Chapter 15

Generation of Astaxanthin Mutants in *Xanthophyllomyces dendrorhous* Using a Double Recombination Method Based on Hygromycin Resistance

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Abstract

Generally two selection markers are required to obtain homozygous mutations in a diploid background, one for each gene copy that is interrupted. In this chapter is described a method that allows the double gene deletions of the two copies of a gene from a diploid organism, a wild-type strain of the *Xanthophyllomyces dendrorhous* yeast, using hygromycin B resistance as the only selection marker. To accomplish this, in a first step, a heterozygous hygromycin B-resistant strain is obtained by a single process of transformation (carrying the inserted *hph* gene). Following, the heterozygous mutant is grown in media with increasing concentrations of the antibiotic. In this way, the strains that became homozygous (by mitotic recombination) for the antibiotic marker would able to growth at higher concentration of the antibiotic than the heterozygous. The method can be potentially applied for obtaining double mutants of other diploid organisms.

Key words: Xanthophyllomyces dendrorhous, Astaxanthin, Recombination, Hygromycin B

1. Introduction

To obtain a homozygous mutant of the *Xanthophyllomyces dendrorhous* yeast strain UCD 67-385, a two-step transformation of the diploid wild-type strain is necessary. However, the molecular tools for transforming *X. dendrorhous* and quickly and reliably selecting mutant strains are insufficient, which has led to the development of the *D*ouble *R*ecombinant *M*ethod (DRM) (1). The DRM is based on the naturally high mitotic recombination frequency of this yeast (as compared to other yeasts, such as *Saccharomyces cerevisiae*), which allows the segregation of some characteristics when the yeast is grown under normal culture conditions. Basically

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the entire process had two steps: In a first step, a heterozygous mutant is generated by insertion of the resistance cassette in one copy of the target gene. In a second step, the heterozygous yeast strain is subcultivated in liquid media supplemented with increasing concentrations of the selective antibiotic. We used hygromycin B because the gene of resistance to this antibiotic shows a doses dependence to confer resistance at different concentrations of the antibiotic. The heterozygous yeast strain is first grown using a low concentration of antibiotic, and after it reaches a high OD is used as the inoculum for the next medium supplemented with higher concentration of the antibiotic. A yeast strain carrying two or more copies of a resistance gene will grow at higher antibiotic concentration that the heterozygous strain carrying only one copy of the selective marker gene. After this procedure is repeated several times, a culture that is enriched in strains homozygous for the selective marker gene is obtained.

Using this method, we obtain mutants of *X. dendrorhous* that are heterozygous and homozygous for the genes *crt*YB, *crt*I, or *crt*S. These mutants were analyzed at molecular level and in relation to production of pigments. As expected, the homozygous mutants are able to grow at higher concentrations of hygromycin B than the heterozygous mutants. The homozygous mutants to *crt*YB and *crt*I genes show an albino phenotype, and homozygous mutants for *crt*S gene develop yellow colonies.

2. Materials

2.1. Strains, Plasmids, and Culture Medium

1. *X. dendrorhous* UCD 67-385 (ATCC, Manassas, VA, USA).
2. *X. dendrorhous* T5 (*crt*I$^+$/*crt*I$^-$ΔRV) (1).
3. *Escherichia coli* DH5α competent cells (Invitrogen, Carlsbad, CA, USA).
4. pBS4 (2) (see Note 1).
5. pL22 (see Note 2).
6. pMN-hph (see Note 3).
7. pXD-*crt*YB::*hph* (see Note 4).
8. pXD-*crt*I::*hph* (see Note 5).
9. pXD-*crt*S::*hph* (see Note 6).
10. pBluescript SK– (Stratagene, La Jolla, CA, USA).
11. Yeast-malt medium (YM): 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1% glucose (3).
12. LB: 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl, adjust pH to 7.0 with 5 N NaOH and sterilize by autoclaving (121°C for 15 min).

13. LB solid: LB and 15 g/L of agar.
14. LB–ampicillin: Add 1 mL of 100 g/L of ampicillin to 1 L LB before use. Store at 4°C.
15. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopiranoside).
16. Hygromycin B.

2.2. PCR-Mediated DNA Amplification and DNA Manipulation

1. 0.8 M KCl.
2. Lysing enzymes (Sigma, St Louis, MO, USA).
3. 10 mM Tris–HCl pH 8.0 and EDTA 25 mM.
4. SDS 10%.
5. Ethidium bromide (0.5 μg/mL).
6. Proteinase K.
7. RNAse A.
8. Saturated phenol pH 8.0.
9. Phenol:chloroform:isoamyl alcohol (25:24:1).
10. Chloroform:isoamyl alcohol (24:1).
11. TE buffer: 10 mM Tris–HCl and 1 mM EDTA pH 8.0.
12. TAE buffer: 2 M Tris base, 0.05 M EDTA pH 8.0, and ~1.56 M glacial acetic acid.
13. Loading buffer (6×): 0.1% xylene cyanol, 0.1% bromophenol blue, 0.5% SDS, 0.1 M EDTA, and 50% glycerol.
14. 6 M KI.
15. Dimethylsulfoxide (DMSO).
16. Glass beads (0.5 mm diameter).
17. Wash buffer GC: 50 mM NaCl, 10 mM Tris–HCl pH 7.5, 2.5 mM EDTA, and 50% v/v ethanol.
18. TBE buffer: 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.0.
19. Saturated basic phenol (pH 8.0).
20. Silica S-5631 (Sigma, St Louis, MO, USA).
21. PBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4.
22. BD solution: 50 mM K_2HPO_4 pH 7.0, and 25 mM dithiothreitol (DTT).
23. STM solution: 270 mM sucrose, 10 mM Tris–HCl, pH 7.5, and 1 mM $MgCl_2$.
24. Glassmilk (see Note 7).
25. λ*Hin*dIII molecular weight marker.
26. 1 kb DNA ladder.
27. *Pfu* polymerase.

