Chapter 33

Combinatorial Genetic Transformation of Cereals and the Creation of Metabolic Libraries for the Carotenoid Pathway

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Abstract

Combinatorial nuclear transformation is used to generate populations of transgenic plants containing random selections from a collection of input transgenes. This is a useful approach because it provides the means to test different combinations of genes without the need for separate transformation experiments, allowing the comprehensive analysis of metabolic pathways and other genetic systems requiring the coordinated expression of multiple genes. The principle of combinatorial nuclear transformation is demonstrated in this chapter through protocols developed in our laboratory that allow combinations of genes encoding enzymes in the carotenoid biosynthesis pathway to be introduced into rice and a white-endosperm variety of corn. These allow the accumulation of carotenoids to be screened initially by the colour of the endosperm, which ranges from white through various shades of yellow and orange depending on the types and quantities of carotenoids present. The protocols cover the preparation of DNA-coated metal particles, the transformation of corn and rice plants by particle bombardment, the regeneration of transgenic plants, the extraction of carotenoids from plant tissues, and their analysis by high-performance liquid chromatography.

Key words: Combinatorial transformation, Metabolic pathway, Metabolic engineering, Carotenoid pathway, Particle bombardment, Transgenic plants, Metabolite profiling

1. Introduction

Combinatorial nuclear transformation is a novel method for the rapid production of populations of transgenic plants wherein each member of the population carries a random sample from a collection of input transgenes (1). We developed this system to dissect and modify the carotenoid metabolic pathway in corn, although in principle, it can be applied to any metabolic pathway or any other complex phenotype requiring the coordinated expression of

Jim M. Dunwell and Andy C. Wetten (eds.), *Transgenic Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 847, DOI 10.1007/978-1-61779-558-9_33,

multiple genes. To demonstrate the principle, we transferred five genes encoding enzymes in the carotenoid biosynthesis pathway, in addition to the selectable marker gene *bar* (encoding phosphinothricin acetyltransferase, which confers resistance to the herbicide glufosinate), into the elite white corn inbred M37W, which is deficient for endosperm carotenoid synthesis. Each gene was controlled by a different, endosperm-specific promoter. We recovered a diverse population of transgenic plants expressing different enzyme combinations and showing distinct metabolic phenotypes that allowed us to identify and complement rate-limiting steps in the pathway and to demonstrate competition between β-carotene hydroxylase and β-carotene ketolase for substrates in four sequential pathway steps (1).

The combinatorial system is potentially very useful because it allows complex pathways to be dissected in a single transformation experiment. Studying and engineering secondary metabolism in plants is difficult when (as is often the case) the pathways are complex, with multiple branches, enzymes that participate in multiple steps and compete for substrates, complex feedback-control mechanisms, and subcellular compartmentalization of enzymes and substrates (2, 3). Traditionally, it has been necessary to dissect such pathways by laboriously modulating the levels of each enzyme, either by introducing a transgene to achieve overexpression or attenuating expression using antisense RNA, cosuppression/RNA interference, or the expression of an intracellular antibody directed against the enzyme. Such methods are often frustrated by the complex way in which pathways are regulated, so it is becoming clear that multistep engineering will be required to dissect complex pathways, i.e., strategies in which partial or complete pathways are overexpressed using multiple transgenes, or where different enzymes are overexpressed or attenuated in the same plant lines (3–5).

Unfortunately, any scheme for multigene engineering that is both comprehensive and rationally designed would quickly become impractical if each transgene combination were deliberately introduced. For example, consider a simple and linear pathway with just four enzymatic steps. A comprehensive dissection in which each enzyme was in turn overexpressed and attenuated, and where all possible different combinations of upregulated and downregulated enzymes were assembled, would require 225 different plant lines (Fig. 1). In each case, at least 10–20 individual transformants would be required to ensure that the sought after combination was recovered because transgene integration is a random process, and even if a transgene is integrated, its expression might be affected by epigenetic phenomena. The number of plants required for higher transgene numbers would quickly become unsupportable because of the exponential increase in the number of different variants required (5, 6).

We used as a model system the South African elite white corn inbred M37W, which lacks carotenoids in the endosperm due to the absence of the enzyme phytoene synthase (PSY1) (Fig. 2) (7, 8).

