Bioscience, Biotechnology, and Biochemistry, 2021, Vol. 85, No. 8, 1899-1909

https://doi.org/10.1093/bbb/zbab109
Advance access publication date: 14 June 2021
REGULAR PAPER

REGULAR PAPER

Analysis of the light regulatory mechanism in carotenoid production in *Rhodosporidium toruloides* NBRC 10032

Khanh Dung Pham,[1] Yuuki Hakozaki,[1] Takeru Takamizawa,[1] Atsushi Yamazaki,[2] Harutake Yamazaki,[3] Kazuki Mori,[4] Sachiyo Aburatani,[5] Kosuke Tashiro,[6] Satoru Kuhara,[7] Hiroaki Takaku,[3] Yosuke Shida,[1,*] and Wataru Ogasawara[1]

[1]Department of Bioengineering, Nagaoka University of Technology, Niigata, Japan; [2]Biological Resource Center, National Institute of Technology and Evaluation (NITE), Chiba, Japan; [3]Faculty of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan; [4]Cell Innovator Co., Ltd., Fukuoka, Japan; [5]AIST-Waseda University Computational Bio Big-Data Open Innovation Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan; [6]Faculty of Agriculture, Kyushu University, Fukuoka, Japan; and [7]Graduate School of Genetic Resource Technology, Kyushu University, Fukuoka, Japan

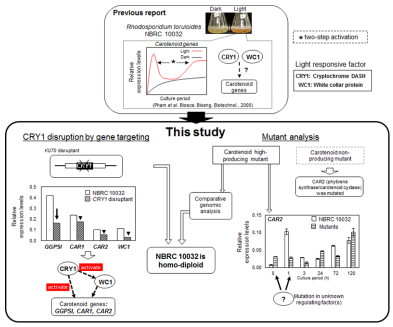
*Correspondence: Yosuke Shida, yshida@vos.nagaokaut.ac.jp

ABSTRACT

Light stimulates carotenoid production in an oleaginous yeast *Rhodosporidium toruloides* NBRC 10032 by promoting carotenoid biosynthesis genes. These genes undergo two-step transcriptional activation. The potential light regulator, Cryptochrome DASH (CRY1), has been suggested to contribute to this mechanism. In this study, based on KU70 (a component of nonhomologous end joining (NHEJ)) disrupting background, *CRY1* disruptant was constructed to clarify CRY1 function. From analysis of *CRY1* disruptant, it was suggested that CRY1 has the activation role of the carotenogenic gene expression. To obtain further insights into the light response, mutants varying carotenoid production were generated. Through analysis of mutants, the existence of the control two-step gene activation was proposed. In addition, our data analysis showed the strong possibility that *R. toruloides* NBRC 10032 is a homo-diploid strain.

Received: 28 April 2021; **Accepted:** 6 June 2021

Graphical Abstract



The aspects of the light regulatory mechanism in the carotenoid production in *Rhodosporidium toruloides* NBRC 10032.

Keywords: *Rhodosporidium toruloides*, carotenogenesis, light response

Carotenoids are important groups of pigments that have over 600 structural compound types. They have 40 carbons in the formula, with oxygenated or nonoxygenated molecules of carotenoids, conjugated double bonds, and rings at the end (Popova and Andreeva 2013; Yuan *et al.* 2015). In general, carotenoids are lipophilic molecules that provide yellowish, orange, or reddish pigmentations (Avalos *et al.* 2017). Carotenoids, including β-carotene, have a strong ability to quench a singlet oxygen and play an important role in protecting against oxidative damage (Moore, Breedveld and Autor 1989; Sakaki *et al.* 2001; Martínez-Cárdenas *et al.* 2018). They are also among the bioactive phytochemicals credited with reducing the risk of degenerative diseases, especially cancer. Hence, carotenoids are in strong demand in cosmetics, foods, feeds, and pharmaceutical industries (Khanafari, Tayari and Emami 2008; Frengova and Beshkova 2009). Carotenoids are naturally synthesized not only by photosynthetic organisms such as plants, algae, and cyanobacteria, but also by nonphotosynthesis organisms such as fungi, bacteria, and archaea (Britton 2004). Carotenoid production from microorganisms has more advantages than that from plants or alga, because the production rate is high, they can be produced from low-cost natural sources and it is less affected by seasonal and geographical factors (Lin, Jain and Yan 2014; Lin *et al.* 2017). Due to its potential and usefulness, production of carotenoids by microorganisms has received more attention recently. However, the carotenoid productivity of microorganisms, and their carotenoid component, depend on culture conditions. Microbial carotenoid production is affected by many physical and chemical stresses, including external factors such as starvation, light, temperature, carbon source, ionizing radiation and osmotic pressure, and internal factors (Alcantara and Sanchez 1999; Belozerskaya and Gessler 2006; Maldonade, Rodriguez-Amaya and Scamparini 2008; Mata-Gómez *et al.* 2014). These factors may exert a singular or a cooperative effect on carotenoid production. Light is one of the important factors in the growth and production of carotenoids (Linden, Ballario and Macino 1997; Khanafari, Tayari and Emami 2008). Because carotenogenesis is a photoprotective mechanism, microorganisms produce carotenoids as an antioxidant performing a role in protecting the cells from light or other environmental stresses (Khanafari, Tayari and Emami 2008; Mata-Gómez *et al.* 2014). The carotenoid yield derived from microorganisms depends on the period and intensity of light exposure. In *Mucor hiemalis*, light illumination raised carotenoid production compared to dark incubation (Khanafari, Tayari and Emami 2008). Increase in the total amount of carotenoids by light irradiation was also observed in *Rhodosporidium glutinis* (Sakaki *et al.* 2001; Yen and Yang 2012; Schneider *et al.* 2013; Zhang, Zhang and Tan 2014). In Phaffia rhodozyma, the carotenoid yields obtained by cultivation in the dark were lower than those obtained under constant illumination cultivation (An and Johnson 1990; Vázquez 2001; Stachowiak and Czarnecki 2007).

Rhodosporidium toruloides is a red yeast known for its ability to accumulate large amounts of lipids in the cells and for its ability to produce carotenoids. Major advances have been made in the identification of main carotenoid biosynthetic pathway genes in *R. toruloides* through biochemical and molecular approaches. However, the carotenoid biosynthesis mechanism, especially the regulation mechanism of carotenogenic genes, is still unknown, largely limiting the efforts to improve carotenoid production in *R. toruloides*. In our previous study we observed 5 times higher carotenoid production in *R. toruloides* NBRC 10032 with exposure to light (Pham *et al.* 2020). In addition, we also deciphered a two-step response in which temporary gene activation occurred early in the cultivation period (1 h) and later (72 h) in the main carotenogenic genes, geranylgeranyl diphosphate synthase, phytoene desaturase, and phytoene synthase/lycopene cyclase (encoding by *GGPSI*, *CAR1*, and *CAR2*, respectively), in the carotenoid biosynthesis pathway. Moreover, *CRY1* (encoding putative Cryptochrome DASH, the blue-light receptor) was expressed exponentially in the early light-exposure period (Pham *et al.* 2020).

In this study, we carried out 2 approaches to unravel the regulatory mechanisms involved in carotenoid production, namely, *CRY1* disruption to analyze its function and comparative genome analysis of mutants to obtain new insights into responses to light. Deletion of *CRY1* using gene targeting revealed that *CRY1* partially contributed to carotenoid production. In addition, comparative genome analysis of high carotenoid-producing mutants resulted in the detection of genes possessing single nucleotide polymorphism (SNP).

