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Rice caryopsis structure in relation to distribution of micronutrients (iron, zinc, β-carotene) of rice cultivars including transgenic indica rice

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ABSTRACT

The transgenic indica rice lines of IR68144 and BR29, developed using endosperm-specific promoters were analyzed for their iron, zinc and β-carotene content in the endosperm. Biochemical analysis clearly revealed the presence of higher accumulation of iron, zinc and β-carotene in transgenic rice grains in comparison with control. Prussian blue staining reaction evidenced the presence of iron in the endosperm cells of transgenic rice grains in comparison with control where iron is restricted only to aleurone and embryo. The rice grain structure of IR64, IR72, IR68144, Swarna, BRRI Dhan 29 (BR29), BR28, Taipai 309 (T309) and New Plant Type-3 (NPT3) indicated that the number of aleurone layers, size of the embryo and size of the caryopsis determines the quantity of important micronutrients (iron, zinc) in the grains. Biochemical analysis revealed that iron and zinc content drastically varies in polished and unpolished rice and among the varieties examined. During the polishing process almost entire aleurone and most part of the embryo is removed which are the main storehouse for major micronutrients. It is estimated that more than 70% of micronutrients are lost during polishing process.

1. Introduction

The structure, chemistry and function of the rice grain and its fraction has been reviewed [1]. The development, structural changes, grain filling, localization of major storage compounds and 2-acetyl-1-pyrroline (2-AP) has been studied [2–4]. Ultra structure of developing embryo and mature rice grain [5–8] and its structure in relation to yield increase have been investigated [9–10]. Tissue specific localization of iron and β-carotene in transgenic rice grain has also been carried out [11–12].

Iron deficiency anemia (IDA) and vitamin A deficiency (VAD) are the major human nutritional disorders especially in Asian countries where rice is the staple food [13–14]. VAD causes symptoms of night blindness, xerophthalmia and keratomalacia which may lead to total blindness. VAD also causes diarrhea, respiratory and childhood diseases. It is estimated that about 124 million children worldwide are deficient of vitamin A, one to two million of which die every year. About a quarter of a million people are reported to go blind every year as a consequence of VAD. To counteract these problems, efforts were made to develop rice with β-carotene and iron [15–18].

Rice is the staple food for more than half of the world population, mainly in developing countries. More than 90% of rice is produced and consumed in South and Southeast Asia and it is an important food crop for Africa, Latin America and Middle East [19]. Rice consuming population is increasing at faster rate and it is estimated that the number of rice consumers may increase considerably by the year 2020 [20]. Rice is one of the cheapest sources of food energy and protein, however it is deficient in many essential micronutrients such as iron and zinc [21]. From various analysis, no rice cultivars have been found producing β-carotene in the endosperm [22]. To overcome micronutrients deficiency, three genes involved in the biosynthesis of β-carotene, namely phytoene synthase (*psy*), phytoene desaturase (*crt*I) and lycopene cyclase (*lcy*) and, for iron enhancement one gene coding for ferritin, an iron storage protein, were introduced in the rice varieties IR68144 and BR29 [16,18,26]. In the present study, we have analyzed the rice grain structure in relation to micronutrients distribution and performed histochemical and biochemical analysis of iron, zinc and β-carotene in transgenic seeds in comparison with the corresponding non-transgenic control.

2. Materials and methods

2.1. Rice seeds

Mature control (non-transgenic) seeds from selected high yielding varieties of IR64, IR72, IR68144, Swarna, BRRI Dhan 29 (BR29), BR28, Taipai 309 (T309) and New Plant Type-3 (NPT3) were

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obtained from the Genetic Resource Centre (GRC) at the International Rice Research Institute (IRRI), Philippines. Seeds from various transgenic homozygous rice lines (with single insert) belong to many different plants of varieties IR68144 and BR29 with enhanced iron, zinc and β-carotene, developed in Genetic Engineering Laboratory at IRRI, Philippines, were used in this study.

2.2. Structural and histochemical studies

Thin (15–20 μm) transverse sections of both polished and unpolished rice caryopsis of above varieties were obtained using vibratome (Vibratome Series 3000 sectioning system, Technical Product International, USA). The sections were stained with 0.1% acridine orange and 1% safranin using standard histochemical procedure [2,3,23]. For localization of iron, Perl's Prussian blue technique was employed. Thin transverse sections of both transgenic and non-transgenic rice grains were stained with 2% potassium ferro cyanide in acidic conditions (usually HCl), which reacts with ferric ions in the tissue to form an insoluble blue color [11,24]. Observations and photography were made by using Carl Zeiss Axioplan-2 microscope under bright-field for iron and fluorescence mode for rice grain structure.

