Chapter 10

Peroxisome Targeting of Lycopene Pathway Enzymes in *Pichia pastoris*

Pyung Cheon Lee

Abstract

Cellular targeting of biosynthetic pathway enzymes is an invaluable technique in metabolic engineering to modify metabolic fluxes towards metabolite of interest. Especially, recombinant carotenoid biosynthesis in yeasts should be balanced with a precursor pathway present in a specific cellular location because yeasts, being eukaryotes, have more defined intracellular location. Here, peroxisomal targeting of lycopene pathway enzymes, CrtE, CrtB, and CrtI, by fusing to peroxisomal targeting sequence 1 (PTS1) in *Pichia pastoris* X-33 is described.

Key words: Cellular targeting, Yeast, Peroxisomal targeting sequence, Lycopene, Metabolic engineering

1. Introduction

In most cases, metabolic pathway engineering of prokaryotic microorganisms, such as *Escherichia coli*, does not require a targeting strategy to send heterologously expressed pathway enzymes into specific cellular location (1–4). However, targeting strategies must be considered when engineering pathways in eukaryotic microorganisms. Eukaryotes such as yeasts have several cellular organelles such as mitochondria (5) and peroxisomes (6–8). 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 (9). When carotenogenic enzymes are heterologously

José-Luis Barredo (ed.), *Microbial Carotenoids From Fungi: Methods and Protocols*, Methods in Molecular Biology, vol. 898,
DOI 10.1007/978-1-61779-918-1_10,

expressed without a proper targeting system they can be randomly located on the membranes of cellular organelles as well as cytoplasmic membranes. The yeast *Pichia pastoris* has a cellular organelle peroxisome where farnesyl diphosphate, an important precursor for carotenoid biosynthesis (10), is known to be generated and present in large amounts (11).

Therefore, to make carotenoid biosynthesis balance with a precursor pathway present in peroxisomes in *P. pastoris*, the proper cellular targeting of carotenoid biosynthetic enzymes is very important. In this study, peroxisomal targeting of lycopene pathway enzymes, CrtE, CrtB, and CrtI, is investigated by fusing to enhanced green fluorescence protein (EGFP) with or without the peroxisomal targeting sequence 1 (PTS1) (12–14) in *P. pastoris* X-33.

2. Materials

Prepare all solution using double-distilled water (DDW) and analytical grade or molecular biology grade reagents. Prepare and store all reagents at 4°C (unless indicated otherwise).

2.1. Plasmids, Strains, and Molecular Biology Grade Chemicals

1. pEGFP (Clonetech, Mountain View, CA, USA).
2. pGAPZB (Invitrogen Corporation, Carlsbad, CA, USA) (see Note 1).
3. *E. coli* JM109 (New England Labs, Beverly, MA, USA).
4. *P. pastoris* X-33 strain (Invitrogen Corporation, Carlsbad, CA, USA) (see Note 2).
5. 100 mg/mL Zeocin™ (Invitrogen Corporation, Carlsbad, CA, USA) (see Note 3).
6. pUCM_CrtE (see Note 4).
7. pUCM_CrtB (see Note 4).
8. pUCM_CrtI (see Note 4).
9. Yeast lytic enzyme (ICN, Solon, OH, USA) (see Note 5).
10. Restriction enzymes *Eco*RI, *Xba*I, and *Avr*II.
11. T4 DNA ligase.
12. Vent DNA polymerases.
13. QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA).
14. QIAGEN Plasmid Mini kit (Qiagen, Valencia, CA, USA).
15. DDW (Qiagen, Valencia, CA, USA).
16. 0.1 mM TE (pH 8.0) (Qiagen, Valencia, CA, USA).

