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The Thom Award Address

Industrial mycology and the new genetics

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SUMMARY

The genetic investigation of fungi has been extended substantially by DNA-mediated transformation, providing a supplement to more conventional genetic approaches based upon sexual and parasexual processes. Initial transformation studies with the yeast *Saccharomyces cerevisiae* provided the model for transformation systems in other fungi with regard to methodology, vector construction and selection strategies. There are, however, certain differences between *S. cerevisiae* and filamentous fungi with regard to type of genomic insertion and the availability of shuttle vectors. Single-site linked insertions are common in yeast due to the high level of homology required for recombination between vectored and genomic sequences, whereas mycelial fungi often show a high frequency of heterologous and unlinked insertions, often in the form of random and multiple-site integrations. While extrachromosomally-maintained or replicative vectors are readily available for use with yeasts, such vectors have been difficult to construct for use with filamentous fungi. The development of vectors for replicative transformation with these fungi awaits further study. It is proposed that replicative vectors may be inherently less efficient for use with mycelial fungi relative to yeasts, since the mycelium, as an extended and semicontinuous network of cells, may delimit an adequate diffusion of the vector carrying the selectable gene, thus leading to a high frequency of abortive or unstable transformants.

INTRODUCTION

For want of a better definition, 'fungi are ornery microbes with creative instincts.' The adjectives 'ornery' and 'creative' apply especially to industrial fungi. The Society for Industrial Microbiology was founded in large part based upon the creative capabilities of fungi. The year was 1949 and the focus was on penicillin. No doubt, when Charles Thom was cataloguing the species of *Penicillium* and *Aspergillus* [92,112], he was impressed by the variety of metabolic activities associated with these fungi as well as by their potential for commercial development. He not only discussed penicillin production by *P. notatum* and related species, but provided details on the elaboration of other types of secondary metabolites, such as the production of organic acids, principally citric acid, the role of these fungi in providing flavor constituents for roquefort and camembert cheeses and their use in oriental food fermentations.

Thom was also aware of the limitations for genetic research with these largely asexual or anamorphic fungi. He devoted a chapter to variation, both between and within species, detailing the general utility of natural as well as 'imposed' variation in selecting strains for commercial use. Thom did not use the term 'mutation', although a program of mutagenesis and screening of survivors was implicit from his writings. The subsequent adoption of mutation/screening programs by industry has been most successful, converting trace amounts of compound into economical yield of product. Keep in mind that Thom's monographic studies predated concepts related to forced heterokaryosis, vegetative incompatibility, and parasexual recombination.

Conventional genetics of fungi typically involve in situ mutagenesis, either spontaneous or induced, and in vivo recombination mediated by a sexual or at least a parasexual cycle. The new genetics or molecular genetics involves in vitro recombination of DNA sequences by restriction enzyme cleavage and ligation and by use of site-directed mutagenesis of isolated nucleotide sequences. DNA that is recombined or mutant by design is then introduced to the cell line via transformation, circumventing a need for mating competence and sexuality.

TRANSFORMATION : INTEGRATIVE VERSUS REPLICATIVE

Today we have at our disposal procedures for in vitro manipulation of genetic material through recombinant DNA (rDNA) technology and the concerted introduction of recombined constructs into fungal systems. As such we are able to draw upon a world gene pool.

Simply defined, genetic transformation is a heritable change in a cell line mediated by the uptake and expression of exogenously supplied DNA. The transformation event may be additive and involve expression of a newly-introduced gene or it may be subtractive and result in disruption or replacement of some resident gene activity. Nucleotide sequences involved in

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the transformation may be inserted into chromosomal or genomic sites (integrative transformation) or they may be located on an extrachromosomal and autonomously replicating element (replicative transformation).

Regardless, the introduced or vectored DNA is typically a hybrid or chimeric molecule, as it carries: (i) a prokaryotic-specific replicon as well as a drug resistance marker for cloning and selection in bacteria and (ii) a selectable marker gene with appropriate expression signals for transformation of the fungus.

Indeed, heterologous gene expression and a wide latitude in promoter recognition among unrelated species are now evident and render it possible to develop transformation schemes for a broad spectrum of less well-studied fungi. To date nearly 100 species of fungi have been transformed, including species with limited or no potential for traditional genetic study [37,38,64].

Integrative vectors may insert DNA sequences at heterologous or homologous genomic sites; transformants are characteristically obtained at rather low frequencies but are relatively stable, even without post-transformational selection. Replicative vectors contain a fungal-specific replicon for autonomous replication to high copy number or, as an alternative, may be variously modified to function as an independent minichromosome; transformants are typically obtained at higher frequencies but are relatively unstable, unless continuous selective pressure is applied.

These general comments on fungal transformation and exceptions to such generalizations will be discussed in the text that follows.

TRANSFORMATION IN YEAST

Two pioneering studies with *Saccharomyces cerevisiae* [9,49] provided the first compelling evidences for fungal transformation and have provided models for the design of transformation experiments with other yeast species and with many filamentous fungi. Both of these early studies (Table 1) used protoplast preparations of strains auxotrophic for leucine. The strains carried mutations at the *leu*2 gene, a gene encoding β-isopropylmalate dehydrogenase. Both studies employed defined chimeric plasmids containing the wildtype allele for *leu*2, and thus selection for transformation was based on prototrophic growth in the absence of leucine. Procedurally, the vector DNA was added to osmotically stabilized protoplasts in the presence of calcium chloride ($CaCl_2$) and the preparation was subsequently treated with polyethylene glycol (PEG), a procedure that is now adopted most commonly in fungal transformation experiments.

Results from these two initial studies with *S. cerevisiae* provided convincing evidences, respectively, for integrative transformation [49] and replicative transformation [9]. The studies employed Southern (1975) hybridization probes for vectored sequences in order to confirm the transformations and to distinguish between integrative (chromosomal) and replicative (extrachromosomal) location of the transforming DNA. Integrative transformation of three types was described

TABLE 1

Yeast transformation

Integrative [49]	vs	**Replicative [9]**
	– protoplasts used (Ca/PEG) **– prototrophic selection**	
Genomic insertion (three types, see Fig. 1)	**– chimeric vector DNA**	Autonomous replication (*ori, ars*)
Low; one/μg DNA	**– frequency**	High; 3000/μg DNA
Stable	**– stability without selection**	Unstable
Indirect	**– recovery in *E. coli***	Direct
Mendelian	**– inheritance**	Non-mendelian

by Hinnen and coworkers [49], although homologous single-site integration was the type most common in the yeast system.

Transformation frequencies vary significantly between integrative and replicative modes of transformation, and this difference was evident from these early studies. Typically, 1–100 (rarely more but less than 1000) transformants are derived per μg DNA per 10^7 protoplasts via integrative transformation, whereas 1000 to several thousand transformants per μg DNA per 10^7 protoplasts are obtained via replicative transformation.

Direct recovery in *E. coli* of the initial transforming vector from a fungal transformant (replicative transformation) was evident in the study by Beggs [9], whereas indirect recovery in *E. coli* of a vector or vector sequences from a transformed fungus (integrative transformation) required restriction enzyme digestion of the genomic DNA of the fungus and subsequent ligation of derived fragments [107].