2.3. Iron and zinc quantification

For quantitative estimation of iron and zinc, both transgenic and control rice grains were dried and de-hulled manually. These were divided into two sets, one kept unpolished while the other was polished for standard milling time using bench-top miller (Kett Electrical Laboratory, Tokyo, Japan). Both polished and unpolished seeds of transgenic and non-transformed control were ground to a fine powder with oscillating mill (VIBRATING SAMPLE MILL (CMT) TI-00, C.M.T. Company LTD, Tokyo, Japan). For each treatment 0.6 g of powder was taken in high-pressure digestion vessel to which 10 ml of $HClO_4$:HNO_3 (1:10) was added and kept overnight for pre-digestion. Pre-digested samples were mixed well by swirling and placed into hot plate. Temperature was gradually increased to 225 °C for digestion. The digested samples were used for the quantification of Fe and Zn by the Inductively Coupled Argon Plasma Optical Emission Spectrometer (ICP-OES) [25].

2.4. Carotenoid extraction and estimation

Mature transgenic and control rice seeds were de-hulled and polished on sand paper with continuous shaking (250 rpm) for 8 h. Polished rice seeds were ground into fine powder with mortar and pestle. Five hundred milligram of powder was soaked in 700 μl of water and incubated at 50 °C for 20 min. Two-millilitre acetone was added for extraction and the mixture was occasionally vortexed and sonicated. The samples were centrifuged for 5 min at 1000 rpm and the supernatant were transferred to clean tubes. Acetone extraction was repeated for two more times or until the supernatant was colorless. The supernatants were combined and half the volume of petroleum ether was added. The mixture was mixed thoroughly and water was added for phase separation. The colored upper layer was taken and dried down in speed vacuum (Maxi Dryer Plus, Heto, Allerod, Denmark). The dried sample was dissolved in 1 ml of petroleum ether and the absorbance was measured using spectrophotometer at 453 nm. Total carotenoid content (μg/g rice) was then calculated.

2.5. HPLC analysis

After the measurement of absorbance, the solution was evaporated to dryness and the dried extract was dissolved in 100 μl of acetone and 40 μl of sample was loaded for HPLC analysis. HPLC analysis was done with Waters Alliance 2690 Separation Module (Waters Corporation, Milford, MA, USA) that was equipped with a Waters 996 photodiode array detector and Waters Millennium^{32} Chromatography Manager. Samples were separated on a Waters YMC Carotenoid™ column (4.6 mm × 250 mm, 5 μm) after passing them through a guard column containing the same material (4.0 mm × 10 mm, 5 μm) and eluted with the following solvent system: solvents A (acetonitrile:tetrahydrofuran:water 10:4:6) and B (acetonitrile:tetrahydrofuran:water 10:8.8:1.2). The column was developed with 100% A for the first 3 min, then a linear gradient to 100% solution B was applied over a period of 7 min and remained at B for 20 min. Peak identification was based on the retention time. Peaks were integrated and the amount of β-carotene was quantified using standard β-carotene calibration curve constructed under the same HPLC conditions. Lutein and β-carotene standards were obtained from Sigma Chemical Company, St. Louis, USA.

3. Results

3.1. Structure of rice caryopsis in relation to distribution of micronutrients

Structure of mature rice caryopsis in relation to micronutrients distribution (especially Fe and Zn) were compared between the varieties of IR68144, IR64, IR72, BR28, BR29, Swarna, Taipei309 (T309) and New Plant Type-3 (NPT3). The comparisons of micronutrients distribution were also made between the transgenic indica rice IR68144 and BR29. The results for structure of rice caryopsis of different varieties are summarized (Fig. 1 1.1–1.12, 1.14–1.17, 1.19, 1.20).