2.2. Culture Media

1. LB: 5 g/L yeast extract, 10 g/L tryptone, and 5 g/L NaCl. Adjust pH to 7.5 with 1 N NaOH. Autoclave on liquid cycle at 15 psi and 121°C for 20 min. Store at 4°C.
2. LB solid: LB and 15 g/L agar before autoclaving. Autoclave on liquid cycle at 15 psi and 121°C for 20 min. Store at 4°C.
3. LB–zeocin: LB and 50 μg/mL zeocin. Add 2,000× zeocin (100 mg/mL) into LB and use it immediately.
4. YPD: 10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose. Autoclave on liquid cycle at 15 psi and 121°C for 20 min. Store at 4°C.
5. YPD solid: YPD and 20 g/L agar before autoclaving. Autoclave on liquid cycle at 15 psi and 121°C for 20 min. Store at 4°C.
6. YPD–zeocin: YPD and 100 μg/mL zeocin. Add 1,000× zeocin (100 mg/mL) into YPD and use it immediately.
7. YPDS: YPD and 1 M sorbitol. Store at 4°C.
8. YPDS–zeocin: YPDS and 100 μg/mL zeocin. Add 1,000× zeocin (100 mg/mL) into YPDS and use it immediately.

2.3. PCR Primers

The complete list of PCR primers designed for this study is shown in Table 1.

1. Forward primer F, e.g., CrtE-F, contains an *Eco*RI site and a Kozak consensus sequence (ATGG) at its 5′ end.
2. Reverse primer R, e.g., CrtE-R, contains a *Xba*I site and a stop codon at its 5′ end.
3. Reverse primer PTS-R, e.g., CrtE-PTS-R, contains a restriction enzyme site, a stop codon, and additional "CAACTTAGA" sequence (peroxisomal targeting sequence 1, PTS1) at its 5′ end. The PTS1 sequence is underlined.
4. Reverse fusion PCR primer I3, e.g., fEEGFP-I3, covers the C terminus of the carotenogenic enzyme with an extra six amino acids (Gly-Gly-Ala-Gly-Gly-Gly: GGT-GGA-GCA-GGA-GGT-GGC) as a spacer.

2.4. PCR

1. PCR machine.
2. 200-μL PCR tube.
3. QIAquick PCR purification kit (Qiagen, Valencia, CA, USA).
4. 10 mM dNTPs mixture.

2.5. Transformation

1. *P. pastoris* X-33 competent cells (Invitrogen Corporation, Carlsbad, CA, USA).
2. Gene Pulser Electroporation system (Bio-Rad Laboratories, Hercules, CA, USA).

Table 1
Primers used in this study. PTS1 sequences are underlined and spacers for fusion proteins are bold

Primers	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-R	GCTCTAGATTACTTGTACAGCTCGTCC
EGFP-PTS-R	GCTCTAGATTACAACTTAGACTTGTACAGCTCGTCC
fEEGFP-I3	**GCCACCTCCTGCTCCACC**ACTGACGGCAGCGAG
fEGFP-I5	**GGTGGAGCAGGAGGTGGC**ATGGTGAGCAAGGGCG
fBEGFP-I3	**GCCACCTCCTGCTCCACC**GAGCGGGCGCTGCC
fEGFP-I5	**GGTGGAGCAGGAGGTGGC**ATGGTGAGCAAGGGCG
fIEGFP-I3	**GCCACCTCCTGCTCCACC**AATCAGATCCTCCAGC
fEGFP-I5	**GGTGGAGCAGGAGGTGGC**ATGGTGAGCAAGGGCG

3. 1-mm Gap width disposable electroporation cuvettes (Bio-Rad Laboratories, Hercules, CA, USA).
4. 4-mm Gap width disposable electroporation cuvettes (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Fluorescence Microscope

1. Leica fluorescence microscope (Leica microsystem, Wetzlar, Germany) with an AxioCamMR camera (Carl Zeiss, Gőttingen, Germany), and MRGrab™ software version 1.0.0.4 (Carl Zeiss, Gőttingen, Germany).
2. Microscope slides and cover slips.
3. SCE buffer (pH 7.0): 0.6 M sorbitol, 0.3 M mannitol, 20 mM K_2HPO_4, 20 mM citric acid, 1 mM EDTA, and 0.1 M β-mercaptoethanol. Store at 4°C.

