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RESEARCH ARTICLE

Genes involved in carotene synthesis and mating in *Blakeslea trispora*

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Abstract Mating of *Blakeslea trispora* and other molds of the order Mucorales requires the interaction of mycelia of opposite sex, (+) and (−), leading to the development of specialized structures and to an enhanced accumulation of β-carotene. Industry obtains β-carotene by co-cultivating appropriate strains of *Blakeslea* ("mated cultures"). Gene transcription in single and mated cultures was assayed by cDNA-AFLP, a technique to observe the differential expression of subsets of mRNA fragments. Overexpression in mated cultures is about ten times more frequent than underexpression. We obtained and sequenced fragments of 97 candidate genes that appeared to be overexpressed during mating and confirmed four of them by reverse transcription and real-time PCR. Comparisons with gene sequences from other organisms suggest functions in carotene biosynthesis (4 genes), energy metabolism (8), cell wall synthesis (1), transfer of acetyl groups (1), and regulatory processes (10). Sodium acetate inhibited sexual overexpression in about two-thirds of the candidate genes and acted as a signal with broad effects on the metabolism and the morphology of mated cultures. Our work offers new materials for the study of carotene biosynthesis and its regulation and for the improvement of carotene production with Mucorales.

Keywords Sexual interaction · Carotene · Transcription profile · AFLP · *Blakeslea*

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Introduction

The mating process of the Mucorales begins with the mutual chemical recognition of mycelia of opposite sex that grow near each other. A succession of dedicated morphological structures culminates in the production of zygospores filled with protoplasm from both interacting mycelia. Attending metabolic changes include an increased accumulation of β-carotene in the mycelia, an increased secretion of trisporoids believed to mediate the mating response, and increased oxygen consumption (Sutter 1987). More new research is needed to fill the many lacunae in our knowledge and to improve carotene production. β-carotene and other carotenoids are used as provitamin, antioxidants and pigments in food and feed, pharmaceuticals, and cosmetics (El-Agamey et al. 2004; Stahl and Sies 2005). Shaken liquid cultures that contain *Blakeslea trispora* strains of

opposite sex ("mated cultures") are industrial sources of β-carotene and lycopene (Ciegler 1965; Mehta et al. 2003; Avalos and Cerdá-Olmedo 2004); the morphological structures of the sexual cycle do not appear under such conditions.

The carotene content of *Blakeslea* is modified by chemicals (Ciegler 1965; Ninet et al. 1969; Lampila et al. 1985; Feofilova et al. 1995; Choudhari et al. 2008), by light (Sutter 1970; Quiles-Rosillo et al. 2005; Kuzina and Cerdá-Olmedo 2007), and by mutations (Mehta and Cerdá-Olmedo 1995; Mehta et al. 2003). The carotene pathway of *Blakeslea* and its structural genes (Mehta and Cerdá-Olmedo 1995; Rodríguez-Sáiz et al. 2004) are very similar to those of the related mold, *Phycomyces blakesleeanus* (Cerdá-Olmedo 2001), but the regulation of carotenogenesis of *Blakeslea* is different, and not as well known, at least in its genetic aspects, as that of *Phycomyces* (Cerdá-Olmedo 1987b, 2001; Avalos and Cerdá-Olmedo 2004; Almeida and Cerdá-Olmedo 2008).

In *Blakeslea* and *Phycomyces* the presence of small amounts of acetate in mated cultures increases the production of sexual structures and inhibits the sexually-enhanced carotene biosynthesis (Kuzina and Cerdá-Olmedo 2006). The same amounts of acetate have no significant effects on single cultures. This provides a simple test to classify genes whose expression is modified by mating into two functional groups, one including the genes for sexual morphogenesis and the other the genes for carotenogenesis.

cDNA-AFLP (complementary DNA amplified fragment length polymorphism) is an RNA fingerprinting technique that allows a semiquantitative comparison of the abundance of a selected subset of mRNA fragments under various conditions (Vos et al. 1995; Bachem et al. 1998; Qin et al. 2005). The technique is robust and reproducible (Breyne et al. 2003; Qin et al. 2005) and its sensitivity is similar to other techniques for gene expression, such as microarrays (Reijans et al. 2003). An advantage of cDNA-AFLP is that it does not depend on pre-existing genomic sequence information. The very few *Blakeslea* genes that have been sequenced include those for the two dedicated enzymes for carotene biosynthesis (Rodríguez-Sáiz et al. 2004), a regulator of responses to light, including carotene biosynthesis (Quiles-Rosillo et al. 2005), and a carotene oxygenase probably involved in trisporoid biosynthesis (Burmester et al. 2007).

