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Transformation technologies for mushrooms

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ABSTRACT: The edible mushrooms *Agrocybe aegerita, Agaricus bisporus, Lentinus edodes, Pleurotus ostreatus* and *Volvariella volvacea* have now been transformed; the first step towards directed genetic modification of these species. In *A. bisporus,* we have evaluated a range of vectors, delivery systems, and *Agrobacterium* transfection. Using protoplasts and selection for hygromycin resistance some transformants were obtained, but they proved unstable. *Agro*-transfections did yield stable transformants. To improve efficiency and utility of markers we are developing novel systems based on *Agaricus* genes and regulatory sequences. An extensive range of promoter cassettes has been built for use with a variety of reporter/marker genes and to assist development of future constructs. The *A. bisporus* orotidine-5'-monophosphate-decarboxylase gene *(URA3)* which has potential for transformation of a *ura3* auxotroph, was cloned and sequenced. Genes for/>ara-aminobenzoic acid can encode sulphonamide resistance and this system was tested in homobasidiomycetes. A mutant A *bisporus* gene that encodes carboxin resistance was cloned, sequenced and used to transform *Coprinus cinereus.*

1 INTRODUCTION

Transformation is a powerful technology whereby genes can be transferred within or between different species and can aid biological analysis in understanding gene function. It may reduce the need for traditional breeding, enabling direct modification of the genome and could be used to either add new genes, or to delete or modify the expression of existing genes.

Possible target genes for transformation include: senescence genes to improve mushroom quality (Burton *et. al.,* 1997); substrate utilisation genes to enhance yields (Woodet*. al,* 1991); and developmental genes to control mushroom fruiting. There are numerous potential pest and disease resistance targets, including genes involved in response to fungal pathogens (Mills *et. al.,* these proceedings), toxicity to insects (White *et. al.,* 1995) and natural pest resistance (White *et. al,* these proceedings). In addition, transformations with mating type genes that regulate inter-strain compatibility can alter breeding behaviour (Challen *et. al.,* 1993).

Transformation has been demonstrated in several cultivated species but the technology has not yet been used to produce commercial transgenic strains. This manuscript briefly reviews progress in transformation of edible mushrooms and describes experiments at HRI with *A. bisporus.*

2 TRANSFORMATION OF MUSHROOMS

Although it is possible to efficiently transform many homobasidiomycete mushrooms most progress has been made with model species e.g. *Coprinus cinereus* (Binninger *et. al.,* 1987),

Schizophyllum commune (Munoz-Rivas *et. al,* 1986). Over the last 10 years however, steady progress has been made in the application of transformation to edible mushrooms.

Byun *et. al.,* (1989) claimed transformation of a *Pleurotus florida* auxotroph using the *Flammulina velutipes Ieu2* gene. Peng *et. al.* (1992) recovered hygromycin resistant colonies *of Pleurotus ostreatus* using pAN7-l (Punt *et. al.,* 1987) which contains the *Escherichia coli hph* gene and regulatory sequences from *Aspergillus nidulans.* However, resistance was attributed to non-integrative, autonomously replicating plasmids and less than 1% of colonies examined contained transforming DNA (Peng *et. al.,* 1992). The bialaphos resistance gene from *Streptomyces hygroscopicus* was coupled with regulatory sequences from *Lentinus edodes* to transform protoplasts *of P. ostreatus* (Yanai *et. al,* 1996); co-expression of the *E. coli* GUS *(uidA)* gene was also shown. A mutant *C. cinereus* tryptophan gene, *trp3*^{ar}, which encodes 5-fluoroindole resistance (Bhattiprolu *et. al.,* 1993) was used to transform *P. ostreatus* and *Volvariella volvacea* (Jia *et. al,* 1998). At HRI genuine *trp3*$^{""}$~ transformants were not recovered in any mushroom crop species tested (Challen & Elliott, 1994) and work with *Coprinus bilanatushad* shown a high level of spontaneous resistance (Bhattiprolu et. *al.,* 1993). Kim *et. al.* (1999) transformed *P. ostreatus* uracil auxotrophs with the *Trichoderma reesei ura3* gene and observed transgene segregation through meiosis.

