Mycoscience 36: 127–133, 1995

Review

Homologous and heterologous gene transfer systems in basidiomycetes

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Accepted for publication 25 January 1995

Key Words——genetic transformation; homologous-heterologous gene expression; Hymenomycetes; Teliomycetes.

Basidiomycetes constitute an important class of fungi with species of interest for basic research, bioconversion, food industry and agriculture. Transformation provides a molecular tool for the study of gene structure and function, the regulation of their expression, and for the genetic engineering of the species which have economic incidence. Of particular interest are the genes involved in the key steps of the life cycle of basidiomycetes, such as the mating type genes controlling plasmogamy between two homokaryons to give rise to the dikaryotic phase, the genes intervening during the differentiation process of the basidiocarp and in the interaction plant-pathogen, and the genes encoding extracellular enzymes usable for substrate conversion. Taking advantage of the knowledge acquired from the transformation of ascomycetes and other filamentous fungi, for both the methodology of transformation (reviewed in Fincham, 1989) and the construction of versatile transformation vectors (Punt et al., 1987), considerable advances in the development of gene tranfer systems in several basidiomycetes species has been made over the past eight years.

The aim of this paper is to survey the species transformed and the markers of selection used, the different procedures to introduce the transforming DNA into recipient strains and their efficiency, and the fate of the transforming DNA. Some examples of different applications of the DNA-mediated transformation systems are given to illustrate how this technology makes several aspects of fungal genetics accessible at a molecular level.

Species and research fields

The basidiomycetes species for which one or several DNA-mediated transformation systems have been reported (Table 1) belong to the Hymenomycetes order, including species of the Aphyllophorales (*Schizophyllum commune*, *Phanerochaete chrysosporium*) and Agaricales class (*Coprinus cinereus*, *Coprinus bilanatus*, *Pleurotus ostreatus*, *Agrocybe aegerita*, *Hebeloma cylindrosporum*, *Laccaria laccata*), and to the Teliomycetes order, with species of the Ustilaginales (*Ustilago* sp.) and Uredinales class (*Uromyces appendiculatus*). All these species are interesting for both basic and applied research: *S. commune* and *Coprinus* sp. are laboratory models for studying morphogenesis and sexual incompatibility (Wessels et al., 1991; Wessels, 1993; Specht et al., 1992; Tymon et al., 1992). *Pleurotus ostreatus* and *A. aegerita* are both edible cultivated mushrooms; *A. aegerita* in particular beneficited of numerous studies dealing with the genetics and molecular biology of basidiocarp differentiation (Meinhardt and Esser, 1981; Salvado and Labarère, 1991; Noël et al., 1991b), mating types (Noël et al., 1991a; Labarère and Noël, 1992) and the mitochondrial genome organization (Moulinier et al., 1992; Barroso et al., 1992). *Hebeloma cylindrosporum* and *L. laccata* are intended to a better understanding of the ectomycorrhizal association (Marmeisse et al., 1992; Barret et al., 1990). *Phanerochaete chrysosporium* is mainly attractive for the production of extracellular ligno-cellulolytic enzymes (Tien, 1989; Alic et al., 1989). Lastly, the phytopathogenic fungi of the Teliomycetes order are basically and economically interesting for the study of the pathogenic process and for a better control of plant disease (Wang et al., 1988).

The genetic markers of transformation

Two types of genetic markers of transformation can be

Table 1. Basidiomycete species transformed and markers of selection used.

