GENETIC ENGINEERING OF FUNGAL CELLS

Margo M. Moore
Department of Biological Sciences, Simon Fraser University, Burnaby, Canada

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Contents

1. Introduction
   1.1. Industrial importance of fungi
   1.2. Purpose and range of topics covered
2. Generation of transforming constructs
   2.1. Autonomously-replicating plasmids
   2.2. Promoters
   2.2.1. Constitutive promoters
   2.2.2. Inducible promoters
   2.3. Selectable markers
   2.3.1. Dominant selectable markers
   2.3.2. Auxotrophic/inducible markers
   2.4. Gateway technology
   2.5. Fusion PCR and Ligation PCR
3. Transformation methods
   3.1. Protoplast formation and CaCl2/PEG
   3.2. Electroporation
   3.3. Agrobacterium-mediated Ti plasmid
   3.4. Biolistics
   3.5. Homo- versus heterokaryotic selection
4. Gene disruption and gene replacement
   4.1. Targeted gene disruption
   4.1.1. Ectopic and homologous recombination
   4.1.2. Strains deficient in non-homologous end joining (NHEJ)
   4.1.3. AMT and homologous recombination
   4.1.4. RNA interference
   4.2. Random gene disruption
   4.2.1. Restriction enzyme-mediated integration (REMI)
   4.2.2. T-DNA tagging using Agrobacterium-mediated transformation (AMT)
   4.2.3. Transposon mutagenesis & TAGKO
5. Concluding statement
Glossary
Bibliography
Biographical Sketch

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-\textit{Saccharomyces} yeasts. There is an extensive literature on genetic and metabolic engineering of \textit{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 \textit{Saccharomyces}, \textit{Schizosaccharomyces} and \textit{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* AMA1 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 Cufetti 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.

<table>
<thead>
<tr>
<th>Constitutive promoters</th>
<th>Gene function</th>
<th>Source DNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pna2/tpi hybrid promoter</td>
<td>neutral amylase + triose phosphate isomerase</td>
<td>Aspergillus nidulans</td>
<td>Olempska-Beer et al. 2006</td>
</tr>
<tr>
<td>gpdA</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Aspergillus nidulans</td>
<td>Punt et al. 1991</td>
</tr>
<tr>
<td>trpC</td>
<td>tryptophan biosynthesis</td>
<td>Aspergillus nidulans</td>
<td>Hamer and Timberlake, 1987</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inducible promoters</th>
<th>Gene function</th>
<th>Source DNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAKA-A amylase</td>
<td>amylase hydrolysis</td>
<td>Aspergillus oryzae</td>
<td>Tada et al. 1991 Christensen 1988</td>
</tr>
<tr>
<td>glaA</td>
<td>glucoamylase</td>
<td>Aspergillus niger var. awamori</td>
<td>Boel et al. 1984</td>
</tr>
<tr>
<td>alcA/AlcR</td>
<td>alcohol dehydrogenase</td>
<td>Aspergillus nidulans</td>
<td>Gwynne et al. 1989</td>
</tr>
<tr>
<td>niiA</td>
<td>nitrite reductase</td>
<td>Aspergillus fumigatus</td>
<td>Amaar and Moore, 1998 Hu et al. 2007</td>
</tr>
<tr>
<td>cbhI</td>
<td>cellobiohydrolase I</td>
<td>Trichoderma reesei</td>
<td>Harkki et al. 1991</td>
</tr>
<tr>
<td>ctr4</td>
<td>high affinity copper transporter</td>
<td>Schizosaccharomyces pombe</td>
<td>Bellemare et al. 2001</td>
</tr>
<tr>
<td>thiA</td>
<td>thiamine biosynthesis</td>
<td>Aspergillus oryzae</td>
<td>Shoji et al. 2005</td>
</tr>
</tbody>
</table>

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 \textit{glaA}.

The alcohol regulon in \textit{Aspergillus nidulans} consists of \textit{alcA} (alcohol dehydrogenase), \textit{aldA} (aldehyde dehydrogenase) and a positive regulator, \textit{alcR}. Glucose repression of all three genes is mediated by the \textit{creA} gene product. In 1995, Fillinger and colleagues showed that ethanol and other substrates stimulate \textit{alcR} whereas poor carbon sources such as lactose or glycerol can derepress the \textit{alcA} gene. The tight regulation of the \textit{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 \textit{A. nidulans alcA} promoter in \textit{Fusarium solani} to drive the expression of an RNAi construct. Use of the \textit{alcA/alcR} system was originally devised by Gwynne and colleagues in 1989 for the production of heterologous proteins by \textit{Aspergillus nidulans} (e.g., alpha interferon-2). In 1996, Gouka used the \textit{exlA} promoter from \textit{Aspergillus awamori} endoxylanase (xylose-inducible) to provide strong expression in \textit{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 \textit{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 \textit{Aspergillus nidulans} and \textit{A. niger}, a hybrid promoter containing the human estrogen response element (ERE) fused to a minimal \textit{S. cerevisiae URA3} TATA element was developed in 2005 by Pachlinger and colleagues to drive expression of a \textit{lacZ} reporter gene. When estrogenic compounds were added as inducers to the medium at picomolar levels, they achieved levels of expression similar to an \textit{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 \textit{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).
<table>
<thead>
<tr>
<th>Function of resistance/nutritional gene</th>
<th>Source organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>benomyl - tub</td>
<td>benomyl-resistant tubulin mutants</td>
<td>Neurospora crassa</td>
</tr>
<tr>
<td>bialophos/phosphinothricin-bar</td>
<td>phosphinothricin acetyltransferase</td>
<td>Streptomyces hygroscopicus</td>
</tr>
<tr>
<td>carboxin - cbx&lt;sup&gt;R&lt;/sup&gt;</td>
<td>carboxin-resistant succinate dehydrogenase mutants</td>
<td>Ustilago maydis</td>
</tr>
<tr>
<td>hygromycin - hph</td>
<td>hygromycin phosphotransferase</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>nourseothricin - nat</td>
<td>nourseothricin acetyltransferase</td>
<td>Streptomyces noursei</td>
</tr>
<tr>
<td>phleomycin/zeocin/bleomycin ble</td>
<td>bleomycin binding protein</td>
<td>Streptothalloteichus hindustanus</td>
</tr>
<tr>
<td>pyrithiamine - ptrA</td>
<td>mutated allele of thiamine biosynthesis gene</td>
<td>Aspergillus oryzae</td>
</tr>
<tr>
<td>amdS</td>
<td>acetamidase</td>
<td>Aspergillus nidulans</td>
</tr>
<tr>
<td>Auxotrophic markers *both are positive-negative selection systems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>niaD</td>
<td>nitrate reductase (assimilatory)</td>
<td>Aspergillus nidulans</td>
</tr>
<tr>
<td>pyrG</td>
<td>orotidine-5(^{\prime})-phosphate decarboxylase</td>
<td>Aspergillus niger</td>
</tr>
</tbody>
</table>

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 *Streptalloteichus 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* in *U. maydis* reduced its pathogenicity to corn, a study by Topp and coworkers in 2002 using several strains transformed with *cbx* 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* 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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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.