The Shii-take, was transformed using the *hph* gene with homologous *L. edodes* regulatory sequences and restriction enzyme mediated DNA integration (Satoe*t. al.,* 1998). In *Agrocybe aegerita* transformants were recovered using electroporation of protoplasts from an *ural* auxotroph and the homologous *URA1* gene (Noel & Labarere, 1994). The same group identified and used homologous promoter-like sequences to transform *A. aegerita* to neomycin resistance (Noelet*. al,* 1995).

Considerable effort has been directed at transformation of *A. bisporus* but early work was unsuccessful (Challen *et. al.,* 1991; Royer & Horgen, 1991; Li & Horgen, 1993; Challen & Elliott, 1994). Several of the vectors used in these studies contained sequences from bacteria and/or lower fungi that may not be effectively transcribed in homobasidiomycetes. However, a group from ATO-DLO, Netherlands reported transformation *of A. bisporus* with pAN7-l and related vectors (Mooibroek *et. al,* 1996; Van de Rhee *et. al,* 1996a). Several other groups had reported no success with pAN7-l (e.g. Royer & Horgen, 1991; Schuren, *pers. comm.).* The ATO-DLO group also reported homologous targeting with an exo-|3-l,3-glucanase gene sequence (Van de Rhee *et. al,* 1996b). Despite this, groups outside of ATO-DLO have had little success using the same methods (see section 3.1).

Virtually all the above protocols used protoplasts and chemical or electroporative mediated uptake of DNA. These protocols are restricted to strains that protoplast and regenerate readily; a severe limitation in *A. bisporus.* Ballistic delivery of transforming DNA (biolistics) is protoplast independent and has potential for mushroom transformation (Moore e*t. al,* 1995). Recently it has been shown that *Agrobacterium tumefaciens* can transfer *hph* sequences to a range of filamentous fungi, including germinating basidiospores of *A. bisporus* (De Groot *et. al,* 1998).

3 *AGARICUS BISPORUS* TRANSFORMATION EXPERIMENTS AT HRI

3.1 *Protoplast mediated transformation*

The transformations developed by ATO-DLO are the basis of a patent application (Mooibroek et. al., 1995). HRI repeated the work, following the protocols rigorously, using the same strain (ATCC 24663), same plasmids (pAN7-l, pA2H & pA2H-Tl; kindly provided by Dr. H. Mooibroek), same media, incubation and selection conditions. No transformants were obtained from 40 individual experiments. In two electroporation experiments with alternative strains (PCI and PC2) *ca.* 500 resistant colonies were recovered (10 µg/ml hygromycin). However, the resistance was not stable and Southern analysis of more than 100 colonies revealed no transforming sequences.

3.2 *Liposomal transformations*

In a modified protocol, protoplasts of PCI were presented with 5 µg/ml *Hindlll* linearised pAN7-l as a complex with DOTAP liposomes (Roche Diagnostics Ltd.). Transfer to complete medium containing 10 µg/ml hygromycin, yielded more than 1200 resistant colonies; equivalent to 240 colonies per µg pAN7-l. Only 3 colonies were observed on no DNA controls. A sample of 29 putative transformants was screened using pairs of PCR primers specific for pUC vector sequences (pVEC18-l: CTTACGCATCTGTGCGGT with pVEC18-2: GAGCGGATA CATATTTGAATG, for a 366 bp product; or pVEC18-3: GATCAAAGGATCTTCTTGAG with pVEC18-4: CCCCTGACGAGCATCACA, for a 587 bp product) and 10 of these yielded one or more of the products (34%). DNA from 93 putative transformants was Southern blotted and probed with pAN7-l: integrative transgenic sequences were identified (e.g. Figure 1) in only 3 samples. The apparent reduction in colonies containing transforming sequences (toca. 3%, equivalent to 7 colonies per µg pAN7-l) indicated transgene instability. Subsequent screening of these transformants using PCR primers specific for the *hph* gene (HYGR1: ATGCCTGAACTCACCGCG with HYGR2: TCGGTTTCCACTATCGGC) did not yield the appropriate 987 bp product. Four months after isolation, these and all other putative transformants tested were no longer hygromycin resistant. Further Southern analyses confirmed that all transgenic sequences had been lost.

3.3 *Agrobacterium mediated transformation*

Patent protection is being sought for the *A. tumefaciens* transformation of filamentous fungi (Beijersbergen *et. al.,* 1998). Based on the claims, we prepared a binary construct (pBIN7.1) containing sequences from pAN7-l. With the appropriate *A. tumefaciens* strain, we used pBINT.l to transform germinating basidiospores of Horst Ul. In controls, the same methods were used to transform *T. reesei* conidia.

