DEVELOPMENT AND CHARACTERIZATION OF TRANSGENIC ELITE INDICA RICE LINES ACCUMULATING β-CAROTENE IN THE ENDOSPERM

Thesis

SUBMITTED TO THE

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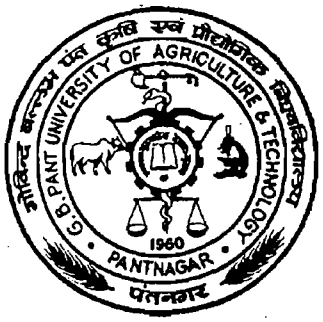

By

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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Delectated

to

my family

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CERTIFICATE

This is to certify that the thesis entitled "Development and Characterization of Transgenic Elite Indica Rice Lines Accumulating β-carotene in the Endosperm" submitted in partial fulfillment of the requirements for the degree of Doctor of philosophy with major in Genetics and Plant Breeding of the College of Post-Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bona fide* research carried out by Mr. Mayank Rai. Id. No. 25653, under our supervision, and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been acknowledged.

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Introduction

"Rice is life"- The theme for International Year of Rice, 2004, reflects the importance of rice as the primary food source, and is drawn from an understanding that rice is essential for food security, poverty alleviation and improved livelihoods, especially in the developing countries. More than 90% of rice is produced and consumed in South and South East Asia where 60% of the global population live (Khush, 1997). India is the second largest producer of rice. Since the green revolution, the total production has increased to a level of 87.0 million tons in 2003-04 as compared to 20.58 million tons in 1950-51. The area under rice cultivation has also expanded from 30.81 to 42.41 million hectares. In 1968 thee were only two districts in India producing more than 2 t/ha of rice, but in 2004, 44% of the districts (103) are realizing more than 2 t/ha of rice yield in the country (Rai, 2004). To ensure the food and much needed nutritional security of the exponentially growing population, further improvements in yield and quality would be needed at a faster rate, which would require the use of both conventional and non-conventional areas of innovative science.

Development in plant biology in the last three decades such as cell culture, molecular biology and plant genetic transformation have opened up new avenues in crop improvement. Thereby allowing the transfer of desirable gene(s) across species and genera (overcoming crossability barriers that limit the scope of conventional breeding) to develop transgenic species with novel traits such as resistance and tolerance of several biotic and abiotic stresses, improved nutritional quality, enhanced grain filling etc. Moreover, advances in genetic transformation techniques provide plant breeders a wide scope to manipulate plants metabolism to improve the quality traits with access to

new and broader gene pools. Transgenic plants can be considered as the most recent development in scientific efforts to genetically improve crops.

Because of its importance as a staple food, rice has become increasingly attractive as a target for molecular biology (Shimamoto, 1995). Rice has 24 chromosomes, and its genome contains only 4.3×10^6 bases (0.6 pg per haploid genome). The whole rice genome has recently been sequenced (Yu *et al.*, 2002; Goff *et al.*, 2002), and more and more information is becoming available everyday about its genes and their functions. The rice genome is one-tenth the size of the human genome and only three times that of *Arabidopsis thaliana*, thus making rice a favorite tool for molecular biology. Due to its small size, rice is considered to be a model plant for genome studies of other cereals (Terada and Shimamoto, 1993). The close relationship among the different cereals makes this approach very promising.

Rice is one of the cheapest sources of food energy and protein. Though rice is an important source of diet for 50% of the total world population predominantly in the developing countries, but it is deficient in many essential micronutrients (such as iron, zinc, and vitamin A). A diet based mainly on milled rice leads to malnutrition, with deficiencies being most severe in iron, lysine, iodine, vitamin A and zinc (FAO, 1993).

Vitamin A is one of the most important micronutrients for the maintenance of life and health. It is involved in several critical functions of the body such as stimulation of growth, proper development of skeletal tissue, normal reproduction, maintenance of rod vision, and preservation of epithelial tissue. The role of vitamin A has also been implicated in quenching of free radicals and preventing oxidative damage, as well as in supporting the human immune system (Bendich, 1989, 1993; Ross, 1992). Furthermore, recent investigations have shown that vitamin A plays a vital role in the protection

against certain types of cancer (Olson, 1992; Ziegler, 1993), the delay of full symptoms of HIV (Santamaria and Bianchi-Santamaria, 1993, Tang *et al.* 1993; Semba *et al.*, 1995a) and the reduced viral transmission from mother to child in HIV infected pregnant women (Semba et al., 1995b).

Vitamin A deficiency (VAD) occurs when the body stores are depleted to the extent that physiological functions are impaired. The most common VAD symptoms are night blindness, conjectival xerosis and bitot's spot. In severe deficiency, corneal changes progress rapidly from xerosis to keratomalasia, resulting in irreversible blindness. Apart from causing eye damage, VAD can also produce systemic effects. The most significant effect is impaired immunocompetence leading to increased risk for morbidity and mortality (Reddy, 2003). Although, the symptoms can be manifested at any age, the children under five years are most susceptible to VAD. Pregnant women are also prone to VAD and their children are also likely to become deficient.

Vitamin A levels are conventionally expressed in terms of μg retinol or retinol equivalent (RE). One μg of β-carotene is considered to be equivalent to 0.167 μg of RE (FAO, 1988). According to the FAO considerations the daily RE uptake for young children (aged 1-6) should be 200 μg on a basal and 400 μg on a safe level. Vitamin A deficiency symptoms are frequent at a level less than 100 μg per day (FAO, 1988). For adults, the corresponding values of basal and safe levels are 300 μg and 600 μg, respectively. A daily uptake below the basal level does not directly lead to symptoms of vitamin A deficiency, until reserves stored in the lever are exhausted.

According to the statistics compiled by UNICEF, the diets of an estimated 124 million children worldwide are deficient in vitamin A (Humphrey *et al.*, 1992). The VAD affected regions include highly populated areas of Asia (India, Bangladesh, Indonesia,

Table 1.1: Regional and global prevalence of vitamin A deficiency and xeropthalmia.(source:http://harvestplus.org/)

Region	Population <5 yrs (000)	Vitamin A deficient		Xeropthalmia	
		Number (000)	%	Number (000)	%
Africa	103,934	33,406	32.1	1,593	1.53
Americas	47,575	8,218	17.3	75	0.16
Eastern Mediterranean	59,818	12,664	21.2	510	0.85
South East Asia	169,909	55,812	33.0	2,026	1.20
Western Pacific	122,006	17,128	14.0	220	0.18
Total	**502,494**	**127,273**	**25.3**	**4,424**	**0.88**

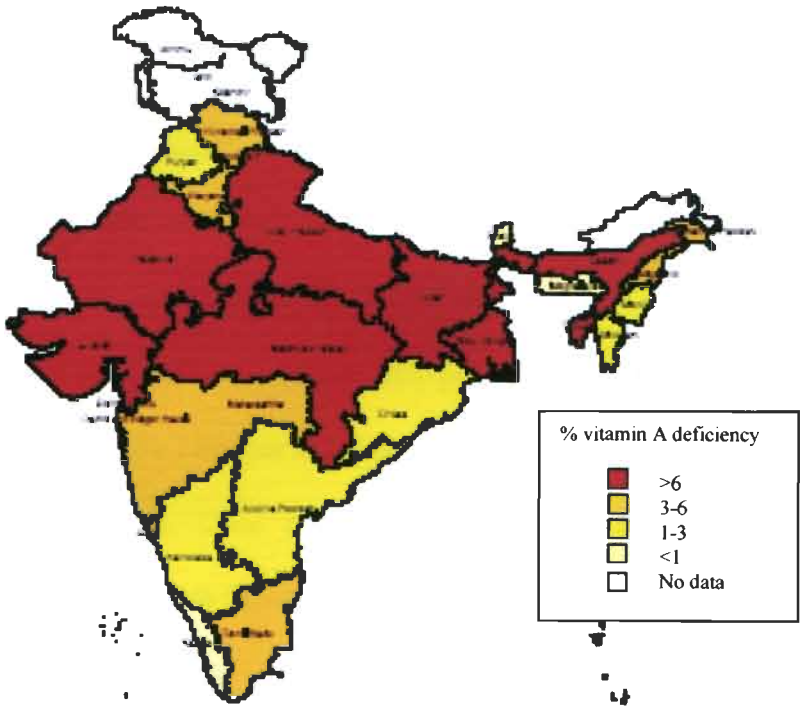


Figure 1.1: Prevalence of vitamin A deficiency in India (based on clinical symptoms) by state for children between ages of one to six. (source: http://www.fao.org/countryprofiles)

Philippines), Africa (Zambia, Tanzania, Sudan), Latin America and the Caribbean (Sommer, 1988) (Table 1.1). In India, the prevalence of Bitot's spot has declined considerably since 1970s, but it is still around 0.7%, which is still higher than the WHO cut off level for a public health problem. Moreover, the national average does not give a full picture because the prevalence rates vary widely, not only between states but also within states (Figure 1.1). Apart from the clinical symptoms, there is very high prevalence of subclinical deficiencies. Community studies carried out in Uttar Pradesh, Andhra Pradesh and Tamil Nadu indicate that 30-50% children have retinol level below 20 μg/dl, the WHO cut off, indicating a public health problem (Reddy, 2004). Surveys show that average intake of vitamin A is around 300 μg in women and 120 μg in children and more than 80% have intakes less than 50% of the RDA.

Several intervention strategies have been developed and used in the past for prevention and control of VAD. These included vitamin A supplementation, food fortification and dietary diversification. The traditional approach to reduce vitamin A deficiency in developing countries has been to supply preschool children with high doses of vitamin A every six months. Even at the start of such program, however, vitamin A rarely reaches more than 80% of the children and this percentage falls with each successive six months period. Furthermore, these programs are fairly expensive and require an educated medical staff as well as extensive infrastructure, which are often not available (Sommer, 1989). In India, the vitamin A supplementation program has been in operation since early 1970s (Reddy, 1994). However, national coverage of all the children has been hard to sustain over time. Although the problem of keratomalasia or night blindness has reduced in the past years, the national goal is not only to prevent blindness but also to eliminate VAD as a public health problem. Vitamin A intake of the

children is less than 50% of RDA even today with a significant proportion of them having clinical and sub-clinical deficiencies.

Another fact, worthy of being noted, is that high doses of vitamin A are known to be teratogenic (Biesalski, 1989), while high amount provitamin A (β-carotene) is considered to be safe, due to the fact that β-carotene conversion into vitamin A is a highly controlled step in the human body (Parker *et al.*, 1993). Experimental doses of up to 180 mg/day have been administered in clinical trials without adverse side effects (Mathews-Roth, 1990).

Dietary improvement is undoubtedly the most logical and sustainable strategy to improve vitamin A status. It traditionally involves the attempt to increase the consumption of green vegetables and suitable fresh fruits. But this approach is more complex, involving a number of factors including accessibility, affordability, and change in dietary habits. Artificial food fortification (micronutrients are added at the time of food processing, for example, cooking oil, sugar, salt etc.), apart from the success story of iodized salt, also suffers from the limitations of recurring costs, and lack of access to the poor who are in greatest need.

Keeping in view the limitations of the traditional interventions measures, it is desirable to deliver the micronutrients like vitamin A on a sustainable basis, through such a vehicle that is a staple food of the target population. But unfortunately, rice, which is the food staple for 50% of the world population, more so in Asia, where VAD is most prevalent, does not accumulate provitamin A in its endosperm (Tan *et al.*, 2004). In fact over dependence on rice for food is considered to be the major cause for such micronutrient deficiencies. However, it has recently been shown that it is possible through genetic engineering to 'bioengineer' β-carotene synthesis pathway into rice

endosperm (Ye *et al.*, 2000). Introducing the endosperm specific carotenogenic pathway into indica rice varieties, which are preferred in South East Asia, would be an elegant way to overcome the problem of VAD on a sustainable basis, as rice would produce provitamin A naturally, and no, or minimal recurrent costs would be involved. Apart from introducing the genes for provitamin A accumulation in the endosperm, it is also important to make sure that the transgenes are expressing; the transgenes are stably inherited in terms of structural integrity and expression; there are no agronomic pay-offs due to the introduction of alien genes; and whether or not there is any loss of provitamin A during milling and cooking, so that a 'laboratory experiment' could take the shape of a viable and sustainable product and create an impact on the health and livelihood of the end users.

Therefore the present study was undertaken considering the following objectives:

1. Genetic transformation of indica rice varieties with transgenes, which would lead to accumulation of β-carotene in the endosperm.
2. Simultaneously, marker assisted introgression of transgenic pathway from transformed japonica line (Taipei 309) into indica background (IR64).
3. Molecular characterization of transgenic lines and their progenies to ensure the stable inheritance of transgenes.
4. Estimation of total carotenoids and β-carotene in the transgenics through biochemical analysis.
5. Estimation of effect of processing (polishing, cooking) on β-carotene level in the seeds.
6. Agronomic and phenotypic evaluation of transgenics.
7. Correlation of molecular, biochemical, agronomic and phenotypic data.

Review of Literature

2.1 Biochemistry of Carotenoids

The carotenoids are one of the most important groups of natural pigments, because of their wide distribution, structural diversity and numerous functions. The structure of carotenoids confers very special and remarkable properties that are the basis of their varied functions and actions in all kinds of living organisms. However, physiological and metabolic functions of carotenoids are still not well understood. The greatest problem is the structural diversity of carotenoids, more than 700 have been characterized. Moreover, individual carotenoids are present naturally in different cis-trans isomeric forms that may affect their biochemistry (O'Neil *et al.*, 1992).

2.1.1 Structure and properties

The carotenoids are polyisoprenoid compounds, which are formed by tail-to-tail linkage of two C_{20} geranylgeranyl molecules. All the carotenoids are produced by variations of the C_{40} skeleton. Carotenoids can be distinguished into carotenes, which are made up of only hydrocarbons (C and H), and xanthophylls (or oxycarotenoids) that have some O-containing groups such as hydroxy, keto and epoxy groups (Oliver and Palou, 2000).

Vitamin A is structurally one half of β-carotene with an added molecule of water at the end of the lateral polyene chain. An unsubstituted β ring with a C_{11} polyene chain is the minimum requirement for vitamin A activity. Thus, β-carotene having two β rings, is assigned 100% vitamin A activity. Other carotenoids, such as γ-carotene, α-carotene, β-cryptoxanthin, α-cryptoxanthin, etc, which have one unsubstituted β ring, would have about half provitamin A activity as compared to β-carotene (Rodriguez-Amaya, 2001).

Apart from the provitamin A activity, carotenoids have been reported to prevent or protect against certain types of cancer, cardiovascular diseases, macular degeneration and cataracts (Olson, 1992; Ziegler, 1993). These functions of the carotenoids have been attributed to their antioxidant property through singlet oxygen quenching and deactivation of free radicals (Paloza and Krinsky, 1992). This ability of carotenoids is related to the conjugated double bond system, and maximum protection is given by those having nine or more double bonds (Foote *et al.,* 1970). The acyclic lycopene was observed to be more effective in oxygen quenching than the bicyclic β-carotene (Di Mascio *et al.*, 1989). In plants, their predominant function is in the mechanism of photosynthesis as a constituent of light harvesting complexes and photosystems to collect light energy and to detoxify excited chlorophyll and oxygen species at high light intensities (Sandmann, 2001).

2.1.2 The biosynthetic pathway- genes and enzymes involved

The carotenoids are part of an immensely diverse group of compounds known as isoprenoids, which originate from a 5-carbon central metabolite Isopentenyl pyrophosphate (IPP). A modular assembly process that produces compounds of 5, 10, 15, 20, or more carbons (in multiple of 5) allows the biosynthesis of the basic skeletons for various isoprenoids with a relatively small number of basic reaction steps (McGarvey and Croteau, 1995). Biosynthesis of carotenoids (Figure 2.1) in plants takes place within plastids, chloroplasts of photosynthetic tissue, and chromoplasts of fruits and flowers. Chloroplyll, tocopherols, plastoquinone, gibbrellins and carotenoids all share precursor, 20 carbon geranylgeranyl pyrophosphate.

Although, efforts had been going on to characterize the enzymes involved in carotenoid biosynthetic pathway since 1970s, they were not very fruitful due to the fact that these

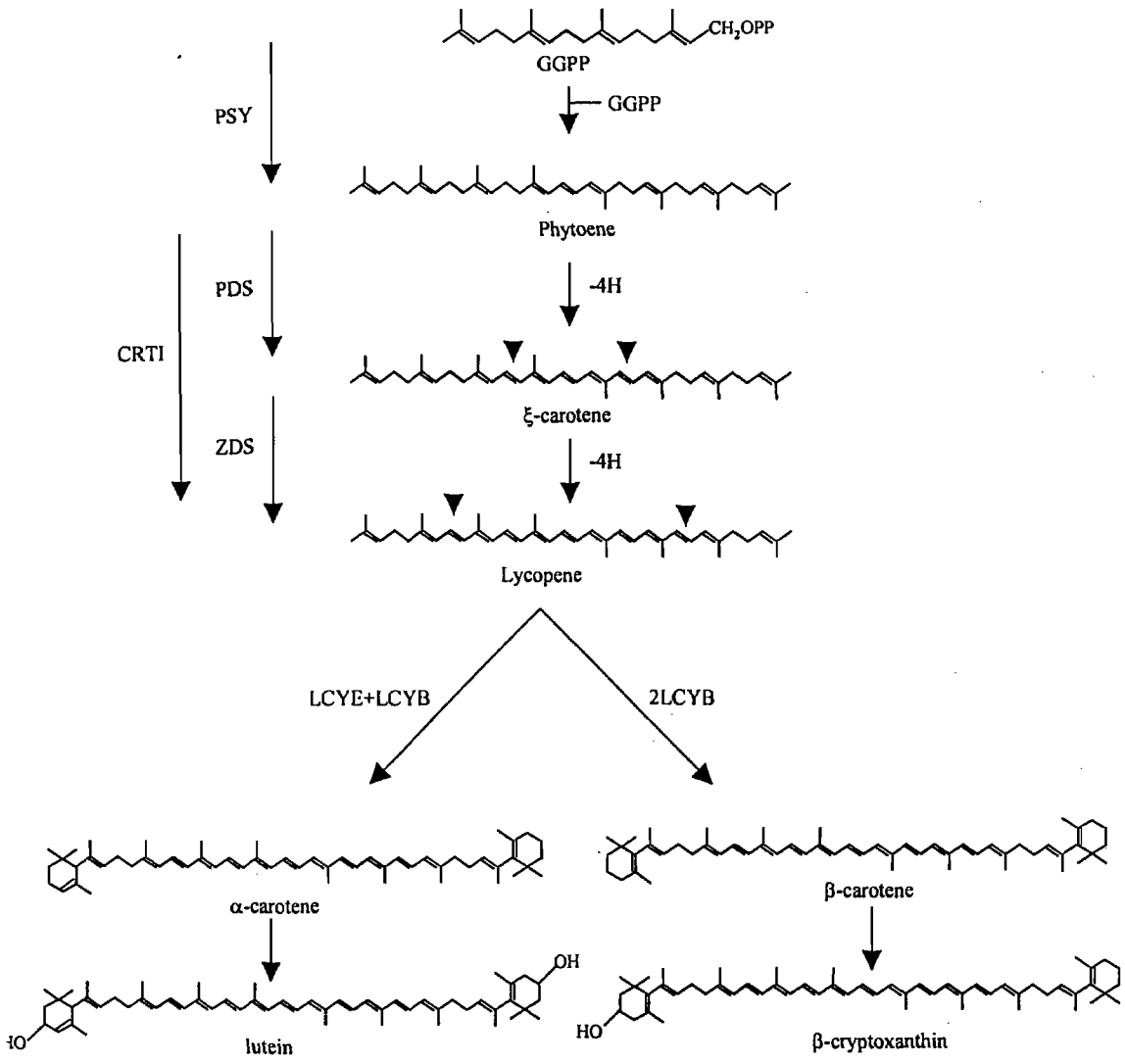


Figure 2.1: A general carotenogenic pathway. GGPP: geranylgeranyl pyrophosphate; PSY: phytoene synthase; PDS and ZDS: phytoene and ξ-carotene desaturase (in plants); CRTI: phytoene desaturase (in bacteria); LCYE:Lycopene ε-cyclase; LCYB: lycopene β-cyclase.

enzymes and most of their products are membrane associated, lipid soluble and present in relatively low amounts (Cunningham and Gantt, 1998). However, in the past decade or so, genes encoding nearly all enzymes involved in carotenoid biosynthesis in green plants have been sequenced and their products characterized. This has been possible due to some pioneering experiments in cyanobacteria and chromoplasts of higher plants like pepper and daffodil.

Armstrong *et al.* (1989; 1990) studied carotenoid biosynthesis (*crt*) mutants in *Rhodobacter capsulatus* using genetic and biochemical techniques. They sequenced an 11kb region encoding eight of the nine genes identified (*crtA, B, C, D, E, F, I, J, K*). This was the first sequence of carotenoid genes reported in any organism. Through there studies involving *in vitro* assays in cell free extracts of the mutants, they proposed that CrtE is the Phytoene synthetase, and CrtB, and possibly CrtJ, are components of Prephytoen pyrophosphate synthetase. However, this study was not complete, as *R. capsulatus* does not synthesize general cyclic carotenoids like β-carotene and zeaxanthin.

Misawa *et al.* (1990) reported the sequence of carotenoid biosynthesis genes in *Erwinia uredovora* and elucidated the carotenoid biosynthesis pathway by functional expression of gene products expressed in *E. coli*. Six open reading frames were found, and by analyzing the carotenoids accumulated in *E. coli* transformants, the pathway was proposed as follows:

Geranylgeranyl $PP_i \xrightarrow{CrtB}$ prephytoene $PP_i \xrightarrow{CrtE}$ phytoene $\xrightarrow{CrtI}$ lycopene $\xrightarrow{CrtY}$ β-carotene $\xrightarrow{CrtZ}$ zeaxanthin $\xrightarrow{CrtX}$ zeaxanthin-β-diglucoside.

They also reported that only one gene product (CrtI) is required to perform four sequential desaturation steps to convert phytoene to lycopene.

In higher plants, several groups reported the sequence and functional expression analysis of phytoene synthase cDNA in different species like pepper (Roemer *et al.*, 1993), tomato (Misawa *et al.*, 1994), and daffodil (Schledz and Beyer, 1996; Scheldz *et al.*, 1996). Al-babili *et al.* in 1996 also reported the sequence of cDNA encoding Lycopene cyclase. Cunningham *et al.* (1996) identified and sequenced the Arabidopsis cDNAs encoding for two enzymes, β and ε Lycopene cyclases, that are responsible for conversion of acyclic lycopene into cyclic carotenoids. They also studied the functional expression of these enzymes in *E.coli*, and concluded that, while β cyclase is capable of adding two β rings to lycopene- forming β -carotene, ε cyclase can add only one, resulting in the formation of δ-carotene. A combination of β and ε cyclase forms α-carotene with one β and one ε ring.

Moreover, a single bifunctional protein carries out phytoene synthase and lycopene cyclase activity in fungi. Its existence was first proposed by Torres-martinez *et al.* in 1980 for *Phycomyces*, and recently it has been shown to be a feature unique to fungal carotenogenesis. The *crtYB* gene of *X. dendrorhous* (Verdoes *et al.*, 1999), *carRP* of *M.circinelloides* (Velayos *et al.*, 2000) and *carRA* of *P. blakesleeanus* (Arrach *et al.*, 2001) have been the most extensively studied. Velayos *et al.* (2000) also reported that the genes *carB* and *carRP* in *M.circinelloides* show a coordinated regulation of their expression by blue light, suggesting a bi-directional mode of transcriptional control.

2.2 Bioengineering the carotenoid pathway

The enormous progress made in the cloning of carotenogenic genes opened up the possibility of modifying and engineering the carotenoid biosynthetic pathway in plants, especially in food crops, considering the importance of carotenoids like β-carotene in

human nutrition and health. Several approaches have been used to increase the level of β-carotene in several plant species and tissues.

Misawa *et al.* (1994) transformed tobacco plants with *E. uredovora* phytoene desaturase gene (*crtI*). They reported that in addition to their resistance towards bleaching herbicides like norflurazon, which interfere with the plant type phytoene desaturase, the transgenic plants showed increased levels of β-carotene and its xanthophylls metabolites in the leaves, while the level of lutein, a xanthophyll derived from α-carotene was reduced.

Rosati *et al.* (2000) were able to enhance the conversion of β-carotene from lycopene, which is normaly present in high amounts in tomato fruit, by transforming tomato with *Arabidopsis* β-*lcy* gene driven by tomato phytoene desaturase promoter. They also reported an increase in the total carotenoid level in the fruit while the leaf carotenoid content was unaltered. In another study, the expression of *E. uredovora crtI* gene driven by CaMV 35S promoter to increase the lycopene content of transgenic tomato fruits unexpectedly resulted in 50% decrease in total carotenoids, mainly at the expense of lycopene while β-carotene increased about three folds (Romer *et al.*, 2000).

When Fray *et al.* (1995) tried to overexpress tomato phytoene synthase by transforming tomato with a copy of fruit specific phytoene synthase under the crontrol of CaMV 35S promoter, it led to the reduced height of transgenic plants due to the reduction (~30 fold) in the levels of gibberellin A_1 (GA_1), while chlorophyll was also reduced. This result highlighted the complexities of manipulating carotenogenic pathway, as the increased flux towards the direction of one product may affect the other essential metabolites, thereby affecting the phenotype. However, in another study Fraser *et al.* (2002) transformed tomato with *E. uredovora* phytoene synthase (*crtB*) gene driven by

fruit specific tomato polygalacturonase promoter and the protein was targeted to chromoplast by tomato phytoene synthase transit sequence. They reported a 2-4 fold higher carotenoid level in the primary transformants, whereas, phytoene, lycopene, β-carotene and lutein levels were also increased by approximately two folds. They did not notice any significant change in the activity of other enzymes in the pathway. This suggests that manipulating carotenogenic pathway in a seed specific, rather than constitutive manner could be the right option.

In *Brassica*, Shewmaker *et al.* (1999) introduced *E. uredovora* phytoene synthase (*crtB*) gene under the control of seed specific *Brassica* napin promoter, and found a 50 fold increase in the total carotenoid level. Surprisingly the predominant compound accumulating in the seeds were α and β-carotene, and not lutein, which is the predominant carotenoid in non-transformed control seed. While sterol levels remained same, tocopherol and chlorophyll levels were significantly reduced in the transgenic seeds.

Engineering β-carotene biosynthetic pathway into rice endosperm was novel in the sense that it was aimed at a tissue that was totally devoid of the pathway, whereas the previous works involved manipulation of already existing pathways in terms of over-expression or under-expression of certain genes. Carotenoids do not accumulate in the rice endosperm; however, the general precursor geranylgeranyl pyrophosphate (GGPP) is present in this tissue.

Burkhardt *et al.* (1997) for the first time demonstrated that it is possible to engineer β-carotene biosynthetic pathway in a non photosynthetic, carotenoid-lacking plant tissue by transforming japonica rice variety T309 with daffodil phytoene synthase gene driven by either CaMV 35S promoter or glutelin (Gt1) seed specific promoter. The transgenic

plants accumulated phytoene in the endosperm. The highest amount of 0.74 μg/g was found in one of the lines having glutelin promoter.

This result was extended to the ultimate β-carotene accumulation by Ye *et al.* (2000), who introduced three genes- a daffodil phytoene synthase (*psy*) gene under the control of seed specific Gt1 promoter, an *E. uredovora* phytoene desaturase (*crtI*) gene (the single enzyme CrtI mediates four desaturation steps, for which two plant enzymes are required) driven by CaMV 35S promoter, and daffodil lycopene β-cyclase (*lcy*) into T309 japonica rice line, which resulted in the accumulation of carotenoids (surprisingly, mostly β-carotene) in the endosperm upto 1.6 μg/g in T_1 seeds. Interestingly, they were also able to produce provitamin A only by introducing the phytoene synthase and phytoene desaturase activities in the absence of heterologous β-cyclase. Al-Babili *et al.* (2001) postulated that this might be due to the positive and negative feedback mechanisms by carotenoid intermediates such as trans-lycopene on endogenous carotenogenic genes.

This proof of concept in an experimental japonica variety was further extended to several widely grown indica rice varieties from different eco-geographical regions of Asia by Datta *et al.* (2003) and popular IRRI variety IR64 (Datta *et al.*, 2000: Hoa *et al.*, 2003).

