Biol Res 41: 93-108, 2008

Genomic organization of the structural genes controlling the astaxanthin biosynthesis pathway of *Xanthophyllomyces dendrorhous*

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ABSTRACT

The cloning and nucleotide sequence of the genes (*idi*, *crtE*, *crtYB*, *crtI* and *crtS*) controlling the astaxanthin biosynthesis pathway of the wild-type ATCC 24230 strain of *Xanthophyllomyces dendrorhous* in their genomic and cDNA version were obtained. The *idi*, *crtE*, *crtYB*, *crtI* and *crtS* genes were cloned, as fragments of 10.9, 11.5, 15.8, 5.9 and 4 kb respectively. The nucleotide sequence data analysis indicates that the *idi*, *crtE*, *crtYB*, *crtI* and *crtS* genes have 4, 8, 4, 11, and 17 introns and 5, 9, 5, 12 and 18 exons respectively. In addition, a highly efficient site-directed mutagenesis system was developed by transformation by integration, followed by mitotic recombination (the double recombinant method). Heterozygote *idi* (*idi*$^{+}$/*idi*$^{-}$::*hph*), *crtE* (*crtE*$^{+}$/*crtE*$^{-}$::*hph*), *crtYB* (*crtYB*$^{+}$/*crtYB*$^{-}$::*hph*), *crtI* (*crtI*$^{+}$/*crtI*$^{-}$::*hph*) and *crtS* (*crtS*$^{+}$/*crtS*$^{-}$::*hph*) and homozygote mutants *crtYB* (*crtYB*$^{-}$::*hph*/*crtYB*$^{-}$::*hph*), *crtI* (*crtI*$^{-}$::*hph*/*crtI*$^{-}$::*hph*) and *crtS* (*crtS*$^{-}$::*hph*/*crtS*$^{-}$::*hph*) were constructed. All the heterozygote mutants have a pale phenotype and produce less carotenoids than the wild-type strain. The genetic analysis of the *crtYB, crtI* and *crtS* loci in the wild-type, heterozygote, and homozygote give evidence of the diploid constitution of ATCC 24230 strains. In addition, the cloning of a truncated form of the *crtYB* that lacks 153 amino acids of the N-terminal region derived from alternatively spliced mRNA was obtained. Their heterologous expression in *Escherichia coli* carrying the carotenogenic cluster of *Erwinia uredovora* result in trans-complementation and give evidence of its functionality in this bacterium, maintaining its phytoene synthase activity but not the lycopene cyclase activity.

Key terms: astaxanthin biosynthesis, *Xanthophyllomyces dendrorhous*, *Phaffia rhodozyma*.

INTRODUCTION

In nature, carotenoids are produced on a large scale: over 100 million tons per year. In addition, these pigments are synthesized in a wide variety of structures by plants, algae, bacteria and fungi. One of them, astaxanthin, is the principal carotenoid responsible for the orange-red color of marine invertebrates, fish, and birds and is primarily produced by phytoplankton and the red basidiomycetous yeast *Xanthophyllomyces dendrorhous* (Andrewes et al., 1976; Johnson and Lewis, 1979; Miller et al., 1976; Johnson, 2003).

In this yeast, astaxanthin, like other carotenoids, is a terpenoid pigment that is produced by the terpenoid biosynthetic pathway from the basic C5-isoterpene unit isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), produced by the isopentenyl pyrophosphate isomerase enzyme encoded by the *idi* gene. In a following step, DMAPP and IPP are condensed to geranyl pyrophosphate, farnesyl, and geranylgeranyl pyrophosphate (GGPP), possibly through the action of farnesyl pyrophosphate synthase and geranylgeranyl pyrophosphate synthase encoded by the *fps*

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Received: June 26, 2007. In Revised form: April 28, 2008. Accepted: May 5, 2008

and *crtE* genes, respectively. Two GGPP molecules are condensed to phytoene by the phytoene synthase enzyme encoded by the *crtYB* gene, representing the first specific step in the carotenoid biosynthesis. Subsequently, via four desaturations by the action of the phytoene desaturase encoded by the *crtI* gene, lycopene is produced, which is converted to β-carotene by the lycopene cyclase activity encoded by the *crtYB* gene after two cyclization reactions. Finally β-carotene is oxidized to astaxanthin by the product of the *crtS* gene.

The *crtYB* and *crtI* genes were isolated from a cDNA library of the strain CBS 6938 of *X. dendrorhous* by heterologous complementation in *Escherichia coli* strains carrying carotenoid biosynthesis genes from the bacteria *Erwinia uredovora* (Verdoes et al., 1999a, Verdoes et al., 1999b). In addition, in the wild-type ATCC 24230 strain of *X. dendrorhous,* two unexpected cDNA of *crtYB* and *crtI* genes have been isolated that correspond to alternative spliced variants, which have numerous stop codons in their sequence (Lodato et al., 2003). The ratio of mature to alternative mRNA for the *crtI* gene decreased as a function of the age of the culture, while the carotenoids content increased (Lodato et al., 2003). Furthermore, the expression at the mRNA level of the carotenoid biosynthesis *idi*, *crtE*, *crtYB*, *crtI,* and *crtS* genes from *X. dendrorhous* was studied by RT-PCR (Lodato et al. 2003; 2004 and 2007). However, little is known about the genomic organization of these genes in this yeast.

In this work, we cloned the *idi*, *crtE*, *crtYB*, *crtI,* and *crtS* genes controlling the astaxanthin biosynthesis pathway and their respective cDNAs and determined their genetic organization by sequence analysis and electrophoretic karyotyping. The function of the *crtYB*, *crtI,* and *crtS* genes was inferred from site-directed mutagenesis.

MATERIALS AND METHODS

Strains, media, plasmid and genomic library

All strains used in this study are listed in Table I and the plasmids in Table II.

The *X. dendrorhous* wild-type strain ATCC 24230 was used to obtain genomic DNA for the construction of the genome library and site-directed mutagenesis. *E. coli* strain DH-5α was used for the propagation of plasmids and the genomic libraries of *X. dendrorhous*. Three genomic libraries of wild-type *X. dendrorhous* were used in this study. The first one contains partially digested *Bam*HI DNA fragments inserted in the *Bam*HI site of the YIp5 vector. The other two partial genomic libraries contain *Eco*RV or *Pst*I DNA fragments in the *Eco*RV or *Pst*I sites of the Bluescript SK- plasmid respectively.

X. dendrorhous wild-type strain was grown at 22 °C in baffled flasks containing YM medium in an orbital shaker at 100 rpm.

The DNA fragment of 3558 bp containing the *crtI* gene of *X. dendrorhous* carried by pL22 plasmid was amplified by PCR using primers PHA1 (5' AAGCTTA AACTGACGTGCC TC 3') and PHA4 (5'GAATTCAGAAAGCAAGAAC 3').

