Metabolic Engineering of the Carotenoid Biosynthetic Pathway in the Yeast Xanthophyllomyces dendrorhous (Phaffia rhodozyma)

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The crtYB locus was used as an integrative platform for the construction of specific carotenoid biosynthetic mutants in the astaxanthin-producing yeast Xanthophyllomyces dendrorhous. The crtYB gene of X. dendrorhous, encoding a chimeric carotenoid biosynthetic enzyme, could be inactivated by both single and double crossover events, resulting in non-carotenoid-producing transformants. In addition, the crtYB gene, linked to either its homologous or a glyceraldehyde-3-phosphate dehydrogenase promoter, was overexpressed in the wild type and a β-carotene-accumulating mutant of X. dendrorhous. In several transformants containing multiple copies of the crtYB gene, the total carotenoid content was higher than in the control strain. This increase was mainly due to an increase of the β-carotene and echinone content, whereas the total content of astaxanthin was unaffected or even lower. Overexpression of the phytoene synthase-encoding gene (crtI) had a large impact on the ratio between mono- and bicyclic carotenoids. Furthermore, we showed that in metabolic engineered X. dendrorhous strains, the competition between the enzymes phytoene desaturase and lycopene cyclase for lycopene governs the metabolic flux either via β-carotene to astaxanthin or via 3,4-didehydrolycopene to 3-hydroxy-3′-4′-didehydro-β-ψ-caroten-4-one (HDCO). The monocyclic carotenoid torulene and HDCO, normally produced as minority carotenoids, were the main carotenoids produced in these strains.

During the last decades fast progress has been made within the field of molecular biology of carotenoid biosynthesis in bacteria, fungi, and plants (reviewed in references 6 and 23). Although more than 600 different carotenoids have been identified in nature, only a few are used industrially. The acyclic carotenoid lycopene, the bicyclic carotenoid β-carotene, and the oxygenated bicyclic carotenoids (xanthophylls) canthaxanthin and astaxanthin are used as food colorants, animal feed additives, and in pharmaceuticals and cosmetics (15). The potential commercial interest for the production of carotenoids and the cloning of genes encoding biosynthetic enzymes has led to all kinds of examples of metabolic pathway engineering. These examples include the overexpression of a gene encoding a rate-limiting enzyme (14, 17), the expression of carotenogenic genes in noncarotenogenic heterologous hosts (12, 18, 20, 32), the increase of the carbon flux into the carotenoid biosynthetic pathway (1, 12, 17, 32), and the combination of genes and modification of catalytic activities in order to improve and/or modify carotenoid biosynthetic pathways (18, 24–26, 32).

So far, the green microalga Haematococcus pluvialis and the heterobasidiomycetous yeast Xanthophyllomyces dendrorhous, the perfect state of Phaffia rhodozyma, are the only microbial systems with commercial potentials for the production of astaxanthin. This oxygenated carotenoid is used as a feed additive in aquaculture to obtain the desired degree of pigmentation of flesh from salmon and trout. Furthermore, when astaxanthin was applied as a nutraceutical, several positive actions on degenerative diseases have been reported (8, 19, 33). The pathway of astaxanthin biosynthesis, as proposed by Andrewes and coworkers (5) is shown in Fig. 1. Several genes involved in the astaxanthin biosynthetic pathway of X. dendrorhous have been cloned and characterized recently (28–31; T. Hoshino, K. Ojima, and Y. Setoguchi, September 2000, Astaxanthin synthetase, European patent application EP 1 035 206 A1; J. C. Verdoes, J. Wery, and A. J. J. van Ooyen, July 1997, Improved methods for transforming Phaffia strains, transformed Phaffia strains so obtained, and recombinant DNA in said methods, International patent application WO 97/23633) and a transformation system has been developed (35, 36).

In this paper, we describe the engineering of the astaxanthin biosynthetic pathway of X. dendrorhous by two different approaches. By specific gene inactivation, the accumulation of intermediates is demonstrated. Furthermore, overexpression of carotenogenic genes led to altered carotenoid production levels and carotenoid compositions.

