Agrobacterium-Mediated Transformation of Indica Rice

Thesis submitted in part fulfilment of the requirement for the degree of
Doctor of Philosophy (Biotechnology)
to
the Tamil Nadu Agricultural University, Coimbatore

by

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(K.K. Kumar)

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Abstract

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Putative transgenic lines of an *indica* rice cultivar, Pusa Basmati1 were evolved using an *Agrobacterium*-mediated transformation protocol and suspension cells or scutellum-derived calli as explants. An *Agrobacterium tumefaciens* strain, LBA4404 harbouring a superbinary plant transformation vector, pTOK233 was used to transform scutellum-derived calli with a transformation efficiency as high as 25.8%. Transfer and incorporation of *hph* or *gusA* gene from the vector was confirmed in T_0 and T_1 lines using histochemical GUS assays and/or PCR analysis. Southern analysis confirmed the presence of *gusA* or *nptII* in T_0 plants and the copy number of *nptII* varied from 1-4. Segregation pattern studied using GUS assays on T_1 plants confirmed incorporation of two or more copies of *gusA*.

A rice chitinase gene, *chi11* which was known to confer resistance against *Rhizoctonia solani*, the rice sheath blight pathogen, was cloned into an *Agrobacterium* binary vector, pCAMBIA1301 which was subsequently mobilized

into EHA105. Another construct (pJRB3), harbouring a CaMV 35S promoter-*chi11*-NOS terminator cassette mobilized into LBA4404, was also used in the present study. Presence of the binary vectors in their hosts, EHA105 or LBA4404 was confirmed by Southern analysis and PCR respectively. Putative transgenic rice lines of Pusa Basmati1 expressing *chi11* were evolved and incorporation of *chi*11 gene confirmed by Western blot analysis. Elite *indica* rice varieties of local importance, ASD16, ADT38, IR50 or White Ponni failed to produce any stable transformants expressing *chi11*.

Introduction

1. Introduction

Rice, *Oryza sativa* L. is the staple food for nearly half the world's population, particularly in East and Southeast Asia. *Indica* and *japonica* rices are the two major subspecies grown in different regions of the world and *indica* rice accounts for 80% of world rice cultivation (Li *et al.*, 1997).

Rice is unique in that it has a small genome of 430 Mbp (Arumuganathan and Earle, 1991) with a chromosome number, 2n=24. Apart from *Arabidopsis*, rice is used as a good model system for the study of plant molecular biology not alone by virtue of its small genome size, but also, of its vast germplasm collection and a well developed classical genetic and restriction fragment length polymorphism (RFLP) maps. Conventional plant breeding methods have greatly contributed to the improvement of both *indica* and *japonica* rice cultivars. Genetic engineering provides an additional opportunity for introducing exotic genes into rice genome, which could not be done hitherto by conventional breeding methods. Therefore, improvement of rice by genetic engineering would be of great importance.

Different transformation methods are used for the genetic improvement of rice. Initially, PEG-mediated transformation of protoplast (Peng *et al.*, 1990) or electroporation-mediated transformation of protoplasts (Toriyama *et al.*, 1988) was used. These two methods appear to suffer from such serious limitations as difficulty in regeneration, sterility, abnormal phenotype and multiple copies of genes integrated. Microprojectile bombardment, which is also called biolistic or particle gun method, is now successfully being used in transforming rice (Christou *et al.*, 1991, 1992; Sivamani *et al.*, 1996).

In the recent past, the monocots and particularly, the graminaceous crop species were considered to be outside the host range of *Agrobacterium*

tumefaciens (Bevan, 1984; De Cleene, 1985). However, transformation methods based on the use of *A. tumefaciens* are still preferred, as *Agrobacterium*-mediated transformation does not require use of protoplasts, and in general, results in higher transformation efficiency and a more predictable pattern of foreign DNA integration rather than any other transformation technique (Chan *et al.*, 1993; Hiei *et al.*, 1994).

Raineri *et al.* (1990) reported the first successful attempt of transforming *japonica* rice with *A. tumefaciens*. In 1993, Chen and coworkers obtained few transgenic rice plants using an *Agrobacterium* system. Hiei *et al.* (1994) subsequently reported a method for an efficient production of transgenic rice plants from *japonica* cultivars using an *A. tumefaciens* superbinary vector system. This evidence was based on a detailed molecular and genetic study of a large number of transgenic plants. Rashid *et al.* (1996) reported the successful application of such a method to Basmati cultivars of *indica* rice. Dong *et al.* (1996) applied a similar method to *javanica* rice. Aldemita and Hodges (1996) showed that immature embryo were also good starting materials for *Agrobacterium*-mediated transformation of Group 1 *indica* cultivars.

Production of rice is affected by various biotic and abiotic constraints of which the diseases caused by fungi, bacteria and viruses are the major ones. Rice sheath blight caused by *Rhizoctonia solani* Kűhn is regarded an internationally important disease second only to rice blast (Dasgupta, 1992). The plant synthesizes a variety of new proteins as a measure of limiting the pathogenic invasion and one of such proteins is chitinase, an enzyme that hydrolysis chitin, an essential component of fungal cell wall. Lin and co-workers (1995) of the Kansas state university was successful in genetically engineering rice plants expressing a rice chitinase gene (*chi11*), which conferred sheath blight resistance.

The present study is done with following objectives:

1. Establishing a suitable *Agrobacterium*–mediated transformation protocol for *indica* rice using the *gusA* and *hph* marker genes.

2. Cloning the rice chitinase gene from the plasmid pCAMBAR*chi11* (a kind gift from Dr S. Muthukrishnan, Kansas state university, USA) into a binary vector pCAMBIA1301 and mobilizing the same into a few *Agrobacterium* helper strains.
3. Transforming certain elite *indica* rice cultivars using the chitinase gene with a view to introgressing resistance against the sheath blight pathogen in rice cultivars known for their consumer-preference.
4. Molecular analysis of transgenic plants using PCR, Southern blotting and/or Western blotting.

Review of literature

2. Review of literature

2.1. Introduction

Agrobacterium tumefaciens is a wound-parasitic soil bacterium that induces crown gall in gymnosperms and dicotyledonous angiosperms. The interaction between *Agrobacterium* and the plant cell is the only known natural example of inter-kingdom exchange of genetic material (Sheng and Citovsky, 1996) and the phenomenon has been described as 'the genetic colonization' (Schell *et al.*, 1979). Zaenen *et al.* (1974) discovered that pathogenic strains of *A. tumefaciens* harboured plasmids of exceptionally large size (more than 300 kbp), now known to be essential for tumour induction. Those plasmids are called Ti (tumour-inducing) plasmids and crown galling is due to transfer of a specific DNA fragment (called T- DNA) from the Ti plasmid to the plant cell (Zeanen *et al.*, 1974). T-DNA region in the Ti plasmid is flanked by border repeats of size 24 bp. After the transfer, T-DNA gets integrated into the plant nuclear genome (Chilton *et al.*, 1977). T-DNA genes usually encode enzyme involved in auxins and cytokinins (Garfinkel *et al.*, 1980) or opine biosynthesis (Murai and Kemp, 1982). Deletion of the '*onc*' genes from within the T-DNA region renders the pathogenic strain non-oncogenic. In spite of that all the genes that are naturally present in T-DNA are inactivated or replaced with other genes, transfer of T-DNA still occurs provided the border repeats remain intact. In recent times, *A. tumefaciens*, unarguably nature's most effective plant genetic engineer has been extensively modified by researchers to allow faster and more specific addition and manipulation of desirable plant genetic traits.

