Engineering the provitamin A beta-carotene biosynthetic pathway into carot...
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13. The AAPK coding sequence was amplified by PCR from the AAPK cDNA clone with the primers 5′-GA-ATCTCCACTACGACGCCGTTTACTTCCG-3′ and 5′-CCGTGCAACCATGGATATGGCATATACAAT-3′. The pAAPK-GFP construct was created by inserting (via Nco I digestion) the amplified AAPK coding sequence downstream of the 35S promoter and upstream of, and in frame with, the GFP coding sequence in the GFP expression vector (pGFP) described in (*17*). To create pAAPK(K43A)-GFP, the lysine at position 43 in the AAPK coding sequence was substituted with an alanine by site-directed mutagenesis {overlapping PCR method; [S. N. Ho, H. D. Hunt, R. M. Horton, J. K. Pullen, L. R. Pease, *Gene* **77**, 51 (1989)]}. Constructs were sequenced to confirm correct junction, orientation, and/or site mutation.
14. Fifteen million *V. faba* guard cell protoplasts were transfected with pGFP, pAAPK-GFP, or pAAPK(K43A)-GFP by polyethylene glycol–mediated DNA transfer (*17*). Protoplasts were lysed and recombinant protein was immunoprecipitated with GFP peptide antibodies (Clontech, Palo Alto, CA) and protein A–Sepharose CL-4B (Amersham Pharmacia Biotech, Picataway, NJ). Immunoprecipitated proteins were assayed for kinase activity using histone III-S (Sigma) as substrate (*4*). No histone phosphorylation by the pGFP control immunoprecipitate was observed. Analogous to native AAPK (*4*), histone phosphorylation by the pAAPK-GFP immunoprecipitate was enhanced when the immunoprecipitate was isolated from ABA-treated guard cells. When the same experiment was performed with pAAPK(K43A)-GFP, histone phosphorylation was reduced.
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16. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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19. *V. faba* leaves were bombarded with DNA-coated [pGFP, pAAPK-GFP, or pAAPK(K43A)-GFP] gold particles (BioRad, Hercules, CA) with the use of a particle delivery system 1000/He (BioRad, Hercules, CA) as described [J. Marc *et al.*, *Plant Cell* **10**, 1927 (1998)]. Stomatal complexes were then assayed for ABA-prevented opening or closure stimulated by ABA, CO_2, or darkness. Conditions for opening experiments were as described [S. M. Assmann and T. I. Baskin, *J. Exp. Bot.* **49**, 163 (1998)] except that the incubation solution was 10 mM MES, 30 mM KCl, pH 6.1 with or without 50 μM [±]-*cis,trans*-ABA. For closure experiments, transformed leaves were illuminated with 0.20 mmol m^{-2} s^{-1} white light for 2.5 hours to open stomata. Epidermal peels were then taken and placed in incubation solution under darkness or treated with 25 μM [±]-*cis,trans*-ABA, or with 700 parts per million (ppm) CO_2 for 1 hour.
20. Abaxial guard cell protoplasts were isolated as described [X.-Q. Wang, W.-H. Wu, S. M. Assmann, *Plant Physiol.* **118**, 1421 (1998)]. Whole-cell patch-clamp experiments were performed as previously established (*22*). Pipette solution contained 100 mM KCl, 50 mM tetraethylammonium, 2 mM $MgCl_2$, 6.7 mM EGTA-$(Tris)_2$, 3.35 mM $CaCl_2$, 10 mM Hepes, pH 7.1 (Tris), and 5 mM Mg-ATP. Bath solution contained 40 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM MES-Tris pH 5.6. Osmolalities were adjusted with sorbitol to 500 mosmol/kg (in the pipette) or 470 mosmol/kg (in the bath). Protein kinase inhibitor K252a (Calbiochem, La Jolla, CA) was prepared as a 2 mM stock in dimethyl sulfoxide (DMSO); DMSO controls showed no effect.
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30. We thank the following for their advice: R. Cyr (provision of GFP expression vector), C. Granger (bombardment experiments), S. Gilroy and S. Ritchie (GFP photography), L. Haubrick (Northern blotting), and J. A. Snyder (CO_2 experiments). We also thank W. Lane and the Harvard Microchemistry Facility for mass spectrometric sequencing. Supported by NSF grant MCB-9874438.