MATERIALS AND METHODS

Molecular techniques and gene cloning. Standard methods were used, unless otherwise indicated, according to Sambrook et al. (22). DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes according to the specifications of the manufacturers. Plasmid DNA from Escherichia coli was isolated by using Qiagen columns (Westburg BV, Leusden, The Netherlands). DNA fragments were purified by using a QIAEX II gel extraction kit. Determination of nucleotide sequences was performed with a Taq DYE primer cycle sequencing kit (Applied Biosystems, Nieuwerkerk aan de IJssel, The Nether-
lands). The digoxigenin (DIG) nonradioactive labeling and detection kit from Roche Diagnostics (Mannheim, Germany) was used in Southern blot analysis. DNA probes were DIG labeled with the DIG PCR labeling kit (Roche Diagnostics). Chromosomal DNA was isolated from sodium dodecyl sulfate-lysed protoplasts of \textit{X. dendrorhous} as described previously (34).

**PCR conditions and primers.** The PCRs were carried out in an automated thermal cycler (Perkin-Elmer Nederland, Nieuwerkerk aan de IJssel, The Netherlands) by using SUPER Taq (HT Biotechnology Ltd., Cambridge, England) under the conditions recommended by the supplier. The standard PCR cycle profile was 5 min at 94°C; 25 to 30 cycles consisting of 1 min at 94°C, 2 min at 50°C, and 2 to 3 min at 72°C; and a final step consisting of 10 min at 72°C. In the recombinant PCR, 0.1 μg of each fragment was used and the total number cycles in the second PCR was reduced to 20. The following primers were used: 5’/Pgpd-Pgpd, 5’-dCCTTACCATCAGCGTCTGGCATATTACC-3’; 3’/Pgpd-crtYB, 5’-dGAGTCCATGCTGTTGC-3’; 5’/crtYB-Nter, 5’-dCGCAATGACGGCTCTGC-3’; 5’/crtI, 5’-dCGCGGATCCGAAGGCGGTCCATAACAGTCATG-3’; 5’/PcrtI, 5’-dGTTCTTTCGCGGATCCACTGACGTGCTCTGCGG-3’; 3’/PcrtI, 5’-dACTCTTACCATCATGGAAAAGAACAGGATCGAAGGCGGTCCATAACAGTCATG-3’; 5’/crtYB-Nter, 5’-dGAGTCCATGCTGTTGC-3’; 5’/crtI, 5’-dACTCTTACCATCATGGAAAAGAACAGGATCGAAGGCGGTCCATAACAGTCATG-3’; 5’/PcrtI, 5’-dGTTCTTTCGCGGATCCACTGACGTGCTCTGCGG-3’; 3’/PcrtI, 5’-dACTCTTACCATCATGGAAAAGAACAGGATCGAAGGCGGTCCATAACAGTCATG-3’. In these primer sequences, the coding regions are indicated by bold letters and the start codons are underlined. Restriction sites, introduced to facilitate subcloning, are double underlined.

**Plasmids, strains, and cultivation conditions.** All plasmids and strains used in this study are summarized in Table 1. The \textit{E. coli} strain XL1-Blue MRF’ was used in all cloning experiments, and \textit{E. coli} strain DM1 was used for plasmid propagation of transformation vectors of \textit{X. dendrorhous}. To construct pPR16 (Fig. 2), the 1.9-kb BamHI-HindIII fragment derived from pPRcrtYB (31), containing the N-terminal portion of the \textit{crtYB} open reading frame, was cloned in the corresponding sites of pPR1 (35) (Fig. 3). To construct pPR19F (forward) (Fig. 3) and pPR19R (reverse), a 1.8-kb EcoRI fragment containing the G418

