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Three Decades of Fungal Transformation

Key Concepts and Applications

Vianey Olmedo-Monfil, Carlos Cortés-Penagos, and Alfredo Herrera-Estrella

Summary

Filamentous fungi include a large, heterogeneous group of heterotrophic organisms with profound influence in human activities. In spite of their economic and scientific relevance, little information is available at the molecular level about their biology. The development of genetic transformation protocols has contributed greatly to the molecular dissection of fungal behavior. The use of this approach in combination with large-scale genome sequencing projects has now provided the basis for gaining insight into the function of fungal genes. This chapter reviews the technology of the transformation of filamentous fungi. The protocols for gene transference by protoplasting/PEG, LiAc, electroporation, biolistics and *A. tumefaciens* are described, and possible mechanisms for transformation are discussed. A brief description of previously reported selection systems is also included. The application of transforming protocols concerning the study of gene function and manipulation are discussed in relation to some fungal species.

Key Words: Gene transference; fungal transformation; selection markers; gene inactivation.

1. Introduction

The potential of molecular genetics has been extensively exploited in the study and manipulation of microorganisms. This has led to the development of new technologies applied to both eukaryotic and prokaryotic species. In this sense, genetic transformation has played a significant role not only in our understanding of life, but also in the enhancement of particular traits. Among microorganisms, the budding yeast *Saccharomyces cerevisiae* has contributed enormously to the improvement of genetic tools

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including the transference of genetic material. Since the first report of yeast transformation in 1978 (***1***), several transformation systems for many other fungi have been reported.

Filamentous fungi, as a group, exhibit unique characteristics due to their cellular organization and their lifestyle. The relative small genome size and the low frequency of redundant DNA have made these organisms very attractive for molecular genetic research. From the Ascomycota, *Neurospora crassa* and *Aspergillus nidulans* have been especially valuable as model organisms, mainly because it is possible to combine molecular approaches with classical genetics. Nowadays, numerous fungal species have a deep impact in human activities. Clearly, the future will see an expansion of the number of fungal species and their products that are commercially exploited. Consequently, molecular genetic technology will play a major role in the adjustment and improvement of products and processes. The application of molecular genetics to fungi has been quite slow because of the need to develop protoplasting techniques, transformation systems, and appropriate vectors. These are still the most important restrictions for some fungal species. Genetic transformation was first achieved in *N. crassa* in 1973 (***2***); this report is now recognized as the earliest for fungi. Traditionally, fungal transformation has relied largely on variations of the same basic protocol. The extent to which this technology can be applied is in direct proportion to the amount of fundamental knowledge of the species concerned.

The information contained in this chapter is intended to be a complementary resource for fungal genomics experts and nonexperts alike, as it puts together recent advances in fungal transformation systems. It includes examination of traditional transformation protocols highlighting the application for relevant fungal species.

2. Transformation Methods

All DNA transference experiments have two separate components: a method for transferring the genetic material to the recipient cells and a selection method for isolating recipient cells that have received the DNA. Most transference methods result in a considerable number of cells receiving the desired material; consequently, very powerful selection techniques are required and frequently selection is the limiting component during genetic transformation. The majority of fungal species are susceptible to transformation. At present, several transformation methods have been described. Most of them have been modified in order to make them easier and more precise for each fungal strain. It is worth noting that the main obstacle in any transformation system is the fungal cell wall.

2.1. Protoplasts/PEG

Generally, a transformation procedure starts with the generation of osmotically sensitive cells (OSCs) or protoplasts. Yeast cells, germinating conidia, or young mycelia are treated with hydrolytic enzyme mixtures in order to remove the fungal cell wall. Accordingly, the enzyme cocktails are a complex combination of β1,3-gluconases and chitinases as predominant activities. In the market there are some commercial products

that have demonstrated to be highly effective in protoplast generation. Helicase and Glusulase are the commercial names of the enzymatic mixture obtained from nail gut; they have been used in protoplast generation of *Cryptoccocus neoformans* and *S. cerevisiae* (***3***) and in the filamentous fungi *N. crassa* (***4***). Zymolase T100 contains concentrated enzymes produced by *Artrobacter luteus.* This product was found to be useful in *S. cerevisiae* generating protoplasts (***5***). Of all commercially available products, Novozyme 234 has been the most popular when protoplasts are required either from yeast or filamentous fungi (***6–8***). Novozyme is composed of enzymes secreted by *Trichoderma atroviridae*. Currently, this enzymatic mixture is commercially available with a different name, Glucanex.

