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Molecular Cloning and Heterologous Expression of the *Agaricus bisporus TRP2* Gene

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ABSTRACT: In filamentous fungi, four genes encode the tryptophan biosynthetic enzymes. One of these, designated here *TRP2*, encodes a trifunctional protein with glutamine amidotransferase, phosphoribosyl anthranilate isomerase (PRAI) and indole glycerol phosphate synthetase (InGP) activities. Trifunctional genes have been cloned from diverse filamentous fungi including *Aspergillus nidulans*, *Coprinus bilanatus*, *Coprinus cinereus*, *Neurospora crassa*, *Phanerochaete chrysosporium* and *Phycomyces blakesleeanus*. Several of these genes have been used to develop transformation systems and some have been expressed in heterologous hosts. The *A. bisporus TRP2* gene was cloned and its heterologous expression evaluated. A cosmid library was prepared and screened by heterologous hybridization with the *C. cinereus trp2* gene; eight overlapping cosmid clones were isolated. Progressive subcloning and hybridization experiments identified a 11.1 kb *Hin*dIII fragment and an internal 2.6 kb *Eco*RI fragment that contained the putative *A. bisporus TRP2* gene. The cosmids and plasmid subclones were introduced into a variety of *Coprinus trp-2* hosts via transformation. Integrative transformants were identified containing the *A. bisporus TRP2* gene but none grew on minimal media. Transformation of an *E. coli trpC1117* mutant confirmed PRAI activity of the *A. bisporus TRP2* clones. The reasons why the *A. bisporus TRP2* gene failed to express in *Coprinus* are not clear. To investigate the possibility that tryptophan biosynthesis in *A. bisporus* could more closely resemble yeasts or prokaryotes where PRAI and InGP enzyme activities are separated, the *A. bisporus* and *C. bilanatus TRP2* genes have been sequenced (unpublished data).

1 INTRODUCTION

In the filamentous fungi, four genes encode the enzymes of the tryptophan biosynthetic pathway that convert chorismate to tryptophan (Hütter *et. al.* 1986). One of these genes, designated here as *TRP2*, encodes a trifunctional protein which has glutamine amidotransferase (GAT), phosphoribosyl anthranilate isomerase (PRAI) and indole glycerol phosphate synthase (InGP) activities. Functionally equivalent genes have been cloned from diverse filamentous fungi including ascomycetes *Aspergillus nidulans* (*trpC*; Yelton *et. al.* 1984) and *Neurospora crassa* (*trp-1*; Schechtman and Yanofsky 1983), basidiomycetes *Coprinus bilanatus* (*TRP2;* Challen *et al.* 1994), *Coprinus cinereus* (*trp2*; Casselton and Fuente Herce 1989), *Phanerochaete chrysosporium* (*trpC*; Schrank *et al.* 1991), *Schizophyllum commune* (*TRP1*; Munoz-Rivas *et al.*1986) and the zygomycete *Phycomyces blakesleeanus* (*TRP1*; Revuelta and Jayram 1987). Several of these genes have been used to develop transformation systems and some were expressed in heterologous hosts, including an *Escherichia coli trpC* auxotroph. A *trp-2* auxotroph of the ink-cap mushroom *C. cinereus* was used to express heterologous but analogous genes from other basidiomycetes (Casselton and Fuente Herce 1989). In a parallel study the *C. cinereus trp2* gene was used for the heterologous transformation of the 2-spored, secondarily homothallic ink-cap, *Coprinus bilanatus* (Burrows *et al.* 1990). Subsequently the *C. bilanatus TRP2* gene was isolated from a cosmid gene library by sib-selection and heterologous expression in a *C. cinereus trp-2* host (Challen *et al.* 1994). These studies demonstrated that it is possible, to exploit heterologous transformation of species where transformation technologies are established, to clone genes from less tractable organisms. As part of a wider strategy to develop mushroom transformation and to expedite the cloning of mushroom genes by expression in a heterologous host we set out to clone the *A. bisporus TRP2* gene and test its expression in heterologous hosts. This paper describes the construction of a mushroom genomic library and the isolation and functional analysis of a putative *TRP2* gene.

