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Heterologous Protein Production by Filamentous Fungi

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Introduction

Filamentous fungi have found application in many technologies. These include the production of antibiotics, enzymes, organic acids, foods and beverages, and polysaccharides. Some species of filamentous fungi are also used in agriculture as biocontrol agents. Genetic manipulation offers the potential for rational strain improvement and for the introduction of new characteristics into existing strains. The ability to manipulate the genomes of filamentous fungi has progressed from being limited to certain laboratory strains of *Neurospora* and *Aspergillus* to encompass a number of commercially important species. Thus, there is considerable opportunity which must be assessed in the light of current understanding of the molecular genetics of filamentous fungi and the commercial reality of the process and the market. This review concentrates on the molecular aspects of heterologous gene expression in filamentous fungi and the production of heterologous proteins.

The attractions of the filamentous fungi as hosts for heterologous protein production are built upon the capacity to transform genetically a number of different species (Esser and Mohr, 1986; Fincham, 1989; Timberlake and Marshall, 1989) and the prodigious abilities of some species to secrete substantial quantities of homologous protein. Also, the enzyme industry has developed the technology for growth of filamentous fungi and for downstream processing. The range of commercially available fungal enzymes is broad, and they are produced from a number of different species (*Table 1*). These enzymes are homologous gene products and the enzyme preparations are available at varying levels of homogeneity. The major fungal enzyme, in terms of bulk, is glucoamylase (amyloglucosidase) (EC 3.2.1.3) and this

Abbreviations: ARS, autonomously replicating sequences; ER, endoplasmic reticulum; MAR, matrix attachment region; NMR, nuclear magnetic resonance; PEG, polyethylene glycol; RIP, repeat-induced point mutation; *tsp*, transcriptional start point; YAC, yeast artificial chromosome.

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enzyme exemplifies the high secreted levels possible: over 20 g l^{-1} in culture media of *Aspergillus niger* (van Brunt, 1986; Ward, 1989a). Such levels are not restricted to this system because equally high levels of cellulase are attainable in cultures of *Trichoderma reesei* (Pourquié *et al.*, 1988). The total world-wide market for industrial enzymes in 1990 was estimated to be *c.* $US1500m (Campbell-Platt and Cook, 1989). These enzymes are used primarily in the food industry (Cheetham, 1985) and fungal enzymes constitute a substantial part of the total enzyme tonnage and value.

Expression of heterologous genes might be desired for the purposes of manipulating the characteristics of the host or for the production of a heterologous target protein. There are a number of examples for each case (van den Hondel, Punt and van Gorcom, 1991). It is apparent, however, that when compared with the successful commercial production of homologous fungal enzymes, equally successful production of heterologous proteins from filamentous fungi has not been achieved. Indeed, it may seem unrealistic to expect a host fungus to express and secrete a heterologous protein with the same ease as a homologous protein. Yields of heterologous proteins might therefore be expected to be lower than the levels achieved with homologous proteins; analysis of the reasons for this difference might be expected to enable some redress. Yield is not the only consideration in determining the success of heterologous protein production although, in comparison with some other host species, filamentous fungi perform very well in permitting high yields of some target proteins, particularly if the gene is derived from another fungus. Another important factor is authenticity of the protein, for example, in its structure (including post-translational modifications) and function. Process considerations determine the financial success of a commercial operation, so the ease of manipulations to purify the target protein to the

Table 1. Homologous enzymes from filamentous fungi (adapted from Godfrey and Reichelt, 1983; Cheetham, 1985)

Enzyme	Species	Uses of enzyme
α- and β-Amylase	*Aspergillus oryzae*, *Aspergillus niger*	Starch liquefaction
Amyloglucosidase (glucoamylase)	*Aspergillus oryzae*, *Aspergillus niger*	Glucose syrup production
Cellulase	*Trichoderma reesei*, *Aspergillus niger*, *A.oryzae*	Fruit and vegetable processing
α- and β-Galactosidase	*Aspergillus niger*, *A.oryzae*	Oligosaccharide (and lactose) hydrolysis
β-Glucanase	*Aspergillus niger*, *A.oryzae*, *Penicillium*	Plant extracts, e.g. fruit juices, flavours
Glucose oxidase	*Aspergillus niger*, *Penicillium*	Anti-oxidant, preservative
Inulinase	*Aspergillus*	Production of sweeteners
Lipase	*Aspergillus niger*, *Mucor*, *Rhizopus*	Leather and wool processing, dairy products, waste treatment
Pectinase	*Aspergillus*, *Rhizopus*, *Trichoderma*, *Penicillium*	Fruit juice clarification, extraction of coffee and spices
Protease	*Aspergillus*, *Mucor*	Meat and fish processing, cheese manufacture
Ribonuclease	*Aspergillus oryzae*, *Penicillium*	Flavouring agent production