The protoplast-obtaining process could be tedious, mainly because cell-wall-free fungal cells are osmotically sensitive and therefore must be handled with care. Protoplasts should be protected at all times by solutions providing osmotic support. Therefore, solutions containing chlorides, magnesium sulfate, mannitol, sorbitol, or sucrose are normally used at concentrations ranging from 0.8 to 1.2 *M* (***9,10***). Once protoplasts are properly formed and protected, they are incubated in a solution mix that includes polyethylene glycol, calcium chloride, and the transforming DNA. Calcium ions could participate in the formation of pores or channels in the cell membrane, which allow internalization of exogenous DNA. On the other hand, PEG promotes cell agglomeration to make DNA uptake easier. The relevance of PEG during transformation has been proven to be crucial (***11***). After calcium-PEG-DNA treatment, protoplasts are transferred to selective media that allow cell wall regeneration and the proper isolation of transgenic lines.

2.2. Lithium Acetate

In theory, it is possible to generate protoplasts from any fungal species but in particular cases this protocol does not work. If it is difficult to obtain protoplasts or if their regeneration is not possible, transformation of intact fungal cells offers an alternative method for those species. It is possible to induce cell permeability by using high concentrations of lithium acetate in combination with PEG. This procedure was initially developed for *S. cerevisiae* transformation (***12***) and has been successfully applied in *N. crassa* (***13***), *Coprinus lagopus* (***14***), and *Ustilago violacea* (***15***). However, the use of this technique has been so far limited to a few species.

2.3. Electroporation

Application of an electric pulse offers an additional alternative to induce transient cell permeability and, consequently, DNA uptake. When cells are exposed to an electric field, polarization of structural components of the cell membrane occurs. If the applied voltage overcomes a threshold level, pores are formed in specific areas, allowing diffusion of molecules (***16***). Electroporation methodology has been applied in both intact cells and protoplasts of different species, from bacteria to mammalian cells, including filamentous fungi (***17–20***). During DNA integration mediated by *in vivo* action of restriction enzymes (REMI), electroporation permits uptake of plasmids together with the selected restriction enzyme (***21***).

2.4. Biolistics

Biolistics involves DNA transference by using microparticles of colloidal gold or tungsten as a vehicle. The DNA-covered particles are shot to the sample at high speed through a special device. Bombardment methodology was originally developed for plant tissues (***22,23***). Subsequently, it was adapted for the transformation of a wide range of cells, such as bacteria (***24***), yeast (***25***), fungal conidia (***26***), and animal cells (***27***). Furthermore, successful chloroplast and mitochondria transformation has been reported by using biolistic protocols (***28–30***). Depending on the cell type, various parameters must be considered before using the biolistic-transformation procedure. Transforming-DNA quantity and microparticle speed are the most important. The required pressure to penetrate without causing irreversible cellular damage is different if tissues, cell cultures, or conidia are bombarded. The required equipment is the limiting factor for the application of biolistic-based transformation protocols. However, it is possible to construct gun devices at low cost if suitable technical knowledge is available.