The *E. coli*-transformed cells were plated on selective Luria-Bertani agar plates (Sambrook et al., 1989) and incubated at 37°C overnight. These plates contained ampicillin (100 μg/ml) for plasmid selection and 40 μl of a 2% solution of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for selection of recombinant clones. The *X. dendrorhous* transformed cells were plated on YM containing 10 μg/ml of hygromicin B.

cDNA synthesis and cloning

For the reverse transcription (RT) reaction, total RNA was purified from cellular pellets obtained from 40 ml aliquots of culture by a modified protocol of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987; Lodato et al., 2004). The total RNA concentration was quantified at 260 nm, and the integrity of the RNA was checked in denaturant agarose gel electrophoresis. The RNA sample was then treated with 1U of DNase I (Roche) in 2.5 mM MgCl2 at 25ºC for 30 min. The reaction was stopped by the addition of EDTA at 2.5 mM final concentration and heating at 65ºC for 15

min. The RT reaction was performed in 25 µl final volume with 3 µg of total RNA, 75 pmoles oligodT15-18, 0.5 mM of dNTPs and 200 U of M-MLV reverse transcriptase (Promega) (Lodato et al., 2003, 2004). The reaction mixture was incubated at 42°C for 60 min and then heated to 65°C for 10 min. For the amplification of double-stranded cDNA of carotenoid biosynthesis genes, PCR reactions were performed according to the experimental conditions previously described (Lodato et al., 2003, 2004) in a final volume of 25 µl containing 2 U of *Pfu* polimerase (Stratagene), 2.5 µl of 10 X *Pfu* buffer, 0.5 µl of 10 mM dNTPs, 1 µl of 50 mM MgC_{l2}, 1 µl of 25 µM of each primer, and 2 µl of RT reaction product, and the final volume was adjusted with nuclease-free water. Amplification was performed in a DNA Thermal Cycler 2400 (Perkin-Elmer) or 2720 ABI Thermal cycler as follows: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, synthesis at 72°C for 3 min and a final extension step at 72°C for 10 min.

TABLE I

Strains of *E. coli* and *X. dendrorhous* created and used in this study

Strains	Genotype or relevant features	Source of reference
E. coli		
DH-5α	F$^-$ ϕ80d *lac*ZΔM15Δ(*lac*ZY-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_k^- m_k^+) *pho*A *sup*E44l- *thi*-1 *gyr*A96 *rel*A1	Sambrook et al.
DS1B	β-carotene producer strain with a yellow phenotype which corresponds to BL21-Gold strain carrying pDS1B	This work
DS1B-ΔcrtB	strain BL21-Gold carrying the plasmid pDS1B-ΔcrtB.	This work
DS1B-ΔcrtY	Strain with a red phenotype, derived from BL21-Gold strain carrying the plasmid pDS1B-ΔcrtBY.	This work
X. dendrorhous		
ATCC 24230	Wild-type	ATCC
T-5	Heterozygote transformant from ATCC 24230 containing an allele of crtI gene with a deletion of 1.1 kb.	This work
T-B2	Homozygote transformant for the 1.1 kb deletion from T-5.	This work
T-I21H	Heterozygote transformant from ATCC 24230 containing an insertion of hph cassette of hygromicin resistance in the ctrI locus.	This work
T-I21H1H	Homozygote double recombinant from T-21H resistant to hygromicin B.	This work
T-YBH1	Heterozygote transfomant resistant to hygromiciun B, containing a partial deletion of crtYB gene and the insertion of the cassette hph	This work
T-YBHH2	Homozygote double recombinant, hygromicin B-resistant, from T-YBH.	This work
T-SH1	Heterozygote hyhBr obtained by transformation of the wild-type strain of *X. dendrorhous* with the cassette crtS::hph.	This work
T-SHH2	Homozygote double recombinant hygBr obtained by MDR from T-SH	This work

TABLE II

Plasmids built and used in this study

Plasmid	Genotype or relevant features	Source of reference
pBluescript SK-	ColE1 ori; Amp^r; cloning vector with blue-white screen	Stratagene
pL22	pBluescript with a 3558 bp PCR product containing the *crtI* gene of *X. dendrorhous*, in the *Eco*RV site.	This work
pL22ΔRV	pL22 containing a deletion of an internal 1.1 kb *Eco*RV fragment of the *crtI* gene	This work
pBAD33	P15 ori; Cam^r; cloning vector	Beckwith Lab.
pDS1B	pBAD33 vector carrying the carotenogenesis genes *crtE*, *crtB*, *crtI* and *crtY* and *crtX* from *Erwinia uredovora*.	This work
pDS1B-Δ*crtB*	Plasmid pDS1B with a deletion of the Eu-*crtB* gene	This work
pDS1B-Δ*crtY*	Plasmid pDS1B with a deletion of the Eu-*crtY* gene	This work
pXD-t*crtYB*	pETOPO expression vector containing cDNA of the truncated *crtYB* gene from alternatively spliced mRNA	This work
pET101/D-TOPO	ColE1 ori; Amp^r; expression vector	Invitrogene
pETOPO-CI	pET101/D-TOPO plus an unspecific DNA fragment	This work
pMN-*hph*	pBluescript SK-containing, in the *Eco*RV site, a cassette of 1.8 kp bearing the *E. coli*-resistant Hygromycin B (*hph*) gene under EF-1a promoter and GPD transcription terminator of *X. dendrorhous* respectively	Niklitschek, 2007
pXD-idi	pBluescript carrying a 4.8 kb *Xho*I fragment containing the *idi* gene of *X. dendrorhous*.	This work
pXD-I10	pBluescript containing a 11.5 kb *Bam*HI DNA fragment carrying the *crtE* gene of *X. dendrorhous*.	This work
pXD-C13	pBluescript containing a 15.8 kb *Bam*HI DNA fragment carrying the *crtI* gene of *X. dendrorhous*.	This work
pXD-33.13	pBluescript containing a 5.9 kb *Eco*RV DNA fragment carrying the *crtYB* gene of *X. dendrorhous*	This work
pXD-C19	pBluescript containing a 4 kb *Pst*I DNA fragment carrying the *crtS* gene of *X. dendrorhous*.	This work
pXD-*idi*::*hph*	pXD-idi with a deletion of a *Hpa*I 1245 bp fragment of the *idi* gene of *X. dendrorhous* and insertion of *hph* cassette from pMN-*hph*.	This work
pXD-*crtE*::*hph*	pXD-I10 with a deletion of an *Eco*RV 1984 bp fragment of *crtE* gene of *X. dendrorhous* and inserted cassette *hph* from pMN-*hph*.	This work
pXD-*crtYB*::*hph*	pXD-33.13 with a deletion of a *HpaI* 1245 bp fragment of the *crtYB* gene of *X. dendrorhous* and an insertion of *hph* cassette from pMN-*hph*.	This work
pXD-*crtI*::*hph*	pL22 with a deletion of a *Eco*RV 1127 bp fragment of the *crtI* gene of *X. dendrorhous* and insertion of *hph* cassette from pMN-*hph*.	This work
pXD-*crtS*::*hph*	pXD-C19 with a deletion of a *Stu*I-*Hpa*I 2479 bp fragment of the *crtS* gene of *X. dendrorhous* and an insertion of *hph* cassette from pMN-hph.	This work

The amplicons of the five genes were separated by electrophoresis in 1% agarose gels in TAE buffer containing 0.5 μg/ml of ethidium bromide followed by purification of each DNA fragment using glassmilk (Boyle and Lew, 1995), mixed with plasmid pBluescript SK previously digested with *Eco*RV and treated with T4 DNA ligase for 16 h at 14°C. Individual ligation mixture reactions for each gene were used to electroporate *E. coli* DH-5α, plated onto LB agar containing 100 μg/ml ampicillin and X-gal. Transformants were selected as white ampicillin-resistant colonies.

