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Identification and characterization of *brt1*, a gene down-regulated during *B*-regulated development in *Schizophyllum commune*

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Abstract To identify genes regulated by the *B* mating-type genes a differential RNA display was established for the homobasidiomycete *Schizophyllum commune* and a gene, *brt1*, was identified. The mRNA concerned is highly abundant in monokaryotic mycelia and is down-regulated in matings in which either the *B* genes alone or else both the *A* and *B* genes are different between the mates. This places the gene *brt1* under the control of the pheromone response system of *S. commune* encoded by the *B* mating-type loci. Sequence analysis revealed similarity to a novel protein family, two members of which have been shown to inhibit translational re-initiation.

Key words *Schizophyllum commune* · Basidiomycete · Mating type · Regulation

Introduction

As in most homobasidiomycetes the tetrapolar mating-type system of the hymenomycete *Schizophyllum commune* FRIES is determined by two independent sets of genes, called *A* and *B* (Kniep 1920, 1922). Both of these are composed of two independent, but redundant, mating-type loci, α and β , (Koltin et al. 1967; Raper et al. 1960). Taking into consideration that each locus confers various specificities

it is estimated that there are more than 23 000 possible mating types in *S. commune* (Koltin et al. 1972). It has been shown, that the *A* α locus, as well as the *A* β locus, codes for transcription factors of the homeodomain family (Stankis et al. 1992; Shen et al. 1996). Activation of either *A* α , *A* β or both loci induces several developmental processes during dikaryon formation, such as nuclear pairing or clamp cell formation (Raper 1966). The *B* mating-type loci contain genes encoding pheromone/receptor-systems homologous to those known from ascomycetes and heterobasidiomycetes. *B* α , as well as *B* β , codes for seven transmembrane domain receptors with similarity to G-protein linked receptors and several putative lipopeptide pheromones (Wendland et al. 1995; Vaillancourt et al. 1997).

It is thought that by analogy to *Saccharomyces cerevisiae* the recognition of appropriate pheromones activates a signal transduction cascade which leads to the transcriptional control of *B*-regulated genes. *B*-activated crossings of *S. commune* typically show a “flat” phenotype, characterized by the lack of aerial mycelium, distorted hyphal growth and multiple branch formation. It is interesting to note that the hydrophobin gene *sc3*, which codes for a small hydrophobic protein thought to stabilize aerial hyphae in monokaryotic mycelia, and a co-expressed gene, *sc15*, which co-purifies with Sc3 protein, are not expressed in *B*-activated crossings (Asgeirsdottir et al. 1995; Lugones 1998; Ruiters et al. 1988). In addition, the nuclear distribution is aberrant with 0–20 nuclei observed in each cell (Niederpruem 1971; Raudaskoski 1973). These phenotypes are thought to be connected to the continuous nuclear migration observed in these matings (Raper 1966) and to the induction of cell-wall lytic enzymes (Wessels 1978).

In order to isolate *B* mating-type-dependent genes we established the differential RNA display (DRD) technique for *S. commune* to identify transcripts differentially regulated by the *B* mating-type genes. Being based on PCR, DRD is an easy and effective technique to screen RNA populations for differentially expressed transcripts (Liang and Pardee 1992; Liang et al. 1995). Using this method we identified a transcript, *brt1*, which is down-regulated in *B*-activated crossings in *S. commune*.

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Table 1 Primers used for DRD and primer extension

T ₁₁ AG	TTTTTTTTTTTTAG
T ₁₁ CA	TTTTTTTTTTTTCA
T ₁₂ GC	TTTTTTTTTTTTGC
DC1	TACACGAGAG
DC2	TCGATACAGG
DC3	GATCAAGTCC
DC4	GATCTAAGGC
DC5	GGTAGTAAGT
BRT-ATGEX	CGGCTTCCATGGGCATTTTG
BRT-EXT	GAGCAGTACACCATCCC

