Genetic Engineering of Filamentous Fungi Progress, Obstacles and Future Trends

Naresh Sharma, Assistant Professor

Department of Engineering & IT, Arka Jain University, Jamshedpur, Jharkhand, India

ABSTRACT: ***Filamentous fungi are widely used in biotechnology as cell factories for the production of chemicals, pharmaceuticals and enzymes. In order to improve their productivities, genetic engineering strategies can be powerful approaches. Different transformation techniques as well as DNA- and RNA-based methods to rationally design metabolic fluxes have been developed for industrially important filamentous fungi. However, the lack of efficient genetic engineering approaches still forms an obstacle for a multitude of fungi producing new and commercially interesting metabolites. This review summarizes the variety of options that have recently become available to introduce and control gene expression in filamentous fungi and discusses their advantages and disadvantages. Furthermore, important considerations that have to be taken into account to design the best engineering strategy will be discussed.***

KEYWORDS: Filamentous fungi, Gene targeting, Genetic, metabolic engineering, RNAi.

1. INTRODUCTION

The ability of filamentous fungi to grow on rather simple and inexpensive substrates as well as their capacity to produce a wide range of commercially interesting metabolites have attracted considerable interest to exploit them as production organisms in biotechnology. Nowadays, filamentous fungi are used in biotechnology[1] as cell factories for a wide range of products. Diverse compounds ranging from simple organic acids to complex secondary metabolites are produced for the use in various market segments.

Due to their exceptional high capacity to express and secrete proteins, filamentous fungi have become indispensable for the production of enzymes of fungal and non-fungal origin. Currently, native or recombinant enzymes are mainly produced by Aspergillus niger, A. oryzae and Trichoderma reesei[2] and also other strains are currently under development (Punt et al., 2002). In addition, filamentous fungi naturally produce an astonishing wealth of secondary metabolites and a few of them are biotechnologically produced and became clinically significant drugs. The β-lactam group of antibiotics, including penicillin and cephalosporin, was the first group that benefited from the progress made in molecular techniques for filamentous fungi. The increasing knowledge of the biosynthesis and molecular genetics of β-lactam antibiotics led to new possibilities to rationally improve production strains and to engineer new biosynthesis pathways (Brakhage and Caruso, 2004) [1]. Further bioactive compounds produced by filamentous fungi and important for human welfare are, for example, cyclosporine A (immunosuppressive agent), lovastatin (cholesterol-lowering agent), taxol (anticancer agent) and griseofulvin (antifungal agent).

2. LITERATURE REVIEW

Other fungal metabolites already produced commercially or potentially valuable in biotechnology involve exopolysaccharides such as pullulan suitable for coating foods or for improved delivery of therapeutic agents (Leathers, 2003) and hydrophobins useful as surface modifiers for (nano) technical and medical applications due to their ability to self-assemble at hydrophilic–hydrophobic interfaces into amphipathic films (Scholtmeijer et al., 2001; Hektor and Scholtmeijer, 2005). Although this is not a complete listing of industrially produced fungal-based compounds, it still reflects the metabolic versatility of filamentous fungi and their importance as cell factories in biotechnology. One important cornerstone for the future of fungal biotechnology will be the improvement of production strains at the molecular level. Genetic and metabolic engineering approaches to both natural and recombinant metabolite producing strains will be powerful tools for improving production levels, producing novel tailored compounds or directing the synthesis of desired products. However, this will only become feasible with the development of efficient methods to introduce and control gene expression in filamentous fungi. The objective of this review is to summarise and discuss the currently available options for

genetic manipulation of filamentous fungi. Furthermore, the question of choosing the most appropriate strategy to genetically engineer a particular process will be addressed. For a specialised review that focuses on functional genomics with filamentous fungi, the readers are directed to (Weld et al., 2006).

3. DISCUSSION

Genetic engineering can be a powerful approach for filamentous fungi in order to increase productivity and to minimize unwanted by-product formation. However, before engineering can become routine, introducing the desired genetic manipulation of the fungus of interest often represents a challenge. Foremost, the establishment of a suitable transformation method is not trivial for many fungi [3]. Moreover, the mode and frequency of individual integration events resulting from homologous or illegitimate recombination is not only dependent on the transformation host itself but also on the applied transformation technique. Thus, designing an engineering strategy first requires consideration of the most suitable transformation method. Since the first report on successful protoplast-mediated transformation (PMT) of the yeast Saccharomyces cerevisiae the use of protoplasts for transformation has been extended to several filamentous fungi. However, the frequency of transformation is extremely low when compared to yields obtained with S. cerevisiae. In order to improve transformation of filamentous fungi, progress has been made over the last years that has resulted in the establishment of alternative methods for fungal transformation such as electroporation, biolistic transformation and Agrobacteriummediated transformation[4]. These methods have especially been shown to be valuable for fungal strains that do not form sufficient numbers of protoplasts or whose protoplasts do not regenerate sufficiently.

