INCREASE THE LEVEL OF γ-ORYZANOL, TOCOPHEROLS AND TOCOTRIENOLS IN RICE BY ISOPRENOID-PATHWAY ENGINEERING

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ABSTRACT

Indica variety IR64 and japonica variety Taipei 309 were transformed by genes involving the isoprenoid pathway. The construct combines the bacterial phytoene desaturase gene <u>crtI</u>, *translationally fused with the pea RuBisCo small subunit transit sequence and under the control of the constitutive 35SCaMV promoter with the daffodil* <u>psy</u> *encoding phytoene synthase, driven by endosperm specific Gt1 promoter. Analysis of T_1, T_2 seeds of the transgenic lines showed that, in addition to β-carotene or provitamin A produced in the transgenic seeds, there was a notable increase with most lines as compared to the wild type in a compound group which was identified as γ-oryzanol. The γ-oryzanol is known to be a very healthy antioxidant compound, claimed to be more efficient than vitamin E (tocopherols and tocotrienols) - another class of isoprenoids present in rice grains. Although these compounds were minor relevance with γ-oryzanol, their amounts seemed to increase in the transgenic lines.*

Keywords: *Rice transformation, γ- oryzanol, vitamin E, tocopherol, tocotrienol*

INTRODUCTION

Rice is consumed by nearly half of the world population. It is the major source of energy in the daily diet of millions of people in developing countries. But rice is a poor source of many essential micronutrients and vitamins, many of the rice eaters are undernourished due to their extreme poverty limiting access to additional nutritious food sources like animal meat and fruits. Conventional breeding was inefficient to improve the nutrition values of rice. Since the first success demonstrated by Ye et al., (2000) to produce the rice lines expressing pro-vitamin A in the endosperm by engineering the three genes needed in the carotenoid pathway into rice, a new approach of biosynthetic pathway engineering for developing rice lines possessing special nutrition values has emerged.

In our study, effort has been made to transform into indica varieties with the isoprenoid-pathway controlled by the two genes, *psy* from daffodil encoding phytoene synthase and *crtI* from *Erwinia uredovara*

encoding phytoene desaturase. These genes plus PMI gene from *Escherichia coli* as a selectable gene encoding phosphomannose isomerase were placed in a single contruct for *Agrobacterium*-mediated transformation. We have observed that the resulting transgenic lines not only expressed beta-carotene in the endosperm but also showed an increase in the amount of γ-oryzanol and vitamin E (tocopherol, tocotrienol). Xu et al. (2001) reported the antioxidant activities of vitamin E (α-tocopherol, α- tocotrienol, γ-tocopherol, and γ-tocotrienol) and γ-oryzanol components purified from rice bran were found significantly antioxidant activity in the inhibition of cholesterol oxidation. They also reported that γ-oryzanol was a more important antioxidant of rice bran in the reduction of cholesterol oxidation than vitamin E and the antioxidant function of these compounds against cholesterol oxidation may to contribute the potential hypocholes-terol property of rice bran. Oxidation products of cholesterol are considered to be mutagenic and carcinogenic compounds (Ansari et al. 1982, Woods and O'Brien 1998).

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In view of the special values of γ-oryzanol and vitamin E to human health, the study is revealing a notable increased amount of these compounds in the transgenic lines developed by isoprenoid-pathway engineering, to facilitate further studies.

MATERIALS AND METHODS

Binary vector and plant transformation

In this study, a single binary vector pCaCar (Hoa et al. 2003) was used for all transformation experiments.

Immature embryos and embryogenic calli derived from immature embryos or mature seeds of the cultivars IR64 and Taipei 309 were inoculated with *A. tumefaciens* strain LBA 4404 (Hoekema et al. 1984). The transformation procedures described by Hoa and Bong (2003) were followed.

DNA isolation and Southern blot analysis

Genomic DNA was isolated from frozen rice leaves following the method by McCouch et al. (1988). Ten micrograms of genomic DNAs were digested with *Eco*RI to detect *psy* or *crtI* and with *Kpn*I for copy number analysis. The restricted DNA was fractionated through a 0.8% agarose gel prior to capillary transfer and immobilization on nylon membranes (Hybond-N+, Amersham). PCR-amplified, DIG-labeled (Boehringer, Rotkreuz, Switzerland) fragments of *psy* (primers: 5`-TACGTAGCAGGAACTG-3`; 5`-CAAACAGGCCACCTGCTAGC-3`) and *crtI* (primers: 5`-GAGTGGGGCGT-3`, 5`-TAACTGCCGCAACCTT-3`) were used as probes. Hybridization, washing and detection were performed following the procedures by Wünn et al.(1996).

