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Review

Heterologous expression of genes in filamentous fungi*

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Isolation of some biologically important proteins from natural sources was found to be too expensive or scarcely possible (human proteins). The problem could be solved by expression of heterologous genes.

Many biologically active proteins have been successfully expressed in filamentous fungi, some of them, however, at a low level. Thus, improvement of this technique appears to be a very important task. The process comprises several steps. Some of them, such as efficient transformation, vector construction, processing of signal sequences, post-translational modifications and secretion of the expressed proteins, have been intensively investigated.

This review presents obstacles and problems encountered in expression of heterologous genes and discusses strategies of development in this area.

Filamentous fungi are very attractive organisms for expression of heterologous genes. Among them the species of *Aspergillus* and *Trichoderma* have been predominantly used as hosts. The industrially important filamentous fungus *T. reesei* is an efficient producer of hydrolases, especially of different cellulolytic enzymes that can degrade native crystalline cellulose to glucose. Some strains of *Trichoderma* secrete up to 40 g l^{-1} of extracellular proteins (Durand *et al.*, 1988). Selected strains of *A. niger* can produce more than 20 g

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Abbreviations: MPD, mannosyl-phospho-dolichol; *DPM1* gene, encoding MPD synthase.

of glucoamylase per litre in industrial fermentations (Berka *et al.*, 1991). The production of such a large amount of proteins by these fungi demonstrates that they have an excellent secretory capacity. Secretion of proteins allows isolation of the product directly from the medium from a continuous culture without breaking cells, and avoiding thus the whole laborious and expensive procedure involved. On the other hand, the yield of heterologous products of filamentous fungi unlike that of the host's proteins, remains often low.

Nonetheless, many laboratories use filamentous fungi for production of heterologous proteins. Some species of *Trichoderma* and *Aspergillus* are used for production of enzymes, antibiotics, food products and biochemicals. Knowledge about the *Trichoderma* genetic system is very limited but genetic engineering has given some important data about the fungus.

Genes coding for *Trichoderma* cellulases, cellobiohydrolases CBHI (Shoemaker *et al.*, 1983; Terri *et al.*, 1983) and CBHII (Chen *et al.*, 1987; Teeri *et al.*, 1987), endoglucanases EGI (Penttila *et al.*, 1986; Van Arsdell *et al.*, 1987), and EGII (Saloheimo *et al.*, 1988), and the *pgk* gene coding for phosphoglycero kinase (Vanhanen *et al.*, 1989) have been cloned and characterised. Also transformation methods (Berges *et al.*, 1989; Durand *et al.*, 1988; Gruber *et al.*, 1990; Mach *et al.*, 1994; Penttila *et al.*, 1987), gene inactivation (Harkki *et al.*, 1991) and targeted integration (Joutsjoki, 1994; Karhunen *et al.*, 1993; Suominen *et al.*, 1993) have been developed.

The progress in these fields allows to create new fungal strains with different useful properties. Results from our laboratory indicate that expression of the *Saccharomyces cerevisiae DPM1* gene (encoding MPD synthase) in *Trichoderma* could increase the level of production/secretion of the protein synthesized by this fungus (Kruszewska, J.S., Butterweck, A.H., Kurzątkowski, W., Migdalski, A., Kubicek, C.P. and Palamarczyk, G., unpublished).

EFFICIENT TRANSFORMATION: THE FIRST STEP IN THE EXPRESSION OF HETEROLOGOUS GENES

Transformation of filamentous fungi still presents a bottleneck in the construction of recombinant strains. Most of the efficient transformation systems in use are exclusively based on auxotrophy and on genetically well studied fungi, *Neurospora crassa* (Case *et al.*, 1979) *A. nidulans* (Ballance & Turner, 1985; Ballance *et al.*, 1983; Tilburn *et al.*, 1983; Yelton *et al.*, 1984) or *A. niger* (Buxton *et al.*, 1985; Hartingsveldt *et al.*, 1987; Kelly & Hynes, 1985). Moreover, there are large numbers of auxotrophic and selectable markers for transformation (Ballance, 1991; May, 1992).

