Biotechnol Lett (2011) 33:313–319
DOI 10.1007/s10529-010-0422-6

ORIGINAL RESEARCH PAPER

High versus low level expression of the lycopene biosynthesis genes from *Pantoea ananatis* in *Escherichia coli*

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Received: 31 August 2010 / Accepted: 17 September 2010 / Published online: 30 September 2010

Abstract The heterologous synthesis of lycopene in non-carotenogenic *Escherichia coli* required the introduction of the biosynthesis genes *crtE*, *crtB*, and *crtI*. Recombinant *E. coli* strains, expressing each lycopene biosynthesis gene from *Pantoea ananatis* using multi-copy plasmid or single-copies after stable chromosomal integration, were cultivated and the formation of lycopene was investigated. The different expression conditions significantly influenced the lycopene formation as well as the growth behaviour. High plasmid expression levels of *crtI* with a single copy background of *crtE* and *crtB* in *E. coli* led to a predominate synthesis of tetradehydrolycopene at 253 μg g^{-1} (cdw).

Keywords Chromosomal integration · Heterologous carotenoid production · Lycopene · Tetradehydrolycopene

Introduction

Carotenoids are ubiquitous with important nutritional and physiological relevance for plants, algae and bacteria. Carotenoids have gained industrial importance as colorant for cosmetic-, food-, and animal feed-products. Recent reports on carotenoids have further demonstrated that carotenoids have manifold beneficial effects on human health (Nagao 2009). The simplest C_{40} carotenoid, lycopene, is an effective antioxidant and has promising biological activity for the prevention of several types of cancer, including breast cancer and prostate cancer (Seren et al. 2008). The genes responsible for the synthesis of lycopene have been isolated from the epiphytic bacterium, *Erwinia,* and plants, and the functions of the carotenogenic genes have been identified (Hundle et al. 1991; Misawa et al. 1990; To et al. 1994; Albrecht et al. 1995; Sandmann 1994). The first substrate of the enzymes encoded by the *Erwinia* carotenoid biosynthesis gene cluster is farnesyl pyrophosphate (FPP) which is the common precursor for the biosynthesis of carotenoids and also of numerous other isoprenoids. The biosynthesis of lycopene in *E. coli* requires the expression of the genes *crtE*, *crtB*, and *crtI*, which utilize the *E. coli* isoprenoid precursor FPP. CrtE is a geranylgeranyl synthase that catalyzed the condensation of FPP and IPP to GGPP and CrtB is a phytoene synthase (Sandmann and Misawa 1992). The phytoene desaturase (CrtI) catalyzed the introduction of double bonds leading to an extended conjugated double bond system. The insertion of four double bonds into phytoene by CrtI from *Pantoea ananatis* (formerly *Erwinia uredovora*) results in the formation of lycopene (Sandmann 2009).

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Due to the increasing demand for carotenoids, improvements have been made in the formation of carotenoids by recombinant bacteria, yeasts, and plants expressing carotenoid biosynthesis genes or gene clusters (Das et al. 2007). In particular, the non-carotenogenic *E. coli* has been widely used as a host for improved carotenoid productivity by transformation with appropriate biosynthesis genes, and methods were developed for increasing the biosynthesis of isoprenoid precursors in *E. coli* (Harada and Misawa 2009).

In this study, the formation of lycopene by heterologous *E. coli* strains expressing each biosynthesis gene from *P. ananatis* either from a multi-copy plasmid or from single copy inserted into the chromosome is presented.

Materials and methods

Bacterial strains, growth conditions, and media

The bacterial strains used were *Escherichia coli* BW25113 lac$^+$ and *E. coli* DH5α (Table 1). If not stated otherwise, they were grown at 37°C in LB medium. Antibiotics used were: 100 μg ampicillin/ml, 50 μg chloramphenicol/ml and 50 μg kanamycin/ml. Difco MacConkey agar was purchased from Nordwald (Hamburg, Germany). Carotenoid standards were provided by the Institute of Technical Biochemistry, University of Stuttgart. All other chemical and reagents are from Sigma-Aldrich or Roth.

