

Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis

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Summary

Rice (*Oryza sativa* L.), the major food staple for more than two billion people, contains neither β -carotene (provitamin A) nor C_{40} carotenoid precursors thereof in its endosperm. To improve the nutritional value of rice, genetic engineering was chosen as a means to introduce the ability to make β -carotene into rice endosperm tissue. Investigation of the biochemical properties of immature rice endosperm using [¹⁴C]-labelled substrates revealed the presence of geranyl geranyl diphosphate, the C_{20} general isoprenoid precursor necessary for C_{40} carotenoid biosynthesis. Phytoene synthase, which condenses two molecules of geranyl geranyl diphosphate, is the first of four specific enzymes necessary for β -carotene biosynthesis in plants. Therefore, the Japonica rice model variety Taipei 309 was transformed by microprojectile bombardment with a cDNA coding for phytoene synthase from daffodil (*Narcissus pseudonarcissus*) under the control of either a constitutive or an endosperm-specific promoter. In transgenic rice plants, the daffodil enzyme is active, as measured by the *in vivo* accumulation of phytoene in rice endosperm. Thus, it is demonstrated for the first time that it is in principle possible to engineer a critical step in provitamin A biosynthesis in a non-photosynthetic, carotenoid-lacking plant tissue. These results have important implications for long-term prospects of overcoming worldwide vitamin A deficiency.

Introduction

Carotenoids are a widely distributed class of natural pigments synthesized in all photosynthetic organisms and

in some non-photosynthetic organisms, such as certain bacteria and fungi (for review, see Goodwin, 1980). Carotenoid biosynthesis represents one intracellularly specialized branch of general isoprenoid metabolism in plants. Whereas sesqui- and triterpenoids are produced in the cytoplasm, mono-, di- and tetraterpenoids are synthesized in plastids (Kleinig, 1989). These pigments are well-known as essential components of the photosynthetic apparatus. Carotenoids are also produced in the chromoplasts of flowers and fruits, where they serve as visual attractants of insects and animals to facilitate pollination and seed dispersal. In some cases, such as maize and potato, carotenoids also can be formed in the amyloplasts of plant storage tissues.

One of the most abundant carotenoids, the tetraterpene β -carotene, is synthesized from the general C_{20} isoprenoid precursor geranyl geranyl diphosphate by four specific plant enzymes (see Figure 1) that form the C_{40} backbone, introduce four conjugated double bonds and finally form two β -ionone rings at the ends of the molecule. The genes coding for these β -carotene synthesizing enzymes have been isolated from a variety of bacteria, fungi and plants (for reviews see Armstrong, 1994; Armstrong and Hearst, 1996; Bartley and Scolnik, 1995).

In mammals, β -carotene and some structurally related compounds have provitamin A character. Dietary β -carotene is converted into vitamin A, also known as retinol, by oxidative cleavage of the central double bond followed by a reduction of the terminal aldehyde. Vitamin A plays an essential role in the normal development of humans (Bendich, 1993, 1994; West Jr *et al.*, 1989) and in other mammals. Recent investigations have furthermore shown that vitamin A quenches free radicals and prevents cellular oxidative damage, as well as supporting the human immune system (Bendich, 1989, 1993; Ross, 1992).

Insufficient dietary (pro)vitamin A leads to severe clinical symptoms. In Southeast Asia alone, it is estimated that 5 million children develop the eye disease xerophthalmia every year, of which 0.25 million eventually go blind (Sommer, 1988). Furthermore, although vitamin A deficiency is not a proximal determinant of death, it is correlated with an increased susceptibility to potentially fatal afflictions including diarrhoea, respiratory diseases and childhood diseases, such as measles (Grant, 1991). According to statistics compiled by UNICEF, improved vitamin A nutrition could be expected to prevent approxi-

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This paper is dedicated to Prof. R. Hütter on the occasion of his retirement.