2.2. *Agrobacterium*-mediated DNA transfer to plant cells

The *Agrobacterium*-plant cell interaction divided into seven steps that include *Agrobacterium*-host cell recognition, transduction of plant signals, activation of *vir* genes, production of T-DNA, transport of T-DNA from the bacterial cell into the host plant cell, T-DNA nuclear transport and integration (Sheng and Citovsky, 1996). Successful genetic colonization by *Agrobacterium* involves the recognition of those plant cells that are competent for transformation. Many of the plant metabolites, including sugars, amino acids and phenolics represent an effective chemo-attractant for agrobacteria (Ashby *et al.*, 1987). Once attracted, bacteria attach to the cell walls of wounded plant cells (Lippincott and Lippincott, 1969). The molecular basis for attachment is not yet clear. Important roles in this process are played by a 14-kDa calcium binding protein called rhicadhesin (Smit *et al.*, 1989) and a cyclic β-1,2 glucan. The *chvA* and *chvB* loci (Douglas *et al.*, 1985) encode proteins involved in the biosynthesis of such a glucan (Zorreguieta *et al.*, 1988) and in its transport into the periplasmic space (Cangelosi *et al.*, 1989) as well.

Agrobacterium has a two-component signal transduction system composed of the virulence proteins, VirA and VirG to sense the signal molecules secreted by wounded plant cells and activate the expression of other *vir* genes (Winans *et al.*, 1994). Wounded plants secrete sap with a characteristic acidic pH (5.0 to 5.8) and a high content of various phenolic compounds, such as lignin and flavanoid precursors. The best characterised and most effective *vir* gene inducers are monocyclic phenolics such as acetosyringone (AS) (Stachel *et al.*, 1985). Signal molecules released by wounded plant cells are recognized by the VirA/VirG regulatory system. The VirA protein is a periplasmic membrane protein that senses specific phenolic compounds (such as acetosyringone and related molecules) synthesised by plant cells (Leroux *et al.*, 1987). The VirA protein also interacts with another protein (ChvE) important for the binding of sugar coinducers. Monosaccharide such as glucose and galactose significantly

increase *vir* gene expression only when AS is limited or absent (Cangelosi *et al.*, 1990). Opines also stimulate the induction of *vir* genes of *A. tumefaciens* at low concentration (Veluthambi *et al.*, 1989). The presence of these phenolic and sugar inducers results in autophosphorylation of VirA, which transfers the phosphate group to VirG protein, thus activating VirG (Jin *et al.*, 1990a). Stimulation of VirA kinase activity leads to the autophosphorylation of a histidine residue in the cytoplasmic C-terminal domain (His-474) (Jin *et al.*, 1990b; Huang *et al.*, 1990) and the subsequent transfer of this high-energy phosphate residue to a conserved aspartic acid residue (Asp-52) in the N-terminal half of VirG (Jin *et al.*, 1990b). The host range of the limited host range strain extended by providing the wild host range (WHR) *vir*A gene (Leroux *et al.*, 1987).

To activate the expression of other *vir* genes, VirG interacts with the *vir* box, a conserved 12-bp sequence in the promoter region of highly inducible *vir* genes (Steck *et al.*, 1988). The fact that binding of VirG to a synthetic *vir* box requires a critical threshold concentration provided additional evidence that VirG acts as a multimer (Powell and Kado, 1990). It is still a matter of controversy that non-phosphorylated VirG also has some basic inducing activity. Several *virG* mutants were generated in which *vir* genes were constitutively active in the absence of VirA inducers (Scheeren-Groot *et al.*, 1994; Jin *et al.*, 1993). In these mutants, VirG protein conformation may mimic that of the phosphorylated wild type, possibly as a consequence of the negative charge introduced by an aspartic acid residue instead of the asparagine at position 54 (Jin *et al.*, 1993). Overexpression of VirG resulted in an acetosyringone-independent increase in *vir* gene expression (Rogowsky *et al.*, 1987; Liu *et al.*, 1992,1993). This pool of VirG, upon phosphorylation, can then more strongly express *virG* in a positively auto-regulated fashion (Mantis and Winans, 1992). The introduction of modified *virA* and *virG* genes encoding constitutively active VirG proteins into Ti-plasmid based binary vector will render the transformation by *Agrobacterium* independent of inducers. This will not only result in enhanced transformation efficiencies (Hansen *et al.*, 1994) but also help to circumvent host range limitations. Thus genetic manipulations that stimulate *vir* gene induction (increase Vir protein

activity) may result in increase T-DNA transfer to the plant. An example of such an increase in the *vir* activity is demonstrated by the hyperactive *virG* gene and protein encoded by the supervirulent Ti plasmid pTiBo542 (or the disarmed derivatives of pTiBo542, pTiEHA101 and pTiEHA105 (Chen *et al.*, 1991; Hood *et al.*, 1986; Jin *et al.*, 1987). Increasing the copy number of *virG* in *A. tumefaciens* had an additional unexpected effect. Increasing the *virG* copy number in *A. tumefaciens* permitted *vir* gene induction in enriched medium and at alkaline pH (Liu *et al.*, 1993). Jin *et al.* (1987) showed that a 2.5 kbp region of plasmid pTiBo542 containing *virG* and the 3' end of *virB* is responsible for the supervirulent phenotype of A281.

The only *cis*-requirement for T-DNA transfer is the presence of two conserved, direct repeats of about 25 bp at both ends of the T-region, the so-called T-DNA borders (Wang *et al.*, 1984; Peralta and Ream, 1985). T-DNA excision is initiated by the concerted action of VirD1 (16.2kDa) and VirD2 (47.5 kDa) encoded by the *virD* locus. The T-strand DNA molecule, which carries genetic information, and its cognate VirD2 and VirE2 protein, which protect the T-strand, shape it into a transferable (thin and unfolded) form and supply specific targeting signals. One molecule of VirD2 is covalently attached to each T-strand (Herrera-Estrella *et al.*, 1988). The T-strand is also associated with VirE2, and ssDNA binding protein (SSB; Citovsky *et al.*, 1988, 1989). The transfer of T-DNA to plant cell requires 10 products of the *virB* operon and *virD4* (Shirasu *et al.*, 1994). There is increasing evidence that in addition to its capping properties the VirD2 protein serves as a 'pilot' protein guiding the T-DNA not only through the bacterial membrane, but also to the plant cell nucleus. VirD2 carries two nuclear localization signals (NLS), one each in the C- and N-terminal half of the molecules (Wang *et al.*, 1990; Tinland *et al.*, 1992). The T-complex passes two bacterial membranes and the plant cytoplasmic membrane and after entry to the nucleus, integrates into plant chromosomal DNA (Zupan and Zambryski, 1995).

A detailed model of the integration process was presented by Gheysen *et al.* (1991), according to which T-DNA integration requires a gap in the target

DNA. Gheysen *et al.* (1987, 1991) showed that T-DNA integration probably occurs *via* illegitimate recombination mediated by host enzymes. The integration sites seemed to be randomly distributed through out the plant genome (Chyi *et al.*, 1986). More recently, evidence has been presented that insertion site is not truly random. T-DNA tagging experiments (Koncz *et al.*, 1989; Herman *et al.*, 1990) as well as investigations of the chromatin structure of T-DNA in transgenic plants (Weising *et al.*, 1990) rather suggested a preferential integration into actively transcribed regions of the genome.