Segregation analysis of the transgenes

The self-pollinated seeds (T$_1$ generation) of the transformants were grown in MS medium (Murashige and Skoog 1962) supplemented with 3% mannose and 0.5% sucrose. The resistant plants scored at two weeks after culture were transferred and grown in the greenhouse for further Southern blot analysis.

Carotenoid extraction and analysis

Dehusked seeds were polished overnight on a shaker at 200-220 rpm in petri dishes equipped with emery paper. Alternatively, we used a commercial grain polisher (Kett, Japan) at a polishing time of 60 s. In contrast to the first method this procedure removes the embryo. Polished seeds (0.5 g) were ground for 60 s to a fine powder using a Micro-Dismembrator (Braun, Germany). The powder in the Teflon capsules was re-suspended in 2ml acetone, and transferred to a glass test tube and 200μg of α-tocopherol acetate were added as an internal standard from an appropriate acetone stock solution. This suspension was sonicated for 10 s, centrifuged and the supernatant collected; the extraction procedure was repeated with another two ml to ensure complete carotenoid extraction. The combined extracts were dried in a Vortex-Evaporator (Haake-Buchler, USA) and resuspended in 500μl acetone. A UV/VIS spectrum was read (700-350nm) and the absorption was determined at 475 nm corresponding to the side-maximum of the carotenoid spectrum obtained. This was done to correct for a certain baseline-drift occurring at shorter wavelengths. Given the relative uniformity of the carotenoid chromophore in transgenic rice (α- or β-carotene derivatives), the absorption at 450 nm could be calculated using a factor of 1.125. Total carotenoid amount was calculated using a molar extinction coefficient of $\varepsilon = 134\ 500\ l^{-1}mol^{-1}cm^{-1}$ averaging for both chromophores. The distribution of carotenoids was then determined by HPLC (see below). Alternatively, omitting photometry, the amount was determined by HPLC using the internal plus an external standard (echinenone, kindly provided by Hoffmann-La Roche, Switzerland). Samples were taken to dryness and dissolved in 70 μl chloroform out of which 50μl were injected into the HPLC system.

For HPLC a Waters (Eschborn, Germany) system was used equipped with a photodiode array detector (model 996) and a fluorescence detector (model 474; excitation 293 nm, emission 324 nm for tocopherol detection). The separation was performed with a C$_{30}$-RP column (YMC Europe) using the solvent system B: MeOH / *tert*-butyl-methyl ether/water (60:12:12, v/v/v) and A: MeOH/*tert*-butyl-methyl ether (1:1 v/v). The

column was developed with a linear gradient from 100% B to 43% B within 45 min then to 0% B within 35 min maintaining the final conditions for another 30 min. Using a maxplot (440-470 nm) carotenoid peaks were integrated at their individual λ_{max} while γ-oryzanol peaks were integrated at 323 nm. Tocopherols were integrated using the fluorescence detector signals. Normalization and quantification: all peaks were normalized relative to the internal α-tocopherol acetate standard to correct for extraction and injection errors. An external standard, echinenone (582ng), run separately, was then used to calculate carotenoid amounts. All carotenoid peaks underwent a second normalization to correct for their individual molar extinction coefficients relative to echinenone (= 1) using: lutein (0.969), β-carotene (0.884), α-carotene (0.791), zeaxanthin (0.891) and β-cryptoxanthin (0.872).

Integrated tocopherol/tocotrienol fluorescence area units, with the aid of calibrated standard solutions (Sigma, Germany), were treated with the following correction factors: α-tocopherol (474 ± 20), β-tocopherol (953 ± 13), γ-tocopherol (966 ± 5), δ-tocopherol (984 ± 12), α-tocotrienol (539 ± 2), β-tocotrienol (910 ± 4), γ-tocotrienol (1101 ± 11), δ-tocotrienol (843 ± 3) ng^{-1}, each. The γ-oryzanol was quantified using a standard solution extracted from a product available in US vitamin shops (γ-oryzanol 200, GNC, USA) applying a conversion factor of 1831 area units / ng.

