# Chapter 6

# Modulation of Carotenoid Accumulation in Transgenic Potato by Inducing Chromoplast Formation with Enhanced Sink Strength

**Joyce Van Eck, Xiangjun Zhou, Shan Lu, and Li Li**

## Abstract

An increasing interest in carotenoids as nutritional sources of provitamin A and health-promoting compounds has prompted a significant effort in metabolic engineering of carotenoid content and composition in food crops. The strategy commonly used in plants is to increase the biosynthetic capacity by altering the carotenogenic enzyme activities. The recent isolation of the *Or* gene from a cauliflower orange mutant has brought a new endeavor for carotenoid enhancement by increasing the sink strength to sequester and store the synthesized carotenoids. Potato as one of the major staple crops usually accumulates low levels of carotenoids. In this chapter, we describe a detailed protocol for metabolic engineering of carotenoids in potato plants with the *Or* gene and the analysis of the *Or* transformants.

**Key words:** Cauliflower *Or* gene, carotenoids, chromoplasts, transgenic potato, sink strength.

## 1. Introduction

Carotenoids are widely distributed in nature, but can only be de novo synthesized in plants (including algae) as well as in some fungi and photosynthetic bacteria. Carotenoids possess numerous health benefits to humans. They have been implicated in reducing the incidence of certain diseases and are the primary dietary sources of provitamin A (1). Vitamin A deficiency represents a major global health problem. The deficiency affects 140–250 million preschool children worldwide and causes one-quarter to a half million of them to become blind every year with half of them dying within 12 months of losing their sight

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(2). Thus, development of food crops rich in carotenoids could help alleviate vitamin A deficiency and maximize the health benefits of carotenoids to a large number of population in the world.

Significant progress has been made in quantitative and qualitative manipulation of carotenoids in food crops (3–5). The strategy commonly employed is to alter the expression of a rate-limiting enzyme or multiple enzymes in the carotenoid biosynthetic pathway in a tissue-specific manner. One of the most successful examples is Golden Rice, which has increased production of β-carotene in rice endosperm from almost nothing to 31 μg/g dry weight, a level adequate to provide the recommended dietary allowance of provitamin A for small children (6).

In 1970s, a natural mutant of cauliflower with high β-carotene accumulation in the curds was found in Canada, and subsequent genetic work showed that this was the result of a single gene (*Or* for *Orange*) mutation (7, 8). Later studies revealed that the massive β-carotene accumulation correlated with biogenesis of chromoplasts (9). Rather than directly controlling carotenoid biosynthesis, the *Or* gene was found to function in triggering the differentiation of proplastids and/or non-colored plastids into chromoplasts (10, 11). Such structures provide deposition sinks to effectively sequester and store large quantities of synthesized carotenoids (12).

Transformation of this cauliflower mutant *Or* gene into white cauliflower leads to high levels of β-carotene accumulation with the formation of chromoplasts in the transgenic curd cells (10). Remarkably, introduction of the *Or* gene under a tuber-specific promoter into potato tubers also conferred carotenoid accumulation in newly formed chromoplasts in a heterologous system (11). Thus, manipulation of chromoplast formation with enhanced sink strength proves as being an alternative and complementary approach for carotenoid enrichment in food crops. In this chapter, we provide a detailed, improved protocol on metabolic engineering of carotenoid accumulation with the *Or* gene in potato tubers.

## 2. Materials

### 2.1. Gene, Promoter, and Vectors

1. Cauliflower *Or* gene (GenBank accession # DQ482460).
2. Potato granule-bound starch synthase (GBSS) promoter (GenBank accession #A23740).
3. pCR2.1 TA cloning vector (K2020-20, Invitrogen) and pBluescript KS(-) vector (Stratagene) for making

intermediate constructs. Binary vector pBI101 (Clontech) with *nptII* gene that confers a kanamycin resistance for vector-only control and for constructing the pBI-GBSS-Or transformation construct.

4. PfuUltra DNA polymerase (Stratagene).

### 2.2. Agrobacterium tumefaciens and Culture Media

1. Strains and selectable markers: the transformation protocol supports a number of variables including the potato lines (*see* **Note 1**), *Agrobacterium tumefaciens* strains (*see* **Note 2**), and the selectable markers (*see* **Note 3**). The binary plasmids containing plant expression cassettes are electroporated into *Agrobacteria*.
2. Luria–Bertani (LB) medium: 10 g/L Bacto-tryptone, 5 g/L yeast extract, and 10 g/L NaCl. Autoclave, then cool to 55°C before adding the appropriate selective agent (dependent upon the vector). Store at 4°C.
3. YM medium: 400 mg/L yeast extract, 10 g/L mannitol, 100 mg/L NaCl, and 200 mg/L $MgSO_4 \cdot 7H_2O$. Autoclave, then cool to 55°C before adding the appropriate selective agent (dependent upon the vector). Store at 4°C.
4. Selective agent: prepare the appropriate agent according to concentration and solubility (*see* **Note 3**). Filter sterilize. Store aliquots at −20°C.

