Journal of Biotechnology 140 (2009) 227–233

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Investigation of cellular targeting of carotenoid pathway enzymes in *Pichia pastoris*

Pyung Cheon Lee [a,b,*], Young geol Yoon [c], Claudia Schmidt-Dannert [d]

[a] *Department of Molecular Science and Technology, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, South Korea*
[b] *Department of Biotechnology, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, South Korea*
[c] *Department of Laboratory Medicine and Pathology and Institute of Human Genetics, University of Minnesota, 420 Delaware St SE, Minneapolis, MN 55455, United States*
[d] *Department of Biochemistry, Molecular Biology and Biophysics, 1479 Gortner Avenue, University of Minnesota, St. Paul, MN 55108, United States*

ARTICLE INFO

Article history:
Received 5 August 2008
Accepted 21 January 2009

Keywords:
Peroxisome-targeting signal
Pichia pastoris
Carotenoid biosynthetic pathway
Metabolic engineering

ABSTRACT

Cellular targeting of lycopene biosynthetic enzymes was investigated in *Pichia pastoris* X-33. Three lycopene pathway enzymes, CrtE, CrtB, and CrtI, were fused to fluorescent EGFPs with or without a peroxisomal targeting sequence (PTS1) and then expressed in *P. pastoris*. When *P. pastoris* was grown in YPD, the PTS1 fusion enzymes were found to be localized in peroxisomes, whereas the enzymes not fused with PTS1 were equally distributed throughout the entire cell. A similar targeting pattern was also observed in *P. pastoris* strains that were grown in peroxisome-proliferating medium, YPOT. Analysis of the fluorescent images of isolated peroxisomes showed that the PTS1 fused enzymes were dominantly present in peroxisomes whereas small amount of the enzymes not fused with PTS1 were non-specifically sent to peroxisomes. These results indicate that PTS1 specifically target lycopene pathway enzymes into peroxisomes and this targeting pathway was strong enough to overcome their inherent targeting program. In conclusion, we first showed that carotenogenic enzymes can be targeted into the specific cellular location of recombinant hosts and this targeting strategy can serve as the basis for the subsequent development of sophisticated pathway engineering in microorganisms.

1. Introduction

Carotenoids, derivatives of isoprenoids, belong to an important class of natural pigments used in biotechnology applications (Lee et al., 2003). These structurally diverse pigment chemicals have different biological functions including coloration, photoprotection, light-harvesting and precursors for many hormones (Sandmann, 2001; Vershinin, 1999). Currently carotenoids are commercially used as food colorants, animal feed supplements, and more recently, as nutraceuticals, and for cosmetic and pharmaceutical purposes. However, only a few carotenoids can be produced commercially by chemical synthesis, fermentation, or isolation from a few abundant natural sources (Johnson and Schroeder, 1995). The increasing industrial importance of carotenoids has led to renewed efforts to develop bioprocesses for the large scale production of a range of carotenoids, including lycopene, β-carotene, and more structurally diverse carotenoids (Cheng, 2006; Lee et al., 2003).

In most cases, metabolic pathway engineering of prokaryotic microorganisms, such as *Escherichia coli*, does not require a targeting strategy to send heterologously expressed enzymes into specific cellular location. However, one must consider targeting strategies when engineering pathways in eukaryotic microorganisms. Eukaryotes such as yeasts have several cellular organelles such as mitochondria (Yoon et al., 2007) and peroxisomes (Poirier et al., 2002). The organelles, where metabolic activities occur, are physically separated from other cellular components by membrane structures. Therefore, the proper cellular location of heterologously expressed pathway enzymes is important and both cellular and cytoplasmic membranes can be putative locations for membrane-bound enzymes to settle in.

Carotenoid pathway enzymes are known to be membrane-associated or membrane-bound (Britton, 1998). When carotenogenic enzymes are heterologously expressed without a proper targeting system they can be randomly located on the membranes of cellular organelles as well as cytoplasmic membranes. Yeast *P. pastoris* has a cellular organelle peroxisome where farnesyl diphosphate (Fig. 1), an important isoprenoid intermediate for carotenoid biosynthesis (Lee et al., 2005), is known to be generated and present in large amounts (Kovacs et al., 2002). Most of the genetic manipulations used in *P. pastoris* are similar to those used in *Saccharomyces cerevisiae*. Furthermore *P. pastoris*

* Corresponding author at: Department of Molecular Science and Technology, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, South Korea. Tel.: +82 31 219 2461; fax: +82 31 219 1610.
E-mail addresses: pclee2000@gmail.com, pclee@ajou.ac.kr (P.C. Lee).