The transformation frequency differs for different markers from 600 transformants/μg of transforming DNA for the *argB* (ornithine carbamoyltransferase) gene of *A. nidulans* used in an arginine auxotroph of *Trichoderma* (John & Peberdy, 1984; Penttila *et al.*, 1987), up to 1500 transformants/μg DNA for the *pyr4* (coding for orotidine-5'-phosphate decarboxylase) gene of *N. crassa*, or *pyrA* of *A. niger* used in a *Trichoderma* uridine auxotroph mutant (Gruber *et al.*, 1990). The highest transformation frequency (1800–2500 transformants/μg DNA) was obtained for the hygromycin B (HmB) resistance gene from *E. coli* (Mach *et al.*, 1994). This gene was expressed under the promoter of the *T. reesei pki1* (pyruvate kinase-encoding) gene (Schindler *et al.*, 1993) and the transcription termination signal of the *cbh2* (cellobiohydrolase encoding) gene (Teeri *et al.*, 1987). Inclusion into the vector of the *ans1* sequence (Ballance & Turner, 1985), an equivalent of *ARS* (autonomously replicating sequence) of *S. cerevisiae*, enhanced the transformation frequency of *A. nidulans*. The efficiency of transformation using the plasmids bearing *ans1* was approx. 5000 stable transformants per microgram of plasmid DNA. Three sequences simi-

lar to *S. cerevisiae ARS*-s were isolated from *T. reesei* (*trs1, 2, 3*) (Belshaw *et al.*, 1997; Laemmli *et al.*, 1992; Roberge & Gasser, 1992). The *Trichoderma trs* sequences enhance transformation frequency (3.6–5-fold) without promoting efficient replication of plasmids in *T. reesei* (Belshaw *et al.*, 1997). Probably the *trs* sequences can enhance recombination of the transforming vector. All these transformation methods lead to the integration of the plasmids into genomic DNA of the host. The integration occurs either by homologous or heterologous recombination at one or several positions in the genome (Jeenes *et al.*, 1991), but, in contrast to the yeast *S. cerevisiae,* the frequency of homologous recombination among filamentous fungi is low (May, 1992). Cassettes are often integrated in tandem at a single chromosomal location (Kelly & Hynes, 1985). An extreme example of multi-copy integration was described by Verdoes *et al.* (1993). A cosmid vector used for the transformation contained four copies of the *A. niger* glucoamylase (*glaA*) gene and the resulting transformants integrated up to 200 copies of this gene.

THE LOCUS OF INTEGRATION INFLUENCES GENE EXPRESSION

To avoid the influence of random integration, the expression cassette can be targeted to a specific locus. Some methods have been reported (Joutsjoki, 1994; Karhunen *et al.*, 1993, Suominen *et al.*, 1993) for one-step gene replacement allowing its homologous integration into the *cbh1* locus of *Trichoderma* using *cbh1* promoter and terminator in a linear plasmid. The clearest system, free from unpredictable effects of chromosomal surroundings, appears to be an episomal expression system (Aleksenko *et al.*, 1996). Filamentous fungi in nature have not been reported to contain circular nuclear plasmids. It is not clear either, whether filamentous fungi possess the enzymatic machinery necessary for replication of small circular DNAs (Cluttersbuck, 1995). On the other hand, mitochondrial plasmids were found to be present in some filamentous fungi, including *Trichoderma*. These plasmids are of two types: circular and linear (Griffiths, 1995).

Plasmids bearing the chromosomal *AMA1* sequence from *A. nidulans* (equivalent of *ARS* sequences in *S. cerevisiae*) are capable of non-chromosomal replication in filamentous fungi. The transformation rate of non-integrative plasmids is higher than in integrative systems and the copy number depends only on the selection method. On the other hand, genes carried by replicating vectors, present in a higher copy number, are not more efficiently expressed than the genes carried by multicopy integrative vectors (Verdoes *et al.*, 1994). Independently replicating vectors might be useful for the expression of poorly expressed genes, but not as a universal method of expression (Aleksenko *et al.*, 1986).

BARRIERS IN HETEROLOGOUS EXPRESSION RESULTING FROM GENE STRUCTURE

Genes of filamentous *Ascomycete* have usually similar promoters, intron splice signals and termination, polyadenylation signals and this, generally, makes them interchangeable. Sometimes during heterologous expression between filamentous fungi, gene regulation can be lost. Significant differences in signal sequences between yeast and filamentous fungi cause difficulties in their cross expression. Yeast seems to require strictly defined sequences for splicing. There are some studies on the importance of the sequences at the sites at which splicing of the immature RNA occurs (Ballance 1986; Gysler *et al.*, 1990; Kempken & Kuck, 1996; Langford & Gallwitz, 1983; Mount, 1982). It is known that defined sequences of pre-mRNA are recognised by elements involved in the splicing process. Many authors attempted to compose consensus se-

quences of 5′ and 3′ splicing sites and splicing signals (Ballance 1986; Gysler *et al.*, 1990; Kempken & Kuck, 1996; Langford & Gallwitz, 1983; Mount, 1982) for filamentous fungi, higher eukaryotes and yeast. In spite of the similarity of conserved sequences in yeast and filamentous fungi the differences between them are sufficient for yeast to fail splicing fungal introns.

