Biotechnol Lett (2012) 34:2077–2082
DOI 10.1007/s10529-012-1004-6

ORIGINAL RESEARCH PAPER

Molecular cloning and functional expression of two key carotene synthetic genes derived from *Blakeslea trispora* into *E. coli* for increased β-carotene production

Jie Sun · Xin-Xiao Sun · Ping-Wah Tang · Qi-Peng Yuan

Received: 23 May 2012 / Accepted: 28 June 2012 / Published online: 14 July 2012
© Springer Science+Business Media B.V. 2012

Abstract *Blakeslea trispora* is used commercially to produce β-carotene. Isopentenyl pyrophosphate isomerase (IPI) and geranylgeranyl pyrophosphate synthase (GGPS) are key enzymes in the biosynthesis of carotenoids. The cDNAs of genes *ipi* and *carG* were cloned from the fungus and expressed in *Escherichia coli*. Greater GGPS activity was needed in the engineered *E. coli* when IPP activity was increased. The introduction of GGPS and IPI increased the β-carotene content in *E. coli* from 0.5 to 0.95 mg/g dry wt.

Jie Sun and Xin-Xiao Sun have contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-012-1004-6) contains supplementary material, which is available to authorized users.

J. Sun · X.-X. Sun · P.-W. Tang · Q.-P. Yuan (✉)
State Key Laboratory of Chemical Resource Engineering, College of Life Science & Technology, Beijing University of Chemical Technology, Beijing 100029, China
e-mail: yuanqp@mail.buct.edu.cn

J. Sun
e-mail: sunjiehh@126.com

X.-X. Sun
e-mail: sunxinxiao@126.com

P.-W. Tang
e-mail: pwtang@yahoo.com

Keywords *Blakeslea trispora* · β-Carotene · Functional expression · Geranylgeranyl pyrophosphate synthase · Isopentenyl pyrophosphate isomerase

Introduction

Carotenoids are tetraterpenoid organic pigments that are widely distributed in nature and have applications as food colorants, animal feed supplements, nutraceuticals, and cosmetic and pharmaceutical fields. As shown in Fig. 1, carotenoids, such as lycopene and β-carotene, are produced by fungi via the mevalonate pathway, which synthesizes isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP) from acetyl-CoA (Bejarano and Cerdá-Olmedo 1992; Disch and Rohmer 1998). IPP isomerase (IPI, EC 5.3.3.2, encoded by *ipi*) catalyzes an essential activation step in isoprenoid metabolism in the conversion of the relatively unreactive IPP to the more reactive electrophile DMAPP (Anderson et al. 1989). Then farnesyl pyrophosphate synthase (FPS, EC 2.5.1.10) catalyzes the biosynthesis of farnesyl pyrophosphate (FPP) by the condensation of two units of IPP and one unit of DMAPP (Homann et al. 1996). Geranylgeranyl pyrophosphate synthase (GGPS, EC 2.5.1.29, encoded by *carG*) catalyzes the formation of GGPP by the condensation of DMAPP and FPP (Jiang et al. 1995).

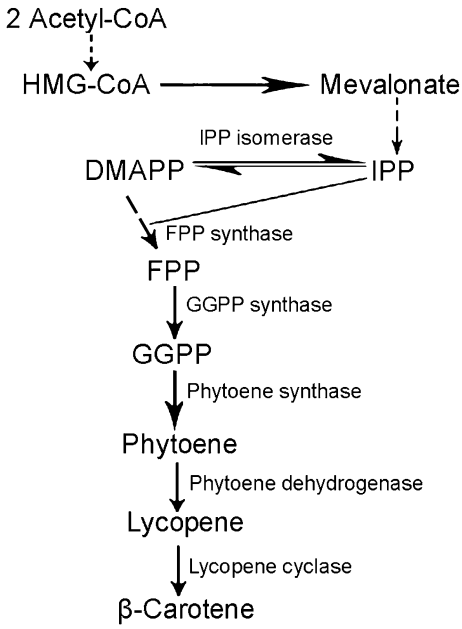

Fig. 1 Pathway of carotenoid biosynthesis in fungi. *HMG-CoA* 3-Hydroxy-3-methylglutaryl-coenzyme A, *DMAPP* dimethylallyl pyrophosphate, *IPP* isopentenyl pyrophosphate, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate

Varied carotenoids are synthesized via the sequential catalyzes with the participation of lycopene cyclase, phytoene synthase, phytoene dehydrogenase, or ketolase (Steiger and Sandmann 2004).