### 2.3. Tissue Culture and Transformation

1. Benzyladenine (BA): 1 mg/mL stock. Dissolve the powder in a few drops of 1 N HCl and then add deionized water to volume. Filter sterilize the stock and store in 1-mL aliquots in sterile 1.5-mL capacity Eppendorf tubes at 4°C.
2. Zeatin riboside (ZR): 1 mg/mL stock. Dissolve the powder in a few drops of 1 N HCl and then add deionized water to volume. Filter sterilize the stock and store in 1-mL aliquots in sterile 1.5-mL capacity Eppendorf tubes at 4°C.
3. Indole-3-acetic acid (IAA): 1 mg/mL stock. Dissolve the powder in a few drops of 1 N KOH and then add deionized water to volume. Filter sterilize the stock and store in 1-mL aliquots in sterile 1.5-mL capacity Eppendorf tubes at 4°C.
4. Naphthaleneacetic acid (NAA): 1 mg/mL stock. Dissolve the powder in a few drops of 1 N KOH and then add deionized water to volume. Filter sterilize the stock and store in 1-mL aliquots in sterile 1.5-mL capacity Eppendorf tubes at 4°C.
5. Selection agent: as appropriate for the selectable marker found in the gene construct (*see* **Note 3**).

6. Carbenicillin: 100 mg/mL stock. Dissolve the powder in deionized water and then filter sterilize. Store as 2.5-mL aliquots at −20°C (*see* **Note 4**).
7. CM medium: 4.3 g/L Murashige and Skoog (MS) salts (Caisson Laboratories, Rexburg, ID), 0.4 mg/L thiamine, 0.1 mg/L myo-inositol, 20 g/L sucrose, and 8 g/L agar. Adjust pH to 5.7 with 1 N KOH and HCl.
8. MS liquid medium: 4.3 g/L MS salts, 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.4 mg/L thiamine-HCl, 0.25 mg/L folic acid, 0.05 mg/L D-biotin, and 30 g/L sucrose. Adjust pH to 5.6 with 1 N KOH and HCl. Autoclave and store at room temperature for up to 1 month.
9. CIM medium: 4.3 g/L MS salts, 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, 0.4 mg/L thiamine, 0.25 mg/L folic acid, 0.05 mg/L D-biotin, 100 mg/L myo-inositol, 30 g/L sucrose, 1 mg/L BA, 2 mg/L NAA (added after autoclaving), and 6 g/L agar. Adjust pH to 5.6 with 1 N KOH and HCl. Autoclave, then cool to 55°C before adding NAA. Pour into approximately 40 Petri-dishes (100 × 20 mm) per liter of medium.
10. 3C5ZR selective medium: 4.3 g/L MS salts, 1 mg/L thiamine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, 100 mg/L myo-inositol, 30 g/L sucrose, 0.1 mg/L IAA (added after autoclaving), 3.4 mg/L ZR (added after autoclaving), 500 mg/L carbenicillin (added after autoclaving), appropriate selection agent (added after autoclaving), and 8 g/L agar. Adjust pH to 5.6 with 1 N KOH and HCl. Autoclave, then cool to 55°C before adding zeatin, IAA, carbenicillin, and the appropriate selection agent. Pour into approximately 40 Petri dishes (100 × 20 mm) per liter of medium.
11. CM selective medium: CM medium containing 500 mg/L carbenicillin (added after autoclaving) and the appropriate selection agent (added after autoclaving). Autoclave, then cool to 55°C before adding the carbenicillin and selection agent. Allot approximately 50 mL of medium per Magenta® GA7 box ([7.7 cm (l) × 7.7 cm (w) × 9.7 cm (h)], PhytoTechnology Laboratories).
12. Glass test tubes [2.5 cm (w) × 15 cm (l), VWR, West Chester, PA].
13. Clear plastic test tube caps.
14. Micropore tape [0.5 inch (1.27 cm); 3 M HealthCare, St. Paul, MN).
15. Sterile paper towels or filter paper.

