Tissue-specific localization of β-carotene and iron in transgenic indica rice (*Oryza sativa* L.)

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Tissue-specific distribution and localization of β-carotene and iron were characterized in transgenic rice seeds by histochemical studies. Histochemical reactions clearly revealed the accumulation of β-carotene in the endosperm of transgenic seeds in comparison with non-transgenic control where no β-carotene could be detected. A similar observation was made for iron-colour reaction in the endosperm. Since histochemical tests can be carried out easily and are fairly specific for determining particular compounds both qualitatively and to some extent quantitatively (based on the intensity of colour), this method could be used for identifying the desirable transgenic material with high β-carotene and iron content.

HISTOCHEMISTRY is a major tool in the localization of trace quantities of substances present in the plant and animal tissues[1–5]. Histochemical techniques and ultrastructural studies have been employed to characterize the structure, development, time of course deposition and distribution of major storage compounds such as protein, starch, lipids, phytin and minerals such as calcium, potassium, iron in rice grain[6–8]. Histochemistry of other cereal grains such as wheat and barley has also been described[9].

Iron deficiency anemia (IDA) and vitamin A deficiencies (VAD) are the major nutritional problems especially among Asian countries. It is estimated that about 30% of the world's population is affected by iron deficiency[10]. Vitamin A deficiency causes symptoms of night blindness, xerophthalmia and keratomalacia, which can lead to total blindness. VAD also causes diarrhoea, respiratory diseases and childhood diseases[11]. About 124 million children worldwide are deficient in vitamin A[12]. Because of VAD about a quarter of a million people go blind and 1 million to 2 million deaths are reported every year among children[13].

Rice is a staple food and no rice cultivar has the capacity to produce provitamin A in the endosperm tissue[14]. Since the conventional interventions have not been quite successful in eliminating these two disorders, the genetic engineering approach was used to develop the first japonica transgenic rice with provitamin A (β-carotene), popularly known as *golden rice*[15]. However, Asian countries with a wide range of climatic zones and agro-ecological variations require highly adapted indica varieties for certain regions. Three genes, namely phytoene synthase (*psy*), phytoene desaturase (*crtI*), and lycopene cyclase (*lcy*) are involved in the biosynthesis of β-carotene and also a gene coding for ferritin, an iron storage protein have now been introduced into many indica rice cultivars[16,17].

Even though many molecular techniques are used for the characterization of the expression of introduced genes in transgenic plants, it is still not possible to detect the expression of transgene product in specific tissues qualitatively and conveniently except by specific histochemical staining reactions. The present investigation aims at characterization of the transgenic rice for transgene product expression in a tissue-specific manner and comparison of distribution of β-carotene and iron in transgenic and non-transgenic control grains.

Seeds of the transgenic rice cultivars produced at the International Rice Research Institute (IRRI), Philippines, were used during this study[16,17]. For the histochemical analysis, various stages of transgenic and non-transgenic control rice grains were sectioned (15–20 μ thickness) using microtome (Vibratome Series 3000 Sectioning System). For the localization of β-carotene in rice endosperm, the Carr–Price reaction (approx. 25 g of antimony trichloride saturated in 100 ml of chloroform + 2 ml of acetic anhydride) was carried out with iodine derivatives under acidic condition to form a blue colour[18].

For the localization of iron, Perl's Prussian blue technique was employed[1]. Thin sections were stained with 2% potassium ferrocyanide in acidic condition (usually hydrochloric acid), which reacts with tissue ferric iron to form an insoluble blue colour. The treatment with acid was done to release ferric ions from tissues. The ferric ions are immediately captured by the replacement of the cation of potassium ferrocyanide, forming insoluble ferric ferrocyanide, which then precipitates. This is an extremely reliable process, quite sensitive and very small amounts of iron can be demonstrated microscopically. Observations and photography were done using Leitz Axioplan-II microscope under the bright-field mode.

Transgenic rice seeds with β-carotene biosynthesis in the endosperm tissue as evidenced by the yellow colour of the endosperm (Figure 1 *a*) were analysed for the localization of β-carotene in the endosperm. The Carr–Price reaction carried out with one section from non-transgenic control grains did not show any colour in the endosperm (Figure 1 *b*) in contrast to the blue colour observed in the case of transgenic grains (Figure 1 *c*). This finding showed correlation with the HPLC analysis showing the carote-