2.3. *Agrobacterium*-mediated transformation of monocotyledons

De Cleene and De Ley (1976) listed two families of monocotyledons, Liliales and Arales, among families that included species susceptible to *A. tumefaciens*. The induction of tumours following inoculation of wild type strain of *A. tumefaciens* has been demonstrated in *Asparagus officinalis* (Hernalsteens *et al.*, 1984), Gladiolus (Graves and Goldman, 1987) and *Dioscora bulbifera* (Schafer *et al.*, 1987). Grimsley *et al.* (1986) developed a new technique, designated "agroinfection" by which the sequence of a viral genome (maize streak virus; MSV) can be introduced into higher plant via *Agrobacterium* with the resultant systemic infection of the plant by virus. They demonstrated that maize could be infected with MSV that had been derived by agroinfection (Grimsley *et al.*, 1987). The integration of T-DNA in chromosome was not demonstrated in these studies.

In the early 1990s, attempts were made to introduce a gene for β-glucuronidase (GUS; Jefferson, 1987) and a drug resistance gene into cereal species. Raineri *et al.* (1990) described the production of transformed cells of the *japonica* cultivar by cocultivation of mature seed embryo with *Agrobacterium*. Kanamycin or G418 resistant cells that expressed GUS were obtained from rice embryo (Raineri *et al.*, 1990). The early studies of *Agrobacterium*-mediated gene transfer into monocotyledons remained controversial for long (Potrykus, 1990).

In 1993, Chen and coworkers obtained a few transgenic rice plants by inoculating immature embryos with a strain of *A. tumefaciens*. They proved the inheritance of the transferred DNA to progeny plants by Southern hybridization, although they analyzed the progeny of only one transformed plant. Hiei *et al.* (1994) subsequently reported the method for efficient production of transgenic rice plants from calli of *japonica* cultivars that were 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 with minor modifications. Dong *et al.* (1996) described the successful application of the method to *javanica* rice. Aldemita and Hodges (1996) showed that the immature embryos were also a good starting material for *Agrobacterium*-mediated transfer of both *indica* and *japonica* varieties.

2.4. *Agrobacterium* vectors for cereal transformation

2.4.1. *Agrobacterium tumefaciens* strains

The wild type *A. tumefaciens* strain was extensively modified by researchers for manipulation of desirable plant genetic traits. The first remarkable development was the removal of wild-type T-DNA from Ti plasmid to create "disarmed strain" such as LBA4404 (Hoekema *et al.*, 1983) and C58C1 (pGV3850) (Zambryski *et al.*, 1983). The *A. tumefaciens* strain, A281 is a wild type "supervirulent" strain with broad host range (Watson *et al.*, 1975). The transformation efficiency of A281 is higher than those of other strains (Hood *et al.*, 1987; Komari *et al.*, 1989). These characteristics are due to a Ti plasmid, pTiBo542 that is harboured by this strain (Jin *et al.*, 1987). Two *Agrobacterium* systems based on pTiBo452 were developed. The first involved the strain, EHA101 (Hood *et al.*, 1986) or EHA105 (Hood *et al.*, 1993), which carries a "disarmed" version of pTiBo542. The second involved a 'superbinary' vector. In

this system, DNA fragment that included *virB*, *virC* and *virG* from the virulence region of pTiBo542 introduced into a small T- DNA carrying binary vector (Komari, 1990). The other widely used *Agrobacterium* strain for cereal transformation is a disarmed octopine strain, LBA4404. The disarmed strain of *Agrobacterium* commonly used for cereal transformation is listed in Table 1.

Table 1. Disarmed strains of *A. tumefaciens*

Strain	Characteristics	Reference
LBA4404	Derivative of *Ach*5 harbouring pAL4404, constructed by the elimination of all of the T-DNA region of pTiAch5	Hoekema *et al.* (1983)
C58C1 (pGV3850)	C58C1 harbouring pGV3850, constructed by the replacement of T-DNA region between both boundaries of pTiC58 with pBR322	Zambryski *et al.* (1983)
EHA101	Derivative of A281 harbouring pEHA101, constructed the replacement of T-DNA region of pTiBo542 with *nptII* gene. The complete elimination of T-DNA boundaries is unconfirmed, supervirulent	Hood *et al.* (1986)
EHA105	Derivative of disarmed strain EHA101, harbouring pEHA101, with the kanamycin (*nptII*) resistance marker within the T-DNA of pEHA101 removed	Hood *et al.* (1993)
AGL1	Derivative of EHA101, harbouring disarmed hyper virulence plasmid pTiBo542 from which T-region DNA sequences have been precisely deleted; recombination minus phenotype with carbenicillin resistance.	Lazo *et al.* (1991)

2.4.2. Cointegrate vectors

Two strategies were developed for the introduction of engineered T-DNA into *A. tumefaciens* which used either cointegrate or binary vector system.

In a cointegrate vector system, an *Escherichia coli* vector that can be integrated into a disarmed Ti plasmid is used to yield what is known as an intermediate vector system (Fraley *et al.*, 1983; Zambryski *et al.*, 1983), also called a cointegrate vector. The intermediate vector contains a small region of homology with the disarmed Ti plasmid and on mobilization into *A. tumefaciens,* the intermediate vector combines with the disarmed Ti plasmid by single homologous recombination and forms the cointegrate vector. The commonest cointegrate vector for plant transformation is pGV3850 (Zambryski *et al.*, 1983).

2.4.3. Binary vectors

The binary vector system exploits the fact that the transfer of T-DNA can occur even if the virulence genes and T-DNA are located on separate replicons in an *A. tumefaciens* cell (Hoekema *et al.*, 1983). Binary vector replicates in both *A. tumefaciens* and *E. coli.* Artificial T-DNA is engineered within the binary vector and then introduced into disarmed *A. tumefaciens* strains, in a binary vector system. Vectors such as pBIN19 (Bevan, 1984), pBI121 (Jefferson, 1987) and pGA482 (An *et al.*, 1988) have been extensively utilized in transformation studies. Most recently developed plant transformation vectors are binary, largely due to the ease of both *in vivo* and *in vitro* DNA manipulation and their higher transformation efficiencies into *Agrobacterium.*

2.4.4. Mobilization of vectors into the *Agrobacterium* strain

The commonest way to introduce plasmids into *A. tumefaciens* is by conjugal transfer using *E. coli* as intermediate (Ditta *et al.*, 1980). Direct DNA transfer into *A. tumefaciens* by freeze-thaw procedure and by electroporation was described by Holsters *et al.* (1978). However, a low frequency of transformation (10^2-10^4/μg DNA) was usually obtained in these studies. Studies of Wen-jun and

Forde, (1989) and of Mattanouich *et al.* (1989) demonstrated that electroporation could be used for *A. tumefaciens* transformation and obtained a frequency of 8-15 x 10^5 transformants/μg DNA. Mersereau *et al.* (1990) used a high-voltage electroporation to transform *A. tumefaciens* strains, A136 and A348, achieving an efficiency of 1-3 X 10^8 transformants/μg DNA. They found transformation frequency was dependent of the electric field strength and the pulse length. Mozo and Hooykaas (1991) reported on the reproducible electroporation of megaplasmids of 200-250 kbp size (Ti and Ti::R772 cointegrates) into *A. tumefaciens*, by using a non-purified mini preparation of plasmid DNA.

2.5. *Agrobacterium*–mediated transformation of rice

Numerous factors are of critical importance in the *Agrobacterium*-mediated transfer of rice. The key factor for successful rice transformation include, explant used for cocultivation, bacterial strain/vector combination, use of *vir* gene inducer such as acetosyringone and use of suitable selectable marker for the selection of transformant.