Materials and methods

Strains, plasmids and media. *S. commune* strains 4-39 (*matA1,1; matB3,2*), 4-40 (*matA4,6; matB1,1*), W22 (*matA1,1; matB3,2*) and 3947 (*matA1,1; matB2,2; ura1*) were grown on minimal medium or complex medium according to Schwalb and Miles (1967) at 30°C. N-limited medium was minimal medium without any nitrogen source. This medium enables wild-type strains to grow for a short time. To obtain surface cultures, *S. commune* strains were grown in liquid media for 3 days, the mycelium was macerated, pelleted, and spread on agar plates which were incubated for another 3–4 days. *Escherichia coli* K12 DH5 α (F⁻, *endA1, hsdRA, supE44, thi-1, recA1, gyrA96, relA1, Φ 80dlacZ Δ M15*) was grown on StdI medium (Merck, Darmstadt, Germany) supplemented with 100 μ g/ml of ampicillin and 40 μ g/ml X-gal when necessary. The vectors pBlue-script SK(+) (Short et al. 1988), pT7/T3 α 18 (Life Technologies) or pIC20H (Marsh et al. 1984) were used for cloning. *E. coli* cells for electroporation in 0.2-mm cuvettes, using a BioRad Gene Pulser at 2.3 kV, 25 μ F and 200 Ω , were prepared according to Calvin and Hanavalt (1988) and Dower et al. (1988). *S. commune* was transfected according to Specht et al. (1988) using *ura1* (pEF1) as a selectable marker (Froeliger et al. 1989). The deletion construct contains a 1.4-kb *Bam*HI/*Bgl*III fragment from pEF1 which was cloned into the *Bam*HI/*Bgl*III restriction sites of pIC20H in the orientation which destroys both sites in pIC-URA1.1. An *Eco*RV/*Xho*I fragment from pIC-URA1.1 was then used to replace the complete *brt1* ORF. The plasmid p Δ BRT1 was linearized with *Bam*HI prior to transfection into strain 3947.

DNA and RNA manipulations. *S. commune* genomic DNA and total RNA were isolated following the protocol of Wendland et al. (1996). Colony and Southern hybridization were performed using standard procedures (Ausubel et al. 1993), Northern hybridization was done according to Beckers et al. (1994) using 20–25 μ g of total RNA. RNA transfer was checked by methylene blue staining (Wilkinson et al. 1990). Double-stranded DNA sequences were determined using the Thermosequense Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham, Buckinghamshire, UK) and the DNA Sequencer Model 4000 (L) (LI-COR; MWG-Biotech, Ebersberg, Germany). The sequence is deposited with Genbank (accession number AF118109). DRD was performed according to Liang and Pardee (1992) using 2 μ g of total RNA from *S. commune* for reverse transcription. Re-amplified bands of interest were re-screened for differential expression using a South-Northern technique (Fabrice et al. 1995). Primers used in DRD reactions and primer extension (BRT-EXT, BRT-ATGEX) are listed in Table 1 and were supplied by MWG-Biotech (Ebersberg, Germany).

Results

Genomic sequences of allelic genes isolated from different *S. commune* strains may vary by 7% even for house-

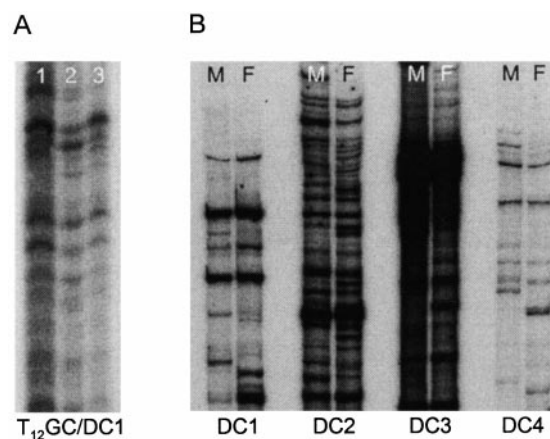


Fig. 1 A, B Differential RNA display (DRD) in *S. commune*. **A** total RNA from surface cultures of the strains 4-39 (lane 1), 4-40 (lane 2) and W22 (lane 3) was used for DRD with the indicated primer combination. **B** DRD from total RNA of surface cultures of the strain 4-40 (lanes M) and the *B*-induced cross W22 \times 4-40 (lanes F) using T₁₁CA for reverse transcription and the indicated second primers