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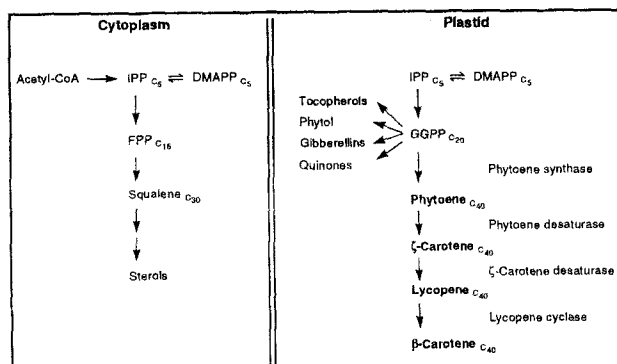


Figure 1. General scheme of isoprenoid and carotenoid biosynthesis in different cellular compartments of plants. IPP: isopentenyl diphosphate, DMAPP: γ,γ -dimethyl-allyl diphosphate, FPP: farnesyl diphosphate, GGPP: geranyl geranyl diphosphate.

mately 1–2 million deaths annually among children aged 1–4 years, and an additional 0.25–0.5 million deaths during later childhood (Humphrey *et al.*, 1992).

For these reasons, it would be desirable to meet the daily requirements for vitamin A by raising the carotenoid levels within staple foods. In Southeast Asia, where rice represents up to 80% of the daily calorie intake (IRRI, 1993), this goal can only be achieved by means of genetic engineering. This approach would have the major advantage that excess dietary β -carotene, in contrast to vitamin A supplements, has no known harmful properties. Rice in its milled form, as it is usually consumed, is characterized by the complete absence of vitamin A and its carotenoid precursors. The milled rice kernel consists exclusively of the endosperm. The carotenoid-containing embryo and the aleurone layer are removed during milling of the rice grain.

As a first step towards the production of β -carotene in rice endosperm, the biochemical blockage in carotenoid biosynthesis has been studied. Consequently, transformation experiments with a recombinant daffodil (*Narcissus pseudonarcissus*) phytoene synthase cDNA have been initiated. Constitutive or endosperm-specific expression of phytoene synthase in rice endosperm leads to the accumulation of large amounts of phytoene, the first C_{40} carotenoid and a key precursor of β -carotene.

Results

Biochemical analysis of immature rice endosperm

Prior to developing a strategy for rice transformation experiments, it was crucial to obtain information on the status of isoprenoid biosynthesis in rice endosperm. *In vitro* incubation of immature endosperm in the presence of radiolabelled isoprenoid precursors yielded information on the biochemical competence of this tissue for prenyl lipid biosynthesis. Incubation experiments with $[1-^{14}C]IPP$ showed that no detectable carotenoids were produced.

However, the formation of geranyl geranyl diphosphate (GGPP), analysed as its corresponding alcohol, geranyl geraniol, was observed (see below). GGPP represents the substrate for the first carotenoid-specific biosynthetic reaction (see Figure 1). Furthermore, the formation of farnesol and squalene, both derived from the cytoplasmic C_{15} and squalene, both derived from the cytoplasmic C_{15} branch of the isoprenoid biosynthesis, was observed. Incubation with more immediate precursors of β -carotene such as $[^{14}C]GGPP$ and $[^{14}C]$ phytoene gave no indication for the presence of later carotenoid-synthesizing enzyme activities (data not shown). Based on these results, transformation experiments using a cDNA coding for the first carotenoid-specific enzyme, phytoene synthase, were performed.

Production of transgenic rice plants

The only plastids present in rice endosperm are amyloplasts. It had been shown earlier that various plastid types, including amyloplasts, are able to import and process nuclear-encoded plastidic proteins (de Boer *et al.*, 1988; Boyle *et al.*, 1986; Strzalka *et al.*, 1987). We therefore decided to use a full-length cDNA representing the nuclear-encoded daffodil *psy* gene for expression in transformed rice. The corresponding daffodil phytoene synthase is normally localized in chromoplasts, a plastid type that develops from chloroplasts in flowers (Schledz *et al.*, 1996). The organization of the plasmids pCPsyH and pGt1PsyH used for stable transformation of rice is shown in Figure 2(a). The expression of the phytoene synthase in construct pCPsyH is regulated by the cauliflower mosaic virus (CaMV) 35S promoter fused to an S1-translational enhancer sequence (Dowson Day *et al.*, 1993; Fütterer *et al.*, 1990) and by a nos 3' polyadenylation sequence. In the construct pGt1PsyH expression of the phytoene synthase is regulated by the endosperm-specific rice glutelin Gt1 promoter (Kim *et al.*, 1993; Okita *et al.*, 1989; Zheng *et al.*, 1993) and by a nos 3' polyadenylation sequence.

For stable transformation of rice, both phytoene synthase cDNA constructs were linked to a CaMV 35S promoter/*aphIV* gene expression cassette to allow hygromycin selection of transformed tissue. DNA was delivered to the scutella of 590 precultured embryos of rice variety Taipei (TP) 309 by microprojectile bombardment using a particle inflow gun constructed according to Finer *et al.* (1992). From these experiments, 87 transgenic plant lines were regenerated, of which 47 (54%) were fertile.