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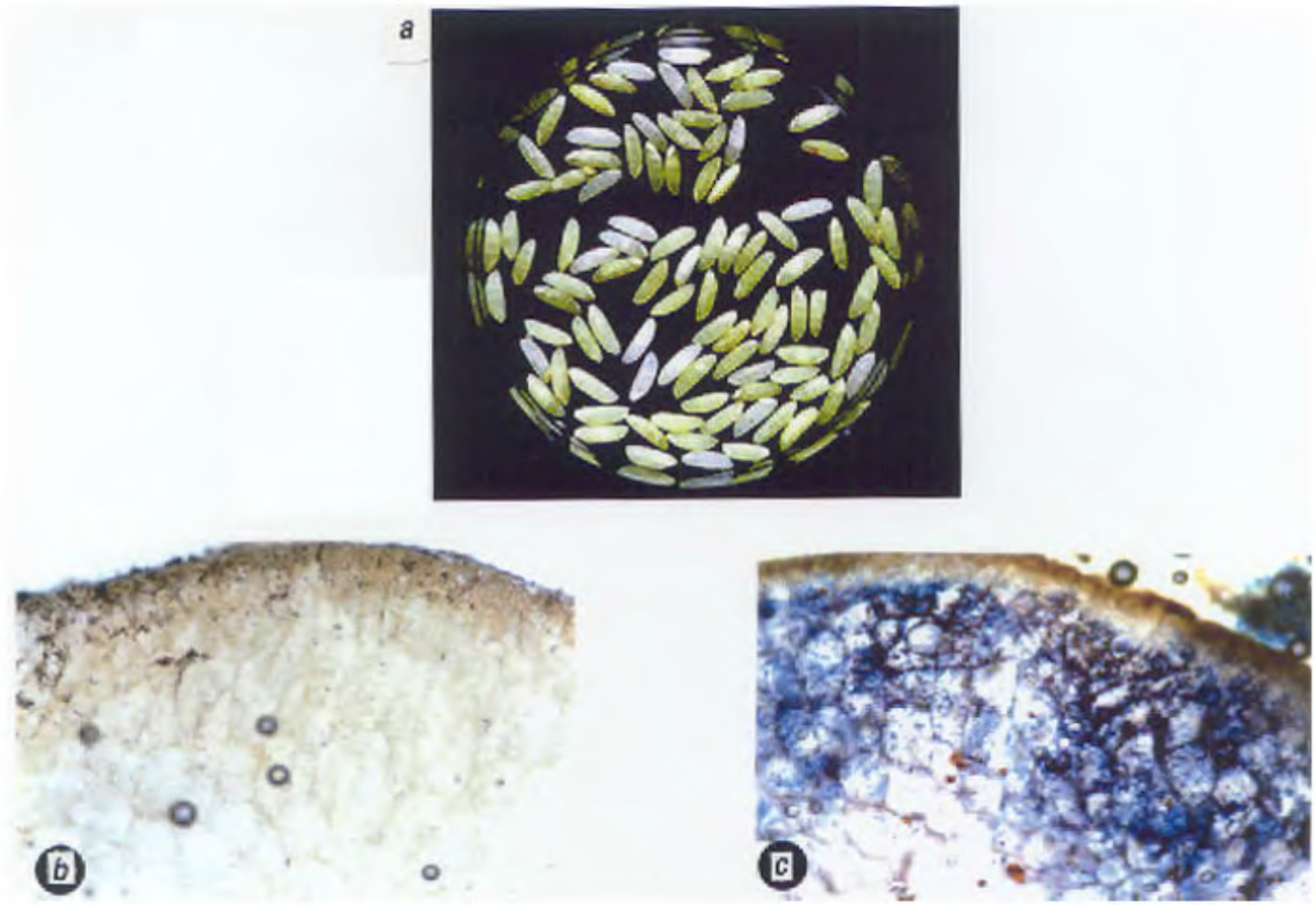

Figure 1. ***a***, Transgenic rice grains (segregating population) accumulating β-carotene in the endosperm appear yellow after polishing whereas non-transgenic ones remain white; ***b***, Transverse section of mature polished non-transgenic control rice grain did not show any colour with Carr–Price test (× 800); ***c***, Transverse section of mature polished transgenic rice grain showing blue colour immediately after the reaction with Carr–Price reagent indicates the accumulation of β-carotene in the endosperm (× 800).

noids including β-carotene peaks only in the transgenic polished rice grains[16].

Despite the high sensitivity of this reaction, the blue colour is very unstable and it slowly disappears. This could be due to the action of chloroform in the Carr–Price reagent, which dissolves most carotenoids to form the colour, and at the same time, dissociates with time.

Perl's Prussian blue reaction with non-transgenic control rice grains showed no detectable iron in the endosperm tissue (Figure 2 *a*), whereas transgenic rice grains showed clear expression of iron in the endosperm tissue (Figure 2 *b*). Besides, the accumulation of iron in the aleurone and embryo of non-transgenic grain was much lower (Figure 2 *c*) than in the transgenic grains (Figure 2 *d*). In normal rice grains, more than 90% of the iron is known to deposit in the aleurone cells. During polishing most of the minerals including iron are removed, leaving almost nothing or in trace quantities. It is important to note that transgenic rice grains accumulate iron in the endosperm cells and iron is present even after polishing which was clearly evident from the present study. The iron content in the rice grain after polishing was determined by inductively coupled plasma argon spectrometry (ICP) and showed much higher levels of iron in transgenic seeds as compared to control seeds described elsewhere[17]. Present histochemical colour reactions show further evidence that additional iron accumulated in transgenic rice endosperm.

