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RESEARCH ARTICLE

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A gene of the opsin family in the carotenoid gene cluster of *Fusarium fujikuroi*

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Abstract Opsins are membrane photoreceptors closely related to the heat-shock proteins of the HSP30 family. Their functions include light-driven ion pumping in archaea and light detection in algae and animals, using the apocarotenoid retinal as a light-absorbing prosthetic group. We describe a gene of *Fusarium fujikuroi*, *carO*, coding for a polypeptide resembling opsins and HSP30-like proteins and contiguous to the genes of the carotenoid pathway, *carRA* and *carB*. Transcription of *carO* is induced by light and is deregulated in carotenoid-overproducing mutants. The same regulation pattern is exhibited by *carRA* and *carB*; and common conserved DNA elements are found in the three promoters. Heat shock resulted in a modest induction of *carO* transcription, similar to the one exhibited by *carB*, confirming a common regulation. Targeted mutagenesis of *carO* produced no apparent phenotypic modification, including no change in the photoinduction of carotenoid biosynthesis.

Keywords *Fusarium* · *Gibberella* · Opsin · Carotenoid · Photoinduction · Fusarin

Introduction

The fungus *Fusarium fujikuroi* (*Gibberella fujikuroi* mating group C) has a complex secondary metabolism, which includes the production of gibberellins (Tudzynski 1999; Avalos et al. 1999), carotenoids (Avalos and Cerdá-Olmedo 1986, 1987), bikaverin (Giordano et al. 1999a) and fusarins (Barrero et al. 1991). Gibberellins are plant hormones (MacMillan 1997) whose growth-promoting properties have found applications in agriculture and the brewing industry. Carotenoids are pigments widespread in nature, synthesized by plants, algae and some bacteria and fungi (Britton et al. 1998; Sandmann and Misawa 2002). Although different in their chemical formulae, carotenoids and gibberellins are terpenoids; and their biosynthetic pathways have in common their initial steps. Other metabolites, such as bikaverin or fusarins, belong to the polyketide family (Barrero et al. 1991; Linnemannstöns et al. 2000b).

The main product of the carotenoid pathway of *F. fujikuroi* is neurosporaxanthin (Avalos and Cerdá-Olmedo 1986, 1987), a xanthophyll with an unusual end-carboxylic group first identified in *Neurospora crassa* (Aasen and Jensen 1965). Neurosporaxanthin is synthesized from geranyl-geranyl pyrophosphate (GGPP) through four enzymatic activities (Fig. 1a). Phytoene synthase produces phytoene from GGPP, phytoene dehydrogenase then introduces five desaturations and carotene cyclase makes a ring at one end. The result of these reactions is torulene, the substrate of a hypothetical oxidase that breaks the molecule, removing five carbon atoms to yield neurosporaxanthin. The first three activities reside in two gene products, coded by the genes *carRA* and *carB* (Linnemannstöns et al. 2002a). A strain accumulating torulene (*carT* mutant), presumably affected in the gene responsible for the conversion of torulene to neurosporaxanthin, has been isolated (Avalos and Cerdá-Olmedo 1987), but the gene *carT* has not been identified. Biosynthesis of neurosporaxanthin is induced by light in wild-type strains (Avalos and Cerdá-Olmedo 1987; Avalos and Schrott 1990) and high amounts of carotenoids are accumulated by deep-pigmented overproducing strains (*carS* mutants) in any growth conditions (Avalos and Cerdá-Olmedo 1987). Carotenoid content correlates with the transcription of

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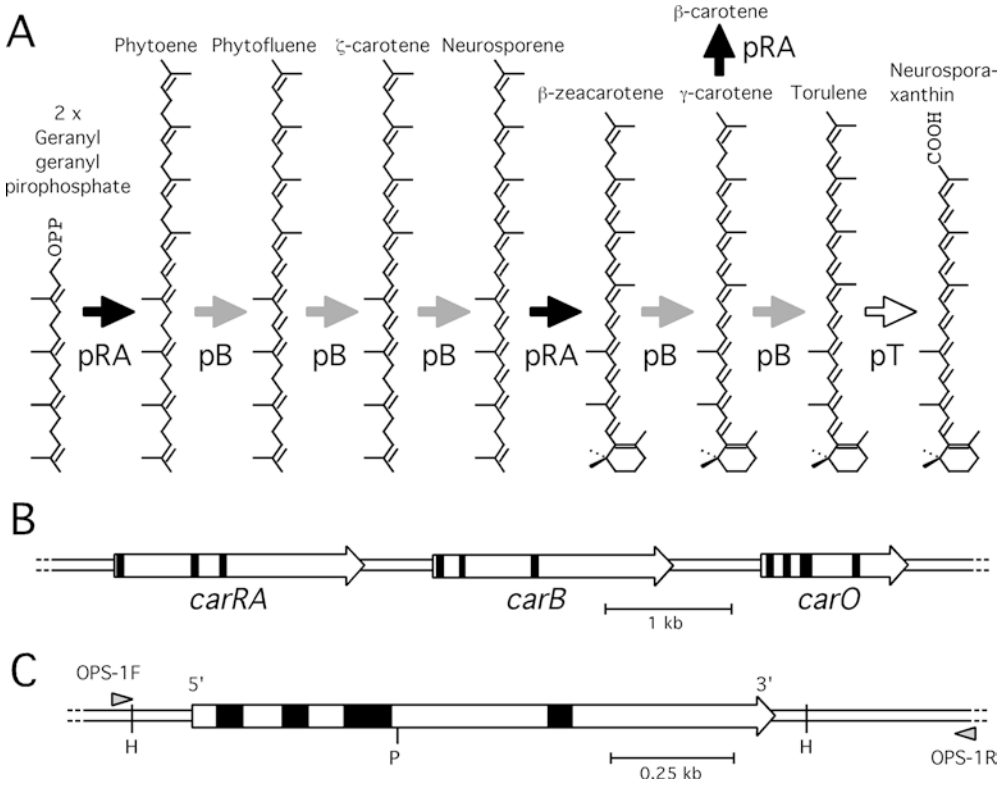

Fig. 1 a–c Biosynthesis of neurosporaxanthin in *F. fujikuroi* and genomic organization of the *car* genes. **a** pRA, pRB and pRT indicate *carRA*, *carB* and *carT* gene products, respectively. *Three different arrows* identify the reactions carried out by each gene product. **b** Map of the DNA region of *F. fujikuroi* showing the relative positions of the genes *carRA*, *carB* and *carO*. The genomic location of *carT* is unknown. **c** Map of the gene *carO* showing the *Pst*I (*P*) and *Hin*dIII (*H*) sites and the positions and directions of the primers ops-1F and ops-1R (*gray arrowheads*). *Black bars* in the genes indicate introns

carRA and *carB*, which is induced by light and by *carS* mutations in the dark (Linnemannstöns et al. 2002a).

