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ORIGINAL ARTICLE

Metabolic engineering of *Dunaliella salina* for production of ketocarotenoids

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Abstract *Dunaliella* is a commercially important marine alga producing high amount of β-carotene. The use of *Dunaliella* as a potential transgenic system for the production of recombinant proteins has been recently recognized. The present study reports for the first time the metabolic engineering of carotenoid biosynthesis in *Dunaliella salina* for ketocarotenoid production. The pathway modification included the introduction of a *bkt* gene from *H. pluvialis* encoding β-carotene ketolase (4,4′β-oxygenase) along with chloroplast targeting for the production of ketocarotenoids. The *bkt* under the control of *Dunaliella* Rubisco smaller subunit promoter along with its transit peptide sequence was introduced into the alga through standardized *Agrobacterium*-mediated transformation procedure. The selected transformants were confirmed using GFP and GUS expression, PCR and southern blot analysis. A notable upregulation of the endogenous hydroxylase level of transformants was observed where the BKT expression was higher in nutrient-limiting conditions. Carotenoid analysis of the transformants through HPLC and MS analysis showed the presence of astaxanthin and canthaxanthin with maximum content of 3.5 and 1.9 µg/g DW, respectively. The present study reports the feasibility of using *D. salina* for the production of ketocarotenoids including astaxanthin.

Keywords *Dunaliella* · Carotenoids · Astaxanthin · Metabolic engineering

Introduction

Carotenoids include a wide range of pigments naturally occurring in all photosynthetic organisms and many non-photosynthetic bacteria and fungi. They provide important photo protective functions and participate in light-harvesting and membrane stabilization (Sui et al. 2013). Among the carotenoids, astaxanthin (3,3-dihydroxy β,β-carotene-4,4-dione) has attracted great attention due to its beneficial effects on human health as well as their utility as food colourant and/or feed additive (Goswami et al. 2010).

Haematococcus pluvialis is known to accumulate high content (3–4 %) of astaxanthin (Hata et al. 2001). *H. pluvialis* cultivation in outdoor conditions is a challenging problem due to many reasons. *H. pluvialis* being a fresh water alga is prone to protozoan infestation and its growth requires low temperature, low light and heterotrophic growth medium which are not considered as cost effective to meet the market demand. Metabolic engineering of the carotenoid biosynthetic pathway in heterologous host systems is a promising approach developed in recent time towards the production of novel carotenoids including

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astaxanthin. The key enzymes involved in astaxanthin biosynthesis from β-carotene, i.e. β-carotene ketolase (BKT) and β-carotene hydroxylase (BKH) (Misawa et al. 1993; Kajiwara et al. 1995), are the main targets for genetic engineering. Metabolic pathway for the synthesis of carotenoids and ketocarotenoids in higher plants and microalgae is shown in Fig. 1.

In vivo production of astaxanthin and other ketocarotenoids by organisms that do not synthesize it in a natural way has been achieved by metabolic engineering in bacteria, algae and higher plants. *Brassica napus* (Shewmaker et al. 1999), *Oryza sativa* (Ye et al. 2000), *Nicotiana tabacum* (Misawa et al. 1993; Mann et al. 2000; Ralley et al. 2004), *Lycopersicon esculentum* (Rosati et al. 2000; Fraser et al. 2002; Huang et al. 2013), *Arabidopsis thaliana* (Stalberg et al. 2003), *Daucus carota* (Jayaraj et al. 2008) and *Solanum tuberosum* (Morris et al. 2006) are some of the plants engineered for ketocarotenoid production. The activity of the endogenous higher plant carotene hydroxylase(s) to act either on β-carotene or heterologous ketolated carotenoids in order to form astaxanthin and its intermediates has been reported by several workers (Mann et al. 2000; Stalberg et al. 2003). Thus, addition of a single enzyme, β-carotene ketolase, is sufficient to obtain high levels of ketocarotenoids in a plant species that normally does not produce these compounds.

Algae have an active isoprenoid metabolism that ensures enough precursors for carotenogenic pathway and provisions for their adequate storage. They are mostly autotrophic and are best candidates for high biomass production in outdoor condition. This offers unique advantages for metabolic engineering of carotenoid biosynthetic pathway in algae when compared to plants and other organisms. In addition, many microalgal strains are considered as GRAS (generally regarded as safe) organisms, which is useful for the production of carotenoids or carotenoids-enriched microalgae for pharmaceutical and feed applications (León-Bañares et al. 2004). For these reasons, they can be adequate hosts for heterologous genes encoding carotenoid biosynthetic enzymes that allow them to overproduce typical carotenoids or produce new ones not usually synthesized by microalgae. Unicellular fresh water alga *Chlamydomonas reinhardtii was* the first alga in which heterologous expression of *bkt* was studied (León et al. 2007) where the production of a novel ketocarotenoid (ketolutein) was observed in transgenic alga. Studies in our lab have reported increased astaxanthin production in transgenic *Haematococcus pluvialis* overexpressing *bkt* gene (Kathiresan et al. 2015). The increased production of astaxanthin in transgenic *H. pluvialis* paved the chance of using *Dunaliella salina* as a better host for *bkt* expression mainly because of its high content of β-carotene which acts as the precursor of astaxanthin biosynthesis. Further the alga being halophilic and autotrophic is also suitable for outdoor cultivation. Also genetic transformation procedures in the alga have been established through various methods (Tan et al. 2005; Geng et al. 2003; Walker et al. 2005a, b; Degui et al. 2002; Sun et al. 2005; Jin et al. 2001;

