Indian Journal of Agricultural Sciences **80** (11): 939–46, November 2010

Inheritance and mapping of transgene in a transgenic ‘Golden Rice’ line of *japonica* rice (*Oryza sativa*) variety ‘Taipei 309’

N K TYAGI[1], B RAMESH[2], T MOHAPATRA[3], K V PRABHU[4] and A K SINGH[5]

Indian Agricultural Research Institute, New Delhi, 110 012

Received: 8 November 2009 ; Revised accepted: 14 August 2010

ABSTRACT

Transgene inheritance is an important consideration for the development of commercially viable transgenic crops. The F_1, F_2 and BC_1F_1 to BC_3F_2 populations of the cross ‘Taipei 309’ × ‘Swarna’ were used to analyze the inheritance of carotenogenic transgene(s) *psy+crtI*. Segregation distortion was observed for the transgene in F_2 population. However, in BC_1F_1 and subsequent backcrosses and their selfed generations, the segregation of transgene positive and transgene negative plants occurred in an expected 1:1 and 3:1 ratio, respectively. In introgression breeding one can often encounter linkage drag due to linkage of some undesirable genetic loci to the gene under transfer thereby affecting the phenotype of the final converted lines. Therefore, mapping and identification of flanking molecular marker linked to the target gene is necessary for elimination of linkage drag. The F_2 population from the ‘Taipei 309’ × ‘Swarna’ cross was used for tagging and mapping of the carotenogenic genes in transgenic ‘Taipei 309’ with 48 polymorphic STMS markers spanning over 12 rice chromosomes for Bulked Segregant Analysis (BSA). The transgene was mapped to the chromosome 4 in the vicinity of the markers RM252, RM3785 and RM7563 at a distance of 10.65 cM, 8.19 cM and 5.73 cM, respectively. The information generated will be useful in integrating transgenic lines into breeding programme.

Key words: BSA, ß-carotene, Golden Rice, Inheritance, Mapping, Microsatellites, Molecular marker, Segregation distortion, Transgene

Rice (*Oryza sativa* L.) is one of the low cost sources of the dietary energy and protein for a vast majority of rural and urban poor of Asian countries including India. Among the cereals, rice protein is considered to be of high biological value because of its high digestibility, relatively better net protein utilization and high lysine content. The rice grain, however, is low in protein quantity and also deficient in many of the micronutrients required for human growth. Vitamin A deficiency (VAD) is a more common nutritional deficiency, prevalent among rice consumers due to the lack of provitamin A carotenoids in the milled rice kernels (Burkhardt 1997). Vitamin A deficiency leads to various disorders and increased disease susceptibility. One of the earliest symptoms of Vitamin A deficiency is night blindness. It is estimated that 2 to 5 million vitamin A-deficient children become blind every year (Al-babli and Bayer 2005). Dietary diversity and supplementation with vitamins and minerals might be the best way to tackle malnutrition but they are impractical in developing countries due to lack of infrastructure and poor economic conditions of the target masses. An alternative approach to tackle the problem is biofortification of rice. Genetic fortification can be achieved either by afresh transformation of *indica* rice varieties using the gene construct or by hybridizing transgenic rice line carrying provitamin A with widely adapted popular rice varieties. The choice of rice varieties for hybridization approach is not limited though it could be a limiting factor in transformation approach.

As a first proof of concept, the prototype of ‘Golden Rice’ was developed in the genetic background of *japonica* rice line ‘Taipei 309’ with the introduction of carotenogenic pathway in the rice endosperm through genetic engineering. Subsequently, to produce ‘Golden Rice’ suitable to deregulation several transformation events were developed.

---

*Based on a part of Ph D thesis of the first author submitted to CCS University, Meerut during 2007

[1] Scientist (e mail: vats30@rediffmail.com), Basmati Export Development Foundation, Modipuram, Meerut, Uttar Pradesh 250 110;

[2] Professor and Head (bandarupalli.ramesh@gmail.com), Department of Genetics and Plant Breeding, CCS University, Meerut, Uttar Pradesh 200 005;

[3] Principal Scientist (tm@nrcpb.org), National Research Centre on Plant Biotechnology, Pusa, New Delhi 110 012;

[4] Head (kvinodprabhu@rediffmail.com), [5] Senior Scientist, (aks_gene@yahoo.com), Division of Genetics

In the public sector, these events were developed using the vector pCaCar, in the genetic background of ‘Taipei 309’ (*japonica*) and ‘IR64’ (*indica*) rice varieties (Datta *et al*. 2003, Hoa *et al*. 2003). In parallel, Syngenta scientists produced many transformation events (GR1) using *javanica* cultivar ‘Cocodrie’ with a modified vector in which *crtI* was under endosperm-specific promoter control in place of *CaMv35S* promoter used by Hoa *et al.* (2003). Transgenic event in the background of ‘Taipei 309’ developed by Hoa *et al.* (2003) has been used in the present study. GR1 events show significantly higher carotenoids content (6 µg/g), as compared to total carotenoid level of 1.2 µg/g of rice endosperm in transgenic ‘Taipei 309’. Further Syngenta scientists focused on optimizing the expression of *psy* gene from different plants, which led to the development of new version of ‘Golden Rice’ (GR2) in the background of American long grain rice variety Kaybonnet sourcing *psy* gene from maize (Paine *et al*. 2005).