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**FIG. 1.** The astaxanthin biosynthetic pathway in \textit{X. dendrorhous} proposed by Andrewes et al. (5). The main carotenoids found after the introduction of additional gene copies of the phytoene desaturase-encoding gene (\textit{crtI}) in the \textit{X. dendrorhous} strains CBS 6938 and PR-1-104 (12) are boxed (this study). Roman numbers (I, II, and III) indicate three potential routes for the formation of torulene from neurosporene.
TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description of</th>
<th>Sources or reference</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
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<tr>
<td>E. coli</td>
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<tr>
<td>XL1-Blue-MRF</td>
<td>Δ(mcrA) 183Δ(mcrCB hasSMR mrr) 173 endA1 supE4 thi-1 recA1 gyrA46 relA1 lac[F' proAB lacZAM15 Tn10]</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>DM1 X. dendrorhous (P. rhodozyma) CBS 6938</td>
<td>F^- dam-13: Tn9(Cm') dcen^- mcrB HsdR^- M^ galI gal2 Ara^- Lac^- Thr^- Leu^- Ton^ Try^ sup^</td>
<td>Gibco BRL</td>
</tr>
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<td>PR-1-104</td>
<td>Wild-type strain (DNA donor strain)</td>
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<td></td>
<td>β-Carotene-accumulating mutant of CBS 6938</td>
<td>13</td>
</tr>
<tr>
<td>Plasmids</td>
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<td>pUC19</td>
<td>General cloning vector for E. coli (Ap)</td>
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<tr>
<td>pMTL22P</td>
<td>General cloning vector for E. coli (Ap)</td>
<td>31</td>
</tr>
<tr>
<td>pPRGDH6</td>
<td>5.5-kb EcoRI fragment carrying the gpd gene of X. dendrorhous in pUC19 (Ap)</td>
<td>28</td>
</tr>
<tr>
<td>pPR1</td>
<td>General transformation vector for X. dendrorhous</td>
<td>35</td>
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<tr>
<td>pPR2TN</td>
<td>Derivative of pPR1 with terminator sequences of the gpd gene of X. dendrorhous and additional unique cloning sites (Ap, G418)</td>
<td>31</td>
</tr>
<tr>
<td>pPRcrtYB</td>
<td>cDNA encoding the phytoene synthase-hyopcone cyclase of X. dendrorhous in pBluescript (Ap)</td>
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<td>pPR10F/R</td>
<td>4.5-kb EcoRI fragment carrying the genomic copy of the crtYB gene in pUC19 (Ap)</td>
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<td>pPR11</td>
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<td>This study</td>
</tr>
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<td>pPR12</td>
<td>Expression cassette (Pgpd crtYB TcrtYB) as BamHI fragment in pMTL22P (Ap)</td>
<td>This study</td>
</tr>
<tr>
<td>pPR13F/R</td>
<td>BamHI fragment (Pgpd crtYB TcrtYB) cloned in corresponding site of pPR2TN (Ap, G418)</td>
<td>This study</td>
</tr>
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<td>pPR16</td>
<td>BamHI-HindIII (crtYBα) fragment cloned in corresponding sites of pPR1 (G418)</td>
<td>This study</td>
</tr>
<tr>
<td>pPR19F/R</td>
<td>Insertion of selection cassette (Pgpd G418 Tgld) on blunt ended (Klenow) EcoRI fragment in the T4 DNA polymerase-treated BsuXI site of pPR10F (crtYBc: Pgpd G418 Tgld) (Ap, G418)</td>
<td>This study</td>
</tr>
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<td>pPR21</td>
<td>crtYB gene of X. dendrorhous as a 4.5-kb BamHI fragment in pMTL22F (Ap)</td>
<td>31</td>
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<tr>
<td>pPR22F/R</td>
<td>4.5-kb BamHI fragment from pPR21 in BamHI site of pPR2TN (Ap, G418)</td>
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<tr>
<td>pPRdpc01</td>
<td>Phytene desaturase-positive cosmid clone from a genomic library of X. dendrorhous (Ap)</td>
<td>30</td>
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<tr>
<td>pPR40F/R</td>
<td>2.8-kb BamHI PCR fragment containing the expression cassette for phytene desaturase (Perti crtYB) cloned in BamHI site of pPR2TN (Ap, G418)</td>
<td>This study</td>
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</table>

* The selective antibiotics are indicated in parentheses.