0168-1656/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2009.01.019

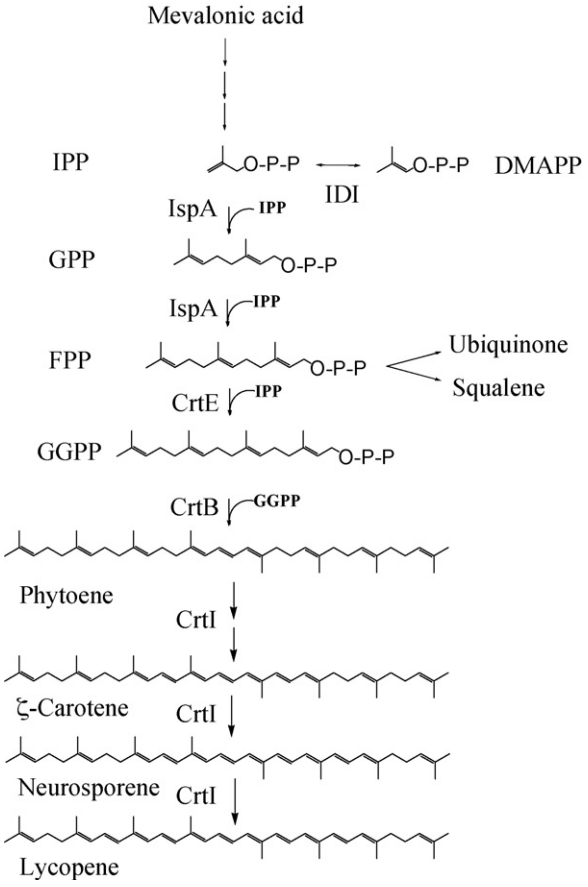

Fig. 1. Simplified engineered lycopene biosynthetic pathway in recombinant *Pichia pastoris*. The enzymes used are IDI, IPP isomerase; IspA, FPP synthase; CrtE, GGPP synthase; CrtB, phytoene synthase; CrtI, phytoene desaturase. The abbreviations used are IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

has a strong preference for respiratory metabolism and thus grows to extremely high cell densities without the accumulation or build-up of high concentrations of ethanol as is typically seen in high-density cultures of *S. cerevisiae* (Cereghino et al., 2002). These advantages of *P. pastoris* make this yeast more interesting and viable in the biotechnological production of hydrophobic compounds such as carotenoids.

Although metabolic engineering of *S. cerevisiae* (Verwaal et al., 2007) and *Candida utilis* (Misawa and Shimada, 1997) typically use heterologously expressed carotenogenic enzymes, the cellular location of engineered carotenoid pathway gene products has not yet been examined. Therefore, in this study, we have constructed *E. coli–Pichia* shuttle plasmids expressing carotenogenic enzyme that are fused to fluorescent EGFP with or without the peroxisomal targeting signal 1 (PTS1). We then examined the cellular locations of the fused carotenogenic enzyme in the recombinant *P. pastoris* grown on different carbon sources by fluorescent microscopy.

2. Materials and methods

2.1. Genes and plasmids

E. coli–Pichia pastoris shuttle plasmid pGAPZB and wild type *P. pastoris* X-33 strain were purchased from Invitrogen Corporation (Carslbad, CA). The gene encoding fluorescent EGFP was amplified from pEGFP (Clontech) using 5′ primers that contained an *Eco*RI site at its 5′ end followed by an optimized Kozak consensus (ATGG), a start codon and a 3′ primer containing an *Xba*I site at its 5′ end that was or was not followed by a PTS1 encoded by CAACTTAGA (Table 1). Carotenogenic enzymes fused to EGFP (CrtE_EGFP_n, CrtE_EGFP_p, CrtB_EGFP_n, CrtB_EGFP_p, CrtI_EGFP_n, and CrtI_EGFP_p) were generated by overlapping PCR as follows: first, the genes *crt*E, *crt*B and *crt*I were amplified from pUC-crtE, pUC-crtB and pUC-crtI with a 5′ primer containing an *Eco*RI site at its 5′ end followed by an optimized Kozak consensus, start codon and a 3′ primer covering the C-terminus of the carotenogenic enzymes with an extra sic amino acids (GlyGlyAlaGlyGlyGly) that was used as a spacer instead of stop codon (Table 1). Next, in the 2nd part of the fusion proteins, the gene encoding EGFP, was amplified using 5′ primers that contained the DNA sequence encoding the six amino acid spacer followed by the N-terminus of EGFP and a 3′ primer containing an *Xba*I site at the 5′ end that was or was not followed by PTS1. Finally the PCR products were purified and then used as templates for the 2nd overlapping PCR with the primer sets described above.

Table 1
Oligonucleotides used in this study.

Oligonucleotide	Sequences (5′->3′)
CrtE-F	GGAATTCAAAATGGCAGTCTGCGCAAAA
CrtE-R	GCTCTAGAGCTTAACTGACGGCAGCG
CrtE-PTS-R	GCTCTAGAGCTTACAACTTAGAAC TGACGGCAGCGAG
CrtB-F	GGAATTCAAAATGGCAGTTGGCTCG
CrtB-R	GCTCTAGAGCCTAGAGCGGGCGCTG
CrtB-PTS-R	GCTCTAGAGCCTACAACTTAGAGAGCGGGCGCTGCC
CrtI-F	GGAATTCAAAATGGCACCAACTACGG
CrtI-R	GCTCTAGAGCTCAAATCAGATCCTCCAG
CrtI-PTS-R	GCTCTAGAGCTCACAACTTAGAAATCAGATCCTCCAGC
EGFP-F	CCGGAATTCAAAATGGTGAGCAAGGGCG
EGFP-PTS-R	GCTCTAGATTACAACTTAGACTTGTACAGCTCGTCC
EGFP-R	GCTCTAGATTACTTGTACAGCTCGTCC
fEEGFP-O5	CGGAATTCAAAATGGCAGTCTGCGCAAAA
fEEGFP-I3	GCCACCTCCTGCTCCACCACTGACGGCAGCGAG
fEGFP-I5	GGTGGAGCAGGAGGTGGCATGGTGAGCAAGGGCG
fBEGFP-O5	CGGAATTCAAAATGGCAGTTGGCTCG
fBEGFP-I3	GCCACCTCCTGCTCCACCGAGCGGGCGCTGCC
fEGFP-I5	GGTGGAGCAGGAGGTGGCATGGTGAGCAAGGGCG
flEGFP-O5	CGGAATTCAAAATGGCACCAACTACGG
flEGFP-I3	GCCACCTCCTGCTCCACCAATCAGATCCTCCAGC
fEGFP-I5	GGTGGAGCAGGAGGTGGCATGGTGAGCAAGGGCG

