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Genetic manipulation of the bifunctional gene, *carRA*, to enhance lycopene content in *Blakeslea trispora*

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ABSTRACT

Blakeslea trispora is an ideal natural source of lycopene. Here, we genetically manipulated the bifunctional protein gene, *carRA*, encoding a phytoene synthase and lycopene cyclase, to enhance lycopene production. *carR*- and *carRA*-knockout and *carA* overexpressing strains were obtained after several rounds of selection. Following combinational fermentation for 6 d, the lycopene content in *carR*-knockout strains increased from 0.47 to 1.40 mg/gDW, while the total carotenoid content decreased from 17.05 to 3.52 mg/gDW compared to the wild type. No carotenoid was produced in *carRA*-knockout strains. In *carA* overexpressing strains, lycopene content, β-carotene, and total carotenoid content increased 2.26-, 1.98-, and 2.02-fold, resepectively, with proportions similar to wild type. Dry weight increased 1.89–1.98-fold in *carR*- and *carRA*-knockout strains compared to the wild type, but not in *carA* overexpressing strains. qRT-PCR analysis revealed that disruption of CarR and CarRA down-regulated carotenoid synthesis genes, especially the isopentenyl pyrophosphate isomerase gene, whereas overexpression of *carA* up-regulated all examined genes, except the ergosterol synthase and the 4-hydroxybenzoate polyprenyl transferase genes. Tripropylamine was shown to target the R domain, rather than the A domain, of CarRA. Furthermore, the possibility of using a *carR*-knockout to obtain high-lycopene *B. trispora* strains for industrial production was explored.

1. Introduction

Lycopene is a natural carotenoid that exhibits strong antioxidant activity [1]. The quenching capability of lycopene for singlet oxygen is over 2-fold that of β-carotene and 100-fold that of vitamin E [2]. Therefore, lycopene has been used extensively as an antioxidant, anticancer agent, and colorant in the pharmaceutical and food industries. *Blakeslea trispora*, a filamentous fungus (class Zygomycetes, order Mucorales), has been proposed as an ideal natural source of lycopene because it is easy to culture and preferentially produces all-trans-lycopene [3]. The positive and negative types of *B. trispora* need to be mated during the fermentation process; the positive type produces trisporic acid and stimulates lycopene synthesis by the negative type [4].

Lycopene is an intermediate of the carotenoid biosynthetic pathway in *B. trispora*. Carotenoid biosynthesis commences with acetyl-CoA and proceeds via the mevalonate (MVA) pathway [5,6] (Appendix A, Fig. S1). Briefly, acetyl-CoA is first condensed to 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), then converted to MVA by HMG-CoA reductase (encoded by *hmgr*), and then to isopentenyl pyrophosphate (IPP), a precursor of isoprenoid biosynthesis. IPP is converted to farnesyl pyrophosphate (FPP) and then to geranylgeranyl pyrophosphate (GGPP). Condensation of two GGPP molecules by phytoene synthase results in phytoene; lycopene is then produced by phytoene dehydrogenase (CarB).

Abbreviations: CarA, phytoene synthase; CarB, phytoene dehydrogenase; *carG*, geranylgeranyl pyrophosphate synthase gene; CarR, lycopene cyclase; *coq2*, 4-hydroxybenzoate polyprenyl transferase gene; CTAB, cetyl trimethyl ammonium bromide; DMAPP, dimethylallyl pyrophosphate; *erg9*, ergosterol synthase gene; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HPLC, high-performance liquid chromatography; *hmgr*, 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene; *ipi*, IPP isomerase gene; IPP, isopentenyl pyrophosphate; *isoA*, farnesyl pyrophosphate synthase gene; MVA, mevalonate; qRT-PCR, semi-quantitative reverse transcription polymerase chain reaction.

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Lycopene can also be cyclized into γ-carotene and β-carotene by lycopene cyclase. In addition, FPP and GGPP can also enter into the ergosterol and ubiquinone synthesis branches, respectively (Appendix A, Fig. S1).

Because lycopene cyclase catalyzes the conversion of lycopene into β-carotene, suppression of its activity would promote lycopene accumulation. Thus, various strategies for preventing the conversion of lycopene into β-carotene have been examined. Mutation breeding is an effective method for generating high-producing *B. trispora* strains. Mutants with lycopene concentrations 55% higher than the parental strain were obtained using the novel atmospheric and room temperature plasma (ARTP) method [7]. Mutants with maximum lycopene yields (26.4–28.8 mg/g and 55–64% higher than the parental strain) were also obtained via N^+ ion implantation [8]. Chemical suppression of lycopene cyclase is another method for increasing the lycopene content in *B. trispora*. Two types of chemicals, amines [9] and nitrogen-containing heterocyclic compounds [10,11], have been shown to be effective cyclase inhibitors that enhance the lycopene content in *B. trispora*. With the advent of DNA-mediated transformation procedures, genetic manipulation of the cyclase gene has recently become a more efficient technique for developing high lycopene transgenic strains. However, such strains have yet to be reported.

