GENETIC ENGINEERING OF FUNGAL CELLS

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Contents

Summary

Filamentous fungi have myriad industrial applications that benefit mankind while at the

same time, fungal diseases of plants cause significant economic losses. The development of effective methods for genetic engineering of these organisms over the past two decades has improved our understanding of the basic biological processes of filamentous fungi. This review provides a summary of the methodology involved in creating transforming DNA constructs, introduction of DNA into filamentous fungi as well as methods for targeted and random gene disruption.

1. Introduction

1.1. Industrial importance of fungi

Fungi have been used for traditional production of wine, beer and cheese for thousands of years. In the past century, their utility to humans has expanded with the synthesis of organic chemicals such as solvents and acids, as well as valuable secondary metabolites such as antibiotics and other pharmaceuticals. A list of products is presented by Lubertozzi and Keasling in their 2009 review. In addition, fungi are a significant source of valuable industrial enzymes; in 2004, Schauer and Borris catalogued more than 80 native enzymes such as proteases, amylases and oxidoreductases. More recently, yeast and some filamentous fungi have proven to be successful alternatives to bacteria for the production of heterologous proteins. An advantage of using fungi for protein production is the GRAS (Generally Regarded as Safe) status of many industrial yeasts and filamentous fungi. Furthermore, recent genetic engineering efforts have altered glycosylation pathways in yeasts to yield glycoproteins with N-glycan structures that more closely mimic those found in mammalian proteins. Recent advances in genetic engineering of fungi are expected to facilitate the introduction of more efficient as well as novel uses of fungi in industrial processes. In addition, because they are eukaryotic cells with a relatively short generation time and small haploid genomes, effective genetic systems have been developed for many fungal species. This has resulted in their use as excellent model systems for basic metabolic processes.

1.2. Purpose and range of topics covered

The purpose of this review is to introduce readers to the basic methods used to genetically engineer fungi from the construction of transforming DNA to methods for high-throughput gene disruption. This article will present methods used for filamentous fungi and non-*Saccharomyces* yeasts. There is an extensive literature on genetic and metabolic engineering of *Saccharomyces cerevisiae* and although many of the methods discussed are applicable to filamentous fungi and other yeast species, there are unique challenges associated with filamentous fungi. These will be the focus of this article.

2. Generation of transforming constructs

2.1. Autonomously-replicating plasmids

In many yeast species including *Saccharomyces*, *Schizosaccharomyces* and *Candida*, shuttle plasmids are available that contain autonomously-replicating sequences (ARS) that permit plasmid replication in yeast. ARS-like sequences on linear plasmids have been identified in many species of filamentous fungi and Katayose and coworkers

demonstrated in 1990 that a 366 bp fragment of a linear mitochondrial plasmid from the basidiomycete, *Lentinus edodes* contained three *S. cerevisiae* ARS consensus sequences. Insertion of this fragment into the yeast integrative plasmid YIp32, permitted its autonomous replication in *S. cerevisiae*. Nevertheless, ARS-containing shuttle vectors have had very limited use in filamentous fungi. Plasmids containing the AMA1 sequence from *A. nidulans* (which contains ARS consensus sequences) were studied in *Penicillium chrysogenum* and the authors found that the stability of the AMA1 plasmids was relatively low (35-75%) and that plasmid integration occurred. Fierro and coworkers showed in 2004 that plasmid vectors containing the *A. nidulans AMA-1* sequence efficiently transformed *Penicillium nalgiovense* and were maintained extrachromosomally. However, mitotic stability (~75%) was not tested after one generation on non-selective media, and in some transformants, recombination occurred between plasmid and chromosomal sequences. In 1998, Aleksenko and Ivanova created autonomous linear plasmids containing human telomeric elements but these were not stable. Because no useful centromeric sequences have been cloned that have yielded stably replicating vectors, transformation is generally carried out using constructs that usually integrate into the fungal genome. Some important features of these constructs are described below.

2.2. Promoters

2.2.1. Constitutive promoters

Strong promoters are essential for the production of industrial enzymes. Several constitutive promoters have been used for this purpose; selected promoters are listed in Table 1. The *Pna2/TPI* hybrid promoter containing triose phosphate isomerase from *A. nidulans* linked 5' to the *A. niger* neutral amylase II promoter has been used for foreign protein expression. The *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter has been extensively used to drive protein expression in fungi. Because the use of homologous promoters can enhance expression levels, the endogenous *gpdA* promoter has been cloned from many different species of fungi. In 2002, Wasylnka and Moore successfully transformed *Aspergillus fumigatus* with a plasmid vector encoding the *sgfp* gene controlled by the *A. nidulans gpdA* promoter; strong constitutive expression of GFP was observed in conidia and hyphae. Interestingly, Redkar et al. reported in 1998 that *gpdA* may be activated by osmotic signals suggesting that the expression level may be modulated by salt concentrations. Similarly, the *trpC* gene promoter from *A. nidulans* (for tryptophan biosynthesis) has been used for strong homologous and heterologous gene expression. The *ToxA* promoter has been used in fungi for green fluorescent protein expression. The ToxA or ToxB promoters were derived by Cuifetti and colleagues in 1997 from protein toxin genes in the plant pathogenic fungus, *Pyrenophora tritici-repentis* and subsequent work in 2005 by Andrie has shown that this promoter is effective in driving *sgfp* expression in a variety of fungi.

2.2.2. Inducible promoters

Inducible promoters provide some control over the expression of introduced genes, particularly for the study of essential genes or for the production of toxic proteins.

Furthermore, in gene knock-out studies, using an inducible promoter may eliminate the requirement for complementation of the mutant strain with the wild type gene. A problem may arise if gene expression in the parent strain is lower than observed in transformed strain in the presence of the repressor; in this case, verification of the phenotype using a null mutant strain is recommended. Many inducible promoters have been used in filamentous fungi and these are listed in Table 1. Ideal promoters are tightly regulated, induce gene expression at low cost and result in high levels of expression after induction.

