Biotechnol Lett (2012) 34:2107–2111
DOI 10.1007/s10529-012-1015-3

ORIGINAL RESEARCH PAPER

Improved β-carotene biosynthesis and gene transcription in *Blakeslea trispora* with arachidonic acid

Xianmei Hu · Jie Sun · Qipeng Yuan

Received: 6 June 2012 / Accepted: 6 July 2012 / Published online: 25 July 2012

Abstract With 0.4 g l^{-1} arachidonic acid (AA) added to the medium after 36 h fermentation, β-carotene production in mated cultures of *Blakeslea trispora* was 73 % higher than that of the control at 690 mg l^{-1}. With the treatment of AA, the transcriptional levels of genes *hmgR*, *carRA* and *carB,* that are involved in carotene biosynthesis, increased by 31, 22 and 39 %, respectively.

Keywords Arachidonic acid · *carRA* · *carB* · β-Carotene · *hmgR*

Introduction

Carotenoids, an abundant group of isoprenoid pigments with wide applications, are synthesized by plants and microorganisms. β-Carotene is used in food and feed products (Choudhari and Singhal 2008). High yields of β-carotene are obtained from the zygomycete mould, *Blakeslea trispora*. *B. trispora* has gained considerable interest because it can efficiently convert inexpensive raw materials and certain industrial waste to high value-added end products (Goksungur et al. 2002).

In fungi, carotenoids are derived via the mevalonate biosynthesis pathway. Mevalonate itself is formed from hydroxymethylglutaryl-CoA by the catalysis of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) which may be the rate-limiting enzyme in sterol and isoprenoid biosynthesis (Wang and Keasling 2002). *hmgR*, *carRA* and *carB* are key genes in carotenoid biosynthesis pathway; *hmgR* encodes HMGR and *carRA* and *carB* are responsible for different enzymes in carotenoid biosynthesis pathway (Fig. 1). Gene *carRA* has two domains, R and A, encoding for lycopene cyclase and phytoene synthase, respectively. *carB* encodes phytoene desaturase (Arrach et al. 2001; Burg and Espenshade 2011).

Arachidonic acid (AA) can induce the genetic expression of *hmgR* and carotene accumulation in plants (Choi et al. 1992; Rodríguez-Concepción and Gruissem 1999). Here, we report for the first time the significant improvement of β-carotene production and gene expression in *B. trispora* cells by the addition of arachidonic acid (AA; 20:4 n-6) to the fermentation medium. The production of β-carotene in AA-treated *B. trispora* cells was measured and the transcriptional levels of genes *hmgR*, *carRA* and *carB* were also investigated in this study.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-012-1015-3) contains supplementary material, which is available to authorized users.

X. Hu · J. Sun · Q. Yuan (✉)
State Key Laboratory of Chemical Resource Engineering, College of Life Science and Technology, Beijing University of Chemical Technology, P.O. Box 75, Beijing 100029, People's Republic of China
e-mail: yuanqp@mail.buct.edu.cn