The glucoamylase gene of *A. awamori* could be expressed in yeast only after removal of introns and provision of a yeast promoter (Innis *et al.*, 1985).

In filamentous fungi introns are present in approximately two-thirds of the genes sequenced so far and they are very short. The *cbh I* gene (cellobiohydrolase I) from *T. reesei* has two introns of 69 and 63 bp (Shoemaker *et al.*, 1983). Introns of filamentous fungi are located mainly in the 5′ region of the genes. Presence of the large number of introns in genes of filamentous fungi contrasts sharply with yeast, where introns are very much the exception in nuclear protein-coding genes. In *S. cerevisiae SAR 1* gene, coding for the small GTP-binding protein which is involved in the secretion pathway, only a single intron was found whereas the *A. niger* gene (*sarA*) contains five introns and the *T. reesei* gene (*sar1*) has four (Veldhuisen *et al.*, 1997). It is noteworthy that the single intron of *S. cerevisiae SAR1* gene, the first intron in the *A. niger sarA* and *T. reesei sar1* genes are located at exactly the same position. Neither the size nor the sequences of the introns are conserved between the three species.

The results of Joutsjoki and Torkkeli (Joutsjoki, 1994; Joutsjoki & Torkkeli, 1992) suggest some important role of introns in regulation of gene expression in filamentous fungi. Glucoamylase P gene of *Hormoconis resinae* containing three introns (Joutsjoki & Torkkeli, 1992) was expressed in *T. reesei* by integration to the *cbh1* locus (Joutsjoki, 1994). The glucoamylases produced by *H. resinae* and by *T. reesei* were identical, suggesting that *T. reesei* is flexible enough to process heterologous introns. Also cDNA of *H. resinae* glucoamylase P was expressed in *T. reesei*. Western blot analysis showed that expression of genomic *gamP* gene of *H. resinae* was more efficient than the corresponding cDNA expression in *T. reesei*. It is well known that introns can play an important role in regulation of transcription in higher eukaryotes in which some transcription regulatory elements were found in the intron sequences (Banerji *et al.*, 1983; Konieczny & Emerson 1987; Rossi & Crombrugghe, 1987).

In higher eukaryotes two elements important for initiation of transcription have been identified. The CAAT box at 70 to 90 bases, and the TATA box at 20 to 40 bases upstream of the major translation start point. In filamentous fungi some genes possess the TATA sequence while others may simply have an AT-rich region, 30–100 bases prior the 5′ translation start point. The situation with CAAT consensus is not clear either. Endo-β-1,6-glucanase gene from *T. harzianum* has two CAAT boxes and also two putative overlapping TATA boxes (Lora *et al.*, 1995). The putative promoter region of *T. reesei cbh 1* contains the TATATATAAA sequence upstream of ATG, followed by a pyrimidine rich sequence (Shoemaker *et al.*, 1983). Analogous sequences are comparable to that of the yeast glycolytic genes promoter region.

Our studies concerning yeast *dpm1* gene (encoding MPD synthase) expression in *T. reesei* pointed to the possibility that the *S. cerevisiae* gene expression occurs under its own promoter (Kruszewska, J.S., Kubicek, C.P. and Palamarczyk, G., unpublished). In addition, the MPD synthase activity was found in the membrane fraction, indicating proper processing of the yeast signal sequence typical of secreted proteins, however the signal sequence was not cut out from the protein.

Generally, there are many examples of heterologous expression of filamentous fungi genes in other filamentous fungi. Also *S. cerevisiae* genes may often be expressed in *Aspergillus* or *Trichoderma* but opposite expression

often failed indicating that filamentous fungi are more flexible in recognising heterologous signal sequences.