In unpolished rice caryopsis both pericarp and aleurone were intact in all the rice varieties. Among the varieties studied, NPT3 and T309 showed maximum number of aleurone layers (3–4 layers) below the ovular vascular bundle region (Fig. 1 1.3,1.5,1.12), minimum number of aleurone layers was observed in BR28, IR72 and BR29 (Fig. 1 1.8,1.11,1.19) and intermediate numbers were observed in IR68144 and Swarna (Fig. 1 1.7,1.17). It was observed that opposite the ovular vascular bundle region single layer of aleurone was present in all the varieties studied (Fig. 1 1.4). In polished rice caryopsis, aleurone layers were completely removed in all the varieties except in T309 and NPT3 where the remnants of aleurone was present just below the ovular vascular bundle region and in the curved regions of caryopsis (Fig. 1 1.2,1.6,1.15).

Transgenic rice with increased iron content was developed for varieties IR68144 and BR29. Transverse section of transgenic rice seeds clearly revealed the enhanced iron in comparison with control as shown in blue color using Prussian blue staining reaction (Fig. 1 1.21–1.24).

3.2. Effect of milling on major micronutrients (iron and zinc) in rice caryopsis

The iron and zinc content in mature polished and unpolished control (non-transgenic) rice grains were compared between eight high yielding rice varieties. Iron and zinc content were also compared between transgenic indica rice IR68144 and BR29. The quantitative estimation of iron and zinc content is summarized in Figs. 2 and 3 and Table 1.

Iron content in unpolished rice seeds ranged from 11 to 18 mg/kg of seed (Table 1). The iron level in variety NPT3 was recorded to be 17 mg/kg of seeds. Among the varieties studied maximum iron content was recorded in variety IR68144 (18 mg/kg) and minimum in variety T309 (11 mg/kg). The variety Swarna and BR28 recorded 14.5 mg/kg and 14 mg/kg, respectively.