	Gene function	**Source DNA**	**Reference**
Constitutive promoters			
pna2/tpi hybrid promoter	neutral amylase + triose phosphate isomerase	*Aspergillus nidulans* *Aspergillus niger*	Olempska-Beer et al. 2006
gpdA	glyceraldehyde-3-phosphate dehydrogenase	*Aspergillus nidulans*	Punt et al. 1991
trpC	tryptophan biosynthesis	*Aspergillus nidulans*	Hamer and Timberlake, 1987
Inducible promoters			
TAKA-A amylase	amylose hydrolysis	*Aspergillus oryzae*	Tada et al. 1991 Christensen 1988
glaA	glucoamylase	*Aspergillus niger var. awamori*	Boel et al. 1984
alcA/AlcR	alcohol dehydrogenase	*Aspergillus nidulans*	Gwynne et al. 1989
niiA	nitrite reductase	*Aspergillus fumigatus*	Amaar and Moore, 1998 Hu et al. 2007
cbhI	cellobiohydrolase I	*Trichoderma reesei*	Harkki et al. 1991
ctr4	high affinity copper transporter	*Schizosacccharomyces pombe*	Bellemare et al. 2001
thiA	thiamine biosynthesis	*Aspergillus oryzae*	Shoji et al. 2005

Table 1: Selected promoters used to drive gene expression in fungi

The TAKA amylase promoter from *Aspergillus oryzae*, developed by Tada et al. in 1991 has been used to drive heterologous protein expression in *A. oryzae*. The promoter is activated by growth on starch as a carbon source and recent work by Ito and coworkers in 2004 characterized AmyR, a transcriptional regulator that activates the genes involved in amylolytic action, including TAKA amylase. Promoters from other *A. oryzae* amylolytic genes such as alpha amylase B (*amyB*) have also been used by Hoshida to drive protein expression in *A. oryzae* during induction by maltose. In 2008, the A. niger *glaA* (glucoamylase A) promoter was analyzed by Ganzlin and Rinas to

determine the complex effect of glucose on promoter function. During these studies, they identified 5-thio-glucose and 2-deoxyglucose as novel and potent inducers of *glaA*.

The alcohol regulon in *Aspergillus nidulans* consists of *alcA* (alcohol dehydrogenase), *aldA* (aldehyde dehydrogenase) and a positive regulator, *alcR*. Glucose repression of all three genes is mediated by the *creA* gene product. In 1995, Fillinger and colleagues showed that ethanol and other substrates stimulate *alcR* whereas poor carbon sources such as lactose or glycerol can derepress the *alcA* gene. The tight regulation of the *alcA* promoter allows a simple phenotypic analysis in defined media promoting induction or complete repression of a gene product. This ability to completely switch off gene expression has permitted the validation of essential genes through analysis of their terminal phenotypes. Ha et al. successfully used in 2006 the *A. nidulans alcA* promoter in *Fusarium solani* to drive the expression of an RNAi construct. Use of the *alcA/alcR* system was originally devised by Gwynne and colleagues in 1989 for the production of heterologous proteins by *Aspergillus nidulans* (e.g., alpha interferon-2). In 1996, Gouka used the *exlA* promoter from *Aspergillus awamori* endoxylanase (xylose-inducible) to provide strong expression in *A. awamori*.

One disadvantage of driving gene expression based on changes in central metabolism is undesirable effects on fungal growth and development. To bypass the potential problems associated with nutritional markers, in 2005 Shoji et al. developed the *thiA* promoter which is transcriptionally repressed by sub-micromolar concentrations of thiamine. However, thiamine repression does not occur when the pH rises above neutral; therefore, culture conditions may affect the effectiveness of this promoter. In *Aspergillus nidulans* and *A. niger*, a hybrid promoter containing the human estrogen response element (ERE) fused to a minimal *S. cerevisiae URA3* TATA element was developed in 2005 by Pachlinger and colleagues to drive expression of a *lacZ* reporter gene. When estrogenic compounds were added as inducers to the medium at picomolar levels, they achieved levels of expression similar to an *alcA* construct. The advantage of this system is that it does not interfere with central metabolism in the host strain. In addition, no special media is required so economical complex media can be used for fungal growth.

2.3. Selectable markers

In the past two decades, a large number of selectable markers have been developed for fungi and most have been effective across a wide range of species. Table 2 lists some of the more commonly-used markers and these are described in more detail below. Markers either complement a nutritional deficiency in an auxotrophic strain, or are dominant selectable markers. Dominant selectable markers are frequently employed because they preclude the need to generate an auxotrophic host strain and so permit the transformation of many strains of one species. Hence, they are particularly useful for uncharacterized strains for which little genetic information is available. These markers are generally cloned into plasmids that can replicate in *E. coli* under the control of appropriate promoters (generally strong constitutive promoters, see section 2.2.1). The Fungal Genetics Stock Centre maintains a repository of useful vectors with either dominant or nutritional markers developed for selection in filamentous fungi (http://www.fgsc.net/plasmid/vector.html).

