Mol Gen Genet (1999) 262: 453–461

ORIGINAL PAPER

J. C. Verdoes · P. Krubasik · G. Sandmann
A. J. J. van Ooyen

Isolation and functional characterisation of a novel type of carotenoid biosynthetic gene from *Xanthophyllomyces dendrorhous*

Received: 20 January 1999 / Accepted: 21 May 1999

Abstract The red heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous* (perfect state of *Phaffia rhodozyma*) contains a novel type of carotenoid biosynthetic enzyme. Its structural gene, designated *crtYB*, was isolated by functional complementation in a genetically modified, carotenogenic *Escherichia coli* strain. Expression studies in different carotenogenic *E. coli* strains demonstrated that the *crtYB* gene encodes a bifunctional protein involved both in synthesis of phytoene from geranylgeranyl diphosphate and in cyclisation of lycopene to β-carotene. By sequence comparison with other phytoene synthases and complementation studies in *E. coli* with various deletion derivatives of the *crtYB* gene, the regions responsible for phytoene synthesis and lycopene cyclisation were localised within the protein.

Key words Phytoene synthase · Lycopene cyclase · Complementation · Astaxanthin · Evolution

Introduction

Carotenoids are important nutritional components for humans and animals. They have antioxidative effects, providing protection against photooxidation and inactivating free radicals (Krinsky 1994). Carotenoids are pigments which are used as food colouring agents, and are fed to salmon, trout and poultry to intensify the colour of the flesh or the egg yolk (Johnson and Schroeder 1995). The most valuable carotenoids commercially are β-carotene and astaxanthin. Both can be synthesised chemically but are also produced by yeast fermentation and in algal cultures (Nonomura 1989). One of the most promising natural sources of astaxanthin is the basidiomycetous yeast *Xanthophyllomyces dendrorhous* (perfect state of *Rhodomyces dendrorhous* or *Phaffia rhodozyma*; Golubev 1995). Its main carotenoid product is astaxanthin in the 3R,3′R-configuration (Andrews and Starr 1976). In the past, strains of *X. dendrorhous* were optimised for astaxanthin production by random mutagenesis (An et al. 1989) and improvement of cultivation conditions. These techniques have apparently been exploited to the limit, yielding strains that produce 2–3 mg of astaxanthin per g dry weight. Strain instability and the tendency of mutants to produce less astaxanthin as a fraction of the total complement of carotenoids are significant problems. A molecular genetic approach involving the cloning of the genes encoding enzymes of astaxanthin biosynthesis and their overexpression offers an alternative to the construction of strains of *X. dendrorhous* with astaxanthin levels that are commercially more attractive. Owing to recent advances in the molecular biology of carotenoid biosynthesis (Sandmann 1994; Armstrong 1997) and the availability of techniques for the transformation of *X. dendrorhous* (Wery et al. 1997, 1998), attempts can now be made to manipulate genetically the carotenoid biosynthetic pathway in this yeast.

As in other organisms (Sandmann 1994), the biogenesis of astaxanthin proceeds from geranylgeranyl diphosphate (GGPP) via the colourless intermediate phytoene and the red compound lycopene. After cyclisation of lycopene to β-carotene, keto groups are introduced at positions 4 and 4′, and hydroxylation occurs at carbons 3 and 3′ of both ionone end groups. Recently we

Communicated by E. Cerdá Olmedo

J. C. Verdoes (✉) · A. J. J. van Ooyen[1]
Division of Industrial Microbiology,
Department of Food Technology and Nutritional Sciences,
Wageningen University, P.O. Box 8129,
6700 EV Wageningen, The Netherlands
E-mail: Jan.Verdoes@imb.ftns.wau.nl
Tel.: +31-317-482302; Fax: +31-317-484978

P. Krubasik · G. Sandmann
Botanisches Institut, Fachbereich Biologie,
J.W. Goethe Universität, PO Box 111932,
D-60054 Frankfurt am Main, Germany

Present address:
[1] Gist-brocades NV, P.O. Box 1,
2600 MA Delft, The Netherlands

cloned the phytoene desaturase-encoding gene of *X. dendrorhous* (Verdoes et al. 1999). Analysis of carotenoid production in a recombinant *Escherichia coli* strain showed that lycopene is the end product of the conversion of phytoene by this enzyme from *X. dendrorhous*. This is very interesting, since no lycopene-accumulating *X. dendrorhous* strains have so far been isolated in mutagenesis screens (Girard et al. 1994; E.A.Johnson, personal communication). In contrast to the case in eubacteria and plants (Sandmann 1994; Armstrong 1997), no lycopene cyclase-encoding genes have yet been isolated from fungi.

Here we describe the cloning and identification of the lycopene cyclase-encoding gene from *X. dendrorhous*. We demonstrate by genetic complementation in *E. coli* that this gene encodes a novel type of carotenoid biosynthetic enzyme, which is responsible for the catalysis of two different steps in the pathway leading to astaxanthin.

Materials and methods

Molecular techniques and gene cloning

Standard methods (Sambrook et al. 1989) were used, unless otherwise indicated. DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes according to the manufacturer's specifications. DNA fragments were analysed on agarose gels and restriction fragments were purified using Elu-Quick (Schleicher and Schuell). Nucleotide sequences were determined using a *Taq* DYE Primer Cycle Sequencing kit (Applied Biosystems). The complete sequence of the *crtYB* gene has been submitted to the EMBL data bases under Accession No. AJ133646. Nucleotide and deduced amino sequences were analysed with the PCGENE program (Intelligenetics). Amino acid sequences were aligned using the programs CLUSTAL and PALIGN. The plasmids pBluescript, pUC19 and a modified pQE32 vector (Qiagen) were used for subcloning experiments. The construction of a cDNA library from an overproducing mutant of *X. dendrorhous* (CBS 6938) and a genomic library of *X. dendrorhous* in a cosmid vector was described previously (Verdoes et al. 1997).

