Genetic transformation in Aspergilli: Tools of the trade

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DNA-mediated transformation is a powerful tool that allows the introduction of specific genetic changes in an organism. Transformation of Aspergilli, acclaimed for their wide use in the industry, has been possible for about two decades now. Several basic and applied problems related to fungal biology have been addressed using this technique. Nonetheless, new markers and strategies for transformation are still being developed for these filamentous fungi. Different methods and markers that are currently available for the transformation of Aspergilli are summarized here. The review also brings out the importance of these transformation systems in analyzing fungal gene function. Aspects of Aspergillus niger transformation are selectively emphasized.

Keywords: Aspergilli, Aspergillus niger, transformation strategies, genetic markers, transformation vectors, gene function

Introduction

Aspergilli are of immense economic and medicinal value and a better understanding of their basic metabolic processes would enable the control and treatment of pathogenic species and in the industrial scenario help in rationalized strain improvement. Genetic transformation is an important tool that allows one to address questions relating to metabolism and gene expression as well as the regulation of these processes. In Aspergilli that reproduce predominantly through asexual mode it is of further value as it provides a means of introducing specific genetic changes.

In filamentous fungi, genetic transformation is usually achieved by integration of the introduced DNA into the genome of the organism. Integration is mostly through heterologous recombination; homologous recombination in fungi occurs only at low frequencies. The lower transformation efficiencies (in relation to yeast or E. coli) observed in filamentous fungi may largely be ascribed to their tough cell wall. Although the problems encountered in transforming Aspergilli are not unique, some of these species are more difficult to transform, than the others. For instance, in A. niger — a) protoplasting is arduous, due to high melanin content of its cell wall; and b) excessive clumping of spores occurs during germination.

The first report of transformation of a filamentous fungus appeared in 1979 when DNA was introduced into protoplasts of Neurospora crassa. Transformation of A. nidulans that followed shortly afterward ushered in an era that has seen rapid advancement in the understanding of these organisms. Although protoplast transformation remains one of the most commonly adopted protocols, several other methods have been developed since then. They are i) Agrobacterium-mediated gene transfer; ii) electroporation of young germings; iii) particle bombardment; and iv) DNA uptake by lithium acetate treated cells. Salient features of these methods are enumerated below. A review of recent literature on the different markers used/available to transform filamentous fungi is presented with an emphasis on Aspergilli. Although there are a number of patents in this general field, this review is largely confined to literature in the public domain barring few exceptions.

Entry of incoming DNA

Transformation of protoplasts

The most routinely used protocol for the transformation of filamentous fungi involves the preparation of protoplasts followed by DNA uptake mediated either by CaCl2/PEG treatment or more recently by electroporation. Protoplasts are prepared from swollen conidia or from young mycelia by enzymatic digestion of their cell walls. For
transformation, protoplasts in osmotic medium containing CaCl₂ are incubated with exogenous DNA and PEG (aids transformation by bringing the DNA and protoplasts together). Highest transformation efficiencies are reported to coincide with germ tube emergence in A. nidulans⁷.

Electroporation
Transformation by electroporation involves exposing cells to short duration high voltage electrical pulses in the presence of exogenous DNA. The electrical pulses induce a reversible permeabilization of the plasma membrane and transient pores are formed enabling the DNA to enter the cell⁸. Electroporation was initially used for the transformation of protoplasts as an alternative to the CaCl₂/PEG method⁹. It was later demonstrated that germinating conidia could also be transformed using this technique. Although the first reports of the procedure included the use of a mild pretreatment with β-glucuronidase to weaken the cell wall⁹, transformation of swollen conidia without the need for pretreatment has been reported for A. niger¹⁰, A. nidulans¹¹ and A. fumigatus¹².

Biolistic transformation
Particle bombardment as a technique for DNA delivery was developed in the late 1980's¹³. In this method, the DNA is first precipitated on small (1 μm) gold or tungsten particles, which are accelerated towards the target in partial vacuum. After particle penetration, the DNA dissociates from the particles and moves to the nucleus, where it gets integrated into the genome. A. nidulans is perhaps the only Aspergillus species that has been transformed using this method¹⁴. This method is generally more useful for the transformation of fungi that are refractory to other methods.

