

Golden rice: introgression, breeding, and field evaluation

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Abstract Considerable progress has been made on the genetic engineering of rice for improved nutritional content involving micronutrients and carotenoid content. Golden Rice, developed by genetic engineering (*Agrobacterium* and biolistic transformation) was used in rice breeding for the transfer of high-nutritional value to the local rice cultivars. Simultaneously, commercial Asian indica rice cultivars were also developed with expression of high-carotenoid levels. The lines were developed based on POSITECH (PMI) selection system or made marker free by segregating out the marker gene from the gene of

interest. Anther culture was used to develop the homozygous stable lines, which could be of much use in further introgress-breeding and in farmer's field. Enhanced carotenoids levels (up to T3 generation) were observed in a number of lines compared to the T0-T1 seeds which could be due to transgeneration effect of growing under greenhouse versus field conditions. However, a few introgressed lines showed less carotenoid levels than the original lines used in the breeding process. Agronomic performance of introgressed lines, non-transgenic controls, and transgenic golden rice (IR64 and BR29) developed at IRRI

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showed acceptable and comparable data under identical limited field conditions (screenhouse data). Syngenta generated a new Golden Rice (US cultivar) containing high level of carotenoids grown in the field at Louisiana, USA is expected to be available to the public domain. Incorporation of genes for carotenogenesis in seeds by transgenesis or by introgression did not change any significant agronomic characteristics in rice plants. The ongoing and future study of bioavailability, quality, larger field testing and freedom to operate will ensure the benefit of Golden Rice to the people who need them most.

Keywords *Oryza sativa* · Golden Rice · Introgress-breeding · Transgenesis · Carotenoids

Introduction

A value added golden yellow rice showing expression of β -carotene and other carotenoids in endosperm developed by genetic engineering is referred to as Golden Rice. Ninety percent of the rice consuming world's population consumes indica rice. The emphasis was therefore given to develop Golden indica rice based on the primary report of Golden rice developed in a Japonica model variety. Deficiencies of vitamin A, iron, and zinc are widespread in Asia and in developing countries, where the main diet is plant-based and the staple food is rice. Rice provides 40–70% of the total calories as a staple food in developing Asian countries. In Southeast Asia, an estimated 250,000 people go blind each year because of vitamin-A deficiency (VAD). Diet is the only source of vitamin A in mammals since they cannot manufacture it on their own. Most of the dietary vitamin A is of plant-food origin in the form of provitamin A that is converted to vitamin A in the body (Sivakumar 1998). So far, none of the screened exotic rice germplasm has shown the presence of β -carotene in polished seeds. Nevertheless, there is tremendous potential for using the genetic variability of carotenoid levels in unpolished rice germplasm (Tan et al. 2004).

More than half of all women and children in South and Southeast Asian countries are anemic. Anemia limits growth and cognitive development

in children and increases the incidence of death among severely anemic women during child-birth. Biofortified rice with enhanced minerals and provitamin A could complement existing nutrition interventions and provide a sustainable and low-cost way of reaching people who do not have access to the normal food market because of their poor economic conditions. Food consumption studies suggest that doubling the iron in rice can increase the iron intake of the poor by 50%. Rice germplasm screening shows that a doubling of iron and zinc in unmilled rice is feasible (Gregorio et al. 2000). The work on combining high iron + Zinc rice with Golden rice is in progress which might help in combating the malnutrition problems at greater levels (Khalekuzzaman et al. 2006).

The present paper discusses progress toward development of improved nutritional content in rice by genetic engineering. The main focus is on the development, breeding, particularly introgression of one event of Golden rice to a recipient cultivar, expression of the carotenoids in the introgressed lines, stability of the carotenoids after cooking and performance of Golden rice in limited greenhouse and field conditions.

Materials And Methods

Several indica cultivars (IR64, BR29, Mot Bui, NHCD, BR28, and IR68144) were used for transformation in conferring β -carotene biosynthesis in rice seeds. However, this report focuses on the two most important Asian indica cultivars, IR64 and BR29. Transformation (biolistic and *Agrobacterium*), regeneration, molecular analysis (PCR, Southern, RT-PCR), event selection, and biochemical analysis (carotenoid profiling by HPLC analysis) were conducted as described earlier (Datta et al. 2003; Vasconcelos et al. 2003; Tan et al. 2004). The pGPTV-bar/Fer vector for iron (Vasconcelos et al. 2003) and pBaal3 and pCaCar (based on mannose selection) were used for β -carotene engineering in rice seeds (Datta et al. 2003, 2006). Anther culture was used to develop the stable homozygous lines (Datta et al. 1990, 2006; Baisakh et al. 2001; Datta 2005).

