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CONTENTS

Practical Section for Growers

The work described in this report is aimed at developing genetic manipulation technology for the mushroom. This technology is of importance in the mushroom because of the advantages it offers over conventional breeding for directed strain improvement, and because of its power in biological analysis. In the strain improvement context it also affords the opportunity to unambiguously mark new/novel spawns and protect them in the market place. The work described was funded by both MAFF (£120K over 3 years) through its Competitive Tender scheme in the Plant Molecular Biology Programme: Development of facile transformation for Crop Plants and the HDC (£30K over 3 years).

Transformation depends on three main components (i) the availability of a suitable selectable marker gene (ii) the ability to deliver this gene, combined with a second gene of agronomic value, into the mushroom (iii) the integration of the transforming genes into the mushroom's DNA and their subsequent expression.

This project has particularly addressed (i) above but also has relevance to (ii) and (iii). Two types of marker genes have been evaluated, two derived from the mushroom itself and therefore expected to be easier to get to work and one based on a bacterial gene. In addition, a molecular tool kit has been created so that DNA sequences which regulate the expression of a gene can be readily tested.

The most significant finding is that a mushroom gene which codes for resistance to the fungicide carboxin is able to transform the ink-cap mushroom *Coprinus cinereus*. This gene is currently being used in transformation experiments with *Agaricus bisporus* and several hundred possible transformants have been obtained.

The ability to transform the mushroom, albeit at very low efficiency and reproducibility, is now well established. Despite concerns about GM and GM foods this technology is likely to make a significant contribution to strain improvement in years to come. In any event, the availability of the technology will impact on all areas of mushroom biology contributing to our understanding of how the mushroom works and how it interacts with its environment in the broadest sense.

Science Section

1. INTRODUCTION

The homobasidiomycete *Agaricus bisporus* (the common mushroom) is the single most valuable crop of protected horticulture with a current farm-gate value of £170M. The application of recombinant DNA technology in this fungus has been limited by the lack of *facile* DNA-mediated transformation. A workable gene transfer system would both enable the development and exploitation of novel strains of *Agaricus* and related species, and provide a powerful tool for biological analysis. In addition, such technology would allow new strains to be "fingerprinted" unambiguously, by the inclusion of covert DNA sequences making possible the generation of royalty income.

Genetic transformation is a process in which exogenous DNA is taken up by a recipient in which it may become incorporated into the genome or exist as an autonomous replicon. A principal complication encountered by pioneering researchers in this area was the presence of an impermeable barrier preventing entry of DNA into the organism, namely the fungal cell wall. The enzymatic removal of this cell wall to produce protoplasts revolutionised transformation techniques and forms the basis of most procedures for the transformation of fungi.

1.1 Transformation Technology

The first fungal transformation was reported by Mishra *et al.* (1973). An inositol auxotrophic strain of *Neurospora crassa* was transformed to prototrophy using the complementary cloned gene. This experiment was not readily reproducible, and there was a low level of spontaneous reversion. A more successful transformation protocol was developed by Hutchinson and Hartwell (1967 - see Fincham 1989). They devised a method for protoplasting the yeast *Saccharomyces cerevisiae* using glusulase, a commercial enzyme, to degrade the cell walls and 1M sorbitol to osmotically stabilise the protoplasts. Hinnen *et al* (1978) used this protocol to successfully transform a *N. crassa leu2* mutant to prototrophy in the presence of calcium chloride. This basic protocol was refined and tailored for different fungal species, and has been successfully applied to many filamentous fungi, including ascomycetes like *A. nidulans* (Tilburn *et al.*, 1983), heterobasidiomycetes such as *Ustilago maydis* (Wang *et al.*, 1988) and the homobasidiomycetes including *C. cinereus* (Binninger *et al.*, 1987). Other approaches to transformation have also been applied to the fungi, including electroporation of protoplasts (Chakraborty *et al.*, 1991) micro-injection (Correa & Hoch, 1993) and particle bombardment (Fungaro *et al.*, 1995).

Attempts have been made to devise a transformation strategy for *A. bisporus* by several research groups. However, to date a reproducible, reliable system has not been developed. Many unsuccessful attempts to transform *A. bisporus* remain unpublished. Li and Horgen (1993) devised a novel helper-experiment, transforming *Ophiostoma ulmi* protoplasts in the presence of *A. bisporus* protoplasts in the hope that non-homologous recombinational enzymes from *O. ulmi* would facilitate the integration of

the DNA into *A. bisporus.* This was unsuccessful, as was the attempt to biolistically transform *A. bisporus* protoplasts and mycelia with constructs containing resistance markers for pleomycin, kanomycin or hygromycin (Li & Horgen, 1993). Moore *et al.,* (1995) report a similar lack of success with biolistics for *A. bisporus* using phleomycin resistance markers although large numbers of spurious phleomycin resistant colonies were obtained. Electroporative transformation of *A. bisporus* protoplasts with the pAN7-1 construct yielded low numbers of hygromycin resistant transformants (van de Rhee *et al.,*1996). Extensive attempts have been made at HRI to reproduce van de Rhee *et al* findings, and are reported here.

An enduring difficulty with protoplast-based transformation systems in *A. bisporus* is the production of viable protoplasts i.e. capable of regenerating a mycelium. There is a system for the protoplasting and regeneration of *A. bisporus* hyphal tissue, devised by Sonnenberg et al (1988), and a system for gill tissue (Chen and Hempp, 1993). However, difficulties producing sufficient quantities of viable protoplasts for transformation have been experienced with both systems, and protoplast viability varies between strains.

The two main elements for successful transformation are (i) effective delivery of DNA and (ii) development of appropriate vectors and these are described next.

1.2 DNA Delivery Systems

In successful transformation systems transforming DNA should integrate into the fungal genome and subsequently be translated and transcribed into a functional protein. The transforming DNA must be able to enter the host nucleus without affecting cell viability. Different methods of DNA delivery have been developed and these include: Polyethylene glycol (PEG)/calcium chloride mediated transformation, electroporation, cationic lioposome-mediated transformation, and accelerated microprojectiles (biolistics). Different organisms require a specific set of transformation parameters, and not every transformation system is applicable to all organisms. Efficient protoplasting and regeneration procedures are the basis of many transformation systems.

1.2.1 PEG/$CaCl_2$ Transformation

This method has been most widely used to transform filamentous fungi (see Fincham 1989 for review) including several Basidiomycetes (Table 1).

Table 1 Basidiomycetes transformed using PEG/CaCl2 treatment of protoplasts.

Basidiomycete Species	Reference
Ustilago maydis	Wang *et al.*, 1988
Ustilago hordai	Holden et al., 1988
Ustilago nigra	Holden *et al.*, 1988
Coprinus cinereus	Binninger *et al*, 1987
Schizophylum commune	Burrows *et al.*, 1990
Schizophylum commune	Munoz-Rivas *et al.*, 1986
Pleurotus ostreatus	Jia *et al.*, 1998
Volvariella volvacea	Jia *et al.*, 1998

Fungal cells are protoplasted with a species specific combination of cell wall degrading enzymes. This removes the cell wall as a barrier to transforming DNA. The type of cell which is lysed is also species dependant. In *A. bisporus* mycelia are normally protoplasted due to the absence of a suitable asexual uninucleate spores. The protoplasts are incubated on ice with the transforming DNA, calcium chloride and polyethylene glycol (PEG) 40000. Calcium ions are believed to assist the uptake of DNA by an active process. PEG promotes the agglutination of the protoplasts ensuring that the DNA is in close association with the cell membranes (Fincham, 1989). The protoplasts are then suspended in a regeneration broth to allow re-synthesis of the cell walls before plating onto selective medium.

This method is effective at transforming different species of *Coprinus* (Binninger *et al.*, 1987, Challen *et al.*, 1994) but remains experimental for *A. bisporus* as most attempts have been unsuccessful.

1.2.2 Electroporative Transformation

Electroporation uses short, high voltage electrical pulses to cause the protoplast cell membranes to become transiently fluid and therefore permeable to transforming DNA. Protoplasts are directly exposed to the transforming DNA whilst suspended in an appropriate buffer. The transformation suspension is exposed to a pulse of electricity generated by electroporative apparatus. The electrical pulse causes the cellular transmembrane voltage to increase dramatically. This in turn increases the electrical energy within the membrane and, combined with the thermal fluctuations present , causes pore formation (Weaver, 1995). Electroporation was used by Van de Rhee *et al.*, (1996) to produce some *A. bisporus* transformants.

1.2.3 Cationic Liposome-Mediated Transformation

Liposomes spontaneously react with DNA, to form a positively charged complex which is attracted to the negatively charged protoplast cell wall. The liposome-DNA complex binds to the cell membrane and releases the DNA into the cell cytoplasm. This avoids cytotoxicity and is therefore highly efficient within optimised systems. There are several theories discussed by Lurquin and Rollo (1993) proposing the mechanism of liposomal transfer to protoplasts, one of which is the direct fusion of the

liposome to the protoplast but there is no supporting evidence that this occurs. Another model proposes that the liposome adsorbs to the protoplast and its contents are transferred across the membrane through transient pores. However, attempts to reproduce this using fluorescent dye have failed. A mechanism of endocytosis seems more likely. Polyethylene glycol with calcium ions can stimulate the uptake of viral RNA into plant cells, but as viral particles are coated in proteins it is more likely that endocytosis into the host is stimulated. This process is termed chemical endocytosis by Lurquin and Rollo (1993) as endocytosis is not a natural phenomenon in plant cells.

Cationic liposomes have not been used extensively in plant DNA transformations. Transformation of the yeast *Schizosaccharomyces pombe* in the presence of Lipofectin (liposome preparation) increases the transformation efficiency 10-50 fold, and facilitates the integration of molecules>500 kb (Allshire, 1990). Success has also been achieved in the higher fungi, as *N. crassa* (Selitrennikoff & Sachs) has been transformed to benomyl and hygromycin resistance, with a 3-4 fold increase in transformation efficiency, liposome technology has been used to transform *A. bisporus* protoplasts and is described in Section 3.2.1. However, these transformants proved unstable.

1.2.4 Biolistic Transformation

The biolistic process was invented by Sanford, Wolf and Allen, and primarily used to accelerate 4μm tungsten micro-projectiles coated in DNA into onion epidermal cells (see Sanford, 1988 for review). The biolistic process has since been constantly refined and modified to suit different species.

Micro-projectiles are typically spherical high density metal (tungsten or gold) particles 0.4-2.0μm in diameter and are coated in transforming DNA (Sanford, 1990). Biolistic particle guns which are used to fire the particles into tissue may be powered by compressed gas or an electrical discharge. An advantage of this technology is that it can be used with a wide range of tissue types (protoplast independent). After osmotic treatment to prevent the cells from bursting upon impact, the target tissue is placed, on the osmotic media, in a vacuum chamber. A helium burst of specific pressure related to the host species is released in to the chamber, which forces the micro-projectiles from the macrocarrier through the stop plate (which prevents the macrocarrier becoming embedded in the agar) and into the target tissue.

A gene gun driven by compressed air was developed at HRI, and was based on a pneumatic device used to transform plant tissue (Moore *et al.*, 1995). The compressed air is released in a controlled manner, and accelerates the particles, which are loaded onto a macrocarrier, up a barrel the macrocarrier impacts upon a stop plates, and the particles are accelerated forwards through an aperture onto the target tissue (Moore *et al.*, 1995). *C. cinereus* and *C. bilanatus* strains have been transformed using this device, and also unstable *A. bisporus* transformants have been obtained.

Many biolistic parameters vary between different host species, e.g. target tissue, helium pressure and particle size. Morrish *et al.*, (1993) considered parameters affecting cereal crop transformation, and proposed that the lack of uniformity in

particle aggregation, coating, dispersal, size, penetration depth, velocity, and quality lead to random results. These problems appear to affect all biolistic transformation systems.

Biolistic transformation has been achieved for some members of the filamentous fungi. An example of this is the apple scab *Venturia inaequalis*, conidia of which were transformed by Parker *et al.*, 1995 (9-17 transformants per 0.8μg DNA). A PDS1000 helium device was used to accelerate tungsten micro-projectiles coated in pOHT DNA (carrying the phosphotransferase gene conferring hygromycin B resistance) at the target tissue.

Moore *et al.*, (1995) state that the successful ballistic transformation of yeasts and lower fungi indicates the possibility of using this protocol to transform *A. bisporus.* They successfully transformed *Coprinus cinereus*, by bombarding both mycelia and oidia of tryptophan auxotrophs, using an air driven gene gun. The rates of transformation were not particularly high.

1.3 Marker Genes and Transforming Vectors

The marker gene is a vital means of identifying and selecting for any putative transformants. Auxotrophic, resistance and reporter gene markers are commonly used methods of identifying transformed cells. Markers for auxotrophy are commonly used in fungal transformation systems. For example, Munoz-Rivas *et al.*, (1986) restored the basidiomycete *Schizophyllum commune* tryptophan auxotrophic strain to prototrophy with the homologous TRP1 sequence. Auxotrophic markers depend upon the existence of mutant strains deficient in one step of a biosynthetic pathway, and therefore require the missing metabolite as a supplement. The wild type gene can be replaced by transformation, and the revertant prototroph can be screened for. Auxotrophs are rare in *A. bisporus* and are difficult to select due to its multinucleate mycelium. However, mutants can be readily created in the ink-cap *Coprinus cinereus* and consequently auxotrophic strains are available in this species. A *C.cinereus* auxotrophic strain (LT2) with a double mutation of the tryptophan synthetase gene *TRP1* has been successfully transformed back to prototrophy (Skrzynia *et al.*, 1989). This method is now used to increase the possibility of transforming *Coprinus* with other marker genes, by co-transformation.

Resistance markers may be used to identify transformants by positive selection. Colonies transformed with a sequence conferring resistance to an antimetabolite to which the wild-type is sensitive can be screened for by plating onto selective medium. This type of marker is widely used in fungal transformation systems. Elliott (1976b) suggested that resistance markers could replace auxotrophic markers as a method of selection in mushroom breeding.

