Fungal Genetics and Biology 47 (2010) 773–781

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Protein–DNA interactions in the promoter region of the *Phycomyces carB* and *carRA* genes correlate with the kinetics of their mRNA accumulation in response to light

Catalina Sanz [a,b], Ernesto P. Benito [a,b], Margarita Orejas [c], María Isabel Álvarez [a], Arturo P. Eslava [a,b,*]

[a] Área de Genética, Departamento de Microbiología y Genética, Universidad de Salamanca, Edificio Departamental, Plaza de los Doctores de la Reina s/n, E-37007, Salamanca, Spain
[b] Centro Hispano-Luso de Investigaciones Agrarias, Universidad de Salamanca, Río Duero 12, Campus de Villamayor, E-37185, Salamanca, Spain
[c] Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Apartado de correos 73, E-46100, Burjassot (Valencia), Spain

ARTICLE INFO

Article history:
Received 17 April 2010
Accepted 19 May 2010
Available online 23 May 2010

Keywords:
Phycomyces blakesleeanus
Carotenoids
Photoinduction
Electrophoretic mobility-shift assay
Phototropic mutants
Carotenoid mutants
Transcriptional regulation

ABSTRACT

Carotene biosynthesis in *Phycomyces* is photoinducible and carried out by phytoene dehydrogenase (encoded by *carB*) and a bifunctional enzyme possessing lycopene cyclase and phytoene synthase activities (*carRA*). A light pulse followed by periods of darkness produced similar biphasic responses in the expression of the *carB* and *carRA* genes, indicating their coordinated regulation. Specific binding complexes were formed between the *carB–carRA* intergenic region and protein extracts from wild type mycelia grown in the dark or 8 min after irradiation. These two conditions correspond to the points at which the expression of both genes is minimal, suggesting that these binding complexes are involved in the down-regulation of photocarotenogenesis in *Phycomyces*. Protein extracts from carotene mutants failed to form the dark retardation complex, suggesting a role of these genes in the regulation of photocarotenogenesis. In contrast, protein extracts from phototropic mutants formed dark retardation complexes identical to that of the wild type.

1. Introduction

In Mucorales species the carotenoid pathway to β-carotene proceeds via three enzymatic steps carried out by the enzymes phytoene synthase, phytoene dehydrogenase and lycopene cyclase. The coding gene for the enzyme phytoene dehydrogenase (*carB*) has been isolated from *Phycomyces blakesleeanus, Mucor circinelloides*, and *Blakeslea trispora* (Ruiz-Hidalgo et al., 1997; Velayos et al., 2000a; Rodríguez-Saiz et al., 2004). The gene named *carRP* in *Mucor* and *Blakeslea* (Velayos et al., 2000b; Rodríguez-Saiz et al., 2004) and *carRA* in *Phycomyces* (Arrach et al., 2001) encodes a protein with separate domains for lycopene cyclase and phytoene synthase. *carB* and *carRA* or *carRP* are adjacent and divergently transcribed (Velayos et al., 2000b; Arrach et al., 2001). In other fungi the genetic organization of these genes is similar to that observed in Mucorales (Verdoes et al., 1999; Arrach et al., 2002; Linnemannstons et al., 2002).

In fungi blue-light can affect metabolism, growth, sexual and asexual development, pigment formation, circadian rhythms and tropisms, among other phenomena (Corrochano and Ávalos, 2010). Some aspects of the above topics have been reviewed with special emphasis in *Neurospora crassa*, an eukaryotic model system for studying the majority of the blue-light responses at a molecular level (Cerdá-Olmedo, 2001; Dunlap and Loros, 2006; Purschwitz et al., 2006; Bahn et al., 2007; Corrochano, 2007; Herrera-Estrella and Horwitz, 2007; Chen et al., 2009; Mehra et al., 2009). In Mucorales blue-light stimulates carotenogenesis in *P. blakesleeanus* (Bergman et al., 1973; Bejarano et al., 1991) and *M. circinelloides* (Navarro et al., 1995; Velayos et al., 1997) and this is due in part to an increased transcription of the structural genes *carB* and *carRA*/*carRP* (Ruiz-Hidalgo et al., 1997; Velayos et al., 2000a,b; Blasco et al., 2001; Almeida and Cerdá-Olmedo, 2008). In *P. blakesleeanus* some of the components responsible for photocarotenogenesis also participate in other light-mediated responses such as phototropism and photomorphogenesis (Bergman et al., 1973; Corrochano and Cerdá-Olmedo, 1992; Cerdá-Olmedo, 2001). All the *Phycomyces* photoresponses require the action of the *madA* and *madB* genes which are homologs to the *N. crassa* light-responsive white collar 1 and white collar 2, respectively (Idnurm et al., 2006; Sanz et al., 2009).

Very little is known about the photoregulation of carotene biosynthesis in Mucorales, with the exception of *M. circinelloides* and *P. blakesleeanus* (Ruiz-Hidalgo et al., 1997; Velayos et al., 2000a,b; Blasco et al., 2001; Almeida and Cerdá-Olmedo, 2008). In *Mucor* there is no well established regulation by the end product

* Corresponding author. Address: Plaza de los Doctores de la Reina s/n, Edificio Departamental, lab. 301, Campus Miguel de Unamuno, Universidad de Salamanca, E-37007 Salamanca, Spain.
E-mail address: eslava@usal.es (A.P. Eslava).

1087-1845/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2010.05.009

β-carotene, but the *crgA* gene product acts as a negative regulator of light-induced carotene biosynthesis (Navarro et al., 2000, 2001; Lorca-Pascual et al., 2004; Murcia-Flores et al., 2007). In *Phycomyces* the phytoene and lycopene contents of *carB* and *carR* mutants is about 40–50-fold greater than that of the β-carotene content of the wild type and this is attributed to a negative regulation of the pathway by its end product (Eslava and Cerdá-Olmedo, 1974; Cerdá-Olmedo, 1987). Moreover, the carotene content is increased about 100 times in mutants of genes *carS*, *carD*, and *carF* (Murillo and Cerdá-Olmedo, 1976; Salgado et al., 1989; Mehta et al., 1997). These genes are thought to mediate the regulation by the end product β-carotene. A general scheme for carotene biosynthesis and its regulation has been proposed very recently (Almeida and Cerdá-Olmedo, 2008).

In *N. crassa*, the two structural carotenogenic genes *al-1* and *al-2* homologs to *carB* and *carRA*/*carRP*, as well as the GGPP synthase encoding gene (*al-3*), are photoinducible (Nelson et al., 1989; Schmidhauser et al., 1990, 1994). A consensus sequence located in the *al-3* gene promoter region is thought to be involved in the regulation of transcription by light. Variations of this sequence (GAAANNTTGCC), named the *al-3* proximal element (APE), (Carattoli et al., 1994) are also present in the promoter region of the genes *carRP* and *carB* of *M. circinelloides* (Velayos et al., 2000b), suggesting a functional role in Zygomycetes. More recently, new light response elements (LRE) required for the light induction of these genes have been described in different photoinducible genes of *N. crassa* (*frq*, *al-3*, and *vvd*), novel *cis*-acting early light response elements (ELREs) and late light response elements (LLREs) has also been described allowing a more global understanding of the regulatory cascade governing the response of the organism to light (Chen et al., 2009). The LRE contain two GATN repeats with a variable distance between them. A consensus sequence of the LRE appears to be GATNC–CGATN, in which N can be any nucleotide, but the same nucleotide is used in both repeats (He and Liu, 2005). LRE type consensus sequences have also been found in several light regulated genes of *Hypocrea jecorina* (Schuster et al., 2007).

