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High-Level Production of Beta-Carotene in *Saccharomyces cerevisiae* by Successive Transformation with Carotenogenic Genes from *Xanthophyllomyces dendrorhous*▽

René Verwaal,[1]† Jing Wang,[1] Jean-Paul Meijnen,[1] Hans Visser,[1]‡ Gerhard Sandmann,[2] Johan A. van den Berg,[1] and Albert J. J. van Ooyen[1]*

Fungal Genomics, Laboratory of Microbiology, Wageningen University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands,[1] and Biosynthesis Group, Molecular Biosciences 213, J. W. Goethe Universität, Siesmayerstrasse 70, P.O. Box 111932, D-60054 Frankfurt, Germany[2]

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To determine whether *Saccharomyces cerevisiae* can serve as a host for efficient carotenoid and especially β-carotene production, carotenogenic genes from the carotenoid-producing yeast *Xanthophyllomyces dendrorhous* were introduced and overexpressed in *S. cerevisiae*. Because overexpression of these genes from an episomal expression vector resulted in unstable strains, the genes were integrated into genomic DNA to yield stable, carotenoid-producing *S. cerevisiae* cells. Furthermore, carotenoid production levels were higher in strains containing integrated carotenogenic genes. Overexpression of *crtYB* (which encodes a bifunctional phytoene synthase and lycopene cyclase) and *crtI* (phytoene desaturase) from *X. dendrorhous* was sufficient to enable carotenoid production. Carotenoid production levels were increased by additional overexpression of a homologous geranylgeranyl diphosphate (GGPP) synthase from *S. cerevisiae* that is encoded by *BTS1*. Combined overexpression of *crtE* (heterologous GGPP synthase) from *X. dendrorhous* with *crtYB* and *crtI* and introduction of an additional copy of a truncated 3-hydroxy-3-methylglutaryl–coenzyme A reductase gene (*tHMG1*) into carotenoid-producing cells resulted in a successive increase in carotenoid production levels. The strains mentioned produced high levels of intermediates of the carotenogenic pathway and comparable low levels of the preferred end product β-carotene, as determined by high-performance liquid chromatography. We finally succeeded in constructing an *S. cerevisiae* strain capable of producing high levels of β-carotene, up to 5.9 mg/g (dry weight), which was accomplished by the introduction of an additional copy of *crtI* and *tHMG1* into carotenoid-producing yeast cells. This transformant is promising for further development toward the biotechnological production of β-carotene by *S. cerevisiae*.

Carotenoids are a class of pigments of commercial interest that have important biological functions. In humans, β-carotene is the precursor of vitamin A; it may function as an antioxidant, has properties protective against cancer, and stimulates the immune system (14, 22, 32). Because carotenoids are colored compounds, they are being used as pigments in the food and feed industries (20). Many carotenoids are being produced by chemical synthesis, which yields products that are pure and cheap (10). Several microorganisms, including fungi, bacteria, and algae, are able to produce carotenoids naturally (12). Biotechnological synthesis of certain carotenoids, by either homologous or heterologous production, may become more and more attractive (15, 17). One example of a carotenoid-producing yeast is the red yeast *Xanthophyllomyces dendrorhous*, which was formerly known as *Phaffia rhodozyma* (9). This yeast mainly produces the carotenoid astaxanthin but also accumulates some β-carotene as an intermediate of the astaxanthin biosynthesis pathway (1, 7, 35). The genes involved in β-carotene production in *X. dendrorhous* have been cloned previously (33, 34). In *X. dendrorhous*, the ergosterol and carotenoid biosynthetic pathways are connected by their utilization of prenyl diphosphates (Fig. 1). Farnesyl diphosphate (FPP) is converted into geranylgeranyl diphosphate (GGPP) by GGPP synthase, which is encoded by *crtE*. Next, the phytoene synthase activity of the bifunctional enzyme CrtYB results in the synthesis of phytoene from two GGPP molecules. Phytoene is subsequently converted into lycopene by four desaturation reactions catalyzed by the enzyme CrtI. Subsequently, two cyclization reactions catalyzed by CrtYB result in the conversion of lycopene into γ-carotene and finally into β-carotene. *X. dendrorhous* mutants with higher carotenoid production capacities have been obtained by chemical mutagenesis (1, 7) or by recombinant DNA technology (35, 39).

The food yeast *Saccharomyces cerevisiae* is widely used in the brewing and fermentation industries; it is generally recognized as safe and can be used for the production of biomass rich in high-quality proteins and metabolites. Furthermore, it has the advantage of easy genetic manipulation with established host-vector systems (2, 21). In order to transform *S. cerevisiae* into a β-carotene-producing organism, precursors of carotenoids should be present. Like *X. dendrorhous*, *S. cerevisiae* is able to produce FPP and converts it into GGPP, the basic building block of carotenoids. Conversion of FPP into GGPP is cata-

* Corresponding author. Mailing address: Fungal Genomics, Laboratory of Microbiology, Wageningen University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands. Phone: 31-6-42255321. Fax: 31-3-17484011. E-mail: Ab.vanOoyen@wur.nl.

† Present address: DSM Food Specialties, 2600 MA Delft, The Netherlands.

‡ Present address: Dyadic Nederland BV, 3704 HE Zeist, The Netherlands.

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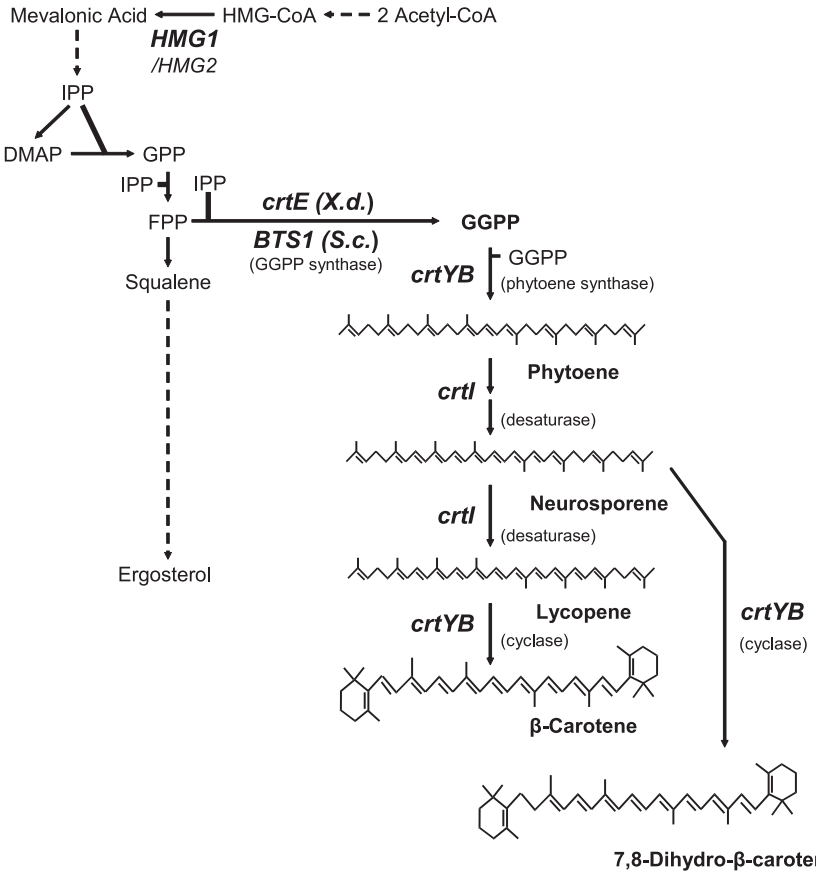

