Engineering the provitamin A (β-carotene) biosynthetic pathway into carotenoid-free rice endosperm

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Vitamin A deficiency causes symptoms ranging from night blindness to those of xerophthalmia and keratomalacia, leading to total blindness. In Southeast Asia, it is estimated that a quarter of a million children go blind each year because of this nutritional deficiency (1). Furthermore, vitamin A deficiency exacerbates afflictions such as diarrhea, respiratory diseases, and childhood diseases such as measles (2, 3). It is estimated that 124 million children worldwide are deficient in vitamin A (4) and that improved nutrition could prevent 1 million to 2 million deaths annually among children (3). Oral delivery of vitamin A is problematic (5, 6), mainly due to the lack of infrastructure, so alternatives are urgently needed. Success might be found in supplementation of a major staple food, rice, with provitamin A. Because no rice cultivars produce this provitamin in the endosperm, recombinant technologies rather than conventional breeding are required.

Immature rice endosperm is capable of synthesizing the early intermediate geranylgeranyl diposphate, which can be used to produce the uncolored carotenoid phytoene by expressing the enzyme phytoene synthase in rice endosperm (7). The synthesis of β-carotene requires the complementation with three additional plant enzymes: phytoene desaturase and ζ-carotene desaturase, each catalyzing the introduction of two double bonds, and lycopene β-cyclase, encoded by the lyc gene. To reduce the transformation effort, a bacterial carotenoid desaturase, capable of introducing all four double bonds required, can be used.

We used Agrobacterium-mediated transformation to introduce the entire β-carotene biosynthetic pathway into rice endosperm in a single transformation effort with three vectors (Fig. 1) (7). The vector pB19p4c combines the sequences for a plant phytoene synthase (psy) originating from daidolfi (9) (Narcissus pseudonarcissus; GenBank accession number X78814) with the sequence coding for a bacterial phytoene desaturase (crt) originating from Erwinia uredovora (GenBank accession number D00087) placed under control of the endosperm-specific gluculin (G1) and the constitutive CaMV (carnation mosaic virus) 35S promoter, respectively. The phytoene synthase cDNA contained a 5'-sequence coding for a functional transit peptide (10), and the crt gene contained the transit peptide (11) sequence of...
the pea Rubisco small subunit (11). This plasmid should direct the formation of lycopene in the endosperm plastids, the site of geranylgeranyl-diphosphate formation.

To complete the β-carotene biosynthetic pathway, we co-transformed with vectors pZPsC and pZLcyH. Vector pZPsC carries psy and crtl as in plasmid pB19hpc, but lacks the selectable marker aphIV expression cassette. Vector pZLcyH provides lycopene β-cyclase from Narcissus pseudonarcissus (12) (GenBank accession number X98796) controlled by the rice glutelin promoter and the aphIV gene controlled by the CaMV 35S promoter as a selectable marker. Lycopene β-cyclase carried a functional transit peptide allowing plastid import (10).

Precultured immature rice embryos (n = 800) were inoculated with Agrobacterium LBA4404/pZPsC and LBA4404/pZLcyH. Hygromycin-resistant plants (n = 50) were analyzed for the presence of psy and crtl genes (Fig. 2). Meganuclease I-Sce I digestion released the −10-kb insertion containing the aphIV, psy, and crtl expression cassettes. Kpn I was used to estimate the insertion copy number. All samples analyzed carried the transgenes and revealed mostly single insertions.

Immature rice embryos (n = 500) were inoculated with a mixture of Agrobacterium LBA4404/pZPsC and LBA4404/pZLcyH. Co-transformed plants were identified by Southern hybridization, and the presence of pZPsC was analyzed by restriction digestion. Presence of the pZLcyH expression cassette was determined by probing I-Sce I and Spe I digested genomic DNA with internal lcy fragments. Of 60 randomly selected regenerated lines, all were positive for lcy and 12 contained pZPsC as shown by the presence of the expected fragments: 6.6 kb for the I-Sce I–excised psy and crtl expression cassettes from pZPsC and 9.5 kb for the lcy and aphIV genes from pZCycH (Fig. 1). One to three transgene copies were found in co-transformed plants. Ten plants harboring all four introduced genes were transferred into the greenhouse for setting seeds. All transformed plants described here showed a normal vegetative phenotype and were fertile.

Mature seeds from T₀ transgenic lines and from control plants were air dried, dehusked, and, in order to isolate the endosperm, polished with emery paper. In most cases, the transformed endosperms were yellow, indicating carotenoid formation. The pB19hpc single transformants (Fig. 2A) showed a 3:1 (colored/noncolored) segregation pattern, whereas the pZPsC/pZLcyH co-transformants (Fig. 2B) showed variable segregation. The pB19hpc single transformants, engineered to synthesize only lycopene (red), were similar in color to the pZPsC/pZLcyH co-transformants engineered for β-carotene (yellow) synthesis.

Seeds from individual lines (1 g for each line) were analyzed for carotenoids by photometric and by high-performance liquid chromatography (HPLC) analyses (13). The carotenoids found in the pB19hpc single transformants accounted for the color; none of these lines accumulated detectable amounts of lycopene. Instead, β-carotene, and to some extent lutein and zeaxanthin, were formed (Fig. 3). Thus, the lycopene α(β)- and β-cyclases and the hydroxylase are either constitutively expressed in normal rice endosperm or induced upon lycopene formation.