All ligations were carried out with the T4 DNA ligase (New England Biolabs, Beverly, MA) using standard methods. The ligation reaction mixtures were directly transformed into *E. coli* JM109 using chemical transformation. Successful transformants were selected for on Luria–Bertani media (LB) (0.5% yeast extract, 1% tryptone and 0.5% NaCl) plates containing 50 μg/ml zeocin. Plates were incubated overnight at 37 °C and colonies were picked and cultured overnight in LB media containing 50 μg/ml zeocin.

2.2. Construction of plasmids expressing fusion enzymes

PCR products containing the EGFP gene with or withoug PTS1 were ligated into the *Eco*RI–*Xba*I sites of pGAPZB, resulting in pGAPZB_EGFP_n and pGAPZB_EGFP_p, respectively (Table 2). Similarly, the carotenogenic genes fused to EGFP with or without PTS1 were ligated into the *Eco*RI–*Xba*I sites of pGAPZB, resulting in six plasmids: pGAPZB_E_EGFP_n, pGAPZB_E_EGFP_p, pGAPZB_B_EGFP_n, pGAPZB_B_EGFP_p, pGAPZB_I_EGFP_n, and pGAPZB_I_EGFP_p, respectively (Table 2). To check if the fused enzymes were functional *in vivo*, pGAPZB_I_EGFP_n and pGAPZB_I_EGFP_p were transformed into recombinant *E. coli* that also expressed pAC_CrtE_CrtB (Lee et al., 2004). The resulting *E. coli* cells were grown in LB containing 50 μg/ml chloramphenicol and 50 μg/ml zeocin as selection markers. The cells were then extracted with acetone and the crude organic extracts were analyzed with HPLC (Lee et al., 2005) to verify the presence of lycopene.

Table 2
Plasmids used in this study.

Plasmid	Descriptions	Source or reference
pGAPZB	Pichia integrative plasmid, zeocin^R	Invitrogen
pEGFP	Source for EGFP gene	Clontech
pUC_CrtE	CrtE in pUCmod	Schmidt-Dannert et al. (2000)
pUC_CrtB	CrtB in pUCmod	Schmidt-Dannert et al. (2000)
pUC_CrtI	CrtI in pUCmod	Schmidt-Dannert et al. (2000)
pAC_CrtE_CrtB	CrtE and CrtB in pACmod	Schmidt-Dannert et al. (2000)
pAC_CrtE_CrtB_CrtI	CrtE, CrtB and CrtI in pACmod	Schmidt-Dannert et al. (2000)
pGAPZB_EGFP_n	EGFP in pGAPZB	This study
pGAPZB_EGFP_p	EGFP_pts in pGAPZB: EGFP with PTS1 at C-terminus	This study
pGAPZB_E_EGFP_n	CrtE_EGFP fusion in pGAPZB	This study
pGAPZB_E_EGFP_p	CrtE_EGFP_pts fusion in pGAPZB	This study
pGAPZB_E_EGFP-n	CrtB_EGFP fusion in pGAPZB	This study
pGAPZB_E_EGFP_p	CrtB_EGFP_pts fusion in pGAPZB	This study
pGAPZB_I_EGFP_n	CrtI_EGFP fusion in pGAPZB	This study
pGAPZB_I_EGFP_p	CrtI_EGFP_pts fusion in pGAPZB	This study

2.3. Pichia transformation

The eight plasmids expressing enzymes with or without PTS1 were linearized with *Avr*II and then transformed into *P. pastoris* X-33 cells by electroporation according to the manufacturer's protocol (Bio-Rad Laboratories, Inc., Hercules, CA). *P. pastoris* transformants were selected on YPDS (1% yeast extract, 2% peptone, 2% dextrose and 1 M sorbitol) plates supplemented with 0.1 mg/ml of zeocin. Plates were incubated at 30 °C until colonies were visible (48–72 h).

2.4. Culture conditions

A single clone from the YPD agar plate was cultured in 10 ml of YPD media in a 125 ml flask for 28 h at 30 °C and 275 rpm and then inoculated into 100 ml of YPD in a 500 ml flask, which was then incubated for 48 h at 30 °C and 275 rpm. Cells were harvested by centrifugation at 3000 × *g* for 5 min, washed with de-ionized water, resuspended in water, and then used as a preculture for further studies. To proliferate peroxisomes of recombinant *P. pastoris*, the washed cells were inoculated into 500 ml baffled flasks containing 100 ml of YPOT (1% yeast extract, 2% peptone, 0.2% oleic acid and 0.02% tween 40).

2.5. Fluorescence microscopy

To analyze EGFP-targeting into peroxisomes, the washed cells were imaged with a Leica fluorescence microscope (Yoon et al., 2007). Images were obtained with an AxioCamMR camera and MRGrab^{TM} software version 1.0.0.4.