FIG. 1. Overview of the ergosterol biosynthetic pathway in *S. cerevisiae* and the carotenogenic pathway in *X. dendrorhous*. The carotenogenic pathway in *X. dendrorhous* consists of GGPP synthase encoded by *crtE*, the bifunctional enzyme phytoene synthase and lycopene cyclase encoded by *crtYB*, and phytoene desaturase encoded by *crtI*. *S. cerevisiae* contains a GGPP synthase, encoded by *BTS1*, which is able to convert FPP into GGPP. *HMG1* encodes HMG-CoA reductase, which is the main regulatory point in the ergosterol biosynthetic pathway in many organisms. IPP, isopentenyl diphosphate; DMAP, dimethylallyl diphosphate; GPP, geranyl diphosphate.

lyzed by GGPP synthase encoded by *BTS1* in *S. cerevisiae* (11; Fig. 1). Therefore, overexpression of only *crtYB* and *crtI* from *X. dendrorhous* in *S. cerevisiae* should generally be sufficient to transform *S. cerevisiae* into a β-carotene-producing organism. Additional overexpression of *crtE* from *X. dendrorhous* or *BTS1* from *S. cerevisiae* might increase GGPP levels and thereby enhance β-carotene production. In an initial effort to produce β-carotene heterologously in *S. cerevisiae*, the carotenogenic genes *crtE*, *crtB*, *crtI*, and *crtY* from the bacterium *Erwinia uredovora* were introduced and overexpressed from episomal vectors with different yeast-derived promoters and terminators for each gene. This resulted in quite low β-carotene production levels of 103 μg/g (dry weight [dw]) (41). Overexpression of the same genes in the food yeast *Candida utilis* resulted in higher β-carotene production levels of 400 μg/g (dw) (18). Additional overexpression of the catalytic domain of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase from *C. utilis*, which is considered to be a key regulatory step in ergosterol biosynthesis in yeasts (4), resulted in a 7.1-fold increase in lycopene content. The effect of HMG-CoA reductase on β-carotene production was not examined (29).

Attempts to produce carotenoids heterologously in *Escherichia coli* by using genes from *X. dendrorhous* resulted in poor enzyme expression and carotenoid production (G. Sandmann, unpublished results). Although heterologous carotenoid production in *S. cerevisiae* has already been studied by using bacterial genes from *E. uredovora*, we expect that β-carotene production levels will be much higher with genes from another yeast species. Therefore, we have constructed a series of carotenoid-producing *S. cerevisiae* strains by successive introduction and overexpression of carotenogenic genes from *X. dendrorhous*.

MATERIALS AND METHODS

Vector construction and transformation. Episomal and integration vectors were constructed as depicted in Fig. 2a. The genes *crtYB*, *crtI*, and *crtE* were amplified with a cDNA library from *X. dendrorhous* as the template (36) and primers indicated in Table 1. The sequences of the carotenogenic genes can be accessed at NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&itool=toolbar). The *BTS1* gene was amplified with genomic DNA prepared from *S. cerevisiae* strain CEN.PK 113-7D and primers indicated in Table 1. Standard PCR conditions and the proofreading enzyme super TAQ plus (SphaeroQ, Gorinchem, The Netherlands) were used in all PCRs, except for site-directed mutagenesis. The amplified PCR products were verified by sequencing. The *crtYB*, *crtI*, *crtE*, and *BTS1* PCR products were BamHI/SalI ligated into vector p426GPD, containing 680 bp of the promoter region upstream of the start codon of the *TDH3* gene and the first 250 bp of the *CYC1* terminator from *S. cerevisiae* (19). Subsequently, *TDH3* promoter-gene-*CYC1* terminator prod-