DNA UPTAKE

Methodology for DNA-mediated transformation of fungal cells involves a sequence of several steps (Table 2). These include: (i) preparation either of protoplasts or of intact cells that are competent for uptake of DNA, (ii) incubation or

TABLE 2

General transformation protocol

- Generation of competent propagules (protoplasts)
- Treatment of propagules with transforming DNA (Ca/PEG, lithium salts, electroporation, biolistics)
- Regeneration of treated propagules on selective medium (antibiotic resistance, prototrophy)
- Isolation of stable transformants
- Analysis of transformants (Southern blot, genetic analysis)

exposure of such propagules to the transforming DNA, (iii) regeneration of the DNA-treated propagules into colony-forming units, and (iv) selection or visual detection of colonies (transformants) that stably incorporate and express the DNA. Transformants are isolated and then analyzed, either by hybridization probes and/or through genetic analysis, to confirm the presence of the introduced DNA.

The most consistent and widespread procedure for fungal transformation, especially with reference to mycelial fungi, is $CaCl_2$/PEG treatment of protoplasts exposed to vector DNA.

Procedures for the preparation of protoplasts employ muralytic enzymes from various sources [82], but the most commonly used enzyme preparation for transformation experiments is Novozyme 234 (Novo Industries, Copenhagen), a commercial enzyme mixture derived from the fungus *Trichoderma viride* [7]. Alternative procedures for DNA uptake, such as electroporation [8,84,88,94,96,98] and biolistics [2,67,115] are gaining acceptance. Electroporation offers the benefit of simple one-step sample preparation, but provides no substantial benefit in terms of increased transformation frequency, and protoplasts are still required for efficient transformation by this procedure. Biolistics is a relatively new procedure, employing specialized equipment and microprojectiles coated with the transforming DNA. Again, this procedure seems no more efficacious in yielding transformants, but it is especially useful with fungi that do not grow well in laboratory culture or that fail to yield regenerative protoplasts in sufficient numbers for transformation experiments.

Lithium salt treatment has been used with some success, initially with intact yeast cells [53], and subsequently with mycelial fungi [13,30,31,131].

VECTOR DEVELOPMENT

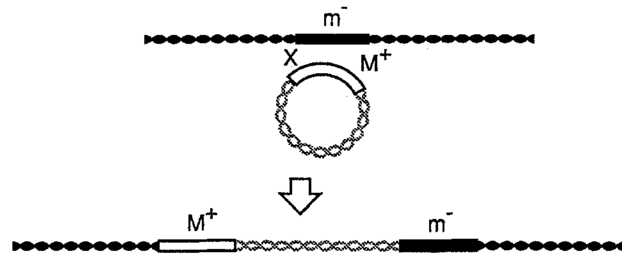
Integrative vectors

The minimal requirements for an integrative vector are: (i) a selectable marker with appropriate regulatory signals for expression in the transformed fungus (eukaryotic part) and (ii) a replicon plus selectable marker for the cloning and recovery of the vector in a bacterial host (prokaryotic part). Vector pAN7-1 is a typical and extensively used vector for integrative transformation (Fig. 2).

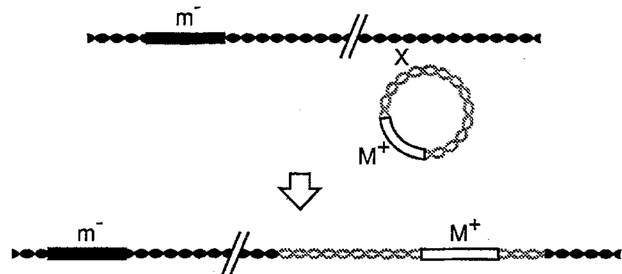
Based on restriction enzyme analysis of DNA from among transformants, coupled with genetic analysis of meiotic progeny from crosses between individual transformants and wild-type, Hinnen and coworkers [49] were able to discern three patterns of integrative transformation in *S. cerevisiae.* These patterns are depicted in Fig. 1 and explained in the figure legend. Their results and data from subsequent studies [78,80] indicated that homologous recombination was the rule for integrative transformation in *S. cerevisiae*, as Type I and III transformants were prevalent and Type II was exceedingly rare [79].

There appears to be far less of a threshold requirement for homologous recombination in the transformation of filamentous fungi, although results have not been consistent in this regard. The relative frequencies among the three patterns of integration vary in these fungi, depending on

I. Homologous (single cross-over/linked insertion)



II. Heterologous (single cross-over/unlinked insertion)



III. Homologous (double cross-over/gene replacement)

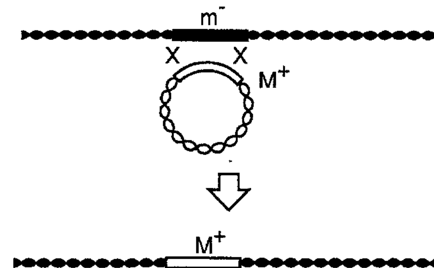


Fig. 1. Patterns of integrative transformation observed in fungi. DNA is illustrated by double-helical representation and is distinguished as chromosomal DNA (closed helix) or vector DNA (open helix). Specific alleles are shown as boxes labelled m^- (closed box) on the chromosome and M^+ (open box) on the vector. Type I integration involves homologous pairing and a single cross-over resulting in linked insertion with M^+ and m^- separated by intervening vector DNA sequences. Type II involves heterologous or ectopic pairing and a single cross-over resulting in unlinked insertion with M^+ and m^- widely separated (as shown) or on different chromosomes (not shown). Type III involves homologous pairing with cross-overs on either side of the m^-/M^+ pairing resulting in a substitutive event in which vector sequences other than M^+ are not integrated into the chromosome and the entire m^- is excised. The Type III pattern depicted is but one example of what may occur, since the insertion, depending on the sites of cross-over may not replace the entire m^- (gene disruption) but substitute a critical or essential sequence within the gene (gene replacement). Type III might also result from a mechanism other than simple cross-overs. Such a mechanism (gene conversion) is discussed by Fincham [37]. Types I, II and III can be distinguished by genetic analysis of meiotic progeny, showing either m^-/M^+ linkage (Type I), divergence in linkage or nonlinkage (Type II) or nonrecovery of the m^- allele (Type III).

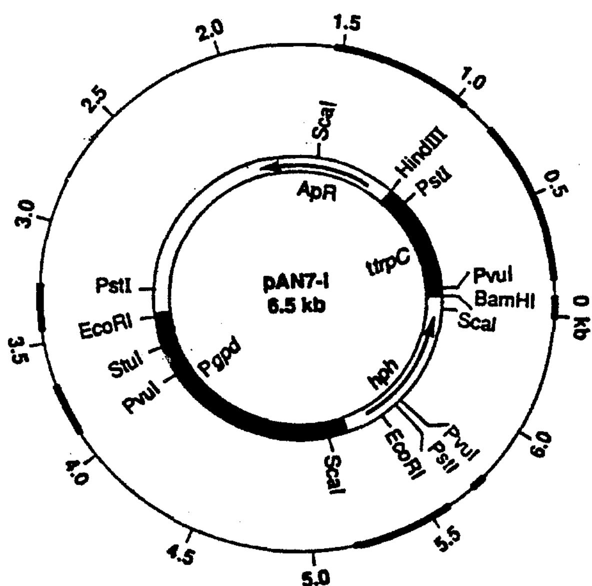


Fig. 2. Restriction enzyme map of the integrative vector pAN7-1 (6.5 kb). This vector (inner circle) contains the *E. coli* hygromycin B phosphotransferase (*hph*) gene fused to expression signals from the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (P*gpd*) and the transcription-termination sequence from the *A. nidulans* tryptophan synthetase (*ttrp*C) gene [89]. Blackened areas of the inner circle represent regions of the plasmid of fungal (*A. nidulans*) origin, whereas unshaded areas are of bacterial (*E. coli*) origin. The gene for resistance to ampicillin (Ap^R), as well as the direction for transcription (arrows) for Ap^R and *hph*, are indicated. The prokaryotic origin of replication (*ori*) of this plasmid is not shown but is located between Ap^R and P*gpd*. Relevant restriction enzyme sites are shown and the alternating thin and thick lines of the outer circle specify interval lengths in kilobases (kb) for adjacent restriction fragments.

the strain and even the marker used [5,10,13,20,30,32,39,42, 44,58,59,70,72,113,119,126,133].