Clustering of genes of the same metabolic pathway is frequent in the genomes of filamentous fungi (Keller and Hohn 1997). An outstanding example is the gibberellin cluster of *F. fujikuroi*, formed by seven coregulated genes (Tudzynski and Hölter 1998; Rojas et al. 2001; Tudzynski et al. 2001). The genes *carRA* and *carB* are contiguous in the *F. fujikuroi* genome (Linnemannstöns et al. 2002a), suggesting a possible linkage of the gene *carT*. In an attempt to identify this gene, we found closely linked to *carB* a gene with similarity to opsins, a family of retinal-binding proteins with ion pump or sensory reception functions (Spudich et al. 2000). Opsins have been investigated in halophilic archaea (where they function as light-driven proton pumps; Spudich 1998), in algae (where they play a role as photoreceptors for phototaxis; Ridge 2002) and in animals (where they act mostly as vision light-absorbing pigments known as rhodopsins; Menon et al. 2001). From the structural point of view, opsins are hydrophobic membrane proteins with a similar tertiary organization determined by seven conserved transmembrane domains. The three-dimensional structure has been resolved for several of these proteins, whose transmembrane alpha-helices form an internal pocket in which retinal is bound. Closely related to opsins is a family of fungal heat-shock proteins with presumed chaperone activity (Zhai et al. 2001), represented by HSP30 from *Saccharomyces cerevisiae*. Despite their similar tertiary structure, these putative chaperones lack a conserved lysine present in the seventh transmembrane domain of opsins, to which retinal is covalently linked. Other proteins belonging to this family are Cvhsp30/1 and Cvhsp30/2 from *Coriolus versicolor* (Iimura and Tatsumi 2002).

Recent genomic analyses in filamentous fungi led to the discovery of novel opsin genes, the first ones found outside of archaea, algae and animals. Two of them, *nop-1* from *N. crassa* (Bieszke et al. 1999a) and *ops* from *Leptosphaeria maculans* (Idnurm and Howlett 2001) have been investigated at the molecular level. Both genes were identified from expressed sequence tag libraries and code for very similar proteins (43% identity) with the seven characteristic transmembrane domains. In the case of Nop-1, retinal binding has been demonstrated and photochemical analyses have revealed that the reactions of this protein upon illumination closely resemble those of bacteriorhodopsin and sensory rhodopsins (Bieszke et al. 1999b; Brown et al. 2001). The biological roles of Nop-1 and Ops remain to be elucidated. Transcription of *nop-1* is absent in young submerged mycelia and is strongly induced in conidia, in sexually differentiated mycelia and in mature cultures grown under light (Bieszke et al. 1999a); and *ops* is highly expressed in mature cultures in either light or dark (Idnurm and Howlett 2001). Loss of function of *nop-1* resulted in no detectable phenotypic alteration, except a light-dependent change in colony morphology observed in the presence of several ATPase inhibitors, particularly oligomycin (Bieszke et al. 1999a). No

targeted mutagenesis has been done with ops in *L. maculans*.

Here, we describe the opsin-like gene of *F. fujikuroi*, its transcriptional regulation and the phenotype of strains carrying a targeted mutated allele. Because of its close linkage to the genes *carRA* and *carB* and its parallel regulation, we conclude that this gene, named *carO*, is a constituent of the *F. fujikuroi* carotenoid gene cluster.

Materials and methods

Strains and culture conditions

IMI58289, FKMC1995 and m567 are wild-type strains of *F. fujikuroi* (*G. fujikuroi* mating population C; O'Donnell et al. 1998). SF1 is a nitrate reductase mutant obtained from FKMC1995 by spontaneous resistance to ClO_3^- through growth selection on minimal medium supplemented with 15 g l^{-1} $KClO_3$ and 1.6 g l^{-1} L-asparagine. Nitrate reductase mutation was deduced from the ability of this strain to grow with either nitrite, hypoxanthine or ammonium, but not with nitrate as nitrogen source (Klittich and Leslie 1988). These strains accumulate traces of carotenoids in the dark and moderate amounts in the light (photoinduction). SG1, SG22 and A06 are carotenoid-overproducing mutants. SG1 and SG22 were obtained from IMI58289 by chemical mutagenesis (Avalos and Cerdá-Olmedo 1987) and A06 from m567 by UV radiation.

For Northern blot analyses, two different growth conditions were used. To investigate the effect of light exposure and *carS* regulatory mutations, the fungal strains were grown in 500-ml Erlenmeyer flasks with 250 ml minimal medium for 4 days at 30°C on an orbital shaker in the dark (Avalos et al. 1985), filtered through filter paper (80 mm diam.) and frozen in liquid nitrogen immediately or after illumination of the mycelial pad for different times under 25 W m^{-2} white light at 30°C. To investigate the effect of heat shock on the wild type and light exposure on the revertants R2 and R3, the strains were grown for 3 days in the dark at 30°C as still submerged cultures in 15-mm Petri dishes containing 100 ml minimal medium with 3 g l^{-1} L-asparagine as the nitrogen source. The mycelia were separated from the medium and frozen in liquid nitrogen immediately, after 1 h exposure to 25 W m^{-2} white light at 30°C, or after incubation of the plates for different times partially submerged in a water bath at either 38°C or 42°C. Dark manipulations were done under safe red light.

For DNA isolation, 250-ml Erlenmeyer flasks with 100 ml minimal medium supplemented with 1 g l^{-1} yeast extract were inoculated with conidia suspensions and incubated for 3 days at 30°C. To obtain protoplasts, 4×10^8 conidia of the strain SF1 were incubated for 14 h in 500-ml Erlenmeyer flasks on an orbital shaker with 200 ml potato/dextrose broth (PDB) at 30°C. All Erlenmeyer flasks were shaken at 200 rpm in the dark.

For analysis of conidia, carotenoid and fusarin production, each strain was grown for 7 days at 22°C or 30°C on minimal agar with 3 g l^{-1} L-asparagine as the nitrogen source. Incubations in the light were done under a battery of white fluorescent lamps, with light intensities of 5 W m^{-2} at 22°C and 25 W m^{-2} at 30°C.

DNA sequence and protein analysis

The *carO* gene was identified in the 6.7-kb *Xba*I genomic segment of plasmid pCB (Fernández-Martín et al. 2000), containing the gene *carB* from the wild-type strain IMI58289. The sequence was determined from overlapping DNA segments subcloned in Bluescript KS + (Stratagene, La Jolla, Calif.). DNA sequencing of recombinant plasmid clones was accomplished by Newbiotechnic (Sevilla, Spain) using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, Calif.). The location of introns was determined through sequences obtained by PCR from cDNA samples obtained from the overproducing mutant SG22 with the Time Saver cDNA synthesis kit (Pharmacia, Uppsala, Sweden). The *carO* sequence has been submitted to the GenBank/EMBL data bank with accession number AJ566362.

Alignments were done with the multi-processor Clustal W program (ver. 1.81), using the server at the Centre for Molecular and Biomolecular Informatics (Nijmegen, The Netherlands).

Computer prediction of phosphorylation targets was carried out with the NetPhos program (ver. 2.0; Blom et al. 1999; http://www.cbs.dtu.dk/services/NetPhos).

A search of conserved DNA motifs in the *carRA*, *carB* and *carO* promoters was done with the AlignACE program (Roth et al. 1998; Hughes et al. 2000; http://atlas.med.harvard.edu/cgi-bin/alignace.pl), using default parameters, except that 12 columns were used in addition to the ten default number. Promoter sequences used in the analyses were 800 bp for *carRA* (5′ sequence from accession number AJ426417), 548 bp for *carB* (3′ sequence from AJ426417 fused to 5′ sequence from AJ426418) and 690 bp for *carO* (3′ sequence from AJ426418 fused to 5′ sequence from AJ566362). Their average G+C contents were 0.43, 0.44 and 0.44, respectively; and the analysis was achieved with a 0.44 fractional background GC content. A search of fungal transcription factor binding sites was done with the MatInspector tool (Genomatix Software, Munich, Germany; Quandt et al. 1995).