For *Phycomyces* there is a large amount of information accumulated about several aspects of its biology, including carotenogenesis, effective methods to isolate mutants, the use of heterokaryons for complementation tests (Ootaki, 1973; Gauger et al., 1980; Suárez et al., 1985; Sanz et al., 2002) and the use of sexual genetic analyses (Álvarez and Eslava, 1983; Orejas et al., 1985, 1987). The disadvantage of this model system is the failure to obtain stable transformants with exogenous DNA. The related fungus *M. circinelloides* can be transformed (Vanheeswijck and Roncero, 1984). However, the lack of both giant macrophores and the sexual cycle limits its use.

In this paper we compare the expression patterns of the *carB* and *carRA* genes after a blue light pulse. They show similar transcript accumulation kinetics, suggesting that transcription of both genes is coordinately regulated by light. By using protein extracts from the wild type, specific retardation complexes formed with DNA motifs within the intergenic region were detected and a good correlation between the formation of the complexes and the kinetics of the *carB* and *carRA* transcripts accumulation was observed. These studies provide the first framework for a more detailed analysis of the DNA–binding proteins and of the trans-activating mechanisms used by light to achieve a coordinated regulation of *carB* and *carRA* gene transcription in *Phycomyces*.

2. Materials and methods

2.1. Culture and light conditions

The strains of *P. blakesleeanus* used in the present work are described in Table 1.

Table 1
Strains of *P. blakesleeanus* used in this work.

Strain	Genotype[a]	Origin[b]
NRRL1555	*(−)*	Wild type
A56	*(+)*	Wild type isogenic strain
C2	*carA5(−)*	NRRL1555 (NTG)
C5	*carB10, geo-10(−)*	NRRL1555 (NTG)
C115	*carS42, mad-107(−)*	NRRL1555 (NTG)
C47	*madA35(−)*	NRRL1555 (NTG)
B24	*madC469(−)*	NRRL1555 (ICR-170)
C111	*madB103(−)*	NRRL1555 (NTG)
A202	*madC469(−)*	A56 × B24
C110	*madE102(−)*	NRRL1555 (NTG)

[a] Mutations labelled *mad* affect the phototropism of the sporangiophore; *car*, carotenoids biosynthesis and *geo*, gravitropism. The mating type is indicated by the symbol *(−)* or *(+)*.

[b] The standard wild type NRRL1555 was obtained originally from the Northern Regional Research Laboratory, USDA, Peoria, IL, USA. Strains with prefix A come from Prof. Arturo P. Eslava (Universidad de Salamanca, Spain); B, from strains isolated at the Max Planck Institute for Molecular Genetics (Berlin); C, from the late Prof. M. Delbrück (California Institute of Technology, Pasadena, CA, USA); S, from Prof. Cerdá-Olmedo (Universidad de Sevilla, Spain). Mutants were isolated by N-methyl-N′-nitro-N-nitrosoguanidine mutagenesis (NTG) or by 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl) aminopropylamino] acridine-2HCl (ICR-170) mutagenesis. Strain A202 was obtained after genetic cross with the isogenic *(+)* strain A56 (Álvarez and Eslava, 1983).

For the growth of *P. blakesleeanus* minimal medium (SIV) was used (Sutter, 1975). For photoinduction studies, plates were inoculated with 10^4 spores and mycelia were grown for 3 days in darkness before being irradiated with broad-band blue light at a fluence of 40 J/m^2 (4 W/m^2 for 10 s). Illumination conditions for expression and DNA–protein interaction studies have been described previously (Velayos et al., 2000a). After irradiation, the mycelia were kept in darkness for different time periods (varying between 2 and 60 min). The mycelia were then collected and frozen in liquid nitrogen and kept frozen until used. Non-irradiated mycelia were also obtained and used for controls. The manipulation of the mycelia was performed under low intensity red light supplied by a red fluorescent lamp (120 cm; TL 40 W/15, Philips) covered with a red plastic filter (Lucita, Rhöm GmbH).

2.2. Standard DNA techniques

The isolation of genomic DNA from *P. blakesleeanus* was performed as previously described (Moller et al., 1992). The PCR amplifications of genomic DNA were carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). To amplify the intergenic region between the genes *carRA* and *carB* the oligonucleotides PRMT1 and PRMT2 were designed. To obtain fragments F7, F8 and F6, oligonucleotide pairs F7U–F7R, F8U–F8R and F7U–F8R were used, respectively (see Table 2 for a description of all the oligonucleotides used in this work). PCR reactions were performed using AmpliTaq Polymerase (Applied Biosystems, Foster City, CA, USA) and genomic DNA. The amplified DNA fragments were purified from agarose gels using the GeneClean Kit (Bio101, Cleveland, Ohio, USA). Other DNA manipulations were carried out using standard methods (Sambrook et al., 1989).

2.3. Preparation of radiolabelled DNA fragments and total protein extracts

DNA fragments were end-labelled with [^{32}P]dCTP using the Klenow fragment of the DNA polymerase (Roche, Indianapolis, IN, USA). To eliminate the unincorporated [^{32}P]dCTP, S300 HR columns (Amersham Pharmacia Biotech, Uppsala, Sweden) were used.

Table 2
Oligonucleotides used in this work.

Primer[a]	Sequences (5′–3′)[b]	Location[c]	Position[d]
F8U	CAAGAAAGACAGACAGACAG	Promoter	−334 to −354
F8R	GTATCTGAGCGGCGCTCATT	Promoter	−613 to −594
F7U	GTTAAAGGATGGCTGTTGTT	Promoter	−241 to −260
F7R	ACTGAACTAGAAGTGATATG	Promoter	−530 to −511
PRMT1	ATAAGTC**GA**CATCTTTGG	Promoter	+13 to −06
PRMT2	GTTTAGGAGGA**T**CCATATT	Promoter	−1378 to −1397
PSP13	TTTTGAGCTCTTAAATGACAGTAAAGGC	*carRA*	+2056 to +2086
PSP1	TACTACTCTGCCTGCGT	*carRA*	+1513 to +1529
cDNA3′	GTCGCCTTCTTAAGAAGTCAAGCTGACGTT	*carB*	+5399 to +5429
PE	AGTTCCACCGGCACCAG	*carB*	+3490 to +3506
EBAct1	TGTTGGAGATGAAGCGC	*actA*	+604 to +620
EBAct2	CATAGTGGTTCCACCAG	*actA*	+1415 to +1398
Q-*carRA*-F	CTGCAACGTCTACACTGCCATT	*carRA*	+1668 to +1690
Q-*carRA*-R	GGTGAGCACGATCGGGATA	*carRA*	+1732 to +1713
Q-*carRA*-T	TGTCTCTCTCCACAAGGGTAACGG	*carRA*	+1688 to +1712
Q-*carB*-F	CCAGGTGTGCGACCACTTT	*carB*	+2867 to +2888
Q-*carB*-R	TGCGCTTGGATGAGGTGAT	*carB*	+2929 to +2910
Q-*carB*-T	CGTCAAGGTTCGTCCCTCTGC	*carB*	+2888 to +2909
Q-*pyrG*-F	CTTCATGAAGGAGTGCGGTACTG	*pyrG*	+585 to +606
Q-*pyrG*-R	TCATGACACCTGGTGTTGGTTT	*pyrG*	+652 to +630
Q-*pyrG*-T	TGCCCAAGACCATCACCCTTG	*pyrG*	+608 to +629

[a] Oligonucleotide name.
[b] When a nucleotide appears in bold it indicates that this position has been changed with respect to the original to introduce a restriction site: in PRMT2 a *Bam*HI (GGAGCC to GGA**T**CC) site was introduced, in PRMT1 a *Sal*I (GTCAGC to GTC**GA**C) site was introduced.
[c] Location in the *carRA–carB* promoter, *carRA* (AJ276965), *carB* (X78434), *actA* (AJ000335) or *pyrG* (X53601) genes.
[d] Position of each nucleotide taking as a reference the start of the gene *carRA* (first nucleotide: +1) except for *actA* and *pyrG* oligonucleotides whose position is referred to their start codon.