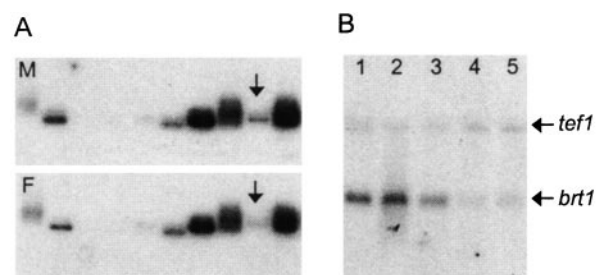


Fig. 2 A, B Identification of the *B*-regulated transcript *brt1*. **A** re-amplified cDNA fragments from DRD reactions were blotted and probed against radioactive cDNA from strain 4-40 (M) or the *B*-activated cross W22 \times 4-40 (F). A cDNA fragment of about 400 bp showing differences in the panels M and F corresponds to *brt1* and is marked with an arrow. The other fragments show no differential expression in the re-screen. **B** Northern analysis using total RNA from liquid cultures of monokaryotic strains 4-39 (lane 1), 4-40 (lane 2), W22 (lane 3), the *B*-induced cross W22 \times 4-40 (lane 4) and the dikaryon 4-39 \times 4-40 (lane 5). The blot was simultaneously probed against the *brt1* cDNA fragment (see above) and *tef1* as a loading control

keeping genes (Lengeler and Kothe 1994). To avoid false positive signals in DRD, isogenic strains had to be used. The isogenicity was first tested by performing DRD with monokaryotic strains which were generated by 30–40 backcrosses to one parental strain (resulting in strains 4-40 and 4-39) or an offspring of these two strains (strain W22). The banding pattern generated with different primer combinations showed about 95–97% identity for all three strains (Fig. 1 A) which is within the accuracy of the DRD method (Liang and Pardee 1992). This result showed both the isogenicity of the strains used and the reproducibility of the DRD method for *S. commune*.

Fig. 3 Nucleotide and derived amino-acid sequence of the *brt1* gene of *S. commune*. The transcription start point (*TSP*; position +1) determined by primer extension analyses and the polyadenylation site (*polyA*) are underlined twice. Putative TATA- and CAAT-elements in the promoter region as well as the conserved internal sequences of the introns are underlined. The positions of the primers used for primer extension experiments are indicated by arrows

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-180 ATCGATCCCA TTGAAATGCG TCCCATATAT CCTCGGATTC CGAGCACAAG CCCCTCCCGC AACGTCCAAG CCGCCGCAAG -101
-100 CGCGCCCGCC ACCCGCCCGC CGCCGGAGGC AAACACTTTC TCTAATCAGC ACAATGCATC CCGCCTCGGA CCCCAGCGCC - 21
                                     -1 +1→TSP
- 20 CCTGATAAAC GGATCATGCG CGGGCCGTGG GGAAGCCTAT CATCGATATA AGGCCTCGGG ACCTCTCGA GGCAGATCA + 60
   1      M P K E A V L T L N A 11
+ 61 GCGTCCGCCC TCTCTATACC TCGCTCAGCT CGTCTGCAAA ATG CCC AAG GAA GCC GTC TTG ACT CTG AAC GCG +133
   12 P P P L P G I Y S Q A I K A G G M V Y C S G 33
+134 CCG CCC CCG CTC CCG GGC ATC TAC TCG CAG GCG ATC AAG GCC GGC GGG ATG GTG TAC TGC TCG GGC +199
   34 A V P M D A K T G K L I D G D V K A H T 53
+200 GCG GTC CCG ATG GAC GCG AAG ACG GGC AAG CTG ATC GAC GGC GAT GTG AAG GCG CAC ACG GTGAGT +265
   54      H Q C I K N L S A I 63
+266 GGGTTGGTT TAGGTGGGG AGGGAGGCTG ATGAGATC TAG CAC CAA TGC ATC AAG AAC CTG AGC GCG ATC +337
   64 L E E A G T S L N N V V K V N V F L S N M 84
+338 CTG GAG GAG GCG GGC ACA AGC CTG AAC AAC GTC GTC AAG GTT AAC GTG TTC CTT TCG AAC ATG GTG +403
   85      D D F A A V N E V 93
+404 CGTTATCCGC CTTTGGAGCG CGCGATGGGT CGCTGATCAT CTCGCGAG GAC GAC TTC GCC GCG GTG AAT GAG GTA +477
   94 Y K E Y W G D V K P C R T C V A V K T L P L 115
+478 TAC AAG GAG TAC TGG GGC GAC GTG AAG CCG TGC CCG ACG TGC GTG GCA GTC AAG ACG CTC CCT CTC +543
  116 N T D V E I E G C I A T Q N * 128
+544 AAC ACC GAT GTC GAG ATC GAG TGC ATT GCA ACC CAA AAC TAA GAGGTGAA TCGTATAGG GATATCTGAG +614
                                     →POLYA
+615 CCTGTATCTT TAGTGGTGT ACTGGAATTT ACCCGTGAT CATGGAGCA CTCGTTGGAC ACCGATCGCC ACCCCATCGC +694
+695 AAGCTCAAGG CCACTGCCC TGAGGGGTGC TCACTGAATG GTTCTCAAAA CTCGACAAAC GACTGCAG +762

