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Marker-free transgenic (MFT) near-isogenic introgression lines (NIILs) of 'golden' indica rice (cv. IR64) with accumulation of provitamin A in the endosperm tissue

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Summary

We have developed near-isogenic introgression lines (NIILs) of an elite indica rice cultivar (IR64) with the genes for β-carotene biosynthesis from dihaploid (DH) derivatives of golden japonica rice (cv. T309). A careful analysis of the DH lines indicated the integration of the genes of interest [phytoene synthase (*psy*) and phytoene desaturase (*crtI*)] and the selectable marker gene (hygromycin phosphotransferase, *hph*) in two unlinked loci. During subsequent crossing, progenies could be obtained carrying only the locus with *psy* and *crtI*, which was segregated independently from the locus containing the *hph* gene during meiotic segregation. The NIILs (BC_2F_2) showed maximum similarity with the recurrent parent cultivar IR64. Further, progenies of two NIILs were devoid of any fragments beyond the left or right border, including the chloramphenicol acetyltransferase (*cat*) antibiotic resistance gene of the transformation vector. Spectrophotometric readings showed the accumulation of up to 1.06 μg total carotenoids, including β-carotene, in 1 g of the endosperm. The accumulation of β-carotene was also evident from the clearly visible yellow colour of the polished seeds.

Keywords: β-carotene, golden indica rice, high-performance liquid chromatography (HPLC), marker-free, near-isogenic introgression lines (NIILs).

Introduction

Vitamin A deficiency (VAD) is one of the principal global nutritional problems associated with night blindness, xerophthalmia and breakdown of the immune system. Available data suggest that 140 million pre-school-aged children and more than seven million pregnant women suffer from VAD every year. Nearly half of all VAD and xerophthalmia occurs in South and Southeast Asia (Sommer and Davidson, 2002). Diet (mainly plant food) is the major source of human vitamin A (in the form of β-carotene or provitamin A). Rice, which feeds two-thirds of the world population, is mainly eaten after milling, and does not contain β-carotene as milling removes the outer aleurone layer of the grain where β-carotene is accumulated (Tan *et al*., 2005). Conventional interventions, such as fortification, distribution as medicine, diet diversification, etc., have been helpful in alleviating VAD to only a minimal extent and are inefficient. Therefore, supplementation with rice bioengineered with provitamin A would be an alternative and sustainable strategy for combating VAD, especially in developing countries, where rice is the staple food. A major scientific breakthrough was the development of golden rice in the background of a japonica cultivar (T309) with the accumulation of β-carotene in the endosperm tissue; this was achieved through the transfer of three genes, *psy*, *crtI* and *lcy*, coding for phytoene synthase, phytoene desaturase and lycopene cyclase, respectively, that are involved in the β-carotene biosynthetic pathway (Ye *et al*., 2000). Subsequently, Datta *et al*. (2003) extended this 'proof of concept' to several indica rice cultivars including IR64. However, in both of these cases, *hph* (coding for hygromycin phosphotransferase), which confers resistance against the antibiotic

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hygromycin, was used as the selectable marker gene. In view of the increasing concern over antibiotic selectable marker genes, a positech™ selection system using phosphomannose isomerase (*pmi*) (Joersbo, 2001) was used successfully to develop golden indica rice in the background of a widely grown popular cultivar of Asia IR64 (Datta *et al*., 2003; Hoa *et al*., 2003) and Kaybonnet (Paine *et al*., 2005).

Nevertheless, the *pmi* gene is also isolated from the bacterium *Escherichia coli*. The markers (in either positive or negative selection) are considered to be undesirable once a transgenic is developed. Although chloroplast engineering is a viable alternative strategy to keep the marker gene in containment (Daniell, 2002), removal of the marker gene from a transgenic plant has been cited as 'good laboratory practice' by many regulatory committees. Consequently, transformation has focused on the generation of selectable marker-free transgenics (MFTs). Several strategies have been developed and adopted to remove selectable marker genes (for a review, see Ebinuma *et al*., 2001; Hohn *et al*., 2001; Hare and Chua, 2002; Puchta, 2003). If the marker gene and the gene(s) of interest (GOI) are integrated in unlinked loci, some progenies in the selfing generation will probably possess only the GOI, with the marker gene segregated out, and the trait can be transferred to a desirable background by conventional breeding. However, if the trait is to be transferred to another genotype, it is time consuming when the processes of screening, production of F_1 plants and subsequent backcrossing to restore the recipient genotype are taken into account. Moreover, homozygous lines are a preferred component of the cross-breeding programme. Although heterozygous transgenic lines can also produce pollen with transgenes, the possibility of null pollen (without transgenes) having an equal likelihood of becoming cross-fertilized results in a lower frequency of transgenic progenies.