Reporter genes confer a quantifiable trait which is readily identifiable, rather than an advantageous trait upon the host. Constitutive expression of the gene must not be present in the host strain. An example of a reporter gene is the *GUS* gene for β-glucoronidase. This gene is widely used in plant and fungal transformation systems.

Wild-type colonies can still survive upon the selective medium, but the transformed colonies can be identified by a distinctive blue colouring. As this is a quantifiable colourimetric assay, the intensity of colouring is directly related to the level of gene expression.

Two molecular approaches have been followed to develop mushroom transformation in this project utilising marker genes: an indirect approach and a direct approach.

1.3.1 Indirect Approach

Novel reporter gene constructs have been exploited to evaluate the activity of a series of homologous and heterologous promoters. A series of vector backbones were designed consisting of a promoter sequence and a terminator sequence. The presence of conserved restriction sites within the construct means that a range of marker gene cassettes can be readily interchanged. Some of these constructs may allow the quantification of promoter activity by assaying protein products.

Successful transformation is more likely if the insert is driven by a promoter recognised by the host's transcriptional machinery, although some systems work with promoterless constructs. It seems more probable that an *A. bisporus* DNA polymerase will recognise and therefore transcribe an *A. bisporus* promoter than a heterologous one unless it is highly conserved. For example, the constructs pBU001 (*C. cinereus TRP1* promoter), pBU003 (*A. bisporus TRP2* promoter), and pBU004 (*A. bisporus GPDII* promoter) all have highly expressed and conserved promoters which drive key fungal enzymes. This means that there is high homology between fungal species. The construct pBU001, despite being a *C.cinereus* promoter, seems equally likely to be transcribed as the other two strong, *A. bisporus* promoters. By evaluating the promoters used in transformation, the strongest promoters can be selected for maximal transformation efficiency.

1.3.1.1. Luciferase

The luciferase gene *luc*$^{+}$ originates from the bioluminescent firefly beetle *Protinus pyralis*. Luciferase is a 62kDa monomer which is enzymatically active upon translation (Promega, 1993). Luciferase catalyses the oxidation of its substrate luciferin in the presence of molecular oxygen, ATP and Mg^{2+} to produce oxyluciferin. This product is in an excited state, and emits a photon of light to revert to the ground state (Murakami *et al.*, 1998). Luciferase has a high specificity for ATP and the highest quantum efficiency of light production for any known chemiluminescent reaction (Promega, 1993). It is therefore the most sensitive system for quantifying promoter activity in a direct assay. The luciferase system is widely used as a molecular marker in both plants, e.g. *Brassica napus* was biolistically transformed with the *luc* gene by Fukuoka *et al.* (1999), and yeast systems, e.g. *Saccharomyces cerevisiae* was transformed using luciferase-encoding RNA's by Russel *et al.* (1999). To date there have been no reports of luciferase expression in the higher fungi.

1.3.1.2. βGlucoronidase (GUS)

The *E. coli* GUSA gene is part of the *gus* operon which encodes proteins allowing the bacterium to use glucoronide substrates in the gut as a carbon source. The *gus* operon consists of a cluster of genes under upstream regulation. Quantitative and qualitative data can be obtained using this reporter gene, and the assay is extremely sensitive. Consequently, this system is widely used to analyse plant (see Martin *et al.*, 1992 for review) and bacterial (see Wilson *et al.*, review) gene expression. The GUS system has been successfully applied to the yeast *Saccharomyces cerevisiae* (Schmitz *et al.*, 1990) and a range of filamentous fungi (Roberts *et al.*, 1989) including the heterobasidiomycete *U. maydis* (Richard *et al.*, 1992).

1.3.1.3 Green Fluorescent Protein

This bioluminescent gene originates from the jellyfish *Aquorae victoria*. In order to improve specific properties of the wild type GFP gene, such as reducing the delay between protein synthesis and fluorophore development, the GFP gene has been synthesised with oligotide-mediated site-directed mutagenesis or optimised codon usage. These improved GFP isoforms display a 42-fold increase in brightness and fluorescence (Jaiwal *et al.*, 1998).

The green fluorescence (λmax 508nm) emitted from the GFP bioluminescence system is caused by a redox reaction involving two associated proteins, aequorin (calcium-binding photoprotein) and coelenterazine (a green fluorescent protein) (Kojima *et al.*, 1997).

GFP is used in plant systems to monitor gene expression and as a genetic marker in living tissue. It has also been successfully expressed in the yeast *S. cerevisiae* (Gurvitt *et al.*, 1999), and the lower fungi (Hardham and Mitchell. , 1998). GFP gene expression is independent of cell location and type, although it may require a specific target site within a cell for efficient post-translational modification (Jaiwal *et al.*, 1998). Cell metabolism is not disrupted by the expression of this construct, and a cluster of cells expressing GFP could be easily identified under a uv. microscope.

1.3.2. Direct approach - Using Heterologous Genes as Selectable Markers – Evaluation of pAN7.1

Prior to work commencing, a group from ATO-DLO in The Netherlands published and filed a patent for a transformation procedure for *Agaricus bisporus* based on the use of the pAN7-1 vector. The pAN7-1 construct is based on the bacterial gene *hph*, and transformation with this vector yields colonies which are resistant to the aminoglycoside antibiotic hygromycin. The expression of the *hph* gene is controlled by a promoter derived from the ascomycete fungus, *Aspergillus nidulans*. This vector had previously been tested for mushroom transformation without success in a number of laboratories.

It was essential to determine whether the patented protocol could routinely produce pAN7-1 transformants in *Agaricus bisporus*. The protocol was therefore evaluated *ad verbatim* and with various minor modifications.

1.3.3 Direct approach - Using Homologous Genes as Selectable Markers

Homologous genes are advantageous as selectable markers for a number of reasons: the host should recognise the sequence as self and therefore not excise the DNA upon entry into the chromosome, and DNA polymerase should recognise and bind to the promoter sequence.

Two mutant *Agaricus* genes, complete with regulatory sequences have been cloned and used in this study. The first is a gene which can confer resistance to the fungicide carboxin. Carboxin is a systemic fungicide which blocks mitochondrial respiration by inhibiting the oxidation of succinate to fumerate by the enzyme succinate dehydrogenase.(SDH). SDH has two sub-units: the flavoprotein and the iron-sulphur protein. Electrons pass from this dimeric protein to the ubiquinone-binding proteins in the electron transport chain. Carboxin blocks this flow of electrons by preventing the redox centre of the iron-sulphur protein from reoxidising (Keon *et al.*, 1995). A carboxin resistance gene isolated from *Ustilago maydis* (Keon *et al.;* 1991) has a modified iron-sulphur protein. Specifically a two base mutation allows the substitution of a leucine residue with a histidine residue within the third cisteine-rich cluster (Keon *et al.,* 1995). This mutation may alter the steric conformation of the cluster, thus allowing the continued flow of electrons in the presence of carboxin. Four carboxin resistant mutants were induced in the secondary homothallic Homobasidiomycete *C. bilanatus* (Challen & Elliott., 1989); only one was stable and behaved in the manner of a single dominant gene. A *C. bilanatus* Lorist library was screened for carboxin resistance using transformation of *C. cinereus,* as well as homologous transformation of *C. bilanatus.* Neither approach was successful (Challen *et al.,* 1991).

Challen & Elliott (1985) produced carboxin resistant mutants in *A. bisporus* using uv mutagenesis. A stable mutant , C54-carb.8 was used to create a cosmid library. Degenerate PCR primers from mutant *A. bisporus* DNA were used to screen the library. Two classes of sequences were identified, and these are used in this study as homologous markers.

The second gene codes for orotidine monophosphate decarboxylase biosynthesis and a mutation in it results in auxotrophy for uracil. Many fungal transformation systems have been based on auxotrophic markers and the orotidine-5'-monophosphate decarboxylase (OMPdecase) gene in particular has proved to be extremely useful. Using this system, mutant recipients can be positively selected for by virtue of their resistance to the toxic compound 5-fluoro-orotic acid (5FOA). In *A. bisporus*, very few auxotrophic mutants have been isolated, of which most exhibit slow or restricted growth. However, one auxotrophic uracil-requiring mutant, N1-*unk*, is available and attempts are described to transform the strain back to prototrophy using the corresponding wild type OMPdecase (*URA3*) gene.

2. MATERIALS AND METHODS

2.1. Strains and Culture Maintenance

2.1.1 *Agaricus bisporus*

The auxotrophs W9-ade his (adenine and histidine), N1-ura and its protoclone derivative PC1 (uracil) were used as target strains for transformation in this study. For mycelial growth the cultures were incubated at 25°C.

Mycelial cultures were retrieved from the HRI liquid nitrogen culture collection. The strains were routinely sub-cultured on complete media. Cultures were also stored at 4°C. *A. bisporus* strains were also maintained as primary, secondary and tertiary macerates. Ten cores of fluffy mycelia were taken from a single culture using a size 6 cork borer. These were suspended in 15ml of ultra pure water and fragmented in a Silverson laboratory homogeniser for 10 seconds. This was termed the primary macerate, and was used to inoculate 10 plates of complete yeast extract medium with compost extract (CYM/CE). After incubation for 1-3 weeks, the mycelial mat from a plate of primary macerate was again resuspended in 15mls of water and homogenised as before. Tertiary macerates are plated into complete yeast extract (CYM) broth and allowed to regenerate for a period of 3-5 overnights before protoplasting.

2.1.2 *Coprinus cinereus*

The *C. cinereus* wild type homokaryons H9 (mating type A_6B_6) and H2 (mating type A_5B_5 were used as target strains for transformation. The *C. cinereus* strain LT2, a double tryptophan auxotroph, was exploited in co-transformation experiments as transformation of *C. cinereus* with the trp1 gene is an established system (Binninger *et al.*, 1987).
C. cinereus cultures were routinely incubated at 37°C.

2.1.3 *Escherichia coli*

E. coli strain DH5α, genotype *supE44 ΔlacU169(Φ80 lacZΔM15) hsdR17 rec1 endA1 gyrA96 thi-1 reclA1* (Hanahan., 1983),was used in this study as a host strain for plasmid vectors. Cultures were incubated at 37°C for growth.

E. coli cultures were stored in micro-porous beads (MicrobankTM, Pro-Lab Diagnostics) at -70°C and -20°C. The beads are used to store the bacteria as they adhere to the surface. L broth (5ml) containing antibiotic was inoculated with *E. coli* and incubated overnight at 37°C. Microbank tubes were then inoculated with 1ml of broth and incubated for 1hour. Excess fluid was removed, and the vials stored at -80 and -40 °C. Beads were streaked out routinely onto L media.

2.1.4 Plasmid vectors and gene constructs

Three types of gene construct were used in this study: constructs carrying a regulatory sequence, the pAN7-1 vector and homologous gene constructs.

1. Regulatory gene constructs

A construct backbone was devised, by the HRI mushroom group in conjunction with Bristol University, into which different promoter, marker gene and terminator

sequences were inserted using restriction enzymes (Fig.1). Ten *A. bisporus* and *C. cinereus* promoters from a variety of constitive and inducible functions were ligated into the construct backbone (Table 2) and cloned into pBluescript. These promoters were quantified for use in transformation systems by inserting different genes with quantifiable protein products into the constructs. This allowed for a direct assay to quantify promoter activity. The genes used in this study were for luciferase (luc^+), β-glucoronidase (GUS) and green fluorescent protein (GFP) activity.

2 Heterologous construct

The pAN7-1 vector containing the *hph* gene encoding for resistance to hygromycin and with *Aspergillus* promoter and terminator sequences was available at HRI.

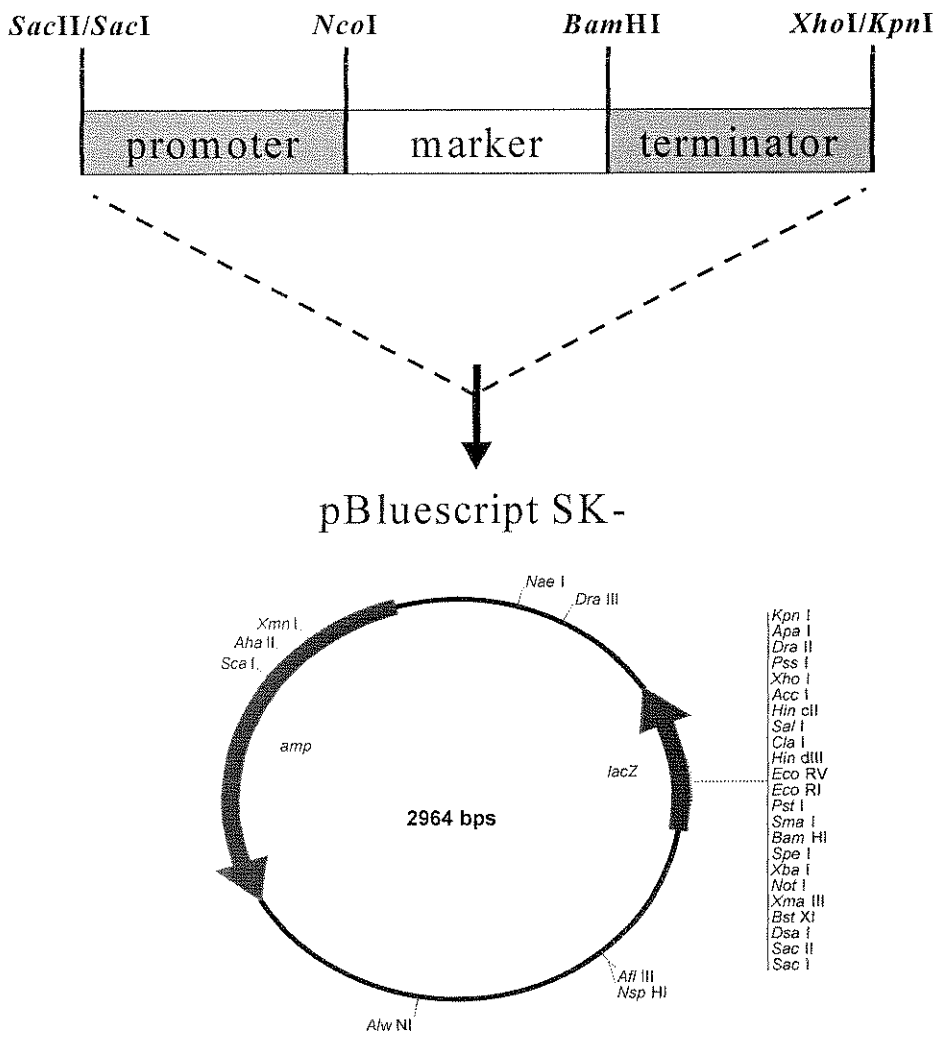


Fig. 1 Schematic of expression cassette developed for the evaluation of mushroom transcription control sequences

Table 2 Promoters quantified for transformation suitability by reporter constructs.