DNA sequence

The nucleotide sequences were determined using a DYEnamic ET terminator Kit Amersham Bioscience in an ABI 3100 Avant genetic analyzer. Each DNA fragment was sequenced by both strands, and the ambiguities were resolved by new sequencing. The sequence data was analyzed using the University of Wisconsin Genetics Computer Group package, version 10.0 (Devereux et al., 1984) and the CLUSTAL W program, version 1.8. (Thompsom et al., 1994).

Pulse field gel electrophoresis

Intact chromosomal DNA of *X. dendrorhous* was prepared in agarose plugs from cells grown to stationary phase in 100 ml of YM medium, washed twice with 50 mM EDTA pH 7.5 and suspended in 1 ml of a solution containing 10 mg Lysing enzyme per ml of 50 mM EDTA, as previously described (Cifuentes et al., 1997). The chromosomal DNA was separated by contour-clamped homogeneous electric fields (CHEF) in a LKB 2015 pulsaphor on a 1% agarose gel at 14°C in 0.1 M TBE buffer or in a Bio Rad DR II equipment on a 0.85% agarose gel at 14°C in 0.5 X TBE respectively (Sambrook et al., 1989).

Southern blot hybridization

Probes were labeled with [^{32}P]α-dCTP by the random primer technique and Southern blot hybridization was performed according to Sambrook et al. (1989).

Transformation of X. dendrorhous

The transformation of the wild-type strain of *X. dendrorhous* was performed by electroporation according to a previously described method (Wery et al., 1997). For the electrotransformation of 60 μl of electrocompetent cells, 10 to 20 μg of DNA was used. The electrocompetent cells were prepared from 200 ml YM culture grown until D.O.660 $_{nm}$ = 1.2. Cells were harvested at 5000 x g for 5 min, and the pellet was suspended in 25 ml of potassium phosphate buffer (50 mM potassium phosphate pH 7, 25 mM DTT) and incubated for 15 min at 21°C. The next step was at 4°C, using all the materials previously refrigerated. The cells were washed twice with 25 ml of STM buffer (270 mM sucrose, 10 mM Tris HCl pH 7.5, 1 mM MgC^{l}). Subsequently, the cells were suspended with 500 μl of STM buffer at 4°C and stored at -70°C. The electroporation conditions were 25 μF, 1000 Ω, 800 V using a BioRad Gene Pulser X Cell with PC and CE Modules. The transformant DNA was linearized with restriction endonucleases according to the cassette type used. The transformant, hygromicin B-resistant colonies were selected on YM with 10 μg/ml hygromicin B. The transformants were identified as *X. dendrorhous* by the presence of dsRNA elements (Castillo and Cifuentes, 1994) and ITS1, 5.8 rRNA gene and ITS2 DNA sequences (Reyes et al., 2004).

RESULTS AND DISCUSSION

Cloning of the structural genes of carotenoids biosynthesis pathway

The structural genes of the astaxanthin biosynthesis pathway were isolated from the genomic libraries of *X. dendrorhous* by PCR using specific primers. Three recombinant plasmids carrying the genes *idi* (pA1), *crtE* (pI10), and *crtI* (pC13) in *Bam*HI fragments of 10.9, 11.5, and 15.8 kb respectively were isolated from a genomic library of *X. dendrorhous* in the YIp5 plasmid. No positive results were

obtained for the *crtYB* and *crtS* genes in this genomic library, possibly by the existence of an internal restriction site in these genes. The *Bam*HI inserts of each plasmid were transferred to the pBluescript SK vector and renamed as pXD-A1 (*idi*), pXD-I10 (*crtE*), and pXD-C13 (*crtI*) respectively (Fig. 1).

To determine a single restriction DNA fragment of *X. dendrorhous* containing either the *crtYB* or *crtS* genes, a genomic restriction map was carried out by hybridization experiments. For this, the DNA of *X. dendrorhous*, in duplicate, was digested with different restriction enzymes, separated by electrophoresis in 0.7% agarose gels, and transferred to a nylon membrane. After hybridization experiments, using radioactive probes for *crtYB* and *crtS* genes, we determined that the *crtYB* gene is contained in an *Eco*RV fragment of 5.9 kb and *crtS* in a 4 kb *Pst*I fragment (data not shown). Using this data, two recombinant plasmids containing a 5.9 kb *Eco*RV insert harboring the *crtYB* (pXD-33.13) and a 4 kb *Pst*I insert harboring the *crtS* (pXD-C19) genes were isolated from the partial genome libraries *Eco*RV and *Pst*I respectively (Fig. 1). In addition, using specific primers for the start and the end codon of each carotenogenesis gene, it was possible to synthesize, by RT-PCR, the respective cDNA of the *idi* (756 bp), *crtE* (1131 bp), and *crtS* (1809 bp) genes, and cloning in the *Eco*RV-restriction site of the pBluescript SK vector. On the other hand, the cDNA of the mature mRNA and the alternative splicing variants of the *crtYB* and *crtI* genes have been previously reported by our group (Lodato et al., 2003).

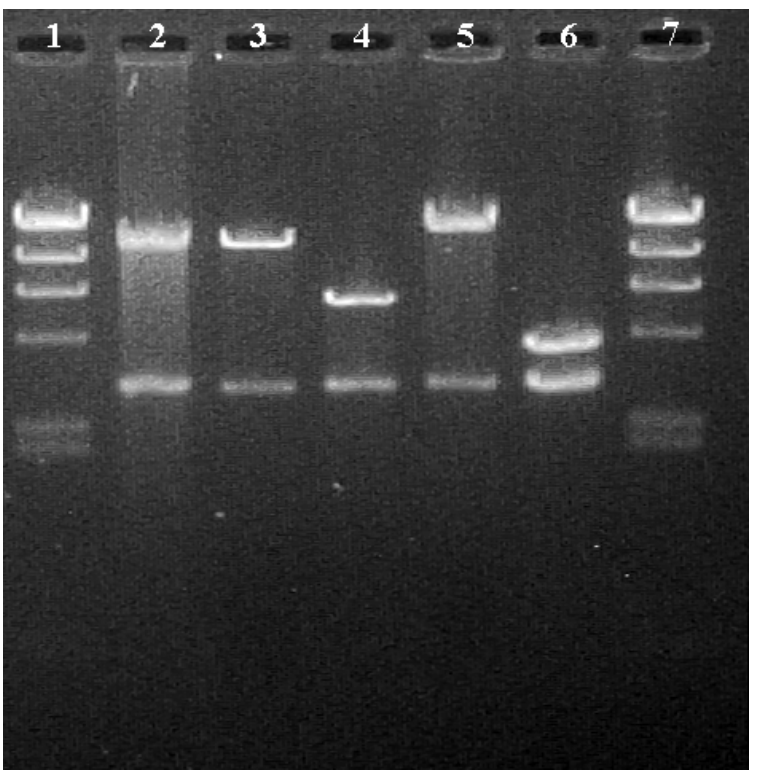

Figure 1: Agarose gel electrophoresis of recombinant plasmids carrying carotenoids biosynthesis genes.
Lanes 1 and 7, Lambda DNA/ *Hin*dIII as size standard; lane 2, pXD-A1 (*idi*); lane 3, pXD-I10 (*crtE*); lane 4, pXD33.13 (*crtYB*); lane 5, pXD-C13 (*crtI*); lane 6, pXD-C19 (*ast*). The plasmids pXD-A1, pXD-I10, and pXD-C13 were digested with *Bam*HI (lanes 2, 3, and 5). The plasmid pXD33.13 was digested with *Eco*RV (lane 4), and plasmid pXD-C19 was digested with *Pst*I (lane 6).