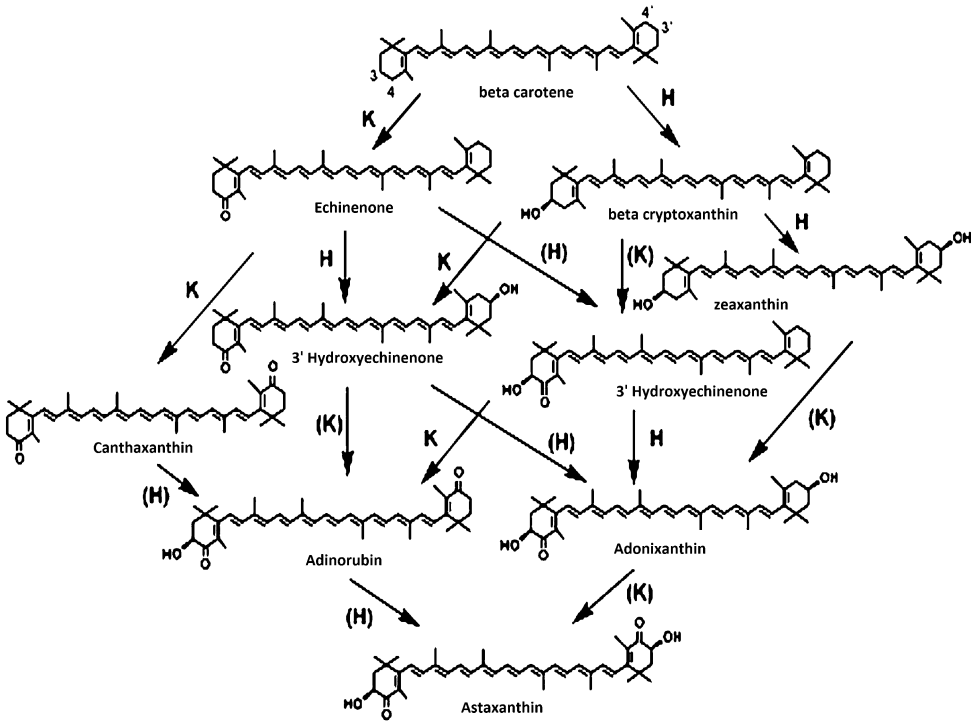

Fig. 1 Schematic representation of the various intermediates that are formed upon the action of β-carotene ketolase and β-carotene hydroxylase in different transgenic systems

Anila et al. 2011). Essentially, the metabolic engineering discussed in the current study is an attempt to extend the existing carotenoid pathway in *D. salina* through a single gene (*bkt*) for production of ketocarotenoids including astaxanthin from β-carotene.

Materials and methods

Microorganisms and culture conditions

D. salina strain V-101 was obtained from the Centre for Advanced Studies in Botany, Madras University, Chennai. Liquid cultures of *D. salina* were maintained in a modified AS100 medium (MAS100) (Vonshak 1986) in which Tris was replaced with $NaHCO_3$ (2 g l^{-1}).

D. salina was also grown for selection and co-cultivation in TAP medium for transformation experiments (Anila et al. 2011) modified to contain sodium chloride at 10 g l^{-1}. The liquid cultures were maintained under 16 h light at an irradiance of 18.75 ± 2.5 µmol m^{-2} s^{-1} at 25 ± 1 °C. The cultures were shaken manually once a day. *D. salina* was also cultured in nutrient-limiting MAS100 (1/4 strength of phosphate and nitrate salts) and De Walnes media (Orset and Young 1999) for carotene induction studies. These cultures were maintained under continuous light intensity of 37.5–43.75 µmol m^{-2} s^{-1}. *E. coli* strain DH5α was used for transformation and plasmid maintenance. All the plasmid constructs were introduced into *Agrobacterium tumefaciens* strain EHA 101 (nopaline type) using the procedure (Höfgen and Willmitzer 1988). Both strains harbouring the binary vector constructs were maintained on LB medium containing 100 mg l^{-1} kanamycin.

Construction of vectors for algal transformation

The binary vector constructs pCAMBIA 1304-CaMV-BKT harbouring genomic clone of *bkt* driven by CaMV promoter was obtained (Kathiresan et al. 2015) and used in the experiments. Genomic DNA clone of *bkt* (1.8 kb size—NCBI accession No. GQ214765) from *H. pluvialis* was used in the above vector construction. RBCS2 promoter region along with transit peptide was amplified from genomic DNA of *D. salina* using modified PCR-based adaptor ligated Genome walking method (Cottage et al. 2001). Primer sequences (Table 1) were designed based on *D. tertiolecta* RBCS2 promoter sequence (NCBI accession No. AY530156). Primers (TF1, TF2 and TR1) were later designed to obtain the full length amplification of promoter along with transit peptide portion based on sequence data of genome walking. The primers TF1 and TF2 were tagged with *Hin*cII and *Kpn*I and TR1 with *Xho*I sites. The amplification using TF1 and TR1 was used for complete promoter construct (1.4 kb) and TF2 and TR1 were used for smaller deletion construct (600 bp) (Fig. S3). Rubisco promoter regions were used to substitute CaMV in the vector construct p1304-CaMV-BKT using the tagged restriction sites (Fig. S2). The RBCS2 promoter along with transit peptide (1.4 kb) was analysed in NCBI-BLAST and was shown to have 98 % similarity with the *D. tertiolecta*. The peptide cleavage site was identified between 62–63 bp (21 amino acids) and 135–136 bp (45 amino acids) after start codon in the sequence.

Agrobacterium-mediated genetic transformation

Three recombinant binary vector constructs and one control vector which were used for *Agrobacterium*-mediated transformation of *D. salina* in the present study are as follows:

1. p1304-RBB-BKT where *bkt* is driven by RBCS2 promoter (1.4 kb) along with chloroplast transit peptide,
2. p1304-RBS-BKT where *bkt* is driven by RBCS2 deletion promoter fragment (0.6 kb) along with chloroplast transit peptide,
3. p1304-CaMV-BKT (*bkt* driven by CaMV promoter) and
4. Control vector p1304.

Agrobacterium-mediated transformation of *D. salina* was carried out using the standard procedure as described by Anila et al. (2011). The selected colonies were grown in MAS100 medium for further confirmation studies. The transformed cells were initially screened for GFP expression and were observed for fluorescence under an excitation filter (BP460-490C) and barrier filter (BA520IF) of a fluorescent microscope (Olympus CKX41) at an excitation wavelength of 488 nm.

Amplification using BKT and RBS primers

The cells of four transformant cultures were harvested by centrifugation at 2000 rpm, and genomic DNA of the transformants was extracted using Genelute plant genomic isolation kit (Sigma-Aldrich, USA). PCR primers BKTF (5′–3′)—TGACTCGAGTGGGCGACACAGT ATCACAT and BKTR (5′–3′)—ACTCTAGAACCAGGTCATGCCA AG were used to amplify a portion of *bkt* from *D. salina* transformed using p1304-CaMV-BKT. A different set of primers was used to confirm *D. salina* transformed using p1304-RBB-BKT and p1304-RBS-BKT constructs. Primers RBF (5′-ACCACTTTACTTTATACGTCC-3′) and

Table 1 Primer sequences for RBCS2 promoter amplification carotene gene expression analysis