Table 2 summarizes main features as well as advantages and disadvantages of these transformation techniques Common to all four techniques is the necessity to optimise every method for the fungal strain of interest and often, only one or two of these methods can be applied to a particular species. Importantly, AMT has been described to be an efficient transformation method for some fungi that were recalcitrant to the other methods described above. However, AMT has also been reported to be less successful or even fails to produce transform ants, e.g. in A. niger. Hence, no general rule can be applied to predict the usefulness of a particular transformation technique for the fungus of interest. Instead, individual species have to be considered independently and the most appropriate method identified and optimised for each strain. Interestingly, mainly single-copy integration events were detected when AMT was used for the introduction of DNA into filamentous fungi such as A. awamori, A. giganteus, Calonectria morganii, F. oxysporum and Suillus bovines. In contrast, when these fungi were transformed by PMT, preferentially multicopy integration events were observed [5].

The impact of the transformation technique on the fate of the transforming DNA can thus have an important influence on the design of a metabolic engineering strategy for a given process. For example, when targeted integration or gene deletion is envisaged, AMT would be the method of choice. Contrary to this, PMT could be the optimal method when multiple copies of a gene of interest should integrate at random sites in the genome. As discussed by Michielse et al. (Michielse et al., 2005b), one possible explanation for the increase in HR frequency obtained by AMT is the fact that A. tumefaciens delivers its DNA to the host as single-stranded DNA, whereas double-stranded DNA[6] is transferred in the case of the other transformation methods. In S. cerevisiae, it has been shown that single-stranded DNA transforms cells at greater HR efficiency than that of double-stranded DNA, suggesting that the recombination machinery[7] has a preferential affinity for single-stranded DNA [8].

2.1 Objective:

1. The knowledge of genetic engineering strategies accumulated so far will provide a valuable framework and opens new avenues for fungi where no genetic manipulation has been achieved yet.
2. The availability of fungal genomes and their better understanding will open new doors to better engineer industrial host strains and will certainly contribute to the removal of bottlenecks.
3. Moreover, new product classes will definitely be developed and manufactured
4. the postgenomic era also calls for new approaches to substantially understand the metabolism, growth and phenotype of filamentous fungi
5. Genome-wide transcription profiling, proteomics and the reconstruction of the complete metabolic networks will provide valuable in-depth insights into cellular processes of filamentous fungi.

2.2 RNA Technologies for Genetic Engineering:

The possibility to encourage or to silence a given metabolic pathway is not only restricted to DNA-based approaches. An alternative strategy circumventing the need for gene targeting and in particular gene deletion would be the use of RNA-based methods[9] that silence gene expression post-transcriptionally. These tools are especially valuable when (i) gene targeting approaches fail, (ii) multiple copies of a gene of interest are present in the genome and/or (iii) isogenes might compensate for the knockout of the deleted gene. Three RNA-based methods antisense RNA, hammerhead ribozymes and RNA interference have been shown to be valuable tools for gene silencing in eukaryotes. Outlines the main principles of the tools and their advantages and disadvantages. Successful gene silencing using artificial antisense constructs have been reported for filamentous fungi. The flow diagram of RNA technology in Figure 1.