X. Hu
e-mail: xianmeihu@126.com

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

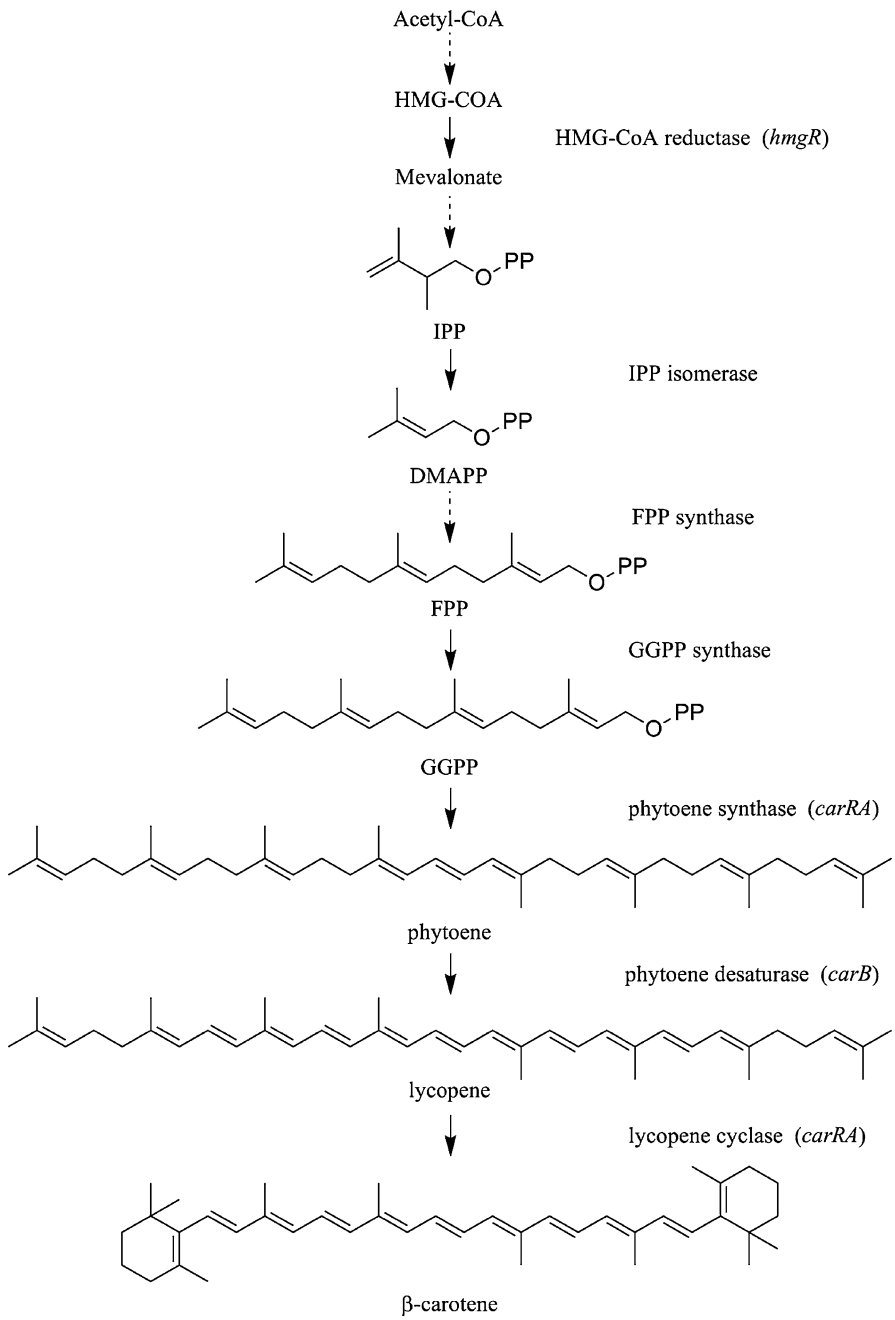

Fig. 1 Scheme of the carotenoid biosynthesis pathway in *B. trispora* *Acetyl-CoA* acetyl coenzyme A, *HMG-CoA* 3-hydroxy-3-methylglutaryl coenzyme A, *IPP* isopentenyl pyrophosphate (diphosphate), *DMAPP* dimethylally pyrophosphate, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate

Materials and methods

Microorganism and culture condition

Blakeslea trispora ATCC14271, mating type (+), and ATCC14272, mating type (−), were grown separately in the seed medium (g l^{-1}): corn starch (40); glucose (20); corn steep liquor (50); KH_2PO_4 (1.5); $MgSO_4$ (0.1); thiamine (0.01); pH 6.5, in 500 ml flasks containing 50 ml medium and shaken at 180 rpm and 28 °C for 40 h. The cultures were used to inoculate the fermentation medium.

Fermentation conditions

The batch cultivation experiments were carried out in 500 ml Erlenmeyer flasks with 50 ml medium including 10 % (v/v) inoculum of ATCC14271(+)/ATCC14272(−)(1:4 v/v) at 220 rpm and 28 °C. The fermentation medium (g l^{-1}): glucose (30); soybean meal (78); KH_2PO_4 (1.5); $MgSO_4$ (0.5); butylated hydroxytoluene (1); soybean oil (32 ml l^{-1}); pH 7.5.

