Chapter 13

Construction and Utilization of Carotenoid Reporter Systems: Identification of Chromosomal Integration Sites That Support Suitable Expression of Biosynthetic Genes and Pathways

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Abstract

In order to metabolically engineer microorganisms to produce compounds of interest, it is often desirable to integrate foreign genes into the chromosome of the host. However, the consequences of these genetic alterations are not always predictable. The use of a reporter system can often assist in determining chromosomal locations for optimal expression of foreign biosynthetic genes. The method described here involves the construction and utilization of promoterless carotenoid transposons, which provides a colorimetric screen for identifying the best chromosomal integration sites for the expression of the genes of interest. The transposons (pUTmTn5::392W and pUTmTn5::392) contain the carotenoid genes required for the production of canthaxanthin and astaxanthin, respectively. Thus, when promoterless transposons insert into the host's genome, the color of the colonies will vary based on their chromosomal location. There is a correlation between the color intensity of the colonies and the expression of the carotenoid transposon. The transposon insertion site can be determined via direct chromosomal sequencing. This sequence information is used to guide the site-specific integration of biosynthetic genes and pathways of interest.

Key words: Reporter systems, Promoterless transposons, Carotenoids, Canthaxanthin, Astaxanthin, Metabolic engineering

1. Introduction

Several of the key C_{40} carotenoids, such as lycopene, β-carotene, canthaxanthin, and astaxanthin, are being produced at commercial scale. These C_{40} carotenoids are being marketed to various industries, including food additives, colorants for beverages, and feed for animal and aquaculture (1). Currently, the majority of carotenoids being sold is made through chemical synthesis or is extracted from various plant sources. However, in recent years, the interest in

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producing these commercially important C_{40} carotenoids via metabolic engineering of microbial hosts has increased significantly (2). Examples of both bacteria, primarily *Escherichia coli* and *Methylomonas* sp., and yeast (*Yarrowia lipolytica*) have been genetically engineered to produce several different carotenoids (3, 4).

The genetic engineering of microbial systems for carotenoid production requires the stable expression of key biosynthetic genes and usually involves the chromosomal integration of pathway genes. However, not every chromosomal location supports gene expression to the same degree. Here, in this work, we describe a method for constructing a carotenoid reporter system (promoterless carotenoid transposon) that enhances the identification of locations within the host's chromosome that supports optimal expression of key biosynthetic genes and pathway (2).

2. Materials

2.1. Sources of C_{40} Carotenoid Gene Clusters

1. *crtEidiXYIBZ* gene cluster from Enterobacteriaceae bacterium DC413 (GenBank accession number DQ090836).
2. *crtW* gene from *Brevundimonas vesicularis* DC263 (GenBank accession number DQ309446).
3. *crtZ* gene from *B. vesicularis* DC263 (GenBank accession number DQ309446).

2.2. DNA Primers

1. Primer MCS.F: 5′AATTCCCGGGACTAGTACGCGTGCGG CCGCCCATGGCATATGTTCGAACCCGGGTACC3′ (see Note 1).
2. Primer MCS.R: 5′GGTACCCGGGTTCGAACATATGCC ATGGGCGGCCGCACGCGTACTAGTCCCGGGAATT3′ (see Note 1).
3. Primer KnAvrIIKpnIBstBI.R: 5′-ATGCTTCGAACGGGTA CCTAGGATGGATGCGTGATCTGATCC-3′ (see Note 2).
4. Primer KnSpeI.F: 5′-TGGCTTCGAACGATGAATTGTG TCTC-3′ (see Note 2).
5. Primer 392KnSeq.R: 5′-GGGACGGCGGCTTTGTTGAA TAAATCG-3′ (see Note 3).
6. Primer 392KnSeq.F: 5′-GTAAGCATCCTGTTCGCTTGCC GCATGG-3′ (see Note 3).

2.3. Strains and Plasmids for Triparental Mating

1. *E. coli* SY327λ *pir* [Δ(*lac-pro*) *arg*E(Ap) *rec*A56 *nal*A Rfr (λ *pir*) π protein for R6Kγ *ori*] (5).
2. *E. coli* DH5α [F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-] (Invitrogen, Carlsbad, CA, USA).

3. *E. coli* DH10B [F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G] (Invitrogen, Carlsbad, CA, USA).
4. pUTmTn5*gfptet* (2).
5. pCR2.1 (Invitrogen, Carlsbad, CA, USA).

2.4. Medium and Antibiotics for Growing Strains

1. LB (Luria–Bertani): Add 10 g of bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl into 1 L of deionized water. Autoclave at 121°C for 15 min.
2. LB-agar: LB and 20 g/L agar. Autoclave at 121°C for 15 min.
3. LB/Kan^{50} agar plates (Teknova, Hollister, CA, USA).
4. LB/Cm^{25} agar plates (Teknova, Hollister, CA, USA).
5. LB/Str^{50} agar plates (Teknova, Hollister, CA, USA).
6. LB/Kan^{50}/Str^{50} (Teknova, Hollister, CA, USA).
7. LB/Amp^{100} (Teknova, Hollister, CA, USA).

2.5. Reagents, Kits, and Equipment

1. Restriction enzymes: *Xma*I, *Spe*I, *Nhe*I, *Bsp*EI, *Xho*I, *Bst*BI, *Mlu*I.
2. X-gal (5-bromo-4-chloro-3-indolyl-b-galactopyranoside) (Invitrogen, Carlsbad, CA, USA).
3. Shrimp Alkaline Phosphatase (SAP) (Affymetric USB Products, Santa Clara, CA, USA).
4. Zymoclean™ Gel DNA Recovery Kit (The Epigenetic Company, Irvine, CA, USA).
5. QIAquick Nucleotide Removal Kit (Qiagen Inc., Valencia, CA, USA).
6. QIAquick Purification Kit (Qiagen Inc., Valencia, CA, USA).
7. QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA, USA).
8. End It™ DNA End Repair Kit (Epicentre Biotechnologies, Madison, Wisconsin, USA).
9. Blue Juice™ Gel Loading Buffer (Invitrogen, Carlsbad, CA, USA).
10. High DNA Mass Ladder (Invitrogen, Carlsbad, CA, USA).
11. Epicentre EZ::TN<Kan-2>(Epicentre Biotechnologies, Madison, WI, USA).
12. Fast-Link™ DNA Ligation Kits (Epicentre Biotechnologies, Madison, WI, USA).
13. 0.7% TBE agarose gels.
14. 0.8% TBE agarose gels.
15. 10× TBE buffer (pH 8.3): Weight 86.4 g of Tris, 44 g boric acid, and 7.36 g of EDTA. Add water and dissolve. Bring to

800 mL with water. For 1× working solution, dilute stock solution 1:10 with sterilized water.

16. Single edge razor.
17. Gel pulser cuvettes (1 mm).
18. Falcon 2059 test tube 14-mL polypropylene round bottom (Becton Dickinson Labware, Franklin Lakes, NJ, USA).
19. Screw-capped freezer vials.
20. Ice/Ice bucket.
21. Sorvall Super T21 with ST-H750 rotor (Sorvall, Newport Pagnell, Buckinghamshire, England).
22. NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).
23. Gradient PCR machine.
24. PCR machine.
25. Gene Pulser.
26. Low UV transilluminator.
27. Incubator.
28. Shaking incubator.
29. Water bath.
30. Microcentrifuge.
31. Gel apparatus.
32. Power supply.