	Function of resistance/nutritional gene	Source organism	Reference
Dominant markers			
benomyl - *tub*	benomyl-resistant tubulin mutants	*Neurospora crassa*	Orbach et al., 1986
bialophos/phosphinothricin-*bar*	phosphinothricin acetyltransferase	*Streptomyces hygroscopicus*	Avalos et al., 1989
blasticidin S - *bsr* *bsd*	blasticidin S deaminase	*Bacillus cereus* *Aspergillus terreus*	Kamakura et al. 1987 Kimura et al.1994
carboxin - cbx^R	carboxin-resistant succinate dehydrogenase mutants	*Ustilago maydis*	Kojic and Holloman, 2000
hygromycin - *hph*	hygromycin phosphotransferase	*Escherichia coli*	Punt et al., 1987
nourseothricin - *nat*	nourseothricin acetyltransferase	*Streptomyces noursei*	Krügel et al., 1993
phleomycin/zeocin/bleomycin *ble*	bleomycin binding protein	*Strepto-alloteichus hindustanus*	Mattern and Punt, 1988
pyrithiamine - *ptrA*	mutated allele of thiamine biosynthesis gene	*Aspergillus oryzae*	Kubodera et al., 2000
amdS	acetamidase	*Aspergillus nidulans*	Tilburn et al. 1983
Auxotrophic markers **both are positive-negative selection systems*			
niaD	nitrate reductase (assimilatory)	*Aspergillus nidulans*	Daboussi et al. 1989
pyrG	orotidine-5'-phosphate decarboxylase	*Aspergillus niger*	Ballance and Turner, 1985

Table 2: Selectable markers used in filamentous fungi

2.3.1. Dominant selectable markers

The parent strain should be tested to establish the minimum inhibitory concentration of these agents as naturally-occurring resistance is common in filamentous fungi. In addition, some antibiotics are less effective at particular salt concentrations and pH ranges. Hence, it is strongly recommended that the resistance of putative transformants picked from selection plates be confirmed using liquid cultures.

The hygromycin phosphotransferase gene from *E. coli (hph*) inactivates the antibiotic, hygromycin B, and has been effective in most systems. Hygromycin B inhibits protein synthesis in both prokaryotes and eukaryotes and in fungi, it is effective at concentrations ranging from 50-250 μg/ml. Several vectors are available; in 1987, Punt and coworkers developed pAN7 that contained the original antibiotic resistance cassette under the control of the *gpdA* promoter with the *A. nidulans trpC* terminator sequence. Other vectors have since been constructed, e.g., pCB1003 in which expression is controlled by the *trpC* promoter. Spontaneous hygromycin resistance can complicate the use of this antibiotic for selection in some fungal species.

Phleomycin and zeocin are members of the bleomycin family of antitumour antibiotics that bind to DNA and in the presence of divalent metal ions, initiate a radical-mediated breakdown of DNA. The antibiotic resistance gene (*ble*) has been cloned from *Streptoalloteichus hindustanus (Sh*) as well as part of the Tn5 determinant in *E. coli*; the Ble protein binds the antibiotic and prevents its interaction with DNA. In 1988, Mattern and colleagues constructed the pAN8-1 vector that contains the *Sh ble* gene under the control of the *gpdA* promoter from *A. nidulans*. Other cassettes have been developed from pUT703; Silar has used pBC-phleo that has the *A. nidulans gpdA* promoter and the *S. cerevisiae CYC1* terminator. In 2007, he and colleagues reported that phleomycin at pH 6.5 was not as effective at inhibiting growth of wild type *A. flavus* compared to pH 7.5.

Phosphinothricin is the active breakdown product of the herbicide, bialophos, which inhibits glutamine synthase in susceptible organisms. Resistance can be conferred by the *bar* gene from *Streptomyces hygroscopicus*. Phosphinothrinocin resistance has been employed in both plants and a wide variety of filamentous fungi as a dominant selectable marker though some species show spontaneous resistance. Ahuia and Punekar have postulated that resistance is via reduced L-phosphinothricin uptake.

Nourseothricin is an aminoglycoside antibiotic that inhibits protein synthesis by a mechanism distinct from that of hygromycin; hence, no cross-resistance between these markers should occur. Resistance is conferred by the *nat* gene product of *Streptomyces noursei* encoding nourseothricin acetyltransferase. Kuck and Hoff developed in 2006 an efficient marker using the *nat-1* gene with the *A. nidulans trpC* promoter and terminator. In 2007, Smith and Smith developed several other vectors that employ different promoters and terminator sequences and these were successfully used to transform *Neurospora crassa* and *Cryphonectria parasitica*.

Benomyl is a fungicide used to control phytopathogenic fungi. Development of resistance to benomyl has been observed with the use of this agent; mutation F167Y in the *N. crassa* beta-tubulin gene was shown to be responsible. This gene was then used by Orbach and coworkers as a dominant selectable marker. *Paecilomyces* spp. have been investigated as biocontrol agents of agricultural pest insects; in 1994, Inglis and colleagues successfully transformed *P. fumosoroseus* and *P. lilacinus* were successfully transformed using the *N. crassa* gene. The advantage of using benomyl resistance for these fungi was the ability to use them along with the application of benomyl-related fungicides resulting in simultaneous control of phytopathogens and insects.

Carboxin is a systemic fungicide and many basidiomycetous fungi have been shown to be resistant to its effects via a mutation in succinate dehydrogenase. In 2000, Kojic and Holloman developed a dominant selection system for the plant pathogenic fungus, *Ustilago maydis*, using the *U. maydis cbx* gene. Although it was reported that the expression of cbx^R in *U. maydis* reduced its pathogenicity to corn, a study by Topp and coworkers in 2002 using several strains transformed with cbx^R found that their pathogenicity on corn was no different from non-transformed controls. In 2009, Shima and coworkers developed a selection system for *Aspergillus parasiticus* based on homologous integration of the shB^{cxr} gene from *Aspergillus oryzae*. Acetate medium was employed in this study because ascomycetes are more susceptible to carboxin in this medium.

Blasticidin S is an aminoacylnucleoside antibiotic that inhibits protein synthesis in both prokaryotes and eukaryotes and resistance genes encoding blasticidin S deaminase have been cloned from bacteria (*bsr, Bacillus cereus*) and fungi (*bsd, Aspergillus terreus*). Yanai and his research team produced in 1991 the first report of successful use of blasticidin S resistance as a positive selection system in filamentous fungi (*Rhizopus niveus)*, using the *B. cereus* deaminase gene under the control of the *glaA* promoter from *Rhizopus oryzae*. Although this system has been employed in plants and mammalian cells, blasticidin S resistance has not been routinely used for positive selection in filamentous fungi.