RESULTS AND DISCUSSION

Two cultivars, namely IR64 (indica) and Taipei 309 (japonica) were transformed. The vector pCaCar (Fig. 1) was used throughout in the present study in the *Agrobacterium*-mediated transformation. Phosphomannose isomerase was used as the selectable marker gene. The construct combines the bacterial phytoene desaturase gene *crtI*, translationally fused with the pea RuBisCo small subunit transit sequence (Misawa et al. 1993) and under the control of the constitutive 35SCaMV promoter with the daffodil *psy*, driven by endosperm specific Gt1-promoter.

To use a combination of only these two structural genes coding for carotenoid biosynthetic enzymes, omitting lcy, is the outcome of the previous findings showing that these were able to reconstitute the entire pathway including the formation of α and β-carotene as well as of derived xanthophylls (Ye et al. 2000).

The T_1 seeds were first phenotypically selected by visible color after polishing. Segregating seed populations with notably yellow grains (about 20% of the transformants) were subjected to carotenoid analysis by HPLC and photometry (see below). The quality of integration and the integration number were judged by Southern hybridization with selected T_0-transformants (Fig. 2). Based on the sum of these data and the carotenoid analysis done with the T_1 seeds, lines were selected to produce self-pollinated progeny (T_2 seeds) for analysis in greater detail.

In the T_2 seeds, in addition of carotenoid content (Fig. 3), a long standing observation is a notable increase with most lines in a compound group which we identified as γ-oryzanol (Fig. 3 and 4). This valuable triterpenoid-ferulate antioxidant occurs in a very steep concentration gradient with high concentrations in the outer layers to much lower concentrations in the endosperm, as seen in a time-course of polishing (data not shown). Vitamin E-compounds (tocopherols and tocotrienols) is another class of isoprenoids present in rice grains. While being of minor relevance with γ-oryzanol, the presence of the embryo needs to be considered here since it contains large amounts of α-tocopherol which is absent from the endosperm where only tocotrienols are found. Their analysis of embryo-deprived grains given in Fig.4 shows that this class of isoprenoid compounds, in contrast to γ-oryzanol, did not change significantly relative to the wild type.

The isoprenoid pathway (triterpenoid), although cytoplasmically localized, is involved together with the phenylpropanoid pathway in delivering ferulate moiety. The γ-oryzanol is a very healthy antioxidant compound, claimed to be more efficient than

vitamin E (Xu et al. 2001, Huang et al. 2002). It remains to be investigated whether all this upregulation takes place in the endosperm alone or in the outer layers of the fruit wall as well, where γ-oryzanol occurs predominantly. In contrast, vitamin E compounds, which are plastid isoprenoids just like carotenoids, remained largely unaffected. Taken together, these are unexpected but beneficial regulative side effects that are all very welcome for the moment. But to get a broader overview of the events occurring, expression profiling is required and has been initiated.
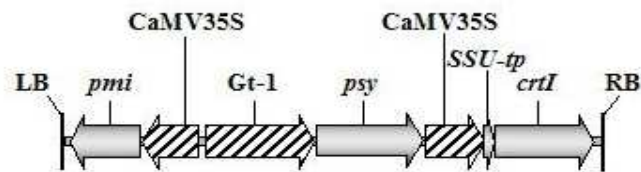
Fig. 1. Scheme of the vector pCaCar. LB, RB: left and right T-DNA borders; GT1 glutelin promoter; *pmi*: phosphomannose isomerase; *psy*, phytoene synthase; *crtI*: bacterial phytoene desaturase, *SSU-tp*: transite peptide of the ribulose-bis-phosphate carboxylase small subunit.
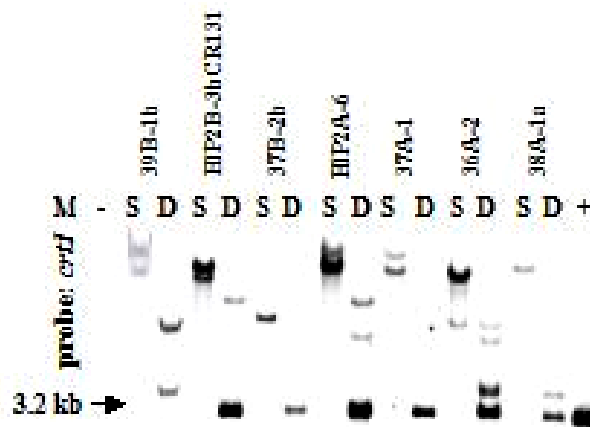