Anther culture, with its unique advantages of rapid fixation of homozygosity, increased selection efficiency and early expression of recessive genes, has proved to be an important component of transgenesis breeding for the rapid development of homozygous transgenics (Baisakh *et al*., 2001) that can be used as the pollen parent in cross-breeding.

As a component of our golden rice research, we attempted to transfer the genes for β-carotene biosynthesis to an elite indica rice cultivar IR64. In this study, we report the development of near-isogenic introgression lines (NIILs) of an elite indica rice cultivar (IR64) through transfer of the genes for β-carotene biosynthesis from dihaploid (DH) golden rice (T309) using transgene-based marker-assisted backcross breeding. To our knowledge, this is the first report to transfer a metabolic pathway from a genetically modified japonica cultivar to elite indica rice by conventional cross-breeding, which is free from a selectable marker gene.

Results

Anther culture

Sixty-one plants were obtained from anther culture of transgenic golden japonica rice (T309). Of these, 51 plants were green and 10 were albinos. Polymerase chain reaction (PCR) analysis showed that 28 (54.9%) plants were positive for the transgenes, which was further confirmed by Southern blot analysis. Southern blot showed a 3.5-kb band corresponding to the *crtI* expression cassette (Figure 1). From 28 transgenic green plants, 10 (35.7%) were identified as DHs on the basis of their phenotype. Three distinct integration patterns were observed in the DH lines. Some of the DH lines (lanes 6, 16, 18 and 19 in Figure 1) contained an additional rearranged band of higher molecular size in addition to the expected 3.5-kb band, whereas others contained either the unexpected rearranged band or both fragments. As expected, all DH lines were positive for all other transgenes, such as *psy*, *hph* and *lcy* genes.

Development of F_1 and isolation of marker-free IR64 NIILs

A large number of seeds were produced from the cross between the DH line (KDHR3-11) and IR64. One hundred

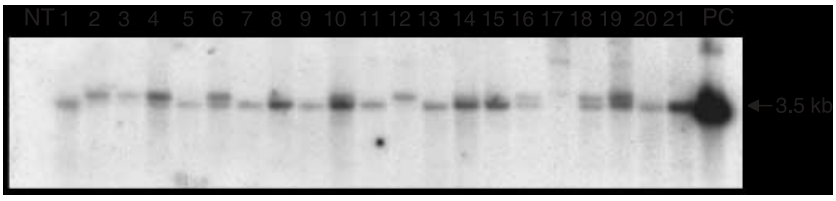

Figure 1 Southern blot showing differential integration patterns amongst the anther culture-derived lines of a single parent, HR3. NT, non-transgenic control; 1–21, anther culture-derived plants of T-309 golden rice (z4b); PC, positive control (a 3.5-kb fragment released by restriction of pBaal2 with *Eco*RI/*Hin*dIII). The genomic DNA was digested with *Eco*RI/*Hin*dIII overnight, run in 1% TAE (40 mM Tris-aretate, 1 mM EDTA)-agarose gel, transferred to Hybond+ nylon membrane, hybridized with a 1.033-kb polymerase chain reaction fragment labelled with (α^{32}P)-dCTP, washed and exposed to X-ray film.

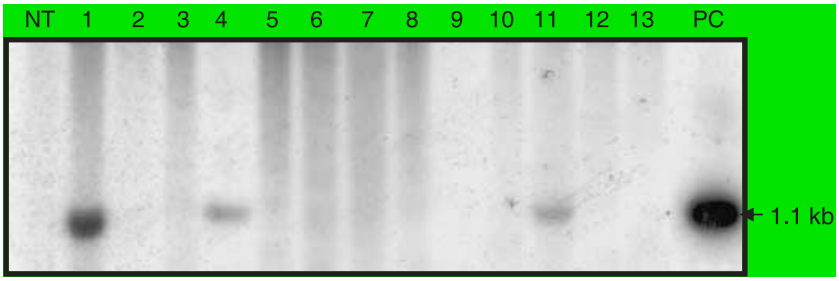

Figure 2 Southern blot analysis of BC_1F_1 plants showing the absence of the 1.1-kb hygromycin phosphotransferase (*hph*) fragment in 10 of 13 plants that were positive for phytoene desaturase (*crtI*). NT, non-transgenic control plants; 1–13, BC_1F_1 plants; PC, positive control (a 1.1-kb fragment from pGL2 on restriction with *Bam*HI). The Southern procedure is the same as that detailed for Figure 1, except that *Bam*HI enzyme was used instead of *Eco*RI/*Hin*dIII.