16. Transfer to soil: Jiffy 7s (42 mm, Griffin Greenhouse Supply, Auburn, NY), 3-gallon pots (Griffin Greenhouse Supply) containing Metro Mix 360 (Griffin Greenhouse Supply), trays without holes, trays with holes, and transparent plastic tray covers (Griffin Greenhouse Supply) or clear plastic bags.

### 2.4. Extraction of DNA from Potato Leaves

1. DNA extraction buffer: 0.1 M Tris–HCl (pH 8.0), 0.05 M EDTA (pH 8.0), 0.5 M NaCl, 1% sodium dodecyl sulfate (SDS), and 1% β-mercaptoethanol (added just prior to use).
2. 5 M potassium acetate.
3. Isopropanol (Sigma).
4. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
5. 70% and 100% ethanol (Pharmco-AAPER).
6. 10 mg/mL RNase A solution. Store in 1-mL aliquots at −20°C.
7. FastPrep instrument (BIO 101 Systems).
8. FisherBrand 2.0-mL conical screw cap tube.

### 2.5. Southern Blot Analysis of Transgenic Potato Plants

1. 20X standard saline citrate (SSC): dissolve 175.3 g NaCl and 88.2 g sodium citrate in $dH_2O$, adjust pH to 7.0. Add deionized water to 1 L.
2. 10% SDS.
3. Hybond-N+ membrane (GE Healthcare).
4. DECAprime II kit (Ambion).
5. $\alpha$-$^{32}$P dCTP (Perkin-Elmer).
6. ULTRAhyb$^{TM}$ ultrasensitive hybridization buffer (Ambion).

### 2.6. RNA Extraction, qRT-PCR, and Northern Blot Analysis of Transgenic Potato Plants

1. Trizol reagent (Invitrogen) for extraction of total RNA from leaf tissue.
2. RNA extraction buffer for extraction of total RNA from potato tubers: 1.0 M Tris–HCl (pH 9.0), 1 M NaCl, 0.2 M EDTA, 2% CTAB, and 1% β-mercaptoethanol (added immediately prior to extraction). Prepare RNA extraction buffer in DEPC-treated water.
3. Phenol/chloroform (1:1). Prepare by mixing phenol and chloroform (Sigma).
4. Chloroform:isoamyl alcohol (24:1) (Biotechnology grade, Amresco).
5. 70% (prepared with DEPC-treated water) and 100% ethanol.
6. 4 M LiCl.

7. DEPC-treated water: Add 0.1% (v/v) DEPC (Sigma) to deionized water. Stir for 1 h. Autoclave after overnight treatment.
8. DNase I (Ambion Inc.).
9. Oligo $dT_{(15–18)}$ and SuperScript III reverse transcriptase (Invitrogen).
10. SYBR Green PCR master mix (Applied Biosystems, Foster City, CA).
11. 384-well clear optical reaction plate (Applied Biosystems).
12. Applied Biosystems 7900HT fast real-time PCR system (Applied Biosystems).
13. 10X MOPS: Add 41.8 g MOPS, 6.8 g sodium acetate, and 20 mL EDTA (0.5 M, pH 8.0) into 1 L deionized $H_2O$, adjust pH to 7.0. Autoclave and keep in the dark.
14. Formaldehyde (ACS grade, VWR).
15. Formamide (for molecular biology, Sigma).

### 2.7. Analysis of Carotenoid Levels in Transgenic Potato Tubers

1. 80% acetone.
2. Ethyl acetate (HPLC grade, Mallinckrodt).
3. Triethylamine (Reagent grade, Fisher).
4. Acetonitrile (HPLC grade, EMD).
5. Nitrogen gas (Airgas).
6. HPLC solvent A: 81% acetonitrile, 9% deionized $H_2O$, 10% ethyl acetate, and 0.1% triethylamine.
7. HPLC solvent B: 80% ethyl acetate, 18% acetonitrile, 2% $H_2O$, and 0.02% triethylamine.
8. Spherisorb ODS2 $C_{18}$ reverse phase column (5-μm particle size) (Waters, Milford, MA).
9. Waters HPLC system with a diode array detector (Waters).

### 2.8. Light Microscopic Study of Chromoplasts

1. Plastid isotonic buffer: 0.33 M sorbitol, 0.1 M Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM NaCl, and 1X plant protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).
2. Olympus BX60 microscope equipped with a Sony CCD color camera.