GENE EXPRESSION

For maximal production of a protein, the gene which encodes this protein should be placed under control of a strong homologous promoter. Normally, such promoters are found in highly expressed genes of the fungus, like the main cellulase gene *cbh1* (Joutsjoki, 1994; Miettinen-Oinonen *et al.*, 1997; Nykanen *et al.*, 1997), *glaA* glucoamylase gene (Carrez *et al.*, 1990) and *gpd* glyceraldehyde-3-phosphate dehydrogenase gene (Juge *et al.*, 1998) in *Aspergillus*.

Regulation of expression of the genes encoding cellulases in *Trichoderma* depends on the promoter and the chromosomal locus. The *Trichoderma* genome comprises six chromosomes. The cellulases genes *cbh1*, *cbh2* and *egl3* (encoding endoglucanase) are located on chromosome II and the *egl1* gene on chromosome VI (Mantyla *et al.*, 1992). The *egl1* structural gene expressed under the *cbh1* promoter gives a higher level of EGI protein (twice as high as under the *egl1* promoter) indicating that the *cbh1* promoter is stronger than the *egl1* one (Harkki *et al.*, 1989). The highest expression of *egl1* was observed when multiple copies of the gene were integrated into the *cbh1* locus. The level of *egl1*-specific mRNA in the single-copy transformant was about 10 times as high as compared to the non transformed host strain, suggesting that the *cbh1* promoter was about 10 times as strong as the *egl1* promoter (Karhunen *et al.*, 1993). Increasing the number of gene copies can enhance production of the target protein. A more or less linear relationship between the number of gene copies (not exceeding 20) and the level of protein production was reported for *A. niger* glucoamylase (Finkelstein *et al.*, 1989; Verdoes *et al.*, 1993), α-amylase of *A. oryzae* (Tada *et al.*, 1989), glucoamylase of *A. oryzae* (Hata *et al.*, 1991) and cellobiohydrolase II of *T. reesei* (Kubicek-Pranz *et al.*, 1991). Some problems appeared with the higher numbers of gene copies (200) when the correlation between copy number and protein synthesis was not linear. This might be explained, in part, by a limited amount of transcription factors or regulatory protein(s) (Verdoes *et al.*, 1993).

If natural promoters with the desired regulatory properties are not available, the regulatory sites existing within the promoter can be mutated. In this way *alc1* and *cbh1* promoters of *A. nidulans* (Hintz & Lagosky, 1993) and *Trichoderma* (Nakari *et al.*, 1994) insensitive to glucose repression were obtained. Isolation of new strong promoters with special regulatory properties was needed since *cbh1* promoter can not be used for protein production in glucose-containing media.

A functional analysis of the *cbh1* promoter of *T. reesei* was performed using a series of deletions and specifically designed alterations. The sequences similar to the binding sites of the glucose repressor MIG1 of *S. cerevisiae* and CRA A/CRE I of filamentous fungi were found (Illmen *et al.*, 1996b). Removal of sequences upstream from nucleotide 500, in relation to the initiator ATG, abolished glucose repression (Illmen *et al.*, 1996a).

Gene expression upon growth on glucose using the mutated *cbh1* promoter would be preferred to cellulose because of easier purification of the product and lower amount of proteases produced by *Trichoderma* in these conditions. In commercial fermentations it is also important that, on using a mutated promoter, protein production could be initiated during earlier phases of the fermentation process (Hintz & Lagosky, 1993).

Sometimes chimeric promoters are constructed including core promoter elements, such as TATA and CAAT motifs from a strong natural promoter, and a suitable repressor or enhancer elements from another promoter (Stanway *et al.*, 1989; Śledziewski *et al.*, 1988). Temperature regulated yeast promot-

ers were constructed in this way (Śledziewski *et al.*, 1988). For example, the operator sequence that binds the repressor protein encoded by the *MATα2* gene expressed in mating-type α cells of *S. cerevisiae*, was inserted into a strong promoter of the constitutive *TPI1* gene (triose phosphate isomerase). This procedure allowed construction of strong hybrid promoters that would be subject to mating-type regulation. The synthesis or activity of *MATα2* repressor protein can be made temperature-sensitive, so that promoters containing the *MATα2* operator sequence can be expressed at one temperature and repressed at another. Cultivation between permissive and restrictive temperature results in an intermediate level of products. Thus, by adjusting the temperature, the level of heterologous product can be altered.