2.3. Genetic transformation: an overview

The initial efforts to transform rice were made in late 1980's (Toriyama *et al.*,1988; Zhang *et al.*, 1988; Shimamoto *et al.*, 1989) and resulted in successful transformation of japonica rice varieties. First fertile homozygous *indica* rice was reported in 1990 using PEG (polyethylenglycol)-mediated protoplast transformation (Datta *et al.*, 1990). The

protoplasts were isolated from embryogenic cell suspension (ECS), derived from calli of anther culture. Peng *et al.* (1992) also reported the production of fertile transgenic *indica* rice. Datta *et al.* (1992) also introduced *bar* gene for herbicide resistance into the protoplasts of modern *indica* variety IR72.

All these early reports used PEG mediated direct DNA dilevery into protoplasts. PEG is an efficient system for protoplast fusion in somatic cell hybridization. PEG stimulates DNA uptake or integration into protoplasts (Shillito *et al.*, 1985) by increasing the permeability of cell membranes. However, this method has a few drawbacks. First, the establishment of embryogenic cell suspension (ECS) is expensive, time and labor consuming, and extremely genotype dependent; second, multiple rearranged gene copies are integrated into the genome; and third, somaclonal variations accumulate in ECS due to the long tissue culture period.

2.3.1 Microprojectile bombardment

The introduction of microprojectile bombardment for the transformation of regenerable tissues (Sanford and Klein, 1987, Christou *et al.*, 1988) offered an easier way to transform recalcitrant higher plants. Sanford (1990) defined particle bombardment as the introduction of substances into intact cells and tissues through the use of high velocity microprojectiles via a mechanism that breaches cell walls and membranes, the principal barriers to cell DNA delivery. Due to the physical nature of the process, there is no biological limitation to the actual DNA delivery process; consequently genotype is not a limiting factor (Christou, 1997).

Small high-density particles are accelerated to high velocity by a particle gun apparatus. The particles penetrate the plant cell wall and membrane, and carry the foreign DNA into the host cells (Klein *et al.*, 1987). A microprojectile should be small enough to

enter a cell of tissue in a non-lethal manner, and should be capable of carrying DNA on its surface or in its interior. Typically, these are made of high-density metals such as gold or tungsten, which are more or less spherical and approximately 1.5-3.0 μm in diameter (Sanford, 1990). However, 1.0 μm gold particles are used in rice transformation in the laboratory at IRRI (Datta *et al.*, 1997).

The advantages of microprojectile bombardment are: a) it is relatively easy to handle, b) cell survives the intrusion of particles and the genes coated onto the particles assume biologically active in the cells, c) target tissue can be as different as yeast, algae pollen, suspension cultures, callus cultures, scutellum, d) particle delivery can also reach deeper cell layers, e) DNA may be transferred without using specialized vectors, f) the introduction of multiple DNA fragments/plasmids can be accomplished by co-bombardment, thus eliminating the necessity of constructing a single large plasmid containing multiple transforming sequences, g) false positives arising from reporter gene expression in *Agrobacterium* is avoided, h) organelle transformation is achived only by particle bombardment.(Potrykus, 1990; Veluthambi *et al.*, 2003).

Methods for preparing DNA / metal mixture have now been standardized. In a standard procedure gold is used as the accelerating particle. DNA is typically loaded onto 1.5-3.0 μm gold bead (Alpha Chemical Inc.) at a rate of up to 40 μg DNA /mg of gold, using $CaCl_2$ and spermidine (Klein *et al.*, 1987) to precipitate the DNA on to the gold. The DNA coated particles are placed as a suspension in a small aqueous volume, on the front end of bullet-like plastic micro-projectile. Shock waves can be created by electric discharge of a drop of water (Christou *et al.*, 1988) or sudden release of compressed air or nitrogen (Morikawa *et al.*, 1989). These systems accelerate a micro-projectile in to a stopping plate or screen.

The particle delivery system PDS-1000 / He (BioRad Laboratories) uses a shock wave generated by the sudden release of compressed helium to accelerate a thin plastic sheet in to a metal screen. The micro-projectiles are dried on to the surface of the carrier sheet from an ethanol suspension.

A number of critical variables have been identified and need to be considered very carefully in involving genetic transformation utilizing particle bombardment (Klein *et al.*, 1992). These factors involve 1. Physical parameters; which include the nature, chemical and physical properties of the metal particle use to carry the foreign DNA into the cells; nature, preparation and binding of DNA onto the particles; and target tissue. 2. Environmental parameters; which include temperature, photoperiod and humidity of donor plants, explants. 3. Bombarded tissues, and biological factors; which include choice and nature of the explant, pre and post-bombardment culture conditions and interaction between introduced DNA and cytoplasmic or nuclear components (Chawla, 2002).

Particle acceleration system as a method of gene delivery in intact cells and tissue system has been enthusiastically employed in the transformation of cereals. Christou *et al.*, (1991) reported success in the recovery of transgenic rice plants from both *japonica* and *indica* varieties using biolistic method.

Many agronomically important genes have been introduced in rice by different groups which include herbicide resistance *bar* gene (Datta *et al*,. 1992; Cao *et al.*, 1992; Ho *et al.*, 2001; Kaur, 2002), *Bt* endotoxin (Alam *et al.*, 1996; 1998; Datta *et al.*, 1998; Nayak *et al.*, 1997; Tu *et al.*, 1998a; Wu *et al.*, 1997), *Xa21* (Tang *et al.*, 1999; Nagadhara *et al.*, 2003), *chitinase* gene for sheath blight resistance (Lin *et al.*, 1995; Baisakh *et al.*, 1999; 2001, Datta *et al.*, 2001), phytoene synthase gene (Burkhardt *et*

al., 1997, *ferritin* gene (Drakakaki *et al.*, 2000; Vasconcelos *et al.*, 2003) and genes for provitamin A synthesis (Datta *et al.*, 2003)

Like other methods, the biolistic method has its few drawbacks. In some cases, the copy number and rearrangement of the introduced DNA is high, thereby rendering transgenic prone to gene silencing (Oard *et al.*, 1996) and causing genomic changes. However, particle bombardment is still considered as a very efficient protocol for production of phenotypically normal, fertile transgenic *japonica* and *indica* rice cultivars (Jain *et al.*, 1996).

2.3.2 *Agrobacterium* mediated transformation:

Agrobacterium tumefaciens is a gram-negative soil bacterium that can genetically transform cells with a segment of DNA which results in crown gall tumors, the tumor provides substances for *Agrobacterium tumefaciens* to grow on the plants. This system of gene transfer is widely used for plant molecular and genetic engineering .The naturally evolved unique ability of *Agrobacterium* to precisely transfer defined DNA sequences to plant cells has been very effectively utilized in the design of a range of plasmid vectors. Three genetic elements, *Agrobacterium* chromosomal gene (*chv*), T-DNA delimited by right border, and left border and Ti plasmid virulence genes (*vir*) constitute the T-DNA transfer machinery. The mechanism governing the transfer of 'T-complex' via the conjugal channel and the role of plant and *Agrobacterium* proteins in T-DNA integration are being intensively studied (Gelvin, 2000, 2003; Zupan *et al.*, 2000).

For transformation Ti plasmid has been altered through genetic modification to create disarmed plasmids or vectors, which can carry any DNA sequence of interest into *Agrobacterium* infected plants, without causing tumor or galls in the host plants (Fraley

et al., 1985; 1986). In a binary system, a "disarmed" Ti plasmid serves as a helper, providing the virulence function, and an artificial T-DNA, which contain the selectable marker gene and gene(s) of interest is placed on the other plasmid (An *et al.*, 1988). The use of such vector for gene delivery to higher plants has several advantages in addition to its convenience and efficiency. It allows the transfer of large segments of DNA with minimal rearrangement and integration of less number of copies into plant chromosomes, and is less expensive (Hamilton *et al.*, 1996; Klee *et al.*, 1987; Dong *et al.*, 1996; Zambryski *et al.*, 1989; Veluthambi *et al.*, 2003).

Several Ti plasmid based vector systems are now available for plant transformation. A typical binary vector comprising of octopine type *vir* helper strain such as LBA4404 (Hoekema *et al.*, 1983) that harbors the disarmed Ach5 Ti plasmid and a binary vector such as pBin19 is very commonly used for plant transformation. The available range of *vir* helper strains has been expanded with the nopaline-type MP90 (Koncz and Schell, 1986) and the L, L-succinamopine-type EHA101 (Hood *et al.*, 1986). The bacterial kanamycin gene was deleted in EHA101 to develop the *vir* helper strain EHA105 (Hood *et al.*, 1993). EHA101 and EHA105, by virtue of harboring the supervirulent *vir* genes, exhibit broader host range and higher transformation efficiency.

Agrobacterium-mediated gene transfer has been less successful in cereals and other monocots, but in the last few years significant progress has been achieved in this direction (Smith and Hood, 1995; Hiei *et al.*, 1994, 1997). It had been believed that *Agrobacterium* infects only dicot plants but not monocots like rice. However, co-cultivation of explants with *Agrobacterium* in the presence of acetosyringone, a *vir* gene inducer, has become a routine exercise in the transformation of recalcitrant monocots such as rice, wheat, barley and maize. Aldemita and Hodges (1996) reported that

preinduction of *Agrobacterium* with 400 μM acetosyringone prior to cocultivation is important for rice transformation.

Reineri *et al.*, (1990) first reported the production of transgenic plants of a *japonica* rice cultivar (Nipponbare) using a wide host range (WHR) supper virulent strain A281 (pTiBo542). Baba *et al.*, (1986) co-cultivate rice protoplasts with *Agrobacterium,* and obtained calli growing on hormone free medium and producing opines. Terouchi *et al.*, (1990) utilized scanning electron microscopy to observe attachment of *Agrobacterium tumefaciens* to the surface of various plants including rice.

Datta *et al.* (1996) used *Agrobacterium* strain LBA4404 harboring plasmid pTOK223 which contain VirB, C and G from Ti plasmid pTiBO542, intron-gus, a hygromycin resistance gene (*hph*), and a gene for kanamycin resistance, for transformation of *japonica* cultivar T309 and *indica* rice cultivars Basmati 122, and IRRI breeding lines IR51500 and IR58.

Komari *et al.* (1996) used a novel super-binary vector that carried two separate T-DNAs, one containing selectable marker gene (*hph*) and the other with *gus* reporter gene, and confirmed the segregation of the two T-DNAs in subsequent progenies. This protocol was found to be highly efficient and the frequency of co-transformation with the two DNAs was greater than 47%. They proposed that such vectors could be used to produce marker free transgenics. They also tested the delivery of two T-DNAs to plants from mixtures of *A. tumefaciens*, but the frequency of transformation was found to be relatively low. For the most part of 1990's, the transformation experiments involved reporter and selectable marker genes, and were aimed at establishing transformation protocol and 'proof of concept'.

However, Cheng *et al.* (1998), for the first time reported agronomically important gene being introduced to rice through *Agrobacterium.* They used *Agrobacterium* strains LBA4404 and EHA105 to introduce *cryIA(b)* and *cryIA(c)* genes into rice for stripped stem borer and yellow stem borer resistance.

Agrobacterium-mediated transformation system has been reported by different groups for *japonica* (Chan *et al.*, 1993; Hiei *et al.*, 1994; Komari *et al.*, 1996; Toki *et al.*, 1997; Cheng *et al.*, 1998 and Yokoi *et al.*, 1998; Ye *et al.*, 2000), and *indica* rice varieties (Aldemita and Hodges, 1996; Datta *et al.*, 1996; Rashid *et al.*, 1996; Baisakh *et al.*, 1999; Datta *et al.*, 2000; Azakanandam, 1999; Datta *et al.*, 2003). The application of this method is still limited as it is amenable only to tissue culture responding genotypes (Datta *et al.*, 1996).

2.3.2.1 Beyond border transfer of plasmid backbone

A unique advantage of *Agrobacterium* T-DNA transfer is the accurate processing of T-DNA between right and left border and its precise transfer and integration into the plant genome. However, there are many reports that non T-DNA portions may also be transferred to the plant genome. Martineau *et al.* (1994) first reported the transfer of binary vector backbone sequences into transgenic plant DNA and questioned the definition of T-DNA. Ramanathan and Veluthambi (1995) also showed that nos-*nptII* cassette, placed outside the T-DNA left border, could be transferred to and confer kanamycin resistance on infected tobacco cells.

Kononov *et al.* (1997) carefully examined the binary vector sequences that could be found in upto 75% of transgenic tobacco plants and concluded that such transfer could result from either skipping the left T-DNA border, or initiation of T-DNA transfer from left border to bring vector backbone sequences into plant cells. They also suggested that

transfer of vector backbone sequences to plants was a natural consequence of the mechanism of VirD2 function.

2.3.3 Selectable marker genes

A key step in obtaining transgenic plants is the selection of transformed calli or plants, specially in plant species like rice, where transformation efficiency is relatively low. Therefore an efficient selectable marker gene must be used in order to establish an efficient transformation system. The three most commonly used marker genes are neomycin phosphotransferase (*nptII*, also called *aphII* or *neo*), hygromycin phosphotransferase (*hpt*, also called *hph* or *aphIV*) and *bar* gene that codes for phosphinothricin acetyl-transferase.

NPTII confers resistance to the amino-glycoside antibiotic Kanamycin. In dicots this marker is routinely used, however, in monocots can be used as a selective agent during protoplast regeneration, but it does not efficiently select transformed calli. In addition, many calli recovered after kanamycin selection were unable to regenerate green plants (Toriyama *et al.* 1986; Zhang *et al.*, 1988).

Hygromycin phosphotransferase, which confers resistance to the amino-glycoside antibiotic Hygromycin B, is another widely used selectable marker for both dicot and monocot transformation (Shimamoto *et al.*, 1989; Datta *et al.*, 1990). The *hpt* gene was originally found in *E.coli* (Blochinger and Diddelmann, 1984) but has been modified for expression in plants (Waldron *et al.*, 1985). To eliminate some internal restriction sites, Zheng *et al.* (1991) further modified and used it to form cassettes for vector construction.

Phosphinothricin, a commercially important herbicide, inhibits glutamine synthase, causing a rapid accumulation of ammonia that leads to plant cell death (Tachibana et al., 1986). The bialaphos (phosphoinothricin derivative, also called basta) resistant *bar* gene

product acetylates phosphinothricin, thereby, rendering it non inhibitory to glutamine synthase. Transgenic tobacco, tomato, potato, and rice plants showed complete resistance to high doses of PPT salt and its derivatives basta and glufosinate ammonia (Cao *et al.*, 1992; Christou *et al.*, 1991; Datta *et al.*, 1992; Chawla et al., 1999).

However, concerns have been raised that the antibiotic and herbicide tolerant marker genes could be transferred into microorganisms and increase the number of resistant pathogens or that horizontal transfer of marker gene into wild relatives may transform them into weedy pest (Dale, 1992; Nap *et al.*, 1992). Recently, a non antibiotic system has been developed, which uses phosphomannose isomerase (*pmi*) gene (Miles and Guest, 1984). It uses mannose as the selective agent, which although, itself has no adverse effects on plant cells, but leads to an accumulation of mannose-6-phosphate, which depletes intracellular stores of inorganic phosphate. This results in inhibition of plant cell growth. Transgenic plants carrying *pmi* gene can detoxify mannose-6-phosphate by conversion to fructose-6-phosphate, in intermediate to glycolysis, via PMI activity. This novel selection agent has been recently reported for the successful selection of sugar beet (Joersbo *et al.*, 1998), maize (Negrotto *et al.*, 2000; Wright *et al.*, 2001) and rice (Lucca *et al.* 2001; Datta *et al.*, 2003; Hoa *et al.*, 2003). Lucca *et al.* (2001) reported a transformation efficiency of upto 41% for selection of rice transformants under mannose selection.

2.3.4 Marker free transgenics

Selectable markers are an integral part of any transformation procedure as they facilitate the selection of transformed cells, thereby increasing the transformation efficiency manifolds. However, the markers (either in positive or negative selection) are considered undesirable once the transgenic is developed. So removal of marker gene

from the transgenic plant has already been stated as a "good laboratory practice (GLP)" by regulatory committee (US Food and Drug Administration, 1998). Consequently, a major focus of transformation has been in the generation of selectable marker free transgenics (MFT). Several strategies like use of cotransformation in biolistic and *Agrobacterium* methods (Tu *et al.*, 2003; Rao *et al.*, 2003; Matthews *et al.*, 2001; Depicker *et al.*, 1985; Petit *et al.*, 1986; De Framond, 1986; Simpson *et al.*, 1986; McKnight *et al.*, 1987; De Block & DeBrouwer, 1991; Komari *et al.*, 1996; De Neve *et al.*, 1997; DeBuck *et al.*, 1998; Daley *et al.* 1998) and sexual crossing, site specific or homologous recombination like Cre/loxP, yeast FLP/FRT, phage lambda attP, and *Zygosaccharomyces rouxii* R/RS systems (Albert *et al.*, 1995; Gleave *et al.*, 1999; Kilby *et al.*, 1995; Zubko *et al.*, 2000; Sugita *et al.*, 2000) have been developed and adopted to remove the selectable marker genes (see recent reviews Puchta 2003; Ebinuma *et al.*, 2001; Hohn *et al.*, 2001). If the marker gene and the gene(s) of interest (GOI) are integrated in the unlinked loci it is likely that some progenies in the selfing generation would possess only the GOI with marker gene segregated out and the trait can be transferred to a desirable background by sexual crossing.

2.4 Transgene integration, organization and expression in plants

The stable inheritance and expression of transgenes over multiple generations is of utmost importance if transgenic plants are to be of long term value to producers and consumers. The transgene expression is influenced by several factors like integrity of individual transgenes, number of transgene copies, and their organization within the transgenic locus. Christou *et al.* (2001) suggested a three-tier hierarchical organization of transgenes. Level 1 comprises of individual transgenes and contiguous transgene copies (either

tandem or inverted repeats, and containing intact and or truncated copies) that concatemerize prior to integration. Level 2 comprises of transgene clusters, i.e., group of transgenes and contiguous transgene copies, interspersed by short regions of genomic DNA that can be amplified through PCR, while level 3 comprises of transgenic clusters separated by megabases of genomic DNA.

PCR amplification and sequencing provide the finest resolution of transgene organization (Kohli *et al.*, 1998,1999). However, Southern blot analysis can also be used to confirm transgene integrity and study second level transgene organization (Kohli *et al.*, 1998,1999; Fu *et al.*, 2000). Restriction enzymes that cut either side of the transgene should release a cassette of diagnostic size that can be used to confirm transgene integrity at the gross level. Restriction enzymes that cut once in the transforming plasmid should provide different hybridizing band for each copy, thus providing an estimate of copy number. Restriction enzymes that do not cut in the transforming plasmid should release the transgenic locus as a single high molecular weight fragment, regardless of number of copies as dictated by the position of restriction sites in the surrounding genomic DNA.

Particle bombardment often generates very large, high copy number transgenic loci, with usually 1-20 copies per locus (Klein *et al.*, 1987; Register *et al.*, 1994). The occurrence of transgene rearrangements following particle bombardment has been widely reported. The analysis of transgene loci in oats (Svistashev *et al.*, 2000; Svistashev and Somers, 2001) has shown that transgene rearrangements can be extensive and extremely complex, with multiple small insertions, inversions and deletions within any transgene locus, plus the presence of filler DNA of uncertain origin.

Kohli *et al.* (1998) proposed that transgene organization in rice transformed through particle bombardment takes place in two phases. The preintegration phase involves concatemerization of transgenes without any interspersed genomic DNA, while the second phase involves the creation of integration hot spots at the original site of integration, facilitating subsequent integration of successive transgenic molecules at the same locus, which may have plant DNA separating the transgenic sequences.

Agrobacterium mediated transformation generally gives rise to lower transgene copy number as compared to direct DNA transfer methods (Kohli *et al.*, 2003). Cheng *et al.* (1997) transformed wheat using both *Agrobacterium* and particle bombardment. Of 26 *Agrobacterium* mediated transformants, more than one third contained a single T-DNA insert, half contained 2-3 copies and the remainder contained 4-5 copies. In contrast from a population of 77 bombarded transformants, only 13 contained a single copy of the transgene. Higher copy number in case of particle bombardment, as compared to *Agrobacterium* mediated transformation, has also been observed in case of rice transformants (K. Datta, personal communication).

There have been several reports of T-DNA rearrangements in transgenic plants. Azhakanandam *et al.* (2000) analyzed the left and right T-DNA border/plant DNA junctions from 20 lines in three rice cultivars. Only two of them contained single non-rearranged inserts. Yin and Wang (2000) also reported that 14% of the rice transformants analyzed contained truncated T-DNA inserts.

Dong *et al.* (2001) used a plasmid that contained three different genes (*hpt*, *bar*, *gus*) on the same T-DNA. Their analysis of 18 transgenic rice lines revealed differences in the copy number of one or more of the three genes in 38% of the cases suggesting that the T-DNA may have undergone rearrangements and truncations.

Kim *et al.* (2003) studied transgene structures in T-DNA inserted rice plants. Out of 171 plants examined 77 carried the vector backbone sequence. They also observed that more than one T-DNA can be integrated into one locus. They estimated that 33% of the transformants carried direct repeats, 21% had inverted repeats with 5' end junctions and 7% carried inverted repeats with 3' end junctions.

In a recent study, Afolabi *et al.* (2004) characterized the integration profile of different T-DNAs in population of transgenic rice plants by using dual binary vector system pGreen/pSoup to carry two different T-DNAs in a single *Agrobacterium* strain. They observed that the four transgenes used integrated at different frequencies in rice genome. They also observed that in around two-third of the co-transformed lines, the transgenes present in the unselected T-DNA were non expressing.

2.5 Marker assisted backcross breeding

Backcross breeding is a well known procedure for the introgression of a target gene from a donor line into the genomic background of a recipient line. The objective is to increase the recipient genome content (RGC) of the progenies. Using molecular information may improve the efficiency of backcross breeding schemes in several ways through marker assisted backcrossing to 1) control the target gene (foreground selection) and 2) control of genetic background (background selection) (Hospital, 2003).

In addition, markers can also be used to estimate RGC in backcross progenies. The most basic estimate is provided by scoring the genotype at a collection of markers over the genome, and then estimating RGC from the ratio of number of markers homozygous for the recipient allele over the total number of markers scored. In general, a few well placed markers (2 to 4 markers on a chromosome of 100cM) should provide adequate coverage of the genome in backcross programs (Visscher, 1996; Servin and Hospital, 2002).

Simple sequence repeats are the most commonly used markers for rice marker assisted rice breeding (Hittalmani *et al.*, 2000; Ribaut *et al.*, 1997; Shen *et al.*, 2001).

2.5.1 Simple Sequence Repeats

Microsatellites or simple sequence repeats (SSRs) are small repeats of one or few tandemly arranged nucleotides spread throughout eukaryotic genomes. The technical efficiency and multiplex potential of SSRs makes them preferable for high throughput mapping, genetic analysis and marker aided selection. SSR markers are co-dominant, multi-allelic and can be readily used to analyze both *indica* and *japonica* germplasm and facilitates the integration of results from independent studies. In addition, the polymorphic nature of many microsatellites is of particular value when analyzing closely related genotypes, as is often the case in breeding programs working within narrowly adapted gene pools (McCouch *et al.*, 2002). Microsatellites consist of tandemly repeated multiple copies of mono-, di-, tri-, or tetra-nucleotide motifs, which are hypervariable and ubiquitously distributed throughout the eukaryotic genome. Microsatellite DNA markers, which can be directly amplified by PCR, have been developed using the unique sequences that flank microsatellites (Weber and May 1989). Recently, Neeraja *et al.* (2003) have reported microsattellite markers, polymorphic between indica and japonica rice varieties.

The present study revealed that the carotenogenic pathway could be successfully be expressed in a tissue specific manner in indica rice background, and that detailed molecular and biochemical characterization of transgenics and their progenies could lead to the identification and release of a stable transgenic product.

Materials and Methods

MATERIALS AND METHODS

3.1 Cultivars for plant transformation

Five popular indica rice cultivars suited to diverse eco-geographical regions of Asia-BR29 (from Bangladesh), Nang Hong Cho Dao and Mot Bui (from Vietnam), IR64 (IRRI-bred elite cultivar), and IR68144 (IRRI-bred high iron and zinc line) were used as targets for genetic transformation. Introgression of transgenes through backcrossing was done in IR64 background, as a case study.

3.2 Plasmid constructs

For transformation experiments involving microprojectile bombardment, three different plasmids were used for co-transformation. The vector pBaal3 contained the daffodil phytoene synthase (*psy*) gene (Burkhardt *et al.*, 1997) under the control of endosperm specific Gt1 promoter, and a bacterial phytoene desaturase (*crtI*) gene driven by constitutive 35S promoter and fused to a transit peptide sequence of pea-Rubisco small subunit (Misawa *et al.*, 1993) to drive the expression of this bacterial gene into the plastids. The lycopene β-cyclase (*lcy*) cDNA subcloned from pCyBlue to the *Kpn*I-*BamH*I site of pGL2 (Gritz and Davies, 1983) under the control of 35S promoter and nopaline synthase terminator to yield the plasmid pTCL6. For selectable marker gene, plasmid pGL2 that carries hygromycin phosphotransferase (*hph*) under CaMV 35S promoter was used (Datta *et al.*, 1990).

Agrobacterium tumefaciens mediated transformation was done using a single binary vector pCaCar (kindly provided by Dr. Peter Beyer), which contained the same expression cassettes for *psy* and *crtI* as in case of pBaal3 together with selectable

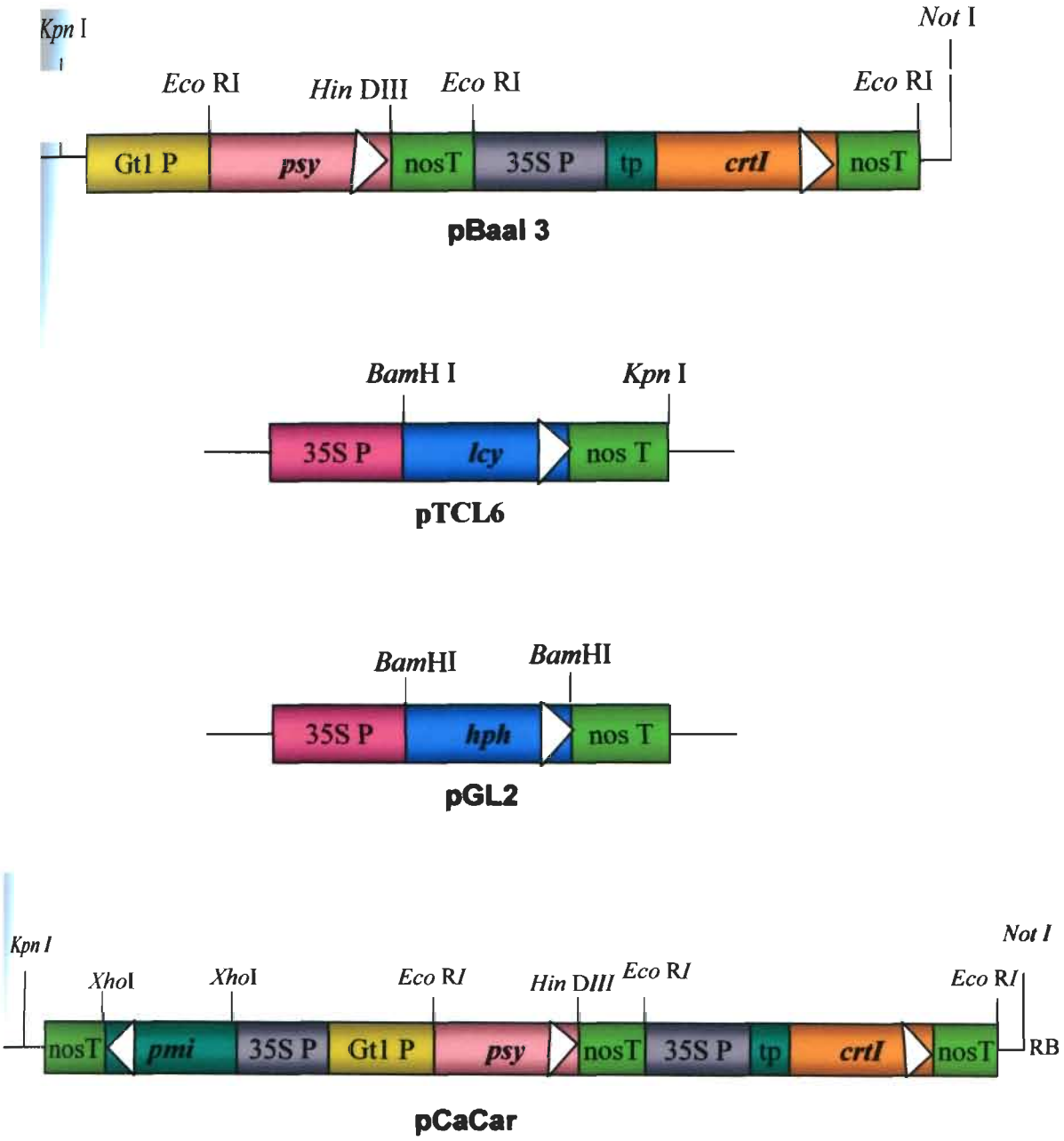


Fig.3.1. Partial maps of constructs used for transformation.

marker *pmi* gene under the control of 35S promoter. This binary vector was transformed into competent cells of *Agrobacterium* strains LBA 4404 (Hoekema *et al.*, 1984) and EHA 101 (Hood *et al.*, 1986). Partial maps of plasmids used for transformation are given in Fig 3.1.