Localization of carotenoids biosynthesis genes in the electrophoretic karyotype

In order to study the genomic organization of the carotenoids biosynthesis genes *crtE*, *crtYB*, *crtI,* and *crtS* of *X. dendrorhous*, assays to determine the location of each gene in the electrophoretic karyotype of the yeast were performed. For this, the intact chromosomal DNA was separated by pulsed field electrophoresis (Cifuentes et al., 1997), using pulses of 90 seconds at 6 V/cm for 24 hours, followed by DNA transfer to nylon membranes and hybridization using radioactive probes of each gene. As is shown in Figure 2, the *crtE* and *crtI* genes were located in the chromosomal band I corresponding to a triplet (Cifuentes et al., 1997). The *crtYB* gene was located in the chromosomal band II, which also corresponds to a triplet. When a pulsed field gel electrophoresis was carried out using pulses of 500 seconds for 48 hours, followed by pulses of 250 seconds for 12 h at 6 V/cm – conditions that separate the chromosomes of the bands I and II – it was observed that the *crtYB* gene is located in two different chromosomal bands (Fig. 2B). In addition, the *crtS* gene was located in two chromosomal bands of the electrophoretic karyotype, using pulses of 90 seconds for 24 hours at 12 V/cm (Fig. 2C). These results could contribute to the evidence supporting the diploid condition and genetic polymorphism of the wild-type strain ATCC 24230 of *X. dendrorhous* as previously described (Hermosilla et al., 2003; Retamales et al., 1998). In fact, electrophoretic karyotype analysis of wild-type strains of *X. dendrorhous* shows a

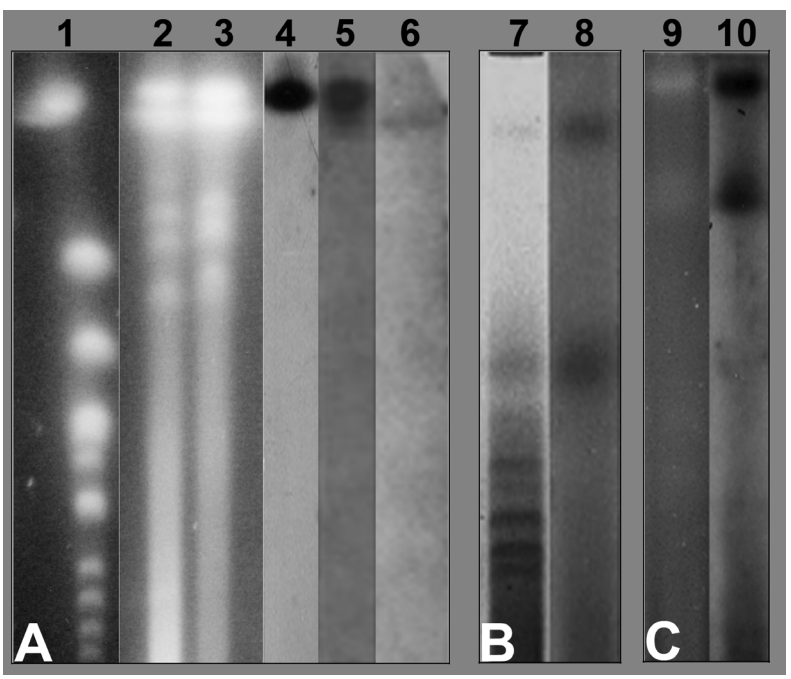

Figure 2: Assignment of cloned carotenoid biosynthesis genes in the chromosome bands of the electrophoretic karyotype of *X. dendrorhous*. (A) Lane 1, CHEF of *S. cerevisiae* AB1380; lanes 2 and 3, CHEF of *X. dendrorhous* ATCC 24230 strain; lane 4, hybridized with *crtE* gene probe; lane 5, hybridized with *crtI* gen probe; lane 6, hybridized with *crtYB* gene probe. (6 V/cm, pulse time 90 seconds for 24 hours) (B): CHEF of *X. dendrorhous* at 6 V/cm with pulse time of 500 seconds for 48 hours, followed by pulses of 250 seconds for 12 hours. Lane 7, ethidium bromide-stained CHEF (negative vision); lane 8, hybridized with *crtYB* gene probe. (C): CHEF of *X. dendrorhous* at 12 V/cm, pulse time of 90 seconds for 24 hours. Lane 9, ethidium bromide-stained CHEF; lane 10, hybridized with *crtS* gene probe.

large chromosomal polymorphism (Cifuentes et al., 1997; Nagy et al., 1997; Adrio et al., 1995) and could explain the fact that both the *crtYB* and *crtS* genes were located on two different chromosomal bands suggesting that their respective homologous chromosomes could be a polymorphic product of chromosomal re-arrangement.

Sequence analysis of the carotenoids biosynthesis genes

The genes *idi*, *crtE*, *crtYB*, *crtI,* and *crtS* and their respective cDNAs were completely sequenced, and the analysis of sequence allowed the establishment of the exon-intron organization (Fig. 3). According to the sequence data, the *idi* gene was located in a *Bam*HI fragment of 10,960 bp, containing 4 introns and 5 exons with a coding region of 756 bp corresponding to a peptide of 251 amino acids. The *crtE* gene was isolated in a *Bam*HI fragment of 11,504 bp, containing 8 introns, and their ORF contained 1,131 bp, which corresponds to a peptide of 376 amino acids. The *crtI* gene was isolated as a *Bam*HI segment of 15,778 bp, containing 11 introns and 12 exons with an ORF of 1,749 bp encoding a peptide of 582 amino acids. The *crtYB* gene is located in an *Eco*RV fragment of 5,917 bp; it contains 4 introns and 5 exons, and its ORF is 2,022 bp, and the peptide encoded has 673 amino acids. Finally the *crtS* gene is located in a *Pst*I fragment of 3,995 bp; it contains 17 introns and 18 exons, and its ORF has 1,674 bp corresponding to a peptide of 577 amino acids. The DNA sequences of the *idi*, *crtE*, *crtYB*, *crtI*, and *crtS* genes and the cDNA sequences from the mRNA of the *crtE* and *crtS* genes of the wild-type strain ATCC 24230 of *X. dendrorhous* have been deposited in the GenBank database under accession numbers DQ235686, DQ012943, DQ016503, DQ028748, DQ002006, DQ016502 and DQ002007, respectively.