Construction of the gene replacement vector

Gene disruption was carried out with plasmid pBN7CB-P, containing the *niaD* gene as a selectable marker and a mutated *carO* allele. To make this plasmid, a 4.4-kb *Hin*dIII–*Pst*I segment containing the *niaD* gene, obtained from plasmid pJN1 (Tudzynski et al. 1996) by

partial *Pst*I digestion, was introduced into the *Hin*dIII–*Pst*I sites of plasmid Bluescript KS^+ (Stratagene) to give plasmid pBSJN1. The mutant allele of gene *carO* was made on plasmid pCB (Fernández-Martín et al. 2000) by deleting four bases in a *Pst*I site located in the 5′ region of the gene (Fig. 1c) by digestion and Klenow treatment of the protruding ends. The *Sal*I–*Not*I 6.7-kb segment of the pCB-derived plasmid containing the non-functional allele of *carO* was cloned into the same sites on the pBSJN1 polylinker to give pBN7CB-P. *Escherichia coli* strain DH5α was used for plasmid propagations; and DNA manipulations were done according to Sambrook et al. (1989).

Transformation and gene replacement

Protoplasts were prepared following Powell and Kistler (1990) with some modifications. Germinated conidia obtained after 14 h incubation of fresh conidia at 30°C in PDB were collected by centrifugation at 12,000 *g* for 15 min, washed with 20 ml OM (1.2 M $MgSO_4$, 5 mM Na_2HPO_4) and resuspended in 20 ml OM in which 2 g Glucanex (Novo Nordisk Ferment, Dittingen, Switzerland) were previously dissolved. The mixture was incubated for 1 h with gentle agitation and checked under a microscope for protoplast formation. The protoplasts were separated from germinated conidia by filtering through a Nylon membrane (Monodur, average pore diam. 50 μm), washed twice with STC (0.8 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris, pH 7.5) and resuspended in STC. The final concentration, 4×10^9 protoplasts ml^{-1}, was adjusted to 2×10^8 protoplasts ml^{-1} with STC and stored at −80°C as 100-μl aliquots.

Protoplast transformation with plasmid pBN7CB-P was carried out as described by Fernández-Martín et al. (2000). Transformed protoplasts were incubated at 30°C on minimal medium; and transformants appeared during the following days. Gene replacement of the wild-type *carO* allele was done as described by Fernández-Martín et al. (2000), except that a counterselection method by chlorate resistance was used to identify strains that had lost the plasmid. Transformants were inoculated by toothpick transfer onto minimal agar supplemented with 15 g l^{-1} $KClO_3$ and 1.6 g l^{-1} L-asparagine. Strains with a functional nitrate reductase are poisoned by the accumulation of ClO_2^- and mycelia lacking nitrate reductase are able to grow (Klittich and Leslie 1988). Conidia were collected from growing mycelia and plated onto minimal medium with L-asparagine as the nitrogen source. The frequency of *niaD*$^-$ mutants was checked by colony transfer to minimal medium with nitrate as the nitrogen source.

Hybridizations and PCR

Genomic DNA was obtained from samples of mycelia harvested by filtration through filter paper, washed with sterile distilled water, frozen in liquid nitrogen and ground into a fine powder in a cold mortar. DNA extractions were done following Giordano et al. (1999b). To investigate integration events, DNA obtained from transformant strains was digested with *Sal*I, separated by agarose gel electrophoresis, transferred onto a Nylon membrane (Hybond-N; Amersham, Little Chalfont, UK), hybridized with a probe labeled with the digoxigenin (DIG) labeling system (Roche Diagnostic, Basel, Switzlerland) and coupled for fluorescent detection on X-ray film (X OMAT S, Kodak). The probe contained a 1.4-kb *Hin*dIII fragment containing the *carO* gene (Fig. 1c). To identify the *carO* allele of *niaD*$^-$ revertants obtained from the transformants, DNA samples were digested with *Pst*I.

Total RNA was isolated from frozen samples with the RNAgents total RNA isolation kit (Promega, Mannheim, Germany). Northern blot hybridizations were carried out according to Di Pietro and Roncero (1998), using the DIG labeling system. RNA transferred to Nylon membranes was stained with methylene blue as described and rRNA bands were used as a load control.

PCR reactions were performed with samples (2 ng) of genomic DNA of the fungal strain with 0.2 mM each deoxynucleoside triphosphate, 1 μM each primer, and 0.5 μl Expand PCR system (Boehringer, Mannheim, Germany). Reaction mixtures were heated at 95°C for 2 min followed by 35 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min) and polymerization (68°C, 5 min), with a final polymerization at 72°C for 10 min in a programmable thermocycler (Perkin–Elmer Cetus 480). Primers used were ops-1F (5′-GGAAAA-TGTGGGATTGAAGC-3′) and ops-1R (5′-AACCTA-CAGAATGTCGTCAG-3′), which resulted in a DNA segment of 1.8 kb containing the whole *carO* gene (Fig. 1c). For cDNA sequencing, PCR segments were obtained with the primers ops-6F (5′-ATGGCTGA-CCACCTTTATGC-3′), coinciding with the start of the coding sequence, and ops-2R (5′-GATCTAGTCG-CCCATTCCTTC-3′), located in the downstream sequence, 20 bases after the stop codon.

Chemical and conidiation analysis

To assay the amounts of carotenoids and fusarin, samples of mycelia were separated from agar and lyophilized before extraction. Carotenoid analyses were done as described by Arrach et al. (2002). Total amounts of colored carotenoids were estimated from maximal absorption spectra in hexane, assuming an average maximal E (1 mg l^{-1}, 1 cm) of 250. Phytoene, neutral colored carotenoids and neurosporaxanthin were determined by Al_2O_3 chromatography. The composition of the neutral carotenoids mixtures was determined by high pressure liquid chromatography.

Fusarin concentrations were calculated as described by Barrero et al. (1991), assuming that the strains investigated accumulate 8Z-fusarin. Conidiation was

calculated under the microscope, using suspensions of conidia obtained by washing the plate surface with 12 ml 15% glycerol, followed by two further washes with 8 ml H_2O.

Results

The sequence of gene *carO*

Partial sequencing of DNA downstream of gene *carB* of *F. fujikuroi* (Linnemannstöns et al. 2002a) revealed the presence of an open reading frame (ORF) coding for a sequence with similarities to opsins. The whole ORF was included in a 6.7-kb sequence previously shown to contain the gene *carB*, coding for phytoene dehydrogenase (Fernández-Martín et al. 2000). A downstream sequence of 2,304 bp containing this ORF was determined and sent to gene databases under accession number AJ566362.

The sequence contains a predicted gene extending along 1,192 bp, including four introns (54, 57, 97, 50 bp), three of which clustering in the 5′ domain of the gene (Fig. 1b,c). With the exception of the intron of 97 bp, the size of the introns is remarkably constant and comparable with those of the two linked genes *carRA* (50, 53, 48 bp) and *carB* (54, 45, 47 bp). The 924-bp ORF codes for a predicted protein of 307 amino acids. The ORF is separated by 690 bp from the stop codon of *carB*, containing the *carO* promoter. The three genes, *carRA*, *carB* and *carO*, are coded by the same DNA strand (Fig. 1b).