Analysis of transgenic R_0 plants

Among the fertile R_0 plants obtained from transformation experiments with either pCPsyH or pGt1PsyH, 27 contained an intact *psy* transgene when total DNA was digested with the appropriate restriction enzymes and analysed by

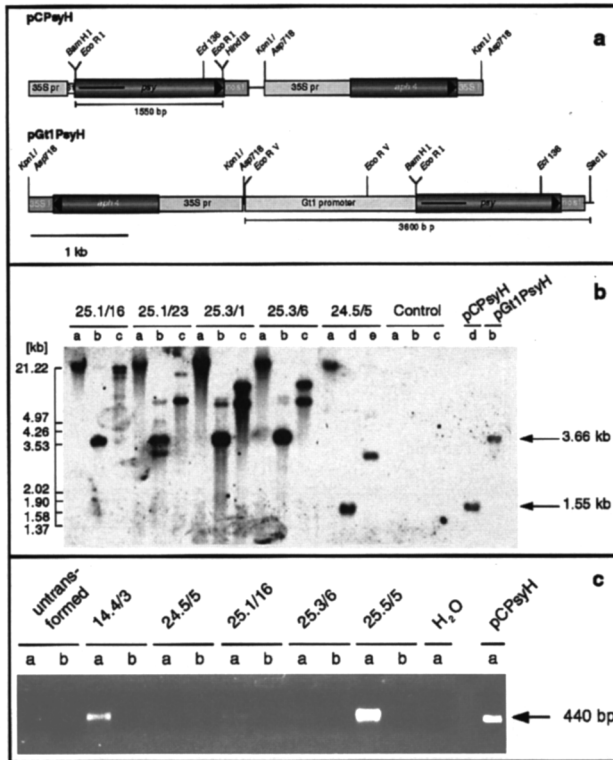


Figure 2. Plasmid constructs and analysis of *psy* gene organization and expression in transgenic plants.

(a) Schematic drawing of the phytoene synthase (*psy*) cDNA driven by the CaMV 35S promoter and the rice glutelin Gt1 promoter in constructs pCPsyH and pGt1PsyH, respectively. The sizes of restriction fragments containing the cDNA are indicated. The fragments used as probes for hybridization are underlined.

(b) Southern blot analysis of selected transformed TP 309 lines. Lines 25.1/16, 25.1/23, 25.3/1 and 25.3/6 were transformed with the plasmid pGt1PsyH, whereas line 24.5/5 was transformed with plasmid pCPsyH (a: undigested DNA, b: Asp718/SacI, c: Asp718, d: EcoRI, e: EcoRI).

(c) RT-PCR of selected TP 309 lines to examine *psy* expression in the endosperm. mRNA from all transgenic lines yielded a primer-dependent fragment (a: with primers; b: without primers). Lines 14.4/3 and 24.5/5 were transformed with plasmid pCPsyH, whereas lines 25.1/16, 25.3/5 and 25.5/5 were transformed with plasmid pGt1PsyH.

Southern blotting (see Figure 2b, lanes b and d). Of these 27, 11 plants were derived from transformation experiments with plasmid pCPsyH and 16 from experiments with plasmid pGt1PsyH. To demonstrate the independence of transformation events, an enzyme that recognised only one restriction site within the transgene was used to produce hybridizing fragments of random length according to the integration site into the nuclear genome (see Figure 2c, lanes c and e). The appearance of additional hybridizing fragments indicated the presence of further rearranged copies of the transgene in the genomes of all plants transformed with plasmid pGt1PsyH.

To determine qualitatively whether or not the integrated and intact *psy* transgenes were transcribed, RT-PCR was used. Template RNA from all of the transgenic plants examined allowed the synthesis of *psy* PCR products of