3. Methods

Promoter probe transposons containing various reporter genes, such as *lac*Z, *lux*AB, and *xyl*E have been used to study the expression of foreign genes insert randomly into its host genome (6). To metabolically engineer *E. coli* for the production of C_{40} carotenoids, a promoterless transposable element based on the Tn5 minitransposon (7, 8) that contain the β-carotene synthetic genes *crtEYIB* (including the isoprenoid gene *idi*) and the β-modification genes *crtWZ* was constructed and used to determine the which chromosomal locations support the best expression of the carotenoid gene cluster (2, 3, 9, 10). The following method can be used to construct the promoter-probe vectors, pUTmTn5-392WKn and pUTmTn5-392Kn, which contain the *crtWEidiYIB* and *crtWZEidiYIB* gene clusters, respectively. The expression of these clusters is necessary for the production of canthaxanthin and astaxanthin.

3.1. Cloning a Multiple Cloning Site into pUTmTn5 Transposon Vector

To increase the number of restriction sites available for cloning into the pUTmTn5*gfptet* transposon vector, add a multiple cloning site (MCS) between the inside end (IE) and outside end (OE) (Fig. 1).

1. Digest pUTmTn5 with *Xma*I using the following digestion mixture: 10 μL of plasmid DNA (~50 ng/μL), 7.5 μL of 10× buffer, 0.75 μL of BSA, 3.0 μL of *Xma*I, and 53.75 μL of sterile dH_2O in a total reaction volume 75 μL.
2. Incubate the digestion mixture at 37°C in a water bath for ~2 h.
3. To dephosphorylate, add 1 U of SAP for every 1 pmol of DNA ends directly to the *Xma*I digestion reaction.
4. Incubate the SAP reaction at 37°C for 60 min.
5. Inactivate/stop the dephosphorylation reaction by heating the reaction at 65°C for 15 min.

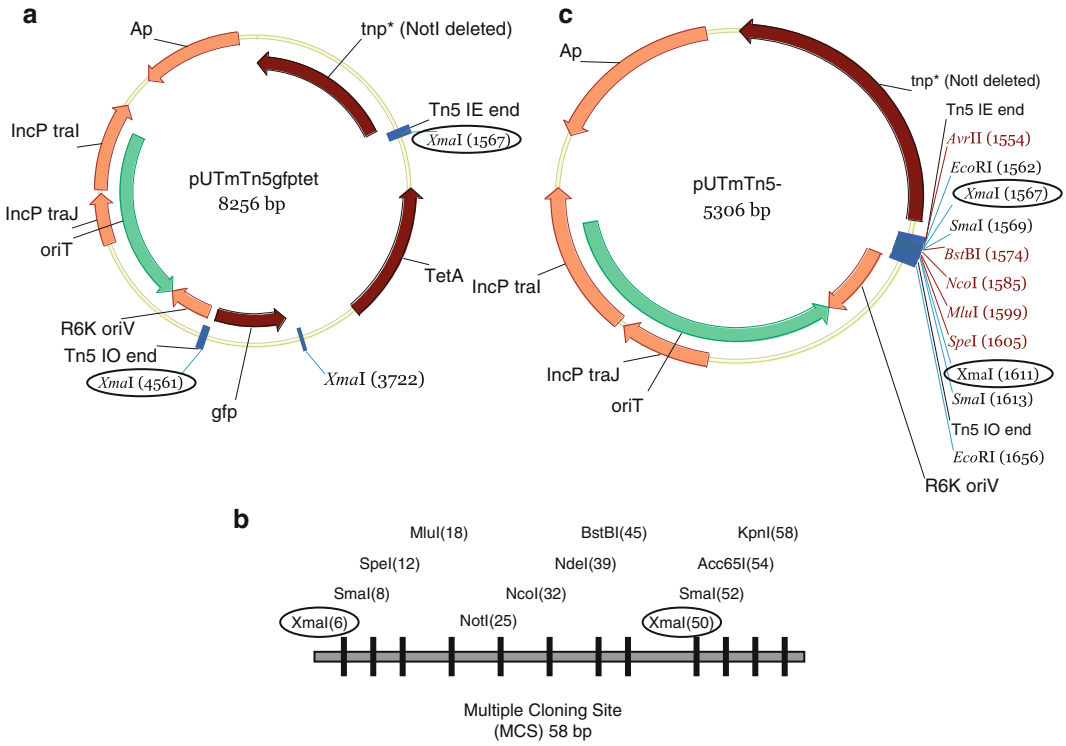

Fig. 1. Preparation of the pUTmTn5 vector backbone for the construction of the promoterless carotenoid transposon. (**a**) Digestion of pUTmTn5gfptet with *Xma*I releases the *gfp* and *tet*A genes from between the Tn5 transposon ends (Tn5 OE and Tn5 IE). (**b**) Formation of a Multiple Cloning Site (MCS) by annealing two 58-nucleotide primers. Twelve restriction sites will make-up the MCS. The restriction sites include *Xma*I, *Sma*I, *Spe*I, *Mlu*I, *Not*I, *Nco*I, *Nde*I, *Bst*BI, *Xma*I, *Sma*I, *Acc*65I, and *Kpn*I. The MCS is digested with *Xma*I. The *Xma*I sites are circled. (**c**) Ligation of the MCS with the *Xma*I-digested pUTmTn5gfptet vector DNA. The resulting vector is pUTmTn5–.

6. Separate the dephosphorylated DNA fragments on a 0.7% TBE agarose gel. As a digestion control, include a lane on the gel of the undigested pUTmTn5 vector.
7. Stain the agarose gel for about 10 min in an ethidium bromide bath.
8. Excise the digested vector fragment from the gel using a single edge razor and a low UV transilluminator to visualize the DNA fragment.
9. To purify the *Xma*I-digested pUTmTn5 using the Zymoclean™ Gel DNA Recovery Kit, add 3 volumes of Agarose Dissolving Buffer to the *Xma*I-digested pUTmTn5 DNA gel sample. Process the DNA sample using a 1.5-mL microcentrifuge tube.
10. Incubate the sample at 55°C for 10 min or until the agarose gel slice dissolves.
11. Transfer the DNA sample to a Zymo-Spin™ Column in a Collection Tube.
12. Centrifuge the spin column at 10,000 × *g* in a microcentrifuge for 30 s.
13. Discard the flowthrough.
14. Add 200 μL of Wash Buffer to the spin column.
15. Centrifuge the spin column at 10,000 × *g* in a microcentrifuge for 30 s.
16. Repeat the wash step.
17. Add 6 μL of water to the column matrix.
18. Transfer the column to a new 1.5-mL microcentrifuge tube.
19. Elute the DNA by centrifugation at 10,000 × *g* for 30 s.
20. Determine the DNA concentration using NanoDrop 1000 spectrophotometer.

To expand the restriction sites available for cloning, add a multiple cloning site (MCS) with eight additional restriction sites to pUTmTn5.

1. Adjust the primers MCS.F and MCS.R concentrations to 100 pmol/μL.
2. To anneal the primers, add 20 μL of each primer to a PCR microfuge tube.
3. Add 60 μL sterile dH_2O to the mixture.
4. Heat the primer–water mixture to 100°C for 5 min.
5. Gradually cool the mixture to 40°C over a 20-min period (after heating to 100°C, turn off the heat block).
6. Transfer the tube to ice.
7. To digest the annealed DNA primers with *Xma*I, prepare the following mixture: 20 μL of annealed DNA primers, 45 μL of

sterile dH_2O, 7.5 μL 10× buffer, 0.75 μL BSA, and 1.0 μL *Xma*I, in a total reaction volume of 75 μL.