We report the first results of a project aimed at the identification and cloning of genes involved in mating and carotene production in *Blakeslea*. We have used the cDNA-AFLP technique to identify 123 mRNA fragments that are differentially expressed during mating and we have confirmed four of them by reverse transcription and real-time PCR.

Materials and methods

Strains and culture conditions

Blakeslea trispora wild-type strains F921 and F986, respectively (−) and (+), were obtained from VKM (All-Russian Collection of Microorganisms, Moscow, Russia). Spores (10^4 per plate) were inoculated on a cellophane disk, 80 mm in diameter, placed on 25-ml minimal agar (Cerdá-Olmedo 1987a) or minimal agar with 10 mmol/l sodium acetate in Petri dishes, 85 mm in diameter. Mated cultures were inoculated with both strains, each contributing one half of the inoculum. Following incubation at 30°C in the dark for 1, 1.5 or 2 days, fresh mycelium was collected, divided into portions of approximately 500 mg wet mass, frozen immediately in liquid nitrogen, and kept at −70°C.

RNA

For cDNA-AFLP, total RNA was extracted by the TRIzol method (Chomczynski and Sacchi 1987). Frozen mycelium was ground to a fine powder under liquid nitrogen and mixed with 5 ml TRIzol reagent (Invitrogen) and 0.5 ml chloroform. The mixture was shaken for 20 s, incubated on ice for at least 15 min, and centrifuged at 12,000*g* for 15 min at 4°C. Two 1-ml samples of the upper, aqueous, phase from each centrifuge tube, containing the RNA, were transferred to new tubes, mixed with 0.5 ml phenol, shaken for 20 s, mixed with 0.5 ml chloroform, shaken again and centrifuged at 12,000*g* for 10 min at 4°C. The upper, aqueous, phase, containing the RNA was mixed with 1 ml isopropanol, incubated at −20°C overnight, and centrifuged at 12,000*g* for 15 min at 4°C for RNA precipitation. The RNA pellet was washed twice by suspension in 1 ml ethanol (750 ml/l), centrifuged at 7,500*g* for 5 min at 4°C, air-dried at room temperature for 5–10 min, and dissolved in 0.1 ml water. To obtain ribonuclease-free water for use in the manipulation of nucleic acids a mixture of 1 ml diethyl pyrocarbonate and 1 l water was shaken, incubated overnight at 37°C and autoclaved.

One microliter of the RNA solution was diluted fivefold in water; 1 µl was used to measure the RNA concentration in a spectrophotometer ND-1000 UV-Vis (Nano Drop, Wilmington, DE, USA) and 3 µl to check the quality by electrophoresis in an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using the kit for the RNA 6000 Nano Assay. Final yield was about 0.7 mg RNA/g fresh mycelium.

PolyA-containing RNA was purified from total RNA (0.50-0.75 mg) by hybridization with a dT oligomer coupled to a solid-phase matrix (Kuribayashi et al. 1988), following the Oligotex mRNA spin-column protocol

(Oligotex mRNA Midi kit, Qiagen, Hilden, Germany). After elution from Oligotex complexes with 200 µl buffer, the mRNA was mixed with 20 µl sodium acetate (3 mol/l, pH 5.2) and 400 µl ethanol (960 ml/l), incubated overnight at −20°C for precipitation, centrifuged, washed and dried as before. Dry mRNA was dissolved in 40 µl water; 37.5 µl of mRNA solution was used for cDNA synthesis, 1 µl was used to measure the concentration, and 1.5 µl to check the mRNA quality as before.

cDNA preparation

For cDNA-AFLP, synthesis of double-stranded cDNA from mRNA was performed following the instructions of the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA), which is based on published methods (Okayama and Berg 1982; Gubler and Hoffman 1983). The first-strand was synthesized by a reverse transcriptase with a mixture of the four nucleotides (25 mmol/l each) and the second-strand by RNase H and DNA polymerase I, all from the same kit (Invitrogen).