β-Carotene extraction and analytical method

The cell mass was filtered through muslin, washed thoroughly with distilled water, and dried at 45 °C under 0.08 MPa vacuum for 48 h and then weighed. Analysis of β-carotene was conducted according to the method of Xu et al. (2006): β-carotene was extracted with petroleum ether and analyzed by HPLC using a Diamonsil C_{18} column (250 mm × 4.6 mm) at 28 °C and acetonitrile/dichloromethane (75:25, v/v) at 1.5 ml min^{-1}. β-Carotene was detected at 450 nm.

Reverse transcription of total RNA and quantitative real-time PCR

Extraction of total RNA in *B. trispora* was done using Trizol. A mixture of total RNA (10 µl, 0.08 µg $µl^{-1}$) was used for reverse transcription with M-MLV reverse transcriptase and random primer according to the manual instruction (Promega. USA). cDNA from this step was stored at −20 °C for using as the template for real-time PCR.

Oligonucleotide primers used in this study are shown in Supplementary Table 1. The mixture for quantitative real-time PCR consisted of cDNA (2.5 µl), real SYBR mixture (10 µl), forward primer (0.3 µl), reverse primer (0.3 µl) and water (6.9 µl). Amplifications were performed on an Eppendorf Mastercycler Gradient PCR instrument with the following temperature profile: a pre-denaturation step of 5 min at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 25 s at 60 °C and elongation for 30 s at 72 °C. Each sample was performed in triplicate. To confirm that only the PCR product of interest had been synthesized, we performed the dissociation curve analysis and subjected the reaction mixtures to agarose gel electrophoresis. Opticon 2 system was used to analyze the amplification results. The transcriptional levels of *hmgR*, *carRA* and *carB* genes obtained by real-time PCR were normalized to the *tef1* gene and compared with the corresponding control (*B. trispora* cells harvested after 3 h following AA treatment, value = 1), following the comparative method of Livak and Schmittgen (2001).

Results and discussion

Effects of AA with different concentrations on dry biomass and β-carotene yield

AA was added to the fermentation medium to determine its effects on dry biomass and β-carotene production in the mated strains of *B. trispora*. The changes of dry biomass and the amount of β-carotene produced in the fermentation medium with different concentrations of AA added to the cultivation medium after 48 h fermentation are shown in Table 1. While the addition of AA to the medium caused an increase of β-carotene production, no significant change of dry biomass was observed. With 0.4 g AA l^{-1}, the yield of β-carotene reached the maximum value (627 mg l^{-1}), which was 53 % higher than the control.

Effects of AA addition to the medium at different times on β-carotene yield

AA (0.4 g l^{-1}) was added to the medium at different times and the fermentation was continued for 84 h (Table 2). The amount of β-carotene produced depended on the time of adding AA. Maximum β-carotene production was obtained when AA was

Table 1 Dry biomass and β-carotene yield of *B. trispora* with different concentrations of AA added after 48 h of fermentation

Concentration of AA (g l^{-1})	Dry biomass (g l^{-1})	β-carotene yield (mg l^{-1})
0	51.8 ± 1.7	410 ± 16.1
0.2	49.5 ± 2.8	501 ± 13.4
0.4	51.7 ± 2.6	627 ± 14.2
0.6	49.3 ± 3.0	565 ± 13.7
0.8	48.5 ± 1.9	488 ± 10.6
1.0	48.8 ± 1.5	427 ± 12.3

The cells were harvested at 84 h. Values are means of triplicate ± standard derivation

Table 2 Dry biomass and β-carotene yield of *B. trispora* with the addition of AA (0.4 g l^{-1}) to the medium after the fermentation at different times

Time of adding AA (h)	Dry biomass (g l^{-1})	β-carotene yield (mg l^{-1})
Control (without AA)	53.4 ± 2.1	399 ± 14.5
0	51.6 ± 1.9	434 ± 16.4
12	51.2 ± 2.3	483 ± 17.2
24	49.5 ± 3.1	551 ± 13.9
36	52.0 ± 1.8	690 ± 15.6
48	50.4 ± 2.4	641 ± 17.7
60	51.2 ± 2.4	575 ± 14.8
72	53.8 ± 1.5	469 ± 16.1

Cells were harvested at 84 h. Values are means of triplicate ± standard derivation

added after 36 h: 690 mg l^{-1}, 73 % higher than the control.