2.5.1. Bacterial strains and vectors

Raineri *et al.* (1990) used a wild-type supervirulent strain, A281 (pTiBo542) to obtain tumorigenic callus tissues of cv. Nipponbare. They also inoculated embryos of cv. Fujisaka5 with LBA4404 (pKIWI105) and obtained a kanamycin-resistant callus. As many as 40% of the embryos inoculated with the LBA4404 produced callus that continued to grow on 150 or even 200 mg/l kanamycin (Raineri *et al.*, 1990). Hiei *et al.* (1994) studied the performance of "supervirulent" strain of *Agrobacterium* in rice transformation. They developed two new types of systems based on pTiBo542 for rice transformation. The first involved the strain, EHA101 (Hood *et al.*, 1986), which carried a "disarmed" version of pTiBo542. The second system involved a "superbinary" vector, pTOK233 into which a DNA fragment from the virulence region of pTiBo542 was introduced.

Hiei *et al.* (1994) compared a normal vector, pIG121Hm, with the superbinary vector, pTOK233, in rice transformation. In their transformation experiments, pTOK233 was as effective or slightly more effective than pIG121Hm with an easy genotype (Tsukinohikari) and it was definitely much more effective with a difficult genotype (Koshihikari). Rashid *et al.* (1996) used the supervirulent strain EHA101 containing the binary vector pIG121Hm to transform Basmati genotypes. Aldemita and Hodges (1996) and Rasul *et al.* (1997) obtained transgenic plants in *indica* genotype by using the strain, LBA4404 harbouring the superbinary vector pTOK233. Dong *et al.* (1996) used two *Agrobacterium* strains, LBA4404 with the superbinary vector, pTOK233 and supervirulent strain, EHA101 with normal binary vector pIG121Hm. They could transform *javanica* rice cultivars, with LBA4404 (pTOK233) suggesting that the presence of additional *vir* sequences may be important for the transformation of some cultivars.

Balconi *et al.* (1998) used a cointegrate vector in a common nopaline strain C58c1 (GV2260::NG3INTGUS), two normal binary vector in two different backgrounds in EHA101 (pMTCA3IG) and LBA4404 (pINTGUS). They also used two superbinary vector in EHA105 (pMT1) and LBA4404 (pTOK233). Some genotype exhibited very high susceptibility to infection with strain EHA101 (pMTCA3IG) and LBA4404 (pTOK233). The least effective strain was supervirulent strain with superbinary vector EHA105 (pMT1).

2.5.2. Choice of explants for agrobacterial infection

Hiei *et al.* (1994) suggested that a 3 week-old, actively dividing, scutellum-derived callus as optimal target for *Agrobacterium* attachment and transformation. They used various types of tissues from rice namely; shoot apices and segments of roots from young seedling, scutella, immature embryos, scutella-derived callus or suspension cells. They detected transient GUS expression upon cocultivation in all tissues. Calli derived from scutellar region was associated with the highest transient expression frequency of 93%. Use of actively growing, embryogenic calli was very important for efficient transformation of rice.

Vijayachandra *et al.* (1995) reported that rice tissues could induce expression of *vir* genes but that induction was greatly enhanced by acetosyringone. They suggest that scutellum and scutellum-derived calli may be the most susceptible tissues of rice for *Agrobacterium*-mediated transformation.

Rashid *et al.* (1996) developed a transformation system in *indica* rice using 3 week-old scutellum-derived calli. They compared the efficiency of transformation of the 3 week-old scutellum-derived callus and a 3 month-old calli. After cocultivation, they could detect transient GUS expression immediately in 3 week-old scutellar-derived callus, while the latter did not show any GUS expression. Aldemita and Hodges (1996) found that the use of immature embryo were critical for the transformation of Group 1 *indica* rice genotypes. In contrast, Hiei *et al.* (1994) demonstrated that immature embryos were a poor starting material for the transformation of *japonica* rice. Aldemita and Hodges (1996) used freshly isolated immature embryos with sizes ranging from 1.5 to 2.5 mm for cocultivation. They showed that smaller embryos were the most susceptible to *Agrobacterium* and resulted in stably transformed callus. Park *et al.* (1996) used shoot apex as explant for transformation of *japonica* rice and obtained very low frequency of transformation. Previously, Gould *et al.* (1991) successfully used shoot apex tissue as a target for *Agrobacterium*-mediated transformation of maize. Wang *et al.* (1997) used 2 to 4 month-old *japonica* rice callus (Taipei 309) and Rasul *et al.* (1997) used seven to 10 day-old scutellar derived callus of *indica* rice for successful transformation. Dong *et al.* (1996) used 3 week-old immature embryo derived callus as the starting material. Cheng *et al.* (1998) used vigorously growing rice calli (1 to 4 month-old) derived from mature or immature embryos of rice cultivars belonging to Italian cultivars of *japonica* type.

2.5.3. Infection and cocultivation

Raineri *et al.* (1990) used preinduced *Agrobacterium* culture with acetosyringone prior to cocultivation. Chan *et al.* (1992) used filtrate from potato suspension culture for the incubation of explant with *Agrobacterium* during

infection process. Chang and Chan (1991) found the filtrate from potato suspension culture (PSC) was essential for transformation of soybean and indicated that potato suspension culture contained many phenolic compounds such as acetosyringone (AS) and sinapic acid (SA).

Hiei *et al.* (1994) showed that addition of glucose, acetosyringone (100 μM), pH 5.2 in the cocultivation medium and a temperature between 22 and 28 °C increased transformation efficiency. They also showed that transformation was not successful when acetosyringone was omitted from the cocultivation medium or cocultivation was carried out at a temperature higher or lower than this range. Rashid *et al.* (1996) found that the inclusion of acetosyringone (50 μM) in the *Agrobacterium* suspension and cocultivation media proved to be indispensable for successful transformation.

Aldemita and Hodges (1996) used preinduced *Agrobacterium* culture for infection. The bacteria were preinduced overnight with 100 to 200 μM acetosyringone (AS) in a PIM2 medium modified slightly from Li *et al.* (1992). The final acetosyringone concentration increased to 200 to 500 μM in the bacterial suspension immediately before cocultivation. Acetosyringone was found to be non-lethal to *Agrobacterium* at concentration greater than 200 μM (Stachel *et al.*, 1985). Normally, *Agrobacterium* T-DNA strand production reached a peak 12-24 h after induction by phenolic compounds. However, *vir* gene activation in these experiments might have been maintained by the continuous presence of acetosyringone (Aldemita and Hodges, 1996)

Park *et al.* (1996) found that preinduction of *Agrobacterium* with acetosyringone or potato suspension extract 2 hour before cocultivation did not improve the efficiency compared to uninduced culture. They found the key factors which increased the transformation, include addition of acetosyringone, extra-wounding treatment of the shoot apex with a hypodermic needle (may adequately stimulate the production of *vir* inducing compound and also favour the penetration of bacteria for infection). All the other successful reports of rice

transformation included acetosyringone in the cocultivation medium (Dong *et al.*, 1996; Wang *et al.*, 1997; Balconi *et al.*, 1998).

Hiei *et al.* (1994) described that culturing calli on a fresh medium prior to agrobacterial infection for 4 d was indispensable for increasing the efficiency of transformation. Rashid *et al.* (1996) used 3 week-old calli directly for cocultivation. Rashid *et al.* (1996) studied the effect of length of cocultivation periods ranging from 1 to 5 d. Calli cocultivated for one day did not show any GUS activity and the activity was prominent in all the calli cocultivated for 2 d or more. As the calli cocultivated for 5 d exhibited GUS activity, the cocultivated tissue was damaged during such prolonged coculture period, they concluded that a cocultivation for 2-3 d was optimal.

