INTRODUCTION OF δ-ENDOTOXIN GENE *cry1Ac* OF *Bacillus thuringiensis* IN BASMATI RICE VARIETIES

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DECLARATION

I, Anil Kumar, hereby declare that the subject matter of the thesis entitled "Introduction of δ-endotoxin gene *cry1Ac* of *Bacillus thuringiensis* in Basmati rice varieties" is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the North-Eastern Hill University for the degree of Doctor of Philosophy in Botany.

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(ANIL KUMAR)

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ABBREVIATIONS

AS	acetosyringone
BPB	bromophenol blue
B_5	Gamborg's medium
bp	basepair
BSA	bovine serum albumin
^0C	degree centigrade
Ci	Curie
cm	centimeter
conc.	concentration
Cont.	continued
CSPD	disodium 3-(4-methoxyspiro{1,2-dioxetane- 3,2'-(5'chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate
CTAB	cetryl-trimethylammonium bromide
cv.	cultivar(s)
DDH_2O	double distilled water
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
e.g.	for example
ELISA	enzyme linked immuno sorbent assay
et al.	*et alia* (Latin; and others)
Fig.	figure
g	gramme
gus	β- glucuronidase
ha	hectare
HCl	hydrochloric acid
hpt	hygromycin phosphotransferase
hrs.	hours
IgG	immuno globulin G
kb	kilobase (s)
KDa	kiloDalton
M	molar
MES	2-(N-morpholino) ethanosulfonic acid
mA	milli ampere
mg l^{-1}	miligram per liter
mg	miligram
min.	minute
lit.	litre
Ltd.	limited

ml	milliliter
mM	milli molar
mm	millimeter
m/sec	miles per second
nm	nanometer
µg	microgram
µl	microliter
No.	number
ng	nanogram
nm	nanometer
O.D.	optical density (absorbance unit)
pg	picogram
pH	hydrogen potential; logarithm of the reciprocal of hydrogen ion concentration i.e. $\log_{10}(1/[H^+]$ or $-\log_{10}[H^+]$
pM	pico mole
pp	page to page
PVDF	polyvinyledene diflouride
RNase	ribonuclease
rpm	revolutions per minute
SD	standard deviation
S.E.	standard error
SDS	sodium dodesyl sulphate
Taq	*Thermus aquaticus*
TBE	tris-Borate-EDTA
TBST	tris buffer saline containing tween-20
T-DNA	transfer deoxy ribonucleic acid
T_m	melting temperature
T_0	parental generation
T_1	first generation
TM	trademark
Tris	tris (hydroxymethyl) aminoethane
U	unit
USA	United Sates of America
UV	ultraviolet light
var.	variety
V	volt
v/v	volume by volume
w/v	weight by volume

Chapter - 1

INTRODUCTION

INTRODUCTION

Cereal grains are amongst the first plants to be domesticated nearly 10,000 years ago when agriculture gradually replaced hunting and gathering during the Neolithic age. In retrospect, the choices made by the Neolithic men have not only proven to be wise and durable, but have also helped in sustaining the development of human civilization by providing valuable sources of food and nutrition. The most important of these are the eight cereal grains, *viz.* wheat, rice, maize, barley, oats, rye, sorghum and pearl millet that are used as food, feed and fodder for human and livestock. Therefore, the genetic improvement of cereals has been a major focus of plant breeding efforts during the past 50 years, resulting in remarkable increases in the yield as well as improvement in the quality of this most important group of food crops (Borlaug and Dowswell, 1988; Vasil, 1990). However, since 1990, growth in the grain harvest has slowed down, although world population continues to increase by 80 million a year with 90% of this increase occurring in developing countries. In order to meet the food requirement of this population growth, world grain production needs to be increased by 26 million tonnes every year (Khush, 1997).

Rice is the most important cereal consumed by over 40% (over 3 billion people) of the world population. More than 90% of the global rice is produced and consumed in Asia, where all three sub-species *viz.* indica, japonica and javanica are grown. Based on the global population growth projections rice production needs to be increased from the present 600 to 850 million tonnes by 2025 to support additional rice consumers. Global rice production this year (2004-05) is estimated to soar to a near record of 611 million

tonnes. India's rice production during 2004-05 is estimated to be 90 million tonnes from 44.5 million hectares. However, a monsoon failure could bring this largely non-irrigated crop down by 10 million tonnes or more from the forecast level, while a well-distributed precipitation could take this production up to 94 million tonnes. There has been tremendous improvement in rice productivity, which has not only made the country self-sufficient but also enables us to export rice in a limited quantity. This improvement has largely been possible through conventional breeding, which has its own limitations. Recombinant DNA technology especially genetic engineering, on the other hand, is capable of overcoming these limitations by transferring gene(s) across sexual barriers. In order to maintain self sufficiency and limited export, we need to add 2.5 to 3 million tonnes of rice every year. This is possible only when efforts in conventional breeding, are supported/supplemented with molecular biology and genetic engineering. Globally, more than 200 million tonnes of rice is lost every year due to abiotic and biotic stresses (Tyagi and Mohanty, 2000). Although, the cost associated with the management practices and chemical control of insect-pests approaches US $10 billion, still there is a 20-30% loss of crop across the globe (Estruch *et al.*, 1997).

Engineering rice for pest resistance is a major challenge; one strategy is being the introduction of *Bacillus thuringiensis* (Bt) crystal insecticidal protein (δ-endotoxin) genes (*cry*) genes. These proteins (Bt-toxins) are highly toxic to lepidopteran, dipteran and coleopteran insect-pests. Among the important insect-pests of rice are Stripped Stem Borer (SSB) (*Chilo suppressalis*), Yellow Stem Borer (YSB) (*Scirpophaga incertulus*) and Leaf folder (*Cnaphalocrocis medanalis* and *Marasima patanalis*) that cause annual losses of around 10 million tonnes (Herdt, 1991).

The first report of *Agrobacterium tumefaciens*-mediated transformation of rice appeared in 1993 (Chan *et al.*). This was followed by a report from Japan in 1994 by Hiei *et al*, demonstrating the production of transgenic rice through *Agrobacterium tumefaciens*-mediated transformation of embryogenic calli. They constructed some unique vector called super-binary vectors, which have additional *vir* genes in the binary plasmid itself. Scutella derived calli and *Agrobacterium tumefaciens* LBA4404 (pTOK233) were found to be the most suitable explants and strain respectively for transformation. After this, there were several reports of rice transformation by *Agrobacterium tumefaciens*-mediated transformation. Cheng *et al.* (1998) obtained a large number of transgenic rice plants through *Agrobacterium tumefaciens*-mediated transformation of different varieties engineered with *cryIA*(*b*) and *cryIA*(*c*) genes, which were codon optimized, Southern, Northern and Western analyses upto R_1 generation were performed to show the integration, inheritance and expression of the transgenes. Use of ubiquitin promoter in their experiments led to high level expression (up to 3% of total soluble protein) of transgene product. The toxic nature of the transgenic plants to SSB and YSB was revealed by insect bioassays. Transformation of japonica rice by *Agrobacterium* was reported by other groups (Yokoi *et al.*, 1997 and 1998; Kang *et al.*, 1998 and Jeon *et al.*, 1999) too. Aldemita and Hodges (1996) obtained transgenic indica as well as japonica rice using immature embryos. Rashid *et al.* (1996), Mohanty *et al.* (1999) and Khanna and Raina (1999) reported successful transformation of elite indica varieties with *Agrobacterium*. Terada *et al.* (2002) studied efficient gene targeting by homologous recombination in rice. Datta *et al.* (2002) pyramided transgenes for multiple resistance in rice by conventional crossing of two transgenic parental lines. Marfa *et al.*

(2002) studied influence of the developmental stage of rice plants expressing the *cry1B* gene on the level of protection against the stripped stem borer. Husnain *et al.* (2002) studied expression of an insecticidal *cryIAb* gene under three different promoters in leaves, stem and panicles to determine organ-specificity in Basmati rice. Bashir *et al.* (2004) recently reported field evaluation and risk assessment of transgenic Basmati rice expressing *cry1Ac* and *cry2A* genes.

However, besides *Agrobacterium*-mediated transformation several other methods of delivery of transgene have been employed. Fujimoto *et al.* (1993) were the first to engineer japonica rice through electroporation with modified delta endotoxin gene (*cry*) from *Bacillus thuringiensis*. Wunn *et al.* (1996) obtained transgenic rice cultivar, IR58 expressing a synthetic *cryIAb* gene driven by 35S promoter through particle bombardment. Feeding inhibition of YSB, SSB and Leaf folder were observed. Nayak *et al.* (1997) reconstructed *cry1Ac* gene and transformed indica rice cultivar IR64 with this synthetic gene. Insect bioassay revealed the resistance nature of transgenic plants to the damage caused by YSB. Rice plants containing *cryIAb* or *cry1Ac* gene have been obtained by using protoplast and particle bombardment methods. Keeping in view that at present US $8 billion is annually spent on insecticides worldwide out of which US $2.7 billion can be replaced by Bt -technology application alone (Tyagi and Mohanty, 2000) thus, Bt research in rice assumes great importance. Until recently, *Agrobacterium tumefaciens*-mediated transformation of monocotyledonous plants, particularly of the cereals, has been considered outside the range of *Agrobacterium*.

However, recent advances in understanding the biology of the infection process (meristematic cells as target tissue, *vir* gene inducing compounds and wide host range strains of *Agrobacterium tumefaciens*), availability of monocotyledonous gene promoters and improved selectable markers have significantly enhanced the opportunities of developing monocotyledonous transformation systems using *Agrobacterium tumefaciens*.

The present investigation was undertaken with following objectives:

1) Introduction of *cry1Ac* gene in elite Basmati rice varieties through *Agrobacterium tumefaciens*-mediated gene transfer utilizing readily available mature seed scutellum-derived calli.

2) Confirmation of transgenecity of the putatively transgenic plants through molecular analysis for the –

   a) presence of transgene, *cry1Ac*

   b) expression of transgene

3) Bioassay of transgenics by insect-feed assay.

4) Inheritance of transgene(s).

Chapter - 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2 Rice– a staple cereal

Rice is the world's most important cereal that feeds more than half of the global population. Rice is of vital importance to the developing countries especially Asia where more than 90% of world's total rice is produced. Rice is a unique crop that is cultivated from under the sea level to high mountains. Rice is planted on about 146.54 million ha (2002-03) annually producing 381.2 million tonnes (2002-03) (http://www.fas.usda.gov/wap/circular/2002/02-09/grains.pdf) of milled rice. *Oryza sativa* and *Oryza glaberrima* are the two cultivated species of genus *Oryza*. Twenty one wild species of *Oryza*, distributed in Asia, Africa, Australia and Central & South America make the genetics of rice an interesting topic that is described below.

2.1 The genus *Oryza* : origin, classification and genetics

2.1.1 The genus *Oryza*

The genus *Oryza* belongs to a member of the grass family *Graminae,* order *Glumifloreae*, class *Monocotyledonae* and division *Angiospermeae*. *Oryza* consists of two cultivated and twenty one wild species. The two cultivated *Oryza* species are *Oryza sativa*, the Asian cultivated rice grown all over the world and *Oryza glaberrima* that is grown on a small scale in West Africa. Twenty one wild species of *Oryza* are scattered in Asia, Africa, Australia and Central and South America. It is estimated that 4,205,000 rice accessions are conserved in various gene banks worldwide and of this, 10% are wild accessions. The size of the national collection ranges from about 40,000 in China to

25,000 in India, 7,000 in U.S. and other countries have smaller collections (Swaminathan, 1984; Khush, 1997; Paroda, 1998). These germplasm are not only utilized as a source of genetic variability in plant breeding programs but also possess important genetic material for resistance to some insect pests (Swaminathan, 1984).

2.1.2 Origin of rice

The archaeological evidences suggest that the genus *Oryza*, to which the cultivated rice belongs, probably originated at least 130 million years ago. The ancient super continent of Gondwana is believed to be the original habitat of this genus. When Gondwana broke up and became Africa, Antarctica, Australia, Malagasy, South America and Southeast Asia, *Oryza* species drifted into different geographic habitats. Today's species of this genus are distributed in all of these continents except Antarctica (Chang, 1976; Swaminathan, 1984; Khush, 1997). Ancient India is undoubtedly one of the oldest regions where cultivation of *O. sativa* began (Khush, 1997). The excavation from Hastinapura revealed that rice had already existed by 1000 BC in Northern India. The oldest carbonized grains found in India date back to about 6750 BC. The second oldest carbonized rough rice grain excavated in 1973 in Hemudu, a village near Ningpo, Central China, is estimated to be 6000-7000 years old (Matsuo *et al.*, 1997; Khush, 1997). The primary center of diversity for *O. glaberrima*, is probably the swampy basin of the upper Niger river, found around 1500 BC and two secondary centers to the South-West near Guinean coast which were formed 500 years later (Khush, 1997).

2.1.3 Brief genetics of rice

O. sativa L. was first reported and presented by Kuwada in 1910,. who demonstrated n=12 (2n=24), the haploid chromosome number. Studies by Audulov in 1931 provide strong evidence that the basic number of chromosome of genus *Oryza* is 12 (Matsuo *et al.*, 1997). The cultivated species of the genus *Oryza*, *O. sativa* and *O. glaberrima* are diploid (2n=24) and posses 12 pairs of chromosomes. They are numbered according to the decreasing order of length at pachytene stage of sexual cell division. Thus, the longest chromosome is number 1, second longest number 2 and the shortest is number 12. Chromosomes of both the cultivated species and closely related wild species are similar and their genomes are designated as AA genome. The chromosomes of other wild species, however, differ from those of cultivated rice and they belong to genomes designated as BB, CC, DD, EE, FF and GG. A few of the tetraploid species have BBCC, CCDD, and HHJJ genomes (Brar and Khush 1997; Khush, 1997). The wild *Oryza* species are a very rich source for improvements to disease and pest resistance (Sitch, 1990; Ratnayaka, 1999). However, due to less homology between *O. sativa* genome and the wild *Oryza* related species transfer of genes between them via breeding is limited due to low cross-ability and reduced recombination between the chromosomes. However, a technique known as embryo rescue has made it possible to transfer genes between distantly related species (Khush, 1997). The wild types and their useful traits are listed in Table 1.

Table 1. Somatic chromosome number, genomic composition and potentially useful traits of *Oryza* species (adapted from Khush, 1997)

Species	2n	Genome type	Distribution	Useful or potentially useful traits[1]
O.sativa* complex				
O.sativa L.	24	AA	World wide	Cultivated rice
O. nivara Sharma et Shastry	24	AA	Tropical and sub-tropical Asia	Resistance to grassy stunt virus, blast, drought tolerance
O. rufipogon Griff.	24	AA	Tropical and sub-tropical Asia	Elongation ability, resistance to BB, source of CMS
O. breviligulata A. Chev. Et Roehr.	24	A^gA^g	Africa	Resistance to GLH, BB, drought tolerance
O. glaberrima Steud.	24	A^gA^g	West Africa	Cultivated rice
O. longistaminata A. Chev. Et Roehr.	24	A^gA^g	Africa	Resistance to BB, drought tolerance
O. meridionalis Ng.	24	A^mA^m	Tropical Australia	Elongation ability, drought tolerance
O. glumaepatula Steud.	24	$A^{gp}A^{gp}$	South and Central America	Elongation ability, source of CMS
***O. officinalis* complex**				
O. punctata Kotschy ex Steud.	24	BB	Africa	Resistance to BPH, Zigzag leafhopper
O. minuta J.S. Pesl. Ex C.B. Presl.	48	BBCC	Philipipines and Papua New Guinea	Resistance to sheath blight, BB, BPH, GLH
O. officinalis Walls ex Watt	24	CC	Tropical and sub-tropical Asia, tropical Australia	Resistance to thrips, BPH, GLH, WBPH
O. rhizomatis Vaughan	24	CC	Sri Lanka	Drought tolerance, rhizomatous

Table 1 Cont.

O. eichingeri A. Peter	24	CC	South Asia and East Africa	Resistance to yellow mottle virus, BPH, WBPH, GLH
O. latifolia Desv.	48	CCDD	South and Central America	Resistance to BPH, high biomass production
O. alta Swallen	48	CCDD	South and Central America	Resistance to striped stemborer, high biomass production
O. grandiglumis (Doell) Prod.	48	CCDD	South and Central America	High biomass production
O. australiensis Domin.	24	EE	Tropical Australia	Drought tolerance, resistance to BPH
O. brachyantha A. Chev. Et Roehr.	24	FF	Africa	Resistance to yellow stemborer, leaf-folder, whorl maggot, tolerance to laterite soil
***O. meyeriana* complex**				
O. granulata Nees et Arn. Ex Watt	24	GG	South and Soth East Asia	Shade tolerance, adaptation to aerobic soil
O. meyeriana (Zoll. Et Mor. Ex Steud.) Baill.	24	GG	South East Asia	Shade tolerance, adaptation to aerobic soil
***O. ridleyi* complex**				
O. longiglumis Jansen	48	HHJJ	Irian Jaya, Indonesia and Papua New Guinea	Resistance to blast, BB
O. ridley Hook. f.	48	HHJJ	South Asia	Resistance to stemborer, whorl maggot, blast, BB
Unknown genome				
O. schlechteri Pilger	48	Unknown	Papua New Guinea	Increased tillering

[1]BPH = Brown Plant Hopper; GLH = Green Leaf Hopper; WBPH = White Backed Plant Hopper; BB = Bacterial Blight; CMS = Cytoplasmic Male Sterility

***All species of *O. sativa* complex have the same genome, AA. However, genomic differences have been observed in samples from different continents. These differences are indicated by adding a superscript to the AA designation, e.g. A^gA^g for *O. glaberrima*.**

Cultivated rice is the model system for cereal genomic research because of its small genome size (450 Mb – the smallest among the cereals), which makes it possible to develop detailed linkage maps and enables large-scale sequencing of expressed sequences. *Oryza sativa* has three subspecies, indica, japonica and javanica. The three subspecies differ in their agronomic characters such as low temperature sensitivity, drought resistance, awn length, first internode length, grain shape and size and photoperiodic response (Oka, 1988). Indica rice is grown in the humid regions of South and Southeast Asia, and China. It is naturally adapted to the monsoon climate, requiring little weed or water control. Plants of this subspecies are usually tall, leafy and respond to fertilizer by producing more vegetative parts rather than grain (Chrispeels and Sadava, 1994). Japonica species are grown in the temperate regions such as Japan, Taiwan and the lower Yangtze valley of China and Korea. These require precise water, weed and pest control, since they are not well suited for the tropics. Their phenotypic characteristics include short stalks, with short or round grains and early maturity. They were initially considered to be indica subspecies due to their morphological characteristics. They were later reclassified as a japonica subspecies based on the isozyme pattern (Glaszmann, 1987). Glaszmann classified the cultivated rice of the Asian countries into six groups on the basis of genetic affinity using isozyme analysis. This classification involves no morphological characters. When the six groups were compared with the varietal groups classified on the basis of morphological characters, Group I corresponded to the indica and Group VI to the japonicas. Javanica rice also belong to Group VI and designated as tropical japonicas and the so-called japonicas are referred to as temperate japonicas (Khush, 1997).

2.2 Genetic manipulation of rice

2.2.1 Conventional methods

Rice is self pollinated crop with less than 5% cross-pollination. Commercial varieties consist of homozygous, pure lines, which breed true-to-type. Natural crossing between commonly existing rice produces new and mixed plant types in the field. The selection of special types from the mixed population was usually, achieved in the past by pure line selection and in few instances by hybridisation (intervarietal, interspecific and intergeneric). This was done not only to suit environmental condition but also for yield. The rice breeding programme using hybridisation was first initiated by Kano in 1904 in Japan (Grist, 1986). The international Rice Research Institute in the Philippines that was set up in the mid-1960s undertook extensive rice breeding programs. Since then, rice breeders of different countries of the world have developed new varieties by crossing selected parents and eventually created many cultivars with improved qualities. Initially, rice breeding programs were undertaken for agronomically important traits, such as tolerance to drought, flood, salinity and soil alkalinity mainly through selection from mixed natural population and through introduction. Now a day's selection is employed for isolation of superior genotypes from segregating population following hybridization (Poehlman and Sleper, 1995). However, the overall objectives include improvement in productivity coupled with disease and pest resistance, tolerance to abiotic stresses, grain quality and nutritional value. Improvement in productivity, the major goal of breeding any crop, is accomplished through efficiently harnessing the high yield technology (use of dwarfing gene Dee-Geo-Woo-Gen; the agent of green revolution and exploitation of

gene blocks governing component traits of Quantitative character-the QTL's) and hybrid rice technology (exploitation of heterosis using hybrid varieties). Resistance to diseases and pest and tolerance to abiotic stress aids in substantially reducing the loss in productivity of rice.

2.2.2 Molecular methods

The advancement of plant molecular biology and biotechnology has enabled us to isolate, characterize and mobilise plant genes across the plant and animal kingdom. Initially dicotyledonous plants were used for transfer/introducing foreign gene(s). However, persistent effort made even the monocots amenable to transformation. The first report of gene transfer in rice appeared in 1987 using direct gene transfer to protoplast and since then several methods have been used to introduce align genes to rice.

2.2.2.1 Direct gene transfer

(i) PEG mediated transformation

It was the first technique to report the successful integration of foreign genes into a plant cell. This procedure revolves around the use of protoplasts and their totipotent ability to regenerate into mature plants. Protoplasts are plant cells whose cell walls have been removed leaving only a plasma membrane around the cells. Protoplasts can be generated by digesting plant tissue with cellulases (purified from fungal extracts) that breaks down the cellulose in the cell walls. This makes them ideal for receiving DNA, as cell walls have proven to be a significant barrier in the transformation systems.

Protoplast-tissue cultures offer a distinct advantage over multicellular culture, as the free cells have a higher efficiency in the recovery of the transgenic products.

Treatment with polyethylene glycol (PEG) is the most common method of delivering foreign DNA into protoplasts. It is still not clear how PEG causes protoplast to take up DNA however it is thought that it induces reversible permeabilization of the plasma membrane, which allows passage of macromolecules (Songstad *et al.*, 1995). The first success of a viable transgenic plant derived from plant protoplasts was reported in 1984 (Paszkowski *et al.*, 1984). However, a few years before researchers described the integration of *Agrobacterium* T-DNA into tobacco cells via PEG treatment of protoplasts (Krens *et al.*, 1982). PEG treated protoplasts for the most part has been abandoned as a genetic delivery system. The lack of interest in this system is primarily due to the low yields of transformants (approximately 1-2%) and the inability of many species to be regenerated into viable plants (approximately 0.1%), making the whole system only 0.0004% effective.

(ii) Electroporation

An alternative to method of permeabilizing a membrane is electroporation, the exposure of cells to intense electric field. This technique, commonly used in bacterial transformation, is also being applied to protoplasts. The electric field causes formation of in the plasma membrane allowing DNA to be up take by the cells. When the field is shut off, the pores close, resealing the cells and trapping the foreign DNA (Fromm *et al.*, 1987). The optimum conditions, including field strength, protoplast density and

culture buffer for a given species of protoplasts have been determined, as this technique has a high mortality rate (50-75%). Alone, electroporation produces fair results. Electroporation-mediated transformation has been achieved on a variety of species and tissue types, but species-specific protoplast regeneration problems still favor the use of other techniques.

(iii) Liposomes

An advancement to PEG mediated transformation is the liposome mediated transformation technique in which foreign DNA is encapsulated in a spherical lipid bilayer termed a liposome (Gad *et al.*, 1990). In the presence of PEG, the host protoplast will bind and envelop the liposome through endocytosis (Fukunaga *et al.*, 1983). After endocytosis, the DNA is free to recombine and integrate with the host genome. The liposomes are formed from neutral lipids similar to those, which compose the plasma membrane and can be produced in a variety of sizes ranging from 30-50 nm with a volume of approximately 2 µl. The DNA is packaged *in vitro* and then combined with the target protoplasts. As with other transformation systems, a variety of vectors including viral vectors can be incorporated in to this system.

(iv) Biolistic/ Gene-gun delivery system

This technique involved loading DNA into a 0.22 caliber shotgun and shooting cells. This "brute force" approach to plant transformation gives the microprojectile technique a unique ability to ~~infect~~ transform any plant tissue. Other transformation delivery systems, including the biological delivery systems, are in many cases inhibited by the

inability to penetrate the cell walls. Mechanical force, generated by high velocity particles, drives the foreign DNA past all biological barriers, allowing for genomic integration. Furthermore, this approach has the unique ability to target all genomes within a cell, including the chloroplast (Ye *et al.*, 1990; Daniell, 1993) and mitochondrial genomes.

Despite its crude nature, this technique requires careful preparation, administration and in many cases tissue regeneration. In the initial experiments, DNA was bound to tiny tungsten particles, approximately one micrometer in diameter and with gunpowder driven piston, fired at the target cell with a velocity of about 430 meters per second. Host cells in the front line are usually destroyed, but just behind, existed an area of cells where the projectiles penetrated the cell without killing them. Some of the cells that survived the bombardment incorporated the DNA from the projectile, into the genome. After successful incorporation, and if the inserted DNA did not knock out any critical gene function, host cells began to express the gene product of the foreign DNA.

Shortly after its discovery, researchers demonstrated the effectiveness of microprojectile-mediated system by successfully transforming monocots, the first of which was Black Mexican Sweet corn (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990). This new ability to transform and regenerate monocot plants marked a significant advance in plant transformation. As more species, including many monocots, were being added to the transformation success list, researchers were concurrently studying and developing particle bombardment transformation, maximizing this technique for both

monocts and dicots (Russel *et al.*, 1992). In the early 1990's, several significant improvements were made to the particle bombardment system. The first of these advances was the DuPont Biolistics device PDS-1000 (Russel-Kikkert, 1993), which replaced the gunpowder driven format with a system that uses high pressure Helium or Nitrogen gas (approximately 150 kg/cm^2) to accelerate the particles. This system achieved a velocity of approximately 440 m/sec, which is needed for penetration. Other devices based on the Biolistic devices and uses of compressed gases were reported having similar results.

A second advancement in microprojectile transformation was an electric discharge particle acceleration device (DPAD). This apparatus employed a completely new design, which uses shock waves to accelerate the particles. The shock waves are generated by the discharge of 14 kV through a small water drop inside a polyvinyl chloride expansion chamber. The force of the shock wave drives a mylar sheet containing particles and precipitated DNA towards a retaining screen. The mylar sheet is stopped by the screen, but the particles and DNA, now at maximum velocity continue past the screen and collide with the target tissue. This technique has been proven effective in several species (Christou *et al.*, 1988; McCaba *et al.*, 1988). In 1990, an alternate form of the DPAD, called the Biolistics PDS-2000 became commercially available. Several modifications, most notable a shock wave generated by compressed Helium allowed for greater and more controlled acceleration (Ye *et al.*, 1990). As technology progressed, other advances, which significantly lowered the cost

of these experiments, were developed. One of these advances involved the use of an air gun (Oard, 1993). A further important improvement was the particle inflow gun (PIG), which uses a solenoid to direct a Helium stream, accelerating particles into an evacuated chamber (Finer *et al*, 1992).

(v) Silicon carbide fibers

In 1990, a new delivery technique was reported that uses silicon carbide fibers to punch holes through cultured plant cells (Kaeppler *et al.*, 1990). The fibers range from 10-80 μm in length and on average 0.6 μm in diameter (Songstad and Somers, 1995). The size, shape and chemical composition of the fibers, makes them capable of puncturing the cells without killing them. The technique is quite simple; plasmid DNA (containing a reporter gene), silicon carbide fibers and cultured plant cells are added to a tube and vortexed vigorously. The mechanical force generated by the vortex drives the fibers into the cell. In Kaeppler's experiment, gene expression was observed in tobacco and corn cultures. Analysis of their experiment suggests that the DNA binds to the fibers and is incorporated into the cells when they are punctured. Although the transformation frequencies are not as high as microprojectile bombardment or *Agrobacterium* transformation, this technique shows much promise for the future. Since 1990, Kaeppler and colleagues have been optimizing this system, which has dramatically increased the transformation frequencies.

The system is rapid, inexpensive and easy to set up and it can work on a variety of cell types. The general concern with this system, although not currently proven, is the

exposure of laboratory technicians to these fibers. Future prospects have proposed the substitution of the silicon carbide fibers for glass wool fibers.

(vi) Microinjection

This technique uses fine glass needles, which inject the foreign DNA directly into the host cell. It gained popularity in the 1940's when the technology became available to generate and manipulate these micro-needles. A unique advantage to microinjection is that one can transfer any amount of DNA from plasmids to chromosomes to whole organelles (Neuhaus and Spangenberg, 1990). This is a rather crude but effective method of transforming plants. Furthermore, techniques have been developed to inject DNA into protoplasts (Crossway *et al.*, 1986), cultured embryonic cell suspensions (Nomura and Komamine, 1986) and multicellular structures. Although it has a fair transformation frequency (20-50%), microinjection is a time consuming process that requires precise equipment and considerable training.

(vii) Electrophoresis

This technique employs the use of a well known technology, gel electrophoresis. First described in 1989, an electric field is used to force the negatively charged foreign DNA into the host cells (Ahokas, 1989). The apparatus is easy to prepare and involves meristematic tissue subtended by two tubes. DNA is mixed with agar, poured into an open-ended tube containing the cathode and agar is poured into the tube containing the anode. Under an optimized electric field, the DNA passes through the agar, onto the

tissue, passes between the cellulose fibers of the cell wall and into the cell. Optimization of this system has revealed that the best conditions are at 0.5 mA and 50 V for 10 minutes (Songstad *et al.*, 1995). On an average, this technique has 55% survival rate and of the survivors, 57% showed significant expression of a reporter gene (Songstad *et al.*, 1995).

(viii) Laser microbeam

The laser microbean technique is not widely used, as successful regeneration of transgenic plants has not been reported. This system uses a 343 nm UV laser through the objective lens of a microscope to introduce foreign DNA into cells (Weber *et al.*, 1989). This technique has also been shown to penetrate chloroplasts making it possible to transform the chloroplast genome (Weber *et al.*, 1989). The laser pokes precise tiny holes in the cells allowing plasmid DNA to be taken up. Once the DNA is incorporated into the cell the holes reseals forming an intact cell. This technique showed that 20% of treated cells were capable of producing colonies on a selection medium, indicating successful incorporation of the foreign DNA (Weber *et al.*, 1989).

(ix) Desiccation

Previous research has shown that dried embryos have the unique ability to take up DNA during rehydration (Topfer *et al.*, 1989). This approach takes advantage of the natural changes that occur within the embryonic plant cell wall during rehydration. Dried embryos can be mixed with a nutrient solution containing the foreign DNA. The

DNA would be taken up as the embryo rehydrates and seedlings can be germinated in the presence of a selection medium to assess the incorporation of the foreign DNA. Due to its simplicity, this property is attractive to those developing new delivery systems and may become more popular in the future.

Of all the methods described above microprojectile mediated gene transfer is the most widely used in plant transformation.

2.2.2.2 *Agrobacterium*-mediated gene transfer

Gene transfer to plants can also be mediated through *Agrobacterium* by many of its species. The bacteria *A. tumefaciens* causes crown gall disease, *A. rhizogenes* causes hairy root disease, *A. rubi* causes cane gall disease and *A. vitis* causes galls on grape and in a few other plant species (Otten *et al.*, 1984). A soil plant pathogenic bacterium, "*A. tumefaciens*" has become the most widely accepted organism for the introduction of foreign genes into plant cells. *A. tumefaciens* naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumors. *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Nester *et al.*, 1984; Binns and Thomashaw, 1988). T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation; and the genes encoding for the synthesis of opines. These compounds, produced by condensation between amino acids and sugars, are

synthesized and excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources. Outside the T-DNA, are located the genes for the opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer. (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995). Virulent strains of *A. tumefaciens* and *A. rhizogenes*, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crow gall and hairy roots, respectively. These strains contain a large megaplasmid which play a key role in tumor induction and for this reason it was named Ti plasmid, or Ri in the case of *A. rhizogenes*. Ti plasmids are classified according to the opines, which are produced and excreted by the tumors they induce. During infection the T-DNA, a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated into the plant chromosome. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. The 30 kb virulence (*vir*) region is a regulon organized in six operons that are essential for the T-DNA transfer (*virA, virB, virD,* and *virG*) or for the increasing of transfer efficiency (*virC* and *virE*) (Hooykaas and Schilperoort, 1992; Zupan and Zambryski, 1995).

2.3 *Agrobacterium tumefaciens*-nature's genetic engineer

2.3.1 Introduction of genes into plants by using *Agrobacterium*

Years before scientists elucidated the molecular mechanism of *Agrobacterium*-mediated transformation of plants, Braun, (1947) proposed the concept of a "tumor-inducing principle" that was stably transferred to and propagated in the plant genome Research in the 1970's resulted in the identification of large plasmids in virulent *Agrobacterium* strains (Zaenen *et al.*, 1974), although we now know that many strains contain plasmids unrelated to virulence. Genetic experiments indicated that a particular class of plasmids, the Ti (and later Ri) plasmids, were responsible for tumorigenesis (Van Larebeke *et al.*, 1974) and that a portion of these plasmids, the T-DNA, was transferred to plant cells and incorporated into the plant genome (Chilton *et al.*, 1977). It was, thus, obvious to propose that Ti plasmids be used as a vector to introduce foreign genes into plant cells. Hoekema *et al.* (1983) and de Frammond *et al.* (1983) determined that the T-region and the *vir* genes could be separated into two different replicons. When these replicons were within the same *Agrobacterium* cell, products of the *vir* genes could act in trans on the T-region to effect T-DNA processing and transfer to a plant cell. Hoekema *et al.* (1983) called this a binary-vector system; the replicon harboring the T-region constituted the binary vector, whereas the replicon containing the *vir* genes became known as the *vir* helper. The *vir* helper plasmid generally contained a complete or partial deletion of the T-region, rendering strains containing this plasmid unable to incite tumors. A number of *Agrobacterium* strains containing non-oncogenic *vir* helper plasmids have been developed, including LBA4404 (Ooms *et al.*, 1981), GV3101 MP90

(Koncz and Schell, 1986.), AGL0 (Lazo *et al.*, 1991), EHA101 and its derivative strain EHA105 (Hood *et al.*, 1993; Hood *et al.*, 1986), and NT1 (pKPSF2) (Palanichelvam *et al.*, 2000). T-DNA binary vectors revolutionized the use of *Agrobacterium* to introduce genes into plants. Scientists without specialized training in microbial genetics could now easily manipulate *Agrobacterium* to create transgenic plants. These plasmids are small and easy to manipulate in both *E. coli* and *Agrobacterium* and generally contain multiple unique restriction endonuclease sites within the T-region into which genes of interest could be cloned. Many vectors were designed for specialized purposes, containing different plant selectable markers, promoters, and poly(A) addition signals between which genes of interest could be inserted, translational enhancers to boost the expression of transgenes, and protein-targeting signals to direct the transgene-encoded protein to particular locations within the plant cell (some representative T-DNA binary vector systems are described in literatures (An *et al.*, 1985; Becker, 1990; Bevan, 1984; Bhattacharyya *et al.*, 1994; Day *et al.*, 1994; Gleave, 1992; Hajdukiewicz *et al.*, 1994; McBride and Summerfelt, 1990; Xiang *et al.*, 1999; Zyprian and Kado, 1990). Hellens *et al.* (2000) provided a summary of many *A. tumefaciens* strains and vectors commonly used for plant genetic engineering. Although the term "binary vector system" is usually used to describe two constituents (a T-DNA component and a *vir* helper component), each located on a separate plasmid, the original definition placed the two modules on different replicons. These replicons do not necessarily have to be plasmids. Several groups have shown that T-DNA, when located in the *Agrobacterium* chromosome, can be mobilized to plant cells by a *vir* helper plasmid (Hoekema *et al.*, 1984; Miranda *et al.*, 1992).

2.3.2 What DNA is transferred from *Agrobacterium* to plants?

T-DNA was initially defined as the portion (the T-region) of the Ti plasmid that was transferred from *Agrobacterium* to plant cells to form crown gall tumors. T-DNA border repeat sequences defined the T-region (Xing *et al.*, 2000), and regions of the Ti plasmid outside these borders were not initially found in tumor cells (Chilton *et al.*, 1977). However, the transfer of Ti-plasmid sequences outside the conventional T-region may at first have been missed because of a lack of known selectable (e.g., tumorigenesis) or screenable (e.g., opine production) markers. Ooms *et al.* (1982) observed the incorporation into plant DNA of regions of the Ti plasmid later shown to be outside the classical T-DNA borders. Ramanathan and Veluthambi (1995) also showed that a *nos-nptII* cassette, placed outside the T-DNA left border, could be transferred to and confer kanamycin resistance on infected tobacco cells. The use of relatively small T-DNA binary vectors made it easier for scientists to evaluate the transfer of "non-T-DNA" regions to plants. Martineau *et al.* (1994) first reported the transfer of binary vector backbone sequences into transgenic plant DNA and questioned the definition of T-DNA. Wenck *et al.* (1997) found that the entire binary vector, including backbone sequences as well as T-DNA sequences, could frequently be transferred to *Nicotiana plumbaginifolia* and *Arabidopsis thaliana* cells. Kononov *et al.* (1997) carefully examined the structure of binary vector backbone sequences that could be found in up to 75% of transgenic tobacco plants and concluded that such transfer could result from either skipping the left T-DNA border when T-DNA was processed from the binary vector or initiation of T-DNA transfer from the left border to bring

vector backbone sequences into plant cells. Considering the previous observation by Durrenberger *et al.* (1989) that *Vir*D2 protein could covalently attach to the 5' end of the non-T-DNA strand, Kononov *et al.* (1997) suggested that transfer of vector backbone sequences to plants was a natural consequence of the mechanism of *Vir*D2 function. Thus, the definition of T-DNA and vector backbone constitutes a semantic argument. It would, thus, appear that the transfer of non-T-DNA sequences to plants may be an unavoidable, but frequently unobserved and untested, result of transformation. Indeed, Frary and Hamilton (2001) observed incorporation of BIBAC plasmid sequences into 9 to 38% of tested tomato transformants. Although the transfer of plasmid backbone sequences may be an unavoidable consequence of the mechanism of *Agrobacterium*-mediated transformation, it may be possible to select against transgenic plants containing this unwanted DNA. Hanson *et al.* (1999) showed that the incorporation of a toxic "killer" gene into the binary vector backbone sequences could severely reduce the percentage of transgenic plants containing such extra sequences. Remarkably, the transformation frequency of tobacco, tomato, and grape plants infected using this modified binary vector did not substantially differ from that of plants infected using a binary vector lacking the killer gene. Because the presence of uncharacterized DNA in transgenic plants is important for regulatory concerns, such an approach may be useful in the future for the production of plants (especially difficult to transform species) with a more highly defined transgenic composition.