2.6. Organelle isolation

The organelles were prepared from cells as described previously (Kaufman et al., 2001). Briefly, yeast cells from a 100-ml culture were grown in YPD media (OD_{600} = 2), harvested, washed with 10 ml of SCE (0.6 M sorbitol, 0.3 M mannitol, 20 mM K_2HPO_4, 20 mM citric acid, 1 mM EDTA and pH 5.8) containing 0.1 M β-mercaptoethanol and digested with 0.5 mg of yeast lytic enzyme (ICN) per gram of cells (wet weight) in SCE (pH 5.8) for 30 min at 30 °C. Cells were washed with 10 ml of SCE (pH 7.0) and osmotically broken (in 15% sucrose, 10 mM Tricine·KOH, 0.2 mM EDTA and pH 7.5). Nuclei and cellular debris were removed by centrifugation (2500 × *g*, 10 min). Organelles including mitochondria and peroxisomes were then pelleted (17,000 × *g*, 15 min), resuspended in 65% (w/v) sucrose/TEN buffer (10 mM Tricine·KOH, 0.1 mM EDTA, 50 mM NaCl and pH 7.5), overlayed with 15, 43, and 55% (w/v) sucrose steps and centrifuged at 25,000 rpm for 1.5 h in a Beckman SW28 rotor. The fraction of peroxisomes was carefully collected from 43 to 55% interface, diluted in 15% sucrose/TEN, and pelleted by centrifugation (17,000 × *g*, 10 min). The organelles were then resuspended in TEN buffer that contained 15% sucrose.

3. Results and discussion

3.1. Construction of peroxisome target system in P. pastoris

Membrane-associated or membrane-bound carotenogenic enzymes (Britton, 1998) can be randomly located on the membranes of cellular organelles as well as the cytoplasmic membranes of *P. pastoris*. Therefore, to investigate where lycopene biosynthetic pathway enzymes will be located in *P. pastoris,* these enzymes were fused to EGFP at the C-terminus. The EGFPs-enzymes were further fused to a peroxisomal targeting sequence (PTS1 and SKL) (McNew and Goodman, 1994) at the C-terminus, resulting in a total of eight plasmids (Table 2). Half of these enzymes, represented by pGAPZB_X_p, were designed to be targeted to peroxisomes,

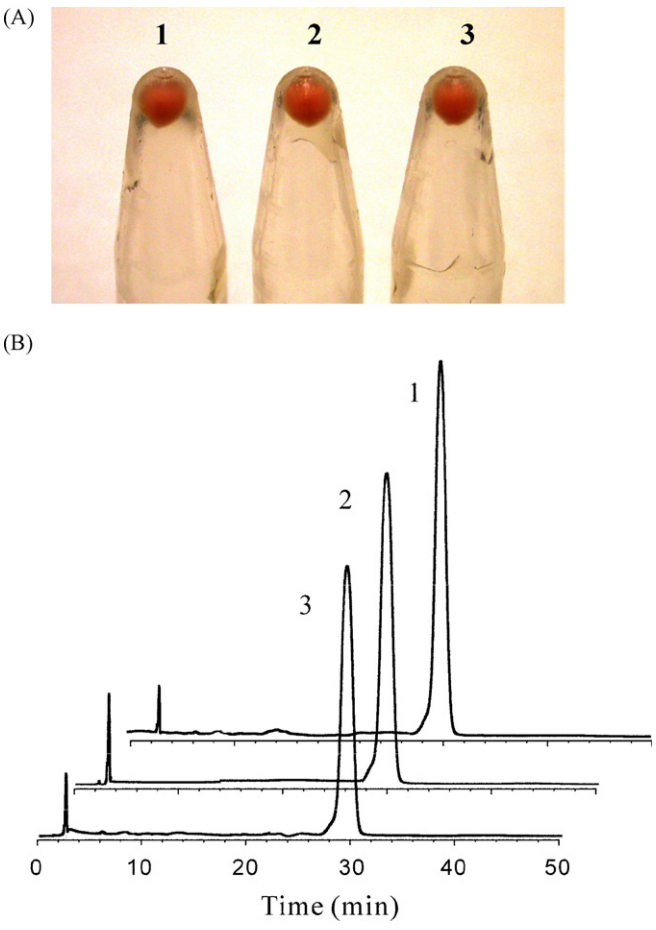

Fig. 2. Functional complementation of CrtI_EGFP and CrtI_EGFP_pts with CrtE and CrtB in *Escherichia coli.* 1, *E. coli* [pAC_CrtE_CrtB_CrtI] as a control; 2, *E. coli* [pAC_CrtE_CrtB + pGAPZB_I_EGFP_n]; 3, *E. coli* [pAC_CrtE_CrtB + pGAPZB_I_EGFP_p]. (A) The pellets of recombinant cells and (B) HPLC analysis of extracts of cells.

whereas the remaining enzymes, represented by pGAPZB_X_n, were not designed for any specific targeting.