The zygomycete *Blakeslea trispora* produces β-carotene as its predominant carotenoid (Kuzina and Cerdá-Olmedo 2007) and is used on an industrial scale to produce β-carotene. IPI and GGPS are key enzymes in the biosynthesis of carotenoids. Thus, genetic manipulation of these two enzymes facilitates the accumulation of carotenoids. To date, only *carRA* and *carB* have been cloned (Rodriguez-Saiz et al. 2004), the full sequences of other important genes in carotenoid biosynthetic pathway of *B. trispora*, including *carG* and *ipi*, remain unknown.

In the present study, full-length cDNAs of *ipi* and *carG* from *B. trispora* were cloned and analyzed. Functional expression of *ipi* and *carG* in *Escherichia coli* was carried out and the end production β-carotene was measured. The present study provides new gene sources for carotene genetic engineering and is helpful for the understanding of the carotene biosynthesis.

Materials and methods

Strains, plasmid, and culture conditions

Blakeslea trispora ATCC 14272 (−) was maintained on potato/dextrose/agar plates. Spores were harvested by rinsing the mature cultures with distilled water. For RNA isolation, 4×10^4 spores were inoculated into 50 ml liquid synthetic mucor medium (SMM, composed of glucose 40 g, asparagine 2 g, KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, thiamine 0.5 mg and 1 l distilled water) containing 1 % (w/v) malt extract and cultured at 28 °C without light.

Competent *E. coli* cells were transformed with plasmid pACCAR16ΔcrtX harboring geranylgeranyl pyrophosphate synthase gene (*crtE*), phytoene synthase gene (*crtB*), phytoene desaturase gene (*crtI*), and lycopene cyclase gene (*crtY*) genes can reconstruct the biosynthetic pathway of β-carotene in *E. coli* (Misawa et al. 1995). The plasmid pACCAR25ΔcrtE carrying crtB, crtI, crtY, zeaxanthin glycosylase gene (crtZ), and β-carotene hydroxylase gene (crtX) was used to confirm the function of *carG* (Sandmann et al. 1993).

5′-RACE

Total RNA was extracted with TRIzol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, USA). An aliquot of 1 μg total RNA was reversely transcribed with 5′-RACE GSP1 and MMLV reverse transcriptase (Promega, Madison, USA). The purified first-strand cDNA was added with polycytimidine using terminal deoxynucleotidyl transferase (TdT) (TaKaRa, Japan) and dCTP. GSP primers are designed based on the EST sequences of *ipi* and *carG* (Genbank accession number JK017326 and JK017328, respectively). The sequences of primers used were shown in Supplementary Table 1. The 5′-RACE PCR was performed twice. The first PCR was carried out with 5′-RACE adaptor and 5′-RACE GSP1. The product was diluted to 500-fold as the template of the second PCR, which was performed with 5′-RACE primer and 5′-RACE GSP2. The PCR product was purified and cloned into pMD18-T vector followed by sequencing.

3′-RACE

Total RNA (1 μg) was used to synthesize cDNA as the template of 3′-RACE PCR with the 3′-RACE adaptor. The 3′-RACE PCR was also performed twice. The primers of the first PCR were 3′-RACE primer and 3′-RACE GSP1. The second PCR was performed with 3′-RACE primer and 3′-RACE GSP2.