Promoter	Construct Name	Original Species	Original Function	Reference
TRP1	pBU001	*C.cinereus*	Tryptophan synthetase catalysing the conversion of indoleglycerol phosphate and serine to tryptophan and glyceraldehyde-3-phosphate.	Skrzynia *et al.*, 1989
Cel1	pBU002	*A.bisporus*	Induces Cellulase production as a regulatory response to cellulose being the primary substrate.	Yague *et al.*, 1994
TRP2	pBU003	*A.bisporus*	Tri-functional protein with glutamine amidotransferase, Phosphoribosyl,anthranilate isomerase and indole glycerol phosphate synthetase activities in the TRP pathway.	Challen *et al.*, 1996
GPDII	pBU004	*A.bisporus*	Glyceraldehyde-3-phosphate dehydrogenase, which is a key enzyme in glycolysis.	Harmsen *et al.*, 1992
Cel3	pBU005	*A.bisporus*	Expression induced by presence of cellulose. 52kDa protein involved in utilisation of cellulose as a growth substrate .	Chow *et al.*, 1994
gdhA	pBU006	*A.bisporus*	$NADP^{+}$dependant glutamate dehydrogenase which catalyses the reversible amination of 2-oxoglutarate to glutamate in nitrogen metabolism.	Shaap *et al.*, 1995
hypA	pBU007	*A.bisporus*	Hydrophobic protein of 8-9kDa which form a protective hydrophobic layer in the cap tissue. Important for cap formation.	Degroot *et al.*, 1996
Lcc1	pBU008	*A.bisporus*	Production of Laccase- glycoprotein whose regulation and production is closely related to fruit body production.	Perry *et al.*,1993
SPR	SPR	*A.bisporus*	Serine protease	
β-tubulin	β-tub	*C.cinereus*	Structural component of tubulin, Involved in cell division.	

3 Homologous plasmids

A carboxin resistant *A. bisporus* stable mutant c54-carb.8 was used to create a cosmid library (Challen *et al.*, 1985). Degenerate PCR primers were used to amplify SDH sequences from *A. bisporus* DNA, which were used to screen the cosmid library. Seven cosmids were identified, and these fell into two distinct classes (Table 3) based on sequence analysis, believed to be wild type and resistant alleles.

Table 3 The two classes of SDH plasmids/cosmids

α GROUP	β GROUP
50-D5	47-E7
21-D5	17-A6
44-D9	34-H1
47-E5	47-E7 β2-1 (subclone of 47-E7)
50-β25 (subclone of 50-D5)	

2.2 Media

Water used in media and buffer preparation was ultra purified by the Elgar Maxima UV water purification system. When necessary, media was solidified by the addition of 1.2% w/v Oxoid technical no. 3 agar, except for minimal medium where 1.0% w/v Oxoid purified agar was used. For sterilisation purposes all media were autoclaved for 15 minutes,15 psi, except for CE/CYM which required 20 minutes.

2.2.1. Mycological media

Complete Yeast Extract Media (CYM Raper *et al.*, 1972)

To make a litre of CYM 0.2 % w/v peptone, 0.2% w/v yeast extract, 0.2% w/v dextrose, 0.05% w/v $MgSO_4.7H_2O$, 0.046% w/v KH_2PO_4 and 0.1% w/v K_2HPO_4 were required.

Malt yeast peptone glucose media (MYPG Challen, 1994)

For 1 litre of medium the following was required: 10g malt extract, 4g yeast extract, 4g peptone, 0.4% w/v D-glucose, 0.05% w/v $MgSO_4.7H_2O$, 0.046% w/v KH_2PO_4 and 10.1% w/v K_2HPO_4.

Minimal media for mushrooms (MMM Raper *et al.*, 1972)

The composition of 1 litre of media was: 0.2% w/v L-asparagine, 2% w/v D-glucose, 0.05% w/v $MgSO_4.7H_2O$, 0.046% w/v KH_2PO_4, 0.1% w/v K_2HPO_4 and 0.013% w/v thiamine.

Compost extract media (CE/CYM)

Compost extract was prepared based on the method described by Xu *et al* (1993). In brief, approximately 400g of phase II mushroom compost was oven dried at 150°C for 3-4h. The dried compost was ground to a powder using a Cyclotec mill no. 1093, added to 1L of cold water and simmered for an hour. After cooling to room temperature, the compost was filtered through Miracloth (Calbiochem Co.) and centrifuged (MSE-HS18, 6 x 250 ml rotor, 5000 solids. This procedure provided approximately 800ml of compost extract, which is stored at -20°C in 400ml aliquots. Concentrated (5x) CYM medium was added to the extract (40ml of 5x CYM to 400ml CE) and made up to 1L.

Protoplast regeneration media

Medium used to regenerate protoplasts were supplemented with 17.i5% w/v sucrose and 0.5% w/v starch.

2.2.2. Antibiotics

Antibiotics for the selection of *E. coli* plasmids were as follows:
Ampicillin - 50 mg/ml stock solution in H2O, 1µl/ml working concentration
Kanamycin - 100 mg/ml stock in H2O, 0.3µl/ml working concentration

2.2.3. Antimetabolites

Carboxin
Stock solutions of carboxin were prepared as 10mg/ml in 100% ethanol stored at 4°C. 1.5µl/ml was added to CYM medium .

X-glucoronidase
A 20mg/ml stock of x-gluc (x-glucoronidase 5-bromo-4-chloro-3-indolyl-glucoronide from Melford chemicals) in dimethyl formamide (DMF) was filter sterilised, stored in the dark (light sensitive) and subsequently added to MMM in a 50µg/ml concentration.

Hygromycin
Hygromycin was incorporated into culture media at a range of concentrations from 5-30µg/ml

2.2.4. Bacterial media

L medium (Little, 1987)
For 1 litre of medium the following was required: 1% w/v tryptone, 0.5% w/v yeast extract and 1% w/v NaCl.

2.3. Protoplasting Buffers and Enzymes

Specific combinations of enzymes and buffers are required for each fungal strain to attempt protoplast production (Table 4).

Table 4. Enzyme and protoplasting buffers used in this study

Strain	Enzyme	Conc. (mg/ml)
A. bisporus PC1	ß-glucanase*	10
A. bisporus N1-URA	Mutanase*	10
A. bisporus W9-ade his	Fluka chitinase*	1
	Horst	1.5
C. cinereus LT2	Onozuka R10*	20
	Sigma chitinase*	1
C. cinereus H2	Onozuka R10*	20
	Novozym*	2.5
	Fluka chitinase*	1
C. cinereus H9	Onozuka R10*	20
	Sigma chitinase*	1

* Enzyme used in combination.

The protoplasting procedure for *C. cinereus* is well established (Binninger *et al.*, 1987; Casselton & Fuente Herce, 1989), and the protoplasts have been used successfully in PEG transformations (Binninger *et al.*, 1987; Challen *et al.*, 1994). However, the availability of the enzyme Novozym used to protoplast *C. cinereus* and *A. bisporus* has been reduced as the manufacturer Interspex Inc has ceased its production. A suitable replacement is required. Sonnenberg *et al.*, 1988 devised a protocol for protoplasting *A. bisporus* hyphal tissue. The activity of a lytic enzyme preparation from a culture filtrate from *Trichoderma harzianum* was compared with that of Novozym 234 for protoplasting *A. bisporus*, and was found to create protoplasts more efficiently at low concentrations. At concentrations of 2-4mg/ml Novozym 234 created 1% of the numbers of protoplasts produced by that of the *T. harzianum.* The *T. harzianum* filtrate has been labelled Horst and is available from Sonnenberg. Although Horst enzyme is very effective at protoplast formation it is expensive and tends to break down the hyphae into tiny hyphal fragments. These may regenerate on the selective medium more readily than protoplasts as they have cell walls and can therefore resist the adverse conditions more successfully, thus creating false positives. Problems with regeneration of hyphal tips for protoplasting remain with *A. bisporus* , as the complete medium which would allow improved growth facilitates the formation of calcium oxalate crystals on the hyphal tips. These crystals are released during protoplasting, and cause protoplast clumping and lysis during pelleting.

2.3.1. *C. cinereus* protoplasting buffers

The *C. cinereus* protoplasting buffers were based on the mannitol osmoticum developed by Binninger *et al.* (1987). The following buffers were used in the protoplasting protocols:

0.2M maleate buffer pH5.5
1.161% w/v Maleic acid

mannitol stock
18.218% w/v mannitol

maleate buffered mannitol transformation buffer (MM)
0.2M maleate buffer pH5.5, 0.4M mannitol stock

Maleate-mannitol $CaCl_2$ transformation buffer (MMC)
0.2M maleate buffer pH5.5, 0.4M mannitol stock, 0.4M $CaCl_2$

2.3.2. *Agaricus bisporus* protoplasting buffers

The *A. bisporus* protoplasting buffers were based on the sucrose osmoticum of Sonnenberg *et al.*, (1988). These are the sucrose-maleate buffer - SM, the sucrose transformation buffer - SMC, and the polyethylene glycol transformation buffer - PEG.

Sucrose-Maleate Buffer (SM)
0.6M sucrose, 0.4M maleate pH 5.5

Sucrose Transformation Buffer (SMC)
0.6M sucrose, 0.4M maleate, 40mM $CaCl_2$

Polyethylene glycol Transformation Buffer (PEG)
0.5% w/v PEG 4000, 10mM Tris pH 7.5, 25mM $CaCl_2$

2.4. PROTOPLASTING PROTOCOLS

2.4.1. *Coprinus cinereus* protoplasts

The protocol for protoplasting *Coprinus* oidia was as follows:

Oidia were released from 8-10-day old mycelial cultures grown on MYPG medium, by pouring on 10ml sterile water and gently scraping with a spreader. After filtration through a 105µ mesh the oidia were pelleted by centrifugation for 8min at 2500rpm (MSE Mistral 1000). The oidia were resuspended in 20-30ml enzyme solution (table 1) and incubated in a water bath at 37°C for 1-4 hours, and mixed by inversion every 15 min. The lysis was monitored by microscopy (x400, phase contrast) every 30 min. When the lysis was judged to have peaked, the protoplasts were filtered and centrifuged as before. The protoplasts were washed thrice in 10 ml of MB buffer, with

centrifugation as before. The pellet was resuspended in 5 ml of MTB buffer. A protoplast count was taken using a haemacytometer. The number of protoplasts required for a transformation is 10^7/ 100µl. Each transformation requires 100µl of cells, suspended in MTB buffer.

2.4.2. *Agaricus bisporus* protoplasts

The protocol for protoplasting *Agaricus* mycelia was as follows:

Tertiary macerates of *Agaricus bisporus,* grown in 4x 50ml CE/CYM broth at 25°C for 3-4 overnights, were harvested by filtration through a 105µ mesh. The mycelia were weighed in a sterile falcon tube. The minimum amount of tissue required was 3-5g. The mycelia were resuspended in filter sterilised enzyme solution (Table 1) at the rate of 1ml of enzyme per 200mg of wet weight tissue. The tube was initially shaken hard to fragment the mycelia and incubated at 25°C in a tissue culture rollacell (NewBrunswick). Cell lysis was monitored as for *C.cinereus*. The protoplasts were filtered through 105µ mesh, centrifuged (MSE Mistral 1000) for 16mins at 3500rpm. The pellet was washed thrice in 10mls of SM buffer (MSE Mistral 1000, 3500g, 10mins) and then resuspended in 5mls of SB. A protoplast count was taken using a haemacytometer as for *C. cinereus.*

2.5. Transformation Protocols

2.5.1 Polyethylene glycol/$CaCl_2$

Protoplasts were prepared using the protocol outlined in (methods 2.4.)
For each transformation 1-10µg of transforming DNA was mixed with 100µl of protoplasts and followed by 25µl of PEG. After incubation on ice for 20 min, a further 1ml of PEG was added, mixed, and incubated for 5mins at RT. The protoplasts were then transferred to either 15 ml of regeneration medium or, after dilution with 2 ml MMC, plated onto an appropriate selective agar in 500µl aliquots.

2.5.2. Electroporative Transformation

Protoplasts were prepared as described in methods 2.4., but resuspended in SEH buffer. The BIO-RAD gene pulser (model 1652098) was set as follows:

0.2cm electrode gap
0.45 kilo volts
25µF capacitance
200Ω resistance

Protoplasts (100µl) and DNA (5µg) were aliquoted into sterile electroporative curvettes (2mm gap, Flowgen) , and placed on ice for 10min before electroporation. The time constant was noted and 1ml of regeneration broth was immediately added. The protoplast solution was transferred to 14ml of regeneration broth in a Petri-plate and incubated at 25°C. Regeneration was monitored. The regenerating protoplasts were then centrifuged at 3000g for 10 min (MSE Mistral 1000), resuspended in 3ml

SM and plated onto selection medium in 500µl aliquots. A protoplast dilution series 10^{-1}- 10^{-5} was plated out in 500µl aliquots on to R medium and incubated at 25°C. Colony counts were taken at the highest colonised dilutions, and from this the protoplast viability per transformation can be calculated.