The functional properties of the fused carotenogenic enzymes were found to be retained when the fused carotenogenic enzyme and wild-type enzymes were co-expressed in *E. coli*. We chose CrtI_EGFP and CrtI_EGFP_pts for this functional complementation because these fusion partners could most significantly affect the multimeric formation of CrtI *in vivo* (Armstrong and Hearst, 1996). The functional complementation made *E. coli* cells turn reddish (Fig. 2A) when CrtI_EGFP and CrtI_EGFP_pts were coexpressed with wild-type CrtE and CrtB in recombinant *E. coli*. In addition, the formation of lycopene was analyzed (Fig. 2B). These results indicate that the fusion enzyme CrtI_EGFP or CrtI_EGFP_pts was functional and could recon-

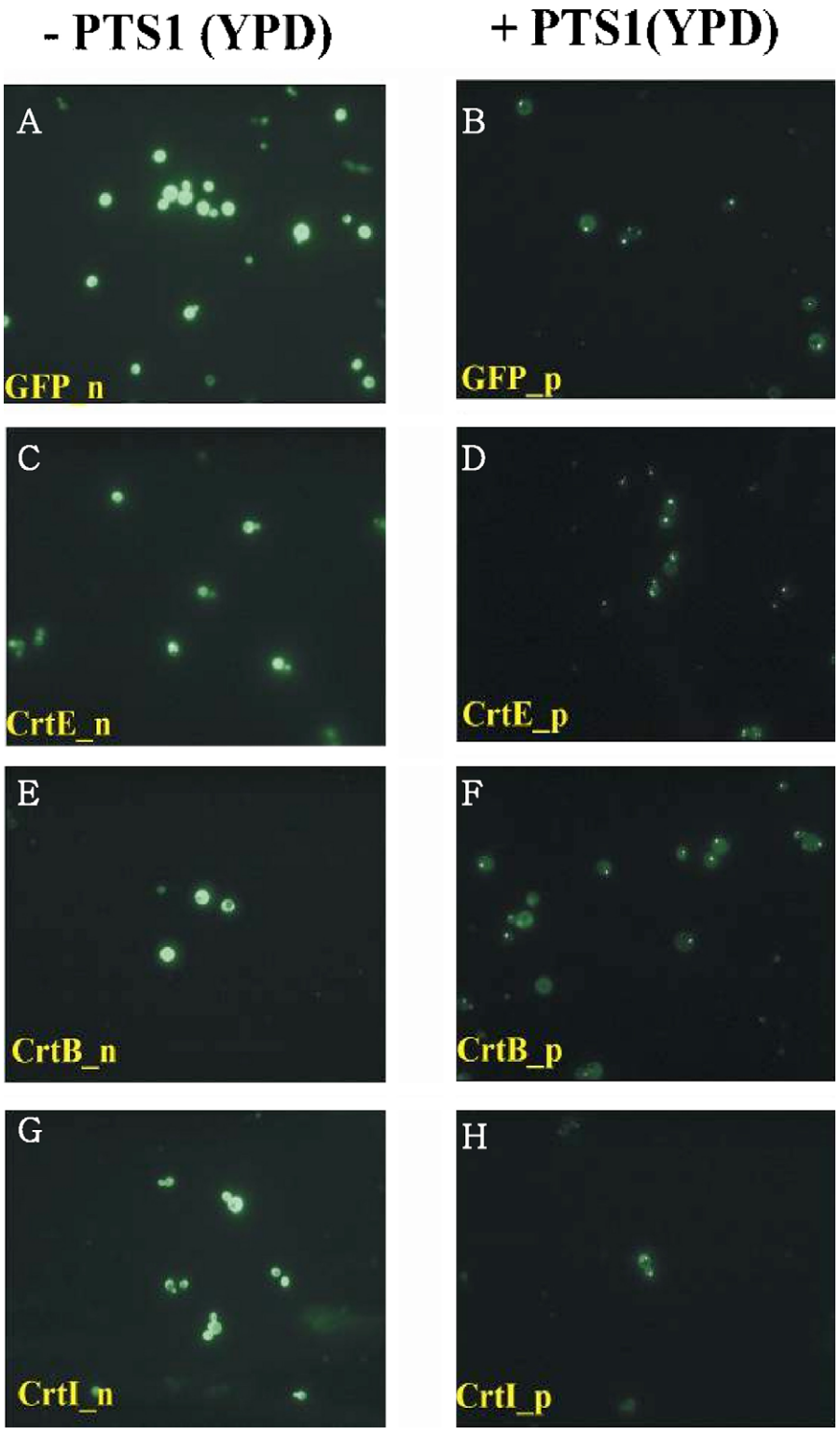

Fig. 3. Localization of control EGFP and the fused carotenogenic enzymes with and without PTS1 in *P. pastoris* grown in YPD medium. GFP_n (A), CrtE_n (C), CrtB_n (E), and CrtI_n (G) represent the enzymes without PTS1 and GFP_p (B), CrtE_p (D), CrtB_p (F) and CrtI_p (H) represent the enzymes with PTS1.

