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Progress in Transformation of the Common Mushroom, *Agaricus bisporus*

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ABSTRACT: Transformation is considered a powerful biotechnological tool to study gene regulation. Recently, an efficient transformation system for homokaryotic *Agaricus bisporus* has become available. Mating with compatible homokaryons results in the formation of transgenic fruitbodies. The system is currently employed to investigate commercially important browning phenomena and to find ways for gene silencing in a heterokaryotic organism with one transformed nuclear type per cell.

1 INTRODUCTION

The cultivation, trade and processing of the common mushroom, *Agaricus bisporus,* spawn and fruitbody production represent important economic activities, especially in the European Community and the United States. The number of commercial strains available to meet specific demands for the market of fresh or processed products, is very limited. Although conventional breeding techniques have been applied successfully for the production of novel strains, the procedure is complicated by the aberrant life-cycle of *A. bisporus* due to the generation of usually heterokaryotic spores with two parental nuclei, that show almost no genetic exchange (Summerbell *et al.* 1989). This property is also the reason for the existence of little genetic variation in commercial strains (Castle *et al.* 1987).

Two recent developments will certainly contribute to improvements of *A. bisporus* breeding programs. The first concerns the discovery of wild isolates that show a tetrasporic life-cycle with only one nuclear type per spore (Kerrigan *et al.* 1994), which appears to be a dominant

trait and therefore, allows inbreeding into normal bisporic strains. The second development concerns the generation of a transformation system for *A. bisporus* (Van de Rhee *et al.* 1994, Van de Rhee *et al.* 1996a). For many years, world-wide attempts to develop a transformation system for common mushroom were not successful (Li and Horgen 1993, Challen and Elliott 1994). The transformation system reported herein is reproducible for an adenine-auxotrophic, homokaryotic *A. bisporus* strain (ATCC 24663, Raper and Raper 1972, Van de Rhee *et al.* 1994). The system was developed using a *Schizophyllum commune ura1* mutant and the wildtype *ura1* gene as a model system (Mooibroek *et al.* 1995a), employing electroporation of protoplasts. The *A. bisporus* transformation system is based on selection with the *E. coli* hygromycin B resistance (*hpt*) gene and it yields a maximum of about 100 transformants per integral transformation experiment, typically using 0.2-1.10^8 protoplasts (Van de Rhee *et al.* 1996a). The primary transformation vector pAN7-1 (Punt *et al.* 1987) contains the *E. coli hpt* gene flanked by *A. nidulans gpdA* promoter and *trpC* terminator sequences. The exchange of the signal sequences with *A. bisporus gpd2* promoter and terminator sequences (Harmsen *et al.* 1992; Van de Rhee *et al.* 1996a) has not resulted in an important increase in transformation efficiency. A homokaryotic and a self fertile heterokaryotic strain, isolated by protoplasting and regeneration of commercial strain Horst U1 (ATCC 62462), also have been subjected to essentially the same protocol and yielded transformants with a lower efficiency than ATCC 24663. Homologous integration via a 3kb genomic *A. bisporus* fragment containing a full-length and a partial exo-ß1,3-glucanase gene is highly efficient (Mooibroek *et al* 1995b, Van de Rhee *et al* 1996b). For the induction of fruitbodies homokaryotic transformants were mated with compatible homokaryons, followed by double selection on minimal medium containing hygromycin B. In all cases the donor DNA sequences were still detectable in the resulting mating products and derived fruitbodies, demonstrating its stability even in the absence of any selective pressure (our unpublished results).