Cloning of the biosynthesis genes in *E. coli*

In order to create an artificial lycopene gene cluster for expression in *E. coli*, the following cloning strategy was used. Modified PCR primers were used to create a *Bgl*II restriction site upstream the start-codon of the gene, plus an artificial RBS and a *Bam*HI or a *Bgl*II site down-stream of the stop-codon. *crtB* was amplified from plasmid pCAR16 (Misawa et al. 1990) using the primers: GATGCTGGAAGAT CTCATATGAATAATCCGTCG and CTTCAGGC GAGGATCCGGGAAAGACATGG. The resulting PCR product was digested by the restriction enzymes *Bgl*II plus *Bam*HI and were ligated into the *Bam*HI-hydrolyzed pCAS30, resulting in plasmid pCAS56. *crtI* was amplified from plasmid pCAR16 using the primers: CATTGCAAGATCTTATGAATTCTCAT CGTTAAAGAGCGAC and CGTTTAGATCTCAT GATTGAGTAACGACGGATTATTCA. The resulting PCR product was digested by *Bgl*II was ligated into the *Bam*HI-hydrolyzed pCAS56, resulting in plasmid pCAS58. Restriction digestions and DNA sequencing were performed for all constructs to verify the orientations and sequences of the cloned biosynthesis genes. Takara PrimeStar DNA polymerase (Lonza, Belgium) was used for amplification. The chromosomal integration of recombinant *crt*-gene expression cassettes and elimination of antibiotic resistants gene cassettes using plasmid pCP20 (Cherepanov and Wackernagel 1995) were conduct as described by Albermann et al. (2010).

Cultivation of *E. coli* cultures and analysis of carotenoids

Recombinant, lycopene-producing *E. coli* strains were cultivated at 30°C in 50 ml LB medium. Plasmid carrying strains were cultivated in the presents of a corresponding antibiotic. IPTG was added at an OD_{600} of 0.3. Cultures with strains that carry plasmid pJOE5559 (unpublished work) were also induced with L-rhamnose at 1.2 mM. Samples (2 ml) were withdrawn from cultures after 24 h. The cells were harvested by centrifugation, washed with cold water, and subsequently extracted with acetone (500 μl) for 15 min at 50°C. Insoluble components of the extract were removed by centrifugation (20,000×*g*). HPLC analysis were performed on Dionex HPLC Instrument installed with a Chromeleon Software, Gina autosampler, P580 pumps, and detection with a UV lamp. Products were analyzed by loading 20 μl supernatant onto a C30-reverse-phase HPLC column (250 mm × 4.6 mm, 5 μm, YMC-Europa GmbH, Dinslaken, Germany) attached with a guard column containing matrix of same material as the column. The solvents used (at 1 ml/min) were A: *tert*-butyl ether (MTBE)/methanol/water (19/80/1, by vol.) and B: MTBE/methanol/water (90/9/1, by vol.). Gradient conditions: equilibration conditions at 60% B (v/v); 0 to 40 min linear gradient from 60% B to 100% B (v/v); 40 to 45 min 100% B (v/v). Standard curves correlating areas to concentration of lycopene and tetradehydrolycopene (TDHL) was used to determine the quantity of these carotenoids in the