Acetamidase (amdS)

Acetamide is a poor nitrogen source for most wild-type fungal strains. Using the homologous gene cloned from *A. nidulans* by Hynes et al. in 1983, *amdS* was then developed as a selectable marker for *A. nidulans* by Tilburn and colleagues; transformants are able to use acetamide as a nitrogen source. Geissen and Leistner used the *A. nidulans amdS* gene as a marker in *Penicillium nalgiovense*. Fungi transformed with *amdS* are sensitive to fluoroacetamide and this was exploited in 2005 by Michielse et al. to inhibit the growth of ectopic transformants that retained the *amdS* gene (the gene would be excised during homologous recombination).

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Ganzlin M, Rinas U. (2008) In-depth analysis of the *Aspergillus niger* glucoamylase (*glaA*) promoter performance using high-throughput screening and controlled bioreactor cultivation techniques. J Biotechnol 135, 266-71. [These authors analyzed the effects of various monosaccharides on *glaA*-driven reporter gene expression, finding that only starch and starch hydrolysis products were inducers but glucose repressed at high concentrations. They also identified glucose analogues that were effective inducers of *glaA*.]

Geisen R and L. Leistner L (1989) Transformation of *Penicillium nalgiovense* with the *amdS* gene of *Aspergillus nidulans*. Curr Genet 15, 307–309. [This paper successfully used the *A. nidulans amdS* gene as a positive selectable marker in the genetically uncharacterized food industry fungus, *P. nalgiovense.*]

Gouka RJ and Hessing JGM, Punt PJ, Stam H, Musters W, and van den Hondel CAMJJ (1996) An expression system based on the promoter region of the *Aspergillus awamori* 1,4-beta-endoxylanase A gene. Appl Microbiol Biotechnol 46, 28–35. [Characterization of the *xylA* promoter from *A. awamori* as a strong inducible promoter for heterologous protein production in *A. awamori*.]

Granado JD, Kertesz-Chaloupková K, Aebi M, Kües U (1997) Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Mol Gen Genet 256, 28-36. [Using several restriction enzymes and varying the protoplast preparation, the authors showed that efficiency of REMI in this basidiomycete was determined by the enzyme type and concentration.]

Gunatilleke IAUN, Arst HN and Scazzocchio C (1975) Three genes determine the carboxin sensitivity of mitochondrial succinate oxidation in *Aspergillus nidulans*. Genet Res 26, 297-305. [Demonstrated using mitochondrial preparations, that three nuclear gene mutations reduced the oxidation of succinate.]

Gwynne DI and Buxton FP, Williams SA, Sills AM, Johnstone JA, Buch JK, Guo ZM, Drake D, Westphal M, and Davies RW (1989) Development of an expression system in *Aspergillus nidulans*. Biochem Soc Trans 17, 338–340. [To increase expression of a human protein using the *alcA* promoter, and use of *alcR* transformation to further increase heterologous protein expression.]

Ha Y-S, Covert SF and Momany M (2006) FsFKS1, the 1,3-β-glucan synthase from the caspofungin-resistant fungus *Fusarium solani.* Eukaryot Cell 5, 1036–1042. [To understand the relative resistance of F. solani to caspofungin, the authors found that FKS1 knockout strains were non-viable but they successfully used RNAi to knock-down expression and identify a phenotype. Expression was driven by the heterologous *alcA* promoter from *A. nidulans*.]

Hamer L, Adachi K, Montenegro-Chamorro MV, Tanzer MM, Mahanty SK, Lo C, Tarpey RW, Skalchunes AR, Heiniger RW, Frank SA, Darveaux BA, Lampe DJ, Slater TM, Ramamurthy L, DeZwaan TM, Nelson GH, Shuster JR, Woessner J, and Hamer JE (2001) Gene discovery and gene function assignment in filamentous fungi. Proc Natl Acad Sci USA 98, 5110-5115. [The authors present their work on TAGKO development and show proof of principle of insertional mutagenesis vectors they created using the phytopathogenic fungi, *Magnaporthe grisea* and *Mycosphaerella graminicola*.]

Harkki A, Mäntylä A, Penttilä M, Muttilainen S, Bühler R, Suominen P, Knowles J, and Nevalainen H (1991) Genetic engineering of *Trichoderma* to produce strains with novel cellulase profiles. Enzyme Microb Technol 13, 227-233. [These authors used the promoter and terminator from *T. reesei* cellobiohydrolase 1 gene to increase expression of endoglucanase in the cellulolytic fungus, *T. reesei*.]

Harrier LA and Millam S (2001) Biolistic transformation of arbuscular mycorrhizal fungi. Molec Biotechnol 18, 25-33.[A review of the use of biolistic transformation of AM fungi.]

Harmsen MC, Schuren FHJ, Moukha SM, van Zuilen CM, Punt HP, and Wessels JGH (1992) Sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase genes from the basidiomycetes *Schizophyllum commune*, *Phanerochaete chrysosporium* and *Agaricus bisporus*. Curr Genet 22, 447–454. [These data explain the low expression levels of genes in basidiomycetes when using ascomycete *gpd* promoter. The authors analyzed the *gpd* genes from these basidiomycetes and compared them to yeast and filamentous ascomycetes.]

He Z-M, Price MS, OBrian GR, Georgianna DR and Payne GA (2007) Improved protocols for functional analysis in the pathogenic fungus *Aspergillus flavus.* BMC Microbiology 7, 104 doi, 10.1186/1471-2180-7-104. [Developed the use of the *ble* gene encoding phleomycin resistance for positive selection in *A. flavus*. In addition, the *N. crassa pyr4* marker was used for selection.]

Hedeler C, Wong HM, Cornell M, Alam I, Soanes DM, Rattray M, Hubbard SJ, Talbot NJ, Oliver SG and Paton, N. (2007) e-Fungi: a data resource for comparative analysis of fungal genomes. BMC Genomics, 8, 426. [The authors have created a single repository of comparative genomic information for more than 30 sequenced fungal genomes accessible via a web interface.]

