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ORIGINAL PAPER

Identification of the gene responsible for torulene cleavage in the *Neurospora* carotenoid pathway

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Abstract Torulene, a C_{40} carotene, is the precursor of the end product of the *Neurospora* carotenoid pathway, the C_{35} xanthophyll neurosporaxanthin. Torulene is synthesized by the enzymes AL-2 and AL-1 from the precursor geranylgeranyl diphosphate and then cleaved by an unknown enzyme into the C_{35} apocarotenoid. In general, carotenoid cleavage reactions are catalyzed by carotenoid oxygenases. Using protein data bases, we identified two putative carotenoid oxygenases in *Neurospora*, named here CAO-1 and CAO-2. A search for novel mutants of the carotenoid pathway in this fungus allowed the identification of two torulene-accumulating strains, lacking neurosporaxanthin. Sequencing of the *cao-2* gene in these strains revealed severe mutations, pointing to a role of CAO-2 in torulene cleavage. This was further supported by the identical phenotype found upon targeted disruption of *cao-2*. The biological function was confirmed by in vitro assays using the purified enzyme, which cleaved torulene to produce β-apo-4′-carotenal, the corresponding aldehyde of neurosporaxanthin. The specificity of CAO-2 was shown by the lack of γ-carotene-cleaving activity in vitro. As predicted for a structural gene of the carotenoid pathway, *cao-2* mRNA was induced by light in a WC-1 and WC-2 dependent manner. Our data demonstrate that CAO-2 is the enzyme responsible for the oxidative cleavage of torulene in the neurosporaxanthin biosynthetic pathway.

Keywords Carotenoid oxygenase · Neurosporaxanthin · CAO-2 · β-apo-4′-carotenal · Light regulation

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Introduction

The fungus *Neurospora crassa* is a leading model for different biological phenomena in lower eukaryotes (Davis 2000), particularly those involved in light sensing and circadian regulation (Linden 2002; Vitalini et al. 2006). *Neurospora* exhibits various photoresponses, such as the entrainment of the circadian clock (Liu 2003), the phototropism of perithecial beaks (Harding and Melles 1983), and the photoinduction of other cellular processes. The latter include the formation of asexual spores, conidia (Lauter et al. 1997), and the biosynthesis of carotenoids, widespread terpenoid pigments produced by all photosynthetic organisms, and by many fungi and non-photosynthetic bacteria (Britton et al. 2004; Hirschberg 2001). *Neurospora* accumulates carotenoids in the mycelia in response to light (Harding et al. 1969; Harding and Turner 1981) and in the conidia in a developmentally regulated manner (Li and Schmidhauser 1995). The massive production of pigmented

conidia is the major reason for the characteristic orange color of the surface cultures of this fungus, a result of the accumulation of the xanthophyll neurosporaxanthin, the end product of the pathway, and variable amounts of intermediate carotenoids.

Current knowledge on the enzymes responsible for carotenoid biosynthesis in *Neurospora* was derived from the genetic analyses of albino mutants, which led to the identification of three *al* genes. A prenyl transferase encoded by the gene *al-3* (Sandmann et al. 1993) synthesizes the carotenoid precursor GGPP (geranylgeranyl diphosphate). The condensation of two GGPP molecules results in the formation of phytoene, the first molecule with a C_{40} carotene structure (Fig. 1). This reaction is catalyzed by the product of the gene *al-2* (Schmidhauser et al. 1994), a bifunctional enzyme with phytoene synthase and *β*-cyclase activities at the carboxy and amino terminal domains, respectively (Arrach et al. 2002). The conjugated system of the colorless phytoene is then extended through sequential desaturation reactions catalyzed by the product of gene *al-1* (Schmidhauser et al. 1990), to form the reddish carotenes lycopene and 3,4-didehydro-lycopene via several intermediates (Hausmann and Sandmann 2000). These molecules are substrates for the *β*-cyclase activity of AL-2, which produces *γ*-carotene and *β*-carotene from lycopene, and torulene from 3,4-didehydrolycopene.

The last step of the pathway is the synthesis of the acidic C_{35} apocarotenoid neurosporaxanthin from the C_{40} precursor torulene, which implies the removal of five carbon atoms and the generation of a carboxy group. In *Neurospora*, no information is presently available on the enzymology of these chemical reactions at the end stage of the pathway. The easy identification of albino mutants contrasts with the less obvious phenotype expected from mutants affected in the conversion of torulene to neurosporaxanthin, which hinders the identification of the gene encoding the responsible enzyme. The use of growth conditions which result in an increase in the relative amount of neurosporaxanthin and a decrease in the one of intermediates (Harding et al. 1984) may alleviate contribute to the isolation of such mutants.

In other organisms, carotenoid cleavage reactions are generally mediated by carotenoid oxygenases, which constitute a new non-heme iron enzyme family common in all taxa (for review see Auldridge et al. 2006; Bouvier et al. 2005; Moise et al. 2005; Wyss 2004). Recently, the crystal structure of a member of this family, the *Synechocystis*-apocarotenoid oxygenase (Ruch et al. 2005), was elucidated at 2.4-Å resolution. The enzyme contains a Fe^{2+}-4-His arrangement at the axis of a seven-bladed *β*-propeller chain fold, covered by a dome formed of six large loops (Kloer et al. 2005).

Carotenoid cleavage products, i.e., apocarotenoids, fulfill key functions in many organisms, as represented by the opsin chromophore retinal (von Lintig and Vogt 2004), the plant hormone abscisic acid (ABA) (Schwartz et al. 1997) and the fungal pheromone trisporic acid (Miller and Sutter 1984). In addition, the pigmentation of several plant tissues and fungi is due to the accumulation of apocarotenoids, e.g., crocetin glycosides in the styles of saffron (*Crocus sativus*; Bouvier et al. 2003) and neurosporaxanthin in