8. Incubate the digestion reaction at 37°C in a water bath for about 3 h.
9. To purify the *Xma*I-digested MCS DNA fragment using the QIAquick Nucleotide Removal Kit, add 10 volumes of Buffer PN to 1 volume of the reaction sample.
10. Mix the solution by moving the micro-centrifuge tube back and forth.
11. Place a QIAquick spin column in the 2-mL collection tube provide in the kit.
12. Apply the sample to the QIAquick column.
13. Centrifuge the column for 60 s at 6,000×*g* in a microcentrifuge.
14. Discard the flowthrough.
15. Put the QIAquick column back into the same collection tube.
16. Wash the column using 750 μL of Buffer PE.
17. Discard the flowthrough.
18. Place the QIAquick column back into the empty collection tube.
19. Centrifuge the column for an additional 60 s at 13,000×*g* in a microcentrifuge.
20. Place the QIAquick column into a clean 1.5-mL microcentrifuge tube.
21. Add 50 μL of Buffer EB to the center of the QIAquick membrane to elute the DNA.
22. Let the column stand for 60 s.
23. Centrifuge the column for 60 s at 13,000×*g* in a microcentrifuge.
24. Determine the DNA concentration using NanoDrop 1000 spectrophotometer.
25. To ligate the MCS into the *Xma*I site of pUTmTn5, set up several ligation reactions with various insert to vector ratios. Reaction #1: 9.75 μL of H_2O, 1.5 μL of ATP, 1.5 μL of ligase buffer, 0.25 μL of vector DNA (40 ng/μL), 0.25 μL of insert DNA (10 ng/μL), and 1.0 μL of ligase in a total volume of 15.0 μL. Reaction #2: 8.75 μL of H_2O, 1.5 μL of ATP, 1.5 μL of ligase buffer, 0.25 μL of vector DNA (40 ng/μL), 2.0 μL of insert DNA (10 ng/μL), and 1.0 μL of ligase in a total volume of 15.0 μL. Reaction #3: 10.75 μL of H_2O, 1.5 μL of ATP, 1.5 μL of ligase buffer, 0.25 μL of vector DNA (40 ng/μL), 0.0 μL of insert DNA (10 ng/μL), and 1.0 μL of ligase in a total volume of 15.0 μL.

26. Incubate the ligation reactions for 5 min at room temperature.
27. Inactivate the ligation reaction by heating the mixture for 15 min at 70°C.

3.2. Preparation of E. coli SY327 Electrocompetent Cells

The pUTmTn5 vector is a conditional replicating plasmid. It has a R6K origin of replication, thus an *E. coli* host that expresses the lambda *pir* gene is required for propagation of the vector. The *E. coli* strain used in this work is strain SY327.

1. Inoculate a single colony into 6 mL of LB broth to generate the seed culture.
2. Use 3 mL of the seed culture to inoculate 200 mL of LB broth in a 500-mL shake flask.
3. Incubate the SY327 cells at 37°C with aeration.
4. Harvest cells at an OD_{600} between 0.5 and 0.7 by centrifugation at 4°C at 4,000 × *g*.
5. Discard the supernatant.
6. Resuspend the cells into 100 mL of ice-cold H_2O, centrifuge at 4,000 × *g* for 15 min, and discard the supernatant.
7. Resuspend the cells into 50 mL of ice-cold H_2O containing glycerol H_2O, centrifuge at 4,000 × *g* for 15 min, and discard the supernatant.
8. Resuspend the cells into 10 mL of ice-cold H_2O containing glycerol H_2O, centrifuge at 4,000 × *g* for 15 min, and discard the supernatant.
9. Resuspend the cells into 1 mL of ice-cold H_2O containing glycerol H_2O, centrifuge at 4,000 × *g* for 15 min, and discard the supernatant.
10. Transfer 150 μL aliquots of the cells into screw-capped freezer vials.
11. Quick-freeze the competent cells using liquid nitrogen and store at –80°C.

3.3. Transformation of the pUTmTn5 + MCS Ligation Reaction into E. coli SY327 Electrocompetent Cells

1. Thaw tubes of competent cells onto ice.
2. Place the 1-mm Gene Pulser cuvettes and the 1.5-mL microcentrifuge tubes onto ice for at least 10 min.
3. Transfer 0.5 μL and 1.0 μL of the DNA ligation reaction from Subheading 3.2 to two mircrocentrifuge tubes.
4. Add 30 μL of the SY327 cells into the tubes containing the ligation reaction. Mix gently by flicking the side of the microcentrifuge tubes. Don't generate bubbles.
5. Transfer the mixture of cells and DNA into the ice-cold cuvette.
6. Set the metrics [voltage (1.8 kV), resistance (200 ohms (Ω)) and capacitance (25 micro Faradays (μF))] of the electroporator.

7. Electroporate the cells. Press both button of the pulser at the same time for ~10 s.
8. Immediately following electroporation, add 400 μL of the SOC recovery medium into the cuvette. Mix by pipetting.
9. Transfer the cells into a 14-mL polypropylene round bottom test tube (Falcon 2059) and incubate the cells at 37°C with aeration (250 rpm) for 90 min.
10. Plate 50 μL and 100 μL the recovered cells onto LB + Amp^{100} agar plates.
11. Incubate the plates at 37°C for several days.

3.4. Evaluation of Colonies (Transformants) Having the pUTmTn5 Vector with the Correct Insert DNA Fragment

1. To isolation of plasmid DNA using the QIAprep Spin Miniprep Kit, streak or patch isolated colonies from Subheading 3.3 onto fresh LB + Amp^{100} agars plates and incubate plates at 37°C for several days.
2. Isolate the plasmid DNA for 5–10 patches (isolates) using the QIAprep Spin Miniprep Kit.
3. Use an inoculating loop to transfer and resuspend a large loop of cells into a 1.5-mL microcentrifuge tube containing 250 μL of Buffer P1. Vortex the cells well to ensure that no clumps of cells remain.
4. Add 250 μL of Buffer P2 and mix well by inverting the tubes 4–6 times. Mix by inverting the tube. Do not vortex at this step, which will result in shearing of the DNA. (Note at this step of the reaction should not exceed 5 min).
5. Add 350 μL of Buffer N3. Mix well and immediately by inverting the tubes 4–6 times.
6. Centrifuge the solution at 13,000 × *g* in the microcentrifuge for 10 min.
7. Transfer the supernatants to a QIAprep spin column.
8. Centrifuge at 13,000 × *g* for 60 s. Discard the flowthrough.
9. Add 500 μL of Buffer PE to the QIAprep spin column to wash the DNA on the column. Centrifuge the column for 60 s at 13,000 × *g*. Discard the flowthrough.
10. Centrifuge the column again for 60 s to remove any remaining wash buffer. This is an important step because any residual ethanol may interfere with subsequent enzymatic reactions.
11. Transfer the QIAprep columns to clean 1.5-mL microcentrifuge tubes.
12. Add 50 μL of Buffer EB to the center of each QIAprep spin column.
13. Let the column stand for 60 s and then centrifuge the QIAprep column for 60 s to elute the plasmid DNA.

14. To digest the plasmid DNA with *Spe*I and *Nhe*I, prepare the following digestion mixture: 5 μL of plasmid DNA, 2.0 μL of 10× buffer, 0.2 μL of BSA, 0.5 *Spe*I (20/μL), 0.5 μL of *Nhe*I (20U/ μL), and 11.8 μL sterile dH_2O to a total reaction volume of 20 μL.
15. Incubate the reaction for 1.5 h at 37°C in a water bath.
16. Separate the DNA fragments on a 0.7% TBE agarose gel. As a digestion control, include a lane on the gel of the undigested pUTmTn5 vector.
17. Stain the agarose gel for ~10 min in an ethidium bromide bath.
18. Excise the digested vector fragment from the gel using a single edge razor (use a low UV transilluminator to visualize the DNA fragment).
19. There should be two DNA fragments with a predicted size of ~4.2 kb and 1.1 kb.
20. To confirm the orientation of the MCS via DNA sequencing, use DNAsequencingprimerspUTmTn5Seq.FandpUTmTn5Seq.R to determine the DNA sequence of two or three candidate plasmids that have the correct restriction patterns.
21. Name the vector pUTmTn5+ or pUTmTn5– depending on the orientation of the MCS.

3.5. Cloning the Promoterless Carotenoid Gene Cluster from pDCQ392W and pDCQ392 into pUTmTn5 Transposon Vector

Please refer to Subheadings 3.3 and 3.4 of Chapter 7 for the methods used to assemble gene clusters pDCQ392W and pDCQ392.

