9 Application of PCR in Fungal Biotechnology

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9.1 Introduction

Filamentous fungi have been utilized for decades in the commercial production of enzymes, antibiotics, and speciality chemicals. Fungi are unparalleled as producers of secreted proteins; *Aspergillus niger*, for example, produces glucoamylase at 20 g l^{-1}. Since the development of transformation methods for filamentous fungi, genetic engineering technology has been applied to fungal systems for the production of heterologous proteins. Members of the genus *Aspergillus*, *Fusarium* and *Neurospora* in particular, have been employed as hosts for the high level secretion of recombinant proteins (Peberdy, 1994; Van den Hombergh *et al.*, 1996). In this chapter, we will discuss the application of various PCR techniques for the characterization, cloning and expression of heterologous genes in industrially important filamentous fungi. Characterization of fungal strains and transformants, as well as the development of suitable fungal hosts for the expression of therapeutic human proteins, will also be discussed.

9.2 Cloning of Conserved Genes by PCR

Several approaches have been used to isolate genes from filamentous fungi (Goosen *et al.*, 1992). A number of fungal genes were cloned by complementation of auxotrophic mutants of *Escherichia coli* or *Saccharomyces cerevisiae*. This approach is limited because fungal expression signals may not be recognized by the host transcriptional or translational machinery, and many fungal genes have introns which cannot be processed

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properly to make a functional mRNA in *E. coli* or yeast. Isolation of fungal genes by complementation of auxotrophic fungal mutants is limited by the low DNA transformation efficiency in filamentous fungal species with the exception of *Aspergillus nidulans* and *Neurospora crassa*. A 'reverse genetics' approach has been used successfully in the cloning of many fungal β-lactam biosynthetic genes (Skatrud, 1992). This approach begins with the purification of the desired enzyme, determination of a partial amino acid sequence, and synthesis of oligonucleotide probes based on the amino acid sequence. A genomic DNA library of this fungal species is then constructed in *E. coli* and screened for clones that hybridize to the DNA probes. Sequencing of the cloned DNA is used to confirm that the DNA sequence matches the known amino acid sequence. For genes which are sufficiently conserved, gene isolation can be achieved in a short period of time by heterologous hybridization with a probe generated from its counterpart gene from another organism. Alternatively, degenerate oligonucleotide primers synthesized from the conserved regions of the available genes, are used to amplify a genomic or a cDNA fragment that can be used as a probe for cloning of the entire gene. This approach has been used successfully for the cloning of the cDNA of the NADPH-cytochrome P450 reductase gene (*cpr*) from *Schizosaccharomyces pombe* (Miles, 1992) as well as the genomic *cpr* gene from *A. niger* (Van den Brink *et al*., 1995), and the cDNA clone of the protein disulphide isomerase gene from *A. niger* (Malpricht *et al*., 1996).

9.2.1 Cloning of a gene from genomic DNA

The NADPH-cytochrome P450 reductase (CPR) serves as an electron donor for the activity of all eukaryotic non-mitochondrial cytochrome P450 mono-oxygenase enzymes. These enzymes catalyse the oxidation of lipophilic chemicals by the addition of one atom of molecular oxygen to the substrate molecule, such as the conversion of benzoic acid to *p*-hydroxy-benzoic acid. Genomic and cDNA clones of the *cpr* gene have been isolated from yeast, rat, rabbit and human genomes. The amino acid sequence of trout and pig CPR have also been determined from the purified protein. Comparison of the deduced amino acid sequences of these proteins shows two highly conserved regions. Miles (1992) used degenerate oligonucleotide primers, based on these conserved sequences, to amplify a 309 bp fragment of the *cpr* gene from *Sc. pombe* and used it as a probe to identify a cDNA clone of the *cpr* gene. Van den Brink *et al.* (1995) used a similar approach to generate a 1.4 kbp PCR-amplified DNA probe and cloned a genomic fragment containing the entire coding region with the 5′ promoter and 3′ untranslated regions of the *cpr* gene from *A. niger*. This method should be generally applicable to the cloning of *cpr* genes from diverse sources when hybridization with heterologous probes fails.