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Engineering the Provitamin A (β-Carotene) Biosynthetic Pathway into (Carotenoid-Free) Rice Endosperm

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Rice (*Oryza sativa*), a major staple food, is usually milled to remove the oil-rich aleurone layer that turns rancid upon storage, especially in tropical areas. The remaining edible part of rice grains, the endosperm, lacks several essential nutrients, such as provitamin A. Thus, predominant rice consumption promotes vitamin A deficiency, a serious public health problem in at least 26 countries, including highly populated areas of Asia, Africa, and Latin America. Recombinant DNA technology was used to improve its nutritional value in this respect. A combination of transgenes enabled biosynthesis of provitamin A in the endosperm.

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 required. 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 diphosphate, which can be used to produce the uncolored carotene 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 *lcy* gene. To reduce the transformation effort, a bacterial carotene 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) (*8*). The vector pB19hpc combines the sequences for a plant phytoene synthase (*psy*) originating from daffodil (*9*) (*Narcissus pseudonarcissus*; GenBank accession number X78814) with the sequence coding for a bacterial phytoene desaturase (*crtI*) originating from *Erwinia uredovora* (GenBank accession number D90087) placed under control of the endosperm-specific glutelin (Gt1) and the constitutive CaMV (cauliflower mosaic virus) 35*S* promoter, respectively. The phytoene synthase cDNA contained a 5′-sequence coding for a functional transit peptide (*10*), and the *crtI* gene contained the transit peptide (*tp*) sequence of

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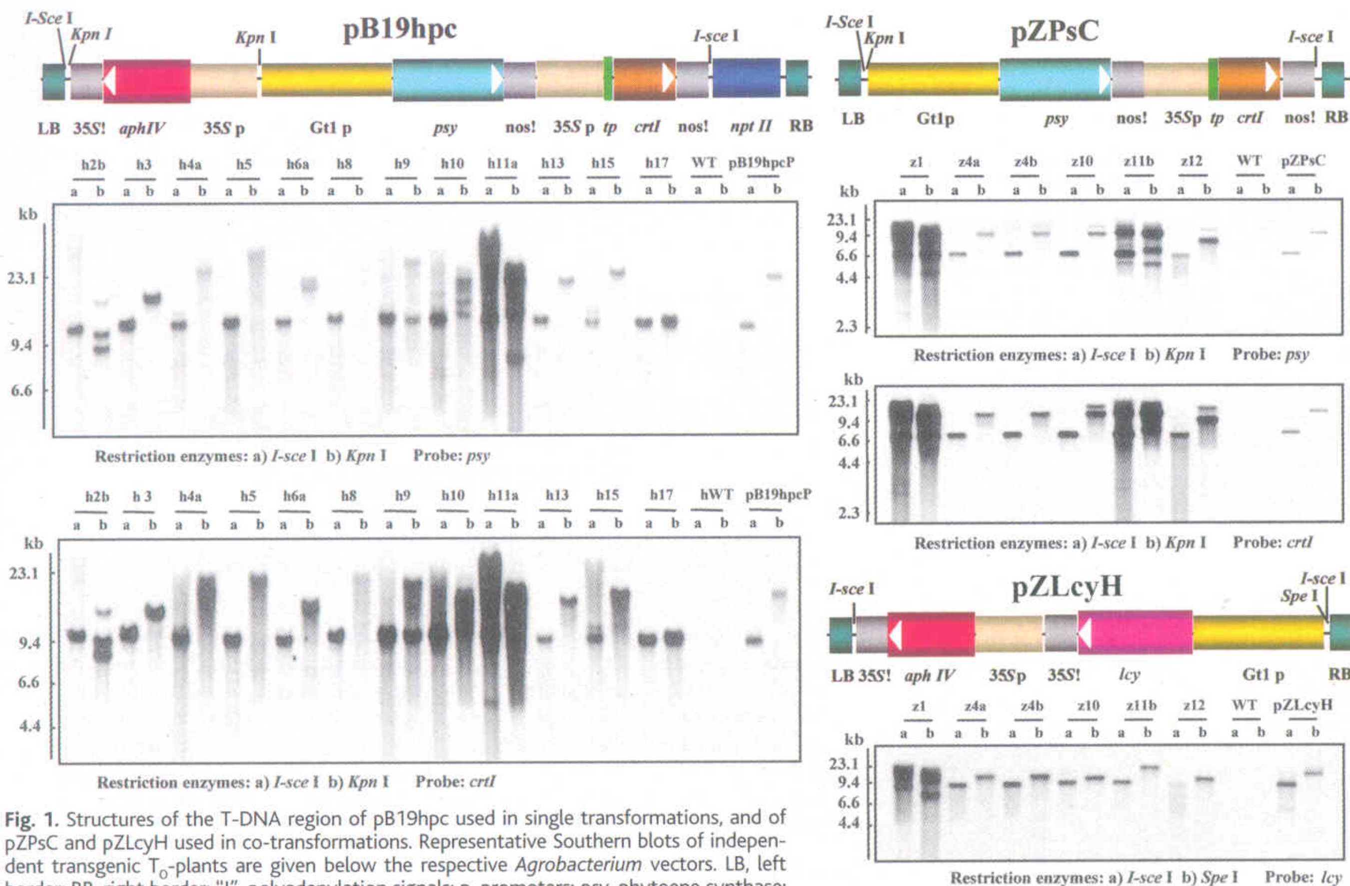