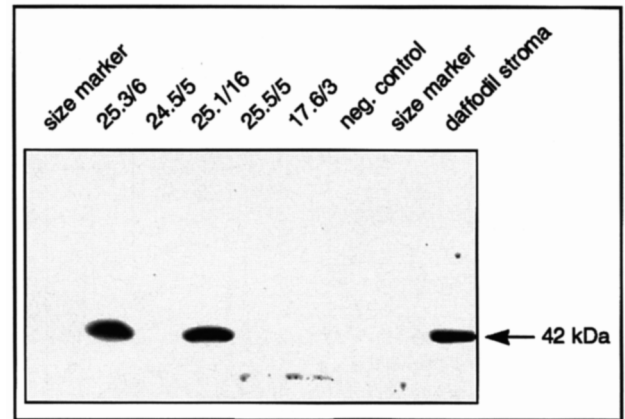


Figure 3. Western blot analysis of phytoene synthase levels in transformed rice plants. TP 309 lines 25.1/16, 25.3/6 and 25.5/5 were transformed with pGt1PsyH, whereas 17.6/3 and 24.5/5 were transformed with pCPsyH. The negative control was an untransformed rice plant, and the positive control was a daffodil stroma extract. The immunoreactive signal corresponding to a molecular weight of 42 kDa represents the mature phytoene synthase.

the expected size, whereas RT-PCR of the untransformed control did not (see Figure 2c), suggesting that the endogenous rice *psy* gene may not be expressed in endosperm of the developmental stage examined.

In order to assess phytoene synthase protein levels in transgenic TP 309 rice lines, immunoblots were performed (see Figure 3), using polyclonal antisera raised against the daffodil phytoene synthase (Schledz *et al.*, 1996). Only some of those rice lines that had been transformed with plasmid pGt1PsyH showed an immunoreactive signal with the expected molecular weight of 42 kDa, corresponding to a mature, plastid-localized phytoene synthase lacking the transit peptide. Carotenoid formation in transgenic rice seeds was studied by pigment extraction, followed by HPLC analysis. Compounds separated were analysed on-line with a photodiode array detector and were identified by comparison with authentic carotene standards. Phytoene was unequivocally identified in several transgenic rice endosperms, particularly those transformed with the Gt1 promoter construct pGt1PsyH. The HPLC chromatogram of pigments extracted from one such line, 25.1/16 (Figure 4b), revealed the highest seed phytoene content (0.74 μ g phytoene per gram dry seed weight) of all transgenic plants tested (Figure 4c). In the control extracts, phytoene was not detectable or was present in trace amounts probably derived from the seed coat (Figure 4a and c). Only two of the lines transformed with construct pCPsyH showed phytoene contents significantly above those of the controls. No carotenoids derived from phytoene could be detected in either the controls or samples obtained from transgenic plants.

Analysis of transgenic *R*₁ and *R*₂ plants

The pGt1PsyH-transformed line with the highest phytoene content, 25.1/16, was chosen for further analysis in the *R*₁

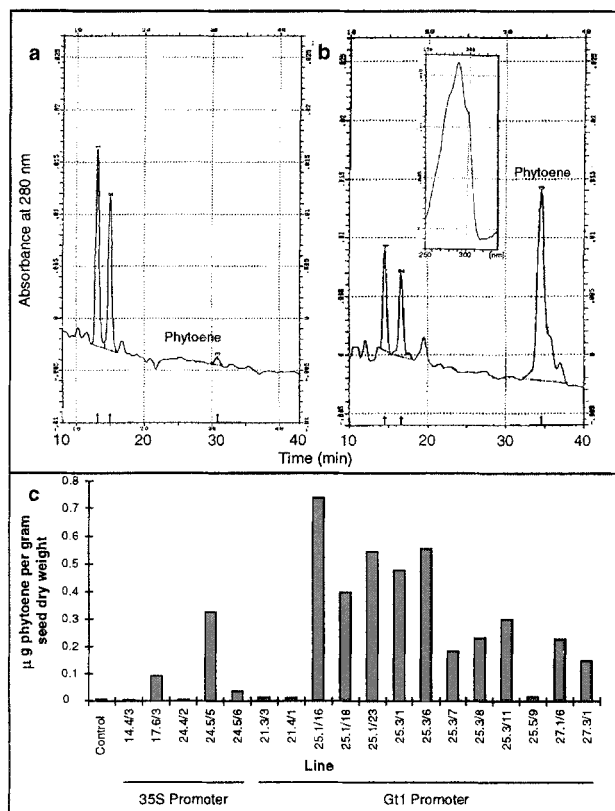


Figure 4. Pigment analysis of transformed rice plants.

(a) HPLC detection at 280 nm of prenyl lipids from seeds of untransformed rice plants, as a negative control. The two peaks at 14.6 and 16.7 min in Fig. 4(a), which were present in all seed extracts, could not be conclusively identified but may represent quinones.

