Patel et al. (2008)
pOT-LUC	Luc	Botrytis	Patel et al. (2008)
pMG2082	GFP-URA3-GFP	Candida	Gerami-Nejad et al. (2009)
pMG1958	Ppck1-GFP	Candida	Gerami-Nejad et al. (2004)
pMG1892	pgal-GFP	Candida	Gerami-Nejad et al. (2004)
pMG1886	pmet3-GFP	Candida	Gerami-Nejad et al. (2004)
pMG1726	CFP-URA3	Candida	Gerami-Nejad et al. (2001)
pMG1648	YFP-URA3	Candida	Gerami-Nejad et al. (2001)
pMG1602	GFP-URA3	Candida	Gerami-Nejad et al. (2001)
pMG1801	CFP-His1	Candida	Gerami-Nejad et al. (2001)
pMG2169	RFP-URA3	Candida	Gerami-Nejad et al. (2009)
pMG1646	GFP-His1	Candida	Gerami-Nejad et al. (2001)
pMG2254	M-cherry-TADH-URA3	Candida	Gerami-Nejad et al. (2009)
pMG1656	YFP-His1	Candida	Gerami-Nejad et al. (2001)
pCAMDsRED	DsRed-Express	Leptosphaeria	Eckert et al. (2005)
gGFP	GFP	Many	Maor et al. (1998)
pVG101	ccg2p-o-luc-I	Neurospora	Gooch et al. (2008)
pHAN1	sgfp, HA	Neurospora	Kawabata and Inoue (2007)
pMF334	RFP	Neurospora	Freitag and Selker (2005)
pMF332	RFP	Neurospora	Freitag and Selker (2005)
pMF331	RFP	Neurospora	Freitag and Selker (2005)

(continued)

Table 15.5 (continued)

Name	Tag	Organism	Reference
pAL3-Lifeact	TagRFP	Neurospora	Berepiki et al. (2010)
pAL4-Lifeact	nat1 TagRFP	Neurospora	Lichius and Read (2010)
pAL5-Lifeact	TagRFP-T	Neurospora	Lichius and Read (2010)
pAL6-Lifeact	TagRFP	Neurospora	Lichius and Read (2010)
pAL12-Lifeact	TagRFP	Neurospora	Lichius and Read (2010)
pYFPN	YFP	Neurospora	Bardiya et al. (2008)
pTH1124.1	YFPC-	Neurospora	Hammond et al. (2011)
pTH1123.1	YFPN-	Neurospora	Hammond et al. (2011)
pTH1117.12	GFP-	Neurospora	Hammond et al. (2011)
pTH1112.8	-YFPN	Neurospora	Hammond et al. (2011)
pTH1111.1	-RFP	Neurospora	Hammond et al. (2011)
pTH1108.2	-YFPC	Neurospora	Hammond et al. (2011)
pMF309	Bml-GFP	Neurospora	Freitag et al. (2004)
pMF280	hH1-GFP	Neurospora	Freitag et al. (2004)
pMF272	GFP	Neurospora	Freitag et al. (2004)
pLUC6 delta BS	lux	Neurospora	Morgan et al. (2003)
pAL10-Lifeact	TagRFP	Neurospora	Lichius and Read (2010)
pRFP-vps-52	RFP	Neurospora	Bowman et al. (2009)
pRFP-cax	RFP	Neurospora	Bowman et al. (2009)
pRFP-nca-1	RFP	Neurospora	Bowman et al. (2009)
pnca-2-GFP	GFP	Neurospora	Bowman et al. (2009)
pRFP-nca-2	RFP	Neurospora	Bowman et al. (2009)
pnca-3-GFP	GFP	Neurospora	Bowman et al. (2009)
pgrp-GFP	GFP	Neurospora	Bowman et al. (2009)
pTH1067.9	GFP	Neurospora	Hammond et al. (2011)
pRFP-grp	RFP	Neurospora	Bowman et al. (2009)
pdpm-GFP	GFP	Neurospora	Bowman et al. (2009)
pVG110	frqp-o-luc-I	Neurospora	Gooch et al. (2008)
pvps-52-GFP	GFP	Neurospora	Bowman et al. (2009)
pYFP	YFP	Neurospora	Bardiya et al. (2008)
pRFP-vam-3	RFP	Neurospora	Bowman et al. (2009)
pRFP-vma-1	RFP	Neurospora	Bowman et al. (2009)
parg-4-GFP	GFP	Neurospora	Bowman et al. (2009)
pAL1	sGFP	Neurospora	Berepiki et al. (2010)
pAL2-Lifeact	tdTomato	Neurospora	Lichius and Read (2010)
pGFP::hph::loxP	GFP	Neurospora	Honda and Selker (2009)
pCCG::N-GFP	GFP	Neurospora	Honda and Selker (2009)
pCCG::C-Gly::GFP	GFP	Neurospora	Honda and Selker (2009)
pYFPC	YFP	Neurospora	Bardiya et al. (2008)
pnca-1-GFP	GFP	Neurospora	Bowman et al. (2009)
pRFP-dpm	RFP	Neurospora	Bowman et al. (2009)