Primer	Primer sequences(5′–3′)
Adapter long arm	CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCC GGGCAGGT
Adapter short arm	ACCTGCCC-NH2
Adapter specific primers	ASP1:GGATCCTAATACGACTCACTATAGGGC
	ASP2:AATAGGGCTCGAGCGGC
Gene specific primer sequence	TR4-GGTCAAGGAGAAGAGAGGTCTGAGG
	TR3-GTTGGGGCAATGACAACAGC
	TR2-CGTATAAAGTAAAGTGGTAA
	TR1-**CTCGAG** CACCATCATCTGGTTGGCGCG
	TF1-**GTCAAC GGTACC** GAGCTGGGACTTGACCACCAAG
	TF2-**GTCAAC GGTACC** TTACTTTGCATGATGCAGCA
PSYF	TCCTCGACCCTCGGACCCTC
PSYR	AGTGCCTGCCACGCGATAGC
PDSF	TCATCTTCGGCCAGAAGTAT
PDSR	CTTCTGGAAGAAGGGCATAC
LCYF	GTGCTGGGTAGATGAGATGC
LCYR	GGTGTATGGCATGGCATACA
BKTF	CATCTCCTTGTACGCCTGGT
BKTR	CAGTGCAGGTCGAAGTGGTA
CHYF	CTACACCACAGCGGCAAGTA
CHYR	GCCTCACCTGATCCTACCAA
ActinF	AGCGGGAGATAGTGCGGGAC
ActinR	AATGCCCACCGCCTCCATGC

BKR (5′-GAGTACAGGAACTCAAGTACAA-3′) amplified 500 bp region from transformants carrying the *bkt* gene fused with Rubisco promoter. PCR was carried out in thermocycler (Eppendorf Thermal cycler, Germany) at an annealing temperature of 58 °C. PCR products were separated on agarose gels (1.2 %), stained with ethidium bromide and documented (Chemidoc XRS, Bio-rad, Germany).

Southern blot of the transformants

Southern blot experiment was carried out separately for p1304-CaMV-BKT and p1304-RBB-BKT/p1304-RBS-BKT transformants due to the difference in probes used.

For the p1304-CaMV-BKT transformants, the PCR amplicon which is a portion of the *bkt* gene (800 bp) was used as probe. The PCR product was purified using PCR purification kit (HiPura, Himedia, Bombay). The fragment was then labelled using a BrightStar Psoralen-Biotin labelling Kit (Ambion Inc, Texas, USA) to be used as probe. 40 µl of genomic DNA (30 µg) from transformed cells was digested for 1 h with *Xho*I and *Xba*I and *Hin*dIII enzymes separately. pCAMBIA1304-BKT and DNA from wild *D. salina* were also digested with *Hin*dIII to be used as positive and negative control.

For p1304-RBB-BKT and p1304-RBS-BKT-transformed *D. salina*, probe (500 bp) was prepared from the amplicons obtained using internal primers RBF and BKR. Genomic DNA of the transformants was digested with *Hin*dIII for southern blot as there is an internal site for the enzyme in both *bkt* and cloned rubisco promoter.

The hybridization was carried out at 58 °C for the transferred nylon membrane for all the southern blots and the blot was visualised using the BioDetect Kit (Ambion, Texas) using BCIP-NBT (Bangalore genei Pvt Ltd, Bangalore) as the substrate.

Expression profile of various carotenogenic genes in the transformants

The primers for the genes involved in carotenoid biosynthesis namely phytoene synthase (PSY), phytoene desaturase (PDS) and lycopene cyclase (LCY) were designed based on *D. salina* nucleotide sequences, and primers for secondary carotenoids synthesizing enzymes namely β-carotene ketolase (BKT) and β-carotene hydroxylase were designed based on *Haematococcus* nucleotide sequences (Table 1). Cells of wild and transgenic *D. salina* in equal cell count were incubated under light intensity of

40 ± 2.5 μmol m^{-2} s^{-1} for a period of 25 days. Each cell lines were grown in MAS100, MAS100 with 1/4 nitrate and phosphate content and De Walnes medium with 1 M NaCl. After harvesting, cells were frozen under liquid nitrogen and subsequently powdered using a mortar and pestle. Then, total RNA was extracted using RNAqueous kit according to instruction manual (Ambion, TX, USA). Possible contaminant genomic DNA in RNA extract was removed using TURBO DNA-free kit (Ambion, Austin, TX). The concentration of total RNA was determined spectrophotometrically at 260 nm. Integrity of RNA was checked by electrophoresis in formaldehyde denaturing gels stained with ethidium bromide. cDNA was synthesized from 1.5 μg of total RNA in a 20 μl final volume, using AMV reverse transcriptase (Sigma-Aldrich, USA) and oligo-dT (18-mer) primer (Ambion, TX, USA). The PCR products obtained were sequenced for further confirmation.

Northern blot analysis of *bkt* gene

Total RNA from cells of wild and transgenic *D. salina* was isolated using RNAqueous RNA isolation kit (Ambion, TX, USA). RNA was later run on 15 % denaturing PAGE containing 7 M urea for 4 h at 5 V cm^{-2}. It was further transferred on to a nylon membrane using turbo transfer method using transfer buffer provided in Northern Max kit(Ambion, TX USA) or electroblotted using semi dry transfer apparatus (Bio-Rad, Germany) using 0.5X TBE. Prehybridization and hybridization were performed using Northern MAX kit following manufacturers' instruction (Ambion, TX USA). The PCR amplicon which is a portion of the *bkt* gene (800 bp) was used as probe for hybridisation. The PCR product was purified using PCR purification kit (HiPura, Himedia, Bombay). The fragment was then labelled using a BrightStar Psoralen-Biotin labelling Kit (Ambion, TX, USA) to be used as probe. Post hybridization washing and detection were carried out using Psoralen-Biotin detection kit following manufacturer's instruction (Ambion, TX, USA).

Carotenoid profiling of the transformants

The carotenoid profiling of the transformants was carried out using analytical procedures like TLC, HPLC and LCMS-APCI.

Cells of wild and transgenic *D. salina* with equal cell count were incubated under light intensity of 40 ± 2.5 μmol m^{-2} s^{-1} for a period of 25 days. Each cell lines were grown in MAS100, MAS100 with 1/4 nitrate and phosphate content and De Walnes medium with 1 M NaCl. After 25 days of incubation under continuous light, cells were harvested by centrifugation. The biomass was lyophilized and weighed. Pigments were extracted from algal cells with 90 % acetone and were passed over anhydrous sodium sulphate to remove moisture content. The pigment was spotted on silica gel 60 F254 *TLC plate* (Merck-Germany) and was separated on acetone:hexane (3:7) solvent system. The bands were compared with that of standard pigments. All operations were carried out under dim light.

HPLC analysis was carried out for the pigment extract using the Shimadzu LC-10AT liquid chromatograph instrument with reverse phase C18 column (Supelco, 25 cm × 4.6 mm). Gradient solvent system consisting of acetone (10 %) and 90 % (v/v) methanol at a flow rate of 1.25 ml min^{-1} was used. The separated carotenoids were identified by comparing retention times and spectra against known standards. The peaks were integrated by Class VP version 6.14 SP1 software (Shimadzu, Singapore) at 476 nm to quantify ketocarotenoids and 445 nm to quantify other carotenoids. Standard β-carotene, astaxanthin and lutein were purchased from Sigma-Aldrich (St. Louis, MO, USA), and canthaxanthin was obtained from ChromaDex, Inc. (SantaAna, CA, USA). PDA spectrum of each carotenoid obtained was also compared to that of standards.

