Appl Biochem Biotechnol
DOI 10.1007/s12010-017-2521-5

Overexpression of DnaJ-Like Chaperone Enhances Carotenoid Synthesis in *Chlamydomonas reinhardtii*

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Received: 17 April 2017 / Accepted: 19 May 2017

Abstract Production of functional carotenoids using microalgae may facilitate the commercialization of anti-aging nutritional supplements. The green alga *Chlamydomonas reinhardtii* uses a non-mevalonate (MEP) pathway for isopentenyl diphosphate (IPP) synthesis. Two enzymes thought to play important roles in this MEP pathway to IPP synthesis are 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and reductase (DXR). DnaJ-like chaperone (Orange protein) is thought to support phytoene synthase, a key enzyme in plant carotenoid synthesis. Genes for Orange (*OR*), *DXS*, and *DXR* were overexpressed via nuclear transformation into *C. reinhardtii*. CDS of *OR*, *DXS*, and *DXR* were amplified and connected with dual promoters of heat-shock protein 70A and ribulose bisphosphate carboxylase small chain 2. Compared with the parental strain, transformant CrOR#2 produced increased lutein and β-carotene (1.9-fold and 1.7-fold per cell, respectively). Transformant CrDXS#1 produced lutein and β-

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Electronic supplementary material The online version of this article (doi:10.1007/s12010-017-2521-5) contains supplementary material, which is available to authorized users.

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Published online: 13 June 2017

carotene at lower per-cell abundances than those for the parental strain. CrDXR#2 transformant produced lutein and β-carotene at higher per-cell abundances than their parental counterpart; however, these transformants produced lutein and β-carotene at lower per-medium abundances than their parental counterparts. These results suggest that OR protein supports phytoene synthase in *C. reinhardtii* and that the phytoene synthesis step is rate-limiting in carotenoid synthesis.

Keywords *Chlamydomonas reinhardtii* · Carotenoid · Orange protein · DXS · DXR · Chaperone

Introduction

Much attention has been focused on microalgal production of functional carotenoids, fatty acids, feed protein, and biofuel. The Chlorophyceae *Haematococcus pluvialis* has been used commercially as a natural source of astaxanthin [1]. Astaxanthin is one of the most powerful known antioxidant carotenoids. It is used in food, dietary supplements, aquaculture, and cosmetics. More than 750 carotenoids, from various organisms, have been reported. Carotenoids derived specifically from microalgae may be desirable natural sources of functional ingredients [2, 3].

Carotenoids are biosynthesized using isopentenyl diphosphate (IPP), dimethylallyl-diphosphate (DMAPP), geranylgeranyl diphosphate, phytofluene, and phytoene. The IPP and DMAPP biosyntheses in eukaryotic phototrophs have been reported to occur through both the mevalonate (MVA) and the non-mevalonate (2-*C*-methylerythritol 4-phosphate, MEP) pathways [4]. Isoprenoid biosynthesis is essential for all living organisms; all plant isoprenoids are synthesized by the MVA pathway in the cytoplasm and the MEP pathway in plastids [5, 6]. Chlorophyceae includes astaxanthin-rich *H. pluvialis*, β-carotene-rich *Dunaliella salina*, and model microalga *Chlamydomonas reinhardtii*; this class utilizes only the MEP pathway for carotenoid synthesis.

In the MEP pathway, pyruvic acid and glyceraldehyde 3-phosphate are converted to IPP and DMAPP via 1-deoxy-D-xylulose 5-phosphate (DXP) and MEP. The first enzyme of the MEP pathway is DXP synthase (EC 2.2.1.7, DXS). The second enzyme, DXP reductase (EC 1.1.1.2267, DXR), catalyzes DXP to MEP. Steps catalyzed by DXS and DXR are thought to be rate-limiting steps in the MEP pathway [4, 7]. To our knowledge, effects of endogenous DXS and DXR gene overexpression on carotenoid biosynthesis in *C. reinhardtii* have not been studied. After enzymatic conversions from IPP and DMAPP to geranylgeranyl diphosphate (C20), phytoene synthase (PSY) binds geranylgeranyl diphosphate to synthesize phytoene (C40).