15.7 Tags

15.7.1 Visualization

Several proteins have been used as target for antibodies when linked to a polypeptide under study. Among them, the GFP protein was localized by immunological reaction using a rabbit anti-GFP antibody (Gordon et al. 2000).

15.7.2 Purification

Numerous protein tags have been developed to facilitate protein purification, and many of these are available in public collections. For example, the FLAG (Honda and Selker 2009), S-TAG (Yang et al. 2004), and HA (Toews et al. 2004) tags are present among many of the most often distributed plasmids from the FGSC collection (Table 15.4). The application of these tags allows rapid protein purification and is having an impact on fungal proteomics (Liu et al. 2009) and on understanding of the fungal protein interactome (Wang et al. 2011). If the distribution of these plasmids (Table 15.4) is a predictor of the impact that they may have, we can expect additional examples of their application to understanding the role of specific proteins in numerous important questions of fungal development, ultrastructure, and environmental interactions.

15.8 Conclusion

15.8.1 Impact

Since 2000, the FGSC has distributed over 3,545 individual plasmid samples. Among these, the most popular plasmids are those for transformation and manipulation of model filamentous fungi (Table 15.4). Similarly, strains for targeted transformation have had a huge impact, and the combined availability of these strains and the plasmids for targeted transformation have allowed the development of a number of systematic and targeted gene deletion programs. This is arguably the biggest advancement in research in filamentous fungi for many decades, and it allows evaluation of the 80–90 % of genes that are not discoverable in traditional mutagenesis programs. In *N. crassa*, a set of mutant strains was generated where nearly every gene has been deleted in a strain engineered to be defective in nonhomologous end joining (Colot et al. 2006). This set was arrayed at the FGSC and has been distributed as arrayed strains to laboratories in the USA, Europe, Asia, and South America (Wiest et al. 2012b). Similar sets have been prepared, although

not in a systematic manner, for *Candida albicans*, *Cryptococcus neoformans*, and, of course, for yeast. There is significant interest in generating similar resources for plant-pathogenic fungi, and a set of 48,000 tagged-integrant strains of *Magnaporthe grisea* were developed and deposited at the FGSC (Betts et al. 2007). Regrettably, the requirement is that every lab that wants to work with these strains receives a permit from the USDA Biotechnology Regulatory Service, which has limited the impact of these materials. Similar permits would be required for genetically engineered strains of any plant pathogen, and until this is resolved, it is unlikely that a systematic gene deletion resource will be made available for these important organisms.

15.8.2 Future Prospects

Most of the molecular tools used for manipulation of filamentous fungi are available from the Fungal Genetics Stock Center. In 2012, the FGSC entered into negotiations with Addgene (http://www.addgene.com), a nonprofit plasmid repository, to deposit many of the most highly requested plasmids. This is both good practice for an active collection (second-site backup is part of the best practice guidelines for microbial germplasm repositories) and increases the impact of each plasmid, by increasing their visibility to potentially new customers.

Additionally, the development of novel organism systems for applications such as biomass deconstruction, pharmaceutical production, biocontrol, or the production of food and fiber will mean that new tools for manipulation and engineering of these fungi will be developed. The sharing of these materials via active well-curated collections assures that these materials will have the greatest impact (Furman and Stern 2011) and that they will be available long after their primary use has been accomplished.

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