SECRETION OF THE HETEROLOGOUS PRODUCT

A secreted protein is synthesized in the form of a precursor with a signal peptide at the N-terminus, necessary to target the protein to the endoplasmic reticulum. A typical fungal signal sequence contains 15–24 hydrophobic amino acids. Fusing of the protein to be secreted with a heterologous signal sequence appears to be a very promising step increasing the yield of the secreted product. Endochitinase of *T. harzianum* was expressed in *T. reesei* that normally does not have the activity of that enzyme. When the endochitinase gene was fused with the *T. reesei* CBH I signal sequence and with its own prepro region, the chimeric gene expression resulted in a 20-fold increase of the secreted product (Margolles-Clark *et al.*, 1996). Different results were obtained on studying expression of the *H. resinae* glucoamylase P (*gamP*) gene in *T. reesei* with natural N-terminal extension of the premature glucoamylase P or the CBHI signal peptide. One copy of the *gam P* gene was integrated into the *cbh1* locus of the host. The use of the natural glucoamylase N-terminal extension led to a higher yield of extracellular enzyme activity than when the signal peptide of CBHI was used (Joutsjoki *et al.*, 1993).

Most of the secreted proteins contain a cleavage site for the Kex2 type protease responsible for hydrolysis of the signal peptide. This enzyme is involved in maturation of endo-β-1,6-glucanase by cutting off a peptide composed of two modules: a signal peptide with a high hydrophobic index (amino acids 1–17) and a second peptide (residues 18–40) which ends with the target sequence for specific Kex 2 protease (Lora *et al.*, 1995; Park *et al.*, 1994). The Kex 2 activity of *T. reesei* has recently been characterised (Goller *et al.*, 1998). Inhibition of the protease activity by specific inhibitor APMSF (aminophenylmethylsulfonylfluoride) coincides with the accumulation of the larger intracellular precursors of cellulases (Goller *et al.*, 1998), suggesting that the preprotein processing is essential for their secretion by *Trichoderma* and *Aspergillus*. Recombinant human lactoferrin was expressed in *A. awamori* as a chimera with the N-terminus of the *A. awamori* glucoamylase gene under the control of the *A. awamori* glucoamylase promoter (Ward *et al.*, 1995). The insertion of a natural protease cleavage site KEX-2, between the glucoamylase and heterologous cDNA, led to efficient processing of the resulting chimeric protein. The protein was secreted to the growth medium in amounts larger than 250 mg per litre. When expression of the lactoferrin gene was under control of the strong promoter of alcohol dehydrogenase (*alcA*) only but without the protease cleavage site, the production of the protein was 5 mg per litre and it was secreted into the medium in only 30% (Ward *et al.*, 1992). Additional mutagenesis of the transformants able to secrete higher amounts of protein (over 250 mg of lactoferrin per litre) led to a significant improvement in the yield of lactoferrin (up to 2 g per litre) (Ward *et al.*, 1995).

PROTEASES INFLUENCE THE YIELD OF SECRETED PROTEINS

Secreted proteases can drastically reduce yields of extracellular proteins. Overexpression of lignin peroxidase (a hemoprotein) did not lead to an increase in protein production although a higher level of specific mRNA was observed (Saloheimo *et al.*, 1989). It was speculated by Saloheimo *et al.* (1989) that, in the absence of heme (*Trichoderma* does not secrete heme proteins) the protein was incorrectly folded and rapidly degraded by proteases of *Trichoderma*.

Integration of multiple copies of the *A. niger pelB* gene (pectin lyaseB, PLB) in the *A. nidulans* genome led to their expression, but the PLB protein was detected only in the medium containing pectin that might have prevented proteolytic breakdown of the enzyme (Kusters-van Someren *et al.*, 1992).

Selection of mutant strains that secrete very little of proteases (Broekhjsen *et al.*, 1993; Mattern *et al.*, 1992) or in which the genes encoding these enzymes have been disrupted, may improve the yield of the secreted protein. There are also some other methods used for protection of proteins against proteases, such as addition of an amino acid-rich supplement (peptone, casamino acids) to the medium or changing the pH of the culture medium (Archer *et al.*, 1990; Cregg *et al.*, 1993).

GLYCOSYLATION OF HETEROLOGOUS PROTEINS

It is well known that post-translational modifications, including glycosylation of proteins play an important role in the efficiency of their secretion.