It is very important to understand the inheritance, segregation and stability of exogenous genes for a given transgenic event. Knowledge of the inheritance and segregation pattern of the transgenes would provide better opportunity to develop transgenic germplasm, strategies to maximize the efficiency of developing improved germplasm, and the ability to breed new varieties carrying these foreign genes. Recent advances in the construction of saturated linkage maps in rice have made it possible to map and tag genes of economic importance with molecular markers including genes for resistance to blast (Liu *et al.* 2005 and Fjellstrom *et al.* 2006), bacterial blight (Yang *et al.* 2003) and for brown plant hopper (Jairin *et al.* 2007). The ‘Golden Rice’ line used as donor in the present study carries 2 transgenes, *psy* and *crtI*, sourced from daffodil and the bacterium *Erwinia uredovora*, respectively, in a single construct. The chromosomal location of the transgene was not known in the donor lines. Further, the stable and Mendelian behaviour of the transgene is prerequisite for the deregulation of transgenic events. Therefore, the present study was undertaken to obtain information on the inheritance of transgene in different crossed and backcross populations and to map the chromosomal location of the transgene so that markers flanking the transgne can be effectively utilized in the elimination of linkage drag, if any, on carrier chromosome. For this purpose, STMS markers were used for bulk segregant analysis (BSA) (Michelmore *et al.* 1991) because of their abundance, co-dominant nature, and known chromosomal location (McCouch *et al*. 2002).

MATERIALS AND METHODS

A total of 9 transgenic lines of ‘Taipei 309’ containing the *psy* and *crtI* genes developed by Hoa *et al.* (2003) were obtained from Dr Hoa, Cuu Long Delta Rice Research Institute, Omon, Cantho,Vietnam under Indo-Swiss collaboration on ‘Golden Rice’ sponsored by Department of Biotechnology, Government of India. Among them, line number 48-67-4-9 CR: 58 contained the highest amount of beta carotene (1.2 µg/g) and used in the present study. These lines were developed through *Agrobacterium*-mediated transformation of the ‘Taipei 309’ *Japonica* rice with the binary vector pCaCar carrying the genes coding for the carotenogenic enzymes *phytoene synthase* (*psy*) and *phytoene desaturase* (*crtI*). The genes were sourced from daffodil (*Narcissus pseudonarcissus*) and the bacterium *E. uredovora*, respectively. The *psy* gene is driven by an endosperm-specific promoter *Gt1* while the *crtI* is under the control of constitutive promoter *CaMv35S*. The T-DNA construct also carries the selectable marker gene *pmi* constitutively expressed through *CaMv35S* promoter. ‘Swarna’ (MTU 7029), a widely-grown, popular *indica* rice cultivar with superior agronomic performance was used as recurrent parent for the transfer of provitamin A trait. Seeds of parental lines were surface sterilized and sown in plastic pots (30 cm×30 cm) containing sterilized artificial medium (agropeat + vermicompost in the ratio of 3:1) in the glass house at National Phytotron Facility (NPF), Indian Agricultural Research Institute (IARI), New Delhi, for multiplication of seeds of parental lines and for making crosses. The temperature in the glass house was maintained at of 30–25°C day-night regime.

For producing F_1 seeds, crosses were made using transgenic ‘Golden Rice’ line 48-67-4-9 CR: 58 as female and ‘Swarna’ as male parent. The F_1 (‘Taipei 309’×‘Swarna’) were raised at NPF, IARI under containment. The F_1 plants were tested for the presence of transgene using transgene-specific primers and for hybridity by using random STMS markers. The F_1 plants were simultaneously selfed and backcrossed to the recurrent parent, ‘Swarna’ to produce the BC_1F_1 population. F_2 population was used for mapping the transgene. Transgene inheritance was also studied at BC_3F_2 populations obtained from single selfed transgenic positive BC_3F_1 plants produced using ‘Swarna’ as recurrent parent.