Agrobacterium-mediated transformation
This method exploits the innate virulence (conferred by the Ti plasmid) of Agrobacterium to achieve transformation. The bacterium is co-cultivated with the host to be transformed in the presence of virulence-inducing molecules like acetosyringone. This leads to the mobilization of T-DNA, a region of the Ti plasmid flanked by imperfect 24 bp direct repeats. The T-DNA subsequently enters the host cell and gets integrated into the genome. In the binary system, the gene of interest (gene to be introduced into the fungus) and a fungal selectable marker are cloned between the direct repeats in an E. coli-compatible plasmid and introduced into Agrobacterium to generate the T-DNA, while the necessary virulence functions are provided in trans from a Ti plasmid devoid of T-DNA¹⁵.

Agrobacterium-mediated DNA transfer in filamentous fungi is possible with both conidia and protoplasts¹⁶,¹⁷. The transforming DNA usually integrates randomly into the host genome. However, this technique has also been used to mediate gene targeting by homologous recombination in A. awamori¹⁸.

Other approaches
Transformation of lithium acetate treated cells, routinely used for yeast and also demonstrated in N. crassa, has not been reported for Aspergilli. However, direct transformation of lysine requiring mutants of Aspergillus ochraceus could be achieved by subjecting the mutant conidia to starvation conditions for 15 days and then treating them with DNA from the wild-type strain¹⁹. This method nevertheless is not commonly used.

Comparison of different methods
The methods described above have their own pros and cons. Transformation of protoplasts necessitates their isolation, which is a laborious procedure requiring great care in experimentation. The competence achieved by the protoplasts varies vastly with the batch of mycolytic enzyme used. A 100-fold slump in transformation frequency on varying the batch of enzyme used has been reported²⁰. This method is also limited due to the low and often variable regeneration frequency of protoplasts. Nonetheless, protoplast transformation remains the most commonly adopted procedure. Electroporation is simpler and provides a good alternative to protoplast transformation; it, however, requires a specialized apparatus and offers no advantage in terms of improved transformation efficiency. Biolistic transformation is expensive and again, requires elaborate apparatus. The method is, however, important in those fungi that are refractory to other transformation protocols.

Transformation mediated by Agrobacterium is the most recent and perhaps the simplest method of all. It is often more effective than the conventional methods.
of protoplast transformation. For instance, use of *Agrobacterium* improved the transformation frequency of *A. awamori* up to 600-fold as compared with protoplast transformation\(^\text{16}\). A comparison of the four different transformation methods has been reported for *Aspergillus giganteus*. *Agrobacterium*-mediated transformation with a 140-fold higher transformation frequency than protoplast transformation was adjudged the best\(^\text{21}\). The ease of the protocol coupled with the wide host range of *Agrobacterium*, which includes several refractory fungi, is anticipated to make this method a hot favourite.

**Markers available for transformation**

In order to select/detect any transformation event it is essential that the transformed cells exhibit a readily observable phenotype that is absent in the untransformed cell. A few markers developed for Aspergilli are analogous to their counterparts in yeast. The markers *pyrG* (*Aspergilli*) and *ura3* (yeast) are based on uracil auxotrophy. However, most markers are unique to filamentous fungi. The different types of genetic markers available to date for the selection of fungal transformants are enumerated below.

### Nutritional markers

An appropriate mutant recipient strain must be available to employ nutritional markers. Selection in case of nutritional markers is based on complementation of a nutritional defect by introduction of the wild type allele\(^\text{22}\). The *amdS* gene is the only exception and it confers the ability to utilize acetamide as the sole carbon/nitrogen source in a wild type background\(^\text{23}\). Table 1 lists the nutritional selectable markers employed so far to transform Aspergilli. Only some of these auxotrophic markers, like *pyrG* and *argB* are in common use.