9.2.2 Cloning of a gene from mRNA

Protein disulphide isomerase (PDI) catalyses the formation, reduction, and isomerization of disulphide bonds in the process of maturation and folding of nascent polypeptide chains in the cell. Comparison of amino acid sequences of PDI proteins from a number of organisms indicates that the PDI enzyme has two highly conserved active sites. Malpricht *et al.* (1996) cloned the PDI cDNA sequences from total RNA isolated from mycelia of *A. niger* NRRL3 in three steps. First, they used two degenerate primers derived from the conserved regions of the two active sites to amplify a 158 bp PCR fragment from single-stranded cDNA which had been transcribed from *A. niger* total RNA. This PCR fragment was cloned and sequenced to verify its identity with the published PDI protein sequences. From the sequence information, a specific 27-mer primer was used as a 5′ primer in a 3′-rapid amplification of cDNA ends (RACE) PCR with single-stranded cDNA and oligo-dT primer to yield a longer cDNA fragment. This fragment consists of the above 158 bp fragment sequence and all of the 3′ sequences extending to the poly(A)-tail (Frohman *et al.*, 1988). The 5′ end of the gene, upstream of the sequenced region, was subsequently cloned by inverse PCR (IPCR) (Huang *et al.*, 1990). Double-stranded cDNA was prepared from full-length mRNA and self-ligated to generate circular templates for PCR. Primers were prepared which were complementary to the 5′ and 3′ ends of the previously amplified cDNA fragment, but oriented outward so that they would amplify the uncharacterized portion of the template. After 25 cycles of PCR, a DNA fragment containing sequences from the 5′ end of the mRNA was synthesized and characterized. By using IPCR, it was possible to determine the sequence of the 5′ region of a specific mRNA molecule without knowing the sequence of the 5′ end in advance. An alternative approach is to use 5′-RACE. Single-stranded cDNA is synthesized from mRNA and C-tailed using terminal deoxynucleotidyl transferase and dCTP. This serves as a template for PCR using oligo-dG and a gene-specific primer. Either method allows full-length cDNA fragments to be generated even with limited nucleotide sequence information (Frohman *et al.*, 1988; Zilberberg and Gurevitz, 1993).

9.3 Expression Vector Construction

Interest in the use of filamentous fungi for the production of both industrial enzymes and therapeutic proteins has increased significantly in recent years. Because fungi can produce large quantities of secreted proteins and many fungal strains are now generally recognized as safe, they can potentially serve as ideal hosts for the expression of heterologous genes. To accomplish this, a transformation protocol must be developed which allows foreign DNA to be introduced into the host in either an integrated or autonomous form.

Then, an expression vector must be constructed which provides the gene of interest with additional DNA sequences to allow its expression in the fungal host. These sequences typically include:

- a strong transcriptional promoter (preferably regulated);
- suitable restriction sites near the promoter to insert heterologous genes;
- a transcriptional terminator;
- a selectable marker.

Optionally, the vector may contain a secretion signal sequence or part of a highly expressed endogenous protein (generally from the gene which is naturally associated with the strong promoter), allowing a foreign gene to be either secreted or expressed as a fusion protein.

PCR is particularly useful in the construction of new expression vectors since most of the components can be derived from existing vectors, but require several changes in the flanking restriction sites. The following example from our laboratory demonstrates how we have easily constructed a rather complex expression system for *Fusarium oxysporum* by PCR.

In the course of developing an enzymatic method to convert penicillin V to 6-aminopenicillanic acid, research scientists at Bristol-Myers Squibb discovered a strain of *F. oxysporum* which produces substantial quantities (1–2 g l^{-1}) of a penicillin V amidase (PVA) enzyme (Fig. 9.1). Expression of the PVA gene is induced by the presence of phenoxyacetic acid (POAc), and the protein is secreted into the medium. To increase production of the enzyme, we cloned the PVA gene and approximately 2 kb of flanking sequences into a vector that contained a phleomycin resistance gene for the selection of fungal transformants. When the transforming plasmid was inserted into the chromosome of a different *F. oxysporum* strain (f. sp. *lycopersici*, ATCC 16322) which has very little endogenous PVA activity and no DNA sequences homologous to the PVA gene, the transformants produced over 1 g l^{-1} of active enzyme. When the host was transformed with a vector containing a tandem repeat (two copies) of the PVA gene, the expression increased to 10 g l^{-1} (Chiang *et al.*, 1996). The protein was secreted and its expression remained POAc-inducible. These characteristics have led us to believe that the PVA promoter and gene can be converted into a component of a valuable expression system in *Fusarium*.