Overexpression of a *D. salina PSY* gene in *C. reinhardtii* leads to an increase in the content of carotenoids [8]. PSY is thought to be a rate-limiting enzyme in carotenoid biosynthesis. The cauliflower Orange (OR) gene encodes a plastid-localized DnaJ-like molecular chaperone with cysteine-rich zinc finger domain that mediates high levels of β-carotene accumulation [9]. Overexpression of *OR* increased carotenoid accumulation and salt stress tolerance in transgenic sweet potato cultures [10]. Overexpression of maize *PSY1* and *Arabidopsis thaliana OR* led to orange-colored rice [11]. OR in *A. thaliana* is considered to be the chief posttranscriptional regulator of PSY for controlling carotenoid biosynthesis [12]. To our knowledge, effects of endogenous *OR* overexpression on carotenoid biosynthesis in *C. reinhardtii* have not been studied.

We overexpressed endogenous DXS, DXR, and OR in *C. reinhardtii* to evaluate the importance of these enzymes in Chlorophyceae carotenoid biosynthesis.

Material and Methods

Microalgal Strains and Culture Conditions

Chlorophyceae *C. reinhardtii* CC-4350 cw15 nit1 nit2 arg7–8 mt + (Matagne strain 302) was obtained from the Chlamydomonas Resource Center and maintained in liquid or 1.5% agar TAP media with 50 mg/L L-arginine at 25 °C, under 50 μmol photons/m^2/s. Light and dark cycles were 14 h:10 h (L/D).

Vector Construction

Candidate coding sequences of 1-deoxy-D-xylulose 5-phosphate synthase (DXS1, Cre07.g356350) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR1, Cre12.g546050) were obtained from the Phytozome v12.0 *C. reinhardtii* v5.5 database (US Department of Energy Joint Genome Institute), and their peptide sequence reliabilities were confirmed by BLASTP (National Center for Biotechnology Information). The *OR* sequence (CPL6, Cre06.g279500) was retrieved using a BLASTP search of the Phytozome v12.0 *C. reinhardtii* v5.5 database, with the *A. thaliana* OR protein sequence (NP_974975) as the query.

Total RNA of *C. reinhardtii* was purified using a FastRNA Pro Red kit (MP Bio, Santa Ana, CA, USA). cDNA was synthesized using the ReverTraAce reverse transcriptase (Toyobo, Osaka, Japan) and the CrDXS-KpnR, CrDXR-OR, and CrOR-OR primers, under thermal conditions of 65 °C for 5 min, 42 °C for 40 min, and 99 °C for 5 min. Primer sequences used in this study are listed in Table S1. Double-stranded DNA of *CrDXS* was amplified with Platinum SuperFi DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and primers CrDXS-EcoF and CrDXS-KpnR (Table S1). PCR conditions are listed in Table S2. Double-stranded DNA of *CrDXR* was amplified by nested PCR with the primer sets of CrDXR-OF and CrDXR-OR, and CrDXR-EcoF and CrDXR-KpnR (Table S1, 2). PCR products were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Double-stranded DNA of *CrOR* was amplified by nested PCR with the primer sets of CrOR-OF and CrOR-OR, and CrOR-XhoF and CrOR-KpnR (Table S1, 2).

The purified PCR fragments of *DXS* and *DXR*, and pChlamy_4 (Thermo Fisher Scientific) were treated with *EcoRI* and *KpnI* (Takara Bio, Kusatsu, Japan), respectively. The purified PCR fragments of *OR* and pChlamy_4 were treated with *XhoI* and *KpnI* (Takara Bio). These digests were connected using Ligation high reagent (Toyobo). Transformation of *Escherichia coli* was conducted with HIT Competent Cells (RBC Bioscience, New Taipei, Taiwan) and 100 μg/mL ampicillin LB agar plates. Ligated vectors of pC4DXS, pC4DXR, and pC4OR were confirmed by colony PCR with the primers pChlamy-2A-F and pChlamy-3UTR-R (Table S1). Sequence analyses of vector inserts were conducted using an Applied Biosystems 3730xl DNA Analyzer (Foster City, CA, USA).