**RESULTS**

Specific inactivation of the crtYB gene. We wanted to establish a specific gene inactivation approach, which is applicable to the construction of strains which are able to accumulate...
FIG. 2. Schematic representation of the specific crtYB gene inactivation approach by single (A) and double (B) crossovers. Prior to the introduction in X. dendrorhous by electrotransformation, the plasmids PR16 and PR19F were linearized with the endonucleases BstXI and EcoRI, respectively. SI, site of insertion (the insert was a blunted EcoRI fragment in a blunted BstXI site) (Table 1).
FIG. 3. Graphical presentation of the transformation vectors (pPR1 and pPR2TN) and carotenogenic expression vectors (pPR13F, pPR22F, pPR19F, and pPR40F). Depending on the orientation of the carotenogenic expression cassette, the vectors were denominated F (forward) or R (reverse) when the carotenogenic gene was transcribed in the same or opposite direction, respectively, as the G418 marker gene. The Sau3A* (not unique) site was created by the ligation of BamHI and BglII sites. SI, site of insertion (the insert was a blunted EcoRI fragment in a blunted BstXI site; details are given in Table 1).
specific carotenoid intermediates of the astaxanthin biosynthetic pathway. For this study, the *crtYB* gene, encoding a bifunctional enzyme involved in the condensation of two geranylgeranyl diphosphate molecules in phytoene and the cyclization of lycopene into β-carotene, was used (31). Previously, an integrative DNA transformation system was developed to introduce multiple copies of a transformation vector, which is selected for by resistance to G418, into the ribosomal DNA of *X. dendrorhous* (35, 36). To inactivate the endogenous *crtYB* gene of *X. dendrorhous* by homologous recombination, two types of knockout vectors were designed (Fig. 2). Vector pPR16 was constructed to inactivate the *crtYB* gene by a single crossover event within the N-terminal portion of this gene. Vector pPR19F was used to disrupt the *crtYB* gene by a double crossover at the *crtYB* locus. After transformation and selection for G418 resistance, both plasmids yielded three white-colored colonies and several colonies with a color phenotype identical to the recipient strain. Southern blot analysis was used to show that, in these latter transformants, the plasmids had integrated in the genome but outside the *crtYB* locus (results not shown). Two white-colored colonies of each transformation experiment were subjected to Southern blot analysis to demonstrate site-specific integration in the endogenous *crtYB* locus (Fig. 4). The transformants CBS 6938(pPR16) C, CBS 6938(pPR19) A, and CBS 6938(pPR19) B showed the expected hybridization pattern. The intensity of the hybridization signal suggested that, in CBS 6938(pPR16) A, multiple DNA fragments were integrated at the *crtYB* locus. As expected, no accumulation of carotenoids was observed when carotenoid extracts from the transformants were analyzed by HPLC. Additionally, 11 red-colored colonies were found among pPR16 transformants. Analysis of the carotenoid composition of some of these colonies indicated the accumulation of low amounts of astaxanthin. Furthermore, the plasmid integrated at the *crtYB* locus, as was found by Southern blot analysis. Apparently, the integration event had resulted in a truncated *crtYB* gene copy (Fig. 2) which retained some of its phytoene synthase and lycopene cyclase activity. With *E. coli*, it was found that the enzymatic activities of such a truncated enzyme are reduced to, respectively, 30 and 15% (31).
Overexpression of the \(\text{crtYB}\) gene in \textit{X. dendrorhous} CBS 6938

To study the effect on astaxanthin biosynthesis and carotenoid composition, the phytoene synthase-lycopene cyclase-encoding gene (\(\text{crtYB}\)) was overexpressed in \textit{X. dendrorhous} wild-type strain CBS 6938. The regulation of carotenoid biosynthesis in \textit{X. dendrorhous} is largely unknown. Some preliminary data (16) suggest that the pathway is regulated by feedback inhibition of the end product astaxanthin. The promoter region of \(\text{crtYB}\) (\(P\text{crtYB}\)) might contain binding sites of regulatory proteins, interaction with which can regulate the transcription of \(\text{crtYB}\). Therefore, the \(\text{crtYB}\) gene was also linked to the promoter region of the glyceraldehyde-3-phosphate dehydrogenase-encoding gene (\(P\text{gpdc}\)) of \textit{X. dendrorhous}. The plasmids \(\text{pPR2TN}\), \(\text{pPR13F}\), and \(\text{pPR22F}\) (Fig. 3) were linearized in their ribosomal DNAs with 
\[\text{SfiI}\] and were introduced in \textit{X. dendrorhous} CBS 6938. The color of a colony of \textit{X. dendrorhous} CBS 6938 or CBS 6938(\text{pPR2TN}) is pink. The color of all transformants of \textit{X. dendrorhous} CBS 6938 carrying integrated copies of \(\text{pPR13F}\) or \(\text{pPR22F}\), hereafter referred to as CBS 6938(\text{pPR13F}) and CBS 6938(\text{pPR22F}), was orange. No visual differences in color were observed when the different CBS 6938(\text{pPR13F}) and CBS 6938(\text{pPR22F}) colonies were compared. This indicates that the carotenoid composition in these strains has been changed due to the introduction of additional copies of the \(\text{crtYB}\) gene. One transformant of CBS 6938(\text{pPR2TN}) and several transformants of CBS 6938 (\text{pPR13F}) and CBS 6938(\text{pPR22F}) were randomly selected. Southern blot analysis demonstrated the integration of multiple copies of the transformation vector in these transformants (Fig. 4). Initially, the spectra of the carotenoid extracts were recorded from 250 to 600 nm. In CBS 6938 and CBS 6938 (\text{pPR2TN}), the absorbance peak was at 471 nm, whereas the absorbance peak for CBS 6938(\text{pPR13F}) and CBS 6938 (\text{pPR22F}) shifted to 465 to 467 and 468 nm, respectively. The carotenoid composition was further analyzed by HPLC. In both types of transformants, an increase in the total amount of carotenoids was found (Table 2). This increase is mainly caused by higher amounts of both \(\beta\)-carotene and echinone. In transformant CBS 6938(\text{pPR13F}) no. 18, an almost twofold increase in the total amount of carotenoids and a slight increase in the specific astaxanthin production were observed. However, in all transformants analyzed, the relative amount of astaxanthin was reduced compared to that of the control.