Browning is an important aspect of *A. bisporus* quality loss, which is caused by mechanical injury, *Pseudomonas tolaasii* infection or by senescence. Brown discolouration has been associated with the transition of latent to active tyrosinase, possibly mediated by serine proteinase. Tyrosinase catalyses the oxidation of phenolic substrates, mainly L-tyrosinase, p-amino-phenol (pAP) and γ-glutamylhydroxy benzene (GHB) to melanins. Work was carried out to clarify which one of the following parameters: tyrosinase activity, phenolic substrate content or protease activity has the highest correlation with the colour and discolouration of mushrooms. There is ample evidence that the actual damage occurs by

mixing of different compounds which are normally located in separate cellular compartments. Preliminary statistical analyses show that tyrosinase activity significantly contributes to the browning reaction (Möller *et al.* 1996). Mushroom tyrosinase has been isolated before and was characterized as a monomeric protein of 43 kDa under denaturing conditions and 47 kDa under native conditions (Gerritsen *et al.* 1994). Many tyrosinase isoforms (about 7) have been detected in mushroom powder using monoclonal and polyclonal antibodies. Also, employing 2D electrophoresis followed by L-DOPA staining about 7 different isoforms have been observed. These results illustrate the multifaceted aspects of the tyrosinase enzyme complex. Some of these isoforms appeared to be growth stage- and tissue-specific. Recent evidence obtained in our laboratory suggests that a latent form of tyrosinase is activated by *P. tolaasii* infection and by its purified toxin tolaasin (Soler Rivas *et al.* 1996). Based on characteristics of *Neurospora crassa* and plant (tomato and faba bean) tyrosinase and *in vitro* activation by SDS and protease via cleavage of a part of the pro-enzyme, a model is proposed for the mechanism of activation of latent (pro-)tyrosinases (Wichers *et al.* 1995).

Based on tyrosinase DNA sequences from a number of other organisms, PCR products (600 bp) and corresponding cDNAs have been isolated from *A. bisporus*, designated *AbPPO1* (1.9 kb) and *AbPPO2* (1.4 kb) (Wichers *et al.* 1995). Another 2.6 kb mRNA has been detected that hybridizes only to the *AbPPO2* probe (our unpublished results).

Currently, the transformation system and available *A. bisporus* tyrosinase cDNAs are employed to investigate processes involved in tyrosinase-mediated browning by a number of approaches, including gene disruption and antisense RNA techniques.

A number of transformants of scientific and potential commercial interest already have been produced. Their characterization has been started and results will be presented on the initial analysis.

2 MATERIALS AND METHODS

2.1 *A. bisporus strains and growth*

Until now three different strains have shown to be transformable: an adenine-auxotrophic homokaryotic strain (ATCC 24663, Raper and Raper 1972), a self fertile heterokaryotic protoclonal derivative (U1mp10) of Horst U1 (ATCC 62462), showing a transient flat colony morphology and rapid differentiation after protoplasting, transformation and regeneration, and a homokaryotic protoclonal derivative of ATCC 62462, U1p8.

Strains were grown on malt extract, mycological peptone agar (MMP, 1.0%, 0.5%, 1.5%, respectively) on top of a cellophane sheet, for 7 to 12 days. Mycelial cultures were then macerated (30 sec) for liquid cultivation in MSG20 (Murashige and Skoog 1962) containing 20 $g.L^{-1}$ glucose, for 3 to 4 days. All incubations were carried out at 24°C.

2.2 *Donor DNA preparation*

DNA manipulations and cloning were carried out using standard procedures and *E. coli* strain JM109 (Sambrook *et al.* 1989). The *hpt* gene from pAN7-1 was altered by site-directed mutagenesis using PCR and restriction sites were introduced or removed for convenient cloning via 5'-*Nco*I and 3' *Bam*HI-sites (Van de Rhee *et al.* 1996a). Based on these characteristics other vectors were also constructed using published *A. bisporus gpd2* promoter and terminator sequences (pA2H and pA2H-T1). The tyrosinase PCR fragments *Abtyr1* and *Abtyr2* (both 600 bp) were cloned in the antisense orientation, similarly as *Nco*I-*Bam*HI fragments. These vectors were used as co-transforming DNAs, but also incorporated in combined constructs with the *hpt* gene/*Abgpd2* promoter cassette. Prior to transformation, vectors were linearized by *Eco*RI or *Hin*dIII restriction enzyme digestion (pA2H and pA2H-T1) or inside the *Abtyr1* and *Abtyr2* sequences to attempt homologous integration and gene disruption.