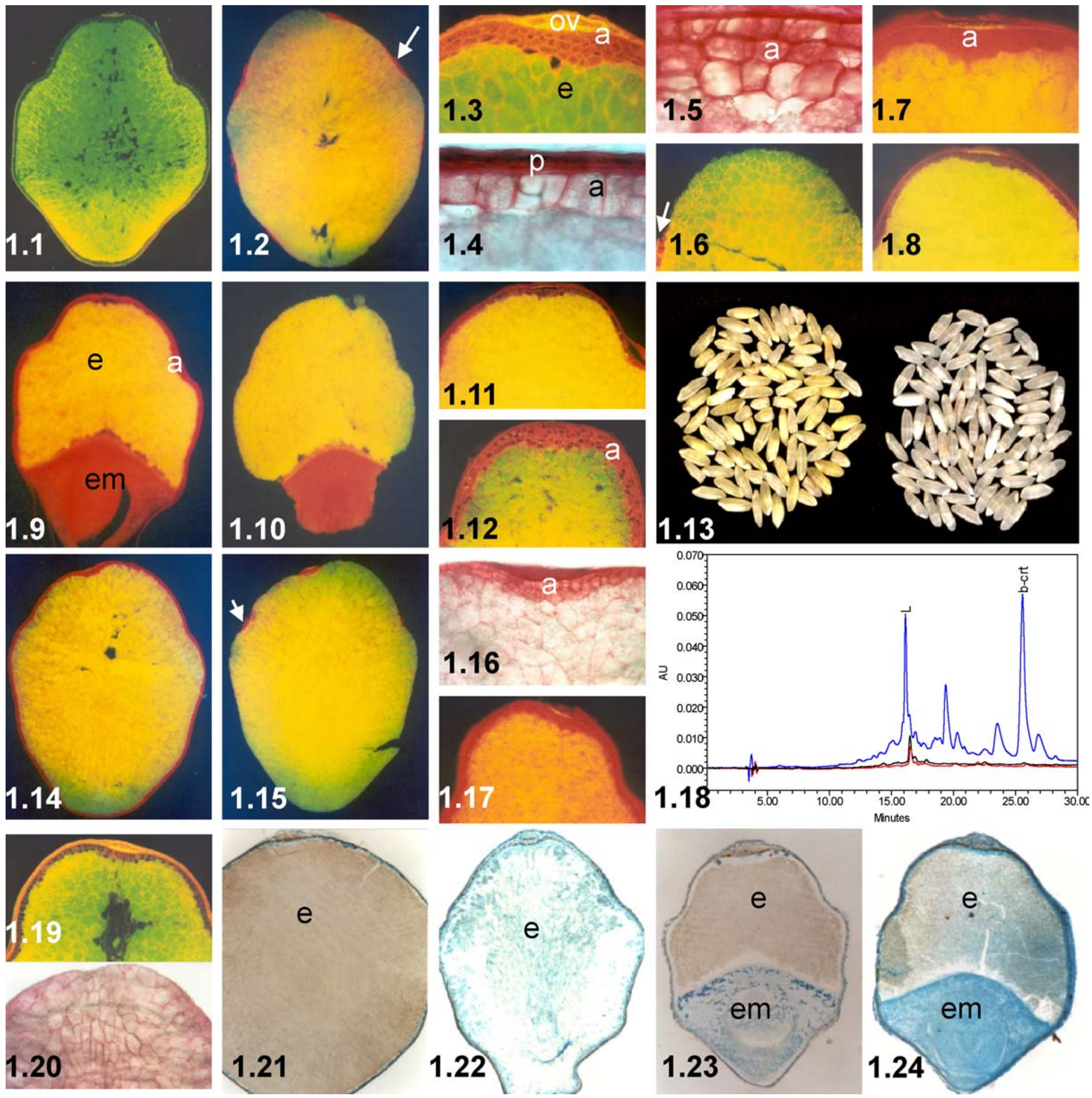


Fig. 1. Transverse sections of rice caryopsis stained with acridine orange (Figs. 1.1–1.3, 1.6–1.12, 1.14, 1.15, 1.17, 1.19) and safranin (Figs. 1.4, 1.5, 1.16, 1.20); Figs. 1.1–1.6. Var. NPT3. Fig. 1.1. Unpolished (X20), Fig. 1.2. Polished (X20), Fig. 1.3. Unpolished, ovular vascular bundle region (X150), Fig. 1.4. Unpolished, opposite to ovular vascular bundle region (X500), Fig. 1.5. Unpolished, just below the ovular vascular bundle (X500), Fig. 1.6. Polished (X75); Fig. 1.7. Var. Swarna, unpolished (X150); Fig. 1.8. Var. BR28, unpolished (X75); Figs. 1.9–1.11. Var. IR72. Fig. 1.9. Unpolished (X20), Fig. 1.10. Polished (X20), Fig. 1.11. Unpolished (X75); Fig. 1.12. Var. Taipei 309, unpolished; Fig. 1.13. Var. BR29 transgenic rice with β–carotene (left) and right (non-transformed control); Figs. 1.14–1.16. Var. IR64. Fig. 1.14. Unpolished (X20), Fig. 1.15. Polished (X20), Fig. 1.16. Unpolished (X75); Fig. 1.17. Var. IR68144; Fig. 1.18. HPLC chromatogram of transgenic BR28 polished (blue), control unpolished (red) and control polished (black); Figs. 1.19 and 1.20. Var. BR29. Fig. 1.19. Unpolished (X150), Fig. 1.20. Polished (X75); Figs. 1.21–1.24. Transverse section of rice caryopsis of var. IR68144 stained for detection of iron (X20), Figs. 1.21 and 1.23. Control (the accumulation of iron in the control grain is restricted to the aleurone and embryo); Figs. 1.22 and 1.24. Transgenics (in transgenic seeds iron is present in entire grain, including in the endosperm). a, aleurone; arrow indicates remnants of aleurone; e, endosperm; em, embryo; L, lutene; β-crt, β-carotene; ov, ovular vascular bundle; p, pericarp. "For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article."

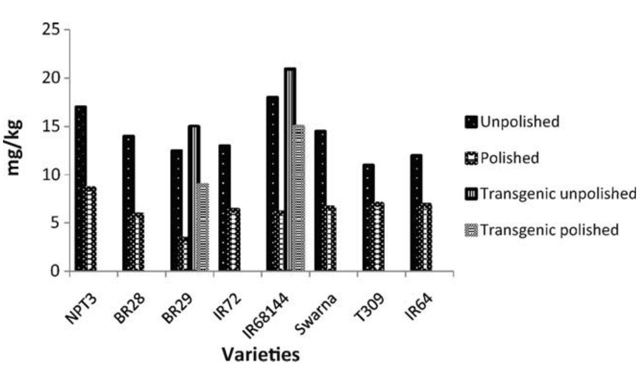


Fig. 2. Iron content in polished, unpolished and transgenic rice seeds.