Regardless, variable integration, with a predilection for Type II insertions, is a common feature among mycelial fungi. It is peculiar that addition of repetitive DNA to vectors often has no effect upon transformation frequency [101,113,125], but an exception has been noted in the case of *Alternaria alternata*, as addition of ribosomal DNA sequences to the vector enhanced transformation twenty-fold [117].

While sequence homology may not be a requisite for vector integration in the filamentous fungi, it is nonetheless apparent that these fungi do recognize homology between DNA sequences. Several lines of evidence support this view. Frequently, tandem repeats of an integrated sequence are observed at genomic sites, indicating either formation of plasmid oligomers in the fungus before integration or an integration of additional plasmid monomers by homologous recombination at the genomic site of initial integration [56,126].

Multiple copy integration, either in the form of tandem repeat sequences or multiple site integration, provides a mechanism for gene amplification potentially quite useful in applied research [50].

Replicative vectors

Replicative vectors, in addition to the requirements outlined for integrative vectors, require a eukaryotic (fungal) replicon. Such vectors, exemplified by yeast-replicating plasmids (YRp) are called shuttle vectors.

The development of systems for replicative transformation has depended largely upon modification of normally integrative vectors through in vitro introduction of replicons that are varied as to type and derivation. In yeast fungi, replicative vectors, based either upon an origin of replication (*ori*) from the 2-μm DNA plasmid of *S. cerevisiae* [9,104] or, alternatively, upon autonomously replicating sequences (*ars*) from the genome of *S. cerevisiae* [109,128], were among the first such vectors to be developed.

Sequences comparable to genomic *ars*-type sequences of *S. cerevisiae* have been identified in several other fungal systems (*Phycomyces blakesleeanus* [93]; *Mucor circinelloides* [97,112,122]; *Aspergillus oryzae* [61]; *Ustilago maydis* [118]). Fragments of mitochondrial DNA (mtDNA), that presumably contained either *ori* or *ars* have also been used in forming replicative vectors for transformation [12,34,62,102,105,106,113,132]. While these *ars*-type sequences are functional as replicons in *S. cerevisiae*, they often do not function as such in the source fungus. It is noteworthy that an *ars* sequence from a linear DNA plasmid associated with the mitochondria of *Nectria haematococca* has been used to develop a replicative vector for transformation of *U. maydis* [98]. Sequences initially considered to be *ori*-type sequences from circular DNA plasmids of mitochondrial origin have also been used to develop replicative vectors for transformation in *Podospora anserina* [106] and *Neurospora crassa* [45,108]. However, the confirmation and sequence identity of such putative replicons await further investigation.

The AT-rich *ars* consensus sequence, (A/T)TTTAT (A/G)TTT(A/T), is estimated to be distributed throughout the *S. cerevisiae* genome in about 400 copies [36,128]. This 11-bp sequence plus associated DNA-bending activity influenced by sequences flanking the *ars* are important for replicative activity [75,127].

Yet another source of replicons for construction of replicative vectors in fungal transformation has involved portions of chromosomes, such as the centrometric (*cen*) region of the *S. cerevisiae* chromosome III [24,74] and the centromere-affiliated (*ans*) sequence from a genomic library of *A. nidulans* [4,15,27]. Unfortunately, *cen* sequences from *S. cerevisiae* do not function in *A. nidulans*, despite the fact that respective 7-bp consensus sequences for one *cen* (TTCCGAA) and *ans* (TTCCTAA) have considerable homology. Centromere-based plasmids of *S. cerevisiae*, however, do function in the congeneric *S. kluyveri* [40].

Vectors that are centromere-based, if also replicative, may be combined with telomeric portions of chromosomes [74,87,88,110] thereby effectively converting the vector into a linearized minichromosome with the potential for autonomous replication. More recently, the introduction of telomeric sequences to the ends of linear extrachromosomally-maintained DNA in the fungus *Crytococcus neoformans* has been reported [33]. The addition of such sequences per se increased

the efficiency, and presumably also the replicative potential, of a linearized vector for transformation of this yeast. Again, the identity and nucleotide sequence of replicons involved in such plasmid constructs remain to be determined. It has been suggested that the mere introduction of the 6-bp telomeric repeat sequence (TTAGGG) may be sufficient to convert an integrative vector into a replicative one carrying some potential for self-replication [14,33,130].

Yeast artificial chromosomes (YAC), initially developed from linearized YRp plasmids, contain both centromeric and telomeric sequences [14,74]. These vectors are maintained at one copy per cell and behave during mitotic growth and through meiosis as stable vectors.

An artificial chromosome must have: (i) at least one *ori* or *ars*, (ii) telomeric sequences at the termini of the linear DNA, and (iii) a centromeric region for division spindle fiber attachment during division. The YAC have proven to be most useful in constructing gene libraries for eukaryotes, especially mammalian systems, since inserts in the order of 1 Mb are clonable, thereby overcoming problems inherent in screening larger cosmid-based libraries [73,81,95,100]. Transformation frequencies using YAC may show size bias for the insert; inserts of approximately 330 kb seem to be an optimal size and the size bias can be minimized by addition of polyamines [25]. Recently, both copy-number control elements [103] and a mammalian-derived *ori* [76] have been incorporated into YAC, improving the prospects for gene amplification through a transformation system based on this type of vector. By inserting a rescue plasmid with a selectable marker into a YAC it is possible to recover fragments from such a construct by homologous recombination [47].

Artificial chromosomes comparable to that of YAC remain to be developed for mycelial fungi. Telomeric DNA has been isolated from *N. crassa* [99]. Recently, telomeric DNA from *P. anserina* has also been isolated and used to form a linearized self-replicating plasmid [54], and a centromeric region of this fungus has been cloned [29]. These results portend the development of an artificial chromosome for use in filamentous fungi.

Despite the extent and scope of varied procedures for in vitro manipulation of vectors in the development of fungal transformation systems, there are rather few examples for in vivo generation and maintenance of replicative transforming molecules among fungal systems. Noteworthy in this regard are the recombinant plasmids with autonomously replicative potential that have been described in three basidiomycetes, *U. violacea* [11,86], *Phanerochaete chrysosporium* [90,91], and *Pleurotus ostreatus* [83]. In *U. violacea* the actual transformation for antibiotic resistance involved integrative events, even though replicative molecules containing initial vector sequences, as well as DNA sequences derived from mitochondria, were identified in certain of the transformants [86]. It is not known if the mitochondria of *U. violacea* harbor linear DNA plasmids similar to those described for *Nectria haematococca* [88] and *Pl. ostreatus* [134] or if *ars*-type sequences from the mitochondrial genome were involved in recombining with the initial vector.