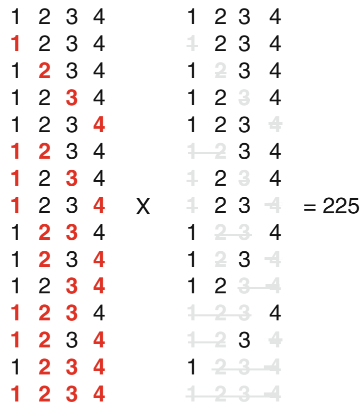

Fig. 1. A “back of the envelope” demonstration of the complexity of comprehensive metabolic analysis, using a theoretical nonbranched pathway with four metabolic steps. Overexpressing all possible combinations of the four enzymes requires 14 separate transgenic lines in addition to the wild type. The same number of combinations is required for comprehensive attenuation. Thus, 225 lines would be required to analyze all possible combinations of enhanced and suppressed enzymes.

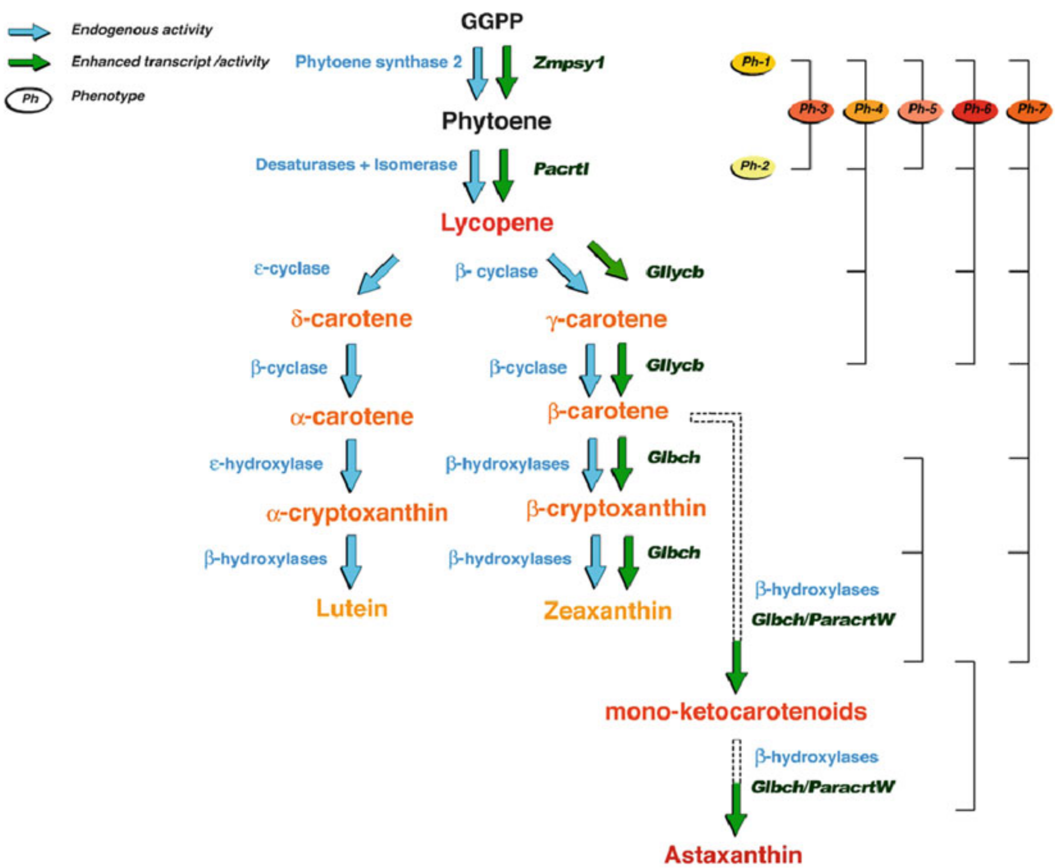

Fig. 2. Reconstruction and extension of the carotenoid biosynthetic pathway in white corn endosperm. On the *right*, seven distinct phenotypes are indicated on the basis of endosperm colour and gene expression in order to dissect the endogenous corn carotenoid biosynthetic pathway and extend it to produce ketocarotenoids. The *bars in the drop-off lines* indicate the enzyme activities present in the different phenotypes.

This provided a blank canvas upon which to study the metabolic capabilities of the input transgenes in different combinations, providing a rapid readout of carotenoid accumulation based on endosperm colour. However, there is nothing in principle preventing the use of other corn varieties and other plants to achieve the same goals, as long as the endogenous levels of metabolites are used as the baseline and fluctuations caused by different transgene combinations are judged against this reference. Therefore, we have recently extended the combinatorial transformation concept to rice, and protocols in this chapter are provided for both corn and rice plants.