Species	Marker[a]	Origin	References
Schizophyllum commune	*TRP1*	*S. commune*	Munoz-Rivas et al., 1986
	hph[b]	*E. coli*	Mooibroek et al., 1990
	URA1	*S. commune*	Schuren et al., 1993
Coprinus cinereus	*TRP1*	*C. cinereus*	Binninger et al., 1987
	acu-7[b]	*C. cinereus*	Mellon et al., 1987
	trp-2	*C. cinereus*	Casselton and de La Fuente Herce, 1989
Coprinus bilanatus	*trp-2*	*C. cinereus*	Burrows et al., 1990
Pleurotus ostreatus	*hph*	*E. coli*	Peng et al., 1992
Agrocybe aegerita	*URA1*	*A. aegerita*	Noël and Labarère, 1994
	Neo^R	*E. coli*	Noël et al., 1995
Hebeloma cylindrosporum	*hph*	*E. coli*	Marmeisse et al., 1992
Laccaria laccata	*hph*	*E. coli*	Barret et al., 1990
Phanerochaete chrysosporium	*ade2, ade5*	*S. commune*	Alic et al., 1989; 1990
	ade1	*P. chrysosporium*	Alic et al., 1991
	Kan^R	*E. coli*	Randall et al., 1989; 1991
	URA3	*P. chrysosporium*	Akileswaran et al., 1993
	ble	*St. hindustanus*	Gessner and Raeder, 1994
Ustilago maydis	*hph*	*E. coli*	Wang et al., 1988
	PYR3	*U. maydis*	Banks and Taylor, 1988
	phleo	*St. hindustanus*	Kinal et al., 1993
	Cbx	*U. maydis*	Keon et al., 1991; Kinal et al., 1993
	sat-1	*E. coli*	Gold et al., 1994
	Ben^R	*U. maydis*	Gold et al., 1994
Ustilago hordei	*hph*	*E. coli*	Holden et al., 1988
Ustilago nigra	*hph*	*E. coli*	Holden et al., 1988
Ustilago violacea	*hph*	*E. coli*	Bej et Perlin, 1989
Uromyces appendiculatus	*GUS*	*E. coli*	Li et al., 1993

[a] *TRP1*, tryptophan synthetase; *acu-7*, isocitrate lyase; *trp-2*, trifunctional protein: glutamine amidotransferase, phosphoribosyl anthranilate isomerase, indole glycerol phosphate synthetase; *URA1 S. commune*, orotidine-5'-phosphate decarboxylase; *URA1 A. aegerita*, dihydroorotate dehydrogenase; *URA3*, orotidylate decarboxylase; *PYR3*, dihydroorotase; *ade2*, adenine biosynthetic pathway; *ade5, ade1*, phosphoribosyl aminoimidazole synthetase; *hph* (alternatively named *HygB* or *NPT*), hygromycin B phosphotransferase; *Neo*^{R}, neomycin phosphotransferase; *Kan*^{R}, kanamycin phosphotransferase; *ble, phleo*, bleomycin/phleomycin resistance; *Cbx*, carboxin resistance; *sat-1*, streptothricin acetyltransferase; *Ben*^{R}, benomyl resistance; *GUS*, β-glucuronidase. Convention for gene symbols (lower or upper case letters) is that used in original papers.

[b] Markers used in cotransformation with the *TRP1* genes.

distinguished according to their origin and the species in which they have been used. The homologous markers are constituted by wild or mutant alleles of genes cloned from the species which should be subsequently transformed. Wild metabolic genes able to complement auxotrophic mutations are most often used. For example, genes of the tryptophane biosynthetic pathway have been used to transform *trp*-mutant strains of *S. commune* and *C. cinereus* and genes from the pyrimidine biosynthetic pathway for *S. commune, A. aegerita, P. chrysosporium* and *U. maydis* (Table 1). Another type of homologous markers are mutant alleles conferring resistance to fungicides, like the *Cbx* and *Ben*^{R} markers of *U. maydis*. The advantage of the homologous markers rests in the fact that they are fully expressed in the transformed strain, and are therefore particularly useful for determining the conditions for the uptake of DNA. On the other hand, the disadvantage lies in the previous selection of mutant strains, either for their use as recipient strain of transformation, or to clone the genetic marker of transformation.

The heterologous markers are constituted by genes belonging to a species different from that to be transformed, derived either from close fungal species or from phylogenically very distant species, such as bacteria for example. They can be functionally subdivided in two groups. One group includes metabolic genes of related basidiomycetous species able to complement auxotrophic mutations. This is for example the case of the *trp-2* gene of *C. cinereus* used in *C. bilanatus*, or the *ade2* and *ade5* genes of *S. commune* used to transform another Aphyllophorale *P. chrysosporium* (Table 1). The other

Table 2. Examples of transformation efficiencies obtained from different transformation procedures using homologous or heterologous genetic markers.