By contrast, the in vivo generated replicative plasmids of *Ph. chrysosporium* were apparently formed through recombination between a low-copy endogenous circular DNA plasmid and the original non-replicative vector [90,91]. The transformants of *Ph. chrysosporium* did not appear to contain integrative vector sequences, unlike results reported for *U. violacea*.

In *Pl. ostreatus* [83,85] two plasmids have been analyzed and share a DNA insert now known to be homologous with a 1.15-kb sequence from P1 bacteriophage [48]. The *Pl. ostreatus* transformants consistently lacked evidence for integration of vector sequences into chromosomal DNA, and if such integration occurred at all, it must have been highly transient [83,85]. These results suggest that the insert sequence of P1 origin somehow functions as a surrogate replicon in *Pl. ostreatus*.

Also noteworthy are examples of replicative molecules in one highly unstable strain of *A. nidulans* [41], in the phytopathogenic *Fusariam oxysporum* [88], and in the zoopathogenic *Histoplasma capsulatum* [129,130]. The plasmid of *A. nidulans* contains a chromosomal-derived insert (*AMA*); it is circular and supercoiled and undergoes in vivo rearrangement. The *AMA* sequence, when introduced into appropriate selection vectors, apparently directed autonomous replication in two other *Aspergillus* species and in *Gibberella fujikuroi*, although only a two-fold increase in transformation frequency was observed [16]. The recombinant plasmids of *F. oxysporum* and *H. capsulatum* were derived through vector and chromosome recombination and contain telomeric sequences. The *F. oxysporum* plasmid contains an *ars*, and although nonreplicative in *S. cerevisiae*, replicates in two related plant pathogens, *N. haematococca* and *Cryphonectria parasitica* [88].

An additional example for a replicative plasmid is the circular plasmid of the zygomycete *Absidia glauca* [18]. This plasmid contains an insert derived from the *SEC*1 genomic locus and can also replicate autonomously in another zygomycete, *Parasitella simplex* [17].

It is assumed that replicative vectors, although extrachromosomal, may be located in the nucleus wherein relevant enzymes for DNA replication are available. The frequent association of centromeric and telomeric sequences with these vectors, effectively improving upon transformation frequency and more stable transmission, is presumably related to an association with the cytoskeletal network. Replicative vectors without such an association may not be stably maintained or transmitted readily through a growing mycelium. This might compromise sufficient and sustained expression of the selectable gene vectored into the mycelium, resulting in formation of minicolonies or even leading to lethal consequences. Filamentous fungi, as opposed to yeasts, may be more prone to lose such vectors, even with selection. This in part may explain the continued lack of success in developing generalized vectors for replicative transformation with mycelial fungi.

PROMOTER RECOGNITION

A fungal transformation vector requires appropriate regulatory sequences to insure proper expression for the introduced

gene(s) of interest. A promoter or transcription-initiation sequence, positioned in the region upstream or 5′ to the ATG start codon of the reading frame, is an absolute requirement. In this 5′ region a signal or secretion sequence may also be required, if the ultimate gene product is to be transported from the cell. At the 3′ end of the gene a transcription-termination sequence beyond the stop codon may be required.

Fungal genes that have been cloned for use in vector construction often carry their native regulatory sequences and it is assumed, a priori, that these homologous expression signals are likely to be more efficient than corresponding heterologous or interspecific signals. This assumption is based on early indications that the yeast *S. cerevisiae* only poorly recognizes mycelial fungal promoters and can, at best, only poorly excise introns from these fungi [52,69].

It is now evident that there is a wide latitude for promoter recognition among the non-yeast ascomycetes and the basidiomycetes, and that other types of regulatory signals may similarly be functional across generic and even higher taxonomic categories [51,66,68]. Since there are rather few studies on this subject, there are few conclusions to be drawn.

A rather detailed study on the activity of promoter and terminator sequences obtained from a range of fungi has been tested with respect to oomycetes [55]. Transient assays using the *uid*A or GUS reporter gene were conducted with *Phytophthora infestans*, *P. megasperma*, and *Achlya ambisexualis*. Oomycete promoters, derived from a heat-shock gene (*hsp* 70) of *Bremia lactucae* and from an actin gene of *P. megasperma*, regulated high GUS accumulation in each of the three oomycetes. By contrast, no activity or very limited activity was evident using promoters from higher fungi (four ascomycetes and one basidiomycete). An oomycete terminator was not absolutely required for GUS accumulation. These results indicate that transcriptional machinery among such lower fungi differs substantially from that of higher fungi, and that sufficient conservation exists to allow vectors to be developed using expression signals from different oomycetes.

BIOTECHNICAL ASPECTS

The application of in vitro genetic methodology, through transformation and related procedures based on recombinant DNA (rDNA), has considerably broadened the prospects for industrial strain improvement. Scientists theoretically have at their disposal a kind of world gene bank, which provides not only an extended catalogue of structural genes but a vast array of regulatory signals—all subject to introduction into cloning vectors by in vitro recombination. Such cloning procedures are supplemental to traditional strategies in biotechnology based on in vivo recombination and random mutation.

This paper has discussed transformation procedures for the fungi and the development of cloning vectors for use with these organisms. These lower eukaryotes combine ease in experimental manipulation with attributes required for eukaryotic molecular cloning. As such, fungi are especially important for industrial processes in which there is a need for an organism that is competent to process and express eukaryotic genes properly, in contrast to prokaryotic systems that can neither splice mosaic genes nor recognize eukaryotic transcription, translation and signal sequences.

The new genetics represents a progression in thinking among molecular geneticists away from strictly basic research opportunities. Much emphasis has been given to yeast systems in this regard. There is also much interest in using mycelial systems, especially species of *Aspergillus*, for the production of heterologous proteins [35,66,114]. The rationale for this is that mycelial fungi: (i) readily recognize intron splice sites of genes from heterologous sources [26], (ii) exhibit high level gene expression [1,22,23,57,77,124] and secretion of product [46,65,123], and (iii) possess strong and widely-recognized promoters such as P*gpd* and *Pamd*S [28,121]. Furthermore, there is considerable industrially-based experience in growing such fungi at production levels.

The potential applications for fungal transformation schemes in biotechnology are many and are evident from recent studies involving heterologous gene expression. Examples are: (i) biocontrol of the chestnut blight fungus, *Cryphonectria parasitica*, by integrating a cDNA copy of an RNA virus that confers hypovirulence [21]; (ii) cloning of specific mating type genes and demonstrating that the products of such genes are DNA-binding proteins that exhibit cross-species function [3,120]; (iii) targeting of a mitochondrial-derived gene from corn into yeast mitochondria and demonstrating expression by dysfunction for oxygen uptake [43]; (iv) intergeneric expression of a gene from *Acremonium chrysogenum* introduced into *Pencillium chrysogenum*, resulting in transgenic production of a cephalosporin in a pencillin production system [19]; (v) expression in yeast of the bacterial *uid*A gene by a heat shock promoter from a higher plant [111]; (vi) coordinate expression of vectored cytochrome *C* genes from yeast, fruit-fly, and rat in a *cyc*C-deficient yeast strain, showing varied and specific post-translational modification for the different genes [60]; (vii) efficient production of murine antibody fragments by *Trichoderma reesei* [77]; (viii) overexpression of cellulases by multicopy integration of heterologous DNA sequences into yeast chomosomes [71]; (ix) hypersection of calf chymosin by *Aspergillus* species by introducing relevant signal sequences [116, 123]; and (x) introduction of a bacterial-derived gene for hygromycin B resistance in the mycorrhizal *Laccaria laccata* [6,63] and the edible mushroom *Pleurotus ostreatus* [64,83]

The various gene transfer systems now available have influenced both basic and applied research, permitting detailed molecular study through the identification, isolation and functional analysis of genes involved in many important biological processes. Transformation procedures transcend dependency upon sexual or parasexual processes for genetic manipulation of fungi.