2.3.3 Transfer of multiple T-DNAs into the same plant cell and generation of "Marker-Free" transgenic plants

Because of concerns regarding the spread of antibiotic resistance genes in nature or the escape of herbicide resistance genes to wild weedy species, scientists have developed several methods to generate marker-free transgenic plants. These plants would initially be selected for resistance to an antibiotic or herbicide, but the selection marker would be removed on subsequent manipulation and plant growth. Several methods have been proposed to eliminate the selection marker from the primary transformant. These include use of a site-specific recombination system, such as Cre-*lox* or Flp-Frt (Albert *et al.*, 1995; Bayley *et al.*, 1992; Dale and Ow, 1990; Lyznik *et al.*, 1996; Odell *et al.*, 1990; Vergunst and Hooykaas, 1998; Vergunst *et al.*, 1998) to remove the marker, transposon-based movement of the selection marker from the initial site of insertion in the plant genome entirely or to another unlinked site from which it can be segregated in subsequent generations (Ebinuma *et al.*, 1997), or the use of multiple T-DNAs which can insert into unlinked sites for future segregation (Hohn *et al.*, 2001; Yoder and Goldsbrough, 1994). Each of these systems has advantages and disadvantages, as for example; excision of marker genes using a site-specific recombination system requires introduction of the site-specific recombinase into plants, either by transformation or by genetic crossing. Segregation of markers can occur only in progeny following the generation of the initial transgenic plant and is limited to species naturally propagated through seed and not those propagated vegetatively. Early research that characterized the integration pattern of T-DNAs in crown gall tumors

indicated that each of the two T-DNAs encoded by an octopine-type Ti plasmid could independently integrate into the plant genome, sometimes in multiple copies (Chilton *et al.*, 1977; DeBeuckeleer *et al.*, 1981; Thomashow *et al.*, 1980). The molecular analyses suggested that these T-DNAs could be integrated into unlinked sites.

These results suggested that co-transformation could be performed to integrate transgenes carried by two different T-DNAs and that perhaps these T-DNAs would segregate in subsequent generations. Three approaches were subsequently used for co-transformation: (i) the introduction of two T-DNAs, each from a different bacterium; (ii) the introduction of two T-DNAs carried by different replicons within the same bacterium; and (iii) the introduction of two T-DNAs located on the same replicon within a bacterium. Early experiments using these various approaches indicated that co-transformation could be a frequent event. An *et al.* (1985) showed that tobacco cells could be co-transformed to two different phenotypes by a single *Agrobacterium* strain containing both a Ti plasmid (phytohormone-independent growth) and a T-DNA binary vector (kanamycin-resistant growth). This experiment represents a "one-strain, two-replicon" approach to cotransformation. When the cells were first selected for kanamycin resistance, 10 to 20% of them also displayed phytohormone- independent growth; when the cells were first selected for phytohormone-independent growth, 60% of the resulting calli were also kanamycin resistant. The authors credited these differing frequencies to the higher copy number (5 to 10) of the binary vector in the bacterium relative to the single copy Ti plasmid. These experiments were extended by de Frammond *et al.* (1986), who showed that fertile transgenic plants could be regenerated from cloned tobacco tissue that was cotransformed by T-DNA from a Ti plasmid and

from a micro-Ti (the one-strain, two-replicon approach). The two DNAs segregated in progeny plants, indicating that the T-DNAs had integrated into genetically separable loci. Other groups have used the one strain, two-replicon approach to generate transgenic plants which initially expressed both T-DNA markers but could subsequently segregate the markers from each other (Daley *et al.*, 1998). The use of two *Agrobacterium* strains to deliver different T-DNAs to the same plant cells was studied by a number of groups (De Buck *et al.*, 2000a; De Buck *et al.*, 1998; De Buck *et al.*, 1999; De Neve, *et al.*, 1997; McKnight *et al.*, 1987). Although co-transfer of T-DNAs to genetically unlinked sites was reported, some authors also reported close linkage of the two different T-DNAs in many instances. It thus remains unclear, which of the three co-transformation protocols will be reproducibly best for the generation of marker-free transgenic plants.

2.3.4 Virulence gene expression and plant transformation

The processing and transfer of T-DNA from *Agrobacterium* to plant cells is regulated by the activity of the *vir* genes. Virulence gene activity is induced by plant wound-induced phenolic compounds such as acetosyringone and related molecules (Delay *et al.*, 1992; Delmotte *et al.*, 1991; Dye *et al.*, 1997; Morris and Morris, 1990; Song *et al.*, 1991). However, there may be instances in which scientists would like to induce *vir* genes to levels higher than that accomplished by plant extracts. Several groups have, therefore, identified *virA* and *virG* mutants that function constitutively, in the absence of phenolic inducers. Constitutive *virA* mutants were characterized by several groups (Ankenbauer *et al.*, 1991; McLean *et al.*, 1994; Pazour *et al.*, 1991).

However, more emphasis has been placed on inducer-independent *virG* mutants, possibly because *virG* functions downstream of *virA*. Extensive genetic studies resulted in the identification of a number of mutations that render the *Vir*G protein active in the absence of phenolic inducing compounds (Han *et al.*, 1992; Pazour *et al.*, 1992). These altered proteins contain mutations that convert either asparagine-54 to aspartic acid (*virGN54D*) or isoleucine-106 to leucine (*virGI106L*). Both of these mutant proteins stimulate a high level of *vir* gene expression, especially when expressed from a high-copy-number plasmid (Gubba *et al.*, 1995). When tested in transient tobacco and maize transformation assays, strains containing the *virGN54D* mutant effected a higher level of transformation than did strains encoding the wild-type *virG* gene (Hansen *et al.*, 1994). An even greater effect was seen when the *virGN54D* allele was harbored on a high-copy-number plasmid; the presence of this mutant gene in *Agrobacterium* increased the transient transformation of rice and soybean two to sevenfold (Ke *et al.*, 2001). Several laboratories have determined the effect of additional copies of wild-type *virG* genes on *vir* gene induction and plant transformation. Rogowsky *et al.* (1987) showed that additional copies of nopaline-type *virG* resulted in increased *vir* gene expression. Liu *et al.* (1993) showed that multiple copies of *virG* altered the pH response profile for *vir* gene induction. Normally, *vir* gene induction is very poor at neutral or alkaline pH or in rich medium; additional copies of *virG* permitted a substantial degree of induction in rich medium even at pH 8.5. Additional copies of *virG* also increased the transient transformation frequency of rice, celery, and carrot tissues (Liu *et al.*, 1992).

2.3.5 T-DNA integration and transgene expression

Plant transformation does not always result in efficient transgene expression. The literature is replete with examples of variable expression levels of transgenes, which frequently did not correlate with transgene copy number (Peach and Velten, 1991). This lack of correspondence was initially attributed to position effects, i.e., the position within the genome into which the T-DNA integrated was credited with the ability of transgenes to express. T-DNA could integrate near to or far from transcriptional activating elements or enhancers, resulting in the activation (or lack thereof) of T-DNA-carried transgenes. T-DNA could also integrate into transcriptionally competent or transcriptionally silent regions of the plant genome. The high percentage (approximately 30%) of T-DNA integration events that resulted in activation of a promoterless reporter transgene positioned near a T-DNA border suggested that T-DNA may preferentially integrate into transcriptionally active regions of the genome. Only integration events that would link the promoterless transgene with an active promoter would result in reporter activity (Koncz *et al.*, 1989). However, a drawback to some of these experiments was that transgenic events may have been biased by the selection of antibiotic resistant plants expressing an antibiotic marker gene carried by the T-DNA. It is not clear whether T-DNA insertions into transcriptionally inert regions of the genome would have gone unnoticed because of lack of expression of the antibiotic resistance marker gene. An obvious way to circumvent the presumed problems of position effect is to integrate T-DNA into known transcriptionally active regions of the plant genome. However, gene targeting in plants by homologous recombination has been at best extremely inefficient (de Groot *et al.*, 1994; Kempin *et al.*, 1997; Miao and Lam, 1995; Offringa *et al.*, 1990;

Offringa *et al.*, 1992; Risseeuw *et al.*, 1997; Risseeuw *et al.*, 1995). An alternative system for gene targeting is the use of site-specific integration systems such as Cre-*lox*. However, single-copy transgenes introduced into a *lox* site in the same position of the plant genome also showed variable levels of expression in independent transformants. Transgene silencing in these instances may have resulted from transgene DNA methylation (Day *et al.*, 2000). Such methylation-associated silencing was reported earlier for naturally occurring T-DNA genes (Hepburn *et al.*, 1983; Van Slogteren *et al.*, 1984). Thus, transcriptional silencing may result from integration of transgenes into regions of the plant genome susceptible to DNA methylation and may be a natural consequence of the process of plant transformation. We now know not only that transgene silencing results from "transcriptional" mechanisms, usually associated with methylation of the transgene promoter (Meyer, 2000), but also that transgene silencing is often "post-transcriptional"; i.e., the transgene is transcribed, but the resulting RNA is unstable (Meins, 2000). Such post-transcriptional gene silencing is frequently associated with multiple transgene copies within a cell. Transgenic plants generated by direct DNA transfer methods (e.g., polyethylene-glycol or liposome-mediated transformation, electroporation, or particle bombardment) often integrate a large number of copies of the transgene in tandem or inverted repeat arrays, in either multiple or single loci (Kohli *et al.*, 1999b). Although *Agrobacterium* mediated transformation usually results in a lower copy number of integrated transgenes, it is common to find tandem copies of a few T-DNAs integrated at a single locus (Jorgensen *et al.*, 1987). Transgene silencing can occur in plants harboring a single integrated T-DNA (Elmayan and Vaucheret, 1996). However, integration of T-DNA repeats, especially 'head-to-head' inverted repeats

around the T-DNA right border, frequently results in transgene silencing (Cluster *et al.*, 1996; Jorgensen *et al.*, 1996; Stam *et al.*, 1997). Thus, a procedure or *Agrobacterium* strain that could be used to generate transgenic plants with a single integrated T-DNA would be a boon to plant molecular biology in general and to the agricultural biotechnology industry in particular. Grevelding *et al.* (1993) noted that transgenic *Arabidopsis* plants derived from a root transformation procedure tended to have fewer T-DNA insertions than did plants derived from leaf disks. However, it is not clear if this observation can be generally applicable to other plant species. Anecdotal information from several laboratories suggests that *Agrobacterium* strains that are less efficient in delivering T-DNA may be more efficient in producing single-copy T-DNA insertions. However, these findings need to be tested rigorously; it is possible that T-DNA copy number may also correlate with the growth state of the bacterium or the plants to be transformed.

2.4 *Agrobacterium*-mediated plant transformation

Twenty-five years ago, the concept of using *A. tumefaciens* as a vector to create transgenic plants was viewed as a prospect and a "wish." Today, many agronomically and horticulturally important species are routinely transformed using this bacterium, and the list of species that is susceptible to *Agrobacterium*-mediated transformation seems to grow daily. In some developed countries, a high percentage of the acreage of such economically important crops as corn, soybeans, cotton, canola, potatoes, and tomatoes is transgenic; an increasing number of these transgenic varieties are or will soon be generated by *Agrobacterium*-mediated, as opposed to particle bombardment-mediated

transformation. There still remain, however, many challenges for genotype-independent transformation of many economically important crop species, as well as forest species used for lumber, paper, and pulp production. In addition, predictable and stable expression of transgenes remains problematic. Several excellent reviews have appeared recently that describe in detail various aspects of *Agrobacterium* biology (Christie, 1997; de la Riva *et al.*, 1998; Gelvin, 2000; Tzfira and Citovsky, 2000; Tzfira *et al.*, 2000; Zupan *et al.*, 2000; Zupan and Zambryski, 1997).

2.4.1 *Agrobacterium*-mediated transformation of monocotyledons

Limited early evidences such as formation of tumors and production of opines in certain monocotyledons, suggest that at least some monocotyledonous species might be susceptible to infection by *A. tumefaciens*. De Cleen and De Ley (1976) listed two families of monocotyledons; Liliales and Arales, among families that included species susceptible to *A. tumefaciens*. Induction of tumors following inoculation of *A. tumefaciens* has been demonstrated in *Asparagus officinales* (Hernalsteens *et al.*, 1984), *Gladiolus* (Graves and Goldman, 1987) and *Dioscora bulbifera* L. (yam) (Schafer *et al.*, 1987) and production of opines by tumors or at sites of inoculation has been detected in monocotyledons that include *Asparagus officinales* (Bytebier *et al.*, 1987; Hernalsteens *et al.*, 1984), *Chlorophytum capense* L. (Liliaceae) (Hooykas-van Slogteren *et al.*, 1984), *Narcissus canaliculatus* (Amaryllidaceae) (Hooykas-van Slogteren *et al.*, 1984) and *Gladiolus* (Graves and Goldman, 1987). Many monocot plant species, including some cultivars of grasses such as maize (Ishida *et al.*, 1996; Frame *et al.*, 2002; Gould *et al.*, 1991), rice (Chan *et al.*, 1992 and 1993; Hiei *et al.*, 1994; Dong *et al.*, 1996; Rashid *et*

al., 1996; Toki, 1997; Khanna and Raina, 2002; Sridevi *et al.*, 2003; Al-Forkan *et al.*, 2004), barley (Tingay *et al.*, 1997) and wheat (Zhang *et al.*, 2000; Amoah *et al.*, 2001), can now be genetically transformed by many *Agrobacterium* strains to the phenotype of antibiotic or phenotypic resistance. However, these plant species do not support the growth of crown gall tumors (Gelvin, 2003). Susceptibility to crown gall disease has a genetic basis in cucurbits (Smarrelli *et al.*, 1986), peas (Robbs *et al.*, 1991), soyabean (Bailey *et al.*, 1994; Mauro *et al.*, 1995; Owens *et al.*, 1984) and grapevines (Szegedi and Kozma, 1984) and even among various ecotypes of *Arabidopsis thaliana* (Nam *et al.*, 1997).

Another type of study of the transfer of T-DNA to monocotyledons was reported by Grimsley *et al.* (1986). *A. tumefaciens* containing the genome of maize streak virus (MSV) in the T-DNA was used to inoculate maize seedlings (Grimsley *et al.*, 1986; Grimsley *et al.*, 1987). The seedlings showed systemic symptoms of viral infection, indicating that T-DNA has been transferred to maize cells. Such introduction of plant infectious agents into plants via *A. tumefaciens* was designated agroinfection. However such studies of agroinfection did not provide any evidence of the integration of T-DNA into the plant genome.

Mooney *et al.* (1991) produced transformed cells from wheat immature embryos that had been co-cultivated with *A. tumefaciens*. Although they were unable to regenerate wheat plants from transgenic callus, they detected T-DNA in callus tissue by Southern hybridization.

The early studies of *Agrobacterium*–mediated transformation of monocotyledons were somewhat controversial. Evidence of integration of T-DNA into host genomic DNA in transgenic plants was demonstrated unequivocally only in *Asparagus officinalis* (Bytebier *et al.*, 1987). Potrykus (1990) presented two critical reviews and suggested that the various groups of researchers might have overlooked the possibility of gene expression by *Agrobacterium* cells that had become attached to plant cells, as well as the transformation of microorganisms that are silently infecting the host plant tissues, however during 1990s several monocots were stably transformed and wheat, rice and maize are the most important cereals included in the list.

2.4.2 *Agrobacterium*-mediated gene transfer to rice

In the past, monocots particularly graminacious crop plants including important cereals like rice and wheat were considered to be recalcitrant to this technology and they were outside the *Agrobacterium* host range. However, transformation methods based on the use of *Agrobacterium* are still preferred in many instances because of the following properties: (i) ease of handling, (ii) higher efficiency, (iii) more predictable pattern of foreign DNA integration, and (iv) low copy number of integration.

Raineri *et al.* (1990) described the production of transformed japonica cultivar by cocultivation of mature embryos with *A. tumefaciens*. Results of Southern blotting indicated the integration of the T-DNA into the plant genome, but no transgenic plants were regenerated. Chan *et al.* (1992) obtained a few transgenic rice plants by inoculating immature embryos with a strain of *Agrobacterium*. They proved the inheritance of the

transformed DNA to progeny plants by Southern hybridization, although they analysed the progeny of only one transformed plant. Hiei *et al.* (1994) subsequently reported a method for efficient production of transgenic rice plants from calli of japonica cultivars that had been cocultivated with *A. tumefaciens*. Their evidence was based on molecular analysis and genetic studies of a large number of transgenic plants and the analysis of sequence of T-DNA junctions in rice. Rashid *et al.* (1996) reported the successful application of such a method to basmati cultivars of indica rice after only minor modifications. In the same year, Dong *et al.* (1996) also described the successful applications of the method to javanica rice. Aldemita and Hodges (1996) showed that immature embryos were also good starting materials for *Agrobacterium*-mediated transformation of indica and japonica varieties. Park *et al.* (1996) reported that the isolated shoot apices are suitable explants for successful application of this method. Mohanty *et al.* (1999) reported the successful application of this method in an elite indica rice variety Pusa Basmati 1 and transmission of the transgene to the R_2 progeny also demonstrated. Khanna and Raina (1999) transformed indica rice cultivars by the *Agrobacterium*-mediated method using binary and super-binary vectors. Chen *et al.* (1998a) developed a protocol for consistent and large-scale production of fertile transgenic rice plants. Yokoi *et al.* (1998) produced chilling tolerance through unsaturation of fatty acids in rice by introducing the *GPAT* gene. Goto *et al.* (1999) obtained rice seeds with iron fortification by using soybean ferritin gene. Ye *et al.* (2000) succeeded in engineering the provitamin A (β-carotene) biosynthetic pathway into carotenoid-free rice endosperm, popularly known as Golden rice. Yara *et al.* (2001) reported production of transgenic rice plants of Taiwanese japonica rice cultivar,

Taichung 65, were obtained by co-cultivating scutellum calli with an *A. tumefaciens* strain, EHA 101, that carried a binary vector harboring the luciferase gene driven by the CaMV35S promoter. Khanna and Raina (2002) obtained Bt-transgenics of elite indica rice breeding lines (IR64, Pusa Basmati 1 and Karnal Local) that were mediated through biolistic or *Agrobacterium*-mediated approaches, with synthetic *cry1Ac* gene (codon optimised for rice) driven by the maize ubiquitin promoter. Initial transformation frequency was in the range of 1 to 2 for particle bombardment while it was comparatively higher (~9%) for *Agrobacterium*. They also reported improved selection procedure, involving longer selection on the antibiotic-supplemented medium, enhanced the frequency of Bt-transformation and reduced the number of escapes. Mohanty *et al.* (2002) obtained transgenics of an elite indica rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress by *Agrobacterium*-mediated approach. Sridevi *et al.* (2003) obtained Pusa Basmati 1 through *Agrobacterium*-mediated transformation of an elite indica rice variety using LBA4404 (pSB1, pMKU-RF2) that harbours chitinase gene (*chl*11) under the control of the maize ubiquitin promoter. Bioassays of T_1 plants revealed enhanced resistance to the sheath blight pathogen in comparison to control plants. Al-Forkan *et al.* (2004) generated morphologically normal, fertile transgenic plants were obtained by co-culturing embryogenic calli of the Bangladeshi indica rice cultivars BR26 and Binni with *A. tumefaciens* strain LBA4404 carrying the super binary vector pTOK233.

2.4.2.1 Key factors involved in *Agrobacterium*-mediated transfer of genes to rice

It is clear that numerous factors are of importance in the *Agrobacterium*

mediated transformation of rice and this multiplicity of factors probably explains why it was initially so difficult to apply this technology to rice. The important factors that effect gene transfer are discussed below.

2.4.2.1.1 Induction of *vir* genes

The bacterium is presumably attracted to a wounded plant in response to signal molecule released by the plant cells to which it then become attached (Hohn *et al.*, 1989; Zambryski, 1992). Wounded tobacco (*Nicotiana* sp.) cells exude phenolic compounds, such as 4-acetyl-2, 6-dimethoxyphenol (acetosyringone) and 4-(2-hydroxyacetyl)-2,6-dimethoxyphenol (α-hydroxyacetosyrinone), which activate *vir* genes on Ti plasmids (Stachel *et al.*, 1985). Bolton *et al.* (1986) found that seven phenolic compounds induced the expression of *vir* genes. These signal molecules appear to be very important in the recognition of suitable hosts by *A. tumefaciens*. Monocotyledons, in particular grasses, appear not to produce these compounds, or if they do, the levels are insufficient to serve as signals (Smith and Hood, 1995).

One of the most commonly used techniques in the transformation of dicotyledonous plants is the addition of phenolic compounds, such as acetosyringone, to co-cultures or bacterial cultures (van Wordragen and Dons, 1992). Their addition is indispensable if the plant does not produce sufficient level of signal molecules. Rice cells might be capable of producing a certain level of signal molecules since Raineri *et al.* (1990) did not add phenolic compounds in their successful attempt to obtain transgenic calli. Vijayachandra *et al.* (1995) reported that rice tissue could induce

expression of *vir* genes but that induction was greatly enhanced by addition for acetosyringone. Thus, the level of signal molecules produced by rice tissue appears to be very limited. Hiei *et al.* (1994) demonstrated that acetosyringone at 100 μM concentration is a key to successful transformation of rice. The level of transient expression of β-glucuronidase (GUS), a marker enzyme, after co-cultivation was extremely low when acetosyringone was omitted. Chan *et al.* (1993) included the medium from suspension culture of potato cells, which is a rich source of phenolic compounds, in the co-cultures. Transfer of T-DNA is initiated at an early stage of co-cultivation in the presence of acetosyringone and pre-treatment of bacteria with acteosyringone is not essential. However, pre-treatment might slightly increase the efficiency of gene transfer (Aldemita and Hodges, 1996).

Other factors during co-cultivation, such as an acidic pH (Alt-Moerbe *et al.* 1988; Turk *et al.*, 1991), culture below 28°C (Alt-Moerbe *et al.*, 1988), and high osmotic pressure (Usami *et al.*, 1988), have also been reported to be important for the expression of *vir* genes. Veluthambi *et al.* (1989) reported that opines stimulated the induction of the *vir* genes. Shimoda *et al.* (1990) and Cangelosi *et al.* (1990) independently reported that a group of aldoses, such as D-glucose and some non-catabolisable sugars markedly enhanced the expression *vir* genes. Hiei *et al.* (1994) confirmed the importance of these factors. Transient expression of GUS in calli derived from scutella after co-cultivation with *Agrobacterium* was strongest when co-cultures were incubated between 22°C and 28°C, and the pH of the co-cultivation medium was between 4.8 to 6.2. The addition of

D-glucose, D-galactose or L-arabonose, and of nopaline did not have any significant synergistic effect in the presence of 100 μM acetosyringone.

2.4.2.1.2 Active cell division and the rice tissue

Early experiments with *Agrobacterium* indicated that wounding of the host plant is required for the development of tumors (Lippincott and Lippincott, 1975). Binns and Thomashaw *et al.* (1988) pointed out the distinct correlation in the literature between the wound-induced division of cells and the competence of such cells to be transformed by *A. tumefaciens*. They proposed that the process related to the synthesis of DNA and cell divisions are required for incorporation of foreign DNA into host genome (Binns and Thomashaw, 1988). Evidence consistent with this hypothesis has been reported in the case of both *Agrobacterium*-mediated transformation and direct transformation (An *et al.*, 1985; Lida *et al.*, 1991; Kudirka *et al.*, 1986; Okada *et al.*, 1986; Valvekens *et al.*, 1988; Wullems *et al.*, 1981). The wound responses of many monocotyledons differ from those of dicotyledons. In many monocotyledons, the cells at sites of wounds tend to be lignified or sclerified without the apparent divisions for cells (Kahl, 1982). Therefore, most monocotyledons are poor host for *A. tumefaciens* (Binns and Thomashaw, 1988; Potrykus, 1990). If the main roles of wounding in the transformation process are the production of *vir*-inducing molecules and the induction of DNA synthesis and rapid division of cells, actively growing tissues from monocotyledons might be transformable in the presence of *vir*-inducing compounds. Reports of the successful transformation of monocotyledons support this hypothesis (Hiei *et al.*, 1994 and Ishida *et al.*, 1996). Tissues consisting of actively dividing cells were co-cultivated with *A. tumefaciens* in

the presence of 100 μM of acetosyringone in such successful studies. Callus cultures are excellent sources of cells for the production of transgenic rice (Dong *et al.*, 1996; Hiei *et al.*, 1994; Rashid *et al.*, 1996). The use of actively growing embryogenic calli is one of the most important factors in efficient transformation. Such calli can be obtained from mature or immature embryos. Others tissues, including shoot apices, immature inflorescences and young roots, might also produce embryogenic calli but have not been tested extensively. Long-term culture does not significantly affect the efficiency of transformation, but risk of so-called somaclonal variations might be associated with such long-term cultures. Unlike, those of japonica rice, the embryogenic calli of indica varieties are poor starting materials for *Agrobacterium*-mediated transformation, probably because indica calli tend to grow very slowly. By contrast, freshly isolated immature embryos consist of actively dividing cells. Thus for the transformation of most japonica and some indica varieties of rice (Rashid *et al.*, 1996), which produced rapidly growing embryogenic calli, callus cultures are the preferred starting materials. Large numbers of pieces of calli can be prepared more easily than immature embryos. For the transformation of other varieties, such as group I indica rice, immature embryos are probably the most suitable materials. Because of having to deal with two different biological elements, many parameters should be tested to satisfy both partners and guarantee a successful outcome. These variables include the use of feeder cells, alternative *Agrobacterium* strains, infiltration of the bacteria, the duration and temperature of co-cultivation.

2.4.2.1.3 Selectable marker genes for selection of transgenics during the culture process

Selection is an important part of the transformation process. In general, the gene of interest is co-delivered with a selectable marker to identify and encourage the growth of recipient cells. During the process of transformation, a specific segment of the vector, T-DNA, which can be engineered to contain a selectable marker and/or genes of interest, is transferred from the bacterium to the host plant cells and inserted into the nuclear genome. Selectable markers usually confer resistance to chemical agents, such as antibiotics or herbicides that inhibit various cellular functions (Wilmink and Dons, 1993). Scorable markers, such as Green Fluorescent Protein (GFP) (Haseloff *et al.*, 1997), are also used for selection. In most cases, some tailoring of the selection process is required for each crop. The gene for neomycin phosphotransferase [*nptII*, also called *aph* (3') *II* or *neo*] was used in many early attempts in direct transformation of rice. This gene confers resistance to the amino-glycoside-antibiotic kanamycin. While kanamycin can be used as a selective agent during regeneration of protoplast, it is not effective for selection of transformed calli. In addition, many calli recovered after kanamycin selections are unable to regenerate into green plants (Ayers and Perk, 1994; Toriyama *et al.*, 1988). G418 is a related amino-glycoside antibiotic that is also inactivated by neomycin phoshotranferase. Not only was selection of transformed calli more efficient using G418 than kanamycin, but calli selected on G418 had a higher frequency of regeneration onto green plants than those selected on kanamycin (Ayers and Perk, 1994). In *Agrobacterium*-mediated transformation stably transformed calli can be produced efficiently with G418, but absence of regenerants, suggest that exposure of

cells to G418 for a long time inhibits regeneration (Aldemita and Hodges, 1996). Another widely used, more effective selectable marker is hygromycin phosphotransferase (*hpt*, *aph-IV* or *hph)*, which confers resistance to the amino-glycoside antibiotic hygromycin. Hygromycin allows clear discrimination between transformed and non-transformed tissues and problems with albinos or the fertility of regenerants have not been reported (Ayers and Perk 1994). The *hpt* gene has been used as a efficient marker gene for selection after *Agrobacterium*- mediated transformation (Aldemita and Hodges, 1996; Dong *et al.*, 1996; Hiei *et al.*, 1994; Rashid *et al.*, 1996). Other potential selective agents include herbicides. Genes have been isolated for resistance to various commercially important herbicides. Among them the *bar* gene for phosphinothricin acetyltransfarase might be the most valuable in Rice (Ayres and Park, 1994). The *bar* gene confer resistance to L-phosphinothricin (PPT), glyphosinate (an ammonium salt of PPT) and bialaphos (a derivative of PPT). The *bar* gene has been successfully used to select transgenic rice calli and plants in number of laboratories (Cao *et al.*, 1992; Christou *et al.*, 1991; Datta *et al.*, 1990; Rathore *et al.*, 1993). Mannose-6-phosphate isomerase (MPI) is a recently developed selectable marker. The enzyme is encoded by *manA* from *E. coli*, which converts the unusable carbon source mannose-6P to fructose-6P. Thus, transformants containing *manA* can grow on mannose as a sole carbon source. This selection has a positive mode of action that encourages the growth of transformed tissues rather than just permitting it. The MPI marker is extremely effective for the selection of transformed sugar beet, (0.94%), maize (>50%) and wheat (25%) (Joersbo *et al.*, 1998).

2.4.2.1.4 Scorable markers

For each of the transformation methods, transient expression experiments, generally performed with a reporter gene, are a preliminary step used to identify conditions that will allow efficient DNA delivery. Several reporter genes are used in plants, including β-glucuronidase, luciferase and genes involved in anthocyanin biosynthesis (Wilmink and Dons, 1993). More recently, the gene for GFP has become an important *in vivo* reporter in plants. When expressed in cells and illuminated with blue light, GFP yields stable bright-green fluorescence, which is easily monitored nondestructively. It can thus be used as a means to visualize the fate of transformed cells over time and rapidly test the influence of various factors through the successive steps of the transformation protocol.

2.4.2.2 Transgene silencing and its amelioration

2.4.2.2.1 Use of matrix attachment regions (MARs)

At present, the generation of single-copy transgenic plants is still somewhat hit and miss. Scientists usually produce a relatively large number of independent transformants and screen them for plants containing a single-copy T-DNA insertion. At best, this can be a time-consuming nuisance. However, for agronomically important species, elite cultivars, or lines that are recalcitrant to transformation, it can become a rate-limiting step. An alternative to this approach may be to generate transgenic plants containing a few copies of T-DNA that are insulated from each other. One proposed mechanism to accomplish this is to flank transgenes within the T-DNA with matrix

attachment regions (MARs). MARs are DNA sequences that either are associated with chromosome "matrices" as isolated or can associate with these matrices in vitro (Hall *et al.*, 1991; Hall and Spiker, 1994; Spector, 1990; Verheijen *et al.*, 1988). Among other properties, they have been ascribed the role of insulating genes within a looped chromatin domain from transcription-activating or silencing effects of neighboring domains. In animal cells, such insulating effects may render transgene expression proportional to transgene copy number (Stief *et al.*, 1989). However, some of the MARs initially used in animal experiments may also have contained enhancer elements, confounding the interpretation of the original experiments (Bonifer *et al.*, 1994; Phi-Van and Stratling, 1996). When they flank transgenes delivered to plants via *Agrobacterium*-mediated transformation, MARs appear to have only a small effect on transgene expression (Han *et al.*, 1997, Levee *et al.*, 1999; Liu and Tabe, 1998; Mlynarova *et al.*, 1994; 1995 and1996; Van der Geest *et al.*, 1994). Larger increases in transgene expression have been observed using particle bombardment-mediated transformation (Allen *et al.*, 1993; Allen *et al.*, 1996; Odell and Krebbers, 1998). However, this increase is generally associated with expression of transgenes in plant cells rather than in whole plants (Tzfira *et al.*, 2002; Vain *et al.*, 1999). It is possible that the higher transgene expression effects of MARs using particle bombardment reflects the higher integrated transgene copy number resulting from this technique as opposed to the relatively lower copy number of integrated T-DNAs delivered by *Agrobacterium* (Allen *et al.*, 2000). As such, it is not clear whether MARs will be, on their own, highly useful for decreasing the silencing of transgenes delivered to plants by *Agrobacterium*-mediated transformation.

2.4.2.2.2 Use of viral suppressors to increase transgene expression

Number of laboratories indicates that some plant viruses, both DNA and RNA viruses, contain genes that suppress gene silencing (Al-Kaff and Covey, 1996; Anandalakshmi *et al.*, 1998; Beclin *et al.*, 1998; Brigneti *et al.*, 1998; Carrington and Whitham, 1998; Covey *et al.*, 1997; Kasschau and Carrington, 1998; Marathe *et al.*, 2000). Several investigators have speculated that viral antisilencing has evolved as a mechanism for viruses to evade a plant's defense through viral gene silencing (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). Regardless of the reason for and mechanism of anti-silencing, viral suppressors of silencing may be useful to mitigate the silencing of transgenes. As indicated in some of the references cited above, viral suppressors of gene silencing can activate a previously silenced stable transgene. One would then wonder whether such silencing suppressors could prevent the silencing of transgenes stably introduced into plants by *Agrobacterium*-mediated transformation. Although this hypothesis has not yet been tested and possible negative consequences (such as increased viral susceptibility) may ensue from the stable incorporation of antisilencing genes into a plant genome, experiments in which viral silencing suppressors have been used to increase the levels of transient expression of *Agrobacterium*-introduced transgenes appear promising.

2.5 *Bacillus thuringiensis*- a potent source of insecticidal genes

2.5.1 History, production and insect host range

The choice of a Bt endotoxin, as the first insecticidal protein for introduction into pants was based on the extensive knowledge gathered about this class of crystal proteins

since 1902. The entomocidal bacterium Bt upon sporulation, normally produces a number of insect toxins including crystalline delta toxin.

This common soil bacterium, most abundantly found in grain dust from soils and other grain storage facilities, was discovered first in Japan in 1901 by Ishawata and then in 1911 in Germany by Berliner (Baum *et al.*, 1999). *Bacillus thuringiensis* first became available as a commercial insecticide in France in 1938 and in the 1950s entered commercial use in the United States. For many years, Bt primarily came in the form of a spray to be applied to crops. The non-persistent nature of the insecticide necessitated many reapplications during early use (van Frankenhuyzen, 1993).

However, more effective ones in 1960's, when various pathogenic strains were discovered with particular activity against different types of insects. For many years, Bt was available only for control of lepidopteran insects, using a highly potent strain *Bacillus thuringiensis* (var. kurstaki). This strain still forms the basis of many Bt formulations. Further screening of a large number of other Bt strain revealed some are active against larva of coleoptera (beetles) or diptera (small flies, mosquitoes). Most of these strains have the same basic toxin structure but differ in insect host range, perhaps because of different degree of binding affinity to the toxin receptors in the insect gut. For example the toxins produced by *Bacillus thuringiensis* var. aizawai have somewhat different toxin from those of Bt. var. krustaki and they are highly specific to lepidoptera, with no effect on other insects. The many commercial studies for control of lepidopteran insects are marketed under various trade names such as Biobit^{R}, Disel^{R} Jawelin^{R} etc. In

contrast, the toxins produced by strain of Bt. Var. israelensis are highly active against simutid blackfly vectors of some tropical diseases and also against fungus, larva and some types of mosquito (*Aedes* sp. But higher toxin doses are needed for control of *Culex* sp. and *Anopheles* sp.). Trade name for these products include Skutal^{R} Vecobac^{R} and Mosquito Attach^{R}.

2.5.2. BT toxins and their classification

Bacillus thuringiensis strains produce two kinds of toxins. The main types are the cry (crystal) toxins encoded by different cry genes and this is how different types of Bt are classified. The second types are the cyt. (cytolytic) toxins which can augment the cry toxins enhancing the effectiveness of insect control.

As per earlier classification there are 34 recognized subspecies of *B. thuringiensis* – some of the most commonly used include subspecies kurstaki (against Lepidoptera), subspecies israelensis (against Diptera, primarily mosquitoes and blackflies), and subspecies tenebrionis (against Leptinotarsa decemlineata, the Colorado potato beetle) (Whalon and McGaughey, 1998). Two general groups of insecticidal crystal proteins made by this wide variety of subspecies have been identified, cyt (cytolysins) and *cry* (crystal delta-endotoxins). Hofte and Whiteley (1989) define four class of c*ry* genes and two classes of c*yt* genes. CryI and cryII toxins are active against lepidopterans, cryII and cryIV against dipterans, and cryIII against coleopterans (Hofte and Whiteley, 1989). While cryIII toxins are produced by subspecies tenebrionis and tolworthi and cryIV by israelensis, generally very little correlation between certain toxins and certain subspecies exists. Cry toxins bind to specific receptors on cells in the

insect midgut. *Cyt* genes are active against dipteran and coleopteran pests, and additionally have shown action against hemipterans (true bugs) and dictyopterans (roaches and termites) (Frutos *et al.*, 1999; Gould and Keeton, 1996). Cyt toxins, unlike cry toxins, do not recognize specific binding sites (Lereclus *et al.*, 1993). These classification criteria presented problems since there is no simple relation between sequence and insecticidal spectrum. In order to avoid inconsistencies, a new nomenclature system has recently been introduced, based solely on sequence homology of the full-length ICP proteins (Crickmore *et al.*, 1998). Currently, this system classifies 169 sequences into 28 Cry classes and 2 cyt classes, based on a phylogenetic tree. Each toxin name is characterized by four ranks (e.g. cry1Ab5) depending on its position in the phylogenetic tree (Figure 1).

Bacillus thuringiensis can be distinguished from related *Bacillus* species by the presence of parasporal crystals that are formed during sporulation. The insecticidal activity of Bt is associated with a parasporal glycoprotein crystal that is synthesized within the organism during its sporulation cycle. Bt subspecies, kurstaki, berliver, abesti and tolworthi, all produce crystals that are similar to each other structurally, biochemically, immunologically and functionally. The parasporal crystal comprises approximately 20-30% of dry weight of the sporulated culture and consists mainly of protein (>90%) and a small amount of carbohydrates (>5%).