Fig. 2. Southern blot analysis. Genomic DNA digested with *Kpn*I as a single cutter (S) and with *Eco*RI as double cutters (D) was analyzed as given in Materials and Methods. The expected transgene band size is 3.2 kb arrow.
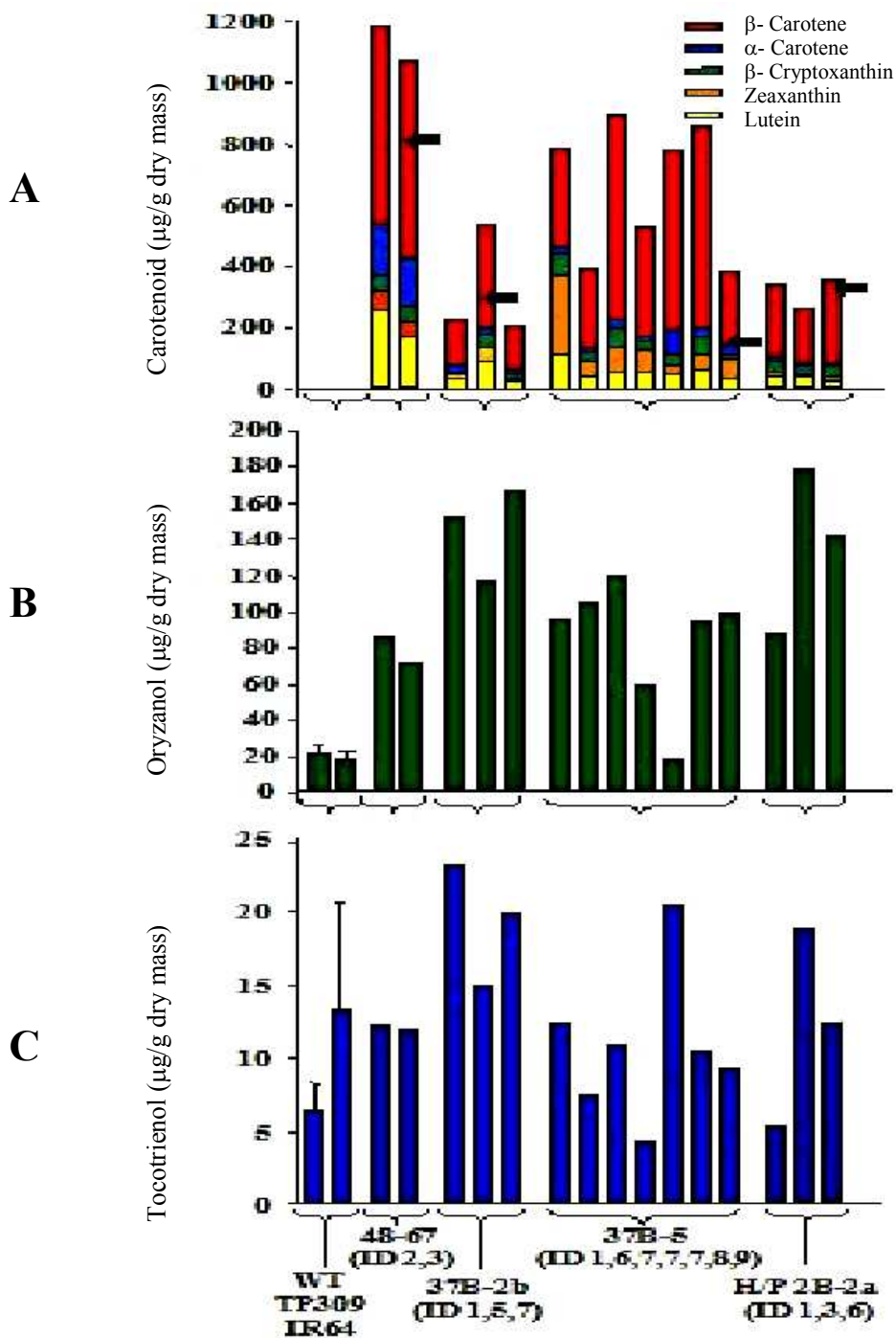
Fig. 3. Analysis of T_2-seeds. A, Carotenoid analysis, the color code for individual carotenoids is given. Arrows indicate the carotenoid amount in the parent T_1-seeds; B, γ-oryzanol determination; C, total tocotrienols.
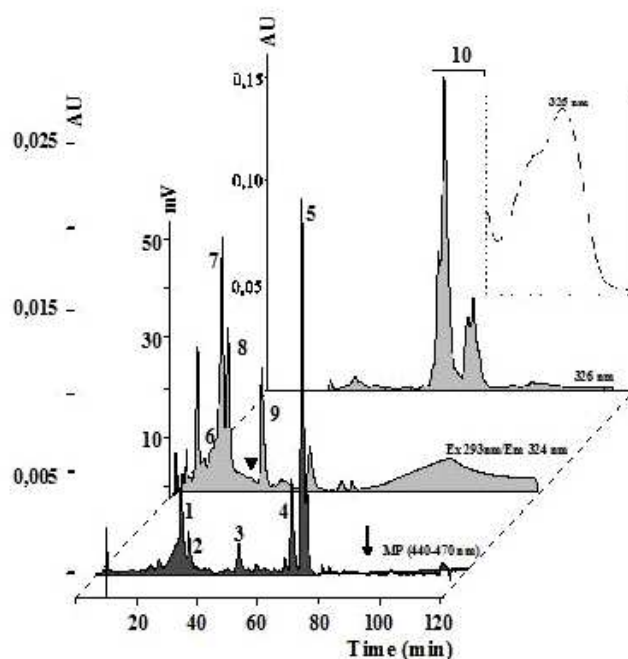
Fig. 4. HPLC analysis of embryo-free T_2 rice seeds (Taipei 48-67-3). Lower trace, carotenoids max-plot (MP; each compound displayed in its indivudual λ_{max}). Arrow indicates the position of lycopene. Middle trace, fluorescence detector response showing Vit E compounds. Arrowhead indicates the position of α-tocopherol appearing in the presence of embryos. Upper trace, detection of γ-oryzanol at 326 nm. Inset displays the γ-oryzanol spectrum. Compounds detected: 1, lutein; 2, zeaxanthin; 3, β-cryptoxanthin, 4, α-carotene, 5, β-carotene; 6, γ-tocotrienol; 7β/δ-tocotrienol; 8, α-tocotrienol, 9 α-tocopherol-acetate as internal standard; 10, γ-oryzanol