1. To prepare the carotenoid plasmids (pDCQ393W and pDC392) ligation into the pUTmTn5 transposon vector, digest the plasmids with *Bsp*EI and *Spe*I (Fig. 2a).
2. To digest pDCQ392W and pDCQ392 with *Bsp*EI, set up the following digestion reaction (see Note 4). 1× Reaction: 5.0 μL of plasmid DNA (50 ng/ μL), 2.0 μL of 10× buffer, 0.2 μL of BSA, 1.0 μL *Bsp*EI (10 U/μL), and 11.8 μL of sterile dH_2O in a total reaction volume of 20.0 μL. 10× Reaction: 50.0 μL of plasmid DNA (5.0 ng/ μL), 20.0 μL of 10× buffer, 2.0 μL of BSA, 10.0 μL *Bsp*EI (10 U/μL), and 118.0 μL of sterile dH_2O in a total reaction volume of 200.0 μL.
3. Incubate the reaction at 37°C in a water bath for 2 h.
4. To purify the *Bsp*EI digested plasmids (pDCQ392W and pDCQ392), add 400 μL of Buffer PBI from the QIAquick purification kit to the 200 μL *Bsp*EI digestion reaction. Pipette to mix (see Note 5).
5. Put the QIAquick spin column into a 2-mL collection tube that is provided in the kit.
6. Apply the sample to the column and centrifuge for 60 s. The DNA will bind to the QIAquick column.

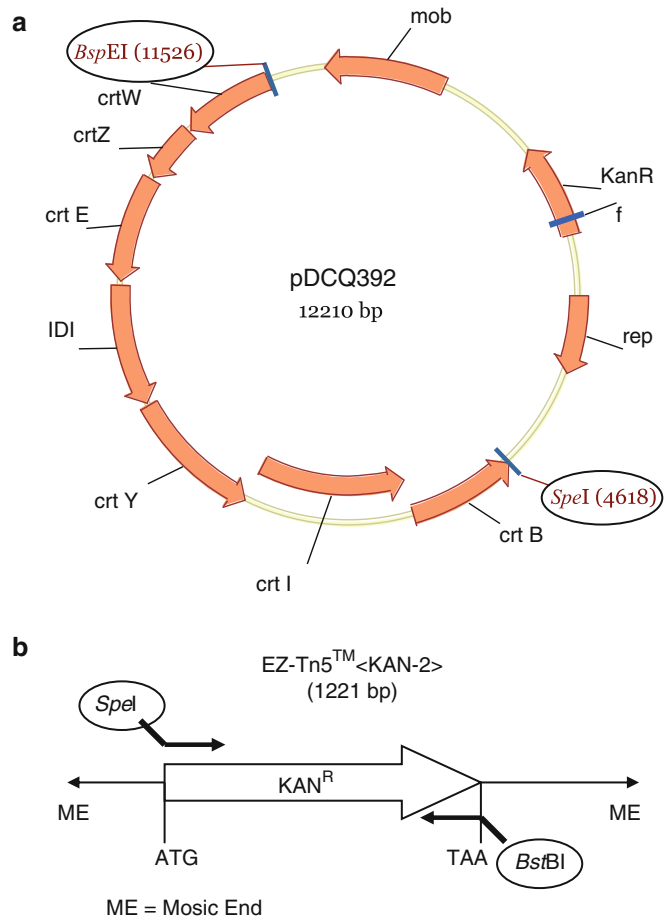

Fig. 2. Preparation of the crt gene cluster for the construction of the promoterless carotenoid transposon. (**a**) The promoterless carotenoid gene cluster is provided by the digestion of pDCQ392 with *Bsp*EI and *Spe*I. The genes present within the carotenoid gene cluster are *crt*W, *crt*Z, *crt*Y, *crt*I, and *crt*B. The isoprenoid gene, *idi*, is also present within the cluster. (**b**) The kanamycin resistance gene is provided through the PCR amplification of the kanamycin gene from the EZ-Tn5™ < KAN-2 > transposon. The PCR primers used are KnBstB.R and KnSpeI.F.

7. Discard the flowthrough.
8. Place the column back into the 2-mL collection tube.
9. Add 750 µL of Buffer PE to the QIAquick column to wash the DNA.
10. Centrifuge the sample for 60 s.
11. Discard the flowthrough.
12. Place the column back into the 2-mL collection tube and centrifuge for an additional 60 s (see Note 6).
13. Place the QIAquick column into a clean 1.5-mL microcentrifuge tube.

14. Elute the DNA from column. Add 30 μL of TE buffer to the center of the QIAquick member and let the column stand for 60 s.
15. Centrifuge the column for 60 s.
16. To blunt-end the *Bsp*EI digested DNA, set-up the following DNA End Repair reaction in a 1.5-mL microcentrifuge tube: 28.0 μL of the *Bsp*EI digested DNA, 5.0 μL of the 10× End-Repair buffer, 5.0 μL of the dNTP mix, 5.0 μL of ATP, 1.0 μL of end-repair enzyme mix, and 6.0 μL of sterile dH_2O in a total reaction volume of 50.0 μL.
17. Incubate the reaction at room temperature for 45 min.
18. Stop the reaction by heating at 70°C for 10 min.
19. To purify the blunt-ended *Bsp*EI digested plasmids, add 100 μL of Buffer PBI from the QIAquick purification kit to the 50 μL blunt-ened *Bsp*EI digestion reaction. Pipette to mix.
20. Repeat steps 5 through 13.
21. To digest the blunt-ended *Bsp*EI digested pDCQ392W and pDCQ392 plasmid DNA with *Spe*I, set up the following digestion reactions: 30 μL of the blunt-ended *Bsp*EI digested DNA, 3.5 μL of 10× buffer, 0.3 μL of BSA, 1.0 μL of SpeI (10 U/μL), and 0.2 μL of sterile dH_2O in a total reaction volume of 35.0 μL.
22. Incubate the reaction at 37°C in a water bath for 2 h.
23. Stop the digestion reaction by heating at 65°C for 10 min.
24. To separate the blunt-end *Bsp*EI digested and *Spe*I digested DNA fragments of pDQ392W and pDQC392 via gel electrophoresis, place the preparative 0.7% TBE agarose gel into the gel box.
25. Fill the gel box with TBE buffer until the gel is submerged.
26. Load lambda *Hin*dIII digested DNA ladder (size marker DNA) in lane one.
27. Add 2 μL of Blue Juice™ tracking dye to each of the DNA.
28. Load the pDQC392W digested DNA sample into lanes two and three.
29. Load the pDQC392 digested DNA sample into lanes four and five.
30. Replace the gel box top, which also attaches the leads. Attach the other end of the leads to the power supply.
31. Run the gel at 100 V for 90 min. Check to make sure that the DNA is moving in the correct direction.
32. To visualize the DNA fragments on a low UV transilluminator.
33. For the pDCQ392W and the pDCQ392 samples, excise the larger DNA fragments from the gel using a single edge razor (see Note 7).

34. To purify the pDCQ392W and pDCQ392 DNA fragments using the Zymoclean™ Gel DNA Recovery Kit, add 3 volumes of Agarose Dissolving Buffer. Process the DNA sample using a 1.5-mL microcentrifuge tube.
35. Incubate the sample at 55°C for 10 min or until the agarose gel slice dissolves.
36. Transfer the DNA sample to a Zymo-Spin™ Column in a Collection Tube.
37. Centrifuge the spin column at 10,000 × *g* in a microcentrifuge for 30 s.
38. Discard the flowthrough.
39. Add 200 μL of Wash Buffer to the spin column.
40. Centrifuge the spin column at 10,000 × *g* in a microcentrifuge for 30 s.
41. Repeat the wash step.
42. Add 6 μL of water to the column matrix.
43. Transfer the column to a new 1.5-mL microcentrifuge tube.
44. Elute the DNA by centrifugation at 10,000 × *g* for 30 s.
45. Determine the DNA concentration using NanoDrop 1000 spectrophotometer. The prepared carotenoid DNA fragment from pDCQ392W and pDCQ392 will be used in a ligation reaction with the prepared pUTmTn5 vector DNA fragment (Subheadings 3.6 and 3.8).