The predicted CarO protein is highly similar to other proteins in the opsins family (Fig. 2). The highest similarity with the proteins available in databases is found with two heat-shock proteins (Cvhsp30/1, Cvhsp30/2) of *C. versicolor*. Of the 307 residues of CarO, 34% are found in the same positions in these two proteins, which differ only in three residues (Iimura and Tatsumi 2002). Significant degrees of identity are also found with the heat-shock protein HSP30 from *S. cerevisiae* (29%), the opsins *ops* from *L. maculans* (29%) and *nop-1* from *N. crassa* (26%) and archaeal bacteriorhodopsins (26% with the one from *Halobacterium salinarum*). Similar or

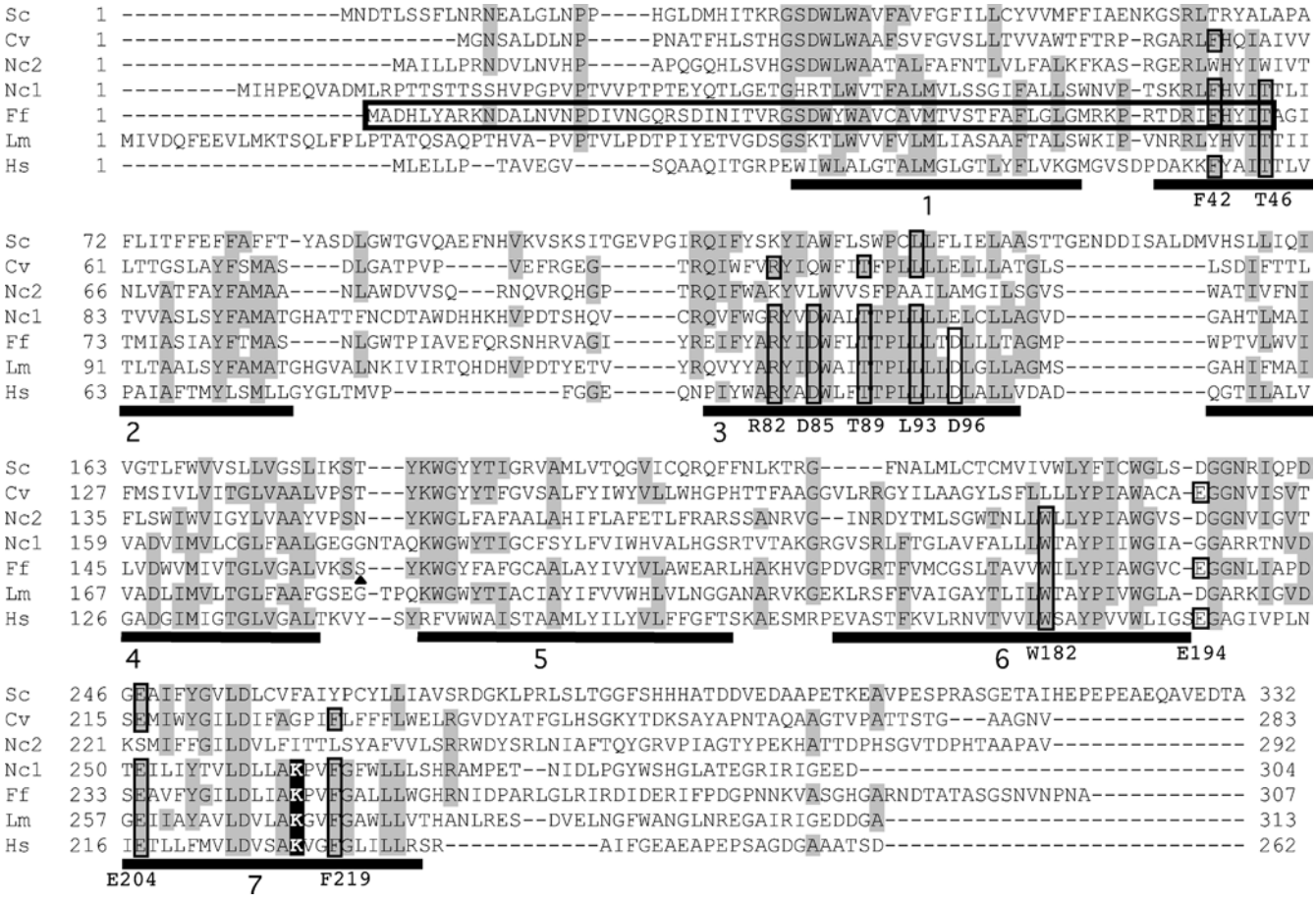

Fig. 2 Comparison of the proteins Hsp30 from *S. cerevisiae* (*Sc*, accession number M93123), Cvhsp30/1 from *C. versicolor* (*Cv*, AB018406), the predicted protein NCU01735.1 from *N. crassa* (*Nc2*, AABX01000725), Nop-1 from the same fungus (*Nc1*, AF135863), CarO (*Ff*, AJ566362), Ops from *L. maculans* (*Lm*, AF290180) and bacteriorhodopsin from *H. salinarum* (*Hs*, M11720). Amino acids coinciding in the same position in at least four proteins are *shaded in gray*. Relevant conserved residues are indicated by the amino acid letter and a number corresponding to its position in the crystallography bacteriorhodopsin protein (Luecke et al. 1998). *Numbered bars* indicate the seven transmembrane domains. The 69 amino acids remaining in the truncated CarO protein are *boxed*. The *small arrowhead* indicates a serine residue predicted as a putative phosphorylation site

lower identity percentages were found when compared with other proteins in this large family (Zhai et al. 2001). The comparison in Fig. 2 includes a second opsin ORF found in the *N. crassa* genome, more similar to CarO than Nop-1 (29% vs 26%). The similarity with bacteriorhodopsin and other opsins indicates the organization of the CarO protein as a seven-helical structure. The proteins segments corresponding to each transmembrane helix in bacteriorhodopsin are highlighted in Fig. 2.

A Blast search of sequences coding for proteins similar to CarO in the genome of *F. graminearum*, the only one from a *Fusarium* species whose sequence has been determined, reveals the presence of three genes coding for proteins with a significant similarity. One of them is homologous to *carO*, as judged by the high percentage of identities (76.8% along a segment of 276 amino acids) and its linkage to *carB* and *carRA* homologues. The second and third coding sequences are less similar (35.5% and 25.3% out of 277 and 255 amino acids, respectively). Parallel Blast searches of genes similar to CarO in the *Aspergillus nidulans* and *Magnaporthe grisea* genomes reveal the presence of a single candidate, that of *M. grisea* with 43.5% identical amino acids along a segment of 264 amino acids and that of *A. nidulans* with 30.5% identical amino acids along 164 amino acids upon alignment with CarO internal segments.

The crystal structure of bacteriorhodopsin has been described at increasing resolution (see, i.e., Pebay-Peyroula et al. 1997) and the residues involved in the proton transfer pathway have been identified (Luecke et al. 1998). Sequence comparison between CarO, Nop-1 and Ops (respectively Ff, Nc1, Lm in Fig. 2) and bacteriorhodopsin (Hs in Fig. 2) shows that the critical amino acids involved in retinal-binding and light-induced isomerization (Asp85, the donor acceptor from the Schiff base; Leu93, Trp182, residues in contact with retinal in the active site) and in proton transfer (arg82, glu204, glu194) are mostly conserved in CarO, Nop-1 and Ops (Fig. 2). The proton donor to the Schiff base, Asp96, and two amino acids contributing to create a hydrophobic environment around Asp96 (Phe42, Phe219) are also conserved. Taken together, these observations strongly suggest that CarO, Nop-1 and Ops have a proton pump activity, probably modulated by light.