2.5.3. Liposomal Transformation

Protoplasts (methods 2.4.) were prepared, and resuspended in SEH buffer. The N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Boehringer Mannheim, cat. No. 1811 177)/DNA mix was prepared as follows: 30µl of DOTAP was added to 70µl of SEH and gently mixed. Transforming DNA (5µg) and SEH were combined to give a total volume of 50µl. The DOTAP and DNA solutions were then combined, mixed, and incubated at RT for 15 min. This allowed the DNA and DOTAP to complex due to ionic attraction.

The DOTAP/DNA mix was then added to 200µl of protoplasts, mixed gently, and were incubated at RT for 15min. The transformation solution was then suspended in 15ml of R broth and were incubated at 25°C. When the protoplasts showed signs of regeneration, the R broth was centrifuged at 3000rpm for 10min (MSE Mistral) and the pellet resuspended in 3ml of SM. The regenerating protoplasts were transferred to selection medium in 500µl aliquots.

2.5.4. Biolistic Transformation

Tertiary *A. bisporus* macerates were spread (1ml) onto sterile discs of cellophane laid on CYM/CE agar plates and incubated overnight at 25°C before bombardment.

Microcarrier Preparation

The following procedure provides micro-carrier for 8x5 bombardments. To wash the micro-particles, 1ml of 70% v/v ethanol was added to 60mg of micro-particles (tungsten or gold) and was vortexed (vortex genie 2) for 3-5min and then incubated at RT for 15min.

The micro-particles were pelleted by micro-centrifuge for 5 sec. The supernatant was discarded. The following washes were repeated thrice:

Sterile water (1ml) was added to the micro-particles, and vortexed for 1min. The particles were allowed to settle for 1min and were then pelleted by micro-centrifuge. The supernatant was removed and discarded.

Sterile 50% v/v glycerol (800µl) was added to the micro-particles to bring the concentration to ca. 60mg/ml. The particles were stored at RT for up to 2 weeks.

Coating DNA onto micro-carriers

This protocol provided enough micro-particles for approx. 5 bombardments. The DNA was coated onto the particles just prior to bombardment. Micro-particles in glycerol were vortexed for 5min to resuspend the particles. 100µl of micro-carriers were

removed, and to this 10µl of DNA, 100µl of $CaCl_2$ and 40µl of spermidine were added, vortexing continuously. The micro-particles were then vortexed again for 2-3 min, allowed to settle for 1min before pelleting by micro-centrifuge for 5sec. The liquid was discarded. The pellet was then washed twice in ethanol; firstly in 280µl of 70% and then in 280µl of 100%. The pellet was finally resuspended in 100µl of 100% ethanol and was vortexed gently at a low speed for 2-3min.

Bombardment Protocol

The following settings were used for the PDS1000 helium gun:

28mm Hg
1cm gap distance
6cm target distance
minimum stop screen
15000psi

The gun was thoroughly cleaned with 100% ethanol to disinfect. The macro-carriers and holders, stopping screen and rupture discs were washed firstly in 70% ethanol secondly in 100% ethanol and then dried in an oven (50°C). The DNA-coated micro-carriers were loaded into the centre of the macro-carriers 10µl at a time, whilst continuously mixing the particles. Each macro-carrier received 20µl of particles. The rupture disc, stopping screen, macro-carrier and holder were all loaded into the gun using forceps. The plates of mycelium were labelled according to the order of bombardment. Control bombardments (2 per experiment) were carried out using macro-carriers with no DNA coating. The bombarded plates were incubated at 25°C overnight, before transferring the cellophane to selection medium.

2.6. Selection/Assay Protocols for Transformants

2.6.1 Lucuferase Assay

The LKB Wallac 1250 luminometer system was used to quantify the light emitted by any cells expressing the luc^+ gene. Luminescence relates directly to the level of luc^+ gene expression and can therefore be used to quantify promoter activity. Regenerating protoplasts in broth were pelleted at 3000rpm for 10min (MSE Mistral 1000) and the supernatant was removed. The cells were transferred to eppendorfs (1.5ml) and were spun for 3min, removing all the liquid carefully. 100µl of *E. coli* containing the luciferase construct PUC-LUC 1X, had been grown overnight in 5ml LBamp broth in a shaker (200rpm, 37°C) was a positive control. The cells were resuspended in 100µl of 1x cell culture lysis reagent. The cells were then ruptured using 0.1mm glass beads from Sigma, to release the protein product. Approx. 100µl of beads was added to the cells, which were then vortexed for 45sec and cooled immediately on ice for 15sec. This was repeated three times. The debris was spun down by micro-centrifuge for 3min. The cell extract supernatant (40µl) was added to 100µl of luc assay reagent in a luminometer curvette and a luminescence reading was performed.

2.6.2 β-Glucoronidase Colorimetric Assay

After regeneration in broth (methods 2.2.1.), the transformation suspension was aliquoted (500µl) on to MM medium containing 50µl/ml x-gluc in DMF(methods 2.2.3.) and incubated in the dark. Colonies expressing the GUS gene were expected to colour blue.

2.6.3. Green Fluorescent Protein (GFP) Assay

Following transformation, mycelial colonies were transferred to an appropriate medium. Ultra violet microscopy (Leitz Wetzlar Dialux 20)was used to screen for colonies expressing GFP. A positive control of an *E. coli* strain DH5α containing a GFP plasmid, expressing GFP streaked onto MM medium was used for comparison.

2.6.4. Carboxin Selection

Post-transformation, regenerating mycelial colonies were plated out in 500µl aliquots onto CYM medium containing 15µg/ml carboxin and was incubated at the relevant temperature. Any regenerating colonies were expressing the gene for carboxin resistanc. These were picked off onto fresh selective medium. Colonies which persisted to grow underwent successive transfers on and off selection to test the stability of the construct integration into the genome.

2.6.5. Selection For Tryptophan Prototrophy

After regeneration in broth following transformation, *C. cinereus* protoplasts were plated in 500µl aliquots onto MM agar, which selected for trp^+ auxotrophs, and incubated at 37°C. This transformation system is established in *C. cinereus* (Binninger *et al.*, 1987) and can therefore be used as a co-transformation system to test new constructs. The trp^+ colonies in co-transformations were picked off MMM and then screened for the second marker gene.

2.6.6. Hygromicin Selection

After regeneration resistant colonies were selected following published protocols. Levels of hygromicin incorporated into culture media varied.

2.6.7 Selection for Uracil Prototrophy

Regenerants were incubated on minimal media i.e. not containing uracil.

2.7. Analyses of Putative Transformants

2.7.1. Fungal Genomic DNA Extraction Protocol

A modification of the protocol developed by Challen *et al* (1995) was used to extract genomic DNA from mycelial cultures. This was to confirm the presence/absence of

the transforming gene. Mycelia were grown in an appropriate broth culture for 1-2 weeks, and the mycelial mats were harvested by squeeze blotting between 3MM paper. The mycelium was then inserted into 1.5ml eppendorfs, rapidly frozen in liquid nitrogen, and was freeze-dried overnight. The freeze-dried mycelium was stored in a dessicator. The dried mycelium was then ground in the eppendorf using an inoculation needle. Extraction buffer (0.4M KCL, 50mM EDTA pH8, 1%v/v Triton X-100) was prepared on the day of extraction. Ribonuclease A (5u/µl)) was added to the buffer, and 700µl of buffer was then added to each ground mycelial sample and mixed thoroughly. The samples were then incubated at 70°C in a water bath for 30min, followed by micro-centrifugation (13000rpm) for 10min. Qiaprep 8 columns were equilibrated in a QIAvac manifold with 300µl of extraction buffer (no RNAse) per column. The mycelial extract in the supernatant was then added to the columns and pulled through via the application of a vacuum. The columns were washed once with 1ml of QIAGEN buffer PB and twice with 1ml of buffer PE. The vacuum was maintained for 1 min after all the buffer had washed through. Excess PE was drained from the manifold, and the DNA was eluted by the application of 100µl of warm 1mM Tris.HCl pH8.0. The DNA was tested on a 0.8% agarose gel, then stored at -20°C.

2.7.2. Southern Blot Analyses

DNA extracted from the putative transformants was digested by Hind III in a 50µl digest for 1hr in a water bath at 37°C. The DNA samples were then run on a 0.8% agarose gel with Hind III and DIG labelled markers. The DNA was then transferred from the gel to a membrane using one of two Southern blotting methods: vacuum blotting or bench blotting.

Vacuum Blotting

The following solutions (1L) were necessary for southern blotting:

Solution I (depurination)	0.25M HCl
Solution II (denaturation)	8.76% w/v 1M NaCl 2.0% w/v 0.5M NaOH
Solution III (neutralisation)	8.76% w/v 1.5M NaCl 0.67%w/v Tris base 7.02%w/v Tris HCl 1mm EDTA

The agarose gel was depurinated in solution I for 15 min, then denatured for 30 min in solution II. Washing in solution III for 30 min neutralised the gel. The gels were rocked gently (HB-SHK 1 Hybaid) during washing steps. A nylon membrane was cut to size, soaked in 20x SSC, and placed on blotting paper soaked in 20x SSC on a hybaid vacuum blot, used as recommended by the manufacturer. The gel was flooded with 20x SSC and was then left under vacuum for 1 h for the DNA transfer to complete. The membrane was oven dried for 1h at 80°C and then cross-linked by u.v. exposure (0.35 j/cm^3).

Bench Blotting

After performing the washes as for vacuum blotting, a modification of Southern`s basic protocol was performed, as described by Sambrook *et al.,* (1989). This method relies upon capillary action to draw the DNA from the gel onto the membrane.

2.7.2.1 Non-Radioactive DNA Labelling (DIG)

Hybridising DNA sequences were identified using non-radioactive digoxigenin-labelled probes. Gel-purified DNA (1µg) was resuspended in 15µl of 1mM Tris pH8.0 and was denatured by boiling for 10min and chilled on ice for 2min. For a 20µl reaction, the following was added (Boehringer Mannheim): 2µl hexonucleotide mix, 2µl dNTP labelling mixture (10x conc.) and 1µl klenow enzyme. This was incubated overnight at 37°C and then inactivated by the addition of 2µl of 0.2M EDTA, followed by incubation for 10 min at 65°C. Salmon testes DNA (150µl) was added to the DIG probe, boiled for 10min to denature the DNA, and then added to 15ml hybridisation solution. The probes were stored at -20°C.

4 Heterologous And Homologous Hybridisation

The protocols used for the hybridisation analysis of Southern blots are as described by Sambrook *et al,* 1989. Different temperature conditions were used for homologous and heterologous hybridisation. Probes were pre-hybridised twice in 15ml of hybridisation solution (5x SSC, 30% w/v 0.1%sarkosyl, 10%v/v 0.02% Sodium Doecyl Sulphate and 2% w/v casein blocking reagent Boehringer Mannheim cat. 1093657) at 65°C for homologous probes and at 57°C for heterologous probes. The probe was denatured by boiling for 10min preceding hybridisation.

Proceeding hybridisation, the membranes probed with homologous probes underwent the following washes:

5 min in 3x SSC, 0.1% v/v SDS at RT followed by 15min at 65°C, 2 washes in 1x SSC, 0.1% v/v SDS at 65°C, two washes in 0.2x SSC, 0.1% v/v SDS.

The washes for heterologous washes were as follows:

Two washes for 15min in heterologous solution I (2x SSC and 0.1% v/v SDS) at 50/57°C, followed by two washes in heterologous solution II (1x SSC and 0.1% v/v SDS) at 50/57°C. A chemiluminescence detection system was used to detect hybridisation of the DIG probes to any homologous/heterologous sequences.

2.7.2.3 Chemiluminescent Detection Of DIG-Labelled Probes

Chemiluminescent detection is a method of visually identifying the homologous and heterologous sequences on a Southern blot which have hybridised with the probe. The following washes were carried out at RT with gentle shaking (HB-SHK 1 Hybaid): 5min in 100ml Immu I solution (100mM Tris pH7.5 and 150mM NaCl), 30 min in 100ml Immu II solution (3% w/v casein in immu I, heated to dissolution in a microwave), 30min in 20ml Immu II solution containing anti-digoxigenin–AP Fab

fragments (1:5000),followed by two 15min washes in 100ml Immu I to remove the unbound conjugate, 5min in 20ml Immu III solution (1M Tris pH9.5, 5M NaCl, 1M $MgCl_2$) to equilibriate the membrane. The membrane was incubated for 5min in 10ml CSPD (chemiluminescent substrate, Boehringer Mannheim) diluted 1:100 in Immu III solution. Excess substrate was removed by blotting for a few seconds, then the membrane was sealed in cling film and incubated at 37°C for 15min. The membranes were then exposed to X-ray film for varying periods of time (15min-overnight). The films were then developed using a hyperprocessor (Amersham Life Science).
To remove probes, membranes were washed briefly in water, then twice in 0.2M NaOH, 0.1% v/v SDS for 15min at 37°C. After a final brief rinse in 2x SSC the membranes were air-dried and stored at RT.

2.7.3 Northern Analysis

2.7.3.1. Extraction Of RNA

Mycelia were grown in CYM broth in a shaking incubator and harvested by squeeze blotting. The mycelia was frozen in liquid nitrogen and stored at -70°C. All glass ware used for the extraction of RNA was washed with DEPC water (0.5µl per 1ml water) before autoclaving to remove all traces of RNAse. The following protocol was used to extract RNA from the frozen tissue:

Frozen mycelia was ground to a fine powder using a pestle and mortar and then gently mixed with 3x w/v extraction buffer (0.2M sodium acetate, 10mM EDTA, 10% w/v SDS, 0.5% w/v β-mercaptoethanol) and 1ml phenol: chloroform isoamylalcohol 3:1 ratio for every 11ml buffer, incubated at 65°C for 15min. This was centrifuged for 35min at 12500rpm (Hermle z 382 k, brake 5, 4°C) and the upper aqueous phase was transferred to sterile centrifuge tubes on ice. An equal volume of chloroform isoamylalcohol 24:1 was added and mixed vigorously, and then centrifuged as before. The upper aqueous phase was decanted and 2M LiCl was added to precipitate the RNA. This was then stored overnight at 4°C.