2.5. Agrobacterium tumefaciens *Mediated Transformation*

Protocols have recently been applied for filamentous fungi that take advantage of the infective strategy of *A. tumefaciens*. This Gram-negative, soil-living bacterium is capable of producing tumors in plants. Tumors are developed as a consequence of the expression of genes transferred from bacteria into the infected plant. This event is an interesting transformation mechanism that occurs in nature. Bacteria transfers T-DNA contained in the Ti (tumor-inducing) plasmid into the genome of plant cells by a conjugation-like process. DNA transfer depends on the expression of genes contained in the Ti plasmid, *vir*-genes, involved in virulence of *A. tumefaciens*. Some of these genes encode proteins, essential for the formation of the T-complex, the actual form of the transferred DNA. The *vir* genes are involved in the formation of the conjugation-like tube or sensing the host. Signaling involves phenolic compounds produced by wounded plants, such as acetosyringone, which induce *vir* gene expression. The T-DNA is flanked by two short direct repeats (25 bp), from which the right border is essential for the process. In nature, the T-DNA contains genes leading to the production of opines, compounds that provide the main nitrogen and carbon sources for *Agrobacterium* (***31***). Thus, T-DNA integration into the plant genome represents an ecological advantage to the bacteria. At the beginning, application of this technology was restricted to dicotyledonous species (***32–35***), natural hosts of the bacteria. Subsequently, protocols were adapted to monocotyledonous plants (***36,37***) and fungi, including yeast (***38–41***). The vehicle for transforming DNA consists of modified versions of the Ti plasmid, where the desired DNA replaces the complete region contained between the T-DNA borders. One of the most important characteristics of this methodology is that it allows random integration of a single copy of the T-DNA. This represents a useful tool in the generation of banks of unique mutants. Moreover, by using PCR technology, it is possible to identify the nucleotide sequence affected by integration of exogenous DNA in the transformed organism (***42***).

3. Selection Markers

Independent of the transformation methodology used, transgenic line selection is one of the most important limitations. In the first event of transformation in the early 1970s, the fungal model selected contained a mutant genetic background. They were auxotrophic for different compounds such as inositol, uracil, and leucin, so screening for transgenic lines was based on the reversion toward wild-type phenotype. There are many well-characterized auxotrophic strains, but they are limited to a few species (*S. cerevisiae*, *N. crassa*, and *A. nidulans*). In all cases, selection markers consist of those genes encoding products that allow transition from mutant to prototrophic phenotype. Thus, the use of selection procedures based on complementation of auxotrophic strains seems to be difficult for other fungal species. Particular auxotrophic mutants could be isolated by positive selection using chemical compounds. Only mutant strains affected in *pyrG* (*A. nidulans*), *pyr4* (*N. crassa*) (***43***), or *ura3* (*S. cerevisiae*) (***44***) genes and consequently, lacking orotidine 5'-monophosphate carboxylase activity are capable to growth in culture media containing a toxic analog of the fluoroorotic acid. In *A. nidulans*, the use of chlorate allows selection of strains affected in nitrate reductase activity, encoded by the *niaD* gene. The isolated mutants are incapable of using nitrate as a sole nitrogen source (***45***). Selective pressure exerted by selenate or fluoroacetate present in culture media is useful in the selection of mutants without ATP sulfurylase (***46***) or acetyl CoA synthetase (***47***) activities, respectively. Finally, in *A. nidulans*, by using fluoracetamide, a toxic analog of acetamide, strains affected in the *amdS* gene may be obtained that are incapable of metabolizing acetamide as a sole nitrogen or carbon source (***48***). Most fungal species cannot metabolize acetamide. This characteristic makes the *amdS* gene a good heterologous selection marker (***49***). Nowadays, many other genes are available as dominant selection markers. They confer resistance to different chemical drugs. **Table 1** shows some of the genes commonly used as heterologous selection markers in filamentous fungi.

The main advantage in the use of selection markers that confer drug resistance is that it is not necessary to have prior knowledge of the genotype of the fungus subjected to transformation. On the other hand, it demands the measurement of the dosage of the drug required to completely inhibit the growth of the wild-type population. In some cases, fungi show natural drug resistance and consequently, high drug concentration is required. However, this kind of selection markers represents the best choice when auxotrophic strains are not available.

4. Fate of DNA

To obtain stable transgenic lines it is not only necessary that DNA gets into the nuclei, but the DNA also must remain unchanged in the genome. This means that the DNA introduced into the transformed cells has to be inherited to the daughter cells after mitotic and/or meiotic divisions. In order to achieve this condition, there are two alternatives: (1) exogenous DNA should replicate in an autonomous way, so vectors containing an origin of replication are required; or (2) stable integration of the exogenous DNA into the chromosomes should occur.