necessary criteria plays a major part in the choice of production host. In many cases secretion of the target protein is advantageous in terms of downstream processing (Saunders *et al.*, 1989) and the filamentous fungi have potential advantages over some competing production hosts in this regard. The advantages of filamentous fungi as hosts for heterologous gene expression, summarized by Saunders *et al.* (1989), are that many species naturally secrete a wide range of proteins, many species are eaten or used in the production of enzymes destined for food use and are thus generally regarded as safe, and there is extensive fermentation and downstream processing experience with industrial species. There is also limited evidence that filamentous fungi glycosylate proteins in a way somewhat analogous to higher eukaryotes, which might be important in the production of proteins encoded by higher eukaryotic genes. Despite these attractions, the number of heterologous proteins available commercially from filamentous fungi is low. We therefore aim in this review to assess the current status of knowledge of gene expression and protein secretion from filamentous fungi and identify the areas requiring further study. Heterologous gene expression in the filamentous fungi has been reviewed by others (Saunders *et al.*, 1989; Ward, 1989a, b; Devchand and Gwynne, 1991; van den Hondel, Punt and van Gorcom, 1991). In many ways these reviews have made our task easier and we have taken the opportunity to make reference to them where appropriate. Some repetition is unavoidable, however, despite our efforts to adopt our own perspective.

Genetic manipulation in filamentous fungi

Since the first report of successful DNA-mediated genetic transformation in *Neurospora crassa* (Mishra and Tatum, 1973) there has been a large increase in research activity in this area. Progress has been made in terms of both the sophistication of vectors used and the numbers of different species in which transformation has been achieved. Nevertheless, such studies are still in their infancy and many questions remain open. Indeed, the conclusions of the early work were not widely accepted until Southern analysis confirmed the integration of exogenous *qa*-2 DNA (the catabolic dehydroquinase gene) into the genome of *N. crassa* (Case *et al.*, 1979). The protoplast-based technique used in this work was adopted from studies in yeast (Beggs, 1978) but was not extended to filamentous fungi other than *N. crassa* until transformation was reported in *Podospora* (Stahl *et al.*, 1982) and *Aspergillus nidulans* (Tilburn *et al.*, 1983). During the remainder of the 1980s reports of successes in other species began to appear and the first comprehensive reviews were written towards the end of the decade (Rambosek and Leach, 1987; Fincham, 1989; Timberlake and Marshall, 1989).

In this section, we attempt to summarize the techniques used for genetic manipulation, to assess the types of vector available, and to list those species which are readily transformed, together with those which are most recalcitrant. We will also discuss the fate of DNA sequences introduced during transformation. An understanding of the mechanisms of integration into the

host genome and subsequent regulation of gene expression is essential for successful heterologous protein production.

TRANSFORMATION TECHNIQUES

The protoplast-based technique used initially for yeast transformation (Beggs, 1978) is still the most widely used of the methods available. In essence, protoplasts are produced via enzymic digestion of the cell walls of germinating conidia or young mycelia in the presence of osmotic stabilizers (e.g. KCl, NaCl, sucrose or sorbitol). The digestion is often achieved using Novozyme 234, although many different enzyme preparations have been effective (Peberdy, 1986). Polyethylene glycol (PEG) and $CaCl_2$ are then used to facilitate transfer of DNA across the plasma membrane, and transformed cells are allowed to regenerate cell walls and produce sporulating colonies on selective media. There are many variations on this basic theme and the relative importance of the various steps is not at all clear; it appears that, as with *Escherichia coli*, in each population of cells there is a small sub-population competent to accept exogenous DNA (Kelly and Hynes, 1985; Mattern *et al.*, 1987; Wernars *et al.*, 1987). However, in filamentous fungi, transformation efficiency is several orders of magnitude lower than in *E. coli* and is probably more affected by protoplasting efficiency than by any other factor so far identified (Fincham, 1989). Attempts have been made in other systems to circumvent the need for protoplasting by use of projectiles (Klein *et al.*, 1987; Fox, Sanford and McMullen, 1988; Johnston *et al.*, 1988), glass beads (Costanzo and Fox, 1988), silicon carbide needles (Kaeppler *et al.*, 1990), lithium ions (Ito *et al.*, 1983; Dhawale, Paietta and Marzluf, 1984) and, most recently, laser beams! With filamentous fungi, electroporation of protoplasts (Ward, Kodama and Wilson, 1989) or of partially digested germinating conidia (Chakrabarty and Kapoor, 1990) look to be the most promising alternative techniques to using PEG-mediated transformation of protoplasts.