Mass spectrometry in APCI positive mode

The carotenoid extract from transformed and wild *D. salina* was analysed by using a Q-TOF ultima mass spectrometer (UK). APCI source was heated at 130 °C and the probe was kept at 500 °C. The corona (5 kv), HV lens (0.5 kv) and cone voltage (30 kv) were optimized. Nitrogen was used as sheath and drying gas at 100 and 300 h^{-1}, respectively. The spectrometer was calibrated in the positive mode and $[M+H]^+$ ion was recorded. Mass spectra of carotenoids were acquired with an m/z 400–600 scan range at 470 nm by a diode array detector and confirmed with respective standards.

Results

The transformants obtained for the four binary vector constructs after *Agrobacterium*-mediated transformation were confirmed initially through GFP expression and PCR. The genomic DNA of p1304-CaMV-BKT transformants amplified using *bkt* primers (BKTF and BKTR) obtained 1.8 kb amplification in the transformants.

In p1304-RBB-BKT and p1304-RBS-BKT transformants, primers RBF and BKR amplified 500 bp region which contains *bkt*-RBSC2 part from the T-DNA of the construct. So, only those transformants harbouring T-DNA

from the respective construct will be amplified with the above set of primers (Fig. 2).

Southern blot of the transformants

Genomic DNA from p1304-CaMV-BKT transformants was digested using *Hin*dIII (lane 2) and *XhoI* and *XbaI* (lane 3). Lane 1 where p1304-CaMV-BKT was digested with *Hin*dIII gave two fragments of size 1.1 and 1.4 kb due to internal *Hin*dIII site (Fig. 3a). The transformants which were digested with same enzyme gave additional bands greater than above mentioned sizes which may be due to digestion by either *XhoI* or *XbaI* outside the right border region. Lane 3 gave the bottom band with size ~1.8 kb which is due to *bkt* region in vector and two more bands of greater sizes due to T-DNA integration. Multiple bands revealed multiple integration of T-DNA. Lane 4 is untransformed *D. salina.*

In order to digest p1304-RBB-BKT and p1304-RBS-BKT transformants, *Hin*dIII enzyme was selected as inside RBCS and BKT region there is internal site for *Hin*dIII. When p1304-RBB-BKT was digested with *Hin*dIII, fragments of sizes 3.2 kb (entire RBCS-BKT-poly A), 1.6, 2.3, 0.7 and 2.4 kb were obtained. Out of these, only fragments 1.6, 2.3 and 2.4 kb can react with the probe which is designed so that it contains part of both RBCS and BKT region (Fig. 3b).

The bands in lane 1 correspond to two fragments of sizes 1.6 and 2.3 kb obtained by the digestion of binary vector p1304-RBB-BKT. The weak signal in lane 2 may be due to part of the RBCS promoter region which is also present in wild *D. salina*. Lanes 3 and 4 are genomic DNA from p1304-RBB-BKT transformants and lanes 5 and 6 from

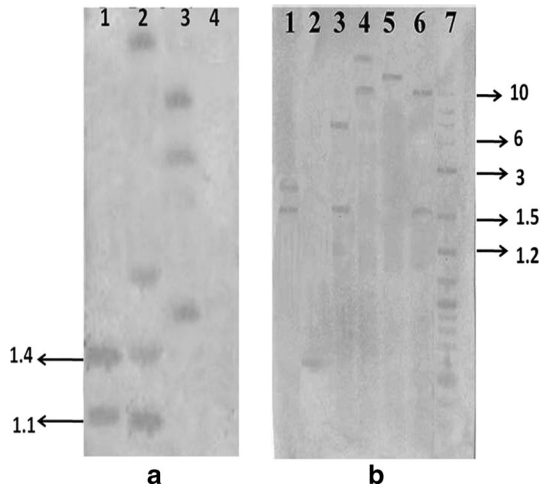

Fig. 3 Southern blot of various *D. salina* transformants with *bkt* gene. **a** p1304-CaMV-BKT transformants *lane 1* pCAMBIA1304-BKT digested with HindIII; *lane 2* DNA from p1304-CaMV-BKT transformants digested with *Hin*dIII; *lane 3* DNA from p1304-CaMV-BKT transformants digested with *Xho*I and *Xba*I; *lane 4* Untransformed *D. salina* DNA digested with *HindIII*. **b** p1304-RBB-BKT and p1304-RBS-BKT transformants: *lane 1* p1304-RBB-BKT binary vector; *lane 2* wild *D. salina*; *lanes 3–4* p1304-RBB-BKT transformants; *lanes 5–6* p1304-RBS-BKT transformants; *lane 7* 10 kb marker

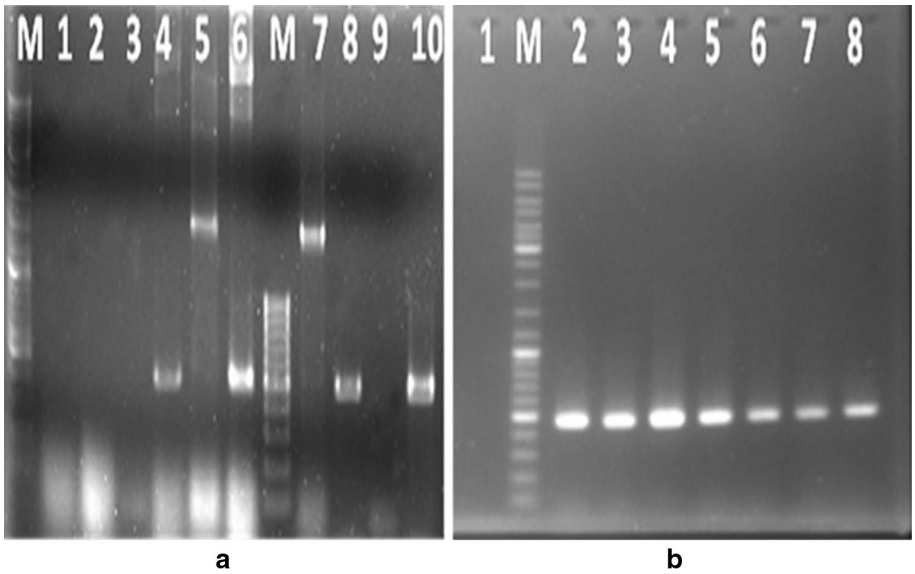

Fig. 2 Gel image of PCR confirmation of transformants. **a** Amplification for p1304-CaMV-BKT transformants using *bkt* primers: *lanes 1*, *3*, *5*, *7* and *9* were amplified using *bkt* primers (1.8 kb). Templates used are *lanes 1*, *2* Wild *D. salina*; *lanes 3*, *4* p1304; *lanes 5*, *6* p1304-CaMV-BKT; *lanes 7*, *8 D. salina* transformed with p1304-CaMV-BKT; *lanes 9*, *10 D. salina* transformed with p1304. **b** Amplification for p1304-RBB-BKT and p1304-RBS-BKT transformants using RBF and BKR primers: *lane M* 10 kb marker; *lane 1* Wild *D. salina*; *lanes 2–4* p1304-RBB-BKT; *lanes 5–8* p1304-RBS-BKT

p1304-RBS-BKT transformants. Lanes 3–6 contain bands of greater sizes than control which clearly indicated integration of T-DNA. Lane 7 is biotinylated marker of 10 kb size.