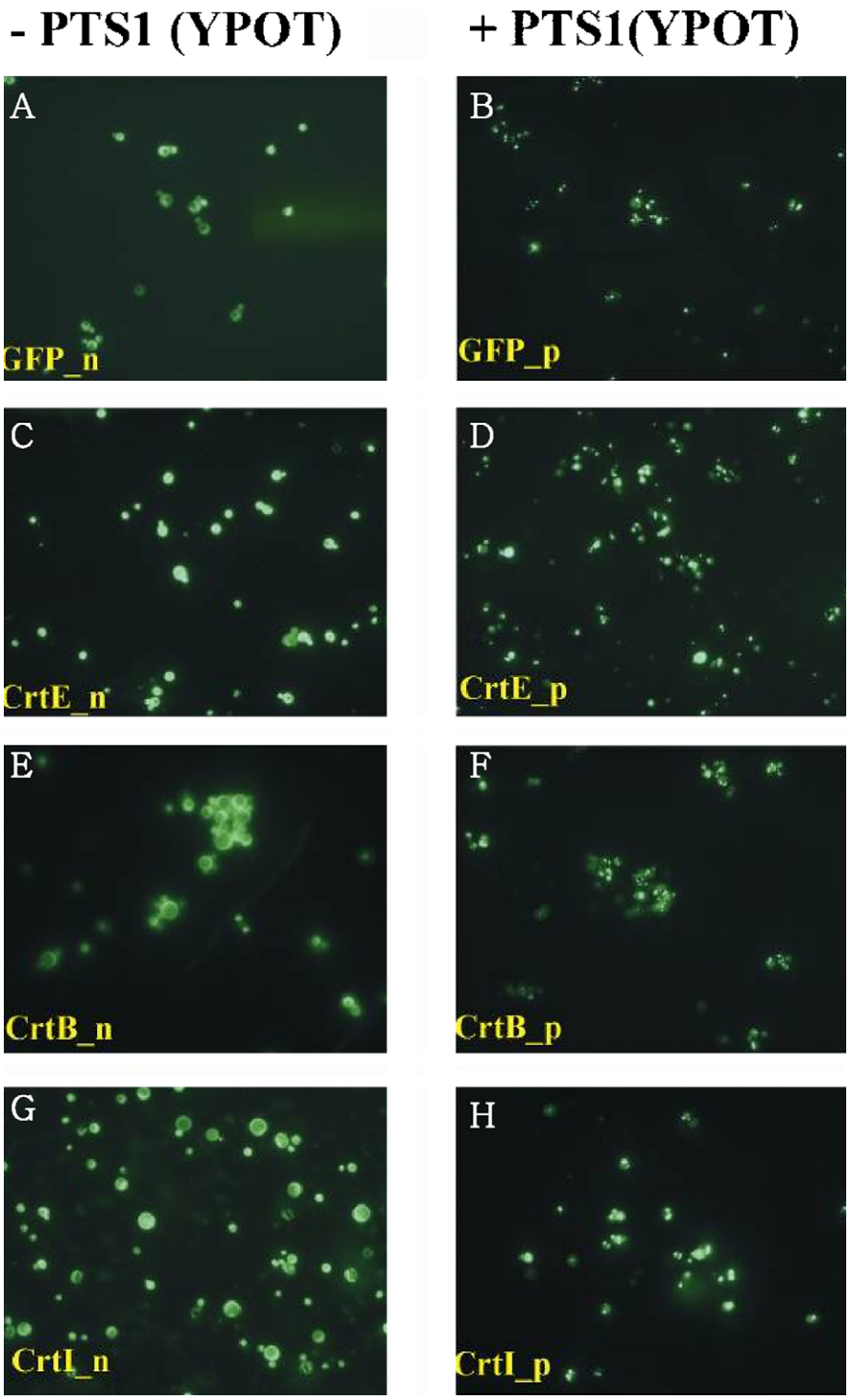

Fig. 4. Localization of the control EGFP and the fused carotenogenic enzymes with and without PTS1 in *P. pastoris* grown in peroxisome-proliferating YPOT medium. GFP_n (A), CrtE_n (C), CrtB_n (E) and CrtI_n (G) represent the enzymes without PTS1 and GFP_p (B), CrtE_p (D), CrtB_p (F) and CrtI_p (H) represent the enzymes with PTS1.

struct the lycopene pathway with wild-type CrtE and CrtB *in vivo*.

3.2. Localization of carotenogenic enzymes

We first determined whether the functional properties of PTS1 were retained *in vivo* by expressing EGFP and EGFP_pts in *P. pastoris*. When EGFP and EGFP_pts were expressed in *P. pastoris* grown in YPD, the fluorescence of the expressed EGFP was found to be evenly distributed throughout the entire cell (Fig. 3A), whereas the fluorescence of the expressed EGFP_pts was intensely localized in a few spots throughout the cell (Fig. 3B) with much weaker fluorescence signals in the intervening regions around cell. This different fluorescence pattern indicates that PTS1 was properly targeted *in vivo*. A similar fluorescence patterns was also observed when carotenogenic enzymes without and with PTS1 were individually expressed in *P. pastoris* grown in YPD. When CrtE_EGFP, CrtB_EGFP, and CrtI_EGFP were expressed, uniform fluorescent intensity was