HOST VECTOR SYSTEMS

While it has been demonstrated that whole, genomic DNA can be effective in achieving transformation (Mishra and Tatum, 1973), convenient handling of transforming DNA necessitated the development of shuttle vectors, i.e. vectors with selectable markers for use in both *E. coli* and fungal hosts. Plasmid vectors containing autonomously replicating sequences (ARS), and thus able to replicate without integrating into the chromosome, have proved valuable in yeast systems (Stinchcomb, Struhl and Davis, 1979) and were naturally sought in filamentous fungi. To date, however, with the exception of nuclear ARS plasmids in *Ustilago* (Tsukuda *et al.*, 1988) and *Mucor circinelloides* (van Heesjwick, 1986), and the mitochondrial plasmids of *Podospora anserina* (Tudzynski, Stahl and Esser, 1980; Stahl *et al.*, 1982) and *N. crassa* (Stohl and Lambowitz, 1983; Stohl, Atkins and Lambowitz, 1984), it has not proved possible to develop such vectors, possibly because of

differences in the organization of origins of replication between species. Nevertheless, filamentous fungal ARS, defined by their ability to function in yeast, do significantly improve transformation frequency during integrative transformation in *N. crassa* and *A. nidulans* (Buxton and Radford, 1984; Ballance and Turner, 1985). Transformation efficiency is improved by at least one order of magnitude but the precise sequences responsible are not known and reasons for their effects are not fully understood. Preliminary data presented recently indicate that rapid progress may now be expected in the development of autonomously replicating plasmid vectors; it would appear that breakthroughs have been made in at least three species of filamentous fungi (Powell and Kistler, 1990; Farman and Leong, 1991; Gems, Johnstone and Clutterbuck, 1991; Robertson *et al.*, 1991).

In the absence of satisfactory maintenance of transforming DNA as plasmids, the main thrust of vector development for filamentous fungi has been in the area of integrating vectors. In contrast to the situation in yeast, where homologous integration events dominate, integration in filamentous fungi has been shown to be both by homologous and heterologous integration. Also, transformants often possess multiple copies of the transforming DNA (*see* p. 332) and high frequencies of co-transformation have been demonstrated where DNA present on a separate plasmid, and not selected for in the initial transformation, has been found integrated in the genomes of up to 80% of transformants (van den Hondel, Punt and van Gorcom, 1991). Recombination frequently results in integration of vector sequences as well as the gene of interest. In some cases it has proved possible to rescue the transforming DNA integrated into the chromosome by taking advantage of *cos* sites present in the original vector. The DNA is packaged into viral particles in a technique known as cosmid rescue (Yelton, Timberlake and van den Hondel, 1985; Weltring *et al.*, 1988).

A large number of selectable markers is now available for use in genetic transformation. These fall into two main groups. In the first, selection depends upon transformation from auxotrophy to prototrophy and thus the strain to be transformed must first be mutated to the appropriate form of auxotrophy. In the second, selection is based on resistance to a given antibiotic and, in principle, any strain normally susceptible to this antibiotic may be transformed. In some cases markers are available which allow for two-way selection, i.e. vectors conferring resistance to a given antibiotic or metabolite result in susceptibility to another. A range of vectors and markers have been used specifically for heterologous protein production and these are discussed further on pp. 340–344.

A more refined type of vector has been developed recently which allows for targeting of the selectable marker to a specific location in the chromosome. Such gene replacement or gene disruption vectors depend on a double cross-over event between linearized vector and the target gene (*see Figure 1*). Also, vectors capable of autonomous replication of megabase (10^6 bp) inserts of exogenous DNA have been developed for yeast (Schlessinger, 1990). These incorporate centromere and telomere sequences and are known as yeast artificial chromosomes (YACs). Telomere sequences have already been

described for some species of filamentous fungi (Powell and Kistler, 1990) and it is likely that artificial chromosome vectors will be available for these organisms in the near future.

The range of species for which genetic transformation has been reported is already very large and is increasing rapidly. For this reason it is very difficult to produce comprehensive lists, and we have confined ourselves to the filamentous fungal species which have been transformed specifically for heterologous protein production (*see Table 2*). In general, where transformation of a particular species has been sought it has been achieved. However, notable exceptions are provided by *Mucor miehei* and *Phytophthora* spp. These failures have been ascribed either to difficulties associated with aneuploidy (*Mucor*: Roncero, 1984) or to unusual promoter structure and function incompatible with promoters used in standard vectors (*Phytophthora*). Recent reports indicate that these problems are now being overcome, at least in the case of *Phytophthora infestans* (Judelson, 1991).