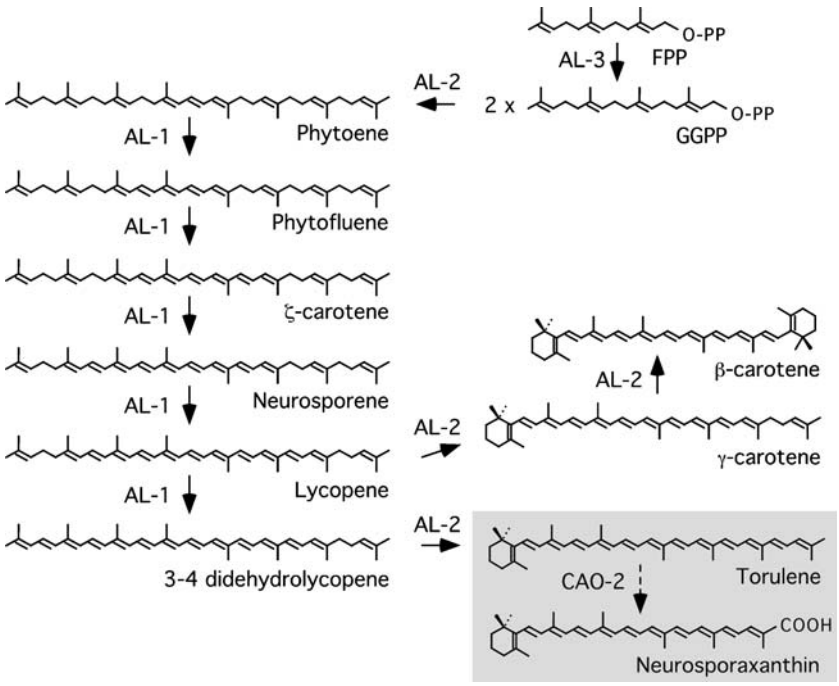

Fig. 1 Carotenoid biosynthetic pathway of *Neurospora*. The gene products responsible for each enzymatic reaction are indicated. AL-2 encodes a bifunctional enzyme responsible for phytoene synthase and *β*-cyclase activities. The *shaded area* emphasizes the torulene to neurosporaxanthin cleavage reaction. The *dashed arrow* indicates that the number of reactions involved is unknown. Attribution of the CAO-2 gene product to torulene cleavage reaction derives from results of this work

Neurospora and other ascomycetes, such as *Fusarium fujikuroi* (Avalos and Cerdá-Olmedo 1987).

In this work, we isolated two novel *Neurospora* mutants accumulating torulene. Allele sequencing in both strains revealed a severe mutation in *cao-2*, a gene encoding a carotenoid oxygenase identified by sequence comparison. A targeted deletion was performed to confirm the function of the *cao-2* gene. In addition, we investigated the enzymatic activity of CAO-2 using heterologously expressed and purified protein, and we compared the expression of *cao-2* with the one of the bifunctional carotenogenic enzyme AL-2. Our data show that CAO-2 is the enzyme responsible for the torulene cleavage reaction leading to neurosporaxanthin in *Neurospora*.

Materials and methods

Strains and growth conditions

The *N. crassa* wild-type Oak Ridge 74-OR23-1A strain and the mutants *wc-1* (FGSC 4395, allele ER45, and FGSC 4398, allele ER53), *wc-2* (FGSC 4408), *mus-51* (FGSC 9717), and *mus-52* (FGSC 9720) were obtained from the Fungal Genetic Stock Center (McCluskey 2003). The two latter mutants are also auxotrophic for histidine.

Genomic DNA and total RNA were extracted from mycelial samples grown for 3 days in Petri dishes at 30°C in 25 ml liquid Vogel's media supplemented with 0.2% Tween 80 to avoid aerial development and 0.5 g l^{-1} L-histidine (Sigma, St. Louis, MO, USA) when required. For carotenoid analyses, incubations were done under the same culture conditions for 2 days in the dark at 30°C, and 1 day in the light at 16°C under white fluorescent light at an intensity of 10 W m^{-2}. In both cases, the plates were inoculated with 10^5 conidia.

Mutagenesis and mutant searches were done as described by Arrach et al. (2002). For phenotypic analysis, the strains were grown as agar cultures on Vogel's medium.

Targeted *cao-2* deletion

The *cao-2* gene, including 1,128 and 418 bp of upstream and downstream regulatory sequences, respectively, was obtained by PCR with the primers 5′-TGATGTATCTG CCGCTGGAG-3′ and 5′-GACGGATCGTTGATGAGA TG-3′ and introduced into the plasmid pGEM T-easy (Promega, Madison, WI, USA) to yield plasmid pLOR1. To interrupt the *cao-2* gene, a 4.1-kb blunt-end DNA fragment containing a hygromycin B resistance cassette consisting of the *hph* gene from *Escherichia coli* under the control of *Aspergillus nidulans* regulatory sequences, was isolated from the plasmid pAN7-1 (Punt et al. 1987) using the restriction enzymes *Hin*dIII and *Bgl*II. This DNA segment was end-filled by Klenow treatment and introduced into pLOR1 digested with *Cla*I and end-filled with T4-polymerase to yield pLOR2. In this plasmid, the hygR cassette replaced 534 and 1,279 bp of the *cao-2* promoter and coding sequences, respectively. For transformation, a 5.45-kb DNA fragment containing the hygR cassette, surrounded by 596 bp of the *cao-2* upstream regulatory sequence and 1,044 bp of the 3′ region of the gene (626 and 418 bp of 3′ coding and non-coding sequence, respectively), was isolated from pLOR2 by *Not*I digestion and purified with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA).

Spheroplasts of the *Neurospora* wild-type strain were prepared as described by Royer and Yamashiro (1992) and transformed with 2 µg of the obtained 5.45 kb DNA fragment following the protocol of Vollmer and Yanofksy (1986). Transformants were selected on hygromycin B-supplemented medium (100 mg l^{-1}), subcultured at least two times under selective conditions and checked for color phenotype. Genomic DNA samples were obtained from selected transformants and analyzed by Southern blot and PCR using the following primers flanking the insertion point in *cao-2*: 5′-ACAGGCCGATGAGCACGACG-3′ and 5′-CAGAACCGAGATAGTGAACC-3′.

Molecular techniques

Genomic DNA was extracted according to Lee and Taylor (1990). Total RNA extractions were performed using the Perfect RNA eukaryotic mini kit (Eppendorf, Hamburg, Germany). Southern and Northern blot hybridizations were performed as described (Youssar et al. 2005). Other DNA manipulations were done according to Sambrook and Russell (2001). The hybridization probes were obtained by PCR using the following primer pairs, *al-2*: 5′-ACTTAC AGACAAAATGGCTG-3′ and 5′-AACCCTACCTCACA AATAGC-3′; *hph*: 5′-TGCCTGAACTCACCGCGACG-3′ and 5′-TATTCCTTTGCCCTCGGACG-3′; *cao-2*: AGCG GAGGGTGTTGAGGAAG-3′ and 5′-CCACGGCCGGT TTCCTTGT-3′. The *al-2* (2 kb) and *cao-2* (0.7 kb) probes were amplified from genomic DNA. The *hph* probe (1 kb) was obtained from the plasmid pAN7-1 (Punt et al. 1987).