A single hygromycin resistant (25 µg/ml), putative transformant (UlAtl) was recovered from one ^4gro-transfection of Ul spores. In a second experiment, seven putative transformants (UlAt2 — 8) were recovered. Resistant colonies appeared 27-32 days into selection. Despite the use of cefotaxime it proved difficult to obtain pure *A. bisporus* cultures post *Agro*-transfection and two of the colonies (UlAtl & 7) were lost. Four of the recovered six colonies exhibited stable resistance and were positive when screened with the *hph* PCR primers (Figure 2) and by Southern analysis (Figure 3). These ylgro-transformants have proved stable in the absence of hygromycin for more than 10 months. Other experiments using Ul and basidiospores from a second commercial strain A12, did not yield stable transformants. Beijersbergen *et. al.* (1998) recovered ten *A. bisporus* transformants from five experiments but the distribution of transformants between experiments was not reported.

4 ALTERNATIVE STRATEGIES

Protoplast and *Agrobacterium* mediated methods have yielded *A. bisporus* transformants, but the efficiency is relatively poor. Current protocols remain based upon the bacterial *hph* gene. In the UK and elsewhere, this marker would not be accepted in transgenic mushrooms. There is a need for alternative markers based upon mushroom sequences, to allay public concerns. In addition, homologous or near-homologous sequences might be expected to improve transformation efficiencies (Casselton & de la Fuente-Herce, 1989; Challen *et. al.,* 1991; Schuren & Wessels, 1994).

4.1 *Mushroom Gene-Promoter cassettes*

To identify efficient promoter sequences, a series of ten interchangeable promoter cassettes have been developed with conserved restriction sites at the 5' and 3' ends. Promoters are based

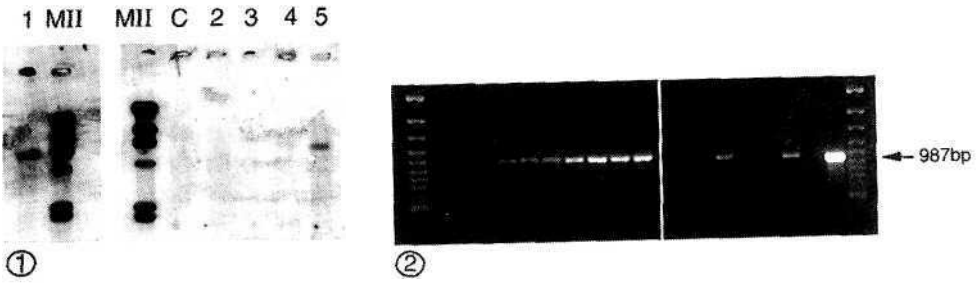

Figure 1. Liposomal-protoplast mediated putative transformants: *Hindlll* DNAs from hygromycin resistant A *bisponis* colonies (1-5) and non-transformed control (C) were probed for pAN7-l. Mil is X-*Hindlll* size marker.

Figure 2. PCR screening of *Agrobacteriwn* mediated putative transformants for *hph* gene sequences. *T. reesei* (left panel) and *A. bisponis* (right); 987 bp product is indicated.

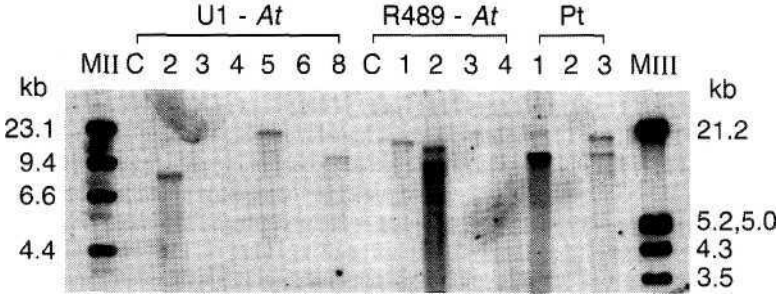

Figure 3. *Agrobacterium* mediated putative transformants: *Bgfil* DNAs from hygromycin resistant *A. bisponis* (Ul-At) and *T. reesei* (R489-AI) were probed for pAN7-l. Protoplast mediated transformants (Pt) and non-transformed controls (C) are compared. Mil and Mill are size markers.