The reaction product (about 200 µl) was mixed with phenol (100 µl) and chloroform (100 µl), shaken and centrifuged at 12,000*g* for 2 min at room temperature. The upper aqueous layer was purified in the same way with chloroform (200 µl). To sediment the cDNA, the purified upper aqueous layer was mixed with 20 µl sodium acetate (3 mol/l) and 400 µl ethanol (960 ml/l), shaken and incubated overnight at −20°C. The precipitated cDNA was centrifuged at 12,000*g* for 15 min at 4°C, washed with ethanol (750 ml/l), air-dried at room temperature and dissolved in 50 µl water.

cDNA-AFLP

cDNA fragments were obtained by digestion of the cDNA with TaqI and MseI restriction enzymes, whose restriction sites are TCGA and TTAA (Fig. 1). Twenty microliter (aproximately 0.4 µg) of cDNA were used for AFLP analysis according to the instructions of the AFLP Expression Analysis kit (LI-COR Biosciences, Lincoln, NE, USA). Both ends of the resulting cDNA fragments were ligated to adapters of known sequence and cohesive ends,

5′-GTAGACTGCGTAC/3′-CATCTGACGCAT GGC, for the TaqI ends and
5′-GATGAGTCCTGAG/3′-CTACTCAGGACTCAT for the MseI ends.

The DNA molecules thus obtained have a TaqI restriction site at one end and a MseI restriction site at the other or either TaqI or MseI restriction sites at both ends. These molecules were replicated by PCR with primers complementary to the adapter sequences. The resulting DNA molecules were further replicated with selective primers, which were the same as before, with an extension of two nucleotides, N_1N_2 for the MseI ends and N_3N_4 for the TaqI ends, respectively; the primer for the TaqI ends was fluorescently labeled with IRDyeIM 700. Fragments with MseI restriction sites at both ends would be replicated but not detected, because of the lack of fluorescence. Out of the 256 possible combinations N_3N_4/N_1N_2, we used the following 62: AC/AC, TG/TC, TC/TG, CA/GA, GT/GT, AG/TG, TC/TC, CT/AG, GA/AG, CT/TG, AC/TC, AC/TG, CA/CT, CA/TG, AC/CT, GA/TG, AC/AG, GT/GA, GT/TC, CT/TC, GA/TC, TC/GA, CT/GA, GA/GA, TC/CT, CT/CT, GA/CT, CA/AG, GT/AG, TC/AG, GT/TG, CA/AC, GT/AC, TC/AC, CT/AC, GA/AC, AC/CA, CA/CA, GT/CA, TC/CA, AT/GT, AG/TT, TG/CG, AT/CG, AG/CG, AT/TT, CG/CG, GC/CG, CC/CG, GG/CG, CC/CC, CG/CC, GC/CC, GG/CC, GC/GC, GG/GC, CC/GG, CC/GG, GC/GG, GG/GG, AT/GC, CC/GC. In each case, the amplified DNA samples were fractionated by electrophoresis in a denaturing polyacrylamide gel. Bands of interest were eluted, reamplified with the same selective primers and sequenced. Fractionation, reamplification and sequencing were performed by PathoFinder B.V. (Maastricht, The Netherlands).

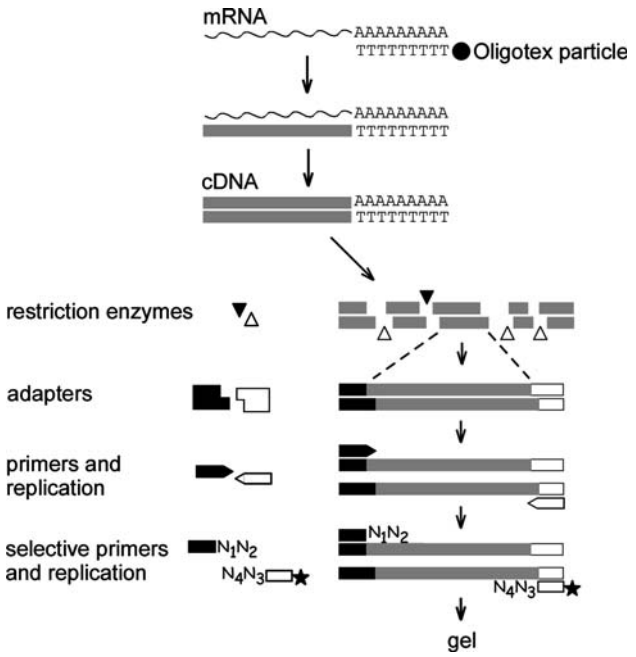

Fig. 1 The cDNA-AFLP method. cDNAs obtained from whole mRNAs were digested with two restriction enzymes, ligated to oligonucleotide adapters complementary to the resulting cohesive ends, and used as templates for the polymerase chain reaction first with primers complementary to the adapters and then with selective primers that amplify only the cDNAs that contain predetermined dinucleotides adjacent to the original restriction sites. One of the selective primers was marked with a fluorescent dye (*star*) to allow the fragments to be seen following fractionation in polyacrylamide gels