Table 1
Dominant Selectable Markers Commonly Used for Transformation in Filamentous Fungi

Marker gene	Source	Resistance	Recipient organism
Hph	*E. coli*	Hygromicin B	*A. nidulans* (***50***) *Magnaporthe grisea* (***51***) *Arthrobotrys oligospora* (***52***) *Trichoderma spp* (***8***)
NphII	Bacterial Tn5	Kanamycin, G418	*Phanerochaete chrysosporium* (***53***) Yeast (***54***)
β-*tubulin*; *tub2*	*N. crassa* *U. maydis* *Trichoderma viride*	Benomyl	*N. crassa* (***55***) *U. maydis* (***56***) *T. viride* (***57***)
OliCR	*A. niger*	Oligomycin	*A. niger* (***58***) *P. chrysogenum* (***59***)
ble; sh-ble	Bacterial Tn5 *S. hygroscopicus*	Bleomycin, phleomycin	*N. crassa*; *A. nidulans* (***60***) *P.chrysogenum* (***61***) *U. maydis* (***56***)
SulII	Enterobacteria	Sulfonamide	*Penicillium crysogenum* (***62***)
Bar	*S. hygroscopicus*	Glufosinate ammonium (Bialaphos)	*Metharhizium anisopliae* (***63***) *Pleurotus ostreatus* (***64***) *N. crassa* (***65***)
CbxR	*U. maydis*	Carboxin	*U. maydis* (***66***)

4.1. Autonomously Replicating Vectors

These vectors are commonly derived from mitochondrial plasmids or contain autonomously replicating sequences (ARSs). Vectors with ARSs promote a high transformation frequency when used in *S. cerevisiae*; for this reason they represent powerful tools in the isolation and cloning of yeast genes (***67***). However, ARSs arising from yeast do not seem to work in filamentous fungi. Many efforts have been made in order to generate truly autonomously replicating vectors in fungi. In some cases, it has been attempted to introduce replicons from fungal plasmids into transformation vectors, but only a few have been reported successful (***68–70***).

Another approach for vector construction is the use of ARSs or sequences that confer a similar function as reported by Balance and Tuner in 1985 (***71***). They made use of three fungal models in order to isolate an *A. nidulans* origin of replication. Hybrid vectors containing genomic *Aspergillus* DNA fragments and the *N. crassa pyr4* gene,

which is poorly expressed in *Saccharomyces*, were constructed. The yeast was transformed with the generated plasmids and unstable transgenic strains that lost the plasmid under nonselective conditions were considered as strains containing autonomously replicating plasmids. The rescued vectors were used to transform *A. nidulans* and, only one, namely ARS1, increased the transformation frequency 50- to 100-fold as compared with the original plasmid lacking this sequence. Although the isolated sequence behaved like an autonomously replicating sequence in *S. cerevisiae*, it was not functional in *Aspergillus*. Thus, sequences may behave like ARS in yeast but are nonfunctional in the organism from which they were isolated. Nevertheless investigations are still aiming at isolating true ARSs in filamentous fungi.

Tsukuda et al. (***72***) reported a 389-bp sequence arising from *Ustilago maydis* similar to yeast ARSs previously reported. The use of this sequence in *U. maydis* generated high plasmid copy number and mitotic instability in the transgenic lines. Van Heeswijck (***73***) and Roncero et al. (***74***) demonstrated the presence of possible ARSs sequences in the *Mucor circinelloides* genome, by transforming a *leu*$^-$ mutant. They obtained a high number of mitotically unstable transgenic strains and it was possible to reisolate the transforming DNA, which was present as an extrachromosomal element. Gems et al. (***75***) reported the AMA-1 sequence from *A. nidulans*, which could be an origin of replication. Transformation using AMA-1-containing plasmids generated high frequencies of unstable transgenic lines. This sequence has been used in *Penicillium crysogenum* (***76***), *Gaeumannomyces graminis* (***77***), and other *Aspergilli*, such as *A.niger* and *A. orizae* (***75***). In spite of all efforts attempting to obtain autonomously replicating systems, neither of the reports has been conclusive. In part, this may be due to the limited occurrence of ARSs among filamentous fungi. An alternative explanation may be that the plasmids used with ARSs did not contain telomeric and centromeric sequences of adequate size, so they could not behave as true chromosomes (***67***). Furthermore, centromeric sequences that provide stability to the replicating vectors in filamentous fungi have not been isolated. In relation to telomeric sequences, it has recently been described that some fungi are capable of adding telomeric repeats to exogenous DNA. This phenomenon seems to be widespread, because it has been recorded in unrelated species, such as the pathogenic yeast *Cryptoccocus neoformans* and filamentous fungi such as *Fusarium oxysporum* (***78***) and *Pestalotiopsis microspora* (***79***). Moreover, telomeric sequences from humans (***80***) or *F. oxysporum* (***78***) have been included in plasmids used for transformation of heterologous systems, showing autonomous replication. Even though at the moment there are not many reliable autonomously replicating vectors for fungal transformation, their development seems to be quite close.