Strains and cultivation conditions

All strains and plasmids used in this study are listed in Table 1. The carotenogenic *E. coli* strains used in the complementation experiments were made by introducing the plasmids pACCRT-EBI$_{Eu}$ and pACCAR25ΔcrtB into XL-Blue-MRF'. The recombinant *E. coli* strains, hereafter referred to as XL[pACCRT-EBI$_{Eu}$], and XL[pACCAR25ΔcrtB], accumulate the carotenoid lycopene and an isoprenoid precursor of carotenoids (GGPP), respectively. In addition a neurosporene-accumulating *E. coli* strain was constructed by transformation with the plasmid pACCRT-EBI$_{Rc}$. All plasmids can be maintained in *E. coli* by growing the transformants in the presence of chloramphenicol (Cm). After introduction of the *X. dendrorhous* cDNA library into the appropriate carotenogenic host, transformed cells were plated on selective Luria-Bertani (LB) agar plates (Sambrook et al. 1989). These plates contained ampicillin (Ap; 100 μg/ml) and Cm (30 μg/ml) for the selection of both plasmids, and isopropyl β-D-thiogalactopyranoside (IPTG; 1 mM) to induce the expression of the cloned cDNAs from the *lac* promoter. The plates were incubated overnight at 37° C and incubation was continued at room temperature until pigmentation of the transformants was observed. For carotenoid analysis, *E. coli* JM101, carrying different combinations of carotenogenic plasmids, was used and grown under appropriate selective conditions in LB medium.

Construction of plasmids

A 4.5-kb *Eco*RI fragment carrying the complete sequence of the *crtYB* gene was isolated from the cosmid vector pPRcrtYBcos1 and cloned in both orientations in the corresponding site of pUC19, yielding pPR10F and pPR10R. To facilitate the subcloning of the gene in a *X. dendrorhous* transformation vector, a recognition site for restriction enzyme *Bam*HI was introduced into this fragment upstream of the promoter region of the *crtYB* gene. Therefore, the 1.7-kb *Hin*dIII-*Bst*XI fragment containing the promoter and encoding the N-terminal region of CrtYB, and the 2.8-kb *Bst*XI-*Bam*HI fragment coding for the C-terminal part of CrtYB and including the terminator (T) sequences were isolated from pPR10F and pPR10R, respectively. These fragments were cloned into the *E. coli* cloning vector pMTL22P (Chambers et al. 1988) digested with *Bam*HI and *Hin*dIII, yielding pPR21. This plasmid was digested with *Bam*HI and the 4.5-kb restriction fragment was purified and cloned in the *Bam*HI site of the *X. dendrorhous* transformation vector pPR2TN, yielding pPR22. The vector pPR2TN is a derivative of pPR2T (Wery et al. 1998). In order to create three unique cloning sites (*Bam*HI, *Hin*dIII and *Not*I) downstream of the terminator (T) sequence of the glyceraldehyde-3-phosphate dehydrogenase encoding gene (*gpd*), the *Bam*HI-*Hin*dIII fragment carrying this sequence was replaced by a variant *Bgl*II-*Hin*dIII fragment. This new T$_{gpd}$ fragment was synthesised by PCR using the primers Tgpd1 (5′-CGC**AG*A*TCT*C*TCCAAACCCTCTCCCCTT-**3′) and Tgpd2 (5′ CGCAAGCTTGGATCCGCGGCCGC**TTGATCAGA TAAAGATAGAGATT-**3′) (restriction sites used for subcloning are underlined, nucleotides that were altered to introduce a new restriction site are italicised, sequences complementary to the *gpd* gene of *X. dendrorhous* are indicated in bold) with pPRGDH6 (Verdoes et al. 1997) as template.

For the construction of *crtYB* deletion plasmids the *crtYB* cDNA was cloned into a modified pQE (Qiagen) vector to make use of its stop codons in all three reading frames. The Cm resistance gene of pQE32 (in addition to its Ap^R marker) was inactivated by digestion with *Msc*I and *Pvu*II, treatment with Klenow enzyme, and religation. The gene conferring Cm^R had to be inactivated to allow complementation with other Cm^R-bearing plasmids. Then, plasmid pPRcrtYB (a cDNA clone of *crtYB* in pBluescript; see Results) was digested with *Bam*HI and *Kpn*I and the fragment carrying the *crtYB* sequence was ligated into *Bam*HI + *Kpn*I-digested pPQE32 to obtain the plasmid pPQE-crtYB. The deletion in pPQE-crtYB/1878 was made by digestion of pPQE-crtYB with *Hin*dIII and religation. The vector pPQE-crtYB/1480 was obtained by PCR with the primer pair gs1 (5′-ACGGGAAAGCTTTCGGTAA-3′; position 1480 in *crtYB* cDNA) and gs2 (5′-CCCTTTCGTCTTCACCTC-3′; upstream of the multiple cloning site of pQE32) and pPQE-crtYB as template. The resulting fragment was treated with Klenow enzyme, digested with *Bam*HI, and ligated into pPQE32 which had been digested with *Hin*dIII, treated with Klenow enzyme and subsequently digested with *Bam*HI. The plasmid pPQE-crtYB/1280 was generated from pPQE-crtYB by digestion with *Sph*I and *Hin*dIII, Klenow treatment, and blunt-end religation. For construction of pPQE-crtYB\514, pPQE-crtYB was digested with *Acc*I, the overhang was partly repaired by Klenow treatment with dATP in the reaction mixture, and digested with *Kpn*I. This fragment was then ligated into pPQE-crtYB which had been digested with *Bam*HI and repaired by Klenow treatment with dATP, dGTP, and dTTP in the reaction mixture, and digested with *Kpn*I.