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Construction of EGFP Systems Targeted to Peroxisomes

1. Amplify the gene encoding fluorescent EGFP_pts from 0.1 to 0.2 μg pEGFP, as a template DNA, using a forward PCR primer EGFP-F and a reverse PCR primer EGFP-PTS-R under these PCR conditions: 95°C/5 min/1 cycle for initial denaturation, 94°C/30 s/29 cycles for denaturation, 50°C/1 min/29 cycles for annealing, 72°C/1 min/29 cycles for extension, 72°C/7 min/1 cycle for final extension. The PCR mixture (50 μL) is as follows: 37.5 μL of DDW, 5 μL of 10× PCR buffer, 5 μL of dNTPs, 1 μL of primer mix, 0.5 μL of DNA polymerase, and 1 μL of template DNA.
2. To amplify the second gene encoding fluorescent EGFP_n, repeat step 1 with primers EGFP-F and EGFP-R.
3. Clean up the PCR products with a QIAquick PCR purification kit (see Note 6), and then elute them with 50 μL DDW (see Note 7).
4. Digest both 1–2 μg PCR products and 2–3 μg pGAPZB in 100 μL reaction volumes with 5 U of *Eco*RI and *Xba*I at 37°C for 5 h.
5. Clean up the enzyme reaction mixtures with a QIAquick Gel Extraction kit, and then elute with ~30 μL DDW.
6. Ligate the digested PCR products into the *Eco*RI and *Xba*I sites of pGAPZB in 20 μL reaction volumes using 1 U of T4 DNA ligase at 16°C for 6 h (see Note 8).
7. Transform 1–4 μL of the ligation mixture into 50 μL *E. coli* competent cells in 1-mm electroporation cuvettes, precooled on ice, by Gene Pulser electroporation system.
8. After transformation, allow the transformants to recover in 1 mL LB at 37°C for 1 h.
9. Plate 25–100 μL the cells on LB–zeocin, prewarmed at 37°C for 1 h, and then incubate the plates at 37°C for 16 h.
10. Inoculate a single colony in 3 mL LB–zeocin and incubate overnight at 37°C.
11. After plasmid miniprep with QIAGEN Plasmid Mini kit, confirm the correct clones by restriction enzyme digestion and DNA sequencing.

3.2. Construction of Carotenogenic Enzyme_EGFP Systems Targeted to Peroxisomes

1. To obtain the first part of fusion proteins CrtX_EGFP_pts or CrtX_EGFP_n, amplify the gene *crtX* with a forward PCR primer CrtX-F and a reverse fusion PCR primer fXEGFP-I3 under the PCR conditions shown in Subheading 3.1, step 1 (see Note 9).

2. To obtain the second part of fusion protein, CrtX_EGFP_pts, which is targeted to peroxisomes, amplify the gene encoding EGFP using a forward fusion PCR primer fEGFP-I5 and a reverse PCR primer EGFP-PTS-R under the same PCR conditions.
3. To obtain the second part of fusion protein, CrtX_EGFP_n, which is not targeted to peroxisomes, repeat step 2 with fEGFP-I5 and EGFP-R.
4. Clean up the PCR products with a QIAquick PCR purification kit and then elute them with 50 μL DDW.
5. To construct CrtX_EGFP_pts or CrtX_EGFP_n, mix the two halves of PCR products together at an equimolar ratio and then do overlapping PCR under the same PCR conditions (see Note 10).
6. Repeat steps 3–11 in Subheading 3.1.

3.3. Pichia Transformation

1. Linearize 1–2 μg plasmid with 5 U of *Avr*II in 100 μL reaction mixture at 37°C for 5 h (see Note 11), heat-inactivate the reaction mixture at 65°C for 30 min, and then clean up with QIAquick Gel Extraction kit.
2. Transform 0.5–1 μg the linearized plasmid into 200 μL *P. pastoris* X-33 competent cells in 4-mm cuvettes that are precooled on ice by Gene Pulser electroporation system.
3. After transformation, allow the transformants to recover in 1 mL YPDS at 30°C for 2 h.
4. Plate 100–200 μL the cells on YPDS–zeocin prewarmed at 30°C.
5. Incubate the plates at 30°C until colonies are visible (see Note 12).

3.4. Culture Conditions

1. Select a single colony on YPDS–zeocin plate, streak it on fresh YPD–zeocin plate, and then incubate the plates at 30°C for 48–72 h.
2. Select a fresh single colony on YPD–zeocin plate and culture in 10 mL YPD–zeocin in a 125-mL flask at 30°C and 275 rpm for 28 h.
3. Harvest cells by centrifugation at 3,000 × *g* for 5 min, washed with 20 mL DDW, resuspended in 10 mL SCE buffer, and then used for further studies.