(b) HPLC analysis of pigments extracted from seeds of the TP 309 line 25.1/16 transformed with the plasmid pGt1PsyH. Detection of separated prenyl lipids was performed at 280 nm. (Insert) On-line analysis of the compound eluting at 34.6 min in the HPLC profile shows the characteristic absorption spectrum of phytoene.

(c) Phytoene contents of seeds from transgenic rice plants expressing daffodil phytoene synthase under the control of the CaMV 35S or rice Gt1 promoter, as determined by integration of HPLC peaks and comparison to different concentrations of a phytoene standard. The untransformed control represents the average of three independent measurements.

and R_2 generations. Selective germination assays were conducted to identify those plants that had retained the *aphIV* transgene during Mendelian segregation of the hygromycin resistance trait. R_2 progeny of two R_1 siblings of this line did not segregate for hygromycin resistance and therefore were considered to be homozygous. Southern blot analysis confirmed the presence of the *psy* transgene in these plants (data not shown). In addition, these experiments showed that the *aphIV* transgene was inherited in a Mendelian manner, revealing an approximate 3:1 segregation ratio. From a total number of 22 R_1 seedlings, six had lost the transgene as a result of Mendelian segregation, whereas 16 were still transgenic. Furthermore, incubation experiments with $[1-^{14}C]$ IIPP and immature

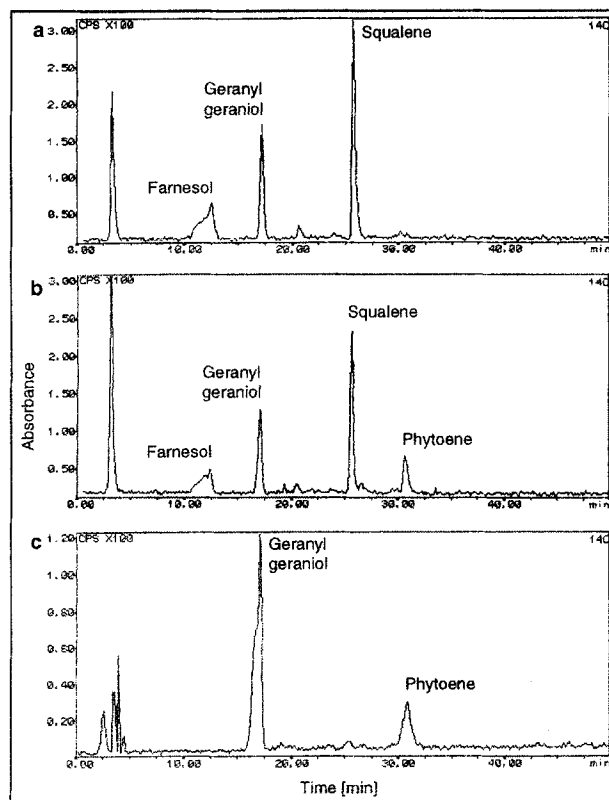


Figure 5. HPLC analysis of prenyl lipids produced during an incubation of immature rice endosperm (12 DAP) in the presence of radioactive isoprenoid precursors.

(a) Negative control, $[1-^{14}C]$ IIPP incubation with untransformed endosperm. In addition to farnesol and squalene, geranyl geraniol, the corresponding alcohol of GGPP, was identified.

(b) $[1-^{14}C]$ IIPP incubation with immature endosperm of a homozygous R_1 plant of line 25.1/16 reveals phytoene formation.

(c) $[^{14}C]$ GGPP incubation with immature endosperm of a homozygous R_2 plant from line 25.1/16. GGPP was not incorporated into farnesol and squalene, as expected, whereas the geranyl geraniol and phytoene peaks were readily detectable.

endosperms of the R_1 plants clearly demonstrated the formation of phytoene (see Figure 5b). Incubation experiments with $[^{14}C]$ GGPP and the immature endosperms of the homozygous R_2 plants also demonstrated the formation of phytoene (see Figure 5c). In neither case, however, were carotenoids derived from phytoene detected.

Discussion

The overexpression of the daffodil phytoene synthase cDNA in transgenic rice endosperm has demonstrated for the first time in plants that it is, in principle, possible to introduce carotenoid production into a normally noncarotenogenic tissue if the necessary biochemical precursors are available. Biochemical analysis of untransformed rice endosperm revealed the presence of GGPP. This isoprenoid C_{20} intermediate is generally accepted to be localized in

plastids (Kleinig, 1989). No carotenoid precursors of β -carotene could be found, however. Therefore, transformation of rice with a phytoene synthase cDNA under the control of an endosperm-specific promoter was expected to lead to the formation of phytoene, the first C₄₀ carotenoid, in this tissue.