Complementation studies and carotenoid analysis

To synthesise GGPP, neurosporene or lycopene, for use as substrates by the *crtYB* gene product, the *E. coli* strain JM101 was transformed with pACCAR-crtE, pACCRT-EBI$_{Rc}$ or pACCRT-EBI$_{Eu}$, respectively. The pBluescript-derived plasmid pPRcrtYB was introduced into these carotenogenic hosts, together with the set of modified pQE32 vectors carrying the complete or deleted versions of the *crtYB* gene. After growth for 3 days at 28° C, cells were harvested and freeze-dried. Carotenoids were extracted either with methanol or acetone and partitioned into 50% ether in

Table 1 Strains and plasmids used in this study

Strain/plasmid	Genotype/relevant features[a]	Source/reference
Escherichia coli		
XL-Blue-MRF′	*Δ(mcrA)183Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[F' *proAB laq^{q}ZΔM15* Tn*10*	Gibco BRL
JM101	*supE thi Δ(lac-proAB)* F'[*traD36 proAB$^+$ lacI^{q}lacZ*ΔM15]	Messing (1983)
X. dendrorhous (*Phaffia rhodozyma*)		
CBS 6938	Wild type	
PR-1-102	White carotenoid biosynthesis mutant of *X. dendrorhous*	Girard et al. (1994)
Plasmids		
pACCAR25ΔcrtB	Insert carries genes encoding GGPP synthase (*crtE*), phytoene desaturase (*crtI*), lycopene cyclase (*crtY*), β-carotene hydroxylase (*crtX*), zeaxanthin glycosylase (*crtZ*) from *Erwinia uredovora* (Cm; **GGPP**)	Misawa et al. (1995)
pACCRT-E	Insert carries the *crtE* gene of *E. uredovora* (Cm; **GGPP**)	Misawa et al. (1994)
pACCRT-EBI$_{Eu}$	Insert carries the *crtE*, *crtB* and *crtI* genes of *E. uredovora* (Cm; **lycopene**)	Misawa et al. (1995)
pACCRT-EBI$_{Rc}$	Insert carries *crtE* and *crtB* genes of *E. uredovora* and the *crtI* gene of *Rhodobacter capsulatus* (Cm; **neurosporene**)	Linden et al. (1991)
pMTL22P	Cloning vector (Ap)	Chambers et al. (1988)
pPR2T	Transformation vector for *X. dendrorhous* (Ap, G418)	Wery et al. (1998)
pPR2TN	Derivative of pPR2T with additional unique cloning sites (Ap, G418)	This study
pPR10F/R	Contains the 4.5-kb *Eco*RI fragment, isolated from pPRcrtYBcos1, carrying the *crtYB* gene in pUC19 (Ap)	This study
pPR21	Contains the *crtYB* gene of *X. dendrorhous* as a 4.5-kb *Bam*HI fragment in pMTL22P (Ap)	This study
pPR22	Contains the 4.5-kb *Bam*HI fragment from pPR21 in the *Bam*HI site of pPR2TN (Ap, G418)	This study
pPRcrtYBcos1	Clone carrying the complete *crtYB* gene (Ap), isolated from the genomic library of *X. dendrorhous*	This study
pPRcrt1	Contains a cDNA insert encoding a putative lycopene cyclase of *X. dendrorhous* in pBluescript (Ap)	This study
pPRcrt2-4	Contains a cDNA encoding a putative phytoene synthase of *X. dendrorhous* in pBluescript (Ap)	This study
pPRcrtYB	Contains a cDNA encoding the phytoene synthase/lycopene cyclase of *X. dendrorhous* in pBluescript (Ap)	This study
pQE32	Cloning vector for *E. coli* (Ap, Cm)	Qiagen
pQE	Derivative of pQE32 with an inactivated Cm resistance gene (Ap)	This study
pPQE-crtYB	Contains a 2.5-kb *Bam*HI-*Kpn*I fragment of pPRcrtYB in the corresponding sites of pQE (Ap)	This study

[a] The selective antibiotics are indicated in *parentheses*; the intermediates of the carotenoid biosynthetic pathway that accumulate in each case are indicated in *bold*

petroleum. The solvent was evaporated, the residue dissolved in acetone or methanol and the carotenoids separated and quantified by HPLC on a Nucleosil C_{18} 3 μm column using acetonitrile:methanol:2-propanol (85:10:5, v/v/v) as the mobile phase. The flow rate was 1 ml/min and the absorbance spectrum was recorded on-line with a Waters 440 photodiode detector. Identification and quantification of carotenoids was carried out by reference to authentic standards.

Results

Isolation of a cDNA clone encoding lycopene cyclase from *X. dendrorhous*

A heterologous complementation approach in a genetically modified *E. coli* strain was applied to clone the lycopene cyclase-encoding gene of *X. dendrorhous*. A cDNA library was constructed using RNA from an astaxanthin-overproducing *X. dendrorhous* strain (Verdoes et al. 1997), and introduced into the lycopene-accumulating *E. coli* strain XL[pACCRT-EBI$_{Eu}$]. It was expected that introduction of a lycopene cyclase-encoding cDNA in XL[pACCRT-EBI$_{Eu}$] would result in a yellow colony, as the red coloured lycopene in the host strain would be converted into β-carotene by the enzyme.

The entire cDNA library of *X. dendrorhous* was introduced into XL[pACCRT-EBI$_{Eu}$] and cells were spread on selective LB agar plates. One out of the approximately 15,000 colonies was found to have a yellow phenotype. The corresponding plasmid, named pPRcrt1, carrying the *X. dendrorhous* cDNA encoding the putative lycopene cylase, was isolated and introduced into JM101[pACCRT-EBI$_{Eu}$]: all transformants had a yellow phenotype. Carotenoids were extracted from one of these JM101[pACCRT-EBI$_{Eu}$ /pPRcrt1] transformants and analysed by HPLC (Fig. 1). Cyclisation of lycopene to γ-carotene with one ionone end group and also to β-carotene with two rings was observed (Fig. 1A). The HPLC profile shows, in addition to the substrate lycopene, the accumulation of γ-carotene, with absorbance maxima and shoulders at 437, 460 and 495 nm,