Real-time PCR

The amounts of mRNAs of known sequences were estimated by real-time PCR following reverse transcription of total RNA (Winer et al. 1999). Cultures were inoculated with 10^5 spores per Petri dish. Total RNA was isolated with the PerfectPure RNA Culture Cell Kit (5 Prime GmbH, Hamburg, Germany) and aliquots (200 ng RNA in 4 µl) were incubated in a Real Time PCR System (Model 7500, Applied Biosystems, Branchburg, NJ, USA) with the reagents for reverse transcription (TaqMan, same provider) and PCR amplification (SYBR Green PCR Master Mix, same provider), following the instructions of the manufacturers. Primers (CGTTTTGGATTCCGGTGATG and GCGTGAGGAAGAGCGTAACC for gene *actA*; TATT GGCGGAACTGCTACTGC and CCCTGATCAAAGCG ATGACC for gene *carB*; TGAATTGAAAGAGTGCTGG AATACC and TGTCATGAACATTGCATTTGCTT for sequence EV825828; TCATCCTACAGGCCAAATCAGA and CGTTCCAACAGCTGAATAAGCA for sequence EV825740; and GTGATAAGGTAACAGAAAGAGAA GTGACA and GAAGTCTCAATAAAGTCACAAGCC AAT for sequence EV825795) were designed with help from the Primer Express Software (same provider). Each result was normalized with the result for the *actA* gene in the same RNA sample following the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Comparison of sequences

To check if two sequences were similar to each other, we aligned them with the application bl2seq from the NCBI webpage (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) and the omission criteria: reward 1 point per match; penalties, −2 per mismatch, −5 per open gap, and −2 per extension gap. We also compared our *Blakeslea* cDNA sequences with the genomes of *Rhizopus oryzae* (http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/Home.html), *Mucor circinelloides* (http://mucorgen.um.es/) and *Phycomyces blakesleeanus* (http://www.es.embnet.org/~genus/phycogenes.html), the conserved protein domain database (http://130.14.29.110/Structure/cdd/cdd.shtml), and the nonredundant database (http://130.14.29.110/BLAST/), both from the NCBI web-page.

Results

cDNA fragment profiles

We have analyzed the relative abundance of cDNA restriction fragments from four cultures grown on minimal agar for 1, 1.5 and 2 days. The four cultures were those of the single (−) strain, the single (+) strain, and two mated cultures of the same strains, one with 10 mmol/l acetate and the other without. Under such conditions (Kuzina and Cerdá-Olmedo 2006), the carotene content in mated cultures increases with age and reaches its maximum, ~8 mg total carotene per g dry mass, or up to 40 times the average of the single cultures, in 1.5–2 days. In the presence of acetate, the maximum carotene content is ~1 mg/g dry mass.

Each cDNA-AFLP experiment (Fig. 1) amplifies a subset of the cDNA restriction fragments, those that contain predetermined dinucleotides at their ends, and provides a set of cDNA fragment profiles (Fig. 2). We obtained 62 out of the 256 possible sets. We observed 163 bands that were consistently more marked in mated than in single cultures in the three time samples examined. A large majority (72%) of these bands were much less marked when acetate was present in the mated cultures.

Seventeen bands were consistently more marked in single cultures than in mated cultures, and presumably contain fragments of genes that are repressed by mating. In eleven of them the effect was independent of whether the mated cultures had acetate or not; in six, the difference was observed only in the presence of acetate; and in one, only in the absence of acetate.

cDNA sequences and genes

We obtained 180 DNA sequences from 89 bands. They have been deposited under accession numbers EV825701 through EV825880. Their quality was variable, some containing many unknown nucleotides. From 58 bands we either had a single sequence or several sequences that were similar. We call these bands homogeneous. The other 31 bands were heterogeneous, having two or more dissimilar sequences. There were a total of 123 different sequences.

The 123 *Blakeslea* sequences were compared with DNA sequences in public databases (Table 1); 70 sequences (about 57%) found no similar ones in any organism. Our sequences are random fragments from expressed genes and need not be the most conserved or the most recognizable fragments of those genes. For the identification of complete genes, we compared the remaining 53 *Blakeslea* sequences with the genomes of *Phycomyces blakesleeanus*, *Mucor circinelloides* and *Rhizopus oryzae*, the only Mucorales whose genomes have been completely sequenced. The closest genome was that of *Rhizopus*. Additionally, each *Blakeslea* sequence was converted to its six putative polypeptide sequences and these were compared to the protein database. Eight *Blakeslea* sequences were similar to fragments of hypothetical genes of unknown function according to the current annotations in the databases. The remaining 45 sequences identified 41 complete genes; two genes were hit twice and one thrice by sequences that were similar to