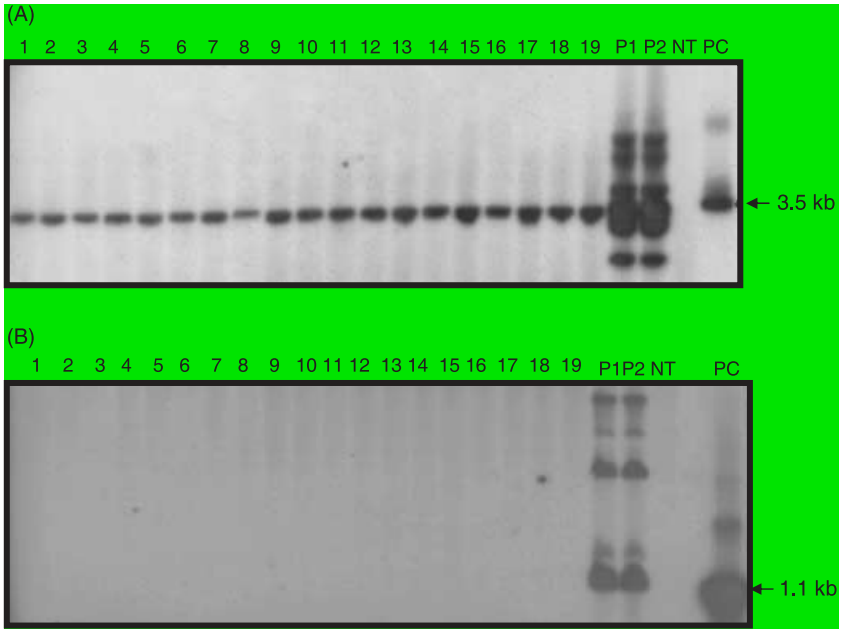

Figure 3 Representative Southern blots of BC_2F_2 introgression plants (lanes 1–19) suggesting homozygosity of the phytoene desaturase (*crtI*) gene (A) and no hybridization signal for the hygromycin phosphotransferase (*hph*) gene (B). NT, non-transgenic control; PC, positive control (as in Figure 1). P1 and P2, two transgenic IR64 indica golden rice lines (64E-26; Datta *et al*., 2003) used as positive control. The Southern procedure is exactly the same as that given in Figure 2. Blot (A) was stripped of *crtI* and reprobed with *hph* to develop blot (B).

putative crossed seeds underwent germination in Petri dishes. Six seeds did not germinate and the rest of the seedlings were subsequently grown in pots in the containment glasshouse. Of the total of 94, 45 F_1 plants were found to be positive for the transgenes by PCR analysis (data not shown), indicating their heterozygosity. The 45 F_1 plants inherited morphological features of both parents. However, almost all of the F_1 plants possessed high sterility, resulting in poor seed setting.

Four healthy F_1 plants were used for backcrossing (BC) with the IR64 female parent. Few BC_1F_1 seeds were obtained as many of the F_1 plants were sterile, which is common for indica × japonica crosses. The BC_1F_1 plants were also initially screened for the transgenes by PCR. Thirteen plants were found to be positive for the *crtI* gene. This was also confirmed by Southern blot analysis for the *crtI* gene (data not shown). However, when the blot was rehybridized with the *hph* gene, interestingly, 10 of the 13 randomly selected plants analysed were negative without any hybridization signal (Figure 2), and in these 10 plants, the *hph* gene was expected to have been removed by recombination in the BC_1F_1 generation. These 10 lines were further studied for their morphological characteristics, seed sterility, etc. Of these 10 BC_1F_1 plants, two were phenotypically closer to IR64. These two lines were again used as pollen parents in the backcross programme with IR64 for another generation and BC_2F_1 seeds were obtained. Fourteen BC_2F_1 plants were grown and allowed one cycle of selfing to produce BC_2F_2 seeds. Further analysis of these BC_2F_2 seed-derived plants confirmed the presence of the *crtI* gene by Southern hybridization (Figure 3A) and, in addition, the *psy* gene by PCR (data not shown). These lines, as expected, did not possess the *hph* gene from the results of either PCR or Southern analysis (Figure 3B). Southern blot analysis revealed that the lines that were negative for *hph* did not show the rearranged copy of *crtI*, nor did they hybridize with *lcy* when used as a probe (data not shown). This indicated that the locus with the gene set of *psy* and *crtI* was different and unlinked to that with the set of the rearranged copy of the *crtI* gene, or to the *hph* and *lcy* genes. Meiosis ensured the independent segregation