The fact that more than one nucleus can co-exist in a single fungal cell may also give rise to problems if transformants are not taken through a uninucleate stage; persistence and subsequent replication of non-transformed nuclei may lead to dilution of transformed nuclei and eventual loss of the selectable marker (Buxton and Radford, 1984), possibly explaining the phenomenon of 'abortive' transformants (Ballance, Buxton and Turner, 1983; Tilburn *et al.*, 1983; Yelton, Hamer and Timberlake, 1984).

FATE OF TRANSFORMING DNA

As noted above, successful DNA-mediated transformation is usually achieved when vectors carrying appropriate promoters and selectable markers integrate into single, haploid nuclei. Depending on the species and method of selection, the integration event may involve homologous or heterologous recombination of one or several copies of the vector at one or several positions in the genome. It is also common in some species for several copies of the vector to become integrated in tandem at a single chromosomal location (Kelly and Hynes, 1985; Wernars *et al.*, 1985; Durrens *et al.*, 1986). Multicopy transformants can also be achieved by co-transformation. Higher copy-number transformants can often, but not always, achieve higher levels of heterologous protein production (Punt *et al.*, 1991; van den Hondel, Punt and van Gorcom, 1991), so the ability to achieve amplification of expression cassettes in industrial fungi is particularly valuable for heterologous protein production. Furthermore, it has been demonstrated that integrative transformants have high mitotic stability, and are apparently unaffected after a great number of mitotic divisions even on non-selective media (Wernars *et al.*, 1985; Kelly and Hynes, 1985; Cullen *et al.*, 1987; Davies, 1991).

Where many copies have become integrated, it has not been determined whether transcription proceeds to the same extent from each copy. It is assumed that 'position effects' noted in other species also come into play in filamentous fungi and such effects are discussed further on p. 344. Their impact on the efficiency of heterologous gene expression is not fully under-

stood but the imprecise nature of the relationship between copy number and protein production indicates that some copies integrate randomly into more active positions in the genome (van Gorcom *et al.*, 1985; Christensen *et al.*, 1988; Ward *et al.*, 1990). Any increase in the number of copies provides a greater chance of integrating into highly active regions of genomic transcription and thus increasing gene expression. Upper limits on what can be achieved by increasing copy number are set by the problems of titrating out essential transcription factors (Hynes *et al.*, 1988) and, in species such as *N. crassa*, by the repeat-induced point mutation (RIP) phenomenon. This occurs at meiosis where, if two copies of a gene are present, they are both mutated and may cease to function. This occurs at high frequency in *Neurospora* and homology as little as 70% between the transforming DNA and a native gene is sufficient to result in a mutant phenotype lacking expression of the gene concerned. It is thought that pairing of introduced and native genes occurs at meiosis since only odd copy-number transformants survive where this happens. This is a useful genetic tool for studying phenotypes of unknown genes (Selker, 1990). Fortunately, from the point of view of strain stability, the phenomenon is much less frequent in other species and has not proved to be a problem in industrial species which, typically, do not undergo meiosis.

MECHANISMS OF INTEGRATION

It has been established that in filamentous fungi circular vector DNA, including plasmid sequences, can become integrated into the genome, in single or multiple copies, by both homologous and heterologous recombination (*Figures 1a* and *1b*). In some species, linearization of vector DNA prior to transformation greatly increases the transformation frequency and the proportion of homologous recombinations, and significantly increases double cross-over events, resulting in the absence of integration of plasmid sequences and hence in simple gene replacement/conversion (*Figure 1c*; Miller, Miller and Timberlake, 1985; Paietta and Marzluf, 1985; Frederick, Asch and Kinsey, 1989; Berka *et al.*, 1990).

The ratio of homologous:heterologous integrations found in different genera appears to vary considerably. As has been mentioned previously, yeast vectors have been found to integrate at predominantly homologous sites (Hinnen, Hicks and Fink, 1978). At the other extreme, *Coprinus* appears to show 96% heterologous integration (Binninger *et al.*, 1987). *Aspergillus* (Tilburn *et al.*, 1983; Yelton, Hamer and Timberlake, 1984), *Ascobolus* (Goyon and Faugeron, 1989) and *Penicillium* (Bull, Smith and Turner, 1988) fall between these extremes with approximately 70% homologous integration. Some exceptions to this rule have been noted recently, e.g. Fowler, Berka and Ward (1990) failed to achieve homologous integration at the *gla*A locus of *A. niger*.

