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Isolation, characterisation and expression patterns of a *RAD51* ortholog from *Pleurotus ostreatus*

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Using degenerated primers for conserved regions of *RecA* homologs we have isolated a gene from *Pleurotus ostreatus* that shows characteristic features of *RAD51* homologs. The encoded amino acid sequence of *P. ostreatus RAD51* (*PoRAD51*) shows greatest sequence similarities with *RAD51* from *Coprinus cinereus* (89% identity). Furthermore the genomic organisation of *PoRAD51* is almost identical to that of *RAD51* from *C. cinereus*. Northern analysis shows that the expression of *PoRAD51* is found in vegetative mycelium, and fruit body tissue, and that it is expressed at elevated levels in lamellae/basidia and following DNA damage. A sporulation deficient mutant strain of *P. ostreatus* (ATTC 58937) showed expression patterns of the *RAD51* gene that are similar those of the normal sporulating strain.

INTRODUCTION

Prokaryotic *RecA* homologs play a central role in homologous pairing and strand exchange during genetic recombination (Roca & Cox 1990, Kowalczykowski 1991, West 1992, Clark & Sandler 1994, Camerini-Otero & Hsieh 1995). Eukaryotic homologs of *RecA* were first discovered in *Saccharomyces cerevisiae* (Basile, Aker & Mortimer 1992, Shinohara, Ogawa & Ogawa 1992, Bishop *et al.* 1992) and later in a variety of eukaryotes. Phylogenetic analyses of eukaryotic *RecA* homologs revealed a gene duplication during evolution which gave rise to two phylogenetic distinct subclasses of *RecA*-like genes, the *RAD51* group and the *DMC1* group (Stassen *et al.* 1997). In yeast *RAD51* is important for double-strand break repair and recombination (Game 1993). The *DMC1* gene of yeast is necessary for meiosis, but appears not to be involved in mitotic DNA repair (Bishop *et al.* 1992). In yeast meiosis, *RAD51* and *DMC1* mutants show very similar phenotypes: both are essential for completion of the meiotic cell cycle and consequently the production of viable spores (Shinohara *et al.* 1992, Bishop *et al.* 1992). But unlike *RAD51* mutants which undergo meiosis and spore formation to some extent (Shinohara *et al.* 1992), cells lacking *DMC1* arrest in meiotic prophase (Bishop *et al.* 1992).

RAD51 protein forms nucleoprotein filaments, on both single- and double-stranded DNA, that resemble the filaments made by *RecA* (Ogawa *et al.* 1993, Sung & Robberson 1995, Bishop *et al.* 1992). *RAD51* can catalyse homologous DNA pairing and strand exchange in an ATP-dependent manner (Sung 1994). During meiosis in yeast *RAD51* and *DMC1* proteins localise on synaptonemal complexes prior to chromosome synapsis in a regulated order, in which *RAD51* is apparently required for the formation of *DMC1* complexes (Bishop 1994). In *S. cerevisiae* transcription of *RAD51* is induced by irradiation with UV-light (Aboussekhra *et al.* 1992) or X-rays (Basile *et al.* 1992), treatment with methyl methanesulfonate (MMS) and during meiosis (Shinohara *et al.* 1992). The *DMC1* transcript is only induced during meiosis (Bishop *et al.* 1992).

RAD51 and *DMC1* homologs have been isolated from organisms as diverse as fungi, animals, and plants (Stassen *et al.* 1997). *In vitro* and *in vivo* studies have shown that across different systems these proteins share common properties and resemble characteristic features of their *S. cerevisiae* homologs.

We are currently studying the processes of meiosis and spore formation in *Pleurotus ostreatus* (oyster mushroom). Worldwide production of this edible mushroom ranges third behind *Agaricus bisporus* (white

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button mushroom) and *Lentinula edodes* (shiitake) (Chang 1996). A drawback in oyster mushroom cultivation is the large number of spores produced by *P. ostreatus* fruit bodies. Mushroom workers can develop an extrinsic allergic alveolitis after inhalation of these spores (Cox, Folgering & van Griensven 1988). Developing a sporeless *P. ostreatus* strain would be an option to eliminate the health risks associated with oyster mushroom cultivation. Sporeless oyster mushroom strains have been described in the scientific literature. Eger (1970) reported a spontaneous mutant strain of *P. ostreatus* (ATTC 58937) and Imbernon & Labarère (1989) generated sporeless strains by mutagenesis. Unfortunately these strains produce low yields making them unattractive for commercial growing and in addition the genetic background of these strains has not been determined.