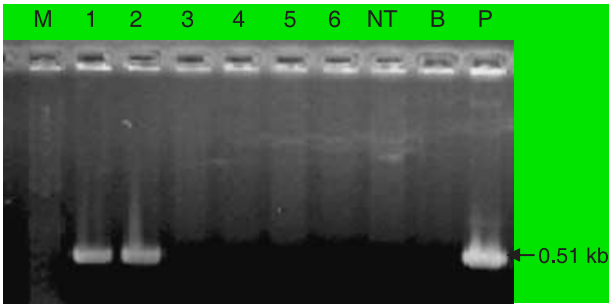

Figure 4 Polymerase chain reaction (PCR) analysis showing the presence of a 0.51-kb amplicon for the chloramphenicol acetyltransferase (*cat*) gene in two plants (lanes 1 and 2) and its absence in four BC_2F_2 plants (lanes 3–6) and in the non-transgenic control (NT). P, plasmid pCACAR having the *cat* gene as positive control; B, blank.

Figure 5 Polished seeds of a homozygous BC_2F_2 derivative of IR64 introgression line 4-2 (left) showing yellow coloration due to the accumulation of β-carotene, as well as other carotenoids, which is absent in the non-transgenic control IR64 (right).

of the two loci, ultimately allowing the subsequent removal of the *hph* and *lcy* genes from some progenies that possessed the *psy* and *crtI* genes. After obtaining an indication of the differential integration patterns of the *crtI* gene amongst the DH lines, we analysed a number of progenies of z4b that also showed the differential integration pattern of *crtI* (data not shown).

Beyond left border transfer of the antibiotic resistance gene

The PCR result (Figure 4) showed that the BC_2F_2 progenies 4-2-32 and 4-2-35 possessed the chloramphenicol acetyltransferase (*cat*) gene for bacterial selection, which was evident from a 510-bp amplicon, whereas the progenies of two BC_2F_1 (10-1 and 10-10) families (i.e. 10-1-19, 10-1-21, 10-10-15 and 10-10-17) did not show any amplification for the *cat* gene, suggesting the absence of any fragment beyond the left border of T-DNA in the latter. There was a similar result for the transfer of any fragment beyond the right border of the T-DNA. Furthermore, Southern analysis using the vector backbone as a probe did not show any hybridization signal in these two lines (data not shown). The vector backbone was released from pBaal3 (Datta *et al.*, 2003) by restriction with *Kpn*I and *Not*I.

Carotenoid quantification

Although the *lcy* gene was co-segregated out together with the *hph* gene, the seeds of the introgression lines with only PSY and CRTI activities accumulated carotenoid products of the entire biosynthetic pathway, including β-carotene and xanthophylls, in the endosperm, as was evident from the yellow colour of the polished seeds (Figure 5). The total carotenoid content, calculated from the absorbance value of the polished seed extract, was almost the same between the progenies with no significant variation. In one of the lines that showed maximum similarity to cultivar IR64 in terms of morphological features with maximum seed setting, the total carotenoids accumulated in the endosperm was 1.06 μg/g of seed powder, which was comparable to the total amount of carotenoid in KDHR3-11 (the DH derivative of z4b) homozygous for the *psy* and *crtI* gene (1.1 μg/g dry mass). On the other hand, the total carotenoid in one of the homozygous lines of z4b carrying all three genes (*psy*, *crtI* and *lcy*) was estimated to be 1.8 μg/g dry mass. High-performance liquid chromatography (HPLC) analysis (Figure 6) showed that β-carotene accounted for as much as 38.7% of the total carotenoid, which amounted to 0.41 μg/g dry mass in the best line selected. However, if all the carotenoids with provitamin A activity, i.e. β-carotene, β-cryptoxanthin and α-carotene (which is not clearly visible in the figure), were taken into account, they made up 87% of the total carotenoids, which translated into 0.912 μg provitamin A/g dry mass.