Parsporal crystal (cry) protein contains the full length 644 (130 KDa) residue protein as the minor component, and a product of bacterial processing with 57 residues removed from the N-terminus as the major component. Delta endotoxin comprises three domains, which are from N- to C- terminus, Domain I, II and III (Li *et al.*, 1991).

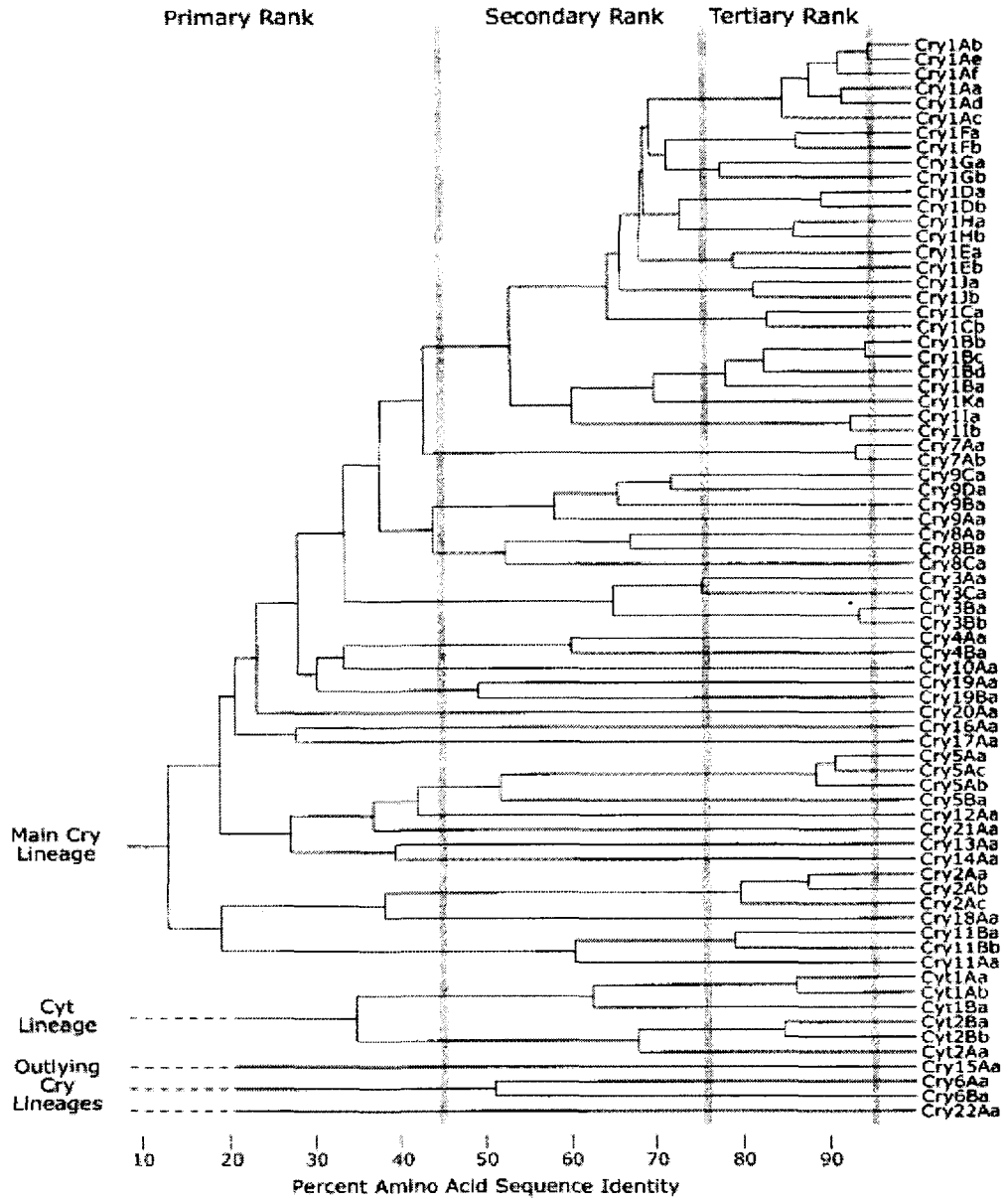

Fig. 1. Phylogram demonstrating amino acid sequence identity among Cry and Cyt proteins. This phylogenetic tree is modified from a TREEVIEW visualization of NEIGHBOR treatment of a CLUSTAL W multiple alignment and distance matrix of the full-length toxin sequences, as described in the text. The gray vertical bars demarcate the four levels of nomenclature ranks. Based on the low percentage of identical residues and the absence of any conserved sequence blocks in multiple-sequence alignments, the lower four lineages are not treated as part of the main toxin family, and their nodes have been replaced with dashed horizontal lines in this figure. (Adapted from Crickmore *et al.*, 1998)

2.5.3 Insect control agents

Bacillus thuringiensis formulation (spore and crystal mixtures) were used as insecticidal sprays in the 1930's, but large scale production started only with the introduction of thuricide^{TM} in the late 1950's and this was followed by similar products from several companies (Beegle and Yamatuoto, 1992). Inspite of their environment-friendly reputation, Bt sprays have never occupied a large share of the insecticide market and are largely used by organic farmers and gardeners. Three factors are responsible for this; lack of stability, failure to penetrate tissues and therefore to reach insects in all parts of the plant and lastly too narrow a specificity. Crystal proteins degrade rapidly in UV light losing their activity. It is therefore necessary to make multiple applications through the growing season, which raises the cost of pesticide treatment. Although some improvements have been made in this area, it remains the biggest single drawback to the use of Bt sprays. Furthermore Bt sprays are non-systemic insecticides and are therefore ineffective against insects that do not come into direct contact with the crystals, such as sap sucking and piercing insects, against root dwelling insect pests or larvae that after hatching rapidly burrow bore into plant tissues. In addition, crops are often subject to predation by a variety of pests that cannot currently be controlled by a single Bt product. The first two problems have been effectively addressed by creating transgenic plants that express the crystal proteins. In these plants, the toxin is continuously produced and protected against degradation and provided it is expressed in the appropriate tissues it will also be ingested by boring larva. The problem of narrow specificity may be overcome by simultaneously expressing genes for protein with different specificities (resistance gene 'stacking' or 'pyramiding'). Because of increasing pressure from the

environmentalists to reduce the use of chemical insecticides the industrials effort to develop improved Bt product is increasing. Microbial insecticides account for only 1.6% share for the world insecticide market, but 95% of these sales involve products based on isolates of Bt which amounts to only 1% of the global insecticide market. The use of these insecticides, transgenic plants will eliminate stability problem and should significantly reduce the cost of insect control. More than 50 crop species, including major field crops such as corn, cotton, soybean, potato and rice have now been successfully transformed (Andrews *et al.*, 1987). Insect resistant plants containing insecticidal crystal proteins (ICPs) coding genes are expected to be among the transgenic varieties to be commercialized during this decade.

2.5.4 Mode of action

The insecticidal crystal proteins (ICPs) are aggregate of a large protein (about 130-140 kDa) that is actually a prototoxin - it must be activated before it has any effect. The crystal protein is highly insoluble in normal conditions, so it is entirely safe to humans, higher animals and most insects. Following ingestion of parasporal crystal, the high alkaline environment (pH 7.5-8.0), the insect midgut activates the prototoxin and causes the crystal to dissolve and release their constituent prototoxins. This toxin is termed as delta toxin. The prototoxin are then subsequently trimmed by gut proteases to an N- terminal, 65-70 KD truncated form- the activated toxin with a molecular weight of approximately 65,000-1,60,000.

The most widely accepted model for the mechanism of the toxin insecticidal action involves; binding of the activated toxin to specific receptors on the villi of midgut

epithelial cells, insertion and piercing of the toxin into the membrane, which results in the formation of an ion channel. This channel disrupts the normal midgut ion flow. The gut is rapidly immobilized, the epithelial cells lyse, the larva stops feeding and the gut pH is lowered by equilibration with the blood pH. The lower pH enables the bacterial spores to germinate and the bacterium can then invade the host, causing a lethal septicaemia.

Chapter - 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Source of seed materials

Seeds of *Oryza sativa* L. subsp. indica var. Pusa Basmati 1, Basmati 370 and Tarori Basmati were procured from field grown healthy plants, dried for one week at 30°C in oven and stored at 10°C in the refrigerator.

3.2 METHODS

3.2.1 Callus induction

Mature seeds of all the above-mentioned rice lines were manually dehusked. Undamaged, uniform, healthy seeds were selected and surface sterilised in 70% (v/v) ethanol for 1 minute and then in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 6-8 minutes, followed by three washings with sterile distilled water. Sterilised seeds were then dried on sterile tissue paper and were plated (15-20 per 9 cm Petri dish) on the following callus induction media:

a) MS salts (Murashige and Skoog, 1962) supplemented with B_5 vitamins, 4 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), 1 mg l^{-1} kinetin, 3% (w/v) sucrose and 0.3% (w/v) phytagel, pH 5.7 (designated as MB, APPENDIX-I).

b) MS salts (Murashige and Skoog, 1962) supplemented with B_5 vitamins, 3 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA (Napthaleneacetic acid), 3% (w/v) sucrose and 0.3% (w/v) phytagel, pH 5.7 (designated as MB1, APPENDIX-I).

c) MS salts (Murashige and Skoog, 1962) supplemented with B_5 vitamins, 300 mg l^{-1} casamino acid, 500 mg l^{-1} L-proline, 3% (w/v) sucrose, 2.5 mg l^{-1} 2,4-D and 0.3% (w/v) phytagel, pH 5.7 (designated as CIM, APPENDIX - I).

Seeds were placed on the medium in such a way that the plumule–radicle side of the seeds was in contact with medium and the scutellum side up. Petri dishes were sealed with parafilm and incubated at 26±2°C in dark for 21 days. Actively proliferating embryogenic calli derived from scutella were then separated from endosperm and sub-cultured on fresh CIM medium for another 5-7 days in dark after fragmenting into 4-5 mm pieces. These vigorously growing, compact, nodular, healthy, embryogenic calli were used for transformation/infection.

3.2.2 Preparation of *Agrobacterium* strain LBA4404 (pSB1, pCAMBAc)

Wild *A. tumefaciens* strain LBA4404 (kindly provided by K.Veluthambi) [harbouring pSB1 vector carrying an extra set of *vir*B, *vir*G and *vir*C (Fig. 2)] to which a binary vector pCAMBAc (Fig. 3) was mobilised by triparental mating. Binary vector pCAMBAc (kindly provided by P. Ananda kumar) is derived from pCAMBIA1304 [(Centre for Application of Molecular Biology to International Agriculture, Canberra, Australia, (CAMBIA)] containing *hpt* (hygromycin phosphotransferase) as plant selection marker and *gus-gfp* fused gene as a reporter gene in the T-DNA region. It also carries *nptII* (neomycin phosphotransferase) gene in the vector backbone (outside T-DNA region) as a bacterial selection marker.

pCAMBIA1304 vector is based on pCAMBIA1301 (bacterial kanamycin resistance marker, plant hygromycin selection marker, pUC18 polylinker in lacZα) which contains *mgfp5*, green fluorescent protein version of the *Aequoria victoria* in translational fusion with *gusA* (N358Q) as a 5′-m*gfp*5-*gus*A-3′ fusion.

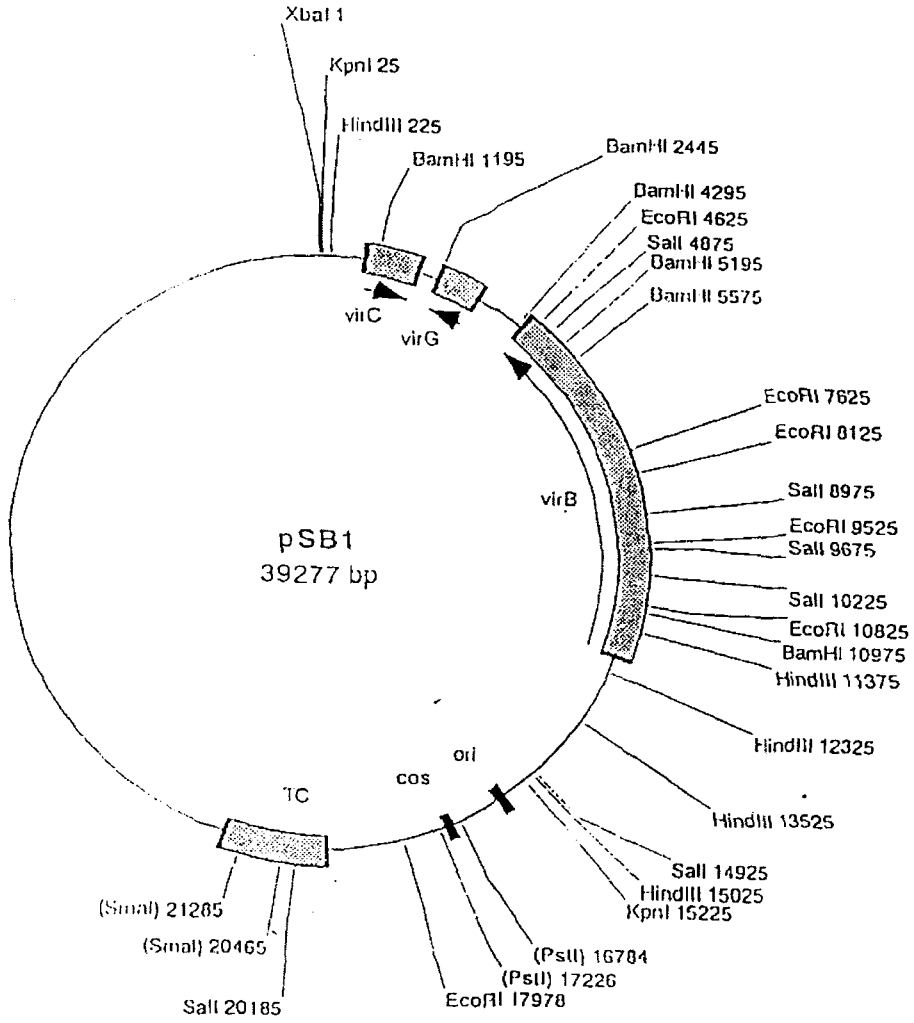

Fig. 2. Map of helper plasmid pSB1 harboured by LBA4404 *Agrobacterium tumefaciens* strain

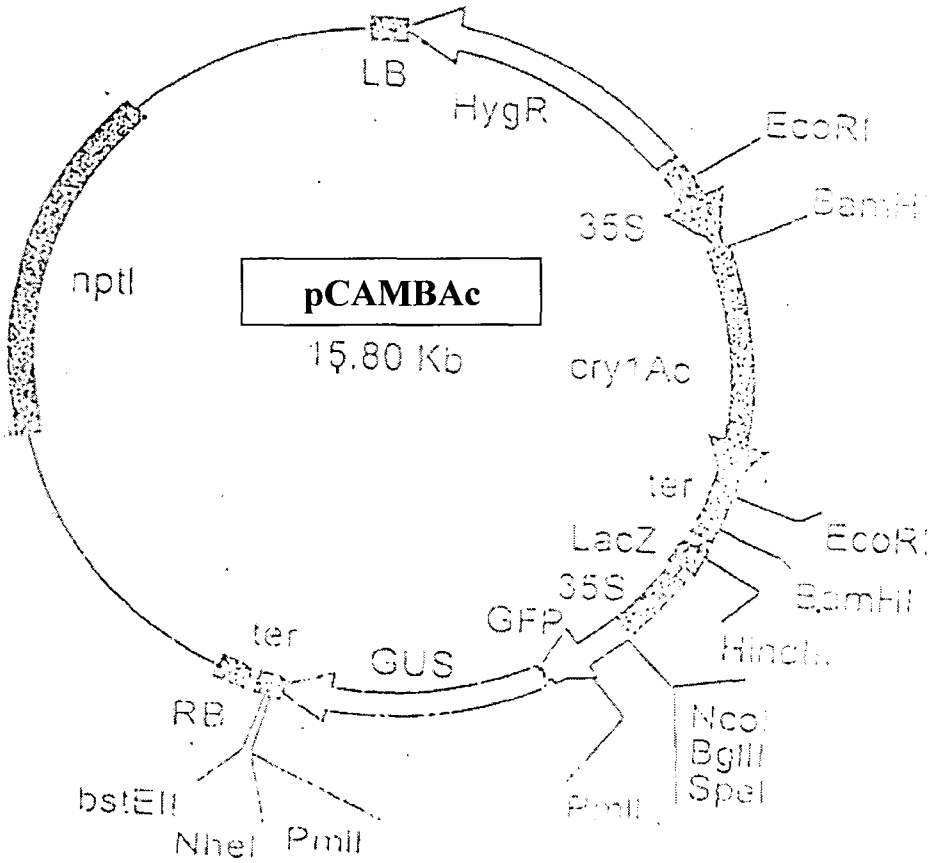

Fig. 3. Plasmid map of pCAMBAc harboured by LBA4404 *Agrobacterium tumefaciens* strain

The vector pCAMBIA1304 exploits the utility of both GUS selection vectors and GFP, despite having a one–protein-one GFP limitation on detection sensitivity. N358Q are intron-less versions of *gusA*.

Codon optimised synthetic *cry1Ac* (1.85 kb) gene along with 35S cassette (35S CaMV promoter and 35S terminator) was subcloned at *EcoRI* restriction site of pCAMBIA1304 in rightward orientation (along the right border direction) creating a new vector which is designated as pCAMBAc (15.8 kb).

3.2.2.1 Preparation of *Agrobacterium* strain LBA4404 (pSB1, pCAMBAc) by triparental mating

3.2.2.1.1 Strains involved in triparental mating

a) *A. tumefaciens* (recipient strain- LBA4404) was grown at 30°C on AB minimal medium (APPENDIX-II) with antibiotic (10 mg l^{-1} tetracycline).

b) *E. coli* harbouring a pCAMBAc plasmid to be mobilised (donor strain) was grown at 37°C on LB (APPENDIX-III) agar medium with antibiotic (50 mg l^{-1} kanamycin).

c) *E. coli* harbouring vector pRK2013 (conjugal helper strain).

3.2.2.1.2 Procedure for triparental mating

The day on which triparental mating was performed was considered as day 1.

Day: (-) 3 (three days before the day of conducting triparental mating)

A. tumefaciens (LBA4404, pSB1) was streaked on AB minimal medium containing the antibiotic (10 mg l^{-1} tetracycline) and incubated at 30°C to get single colonies.

Day: (-) 1 (a day before the day of conducting triparental mating)

E. coli harbouring pRK2013 was streaked to get single colonies on LB agar medium with 50 mg l^{-1} kanamycin and *E. coli* harbouring the pCAMBAc plasmid i.e. donor strain was streaked for single colonies on LB agar medium with antibiotic 50 mg l^{-1} kanamycin and incubated at 37°C.

Day: (+) 1

A plate with plain YEP (Yeast Extract Peptone, APPENDIX-IV) agar medium was prepared. One colony each from *E. coli* (pRK2013), *E. coli* harbouring the plasmid (pCAMBAc) to be mobilised and *A. tumefaciens* (the recipient-LBA4404) patched separately but very close to each other on the YEP medium containing plate. Using a sterile loop, all the three bacterial strains were mixed very well. The plate was left at 30°C for 12-18 hrs.

Day: (+) 2

After mating, the bacteria on the YEP medium were scrapped and suspended in 1.0 ml of 0.9% NaCl. A serial dilution was performed by transferring 0.1 ml of the bacterial suspension into 0.9 ml NaCl (10^{-1} dilution). Likewise four or five dilutions (upto $10^{-4}/10^{-5}$) were prepared. From each dilution 100 µl was plated on AB minimal medium containing antibiotic of donor (50 mg l^{-1} kanamycin) and recipient (10 mg l^{-1} tetracycline) both, and spread uniformly using a triangular-glass rod. The plates were incubated at 30°C for 3-5 days.

Day: (+) 6

At one or two dilutions, single colonies appeared on AB plates. These colonies

were of *A. tumefaciens* into which the donor plasmid had been transferred.

Day: (+) 7

Six to eight colonies of *A. tumefaciens* were streaked to get single colonies on AB minimal medium containing appropriate antibiotics (50 mg l^{-1} kanamycin and 10 mg l^{-1} tetracycline). Single colonies were seen after 4 days. Then these single colonies were patched and maintained as a master plate. In order to confirm the transfer of donor plasmid, total DNA was extracted and subjected to non-radioactive southern blotting.

3.2.3 Preparation of *A. tumefaciens* culture

A. tumefaciens LBA4404 (pSB1, pCAMBAc) was streaked on AB minimal medium supplemented with 50 mg l^{-1} kanamycin and 10 mg l^{-1} tetracycline (*Agrobacterium* selection medium) and incubated at 28°C for colonies to appear. A single colony was transferred to 5 ml AB liquid minimal medium containing the antibiotics (50 mg l^{-1} kanamycin and 10 mg l^{-1} tetracycline) and the cultures were incubated at 28°C, 220 rpm in dark for overnight. This culture was then transferred to 50 ml AB liquid minimal medium containing same selection antibiotics and left for 12 hrs. The bacterial suspension at early log phase was then subjected to centrifugation in a sterile polypropylene tube and was spun at 3,800 rpm for 10 min. The supernatant was discarded and pellet was resuspended in AA-AS medium (APPENDIX-V) and the O.D. of this suspension (*A. tumefaciens* culture) was adjusted to 1-1.2 at 600 nm. The temperature of this culture was maintained at 23±1°C throughout. This culture was used for infection of sub-cultured embryogenic calli.

3.2.4 Infection and co-cultivation

The subcultured calli were transferred to a 150 ml conical flask containing the *A. tumefaciens* LBA4404 (pSB1, pCAMBAc) culture and left for 10 min. The bacterial suspension was then decanted and the treated calli were transferred to Petri dish containing sterile filter paper for a brief period to allow partial drying to remove excess adhering of bacterial suspension. The calli were then transferred to Petri dish containing co-cultivation medium (semi-solid CIM medium) overlaid with two Whatman no.1 filter paper circles soaked in 2 ml of AA-AS liquid medium and were incubated in diffused light of 18 hrs. photoperiod at 23±1°C for 84 hrs. On completion of 84 hrs. of co-cultivation period, the calli were washed four times with sterile CIM medium containing 500 mg l^{-1} of cefotaxime and then blotted dry on a sterile tissue paper to remove excess moisture. The calli were then transferred to post co-cultivation medium (CIM medium containing 350 mg l^{-1} cefotaxime) in dark at 26±1°C for 7-8 days.

3.2.5 Selection of putatively transformed calli

After 7-8 days of post co-cultivation the calli were subjected to first phase of selection for 16-18 days in CIM medium supplemented with 50 mg l^{-1} hygromycin along with 350 mg l^{-1} cefotaxime in dark at 28°C. After Ist phase of selection, the calli were subcultured for 3 weeks to fresh CIM selection medium with enhanced concentration of hygromycin (60 mg l^{-1}) and optimum concentration of cefotaxime (250 mg l^{-1}). This step was repeated once more as third selection phase.

This step was repeated again with reduced concentration of cefotaxime (150 mg l^{-1}) after completion of third phase. Data were recorded to determine percentage of calli

proliferated in the selection medium. Finally the callus, which proliferated during the course of selection phases, was transferred to regeneration medium (MS-KN_3) supplemented with 50 mg l^{-1} of hygromycin and 75 mg l^{-1} of cefotaxime.

All the above selection phases were carried out in dark including initial 2 weeks of regeneration selection phase at 28°C.

3.2.6 Assay for GUS activity

3.2.6.1 GUS histochemical staining

Since *gus* is absent in most plants, the *E. coli* β-glucuronidase is used as a reporter gene in the plant transformation vectors (Jefferson, 1987). The histochemical substrate for *gus* is 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohaxamide salt.

3.2.6.2 GUS reaction

The enzyme β-glucuronidase cleaves the susbstrate X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohaxamide salt) into glucuronic acid and indoxyl derivative. The indoxyl derivative dimerises and is then oxidised to form insoluble, highly coloured indigo dye (dichloro-dibromo indigo). Although atmospheric oxygen stimulates the dimerisation, the oxidation catalyst potassium ferricyanide and ferrocyanide present in the reaction mixture, accelerate the process.

3.2.6.3 GUS assay

GUS expression was assayed with X-Gluc as substrate (Jefferson, 1987). Explants (callus, leaves and roots) were washed in phosphate buffer (APPENDIX -VI) (39 ml of 0.2 M monobasic sodium phosphate and 61 ml of dibasic sodium phosphate

and volume was made up to 400 ml) and then incubated at 37°C in phosphate buffer with 1% Triton X-100 for 1 hr. The explants were then transferred to X-gluc staining solution (1 mM) after giving brief vacuum infiltration and were then incubated at 37°C for 16 to 24 hrs.

The following data were recorded for all the three Basmati varieties:

a) Transient transformation rate immediately after co-cultivation.

b) Stable transformation rate after final (4^{th}) phase of callus selection.

3.2.7 Partial desiccation of hygromycin resistant callus

The healthy looking hygromycin resistant calli after completion of callus selection phases and prior to regeneration phase were subjected to dehydration stress. The callus were transferred to empty sterile petriplate and sealed with parafilm and placed in dark for 36 hrs. at 28±1°C.

3.2.8 Plant regeneration from callus

Five different plant regeneration media were studied for efficient plant regeneration for 21 days old control (untransformed) callus of all the three Basmati varieties. The different regeneration media were:

a) MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 1 mg l^{-1} BAP (6-benzylamino purine), 0.5 mg l^{-1} NAA and 1.2% agarose, pH 5.7 (designated as MS-BN_1).

b) MS salts (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 2 mg l^{-1} BAP, 0.75 mg l^{-1} NAA and 1.2% (w/v) agarose, pH 5.7 (designated as MS-BN_2).

c) MS salts (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 1 mg l^{-1} kinetin, 0.25 mg l^{-1} NAA and 1.2% agarose, pH 5.7 (designated as MS-KN_1).

d) MS salts (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 3 mg l^{-1} kinetin, 1.0 mg l^{-1} NAA and 1.2% agarose, pH 5.7 (designated as MS-KN_2).

e) MS salts (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 3 mg l^{-1} kinetin, 0.5 mg l^{-1} NAA and 1.2% agarose, pH 5.7 (designated as MS-KN_3).

The calli after desiccation phase were transferred to the standardised regeneration medium, MS-KN_3 for 15 days in dark and then transferred to light under 16 hr. photoperiod at 26±2°C for 3-4 weeks. The shoot regeneration frequencies were recorded as percentage of scutellum derived hygromycin resistant calli that produced one or more shoots. Putatively transformed hygromycin resistant calli originated from its different source calli were maintained separately as a result of unique independent transformation event i.e. descendents from each hygromycin resistant calli produced and proliferated during the course of selection phases were maintained and marked separately. All the healthy looking normal green shoots regenerated from the calli were transferred to half strength MS medium in a culture tubes and magenta boxes. Shoots regenerated from single callus were maintained in single Magenta boxes i.e. where each plant represents similar transformation event. Plants were maintained for 10-15 days in light.

3.2.9 Establishment of regenerated plants in glasshouse

Plants with profuse roots attaining a length of 10 cm were taken out and washed thoroughly under running tap water to remove any adhering medium. The plants were

then directly transferred to pots with Farm Yard Manure (FYM) mixture and soil and watered heavily. The planted shoots were covered with inverted Jam Jar bottle for 5 days in green house for maintaining high relative humidity and rapid acclimatization. The Jam Jar bottle was removed from pot after confirming that the established plant had added a new leaf.

3.2.10 Molecular analyses

3.2.10.1 Miniscale plasmid isolation from *E. coli*

Two ml culture of *E. coli* (harbouring pCAMBAc vector) was grown overnight in liquid LB medium supplemented with 50 mg l^{-1} kanamycin at 37°C in shaker at 220 rpm till log phase (checked by turbidity of the culture). The bacterial cells were pelleted down in a Table-top centrifuge (Sigma Laborzenrigugen GMBH, Germany) at 8,000 rpm for 5 min. The supernatant was decanted and the microfuge tube containing the pellet was placed in ice (optional). The pellet was resuspended in 100 µl of solution I (APPENDIX-VII) by brief vortexing to ensure complete mixing of pellet. Two hundred ml of solution II (APPENDIX-VII, prepared fresh) was immediately added and placed in ice for 15 min. Mixing was done by gentle inversion of the tubes. One fifty ml of ice-cold solution III (APPENDIX -VII) was then added and mixed gently by inversion. The tube was then placed in ice for another 25 min. The content was then spinned down in a microfuge for 10 min. at 10,000 rpm. The supernatant was transferred to new tube, to it 300 µl of neutral phenol: chloroform mix was added and centrifuged for 5 min. at 10,000 rpm. The aqueous phase (upper layer) was then carefully transferred to fresh microfuge tube without disturbing the interphase. To this 300 µl of isopropanol was

added and gently mixed and left at room temperature for 2 min. The contents were then centrifuged at 12,000 rpm for 10 min. and supernatant was discarded immediately. To the pellet, 100 µl of TES (APPENDIX-VII) along with 2 µl of RNase (from 5 µg ml^{-1} stock) was added and mixed by gentle tapping followed by a brief spinning. It was then incubated at 37°C in water bath for 10 min. To this 50 µl of neutral phenol : chloroform mix was added and spinned for 5 min. at 11,000 rpm. The aqueous phase was collected, and transferred to new fresh tube, to this $1/10^{th}$ volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of 95% cold ethanol were added and mixed by inverting the tube. The tube was then placed in -20°C overnight. The contents were then centrifuged in a refrigerated microfuge for 10 min. at 12,000 rpm and supernatant was decanted. To this 500 µl of 70% ethanol was added and centrifuged for 10 min. The pellet was then dried in speed vacuum for 10 min. and was finally dissolved in 20 µl of 0.1X TE (pH 8.0).

3.2.10.2 Isolation of total DNA from *A. tumefaciens* LBA4404 (pSB1, pCAMBAc)

Isolation of total DNA from *A. tumefaciens* as described by Chen and Kao (1993). *A. tumefaciens* at early log phase were collected in microfuge tube and pelleted down at 10,000 rpm for 2 min. Supernatant was decanted and 200 µl of lysis buffer [400 mM Tris acetate (pH 7.8), 20 mM sodium acetate, 1 mM EDTA, 1% SDS] was added. The pellet was vortexed and resuspended in lysis buffer by pipetting. To this 66 µl of 5 M NaCl was added and mixed thoroughly by gentle inversion. The content was then placed at –20°C for 10 min. and then was spinned at 12,000 rpm for 10 min. The supernatant was transferred to fresh microfuge tube and equal volume of phenol : chloroform was added to it. The content was gently mixed by inverting tube for at least

50 times and then centrifuged for 12,000 rpm for 3 min. The supernatant was then transferred to new microfuge tube and to it 2.5 volumes of 95% (v/v) ethanol was added and left at –20°C overnight for precipitation of DNA. Content was then spinned and washed twice with 70% (v/v) ethanol. The pellet was vacuum dried for 10 min. and dissolved in 50 µl of 0.1X TE.

3.2.10.3 Isolation of plant DNA and its purification

3.2.10.3.1 Isolation of plant DNA

Two methods developed originally by Dellaporta *et al.* (1983), and Murray and Thompson (1980) are being commonly adopted with certain modifications for isolating high molecular weight DNA from small amount of plant tissue. In the present investigation a modified Murray and Thompson (1980) protocol as described below worked well.

Two to three gram of fresh tender leaves was ground in liquid nitrogen to fine powder using pre-chilled mortar and pestle. After grinding to fine powdery texture, it was immediately transferred to a 50 ml polypropylene centrifuge tube containing 12 ml of pre-warmed (65°C) extraction buffer (APPENDIX-VIII). Samples were then thoroughly mixed by gentle inversion to form homogenous suspension and were incubated at 65°C for 45 min. in the water bath (B. Braun, Singapore) with occasional mixing by gentle swirling. The samples were then transferred to ice immediately for 10 min. To each sample, 8 ml of chloroform: isoamylalcohol (24:1) (v/v) were added and mixed by gentle inversion for 10-15 min. to form emulsion. The contents were then centrifuged at 8,000 rpm for 10 min. at 4°C. The aqueous phase (upper phase) was then

transferred to new polypropylene tube using a wide-cut 1 ml pipette tip. To this 2/3 (v/v) cold isopropanol (stored at -20°C) was added and mixed quickly by gentle inversion. DNA was spooled using wide bored 1 ml pipette tip and transferred to 2 ml microfuge tube. The microfuge tubes were then briefly centrifuged at 10,000 rpm to allow pelleting of DNA and the aqueous phase was pipetted out. Alternatively if the DNA appeared flocculent, the contents were centrifuged at 10,000 rpm for 2 min. and the supernatant was discarded. The DNA pellet was then washed with 70% ethanol (1 ml) for 5-10 min. and the pellet was allowed for drying at room temperature or incubated at 37°C for 15 min. and then dissolved in 500 µl of 0.1X TE buffer (APPENDIX- IX).

3.2.10.3.2 Purification of DNA

Major contaminants in crude DNA preparation are RNA, proteins and carbohydrates. Inclusion of CTAB in DNA extraction buffer helps in eliminating polysaccharides from DNA preparation to a large extent. The RNA was removed by treating the sample with RNase (APPENDIX-VIII) and incubated at 37°C for one hr. in water bath. Equal volume of phenol : chloroform mix was added and mixed thoroughly by gentle inversion. The contents were then centrifuged at 12,000 rpm for 2 min. at room temperature. The aqueous (upper) phase was carefully pipetted out to fresh tube using wide-bored 1 ml tip without disturbing the interphase. To this 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of absolute ice cold alcohol were added and mixed by gentle inversion to precipitate the DNA or in case DNA is trapped in bubbles then incubated at -70°C for 10 min. The content was then centrifuged at 10,000 rpm for 5 min. at 4°C to pellet down the DNA. Supernatant was carefully decanted. The pellet

was washed with 70% (v/v) cold ethanol, centrifuged and supernatant was discarded and air-dried. The pellet was dissolved in 50-100 µl of 0.1X TE buffer.

3.2.10.4 Quantification of DNA

Reliable measurement of DNA concentration is important for many applications in molecular biology including complete digestion of DNA by restriction enzymes and amplification of target DNA by polymerase chain reaction. DNA quantification was carried out by spectrophotometric (Bio-rad, Smart Spec 3000) analyses and agarose gel analyses.

3.2.10.4.1 Spectrophotometric measurement

One ml of 0.1X TE buffer in a cuvette was taken and absorbance was recorded with spectrophotometre at A260 nm / A280 nm as well as at A280 nm / A260 nm and served as a blank against which quantification of DNA samples was measured. Two µl of DNA was added to 998 µl of 0.1X TE buffer, mixed thoroughly and absorbance was recorded at A260 nm / A280 nm. DNA concentration was estimated by employing the following formula

$$\text{Amount of DNA } (\mu g\ \mu l^{-1}) = \frac{(OD)_{260} \times 50 \times \text{dilution factor}}{1000}$$

3.2.10.4.2 Gel analyses

The best way to check for concentration and integrity of DNA is to run the samples on an agarose gel (0.8%) with the known amount of uncut λ phage DNA. Agarose gel (0.8%) was casted in TBE (90 mM Tris HCl, 90 mM boric acid and 2 mM Na_2EDTA) buffer. Gel was run at 50V (5 V cm^{-1} length of the gel). Following

completion of run, the gel was stained with ethidium bromide solution (0.5 μg ml^{-1}) for 10-15 min., subsequently washed with distilled water and visualised under UV light (UV transilluminator).

3.2.10.5 Synthesis of primers

The *cry1Ac* (β-endotoxin gene situated approximately in the middle of T-DNA) gene, *hpt* (hygromycin phosphotransferase gene-the plant selection marker, situated at left border of T-DNA) gene and *gus* (β-glucuronidase gene-the histochemical marker situated at right border of T-DNA) gene were used for screening of putative transgenics of T_0 and T_1 generations through PCR. Specific oligonucleotides (Table 2) for all these genes were synthesized (by Sigma-Genosys Ltd, Texas) to act as primers in Polymerase Chain Reaction (PCR).

Table 2: Description of different gene probes/primers, number of nucleotides, Tm value and their sequence

Primer	No. of nucleotides	Sequence (5′⟶3′)	T_m value
CRY forward	24	CCCAGAAGTTGAAGTACTTGGTGG	66.5
CRY reverse	24	CCGATATTGAAGGGTCTTCTGTAC	63.5
HPT forward	25	CGACCTGATGCAGCTCTCGGAGGGC	79.6
HPT reverse	25	CGATTGCGTCGCATCGACCCTGCGC	84.4
GUS forward	25	GGAAGTGATGGAGCATCAGGGCGGC	80.2
GUS reverse	25	CAGCCCGGCTAACGTATCCACGCCG	75.6

3.2.10.6 Screening of putative transformants by PCR

Standard PCR was performed using a programmable thermal cycler (MJ Research). The reaction was carried out using total DNA as substrate from 30 putative transformed plants from each of the varieties of Pusa Basmati 1, Basmati 370 and Tarori Basmati. The amplification reaction mixture was dispensed in 48 well PCR plate consisted of thermostable Taq DNA polymerase (Sigma, USA), 10X PCR reaction buffer without $MgCl_2$ (Sigma, USA), 10 mM dNTP mixture (MBI, Fermentas), 50 mM $MgCl_2$ (Sigma, USA), forward and reverse primers (Sigma Genosys, USA), template DNA (from putative transgenics) and total volume was made upto 25 µl with sterlised ultrapure water.

The concentration of each component of amplification reaction mixture is given in Table below:

Table 3: Composition of PCR master mix

Reagents	Stock conc.	Final conc.	Final volume (25 µl)
PCR buffer	10X	1X	2.5 µl
$MgCl_2$	50 mM	3 mM	1.5 µl
dGTP	10 mM	120 µM	0.3 µl
dCTP	10 mM	120 µM	0.3 µl
dATP	10 mM	120 µM	0.3 µl
dTTP	10 mM	120 µM	0.3 µl
Taq polymerase	5U μl^{-1}	1 U	0.2 µl
Forward primer		1 pM μl^{-1}	1 µl
Reverse primer		1 pM μl^{-1}	1 µl
Template DNA		50-75 pg	2 µl
Sterilised ultra pure water			15.6 µl

To avoid contamination, amplification reaction mixture preparation was carried out in laminar flow cabinet.