Table 1 Strains and plasmids

Strains and plasmids	Relevant genotype or sequence	Source
E. coli DH5α	F^-, ϕ80d, *lacZ*ΔM15, *endA*1, *recA*1, *hsdR*17($r_K^- m_K^-$), *supE*44, *thi*-1, *gyrA*96, *relA*1, Δ(*lacZYA-argF*)U169	Laboratory strain
E. coli BW25113 *lac*$^+$	$rrnB_{T14}$, *hsdR*514, $\Delta araBAD_{AH33}$, $\Delta rhaBAD_{LD78}$	Albermann et al. (2010)
E. coli BW-EI	*E. coli* BW25113 *lac*$^+$, *malEFG::crtE, xylAB::crtI*	This study
E. coli BW-BE	*E. coli* BW25113 *lac*$^+$, *malEFG::crtE, fucIK ::crtB*	Albermann et al. (2010)
E. coli BW-BI	*E. coli* BW25113 *lac*$^+$, *xylAB::crtI, fucIK ::crtB*	This study
E. coli BW-Lyco	*E. coli* BW25113 *lac*$^+$, *malEFG::crtE, fucIK ::crtB, xylAB::crtI*	This study
pKD46	P_{araB} γ β exo (red recombinase), Amp^R	Datsenko and Wanner (2000)
pCP20	FLP^+, λ $cI857^+$, λ p_R Rep^{ts}, Amp^R, Cm^R	Cherepanov and Wackernagel (1995)
pCAR25	pUC19, *P. ananatis crtEXYIBZ* gene cluster	Misawa et al. (1990)
pJOE5559	pJeM1, P_{RhaBAD} -*dxs-idi-ispA*, Km^R	J. Altenbuchner, Universität Stuttgart (unpublished)
pJF119ΔN	cloning vector, RBS, P_{tac}, Amp^R	Albermann et al. (2008)
pCAS58	pJF119ΔN, *P. ananatis crtEBI* genes, Amp^R	This study
pCAS30	pJF119ΔN, *P. ananatis crtE* gene, Amp^R	Vallon et al. (2008)
pCAS30-FRT-*cat*-FRT	pJF119ΔN, *P. ananatis crtE* gene, FRT-sites, Amp^R, Cm^R	Vallon et al. (2008)
pJF119-crtB	pJF119ΔN, *P. ananatis crtB* gene, Amp^R	Albermann et al. (2010)
pJF119-crtB-FRT-*kan*-FRT	pJF119ΔN, *P. ananatis crtB* gene, FRT-sites, Amp^R, Km^R	Albermann et al. (2010)
pJF119-crtI	pJF119ΔN, *P. ananatis crtI* gene, Amp^R	Albermann et al. (2010)
pJF119-crtI-FRT-*cat*-FRT	pJF119ΔN, *P. ananatis crtI* gene, FRT-sites, Amp^R, Cm^R	Albermann et al. (2010)

samples. HPLC–MS analysis was carried out on a HPLC coupled to a UV-detector and a mass spectrometer (LCMS-2010EV/dual ion source DUIS2010, Shimadzu, Germany), using identical conditions as described above.

For the semi-preparative isolation of TDHL, *E. coli* BW-EB pJF-crtI was cultivated in 400 ml minimal medium (3 g KH_2PO_4 l^{-1}, 12 g K_2HPO_4 l^{-1}, 5 g $(NH_4)_2SO_4$ l^{-1}, 0.3 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 0.015 g $CaCl_2 \cdot 2H_2O$ l^{-1}, 0.1 g NaCl l^{-1}, 5 g Glucose monohydrate l^{-1}, 15 ml $FeSO_4 \cdot 7H_2O$/sodium citrate l^{-1} (from a solution of 7.5 g $FeSO_4 \cdot 7H_2O$ l^{-1} and 100 g sodium citrate l^{-1}), 7.5 mg thiamine l^{-1}, 33 ml trace elements l^{-1}) at 30°C. The culture was induced with 0.5 mM IPTG at OD_{600} of 0.3. After 24 h, the cells were harvested by centrifugation, washed with cold water, and subsequently extracted twice with 20 ml acetone by vigorous shaking. Insoluble components of the extract were removed by centrifugation (20,000×*g*). Acetone was removed under a stream of nitrogen and the residual material was dissolved in MTBE. TDHL was isolated by HPLC (several runs) using the same device as for analysis. The mobile phase was MTBE/methanol/water (90/9/1, by vol.) at 1.2 ml min^{-1} for 20 min isocratic; 200 µl as injection volume. TDHL fractions were collected, dried under a stream of nitrogen and subsequently dissolved in acetone. TDHL content in acetone solution was quantified by measuring the absorbance at 508 nm. Concentration of TDHL was determined using an extinction coefficient (5933 $(g/ml)^{-1}$ cm^{-1}, Unrean et al. 2010).