By extending the potential for 'imposed' variation through rDNA technology, it is hoped that industrial fungi will become less 'ornery' and even more 'creative'.

REFERENCES

1 Allison, D.S., M.W. Rey, R.M. Berka, G. Armstrong and N.S. Dunn-Coleman. 1992. Transformation of the thermophilic fungus

Humicola grisea var. *thermoidea* and overproduction of *Humicola* glucoamylase. Curr. Genet. 21: 225–229.
2 Armeleo, D., G.-N. Ye, T.M. Klein, K.B. Shark, J.C. Sanford and S.A. Johnston. 1990. Biolistic nuclear transformation of *Saccharomyces cerevisiae* and other fungi. Curr. Genet. 17: 97–103.
3 Arnaise, S., D. Zickler and N.L. Glass. 1993. Heterologous expression of mating-type genes in filamentous fungi. Proc. Natl Acad. Sci. USA 90: 6616–6620.
4 Ballance, D.J. and G. Turner. 1985. Development of a high-frequency transforming vector for *Aspergillus nidulans*. Gene 36: 321–331.
5 Ballance, D.J. and G. Turner. 1986. Gene cloning in *Aspergillus nidulans*: isolation of the isocitrate lyase gene (*acu*D). Mol. Gen. Genet. 202: 271–275.
6 Barrett, V., R.K. Dixon and P.A. Lemke. 1990. Genetic transformation of a mycorrhizal fungus. Appl. Microbiol. Biotechnol. 33: 313–316.
7 Beach, D. and P. Nurse. 1981. High-frequency transformation of the fission yeast *Schizosaccharomyces pombe*. Nature 290: 140–142.
8 Becker, D.M. and L. Guarente. 1991. High-frequency transformation of yeast by electroporation. Method. Enzymol. 194: 182–187.
9 Beggs, J.D. 1978. Transformation of yeast by a replicating hybrid plasmid. Nature 275: 104–109.
10 Begueret, J., V. Razanamparany, M. Perrot and C. Barreau. 1984. Cloning gene *ura*5 for the orotidylic acid pyrophosphorylase of the filamentous fungus *Podospora anserina*: transformation of protoplasts. Gene 32: 487–492.
11 Bej, A.K. and M.H. Perlin. 1989. A high efficiency transformation system for the basidiomycete *Ustilago violacea* employing hygromycin resistance and lithium-acetate treatment. Gene 80: 171–176.
12 Beri, R.K., E.L. Lewis and G. Turner. 1988. Behaviour of a replicating mitochondrial DNA sequence from *Aspergillus amstelodami* in *Saccharomyces cerevisiae* and *Aspergillus nidulans*. Curr. Genet. 13: 479–486.
13 Binninger, D.M., C., Skrzynia, P.J. Pukkila and L.A. Casselton. 1987. DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J. 6: 835–840.
14 Blackburn, E.H. and J.W. Szostak. 1984. The molecular structure of centromeres and telomeres. Annu. Rev. Biochem. 53: 163–194.
15 Boylan, M.T. and M.J. Holland and W.E. Timberlake. 1986. *Saccharomyces cerevisiae* centromere CEN II does not induce chromosome instability when integrated into *Aspergillus nidulans* genome. Mol. Cell. Biol. 6: 3621–3625.
16 Bruckner, B., S.E. Unkles, K. Weltring and J.R. Kinghorn. 1992. Transformation of *Gibberella fujikuroi*: effect of the *Aspergillus nidulans* AMA1 sequence on frequency and integration. Curr. Genet. 22: 313–316.
17 Burmester, A. 1992. Transformation of the mycoparasite *Parasitella simplex* to neomycin resistance. Curr. Genet. 21: 121–124.
18 Burmester, A., A. Wostemeyer, J. Arnau and J. Wostemeyer. 1992. The SEG1 element: a new DNA region promoting stable mitotic segregation of plasmids in the zygomycete *Absidia glauca*. Mol. Gen. Genet. 235: 166–172.
19 Cantwell, C., R. Beckmann, P. Whiteman, S.W. Queener and E.P. Abraham. 1992. Isolation of decaceto-xycephalosporin C from fermentation broths of *Pencillium chrysogenum* transformants: construction of a new fungal biosynthetic pathway. Proc. R. Soc. Lond. Ser. B Biol. Sci. 248: 283–289.
20 Case, M.E., M. Schweizer, S.R. Kushner and N.H. Giles. 1979. Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. Proc. Natl Acad. Sci. USA 76: 5259–5263.
21 Choi, G.H. and D.L. Nuss. 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science 257: 800–803.
22 Choi, G.H., D.M. Pawlyk, B. Rae, R. Shapira and D.L. Nuss. 1993. Molecular analysis and over-expression of the gene encoding endothiapepsin, an aspartic protease from *Cryphonectria parasitica*. Gene 125: 135–141.
23 Christiansen, T., H. Woeldike, E. Boel, S.B. Mortensen, K. Hjortshoej, L. Thim and M.T. Hansen. 1988. High level expression of recombinant genes in *Aspergillus oryzae*. Bio/Technol. 6: 1419–1422.
24 Clarke, L. and J. Carbon. 1980. Isolation of the centromere-linked *CDC*10 gene by complementation in yeast. Proc. Natl Acad. Sci. USA 77: 2173–2177.
25 Connelly, C., M.K. McCormick, J. Shero and P. Hieter. 1991. Polyamines eliminate an extreme size bias against transformation of large yeast artificial chromosome DNA. Genomics 10: 10–16.
26 Connerton, I.F., J.R.S. Fincham, R.A. Sandman and M.J. Hynes. 1990. Comparison and cross-species expression of the acetyl-CoA synthetase genes of the ascomycete fungi, *Aspergillus nidulans* and *Neurospora crassa*. Mol. Microbiol. 4: 456–460.
27 Cullen, D., L.J. Wilson, G.L. Grey, D.J. Henner and D.J. Ballance. 1987. Sequence and centromere proximal location of a transformation enhancing fragment *ans*1 from *Aspergillus nidulans*. Nucl. Acids Res. 15: 9163–9175.
28 Davis, M.A., C.S. Cobbet and M.J. Hynes. 1988. An *amd*S-*lac*Z fusion for studying gene regulation in *Aspergillus*. Gene 63: 199–212.
29 Debuchy, R., E. Coppin-Raymal, D. LeCoze and Y. Brygoo. 1988. Chromosome walking toward a centromere in the filamentous fungus *Podospora anserina*: cloning of a sequence lethal to a two copy state. Curr. Genet. 13: 105–111.
30 Dhawale, S.S. and G.A. Marzluf. 1985. Transformation of *Neurospora crassa* with circular and linear DNA and analysis on the fate of the transforming DNA. Curr. Genet. 10: 205–212.
31 Dickman, M.B. 1988. Whole cell transformation of the alfalfa fungal pathogen *Colletotrichum trifolii*. Curr. Genet. 14: 241–246.
32 Durrens, P., P.M. Green, H.N. Arst Jr and C. Scazzocchio. 1986. Heterologous insertion of transforming DNA and generation of new deletion associated with transformation in *Aspergillus nidulans*. Mol. Gen. Genet. 203: 544–549.
33 Edman, J.L. 1992. Isolation of telemere-like sequences from *Cryptococcus neoformans* and their use in high-efficiency transformation. Mol. Cell. Biol. 12: 2777–2783.
34 Esser, K., U. Kuck, C. Land-Hindricks, P. Lemke, H.D. Osiewacz, U. Stahl and P. Tudzynski. 1986. Plasmids of Eukaryotes. 124 p, Springer-Verlag, Berlin.
35 Esser, K. and G. Mohr. 1986. Integrative transformation of filamentous fungi with respect to biotechnological application. Process. Biochem. 21: 153–159.
36 Fangman, W.L. and B.J. Brewer. 1991. Activation of replication origins within yeast chromosomes. Annu. Rev. Cell Biol. 7: 375–403.
37 Fincham, J.R.S. 1989. Transformation in fungi. Microbiol. Rev. 53: 148–170.
38 Finkelstein, D.B. 1992. Transformation. In: Biotechnology of Filamentous Fungi (Finkelstein, D.B. and C. Ball, eds), pp. 113–156, Butterworth-Heinemann, Boston.
39 Froeliger, E.H., A.M. Munos-Rivas, C.A. Specht, R.C. Ullrich and C.P. Novotny. 1987. The isolation of specific genes from the basidiomycete *Schizophyllum commune*. Curr. Genet. 12: 547–554.
40 Fujimura, H.-A. 1991. Transformation of the yeast *Saccharomyces*