3.3 Rice transformation

Transformation of the target varieties was done using both, biolistic as well as *Agrobacterium* mediated methods. Immature embryos and mature seeds were used as explants in all the varieties for tissue culture process. The schematic procedure depicted the generalized transformation processes is shown in Fig. 3.2.

3.3.1 Sterilization and callus induction using immature embryo

Rice panicles were collected 12 days after pollination from screen house and immature grains at milk stage were selected and rinsed with 70% ethanol for 1 minute and surface sterilized with 50% (v/v) Chlorox for 90 min in a vacuum with continuous stirring. Grains were washed with sterile distilled water three times and to remove the immature embryos from the spikelet, a gentle cut was made through the palea and lemma above the base of the floret. Using the dull edge of the scalpel, middle of the floret was gently pressed to facilitate the embryo to come out from the cut end. These immature embryos were either placed on callus induction medium MS (Murashige and Skoog, 1962) + 3% sucrose/maltose + 2mg/l 2,4-D), or were directly used as explants for bombardment after 16-18 h preculture at 27°C in a dark room (Datta *et al.*, 1990). Immature embryos were arranged with scutella side up in the center of the petridishes (90x15mm) containing 20ml MS + 30g/l of Maltose (Murashige and Skoog, 1962).

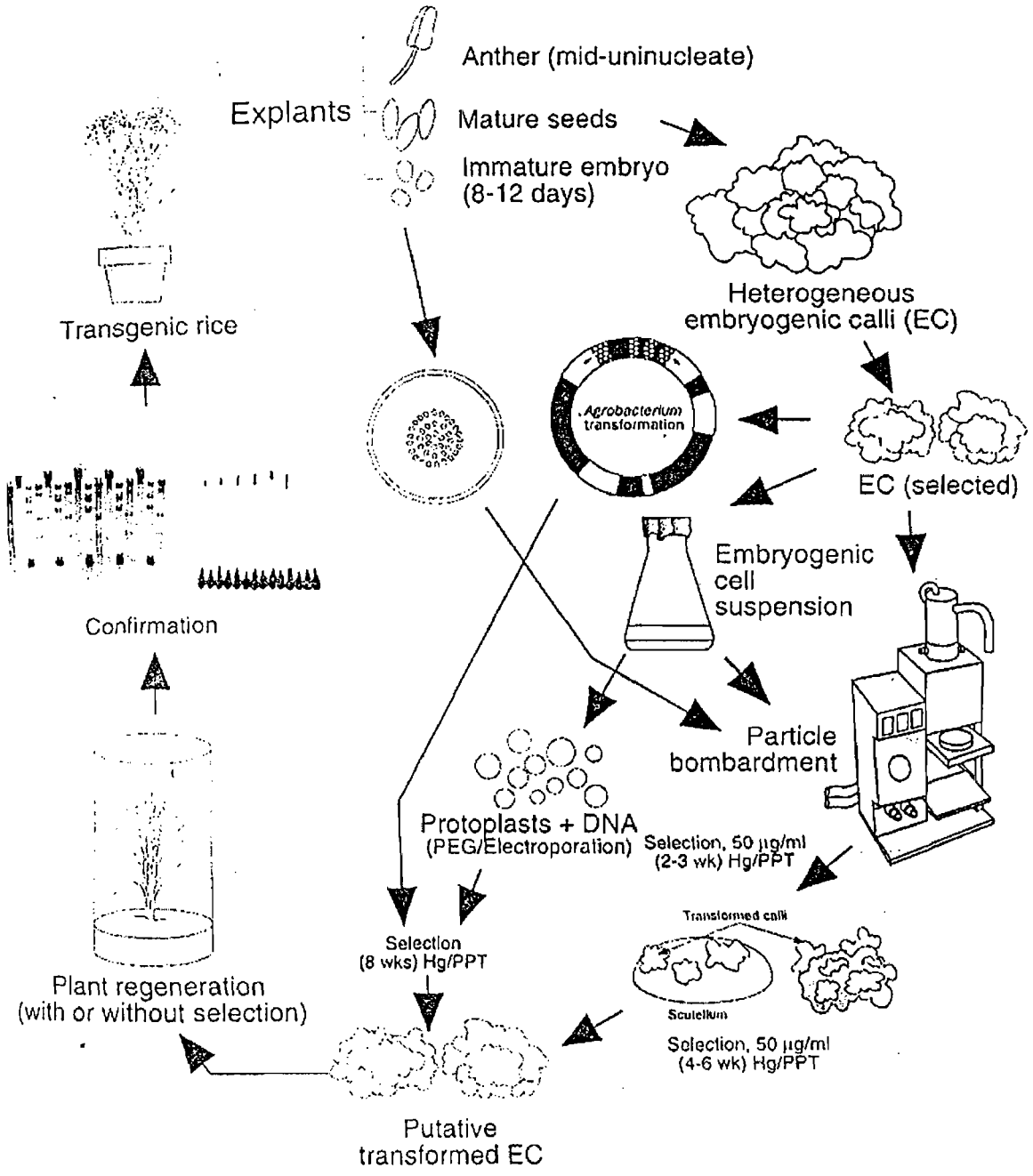


Figure 3.2: A schematic presentation of the development of transgenic indica rice using biolistic, *Agrobacterium*, and protoplast methods (Datta *et al*, 1997).

3.3.2 Sterilization and callus induction using mature seeds

Mature seeds were dehulled and washed in 70% ethanol for 1 min and then sterilized with 50% chlorox for 30 min. Seeds were washed and rinsed with sterile distilled water 3-4 times and inoculated on callus induction medium and incubated in the dark at 27°C. After 2-3 week, embryogenic calli were separated from the endosperm and inoculated onto fresh medium for callus proliferation. These were used as explants for biolistic transformation by arranging them in the osmoticum medium (MS + 30g/l maltose with 2% sorbitol and 0.2M mannitol) as described previously by Datta *et al.*, 1997. 3-4 week old embryogenic calli were also used as explants for *Agrobacterium* mediated transformation.

3.3.3 Biolistic transformation

3.3.3.1 Preparation of plasmid DNA coated with gold particles

A mixture of pGL2 : pTCL6 : pBaal3 in the ratio of 1:2:2 was used for particle bombardment. The coating of gold particles (spherical gold powder) with plasmid DNA was prepared for transformation following the method of Klein *et al.*, 1988a. The precipitation mixture consisted of 10 mg of gold particles with diameters of approximately 1.5-3.0 μM, 25 μg of plasmid DNA, 2.5 M cold $CaCl_2$ and 0.1M cold spermidine in a total volume of 350 μl. The mixture was washed for 2 minutes with 70% and 100% ethanol respectively and centrifuged at 13,000 rpm for 2-3 minutes. The DNA coated gold particles were finally suspended in 60 μl of 100 % ethanol. Ten μl of DNA-gold particle mixture was then evenly dispensed on to the center of each micro-projectile carrier disc and allowed to dry before bombardment.

3.3.3.2 Particle bombardment and selection

Immature embryos, calli derived from immature embryo or mature seeds were used for transformation. Prior to bombardment immature embryos or calli were arranged in the center of petri-plate containing bombardment media (MS + 2 % sorbitol), and were bombarded with particle gun.

The gold-particles were released with a pressure of 1300/1500 pounds per square inch (psi) of helium. After bombardment, the explants were kept in the dark at 27 °C for overnight in the same medium.

The next day, the bombarded explants were transferred to callus induction medium supplemented with 50 mg/ml Hygromycin B (Datta *et al.*, 1997). The plates were kept in dark at 27°C for two weeks, after which, the proliferating calli under selection were transferred to a fresh selection medium. This process was repeated for 4-5 selection cycles.

3.3.4 *Agrobacterium* mediated transformation

Three days prior to the cocultivation experiment, 3-4 weeks old embryogenic calli were subcultured to fresh callus induction medium to induce fresh growth of calli. *Embryogenic calli* of approx. 5mm size were selected for transformation experiments.

3.3.4.1 Growth of *Agrobacterium* strains for cocultivation experiments

Agrobacterium tumefaciens strains LBA 4404 and EHA 101 were streaked on LB agar plates supplemented with 25mg/l of chloramphenicol and were incubated at 28°C. Large single colonies were picked and grown in LB liquid medium, supplemented with 20mg/l of chloramphenicol, at 28°C overnight (20-22 hours) prior to the day of cocultivation. The culture should reach a final titer of about 1-2 x 10^9 cfu/ml (optical

density: OD600 = 0.8). Bacterial cells were washed twice with $MgSO_4$ (10 mM) by centrifugation and the optical density (OD600) of the bacterial cultures was adjusted to 0.8 by resuspending N6 liquid medium supplemented with 200μM acetosyringone (sigma-aldrich) (Hiei *et al.*, 1996; Datta *et al.*, 2002).

3.3.4.2 Cocultivation and selection

The embryogenic calli were immersed in bacterial suspension and placed into the vaccum chamber. Two cycles of vaccum (0.4-0.6 atm) with a duration of 5 min each were applied. The calli were transferred to the cocultivation medium (solid MS medium with 2 mg/l 2,4-D and 200 μM acetosyringone) after keeping briefly on Wattman filter paper to remove the excess bacterial liquid culture. The plates were incubated at 28°C in dark for three days (Datta *et al.*, 2002). After cocultivation the calli were washed thoroughly in sterile water containing 250 mg/l cefotaxime (GIBCO-BRL) and transferred to callus induction medium supplemented with 250mg/l cefotaxime to allow the calli to revive.

After 4 days the calli were transferred to *pmi* selection medium (MS + 2mg/l 2,4-D + 1% D(+) mannose + 2% sucrose) and kept in dark for 14 days. In subsequent selection cycles the mannose to sucrose ratio was increased gradually to 1.5% + 1.5%, 2% + 1% and 3% + 0.5% depending upon the response of the calli to the selection pressure.

3.3.5 Plant regeneration and cultural management of regenerants

The selected hygromycin and mannose resistant embryogenic calli were transferred to the pre-regeneration medium (MS + 1.5 mg/l BAP + 0.5 mg/l NAA + 0.5 mg/l Kinetin + 2% sorbitol). The calli were kept in the dark at 27°C for further 2 weeks. The calli were then carefully transferred to 50 ml flasks containing regeneration medium (MS +

1.5 mg/l BAP + 0.5 mg/l NAA + 0.5 mg/l Kinetin). The cultures were incubated in 16h photoperiod of 3,000-lux intensity at 26°C. Three to four week old plantlets were transferred to MS basal medium (without hormone) for rooting. The plantlets with vigorous roots were transferred to Styrofoam boards with holes in a plastic tray containing Yoshida culture solution (Yoshida *et al.*, 1976) and were kept for two weeks. The regenerants were finally transferred to the plastic pots with soil in the transgenic green house with a day/night temperature regime of 29 / 23 °C (Datta *et al.*, 1997).

3.4 Molecular analysis of transgenic plants

3.4.1 Polymerase chain reaction (PCR) analysis

The putative transgenics were screened initially for the presence of transgene(s) by polymerase chain reaction. Genomic DNA was extracted using 3-5 cm fresh leaf sample collected from the putative transgenic plants and were grounded on the ceramic plate with 400 µl of PCR extraction buffer, further 400 µl of extraction buffer was added to the leaf paste. 400 µl of extract was collected into a 1.5 ml eppendorf tube and 400 µl of 24:1 chloroform:iso-amylalcohol was added and the samples were mixed gently. The tubes were then centrifuged for 30 seconds @ 13,000 rpm. The supernatant was transferred into new eppendorf tube and 800 µl of absolute ethanol was added to each tube and after mixing well was centrifuged for 2 min @ 13,000 rpm. The pellet was washed with 70% ethanol and after drying the pellet 30-45 µl of sterile distilled water was added to the pellet, according to the relative pellet size.

For the analysis of integration of transgenes, the following primer pairs were used:

psy F: tggtggttgcgatattacga, *psy* R: accttcccagtgaacacgtc
crtI F: ggtcgggcttatgtctacga, *crtI* R: atacggtcgcgtagttttgg
lcy F: ccaatccccagaaccctaat, *lcy* R: ctcgctaccatgtaacccgt
hph F: tacttctacacagccatc, *hph* R: tatgtcctgcgggtaaat

One hundred ng of genomic DNA isolated from the putative transgenic rice plants, the negative control (non-transformed) plants, and DNA from respective transforming plasmids (positive control) were used as template for PCR. The PCR reaction mixture consisted of 100 ng of template DNA, 50 ng each of primers, 0.16 mM dNTPs, 1X PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, and 15 mM $MgCl_2$), and one unit of *Taq* DNA polymerase in a final volume of 25 µl. The PCR reaction was performed in a thermocycler (MJ Research) under following conditions:

- Step 1 (denaturation)	94°C	5 min	
- Step 2 (denaturation)	94°C	30second	36 cycles
- Step 3 (annealing)	55°C	30 second	
- Step 4 (elongation)	72°C	1 min	
- Step 5 (final extension)		5 min	

PCR products were separated in one percent agarose gel (in 1X TAE electrophoresis buffer) containing 0.5 µg/ml ethidium bromide. The separated PCR products were visualized under UV light and photographed.

3.4.2 Southern hybridization

Southern analyses were done to verify the PCR results (wherever done), and for determining the copy number and site(s) of integration of the transgenes. Genomic DNA was isolated following the method of Datta *et al.*, 1997. Leaf samples (1-2 g) were collected from green house grown plants and were shock-frozen in liquid nitrogen and grounded to a fine powder using mortar and pestle. The leaf powder was immediately transferred to 50 ml Falcon tube and was kept on ice. 15 ml of DNA extraction buffer and 2.0 ml of 20% SDS (sodium dodecyl sulphate) was added to each

tube and mixed well and incubated at 65°C for 30 minutes for cell lysis. Then 5 ml of potassium acetate was added and samples were kept at 4 °C for another 30 minutes followed by centrifugation at 4°C for 30 min at 3,500 rpm. Supernatant filtered through a miracloth was added to 10 ml of cold isopropanol in new 50 ml Falcon tube, and was mixed gently. The samples were again kept at –20°C for one hour and centrifuged at 3,500 rpm at 4°C for 30 min. Supernatant was discarded and the pellet dried for 10-20 min. The pellet was dissolved with 700 µl distilled water and was transferred to 1.5 ml eppendorf tubes, and 500 µl of 24:1 chloroform and iso-amylalcohol was added and the samples were mixed gently to remove the proteins and other impurities. The tubes were then centrifuged at 13,000 rpm at 4°C for 10 min. Upper most aqueous layer was transferred to new 1.5 ml eppendorf tube, and 75 µl of 3M sodium acetate and 500 µl of cold isopropanol was added, mixed gently until DNA precipitation and then centrifuged for 2 minutes. The pellet was washed with 70% ethanol and dried. Obtained DNA was dissolved in sterilized distilled water (100-150 µl) according to the quantity of DNA pellet. DNA concentration was checked with spectrophotometer or gel electrophoresis.

Ten µg aliquots of total DNA were digested overnight at 37°C with different restriction enzymes depending upon the purpose of the study (Table 3.1). The total volume of digestion reaction was 41 µl. One µl of each sample was checked for digestion in 1% (w/v) agarose gel in 1X TAE buffer stained with ethidium bromide (0.5 µg/ml) and visualized under UV. The digested DNA was electrophoresed at 20 Volts overnight on 1% (w/v) agarose gel. The gel was depurinated in 0.25 N HCl for 10 min followed by equilibration in 0.4M NaOH for 20 min. The DNA fragments were transferred from gel to Hybond-N^+ nylon membrane under alkaline (0.4M NaOH) denaturation conditions

(Amersham, Arlington Heights, IL) according to manufacturers instruction. The membrane was prehybridized for at least one hour in 50 ml of hybridization buffer (5X SSPE, 5X Denhardt's solution, 0.5% SDS), into which sheared single stranded salmon sperm DNA (10 μg/ml) was added for blocking. Specific PCR amplified fragments from the transforming plasmid were radiolabeled with (α-^{32}P)dCTP using the Rediprime Labeling Kit (Amersham, Arlington Heights, IL) and were used as hybridization probes. After hybridization for overnight, the membranes were washed three times each in wash 1 (2X SSC, 0.1% SDS) for 20 min followed by wash 2 (1X SSC, 0.1% SDS) for 30 min, and wash 3 (0.5X SSC, 0.1% SDS) for 15-30 min. The filters were wrapped with saranwrap after removing the excess wash solution and exposed to X-ray film for overnight at -80°C depending on the radioactive count by a Geiger muller counter.

Table 3.1: List of restriction enzymes used for Southern analysis

Enzyme	Remarks	Size (kb)
*Eco*RI	Releases diagnostic fragments for *crtI* and *psy*	*crtI*: 3.2 *psy*: 1.5
*Kpn*I	Single cutter in pCaCar; for copy No. study	Variable
*Bst*EII	Non-cutter in pCaCar; to study No. of integration loci	Variable
*Kpn*I + *Bam*I	Releases diagnostic fragment for *lcy*	1.8
*Xho*I	Releases diagnostic fragment for *pmi*	1.2
*Bam*HI	Releases diagnostic fragment for *hph*	1.1

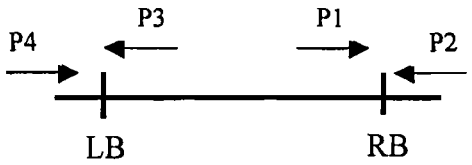
3.4.3 Detection of beyond T-DNA border transfer

To detect whether any Beyond T-DNA border transfer during *Agrobacterium* mediated transformation has occurred or not, specific primers were used to amplify T-DNA –

backbone junctions (Hoa *et al.*, 2003). Additionally, primer pair specific to chloramphenicol acetyltransferase (*cat*) gene (marker gene present in pCaCar backbone) was also used. The primer sequences used are as follows:

Right border: Primer 1: 5'cagcgtactgatgctccaag3'

Primer 2: 5'aaacctttcacgccctttt3'



Left border: Primer 3: 5'cgctattgctgaatgtggtg3'

Primer 4: 5'ctgcctgtatcgagtggtga3'

cat gene: F: 5'gcagtcgccctaaaacaaag3' and R: 5'atcacaacggcatgatgaa3'

Components for the PCR reaction were same as mentioned in section 3.6.1. After the initial denaturation step at 94°C for 5 minutes, the PCR reactions were performed for 36 cycles including 30 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C, and one minute of polymerization at 72°C. The 36 cycles were followed by final extention at 72°C for 5 min.

3.4.4 Reverse transcription PCR (RT-PCR)

Total RNA was isolated from leaves of transgenic plants using the RNAeasy extraction kit (Qiagen, Germany). RT-PCR for *crtI* was performed using the Qiagen single step RT-PCR kit. The PCR conditions were same as described in section 3.6.1, except for an additional 30 minutes reverse transcription step at 50^0C in the beginning. The amplified products were electrophoresed on 1.2% TAE-agarose gel, and photographed under UV light.

3.4.5 Total carotenoids extraction and HPLC analysis

The carotenoids extraction and HPLC analysis was performed as reported by Tan *et al.*, 2004), with some modifications. For extractions without saponification, 0.5 g of

polished seed powder was soaked in 500µl of water for 20 min. 1 ml of methanol was added to rice powder and vortexed intermittently for 10 min, after which 1ml of tetrahydrofuran (THF) was added and mixed by vortexing and sonication. The mixture was centrifuged and the supernatant was collected in a separate tube. The procedure was repeated twice, or until complete decolorization. To the pooled extract, a half volume of petrol-ether/diethyl ether (1:1) was added, followed by water until phase separation. The epiphase was dried down in a Speed-Vac dryer (Maxi Dryer Plus, Heto, Allerod, Denmark). The dried samples were again dissolved in equal volume of petroleum ether (500 µl) and a pinch of sodium sulphate was added to absorb any residual water. The samples were then centrifuged and supernant recovered. The O.D. of the samples was measured at 450 nm wavelength and the concentration of total carotenoids was calculated according to the following formula:

$$\text{Total carotenoids } (\mu g/g) = (A \times \text{volume} \times 10^4) / (2500 \times \text{sample weight})$$

Where, A = absorbance (at 450 nm); volume = 500 µl

2500 is the absorbance coefficient of carotenoids.

The samples were dried again and stored at –20°C or dissolved in 50 µl acetone for HPLC analysis (40 µl was injected).

HPLC analysis was done by using a Waters Alliance 2690 Separation Module (Waters Corporation, Milford, MA, USA) that was equipped with a Waters 996 photodiode array detector. The detector could record the spectra of elutants in a specified wavelength range and monitor the chromatogram at selected wavelengths simultaneously. The software associated with the photodiode array detection system and used to process the spectral data was the Waters Millennium[32] Chromatography Manager. Samples were separated on a Waters Symmetry C_{18} column (4.6 x 250 mm, 5 µm) after

passing a guard column of the same material (3.9 x 20 mm, 5 μm) and eluted with the following set of solvent systems.

The solvent systems used in the HPLC are: A (acetonitrile:tetrahydrofuran:water :: 10:4:6) and B (acetonitrile:tetrahydrofuran : water :: 10:8.8:1.2). The column was developed with 100% A for the first 3 minutes, then a linear gradient to 100% B in 7 minutes and it remained at 100% B for 20 minutes. The column was conditioned for 10 minutes between samples.

Peak identification was based on retention time, main absorption maxima and spectral shape (expressed as peak II-to-peak III ratio, which was the ratio of the height of the main absorbance maximum versus the height of the longest wave maximum) as compared with the corresponding standards under the same separation conditions (Table 2). Normalized spectra were compared with that of the corresponding standard for verification. Peaks were smoothed when necessary. Spectral information on each peak was obtained from the photodiode array detector.

Lutein and β-carotene standards (Sigma-Aldrich Co.) were used externally for peak identification. All chemicals, either HPLC or A.C.S. grade, were from J.T. Baker (Phillipsburg, NJ, USA). Tetrahydrofuran was stabilized with butylated hydroxytoluene (BHT, 0.01%).

3.5 Development of transgenic introgression lines

Doubled haploid transgenic japonica (T309) line (Baisakh *et al.*, 2001) was used as the donor parent for the introgression of transgenes into IR64 (recurrent parent) background. The backcrossing program is outlined in fig 3.3.

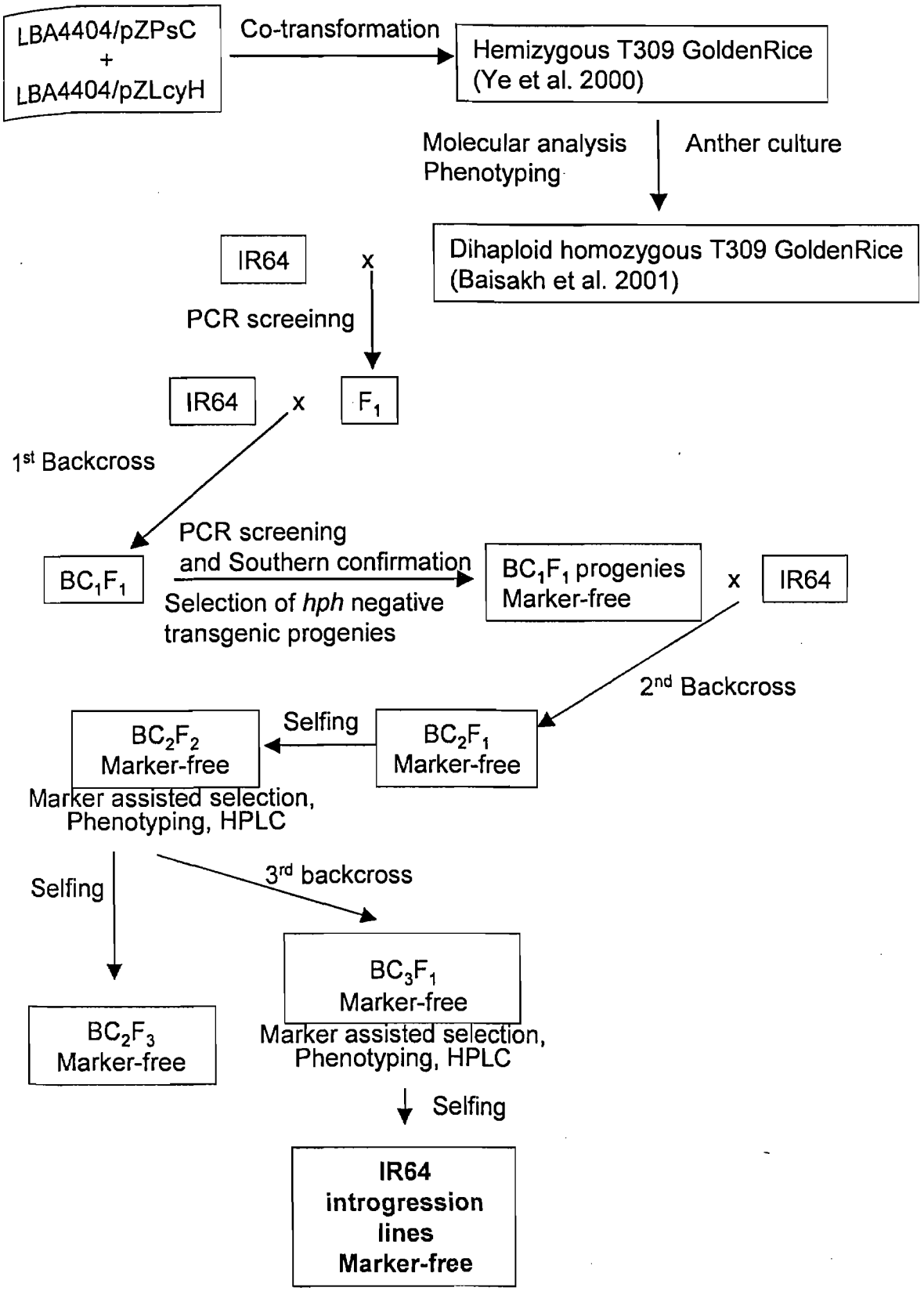


Figure 3.3: Outline of backcross breeding program.

3.5.1 Crossing

The crossing work was carried out in CL4 containment facility at IRRI. Staggered planting was done for the donor and recipient parent at one-week intervals to ensure concurrent flowering. IR64 was used as the female parent for F_1 and subsequent backcrosses. Emasculation of the female parent was done using vacuum suction in late afternoon, one day prior to the crossing day, and the panicles were bagged. The next day, the emasculated panicles were pollinated with pollen from male parent at the time of maximum anthesis (10AM-12 noon). The panicles were bagged again and the crossed seeds were harvested 25 days after pollination.

3.5.2 Marker assisted selection

Transgene introgressions were selected through PCR screening using transgene specific primers for *crtI*, and Southern analysis as mentioned in the previous sections. The background selection was performed on the basis of phenotype, assisted by molecular markers.

3.5.2.1 DNA fingerprinting of BC_2F_2 lines

The DNA fingerprinting of the BC_2F_2 progenies including the donor DH and IR64 (recurrent) parental lines were performed with the SRILS Uniprimer™ kit I (SLB, Seoul, Korea) containing twelve universal rice primers (URPs) as per the manufacturer's instruction. The PCR reaction mixture consisted of 150 ng of primer, 200 µM dNTPs mix, 1X PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 15 mM $MgCl_2$), 2.5 unit of *Taq* DNA polymerase, and 100 ng of genomic template DNA in a final volume of 30 µl. The PCR was performed in programmed thermodyne (MJ Research) 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-agrose gel and viewed under UV-transilluminator. The total number of unique scorable bands generated using 12 URPs for all the samples studied were scored as present (1) or absent (0). Genetic similarity between samples was calculated using the program similarity for qualitative data (SIMQUAL), NTSYS–PC. Clustering analysis was performed by the UPGMA method and dendrograms were generated using SHAN subroutine of NTSYS – PC (Sokal and Sneath, 1963).