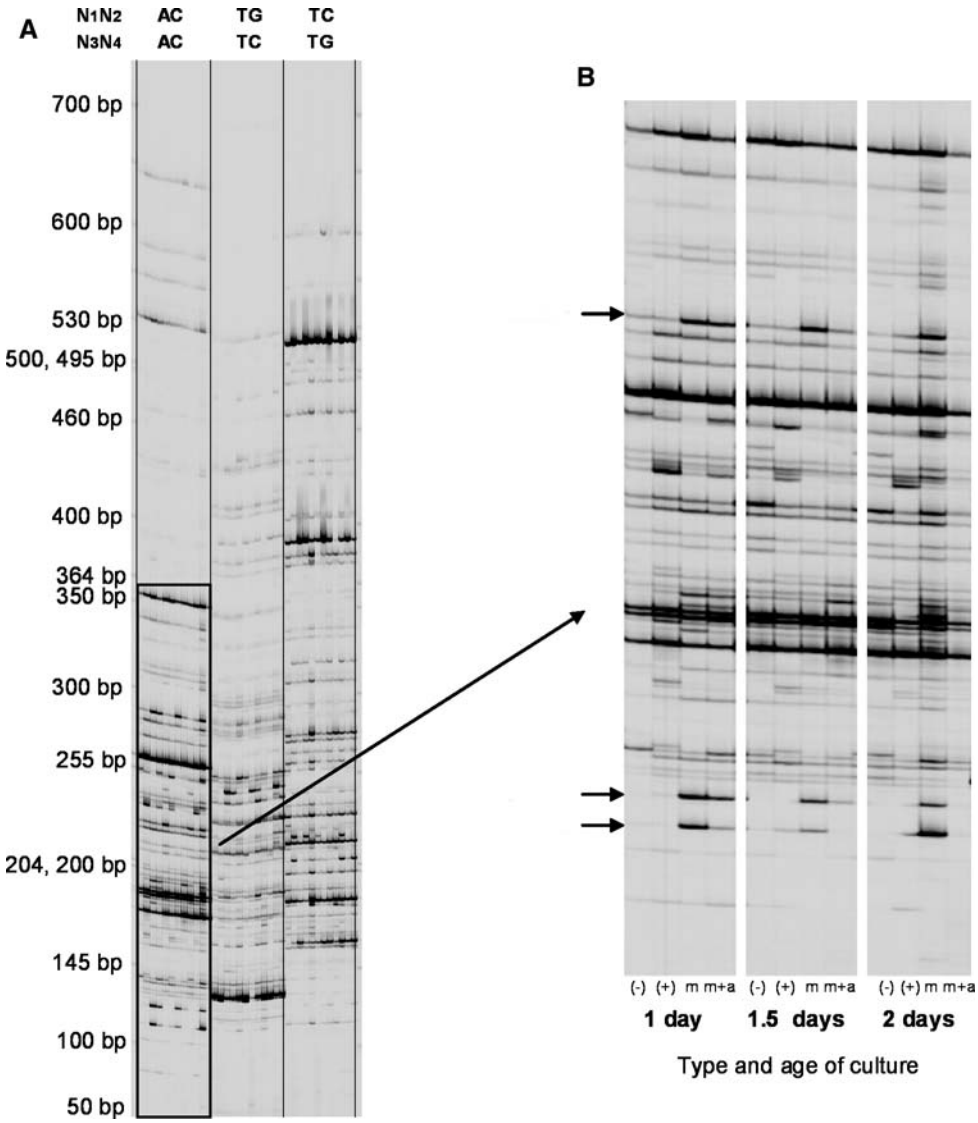

Fig. 2 **a** Fluorescent images of polyacrylamide gels that contain the results of three experiments with the dinucleotide combinations of selective primers indicated at the *top*. **b** Part of **a** indicating the source of the RNA in each lane and several bands of interest. Single cultures are indicated by (−) and (+); mated cultures, by m, and mated cultures with acetate, by m+a. The band marked by an *arrow* down in the Figure contained a fragment of gene *carB*; the *upper* marked band contained a fragment sequence similar to genes for tRNA synthetase

different parts of the gene. Information on all the sequences is presented in Table 2 (Supplementary material).

mRNA quantification by RT-PCR

Reverse transcription and real-time PCR confirmed that transcription of four genes, a sugar transporter, a RAS protein, a serine/threonine protein kinase, and a phytoene dehydrogenase, was enhanced by mating (Fig. 3). In these experiments, the plating density had to be increased and this change may have some influence on the results. Two days after spore germination, cultures are in the process of differentiation (Corrochano and Cerdá-Olmedo 1991) and were not used. The transcription of gene *carB* (for phytoene dehydrogenase) was enhaced by mating and this enhancement was suppressed by acetate. The sexual overexpression of the three other genes was not suppressed by acetate.