Protein secretion in *Trichoderma* was influenced *in vivo* by the same factors which affect the activity of MPD synthase (Kruszewska *et al.*, 1990), a key enzyme in the O-glycosylation pathway in this fungus (Kruszewska *et al.*, 1989). *Trichoderma* grown in media supplemented with choline or Tween 80 secreted elevated amounts of proteins and revealed higher activity of MPD synthase. This result suggests an important role for MPD synthase activity in protein secretion by *Trichoderma*. Addressing this question we tried to increase the MPD synthase activity by overexpression of yeast *DPM1* gene (coding for MPD synthase) in *Trichoderma*. Heterologous expression of the yeast *DPM1* gene led to an elevated activity of MPD synthase and six-fold increase in the amount of CBH I relative to that of the parental strain (Kruszewska *et al.*, 1997). It is noteworthy that the CBH I protein that was secreted in higher amounts was not hypoglycosylated. Thus the glycosylation machinery had to work more intensively, on glycosylating the higher amount of protein secreted by the *Trichoderma DPM1* transformants. The effect of *DPM1* overexpression is difficult to interpret since MPD (the product of MPD synthase) is involved in three vital processes (Orlean, 1990; Tanner & Lehle, 1987): O- and N-glycosylation and synthesis of sugar moiety of phosphatidylinositol (PI) anchor, which anchors some proteins in the membranes. It is not clear which of these processes (if any) is affected by *DPM1* overexpression in *Trichoderma* and what is the relationship between that process and protein secretion.

Another problem in expression of the heterologous genes is that of the identity of the glycosylated product, which is particularly important with regard to proteins used for therapeutic purposes. *Trichoderma* and *Aspergillus* strains naturally secrete a wide range of glycoproteins that makes these systems especially attractive for production of heterologous glycoproteins.

Two asparagine residues of *A. niger* glucoamylase are glycosylated. They are located in the catalytic domain carrying oligomannosyl glycans. Also *Aspergillus* invertase is heavily glycosylated. Detailed studies on *Trichoderma* cellobiohydrolase I have shown the presence of N-glycans in the catalytic domain and of O-

mannosidic chains in the linker region. *Trichoderma* genes encoding cellobiohydrolases were expressed in *S. cerevisiae* under the *pgk* (phosphoglycero kinase) promoter. Western blot analysis of the subcellular localisation of CBH I and CBH II produced by yeast revealed that, during the mid-exponential phase of growth, both proteins remained cell-associated. In the late exponential and stationary phases they were secreted to the culture medium.

The yeast-produced CBH I and CBH II were larger than the native enzymes synthesized by *Trichoderma*. Endo H (endoglucosidase H) treatment increased the electrophoretic mobility of the recombinant CBH I, indicating that the sugar residues were of the hypermannosylated yeast type, and were N-glycosidically linked to the protein. No differences in molecular mass were detected after Endo H treatment of native CBH I produced by *Trichoderma* (Penttila *et al.*, 1988) although three N-glycosylation sites in the enzyme were glycosylated (Salovuori *et al.*, 1987). Specific activity of the recombinant enzyme, the yeast-made CBH II towards beta-glycan corresponded to 65% of the activity of the native *Trichoderma* enzyme. Hyperglycosylation of recombinant CBH II reduced its affinity towards crystalline cellulose. Poor binding to cellulose resulted in CBH II aggregating more readily than the *Trichoderma* enzyme (Penttila *et al.*, 1988). Thus, as it could be expected, the heterologous protein acquired the glycosylation pattern typical of the host organism. Draborg *et al.* (1996) obtained similar results with *T. harzianum* endochitinase secreted by *S. cerevisiae*. The recombinant enzyme was secreted in two forms of relative molecular mass of 43000 and 44000, both these values being higher than that for the native *T. harzianum* chitinase (41000). This result could be explained in part by different N-glycosylation of the heterologous protein.

Thus, *Trichoderma* and *Aspergillus* were used more successfully than yeast as hosts for production of heterologous glycoprotein.