The major obstacle in adapting a recessive marker for use in a new species is the non-availability of suitable mutant recipients. Counter selectable or bi-directional markers, like *pyrG*, *niaD* and *sC* provide a two-way selection and make the isolation of mutants more convenient\(^\text{39-41}\). For example, *pyrG* mutants that are auxotrophic for uracil/uridine can be selected through their resistance to 5-fluoroorotic acid (5-FOA). These markers allow the selection of both the presence and absence of the marker gene function as they have readily identifiable phenotypes. Transformation can thus be scored in either direction; transformation of *pyrG* to *pyrG*\(^+\) (uracil auxotrophy

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**Table 1—Nutritional markers used in *Aspergillus* transformation**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Encoded function</th>
<th>Selection</th>
<th>Species transformed(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>acuD</em></td>
<td>isocitrate lyase</td>
<td>acetate utilization</td>
<td><em>A. nidulans</em>(^2^4)</td>
</tr>
<tr>
<td><em>amdS</em></td>
<td>acetamidase</td>
<td>acetamide utilization</td>
<td><em>A. nidulans</em>(^2), <em>A. niger</em>(^2^3), <em>A. oryzae</em>(^2^5), <em>A. terreus</em>(^2^6), <em>A. ficuum</em>(^2^7)</td>
</tr>
<tr>
<td><em>argB</em></td>
<td>ornithine carbamyltransferase</td>
<td>arginine prototrophy</td>
<td><em>A. nidulans</em>(^2^8), <em>A. oryzae</em>(^2^9), <em>A. terreus</em>(^3^0), <em>A. niger</em>(^3^1)</td>
</tr>
<tr>
<td><em>prn</em></td>
<td>proline catabolism</td>
<td>proline utilization</td>
<td><em>A. nidulans</em>(^2^2)</td>
</tr>
<tr>
<td><em>qutE</em></td>
<td>catabolic quinate dehydrogenase</td>
<td>quinate utilization</td>
<td><em>A. nidulans</em>(^3^3), <em>A. nidulans</em>(^3^4)</td>
</tr>
<tr>
<td><em>riboB</em></td>
<td>not known</td>
<td>riboflavin prototrophy</td>
<td><em>A. oryzae</em>(^3^5)</td>
</tr>
<tr>
<td><em>adeA</em></td>
<td>phosphoribosylaminomimidazole carboxylase</td>
<td>adenine prototrophy</td>
<td><em>A. nidulans</em>(^5)</td>
</tr>
<tr>
<td><em>adeB</em></td>
<td>phosphoribosylaminomimidazole carboxylase</td>
<td>adenine prototrophy</td>
<td><em>A. oryzae</em>(^3^7)</td>
</tr>
<tr>
<td><em>trpC</em></td>
<td>trifunctional enzyme of tryptophan biosynthesis</td>
<td>tryptophan prototrophy</td>
<td><em>A. nidulans</em>(^5), <em>A. niger</em>(^3^6)</td>
</tr>
<tr>
<td><em>hemA</em></td>
<td>5-aminolevulinate synthase</td>
<td>5-aminolevulinic acid prototrophy</td>
<td><em>A. oryzae</em>(^3^7)</td>
</tr>
<tr>
<td><em>pkiA</em></td>
<td>pyruvate kinase</td>
<td>sucrrose utilization</td>
<td><em>A. nidulans</em>(^2^8)</td>
</tr>
</tbody>
</table>

\(a\) - The references listed mostly represent the first reported use of the marker; this list however is not exhaustive.
to prototrophy) and pyrG to pyrG (5-FOA sensitivity to resistance) are both possible. A list of two-way selection markers is given in Table 2.

**Resistance markers**

These dominant markers confer an additional property (resistance to antibiotics/inhibitors) to the wild-type strain and hence obviate the need for an appropriate mutant recipient. This becomes important when wild type strains need to be transformed or while dealing with new strains about which little is known. Also, when gene disruption/gene replacement is desired, it is useful to employ a dominant marker since the absence of a corresponding allele in the recipient eliminates the possibility of homologous recombination at the marker locus. Inhibitor resistance markers known to date\textsuperscript{27,57-73} are summarized in Table 3. However, these markers may have their own limitations such as — i) markers like OliC may be species-specific; ii) a high natural resistance to the antibiotic, if observed, gives high background; iii) an allele for resistance may have to be isolated; and iv) the resistant allele may not show significant dominance over the wild-type allele to be of practical value.