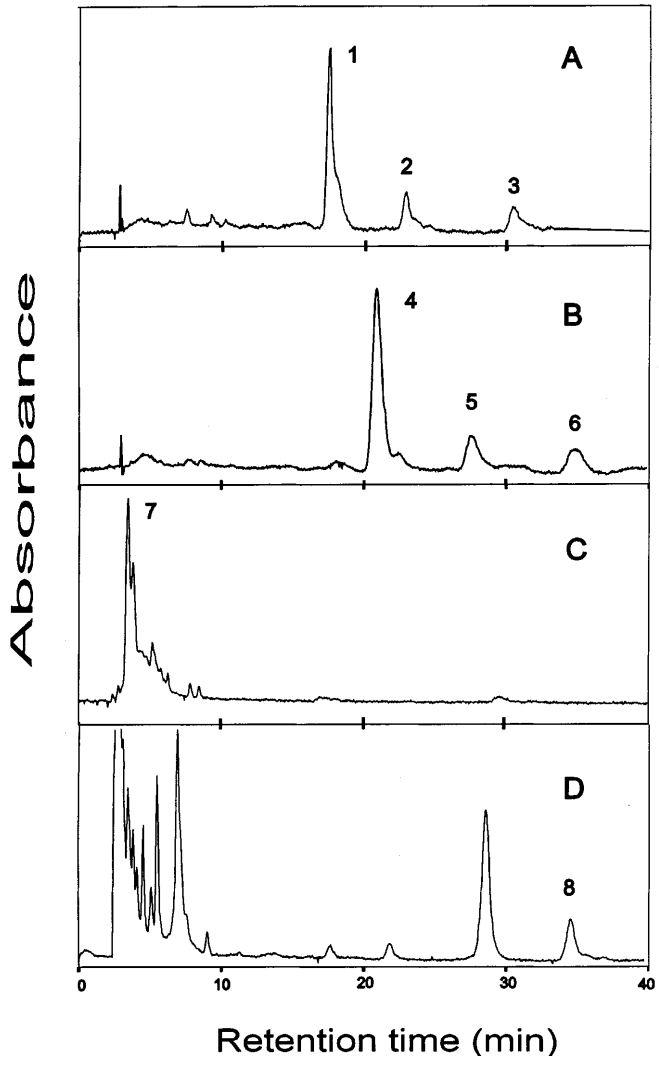


Fig. 1A–D HPLC profile of carotenoid products formed on expression of *X. dendrorhous* cDNAs encoding a putative lycopene cyclase (pPRcrt1) or a putative phytoene synthase (pPRcrt2) in different carotenogenic strains of *E. coli* JM101. pPRcrt1 was co-expressed together with pACCRT-EBI$_{Eu}$ (**A**) or pACCRT-EBI$_{Rc}$ (**B**), and pPRcrt2 was expressed together with pACCAR25ΔcrtB (**C**) and pACCRT-E (**D**). Similar results were obtained when pPRcrt1 or pPRcrt2 was replaced by pPRcrt2 or pPRcrt1, respectively. Co-chromatography was used to identify peak 1 with lycopene, peak 2 with γ-carotene, peak 3 with β-carotene, peak 4 with neurosporene, peak 5 with β-zeacarotene, peak 6 with 7,8-dihydro-β-carotene, peak 7 with zeaxanthin diglucoside and peak 8 with phytoene

and β-carotene with maxima at 425, 450, and 475 nm, resulting from lycopene cyclase activity. In another complementation experiment using pACCRT-EBI$_{Rc}$, a neurosporene-accumulating background was established (Linden et al. 1993). Under these conditions introduction of the putative lycopene cyclase on the plasmid pPRcrt1 resulted in conversion of neurosporene to β-zeacarotene (shoulders or maxima at 407, 425 and 452 nm) and 7,8 dihydro-β-carotene with the same absorbance maxima as shown in the HPLC profile in Fig. 1B.

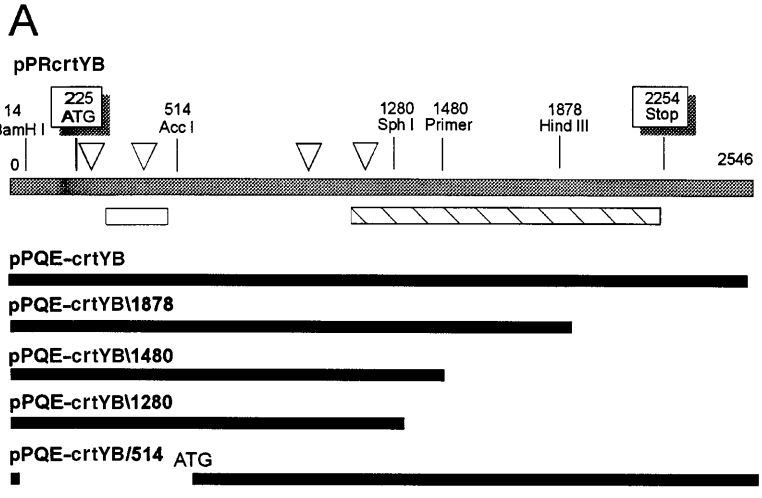
Functional assignment of a second catalytic function to the product of the lycopene cyclase-encoding cDNA

A restriction map of pPRcrt1 was constructed (Fig. 2) and the complete nucleotide sequence of the cDNA fragment was determined. The insert is 2502 nucleotides long, including a polyA tail of 20 nucleotides and an ORF encoding a 673-residue protein with a predicted molecular mass of 74.7 kDa. The deduced amino acid sequence was compared to protein sequences in the databases. Surprisingly, the C-terminal part (amino acids 295–673) of the polypeptide showed clear homology to phytoene synthases, but not to lycopene cyclases from other species. We hypothesised that in *X. dendrorhous* phytoene synthase and lycopene cyclase are produced as a hybrid protein. This hypothesis was tested in two ways. Firstly, we isolated independent cDNAs encoding phytoene synthase by heterologous complementation of another carotenogenic *E. coli* strain. Secondly, the effect of co-expression of the putative lycopene cyclase-encoding cDNA (pPRcrt1) on the carotenoid profile in other carotenogenic hosts was analysed.

Three yellow colonies were identified among 25,000 white colonies after the introduction of the cDNA library of *X. dendrorhous* into XL[pACCAR25ΔcrtB]. This *E. coli* host strain was obtained by introducing the plasmid pACCAR25ΔcrtB, which contains all the *Erwinia uredovora* genes required for the synthesis of zeaxanthin diglucoside (Misawa et al. 1995), except the phytoene synthase-encoding gene (*crtB*), inserted in XL-Blue-MRF'. By restriction analysis it was shown that the plasmids isolated from the three yellow colonies, which were named pPRcrt2–4 and carried a cDNA encoding a putative phytoene synthase, were identical to each other and were indistinguishable from the plasmid pPRcrt1, which harbours the cDNA encoding the putative lycopene cyclase. The formation of a biologically active phytoene isomer by the putative phytoene synthase and its subsequent conversion to desaturated products was demonstrated by HPLC analysis (Fig. 1C). In the yellow transformants of JM101[pACCAR25ΔcrtB/pPRcrt2], zeaxanthin diglucoside (absorption maxima and shoulders at 425, 450 and 475) was indeed produced.