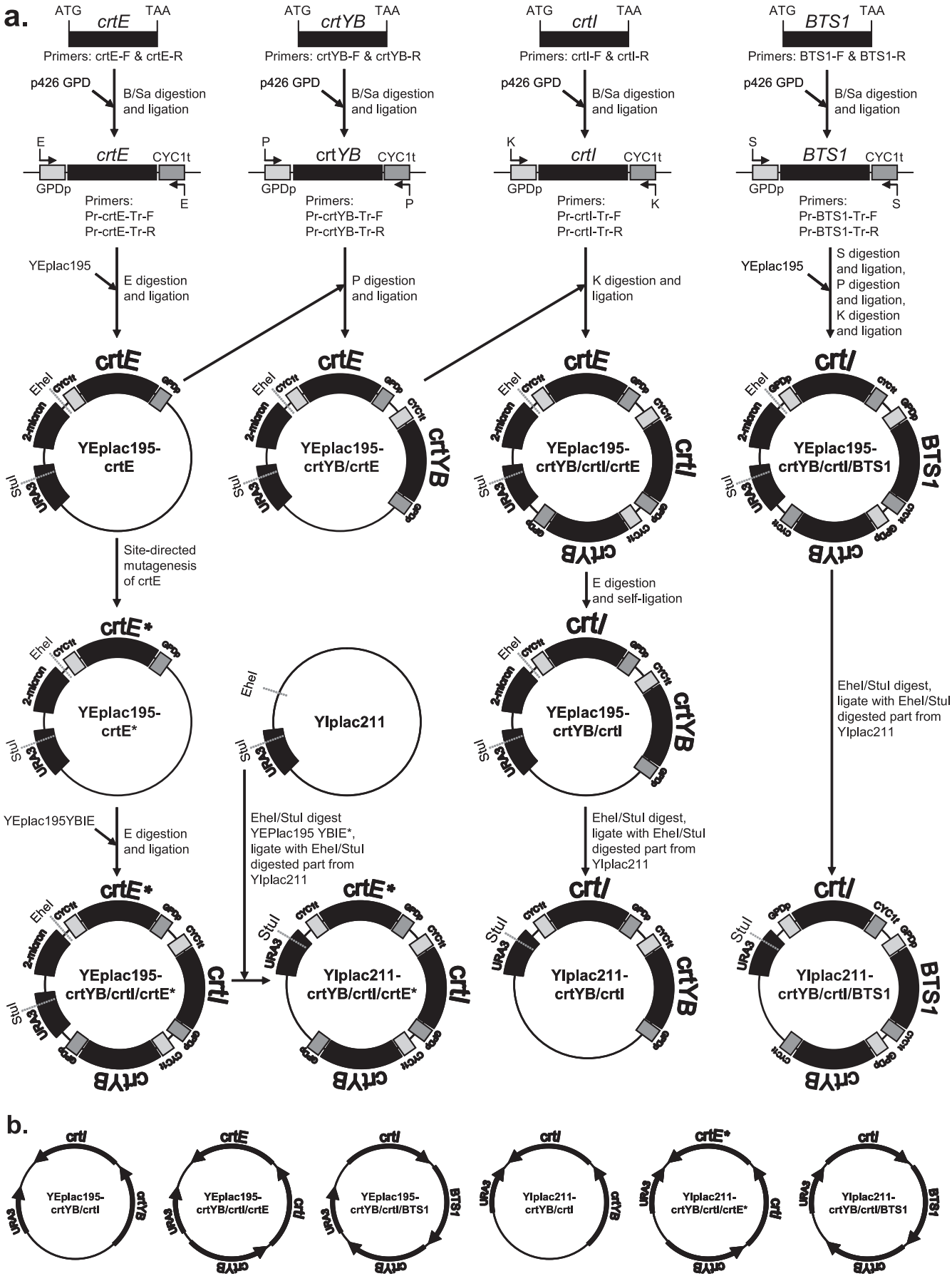
a.
ATG
TAA
crtE
Primers: crtE-F & crtE-R
p426 GPD
B/Sa digestion and ligation
E
CYC1t
GPDp
Primers: Pr-crtE-Tr-F Pr-crtE-Tr-R
YEplac195
E digestion and ligation
YEplac195-crtE
Site-directed mutagenesis of crtE
crtE*
YEplac195-crtE*
YEplac195YBIE
E digestion and ligation
YEplac195-crtYB/crtI/crtE*
crtYB
P
Primers: crtYB-F & crtYB-R
Primers: Pr-crtYB-Tr-F Pr-crtYB-Tr-R
P digestion and ligation
YEplac195-crtYB/crtE
EheI/StuI digest YEPlac195 YBIE*, ligate with EheI/StuI digested part from YIplac211
YIplac211
YIplac211-crtYB/crtI/crtE*
crtI
K
Primers: crtI-F & crtI-R
Primers: Pr-crtI-Tr-F Pr-crtI-Tr-R
K digestion and ligation
YEplac195-crtYB/crtI/crtE
E digestion and self-ligation
YEplac195-crtYB/crtI
EheI/StuI digest, ligate with EheI/StuI digested part from YIplac211
YIplac211-crtYB/crtI
S
BTS1
Primers: BTS1-F & BTS1-R
Primers: Pr-BTS1-Tr-F Pr-BTS1-Tr-R
S digestion and ligation, P digestion and ligation, K digestion and ligation
YEplac195-crtYB/crtI/BTS1
EheI/StuI digest, ligate with EheI/StuI digested part from YIplac211
YIplac211-crtYB/crtI/BTS1
b.
YEplac195-crtYB/crtI
YEplac195-crtYB/crtI/crtE
YEplac195-crtYB/crtI/BTS1
YIplac211-crtYB/crtI
YIplac211-crtYB/crtI/crtE*
YIplac211-crtYB/crtI/BTS1

TABLE 1. PCR and qPCR primers used in this study and referred to in Fig. 2[a]

Primer	Sequence (5′ to 3′)
For PCR	
crtYB-F	CGCGGATCCATGACGGCTCTCGCATATTAC
crtYB-R	TGCGGTCGACTTACTGCCCTTCCCATCCGC
crtI-F	GCGGGATCCATGGGAAAAGAACAAGATCAGG
crtI-R	TGCGGTCGACTCAGAAAGCAAGAACACCAACG
crtE-F	CGCGGATCCATGGATTACGCGAACATCCTC
crtE-R	TGCGGTCGACTCACAGAGGGATATCGGCTAG
BTS1-F	CGCGGATCCATGGAGGCCAAGATAGATGAG
BTS1-R	TGCGGTCGACTCACAATTCGGATAAGTGGTCT
Pr-crtYB-Tr-F	AAAACTGCAGCAGTTCGAGTTTATCATTATCAAT
Pr-crtYB-Tr-R	TTTTCTGCAGGCGGCCGCGCAAATTAAAGCCTTCGAGCG
Pr-crtI-Tr-F	CGGGGTACCCAGTTCGAGTTTATCATTATCAAT
Pr-crtI-Tr-R	GCCGGTACCGGCCGGCCGCAAATTAAAGCCTTCGAGCG
Pr-crtE-Tr-F	GGAATTCCAGTTCGAGTTTATCATTATCAAT
Pr-crtE-Tr-R	CGAATTCGGCGCGCCGCAAATTAAAGCCTTCGAGCG
Pr-BTS1-Tr-F	TCCCCCGGGCAGTTCGAGTTTATCATTATCAAT
Pr-BTS1-Tr-R	AGGCCCGGGGGCGCGCCGCAAATTAAAGCCTTCGAGCG
crtE (551 A-to-G mutation)-F	CCATAGAGGGCA**G**GGCCTGGAGCTATTC
crtE (551 A-to-G mutation)-R	GAATAGCTCCAGGCC**C**TGCCCTCTATGG
tHMG1-F	GGACTAGT**ATG**GACCAATTGGTGAAAACTGAAG
tHMG1-R	CCGCTCGAGTTAGGATTTAATGCAGGTGACG
Pr-tHMG1-Tr-F	AAAACTGCAGCAGTTCGAGTTTATCATTATCAAT
Pr-tHMG1-Tr-R	TTTTCTGCAGGCGGCCGCGCAAATTAAAGCCTTCGAGCG
For qPCR	
ACT1-F	GCCTTGGACTTCGAACAAGA
ACT1-R	CCAAACCCAAAACAGAAGGA
crtYB-F	TAGTCGCCTACGCAGAGGAT
crtYB-R	TCTCTCCGACGTCTCCTTTC
crtI-F	GGGCTGACTTAGTTGGTGGA
crtI-R	TTGTGTCGAATGATCCCTTG
crtE-F	CACAGAAACCCACTCATTCG
crtE-R	GTTCTTCCCCGTTCTTCCTT
BTS1-F	CGAAAGGTCAAACTGAGCAAC
BTS1-R	GCGAAGCCAAATCAGGTAAA