Pre-treatment of tissue, by wounding or enzymatic digestion of cell walls was found to be essential in earlier studies (Chan *et al.*, 1993; Raineri *et al.*, 1990). Such a pre-treatment was not indicated in the successful transformations reported by several other workers in the later years (Hiei *et al.*, 1994; Rashid *et al.*, 1996; Aldemita and Hodges, 1996)

2.5.4. Selection of transformants

In general, the choice of marker genes and of selective agents has a major influence on the efficiency of rice transformation. The gene for neomycin phosphotransferase (*nptII*) was used in many earlier attempts to transform rice. Raineri *et al.* (1990) found that transfer of mature seed embryos immediately after cocultivation onto semi-selective levels of kanamycin (50 mg/l) favored better selection of the transformants on higher levels of kanamycin (100-150 mg/l) during the later periods of selection. They found that as many as 40% of the embryos inoculated with LBA4404 (pKIWI105) produced callus that continued to grow on 150 or even 200 mg/l kanamycin and such calli were classified as kanamycin-resistant. However, these workers failed to generate normal rice plants from transgenic tissues, perhaps because of the high levels of kanamycin

employed for selection. Chan *et al.* (1993) successfully produced a few transgenic plants from *japonica* rice using the *nptII* marker on G148 (a amino-glycoside antibiotic related to kanamycin; also inactivated by neomycin phosphotransferase) as selection agent. Although Aldemita and Hodges (1996) obtained both transient and stable β-glucuronidase (GUS) expression, but plant regeneration following selection on G418 did not occur at all.

Another widely-used and a more effective selectable marker is the gene encoding hygromycin phosphotransferase (*hph*), which confers resistance to the amino-glycoside antibiotic, hygromycin. Hygromycin allows clear discrimination between transformed and nontransformed rice tissues and there have been fewer albinos (Ayres and Park, 1994). The *hph* gene was used as an efficient marker gene for selection for *Agrobacterium*-mediated transformation of *indica, japonica* or *javanica* rices (Aldemita and Hodges, 1996; Rashid *et al.*, 1996; Hiei *et al.*, 1994 and Dong *et al.*, 1996). Aldemita and Hodges (1996) cocultivated rice immature embryos with the strain LBA4404 (pTOK233), grew them for 3 wk without selection, cut the calli 1 to 2 mm pieces of and transferred them onto N_6 medium containing 30 mg/l hygromycin for 3 wk during selection. Hygromycin resistant calli thus obtained regenerated without hygromycin selection during regeneration phase and about 50% of the populations regenerated were found to be positive. Rasul *et al.* (1997) used hygromycin at 30 mg/l for selection of transformants of the *indica* rice variety, Binantoa and obtained only a 4% of the regenerated plantlets were alive on a selective rooting medium. Their studies indicated a greater percentage of transgenic escapes even though all the nontransformed calli died at the same concentration. Such transformation escapes ranging from 12-66% was also reported by Dong *et al.* (1996) in their attempts to transform *javanica* rice cultivars. Dong *et al.* (1996) showed that inclusion of hygromycin (50 mg/l) in the regeneration and rooting media greatly favored the production of transgenic plants that stably expressed the *hph* gene.

Wang *et al.* (1997) used a binary vector containing hygromycin resistance genes and castor bean catalase-1 (CAT-1) gene intron or a *Parasponia andersoni*

haemoglobin gene intron in the coding region. Insertion of an intron, completely abolish the expression of the gene in *Agrobacterium*, rendering it susceptible to hygromycin (Wang *et al.*, 1997). Use of these modified binary vectors minimized the overgrowth of *Agrobacterium* during plant transformation. Both the introns in the *hph* coding sequence not only maintained the selection efficiency of the *hph* gene, but also substantially increased the frequency of rice transformation (Wang *et al.*, 1997). The CAT-1 intron has been reported previously to enhance gene expression in rice up to 90-fold (Tanaka *et al.*, 1991). Wang *et al.* (1997) found LBA4404 and EHA105 were effectively controlled by cefotaxime (250 mg/l) and Timentin™ (150 mg/l) respectively.

bar encoding resistance to phosphinothricin has been successfully used to select transgenic rice calli and plants in a number of laboratories the world over (Cao *et al.*, 1992; Christou *et al.*, 1991; Datta *et al.*, 1992 and Toki, 1997). Park *et al.* (1996) used *bar* for the selection of cocultivated shoot apex driven by an Actin1 promoter or CaMV 35S promoter.

2.5.5. Transgene copy number and *Agrobacterium*-mediated transformation of rice

In their attempts to transform *japonica* rice, Hiei *et al.* (1994) evolved a large number of transgenic rice lines exhibiting complete sterility to full fertility. Nevertheless, Hiei *et al.* (1994) observed the majority (about 70%) of transformants produced as many seeds as seed-derived control plants. The majority of the progeny showed clear segregation for GUS-positive and GUS-negative plants and the estimated number of loci were consistent with those estimated on the basis of resistance to hygromycin. The estimated number of loci was smaller than the number of copies of the genes that were measured by Southern analysis. It was likely that more than two copies of genes were integrated on a chromosome of such a plant. The copy number of integrated genes varied from one to six (Hiei *et al.*, 1994).

Rashid *et al.* (1996) obtained a large number of morphologically normal, fertile transgenic plants in Basmati cultivars. Integration of foreign genes into the genome of the T_0 plants was confirmed by Southern analysis. *gusA* and *hph* genes were inherited and expressed in T_1 progeny. Albinos appeared in 11% of the plants and they failed to survive in soil. However, 30% hygromycin resistant plants exhibited no GUS activity in Basmati 370. In Basmati 385, all hygromycin resistant plants showed GUS activity. Lack of GUS activity and hygromycin resistance might indicate that the foreign DNA was either absent or present but non-functional (Rashid *et al.*, 1996)

Aldemita and Hodges (1996) obtained transgenic lines of both the *japonica* (Radon) and *indica* varieties (IR72 and TSC10) and the plants obtained were self-fertile and comparable in this respect to seed-grown plants. Southern analyses indicated two to three copies of the gene was integrated in most transformants. Park *et al.* (1996) observed inactivation of *bar* gene in the R_2 progeny. Southern analysis of the inactivated progeny plant suggested that the three *Bam*HI sites located in the inserted T-DNA region were extensively methylated (hypermethylation). There are several reports on suppression or silencing of homologous gene in transgenic plants that have either retransformed with the same bacterial gene or transformed with extra copies of endogenous plant gene (Finnegan and McElroy, 1994). Dong *et al.* (1996) produced transgenic *javanica* rice lines that were phenotypically normal and fertile. Southern analysis of those lines showed that one or two copies of the T-DNA insert were present. Progeny analysis of lines bearing two copies showed cosegregation indicating that they were located relatively closely on the same chromosome (3:1).

Wang *et al.* (1997) observed that transgenic plants expressing *hph* with an intron exhibited low-copy number (1-3) patterns coupled with substantially more mRNA of predicted size, while plants transformed with the intronless transgene had more complex high copy number patterns. They also indicated that the transgenic plants with many copies of the transgene were more likely to show gene silencing than plants with 1-3 copies. Four out of the 5 high copy number

lines had low levels of mRNA whereas the other low copy number lines produced substantial amount of *hph* mRNA (Wang *et al.*, 1997).