The pZPsC/pZLcyH co-transformants had a more variable carotenoid pattern ranging from phenotypes similar to those from single transformations to others that contain β-carotene as almost the only carotenoid. Line z11b is such an example (Fig. 3C and Fig. 2B, panel 2) with 1.6 µg/g carotenoid in the endosperm. However, reliable quantitations must await homogenous lines with uniformly colored grains. Considering that extracts from the sum of (colored/
noncolored) segregating grains were analyzed, the goal of providing at least 2 μg/g provitamin A in homogenous lines (corresponding to 100 μg retinol equivalents at a daily intake of 300 g of rice per day), seems to be realistic (7). It is not yet clear whether lines producing provitamin A (β-carotene) or lines possessing additional zeaxanthin and lutein would be more nutritious, because the latter have been implicated in the maintenance of a healthy mucosa within the retina (14).

References and Notes

8. Three vectors—pUC18, pZP100, and pBIN19(15–17)—were digested with Eco RI and Hind III and a synthetic linker flanking by meganuclease I-Sce I including Kpn I, Not I, and Sma I (5'-AATTCATTACCCCTGTTAATCCTACCGCGCCGGCCCCGTTACCACTCCTCCTAA-3') and (5'-AGCTTGAATACCAAGGGTAAATGACCGCGCCGGCGTTAGGATACCGGTAACTG-3') were introduced, forming pUC18, pZP100, and pBIN19, respectively. An intermediate vector was made by insertion of the ctri expression cassette excised from Hind III/Eco RI digested pUC14, originally derived from pYRE4 (11), into pBluescript KS with Hind III/Eco RI digestion, followed by insertion of psy expression cassette from Sac II-blunted/Kpn I-digested pOT1psyI (7) into the Kpn I/Xho I-blunted previous vector. Finally, ctri and psy expression cassettes were isolated with Kpn I/Nco I digestion and inserted into Kpn I/Nco I-digested pUC18M and designated as pBNA3. pBNA19 was made by insertion of a Kpn I fragment originally from pCB8000 (12) containing psy selectable marker gene into pBNA3, followed by digestion of the I-Sce I fragment of the resulting plasmid and insertion into I-Sce I-digested pBIN19M. PC25 was obtained by insertion of the I-Sce I fragment of pBNA3 bearing the psy and ctri genes into I-Sce I-digested pZP100M. The three vectors were separately electroporated into Agrobacterium tumefaciens LBA4404 (19) with corresponding antibiotic selection. Callus induction: Immature seeds of japonica rice cultivar TF 329 at milk stage were collected from greenhouse-grown plants, surface-sterilized in 70% ethanol (v/v) for 1 min, incubated in 0.3% calcium hypochlorite for 1 h on a shaker, and rinsed three to five times with sterile distilled water. Immature embryos were then isolated from the sterilized seeds and cultured onto NB medium [N6 salts and 85 vitamins, supplemented with 30 g/l maltose, 500 mg/l proline, 300 mg/l casein hydrolysate, 500 mg/l glutamine, and 2 mg/l L-asparagine (pH 5.8)]. After 4 to 5 days, the coleoptiles were removed, and the swelled scutella were subcultured onto fresh NB medium for 3 to 5 days until inoculation of Agrobacterium. Transformation: 1-week-old precultured immature embryos were immersed in Agrobacterium tumefaciens LBA 4404 cell suspension as described (20). For co-transformation, LBA4404/pZP100C optical density at 600 nm (OD600) = 2.0 mixed with an equal volume of LBA4404/pZP100C (OD600 = 1.0) was used for inoculation after acetylsyringone induction. The inoculated precultured embryos were co-cultivated on NB medium supplemented with 200 mM acetosyringone for 3 days, subcultured on recovery medium (NB with 250 mg/l cefotaxime) for 1 week and then transferred onto NB selection medium in the presence of 30 mg/l hygromycin and 250 mg/l cefotaxime for 4 to 6 weeks. Transgenic plants were regenerated from recovered resistant calli on NB medium supplemented with 0.5 mg/l NAA and 3 mg/l BAP in 4 weeks, rooted and transferred into the greenhouse.

5. Dehulled seeds were polished for 6 hours with emery paper on a shaker. The endosperm obtained was ground to a fine powder and 1 g was extracted repeatedly with acetone. Combined extracts were used to record the ultraviolet-visible spectrum, allowing quantification using an e1%1cm of 134,000 for β-carotene. The samples were dried and the residue quantitatively applied in 3 spectrophotometer to HPLC for analysis using a photodiode array detector (Waters) and a C18 reversed-phase column (YMC Europe GmbH) with the solvent system A [methanol: tert-butyl/methyl ether (1:1, v/v)] and system B [methanol:tert-butyl/methyl ether (1:1, v/v)] used, a gradient of 100% B to 4% B within 25 min, then to 0% B within a further 75 min. Final conditions were maintained for an additional 10 min. Photometric quantifications were re-examined using HPLC with synthetic all-trans lycopene as an external standard.
13. The ctri gene fused to the transit peptide sequence was kindly provided by N. Misawa (Kihon Co., Ltd., Japan). We thank W. Dong and P. Burkhardt for their valuable contributions. S. Klarer, K. Konja, and U. Schneider-Ziebert for skillful technical assistance, and R. Cassada for correcting the English version of the manuscript. Supported by the Rockefeller Foundation (1993–1996), the European Community Biotech Program (FAIR CT96, 1996–1999) (P.B.), the Swiss Federal Office for Education and Science (I.P.), and by the Swiss Federal Institute of Technology (1993–1996).
15 July 1999; accepted 19 November 1999.