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DRD then was performed using the monokaryotic strain 4-40 versus a *B* induced “flat” crossing (4-40×W22, Fig. 1B). Out of 1000 fragments generated with the listed primers (see Table 1) 50 cDNA fragments of interest were isolated. The re-screening of these fragments identified *brt1* as a differentially expressed gene showing higher amounts of transcript in the monokaryon (Fig. 2A). This was confirmed by Northern experiments using the constitutively expressed gene *tefl* (approximately 1800 bp) as a loading control (Wendland and Kothe 1997). Expression of *brt1* (approximately 700 bp) was shown to be high in the three monokaryotic strains and low in a semi-compatible *B*-activated cross as well as in the dikaryon (Fig. 2B).

The transcript accordingly was named *brt1* for *B*-regulated transcript. The down-regulation of *brt1* takes place within 3–6 h after mating which could be shown in a subsequent Northern analysis (data not shown). A better resolution of the time scale for the down-regulation could not be obtained due to the poor synchrony of the hyphae for the mating reaction.

Using the cloned cDNA fragment from the DRD the chromosomal region of *brt1* was identified from a partial genomic library by colony hybridization. On a 5.5-kb *Bam*HI fragment the gene could be localized to a 0.9-kb *Cla*I/*Pst*I fragment which was sequenced. A single open reading frame was identified coding for a protein of 128 amino acids. Two small introns within the ORF were identified in comparison to the cDNA sequence which also identified the polyadenylation site. The transcriptional start site was identified by primer extension. The determined chromosomal structure of *brt1* is shown in Fig. 3.

No cross hybridization to other genes was observed even if lower hybridization temperatures were used, which makes *brt1* a single-copy gene (data not shown).

Using the cloned 5.5-kb chromosomal *Bam*HI fragment a *brt1* deletion construct was generated replacing *brt1* by the selectable marker *ura1*. Only 51 bp of the nontranslated leader and 55 bp of the *brt1* trailer were retained in the construct. From 50 uracil prototrophic transformants 1 could be identified by Southern analysis showing the expected signal pattern for the replacement of the chromosomal *brt1* copy by the selection marker. The inactivation of

brt1 was confirmed by Northern analysis which yielded no *brt1* transcript in the deletion strain KBLΔB1. No phenotype was detectable in colony or hyphal morphology on any medium tested (N-limited, minimal or complex media) nor in either crossings activated for *B* or in fully compatible mating reactions. Also, neither staining of cell-wall components with calcofluor nor staining nuclear DNA with DAPI showed any differences between the deletion strain and the recipient strain 3947.

Discussion

In this paper we describe the identification of *brt1* by means of differential RNA display. This gene is specifically down-regulated in *B*-activated crossings and codes for a putative protein of 128 amino acids.