Materials and methods

Yeast strain and culture condition

The *R. toruloides* NBRC 10032 (a strain with high lipid production) was obtained from the Biological Resource Center of the

National Institute of Technology and Evaluation (NITE). *R. toruloides* was grown on yeast peptone D-glucose (YPD) agar plates (1% yeast extract, 1% peptone, 2% D-glucose, 2% agar [Nacalai Tesque]) at 30 °C for 4 days, and maintained at 4 °C. YPD broth was used for precultivation, and LS10 broth (0.805% powder yeast extract [Kyokuto Seiyaku Co.], 15% D-glucose, 0.047% NH_2CONH_2, 0.15% $MgSO_4{\cdot}7H_2O$, 0.04% KH_2PO_4, 1.9 × 10^{-6} mM $ZnSO_4$, 1.22 × 10^{-4} mM $MnCl_2$, 1 × 10^{-4} mM $CuSO_4$, and 1.5 mM $CaCl_2$ [Nacalai Tesque]) was used as the main culture. Yeast cells were precultivated for 24 h in YPD broth in L-shaped test tubes and were then cultured in 200 mL Erlenmeyer flasks in 50 mL LS10 medium at an initial optical density (OD) of 660 nm = 0.1. Experiments were performed in the same dark room. Light condition was established by employing a blue LED light irradiation unit (LC-LED 470B, Taitec) with 80 μmol m^{-2} s^{-1} photon, and dark condition was set by wrapping the tubes in 2 layers of aluminum foil (24 μm thick in total). An orbital shaker set at 13.61 rad/s was used for all cultures.

Construction of DNA fragment for yeast transformation

The construction of a *URA3* disruption cassette was carried out as follows: The DNA fragment containing *URA3* and its approximate 1.5 kbp of 5′- and 3′-flanking regions was amplified by polymerase chain reaction (PCR) with primer set URA3_all_Fw and URA3_all_Rv. The *R. toruloides* genome was used as a template. The amplicon and the linearized pUC118 vector at the EcoRI site were assembled to obtain pUC-*URA3*. pUC-*URA3* was opened by inverse PCR with primer set pUCURA3_inV_Fw and pUCURA3_inV_Rv, which annealed just upstream of the start codon and just downstream of the stop codon of *URA3*, respectively. The codon-optimized *hph* gene encoding hygromycin B phosphotransferase from *E. coli* was used, and opened pUC-*URA3* was assembled to obtain pUC-Δ*URA3*::*hph*. A DNA fragment containing *URA3* upstream region, *hph*, and *URA3* downstream region was released by EcoRI treatment and used for transformation. In this fragment, *hph* was driven by the *URA3* promoter.

The construction of a *KU70* disruption cassette was carried out as follows: A DNA fragment containing *URA3*, its 1.0 kbp upstream region and 500 bp downstream region were amplified by PCR with gene-specific primers by using *R. toruloides* genomic DNA as a template. DNA fragments of partial *KU70* (start codon to 1.5 kbp), *KU70* upstream region (−500 bp to +1 bp) and 1.5 kbp downstream region were also amplified by using fragment-specific primers. These amplicons were assembled into a HindIII site of pUC118 in the order of partial *KU70*, *URA3*, *KU70* upstream region, and *KU70* downstream region. From the resulting plasmid, pUCΔ*KU70*::*URA3* was excised by BamHI and HindIII and the released *KU70* disruption cassette was used for transformation.

The construction of the *CRY1* disruption cassette was carried out similar to the *KU70* disruption cassette. *CRY1* partial open reading frame (ORF) (start codon to +1.5 kbp), *URA3*, *CRY1* upstream region (−500 to +1 bp), and 1000 bp *CRY1* downstream region were amplified by PCR and assembled in a BamHI site of pUC118 to obtain pUCΔ*CRY1*::*URA3*. The CRY1 disruption cassette was released by HindIII and AseI and used for transformation. PCR primers used in the construction of each disruption cassette are listed in Table S1 and illustration of constructed plasmids were showed in Figure S1. As enzymes for assembly of DNA fragments, a Gibson assembly system or NEBuilder HiFi DNA assembly (New England Biolabs) was used in all experiments. The plasmids and strains that were used in this study are shown in Table S2.

Yeast transformation

The NBRC 10032 strain was used for transformation using the electroporation method reported by (Takaku *et al.* 2020) with little modification; 2 M LiAc and 1 M DTT were used, with 0.75 kV, 800 Ω, and 25 μF power conditions. After electroporation, restored cells were streaked on a YPD plate containing 100 mg L^{-1} Hygromycin B for hygromycin resistance, YNB for uracil autotroph, or YNB containing 10 mM 5-Fluoroorotic acid (5-FOA) and 10 mM uracil for *URA3* disruption.

Ultraviolet irradiation mutagenesis and selection

To generate a random mutation in the genomic DNA of *R. toruloides*, cells in the exponential growth phase were mutagenized by ultraviolet (UV) light irradiation, using a UV Crosslinker (CL-1000, UVP, LLC) under the following conditions: 254 nm, 100 V, 0.8 A, 8 W × 5 (UV discharge tube), × 100 μJ cm^{-2}. *R. toruloides* NBRC 10032 was cultivated in an L-shaped test tube with 5 mL YPD broth at 30 °C. After cultivation for 24 h, cells were collected, washed once, and suspended in sterile 0.9% (w/v) NaCl solution. Next, 5 mL of 10^6 cells mL^{-1} on a 90 mm diameter petri plate (As One) was irradiated by UV light for 30 s, which corresponded to approximately 0.1% of the survival ratio. Then, 0.2 mL × 10^3 living cells were placed on the YPD agar medium plates and incubated under dark condition at 30 °C for 5 days to check the color of the colonies.

Genome analysis of *Rhodosporidium toruloides* mutants

For the preparation of genomic DNA, yeast cells were inoculated into an L-shaped test tube containing 5 mL of YPD medium and cultivated at 30 °C for 24 h. Then, 1 mL of the culture solution was collected by centrifugation and the cells were frozen with liquid nitrogen. Cells were disrupted by a multibead shocker (Yasui Kikai) and suspended in an extraction buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA·2Na, 1% SDS). After the addition of 3 M potassium acetate, the mixture was centrifuged and the supernatant was purified through RNase treatment, phenol–chloroform treatment, and ethanol precipitation. Finally, the precipitated genomic DNA was dissolved in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA).

Genome sequences of mutants were sequenced by the Illumina MiSeq platform. Sequence reads of mutants were trimmed by trimmomatic 0.36 and mapped to the genome sequence of NBRC 10032 that was determined previously (Accession Nos. BJWK01000001 to BJWK01000039) by bwa 0.7.2. SNP call was carried out by samtools 2.8 and varscan 2.4.0. SNP annotation was carried out by snpEff 4.3q.

Biochemical analysis

The dry cell weight (DCW) was quantified in lyophilized cells derived from 1 mL of broth.

Carotenoids were extracted from dry cells in 100% acetone using a multibead shocker (Yasui Kikai), as described in (Pham *et al.* 2020). Carotenoid production was calculated as the equivalent of β-carotene (Wako Pure Chemical Industries Ltd.) with absorbance at 488 nm, using a microplate reader (Infinite M200 Pro, Tecan).

A Southern analysis was performed using Alkphos Direct™ Labeling and the CDP-star Detection System (GE Healthcare Life Science) in accordance with the manufacturer's instructions.

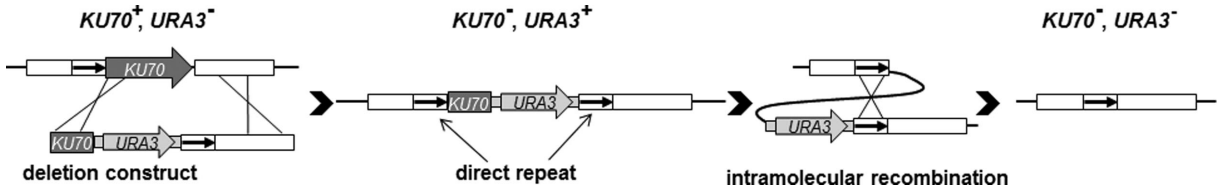


Figure 1. Schematic representation of *KU70* disruption. *URA3* is the selection maker. The position of the homologous recombinants is indicated.

Chromosomal DNA was digested by appropriate restriction enzymes and was hybridized with a gene-specific probe.

Quantitative reverse transcription polymerase chain reaction

Total RNA extraction was performed by hot phenol method and purification of total RNA was carried out using Illustra RNAspin (GE HealthCare Life Science) in accordance with the manufacturer's instructions. Quantitative real-time PCR was performed using Light Cycler 480 SYBR Green I Master Mix (Roche Applied Science) as described previously (Pham *et al.* 2020). The relative expression level was calculated as the ΔCt power of 2, in which the ΔCt value was obtained by subtracting the Ct value of the housekeeping gene (*ACT1*, the gene encoding actin) from that of the target gene. The PCR primers for expression analysis are listed in Table S3.