Overexpression of \(\text{crtYB}\) expression cassettes in PR-1-104

The plasmids \(\text{pPR2TN}\), \(\text{pPR13F}\), and \(\text{pPR22F}\) were also introduced in a carotenoid biosynthetic mutant of \textit{X. dendrorhous} CBS 6938. This strain, PR-1-104, does not produce xanthophylls but accumulates \(\beta\)-carotene (13). After introduction of the plasmids in strain PR-1-104, several G418-resistant colonies were isolated. The color phenotype of all \(\text{pPR13F}\)- and \(\text{pPR22F}\)-derived colonies was different from that of the host strain and PR-1-104(\text{pPR2TN}) transformants, indicating that the carotenoid composition had changed. All PR-1-104 (\text{pPR13F}) and PR-1-104(\text{pPR22F}) transformants displayed a bright yellow color, and therefore, two colonies of each were randomly selected for further analysis. In all PR-1-104 transformants, only the accumulation of one carotenoid, \(\beta\)-carotene, was observed (Table 2). No significant differences in production levels were determined between the two expression signals.

\begin{table}
\centering
\small
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Carotenoid & Specific amount (\(\mu\text{g/g dry weight}\)) & (\% relative distribution) of carotenoid in strain* &
\hline
 & CBS 6938(\text{pPR2TN}) & CBS 6938(\text{pPR22F}) & CBS 6938(\text{pPR13F}) & CBS 6938(\text{pPR13F}) & CBS 6938(\text{pPR13F})
\hline
Astaxanthin & 84 ± 11 (33) & 78 ± 22 (24) & 48 ± 4 (16) & 70 ± 13 (26) & 95 ± 29 (21)
\hline
Phoenicoxanthin & 50 ± 7 (19) & 43 ± 30 (15) & 43 ± 1 (14) & 43 ± 11 (16) & 59 ± 21 (13)
\hline
HO-echinone & 20 ± 2 (8) & — (0) & 4 ± 5 (1) & — (0) & — (0)
\hline
Echinone & 31 ± 3 (12) & 88 ± 7 (33) & 114 ± 4 (38) & 78 ± 24 (28) & 181 ± 41 (39)
\hline
\(\beta\)-Carotene & 28 ± 7 (11) & 40 ± 3 ± 15 & 69 ± 9 (23) & 50 ± 3 ± 18 & 113 ± 31 (24)
\hline
HDCO & 39 ± 2 (15) & 23 ± 1 (9) & 18 ± 2 (6) & 31 ± 11 (11) & 17 ± 2 (4)
\hline
Torulene & 5 ± 3 (5) & — (0) & 7 ± 10 (2) & — (0) & — (0)
\hline
Total & 256 ± 12 (100) & 271 ± 42 (106) & 302 ± 13 (118) & 273 ± 13 (107) & 465 ± 24 (182)
\hline
\end{tabular}
\caption{Carotenoid composition of \textit{X. dendrorhous} strains CBS 6938 and PR-1-104 overexpressing the phytoene synthase-lycopene cyclase-encoding gene by using homologous (\text{pPR22F}) and heterogeneous (\text{pPR13F}) expression signals*