**Transformation vectors**

Two kinds of DNA vectors are available for the transformation of filamentous fungi — i) the integrative vector, which introduces the DNA into the genome; and ii) the autonomously replicating vector, which allows an extra-chromosomal existence of the introduced DNA. Transformation in filamentous fungi is usually performed with the former class of vectors due to problems of instability in transformants obtained through autonomously replicating vectors. This instability primarily arises due to unequal partitioning of plasmids during cell division. A proportion of the daughter cells may, therefore, lack the plasmid. Many filamentous fungi being coenocytic, the selection cannot be made stringent enough to allow the division of only plasmid-bearing cells/nuclei resulting in a heterokaryon and hence the instability. Even in the case of stably integrated DNA it is most often necessary to isolate the transformed nucleus by repeated single-spore isolation.

**Autonomously replicating vectors**

Recombination efficiency largely determines the frequency of integrative transformation. Autonomously replicating plasmids provide a successful alternative to integrative transformation in bacteria/yeast. Development of a convenient vector system based on naturally occurring fungal replicative plasmids however has so far not been possible. The only plasmids found naturally are mitochondrial plasmids and attempts to adapt these as molecular tools for fungal recombinant technology have not

### Table 2—Bidirectional markers reported for Aspergilli

<table>
<thead>
<tr>
<th>Marker</th>
<th>Encoded function</th>
<th>Selection</th>
<th>Species transformed\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>acuA</td>
<td>acetyl CoA synthase</td>
<td>• acetate utilization • fluoroacetate resistance</td>
<td>A. nidulans\textsuperscript{42}</td>
</tr>
<tr>
<td>niaD</td>
<td>nitrate reductase</td>
<td>• nitrate utilization • chlorate resistance</td>
<td>A. nidulans\textsuperscript{43}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• uridine/uracil prototrophy • 5-FOA resistance</td>
<td>A. parasiticus\textsuperscript{44}</td>
</tr>
<tr>
<td>pyrG</td>
<td>OMP decarboxylase</td>
<td>• sulfate utilization • selenate resistance</td>
<td>A. oryzae\textsuperscript{49}</td>
</tr>
<tr>
<td>pyr4</td>
<td>OMP decarboxylase</td>
<td>• uridine/uracil prototrophy • 5-FOA resistance</td>
<td>A. oryzae\textsuperscript{49}</td>
</tr>
<tr>
<td>sC</td>
<td>ATP sulfurylase</td>
<td>• sulfate utilization • selenate resistance</td>
<td>A. nicotianus\textsuperscript{41}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The references listed mostly represent the first reported use of the marker; this list, however, is not exhaustive.

\textsuperscript{b} unpublished data from our group
been very successful. The search for chromosomal replicators in *A. nidulans* led to the discovery of two kinds of DNA elements — i) sequences that lead to vector rearrangement and multimerization and eventually result in chromosomal integration. Examples of such sequences are *ans1* and MATE elements; and ii) sequences that can support autonomous replication of a plasmid stably without vector rearrangements; AMA1 is an example. The increased transformation frequency achieved by AMA1 element has been exploited to improve transformation frequencies and also for cloning purposes. Plasmids bearing AMA1 have been co-transformed along with libraries and also genomic DNA to clone genes by direct complementation. AMA1 based vectors also offer the advantage of providing an expression system free from position effects associated with integrative transformants and have been used for gene expression studies.