Finally, the phytoene synthase function of the cDNA encoding the putative lycopene cyclase was demonstrated (Fig. 1D) by its expression in a GGPP-accumulating *E. coli* strain, JM101[pACCRT-E]. Introduction of pPRcrt1 into JM101[pACCRT-E] resulted in the total conversion of GGPP into phytoene (82.4 μg/g dry mass), which is absent in a control transformant carrying the plasmid without an insert. The phytoene peak had the typical main absorbance maximum at 285 nm with shoulders at 275 and 295 nm.

From these data, we conclude that in *X. dendrorhous* phytoene synthase and lycopene cyclase activities are encoded by one gene, now designated as *crtYB*.



B

C

	PHYTOENE SYNTHASE ACTIVITY	LYCOPENE CYCLASE ACTIVITY		
	Zeaxanthin diglucoside (μg/g dry mass)	Lycopene (μg/g dry mass)	γ-carotene	β-carotene
pPQE-crtYB	76.6	20.5	15.2	64.4
pPQE-crtYB\1878	12.2	71.2	16.6	12.2
pPQE-crtYB\1480	0	98.7	0	0
pPQE-crtYB\1280	0	94.3	0	0
pPQE-crtYB/514 ATG	21.2	89.6	0	0

Fig. 2A–C Analysis of phytoene synthase and lycopene cyclase activities after complementation with various *crtYB* deletion constructs. **A** Restriction map of the insert in pPRcrtYB. The numbers *above* the restriction sites refer to positions in the *crtYB* cDNA inserted in pBluescript (pPRcrt). The *triangles* indicate the positions of the introns in the genomic sequence. The *open boxes* indicate the regions of CrtYB that are homologous to other protein sequences (see Fig. 3). The deletion derivatives used to localise enzyme domains are indicated *below* the map. **B** Phytoene synthase activity encoded by the indicated deletion derivatives was assayed by measuring the formation of zeaxanthin diglucoside (μg/g dry mass) in *E. coli* JM101[pACCAR25ΔcrtB]. **C** Lycopene cyclase activity was determined from the rate of conversion of lycopene into the cyclic carotenoids γ- and β-carotene in *E. coli* JM101[pACCRT-EBI$_{Eu}$]

Sequence analysis of the *crtYB* gene

The CrtYB-encoding cDNA insert was used as a probe to screen a genomic cosmid library of *X. dendrorhous* (Verdoes et al. 1997) and several positive cosmids were detected. Southern analysis indicated that a single 4.5-kb *Eco*RI fragment contained the region of homology. This fragment was also detected on blots containing *X. dendrorhous* genomic DNA digested with *Eco*RI. This fragment was subcloned from one of the positive cosmids, named pPRcrtYBcos1, into pUC19, yielding pPR10F.

The complete nucleotide sequence of the *crtYB* gene and its flanking regions was determined (results not shown). Introns were localised by comparing the genomic and cDNA sequences. The structural gene includes four introns and consists of 2385 nucleotides. The intervening sequences were found in the N-terminal half of the polypeptide (Fig. 2) and varied in size between 72 and 111 nucleotides. The introns contained similar 5′, internal, and 3′ splice signal sequences to those found in other *X. dendrorhous* genes (Verdoes et al. 1997). The C-terminal domain of CrtYB showed clear homology to the phytoene synthases of bacteria, cyanobacteria and plants. In most organisms phytoene synthases are about 300–400 amino acids long, including targeting sequences. In *Neurospora crassa* the phytoene synthase is 602 residues long, similar to the size of the *X. dendrorhous* enzyme (673 amino acids). Although the degree of overall sequence homology between CrtYB and other phytoene synthases is low, regions with significantly higher sequence homology can be observed (Fig. 3A). Its closest relative is the phytoene synthase of *N. crassa*, which was found to have 28% homology (17% identity) over the complete length of the protein (Fig. 3A). In addition, clear homology (29% homology; 18% identity) was found between the first 95 amino acids of CrtYB and Orf7 of the *Myxococcus xanthus* carotenoid biosynthesis cluster (Fig. 3B; Botella et al. 1995).

Localisation of the phytoene synthase and lycopene cyclase domains by truncation of *crtYB*

The sections of the *crtYB* cDNA encoding the phytoene synthase and lycopene cyclase activities were localised by truncation from the 3′- and 5′-ends. First, the region with high homology to bacterial phytoene synthase-encoding genes (Fig. 3A, residues 295–673) was shortened by about 15, 30 and 70% by truncating the cDNA at positions 1878, 1480 and 1280 in the gene, as indicated in Fig. 2A. In the first case, phytoene synthesis, as measured by complementation with pACCAR25ΔcrtB and product analysis, decreased to about 30%; whereas deletions of the 3′-end from positions 1280 and 1480 resulted in complete loss of phytoene synthesis activity (Fig. 2B). In order to locate the region important for lycopene cyclisation within CrtYB, complementation with the deletion plasmids was also carried out in *E. coli* strains that accumulate lycopene, and the resulting cyclisations products γ-carotene and β-carotene were quantified (Fig. 2C). In the presence of the full-length *crtYB* cDNA about 80% of the lycopene was cyclised to γ-carotene and – preferentially – β-carotene. Of all the truncated plasmids only the one that extended to position 1878 yielded small amounts of the products, with almost equal levels of mono- and dicyclic carotenes. Truncation of the 5′-end of the *crtYB* cDNA at position 514 resulted in