Bioinformatic analysis

The 3′-end, core fragment and 5′-end sequences of *ipi* and *carG* were assembled using the DNAMAN software package (Version 5.2.2, Lynnon Biosoft, Canada). Bioinformatic analysis of the deduced amino acid sequence of IPI and GGPS from *B. trispora* was carried out online (http://www.ncbi.nlm.nih.gov and http://cn.expasy.org). The phylogenetic trees for IPIs and GGPSs were constructed by using the neighbor-joining method of MEGA4, and the bootstrap values were produced with 1,000 repeats (Tamura et al. 2007).

Expression of *ipi* and *carG* in *E. coli*

The coding regions of *ipi* and *carG* were amplified using the primers as shown in Supplementary Table 2,

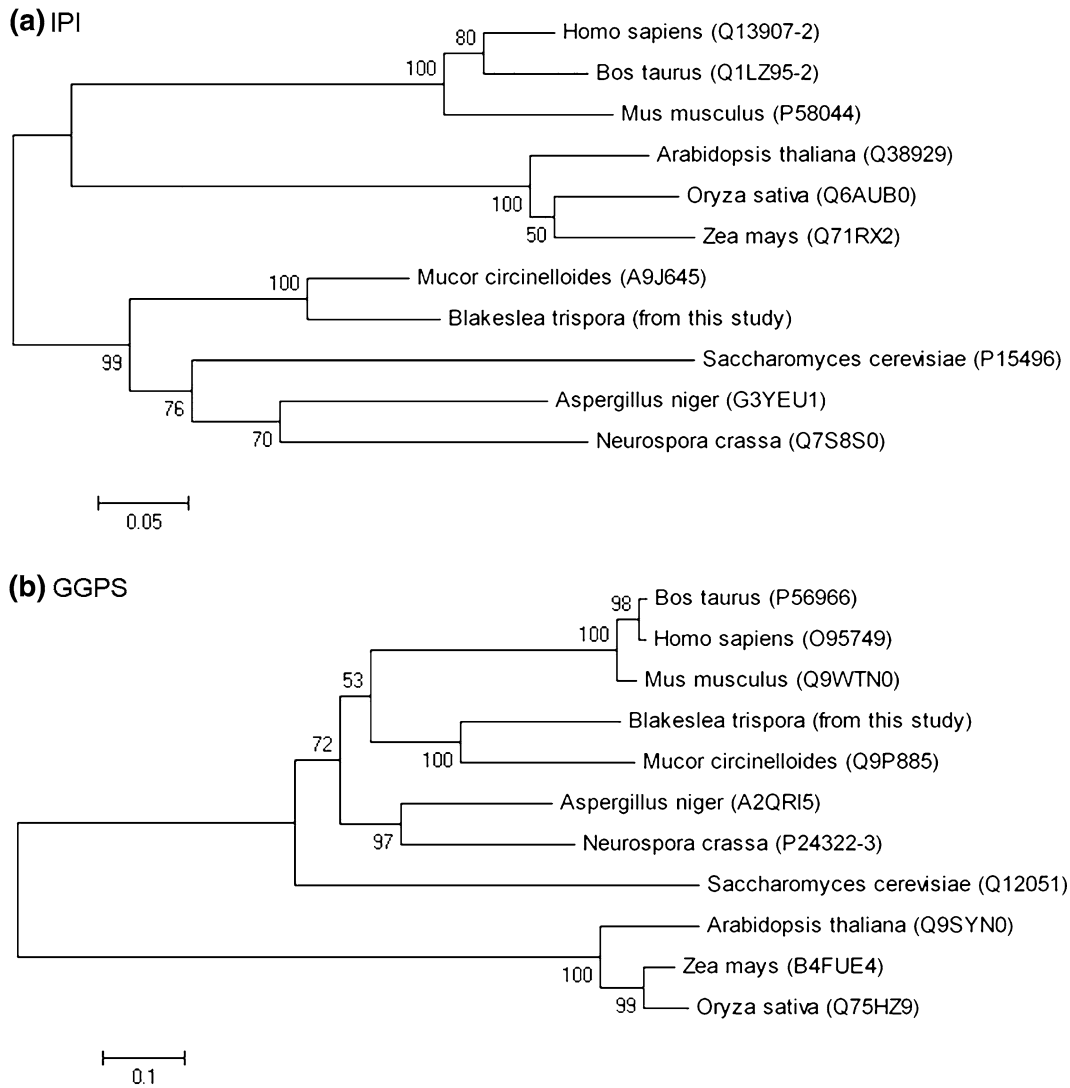

Fig. 2 Phylogenic tree analyses of IPIs (**a**) and GGPSs (**b**) from different organisms constructed by the neighbor-joining method. The protein accession number was obtained from the website http://www.uniprot.org