Table 1
Primers used in this work

Primer	Sequence 5′→3′	Description	Position on the gene
*gpd*T-F	AGGCCTACGGTTCTCTCCAA	Forward *gpd* terminator	Y08366 2.431–2.452→
*gpd*T-R	ATGAGAGATGACGGAGATG	Reverse *gpd* terminator	Y08366 ←2.798–2.816
PEF-F-EV	GATATCGGCTCATCAGCCGAC	Forward promoter *TEF*-1α with *EcoRV* site	953–971→
PEF-R-Hyg	GCTTTTTCATGGTGAAGC TGT TCGAGATAG	Promoter *TEF*-1α reverse with *hph* gene adapter	←1,328–1,347
Hyg-F-PEF	CAGCTTCACCATGAAAAAGCCT GAACTCACC	Forward *hph* gene with Promoter *TEF*-1α adapter	1.174–1.189→
Hyg-R-*gpd*T	CCGTAGGCCTCTATTCCTTTGC CCTCGGAC	Reverse *hph* gene with adapter of terminator *gpd*	←2.183–2.202
*gpd*T-F-Hyg	AAAGGAATAGAGGCCTACGGTT CTCTCCAA	Forward terminador*gpd* with adapter gen *hph*	1.511–1,540→
Hyg-F	ATGAAAAAGCCTGAACTCACC	Forward *hph* gene	1.183–1.189→
Hyg-R	CTATTCCTTTGCCCTCGGAC	Reverse *hph* gene	←2.183–2.193
*gpd*T-R-EV	GATATCATGAGAGATGAC GGAGATG	Reverse terminator *gpd* with *EcoRV* site	←1.894–1.918
EF-aD	GGATCCTTCAAGTACGC	*TEF*-1α	1.501–1.517→
EF-aR	ACGTTCTTGACGTTGAA	*TEF*-1α	←2.531–2,547
C13D5	CGAGGCTTACCTTGTCTCTC	Forward *crt*I gene	889–918→
Pha4-8R	GAAAGCAAGAACACCAACGG	Reverse *crt*I gene	←5.024–5.043

28. M-MulV reverse transcriptase.
29. Restriction endonucleases.
30. Primers (see Table 1).
31. Geneclean Kit (MP Biomedicals, Solon, OH, USA).
32. 1D Image Analysis Software version 2.0.1 (Kodak Scientific Image System, Rochester, NY, USA).

2.3. HPLC Analyses

1. High performance liquid chromatography (HPLC) with diode array detector.
2. Column Lichrospher RP18 125-4 (Merck, Darmstadt, Germany).

3. Mobile phase: acetonitrile:methanol:isopropanol (85:10:5).
4. Carotenoids standards (Sigma, St Louis, MO, USA) (see Note 8).

3. Methods

The methods described below are used for the selection, cloning, and construction of the hygromycin B resistance cassette.

3.1. Genomic DNA purification

The protocol described here is optimized for obtaining a great yield of genomic DNA with the minimum degradation as possible.

1. Grow the yeast in 500–1,000 mL of YM medium at 22°C for 4–5 days.
2. Centrifuge at 7,000×*g* for 10 min, discard the supernatant, and wash the cell pellet with 200 mL of 0.8 M KCl. Then centrifuge at 7,000×*g* for 10 min, discard the supernatant, and resuspend cell pellet in 16–20 mL of 0.8 M KCl.
3. Divide the cells in four flasks (4–5 mL each) and add 1 mL of 20 mg/mL of lysing enzymes. Incubate at 22°C with gentle agitation for 12–16 h.
4. Centrifuge at 4,000×*g* for 5 min, and resuspend in 6 mL of Tris–HCl 10 mM pH 8.0 and EDTA 25 mM.
5. Add SDS 10% to reach a final concentration of 1% and incubate at room temperature for 5 min.
6. Add 50 μL of 20 mg/mL of proteinase K and incubate at 55°C for 1 h.
7. Add 10 μL of 10 mg/mL of RNAse A and incubate at room temperature for 10 min.
8. Add 1 volume of saturated phenol pH 8.0 and mix gently.
9. Centrifuge at 4,000×*g* for 5 min and transfer the aqueous phase to a new tube.
10. Add 2 mL of 10 mM Tris–HCl and 25 mM EDTA to the remnant phenolic phase and mix gently.
11. Centrifuge at 4,000×*g* for 4 min and transfer the aqueous phase to the tube containing the previously collected aqueous phase.
12. Wash twice with saturated basic phenol.
13. Wash with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1).
14. Centrifuge at 4,000×*g* for 4 min and transfer the aqueous phase to a new tube.
15. Wash with 1 volume of chloroform:isoamyl alcohol (24:1).

16. Centrifuge at 4,000 × *g* for 4 min and transfer the aqueous phase to a new tube.
17. Add 2.5 volumes of ethanol and mix gently to form a "DNA ball," get it out with a pipette and put it in a new tube.
18. Wash the "DNA ball" with ethanol 70%.
19. Let the "DNA ball" dry and resuspend in TE buffer.
20. Store the DNA at –20°C until it is used.