About 5 mg of protein extracts were obtained from wet mycelia grown for 3 days on solid minimal medium (supplemented as needed) as described (Pérez-Esteban et al., 1993). After precipitation residual ammonium sulphate was eliminated using PD-10 columns (Amersham Pharmacia Biotech, Uppsala, Sweden). The quantity of protein was calculated (Bradford, 1976) using lysozyme as standard and the aliquots were maintained at −80 °C until their use.

2.4. Electrophoretic mobility-shift assays (EMSAs)

The binding assays were performed with the labelled DNA fragments in the presence of a 3000 times excess (in mass) of the non-competitive inhibitor poly(dI–dC) (Amersham Pharmacia Biotech, Uppsala, Sweden) and different quantities of protein extracts. The reactions were carried out, essentially, as previously described (Pérez-Esteban et al., 1993; Orejas et al., 1995) using A50 buffer. Competitor fragments were included as indicated. After the incubation the samples were loaded in vertical gels of 4.1% acrylamide (acrylamide:bis-acrylamide 29:1) in 0.5× TBE. The gels were run at 4 °C and 200 V, vacuum dried for 2 h at 60 °C (in a BIO-Rad Model 483 Salb dryer) and exposed to auto radiographic films (Kodak X-OMAT AR o BioMax MR) for a variable time depending on the signal emitted.

2.5. RNA extraction and northern analyses

In all the cases 3-day-old mycelia kept at −80 °C were used for RNA extraction. Total RNA was isolated as previously described (Velayos et al., 2000a). The RNA concentration was determined spectrophotometrically. RNA samples were electrophoresed in MOPS/formaldehyde buffer and transferred to Hybond N+ membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Labelling, hybridization and immunological detections were carried out using the non-radioactive digoxygenin (DIG) labelling and hybridization system (Roche, Indianapolis, IN, USA) following the supplier's recommendations. For PCR labelling of the *P. blakesleeanus carB*, *carRA* and of the *Botrytis cinerea actA* probes, oligonucleotide pairs cDNA3′-PE, PSP13–PSP1 and EBAct1–EBAct2 were used, respectively (Table 2). After autoradiography, the films were analyzed with an Image Acquisition Console and the Whole Band Analyzer software (Bio Image; MilliGen/Biosearch, Ann Arbor, Mich., USA).

2.6. Real-time PCR analyses

Total RNAs were treated with RNase-free DNase (Ambion, Applied Biosystems, California, USA) to avoid DNA contamination. These samples were employed to perform RT-PCRs with the "Superscript™ First-Strand Synthesis System for RT-PCR" (Invitrogen, Life Technologies Corporation, California, USA) following the manufacturer's recommendations. RT-PCR was performed in an ABI-PRISM 7000 thermocycler (Applied Biosystems, California, USA), with the following cycling conditions: a first step at 50 °C for 2 min to allow for uracil-N-gycosylase cleavage, a second step at 95 °C for 4 min to activate the AmpliTaq Gold polymerase, and 40 amplification cycles consisting of 15 s at 95 °C and 1 min at 60 °C. Primers and internal fluorescent probes for the TaqMan system (Applied Biosystems, California, USA) were designed with the aid of Primer Express software 1.0 (Applied Biosystems). The primers used were: Q-*carB*-F and Q-*carB*-R, which specifically amplify a 62 bp fragment from the *carB* gene coding region; Q-*carRA*-F and Q-*carRA*-R, which amplify a 68 bp fragment from the *carRA* gene; and Q-*pyrG*-F and Q-*pyrG*-R, which amplify a 72 bp fragment from the *pyrG* coding region. The *carB* (Q-*carB*-T) and *carRA* (Q-*carRA*-T) Taqman fluorescent probes were labelled with the fluorescent reporter VIC whereas the *pyrG* Taqman fluorescent probe (Q-*pyrG*-T) were labelled with the fluorescent reporter FAM. To generate the standard curves, genomic DNA was serially diluted by a factor of 10 and aliquots of the dilutions were used in standard RT-PCRs. Each value determination was repeated three times. The concentration of unknown samples was calculated with the ABI-Prism 7000 SDS software, which creates threshold cycle values (Ct) and extrapolates relative levels of PCR product from the standard

curve. The expression levels of the *carB* and *carRA* genes were determined for both conditions, dark (grown in complete darkness for 3 days) and light (grown in complete darkness for 3 days and then irradiated with blue light at 40 J/m^2 and kept in darkness for 2 min before collection) in the wild type and in different *car* mutants. For each of the strains and the light conditions (dark or light) tested, *carB* and *carRA* expression data were normalized against the expression of the endogenous control gene (*pyrG*) according to the quotient (expression of *carB* or *carRA*/expression of *pyrG*). In addition, for each strain the light/dark ratio was calculated according to the quotient [(expression of *carB* or *carRA* in light)/(expression of *pyrG* in light)]/[(expression of *carB* or *carRA* in dark)/(expression of *pyrG* in dark)]. Three RT-PCRs were performed with each cDNA sample to reduce reaction variability.

3. Results and discussion

3.1. Light-induced similar biphasic kinetics of carB and carRA mRNA accumulation

Previous studies have shown that the fluence response curve of the *P. blakesleeanus carB* mRNA induction has a biphasic character after irradiating mycelia either with continuous white light or with a blue light pulse followed by darkness (Velayos et al., 2000a). To investigate whether this mechanism controlling blue light induction of the *carB* gene expression also controls the expression of its linked and divergently transcribed gene *carRA*, or if there are differences in the regulation of the expression of these genes, we have extended these studies to the *carRA* gene. Northern blot analyses were used to compare the kinetics for the accumulation of the *carB* and *carRA* mRNAs over time in response to a light stimulus (pulse). Wild type mycelia from plates incubated for 3 days in darkness were irradiated with blue light at a fluence of 40 J/m^2 (4 W/m^2 for 10 s) and then maintained in darkness for periods of 2, 4, 6, 8, 10, 20, 40, and 60 min before being frozen in liquid nitrogen. Total RNA from each sample was extracted and the accumulated levels of the *carB* and *carRA* gene transcripts were quantified after hybridization. The expression data obtained were normalized against the expression of the constitutively expressed gene *act1* quantified from the hybridization signals obtained using a DNA fragment derived form the *B. cinerea actA* gene as a probe.

Low levels of expression of the *carB* and *carRA* genes could be detected in mycelia cultured in darkness (Fig. 1B and C), but a blue light pulse induced the expression of both genes giving rise to a biphasic induction response (Fig. 1A–B). After irradiation, the amount of both transcripts increased very rapidly, reaching a first maximum (a ~5-fold increase for the *carB* gene and a 7-fold increase for the *carRA* gene) within 2 min. The expression of both genes decreased afterwards, but a second transient increase of the *carB* and *carRA* mRNAs (approximately 6-fold and 9-fold increase, respectively) was observed shortly after, with a peak of expression at about 20 min after irradiation. The mRNA levels then decreased to near basal levels after 1 h of incubation in darkness. Both, the expression patterns over time in response to the light stimulus and the levels of expression of the two genes at each time point considered are very similar, suggesting a coordinated regulation of the expression of both genes by light. Bearing in mind the structure of the two genes, probably sharing regulatory elements in the common intergenic region, the similarities of the photocontrol of *carB* and *carRA* are not surprising. A similar transient expression of light responding genes has been also observed in *M. circinelloides carB* and *carRP* (Velayos et al., 2000a,b).