Amplification conditions (for *hpt, cry* and *gus* genes) are given below:

Cycle 1:	Denaturation (94 °C)	4 min.
	Primer annealing (55°C)	1 min.
	Primer extension (72°C)	2 min.
Cycle 2-29:	Denaturation (94°C)	1 min.
	Primer annealing (55°C)	1 min.
	Primer extension (72°C)	2 min.
Cycle 30:	Primer extension (72°C)	7 min.

3.2.10.6.1 Agarose gel electrophoresis of PCR product

Three µl of tracking/loading dye (MBI, Fermentas) was added to each PCR product of 25 µl volume. Total volume of all the samples was dispensed in 0.8% (w/v) agarose (Sigma, USA) horizontal slab gel in TBE buffer and the Gene ruler^{TM} 1 kb DNA ladder (MBI, Fermentas) was used as marker. The gel was run for 1 hr. at 60 V. Upon completion of run the gel was then stained with ethidium bromide (0.5 mg l^{-1}) in TBE buffer. The amplified fragments were photographed using documentary system (Syngene, Bioimaging system).

3.2.10.7 Southern hybridization analyses

3.2.10.7.1 Southern blot analyses by non-radioactive labelling method

3.2.10.7.1.1 Restriction digestion of *E. coli* plasmid (pCAMBAc) and total DNA of *Agrobacterium* transformants LBA4404 (pSB1, pCAMBAc) with *EcoRI* restriction enzyme

Three µg of pCAMBAc plasmid and 15 µg of *Agrobacterium* DNA was digested in 40 µl reaction mixture with 30 units and 80 units of enzyme at 37°C in water bath for 2 hrs. and 16 hrs. respectively. To check complete digestion, 5 µl of the reaction mixture was electrophoresed in a 0.8% (w/v) agarose gel for 1 hr. at 60 V. The gel was stained with ethidium bromide for 30 min. and DNA bands were visualised and stored in gel documentation system.

3.2.10.7.1.2 Agarose gel electrophoresis of digested DNA

Restricted DNA was fractionated on 1% (w/v) agarose horizontal slab gel in TBE buffer for 12-16 hrs. The gel was stained with ethidium bromide in 0.5X TBE buffer for 30 min. and then the image was stored in gel documentation system.

3.2.10.7.1.3 Blotting of DNA onto nylon membrane by upward capillary transfer

After gel documentation analyses the gel was placed in a tray containing 2 volumes of 0.25 M HCl for 5 to 8 min. with gentle agitation to facilitate depurination of DNA (acid treatment can increase the efficiency of blotting of DNA species that are larger than 8 kb length). The HCl solution was then replaced with 4 volumes of

denaturing solution (20X SSC) (APPENDIX-X) to denature double stranded DNA molecules into single stranded ones for later hybridization with a probe. The denaturation process was allowed until the yellow colour of tracking dye changed to blue. Meanwhile a positively charged Zeta probe membrane (Bio-rad) of the same size as that of the gel and two pieces of 3 mm Whatman filter paper were prepared. The membrane filter was marked at the upper right corner with a pencil as a positional marker. The denatured gel was rinsed twice with 2 volumes of distilled water and then the gel was transferred to 4 volumes of neutralization buffer to partially neutralize the gel condition. The neutralization was allowed to proceed for 30 min. at room temperature with gentle shaking.

A clean glass plate was placed on top of a tray containing 250 ml of 20X SSC buffer (APPENDIX- X). Pre-soaked with 20X SSC buffer, a large piece of 3 mm Whatman filter paper wick was placed on a glass plate in such a way that the ends are dipped well into 20X SSC buffer. The gel was then laid on these wet sheets in such a way that the bottom even side of the gel faces up. Air bubbles underneath were removed by rolling a pipette over the gel. The Nylon membrane exactly the size of the gel was then carefully laid on the gel. The marked side of the membrane was kept in such a way that it faced the gel. The membrane was wetted with 20X SSC buffer and bubbles between the membrane and the gel was removed by repeated lifting up and laying down the membrane. The gel was then overlaid with two pieces of 3 mm Whatman filter paper followed by 10-15 cm of paper towel stack. A glass plate was placed on top of the paper towel stack followed by a half kilogram of weight on it. The blot was then removed after

overnight transfer and rinsed briefly (30 seconds) in 2X SSC. The blot was then air-dried and UV induced cross-linking was performed for 30 to 60 seconds.

3.2.10.7.1.4 Preparation of non-isotopic probe by Random Primer Digoxigenin labelling of double stranded DNA

500 ng of template DNA was denatured in a microcentrifuge tube by boiling for 10 min., then the tube was quickly chilled in ice for 5 min. Final volume was made upto 15 µl with sterile double distilled water. To this 2 µl of 10X dNTPs mixture containing DIG-dUTP and 1 µl of Klenow enzyme (2 units μl^{-1}) was added. This mixture was then incubated at 37°C overnight in waterbath. After the labeling was complete the reaction was stopped by adding 2 µl of 0.2 M EDTA and subsequently precipitated by adding 2.5 µl of 4 M $LiCl_2$ and 75 µl of 100% ethanol (pre-chilled). The mixture was left at -20°C for overnight. After overnight incubation the mixture was centrifuged at 12,000 rpm for 10 min. at 4°C followed by washing with 50 µl of 70% ethanol at 12,000 rpm for 5 min. at 4°C. The supernatant was discarded and air-dried. The pellet was then dissolved in 50 µl of 0.1X TE buffer (pH 8.0).

3.2.10.7.1.5 Pre-hybridization

The nylon membrane was placed inside the hybridization bottle and 20 ml of pre-hybridization solution (5X SSC, 0.1% N-Lauryl sarcosine, 0.02% SDS, 1% blocking solution) (APPENDIX-X) was added and incubated at 65°C for 1 hr. in a hybridization oven. Meanwhile double stranded DNA probe (prepared in step 3.2.13.1.4) was denatured to single stranded DNA in boiling water for 10 min. and then immediately chilled in ice for 10 min. followed by brief spin.

3.2.10.7.1.6 Hybridization

The probe was diluted by adding 1 ml of pre-hybridization solution. The 19 ml of pre-hybridization buffer from the hybridization bottle was replaced by fresh 19 ml of pre-warmed hybridization buffer and the diluted probe was added to it. The hybridization bottle was placed back into the hybridization oven and allowed to proceed for 16 hrs. at 65°C.

3.2.10.7.1.7 Post-hybridization washes

Post hybridization washes as a measure of high stringency were done at 65°C for 30 min. with 2X SSC, 0.1% (w/v) SDS then with 0.5X SSC, 0.1% SDS and lastly with 0.1X SSC, 0.1% SDS. After washing, the temperature of hybridization oven was brought down to 37°C steadily.

3.2.10.7.1.8 Detection procedure

After hybridization and stringency washes, membrane was rinsed with wash buffer (APPENDIX-X) (maleic acid buffer + 0.3% Tween 20 added freshly to autoclaved maleic acid buffer) at 37°C (20 ml per 100 cm^2). Wash buffer was decanted and replaced by 1X blocking solution (36 ml of maleic acid buffer + 4 ml of 10% blocking solution) (APPENDIX-X) and kept in the oven for 1 hr. at 37°C. Blocking solution was then decanted and 20 ml of 1X blocking solution containing 2 ml of anti DIG-AP conjugate antibody (1:10,000 dilution) were used and incubated for 1 hr. at room temperature with gentle agitation. The membrane was then transferred to clean

tray containing wash buffer. The membrane was washed twice for 15 min. at room temperature followed by washing with detection buffer (APPENDIX-X) for 5 min. The nylon membrane was then subjected to chemiluminence detection.

3.2.10.7.1.9 Chemiluminence detection

Meantime the 20 µl of chemiluminescent substrate (CSPD) was mixed in 2 ml of detection buffer. The substrate is photosensitive hence covered with aluminium foil. The nylon membrane was placed in polythene bag and substrate (2 ml solution) was evenly spread by rolling pipette over a nylon membrane. The hybridised probe was detected by alkaline phosphatase conjugate anti-DIG antibody and visualised using enzyme activated chemiluminescent substrate. Filter was exposed to Hyperfilm (Amersham) in an X-ray cassette with an intensifying screen for 15 min. to 1 hr. depending on the intensity of signal.

3.2.10.7.2 Southern blot analyses by radioactive labeling method

3.2.10.7.2.1 Plant DNA extraction from putative transgenics and its restriction digestion with *Hind*III enzyme

Ten µg of plant DNA was isolated (as described in step 3.2.9.3 and 3.2.9.4) from putative transgenics of three Basmati varieties. Ten T_0 plants of each Basmati varieties namely Pusa Basmati 1 (6a, 12a, 13a, 15a, 20a, 3b, 4b, 15b, 23b and 24b), Basmati 370 (3a, 5a, 17a, 19a, 2b, 9b, 10b, 12b, 17b and 18b) and Tarori Basmati (1a, 7a, 11a, 12a, 24a, 1b, 7b, 11b, 13b and 21b) were considered for southern blot analyses. However,

few of the T_1 plants obtained from selfing of T_0 plants (southern positive) were also considered for southern blot namely Pusa Basmati 1 (12a-1, 20a-1 and 15b-1), Basmati 370 (12b-1, 12b-2, 12b-3, 12b-4 and 17b-1) and Tarori Basmati (12a-1, 12a-2, 12a-3, 12a-4, 12a-5 and 12a-6). Ten µg of DNA was digested with 80 units of enzyme at 37°C in water bath for 16 hrs.

3.2.10.7.2.2 Probe preparation

Thirty ng (2 µl) of probe (*cry1Ac* insert of 1 kb length) DNA was taken and volume was made upto 28 µl with sterile distilled water. Then 5 µl of random primer was added to make up the final volume to 33 µl (all operations were carried out in ice). The contents were mixed by brief spin. A hole was made on the microfuge tube lid with a fine needle. The microfuge tube was then placed in boiling water bath for 5 min. The tube was then transferred to hot water (70°C) and allowed it to cool down to room temperature gradually. To the content 10 µl of labelling mix, 5 µl of α-^{32}P dCTP (3000 Ci mM^{-1}) and 2 µl of Klenow fragment (4-5 units) were added and mixed well by tapping. The contents were then briefly centrifuged and placed in water bath at 37°C for 30 min. The reaction was stopped by adding nick translation dye (6 mg of Blue dextran and 1 mg of Orange–G to 1 ml of 0.5 M EDTA).

3.2.10.7.2.3 Purification of labeled DNA

The labeled probe generated was purified to remove free polymers or unincorporated radioactive precursors and oligomers, which is retained in the column chromatography prepared with Sephadex® G-50 (0.4 gm of Sephadex G-50 in 6 ml of

column buffer, autoclaved and stored at 4°C). Total volume of probe was then added to sephadex column when column buffer [1 M NaCl, 2 M Tris (pH 7.0) and 0.5 M EDTA) reaches the upper meniscus of sephadex. Column buffer was then slowly dispensed by the side of the wall. The blue fraction of elute was collected and denatured in boiling water for 5 min. and then immediately cooled in ice prior to adding to pre-hybridization buffer for hybridization.

3.2.10.7.2.4 Pre-hybridization and hybridization

Membrane was pre-hybridised for 1 hr. at 65°C in 30 ml of pre-hybridization solution [0.5 M Na_2HP0_4, 7% SDS and 1 mM EDTA (pH 7.0)]. The solution was replaced by fresh pre-hybridization solution (now termed as hybridization solution) containing the probe (blue fraction elute from sephadex G-50) and was incubated at 65°C for 24 hrs. in hybridization oven for hybridization.

3.2.10.7.2.5 Post hybridization washes (high stringency wash) and autoradiography

Hybridization solution was discarded and membrane was briefly rinsed with 30 ml of 2X SSC buffer, 0.1% SDS followed by three washes with 40 ml of each of 3X SSC, 0.1% SDS, 0.5X SSC, 0.1% SDS and 0.3X SSC, 0.1% SDS one after another for 30 min. at 65°C. Final rinse was given briefly with 2X SSC at room temperature. The nylon filter was kept moist in a plastic bag for autoradiography to prevent irreversible binding of the probe.

Autoradiography of this damp nylon filter was performed at –70°C for various exposures times using X-ray (Sigma, Kodak X-OMAT) film with intensifying screen.

The film was developed in 500 ml of developer and fixer (PREMIER band marketed by Allied photographer India ltd.) as specified by the company.

3.2.10.8 Western blotting

Protein from putatively transformed plants was isolated as described by Koziel *et al.* (1993). Young leaves (500 mg) were ground to a fine powder with liquid nitrogen and homogenised with 5 ml 1X PBS extraction buffer (APPENDIX-XI). The extract was centrifuged at 10,000 rpm at 4°C for 10 min. and supernatant was collected. The total protein quantity was estimated by Bradford method. Approximately 10 µg was separated on an 8% polyacrylamide gel containing SDS (APPENDIX-XII) using the mini gel apparatus (Biorad). After pouring 8% separating gel between two thin glass plates placed on levelled stand, a thin layer of distilled water was added and allowed for polymerisation. Once separating gel was polymerised, 5% stacking gel (APPENDIX-XII) of 2 cm in height was poured and a comb of ten well capacity was placed at the top to produce well. Tris glycine buffer (APPENDIX-XIII) was used as electrode buffer. The prepared sample (15 µl sample + 5 µl dye + 3 µl DTT) was boiled for 4 min. and then dipped in ice immediately. After loading the sample the gel was run at 60 V till the dye reached the bottom of the gel. When the dye front reached the bottom of the gel, apparatus was disassembled and gel plates were gently pried apart. Stacking gel was discarded.

The resolving gel was then assembled in sandwich fashion for transferring protein to PVDF membrane as per instruction manual of Biorad.

The protein was transferred to PVDF membrane using Mini Transblot Electrophoretic Transfer cell (Biorad 1700-1900). The protein transfer was carried out for overnight run at 30 V, 90 mA in glycine transfer buffer (APPENDIX-XIII). After overnight transfer, the membrane was washed with Tris-buffer saline containing Tween-20 (APPENDIX-XIV) for 5 min. with gentle shaking. The membrane was then transferred to blocking solution (TBST + 3% BSA) for 1-2 hr. with gentle shaking at room temperature. The membrane was then again washed for 5 min. with TBST containing 3% BSA. This washing step was repeated 5 times. After washing, the membrane was incubated with primary antibody solution [12.5 ml of 150 mM NaCl, 50 mM Tris (pH 7.5) + rabbit anti-cry1Ac antiserum in 1:100, v/v + 0.5% BSA] for overnight at 4°C and then for ½ an hr. at 40 rpm. The membrane was washed with TBST for 5 min. after overnight incubation. This washing step was repeated 5 times. The membrane was then incubated in secondary antibody (goat anti-rabbit IgG-Alkaline phosphatase conjugate) at 1:500 dilution for 1 hr. at 40 rpm. The membrane was then washed for 5 times with TSBT containing 0.3% BSA. Protein antibody complex was developed by adding ready to use substrate BCIP (5-bromo-4-chloro-3-indyl phosphate) from Bangalore Genei, India. The reaction product, a bluish grey to black precipitate became visible within 10 min. at room temperature with gentle shaking.

3.2.10.9 ELISA

ELISA was carried out for T_1 transgenics of three Basmati varieties to know the level of Bt-toxin expression. DesiGen Quan-T ELISA 96-well plate kit (DG040) was used for quantitative estimation of Cry1Ac protein as per manufacturer instruction

(Mahyco, India). The DesiGen cry1Ac plate is designed for quantitative laboratory detection of cry1Ac protein in leaf samples. The DesiGen 96-well plate works as an Enzyme-linked Immuno Sorbent Assay (ELISA). Tissue extracts (100 µg of total protein: quantified by Bradford method) was added to wells coated with antibodies raised against cry1Ac protein. Cry1Ac protein residues in the plant sample bind to the antibodies. A secondary antibody against cry1Ac protein is added to the wells. After the wash step, a third antibody, labeled with an enzyme (alkaline phosphatase-conjugated affinity pure Donkey Anti-Goat IgG) is used to detect cry1Ac protein in the sample via a colour reaction (deep yellow color) formed by action of alkaline phosphatase on a substrate p-nitrophenylphosphate (pNPP). The concentration (% of total soluble protein) of the antigen present in each treatment samples was judged as average of five replications after measuring at 405 nm in ELISA/Microplate reader (TECAN, SUNRISE Absorbance Reader, Austria).

3.2.11 Study of inheritance for the hygromycin resistance gene in transgenic plants of T_1 (obtained from selfing of T_0 plants) and its progeny (T_2)

Segregation of the transgene in T_1 plants (that were southern positive for *cry1Ac* gene during their T_0 generations) and some of its T_2 plants was studied to follow the inheritance of the transgene and to determine transgene copy numbers (loci) and also the homozygous lines. Seeds obtained from selfed T_0 plants and some of its T_1 plants were germinated and scored for hygromycin resistant growth.$\chi 2$ test was performed to check the segregation pattern.

3.2.12 Assessment of agronomic characters in transgenic plants

Five numbers of plants of each T_0, T_1 and T_2 transgenic Basmati varieties were assessed for agronomic characters, namely plant height, flag leaf length, number of panicle bearing tillers, panicle length, number of spikelets/panicle, spikelet fertility and 100 seed weight.

3.2.13 Insect bioassay

Bioassays were conducted with first instar neonate larvae from field collected moths of YSB (yellow stem borer). Moths as well as first instar larvae were collected from rice field of G. B. Pant University, India. Bioassays were carried out for those T_1 plants, which were southern positive during their T_0 generation. Cut stems were selected from Greenhouse grown healthy plants during their booting stage (initial reproductive stage). Cut stem bioassay method of Khanna and Raina (2002) was followed in the present investigation. Larvae mortality of YSB was recorded after 4 days of infestation.

3.2.13.1 Cut stem bioassay

Cut stems for bioassay were taken from the base of the plant when their vegetative stage coincided with booting stage. A moistened filter paper was placed in disposable petriplates on which four cut stems (T_1 plants that were southern positive during their T_0 generation) were placed and infected with twenty neonate larvae. This experiment was carried out in two replications. The Petri dishes were then sealed and incubated at 26-28°C with alternative 16 hr. dark/light photoperiod. Cut stems were evaluated for larval growth and mortality after 4 days of infestation.

Chapter - 4

RESULTS

RESULTS

4.1 Callus induction

4.1.1 Standardisation of media for *Agrobacterim*-mediated transformation

Three different media *viz.* MB, MB1 and CIM (Table 4) were employed in the present study for callus induction from seed scutellum. Varied callus induction efficiency was recorded among the three callus induction media. Two types of calli were observed *viz.* embryogenic and non-embryogenic.

Table 4: Response of different callus induction media

Varieties	No. of seeds inoculated	No. of seeds showing callus induction in different media					
		MB	Mean±SD	MB1	Mean±SD	CIM	Mean±SD
Pusa Basmati 1	100	72	67.4±3.97	80	76±2.34	95	95.4±3.28
	100	65		74		98	
	100	68		76		98	
	100	62		75		90	
	100	70		75		96	
Basmati 370	100	64	69.2±4.65	82	79.4±4.15	96	96±1.41
	100	65		85		94	
	100	70		79		96	
	100	72		76		96	
	100	75		75		98	
Tarori Basmati	100	64	56.8±5.76	70	74.4±3.28	90	89.2±2.28
	100	60		72		92	
	100	52		76		86	
	100	50		78		88	
	100	58		76		90	

The embryogenic calli were pale yellow, compact, dry and globular in appearance. In contrast, non-embryogenic calli were unorganized with brownish watery appearance. The highest callus induction percentage was recorded in CIM medium for all the three Basmati rice varieties followed by MB1 and MB. Pusa Basmati 1 showed the highest callus induction in CIM medium (95.4%) followed by MB1 (76%) and MB medium (67.4%). In Basmati 370 highest callus induction was observed in CIM (96%) medium followed by MB1 (79.4%) and MB medium (69.2%). Similarly, Tarori Basmati recorded highest callus induction in CIM medium (89.2%) followed by MB1 (74.4%) and MB medium (56.8%) (Table 4).

CIM medium was therefore selected for callus induction, since it out-performed the other two media in terms of callus induction efficiency.

4.1.2 Callus proliferation and establishment

After plating seeds on CIM medium, embryos germinated within 2-3 days and produced shoot of 4-5 cm in length after 7-9 days of plating. Scutellum-derived calli were visible after 5-7 days of seed inoculation. However, growth of the shoots ceased and the shoots became partially brown as callus started becoming prominent. Within 18-21 days of seed plating the scutelluar calli became prominent, compact, pale yellow, globular and embryogenic (Fig. 4a, 5a, 6a & 7a).

4.1.3 Subculture of scutellum-derived callus

Embryogenic calli induced from the scutella in CIM medium were detached from the seed endosperm after 21 days and fragmented into small calli of 4-5 mm and were

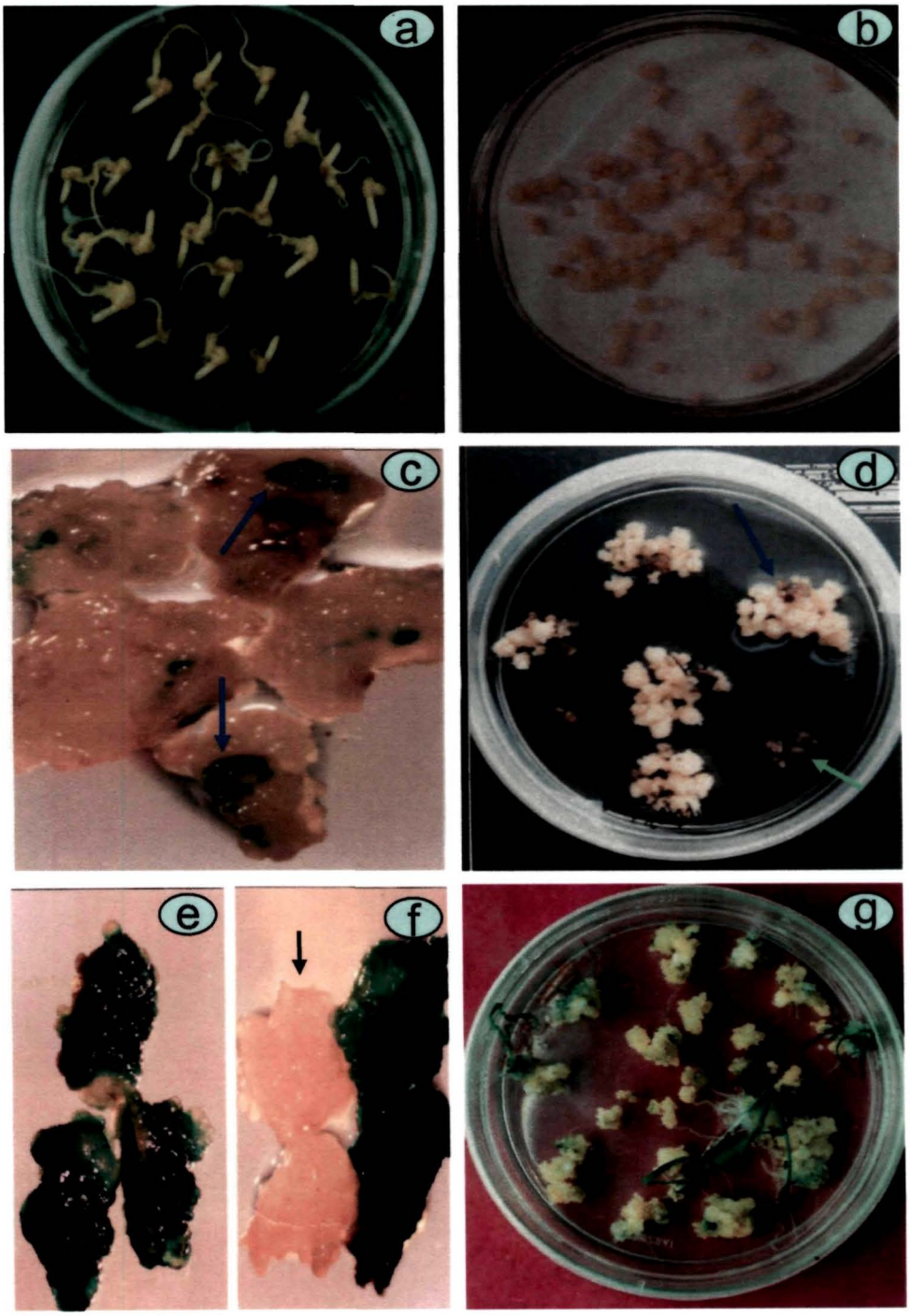

Fig 4. Contd →

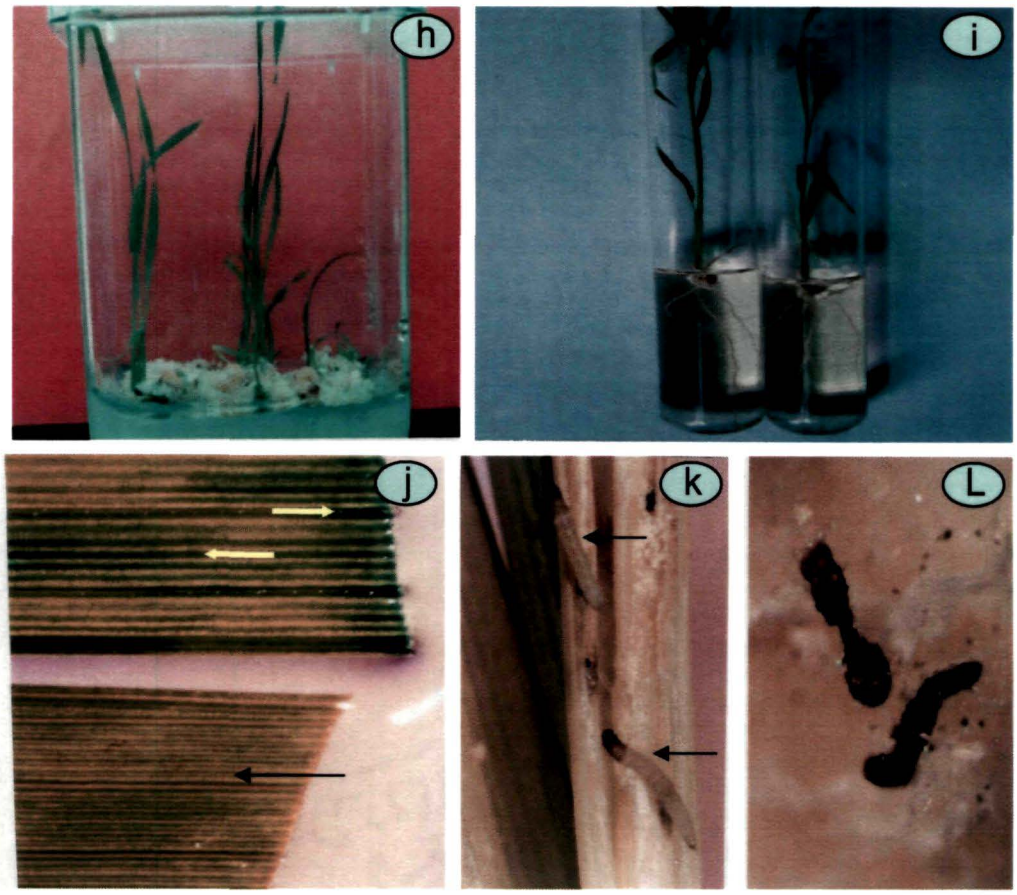

Fig. 4. Production of Bt-transgenic Basmati rice through *Agrobacterium*-mediated transformation **(a)** Scutellum-derived calli induction from seed explants **(b)** Co-cultivation of *Agrobacterium* treated calli for 3-4 days in dark **(c)** Detection of putatively transformed calli by transient *gus* (reporter gene) expression as blue spots (indicated by arrows) **(d)** Proliferation of hygromycin resistant calli (indicated by blue arrow) and death of untransformed calli (indicated by green arrow) **(e-f)** Detection of stable transformation by *gus* gene expression (arrow indicating the control calli) **(g-h)** Regeneration from putatively transformed calli in regeneration medium containing hygromycin (50 mg l^{-1}) and cefotaxime (75 mg l^{-1}) **(i)** Rooting of regenerated plants in rooting medium containing hygromycin (50 mg l^{-1}) and cefotaxime (250 mg l^{-1}) **(j)** GUS expression in leaf of regenerated transformed plants (yellow arrow indicates *gus* expression sectors) whereas the control (indicated by black arrow) leaf shows complete absence of *gus* gene expression **(k)** A second instar larvae of yellow stem borer feeding on control cut stems (indicated by arrow) **(l)** Death of first instar larvae while feeding on putatively transformed plant producing *cry1Ac* endotoxin as a result of expression of *cry1Ac* gene.

Fig. 5. *Agrobacterium*-mediated transformation of rice cv. Pusa Basmati 1 from embryogenic calli of mature seed-derived scutella. **(a)** Scutellum-derived calli obtained from mature seeds **(b)** Proliferation of hygromycin resistant calli during third selection phase in CIM medium supplemented with hygromycin (60 mg l^{-1}) and cefotaxime (250 mg l^{-1}) **(c-d)** Plantlets regenerated from hygromycin resistant calli after transfer to $MSKN_3$ medium containing hygromycin (50 mg l^{-1}) and cefotaxime (75 mg l^{-1}) **(e)** Insect bio-assay of T_1 putative transformants expressing *cryIAc* gene recorded after 4 days of feeding on cut stems **(f)** Segregation of hygromycin gene in the progenies of T_1. Non-transgenic seeds failed to germinate while their counter-part transgenic T_1 seeds germinated on half strength MS medium supplemented with hygromycin (50 mg/l).

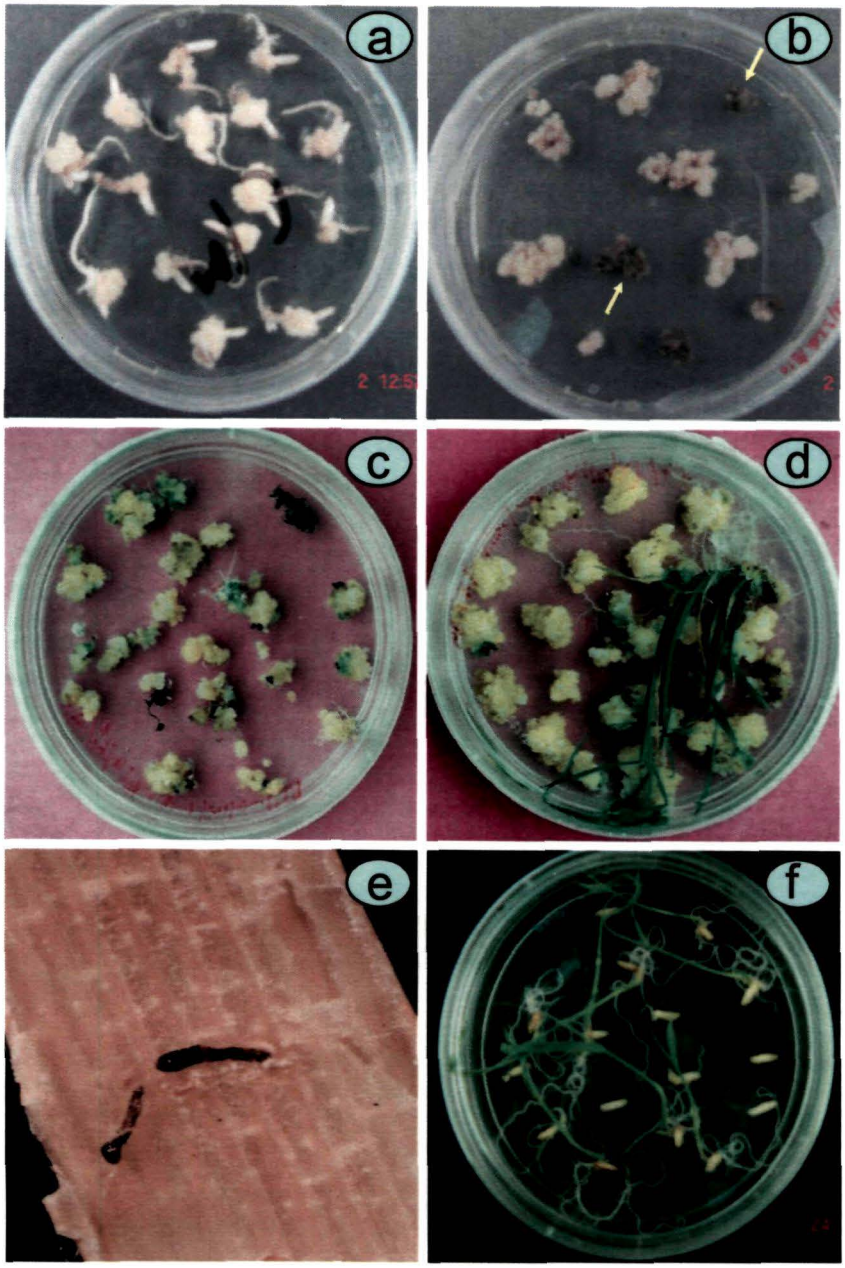

Fig. 6. *Agrobacterium*-mediated transformation of rice cv. Basmati 370 from embryogenic calli of mature seed-derived scutella **(a)** Scutellum-derived calli obtained from mature seeds **(b)** Proliferation of hygromycin resistant calli during third selection phase in CIM medium supplemented with hygromycin (60 mg/l). Arrows indicates death of untransformed calli **(c-d)** Plantlets regenerated from hygromycin resistant calli after transfer to $MSKN_3$ medium containing hygromycin (50 mg/l) and cefotaxime (75 mg l^{-1}) **(e)** Insect bio-assay of T_1 transformant expressing *cryIAc* recorded after 4 days of feeding on cut stems **(f)** Segregation of *hpt* gene in the progeny of T_1. Non-transgenic seeds failed to germinate while their counter-part transgenic T_1 seeds germinated on half strength MS medium supplemented with hygromycin (50 mg/l).

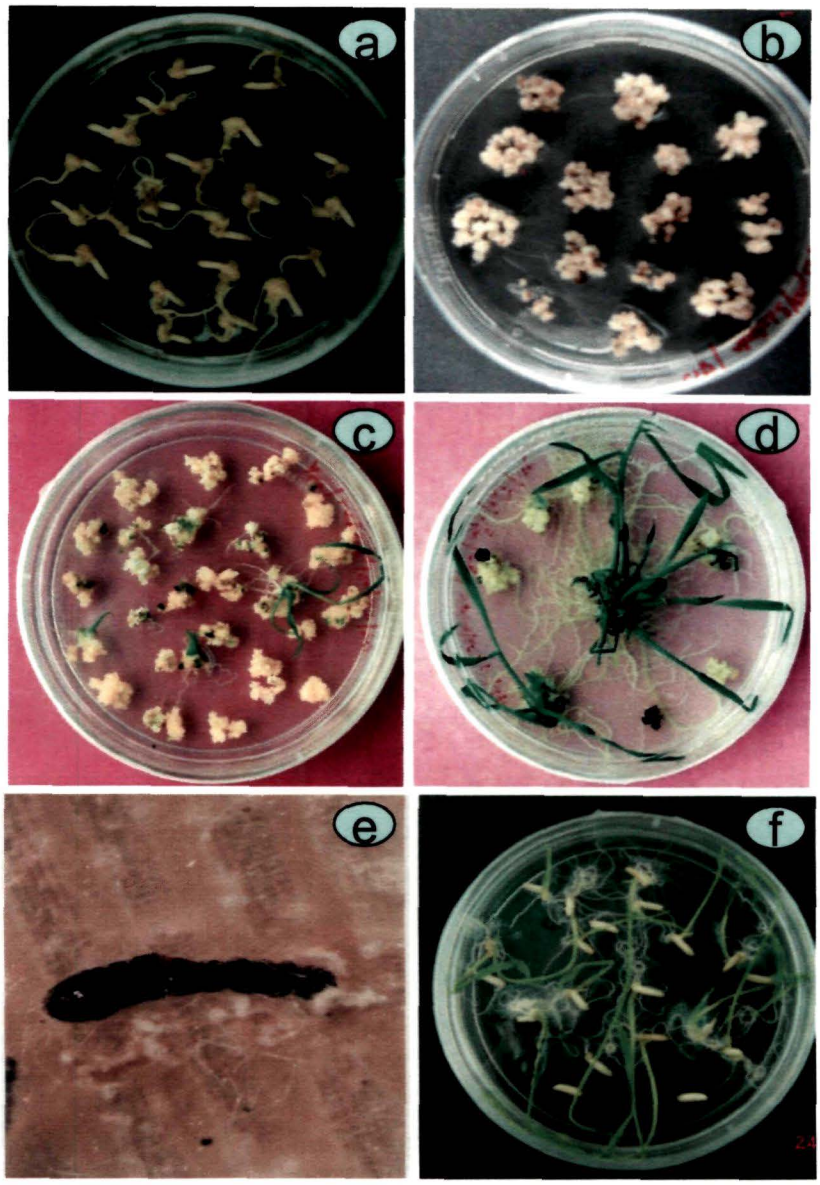

Fig. 7. *Agrobacterium*-mediated transformation of rice cv. Tarori Basmati from embryogenic calli of mature seed-derived scutella. **(a)** Scutellum-derived calli obtained from mature seeds **(b)** Proliferation of hygromycin resistant calli during third selection phase in CIM medium supplemented with hygromycin (60 mg l^{-1}) and cefotaxime (250 mg l^{-1}) **(c-d)** Plantlets regenerated from hygromycin resistant calli after transfer to $MSKN_3$ medium containing hygromycin (50 mg l^{-1}) and cefotaxime (75 mg l^{-1}) **(e)** Insect bio-assay of T_1 putative transformants expressing *cryIAc* recorded after 4 days of feeding on cut stems **(f)** Segregation of *hpt* gene in the progenies of T_1. Non-transgenic seeds failed to germinate while their counter-part transgenic T_1 seeds germinated on half strength MS medium supplemented with hygromycin (50 mg/l).

then subcultured to fresh CIM medium for another 5-7 days in dark. Subculture of these calli in fresh CIM medium induced proliferation from exposed regions as well.