In *B. trispora*, lycopene cyclase and phytoene synthase, are thought to be encoded by the bifunctional enzyme gene *carRA*. The gene was first cloned in 2004; it is 1894 bp in size with a single 70 bp intron [12]. *B. trispora* CarRA is 67% similar to *Mucor circinelloides* CarRP, 55% similar to *Phycomyces blakesleeanus* CarRA, 31% similar to *Xanthophyllomyces dendrorhous* CrtYB, and 28% similar to *Neurospora crassa* AL-2 [12]. CarRA contains a putative protease cleavage site at residues 241–246 (AQAILH), which could split the protein at residues 243 and 244 into two separate functional molecules. The R domain at the N terminus functions as a lycopene cyclase, while the A domain at the C terminus functions as a phytoene synthase [12]. Mutations in one domain of a bifunctional enzyme have been reported in other fungi. When the P domain of *M. circinelloides* CarRP was disrupted, the R domain remained functional; the resulting *carR* mutant accumulated lycopene and exhibited a red color [13]. Defective AL-2 cyclase activity resulted in increased lycopene accumulation in *N. crassa* [14]. Furthermore, the *P. blakesleeanus carR* mutant accumulated the highest reported lycopene content (approximately 1.5 mg/g dry mass) [15]. However, it is unclear whether the two domains of *B. trispora* CarRA function independently, as in CarRP and AL-2, and whether disruption of CarR would increase lycopene production.

In this study, we developed *B. trispora* ATCC 14271(+) and 14272(−) strains in which *carR* and *carRA* were knocked out using homologous recombination technology and characterized the effects of CarR and CarRA disruption on carotenoid content, growth status, and carotenoid synthesis pathway gene expression. To the best of our knowledge, this is the first study to manipulate *carRA* in *B. trispora* using homologous recombination technology. The results of this study will further our understanding of the *carRA* regulatory mechanisms involved in carotenoid synthesis.

2. Materials and methods

2.1. Strains and culturing conditions

B. trispora ATCC 14271(+) and 14272(−) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured as previously described [16]. *Escherichia coli* DH5α-FT, plasmid pAN7-1, and pAcGFP1 were maintained at our laboratory. pMD18-T was purchased from Dalian TaKaRa Biotechnology (Co., Ltd., Dalian, China). *E. coli* strains were cultured in LB medium at 37 °C and 180 r/min.

2.2. Construction of carR- and carRA-knockout vectors

Plasmids p18-AG and p18-G (Appendix A, Fig. S2) for the knockout of *carR* and *carRA*, respectively, were constructed as described below. P_{carRA} (0.6 kb) and T_{carRA} (1.2 kb), the promoter and terminator of *carRA* (AY884174.1), were amplified from *B. trispora* genomic DNA using primer pairs RA-P-F/RA-P-R and RA-T-F/RA-T-R (Appendix A, Table S1), respectively. *AcGFP* (AY233272.1; 0.8 kb) was amplified from plasmid pAcGFP1, which contains a highly stable green *Aequorea coerulescens* fluorescent protein (GFP) gene [17], using primer pair AcGFP1-F/AcGFP1-R. The *AcGFP* fragment was then inserted into the *Eco*RV site between P_{carRA} and T_{carRA} to generate p18-G. The *carA* fragment (1.1 kb) was amplified from genomic DNA using primer pair CarA-F/CarA-R and then inserted into the *Sal*I-*Kpn*I sites upstream of *AcGFP* on p18-G to generate p18-AG. Because P_{carRA} and T_{carRA} are completely homologous to the *B. trispora* genome, p18-G was targeted to knock out *carRA* between P_{carRA} and T_{carRA}. When p18-AG was used to knock out *carRA*, *carA* would be introduced. In this case, p18-AG was targeted to knock out only *carR* in *B. trispora* genome.

2.3. Preparation of protoplasts

Mycelia were collected following culturing on seed medium [16] for 60 h (28 °C, 180 r/min) and centrifuged at 2000*g* for 5 min. The mycelia (0.2 g) were dispersed with a magnetic stirrer and incubated with enzymatic solutions (described below, dissolved in 0.6 mol/L sucrose) [18] for 1.5 h at 28 °C, under dark conditions. In order to achieve high-quality isolation, nine combinations of four enzymes (lysozyme, lyticase, snail enzyme, and cellulose) at three concentrations (0%, 1.5%, and 3%) were used according to orthogonal design $L_9(3^4)$. The mixture was then filtrated through 200 and 500 mesh screens [19] and centrifuged at 2655*g* for 5 min. The precipitate was then resuspended in 600 μL sucrose solution (0.6 mol/L) and filtrated and centrifuged twice more, as described above. The resulting protoplasts were diluted to 10^7 cells/mL by adding an osmotic stabilizer (0.6 mol/L sucrose solution) and then used for electronic transformation.

2.4. Transformation and regeneration

Transformation was performed according to the method described by Murray et al. [20] with some modification. The linearized (*Eco*RI) p18-AG or p18-G vector (0.5 μg) was introduced together with 0.5 μg pAN7-1 (a suicide plasmid carrying a hygromycin resistance gene) into 200 μL protoplasts by electroporation using three 0.5 ms pulses at 0.4 kV/cm (the first interval was 2 s and the second interval was 3 s). The protoplasts were then transferred into 940 μL regeneration medium (seed medium with 20% sucrose) for 1.5 h (28 °C, 180 r/min), then plated on solid seed medium with 100 μg/mL hygromycin and incubated at 28 °C under dark conditions for 1–2 d. The regenerated mycelia were transferred to solid seed medium for 3 d. Single mycelia were picked and cultivated on fresh solid medium. During the transfer process, the mycelia were checked under a fluorescent microscope (Olympus BX41, Olympus Co., Tokyo, Japan) to determine whether it displayed green fluorescence.

2.5. Molecular analysis, carotenoid content, and antioxidant enzyme activities

Strains displaying fluorescence were selected for amplification. Genomic DNA was extracted using the cetyl trimethyl ammonium