Transformation

One microgram each of pC4DXS, pC4DXR, and pC4OR was linearized by *ScaI* (New England Biolabs, Ipswich, MA, USA) and transformed into *C. reinhardtii* CC-4350 at 3.3×10^8 cells/mL, using the glass bead method [13]. Treated cells were spread on agar plates containing 10 mg/L Zeocin and 50 mg/L L-arginine. Fast growing colonies were checked for

transformation by colony PCR with Chelex-100 DNA purification, and the primers pCh4-V5HIS-F and pCh4-Term-R (Table S1) [14]. Overexpressed CrOR protein contained a 6-histidine tag. His-tagged CrOR protein was purified with MagneHis Ni-Particles (Promega, Madison, WI, USA) and quantified using Bio-Rad Protein Assay Dye Reagent Concentrate (Hercules, CA, USA), with bovine serum albumin as a standard.

Growth Measurement and Carotenoid Analysis

Cell concentrations for microalgal cultures were measured using a Burker-Turk hemocytometer and a microscope. *C. reinhardtii* cells at an initial concentration of 0.5–1.0 × 10^5 cells/mL were cultured in TAP medium for 3–7 days. Carotenoids were extracted from the cell pellet by vortexing in 5 mL chloroform-methanol (2:1 *v*/*v*). Lutein and β-carotene concentrations were measured using HPLC with a CrestPak C18T-5 column (JASCO, Hachioji, Japan) and absorbance at 450 nm. The mobile phase began as acetonitrile-methanol-pure water (75:15:10) containing 0.1% ammonium acetate (A) and ended as ethyl acetate-methanol (30:70) containing 0.1% ammonium acetate (A). The mobile phase followed a gradient over 20 min, from A/B = 100:0 to A/B = 0:100. These experiments were independently conducted in triplicate. Student's *t* tests were performed at the 0.01 and 0.05 significance levels.

Results

Transformation of *OR*

Agarose gel electrophoresis of the *CrOR* PCR product revealed a 0.9-kb band (Fig. 1). The constructed vector map of pC4OR is shown in Fig. 2. The determined sequence of ligated pC4OR corresponded with the *CPL6* sequence in the Phytozome v12.0 *C. reinhardtii* v5.5 database; therefore, *CrOR*-CDS was successfully amplified from *C. reinhardtii* total RNA. Base 456 of the determined sequence for *CrOR*-CDS was G; this site is A in the *CPL6* sequence from the Phytozome v12.0 *C. reinhardtii* v5.5 database. This mutation is synonymous, since residue 152 is leucine in both peptide sequences. Glass bead transformation of pC4OR produced two Zeocin-resistant colonies (CrOR#1, #2). Colony PCR indicated that CrOR#2 contained full-length *CrOR*-CDS (Fig. 1). The overexpressed OR protein contained a histidine tag. His-tagged OR protein was purified using nickel beads and quantitated as 1.4% of total cell protein in *C. reinhardtii*.

Figure 3 shows lutein and β-carotene content per cell and per milliliter medium, for the CrOR#2 strain and the untransformed control strain. After 72 h of incubation in TAP medium, the lutein contents per cell and per milliliter medium for CrOR#2 were significantly higher than those for the control (1.9- and 1.5-fold higher, respectively). The β-carotene contents per cell and per milliliter medium for CrOR#2 were significantly higher than those for the control (1.7- and 1.3-fold higher).

Transformation of *DXS*

Agarose gel electrophoresis of the *CrDXS* PCR product revealed a 2.2-kb band (Fig. 4). The constructed vector map of pC4DXS is shown in Fig. 2. The determined sequence of pC4DXS corresponded with the *DXS1*-CDS sequence in the Phytozome v12.0 *C. reinhardtii* v5.5