Results And Discussion

Rice transformation for improved nutrition

Brown rice is rich in micronutrients including carotenoids (Tan et al. 2004). However, this rice is rarely consumed and polishing of the rice grain brings about a considerable loss of micronutrients by removing the outer layers. Expression of the soybean *ferritin* gene under the control of the glutelin promoter in rice has proven to be effective enhancing grain nutritional levels, not only in brown grain but also in polished grain (Goto et al. 1999; Vasconcelos et al. 2003; Khalekuzzaman et al. 2006). A similar approach has been utilized in developing Golden Rice (Ye et al. 2000; Hoa et al. 2003; Datta et al. 2003, 2006; Parkhi et al. 2005; Paine et al. 2006).

Zinc deficiency has been associated with complications in pregnancy and delivery, as well as growth retardation, congenital abnormalities, and retarded neuro-behavioral and immunological development in the fetus (Vasconcelos et al. 2003). Interestingly, we found that, along with enhanced iron, zinc levels were likewise increased in our transgenic ferritin rice. We reported earlier 27.9 $\mu\text{g/g}$ iron and 55.5 $\mu\text{g/g}$ zinc in unpolished transgenic rice seeds (Vasconcelos et al. 2003). This accounts for a two to threefold increase in iron compared with that of the control. The high levels of concentration of iron and zinc remained in rice grains after polishing (12–15 $\mu\text{g/g}$ iron in transgenic seed in the T3 generation versus 5–7 $\mu\text{g/g}$ in control seed). Iron and zinc levels in rice grain may vary because of soil properties,

weather, and some unknown factors (Gregorio et al. 2000).

Vitamin A plays an important role in a wide variety of physiological functions in human beings. Biosynthesis of carotenoids in plants takes place within the plastids, chloroplasts of photosynthetic tissues and chromoplasts of fruits and flowers. Chlorophyll, tocopherols, plastoquinone, phyloquinone, gibberellins, and carotenoids all share a common biosynthetic precursor, geranylgeranyl/diphosphate (GGPP), which is derived from a plastidic isoprenoid metabolism. The genes necessary for the four enzymes (phytoene synthase, phytoene desaturase, α -carotene desaturase, and lycopene cyclase) catalyze to complete the pathway toward β -carotene (provitamin A) biosynthesis from GGPP (Misawa et al. 1990). Conventional interventions (distribution, fortification, diet diversification) have been helpful in defeating VAD but are not effective enough. Conventional plant breeding to alter, modify, or introduce this biosynthetic machinery into the target endosperm tissues in rice has been impossible to date, as endosperm-active carotenoid biosynthetic genes have not been found thus far in the available rice gene pool (Tan et al. 2004; Fig. 2). Thus, the transgenic approach offers the most realistic way to improve rice by incorporating genes, originating from sources other than rice, into rice and showing β -carotene expression in rice seeds (Figs. 1, 2). Based on the proof of concept of β -carotene rice in japonica rice published by Ye et al. (2000), we carried out this work in indica rice. Several tropical indica cultivars (BR29, IR64) were transformed and showed

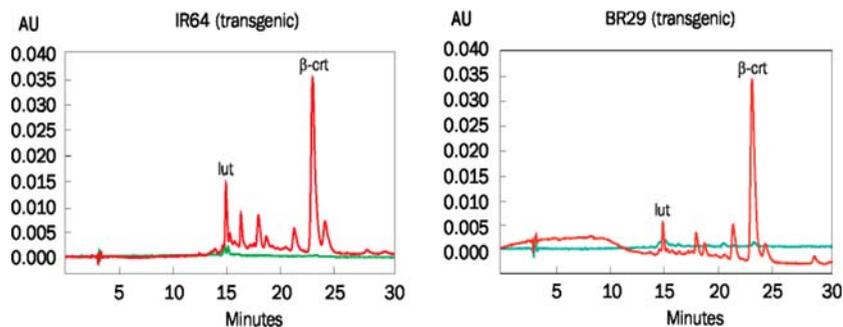


Fig. 1 Carotenoids profile of selected rice cultivars (IR64 and BR29) by HPLC analysis Carotenoid profile for selected rice cultivars under both polished and unpolished

conditions. Curves in red = polished seeds. Curves in blue = unpolished seeds. IR64 and BR29 are genetically engineered Golden indica rice (*lut* lutein, *b-crt* β -carotene)