The sequences of the *cao-2* alleles from the red mutants JA1 and JA2 were obtained from three overlapping fragments covering the whole coding region, amplified with the primers 5′-AGCGGAGGGTGTTGAGGAAG-3′ and 5′-CCACGGCCGGTTTCCTTGTC-3′, 5′-GCATGTCAA CCCTCCTGGAC-3′ and 5′-TAGAGGGAGTGGATGAA CTC-3′, and 5′-CATCTGACATCAACCTCTAC-3′ and 5′-CAGAACCGAGATAGTGAACC-3′. PCR reactions were performed using the Triplemaster PCR system (Eppendorf AG) to enhance the fidelity of the amplification. The sequences of the three fragments were determined from

both DNA strands of at least two independent PCR products using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Carotenoid analyses

Mycelial samples were frozen and lyophilized. Extraction was performed by homogenizing about 0.1 g of dry mycelium in 2 ml Eppendorf tubes in a Mini-Beadbeater (Biospec Products Inc., Bartlesville, OK, USA) containing 1 ml acetone and about 0.1 g of sand. Beating cycles lasted no more than 30 s to avoid sample overheating. Samples were then centrifuged and the supernatants were collected. The extraction was repeated until bleaching of the sample and the supernatants were combined and vacuum-dried. Spectra for the polar and neutral carotenoid fractions were obtained as described by Thewes et al. (2005). HPLC analyses were achieved as described by Arrach et al. (2000). Peak identifications were based on UV/Vis-absorption spectra (neurosporene and neurosporaxanthin) and on former analyses with carotenes obtained from *Phycomyces blakesleeanus* (γ-carotene and β-carotene, Kuzina and Cerdá-Olmedo 2006) and from *F. fujikuroi* (torulene, Prado-Cabrero et al. 2007a).

Cloning of *cao-2* for *E. coli* expression

Five micrograms of total RNA from the *Neurospora* wild type strain were used for cDNA synthesis using SuperScript™ RnaseH$^-$ reverse transcriptase (Invitrogen, Paisley, UK) following the instructions of the manufacturer. Two microliters of the obtained cDNA were then used for the amplification of *cao-2* using the primers 5′-ATGAGT CCGCACGAGGTGATCGGC-3′ and 5′-TCAGTACTCC GGGCCCTCAACCCT-3′. The obtained PCR product was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and cloned into the pCR2.1®-TOPO® (Invitrogen) vector to yield pCR-Cao2. The nature of the product was verified by sequencing.

Protein expression and purification

To express CAO-2 as a GST fusion protein, the corresponding cDNA was excised as an *Eco*RI-fragment from pCR-Cao2 and ligated into *Eco*RI-digested and alkaline phosphatase-treated pGEX-5X-1 (Amersham Biosciences) to yield pGEX-Cao2. Subsequently, *E. coli BL21* cells were transformed with pGEX-Cao2, grown at 28°C in 2X YT-medium and induced at an OD_{600} of 0.5 with 0.2 mM IPTG. After incubation for additional 4 h at 28°C, cells were harvested by centrifugation, and the fusion protein was then purified using glutathione-sepharose 4B (Amersham Biosciences) according to the instructions of the manufacturer. CAO-2 was then released by overnight treatment with the protease factor Xa in PBS containing 0.1% (v/v) Triton X-100 at room temperature, according to the instructions of the manufacturer (Amersham Biosciences). Purification steps and protein expression were analyzed by SDS-PAGE. The control strain carried a plasmid expressing only GST.

Enzyme assays

β-apo-4′-carotenal was kindly provided by BASF, Ludwigshafen, Germany, and highly pure γ-carotene was obtained from Carotenature, Lupsingen, Switzerland. Torulene was purified from carotenoid extracts of the *F. fujikuroi* mutant SG68 by preparative HPLC (Prado-Cabrero et al. 2007a). Enzyme assays and HPLC-analyses were performed according to Prado-Cabrero et al. (2007a). Substrates were quantified spectrophotometrically at their individual λ_{max} using extinction coefficients calculated from E1% (Barua and Olson 2000). Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Sequence analyses

Blast analyses were done through the NCBI server (www.ncbi.nlm.nih.gov/blast/). BlastP was carried out against the non-redundant Swissprot database. Alignments were achieved with the Clustal X 1.63b program (National Center for Biotechnology Information, Bethesda, MD, USA). Corrections for multiple substitutions were applied for phylogenetic analyses. DNA sequences and *Neurospora* genome information were obtained through the server www.broad.mit.edu/annotation/fungi/neurospora/ of the Broad Institute, Cambridge, MA, USA.

Results

Isolation and phenotype of reddish mutants

The elevated amounts of intermediate carotenoids accumulated by *Neurospora* under standard laboratory conditions hinder the identification of mutants affected in later steps of the pathway. However, the proportion of neurosporaxanthin increases upon illumination at low temperature of a dark-grown culture (Harding et al. 1984), facilitating the identification of such mutants. UV-exposed conidia were incubated on sorbose agar in the dark and transferred to the light at low temperature before conidia production. Under these conditions, the white colonies acquire a deep-orange pigmentation within 24 h. A screening for mutants exhibiting color alterations allowed the identification of several mutants with a pale reddish color. Preliminary analyses showed the accumulation of a carotenoid mixture in which neurosporaxanthin was not detected. The pale pigmentation

of the surface cultures (one example shown in Fig. 2a) turned into a reddish color upon illumination at low temperature under submerged conditions (Fig. 2b), markedly different from the orange color of wild-type mycelia.

Two independent mutants (JA16 and JA17) were chosen for carotenoid analysis under growth and illumination conditions leading to high neurosporaxanthin accumulation. As shown in Fig. 2c, both mutants contained only traces of carotenoids in the polar fraction. In contrast, more than 80% of the carotenoids accumulated by the wild type were polar and exhibited the typical spectrum of neurosporaxanthin, with absorption maximum at 477 nm. The neutral fraction spectra of the mutants resembled the one of torulene, with the characteristic three-peaks shape and absorption maximum at 487 nm. The accumulation of a high proportion of torulene was confirmed by HPLC analyses (see Fig. 5).

Identification of two *Neurospora* genes from the carotenoid oxygenase family

The carotenoids accumulated by the mutants JA16 and JA17 suggest that they are affected by the gene involved in the oxidative cleavage of torulene to neurosporaxanthin. Taking advantage of the sequenced genome, we identified two genes encoding putative carotenoid oxygenases in *Neurospora*. The predicted enzymes, named here CAO-1 and CAO-2, consist of 526 and 649 amino acids, respectively, with significant homology to other well-known enzymes from this family of carotenoid cleaving enzymes (sequences aligned in Fig. 3 with the human enzyme BCDO1 as a representative example).