Transformation procedure	Species	Marker[a]	Transformation efficiency[b]	Reference
PEG/$CaCl_2$	*S. commune*	*TRP1*	1000	Specht et al., 1988
	P. chrysosporium	*Ade1*	300	Alic et al., 1991
	C. cinereus	*TRP1*	100	Binninger et al., 1991
	P. ostreatus	*HPT*	50	Peng et al., 1992
	H. cylindrosporum	*HPT*	5	Marmeisse et al., 1992
	U. maydis	*HPT*[c]	1000	Wang et al., 1988
	U. maydis	*pyr3*	200	Banks and Taylor, 1988
Electroporation	*P. ostreatus*	*HPT*	50	Peng et al., 1992
	A. aegerita	*URA1*	50	Noël and Labarère, 1994
	A. aegerita	*Neo*^{R}	5	Noël et al., 1995
LiOAc	*C. cinereus*	*TRP1*	10	Binninger et al., 1987
	U. violacea	*HPT*	80	Bej and Perlin, 1989
Particle bombardment	*U. appendiculatus*	*GUS*	$8.2\ 10^{-4}$	Li et al., 1993

[a] See legend of Table 1.
[b] The maximal transformation efficiency is expressed as the number of transformants per μg of DNA, irrespective of the number of viable cells. In the case of particle bombardment, the efficiency is given as the frequency of cells transformed per 1.6 μg of DNA.
[c] Vector linearized.

group comprises genes encoding antibiotic resistance, whose utilization is more widespread in transformation experiments. These genes are of bacterial origin and should be placed under the control of fungal transcriptional signals to be expressed in the transformed cells. For this purpose, the versatile vector pAN7.1 (Punt et al., 1987) containing the *E. coli* hygromycin phosphotransferase gene (*hph*) fused to the *Aspergillus nidulans gpdA* (glyceraldehyde phosphate dehydrogenase) promoter and *trpC* (tryptophan synthetase) terminator sequences has been widely used, e.g. for the transformation of *S. commune, P. ostreatus, H. cylindrosporum* and *L. laccata*. In *Ustilago* sp., the *hph* and *sat-1* genes were fused to an *U. maydis* heat shock gene promoter (*HSP70*), or to a *Cochliobolus heterostrophus* promoter, and in *P. chrysosporium*, the phleomycin-resistance gene to a histone *H4* promoter. Of particular interest are the *Kan*^{R} gene, which is expressed from its own promoter in *P. chrysosporium*, and the *GUS* gene expressed from the cauliflower mosaic virus 35S gene promoter in *U. appendiculatus*. In the case of *A. aegerita*, the *Neo*^{R} gene functions under the control of an *Agrocybe* promoter-like DNA sequence, which was previously cloned in *E. coli* upon its ability to drive expression of different structural antibiotic-resistance genes in bacterial cells. The antibiotic-resistant markers possess the advantage to be potentially expressed in any wild sensitive genetic context. However, problems may arise from their expression level in the transformed cells, either because of the origin and strength of the promoter used, or because of excessive methylation of the structural gene in the fungus (Mooibroek et al., 1990). This can be overcome by the development of homologous gene-reporter systems, as described in *S. commune* (Schuren et al., 1993).

Transformation procedures and transformation efficiencies

The most commonly used procedure for transformation consists in the treatment of protoplasts for most species, or spheroplasts in the case of Ustilaginales, with $CaCl_2$ (from 10 to 50 mM, typically 25 mM) and polyethylene glycol (PEG 4000, 25-60% w/v) in the presence of the transforming DNA. Protoplasts can be isolated from young mycelium, or from ungerminated or germinating basidiospores and, when possible, oidia. The transformation efficiencies given by this method (Table 2) vary generally from 1 to 100 transformants per μg of DNA. In the case of the *ade1* homologous tranformation system of *P. chrysosporium*, an efficiency of 300 transformants per μg of DNA was reported (Alic et al., 1991). From the accurate analysis of the parameters influencing transformation efficiencies, e.g. $CaCl_2$, PEG, protoplasts and DNA concentrations, or the procedures to isolate protoplasts, an efficiency as high as 1000 transformants per μg of DNA has been obtained in *S. commune* (Specht et al., 1988). For the Ustilaginales, efficiencies of the same order (10 to 1000 transformants per μg DNA) have been reported (Holden et al., 1988; Banks and Taylor, 1988; Wang et al., 1988).