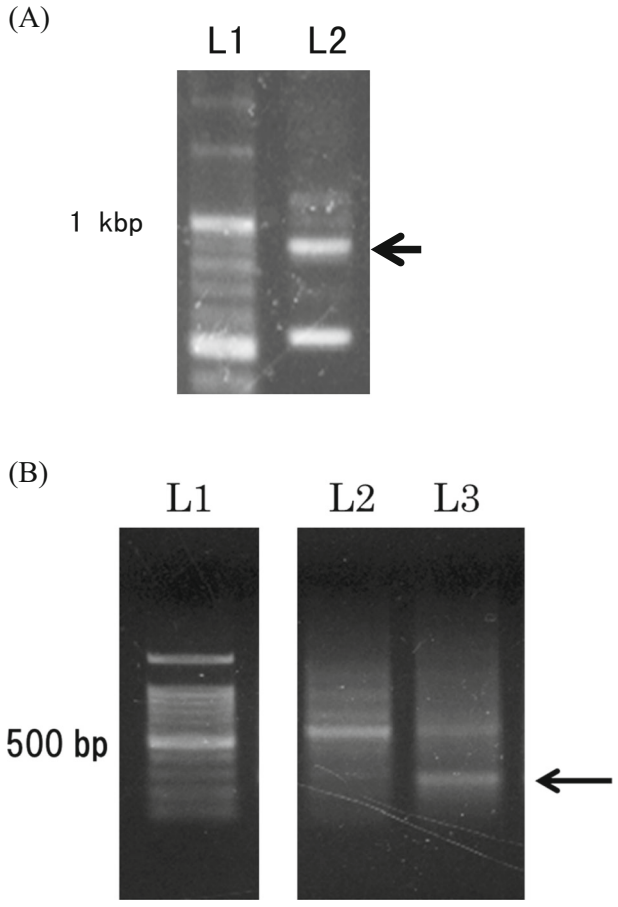

Fig. 1 Agarose gel electrophoreses of PCR products. **a** *Arrow* indicates a PCR product of *CrOR* at 0.9 kbp. *L1* marker, *L2* PCR products from *C. reinhardtii* cDNA. **b** *Arrow* indicates a colony PCR product (272 bp) amplified pC4OR terminator range with transformed *C. reinhardtii* cells of CrOR#1 and #2 as DNA templates. *L1* marker, *L2* colony PCR products of CrOR#1, *L3* colony PCR products of CrOR#2

database; therefore, *CrDXS*-CDS was successfully amplified from *C. reinhardtii* total RNA. Glass bead transformation of pC4DXS produced 5 Zeocin-resistant colonies (CrDXS#1-#5). Colony PCR indicated that CrDXS#1 contained full-length *DXS*-CDS.

Figure 5 shows lutein and β-carotene content per milliliter medium and per cell for the CrDXS#1 strain and the untransformed control strain. The lutein content per cell for CrDXS#1 was significantly lower than that for the control. The lutein content per milliliter medium for CrDXS#1 was significantly higher than that for the control. The β-carotene content per cell for CrDXS#1 was significantly lower than that for the control. The β-carotene content per milliliter medium did not significantly differ between the CrDXS#1 and control strains.

Transformation of *DXR*

Agarose gel electrophoresis of *CrDXR* nested PCR product revealed a 1.3-kb band (Fig. 6). The constructed vector map of pC4DXR is shown in Fig. 2. The analyzed sequence of

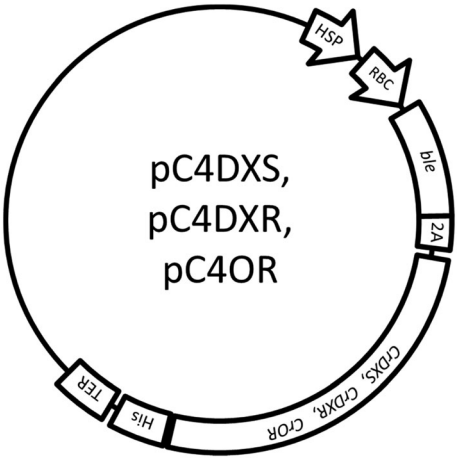

Fig. 2 Constructed vector map of pC4OR, pC4DXS, and pC4DXR. *HSP CrHSP70A* promoter, *RBC CrRBCS2* promoter, *ble* zeocin resistance gene, *2A* FMDV 2A peptide, *His* 6xHis tag, *TER CrRBCS2* terminator

pC4DXR corresponded with the *DXR1*-CDS sequence in the Phytozome v12.0 *C. reinhardtii* v5.5 database. The *CrDXR* coding sequence was successfully amplified from *C. reinhardtii* total RNA. Glass bead transformation of pC4DXR produced 11 Zeocin-resistant colonies (CrDXR#1-#11). Colony PCR indicated that CrDXR#1-#4 contained full-length *DXR*-CDS.