2. Materials

2.1. Plant Material

1. The transformation protocol for corn (*Zea mays* L.) plants (Subheading 3.1) is optimized for cultivar M37W, but other cultivars can also be used (9). Plants should be maintained in a climate-controlled growth chamber until fully acclimated, with a day/night temperature of 28/24°C, 70%/85% humidity, and a 14-h photoperiod. The plants should then be transferred to a greenhouse (24–28°C, 70% humidity) until seeds are available for collection.
2. The transformation protocol for rice (*Oryza sativa* L.) plants (Subheading 3.2) is suitable for many different cultivars (10). Plants should be grown under the same conditions described above for corn.

2.2. Handling Plants

1. Controlled environment room.
2. Plant culture room with illumination system.
3. Laminar flow hood.
4. Stereomicroscope with light (for corn and rice transformation).
5. Fine forceps (two pairs) and scalpel.
6. 30°C/37°C incubator.
7. Whatman 3MM filter paper.
8. Rotary shaker.
9. Petri dishes.
10. Falcon tubes.
11. Mortar and pestle.
12. Parafilm.
13. Sterile, double-distilled water.
14. 70% (v/v) Ethanol.

15. 20% and 50% (w/v) Sodium hypochlorite solutions.
16. Autoclave and/or 0.2 μm sterile filter.
17. pH meter.

2.3. Growth Media and Additives

2.3.1. Basic Components

1. MS (Murashige and Skoog) salts (11) with minimal organics (see Note 1).
2. Sucrose (tissue culture grade).
3. Gellan gum (e.g., Gelrite or Phytagel) (see Note 2).
4. Agar (see Note 2).
5. Mannitol (Sigma).
6. Sorbitol (Sigma).
7. KOH for pH adjustment.

2.3.2. Additives and Stock Solutions (see Note 3)

1. Hygromycin B (Sigma). Stock solution: 50 mg/mL in H_2O (see Note 4).
2. Phosphinothricin (PPT) supplied as glufosinate-ammonium powder (Sigma). Stock solution: 10 mg/mL in H_2O (see Note 4).
3. 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma). Stock solution: 5 mg/mL in ethanol.
4. 6-Benzylaminopurine (6-BAP) (Sigma). Stock solution: 1 mg/mL in DMSO.
5. α-Naphthaleneacetic acid (NAA) (Sigma). Stock solution: 1 mg/mL in H_2O.

2.3.3. Media Composition

All media should be prepared immediately before use with ultrapure water (>18 MΩ/cm) and autoclaved or filter-sterilized (0.2 μm). Heat-sensitive components (selective agents, phytohormones) must be added after cooling to below 50°C.

Corn Transformation

1. Basic MS medium for corn (see Note 5). Make up MS salts and minimal organics as shown in Table 1 or prepare from a ready-mixed powder (see Note 1).
2. MSHc medium (MS with phytohormones for corn): basic MS medium for corn supplemented with 20 g/L sucrose, 4 g/L gellan gum, and 2.5 mg/L 2,4-D.
3. MSOc (MS osmoticum medium for corn): MSHc supplemented with 0.2 M mannitol and 0.2 M sorbitol.
4. MSSc (MS selection medium for corn): MSHc supplemented with 3 mg/L PPT.
5. MSRc (MS regeneration medium for corn): basic MS medium for corn supplemented with 30 g/L sucrose, 4 g/L gellan gum, 10 mg/L 6-BAP, 0.25 mg/L 2,4-D, and 3 mg/mL PPT.

Table 1
MS salts and minimal organics (11). All stocks should be stored at 4°C. The macroelements, microelements, and iron can be added before autoclaving, but the organic compounds should be added after cooling

Ingredients	MS
Macroelements (mg/L)	
Make up as a 10× stock in sterile distilled water	
$CaCl_2 \cdot 2\ H_2O$	440
KH_2PO_4	170
KNO_3	1,900
$MgSO_4 \cdot 7H_2O$	370
Microelements (mg/L)	
Make up as a 100× stock in sterile distilled water	
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.025
H_3BO_3	6.2
KI	0.83
$MnSO_4 \cdot 4H_2O$	22.3
$Na_2MoO_4 \cdot 2H_2O$	0.25
$ZnSO_4 \cdot 7H_2O$	8.6
Iron (mg/L)	
Make up as 100× stock in sterile distilled water	
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA \cdot 2H_2O$	37.3
Organic components (mg/L)	
Make up as 100× stock in sterile distilled water. Filter-sterilize	
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Glycine	2
Myoinositol	100
Nicotinic acid	0.5
Casein hydroxylate	1,000

6. MSRMc (MS rooting medium for corn): basic MS medium for corn supplemented with 30 g/L sucrose, 4 g/L gellan gum, and 3 mg/L PPT. This medium is similar to MSRc but contains no phytohormones.