Results and discussion

To investigate the effect of single- and multi-copy expression of *crtE*, *crtB*, and *crtI* from *P. ananatis* on lycopene biosynthesis in *E. coli*, strains with all different combinations of two chromosomal and one plasmid-encoded *crt*-gene were constructed. The expression of each gene is controlled by an IPTG-

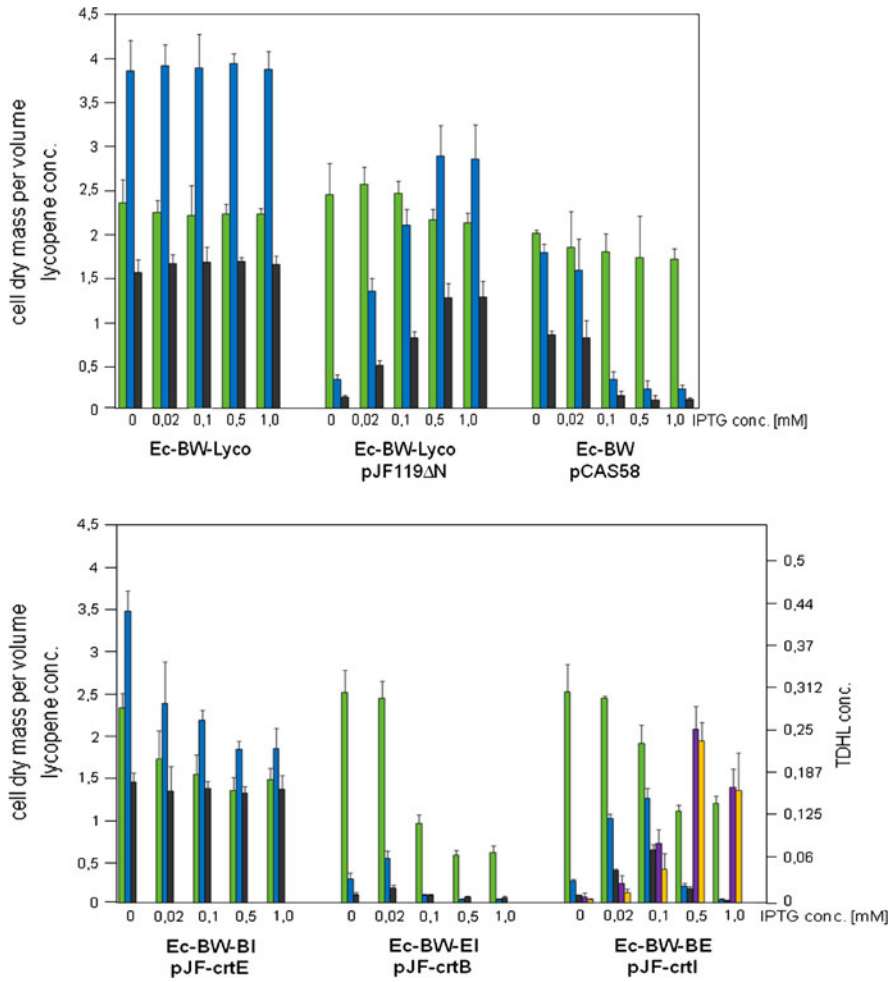


Fig. 1 Formation of lycopene and tetradehydrolycopene (TDHL) by recombinant *E. coli* strains upon different inductor (IPTG) concentration. *Green bars*: cellular dry weight per volume (g cdw l^{-1}); *blue bars*: lycopene concentration (mg l^{-1}); *black bars*: lycopene concentration (mg g^{-1} cdw); *purple bars*: TDHL concentration (mg l^{-1}); *yellow bars*: TDHL concentration (mg g^{-1} cdw). Values represent the average of $\geq$ 3 independent cultivations