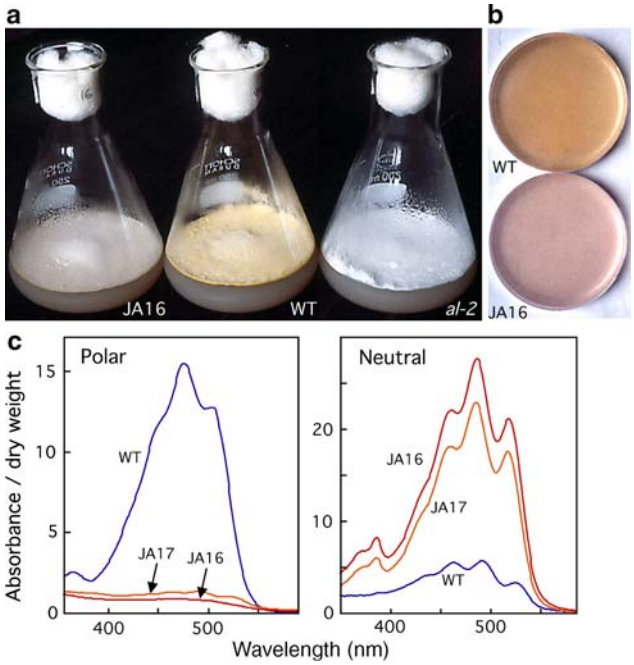

Fig. 2 Phenotype of torulene-accumulating mutants. **a** Flask cultures of JA16 and the wild type compared with an albino *al-2* mutant grown for 5 days at 22°C in the light. **b** Petri dish cultures of the wild type and mutant JA16 incubated for 3 days at 30°C in the dark followed by 1 day at 16°C in the light. **c** Absorption spectra of the polar and neutral carotenoid fractions from the wild type (*blue*) and the mutants JA16 (*red*) and JA17 (*orange*) under the conditions shown in **b**

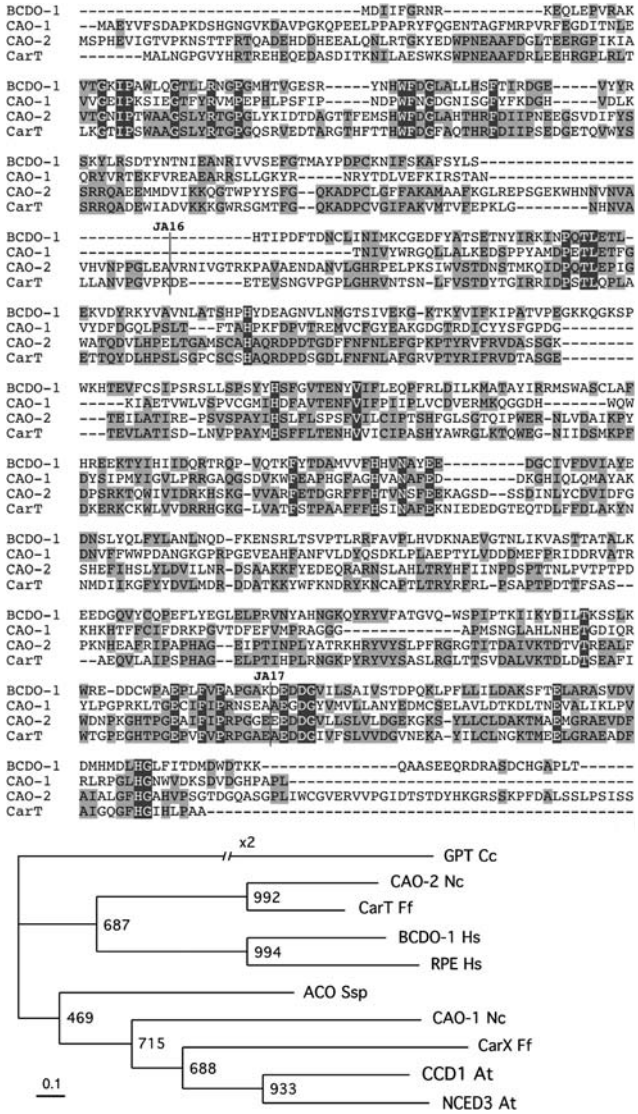

Fig. 3 Sequence analysis of the predicted CAO-1 and CAO-2 proteins from *Neurospora*. **a** Alignment of both protein sequences with the human *β*,*β*-carotene 15,15′-dioxygenase (BCDO-1, accession No. Q9HAY6) and CarT from *F. fujikuroi* (accession No. AM418467). Coincident amino acids in at least two of the proteins are *shaded*. Break positions for the truncated CAO-2 proteins from the JA16 and JA17 mutants are indicated. **b** Phylogram of CAO-1, CAO-2 and representative proteins of the carotenoid oxygenase family (*Coprinus cinereus* GPT protein, accession number O13438; *F. fujikuroi* CarT protein, AM418467; human *β*,*β*-carotene 15,15′-dioxygenase [BCDO-1], Q9HAY6; human RPE protein, Q16518; *Synechocystis sp.* Lignostilbene-*α*,*β*-dioxygenase [ACO], P74334; *F. fujikuroi* CarX protein, AJ854252, and *Arabidopsis thaliana* carotenoid 9,10-9′10′ cleavage dioxygenase [CCD1], 065572, and 9-*cis*-epoxycarotenoid dioxygenase [NCED3], Q9LRR7)

Recently, we characterized a retinal-forming, β,β-carotene 15,15′-dioxygenase (CarX) from *F. fujikuroi* (Prado-Cabrero et al. 2007b), encoded in the *car* gene cluster, which also harbors the structural genes of the carotenoid pathway *carB* and *carRA* (Thewes et al. 2005). Based on sequence homology, we have also identified a second carotenoid oxygenase (CarT) in *Fusarium*. In vitro characterization pointed to CarT as the torulene-cleaving enzyme in neurosporaxanthin biosynthesis (Prado-Cabrero et al. 2007a). To get an indication of the function and origin of the CAO proteins, a phylogenetic analysis was carried out with CAO-1 and CAO-2 in comparison with CarX, CarT and several representative carotenoid oxygenases (Fig. 3). The results show that (a) CAO-1 and CAO-2 are presumably the orthologues of CarX and CarT from *F. fujikuroi*, respectively, and (b) that CAO-1/CarX are closer by sequence to carotenoid oxygenases of plant and bacterial origin, while CAO-2/CarT are closer to animal enzymes related to retinal production and visual cycle.