Rice Transformation

1. Basic MS medium for rice (see Note 6). Make up MS salts and minimal organics as shown in Table 1 or prepare from a ready-mixed powder (see Note 1).
2. MSHr (MS with phytohormones for rice): basic MS medium for rice supplemented with 30 g/L sucrose, 5 g/L gellan gum, and 2.5 mg/L 2,4-D.

3. MSOr (MS osmoticum medium for rice): basic MS medium for rice supplemented with 30 g/L sucrose, 3 g/L gellan gum, 2.5 mg/L 2,4-D, and 0.2 M mannitol.
4. MSSr (MS selection medium for rice): MSHr supplemented with 30 mg/L hygromycin B.
5. MSRr (MS regeneration medium for rice): basic MS medium for rice supplemented with 0.2 M maltose, 4 g/L gellan gum, 3 mg/L 6-BAP, 0.5 mg/L NAA, and 30 mg/L hygromycin B.
6. MSRMr (MS rooting medium for rice): basic MS medium for rice supplemented with 10 g/L sucrose, 3 g/L gellan gum, 2 g/L agar, and 30 mg/L hygromycin B. This medium contains no phytohormones.

2.4. Materials for Transformation

1. Stock solution of plasmids carrying the metabolic transgenes (see Notes 7 and 8).
2. Stock solution of plasmid DNA carrying selectable marker gene (see Note 9).
3. Particle accelerator gun and accessories (Bio-Rad or homemade devices) (see Note 10).
4. Gold particles (see Note 11).
5. 100 mM Spermidine.
6. 25% PEG (MW 8,000).
7. 100% Ethanol.
8. TE buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
9. 2.5 M $CaCl_2$.
10. Sonicator.

2.5. Analysis of Carotenoids

2.5.1. Equipment

1. ACQUITY Ultra Performance LC™ system (Waters, Milford, MA, USA) linked to a PDA 2996 detector (Waters, Milford, MA, USA). MassLynx™ software version 4.1 (Waters, Milford, MA, USA).
2. UNICAM UV/Vis spectrophotometer UV-2.
3. Mortar and pestle.
4. Separation funnel.
5. Gravity funnel.
6. Steel tube.
7. Quartz cuvette.
8. Whatman Grade No. 40 quantitative filter paper (Schleicher & Schuell).
9. Durapore polyvinylidene fluoride (PVDF) membrane filters (Millipore, Bedford, MA, USA).

2.5.2. Chemicals

All chemicals are analytical grade unless otherwise specified. Aqueous solutions must be prepared with ultrapure water. Solvents should be filtered and degassed before use (we recommend 0.22-μm Durapore PVDF Membrane Filters).

1. β-Carotene (Sigma–Aldrich Fine Chemicals).
2. Zeaxanthin (Sigma–Aldrich Fine Chemicals).
3. Lutein (Sigma–Aldrich Fine Chemicals).
4. Lycopene (Sigma–Aldrich Fine Chemicals).
5. Astaxanthin (Sigma–Aldrich Fine Chemicals).
6. Methanol (B.T. Baker).
7. Acetonitrile (B.T. Baker).
8. Hexane (B.T. Baker).
9. 2-Methoxy-2-methylpropane (methyl *tert*-butyl ether; B.T. Baker).
10. Ethoxyethane (diethyl ether; B.T. Baker).
11. Oxacyclopentane (tetrahydrofuran; B.T. Baker).
12. Propanone (acetone; B.T. Baker).
13. NaCl (Panreac).

3. Methods

3.1. Transformation of Corn Plants

The manipulation of seeds, explants, and callus should be carried out using sterilized tools and equipment in a laminar flow hood to avoid microbial contamination. We recommend that all glassware is cleaned with double-distilled water (no detergents), covered with aluminum foil (see Note 12) and sterilized at 180°C for 20 h.