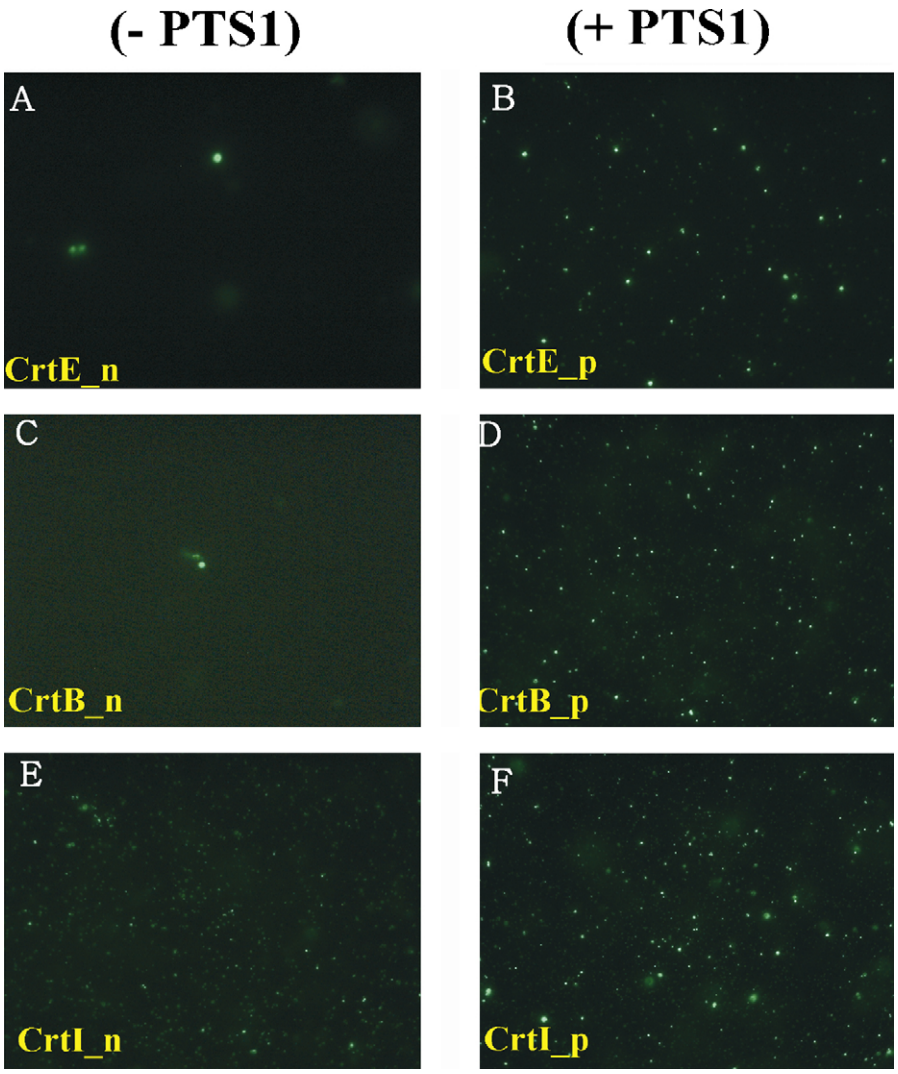

Fig. 5. Isolated peroxisomes of recombinant *P. pastoris* cells expressing carotenogenic enzymes with and without PTS1, grown in YPOT medium. CrtE_n (A), CrtB_n (C) and CrtI_n (E) represent the enzymes without PTS1 and CrtE_p (B), CrtB_p (D) and CrtI_p (E) represent the enzymes with PTS1.

observed throughout the entire cell, indicating that the fusion enzymes were evenly distributed in the cells (Fig. 3C, E and G). Thus, there was no difference in the fluorescence patterns between cells expressing EGFP (Fig. 3A) alone and cells expressing carotenogenic enzyme fused to EGFP (Fig. 3C, E and G). In contrast, when the fusion enzymes that contained PTS1 were expressed in *P. pastoris* grown in YPD, very intense fluorescent signals were observed in a few small spots throughout the cell (Fig. 3D, F and H), which was very similar to the fluorescent pattern observed when EGFP_pts was expressed (Fig. 3B). The reason that only a small number of spots were observed in Fig. 2 was because *P. pastoris* has only a small number of peroxisomes when grown in YPD (Lazarow and Fujiki, 1985). Therefore, these combined results strongly indicate that the four enzymes containing PTS1 (EGFP_pts, CrtE_EGFP_pts, CrtB_EGFP_pts and CrtI_EGFP_pts) were properly targeted to peroxisomes via PTS1 in *P. pastoris* grown in YPD. The successful targeting demonstrated here suggests that PTS1 was strong enough to overcoming the inherent targeting program of carotenogenic enzymes.

3.3. Effect of peroxisome proliferation conditions

P. pastoris has the capability of proliferating peroxisomes when grown in oleic acids or methanol (Gould et al., 1992) but not in glucose-containing medium such as YPD. As shown in Fig. 3, *P. pastoris* grown in YPD was found to have one or two peroxisome. However, the effects of peroxisome proliferation on the targeting pathways in *P. pastoris* have not yet been investigated. Therefore, we wanted to determine whether the targeting of the lycopene pathway enzymes would be affected by peroxisome-proliferating conditions. To examine this we conducted the same experiments as above under peroxisome-proliferating conditions. From these experiments we found that the fusion enzymes could still be properly localized in extended sizes and number of peroxisomes in *P. pastoris* grown in peroxisome-proliferating medium, YPOT. As shown in Fig. 4, very strong fluorescence signals were observed in several spots throughout the cells expressing the enzymes fused to PTS1 (Fig. 4B, D, F and H), whereas the fluorescence was evenly distributed throughout the entire cell in cells that expressed the enzymes without PTS1 (Fig. 4A, C, E and G). As previously reported, *P. pastoris* grown in the oleic acid-containing medium (YPOT) had a larger number of peroxisomes, which is indicative of peroxisome proliferation (Lazarow and Fujiki, 1985). The intense fluorescent spots shown in Fig. 4 clearly indicate that the expressed enzymes were properly targeted into extended sizes and number of peroxisomes. The successful targeting demonstrated in these results also indicates that PTS1 was functional in sending the carotenogenic enzymes to peroxisomes under peroxisomal-proliferating conditions.