2 MATERIALS AND METHODS

2.1 *Mushroom strains, media, plasmids and transformations*

The carboxin resistant mutant of *A. bisporus* designated C54-*carb*.8 (Challen and Elliott 1987) was used in the construction of the genomic library. Auxotrophic monokaryons of *C. bilanatus* Cb1-t2 (*trp-2*; Burrows

et al. 1990; ATCC 90221) and *C. cinereus* FM2 (*trp-2.1*; Casselton and Fuente Herce 1989; ATCC 96626) and AL1 (*trp-1.1, 1.6, trp-2.1*, Casselton and Fuente Herce, 1989) were used as heterologous basidiomycete hosts. Complete, minimal and supplemented minimal media were as previously described (Challen *et al.* 1994, Challen and Elliott 1994). The following *Coprinus* plasmids were used in these studies: pAK3 which contains the *C. cinereus trp2* gene within a 6.5 kb *Xba*I-*Eco*RI fragment (Kispert and Casselton unpublished, Burrows *et al.* 1990); pHIONA8 which contains a 3.5 kb *Eco*RI subclone from within pAK3 (Mellon and Casselton unpublished); pCBT2-S5 which contains the *C. bilanatus TRP2* gene within a 6.2 kb *Sac*I fragment (Challen *et al.* 1994); pCC1001 which contains the *C. cinereus TRP1* gene (Skrzynia *et al.* 1989) within a 6.5 kb *Pst*I insert. Protocols used for transformations were as described by Challen *et. al.* (1994).

2.2 *Escherichia coli strains, media and plasmids*

E. coli strain 1046, a variant of the *recA*$^-$ strain ED8767 (Cami and Kourilsky 1978, Gibson *et al.* 1987) was used as the host for cosmid cloning. The vector pUC18 (Gibco-BRL) and *E. coli* strain DH5α (Hanahan 1983) were used for subcloning *TRP2* fragments with standard protocols (Sambrook *et al.* 1989). The auxotrophic strain JA300 (*trpC1117*, ATCC 33588) was used to test for heterologous expression of *TRP2* genes. *E. coli* cells were routinely grown in the L media described by Little (1987). Cosmid clones for cryopreservation (-80°C) were grown in 96 broth (Challen *et al.* 1994). TRP$^+$ transformants were selected on the glucose minimal (GM) medium described by Munoz-Rivas *et al.* (1986).

2.3 *DNA manipulations*

High molecular weight, genomic DNA of *A. bisporus* was extracted from C54-*carb*.8 mycelia using the method described for *C. cinereus* (Mutasa *et al.* 1990). Small scale extraction of genomic DNAs were carried out using the method of Zolan and Pukkila (1986). Standard protocols were used for restriction enzyme digests and Southern blotting (Sambrook *et al.* 1989). Hybridising sequences were detected with non-radioactive digoxigenin probes (Boehringer Mannheim 1989) using previously described conditions to detect homologous and heterologous sequences (Challen *et al.* 1994)

2.4 *Construction and screening of A. bisporus genomic library*

The genomic library was constructed in the 8153 bp cosmid vector Lawrist, which contains two *cos* sites, two drug resistance markers kanamycin and ampicillin, a λ-origin of replication and offers alternative *Bam*HI and *Hin*dIII cloning sites flanked by SP6 and T7 promoters (P.F.R. Little, unpublished and pers. comm.). Lawrist was formed by fusing the LoristX cosmid (5386 bp, Speek *et al.* 1988) with a modified pUC vector (2767 bp). Details of the methods used in cosmid cloning were essentially as described by Little (1987) and Little and Jackson (1987). Inserts of C54-*carb*.8 were prepared by partial digestion of high molecular weight DNA with *Hin*dIII and treated with calf intestinal alkaline phosphatase (Sodhi 1992). Genomic inserts were ligated with Lawrist that had been linearized with *Sca*I (which inactivates ampicillin resistance), phosphatased and subsequently restricted at the *Hin*dIII cloning site. Recombinants were selected on L agar containing 30 µg/ml ai kanamycin (Sigma K4000) only. An ordered library was prepared by the isolation of 8288 individual clones into microtitre plates. Additional filter based libraries were constructed by plating an estimated 17,255 recombinants onto seven Pall Biodyne filters (BNNG 132, *ca.* 13 cm diameter).