Computer analysis of the protein identifies a predicted phosphorylation site in the second serine located in the internal loop between transmembrane domains 4 and 5 (indicated by an arrowhead in Fig. 2), suggesting that the activity of the protein can be regulated by a serine kinase.

Transcription of *carO*

Total RNA samples were obtained from cultures of the wild-type IMI58289 incubated in the dark and for increasing times in the light. Northern analyses show a low transcription of the gene *carO* and a strong induction by light, with a maximal mRNA amount after 1 h illumination and decreasing amounts after longer light exposures (Fig. 3, left). A single transcript is found on the gel whose size (approx. 1 kb, as judged by its relative position with ribosomal RNA) fits with the predicted size of the gene. The light induction pattern coincides with those of the *carRA* and *carB* genes, indicating a coordinated regulation. Film overexposure did not allow the detection of any signal of *carRA* and *carO* in the wild-type sample grown in the dark, denoting a very low expression of these genes. In contrast, a feeble band was detected for the gene *carB*, suggesting a less strict regulation.

Expression of *carO* was compared in two different wild-type strains and three carotenoid-overproducing mutants grown in the dark. Previous results showed that genes *carB* and *carRA* are expressed at high levels in dark-grown cultures of overproducing mutants (Linnemannstöns et al. 2002a). The gene *carO* is also deregulated in the dark in these mutants (Fig. 3, right), confirming a shared regulation of *carRA*, *carB* and *carO*.

Because of the high similarity of CarO with the Cvhsp30 proteins from *C. versicolor* and HSP30 from

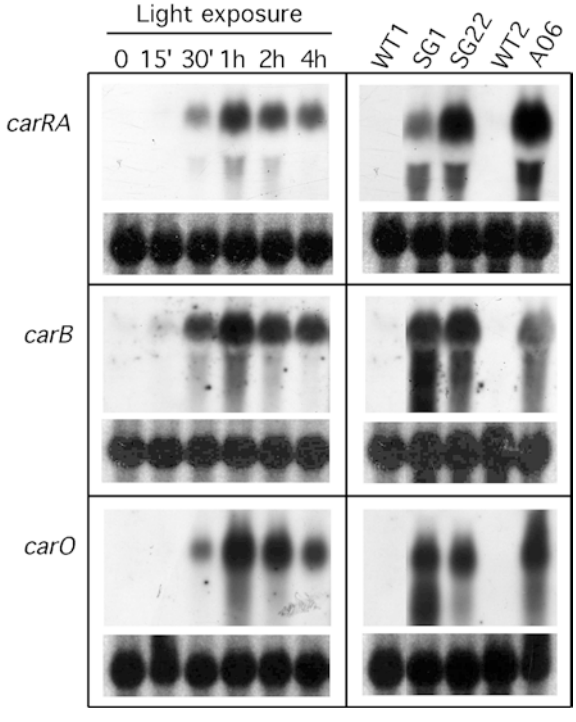

Fig. 3 Expression of *carO*, *carRA* and *carB* genes under different regulatory conditions for carotenogenesis. *Left panels* Northern blots of total RNA from the wild-type IMI58289 grown in the dark and exposed for different times to 25 W m^{-2} white light. *Right panels* Northern blots of total RNA from the wild-types IMI58289 (*WT1*) and M567 (*WT2*) and three carotenoid-overproducing mutants grown in the dark. The probes are indicated on the left. SG1 and SG22 were obtained from IMI58289 and A06 from M567. rRNA bands are shown below each panel as a load control

S. cerevisiae, the effect of heat shock on *carO* expression was investigated. Incubation for 1 h at high temperature activates transcription of the genes for different heat-shock proteins in *N. crassa* (Mohsenzadeh et al. 1998). Growth of *F. fujikuroi* is optimal between 26°C and 30°C, severely impaired at 37°C and absent at 40°C. No induction of *carO* transcription was appreciated after 1 h incubation at either 38°C or 42°C (Fig. 4), but a slight induction was detected upon 2 h incubation at 42°C. A comparable heat-shock induction was exhibited by the *carB* gene, providing additional evidence for a common regulation of *carO* with the genes of the carotenoid pathway. In both cases, the heat-shock induction was minor compared with the one produced by 1 h light exposure.

Analysis of the promoter sequences

The coordinated expression of the three genes suggests that their promoters are recognized by common regulatory proteins and allow prediction of the occurrence of similar regulatory elements in their DNA sequences. A computer search for *cis*-acting regulatory DNA elements recognized by fungal *trans*-acting factors (TRANSFAC database; Matys et al. 2003) in the *carRA*, *carB* and *carO* upstream sequences shows the presence of recognition sites for several proteins. Among them, only the CAATT box, recognized by the HAP1/2/3 yeast proteins (Mantovani 1998), is present in the three promoters (Fig. 5a). An alternative computer search for unknown repeated DNA motives reveals the presence of different conserved sequence elements in the three promoters (Fig. 5b). None of the best matches coincide or overlap with the CAATT boxes. Conserved promoter sequences of photoinducible *N. crassa* genes, such as the APE elements found in *al-1*, *al-3*, *ccg-2* and *con-10* (Carattoli et al. 1994), or sequences reminiscent of the consensus CGACGCACGAGCT elements found in *al-1*, *al-2* and *al-3* (Schmidhauser et al. 1994) were not found in the *car* genes of *F. fujikuroi*.

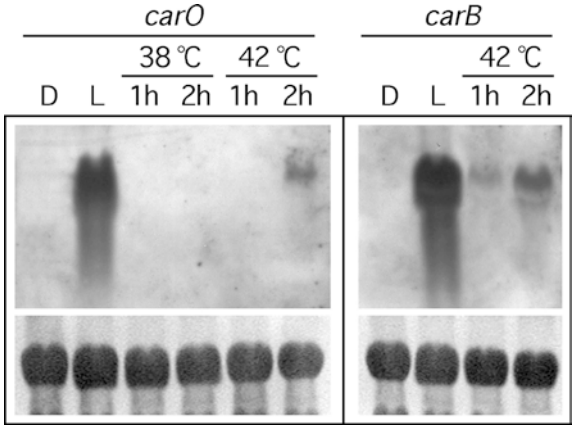

Fig. 4 Effect of light and heat shock on the expression of genes *carO* and *carB*. Northern blots of total RNA from the wild-type FKMC1995 grown in the dark and then exposed either for 1 h to 25 W m^{-2} white light or for the indicated times to either 38°C or 42°C. rRNA bands are shown below each panel as a load control

Gene disruption of *carO*

A gene disruption vector was constructed containing a four-base deletion in the *Pst*I site close to the start codon (Fig. 1c). The mutation results in a predicted truncated polypeptide with the first 69 residues of the wild-type protein (Fig. 2), followed by three random residues