It is assumed that the initial events of integration involve repair of double-strand breaks, perhaps involving mechanisms such as those that occur following X-ray damage. Many models have been proposed to explain repair of double-strand breaks, either with or without accompanying cross-over

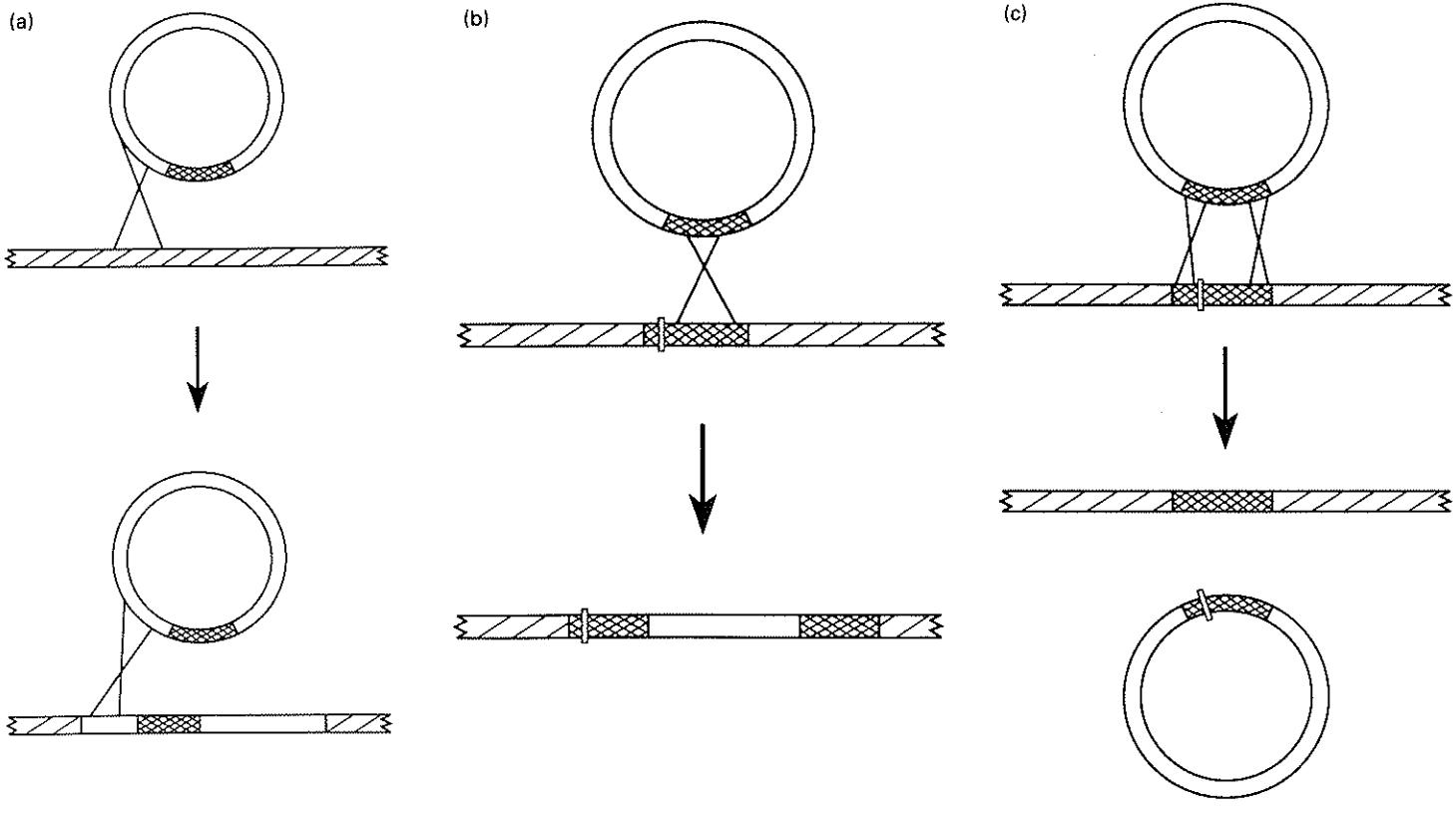

Figure 1. Models of integrative transformation. (a) Via a single cross-over at a non-homologous locus followed by a homologous recombination leading to tandem, multicopy insertions. (b) Via a single cross-over at a homologous locus. In this case, the genomic copy of a gene, carried also on the plasmid vector, is marked. Further rounds of recombination lead to multicopy insertions, as in (a). (c) Gene replacement mediated by a double cross-over event at the homologous locus. The original genomic copy (marked) is subsequently lost since the plasmid contains no autonomously replicating sequences.