The precipitated RNA was pelleted by centrifugation (Hermle, 12500rpm, brake 5, 4°C) for 20min and the pellet was washed with 3M sodium acetate pH5.2 to remove all traces of DNA. This was repeated once. The pellet was washed twice in 70% EtOH and dried in a dessicator (nitrogen stream) for 10min before resuspending in 1ml DEPC water at 65°C for 15min. The RNA was stored at -4°C until required.

2.7.3.2. Formaldehyde Agarose Gel Electrophoresis

RNA samples were prepared for electrophoresis by the addition of RNA to 10x MOPS [3-(N-morpholino)proanesulfonic acid] 10% (0.4M MOPS, 100mM sodium acetate, 10mM EDTA), formaldehyde 17.5%, formamide 50% and 1/10 volume RNA dye (glycerol 50%, bromophenol blue 0.2% and 5mM sodium phosphate buffer pH6.8) in siliconised eppendorfs. The final volume was calculated by dividing the volume containing the RNA by 22.5 then multiplying by 100. The samples were then denatured by heating to 55°C for 15 min followed by cooling on ice for 5min before loading. A 120ml formamide gel was prepared by melting 1.2g agarose in 105ml DEPC water in a microwave, cooling, and then adding 12ml 10x MOPS and 2ml

formaldehyde. The gel running buffer was 1x MOPS buffer and 1.8% formaldehyde, and the gel was run at low voltage.

2.7.3.3. Northern Blotting

RNA samples from the formamide agarose gel were transferred onto positively charged membranes (Boehringer Mannheim) by northern blot transfer. No washes were required before transfer by bench blotting overnight. Immobilised RNA was visualised by soaking in 0.5M sodium acetate and 0.004% methylene blue for 10min, followed by washing in water for 10min.

2.7.3.4. Northern Hybridisation

Radiolabelled P^{32} were used to hybridise with the homologous sequences on the membranes. The membranes were prehybridised at 42°C (50% formatted, 5x SSPE, 2x Denhardt`s reagent, 0.1% SDS) for 1-2 h. The denatured probe was added directly to the prehybridisation solution and incubation was continued for 16-24h The filter was then washed once in 1x SSC, 0.1% SDS for 20min and then three times in 0.2x SSC, 0.1% SDS at 68°C for 20min. The filter was exposed to X-ray film at -70°C with an intensifying screen for 24-48h before developing.

The probe was removed by washing for 1-2h at 65°C in 5mM Tris-HCl (pH8), 2mM disodium EDTA and 0.1x Denhardt's solution followed by air drying.

3. RESULTS

3.1. Indirect Approach

3.1.1. *Coprinus cinereus* transformations with the luciferase construct

The luc $^{+}$ constructs pBU001-8 were each tested in *C. cinereus* strains H2 and H9. To date 44 PEG/$CaCl^{2}$ protoplast transformations have been performed. *E. coli* strain DH5α transformed with the PUC-LUC LX construct for luciferase was used as a positive control (lucifease expression was <160 units). To date, no evidence of luc $^{+}$ expression in *C. cinereus* has been detected by luminescence.

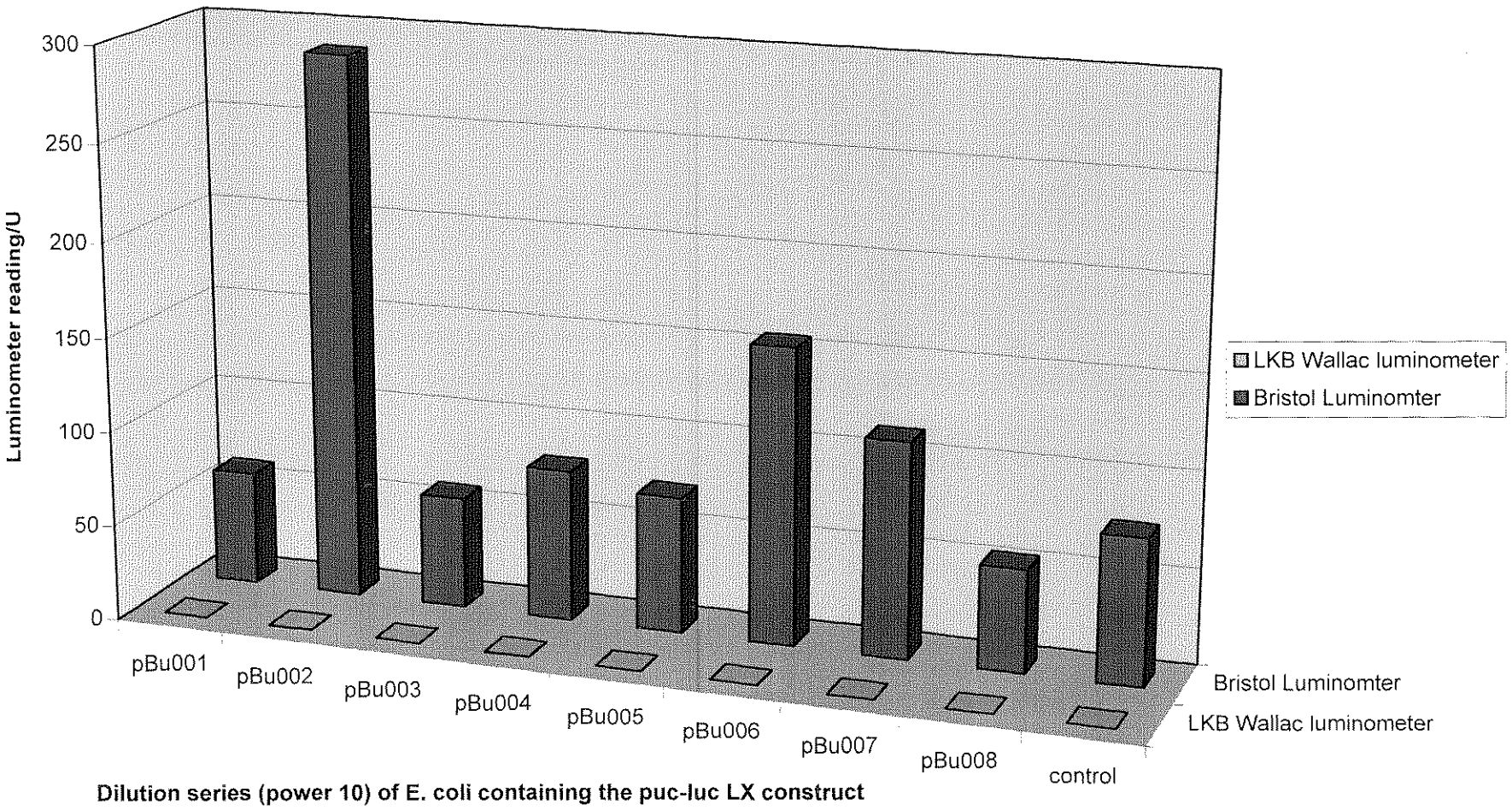
To determine if the sensitivity of the LKB Wallac luminometer was too low to detect poor expression of the luc $^{+}$ gene, readings were compared with those taken from the Lumat LB9507 luminometer. Readings were taken from 18 H9 transformations regenerated for 1 and 2 overnights. A series of *E. coli* PUC-LUC LX dilutions (10^{1} - 10^{9}) were used to directly compare the relative sensitivities of the two luminometers (Fig.2). The Lumat LB9507 luminometer was shown to be 10x more sensitive than the LKB Wallac model, but the light readings from the *C. cinereus* protoplasts in relation to the positive control were negligible.

In order to determine if the lack of success with these luc^{+} constructs is due to non-integration, or integration without transcription, 4 TRP/luc $^{+}$ co-transformations have been carried out. The *C. cinereus* trp auxotrophic strain LT2 was used as a co-trasformation host. For each PEG/CaCl2 transformation, pCc1001 DNA (trp construct) and luc $^{+}$ construct pBU001-8 DNA was used .The protoplasts were regenerated in MMMR broth for two overnights at 37°C, before resuspension in MTB and plating onto MMM agar. After incubation for 5 overnights, trp $^{+}$ transformants were observed on the MMM agar (Table 5). A proportion of these trp $^{+}$ transformants were isolated (up to 200) and transferred to fresh MMM agar. DNA was extracted from up to 50 colonies of each construct, with a view to performing PCR using luc $^{+}$ specific primers. The luc $^{+}$ primers will be used to find evidence of integrative transformation. RT PCR will be performed on luc $^{+}$ colonies to investigate transcription of the gene.

3.1.2 *Agaricus bisporus* Transformations With The Luciferase Constructs

A. bisporus transformations were also performed using the pBU001-8, SPR and pBtub constructs. To date a total of 24 PEG/CaCl2 transformations have been carried out using the commercial mushroom strains C38 (brown cap) and A12 (white cap). For each experiment, 10g of gill tissue from young fruit bodies was excised and protoplasted with 10mg/ml of Novozyme 234 in SM. Table 5 displays the protoplast yields per gram of tissue lysed. There was no obvious difference between the protoplast yields of the two strains used.

Fig 2 Comparative sensitivities to luciferase activity of two luminometers



Dilution series (power 10) of E. coli containing the puc-luc LX construct

After transformation, the protoplasts were suspended in 20ml of MMMR for regeneration overnight. The protoplasts were then lysed using the freeze/thaw cycle method and assays performed with the LKB Wallac luminometer. As with *C. cinereus* expression of the luc^+ gene could not be detected in *A. bisporus.*

In a single experiment, 5 electroporative transformations were carried out using protoplasts obtained from the commercial strain A12 gill tissue (10mg/ml Novozyme 234, $5.7x10^6$ protoplasts/g tissue, $1.35x10^7$ protoplasts/100µl) using luc^+ construct DNA. After electroporation the protoplasts were incubated overnight in R broth. The protoplasts underwent the freeze/thaw cycle before readings were taken as before. No luc^+ transformants were identified.

Table 5 Protoplast yields of *Agaricus bisporus* gill tissue PEG/CaCl2 transformations with the luciferase constructs.

Expt.	**Strain**	**Protoplasts/g Tissue**	**Protoplasts / Transformation**	**DNA/ μg Per Transformation**
1	C38	$1.25x10^7$	$4.16x10^7$	10
2	A12	$1.05x10^7$	$1.05x10^8$	10
3	C38	$1x10^7$	$1x10^8$	10
4	A12	$1.6x10^6$	$1x10^8$	10
5	C38	$1x10^6$	$6.2x10^7$	10
6	A12	$1.37x10^7$	$1.0x10^8$	10
7	C38	$5.7x10^6$	$4.4x10^7$	10

3.1.3 *Coprinus cinereus* Transformations With The β-Glucoronidase Constructs

The *C. cinereus* strains used in this study were tested for constitutive GUS expression by growing mycelial plugs on MMM-xgluc agar in the dark. All strains tested negatively and are therefore suitable for use as hosts for the GUS gene.
A total of 11 PEG/$CaCl_2$ transformations were carried out on oidial (Table 6); protoplasts from the *C. cinereus* strains H2 (4 transformations) and H9 (7 transformations). No DNA controls were included in each experiment. After an overnight incubation, the protoplasts were plated out onto MMM-xgluc agar and incubated in the dark. No GUS activity was detected. The viability of *C. cinereus* protoplasts must be over the 10^4 threshold before any regeneration is expected (Binninger *et al.*, 1987). The viability data for these experiments is therefore low, although regeneration did occur as white colonies were observed on all MMM-xgluc plates except the control.

In order to determine if the GUS constructs are integrating and not being expressed, two co-transformation experiments were carried out. The protoplasts of *C. cinereus* LT2 strain were co-transformed with the pCc001 plasmid and 6 gus constructs (Table 7) in two separate experiments. trp^+ co-transformants were screened for the expression of the gus gene using MMM-xgluc agar. About 200 trp^+ transformants of each construct from each experiment were transferred onto x-gluc medium. No GUS expression was identified.

3.1.4 *Agaricus bisporus* Electroporative Transformations With GUS Constructs

A tertiary macerate of the *A. bisporus* auxotrophic strain W9-ade his was protoplasted using Horst (1.5mg/ml) enzyme to release $6x10^7$ protoplasts/g tissue. These protoplasts were used in 4 electroporative transformations ($3.75x10^8$ protoplasts per transformation) with the GUS constructs pgus001, pgus004, pgus005 and pgus008. After electroporation, the protoplasts were incubated for 5 overnights in R broth before plating onto MMM- ade his x-gluc. Protoplast viability was $1x10^3$ and the regeneration was $3.33x10^{-4}\%$. Assuming that the regenerative frequency of *A. bisporus* is comparable to that of *C. cinereus*, then this figure was too low to expect the recovery of any transformants. It is probable that *A. bisporus* does not regenerate as easily as *C. cinereus* due to its recalcitrance as a laboratory organism. Blue colonies were not observed.

3.1.5. *Coprinus cinereus* Transformations With GFP Constructs

A preliminary transformation has been performed on the *C. cinereus* strain H9. Protoplasted oidia were transformed via PEG/CaCl2 with pGFP001, pGFP003, pGFP004 and pGFP008 ($1.57x10^8$ protoplasts per transformation, 0.74% regeneration). Following regeneration for two nights, the protoplasts from each transformation were plated onto MMM agar and incubated. Regenerated colonies were analysed using uv microscopy to look for green fluorescence; to date none has been observed.

Table 6 *Coprinus cinereus* PEG/$CaCl^2$ transformations with GUS constructs.