inducible tac-promoter. The chromosomal insertion of the heterologous expression cassettes were conducted by a method described recently (Albermann et al. 2010). This method is based on the λ-Red-recombineering technique (Datsenko and Wanner 2000; Zhang et al. 1998). The method allows for a fast and accurate screening of the recombination event by differential MacConkey agar medium. The following strains were received after integration of two individual *crt*-expression cassettes into the chromosome of *E. coli* BW25113 lac^+: *E. coli* BW-EB, *E. coli* BW-EI, *E. coli* BW-BI (Table 1). To allow the biosynthesis of lycopene in these strains, each of the strains was transformed with the multi-copy expression vector pJF119ΔN carrying the respective *crt*-gene, so that each strain carries the heterologous genes *crtE*, *crtB*, and *crtI*, resulting in *E. coli* BW-EB pJF119-crtI, *E. coli* BW-EI pJF119-crtB, and *E. coli* BW-BI pJF119-crtE, respectively. These strains were cultivated in LB medium and the cultures were induced with different concentrations of IPTG (0–1 mM). *E. coli* BW-Lyco, that contains all three lycopene biosynthesis genes on the chromosome, *E. coli* BW-Lyco pJF119ΔN, and *E. coli* BW25113 lac^+ pJF119-crtEBI were used as strains for comparison.

The lycopene content per volume as well as per g cell dry wt are given in Fig. 1. *E. coli* BW-Lyco, which expresses *crtE*, *crtB*, and *crtI* from individual chromosomal copies, had the highest lycopene formation (3.9 ± 0.1 mg l^{-1}; 1.65 ± 0.12 mg g^{-1} cdw). The enhanced expression of *dxs*, *idi*, and *ispA* by *E. coli* BW-Lyco pJOE5559 resulted in the formation of 8.72 ± 0.21 mg l^{-1}, 4.45 ± 0.32 mg g^{-1} cdw lycopene showing that the enzyme activities of CrtE, CrtB, and CrtI expressed by single chromosomal gene copies