4.2 *A. tumefaciens*-mediated infection of subcultured callus

The embryogenic proliferating calli were infected with *Agrobacterium tumefaciens* LBA4404 (pSB1, pCAMBAc) and co-cultivated in dark for 84 hours. Occasionally, gelly like bacterial overgrowth was noticed in co-cultivated calli. Such calli were usually discarded, since even after thorough washing bacterial growth could not be eliminated.

4.3 Standardisation of concentration of acetosyringone (AS) for enhancing transient GUS expression in *Agrobacterium tumefaciens* infected scutellar calli

Scutellar calli immediately after 5-7 days of subculture were co-cultivated with *Agrobacterium* LBA4404 (pSB1, pCAMBAc) in AA medium containing different concentration of AS *viz.* 50 µM, 100 µM, 200 µM and 300 µM (Table 5) to find out the optimum AS concentration at which the best transient GUS expression rate could be achieved. After 84 hours of co-cultivation, the calli from each treatment were subjected to GUS histochemical staining for standardisation of the best transient GUS expression for each variety.

Table 5: Effect of different concentrations of acetosyringone in influencing GUS expression for 25 number of co-cultivated calli in each treatment

Varieties	Acetosyringone concentration in co-cultivation medium (μM)	No of callus showing transient GUS expression (%)	Mean ± SD
Pusa Basmati 1	50	50 50 60	53.3±5.77
	100	65 45 60	56.6±10.40
	200	75 80 60	71.6±10.40
	300	30 40 60	43.3±15.27
Basmati 370	50	30 30 30	30±0
	100	30 30 35	31.6±2.88
	200	40 60 40	46.6±11.54
	300	55 55 50	53.3±2.88
Tarori Basmati	50	50 50 40	46.6±5.77
	100	65 60 55	60.0±5.0
	200	45 30 30	35±8.66
	300	50 30 30	36.6±11.54

The data (Table 5) of transient GUS assay showed that 200 μM of AS was the best for Pusa Basmati 1 exhibiting mean value of 71.6%, 300 μM was the best for

Basmati 370 exhibiting mean value of 53.3% and 100 μM acted best for Tarori Basmati exhibiting mean value of 60.0% for transformation.

After standardising the best AS concentration for each variety, five independent experiments were carried out for each of the three varieties. AS concentration of 200 μM for Pusa Basmati 1 exhibited mean value of 75.6%, 300 μM for Basmati 370 exhibited mean value of 58.8% and 100 μM for Tarori Basmati exhibited mean value of 48.4% for transient GUS assay (Table 6).

Table 6: Efficiency of standardised AS concentration in inducing transient GUS expression

Varieties	Acetosyri-ngone concentration (μM)	No. of calli selected for transient GUS assay	No. of calli showing transient GUS expression	Percentage of calli showing transient GUS expression	Mean±SD
Pusa Basmati 1	200	12	8	66	75.6±8.98
		25	20	80	
		25	17	68	
		25	19	76	
		25	22	88	
Basmati 370	300	12	6	50	58.8±9.12
		25	18	72	
		25	14	56	
		25	13	52	
		25	16	64	
Tarori Basmati	100	12	6	50	48.4±7.53
		25	12	48	
		25	10	40	
		25	11	44	
		25	15	60	

4.4 Proliferation of putatively transformed hygromycin resistant calli

The transformed region of calli started dividing and proliferating approximately from 10-15 days of plating on the 1st selection media. The rapidly dividing transformed sectors of calli were dissected away from the mother tissue after it attained an

approximate size of 3±1 mm. These calli were then transferred to fresh selection medium. The proliferating calli were yellowish and grew rapidly in selection medium forming compact small clumps/nodular pearl-like aggregation that appeared mostly yellowish white. These pearl-like small aggregations of calli on sub-culturing for two phases of selection led to formation of large mass of globular pale yellow calli by the end of 3rd and/or 4th selection (Fig. 4d, 5b, 6b, 7b). Major sector of treated calli that remained untransformed, subsequently died by the end of 3rd selection giving a dark brown to black colored tissue aggregates (Fig. 4d & 6b). During the selection process each transformed sector of calli produced 5-25 numbers of calli clumps. Calli that proliferated from the parental callus were maintained as a single callus line and accordingly marked to distinguish from other callus lines. Hence, callus mass produced during selection phases of each calli line represented similar single point transformation event.

The untransformed control calli proliferated poorly during selection phases and completely seized by 3rd selection. These calli became brown or black and ultimately died.

The response for the production of hygromycin resistant calli in selection medium (CIM medium supplemented with 50 or 60 mg l^{-1} of hygromycin and varied cefotaxime levels) of all the three Basmati varieties infected with *Agrobacterium tumefaciens* was recorded (Table 7). Five independent experiments were carried out for each variety. Percentage of proliferated hygromycin resistant calli of five independent experiments for each variety, varied from 79.5% to 90.6% in case of Pusa Basmati 1, 72.0% to 82.6% in case of Basmati 370 and 50.6% to 59.0% in case of Tarori Basmati.

A high frequency of hygromycin resistant calli *viz.* 84.2% (327 out of 388 calli plated) in Pusa Basmati 1, 77.3% (300 out of 388 calli) in Basmati 370 and 55.5% (216 out of 388 calli plated) in Tarori Basmati was recorded (Table 7).

Table 7: Proliferation rate of infected calli in CIM medium supplemented with 50 or 60 mg l^{-1} of hygromycin

Varieties	No. of calli treated	No. of calli plated on hygromycin (A)	No. of hygromycin resistant calli (B)	Total no. of hygromycin resistant calli	Percentage of hygromycin resistant calli (Bx100/A)	Mean (%)
Pusa Basmati 1	100	88	70	327	79.5	84.2
	100	75	62		82.6	
	100	75	65		86.6	
	100	75	62		82.6	
	100	75	68		90.6	
Basmati 370	100	88	66	300	75.0	77.3
	100	75	58		77.3	
	100	75	60		80.0	
	100	75	62		82.6	
	100	75	54		72.0	
Tarori Basmati	100	88	52	216	59.0	55.5
	100	75	38		50.6	
	100	75	42		56.0	
	100	75	40		53.3	
	100	75	44		58.6	

4.5 GUS assay for determination of stable transformation efficiency

Sections from calli that survived and proliferated till the 4th selection phase and exhibited a typical globular morphology were used to determine stable transformation rate. After a rigorous selection period of 11 weeks, the calli displayed uniform blue

precipitation for stable *gus* gene expression (Fig. 4e & 4f), rather than chimeras when assayed for transient GUS expression (Fig. 4c). The mean stable transformation rate was observed to be 66% for Pusa Basmati 1, 60% for Basmati 370 and 56% for Tarori Basmati (Table 8).

Table 8: Assessment for stable GUS expression

Varieties	No. hygromycin resistant calli for GUS assay	No. of stable GUS positive calli	Percentage of stable GUS positive calli	Mean±SD
Pusa Basmati 1	10	7	70	66±5.44
	10	6	60	
	10	7	70	
	10	6	60	
	10	7	70	
Basmati 370	10	6	60	60±7.07
	10	5	50	
	10	6	60	
	10	6	60	
	10	7	70	
Tarori Basmati	10	6	60	56±5.47
	10	5	50	
	10	6	60	
	10	5	50	
	10	6	60	

4.6 Plant regeneration

4.6.1 Standardisation of regeneration media

Five regeneration media formulations were tried *viz.* MS-BN_1, MS-BN_2, MS-KN_1, MS-KN_2 and MS-KN_3 (Table 9). MS-KN_3 was found to be the best for

regeneration of 21 days mature seed scutellum derived calli of all the three varieties with a regeneration value of 73% for Pusa Basmati 1, 84% for Basmati 370 and 60% for Tarori Basmati.

Table 9: Response of different regeneration media

Varieties	No. of callus plated	% regeneration									
		MS-BN_1	Mean (%)	MS-BN_2	Mean (%)	MS-KN_1	Mean (%)	MS-KN_2	Mean (%)	MS-KN_3	Mean (%)
Pusa Basmati 1	50	30	27 (54)	32	28 (56)	32	30 (61)	34	32 (64)	38	36 (73)
	50	24		24		29		30		35	
Basmati 370	50	20	24 (48)	26	25 (50)	26	25 (50)	29	29 (61)	40	42 (84)
	50	28		24		24		30		44	
Tarori Basmati	50	18	19 (38)	16	19 (38)	20	22 (44)	26	25·(50)	28	30 (60)
	50	20		22		24		24		32	

4.6.2 Plant regeneration from hygromycin resistant calli

After a course of four selection phases, the putatively transformed hygromycin resistant calli were transferred to regeneration selection medium (MS-KN_3) supplemented with 50 mg l^{-1} of hygromycin and 75 mg l^{-1} of cefotaxime. The hygromycin resistant calli produced creamy white compact embryo like structures after 2 weeks of incubation in dark which on transfer to light immediately differentiated to green plantlets (Fig. 4g, 4h, 5c, 5d, 6c, 6d, 7c & 7d). Very few of them produced new sector of white callus during dark period, which on transfer to light immediately differentiated into cluster of plantlets. Occurrence of green pigmentation was more prominent in small calli in comparison to those in big callus clumps. On the other hand, few calli produced albino plants in dark, which on transfer to light immediately became green in 1-2 days. Occasionally, some calli that showed green pigmentation did not differentiate into shoot(s) and roots even after prolonged incubation in light for more

than a month and this feature was more prominent when unregenerated calli were subcultured to fresh regeneration medium.

Table 10: Plant regeneration efficiency in MS-KN_3 medium from hygromycin resistant calli

Varieties	No. of hygromycin resistant calli	No. of calli regenerated into plants	Total no. of calli regenerated	Regeneration frequency		No. of plantlets regenerated	Total no. of plants regenerated
				(%)	Mean		
Pusa Basmati 1	70	42		60.0		66	
	62	40		64.5		62	
	65	40	208	61.5	63.6	60	310
	62	42		67.7		60	
	68	44		64.7		62	
Basmati 370	66	48		72.7		62	
	58	44		75.8		58	
	60	40	214	66.6	71.3	56	294
	62	42		67.7		60	
	54	40		74.0		58	
Tarori Basmati	52	30		57.6		46	
	38	20		52.6		40	
	42	25	124	59.5	57.3	36	186
	40	24		60.0		34	
	44	25		56.8		30	

All the healthy looking green regenerated plantlets were transferred to culture tubes and magenta boxes for profuse rooting (Fig. 4i). Plants were maintained in culture tubes for 20-25 days in light.

A total of 310 plants of Pusa Basmati 1 from 327 hygromycin resistant calli lines with an average regeneration rate of 63.6% (considering number of calli regenerated into plants), 294 plants of Basmati 370 from 300 hygromycin resistant calli lines at an average rate of 71.3% and 186 plants of Tarori Basmati from 216 hygromycin resistant calli lines at an average rate of 57.3% were produced (Table 10).

4.7 PCR analyses of putative transformants

4.7.1 Analyses for transgene(s) integration

Genomic DNA from putative transformants of Pusa Basmati 1, Basmati 370 and Tarori Basmati was checked for presence of *cry1Ac* gene (Fig. 8c, 9c & 10c) and its flanking sequence *hpt* gene (Fig. 8a, 9a & 10a), the left T-DNA border and *gus* gene (Fig. 8b, 9b & 10b), the right T-DNA border. Instead of five, four sets of independent experiments for each variety were considered for transgene analyses (Table 11) for ease in handling.

The various combinations of three genes present in T-DNA region for their absence and presence in T_0 Basmati rice varieties are presented in Table 11. Out of 96 plants analysed 69.7%, 70.8% and 63.5% of Pusa Basmati 1, Basmati 370 and Tarori Basmati respectively were hygromycin resistant plants, which were also co-transformed with the non-selectable marker *cry1Ac* gene and *gus* reporter gene (Table 11) suggesting complete integration of T-DNA region. On the contrary mere 3.12%, 2.08% and 2.08% were found to be null for the T-DNA region for primary transformants of Tarori Basmati, Pusa Basmati 1 and Basmati 370 respectively.

It is assumed that *gus* positive transgenic plants are also positive for presence of *gfp* gene, since *gfp* gene is translationally fused with *gus* gene.

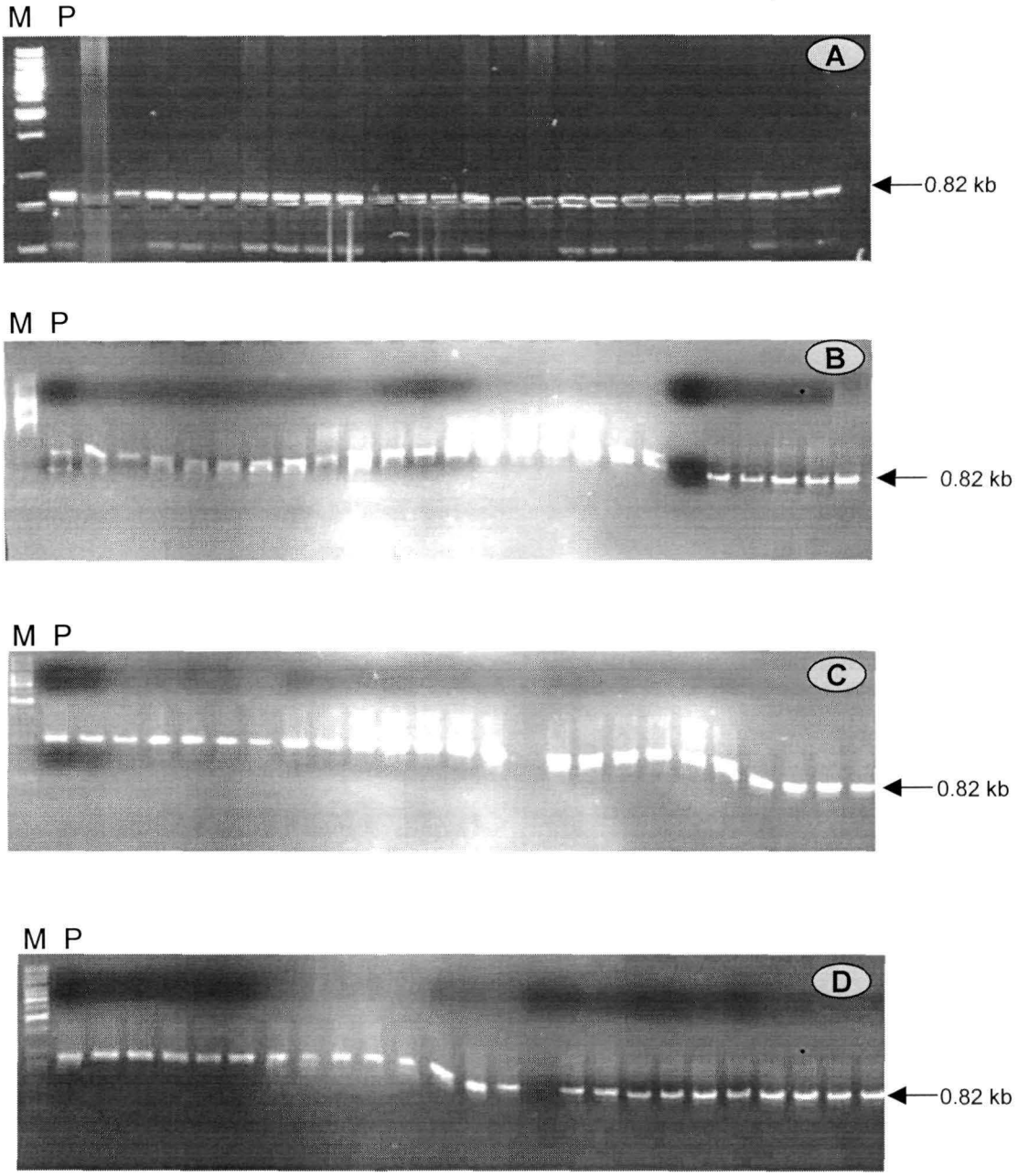

Fig. 8a. PCR analyses of putative transgenic (T_0) plants of Basmati 370 screened for the presence of *hpt* gene (0.82 kb) in four independent experiments namely A, B, C and D. 'M' designates positions and sizes of 1 kb mass ruller while 'P' designates *hpt* gene amplification from plasmid pCAMBAc.

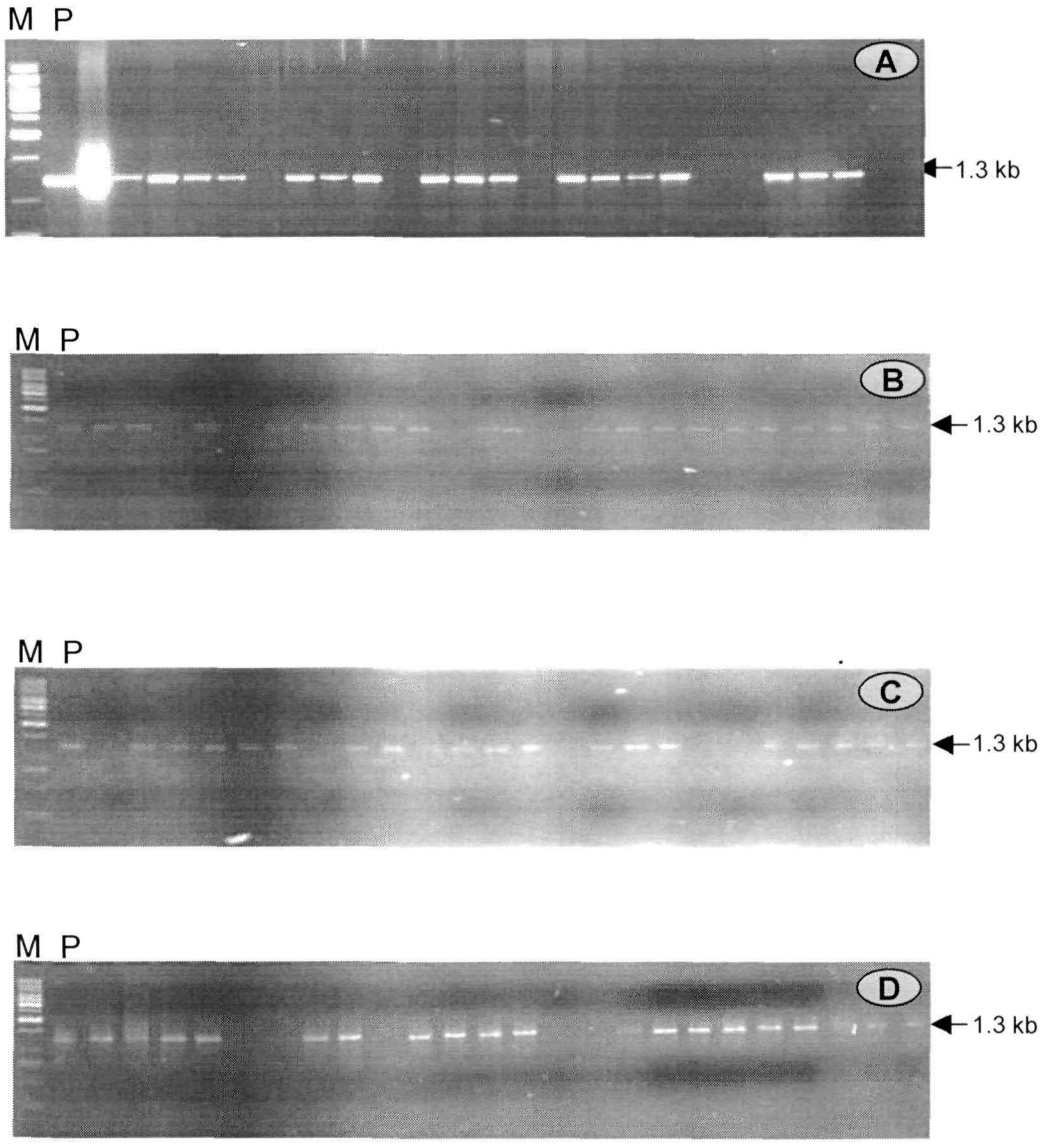

Fig. 8b. PCR analyses of putative transgenic (T_0) plants of Basmati 370 screened for the presence of *gus* gene (1.3 kb) in four independent experiments namely A, B, C and D. 'M' designates positions and sizes of 1 kb mass ruller while 'P' designates *gus* gene amplification from plasmid pCAMBAc

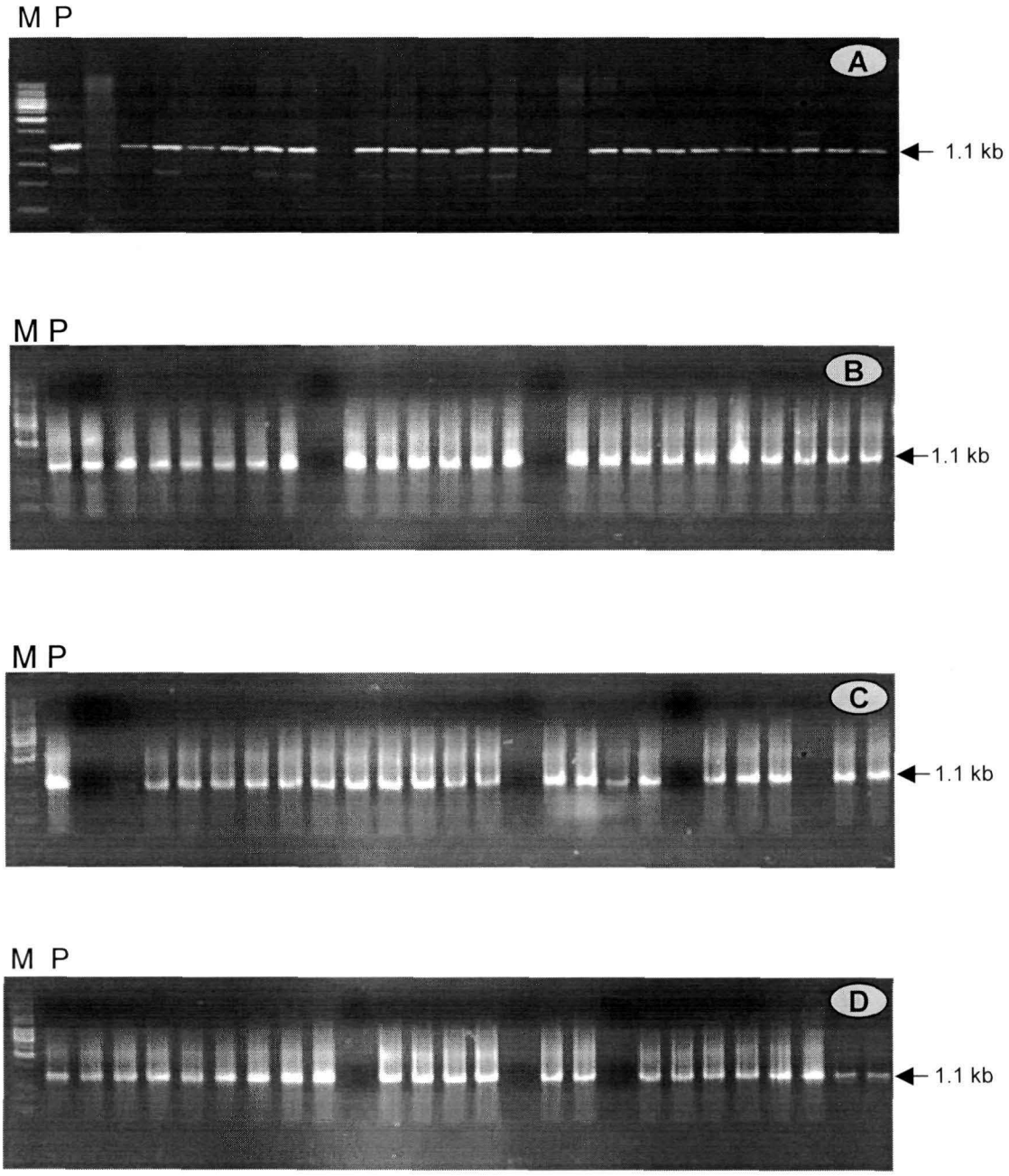

Fig. 8c. PCR analyses of putative transgenic (T_0) plants of Basmati 370 screened for the presence of *cryIAc* gene (1.1 kb) in four independent experiments namely A, B, C and D. 'M' designates positions and sizes of 1 kb mass ruller while 'P' designates *cryIAc* gene amplification from plasmid pCAMBAc.

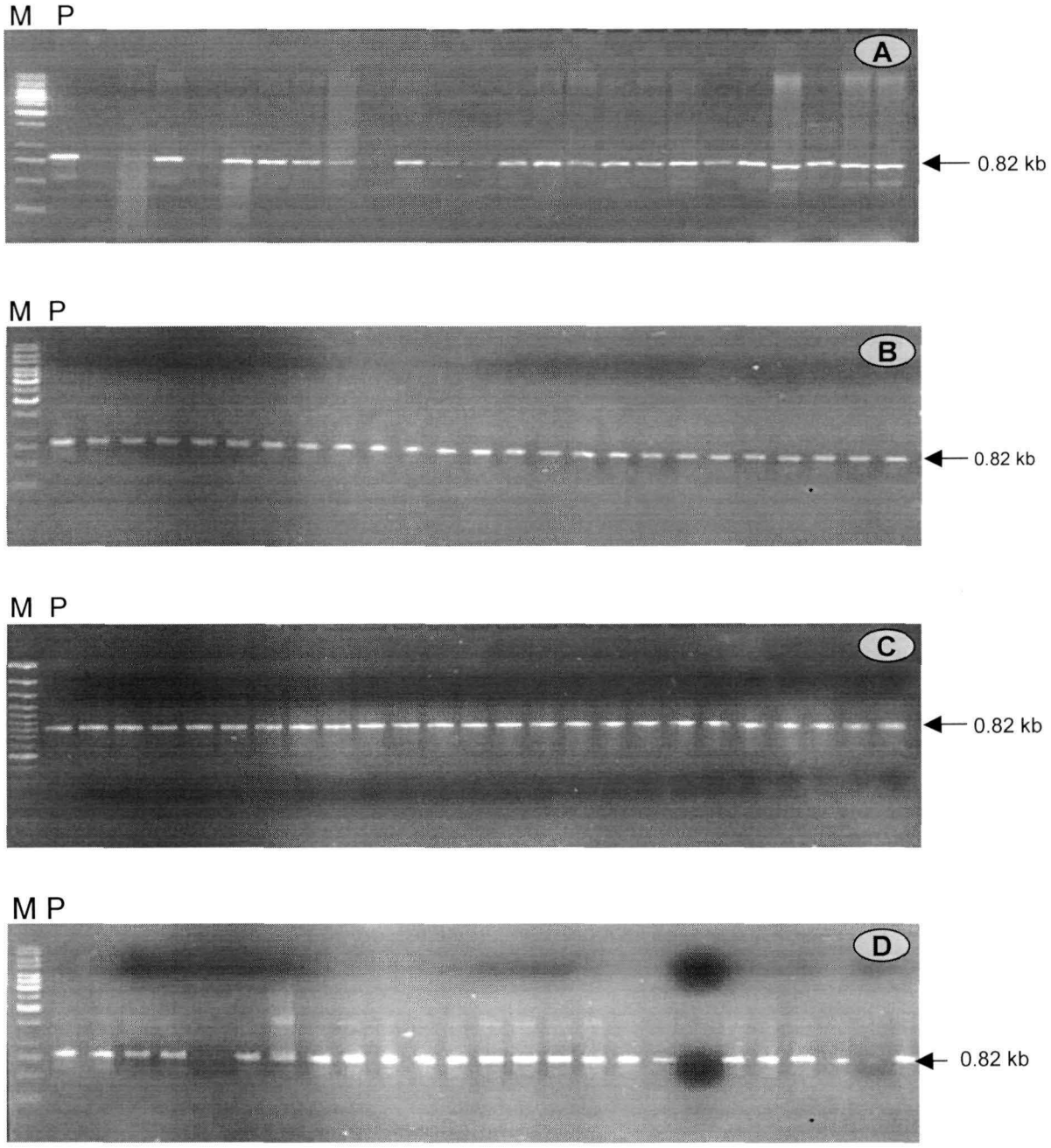

Fig. 9a. PCR analyses of putative transgenic (T_0) plants of Pusa Basmati 1 screened for the presence of *hpt* gene (0.82 kb) in four independent experiments namely A, B, C and D. 'M' designates positions and sizes of 1 kb mass ruller while 'P' designates *hpt* gene amplification from plasmid pCAMBAc

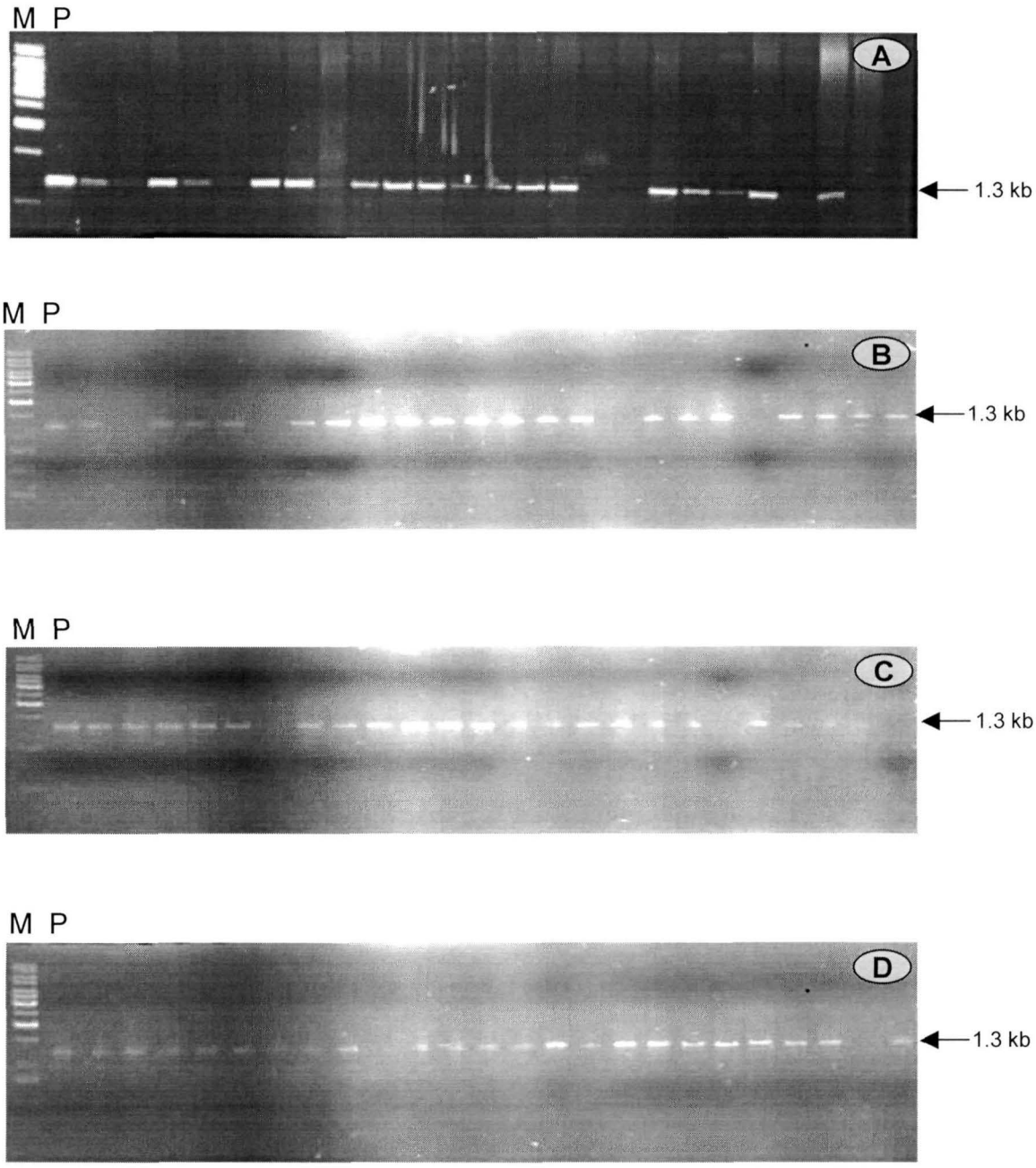

Fig. 9b. PCR analyses of putative transgenic (T_0) plants of Pusa Basmati 1 screened for the presence of *gus* gene (1.3 kb) in four independent experiments namely A, B, C and D. 'M' designates positions and sizes of 1 kb mass ruller while 'P' designates *gus* gene amplification from plasmid pCAMBAc.

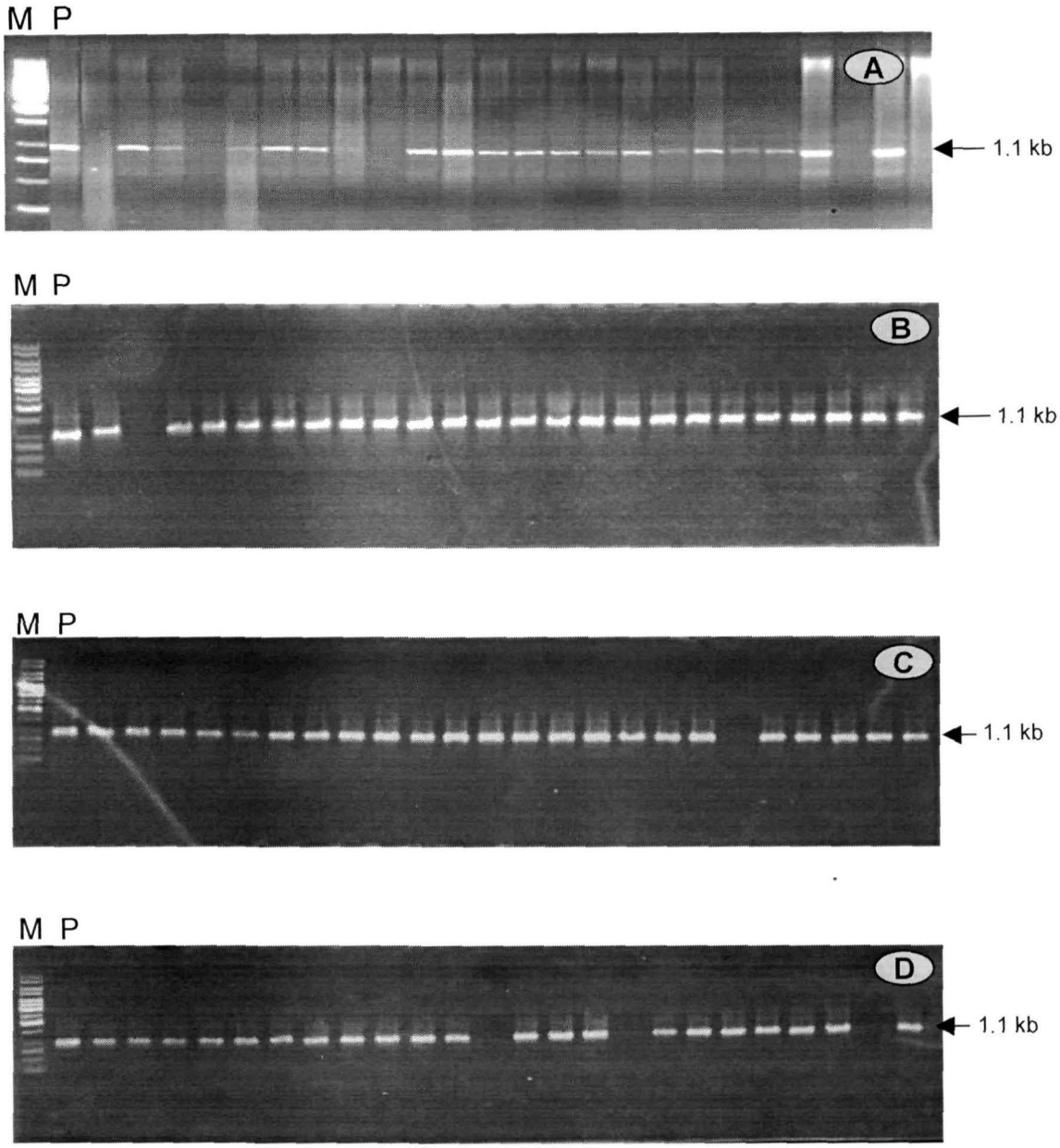

Fig. 9c. PCR analyses of putative transgenic (T_0) plants of Pusa Basmati 1 screened for the presence of *cryIAc* gene (1.1 kb) in four independent experiments namely A, B, C and D. 'M' designates positions and sizes of 1 kb mass ruller while 'P' designates *cryIAc* gene amplification from plasmid pCAMBAc

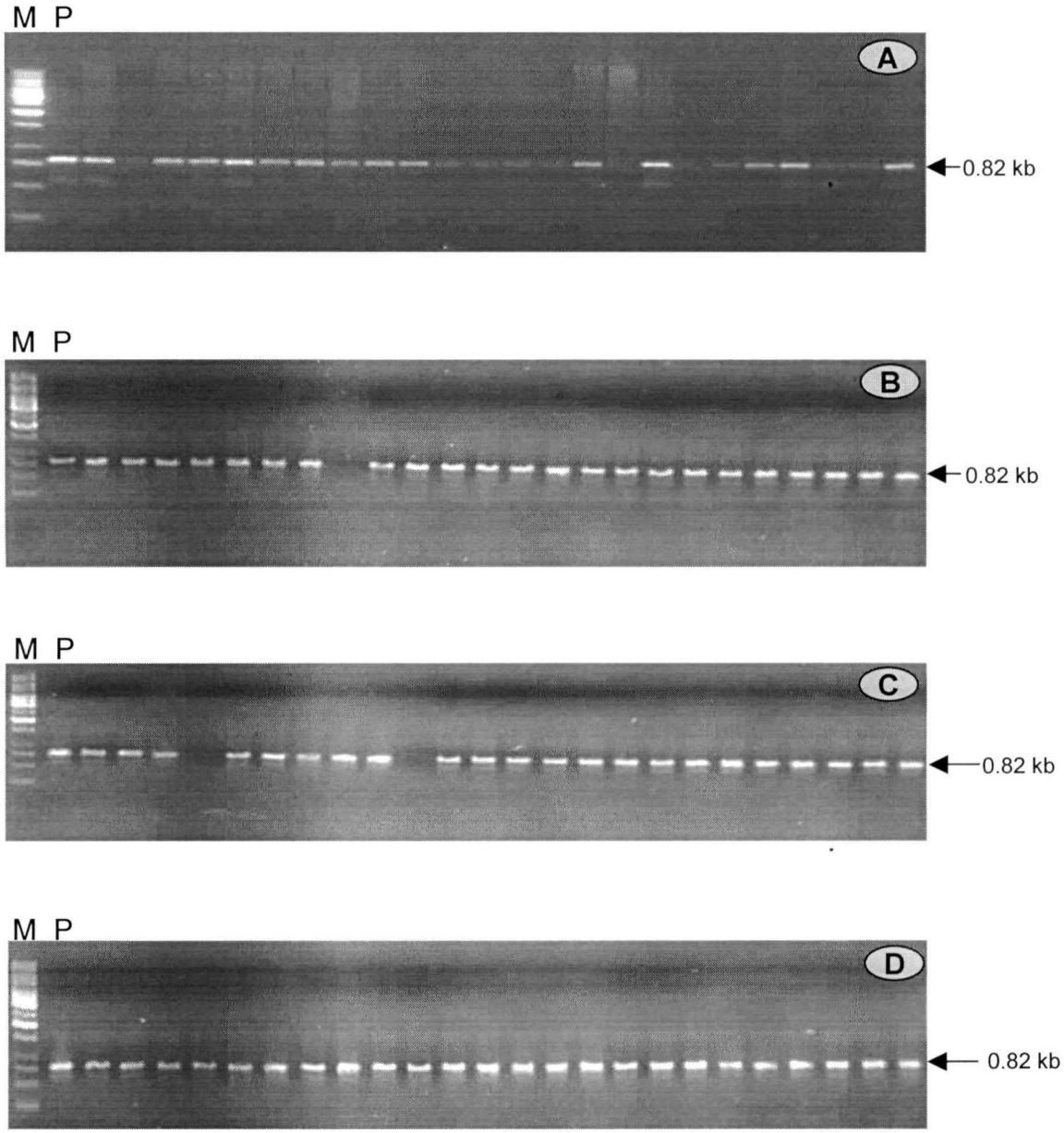

Fig. 10a. PCR analyses of putative transgenic (T_0) plants of Tarori Basmati screened for the presence of *hpt* gene (0.82 kb) in four independent experiments namely A, B, C and D. 'M' designates positions and sizes of 1 kb mass ruller while 'P' designates *hpt* gene amplification from plasmid pCAMBAc.