3.1.1. Preparation of Corn Explants for Bombardment

1. Collect cobs from corn plants in the greenhouse. Immature embryos are required for the following protocol (see Note 13).
2. Wash cobs in 70% ethanol and then 20% sodium hypochlorite for 20 min on a rotary shaker.
3. Transfer work to the laminar flow hood.
4. Rinse cobs at least three times in sterile distilled water.
5. Excise the immature embryos using fine forceps.
6. Place the embryos, scutellum-uppermost on Petri dishes containing MSHc medium.
7. Incubate the plates in darkness at 24°C for 4–6 days.
8. Four hours before bombardment, transfer embryos to MSOc medium (see Note 14).

3.1.2. Preparation of DNA-Coated Gold Particles for Bombardment

1. Prepare high-quality plasmid DNA at a concentration of 10 mg/mL (see Note 8).
2. Mix 10 mg of gold particles with 5 μg of the selectable marker plasmid and 15 μg of each plasmid carrying the carotenogenic transgene. Add TE buffer to 100 μL. Vortex for 30 s.
3. Add 100 μL of 100 mM spermidine to protect the DNA during the precipitation process. Vortex for 30 s.
4. Add 100 μL of 25% PEG (MW 8,000) and vortex for 30 s.
5. Slowly add 100 μL of 2.5 M $CaCl_2$ with continuous vortexing. When all the $CaCl_2$ has been added, vortex for a further 10 min.
6. Centrifuge at 5,000 × *g* for 30 s and discard supernatant.
7. Wash with 200 μL 100% ethanol and centrifuge at 5,000 × *g* for 30 s.
8. Add 100 μL of 100% ethanol and sonicate briefly to break up clumps of particles.
9. Spot 5–10 μL of the suspension onto the center of a carrier disc and allow to air dry.

3.1.3. Bombardment Using Bio-Rad Helium Gun (PDS 1000/He)

1. Follow manufacturer's guidelines for loading carrier disc into the barrel of the helium gun.
2. Place plant material in the targeting chamber.
3. Bombard plant material at 900–1,300 psi (see Note 15).
4. Bombard embryos twice with a 4-h interval between bombardments (maintain the embryos in darkness at 24°C on MSO during the interval).

3.1.4. Selection and Regeneration of Transgenic Corn Plants

1. Transfer bombarded embryos to MSHr medium. Incubate in darkness at 24°C for 2 days.
2. For the induction of transgenic embryogenic callus, transfer embryos to MSSc medium and incubate in darkness at 24°C for 2 weeks.
3. Subculture any callus recovered under selection at 2-week intervals on fresh MSSc medium in darkness at 24°C (see Note 18).
4. At the end of the third round of selection, transfer explants to MSRc medium for regeneration. Incubate plates at 24°C under low light (100 μmol/m^2/s) for the first 10 days and stronger light (130 μmol/m^2/s) for the next 10 days, with an 18-h photoperiod.
5. For rooting, transfer regenerating callus to MSRMc medium under the stronger light conditions described above.

6. When transgenic corn plantlets are approximately 3–4 cm long, transfer them to soil (see Note 18).
7. Maintain transgenic corn plants in a growth chamber with day/night temperature and humidity conditions of 28/24°C and 70/85%, with a 14-h photoperiod for 3 months. Then transfer to greenhouse conditions (24–28°C, 70% humidity, sunlight, and a 14-h photoperiod).

3.2. Transformation of Rice Plants

As discussed for corn, carry out the manipulation of seeds, explants, and callus using sterilized tools and equipment in a laminar flow hood to avoid microbial contamination. We recommend that all glassware is cleaned with double-distilled water (no detergents), covered with aluminum foil (see Note 12) and sterilized at 180°C for 20 h.

3.2.1. Preparation of Rice Explants for Bombardment

1. Collect seeds from rice plants in the greenhouse. Immature embryos can be used for transformation, but we recommend the following protocol that uses mature seed–derived callus as it is more efficient (see Note 16).
2. Dehusk the seeds using a mortar and pestle and collect them in a 50-mL Falcon tube (see Note 17).
3. Sterilize the seeds in 100% ethanol for 2 min, rinse three times in distilled water (optional), and then wash in 50% sodium hypochlorite for 20 min.
4. Transfer to a laminar flow hood and rinse the seeds three times with sterile distilled water and blot dry on Whatman 3MM paper.
5. Place the seeds on MSHr medium (20 seeds per Petri dish) and check for contamination daily. Discard contaminated seeds and transfer the remainder to fresh dishes.
6. After 1 week in culture, excise mature embryos from the surviving seeds and place them, scutellum-uppermost, on MSHr medium.
7. Incubate the plates in darkness at 24°C for 1 day.
8. Four hours before bombardment, transfer embryos to MSOr medium (see Note 14).