3.4. Isolated peroxisomes showing fluorescence

To ensure that the intense fluorescent spots shown in Figs. 3 and 4 were related to peroxisomes that contained the targeted fusion enzymes, we isolated crude peroxisomes from both *P. pastoris* expressing the three fusion lycopene enzymes with and without PTS1, which were grown in YPOT, by sucrose-gradient centrifugation as described in Section 2. Analysis of the fluorescence microscopy images of the isolated peroxisomes clearly demonstrates that the enzymes fused to PTS1 were present at high concentrations in the isolated peroxisomes (Fig. 5B, D and F), whereas only trace amounts of the enzymes without PTS1 were found in the peroxisomes (Fig. 5A, C and E). These results indicate that the enzymes without PTS1 were not targeted into peroxisomes although small percent of them seemed to be non-specifically sent to peroxisomes.

In conclusion, we clearly demonstrated in this study that carotenogenic enzymes can be targeted to and localized in cellular organelles of recombinant hosts. These results also show that PTS1 was strong enough to overcome the carotenogenic enzymes inherent targeting program and send them to the peroxisomes. Thus, this targeting study can serve as the basis for the subsequent development of sophisticated pathway engineering in microorganisms.

Acknowledgements

The authors gratefully acknowledge support from the Defense Advanced Research Projects Agency (DARPA-BIOS N66001-02-1-8928). PC Lee was also supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2007-313-D00257) and by the Korea-Australia Collaborative Research Project on the Development of Biomass-Based Bioprocess Platform (10030795) from the Korean Ministry of Knowledge Economy.

References

Armstrong, G., Hearst, J., 1996. Carotenoids. 2. Genetics and molecular biology of carotenoid pigment biosynthesis. FASEB J. 10 (2), 228–237.

Britton, G., 1998. Overview of carotenoid biosynthesis. In: Britton, G. (Ed.), Carotenoids: Biosynthesis and Metabolism. Birkhauser, Basel, pp. 13–147.

Cereghino, G.P., Cereghino, J.L., Ilgen, C., Cregg, J.M., 2002. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris* heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. Curr. Opin. Biotechnol. 13 (4), 329–332.

Cheng, Q., 2006. Structural diversity and functional novelty of new carotenoid biosynthesis genes. J. Ind. Microbiol. Biotechnol. 33 (7), 552–559.

Gould, S.J., McCollum, D., Spong, A.P., Heyman, J.A., Subramani, S., 1992. Development of the yeast *Pichia pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. Yeast 8 (8), 613–628.

Johnson, E., Schroeder, W., 1995. Microbial carotenoids. Adv. Biochem. Eng. Biotechnol. 53, 119–178.

Kaufman, B.A., Newman, S.M., Hallberg, R.L., Slaughter, C.A., Perlman, P.S., Butow, R.A., 2001. In organello formaldehyde crosslinking of proteins to mtDNA: identification of bifunctional proteins. Proc. Natl. Acad. Sci. U.S.A..

Kovacs, W.J., Olivier, L.M., Krisans, S.K., 2002. Central role of peroxisomes in isoprenoid biosynthesis. Prog. Lipid Res. 41 (5), 369–391.

Lazarow, P.B., Fujiki, Y., 1985. Biogenesis of peroxisomes. Annu. Rev. Cell Biol. 1, 489–530.

Lee, P.C., Mijts, B.N., Petri, R., Watts, K.T., Schmidt-Dannert, C., 2004. Alteration of product specificity of Aeropyrum pernix farnesylgeranyl diphosphate synthase (Fgs) by directed evolution. Protein Eng. Des. Select. 17 (11), 771–777.

Lee, P.C., Momen, A.Z.R., Mijts, B.N., Schmidt-Dannert, C., 2003. Biosynthesis of structurally novel carotenoids in *Escherichia coli*. Chem. Biol. 10 (5), 453–462.

Lee, P.C., Petri, R., Mijts, B.N., Watts, K.T., Schmidt-Dannert, C., 2005. Directed evolution of *Escherichia coli* farnesyl diphosphate synthase (IspA) reveals novel structural determinants of chain length specificity. Metab. Eng. 7 (1), 18–26.

McNew, J.A., Goodman, J.M., 1994. An oligomeric protein is imported into peroxisomes in vivo. J. Cell Biol. 127 (5), 1245–1257.

Misawa, N., Shimada, H., 1997. Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. J. Biotechnol. 59 (3), 169–181.

Poirier, Y., Erard, N., MacDonald-Comber Petetot, J., 2002. Synthesis of polyhydroxyalkanoate in the peroxisome of *Pichia pastoris*. FEMS Microbiol. Lett. 207 (1), 97–102.

Sandmann, G., 2001. Carotenoid biosynthesis and biotechnological application. Arch. Biochem. Biophys. 385 (1), 4–12.

Schmidt-Dannert, C., Umeno, D., Arnold, F.H., 2000. Molecular breeding of carotenoid biosynthetic pathways. Nat. Biotechnol. 18 (7), 750–753.

Vershinin, A., 1999. Biological functions of carotenoids—diversity and evolution. Biofactors 10 (2–3), 99–104.

Verwaal, R., Wang, J., Meijnen, J.-P., Visser, H., Sandmann, G., van den Berg, J.A., van Ooyen, A.J.J., 2007. High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from Xanthophyllomyces dendrorhous. Appl. Environ. Microbiol. 73 (13), 4342–4350.

Yoon, Y.G., Haug, C.L., Koob, M.D., 2007. Interspecies mitochondrial fusion between mouse and human mitochondria is rapid and efficient. Mitochondrion 7 (3), 223–229.