2.3 *Transformation procedure*

MSG20-grown mycelium was collected by filtration, rinsed with water and 0.6 M sucrose and incubated in 40 ml 0.6 M sucrose containing 10 $mg.mL^{-1}$ Novozyme 234 (InterSpex Products, USA, or Sigma) for 1.5 to 3 h at 24°C and 50 rpm. Protoplasts were purified by filtration and pelleted by centrifugation (30 min, 2000 g at 4°C). Protoplasts were washed twice with ice-cold 0.6 M sucrose and once with ice-cold electroporation buffer (0.6 M sucrose, 1 mM EDTA, 1 mM Hepes, pH 7.0) and pelleted by centrifugation (5 min, 2000 g at 4°C). Protoplasts (10^7 to 10^8 for ATCC 24663; 10^6 to 10^7 for U1p8 and U1mp10) resuspended in 100 µL ice-cold electroporation buffer were electroporated using the BioRad Gene Pulser (0.2 cm electrode gap, 0.45 kV, 25 µF, 200 Ω) in the absence or presence of DNA (10 µg Qiagen-purified plasmid DNA, 1 $\mu g.\mu L^{-1}$, either non-digested or linearized by restriction enzyme digestion, phenol-purified and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). After addition of DNA, protoplasts were held on ice for 30 sec before electroporation. Immediately after pulse delivery, protoplasts were mixed with 10 mL CMPS medium containing 100 $\mu g.mL^{-1}$ cefotaxim and incubated for

three days at 24°C. To estimate viability, small samples of protoplasts were mixed with 1% low gelling point agarose in 0.6 M sucrose (LMPS) at 38°C and poured over CMPS medium with 1.5% agar. Average regeneration percentages were 1% before electroporation and 0.2% to 0.5% after electroporation of ATCC 24663 and U1mp10 or U1p8. Protoplasts regenerated at least ten times more frequently in liquid CMPS than on solid medium, but clustering of regenerates in liquid CMPS hampered determination of the regeneration percentage. For selective plating, ATCC 24663, U1mp10 and U1p8 regenerates in liquid CMPS were mixed with 10 ml 2% LMPS (38°C). Portions of 5 mL were poured over Petri dishes with 20 mL selective medium, consisting of DT80 medium (Sonnenberg *et al.* 1988) with 1.5% agar, 20 $\mu g.mL^{-1}$ adenine (ATCC 24663 only) and 10 or 25 $\mu g.mL^{-1}$ hygromycin B (Duchefa), followed by incubation at 24°C. Regenerates of protoplasts electroporated in the absence of DNA never showed growth on medium containing 25 $\mu g.mL^{-1}$ hygromycin B. Occasionally, growth was observed on 10 $\mu g.mL^{-1}$ hygromycin B, when the plating density was very high, as has been observed by others (Royer and Horgen 1991). For U1mp10 regenerates selective plating was carried out on DT80 medium containing 25 and 50 $\mu g.mL^{-1}$ hygromycin B and on B5 medium (Duchefa; Gamborg *et al.* 1968; using 20 $g.L^{-1}$ glucose as carbon-source) with 50 and 100 $\mu g.mL^{-1}$ hygromycin B. Colonies appeared after 1 to 2 weeks.

2.4 *Analysis of transformants*

DNA was isolated from freeze-dried fruit-bodies or mycelium grown on MMP agar medium with cellophane according to Raeder and Broda (1985). Southern blot analyses were performed with 1 µg DNA per sample using standard procedures (Sambrook *et al.* 1989). The *hpt* gene was labeled using digoxigenin-dUTP and detection was performed using Lumigen PPD chemiluminescence following manufacturer's instructions (Boehringer Mannheim).