Discussion

Efficiency of the method

We processed about one fourth of all the cDNA restriction fragments since we used 62 selective primers combinations out of the 256 possible ones that would contain all of them. The cDNA restriction fragments in our experiments should represent the most of the expressed genes, because each gene, depending on its length and sequence, should give rise to several different restriction fragments.

The efficiency of the method can be calculated as follows. To be replicated by PCR the cDNA must contain at least once in each strand one of the sequences $T = TCGAN_3N_4$ (the TaqI site with its additional selective dinucleotide) or $M = TTAAN_1N_2$ (the MseI site with its additional selective dinucleotide), so that primers will be bound to both strands. Let us call "forward sequences"

Table 1 Comparisons of *Blakeslea* cDNA sequences with sequences of other organisms available in databases

GenBank access number	Fragment length (bp)	Query sequence	Database	Protein/domain identification number	Protein/domain	Expectation value	Score (bits)	
Induced by mating with and without acetate								
EV825707*	221	F	R	RO3G_07144.1	*O*-mannosyl transferase	−16	81	Cell wall synthesis
EV825805*	233	F	R	RO3G_06770.1	Gamma-butyrobetaine hydroxylase	−4	40	Energy metabolism
EV825809	522	F	R	RO3G_07611.1	Glycosyl hydrolase and carbohydrate binding domain	−38	139	
EV825798	484	G	nr	CA847691	MkpA (tyrosine/serine/threonine phosphatase activity)	−17	90	Regulatory proteins
EV825709*	269	G	cdd	32878	Membrane protein involved in colicin uptake	−7	51	
Induced by mating, but not in the presence of acetate								
EV825715	91	F	nr	AY176663.1	Phytoene dehydrogenase (CarB protein)	−4	42	Carotene synthesis
EV825782	281	F	nr	AY176663.1	Phytoene dehydrogenase (CarB protein)	−133	451	
EV825822	261	F	nr	AY176663.1	Lycopene cyclase/phytoene synthase (CarRA protein)	−67	255	
EV825753*	285	F	R	RO3G_04292.1	HMG-CoA reductase	−8	39	
EV825819	116	F	nr	AJ496299.1	Farnesyl pyrophosphate synthase	−9	66	
EV825816	512	G	nr	NP_346206.1	Cell wall surface anchor protein	−44	178	Cell wall synthesis
EV825711	446	F	R	RO3G_17086.1	ATP synthase subunit α	0.0	253	Energy metabolism
EV825785*	419	F	R	RO3G_13066.1	ATP-binding region family	−12	65	
EV825775	289	F	R	RO3G_11939.1	Fructose-bisphosphate aldolase	−46	177	
EV825811	179	F	R	RO3G_05293.1	Sugar (and other) transporter	−10	60	
EV825828*	375	F	R	RO3G_08024.1	Sugar (and other) transporter	−47	184	
EV825876	245	F	R	RO3G_08419.1	Acyl-CoA dehydrogenase	−14	73	
EV825752*	626	F	R	RO3G_04977.1	Acetyl-CoA carboxylase	−36	147	
EV825750*	271	F	R	RO3G_09011.1	Phosphoinositide 3-kinase (PIK) domain and Phosphatidylinositol 3- and 4-kinase	−18	85	
EV825761*	294	F	R	RO3G_12365.1	Pyruvate decarboxylase	−16	78	
EV825704	292	F	R	RO3G_07889.1	WD domain, G-beta repeatet protein family	−10	45	Regulatory proteins
EV825740	238	F	R	RO3G_05706.1	Serine/threonine protein kinase	−12	60	
EV825744	516	F	R	RO3G_06455.1	tRNA synthetases class I	−8	38	
EV825793	251	F	R	RO3G_13853.1	Reverse transcriptase and integrase core domain	−21	97	
EV825825	141	F	R	RO3G_08106.1	Reverse transcriptase and integrase core domain	−4	42	
EV825801*	425	F	R	RO3G_12462.1	Reverse transcriptase and integrase core domain	−25	107	
EV825795	183	F	R	RO3G_05138.1	RAS protein	−19	89	
EV825859	578	F	R	RO3G_05142.1	Diaphanous FH3 and formin homology 2 domains	−31	82	
EV825844	149	G	cdd	30094	Kinesin motor domain, KIF4-like subfamily	−77	286	
EV825880	360	F	R	RO3G_04617.1	Ferric reductase-like transmembrane component	−21	97	
EV825771*	509	F	R	RO3G_14591.1	Acetyltransferase (GNAT)	−7	48	Transfer of acetyl groups