Expression profile of various carotenogenic genes in the transformants

The expression of various carotenogenic genes revealed that there was no significant upregulation in the initial genes viz: PSY, PDS and LCY of the carotenoid pathway in control as well as transformants. The expression of BKT and CHY was higher in transformants with RBCS promoter constructs, particularly with the RBCS deletion construct. There is an upregulation in the endogenous hydroxylase level in the transformants, where the BKT expression was higher. It is also notable that among all the carotenoid genes, upregulation of BKT and CHY was significantly higher only in nutrient-limiting conditions (Fig. 4). Wild and p1304 transformed *D. salina* was showing similar expression profile. In transformants with p1304-CaMV-BKT construct, although expression of BKT was observed in lower amounts, no subsequent increase in CHY level was noted.

Relative band intensities revealed that expression of BKT is 2 and 3 fold higher in De Walnes and nutrient-limiting media when compared to MAS100 medium. Expression of all carotenoid genes was compared taking β-actin as the standard. rRNA is also presented in the gel for comparison.

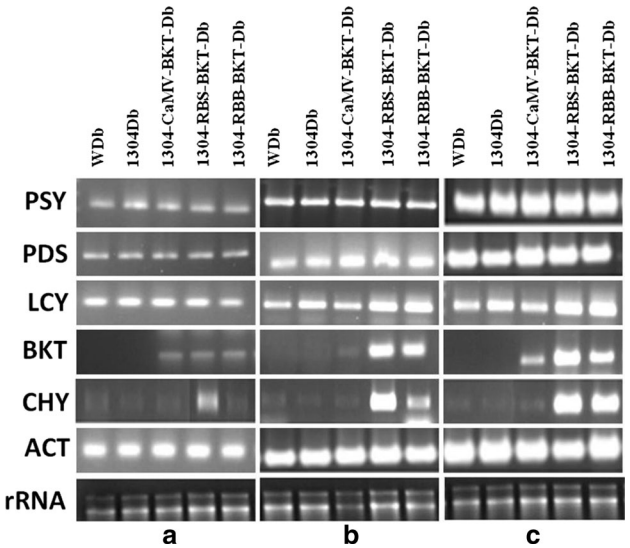

Fig. 4 Expression profile of various carotenoid genes in wild and transformant. *D. salina* in control and nutrient-limiting conditions. Various media used are MAS100 (**a**), De Walnes (**b**) and MAS100 with 1/4 strength phosphate and nitrate (**c**). rRNA is presented as the control. (*PSY* phytoene synthase, *PDS* phytoene desaturase, *LCY* lycopene cyclase, *BKT* β-carotene ketolase, *CHY* β-carotene hydroxylase, *ACT* β-actin)

Northern blot analysis of *bkt* gene

Total RNA from all BKT transformant constructs (p1304-CaMV-BKT, p1304-RBB-BKT and p1304-RBS-BKT) were used for northern analysis (Fig. 5). Transformants with p1304-RBS-BKT construct showed the highest expression levels of *bkt*, while transformants with p1304-CaMV-BKT had low expression levels.

Carotenoid profiling of the transformants

TLC analysis of carotenoid pigments extracted from transformed and control *D. salina* showed the presence of astaxanthin in p1304-RBS-BKT (lane 4, Fig. 6) and p1304-RBB-BKT (lane 5, Fig. 6) transformants. The concentration of astaxanthin was higher in p1304-RBS-BKT transformant when compared to p1304-RBB-BKT. Astaxanthin was absent in wild *D. salina* and transformants with p1304 and p1304-CaMV-BKT constructs.

HPLC analysis showed the presence of astaxanthin and canthaxanthin in transformed *D. salina* (Fig. 7) in addition to the other carotenoids. HPLC analysis revealed that in wild and transformed *D. salina*, lutein and β-carotene were the major carotenoids. Additionally, peaks of astaxanthin and canthaxanthin were detected in p1304-RBB-BKT and p1304-RBS-BKT transformants (Fig. 7). Estimation of carotenoids in transformants indicates that the highest concentration of astaxanthin is found in p1304-RBS-BKT transformants followed by p1304-RBB-BKT transformants (Table 2). PDA spectrum of peaks 1 and 4 was similar to that of standards confirming the presence of astaxanthin and canthaxanthin, respectively (Fig. S2). Astaxanthin and canthaxanthin were completely absent in p1304-CaMV-BKT transformants. Zeaxanthin content in transformants (p1304-RBB-BKT and p1304-RBS-BKT) was lower than the control (Table 2). Carotenoid profile was similar in wild, p1304 and p1304-CaMV-BKT transformants. The

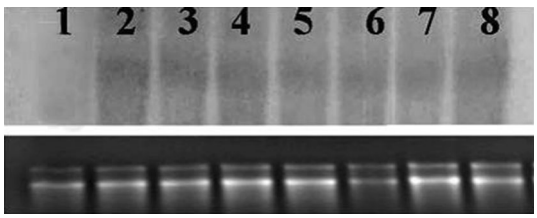

Fig. 5 Northern blot analysis with *bkt* probe in wild (*lane 1*) and transformed. *D. salina* (*lanes 2–8*). *Lanes 2–4* are p1304-RBS-BKT transformants; *lanes 5–6* are p1304-CaMV-BKT transformants and *lane 7–8* are p1304-RBB-BKT transformants. *Lower panel* shows RNA loading control