We therefore aimed to isolate genes expressed during meiosis. These sequences could be used as markers in breeding, or as targets for knock-out experiments in the process to generate non-sporulating mushroom strains.

Here we describe the genomic and cDNA sequence of a *RAD51* ortholog from a commercially grown *P. ostreatus* strain (Somycel 3015). We studied the expression patterns of *RAD51* in the normal sporulating commercial strain and compared them with the expression patterns of *RAD51* in the sporeless mutant strain ATTC 58937.

MATERIAL AND METHODS

Strains and cultivation

The commercial strain *Pleurotus ostreatus* Somycel 3015 and the sporeless mutant strain ATCC 58937 were grown on MMP-medium (1% malt extract, 0.5% mycologic peptone, 10 mM KMOPS (3-[N-morpholino] propanesulfonic acid) pH 7.0, 1.5% agar) at 24 °C. For DNA and RNA isolation mycelium was grown on MMP-plates covered with cellophane disks. Lab scale fruiting experiments were performed according to Baars *et al*. (2000). For MMS treatment mycelium was grown overnight in liquid medium. Samples were taken before MMS addition and 1, 2, 4, 6 and 16 h following treatment with 0.02% MMS (Acros Organics, The Netherlands).

Bacterial strains and growth conditions

Escherichia coli strain DH5α was used for cloning, amplification and maintenance of plasmids. *E. coli* DH5α was grown in LB-medium (10 g l^{-1} casein, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, 15 g l^{-1} agar). *E. coli* strain LE 392 was used as host strain for bacteriophage λ-EMBL3. For bacteriophage λ-EMBL3 infection *E. coli* LE392 was grown in LM-medium (LB-medium supplemented with 0.2% maltose and 10 mM $MgSO_4$). *E. coli* strain XL1-Blue MRF was used as host strain for bacteriophage λ-Uni-ZAP XR. *E. coli* XL1-Blue MRF was grown on LB-medium supplemented with 12.5 μg/ml tetracycline for general purposes and for bacteriophage λ infection in LM-medium.

Cloning and DNA manipulations

All standard DNA manipulations and cloning were performed according to Sambrook, Fritsch & Maniatis (1989), or following manufacturer's instructions.

Genomic DNA and cDNA libraries

Throughout this work a EMBL3 λ genomic DNA library was used. This library was made from genomic DNA from PK22, a homokaryotic protoclone isolated from *Pleurotus ostreatus* (Somycel 3015) by protoplasting.

The cDNA library used in this study is a λ-Uni-ZAP (Stratagene) library made from lamellae of *P. ostreatus* (Somycel 3015) sporophores as starting material.

PCR with degenerated oligonucleotide primers

PCR was carried out in 25 μl containing 0.3 μg of *Pleurotus ostreatus* (Somycel 3015) genomic DNA, 5 μM of primers A and B, 0.2 mM dNTP and 0.3 units Super TAQ (HC) (HT Biotechnology) in buffer prepared as recommended by HT Biotechnology. The first PCR consisted of an initial denaturation step for 5 min at 94 °, followed by a touch-down PCR with the following parameters: denaturation for 1 min at 94 °, annealing starting at 65 ° with a decrease of 2 ° after every second cycle, amplification for 2 min at 72 °. After reaching an annealing temperature of 43 ° 10 cycles under the following conditions were performed: 1 min 94 °, 2 min 43 °, 2 min 72 °. The final cycle was followed by an additional 5 min at 72 °. For a second amplification 5 μl of the first PCR were used as template for a PCR under the same conditions as described above. Primer A is the oligonucleotide 5′-GGNGARTTYMGNWSNGGNA-AR-3′ and primer B is 5′-YTCNCCTCKNCCTSW-RWARTC-3′, where N is A, C, G or T; K is G or T; M is A or C; R is A or G; S is C or G; W is A or T; Y is C or T. Primer A is the sense strand and primer B is the antisense strand primer. PCR products were cloned in the vector pGEM-T (Promega) under conditions recommended by Promega.