Transformation by protoplast electroporation was also reported in *P. ostreatus* (Peng et al., 1992) and in *A. aegerita* (Noël and Labarère, 1994; Noël et al., 1995). In electric field strengths varying from 2.2 to 2.8 kV/cm for *P. ostreatus*, and from 2.25 to 5.5 kV/cm for *A. aegerita*, the transformation efficiencies ranged in both species from 1 to 50 transformants per μg of DNA. Although no difference in transformation efficiency could be observed

between electroporation and the $CaCl_2$-PEG method with *P. ostreatus*, electroporation was the sole technique allowing selection of transformants in *A. aegerita* (Labarère et al., 1993).

An alternative to the transformation of protoplasts is the treatment of intact sexual or asexual spores with 100 mM lithium acetate. In *C. cinereus*, the transformation efficiency of one transformant per 10^7 oidia makes this technique only usable with non-revertant mutations (Binninger et al., 1987). In contrast, the lithium acetate treatment was efficient with sporidia of *U. violacea* for which 60–80 transformants per μg of DNA has been reported (Bej and Perlin, 1989).

A fourth technique has been used for the transient expression of the *GUS* gene in urediniospores of *U. appendiculatus* by DNA-coated gold particule bombardment (Li et al., 1993). Because of the particular experimental conditions, the transformation efficiency ($8.2\ 10^{-4}$ cells expressing *GUS*/1.6 μg of DNA) cannot be compared to the three other methods.

In addition to the transformation procedure itself, several other parameters influence the transformation efficiency. The use of antibiotic-resistance markers, whose expression is driven by heterologous promoters, may lead to transformation efficiencies 10 times lower than with homologous markers (Mooibroek et al., 1990; Noël et al., 1995). The form under which the transforming DNA is delivered may also play a role. A slight increase in transformation frequency was reported in *C. cinereus* by linearization of the transformation vector outside the *TRP1* marker of selection (Binninger et al., 1991). Likewise, a 20-fold increase in transformation efficiency was reported in *U. maydis* by linearizing the plasmid DNA in the 5′ region of the *HSP70* promoter used to drive expression of the *hph* gene (Wang et al., 1988).

Fate of the transforming DNA

Depending on the vector used for transformation, the transforming DNA may undergo integration into the recipient chromosome, or may be maintained as an extrachromosomal form into the recipient strain.

Integrative events Like previously reviewed in filamentous fungi (Fincham, 1989), transformation in basidiomycetes leads generally to integration of the vector into the genomic DNA of the recipient strain by recombination. The more comprehensive studies about the fate of the transforming DNA were realized in *C. cinereus* (Binninger et al., 1987) and *U. maydis* (Wang et al., 1988), which can be chosen as examples to survey the different types of integration events in basidiomycetes. Among 92 *TRP1* transformants of *C. cinereus*, 5% had integrated the vector DNA at the resident *trp1* locus (homologous integration), and 95% somewhere else in the genome (ectopic integration). In the case of homologous integration, 75% of the transformants showed tandem duplications of the integrated vector, and gene replacement was observed in only one transformant (Binninger et al., 1991). In the case of ectopic integration, 96% of the transformants had integrated vector sequences in a single site and 4% in multiple sites. Integration of the vector DNA in tandem arrays was reported for 14% of the ectopic transformants (Binninger et al., 1987). Similar data about the nature and the frequency of the integration events were reported in *U. maydis* with the *hph* gene driven by the *HSP70* promoter. Homologous integration at the *HSP70* locus occurred in 18% of the transformants (of which 80% possessed multiple copies) and ectopic integration in 82%, of which 87% had multiple copies, the half having undergone deletions or molecular rearrangements (Wang et al., 1988). In the case of the *Neo*^{R} heterologous marker used to transform *A. aegerita*, the 0.9 kb promoter-like fungal sequence cloned upstream the bacterial structural gene seems to play a role in the targeted integration of the vector, indeed, 25% of the transformants analysed appeared to have integrated the transforming vector at the locus from which the promoter-like DNA sequence was derived (Noël et al., 1995). In other studies using homologous markers of transformation, the few number of transformants analysed in most cases did not allow to show targeted integration of the transforming DNA by homologous recombination at the resident locus, and ectopic integration prevails in most of the species transformed.