Hinnen A, Hicks JB and Fink CR (1978) Transformation of yeast. Proc Natl Acad Sci USA 75, 1929-1933. (The first *S. cerevisiae* transformation based on a ColE1 plasmid; both ectopic and homologous integration into the yeast genome were demonstrated.)

Hissen AHT, Wan ANC, Warwas ML, Pinto LJ and Moore MM (2005) The *Aspergillus fumigatus* siderophore biosynthetic gene *sidA*, encoding L-ornithine-N^5-oxygenase, is required for virulence. Infect Immun 73, 5493–5503. [This study used electroporation of swollen conidia to transform *A. fumigatus* with linear constructs.]

Hoshida H, Fujita T, Murata K, Kubo K, Akada R. (2005) Copper-dependent production of a *Pycnoporus coccineus* extracellular laccase in *Aspergillus oryzae and Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 269, 1090-1097. [To develop *A. oryzae* as a heterologous host for laccase production, the authors successfully expressed laccase using the *amyB* promoter.]

Hu W, Sillaots S, Lemieux S, Davison J, Kauffman S, Breton A, Linteau A, Xin C, Bowman J, Becker J, Jiang B, and Roemer T (2007) Essential gene identification and drug target prioritization in *Aspergillus fumigatus*, PLoS Pathogens 3, e24. [The authors developed a conditional replacement promoter construct

containing the *pNiiA* promoter from *A. fumigatus* (regulated by nitrate and ammonium) and the *pyrG* selectable marker flanked by endogenous promoter flanking regions. They identified 250 essential genes in *A. fumigatus*.]

Hua-Van A, Pamphile JA, Langin T, and Daboussi MJ (2001) Transposition of autonomous and engineered impala transposons in *Fusarium oxysporum* and a related species. Mol Gene Genet 264, 724-231. [This work describes the transposition of impala in *F. oxysporum* and showed that even divergent genetic elements can activate a defective (transposase-deficient) *impala* transposon, and that *impala* can transpose in a heterologous host (*F. moniliforme*).]

Hynes MJ, Corrick CM, and King JA (1983) Isolation of genomic clones containing the *amdS* gene of *Aspergillus nidulans* and their use in the analysis of structural and regulatory mutations. Mol Cell Biol 3, 1430-1439. {Describes the fine-scale mapping of the *A. nidulans* acetamidase (*amdS*) gene as well as the 5'-regulatory regions.]

Inglis PW Tigano MS., Valadares-Inglis MC. (1999) Transformation of the entomopathogenic fungi, *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus* (Deuteromycotina: hyphomycetes) to benomyl resistence. Genet Mol Biol [serial on the Internet]. 22: 119-123. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1415-47571999000100023&lng=en. doi: 10.1590/S1415-47571999000100023. [Stable homologous recombination in *Paecilomyces* spp. using the *N. crassa* beta tubulin gene to create strains resistant to benomyl.]

Inoue I, Ohara T, Namiki F and Tsuge T (2001) Isolation of pathogenicity mutants of *Fusarium oxysporum* f. sp. melonis by insertional mutagenesis. J Gen Plant Pathol 67, 191-199. [The authors used REMI to successfully generate mutant strains by transforming with a linear construct encoding *hph*; however, no increase in transformation frequency was afforded by including up to 200 units of various restriction enzymes.]

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Kadotani N, Nakayashiki H, Tosa Y, Mayama S (2003) RNA silencing in the phytopathogenic fungus, *Magnaporthe oryzae*. Mol Plant-Microbe Int 16, 769-776. [The authors demonstrated using an eGFP - expressing strain, that hairpin RNAi constructs containing the eGFP gene reduced eGFP gene expression.]

Kahmann R and Basse C (1999) REMI (Restriction Enzyme Mediated Integration) and its impact on the isolation of pathogenicity genes in fungi attacking plants. Eur J Plant Pathol 105, 221–229. [A brief review of the use of REMI as a method for identifying virulence genes in phytopathogenic fungi.]

Kamakura T, Kobayashi K, Tanaka T, Yamaguchi I, Endō T (1987) Cloning and expression of a new structural gene for blasticidin S deaminase, a nucleoside aminohydrolase. Agric Biol Chem 51, 3165-3168. [The original publication on the cloning of the *B. cereus bsr* gene and its expression in *E. coli* and *B. subtilis*.]

Katayose Y, Kajiwara S and Shishido K (1990) The basidiomycete *Lentinus edodes* linear mitochondrial DNA plasmid contains a segment exhibiting a high autonomously replicating sequence activity in *Saccharomyces cerevisiae*. Nucl Acids Res 18, 1395-1400. [Isolation and characterization of linear plasmid sequences from *L. edodes* that contained ARS sequences that were functional in *S. cerevisiae* when cloned into a yeast expression vector.]

Kelly R, Miller SM, Kurtz MB (1988) One-step gene disruption by co-transformation to isolate double auxotrophs in *Candida albicans*. Mol Gen Genet 214, 24-31. [A linear DNA fragment was successfully co-transformed with a URA-ARS vector in *C. albicans*.]

Keon JP, White GA, Hargreaves JA (1991) Isolation, characterization and sequence of a gene conferring resistance to the systemic fungicide carboxin from the maize smut pathogen, *Ustilago maydis*. Curr Genet 19, 475–481. [Identification of a mutated form of succinate dehydrogenase FeS-subunit as the gene conferrring carboxin resistance in resistant strain of *U. maydis*.]

Keränen S and Penttilä M (1995) Production of recombinant proteins in the filamentous fungus *Trichoderma reesei*. Curr Opin Biotechnol 6, 534-537. [A review of heterologous protein expression in *T.*

reesei and a description of the utility of the *cbh1* promoter to drive protein expression.]