Microprojectile bombardment of the scutella of 590 pre-cultured immature zygotic TP 309 embryos gave rise to 87 transgenic plants, yielding a transformation efficiency of 11.2%. Only 54% of these plants, however, turned out to be fertile, despite the short tissue culture period of the method used. The constitutive CaMV 35S promoter in combination with the phytoene synthase cDNA may, however, have led to pleiotropic effects, resulting in reduced fertility of the pCPsyH-transformed lines. Metabolic disturbances, such as an alteration in gibberellin levels (see Figure 1), have previously been seen in transgenic tomato plants that constitutively overexpress phytoene synthase (Fray *et al.*, 1995).

Interestingly, most of the pCPsyH-transformed plants analysed had only one copy of the transgene, whereas all 16 of the pGt1PsyH-transformed plants contained at least one intact copy, as well as additional rearranged copies of the transgene.

Qualitative RT-PCR analysis of RNA isolated from mature transgenic seeds of both pCPsyH and pGt1PsyH transformants demonstrated the presence of *psy* transcripts. In the case of transformants that express phytoene synthase under the control of the CaMV 35S promoter, it cannot be excluded that the RT-PCR signal is derived from expression of the *psy* transgene in the seed coat, a photosynthetically active tissue prior to seed ripening that thereafter still contains traces of carotenoids. Consumed rice, however, is mechanically processed and thereby freed of the seed coat. In our RT-PCR analysis of rice seeds, although the embryo had been removed the seed coat was still present at the time of RNA isolation. The expression of the rice *Gt1* promoter is, however, known to be restricted to the endosperm (Kim *et al.*, 1993; Zheng *et al.*, 1993). Therefore, the *psy* RT-PCR signal found in RNA from pGt1PsyH-transformed plants is clearly assignable to this tissue. The absence of a PCR product with the untransformed controls suggests that the rice *psy* gene may not be expressed in endosperm tissue. We note, however, that no sequence data are available for the rice *psy* gene and that we have used PCR primers derived from the daffodil *psy* sequence. Therefore, it is entirely possible that we would not have detected rice *psy* expression with these primers. Nevertheless, untransformed rice endosperm does not produce phytoene.

Western blot analysis of mature transgenic seeds yielded immunoreactive signals of 42 kDa, corresponding to a mature, plastid-localized phytoene synthase, only in

some pGt1PsyH transformants. In pCPsyH- and other pGt1PsyH-transformed lines, the amounts of the protein present may be too low to produce an immunoreactive signal. In the former case, this would be in accordance with the generally low expression level of the CaMV 35S promoter in rice endosperm tissue (Shimamoto *et al.*, 1989). Seeds of several pGt1PsyH-transformed lines that did not contain immunodetectable phytoene synthase produced considerable amounts of phytoene, as did two of the pCPsyH-transformed lines. Interestingly, there are several examples known in the literature in which expression of carotenoid biosynthesis genes in transformed bacteria alters carotenoid content in the absence of immunologically detectable gene products (Bartley and Scolnik, 1989; Bartley *et al.*, 1990). Carotenoid biosynthesis enzymes in higher plants are, in general, present in extremely low amounts that nevertheless suffice for pigment accumulation (e.g. Al-Babili *et al.*, 1996; Schledz *et al.*, 1996).

No phytoene synthase activity could be detected in immature rice endosperm after *in vitro* incubation with [¹⁴C]GGPP. Furthermore, none of the untransformed control plants tested yielded a phytoene synthase immunoreactive signal nor a *psy* RT-PCR signal. Therefore, it could be that the transcription of the endogenous phytoene synthase gene(s) is blocked in developing rice endosperm tissue. Phytoene desaturase enzyme activity (see Figure 1) also seems to be absent, as *in vitro* incubation experiments with [¹⁴C]phytoene did not result in the production of ζ -carotene. In addition, although phytoene accumulated in the transgenic rice endosperms that overexpressed daffodil phytoene synthase, no later carotenoids could be detected. It is not yet clear whether the other two enzymes necessary to produce β -carotene, ζ -carotene desaturase and lycopene cyclase, are present in rice endosperm.