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SUMMARY IN VIETNAMESE

Gia tăng hàm lượng γ-oryzanol, tocopherols và tocotrienols ở lúa bằng chuyển nạp gen điều khiển sinh tổng hợp isoprenoid

Giống lúa indica IR64 và giống lúa japonica Taipei 309 được chuyển nạp các gen điều khiển sinh tổng hợp isoprenoid. Vector mang gen *crtI* từ vi khuẩn *Erwinia uredovora* điều khiển tổng hợp phytoene desaturase được kiểm soát bởi đoạn khởi động (promoter) 35SCaMV và gene *psy* từ hoa của cây thủy tiên (daffodil) điều khiển tổng hợp phytoene synthase được kiểm soát bởi đoạn khởi động chuyên biệt cho phôi nhũ Gt1. Phân tích hạt T_1, T_2 của cây lúa biến đổi gen cho thấy, ngoài chất β-carotene hay pro-vitamin A được tổng hợp ở hạt, còn có một sự gia tăng đáng kể các hợp chất γ-oryzanol và vitamin E (tocopherols và tocotrienols) ở các hạt của dòng lúa biến đổi gen so với đối chứng. γ-oryzanol được biết là hợp chất rất hữu ích đối với sức khoẻ con người, tác dụng y học của γ-oryzanol còn hữu hiệu hơn vitamin A. Kết quả nghiên cứu này mở ra hướng chuyển nạp gen trong tạo/tăng các hợp chất có giá trị dinh dưỡng đặc biệt ở lúa rất đáng được quan tâm.