Figure 7 shows lutein and β-carotene contents per milliliter medium and per cell in the CrDXR#1, #2, and #4 strains, and the untransformed control strain. The lutein contents per cell for CrDXR#2 and #4 were significantly higher than that for the control, after 72 h of incubation in TAP medium. On the other hand, the lutein contents per milliliter medium for

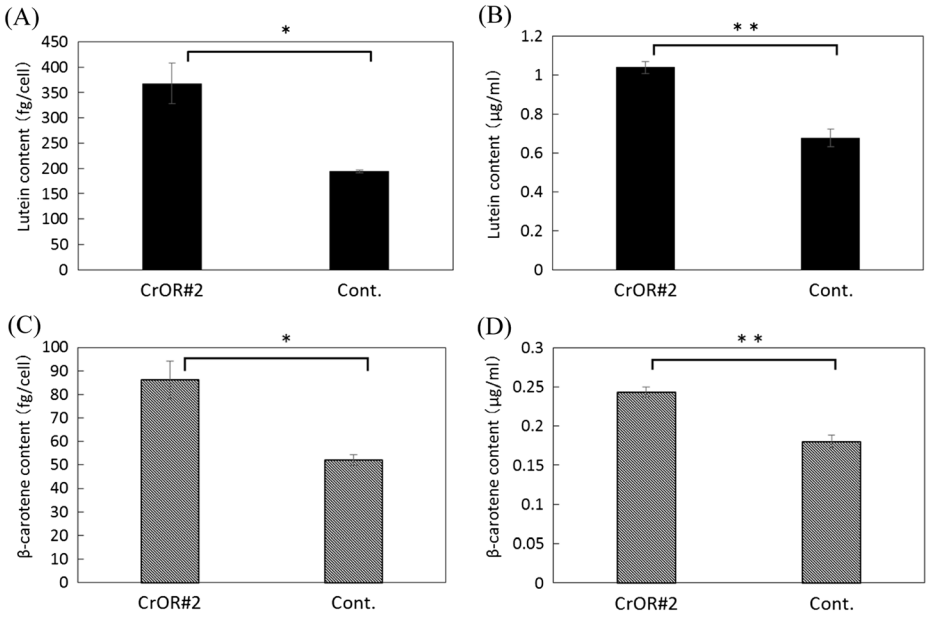

Fig. 3 Lutein and β-carotene contents of transformant CrOR#2 and the untransformed original strain, cultured with TAP medium. **a** Lutein content per cell. **b** Lutein content per volume of medium. **c** β-Carotene content per cell. **d** β-Carotene content per volume of medium. *mean $p < 0.05$ by Student's t test

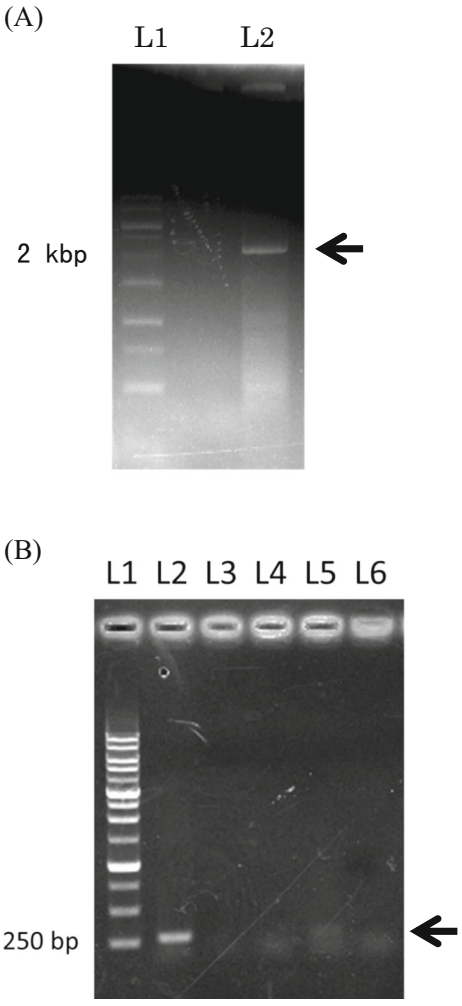

Fig. 4 Agarose gel electrophoreses of PCR products. **a** *Arrow* indicates a PCR product of *CrDXS* at 2.2 kbp. *L1* marker, *L2* PCR product. **b** *Arrow* indicates PCR products (272 bp) amplified pC4DXS terminator range, with transformed *C. reinhardtii* cells of CrDXS#1–#5 as DNA templates. *L1* marker, *L2* colony PCR products of CrDXS#1, *L3* colony PCR products of CrDXS#2, *L4* PCR products of CrDXS#3, *L5* PCR products of CrDXS#4, *L6* PCR products of CrDXS#5

CrDXR#2 and #4 were significantly lower than that for the control. The β-carotene content per cell for CrDXR#4 was significantly higher than that for the control, and the β-carotene contents per milliliter medium for CrDXR#2 and #4 were significantly lower than that for the control.