3.2. Purification of DNA from Agarose Gel

1. Add loading buffer (6×) to samples and subject them to 1% agarose gel electrophoresis in TAE buffer (see Note 9).
2. Stain with ethidium bromide and visualize under UV light.
3. Excise the band of interest from the gel (see Note 10); place in an Eppendorf tube and add 3 volumes of 6 M KI.
4. Incubate tubes at 55°C until the gel fragments completely dissolve.
5. Cool to room temperature and mix with 10 µL of glassmilk.
6. Incubate on ice for 10 min and centrifuge at 10,000 × *g* for 30 s.
7. Discard the supernatant, and wash the pellet three times with 500 µL of wash buffer GC, centrifuging at 10,000 × *g* for 30 s between washing steps.
8. Remove the wash buffer and resuspend the pellet in 15–20 µL of nuclease-free water.
9. Incubate at 55°C for 10 min.
10. Centrifuge at 10,000 × *g* for 20 s, and transfer the supernatant containing the nucleic acids to a new tube.

3.3. PCR Amplification of the TEF-1α Promoter from X. dendrorhous

Based on studies in filamentous fungi and yeasts (4), we decided to use the promoter of the gene encoding the translation elongation factor 1-α (*TEF*-1α) of *X. dendrorhous*.

1. To design the primers for the cloning of the *TEF*-1α promoter, compare all available coding sequences for fungal *TEF*-1α, and identify the most conserved regions by multiple alignments.
2. Use the two highly conserved regions to design the primers. Primers designed are shown in Table 1.
3. Amplify by PCR using the primers EF-aD and EF-aR and genomic DNA from *X. dendrorhous*, and this standard PCR reactions: 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.
4. Check the amplicons by agarose gel electrophoresis. In our model a unique 1 kb amplicon was obtained.

3.4. Cloning the TEF-1α Promoter from X. dendrorhous from a DNA Library

1. Use the primers EF-aD and EF-aR (see Table 1) to screen for the *TEF*-1α gene in an *X. dendrorhous* gDNA library constructed with *Bam*HI fragments (1).
2. Prepare a PCR reaction solution as follow: In a PCR tube mix 18.8 μL of nuclease-free water, 2.5 μL of *Taq* buffer (10×), 0.5 μL of dNTPs mix 10 mM, 1 μL of $MgCl_2$ 50 mM, 1 μL of each primer 25 mM, and 0.2 μL of *Taq* DNA polimerase (5 U/μL).
3. Using a toothpick take out the colony to analyze and suspend in PCR reaction solution.
4. Proceed to the amplification with the following program: Initial denaturation at 95°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C × 3 min; and final elongation at 72°C for 10 min.
5. Check the PCR products by agarose gel electrophoresis.
6. Grow the positive clones in LB medium at 37°C for 16 h.

Purify the plasmid DNA by a kit, and sequence the DNA fragment by two strands and resolve any ambiguities by resequencing.

The contig of two positive clones (pBR60 and pTEF2-2) obtained for *TEF*-1α gene is shown in Fig. 1. The sequence analysis reveals that the *TEF*-1α gene is in a 14,107 bp DNA fragment containing *Bam*HI cleavage site. The ATG start codon, promoter region, and putative regulating boxes are defined (see Fig. 2).

3.5. Cloning of Transcription Terminator

To construct the hygromycin B resistance cassette, the transcription terminator of the glyceraldehyde-3 phosphate dehydrogenase-encoding gene, *gpd*, is used because it has been successfully used in other basidiomycetes yeast expression systems. It is necessary to obtain the sequence for the *X. dendrorhous gpd* gene before its translation stop codon-containing downstream region (5).

1. Use the primers gpdT-F and gpdT-R (see Table 1) to amplify the *gpd* terminator using genomic DNA of *X. dendrorhous.*
2. Separate the PCR products by agarose gel electrophoresis and purify the amplicon (~390 pb) from gel.
3. Mix the purified amplicon with plasmid pBluescript SK previously digested with *Eco*RV.
4. Add 5 U of T4 DNA ligase and incubate for 16 h at 14°C.
5. Desalt the reaction mix by dialysis.
6. Mix the desalted DNA with electrocompetent *E. coli* DH5α, apply electroshock (25 μF, 200 Ω, 2.5 kV), and suspend in 1 mL of LB medium.
7. Seed 100 μL of cell suspension onto LB agar plate supplemented with 100 μg/mL of ampicillin and 80 μg/mL of X-gal.