Phenotypic characters of the F_1 plants and backcross progenies

The F_1 plants were intermediate type with regard to morphological features, which is a characteristic of indica × japonica crosses. The leaves were broader and dark green and the grains were medium bold and shorter, than IR64, with or without awns. The sterility percentage was quite high, which resulted in the recovery of few BC_1F_1 seeds. However, in subsequent generations of backcrossing, the restitution of IR64 characters was prominent in the progenies. The fertility, seed setting and panicle and plant features were almost the same as in IR64 in the BC_2F_2 progenies. Two of the NILs were homozygous for *crtI* and *psy* with maximum phenotypic similarity to IR64, and were subjected to another backcross generation resulting in

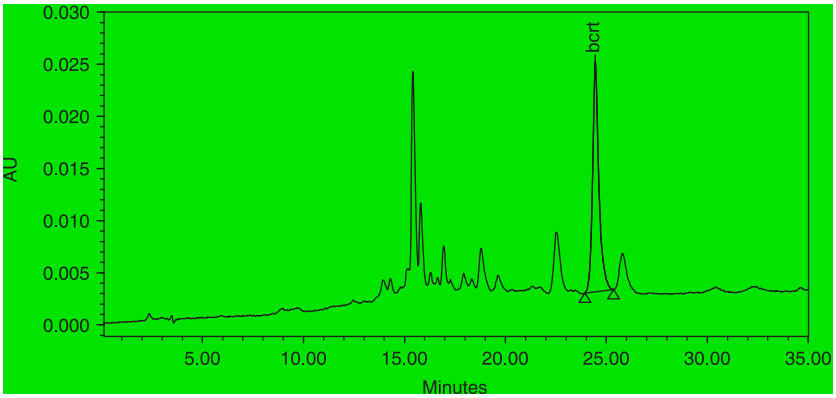

Figure 6 A representative high-performance liquid chromatography (HPLC) profile of the carotenoid extract of polished seeds of a BC_2F_2 IR64 near-isogenic introgression line (NIIL) showing a β-carotene peak (flanked with arrows) as well as other carotenoid peaks.

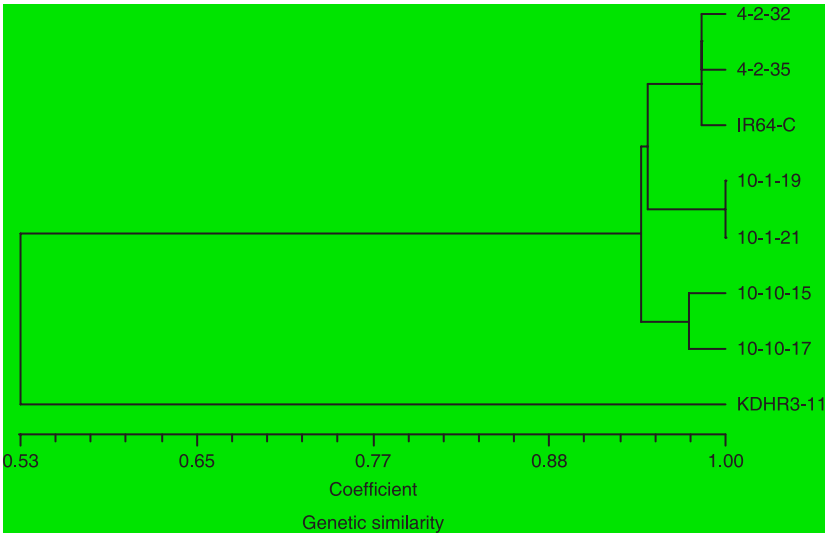

Figure 7 Dendrogram showing the genetic similarity tree between the IR64 near-isogenic introgression lines (NIILs), wild-type IR64 (IR64-C) and donor KDHR3-11.

IR64 near-isogenic lines (NILs). The BC_3F_2 plants were identical to IR64 with no statistically significant difference in agronomic features (data not shown).