DNA Sequences

All sequencing reactions were performed by BaseClear (Leiden). The GenBank accession number for *PoRAD51* is AJ430697.

Northern blot analysis

Total RNA was isolated from tissue or mycelium after freezing in liquid nitrogen and grinding the sample

using a mortar and pestil. About 100 mg of the powdered samples were transferred to a tube containing 3 ml extraction buffer (100 mM Tris/HCl pH 9.0, 10 mM EDTA, 1% w/v SDS). Subsequently the samples were extracted once with PCI (phenol/chloroform/isoamylalcohol, 25:24:1) and two times with CI (chloroform/-isoamylalcohol, 24:1). For phase separation the tubes were centrifuged at 15000 ***g***, 4 ° for 10 min. The RNA was precipitated with 2 M LiCl at 4 ° overnight. RNA was sedimented by centrifugation and washed twice with 80% v/v ethanol. The isolated RNA was dissolved in H_2O and stored in portions of 30 µg at −20 °. For gel electrophoresis the RNA was denatured with glyoxal/DMSO (1 M glyoxal, 45% (v/v) DMSO, 30 mM Bis–Tris, 10 mM PIPES, 1 mM EDTA, 0.1% (w/v) bromophenolblue, pH 6.5) for 1 h at 55 ° and separated in a 1% BTPE-agarose gel (30 mM Bis-Tris, 10 mM PIPES, 1 mM EDTA, pH 6.5). The RNA was blotted onto Hybond N nylon membranes by capillary transfer using 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Blots were crosslinked 5 min with UV-light and used for hybridisations after baking for 2 h at 80 °. UV-shadowing to visualise rRNA on the Nylon membranes was performed according to Mikosch (1999). Northern hybridisation was performed according to the standard protocol of the Boehringer-Mannheim DIG (Digoxygenine) System for filter hybridisation's using DIG-labelled RNA probes. Single strand probes were generated from cDNA containing plasmids using T7 and T3 RNA-polymerases and the Boehringer Mannheim DIG RNA labelling Mix. To generate run-off transcripts the plasmids were digested on one side of the cDNA inserts prior to in vitro transcription according to the manufacturer's protocol. The coding strands of cDNA's were used as negative controls.

5′-RACE

To identify the 5′-end of transcripts the SMART RACE cDNA amplification Kit (Clontech) was used. 1 µg of total RNA was used as starting material and a primer annealing about 120 bp downstream of the longest cDNA sequence was used for 5′-amplification according to the manufacturer's protocol. The primer had the sequence: ATTCGACTGTGTTCAG.

RESULTS AND DISCUSSION

Isolation and characterisation of a RAD51 *homolog*

We designed degenerated primers based on conserved regions of *RecA* homologs from fungal, mammalian and plant origin. These primers were used to amplify a fragment of a *RAD51* homolog from chromosomal DNA of *Pleurotus ostreatus* Somycel 3015. An amplification product of 400 bp was found that encodes a putative protein with homology to known *RAD51* proteins. Database searches using the sequence analysis program BlastX (Altschul *et al*. 1997) revealed that this sequence encodes a polypeptide that is about 70% identical to *RAD51* proteins from *Coprinus cinereus*, *Saccharomyces cerevisiae* and other *RAD51* homologs. This PCR product was used as probe to screen a *P. ostreatus* EMBL3 genomic library, and a cDNA library made from lamellae as starting material. From the libraries two overlapping gDNA clones spanning 2.7 kb and a cDNA with a 1.2 kb insert were isolated. To identify the transcription start of the *P. ostreatus DMC1*, 5′-RACEs were performed using different primers based on the cDNA sequence. Screening a large number of RACE products, we succeeded in extending the transcript only 48 bp beyond the start codon. Equally short distances between the end of the longest transcript and the start codon have been reported for *C. cinereus* and *P. ostreatus DMC1* and *C. cinereus RAD51* (Nara *et al*. 1999, Mikosch, Sonnenberg & van Griensven 2001, Stassen *et al*. 1997). As shown in Fig. 1, the cDNA sequence contained a 1023 bp ORF encoding the *P. ostreatus* ortholog of the *S. cerevisiae RAD51*, the deduced protein sequence contained 340 amino acid residues. Homology studies of the deduced protein sequence using BlastP (Altschul *et al*. 1997) revealed that the *P. ostreatus* sequence shows a high degree of amino acid identify to the *C. cinereus RAD51* (89% identity), the *Schizosaccharomyces pombe RAD51* (73% identity) and the mouse *RAD51* (72% identity) proteins. Furthermore the characteristic nucleotide binding domains of the *RecA* family were present within the predicted protein sequence (Fig. 1). Therefore the sequence was designated *PoRAD51* (*Pleurotus ostreatus RAD51*).