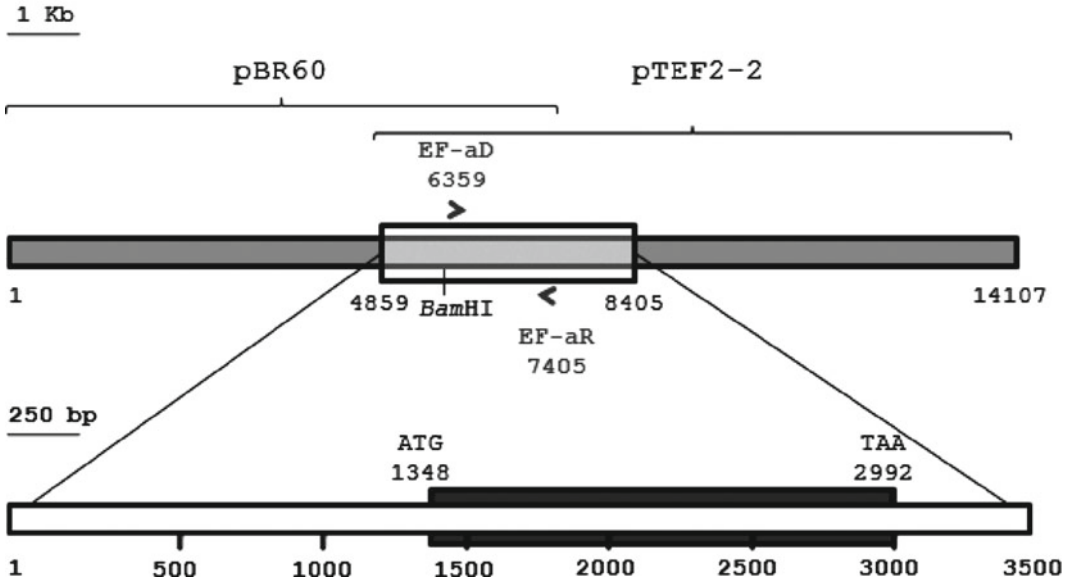

Fig. 1. Schematic representation of the sequences of the pBR60 and pTEF2-2 clones. The EF-aD and EF-aR primer binding sites are shown. A 3,547 bp DNA sequence that included 1,500 bp upstream of the EF-aD primer binding site, a 1,047-bp fragment amplified by the EF-aD and EF-aR primers, and 1,000 bp downstream of the EF-aR primer binding site was compared against GeneBank databases.

TCGCCAAGGTCGGTGGTTGGGTCTCTGAAAAAATTCCGCGAGAGAACGCATCTCGCGCAGGGAAGG
CGATCCTCTCTCGGATCGCGTGGGAGGAAGATGCTGACGTGAAACGGCTACGAGCTGAGCATTTCA
GCTTCTGGCAGAGCTGGGTGATCAAAAAAAGGTTAGGCTTGTTGGCAATCCTTTTTGGATTAGCAA
CAGCAGCAGGGGCCTGGAAAGGCAAAAGATACAAGCAAGCGATCCTTTCTCGATTGCGTGGAGGAA
GATGTTACGTGAACGGGTACGAGTGAGCATTTCAGCTTCTGGCAGAGTGGGTGATCAAAAAAAGGT
TAGGCTTGTTGGCAATCCTTTTTGGATTAGCAACACAGCAGGGGCCTGGAAATGGCAAGTATTTAG
GTGACGGCCTTAGTTACAATTATCAAAATGTTAAACTAGAAAGTATTCTATGTATAATTAATTTCA
TCCTCCTTTCTCTCCTTCTGCGTAGCTGATGATGGGACCTGCCGTTTTGTGTCAGCATGAAGGCAC
ATTTCGTACAATGAGCTGTGGTATTTAGGTGACGGCCTTAGTTACAATTATCAAAATGTTAAACTA
GAAAGTATTCTATGTATAATTAATTTCATCCTCCTTTCTCTCCTTCTGCGTAGCTGATGATGGGAC
CTGCCGTTTTGTGTCAGCATGAAGGCACATTTCGTACAATGAGCTGTGTTGAACGAGGAAGACAAA
ATTCAAGCAATATACATAGTAGGGTGTAGAGAGAAAAAGCCGACTTTCGACTGAAACTAGCTGTTT
GGCCAAGACTAACAGCACAAATGCTTCTGAACATATCTCGATCATGCGTAACTGGGGGGCTCATCA
GCCGACAGTTCATCCGACACAAGCTCTTTGCCTAGATCGTCAAACGATCGACATCGACACGAGGTT
TGACATATCTCGATCATGGGTAACTGGGG*GCTCATCAGCCGAC*AGTTCATCCGACACAAGCTCTTT
GCCTAGATCGTCAAACGATCGACATCG**ACACGAAAACAAAT**CCCGATCAGTCAATCAGTAG**AACTG**
CCGAAGAATGAAACTGAAGACTGCTGTGACACGTGAC**TATAGAA**GCGGTGTCATCTGACTTGCGAA
T**T**TGCTTGTACAAAAGTCAGGTTGGCTGATTCGCTCGCGTTGTCGACAAGAATTAGGAAA**CCTCAT**
TTCCAGCCTTCCTTTTTTTCCCCTCAGAACACCTCACATCTTCTCACAAAAAAGTAAGATATTTTC
ACTTGGAGTTGGTGGATTTGGCGACTGAATGGAGAGACAGAAGCTTGATACTGATAAGACACTCTT
TTGTAATCTATCTCGAACAGCTTCAAA**ATG**

Fig. 2. Sequence analysis of the 3.5 kb region containing the *TEF*-1α gene. The sequence analysis identified the ATG translation start codon at position 1,348. The putative TATA box is located at position 1,094, the transcription start site is at position 1,124, the putative RPG box is located between positions 1,018 and 1,031, the HOMOL1 box is located between positions 1,052 and 1,064, and a CT-rich region is located between positions 1,183 and 1,213. The binding sites of the *TEF*-1α gene primers, EF-aD and EF-aR, are also shown.

8. Incubate the plates at 37°C for 16 h.
9. Select the white colonies (harboring the recombinant plasmid).

In this way we obtained the plasmid pBgpdT harboring the terminator of *gpd* gene, which was confirmed by sequencing.