3.5.2.2 Estimation of recipient genome in BC_3F_1 using SSRs

A total of 104 SSR primer pairs, spread over all the 12 chromosomes, were screened for polymorphism between T309 and IR64 (Table 3.2). The cocktail for PCR amplification of respective SSR fragments was prepared as follows:

Reaction mixture (15µl) contained:

Stock	Aliquot	Final concentration
DNA (50ng)	- 1.0 µl	50.0 ng
dNTPs (1mM)	- 1.5 µl	100.0 µM
Forward Primer (5µM)	- 0.6 µl	0.2 µM
Reverse Primer (5µM)	- 0.6 µl	0.2 µM
Taq DNA polymerase (5 units/µl)	- 0.1 µl	0.02 units
Buffer (10X)	- 1.5 µl	1X
Sterile distilled water	- 9.7 µl	
Total	- 15.0 µl	

Table 3.2 : List of SSR primers used for screening of polymorphism between T309 and IR64

Chr1	RM495	RM428	RM259	RM212	RM568	RM9	RM104	RM315	RM486		
Chr2	RM485	RM555	RM425	RM207	RM250	RM29	RM138	RM240	RM438	RM525	RM535
Chr3	RM22	RM282	RM218	RM426	RM565	RM16	RM135	RM487	RM489	RM504	RM545
Chr4	RM335	RM261	RM252	RM470	RM567	RM119	RM131	RM451	RM471		
Chr5	RM507	RM413	RM289	RM164	RM334	RM161	RM305				
Chr6	RM540	RM584	RM541	RM162	RM400	RM461	RM494	RM510	RM587		
Chr7	RM481	RM445	RM336	RM478	RM420	RM427	RM455				
Chr8	RM408	RM38	RM310	RM404	RM308	RM281	RM331				
Chr9	RM444	RM321	RM566	RM288	RM215	RM321	RM108	RM278			
Chr10	RM222	RM467	RM258	RM484	RM590	RM474	RM484	RM269	RM271	RM496	RM304
Chr11	RM206	RM229	RM260	RM286	RM457	RM536	RM287	RM441			
Chr12	RM4A	RM277	RM19	RM270	RM519	RM415					

The PCR reaction was performed in a thermocycler (MJ Research) under following conditions:

- Step 1 (denaturation)	94°C	5 min	
- Step 2 (denaturation)	94°C	45 second	35 cycles
- Step 3 (annealing)	55°C	45 second	
- Step 4 (elongation)	72°C	1 min	
- Step 5 (final extension)		5 min	

After PCR amplification, products were separated by electrophoresis on 3% agarose gels together with 1kb plus DNA marker. The gels were stained with ethidium bromide and visualized under UV.

3.6 Polishing and cooking of transgenic rice seeds

Since the amount of transgenic seeds available for carotenoid estimation was less, polishing of transgenic seeds was done in petriplates coated with emery paper, by shaking the petriplates for 10 hours on a rotary shaker. This methods avoids breakage of seeds while polishing, so that the selection of yellow seeds is easier.

To study the effect of cooking on β-carotene content in transgenic rice endosperm, 0.5g per replication of rice grains were cooked in 2ml (cook 1) and 4ml (cook 2) of water in test tubes for 17min at 100^0C. While 2ml of water was optimum for cooking, the excess water in cook 2 was drained after cooking. The total carotenoid extraction (Section 3.6.5) was performed in the same test tubes to avoid losses.

3.7 Evaluation of agronomic performance of transgenic rice

Transgenic IR64 homozygous lines were evaluated for their agronomic performance vis-à-vis non-transgenic control under screenhouse condition. The experimental plot

was divided into 10 subplots of equal size (2 m x 6 m). Eight subplots were assigned to two independent transgenic lines (four each) and two subplots were assigned to control plants. Each subplot was transplanted with 100 plants, with a distance of 25 cm between plants. Homozygosity of the transgenic progenies was confirmed by both PCR and Southern analysis. Agronomic data on nine yield component characters were recorded for ten randomly selected plants from each subplot, and were used for analysis of variance (ANOVA) using SPSS software.

Results

4.1 Genetic transformation

Five popular indica rice cultivars (BR29, IR64, IR68144, Nang Hong Cho Dao and Mot Bui) were successfully transformed using biolistic and *Agrobacterium* mediated transformation methods. The summary of the transformation experiments is presented in Table 4.1.

Immature embryos and embryogenic calli (Fig 4.1 a and b) were used as explants for transformation experiments. Both, hygromycin and mannose based selection systems proved to be efficient for rice transformation. Among the five cultivars used, BR29 was most responsive for callus induction and plant regeneration as well as for transformation. In case of *Agrobacterium* mediated transformation, strain EHA101 proved to be more efficient than LBA4404.

After four to five selection cycles under hygromycin (Fig 4.1 c and d) or mannose (Fig 4.1 e) the putative transformed calli were transferred to preregeneration medium in the dark, followed by regeneration medium under light, which induced the calli to differentiate into plantlets in about 15 days (Fig 4.2 a). These plantlets were transferred to MS basal medium for rooting (Fig 4.2 b). Plantlets with well grown roots were transferred after 15 days to Yoshida solution for hardening (Fig 4.2 c), and were subsequently transferred to soil in CL4 transgenic containment facility and grown till maturity.

Most of the primary transgenics showed normal phenotype in terms of morphological characters, fertility, etc. However, some of the plants did show abnormal phenotype and

Table 4.1: Summary of transformation experiments

Cultivar	System of transformation	Vectors/genes of interest	Selectable marker used	No. of plants analyzed	PCR/Southern positive plants
BR 29	Agrobacterium	EHA101/pCacar	*pmi*	304	221
		LBA4404/pCacar	*pmi*	263	57
	Biolistic	*psy, crtI, lcy*	*hpt*	759	396
		psy, crtI	*hpt*		12
IR 64	Agrobacterium	LBA4404/pCacar	*pmi*	100	10
	Biolistic	*psy, crtI*	*hpt*	36	3
IR 68144	Agrobacterium	LBA4404/pCacar	*pmi*	34	1
Nang Hong Cho Dao	Biolistic	*psy, crtI, lcy*	*hpt*	15	3
Mot Bui	Biolistic	*psy, crtI, lcy*	*hpt*	13	2

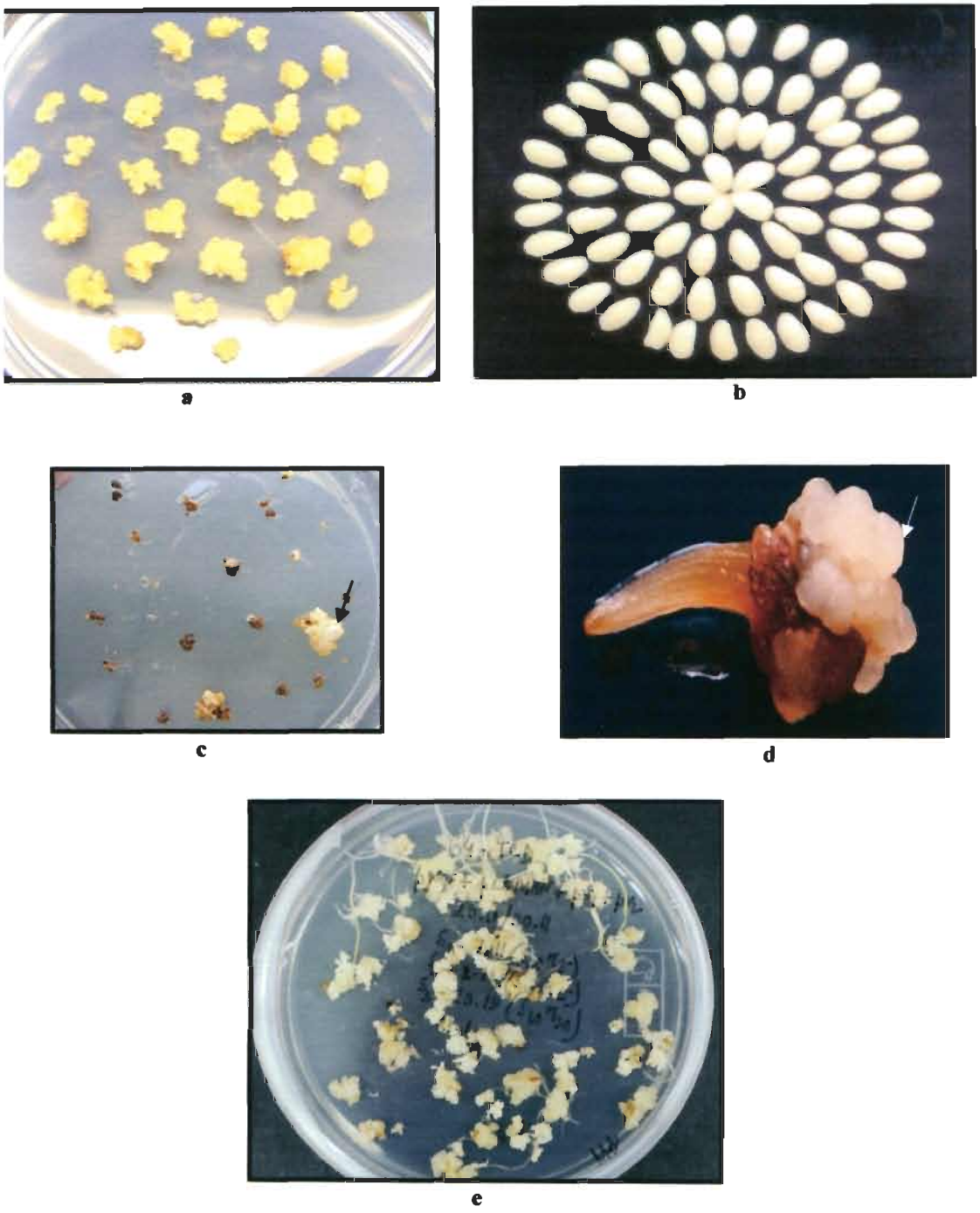


Figure 4.1: The explants used for transformation and their selection. a) four weeks old embryogenic calli for *Agrobacterium* mediated transformation; b) immature embryos arranged for particle bombardment; c and d) Putative hygromycin resistant calli proliferating under selection; e) putative mannose resistant calli in pre-regeneration medium under selection.

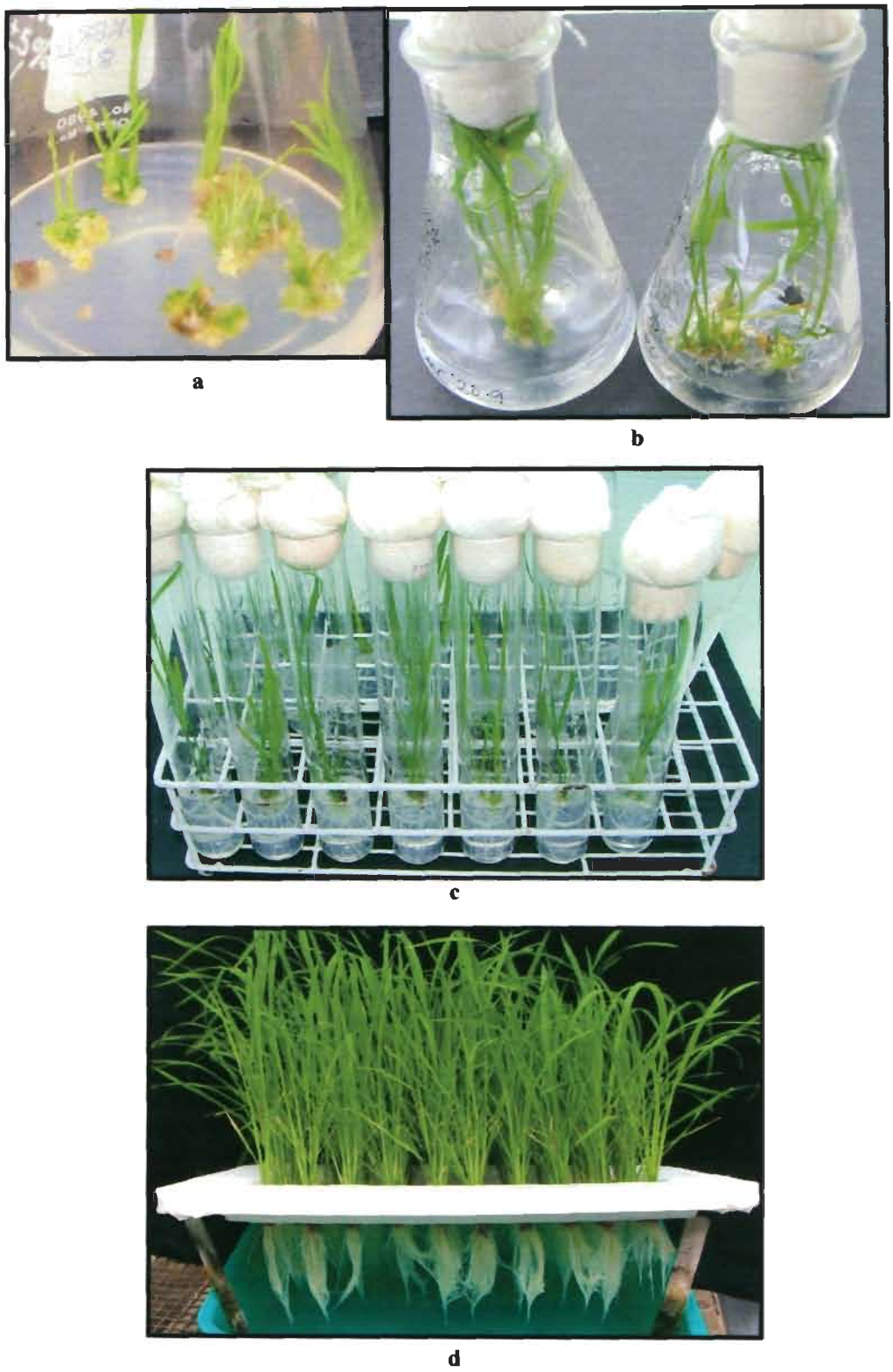


Figure 4.2: Regeneration of putative transgenic plants. a) shoot initiation in regeneration medium; b) Regeneration of plantlets; c) plantlets in rooting medium; d) hardening of putative transgenics in Yoshida solution.

Figure 4.3: Polished seeds of transgenic lines showing differential expression of transgenes.

increased sterility. These plants were not advanced to the next generation. Polished mature seeds showed yellow color, indicating the accumulation of β-carotene in the endosperm. Moreover, the transgenic lines showed differences in the intensity of seed color, indicating differential expression of transgenes (Fig. 4.3).

PCR analysis was conducted on all the putative transgenics, which was then followed by Southern analysis (data not shown) to confirm the integration of transgenes. A total of 278 BR29 transgenics were developed through *Agrobacterium* mediated transformation, using *pmi* selection. Detailed molecular and biochemical analysis of T_0 and T_1 generations was done on selected events of transgenic BR29 (designated at SKBR), which are detailed in the following sections.

4.2 Characterization of T_0 plants

4.2.1 Southern analysis

Twenty PCR positive T_0 plants were analysed by Southern hybridization for there integration pattern, copy number and number of loci by using different enzymes as mentioned in chapter 3 (Fig. 4.4). Out of these, 10 transgenics showed simple integration pattern with an expected size band (3.28 kb) for *crtI* gene, while eight transformants possessed extra bands in addition to the expected size, indicating the presence of rearranged copies of the transgene in the genome. Transgenic line number SKBR 368 was found to be negative (Fig. 4.4A). Copy number among the transformants, as determined by a single cutter enzyme *Kpn*I, ranged from single to four copies. The maximum number of copies (4) were detected in line SKBR 378 (Fig. 4.4B). The number of independent transgenic loci, as detected by the non-cutter enzyme *Bst*EII, also ranged from one to four, and again SKBR378 was the line having *crtI* integrated at four loci (Fig. 4.4C). When the same blots were stripped and

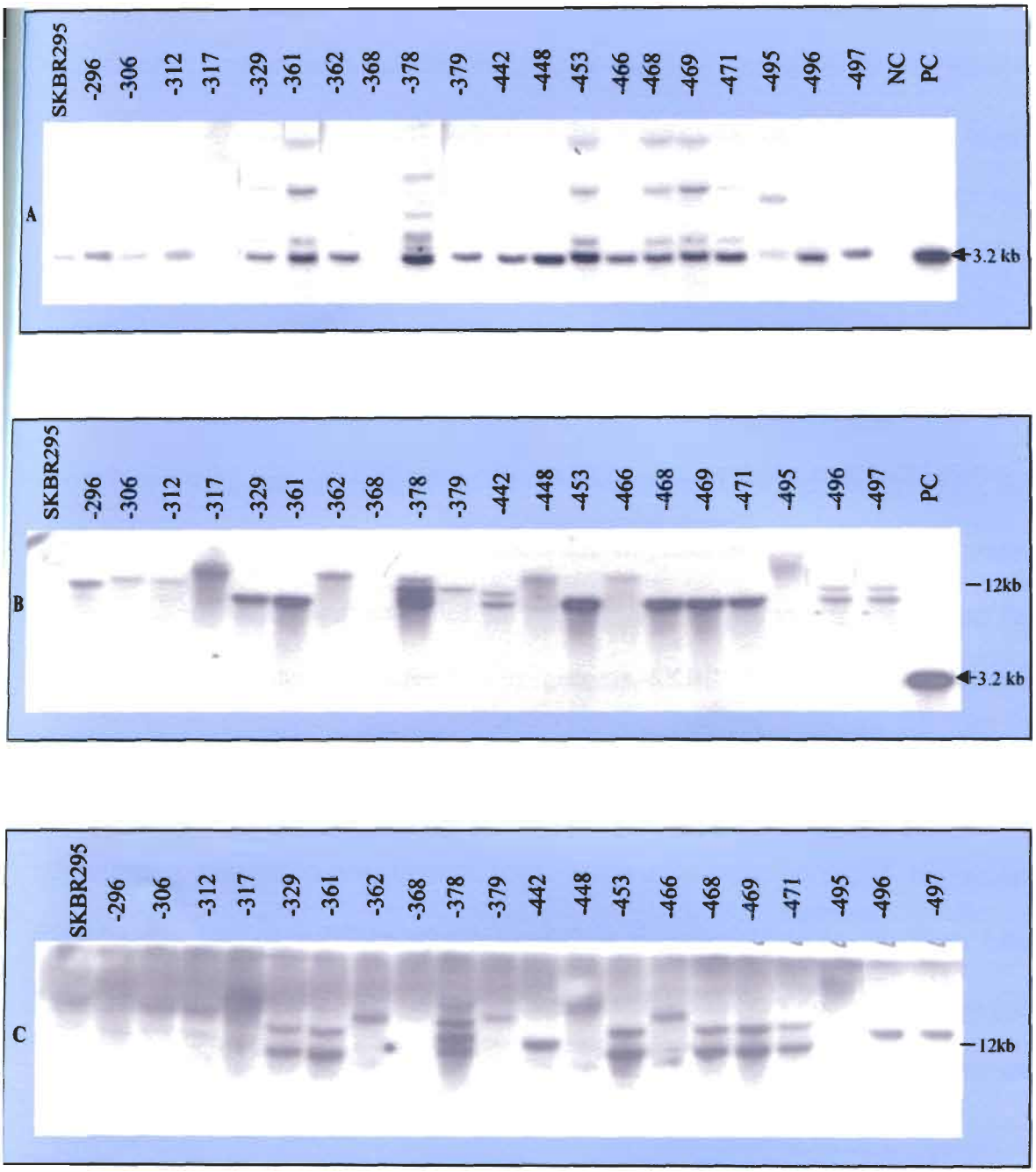


Figure 4.4: Southern blot analysis of T_0 plants for *crtI* gene. DNA digested with A) *Eco*RI for integration pattern; B) *Kpn*I for copy number; C) *Bst*EII for sites of integration and hybridized with P^{32} labelled PCR amplified *crtI* probe.

hybridized with the *psy* probe (Fig. 4.5), only a single band of expected size (1.5 kb when digested with *EcoR*I) was observed in all the lines, except in line SKBR448, where one extra band was present, indicating the presence of rearranged copies of *psy* (Fig. 4.5A). The number of integration loci for *psy* was found to be similar to that observed in case of *crtI* (Fig. 4.5B).

4.2.2 Detection of beyond T-DNA border transfer

The T_0 plants were analyzed for any beyond T-DNA border transfer, which may have taken place during transformation (Fig. 4.6). Primers specific for right border (RB), left border (LB) and *cat* gene were used for PCR amplification. Out of 20 T_0 plants analyzed, SKBR378 showed amplification with all the three primers, indicating that the whole plasmid may have integrated into the genome. SKBR379 showed the integration of only right border. Interestingly, two lines, SKBR317 and 448 possessed *cat* gene but did not give any amplification with LB and RB primers. This result was confirmed by rehybridizing the membrane used in Fig. 4.4C with PCR amplified *cat* probe. Hybridization was observed in all the three PCR positive plants for *cat* gene. Line SKBR378 in this blot also showed that *cat* was integrated only at one locus, while *crtI* integrated at four loci. Results of molecular characterization of T_0 plants are summarized in Table 4.2.

4.2.3 Carotenoid quantification

The total carotenoid level in polished seeds of the T_0 plants analyzed ranged from 0.338 μg/g in SKBR466, to a maximum of 1.168 μg/g in SKBR361. The β-carotene level, as determined by the percentage area of the peak, ranged from 0.125 μg/g in SKBR471 to 0.558 μg/g in SKBR361. HPLC peaks corresponding to β-cryptoxanthin, α-carotene

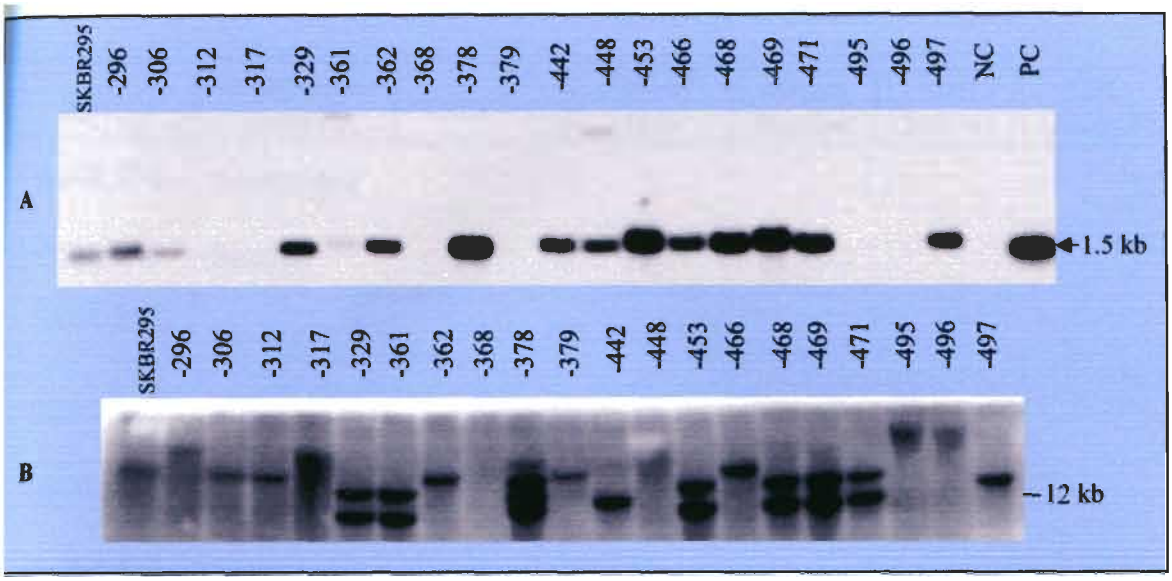


Figure 4.5: Southern blot analysis of T_0 plants for *psy* gene DNA digested with A) *Eco*RI for integration pattern; B) *Bst*EII for sites of integration and hybridized with P^{32} labelled PCR amplified *psy* probe.

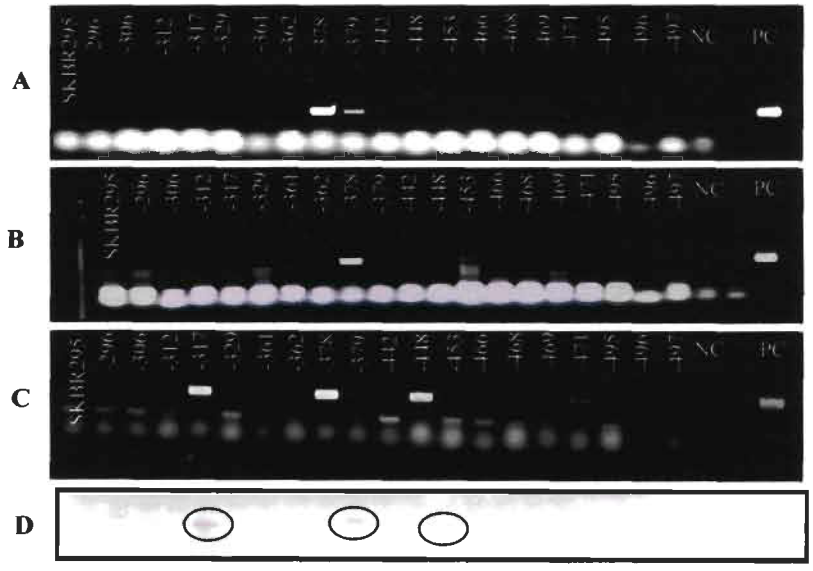
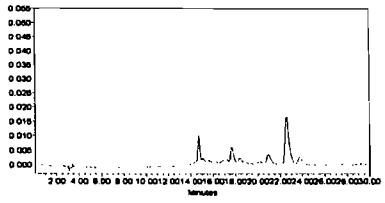
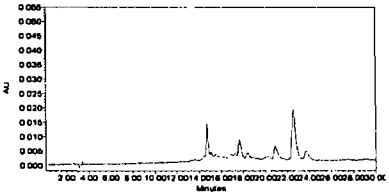
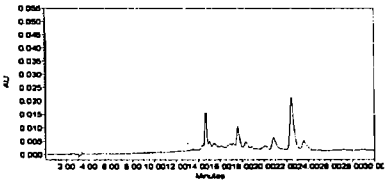
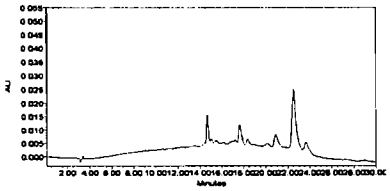
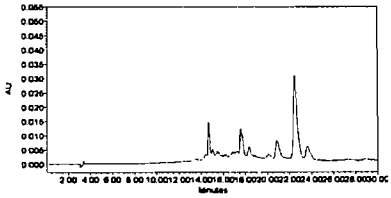
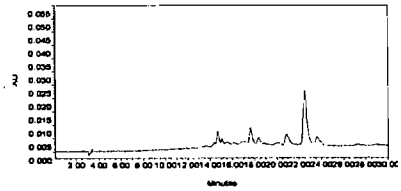
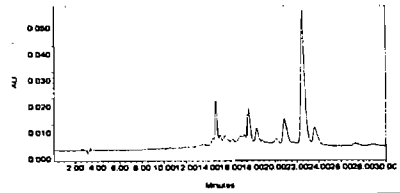


Figure 4.6: PCR analysis to detect beyond border transfer. A) Right border; B) Left border; C) *cat* gene; D) Southern blot hybridized with radiolabelled *cat* gene probe (lines SKBR317, 378, 448 showing hybridization).