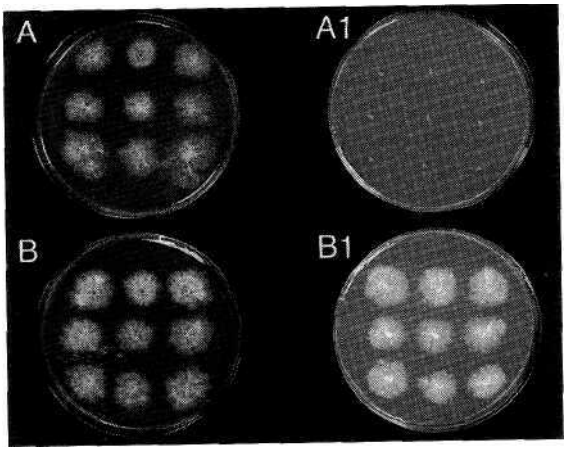

Figure 4. Carboxin resistance in A *bisporus.* Parent (A) and mutant (B) strains grown on minimal media Parent (Al) and mutant (Bl) strains grown on minimal media with carboxin.

on 5'-UTRs of the *C. cinereus* genes 77^7 (Skrzynia *et. al,* 1989), *$-tub* (Matsuo *et. al,* 1999) and eight *A. bisporus* sequences which include constitutively expressed, regulated and tissue specific promoters. Marker cassettes have been prepared for three reporter genes, firefly luciferase, GUS, green fluorescent protein *(GFP)* and the positively selectable *hph.* Expression of marker genes has been tested in *C. cinereus.* Co-transformants were generated harbouring each of the three reporter genes but despite stable chromosomal integration, gene expression was not detected. Transcript analysis is in progress and rt-PCR has revealed full-length products for *GFP* only.

4.2 *Agaricus genes as selectable markers*

Three genes that can be used directly as transformation markers have been studied; two from biosynthetic pathways (uracil and /wra-aminobenzoic acid) and a third encoding fungicide resistance.

4.2.1 *Uracil genes*

Few *A. bisporus* auxotrophs are available, most are poor growing and little is known of their genetics (Elliott, 1979). However one auxotroph, C63-«*r*a, with a dysfunctional orotidine-5'-monophosphate decarboxylase (OMPdecase) does grow well and also protoplasts (Challen & Elliott, 1994). Genes encoding OMPdecase have been used to develop transformation in numerous fungi including; *Phanerochaete chysosporium* (Akileswaran *et. al.,* 1993), *Phycomyces blakesleeanus* (Diaz-Minguez *et. al.,* 1990), *S. commune* (Froeliger *et. al.,* 1987), and the mushroom *P. ostreatus* (Kim *et. al.,* 1999).

Using PCR primers for conserved OMPdecase motifs, internal fragments were amplified from cloned *S. commune URA1* and *P. blakesleeanus pyrG* genes. These products hybridised with two clones in *an A. bisporus* cosmid library. Sequencing within these revealed a 932 bp *A. bisporus* OMPdecase gene *(URA3)* which has potential for transforming *ura3* auxotrophs. Complementation with *URA3* would not work if the *C63-ura* auxotrophy were not a single gene mutation. It should however, be possible to exploit *the A. bisporus URA3* in gene disruption experiments combined with selection on 5-fluoro-orotic acid (Boeke *et. al.,* 1984).

4.2.2 *para-aminobenzoic acid*

In the yeast, *Saccharomyces cerevisae,* the *pabA* gene encodes the folic acid precursor *para*-aminobenzoic acid and multiple copies confer sulphonamide resistance (Edmane*t. al.,* 1993). Transforming with mushroom *pab* genes might enable positive selection on media containing sulphonamides. To test this hypothesis in homobasidiomycetes, the *Coprinus bilanatus pabl* gene was identified by heterologous hybridisation with the *C. cinereus pabA* gene (Granado *et. al.,* 1997). Transformation of a *C. cinereus pab-* auxotroph with C. *bilanatus pabl* restored prototrophy and some transformants were resistant to sulphanilamide (500 µg/ml). Direct selection for sulphonamide resistance also yielded colonies and Southern analysis revealed multiple copies *ofpabl.* To develop this approach in *Agaricus,* a putative *Agaricus bitorquis pab* gene was identified by hybridisation with *C. cinereus pabA,* and by transformation of the *C. cinereus pab-* auxotroph with cosmid pools from an^4. *bitorquis* library (Calvo-Bado, 1999). It remains to be determined whether the *A. bitorquis pab* gene can confer sulphonamide resistance.