Expression of human lactoferrin (hLF) gene in *A. nidulans* led to the production of a biologically active protein. The functions proposed for lactoferrin, an iron bound glycoprotein, include protection against microbial infection, cellular growth promotion and regulation of intestinal iron homeostasis (Arnold *et al.*, 1977; Hu *et al.*, 1990). Lactoferrin secreted by *A. nidulans* had an iron binding capacity similar to that of the authentic human protein (Ward *et al.*, 1992). The linkage and extent of glycosylation of the recombinant protein were similar to those of the human breast milk lactoferrin. Differences were observed only in specific carbohydrate residues. Bovine lactoferrin contains additional glycans of the high-mannose type, absent in human protein but found in the recombinant one secreted by *A. nidulans* (Derisbourg *et al.*, 1990). Oligosaccharides of filamentous fungi resemble the high mannose structure found in animal rather than in yeast cells (Salovuori *et al.*, 1987). Thus it can be expected that filamentous fungi have a potential for modification of heterologous proteins into the animal type glycoconjugates (Keranen & Penttila, 1995). Human α-interferon, human tissue plasminogen activator, epidermal growth factor, growth hormone, interleukin-6 and corticosteroid-binding globulin expressed in *A. nidulans* seemed to be correctly glycosylated in contrast to the hyperglycosylation observed in *S. cerevisiae* (Devchand & Gwynne, 1991). Excessive glycosylation is thought to be a result of delay in protein transport and then saturation of the secretion pathway. A different degree of glycosylation may affect the stability and half-life of the protein, change its activity or affinity towards some substrates. On the other hand, no direct correlation between the extent of glycosylation and the level of secretion was found (Aho 1991). Although the basic glycosylation mechanism is conserved among eukaryotes, the glycoprotein pattern varies from one species to another and, moreover, within the same organism it is diverse in different pro-

teins. Although there are well-documented examples of the significance of carbohydrate structure for function and secretion of various proteins, no easy generalisation can be made.

EXPRESSION OF HETEROLOGOUS GENES – A METHOD FOR GENE CLONING

The heterologous gene expression might be the easiest way to clone genes. We can find examples of cloning genes using a host that normally does not have the equivalent gene. The appropriate clone can be found after transformation looking for some new properties normally not existing in the host cells. Cloning of the *A. niger* invertase gene by expression in *T. reesei* gives us the best example (Berges *et al.*, 1993). *Trichoderma* species lack invertase and are unable to utilise sucrose as a carbon source. In contrast, *A. niger* produces two glycosylated forms of the enzyme. This permitted the cloning of an *A. niger* invertase gene by complementation of the sucminus phenotype of *T. reesei*. In addition, the cloned *suc1* gene from *A. niger* can be used as a dominant selectable marker for transformation of *Trichoderma* bearing specific auxotrophic mutations.

This was an exceptional event as *Trichoderma* is used but rarely for cloning of genes by heterologous expression. The most convenient organisms very often used for these purposes are yeast mutants. Human GlcNAc-1P transferase has been cloned in 1998 by complementation of the conditional lethal *S. cerevisiae* strain (Eckert *et al.*, 1998). In our laboratory the *Trichoderma mpg1* gene coding for α-D-mannose-1-phosphate guanyltransferase was isolated as a suppressor of the *S. cerevisiae* mutation in the *DPM1* gene encoding mannosyl-phospho-dolichol synthase (Kruszewska *et al.*, 1998).

CONCLUSIONS

Generally, numerous heterologous genes are successfully expressed in filamentous fungi, however, some of them at a low level. Filamentous fungi cope rather well with heterologous intron processing, post-translational modifications (glycosylation) and secretion. On the other hand, there are many steps on the way from vector construction to the final product of heterologous expression which could be improved. Years of studies have given lots of useful informations and indicated directions for future investigations.

In general, an increase in the number of gene copies integrated in the host genome causes elevated protein production but, for optimal gene expression the site of integration is also important.

However, there may be obstacles at further steps of expression of heterologous genes resulting in a low level of protein production. Improvement of the gene expression can be achieved by placing it under control of an expression signal derived from highly expressed genes. Also the secretion process (Palamarczyk *et al.*, 1998), with proper signal sequences and a cleavage site for KEX-2 protease offeres a large possibilities of improvement.

Studies on protein glycosylation (Palamarczyk *et al.*, 1998) occuring during passage of protein through the secretory system could give some answers important for assessing the identity of the heterologous product.

Cloning of genes coding for enzymes of the glycosylation pathway (*mpg1* of *T. reesei* (Kruszewska *et al.*, 1998), *dpm1* of *T. reesei* (Kruszewska, J.S., Saloheimo, M., Migdalski, A., Penttila, M. and Palamarczyk, G., unpublished)) in filamentous fungi could help in understanding not only the glycosylation but also the secretion mechanism (Kruszewska *et al.*, 1997). At the same time we should not neglect the problems concerning the stability of

heterologous mRNA, protein degradation and improvement of growth conditions.

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