Table 1 continued

GenBank access number	Fragment length (bp)	Query sequence	Database	Protein/domain identification number	Protein/domain	Expectation value	Score (bits)	
Repressed by mating with and without acetate								
EV825803*	256	F	R	RO3G_11939.1	Fructose-bisphosphate aldolase	−13	69	Energy metabolism
EV825802*	242	F	R	RO3G_01139.1	CotH (spore coat assembly) domain	−6	45	Cell wall synthesis
EV825850	423	G	cdd	48664	VHS domain	−39	158	Regulatory proteins
EV825865	567	F	R	RO3G_12879.1	EXTL2, alpha−1,4-*N*-acetylhexosaminyltransferase	−35	146	
Induced by mating in the presence of acetate								
EV825849	151	F	R	RO3G_16531.1	Nuclear export factor GLE1	−9	56	Regulatory proteins
EV825871	436	F	R	RO3G_01753.1	Eukaryotic protein of unknown function DUF914	−32	131	
Repressed by mating in the presence of acetate								
EV825858	91	F	R	RO3G_04997.1	Rare lipoprotein A (RlpA)-like double-psi beta-barrel	−7	52	Membrane structure and function maintenance

The expectation value is the number of alignments that are expected to occur by chance in a database search with similarity scores to the query equal to or better than that of the result sequence. It is indicated as an exponent of 10. The scores were calculated by using the defect criteria (BLOSUM62). "No similarity" indicates expectation values over 10^{-3} in all searches. The sequences obtained from heterogeneous bands are marked with an *asterisk*

Query sequences used: *F* sequence of *Blakeslea* cDNA fragment, *G Rhizopus* whole gene sequence identified as similar to the F fragment sequence of *Blakeslea*. Databases used: *R Rhizopus oryzae* database (Broad Institute, MIT, USA), *cdd* conserved protein domain database from NCBI web-page, *nr* nonredundant database from NCBI web-page

Fig. 3 Effect of sexual activity on the amounts of mRNA of four genes: *carB* (phytoene dehydrogenase, *squares*), sequence EV825828 (putative sugar transporter, *circles*), sequence EV825795 (putative RAS protein, *triangles*), and sequence EV825740 (putative serine/threonine protein kinase, *diamonds*). Mean results of four Real-Time PCR following reverse transcription of RNA from two independent sets of cultures grown for 1 day (*open symbols*) or 1,5 days (*closed symbols*). All measurements were calibrated with those of gene *actA* (for actine). The values for mated cultures and for mated cultures with acetate were divided over the values for single cultures of the (–) strain in the same experiment

those present in one of the strands and "reverse sequences" those found in the other. It is also necessary that at least a pair of forward and reverse sequences converge (i.e., the forward sequence must be "upstream" of the reverse sequence). Finally, at least one of the sequences present in either strand must be a T sequence because otherwise the product would not be fluorescently labeled and would not be detected.

Let us consider, for example, gene *carB*, whose cDNA sequence contains 1,746 nucleotides (Rodríguez-Sáiz et al. 2004) with a GC content of 40%. This is also the average GC content in the exons of the *Blakeslea* genes available in public databases. In this example both selective dinucleotides contain one G/C and one A/T. The average frequency of sequence T in each strand of a DNA fragment of the size and composition of the *carB* cDNA would be 0.376 (the product of the length and the probability of each nucleotide). The number of T sequences in each strand would follow a Poisson distribution of this mean. In the same way, the frequency of M sequence would be 0.846.

The probability that at least one fragment of a gene like *carB* will be amplified with a combination of selective dinucleotides is $P = 23\%$. With 62 similar combinations of selective dinucleotides the probability of absence would be $(1 - P)^{62}$, about $10^{-5}\%$.

The real escape frequency would depend on the technical quality of the experiments: complete retrotranscription, complete DNA digestions, and precise and efficient PCR

amplifications. If everything is correct, at least one fragment of gene *carB* would be expected to be found in about 13% of the experiments in our set. We found it in three experiments, not far from our expectation, particularly if we consider that sequences were obtained from only 89 out of 163 bands that interested us.