Linear plasmids with human telomeric ends allow extra-chromosomal maintenance in *A. nidulans* and also improve the efficiency of transformation. The search for chromosomal replicators in *A. nidulans* led to the discovery of two kinds of DNA elements — i) sequences that lead to vector rearrangement and multimerization and eventually result in chromosomal integration. Examples of such sequences are *ans1* and MATE elements; and ii) sequences that can support autonomous replication of a plasmid stably without vector rearrangements; AMA1 is an example. The increased transformation frequency achieved by AMA1 element has been exploited to improve transformation frequencies and also for cloning purposes. Plasmids bearing AMA1 have been co-transformed along with libraries and also genomic DNA to clone genes by direct complementation. AMA1 based vectors also offer the advantage of providing an expression system free from position effects associated with integrative transformants and have been used for gene expression studies.

**Table 3—Markers conferring inhibitor resistance**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Encoded function</th>
<th>Mode/ target of action</th>
<th>Species transformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>ben<em>A</em></td>
<td>benomyl-resistant β-tubulin</td>
<td>inhibits mitosis</td>
<td><em>A. nidulans</em>&lt;sup&gt;57&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>ble</em>b</td>
<td>phleomycin-binding protein</td>
<td>causes DNA scission</td>
<td><em>A. nidulans</em>&lt;sup&gt;60&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>hph</em>b</td>
<td>hygromycin B phosphotransferase</td>
<td>causes errors in protein translation</td>
<td><em>A. nidulans</em>&lt;sup&gt;61&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>oliC</em>a</td>
<td>oligomycin-resistant mitochondrial ATP synthase</td>
<td>inhibits ATP synthase</td>
<td><em>A. nidulans</em>&lt;sup&gt;67&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>ptrA</em>a</td>
<td>not known</td>
<td>thiamine antagonist</td>
<td><em>A. oryzae</em>&lt;sup&gt;69&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>sur</em>a</td>
<td>sulfonylurea-resistant acetlactate synthase</td>
<td>inhibits branched chain amino acid biosynthesis</td>
<td><em>M. grisea</em>&lt;sup&gt;71&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>bar</em>b</td>
<td>phosphinothricin acetyl transferase</td>
<td>inhibits glutamine synthetase</td>
<td><em>N. crassa</em>&lt;sup&gt;73&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*a* - Resistant alleles of fungal genes, *b* - bacterial genes expressed under fungal promoters, *c* - unpublished data from our group

An integrative vector typically has the following components: i) a marker for selection in the fungus; ii) bacterial plasmid sequences that enable selection and propagation of the plasmid in *E. coli*; and iii) the gene of interest that is to be introduced into the fungus. The vector on transformation gets integrated into the genome either by heterologous or homologous recombination. Illegitimate or homologous recombination becomes possible when there are extended lengths of sequence homology between the vector and the genome (Fig. 1). An increase in the length of homologous sequence in the vector enhances the frequency of homologous integration.

**Analysis of gene function**

Transformation opens up the possibility of several approaches to study the function and regulation of a gene. Potential fungal promoters can be analyzed by the use of reporter genes. The function of the gene itself may be investigated by studying the effects of its deletion or over-expression. Anachronic
expression of a gene can also provide valuable information about its function. Such approaches require the *in vitro* manipulation of a gene followed by its transformation into an appropriate recipient strain.

**Gene disruption or replacement**

Deletion or disruption of a gene requires homologous recombination of the introduced DNA with the genome. Integration by single crossover between the internal gene region and the homologous genomic region will ultimately lead to the formation of two incomplete copies of the target gene. Alternatively, a construct can be made in which the selectable marker is flanked by sequences of the gene to be disrupted. A double crossover between the incoming plasmid and the genome would result in disruption of the target gene (Fig. 1). Again, a gene replacement is achieved by a precise double crossover event. The efficiency of these processes is limited by the preponderance of illegitimate recombination in filamentous fungi. Considerable variability in the efficiency of targeting is seen from organism to organism and even with different targets within the same organism. For instance, it was found that targeting of the *niaD* locus was five times more efficient than that of the *amdS* locus in *A. nidulans*.