RNA was isolated from MMP-grown mycelia (Van Tunen *et al.* 1988). Northern blotting was performed with approximately 10 µg total RNA per sample using standard procedures (Sambrook *et al.* 1989) with the ^{32}P-labeled *hpt* gene and exon 9 from *gpd2* as probes. RNA size markers were from Gibco BRL.

For the initial analyses of tyrosinase antisense transformants slot blot hybridizations are currently being performed using the *hpt*-probe, the *A. bisporus gpd2* probe and strand-specific *Abtyr1* and *Abtyr2* probes to show the presence of antisense transcripts. Furthermore, PCR analysis is being performed with primers that anneal outside the 600 bp fragments of

the *AbPPO1* and *AbPPO2* genes in order to assess possible homologous integration and, thus, gene disruption.

2.5 *Mating and heterokaryosis*

Homokaryotic ATCC 24663 transformants were mated with a homokaryotic protoclonal derivative of U1 (H97p5) on MMP agar medium by placing mycelial inocula 0.5 to 1 cm apart. After incubation at 24°C for 1 to 2 weeks, inocula were taken from the interaction zone, then transferred to minimal medium (Yeast Nitogen Base from Difco) containing 25 $\mu g.mL^{-1}$ hygromycin B, again grown for 1 to 2 weeks at 24°C and finally grown on MMP agar medium for further propagation.

2.6 *Small-scale fruitbody induction*

U1mp10 transformants and mating products of ATCC 24663, grown on MMP plates with cellophane and including primary colonies and derived sectors, were used for small-scale fruit-body production, essentially according to San Antonio (1971). Fruitbodies were harvested before cap opening to prevent release of transgenic spores.

3 RESULTS

Using different donor DNA constructs, 167 putative ATCC 24663 transformants and co-transformants potentially containing antisense tyrosinase constructs were obtained. At a hygromycin B concentration of 10 $\mu g.mL^{-1}$ a minor fraction (μ 10%) of the colonies are so-called false positives or escapes, which are not found at a concentration of 25 $\mu g.mL^{-1}$. Table 1 shows an overview of currently available putative transformants, most of them still being in the initial phase of analysis by slot-blot hybridization with the *hpt*-probe. In some transformants from the GKII- GKIIe- and MIs-series, that were obtained first, more detailed analysis by Southern and Northern blot analysis have shown the presence of the *hpt*-gene and additional copies of *Abtyr1* or *Abtyr2*. In one transformant clearly aberrant *Abtyr2* transcripts were detected both in the primary transformant (GKII.10-2) and in the derived heterokaryon. The heterokaryon was obtained after mating and double selection with homokaryon H97p5 (derived from H97 from Horst U1, Horst Mushroom Experimental Station, Horst, The Netherlands). The GKII.10-2 transformant did not show any reduced level of the sense *AbPPO2* mRNA, either in vegetative mycelium or in derived fruitbodies. In future studies, a subset of the available putative transformants will be subjected to fruitbody production and further analyses.

Table 1. Screening of potential ATCC 24663 anti-tyrosinase-transformants.[a]