3.2. The carRA–carB intergenic region

The *carRA* and the *carB* genes are divergently orientated and their respective initiation codons are separated by 1379 nt (Arrach et al., 2001). Elements controlling their expression, in particular sequences involved in light responses, are expected to be located within this intergenic region and, given the similarities in the expression patterns of the two genes, it might be expected that both genes share common regulatory elements. In order to identify putative cis-acting elements that could be important for blue light regulation, the sequence of the *carRA–carB* intergenic region was

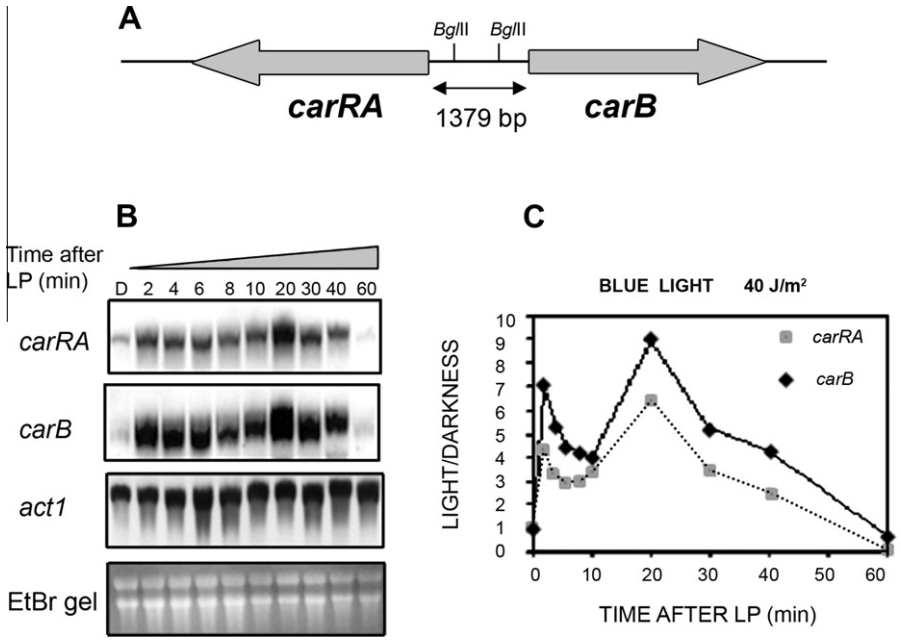

Fig. 1. Northern blot analysis of the expression the *P. blakesleeanus carRA*, *carB* and *act1* genes in response to light. (A) Organization of the Phycomyces *carRA–carB* region. (B) Filters with RNA samples obtained from mycelia irradiated with a blue light fluence of 40 J/m^2 and collected at the times indicated in each line after the light pulse, were hybridised with probes derived from the *P. blakesleeanus carRA* and *carB* genes and from the *B. cinerea actA* gene. Ethidium bromide staining was used check RNA quality and to evaluate RNA loading. (C) Accumulation levels of the *carRA* and *carB* transcripts from irradiated mycelia at the indicated time points referred to their accumulation in the darkness control, D, after normalization using the signals of the *Phycomyces* constitutively expressed *act1* gene. LP: Light Pulse.

analyzed searching for motifs (such as GATA, LRE, APE), which have been shown to play a role in blue-light responses in the promoter of light regulated fungal genes (Fig. 2A).

The GATA-box is known to be a target for the GATA type zinc finger transcription factors (Scazzocchio, 2000). The APE motif (consensus 5′-GAANNTTGCC-3′) has been shown to mediate the transcriptional light response of the *alb-3* gene (Carattoli et al., 1994). In the promoters of the *N. crassa frq*, *al-3*, and *vvd* genes a consensus sequence was identified for light responsive elements (LRE) (He and Liu, 2005). Analysis of the *carRA–carB* intergenic region made it possible to identify eight copies of the consensus GATA-box that could be bound by the *P. blakesleeanus* WC-1 and WC-2 homologs MADA and MADB, five of them are located in the *carRA* initiation region, one occupying a central position in the intergenic region and two in the *carB* proximal region (Fig. 2A). No perfect match of the *N. crassa* APE motif was found. In *M. circinelloides* the consensus sequence for the APE-like elements have been suggested to be $GRAN_{(1-6)}TTGYY$ (Velayos et al., 2000a) and up to four copies of this consensus sequence could be identified in the *Phycomyces carRA–carB* intergenic region, one of them close to the *carRA* gene initiation region and three close to the *carB* gene initiation region (Fig. 2A). Finally, a putative LRE sequence was identified between positions −200 and −191 from the *carB* gene translation initiation codon. Interestingly, a perfect match of the light response region (sequence ATGAARA) has been identified in the light regulated gene encoding the β-subunit of the chloroplast glyceraldehyde 3-phosphate dehydrogenase from *Arabidopsis thaliana* (Jeong and Shih, 2003). Two additional motifs, EUM1 (5′-CTGTGC-3′) and EUM2 (5′-ACCTTGAC-3′), which could be involved in the light response in fungi, have been proposed on the basis of their strong conservation in the promoter regions of the *N. crassa vvd* gene encoding the light desensitization protein VIVID and of its *H. jecorina* ortholog *env1* (Schmoll et al., 2005). No EUM1 or EUM2 matching sequences were identified in the *Phycomyces carRA–carB* region.

3.3. Protein–DNA interactions in the promoter region of carB and carRA genes

In order to determine whether protein–DNA interactions between any of the DNA elements indicated above, or other elements, and a regulatory protein, or a regulatory protein complex present in *Phycomyces* mycelia occur in response to light we performed a series of gel mobility-shift assay (EMSAs) experiments. A first set of experiments was designed, on the one hand to determine the growth and physiological conditions under which possible regulatory proteins accumulate in the mycelia and, on the other hand, to delimit the region of the promoter where the sequences to which the regulatory protein or proteins bind are located.

Protein extracts from mycelia grown in the same conditions as those used for the Northern experiments were isolated at time points during which minimal expression (mycelia incubated in permanent darkness, mycelia collected 8 min after irradiation and mycelia collected 60 min after irradiation) and maximal expression (mycelia incubated 2 min and 20 min after irradiation) of the *carRA* and *carB* genes were detected. Several labelled overlapping DNA fragments (F1–F5) derived from the *carRA–carB* intergenic region were then prepared (Fig. 2A). In all the experiments for each DNA fragment and illumination condition, two different amounts of protein, 3 μg and 5 μg, were used. As a control a third reaction mixture with no protein was included.

Protein extracts of *P. blakesleeanus* formed gel retardation complexes with fragments F2 and F4 (Fig. 2B). No complexes were formed with fragments F1, F3 and F5 (Fig. 2B), indicating that the putative regulatory protein binding motifs identified by *in silico* analysis within these three fragments are not being recognised by proteins accumulating under the physiological conditions tested. It is in the overlapping region of fragments F2 and F4 where *in vitro* functional DNA motifs are recognised by binding proteins. This overlapping DNA fragment is 315 nt long and occupies a central position within the *carRA–carB* intergenic region, between positions −1111 and −796 relative to the *carB* ATG codon. The retardation complexes are formed only in the samples containing protein extracts from mycelia grown in the dark or collected 8 min after being irradiated (Fig. 2B). Comparison of the patterns of the *carRA* and *carB* transcripts accumulation (Fig. 1B) and the pattern of retardation complexes formation (Fig. 2B) shows that there is a good correlation between the lowest levels of transcript accumulation and the ability of the protein/s to bind the DNA fragments *in vitro*. Very interestingly, the protein extracts obtained from mycelia grown in the dark formed a high mobility gel retardation complex (HMC), whereas proteins from mycelia collected 8 min after the light pulse formed a low mobility complex (LMC).