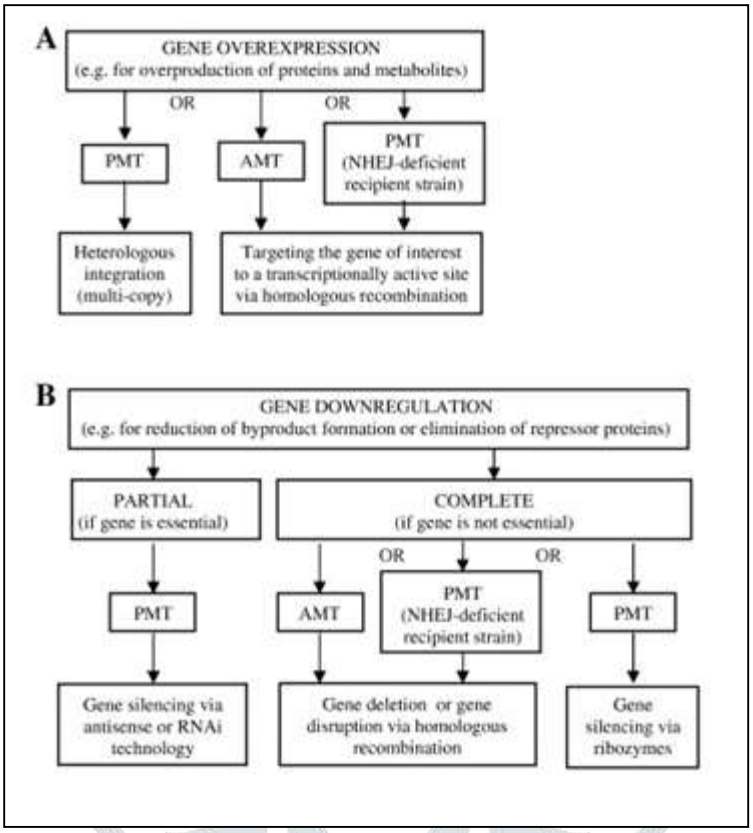

Figure1: RNA Technology in Genetic Engineering

For example, an antisense construct displaying homology to two different genes coding for extracellular carboxypeptidases was transferred by PMT into the genome of A. oryzae. The construct, found to be randomly integrated in multiple copies into the genome, resulted in about 70% reduced protease activities. This antisense strategy improved the capacities of A. oryzae as host for heterologous protein expression as more stable and higher level of human lysozyme were detected. Similarly, antisense silencing of the protease aspergillopepsin B achieved a reduction of 10–70% of protease levels and resulted in a 30% increase in heterologous thaumatin[10] production in A. awamori remarkably, there has been no antisense-mediated reduction of gene expression to zero levels reported to date, indicating that the efficiency of this strategy is limited. However, in view of the fact that a complete knockdown of a particular gene can be lethal to the fungus of interest, attenuation of its expression by the antisense tool can still be a powerful approach. For instance, the wide-domain transcription factor CreA, the key component of carbon catabolite repression in Aspergillus negatively regulates a number of industrially important enzymes. As partial suppression of creA expression in A. nidulans by its antisense molecule (about 50% reduced expression was estimated) yielded in partial alleviation of glucose repression and thereby in a substantial increase of the productivities of intra- and extracellular glucoserepressible enzymes. Most importantly, growth characteristics of A. nidulans were not affected.

Catalytic RNA molecules, also referred to as ribozymes, offer an alternative strategy for gene silencing approaches. The hammerhead ribozyme is the smallest, best-known and most widely used class of RNA-based enzymes. The substrate-recognition arms can be engineered so that the arms are complementary to any chosen

RNA target, enabling the ribozyme to bind to its target. The cleavage of the substrate RNA occurs immediately adjacent to a "NUX"triplet, where N represents any base and X can be A, U, or C. The applicability of a ribozymebased technology as tool for effective post-transcriptional suppression of gene expression has been shown for bacterial, yeast, plant and mammalian systems. Most recently, a proof of principle for the use of hammerhead ribozymes to control gene expression in filamentous fungi was given. As model organism, A. giganteus was used as for these fungus gene deletions were not feasible due to the lack of HR (Meyer et al., 2002). A reporter-based model system using the β-glucuronidase transcript (uidA) as the target mRNA was employed. This system was used to validate the activity of seven in silico selected hammerhead ribozymes targeting different sites within the uidA mRNA[11]. All ribozymes tested were able to reduce the reporter activity in A. giganteus (up to a maximum of 100%), demonstrating that ribozyme technology is indeed a useful tool for fungal metabolic engineering and can also be used to completely silence a gene of interest.

4. CONCLUSION

This concept of 'systems biology' and the knowledge deduced will significantly improve industrial fermentation processes and will further increase the value of filamentous fungi in the future of biotechnology. Guidelines for the design of genetic engineering approaches for filamentous fungi aiming at over expression (A) or down regulation (B) of a gene of interest. PMT: Protoplast-mediated transformation; AMT: Agro bacterium mediated transformation; NHEJ: no homologous end joining pathway. New product classes will definitely be developed and manufactured. However, the postgenomic era also calls for new approaches to substantially understand the metabolism, growth and phenotype of filamentous fungi. Genome-wide transcription profiling, proteomics and the reconstruction of the complete metabolic networks will provide valuable in-depth insights into cellular processes of filamentous fungi.

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