Total genomic DNA was isolated from powdered leaf tissue from each sample by micro extraction method described by Prabhu *et al.* (1998). The DNA was quantified by analyzing it on 0.8% agarose gel with diluted λ DNA as standard. The *psy* primers described by Hoa *et al.* (2003) were used for transgenic analysis. The primer pairs were synthesized from Operon Technology Inc. USA. The PCR reactions were carried out using sterile 96 well PCR plates obtained from Axygen Scientific Inc. Union City, CA, USA. The master mix consisted of 25 ng of genomic DNA, 0.2 U of Taq DNA polymerase, 1X PCR assay buffer without $MgCl_2$, 1.75 mM $MgCl_2$, 12 ng each of forward and reverse primers and 200 µM of dNTP mix. The reaction volume was made up to 10 µl using sterile double distilled water. The entire exercise was carried out over ice and the PCR plate was immediately loaded in the thermal cycler (PT-200 MJ Research) to prevent the amplification of non-specific products. The PCR reaction process was as follows: 94°C pre-denaturation for 5 min., 94°C denaturation for 1 min., annealing at 58°C for *psy* and 55°C for microsatellite markers for 1 min., and primer extension at 72°C for 2 min. for 35 cycles of polymerization and final primer extension at 72°C for 7 min. The amplification product was preserved in the refrigerator at 4°C. The amplified PCR products using gene-specific primers were resolved on 2% agarose gel in 1× TAE buffer and the amplified PCR products using microsatellite markers were resolved on 3% Metaphor^{TM} agarose (Cambrex Bioscience, Rockland Inc. USA) using 1× TBE buffer. The gel was run for at least 2 hr for agarose and 4 hr for metaphor at 70 volts using power pack 1000 (BIO-RAD, USA). The gel was stained in ethidium bromide solution (1 ug/ml) for about 30 min. and visualized under UV transilluminator in a gel documentation system (Alpha Innotech Corporation, USA).

A F_2 mapping population of 244 individuals was generated by crossing 'Swarna' with transgenic 'Golden Rice' line 48-67-4-9 CR: 58 available in the genetic background of rice variety 'Taipei 309'. BSA was carried out following the procedure described by Michelmore *et al.* (1991). Transgenic positive and negative plants were identified in F_2 population through PCR amplification of the *psy* gene and equal amount of DNA (10 µl) from the 10 individual plants in each group were used for constituting transgene positive and transgene negative F_2 bulks. A set of 294 STMS markers covering the entire 12 rice chromosomes were screened for polymorphism between 'Swarna' and transgenic 'Taipei 309'. The bulks were then screened with the polymorphic STMS markers (Table 1) identified through parental polymorphism survey. Parents including transgene donor 'Taipei 309' and recurrent parent 'Swarna' and F_1 and transgene positive and transgene negative F_2 bulks were screened with polymorphic STMS markers to identify the putative marker linked to the transgene. The STMS primer sequences were obtained from the publications of the Temnykh *et al.* (2000), McCouch *et al.* (2002) and the Gramene SSR marker resource data base (www.gramene.com).

The microsatellite markers that distinguish parents and bulks were considered putatively linked to the transgene. The putative microsatellite markers were used to analyze the individual plants constituting each bulk and further tested on the remaining F_2 individuals. The transgene positive individuals showing homozygosity of recurrent parent-specific allele of the putative marker locus were considered recombinants. Similarly, the transgene negative F_2 individuals showing homozygosity for donor parent allele or heterozygosity at putative linked marker locus were also considered as recombinants. Linkage analysis was done using the recombination frequency between marker and transgene at each locus. The goodness of fit of the observed segregation ratio for the transgene was tested for the pattern expected for a single dominant gene using classical chi-square (χ^2) test.

Table 1 Chromosome-wise list of polymorphic markers identified

Ch.no.	Polymorphic markers	Product size (bp)	
		'Taipei 309'	'Swarna'
1	RM129	220	190
	RM259	180	170
	RM572	170	200
	RM1282	165	185
	RM5336	125	115
2	RM16	190	200
	RM145	200	180
	RM525	125	100
	RM3762	115	90
	RM5460	190	175
3	RM489	260	240
	RM565	190	170
	RM1038	170	185
	RM1284	160	175
	RM5474	175	150
4	RM252	200	225
	RM273	200	210
	RM3785	140	150
	RM7563	147	140
5	RM274	160	175
	RM334	170	200
	RM440	175	210
	RM1054	175	160
6	RM253	135	125
	RM276	140	110
	RM494	200	175
7	RM214	190	120
	RM234	150	170
	RM505	210	185
8	RM44	110	80
	RM152	160	140
	RM556	100	90
	RM1235	100	110
9	RM201	150	170
	RM257	175	150
	RM288	125	130
	RM444	170	180
10	RM184	225	215
	RM216	130	125
	RM258	140	170
	RM271	100	120
11	RM206	200	160
	RM286	125	100
	RM1812	150	120
12	RM17	175	200
	RM313	120	125
	RM277	125	135
	RM3331	140	120

RESULTS AND DISCUSSION

Inheritance of transgene (psy+crtI)