kluyveri by *Saccharomyces cerevisiae* based plasmids. FEMS Microbiol. Lett. 82: 149–152.

41 Gems, D., I.L. Johnstone and A.J. Clutterbuck. 1991. An autonomously replicating plasmid transforms *Aspergillus nidulans* at high frequency. Gene 98: 61–67.

42 Giasson, L., C.A. Specht, C. Milgrim, C.P. Novotny and R.C. Ullrich. 1989. Cloning and comparison of Aα mating-type alleles of the basidiomycete *Schizophyllum commune*. Mol. Gen. Genet. 218: 72–77.

43 Glab, N., P.X. Petit and P.P. Slonimski. 1993. Mitochondrial dysfunction in yeast expressing cytoplasmic male sterility T-*urf*B gene from maize: analysis at the population and individual cell level. Mol. Gen. Genet. 236: 299–308.

44 Goyon, C. and G. Faugeron. 1989. Targeted transformation of *Ascobolus immersus* and *de novo* methylation of the resulting duplicated DNA sequences. Mol. Cell. Biol. 9: 2818–2827.

45 Grant, D.M., A.M. Lambowitz, J.A. Rambosek and J.A. Kinsey. 1984. Transformation of *Neurospora crassa* with recombinant plasmids containing the cloned glutamate dehydrogenase (*am*) gene: evidence for autonomous replication of the transforming plasmid. Mol. Cell. Biol. 4: 2041–2051.

46 Gwynne, D.I. and M. Devchand. 1992. Expression of foreign proteins in the genus *Aspergillus*. In: Aspergillus: Biology and Industrial Application (Bennett, J.W. and M.A. Klitch, eds), pp. 203–214, Butterworth-Heinemann, Boston.

47 Hermanson, G.G., M.F. Hoekstra, D.L. McElligott and G.A. Evans. 1991. Rescue of end fragments of yeast artificial chromosomes by homologous recombination in yeast. Nucl. Acids Res. 19: 4943–4948.

48 Herzog, R., N.K. Singh, C. Schmidt and P.A. Lemke. 1995. Presence of a Pl bacteriophage sequence in transforming plasmids of *Pleurotus ostreatus*. Curr. Genet. 27: 460–465.

49 Hinnen, A., J.B. Hicks and G.R. Fink. 1978. Transformation of yeast. Proc. Natl Acad. Sci. USA 75: 1929–1933.

50 Hunter, G.D., C.R. Bailey and H.N. Arst Jr. 1992. Expression of a bacterial aspartase gene in *Aspergillus nidulans*: an efficient system for selecting multicopy transformants. Curr. Genet. 22: 377–383.

51 Hynes, M.J. 1989. Complementation of an *Aspergillus nidulans* mutation by a gene from the basidiomycete *Coprinus cinereus*. Exp. Mycol. 13: 196–198.

52 Innis, M.A., M.J. Holland, P.C. McCabe, G.E. Cole, V.P. Wittman, R. Tal, K.W. Watt, D.H. Gelfand, J.P. Holland and J.H. Meade. 1985. Expression glycosylation and secretion of an *Aspergillus* glucoamylase by *Saccharomyces cerevisiae*. Science 228: 21–26.

53 Ito, H., Y. Fukuda, K. Murata and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153: 163–168.

54 Javerzat, J.P., V. Bhattacherjee and C. Barreau. 1993. Isolation of telomeric DNA from the filamentous fungi *Podospora anserina* and construction of a self-replicating linear plasmid showing high transformation frequency. Nucl. Acids Res. 21: 497–504.

55 Judelson, H.S., B.M. Tyler and R.W. Michelmore. 1992. Regulatory sequences for expressing genes in oomycete fungi. Mol. Gen. Genet. 234: 138–146.

56 Kelly, J.M. and M.J. Hynes. 1985. Transformation of *Aspergillus niger* by the *amd*S gene of *Aspergillus nidulans*. EMBO J. 4: 475–479.

57 Khanh, N.O., K. Leidinger, H. Albrecht, E. Ruttkowski and M. Gottschalk. 1992. Effects of promoters on the enhancement of pectin methyl esterase expression in *Aspergillus niger*. Biotechnol. Lett. 14: 1047–1052.

58 Kim, S.Y. and G.A. Marzluf. 1988. Transformation of *Neurospora crassa* with the *trp*-1 gene and the effect of the host strain upon the fate of the transforming DNA. Curr. Genet. 13: 65–70.

59 Kinsey, J.A. and J.A. Rambosek. 1984. Transformation of *Neurospora crassa* with the cloned *am* (glutamate dehydrogenase) gene. Mol. Cell. Biol. 4: 117–122.

60 Koshy, T.I., T.L. Luntz, E.A.E. Garber and E. Margoliash. 1992. Expression of recombinant cytochromes C from various species in *Saccharomyces cerevisiae*: post-translational modifications. Protein Expression Purif. 3: 441–452.

61 Kudo, T., A. Okoshi, R. Usami and K. Horikoshi. 1993. Cloning an autonomous replication sequence from *Aspergillus oryzae*. Starch 45: 209–211.

62 Lazarus, C.M. and H. Kuntzel. 1981. Anatomy of amplified mitochondrial DNA in 'ragged' mutants of *Aspergillus amstelodami*: excision points within protein genes and a common 215-bp segment containing a possible origin of replication. Curr. Genet. 4: 99–107.

63 Lemke, P.A., V. Barrett and R.K. Dixon. 1991. Procedures and prospects for DNA-mediated transformation of ectomycorrhizal fungi. Methods Microbiol. 23: 281–293.

64 Lemke, P.A. and M. Peng. 1995. Genetic manipulation of fungi by transformation. In: The Mycota II. Genetics and Biotechnology (Kuck, U., ed.), pp. 109–139, Springer, Berlin.

65 Leong, S.A. 1989. Molecular genetic approaches to the study of fungal pathogenesis. Annu. Rev. Phytopathol. 27: 463–481.