Fig. 2 Golden indica BR29 (control at *left* and DH transgenic line at *right*) showing expression of beta-carotene in rice seeds after polishing. Transformation was carried out by *Agrobacterium* with mannose selection (PMI) system



expression of β -carotene genes (Table 1; Datta et al. 2003, 2006; Parkhi et al. 2005; Figs. 1, 2). *Agrobacterium*-mediated transformation along with the POSITECH™ (mannose selection) system works well with several cultivars. Expression of β -carotene varies among cultivars, as it was found that IR64 exhibited less expression compared with BR29. Differential expression of carotenoids, plant varietal backgrounds and the selection of the particular event of transgenic lines might attribute to such variations.

Introgression breeding and development of homozygous lines

Transgenic T309 line containing genes for carotenoids and selectable marker genes (Ye et al. 2000) was used for anther culture to develop the homozygous lines (Baisakh et al. 2001). Homozygous lines (DH line, KDHR3-11 from Z4b

containing 1.1 $\mu\text{g/g}$ carotenoids) were preferred as the best materials available at that time for cross breeding to transfer the genes/traits into the desirable cultivars. Eventually, introgressed IR64 lines were developed using the selected DH lines described in details elsewhere (Baisakh et al. 2006).

Phenotypic characters of the F1s and Backcross progenies

The F1 plants were intermediate type with regard to morphological features, which is a characteristic of indica \times japonica crosses. The leaves were broader and dark green and the grains were medium bold and shorter, with or without awns. The sterility percentage was quite high, which resulted in recovery of fewer BC1F1 seeds. However, in the subsequent generations of backcrossing, the restitution of IR64 characters was prominent in the progenies. The fertility, seed set,

Table 1 Golden indica rice obtained by *Agrobacterium*-mediated transformation and the lines used for introgression

Cultivars	Agro-strain used/breeding	Vector	Number of plants analysed	Number of southern positive plants	Carotenoid levels (TA) ($\mu\text{g/g}$)
BR29 (Indica)	EHA101	pCacar	304	221	9.34 ^a
	LBA4404 Transformation	pCacar	263	57	
IR64 (Indica)	LBA4404 Transformation	pCacar	100	10	2.32 ^a
T309 (Japonica)	Transformation	NA	NA	2	1.10 ^b
KDHR3-11	Introgression	NA	NA		1.06 ^c

NA not applicable, TA total amount

^a Selected lines after transformation, Datta et al. (2006)

^b Ye et al. (2000)

^c Selected introgressed line derivative from the original line described by Ye et al. (2000)

and panicle and plant features were almost the same as in IR64 in the BC2F2 progenies. Two of the near-isogenic lines (NILs) were homozygous for *crtI* and *psy* with maximum phenotypic similarity to IR64, and were subjected to another backcross generation resulting in IR64 NILs. The BC3F2 plants were identical to IR64 with all agronomic features measured showing no statistically significant differences (Table 2).

Genetic fingerprinting for similarity analysis of the IR64 NILs

The data on the total number of bands generated by the 12 URPs (universal rice primers) were recorded for six BC2F2 transgenic progenies (two plants from each family) and the two parental lines (IR64 recurrent-IR64C and donor KDHR 3–11). The size of the amplified products by RAPD analysis varied from 300 to 3,500 bp. A total of 86 amplified products were generated with the 12 random primers. Based on the mean length of amplified sequence of 1,200 kb/primer, it could be estimated a total of 14,451.35 kb of the rice genome has been spanned by the primers. This translates into 3.36% of the 4.3-Mb haploid rice genome. All the primers tested generated a nearly identical banding pattern among the introgression lines with the recipient parent IR64. However, introgression lines 10–1 and 10–10 showed a polymorphic band with line 4–2 by URP4, and lines 10–1 and 10–10 from a single family showed polymorphism by URP10 and URP12. URP8 generated a distinct polymorphic