Because the two-copy PVA gene vector produces much higher levels of expression, we decided to create a *Fusarium* expression system with two component plasmids for the easy construction of two-gene expression vectors. One component, pWB20 (the 'cassette' vector, Fig. 9.2), allows a heterologous gene to be inserted into the PVA gene at a variety of locations. Depending upon the insertion site, the gene can be expressed directly, or fused to the PVA secretion signal sequence, or expressed as a fusion protein. After the gene is inserted, an expression cassette containing the gene plus the PVA promoter and transcriptional terminator can be cut out with either of two restriction enzymes (*Eco*RI or *Xba*I). A second component, pSJC62 (the 'resistance'

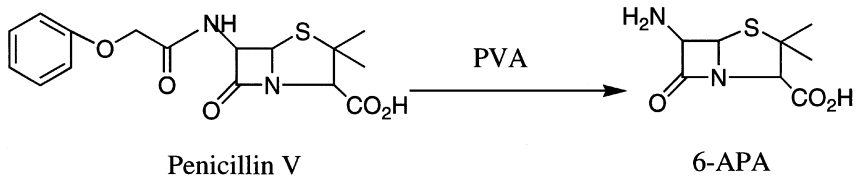

Fig. 9.1. Enzymatic conversion of penicillin V to 6-aminopenicillanic acid (6-APA).

vector, Fig. 9.2), contains the gene coding for phleomycin resistance and unique *Eco*RI and *Xba*I restriction sites on opposite sides of the gene. Two copies of the expression cassette can be inserted into these sites on pSJC62 to generate four different combinations. These copies are not true tandem repeats since they are separated by the phleomycin resistance gene. In order to generate tandem repeats we engineered an overlapping *dam*-methylation site (GATC) at one of the *Xba*I sites of the pWB20 cassette vector. The vector is initially grown in a *dam*$^-$ *E. coli* host so that the cassette can be cut out with *Xba*I. After the first copy of the cassette is inserted into the pSJC62 resistance vector, it is grown in a *dam*$^+$ *E. coli* host which prevents cleavage of one of the *Xba*I sites. A second copy of the expression cassette is now easily inserted into the one remaining *Xba*I site to produce a true tandem repeat.

The cassette vector, pWB20 was created from the vector pFO20, which contains the PVA gene plus 1.2 kb of upstream (promoter region) and 0.8 kb of downstream (transcriptional terminator region) DNA sequences inserted into the popular cloning vector pUC19. To convert pFO20 to pWB20, it was necessary to remove six restriction sites, add four new restriction sites (one with an overlapping *dam* methylation site), and to modify the translation start

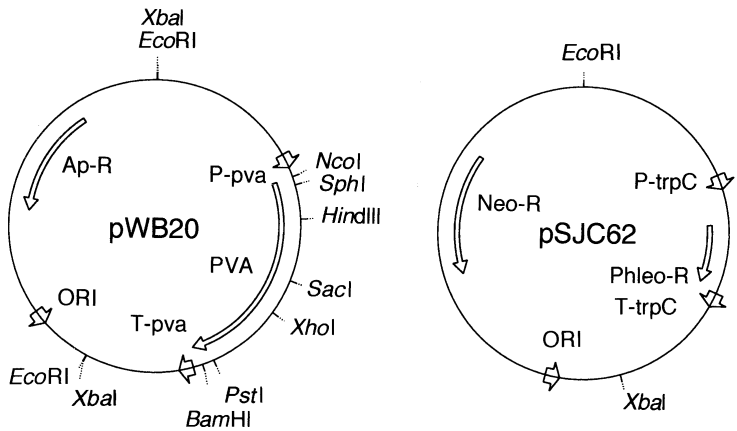

Fig. 9.2. Plasmid maps of pWB20 and pSJC62.