## 3. Methods

### 3.1. Making Or Transformation Construct

The *Or* construct contains the *Or* genomic DNA under the control of potato granule-bound starch synthase (GBSS) promoter (*see* **Note 5**).

1. Cloning *GBSS* promoter sequence from potato. Potato DNA is used as template to amplify the *GBSS* promoter sequence using PfuUltra DNA polymerase with a forward primer (5′-GATCTGACAAGTCAAGAAAATTGCCATTG AAG-3′) and a reverse primer containing an *Nco*I site (5′-TACCATGGTGATGTGTGGTCTACAAAAAGGGGA ATC-3′). The amplified product is cloned into the TA cloning vector pCR2.1 to produce pTA-GBSS and sequenced.
2. Making intermediate construct of pBS-GBSS. pTA-GBSS is digested with *Hind*III and *Nco*I. *GBSS* fragment is ligated into pBluescript KS(-) to produce pBS-GBSS.
3. Cloning cauliflower *Or* gene. The *Or* genomic DNA starting from the ATG start codon is amplified using PfuUltra DNA polymerase with a forward primer containing an *Nco*I site (5′-TACCATGGGATCTTGTTTGGGTAGGATCTTG-3′) and a reverse primer containing a *Sac*I site (5′-TGAGA GCTCAGACAACCGCTGCGAGAATG-3′). The 7.5 kb amplified product is cloned into the TA cloning vector pCR2.1 to produce pTA-Or construct and digested with *Hind*III to check the characteristic digestion pattern (*see* **Note 6**).
4. Making intermediate construct of pBS-GBSS-Or. pBS-GBSS and pTA-Or plasmids are digested with *Nco*I and *Sac*I, respectively. The *Or* fragment is ligated downstream of the *GBSS* promoter to produce pBS-GBSS-Or.
5. Generating the transformation construct of pBI-GBSS-OR. The GBSS-Or fragment is digested out of pBS-GBSS-Or plasmid with *Sal*I and *Sac*I and cloned into the binary vector pBI101 to generate the final construct of pBI-GBSS-OR for transformation. The nucleotide sequences of cloning sites are confirmed by DNA sequencing.

### 3.2. Potato Transformation

1. In vitro stock plants are maintained in test tubes containing 10 mL of CM medium as a source of internode segments for transformation (*see* **Note 7**).
2. Day 1, streak the *Agrobacterium* strain containing the binary plasmid of interest onto a plate of LB medium containing the appropriate selection agent. Incubate at 28–30°C for 48 h.
3. Day 3, select four well-formed *Agrobacterium* colonies to initiate a 50 mL culture in YM selective medium. Maintain at 28–30°C in a shaking incubator at 250 rpm.
4. Day 4, check the $OD_{600}$ of the liquid culture. The $OD_{600}$ needs to be at 0.4–0.6. Should the culture overgrow an

$OD_{600}$ of 0.6, dilute the culture with YM medium until the reading is below 0.5, and then grow about 1 h before rechecking the $OD_{600}$. Centrifuge the culture at 8,000 rpm for 10 min at room temperature before re-suspending the pellet in 50 mL MS liquid medium.

5. For each experiment or gene construct, cut approximately 100 stem internode segments of 0.5–1 cm in length from 6-week-old in vitro stock plants. Place internode segments on CIM medium plate during cutting.
6. Incubate approximately 30 internode segments in 50 mL of prepared *Agrobacterium* inoculum for 10 min, agitating occasionally (*see* **Note 8**).
7. Pipette off the *Agrobacterium* inoculum and transfer the internode segments to sterile filter paper or paper towels to remove excess inoculum.
8. Place infected internode segments back onto CIM medium plate and incubate at 19°C in the dark for 48 h (*see* **Note 9**).
9. Day 6, transfer to 3C5ZR selective medium containing 500 mg/L carbenicillin in Petri dishes (*see* **Note 4**). Seal the plates with micropore tape and incubate at 24°C ± 1°C under 16 h light/ 8 h dark at 45 μmol photons/$m^2$s. Transfer weekly for 1 month to fresh 3C5ZR selective medium. After 1 month, transfer every 10–14 days.
10. After approximately 8 weeks (depends upon the potato cultivar), excise regenerated shoots (1.5 cm long) and transfer into CM selective medium in Magenta® GA7 boxes (*see* **Note 10**). Cut only one transformant from each end of a stem segment (*see* **Note 11**). Culture five shoots per box.
11. After 1 month, propagate only the well-rooted plantlets by cutting a nodal segment (section containing an internode) and transferring again to CM selective medium in Magenta® GA7 boxes (*see* **Note 12**).
12. Use PCR, Southern blot or technique of choice to verify that the rooted plants contain the transgene. Confirmed transformants should be maintained on CM medium in test tubes and propagated by nodal segments every 6–8 weeks.
13. If needed, well-rooted 3- to 4-week-old in vitro plants can be transferred into soil. At least 2 h before transferring, place a tray with holes inside a tray without holes, then place the number of Jiffy 7s needed in the tray. Fill the tray with water and allow the Jiffy 7s to absorb the water. When the Jiffy 7s are completely expanded and water soaked, they are ready to use.