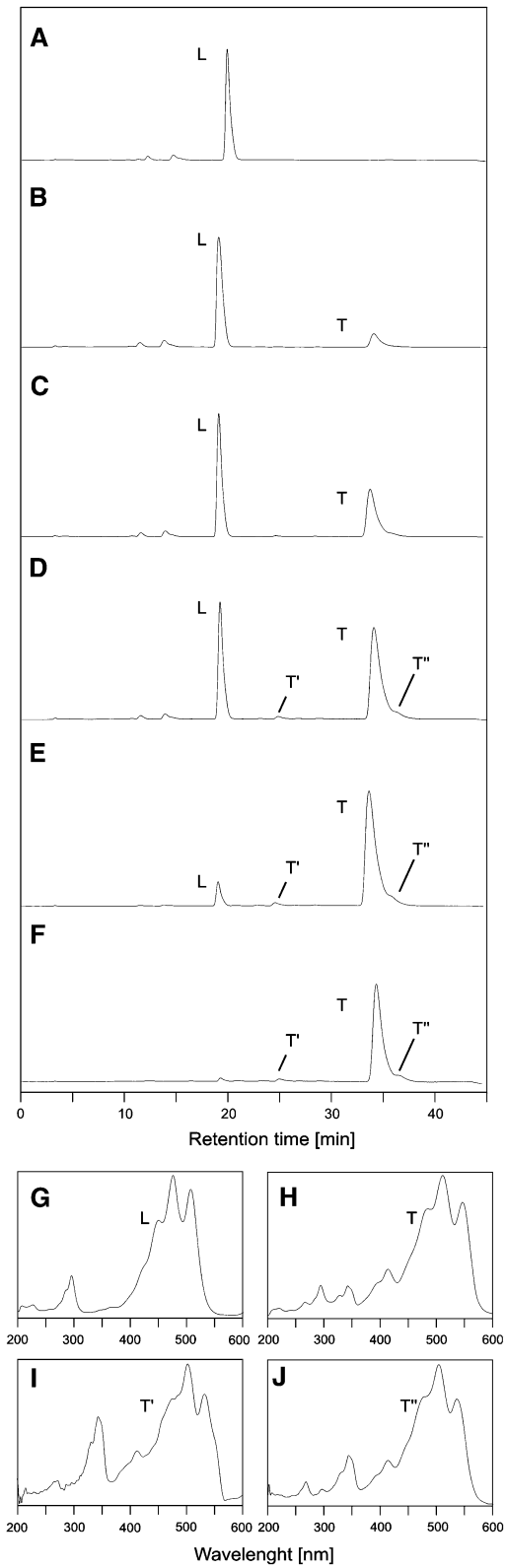
is sufficient for the conversion of FPP to lycopene even under conditions with increased supply of isoprenoid precursors. The induction of these strains with IPTG had no significant effect either on growth or on lycopene formation. *E. coli* BW-Lyco pJF119ΔN, carrying an additional *lacI* gene on the plasmid, showed an increase in lycopene formation after increasing the inducer concentration showing that the expression of the chromosomal *lacI* is not sufficient to repress the tac-promoter under the given conditions. A higher LacI concentration, as given in *E. coli* BW-Lyco pJF119ΔN, led to an IPTG-inducible expression of the heterologous genes.

Expression of lycopene biosynthesis genes by an artificial, plasmid encoded *crtEBI*-operon (*E. coli* BW pCAS58) yielded 1.77 ± 0.23 mg l^{-1}, 0.84 ± 0.11 mg g^{-1} (cdw) lycopene without induction. The amount of lycopene decreased upon induction by IPTG. The plasmid expression of *crtE* by *E. coli* BW-BI pJF119-crtE under different inducer concentrations led to a similar amount of lycopene per cell (1.35 ± 0.08 mg g^{-1} cdw) but the total biomass decreased due to the high expression of *crtE*. The higher lycopene content in *E. coli* BW-BI pJF119-crtE compared to *E. coli* BW-Lyco pJF119ΔN in cultures without induction indicates that the CrtE catalyzed reaction is the most limiting step in the conversion of FPP to lycopene. The *crtB* plasmid expression by *E. coli* BW-EI pJF119-crtB led to a decrease in growth as well as on lycopene formation. The CrtB product phytoene was not detected in the cell extract. Growth retardation may occur due to the interference of CrtB with the formation of prenylquinones in *E. coli* (Neudert et al. 1998), but the low biomass and lycopene amount per cell in the stationary phase (after 24 h) in induced cells (>0.1 mM IPTG) suggests that the CrtB protein also being toxic or a protein burden to *E. coli*. This is also supported by the observation that *E. coli* BW-EI pJF119-crtB pJOE5559, which showed increased formation of FPP by the additional copies of *dxs*, *idi*, and *ispA*, had reduced growth under high *crtB* expression conditions (data not shown).