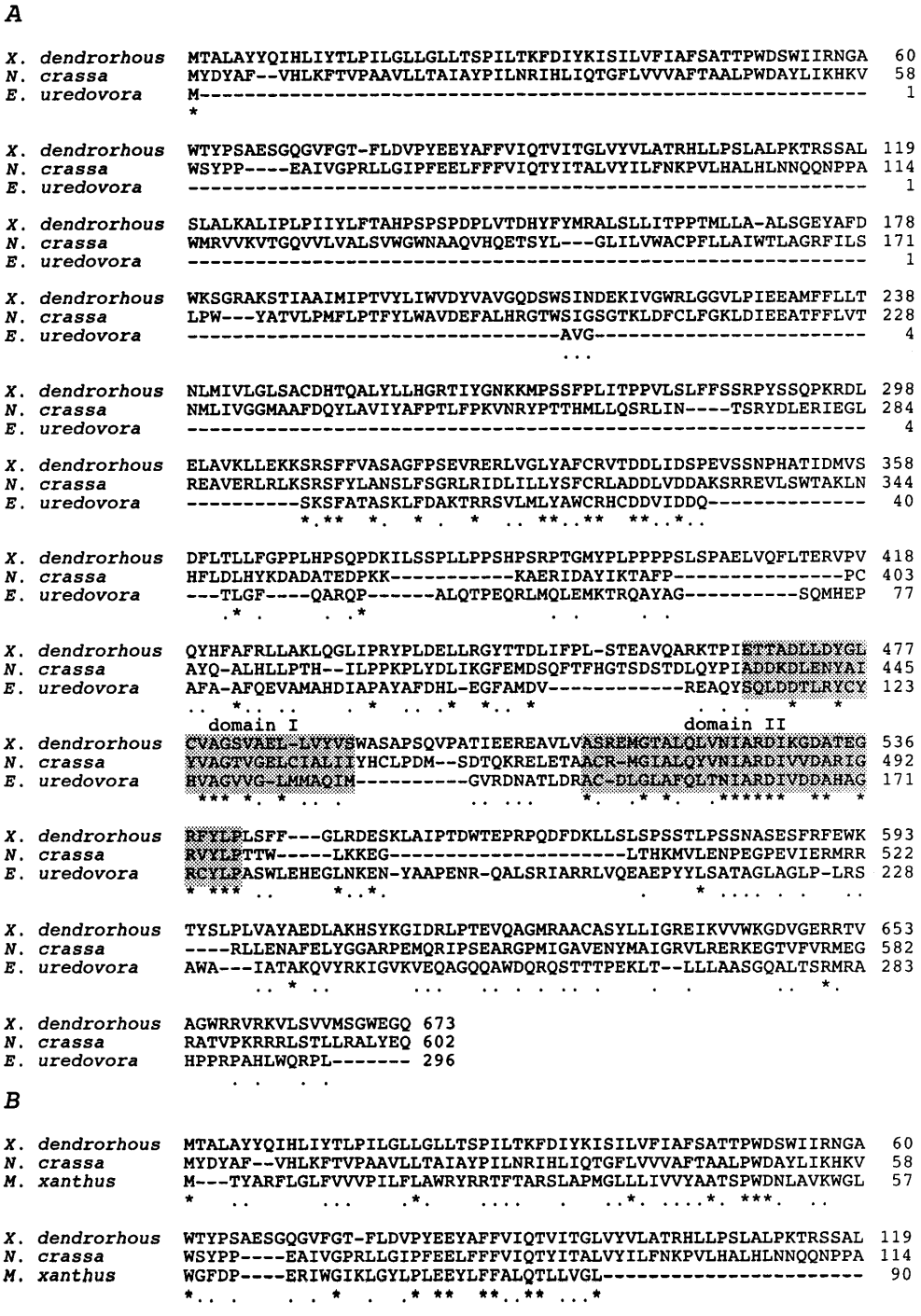


Fig. 3A Alignment of the deduced amino acid sequence encoded by the complete *crtYB* gene from *X. dendrorhous* with those of phytoene synthases from *N. crassa* and *E. uredovora*. **B** Comparison of the N-terminal domain of CrtYB from *X. dendrorhous* with those of the phytoene synthase from *N. crassa* and Orf7 from *M. xanthus*. Residues that are perfectly conserved or well conserved are indicated by asterisks and dots, respectively. Gaps introduced by the sequence analysis software are shown by *dashes*. Two well conserved domains that are also found in other phytoene synthases and squalene synthases are *boxed*. The genomic sequence of the *crtYB* gene of *X. dendrorhous* is available in the EMBL database under Accession No. AJ133646

total loss of lycopene cyclisation activity. This deletion also affected phytoene synthesis activity considerably and only about 30% of cyclisation products were obtained, compared to the full-length insert.

Manipulation of carotenoid biosynthesis in *X. dendrorhous*

The *crtYB* gene was used to complement a white mutant strain of *X. dendrorhous* named PR-1-102 (Girard et al. 1994). This strain, obtained after mutagenesis, showed no carotenoid biosynthesis and is most probably affected in phytoene synthase activity. The 4.5-kb *Eco*RI fragment containing the complete coding sequence of CrtYB was subcloned into a transformation vector for *X. dendrorhous*. This yielded the vector pPR22 (see Materials and methods), which was linearised with *Sfi*I and introduced into PR-1-102 by electroporation (Wery et al. 1998). A large number of G418-resistant, orange-coloured transformants were obtained. Several transformants were selected for further analysis. Southern

analysis of genomic DNA isolated from these transformants indicated that multiple copies of the transforming plasmid were present in each. Astaxanthin production could also be demonstrated by HPLC analysis (results not shown).

Discussion

The cloning and characterisation of a gene from *X. dendrorhous* that encodes two carotenoid biosynthetic activities is described. The gene, encoding phytoene synthase and β-carotene-synthesizing lycopene cyclase (CrtYB) of *X. dendrorhous* is designated *crtYB*. Both enzymatic activities were detected in *E. coli* after expression of the corresponding cDNA (Fig. 1). It is very unlikely that a splicing event at the mRNA level results in the synthesis of two distinct proteins. All lycopene cyclase or phytoene synthase encoding cDNAs (pPR-crt1–4) were isolated as full-length clones encoding both activities. Furthermore, Northern analysis of total RNA from *X. dendrorhous* indicated the presence of only one *crtYB* transcript of about 2.5 kb (results not shown). Although the possibility of post-translational modification in *X. dendrorhous* cannot be excluded, our finding that common regions of the protein are important for both activities (Fig. 2) indicates the expression of a single, hybrid protein.