**Gene replacement strategies**

The ability to counter-select the mutant in two-way markers (Table 2) has been exploited to construct disruption cassettes that allow the selection of successive disruptions with the same marker. The pyrG blaster used in *A. fumigatus* consists of the pyrG gene flanked by a direct repeat encoding neomycin phosphotransferase. After disruption, recombination between the direct repeats leading to excision of the pyrG gene was selected in the presence of 5-FOA allowing the use of this marker for the next round of disruption.

High frequencies of homologous recombination can be achieved by increasing the length of homologous DNA flanking the marker. The Red{αβγ} system, which promotes recombination in *E. coli*, has been exploited to generate suitable constructs (disrupted cosmids) for targeted transformations in *A. nidulans* enabling gene disruption efficiencies of greater than 50 per cent.

Often the lack of suitable restriction sites poses problems in building the disruption construct. The GPS-1 system (Genome priming system kit, from New England Biolabs, Beverly) for *in vitro* transposon mutagenesis allows the random insertion of a selectable marker into the target gene. This eliminates the need for suitable restriction sites into which the marker can be inserted. The argB gene of *A. fumigatus* was disrupted *in vitro* by the pyr4 gene of *N. crassa* using this method. The split-marker deletion method, based on PCR, also minimizes the efforts involved in generating deletion constructs. However, this has so far been employed only in *Cochliobolus heterostrophus* and *Gibberella zeae*.

The use of anti-sense RNA is fast becoming an alternative to gene disruption. The silencing of *pepB* gene of *A. awamori* to study its effect on thaumatin production and characterization of *pgmB* in *A. nidulans* are two such examples. This technique could be valuable in organisms where homologous recombination is inefficient. The use of anti-sense RNA shuts off gene expression only partially, thereby allowing the functional characterization of essential genes as well — something that is not possible with gene disruption. For industrial applications, however, when gene functions are knocked out with the objective of strain improvement, it is often preferable to delete/disrupt the gene.

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**Fig. 1**—Patterns of integrative transformation observed in fungi (Adapted from ref. 82). (A): Homologous (single cross over/linked insertion); (B): heterologous (single cross over/unlinked insertion); and (C): homologous (double cross over/gene replacement). [DNA is illustrated as horizontal bars and is distinguished as chromosomal DNA (hatched bars) or vector DNA (open bars). Specific alleles are shown as boxes labeled m' (thatched box) and M' (black box). DNA length has not been represented to scale]
Promoter analysis

The study of 5’ sequences required for gene regulation can readily be studied by the use of heterologous reporter genes. The reporter gene activities can be conveniently assayed to reflect the activity of the promoters and/or associated regulatory elements. The most commonly used reporter is the lacZ gene encoding a β-galactosidase. A mini-promoter fused to this reporter is also available for determining the regulatory effects of inserted sequences.

The uidA gene encoding β-glucuronidase is another commonly used reporter. Analysis of enolase promoter of A. oryzae using uidA and gpd promoter of A. nidulans using lacZ are examples of the use of reporter genes. More recently, the glucose oxidase gene (goxA) of A. niger has been adapted for use as a reporter in T. atroviride, T. harzianum and also in A. aculeatus. The green fluorescent protein has also been used as a reporter to monitor gene expression and for promoter studies in Aspergilli. This reporter allows the in vivo observation of its expression and gfp-tagged proteins have been employed for specific organelle labeling, protein localization and gene regulation analysis.

Expression studies

The unmatched capacities of fungi to secrete enzymes make them ideal candidates to produce homologous/heterologous proteins at commercially viable levels. A major feature of an efficient expression system is the use of strong promoters. Some examples include the use of alcA and gpd promoters of A. nidulans and the glaA promoter of A. niger. An earnest hunt for other strong promoters is on to achieve expression of exogenous genes/gene fusions and promoter swaps in Aspergilli. A patent employing the A. awamori gdhA gene promoter to drive expression of foreign genes in filamentous fungi is reported. Genes under the control of weak promoters may be fused to strong constitutive promoters, if uncontrolled over-expression is desired. For regulated expression, inducible promoters, such as alcA or bphA can be used instead.