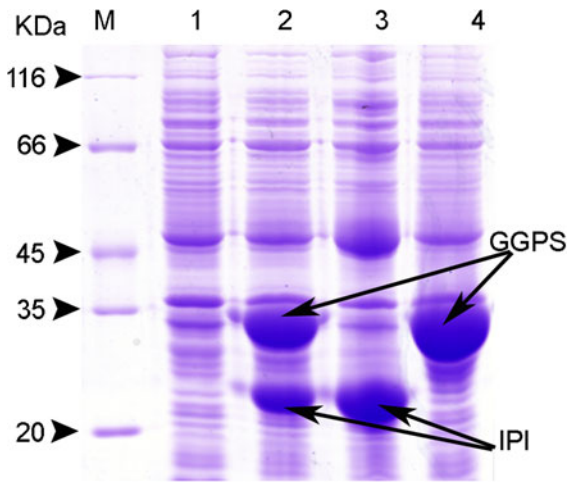

Fig. 3 SDS-PAGE of BtIPI and BtGGPS produced by *E. coli*. *Lane M* Protein molecular weight marker, *lane 1* proteins from *E. coli* with pET22b, *lane 2* proteins from *E. coli* with pET22b-BtGGPS-BtIPI, *lane 3* proteins from *E. coli* with pET22b-BtIPI, *lane 4* proteins from *E. coli* with pET22b-BtGGPS. 0.01 volumes of overnight cultures of the positive colonies was inoculated into fresh LB medium. *E. coli* was grown at 37 °C. 0.2 mM IPTG was added when OD600 reached 0.5 and *E. coli* was grown at 30 °C for 4 h

and then introduced into the expression vector pET22b (Invitrogen, Carlsbad, USA) through restriction enzymes *Nde*I and *Bam*HI, namely pET22b-BtIPI and pET22b-BtGGPS, respectively. The cDNA of *ipi* was also inserted into pET22b-BtGGPS through restriction enzymes *Bam*HI and *Sac*I, namely pET22b-BtGGPS-BtIPI. The intergenic sequence (GGATCCTAAGAAGGAGATATACAT) between *carG* and *ipi* in the plasmid pET22b-BtGGPS-BtIPI contained a *Bam*HI cutting sites (GGATCC) and a ribosome bind site (AAGGAG). The resulting plasmids were transformed into *E. coli* strain BL21 (DE3) to express BtIPI and BtGGPS. SDS-PAGE was performed to confirm the construction of plasmids. After electrophoresis, the gel was stained with Coomassie Brilliant Blue.

The plasmids pET22b-BtIPI, pET22b-BtGGPS, and pET22b-BtGGPS-BtIPI were transformed into *E. coli* strain BL21 (DE3) harboring the plasmid pACCAR16ΔcrtX or pACCAR25ΔcrtE to investigate the function of *ipi* and *carG* from *B. trispora*. The positive clones were inoculated onto the Luria-Bertani (LB) agar medium with chloromycetin and ampicillin to observe the color of colonies.

β-Carotene extraction and chemical analysis

To measure β-carotene content, an overnight culture of the positive colonies was inoculated into fresh LB medium at 1 % (v/v). After 2 days, the culture was centrifuged, resuspended in acetone, incubated at 50 °C for 20 min in the dark, and then centrifuged to recover the supernatant with the pigment. The contents of β-carotene in the acetone extracts were measured by HPLC (Tang et al. 2008).