site. Although introducing this number of changes appears daunting, it was easily accomplished by PCR with carefully designed primers. The strategy is outlined in Fig. 9.3. Firstly, an *Nco* I site was removed from the transcriptional terminator by digestion, filling-in, and religation. Next, PCR primers were made to amplify either the promoter region or the coding and terminator regions. The primers were 29–33 bp long and their 3′ ends provided 14–19 bases of homology to the PVA gene. The 5′ sequences of the primers were designed to create all of the new restriction sites needed for pWB20. For the annealing step of the PCR, the T_m is about 50°C when the template is the pFO20 vector (since only the 3′ end of the primer hybridizes). After the initial rounds of PCR, however, the newly synthesized template will be complementary to the entire primer and the T_m will increase to 80–90°C. To optimize the specificity of the PCR, it was carried out in two stages. Firstly, a PCR with an annealing temperature of 48°C was run for six cycles to generate a small amount of newly synthesized template. This was followed by a two-step PCR (94° and 70°C) for 35 cycles to amplify the fragments. The PCR-generated fragments were then digested with restriction enzymes to produce 'sticky' ends and ligated into a modified pUC19 vector (pUC19EB) which had most of the polylinker region removed. The resulting pWB20 vector is now being developed to express heterologous genes in its *Fusarium* host.

9.4 Site-directed Mutagenesis by PCR

After an expression vector (usually a plasmid) has been constructed, it is often desirable to introduce mutations into the vector either to alter the properties of an expressed gene product or to increase its level of expression. In some cases, the desired mutation will be very specific (for example, the introduction of new restriction sites or changing a single amino acid at the active site of an enzyme). In other cases, it may be preferable to introduce random mutations into a small region of the vector (for example, when trying to increase the strength of a promoter). In either case, the most convenient way to mutagenize specific sites or regions of the vector is by PCR. Several of the most commonly used procedures will be described below.

9.4.1 Introduction of specific mutations

All of the procedures to create non-random mutations begin with the synthesis of PCR primers which contain the desired mutation. Either a region of the plasmid or the entire plasmid is amplified, and the vector containing the mutation is reconstructed.

Inverse PCR (IPCR) and enzymatic inverse PCR (EIPCR)

An easy way to mutagenize a circular plasmid, inverse PCR procedures require only two primers to the complementary strands of the plasmid.

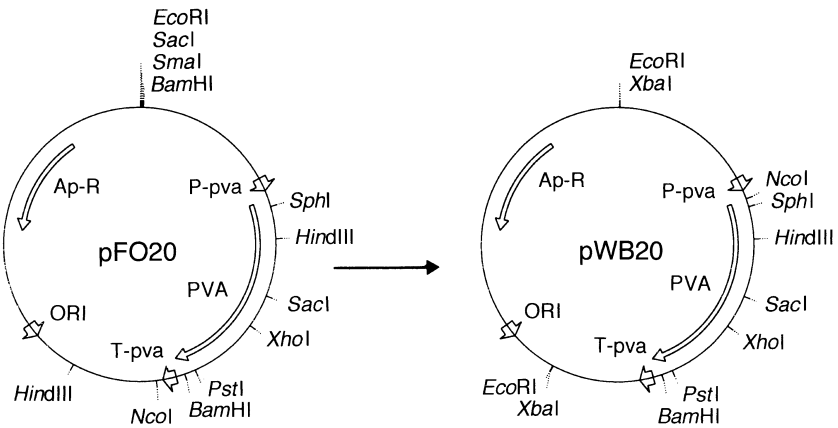

Fig. 9.3. pWB20 Construction strategy.

1. Cut pFO20 with *Nco*I, fill in, and religate (= pFO20ΔNco). [Removes unwanted *Nco*I site from the PVA terminator region.]
2. Cut pUC19 with *Eco*RI + *Hind*III, add a linker, and ligate (= pUC19EB). Cut the plasmid with *Eco*RI and remove 5′-phosphates. [Removes all of the polylinker region, keeping only the *Eco*RI site and adding a *Bgl*II site.]
3. PCR the promoter region of pFO20ΔNco and cut the product with *Eco*RI + *Nco*I. [Adds an *Eco*RI and *Xba*I (with overlapping *dam* methylation) site to the 5′ end and an *Nco*I site at the translation start site.]
4. PCR the coding and terminator region of pFO20ΔNco and cut the product with *Eco*RI + *Nco*I. [Adds an *Eco*RI and *Xba*I site to the 3′ end, an *Nco*I site at the translation start site, and adds one amino acid to the N terminus of PVA.]
5. Ligate the three fragments from steps 2–4. [If the correct fragments come together, pWB20 is produced. The correct construct is identified by restriction analysis.]