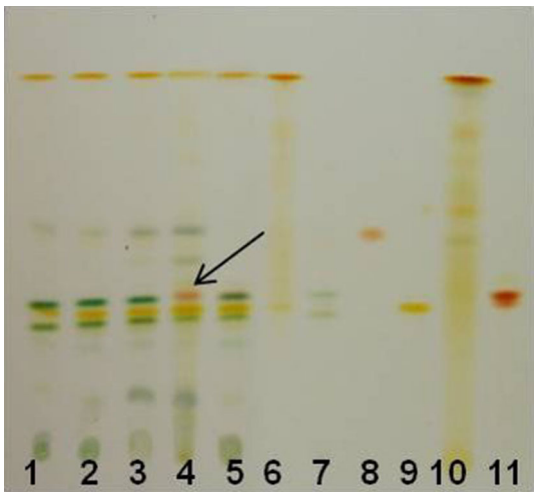

Fig. 6 TLC analysis of carotenoids extracted from wild and transformed *D. salina*. *Lane 1* Wild *D. salina*; *lane 2 D. salina p1304*; *lane 3 D. salina* (p1304-CaMV-BKT); *lane 4 D. salina* (p1304-RBS-BKT); *lane 5* D. *salina* (p1304-RBB-BKT); *lane 6 H. pluvialis* (astaxanthin accumulation stage); *lane 7 H. pluvialis* (growth stage); *lane 8* Canthaxanthin; *lane 9* Lutein; *lane 10* β-carotene; *lane 11* Astaxanthin (*arrow* in *lane 4* indicates astaxanthin band)

other carotenoids such as β-cryptoxanthin, violaxanthin and neoxanthin as analysed by C30 column showed no variation in both control and transformants (data not shown). Maximum content of astaxanthin observed in p1304-RBS-BKT transformant is 3.5 μg g^{-1} dry weight of biomass (Table 2).

Mass spectrometry in APCI positive mode

The carotenoid extract from p1304-RBB-BKT and p1304-RBS-BKT transformants grown in nutrient-limiting medium showed peaks 2 and 3 which are $[M+H]^+$ ions of canthaxanthin and astaxanthin, respectively (Fig. 8). Peak 1(β-carotene) was present in wild and transformed *D. salina*. The mass of canthaxanthin, astaxanthin and β-carotene were compared with that of standards.

Discussion

Metabolic engineering is a new approach for the understanding and utilization of metabolic processes. Astaxanthin is one among the major carotenoids being explored through this approach due its high demand. In the present study, metabolic engineering of *D. salina* was carried out to alter its carotenoid biosynthetic pathway for the production of ketocarotenoids through the introduction of β-carotene ketolase (*bkt*) gene from *H. pluvialis*.

Astaxanthin synthesis in *H. pluvialis* follows the pathway exhibited by marine bacteria. In *H. pluvialis*, β-carotene ketolase (BKT) is the major enzyme involved in the production of ketocarotenoids including astaxanthin by catalysing the conversion of β-carotene to echinenone initially and then to canthaxanthin. Further, the pathway proceeds with the conversion of canthaxanthin to astaxanthin by the use of β-carotene hydroxylase (BKH)

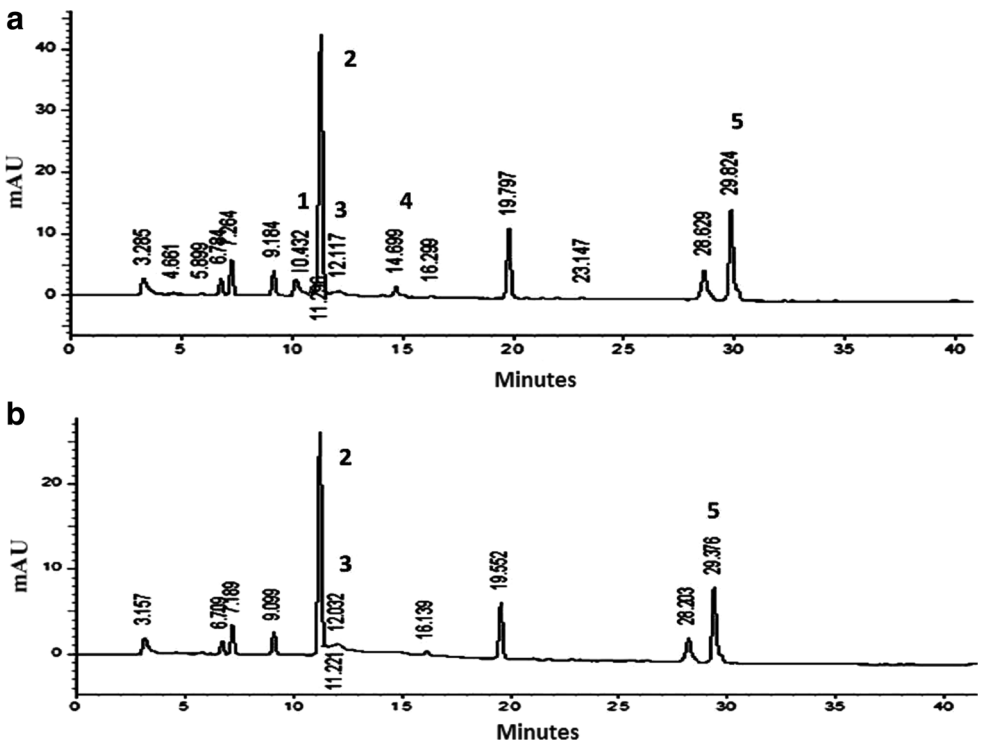

Fig. 7 HPLC separation of carotenoids from wild (**a**) and p1304-RBS-BKT (**b**) transformed *D. salina*. Astaxanthin (peak *1*), lutein (peak *2*), zeaxanthin (peak *3*), canthaxanthin (peak *4*) and β-carotene (peak *5*) are marked

Table 2 Carotenoid content of wild and *bkt*-transformed *D. salina*

D. salina	Lutein[a]		Zeaxanthin		β-carotene		Astaxanthin		Canthaxanthin	
	AS	NL	AS	NL	AS	NL	AS	NL	AS	NL
Wild	2400	1400	5.9	4.4	300	810	0	0	0	0
p1304	2230	1080	5.5	4.2	320	890	0	0	0	0
p1304-CaMV-BKT	2100	700	4.9	4.1	334	795	0	0	0	0
p1304-RBS-BKT	1841	640	2.5	2.3	349	514	0	3.5	0	1.9
p1304-RBB-BKT	1912	782	3.5	3.0	310	575	0	1.4	0	0.5

[a] Contents are expressed in μg/g on dry weight basis and each value is the average values from three transformant lines. *AS* MAS100 medium, *NL* nutrient-limiting medium with 1/4 strength phosphate and nitrate content