A further effect on the plasmid overexpression of a single *crt*-gene was observed by the expression of *crtI*. Cultivation of *E. coli* BW-BE pJF119-crtI yielded, beside lycopene, predominantly in the formation of 3,4,3′,4′-tetradehydrolycopene (Fig. 2), which was identified by LC–MS analysis (M^+ 532) and UV/visible spectroscopy (Fig. 2h). For the quantification of TDHL, *E. coli* BW-BE pJF119-crtI was cultivated in minimal medium and the extracted TDHL was isolated by semi-preparative HPLC (Fig. 2f). The concentration of TDHL in acetone was determined by photospectroscopy [extinction coefficient 5933 $(g/100\ ml)^{-1}$ cm^{-1}, Unrean et al. 2010]. The amount of TDHL in LB cultures of *E. coli* BW-BE pJF119-crtI increased upon IPTG induction (Figs. 1, 2). The highest TDHL concentration was after induction with 0.5 mM IPTG (253 ± 33 μg g^{-1} cdw, 59% of all carotenoids). Induction with 1 mM IPTG gave the highest percentage of TDHL with 80% (169 ± 27 μg g^{-1} cdw). Extracts of cultures with a high TDHL content as well as the purified TDHL samples also reveal the existence of two further isomeric forms of TDHL (M^+ 532) as indicated in Fig. 2. The formation of TDHL was not observed in any of the other tested lycopene producing strains, in which *crtI* is expressed from single copy or by the plasmid encoded gene cluster (pCAS58).

For the CrtI-type phytoene-desaturase from *P. ananatis* and other organisms, the general ability to carry out up to six desaturation reactions on phytoene has been shown (Linden et al. 1991; Sandmann 2009). Additionally, the ratio of substrate and desaturase can further define the degree of phytoene desaturation (Stickforth and Sandmann 2007). Sandmann and co-workers used a two plasmid system for the differentiated expression of *crtEB* and *crtI* in *E. coli*, which resulted in TDHL at approx. >10% of all carotenoids. Here, the reported combination of single-copy (*crtE*, *crtB*) and multi-copy (*crtI*) expression led to a TDHL portion of up to 80% of the *E. coli* synthesized carotenoids which verifies that the kinetics of CrtI from *P. ananatis* can determine the degree of desaturation. By the use of directed evolution methods a CrtI-mutant was generated that led, compared to the wild type enzyme, to a partly conversion of lycopene to TDHL in *E. coli* (Schmidt-Dannert et al. 2000). Due to the lack of biochemical data it is not yet clarified if the mutein has an increased catalytic activity or if the amino acid exchanges lead to a higher protein overproduction or stability that caused the formation of TDHL.

This report demonstrates that the expression level of heterologous biosynthesis pathways may have a significant impact on the product yield as well as on



◀ **Fig. 2** HPLC-analysis of carotenoids in extracts from recombinant *E. coli* strains after cultivation for 24 h in LB medium. **a** *E. coli* BW-Lyco, **b** *E. coli* BW-BE pJF119-crtI w/o IPTG, **c** *E. coli* BW-BE pJF119-crtI + 0,05 mM IPTG, **d** *E. coli* BW-BE pJF119-crtI + 0,2 mM IPTG, **e** *E. coli* BW-BE pJF119-crtI + 1 mM IPTG. (*f*) HPLC analysis of TDHL after purification. UV/Vis spectrum of lycopene **g**, of TDHL **h**, of TDHL isomer T′ **i**, of TDHL isomer T″ (*j*). *L* lycopene, *T* tetradehydrolycopene

product specificity. The expression of single chromosomal copies of either *crtE*, *crtB* or *crtI* leads to an about 90% higher yield of lycopene compared to the plasmid expressed gene cluster. *E. coli* BW-BE pJF119-crtI produced TDHL as the predominant carotenoid, due to the high expression of *crtI*, which opens the opportunity for a preparative synthesis of this rare carotenoid.

In conclusion, this report demonstrates that an efficient heterologous in vivo biosynthesis of carotenoid compounds requires an adapted expression of the heterologous genes to avoid metabolic burden effects and unspecific side reactions.

Acknowledgments The author is grateful to Holger Beuttler (Universität Stuttgart) for LC–MS measurements and for providing carotenoid standards. The author thanks Josef Altenbuchner (Universität Stuttgart) for providing plasmid pJOE5559. This work was supported by the Deutsche Forschungsgemeinschaft (SFB706, TP B3).

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