Extrachromosomally maintained plasmids Several reports have mentioned the presence of extrachromosomally maintained vector-derived DNA sequences in transformants. Even using integrative transformation vectors, no transformant by integration could be obtained in *P. chrysosporium* with vectors carrying the *Kan*^{R} gene (Randall et al., 1991) or a lignin peroxydase gene disrupted by the *Kan*^{R} gene (Randall and Reddy, 1991). Both vectors undergo replication in transformants; one of them had acquired a DNA sequence derived from an endogenous extrachromosomal DNA element of *P. chrysosporium* (Randall et al., 1991). Likewise, no integrative event could be obtained with the pAN7.1 vector in *P. ostreatus* (Peng et al., 1992). The vector had recombined in vivo with a chromosomal fragment supposed to support replication of the plasmid in transformants, eventhough the nucleotide sequence of this fragment did not provide direct evidence for the presence of an ARS (Autonomous Replicating Sequence) ~ type element (Peng et al., 1993). In two other studies on *U. violacea* (Bej and Perlin, 1989) and *A. aegerita* (Noël and Labarère, 1994), both integrative events and vector-derived extrachromosomal molecules were characterized. In *U. violacea*, these molecules were shown to derive from the primary transformation vector, either unmodified or which had acquired mitochondrial DNA sequences (Bej and Perlin, 1991). In *A. aegerita*, all the extrachromosomal plasmidic forms rescued from transformants had suffered deletions in the *URA1* selection marker and were generated, in all likelihood, by the permanent excision of primarily integrated vector sequences (Noël and Labarère, 1994). In contrast to the well characterized ARS sequence isolated in *U. maydis* (Tsukuda et al., 1988), and exception made for *P. chrysosporium*, in which experimental data demonstrated replication of the plasmids in the fungus, there is no in-

dication for the involvement of ARS-type sequences in the other transformation systems.

Applications of transformation

The applications of transformation in basidiomycetes are yet effective, by the cloning of metabolic and fungicide-resistance genes, the study of heterologous gene expression, and the isolation and functional analysis of developmentally regulated genes.

Cloning of metabolic genes With high-frequency transformation vectors, several metabolic genes have been cloned directly by transforming mutant strains of the fungus with genomic banks. The plasmid carrying a gene able to restaure a prototroph phenotype is recovered from transformants either by DNA digestion/ligation and back transformation of *E. coli* (Froeliger et al., 1987), by sib-selection (Pukkila and Casselton, 1991) or by using autoreplicative vectors when available (Fotheringham and Holloman, 1989). This allowed isolation of the *ADE2, ADE5, PAB1, TRP1* and *URA1* genes of *S. commune* (Froeliger et al., 1987), the *TRP-3, ADE-8* and *PAB-1* genes of *C. cinereus* (Pukkila and Casselton, 1991), and the *LEU1* gene of *U. maydis* (Fotheringham and Holloman, 1989).