[a] Underlined letters indicate restriction enzyme cleavage sites corresponding to the primer description. Bold letters indicate the A-to-G mutation in the *crtE* gene. Bold and underlined letters indicate the start codon created for *tHMG1*.

ucts were amplified by PCR with the primers indicated in Table 1 (Isogen Life Science, IJsselstein, The Netherlands). To construct episomal expression vectors, *TDH3p-crtE-CYC1t* was EcoRI ligated into vector YEplac195 (6). Next, *TDH3p-crtYB-CYC1t* was PstI ligated and *TDH3p-crtI-CYC1t* was KpnI ligated into YEplac195E, resulting in vector YEplac195YB/I/E. To construct YEplac195YB/I/BTS1, in sequential order *TDH3p-BTS1-CYC1t* was SmaI ligated, *TDH3p-crtYB-CYC1t* was PstI ligated, and *TDH3p-crtI-CYC1t* was KpnI ligated into YEplac195. To construct YEplac195YB/I, YEplac195YB/I/E was restricted with EcoRI and the remaining vector was self-ligated. For targeted integration into the *ura3-52* locus, integrative vectors were constructed (6). Targeted integration into the *ura3-52* locus requires linearization of vector YIplac211 with StuI. The StuI restriction site present in the coding sequence of *crtE* was eliminated by site-directed mutagenesis. A at position 551 was mutated to G with vector YEplac195E as the template and the primers indicated in Table 1 (for the procedure used, see reference 37). *THD3p-crtE*-CYC1t* (* denotes the A-to-G mutation) was EcoRI ligated into YEplac195YB/I/E restricted with EcoRI. The 2μm part of YEplac195 was eliminated by ligating a 694-bp EheI/StuI fragment from vector YIplac211 into YEplac195YB/I/E*, YEplac195YB/I/BTS1, and YEplac195YB/I, which were restricted by EheI/StuI (6). In this manner, tandem head-to-tail orientation of the genes introduced was maintained. All promoter-gene-terminator fusions were indeed present in tandem head-to-tail orientation on the YEplac and YIplac expression vectors, as determined by restriction analysis and shown schematically in Fig. 2b. The YIplac211 vectors containing carotenogenic genes were linearized with StuI and transformed into strain CEN.PK 113-5D to create strains YB/I/BTS1 and YB/I/E. Single-copy integration of the constructs was confirmed by Southern blotting by standard laboratory procedures (27). To create vector YIplac128 *crtI*, vector YEplac195YB/I was restricted with KpnI and the *TDH3p-crtI-CYC1t* fragment was ligated into YIplac128 (6). For overexpression of the catalytic domain of HMG-CoA reductase (*tHMG1* for truncated *HMG1*), the 1,575-bp C-terminal part of *HMG1* was amplified with primers tHMG1-F and tHMG1-R and *S. cerevisiae* genomic DNA as the template. In the forward primer, a start codon (ATG) was included. Amplified t*HMG1* was SpeI/XhoI ligated into vector p426 GPD (19). Subsequently, *TDH3p-tHGM1-CYC1t* was amplified with primers Pr-tHMG1-Tr-F and Pr-tHMG1-Tr-F and PstI ligated into YIplac204 (6) to create YIplac204 *tHMG1*. To create strain YB/I/E + extra I, YIplac128 *crtI* was linearized with ClaI for targeted integration into the *leu2,3-112* locus. To create strain YB/I/E + tHMG1, YIplac204 *tHMG1* was linearized with EcoRV to target integration into the

FIG. 2. Construction of expression vectors used in this study. (a) Scheme representing the construction of the different vectors used in this study. GPDp is the 680-bp sequence of the *TDH3* promoter, and CYC1t is the 250-bp sequence of the *CYC1* terminator. Restriction enzyme abbreviations: B, BamHI; Sa, SalI; E, EcoRI; P, PstI; K, KpnI; S, SmaI. Sequences used for primers are indicated in Table 1. Integration into the *ura3-52* locus requires linearization of the YIplac211 constructs with StuI. Because *crtE* contains a StuI restriction site, A at position 551 was changed into G by site-directed mutagenesis (indicated by an asterisk). See Materials and Methods for details concerning the cloning strategy used. (b) Orientation of the carotenogenic genes in the vectors used in this study, as determined by restriction analysis. All genes are present in tandem head-to-tail orientation on the expression vectors.

TABLE 2. Yeast strains and plasmids used in this study[a]