Following the same estimation, the total number of detectable genes would be about 5 times the number of bands detected in each experiment. This means that in practice our method screened only about 550 genes of *Blakeslea*, presumably those with abundant transcripts. This is a small proportion of the more than 10,000 genes likely to be present in the *Blakeslea* genome. Future efforts should be devoted to detect a larger fraction of the transcriptome, for example, by increasing the visibility of the bands and the sequencing efficiency.

Based on the sequence of *carB* gene of *Blakeslea* (Rodríguez-Sáiz et al. 2004) and the selective primer combinations used in our analysis, we should have found three different fragments of this gene: a fragment of 112 bp amplified with the selective primer combination N_1N_2 = AC and N_3N_4 = AC, a fragment of 260 bp with N_1N_2 = AT and N_3N_4 = GT, and a fragment of 37 bp with N_1N_2 = CA and N_3N_4 = CA. We found the first two fragments at the expected position in the gel (sequences EV825715/EV825716 and EV825782/EV825783, respectively). This showed that the cDNA-AFLP method was working efficiently. We did not detect the third fragment presumably because of its short length and its expected location, inadequate for efficient identification and elution of bands.

Identification of detected genes

The conjectures on gene function (Table 1) based on the similarity of our sequences to genes from other organisms suggest that mating causes many metabolic changes. The enhanced carotene production during mating was of particular interest. Fewer *Blakeslea* genes were repressed by mating than induced by it. This is indicated by the relatively few bands that were consistently inhibited in mated cultures in comparison with single cultures and suggests that few processes occur in single cultures that do not occur in mated cultures. Those are probably related to the production of vegetative fruiting bodies (sporangiophores), which are formed only in single cultures and are repressed by mating, while zygospores are formed only in mated cultures. Most of the genes used by the related fungus *Phycomyces* for vegetative structures are used for sexual structures as well as indicated by the finding that all 13 mutants isolated for their inability to produce sporangiophores did not produce zygospores either (Gutiérrez-Corona and Cerdá-Olmedo 1988).

Some of the bands were seen in single cultures of one sex, but not in those of the other. These differences may not reflect a sexual difference, but the broad diversity of the natural strains of *Blakeslea*.

Our candidate genes (Table 1; Table 2 in Supplementary material) must be confirmed by further experiments. The majority component in each band, whose abundance is influenced by mating, is mixed with additional nonfluorescent molecules, which may be relatively frequent (MseI-MseI restriction fragments), and with fluorescent minority fragments. It is thus possible that a sequence corresponds to one of the contaminant molecules. This is probably rare for the sequences from the 58 homogeneous bands and more frequent for the 31 heterogeneous bands from which we obtained 67 different sequences.

Reverse transcription and real-time PCR have confirmed that mating enhances the transcription of the four different sequences that were tested (Fig. 3). Sequence comparisons with genes from other organisms suggest functions for three of them: a sugar transporter, a RAS protein, and a serine/threonine protein kinase. Sugar transporters are essential for mating (Kasper et al. 2008; Niederberger et al. 1998). Ras protein regulates two distinct pathways in yeasts: one that controls mating through a Byr2-mitogen-activated protein kinase cascade and one that signals through Scd1-Cdc42 to maintain elongated cell morphology (Onken et al. 2006). Activation of protein kinases transcription occurs subsequent to the activation of the mating pathway in yeasts (Buehrer and Errede 1997). This variety of genes with different functions involved in mating agrees with an old hint that sexual activity causes broad metabolic changes, the observation that *Phycomyces* increases its energy metabolism during mating, consuming up to 50% more oxygen (Burnett 1953). This is now confirmed by our finding that the mRNAs of many *Blakeslea* genes are more abundant during mating than in single cultures. The effect on most of these genes is abolished by acetate, added in small amounts that cannot match the acetyl-CoA made from the abundant glucose in the medium. Acetate is thus likely to act as a signal with broad effects on the metabolism of mated cultures, and not just carotene production. Our results make those changes accessible to research by pointing at concrete genes.

The sexual regulation of the gene responsible for phytoene dehydrogenase was already known. In mated cultures the transcripts of *carB* and *carRA*, the structural genes for carotenogenesis, are more abundant than in single cultures (Almeida 2005; Schmidt et al. 2005; Almeida and Cerdá-Olmedo 2008) and this increase is inhibited by acetate. The amounts of these transcripts correlate with the carotene contents in mated and single cultures and provide a sufficient explanation for them (Kuzina and Cerdá-Olmedo 2006). Strong activations in *Phycomyces* gene function do

not always involve changes in gene transcription. For example, the increased carotene contents in overproducing mutants and in wild types exposed to retinol or dimethyl phtalate occur without changes in the transcripts of the *car* structural genes (Almeida and Cerdá-Olmedo 2008).

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