2.5.6. Rice genotypes used in *Agrobacterium*-mediated transformation

Among the eight *japonica* cultivars screened for their response to tumorigenicity, Raineri *et al.* (1990), found only two genotypes namely, Nipponbare and Fugisaka5 were susceptible to *Agrobacterium*. Hiei *et al.* (1994) reported that among various *japonica* rice cultivars they tried, Tsukinohikari, Asanohikari, and a poor tissue culture-responsive variety Koshihikari, could be transformed quite efficiently by *Agrobacterium*. Taichung Native1, an *indica* rice could successfully be transformed by *Agrobacterium* (Chan *et al.*, 1992). Rashid *et al.* (1996) used the *indica* rice cultivars, Basmati370 and Basmati385 in their experiments and obtained 22% and 4.8% stable transformed plants respectively, while Basmati6129 could not be transformed at all. Aldemita and Hodges (1996) obtained a high transformation efficiency of about 27% in a *japonica* variety, Radon while it was only from 1-5% when IR72 or TSC10 were used. Park *et al.* (1996) used shoot apices of a tropical *japonica* rice variety, Maybelle in their *Agrobacterium*-mediated transformation experiments. Rasul *et al.* (1997) used a highly regenerable *indica* rice variety, Binantoa for transformation and obtained a 2% transformation efficiency. Dong *et al.* (1996) evolved transgenic *javanica* rice lines of Gulfmont and Jefferson with a transformation efficiency of 2.8%.

2.6. Transformation of rice with agronomically important genes

2.6.1. Agronomically useful genes

Ku *et al.* (1999) using an *Agrobacterium*-mediated transformation system, introduced an intact gene encoding maize phosphoenol pyruvate carboxylase (PEPC) involved in catalyzing an initial fixation of atmospheric CO_2 in the C_4 plant (maize) into *japonica* rice (C_3 plants) varieties, Kita-ake and Nipponbare About 15% of the transgenic lines exhibited 30- to110-fold higher PEPC activities

and the enzyme accounted for up to 12% of the total soluble leaf protein. Goto *et al.* (1999) introduced a soybean ferritin gene (iron-storage protein) into a *japonica* rice cultivar, Kita-ake by *Agrobacterium*-mediated transformation. The ferritin gene was expressed under the control of a rice-seed-storage protein glutelin promoter, GluB-1 to mediate accumulation of iron specifically in the grain. Goto *et al.* (1999) also indicated that the transgenic seeds stored up to three times more iron than the normal seeds.

2.6.2. Pest resistance genes

Currently, there are two major candidate genes used in genetic engineering of rice for insect resistance. They are insecticidal crystal protein genes from *Bacillus thuringiensis* (*Bt*) and plant-derived insecticidal proteinase genes. Fujimoto *et al.* (1993) cloned a *cry1A(b)* gene into rice protoplasts. Wunn *et al.* (1996) reported introduction of a synthetic *cry1A(b)* gene into rice through the biolistic method of transformation. Other insecticidal proteins such as lectins and α-amylase inhibitors have also begun to receive more attention (Chrispeels and Raikher, 1991; Shade *et al.*, 1994).

Cheng *et al.* (1998) transformed mature/immature embryo-derived calli of nine rice cultivars with *cry1A(b)* and *cry1A(c)* and evolved over 2,600 transgenic rice plants from 500 independently selected callus lines. Rice lines expressing *cry1A(b)* or *cry1A(c)* was obtained by particle bombardment method. However, the number of plants obtained and levels of toxin protein achieved in these studies were still very low (Wunn *et al.*, 1996; Nayak *et al.*, 1997). In approximately 10% of these plants, the toxin levels were as high as 3% of the total soluble protein (Cheng *et al.*, 1998). This is 10 to100 times higher than the *cry1A(b)* and *cry1A(c)* contents in the previously reported transgenic rice plants (Wunn *et al.*, 1996). Insect feeding assay with R_1 plants showed resistance to major rice insect pests namely, striped stem borer (*Chilo suppressalis*) and yellow stem borer (*Scirpophaga incertulas*) with mortality rates of 97-100% within 5 d after infestation (Cheng *et al.*, 1998).

Duan *et al.* (1996) introduced a potato proteinase inhibitor II gene (*pin2*) into several *japonica* rice varieties (Nipponbare, Tainung67 and PO4) by particle bombardment and regenerated a large number of fertile transgenic plants. Wound-inducible expression of the *pin2* gene driven by its own promoter together with the first intron of the rice Actin1 gene (*act1*) resulted in higher levels accumulation of the PINII protein in the transgenic plants. Expression of the *pin2* was regulated by its own promoter and 3' terminator sequence. Actin1 intron was inserted between the *pin2* promoter and *pin2* coding region (McElroy *et al.*, 1990). In unwounded plants, a low level of PINII protein (approximately 0.05% of total soluble proteins) was detected in leaves, stem and roots. In wounded leaves, analysis made 20 h after wounding showed that PINII protein levels increased 10 to 20-fold (0.5–2.0% of total soluble proteins). Preliminary bioassays for insect resistance showed that transgenic rice plants expressing the potato proteinase inhibitor gene had increased resistance to one major species of rice insect pest, the pink stem borer (Duan *et al.*, 1996).

Snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) has been shown to be toxic to rice brown planthopper (*Nilaparvata lugens*; BPH) when administered in an artificial diet (Powell *et al.*, 1993, 1995). Rao *et al.* (1998) transformed several *indica* rices (ASD16, M5 and M12) and *japonica* rices (cv. Radon and Nortai) with the *gna* gene driven by a phloem-specific promoter (from the rice sucrose synthase RSS1 gene) and by a constitutive promoter (the maize ubiquitin gene promoter) to confer resistance against a sap-sucking insect pest (BPH). Immuno-histochemical localization study showed the expression of *gna* under RSS1 promoter was phloem-specific. Insect bioassays and feeding studies showed that GNA expression in transgenic rice plants resulted in a decreased survival and had a deterrent effect on feeding while reducing the overall fecundity of the insects. Rao *et al.* (1998) demonstrated that rice transformed with GNA conferred a partial protection against BPH.

2.6.3. Disease resistance genes

Plants respond to attack by pathogenic microorganisms by the induction of expression of a large number of genes encoding diverse proteins many of which are believed to have a role in defense (Linthorst, 1991). These proteins are commonly referred to as pathogenesis-related (PR) proteins. The PR-proteins are further subdivided into PR1, PR2 (β-1, 3 glucanases), PR-3 (chitinases), PR4, PR5 (thaumatin-like protein) and PR6 (protease inhibitor) proteins. Among the PR-proteins, chitinases have been studied most intensively for their role in plant's defence against pathogens.

Chitin is a critical component of fungal cell walls of most fungi and of the exoskeleton of insects. Various chitinase preparations were found to inhibit growth of many fungi *in vitro* by causing lysis of their hyphal tips, especially in combination with β-1-3-glucanase (Mauch *et al.*, 1988). Endochitinases, which randomly hydrolyze internal β-1, 4-linkages of chitin-releasing oligosaccharides of GlcNAc (Boller *et al.*, 1983) are the most extensively studied chitinases in plants. In general, plant endochitinases are proteins of 25-35 kDa in size, which occur as monomers and have either a high or low isoelectric point (Boller, 1988). Chitinase activity markedly increases in plant after infection with fungal, bacterial and viral pathogens (Collinge and Slusarenke, 1987).

Broglie *et al.* (1991) transformed tobacco with a chitinase gene and the constitutive expression of the chitinase in transgenic tobacco plants was shown to result in an increased ability to survive in soil infested with fungal pathogen and delayed development of disease symptom. Lin *et al.* (1995) developed transgenic rice plants that express a rice chitinase gene constitutively and high level of chitinases in transgenic rice lines indicated an increased resistance to infection by the sheath blight pathogen, *Rhizoctonia solani.*

So far, only a few defense genes have been cloned into plants and still fewer into monocots like rice. Transformation of rice with a number of putative defense genes is being tried the world over. Different forms of chitinase genes

from rice (Huang *et al.*, 1991) and barley (Leah *et al.*, 1991); β-1, 3-glucanase genes from tobacco and barley (Leah *et al.*, 1991) and osmatin-like protein (AP24) from tobacco (Singh *et al.*, 1989) have been tried in plant transformation. Lin *et al.* (1995) reported genetic engineering of rice for resistance to sheath blight caused by *R. solani* using a rice chitinase gene.