Genetic fingerprinting for similarity analysis of the IR64 NIILs

The data on the total number of bands generated by the 12 universal rice primers (URPs) were recorded for six BC_2F_2 transgenic progenies (two plants from each family) and the two parental lines (IR64 recurrent–IR64C and donor–KDHR3-11). The data on the presence and absence of DNA bands were used to produce the dendrogram. The size of the amplified products by RAPD (Random Amplified Polymorphic DNA) analysis varied from 3500 to 300 bp. Eighty-six amplified products were generated with the 12 random primers. Based on the mean length of the amplified sequence of 1200 kb/primer, it could be estimated that a total of 14 451.35 kb of the rice genome had 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 amongst 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 (KDHR3-11). On average, the introgression lines and the IR64 control rice plants shared more than 90% of monomorphic bands generated by all 12 URPs tested.

The genetic similarity tree produced from the fingerprinting data of the introgression lines and parental lines is shown in the dendrogram (Figure 7). The similarity values for six of the introgression lines used (4-2-32, 4-2-35, 10-1-19, 10-1-21, 10-10-15, 10-10-17) to IR64C (recurrent parent control) ranged from 0.944 to 0.984 (data not shown). Two major clusters were evident with all the introgression lines, together with IR64C in one and KDHR3-11 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 present BC generation. This suggested that the introgression of the two genes (*psy* and *crtI*) capable of installing the entire carotenogenic pathway did not alter the genome of IR64 significantly. Similarly, the homozygous lines of original golden rice (T-309) did not show any significant difference in yield when expressing the genes in the carotenoid pathway.

Discussion

The use of selectable marker genes is an integral part of the transformation process, allowing the selection of transgenic calli/tissues. However, second-generation transgenics using markers with possibly no harmful biological activities, or completely devoid of a selectable marker, would be an important component of a crop improvement programme through direct commercial deployment, to further engineer multiple and complex traits, or for use in conventional cross-breeding programmes.

Different strategies with their own merits and demerits have been proposed to produce MFT plants. Nonetheless, co-transformation still seems to be the simplest strategy and can be accomplished in several ways through the use of different delivery systems. This takes advantage of the *Agrobacterium*-mediated transfer of two different T-DNAs, bearing the GOI and the selectable marker gene, respectively, and their subsequent integration at sufficiently unlinked sites in the primary transformant, followed by genetic segregation in the progenies. The two T-DNAs can be borne by a single binary vector (two T-DNA system; Komari *et al*., 1996; Matthews *et al*., 2001), two binary vectors in the same *Agrobacterium* cell (Daley *et al*., 1998) or two different vectors carried by different *Agrobacterium* strains (De Neve *et al*., 1997).

In our study, the integration pattern for *crtI* was revealed to be different amongst the DHs by Southern analysis (Figure 1). This led us to presume that the lines might contain a rearranged fragment of *crtI* in different parts of the chromosome in the original material (z4b) used for anther culture, in which the pollen gamete carrying three different types (both intact and rearranged copy, only intact copy and only rearranged copy) had an equal likelihood of developing into DH lines. Further analysis showed that this additional rearranged fragment was tightly linked with the *hph* and *lcy* genes, which were situated in a different T-DNA. Such rearrangement in T-DNA integration has been reported previously in rice, and could possibly be due to a short and truncated T-DNA generated during or after integration of the full-length T-DNA into the genome (Datta *et al*., 2000).

The amenability of T-DNAs to segregate independently is determined by their relative site of integration in the plant genome. The integration of the two T-DNAs in two different, genetically distant, unlinked loci resulted in the isolation of backcross progenies without the locus that harboured the *hph* marker gene. The type of *Agrobacterium* strain used for the introduction of the T-DNA into the plant genome influences its organization (De Block and Debrouwer, 1991). The octopine-derived strains (LBA4404) used in the initial development of japonica golden rice (Ye *et al*., 2000) might have favoured the integration of multiple T-DNAs at unlinked loci, relative to the nopaline-derived *Agrobacterium* strains that usually favour insertion at genetically linked loci. We isolated MFT golden rice in the first backcross progenies, and subsequent backcrossing was performed until BC_2, with theoretically 87.5% introgression from IR64 used as the recurrent recipient parent. The fingerprinting results with 12 URP RAPD primers that are known to generate maximum polymorphism amongst rice cultivars (Kang *et al*., 2001) matched the phenotypic characteristics of the introgressed NILs *vis-à-vis* the recurrent IR64C. As has been observed in our earlier studies (Datta *et al*., 2003) and those of others (Ye *et al*., 2000; Hoa *et al*., 2003), and in the IR64 NIILs, the ability of the transgenics to accumulate β-carotene with only the *psy* and *crtI* genes can be attributed to a feedback signalling loop leading to the activation of the rice endogenous carotenoid biosynthesis gene through the delivery of all-*trans*-configured lycopene by bacterial desaturase (CRTI), compared with the delivery of poly-*cis*-lycopene by two plant desaturases (Schaub *et al*., 2005). Moreover, the IR64 NIILs accumulated more carotenoid (0.941 μg/g dry mass) than IR64 golden rice (developed earlier with *pmi* as the selectable marker gene) (0.670 μg/g dry mass, Datta *et al*., 2003; 0.80 μg/g dry mass, Hoa *et al*., 2003).