66 Leong, S.A. and R.M. Berka. 1991. Molecular Industrial Mycology. 292 p, Marcel Dekker, New York.

67 Lorito, M., C.K. Hayes, A. Di Pietro and G.E. Harman. 1992. Biolistic transformation of *Trichoderma harzianum* and *Gliocladium virens* with plasmid and genomic DNA. Curr. Genet. 24: 349–356.

68 Marmeisse, R., G. Gay, J.C. Debaud and L.A. Casselton. 1992. Genetic transformation of the symbiotic basidiomycete *Hebeloma cylindrosporum*. Curr. Genet. 22: 41–45.

69 McKnight, G.L., H. Kato, A. Upshal, M.D. Parker, G. Saari and P.J. O'Hara. 1985. Identification and molecular analysis of a third *Aspergillus nidulans* alcohol dehydrogenase. EMBO J. 4: 2093–2099.

70 Mellon, F.M. and L.A. Casselton. 1988. Transformation as a method of increasing gene copy number and gene expression in the basidiomycete fungus *Coprinus cinereus*. Curr. Genet. 14: 451–456.

71 Mochizuki, D., K. Miyahara, D. Hirato, H. Matsuzaki, T. Hatano, S. Kukui and T. Miyakawa. 1994. Overexpression of cellulolytic enzymes by delta-sequence-mediated multicopy integration of heterologous DNA sequences into the chromosomes of *Saccharomyces cerevisiae*. J. Ferm. Bioeng. 77: 468–473.

72 Mohr, G., D. Wilmanska and K. Esser. 1989. Analysis of *Aspergillus niger* transformants for single site integration and vector recombination. Appl. Microbiol. Biotechnol. 32: 160–162.

73 Moir, D.T., T.E. Dorman, A.P. Smyth and D.R. Smith. 1993. A human genome YAC library in a selectable high-copy-number vector. Gene 125: 229–232.

74 Murray, A.W. and J.W. Szostak. 1983. Construction of artificial chromosomes in yeast. Nature 305: 189–193.

75 Nakajima, M., Q.I. Sheikh, K. Yamaoka, Y. Yui, Y. Kajwaras and K. Shishido. 1993. Bending of DNA segments with *Saccharomyces cerevisiae* autonomously replicating sequence activity, isolation from basidiomycete mitochondrial linear plasmids. Mol. Gen. Genet. 237: 1–9.

76 Nonet, G.H. and G.M. Wahl. 1993. Introduction of YACs containing a putative mammalian replication origin into mammalian cells

can generate structures that replicate autonomously. Somatic Cell. Mol. Genet. 129: 171–192.

77 Nyyssonen, E., M. Penttila, A. Harkki, A. Saloheimo, J.K.C. Knowles and S. Keranan. 1993. Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. Bio/Technol. 11: 591–595.

78 Orr-Weaver, T.L. and J.W. Szostak. 1983a. Yeast recombination: the association between double-strand gap repair and crossing-over. Proc. Natl Acad. Sci. USA 80: 4417–4421.

79 Orr-Weaver, T.L. and J.W. Szostak. 1983b. Multiple tandem plasmid integration in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 3: 747–749.

80 Orr-Weaver, T.L., J.W. Szostak and R.J. Rothstein. 1981. Yeast transformation: a model system for the study of recombination. Proc. Natl Acad. Sci. USA 78: 6354–6358.

81 Pavan, W.J., P. Hieter and R.H. Reeves. 1990. Generation of deletion by targeted transformation of human-derived yeast artificial chromosomes. Proc. Natl Acad. Sci. USA 87: 1300–1304.

82 Peberdy, J.F. 1989. Fungi without coats—protoplasts as tools for mycological research. Mycol. Res. 93: 1–20.

83 Peng, M., N.K. Singh and P.A. Lemke. 1992. Recovery of recombinant plasmids from *Pleurotus ostreatus* transformants. Curr. Genet. 22: 53–59.

84 Peng, M., P.A. Lemke and J.J. Shaw. 1993a. Improved conditions for protoplast formation and transformation of *Pleurotus ostreatus*. Appl. Microbiol. Biotechnol. 40: 101–106.

85 Peng, M., P.A. Lemke and N.K. Singh. 1993b. A nucleotide sequence involved in replicative transformation of a filamentous fungus. Curr. Genet. 24: 114–121.

86 Perlin, M.H., A.K. Bej, O.H. Will III and R.T. Jacob. 1990. Introduction and maintenance of prokaryotic DNA in *Ustilago violacea*. J. Ind. Microbiol. 5: 355–364.

87 Perrot, M., C. Barreau and J. Begueret. 1987. Nonintegrative transformation in the filamentous fungus *Podospora anserina*: stabilization of a linear vector by the chromosomal ends of *Tetrahymena thermophila*. Mol. Cell. Biol. 7: 1725–1730.

88 Powell, W.A. and H.C. Kistler. 1990. In vivo rearrangement of foreign DNA by *Fusarium oxysporum* produces linear self-replicating plasmids. J. Bacteriol. 172: 3163–3171.

89 Punt, P.J., R.P. Oliver, M.A. Dingemanse, P.H. Pouwels and C.A.M.J.J. van den Hondel. 1987. Transformation of *Aspergillus nidulans* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56: 117–124.

90 Randall, T., T. Rao and C.A. Reddy. 1989. Use of a shuttle vector for the transformation of the white rot basidiomycete, *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. 161: 720–725.

91 Randall, T., C.A. Reddy and K. Boominathan. 1991. A novel extrachromosomally maintained transformation vector for the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Curr. Genet. 21: 255–260.

92 Raper, K.B. and C. Thom. 1994. A Manual of the *Penicillia*. 875 p, Waverly Press, Baltimore.

93 Revuelta, J.L. and M. Jayaram. 1987. Transformation of *Phycomyces blakesleeanus* to G418 resistance by an autonomous replicating plasmid. Proc. Natl Acad. Sci. USA 83: 7344–7347.

94 Richey, M.G., E.T. Marek, C.L. Schardl and D.A. Smith. 1989. Transformation of filamentous fungi with plasmid DNA by electroporation. Phytopathology 79: 844–847.

95 Riethman, H.C., R.K. Moyzis, J. Meyne, D.T. Burke and M.V. Olsen. 1989. Cloning human telomeric DNA fragments into *Saccharomyces cerevisiae* using a yeast-artificial-chromosome vector. Proc. Natl Acad. Sci. USA 86: 6240–6244.

96 Rohrer, T.L. and S.K. Picataggio. 1992. Targeted integrative transformation of *Candida tropicalis* by electroporation. Appl. Microbiol. Biotechnol. 36: 650–654.

97 Roncero, M.I.G., L.P. Jepsen, P. Stroman and R. van Heeswijck. 1989. Characterization of a *leu*A gene and an *ARS* element from *Mucor circinelloides*. Gene 84: 335–343.

98 Samac, D.A. and S.A. Leong. 1989. Characterization of the termini of linear plasmids from *Nectria haematococca* and their use in construction of an autonomously replicating transformation vector. Curr. Genet. 16: 187–194.

99 Schechtman, M.G. 1987. Isolation of telomeric DNA from *Neurospora crassa*. Mol. Cell. Biol. 7: 3168–3177.

100 Schlesinger, D. 1990. Yeast artificial chromosomes: tools for mapping and analysis of complex genomes. Trends Genet. 6: 248–258.

101 Skatrud, P.L., S.W. Queener, L.G. Car and D.L. Fisher. 1987. Efficient integrative transformation of *Cephalosporium acremonium*. Curr. Genet. 12: 337–348.