On the other hand, the genes *crtYB* and *crtI,* in addition to the mature mRNA, produce variants of alternative splicing – amRNA *crtYB* and amRNA *crtI,* respectively – as previously reported by our group, suggesting that the splicing to mature or alternative messenger could regulate the cellular concentration of both enzymes depending on the physiological or environmental conditions (Lodato et al., 2003). In both genes, the variants of alternative splicing lead to the formation of premature stop codons in the translation of the three reading frames. However, a correct reading frame of both transcripts may be reestablished downstream from the start codon. In the case of the *crtI* messenger, if the translation could start internally in the alternative messenger, a truncated phytoene desaturase enzyme without 81 amino acids in the N-terminal of the protein could be synthesized from amRNA *crtI*. However, the N-terminal of the phytoene desaturase of *X. dendrorhous* has a dinucleotide binding motif that is conserved in the phytoene desaturases (Lodato et al., 2003), indicating that it is unlikely that an enzyme lacking 81 aminoacids in the N-terminal could remain active. In the case of the *crtYB* gene, the analysis of the nucleotide sequence of the amRNA indicates that downstream from the initial start codon, an open reading frame is re-established that could be translated to a protein that lacks the first 153 aminoacids of the N-terminal, which constitutes part of the lycopene cyclase domain of the enzyme phytoene-β-carotene synthase (Verdoes et al., 1999b). To determine if the truncated CRTYB has the lycopene cyclase or phytoene synthase activity, a heterologous complementary system was elaborated in *E. coli*. For this, three *E. coli* strains were constructed that carried three different carotenogenesis gene arrangements from *E. uredovora* (DS1B, having the wild-type cluster of carotenogenic genes of *E. uredovora*; DS1B- Δ*crtB* bearing a deletion of the *Eu-crtB* gene; and DS1B-Δ*crtY* bearing the cluster with a deletion of *Eu-crtY* gene, respectively). These were transformed with the pXD-t*crtYB* (Table II) vector carrying cDNA of the truncated version of the gene *crtYB* that specifies a protein of 520 aminoacids. Results show that the truncated gene may weakly complement the function of the phytoene synthase in the *E. coli* bearing a deletion of

the *Eu-crtB* of *E. uredovora*, but not lycopene cyclase in the *E. coli* strain carrying a deletion of the *Eu-crtY* gene of *E. uredovora* (Table III). These results suggest that the lack of the first 153 amino acids completely alters the lycopene cyclase domain of this bifunctional enzyme encoded by *crtYB* gene of *X. dendrorhous*. However, the phytoene synthase activity is maintained, although the levels of activity are reduced in comparison to the gene *Eu-crtB* of *E. uredovora*. If the alternative splicing of the mRNA of the gene *crtYB* fulfills a regulatory role in the carotenoids biosynthesis pathway of *X. dendrorhous*, the existence of a gene product with a single activity, phytoene synthase, could change the composition of intermediates and thus exert a regulatory role in the pathway, a mechanism which should be studied to identify all the processes involved in the carotenogenesis.

Additionally, a blast analysis of the deduced amino acid sequence of the astaxanthin synthase enzyme, the product of the *crtS* gene, shows a high level of similarity with cytochrome P450 hydroxylase. In other organisms that synthesize astaxanthin, such as the microalgae *H. pluvialis* and the bacteria *Agrobacterium auriantiacum* (Misawa et al., 1995) or *Paracoccus haeundaensis* (Lee and Kim, 2006), the step from β-carotene to astaxanthin using several ketocarotenoids as intermediates are controlled by two genes. The gene *crtW* that codes for the enzyme β-carotene ketolase catalyzes the introduction of the keto groups, while the gene *crtZ* that codes for the enzyme β-carotene hydroxylase catalyzes the incorporation of the hydroxyl groups in positions C3 and C3'. However, in *X. dendrorhous,* the step from β-carotene to astaxanthin is controlled by a single

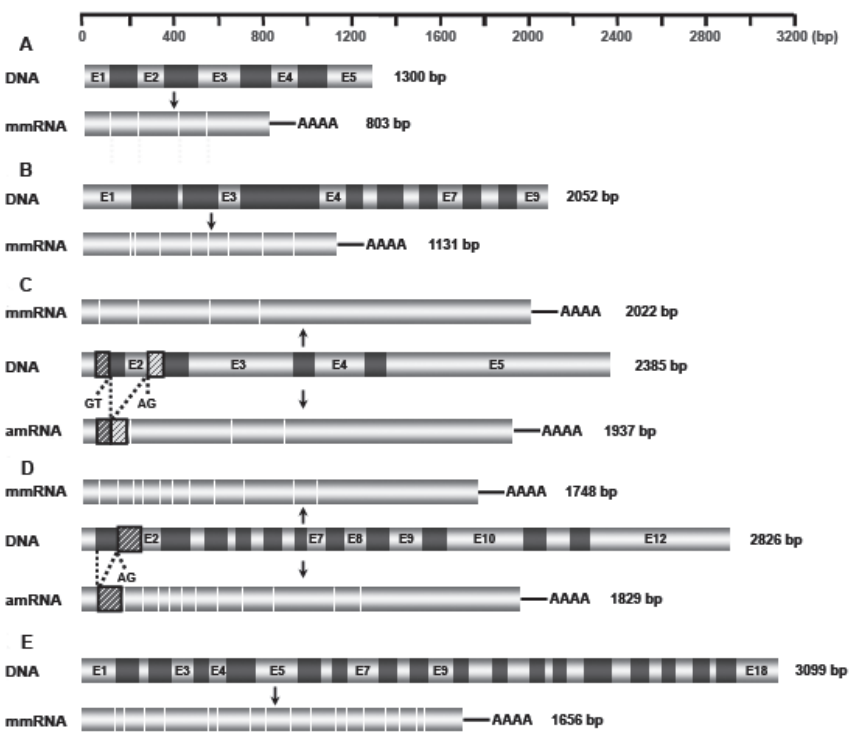

Figure 3: Schematic representation of the structure of genes controlling the astaxanthin biosynthesis pathway of *X. dendrorhous*. (A) *idi*; (B) *crtE*; (C) *crtYB*; (D) *crtI*; (E) *crtS*. DNA: genomic sequence of gene; mRNA: messenger RNA; mmRNA: mature messenger RNA; amRNA: alternative splicing RNA. Gray boxes: exon regions. Dark gray boxes: intron regions. Gray striped boxes: exon region involved in alternative splicing. Dark gray striped boxes: intron region involved in alternative splicing.

gene, *crtS,* that codes for the enzyme astaxanthin synthase, which is a cytochrome P450 hydroxylase-type enzyme, subfamily 3A (Ojima et al., 2006), which catalyzes all the reactions to convert β-carotene to astaxanthin. However, it has recently been suggested that CRTS would require an additional gene function, which could play a possible auxiliary role (Martín et al., 2008). This explains the inability to obtain mutants blocked in same step between β-carotene and astaxanthin that accumulate echinenone, 3-hydroxy-echinenone or phoenicoxanthin.