The highest phytoene content measured was obtained with one of the pGt1PsyH transformants (0.74 μ g phytoene per gram dry seed weight). The final goal of 2 μ g β -carotene per gram dry seed weight, to provide the minimum daily provitamin A requirements for young children (100 μ g retinol equivalents), thus seems to be realistic. The transgenic seeds analysed here were derived from an R₀ plant and therefore represent a heterozygous population due to Mendelian segregation.

Thus, it has been demonstrated that it is possible to initiate β -carotene biosynthesis in rice endosperm. The expression of phytoene synthase leads to the formation of the first carotenoid-specific intermediate, phytoene, in rice seeds. Experiments are currently underway to transform rice plants with cDNAs encoding the remaining enzymes (see Figure 1) needed to produce β -carotene in the rice endosperm.

Experimental procedures

Biochemical analysis of untransformed rice endosperm

The endosperm of 15 immature caryopses (10–15 days after pollination, DAP) was suspended in 500 µl incubation buffer (100 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM DTE) and homogenized by sonification. After the addition of 1 µCi [1-¹⁴C]IPP (Amersham), ATP (2 mM) and MnCl₂ (1 mM), the mixture was incubated at 27°C for 4 h. After alkaline phosphatase treatment (Boehringer), prenyl lipids were extracted with chloroform/methanol (2:1, v/v) and redissolved in chloroform. Radioactive compounds were directly analysed by reverse phase HPLC employing a C₁₈ column (ET 250 Nucleosil 100–5 C₁₈, Machery and Nagel) with a high-pressure liquid chromatograph (Waters) using H₂O/methanol (3:1, v/v) and acetonitrile/tetrahydrofuran (1:1, v/v) in a 60-min gradient as described previously (Beyer and Kleinig, 1992).

Plasmid constructs

All plasmids used were pUC derivatives. Plasmid pCPsyH contains a daffodil phytoene synthase cDNA regulated by a modified CaMV 35S promoter and a nos polyadenylation sequence. The phytoene synthase cDNA was inserted as a *Bam*HI/*Hind*III fragment into this plasmid. For the selection of transgenic rice tissue, the *aph*IV gene from *E. coli*, placed under the control of the CaMV 35S promoter and the CaMV polyadenylation sequence, was inserted as a *Kpn*I fragment from plasmid pSBHI (Wünn *et al.*, 1996). The plasmid pGt1PsyH was constructed by inserting the phytoene synthase cDNA and adjacent nos polyadenylation sequence from pCPsyH as a *Bam*HI/*Asp*718 fragment into plasmid pKS1 (Okita *et al.*, 1989), containing the rice Gt1 promoter cloned into vector pBSK-. For the selection of transgenic rice tissue, the plasmid was modified with the same *aph*IV expression cassette as that used for plasmid pCPsyH.

Rice transformation

Immature embryos of the Japonica variety Taipei 309 were aseptically isolated 10–14 DAP from greenhouse-grown plants (day: 12 h, 28°C, 80% humidity; night: 12 h, 21°C, 60% humidity) and plated scutellum side up on solid MS medium (Murashige and Skoog, 1962) containing 3% sucrose, 2 mg l⁻¹ 2,4-D and 50 mg l⁻¹ cefotaxime (MS1). After 4–6 days (26°C, darkness), embryos were transferred to solid MS medium containing 10% sucrose, 2 mg l⁻¹ 2,4-D and 50 mg l⁻¹ cefotaxime (MS2) and subjected within 1 h to microprojectile bombardment with a particle inflow gun. Plasmids (5 µg) were precipitated on 1–3 µm gold particles (Aldrich) as described elsewhere (Vain *et al.*, 1993). Gold particles (400 µg per bombardment) were accelerated to the target with a particle inflow gun (Finer *et al.*, 1992) at a pressure of 6 bar. Embryos were placed 16 cm below the syringe filter. Twenty-four hours post-bombardment, embryos were transferred to fresh MS1 plates and incubated at 26°C in the dark. After another 6 days, embryos were subjected to selection on MS1 medium containing 30 mg l⁻¹ hygromycin B. The tissue was subcultured in weekly intervals. Resistant calli were transferred to solid R₂ regeneration medium (Ohira *et al.*, 1973) supplemented with 2% sucrose, 3% sorbitol, 30 mg l⁻¹ hygromycin B, 1 mg l⁻¹ zeatin, 0.5 mg l⁻¹ IAA, MS vitamins and 0.65% agarose. The callus tissue was maintained at 28°C with 12 h of light subsequent to air-drying in a laminar flow for 5–10 min in order to enhance shoot formation (Rancé *et al.*, 1994). The calli were subcultured every 3 weeks until shoots

had reached a length of 2–3 cm, at which time they were transferred to half-strength MS rooting medium without hormones, supplemented with 1.5% sucrose and 0.3% gelrite®. After cultivation for another 2–4 weeks, plantlets were transferred directly to the greenhouse and planted in soil.