Results

Establishment of gene targeting in NBRC 10032

Our previous investigation revealed that the expression of *CRY1* of NBRC 10032 increased after 1 h of light exposure and that there was a two-step response by light (in which gene activation occurred early in the cultivation period [1 h] and later [72 h] in the *CRY1* gene). These results suggested a role for *CRY1* in carotenoid production (Pham *et al.* 2020). For further evaluating the function of *CRY1* on carotenoid production, we constructed the *CRY1* deleted strain. Since gene targeting of NBRC 10032 has not been established until now, we first attempted to construct a host strain. Generally, gene targeting frequency is extremely low in *Rhodosporidium* sp., because of the nonhomologous end joining (NHEJ) system. Therefore, the *KU70* gene that was involved in NHEJ was deleted to increase the efficiency of homologous recombination. To carry out multiple gene disruptions, we used *URA3* encoding orotidine 5′-phosphate decarboxylase as the selection marker for transformation in NBRC 10032. By using a *URA3* marker and carrying out positive selection by a uridine auxotroph and counter selection by 5-fluoroorotic acid resistance, we were able to obtain *CRY1* disruption following the *KU70* disruption (Figure 1). In order to obtain a *URA3* disruption of NBRC 10032, first a DNA fragment containing the 5′-flanking region of *URA3*, the hygromycin resistance gene, and the 3′-flanking region of *URA3*, was introduced into NBRC 10032. From more than 800 hygromycin resistants, three 5′-FOA nonsensitive strains were obtained. Using Southern analysis, it was confirmed that 1 of the 3 strains was a homozygous *URA3* disruption (Figure S2), and this strain was named dURA3. Next, we used dURA3 as the parental strain and *URA3* as the selection marker. The DNA fragment for transformation consisted of a partial *KU70* ORF, *URA3*. The 3′-flanking region of *KU70* and the 5′-upstream region of *KU70* that was used as a direct repeat was inserted between *URA3* and the 3′-flanking region of *KU70*. In the transformation of dURA3 using this fragment, if homologous integration had taken place at the *KU70* locus, *URA3* would be looped out by intramolecular homologous recombination (Figures 1 and 2b). Therefore, only homologous recombinants could be obtained by selection by 5-FOA. However, colony-PCR of 5-FOA resistant strain showed 2 amplicons that were 3.1 kbp and 6.2 kbp, expected as *KU70* deletion and native *KU70* bands, respectively (Figure 2a, dKU70-1). When dKU70-1 was transformed by the same DNA fragment, the 5-FOA resistant strain showed only 1 amplicon (Figure 2a, dKU70-2). These results led us to suspect that NBRC 10032 might be a diploid strain. The homozygosity of *KU70* disruption was confirmed by Southern analysis (Figure S3) and the *KU70* disruptant was named dKU70. Finally, *CRY1* was disrupted using dKU70 as the host strain. DNA fragment for *CRY1* disruption was constructed in the same manner as for *KU70*. Similar to the *KU70* disruption experiment in which 2 transformation steps were necessary, the construction of *CRY1* disruptant also involved 2 steps to obtain a homozygote of *CRY1* disruption. Finally, the homozygosity for *CRY1* disruption was confirmed by Southern analysis and the strain was named dCRY1 (Figure S4). With regards to the acquisition of gene disruptions, the frequencies of obtaining a homologous recombinant were 4.5% and 85% for the *KU70*-disrupted strain and the *CRY1*-disrupted strain, respectively.

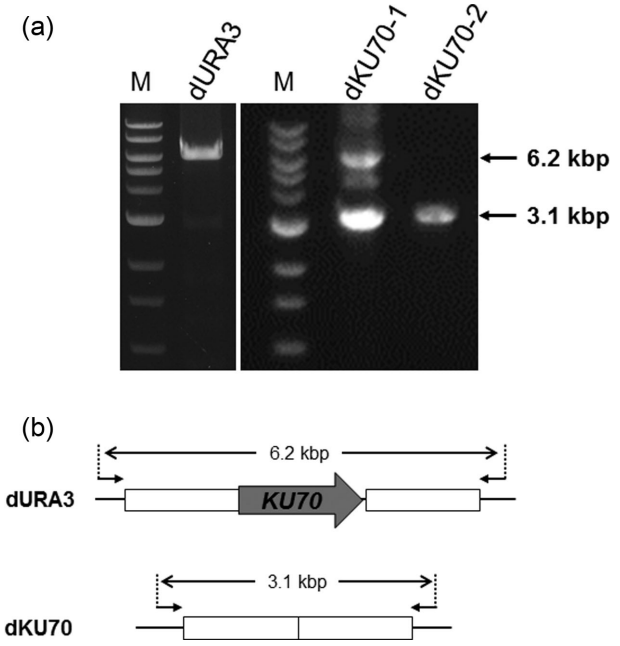


Figure 2. Results of *KU70* deletion. (a) PCR analysis. (b) The PCR amplification region.

Effect of *CRY1* disruption on carotenoid production

To analyze the effect of *CRY1* disruption on carotenoid production, we cultivated dCRY1 and parent strain (PS) NBRC 10032

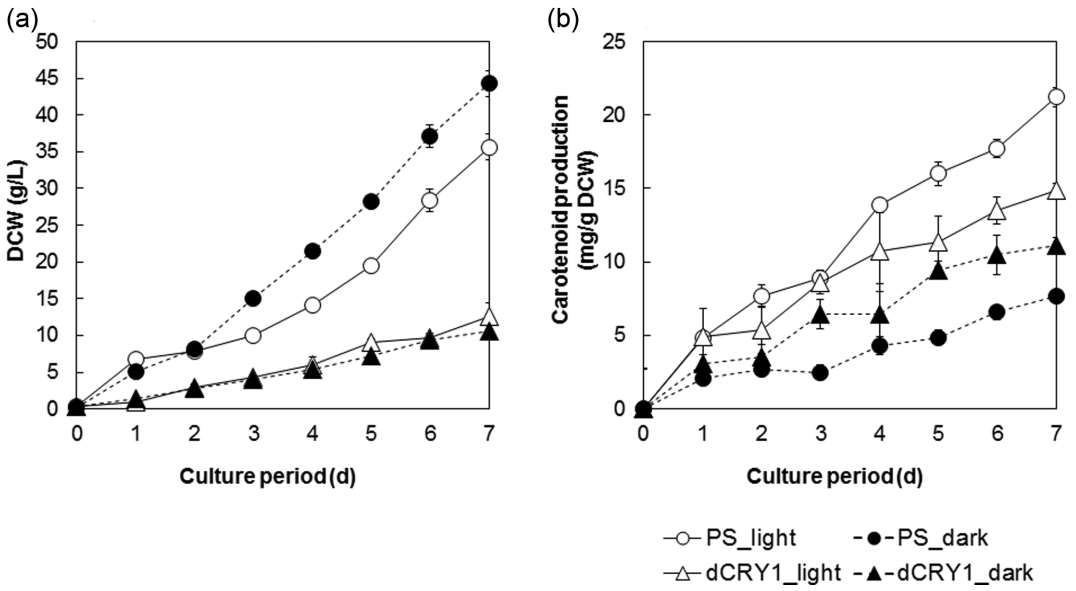


Figure 3. Growth rate (a) and carotenoid production (b) of *CRY1* deletion mutant dCRY1. Growth rate (a) and carotenoid production (b) were compared between the dCRY1 strain (triangles) and the PS strain (circles) under light (open) and dark (filled) conditions. Dry cell weight (DCW) was determined by g/L for the growth rate. Carotenoid production was determined by mg/g DCW depending on the β-carotene standard. Culture periods are represented in days (d). Error bars show SD derived from triplicate biological experiments.