The amino acid sequence deduced from the *crtYB* gene sequence was found to resemble in part known phytoene synthases from bacteria and plants (Sandmann 1994; Fig. 3). As described for *N. crassa* (Schmidhauser et al. 1994), the *crtYB* gene product includes a 5′-extension of about 300 amino acids which is absent in all other non-fungal phytoene synthase-encoding genes (Fig. 3). The 3′ region of *crtYB* is responsible for phytoene synthase activity and the 5′ part carries the coding region for the lycopene cyclisation activity (Fig. 2). Two well conserved domains, found in phytoene synthases as well as squalene synthases from other organisms (Botella et al. 1995), are also present in the phytoene synthase segment of CrtYB (Fig. 3). Deletion of this region results in the loss of all phytoene synthase activity, whereas the region downstream of this domain is important but not essential for enzymatic activity (Fig. 2). So far, despite several efforts, no lycopene-accumulating *X. dendrorhous* strain has been isolated. The unique characteristics of the lycopene cyclase-encoding gene of *X. dendrorhous* provide a further explanation for this failure, in addition to those discussed by Girard and coworkers (1994). Apparently, mutations in the *crtYB* gene much more frequently affect phytoene synthesis than lycopene cyclisation and therefore result in mutants that accumulate the colourless GGPP instead of the red lycopene.

The only sequence that shows similarity to the first 300 amino acid residues of CrtYB was found in the corresponding region of the phytoene synthase from *N. crassa* and the product of the *orf7* found in a carotenogenic gene cluster from *M. xanthus* (Botella et al. 1995). Assignment of functions to carotenogenic genes in *M. xanthus* was based on the analysis of the effect of transposon insertions and on sequence homology. Considering the significant homology with the lycopene cyclase of *X. dendrorhous* we postulate that Orf7 of *M. xanthus* is involved in cyclisation. Based on the accumulation of non-cyclic carotenoids in a mutant bearing a Tn*5* insertion in *orf6*, a carotene cyclase function was assigned to that gene (Botella et al. 1995). This would indicate the presence of two carotene cyclase-encoding genes in *M. xanthus*. Alternatively, the transposon in the gene encoding Orf6 might affect the transcription and/or translation of the gene encoding Orf7 and this could account for the fact that the insertion mutant accumulates non-cyclic carotenes. If only a single cyclase-encoding gene is present, the product of the gene designated *orf6* might be involved in an as yet unidentified enzymatic conversion step before the cyclisation reaction. The overall similarity between the sequences from *N. crassa* and *X. dendrorhous* considered here (Fig. 3) suggests that the phytoene synthase gene of *N. crassa* might also encode a bifunctional enzyme.

The *crtYB*-encoded lycopene cyclase of *X. dendrorhous* converts lycopene into the intermediate γ-carotene and further to β-carotene (Fig. 2). Recently it was shown that the non-homologous lycopene cyclases from the bacterium *E. uredovora* and the plant *Capsicum annuum* are also able to cyclise a 7,8-dihydro-ψ end group (Takaichi et al. 1996). In the complementation experiments with *crtYB,* we observed a similar phenomenon. Neurosporene was converted to the monocyclic β-zeacarotene (cyclisation of its ψ end group) and the resulting 7,8-dihydro-ψ end group was converted to 7,8-dihydro-β-carotene (Fig. 1B). This may well be a general feature of all lycopene cyclases.

The bifunctional nature of CrtYB is quite distinct from that of other carotenogenic enzymes, like phytoene desaturases or lycopene cyclases, which catalyse multiple desaturation sequences (Linden et al. 1991) or cyclisation of both ends of a symmetrical molecule (Schnurr et al. 1996), respectively. To date, the similarities among the lycopene cyclase genes from bacteria and plants are very low (Hugueney et al. 1995). A third type of lycopene cyclase that synthesises cyclic carotenoids must be postulated to occur in the cyanobacterium *Synechocystis* 6803. Although the genome of this strain has been sequenced completely (Kotani et al. 1995) no gene related to known lycopene cyclase genes could be detected. However, the N-region of CrtYB is completely unrelated to all known lycopene cyclases. This part of CrtYB may therefore have a common ancestor with another unrelated lycopene cyclase gene.

Fusion of genes may occur as a result of recombination processes. An example of how a carotenogenic fusion gene could originate by chromosomal rearrangement was recently discovered in a strain of *Rubrivivax gelatinosous* (Ouchiane et al. 1997). Based on the results of Ouchiane et al. (1997) and our results shown in

Fig. 2, one can speculate that the following scenario could account for the development of the bifunctional *crtYB* gene in *X. dendrorhous*. It is proposed that in a first step, the section of a gene encoding the active site of a lycopene cyclase was fused to the gene encoding phytoene synthase. In the resulting enzyme, the structure of phytoene synthase is essentially conserved. This phytoene synthase-related part of the fusion protein with its tertiary structure and its membrane-spanning regions were important in integrating the new bifunctional enzyme into the membrane and retaining a favourable conformation for catalysis. Such an event could have occurred in an ancestor of basidiomycetous and ascomycetous fungi.

In contrast to the case in *N. crassa,* where a broad range of colour mutants have been isolated and characterised, lycopene accumulates in various carotenoid-deficient mutants of the Mucorales (Cerda-Olmedo and Torres-Martinez 1979; Mehta and Cerda-Olmedo 1995; Ruiz-Hidalgo et al. 1997) – from which the Ascomycetes and Basidiomycetes evolved. This may be taken as an indication that in some Mucorales a distinct lycopene cyclase gene could exist. However, mutations affecting lycopene cyclase in *Phycomyces* are closely linked to a special albino phenotype which is characterized by the lack of all carotenoids and could be caused by a mutation in phytoene synthase (Torres-Martínez et al. 1980). This may hint at the presence of a combined phytoene synthase/lycopene cyclase, as in *X. dendrorhous*, and makes it very interesting to find out whether this gene from Mucorales is organised similarly to *crtYB* or is related only to the lycopene cyclase-encoding part of *crtYB*. The answers to these questions should provide a better understanding of the phylogeny of *crtYB* and the evolution of lycopene cyclase genes.