Over-expression is often used as a tool to understand gene function. For example, the roles of NUDE and NUDF proteins of A. nidulans were probed by over-expression. The over-expression of cita (encodes citrate synthase) in A. niger provided insights into points of flux control during citric acid fermentation. Inducible promoters allow the expression of genes under conditions when they are not normally expressed. This has been used to gain information about the function of developmental genes. The role of br1A, abA, and wetA genes in asexual sporulation of A. nidulans was analyzed by this approach using the inducible alcA promoter.

High-level expression of genes can be achieved by the introduction of multiple copies by transformation. Although increased copy number often leads to increased levels of the introduced gene product/activity, a one-to-one correlation is not usually observed. This is possibly due to – a) the effect of the site of integration on expression; and b) the limitations of post translational processing. Over-expression strategies are employed in the industry to obtain high yields of desired enzymes or other metabolites. Certain Aspergilli like A. niger, A. oryzae and A. awamori are also favourite hosts for heterologous protein production. This is mainly because of their great secretory potential and their ability to process other fungal, and even mammalian, introns and glycosylation signals. In general, the yield of mammalian proteins is much lower as compared to heterologous fungal proteins. Some of the mammalian proteins that have been successfully expressed in Aspergilli include human lactoferrin, chymosin, human interferon α-2, human tissue plasminogen activator and human interleukin-6.

Apart from their commercial importance as producers of several enzymes and secondary metabolites, Aspergilli also serve as model organisms for study of eukaryotic processes. The disruption of areA gene to deduce its role in nitrogen metabolite repression and that of aflJ gene to understand its function in aflatoxin biosynthesis are only two illustrative examples of the use of gene disruption in analysis of gene function.

Aspergillus niger: An industry paradigm

Exploitation of Aspergilli began early with the use of A. niger (citric acid) and A. oryzae (taka-amylase) in the fermentation industry. Although several other fungi are currently in use, A. niger remains a favourite. This may be attributed to its GRAS status, great secretory potential and the wealth of information accrued on its fermentation. The published literature, however, has been partial to A. nidulans — the most ‘academic’ of the Aspergillus species — owing to its well-defined genetics. Indeed most molecular tools
initially developed for \emph{A. nidulans} were used in \emph{A. niger}. A dearth of direct data on \emph{A. niger} may just be notional as much of the research on this species is confined to commercial establishments (http://www.dsm.com), where such knowledge gained is proprietary. Nonetheless, with the advent of transformation systems for \emph{A. niger}, analysis of gene function and regulatory mechanisms are now feasible.

An updated compendium of gene function analyses in \emph{A. niger}, through gene disruptions (Table 4) and protein over-expression studies (Table 5), is presented here. The genetic transformation strategy is also exploited in the industry for strain improvement. For instance, the \emph{oah} gene (Table 4) was disrupted to eliminate formation of oxalate, an undesirable by-product during acidogenesis\textsuperscript{116}. Similarly, the deletion of \emph{goxA} gene greatly reduced the level of sodium gluconate, thereby minimizing the need for expensive waste handling\textsuperscript{117}.