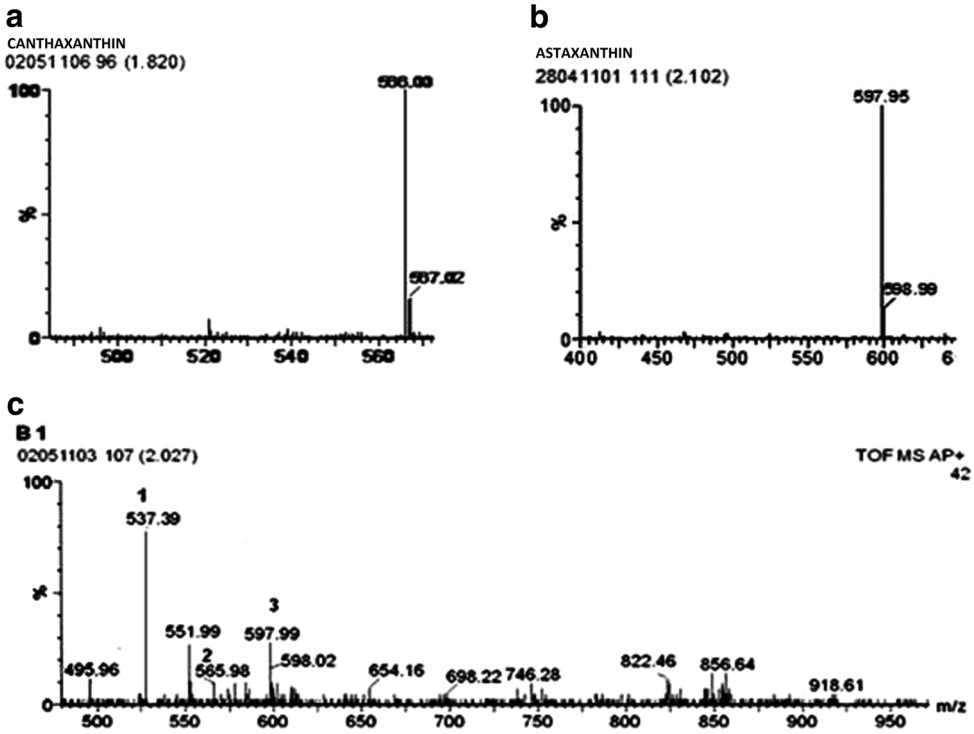

Fig. 8 MS analysis carotenoid extract from transformants in APCI positive mode. **a**, **b** is the spectra of canthaxanthin and astaxanthin, respectively. **c** is the MS spectra of carotenoids extracted from p1304-RBS-BKT transformants. Peaks *1*, *2* and *3* denote β-carotene, canthaxanthin and astaxanthin, respectively

(Hirschberg 2001). Many of the higher plants and algae lack *bkt,* while hydroxy derivatives of carotenoids are present in most of them. Therefore, expression of BKT is the preferred method for astaxanthin production in most of the transgenic systems.

Light is a major environmental factor favouring β-carotene accumulation in *Dunaliella* which is also the precursor for ketocarotenoid formation and substrate for *bkt*. In *Dunaliella*, however, β-carotene accumulation is observed in interthylakoidal membranes of the chloroplast and thus it became imperative to target BKT to chloroplast by the addition of a chloroplast transit peptide preceding the *bkt* gene in the present study. A similar approach was also made in transgenic Carrot, *Lotus japonicus*, *Chlamydomonas*, Tobacco, etc. where *bkt* was targeted to chromoplasts using chloroplast transit peptide for ketocarotenoids production (Jayaraj et al. 2008; Suzuki et al. 2007; León et al. 2007; Mann et al. 2000). To validate the location of β-carotene accumulation, p1304-CaMV-BKT which lacks a signal peptide was kept as a control during transformation in the present study. It was observed that even though *bkt* expression was present, ketocarotenoid formation was completely absent in p1304-CaMV-BKT transformants. Mulders et al. (2014) have reviewed the possible mechanism of involving the channelling of β-carotene to cytoplasm as a sink to increase the rate of β-carotene formation and subsequent ketocarotenoid formation in microalgae. However, as in *Dunaliella* β-carotene formation is strictly restricted to inter thylakoid membrane space, the targeting of *bkt* to chloroplast was irremissible.

The ability of the *Dunaliella* RBCS2 promoter regulatory regions for driving expression of the *ble* selectable marker gene was assessed in *C. reinhardtii*, in which they also carried out a promoter deletion analysis to determine

the optimal promoter length (Walker et al. 2005a, b). The work also reported that optimal promoter length for maximum expression from the *D. tertiolecta* RBCS1 was 300 bp based on which a deletion construct (p1304-RBS-BKT) was also prepared in the present study along with the full length promoter (p1304-RBB-BKT). Highest production was reported in p1304-RBS-BKT transformants. In the present study, RBCS2 promoter region from *D. salina* was used in two different lengths of 1.25 and 0.45 kb for expression of *bkt*. Both promoter constructs were followed by a 150 bp region of chloroplast transit peptide of RBCS2 which just preceded start codon of *bkt*. The deletion construct of RBCS2 promoter with 300 bp from *D. tertiolecta* was previously reported to drive expression 4.5-fold higher than full length promoter (Walker et al. 2005a, b). The deletion construct of RBCS2 with a promoter region of 450 bp was made use in the p1304-RBS-BKT construct which gave threefold higher production of astaxanthin. The ketocarotenoid production in the transformants accounted to a maximum of 3 % of total carotenoids in the present study. Total carotenoid content of wild and transformed *D. salina* remained almost same in the present study. A similar observation was also made in transformed *L. japonicus*, where low ketocarotenoids accumulating transgenic lines (3–15 %) had no significant increase in total carotenoid content (Suzuki et al. 2007).

The present study used the genomic clone of *bkt2* from *H. pluvialis*. Since the ketolases known to date prefer an unsubstituted β-ionone ring, astaxanthin synthesis is possible only when ketolase concentration in transgenic plants is significantly higher than the endogenous hydroxylase level (Zhu et al. 2007). Various *bkt* genes have different degrees of affinity for substrates, such as β-carotene and zeaxanthin. In transgenic *A. thaliana,* it was shown that only ketolases with high activity of converting zeaxanthin to astaxanthin trigger accumulation of large amounts of astaxanthin (Zhong et al. 2011). They compared the efficiencies of different *bkt* from *Chlamydomonas reinhardtii*, *Chlorella zofingiensis* and *Haematococcus pluvialis*, and observed that *bkt* from *H. pluvialis* which belongs to *bkt3* category is least efficient in ketolating zeaxanthin. Unlike other ketolases which prefer only β-carotene as the substrate, *bkt2* is reported to utilize both β-carotene and its 3-hydroxy β-carotene derivative zeaxanthin as the substrate (Kajiwara et al. 1995). In the present study, *bkt2* was used and substantiating previous reports, the activity of *bkt2* was evidenced in the formation of canthaxanthin as well as in the conversion of zeaxanthin to astaxanthin. There was a significant decrease in zeaxanthin content of transformants when compared to the control suggesting that astaxanthin formation is possibly through zeaxanthin pathway. Further intermediates in the canthaxanthin pathway viz adonirubin were absent in the transformants which indicate that intrinsic hydroxylases do not have substrate specificity towards canthaxanthin.