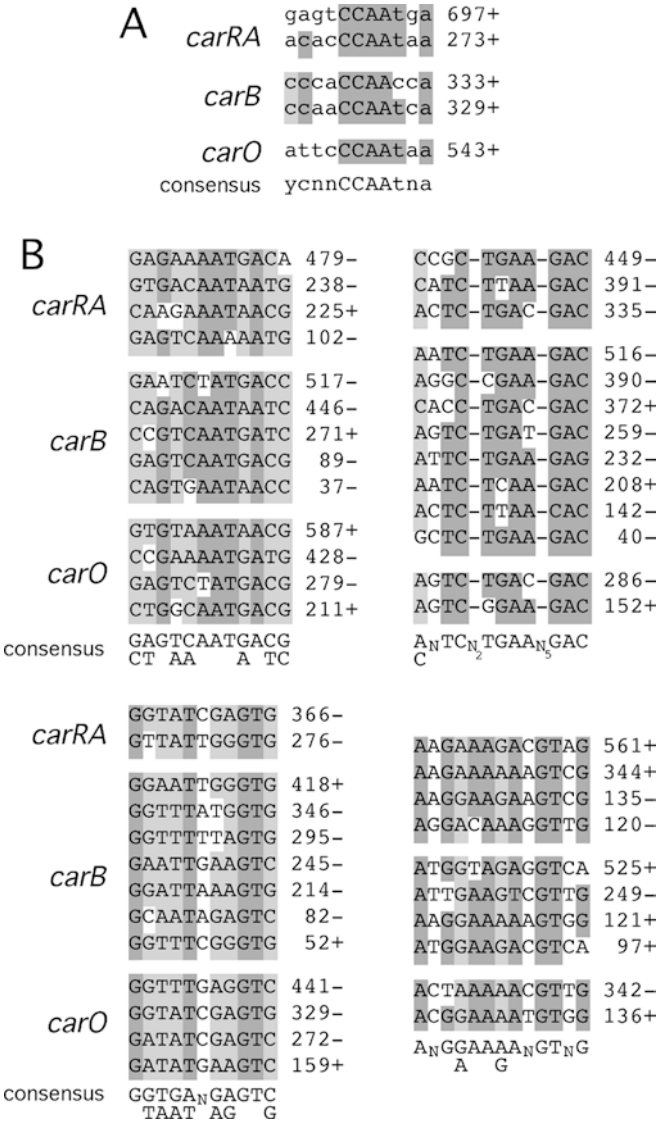

Fig. 5 a,b Conserved sequence elements. **a** Matches for the yeast factor complex HAP2/3/5 binding sites found with the MatInspector program in the *carRA*, *carB* and *carO* promoters. The core sequence is indicated in *capital letters*. **b** Examples of conserved sequence elements in the promoters of *carRA*, *carB* and *carO* found with the AlignACE program. *Numbers* indicate the distance from the first base of the sequence to the start codon. *Plus* forward strand, *minus* reverse strand

preceding an early stop codon in the new reading frame. Transformation was carried out on a *niaD* mutant, using the wild-type *niaD* gene as a selectable marker. Previous results indicated a high frequency of homologous recombination in *F. fujikuroi* (Fernández-Martín et al. 2000). The plasmid contains two sequences through which homologous recombination may occur. In agreement with this, Southern analysis of DNA from 11 transformants probed with the *carO* sequence showed the predominance of two different patterns of bands (Fig. 6), indicating two kinds of integration events. Six transformants (T2, T3, T5, T7, T10, T11) showed the wild-type 14.0-kb band and an additional band at 11.8 kb, as expected from the integration of the plasmid through the *niaD* sequence. In one of the transformants (T2) the lower band has a higher intensity, as expected by multiple tandem integration of the vector. This also seems to be the case in the transformant T8, which exhibited an additional band of high molecular weight, suggesting an additional ectopic integration event.

The homologous integration of the plasmid through the 6.7-kb sequence of the *car* genes would result in the loss of the 14-kb wild-type band and the generation of two bands above 8.7 kb and 9.7 kb. In four transformants (T1, T4, T6, T9) the wild-type band was replaced by a single band of about 13 kb, suggesting a similar size of the two newly generated restriction fragments. These transformants are expected to contain two tandem copies of the *carO* gene separated by plasmid DNA, one of them with the four-base deletion. To obtain a mutant strain of *carO*, a recombination event must occur through the duplicated sequences liberating the plasmid with the *niaD* and *carO* wild-type alleles, a procedure previously used to obtain a *carB* mutant (Fernández-Martín et al. 2000). To detect spontaneous plasmid loss, we selected *niaD*$^-$ reversion by resistance to the nitrate analogue, chlorate.

When a mycelial fragment from a wild-type colony is transferred to chlorate-supplemented medium, spontaneous sectors lacking nitrate reductase activity eventually appear. Growth tests of the sectors reveal different phenotypes, explained by loss of nitrate reductase either by mutations in the structural gene *niaD*, in one of the genes for the synthesis of the molybdenum cofactor, or in a regulatory gene (Klittich and Leslie 1988). In contrast, mycelial fragments of the *niaD*$^+$ transformants T1, T4, T6 and T9 exhibited normal growth on chlorate medium, suggesting a high frequency of plasmid loss by homologous recombination. Unexpectedly, only about 1% of the colonies obtained from conidia formed by these chlorate-resistant mycelia were actually *niaD*$^-$. The remaining 99% grew at different rates on nitrate as the sole nitrogen source.

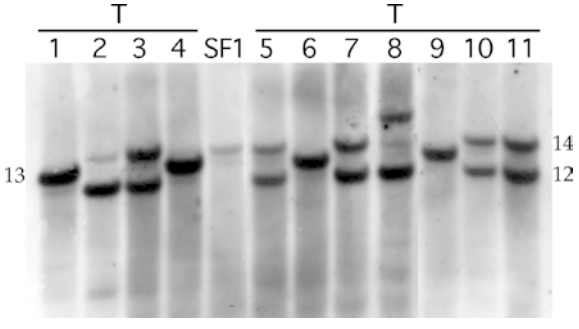

Fig. 6 Characterization of *niaD*$^+$ transformants strains. Southern blot of genomic DNA from the *niaD*$^-$ strain SF1 and 11 *niaD*$^+$ transformants digested with *Sal*I and hybridized with the *carO* gene probe. Relevant fragment sizes (in kilobases) are shown at both sides of the panel

Four *niaD*$^-$ revertant strains, R1–R4, obtained from transformants T1 (R1), T4 (R2, R3) and T9 (R4), were chosen for further investigation. The presence of the mutated allele in these strains was checked by PCR (Fig. 7a) and by Southern blots of *Pst*I-digested DNA samples (Fig. 7b). Two revertants (R1, R2) contained the wild-type allele and the others (R3, R4) the non-functional one. The presence of a single *carO* allele in each strain and the inability to assimilate nitrate suggest that the four revertants contain a single copy of the gene. The occurrence of the four-base deletion in the *carO* gene of the revertants R3 and R4 was confirmed by sequence analysis.

Phenotype of carO$^-$ mutants

Carotenoid production is similar in the *carO*$^+$ and *carO*$^-$ revertants in different growth conditions (Fig. 7c). Column chromatography and HPLC analysis of the carotenoids accumulated in the light by both kinds of strains revealed very similar mixtures, containing neurosporaxanthin (50–75%), torulene and other intermediary carotenes in lower amounts. Only traces of carotenoids were found in dark-grown cultures. Accordingly, the *carO* mutation has no effect on the regulation of the carotenoid genes, as shown by the normal light induction of *carB* transcription in *carO*$^+$ and *carO*$^-$ revertants (Fig. 7d). A similar result was obtained when the RNA samples were probed with the *carO* gene. As expected, the 4-bp deletion in the *carO* gene has no consequence on its own transcription.