4.2. Integration of DNA Into Chromosomes

Depending on the purpose of obtaining a particular transgenic line, it may be more adequate to obtain strains with the transformed DNA integrated into their genome, avoiding the need of maintaining selective pressure or strains in which DNA may be lost upon removal of the pressure. However, in most cases stable integration of transforming DNA into chromosomes is desired (i.e., the generation of banks of mutants). Consequently, the majority of plasmids used in transformation of filamentous fungi do

not contain an origin of replication. These vectors integrate into the genome at either homologous or heterologous sequences.

Integrative events are separated into three main classes (***1***). Class I includes integration processes that occurred into a region of homology within the genome; this is known as homologous recombination. The Class II group's integration events have opposite characteristics to those already mentioned; it includes those where there is not apparent homology between the transforming DNA and its integration site into the recipient genome. This integration type is known as heterologous recombination. The mechanism through which heterologous recombination takes place is not well understood. Apparently, short sequences with fortuitous identity are enough to allow DNA integration (***81***). Class III contains those integration events where transforming and endogenous DNA interact and only DNA with high homology is integrated, replacing part of the recipient genome. Thus, it is not possible to detect plasmid sequences in the transformed cells. This process is known as double homologous recombination. It is a useful tool that allows direct integration toward particular sequences generating gene conversion. In some cases, the integration event gives mitotic stability to the transforming DNA but it is still meiotically unstable, just as has been observed in *N. crassa* (***82***) and *A. nidulans* (***83***). In the case of fungi lacking a sexual phase, this instability does not represent any problem.

Integration of multiple plasmid copies is a characteristic commonly observed in transgenic lines. Tandem integrations could be generated by two mechanisms: free plasmids form circular oligomers by homologous recombination and then all copies are integrated into the genome. Alternatively, single plasmid increases the homology with copies of the plasmid making a second integration event easier (***10***).

In order to increase the frequency of transformation, some protocols recommend the use of lineal plasmids. Nevertheless, the results obtained by using them are contrasting, depending on the fungal species and the method of transformation. In the case of *S. cerevisiae* and *U. maydis*, lineal DNA gives higher efficiencies of transformation than circular plasmid (***84,85***). In *N. crassa* the results are similar whether lineal or circular DNA is used (***86***). It is worth noting that in some fungal species transformation with linearized versions of the plasmid increases the frequency of homologous integration and consequently gene conversion, which in some cases is desirable (***87,88***).

5. Applications of Transformation

5.1. Gene Inactivation

Classical fungal genetic approaches for gene identification rely on inactivation of a gene leading to a recognizable phenotype. This approach continues to be extremely useful, yielding mutations that result in obvious phenotypes reflecting the role of the corresponding gene. The process of identifying a gene's function subsequent to establishing its DNA sequence is known as reverse genetics, for which a number of methods have been explored. These methods include homologous recombination, insertional mutagenesis, and gene silencing.

High-throughput sequencing projects are providing large quantities of genomic and

EST sequences for a considerable number of fungal species. This newly compiled database confronts the scientific community with the problem of understanding the function of thousands of previously unknown genes. Comparative analysis using the gene-bank database can designate putative functions by association to earlier identified genes, but only the characterization of mutants can offer definitive answers. In most fungal systems, knockout mutations are now routinely obtained by promoting the homologous recombination of null gene constructs with the genomic wild-type sequence by using the appropriate transformation system. Gene knockouts, or null mutations, are important because they provide a straight practice to determining the function of a gene product. Gene inactivation by homologous recombination has been used in all major groups of fungi, including different zygomycetes, ascomycetes, and basidiomycetes, in addition to several deutermycetes. However, these processes cannot be applied as such for high-throughput mutagenesis.