Transgenesis, coupled with conventional cross-breeding, could make use of the transgenes as the markers for an interrupted backcrossing, thereby reducing the time required in a conventional process up to at least BC_6 or BC_7 to obtain NIILs. Conventional crossing involving a transgene(s)-based marker-aided selection strategy has been followed to combine multiple transgene(s) for disease and insect resistance (Datta *et al*., 2002). A similar type of approach for the isolation of MFT potato and the subsequent transfer of this trait into a desirable cultivar through backcrossing was advocated in Matthews *et al*. (2001).

In rice, a few reports have documented the development of transgenic plants free from selectable marker genes, such

as Komari *et al*. (1996) with the use of the two-T-DNA system and two-strain approach and Lu *et al*. (2001) using a double right-border binary vector approach. Recently, marker-free golden rice has also been reported by the use of two binary vectors in the same *Agrobacterium* strain (Parkhi *et al*., 2005).

Selectable marker-free *Bt* transgenics have also been recovered through the co-transformation of a vector with the *Bt* expression cassette and a vector carrying the *hph* cassette via the biolistic transformation method (Tu *et al*., 2003).

Our study indicates that the isolation of segregants without a marker gene is feasible through the use of different vectors in a co-transformation experiment by either *Agrobacterium* (in this case) or biolistic (as mentioned above) transformation. A detailed molecular analysis of the primary transformants and the immediate segregating population, and the screening of a large number of segregants, are the key to isolating such an event without the marker gene.

The agronomic performance and stability of the β-carotene content of these 'clean' transgenic golden IR64 NIILs, free from a plant selectable marker gene and bacterial selection antibiotic resistance gene, have been evaluated in the screen house and in field conditions, and showed no significant difference between the transgenics (NIILs and the original T-309 golden rice) and their respective non-transgenic controls (M. Rai *et al*., unpublished results). These final clean products would represent no threat to open field conditions or human consumption, and thus could safely be transferred to national programmes for further breeding or for direct use with deregulation.

Experimental procedures

Plant materials and development of DHs

The transgenic seeds of T309 plants transformed with two different plasmids, pZPsC carrying *psy* and *crtI* genes, and pZLycH carrying *lcy* and *hph* genes, each harboured in a separate *Agrobacterium tumefaciens* strain LBA4404 (Ye *et al*., 2000), were provided by Professor Ingo Potrykus, ETH, Zurich, Switzerland, under the golden rice network programme. The segregating population of golden rice (T309) plants was grown in the containment glasshouse facilities of the International Rice Research Institute (IRRI), Philippines, and was screened at the seedling stage for the presence of the transgenes, including *hph*, by PCR (as described below). The immature panicles from the PCR-positive golden rice plants at the booting stage were used for anther culture (Baisakh *et al*., 2001). The green plantlets were transferred to the containment glasshouse and were identified for their ploidy levels by phenotypic markers.

PCR and Southern blot analysis

The PCR primers and conditions were essentially the same as described previously (Baisakh *et al*., 2001; Datta *et al*., 2003). The DNA was isolated from leaves, 2–3 cm in length, collected from 1-month-old plants growing in the containment glasshouse by a microprep method, and 50–100 ng of DNA was used as a template for PCR. The primer sequences specific to the transgenes used were as follows: *psy* F, 5′-tggtggttgcgatattacga-3′; *psy* R, 5′-accttcccagtgaacacgtc-3′; *crtI* F, 5′-ggtcgggcttatgtctacga-3′; *crtI* R, 5′-atacggtcgcg-tagttttgg-3′; *lcy* F, 5′-ccaatccccagaaccctaat-3′; *lcy* R, 5′-ctcgctaccatgtaacccgt-3′; *hph* F, 5′-tacttctacacagccatc-3′; *hph* R, 5′-tatgtcctgcgggtaaat-3′.