A number of rice plants (*japonica*) containing genes that code for complete or mutated rice tungro bacilliform virus (RTBV) protein were evolved by Potrykus *et al.* (1996). A rice bacterial blight resistance gene, *Xa21* was cloned into a *japonica* rice (Song *et al.*, 1995; Ronald *et al.*, 1996). Zhang *et al.* (1998) transformed the *indica* (Group I) rice varieties IR64, IR72, hybrid restorer lines Minghui63 and BG90-2 by micro bombardment of embryogenic suspension with the *Xa21* gene and *hph* gene for resistance to hygromycin. They obtained 55 lines of transgenic R_0 plant, out of which six lines displayed high levels of resistance to the pathogen (*Xoo* race 6). Resistant lines exhibited a significant reduction in the severity of bacterial blight symptoms. The transgenic plants also showed greater resistance to the bacterial blight pathogen as compared to that exhibited by IRBB21 (*Xa21* introgressed into the IR24 background).

Datta *et al.* (1999) transformed the *indica* rice cultivars, Chinsurah Boro II, IR72 and IR51500 with the 1.1 kbp *tlp* gene (encoding the thaumatin-like protein) construct by PEG-mediated direct gene transfer to protoplasts and by biolistic transformation using immature embryos as explants. A bioassay of transgenic plants showed enhanced resistance to sheath blight pathogen, *Rhizoctonia solani*, as compared to control plant.

2.6.3.1. Rice chitinase genes

Using a barley endochitinase cDNA as the probe, Haung *et al.* (1991) screened a rice genomic library of *Oryza sativa* L. var. *japonica* (Clontech, CA) and isolated five endochitinase clones. A 4.0 kbp *SalI* fragment that contained an endochitinase gene was sequenced. The rice chitinase gene isolated by Huang *et*

al. (1991) encode a protein whose deduced amino acid sequence shared 69%, 70% and 78% identity with those chitinase genes isolated from tobacco (Shinshi *et al.*, 1990), bean (Broglie *et al.*, 1989) and barley (Swegle *et al.*, 1989) respectively. Lin *et al.* (1995) transformed the *indica* variety (Chinsurah BoroII) with a 1.1 kbp rice chitinase gene driven by CaMV 35S promoter. The presence of a chimeric chitinase gene in T_0 and T_1 transgenic rice plants was detected by Southern blot analysis. Western blot analysis of the transgenic plants and their progeny revealed the presence of two bands with molecular weight of 30 and 35 kDa. The degree of resistance displayed by the transgenic plants to sheath blight pathogen, *R. solani* was correlated with the level of chitinase expression. Western blot analysis of extracts of uninfected transgenic plants showed the presence of one 35kDa protein with different level of chitinase accumulation and missing in few T_0 plants (Lin *et al.*, 1995).

Nishizawa *et al.* (1999) transformed the *japonica* rice varieties, Nipponbare and Koshihikari by *Agrobacterium*-mediated transformation method with rice class I chitinase genes, *Cht*-2 or *Cht*-3, under the control of an enhanced CaMV 35S promoter. Transgenic lines of either chitinase genes showed significantly a high level of resistance to the rice blast pathogen, *Magnoporthe grisea.*

Materials and Methods

3. Materials and methods

3.1. Transformation of callus derived from rice suspension culture with LBA4404 (pTOK233)

3.1.1. Bacterial strain and plasmid

In the present study *A. tumefaciens* strain LBA4404, harbouring a binary vector pTOK233 (Hiei *et al.*, 1994), a gift of Japan Tobacco Company, Tokyo, was used. The physical map of pTOK233 is presented in Fig. 1.

3.1.2. Explant

Manually dehusked seeds of ASD16, CO46, IR50, TKM9, TKM10 and Pusa Basmati1 (A gift of the Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore) were surface sterilized with 70% ethanol for 3 min. followed by 0.1% mercuric chloride for 5 min and washed with sterile distilled water. Sterilised seeds were plated onto Murashige and Skoog (MS) medium (Murashige and Skoog, 1962; Appendix 1) containing 2.5 mg/l 2,4-D (callus induction medium) and incubated in the dark at 27 °C. Calli obtained after 30 d were subcultured once again onto a fresh callus induction medium. Approximately 500 mg friable calli were transferred to 100 ml conical flasks containing 25-30 ml of the modified R_2 medium (Ohiro *et al.*, 1973; Appendix 2) containing 2 mg/l of 2,4-D. The flasks were incubated on a rotary shaker (175 rpm) in dark at 25±2 °C. Subculturing of suspension culture was done once in a week into fresh medium. Generally, the establishment of suspension culture took 7-8 weeks.

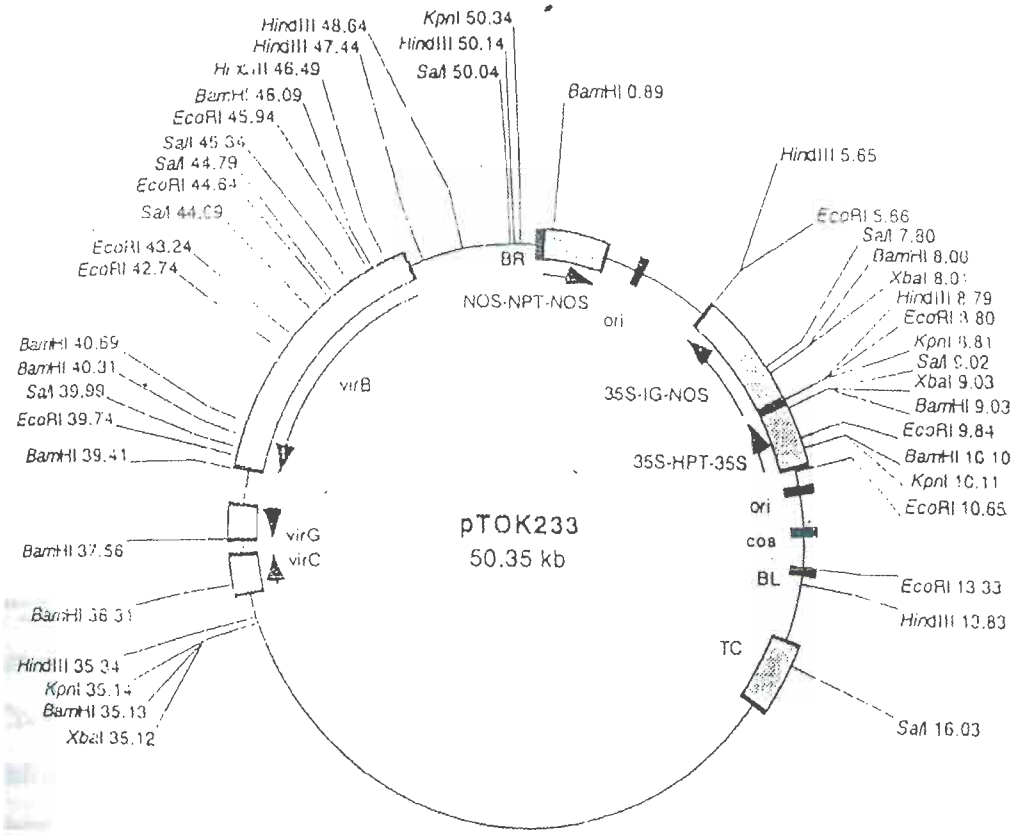

Fig.1. Physical map of pTOK233

3.1.3. Preparation of bacterial suspension

A. tumefaciens strain, LBA4404 (pTOK233) streaked onto AB (Chilton *et al.*, 1974; Appendix 3) agar plate containing hygromycin (50 mg/l) and kanamycin (50 mg/l) was grown at 28 °C for 3-4 d. A loopful of *Agrobacterium* was inoculated into AB broth containing hygromycin (50 mg/l) and grown to A_{600} =1 at 28 °C in a shaker (220 rpm). This culture was centrifuged and the resultant pellet was resuspended in an equal volume of AAM medium (Appendix 4) containing 100 µM acetosyringone. This bacterial suspension was used for cocultivation.