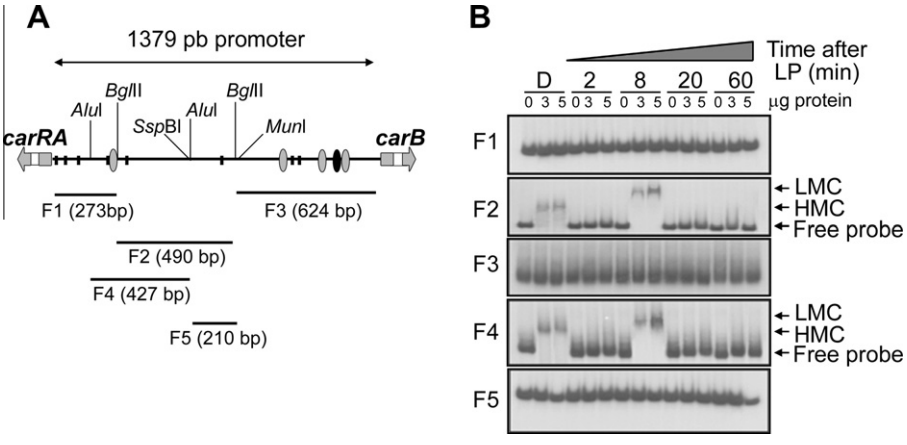

Fig. 2. (A) Schematic representation of the 1379 bp *carRA–carB* intergenic region of *P. blakesleeanus*. Positions of DNA sequence motifs resembling motifs which have been shown to play a role in blue-light responses in the promoters of light regulated genes in fungi and plants are presented: GATA motifs are represented by a thin vertical black box, APE-like elements by a light grey oval, the unique LRE element by a black oval. Below the scheme, the length and positions covered by the different DNA fragments used in the EMSAs experiments are shown. (B) Gel retardation assays for the characterization of DNA–protein interactions in the *carRA–carB* intergenic region. Total protein extracts from *P. blakesleeanus* wild type mycelia collected after being grown in darkness (D), or after being irradiated with blue light and maintained in darkness for 2, 8, 20 and 60 min, and different [^{32}P]-labelled DNA fragments (F1–F5) of the *carRA–carB* promoter were added to the reaction mixture. Reaction conditions are described in Section 2. Protein extract was omitted in the reaction mixtures resolved in lanes with 0 μg. LMC (from low mobility complex) indicates the complexes formed with protein extracts obtained from mycelia keep 8 min in dark after the blue light irradiation. HMC (from high mobility complex) indicates the complexes formed with protein extracts obtained from mycelia grown in permanent darkness.

These results suggest that *P. blakesleeanus* possesses trans-acting transcription factors that interact with DNA sequences in the promoter regions of the *carRA* and *carB* genes during both dark and light conditions and that the corresponding complexes are formed transiently.

The formation of the HMC complex in the dark, its quick disappearance after the light pulse (Fig. 2B) and the concomitant fast increase of *carB* and *carRA* transcripts are conspicuous (Fig. 1). Taken together these results strongly suggest that in the absence of light a negatively acting protein/s are involved in the establishment of the HMC complex to preclude transcription of both carotenogenic genes. After the light pulse, the repressor would be inactivated probably due to a post-translational modification in such a way that binding to its target is no longer allowed and the transcription of the genes would become de-repressed and/or induced. The decrease of expression after 2 min seems to be due to a specific mechanism of very fast mRNA degradation "turnover" (Blasco et al., 2001). We then focused on the analysis of the HMC complex and experiments are in progress on the characterization of the LMC complex.

To further delimit the target sequence of the DNA-binding activity needed to form the HMC complex, the overlapping region between fragments F2 and F4 was amplified by PCR and fragment F6 (315 bp) was isolated (see Fig. 3A). As expected, the formation of the HMC retardation complex was detected when using fragment F6 and protein extracts from mycelia grown in the dark (Fig. 3B). Surprisingly, no binding was observed with either fragment F7 (215 bp) or F8 (218 bp). These two overlapping fragments are derived from F6 and each one lacks ~100 bp from either the *carRA* proximal extreme (fragment F8) or from the *carB* proximal extreme (fragment F7) of fragment F6 (Fig. 3A). When the two fragments, F7 and F8, were added to the same reaction mixture the HMC complex was not formed, either (Fig. 3B). In order to further confirm that the binding between the fragment F6 and the protein/s that form the HMC complex was specific, competition experiments using the unlabelled F6 fragment were carried out (Fig. 3C). A 10-fold molar excess of the unlabelled F6 DNA fragment partially competed with labelled fragment F6. When the concentration of the specific competitor fragment increased to 20-fold or 30-fold the formation of the retardation complex was almost totally precluded.

These results map the DNA binding motifs within fragment F6 and suggest that for the binding to occur two separated motifs need to be linearly arranged to efficiently interact with a protein or a group of proteins to form the HMC complex. This F6 fragment has a GC content of 32.2%, very similar to the GC content of the whole intergenic region (34.3%). Occupying a central position within this fragment a 33 nt long poly(dA–dT) segment can be identified between positions 128 and 160 relative to the *Bgl*II site at its left border (Fig. 3A). The presence of two motifs similar in sequence and of high AT content at each end of the F6 fragment can be highlighted: the first one with the sequence 5′-TATCTCTTTTTTGTA-3′ and starting at position +37 from the *carRA* proximal extreme of F6, which contains a putative GATA motif, and the second one with the sequence 5′-ATCCTCCTTTTTGCA-3′ ending at position +72 upstream of the *carB* proximal extreme of F6. This structural organization has been described (Helmreich, 2001).

3.4. Structural and regulatory carotenogenic gene products are essential for the formation of the HMC complex

In *P. blakesleeanus* carotenogenesis is thought to be regulated by the end product, β-carotene (Eslava and Cerdá-Olmedo, 1974; Cerdá-Olmedo, 1987). In addition, several carotenoid regulatory genes have been described (Navarro et al., 2000, 2001; Lorca-Pascual et al., 2004; Schmoll et al., 2005; Dunlap and Loros, 2006; Idnurm et al., 2006; Murcia-Flores et al., 2007; Sanz et al., 2009). We therefore decided to investigate whether the gene products of *carB* and *carRA* and the gene product of the regulatory gene *carS* participate in the formation of the HMC complex. To this end, a new set of EMSA experiments was performed using protein extracts obtained from the wild type (NRRL1555) and from strains C2 (*carA*), C5 (*carB*) and C115 (*carS*). For protein purification, mycelia were grown in continuous darkness for 72 h. Protein extracts were then prepared and used in gel retardation assays with the radiolabeled fragment F6. The HMC retardation complex was only formed in the wild type, while in all the carotenoid mutant strains it was not (Fig. 4A), indicating that the proteins encoded by the structural *carRA* and *carB* genes and by the regulatory *carS* gene

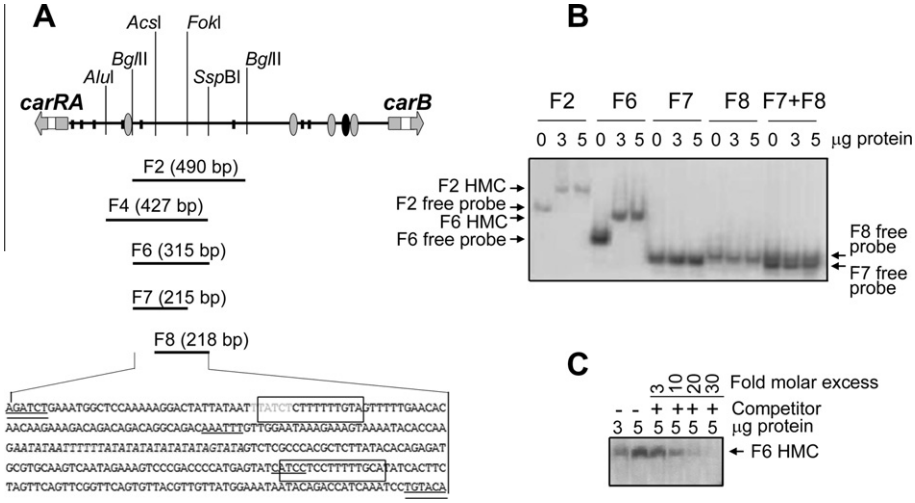

Fig. 3. Gel retardation assays for the characterization of DNA–protein interactions in the *carRA–carB* promoter. (A) Scheme of the *carRA–carB* intergenic region and locations of fragments F2, F4, F6, F7 and F8 within this region. The nucleotide sequence of fragment F6 is shown. The *Bgl*II site at its left border and the *Ssp*B1 site at its right border are double underlined. The internal *Acs*I and *Ssp*B1 sites are underlined. The only GATA-box identified within this region is presented in grey. The two sequences inside a black box indicate the two motifs similar in sequence and of high AT content each one near one end of the F6 fragment. The central poly(dA–dT) sequence is written in italics. (B) Gel retardation assay with total protein extract from wild type mycelia grown in darkness and labelled DNA fragments F2, F6, F7, F8 and F7 + F8. Arrows indicate the high mobility complexes (HMC) and free probes. (C). Competition experiment. The reaction mixtures resolved in the last four lines contained increasing amounts of the unlabelled F6 DNA fragment as specific competitor.