Yeast strain or plasmid	Relevant features
Yeast strains	
CEN.PK 113-7D[b]	*MAT***a** *SUC2 MAL2-8*^{c}
CEN.PK 113-5D[b]	*MAT***a** *SUC2 MAL2-8*^{c} *ura3-52*
CEN.PK 113-6B[b]	*MAT***a** *SUC2 MAL2-8*^{c} *ura3-52 leu2,3-112 trp1-289*
Episomal vector transformants	
YB/I	CEN.PK 113-5D + YEplac195 YB/I
YB/I/BTS1	CEN.PK 113-5D + YEplac195 YB/I/BTS1
YB/I/E	CEN.PK 113-5D + YEplac195 YB/I/E
YB/I/E*	CEN.PK 113-5D + YEplac195 YB/I/E*
Integrative vector transformants	
YB/I/BTS1	CEN.PK 113-5D + YIplac211 YB/I/BTS1
YB/I/E	CEN.PK 113-5D + YIplac211 YB/I/E*
YB/I/E + extra I	CEN.PK 113-6B + YIplac211 YB/I/E* + YIplac128 *crtI*
YB/I/E + tHMG1	CEN.PK 113-6B + YIplac211 YB/I/E* + YIplac204 *tHMG1*
YB/I/E + tHMG1 + extra I	CEN.PK 113-6B + YIplac211 YB/I/E* + YIplac204 *tHMG1* + YIplac128 *crtI*
Plasmids	
YEplac195 YB/I	YEplac195 *TDH3p-crtYB-CYC1t*; *TDH3p-crtI-CYC1t*
YEplac195 YB/I/BTS1	YEplac195 *TDH3p-crtYB-CYC1t*; *TDH3p-crtI-CYC1t*; *TDH3p-BTS1-CYC1t*
YEplac195 YB/I/E	YEplac195 *TDH3p-crtYB-CYC1t*; *TDH3p-crtI-CYC1t*; *TDH3p-crtE-CYC1t*
YEplac195 YB/I/E*	YEplac195 *TDH3p-crtYB-CYC1t*; *TDH3p-crtI-CYC1t*; *TDH3p-crtE*-CYC1t*
YIplac211 YB/I/BTS1	YIplac211 *TDH3p-crtYB-CYC1t*; *TDH3p-crtI-CYC1t*; *TDH3p-BTS1-CYC1t*
YIplac211 YB/I/E*	YIplac211 *TDH3p-crtYB-CYC1t*; *TDH3p-crtI-CYC1t*; *TDH3p-crtE*-CYC1t*
YIplac128 *crtI*	YIplac128 *TDH3p-crtI-CYC1t*
YIplac204 *tHMG1*	YIplac204 *TDH3p-tHMG1-CYC1t*

[a] The asterisk indicates the 551A-to-G mutation in the *crtE* coding sequence. Functional genes are in uppercase italics; nonfunctional ones are in lowercase italics. Promoters and terminators are represented as follows: *TDH3p*, *TDH3* promoter; *CYC1t*, *CYC1* terminator. For targeted integration, integrative (YIplac) plasmids were transformed as described in Materials and Methods.

[b] Institut für Mikrobiologie, J. W. Goethe Universität, Frankfurt am Main, Germany.

trp1-289 locus. The linearized vectors were transformed into *S. cerevisiae* strain CEN.PK 113-6B previously transformed with YIplac211 YB/I/E*. To create strain YB/I/E + tHMG1 + extra I, strain YB/I/E + tHMG1 was transformed with ClaI-linearized YIPlac128 *crtI* for targeted integration into the *leu2,3-112* locus. All constructs were transformed into yeast by electroporation (3). The constructs created in this study are indicated in Table 2.

Strains and media. The *S. cerevisiae* strains used in this study are indicated in Table 2. During batch culture, experiments yeast cells were grown on yeast nitrogen base (YNB) without amino acids (Difco, Boom, The Netherlands), supplemented with the appropriate amino acids when required and 2% (wt/vol) D-glucose. Cells were grown at 225 rpm and 30°C in a shaking incubator.

Carotenoid analysis. Cells were resuspended in 1 ml sterile water, 1 g 0.50- to 0.75-mm glass beads was added, and cells were broken by vortexing for 3 min. A 2.5-ml volume of 0.2% (wt/vol) pyrogallol dissolved in methanol was added, and cells were vortexed for 10 s. After adding 1.25 ml 60% (wt/vol) KOH and vortexing for 10 s, the cells were incubated for 1 h at 75°C with vortexing every 15 min for saponification. Next, an appropriate amount of hexane was added to extract the carotenoid fraction. The tubes were centrifuged for 5 min at 2,800 rpm, and 1 ml of the hexane was pipetted into a cuvette. Absorption between 550 and 400 nm was monitored on a Shimadzu UV-2501 PC spectrophotometer (Shimadzu, Duisburg, Germany) to measure the amounts of colored carotenoids. A standard curve was determined by measuring known β-carotene concentrations. The total colored carotenoid concentration was calculated by the following formula: total carotenoids (in $\mu g\ g^{-1}$ [dw]) = (A_{449} × ml hexane)/(0.2072 × g [dw]). An aliquot of the total carotenoid extractions was evaporated under nitrogen in the dark for subsequent analysis of the carotenoid composition by high-performance liquid chromatography (HPLC). HPLC separation and quantization were performed on a Nucleosil C_{18} 3-μm column eluted isocratically with acetonitrile–methanol–2-propanol (50:40:10, vol/vol) at a flow rate of 1 ml min^{-1} (30). The separated carotenoids were detected with a Kontron 440 diode array detector and spectra were directly recorded online. Reference compounds for identification and quantitation were generated in transgenic *E. coli* cells (28). The dw of a sample was measured by taking the same culture volume as used for the carotenoid extraction, drying it overnight at 80°C, and measuring the weight of the dried yeast cells on an analytical balance.

qPCR studies. Total RNA was isolated as previously described (38). To ensure similar amounts of starting materials, total RNA concentrations were carefully measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc.). Next, chromosomal DNA was degraded by treatment with DNase I (amplification grade; Invitrogen) according to the manufacturer's instructions. Subsequently, cDNA was amplified with an Omniscript RT kit (QIAGEN, Venlo, The Netherlands) with the following modifications. A 10 mM deoxynucleoside triphosphate stock (Promega) and an Oligo-dT18 primer (Isogen Life Science, IJsselstein, The Netherlands) were used in the reaction mixtures. Pipetting was performed by a pipetting robot (Corbett Robotics), with the amplified cDNA, the quantitative PCR (qPCR) primers as indicated in Table 1, and ABsolute QPCR SYBR green mix (ABgene). These qPCR primers were designed with Primer3 (26). The qPCRs were performed in a Rotor Gene RG-3000 real-time PCR machine (Corbett Research). To correct for differences in the amounts of starting materials, *ACT1* was chosen as a reference housekeeping gene. The results are presented as ratios of gene expression between the target gene (gene of interest) and the reference gene, *ACT1* (23).