Additionally, after nitrosoguanidine (NTG) mutagenesis, we isolated the mutant strain atx5, that shows a yellow phenotype as a result of β-carotene accumulation (Cifuentes et al., 1997). To discover if the mutation of atx5 affects the *crtS* gene, a 5,920 bp fragment containing the complete *crtS* gene of strain atx5 was sequenced (GenBank accession number EU713462). The sequence analysis indicates there is only a single base change that corresponds to a guanine (G) in position 376, from the ATG of the genome version, to an adenine (A). This change is located in exon 3, 12 nucleotides from the limit between exon 2 and 3. This transition mutation provokes a change from the Gly to Asp in position 60 of the CRTS protein. According to the literature, the location of this change does not affect important sites for the correct functioning of astaxanthin synthase, such as the oxygen-binding site, binding domain to the Heme group, or the ESLR domain involved in the maintenance of the tridimensional structure (Álvarez et al., 2006). However, since G376A (Gly 60 to Asp) is the only change observed in strain atx5 and considering that glycine is an amino acid that given its short hydrophobic lateral chain, makes no real contributions to the hydrophobic interactions and that Aspartic is a negatively charged amino acid, this change could have not affected the activity of astaxanthin synthase. The aforementioned, coupled with six out of six unsuccessful attempts to transform strain atx5 using as donor DNA the complete wild-type *crtS* including the promoter region, suggests the involvement of at least another gene, complementary to the *crtS* gene, in the conversion of β-carotene to astaxanthin in *X. dendrorhous*.

Directed mutagenesis of crtI, crtYB, and crtS in X. dendrorhous

The cDNA of the genes *crtYB* and *crtI* have been cloned by heterologous complementation in *E. coli*. The *crtI* gene of *X. dendrorhous* is expressed in *E. coli,* which synthesizes phytoene due to the plasmid carrying the genes *crtE* and *crtB* of

TABLE III

Heterologous complementation analysis of truncated *crtYB* gene in *E. coli* carrying carotenoids biosynthesis genes of *E. uredovora*

Strain	Plasmid A	Plasmid B	Carotenoid (ug/g of biomass)	Produced	Phenotype
BL21 Gold	-	-	none	-	albino
BL21 Gold	pETOPO-CI	-	none	-	albino
DS1B	pDS1B	-	β-carotene	1226	yellow
DS1B-Δ*crtB*	pDS1B-Δ*crtB*	-	none	-	albino
DS1B-Δ*crtB*	pDS1B-Δ*crtB*	pXd-t*crtYB*	β-carotene	139	yellow
DS1B-Δ*crtY*	pDS1B-Δ*crtY*	-	Lycopene	849	Red
DS1B-Δ*crtY*	pDS1B-Δ*crtY*	pXd-t*crtYB*	Lycopene	859	Red

E. uredovora, giving lycopene as producer of the desaturation reactions of phytoene (Verdoes et al., 1999a). Similarly, the cDNA of the gene *crtYB* of *X. dendrorhous* is expressed in different carotenogenic strains of *E. coli,* providing evidence that it codes for a bifunctional protein with phytene synthase and lycopene cyclase activity (Verdoes et al., 1999b). However, the functionality of these genes in *X. dendrorhous* has not been directly demonstrated. Therefore, the effect of mutations in these genes in the carotenoids synthesize in *X. dendrorhous* was determined. For the study of the functionality of the *crtI* gene, the wild-type strain was transformed with the plasmid pL22ΔRV, harboring a 1.1 kb deletion of the *crtI* gene, linearized with *Bam*HI and *Xho*I. A total of 8 colonies with a pale phenotype were obtained, and one of these, named T5, was selected, and the presence of the 1.1.kb deletion of the *crtI* gene was confirmed by PCR using specific primers PHA1 and PHA4. In addition, the PCR analysis also showed the presence of a wild-type copy of the *crtI* gene, indicating that this strain was heterozygous for the *crtI* locus, it is $crtI^{+}/crtI^{-}$, with this $crtI^{-}$ allele being a deletion of 1.1 kb *Eco*RV fragment of the gene ($crtI^{\Delta RV}$ allele), which was integrated in the chromosomal *crtI locus* by a double recombination or double crossover event and gives evidence that the ATCC 24230 wild-type strain is diploid. Subsequently, the heterozygote T5 strain was transformed with the same lineal DNA plasmid pL22ΔRV, obtaining a large number of white (albino) colonies and few red colonies, some of which showed sectors (wild-type red and pale phenotype). It is possible that these colonies arose by a mitotic recombination event between heterozygote chromosomes in the T5 transformant, reverting to the wild-type condition of red phenotype. Similarly, transformation of the heterozygote T5 strain homozygote double transformants for the deletion mutation $crtI^{\Delta RV}$ ($crtI^{\Delta RV}$ / $crtI^{\Delta RV}$) of albino phenotype arise. However, the high number of albino transformants could be a result of two events, one by transformation by integration and the other by mitotic recombination between homologous chromosomes of the heterozygote strain, generating albino homozygotes in both cases. Once the homozygotization has occurred, the wild-type revertant or albino double mutant becomes genetically stable. These results suggest that homozygote mutants can be obtained from the wild-type *X. dendrorhous* strain through a single genetic transformation event followed by mitotic recombination which we have called the double recombinant method (DRM). From the results shown above, the DRM was developed to obtain deletion homozygote mutants of the gene *crtI* and insertion of a hygromycin B-resistant cassette. For this, cells of the wild-type strain were transformed with the plasmid pXD-*crtI*::*hph* previously linearized with *Bam*HI and *Xho*I restriction enzymes. As a result, 15 heterozygote pale colonies resistant to 10 μg/ml of hygromicin B were obtained. The genetic analysis of these colonies by PCR using specific primers for the *crtI* and the *hph* genes and their DNA sequence, indicates that they all bear the *hph* gene in the *crtI* locus in addition to a wt allele of the *crtI*. Cells of one heterozygote pale hygromicin B-resistant colony, named T-I21H, showing the $crtI^{+}/crtI$::*hph* genotype, were grown in 50 ml flasks containing 10 ml of YM plus 10 μg/ml of hygromicin B for 3 days. Then, a 0.3 ml aliquot of the culture was diluted 100 times in a 125 ml flask with 30 ml of YM medium supplemented with 50 μg/ml of hygromycin B. After incubation for five days at 22°C, a transition from a paler culture was observed, with a pigmentation of approximately 10% of the wild-type, resulting in a white culture. Dilutions were plated on solid YM containing 10 μg/ml hygromicin B to give between 200 to 500 colonies per dish. Over 90% of colonies developed were white and hygromicin B-resistant. The genetic analysis by PCR shows that these colonies (represented by T-I21H1H) were homozygote for the mutation by insertion of the *hph* gene in *crtI* locus; it contains the allele $crtI^{-}$::*hph* on both homologous chromosomes ($crtI^{-}$::*hph*/ $crtI^{-}$::*hph*). The pigment content analysis by

HPLC showed that they only produced phytoene, which accumulates due to the absence of the enzyme phytoene desaturase that prevents its conversion to lycopene (Fig. 5 B and C).