Inheritance of transgene was studied in F_2, BC_1F_1, BC_2F_1, BC_2F_2, BC_3F_1 and BC_3F_2 generations of the cross 'Taipei 309' × 'Swarna' using 'Swarna' as recurrent parent. Data on transgene segregation along with p value for expected segregation ratio of 1 : 1 in backcross and 3 : 1 in F_2 and BC_nF_2 generations are presented in Table 2. A representative gel picture showing amplification profile of transgene positive and negative individuals in F_2 population using *psy* primers is presented in Fig 1. The goodness of fit for the observed number of transgene positive and negative plants was tested against the expected segregation ratio for a single gene using χ^2 test. Out of 244 F_2 plants, 212 were transgenic positive while 32 were transgenic negative. The observed ratio deviated significantly from expected ratio of 3 : 1. The results obtained clearly indicated the occurrence of meiotic drive or segregation distortion in the population in favour of the individuals carrying the transgene. However, in the BC_1F_1 population comprising of 115 plants, tested for the presence of transgene using *psy* and reaction A primers, only 61 plants were found transgenic positive, whereas the rest 54 plants were transgene negative. In this case the data showed a good fit to the expected 1 : 1 ratio. Similarly, 100 BC_2F_{1-67} individuals segregated in 52 transgene positive and 48 transgene negative, showing good fit to 1:1 ratio. In BC_3F_1, out of 73 plants analyzed, 42 were transgene positive and 31 were transgene negative, the data fitted to the expected 1 : 1 ratio with a p value of ≥ 0.30. Interestingly, in BC_2F_2 and BC_3F_2 generations, unlike F_2, no segregation distortion was observed for the transgene segregation, the data fitted well to the expected 3 : 1 ratio (Table 2). Based on comparison of F_2 and backcross generations with respect to transgene segregation, it was apparent that transgene segregation was influenced by the transmission of gametes from male parent. The recovery of recurrent parent genome during backcrossing also seems to have profound influence on normalization of transgene segregation behaviour.

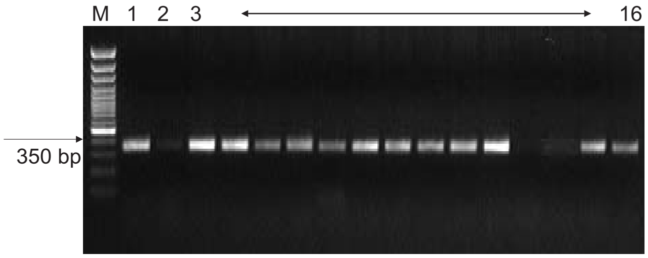

Fig 1 Segregation pattern of F_2 plants for the transgene-specific marker *psy*
M=100 bp ladder, 1= 'Taipei 309', 2='Swarna', 3–16=F_2 plants

Transgene loci introduced into higher plant species frequently display unpredictable pattern of inheritance and expression (Bregitzer and Tonks 2003). Transgene instabilities either of inheritance or expression complicate the identification, selection and use of transgenic lines. Therefore, the information regarding the inheritance pattern of the transgene in different genetic background is essential to ensure the stability of the transgene in the final product. Elaborate attempts were made in the present study to establish the inheritance pattern of the transgene of 'Golden Rice' line 'Taipei 309' using F_2 and backcross generations. The results obtained clearly indicate high degree of segregation distortion in favour of the transgene positive plants in F_2 generation (Table 2). However, interestingly, in BC_1F_1 produced using 'Swarna' as male parent, segregation of transgene positive and transgene negative plants occurred in an expected 1 : 1 ratio. Comparison of inheritance pattern in F_2 vis-à-vis BC_1F_1 clearly indicated that the transgene positive and negative gametes are transmitted in equal frequency from female side, while transgene positive gametes are transmitted in higher frequency from male side. Since in BC_1F_1, when 'Swarna' was used as male parent, there is no gametic competition as all gametes are transgene negative. If transgene positive and negative gametes were transmitted in unequal proportion from female side in BC_1F_1, the expected segregation ratio of 1 : 1 could not have been realized. While in F_2, being selfed generation, both female and male sides carry hemizygosity for transgene and in such a case, transmission of transgene positive gametes from male side in higher frequency result in segregation distortion. The

Table 2 Transgene segregation pattern in selfed and backcross generations of the cross 'Taipei 309' × 'Swarna'

Generation	Total no. of plants	Transgene positive (+)	Transgene negative (–)	Expected segregation ratio	χ^2 value	P value
F_2	244	212	32	3 : 1	18.37	<0.001
BC_1F_1	115	61	54	1 : 1	0.42	>0.50
BC_2F_{1-67*}	100	52	48	1 : 1	0.16	>0.50
BC_2F_{1-71*}	48	28	20	1 : 1	1.32	>0.30
BC_2F_2	138	107	31	3 : 1	0.46	>0.50
BC_3F_1	73	42	31	1 : 1	1.64	>0.30
BC_3F_2	125	96	29	3 : 1	0.21	>0.50

*Indicate the BC_2F_1 plants originating from plants BC_1F_{1-67} and BC_1F_{1-71}

Table 3 Nucleotides sequence of the polymorphic microsatellite markers linked to the transgene in the ‘Golden Rice’ line ‘Taipei 309’

Microsatellite marker	Primer sequence	Fragment size (bp)	
		‘Taipei 309’	‘Swarna’
RM252	F TTCGCTGACGTGATAGGTTG R ATGACTTGATCCCGAGAACG	200	225
RM3785	F ACCTTTTCTTGGCTTGAGGG R GCTTTTGCTACTTTTGGGGG	125	140
RM7563	F CCACCGCTCGTAGAAAAAAC R GGGTTGAGATGCCTGTGC	147	140

F, Forward primer; R, reverse primer

F_1 pollen grains, when checked for fertility through acetocarmine staining, were found to be uniformly fertile indicating that pollen sterility is not involved in differential transmission of transgene.