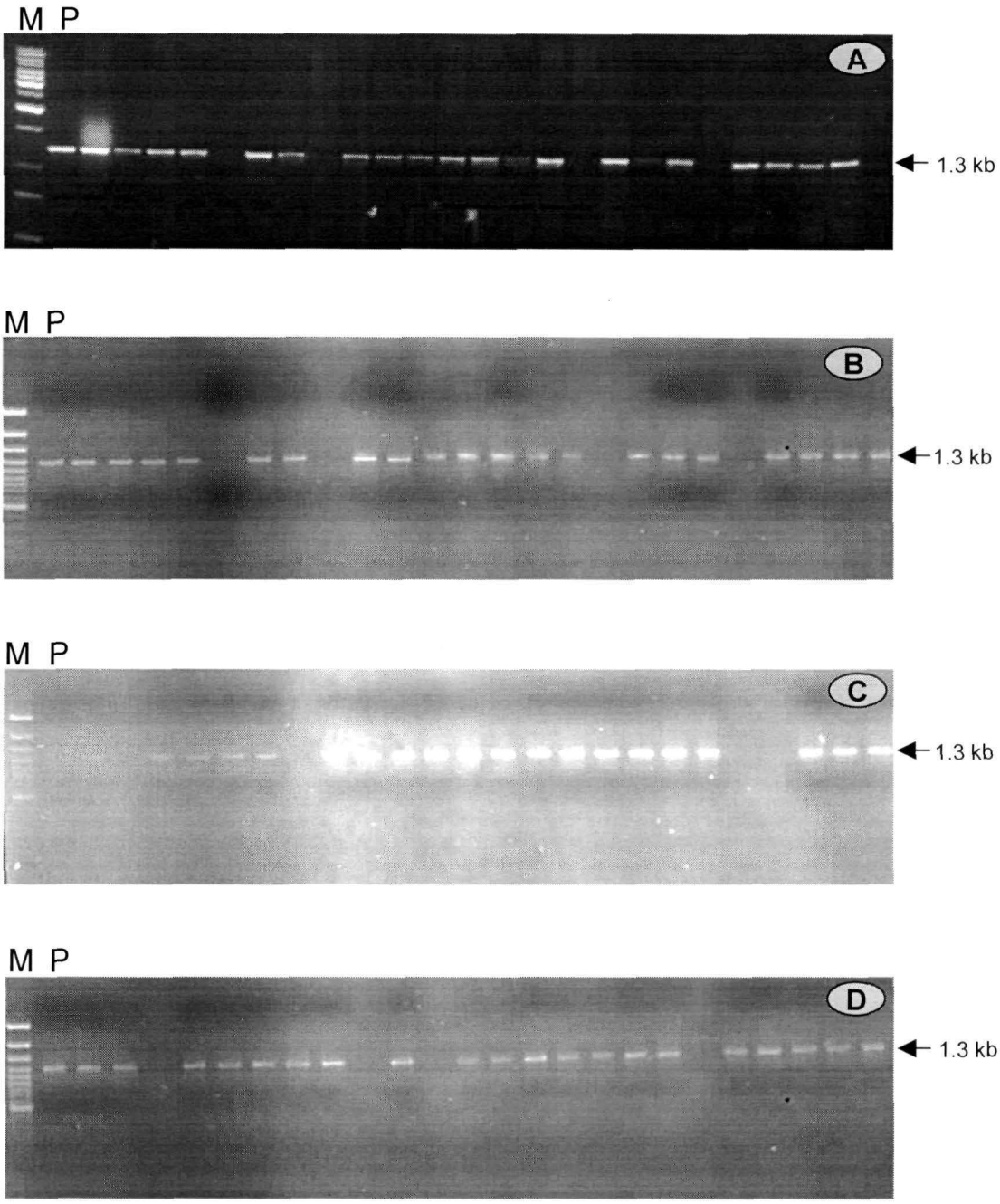

Fig. 10b: PCR analyses of putative transgenic (T_0) plants of Tarori Basmati screened for the presence of *gus* gene (1.3 kb) in four independent experiments namely A, B, C and D. 'M' designates positions and sizes of 1 kb mass ruller while 'P' designates *gus* gene amplification from plasmid pCAMBAc.

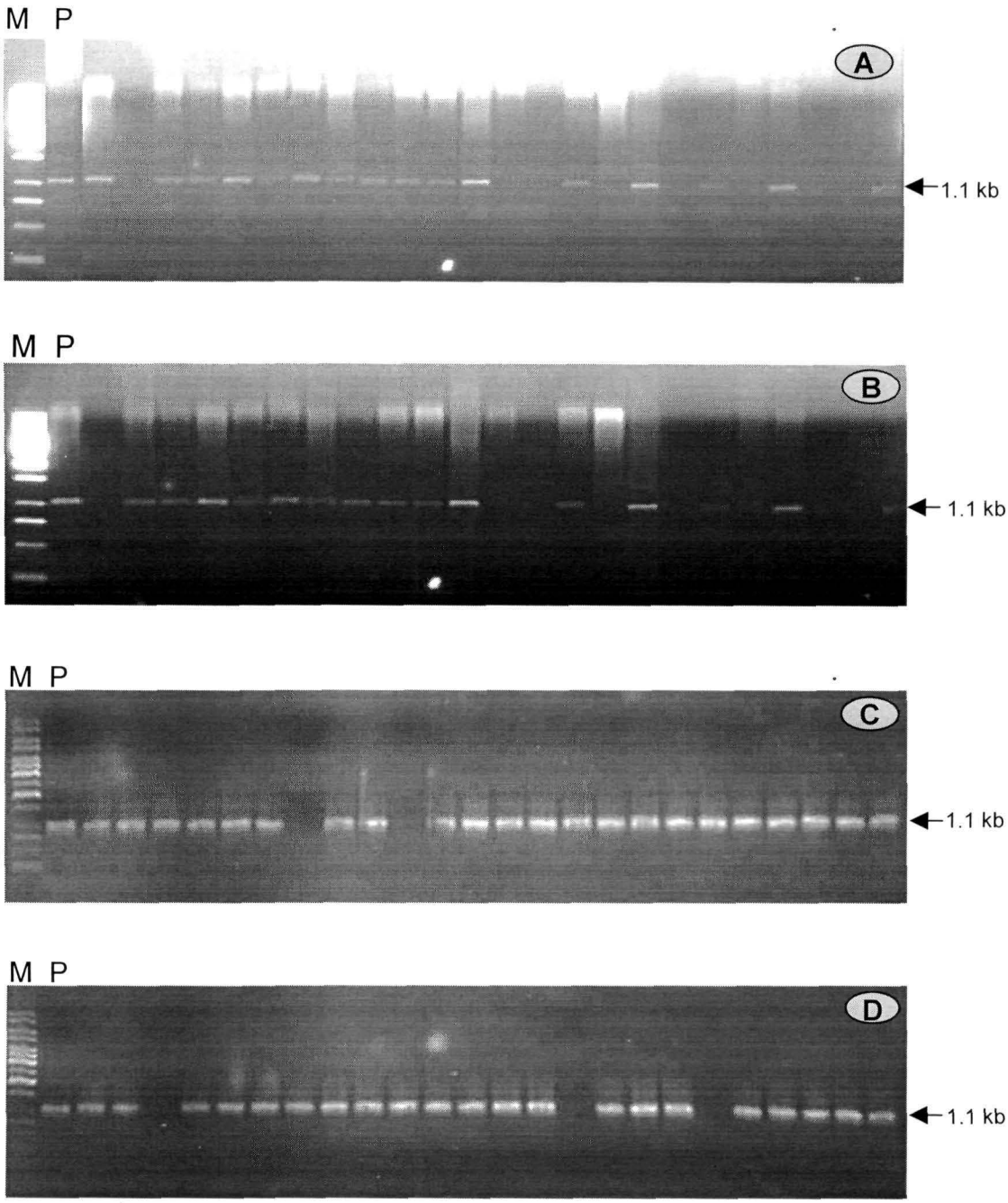

Fig. 10c. PCR analyses of putative transgenic (T_0) plants of Tarori Basmati screened for the presence of *cryIAc* gene (1.1 kb) in four independent experiments namely A, B, C and D. 'M' designates positions and sizes of 1 kb mass ruller while 'P' designates *cryIAc* gene amplification from plasmid pCAMBAc

Table 11: Results of PCR analyses of four independent experiments (A, B, C & D) for each of the Basmati varieties with and without transgene

Varieties	Independent experiments	No. of plants	*cry*$^+$ *gus*$^+$ *hpt*$^+$	*cry*$^+$ *gus*$^+$ *hpt*$^+$ (%)	*cry*$^+$ *hpt*$^+$ *gus*$^-$	*cry*$^+$ *hpt*$^+$ *gus*$^-$ (%)	*cry*$^+$ *hpt*$^-$ *gus*$^-$	*cry*$^+$ *hpt*$^+$ *gus*$^-$ (%)	*cry*$^+$ *hpt*$^-$ *gus*$^+$	*cry*$^+$ *hpt*$^-$ *gus*$^+$ (%)	*cry*$^-$ *hpt*$^+$ gus$^+$	*cry*$^-$ *hpt*$^+$ *gus*$^+$ (%)	*cry*$^-$ *hpt*$^+$ *gus*$^-$	*cry*$^-$ *hpt*$^+$ *gus*$^-$ (%)	*cry*$^-$ *hpt*$^-$ *gus*$^-$	*cry*$^-$ *hpt*$^-$ *gus*$^-$ (%)	*cry*$^-$ *hpt*$^-$ *gus*$^+$	*cry*$^-$ *hpt*$^-$ *gus*$^+$ (%)
Pusa Basmati 1	A	24	12	69.7	3	10.4	2	2.08	2	7.2	0	2.08	2	3.12	0	2.08	3	3.1
	B	24	17		3		0		3		0		0		1		0	
	C	24	21		2		0		0		0		1		0		0	
	D	24	17		2		0		2		2		0		1		0	
Basmati 370	A	24	15	70.8	6	14.5	0	0	0	1.04	3	7.29	0	4.16	0	2.08	0	0
	B	24	18		3		0		1		1		1		0		0	
	C	24	17		2		0		0		2		2		1		0	
	D	24	18		3		0		0		1		1		1		0	
Tarori Basmati	A	24	11	63.5	2	8.33	0	1.04	2	3.12	0	1.04	1	11.4	3	3.12	5	6.2
	B	24	13		3		1		0		0		7		0		0	
	C	24	18		3		0		1		0		1		0		1	
	D	24	19		2		0		0		1		2		0		0	

4.7.2 Analyses for T-DNA border sequence for integration

Analyses of border region of T-DNA revealed that the left border (LB) region was more conserved in all the three Basmati rice varieties with an average of 85.4%, 96.87 and 86.45% in Pusa Basmati 1 and Basmati 370 and Tarori Basmati respectively. On the other hand right border (RB) region was found to be comparably less conserved with an average of 82.29%, 79.6% and 73.95% in Pusa Basmati 1, Basmati 370 and Tarori Basmati respectively (Table 12). The non-selectable marker gene (*cry1Ac*) was found to be in 87.5%, 86.45% and 78.12% in primary transformants of Pusa Basmati 1, Basmati 370 and Tarori Basmati respectively.

Table 12: PCR analyses for presence of T-DNA border sequence (*hpt* & *gus* genes) and non selectable marker (*cry1Ac* gene)

Varieties	Independent experiments	No. of plants exhibiting left border T-DNA sequence (*hpt*$^+$)	% of plants exhibiting left border T-DNA sequence	No. of plants exhibiting right border T-DNA sequence (*gus*$^+$)	% of plants exhibiting right border T-DNA sequence	No. of plants exhibiting non selectable marker (*cry*$^+$)	% of plants exhibiting non selectable marker
Pusa Basmati 1	A	17	85.4	17	82.29	19	87.5
	B	20		20		23	
	C	24		21		21	
	D	21		21		21	
Basmati 370	A	24	96.87	18	79.16	21	86.45
	B	23		20		22	
	C	23		19		19	
	D	23		19		21	
Tarori Basmati	A	14	86.45	18	73.95	15	78.12
	B	23		13		17	
	C	22		20		22	
	D	24		20		21	

4.8 Analyses for transformation frequency

All the three varieties were evaluated for transformation frequency. The regeneration frequency varied from 47.7 to 53.3% for Pusa Basmati 1, 47.7-58.6% for Basmati 370 and 26.6-34.0% for Tarori Basmati (Table 13). The percentage mean transformation frequency obtained for Pusa Basmati 1 was 52.10%, 54.48% for Basmati 370 and 30.84% for Tarori Basmati.

Table 13: Putative transformation frequency based on PCR analyses

Varieties	Indepe-ndent experi-ments	No. of callus plated in hygromycin (A)	No. of callus regenerated into plants		No. of plants selected for PCR analyses	Transform ed plants in context to (C)	Transformatio n frequency (DxBx100/C)/ A	
			(B)	%	(C)	(D)	%	Mean
Pusa Basmati 1	A	88	42	47.7	24	23	45.7	52.10
	B	75	40	53.3	24	24	53	
	C	75	40	53.3	24	24	53	
	D	75	42	47.7	24	24	56	
Basmati 370	A	88	48	54.5	24	23	52.2	54.48
	B	75	44	58.6	24	24	58.6	
	C	75	40	53.3	24	24	53	
	D	75	42	47.7	24	23	53.6	
Tarori Basmati	A	88	30	34.0	24	24	34.0	30.84
	B	75	20	26.6	24	24	26.6	
	C	75	25	33.3	24	23	31.9	
	D	75	24	32.0	24	23	30.6	

4.9 GUS expression in T_1 and primary transformants

Different organs of T_1 plants *viz.* roots (Fig. 11a & 11b), seedling leaf (11e & 11f) and seeds with an embryo (Fig. 11c & 11d) were tested for GUS expression while only

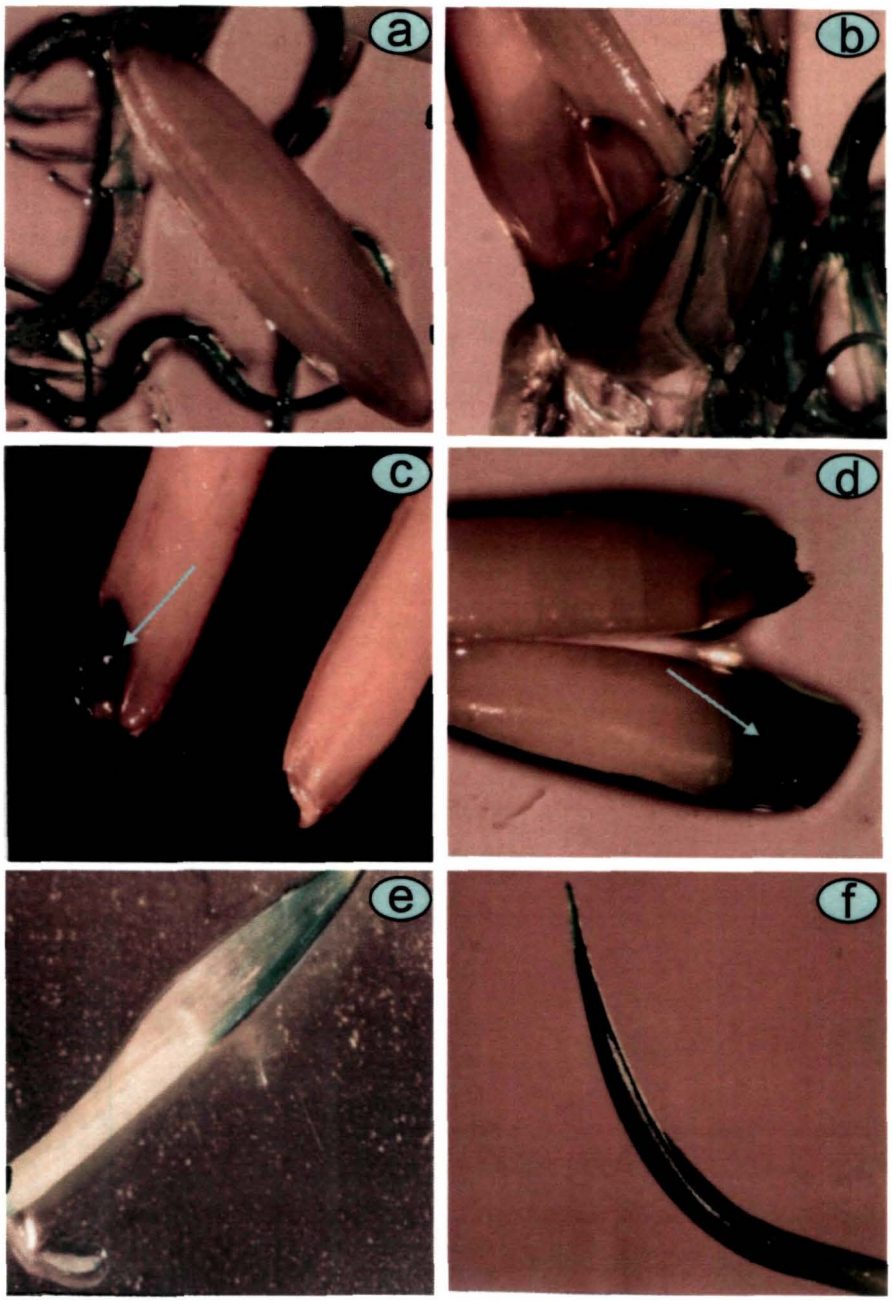

Fig. 11. GUS staining of T_1 seeds and seedling parts obtained from selfing of T_0 transformants. Seedling roots (a) & (b), seed embryo (arrow indicates intense GUS stain) with counterpart control seed embryo (c) & (d), seedling leaf (e) and seedling leaf tip (f) of transgenics.

matured leaves (Fig. 4j) were analysed in case of primary transformants. Blue color precipitation was prominent at the cut ends of leaf pieces, roots and seed embryos.

4.10 Analyses of the T_0 transgenic plants for *cry1Ac* gene integration

Integration and copy number determination was done by Southern blot analyses. DNA was extracted from leaf tissue of 10 T_0 putative transgenic plants of Pusa Basmati 1, Basmati 370 and Tarori Basmati. Internal coding sequence of *cry1Ac* gene of 1.1 kb long insert was used as a probe. Genomic DNA samples were digested with *Hin*dIII which linearises the plasmid, hence was ideal restriction enzyme for determining copy number in plants. *Hin*dIII selects one restriction site from the integrated plasmid T-DNA in the plant genome and one from plant genome nearest to the site of T-DNA integration. Thus the junction fragments with a portion of the T-DNA and a portion of the plant DNA will hybridise to the *cry1Ac*.

Southern analyses of putative transgenic plants of the three varieties used in the present investigation is presented in Table 14.

4.10.1 Southern analyses of T_0 Pusa Basmati 1 putative transgenic plants

Genomic DNA analysed from ten T_0 Pusa Basmati 1 plants showed presence of mostly one copy. However, two copies were recorded in one of the plants. In contrast two of the putative transgenics showed absence of *cry1Ac* gene fragment (Fig. 12, lane 7&11, Table 14). The putative T_0 plants of Pusa Basmati 1 showed presence of junction fragment ranging from 3 kb-8 kb. Plant no. 6a (lane 2), 12a (lane 3), 13a (lane 4), 15a

(lane 5), showed strong single hybridisation bands while 20a (lane 6) showed single weak junction fragment of similar molecular weight of approximately of 8 kb (Fig. 12). Plant numbers 4b (lane 8) and 23b (lane 10) showed similar single gene insertion of approximately 5.8 kb. Plant number 15b (lane 9) on hybridisation produced two junction fragments of approximately 6.2 kb and 3 kb (Fig. 12, Table 14). Plant numbers 3b (lane 7) and 24b (lane 11) did not produced any hybridization signal (Fig. 12).

4.10.2 Southern analyses of T_0 Basmati 370 putative transgenic plants

Genomic DNA analysed from ten T_0 Basmati 370 plants also depicted presence of mostly single copy insertions. Five plants *viz.* 19a (lane 15), 12b (lane 19), 17b (lane 20) and 18b (lane 21) showed strong single band while 17a (lane 14) showed very weak signal of single hybridisation band (Fig. 12, Table 14). Plant no. 19a (lane 15) showed a band of approximately 8 kb, plant no. 12b (lane 19) and 18b (lane 21) showed a single band of approximate size of 5.8 kb while plant 17b showed a single band of approximate length of 4 kb. Plant 2b (lane 16) and 9b (lane 17) both exhibited two bands of similar size of 6.2 kb and 3 kb. Plant 5a (lane 13) exhibited three bands of 8 kb, 6.2 kb and 3 kb. Plant 3a (lane 12) exhibited two bands of 10 kb and 8 kb size approximately. Plant 10b (lane 18) showed intense signal near the well (Fig. 12).

4.10.3 Southern analyses of T_0 Tarori Basmati putative transgenic plants

Southern blot of ten T_0 Tarori Basmati putative transgenic plants revealed that three plants among them *viz.* 1a (lane 2), 7a (lane 3) and 11a (lane 4) showed presence of

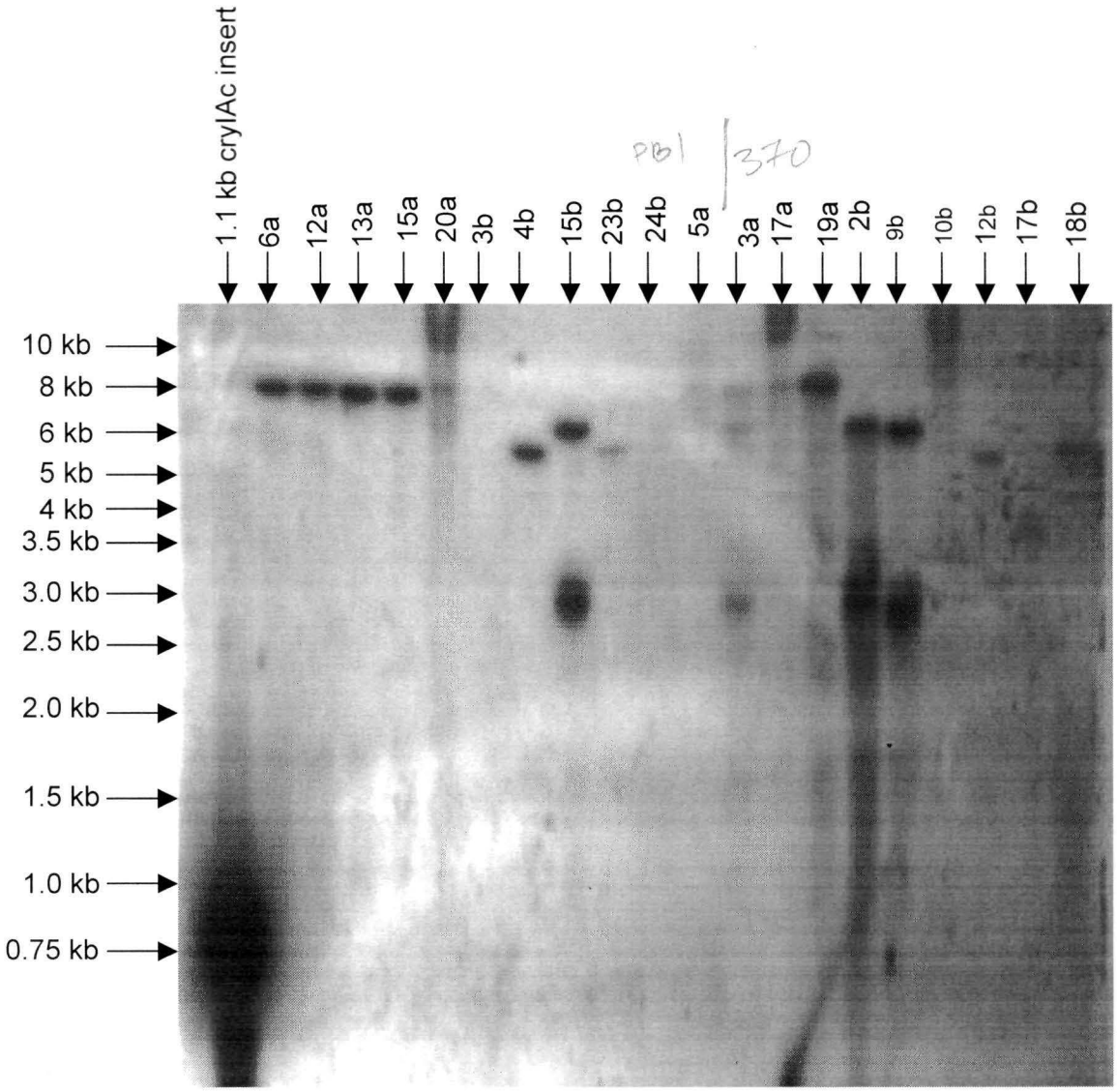

Fig. 12. Southern blot of two Basmati varieties Pusa Basmati 1 and Basmati 370 regenerated from hygromycin resistant calli. DNA (10 µg) was digested with *Hin*dIII and separated on 0.8% agarose gel and hybridised to [α-32P]dCTP-labelled *cryIAc* 1.1 kb coding sequence. Twenty independent T_0 plants are shown in lanes 2-21 of which Pusa Basmati 1 lines are 6a, 12a,13a, 15a, 20a, 3b, 4b, 15b, 23b and 24b in lane 2-11 respectively and Basmati 370 lines are 3a, 5a, 17a, 19a, 2b, 9b, 10b, 12b, 17b & 18b in lane 12-21 respectively. Lane 1 depicting *cryIAc* gene insert of 1.1 kb. Positions and sizes of 1 kb gene ruller (left) are marked.

Table 14: Southern analyses of T_0 putative transgenic plants for presence of *cry1Ac* gene

Varieties	Well no.	Plant no.	No. of *cry* gene inserts	Type of bands according to molecular weight (kb)	Western analyses
Pusa Basmati 1	1	*cry1Ac* gene probe	1	1.1	
	2	6a	1	8	
	3	12a	1	8	+
	4	13a	1	8	
	5	15a	1	8	
	6	20a	1	8	+
	7	3b	-	-	
	8	4b	1	5.8	+
	9	15b	2	6.2 & 3	+
	10	23b	1	5.8	
	11	24b	-	-	
Basmati 370	12	3a	2	10 & 8	
	13	5a	3	8, 6.2 & 3	
	14	17a	1	8	
	15	19a	1	8	+
	16	2b	2	6.2 & 3	
	17	9b	2	6.2 & 3	+
	18	10b	Smear	-	
	19	12b	1	5.8	+
	20	17b	1	4	
	21	18b	1	5.8	+
Tarori Basmati	1	*cry1Ac* gene probe	1	1.1	
	2	1a	1	6.2	
	3	7a	1	5.8	+
	4	11a	1	5.8	+
	5	12a	2	6.2 & 3	+
	6	24a	-	-	
	7	1b	2	6.2 & 3	
	8	7b	-	-	
	9	11b	2	6.2 & 3	
	10	13b	-	-	
	11	21b	2	6.2 & 3	

strong signal of single band (Fig. 13). Plant no. 1a showed a band size of 6.2 kb while 7a and 11a possessed a band of smaller size of approximately 5.8 kb (Fig. 13). Plant no. 12a (lane 5), 1b (lane 7), 11b (lane 9) and 21b (lane 11) exhibited two bands of approximately 6.2 kb and 3 kb size. Plant no. 24a (lane 6), 7b (lane 8) and 13b (lane 10) were poorly digested and showed intense signal near the well accompanied with little smearing (Fig. 13, Table 14).

4.11 PCR analyses of T_1 hygromycin resistant plants for integration of *cry1Ac*, *hpt* and *gus* genes

All the T_1 hygromycin resistant plants of Pusa Basmati 1, Basmati 370 and Tarori Basmati, which were positive for *cry1Ac* gene in T_0 generation, were analysed for the presence of *cry1Ac* and *hpt* gene by PCR. All these plants irrespective of varieties showed presence of discrete band of *cry1Ac* (1.1 kb), *hpt* (0.82 kb) and *gus* (1.3 kb) gene (Fig. 14).

4.12 Analyses of the T_1 transgenic plants for *cry1Ac* gene integration and segregation

Different T_1 individuals (obtained by selfing of T_0 plants) of Pusa Basmati 1 (plant no. 12a-1, 20a-1, 15b-1), Basmati 370 (plant no. 12b-1, 12b-2, 12b-3, 12b-4 and 17b-4) and Tarori Basmati (12a-1 to 12a-6) that were resistant to hygromycin as well as Southern positive for *cry1Ac* gene in their T_0 generation were checked for presence of transgene *cry1Ac*. The results are presented in Fig. 15 and Table 15.

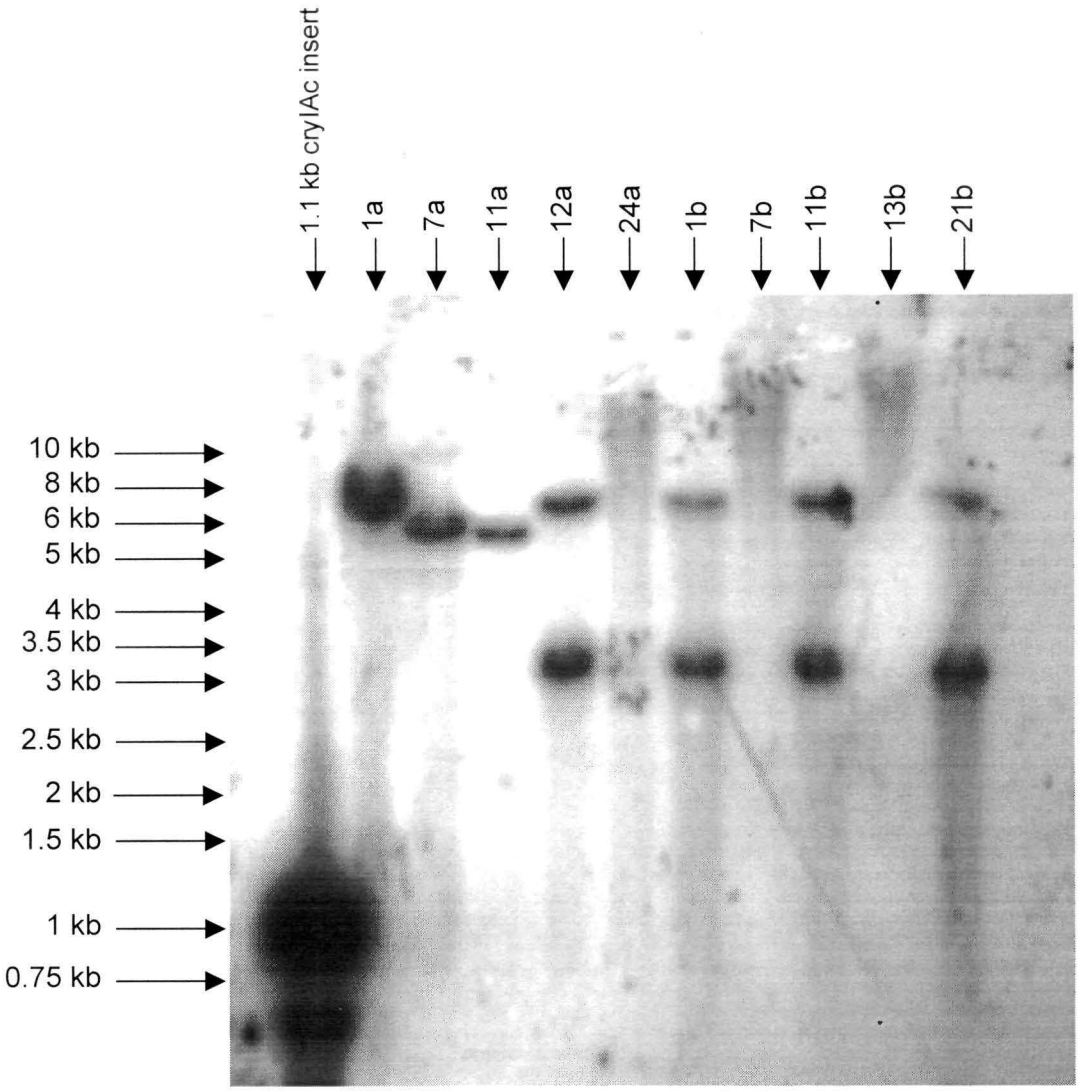

Fig. 13. Southern blot of primary transformants of Basmati var. Tarori Basmati regenerated from hygromycin resistant calli. DNA (10 µg) was digested with *Hin*dIII and separated on 0.8% agarose gel and hybridised to [α-32P]dCTP-labelled *cry1Ac* 1.1 kb coding sequence. Ten independent T_0 transformants of Tarori Basmati are 1a, 7a, 11a, 12a, 24a, 1b, 7b, 11b, 13b and 21b are shown in lanes 2-11 respectively. Lane 1 depicting *cryIAc* gene insert of 1.1 kb insert. Positions and sizes of 1 kb ruller (left) are marked

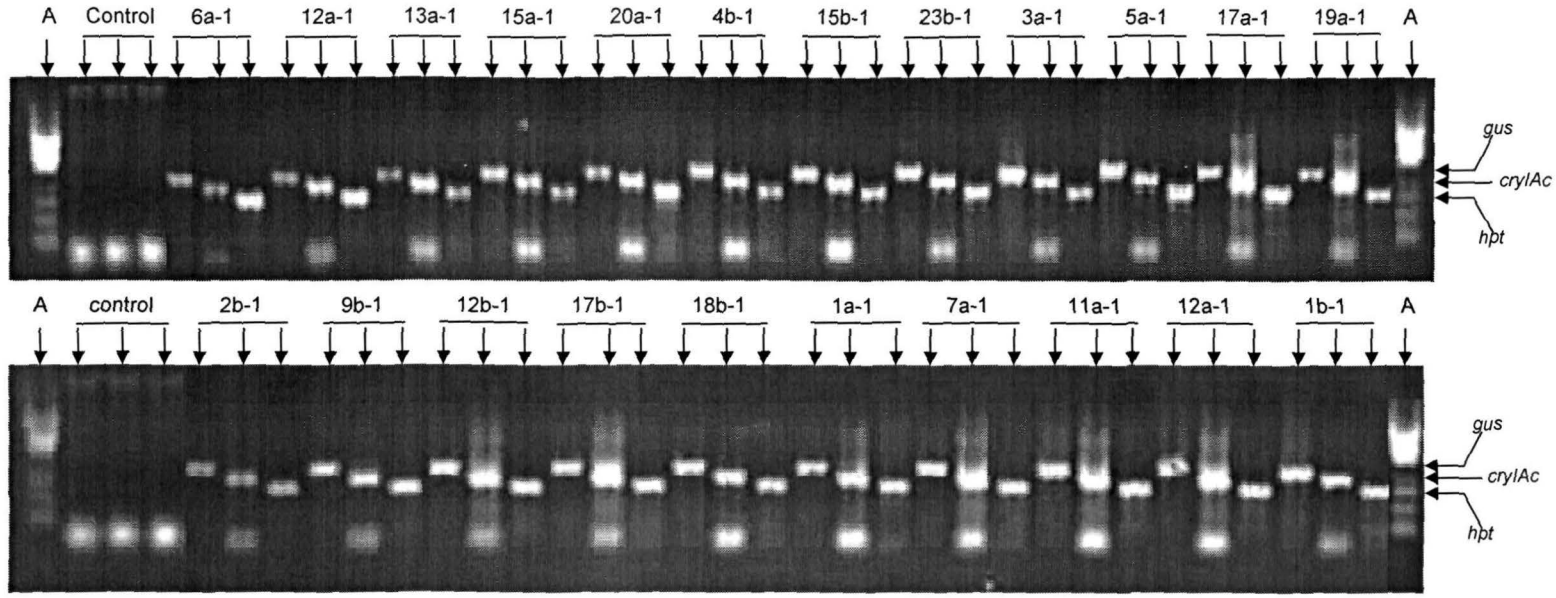

Fig. 14: PCR analyses of some of the T_1 hygromycin resistant plants of cv. Pusa Basmati 1, Basmati 370 and Tarori Basmati for *gus* gene (1.3 kb), *cryIAc* gene (1.1 kb) and *hpt* gene (0.82 kb). 'A' indicates 1 kb mass ruler.