Kimura M, Kamakura T, Tao QZ, Kaneko I, and Yamaguchi I (1994) Cloning of the blasticidin S deaminase gene (BSD) from *Aspergillus terreus* and its use as a selectable marker for *Schizosaccharomyces pombe* and *Pyricularia oryzae.* Molec Gen Genet 242, 121-129. [The eukaryotic blasticidin resistance gene was characterized and compared to the gene from *B. cereus* (*bsr*); only *bsd* functioned as a selectable marker in the ascomycete fungus, *Pyricularia*.]

Klinner U and Schäfer B (2004) Genetic aspects of targeted insertion mutagenesis in yeast. FEMS Microbiol Rev 28, 201-223. [The efficiency of targetted integration of various vectors in non-*Saccharomyces* yeasts is described as well as mechanisms of integration.]

Kojic M, Holloman W (2000) Shuttle vectors for genetic manipulations in *Ustilago maydis*. Can J Microbiol 46, 333–338. [The authors described various shuttle vectors based on pUC19 for selection using geneticin, carboxin, nourseothricin, or hygromycin, which either integrate or are autonomously replicated.]

Kooistra R, Hooykaas PJJ and Steensma HY (2004) Efficient gene targetting in *Kluyveromyces lactis*. Yeast 21, 781-792. [Cloning and knockout of the genes involved in NHEJ in *K lactis* (a *ku80* mutant) resulted in a dramatic rise in targetted integration to >97% independent of the length of flanking regions.]

Kück, U., and Hoff B 2006. Application of the nourseothricin acetyltransferase gene (*nat1*) as a dominant marker for the transformation of filamentous fungi. Fungal Genet. Newsl. 53, 9-11. [The construction of a transforming vector containing the bacterial *nat1* gene driven by the *A. nidulans trpC* promoter, and its validation as a selectable marker in *Sordaria macrospora* and *Acremonium chrysogenum.*]

Kuwayama H, Obara S, Morio T, Katoh M, Urushima H, Tanaka Y (2002) PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. Nucleic Acids Res 30, e2. [Their method using fusion PCR to create, without cloning, constructs with long flanking regions for transformation of the amoeba, *Dictyostelium dicoideum.*]

Lacroix B, Tzfira T, Vainstein A and Citovsky V (2006) A case of promiscuity: *Agrobacterium*'s endless hunt for new partners. Trends Genet 22, 29-37. [A review of the mechanisms of AMT in non-plant organisms including fungi, and the utility of AMT in genetic manipulation.]

Lee M-H and Bostock RM (2006) *Agrobacterium* T-DNA-mediated integration and gene replacement in the brown rot pathogen *Monilinia fructicola*. Curr Genet 49, 309–322. [The successful use of AMT in targetted gene disruption in the ascomycete phytopathogen, *M. fructicola* using the *npt*II cassette, and the difficulties in obtaining homokaryotic transformants from a multinucleate fungus.]

Liu YG, Mitsukawa LN, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8, 457–463. [The use of TAIL-PCR to map the genomic sequences flanking the insertion of T-DNA in plants transformed using AMT.]

Loppnau P, Tanguay P., and Breuil C. (2004) Isolation and disruption of the melanin pathway polyketide synthase gene of the softwood deep stain fungus, *Ceratocystis resinifera*. Fungal Genet Biol 41, 33-41. {Successful use of AMT with 24-hour germinated conidia in the ascomycete *C. resinifera*, for targetted gene disruption.]

Lubertozzi D and Keasling JD (2009) Developing *Aspergillus* as a host for heterologous expression. Biotechnol Adv 27, 53-75. [A review of the past, present and potential future uses of *Aspergillus* spp. as hosts for heterologous protein expression, with particular emphasis on the production of secondary metabolites.]

Magnani E, Bartling L, Hake SC (2006) From GATEWAY to MULTISITE GATEWAY in one recombination event. BMC Molec Biol 7, 46.[The authors created a vector, pDONR-R4-R3 which when recombined with a single site Gateway destination vector, will convert it to a vector that will accept up to three Gateway entry clones.]

Manivasakam P and Schiestl RH (1998) Nonhomologous end joining during restriction enzyme-mediated DNA integration in *Saccharomyces cerevisiae*. Mol Cell Biol 18, 1736–1745. {An analysis of the types of restriction enzymes and sequence requirements that promoted integration using REMI, as well as the genomic location of integration.]

Mattern JE, Punt PJ, van den Hondel CAMJJ (1988) A vector of *Aspergillus* transformation conferring phleomycin resistance. Fungal Genet Newslett 35, 25. [Description of the construction of pAN8-1, a plasmid containing the bacterial *ble* gene, and the transformation of *A. nidulans* and *A. niger*.]

May GS (1992) Fungal technology. In, Applied Molecular Genetics of Filamentous Fungi. Kinghorn JR and Turner G, eds., Glasgow, Blackie Academic and Professional, pp 1-27. [A review of methods in fungal biotechnology with an emphasis on genetic manipulation.]

Meyer V, Mueller D, Strowig T, and Stahl U (2003) Comparison of different transformation methods for *Aspergillus giganteus*. Curr Genet 43, 371-377. [The authors compared four different methods of transformation: protoplast transformation using PEG/$CaCl_2$, electroporation, biolistic transformation, and AMT. AMT improved transformation frequencies >100-fold compared to PEG-mediated transformation.]

Michielse CB, Arentshorst M, Ram AFJ, van den Hondel CAMJJ (2005) *Agrobacterium*-mediated transformation leads to improved gene replacement efficiency in *Aspergillus awamori*. Fungal Genet Biol 42, 9-19. [Compared AMT-mediated transformation of *A. awamori* with a PEG/$CaCl_2$ method, and found that adding a second selectable marker (*amdS*) to the disruption construct doubled the proportion of gene replacement events.]