3.2.2. Bombardment Using Bio-Rad Helium Gun (PDS 1000/He)

1. Prepare DNA-coated gold particles as described in Subheading 3.1.2.
2. Follow manufacturer's guidelines for loading carrier disc into the barrel of the helium gun.
3. Place plant material in the targeting chamber.
4. Bombard plant material at 900–1,300 psi (see Note 15).

5. Bombard embryos twice with a 4-h interval between bombardments (maintain the embryos in darkness at 24°C on MSOr during the interval).

3.2.3. Selection and Regeneration of Transgenic Rice Plants

1. Transfer bombarded embryos to MSHr medium. Incubate in darkness at 24°C for 1 day.
2. For the induction of transgenic embryogenic callus, transfer embryos to MSSr medium and incubate in darkness at 24°C for 2 weeks.
3. Subculture any callus recovered under selection at 2-week intervals on fresh MSSr medium in darkness at 24°C (see Note 18).
4. After the third round of selection, transfer explants to MSRr medium for regeneration. Incubate plates at 24°C under low light (100 μmol/m^2/s) for the first 10 days and stronger light (130 μmol/m^2/s) for the next 10 days, with an 18-h photoperiod.
5. For rooting, transfer regenerating callus to MSRMr medium under the stronger light conditions described above.
6. When transgenic rice plantlets are approximately 10-cm long, transfer them to soil (see Note 19).
7. Maintain transgenic rice plants in a growth chamber with day/night temperature and humidity conditions of 28/24°C and 70/85%, with a 14-h photoperiod for 3 months. Then transfer to greenhouse conditions (24–28°C, 70% humidity, sunlight, and a 14-h photoperiod).

3.3. Carotenoid Analysis by HPLC

3.3.1. Carotenoid Extraction

1. Freeze-dry seeds before commencing extraction.
2. Grind the dry samples into fine powder using a mortar and pestle, washing the equipment between samples to avoid cross-contamination.
3. Transfer 50–100 mg of each sample into a steel tube (see Note 20).
4. Add 15 mL (1:1 v/v) tetrahydrofuran:methanol and cover with aluminum foil.
5. Transfer to a water bath and incubate at 60°C for 20 min.
6. Cool at room temperature for 10–15 min.
7. Transfer the extract to a separation funnel. Line a gravity funnel with filter paper then filter the carotenoid extract into the separation funnel to remove starch.
8. Transfer filter back to the steel tube, add 5 mL acetone, and incubate for 5 min.
9. Pour the remaining extract carefully in the separation funnel.

10. Add 15 mL (9:1 v/v) hexane:diethyl ether. Invert the separation funnel to mix and vent to remove vapor.
11. Add 20 mL of saturated NaCl and repeat the mixing and venting step.
12. Allow the layers to separate (~1 min).
13. Remove lower layer.
14. Add saturated NaCl as above and repeat the mixing and venting step.
15. Allow the layers to separate and again remove the lower layer.
16. Dry the organic phase under N_2 in a block at 37°C until the volume is 5 mL.
17. Transfer 1 mL of (9:1 v/v) hexane:diethyl ether to a cuvette and use this to set the baseline absorbance of the spectrophotometer at 450 nm.
18. Measure the absorbance of 1 mL of the organic phase from the sample.
19. Pour the sample back into the tube and leave it under N_2 for further drying.
20. When the sample is completely dry, fill the tube with argon gas and close tightly with a rubber stopper.
21. Cover the tube with aluminum foil and store at –80°C.

3.3.2. Chromatography

1. For liquid chromatography, the solvent is a 70:50:30 (v/v) mixture of acetonitrile, acetone, and methanol. Filter the solvent through a 0.22-μm Durapore PVDF Membrane Filter before use. Each extract sample for analysis should be dissolved in 300 μL of the filtered solvent.
2. Perform the separation of lutein and zeaxanthin using a YMC C_{30} carotenoid 3-μm, 2.0×100-mm HPLC column (Waters, Milford, MA) with a mobile phase comprising solvent A (methanol:water, 80:20, v/v) and solvent B (100% methyl *tert*-butyl ether).
3. Warm the sample to 25°C and inject 10 μL into the column, which should also be thermostatically maintained at 25°C. Set the flow rate to 0.3 mL/min.
4. Set the gradient program as follows: initial conditions 97% solvent A and 3% solvent B for 6 min; change with a linear gradient to 62% solvent A and 38% solvent B in 1 min, hold for 8 min; change with a linear gradient to 32% solvent A and 68% solvent B in 1 min, hold for 10 min; then return to initial conditions in 4 min, followed by equilibration for 5 min.
5. Perform the separation of all other carotenoids using a reversed phase column (ACQUITY UPL® BEH 300 Å C_{18}, 1.7 μm,

2.1 × 150 mm; Waters, Milford, MA), with a mobile phase comprising solvent A (acetonitrile:methanol, 70:30, v/v) and solvent B (100% water).