In rice, segregation distortion for native gene(s) owing to location of gene(s) in the genomic region that is prone to segregation distortion, gamete sterility governed by the sterility locus (*S* locus) as in *indica* × *japonica* crosses (Schimenti 2000, vanBoven and Weissing 2001) and the differential viability of the cross (hybrid) seeds or seedlings and gametophytic selection genes that select male gametes during fertilization (Matsushita *et al*. 2003) have been reported. There are several reports of non-Mendelian segregation of transgene (s) in other crops. In case of cotton, segregation distortion for exogenous *Bt* gene was reported in F_2 populations derived from transgenic regenerative plants and conventional cotton lines (Sachs *et al*. 1998). Poor transmission of integrated transgene to progeny in some transgenic lines had been observed in maize (Register *et al*. 1994) and in barley (Cho *et al.* 1999). However, in the present study, the observed segregation distortion was due to the excess of transgene positive plants. Similar observations were reported for the predominance of the transgenic plants in the segregating generations in barley for herbicide resistance (bar) and barley yellow dwarf virus coat protein (BYDVCp) genes (Bergitzer and Tonks 2003).

A hybrid sterility gene locus S9 is reported linked to RM 5586 at 19.71 Mb position on chromosome 4, whereas the transgene mapped in the present study has been found linked with RM7563 located at a physical position of 22 Mb. However, we did not observe any hybrid sterility indicating that S9 locus may not be important in this case. Therefore, the segregation distortion for transgene observed in the present study may not be attributed to inter sub-specific hybrid sterility. Further, the inter sub-specific hybrid sterility loci may not be important in the present case because the parent ‘Swarna’ was developed using Mahasuri as a parent which in turn is a product of *indica/japonica* hybridization and it is possible that hybrid sterility gene loci might be fixed for same allele in both parents ‘Taipei 309’ and ‘Swarna’ and therefore, no inter sub-specific sterility.

Identification of microsatellite markers linked to transgene through bulked segregant analysis

A set of 48 STMS markers showing polymorphism between the parental lines ‘Taipei 309’ and ‘Swarna’ were used for identifying markers associated with the transgene through BSA. Ten transgenic positive and equal number of transgenic negative plants of F_2 population from the cross ‘Taipei 309’× ‘Swarna’ were used for constituting the transgene positive and negative bulks. BSA was performed using parents, F_1 and the two bulks with 48 polymorphic

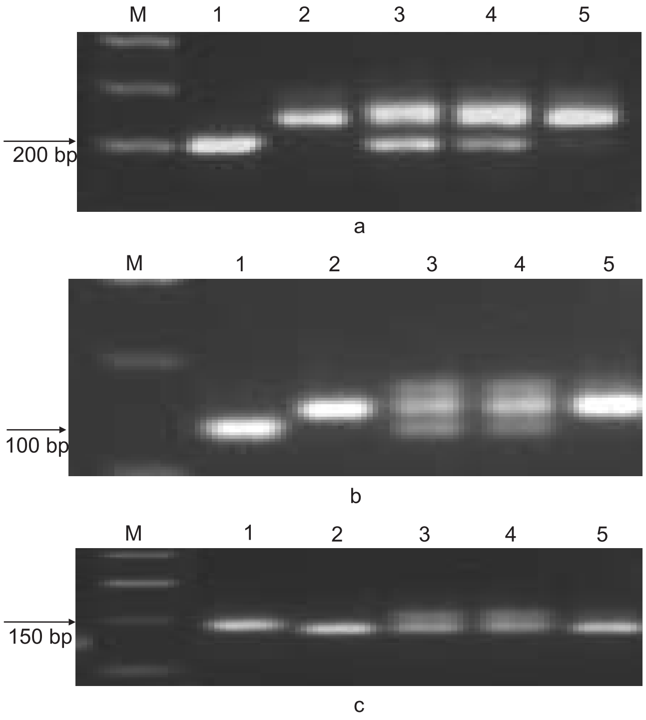

Fig 2 Identification of putative STMS markers associated with transgene through BSA: (*a*) RM252 (*b*) RM3785 and (*c*) RM7563 markers
M, 50 bp ladder; 1, ‘Taipei 309’; 2, ‘Swarna’; 3, F_1; 4, transgene positive bulk; 5, transgene negative bulk