Subsequently, the double recombinant method was used to obtain mutants of genes *crtYB* and *crtS*, inserting the hygromycin B-resistance cassette in each gene. In an initial step, the wild-type strain ATCC 24230 of *X. dendrorhous* was transformed, in parallel, with the *crtYB*::*hph* cassette from plasmid pXD-*crtYB*::*hph* linearized with the enzyme *Xho*I and with the cassette *crtS*::*hph* from the plasmid pXD-*crtS*::*hph* linearized with *Sma*I. Five heterozygote transformants of the gene *crtYB* (*crtYB*$^{+}$/*crtYB*$^{-}$::*hph*) and 12 heterozygote transformants for the gene *crtS* (*crtS*$^{+}$/*crtS*$^{-}$::*hph*) were obtained. One colony of each transformant type (T-YBH1 and T-SH1, respectively) was selected and grown in conditions for enrichment of mitotic recombination, after which thousands of albino (*crtYB* -/-, non-pigment producing) or yellow (*crtS* -/-, β-carotene accumulating) colonies were observed, respectively. Similarly to what was observed with the mutants of the gene *crtI*, in genes *crtYB* and *crtS*, more than 90% of the colonies were homozygous after the application of DRM.

In addition, the wild-type and the heterozygote and homozygote strains affected in the *crtI*, *crtYB,* and *crtS* genes were analyzed for their cellular production and composition of carotenoids by HPLC. In all heterozygote strains, T-I21H (crtI +/-), T-YBH1 (crtYB +/-) and T-SH1 (crtS +/-), the production of total carotenoids is less than in the wild-type. However, its composition was similar (Fig. 5 B, D and F). On the other hand, both the homozygote T-I21H1H (*crtI* -/-) and T-YBHH2 (*crtYB* -/-) do not produce colored pigments and are albino in phenotype (Fig. 5 C and E) and T-SHH2 (*crtS* -/-) produces β-carotene and was yellow in phenotype (Fig. 5 G). However, the *crtI* -/- homozygote produces and accumulates the uncolored carotenoid phytoene (peak 10 in box of Fig. 4) because this mutant does not synthesize the phytoene desaturase enzyme that transforms phytoene to lycopene, accumulating the former carotenoid. However, in the homozygote *crtYB* -/- strain, its albino phenotype is a product of the inability to synthesize phytoene due to the lack of the enzyme phytoene synthase. In addition, the *crtS*-/- homozygote strain produces and accumulates β-carotene (peak 9 in Fig. 4). These results provide clear evidence of the functionality of the genes *crtYB, crtI,* and *crtS* of *X. dendrorhous* and confirm the previous data obtained from heterologous complementation (Verdoes et al., 1999a; 1999b). In addition, it was also possible to obtain heterozygous transformants for the genes *idi* (*idi*$^{+}$/*idi*$^{-}$::*hph*) and *crtE* (*crtE*$^{+}$/*crtE*$^{-}$::*hph*), however, the respective homozygotes using DRM or by transformation of the heterozygotes were not obtained, suggesting a possible essential role for the products of both genes in *X. dendrorhous*.

Finally, in *X. dendrorhous*, the astaxanthin biosynthesis from IPP could be controlled by five structural genes, *idi*, *crtE*, *crtYB*, *crtI,* and *crtS*, while other organisms such as plants, algae, and bacteria, require a greater number of genes. The intermediaries from isopentenyl pyrophophate to astaxanthin, in the main biosynthetic pathway of *X. dendrorhous*, except HDCO, DCD, HDC (Andrewes et al., 1976; An et al., 1999) are: IPP ↔ DMAPP → GPP → FPP → GGPP → phytoene → phytofluene → ζ-carotene → neurosporene → lycopene → β-carotene → echinenone → 3-hydroxy-echinenone → phoenicoxanthin → astaxanthin. In *X. dendrorhous*, as in all fungi and eubacteria, the four desaturations required to convert phytoene into lycopene are synthesized by phytoene desaturase, controlled by the *crtI* gene (Verdoes et al., 1999a). However, in plants and algae, two gene products are required in this step, a phytoene desaturase that converts phytoene to ζ-carotene and ζ-carotene desaturase that transforms ζ-carotene into lycopene. In addition, in *X. dendrorhous* in the phytoene synthesis from GGPP and the β-carotene production from lycopene, two enzymatic reactions are catalyzed by a bi-functional gene product with phytoene synthase and lycopene

cyclase activity (Verdoes et al., 1999b), which differs from plants, algae, and bacteria in which two independent gene products are required. The four steps to convert β-carotene to astaxanthin in *X. dendrorhous* are catalyzed by the *crtS* gene product, a protein of 557 amino acids in which the sequence analysis by blast suggests a cytochrome P450 in accordance with that previously described (Hoshino et al., 2000; Ojima et al., 2006). This is consistent with the first report that cytochrome P450 was involved with astaxanthin production in *X. dendrorhous* (An et al., 1989, Johnson 2003). Other organisms require at least two genes, *crtW* and *crtZ,* encoding a β-carotene oxygenase and a β-carotene hydroxylase respectively. Knowledge about the regulation of the expression of genes controlling the carotenogenesis in *X. dendrorhous* is limited. In fact, in previous works, it was determined that the *crtI* mRNA and *crtYB* mRNA levels decreased during the stationary phase of *X. dendrorhous* (Lodato et al., 2003). However, the carotenoid production differences between wild-type and astaxanthin-overproducing strains of *X. dendrorhous* could not be explained by a difference in the carotenogenic transcript at the stationary phase of growth (Lodato et al., 2007; 2004). The knowledge of the genomic organization of the structural genes controlling the carotenogenesis and the homozygous and heterozygous mutants obtained carrying deletions of each gene