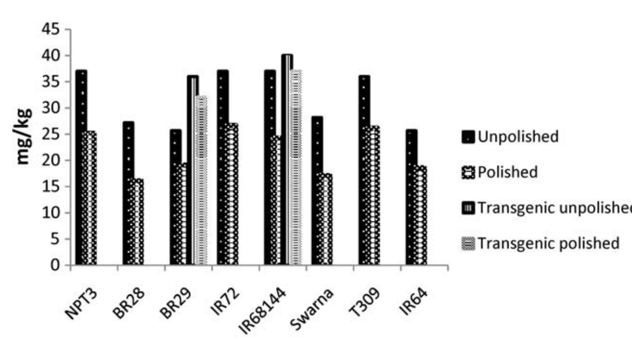


Fig. 3. Zinc content in polished, unpolished and transgenic rice seeds.

Table 1
Iron and zinc content in transgenic and non-transgenic rice.

Varieties		Iron (mg/kg)		Zinc (mg/kg)	
		UP	P	UP	P
Non-transgenic	NPT3	17	8.5	37	25
	BR28	14	5.8	27	16
	BR29	12.5	3.3	25.5	19
	IR72	13	6.3	37	26.5
	IR68144	18	6.0	37	24
	Swarna	14.5	6.5	28	17
	T309	11	6.9	36	26
	IR64	12	6.8	25.5	18.5
Transgenic	BR29	15	8.9	36	32
	IR68144	21	15	40	37

UP-unpolished, P-polished

In polished rice caryopsis, higher level of iron was detected in variety NPT3 (8.5 mg/kg) and minimum was in variety BR29 (3.3 mg/kg). Intermediate level (6–6.9 mg/kg) of iron was recorded in the remaining varieties studied (Table 1; Fig. 2).

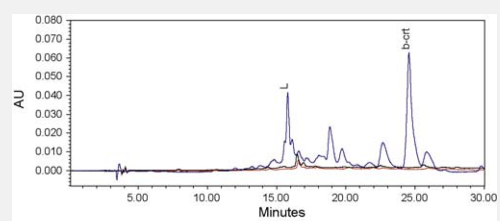
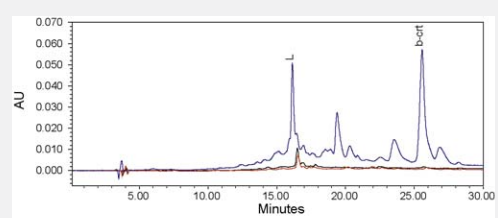
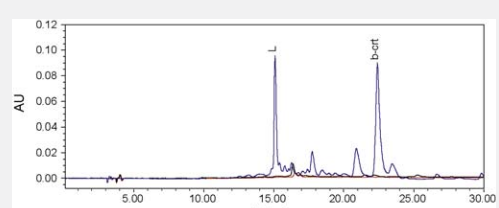
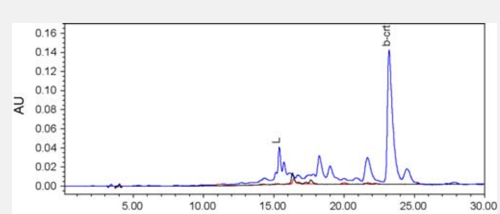
Similarly, zinc in unpolished rice grain ranged from 25.5 to 37 mg/kg of seeds. The higher level of zinc was recorded in varieties NPT3, IR72, IR68144 (37 mg/kg) and T309 (36 mg/kg). Minimum amount of zinc was recorded in varieties BR29 and IR64. Zinc content in polished rice grain ranged from 16 mg/kg in BR28 to 26.5 mg/kg in IR72 (Table 1; Fig. 3).

Among the transgenics developed, the unpolished IR68144 rice showed the maximum iron content of 21 mg/kg and in BR29 it was 15 mg/kg. Iron content in polished rice grains of IR68144 and BR29 were 15 and 8.9 mg/kg, respectively. Zinc content of unpolished transgenic rice seeds of IR68144 was 40 mg/kg, while in BR29 it was 36 mg/kg. Polished rice grains of IR68144 and BR29 recorded 37 and 32 mg/kg of zinc, respectively.