3.5. Fluorescence Microscopy

1. Drop 5 μL cells in SCE buffer on a glass plate and then carefully put a coverslip on the spot.
2. Check fluorescence of a single cell under a Leica fluorescence microscope with an AxioCamMR camera and MRGrab™ software version 1.0.0.4.
3. Take snapshots (see Note 13).

4. Notes

1. Invitrogen Corporation (http://www.invitrogen.com) sells several *E. coli–P. pastoris* shuttle vectors and *Pichia* strains that are right for your experiments. Thus, you can choose any vector or strain or combination for your experimental purposes.
2. "*Pichia* Protocols" in Methods in Molecular Biology, edited by D. R. Higgins and J. Cregg, will be helpful to be familiar to *Pichia* and its expression system. It collects key experimental procedures for use of the *P. pastoris* Expression System.
3. Zeocin™ is very sensitive to light, high ionic strength, and pH. Reduction of NaCl concentration to 5 g/L in LB and adjustment of the pH to 7.0–7.5 are recommended.
4. Plasmids carrying the genes encoding carotenogenic enzymes can be obtained from researchers working on carotenoid pathway engineering. Otherwise, these genes can be obtained by PCR amplification using specific primers.
5. We use the yeast lytic enzyme from ICN, but other yeast cell wall-degrading enzymes are available commercially.
6. If there are nonspecific PCR products, a QIAquick Gel Extraction kit (Qiagen) can be used instead of a PCR purification kit.
7. DDW is recommended if the purified PCR product is immediately used for restriction enzyme digestion. If not, 0.1 mM TE (pH 8.0) should be used.
8. Molar ratio of a PCR product (insert DNA) to pGAPZB (vector DNA) needs to be optimized.
9. X in CrtX, pUC-crtX, CrtX-F, and fXEGFP-I3 is E, B, or I in this study.
10. PCR condition may need to be optimized, especially annealing temperature.
11. Whether genes of interest have the same restriction site should be checked. If the enzyme site is present, site-directed mutagenesis PCR will be needed to remove the restriction enzyme site of the gene.
12. It takes usually 48–72 h for cells to grow on YPDS–zeocin.
13. You can see fluorescence of recombinant *P. pastoris* cells as shown in Fig. 1.