under light and dark conditions and analyzed the carotenoid production. The dCRY1 strain had a lower growth rate than that of PS under both dark and light conditions. The carotenoid productivity of dCRY1 was higher than that of PS in the dark, but lower than that of PS in the light (Figure 3). For the gene expression analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR), genes in the carotenoid biosynthesis pathway, such as, *GGPSI* encoding geranylgeranyl diphosphate synthase, type III, *CAR2* encoding phytoene synthase/lycopene cyclase, and *CAR1* encoding phytoene desaturase responding to light (Pham *et al.* 2020) were selected (Figure 4). In addition, white-collar is known as the photoreceptor for blue light and plays the role in carotenoid production (Linden, Ballario and Macino 1997; Froehlich *et al.* 2002; Iigusa, Yoshida and Hasunuma 2005; Estrada and Avalos 2008). This protein was also reported in cooperative participation with Cryptochrome DASH into carotenoid biosynthesis in *Fusarium fujikuroi* (Castrillo and Avalos 2015). Therefore, WC1 that is a white-collar in NBRC 10032 encoded by *WC1* was selected to analyze in the *CRY1* deletion strain. Under the dark condition, reduced gene expression compared to PS was observed in *GGPSI* of dCRY1 from 0 to 24 h experiment period (Figure 5a). Although the significant difference was not observed in *CAR1* gene between dCRY1 and PS (Figure 5b), higher expression was detected in *CAR2* (Figure 5c). In the case of light condition, the expression of *GGPSI*, and *CAR1* genes in dCRY1 were lower than that in PS except 3 h cultivation (Figure 5e and f). Similarly, lower expression was also observed in *CAR2* gene of dCRY1 at 1h light exposure comparing to PS (Figure 5g). The two-step light response was not observed in the *GGPSI* gene in the dCRY1 strain (Figure 5e). Although a two-step light response was observed in the *CAR1* and *CAR2* genes, that response was somewhat muted compared to that of PS (Figure 5f and g). *CAR1* and *CAR2* expression levels of dCRY1 increased at 1 h, but by less than that of PS, and then gradually decreased until the 24 h experiment period was concluded instead of the dramatic decrease seen in PS (Figure 5h and g). The expression of the *WC1* gene in dCRY1 was lower than that in PS under both light and dark conditions at before 24 h experiment period (Figure 5d and h). No peak of expression was observed in *WC1* by light indicated that the light response also was disrupted in the *WC1* gene of the dCRY1 strain.

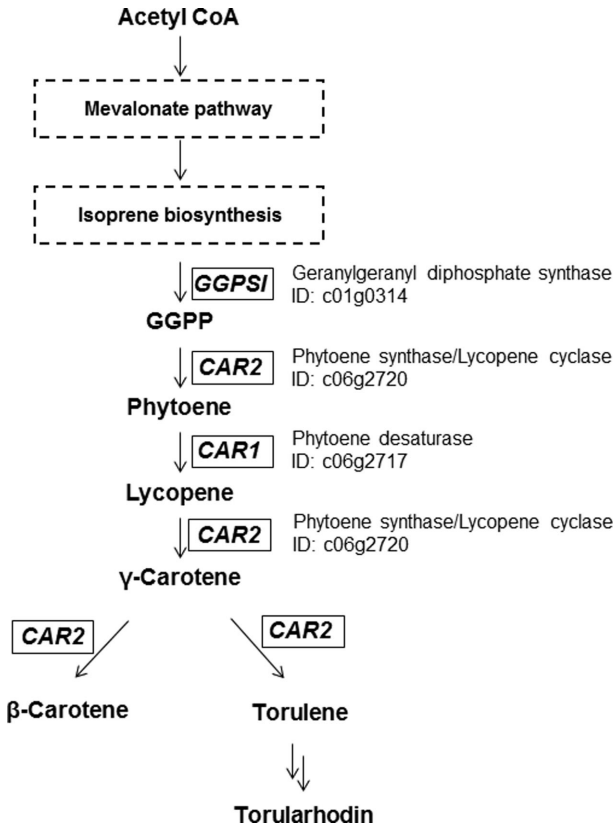


Figure 4. The studied genes in the carotenoid biosynthesis pathway. *GGPSI* (geranylgeranyl diphosphate synthase, type III), *CAR2* (phytoene synthase/lycopene cyclase), and *CAR1* (phytoene desaturase).

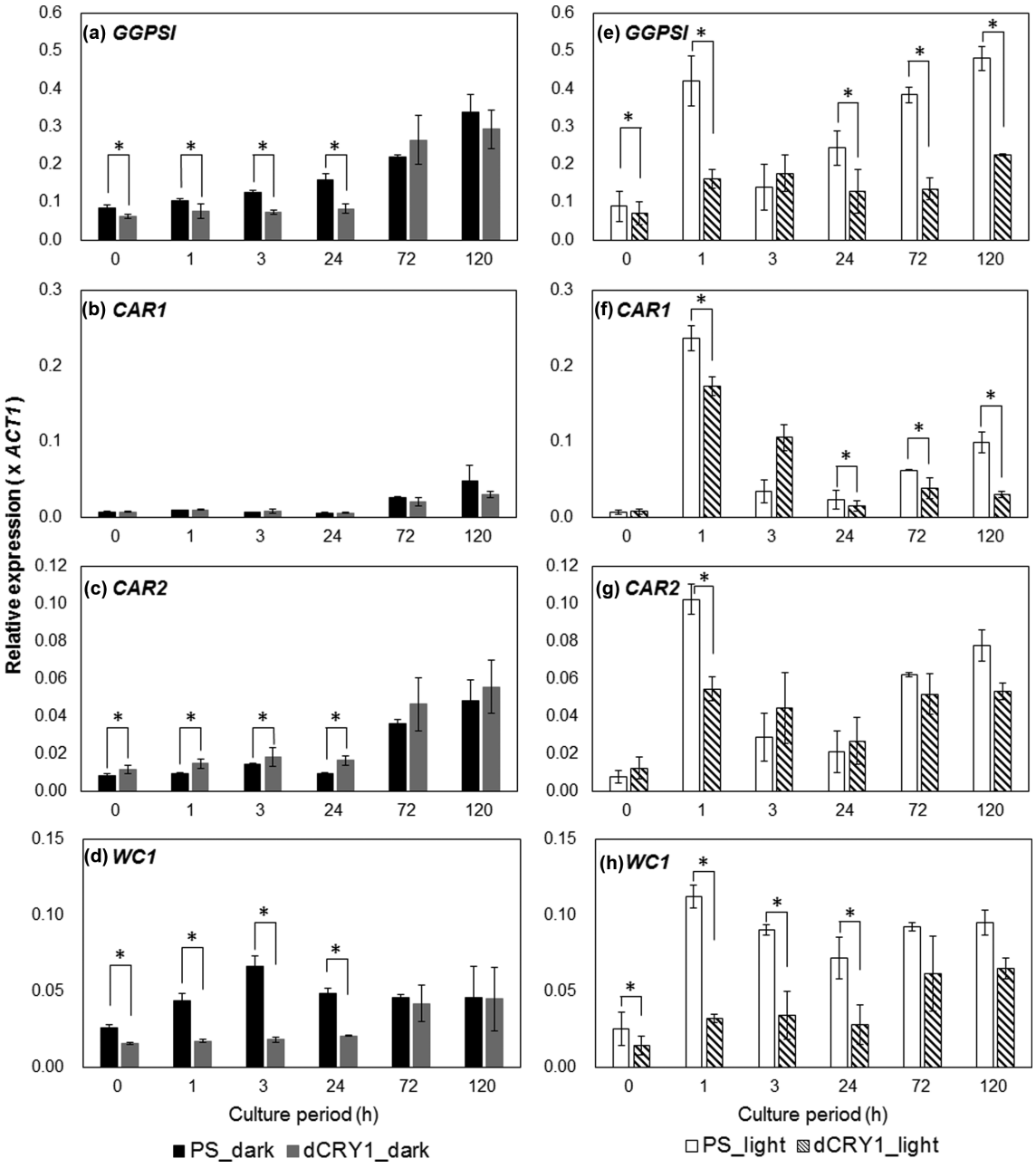


Figure 5. Gene expression of *GGPSI*, *CAR1*, *CAR2*, and *WC1* of dCRY1. (a) *GGPSI*, (b) *CAR1*, (c) *CAR2*, and (d) *WC1*. Left: Gene expression of mutants under dark condition. Filled black and dark gray bars represent PS and dCRY1 respectively. (e) *GGPSI*, (f) *CAR1*, (g) *CAR2*, and (h) *WC1*. Right: Gene expression of mutants under light condition. Unshaded and shaded bars represent PS and dCRY1 respectively. Error bars show SD derived from triplicate biological experiments. Student's *t*-test was used to determine the statistically significant differences ($^{*}P \leq .05$).