Migheli Q, Laugé R, Davière JM, Gerlinger C, Kaper F, Langin T, and Daboussi M-J (1999) Transposition of the autonomous Fot1 element in the filamentous fungus *Fusarium oxysporum*. Genetics 151, 1005-1013. [This paper provides the first description of an autonomous transposable element in filamentous fungi.]

Nakayashiki H and Nguyen QB (2008) RNA interference: roles in fungal biology. Curr Opinion Microbiol, 11, 494–502. [A review of RNA silencing in filamentous fungi including current methods and a list of publications on the use of RNAi in various fungal species.]

Nielsen JB, Nielsen ML and Mortensen UH (2008) Transient disruption of non-homologous end-joining facilitates targeted genome manipulations in the filamentous fungus *Aspergillus nidulans*. Fungal Genet Biol 45, 165–170. [The authors transiently disrupted the *nku* gene by using the *pyrG* as a counterselectable marker, flanked by direct repeats; this will allow restitution of NHEJ activity once the desired genetic changes have been completed.]

Nielsen ML, de Jongh WA. Meijer SL, Nielsen J, and Mortensen UH (2007) Transient marker system for iterative gene targeting of a prototrophic fungus. Appl Environ Microbiol, 73, 7240–7245. [This paper describes an approach similar to the one used for the ku gene in the 2008 publication, except that here the focus is on restoring an auxotrophic strain to wild type once genetic manipulations are done.]

Ninomiya Y, Suzuki K, Ishii C, and Inoue H (2004) Highly efficient gene replacement in *Neurospora* strains deficient for non-homologous end joining. Proc Natl Acad Sci USA 101, 12248-12253. [The first study with *Neurospora crassa* in which the deletion of *ku70* and *ku80* homologues resulted in a dramatic increase in homologous recombination. The sensitivity of the ku knockout strains to various agents is also presented.]

Nitta Y, Miyazaki Y, Nakamura M, Iimura Y, Shishido K, Kajita S, and Morohoshi N (2004) Molecular cloning of the promoter region of the glyceraldehyde-3-phosphate dehydrogenase gene that contributes to the construction of a new transformation system in *Coriolus versicolor*. Mycoscience 45, 131–136. [The construction of a transforming plasmid in the basidiomycete *C. versicolor* in which the *C. versicolor gpd* gene drives expression of *hph*.]

Olempska-Beer ZS, Merker RI, Ditto MD, and DiNovi MJ (2006) Food-processing enzymes microorganisms—a review. Regul Toxicol Pharm 45, 144–158. [This paper reviews the safety of microorganisms used as heterologous hosts for food processing enzymes as well as some regulatory issues. The construction of recombinant strains and methods to improve enzyme properties are also discussed.]

Orbach MJ, Porro B and Yanofsky C (1986) Cloning and characterization of the gene for beta-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Mol Cell Biol. 6, 2452-2461. [The authors established the molecular basis for benomyl resistance in the *N. crassa beta* tubulin gene and developed the gene as a selectable marker for transformation of *N. crassa*.]

Ozeki, K., Kyoya, F., Hizume, K., Kanda, A., Hamachi, M. and Nunokawa, Y. (1994) Transformation of intact *Aspergillus niger* by electroporation. Biosci Biotechnol Biochem 58, 2224-2227. [This paper

studied the variables affecting transformation efficiency of *A. niger* by electroporation of germinated conidia with and without enzymatic cell wall lysis, and compared this with the efficiency of transformation of protoplasts with $CaCl_2$-PEG.]

Punt, PJ, Oliver, RP, Dingemanse, MA, Pouwels, PH, and van den Hondel, CA (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* 56, 117–124. [The first description of the use of the *hph* gene in *Aspergillus* spp. creating a dominant selectable marker for transformation and co-transformation.]

Punt PJ, Zegers ND, Busscher M, Pouwels PH, and van den Hondel CAMJJ (1991) Intracellular and extracellular production of proteins in *Aspergillus* under the control of expression signals of the highly expressed *Aspergillus nidulans gpdA* gene. J Biotechnol 17, 19-34. [A comparison of the homologous *gpdA* promoter with two heterologous promoters for intracellular and secreted protein expression in *A. nidulans*.]

Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J and van den Hondel C (2002) Filamentous fungi as cell factories for heterologous protein production. Trends Biotechnol 20, 200-206. [A review of the molecular tools available for optimizing heterologous protein production in filamentous fungi with an emphasis on production of commercially-relevant protein concentrations.]

Redkar RJ, Herzog RW, and Singh NK (1998) Transcriptional activation of the *Aspergillus nidulans gpdA* promoter by osmotic signals. Appl Environ Microbiol 64: 2229-2231. [Although the *gpdA* promoter is considered by most to be constitutive, these authors report that sodium chloride concentration affected the transcriptional activity of an *A. nidulans gpd*A-driven reporter genes in *N. crassa*.]

Romero B. Turner G. Olivas I, Laborda F and Ramón De Lucas J (2003) The *Aspergillus nidulans alcA* promoter drives tightly regulated conditional gene expression in *Aspergillus fumigatus* permitting validation of essential genes in this human pathogen. Fungal Genet Biol 40, 103–114. [This report describes the expression of the *A. fumigatus nudC* gene required for survival under the control of the *A. nidulans alcohol dehydrogenase (alcA)* promoter.]

Sánchez, O. and Aguirre, J. (1996) Efficient transformation of *Aspergillus nidulans* by electroporation of germinated conidia. Fungal Genet Newsl 43, 48-51. [Assessed the effect of conidial germination time and conidial freeze-thawing on transformation efficiency by electroporation.]

Schauer F, Borriss R. (2004) Biocatalysis and biotransformation. In: Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine. Tkacz JS, Lange L, eds., Kluwer Academic/Plenum Publishers; 2004. p.237–275. [This review provides a list of industrially important fungal enzymes divided into six major IUPAC classes as well as a discussion of fungal enzymes involved in both biodeterioration and bioremediation.]

Schiestl RH and Petes TD (1994) Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 88, 7585-7589. [The first example of REMI using *S. cerevisiae*.]