Food-based micronutrients may be a preferable solution to reduce malnutrition since food fortification and oral delivery of vitamin A and iron are difficult in developing countries, mainly due to the inadequate infrastructure and lack of affordability by the poor[19,20]. Transgenic indica rice with β-carotene and iron may help in reducing malnutrition, especially VAD and IDA, in Asian countries where rice is the staple food. This histochemical survey and localization of iron and β-carotene in transgenic rice provides a broad framework of reference for understanding transgene expression in a tissue-specific

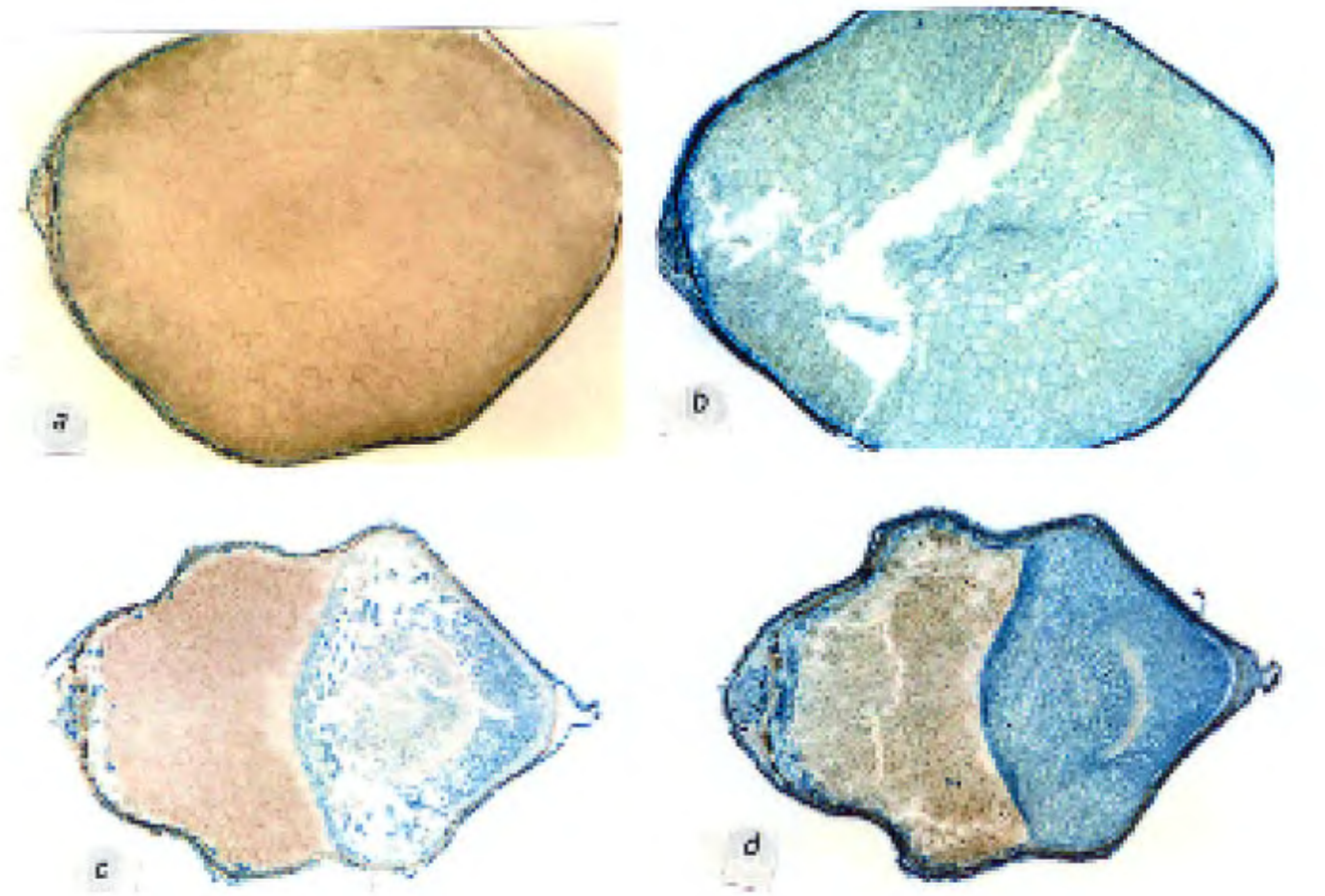

Figure 2. ***a***, Transverse section of mature non-transgenic control rice grain (cv. IR68144) showing no detectable iron in the endosperm tissue, × 160; ***b***, Transverse section of mature transgenic rice grain (cv. IR68144) showing clear accumulation of iron in the endosperm tissues as shown by blue colour, × 160; ***c***, Transverse section of mature non-transgenic rice grain passing through embryo region showing low concentration of iron in the embryo when compared to transgenic rice grain but no detectable iron in the endosperm, × 160; ***d***, Transverse section of mature transgenic rice grain showing higher accumulation of iron in the embryo and endosperm, × 160.

manner as well as the time and place of deposition of iron and β-carotene within the caryopsis.

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