Fig. 1. Structures of the T-DNA region of pB19hpc used in single transformations, and of pZPsC and pZLcyH used in co-transformations. Representative Southern blots of independent transgenic T_0-plants are given below the respective *Agrobacterium* vectors. LB, left border; RB, right border; "!", polyadenylation signals; p, promoters; *psy*, phytoene synthase; *crtI*, bacterial phytoene desaturase; *lcy*, lycopene β-cyclase; *tp*, transit peptide.

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 *crtI*, 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 rice glutelin promoter and the *aphIV* gene controlled by the CaMV 35*S* 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/pB19hpc. Hygromycin-resistant plants (n = 50) were analyzed for the presence of *psy* and *crtI* genes (Fig. 2). Meganuclease I–Sce I digestion released the ~10-kb insertion containing the *aphIV*, *psy*, and *crtI* 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 cassettes 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 *crtI* 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 T_0 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(ε)- 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 homozygous lines with uniformly colored grains. Considering that extracts from the sum of (colored/

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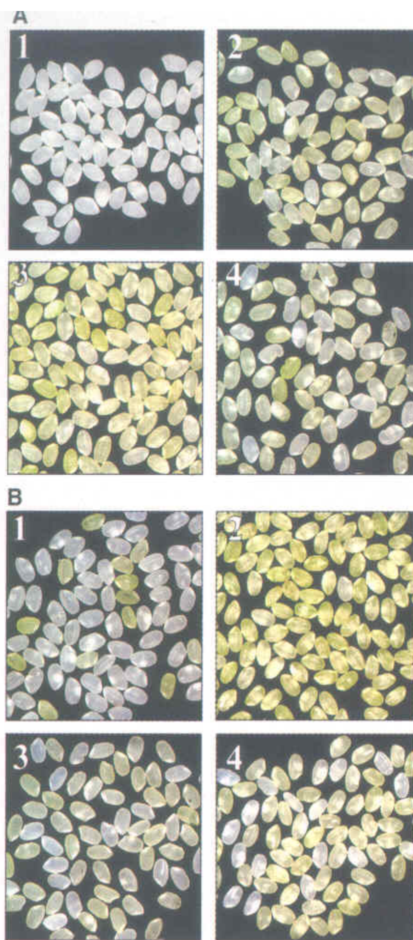
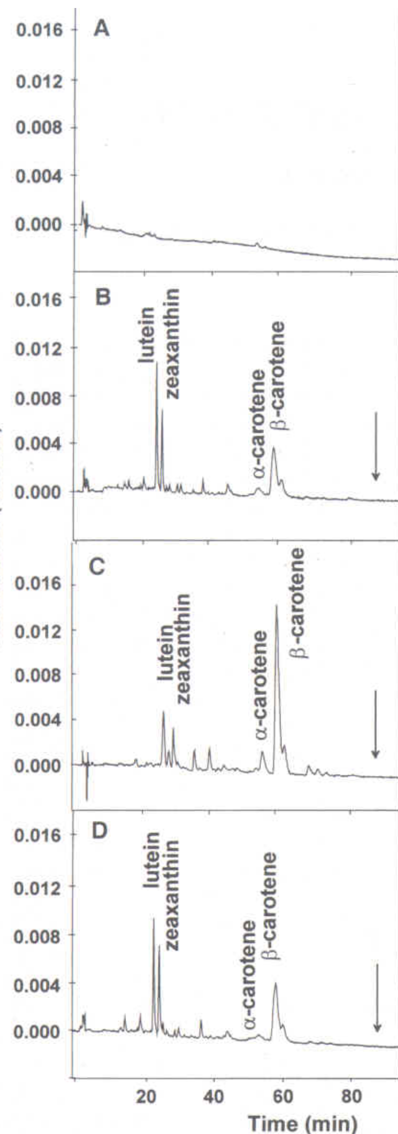