Their 5′ ends are adjacent to each other, and one of the primers contains the desired mutation (Helmsley *et al.*, 1989). PCR with these primers will amplify the entire plasmid as a linear molecule, and this can then be recircularized with T4 DNA ligase and transformed into an *E. coli* host. Because the ends of the molecule are not always precisely blunt, it is often necessary to sequence the junction to confirm that no additional mutations have been introduced. A better way to accurately recircularize the plasmid is to use overlapping complementary primers containing a unique restriction site and digest the ends with the restriction enzyme before ligation (EIPCR). If a unique restriction site is located close enough to the mutation site, complementary primers containing both the restriction site and the mutation can be used for the PCR. Even if there are no restriction sites close to the mutation site, a unique class 2s restriction site whose recognition site is 5′ to the cutting site can be added as a 5′ extension to both primers

(Stemmer and Morris, 1992). Digestion of the amplified DNA with the class 2s restriction enzyme removes the recognition site and leaves complementary overhangs which are part of the original plasmid sequence. When ligated into a circle, the new plasmid is identical to the old one except for the introduced mutations.

Recombination PCR (RPCR)

Similar to IPCR, an entire plasmid is amplified with complementary primers which contain the desired mutation. Instead of recircularizing the plasmid *in vitro*, the linear DNA is transfected directly into competent *E. coli* cells (Jones and Howard, 1991). The host cells (including *recA*$^-$ strains) contain recombinational repair enzymes which can recircularize the plasmid *in vivo.* Although initially simpler than IPCR or EIPCR, some errors can be introduced during this recombination, so further characterization of the plasmid is often advisable.

Overlap PCR

Introducing a mutation into the middle of a linear fragment of DNA requires four PCR primers. Two primers are complementary to the ends of the fragment, and two are complementary to each other and contain the desired mutation. Initially, two separate PCRs are carried out which amplify the two halves of the fragment. If the overlapping region containing the mutation also contains a unique restriction site, the two fragments can simply be digested with the enzyme and ligated together. If not, one alternative is to add a class 2s restriction site to the 5′ ends of the primers containing the mutation (Tomic *et al.*, 1990). When digested with the enzyme, the recognition sites are removed and the complementary overhangs contain only sequences from the original DNA fragment plus the mutation. A second alternative is to allow the overlapping region of the two fragments to prime each other in a third PCR reaction (Ho *et al.*, 1989). The two halves of the fragment are combined with the two primers which are complementary to the ends of the original DNA fragment and the PCR is repeated to regenerate the original fragment with the desired mutation. This approach can also be used to fuse together two unrelated gene fragments.

9.4.2 Introduction of random mutations (mutagenic PCR)

Depending upon the application, it may be preferable to introduce random mutations over a small region of an expression vector rather than create a larger number of plasmids with specific mutations. Mutagenic PCR takes advantage of the fact that native *Taq* DNA polymerase has an unusually high error rate (approximately 10^{-4} per nucleotide incorporated). This rate can be further increased by manipulating the concentration of $MgCl_2$, $MnCl_2$, and dNTP in the PCR reaction mix (Cadwell and Joyce, 1995). By carrying out a mutagenic PCR on a small region of an expression vector and subsequently

subcloning the fragment back into the vector, a large number of random mutations in a small region of the plasmid can easily be generated.

9.5 Characterization of High-producing Industrial Strains

Fungi have been employed in the fermentation industry for over 100 years. Traditional strain improvement methods such as random mutagenesis and selection have led to tremendous increases in the productivity of these organisms. The genetic changes that have resulted in these improvements, however, remain largely uncharacterized. PCR applications such as reverse transcription PCR (RT–PCR) and differential display RT–PCR (DDRT–PCR) enable us to identify genes which have altered expression levels in higher-producing organisms. Identifying these mutations enables the development of rational strategies for the genetic engineering of these strains for further improvements in productivity. Furthermore, knowledge gained regarding the genetic changes in one strain improvement programme may also help to speed productivity improvements of other related products. PCR can then be used for the identification and characterization of these genetically engineered fungi.

9.5.1 Characterization and quantitation of mRNA

RT–PCR is a technique for analysing gene expression by specific amplification of cDNA which has been synthesized from mRNA (reverse transcription). It can be used simply to detect the presence of a specific RNA transcript, or when a competitive template of a known quantity is added to the reaction, RT–PCR can be used to quantitate expression levels in RNA samples. The competitive template should be as similar to the cDNA target as possible. A cloned genomic fragment provides a good competitor if the PCR primers are chosen so that they span an intron. The amplified products can then be distinguished by the difference in size between the genomic and cDNA sequences. The advantage of RT–PCR over Northern blotting is that a smaller amount of total RNA of a lesser quality is required and further purification of poly(A)-tailed mRNA is not necessary. This is particularly helpful when dealing with fungal organisms from which RNA is difficult to extract.