RESULTS AND DISCUSSION

Transformation with episomal vectors. To determine whether *S. cerevisiae* is able to produce carotenoids, carotenogenic genes from *X. dendrorhous* were initially overexpressed from episomal vectors. Expression of each of the genes introduced was controlled by the constitutive *TDH3* promoter and the *CYC1* terminator (19). Overexpression of *crtYB* and *crtI* with YEplac195 resulted in faintly yellow transformants. Additional overexpression of the GGPP synthase *BTS1* from *S. cerevisiae* or *crtE* from *X. dendrorhous* resulted in orange cells. When grown in YNB 2% glucose medium, all transformants reached optimal densities similar to the optical density of wild-type cells, indicating that carotenoid production in *S. cerevisiae*

TABLE 3. Carotenoid compositions of *S. cerevisiae* strain transformed with the episomal vector YEplac195 containing different carotenogenic genes

Carotenoid	Specific amt (μg/g [dw]) (% distribution) of carotenoids[a]		
	YB/I	YB/I/BTS1	YB/I/E
Phytoene	1 (33)	61 (73)	310 (64)
Neurosporene	—[b]	—	13 (3)
Lycopene	—	—	79 (16)
β-Carotene	2 (67)	13 (16)	85 (17)
7,8-Dihydro-β-carotene	—	9 (11)	—
Total	3	83 (28)	487 (162)

[a] The data are averages of two independent cultures. The *n*-fold increases in total carotenoid production levels compared to that of strain YB/I are in parentheses in the bottom row (the value for strain YB/I is set at 1).
[b] —, not detected.

does not influence growth (data not shown). After centrifugation, cells overexpressing *crtYB* and *crtI* were faintly yellow, cells overexpressing *crtYB*, *crtI*, and *BTS1* were yellow, and cells that overexpressed *crtYB*, *crtI*, and *crtE* were orange. HPLC studies confirmed that carotenoids were produced in cells overexpressing *crtYB* and *crtI* (3 μg total carotenoids/g [dw]) and that additional overexpression of *BTS1* resulted in a 28-fold increase in carotenoid production (83 μg/g [dw]). Overexpression of *crtYB*, *crtI*, and *crtE* resulted in 162-fold higher total carotenoid levels compared to cells overexpressing *crtYB* and *crtI* (487 μg/g [dw]; Table 3). It was expected that overexpression of carotenogenic genes from *X. dendrorhous* would yield *S. cerevisiae* cells producing high levels of pure β-carotene. However, HPLC studies showed that besides β-carotene, cells overexpressing *crtYB* and *crtI* also produced phytoene and cells overexpressing *crtYB*, *crtI*, and *BTS1* additionally produced phytoene and dihydro-β-carotene (a cyclization product of neurosporene). Phytoene, neurosporene, and lycopene were also accumulated in cells overexpressing *crtYB*, *crtI*, and *crtE* (Table 3).

It was observed that about 10% of the cells transformed with episomal vectors lost their color after growth for 3 days in YNB medium and subsequent growth for 3 days on agar plates (data not shown). This result suggested instability of the strain or instability of the expression vector. Episomal expression vectors tend to be structurally unstable, especially when they contain large inserts (42). This was indeed confirmed by extraction of the expression vectors containing carotenogenic genes from white yeast cells and subsequent restriction analysis to determine the presence of the introduced genes on the vector (data not shown). To avoid the problem of instability, integrative vectors were used to generate a new series of transformants.

Transformation with integrative vectors. To create genetically stable carotenoid-producing *S. cerevisiae* cells, integrative vectors were constructed as indicated in Fig. 2. Because integration into the *ura3-52* locus required linearization of the vectors with StuI, which is present within the coding sequence of *crtE*, the StuI restriction site within the *crtE* gene was changed by site-directed mutagenesis. This mutation did not influence carotenoid accumulation (data not shown). Constructs containing different combinations of carotenogenic genes were integrated into genomic DNA. Integration of *crtYB* and *crtI* resulted in faintly yellow colonies, additional integration of *crtE* resulted in orange cells and integration of *crtYB*, *crtI*, and *BTS1* resulted in yellow cells. Transformants were grown overnight in YNB medium and subsequently streaked onto nonselective agar plates. Less than 0.5% of the cells lost their color after 3 days of incubation, indicating that the stability of carotenoid-producing *S. cerevisiae* cells is greatly increased by genomic integration of carotenogenic genes. Because YB/I cells produced very low levels of carotenoids, this strain was excluded from further studies. To determine the exact composition of the accumulated carotenoids, cells were grown for 72 h in liquid cultures and HPLC studies were performed (Table 4). The growth properties of YB/I/E and YB/I/BTS1 cells were similar compared to those of wild-type cells (data not shown). Carotenoid production levels were higher in cells containing integrated carotenogenic genes compared to expression from episomal vectors (Tables 3 and 4). Copy numbers of episomal (YEplac) vectors are, in general, higher compared to those of integrative (YIplac) vectors (25), suggesting that higher protein levels and hence higher carotenoid production levels should be obtained. Results similar to ours were obtained with *E. coli*, where higher β-carotene production was obtained with a low-copy-number vector compared to a high-copy-number vector (13). The use of high-copy-number plasmids increases the demand for nucleotides during plasmid replication and might result in metabolic burden issues (8), resulting in decreased carotenoid production levels. Furthermore, it was observed that total carotenoid levels were higher

TABLE 4. Carotenoid compositions of *S. cerevisiae* strains containing integrated carotenogenic gene overexpression cassettes and an additional *crtI* overexpression cassette, an additional *tHMG1* overexpression cassette, or both

Carotenoid	Specific amt (μg/g [dw]) (% distribution) of carotenoids[a]				
	YB/I/BTS1	YB/I/E	YB/I/E+tHMG1	YB/I/E+I	YB/I/E+tHMG1+I
Phytoene	476 (94)	1,323 (86)	10,302 (92)	682 (29)	5,380 (48)
Neurosporene	3 (0.5)	5 (0.5)	48 (0.5)	75 (3)	—[b]
β-Carotene	15 (3)	141 (9)	501 (4.5)	1,627 (68)	5,918 (52)
β-Zeacarotene	4 (0.5)	16 (1)	109 (1)	—	—
7,8-Dihydro-β-carotene	10 (2)	60 (3.5)	262 (2)	—	—
Total	508 (33)	1,545 (100)	11,222 (726)	2,384 (154)	11,298 (731)

[a] The data are averages of two independent cultures, except for YB/I/E+tHMG1+I, which are triplicates. The relative total carotenoid production levels compared to that of the YB/I/E strain are in parentheses in the bottom row (the value for strain YB/I/E is set at 100%).
[b] —, not detected.