band in line 4–2 vis-à-vis lines 10–1 and 10–10. All the primers generated polymorphic bands in the introgression lines compared to the japonica donor (KDHR 3–11). On average, the introgression lines and the IR64 control rice plants shared more than 90% of monomorphic bands generated by all 12 URPs tested. A genetic similarity tree was produced from the fingerprinting data of introgression and parental lines (data not shown). The similarity values for six introgression lines used (4–2–32, 4–2–35, 10–1–19, 10–1–21, 10–10–15, 10–10–17) with IR64C (recurrent parent control) ranged from 0.944 to 0.984 (data not shown). Two major clusters were evident with all the introgression lines along with IR64C in one and KDHR3–1 1 in the other. Lines 4–2–32 and 4–2–35 showed maximum closeness to IR64C, forming one subcluster, followed by 10–1–19 and 10–1–21 in the second subcluster and 10–10–15 and 10–10–17 in the third. The differences between the introgression lines in a subcluster were negligible and would be further minimized in the next BC generation. This suggested that the introgression of the two genes (*psy* and *crtI*) capable of installing the entire carotenogenic pathway only resulted in minor genome changes in IR64 (Baisakh et al. 2006). Introgressed IR64 from original japonica rice (T309) also showed lower carotenoid levels (Baisakh et al. 2006; Table 1). Efforts have been made to select a transgenic event with a simple and low-copy transgene for a Mendelian 3:1 segregation pattern and eventually to develop homozygous lines with acceptable agronomic performance.

Table 2 Agronomic performance of transgenic Golden rice (cv. IR64) vis-à-vis the IR64 control

Characters	Transgenic		Control		<i>F</i> -value (transgenic versus control)
	Mean	SEM±	Mean	SEM±	
Plant height	107.13	0.745	108.80	1.733	0.950 ^{ns}
Number of panicles per plant	9.13	0.358	8.65	0.539	0.391 ^{ns}
Number of grains per panicle	88.81	2.460	86.05	5.558	0.242 ^{ns}
Number of unfilled spikelets per panicle	34.16	1.364	28.75	3.312	2.881 ^{ns}
Spikelet fertility (%)	71.46	1.078	74.67	2.635	1.627 ^{ns}
1000 grain wt (g)	25.86	0.168	25.77	0.223	0.060 ^{ns}
Biological yield per plant p (g)	109.25	5.953	98.98	9.309	0.702 ^{ns}
Grain yield per plant (g)	13.49	0.661	13.74	1.350	0.030 ^{ns}
Harvest index per plant (g)	13.66	0.610	14.86	1.290	0.770 ^{ns}

^{ns} non-significant at *p* (0.05 (Rai et al. 2002)

Inheritance and possible transgeneration carotenoids levels in T3 plants

For each line selected to advance to the next generation, 60 T1 progenies were grown to study the inheritance of the integrated genes, identification of putative homozygous lines and their differential carotenoid expression level in the seeds (T2). PCR and Southern blot analyses of individual plants of each line showed single-locus Mendelian segregation (3:1) to a variable segregation pattern. This represents the possibility of transgene insertion into one or more loci. Simple gene integration patterns detected in the T0 generation were maintained in T1, while rearranged patterns in T0 were resolved into a mixture of simple and rearranged gene-integration patterns. Southern blot analyses showed the integration of *crtI* gene (3.28 kb) in 20 individual T1 progenies of one IR64 line (SK64–560) (data not shown). After harvesting, the dried seeds of individual progeny (T2) of each line were polished to assess directly by visual examination of yellow color. Estimation of carotenoid of the polished seeds by spectrophotometer and carotenoid profiling by HPLC analysis showed a wide variation in the amount of carotenoid expression in seeds (T2) of individual progeny (T1) of each line. Enhanced carotenoid levels were observed in many of the T2 seeds when compared with their respective T1 seeds. Such enhanced expression was attributed due to the post-transgeneration positive effect on carotenoids biosynthesis in rice seeds. We have also noted the enhanced chlorophyll biosynthesis in some lines (unpublished data).