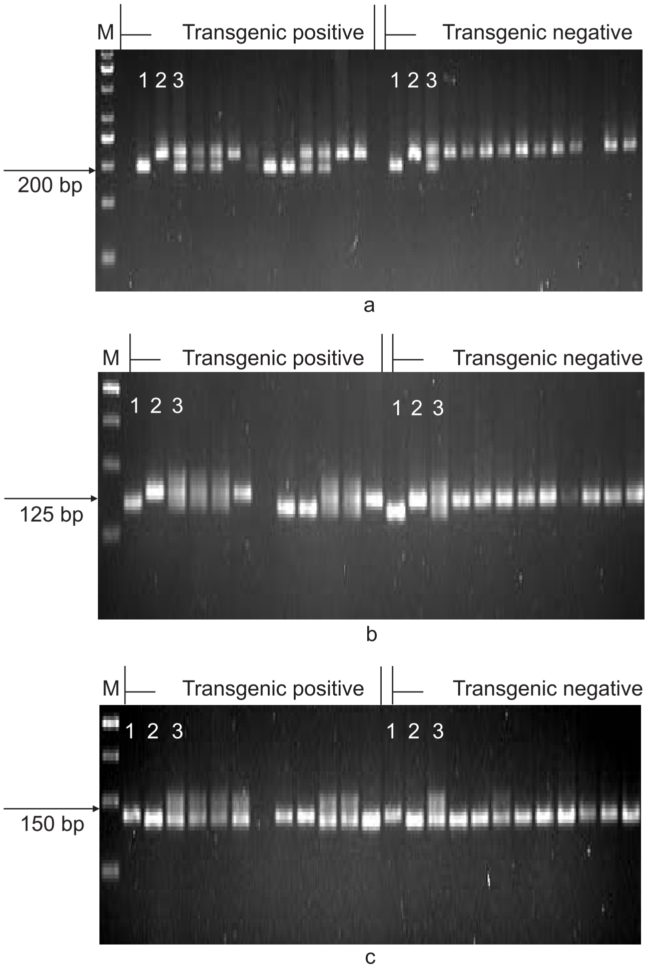

Fig 3 Screening of transgene positive and negative F_2 plants constituting bulk using putatively linked microsatellite markers - RM252 (*a*), RM3785 (*b*), and RM7563 (*c*)
M, 50 bp ladder; 1, 'Taipei 309'; 2, 'Swarna'; 3, F_1

markers providing whole genome coverage. In BSA, the marker RM252, which amplified a fragment of 200 bp and 225 bp in 'Taipei 309' and 'Swarna', respectively, showed clean polymorphism between bulks, showing heterozygous pattern in transgene positive bulk and homozygotes for 'Swarna' allele in transgene negative bulk. This marker (RM252) is located on the chromosome 4 at a physical distance of 24.86 Mb. Considering the physical location of the RM252, 14 other STMS primer pairs flanking both sides of RM252 covering a length of ~ 4 Mb were synthesized. Among these, RM3785 and RM7563 at physical distance of 23.74 Mb and 22.37 Mb, respectively, were found polymorphic between parents as well as the bulks. The primer sequences and fragment sizes amplified using these 3 polymorphic markers putatively linked to the transgene are presented in Table 3. RM3785 amplified a product of 125 bp in 'Taipei 309' and 140 bp in 'Swarna', while products of 147 bp and 140 bp were amplified in 'Taipei 309' and 'Swarna', respectively using RM7563. The gel pictures showing results of BSA using RM252, RM3785 and RM7563 primers/markers are given in Fig 2. These markers were further used to genotype the individual plants constituting the transgene positive and negative bulks (Fig 3). The pattern obtained clearly indicated the association of these markers with the transgene. Out of 10 plants analyzed in transgene positive bulk, 3 for RM252 and 2 each for RM3785 and RM7563 were recombinants, while in transgene negative bulks no recombination was obtained with any of the marker.

Mapping of transgene using informative markers

A total of 244 individual F_2 plants were used for genotyping with the 3 microsatellite markers, viz RM252, RM3785 and RM7563, which were identified as putatively linked to transgene based on BSA. The goodness of fit of segregation ratio for each marker locus was tested using χ^2 test against the expected genetic ratio of 1 : 2 : 1. The observed number of plants, χ^2 value and probability of goodness of fit for each marker is presented below.

Marker	No. of individuals			χ^2	P
	Homozygous for 'Taipei 309'	Heterozygous	Homozygous for 'Swarna'	value	value
RM252	66	133	45	5.58	0.05
RM3785	72	128	44	7.00	0.02
RM7563	78	128	38	9.76	0.01

It is evident from the results obtained that observed ratio did not show a good fit with expected ratio with respect to all the 3 markers, indicating segregation distortion, which limited the use of software like Mapmaker version 3 for linkage analysis. The co-segregation analysis of individual markers using the markers and transgene genotype of 244 individual F_2 plants was carried out with the help of recombination frequency between marker and transgene at each locus. Based on the data on recombination frequency, the marker loci RM252, RM3785 and RM7563 were mapped at distances of 10.65 cM, 8.19 cM and 5.73 cM, respectively from the transgene locus (Fig 4). The linkage analysis with the microsatellite marker RM252 identified 27 recombinants out of the 244 F_2 individuals tested. Similarly, RM3785 and RM7563 markers identified 20 and 14 recombinants out of the 244 F_2 individuals (Table 4).