The β-carotene is a secondary metabolite that showed a sharp increase after the rate of cell growth began to diminish (Sun et al. 2007). The biomass increased rapidly, accompanying a rapid consumption of glucose (Fig. 2). After 36 h, the rate of cell growth diminished. The cells gradually turned yellow and the maximum β-carotene production was obtained after 84 h. Therefore, it was effective to add AA at 36 h into the medium when cells begin to synthesize β-carotene.

Quantitative analysis of transcriptional levels of *hmgR*, *carRA* and *carB*

In order to gain some insight into the mechanism of AA regulating β-carotene biosynthesis in *B. trispora*, changes of transcriptional levels of *hmgR*, *carRA* and *carB* genes were investigated in this work. *B. trispora* cells cultivated for 36 h were treated with 0.4 g AA l^{-1} and then cells were harvested after 3, 6, 9, 15 and 24 h following AA treatment. The changes in the levels of the compounds in the isoprenoid biosynthetic pathways are correlated with changes in the activities of HMGR and subsequent enzymes in the pathways leading to their synthesis (Choi et al. 1994). The expression of genes *hmgR*, *carRA* and *carB* was examined by real-time PCR. In the present study, all the transcriptional levels of *hmgR*, *carRA* and *carB* were induced by treatment of AA and a significant increase of β-carotene was obtained. Effects of AA on genes *hmgR*, *carRA* and *carB* transcriptional levels are shown in Fig. 3.

For the gene, *hmgR*, the mRNA level increased gradually until the maximum 4.4-fold at 6 h, while that in the control was 3.4-fold, then a decrease of mRNA content was observed (Fig. 3a). For genes *carRA* and *carB*, the maximum mRNA levels were obtained at 9 h after AA addition (Fig. 3b and c). Compared with the control, the mRNA contents of *carRA* and *carB* increased by 2.2- to 2.7-fold and 3.4- to 4.7-fold, respectively. Savchenko et al. (2010) found that AA, as the signaling molecule, could induce the expression of genes whose promoters contain the conserved RSRE (the rapid stress response element) motif. In *B. trispora*, AA might induce the expression of *hmgR*, *carRA* and *carB* as a signaling molecule as occurs in plants. However, that whether AA regulates the transcriptional levels of *hmgR*, *carRA* and *carB* through specific transcription factors needs further investigation.

In conclusion, AA improved production of β-carotene in the fermentation by transcriptional

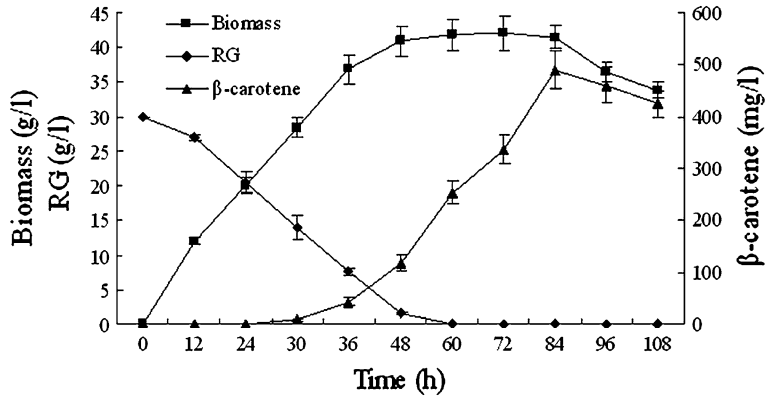

Fig. 2 Time-course of fermentation process by *B. trispora* without AA treatment *RG* residual sugar Values are means of triplicate ± standard derivation