Comparison of the *P. ostreatus* genomic and cDNA sequences revealed that the ORF is interrupted by 5 introns (Fig. 1) ranging in size from 49 to 66 bp, a size consistent with those found in other fungi (Gurr, Unkles & Kinghorn 1987). A comparison of the exon intron distribution showed that the 5 introns of *C. cinereus RAD51* (Stassen *et al*. 1997) almost exactly match with the position and size of the introns in *PoRAD51* (Fig. 2) underlining the close evolutionary relation of these two basidiomycete *RAD51* orthologs. A matching exon intron organisation has also been found for the *DMC1* genes of *P. ostreatus* and *C. cinereus* (Mikosch *et al*. 2001).

A search in the yeast promoter database (SCPD, http://cgsigma.cshl.org/jian/) revealed the presence of a consensus regulatory sequence (*UASRAD*) in the promoter region of *PoRAD51*, that has been reported for genes involved in DNA repair, i.e. *RAD6* and *RAD18* of yeast (Xiao *et al*. 2000). An extended BlastX (Altschul *et al*. 1997) analysis of the genomic clone of *PoRAD51* identified a putative second ORF (600 bp) in the 3′ untranslated region (UTR) of *PoRAD51*. This partial sequence is showing significant homology (58% identity) to the C-terminus of phosphoglycerate dehydrogenase (*SERA*) from yeast (GenBank: accession no. U18839, SwissProt no. P40054). Interestingly the two genes lie in tail to tail orientation and seem to share