Fig. 2. Phenotypes of transgenic rice seeds. Bar, 1 cm. (**A**) Panel 1, untransformed control; panels 2 through 4, pB19hpc single transformants lines h11a (panel 2), h15b (panel 3), h6 (panel 4). (**B**) pZPsC/pZLcyH co-transformants lines z5 (panel 1), z11b (panel 2), z4a (panel 3), z18 (panel 4).



g. 3. The carotenoid extracts from seeds (1 g each line) were subjected quantitatively to LC analysis. (**A**) Control seeds, (**B**) line h2b ngle transformant), (**C**) line z11b (co-trans-mant), and (**D**) z4b (co-transformant). The e of lycopene elution in the chromatogram is licated by an arrow.

noncolored) segregating grains were analyzed, the goal of providing at least 2 μg/g provitamin A in homozygous 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 additionally zeaxanthin and lutein would be more nutritious, because the latter have been implicated in the maintenance of a healthy macula within the retina (*14*).

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8. Three vectors—pUC18, pPZP100, 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′-AATTCATTACCCTGTTATC-CCTACCCGGGCGGCCGCGGTACCATTACCCTGTTA-TCCCTAA-3′) and (5′-AGCTTTAGGGATAACA-GGGTAATGGTACCGCGGCCGCCCGGGTAGGGAT-AACGGGTAATG-3′) were introduced, forming pUC18M, pPZP100M, and pBin19M, respectively. An intermediate vector was made by insertion of the *crtI* expression cassette excised from Hind III/Eco RI–digested pUCET4, originally derived from pYPIET4 (*11*), into pBluescriptKS with Hind III/Eco RI digestion, followed by insertion of *psy* expression cassette from Sac II–blunted/Kpn I–digested pGt1psyH (*7*) into the Kpn I/Xho I–blunted previous vector. Finally, *crtI* and *psy* expression cassettes were isolated with Kpn I/Not I digestion and inserted into Kpn I/Not I–digested pUC18M and designated as pBaal3. pBin19hpc was made by insertion of a Kpn I fragment originally from pCIB900 (*18*) containing *aphIV* selectable marker gene into pBaal3, followed by digestion of the I-Sce I fragment of the resulting plasmid and insertion into I-Sce I–digested pBin19M. pZPsC was obtained by insertion of the I-*SceI* fragment of pBaal3 bearing the *psy* and *crtI* genes into I-Sce I–digested pPZP100M. pZLcyH was constructed by digestion of pGt1LcyH with I-Sce I and insertion of the resulting fragment, carrying *lcy* and *aphIV*, into I-Sce I–restricted pPZP100M. The three vectors were separately electroporated into *Agrobacterium tumefaciens* LBA4404 (*19*) with corresponding antibiotic selection. Callus induction: Immature seeds of japonica rice cultivar TP 309 at milk stage were collected from greenhouse-grown plants, surface-sterilized in 70% ethanol (v/v) for 1 min, incubated in 6% calcium hypochloride for 1 hour 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 B5 vitamins, supplemented with 30 g/l maltose, 500 mg/l proline, 300 mg/l casein hydrolate, 500 mg/l glutamine, and 2 mg/l 2,4-D (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/pZPsC [optical density at 600 nm (OD_{600}) = 2.0] mixed with an equal volume of LBA4404/pZLcyH (OD_{600} = 1.0) was used for inoculation after acetonsyrigone induction. The inoculated precultured embryos were co-cultivated onto NB medium supplemented with 200 mM acetonsyringone 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.
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13. Dehusked 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 ε_{450nm} 134,000 l^{-1} mol^{-1} cm^{-1} for β-carotene. The samples were dried and the residue quantitatively applied in 30 μl chloroform to HPLC for analysis using a photodiode array detector (Waters) and a C_{30} reversed-phase column (YMC Europe GmbH) with the solvent system A [methanol: *tert*-butylmethyl ether (1:1, v/v)] and system B [methanol:*tert*-butylmethyl ether:H_2O (6:1.2:1.2, v/v/v)], using a gradient of 100% B to 43% 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 by HPLC using synthetic all-*trans* lycopene as an external standard.
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