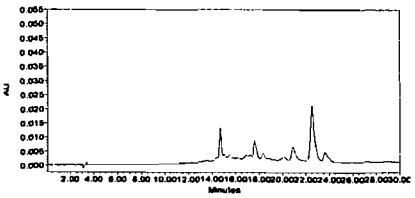
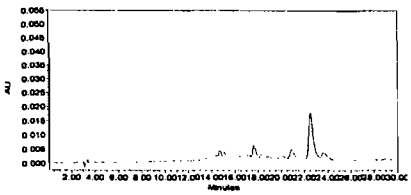
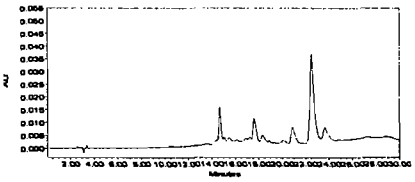
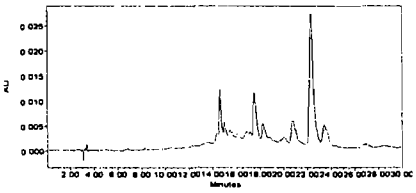
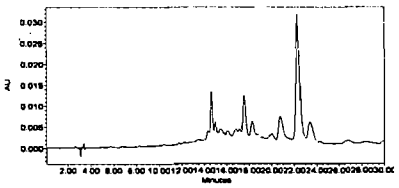
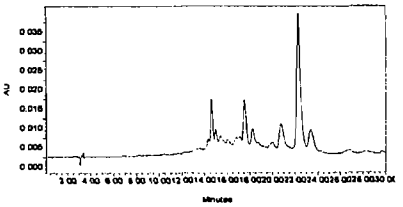
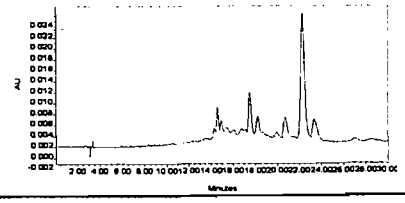
Table 4.2: Summary of molecular characterization of T_0 plants

Line	Integration pattern	Copy No.	No. of loci	Right border	Left border	Cat marker
SKBR 295	Rearranged	1	2	–	–	–
SKBR 296	Simple	2	2	–	–	–
SKBR 306	Simple	1	2	–	–	–
SKBR 312	Simple	1	2	–	–	–
SKBR 317	--	1	3	–	–	+
SKBR 329	Rearranged	1	2	–	–	–
SKBR 361	Rearranged	1	2	–	–	–
SKBR 362	Simple	1	1	–	–	–
SKBR 378	Rearranged	4	4	+	+	+
SKBR 379	Simple	1	1	+	–	–
SKBR 442	Simple	2	1	–	–	–
SKBR 448	Simple	1	1	–	–	+
SKBR 453	Rearranged	1	2	–	–	–
SKBR 466	Simple	1	1	–	–	–
SKBR 468	Rearranged	1	2	–	–	–
SKBR 469	Rearranged	1	2	–	–	–
SKBR 471	Rearranged	1	2	–	–	–
SKBR 495	Rearranged	--	--	–	–	–
SKBR 496	Simple	2	1	–	–	–
SKBR 497	Simple	2	1	–	–	–

Table 4.3: HPLC chromatograms of T_1 seeds showing carotenoid profile

Name	Total Carotenoi (ug/g)	HPLC (450nm)	Beta-Carote. (ug/g) by curve	β-crt peak %	β-cry + α-crt %	Lutein Peak %
SKBR-295	0.568		0.182	44.59	19.85	13.59
SKBR-296	0.756		0.194	38.52	19.51	14.39
SKBR-306	0.628		0.210	39.86	21.04	14.57
SKBR-312	0.632		0.255	46.63	19.37	11.32
SKBR-317	0.860		0.313	40.67	19.83	9.87
SKBR-329	0.536		0.214	49.84	19.42	7.44
SKBR-361	1.168		0.558	47.84	17.93	8.25

Contd....

SKBR-362	0.588		0.209	43.80	20.68	13.34
SKBR-378	0.452		0.192	54.72	18.61	4.55
SKBR-379	0.904		0.383	47.85	17.69	10.36
SKBR-453	0.56		0.14	41.58	18.71	9.64
SKBR-466	0.388	—	—	—	—	—
SKBR-468	0.628		0.153	42.01	19.27	9.32
SKBR-469	0.64		0.186	41.97	20.38	8.52
SKBR-471	0.50		0.125	48.78	18.41	6.43

and lutein could also be identified in the chromatogram. Per cent area of β-cryptoxanthin + α-carotene, that also have provitamin A activity, ranged from 17.69 to 21.04. The carotenoid profiles of T_0 plants are summarized in Table 4.3.

4.3 Analysis of T_1 progenies

A separate set of 15 SKBR lines was selected for analysis of transgene behaviour in the segregating T_1 progenies. HPLC analysis was done on T_1 seeds except for lines SKBR1, 12 and 13, where expression was studied in T_1 progenies T_2 seeds. These three lines were studied in more detail, and are discussed separately.

4.3.1 T_1 Progeny analysis of SKBR1, 12, 13

The PCR analysis of T_1 progenies of line SKBR1 showed a segregation ratio of 15:1 (9:3:3:1) for *crtI* (Fig. 4.7). The same segregation pattern was observed in progenies of SKBR13, suggesting the presence of at least two unlinked *crtI* loci. However, for SKBR12, out of 60 T_1 plants, only 10 were positive for *crtI*. The southern analyses for integration pattern, copy number and site of integration confirmed that SKBR1, 12 and 13 were clones. Although, SKBR12 was derived from the same transformed event designated as SKBR1 and 13, it was showing unique meiotic behavior. Southern analysis of SKBR13 T_1 progenies showed the presence of rearrangements (Fig. 4.8A) with at least two intact copies of *crtI* (Fig. 4.8B), which were integrated at two separate loci in the genome (Fig. 4.8B). Genetic recombination between these loci resulted in the presence of non-parental types in the progeny. Out of 83 (PCR positive) SKBR1 and 13 T_1 progenies analyzed by southern, 57 showed parental type (having both the loci), 15 had locus 1 only, while 11 plants carried only locus 2. This data showed that two unlinked loci were integrated in this event. Close examination of the T_1 Southern blots

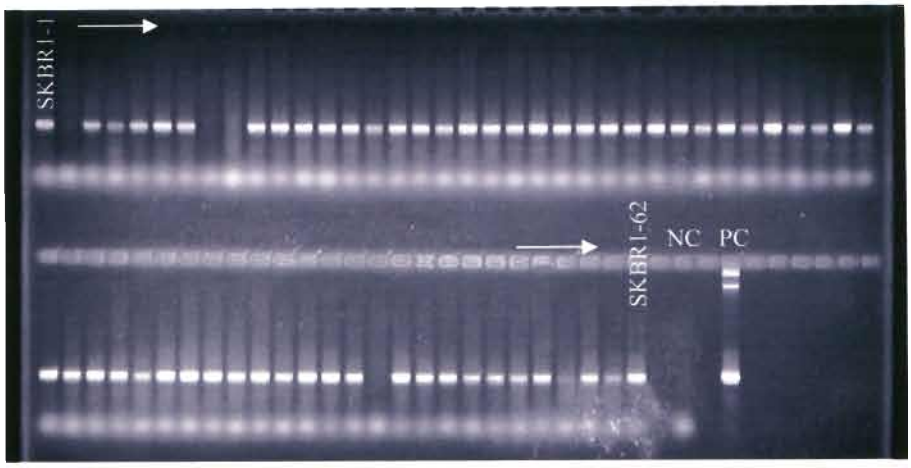


Figure 4.7: PCR analysis of SKBR 1 T_1 progeny showing a segregation ratio of 15:1 NC =negative control; PC = positive control

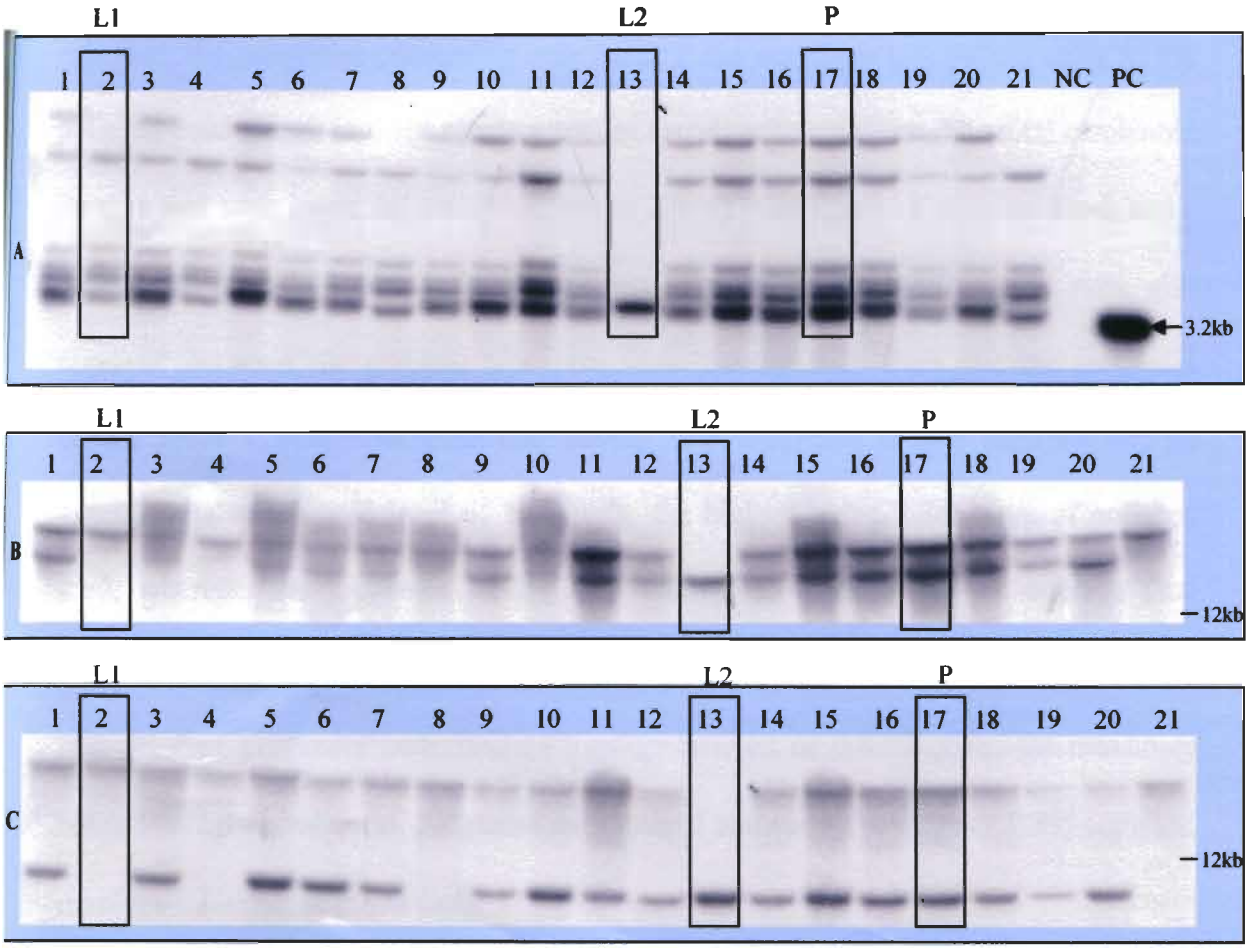


Figure 4.8: Southern blot analysis of SKBR 13 T_1 progeny plants showing independent assortment of two transgenic loci. L1: locus one; L2: locus 2; P: parental. DNA was digested with A) *EcoR*I for integration pattern; B) *Kpn*I for copy number; C) *BstE*II for sites of integration and hybridized with P^{32} labelled PCR amplified *crtI* probe.

for *crtI* elucidated that locus 1 constituted of an intact copy with some rearrangements, and locus 2 had a single copy with no rearrangements.

The nitrocellulose membranes were stripped and rehybridized with *psy* and *pmi* probes (Fig. 4.9). Interestingly, Southerns for *psy* showed that it had integrated at locus 1 but not at locus 2, indicating that at locus 2, T-DNA transfer was not complete during the transformation event. Selectable marker *pmi* was found to be present at both the loci.

At maturity, the T_1 plants were harvested separately, and polished seeds of selected plants were analyzed for their total carotenoid and β-carotene content (Fig. 4.10). As expected, the progenies possessing only locus 2 did not show any yellow color in the polished endosperm, as these plants were not carrying the *psy* gene. The total carotenoid level in one such plant, SKBR12-36, was as low as 0.162 μg/g of polished seed, and a β-carotene level of 0.008 μg/g of polished seed. Significant differences were also observed between the parental type progenies (having both loci) and the progenies having locus 1 only. The progenies having *crtI* integrated at two sites showed an average total carotenoid level of 1.81 μg/g, the highest being 2.268 μg/g of polished seed, whereas, the progenies having only transgenic locus 1 had an average total carotenoid level of 1.124 μg/g, with a high of 1.48 μg/g. Similarly, for β-carotene, the parental type progenies possessed an average amount of 0.8272 μg/g, the maximum being 1.067 μg/g, whereas, progenies with locus 1 showed an average of 0.399 μg/g and maximum amount of 0.678 μg/g.

4.3.2 Analysis of other T_1 progenies

Twelve T_1 progenies, apart from SKBR1, 12 and 13, were grown in the transgenic screenhouse to study their segregation pattern. PCR analysis was done on about 50

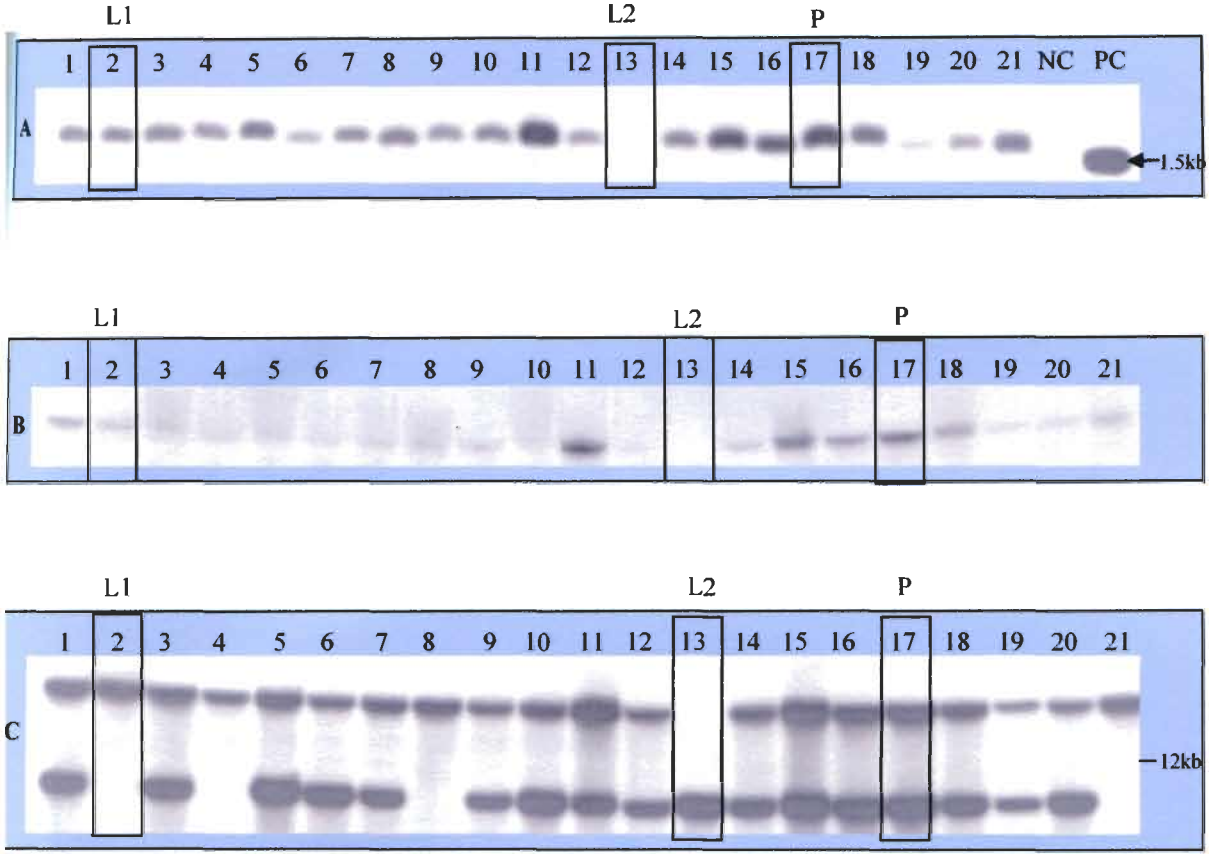
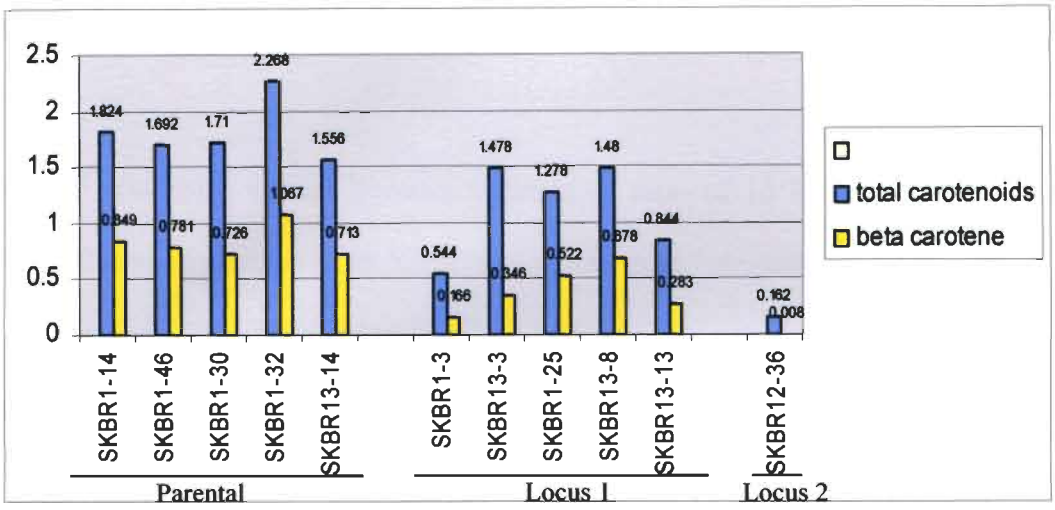
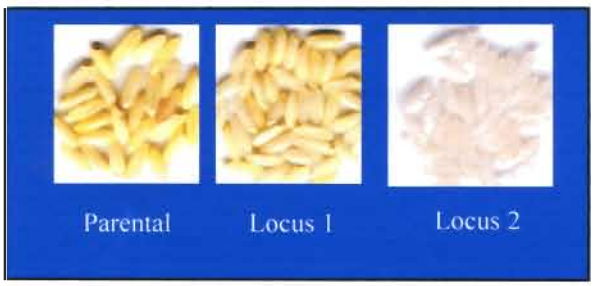


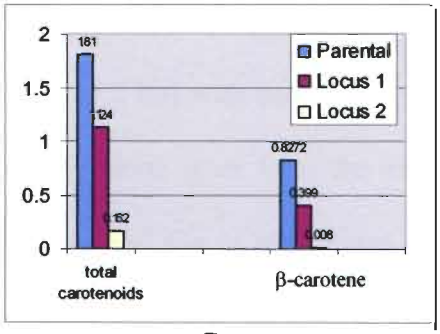
Figure 4.9: Southern blots showing A) expected size of *psy* gene (DNA digested with *Eco*RI); B) copy number of *psy* gene (DNA digested with *Kpn*I); and integration loci for *pmi* gene (DNA digested with *Bst*EII) in the T1 progeny of SKBR 13.



A



B



C

Figure 4.10: Estimation of carotenoid accumulation in the seeds of T_1progenies of SKBR1 and 13. A) Total carotenoid and β-carotene levels in polished seeds of individual T_1 plants. B) Polished seeds of SKBR13 T_1 progeny plants showing segregating differential yellow color. C) Average carotenoid and β-carotene levels in three types of T1 progenies.

plants from each progeny to study the segregation ratio, followed by Southern analysis using different enzymes to study their segregation pattern in terms of integration, copy number and number of loci of the transgenes (Fig. 4.11). Simultaneously, the T_1 seeds were also used for the total carotenoids and β-carotene quantification through HPLC (Table 4.4).

SKBR53 showed a typical bi-locus segregation ratio of 15:1. Southern blot analysis indicated it to be a clone of SKBR13, having the same integration pattern, copy number and two segregating loci as in case of SKBR13 T_1 progeny. However, the HPLC analysis of T1 seeds resulted in a total carotenoid level of 0.984 μg/g and a β-carotene level of 0.381 μg/g of polished seed which was significantly less than that of SKBR 13.

PCR analysis of SKBR56 progeny gave a positive to negative ratio of 37:10 (~3:1). For Southern, out of 22 T_1 progeny plants analyzed for integration pattern, most of them showed a parental type simple integration for *crtI* gene (3.2 kb) with no rearrangements except for three plants which had some rearranged fragments apart from the expected 3.2 kb band. Interestingly, the Southern for copy number and site of integration did not show any variation between the two types of progenies. Two copies and two sites of integration could be detected in all the progenies when the DNA was digested with *Kpn*I and *BstE*II, respectively. This indicated that the rearrangements were taking place within locus, and were not affecting the overall size of the locus/loci. Also, the two loci were tightly linked and behaving as a single transgenic locus in terms of segregation. The total carotenoid and β-carotene levels in T_1 polished seed were found to be 0.444 and 0.146 μg/g, respectively.

SKBR89 T_1 progenies showed a segregation ratio of 25:27 (positive : negative) which suggested that the transgenic locus/loci was being "knocked out" during meiosis, which

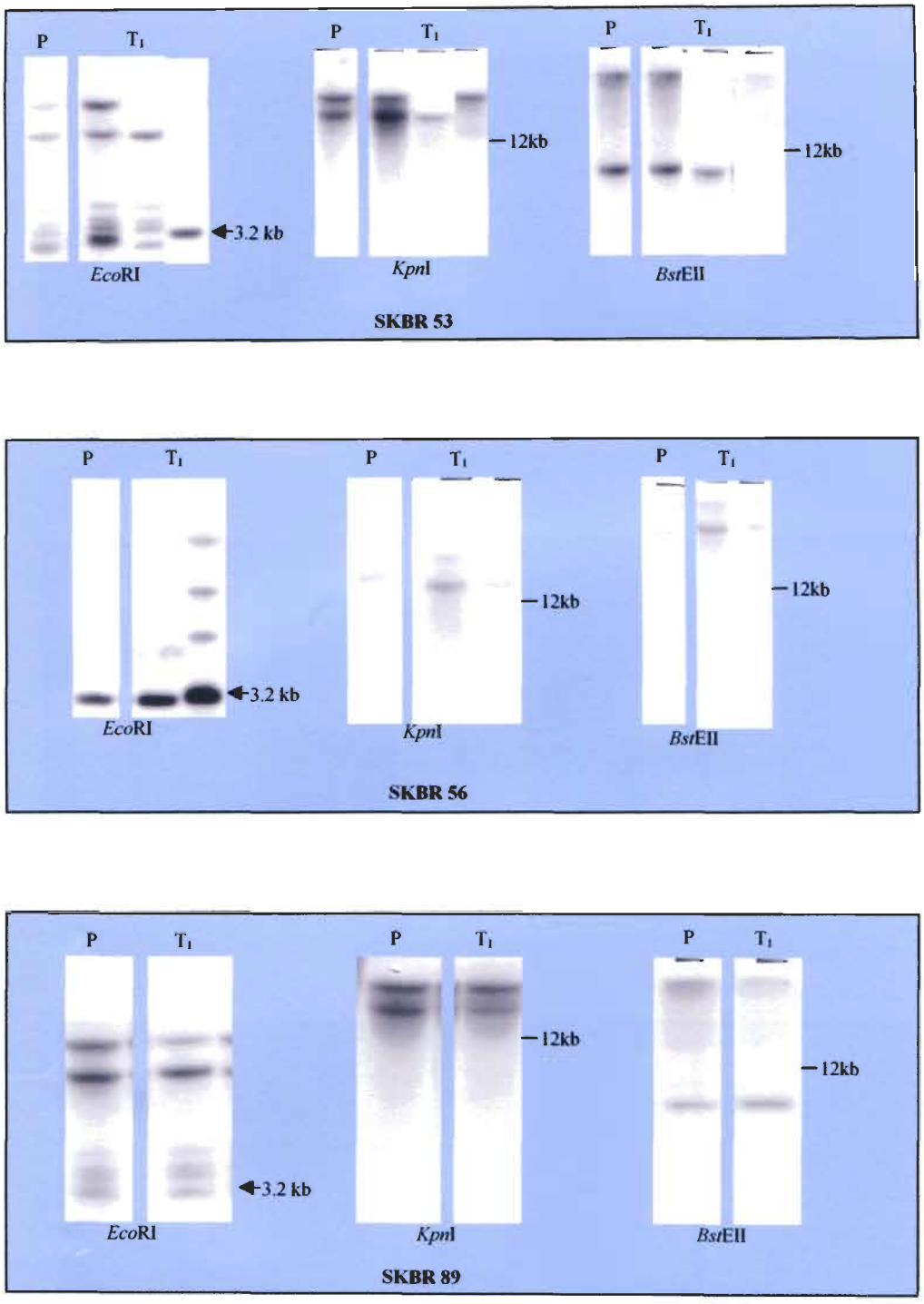
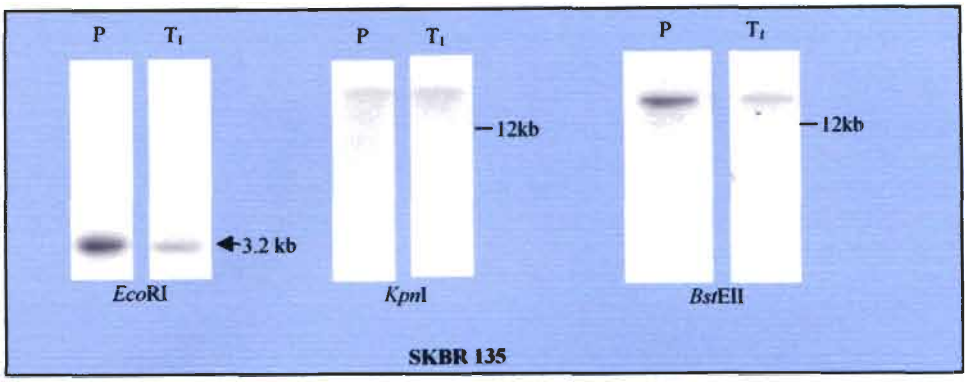
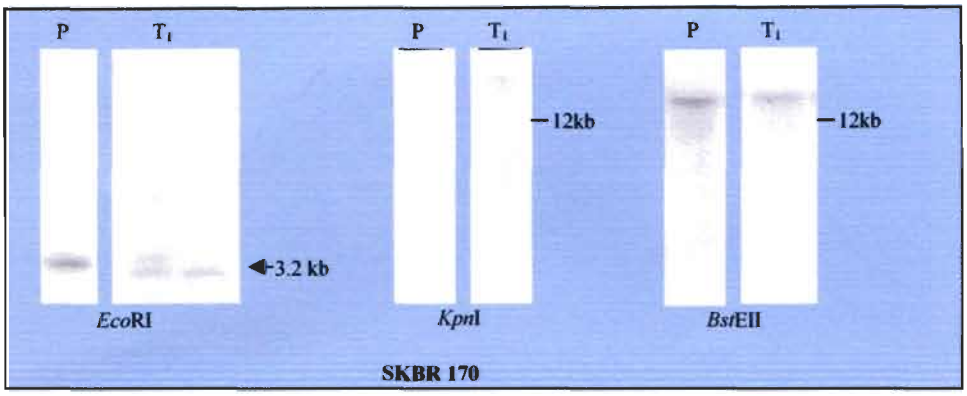


Figure 4.11: Southern analysis of T1 progenies for integration pattern (*Eco*RI), copy number (*Kpn*I), site(s) of integration (*Bst*EII). P = parental banding pattern; T_1= banding pattern(s) found in T_1 progenies.