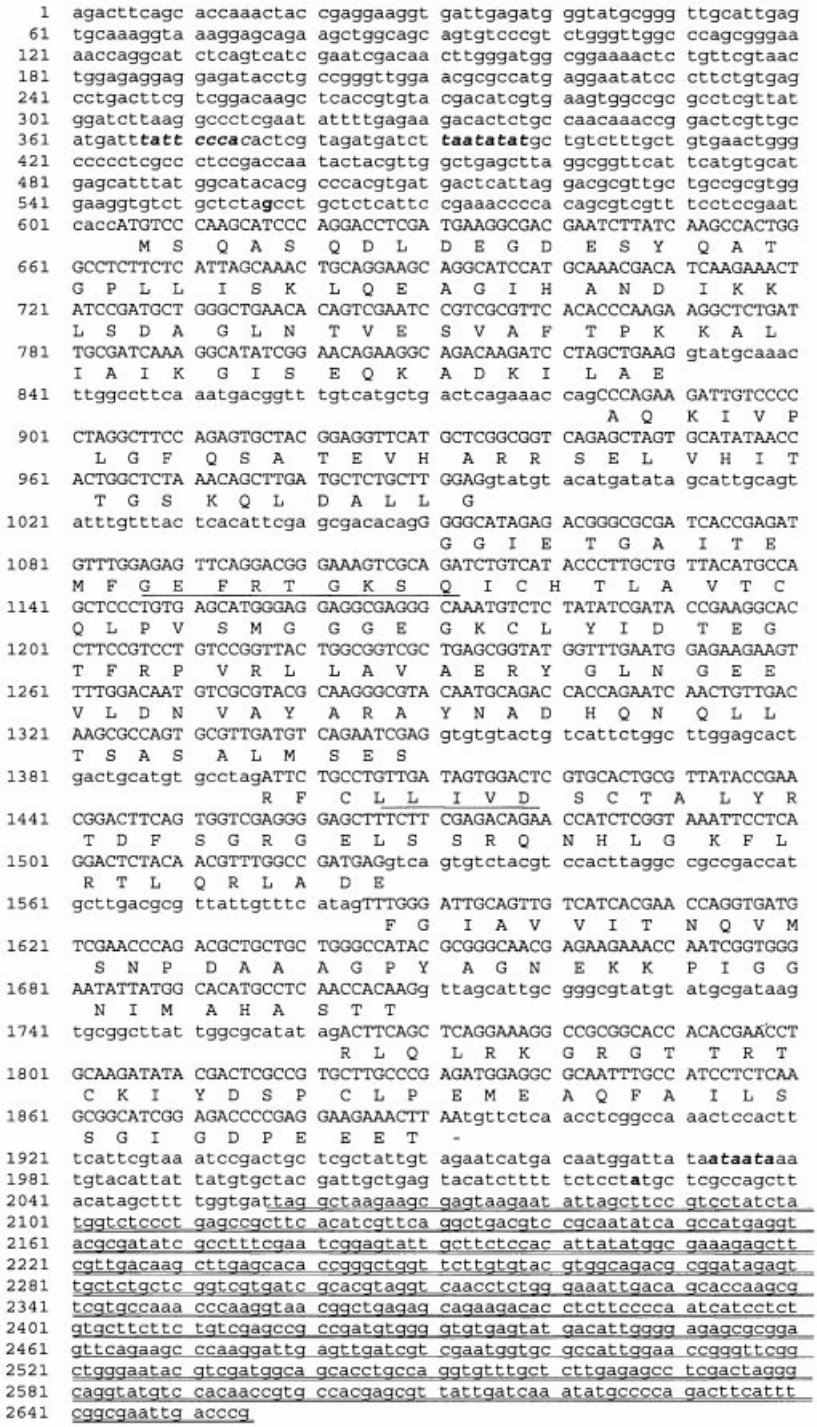

Fig. 1. Sequence of *PoRAD51*. Capitals indicate coding sequences, introns and non coding sequences are represented by small letters. The single letter designation for the amino acids encoded by the ORF are placed under the third base of a codon. The UASRAD upstream activating element (tattcccac), core promoter element (tatatat) and polyadenylation signal (ataata) are bold italic. The 5′ end of the mRNA and the beginning of the poly(A) tail are indicated in bold. ATP binding motifs are underlined. Double underlined the partial sequence of a putative gene on the complementary strand (see text).

the same terminator region of only 170 bp (Figs 1–2). In *S. cerevisiae* the two genes for *RAD51* and *SERA* are located on chromosome V, but not in direct proximity.

Northern analysis

In yeast and *Coprinus cinereus*, the *RAD51* gene is constitutively expressed and up-regulated after DNA damage and during meiosis (Shinohara *et al*. 1992, Stassen *et al*. 1997). To determine the expression pattern of *PoRAD51* total RNA was isolated from vegetative mycelium, fruit body stipes and lamellae/basidia of *Pleurotus ostreatus*. The *PoRAD51* transcript was detected in all samples, with a higher expression level in lamellae/basidia, indicating an induction of *PoRAD51* during meiosis (Fig. 3). The yeast *RAD51*

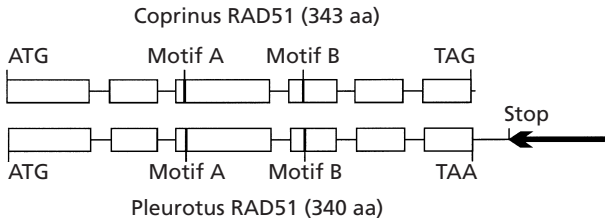

Fig. 2. Comparison of the genomic organisation of *RAD51* from *Coprinus* and *Pleurotus*. Lines represent introns and boxes exons. The arrow shows the direction of a putative gene found downstream of *PoRAD51*. Introns 1, 2, 3 and 5 in the *Pleurotus* sequence are shifted by one codon (5′ direction) compared to the *Coprinus* sequence and intron 4 has exactly the same position. The introns in the *Pleurotus* and *Coprinus* sequence have the same length.