In the case of the β-lactam antibiotics produced by *Penicillium chrysogenum* and *Acremonium chrysogenum*, most of the genes involved specifically in the biosynthetic pathway have been cloned (Martin *et al.*, 1994). In the higher producing strains, accumulation of beneficial mutations has increased the expression of these genes dramatically. Mutations in promoter regions can increase expression levels and alter the pattern of regulation that these genes display. Mutations in the coding region can affect the activity of the β-lactam biosynthetic enzymes, resulting in increased productivity of the

organism. One approach for analysing the mutations accumulated through the course of strain improvement uses PCR to amplify a defined region of genomic DNA from a number of different strains followed by a non-isotopic RNase cleavage assay to locate mutations (Myers *et al.*, 1985). This technique locates the position of mutations so that sequencing can be limited to regions where mutations have been previously been identified. PCR primers designed to amplify a genomic region of 100–1000 bp in length are used to amplify a specific promoter or coding region from a wild-type or early development 'reference strain' and from higher-producing strains. These amplified products can be cloned into a vector between two different bacteriophage promoters. Separate sense and antisense transcripts can be generated by adding one of two different bacteriophage RNA polymerases. The sense strand amplified from the reference strain is annealed to the antisense transcripts generated from the high-producing strains. Mismatches in the complementary RNA templates will result in positions susceptible to RNase cleavage. By sizing the products on an agarose gel, the positions of these mismatches can be mapped, indicating the location of mutations incurred during strain improvement.

9.5.2 Differential display of fungal mRNA

The comparison of gene expression in fungal cells at various stages of differentiation has traditionally been performed by subtractive hybridization (Zimmerman *et al.*, 1980). This technique is time consuming and requires the isolation of high quality mRNA. DDRT–PCR was developed to analyse a broad spectrum of expressed genes and to detect differences in expression levels between cell types (Appleyard *et al.*, 1995). DDRT–PCR relies on the use of short random primers that non-specifically amplify fragments (<1000 bp) of cDNA. Differences in the expression of various genes can be determined by comparison of the DNA banding pattern observed upon amplification of different RNA samples. For example, antibiotics are produced by fungal organisms as secondary metabolites; specific conditions such as environmental stress are required for antibiotic production. DDRT–PCR of RNA isolated from cultures grown at either logarithmic phase (trophophase) or producing phase (idiophase) can be used to identify the genes that are expressed during the synthesis of antibiotics. These genes, related to antibiotic synthesis, are good targets for cloning and overexpression (Usher *et al.*, 1992). For a company with a long history of strain improvement, analysis of high- and low-producing strains by this method can determine the genetic changes resulting in higher productivity. The DDRT–PCR and RT–PCR techniques are described in detail in other chapters of this book.

9.5.3 Characterization of transformants and integration sites

PCR is useful for identifying and characterizing fungal transformants. Because many selection systems for fungal transformation are not 100%

selective, abortive transformants due to the transitory uptake of transforming DNA can outgrow the selection. Fungal transformation in industrial applications is almost exclusively integrative. Therefore, to identify true transformants, genomic DNA is first isolated from putative transformants and a dot-blot or PCR amplification of the integrated DNA is performed. Nowak *et al.* (1995) used PCR to confirm that a linear DNA fragment which contains a mutant tubulin gene and lacks any bacterial sequences can confer resistance to benomyl and is sufficient for transformation of *Acremonium chrysogenum*.

The expression level of a heterologous gene which has been integrated into a fungal chromosome is often determined by the site of integration as well as by any regulatory sequences on the expression vector. A gene integrated into a transcriptional 'hot spot' can express at higher levels than the same gene inserted elsewhere on the chromosome. If a putative hot spot is identified after the random insertion of a vector, it may be desirable to isolate the flanking sequences from the host chromosome. These sequences could then be added to future expression vectors to direct integration to the hot spot by homologous recombination. Inverse PCR can be used to amplify the host DNA sequences flanking integrated transforming DNA. A Southern blot of the genomic DNA is first performed to identify restriction fragments which contain the integrated DNA and flanking genomic DNA. Sometimes a single fragment will contain both the upstream and downstream sequences, otherwise two separate fragments may be used for amplification of each flanking region. Once the restriction enzyme is chosen, genomic DNA is digested, diluted, and ligated so that the restriction fragments circularize. This material will serve as the template for the amplification. Unlike standard PCR where the primers are designed so that they amplify the sequences between them, primers for inverse PCR are designed to extend their template in outward directions. When annealed to the circular template, these primers will amplify a fragment that contains the sequences flanking the primers until they join at the restriction site used for cleavage of the genomic DNA. This amplified product can then be cloned and sequenced.