14. Drain the excess water from the tray and orient the Jiffy 7s, so that the holes face upward. Enlarge the hole slightly at the top of Jiffy 7 to make it easier to place the plant.
15. Remove a plant from a test tube. Be careful to not break off the roots (*see* **Note 13**). Wash all medium from the roots in tepid water by gently rubbing the roots (*see* **Note 14**).
16. Transfer the plant to a Jiffy 7. Make certain that all the roots are covered. Transfer the plant back to the tray with a label. Continue until all the plants are transferred. Cover with plastic dome (*see* **Note 15**).
17. Add water to a depth of approximately 1.5 cm. Cover with transparent plastic dome.
18. Place in a shaded area of a greenhouse (*see* **Note 16**).
19. Plants are acclimated over a 2-week period in trays covered with transparent, plastic domes. They remain completely covered for the first week and then the lids are gradually lifted over the course of the second week and completely removed at the end of that week. The tray of plants is removed from the tray without holes, and the plants are watered as needed. When the roots extend to approximately 1–2 cm beyond the Jiffy 7s, the plants are transferred to 3-gallon pots containing Metro Mix 360 and grown at 20–22°C in a greenhouse.
20. Potato tubers are harvested in approximately 3 months. The *Or* transgenic tubers show orange-yellow flesh color (**Fig. 6.1**).

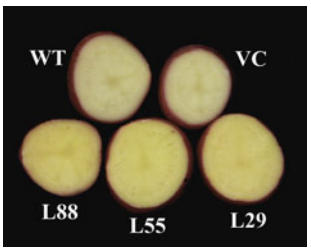


Fig. 6.1. Cross sections of potato tubers. The flesh color is associated with carotenoid accumulation. WT, wild type; VC, vector-only control; L29, L55, and L88, individual *Or* transgenic lines.

### *3.3. Extraction of Genomic DNA from Transgenic Potato Plants*

Many protocols for plant genomic DNA extraction are available. The procedure described here is a protocol based on the method of Dellaporta et al. (13) and produces good-quality DNA for most of the downstream applications. A larger quantity of DNA can be obtained by proportional increase of starting plant materials and volumes of reagents.

1. Harvest young potato leaves (~100 mg) into a 2.0-mL conical screw cap tube. Freeze in liquid nitrogen.
2. Quickly include one ceramic sphere (Qbiogene) per tube and crush leaves into powder for 10 s in a FastPrep instrument (*see* **Note 17**).
3. Remove ceramic sphere and add 750 μL of the DNA extraction buffer.
4. Incubate at 65°C for 10 min.
5. Add 150 μL of 5 M potassium acetate, vortex to mix, and incubate on ice for 15 min.
6. Centrifuge at 12,000 rpm for 10 min.
7. Label new set of microcentrifuge tubes and add 850 μL of isopropanol to each tube.
8. Transfer 850 μL supernatant to each new tube, mix well, and centrifuge at 12,000 rpm for 10 min to pellet DNA.
9. Pour off supernatant and add 1 mL 70% ethanol to each tube.
10. Mix and centrifuge at 12,000 rpm for 5 min.
11. Pour off the supernatant and air-dry the DNA samples on the bench for about 30 min (*see* **Note 18**).
12. Dissolve DNA samples in 40 μL TE buffer containing RNase A (20 μg/mL).
13. Incubate at 37°C for 30 min to digest RNA.
14. Use 2 μL DNA sample to measure DNA concentration at $OD_{260}$.

### *3.4. Analysis of Or Transgenic Potato Plants by PCR and Southern Blot Analysis*

Positive transformants are identified by PCR amplification of the *Or* transgene using primers of BoOrF1 (5′-ATCTC GAGGGAATCAAAGGAAGGGA-3′) and BoOrR1 (5′-CGACG AAACAAGATCTTCTTGC-3′) as well as of the selective marker, *nptII* gene, using primers nptIIF1 (5′-GGGTGGAGAGGCTAT TC-3′) and nptIIR1 (5′-GAAGGCGATAGAAGGCG-3′).