Donor DNA	Restriction enzyme	Codename transformant	Number of colonies isolated	Comment
pA2H + pA2HαAb1	*Sph*I *Sph*I	GKI	5	Northern slot-blot-analysis in progress
pA2H + pA2HαAb1	*Eco*RI *Sal*I	GKIs	3	GKIs.10-1 and GKIs.10-2: both *hpt*,
pA2H + pA2HαAb2	*Eco*RI *Eco*RI	GKII	7	GKIs.10-2: *Abtyr1*-sequences demonstrated GKII.10-1, GKII.10-2 and GKII.25-1: *hpt*, GKII.10-2, GKII.25-1: *Abtyr2*, GKII.10-2: aberrant *Abtyr2*-transcripts, In fruitbodies after mating between H97p5 and GKII.10-1, GKII.10-2, GKII.25-1: *hpt* demonstrated
pA2H + pA2HαAb2	*Eco*RI *Eco*RV	GKIIe	1	GKIIe.10-1: *hpt* and *Abtyr2* demonstrated
pA2H + pA2HαAb1 + pA2HαAb2	*Sph*I *Sph*I *Sph*I	GKIII	31	Northern slot-blot-analysis in progress
pαP1H	*Sal*I	MIs	2	MIs.10-1 and MIs.25-1: *hpt* and *Abtyr1* demonstrated
	*Not*I	MIn	4	Northern slot-blot-analysis in progress
pαP1H pvP2H	*Not*I	MIIn	0	Northern slot-blot-analysis in progress
pαP1H + pαP2H	*Not*I *Not*I	MIIIn	38	Northern slot-blot-analysis in progress
pαP1H + pαP2H	*Sal*I *Eco*RV	MIIIse	68	Northern slot-blot-analysis in progress
pA2H + pA2HαAb1 + pA2H + pA2HαAb2	*Sph*I *Sph*I *Eco*RI *Eco*RI	GKIIIspe	8	Northern slot-blot-analysis in progress

[a]Transformants of ATCC 24663 were produced with plasmid pA2H comprising the selectable *hpt*-marker in combination with antisense constructs pA2HαAb1 and pA2HαAb2. The latter contain *Abtyr1* and *Abtyr2*, respectively, in the antisense orientation. All three constructs contain the *Abgpd2*-promotor and *AntrpC*-terminator sequences. The *Abtyr1* en *Abtyr2*-sequences comprise 600 bp of the *AbPPO1* and *AbPPO2* cDNA sequences, which are located between the two copper-binding domains of the PPO1 and PPO2 polypeptides. Plasmids pαP1H and pαP2H are co-integration constructs consisting of pA2H plus the antisense *Abtyr1*- or *Abtyr2*-cassettes, respectively. Prior to transformation the donor DNA was linearized using the restriction enzymes indicated in order to improve the co-transformation efficiency according to Judelson (1993). All enzymes indicated cut outside the essential cassette elements, except *Sal*I, which is a primer-coded artificial restriction site in *Abtyr1*, not present in the endogenous *AbPPO1* sequence, and *Eco*RV, which is endogenous in *AbPPO2* and *Abtyr2*. Transformants were obtained via the standard transformation protocol for ATCC 24663 and were isolated at a hygromycin B-selective pressure of 10 or 25 $\alpha g.mL^{-1}$.

4 DISCUSSION

Since the transformable strain (ATCC 24663) is a homokaryon, mating with a compatible homokaryon is necessary for heterokaryosis and fruitbody induction. The transgenic heterokaryon obtained contains a transformed and a non-transformed nuclear type. In this situation, gene disruption may not be applicable in order to reduce the expression of tyrosinase, because the non-transformed nucleus may complement the transformed nucleus. However, it cannot be ruled out that gene silencing in one nucleus will affect gene dosage and, thus, tyrosinase activity. This aspect, however, is also complicated by the normal occurrence of tyrosinase in a latent form. It cannot be foreseen, whether reduced levels of latent tyrosinase will also result in a lower enzyme activity upon activation. The same is true for the antisense approach.

In addition to attempts for gene disruption, the antisense approach has been chosen. One of the possible antisense mechanisms involves the inhibition of translation by binding to ribosomes. It has recently been shown that replication of a viral RNA carrying an antisense carotenoid biosynthesis gene completely inhibited expression of the endogenous gene in *Nicotiana benthamiana* plants (Kumagai *et al.* 1995). This suggests that antisense RNA can act in the cytoplasm to turn off gene expression. Thus, inhibition of gene expression in *A. bisporus* fruitbodies may require transformation of only one nuclear type with the antisense construct. Furthermore, such experiments could contribute to our understanding of the mechanism and cellular site of action of gene inhibition by antisense RNA.

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