3.6. Cloning the Kanamycin Resistance Gene from the Epicentre EZ::TN < Kan-2 > into pUTmTn5 Transposon Vector

PCR amplification of the kanamycin resistance gene from EZ-TN™ < Kan-2>

1. To amplify the kanamycin gene present in the EZ::TN < KAN2 > transposon, use PCR primers KnBstBI.R and KanSpeI.F (Fig. 2b).
2. Assemble the following PCR mixture for each PCR reaction: 1× Reaction: 0.5 μL of EZ-TN™ < KAN2 > DNA, 45.0 μL of Supermix HF, 1.0 μL of Primer KnBstBI.R (20 μM), 1.0 μL of KnSpeI.F (20 μM), and 2.5 μL of sterile dH_2O for a total reaction volume of 50 μL.
3. Distribute 20.0 μL of the batch PCR mix into seven thin-wall PCR tubes.
4. Cover the PCR tubes with PCR caps.
5. Place the PCR tubes in to the gradient PCR machine in the following columns: Column 4 at 51.8°C, Column 6 at 54.2°C, Column 8 at 56.9°C, Column 9 at 58.4°C, Column 10 at 59.4°C, Column 11 at 59.8°C, and Column 12 at 60.1°C.
6. To check the efficiency of the PCR reaction by gel electrophoresis, load 3 μL of each PCR reaction on a 0.8% TBE agarose gel.

7. Fill the gel box with TBE buffer until the gel is submerged.
8. Load lambda *Hin*dIII digested DNA ladder (size marker DNA) in lane one.
9. Add 2 μL of Blue Juice™ tracking dye to each of the DNA .
10. Load the pDQC392W digested DNA sample into lanes two and three.
11. Load the pDQC392 digested DNA sample into lanes four and five.
12. Replace the gel box top, which also attaches the leads. Attach the other end of the leads to the power supply.
13. Run the gel at 100 V for 60 min. Check to make sure that the DNA is moving in the correct direction.
14. To visualize the PCR products, place the agarose gel on a UV transilluminator.
15. To clone the desirable PCR products into a TOPO™ vector, set-up the following TOPO reaction: 1.0 μL of fresh PCR product, 1.0 μL of dilute salt solution, 1.0 μL of TOPO vector, and 3.0 μL of sterile dH_2O to a final reaction volume of 6.0 μL.
16. Mix gently and incubate the reaction components for 5 min at room temperature.
17. Place the TOPO cloning reaction on ice.
18. Prepare for the TOPO transformation reaction. Warm the SOC medium to room temperature. Warm the LB/Kan^{50} agar plates at 37°C for 30 min. Spread 40 μL of X-gal (40 mg/mL) onto each LB/Kan^{50} agar plate and incubate the plates at 37°C until time to plate the transformation mixture. Thaw on ice one vial of One Shot cells for each transformation.
19. Add 2 μL of the TOPO Cloning reaction into one vial of the One Shot chemically competent *E. coli* cells and mix gently.
20. Incubate the transformation on ice for 15 min.
21. Heat-shock the cells by placing in a water bath heated to 42°C for 30 s. Do not shake.
22. Immediately transfer the tubes to ice.
23. Add 250 μL of the room temperature SOC medium.
24. Cap the tube tightly and shake the tube horizontally at 37°C for 60 min.
25. Plate 10 μL and 50 μL of the transformation mixture onto LB/Kan^{50}/X-gal agar plate and incubate plates overnight at 37°C.
26. Streak five white colonies for plasmid isolation onto LB/Kan^{50} agar plates and incubate the plates overnight at 37°C (see Note 8).

27. Isolate the plasmid DNA using the QIAprep Spin Miniprep kit. Follow steps 2–13 as described in Subheading 3.3.
28. To analyze the candidate plasmids, digest the DNA with *Xho*I using the following digestion mixture: 5.0 μL of miniprep DNA, 2.0 μL of 10× buffer, 0.2 μL of BSA, 1.0 μL of *Xho*I (10U/μL), and 11.8 μL for a total reaction volume of 20.0 μL.
29. Incubate the reaction for 1.5 h at 37°C in a water bath.
30. Separate the DNA fragments on a 0.8% TBE agarose gel (see Note 9).
31. Name the TOPO vector having the correct insert DNA fragment in the reverse orientation pCR2.1Kn^R.
32. To prepare the kanamycin resistance gene for ligation into the pUTmTn5 transposon vector,digest pCR2.1Kn^R with *Bst*BI and *Spe*I in the following digestion reaction: 1× reaction: 5.0 μL of the pCR2.1Kn^R (50 ng/μL), 2.0 μL of 10× buffer, 0.2 μL BSA, 1.0 μL of *Bst*BI (10 U/μL), 1.0 μL *Spe*I (10 U/μL), and sterile dH_2O for a total reaction volume of 20.0 μL.
33. Incubate the reaction at 37°C in a water bath for 2 h.
34. To separate the *Spe*I and *Bst*BI digested DNA fragments of pCR2.1Kn^R via gel electrophoresis, follow steps 35–40 below.
35. Fill the gel box with TBE buffer until the gel is submerged.
36. Load lambda *Hin*dIII digested DNA ladder (size marker DNA) in lane one.
37. Add 2 μL of Blue Juice™ tracking dye to 20 μL of the *Spe*I and *Bst*BI digested pCR2.1Kn^R DNA sample.
38. Load the DNA sample onto a 0.8% TBE agarose gel.
39. Replace the gel box top, which also attaches the leads. Attach the other end of the leads to the power supply.
40. Run the gel at 100 V for 60 min. Check to make sure that the DNA is moving in the correct direction.
41. To visualize the PCR products, place the agarose gel on a UV transilluminator.
42. Excise the smaller DNA fragment from the gel using a single edge razor for the pCR2.1Kn^R DNA sample. The pCR2.1Kn^R DNA fragment containing the kanamycin resistance genes is ~1.0 kb.
43. To purify the pCR2.1Kn^R Kanamycin resistance DNA fragment, use the Zymoclean™ Gel DNA Recovery Kit
44. Add 3 volumes of Agarose Dissolving Buffer to the excised *pCR2.*1Kn^R DNA gel sample. Process the DNA sample using a 1.5-mL microcentrifuge tube.
45. Incubate the sample at 55°C for 10 min or until the agarose gel slice dissolves.

46. Transfer the DNA sample to a Zymo-Spin™ Column in a Collection Tube.
47. Centrifuge the spin column at 10,000 × *g* in a microcentrifuge for 30 s.
48. Discard the flowthrough.
49. Add 200 μL of Wash Buffer to the spin column.
50. Centrifuge the spin column at 10,000 × *g* in a microcentrifuge for 30 s.
51. Repeat the wash step.
52. Add 6 μL of water to the column matrix
53. Transfer the column to a new 1.5-mL microcentrifuge tube.
54. Elute the DNA by centrifugation at 10,000 × *g* for 30 s.
55. Determine the DNA concentration using NanoDrop 1000 spectrophotometer.
56. The prepared kanamycin resistance DNA fragment from *pCR2.1KnR* will be used in a ligation reaction with the prepared pUTmTn5 vector DNA fragment (Subheading 3.7) and the prepared pDCQ392W and pDCQ392 cartenoid DNA fragments (Subheading 3.5) in Subheading 3.8.