The *carO* mutation did not affect fusarin production, which was found to be influenced by light. Fusarin Z is produced by *F. fujikuroi* in large amounts at high temperature in the dark (Barrero et al. 1991). Strains R2 (*carO*$^+$) and R3 (*carO*$^-$) accumulated about 2 mg g^{-1} dry weight at 22°C and 10 mg g^{-1} at 30°C in the dark, but only about 0.15 mg g^{-1} and 0.25 mg g^{-1}, respectively, in the light. A similar light repression of fusarin biosynthesis was exhibited by strains R1 and R4, confirming the lack of effect of the *carO* mutation on this photoresponse.

Light induction of conidiation was reported in a strain of *F. fujikuroi* (Avalos et al. 1985). The *niaD*$^-$ strain used for the targeted mutagenesis, SF1, shows variations in the conidiation level in different experiments, but

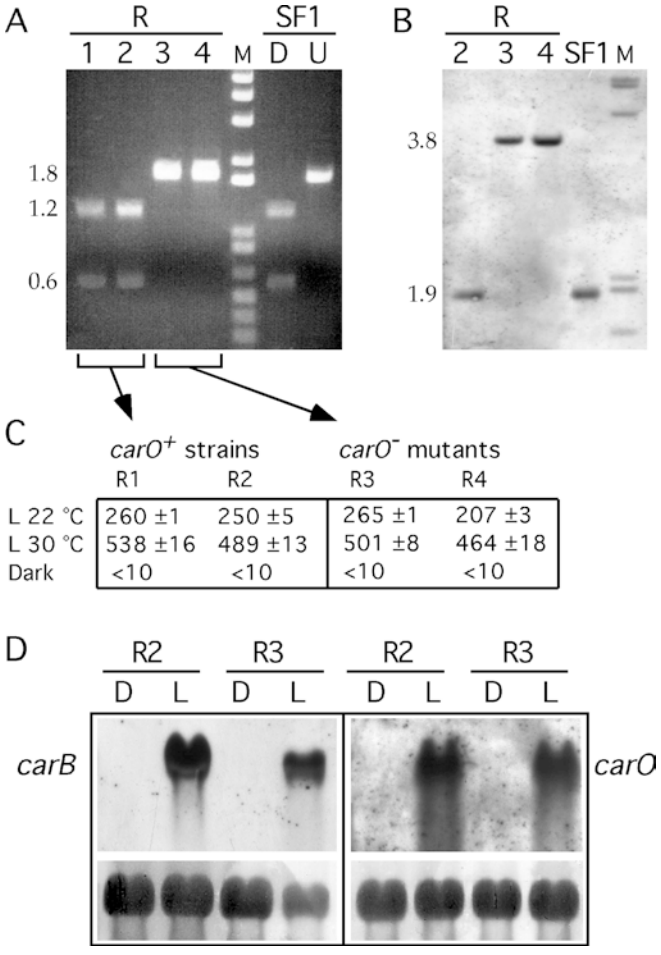

Fig. 7 a–d Characterization of *niaD*$^-$ revertant strains. **a** Electrophoretic separation of DNA products obtained by PCR replication with primers ops-1F and ops-1R on genomic DNA from SF1 and four *niaD*$^-$ revertants (*R1–R4*). The samples were digested with *Pst*I, except one of the samples obtained from SF1 (*D* digested, *U* undigested). *carO* alleles differ in the presence of the *Pst*I restriction site. *M* indicates size markers (kilobases). **b** Southern blot of genomic DNA from the *niaD*$^-$ strain SF1 and three *niaD*$^-$ revertants (*R2–R4*) digested with *Pst*I and hybridized with the *carO* gene probe. The 3.8-kb band corresponds to the sum of two 1.9-kb fragments produced by the *Pst*I digestion of SF1 DNA. **c** Carotenoids accumulated in the light or in the dark at 22°C or 30°C by four *niaD*$^-$ revertants produced by plasmid loss. Data (μg g^{-1} dry wt) are averages from two determinations. Standard deviation is shown when relevant. **d** Effect of light on expression of genes *carB* and *carO* in the revertant strains R2 and R3. Northern blots of total RNA from mycelia grown in the dark and exposed for 1 h to 25 W m^{-2} white light. rRNA bands are shown below each panel as a load control

conidiation was always similar or lower in the same experiment in the light than in the dark, indicating a lack of light induction of this developmental process in this strain. Conidiation was investigated in the *carO*$^+$ and *carO*$^-$ strains, but no effect of the *carO*$^-$ mutation could be identified.

A light-dependent alteration of the colony morphology was found in *N. crassa* in the presence of 100 μg l^{-1} oligomycin (Bieszke et al. 1999a). To check whether a similar effect is found in *F. fujikuroi*, the revertant strains were grown on solid cultures supplemented with the same oligomycin concentration. After 4 days incubation at 22°C, the diameter of the colonies grown on oligomycin-supplemented media was one-third that of the colonies grown on the control plates. A comparison between the *carO*$^+$ and *carO*$^-$ strains revealed the same oligomycin sensitivity and no difference in morphology or pigmentation.

Discussion

We have found an opsin-like gene linked to the genes responsible for the synthesis of torulene in *F. fujikuroi*, *carRA* and *carB*, sharing with them a common transcriptional regulation. The protein coded by this gene, called *carO*, is similar to the proteins of a family of sensory opsins, such as rhodopsin and bacteriorhodopsin, and fungal heat-shock putative chaperons. The highest similarity was found with two nearly identical heat-shock proteins of *C. versicolor* (Iimura and Tatsumi 2002). Despite a lower sequence similarity, CarO seems functionally closer to light-absorbing opsins, as indicated by the presence of the conserved lysine residue responsible for the Schiff base linkage to retinal, absent from the heat-shock proteins of this structurally related family. CarO is also similar to the recently discovered opsins Nop-1 from *N. crassa* (Bieszke et al. 1999a) and Ops from *L. maculans* (Idnurm and Howlett 2001).

The increasing number of fungal species for which the genome sequence is available confirms the ubiquity of such genes in fungi. A second predicted opsin-like gene is found in the *N. crassa* genome (Fig. 2) and two additional ones are found in the *F. graminearum* genome. One of them contains the conserved lysine residue (amino acid 246 of CarO), and thus could have a hypothetical light-absorbing role in the cell. Its eventual presence and function in *F. fujikuroi* will be the subject of future experiments. In contrast, only a coding sequence for an opsin-like protein is found in the genomes of *A. nidulans* and *M. grisea*.

Targeted mutagenesis of *carO* was carried out through a two-step disruption method, previously used to obtain a mutation of the gene *carB* (Fernández-Martín et al. 2000). The method requires the sequential identification of transformants that have integrated the vector in the target region, strains derived from them which have lost the vector and, among the latter, those containing the mutated allele of the gene. The first step is favored by the high frequency of homologous recombination in this fungus (Fernández-Martín et al. 2000). To facilitate the subsequent step, we used nitrate reductase as a selectable marker (Tudzynski et al. 1996), which allows positive selection by growth on nitrate as the nitrogen source, and counterselection by growth on chlorate-supplemented medium. The identification of strains losing the integrated vector was hindered by the abundance of strains growing in the presence of chlorate but conserving a residual nitrate

reductase activity, as implied by their slow growth on nitrate as the sole nitrogen source. This could be attributed to the occurrence in *F. fujikuroi* of a mechanism inactivating duplicated sequences, such as the one in *N. crassa* known as "quelling" (Pickford et al. 2002), which acts at the transcriptional level and is partially reversible.