Analysis of transgenic rice plants

Southern blot analysis. DNA was extracted from hygromycin resistant rice plants using the CTAB (N-lcetyl-N,N-trimethylammonium bromide) method (Murray and Thompson, 1980) and digested with appropriate restriction enzymes. DNA fragments were separated in 0.8% agarose gels, transferred to nylon membranes (Boehringer) and cross-linked by baking for 30 min at 120°C. A PCR-amplified, DIG-labeled (Boehringer) 440-bp fragment of the coding region of the *psy* cDNA was used as a probe. Hybridization, washing and detection were performed as described previously (Wünn *et al.*, 1996).

Qualitative RT-PCR analysis. Total RNA was extracted from dehusked, mature transgenic rice seeds from which the embryo had been removed. Five caryopses were ground to a fine powder under liquid nitrogen. RNA was extracted with an RNeasy® kit (Qiagen) according to the instructions of the manufacturer and subsequently treated with RNase-free DNase (Boehringer). One hundred nanograms of RNA were used as a template for cDNA synthesis with a daffodil *psy* 3' primer 5'CCCAAATGGCTCTCTCCTC3' at 42°C for 30 min. The total volume of 10 µl was used for PCR with the daffodil *psy* 5' primer 5'GAGATCCCTATCCGCCTTGG3' in a final volume of 50 µl. The cDNA was denatured at 93°C for 1 min followed by 28 amplification cycles of primer annealing at 55°C, extension at 72°C and denaturation at 93°C (1 min each). Five microlitres of each reaction were analysed on a 1.4% agarose gel to assay for the presence of the expected PCR fragment of 440 bp.

Western blot analysis. Mature transgenic seeds prepared as for RT-PCR analysis were ground to a fine powder with a micro-dismembrator (B. Braun, Melsungen, Germany). The powder was suspended in H₂O, and ethanol was added to a final concentration of 27%. Starch was pelleted by centrifugation, and the supernatant was lyophilized and redissolved in a 1:1 (v/v) mixture of incubation buffer (see above) and sample buffer (65 mM Tris-HCl, pH 6.8, 5% SDS w/v, 5% β-mercaptoethanol v/v, 20% glycerol v/v, 0.1% glycerol). The samples were briefly heated, analysed by SDS-PAGE on a 10% gel and transferred electrophoretically to a nitrocellulose membrane. The primary antibodies used were raised in rabbits injected with the recombinant daffodil phytoene synthase overexpressed in insect cells with the aid of the baculovirus system (Schledz *et al.*, 1996). The detection of phytoene synthase was performed with an ECL chemiluminescence Western blotting kit (Amersham), according to the instructions of the manufacturer.

Biochemical analysis

Fifty mature transgenic rice seeds, prepared as for RT-PCR analysis, were ground to a fine powder with a micro-dismembrator. Prenyl lipids were extracted with chloroform/methanol (3:1, v/v), treated with ethanolic KOH for saponification at 55°C for 1 h, and thereafter extracted with petroleum ether. The solvent was evaporated, and the residue was redissolved in chloroform. This solution was spotted on a silica gel plate and developed with petroleum ether/diethyl ether/acetone (40:10:10, v/v/v). The solvent

front was extracted with acetone, the solvent evaporated, and the residue redissolved in chloroform prior to analysis with an isocratic HPLC system employing acetonitrile as the solvent at a flow rate of 1 ml min⁻¹ using a C₁₈ column, as described above. Phytoene was identified by its characteristic UV absorption spectrum (Schledz *et al.*, 1996) and by comparison of the retention time to an authentic phytoene standard.

Growth of R₁ and R₂ transgenic plants

In order to obtain for further cultivation those R₁ and R₂ progenies that contained the transgene, seedlings were grown on a selective medium containing 20 mg l⁻¹ hygromycin B. Hygromycin-resistant plants were transferred to soil. The immature endosperms of these plants were biochemically analysed as described above for untransformed tissue, with the exception that 0.3 µCi of [1-¹⁴C]GGPP were also tested as a substrate for phytoene production.

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