The chromosomal location and mapping of transgene has some important applications in the marker assisted backcross-breeding programme. The first and foremost is the identification of suitable STMS markers flanking the transgene for elimination of the linkage drag on carrier chromosome. Because SSR markers are co-dominant, multi-allelic and available at a high density in the rice genome (one SSR for every 157 kb, McCouch *et al.* 2002), these markers can be used for identifying the target region associated with trait of interest. The availability of high

Table 4 Recombination frequency in F_2 individuals with 3 linked markers

Marker	Homozygous for 'Taipei 309' allele but transgene negative	Heterozygous but transgene negative	Homozygous for 'Swarna' allele but transgene positive	Total number of recombinants	Recombination frequency (%)
RM252	1	6	20	27	10.65
RM3785	1	5	14	20	8.19
RM7563	1	5	8	14	5.73

density molecular map of rice based on STMS markers greatly facilitated the mapping of transgene (ß-carotene) to a specific chromosome region. In the present study, transgene mapping was successfully carried out in the transgenic parent 'Taipei 309' using an F_2 mapping population through BSA approach. The data obtained using the STMS markers clearly demonstrated that the transgene was located on chromosome 4 and showed linkage with the marker RM252 (10.65 cM). Availability of the complete rice genome sequence data base (http://www.ncbi.nlm.nih.gov and http://www.gramene.org) facilitated selection of additional markers flanking RM252, which were used for narrowing down the gap further. Among the polymorphic markers used for BSA, RM3785 and RM7563 were found informative and mapped at a distance of 8.19 cM and 5.73 cM from the transgene, respectively. It has been found that the markers RM3785 and RM7563 are located on one side of transgene integration locus on chromosome 4. Although BSA was done using markers flanking the both sides of RM252 but no SSR markers were found flanking the gene integration site on the other side. BSA is generally employed for tagging the genes of interest. BSA approach for molecular tagging in transgene is much robust as the bulks are constituted based on presence or absence of transgene as indicated by gene-based markers as against constitution of bulks based on extreme phenotypes of the trait in most of the cases where tagging has been carried out using BSA. BSA has been employed by different workers for molecular tagging using different marker systems in previous studies in rice (Huang *et al*. 2001, Yang *et al*. 2002 and Chen *et al*. 2007) and barley (Salvo-Garrido *et al*. 2004). When mapping population is readily available, BSA approach for mapping transgene is simpler as compared to other molecular approaches used for transgene location such as inverse PCR, which would require amplification of outward region, cloning, sequencing and BLAST search as used by Paine *et al*. (2005). However, the genetic distance between transgene and markers obtained in the present study may not be a true reflection of actual genetic distances because of the segregation distortion observed for transgene. Under such a situation, inverse PCR approach would reflect actual physical position of the transgene and facilitate selection of polymorphic STMS markers closely located in the flanking region of transgene, which could be effectively used for elimination of linkage drag.

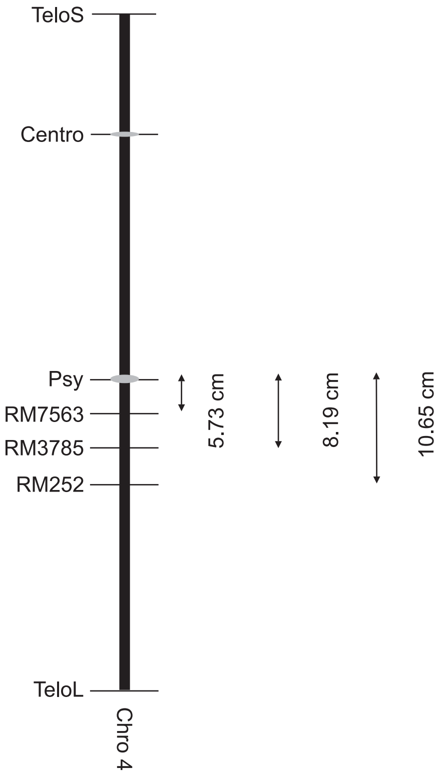

Fig 4 Linkage map showing the genetic distance of linked markers in relation to the transgene (*psy+crtI*)

ACKNOWLEDGEMENT

The authors are grateful to the Department of Biotechnology, Government of India for the financial support to the project entitled 'Development of pro-vitamin A rich *indica* rice lines through marker assisted backcross breeding', under which the present study was carried out.

REFERENCES

Al-Babili S and Beyer P. 2005. Five years on the road-five years to go? *Trends in Plant Science* **10**(12): 555–73.

Bregitzer P and Tonks D. 2003. Inheritance and expression of transgenes in barley. *Crop Science* **43**: 4–12.

Burkhardt P, Beyer P, Wonn J, Kloti A, Armstrong G A, Schledge M, Vonlinting J and Potrykus I. 1997. Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of pro-vitamin A biosynthesis. *The Plant*

Journal **44** (5): 1071–8.