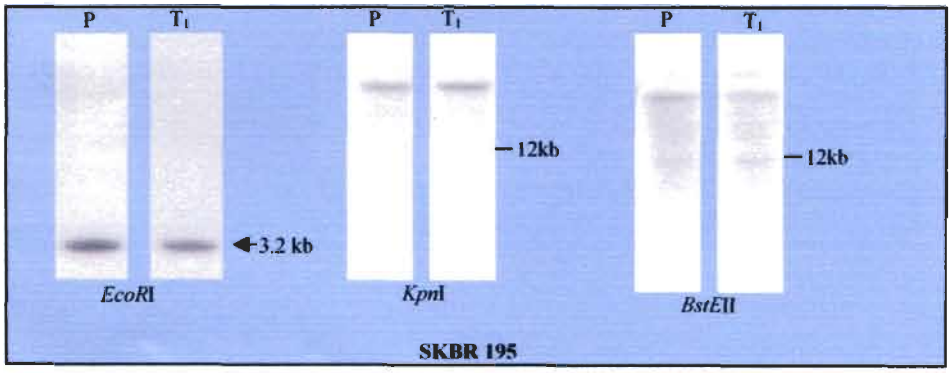
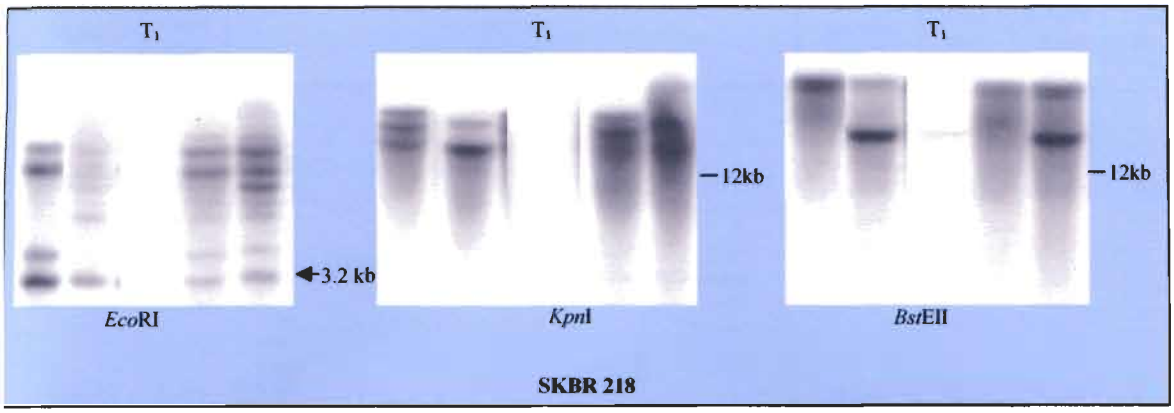
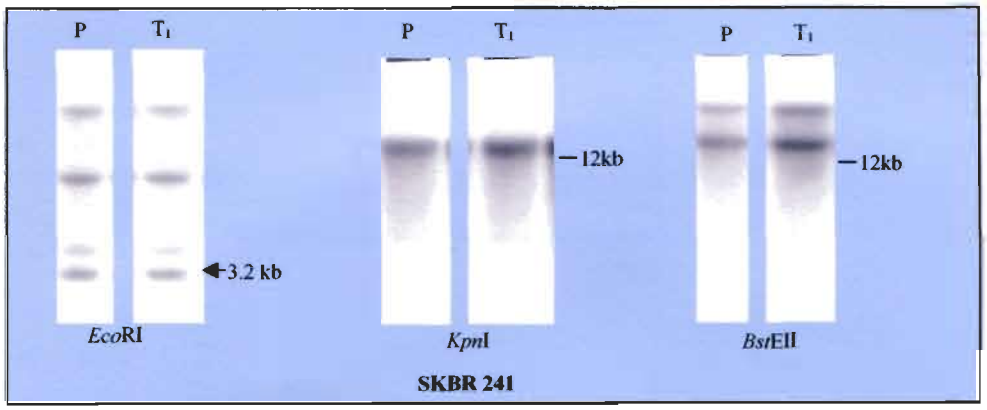


Figure 4.11: continued…





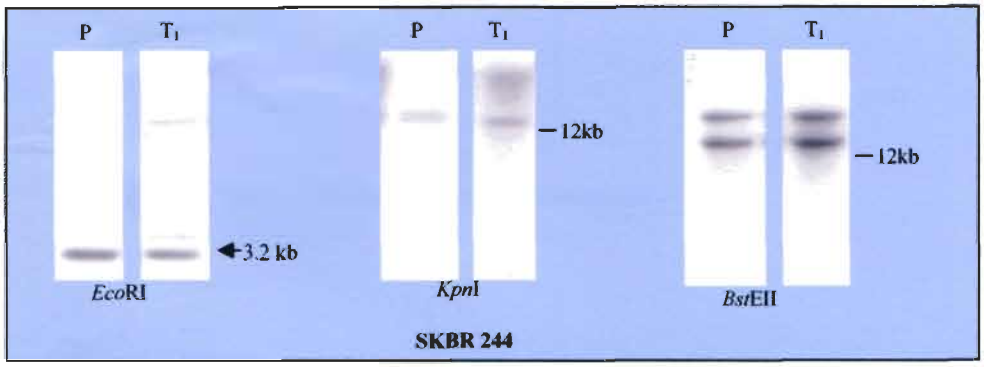
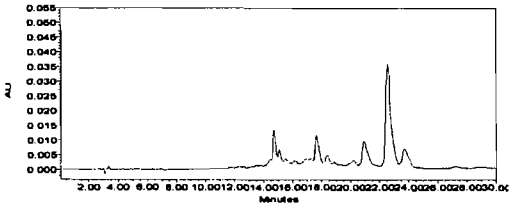
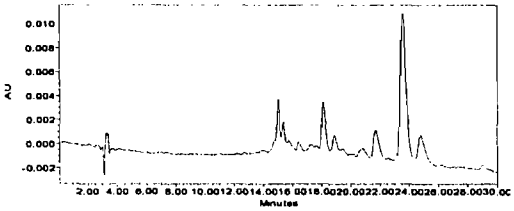
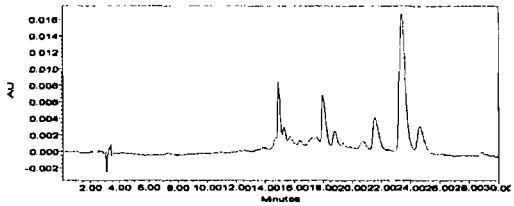
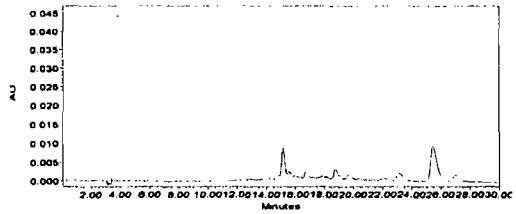
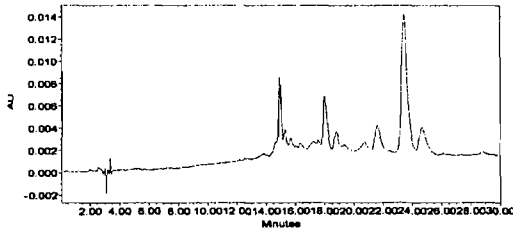
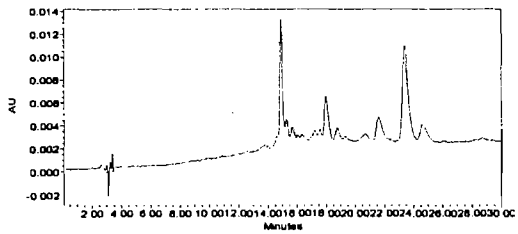
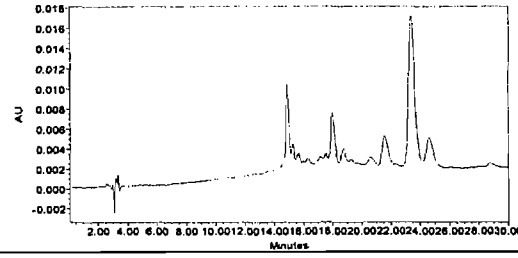
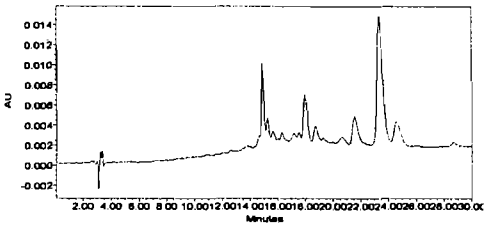
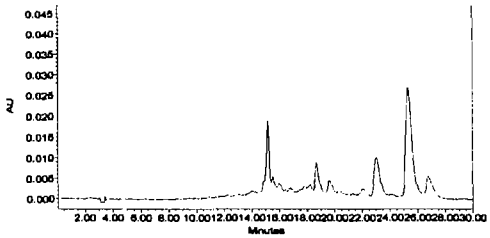
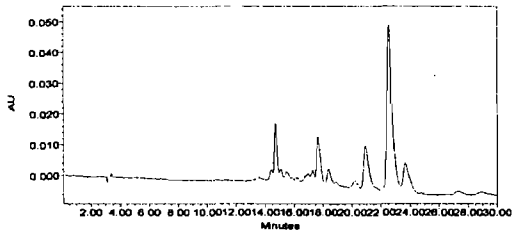
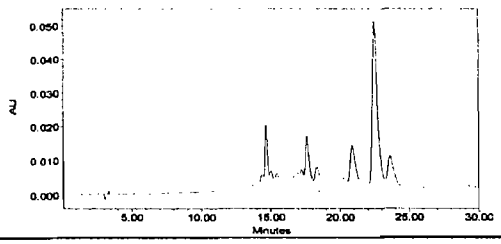


Figure 4.11: continued…

Table 4.4: HPLC analysis of T_1 seeds showing carotenoid profile

Name	Total Carot enoi (ug/g)	HPLC (450nm)	Carote. (ug/g) by curve	β-crt peak %	β-cry + α-crt %	Lutein Peak %
SKBR-53	0.984		0.381	41.15	19.39	8.60
SKBR-56	0.444		0.146	53.46	21.86	8.86
SKBR-61	0.64		0.21	46.53	20.75	10.51
SKBR-89	0.536		0.134	–	–	–
SKBR-135	0.464		0.156	50.02	19.54	12.12
SKBR-136	0.404		0.1	39.13	20.02	23.74
SKBR-170	0.584		0.197	49.13	18.54	12.16

Contd....

SKBR-195	0.556		0.161	45.53	19.36	13.12
SKBR-218	1.312		0.328	–	–	–
SKBR-241	1.584		0.612	44.95	19.34	7.78
SKBR-244	1.004		0.550	44.28	19.42	8.24

may be possible as the transgene locus/loci were still in a hemizygous state. Southern blot analysis showed that all the T_1 progenies analyzed, possessed the parental banding pattern when the DNA was digested with *EcoRI*. In all the progenies analyzed for Southern, two copies and two integration sites could be detected which were not assorting independently. The polished T_1 seeds had a total carotenoid and β-carotene level of 0.536 and 0.134 μg/g, respectively.

The 64 T_1 plants of SKBR135 analyzed for PCR gave a segregation ratio of 46 positive:18 negative, which suggested the presence of a single transgenic locus. This was confirmed by Southern analysis, which showed a single expected size band for *crtI* when the DNA was digested with *Eco*RI. Southerns with *Kpn*I and *Bst*EII elucidated that all the progenies had single copy and a single site of integration for *crtI*. The total carotenoid level in polished T_1 seeds was 0.464 μg/g and β-carotene amount was 0.156 μg/g.

Similar to SKBR135, T_1 progenies of SKBR170 showed a segregation ratio of 46:16 (3:1). Southern analysis with enzymes *Kpn*I and *BstE*II revealed that the progenies had one copy that was integrated at a single site in the genome. However, Southern using *EcoR*I revealed that one of the progenies had rearrangements within the transgenic locus, which was not changing the overall size of the locus; a case similar to the three progeny plants of SKBR 56. The carotenoids and β-carotene levels in the T_1 seeds of SKBR 170 were found to be 0.584 and 0.197 μg/g, respectively.

T_1 progenies of SKBR195 showed a nonMendelian segregation ratio of 37:20. Southern analysis with *EcoR*I revealed a single expected size band for *crtI* in all the progeny plants. All progenies were found to have two copies of *crtI* . The polished T_1 seeds had a total carotenoid and β-carotene level of 0.556 and 0.161 μg/g, respectively.

SKBR218 T_1 progenies gave a positive : negative segregation ratio of 26:24, which indicated that the transgenic locus/loci were unstable in the hemizygous condition, and were being knocked out during meiosis. This was confirmed by Southern analysis that showed five different types of banding patterns in the PCR positive progenies analyzed. The copy number and integration loci varied from one to three among the progenies. It could not be established whether this variation was due to the independent segregation of the transgenic loci or because of the rearrangements within the transgenic locus, as the population size analyzed by Southern was small. However, the total carotenoid and β-carotene levels in the T_1 seeds were found to be quite high, i.e. 1.312 and 0.328 μg/g, respectively.

PCR analysis of SKBR241 and 244 T_1 progenies revealed similar segregation ratios (33:18 and 37:19, respectively) and their Southern analysis also showed similar banding patterns. Both progenies showed rearrangements in addition to the expected size of *crtI*. A single copy and two sites of integration could be detected in both the progenies and were of same size, which confirmed that they were clones. Further, it may be possible that at one locus, only rearranged copies of *crtI* were present, which could not be detected by *Kpn*I. The two loci were not found to be segregating independently in the progeny. The total carotenoid levels in the polished T1 seeds of SKBR241 and 244 were found to be 1.584 and 1.004 μg/g, respectively, while the amount of β-carotene was 0.612 and 0.550 μg/g, respectively.

4.3.3 Reverse Transcriptase PCR (RT-PCR)

RT-PCR analysis was conducted using one representative positive sample from each of the T1 progenies, to compare these lines in terms of transcription levels (Fig. 4.12).

Differential cDNA amplification was observed between the lines. In most of the cases, the amount of cDNA amplified was consistent with the level of total carotenoids in the seeds.

4.3.4 Detection of beyond T-DNA border transfer

To detect any beyond T-DNA border transfer that may have taken place during transformation, PCR reactions were carried out using specific primers to amplify right border, left border and *cat* gene fragments (Fig. 4.13). The results revealed that in transgenic lines SKBR56, 57 and 61, all the three fragments were amplified, indicating that the whole plasmid may have integrated into the genome. Also, in SKBR1, 53 and 89, only the fragment specific to right border could be detected. In all the other lines analyzed, the T-DNA transfer was precise, as no amplification could be detected with all the three sets of primers.

4.4 Development of IR64 introgression lines with β-carotene biosynthetic pathway in the endosperm through marker assisted backcross breeding

Staggered planting of T309 double haploid transgenic material (donor parent) and IR64 was done in order to achieve concurrent flowering. Sufficient number of crosses were made and a large number of seeds were produced from the cross between T309 DH transgenic donor line and IR64. One hundred cross seeds were germinated in petri dishes of which six did not germinate and the rest of the seedlings were subsequently grown in the pots in the containment greenhouse.

4.4.1 F_1 and Backcross progeny analysis

The transgenic status of F_1 was confirmed through PCR for CrtI(data not shown). The F_1 plants were also clearly evident from their intermediate morphological identity. However many of the F_1 plants possessed high sterility, resulting in poor seed setting.

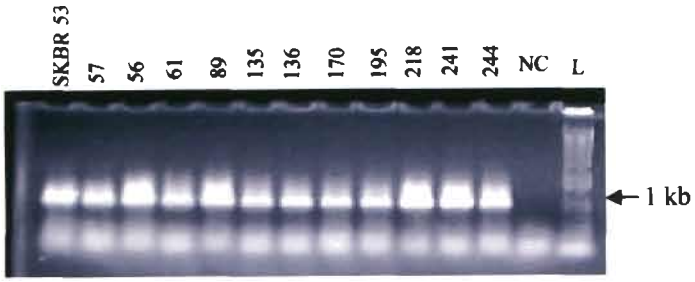


Figure 4.12: RT-PCR amplified cDNA showing differential expression of *crtI* in the leaves of transgenic lines. NC: negative control; L: 1kb DNA ladder

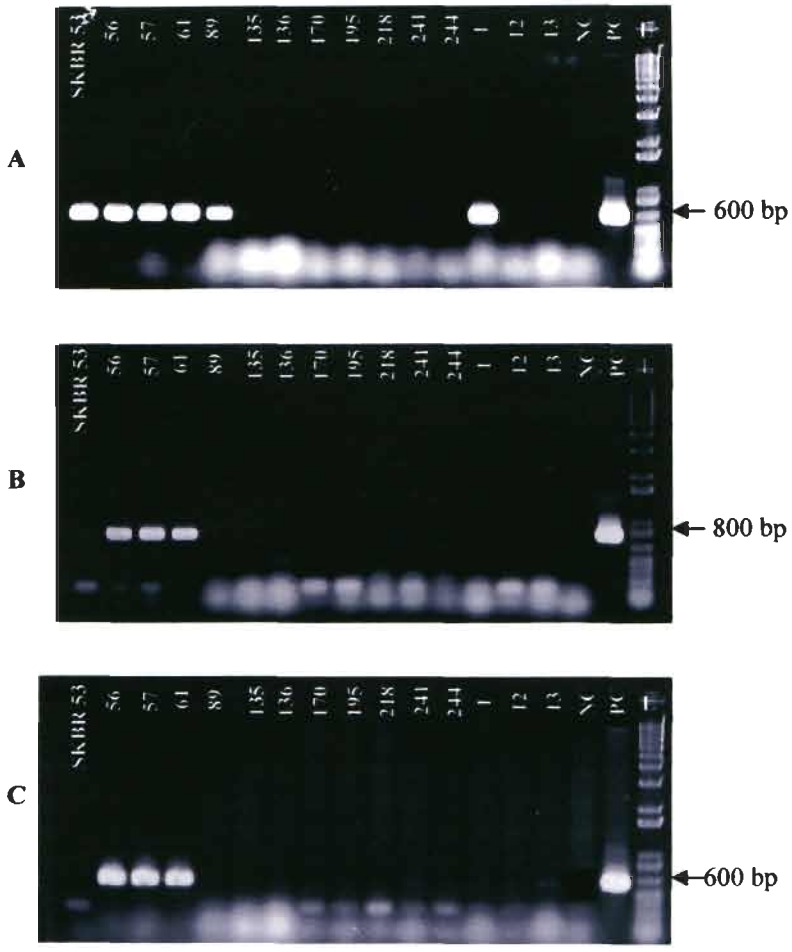


Figure 4.13: PCR analysis to detect beyond border transfer. A) Right border; B) Left border; C) *cat* gene. NC= negative control; PC+ positive control; L= 1 kb plus DNA ladder

Four healthy F_1 plants were used for the backcrossing with IR64 female parent. A few number of BC_1F_1 seeds were obtained as many of the F_1s were sterile which is very characteristic of indica-japonica cross. The BC_1F_1 plants were also initially screened for the transgenes by PCR. Sixteen plants were found to be positive for the *crtI* gene. This was also confirmed by the Southern blot analysis for *crtI* gene (Fig. 4.14.A). However, when the blot was rehybridized with *hph* gene, interestingly the plants were found to be negative without any hybridization signal (Fig. 4.14.B). This result was a possibility as the original transformation of the T309 line was done with two plasmids- one harbouring *psy* and *crtI* expression cassettes and the other having *hph* selectable marker gene and *lcy* gene. During co-transformation these two plasmids may have integrated at unlinked loci in the genome, which resulted in their separation during backcrossing. Similarly, it was confirmed that *lcy* gene was also absent in these lines. This was verified by using the same genomic DNA for Southern and hybridizing with PCR amplified *lcy* fragment as a probe (Fig. 4.14.C). All the *hph* negative plants were found to be negative for *lcy* also. These 16 lines were studied for their morphological characteristics, seed sterility...etc. Out of these 16 BC_1F_1 plants, two plants appeared to be similar to IR64 phenotypically. These two plants were again used as pollen parents in the backcross program with IR64 for another generation and BC_2F_1 seeds were obtained. Further analysis of these BC_2F_1 seed-derived plants confirmed the presence of the *crtI* gene by Southern and also *psy* by PCR (data not shown). These marker-free BC_2F_1 progenies were advanced to BC_2F_2 and BC_2F_3 for molecular, phenotypic and agronomic characterization, and to make the transgenes homozygous. Although one of the BC_2F_2 lines was found to be homozygous for *crtI* and *psy* (Fig. 4.15), another backcross was performed to increase the IR64 background in the marker free

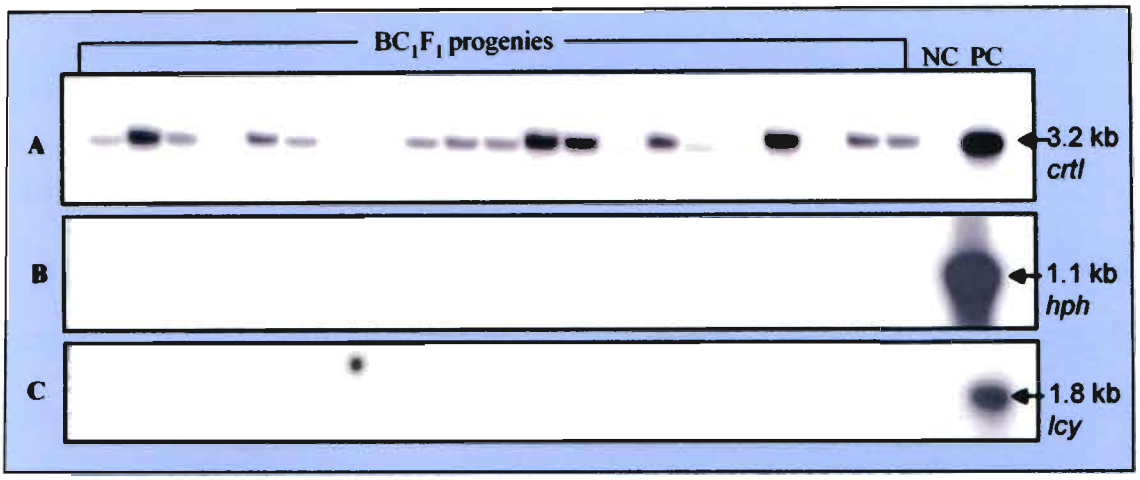


Figure 4.14. Southern analysis of BC_1F_1 backcross progenies showing the presence of *crtI* (A), but the absence of *hph* (B) and *lcy* (C) genes. NC= negative control; PC= positive control.

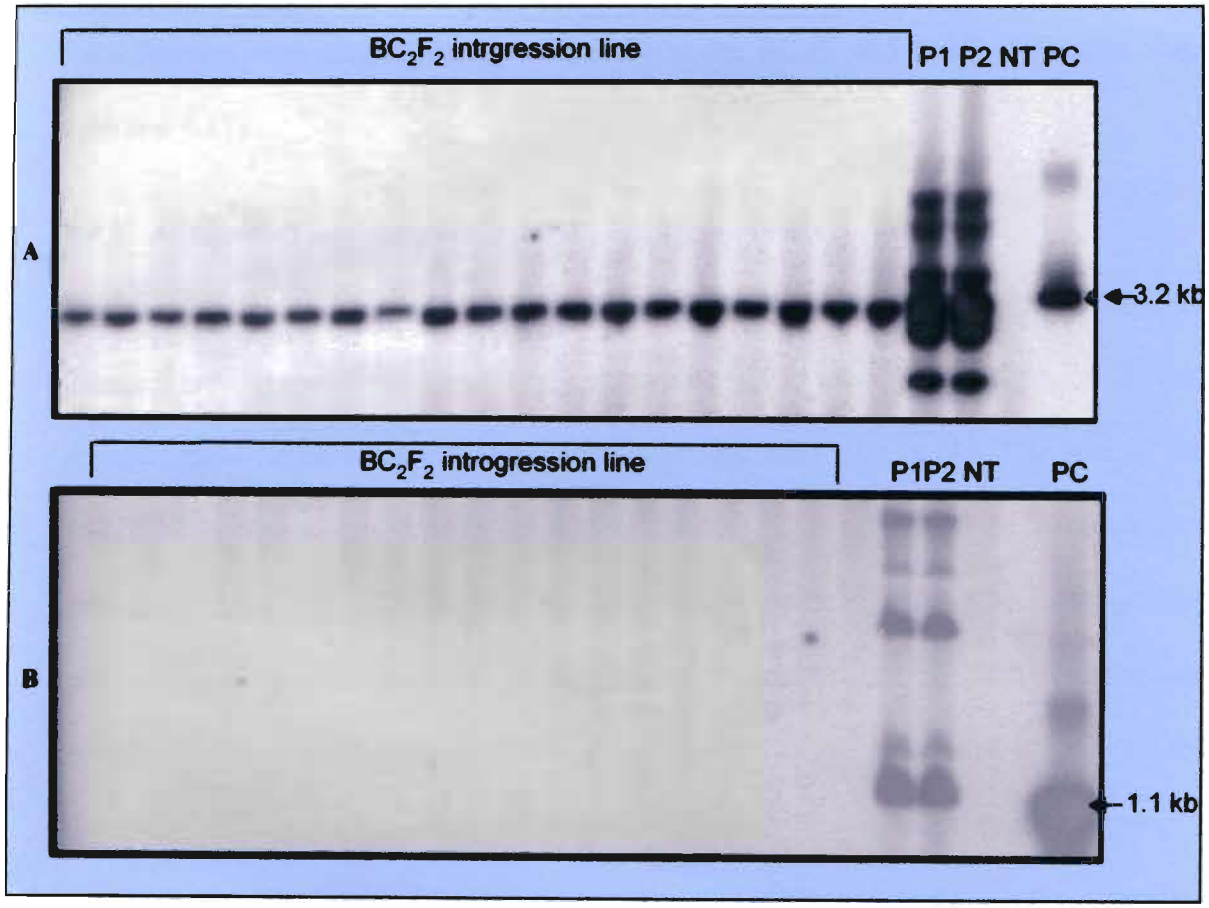


Figure 4.15. Southern blot analysis of BC2F2 line homozygous for *crtI* gene (A) and lacking *hph* marker gene (B) P1, P2= positive plant control; NT= non transformed control; PC= positive control.

introgression lines that could ultimately be fixed by a selfing generation or anther culture.

4.4.2 Phenotypic characters of the F_1s, and Backcross progenies

The F_1 plants were intermediate type with regard to morphological features that are characteristic features of the indica x japonica crosses. The leaves were broader, 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 less number of BC_1F_1 seeds. However in the 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 IR64 in the BC_2F_2 and BC_3F_1 progenies (Fig. 4.16 and 4.17).

4.4.3 Analysis of the transgenic BC_2F_2 lines using SRILs

The data on the total number of bands generated by the twelve URPs (Universal Rice Primers) were recorded for three BC_2F_2 transgenic family (two plants each) and the two parental lines (IR64 recurrent and T3.9 donor DH line) (Fig. 4.18). The size of the amplified products varied from 35,000 to 300 bp. Out of a total of 77 fragments amplified from the 12 primers, 50 were polymorphic (Table 4.5). The percent polymorphism for the individual primers ranged from 25% for URP12 to 100% in case of URP4. The data of presence and absence of DNA bands were used to generate the dendrogram (Fig. 4.19). The dendrogram showed two distinct groups having a similarity coefficient of 0.53. Group 1 consisted of all the introgression lines, together with IR64, whereas, group 2 consisted of T309 transgenic line. Group 1 was again

Figure 4.16: Marker free transgenic BC_3F_1 introgression lines showing phenotypic similarity to the recurrent parent IR64.

Figure 4.17: Gradual restitution of IR64 panicle phenotype in successive backcross generations

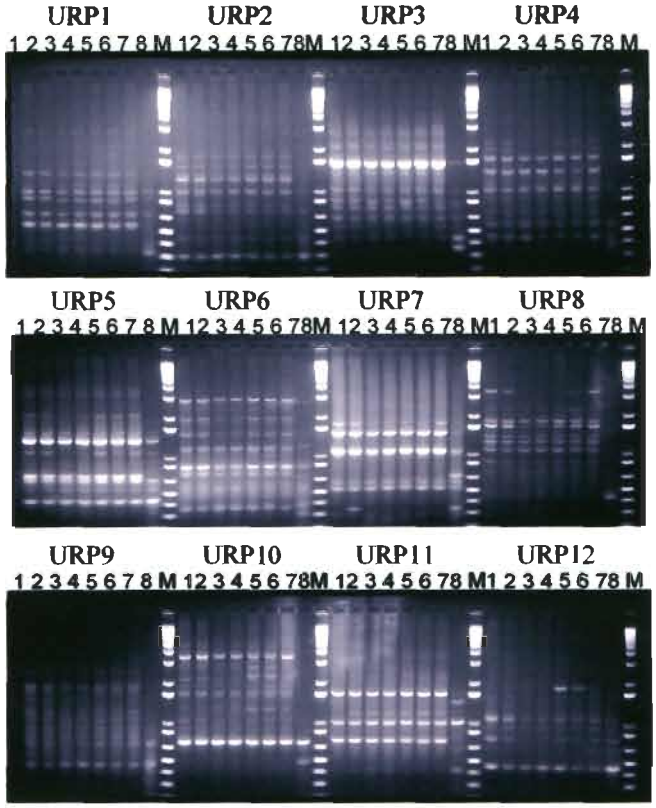


Figure 4.18. Gel pictures showing banding pattern of BC_2F_2 progenies (lanes 1-6) as compared to parents IR64 (lane 7) and T309 (lane 8), using universal rice primers (URP 1-12). M = 1 kb plus DNA marker.