Results and discussion

Cloning of the full-length cDNA of *ipi* and *carG*

The full-length cDNAs of *ipi* and *carG* were isolated by PCR and subsequently confirmed by sequencing. The sequences of cDNAs of *ipi* and *carG* and deduced amino acid sequences were shown in Supplementary Fig. 1. Analysis of the *ipi* cDNA sequence indicated the presence of an open reading frame (ORF) of 624 bp, 5′ untranslated region of 26 bp, 3′- untranslated region of 71 bp, and a poly (A) tail of 22 bp. The ORF encodes a 207-amino acid polypeptide with a theoretical pI of 5.46 and a theoretical molecular mass of 24.1 kDa. The nucleotide sequences of the *ipi* cDNA have been deposited in the GenBank data base under accession number JQ867267.

The results also indicated that the *carG* cDNA sequence comprised an ORF of 963 bp, 5′- untranslated region of 34 bp, 3′- untranslated region of 62 bp, and a poly(A) tail of 11 bp. The ORF of *carG* encodes a 320-amino acid polypeptide with a theoretical pI of 5.81 and a theoretical mass of 36.6 kDa. The nucleotide sequences of the *carG* cDNA have been deposited in the GenBank data base under accession number JQ289995.

Bioinformatic analysis

The deduced amino acid sequences of BtIPI and BtGGPS were submitted to National Center for Biotechnology Information (NCBI) for protein blast searching against non-redundant protein sequences database. The results indicated that BtIPI belonged to the Nudix hydrolase superfamily and BtGGPS belonged to the isoprenoid biosynthesis C1 superfamily. As shown in Fig. 2, phylogenetic study of IPI and