6. Warm the sample to 25°C and inject 5 µL into the column, which should be thermostatically maintained at 32°C. Set the flow rate to 0.4 mL/min.
7. Set the gradient program as follows: initial conditions 80% solvent A and 20% solvent B for 2 min; change with linear gradient to 100% solvent A in 1 min, hold for 9 min; then return to initial conditions in 0.1 min, followed by equilibration for 2 min.
8. Monitor absorbance at 450 nm.
9. Identify carotenoids by monitoring the following parameters: order of elution from the column, ultraviolet and visible spectra, and the spectral fine structure (%III/II) (12) with literature data (13) and with that of the authentic standards. Also, use those standards for quantitation in combination with the extinction coefficients (13).

4. Notes

1. The ingredients for these basic media are listed in Table 1, although, ready-mixed powders can be purchased from many suppliers (e.g., Gibco-BRL, Flow Labs, Sigma).
2. Two gelling agents are used in the rice transformation protocol because various studies have shown that the "stiffness" of the medium affects callus growth and the efficiency of shoot and root formation (14).
3. Some chemicals may require warming to 37°C and/or long stirring before they dissolve. Before use, briefly vortex all stock solutions.
4. The choice of selective agent depends on the selectable marker used for transformation. Hygromycin B is preferred in rice because kanamycin is not as effective. Hygromycin B is used with the marker *hpt* (from *Klebsiella* spp., encoding hygromycin B phosphotransferase). PPT selection is used with the markers *bar* or *pat*, both of which encode phosphinothricin acetyltransferase.
5. Immature M37W corn embryos must be cultured on semisolid nutritive media based on MS salts (9). The basic MS medium for corn contains the salts and organic compounds listed in Table 1. Several variants are required for different stages of growth, transformation, and regeneration, all of which contain sucrose and a gelling agent (gellan gum) and some of which

contain additional components. The sucrose and gelling agent should be mixed with the basic medium prior to autoclaving, but labile reagents such as the phytohormones and selection agents should be added when the autoclaved medium has cooled below 50°C. It is useful to make up basic MS salts and minimal organics with 4 g/L gellan gum and 20 g/L sucrose for the MSHc, MSOc, and MSSc media and to make a second batch with 4 g/L gellan gum and 30 g/L sucrose for the MSRc and MSMRc media. Add the sucrose first, and before adding the gelling agent, adjust the pH to 5.8 with 1 M KOH. MSHc is the basic MS salts and organics recipe supplemented with 20 g/L sucrose, 4 g/L gellan gum, and 2.5 mg/L 2,4-D to promote callus growth. An osmoticum medium (MSOc) is used prior to bombardment, and this is MSHc supplemented with 0.2 M mannitol and 0.2 M sorbitol (see Note 14). A selection medium (MSSc) is required to isolate transgenic callus expressing the *bar* marker gene, and this is MSHc supplemented with 3 mg/L PPT. The regeneration medium (MSRc) is the basic MS salts and organics recipe supplemented with 30 g/L sucrose, 4 g/L gellan gum, 0.25 mg/L 2,4-D, 10 mg/L 6-BAP, and 3 mg/L PPT to encourage the growth of transgenic shoots. The rooting medium (MSRMc) is similar to MSRc but contains no phytohormones.

6. Mature rice embryos are cultured on media based on MS salts (15, 16). The basic MS medium for rice contains the salts and organic compounds listed in Table 1. Several variants are required for different stages of growth, transformation, and regeneration, all of which contain a carbon source (sucrose or maltose) and a gelling agent (gellan gum or gellan gum with agar; see Note 2) and some of which contain additional components. The sugar and gelling agent should be mixed with the basic medium prior to autoclaving, but labile reagents such as the phytohormones and selection agents should be added when the autoclaved medium has cooled below 50°C. Add the sugar first, and before adding the gelling agent, adjust the pH to 5.8 with 1 M KOH. MSHr is the basic MS salts and organics recipe supplemented with 30 g/L sucrose, 5 g/L gellan gum, and 2.5 mg/L 2,4-D to promote callus growth. An osmoticum medium (MSOr) is used prior to bombardment, and this is similar to MSHr, but contains a slightly lower concentration of gelling agent (3 g/L gellan gum; see Note 2) plus 0.2 M mannitol (see Note 14). A selection medium (MSSr) is required to isolate transgenic callus expressing the *hpt* marker gene, and this is MSHr supplemented with 30 mg/L hygromycin B. The regeneration medium (MSRr) is the basic MS salts and organics recipe supplemented with 0.2 M maltose, 4 g/L gellan gum, 5 mg/L NAA, and 3 mg/L 6-BAP to encourage the growth of shoots and 30 mg/L hygromycin B