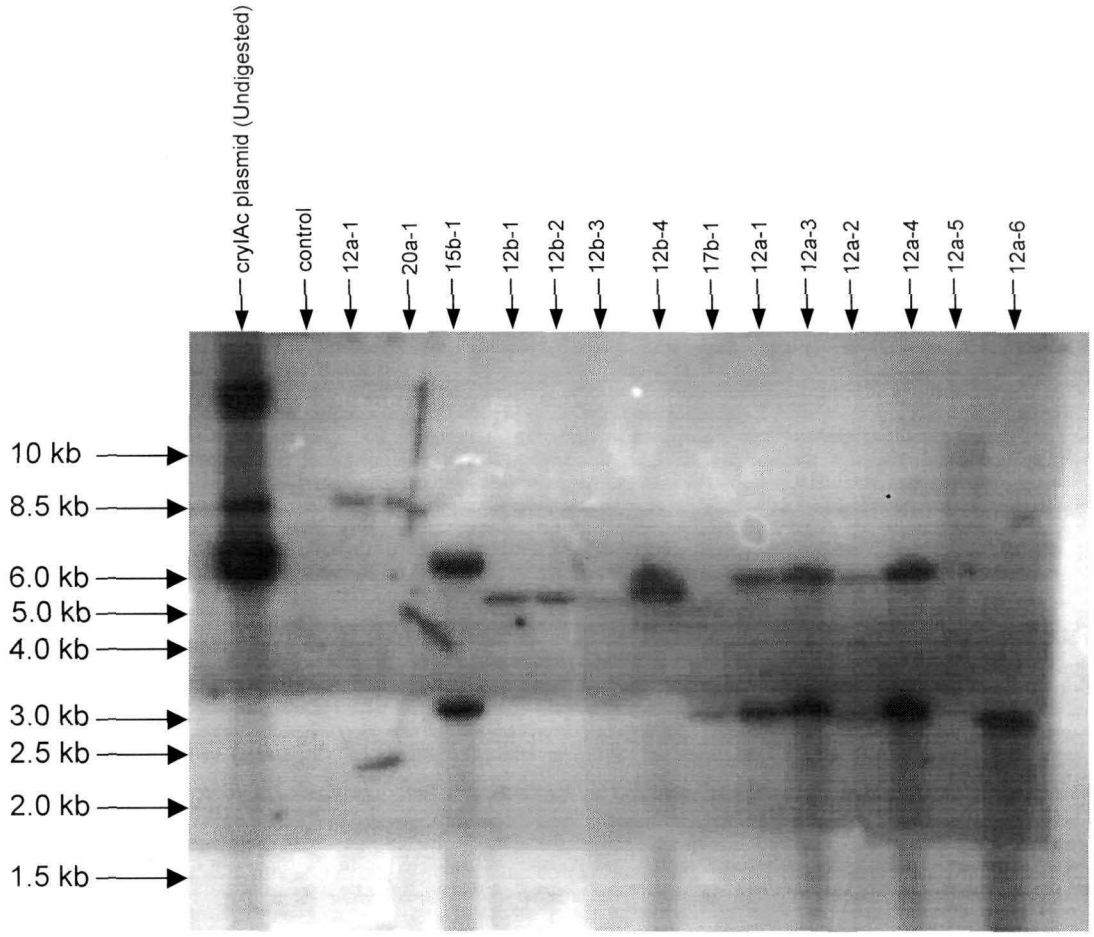

Fig. 15. Southern blot of T_1 generation of three Basmati rice varieties *viz*. Pusa Basmati 1, Basmati 370 and Tarori Basmati obtained from T_0 seeds. DNA (10 µg) was digested with *Hin*dIII and separated on 0.8% agarose gel and hybridised to [α-32P]dCTP-labelled *cry1Ac* 1.1 kb coding sequence. Lane 1 depicts undigested pCAMBAc plasmid and lane 2 as control (10 µg of DNA from Pusa Basmati 1, Basmati 370 and Tarori Basmati). Lane 3,4 and 5 are T_1 progenies of Pusa Basmati 1, lane 6-10 are T_1 progenies of Basmati 370 and lane 11-16 are T_1 progenies of Tarori Basmati. Positions and sizes of 1 kb ruller (left) are marked.

Table 15: Southern analyses for T_1 transgenic plants of three Basmati rice varieties for integration and segregation of *cry1Ac* gene

Varieties	Well no.	Plant No.	No. of *cry1Ac* gene inserts	Type of bands
Undigested plasmid	1	Undigested plasmid	3	6.2, 8.5 & 14.5
CONTROL	2	-	0	-
Pusa Basmati 1	3	12a-1	1	8
	4	20a-1	2	8 & 2.2
	5	15b-1	2	6.2 & 3
Basmati 370	6	12b-1	1	5.8
	7	12b-2	1	5.8
	8	12b-3	1	5.8
	9	12b-4	1	5.8
	10	17b-1	1	3.0
Tarori Basmati	11	12a-1	2	6.2 & 3
	12	12a-2	2	6.2 & 3
	13	12a-3	2	6.2 & 3
	14	12a-4	2	6.2 & 3
	15	12a-5	1	3
	16	12a-6	1	3

4.12.1 Southern analyses of T_1 plants of Pusa Basmati 1

Southern analyses revealed that plant no. 12a-1 in lane 3 possessed a band of approximately 8 kb (Fig. 15) exactly like its parent (Fig. 12, lane 3) while 20a-1 possessed two bands (lane 4), one of which is very similar to its parent (Fig. 12, lane 6) of approximate size of 8 kb and the another one approximately of 2.2 kb size. The lane 5 (Fig. 15) showed presence of two bands in T_1 plant 15b-1 (progeny of T_0 plant no. 15b), which are similar to its parent with respect to lower band of 3 kb as well as its upper band of (6.2 kb) (Fig. 12, lane 9) (Table. 11).

4.12.2 Southern analyses of T_1 plants of Basmati 370

Southern blot of four T_1 plants no. 12b-1 to 12b-4 revealed that the one band (5.8 kb) present in their parent (Fig. 12, lane 19) was intact in their progenies (Fig. 15, lane 6,

7, 8 and 9), while the plant no. 17b-1 (Fig. 15, lane 10) showed presence of low molecular weight 3 kb band instead of 4 kb as present in its parent (Fig. 12, lane 20, Table 15).

4.12.3 Southern analyses of T_1 plants of Tarori Basmati

Southern analyses of six T_1 plants no. 12a-1 to 12a-6 (progenies of T_0 plant no. 12a, Fig. 13, lane 5) revealed that the two bands of 6.2 kb and 3 kb that were present in the parent, and they were intact in their four progenies (lane 11-14) while two of them showed only one band of 3 kb (lane 15 and 16).

4.13 Western analyses for expression of *cry1Ac* gene

A total of eleven blot T_0 Southern positive plants for *cry1Ac* were selected for immunoblot assay from all the three varieties (Table 10). Four plants of Pusa Basmati 1 namely 12a (lane 3), 20a (lane 6), 4b (lane 8) and 15b (lane 9), four plants of Basmati 370 namely 19a (lane 15), 9b (lane 17), 12b (lane 19) and 18b (lane 21) while only three plants of Tarori Basmati *viz.* 7a (lane 3), 11a (lane 4) and 12a (lane 5) were selected. All the eleven plants were found to be positive of which two of the Basmati 370 T_0 plants *viz.* 19a and 9b and two of the Tarori Basmati plants namely 11a and 12a showed high levels of cry1Ac protein of 67 KDa in leaf samples (Fig. 16).

In the present investigation, T_2 plants, which were tested hygromycin resistant, some of them tested for presence of Bt toxin were found to be positive, confirmed by the presence of Test line, an immunoreactive product, in DesiGen Xpresstrips (Fig. 17).

4.14 Inheritance of the *cry1Ac* gene in T_1 and its T_2 progeny

Segregation of the transgene in T_1 (obtained by selfing of T_0 plants) and T_2

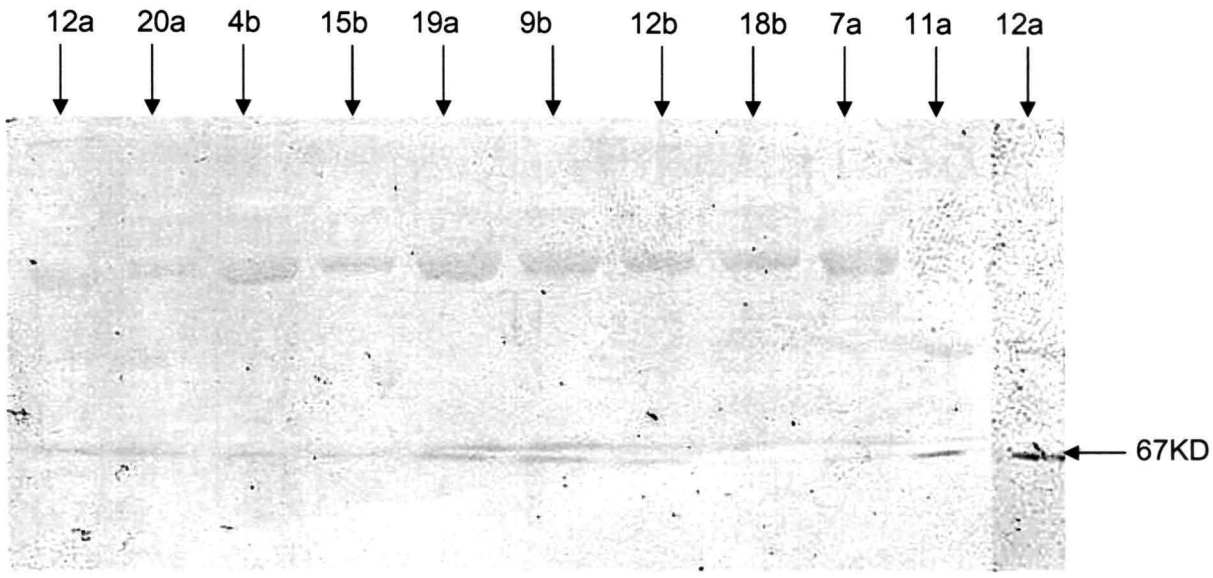

Fig. 16. Western blot of T_0 transformants of Pusa Basmati 1, Basmati 370 and Tarori Basmati transformants. Ten microgram aliquots of total protein were loaded in each lane and separated by SDS-PAGE in 8% polyacrylamide gel. Protein extracted from the leaves of putatively transformed 12a, 20a, 4b and 15b of Pusa Basmati 1 plants was loaded in the lanes 1,2,3 and 4 respectively, similarly 19a, 9b, 12b and 18b of Basmati 370 in lanes 5,6,7 and 8 respectively and 7a, 11a & 12a in lanes 9, 10 and 11 of Tarori Basmati plants respectively.

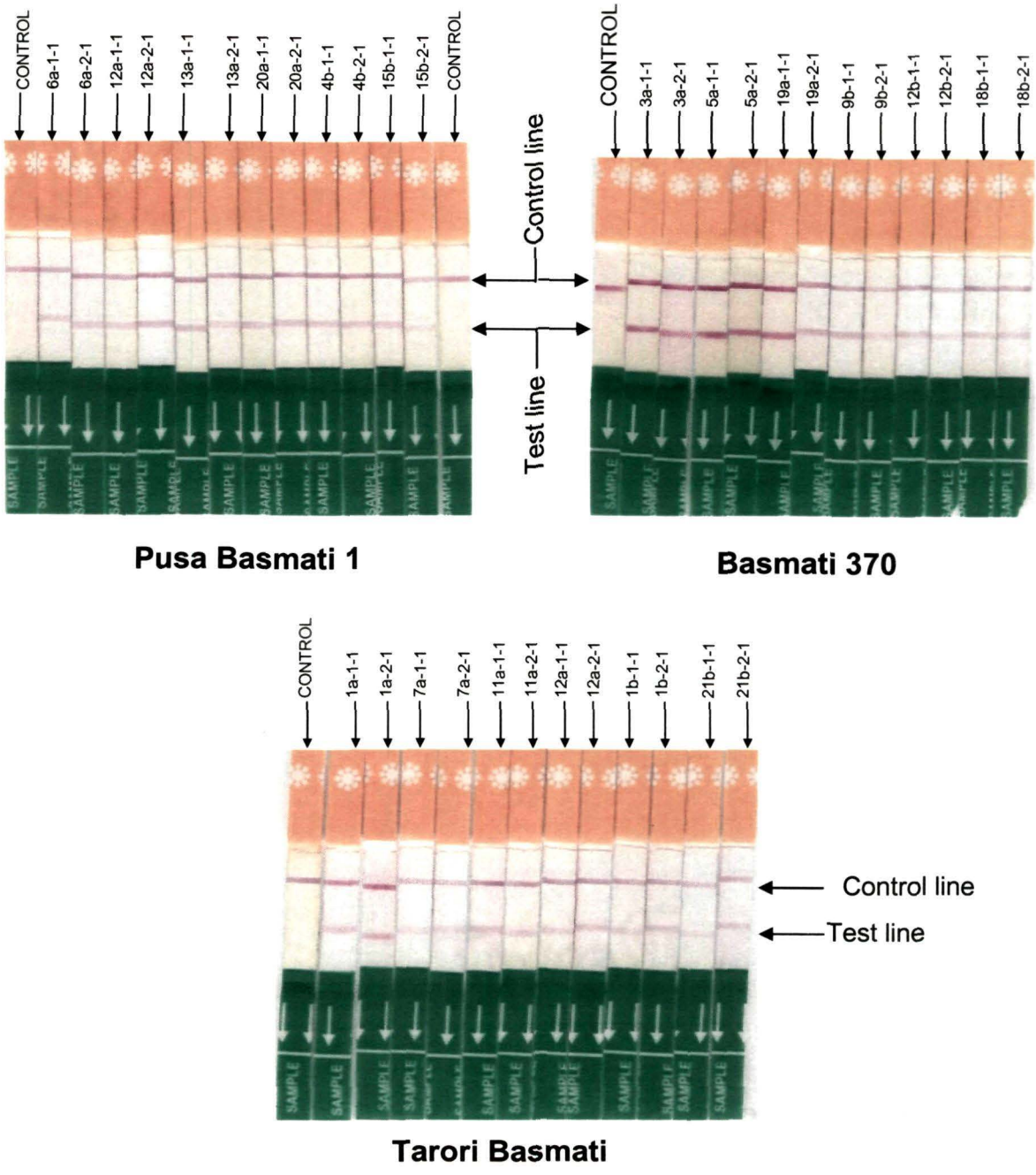

Fig. 17. Xpresstrips analysis for presence of CryIAc toxin in T_2 plants of Pusa Basmati 1, Basmati 370 and Tarori Basmati.

(obtained by selfing of T_1 plants) was studied to know the inheritance of the transgene [*cry1Ac* gene through Southern blot and hygromycin resistance through their ability to germinate and survive on hygromycin containing medium (Fig. 5f, 6f & 7f)] and to determine the transgene copy number(s) (number of integration sites) with respect to loci. Seeds of primary transformants (T_0 plants) obtained by selfing were germinated and scored for hygromycin resistance (Table 16) to know the segregation pattern. Similar study was also carried for T_2 plants, (obtained from selfing of T_1 plants) and segregation pattern was also recorded (Table. 17).

Table 16: Segregation pattern of the hygromycin resistance in the T_1 plants (obtained from selfed T_0 lines)

Variety	Parents plant no. (T_0)	No. of seeds inoculated (T_1)	No. of seeds germinated (T_1)	No. of seeds died (T_1)	Chi-square value	P-value	T-DNA insert (T_0)
Pusa Basmati 1	6a	36	26	10	0.148	>0.7	1
	12a	35	24	11	0.771	>0.3	1
	13a	30	22	8	0.044	>0.7	1
	15a	42	27	15	2.571	>0.1	1
	20a	31	26	5	1.301	>0.2	1
	4b	25	19	6	0.013	>0.9	1
	15b	28	22	6	0.19	>0.5	2
	23b	26	20	6	0.051	>0.7	1
Basmati 370	3a	30	24	6	0.4	>0.5	2
	5a	26	20	6	0.051	>0.7	3
	17a	27	21	6	0.111	>0.7	1
	19a	26	20	6	0.051	>0.7	1
	2b	32	27	5	1.5	>0.2	2
	9b	21	16	5	0.016	>0.7	2
	12b	22	17	5	0.061	>0.7	1
	17b	22	18	4	0.545	>0.3	1
	18b	34	30	4	3.176	>0.05	1
	10b	35	24	11	0.771	>0.3	Smear
Tarori Basmati	1a	40	35	5	3.333	>0.05	1
	7a	41	31	10	0.008	>0.09	1
	11a	46	36	10	0.261	>0.5	1
	12a	47	42	5	5.17	>0.01	2
	1b	36	26	10	0.148	>0.7	2
	11b	45	34	11	0.007	>0.9	2
	21b	28	18	10	1.174	>0.1	2

Chi-square analyses was performed for all the three varieties (Table. 16) for 3:1 Mendelian segregation ratio in T_1 and T_2 putative transgenics except for plant no. 15b-1-1 (Table 17) of Pusa Basmati 1, which showed 4:0 Mendelian segregation ratio.

Table 17: Segregation pattern for hygromycin resistance in the T_2 seedlings obtained from selfing of T_1 plants

Varieties	Plant no.	No of seeds inoculated	No. of seeds germinated	No. of seeds died	Chi square value	P- value
Pusa Basmati1	12a-1-1	30	23	7	0.044 (3:1)	>0.7
	12a-2-1	27	20	7	0.012 (3:1)	>0.7
	20a-1-1	33	27	6	0.818 (3:1)	>0.7
	20a-2-1	27	19	8	0.309 (3:1)	>0.9
	15b-1-1	34	34	0	0 (4:0)	>0.99
	15b-2-1	17	15	2	1.588 (3:1)	>0.3
	15b-3-1	33	29	4	2.919 (3:1)	>0.7
	4b-1-1	48	39	9	1 (3:1)	>0.001
	4b-2-1	23	20	3	1.754 (3:1)	>0.5
Basmati3 70	12b-1-1	32	24	8	0 (3:1)	>0.5
	12b-2-1	28	22	6	0.19 (3:1)	>0.5
	12b-3-1	36	26	10	0.148 (3:1)	>0.7
	12b-4-1	35	24	11	0.771 (3:1)	>0.3
	12b-5-1	31	26	5	1.301 (3:1)	>0.2
	17b-1-1	25	19	6	0.013 (3:1)	>0.9
	17b-2-1	28	22	6	0.19 (3:1)	>0.5
	19a-1-1	26	20	6	0.051 (3:1)	>0.7
	19a-2-1	28	21	7	0 (3:1)	>0.95
	9b-1-1	32	24	8	0 (3:1)	>0.95
	9b-2-1	30	24	6	0.4 (3:1)	>0.5
	9b-3-1	26	20	6	0.051 (3:1)	>0.7
	5a-1-1	27	21	6	0.111 (3:1)	>0.7
	5a-2-1	26	20	6	0.051 (3:1)	>0.7
Tarori Basmati	12a-1-1	21	16	5	0.016 (3:1)	>0.7
	12a-2-1	22	17	5	0.061 (3:1)	>0.7
	12a-3-1	22	18	4	0.545 (3:1)	>0.3
	12a-4-1	32	24	8	0 (3:1)	>0.95
	12a-5-1	34	24	10	0.353 (3:1)	>0.5
	12a-6-1	36	24	12	1.333 (3:1)	>0.3
	1a-1-1	27	19	8	0.309 (3:1)	>0.5
	1a-2-1	28	19	9	0.762 (3:1)	>0.3
	7a-1-1	35	22	13	2.752 (3:1)	>0.1
	7a-2-1	36	21	15	5.333 (3:1)	>0.01
	11a-1-1	34	34	4	3.176 (3:1)	>0.05
	11a-2-1	30	25	5	1.111 (3:1)	>0.2

4.15 Morphological characterization and comparison of putative transgenics with their controls

Putative transgenics (T_0, T_1 and T_2) of all the three Basmati varieties displayed more or less similar morphological characters as their counterpart control plants with a few exceptions. Pusa Basmati 1 and Tarori Basmati differed significantly from their control during their T_1 generation with respect to plant height only. No difference was observed in any other morphological characters. Basmati 370 putative transgenics did not showed any significant variation for any of the morphological characters studied.

Table 18: Assessment for agrobotanic characters of three indica Basmati rice transgenic varieties

Varieties	Generati-ons	Plant height (cm)	Flag leaf length (cm)	Tiller numbers	Panicle length (cm)	Spikelets/ Panicle	Fertile spikelets	100 seed weight (gm)
		Mean±SE	mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Pusa Basmati 1	Control	101.2±2.41	28.1±0.5	16±0.7	22.8±0.3	117.4±3.6	81.2±2.8	2.40±0.03
	T_0	107.4±2.85	28.6±1.0	16.6±0.4	23.6±0.4	108.2±4.7	78.6±1.8	2.38±0.04
	Control	115.8±1.68	28±0.3	16.4±0.5	22.2±0.3	113.8±4.5	77.8±1.3	2.34±0.02
	T_1	*120.8±1.2	28.4±0.5	16.4±0.5	23±0.31	118.8±4.2	74.8±0.8	2.33±0.02
	Control	121±2	28±0.54	16.8±0.3	22.4±0.2	117±2.56	73.4±1.0	2.34±0.01
	T_2	121.6±1.20	29.4±0.8	16±0.7	22.8±0.4	116.8±2.7	74.2±1.9	2.34±0.01
Basmati 370	Control	100.8±3.21	28.8±0.58	16.6±0.9	22.2±0.4	102±3.68	73.8±1.3	2.52±0.03
	T_0	106.4± 3.9	28.2±0.58	16.4±0.7	22.8±0.4	102.8±3.4	77.6±3.1	2.45±0.03
	Control	117.6±2.31	30.2±0.7	15.6±0.7	22±0.31	98.4±4.61	72.8±1.4	2.63±0.05
	T_1	120± 1.41	29±0.54	16.2±0.6	22.8±0.2	99.8±4.89	71.4±1.7	2.61±0.03
	Control	116.4±0.92	29.6±0.5	17.2±1.0	22±0.44	101.6±1.4	75.6±3.9	2.69±0.05
	T_2	116.6±0.74	30.4±0.8	16.4±0.7	22.6±0.2	97.8±5.37	77.8±1.5	2.58±0.02
Tarori Basmati	Control	124.6±2.03	30.8±0.7	15±0.44	25±0.54	105±4	75.6±2.7	2.34±0.03
	T_0	124.6±3.96	32.2±0.8	14.4±0.5	24.6±0.2	107±2.77	76.8±1.1	2.31±0.04
	Control	134.2±1.42	31±0.7	14.8±0.4	25.2±0.4	103.6±2.8	81±1.8	2.34±0.02
	T_1	135.2±1.59	*33.2±0.6	14.6±0.4	26±0.31	100.8±3.3	76.6±2.7	2.28±0.01
	Control	130.6±2.22	30.4±0.9	15.4±0.5	24±0.31	101.4±7.3	76.6±1.0	2.34±0.03
	T_2	135.8±1.68	31.8±0.58	15.8±0.6	24.6±0.2	106.8±2.4	80.8±3.2	2.31±0.02

* Significantly differ at 5% level by student t-test using two-sample assuming equal variance

4.16 Bioassay and ELISA for resistance to YSB neonate larvae

4.16.1 Bioassay and ELISA of control plants (Pusa Basmati 1, Basmati 370 and Tarori Basmati)

The bioassay of cut stems of control plants indicated larvae mortality of 0-5% in Pusa Basmati 1, 5% in Basmati 370 and also 5% in case of Tarori Basmati considering individual replication. This mortality rate may be due to improper handling. ELISA was carried out for controls of each variety of Basmati and their titre value was adjusted to zero. All the survived larvae progressed to second stage instar and were found feeding at the nodal regions, when the cut stems were split apart.

4.16.2 Bioassay and ELISA of Pusa Basmati 1 putative transformants

Cut stem bioassay of T_1 generation of Pusa Basmati 1 revealed that number of larvae recovered dead from split cut stems was significantly higher than control (0-5%) plants (Table 19). Considering the larvae recovered dead from split stem (Table 19), the mortality rate ranged from a minimum of 88.5% in case of 13a-1 to the maximum of 100% in case of 15b-1. The ELISA titre value of T_1 transformed plants of Pusa Basmati 1 varied from 0.15% (plant no. 20a-1) to 0.24% (plant no. 15b-1) of the total soluble leaf protein and showed resistance to yellow stem borer with mortality ranging from 88.5% (plant no.13a-1) to 100% (15b-1) (Table 19).

Table 19: Cut stem insect bioassay of T_1 transgenic plants of three indica Basmati rice varieties

Varieties	Plant no.	No. of larvae per cut stem assay	No. of larvae found dead in split stem		Mortality in split stem (C)		ELISA titre value (% of soluble protein)
			Dead (A)	Alive (B)	(%)	Mean	
Pusa Basmati 1	Control	20	1	19	5	2.5	0.00
		20	0	20	0		
	6a-1	20	16	2	88.8	91.6	0.20
		20	17	1	94.4		
	12a-1	20	15	2	88.2	91.1	0.18
		20	16	1	94.1		
	13a-1	20	18	1	94.7	88.5	0.16
		20	14	3	82.3		
	20a-1	20	16	2	88.8	91.7	0.15
		20	18	1	94.7		
	4b-1	20	17	0	100	94.1	0.19
		20	15	2	88.2		
	15b-1	20	18	0	100	100	0.24
		20	16	0	100		
Basmati 370	Control	20	1	19	5	5.0	0.00
		20	1	19	5		
	3a-1	20	14	2	87.5	90.9	0.16
		20	17	1	94.4		
	5a-1	20	16	1	94.1	97.0	0.18
		20	17	0	100		
	19a-1	20	18	0	100	97.2	0.21
		20	17	1	94.4		
	9b-1	20	17	1	94.4	97.2	0.23
		20	18	0	100		
	12b-1	20	17	0	100	97.0	0.19
		20	16	1	94.1		
	18b-1	20	18	0	100	94.1	0.15
		20	15	2	88.2		
Tarori Basmati	Control	20	1	19	5	5.0	0.00
		20	1	19	5		
	1a-1	20	16	2	88.8	91.7	0.18
		20	18	1	94.7		
	7a-1	20	16	0	100	93.7	0.16
		20	14	2	87.5		
	11a-1	20	16	1	94.1	94.2	0.20
		20	17	1	94.4		
	12a-1	20	18	0	100	94.4	0.21
		20	16	2	88.8		
	1b-1	20	17	2	89.4	89.1	0.18
		20	16	2	88.8		
	21b-1	20	17	2	89.4	92.0	0.18
		20	18	1	94.7		

The larvae recovered dead from outside the cut stem after 4 days of infestation, were not considered for larvae mortality but as an escape.

4.16.3 Bioassay and ELISA of Basmati 370 putative transformants

The bioassay of T_1 generation of Basmati 370 at about booting stage revealed that number of larvae recovered dead from split cut stem was significantly higher (85-95%) than control (5%) plants (Table 19). Considering only the larvae recovered dead from split stem (Table 19 larvae mortality rate ranged from a minimum value of 90.95% in case of 3a-1 to the maximum of 94.1% in case of 18b-1. ELISA titre value varied from 0.15% (plant no. 18b-1) to 0.23% (plant no. 9b-1) of the total soluble protein and showed resistance to yellow stem borer with mortality rate ranging from 90.9% (3a-1) to 97.2% (19a-1 and 9b-1) (Table 19).

4.16.4 Bioassay and ELISA of Tarori Basmati putative transformants

The bioassay of T_1 generation of Tarori Basmati at about booting stage revealed that number of larvae recovered dead from split cut stem was significantly higher than control (5%) plants (Table 19). Considering only the larvae recovered dead from split stem (Table 19) larvae mortality rate ranged from a minimum value of 87.5% in case of 7a-1 to the maximum of 100% in case of 12a-1 and 7a-1. ELISA titre value varied from 0.16% (plant no. 7a-1) to 0.21% (plant no. 12a-1) of the total soluble protein and showed resistance to yellow stem borer with mortality rate ranging from 89.1% (1b-1) to 94.4% (12a-1) (Table 19).

Chapter - 5

DISCUSSION

DISCUSSION

5.1 Callus induction and maintenance of embryogenicity

Induction of embryogenic calli and maintenance of embryogenecity are vital pre-requisites for *Agrobacterium tumefaciens*-mediated transformation through callus. In the present investigation, a reproducible embryogenic callus induction as well as plant regeneration system was established. Different explants sources *viz.* mature seeds, immature embryos, leaf bases and stem bases had been used by various researchers for induction of callus. It has been observed that source of explants has significant bearing on callus induction and embryogenicity. Immature embryos and mature seed-derived embryos have been found to be the most suitable explants. However, mature embryos have also been used frequently as a source of explants due to their availability round the year. In the present investigation, embryo derived scutellum calli were used for *A. tumefaciens*-mediated transformation experiments.

Embryogenic calli were induced from mature seeds on CIM, MB and MB1 medium. Chemical composition of callus induction media were found to influence the frequency of embryogenic calli induction in all the three varieties. Among the three media tested for calli induction, CIM was found to be the best for all the three varieties. Increase in callus induction percentage in CIM over MB and MB1 is presumably due to presence of proline and casamino acid. Inclusion of casamino acid in callus induction medium has been reported to be beneficial in induction of embryogenic calli in japonica (Hiei *et al.*, 1994; Toki, 1997) as well as in indica rice varieties (Zhang *et al.*, 1996; Saharan *et al.*, 2004; Mohanty *et al.*, 1999). Thus, effectiveness of CIM medium in

callus induction is in agreements with the earlier reports for Tarori Basmati, IR-64 (Khanna and Raina, 1999) and for Pusa Basmati 1 (Khanna and Raina, 1999; Sridevi *et al.*, 2003). The inclusion of L-proline in the callus induction medium has also been reported to be effective in initiation and maintenance of embryogenicity of the calli (Datta *et al.*, 1992; Kishor *et al.*, 1999; Saharan *et al.*, 2004).

5.2 Subculture of calli for *Agrobacterium*-mediated transformation

Subculture of calli to fresh medium is known to revive potential of the callus for accelerated cell division and helps in maintenance of callus embryogenicity. Detaching calli after 21 days of induction from the scutellum and cutting into pieces of 4-5 mm, which created wounding, and subsequent subculturing to fresh medium enhanced callus proliferation. The wounded/exposed portions as well as newly proliferated calli were found to be more susceptible to *Agrobacterium tumefaciens* infection. Such wounding followed by preculture of scutellar calli for few days has been reported to enhance the rate of cell division (An, 1985) and thereby making the tissue more competent for *Agrobacterium tumefaciens* infection (Vijayachandra *et al.*, 1995). Although many dicotyledonous plant cells had been transformed by *Agrobacterium*, only cells that have been wounded are reported to be susceptible (Stachel *et al.*, 1985). Pre-treatment of tissues, for example, by wounding or enzymatic digestion of cell walls has been reported to be essential for enhancing the rate of transformation in many cases (Chan *et al.*, 1993; Mooney *et al.*, 1991 and Raineri *et al.*, 1990). Key factors like presence of *vir* gene inducing compounds, monocot promoters and extra-wounding also play vital role in increasing the frequency of transgenic rice production (Park *et al.*, 1996). Hiei *et al.* (1994) reported that calli were excised from the seeds 5 days after plating on callus

induction medium and after 3 weeks of subculture it was again subcultured on fresh callus induction medium for another 4 days without dissecting the big clump of calli. However, they selected only rapidly proliferating calli (1-2 mm) for transformation experiments. Vijaychandra *et al.* (1995) also reported that use of *vir* gene inducing compounds like acetosyringone and tissue susceptibility to infection like scutellum and scutellum-derived calli are instrumental in successful transformation of monocots by *Agrobacterium tumefaciens*. They used similar protocol of Hiei *et al.* (1994) except that they cut out big callus clumps into 4 to 6 pieces before subculturing. Similar protocol was followed by Rasul *et al.* (1997) also. They cut 7 to 10 days old scutellar calli that were further broken into fragments before infection. Subculture of calli for four days prior to co-cultivation was found to be very useful for the selection of fast growing calli for transformation (Mohanty *et al.*, 1999). Our protocol is similar to that of Al-Forkan *et al.* (2004) except that they used 4 weeks old embryogenic calli initiated directly from mature seed scutellum while we used 3 weeks old embryogenic calli. These calli were then fragmented into 4-5 mm segments and subcultured for another 4 days while in the present investigation it was subcultured for 5-7 days. Numerous reports on *Agrobacterium tumefaciens*-mediated transformation of rice document that calli initiated on medium containing 2,4-D were transformed by *Agrobacterium tumefaciens* very efficiently (Aldemita and Hodges, 1996; Uze *et al.*, 1997; Rashid *et al.*, 1996; Dong *et al.*, 1996; Rasul *et al.*, 1997; Terada *et al.*, 2002 and 2004, Vijayachandra *et al.*, 1995; Hiei *et al.*, 1994; Khanna and Raina, 1997, 1999 and 2002; Yara *et al.*, 2001; Pipatpanukul *et al.*, 2004; Sridevi *et al.*, 2003; Mohanty *et al.*, 1999). However, Khanna an Raina (1999), Mohanty *et al.* (1999) and Sridevi *et al.* (2003) reported that callus

induction duration for 3 weeks before subculturing for 4 days on fresh medium enhances *Agrobacterium tumefaciens*-mediated transformation rate. Khanna and Raina (1999) selected actively proliferating embryogenic calli of 2-3 mm size for subculturing while Sridevi *et al.* (2003) and Mohanty *et al.* (1999) selected the whole clumps of calli of Pusa Basmati 1 for subculture without dissecting into smaller clumps for infection. Apart from scutellum calli detachment from seed endosperm as initial wounding, an extra wounding by cutting the calli into 4-5 mm pieces during the subculture process may have greatly influenced the bacterial infection as reflected by production of high percentage of hygromycin resistant calli. Park *et al.* (1996) reported that extra-wounding treatment favoured the penetration of bacteria into the rice shoot apex or provided access to susceptible cells, including the germline cells. Therefore, extra-wounding treatment appeared to allow successful transfer and establishment of T-DNA to the cell genome possibly by inducing production of *vir* gene inducing compounds.

5.3 Co-cultivation, effect of acetosyringone and transient GUS assay

After 5-7 days of subculture, the calli were infected with *Agrobacterium tumefaciens* LBA4404 (pSB1, pCAMBAc) at various concentrations of AS. In the present investigation, increase in AS concentration did not necessarily enhance the infectivity as shown by transient GUS assay. The data recorded for transient GUS assay suggested that 200 µM concentration of AS worked best for Pusa Basmati 1 exhibiting mean value of 71.6%, 300 µM concentration of AS worked best for Basmati 370 exhibiting mean value of 53.3% and 100 µM concentration of AS worked best for Tarori Basmati exhibiting mean value of 60%. These results are in agreement with that of Al-

Forkan *et al.* (2004). They found that 100 μM concentration of AS induced maximum GUS activity in calli of indica cultivars *viz.* BR26 and Binni with 65% and 88% frequencies respectively. However, GUS activity was less with 50 μM (33% in BR26; 42% in Binni), 200 μM (51% in BR26; 57% in Binni) and 400 μM (28% in BR26; 49% in Binni) of AS. A similar result was also reported by Aldemita and Hodges, (1996) based on counting the transient *gus* gene expression in immature rice embryo-derived calli of indica and japonica varieties. They concluded that transient GUS activity decreases irrespective of increase of AS concentration beyond 400 μM during co-cultivation. Terada *et al.* (2004) also reported similar results during transformation of embryogenic calli derived from mature seeds of japonica cv. Nipponbare. They experimented with various concentrations ranging from 100-400 μM AS during co-cultivation to increase transformation frequency. Among the AS concentration tested, 200 μM appeared to be the best while 300 μM and 400 μM concentration reduced GUS activity. However, contrary reports also exist indicating higher AS levels in the co-cultivation medium raised the frequency of GUS positive calli from about 40% (at 60 μM of AS) to more than 60% (at 500 μM of AS) in indica varieties. A further increase to about 90% or more was achieved by increasing the level of AS in the pre-induction medium (Khanna and Raina, 1999). Use of exogenous AS is known to enhance transformation efficiency in certain dicots also and has been found to be one of the factors affecting transformation in monocots, such as corn (Gould *et al.*, 1991; Ishida *et al.*, 1996), rice (Aldemita and Hodges, 1996; Chen *et al.*, 1998a and 1998b; Cheng *et al.*, 1998; Hiei *et al.*, 1994; Rashid *et al.*, 1996) and wheat (Cheng *et al.*, 1997), though it was not found necessary in barley (Tingay *et al.*, 1997). Extremly lower or higher

concentration of AS reduced transient GUS activity, indicating that this compound may be bacteriostatic at higher concentrations (Sheng *et al.*, 1996), or unable to act as signal molecule at lower concentrations (Hiei *et al.*, 1994).