Strain	Expt.	Construct	Protoplasts /Transformation	Viability / Transformation	DNA/ μg per Transformation
H9	1	pgus001	$1.9x10^7$	No data	5
H9	2	pgus001	$2.7x10^7$	No data	5
H9	3	pgus001	$3.4x10^7$	No data	5
H9	4	pgus001 pgus004 pgus005 pgus008	$2.2x10^7$	$1.4x10^4$	5
H2	5	pgus001 pgus004 pgus005 pgus008	$1.7x10^7$	$1.9x10^3$	5

Table 7 Summary of *Coprinus cinereus* strain LT2 trp^+/GUS co-transformations

Expt.	Primary Construct	Secondary Construct	Protoplasts/ Transformation	DNA/ μg Transformation	Viability Transformation	No. trp^+ colonies	No. trp^+ colonies/ μg DNA
1	pCc1001	pCc1001	$1.9x10^7$	2	$6.3x10^4$	210	105
	pCc1001	pgus001	$1.9x10^7$	5	$6.3x10^4$	0	0
	pCc1001	pgus004	$1.9x10^7$	0.9	$6.3x10^4$	160	177.8
	pCc1001	pgus005	$1.9x10^7$	4.93	$6.3x10^4$	141	28.6
	pCc1001	pgus008	$1.9x10^7$	13	$6.3x10^4$	105	8
	pCc1001	ϕ	$1.9x10^7$	0	$6.3x10^4$	30	0
2	pCc1001	pcc1001	$1.1x10^7$	2	No data	550	275
	pCc1001	Pgus001	$1.1x10^7$	5	No data	543	108.6
	pCc1001	Pgus004	$1.1x10^7$	0.9	No data	505	561
	pCc1001	Pgus005	$1.1x10^7$	4.93	No data	452	
	pCc1001	pgus008	$1.1x10^7$	13	No data	403	31
	pCc1001	ϕ	$1.1x10^7$	0	No data	0	0

3.2 Direct Approach

3.2.1 *Agaricus* transformation with the pAN7.1 construct

In over 30 experiments based on the Dutch protocol no transformants were obtained. Using an alternative DNA delivery system, cationic liposomes, in conjunction with the pAN7-1 vector, more than 800 putative transformants were obtained. The presence of vector DNA was determined by PCR screening (see later) and by Southern blotting. From a sample of 93 putatives tested by Southern analysis, only 3 were found to contain transforming DNA. During the course of subculturing these colonies lost their resistance to hygromycin and this was most likely due to the elimination of transforming DNA. Subsequent experiments have failed to recover transformants, and selection for hygromycin resistance has been problematic with marked variation in expression of resistance. Work in other laboratories has failed to validate the Dutch protocol. It appears irreproducible and therefore is of little practical use.

Homokaryotic strains N1-*unk* (also known as C82-*ura*) and W9-*ade his* (also known as ATCC24663) were recovered from liquid nitrogen and their respective genotypes were confirmed by growth tests on minimal media and minimal media supplemented with appropriate auxotrophic requirements. In addition, the W9-*ade his* strain was obtained directly from the Dutch group, tested and then used in subsequent transformation experiments.

For each mushroom strain, optimum protoplasting conditions were determined. Although very similar, the quantity of Novozyme 234 protoplasting enzyme and the length of exposure to the enzyme were altered to ensure that the maximum number of protoplasts were obtained from each strain. From the N1-*unk* strain, individual protoclones, called Pc1, Pc2, Pc3 and Pc4, were isolated. It was found that Pc1 (and to a lesser extent Pc4) protoplasted exceptionally well, whereas Pc2 and Pc3 did not protoplast very well at all. Future transformation experiments will therefore focus on the auxotrophic protoclones Pc1 and Pc4.

A number of additional cell wall digesting enzymes were tested for improved activity, but it appears that Novozyme 234 is the most effective.

It was found that levels of hygromycin used needed to be modified, as it seemed that non-transformed recipient strains of *A. bisporus* had an inherent resistance (in some cases up to 30ig/ml hygromycin) to the antibiotic. Furthermore, four confirmed *Agaricus* transformants were obtained from the Dutch group in order to study the level of hygromycin resistance. This not only allowed insights into the levels of resistance expected in true transformants, but also provided positive controls for a PCR-based assay developed (see below).

Putative hygromycin resistant transformants were obtained on a number of occasions, but besides the 3 transiently expressed colonies discussed above, none have persisted when transferred to fresh selective media. Those which did show weak growth when transferred failed to produce positive signals on genomic DNA blots following hybridization with DIG labelled pAN7-1.

A PCR-based assay has been developed to allow analysis of large numbers of putative liposome-mediated transformants. Primers HYGR1 and HYGR2 designed against pAN7-1 vector sequence and shown below were used to amplify a specific 969 bp fragment by PCR. Using the confirmed *Agaricus* transformants obtained from ATO-DLO as positive controls, the PCR reaction was optimized. However, since the Dutch protocol has proved irreproducible and putative hygromycin resistant transformants are rarely observed, this assay has been of little practical use to date.

HYGR1: 5'-ATG CCT GAA CTC ACC GCG-3'

HYGR2: 5'-TCG GTT TCC ACT ATC GGC-3'

Nucleotide sequences of primers HYGR1 and HYGR2.

Cloning and testing the *A. bisporus* URA3 gene.

Sequence information from previously cloned OMPdecase genes from other fungal species suggested the genes of *Schizophyllum commune* and *Phycomyces blakesleeanus* are likely to be closest in structure to that of the mushroom. Initial attempts to clone the *A. bisporus URA3* gene by probing with the entire OMPdecase genes of *S. commune* and *P. blakesleeanus* were unsuccessful as non-specific signals were obtained. Specific PCR oligonucleotide primer pairs were therefore designed against a highly conserved region of these two genes; for *S. commune*, ScURA1 and ScURA2 and for *P. blakesleeanus*, PbURA1 and PbURA2. Primer sequences are shown below.

ScURA1: 5'-ATT TCC TCA TCT TTG AGG A-3'
ScURA2: 5'-TGC CCC ATC CCA TCG CCC TT-3'

Nucleotide sequences of primers ScURA1 and ScURA2.

PbURA1: 5'-ATT TTT TGA TCT TCG AGG ATC GC-3'
PbURA2: 5'-TGC CCA AGA CCA TCA CCC TT-3'

Nucleotide sequences of primers PbURA1 and PbURA2.

These primers were used against heterologous *A. bisporus* genomic DNA as a template and two DNA fragments were preferentially amplified. Sequencing of these fragments showed no homology with known OMPdecase sequences. Similarly, no OMPdecase-

specific fragments were produced when RT-PCR technology was used in conjunction with these primers. Ultimately, both pairs of primers were used to homologously amplify the highly conserved region of the respective OMPdecase genes. These amplified products (called ScURA and PbURA) were DIG labelled and used to probe the cosmid-based library of *A. bisporus*. Using ScURA, three cosmid clones were identified which contain hybridising sequences i.e. are putative sequences of the *Agaricus bisporus* OMPdecase gene; no positive cosmid clones were identified using the PbURA probe. In addition, further probing with a subclone derived from one of the ScURA-specific cosmids identified a further two overlapping clones from the library.

After extensive optimization of PCR conditions, primers ScURA1 and ScURA2 amplified two specific fragments (*fast* and *slow* fragments) when genomic *Agaricus* DNA was used as a template. However, when these fragments were cloned, sequenced and analyzed, no homology was found to other fungal OMPdecase genes. The PbURA1 and PbURA2 primers did not amplify any fragments despite a variety of reaction conditions tested.

A highly conserved region of the *S. commune URA1* gene (cloned into vector pEF2) has been successfully amplified using the ScURA1 and ScURA2 primer set. By comparison with the *Schizophyllum* sequence, the expected size of the amplified PCR fragment was 451 bp. A fragment of approximately this size was generated, DIG labelled and used to probe an *A. bisporus* genomic library. Preliminary screening of pooled library DNA produced three possible positive signals from pools 15, 27 and 49, the latter of these giving strongest hybridization to the probe. These pools were reprobed and this time individual hybridizing cosmid clones cos15D3, cos27H12 and cos49E9 were identified.

Cosmid DNA was extracted from these clones, restricted with *Hind*III (the site into which DNA is inserted into cosmid vector Lawrist) and subjected to Southern blotting, before being probed again with ScURA. In one of the clones, cos49E9, a fragment of about 4 kb in size was identified; no signals were obtained from clones cos15D3 or cos27H12. It is believed that this DNA fragment harbours the *A. bisporus URA3* gene, either in its entirety or in a truncated form.

After purification, the 4 kb *Hind*III OMPdecase-specific fragment of cosmid cos49E9 was (i) DIG labelled to produce probe 49E9H5 and (ii) subcloned into *Hind*III-digested pBluescript SK II+ to create the plasmid construct pURA-49E9H5. The DIG labelled fragment was used to rescreen the *Agaricus* library to isolate all overlapping clones in case the cosmid cos49E9 (and plasmid pURA-49E9H5) contained only truncated versions of the *URA3* gene. Following hybridizations of the library pools with probe 49E9H5, two additional cosmids, cos5F4 and cos32B1, were eventually identified that showed strong probe homology. Upon probing *Hind*III-digested DNA from these clones with the original ScURA PCR probe, a band of about 4 kb was observed in both cases. In addition, a further band of about 10 kb in size was present in these two cosmids (but not cos49E9) when *Hind*III-digested DNA was probed with the entire *S. commune URA1* gene in plasmid pEF2. This would suggest that the *A. bisporus* OMPdecase gene contains an internal *Hind*III site, and that in cos49E9 the gene is truncated at this point, whereas in cos5F4 and cos32B1 the gene and upstream control regions are present.

Since the *A. bisporus URA3* gene has been cloned using alternative methods, it was not necessary to employ RT-PCR technologies. However, in the course of the research, the

technique was attempted in order to isolate cDNAs corresponding to the *URA3* gene. An RT-PCR product was produced and cloned using the ScURA primers, but upon sequencing was found not to share any homology with other OMPdecase genes.

The *fast* and *slow* PCR products produced using *A. bisporus* genomic DNA as a template with the ScURA primers were cloned and sequenced, but did not show OMPdecase homology following GCG analysis.

3.2.2 *Coprinus cinereus* Transformations With The SDH Constructs

Initial PEG/CaCl2 transformations (7) of the *C. cinereus* strains H9 and H2 with the 4 α group cosmids 50-D5, 21-D5, 44-D9, 47-E5 and the plasmid subclone 50-β25, and the 3 β group cosmids 47-E7, 17-A6 and 34-H1 yielded 20 carboxin resistant putative transformants (Table 8). These putatives were retrieved from both groups and both *C. cinereus* strains. To test for stability the putatives were subcultured through six successive transfers alternating between the absence and presence of carboxin. If the colony persisted upon the medium after a 1-2 week period, it then underwent a further transfer, or failing this was classified as having lost resistance. Two colonies lost resistance upon the second transfer to carboxin medium.

Genomic DNA was extracted from 13 resistant isolates (5 α group, 7 β group and 1 no DNA control) and Southern blotted and probed with (I) DIG labelled Lawrist vector and (II) 6kb 50-β25 Hind III fragment (Fig. 3). Hybridising fragments were detected for 6 putative transformants at the 6kb mark using the 50-β25 Hind III fragment. Hybridisation was also detected within the same individuals, at the 2.8kb mark when probed with the DIG-labelled vector (Fig. 4). .None of the carboxin resistant colonies transformed with the A construct hybridised with either probe.

In an attempt to confirm that the *A. bisporus* SDH construct DNA was causing the carboxin resistance in these *C. cinereus* colonies, Northern analysis was conducted. The same carboxin resistant colonies were grown in CYM broth in the presence and in the absence of carboxin, in a shaking incubator. This was to screen for constitutive expression of the integrated construct DNA. RNA was extracted from these colonies and Northern blotted. The RNA was visualised using methylene blue stain (Fig. 5). Approximately equal amounts of RNA were present for each colony. The Northern blot was probed with P^{32}-labelled 6kb 50-β25 Hind III construct DNA. Although an equal amount of RNA was blotted for each individual colony, the levels of hybridisation varied. Due to weak hybridisation with transformant 11 (H9 transformed with 47-E7) a fourfold amount of RNA was blotted and probed as before. This revealed a hybridisation signal for this colony grown in either the presence and absence of carboxin (Fig. 5). The levels of hybridisation between RNA samples taken from the same colony but grown in different conditions are approximately equal for all colonies with the exception of transformant 3 (H2 transformed with 17-A6 construct). This individual shows higher levels of transcription when grown in the presence of carboxin, than in the absence.

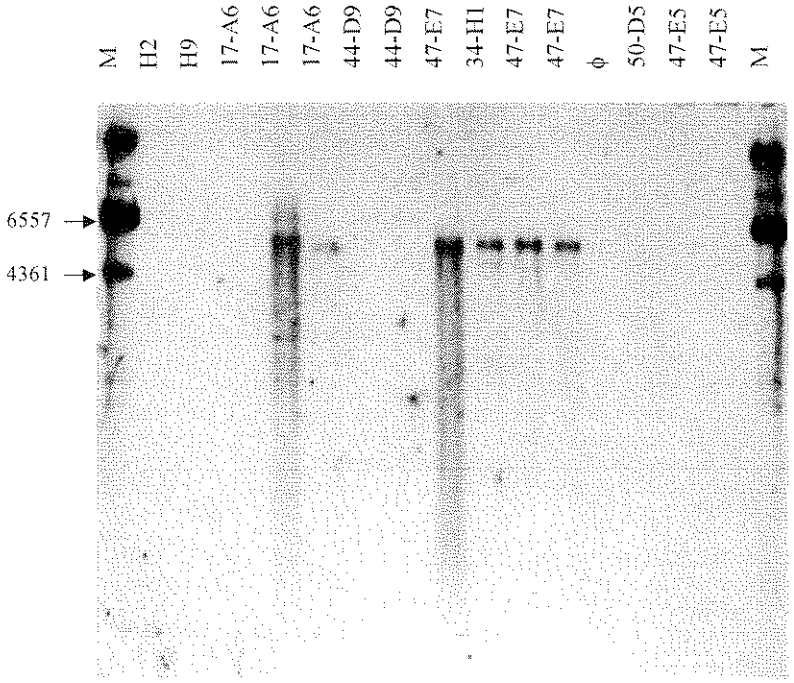


Fig. 3 Southern analysis of *Coprinus cinereus* carboxin resistant colonies. Homologous hybridisation with DIG-labelled Lawrist.