#### *3.4.1. PCR Analysis*

#### *3.4.2. Southern Blot Analysis of the Or Transgene Copy Numbers in Or Transformants*

1. Digest 10 μg of genomic DNA with a proper restriction enzyme in a 50 μL reaction mixture at 37°C overnight.
2. Separate the digested DNA on a 0.8% agarose gel and transfer DNA onto a Hybond-N+ membrane following the manufacturer's instruction.
3. Radiolabel DNA probe with $\alpha$-$^{32}$P dCTP using DECAprime II kit according to the manufacturer's instruction (*see* **Note 19**).

4. Prehybridize the membrane in ULTRAhyb$^{TM}$ ultrasensitive hybridization buffer at 42°C for 1 h and then hybridize overnight after adding the radiolabeled probes.
5. Wash the membrane twice with 2X SSC plus 0.5% SDS at 42°C for 20 min and then twice with 0.2X SSC plus 0.5% SDS at 42°C for 20 min.
6. Detect the signals by autoradiography or PhosphorImager.

### 3.5. Examination of the Expression of Or Transgene and Other Genes in Potato Tubers by qRT-PCR and Northern Blot Analysis

The *Or* transgene is driven by the tuber-specific promoter *GBSS*. It is essential to examine *Or* expression in transgenic tubers. Since potato tubers are starch and phenolics-rich storage tissues, it is difficult to extract RNA without contamination of polysaccharides and other phenolic compounds. Here we describe a modified phenol/chloroform/LiCl method to extract good-quality total RNA from potato tubers (14) (*see* **Note 20**).

#### 3.5.1. Total RNA Extraction from Potato Tubers

1. Homogenize 2 g of potato tuber in liquid nitrogen, add 5 mL of the RNA extraction buffer, and grind for a few more minutes.
2. Transfer the homogenate into 50-mL centrifuge tube. Incubate at 65°C for 15 min.
3. Add 5 mL phenol/chloroform (1:1) and vortex for 30 s. Centrifuge for 10 min at 12,000 rpm.
4. Transfer the upper phase into a new tube and add equal amounts of chloroform: isoamyl alcohol (24:1). Vortex to mix.
5. Centrifuge at 12,000 rpm for 10 min.
6. Repeat steps 4 and 5.
7. Transfer the upper phase into a new tube, add 3 mL isopropanol, and incubate at room temperature for 10 min.
8. Centrifuge at 12,000 rpm for 15 min at 4°C to precipitate RNA.
9. Rinse the pellet with 3 mL of 70% ethanol, and air-dry at room temperature.
10. Re-suspend the pellet in 1 mL of DEPC-treated $H_2O$.
11. Add an equal volume of 4 M LiCl and incubate on ice over 3 h.
12. Recover RNA by centrifugation at 12,000 rpm for 15 min at 4°C (*see* **Note 21**).
13. Rinse the RNA pellet with 1 mL of 70% EtOH, air-dry, and dissolve in 100–200 μL of DEPC-treated $H_2O$.
14. Measure RNA concentration at $OD_{260}$ and determine RNA quality using the ratio of $OD_{260}$ over $OD_{280}$ (*see* **Note 22**).

*3.5.2. qRT-PCR Analysis*

1. Total RNA (5 μg) is treated with DNase I and reverse transcribed using oligo $dT_{(15–18)}$ as primer and SuperScript III reverse transcriptase to generate the first-strand cDNA following the manufacturer's instruction (Invitrogen).
2. Dilute the synthesized cDNA to 100 μL.
3. Normalize cDNA concentration in different samples based on the amplification of *Actin* using primers: actinF (5′-GCTTCCCGATGGTCAAGTCA-3′) and actinR (5′-GGATTCCAGCTGCTTCCATTC-3′).
4. Add 2 μL cDNA, 5 μL SYBR Green PCR master mix, 0.5 μL each of 10 μM gene-specific primers, and $H_2O$ to total 10 μL of reaction volume in a 384-well qRT-PCR plate.
5. Reaction mixtures are amplified in an Applied Biosystems 7900HT fast real-time PCR system. Thermal cycling conditions consist of a first step of denaturation at 95°C for 10 min, followed by 40 cycles of denaturation for 15 s at 95°C, and annealing/extension for 1 min at 60°C.
6. Relative expression levels were calculated using the $\Delta\Delta C_T$ method (http://www.appliedbiosystems.com) as described by Lyi et al.(15).