The present study is the first report in *Dunaliella* for manipulation of carotenoid pathway. Except in C. *reinhardtii*, all other studies have reported astaxanthin production in transformants. It is evident from the HPLC and MS results that *bkt2* used in the present study is capable of converting the β-ionone rings of β-carotene into 4(4′)-keto β-ionone rings to produce canthaxanthin probably via echinenone as well as in the formation of astaxanthin via the ketolation of zeaxanthin. PDA spectrum of the new peaks in transformants clearly confirmed the production of canthaxanthin and astaxanthin. Lutein levels in transformants were lower when compared to wild types as in previous studies (Huang et al. 2013). They also reported lutein as a global down regulator of carotenogenesis. TLC analysis also confirmed the production of astaxanthin. Although canthaxanthin was detectable through HPLC analysis, the intermediate echinenone was not observed in the present transformants due to low production of ketocarotenoids. However, zeaxanthin content in the transformants was significantly lower when compared to control indicating the formation of astaxanthin via zeaxanthin. It is noteworthy that transformants were stable and gave a similar HPLC pattern even after a period of 4 years.

H. pluvialis bkt2 gene was previously expressed in *A. thaliana* seeds and 4-ketolutein, adonirubin, and canthaxanthin (8 % of total carotenoids) was observed as the major keto derivatives rather than astaxanthin (Stalberg et al. 2003). Even though *bkt2* is reported to ketolase zeaxanthin, the low substrate specificity to use hydroxylated carotenoids may be the reason for their reduced astaxanthin conversion activity (Misawa et al. 1995a, b). In addition, the endogenous hydroxylases compete with the transgene for β-carotene to produce zeaxanthin. In the present study, *bkt2* acts both as a carotene ketolase and zeaxanthin ketolase, which effectively reduced the concentration of final product, astaxanthin. It is previously reported that only ketolases with high activity of converting zeaxanthin to astaxanthin trigger transgenic *Arabidopsis* to accumulate large amounts of astaxanthin. Astaxanthin formed in the present study may be produced by the ketolation of zeaxanthin rather than β-carotene as evidenced by the decrease in zeaxanthin content in the transformants (Table 2). Efficient ketolation of zeaxanthin (Zhong et al. 2011) or simultaneous expression of an additional carotenoid hydroxylase gene along with the introduced *bkt* (Ralley et al. 2004) are the alternate approaches to further increase the astaxanthin content. It was also suggested that the combination of enzymes used in pathway engineering plays a crucial role in high-yield production of astaxanthin in plants as the key enzymes BKT and BKH compete for single substrate, i.e. β-carotene (Huang et al. 2013).

Mann et al. (2000) have reported that the β-carotene ketolase can function in conjunction with the intrinsic β-carotene hydroxylase, which adds hydroxyls at C3 and C3′, to produce astaxanthin. Chloroplasts exhibit high BKH activity and therefore are rich in hydroxyl carotenoids. The upregulation of endogenous β-carotene hydroxylase in the presence of β-carotene ketolase was also reported by Jayaraj et al. (2008) in carrot transformants with *bkt* gene. Corroborating the previous reports, a distinct upregulation of β-carotene hydroxylase up to 2–3 fold was observed in p1304-RBB-BKT and p1304-RBS-BKT transformants when compared to wild *D. Salina* (Fig. 4).

Stress conditions including culture conditions and media constituents are reported to have effect on carotenogenesis in *Dunaliella*. In *Dunaliella*, combination of stress factors leads to a higher accumulation of β-carotene than when the cells experience a single stress (Ben Amotz and Avron 1983). A no. of stress conditions like high salinity (Arun and Singh 2013), high irradiance (Lamers et al. 2010), nitrate starvation (Lamers et al. 2012), high salinity and irradiance stress (Ben Amotz and Avron 1983) and nitrate and sulphate starvations (Becker 2004; Dipak and Lele 2005) were reported to increase the β-carotene content in *Dunaliella*. The steady-state levels of transcripts encoding phytoene synthase and phytoene desaturase were reported to increase substantially in *D. salina* cells when shifted to high light or salt under nutrient-limiting conditions. Among the stress conditions, nutrient limitation was the main regulatory factor for carotenoid accumulation in *D. salina* (Coesel et al. 2008). Substantiating the previous reports carotenogenesis in the present study was carried out under multiple stress conditions of continuous light and nutrient stress. An upregulation of all the carotenoid genes was observed in nutrient-limiting MAS100 medium when compared to MAS100 and De Walne's media (Fig. 4). The salinity levels in MAS100 and De Walne's media were found to be 50 and 37 ppt, respectively. The cumulative stress effect of nutrient limitation with higher salinity could be the possible reason for higher expression of carotenoid genes in nutrient-limiting MAS100 medium resulting in the production of astaxanthin and canthaxanthin. A similar approach was carried out in *H. pluvialis* transformants where they used salinity stress conditions for enhanced astaxanthin production (Kathiresan et al. 2015).

The present study proved the functionality of cloned *bkt2* in *D. salina* as evidenced by the production of canthaxanthin and formation of astaxanthin. The transformants were analysed by various molecular methods like PCR, southern blot, northern blot etc. through which the integration of *bkt* in transformants was confirmed. The probe used in southern blot analysis of transformants with rubisco transformants contained both RBCS2 region and BKT region from the vector p1304-RBB-BKT to avoid false hybridisation as RBCS2 region is also present in wild *Dunaliella*. The banding pattern of transformants was distinct and different from wild and control plasmid.

Dunaliella is well known for accumulation of high β-carotene under stress conditions like high light and high salinity. Hence it is suggested as a suitable model system for genetic manipulations of carotenogenesis by expression of *bkt* (León et al. 2007). Although the system proved to be feasible for the production of astaxanthin, low production of astaxanthin may be attributed to the low light intensity provided during the experiment. Irradiance stress is the major factor contributing to the over production of β-carotene in *Dunaliella*. At low light intensity, only nitrogen starvation is reported to cause over production of β-carotene in a bioreactor (Lamers et al. 2010). When compared with the massive accumulation of β-carotene in *Dunaliella*, it is evident that the second stage of β-carotene accumulation was not induced in the transformants. The minor increase in the carotenoid content is because of photoacclimation rather than over production of carotenoids (Mulders et al. 2014; Lamers et al. 2008). Extensive studies are therefore required under high β-carotene producing conditions in *Dunaliella* to optimize the production of astaxanthin in present transformants. The present study is the first report in genetic manipulation of carotenoid biosynthetic pathway in *D. salina* for the production of ketocarotenoids including astaxanthin. The study has clearly demonstrated the feasibility of astaxanthin production in *Dunaliella* transformants which were transformed using *bkt* from *H. pluvialis*. The present study may be thus considered as a beginning for utilization of *D. salina* for the production of valuable carotenoids through metabolic engineering of carotenoid biosynthetic pathway.

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