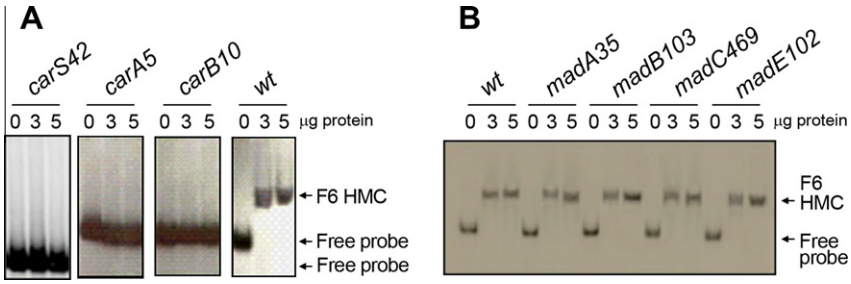

Fig. 4. Gel retardation assays for the characterization of DNA–protein interactions in the *carRA–carB* genes promoterintergenic region in *car* and *mad* mutants. (A) Total protein extracts from *P. blakesleeanus* wild type (wt) and from strains C2 (*carA5*), C5 (*carB10*) and C115 (*carS42*) grown in darkness were added to the reaction mixture together with labelled DNA fragment F6. (B) Total protein extracts from *P. blakesleeanus* wild type (wt) and from strains C47 (*madA35*), C111 (*madB103*), A202 (*madC469*) and C110 (*madE102*) grown in darkness were added to the reaction mixture together with labelled DNA fragment F6. In both panels, in the upper part of each lane the amount of protein extracts employed is shown. Arrows indicate High Mobility retardation Complexes (HMC) and free probes.

are needed directly or indirectly for the formation of the putative dark repressor complex.

3.5. Blind mutants (genotype mad) retained the ability to form the HMC complex

In order to know if MAD proteins, which mediate almost all known light responses in *Phycomyces* including photocarotenogenesis (Idnurm et al., 2006; Sanz et al., 2009), play a role in the formation of the HMC complex, protein extracts from cultures of blind *P. blakesleeanus* mutant strains grown in darkness affected in different genes (*madA*, *madB*, *madC*, and *madE*) (see Table 1) were used to assay protein binding to the F6 labelled DNA fragment. No difference in the formation of the HMC complex could be detected when comparing the *mad* mutant strains and the wild type (Fig. 4B). These results suggest that the MADA, MADB, MADC and MADE proteins are not involved in the formation of the darkness HMC complex. However, the possibility that these MAD proteins could be involved in the activation of transcription by light through an additional regulation mechanism not detected with our experimental conditions cannot be excluded.

3.6. Structural and regulatory carotenogenic mutants are impaired in the carRA and carB genes regulation by light

As the products encoded by *carRA*, *carB* and *carS* genes seem to be essential for the formation of the putative regulatory HMC complex in the dark, the light response capacity of the strains altered in these genes was determined by analyzing the transcription level of the *carRA* and *carB* genes in both light and dark conditions. The same strains tested for the formation of the HMC complex in dark conditions were used. All of them were incubated in the dark during 72 h. Then, mycelia were collected from plates and frozen. Additional plates from each strain were irradiated with a light pulse (4 W m^{-2} for 10 s) and returned to dark conditions. Mycelia were collected 2 min later. For each strain the expression levels of the *carRA* (Fig. 5A) and *carB* (Fig. 5B) genes in both conditions were estimated by Quantitative PCR relative to the expression of the *pyrG*, a constitutively expressed gene (Díaz-Mínguez et al., 1990). For each gene in each strain the light/darkness ratio was then established (Fig. 5C), which provides an indication of its ability to be light-induced. As shown in Fig. 5C, the light/darkness ratio in the wild type for the expression or the *carRA* and *carB* genes is about 4.5–5.0, the expression of these genes being about five times stronger after the light pulse than in darkness. In all carotenogenic mutant strains analyzed the light/darkness ratio was significantly lower, indicating that in all of them the ability of the *carRA* and *carB* genes to be light-induced is markedly reduced. A consideration of the expression data for each gene indicates that this reduction is determined by different means. In strains C2 and C5 there seems to be an increase in the darkness expression of both genes in comparison to the wild type. This increase could be due to the loss in these two strains of the *carRA* and *carB* genes darkness repression characteristic of the wild type as a consequence of the incapacity to form the HMC complex observed in both mutant strains. However the two mutants behave differently in response to the light pulse since in the C2 strain the *carRA* and *carB* genes transcriptions are still induced by light, while in the C5 strain this ability is lost. In the C115 strain (*carS*) the situation observed for the expression of *carRA* and *carB* genes differs from that observed

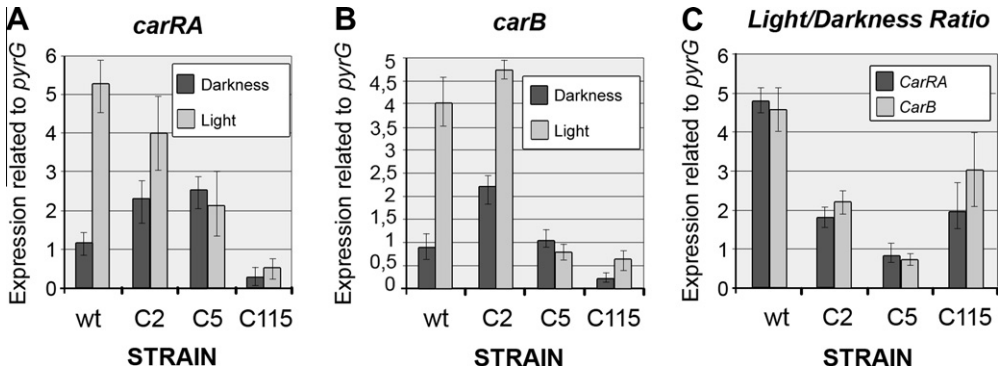

Fig. 5. Real Time PCR Analysis of the *carRA* and *carB* gene expression in the wild type and in different *car* mutants under blue light (40 J/m^2) or dark growth conditions. The values are means and standard errors for three independent experiments. (A) *carRA* expression analysis related to the *pyrG* expression in strains wt (*NRRL1555*), C2 (*carA5*), C5 (*carB10*), and C115 (*carS42*). (B) *carB* expression analysis related to the *pyrG* expression in strains wt (*NRRL1555*), C2 (*carA5*), C5 (*carB10*) and C115 (*carS42*). (C) Light/Darkness ratio of the *carRA* and *carB* genes expression related to the *pyrG* gene expression in strains wild type (*NRRL1555*), C2 (*carA5*), C5 (*carB10*) and C115 (*carS42*).

in the mutants altered in the carotenogenic structural genes. The *carRA* and *carB* expression levels are much lower than those observed in the wild type both in the dark and after the light pulse, but they became a slightly higher upon light exposure, indicating that in this strain some level of activation by light is retained.