*3.5.3. Northern Blot Analysis*

1. Total RNA (10 μg) is separated on a 1.2% agarose gel containing formaldehyde and transferred onto a Hybond-N+ membrane.
2. Preparation of probe, hybridization, washing, and signal detection are conducted as described above for Southern blot analysis. **Figure 6.2** shows the expression of *Or* transgene in transgenic potato tubers (*see* **Note 23**).

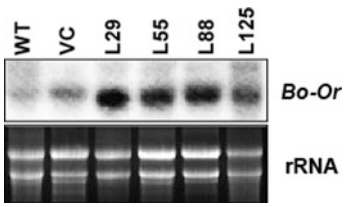


Fig. 6.2. Northern blot analysis of the non-transgenic and transgenic potato tubers. Total RNA (10 μg) was fractionated on 1.2% formaldehyde-containing agarose gel and then hybridized to cauliflower *Or* probe. The ethidium bromide-stained rRNA is shown as a loading control. WT, wild type; VC, vector-only control; L29, L55, L88, and L125, individual *Or* transgenic lines.

### *3.6. Analysis of Carotenoid Content in Transgenic Potato Tubers by HPLC*

1. Fine grind 500 mg of potato tubers in 800 μL 80% acetone with a mortar and pestle (*see* **Note 24**). Transfer homogenate into a 2.2-mL capacity centrifuge tube (*see* **Note 25**).
2. Add 500 μL ethyl acetate and vigorously vortex for 30 s to extract carotenoids.

3. Partition the extract by adding 400 μL water and vortex again.
4. Centrifuge at 12,000 rpm for 5 min.
5. Carefully transfer the upper phase into a new tube and dry under stream of nitrogen gas.
6. Re-suspend the dried samples in 200 μL ethyl acetate (*see* **Note 26**).
7. Centrifuge at 12,000 rpm for 10 min and transfer 100 μL sample into a sample vial for HPLC analysis.
8. Sample (40 μL) is injected automatically and separated on a Spherisorb ODS2 $C_{18}$ reverse phase column using a Waters HPLC system with a diode array detector.
9. Pigments are separated by a linear gradient between solvents A and B over 35 min at a flow rate of 1.0 mL/min. Elution program is 0 min at 100% A and 0% B; 1–25 min with a linear gradient to 0% A and 100% B; 25–30 min at 0% A and 100% B; and 30–35 min at 100% A and 0% B. The detector records absorbance spectra from 280 to 530 nm.

### *3.7. Light Microscopic Analysis of Chromoplasts*

While it is possible to observe chromoplasts from freshly prepared hand sections of cauliflower orange mutant tissues with a light microscope (9), it is difficult to observe them directly from hand sections of fresh transgenic potato tubers due to the presence of a large number of amyloplasts. Therefore, fresh tissue (*see* **Note 27**) of potato tubers is gently homogenized first to remove large amyloplasts.

1. Fresh tissue of transgenic potato tubers is cut with a razor blade into small pieces and gently homogenized in a plastid isotonic buffer.
2. Centrifuge at 100 *g* for 2 min.
3. Upper suspension is dropped on a microscope slide under a cover slip and examined with an Olympus BX60 microscope equipped with a Sony CCD color camera. Images are recorded.
4. Large numbers of carotenoid-sequestering structures from broken chromoplasts can be observed at the edge of the cover slip.

## 4. Notes

1. Potato lines: Desiree, Russet Burbank, Yema de Huevo, and Shepody.
2. Two *Agrobacterium* strains we have used are LBA4404 and EHA105. We do not see any significant difference in the

number of transgenic plants recovered using these bacteria, but each has advantages and disadvantages. The strain LBA4404 is our preferred strain in spite that it is *recA* positive, and constructs must be checked regularly for recombination events. The EHA105 strain is *recA* negative and more virulent than LBA4404. As a result, it is often difficult to eradicate. This can lead to overgrowth of the bacteria on the explants and kill the explants.