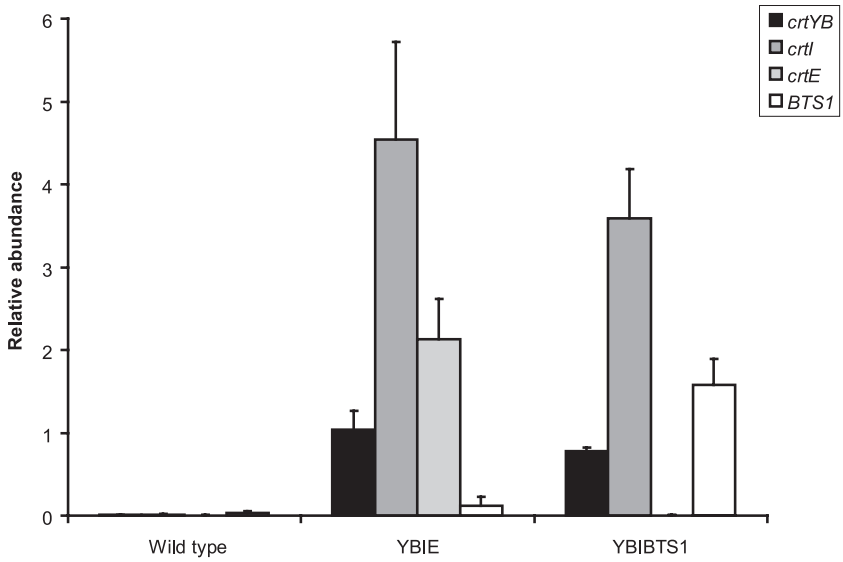

FIG. 3. qPCR studies to determine overexpression of carotenogenic genes in carotenoid-producing yeast strains. Three cultures of each strain, the wild type, YB/I/E, and YB/I/BTS1, were inoculated in YNB with 2% glucose at the same optical density. Six hours after inoculation, samples were taken and further processed for cDNA synthesis. qPCR experiments were performed to determine the relative abundances of *crtYB*, *crtI*, *crtE*, and *BTS1* in each strain with respect to that of *ACT1*, which encodes actin and served as an internal control. The data represent the average and standard deviation of three independently grown cultures.

in YB/I/E cells compared to YB/I/BTS1 cells (Tables 3 and 4). This might be caused by differences in substrate specificity for CrtE and Bts1. It has been reported that CrtE from *E. uredovora* uses both FPP and GPP as substrates (40), which might be similar for CrtE from *X. dendrorhous*. Only FPP can serve as a substrate for Bts1 (11). The ability to use both FPP and GPP as precursors might lead to higher carotenoid production levels. In both strains, about 90% of the produced carotenoids consisted of phytoene, whereas small amounts of neurosporene, β-zeacarotene, and 7,8-dihydro-β-carotene were also present (Table 4). It has been shown that overexpression of lycopene cyclase (*crtY*) from *E. uredovora* or *Capsicum annuum* in carotenoid-producing *E. coli* cells resulted in production of the bicyclic carotenoid 7,8-dihydro-β-carotene via monocyclic β-zeacarotene and neurosporene (31). Formation of these carotenoids in our carotenoid-producing *S. cerevisiae* strains is probably the result of the high cyclase activity of CrtYB (Fig. 1).

Accumulation of intermediates indicated that the flux through the carotenogenic pathway was not fully efficient. The high levels of phytoene and other intermediates with a degree of desaturation lower than that of β-carotene in YB/I/BTS1 and YB/I/E cells suggest that the phytoene desaturation reaction might be the bottleneck in heterologous β-carotene production by *S. cerevisiae*. This could be caused by poor transcription or translation efficiency of *crtI* or poor activity of the phytoene desaturase protein in *S. cerevisiae*. Accumulation of intermediates was not observed in the production of carotenoids in *E. coli* with the carotenogenic genes from *E. uredovora* (16). However, in *S. cerevisiae* cells overexpressing carotenogenic genes from *E. uredovora* on episomal vectors, intermediates also accumulated; 78% of the total carotenoids consisted of β-carotene, 11% accumulated as phytoene, and 11% accumulated as lycopene (41). Apparently, phytoene desaturation becomes a rate-limiting step in heterologous β-carotene production by *S. cerevisiae* when using carotenogenic genes from *X. dendrorhous* or *E. uredovora*. Accumulation of phytoene was also observed in a lycopene-producing *C. utilis* strain transformed with carotenogenic genes from *E. uredovora* (18). It was suggested that yeast membrane environments in which the conversion of phytoene into lycopene is likely to occur are not suitable for an efficient desaturation reaction. Suitable electron carriers, required for the dehydrogenation reaction, might be absent. It was presumed that active proteins would be present because of the presence of carotenogenic gene transcripts (18).

To determine whether the integrated carotenogenic genes in our strains were correctly expressed, qPCR studies were performed (Fig. 3). In wild-type cells, no signal was obtained with primers to detect the expression of *crtYB*, *crtI*, and *crtE*, whereas the GGPP synthase *BTS1* was expressed at a low level. In YB/I/E cells, quite high expression of *crtYB*, *crtI*, and *crtE* and low expression of *BTS1* were detected. In YB/I/BTS1 cells, no expression of *crtE* was detected, whereas *crtYB*, *crtI*, and *BTS1* were expressed at high levels compared to the housekeeping gene *ACT1*, which encodes actin. In both YB/I/E and YB/I/BTS1 cells, the relative expression levels of *crtI* were the highest of the carotenogenic genes introduced. The results from the qPCR studies indicated that the high phytoene levels were presumably not caused by poor *crtI* transcription efficiency.

Additional overexpression of *crtI* in strain YB/I/E. Although high accumulation of phytoene was not caused by low *crtI* transcription levels, we tested whether additional overexpression of the *crtI* gene and integration at another locus in YB/I/E cells would have an effect on phytoene and β-carotene levels. The *TDH3p-crtI-CYC1t* cassette was integrated into the *leu2,3-112* locus in YB/I/E cells. An increased copy number might result in higher levels of the phytoene desaturase protein and