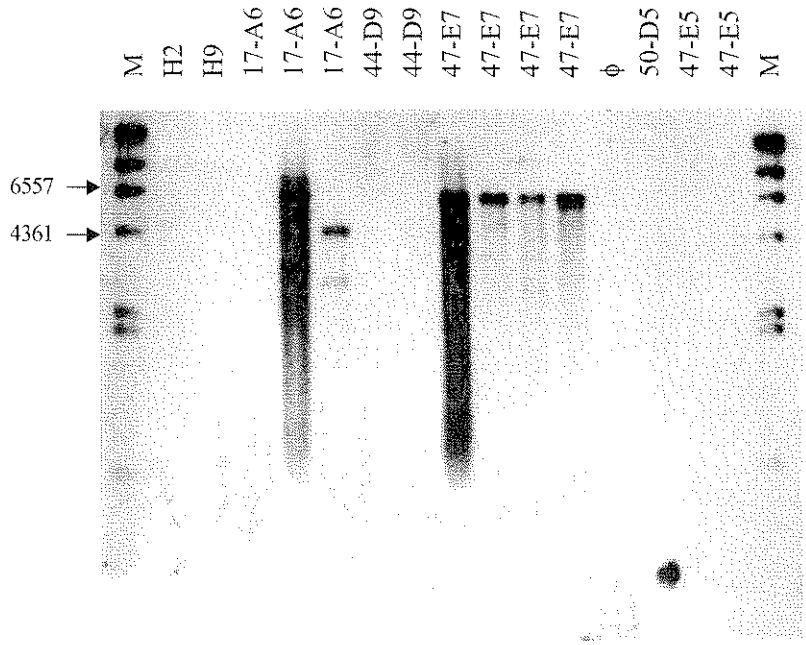


Fig. 4. Southern analysis of *Coprinus cinereus* carboxin resistant colonies. Homologous hybridisation with DIG-labelled 50-β25 insert fragment.

Fig. 5 Northern analysis of carboxin resistant *Coprinus cinereus* colonies grown in the presence (+) and absence (-) of carboxin (top)
RNA from carboxin resistant *Coprinus cinereus* colonies stained with methylene blue (bottom)

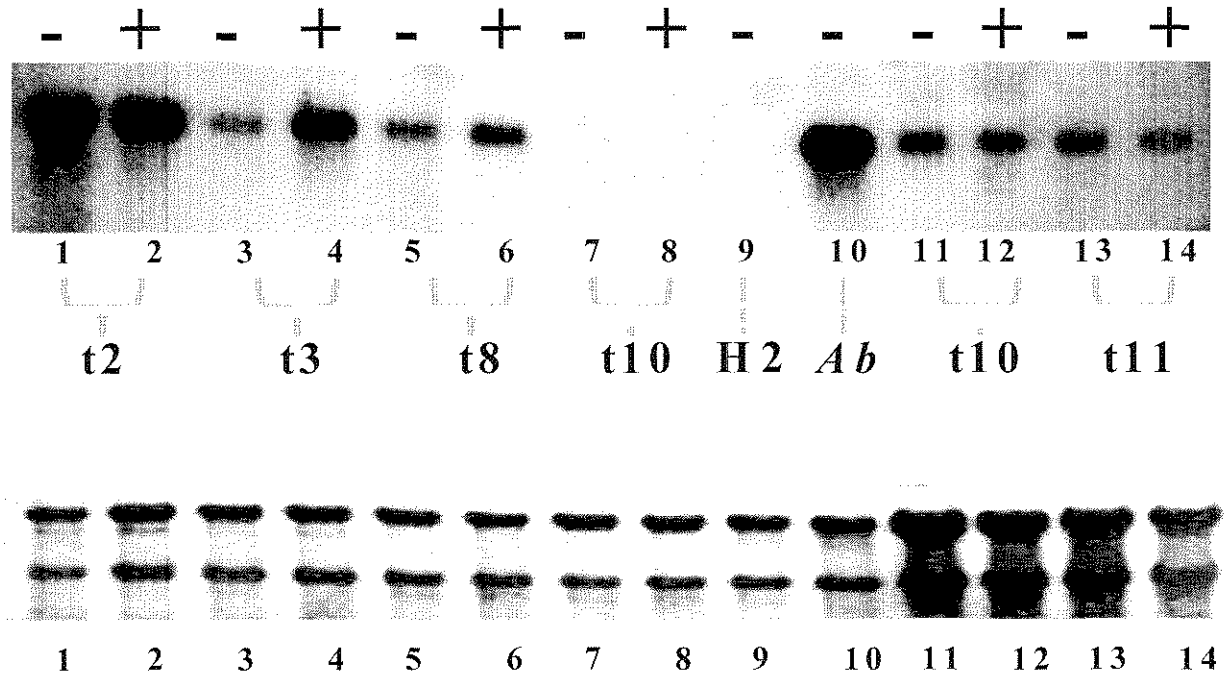


Table 8 *Coprinus cinereus* putative SDH transformants.

Expt.	Strain	Construct	SDH group	No. of Carb^r colonies	Stability of Carb^r colonies	No. Carb^r colonies/μg DNA	DNA/μg per Transformation
1	H9	50-β25	α	1	unstable	0.03	31.5
1	H9	47-E5	α	2	stable	0.09	21.9
2	H9	50-D5	α	1	stable	0.09	11
2	H9	47-E7	β	2	stable	1	2
3	H9	47-E7	β	1	stable	0.5	2
4	H2	17-A6	β	3	stable	0.3	9.6
4	H2	44-D9	α	2	stable	0.17	11.5
5	H2	47-E7	β	2	stable	0.18	11
5	H2	34-H1	β	1	stable	0.06	18
6	H9	47-E7	β	4	stable	0.36	11
6	H9	ϕ	α	1	stable	0	0
7	H2	50-β25	α	1	unstable	0.03	31.5

Two further transformation experiments were performed using only the β SDH constructs. Transformants began to appear upon the selective plates one week after transformation. A total of 151 carboxin resistant colonies were observed. The majority of these colonies were from the H2 transformation (Table 9). Sample putative transformants (134) were successively subcultured alternating between the presence and absence of carboxin as before, to test stability. Two individuals died on the first transfer to carboxin medium, 7 on the second transfer and 4 on the third. Thirty-five colonies grew poorly upon the second transfer to carboxin but still continued to make some growth.

The regeneration frequency was good for both these experiments so transformants could be expected. DNA was extracted from 18 of the stable carboxin resistant colonies and southern blotted. This was to confirm the presence of the β SDH construct. The blots were probed with the 6.2kb *A. bisporus* SDH fragment, the 5.4kb Lawrist cosmid vector sequence and the pBluescript plasmid vector sequence. It was observed that 56 of the carboxin resistant colonies southern blotted had homology with both the probes (Figs. 6 and 7). Evidence for multiple integration events was seen in transformants, lane 6 of H9 Fig.4 and lanes 2,3,5,7,14, and15 on H2 Figs. 6 and 7. More than one band is visible in these lanes, as well as the 6kb intact *A. bisporus* fragment. This is probably due to the partial integration of this sequence into different sites within the genome.

3.2.3 *Agaricus bisporus* Transformations With The SDH Constructs

A total of 51 individual electroporative transformations of *A. bisporus* protoplasts with the SDH β constructs have been performed. These have yielded no carboxin resistant colonies. The viability has only been higher than $1x10^5$ for one experiment. Therefore recovery of transformed colonies cannot be realistically be expected for the other experiments as the regeneration frequency will have been far to low.

Hyphal macerates of PC1 and N1-ura strains were also used in 7 biolistic transformations with the β plasmid 47-E7 β2-1. High levels of contamination caused problems with this biolistic protocol. Two transformations yielded a large number of slow growing carboxin resistant colonies. These colonies were discrete and fluffy in morphology. A proportion of the colonies (121) were transferred onto fresh carboxin media. The growth rate of these colonies was found to be considerably slower than the genuine C54-carb 8(7) mutant. The carboxin resistant N1-ura colonies took two weeks to grow to the size that C54-carb 8 grew in 4 days. Two carboxin resistant colonies were observed on the unshot control plates. The least well growing resistant colonies grew very poorly on transfer and did not have the fluffy morphology. The faster growing colonies will be transferred on and off selection to test the stability of the resistance.

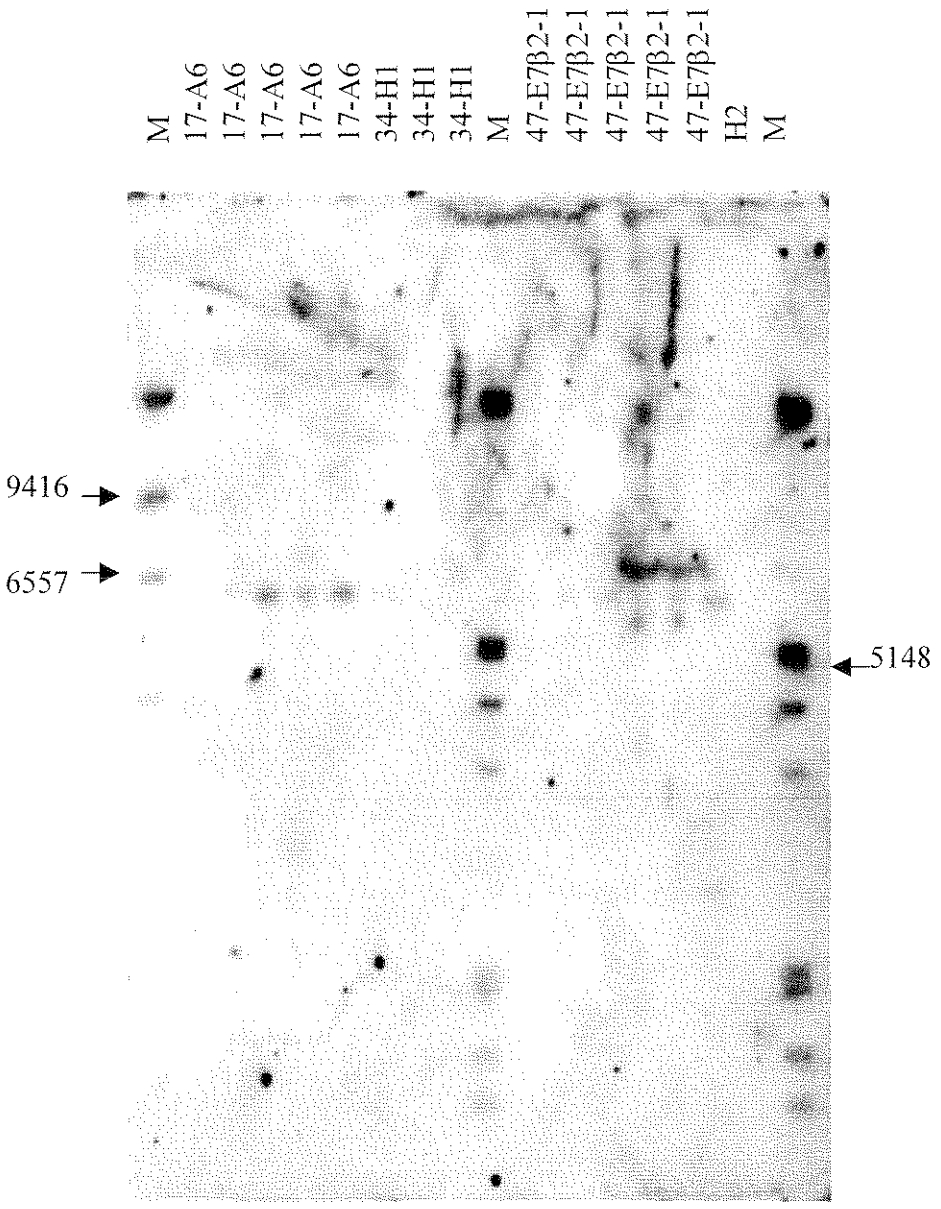


Fig.6. Southern analysis of *Coprinus cinereus* strain H2 carboxin resistant colonies. Homologous hybridisation of DIG-labelled 50-β25 insert fragment.