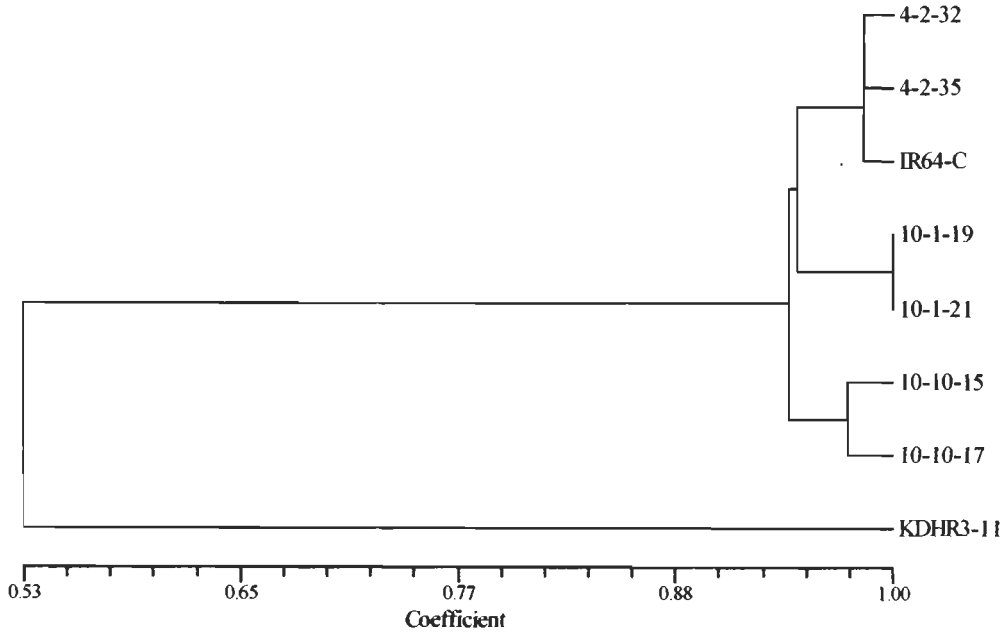


Figure 4.19: Dendogram depicting the similarity coefficients among BC_2F_2 lines and parents IR64 and transgenic T309 (KDHR3-11)

Table 4.5 : Summary of bands scored by the use of Universal rice primers

Primers	Total no. of bands	Polymorphic	Monomorphic	% polymorphism.
URP1	5	2	3	40
URP2	8	6	2	75
URP3	7	4	3	57.14286
URP4	7	7	0	100
URP5	6	3	3	50
URP6	6	3	3	50
URP7	8	7	1	87.5
URP8	5	3	2	60
URP9	6	2	4	33.33333
URP10	7	6	1	85.71429
URP11	8	6	2	75
URP12	4	1	3	25
Total	77	50	27	

divided into three subgroups that were differentiating the three BC_2F_2 families. BC_2F_2 line 4-2 was found to be closest to IR64.

In all the primers tested the introgression lines showed identical banding pattern with the recipient parent IR64. However, introgression line 10-1 and 10-10 produced polymorphic band with 4-2 by URP4, and 10-1 and 10-10 from a single family showed polymorphism by URP10 and URP12. On an average, the introgression lines and the wild type IR64 rice plants shared more than 90% monomorphic bands (Fig. 4.18).

4.4.4 Microsatellites based estimation of IR64 genome content in BC_3F_1

Out of 300 BC_3F_1 plants grown, 12 plants were selected on the basis of their phenotypic similarity to the recurrent parent. These 12 plants were screened with microsatellite markers polymorphic between IR64 and T309 donor. Out of total 104 markers screened, 39 were found to be polymorphic. These 39 markers were distributed throughout the 12 rice chromosomes (Fig. 4.20). The maximum (eight) number of polymorphic markers were located on chromosome 2, while only one polymorphic marker each could be found on chromosome 11 and 12. Most of the marker loci were found to be homozygous for the recurrent parent (Fig. 4.21), however hetrozygotes and homozygous loci for donor parent were also found. The number of microsattellites similar to IR64 ranged from 29 to 32 among the progenies. Plants I4T70, 71, 75, 80 and 114 showed maximum similarity to IR64, with 32 microsattellite loci homozygous for IR64, three homozygous for T309, and four heterozygotes (Table 4.6). In terms of percentage allele component, these lines had 87.18% IR64 background. This result may not be consistent with the theoretical percentage of recurrent parent in BC_3F_1 generation, but sufficiently depicted the restitution of IR64 background, and using the heterozygous markers (four) in subsequent selfing generation to select for IR64

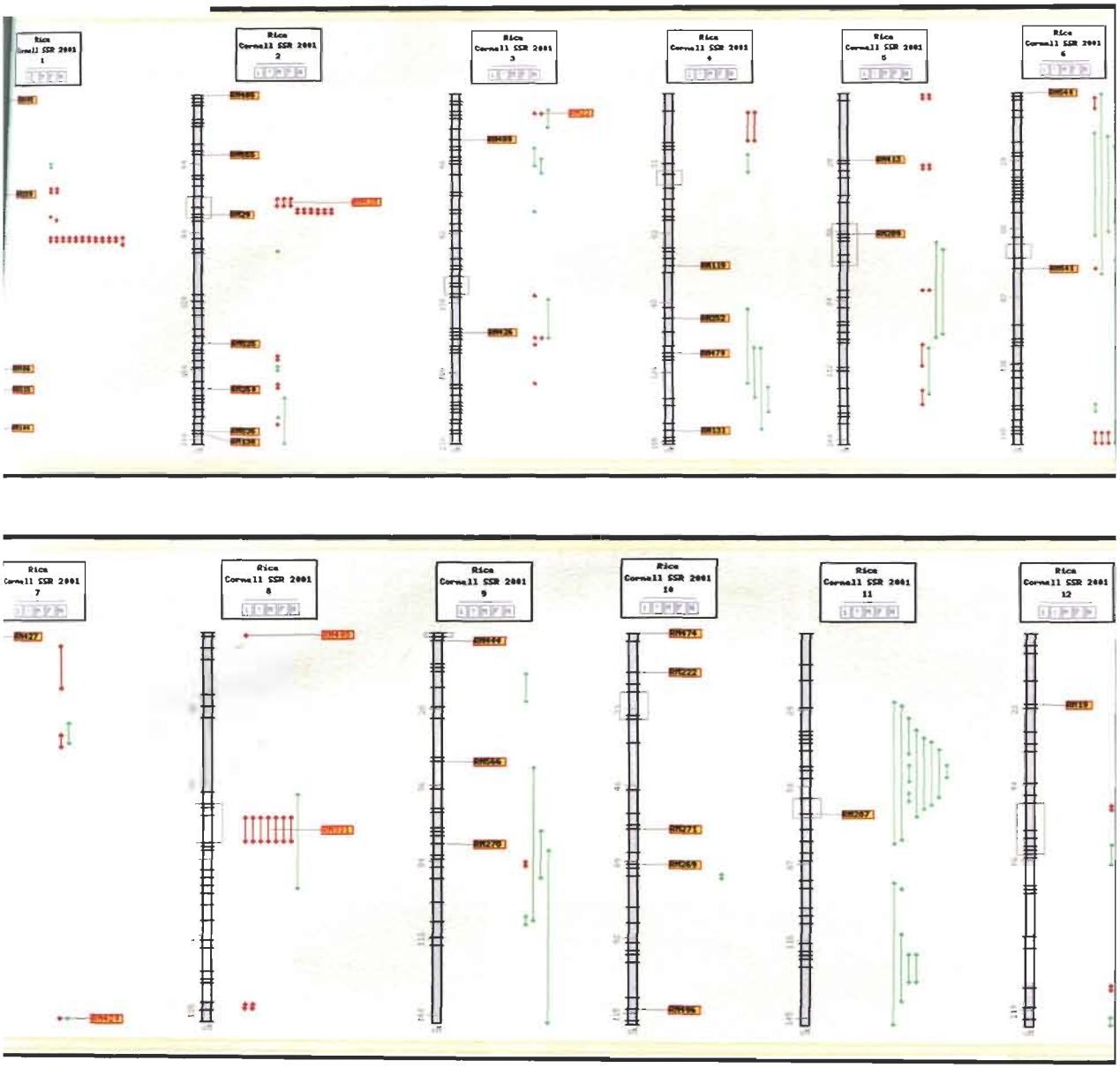


Figure 4.20: Physical map of rice chromosomes depicting position of microsatellites polymorphic between IR64 and T309

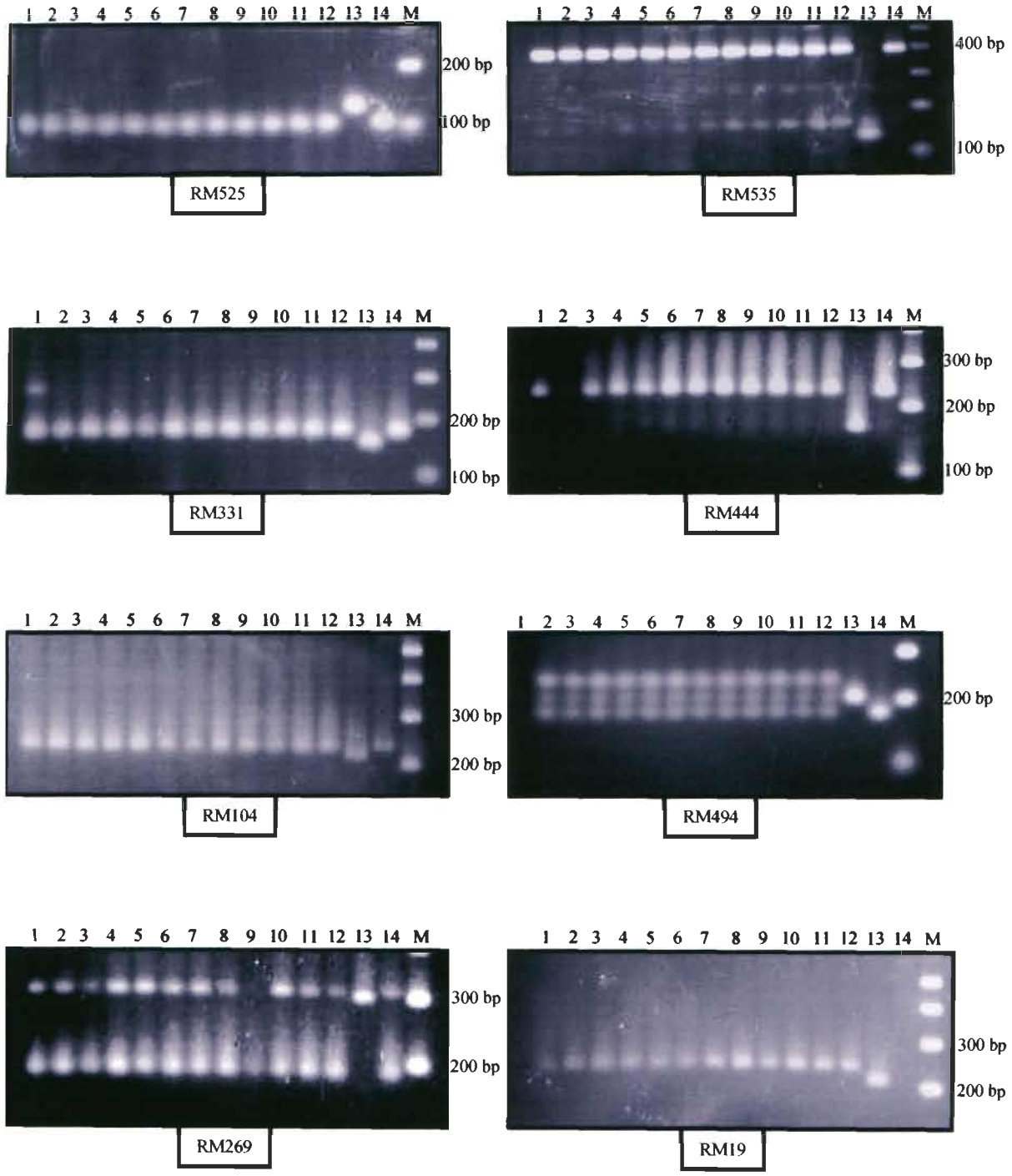


Figure 4.21: Gel picture showing amplification of microsatellite loci in BC3F1 progenies (lanes 1-12) as compared to parents T309 (lane 13) and IR64 (lane 14) .
M= 1kb plus DNA marker

Table 4.6: Microsatellite markers in BC3F1 progenies

BC3F1 progenies →		I4T 70	I4T 71	I4T 72	I4T 75	I4T 80	I4T 85	I4T 87	I4T 96	I4T 106	I4T 109	I4T 114	I4T 124
Chr1	RM495	A	A	-	A	A	A	A	A	A	A	A	A
	RM259	A	A	A	A	A	A	A	A	A	A	A	A
	RM486	A	A	A	A	A	A	A	A	A	A	A	A
	RM315	B	B	B	B	B	B	B	B	B	B	B	B
	RM104	A	A	A	A	A	A	A	A	A	A	A	A
Chr2	RM485	B	B	B	B	B	A		B	B	B	B	B
	RM555	A	A	A	A	A	A	A	A	A	A	A	A
	RM438	A	A	A	A	A	B	B	A	B	A	A	B
	RM29	A	A	A	A	A	A	B	-	B	A	A	B
	RM525	A	A	A	A	A	A	A	A	A	A	A	A
	RM250	A	A	A	A	A	A	A	-	A	A	A	A
	RM535	A	A	A	A	A	A	A	A	A	A	A	A
	RM138	A	A	A	A	A	A	A	A	A	A	A	A
Chr3	RM22	A	A	A	A	A	A	A	A	A	A	A	A
	RM489	H	H	H	H	H	H	H	H	H	H	H	H
	RM426	A	A	A	A	A	A	A	A	A	A	A	A
Chr4	RM119	A	A	A	A	A	A	A	A	A	A	A	A
	RM252	H	H	H	H	H	H	H	H	H	H	H	A
	RM470	A	A	A	A	A	A	A	A	A	A	A	A
	RM131	A	A	A	A	A	A	A	A	A	A	A	A
Chr5	RM413	A	A	A	A	A	A	A	A	A	A	A	B
	RM289	A	A	A	A	A	A	A	A	A	A	A	A
Chr6	RM540	B	B	B	B	B	B	B	B	B	B	B	A
	RM541	A	A	A	A	A	A	A	-	A	-	A	A
	RM494	H	H	H	H	H	H	H	H	H	H	H	H
Chr7	RM427	A	A	A	A	A	A	A	A	A	A	A	A
	RM420	A	A	A	A	A	A	A	A	A	A	A	A
	RM481	H	H	H	H	H	H	H	H	H	H	A	H
Chr8	RM408	A	A	A	A	A	A	A	A	A	A	A	A
	RM331	A	A	A	A	A	A	A	A	A	A	A	A
Chr9	RM444	A	A	A	A	A	A	A	A	A	A	A	A
	RM566	A	A	A	A	A	A	A	A	A	A	A	A
	RM278	A	A	A	A	A	A	A	A	A	A	A	A
Chr10	RM474	A	A	A	A	A	A	A	A	H	A	H	A
	RM222	A	A	A	A	A	A	A	A	A	A	A	A
	RM269	A	A	A	A	A	A	A	A	A	A	A	A
	RM496	A	A	A	A	A	A	A	A	A	A	A	A
Chr11	RM287	A	A	A	A	A	A	A	A	A	A	A	A
Chr12	RM19	A	A	A	A	A	A	A	A	A	A	A	A
Total	**A**	32	32	31	32	32	31	30	29	30	31	32	31
	B	3	3	3	3	3	4	5	3	5	3	3	5
	H	4	4	5	4	4	4	4	4	4	4	4	3

A= homozygous for IR64; B=homozygous for T309; H= heterozygous

homozygotes can sufficiently increase the IR64 complement. Alternatively one further backcross can also be done.

4.4.5 Carotenoid quantification of BC_2F_3 and BC_3F_1 generations

Even the *lcy* gene was co-segregated out along with *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 in the endosperm as was evident from the yellow color of the polished seeds. Amount of total carotenoids and β-carotene levels were estimated by spectrophotometer and HPLC analysis, respectively. The total carotenoid content as calculated from the absorbance value of the polished seed extract of the BC_2F_3 generation ranged from a maximum of 1.164 μg/g to a minimum of 0.497 μg/g. This was significantly lower than the amount found in T309 donor parent which was1.56 μg/g. Similar was the case with the amount of β-carotene in the progenies. It could be argued that this reduction was due to the absence of *lcy* gene in the progeny. It could also be possible that the interaction of transgenes and their products with the host background plays a role in the final accumulation of β-carotene, and since the background was changing due to backcrossing, it may be leading to the reduction in the β-carotene levels. The later view was fortified by the HPLC analysis of BC_3F_2 seeds. While both BC_2F_3 and BC_3F_1 were lacking the *lcy* gene, the total carotenoids and β-carotene levels further reduced, the maximum being 0.74 and 0.145 μg/g, respectively (Fig. 4.22 and 4.23). **Therefore this result suggests that the interaction of transgenes and their products with the host genome has a role to play in the carotenoid biosynthetic pathway.**

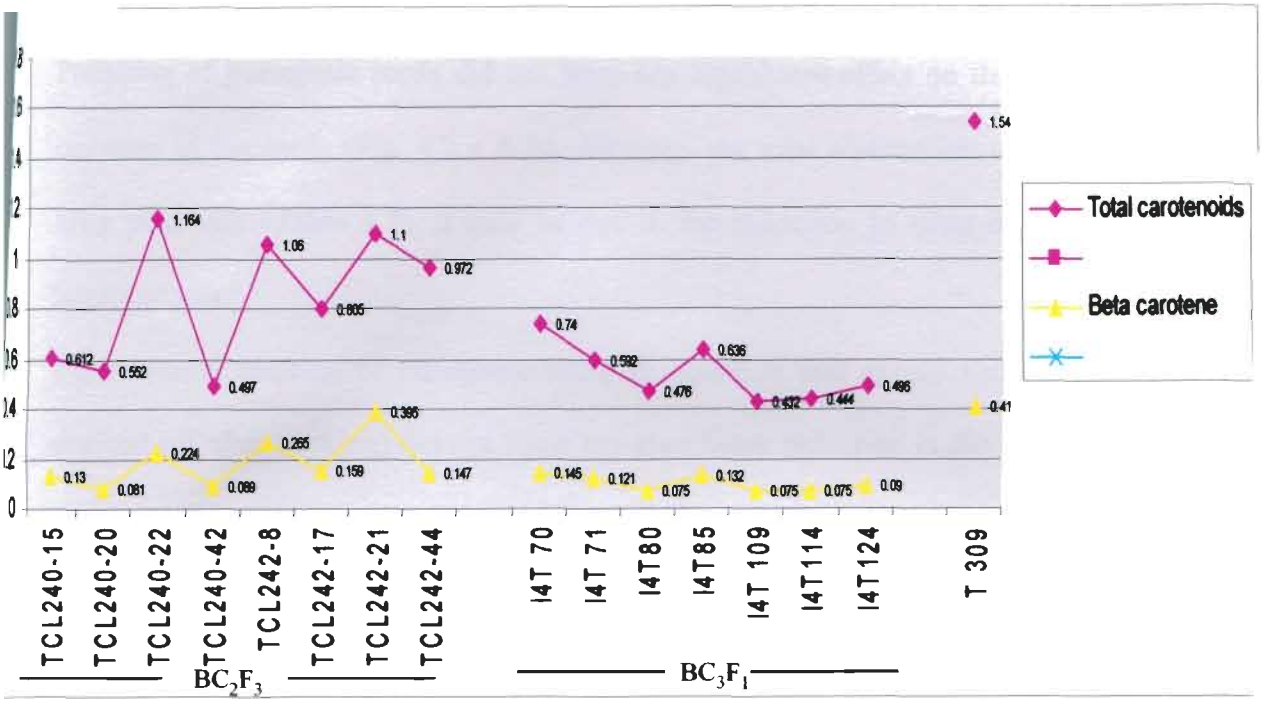


Figure 4.22: Graph showing successive reduction of total carotenoids and β-carotene levels in BC_2F_3 and BC_3F_1 generations as compared to transgenic T309 (donor parent).

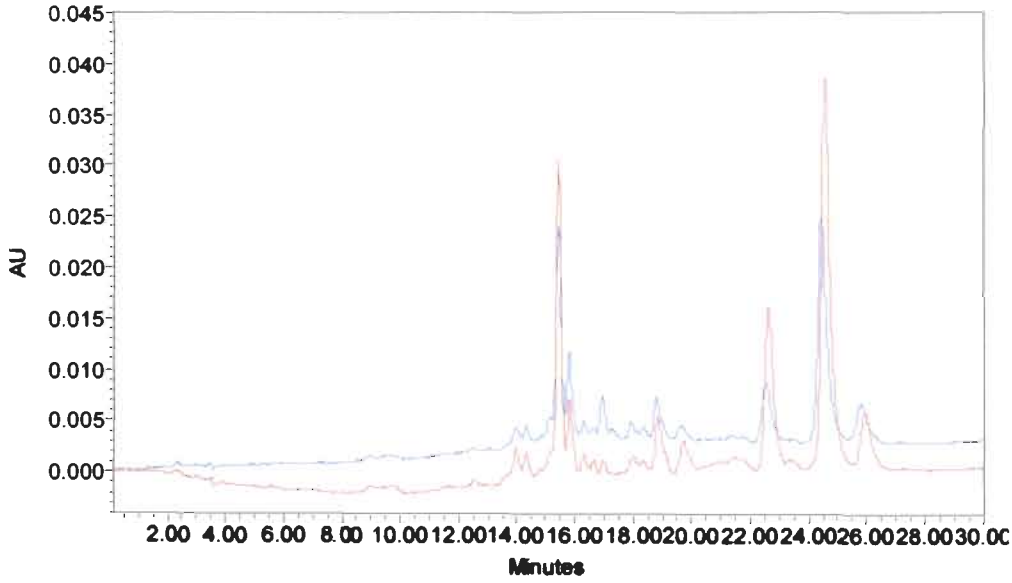


Figure 4.23: HPLC chromatogram showing carotenoid profile of transgenic introgression line TCL242-44 (blue) as compared to double haploid transgenic T309 (red)

4.5 Effect of polishing and cooking on carotenoids

Polishing of transgenic seeds did not have any significant effect on the amount of β-carotene in the seeds (Fig. 4.2 a & b). Although the total carotenoids reduced slightly after polishing (Table 4.7), it may be due to the reduction in other carotenoids like xanthophylls.

Furthermore, cooking of transgenic seeds in optimum and excess water (which was drained off after cooking) did not show any significant reduction in the amount of total carotenoids and β-carotene (Fig. 4.24,c,d,e; Table 4.7), indicating that the carotenoids were thermostable.

4.6 Agronomic performance of homozygous IR64 transgenic rice vis-à-vis control

The experimental plot was divided into 10 subplots of equal size (2 m x 6 m). Eight subplots were assigned to two independent transgenic lines (four each) and two subplots were assigned to control plants. Each subplot was transplanted with 100 plants, with a distance of 25 cm between plants (Fig. 4.27). Homozygosity of the transgenic progenies was confirmed by both PCR (data not shown) and Southern analysis (Fig. 4.26), which showed a 3.2-kb fragment corresponding to the *crtI* expression cassette. Apart from this expected fragment, other bands were also observed, indicating the integration of rearranged copies of the *crtI* gene into the genome, which is not uncommon in transgenics produced through biolistic transformation. Agronomic data on nine characters were recorded for ten randomly selected plants from each subplot, and were used for analysis of variance (ANOVA) using SPSS software. For all the characters analyzed, the variance between transgenics (pooled) and the control was statistically nonsignificant (Table 4.7), indicating that the transgenic is similar to the control in

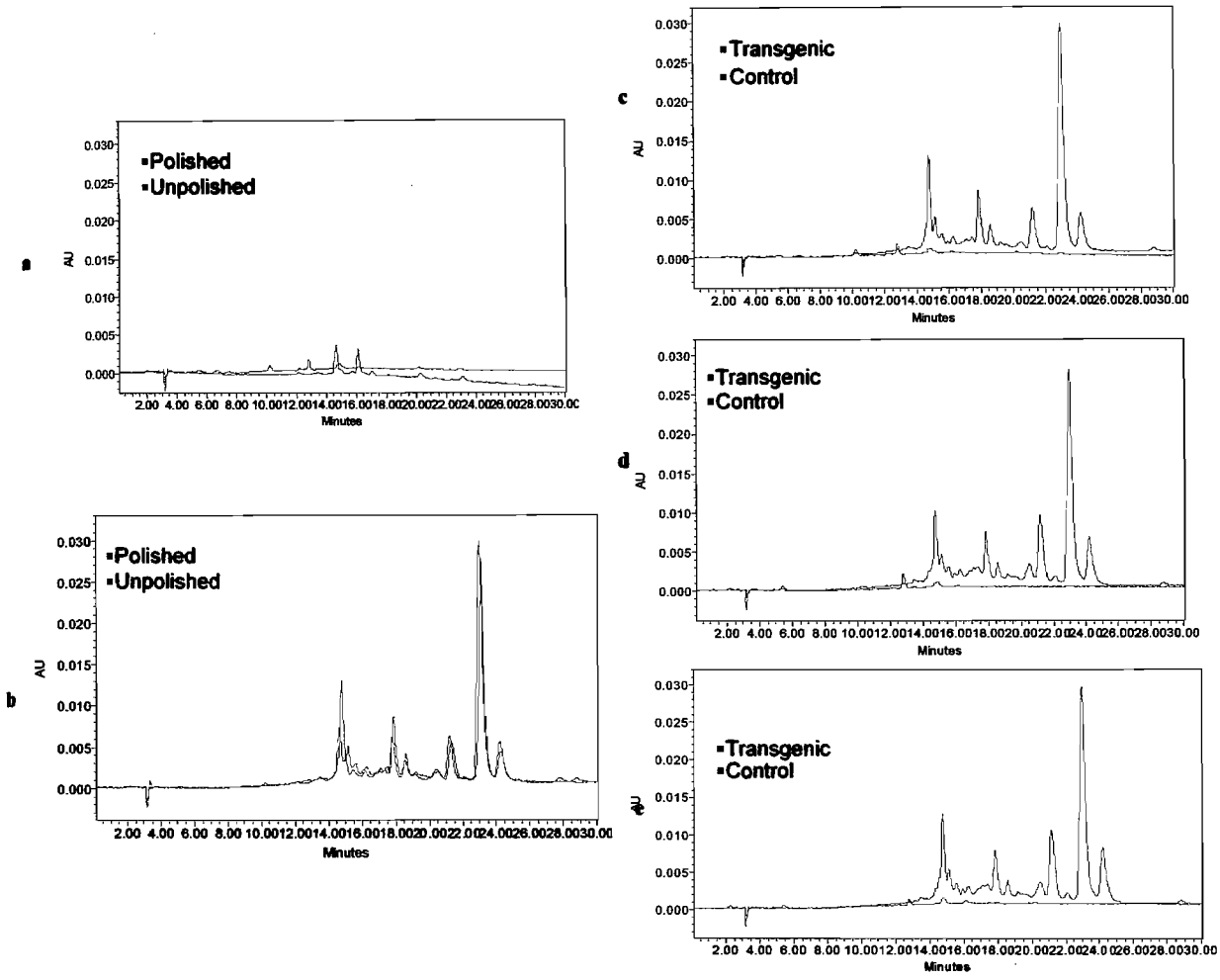


Figure 4.24: HPLC profiles of (a) BR29 control grains; (b) BR29 transgenic grains; (c) grains without cooking: (d) grains cooked optimum water; (e) grains cooked in excess water.

Table 4.7: Effect of polishing and cooking on total carotenoid and β-carotene levels in transgenic seeds.

Treatments	Total carotenoids (µg/g)	β-carotene (µg/g)
Transgenic unpolished uncooked	1.202	0.273
Transgenic unpolished cook1	1.242	0.202
Transgenic unpolished cook2	1.356	0.220
Transgenic polished uncooked	1.09	0.268
Transgenic polished cook1	1.05	0.250
Transgenic polished cook2	1.032	0.261

Figure 4.25: Transgenic Golden indica rice (T) and control rice (cv. IR64; C) showing uniformity in overall phenotype (left panel) and grain filling (right panel) grown under screenhouse conditions at IRRI, Philippines.

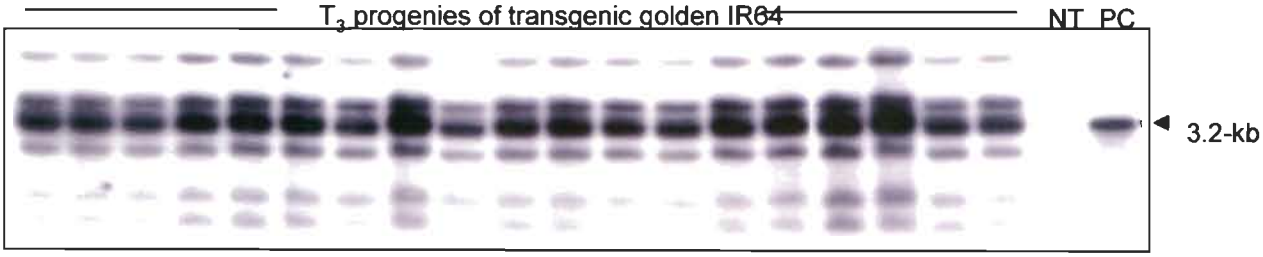


Figure 4.26: Southern blot showing homozygous progenies of Golden indica rice (cv. IR64) with integration of a 3.8-kb fragment corresponding to the *crtI* expression cassette. NT = nontransformed control, PC = positive control.
Genomic DNA was digested with *Eco*RI, electrophoresed, blotted to a nylon membrane, and hybridized using a (^{32}P-αdCTP)-labeled PCR-generated fragment of *crtI*.