The homology of CAO-2 to CarT from *F. fujikuroi* (sequences aligned in Fig. 3) points to this enzyme as responsible for the oxidative cleavage of torulene in *N. crassa*. Therefore, the *cao-2* alleles of the mutants JA16 and JA17 were cloned and sequenced. The comparison with the wild-type allele revealed, in both cases, frameshift mutations in the *cao-2* coding sequence. The JA16 allele exhibited an insertion of a G at position +630, possibly leading to the synthesis of a truncated 189 amino acids polypeptide (Fig. 3) followed by 24 random amino acids. The resulting protein would lack 70% of the CAO-2 sequence, and therefore, it should show total loss of function. The JA17 allele contained a G insertion at position +1699, resulting in the replacement of the last 65 residues of the protein (Fig. 3) by four unrelated amino acids (GRGR). Although most of the original protein sequence is still present, the mutation causes the loss of one of the histidine residues (position 630) which are supposed to coordinate the Fe^{2+} co-factor in the reaction center, as shown by the crystal structure of the homologous SynACO from *Synechocystis* (Kloer et al. 2005). Therefore, the JA17 mutation is consistent with a total loss of function of CAO-2 and a phenotype indistinguishable from the one of JA16 (Fig. 2).

Generation of targeted *cao-2* null mutants

To confirm that the phenotype of the mutants JA16 and JA17 was due to the mutations found in the *cao-2* gene, targeted deletion of *cao-2* was performed. For this purpose, the strains *mus-51* and *mus-52* were chosen because of their efficient homologous recombination (Ninomiya et al. 2004). A linear fragment carrying the HygR cassette surrounded by 5′ and 3′ *cao-2* sequences (Fig. 4a) was constructed and

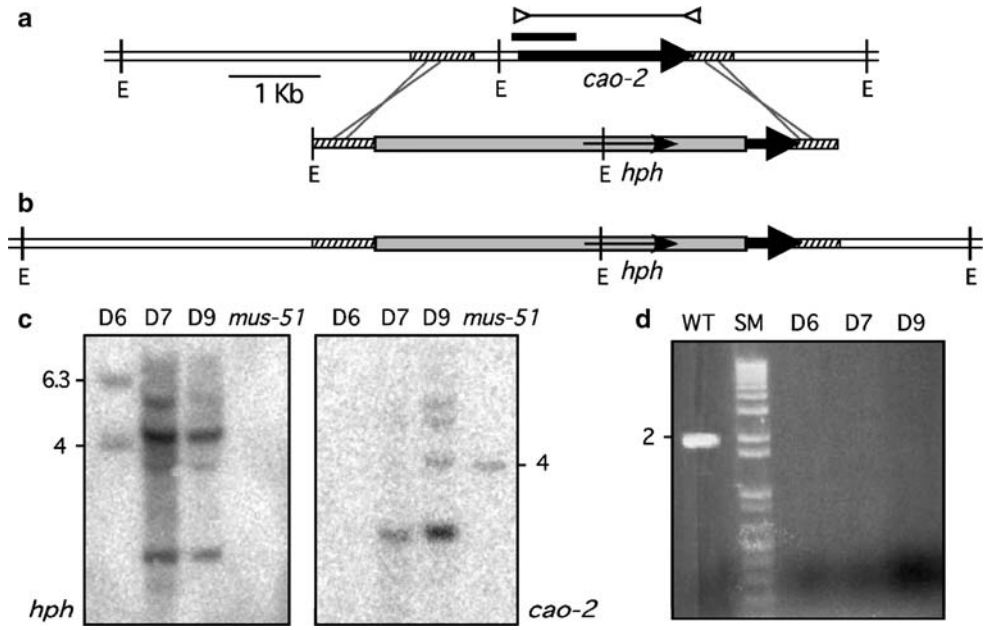

Fig. 4 Generation of Δ*cao-2* mutants. **a** Physical map of the *cao-2* region and expected double-recombination event leading to *cao-2* replacement. *Dashed segments* indicate non-coding *Neurospora* DNA present in the plasmid. The probe and expected PCR product (*thick bar* and *arrowheads-delimited bar*, respectively), mentioned in panels C and D are represented on *top*. **b** Physical map of the predicted *cao-2* deletion. **c** Southern blots of genomic DNA from the control strain and three selected transformants digested with *Eco*RI and hybridized with either the *hph* (*left panel*) or the *cao-2* (*right panel*) gene probes. *Eco*RI restriction sites are shown on the physicals maps above. The *numbers* by each panel show sizes (kb) of relevant DNA fragments. **d** PCR amplification of gene *cao-2* from DNA of the wild type and the strains shown in the Southern blots on the *left* SM, size markers

used for transformation of the *mus* strains. A set of eight transformants was checked for pigmentation and carotenoid content under illumination at low temperature, as described above. The disruption of *cao-2* led to changes in the carotenoid content, as indicated by the reddish color of several transformants, similar to the ones of the JA16 and JA17 mutants. Three strains, *D6* and *D9*, obtained from the *mus-52* strain, and *D7*, obtained from *mus-51*, were chosen for detailed molecular and phenotypic analyses.

Southern blot experiments showed that the three transformants contain *hph* sequences (Fig. 4c). However, only the strain *D6* exhibited the hybridization pattern expected from the gene replacement event (Fig. 4b), which should result in two bands upon *Eco*RI digestion. This indicated that the *cao-2* gene was deleted in this strain (Fig. 4c, right panel). The other two strains (*D7* and *D9*) displayed a more complex pattern, since they contained more than two *hph* bands. In addition, *cao-2* sequences were detected in their genomic samples. However, the restriction pattern of the *cao-2* sequences differed markedly from the one of the control strain. In addition, attempts to amplify the whole *cao-2* gene did not lead to detectable products (Fig. 4d) indicating DNA reorganizations affecting the integrity of this gene in the two strains.

The *D6* strain showed a phenotype similar to the ones of mutants JA16 and JA17 in the accumulation of a high proportion of neutral carotenoids (Fig. 5a). The UV-Vis-spectrum of the neutral fraction is coherent with a high content of torulene with absorption maximum at 487 nm, as expected from all-*trans*-torulene in the same solvent (Davies 1976). The polar fraction contained small amounts of carotenoids exhibiting, like in the *JA* mutants, a torulene-like UV-Vis-spectrum that differed markedly from the one of neurosporaxanthin (see wild type and *mus-52* spectra above and JA17 for comparison). The carotenoids accumulated by the transformants *D7* and *D9* were very similar to those of *D6*. In contrast, the wild type as well as the parental strains *mus-52* and *mus-51* (the latter not shown) contained large amounts of polar carotenoids, showing the typical neurosporaxanthin UV-Vis-spectrum, and minor amounts of neutral carotenes.