A CAAT consensus sequence is present in the promoter region of *brt1* (–49, see Fig. 3). These elements are thought to be involved in high-level basal transcription in mammals and ascomycetes (Gurr et al. 1987; Montague 1987). The *brt1* gene shows strong signals in Northern experiments in monokaryotic mycelia, which may indicate that there is indeed a high basal-transcription level. This is in accordance with the high G+C content of 65.5% in the coding region (88.3% in wobble positions) and the reduced number of codons used (40) which are both known to be typical of highly expressed genes in many organisms. There is no strong TATA-element except for an AT-rich region around position –15 which is seen in many basidiomycete and ascomycete genes (Gurr et al. 1987).

The predicted protein of 128 amino acids, with a molecular mass of 13 733 Da, did not reveal any obvious functional parameters. A search of the SWISS Prot database revealed amino-acid sequence similarities to a novel protein family with the identification YER057c/YJGF (Fig. 4). These proteins of approximately 15 kDa show high conservation from bacteria to eukaryotes like fungi and mammals, and to archaea. The highest identity (56%) to Brt1 is seen with a putative protein from *Schizosaccharomyces pombe* which shows up to 74% similarity if con-

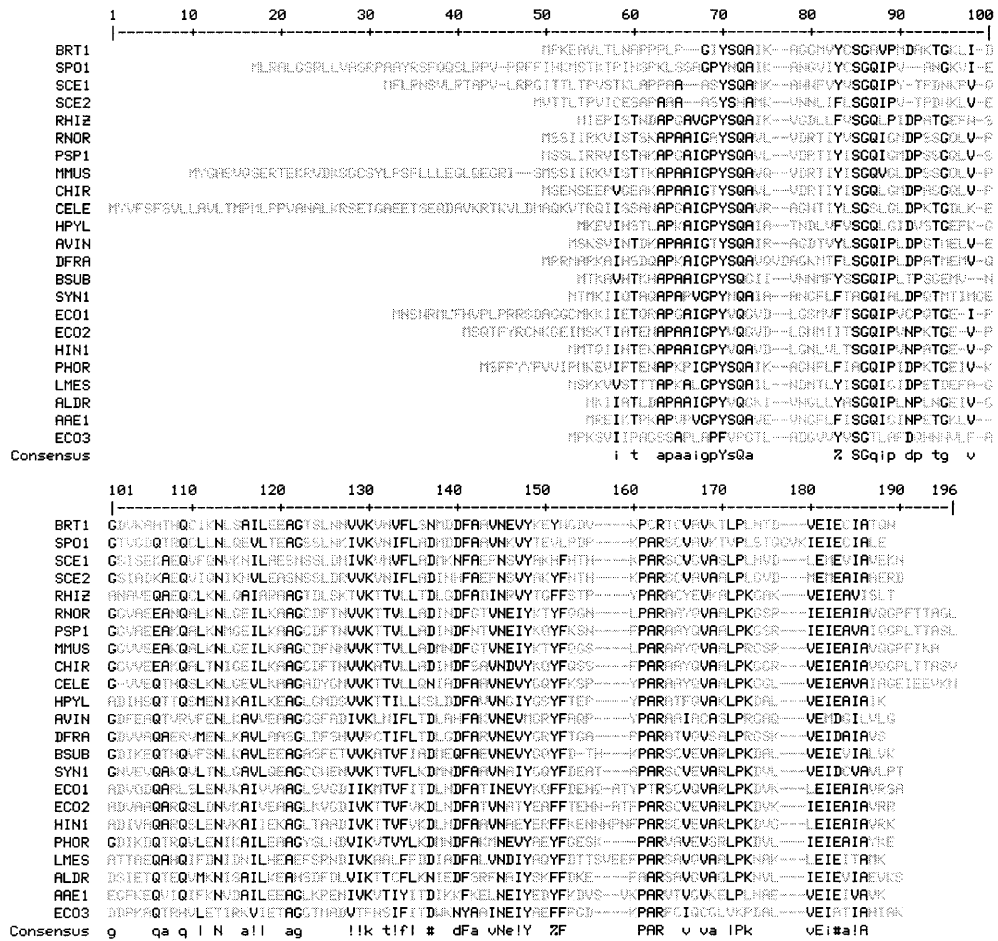


Fig. 4 Multiple sequence alignment of the amino-acid sequences of the YER057c/YJGF protein family members. The alignment was performed with MultAlin according to Corpet (1998) using standard features. Residues showing more than 70% identity in all proteins are shown in *bold face*. ! = IV, \$ = LM, % = FY, # = NDQE. BRT1 (*S. commune*), SPO1 (*S. pombe*, Wood et al. 1998), SCE1/2 (*S. cerevisiae*, Barrell et al. 1994; Dietrich et al. 1997), RH12 (*Rhizobium*, Freiberg et al. 1997), RNOR (*Rattus norvegicus*, Levy-Favatiar et al. 1993; Oka et al. 1995), HSAP (*Homo sapiens*, Schmiedeknecht et al. 1996), MMUS (*Mus musculus*, Samuel et al. 1997), CHIR (*Capra hircus*, Cecilian et al. 1996), CELE (*Caenorhabditis elegans*, Waterston 1995), HPYL (*Helicobacter pylori*, Tomb et al. 1997), AVIN (*Azotobacter vinelandii*, Joerger et al. 1989), DFRA (*Myxococcus xanthus*, Harris et al. 1998), BSUB (*Bacillus subtilis*, Ogasawara et al. 1994), SYN1 (*Synechocystis*, Chavez et al. 1995), ECO1/2 (*E. coli*, Blattner et al. 1997), HIN1 (*Haemophilus influenzae*, Fleischmann et al. 1995), PHOR (*Pyrococcus horikoshii*, Kawarabayasi et al. 1998), LMES (*Leuconostoc mesenteroides cremoris*, Cavin et al. 1996), ALDR (*Lactococcus lactis*, Goupil-Feuillerat et al. 1997) and AAE1 (*Aquifex aeolicus*, Deckert et al. 1998)