3.7. Prepare the Transposon Vector (pUTmTn5) for Digestion with the First Restriction Endonuclease (MluI)

The digestion of pUTmTn5 with restriction endonucleases *Bst*BI and *Mlu*I will be carried out in separate reactions because the ends of the *Mlu*I digested DNA fragment will be blunt-ended prior to digestion with the second restriction enzyme.

1. To digest pUTmTn5 with *Mlu*I, set-up the following digestion reaction: 5.0 μL of pUTmTn5 (50 ng/μL), 2.0 μL of 10× buffer, 0.2 μL of BSA, 1.0 μL of *Mlu*I, and 11.8 μL of sterile dH_2O for a final reaction volume of 20.0 μL.
2. Incubate the reaction at 37°C in a water bath for 2 h.
3. To blunt-end the *Mlu*I digested DNAs, use the End It™ DNA End Repair Kit.
4. To the 28.0 μL of purified *Mlu*I digested DNA, add the following digestion components: 5.0 μL of 10× End-Repair buffer, 5.0 μL of dNTP mix, 5.0 μL of ATP, 1.0 μL of End-Repair Enzyme mix and 6.0 μL of sterile dH_2O in a total reaction volume of 50.0 μL.
5. Incubate the reaction at room temperature for 45 min.
6. Stop the reaction by heating at 70°C for 10 min.
7. To purify the blunt-ended *Mlu*I digested pUTmTn5 vector DNA, add 100 μL of Buffer PBI from the QIAquick purification kit to the 50 μL blunt-ended *Mlu*I digestion reaction. Pipette to mix.

8. Check to see if the color of the mixture remains yellow. If the mixture is orange or violet, add 10 μL of 3 M sodium acetate (pH 5) to the sample. Pipette to mix.
9. Put the QIAquick spin column into a 2-mL collection tube that is provided in the kit.
10. Apply the sample to the column and centrifuge for 60 s. The DNA will bind to the QIAquick column.
11. Discard the flowthrough.
12. Place the column back into the 2-mL collection tube.
13. Add 750 μL of Buffer PE to the QIAquick column to wash the DNA.
14. Centrifuge the sample for 60 s.
15. Discard the flowthrough.
16. Place the column back into the 2-mL collection tube and centrifuge for an additional 60 s (see Note 6).
17. Place the QIAquick column into a clean 1.5-mL microcentrifuge tube.
18. Elute the DNA from column. Add 30 μL of TE buffer to the center of the QIAquick member and let the column stand for 60 s.
19. Centrifuge the column for 60 s.
20. To digest the blunt-ended *Mlu*I digested pUTmTn5 DNA with *Bst*BI, set-up the following digestion reaction: 30 μL of the blunt-ended *Bst*I digested DNA, 3.5 μL of 10× buffer, 0.3 μL of BSA, 1.0 μL of *Bst*BI (10 U/μL), and 0.2 μL of sterile dH_2O in a 35 μL total reaction volume.
21. Incubate the reaction at 37°C in a water bath for 2 h.
22. Stop the digestion reaction by heating at 65°C for 10 min.
23. To purify the DNA using the QIAquick Nucleotide Removal Kit, add 10 volumes of Buffer PN to 1 volume of the reaction sample.
24. Mix the solution by moving the microcentrifuge tube back and forth.
25. Place a QIAquick spin column in the 2-mL collection tube provide in the kit.
26. Apply the sample to the QIAquick column.
27. Centrifuge the column for 60 s at 6,000×*g* in a microcentrifuge.
28. Discard the flowthrough.
29. Put the QIAquick column back into the same collection tube.
30. Wash the column using 750 μL of Buffer PE.
31. Discard the flowthrough.

32. Place the QIAquick column back into the empty collection tube.
33. Centrifuge the column for an additional 60 s at 13,000 × *g* in a microcentrifuge.
34. Place the QIAquick column into a clean 1.5-mL microcentrifuge tube.
35. Add 50 μL of Buffer EB to the center of the QIAquick membrane to elute the DNA.
36. Let the column stand for 60 s.
37. Centrifuge the column for 60 s at 13,000 × *g* in a microcentrifuge.
38. Determine the DNA concentration using NanoDrop 1000 spectrophotometer. The prepared pUTmTn5 vector DNA will be used in a ligation reaction with the prepared carotenoid insert DNA from pDCQ3929W and pDCQ392 (Subheadings 3.5 and 3.8).

3.8. Ligation of the Linearized Transposon Vector, pUTmTn5, the Insert DNA Fragment Containing the Kanamycin Resistance Gene from pCR2.1Kn^R and the Insert DNA Fragments Containing the Promoterless Carotenoid Genes from pDCQ392W and pDCQ392

To ligate the pUTmTn5 vector DNA fragment to either the pDCQ392W or pDCQ392 DNA fragments, each of which contains a promoterless set of carotenoid genes and the kanamycin resistance gene, use the Epicentre FastLink™ DNA Ligation Kit. The carotenoid genes present on the pDCQ392W fragment are *crt*WE*idi*YIB and when expressed from a suitable promoter produces canthaxanthin. In contrast, the genes present on the pDCQ392 fragment are *crt*WZE*idi*YIB. When expressed the *crt*WZE*idi*YIB gene cluster synthesizes of astaxanthin. The ligation of pUTmTn5 with the *crt*WE*idi*YIB gene cluster will result in the generation a promoterless canthanxanthin transposon vector that should be named pUTmTn5::392W. A promoterless astaxanthin transposon will also be constructed through the ligation pUTmTn5 with the promoterless *crt*WZE*idi*YIB gene cluster. This plasmid should be named pUTmTn5::392 (Fig. 3).

1. To ligate [pUTmTn5 + pDCQ392W + Kan] and [pUTmTn5 + pDCQ392+ Kan], set-up the following ligation reaction: 1.5 μL of vector DNA (pUTmTn5), 1.5 μL of insert DNA #1 (Kan^R), 1.5 μL of insert DNA #2 (pDCQ392W or pDCQ392), 1.5 μL of 10 mM ATP, 1.5 μL of 10× Fast-Link Ligation buffer, 1.0 μL of Fast Link DNA ligase, and 5.0 μL of sterile dH_2O in a 15.0 μL total reaction volume (see Note 10).
2. Incubate the ligation reaction for 15 min at room temperature.
3. Inactivate the Fast-Link DNA ligase by heating the reaction to 70°C for 15 min in a water bath.
4. Centrifuge the ligase reaction in microcentrifuge to remove the condensation from the lid.