102 Skatrud, P.L., A.J. Tietz, T.D. Ingolia, C.A. Cantwell, D.L. Fisher, J.L. Chapman and S.W. Queener. 1989. Use of recombinant DNA to improve production of cephalosporin C by *Cephalosporium acremonium*. Bio/Technology 7: 477–485.

103 Smith, D.R., A.P. Smyth, W.M. Strauss and D.T. Moir. 1993. Incorporation of copy-number control elements into yeast artificial chromosomes by targeted homologous recombination. Mamm. Genome 4: 141–147.

104 Som, T., K.A. Armstrong, F.C. Volkert and J.R. Broach. 1988. Autoregulation of 2 μm circle gene expression provides a model for maintenance of stable plasmid copy levels. Cell 52: 27–37.

105 Stahl, U., E. Leitner and K. Esser. 1987. Transformation of *Penicillium chrysogenum* by a vector containing a mitochondrial origin of replication. Appl. Microbiol. Biotechnol. 26: 237–241.

106 Stahl, U., P. Tudzynski, U. Kuck and K. Esser. 1982. Replication and expression of a bacterial mitochondrial hybrid plasmid in the fungus *Podospora anserina*. Proc. Natl Acad. Sci. USA 80: 1058–1062.

107 Stinchcomb, D.T., M. Thomas, J. Kelly, E. Selker and R.W. Davis. 1980. Eukaryotic DNA segments capable of autonomous replication in yeast. Proc. Natl Acad. Sci. USA 77: 4559–4563.

108 Stohl, L.L. and A.M. Lambowitz. 1983. Construction of a shuttle vector for the filamentous fungus *Neurospora crassa*. Proc. Natl Acad. Sci. USA 80: 1058–1062.

109 Struhl, K., D.T. Stinchcomb, R. Davis and W. Scherers. 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl Acad. Sci. USA 76: 1035–1039.

110 Szostak, J.W. and E.A. Blackburn. 1982. Cloning yeast telomeres on linear plasmid vectors. Cell 29: 245–255.

111 Takahashi, T., N. Yabe and Y. Komeda. 1993. Expression in yeast of a fusion gene composed of the promoter of a heat-shock gene from *Arabidopsis* and bacterial gene for β-glucoronidase. Plant Cell. Physiol. 34: 161–164.

112 Thom, C. and K.B. Raper. 1945. A Manual of the *Aspergilli*. 372 p, Waverly Press, Baltimore.

113 Tilburn, J., C. Scazzocchio, G.G. Taylor, J.H. Zabicky-Zissima, R.A. Lockington and R.W. Davis. 1983. Transformation by integration in *Aspergillus nidulans*. Gene 26: 205–221.

114 Timberlake, W.E. and M.A. Marshall. 1989. Genetic engineering of filamentous fungi. Science 244: 1313–1317.

115 Toffaletti, D.L., T.H. Rude, S.A. Johnston, D.T. Durack and J.R. Perfect. 1993. Gene transfer in *Cryptococcus neoformans* using biolistic delivery of DNA. J. Bacteriol. 175: 1405–1411.

116 Tsuchiya, K., K. Gomi, K. Kitamoto, C. Kunagoi and G. Tamura.

1993. Secretion of calf chymosin from the filamentous fungus *Aspergillus oryzae*. Appl. Microbiol. Biotechnol. 40: 327–332.
117 Tsuge, T., S. Nishimura and H. Kobayashi. 1990. Efficient integrative transformation of the phytopathogenic fungus *Alternaria alternata* mediated by the repetitive rDNA sequences. Gene 90: 207–214.
118 Tsukuda, T., S. Carleton, S. Fotheringham and W.K. Holloman. 1989. Isolation and characterization of an autonomously replicating sequence from *Ustilago maydis*. Mol. Cell. Biol. 8: 3703–3709.
119 Turcq, B. and J. Begueret. 1987. The *ura5* gene of the filamentous fungus *Podospora anserina:* nucleotide sequence and expression in transformed strains. Gene 53: 201–209.
120 Turgeon, B.G., H. Bohlmann, L.M. Ciuffetti, S.K. Christiansen, G. Yang, W. Schafer and O.C. Yoder. 1993. Cloning and analysis of the mating type genes from *Cochliobolus heterostrophus*. Mol. Gen. Genet. 238: 270–284.
121 van Gorcom, R.F.M., P.H. Pouwels, T. Goosen, J. Visser, H.W.J. van den Broed, J.F. Hamer, W.E. Timberlake, C.A.M.J.J. van den Hondel. 1985. Expression of an *Escherichia coli* β-galactosidase fusion gene in *Aspergillus nidulans*. Gene 40: 99–106.
122 Van Heeswijk, R. 1986. Autonomous replication of plasmids in *Mucor* transformants. Carlsberg Res. Commun. 51: 433–443.
123 Ward, M. 1991. Chymosin production in *Aspergillus*. In: Molecular Industrial Mycology (Leong, S.A. and R.M. Berka, eds), pp. 83–106, Marcel Dekker, New York.
124 Ward, M., K.H. Kodama and L.J. Wilson. 1989. Transformation of *Aspergillus awamori* and *A. niger* by electroporation. Exp. Mycol. 13: 289–293.
125 Wernars, K., T. Goosen, B.M.J. Wennekes, J. Visser, C.J. Bos, H.W.J. van den Broek, R.F.M. Gorcom, C.A.M.J.J. van den Hondel and P.H. Pouwels. 1985. Gene amplification in *Aspergillus nidulans* by transformation with vectors containing the *amd*S gene. Curr. Genet. 9: 361–368.
126 Wernars, K., T. Goosen, K. Sewart and H.W.J. van den Broek. 1986. Genetic analysis of *Aspergillus nidulans amd*S^+ transformants. Mol. Gen. Genet. 205: 312–317.
127 Williams, J.S., T.T. Eckdahl and J.N. Anderson. 1988. Bent DNA function as a replication enhancer in *S. cerevisiae*. Mol. Cell. Biol. 8: 2763–2769.
128 Williamson, D.H. 1985. The yeast *ARS* element, six years on: a progress report. Yeast 1: 1–14.
129 Woods, J.P. and W.E. Goldman. 1992. In vivo generation of linear plasmids with addition of telomeric sequences. Mol. Microbiol. 6: 3603–3610.
130 Woods, J.P. and W.E. Goldman. 1993. Autonomous replication of foreign DNA in *Histoplasma capsulatum*: role of native telomeric sequences. J. Bacteriol. 175: 636–641.
131 Worsham, P.L. and W.E. Goldman. 1990. Development of a genetic transformation system for *Histoplasma capsulatum*: a complementation of uracil auxotrophy. Mol. Gen. Genet. 221: 358–362.
132 Yamamoto, H., Y. Yoshida, Y. Kitamura and J. Sekiguchi. 1991. Autonomously replicating fragments of mitochondrial DNA from *Penicillium urticae* in *Saccharomyces cerevisiae*. J. Ferment. Bioeng. 71: 275–277.
133 Yelton, M.M., J.E. Hamer and W.E. Timberlake. 1984. Transformation of *Aspergillus nidulans* by using a *trp*C plasmid. Proc. Natl Acad. Sci. USA 81: 1470–1474.
134 Yui, Y., Y. Katayose and K. Shishido. 1988. Two linear plasmid-like DNA elements simultaneously maintained in *Pleurotus ostreatus*. Biochim. Biophys. Acta 951: 53–60.