Discussion

Orange-colored mutant cauliflower (*Brassica oleracea*) produced a relatively high amount of β-carotene, and the mutated gene was designated as the Orange (*OR*) gene [9]. Overexpression of the cauliflower *OR* transgene enhanced carotenoid accumulation more than 5-fold in transgenic potato tubers, compared with that in untransformed tubers [15]. The cauliflower *OR* encodes a DnaJ cysteine-rich domain-containing chaperone protein that mediates high levels of β-carotene accumulation. In the present study, overexpression of endogenous *OR* in

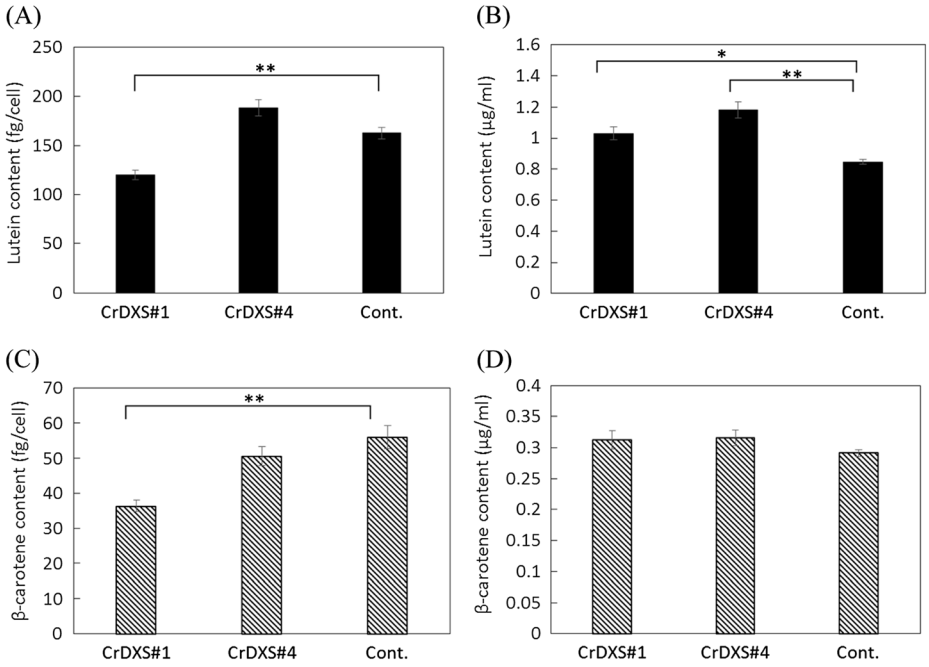

Fig. 5 Lutein and β-carotene contents of transformants CrDXS#1, #4, and the untransformed original strain, cultured with TAP medium. **a** Lutein content per cell. **b** Lutein content per volume of medium. **c** β-Carotene content per cell. **d** β-Carotene content per volume of medium. Asterisks (* and **) mean $p < 0.05$ and $p < 0.01$ by Student's *t* test, respectively

C. reinhardtii led, respectively, to 1.9-fold and 1.7-fold increases in the lutein and β-carotene content per cell, relative to that seen in control cells. These results indicate that *CPL6*, in the *C. reinhardtii* nuclear genome, encodes the OR protein.

Overexpression of OR protein led to carotenoid accumulation in sweet potato and rice [10, 11]. Overexpression of the *A. thaliana* OR protein did not change the transcriptional level of PSY; however, the abundance of active PSY proteins increased [12]. The AtOR protein is the chief posttranscriptional regulator of phytoene synthase in controlling carotenoid biosynthesis. Similarly, the CrOR protein could be the chief posttranscriptional regulator of phytoene synthase, controlling carotenoid biosynthesis in cooperation with PSY in *C. reinhardtii*.

pC4OR, pC4DXS, and pC4DXR include dual promoters of heat-shock protein 70A (HSP70A) and ribulose bisphosphate carboxylase small chain 2 (RBCS2). The promoter of HSP70A is thought to inhibit transgene silencing [16]. These dual promoters control ble and target CDS connected by foot and mouth disease virus (FMDV) 2A peptide to easily screen strongly expressing transformants [17]. CrOR#2 transformed with pC4OR produced His-tagged protein at 1.4%; therefore, OR protein was strongly expressed by pC4OR, and transgene silencing was relatively avoided.