As previously found for *carRA* and *carB* (Linnemannstöns et al. 2002a), *carO* expression is photoregulated. Activation of *carO* transcription by light follows the same pattern as *carB* and *carRA*, with a peak of expression after 1 h illumination and a subsequent decrease. The *nop-1* of *N. crassa* is also induced by light, but only under certain developmental conditions (Bieszke et al. 1999a). Despite this difference, a lack of phenotypic effect of the *nop-1* mutation in *N. crassa* suggests that this gene and *carO* are homologues. A connection of *carO* with carotenogenesis in *F. fujikuroi* is pointed out by its linkage with *carB* and *carRA* and by the transcriptional derepression of the three genes in the carotenoid-overproducing mutants. Such a connection would not be evident in *N. crassa*, where *nop-1* is not linked to any of the three carotenoid genes, *al-1*, *al-2* or *al-3*, spread on different locations on the *N. crassa* genome and where no deregulated mutants producing large amounts of carotenoids in the dark have been identified. Light induction is not enough to establish a relation with carotenogenesis, since up to 3% of the *N. crassa* genes are photoinducible (Lewis et al. 2002). Moreover, *nop-1* is not induced by light in young submerged mycelia (Bieszke et al. 1999a), suggesting a developmental regulation independent of carotenogenesis.

The similarity of CarO with heat-shock proteins of the opsin family suggests a role in the *F. fujikuroi* heat-response. A certain transcriptional induction was appreciated in *carO* upon thermal shock, but this response was also exhibited by the *carB* gene; and it is minor compared with the strong transcriptional induction produced by light. This result is not conclusive about an eventual role of *carO* in the heat-shock response mechanisms of *F. fujikuroi*, but provides further support to a common regulation with the structural genes of the carotenoid pathway.

The coordinated transcription of *carRA*, *carB* and *carO* indicates that the three genes form a gene cluster governed by shared regulatory proteins, including a repressor (CarS) and probably others mediating light activation. In agreement with this conclusion, computer analysis of their promoters identifies conserved DNA sequence elements in the three genes. Some of them coincide with sequence elements found previously in the *carRA* and *carB* promoters with a different computer approach (Linnemannstöns et al. 2002a). Photoinduction of the carotenoid genes *al-1*, *al-2* and *al-3* of *N. crassa* is mediated by the WC complex, formed by WC-1/WC-2 dimers (Talora et al. 1999). A conserved regulatory sequence (APE element), identified by mutational and deletion analysis as responsible for the photoinduction of *al-3*, was also found in other photoinduced genes of *N. crassa* (Carattoli et al. 1994). Similar sequences were identified in the photoinduced photolyase gene from *Trichoderma harzianum* (Berrocal-Tito et al. 1999) and in the *carRP*/*carB* regulatory sequence of *Mucor circinelloides* (Velayos et al. 2000a, b). A different 13-bp consensus sequence, unrelated to the APE element, was found in the *al-1*, *al-2* and *al-3* promoters (Schmidhauser et al. 1994). None of them were found in the promoters of the three *car* genes of *F. fujikuroi*, suggesting differences at the molecular level between the photoinduction mechanisms of *Fusarium* and *Neurospora*.

Mutants carrying a frameshift deletion of *carO*, leading to the loss of 77% of the wild-type protein, have no apparent phenotypic effect. Apart from photoreactivation after UV damage (Avalos et al. 1985), the only photoresponse investigated in *F. fujikuroi* is the light induction of carotenogenesis (Avalos and Cerdá-Olmedo 1987; Avalos and Schrott 1990). We have found in addition a photorepression of fusarin biosynthesis, produced in high amounts at high temperature (Barrero et al. 1991). None of these photoresponses is altered in the *carO* mutants. A lack of expression of *carO* in the dark suggests that a difference would be expected only in the light. We were not able to find any effect, including the light-dependent morphological alteration of aerial growth found in *N. crassa* in the presence of oligomycin (Bieszke et al. 1999a). This is not surprising, since *F. fujikuroi* and *N. crassa* exhibit different growth patterns on surface cultures. The former grows as compact slow-growing colonies that take more than a week to extend over a Petri dish. The latter develops as thin fast-growing mycelial pads spreading aerial hyphae and filling the surface of a Petri dish in less than 3 days. *N. crassa* conidiates abundantly and the conidiation is induced by light. Our strain of *F. fujikuroi* produces fewer conidia and the amount does not increase in the presence of light.

The regulatory connection between *carO* and the genes of the carotenoid pathway could be related with the need for retinal in its function. The presence of the conserved lysine residue responsible for the Schiff base linkage to retinal and the similarity to the *nop-1* of *N. crassa* suggest that *carO* codes for an opsin which binds retinal as a prosthetic group. In addition to neurosporaxanthin, the main end-product of its carotenoid pathway, *F. fujikuroi* accumulates low amounts of beta-carotene (Avalos and Cerdá-Olmedo 1987) that might serve as a source for the synthesis of retinal. Alternatively, retinal could be produced by *F. fujikuroi* from neurosporaxanthin or other carotenoid by means of a novel enzyme activity. The coordinated expression with *carRA* and *carB* suggests a coupling of CarO and retinal biosyntheses. This is the case for bacteriorhodopsin formation in *H. salinarum*. The elimination of the gene *carY*, required for beta-carotene biosynthesis, results in the loss of both retinal and bacteriorhodopsin in this archaeon (Peck et al. 2002).

Our work leaves open the question of the function of opsin-like proteins in fungi. A biochemical approach strongly points toward a role for an opsin as the photoreceptor responsible for the phototaxis of the zoospores of the chytridiomycete *Allomyces reticulatus* (Saranak and Foster 1997), but the gene responsible for this opsin has not been investigated. A chemical block of carotenogenesis in this fungus reduces severely the photoresponse. Phototropism is one of the several photoresponses known in the zygomycete *Phycomyces blakesleeanus* (Corrochano and Cerda-Olmedo 1992), but none of them is affected when carotenoid biosynthesis is blocked either by mutation or chemical inhibition. Photoinduced phytoene biosynthesis is found in a mutant of *F. fujikuroi* with a total loss of phytoene dehydrogenase (Fernández-Martín et al. 2000). This is not only in agreement with the normal carotenoid photoinduction shown by the *carO*$^{-}$ mutants, but discards a retynilidene protein as photoreceptor for this photoresponse.

Photoregulation of *nop-1* and *carO* indicates a light-dependent function, probably different from that of *ops* in *L. maculans*, as suggested by the lack of effect of light on its expression. Carotenoid biosynthesis has not been reported in this fungus, as expected if the Ops activity requires retinal as a prosthetic group. The protein similarity of CarO with archaeal opsins suggests a light-driven proton pump activity, but its in vivo role remains to be elucidated. The lack of phenotype of the *carO* mutation might also be explained by functional redundancy. The occurrence of a second opsin in *Fusarium* whose predicted protein contains the conserved lysine responsible for the Schiff base linkage makes plausible this possibility. Future experiments on the function of this second gene, the effect of different environmental conditions on *carO* expression and the effect of the *carO* mutation on expression of other genes in the light should contribute to understand the biological role of these genes in *F. fujikuroi* and other filamentous fungi.

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