The putative *A. bisporus TRP2* gene was located by heterologous hybridization with the 3.5 kb *Eco*RI fragment from pHIONA8. Preliminary screens were with Southern blotted *Hin*dIII restricted, pooled DNA from 56 microtitre plates (96 clones each, *ca.* 5376). Individual positive clones were identified from colony blots of microtitre plates.

3 RESULTS

3.1 *The A. bisporus libraries*

Using the cosmid vector Lawrist an assortment of gene libraries were prepared from genomic DNA of C54-*carb*.8. The average size of *A. bisporus* inserts was determined at *ca.* 37 kb (Sodhi 1992). Using the formulae of Clarke and Carbon (1976) and an estimated genome size of 3.42×10^7 bp (Arthur *et al.* 1982) a library of 4254 clones should give a 99% probability that any one gene is represented at least once. The numbers of clones isolated (8288-17,255) represents a 2-4 fold redundancy for the heterokaryotic (2n) genome of C54-*carb*.8. The representative nature of the library has been confirmed by the isolation of several independent clones containing cellulase and laccase genes (Sodhi 1992, Perry *et al.* 1993, Yagüe *et al.* in press).

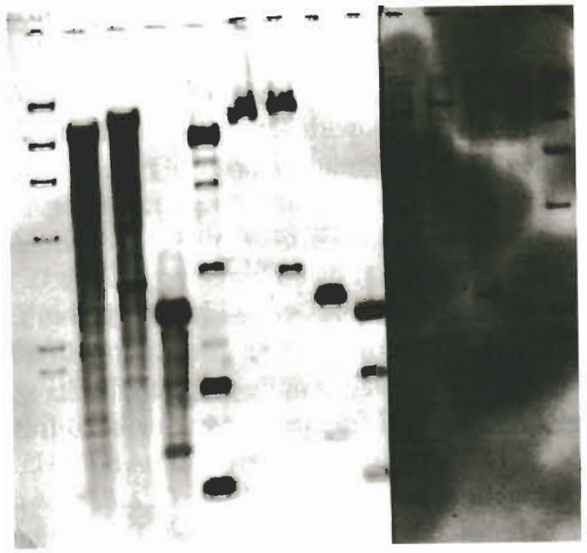

Fig. 1. Composite Southern blot showing hybridization of *C. cinereus trp2* probe to *A. bisporus* DNA. Lane contents from left to right are: λ-*Hin*dIII molecular marker; cosmid clone LA4G8 restricted with *Hin*dIII, *Pst*I, *Eco*RI, *Sac*I; cosmid clone LA12C3 restricted with *Hin*dIII, *Pst*I, *Eco*RI, *Sac*I; Genomic DNA of C54-*carb*.8 restricted with *Hin*dIII, *Pst*I, *Eco*RI, *Sac*I; and λ-*Hin*dIII molecular marker.