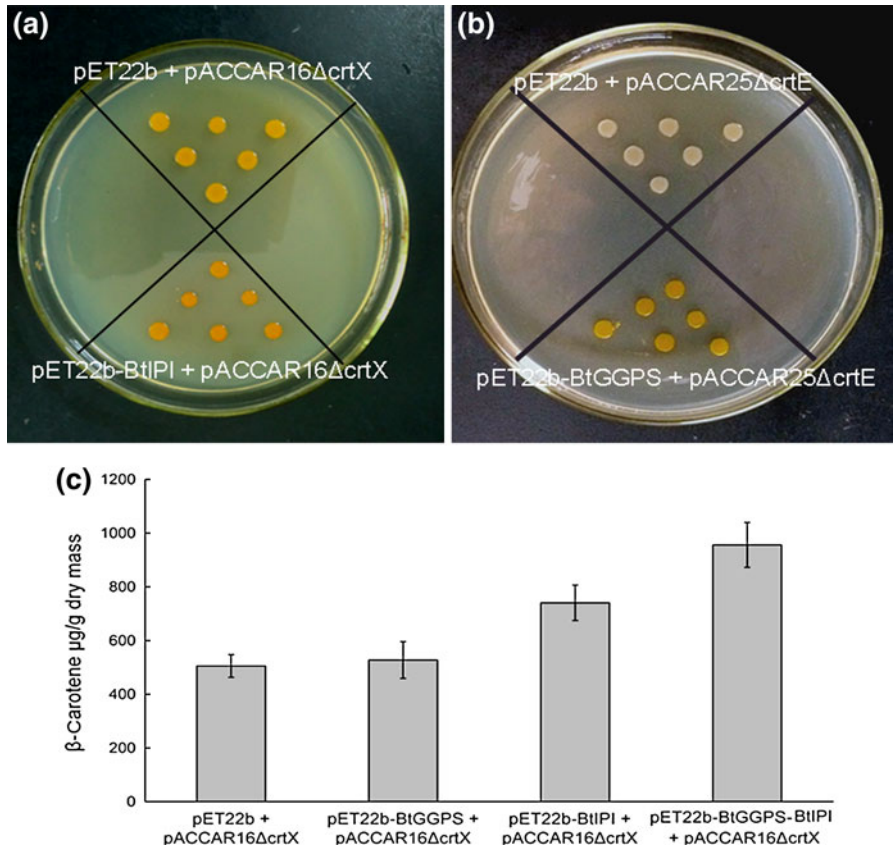

Fig. 4 Functional expression of BtIPI and BtGGPS in *E. coli*. **a** *E. coli* BL21 (DE3) harboring pACCAR16ΔcrtX was transformed with BtIPI. **b** *E. coli* BL21 (DE3) harboring pACCAR25ΔcrtE was transformed with BtGGPS. The positive clones were inoculated onto LB agar medium with chloromycetin and ampicillin at 25 °C for 4 days. **c** *E. coli* BL21 (DE3) with pACCAR16ΔcrtX, BtIPI, and BtGGPS produced different levels of *β*-carotene. *β*-Carotene was extracted with acetone from *E. coli* grown in LB liquid medium at 28 °C for 2 days. The contents of *β*-carotene were measured. Values are the mean ± standard error of three independent experiments

GGPS from 11 species of eukaryotic organisms, including fungi, plants and animals, indicates that IPI from *B. trispora* are clustered with other fungi, however, GGPSs from Mucorales has closer relationships with mammals than other fungi.

Functional expression of IPI and GGPS from *B. trispora*

Escherichia coli cells do not produce *β*-carotene but can produce IPP and DMAPP, which are the essential precursors of carotenoids (Rohmer et al. 1993). The nucleotide sequence of the carotenoid biosynthesis genes in *Erwinia uredovora* were cloned and used to construct the plasmid pACCAR16ΔcrtX (Misawa et al. 1995). *E. coli* harboring pACCAR16ΔcrtX can produce *β*-carotene.

To observe the stimulation of BtIPI and BtGGPS on carotenoid biosynthesis, the expression vectors pET22b-BtIPI, pET22b-BtGGPS, and pET22b-BtGGPS-BtIPI were constructed. BtIPI and BtGGPS were expressed in *E. coli* BL21 (DE3) and showed in SDS-PAGE map (Fig. 3). In the present study, *E. coli* harboring pACCAR16ΔcrtX and pET22b-BtIPI are more orange than the bacterial clones of *E. coli* harboring pACCAR16ΔcrtX and pET22b (Fig. 4a). This phenomenon was consistent with the previously researches (Sun et al. 1998). The result demonstrated that in the engineered *E. coli* BtIPI promoted the biosynthesis of *β*-carotene and confirmed that the cDNA of *ipi* cloned in this study encoded the activated IPI protein.

We used *E. coli* strain BL21 (DE3) to test whether BtGGPS encoded the anticipated functional enzyme in the color complementation assay. The *crtE* encoding GGPS had been deleted in the plasmid pACCAR25ΔcrtE. *E. coli* containing plasmid pACCAR25ΔcrtE and a functional GGPS gene can accumulate the yellow carotenoid, zeaxanthin, and its glucoside derivatives and forms yellow colonies. *E. coli* only containing plasmid pACCAR25ΔcrtE cannot accumulate carotene and thus forms white colonies. When pET22b-BtGGPS was co-transformed into *E. coli* BL21 with the plasmid pACCAR25ΔcrtE,

the bacterial colonies turned from white to yellow, indicating that the cDNA of *carG* cloned in this study encoded the activated GGPS protein (Fig. 4b).

Interestingly, we failed to observe an increase in β-carotene when BtGGPS was introduced into *E. coli* harboring pACCAR16ΔcrtX (Fig. 4c), suggesting that GGPS activity from pACCAR16ΔcrtX is sufficient for β-carotene production. Moreover, as compared with *E. coli* with the introduced BtIPI, the β-carotene content in *E. coli* with the introduced BtGGPS and BtIPI together increased by 29 % after 48 h, indicating that more GGPS activity was needed for the increased upstream metabolic flux in *E. coli* harboring pACCAR16ΔcrtX. Thus, it was necessary to express IPI and GGPS together in for carotene production. Introducing BtGGPS and BtIPI increased the β-carotene content in *E. coli* by 89 % to approx. 950 μg/g cell dry wt. (Fig. 4c). To observe the connection between the effects of IPI and GGPS from *B. trispora* in engineering *E. coli*, these two enzymes were leakily expressed without IPTG addition in this study. Further improvement of β-carotene production by the strain can presumably be achieved by optimization of IPTG concentration, culture period, and medium component.