The use of antibiotics has great advantages for the selection of transformants, a critical step in obtaining genetically modified organisms. Currently, *X. dendrorhous* transformant strains are selected using the antibiotic geneticin (G-418). However, the diploid nature of the *X. dendrorhous* UCD 67-385 strain requires the use of other selection markers. The antibiotic hygromycin B is compatible with geneticin, making this mix a good choice for selection (6). We used the *E. coli hph* gene encoding the hygromycin B phosphotransferase enzyme as a resistance marker (7). For this reason, we cloned the *hph* gene from the plasmid pBS4 for our selection system.

3.6. Construction of the Hygromycin B Resistance Cassette

1. Mix equimolar amounts (100–300 ng) of DNA fragment corresponding to the *TEF*-1α promoter and the *hph* resistance gene in a final volume of 20.8 μL. Add 2.5 μL of *Taq* buffer (10×), 0.5 μL of dNTPs mix 10 mM, 1 μL of $MgCl_2$ 50 mM, and 0.2 μL of *Taq* DNA polymerase (5 U/μL). Perform an elongation reaction with the following program: Initial denaturation at 95°C for 1 min, 10 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, synthesis at 72°C for 1 min, and a final extension step at 72°C for 10 min.
2. Use 10 μL from the elongation reaction as a template to amplify the full fragment by PCR using the primers PEF-F-EV and Hyg-R-*gpd*T (see Table 1).
3. Separate PCR products by agarose gel electrophoresis and purify the amplicon (1.5 kb) from gel.
4. Mix the amplicon obtained with *gpd* gene terminator region and perform an elongation reaction.
5. Use 10 μL from the elongation reaction as a template to amplify the full fragment by PCR using the primers PEF-F-EV and gpdT-R-EV (see Table 1).
6. Separate by agarose gel electrophoresis and purify the amplicon from it.
7. Ligate into the *Eco*RV site of pBluescript SK–, desalt, transform into *E. coli* DH-5α, and select recombinant clones as described above.

A schematic representation of the construction of the resistance cassette is shown in Fig. 3. A plasmid named pMN-Hyg containing the desired construction is obtained.

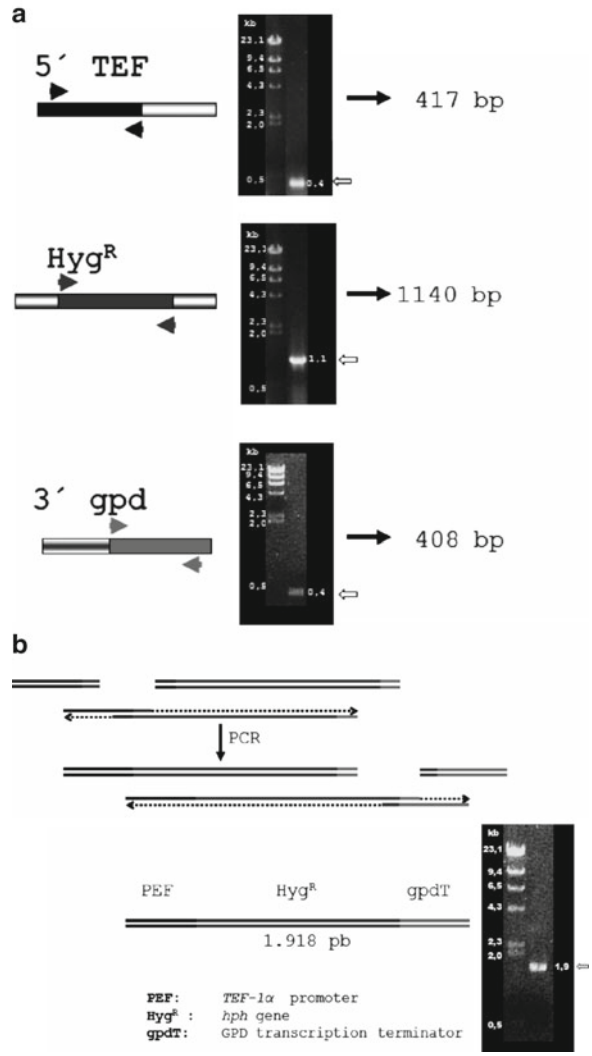

Fig. 3. Construction of *X. dendrorhous* hygromycin B resistance cassette. (**a**) Amplification of the *TEF*-1α gene promoter region with the primers PEF-F-EV and PEF-R-Hyg, amplification of the *hph* gene with the primers Hyg-F-PEF and Hyg-R-gpdT, and amplification of the *gpd* gene transcription termination region with the primers gpdT-F-Hyg and gpdT-R-EV. (**b**) Hygromycin B resistance cassette construction through fragment union by elongation and a subsequent PCR amplification. Molecular weight markers λ*Hin*-dIII (λ) are indicated. The size of the amplified products are indicated by *arrows.*

3.7. Insertion of the Hygromycin B Resistance Cassette into the crtI Locus by Transformation of X. dendrorhous

To check the functionality of the hygromycin B resistance cassette in *X. dendrorhous*, it was used to the replacement of *crt*I, a gene involved in the biosynthesis of astaxanthin. In this way, the transformants obtained will be resistant to hygromycin B and will display different coloration respect to parental strain.