Isolation of high carotenoid-producing mutants

In parallel with the *CRY1* disruption, we carried out mutation analysis to identify the other light-response factors. When *R. toruloides* NBRC 10032 was cultivated in continuous darkness, carotenoid production was much lower than that under continuous light irradiation. By analyzing carotenoid nonproducing mutants in the light, it was expected that the light responsive factor(s) would be mutated. Only 1 mutant through UV mutagenesis and screening by colony color was obtained. However, it was clarified that *CAR2* was mutated (data not shown). *CAR2* encodes phytoene synthase/carotene cyclase that is important enzyme for carotenoid biosynthesis (Figure 4) explained the noncarotenoid in this strain. Therefore, only high carotenoid-producing mutants in the dark were continued the experiments. By analyzing high carotenoid-producing mutants, it was expected that factor(s) repressed carotenoid gene expression were mutated. To obtain high carotenoid-producing mutants in the dark condition, UV mutagenesis was carried out. After UV irradiation, among about 3×10^4 isolated colonies growing in the dark, some colonies showed intense color compared to NBRC 10032, which showed a faint pink color. Mutant colonies showed a darker red color, suggesting that in the dark condition they were high carotenoid-accumulating mutants. After the first visual screening, the darker color colonies were subcultured on

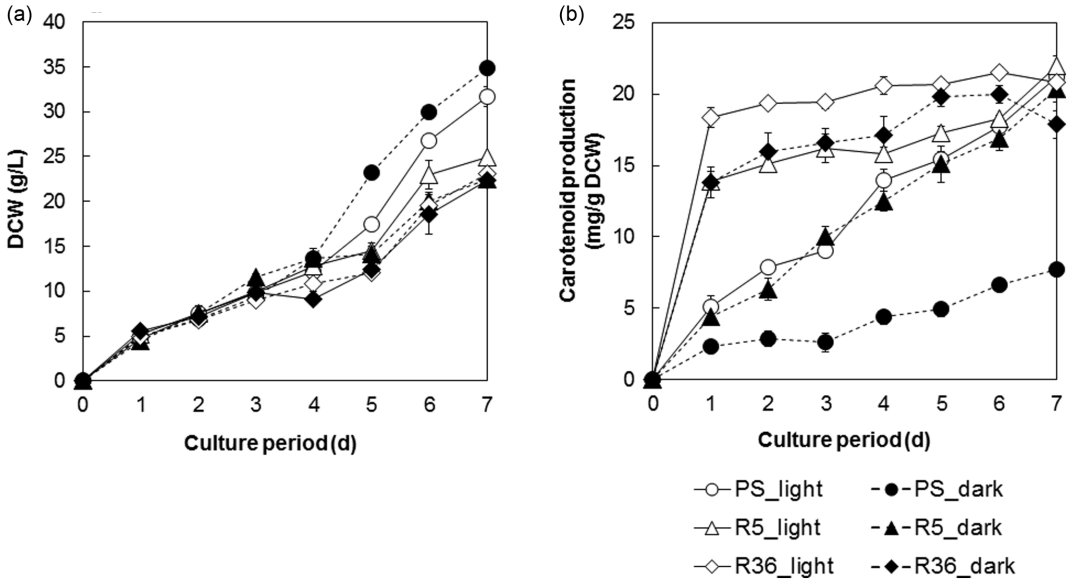


Figure 6. Growth rate (a) and carotenoid production (b) of high carotenoid-producing mutants. Growth rate (a) and carotenoid production (b) were compared between PS (circles) R5 (triangles) and R36 (diamonds) under light (open) and dark (filled) conditions. DCW was determined by g/L for the growth rate and carotenoid production was determined by mg/g DCW depending on the β-carotene standard. Culture periods are represented in days (d). Error bars show SD derived from triplicate biological experiments.

new plates. The 30 darkest red colonies were picked up and carotenoid production was analyzed. Two mutants, R5 and R36, were chosen as the high carotenoid-producing mutants, since the 2 mutants showed higher and faster carotenoid production than PS or other mutant strains (Figure S5) under dark condition. The color of the mutant colonies was showed in the Figure S6a. Microscopic analysis did not show significant morphological differences between PS and the mutants (Figure S6b). Mutant strains were subcultured several times to confirm their stability before investigating the growth rate, carotenoid productivity, and gene expression.

Growth rate and carotenoid production of high carotenoid-producing mutants

Growth phenotypes of mutants were compared by culturing them under both dark and light conditions. The cell growth of R5 and R36 was lower than that of PS in both conditions. However, R5 and R36 showed higher carotenoid production than PS (Figure 6). Under the dark condition, carotenoid production of R5 and R36 was approximately twice as much and 6 times as much, respectively, as that of PS at 1-day incubation. Thereafter, carotenoid production of R36 was still higher but was negligibly increased and was at its highest at 6 days of incubation, at approximately 20 mg g^{-1} DCW. The R5 mutant increased its carotenoid productivity faster than PS and attained its highest carotenoid production at 7 days of cultivation, with 20.3 mg g^{-1} DCW. That was over twice as much as PS at 7 days of incubation. Moreover, the higher carotenoid-producing phenotype of R5 and R36 was also detected in the light condition. Both R5 and R36 produced high levels of carotenoids in the early culture period (after just 1 day of cultivation) (Figure 6). The R36 mutant attained high carotenoid production, near the maximum carotenoid capacity yield, at just 1 day of cultivation in both dark and light conditions. The same result was found in the carotenoid productivity of R5 with only light cultivation. Altogether, carotenoid production of both mutants was enhanced not only in the dark but also in the light condition.

The expression of carotenoid genes in the high carotenoid-producing mutants

As the carotenoid production of R5 and R36 was higher than that of PS, we analyzed the expression of genes encoding the carotenoid biosynthesis pathway in these 2 mutants. From the carotenoid biosynthesis pathway (Figure 4), *GGPSI*, *CAR2*, and *CAR1* were selected. *WC1* encoding white-collar and *CRY1* encoding Cryptochrome DASH, the putative photoreceptor, were also selected. Mutants and PS were precultured for 24 h in the dark, and the growing cells were then transferred into the new medium and cultivated under a continuous light or dark condition. Light enhanced the gene expression of *GGPSI*, *CAR1*, and *CAR2* of PS. Two-step activation was also detected, which dramatically increased the expression level at 1 h, and then the second activation after 72 h was observed in PS (Figure 7).

In the R5 mutant, expression level of the *GGPSI*, *CAR1*, and *CAR2* genes was higher than that in PS in the dark conditions in the first 24 h experiment period (Figure 7a-c). The expression level of *GGPSI*, *CAR1*, and *CAR2* genes (especially *CAR1* and *CAR2*) dramatically increased at 1 h of light exposure (Figure 7f-h). The expression of *CRY1* gene of R5 was substantially increased at 1 h of light exposure, surpassing that of PS at the same light exposure time that was twice fold higher expression gene as compared to PS (Figure 7i). Additionally, *CRY1* expression level at 1 h by light exposure was 15-fold compared to 0 h in the R5, whereas it was 5-fold in the PS (Figure 7i). For *WC1* expression, significant differences were not observed between R5 and PS in the dark (Figure 7e). Gene activation by light at 1 h of cultivation was also observed in *WC1* expression in R5 (Figure 7j), however, the increase was not as remarkable as those of *CAR1*, *CAR2*, or *CRY1* genes in the light. Gene activation at a later cultivation time was also detected in these genes.