* In PR-1-104 and its derivatives, only \(\beta\)-carotene is produced. Total amounts of \(\beta\)-carotene were as follows (results are in micrograms per gram [dry weight], with % relative distribution in parentheses): PR-1-104(\text{pPR2TN}) no. 1, 308 ± 28 (100); PR-1-104(\text{pPR22F}) no. 1, 407 ± 8 (132); PR-1-104(\text{pPR22F}) no. 2, 418 ± 3 (136); PR-1-104(\text{pPR13F}) no. 1, 412 ± 26 (134); PR-1-104(\text{pPR13F}) no. 2, 372 ± 11 (121).

All strains were cultivated in duplicate, and the indicated numbers are the averages of two cultures (the value for the strain with the empty cloning vector \(\text{pPR2TN}\) is set at 100).

—, not detected.

\textit{For more information on Table 2, please refer to the original text for detailed explanations and interpretations.\end{table}}
**DISCUSSION**

The potential of X. dendrorhous as a microalgal source of astaxanthin was recognized soon after the isolation of this yeast by Hermann Phaff and coworkers (12). So far, strategies to improve astaxanthin production in X. dendrorhous have been based on classical mutagenesis and selection (2, 9, 13) or the improvement of fermentation conditions (4, 11). The expression of the introduced phytoene desaturase-encoding gene or a mutated CRTYB gene in X. dendrorhous has been isolated by classical mutagenesis yet (13). An additional advantage of this method has been the isolation of mutants lacking the CRTYB gene, by either phage transduction or chemical mutagens (13). A heterologous phytoene desaturase-encoding gene or a mutated CRTYB gene copy of X. dendrorhous that lacks the CRTYB gene product, as it possesses both phytoene desaturase-negative and lycopene cyclase activity (31). Two white strains, PR-1-120 and PR-1-139, were selected. The results of the analysis of carotenoid extracts are shown in Table 3. In most of the transformants of PR-1-104, four strains, hereafter referred to as PR-1-104(PR40F) no. 4 and no. 11 and PR-1-104(PR40R) no. 3 and no. 14 were selected. The results of the analysis of carotenoid extracts are shown in Table 3. In most of the transformants, the relative production levels compared to that of the control strain CBS 6938(pPR2TN) are indicated in parentheses. For the PR-1-104 strains, the relative production levels are compared to those of the control strain PR-1-104(pPR2TN) and are indicated in parentheses.
struct a lycopene-accumulating \textit{X. dendrorhous} strain. However, we have found that these mutants have retained some of the lycopene cyclase activity (J. C. Verdoes, unpublished data). Engineered strains such as CBS 6938(pPR16) or CBS 6938 (pPR19F), with inactivated \textit{crtYB} genes and displaying no lycopene cyclase activity, are much more defined and are therefore better as starting material for such an approach.

One possibility for the improvement of the metabolic productivity of an organism is genetic modification. This strategy can be successful when an increase of the flux through a pathway is achieved by, e.g., the overproduction of the rate-limiting enzyme, an increase of precursors, or the modification of the regulatory properties of enzymes. The isolation of several carotenogenic genes of \textit{X. dendrorhous} enabled us to study the effect of their overexpression on carotenoid biosynthesis. Overexpression of the chimeric \textit{crtYB} gene either under control of the \textit{gpd} or \textit{crtYB} promoter leads to a different carotenoid composition in both the wild type and a \textit{β-carotene-accumulating X. dendrorhous} strain (Table 2). In transformant CBS 6938(pPR13F) no. 18, the total number of carotenoids increased by 82%. Although the absolute astaxanthin content was higher than in the wild-type strain, the relative amount decreased. This is the result of a 270% increase in the amount of \textit{β-carotene} and echinone. A similar increase was observed in all transformants of CBS 6938 carrying additional \textit{crtYB} gene copies. Furthermore, in the transformants of CBS 6938, the relative amount of monocyclic carotenoids, e.g., torulene and HDCO, is reduced by at least 50%. No accumulation of carotenoids other than \textit{β-carotene} was observed when the \textit{crtYB} gene was overexpressed in a \textit{β-carotene-accumulating X. dendrorhous} strain. Compared to the control strain, a small but significant increase in the total amount of \textit{β-carotene} was observed in all transformants.