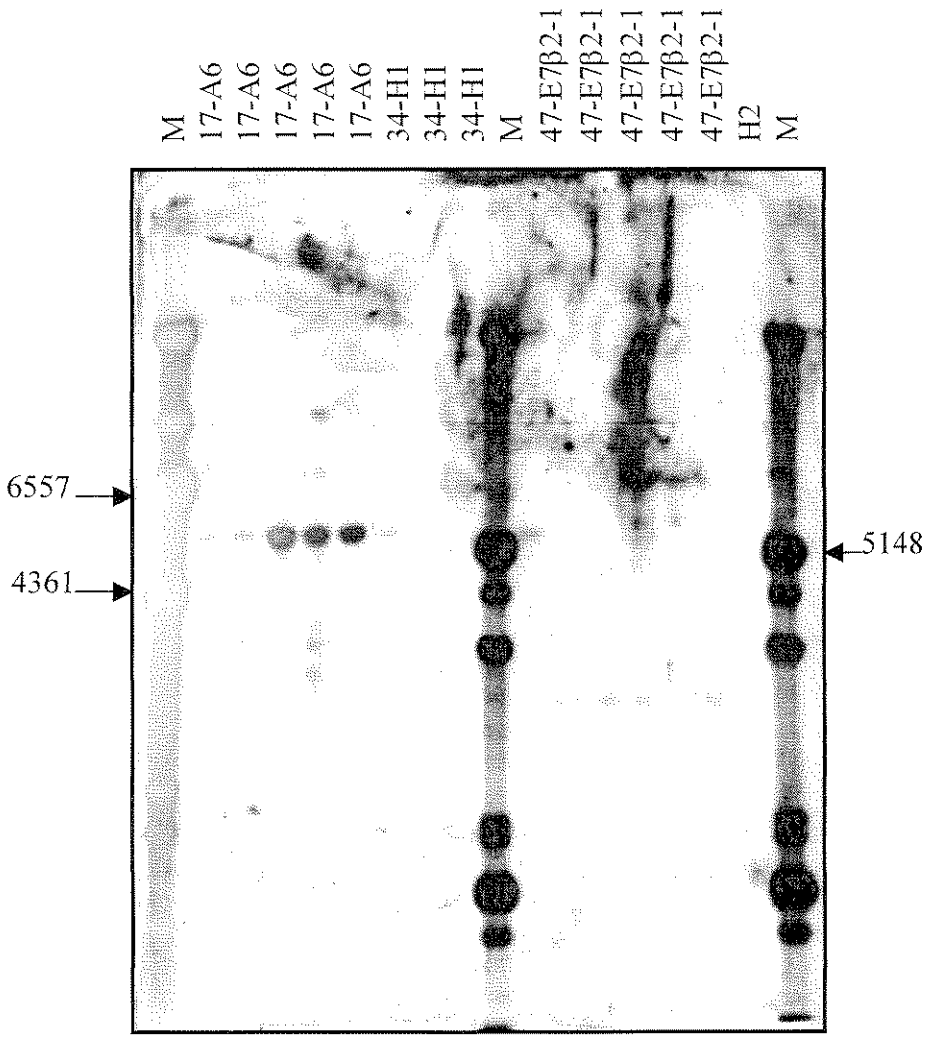


Fig .7 Southern analysis of *Coprinus cinereus* strain H2 carboxin resistant colonies. Homologous hybridisation of sequences with DIG labelled Lawrist.

Table 9 Carboxin resistant colonies of *Coprinus cinereus* transformed with the *Agaricus bisporus* β SDH constructs

Expt.	Strain	SDH Construct	Carb^{r} colonies	No. carb^{R} colonies/μg DNA	DNA μg/ transformation	No. viable protoplasts/ transformation	Regeneration Frequency %	% Stable Carb ^{r} Colonies
1	H2	17-A6 34-H1 47-E7-β2-1 no DNA control	62 7 55 0	4.35 0.19 9.17 0	14.25 37.25 6 0	$36x10^{5}$	2.4	96.8 100 80
2	H9	17-A6 34-H1 47-E7 β2-1 no DNA control	6 7 8 0	0.42 0.18 1.33 0	14.25 37.25 6 0	$2.8x10^{6}$	1.4	100 100 100

Mutation rate $1x10^{5}$ and $1x10^{6}$ for H2 and H9 experiments, respectively.

4. CONCLUSIONS

Transformation of the mushroom *A. bisporus* has proved extremely challenging. Transformants have only been recovered at low transformation frequencies (Van de Rhee *et al.*, 1996). Hyphal protoplasts transformed with the hygromycin resistance gene, yielded between 1-5 transformants per 10^5-10^6 protoplasts using 10μg of PAN 7-1 DNA (van de Rhee *et al.*, 1996). Attempts to reproduce this technology elsewhere have not proved successful; PAN7-1 transformants recovered at HRI were not stable, excising the transforming DNA after several subcultures (Challen, *pers comm.*). De Groot *et al* (1998)transformed *A. bisporus* 'conidia' (presumably basidiospores) to hygromycin resistance using *Agrobacterium-tumefaciens*. Again the transformation rates were very low (1 transformant per 10^7 spores) and there is some doubt about transformant stability.. Transformation of *A. bisporus* is therefore possible, but the transformants may be unstable and the technologies are difficult to reproduce.

4.1. Protoplasting *Agaricus*

An efficient protocol for protoplasting *Agaricus* hyphal fragments and gill tissue is required for the protoplasting transformation systems used in this study. Sonnenberg *et al* (1988) devised a protocol for protoplasting hyphal macerates, and Chen & Hempp (1993) for gill tissue. However, difficulties were experienced in this study both with the regrowthof tertiary hyphal macerates for protoplasting, and with the release of protoplasts. Gill tissue protoplasts have previously been shown to regenerate poorly (Challen and Elliott, 1994) and were again found unstable in this study. Some attempts were made to optimise protocols. Different pre-protoplasting incubation periods and enzyme combinations were tested. Due to the withdrawl of Novozym 234 (Interspex Inc), a suitable replacement was required for protoplasting hyphal macerates. It was determined that production of viable protoplasts is dependant upon the age of tertiary macerate, enzyme combination and period of protoplasting. Different *A. bisporus* strains require different combinations of these parameters to optimise viable protoplast production.

4.2 Indirect Approach

A series of interchangeable promoter marker gene constructs were tested in this study. A range of *A. bisporus* and *C. cinereus* promoters from both constitutive and inducible genes have been or are being used to test expression of luc^+, GUS, and GFP genes.

It is expected that the *A. bisporus* or *C. cinereus* promoters should be recognised by their own DNA polymerases. The construct pBU001 is driven by the *C. cinereus* TRP1 (tryptophan synthetase) promoter. The TRP1 promoter is expected to be read when inserted into *C. cinereus* as this gene has been used to successfully in transformations (Binninger *et al.*, 1987). However, the TRP1 gene is believed to be upregulated by the availability of tryptophan. In the presence of tryptophan the transgene may be present and functional, but not expressed; steps have been taken to ensure the absence of tryptophan in the selection medium. However, it is possible that

the TRP1 gene may be expressed at low levels by a weak promoter, with transcription terminating when a minimal amount of protein has been produced. This may also occur with the TRP2 promoter, also involved in the trytophan biosynthetic pathway. The *A. bisporus* Cel1 and Cel3 promoters are also regulated, by the external levels of cellulase substrate.

Glyceraldehyde-3-phosphate dehydrogenase is a key enzyme in glycolysis and is therefore highly conserved between fungal species. A homologous GPD promoter has successfully driven the expression of a heterologous transgene in the homobasidiomycete *Schizophyllum commune* (Schuren, Harmsen and Wessels, 1993, Schuren and Wessels, 1998). In the same study Ascomycetous regulatory sequence failed to transcribe. This suggests that homologous regulatory sequences are more likely to be successful in the development of transformation systems of homobasidiomycetes. The *A. bisporus* promoters are therefore a good candidates as promoters for *A. bisporus* marker gene constructs.

The luciferase reporter gene luc^+ is widely used for the study of gene expression in plants as the assay is technically easy and extremely sensitive (Promega, 1993). The LUC gene has been used in both plant transformations (Welsh & Kay, 1997) and yeast transformations (Vieites *et al.*, 1994). However, in this study no positive results were obtained for either *C. cinereus* or *A. bisporus*.

The GUS colorimetric marker gene has been used to report transformation of cereal fungi (Forbes *et al*, 1998; Stleger *et al.*, 1995), including the heterobasidiomycete *U. maydis* (Richard *et al*, 1992). An absence of background GUS activity in *C. cinereus* and *A. bisporus* would allow use of the marker. However, no positive results were obtained from the 18 transformations performed with *C. cinereus*.

GFP marker gene construct transformations are only in the preliminary stages, but the negative results so far are in accordance with those of the luc^+ and GUS constructs. The gene for GFP has been successfully expressed in filamentous fungi including the Ascomycete *Aspergillus nidulans* (FernandezAbalos *et al.*, 1998) and the heterobasidiomycete. *U. maydis* (Spellig *et al.*, 1996).

The lack of success in *C. cinereus* with these novel genes may have been in part, due to low viability of protoplasts. Similarly , the *A. bisporus* viability rates were low ($<1x10^{-5}$ viable protoplasts per transformation).As *A. bisporous* is a more difficult organism to manipulate in the laboratory, a lower protoplast viability is expected, therefore more protoplasts are required to reach similar viability levels as *C. cinereus*. However, the viability frequency of the *C. cinereus* co-transformation was sufficient to enable recovery of trp^+ transformants. Transformants expressing the luc^+, GUS and GFP genes were not recovered. This suggests that there may be a problem with the integration and/or expression of the marker genes.

Possible explanations for the failure of marker gene expression are proposed below:

The construct may fail to integrate into the hosts genome with the regulatory sequence intact. Alternatively integration into house keeping genes may cause cell death. The construct may integrate successfully but is subsequently recognised as non-self and is excised by the host. There is already some evidence for this phenomenom in *A. bisporus* (Challen, *pers. Comm.*). The host may modify the transgene, for example, by

glycosylation, such that DNA polymerases either fail to recognise and transcribe it, or that a truncated protein is produced (Royer & Horgen, 1991). The transgene may successfully integrate, be transcribed, but there is a problem with post-translational modification of the foreign protein resulting in misfolding and disfunction. It has also been shown that some heterologous DNA`s are incorrectly transcribed in Homobasidiomycetes resulting in truncated mRNA`s (Schuren *et al.*, 1998). This may be due to the presence of AT-rich sequences which may encode polyadenylation or early truncation of the transcript.

In order to determine if the GUS, luc^+, and GFP genes have been transcribed, PCR will be performed upon the co-transformants expressing the primary construct, to identify transformants with integrated copies of the marker gene. RT PCR analysis will be carried out on the RNA of PCR positive colonies. If the predicted transcription has taken place, a western blot may be used to identify whether the appropriate proteins have been produced.

4.3. Direct Approaches

Whilst transformation with pAN7.1 has been achieved this vector does not seem to give consistent results and does not have the potential advantages of carboxin selection as discussed below.

Complementation for uracil prototrophy requires further evaluation. Ideally cloning the OMP-decase gene from the N1-unk mutant followed by sequencing should be carried out to ensure that the resident OMP-decase gene is mutant.

Carboxin is a systemic fungicide which is the active ingredient of commercial seed anti-fungal treatments (Dekeyser & Davis, 1998). Flutolanil (α, α, α-trifluoro-3`-isopropoxy-o-toluanilide) is a compound which is not related to carboxin, but also inhibits the function of the iron-sulphur sub-unit of the SDH enzyme (Oita *et al.*, 1997). A gene for flutolanil resistance from *Schizophyllum commune* was isolated and used to successfully transform *C. cinereus* (Oita *et al.*, 1997). However, in these experiments apparent transformation rates were low. Molecular evidence supporting transformation was absent and the resistance tended to be transient.

The reproducible recovery of stable carboxin resistant colonies in *C. cinereus* after transformation with the *A. bisporus* SDH β gene indicated that the SDH gene was heterologously expressed. This was confirmed by Southern analysis. Sequence analysis suggests that the *A. bisporus* α SDH gene is the wild type allele and that the β SDH allele is a mutant type, and the recovery of these transformants confirms this. In *U. maydis* specific mutations enabled a change in steric conformation (Broomfield *et al.*, 1992). The changes thus far seen in the *A. bisporus* SDH sequence are different to those characterised in *U. maydis* resistant mutants (Keon *et al.*, 1991, Broomfield *et al.*, 1992).

Southern analysis of carboxin resistant *C. cinereus* transformants indicated the occurrance of multiple partial integrations of transforming DNA. This highlights the random nature of DNA integration into non-homologous sites. Multiple copies of the SDH gene may integrate into the same genome at different sites, either intact or as

partial insertions. Some of the copies may integrate into house- keeping genes, thus causing the protoplast to die, or at least hindering growth. Tandem integration of transforming DNA may also occur. A low level of spontaneous mutation was observed. The occurrence of spontaneous mutants and spurious transformants are a recognised problem in transformation systems using positive selection. Spurious transformants are colonies which attempt to survive on selection medium by physiological adaptation. These colonies usually do not survive successive subculture. Li *et al.* demonstrated (1993) that a system previously used to successfully transform rust urediospores only gave false positives when performed on *A. bisporus*. They suggest that as recombination events during meiosis were rare, recombinational enzymes could be limiting, thus affecting the integration of the transforming DNA into the host genome. Moore et al, 1995 also produced false positives when using a biolistic system that transforms *C. bilanatus*, using *A. bisporus* mycelia and lamellae as target tissue. Bhattiprolu *et al.*, (1993) used a secondary screen based on the increased tolerance of genuine transformants to higher levels of 5-fluoroindole antimetabolite to eliminate this problem. However, the levels both of spurious regenerating colonies and spontaneous mutations are at an acceptable level in this study, and do not warrant an increase in carboxin concentration.

Northern analysis revealed that colonies transformed with the β SDH construct were transcribing the *A. bisporus* gene. The analysis of RNA extracted from colonies grown in the presence or absence of carboxin suggests that in general expression of the heterologous gene is not regulated. The possible regulation of SDH expression in one transformant is interesting and merits further analysis. In that transformant, the gene appears to be up-regulated by the presence of carboxin. The difference observed in transcriptional levels may be due to the post-translational degradation or modification of the transcript.

The presence of many carboxin resistant colonies on selection plates after biolistic transformation of *A. bisporus* indicated the possible integration and expression of the βSDH construct. The regeneration of these hyphal macerates is surprisingly high as the random nature of the biolistic process renders it less efficient than protoplasting protocols. The growth of biolistic carboxin resistant colonies was very slow in comparison to the original *A. bisporus* carboxin resistant mutant c54-carb 8. In a few cases this could be due to the transgene integration into 'house keeping' genes, but not for the large number of colonies observed. Poor expression of the transgene may be a more reasonable explanation. Only two colonies were found on no DNA control plates, which indicates a low level of spontaneous mutation. This does not, however, explain the presence of all the other carboxin resistant colonies. Challen & Elliott (1987) experienced extensive problems with *A. bisporus* spontaneous mutants to the fungicide benodanil, and suggested that these were transient physiological adptations to antimetabolite tolerance.

The results obtained in this study indicate that the β SDH gene is a suitable candidate for transformation of *A. bisporus*.

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