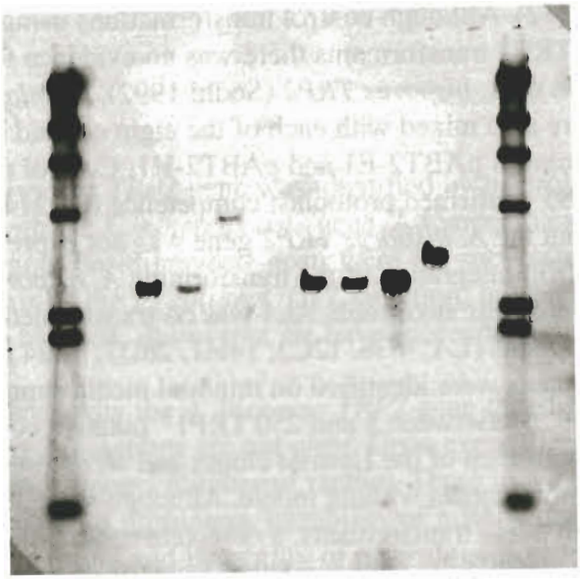

Fig. 2. Southern analysis of *C. cinereus* AL1 co-transformants probed with *A. bisporus TRP2* probe. Lane contents from left to right are: λ-*Hin*dIII molecular marker; *Eco*RI digests of genomic DNA from ten TRP1$^+$ transformants T2-227, T2-14, T2-33, T2-150, T4-99, T4-114, T4-152, T8-54, T8-14, T2-230; and λ-*Hin*dIII molecular marker. Eight of the transformants contained hybridising sequences consistent with the integration of transforming *A. bisporus* DNA. Transformants T2-227 and T2-230 were negative.

3.2 *A. bisporus TRP2 clones*

The *C. cinereus trp2* probe successfully hybridized to *A. bisporus* DNA (Fig. 1). Hybridization at 57°C with sequential washes at 50°C (2 x SSC and 1 x SSC) were required to reduce levels of non-specific hybridization. These conditions were used for all subsequent screenings. Eight cosmid clones (designated LA: 4G8, 12C3, 14C6, 14H1, 28D7, 32E4, 32F12, and 42B12) were identified which showed homology to the *C. cinereus trp2* probe. Single hybridising fragments were identified from *Eco*RI (*ca.* 2.6 kb) and *Hin*dIII (*ca.* 11.1 kb) digests that correspond directly to fragments identified through Southern analysis of genomic DNA (Fig. 1, Sodhi 1992). The *Eco*RI and *Hin*dIII fragments were isolated from LA4G8 and subcloned into pUC18 to yield pABT2-E1 and pABT2-H1 respectively. These fragments had high homology with the *C. cinereus trp2* probe (Sodhi 1992) as determined by their ability to hybridize at the stringent conditions used for homologous probes.

3.3 *Heterologous transformations*

Twenty-two separate protoplast mediated transformations were performed using *C. cinereus* FM2 and four recombinant clones (LA: 28D7, 32E4, 32F12 and 42B12). Although control transformations using pAK3 consistently yielded TRP^+ transformants there was no evidence for the heterologous expression of *A. bisporus TRP2* (Sodhi 1992). *C. bilanatus* Cb1-t2 protoplasts were also mixed with each of the eight cosmid clones identified or the subclones pABT2-E1 and pABT2-H1. Control transformations using pCBT2-S5 confirmed protoplast competence (24-214 TRP^+ transformants/μg) but the *A. bisporus TRP2* gene was not expressed.

To confirm integration of the transforming *A. bisporus* DNA, the double *trp- C. cinereus* auxotroph, AL1 was co-transformed with pCC1001 and six recombinants (LA: 4G8, 12C3, 14H1, 28D7, 32E4 and 42B12). *TRP1* transformants were identified on minimal media supplemented with indole (Tilby 1976). Between 5 and 250 $TRP1^+$ putative co-transformants were isolated with each of the Lawrist clones and were tested for $TRP2^+$ activity on minimal media without indole. Although some leaky growth was observed in a few transformants, a complete restoration of prototrophy was not observed. Colonies showing either leaky or no growth on minimal media were tested by Southern analysis with an 0.8 kb *Eco*RI - *Sac*I fragment from pABT2-E1. Of a total of 138 transformants screened eight were found to contain transforming sequences consistent with the integration of full length copies of the *A. bisporus TRP2* gene (Figure 2). Only two of these positives, T2-33 and T2-150, made leaky growth on minimal media

and it seems unlikely that any of these transformants were, even partially, heterologously expressing the *A. bisporus TRP2* gene.