9.6 Future Applications

9.6.1 Efficient secretion of heterologous proteins

The efficiency of protein secretion is protein-specific and recombinant proteins are often difficult to secrete at high levels in fungal hosts. The effort at Genencor to produce bovine chymosin in *A. nidulans* and *A. awamori* demonstrated that transcription was not a limiting factor in chymosin production but that secretion may have been inefficient (Ward *et al.*, 1990). The productivity and secretion of the chymosin protein was significantly improved by engineering a fusion protein product which links the cDNA

encoding bovine prochymosin in frame to the last codon of the *A. awamori* glycoamylase gene. The produced chymosin is autocatalytically released at pH 2 from the fusion protein after secretion (Ward *et al*., 1990). This work demonstrated that secretion of the fusion protein is more efficient than for heterologous protein alone. If autocatalytic release does not occur in the fungal system, a cleavable linker peptide can be engineered between the proteins to allow separation of the products for purification. Site-specific mutagenesis by PCR or overlap PCR (Pont-Kingdon, 1994) can be used in the construction of fusion vectors and the generation of a cleavable linker.

9.6.2 Host improvement for heterologous gene expression

The ability of filamentous fungi to secrete large quantities of protein has made them an attractive host for the production of recombinant proteins. For the proper expression of therapeutic human proteins in filamentous fungi, several aspects need to be considered in addition to the efficient expression of heterologous genes:

- stability of the secreted preteins in the fermentation media;
- glycosylation pattern/ biological activity of the secreted proteins;
- 'generally regarded as safe' (GRAS) strain development.

Proteinase-deficient mutants

A recombinant protein secreted into the fermentation medium may be degraded by extracellular fungal proteinases. The aspartic proteinase of *A. awamori* had been shown to degrade secreted bovine chymosin and the gene encoding this enzyme was cloned (Berka *et al*., 1990). Deletion of the native aspartic proteinase gene in *A. awamori* resulted in a significant reduction in overall extracellular proteinase activity. The production of chymosin was improved seven-fold by combining expression in the proteinase-deficient mutant with a classical strain improvement programme (Dunn-Coleman *et al*., 1991). Based on the deduced amino acid sequence, *A. awamori* aspartic proteinase shares homology with other fungal aspartic proteinases (Berka *et al*., 1990). Cloning of conserved protease genes in other fungal hosts by PCR could be performed as discussed earlier. Aspartic proteinase-deficient mutants could then be constructed by gene disruption or deletion. A number of genes encoding other fungal proteinases have been isolated (Van den Hombergh *et al.*, 1996), and some of these genes are highly conserved in fungi. Cloning of these genes can be facilitated by PCR and used to construct proteinase-deficient mutants.

Glycosylation engineering

The correct carbohydrate composition of many human therapeutic proteins is essential *in vivo* for their solubility, stability, and biological activity, as well as to avoid recognition by the human immune system. The recombinant mammalian proteins produced in yeast usually have a large number of

mannose residues (hyperglycosylation) which alter their serum half-life and antigenicity in mammals (MacKays, 1987). The glycosylated recombinant mammalian proteins produced in filamentous fungi usually co-migrate with the natural protein in SDS–PAGE gels, indicating no excess glycosylation. Upshall *et al*. (1987) have shown that human tissue plasminogen activator (tPA) produced in *A. nidulans* is correctly processed at the amino terminus and is not over-glycosylated. Ward *et al*. (1992) have also demonstrated that recombinant human lactoferrin expressed in *A. oryzae* has the same level of glycosylation as the authentic lactoferrin present in human milk. However, the nature and chemical composition of the carbohydrates added to these recombinant proteins derived from different fungal expression systems have never been characterized. When the cDNA encoding tPA was expressed by a strong fungal promoter in *A. nidulans*, the recombinant tPA protein was overglycosylated (Upshall *et al*., 1991). The authors suggested that in the high-yielding transformants, the translation of mRNA proceeds faster than the ability of the host cell to process and to translocate the protein extracellularly. The intracellular accumulation of this protein in the Golgi apparatus results in the continued addition of excess carbohydrate side-chains. A similar observation of over-glycosylation of recombinant proteins has been observed in the high-level expression of an aspartic proteinase derived from *Rhizomucor miehei* in *A. oryzae*, although the biological activity was not affected (Christensen *et al.*, 1988). Therefore, in order to achieve the correct level of glycosylation, high-level expression of mammalian glycoproteins is not always desirable.