We can conclude that, compared to the wild type, strains C2, C5, and C115 show significant alterations of the *carRA* and *carB* expression levels under the analyzed illumination conditions. These results imply that the genes altered in these strains seem to be necessary to maintain the capacity of *carRA* and *carB* genes to be regulated by light. This observation is consistent with the results obtained in the EMSA assays where it was also found that the products encoded by *carRA*, *carB* and *carS* genes are essential for the formation of the putative regulatory HMC complex in the dark. The fact that the complex HMC is formed in the absence of light, when *carRA* and *carB* gene expression is minimal indicates a possible repressor effect of the HMC complex in the dark. This is in agreement with the results obtained in the RT-PCR experiment in which it appears that the mutations present in strains C2 and C5 determine an increase in the *carRA* and *carB* expression levels in the dark, so that the proteins encoded by the genes altered in these strains would be necessary to maintain repression in darkness possibly due to its role in the formation of the HMC complex.

The strains tested (C2 and C115) maintain some capacity for the photoinduction of *carRA* and *carB*, pointing to the existence of additional regulation mechanism/s. These mechanisms could possibly act through the activation of carotenogenesis in light conditions. It may be assumed that in *P. blakesleeanus* a similar situation to that observed in its relative *M. circinelloides* might exist. In this system it has been found that in the *crgA* mutant (altered in one way of regulation that exerts a repression of the carotenoids synthesis in dark conditions) light is still able to activate the *carB* gene transcription (Navarro et al., 2001), meaning that regulation of carotenogenesis in these fungi is a complex process that may involve several regulatory mechanisms. In the *carRA–carB* promoter region several putative regulatory sequences have been detected for which we have not observed binding of transcriptional regulatory complexes in the conditions analyzed in this work. It cannot be discarded that some of those elements, or elements specific to our system, which do not match previously described consensus elements, could play a role in some of these additional regulatory mechanisms which could operate under particular physiological conditions. Further analysis under different irradiation conditions or with different experimental approaches could provide new data on these additional mechanisms for regulating the expression of *carRA* and *carB* genes by blue light.

Acknowledgments

This work was supported by Grants GR64 (Junta de Castilla y León, Spain) and AGL2005-02542 (Spanish Ministerio de Educación y Ciencia/FEDER).We thank Raúl Martín-Domínguez and Luís Miguel Hernández-Sánchez for useful discussions and suggestions and Fernando Díez-Martín for excellent technical assistance.

References

Almeida, E.R., Cerdá-Olmedo, E., 2008. Gene expression in the regulation of carotene biosynthesis in *Phycomyces*. Curr. Genet. 53, 129–137.

Álvarez, M.I., Eslava, A.P., 1983. Isogenic Strains of *Phycomyces blakesleeanus* suitable for genetic analysis. Genetics 105, 873–879.

Arrach, N., Fernández-Martín, R., Cerdá-Olmedo, E., Ávalos, J., 2001. A single gene for lycopene cyclase, phytoene synthase, and regulation of carotene biosynthesis in *Phycomyces*. Proc. Natl. Acad. Sci. USA 98, 1687–1692.

Arrach, N., Schmidhauser, T.J., Ávalos, J., 2002. Mutants of the carotene cyclase domain of *al-2* from *Neurospora crassa*. Mol. Genet. Genomics. 266, 914–921.

Bahn, Y.S., Xue, C.Y., Idnurm, A., Rutherford, J.C., Heitman, J., Cárdenas, M.E., 2007. Sensing the environment: lessons from fungi. Nat. Rev. Microbiol. 5, 57–69.

Bejarano, E.R., Ávalos, J., Lipson, E.D., Cerdá-Olmedo, E., 1991. Photoinduced accumulation of carotene in *Phycomyces*. Planta 183, 1–9.

Bergman, K., Eslava, A.P., Cerdá-Olmedo, E., 1973. Mutants of *Phycomyces* with abnormal phototropism. Mol. Gen. Genet. 123, 1–16.

Blasco, J.L., Roessink, D., Iturriaga, E.A., Eslava, A.P., Galland, P., 2001. Photocarotenogenesis in *Phycomyces*: expression of the *carB* gene encoding phytoene dehydrogenase. J. Plant Res. 114, 25–31.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Carattoli, A., Cogoni, C., Morelli, G., Macino, G., 1994. Molecular characterization of upstream regulatory sequences controlling the photoinduced expression of the *albino-3* Gene of *Neurospora-Crassa*. Mol. Microbiol. 13, 787–795.

Cerdá-Olmedo, E., 1987. Carotene. In: Cerdá-Olmedo, E., Lipson, E.D. (Eds.), Phycomyces. Cold Spring Harbor, New York, pp. 199–222.

Cerdá-Olmedo, E., 2001. *Phycomyces* and the biology of light and color. FEMS Microbiol. Rev. 25, 503–512.

Chen, C.H., Ringelberg, C.S., Gross, R.H., Dunlap, J.C., Loros, J.J., 2009. Genome-wide analysis of light-inducible responses reveals hierarchical light signalling in *Neurospora*. EMBO J. 28, 1029–1042.

Corrochano, L.M., 2007. Fungal photoreceptors: sensory molecules for fungal development and behavior. Photochem. Photobiol. Sci. 6, 725–736.

Corrochano, L.M., Ávalos, J., 2010. Light sensing. In: Borkovich, K.A., Ebbole, D.J. (Eds.), Cellular and Molecular Biology of Filamentous Fungi. ASM Press, Washington, DC, pp. 415–441.

Corrochano, L.M., Cerdá-Olmedo, E., 1992. Sex, light and carotenes: the development of *Phycomyces*. Trends Genet. 8, 268–274.

Díaz-Mínguez, J.M., Iturriaga, E.A., Benito, E.P., Corrochano, L.M., Eslava, A.P., 1990. Isolation and molecular analysis of the orotidine-5′-phosphate decarboxylase gene (*pyrG*) of *Phycomyces blakesleeanus*. Mol. Gen. Genet. 224, 269–278.

Dunlap, J.C., Loros, J.J., 2006. How fungi keep time: circadian system in *Neurospora* and other fungi. Curr. Opin. Microbiol. 9, 579–587.

Eslava, A.P., Cerdá-Olmedo, E., 1974. Genetic control of phytoene dehydrogenation in *Phycomyces*. Plant Sci. Lett. 2, 9–14.

Gauger, W., Peláez, M.I., Álvarez, M.I., Eslava, A.P., 1980. Mating type heterokaryons in *Phycomyces blakesleeanus*. Exp. Mycol. 4, 56–64.

He, Q., Liu, Y., 2005. Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation. Genes Dev. 19, 2888–2899.

Helmreich, J.M., 2001. Machinery of gene transcription. In: Inc, O.U.P. (Ed.), The Biochemistry of Cell Signalling. Oxford University Press Inc., New York, pp. 157–171.

Herrera-Estrella, A., Horwitz, B.A., 2007. Looking through the eyes of fungi: molecular genetics of photoreception. Mol. Microbiol. 64, 5–15.

Idnurm, A., Rodríguez-Romero, J., Corrochano, L.M., Sanz, C., Iturriaga, E.A., Eslava, A.P., Heitman, J., 2006. The *Phycomyces madA* gene encodes a blue-light photoreceptor for phototropism and other light responses. Proc. Natl. Acad. Sci. USA 103, 4546–4551.

Jeong, M.J., Shih, M.C., 2003. Interaction of a GATA factor with *cis-acting* elements involved in light regulation of nuclear genes encoding chloroplast glyceraldehyde-3-phosphate dehydrogenase in *Arabidopsis*. Biochem. Biophys. Res. Commun. 300, 555–562.

Linnemannstons, P., Prado, M.M., Fernandez-Martin, R., Tudzynski, B., Avalos, J., 2002. A carotenoid biosynthesis gene cluster in *Fusarium fujikuroi*: the genes *carB* and *carRA*. Mol. Genet. Genomics 267, 593–602.

Lorca-Pascual, J.M., Murcia-Flores, L., Garre, V., Torres-Martínez, S., Ruiz-Vázquez, R.M., 2004. The RING-finger domain of the fungal repressor *crgA* is essential for accurate light regulation of carotenogenesis. Mol. Microbiol. 52, 1463–1474.