to prevent the emergence of "escapes" (nontransgenic cells that survive under selection). Maltose has been shown to promote green tissue growth better than sucrose and other carbon sources in a number of plant tissue culture systems (17–19), and we have found this also applies to callus derived from mature rice embryos. The rooting medium is similar to MSRr but replaces maltose with 10 g/L sucrose and contains no phytohormones.

7. We used five different endosperm-specific promoters in our pilot system (1), but we have found that using the same promoter for each transgene is just as effective (20).
8. Only high-quality supercoiled plasmid DNA should be used. We find that QIAGEN Maxiprep plasmid kits are satisfactory for this purpose. The eluted plasmid DNA should be precipitated with ethanol and redissolved in water to a concentration of 10 mg/mL. Check the concentration and purity using a Nanodrop 1000 UV spectrophotometer.
9. The selectable marker gene is provided on a separate plasmid. All transgenic plants carry this marker even though the recovery of other transgene combinations is random. This does not mean the marker is any more likely to integrate but reflects the fact that transgenic plants are propagated under selection, so only those carrying the marker survive.
10. A Bio-Rad pneumatic helium gun, model PDS 1000/He, is described here, but other devices can be used including homemade ones. When considering investing in or hiring a PDS 1000/He particle gun, the manufacturers will advise on setting up and operating procedures. Bottled helium gas, carrier sheets, rupture discs, and stopping sheets are available from the same supplier.
11. Tungsten particles are less expensive than gold ones, but we find gold particles more efficient for transformation, possibly because tungsten particles are less uniform in size and are more reactive than gold, resulting in more damage to the target tissue (reviewed in ref. 21). The size of the particles also influences transformation efficiency. Larger particles are generally faster and penetrate further but cause more damage. For rice and corn transformation, we recommend particles 0.7–0.9 μm in diameter.
12. The use of expensive covers for the culture vessels is not necessary. Aluminum foil is easily costerilized and provides a satisfactory safety level against contamination.
13. The optimal age of immature corn embryos for transformation is 14 days. The maturity of the embryos can be determined by their size (immature embryos will be 0.8–1 mm in length).
14. Osmoticum treatment has been shown to improve the efficiency of particle bombardment. The exact basis is unclear.

It may remove water droplets from the seed surface that deflect the metal particles or it may change the physiology of the cell (e.g., plasmolysis), making it more amenable to transformation (22). We use mannitol for this purpose in both corn and rice, although the corn protocol also includes sorbitol as this has been shown to stimulate the growth of embryogenic callus (23).

15. The optimal pressure and distance between the gun and the target needs to be determined empirically for each type of explant. For optimization, a transient expression assay is useful. Bombard the explant with a DNA construct comprising the visible marker *gusA* driven by a strong and constitutive promoter such as corn *Ubi-1*. Optimal transformation occurs when many small spots of GUS activity are detected 1–2 days after bombardment. If there are large sectors of GUS-positive tissue, this usually means the particle velocity is too high and the tissue is dead. Reduce the pressure or increase the distance to the target and try again.
16. The maturity of rice embryos can be estimated by pressing the seeds between the fingers. Immature seeds leave a milky liquid residue on the fingers whereas mature seeds do not. Callus derived from mature seeds is more efficient for transformation, but mature seeds are tougher and need to be dehusked with sandpaper.
17. Handle the dehusked seeds very carefully from this stage onward and use very gentle agitation.
18. Here, the aim is to preserve any embryogenic callus produced within the scutellar region of the mature embryos. Dead callus, which can be recognized by its brown or black colour, should be discarded at each subculture to prevent the proliferation of escapes.
19. We use a 4:2:1 mix of topsoil, sand, and pouzzelane (EuroPouzzelane, Lattes, France).
20. The amount depends on the colour intensity of the samples. For pale samples, extract 100 mg. For darker samples, it is better to extract 50 mg.

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