Heterologous gene expression studies Heterologous gene expression through transformation may be useful for identifying mutations or the product of cloned genes, for functional studies or to have insights about the phylogenetic distances of different taxa. Successful transformation of *P. chrysosporium* adenine auxotrophs was achieved with the *ADE2* and *ADE5* genes of *S. commune* (Alic et al., 1989, 1990). Likewise, a *trp-2* mutant of *C. bilanatus* has been transformed with the wild *trp-2* gene of *C. cinereus* (Burrows et al., 1990) and a *trp-2* mutant of *C. cinereus* could be complemented with the *TRP1* and *TRPC* genes of *S. commune* and *P. chrysosporium*, respectively (Casselton and de La Fuente Herce, 1989). Such a possibility to interchange genes among basidiomycetes led to interspecies functional studies, as reported for the expression of the *A* mating type gene of *C. cinereus* in *C. bilanatus* (Challen et al., 1993), and the study of the conservation of the *b* mating type gene complex in bipolar and tetrapolar smut fungi (Bakkeren and Kronstad, 1993). In contrast to the possibility to interchange genes between basidiomycetes species, the attempts to make express ascomycetous genes in basidiomycetes have failed (Casselton and de La Fuente Herce, 1989), whereas the expression of basidiomycetous genes in ascomycetes has already been reported (Hynes, 1989; Kronstad et al., 1989; Alic et al., 1990).

Cloning of developmentally regulated genes and functional analysis Certainly one of the most interesting application of the transformation systems developed in basidiomycetes has been the identification and the isolation of the *A* mating type genes of *S. commune* (Giasson et al., 1989) and *C. cinereus* (Mutasa et al., 1990), and of the *b* mating type genes of *U. maydis* (Schultz et al., 1990). The molecular characterization of the *A* mating type region revealed a complex structure involving several genes in both *S. commune* and *C. cinereus* (Stankis et al., 1992; Kües et al., 1992) encoding proteins with homeodomains typical of transcriptional factors, as also reported in *U. maydis* (Schultz et al., 1990). Functional analysis by transformation allowed to determine the function of these genes in the regulation of both asexual and sexual developmental pathway of hymenomycetes (Tymon et al., 1992; Specht et al., 1992), and in the threshold and maintenance of the pathogenic process in *U. maydis* (Schultz et al., 1990).

Functional studies of genes that cannot be directly used as markers to screen transformants may be achieved by cotransformation, which can be easily obtained in basidiomycetes, with an average efficiency of 70% of cotransformants (Mellon et al., 1987; Casselton and de La Fuente Herce, 1989; Mooibroek et al., 1990; Marmeisse et al., 1992). Unfortunately, many efforts remain to do to in hymenomycetes for the study of well-defined genetic mutation or the creation of null mutation by one-step gene disruption, as described in *U. maydis* (Fotheringham and Holloman, 1989; Kronstad et al., 1989), in part because it appears difficult to enhance the frequency of homologous integration (Binninger et al., 1991).

Others genes involved in fundamental pathways of the life cycle of basidiomycetes have been isolated, such as the *FRT1* gene of *S. commune* which induces the development of fruit bodies in homokaryotic strains (Horton and Raper, 1991), and the *REC1* gene controlling recombination in *U. maydis* (Tsukuda et al., 1989). The isolation of mutant alleles conferring resistance to fungicides in *U. maydis* may prove useful to better understand the mode of action and the targets of the fungicides used to control phytopathogenic fungi (Keon et al., 1991; Kinal et al., 1993; Gold et al., 1994).

Besides their applications in basic domains, transformation of the basidiomycete species with economic incidence in the food industry constitutes an alternative tool to target genetic improvement, and to reach a better knowledge of the control of basidiocarp differentiation. For example in *A. aegerita*, transformation will be helpful to gain further information on the function and regulation of the cloned morphogenetic genes (Salvado and Labarère, 1991) and to confirm at the molecular level the mating type switching phenomenon demonstrated by genetic analyses (Labarère and Noël, 1992). From a general point of view, it appears that the great diversity of transformation systems and genetic markers now available in basidiomycetes constitutes performant molecular tools to elucidate the genetic and molecular mechanisms involved in fungal differentiation.

Acknowledgements——The preparation of this review was supported by grants from the Conseil Scientifique de l'Université de Bordeaux II, the Conseil Régional d'Aquitaine and the Institut National de la Recherche Agronomique.

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