Overexpression of the phytoene desaturase-encoding gene (\textit{crtI}) of \textit{X. dendrorhous} affected the ratio between bicyclic and monocyclic carotenoids in both CBS 6938 and PR-1-104 (Table 3). In the control strain, more than 84% consisted of bicyclic carotenoids. However, this number is reduced to less than 50% in the transformants. In transformants of CBS 6938, the main carotenoid is HDCO (Fig. 1). Furthermore, the relative astaxanthin content is decreased twofold and that of \textit{β-carotene} and echinone is decreased by a factor 3. Introduction of \textit{crtI} gene copies in the \textit{β-carotene-accumulating strain PR-1-104 has a negative effect on the \textit{β-carotene} production and the total carotenoid production. In PR-1-104(pPR40R) no. 3, there was an increase in the specific and relative amounts of the monocyclic carotenoid torulene by factors of 2 and 5, respectively (Table 3).

Recently, An et al. (3) proposed the presence of a monocyclic carotenoid pathway in \textit{X. dendrorhous} in addition to the dicyclic one proposed by Andrews et al. (5). The fact that torulene is the end product in a \textit{β-carotene-accumulating strain carrying multiple copies of the phytoene desaturase-encoding gene (\textit{crtI}) suggests that the enzymes that convert \textit{β-carotene} into astaxanthin are the same ones that convert echinone into HDCO (Fig. 1). Apparently, these enzymes have a broad substrate range and can accept both monocyclic and bicyclic carotenoids. In some of the carotenoid-hyperproducing mutants studied by An et al. (3), one or more mutations affecting phytoene synthase activity and/or \textit{crtI} gene expression may explain the observed increased levels of monocyclic carotenoids.

The carotenoid biosynthetic enzymes of \textit{X. dendrorhous} are specific only to certain regions of the substrate molecule. It was shown previously that neurosporene is also a substrate for the cyclase moiety of \textit{crtYB} in a heterologous genetic background.
Overexpression of the crtYB gene, encoding the bifunctional carotenogenic enzyme, in CBS 6938 resulted in the accumulation of the intermediates β-carotene and echinone (Table 2). When the flux towards β-carotene was reduced by the introduction of additional copies of the phytoene desaturase-encoding gene, a decrease in the amounts of these two compounds was observed (Table 3). These results indicate that, under overexpression of the crtYB gene, the oxygenation reactions (e.g., of β-carotene and echinone) are limiting in the pathway to astaxanthin. The increase of lycopene cyclase activity also resulted in a decrease of the carotenoids derived from 3,4-didehydrolycopene like torulene and HDCO.

A decisive reaction for the formation of monocyclic or bicyclic products is the desaturation sequence to lycopene and further on to 3,4-didehydrolycopene. In the nontransformed strain, cyclization of lycopene, which directs the metabolic flux towards astaxanthin, is the dominating reaction. However, when the gene encoding phytoene desaturase is overexpressed, the five-step desaturation to 3,4-didehydrolycopene is intensified, resulting in an accumulation of torulene and HDCO as subsequent products (Table 3). Apparently, the strength of the crtI expression, i.e., the amounts and activities of phytoene desaturase present, determine the number of double bonds to be introduced by the desaturase. It can be concluded from the results of the crtI and crtYB transfectants that, in X. dendrorhous, the competition between desaturase and cyclase for lycopene governs the metabolic flux either via β-carotene to astaxanthin or via 3,4-didehydrolycopene to HDCO. This indicates that a change in the ratios of carotenogenic enzymes in X. dendrorhous by either induced mutations or metabolic engineering may affect the amounts and composition of carotenoids. We propose that, like in Phycomyces blakesleeanus (10), the carotenogenic enzymes of X. dendrorhous are present in a complex (Fig. 5). Increased levels of the phytoene desaturase might alter the sequence of reactions and therefore the end products that are formed. From this viewpoint, it might be important, in order to optimize astaxanthin production, to overexpress multiple carotenogenic genes in such a way, e.g., by coregulated expression, that the ratios are not affected.

It is anticipated that ultimately, by using the methods presented in this study and by a combination of overexpression and deletion of specific carotenoid biosynthetic genes, the carotenoid content in X. dendrorhous can be altered significantly and can be directed to produce a specific carotenoid in higher amounts.

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REFERENCES