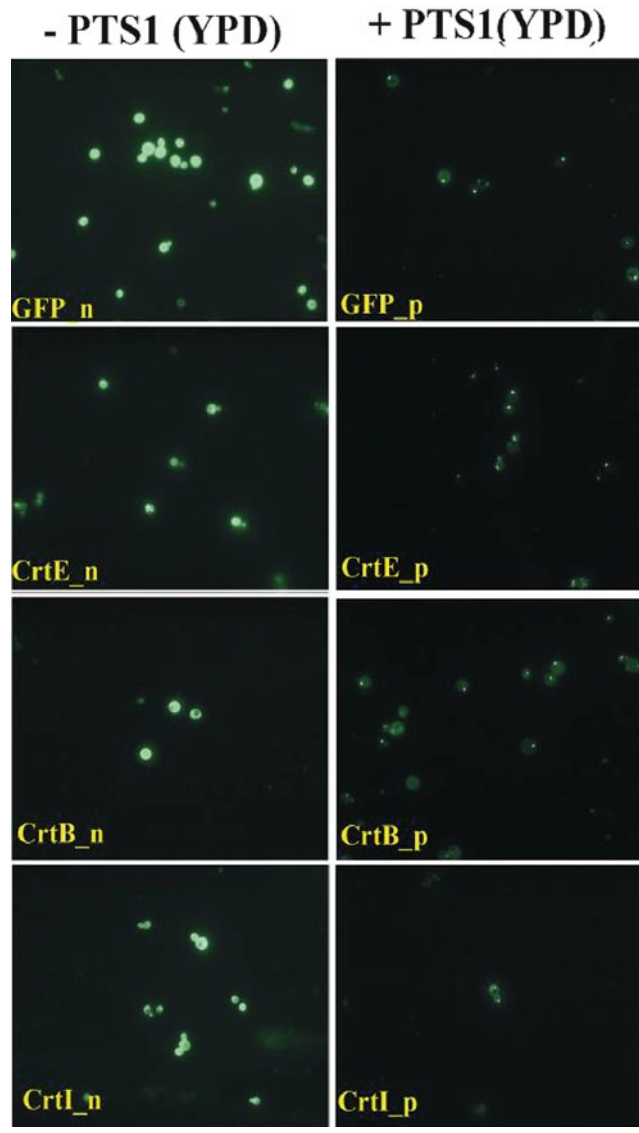

Fig. 1. Localization of control EGFP and the fused carotenogenic enzymes with (+PTS1) and without (–PTS1) PTS1 in *P. pastoris* grown on YPD. GFP_n, CrtE_n, CrtB_n, and CrtI_n represent the enzymes without PTS1 and GFP_p, CrtE_p, CrtB_p, and CrtI_p represent the enzymes with PTS1. Reproduced from ref. 8 with permission from Elsevier.

Acknowledgments

This work was supported by Mid-career Researcher Program through NRF funded by MEST (2011-0018057). Many parts of this manuscript was obtained from a reprint from Journal of Biotechnology, 140/3–4, Pyung Cheon Lee, Young geol Yoon,

Claudia Schmidt-Dannert, Investigation of cellular targeting of carotenoid pathway enzymes in *P. pastoris*, 227–233, Copyright (2009), with permission from Elsevier.

References

1. Chae HS, Kim KH, Kim SC et al (2010) Strain-dependent carotenoid productions in metabolically engineered *Escherichia coli*. Appl Biochem Biotechnol 162:2333–2344
2. Kim JR, Kong MK, Lee SY et al (2010) Carbon sources-dependent carotenoid production in metabolically engineered *Escherichia coli*. World J Microbiol Biotechnol 26:2231–2239
3. Kim SH, Park YH, Schmidt-Dannert C et al (2010) Redesign, reconstruction, and directed extension of *Brevibacterium linens* carotenoid pathway in *Escherichia coli*. Appl Environ Microbiol 76:5199–5206
4. Lee PC, Momen AZR, Mijts BN et al (2003) Biosynthesis of structurally novel carotenoids in *Escherichia coli*. Chem Biol 10:453–462
5. Yoon YG, Haug CL, Koob MD (2007) Interspecies mitochondrial fusion between mouse and human mitochondria is rapid and efficient. Mitochondrion 7:223–229
6. Bhataya A, Schmidt-Dannert C, Lee PC (2009) Metabolic engineering of *Pichia pastoris* X-33 for lycopene production. Process Biochem 44:1095–1102
7. Poirier Y, Erard N, MacDonald-Comber Petétot J (2002) Synthesis of polyhydroxyalkanoate in the peroxisome of *Pichia pastoris*. FEMS Microbiol Lett 207:97–102
8. Lee PC, Yoon YG, Schmidt-Dannert C (2009) Investigation of cellular targeting of carotenoid pathway enzymes in *Pichia pastoris*. J Biotechnol 140:227–233
9. Britton G (1998) Overview of carotenoid biosynthesis. In: Britton G (ed) Carotenoids: biosynthesis and metabolism, 1st edn. Birkhäuser, Basel
10. Lee PC, Petri R, Mijts BN et al (2005) Directed evolution of *Escherichia coli* farnesyl diphosphate synthase (IspA) reveals novel structural determinants of chain length specificity. Metab Eng 7:18–26
11. Kovacs WJ, Olivier LM, Krisans SK (2002) Central role of peroxisomes in isoprenoid biosynthesis. Prog Lipid Res 41:369–391
12. McNew JA, Goodman JM (1994) An oligomeric protein is imported into peroxisomes in vivo. J Cell Biol 127:1245–1257
13. Lazarow PB, Fujiki Y (1985) Biogenesis of peroxisomes. Annu Rev Cell Biol 1:489–530
14. Gould SJ, McCollum D, Spong AP et al (1992) Development of the yeast *Pichia pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. Yeast 8:613–628