Table 4.8: Agronomic performance of transgenic Golden rice (cv. IR64) vis-à-vis the IR64 control

Characters → / Treatments ↓		Plant height (cm)	No. of panicles per plant	No. of grains per panicle	No.of unfilled spikelets per panicle	Spikelet fertility (%)	1,000-grain weight (g)	Biological yield per plant (g)	Grain yield per plant (g)	Harvest index (%)
TRANSGENIC	Mean	107.13	9.13	88.81	34.16	71.46	25.86	109.25	13.49	13.66
	SEm±	0.745	0.358	2.460	1.364	1.078	0.168	5.953	0.661	0.610
CONTROL	Mean	108.80	8.65	86.05	28.75	74.67	25.77	98.98	13.74	14.86
	SEm±	1.733	0.539	5.558	3.312	2.635	0.223	9.309	1.350	1.290
F-Value (transgenic vs. control)		0.950^{ns}	0.391^{ns}	0.242^{ns}	2.881^{ns}	1.627^{ns}	0.060^{ns}	0.702^{ns}	0.030^{ns}	0.770^{ns}

ns= nonsignificant at $p \leq 0.05$

terms of yield traits. The overall phenotype and panicle characteristics of the transgenics were also similar to those of the control (Fig. 4.25). Similarly, no intra-transgenic variation was observed in the entire population of 800 transgenic progenies grown in screenhouse condition (data not shown). This substantiated that incorporation of the β-carotene biosynthetic pathway in rice endosperm did not cause any alteration in the agronomic traits of the transgenic plants.

Discussion

DISCUSSION

Rice grain, being the single major source of calories to the majority of population in south and Southeast Asia, where micronutrient deficiencies like vitamin A deficiency (VAD) are most prevalent, could prove to be an ideal vehicle for the delivery of micronutrients such as provitamin A to the people who need it the most on a low-cost sustainable basis. However, screening of primary and secondary gene pools has shown that the polished rice does not contain any trace of provitamin A (Tan *et al.*, 2004). Therefore, it is imperative to bioengineer the provitamin A (β-carotene) biosynthetic pathway into rice endosperm, specially indica rice that feeds more than two billion people, and to ensure that the transgenes and the phenotype is inherited stably in the subsequent generations, so that the bio-fortified rice could reach the farmers and the consumers.

To direct the accumulation of β-carotene in the endosperm, phytoene synthase driven by endosperm-specific glutelin promoter, phytoene desaturase under the control of CaMV 35S promoter and fused sequence for plastid specific transit peptide, and lycopene cyclase driven by CaMV 35S promoter were used for transformation (Ye *et al.*, 2000). Since it became evident that transgenic *lcy* was not required for β-carotene synthesis in the endosperm (Ye *et al.*, 2000; Datta *et al.*, 2003), it was not used in the later experiments with *Agrobacterium* mediated transformation. Cultivars BR 29, IR64, IR 68144, NHCD and Mot Bui were used for transformation, so that these varieties could directly be introduced into the target areas, thereby saving time and cost in further breeding.

A large number of transgenic plants were produced using biolistic transformation under hygromycin selection system. However, further characterization of these lines could not be continued as per the directions of the Chairman of humanitarian board on golden rice. Positech^{TM} selection with *pmi* under a novel promoter was used as an alternative to *hph*, in case of *Agrobacterium* mediated transformation. The *pmi* selection system proved to be successful and efficient in generating large number of indica rice transformants. This selection system has also been successfully used in several other crops (Joersbo *et al.*, 1998; Negrotto *et al.*, 2000; Wright *et al.*, 2001). The present non-antibiotic selection system could be an advantage for addressing the public concern and obtaining acceptance for transgenic nutritional rice (Datta *et al.*, 2000).

Agrobacterium strain EHA101 proved to be more efficient for rice transformation, as compared to LBA4404. This may be due to the fact that EHA101 harbors additional super-virulent *vir* genes, which could increase the transformation efficiency (Hood *et al.*, 1986; Rashid *et al.*, 1996).

Most of the primary transformants showed normal phenotype and fertility as compared to the non-transgenic control. Less than 10% of the T_0 plants showed abnormal phenotype or low fertility. This could be attributed to somaclonal variation (a phenomenon often observed in *in vitro* culture), and not necessarily to alien gene integration or expression. Such lines should be discarded, or alternatively, be backcrossed with the control plant to restitute the normal phenotype.

It has been a general consensus that *Agrobacterium* mediated transformation leads to simple integration, low copy number of transgenes in the host genome, with minimal rearrangements (Veluthambi *et al.*, 2003; Kohli *et al.*, 2003), perhaps the T-DNA is protected from degradation by its association with *Agrobacterium* virulence proteins.

However, there have been several reports of T-DNA rearrangements in dicots (Deroles and Gardner, 1988; Offringa *et al.*, 1990; Puchta *et al.*, 1992). In the present investigation, almost 50% of the transgenic lines studied showed rearrangements for *crtI*, with the copy number and sites of integration ranging from one to four. Interestingly, almost all the lines showed simple integration for *psy* gene, in spite of it being on the same T-DNA as *crtI*. This may be due to the fact that the *crtI* fragment released upon digestion with *EcoR*I contained CaMV 35S promoter, which has been reported by Kohli *et al.* (1999) to cause microhomology mediated illegitimate recombination, thereby leading to rearrangements, in transgenic rice transformed through particle bombardment. The CaMV 35S promoter contains a 19 bp imperfect palindromic sequence that forms a hairpin secondary structure, which may stimulate illegitimate recombination events. Similar seems to be the case with T-DNA inserts. The disparity in the integration pattern of *psy* and *crtI* also suggest that rearrangements may not involve the entire T-DNA.

All the 35 transgenic lines developed through *Agrobacterium* mediated transformation were analyzed for beyond border transfer of plasmid backbone. Majority of the lines were found to be lacking any beyond border transfer. Four lines showed only right border integration, two lines only had *cat* gene, a bacterial selectable marker whereas four lines showed integration of the entire three- right border, left border and *cat* gene,. Beyond T-DNA border transfer has been reported by several workers in different crops (Martineau *et al.*, 1994; Ramanathan and Veluthambi, 1995; Kononov *et al.*, 1997). Since the backbone contains bacterial antibiotic resistance markers, such beyond border transfers may be avoided to address the negative public perception of using antibiotic selectable marker gene. Such transformants should be carefully identified and discarded.

Fifteen SKBR lines were selected for the study of transgene inheritance in T_1 progenies.SKBR1, 12 and 13, which were studied in detail, showed the same banding pattern with all the three enzymes used for Southern, confirming that they were clones. However, while the T1 progenies SKBR 1 and 13 were showing a typical bi-locus segregation ratio of 15:1 for *crtI* gene, SKBR 12 showed a highly negatively skewed segregation. It suggests the occurrence of some somaclonal changes that may have taken place after the transformation event.

The study of SKBR1 and 13 T_1 progenies was interesting in two more aspects. Firstly, while *crtI* had integrated at two independently segregating loci, *psy* was present at only one locus, which is a clear evidence that partial T-DNA transfer had taken place at one of the loci. Truncated T-DNA inserts have also been reported by Yin and Wang (2000) and Dong *et al.* (2001). Secondly, it was evident that the progenies with two loci of *crtI* were accumulating more β-carotene in the endosperm than the progenies with only one locus of *crtI*. Since *psy* was not variable in both the cases, it suggests that that the "dosage effect" of two copies of *crtI* had a role to play in higher accumulation of β-carotene.

The 12 other T_1 progenies studied showed variable segregation pattern for *crtI* gene. While some of the progenies were showing typical Mendelian single locus (SKBR56, 135, 170) and bi-locus (SKBR1, 13, 53) segregation, others were segregating in a non-Mendelian fashion. Two scenarios could be proposed for the skewed segregation. First, since the transgenic locus in a hemizygous condition, it may be large enough to cause problems during homologous pairing of chromosomes, thereby being "looped-out" during meiosis. Secondly, it may be possible, especially in the progenies giving a segregation ratio close to 2:1, that the transgene had integrated at a single locus in an

essential gene causing a lethal homozygous phenotype (Burkhardt, 1996). However, if a larger population is grown the 2:1 ratio may actually turn out to be 3:1.

Southern blot analysis of the T_1 progenies revealed that, in most of the cases the transgenes were being stably inherited in terms of integration pattern, copy number and number of loci. Apart from SKBR1, 13, and 53, in all the lines that were showing two loci of integration, the loci co-segregated in the T1 progenies, indicating that the loci were tightly linked. It has been widely reported, both in case of biolistic and *Agrobacterium* mediated transformation, that the transgenic loci are usually clustered together, interspersed with genomic DNA of size ranging from hundreds to thousands base pairs, rather than megabase pairs (Kohli *et al.*, 1998; Pawlowski and Somers, 1998; Debuck *et al.*, 1999; Kumar and Fladung, 2000).

However, in case of three lines *viz.* SKBR56, 170 and 218, the T_1 progenies showed variation with respect to parents, which could not be attributed to Mendilian segregation (unlike SKBR 1, 13 and 53). In case of SKBR 56 and 170, a few T_1 plants showed rearranged bands, as opposed to simple integration in the parent, when Southern analysis was done using *EcoR*I, while the copy number and locus integrity remained unchanged. Whereas, the T_1 progenies of SKBR218 showed variation for integration pattern, as well as copy number and number of loci, indicating that the transgenic locus was highly unstable. Homologous recombination events at the genome level have also been shown to 'induce' intrachromosomal and/or interchromatid crossover or gene conversion events in the neighbouring transgenic sequences (Peterhans *et al.*, 1990; Tovar *et al.*, 1992).

Carotenoids profiles were analyzed from the polished T_1 seeds of all the 35 transgenic lines. The total carotenoid content ranged from 0.388 μg/g of seed in SKBR466 to

1.584 μg/g in SKBR 241. The amount is expected to be higher once homozygosity is achieved, as was evident from the amount recorded from the T_2 seeds of SKBR1-32, which was more than 2 μg/g. The β-carotene level varied from 0.1 μg/g in SKBR136 to 0.612 μg/g in SKBR241. Moreover, HPLC peaks could also be detected for α-carotene and β-cryptoxanthin, which also have about 50% of provitamin A activity as compared to β-carotene (Rodriguez-Amaya, 2001). Therefore, the amount of these carotenoids should also be accounted for while calculating in terms of provitamin A content available in the rice endosperm.

Interestingly, all the lines showing high carotenoid content were having more than one copy of *crtI*. However, all the lines having high copy number did not show high carotenoid accumulation. This observation, together with the study on T_2 seeds of SKBR1 and 13 present a strong case for the dosage effect of multiple copies of a transgene. The dosage effect due to higher copy numbers has been reported to lead to higher expression (Hobbs *et al.*, 1993). Although a single or low copy number of transgene is desirable, the possibility of silencing of a single copy gene has also been documented (Elmayan and Vaucheret, 1996; Fu *et al.* 2000), besides the frequent co-suppression and inactivation of multiple copies of transgenes (Vaucheret *et al.*, 1998). However, with other factors like transgene integrity and spatial integration of transgene in the host genome (position effect) and interaction of transgene with the host genome affecting the expression levels, a more detailed study is required to establish the relation between transgene copy number and expression.

Marker assisted backcross breeding was successfully used to introgress the transgenes for β-carotene synthesis into IR64 background, using the double transgenic haploid T309 (Baisakh *et al.*, 2001) as the donor parent. The original transformation of T309

was done using two separate Ti plasmids, one harboring *psy* and *crtI*, and the other having *lcy* and the selectable marker *hpt* Ye *et al.*, 2000). Apparently, the two plasmids integrated at unlinked loci in the genome, and thus the *hpt* and *lcy* genes could be segregated out during backcrossing leading to the recovery of marker free progenies with only *crtI* and *psy*. Co-transformation strategy has been used successfully by several workers to produce marker free transgenics (Tu *et al* 2003; Rao *et al.* 2003; Matthews *et al.* 2001; Depicker *et al*, 1985. Petit *et al.* 1986; De Framond 1986. Simpson *et al.* 1986; McKnight *et al.* 1987; De Block & DeBrouwer 1991; Komari *et al.* 1996; De Neve *et al.* 1997; DeBuck *et al.* 1998; Daley *et al.* 1998). The progenies with only *psy* and *crtI* still accumulated β-carotene in the endosperm, as has been reported previously (Ye *et al.* 2000; Datta *et al.*, 2003; and Hoa *et al.*, 2003.

Molecular markers were used in BC_2F_3 and BC_3F_1 generations to assess the genome contribution from the recurrent parent in the progenies. Microsatellites marker based estimate of recipient genome content of the 12 selected lines was found to be 87.1% of IR-64. Though the representation of markers across the chromosomes was not sufficient, the previous studies reported that a few well placed markers (2 – 4 markers on a chromosome of 10cM) provide adequate coverage of the genome in backcross programs (Visscher, 1996; Servin and Hospital, 2002). As the microsatellite markers used in the present study are optimally positioned along with chromosome, their use was helpful in the efficient selection process.

Significantly, it was observed that the total carotenoids and β-carotene levels reduced in the successive generations of backcrossing as more and more IR64 background was restituted. In another backcross study, in which transgenic *Bt* gene was introgressed into several indica rice backgrounds, no such difference among the progenies and the donor

parent, in terms of Bt protein amount, was observed (unpublished data). However, unlike in case of *Bt* which is an 'independent' protein, carotenoid biosynthesis pathway is a small part of an entire network of pathways originating from a central compound isopentenyl pyrophosphate (IPP) (Cunningham and Gantt, 1998). There have been several reports of interaction of transgene products with the host genes and enzymes of the carotenogenic and related pathways (Fray *et al.*, 1995; Shewmaker *et al.*, 1999; Al-Babili *et al.*, 2001). Therefore, it is possible that when a transgenic locus is transferred to another diverse background through backcrossing, these interactions change, leading to variation in the amount of the final product, ie., total carotenoids and β-carotene.

The HPLC estimates of carotenoid levels from the unpolished and polished seeds of transgenics did not show any significant differences for β-carotene, indicating that its accumulation was confined to the endosperm..Although some variations were observed for some minor peaks, which may be xanthophylls or chlorophyll. Cooking polished transgenic seed in optimum and excess water also did not have any effect on the amount of total carotenoids and β-carotene. Although, in another study (Datta *et al.*, 2003), a minimal loss in some xanthophylls after cooking was observed. Due to the lack of sufficient transgenic seeds, these experiments were carried out on small scale, and may not sufficiently represent the actual cooking process. Therefore, a large scale cooking experiment, once homozygous lines are achieved, is desirable. Moreover, effect of other factors such as time and conditions of storage, time of milling, etc. should also be tested, as they also may lead to loss of carotenoids.

Apart from assuring the stability of integration and expression of transgene, it is also imperative to make sure that there are no agronomic pay-offs due to the introduction of transgenes in the host genome. Agronomic performance of transgenic IR64

homozygous line, vis-à-vis non-transgenic control, was evaluated under screenhouse conditions. The transgenics showed normal phenotype and were at par with the non-transgenic in terms of yield component traits. This substantiated that manipulation of the β-carotene biosynthetic pathway did not cause any alteration in the agronomic traits of the transgenic plants. Under field conditions also, transgenic *Bt* hybrids did not show any phenotypic trade-off because of the *Bt* transgene. Rather, they had an approximately 28% yield advantage because of protection against yellow stem borer and leaffolder (Tu *et al.* 2000b). Similar work has also been conducted with a selected event of *Xa21*-IR72 rice showing comparable results with the nontransgenic IR72 (Tu *et al.*, 2000a).

The production of transgenic indica rice with endosperm specific accumulation of β-carotene has successfully been achieved. Transgenic lines SKBR1, 13, 241 and 244 could be identified as the best lines in terms of high expression, stable inheritance of transgene structure and expression, Mendelian segregation ratio of the transgenes, and good phenotype.

However, further improvements may be possible by modification of transformation constructs and use of different endosperm specific promoters (globulin, glutelin, prolamin). The use of a mammalian methyl transferase gene, which converts β-carotene into vitamin A, has also been proposed for the direct accumulation of vitamin A in the endosperm (P. Beyer, personal communication). In view of daily dietary requirements, an increase in the amount of carotenoids, especially β-carotene and others, such as β-cryptoxanthin, that are converted into vitamin A would be desirable, although the current level of carotenoids in transgenic seeds might already be sufficient to prevent vitamin A malnutrition on the basis of daily diet of 300g of rice (R. Russel, personal communication).

Once homozygosity is achieved, large-scale multilocational field trials should be conducted to assess the performance of these lines under field conditions. Although, a planned study was not conducted, it has been observed, and is highly likely that the carotenoid levels are affected by environmental factors like water stress, fertilization, time of harvesting, etc. In vitro tests and human feeding trials also need to be conducted to assess the bioavailability and nutritional value of β-carotene delivered through rice. These lines could be incorporated into the varietal development programs and transgenic trait could be transferred to other widely grown genetic backgrounds. Further, this transgenic trait could also be stacked together with other nutritionally and agronomically important transgenic traits like high iron and lysine content, disease and pest resistance, abiotic stress tolerance etc., so that maximum impact and benefits could be achieved at the farmer's field and at the consumer level.

Summary and Conclusion

Chapter 6 SUMMARY AND CONCLUSION

Considering that bioengineered indica rice with β-carotene accumulation in the endosperm could prove to be a sustainable and cost effective solution to the prevailing vitamin A deficiency problem of South and South East Asia in particular and the world in general, this study was undertaken to introduce the carotenogenic pathway into popular indica rice backgrounds through genetic transformation and marker assisted backcross breeding. The study also aimed at the molecular, biochemical and agronomic characterization of the transgenic lines and their progenies.

A large number of transgenics were produced through biolistics and *Agrobacterium* mediated transformation methods, using hygromycin and mannose selection systems, respectively. Most of the T_0 plants showed normal phenotype and fertility. Selected lines transformed through *Agrobacterium* using *pmi* as the selectable marker were characterized on molecular and biochemical basis.

Among the 20 T_0 plants analyzed by Southern, 50% showed simple integration of *crtI* gene, while the rest were having rearranged fragments. However, all lines except one were showing simple integration of *psy* gene. The copy number and transgenic loci in the primary transgenics varied from one to four.

Transgene segregation pattern in the T_1 progenies varied from Mendelian monogenic (3:1) and digenic (15:1) to non Mendelian segregation ratios. Southern blot analysis showed that the transgenic loci were stably inherited in the T_1 progenies in terms of structural integrity, except in case of lines SKBR 56, 170 and 218, which showed non-parental variations. In most of the lines, multiple transgenic loci were closely linked,

and co-segregated in the T_1 progenies. The only exceptions were SKBR 13 and its clones, which had *crtI* integrated at two unlinked loci that assorted independently. The analysis of SKBR 13 progenies also elucidated the partial integration of T-DNA at one locus. Correlation of Southern data and β-carotene estimation through HPLC indicated the 'dosage effect' of the two copies of *crtI*.

Beyond T-DNA border transfer was detected in 10 out of 35 lines studied. RT-PCR confirmed the expression of *crtI* gene at the transcriptional level. In most of the cases, the amount of cDNA amplified was consistent with the level of total carotenoids in the seeds.

Total caroteniod and β-carotene estimation on polished T_1 seeds was done using spectrophotometer and HPLC, respectively. The highest amounts were recorded in lines SKBR 13, 218, 241 and 244. One of the T1 progenies of SKBR 1 (SKBR 1-32) showed a total carotenoid level of 2.268 μg/g. Interestingly, all the lines showing high carotenoid accumulation possessed multiple copies of transgenes. Transgenic lines SKBR 1, 13, 241 and 244 were identified as the best lines in terms of high expression, stable inheritance of transgene structure and expression, Mendelian segregation ratio of the transgenes, and good phenotype.

Apart from the transformation experiments, the transgenes were also introgressed into IR64 background through backcross breeding using transgenic T309 japonica rice line as the donor parent. Marker free progenies having *psy* and *crtI* genes could be recovered in BC_1F_1 generation. Use of molecular markers in BC_2F_2 (SRILs) and BC_3F_1 (microsatellites) confirmed the restitution of IR64 background. Interestingly, the amount of total carotenoids and β-carotene declined successively in BC_2F_3 and BC_3F_2

seeds as compared to donor parent, suggesting the effect of host background on the expression of transgenes.

Polishing did not have any effect on the amount of β-carotene and other major carotenoids in the transgenic seeds, confirming the endosperm specific functioning of the bioengineered pathway. Furthermore, no significant reduction in total carotenoids and β-carotene levels was observed in polished transgenic seeds upon cooking under optimum and excess water.

Agronomic performance of transgenic IR64 homozygous line, vis-à-vis non-transgenic control, was evaluated under screenhouse conditions. The transgenics showed normal phenotype and were at par with the non-transgenic in terms of yield component traits, indicating that there were no apparent trade offs due to the introduction of the endosperm specific carotenogenic pathway.

Conclusion

The stable transgenic lines produced through genetic transformation using non-antibiotic selectable marker, and marker free lines produced through backcross breeding, in popular indica rice backgrounds could be advanced for large scale multilocational yield trials, and eventually could be incorporated into further breeding and varietal development programs.

Moreover, as the present study was conducted on relatively small/segregating sample/ population size, a more extensive as well as intensive study could fortify these results, or highlight certain new findings that eluded the scope of this study. Furthermore, bioavailability and bioefficacy studies of β-carotene in transgenic rice seeds need to be conducted, before the final product is released for commercial production.

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Appendices

Appendix I

Composition of MS medium

Bottle	AMOUNT TO TAKE			
	Component	Stock solution (g/l)	Per liter of preparation (ml)	Final conc. (mg/l)
MSI	NH_4NO_3	82.5	20	1650.0
	KNO_3	95.0		1900.0
MSII	$MgSO_4.7H_2O$	37.0	10	370.0
	$MnSO_4.4H_2O$	2.23		22.3
	$ZnSO_4$	1.058		10.6
	$CuSO_4.5H_2O$	0.0025		0.025
MSIII	$CaCl_2.H_2O$	44.0	10	440.0
	KI	0.083		0.83
	$CoCl_2.6H_2O$	0.0025		0.025
MSIV	KH_2PO_4	17.0	10	170.0
	H_3BO_3	0.62		6.2
	$Na_2MoO_4.2H_2O$	0.025		0.25
MSV	$FeSO_4.7H_2O$	2.785	10	27.85
	$Na_2EDTA.2H_2O$	3.725		37.25
mg/100ml				
Vitamins	Nicotinic acid	100	5	5.0
	Pyridoxine HCL	100		5.0
	Thiamine HCL	20		1.0
	Glycine	400		20.0
Hormone	2,4-D	10	20	2.0
Myo-inositol		100		
Sucrose/Maltose				30000.0
Agar				8000.0
pH	5.6 to 5.8			

Appendix II

Yoshida Culture Solution

Component	Stock Concentration (g/10L)
$MgSO_4.7H_2O$	3240.0
NH_4NO_3	914.0
$CaCl_2.2H_2O$	886.0
K_2SO_4	714.0
$NaH_2PO_4.2H_2O$	403.0
H_3BO_3	300.0
$FeCl_3.6H_2O$	77.0
$MnCl_2.4H_2O$	15.0
$ZnSO_4.7H_2O$	0.35
$CuSO_4.5H_2O$	0.31
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.74

Take 10 ml from stock of each component for every 8 liters final volume of culture solution.

Appendix III

DNA isolation

1. Extraction buffer

Final Concentration	**1000 ml**
100 mM Tris-Cl, pH 8.0	100 ml 1 M Tris-Cl, pH 8.0
50 mM EDTA pH 8.0	100 ml 0.5 M EDTA, pH 8.0
500 mM NaCl	125 4M NaCl
10 mM mercaptoethanol	700 ul

2. 20% SDS (sodium dodecyl sulphate)

Dissolve 200 g of electrophoretic grade SDS in 900 ml of nano-pure water. Heat to 68^0c to assist dissolution. Adjust pH to 7.2 by adding a few drops of concentrated HCl. Adjust volume to 1.0 liter with nanopure water.

3. 5M Potassium acetate

Dissolve 490.7 g of potassium acetate into 500 ml distilled water. Adjust volume to 1 liter with distilled water.

4. RNAase (10 mg/ml)

1g/100 ml of distilled water

5. 3M Sodium acetate, pH 5.2

408.24g sodium acetate, trihydrate. Adjust pH using glacial acetic acid. Nanopure water to 1.0 liter.

Appendix IV

Southern blot and hybridization

1. 250 mM HCl

   Add 2038 ml of 12N HCl to 700 ml distilled water. Adjust to 1 liter with distilled water.

2. Alkali transfer buffer (0.4 M NaOH)

   Dissolve 16 g NaOH pellets in 900 ml distilled water. Adjust to 1 liter with distilled water.

3. 50X TAE

   To make 1 liter:

   24.2g Trizma Base

   57.1 ml glacial acetic acid

   200 ml 0.5 mM EDTA

4. EtBr stock (10 mg/ml ethidium bromide)

   Dissolve 1g in 100 ml distilled water

5. 20X SSC

   175.3 g NaCl

   88.2 g Sodium citrate

   800 ml nanopure water

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ABSTRACT

Transgenics for endosperm specific expression of β-carotene synthesis pathway were produced in the background of several popular indica rice cultivars(BR29, IR64, IR68144, Nang Hong Cho Dao and Mot Bui) through biolistic and *Agrobacterium* mediated transformation using hygromycin and mannose (using non-antibiotic selectable marker *pmi*) based selection systems, respectively. 35 transgenic BR29 lines produced through *Agrobacterium* mediated transformation were characterized on molecular, biochemical and phenotypic level. 15 lines were studied for the segregation pattern of transgenic loci in the T_1 progenies.

Among the 20 T_0 plants analyzed by Southern, 50% showed simple integration of *crtI* gene, while the rest were having rearranged fragments. However, all lines except one were showing simple integration of *psy* gene. The copy number and transgenic loci in the primary transgenics varied from one to four.

Transgene segregation pattern in the T_1 progenies varied from Mendelian monogenic (3:1) and digenic (15:1) to non Mendelian segregation ratios. Southern blot analysis showed that the transgenic loci were stably inherited in the T_1 progenies in terms of structural integrity, except in case of lines SKBR 56, 170 and 218, which showed non-parental variations. In most of the lines, multiple transgenic loci were closely linked, and co-segregated in the T_1 progenies. The only exceptions were SKBR 13 and its clones, which had *crtI* integrated at two unlinked loci that assorted independently. The analysis of SKBR 13 progenies also elucidated the partial integration of T-DNA at one locus. Correlation of Southern data and β-carotene estimation through HPLC indicated the 'dosage effect' of the two copies of *crtI*.

Beyond T-DNA border transfer was detected in 10 out of 35 lines studied. RT-PCR confirmed the expression of *crtI* gene at the transcriptional level. In most of the cases, the amount of cDNA amplified was consistent with the level of total carotenoids in the seeds.

Total caroteniod and β-carotene estimation on polished T_1 seeds was done using spectrophotometer and HPLC, respectively. The highest amounts were recorded in lines SKBR 13, 218, 241 and 244. One of the T1 progenies of SKBR 1 (SKBR 1-32) showed a total carotenoid level of 2.268 μg/g. Interestingly, all the lines showing high carotenoid accumulation possessed multiple copies of transgenes. Transgenic lines SKBR 1, 13, 241 and 244 were identified as the best lines in terms of high expression, stable inheritance of transgene structure and expression, Mendelian segregation ratio of the transgenes, and good phenotype.

Apart from the transformation experiments, the transgenes were also introgressed into IR64 background through backcross breeding using transgenic T309 japonica rice line as the donor parent. Marker free progenies having *psy* and *crtI* genes could be recovered in BC_1F_1 generation. Use of molecular markers in BC_2F_2 (SRILs) and BC_3F_1 (microsatellites) confirmed the restitution of IR64 background. Interestingly, the amount of total carotenoids and β-carotene declined successively in BC_2F_3 and BC_3F_2 seeds as compared to donor parent, suggesting the effect of host background on the expression of transgenes.

Polishing did not have any effect on the amount of β-carotene and other major carotenoids in the transgenic seeds, confirming the endosperm specific functioning of the bioengineered pathway. Furthermore, no significant reduction in total carotenoids and β-carotene levels was observed in polished transgenic seeds upon cooking under optimum and excess water.

Agronomic performance of transgenic IR64 homozygous line, vis-à-vis non-transgenic control, was evaluated under screenhouse conditions. The transgenics showed normal phenotype and were at par with the non-transgenic in terms of yield component traits, indicating that there were no apparent trade offs due to the introduction of the endosperm specific carotenogenic pathway.

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