Chen Q H, Wang Y C, Li A N, Zhang Z G and Zheng X B. 2007. Molecular mapping of two cultivar-specific avirulence genes in the rice blast fungus *Magnaporthe grisea*. *Molecular Genetics and Genomics* **277**: 139–48.

Cho M J, Choi H W, Buchanan B B and Lemaux P G. 1999. Inheritance of tissue-specific expression of barley hordein promoter uidA fusions in transgenic barley plants. *Theoretical and Applied Genetics* **98**: 1253–62.

Datta K, Baisakh N, Oliva N, Torrizo L, Abrigo E, Tan J, Rai M, Rehana S, Al-Babili S, Beyer P, Potrykus I and Datta S K. 2003. Bioengineered golden *indica* rice cultivars with ß-carotene metabolism in the endosperm with hygromycin and mannose selection systems. *Plant Biotechnology Journal* **11**: 81–90.

Fjellstrom R, McClung A M and Shank A R. 2006. SSR markers closely linked to the *Pi-Z* locus are useful for selection of blast resistance in a broad array of rice germplasm. *Molecular Breeding* **17**: 149–57.

Hoa T T C, Al-Babili S, Schaub P, Potrykus I and Beyer P. 2003. Golden *indica* and *japonica* rice lines amenable to deregulation. *Plant Physiology* **133**: 161–9.

Huang Z, Shu L, Lix and Zhang Q. 2001. Identification and mapping of two brown planthopper resistance genes in rice. *Theoretical and Applied Genetics* **102**: 929–34.

Jairin J, Phengrat K, Teangdeerith S, Vanavichit A and Toojinda T. 2007. Mapping of a broad-spectrum brown planthopper resistance gene, *Bph3*, on rice chromosome 6. *Molecular Breeding* **19**: 35–44.

Liu X Q, Wang L, Chen S, Lin F and Pan Q H. 2005. Genetic and physical mapping of *Pi36 (t)*, a novel rice blast resistance gene located on chromosome 8. *Molecular Genetics and Genomics* **274** (4): 394–401.

Matsushita S, Takashi I, Fukuta Y, Agaki E, Kobayashi S, Osaki M and Yamagishi M. 2003. Characterization of segregation distortion on chromosome 3 induced in wide hybridization between indica and *japonica* type rice varieties. *Euphytica* **134**: 27–32.

McCouch S R, Teytelman L, Xu Y, Lobos K B, Clare K, Walton M, Fu B, Maghirang R, Li Z, Zing Y, Zhang Q, Kono I, Yano M, Fjellstrom R, Declerck G, Schneider D, Cartinhour S, Ware D and Stein L. 2002. Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.) *DNA Research* **9**: 199–207.

Michelmore R W, Paran I and Kesseli R V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Sciences*, USA **88**: 9828–32.

Paine J A, Shipton C A, Chaggar S, Howells R M, Kennedy M J, Vernon G, Wright S Y, Hinchliffe E, Adams J L, Silverstone A L and Drake R. 2005. Improving the nutritional value of golden rice through increased pro-vitamin A content. *Nature Biotechnology* **23**(4): 482–7.

Prabhu K V, Somers D T, Rakow G and Gugel R K. 1998. Molecular markers linked to white rust reisstance in mustard *Brassica juncia*. *Theoretical and Applied Genetics* **97**: 865–70.

Register J C, Peterson D J, Bell P J, Paul Bullock W, Evans I J, Frame B, Greenland I J, Higgs N S, Jepson I, Jiao S, Lewnau C J, Sillick J M and Wilson H M. 1994. Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. *Plant Molecular Biology* **25**: 951–61.

Sachs E S, Benedict J H, Stelly D M, Taylor J F, Altman D W, Berberich S A and Davis S K. 1998. Expression and segregation of genes encoding *Cry1Ac* insecticidal proteins in cotton. *Crop Science* **38**: 1–11.

Salov-Garrido H, Travella S, Bilham L J, Harwood W A and Snape J W. 2004. The distribution of transgene insertion sites in barley determined by physical and genetic mapping. *Genetics* **167**: 1317–79.

Schimenti J. 2000. Segregation distortion of mouse haplotypes: the molecular basis emerges. *Trends in Genetics* **16**: 240–43.

Temnykh S, Park W D, Ayres N, Cartinhour S, Hauch N, Lipovich Y G, Ishii T and McCouch S R. 2000. Mapping and genome organisation of microsatellite sequencs in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics* **100**: 697–712.

Van Boven M and Welssing F J. 2001. Competition at the mouse t complex: rare alleles are inherently favoured. *Theoretical Population Biology* **60**: 343–58.

Yang H, Xiang R, Weng Q, Zhu L and Guangcum H. 2002. Molecular mapping and genetic analysis of a rice brown planthopper (*Nilaparvata lugens* Stal) resistance gene. *Hereditas* **136**: 39–43.

Yang Z, Sun X, Wang S and Zhang Q. 2003. Genetic and physical mapping of a new gene for bacterial blight resistance in rice. *Theoretical and Applied Genetics* **106**: 1467–72.