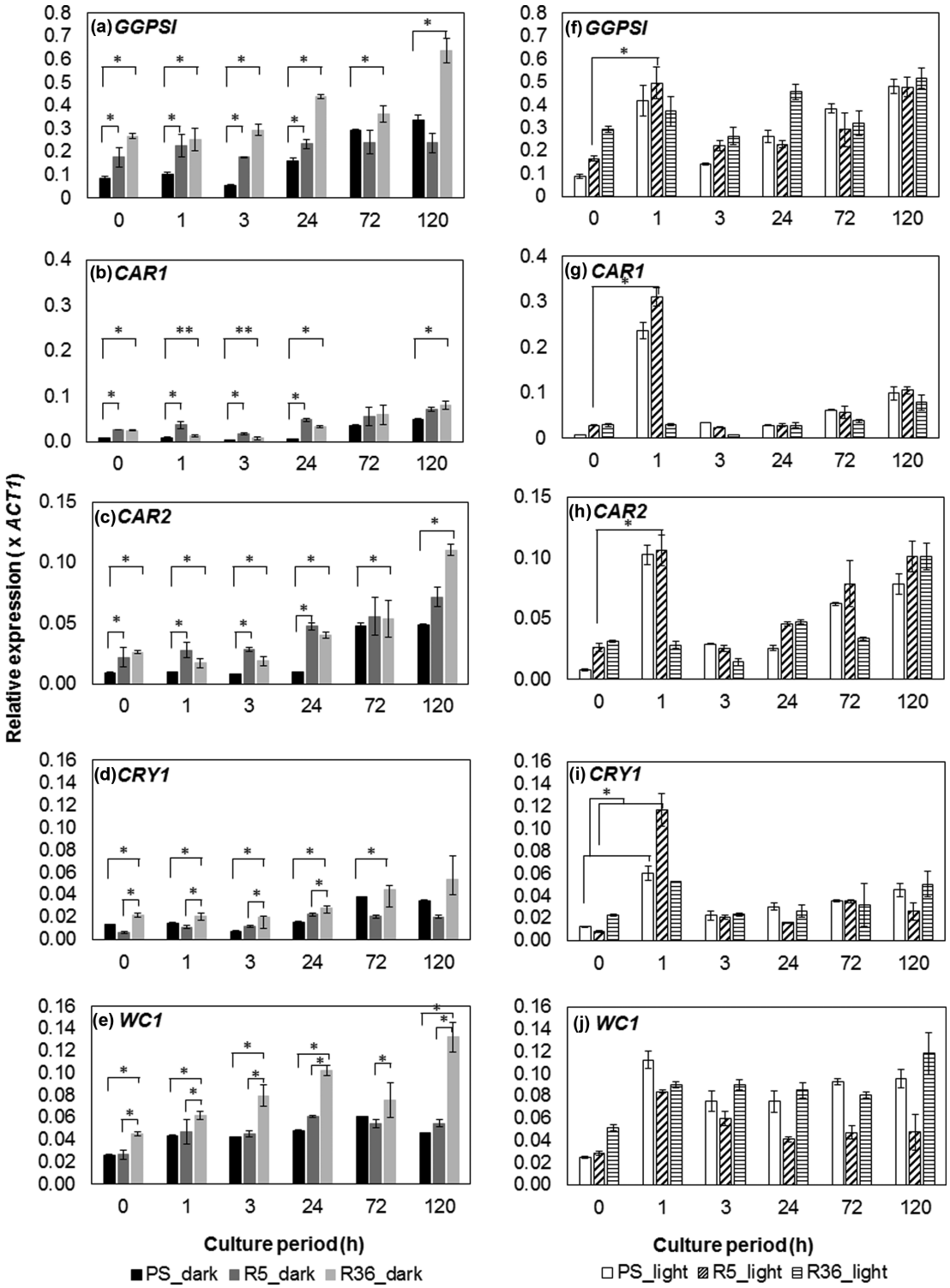


Figure 7. Effect of light irradiation on gene expression. (a) *GGPSI*, (b) *CAR1*, (c) *CAR2*, (d) *CRY1*, and (e) *WC1*. Left, gene expression of mutants under dark condition. Black, dark gray, and light gray bars represent PS, R5, and R36, respectively. (f) *GGPSI*, (g) *CAR1*, (h) *CAR2*, (i) *CRY1*, and (j) *WC1*. Right: Gene expression of mutants under light condition. Unshaded, diagonally shaded, and horizontally shaded bars represent PS, R5, and R36, respectively. Culture periods are represented in hours (h). Error bars show SD derived from triplicate biological experiments. Student's *t*-test was used to determine the statistically significant differences ($^{*}P \leq .05$; $^{**}P \leq .1$).

In the case of the R36 mutant, increased expression compared to PS of all tested genes was evident in the dark condition (Figure 7a-e). In the light condition, the two-step light response remained in the *GGPSI* gene but was absent in *CAR1* and *CAR2* genes (Figure 7f-h). For *CRY1*, gene expression of R36 was higher than that of PS in the dark (Figure 7d) but was almost same level as that of PS in the light (Figure 7i). On the other hand, *WC1* expression in the dark condition was higher than that of R5 and PS in the dark conditions (Figure 7e). In addition, the expression of *WC1* increased by light exposure (data at 1 h light exposure) and it was maintained until 120 h (Figure 7j).

Comparative genome analysis

To screen genetic mutations contributing to the phenotypes of R5 and R36 mutants, genome sequences of these mutants were sequenced by the MiSeq platform. Sequence reads of mutants were mapped to the genome sequence of NBRC 10032 that was determined previously (Accession No. BJWK01000001 to BJWK01000039). Analysis showed 118 and 34 SNPs for R5 and R36, respectively, in the coding region (Table S4a-c). Surprisingly, almost every mutated point observed was heterozygous, in which about half of the reads covering a mutation point supported the references, while the remaining reads supported mutations. We suspected if the genome of the parental strain was contaminated, but further analysis using Sanger method sequencing of the genomic DNA confirmed the mutant heterogeneity (data not shown). In combination with the results of gene targeting experiments, these results suggested that NBRC 10032 is a homozygous diploid. The R36 mutant only had 2 mutated genes with all reads supporting a mutation. One of the 2 genes was *FAS1* encoding fatty acid synthase. For the R5 mutant, there was no mutation point in which all reads supported a mutation. NBRC 10032 was registered as mating type A at NITE. However, this strain did not produce teliospore showing pseudohyphae when grown on a sporulation medium (Figure S7a). Surprisingly, when this strain and NBRC 0880 as mating type a were crossed, hyphal growth and teliospore formation were detected (Figure S7b).

Discussion

In this study, we used 2 methods, gene targeting and mutagenesis analysis, concurrently for unraveling carotenoid production as a response to light in *R. toruloides* NBRC 10032. For gene targeting analysis, we established a gene recombination system in NBRC 10032. To avoid the NHEJ effect, we constructed a *KU70* disruptant of NBRC 10032 by a *URA3* marker recycling system based on the uracil autotroph and 5′-FOA resistance. KU70 is one of the components of NHEJ and studies have reported *KU70* disruption leading to high-frequency homologous recombination in many fungi (Heidenreich *et al.* 2003; Choquer *et al.* 2008; Catalano *et al.* 2011; Feng *et al.* 2012; Verbeke, Beopoulos and Nicaud 2013; Huang *et al.* 2017; Dai *et al.* 2019). In the case of *R. toruloides*, high-frequency gene targeting was reported for the ATCC 10 657 strain (Koh *et al.* 2014). Our results also showed high-frequency homologous recombination of the targeted gene in the homozygous *CRY1* disruptant.

Although *CRY1* disruption led to weakened carotenoid production under both light and dark conditions, light-stimulated carotenoid production was maintained. In addition, *CRY1* deficiency affected carotenoid biosynthesis genes, which showed lower expression compared to PS. However, the two-step activation of gene expression that is seen in *R. toruloides* NBRC 10032 was maintained. Moreover, *WC1* expression was also lower in the *CRY1* disruptant. In *Neurospora crassa*, the white-collar complex (WCC), a protein complex formed by WC1 and WC2 proteins known as the primary photoreceptor system for blue light, promoted carotenoid biosynthesis by light irradiation (He *et al.* 2002; Iigusa, Yoshida and Hasunuma 2005; Belozerskaya *et al.* 2012). But other reports mentioned Cryptochrome DASH (encoded by *CRY1*), which has been identified as a blue light receptor plays the role in promoting the carotenoid production (Ahmad *et al.* 1998; Facella *et al.* 2006; Froehlich *et al.* 2010; Yu *et al.* 2010; Castrillo and Avalos 2015). The WcoA, white-collar protein in *F. fujikuroi*, was reported to play a role in carotenoid biosynthesis with light signaling acting in tandem with CryD (flavin photoreceptor, the DASH cryptochrome) (Castrillo and Avalos 2015). Thus, the lower expression level of *WC1* in the *CRY1* disruptant compared to PS under the same light or dark condition might be the result of repressed WC1 action by CRY1. The overall reduced expression in the carotenoid biosynthesis genes in the *CRY1* disruptant suggested that CRY1 might directly regulate *GGPSI*, *CAR1*, and *CAR2* or might regulate carotenoid genes through the *WC1* gene. Cryptochrome was defined as a photolyase-like protein with no DNA repair activity but was known to have a blue light photoreceptor function (Chaves *et al.* 2011). In *N. crassa*, the expression of the *CryD* gene is strongly induced by light through WCC, which plays a central role in the control of circadian rhythmicity (Dunlap and Loros 2004; Chen and Loros 2009). The regulatory role of CryD also affects the synthesis of other metabolites as suggested by the differential pigmentation of Δ*CryD* strain cultivated in light (Castrillo, García-Martínez and Avalos 2013). Our data showed that CRY1 and WC1 are important for carotenoid production in NBRC 10032. However, since the light response still remained in the dCRY1 strain, the other factor(s) might control the two-step gene activation by light.