HPLC-analyses confirmed the spectrophotometric analyses and provided more detailed information. The absorption chromatograms of the wild type and the parental strains *mus-51* and *mus-52* were almost identical (see Fig. 5b for *mus-52*). In the HPLC system used, the pool of polar carotenoids eluted between 4 and 8 min. In these strains, the spectrum of the compounds eluted within this time interval showed variations (see examples 1 and 1′), and suggested the presence of neurosporaxanthin as a major substance eluted (spectrum 1). The three strains contained also minor amounts of γ-carotene (peak 3) and a carotene tentatively identified as neurosporene (peak 2). Torulene, which overlapped with peak 2, occured also in low amounts, as indicated by the UV/Vis spectrum. According to the spectrophotometric analyses, the HPLC chromatograms of the mutants JA16 and JA17 (JA17 shown in Fig. 5b) and in the three transformants (*D6* shown in Fig. 5b), were similar to each other and revealed the absence of neurosporaxanthin. Instead, two minor compounds (peaks 3 and 4) were detected, showing a neurosporaxanthin-like elution pattern. However, the UV/Vis spectra of these compounds resembled rather that of torulene with a maximal absorption at a longer wavelength. HPLC analyses revealed also the occurrence of large amounts of torulene (Fig. 5b, peak 5) and minor amounts of γ-carotene and β-carotene

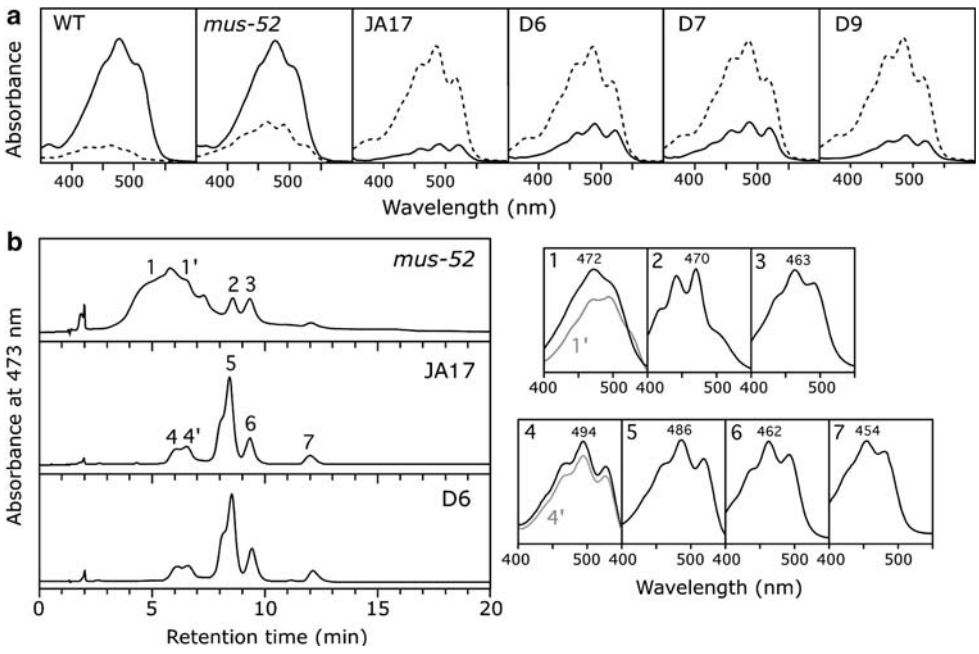

Fig. 5 Carotenoid analysis of Δ*cao-2* mutants and control strains. **a** Absorption spectra of the polar (*continuous line*) and neutral carotenoids (*dashed lines*) fractions from the wild type, the parental strain *mus-52*, the mutant JA17 and three Δ*cao-2* strains. **b** HPLC profiles at 473 nm of the carotenoid mixtures extracted from selected strains shown above. The wild type and *mus-51* profiles and the D7 and D9 profiles were very similar to those of *mus-52* and D6, respectively. Spectra and absorption maxima of relevant peaks are shown on the right. Peaks 1, 3 (same as 6), 5, and 7 were identified as neurosporaxanthin, torulene, γ-carotene and β-carotene, respectively

(Fig. 5b, peaks 6 and 7, respectively); the latter was also detected in low amounts in the parental strain.

Regulation of *cao-2*

Given that *cao-2* is a gene needed for the oxidative cleavage of torulene in the neurosporaxanthin biosynthetic pathway, it could be expected that this gene is also co-regulated with the ones involved in the earlier steps of the pathway. To prove this hypothesis, Northern blot analyses were performed. As shown in Fig. 6, *cao-2* exhibited the same transcriptional pattern as *al-2*, which encodes the phytoene synthase/β-cyclase. The mRNA levels for both genes were very low in vegetative mycelia grown in the dark, and increased markedly upon illumination. Furthermore, illumination did not lead to any detectable increase of the *cao-2* and *al-2* mRNA amounts in *wc-1* or *wc-2* mutants, confirming that *cao-2* photoinduction is mediated by the WC-1/WC-2 photoreceptor system, as reported for other light-regulated genes of *Neurospora* (Li and Schmidhauser 1995; Nelson et al. 1989; Schmidhauser et al. 1994).

A short sequence of the *al-3* promoter was formerly identified by deletion and mutagenesis analysis as the one responsible for transcriptional photoinduction (Carattoli et al. 1994). Similar conserved sequences were found in the promoters of other photoinducible genes from *Neurospora*, defining the consensus GAA(N_{1-8})TTGCC, known as APE element. At least three putative APE-like elements were identified in the *cao-2* promoter: TAA(N_2)TTGCC at position −268, GATNTTGCA at position −227 and GAA(N_7)TTGCA at position −126.

In vitro activity of the CAO-2 protein

To obtain further evidence on the biological role of CAO-2 in the *Neurospora* carotenoid pathway, we investigated its enzymatic activity in vitro. For this purpose, CAO-2 was expressed as a GST fusion protein, purified, released with the protease factor Xa (Fig. 7a), and then used for in vitro assays with torulene as a substrate. As can be seen in the HPLC analysis (Fig. 7b, c), CAO-2 converted torulene into a new carotenoid product. The reaction was almost quantitative, since only traces of the substrate were detected after the incubation. However, the spectra and absorption maxima of the product were different from those of neurosporaxanthin. Given that carotenoid oxygenases usually generate aldehyde radicals in their cleavage products, and assuming that the cleavage reaction to produce neurosporaxanthin should be achieved at the C4′–C5′ double bond, the predicted torulene cleavage product would be β-apo-4′-carotenal, i.e., the aldehyde counterpart of the acidic neurosporaxanthin. As shown in the HPLC analysis (Fig. 7b, c), UV/Vis spectrum and elution pattern of the CAO-2 product were identical to those of a β-apo-4′-carotenal standard. These data confirm that CAO-2 catalyzes the cleavage reaction in neurosporaxanthin biosynthesis (Figs. 1, 7d).