One transgenic plant, SKBR-244–26, contained total carotenoids 9.34 $\mu\text{g/g}$ of polished seeds and 3.92 $\mu\text{g/g}$ of β -carotene, the highest value obtained from BR29 or any indica rice so far (Datta et al. 2006; Table 3). Earlier papers reported the amount of carotenoids in transgenic indica rice was 1–3 $\mu\text{g/g}$ (Datta et al. 2003; Hoa et al. 2003; Parkhi et al. 2005). About 4–5 $\mu\text{g/g}$ carotenoids may provide more than 60% of the RDA (Recommended Daily Allowances) by the ICMR/WHO (B. Sivakumar, NIN, Hyderabad, personal communication). In case of IR64 the highest value of total carotenoids obtained in T2 seeds of

Table 3 Variation of carotenoid content in different progenies of selected lines

	Parent number	T0 ($\mu\text{g/g}$)	Progenies T1 ($\mu\text{g/g}$)				
			1	2	3	4	5
1	SKBR29–3	1.33	2.56	2.9	4.68	nd	nd
2	SKBR29–11	1.80	1.73	3.21	2.2	4.05	nd
3	SKBR29–13	1.60	2.04	2.63	4.21	4.46	4.49
4	SKBR29–216	1.254	2.24	3.05	3.15	3.63	4.12
5	SKBR29–217	2.560	4.56	4.98	6.43	6.59	7.55
6	SKBR29–218	1.312	2.38	3.91	4.56	6.08	nd
7	SKBR29–234	2.03	3.43	4.1	4.57	nd	nd
8	SKBR29–240	1.434	4.62	5.12	5.79	nd	nd
9	SKBR29–241	1.584	3.37	3.44	4.31	nd	nd
10	SKBR29–244	1.004	4.3	4.54	4.59	6.77	9.34
11	SK6429–561	0.592	1.03	1.47	2.32	1.16	1.38
12	SK6429–562	0.948	1.05	1.94	nd	nd	nd
13	SK6429–560	0.748	1.08	1.32	1.25	1.08	0.76

nd not done; Datta K et al. (2006)

SK64–561–8 was 2.32 $\mu\text{g/g}$ of polished seeds. However, in many lines the carotenoid accumulation was much lower in their respective parents as shown in Table 2. The carotenoid profile of the transgenic seeds showed the presence of lutein, β -cryptoxanthin and α -carotene (Fig. 1).

Evaluation and agronomic performance of Golden BR29 rice in Bangladesh

BR29 is a popular high-yielding commercial rice variety in Bangladesh. This cultivar has been evaluated and accepted successfully in several Asian countries through INGER and is also being adapted in some countries, including India. Two BR29 Golden rice lines (SKBR-3–7 and SKBR-7–11) developed at IRRI (Datta et al. 2006) were transferred to Bangladesh for field evaluation. First year study conducted at containment greenhouse at BRRI showed substantive equivalence of Golden BR29 and control seed derived BR29 plants. Further field study is on progress with the support from the BARC (Govt. of Bangladesh). Golden rice (US-variety) developed by Syngenta (Paine et al. 2005) was evaluated in AgCenter Rice Research Station at Louisiana, USA in 2004–2005 (AgBioworld 2004). Golden rice was found similar in various agronomic characters such as plant height, days to flowering, seed set, 100-seed weight and total biomass as compared to similar control varieties when grown side by side.

Golden IR64 grown in soil in greenhouse conditions at IRRI, Los Banos, Philippines and Golden BR29 at BRRI, Gazipur, Bangladesh showed similar results. Freedom to operate to use the Golden Rice in Southeast Asia could expedite the research, breeding and introgression of the traits in the local varieties and eventually acceptance and use by the consumers.

Bioavailability and future study

The bioavailability of β -carotene needs to be studied. Since ferritin is used as a natural source of iron in the early development of animals and plants, ferritin-iron bioavailability is well accomplished. In fact, iron in transgenic rice with ferritin has already been tested in deficient rats, and rice diets were as effective as the FeSO_4 diet in replenishing hematocrit (Murray-Kolb et al. 2002). Further studies suggest that a relatively small supplement of vitamin A or β -carotene can double the absorption of endogenous nonhaem iron from cereals (Graham and Rosser 2000). Thus, β -carotene-enriched rice helps reduce VAD and protect against iron-deficiency anemia. Bio-fortified rice with enhanced nutrition (β -carotene, iron, zinc, and lysine) along with other superior agronomic traits and yield could support the food-health and livelihood of the millions of people who need them most. Currently, Syngenta generated Golden Rice (US cultivar) is being field evaluated by the LSU Rice station at Louisiana. The work on Golden BR29 (indica type commercial Asian rice) developed by IRRI (Datta et al. 2006) is in progress at BRRI, Bangladesh which might provide valuable information for the future use of the selected materials.

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