Shima Y, Ito Y, Kaneko S, Hatabayashi H, Watanabe Y, Adachi Y and Yabe K (2009) Identification of three mutant loci conferring carboxin-resistance and development of a novel transformation system in *Aspergillus oryzae*. Fungal Genet Biology 46, 67-76. [Identification of mutations in succinate dehydrogenase (*sdh*) subunits that confer carboxin resistance in *A. oryzae*, and development of a transformation construct using the *sdh* genes with their endogenous promoter in *A. parasiticus*.]

Shoji J, Maruyama J, Arioka M and Kitamoto K (2005) Development of *Aspergillus oryzae thiA* promoter as a tool for molecular biological studies. FEMS Microbiol Lett 244, 41-46. [The authors developed an alternative regulatable promoter for *A. oryzae* that does not require change in nutrient for induction. *A. oryzae egfp* gene expression driven by the *thiA* promoter was regulated by thiamine concentration in the medium.]

Silar, P (1995) Two new easy to use vectors for transformation. Fungal Genet. Newsl 42, 73. [Development of vectors encoding phleomycin or hygromycin resistance driven by *A. nidulans gpd* promoter or the *N. crassa cpc-1* promoter, respectively.]

Simon JR, Moore PD. (1987) Homologous recombination between single-stranded DNA and chromosomal genes in *Saccharomyces cerevisiae*. Mol Cell Biol 7, 2329–2334.

Smith JE (1994) *Aspergillus*, Plenum, New York, pp 41-76. [The authors examined the importance of single- versus double-stranded transforming DNA in promoting homologous recombination in *S. cerevisiae*.]

Smith RP and Smith ML (2007) Two yeast plasmids that confer nourseothricin-dihydrogen sulfate and hygromycin B resistance in *Neurospora crassa* and *Cryphonectria parasitica*. Fungal Genetics Newsletter 54, 12-13. [The authors validated the use of these plasmids in two fungi as dominant selection markers and for forcing heterokaryons.]

Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh N, Osmani SA and Oakley BR (2006) Fusion PCR and gene targetting in *Aspergillus nidulans*. Nature Protocols 1, 3111-3120. [A detailed description of one method for creating linear constructs for transformation using fusion PCR.]

Tada S, Gomi K, Kitamoto K, Takahashi K, Tamura G, Hara S (1991) Construction of a fusion gene comprising the Taka-amylase A promoter and the *Escherichia coli* beta-glucuronidase gene and analysis of its expression in *Aspergillus oryzae*. Mol Gen Genet 229, 301-306. [A detailed analysis of the induction of expression of a reporter gene by the TAA promoter using a variety of sugar substrates.]

Takeno S, Sakuradani E, Tomi A, Inohara-Ochiai M, Kawashima H, Ashikari T and Shimizu S (2005) Improvement of the fatty acid composition of an oil-producing filamentous fungus, *Mortierella alpina* IS-4, through RNA interference with D12-desaturase gene expression. Appl Environ Microbiol 71, 5124-5128. [The authors showed that RNAi-mediated silencing operates in the basidiomycete, *Mortierella alpina* and that introduction of the transforming RNAi construct could be done using biolistics.]

Takken FL, Van Wijk R, Michielse CB, Houterman PM, Ram AF, Cornelissen BJ (2004) A one-step method to convert vectors into binary vectors suited for *Agrobacterium*-mediated transformation. Curr Genet 45, 242-248. [This report describes an efficient method to convert BACs into binary BACs for use in complementation; the method was validated using BACs containing fungal DNA and integration into two fungal genera.]

Te'o VSJ, Bergquist PL and Nevalainen KMH (2002) Biolistic transformation of *Trichoderma reesei* using the Bio-Rad seven barrels Hepta Adaptor system. J Microbiol Meth 51, 393-399. [A detailed protocol for biolistic transformation of conidia for targetted gene disruption using linear or circular DNA.]

Tilburn J, Scazzocchio C, Taylor GG, Zabicky-Zissman JH, Lockington RA, Davies RW (1983) Transformation by integration in *Aspergillus nidulans*. Gene 26, 205–221. [An early paper describing the transformation of *A. nidulans* protoplasts using a PEG/CaCl2 method with plasmids containing the *amdS* gene.]

Toews MW, Warmbold J, Konzack S, Rischitor P, Veith D, Vienken K, Vinuesa C, Wei H, Fischer R (2004) Establishment of mRFP1 as a fluorescent marker in *Aspergillus nidulans* and construction of expression vectors for high-throughput protein tagging using recombination in vitro (GATEWAY). Curr Genet 45, 383–389. [The authors generated a set of plasmids using the GATEWAY system that contain the *alcA* promoter and the argB selectable marker. These can be used for insertion of PCR products for tagging with a number of fluorescent marker genes as well as hemagglutinin.]

Topp CN, Ruiz-Herrera J, Martínez-Espinoza, AD, Gold SE (2002) Integration of the gene for carboxin resistance does not impact the *Ustilago maydis*–maize interaction. Curr Microbiol 44, 67–70. [A follow up study from an earlier one published by Ruiz-Herrara to demonstrate that integration of the *cbx* gene into *Ustilago maydis* does not alter its pathogenicity; therefore, carboxin resistance remains a suitable dominant marker gene in *Ustilago*.]

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Biographical Sketch

Margo M. Moore obtained her B. Sc. in Biochemistry and her Ph.D. in Pharmacology at the University of British Columbia. She then did post-doctoral studies for two years at the Karolinska Institute and has been on the faculty of Simon Fraser University since 1990. She is currently a professor in the Department of Biological Sciences. Dr. Moore's research on fungi initially focussed on the ability of filamentous fungi to metabolize naturally occurring polyaromatic compounds. More recently, her laboratory has investigated virulence mechanisms in the opportunistic fungal pathogen, *Aspergillus fumigatus.*