3.3. Estimation of β-carotene in transgenic and non-transgenic rice

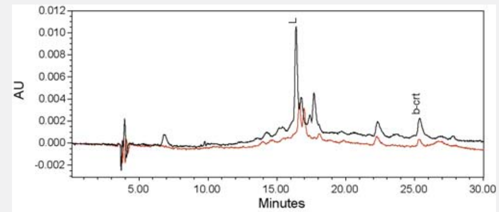
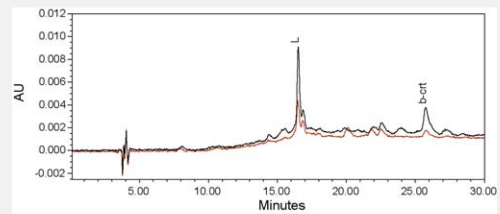
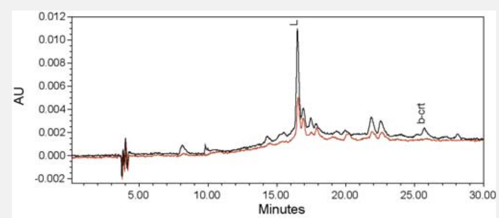
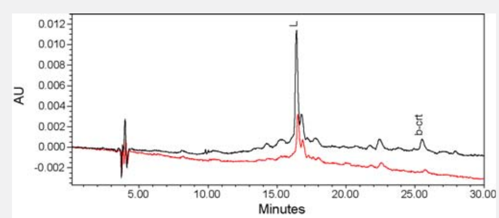
The transgenic lines MFP-16-34, BMF-5-2, SK64-561-8 and VPIR68-43 are developed by transforming BR29, BR28, IR64 and IR68144 respectively [16,26], for accumulation of carotenoids in the seed endosperm (Table 2). These transgenic lines are transformed with two important genes operated in carotenoid biosynthesis pathway, *psy* for phytoene synthase (from *Narcissus pseudonarcissus*), driven by the endosperm-specific Glutelin promoter and *crt*I for phytoene desaturase (from *Erwinia uredovora*), linked to the CaMV 35S promoter and fused to the coding sequence for the transit peptide of Rubisco [27,28]. Transgenic lines, MFP-16-34 and BMF-5-2 are developed by using hygromycin selection, while line SK64-561-8 was developed by

Table 2
HPLC chromatograms (A450) of polished (P) and unpolished (UP) BR28, BR29, IR64 and IR68144 with their corresponding polished golden rice transgenic lines.

Name	Total Carotenoid (ug/g)	β-Carotene (ug/g) by curve	HPLC (450 nm)
BR29 (P)	0.08	0	
BR29 (UP)	0.10	0.04	
MFP-16-34	1.58	0.40	
BR28 (P)	0.08	0	
BR28 (UP)	0.13	0	
BMF-5-2	1.28	0.36	
IR64 (P)	0.01	0	
IR64 (UP)	0.01	0.04	
SK64-561-8	2.32	0.96	
IR68144 (P)	0.07	0	
IR68144 (UP)	0.15	0.04	
VPIR68-43	2.10	0.88	

Note: Blue = golden rice, Black = unpolished control, Red = polished control.

Table 3
HPLC chromatograms (A450) of polished (P) and unpolished (UP) NPT, IR72, Swarna and T309.

Name	Total Carotenoid (ug/g)	β-Carotene (ug/g) by curve	HPLC (450nm)
NPT (P)	0.07	0	
NPT (UP)	0.14	0.04	
IR72 (P)	0.09	0	
IR72 (UP)	0.10	0.05	
Swarna (P)	0.06	0	
Swarna (UP)	0.12	0.04	
T309 (P)	0.06	0	
T309 (Up)	0.12	0.04	

Note: Black = unpolished control, Red = polished control.

using mannose selection. Both HPLC and spectrophotometric analysis showed the clear presence of β-carotene in transgenic rice lines (Fig. 1 1.18; Table 2).

Among the eight varieties studied (IR64, IR72, IR68144, Swarna, BRRI Dhan 29 (BR29), BR28, Taipai 309 (T309), New Plant Type-3 (NPT3)) for the estimation of β-carotene in matured control seeds (non-transgenic), none of them (both polished and unpolished) showed any presence of β-carotene (Table 3). However, minute quantity of lutene was detected. The transgenic rice seeds with β-carotene were developed for the varieties BR29, BR28 and IR68144 where the polished seeds showed golden yellow color in comparison to the white non-transformed control (Fig. 1 1.13).