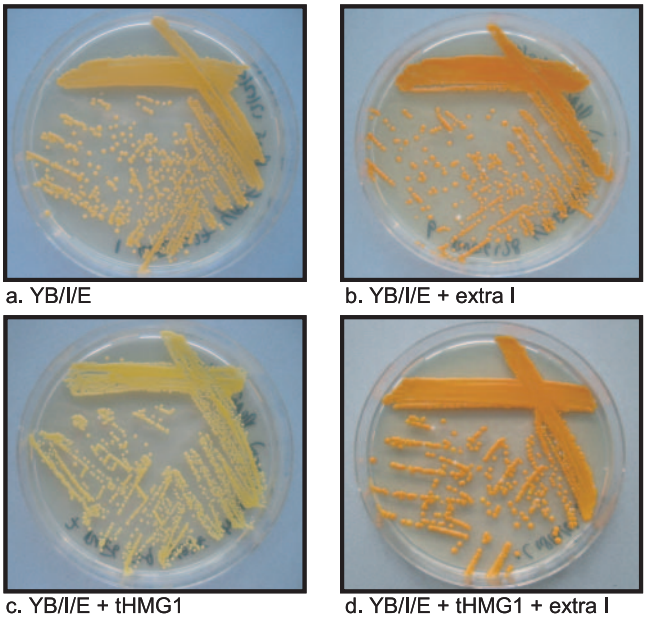

FIG. 4. Colors of different carotenoid-producing *S. cerevisiae* mutants. All strains were initially transformed with YIplac211 *crtYB*/*crtI*/*crtE** to obtain carotenoid-producing cells and subsequently with the integrated vectors containing the indicated genes. (a) Strain YB/I/E, CEN.PK 113-5D; (b) strain YB/I/E+extra I, CEN.PK 113-6B transformed with YIplac204 *crtI*; (c) strain YB/I/E+tHMG1, CEN.PK 113-6B transformed with YIplac128 *tHMG1*; (d) strain YB/I/E+extra I+tHMG1, CEN.PK 113-6B transformed with YIplac128 *crtI* and YIplac204 *tHMG1*. Individual transformants were grown for 2 days on YNB–2% glucose medium supplemented with amino acids where required and streaked onto yeast extract–peptone–2% glucose plates. After 3 days of incubation at 30°C, the plates were photographed.

could lead to more efficient conversion of phytoene into downstream products of the carotenogenic pathway. The transformants displayed a more orange color compared to that of YB/I/E cells (Fig. 4a and b). Subsequent HPLC studies revealed that overexpression of *crtI* in strain YB/I/E resulted in a 1.5-fold increase in the total carotenoid levels compared to those of strain YB/I/E (Table 4). The major difference was a decrease in phytoene accumulation (86% to 29% of the total carotenoid levels) and an increase in β-carotene accumulation (9% to 68% of the total carotenoid levels), yielding β-carotene levels of around 1.5 mg/g (dw). Apparently, additional overexpression of *crtI* in a strain overexpressing *crtYB*, *crtI*, and *crtE* resulted in increased desaturation of phytoene and greatly improved the flux toward β-carotene. Possibly, the amount of CrtI protein should reach a certain level in order to efficiently convert phytoene into lycopene in the membrane environment of *S. cerevisiae*. This level might be reached after additional introduction and overexpression of the *crtI* gene.

Overexpression of the catalytic domain of *HMG1* in strain YB/I/E. It has been shown that the flux through the ergosterol biosynthetic pathway, which is related to the carotenoid pathway by prenyl diphosphate utilization, can be increased by overexpression of the catalytic domain of HMG-CoA reductase (*tHMG1*) in *S. cerevisiae* (5, 24). Furthermore, overexpression of *tHMG1* from *C. utilis* in *C. utilis* cells heterologously producing lycopene resulted in increased lycopene production, probably by increasing the supply of precursors (29). Therefore, it was determined whether overexpression of *tHMG1* could increase carotenoid production in carotenoid-producing *S. cerevisiae* cells. For this purpose, the *TDH3p-tHMG1-CYC1t* cassette was integrated into the *trp1-289* locus in YB/I/E cells. Transformation of *tHMG1* resulted in a clear color difference; YB/I/E cells were orange, and YB/I/E+tHMG1 cells were yellow (Fig. 4a and c). Overexpression of the catalytic domain of Hmg1 results in a sevenfold increase in total carotenoid levels compared to those of YB/I/E cells (Table 4). This increase in total carotenoid accumulation is largely caused by a massive increase in phytoene levels, up to 10 mg/g (dw), which suggested that desaturation of phytoene is limiting without additional *crtI* overexpression.

Overexpression of *tHMG1* and additional overexpression of *crtI* in strain YB/I/E. The results obtained so far suggest that combined overexpression of *crtI* and *tHMG1* in carotenoid-producing *S. cerevisiae* cells results in a strain that efficiently produces high β-carotene levels. Additional overexpression of *crtI* in strain YB/I/E+tHMG1 indeed increased the flux through the carotenogenic pathway and improved β-carotene production levels (Table 4). Phytoene levels decreased from 10.3 mg/g (dw) in strain YB/I/E+tHMG1 to 5.4 mg/g (dw) in strain YB/I/E+tHMG1+I, whereas β-carotene levels increased from 0.5 mg/g (dw) to 5.9 mg/g (dw). Total carotenoid accumulation levels were similar in strain YB/I/E+tHMG1 and strain YB/I/E+tHMG1+I (11 mg/g [dw]). The transformants were more orange than YB/I/E+tHMG1 cells (Fig. 4c and d). The absence of intermediates with a lower degree of desaturation than β-carotene (neurosporene, β-zeacarotene, and 7,8-dihydro-β-carotene) in strain YB/I/E+tHMG1+I indicated that the flux through the carotenogenic pathway was more efficient compared to that in strain YB/I/E+tHMG1. Although strains producing high levels of β-carotene were created by overexpression of *tHMG1* and additional overexpression of *crtI* in strain YB/I/E, still relatively high phytoene levels accumulated, suggesting that a limitation at the level of phytoene desaturation still exists. Possibly, fine-tuning of *tHMG1* expression levels to more efficiently control phytoene production might further increase β-carotene production levels.

Concluding remarks. In summary, we have been able to construct *S. cerevisiae* strains that produce various amounts of carotenoids by integration and overexpression of carotenogenic genes from *X. dendrorhous*. We succeeded in the construction of a strain producing 5.9 mg β-carotene/g (dw), which is 57-fold more than previously reported for heterologous β-carotene production in *S. cerevisiae* (41). This was achieved by overexpression of the catalytic domain of *HMG1* from *S. cerevisiae* and additional overexpression of the *crtI* gene from *X. dendrorhous* in carotenoid-producing *S. cerevisiae* cells transformed with carotenogenic genes from *X. dendrorhous*. Optimizing the culturing conditions, for instance, by growing the strains in large volumes under controlled fermentor conditions, might further increase β-carotene yields. This approach was successful for engineered β-carotene-producing *E. coli* strains (13). Possibly, β-carotene production levels can be further increased by chemical mutagenesis. This strategy has resulted in *X. dendrorhous* strains with increased astaxanthin production levels (1). The strains producing high β-carotene

levels are promising for further development toward the biotechnological production of β-carotene by *S. cerevisiae*.

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