To investigate the specificity of the cleavage site, we incubated CAO-2 with γ-carotene, which differs from torulene only in that it is not desaturated at the C4′–C5′ bond (shaded in the torulene molecule shown in Fig. 7d), which is presumably a prerequisite for site identification by the enzyme. In contrast to the efficient conversion of torulene, no cleavage activity was observed upon incubation with γ-carotene (Fig. 7b, c), indicating a high specificity of CAO-2 in cleaving the C4′–C5′ double bond.

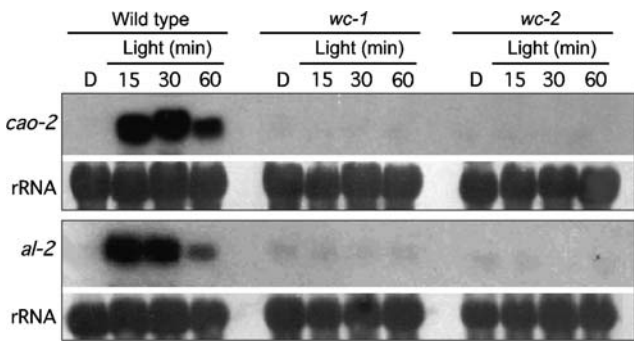

Fig. 6 Expression of the gene *cao-2*. Northern blots of total RNA isolated from the wild type and mutants *wc-1* (allele ER53) and *wc-2* grown in the dark (D), or following incubation in the light for the indicated times. The same result was obtained with a different *wc-1* allele (ER45). Expression of the gene *al-2* is shown for comparison. rRNA bands are shown below each panel as load controls

Discussion

Carotenoid biosynthesis has been the object of research in *Neurospora* from both the genetic and biochemical points of view. Despite the attention given, only genes whose mutations result in the absence of colored carotenoids, *al-1*, *al-2* and *al-3*, were known so far (Sandmann et al. 1993; Schmidhauser et al. 1990, 1994). The identification of these genes, a pioneering work in the research of fungal carotenogenesis, opened the way to the cloning of the homologous genes in other unrelated fungi, such as the zygomycete *P. blakesleeanus* or the basidiomycete *Xanthophyllomyces dendrorhous* (Avalos and Cerdá-Olmedo 2004), a task facilitated by the sequence conservation of the encoded enzymes. In *Neurospora*, the three *al* genes are responsible for the synthesis of torulene, a late intermediate carotenoid in the pathway. However, little attention has been paid to the gene responsible for the late step, the conversion of the C_{40} molecule into the C_{35} apocarotenoid neurosporaxanthin. This omission must be attributed to the lack of mutants blocked in this biosynthetic step. Indeed, the

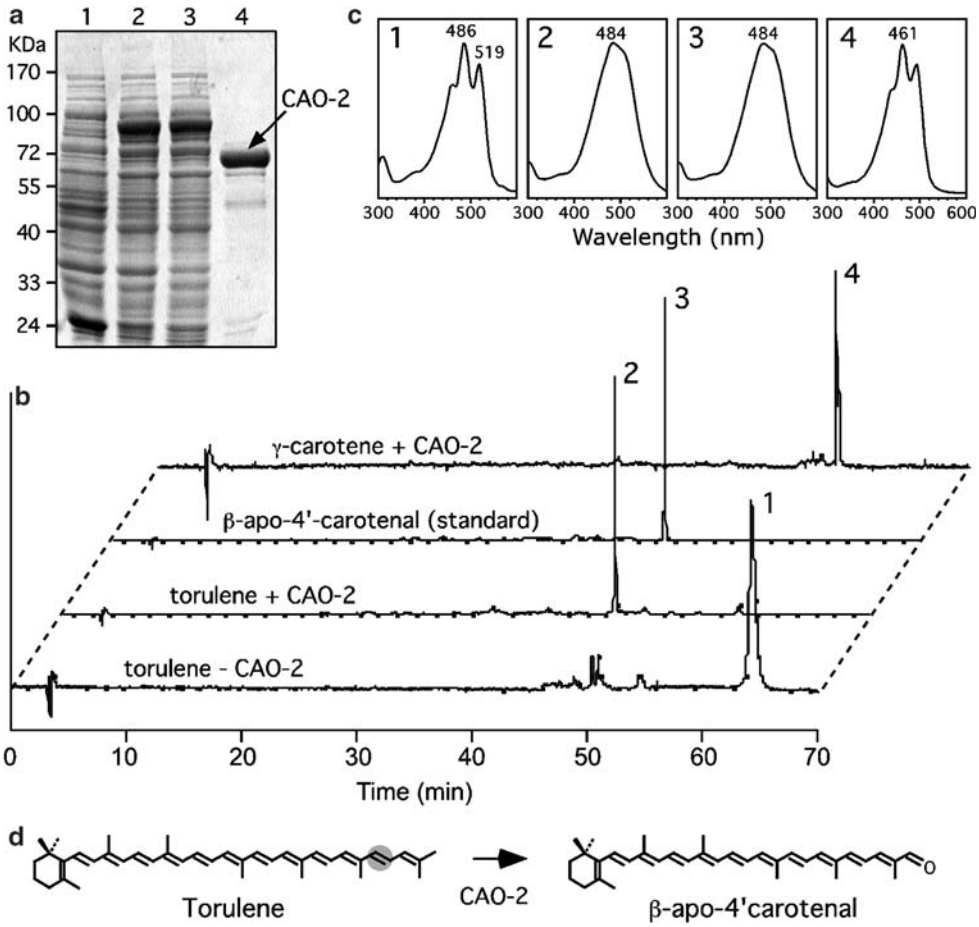

Fig. 7 In vitro activity of purified CAO-2 protein. **a** CAO-2 Purification. 1: control for lane 2; 2: coomassie-stained SDS gel showing total cell lysate; 3: soluble protein fraction; 4: purified CAO-2 released by cleavage with factor Xa. Control samples were obtained from cells expressing only GST. The positions of the molecular size markers are shown on the *left*. **b** HPLC-analyses of the in vitro assays (front to back): incubation of torulene (peak 1) with CAO-2 resulted in the formation of a product (peak 2) identical in elution pattern and UV/Vis spectrum to *β*-apo-4′-carotenal (peak 3). Incubation of *γ*-carotene (peak 4) with CAO-2 resulted in no product formation. **c** Spectra and absorption maxima of peaks 1–4. **d** Reaction catalyzed by CAO-2

molecular identification of the *al* genes was preceded by the identification and genetic analysis of the corresponding mutants.