Overexpression of the *PSY* gene from *Chlorella zofingiensis*, by nuclear transformation, enhanced lutein content by 2.2-fold relative to that for untransformed *C. reinhardtii* cells [18]. Overexpression of the *D. salina PSY* in *C. reinhardtii* was reported to increase the lutein and β-carotene contents by 2.6-fold and 1.25-fold compared to those of control cells [8]. These studies suggested that PSY is a rate-limiting enzyme in *C. reinhardtii*. Simultaneous

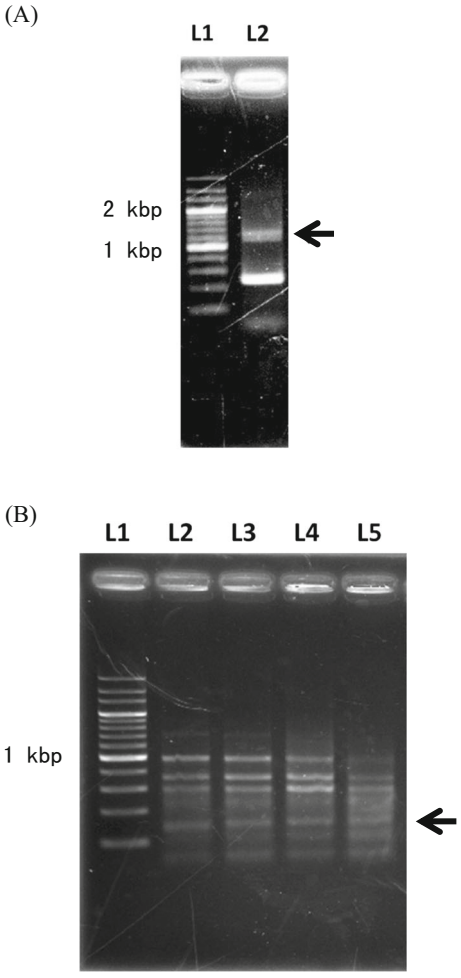

Fig. 6 Agarose gel electrophoreses of PCR products. **a** *Arrow* indicates a PCR product of CrDXR at 1.3 kbp. *L1* marker, *L2* PCR product. **b** *Arrows* indicate PCR products (272 bp) amplified pC4DXR terminator range with transformed *C. reinhardtii* cells of CrDXR#1–#4 as DNA templates. *L1* marker, *L2* colony PCR products of CrDXR#1, *L3* colony PCR products of CrDXR#2, *L4* colony PCR products of CrDXR#3, *L5* colony PCR products of CrDXR#4

overexpression of *PSY* and *OR* is a promising molecular breeding direction to further enhance carotenoid synthesis in *C. reinhardtii*.

In *A. thaliana*, DXS is the chief regulatory enzyme of the MEP pathway, and its overexpression led to only modest increases in isoprenoid end products [19]. An investigation of DXS, DXR, and hydroxymethylbutenyl diphosphate synthase (HDS), three of the seven MEP pathway enzymes, in tomato suggested that DXS is limiting for carotenoid biosynthesis [20–22]. In rice, simultaneous overexpression of *DXS* and *PSY* enhanced carotenoid production relative to overexpression of only *PSY* [11]. In *H. pluvialis*, the *DXS* transcription level is reported to increase under nitrogen-poor or high light conditions [23]. To our knowledge, effects of *DXS* overexpression on microalgal carotenoid synthesis have not been reported. The present results suggest that overexpression of *DXS* by itself does not enhance carotenoid synthesis in *C. reinhardtii*.