PCR was performed under initial denaturation for 5 min at 94 °C, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 1 min and final extension at 72 °C for 5 min. The PCR products were resolved in 1% TAE-agarose gel and the view was captured in an Alpha Innotech Imager (San Leandro, CA).

For Southern blot analysis, total genomic DNA was isolated from freshly collected leaves of the PCR-positive plants following the modified procedures of Dellaporta *et al*. (1983). Ten micrograms of DNA were digested overnight with restriction enzymes corresponding to the transgenes (see figures for details), run in 1% TAE-agarose gel, transferred to nitrocellulose membrane, hybridized, washed and exposed to X-ray film according to Datta *et al*. (2000). The (α^{32}P)-dCTP-radiolabelled PCR-generated fragments of the genes (0.957 kb for *psy*, 1.079 kb for *crtI*, 1.033 kb for *lcy* and 0.763 kb for *hph*) were used as hybridization probes.

PCR analysis for beyond border transfer

The transformation vector pZPsC that contained both *psy* and *crtI* expression cassettes in the T-DNA carried an antibiotic resistance gene (*cat*) for multiplication of *Agrobacterium* in chloramphenicol selection. To check the transfer of fragments beyond the left border of T-DNA (T_L), PCR was performed with the following *cat*-specific primers: *cat* F, 5′-atcccaatggcatcgtaaag-3′; *cat* R, 5′-ccttgtcgccttgcgtataa-3′.

PCR was performed under denaturation at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min, and a final elongation at 72 °C for 5 min. For the fragment

outside right border, the PCR primers and conditions were the same as described previously (Hoa *et al*., 2003).

Development of F_1 and IR64 NIILs

Of the many F_1 seeds obtained from the cross between IR64 and the DH T-309 transgenic golden rice (KDHR3-11), 100 seeds were germinated in agar medium in Petri dishes and 1-week-old seedlings were transferred to pots in the containment glasshouse. The F_1 plants were screened for the presence of transgenes, and a few selected plants were used as pollen parents (donor) in backcross breeding with IR64 as a female recurrent parent. The BC_1F_1 plants were screened for transgenes, including the *hph* gene. Seed progenies were grown to maturity in the containment glasshouse, and the process was repeated for two backcross generations with transgene-based screening. A selfing generation was allowed for the progenies from BC_2F_1 with morphological proximity to IR64, resulting in BC_2F_2 plants. The materials are now in the BC_3F_2 generation and are being used for further pyramiding of the transgene (*ferritin*) for high iron storage in the endosperm.

Quantification of β-carotene and HPLC

The extraction of total carotenoids was performed from 0.5 g of seeds from the BC_2F_2 progenies, and the extract was subjected to further HPLC analysis as described by Tan *et al*. (2005).

Spectrophotometer absorbance was measured at 450 nm. The total carotenoid (μg/g) was calculated from the spectrophotometer reading, taking the dilution and the factor 1.125 into account. The amount of β-carotene was estimated by integration of its peak with respect to the total area of carotenoids.

Comparative genetic fingerprinting of the introgression transgenic lines and parental lines

The DNA fingerprinting of the BC_2F_2 progenies, including the donor KDHR3-11 and IR64 (recurrent) parental lines, was performed with 12 URPs of the SRILS Uniprimer™ kit I (SLB, Seoul, South Korea) following the manufacturer's instructions. The PCR reaction mixture consisted of 150 ng of primer, 200 μM dNTPs mix, 1 × PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 15 mM $MgCl_2$), 2.5 units of Taq DNA polymerase and 100 ng of genomic template DNA in a final volume of 30 μL. PCR was performed in programmed thermocycler (MJ Research, Wiltham, MA) under the following conditions: 4 min at 94 °C, 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final step of 72 °C for 7 min. The PCR products were resolved in 1.2% TAE-agarose gel and viewed under a UV transilluminator.

All the unique bands generated using 12 URPs for all the samples studied were scored as present (1) or absent (0). Genetic similarity between the samples was calculated using the program 'Similarity for Qualitative Data' (SIMQUAL), with NTSYSPC. Similarity coefficients were estimated using DICE and dendrograms were generated using the SHAN subroutine of NTSYSPC version 2.1.1L.

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