Under standard growth conditions, the identification of *Neurospora* mutants blocked in late steps of the carotenoid pathway is hindered by the accumulation of a complex carotenoid mixture, with relatively low neurosporaxanthin content. However, this xanthophyll becomes predominant in mycelia grown in the dark at standard incubation temperature (30–34°C) and transferred to light at low temperature (Harding et al. 1984). Its proportion reaches up to 90% at 8°C (Arrach et al. 2002), a temperature at which the fungus is hardly able to grow but shows an unexpectedly efficient carotenoid biosynthetic activity (Harding 1974). Scrutiny of colonies surviving to UV mutagenesis under these unusual culture conditions allowed the identification of mutants blocked in the cyclase activity of the bifunctional protein encoded by the gene *al-2* (Arrach et al. 2002). The same protocol allowed us to identify colonies with a pale reddish pigmentation, easily recognized amongst the deep orange-colored wild-type colonies. The analysis of the carotenoid content of cold-illuminated cultures of two such mutants revealed the accumulation of high amounts of torulene, making them a valuable tool to identify the gene responsible for the torulene cleavage reaction.

The pale color exhibited by surface cultures of these mutants suggests a high instability of torulene in the cell. Similarly, cultures of the torulene-accumulating mutant SG68 of *F. fujikuroi* (Avalos and Cerdá-Olmedo 1987) gradually lose their pigmentation within 24–48 h, while its neurosporaxanthin-accumulating parental strain retains a deep-orange pigmentation for longer incubation periods (unpublished observations). No information is available on the role of the terminal carboxy group of neurosporaxanthin, absent in the former intermediates of the pathway, but we might infer that it contributes to its stability or appropriate storage in these fungi. The observed instability of all-*trans*-torulene may explain the occurrence of torulene-like compounds in the polar fractions of the mutants or the Δ*cao-2* transformants, identified in the HPLC chromatograms.

Identification of the torulene-cleaving enzymatic activity was also tried through a genomic approach. Since the cloning of the first gene, coding for a carotenoid-cleaving enzyme (Schwartz et al. 1997), the identification of an increasing number of related oxygenases has led to the establishment of a family of carotenoid-metabolizing enzymes (for review see

Auldridge et al. 2006; Bouvier et al. 2005; Moise et al. 2005; Wyss 2004). They have been found in animals, plants, lower eukaryotes and prokaryotes, giving strong evidence to the existence of a very ancient group of proteins that evolved to carry out a diversity of carotenoid-cleaving reactions. Based on our recent characterization of two carotenoid-cleaving enzymes from *Fusarium* (CarX and CarT; Prado-Cabrero et al. 2007a, b), we screened the *Neurospora* proteome for similar enzymes, and identified two candidates, which we named CAO-1 and CAO-2, based on their expected role as carotenoid oxygenases.

Our alignments suggest that CAO-1 is the ortholog of CarX from *F. fujikuroi*, shown to cleave β-carotene into retinal (Prado-Cabrero et al. 2007a). This is an expected enzymatic activity present in *Neurospora*, as indicated by the ability of the opsin NOP-1 to bind retinal as a chromophore (Bieszke et al. 1999b). If CAO-1 is the *Neurospora* counterpart of the animal vitamin A forming enzymes (von Lintig and Vogt 2004; von Lintig and Wyss 2001), disruption of this gene should result in the light-dependent morphological alteration exhibited by the *nop-1* mutant when grown in the light in the presence of oligomycin (Bieszke et al. 1999a). This hypothesis about CAO-1 function still awaits experimental confirmation.

Sequencing of the *cao-2* alleles from two torulene-accumulating mutants revealed frameshift mutations leading to truncated proteins. Therefore, we hypothesized that CAO-2 is the torulene-cleaving enzyme in *Neurospora*. In agreement with a role in carotenoid biosynthesis, the transcription of *cao-2* is co-regulated together with the *al* genes, as shown by the rapid WC-dependent induction of *cao-2* mRNA upon illumination of vegetative mycelia. Moreover, we identified putative WC-binding elements in the *cao-2* promoter. The association of the *cao-2* gene to the torulene-cleaving reaction was conclusively demonstrated by the identical phenotype displayed by the targeted Δ*cao*-2 mutants. This experimental approach was facilitated by the use of *Neurospora* strains deficient in non-homologous recombination (Ninomiya et al. 2002), a trait with no predictable connection to carotenoid metabolism.

To confirm the function of CAO-2, we showed that the purified CAO-2 cleaves torulene at the C4′–C5′ double bond to form β-apo-4′-carotenal in vitro. The enzyme exhibited a high specificity for this cleavage site, since no other product could be detected, and it did not recognize γ-carotene, which is identical to torulene except for the lack of the C4′–C5′ double bond. The investigation of γ-carotene-cleavage activity appeared necessary, since this monocyclic carotene is accumulated as a side product in *Neurospora*, and it is the precursor of β-carotene, which is found in low amounts in *Neurospora* mycelia.

Our results are substantiated by our recent work on *carT* from *F. fujikuroi* (Prado-Cabrero et al. 2007a). Both fungi produce torulene through different intermediates, via β-zeacarotene in *F. fujikuroi* (Avalos and Cerdá-Olmedo 1987), and via 3,4-didehydrolycopene in *Neurospora* (Hausmann and Sandmann 2000). In contrast to *Neurospora*, the proportion of neurosporaxanthin in *F. fujikuroi* is high under standard growth conditions in the light in the wild type, and under any culture conditions in deregulated mutants (Avalos and Cerdá-Olmedo 1987). In this fungus, a mutation in the gene *carT* resulted in torulene accumulation, while the CarT protein also showed torulene-cleaving activity. As indicated by the phylogenetic analysis, *carT* and *cao-2* are orthologues.

The catalytic activity of carotenoid oxygenases leads usually to the formation of aldehyde products. Accordingly, CAO-2 formed β-apo-4′-carotenal, the corresponding aldehyde of neurosporaxanthin. It could be assumed that the CAO-2 product is the substrate of an aldehyde oxidoreductase or oxidase, which converts it into the acidic neurosporaxanthin. Similar scenarios take place during the formation of the plant hormone ABA (Nambara and Marion-Poll 2005) and the saffron pigment crocetin (Bouvier et al. 2003). In contrast to carotenoid oxygenases, there is no well-defined group of enzymes recognizing aldehyde groups on carotenoid substrates. Systematic mutation programs based on the *Neurospora* genome annotation are currently in progress. The identification of β-apo-4′-carotenal-accumulating strains in the phenotypic screenings should reveal the responsible enzyme.

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