4.2.3 *Carboxin resistance*

The systemic fungicide, carboxin, is particularly active against basidiomycetes and inhibits the mitochondrial enzyme succinate dehydrogenase (SDH). Mutations in the iron sulphur protein subunit (SDH-Ip) confer carboxin resistance in some fungi (Keen *et. al.,* 1991; Broomfield & Hargreaves, 1992; Skinner *et. al,* 1998). Carboxin resistant mutants *ofA. bisporus* (Figure 4) have been produced at HRI (Challen & Elliott, 1987). Resistance in one of these was a single gene dominant; the cloned gene could therefore function as a selectable marker.

Degenerate PCR primers designed within conserved cysteine rich clusters of SDH (Broomfield & Hargreaves, 1992), were used to amplify a 600 bp product from *A. bisporus* genomic DNA. Screening the *C54-carb.8* cosmid library (Challenet. *al,* 1996) with the 600 bp product identified seven cosmids which were divided into two classes (a wild type and p mutant type alleles) by DNA sequencing. The 804 bp *A. bisporus SDH-Ip* gene contains at least five introns. Specific differences were identified between the a and p nucleotides and predicted amino acids. The a and (3 cosmids and *SDH-Ip* subclones were used in transformation experiments with *C. cinereus.* Significantly higher numbers of carboxin resistant (15 µg/ml), putative transformants, were recovered using the p clones than compared to a clones. In Southerns, only p transformed colonies contained heterologous *SDH-Ip* sequences. Northern analysis confirmed transcription of the *A. bisporus* gene in *C. cinereus.* Use of the P *SDH-Ip* gene for transformation of *A. bisporus* and other homobasidiomycete mushrooms is being assessed. Biolistic delivery of the p clones to PCI and a second *A. bisporus* strain B444 has yielded carboxin resistant, putative transformants, which are being evaluated.

Novel binary plasmids containing the *A. bisporus* p *SDH-Ip* gene are being developed in order to exploit *A*gro-transfection. Carboxin resistance genes are also being used to develop transformation of P. *ostreatus* (Honda *et. al.,* 1999; and these proceedings).

5 CONCLUSIONS

The edible mushrooms *A. aegerita, A. bisporus, L. edodes, P. ostreatus* and *V. volvacea* have been transformed by a variety of methods.

In *A. bisporus,* pAN7-l and related vectors have been used with electroporation or liposomal transfection of protoplasts and *Agrobacterium* mediated transfection. There have been problems in using the protoplast techniques in different laboratories and there is some evidence *forhph* transgene instability. y4gro-transfection of *A. bisporus* has been used successfully by ourselves and other laboratories (Romaine, *pers. comm.;* Mikosch *et al.,* these proceedings). The technique may have application in the transformation of other mushrooms e.g. *P. ostreatus* (Beijersbergen *et. al,* 1998) and is likely to gain increasing use. However, the existing techniques may not yield further improvements in efficiency and the *hph* gene may not be acceptable in commercial spawns. HRI continues to develop systems based *onAgaricus* genes and regulatory sequences. Our series of interchangeable promoter and marker cassettes will assist future vector development. Three specific genes have been cloned to extend choice and utility of markers. The *URA3* gene is most limited as only specific auxotrophs can be transformed. Despite this, systems based on auxotrophy yield fewer spurious transformants than resistance markers and have been used extensively in the biological analysis of fungi.

Two positively selectable markers are being developed. Multiple copies of *para*-aminobenzoic acid genes can encode sulphonamide resistance; as a first step in developing this strategy for edible mushrooms we have cloned an *A. bitorquis pab* gene. A mutant^, *bisporus SDH-Ip* gene that encodes carboxin resistance has been cloned and sequenced. This gene may have general utility as a selectable marker for homobasidiomycete transformation and its efficacy has been demonstrated by transformation of *C. cinereus.* The carboxin resistance marker may improve efficiency of ^(gro-transfection.

Future experiments with the cloned *A. bisporus* genes and promoter cassettes should determine whether transformation efficiencies could be improved using homologous sequences.

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