Acknowledgements This work was supported by a grant to G.S. from the Deutsche Forschungsgemeinschaft. We thank Dr P. Girard for the carotenoid biosynthetic mutant PR-1-102 of *X. dendrorhous*.

References

An G-H, Bielich J, Auerbach R, Johnson EA (1989) Isolation and characterization of carotenoid hyperproducing mutants of yeast by flow cytometry and cell sorting. Biotechnology 9:70–73

Andrews AG, Starr MP (1976) (3R,3′R)-Astaxanthin from the yeast *Phaffia rhodozyma*. Phytochem 15:1009–1011

Armstrong GA (1997) Genetics of eubacterial carotenoid biosynthesis: a colorful tale. Annu Rev Microbiol 51:629–659

Botella JA, Murilla FJ, Ruiz-Vazquez R (1995) A cluster of structural and regulatory genes for light-induced carotenogenesis in *Myxococcus xanthus*. Eur J Biochem 233:238–248

Cerdá-Olmedo E, Torres-Marinez S (1979) Genetics and regulation of carotene biosynthesis. Pure Appl Chem 51:631–637

Chambers SP, Prior SE, Barslow DA, Minton NP (1988) The pMTL *nic*⁻ I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene 68:139–149

Girard P, Falconnier B, Bricout J, Vladescu B (1994) β-Carotene producing mutants of *Phaffia rhodozyma*. Appl Microbiol Biotechnol 41:183–191

Golubev W (1995) Perfect state of *Rhodomyces dendrohous* (*Phaffia rhodozyma*). Yeast 11:101–110

Hugueney P, Badillo A, Cehn H-C, Klein A, Hirschberg J, Camara B, Kuntz M (1995) Metabolism of cyclic carotenoids: a model for the alteration of this biosynthetic pathway in *Capsicum annuum* chromoplasts. Plant J 8:417–424

Johnson EA, Schroeder WA (1995) Microbial carotenoids. In: Fiechter A (ed) Adv Biochem Eng Biotechnol 53:119–178

Kotani H, Tanaka A, Kaneko T, Sato S, Sugiura M, Tabata S (1995) Assignment of 82 known genes and gene clusters on the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. DNA Res 2:133–142

Krinsky NI (1994) The biological properties of carotenoids. Pure Appl Chem 66:1003–1010

Linden H, Misawa N, Chamovitz D, Pecker I, Hirschberg J, Sandmann G (1991) Functional complementation in *Escherichia coli* of different phytoene desaturase genes and analysis of accumulated carotenes. Z Naturforsch 46c:160–166

Linden H, Vioque A, Sandmann G (1993) Isolation of a carotenoid biosynthesis gene coding for ζ-carotene desaturase from *Anabaena* PCC7120 by heterologous complementation. FEMS Microbiol Lett 106:99–104

Mehta BJ, Cerdá-Olmedo E (1995) Mutants of carotene production in *Blakeslea trispora*. Appl Microbiol Biotechnol 42:836–838

Messing J (1983) New vectors for M13 cloning. Methods Enzymol 101:20-78

Misawa N, Truesdale MR, Sandmann G, Fraser PD, Bird C, Schuch W, Bramley P (1994) Expression of a tomato cDNA coding for phytoene synthase in *Escherichia coli*, phytoene formation in vivo and in vitro, and functional analysis of the various truncated gene products. J Biochem 116:980–985

Misawa N, Satomi Y, Kondo K, Yokoyama A, Kajiwara S, Daito T, Ohtanai T, Miki W (1995) Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. J Bacteriol 177:6575–6584

Nonomura AM (1989) Industrial biosynthesis of carotenoids. In: Krinski NI, Mathews-Roth MM, Taylor RF (eds) Carotenoid chemistry and biology. Plenum Press, New York, pp 365–375

Ouchaine S, Picaud M, Vernotte C, Astier C (1997) Photooxidative stress stimulates illegitimate recombination and mutability in carotenoid-less mutants of *Rubrivivax gelatinosus*. EMBO J 16:4777–4787

Ruiz-Hidalgo MR, Benito EP, Sandmann G, Eslava AP (1997) The phytoene dehydrogenase gene of *Phycomyces*: regulation of its expression by blue light and vitamin A. Mol Gen Genet 253:734–744

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sandmann G (1994) Carotenoid biosynthesis in microorganisms and plants. Eur J Biochem 223:7–24

Schmidhauser T, Lauter F, Schumacher M, Zhou W, Russo VEA, Yanofsky C (1994) Characterization of *al-2*, the phytoene desaturase gene of *Neurospora crassa*: cloning, sequence analysis and photoregulation. J Biol Chem 269:12060–12066

Schnurr G, Misawa N, Sandmann G (1996) Expression, purification and properties of lycopene cyclase from *Erwinia uredovora*. Biochem J 315:869–874

Takaichi S, Sandmann G, Schnurr G, Satomi Y, Suzuki A, Misawa N (1996) The carotenoid 7,8-dihydro-ψ end group can be cyclized by the lycopene cyclases from the bacterium *Erwinia uredovora* and the higher plant *Capsicum annuum*. Eur J Biochem 241:291–296

Torres-Martínez S, Murillo FJ, Cerdá-Olmedo E (1980) Genetics of lycopene cyclization and substrate transfer in ß-carotene biosynthesis in *Phycomyces*. Genet Res 36:299–309

Verdoes JC, Wery J, Boekhout T, van Ooyen AJJ (1997) Molecular characterization of the glyceraldehyde-3-phosphate

dehydrogenase gene of *Phaffia rhodozyma*. Yeast 13:1231–1242

Verdoes JC, Misawa N, van Ooyen AJJ (1999) Cloning and characterization of the astaxanthin biosynthetic gene encoding phytoene desaturase of *Xanthophyllomyces dendrorhous*. Biotechnol Bioeng 63:750–755

Wery J, Gutker D, Renniers ACHM, Verdoes JC, van Ooyen AJJ (1997) High-copy-number integration into the ribosomal DNA of the *yeast Phaffia rhodozyma*. Gene 184:89–97

Wery J, Verdoes JC, van Ooyen AJJ (1998) Efficient transformation of the astaxanthin-producing yeast *Phaffia rhodozyma*. Biotech Technol 12:339–405