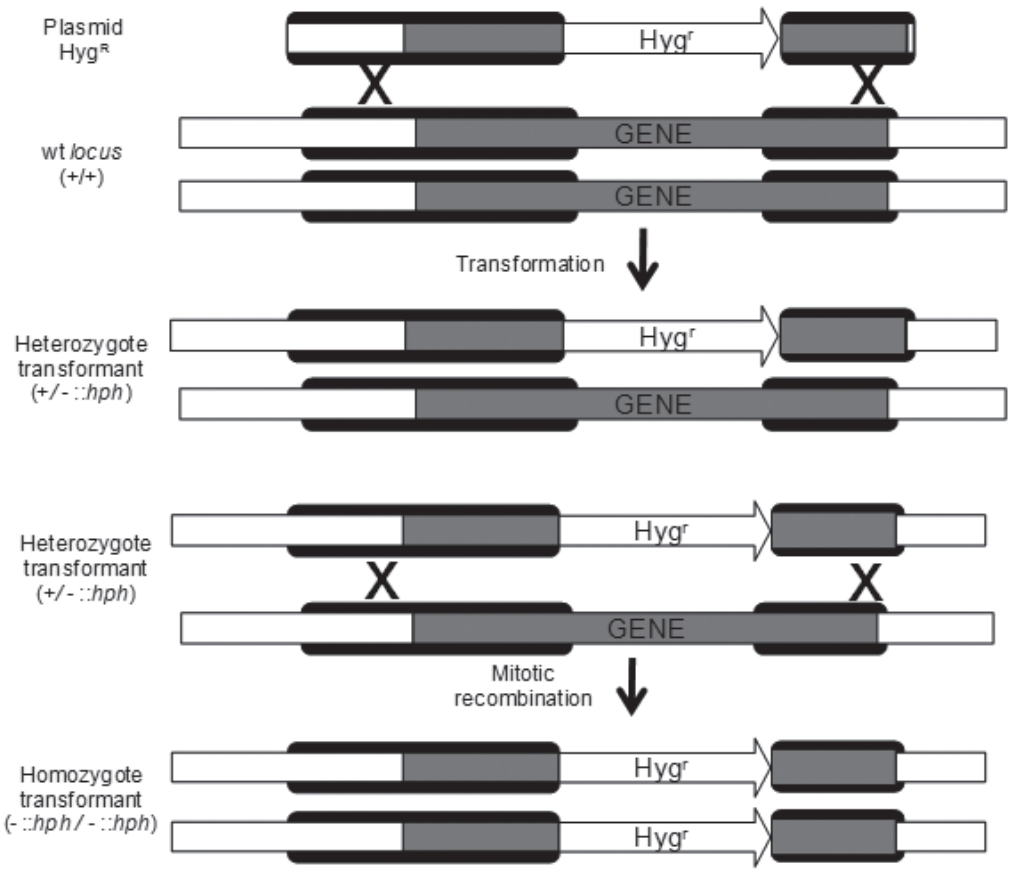

Figure 4. Schematic representation of the Double Recombinant Method in *X. dendrorhous*. The locus of interest is represented in gray. The gDNA that flanks the locus of interest is represented in white. The potentially recombinant gDNA region is represented in black. The hygromycin B resistant cassette of *X. dendrorhous* is represented as an arrow. GENE: represents any one of the five carotenogenesis genes (*idi*, *crtE*, *crtYB*, *crtI* and *crtS*) of the yeast.

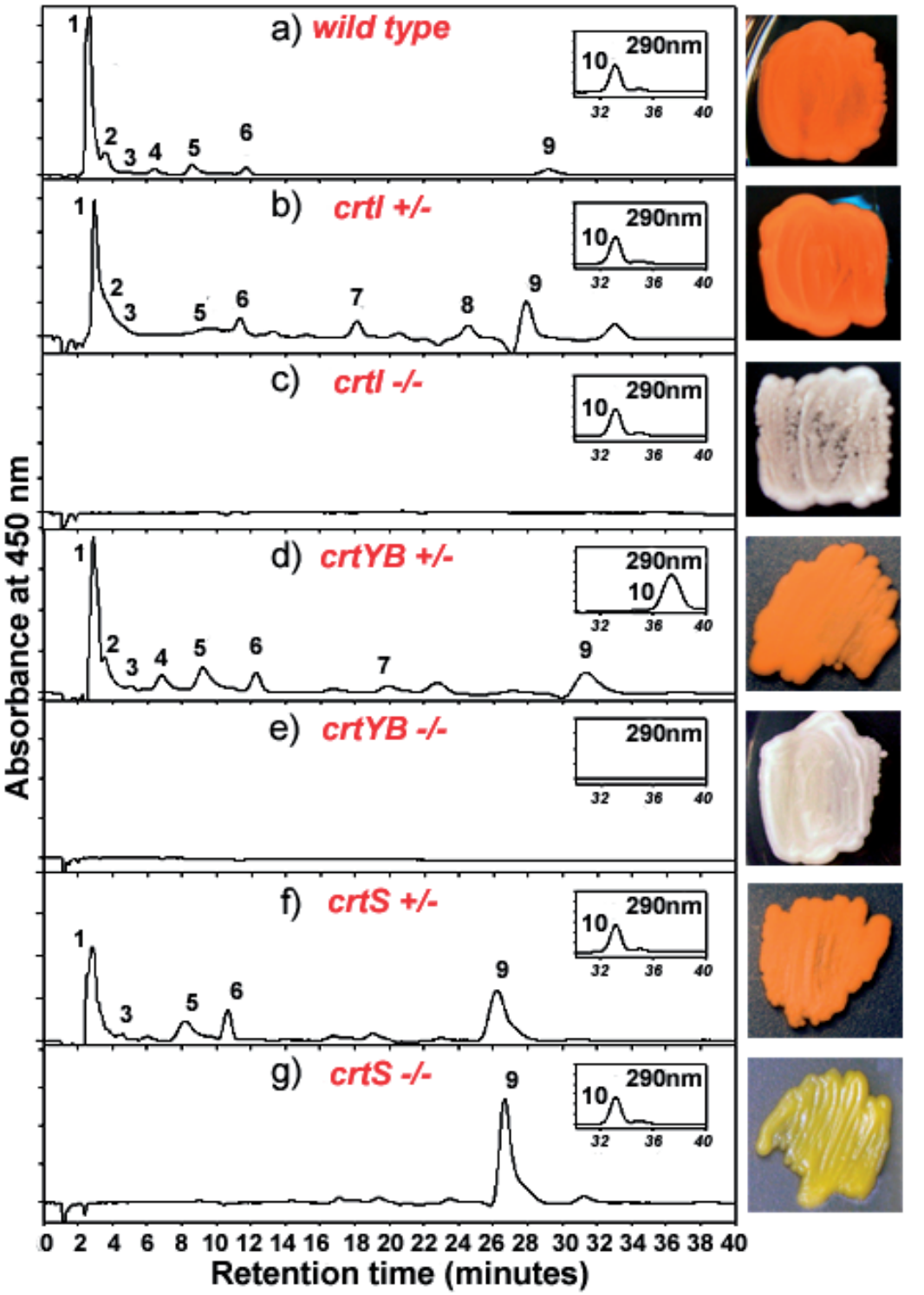

Figure 5: HPLC analysis of the pigment content of wild-type and heterozygous and homozygous strains of *X. dendrorhous*. The symbols: (+/-) represents the heterozygous and (-/-) the homozygous genotype of the strains. The numbers represent the different carotenoids intermediates as follow: 1, astaxanthin; 2, phoenicoxanthin; 3, canthaxanthin; 4, 3-hydroxy-4-ketotoruleno; 5, hydroxyl-keto-γ-carotene; 6, hydroxyl echinenone; 7, echinenone; 8, neurosporene; 9, β-carotene; 10, phytoene.

provides the genetic tools that will permit the study of the mechanism involved in the genetic regulation of the carotenoids biosynthesis pathway of *X. dendrorhous*. In addition, these site-specific mutants would allow the study of the gene dose effect and how it participates in the final expression of the structural genes of carotenogenesis and its regulation.

ACKNOWLEDGEMENTS

This work was supported by Fondecyt 1040450, Mecesup UCH 0106, through a graduate scholarship to Jennifer Alcaino and Mauricio Niklitschek; Fundación María Ghilardi, through a graduate scholarship to Carla Lozano; CONICYT through a graduate scholarship to Andrés Marcoleta; and Deutscher Akademischer Austanschdienst (DAAD) through a graduate scholarship to P. Lodato.

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