1. Grow *X. dendrorhous* in 500–1,000 mL of YM medium at 22°C, until an OD_{600} of 1.2 is reached.

2. Centrifuge at 4,000 × *g* for 5 min, suspend in 25 mL of BD solution, and incubate at 22°C for 15 min.
3. Wash the cells twice with 25 mL of cold STM solution and suspend in 1 mL of STM solution (see Note 12).
4. Mix 60 μL of electrocompetent cells with 10–20 μg of "transforming DNA" (see Note 13), deposit in the electroporation cuvette, and apply the electric pulse (125 μF, 600 Ω, and 0.45 kV).
5. Add to the cuvette 1 mL of YM medium, resuspend the cells well, and transfer to a sterile tube.
6. Incubate at 22°C for 5 h, seed aliquots of 100 μL onto YM plates supplemented with 10 μg/mL of hygromycin B, and incubate at 22°C until development of colonies.

In a first step, we used the pIH plasmid (see Note 11) to transform a wild-type (*crt*I$^+$/*crt*I$^+$) *X. dendrorhous* strain. A hygromycin B-resistant transformant was obtained and selected because it has a level of pigmentation lower than the parental strain (pale phenotype). Genomic DNA was extracted from clones, and used in PCR reactions with: (1) PEF-F-RV and gpdT-R-RV primers that amplify the whole cassette (1,900 pb); (2) Hyg-F and Hyg-R primers that amplify the *hph* gene (1,100 pb); (3) C13D5 and Hyg-R primers that would amplify a 5′ fusion region of *hph* and *crt*I genes (2,283 bp); and (4) pha4(8R) and Hyg-F primers that would amplify a 3′ fusion region of *hph* and *crt*I genes (2,990 bp). Results obtained from PCR reactions indicate that the pale transformants have the hygromycin B cassette integrated in one copy of *crt*I gene, therefore, are heterozygous mutants (*crt*I$^+$/*crt*I$^-$). These results strongly suggest a gene dose-dependency in the biosynthesis of astaxanthin, because the presence of only one allelic copy reduced the pigmentation Fig. 4.

3.8. Generation of Homozygous Mutants of X. dendrorhous

In a second step, the heterozygous mutant is transformed into homozygous mutant. For that the DRM is developed (1). The basis of the method is that cells which have a mitotic recombination event in the *crt*I locus will be homozygous for the mutation, increasing the copy of the *hph* gene and therefore its resistance to the antibiotic.

1. Grow the heterozygous strains at 22°C in YM medium supplemented with 50 μg/mL of hygromycin B, until late exponential phase (OD_{600} 8–10).
2. Use this culture to inoculate a new YM media containing 100 μg/mL of hygromycin B and incubate at 22°C until late exponential phase.
3. Repeat the procedure of subcultivation. In each step increase the concentration of antibiotic twice, increasing at twofold, until 800 μg/mL is reached.

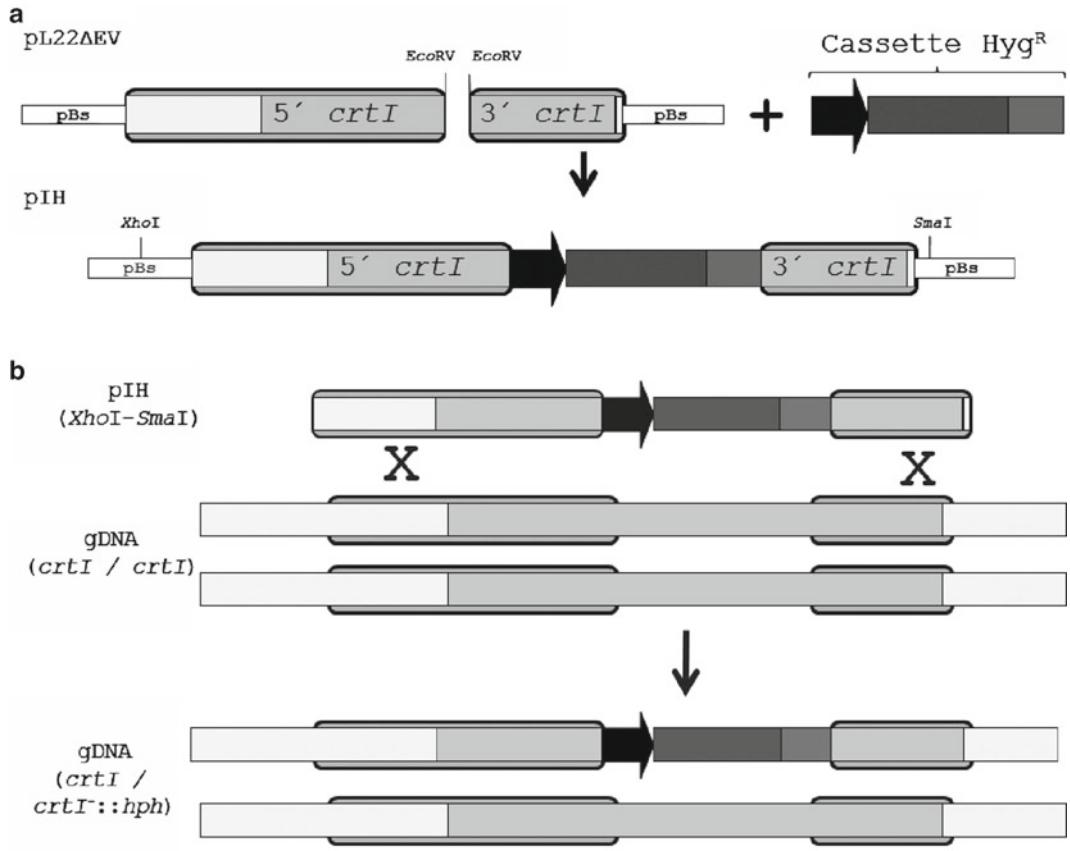

Fig. 4. Schematic representation of insertion of hygromycin B resistance cassette into *crt*I locus of *X. dendrorhous*. (**a**) The *X. dendrorhous* hygromycin B resistance cassette is inserted in the *Eco*RV site of the plasmid pL22ΔRV, resulting in the plasmid pIH that is used to transform *X. dendrorhous*. (**b**) The mechanism of the integration of the hygromycin B resistance cassette in the *crt*I locus by homologous recombination.