Tobacco leaves transformed with *DXR* from *Synechocystis* sp. produced elevated contents of β-carotene, lutein, and antheraxanthin [24]. Overexpression of *DXR* in some plants enhanced isoprenoid production including carotenoids [25–27]. To our knowledge, effects of *DXR* overexpression on microalgal carotenoid synthesis have not been reported. The lutein

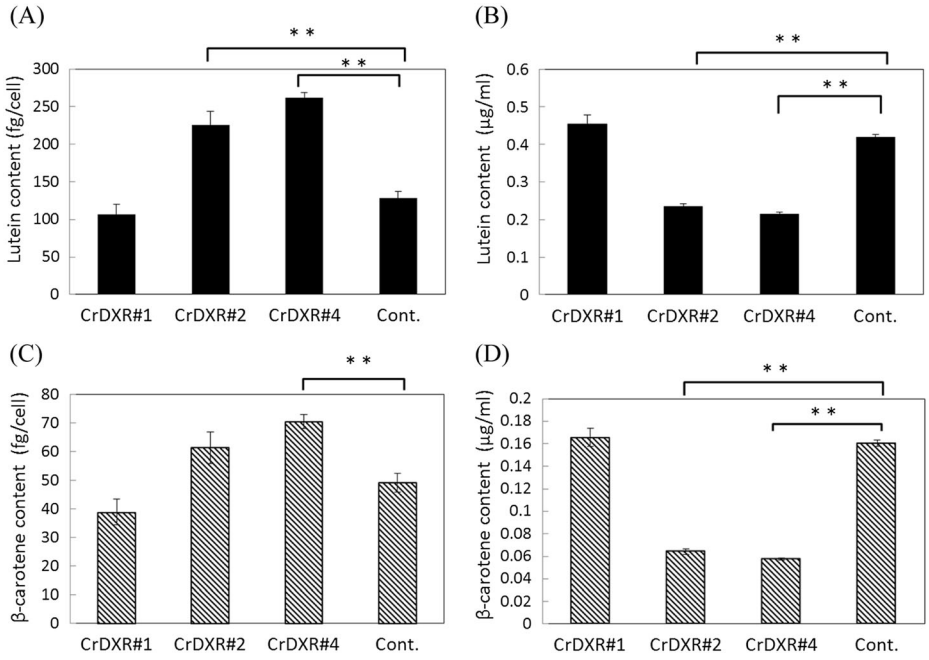

Fig. 7 Lutein and β-carotene contents of transformants CrDXR#1, #2, and #4, and the untransformed original strain, cultured with TAP medium. **a** Lutein content per cell. **b** Lutein content per volume of medium. **c** β-Carotene content per cell. **d** β-Carotene content per volume of medium. Asterisks (* and **) mean $p < 0.05$ and $p < 0.01$ by Student's t test, respectively

contents per cell of CrDXR#2 and #4 are significantly higher than that of the control. On the other hand, the lutein contents per medium for CrDXR#2 and #4 were significantly lower than that for the control. There is a possibility that overexpression of *DXR* in *C. reinhardtii* has a positive effect on carotenoid content per cell and a negative effect on cell growth. In general, overexpressions of even endogenous proteins lead to unbalanced proteome in the transformed cells; therefore, somewhat decrease in growth rate could be caused by this negative effect. There is a possibility that overexpression of particularly *DXR* has an inhibitory effect on growth in *C. reinhardtii*. Further investigation is necessary to understand the cell growth in the *DXR* transformants.

DXS enzymatic activity is reportedly suppressed by feedback inhibition of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) [28]. Rather than DXS, reinforcements of geranyl diphosphate synthase, farnesyl diphosphate synthase, and geranylgeranyl diphosphate synthase could be necessary to enhance further carotenoid synthesis. In the MEP pathway, overexpression of 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) gene enhanced diterpenoid tanshinone biosynthesis in *Salvia miltiorrhiza* [29]. So far, there is a possibility that overexpression of *HDR* enhances carotenoid synthesis in *C. reinhardtii*.

In this study, genes for Orange (*OR*), *DXS*, and *DXR* were overexpressed via nuclear transformation into *C. reinhardtii*. Compared with the parental strain, transformant CrOR#2 produced increased lutein and β-carotene (1.9- and 1.7-fold per cell, respectively). In conclusion, our study suggests that OR protein supports phytoene synthase in *C. reinhardtii* and that the phytoene synthesis step is rate-limiting in carotenoid synthesis.

Acknowledgements The authors would like to thank Prof. T. Sugawara and Assist. Prof. Y. Manabe, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, for their support in the carotenoid analyses.

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