\textbf{Envoi}

In this era of complete genome sequencing, Aspergilli have not lagged behind. The entire genome sequence for \emph{A. nidulans}, the model fungus, is now available. Sequencing of \emph{A. fumigatus} is nearing completion. Some parts of the \emph{A. terreus} and \emph{A. parasiticus} genomes have also been sequenced. These genome databases may be accessed online (http://www.ncbi.nih.gov/Entrez/index.html). The sequencing of \emph{A. niger} genome is complete (http://www.dsm.com/en_US/html/dfs/genomics_access_arr.htm), but the data is not freely available. An efficient genetic transformation system is really a prerequisite for functional analysis of genes when sequence data is forthcoming. Significant strides have been made in the transformation of Aspergilli in terms of protocols for DNA entry and markers for selection. Some aspects of this endeavor still remain more of an art than science. Targeted genetic changes, gene disruption and gene replacement in filamentous fungi are currently possible. However a need for extensive screening from often a difficult background growth remains a challenge. Given the enormous interest in their exciting life cycles and a longstanding industrial value, simple and robust protocols to obtain well defined fungal transformants will continue to emerge.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Table 4—Genes disrupted in} \emph{A. niger} & \textbf{Encoded function} & \textbf{Marker} \\
\hline
\textbf{Disrupted gene} & \textbf{Encoded function} & \textbf{Marker} \\
\hline
\textit{pepA}, \textit{pepB}, \textit{pepE} & acid proteases & \textit{argB}_{\text{nidulans}} \textsuperscript{113} \\
\textit{pacC} & \textit{pH}-dependent regulatory gene & \textit{argB}_{\text{nidulans}} \textsuperscript{114} \\
\textit{pkaR} & regulatory subunit of cAMP-dependent protein kinase & \textit{argB}_{\text{nidulans}} \textsuperscript{115} \\
\textit{areA} & transcriptional regulator & \textit{argB}_{\text{niger}} \textsuperscript{111} \\
\textit{argB} & ornithine transcarbamylase & \textit{pyrG}_{\text{niger}} \textsuperscript{31} \\
\textit{oah} & oxaloacetate hydrolase & \textit{pyrG}_{\text{niger}} \textsuperscript{116} \\
\textit{trpC} & trp biosynthesis (multifunctional enzyme) & \textit{pyrG}_{\text{niger}} \textsuperscript{36} \\
\textit{gaxA} & glucose oxidase & \textit{pyrG}_{\text{niger}} \textsuperscript{117} \\
\textit{pkaC} & catalytic subunit of cAMP-dependent protein kinase & \textit{pyrG}_{\text{niger}} \textsuperscript{115} \\
\textit{srgA} & small GTPase & \textit{pyrG}_{\text{oryzae}} \textsuperscript{118} \\
\textit{nicB} & nicotine utilization & \textit{pyrG}_{\text{oryzae}} \textsuperscript{78} \\
\textit{aglA} & \alpha\text{-galactosidase} & \textit{pyrG}_{\text{oryzae}} \textsuperscript{119} \\
\textit{kexB} & Endoprotease & \textit{pyrG}_{\text{oryzae}} \textsuperscript{120} \\
\textit{nuo51} & NADH:ubiquinone oxidoreductase & \textit{Hph} \textsuperscript{121} \\
\textit{tpsA} & trehalose-6-phosphate synthase & \textit{hph} \textsuperscript{85,122} \\
\textit{cpcA} & transcriptional regulator & \textit{ble} \textsuperscript{123} \\
\hline
\textbf{a} - The subscript represents the organism from which the marker gene was derived
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Table 5—Over-expression studies in} \emph{A. niger} & \textbf{Encoded function} & \textbf{Process studied} \\
\hline
\textbf{Promoter used} & \textbf{Gene expressed} & \textbf{Process studied} \\
\hline
\textit{pfk} & \textit{pfk} & phosphofructokinase & citric acid production\textsuperscript{124} \\
\textit{pki} & \textit{pki} & pyruvate kinase & citric acid production\textsuperscript{106} \\
\textit{citA} & \textit{citA} & citrate synthase & NADPH production\textsuperscript{125} \\
\textit{gsdA} & \textit{gsdA} & glucose-6-phosphate dehydrogenase & unfolded protein response\textsuperscript{100} \\
\textit{gpdA} & \textit{hacA} & transcriptional regulator & heterologous manganese peroxide production\textsuperscript{126} \\
\textit{glaA} & \textit{clxA} & calnexin, chaperone & cyclosporin A sensitivity\textsuperscript{127} \\
\textit{cypA} & \textit{cypA} & cyclophilin & protein secretion\textsuperscript{128} \\
\textit{glaA} & \textit{pdiA} & protein disulfide isomerase A & fungal morphogenesis\textsuperscript{129} \\
\textit{pkaC} & \textit{pkaC} & catalytic subunit of cAMP-dependent protein kinase & \\
\hline
\end{tabular}
\end{table}
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References
3. Case M E, Schweizer M, Kushner S R & Giles N H (1979) Proc Natl Acad Sci (USA) 76, 5259-5263