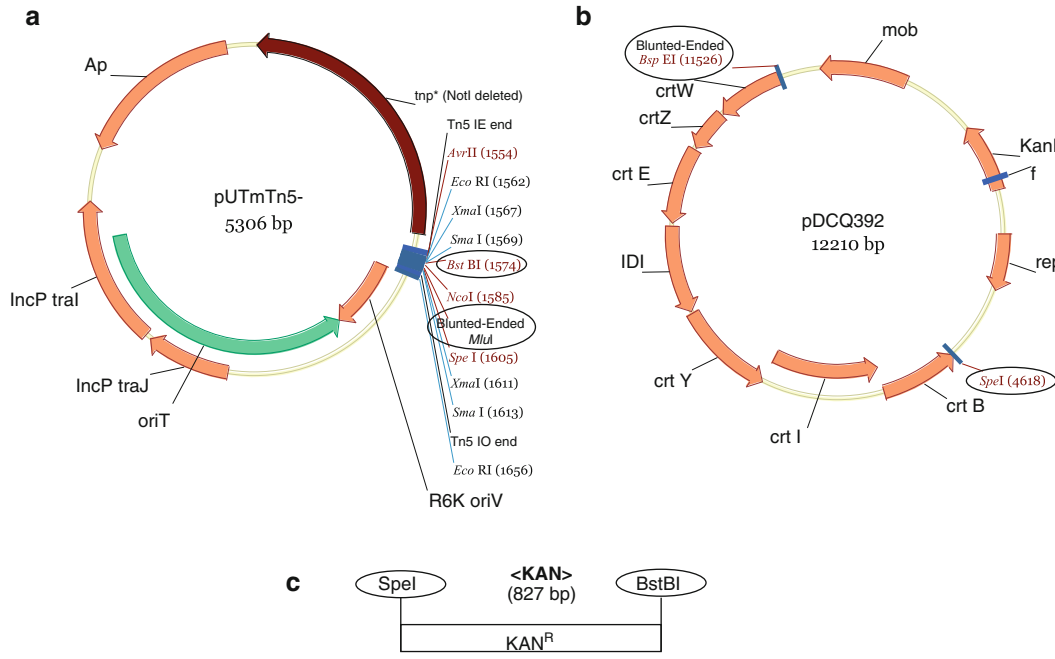

Fig. 3. Components of the ligation reaction in the formation of pUTmTn5::392. (**a**) The pUTmTn5– vector is prepared for the ligation reaction by digestion with *Bst*BI and *Mlu*I. The plasmid is digested with *Mlu*I first and blunt-ended prior to digestion with *Bst*I. (**b**) The carotenoid genes were prepared for the ligation reaction by digestion of pDCQ392 with *Spe*I and *Bsp*EI. Digestion of pDCQ392 occurs first with *Bsp*EI, which is blunt-ended prior to digestion with *Spe*I. (**c**) The kanamycin gene is prepared for the ligation reaction by the digestion of the kanamycin PCR product with *Spe*I and *Bst*BI.

5. Transform *E. coli* SY327 electrocompetent cells with 1.5 μL of the ligation reaction accord to the method outlined in Subheading 3.3.
6. Plate the transformation mixture on LB/Kan^{50} agar plates and incubate the plates at 37°C.
7. Inoculate individual colonies into 5 mL of LB/Kan^{50} broth and grow the cultures overnight with aeration (shaking at 250 rpm).
8. Harvest the cells by centrifugation at 4,000 × *g* for 10 min. Discard the supernatant.
9. To isolate the plasmid DNA using the QIAprep Spin Miniprep Kit, resuspend the pellet in 250 μL of Buffer P1. Vortex the cells well to ensure that no clumps of cells remain.
10. Add 250 μL of Buffer P2 and mix well by inverting the tubes 4–6 times. Mix by inverting the tube. Do not vortex at this step, which will result in shearing of the DNA.
11. Add 350 μL of Buffer N3. Mix well and immediately by inverting the tubes 4–6 times.
12. Centrifuge the solution at 13,000 × *g* in the microcentrifuge for 10 min.

13. Transfer the supernatants to a QIAprep spin column.
14. Centrifuge at 13,000 × *g* for 60 s. Discard the flow-though.
15. Add 500 μL of Buffer PE to the QIAprep spin column to wash the DNA on the column. Centrifuge the column for 60 s at 13,000 × *g*. Discard the flowthrough.
16. Centrifuge the column again for 60 s to remove any remaining wash buffer (see Note 6).
17. Transfer the QIAprep columns to clean 1.5 mL microcentrifuge tubes.
18. Add 50 μL of Buffer EB to the center of each QIAprep spin column.
19. Let the column stand for 60 s and then centrifuge the QIAprep column for 60 s to elute the plasmid DNA.
20. To confirm that the construction of plasmids pUTmTn5::392 W and pUTmTn5::392 are correct, digest the plasmid DNA with *Bst*BI and *Spe*I in the following digestion reaction: 1× reaction: 5.0 μL of the pCR2.1Kn^R (50 ng/μL), 2.0 μL of 10× buffer, 0.2 μL BSA, 1.0 μL of *Bst*BI (10 U/μL), 1.0 μL *Spe*I (10 U/μL), and sterile dH_2O for a total reaction volume of 20.0 μL.
21. Incubate the reaction at 37°C in a water bath for 2 h.
22. To separate the *Spe*I and *Bst*BI digested DNA fragments of pCR2.1Kn^R via gel electrophoresis, follow steps 26–29 below.
23. Fill the gel box with TBE buffer until the gel is submerged.
24. Load lambda *Hin*dIII digested DNA ladder (size marker DNA) in lane one.
25. Add 2 μL of Blue Juice™ tracking dye to 20 μL of the *Spe*I and *Bst*BI digested DNA sample.
26. Load the DNA sample onto a 0.8% TBE agarose gel.
27. Replace the gel box top, which also attaches the leads. Attach the other end of the leads to the power supply.
28. Run the gel at 100 V for 60 min. Check to make sure that the DNA is moving in the correct direction.
29. To visualize the DNA fragments, place the agarose gel on a UV transilluminator (see Note 11).

3.9. Transfer of pUTmTn5::392W and pUTmTn5::392 into E. coli Strain TOP10 via Conjugation (Triparental Mating)

The reporter plasmids (pUTmTn5::392W and pUTmTn5::392) prepared in Subheading 3.8 will be transferred from the donor *E. coli* strains into recipient *E. coli* strains containing the promoterless carotenoid genes through conjugation or specifically triparental mating. The donor plasmid, which harbors the promoterless carotenoid plasmid, has several important attributes. The donor plasmid contains the transfer functions necessary for mobilization from

one strain to another. These include *tra*J, *tra*I, and *ori*T. The origin of replication (*ori*V) of the host plasmid is of the R6K type. The plasmid is able to replicate in lambda *pir* containing strains, such as SY327. However, it is incompatible with many commonly used strains. In these cases, pUTmTn5::392 W and pUTmTn5::392 will serve as a suicide vector. To follow the movement of the transposable element, the gene that confers resistance to kanamycin is positioned between the ends of transposable element. A key feature of the donor plasmid is the presence of a promoterless synthetic cluster of carotenoid genes. The carotenoid genes present on the pUTmTn5::392 W vector are *crt*B, *crt*I, *crt*Y, *idi*, *crt*E, and *crt*W. The expression of this synthetic operon allows for the production of canthaxanthin, which is an orangish pigment. The carotenoid genes present on the pUTmTn5::392 vector are *crt*B, *crt*I, *crt*Y, *idi*, *crt*E, *crt*Z, and *crt*W. The expression of this synthetic operon allow for the production of astaxanthin, which is reddish-orange in color. The intensity of the color is related to the level of expression of the carotenoid genes.

3.10. Growth of E. coli Donor, Helper, and Recipient Cells

1. Inoculate isolated colonies of the donor strain [*E. coli* SY327λpir (containing pUTmTn5::392 W or pUTmTn5::392)] into 5 mL of LB broth containing 50 μg/μL kanamycin in round bottom disposable test tubes.
2. Inoculate isolated colonies of the helper strain [*E. coli* DH5α (containing conjugative plasmid pRK2073)] into 5 mL of LB broth containing 25 μg/μL chloramphenicol in round bottom disposable test tubes.
3. Inoculate isolated colonies of the recipient strain [*E. coli* TOP10] into 5 mL of LB broth in round bottom disposable test tubes.
4. Grow the *E. coli* strains overnight (~12–16 h) at 30°C with aeration.
5. Harvest the cells from the *E. coli* donor and helper strains by centrifugation for 10 min (3,500 × *g*).
6. Discard the supernatant and resuspend both the *E. coli* donor and helper strains in equal volumes of LB broth (see Note 12).
7. Mix the washed *E. coli* donor/helper cells together and incubate the cells in stationary position at 30°C for ~2 h (see Note 13).
8. Harvest the *E. coli* donor and helper cells by centrifugation for 10 min (3,500 × *g*).
9. Remove as much of the liquid as possible, but do not allow the cells dry out.
10. Mix the damp *E. coli* donor/helper cell pellets with recipient cells during the triparental mating (Subheading 3.11).