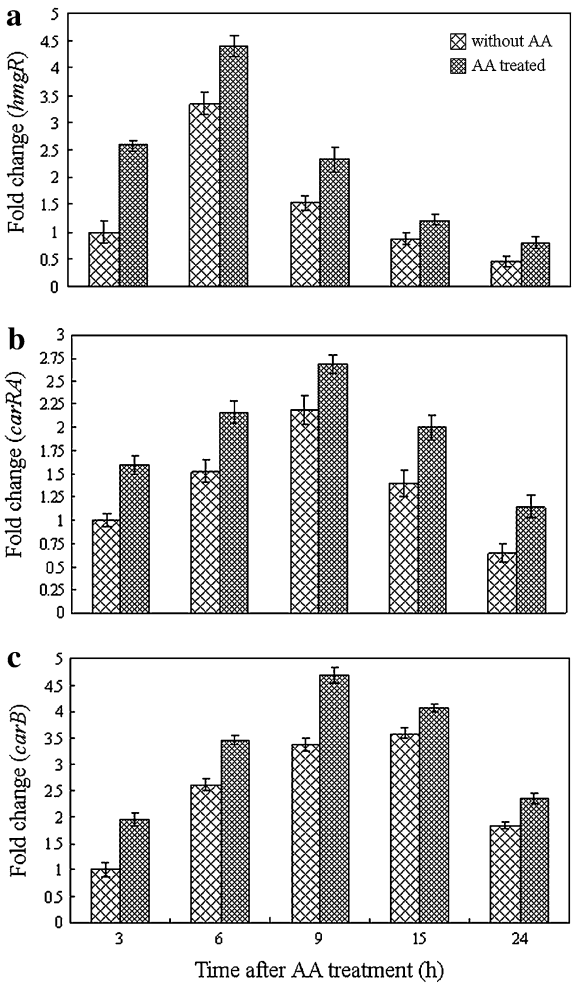

Fig. 3 Change of *hmgR* (**a**), *carRA* (**b**) and *carB* (**c**) transcriptional levels in *B. trispora* cells (with and without AA treatment). In AA-treated groups, AA (0.4 g l^{-1}) was added to the medium after 36 h of fermentation. All results were normalized to the *tef1* transcriptional level and relative to the transcriptional level of the corresponding control (*B. trispora* cells harvested after 3 h following AA treatment, value = 1). Values are means of triplicate ± standard derivation

regulation. The addition of optimal concentration of AA after 36 h of cultivation enhanced β-carotene accumulation and induced the transcriptional levels of *hmgR*, *carRA* and *carB*. The results provide a new stimulator for carotene production in *B. trispora*.

Acknowledgments The authors would like to acknowledge the financial support of the National Natural Science Foundation of China (No. 20976009 and 21176018).

References

Arrach N, Fernández-Martín R, Cerdá-Olmedo E, Avalos J (2001) A single gene for lycopene cyclase, phytoene synthase, and regulation of carotene biosynthesis in *Phycomyces*. Proc Natl Acad Sci USA 98:1687–1692

Burg JS, Espenshade PJ (2011) Regulation of HMG-CoA reductase in mammals and yeast. Prog Lipid Res 50:403–410

Choi D, Ward BL, Bostock RM (1992) Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phtophthora infestans* and to its elicitor arachidonic acid. Plant cell 4:1333–1344

Choi D, Bostock RM, Avdinshko S, Hildebrand DF (1994) Lipid derived signals that discriminate wound- and pathogen-responsive isoprenoid pathway in plant: methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. Proc Natl Acad Sci USA 9:2329–2333

Choudhari S, Singhal R (2008) Media optimization for the production of β-carotene by *Blakeslea trispora*: a statistical approach. Bioresour Technol 99:722–730

Goksungur Y, Mantzouridou F, Roukas T (2002) Optimization of the production of β-carotene from molasses by *Blakeslea trispora*: a statistical approach. J Chem Technol Biotechnol 77:933–943

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Rodríguez-Concepción M, Gruissem W (1999) Arachidonic acid alters tomato *HMG* expression and fruit growth and induces 3-hydroxy-3-methylglutaryl coenzyme A reductase-independent lycopene accumulation. Plant Physiol 119:41–48

Savchenko T, Walley JW, Chehab EW et al (2010) Arachidonic acid: an evolutionarily conserved signaling molecule modulates plant stress signaling networks. Plant Cell 22: 3193–3205

Sun Y, Yuan QP, Vriesekoop F (2007) Effect of two ergosterol biosynthesis inhibitors on lycopene production by *Blakeslea trispora*. Process Biochem 42:1460–1464

Wang GY, Keasling JD (2002) Amplification of HMG-CoA reductase production enhances carotenoid accumulation in *Neurospora crassa*. Metab Eng 4:193–201

Xu F, Yuan QP, Dong HR (2006) Determination of lycopene and β-carotene by high-performance liquid chromatography using sudan I as internal standard. J Chromatogr B 838:9–44