Mehra, A., Baker, C.L., Loros, J.J., Dunlap, J.C., 2009. Post-translational modifications in circadian rhythms. Trends Biochem. Sci. 34, 483–490.

Mehta, B.J., Salgado, L.M., Bejarano, E.R., Cerdá-Olmedo, E., 1997. New mutants of *Phycomyces blakesleeanus* for beta-carotene production. Appl. Environ. Microbiol. 63, 3657–3661.

Moller, E.M., Bahnweg, G., Sandermann, H., Geiger, H.H., 1992. A simple and efficient protocol for isolation of high-molecular-weight DNA from filamentous fungi, fruit bodies, and infected-plant tissues. Nucleic Acids Res. 20, 6115–6116.

Murcia-Flores, L., Lorca-Pascual, J.M., Garre, V., Torres-Martínez, S., Ruiz-Vázquez, R.M., 2007. Non-AUG translation initiation of a fungal RING finger repressor involved in photocarotenogenesis. J. Biol. Chem. 282, 15394–15403.

Murillo, F.J., Cerdá-Olmedo, E., 1976. Regulation of carotene synthesis in *Phycomyces*. Mol. Gen. Genet. 148, 19–24.

Navarro, E., Sandmann, G., Torres-Martínez, S., 1995. Mutants of the carotenoid biosynthetic-pathway of *Mucor circinelloides*. Exp. Mycol. 19, 186–190.

Navarro, E., Ruiz-Perez, V.L., Torres-Martínez, S., 2000. Overexpression of the *crgA* gene abolishes light requirement for carotenoid biosynthesis in *Mucor circinelloides*. Eur. J. Biochem. 267, 800–807.

Navarro, E., Lorca-Pascual, J.M., Quiles-Rosillo, M.D., Nicolás, F.E., Garre, V., Torres-Martínez, S., Ruiz-Vázquez, R.M., 2001. A negative regulator of light-inducible carotenogenesis in *Mucor circinelloides*. Mol. Genet. Genomics 266, 463–470.

Nelson, M.A., Morelli, G., Carattoli, A., Romano, N., Macino, G., 1989. Molecular-cloning of a *Neurospora crassa* carotenoid biosynthetic gene (*albino-3*) regulated by blue-light and the products of the white-collar genes. Mol. Cell. Biol. 9, 1271–1276.

Ootaki, T., 1973. New method for heterokaryon formation in *Phycomyces*. Mol. Gen. Genet. 121, 49–56.

Orejas, M., Suárez, T., Eslava, A.P., 1985. Helper strains for shortening the dormancy in *Phycomyces blakesleeanus*. Curr. Genet. 9, 369–372.

Orejas, M., Peláez, M.I., Álvarez, M.I., Eslava, A.P., 1987. A genetic map of *Phycomyces blakesleeanus*. Mol. Gen. Genet. 210, 69–76.

Orejas, M., Espeso, E.A., Tilburn, J., Sarkar, S., Arst Jr., H.N., Peñalva, M.A., 1995. Activation of the *Aspergillus PacC* transcription factor in response to alkaline ambient pH requires proteolysis of the carboxy-terminal moiety. Genes Dev. 9, 1622–1632.

Pérez-Esteban, B., Orejas, M., Gómez-Pardo, E., Peñalva, M.A., 1993. Molecular characterization of a fungal secondary metabolism promoter: transcription of the *Aspergillus nidulans isopenicillin N synthetase* gene is modulated by upstream negative elements. Mol. Microbiol. 9, 881–895.

Purschwitz, J., Muller, S., Kastner, C., Fischer, R., 2006. Seeing the rainbow: light sensing in fungi. Curr. Opin. Microbiol. 9, 566–571.

Rodríguez-Saiz, M., Paz, B., De La Fuente, J.L., López-Nieto, M.J., Cabri, W., Barredo, J.L., 2004. *Blakeslea trispora* genes for carotene biosynthesis. Appl. Environ. Microbiol. 70, 5589–5594.

Ruiz-Hidalgo, M.J., Benito, E.P., Sandmann, G., Eslava, A.P., 1997. The *phytoene dehydrogenase* gene of *Phycomyces*: regulation of its expression by blue light and vitamin A. Mol. Gen. Genet. 253, 734–744.

Salgado, L.M., Bejarano, E.R., Cerdá-Olmedo, E., 1989. Carotene-superproducing mutants of *Phycomyces blakesleeanus*. Exp. Mycol. 13, 332–336.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

Sanz, C., Álvarez, M.I., Orejas, M., Velayos, A., Eslava, A.P., Benito, E.P., 2002. Interallelic complementation provides genetic evidence for the multimeric organization of the *Phycomyces blakesleeanus phytoene dehydrogenase*. Eur. J. Biochem. 269, 902–908.

Sanz, C., Rodríguez-Romero, J., Idnurm, A., Christie, J., Heitman, J., Corrochano, L.M., Eslava, A.P., 2009. *Phycomyces* MADB interacts with MADA to form the primary photoreceptor complex for fungal phototropism. Proc. Natl. Acad. Sci. USA 106, 7095–7100.

Scazzocchio, C., 2000. The fungal GATA factors. Curr. Opin. Microbiol. 3, 126–131.

Schmidhauser, T.J., Lauter, F.R., Russo, V.E.A., Yanofsky, C., 1990. Cloning, sequence, and photoregulation of *al-1*, a carotenoid biosynthetic gene of *Neurospora crassa*. Mol. Cell. Biol. 10, 5064–5070.

Schmidhauser, T.J., Lauter, F.R., Schumacher, M., Zhou, W.B., Russo, V.E.A., Yanofsky, C., 1994. Characterization of *al-2*, the *phytoene synthase* gene of *Neurospora crassa*. Cloning, sequence-analysis, and photoregulation. J. Biol. Chem. 269, 12060–12066.

Schmoll, M., Franchi, L., Kubicek, C.P., 2005. Envoy, a PAS/LOV domain protein of *Hypocrea jecorina* (Anamorph *Trichoderma reesei*), modulates *cellulase* gene transcription in response to light. Eukariot. Cell 4, 1998–2007.

Schuster, A., Kubicek, C.P., Friedl, M.A., Druzhinina, I.S., Schmoll, M., 2007. Impact of light on *Hypocrea jecorina* and the multiple cellular roles of ENVOY in this process. BMC Genomics 8, 449.

Suárez, T., Orejas, M., Eslava, A.P., 1985. Isolation, regeneration, and fusion of *Phycomyces blakesleeanus* spheroplasts. Exp. Mycol. 9, 203–211.

Sutter, R.P., 1975. Mutations affecting sexual development in *Phycomyces blakesleeanus*. Proc. Natl. Acad. Sci. USA 72, 127–130.

Vanheeswijck, R., Roncero, M.I.G., 1984. High-frequency transformation of *Mucor* with recombinant plasmid DNA. Carlsberg Res. Comm. 49, 691–702.

Velayos, A., López-Matas, M.A., Ruiz-Hidalgo, M.J., Eslava, A.P., 1997. Complementation analysis of carotenogenic mutants of *Mucor circinelloides*. Fung. Genet. Biol. 22, 19–27.

Velayos, A., Blasco, J.L., Álvarez, M.I., Iturriaga, E.A., Eslava, A.P., 2000a. Blue-light regulation of phytoene dehydrogenase (*carB*) gene expression in *Mucor circinelloides*. Planta 210, 938–946.

Velayos, A., Eslava, A.P., Iturriaga, E.A., 2000b. A bifunctional enzyme with lycopene cyclase and phytoene synthase activities is encoded by the *carRP* gene of *Mucor circinelloides*. Eur. J. Biochem. 267, 5509–5519.

Verdoes, J.C., Krubasik, K.P., Sandmann, G., van Ooyen, A.J., 1999. Isolation and functional characterisation of a novel type of carotenoid biosynthetic gene from *Xanthophyllomyces dendrorhous*. Mol. Gen. Genet. 262, 453–461.