4. Discussion

The nutrients are transported to the endosperm through single ovular vascular trace present on the ventral side of the ovary. In early stage of development of rice caryopsis, solutes enter through the chalaza into the nucellar projection and then into the endosperm. At later stages, transport occurs through the nucellar epidermis, centripetally towards endosperm, a unique way in which the nutritional components are distributed in the rice caryopsis [3]. In mature rice grain, aleurone and embryo are the major store-house for lipids. It also stores phytin granules which contain abundant calcium, potassium and iron [2]. The phytin granules consist of myoinositol hexaphosphate and associated cations. Calcium and iron are also present in scutellar cells. The sodium cobaltinitrite reagent is used for the localization of K^+ associated with phytin in aleurone [2,3]. The polishing of rice grain, however, is an essential process which is carried out by all rice industries and commercial farmers to remove the oil rich aleurone layer that would otherwise make the rice seed rancid during long storage [29,30]. The present study of structure of rice caryopsis in relation to distribution of micronutrients in mature polished and unpolished rice grains of eight high yielding rice varieties IR68144, IR64, IR72, BR28, BR29, Swarna, Taipei309 (T309) and New Plant Type-3 (NPT3) indicates that normal polishing process removes almost all the aleurone and embryo, which is the main storehouse for major micronutrients [2,3,12].

In nature carotenoids are present in all green tissues of plants. Carotenoids especially β-carotene is not present in endosperm of rice, the edible part. In transgenic rice, the carotenoid gene has been driven by endosperm-specific promoter, which drive the expression and deposition of carotenoids in the endosperm [16,26].

The requirement of iron for human body varies with age and body weight. Infants need 0.55 mg/day, while, children of age one to ten required 0.22–0.32 mg/day. Males of age more than ten years required 0.45–0.60 mg/day, while female needs 0.35–0.55 mg/day [31]. The requirement of zinc for human body varies with age, body weight and bioavailability. Considering the high bioavailability of this mineral infant (up to 12 month) requires 66–200 (μg/kg body weight/day) and children of one to ten years age required 90–138 (μg/kg body weight/day). The males of 10–18 age required 61–80 (μg/kg body weight/day), while, females of same age group need 56–68 (μg/kg body weight/day). Adult males of 18+ of age required 43 (μg/kg body weight/day), while, females of same age group needs 36 (μg/kg body weight/day) [31]. Vitamin A (carotenoids) requirement is calculated as retinol equivalency (RE) μg/day and one international unit (IU) of retinol is equal to three IU of β-carotene. The requirement of RE varies with health condition of human. The mean RE requirement for infants (up to 12 months) are 180–190 μg/day and for children up to age of nine is 200–250 μg/day. Adolescents (age 10–18 years) needs 330–400 μg RE per day, while, adults above 18 needs 300 μg RE per day. Pregnant and lactating women need higher RE/day that is 370 and 450 μg/day [31].

The quantitative estimation of iron and zinc content in mature polished, unpolished control rice grains indicated that more than 70% of micronutrients are removed during the polishing process. To solve the problem of micronutrients loss during the polishing process, transgenic rice with enhanced iron and zinc were produced using endosperm-specific promoters [18] and the transgenic rice grains analyzed in the present study showed the enhanced iron and zinc in the endosperm after polishing.

The Prussian blue staining reactions clearly revealed the presence of iron in the endosperm cells of both transgenic and non-transgenic rice grains. When thin sections of rice grains were placed in the solution of potassium ferro cyanide in acidic conditions, the potassium ferro cyanide reacts with tissue ferric ions to form an insoluble blue color. The treatment with acid was done to release ferric ions from the tissues. Ferric ions are immediately captured by the replacement of the cation of potassium ferro cyanide, forming insoluble ferric ferro cyanide, which then precipitates. This is an extremely reliable process, quite sensitive and very small amounts of iron can be demonstrated microscopically. The intensity of the color gives an indication for the qualitative amount only [11,12,18].

The structural, histochemical and biochemical studies on eight high yielding rice cultivars revealed that the micronutrients are mainly located in the aleurone layers and embryo and not in the endosperm cells [2,3]. Also, this study clearly revealed that the number of aleurone layers is directly proportional to the major micronutrients content and that more than 70% of micronutrients is lost during polishing process when aleurone is removed. It is very appropriate to enhance micronutrients in the endosperm cells to avoid the loss of micronutrients during polishing. In the present study the transgenic rice with enhanced iron, zinc and β-carotene were analyzed for the varieties IR68144, BR28 and BR29. However, further field evaluation of transgenic rice, bioavailability of iron, zinc and β-carotene and stability of transgene expression in the field conditions will allow the release of these varieties for farmer's cultivation and for consumption.

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