In the mutagenesis approach, our purpose was to obtain mutants with high carotenoid production under a dark condition to enable us to decipher the production mechanism of carotenoids better because mutants that produce high carotenoids in the dark were expected to possess mutations in repressing mechanism of carotenogenic genes. We obtained several high carotenoid-producing mutants. Among them, strains R5 and R36 showed the highest carotenoid production. However, their phenotypes exhibited a few differences. Carotenoid production was significantly increased on day 1 in R5 and then showed gradual increase in the light, whereas it increased gradually over time in the dark. In the case of R36, carotenoid production reached almost the maximum amount on day 1, under both light and dark conditions. This suggested that the effect of mutated genes affecting carotenoid production might differ in each strain. The high carotenoid production under light condition indicates that mutations in the genomes of both these high carotenoid-producing strains might happen in a repressing mechanism and this mechanism might also affect carotenoid production in light. Enhancement of carotenoid production in R5 and R36 was supported at the transcriptional level as revealed by the higher expression amounts of *GGPSI*, *CAR1*, and *CAR2* compared to those of PS in both strains. The important genes in carotenoid biosynthesis are phytoene synthase and carotene cyclase, which are encoded by 2 separate genes in bacteria and plants but are encoded by a single gene in fungi such as *crtYB*, in the basidiomycete *Xanthophyllomyces dendrorhous*, *carRA* and *carRP* in the zygomycetes *Phycomyces blakesleeanus* and *Mucor circinelloides*, respectively, and *al-2* in the ascomycete *Neurospora crassa* (Schmidhauser *et al.* 1994; Velayos, Eslava and Iturriaga 2000; Lodato *et al.* 2007; Sanz *et al.* 2011). In *X. dendrorhous*,

over-expression of *CrtYB* in the wild type showed enhanced production of carotenoids (Lodato *et al*. 2007). Although enzymatic characterization had not yet been carried out for *CAR2* from NBRC 10032, it had a high amino acid identity with these bifunctional enzymes. Previously, Jiao *et al*. reported that the *CAR2* disruptant of *R. toruloides* NP11 showed a phenotype with the white color colony (Jiao *et al*. 2019). Therefore, one reason for R5 and R36 to show high carotenoid production could be a higher expression of *CAR2*. On the other hand, the 15-fold higher expression of *CRY1* gene in R5 (1 h compared to 0 h) might contribute to high carotenoid yield at the short time of light exposure. This was supported by the gene targeting analysis that was suggested that CRY1 activated carotenogenic genes. The higher expression in all tested genes in the dark and light condition of R36 than that of PS in just only 0 h might resulted in highest carotenoid production of R36 in both dark and light conditions. We also tried to obtain carotenoid nonproducing mutants because analysis of that mutant may directly lead to the factor controlling carotenoid production. However, genomic analysis of noncarotenoid producing mutant revealed the frameshift mutation of *CAR2* encoding phytoene synthase/carotenoid cyclase that plays the role in the carotenoid biosynthesis. Thus, there was high possibility that loss of function of CAR2 contributed to the noncarotenoid production in this mutant.

In the view point of two-step gene activation of carotenogenic genes, R5 showed a similar gene expression profile to PS, but this phenomenon was only partially observed in R36 (*GGPSI*, *WC1*, and *CRY1*). Therefore, the existence of other factor(s) regulating two-step gene activation. Although comparative genome analysis to determine the SNPs in R5 and R36 genomes should lead to important insights for unraveling this mechanism, we encountered the unexpected barrier that NBRC 10032 was a diploid strain. From the induction of teliospore formation and sexual cross, it was expected that NBRC 10032 was a diploid without sporulation ability, possessing a haploid life cycle.

Conclusion

To analyze the effects of the potential regulator *CRY1* in carotenoid production, we established a gene targeting system in this strain and analyzed the effect of *CRY1* on carotenoid production. Our results using a *CRY1* disruptant suggested that *CRY1* might directly regulate *GGPSI*, *CAR1* and *CAR2* or may regulate carotenoid genes through the *WC1* gene. Through mutagenesis, obtained 2 high carotenoid-producing mutants showed the contribution of CRY1 for carotenoid production and the existence of other factor regulating two-step light response of carotenogenic genes. Therefore, these mutants are worth investigating further. In addition, we also found the strong possibility that NBRC 10032 was a diploid strain. Further investigation is needed to better understand light-stimulated carotenoid production in this yeast.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material. Genomic analysis is available in DNA Data Bank of Japan (DDBJ), accession nos. BJWK01000001 to BJWK01000039.

Author contribution

KD.P. conceived of this study and coordinated the manuscript draft and revision. Y.H. and T.T. executed the experimental work and data analysis. A.Y. executed the sexual cross-analysis of *R. toruloides*. K.M., S.A., K.T., and S.K. executed with genome data analysis. H.Y. and H.T. supported the manipulation of oleaginous yeast and data analysis. Y.S. and W.O. participated in the design of the study and critically revising of this article. All authors read and approved the final manuscript.

Funding

This work was supported by the New Energy and Industrial Technology Development Organization (NEDO) Project under Grant P16009 and the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

Ahmad M, Jarillo JA, Smirnova O *et al*. The CRY1 blue light photoreceptor of *Arabidopsis* interacts with phytochrome a in vitro. *Mol Cell* 1998;**1**:939-48.

Alcantara S, Sanchez S. Influence of carbon and nitrogen sources on *Flavobacterium* growth and zeaxanthin biosynthesis. *J Ind Microbiol Biotechnol* 1999;**23**:697-700.

An GH, Johnson EA. Influence of light on growth and pigmentation of the yeast *Phaffia rhodozyma*. *Antonie Van Leeuwenhoek* 1990;**57**:191-203.

Avalos J, Pardo-Medina J, Parra-Rivero O *et al*. Carotenoid biosynthesis in *Fusarium*. *J Fungi* 2017;**3**:39.

Belozerskaya TA, Gessler NN. Oxidative stress and differentiation in *Neurospora crassa*. *Microbiology* 2006;**75**:427-31.

Belozerskaya TA, Gessler NN, Isakova EP *et al*. *Neurospora crassa* light signal transduction is affected by ROS. *J Signal Transduct* 2012;**2012**:1-13.

Britton G. Britton G, Liaen-Jensen S Pfander H (eds.). *Carotenoids Handbook*, 1st ed. Basel: Birkhäuser, 2004.

Froehlich AC, Liu Y, Loros JJ *et al*. White collar-1, a circadian blue light photoreceptor, binding to the frequency promoter. *Science* 2002;**297**:815-9.

Castrillo M, Avalos J. The flavoproteins CryD and VvdA cooperate with the white collar protein WcoA in the control of photocarotenogenesis in *Fusarium fujikuroi*. *PLoS One* 2015;**10**:1-22.

Castrillo M, García-Martínez J, Avalos J. Light-dependent functions of the *Fusarium fujikuroi* CryD DASH cryptochrome in development and secondary metabolism. *Appl Environ Microbiol* 2013;**79**:2777-88.

Catalano V, Vergara M, Hauzenberger JR *et al*. Use of a non-homologous end-joining-deficient strain (delta-*ku70*) of the biocontrol fungus *Trichoderma virens* to investigate the function of the laccase gene lcc1 in sclerotia degradation. *Curr Genet* 2011;**57**:13-23.

Chaves I, Pokorny R, Byrdin M *et al*. The cryptochromes: blue light photoreceptors in plants and animals. *Annu Rev Plant Biol* 2011;**62**:335-64.