3. Selective agents used include kanamycin (PhytoTechnology Laboratories) and bialaphos (PhytoTechnology Laboratories). The optimal concentration for each selective agent is dependent on the potato line. We have used kanamycin at 50 and 75 mg/L and bialaphos at 2 and 3 mg/L. No significant difference has been seen in transformation efficiencies between these selection agents; however, regeneration of putative transformants is slower on bialaphos than on kanamycin.
4. Substitute 300 mg/L timentin (PhytoTechnology Laboratories) if carbenicillin cannot be acquired.
5. Other tuber-specific promoters can also be used such as patatin B33 promoter.
6. Digestion of the *Or* genomic DNA in a plasmid from ATG start codon to *Sac*I site with *Hind*III generates DNA fragments of 1.8, 1.4, 1.0, 0.9, 0.7, and 0.3 kb, and the size of vector.
7. We have found that it is important to use test tubes with clear caps for maintenance of individual potato plants as a source of internode segments for transformation. Individual plants maintained in test tubes are more vigorous than multiple plants that are maintained in Magenta® GA7 boxes, which produce plants with thin stems. We have noticed that internode segments from plants with the thickest stems result in the highest transformation efficiency (50–60%).
8. A longer period of incubation may cause water soaking damage to cells.
9. Lower co-cultivation temperatures have been shown to increase the transformation efficiency for some crops (16, 17). This has not been reported for potato; however, we have seen increased recovery of transformed lines at low temperature following expression of a gene that exhibits a negative effect on transformation efficiency.
10. We have found that the transformation efficiency (percent of infected stem segments that give rise to transformants) varies with different cultivars, selectable markers, and introduced genes. Expression of some transgenes decreases the

number of transformed lines recovered. A vector-only construct (no gene of interest, only the vector with a selectable marker) should be included as a transformation control to determine if the introduced gene has such an effect.

11. We do not take multiple transformants from each end of a stem segment because these could be sister clones instead of independent transformation events. After harvesting a shoot, we then cut off the end of the stem segment to prevent regeneration of shoots from that end, which helps to keep track of the regions where shoots have been taken.
12. When selecting shoots to propagate to a second round of selective rooting medium, be certain that plantlets have roots growing from the cut region of the stem in the medium but not growing from an area of a stem above the medium. We have found that plants with roots coming from the stem above the medium are escapes (not transformants).
13. We gently tap the bottom of the test tube to dislodge the medium. To remove the plant, we grasp the bottom with forceps and slowly pull the plant from the test tube.
14. Washing the medium away from the roots reduces the chance of adverse bacterial and fungal growth that may kill the plantlet once it is placed in soil.
15. It is important to cover the plants immediately after transfer to Jiffy 7 to prevent wilting.
16. If new transfers are placed in direct sunlight, heat will build up under the cover and kill the plants.
17. Work fast to make sure leaf material remains frozen during the crushing process. Return sample tubes back to liquid nitrogen container right after taking them out from FastPrep instrument.
18. To reduce drying time, liquid remaining in the centrifuge tube can be removed gently with a micropipette.
19. The fragment corresponding to 265–904 nt of cauliflower *Or* mRNA (DQ482459) is amplified by PCR with the primers 5′-CGACGAAACAAGATCTTCTTGC and 5′-GGAATCAAAGGAAGGGA. PCR product is purified with QIAquick PCR purification kit (QIAGEN) and then 100 ng DNA is used for probe labeling.
20. A number of other RNA isolation methods can also be followed to extract good-quality RNA from potato tubers, such as a method by Kumar et al. (18) and the RNeasy plant mini kit (QIAGEN). Total RNA from leaves is extracted using Trizol reagent.

21. Steps 10–12 can be repeated to obtain high-quality RNA.
22. The ratio of $OD_{260}$ over $OD_{280}$ should be from 1.8 to 2.0, which indicates good-quality RNA.
23. Equal loading can be verified by ethidium bromide-staining rRNA or by hybridization to an *Actin* probe**.**
24. Extraction should be performed under dim light to minimize degradation and isomerization of carotenoids.
25. It is very important to grind the tissue well in order to fully extract pigments. We usually first grind 500 mg of potato tubers with 500 μL 80% acetone, transfer the homogenate into a 2.2-mL centrifuge tube, and then use another 500 μL 80% acetone to rinse the mortar to ensure full transfer of the sample into a tube. The final extract volume should be around 800 μL due to evaporation of acetone during homogenization.
26. If saponification is needed, the dried sample is re-suspended in 200 μL 10% KOH in MeOH and saponified overnight at 4°C. Re-extract the sample with equal volume of ethyl acetate and then dry it down under a stream of nitrogen gas.
27. It is easier to observe intact chromoplasts from fresh tissues with a light microscope. Although long-term storage results in increased levels of total carotenoids (11), most of the chromoplasts are not intact.

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