4. Collect aliquots of each culture, seed onto YM plates containing hygromycin B and incubate at 22°C until development of colonies.

The procedure and the results obtained are schematized in Fig. 5. As the subcultivations are made, the coloration of the colonies obtained varies from pale phenotype (heterozygous mutant) to albino phenotype (homozygous strains). Molecular analysis to confirm the homozygous mutants are made as described above. In this way the homozygous mutants *crt*YB⁻::*hph*/*crt*YB⁻::*hph*, *crt*I⁻::*hph*/*crt*I⁻::*hph* and *crt*S⁻::*hph*/*crt*S⁻::*hph* are obtained.

3.9. Pigment Extraction and Analysis

1. Centrifuge 50 mL of the yeast culture at 7,000 × *g*.
2. Wash with 1 mL of water and suspend in 1 mL of water.
3. Add 0.5 mL of glass beads (0.5 mm diameter) and vortex for 2 min.

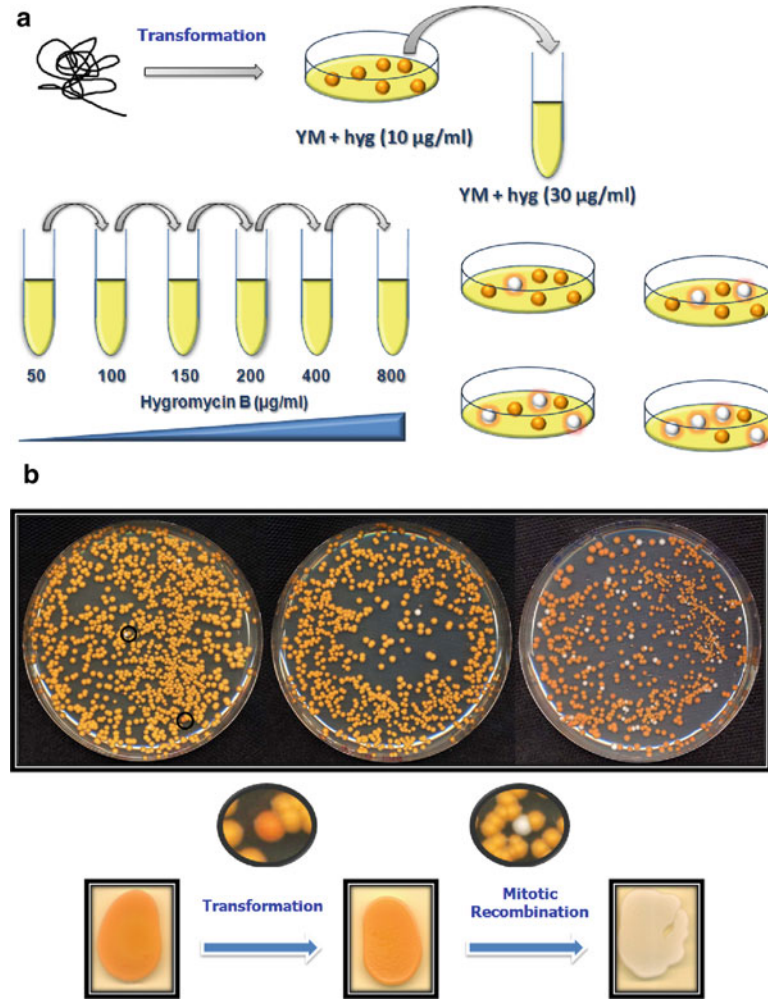

Fig. 5. Schematic representation of the *X. dendrorhous* DRM protocol. (**a**) The hygromycin B-resistant heterozygous strains are grown in liquid media with increasing concentrations of the antibiotic, and then plated on YM with 15 µg/mL of hygromycin B. (**b**) Petri dishes with colonies grown at 50, 100, and 150 µg/mL of hygromycin B.

4. Incubate on ice for 2 min and add 1 mL of acetone.
5. Vortex for 2 min and centrifuge at 12,000 × *g* at 4°C.
6. Transfer the organic phase to a new tube.
7. Add 2 mL of acetone to the cellular pellet and repeat the steps 5 and 6.
8. Repeat step 7 until the cellular pellet is completely discolored and join all organic phases collected in the same tube.
9. Add 1/5 volume of petroleum ether and vortex for 2 min.
10. Centrifuge at 12,000 × *g* for 3 min and transfer the petroleum ether phase to a new tube.

11. Determine the volume of petroleum ether obtained and determine OD_{474}.
12. Calculate the amount of total carotenoid pigments according to the following formula (3):

$$TCP = OD_{474} \times V_f \times 10^4 \,/\, 2{,}100 \times P$$

where TCP is the total carotenoids pigments (μg/g of sample), OD_{474} the absorbance at 474 nm, V_f the final volume of petroleum ether, P the grams of sample and the value 2,100 is the molar extinction coefficient of astaxanthin.