Insertional mutagenesis is an alternative method for large-scale reverse genetics and is based on the insertion of foreign DNA into the gene of interest by using mobile elements of various origins. Individual clones carrying a particular insertional-DNA mutation can then be easily identified by a polymerase chain reaction (PCR) method (***89***). *A. tumefaciens* T-DNA and transposable elements appear to be the most suitable methods for generating insertional mutations in filamentous fungi (***90,91***). Heterologous transposition of retrotransposons *impala* and MAGGY from *F. oxysporum* and *M. grisea*, respectively, have been reported in different fungal hosts (***92–94***). Methods for insertional mutagenesis were extended to include restriction-enzyme-mediated integration (REMI). This technique, originally described in *S. cerevisiae* (***21***), involves the addition to the transformation reaction of an active restriction enzyme that efficiently targets the transforming DNA to corresponding restriction site. In fungi, REMI can be used in combination with both protoplast- (***95***) and electroporation-mediated transformation (***96***). The REMI technique has been extensively used among plant pathogens, that including *C. heterostrophus* (***95***), *U maydis* (***97***), *M. grisea* (***98***), and *C. graminicola* (***99***).

Techniques established on posttranscriptional gene silencing (PTGS) appear promising for the study of fungal gene function. PTGS based on the expression of an antisense RNA molecule has been successfully used in *C. albicans* (***100***), *C. neoformans* (***101***), and *Aspergillus* species (***102***). Similarly, when a small double-stranded RNA corresponding to a sense sequence of an endogenous mRNA is introduced into a cell, the cognate mRNA is degraded and the gene silenced. This type of PTGS, called RNA interference (RNAi), was first discovered in *C. elegans* (***103***) and has been recently used in the specific inhibition of genes involved in capsule synthesis in *C. neoformans* (***104***). Despite these promising data, gene inactivation through such methods has not been extensively tested in fungi, so the interpretation is sometimes complex.

5.2. Gene Regulation

The use of gene fusions with a reporter gene in combination with the proper transformation system allows gene activity to be monitored. This procedure was first used in bacteria 24 yr ago (***105***). In this way genes could be recognized based on their pattern of expression over time or under a selected condition. Several reporter genes have been

successfully applied in fungi (***106–110***). The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has proven to be a powerful tool in fungal genetic transformation studies, although modifications to the original sequence were required to obtain GFP versions that fluoresce proficiently in fungal cells (***111***). GFP-expressing transformants of *U. maydis*, *C. albicans*, and *T. atroviride* have been evaluated *in situ* during interaction with their respective hosts (***111–114***).

5.3. Gene Improvement

Genetic transformation seems to be an essential step by which useful traits are engineered into fungi. Species belonging to the genus *Trichoderma* (***115***), *Aspergillus* (***116***) and *Penicillium* (***117***) have been studied extensively in order to understand and manipulate enzyme production and secondary metabolism. Thus, recombinant DNA technologies could be successfully applied to most commercially useful proteins. Yeast and some filamentous fungi are already well established in fermentation procedures and are able to secrete glycosylated and modified proteins to provide the biologically active product (***118,119***).

6. Conclusions

Several powerful methods for studying fungal biology have been developed in recent years, many of which have been used in combination with transformation systems. Technical improvements to basic transformation protocols, in particular selection markers, have allowed the manipulation of a significant number of fungal species. Each of the transformation systems discussed in this chapter has its own advantages and disadvantages, but most of them are potentially suitable for any given species. No general rule could be applied for the transformation efficiency, although factors such as transformation method, vector, host strain, and selection system might influence it.

In the near future, the sequences of numerous genes will be available; consequently the assignment of their biological function is one of the most challenging goals in fungal biology. It is likely that genetic transformation will continue playing a major role in the next phase of fungal genomics—determining gene function.

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