analysis because of their solubility in perchloric or trifluoroic acid. Oka et al. (1995) and Schmiedeknecht et al. (1996) have shown that rat and human homologues to Brt1 have an inhibitory effect on translational re-initiation in vitro. This is the first indication of a possible function for these proteins as translational regulators. Since no kinase activity could be found it seems unlikely that these proteins act similarly to the eIF-2a kinases known to be regulators of translational re-initiation (for review see Samuel 1993). Therefore, it will be interesting to see whether this new protein family shows RNA-binding activity, another possibility of regulating translation. The localization of the proteins either in the nuclei or the cytoplasm (Oka et al. 1995; Schmiedeknecht et al. 1996) would be in accordance with proteins as RNA-binding proteins.

The YER057c/YJGF protein family may thus be involved in the adjustment of the activities of isozymes, or proteins of redundant function, according to physiological needs. Such physiological changes are also expected during mating. In *S. cerevisiae* protein metabolism is changed completely during sporulation (Betz and Weiser 1976). This is also true during dikaryon formation in fungi (de Vries et al. 1980; Elliot 1994). Thus, metabolic changes are likely to occur dependent on the activation of mating-type genes which would place *brt1* in a cascade involved

servative amino-acid exchanges are allowed. Furthermore, the search of the database identified nine additional proteins which show a lower similarity (<50%) to Brt1. This might indicate that there are further protein families with a similar function but differing specificity.

With few exceptions the proteins are known from genome sequencing projects but their function is unknown. Homologous proteins are easily accessible to biochemical

in adjusting metabolic pathways to the needs of cells responding to the pheromone signal of a mate.

Upstream of *brt1* a novel peptide transporter has been recently identified which is also under the control of the mating-type system. The gene involved is induced in fully compatible matings when both A- and B-regulated development are turned on. Both genes share a common sequence element in their 3' non-translated regions which possibly means that Brt1 regulates its own expression as well as the expression of the adjacent transporter gene via the 3' trailer. Further experiments, including deletion of the sequence element in the RNA trailer and heterologous expression of *brt1*, will be necessary to investigate this interesting possibility.

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