13. Dry the sample of petroleum ether under nitrogen and dissolve in 100 μL of acetone.
14. Separate the pigment by HPLC, using a RP-18. Lichrocart 125-4 column, in isocratic conditions using a solution of aceto nitrile:methanol:isopropanol (85:10:5) as mobile phase, at a flux of 1 mL/min.
15. Detect the pigments at 474 nm and determine the absorbance spectrum of the samples collected.
16. Identify the pigments according to retention time and absorbance spectrum.

The analysis of pigment of different mutants of *X. dendrorhous* is shown in Fig. 6.

4. Notes

1. pBS4 contains the *hph* gene.
2. pL22 is pBluescript SK– containing a 3,558-bp amplicon carrying the *crt*I gene of *X. dendrorhous.*
3. pMN-hph is pBluescript SK– containing, in the *Eco*RV site, a 1.8-kb cassette encoding the *Escherichia coli hph* hygromycin B resistance gene.
4. pXD-crtYB::*hph* is pBluescript containing a 5.9-kb *Eco*RV fragment carrying the *crt*YB gene with a deletion of a *Hpa*I 1,245 bp segment and an insertion of an *hph* cassette from pMN-*hph.*
5. pXD-*crt*I::*hph* is pL22 with a deletion of a 1,127 bp *Eco*RV fragment of the *crt*I gene and an insertion of an *hph* cassette from pMN-*hph.*
6. pXD-*crt*S::*hph* is pBluescript containing a 4 kb *Pst*I fragment carrying the *crt*S gene with a deletion of a *Stu*I-*Hpa*I 2,479 bp fragment and an insertion of an *hph* cassette from pMN-*hph.*

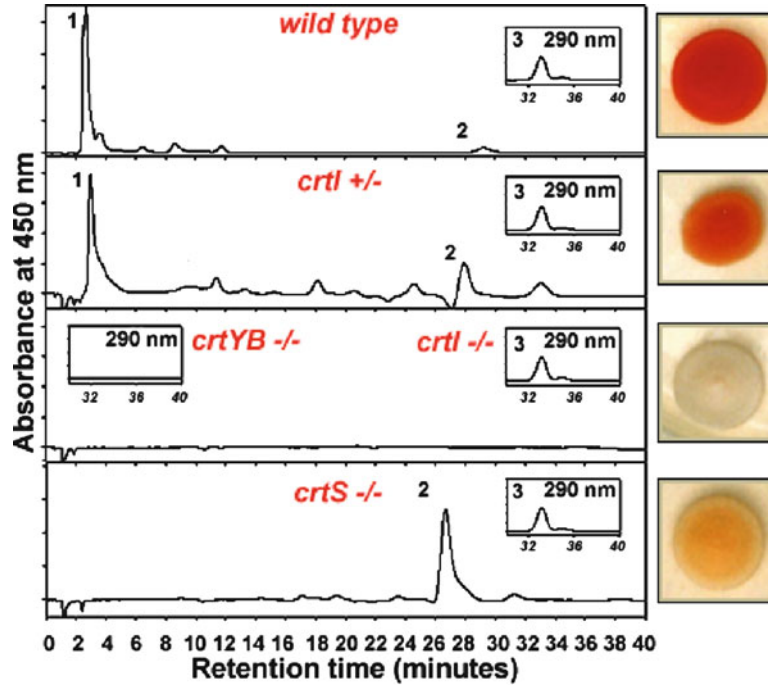

Fig. 6. RP-HPLC analysis at 450 nm of carotenoids from the parental wild type, heterozygous, and homozygous mutant strains. The numbers indicate the different carotenoids as follows: (1) astaxanthin, (2) beta-carotene, and (3) phytoene (290 nm). The strain genotype that corresponds to each chromatogram is indicated.

7. Glassmilk: Dissolve 1 g of silica in 10 mL of PBS buffer, allow the silica to settle for 2 h, remove the supernatant (containing fine particulate matter), and add 10 mL of PBS. Repeat the procedure twice. Centrifuge at 2,000×*g* for 2 min and resuspend the silica in 10 mL of 3 M NaI. Store at 4°C in the dark.
8. Each pigment is identified by comparison with specific standards based on retention time and absorption spectrum (8).
9. The purification of fragments from TBE-based gels is more complex than from TAE-based gels.
10. Only the section of gel that contains the nucleic acids should be excised. This facilitates the dissolving of the gel and allows the use of several gel pieces in a single tube, increasing the amount of DNA recovered.
11. Plasmid pMN-Hyg is digested with the *Eco*RV enzyme releasing the 1,912-bp *X. dendrorhous* hygromycin B resistance cassette. This fragment is purified by Geneclean and inserted into the *Eco*RV site of the pL22ΔRV plasmid, creating the pIH plasmid (see Fig. 4). The plasmid pL22ΔEV carries a *crt*I gene mutated by deletion of a fragment of 1,128 between *Eco*RV restriction sites.

12. Do not store the *X. dendrorhous* electrocompetent cells for long period of time. Use preferably just after production.
13. For high transformation efficiency, the DNA used for transformation must be lineal. In our case we use the pIH plasmid digested with *Xho*I and *Sma*I; these digestions release a DNA fragment containing the cassette.

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