Conclusions

Two carotene synthetic genes, encoding IPI and GGPS from *B. trispora,* were cloned, characterized and functionally identified. Functional expression confirmed that the cloned cDNAs could encode activated proteins. The effect of exogenous GGPS activity on carotene biosynthesis depends on the increase of IPP activity in *E. coli* with plasmid pACCAR16ΔcrtX. The results will facilitate the unveiling of the carotene biosynthesis at the molecular level and provide two new candidate genes for metabolic engineering of carotene.

Acknowledgments We thank Dr. Misawa (Ishikawa Prefectural University, Ishikawa, Japan) for providing plasmid pACCAR16ΔcrtX and Dr. Kawamukai (Shimane University, Matsue, Japan) for providing plasmid pACCAR25ΔcrtE. This work was supported in part by the grants from the National Natural Science Foundation of China (No. 20976009 and 21176018).

References

Anderson MS, Muehlbacher M, Street IP, Proffitt J, Poulter CD (1989) Isopentenyl diphosphate: dimethylallyl diphosphate isomerase. An improved purification of the enzyme and isolation of the gene from *Saccharomyces cerevisiae*. J Biol Chem 264:19169–19175

Bejarano ER, Cerdá-Olmedo E (1992) Independence of the carotene and sterol pathways of Phycomyces. FEBS Lett 306:209–212

Disch A, Rohmer M (1998) On the absence of the glyceraldehyde 3-phosphate/pyruvate pathway for isoprenoid biosynthesis in fungi and yeasts. FEMS Microbiol Lett 168:201–208

Homann V, Mende K, Arntz C, Ilardi V, Macino G, Morelli G, Bose G, Tudzynski B (1996) The isoprenoid pathway: cloning and characterization of fungal FPPS genes. Curr Genet 30:232–239

Jiang Y, Proteau P, Poulter D, Ferro-Novick S (1995) BTS1 encodes a geranylgeranyl diphosphate synthase in *Saccharomyces cerevisiae*. J Biol Chem 270:21793–21799

Kuzina V, Cerdá-Olmedo E (2007) Ubiquinone and carotene production in the Mucorales *Blakeslea* and *Phycomyces*. Appl Microbiol Biotechnol 76:991–999

Misawa N, Kajiwara S, Kondo K, Yokoyama A, Satomi Y, Saito T, Miki W, Ohtani T (1995) Canthaxanthin biosynthesis by the conversion of methylene to keto groups in a hydrocarbon beta-carotene by a single gene. Biochem Biophys Res Commun 209:867–876

Rodriguez-Saiz M, Paz B, De La Fuente JL, Lopez-Nieto MJ, Cabri W, Barredo JL (2004) *Blakeslea trispora* genes for carotene biosynthesis. Appl Environ Microbiol 70:5589–5594

Rohmer M, Knani M, Simonin P, Sutter B, Sahm H (1993) Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem J 295:517–524

Sandmann G, Misawa N, Wiedemann M, Vittorioso P, Carattoli A, Morelli G, Macino G (1993) Functional identification of al-3 from *Neurospora crassa* as the gene for geranylgeranyl pyrophosphate synthase by complementation with crt genes, in vitro characterization of the gene product and mutant analysis. J Photochem Photobiol, B 18:245–251

Steiger S, Sandmann G (2004) Cloning of two carotenoid ketolase genes from *Nostoc punctiforme* for the heterologous production of canthaxanthin and astaxanthin. Biotechnol Lett 26:813–817

Sun Z, Cunningham FX Jr, Gantt E (1998) Differential expression of two isopentenyl pyrophosphate isomerases and enhanced carotenoid accumulation in a unicellular chlorophyte. Proc Natl Acad Sci USA 95:11482–11488

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Tang Q, Li Y, Yuan QP (2008) Effects of an ergosterol synthesis inhibitor on gene transcription of terpenoid biosynthesis in *Blakeslea trispora*. Curr Microbiol 57:527–531