Chen C, Loros JJ. *Neurospora* sees the light: light signaling components in a model system. *Commun Integr Biol* 2009;**2**:448-51.

Choquer M, Robin G, Pêcheur Le P *et al*. *Ku70* or *Ku80* deficiencies in the fungus *Botrytis cinerea* facilitate targeting of genes that

are hard to knock out in a wild-type context. *FEMS Microbiol Lett* 2008;**289**:225-32.
Dai Z, Pomraning KR, Deng S *et al*. Deletion of the *KU70* homologue facilitates gene targeting in *Lipomyces starkeyi* strain NRRL Y-11558. *Curr Genet* 2019;**65**:269-82.
Dunlap JC, Loros JJ. The *Neurospora* circadian system. *J Biol Rhythms* 2004;**19**:414-24.
Estrada AF, Avalos J. The white collar protein WcoA of *Fusarium fujikuroi* is not essential for photocarotenogenesis, but is involved in the regulation of secondary metabolism and conidiation. *Fungal Genet Biol* 2008;**45**:705-18.
Facella P, Lopez L, Chiappetta A *et al*. *CRY-DASH* gene expression is under the control of the circadian clock machinery in tomato. *FEBS Lett* 2006;**580**:4618-24.
Feng J, Li W, Hwang SF *et al*. Enhanced gene replacement frequency in *KU70* disruption strain of *Stagonospora nodorum*. *Microbiol Res* 2012;**167**:173-8.
Frengova GI, Beshkova DM. Carotenoids from *Rhodotorula* and *Phaffia*: yeasts of biotechnological importance. *J Ind Microbiol Biotechnol* 2009;**36**:163-80.
Froehlich AC, Chen CH, Belden WJ *et al*. Genetic and molecular characterization of a cryptochrome from the filamentous fungus *Neurospora crassa*. *Eukaryotic Cell* 2010;**9**:738-50.
He Q, Cheng P, Yang Y *et al*. White collar-1, a DNA binding transcription factor and a light sensor. *Science* 2002;**297**:840-3.
Heidenreich E, Novotny R, Kneidinger B *et al*. Non-homologous end joining as an important mutagenic process in cell cycle-arrested cells. *EMBO J* 2003;**22**:2274-83.
Huang Q, Cao Y, Liu Z *et al*. Efficient gene replacements in *ku70* disruption strain of *Aspergillus chevalieri* var. *intermedius*. *Biotechnol Biotechnol Equip* 2017;**31**:16-22.
Iigusa H, Yoshida Y, Hasunuma K. Oxygen and hydrogen peroxide enhance light-induced carotenoid synthesis in *Neurospora crassa*. *FEBS Lett* 2005;**579**:4012-6.
Jiao X, Zhang Y, Liu X *et al*. Developing a CRISPR/Cas9 system for genome editing in the basidiomycetous yeast *Rhodosporidium toruloides*. *Biotechnol J* 2019;**14**:1-7.
Khanafari A, Tayari K, Emami M. Light requirement for the carotenoids production by *Mucor hiemalis*. *Iran J Basic Med Sci* 2008;**11**:25-32.
Koh CMJ, Liu Y, Moehninsi *et al*. Molecular characterization of *KU70* and *KU80* homologues and exploitation of a *KU70*-deficient mutant for improving gene deletion frequency in *Rhodosporidium toruloides*. *BMC Microbiol* 2014;**14**:50.
Lin X, Gao N, Liu S *et al*. Characterization the carotenoid productions and profiles of three characterization the carotenoid productions and profiles of three *Rhodosporidium toruloides* mutants from *Agrobacterium tumefaciens*-mediated transformation. *Yeast* 2017;**34**:335-42.
Lin Y, Jain R, Yan Y. Microbial production of antioxidant food ingredients via metabolic engineering. *Curr Opin Biotechnol* 2014;**26**:71-8.
Linden H, Ballario P, Macino G. Blue light regulation in *Neurospora crassa*. *Fungal Genet Biol* 1997;**22**:141-50.
Lodato P, Alcaíno J, Barahona S *et al*. Expression of the carotenoid biosynthesis genes in *Xanthophyllomyces dendrorhous*. *Biol Res* 2007;**40**:73-84.
Maldonade IR, Rodriguez-Amaya DB, Scamparini ARP. Carotenoids of yeasts isolated from the Brazilian ecosystem. *Food Chem* 2008;**107**:145-50.
Martínez-Cárdenas A, Chávez-Cabrera C, Vasquez-Bahena JM *et al*. A common mechanism explains the induction of aerobic fermentation and adaptive antioxidant response in *Phaffia rhodozyma*. *Microb Cell Fact* 2018;**17**:1-16.
Mata-Gómez LC, Montañez JC, Méndez-Zavala A *et al*. Biotechnological production of carotenoids by yeasts: an overview. *Microb Cell Fact* 2014;**13**:1-11.
Moore MM, Breedveld MW, Autor AP. The role of carotenoids in preventing oxidative damage in the pigmented yeast, *Rhodotorula mucilaginosa*. *Arch Biochem Biophys* 1989;**270**:419-31.
Pham KD, Shida Y, Miyata A *et al*. Effect of light on carotenoid and lipid production in the oleaginous yeast *Rhodosporidium toruloides*. *Biosci Biotechnol Biochem* 2020;**84**:1501-12.
Popova AV, Andreeva AS. Carotenoid–lipid interactions. *Advances in Planar Lipid Bilayers and Liposomes*. Amsterdam: Elsevier, Vol. **17**. 2013, pp. 215-36.
Sakaki H, Nakanishi T, Tada A *et al*. Activation of torularhodin production by *Rhodotorula glutinis* using weak white light irradiation. *J Biosci Bioeng* 2001;**92**:294-7.
Sanz C, Velayos A, Álvarez MI *et al*. Functional analysis of the *Phycomyces carRA* gene encoding the enzymes phytoene synthase and lycopene cyclase. *PLoS One* 2011;**6**:e23102.
Schmidhauser TJ, Lauter FR, Schumacher M *et al*. Characterization of *al-2*, the phytoene synthase gene of *Neurospora crassa*. *J Biol Chem* 1994;**269**:12060-6.
Schneider T, Graeff-Hönninger S, French WT *et al*. Lipid and carotenoid production by oleaginous red yeast *Rhodotorula glutinis* cultivated on brewery effluents. *Energy* 2013;**61**:34-43.
Stachowiak B, Czarnecki Z. Effect of light on carotenoid yield in fed cultures of *Phaffia rhodozyma* CBS 5626. *Polish J Food Nutr Sci* 2007;**57**:129-31.
Takaku H, Miyajima A, Kazama H *et al*. A novel electroporation procedure for highly efficient transformation of *Lipomyces starkeyi*. *J Microbiol Methods* 2020;**169**:105816.
Vázquez M. Effect of the light on carotenoid profiles of *Xanthophyllomyces dendrorhous* strains (formerly *Phaffia rhodozyma*). *Food Technol Biotechnol* 2001;**39**:123-8.
Velayos A, Eslava AP, Iturriaga EA. A bifunctional enzyme with lycopene cydase and phytoene synthase activities is encoded by the *carRP* gene of *Mucor circinelloides*. *Eur J Biochem* 2000;**267**:5509-19.
Verbeke J, Beopoulos A, Nicaud JM. Efficient homologous recombination with short length flanking fragments in Ku70 deficient *Yarrowia lipolytica* strains. *Biotechnol Lett* 2013;**35**:571-6.
Yen HW, Yang YC. The effects of irradiation and microfiltration on the cells growing and total lipids production in the cultivation of *Rhodotorula glutinis*. *Bioresour Technol* 2012;**107**:539-41.
Yu X, Liu H, Klejnot J *et al*. The cryptochrome blue light receptors. *Arab B* 2010;**8**:e0135.
Yuan H, Zhang J, Nageswaran D *et al*. Carotenoid metabolism and regulation in horticultural crops. *Hortic Res* 2015;**2**:15036.
Zhang Z, Zhang X, Tan T. Lipid and carotenoid production by *Rhodotorula glutinis* under irradiation/high-temperature and dark/low-temperature cultivation. *Bioresour Technol* 2014;**157**:149-53.