5.4 Transformation frequency

5.4.1 On the basis of hygromycin resistant calli

The experiments in the present investigation revealed that the percentage of calli that proliferated on hygromycin containing medium from five independent experiments for each variety varied from 79.5-90.6% in case of Pusa Basmati 1, 72-82.6% in case of Basmati 370 and 50.6-59% in case of Tarori Basmati. This is in agreement with the results of Sridevi *et al.* (2003) who reported proliferation in 94% of the *Agrobacterium tumefaciens*-infected calli of Pusa Basmati 1 on hygromycin containing medium using similar *Agrobacterium tumefaciens* strain LBA4404 (pSB1 harbouring pMKU-RF2) but with different binary vector (pMKU-RF2 is a derivative of pCAMBIA1301) that contain rice chitinase gene (*chi*11) under maize *Ubi*1 promoter. Khanna and Raina (1999) reported 75.6% transformation frequency with *Agrobacterium tumefaciens* strain LBA4404 harbouring pTOK233 vector to 90.6% with *Agrobacterium tumefaciens* strain AGL1 harbouring pCAMBIA1301 in IR-64 and 60.3%% with *Agrobacterium tumefaciens* strain AGL1 harbouring pCAMBIA1301 to 63.2% frequency with *Agrobacterium tumefaciens* strain LBA4404 harbouring pTOK233 vector in Tarori Basmati. In contrast, Mohanty *et al.* (1999) reported 44-61% tranformation frequency in Pusa Basmati 1 which could be due to difference in co-cultivation medium and concentration of AS.

5.4.2 On the basis of stable GUS staining

Uniform blue color throughout hygromycin resistant callus after final callus selection phase indicated that the calli were co-transformed with *hpt* and *gus* gene. Stable GUS expression ranged from 60% in Basmati 370 to 56% in Tarori Basmati and 66% in Pusa Basmati 1. Khanna and Raina (1999) reported comparatively higher transformation efficiency ranging from 68.4 to 75% in Tarori Basmati which might have been due to stable expression of GUS gene of super binary vector pTOK233 while Sridevi *et al.* (2003) reported little lower stable transformation rate of 55% in Pusa Basmati 1, which might be due to appearance of escapes and silencing of GUS transgene. The lack of GUS activity indicates that the foreign DNA was either absent or present but non-functional (Rashid *et al.*, 1996).

5.4.3 On the basis of plant regeneration frequency

The percentage mean transformation frequency obtained for Pusa Basmati 1 was 52.10%, 54.48% for Basmati 370 and 30.84% for Tarori Basmati (Table 13). Transformation frequency in the present investigation is higher than the reported frequencies. Khanna and Raina (2002) reported transformation frequency as high as 10.3% for Pusa Basmati 1 and 2.3% for Tarori Basmati on the basis of PCR positive plants regenerated from calli used for transformation. This high transformation frequency is mainly due to appearance of negligible escapes and high plant regeneration frequency.

Partial desiccation of hygromycin resistant calli presumably was one of the factors in attaining high plant regeneration frequencies. Partial desiccation has been

found promotive to plant regeneration (Diah and Bhalla, 2000; Chand and Sahrawat, 2001). The results of Saharan *et al.* (2004) demonstrated that partial desiccation (48 hr.) gave maximum shoot regeneration in two indica rice cultivars with 8-10 numbers of shoots per callus. They also reported that regeneration frequency was higher by 1.2 to 5.6 fold in indica rice cultivar with 48 hr desiccation as compared to 0 hr desiccation. In the present investigation, the plant regeneration frequencies of 63.6%, 71.3% and 57.3% for Pusa Basmati 1, Basmati 370 and Tarori Basmati respectively were recorded from the hygromycin resistant calli (Table 10). This is due to maintenance of embryogenicity in the rapidly proliferated hygromycin resistant calli. Each transformed sector of infected calli produced 5-25 numbers of calli clumps during the course of selection phases, depicting its single point origin and transformation event. Hence, a single plant regenerated from each such calli clumps of single transformation event were scored as 100% regeneration frequency. Extensive proliferation in the present experiment has greatly facilitated high regeneration frequency more or less similar to regeneration rate from 21 days old control callus. Use of correct concentration of plant selection marker– the hygromycin antibiotic, was found to be essential to avoid development of undesirable escapes. Increasing the concentration of hygromycin from the optimum concentration of 50 mg l^{-1} to 60 mg l^{-1} during last three phases of callus selection and regaining its original concentration of 50 mg l^{-1} during regeneration phase had presumably avoided appearance of escapes. Consequently, less than 2-3% escapes were recorded in the present investigation. In addition, reduced concentration of cefotaxime from 350 mg l^{-1} in first phase to 250 mg l^{-1} during second and third phase and then to 150 mg l^{-1} during final callus selection phase. The cefotaxime level was further reduced

to 75 mg l^{-1} during regeneration phase. Such regime of cefotaxime markedly reduced its ill effect on regeneration potentiality.

Higher concentrations of antibiotics required in many cases inhibit regeneration of the plant tissues (Barrett and Cassells, 1994). The capability of carbenicillin and cefotaxime in controlling the growth of *Agrobacterium tumefaciens* on the regeneration of the calli of rice *cv.* RD6 showed that there was strong inhibition of the regeneration potential (Pipatpanukul *et al.*, 2004). They reported complete inhibition of regeneration in indica *cv.* RD6 at the concentration of 250 mg l^{-1} of both carbenicillin and cefotaxime. The reduced regeneration capacity was also reported by Nauerby *et al.* (1997) in *Nicotiana tabacum* due to application of cefotaxime. They reported that 500 mg l^{-1} cefotaxime and 1000 mg l^{-1} carbenicillin played an inhibitory effect on regeneration of *Nicotiana tabacum*. Hence, in the present investigation, the application of increased concentration (350 mg l^{-1}) of cefotaxime initially and then its reduction (to 150 mg l^{-1}) during fourth selection phase and further at regeneration stage (to 75 mg l^{-1}) had possibly conditioned the calli to regain its original regeneration potential. The higher regeneration rates reported here are also due to the induction of long-term morphogenically potential embryogenic callus and large numbers of calli clumps produced from each transformed sector of calli. In addition, early application of high selection pressure of hygromycin had markedly reduced the appearance of escapes by facilitating growth of transformed cells and inhibiting growth of untransformed cells.

Absence of escapes with few exceptions are evident from the PCR data and southern blot analyses explained later.

5.5 Molecular analyses of putative transgenics

5.5.1 PCR analyses for all the marker genes present in the T-DNA

Out of 96 hygromycin resistant plants analysed for the presence of the three genes *viz. cry1Ac*, *hpt* and *gus* by PCR; 69.7%, 70.8% and 63.5 % of Pusa Basmati 1, Basmati 370 and Tarori Basmati respectively were positive for all three genes (Table. 11) suggesting complete integration of T-DNA region. On the contrary, mere 2.08%, 2.08% and 3.12% of Pusa Basmati 1, Basmati 370 and Tarori Basmati, respectively, were found to be null for the T-DNA region.

Analyses for border region of T-DNA revealed that the left border (LB) region was more conserved in all the three Basmati varieties with an average of 85.4%, 96.87% and 86.45% in Pusa Basmati 1, Basmati 370 and Tarori Basmati, respectively, than right border (RB) region. The RB region was conserved with an average frequency of 82.29%, 79.16% and 73.95% in Pusa Basmati 1, Basmati 370 and Tarori Basmati respectively (Table 12). The middle non-selectable gene (*cry1Ac*) was found to be present with a frequency of 87.5%, 86.45% and 78.12% in Pusa Basmati 1, Basmati 370 and Tarori Basmati, respectively. Khanna and Raina (2002) reported that *Agrobacterium tumefaciens*-mediated transformation resulted in 33.3% (Tarori Basmati) to 35.7% (Pusa Basmati 1) production of the hygromycin resistant plants which contained *cry1Ac* gene while 14.1% (Pusa Basmati 1) of regenerants carried one of the two genes tested; *hpt* or *cry1Ac*. However, 50% (Pusa Basmati 1) to 66.6% (Tarori Basmati) of the hygromycin

resistant regenerants did not carry either of the gene. Such high number of escapes in their experiments may be due to low selection pressure of hygromycin during callus selection and regeneration phase and also may be due to two rounds of short callus selection phase (2 weeks each). In contrast, in the present investigation callus selection phase was of 11 weeks, which presumably led to appearance of reduced number of escapes (a mere 2.08%, 2.08% and 3.12% of Pusa Basmati 1, Basmati 370 and Tarori Basmati respectively). Such predominance of LB region over RB region suggests that even for *Agrobacterium tumefaciens*-mediated transformation system, all the genes on T-DNA do not get transferred as an intact insert. Similar observation has been also made earlier in rice (Cheng *et al.*, 1998). Sridevi *et al.* (2003) also reported the similar phenomenon in *Agrobacterium tumefaciens*-mediated transformation of indica rice var. Pusa Basmati 1. They observed that in many putative transformants with multiple T-DNA copies, the number of junction fragments for the *hpt* gene (the left border region) was more than those for the *gus* gene (the right border region) through Southern analyses. A similar observation was also reported in rice by Mohanty *et al.*, 1999. Jeon *et al.* (2000) examined 34 putative transgenic rice plants of which, 11 lines carried a single copy of the *gus* gene and 13 carried a single copy of the *hpt* gene. The remaining lines carried both the gene in two or more copy number analysed through DNA blotting. This result suggests higher probability of integration of *hpt* gene (LB region) than the *gus* gene (RB region). De Buck *et al.* (2000b) further strengthened this observation in *Agrobacterium tumefaciens*-mediated transformation of Arabidopsis and Tobacco. They observed that LB vector junctions were more frequent than RB vector junctions. Their transformants generally showed either left T-DNA end junctions or left and right T-

DNA vector junctions, but rarely a right T-DNA vector junction alone. Alternatively, formation of head to head, inverted T-DNA dimmers around the right border would also explain the detection of lesser number of junction fragments around the T-DNA border (de Neve *et al.*, 1997).

5.5.2 Analyses of *cry1Ac* gene

Southern blot was performed to confirm the stability of integration of the genetic sequence over plant generations and to estimate transgene copy number. DNA hybridization was performed for presence of *cry1Ac* gene in putative transformants and progenies of the confirmed transgenic plants. In the present investigation *Hin*dIII restriction enzyme was selected for digestion of putative transgenic plant DNA which restricted T-DNA once and the other *Hin*dIII restriction site was in the plant genomic sequence, thereby facilitating correct estimation of *cry1Ac* copy number. In the present *Agrobacterium tumefaciens*-mediated transformation experiments, 1-3 copy of *cry1Ac* gene was detected through Southern blot which proved insertion of the *cry1Ac* gene into the rice nuclear DNA. The primary transformants of var. Pusa Basmati 1 viz. plant no. 6a, 12a, 13a and 15a and 12a, 1b, 11b and 21b of Tarori Basmati are probably are clones and are results of similar transformation events. The primary transformants of all the three varieties showed mostly single copy number, however, few transformants also showed two copy numbers of varying sizes ranging from 3 kb to 10 kb while only one plant of Basmati 370 *viz.* plant no. 5a showed presence of 3 copy number. Such integration of transgene (*cry1Ac* gene) in low copy number reduces the chances of anomalies associated with gene integration such as complicated patterns of integration (Hiei *et al.*, 1994; De Block *et al.*, 1997). Similar rearrangement of T-DNA

during the integration process in *Agrobacterium tumefaciens*-mediated transformation has been reported by various researchers (Iyer *et al.*, 2000; Yara *et al.*, 2001; Khanna and Raina, 1999; Mohanty *et al.*, 1999) in rice. In contrast, particle bombardment produces between 1 and more than 20 hybridizing bands of the transgene as revealed by Southern analyses of transformants (Wakita *et al.*, 1998).

5.5.3 Analyses of T_0 and T_1 plants for T-DNA integration

The PCR derived insert (1.1 kb fragment) from the coding sequence of *cry1Ac* gene was used as probe for left border analyses because *Hin*dIII was expected to cleave at one of the internal site within the T-DNA and at another site from the plant DNA nearest to the site of T-DNA integration. Thus, junction fragments with a portion of the T-DNA (left border sequence) and a portion of plant DNA will hybridise to the probe. Left border junction fragments that hybridise to 1.1 kb *cry1Ac* probe are expected to be longer than 5.0 kb which is the distance between the *Hin*dIII site in the T-DNA and the immediate site near to right T-DNA border. 33% of the primary transformants showed two bands *viz.* 6.2 kb & 3 kb or 10 kb & 8 kb. High molecular weight bands (6.2 kb, 8 kb & 10 kb) indicate integration of left border T-DNA region along with part of genome from the plant. Band of 5.8 kb indicates immediate presence of plant *Hin*dIII site near the right border junction sequence while 8 kb and 10 kb bands suggest that the T-DNA has also integrated at some other site. However, there are also occurrence of bands of low molecular weight i.e. 3 kb and 4 kb which suggest that scrambling and deletions of the T-DNA could have occurred at the left border prior to T-DNA integration. Our results are similar to that of Sridevi *et al.* (2003). They reported frequent occurrence of T-DNA scrambling and deletion at left border before the integration process. Presence of

single band like 6.2 kb and 8 kb indicates insertion of T-DNA in an unrearranged manner. However, presence of 10 kb fragment also indicates insertion of T-DNA in an unrearranged manner but it may also be due to integration of T-DNA in the form of inverted repeats with the 5′-end junctions. This type of inverted repeats with 5′-end junctions has been reported by Kim *et al.* (2003) in 21% of the transformants.

Progeny (T_1) analyses of some of the primary transformants revealed that most of the progenies inherited the T-DNA integration pattern like that of their parents. However, plant no. 20a-1 var. Pusa Basmati 1 showed presence of an extra band of 2.7 kb. This phenomenon in the progeny (showing hybridization signal of unexpected sizes) is possibly due to DNA rearrangements. Similar observations have also been recorded by Khanna and Raina (2002). In the present investigation, all the progenies of plant no. 12b var. Basmati 370 exhibited stable inheritance of transgene except the progeny of plant no. 17b-1 which showed presence of 3 kb gene fragment rather than 4 kb as present in its parent. This may be due to reconstitution of the same transgene or loss of earlier restriction site. Such rearrangements were also reported by Kohli *et al.* (1998). In their experiments, they suggested that the appearance of larger sized hybridization bands in Southern blots are due to loss of predicted restriction sites. They also reported that such rearrangement is more common in direct gene transfer technology which leads to frequent integration of T-DNA in host genome as multiple copies in form of direct or inverted repeats. In case of Tarori Basmati, four progeny of 12a showed stable inheritance and integration pattern of T-DNA like their parent *viz.* 6.2 and 3 kb. Conserved band profiles in the lines containing two inserts indicate that all the T-DNA inserts present in the lines are located relatively close to each other on the same

chromosome. However, T_1 progeny *viz.* plant no. 12a-5 and 12a-6 showed presence of only one band of 3 kb. This can be explained on the basis of occasional gene-rearrangement or crossing over and segregation between the two-transgene loci. Such phenomenon has been reported by Tu *et al.* (1998). In their experiments, out of 29 progenies, one progeny deviated from others in possessing only one band instead of two. This phenomenon was explained to be due to recombination event that had occurred between the expected and rearranged copies of the transgenes of the progeny plants.

5.5.4 Expression of Bt-toxin

The expression levels of the Bt toxin gene in the transgenic rice plants were checked through protein immunoblot analyses of T_0 and T_1 plants. Western blot indicated that the delta-endotoxin protein, product of *cry1Ac* gene, expressed in all the lines tested in the present investigation. Western blots of protein extracts from all the 11 transgenic plants of T_0 and their T_1 were found positive. However, some T_0 plants *viz.* 19a and 9b of Basmati 370 and 11a and 12a of Tarori Basmati showed comparably higher amounts of Cry1Ac protein as shown by relatively intense band of 67 KDa. Varying expression levels in the different transformants reflected differential expression of the transgene that could be either due to the varying copy numbers of the gene and its insertion at different sites in rice genome. Maqbool and Christou (1999) reported that transgene integration might be a random process, as indicated by the multiple and unique banding pattern in Southern blots. Such banding pattern of transgene could produce position effects, which might have been responsible for variations in protein expressions. Khanna and Raina (2002) also observed similar phenomenon. They pointed out that transgene copy number did not correlate generally with the Bt-protein estimates.

A plant containing higher copy number may not necessarily show higher Bt-titer or vice versa. Similar observations were also made by Tu *et al.* (1998) in biolistic transformation of elite indica rice cultivar IR72 with hybrid *Bt* toxin

In the present investigation, T_2 plants, which were tested hygromycin resistant, were also found positive for presence of Bt toxin, which is confirmed by the presence of Test line, an immunoreactive product, in DesiGen Xpresstrips. The Bt toxin positive plants of T_2 plants confirm stable inheritance of transgene and its proper transcription and subsequent translation into active protein.

5.5.5 Level of Bt toxin expression vis-à-vis insect mortality

In the present investigation, the higher ELISA titre value of 0.24% (plant no. 15b-1 of Pusa Basmati 1) caused 100% mortality of neonate larvae. In contrast, lower titre value of 0.15% (in plant no. 20a-1 of Pusa Basmati 1 and 18b-1 of Basmati 370) caused larval mortality ranged from 91.7% in Pusa Basmati 1 to 94.1% in Basmati 370. This is in agreement with the observation of Cheng *et al.* (1998). They reported ELISA titre value, as high as 0.31% and as low as 0.23%. However, both the level caused 100% larval mortality in japonica rice variety. Khanna and Raina (2002), reported that BT protein content of 0.24% caused 100% mortality whereas 0.16% caused 81.81% larval mortality. In the present investigation, increase in ELISA titre value did not strictly increased the larval mortality as for example, ELISA titre value of as high as 0.20% causes mean larval mortality of 91.6% in plant no. 6a-1 of Pusa Basmati 1, whereas ELISA titre value of as low as 0.16% causes mean larval mortality of 93.7% (plant no.

7a-1 of Tarori Basmati). This phenomenon is also found in the var. Pusa Basmati 1 where ELISA titre value of as high as 0.20% causes mean larval mortality of 91.6% in plant no. 6a-1, whereas ELISA titre value of as low as 0.15% caused mean larval mortality of 93.7% in plant no. 20a-1.

5.6 Inheritance of *cry1Ac* gene

Genetic analyses of the T_1 and T_2 plants for the expression of transgene demonstrated stable integration of T-DNA into the rice nuclear DNA. The Bt toxin production along with hygromycin resistance was transmitted through the sexual generation to T_1 and T_2 in all the lines tested. Segregation ratio of the transgene in the T_1 progeny (obtained from selfing of T_0 plants) indicated that the inheritance followed Mendelian segregation pattern of 3:1 for a single dominant, where hygromycin resistance (Hyg^R) and hygromycin susceptible (Hyg^S) plants (in the T_1 generation) were in the ratio of 3:1 due to hemizygous state of the transgene. Such segregation ratio has also been reported by Cheng *et al.* (1998) in japonica var. Nipponbare; Azhakanandam *et al.* (2000) in japonica cultivar Taipei 309; Rashid *et al.* (1996) in Basmati 370 and 385 and Mohanty *et al.* (1999) and Sridevi *et al.* (2003) in Pusa Basmati 1. In the present investigation plant no. 12a showed a chi-square value of 5.17 indicating that it did not follow Mendelian segregation ratio of 3:1. This may be due to integration of the transgene at two sites i.e. at the two different loci and are not linked and hence segregated due to crossing over between the transgenes, and this might be responsible for not fitting in 3:1 segregation ratio. This assumption is also clearly supported by segregation of 6.2 and 3 kb band in two of their progeny out of five analysed. Such

deviation from 3:1 Mendelian ratio was also reported by Mohanty *et al.* (1999 & 2002) and Rashid *et al.* (1996).

On the other hand, segregation ratio in the T_2 progeny (obtained from selfing of T_1 plants) checked for hygromycin resistance showed 3:1 segregation ratio except a homozygous Pusa Basmati 1 transgenic line (plant no. 15b-1-1) which segregated in 4:0 Mendelian ratio. The high co-segregation rate of *hpt* and *cry1Ac* gene expression in our experiments further confirmed the low degree of T-DNA rearrangement as indicated by PCR analyses and segregation during T_1 and T_2 generation.

5.7 Bioassay for evaluation of biological activity of the toxin peptide in transgenic rice plants

Insecticidal activity of the transgenic rice plants was investigated to determine whether *cry1Ac* gene expression confers protection against insect predation of Yellow Stem Borer (YSB). Entomocidal activity of the toxin peptide in the cut stem tissues of the six T_1 transgenic plants of each variety indicated as high as 100% mean mortality (Plant no. 15b-1, whose parents were Southern and Western positive) to as low as 88..5% (Plant no. 13a-1) in Pusa Basmati 1 within 4 days of feeding on cut stems. Insecticidal crystal protein produced by plant no. 15b-1 homozygous line showed detrimental effects on the larvae after feeding on cut stems. These larvae did not survive or were severely stunted as indicated by the typical symptom of complete browning starting from pronotom, shrinking of size and ultimately complete necrosis leading to

death. Thus, Bt-protein produced in the transgenic plants caused a very rapid cessation of larval feeding activity and subsequent inhibition of their development. The high rate of mortality indicates stable production of toxin due to stable integration and inheritance of T-DNA to the progenies. On the contrary, larvae fed with control stems attained an advanced developmental stage.

Chapter - 6

SUMMARY

SUMMARY

In the present investigation embryogenic calli were produced from mature seed scutella of three Basmati varieties (Pusa Basmati 1, Basmati 370 and Tarori Basmati). Of three callus induction media tried, CIM medium outperformed the other two media (MB and MB1) in embryogenic callus induction percentage.

Inclusion of casamino acid and proline in the callus induction media proved to be vital in markedly enhancing the production, maintenance and multiplication of embryogenic calli.

Standardization of acetosyringone concentration was found to be critical step for establishing maximum transformation efficiency. However, it was found that increase in the concentration of acetosyringone does not always increase transformation rate as indicated by transient GUS assay. Tarori Basmati was found to be comparatively less susceptible to *Agrobacterium tumefaciens*-mediated transformation than Pusa Basmati 1 and Basmati 370 as indicated by transient GUS assay, percentage of hygromycin resistant calli and stable transformation rate by GUS assay.

Embryogenic calli after co-cultivation at 23±1°C with *A. tumefaciens* culture (harvested at early log phase) and kept in diffuse light for 84 hours followed by post co-cultivation in CIM medium (supplemented with 350 mg l^{-1} of cefotaxime) for 7-8 days in dark. Such, post co-cultivation presumably provided opportunity to all cells (whether transformed or not) to revive and divide after co-culture shock and effectively kills the *Agrobacterium tumefaciens*.

After post co-cultivation the calli were exposed to selection medium supplemented with hygromycin at 60 mg l^{-1} (during the last three callus selection phases) rather than standard 50 mg l^{-1}. This allowed selective proliferation of only transformed sector of calli and markedly prevented the appearance of the escapes. However, higher hygromycin concentration resulted in slight reduction (10-15%) in production of hygromycin resistant calli.

Most of the hygromycin resistant calli were truly transformed as indicated by high percentage of stable GUS positive calli (the mean stable transformation rate was 66% for Pusa Basmati 1, 60% for Basmati 370 and 56% for Tarori Basmati) and by molecular analysis of regenerated plants. Our results on stable transformation rate through GUS assay are higher than reported earlier by Sridevi *et al.* (2003).

Hygromycin resistant calli were then transferred to regenerated medium ($MSKN_3$) supplemented with reduced concentration of cefotaxime of 75 mg l^{-1}. The regeneration frequency from the transformed calli more or less was similar to that of control calli. The regeneration frequency of 63.6%, 71.3% and 57.23% for Pusa Basmati 1, Basmati 370 and Tarori Basmati respectively was obtained. The transformation frequency in terms of plant regeneration obtained for Pusa Basmati 1 was 51.92%, 54.35% for Basmati 370 and 30.7% for Tarori Basmati are the highest so far reported in Basmati varieties.

PCR analysis revealed that mere 3.12% for Tarori Basmati and 2.08% for Pusa Basmati and Basmati-370 are negative for integration of T-DNA i.e. these percentage of plants are negative for all the three genes *viz. hpt* (left border), *cry1Ac* (desired gene) and

gus (right border). These data suggested that the regenerated plants were mostly true transformants with rare occurrence of escapes. However, data on Southern blot suggested that not all PCR positive plants are positive when probed with *cry1Ac* gene insert. Hence all PCR positive plants may not be treated as truly putative transformants. Thus, these PCR positive plants should also be screened with *hpt* and *gus* gene probe to prove their authenticity of being true transformants. The present investigation assumes that those putative transformants which are *gus* positive are also *gfp* positive provided that the integration of T-DNA right junction fragment has integrated as such without re-arrangements, silencing and truncation. Southern blot using 1.1 kb probe of *cry1Ac* gene revealed the integration of *cry1Ac* gene and presence of fragments longer than 5.1 kb in majority of the transformants as expected. Shorter fragments (smaller than 5.1 kb) for example, 3 kb and 4 kb occurred at low frequency which indicated that rearrangements also had occurred within the T-DNA region.

The expression of cry1Ac toxin by the presence of 67 kDa protein in Western blot in primary transformants of all the three varieties, presence of prominent band of Test line in Desigen Xpresstrips analysis of their progeny (T_1) and presence of cry1Ac protein in substantial amount (0.15-0.24% of total soluble protein) in ELISA confirmed the expression of *cry1Ac* gene.

Cut stem insect bioassay of T_1 transformants of all the three varieties revealed high level of larval motility, indicating that protein is efficiently expressed and produced in sufficient amount required to cause 88.5-100% mortality in Pusa Basmati 1, 90.9-97.2% in Basmati 370 and 89.1-94.4% in Tarori Basmati. Expression of *cry1Ac* gene as

revealed by Western blot and ELISA, and encouraging results of bioassay further strengthened the versatile feasibility of our experiments.

CONCLUDING REMARK

Standardisation of high throughput *Agrobacterium tumefaciens*-mediated transformation method in the present investigation is expected to upgrade the plant transformation technology. A high transformation frequency in the present investigation is utmost requirement of any plant transformation based technology. This high throughput transformation technology will reduce the effort (required for generation of high number of transformants) and also the wastage of time involved in screening of true transformants and costly plant selection antibiotics (required in screening for true transformants). High efficiency transformation technology will have a rapid application in enhancing the frequency of T-DNA insertional mutagenesis and hence in turn will aid in T-DNA mediated gene trapping, promoter trapping, study of gene function and in advancement of functional genomics etc.

Chapter - 7

APPENDICES

APPENDICES

APPENDIX-I: Callus induction media used in the present investigation (modified from Murashige and Skoog, 1962)

Components	Concentration (mg l^{-1})		
	CIM	MB	MB1
Micronutrients			
NH_4NO_3	1650	1650	1650
KNO_3	1900	1900	1900
$CaCl_2.H_2O$	440	440	440
$MgSO_4.7H_2O$	370	370	370
KH_2PO_4	170	170	170
Macronutrients			
KI	0.830	0.830	0.830
$CoCl_2.6H_2O$	0.025	0.025	0.025
H_3BO_3	6.200	6.200	6.200
$Na_2MoO_4.2\ H_2O$	0.250	0.250	0.250
$MnSO_4.4\ H_2O$	22.30	22.30	22.30
$CuSO_4.5\ H_2O$	0.025	0.025	0.025
$ZnSO_4.7\ H_2O$	8.600	8.600	8.600
$FeSO_4.7\ H_2O$	27.850	27.850	27.850
Na_2EDTA	37.250	37.250	37.250
B_5 vitamins			
Inositol	100	100	100
Nicotinic acid	1.0	1.0	1.0
Pyridoxine HCl	1.0	1.0	1.0
Thiamine HCl	10.0	10.0	10.0
Amino acid			
L-proline	500	-	-
Complex amino acid			
Casamino acid	300	-	-
Carbon source			
Sucrose	30,000	30,000	30,000
Growth Regulators			
2,4-D	2.5	4.0	3.0
NAA	-	-	0.5
Kinetin	-	1.0	-
pH 5.7			

CIM, MB and MB1 were made up as a single strength solution in distilled water and autoclaved at 121^{0}C for 20 minutes and solidified with 0.3% (w/v) phytagel.

APPENDIX II: composition of AB medium used for culture of *Agrobacterium tumefaciens* (Chilton *et al.*, 1974)

Components	Concentration
Component A: AB buffer solution (20X)	100 ml
K_2HPO_4 (anhydrous)	6.0 g
NaH_2PO_4 (anhydrous)	2.0 g
OR	
NaH_2PO_4 (hydrated)	2.6 g
Component B: AB salts (20X)	100 ml
NH_4Cl	2.0 g
$MgSO_4.7H_2O$	0.6 g
KCl	0.3 g
$CaCl_2$ (anhydrous)	0.3 g
$FeSO_4.7H_2O$	0.005 g

Each component "A" & "B" was dissolved separately in 50 ml of double distilled water. Both the salts were then mixed and autoclaved at 121°C for 20 minutes. For preparation of 500 ml AB medium with agar, 2.5 g of glucose was dissolved in 450 ml of double distilled water with 7.5 g of agar. The medium was autoclaved at 121°C for 20 minutes. After the medium cools to about 50-60°C, 25 ml of 20X AB buffer and 25 ml of AB salts were added and poured into the plates.

APPENDIX III: Composition of LB medium used for culture of *E.coli* (Luria-Bertani, 1960)

Components	Concentration ($g\ l^{-1}$)
Tryptone	10
Yeast extract	5
NaCl	10
	15
pH 7.2	

LB medium was made up as a single strength solution in double distilled water and autoclaved at 121°C for 20 min. and solidified with 1.5% (w/v) agar.

APPENDIX IV: Composition of YEP medium used for culture of *E.coli* and *Agrobacterium tumefaciens* used for triparental mating (An, *et al.*, 1988)

Components	Concentration ($g\ l^{-1}$)
Yeast Extract	10
Peptone	10
NaCl	5.0
pH 7.0	

YEP medium was made up as a single strength solution in double distilled water and autoclaved at 121°C for 20 min. and solidified with 1.5% (w/v) agar.

APPENDIX V : Composition and preparation of AA-AS medium (Rashid *et al.*, 1996)

Components	Concentration (mg l^{-1})
Macronutrients	
$CaCl_2.H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Micronutrients	
KI	0.830
$CoCl_2.6H_2O$	0.025
H_3BO_3	6.200
$Na_2MoO_4.2\ H_2O$	0.250
$MnSO_4.4\ H_2O$	22.30
$CuSO_4.5\ H_2O$	0.025
$ZnSO_4.7\ H_2O$	8.600
$FeSO_4.7\ H_2O$	27.850
Na_2EDTA	37.250
KCl	2.95
Vitamins	
Inositol	100
Nicotinic acid	1.0
Pyridoxine HCl	1.0
Thiamine HCl	10.0
Amino acid	
Glycine	7.5
L-Glutamine	876
L-Arginine	174
L-Aspartic acid	266
Carbon source	
Sucrose	20,000
Growth regulator	
2,4-D	1.0
pH 5.7	

The amino acids were dissolved separately and to it 10 mM MES was dissolved and then filter sterilized before adding to the autoclaved medium along with acetosyringone.

APPENDIX VI: Reagents preparation for GUS histochemical staining (Jefferson *et al.*, 1987)

1. Preparation of phosphate buffer

A. Preparation of monobasic sodium phosphate, 0.2 M stock, 200 ml.

6.24 g of $NaH_2PO_4.2H_2O$ was dissolved in 150 ml DDH_2O and volume was made upto 200 ml with DDH_2O and autoclaved at 121^0 C for 20 minutes.

B. Preparation of Dibasic Sodium Phosphate, 0.2 M stock. 200 ml

5.678 g of Na_2HPO_4 was dissolved in 150 ml DDH_2O and volume was made upto 200 ml with DDH_2O and autoclaved at 121^0 C for 20 minutes.

39 ml from stock A and 61 ml of stock B was mixed and volume was made upto 400 ml with autoclaved DDH_2O.

2. Preparation of 0.5 M EDTA (pH 8.0)

18.6 g of EDTA was dissolved in 69 ml DDH_2O and the pH was adjusted to 8.0 using 10N NaOH. The volume was made upto 100 ml with DDH_2O and autoclaved at 121^0 C for 20 minutes.

3. Preparation of 10 N NaOH

8.0 g of NaOH was dissolved in 15 ml of DDH_2O and the volume was made upto 20 ml with DDH_2O.

4. Preparation of 100 mM of potassium ferricyanide

3.3 mg of ferricyanide was dissolved in 100 µl of phosphate buffer.

5. Preparation of 100 mM of potassium ferrocyanide

4.22 mg of potassium ferrocyanide was dissolved in 100 µl phosphate buffer.

6. Preparation of GUS histochemical reagent

Components (Stock concentration)	Final volume
Sodium phosphate buffer, 50 mM (pH 7.0)	40 ml
EDTA, 0.5 M (pH 7.0)	100 µl
Potassium ferricyanide, 100 mM	50 µl
Potassium ferrocyanide, 100 mM	50 µl

GUS histochemival reagent was prepared as single strength and volume was made upto 50 ml with 50 mM phosphate buffer (pH 7.0).

7. Preparation of X-Gluc stock solution (2 mM)

5.2 mg of X-Gluc was disolved in 50 µl of DMSO (dimethyl sulphoxide) and volume was made upto 5 ml in ice with histochemical reagent.

Contd. ....

8. **Preparation of X-Gluc staining solution (1 mM)**

Components (Stock conc.)	Working conc.	Final volume
Methanol	20%	2 ml
X-Gluc (2 mM stock)	1 mM	5 ml
Phosphate buffer (60 mM)	50 mM	3 ml

APPENDIX VII: Reagents for Plasmid isolation

1. **Solution I**

Components	Concentration
Tris	25 mM
EDTA	10 mM
Glucose	50 mM

pH 8.0

Solution I was made up as a single strength solution in double distilled water and autoclaved at 121°C for 20 min.

2. **Solution II (freshly prepared before use)**

Components	Concentration
NaOH	0.2 M
SDS	1%

0.4 N NaOH was prepared in distilled water and stored in a plastic reagent bottle. 2% SDS was prepared in autoclaved distilled water. Both of them were mixed in 1: 1 ratio before use.

3. **Solution III**

Potassium acetate 3 M (pH 5.5). The pH was adjusted with glacial acetic acid.

4. **TES**

Components	Concentration
Tris	10 mM (pH 8.0)
EDTA	1 mM (pH 8.0)
NaCl	100 mM

pH 8.0

TES components were dissolved in distilled water and autoclaved at 121°C for 20 min.

5. **Sodium acetate**

Sodium acetate 3 mM (pH 5.2)

Contd. ....

APPENDIX VIII: Reagents for Plant DNA extraction (Murray and Thompson, 1980)

A. Composition of DNA extraction buffer

Components	Concentration
Tris (pH 8.0)	100 mM
NaCl	1.4 M
EDTA (pH 8.0)	20 mM
CTAB	2.0 %
DDH_2O	70 ml

DNA extraction buffer was was made up as a single strength solution in double distilled water and autoclaved at 121°C for 20 min. and 2.0% CTAB along with 0.2% of β-Mercaptoethanol were then added and dissolved.

B. Preparation of RNase

10 mg RNase was dissolved in 1 ml of 10 mM Tris-HCl (pH 8.0) and 15 mM NaCl in 1.5 ml microfuge tube. The tube with the contents was placed in boiling water at 100°C for 15 min and then slowly allowed to cool at room temperature.

APPENDIX IX: Composition of 0.1 X TE buffer

Components	Stock	Working solution
Tris HCl	1 M (pH8.0)	50 µl
EDTA	5 M (pH8.0)	20 µl
Sterile DDH_2O		98.8 ml

Components of 0.1X TE buffer was dissolved in double distilled water and autoclaved at 121°C for 20 min.

APPENDIX X: Preparation of reagents for non-isotopic Southern blotting

1. **Malic acid buffer**
   0.1 M Maleic acid
   0.15 NaCl
   pH 7.5 was adjusted with strong NaOH and then autoclave at 121^0 for 20 min.
2. **Washing buffer**
   0.3% Tween 20 was added to autoclaved Malic acid buffer.
3. **Blocking solution (10%)**
   10% (w/v) in Maleic acid buffer.
4. **Detection buffer**
   0.1 M Tris HCl
   0.1 M NaCl
   pH 9.5

50 mM of $MgCl_2$ was added to it after autoclaving the above solution.

Contd. ....

5. **20X SSC**

Components	Concentration ($g\ l^{-1}$)
Sodium chloride	175.3
Sodium citrate	88.2

The pH was adjusted to 7.0 with concentrated HCl and autoclaved at 121°C for 20 min.

6. **Hybridization solution (freshly prepared before use):-**
5X SSC
0.1% N-lauryl sarcosine
0.02% (w/v) SDS
1% blocking solution

APPENDIX XI: Composition of Protein Extraction Buffer (phosphate buffer saline) (Koziel *et al.*, 1993)

Components	Concentration ($g\ l^{-1}$)
NaCl	8.0
KH_2PO_4	0.24
KCl	1.44
Na_2HPO_4	1.44

Volume was made upto 1 litre with double distilled water and pH was adjusted to 7.4 with 1N HCl.

APPENDIX XII

A. **Composition of 8% resolving gel for Tris-Glycine SDS-PAGE (Laemmli, U. K., 1970)**

Component	For volume of 40ml
DDH_2O	17.5ml
30% acrylamide mix	11.7 ml
1.5 M Tris (pH 8.8)	10.0 ml
10% SDS	0.4 ml
10% Ammonium persulphate	0.4 ml
TEMED	0.024 ml

Volume was made upto 40 ml with double distilled water

B. 5% stacking gel for Tris-Glycine SDS PAGE (Laemmli, U. K., 1970)

Components	For volume of 40ml
H_2O	6.5 ml
30% acrylamide mix	1.3 ml
1.0 M Tris (pH 6.8)	1.0 ml
10% SDS	0.08 ml
10% Ammonium persulphate	0.08 ml
TEMED	0.008 ml

Volume was made upto 40 ml with double distilled water

C. 2X SDS loading gel buffer

Components	Concentration
Tris-HCl (pH 6.8)	100 mM
DTT	200 mM
SDS	4.5%
BPB	0.2%
Glycerol	20%

The components were prepared in double distilled water.

APPENDIX-XIII: Composition of Tris Glycine buffer

Components	Concentration
Glycine	39 mM
Tris base	48 mM
SDS	0.037%
Methanol	20%

APPENDIX-XIV: Composition of Tris Buffer Saline (TBS) (Burnette, W.N., 1981)

Components	Concentration
Tris HCl (pH 7.5)	10 mM
NaCl	150 mM

Tween 20 @ 0.05% was added to above autoclaved TBS buffer (TBST)

Chapter - 8

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