There is an increasing understanding of the biosynthetic pathways of protein glycosylation and the relationship between glycosylation and the biological activity of various proteins (Stanley, 1992; Jenkins and Curling, 1994). With this basic knowledge, a filamentous fungal host can be engineered by removing the fungal glycosyltransferases and adding the desired mammalian glycosyltransferases. Cloning, inactivation, and replacement of these genes can be achieved by applying PCR technology. Recently, Borsig *et al*. (1995) have demonstrated the expression of a functional human α-2,6(*N*)-sialyltransferase enzyme in *S. cerevisiae*. Theoretically, the same gene could be expressed in filamentous fungal cells.

GRAS strain development

For the production of recombinant proteins or metabolites by fungal organisms, it is important to use a fungal host which has a GRAS status. One of the most important factors that is required to recognize a fungal strain as safe is the absence of mycotoxins which are toxic to humans, animals, or plants. A number of fungal strains used for antibiotic and food industries are considered GRAS strains by the Food and Drug Administration (FDA) in the United States. These include *A. awamori*, *A. oryzae*, *A. chrysogenum*, *P. chrysogenum* and *Fusarium graminerarum* A3/5. If a mycotoxin-producing fungus must be used to produce a desirable product, the cloning and

subsequent inactivation of genes encoding for one or more key enzymes in the mycotoxin biosynthetic pathway could be used to generate new GRAS strains. PCR will undoubtedly serve as a key technology for cloning and characterizing these genes.

9.7 Summary

This chapter details the many uses of PCR technology in the genetic engineering of industrial fungi for the commercial production of antibiotics, enzymes, secreted proteins and speciality chemicals. One of the major activities of industrial biotechnology process improvement laboratories is to overexpress the desired antibiotic, enzyme, or therapeutic protein using modern genetic engineering strategies through the construction of efficient expression vectors. PCR technology appears to be particularly useful in the development of improved vectors since most of the essential expression components are derived from existing vectors but require changes in flanking restriction sites. One of the highlights of this chapter is a detailed description of how PCR technology was used to overproduce penicillin V amidase (PVA) in engineered strains of *F. oxysporum*. When this host was transformed with a vector containing a tandem repeat representing two copies of the PVA gene, a nearly ten-fold improvement in enzyme production was noted.

PCR has also been shown to be a convenient procedure to mutagenize specific regions of an expression vector. Examples are described for the introduction of specific mutations using IPCR and EIPCR. The utility of RPCR, overlap PCR, and the use of PCR for the introduction of random mutations have also been highlighted in this chapter.

Another important application of PCR in fungal biotechnology is its use in characterizing high-producing industrial strains. Traditional strain improvement methodologies, including random mutagenesis and directed selection, have resulted in significant over-production of commercial metabolites. However, the genetic basis for these improvements remains largely uncharacterized. PCR strategies, including RT–PCR and differential DDRT–PCR, enable biotechnologists to identify discrete genes that may be tied to increased expression in higher-producing strains. These altered genes now provide for rational strategies for further improvements in strain productivity.

PCR applications will play even more important roles in future fungal biotechnology research and development programmes. Strategies have been described in this chapter on the efficient secretion of bovine chymosin through the fusion of the chymosin gene to the last codon of the *Aspergillus awamori* glucoamylase gene. Site-directed mutagenesis by PCR can be utilized for the generation of fusion vectors and cleavable linker protein products. Lastly, future uses of PCR and site-directed mutagenesis include the development of potential commercial, non-toxin-producing GRAS

fungal strains for the development of proteinase-deficient fungal isolates and for the development of fungal strains producing proteins with desirable changes in their protein glycosylation process.

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