To test the functionality of the *A. bisporus TRP2* gene *E. coli* JA300 was transformed with pABT2-E1, pABT2-H1 and the *C. bilanatus TRP2* gene pCBT2-S5. Transformants were primarily selected by ampicillin resistance before transfer to GM agar plus ampicillin. Large numbers of TRP^+ colonies were obtained from transformations using the *A. bisporus* and *C. bilanatus* plasmids (Table 1). Plasmid DNAs were isolated from six of each of the three classes of *E. coli* TRP^+ transformants (pCBT2-S5, pABT2-E1, pABT2-H1). Miniprep digests with appropriate enzymes were Southern blotted and probed with the appropriate *TRP2* gene. All prototrophic colonies tested contained transforming plasmids.

Table 1. Numbers of *E. coli* JA300 TRP^+ transformants recovered using *A. bisporus* (pABT2) and *C. bilanatus* (pCBT2) plasmids. Media were supplemented with ampicillin (amp) where appropriate.

Transforming DNA	L (+amp)	GM(+amp)	GM (no amp)
no DNA	0	0	0
pCBT2-S5	1×10^8	7×10^7	5×10^9
pABT2-E1	2×10^9	8×10^8	2×10^9
pABT2-H1	2×10^7	9×10^5	6×10^8

4 DISCUSSION

A putative *A. bisporus TRP2* gene was identified by heterologous hybridization with the *C. cinereus trp2* gene. Attempts to express the gene in two heterologous basidiomycete hosts were not successful. Complementation of the *E. coli trpC1117* mutation, in which the production of PRAI is blocked, shows that we have succeeded in cloning a functional gene.

The reasons why the *A. bisporus TRP2* gene failed to complement the *Coprinus trp-2* mutations are not clear. Gene expression in heterologous hosts depends upon a number of factors. These include poor expression from heterologous promoters, instability of mRNA, failure to splice introns, poor translation and instability of heterologous proteins. Although integrative transformation was confirmed in our experiments, analysis of mRNA was not conducted and it is conceivable that *A. bisporus* sequences were not successfully transcribed.

Tryptophan biosynthesis appears similar in all organisms hitherto characterized and involves seven catalytic activity domains (Hütter *et. al.*

1986). There are however differences in the genetic organization of this pathway between diverse microorganisms. In the ascomycete *N. crassa* four unlinked genes encode five enzymes that specify the seven catalytic activities (Schechtman and Yanofsky 1983). The *N. crassa trp1* gene encodes a trifunctional protein with activity domains for GAT, InGP and PRAI. Functionally equivalent genes have been cloned from the filamentous fungi described in Section 1 and where examined each encodes a protein with identically organized domains (NH_2-GAT-InGP-PRAI-COOH). Among the fungi examined to date, only the yeast *Saccharomyces cerevisiae* encodes PRAI from a single gene (Hütter *et. al.* 1986). In *E. coli* five genes are organized in a single operon and a bifunctional *trpC* gene encodes for InGP and PRAI (Yanofsky *et. al.* 1981).

Given the above information it is surprising that the *A. bisporus TRP2* gene did not complement *trp-2* mutations in *Coprinus*. A possible explanation is that the genetic regulation of tryptophan biosynthesis in *A. bisporus* differs from that described for other filamentous fungi. This hypothesis is supported by the fact that recombinant clones with separate PRAI and InGP activities have been previously identified in *A. bisporus* (Wach 1988, Wach *et. al.* 1989). To further investigate this possibility the *TRP2* genes from both *A. bisporus* and *C. bilanatus* have been sequenced at HRI (details to be published elsewhere). Analyses of nucleic acid and deduced polypeptide sequences should reveal the catalytic activity domains present in these two genes and enable us to determine whether the failure to heterologously express *A. bisporus TRP2* in *Coprinus* is due to differences in gene structure.

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