3.11. Triparental Mating for E. coli

1. Grow the donor, helper, and recipient strains as stated in Subheading 3.10.
2. Resuspend the *E. coli* donor/helper cell pellets by adding 40 μL of the washed and concentrated recipient cells.
3. Mix the donor/helper/recipient cells together by moving the cells up and down in a 200 μL pipette tip.
4. Transfer the cell mixture to a single spot (the size of a nickel) onto a LB agar plates that do not contain antibiotics.
5. Incubate the LB plates containing the spots of *E. coli* cells overnight at 30°C.
6. Scrape the cells off the plate using an inoculating loop and resuspend the cells into 1.0 mL of LB broth.
7. Plate 10 μL, 50 μL and 100 μL of cells LB agar plates containing Kan^{50} μg/mL and Str^{50} μg/mL, which selects for recipient cells containing either the mTn5::392 W or the mTn5::392 transposon. Store the remainder of the cells at 4°C.
8. Incubate the plates at 30°C. Determine the appropriate amount of cells to plate on each plate (see Note 11).
9. Plate the reminder of the resuspended cell in the appropriate amounts onto fresh LB/Kan^{50}/Str^{50} agar plates.
10. Screen the colonies for those having the darkest hue (see Note 14).
11. Pick the colonies of interest and streak them twice to obtain will isolated colonies.
12. Determine the carotenoid levels produced (Subheading 3.12).

3.12. Analysis of Canthaxanthin and Astaxanthin in E. coli

To determine the levels of canthaxanthin or astaxanthin produced in strains having the darkest visual pigmentation, use the methods outlined in Subheading 3.5 of Chapter 7.

3.13. Determining the Chromosomal Location of Transposons mTn5::392 W and mTn5392 Within the Chromosome of E. coli Strain TOP10 via Direct Sequencing

1. To determine the chromosomal location of the carotenoid transposons, use the sequencing primers 392KnSeq.R and 392KnSeq.F (see Note 3) in the following DNA sequencing reaction: 3 μg/reaction of purified genomic DNA, 16 μL of BigDye v3.1 sequencing reagent, 3 μL of 10 μM Sequencing primer, 1 μL of Thermofidelase, and 12 μL of Molecular biology grade water (Fig. 4).
2. Perform sequencing reaction using the following thermal-cycling procedure: heat the sample for 3 min at 96°C; next 200 cycles [30 s at 95°C, 20 s at 55°C, and 2 min at 60°C]; store samples at 4°C.
3. To remove the unincorporated dideoxynucleoside triphosphates, use the QIAquick Nucleotide Removal kit prior to sequencing.

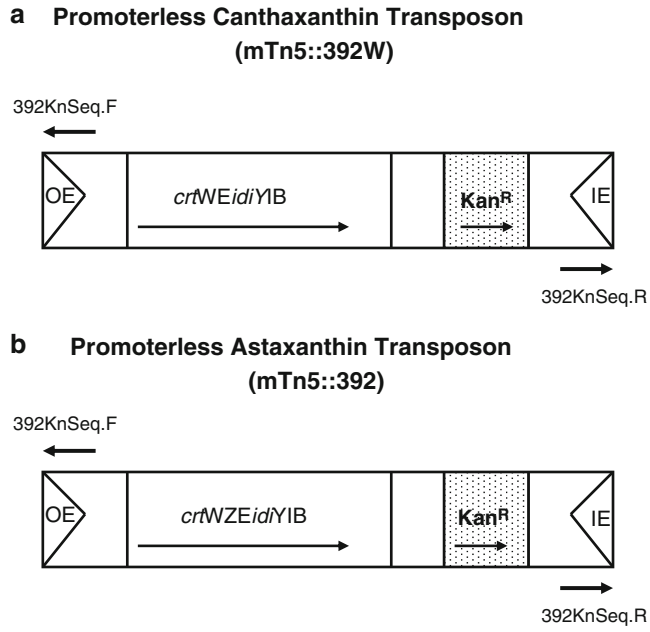

Fig. 4. Diagrams of the promoterless canthaxanthin (Tn5::392 W) and the promoterless astaxanthin (Tn5::392) transposons. (**a**) The Tn5::392 W transposon contains the carotenoid gene cluster (*crt*WE*idi*YIB) from pDCQ392W. (**b**) The Tn5::392 transposon contains the carotenoid gene cluster (*crt*WZE*idi*YIB) form pDCQ392. Both transposons have the following features. The triangles represent the mosaic ends of the Tn5 element. OE represents outside end and IE represents inside ends. Kan^R indicates the kanamycin resistance gene. The arrows at the ends of the transposons depict the DNA sequencing primers that are used to determine the location of the promoterless carotenoid transposons within the host's genome.

4. To purify the DNA sequencing reaction, add 10 volumes of Buffer PN to 1 volume of the reaction sample.
5. Mix the solution by moving the microcentrifuge tube back and forth.
6. Place a QIAquick spin column in the 2-mL collection tube provide in the kit.
7. Apply the sample to the QIAquick column.
8. Centrifuge the column for 60 s at 6,000 × *g* in a microcentrifuge.
9. Discard the flowthrough.
10. Put the QIAquick column back into the same collection tube.
11. Wash the column using 750 μL of Buffer PE.
12. Discard the flowthrough.
13. Place the QIAquick column back into the empty collection tube.
14. Centrifuge the column for an additional 60 s at 13,000 × *g* in a microcentrifuge.

15. Place the QIAquick column into a clean 1.5-mL microcentrifuge tube.
16. Add 50 μL of Buffer EB to the center of the QIAquick membrane to elute the DNA.
17. Let the column stand for 60 s.
18. Centrifuge the column for 60 s at 13,000 × *g* in a microcentrifuge.
19. For each sequencing reaction mixture, transfer 40 mL to a well of a prespun 96-well cleanup plate.
20. Spin the plate for 5 min at 5,000 × *g*.
21. Place the cleaned up reaction directly onto an Applied Biosystems 3700 DNA sequencer.
22. Sequence the DNA sample by automatic base calling.

4. Notes

1. Multiple Cloning Site (MCS). 58-nucleotide DNA primer and its reverse complement.
2. DNA PCR primers for amplification of the kanamycin gene.
3. DNA sequencing primers for direct chromosomal sequencing.
4. The digestion of pDCQ392W and pDCQ392 with restriction endonucleases *Bsp*EI and *Spe*I will be carried out in separate reactions because the buffering conditions needed for the two enzymes are incompatible.
5. Check to see if the color of the mixture remains yellow. If the mixture is orange or violet, add 10 μL of 3 M sodium acetate (pH 5) to the sample. Pipette to mix.
6. It is very important to remove all residual Buffer PE from the sample. The ethanol component of the buffer may interfere with subsequent reactions.
7. The pDCQ392W and the pDCQ392 DNA fragments containing the carotenoid genes should be approximately 6.4 kb and 6.9 kb, respectively.
8. Do not pick the dark blue colonies, which represent the TOPO cloning vectors that do not contain an insert DNA.
9. When the PCR fragment ligated in the reverse orientation in the TOPO vector, the expected size of the *Xho*I DNA fragments are ~4.0 kb (vector backbone) and ~0.94 kb (insert DNA).
10. Use a vector to insert molar ratio of 1:2 for each experimental reaction and set up a control reaction that does not contain

ligase (No Ligase Control) to detect the level of background colonies.

11. The expected sizes of the DNA fragments are 5.3 kb (Vector backbone), 0.8 kb (Kan gene) 6.5 kb (*crt*WE*idi*YIB genes), and 6.9 kb (*crt*WZE*idi*YIB genes).
12. This step serves to remove the antibiotics, which could interfere with the growth of the recipient cells.
13. During the liquid mating, some cells will acquire both the donor and helper plasmids.
14. Allow the plates to incubate until the colonies are pigmented. Some of the cells should appear orange or red due to the insertion of the promoterless carotenoid transposon behind a chromosomal promoter. The pigment produced is either canthaxanthin or astaxanthin.

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