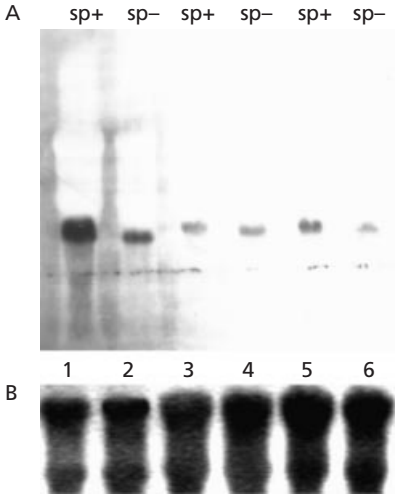

Fig. 3. A, Northern hybridisation using the *PoRAD51* cDNA as probe: sp+ = sporulating strain, sp− = sporeless strain. Lanes 1+2 RNA from lamellae/basidia, lane 3+4 RNA from fruit body stipes, lane 5+6 RNA from vegetative mycelium, each lane 30 μg of total RNA. B, Loading control. Same blot as in A. Visualisation of the rRNA bands on the nylon membrane by UV-shadowing.

gene is essential for meiosis and spore formation. *RAD51* mutant yeast strains show a dramatic reduction of spore production (Shinohara *et al*. 1992). Previously, a sporeless *P. ostreatus* strain (ATCC 58937) has been described by Eger (1970) and Leal Lara (1978). So far, no genetic analysis of this strain has been published and the mutation responsible for the sporeless phenotype has not been characterised. We were, therefore, interested in the expression of *PoRAD51* in this mutant strain.

As can be seen in Fig. 3, the expression of *PoRAD51* in the sporeless strain is somewhat lower than in the normal sporulating strain, but expression of the gene is clearly induced during meiosis. Although the mutant genotype needs to be clarified, it seems unlikely that this lower expression is connected to the absence of spores in the mutant strain.

To investigate if *PoRAD51* expression is induced by DNA damage, we incubated mycelium from *P. ostreatus*

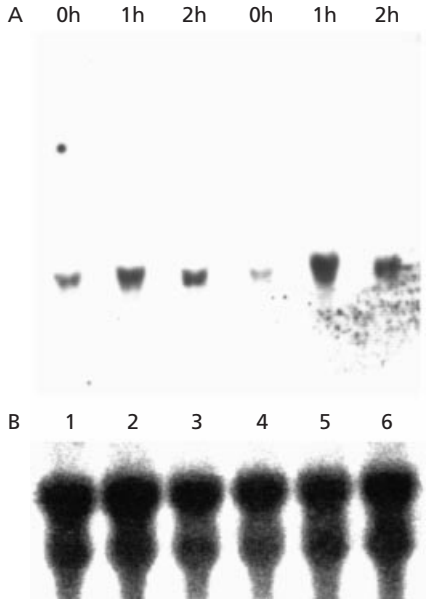

Fig. 4. A, Northern hybridisation following MMS treatment (0.2%) using the *PoRAD51* cDNA as probe: Lanes 1, 2 and 3 RNA from *Pleurotus ostreatus*, lane 4, 5 and 6 RNA from the Sporulation mutant (ATCC 58937), each lane 30 μg of total RNA. 0 h before MMS treatment, 1 h and 2 h after addition of MMS. B, Loading control. Same blot as in A. Visualisation of the rRNA bands on the nylon membrane by UV-shadowing.

and the mutant strain in the presence of 0.02% MMS. As can be expected for *RAD51* orthologs *PoRAD51* expression is induced following MMS treatment in both strains (Fig. 4). This underlines the previous conclusion that a *PoRAD51* detect is probably not responsible for the mutant phenotype.

PoRAD51-deficient mutants are required to elucidate the role of this gene in *Pleurotus*. Currently we are examining the possibility to generate *RAD51* knock-out mutants in *P. ostreatus* via transformation.

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