3.1.4. Cocultivation and Selection

Embryogenic calli derived from suspension culture was pretreated with carborundum (Carborundum Universal, India; 320 grit) by vortexing them together for 30 sec. Pretreated calli were immersed in *Agrobacterium* culture for 15 min and transferred to MS cocultivation medium (MS medium containing 2.5 mg/l 2,4-D, 10 g/l glucose, 300 mg/l casamino acid, 100 µM acetosyringone) overlaid with a Whatman No. 1 filter paper wetted with AAM medium. Cocultivation was done for 3 d at 25 °C. After three days, calli were washed in cefotaxime solution (250 mg/l) 3 or 4 times, blot dried and transferred to MS medium containing 2.5 mg/l 2,4-D, 50 mg/l hygromycin and 250 mg/l cefotaxime (Selection medium) and kept at 27 °C. Actively growing pieces of calli were transferred to fresh selection medium every 3 wk. Selection was repeated for 3 to 4 times and the selected calli were transferred to a regeneration medium (MS medium, 3mg/l BAP, 0.5 mg/l NAA, 250 mg/l cefotaxime and 50 mg/l hygromycin). The emerging shoots were transferred to MS rooting medium (half strength MS basal salts, MS vitamin, 10 g/l sucrose) containing 40 mg/l hygromycin.

3.1.5. Transient GUS expression

A portion of cocultivated calli was incubated in X-Gluc solution (Appendix 5) at 37 °C overnight.

3.1.6. Polymerase Chain Reaction

Polymerase chain reaction was performed as recommended by Sambrook *et al.*, (1989) with a few modifications. The reaction was performed with 100 ng of genomic DNA extracted from transformed suspension culture derived calli (modified CTAB method; Porebski *et al.*, 1997; Appendix 6) or 1 ng of plasmid DNA in a 25 µl reaction mixture containing 10 mM Tris-HCl (pH8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (W/V) gelatin, 200 µM each of dNTPs, 1 µl each of forward and reverse primer and 2 units of *Taq* DNA polymerase. The amplification was performed in a PTC-100 Minicycler (MJ Research, USA) with following temp profile: pre-incubation at 94 °C for 5 min leading to 30 cycles of melting at 94 °C for 30 sec, annealing at 52 °C for 1 min and synthesis at 72 °C for 1.5 min, followed by an extension at 72 °C for 5min. After amplification, 10 µl of the reaction was used for electrophoretic analysis on a 1.2% agarose gel.

3.2. Transformation of *indica* genotypes with LBA4404 (pTOK233) using mature seeds derived callus as explants

3.2.1. Cocultivation and recovery of transgenic plants

Sterilised mature seeds of Pusa Basmati1, ASD16, IR50, ADT38 and White Ponni were cultured on MS callus induction medium (MS medium supplemented with 2.5 mg/l 2,4-D) for 21 d for callus induction. Embryogenic calli were subcultured onto a fresh callus induction medium for 4 d before cocultivation. Cocultivation was done as described in Section 3.1.3 and 3.1.4 with the exception of carborundum pre-treatment. A few randomly selected pieces of cocultivated calli were incubated in X-Gluc solution. Cocultivated calli were transferred to an MS selection medium (MS basal salts with 2.5 mg/l 2,4-D, 250

mg/l cefotaxime and 50 mg/l hygromycin). Actively growing pieces of calli were subcultured onto a fresh selection medium for every 3 wk. After four rounds of selection on hygromycin, the callus lines were tested for stable GUS expression by incubating a portion of callus lines in X-Gluc solution. The callus lines were transferred onto the regeneration medium (MS medium with 3 mg/l BAP, 0.5 mg/l NAA, 250 mg/l cefotaxime and 50 mg/l hygromycin) and kept under dark for 2 wk followed by light (110-130 $\mu M/m^2/S$) with 16 h photoperiod. The emerging shoot buds were transferred to an MS rooting medium containing 40 mg/l hygromycin.

3.2.2. Biochemical and Molecular analyses of transgenic plants

3.2.2.1. GUS assay

Leaf bits and root bits excised from putatively transformed plants were incubated in X-Gluc solution (Appendix 5) at 37 °C overnight with a view to demonstrate stable GUS expression in transformed plants.

3.2.2.2. PCR assays

Polymerase chain reaction was performed with genomic DNA extracted from putatively transformed Pusa Basmati1 plants, following the method described in Section 3.1.6.

3.2.2.3. Southern hybridization analysis

Ten micrograms of genomic DNA extracted from putative transformants, untransformed control plant and 20, 50, 100 ng of total DNA from *Agrobacterium* strain LBA4404 (pTOK233) were digested with *Hin*dIII, electrophoresed in 1% agarose gel and transferred onto a nylon membrane, as described by Sambrook *et al.* (1989) (Appendix 7).

3.2.2.3.1. Experiment I

This experiment was conducted with a view to demonstrate the integration of transgene in plants. The blot was probed with a α^{32}P-dCTP-labelled

3.1 kbp fragment consisting of *gusA* gene (obtained 3.1 kbp fragment from pIG221 (Ohta *et al.*, 1990) by digestion with *Hin*dIII and *Eco*RI). The blot was subsequently autoradiographed.

3.2.2.3.2. Experiment II

This experiment was conducted with a view to estimating the number of copies of transgenes in putative transformants. The blot was probed with a α^{32}P-dCTP labelled 2.0 kbp fragment consisting of *nptII* gene (obtained 2.0 kbp fragment from plasmid pGA472 (An, 1986) by digestion with *Bam*HI and *Hin*dIII). The blot was subsequently analysed by autoradiography using x-ray films (Konica, India).

3.2.3. Progeny analysis

Histochemical GUS assays were carried out, as described elsewhere, with the seeds collected from T_0 plants and leaves collected from T_1 plants.

3.3. Transformation of rice genotypes with rice chitinase gene, *chi11*

3.3.1. Construction of *Agrobacterium* binary vector containing *chi11*

The plasmid pCAMBAR*chi11* (containing a 3.2 kbp fragment comprising *chi11* cDNA flanked by ubiquitin promoter and polyA terminator; a gift from Dr. S. Muthukrishnan, Kansas state university, USA) and pCAMBIA1301 (a cloning vector; a gift from Centre for Application of Molecular Biology to International Agriculture, Canberra, Australia) were isolated following the alkali lysis method (Birmboim and Doly, 1979). Maps of pCAMBAR*chi11* and pCAMBIA1301 are presented in Fig. 2 and 3.

The 3.2 kbp *chi11* cassette containing a rice chitinase gene, ubiquitin promoter and polyA terminator was released by digesting pCAMBAR*chi11* with *Hin*dIII. The product was run on a 0.7% agarose gel. The gel region containing the DNA band of interest was excised with a sterile and clean razor blade and transferred to a pre-treated dialysis bag. The bag was placed in a gel tank

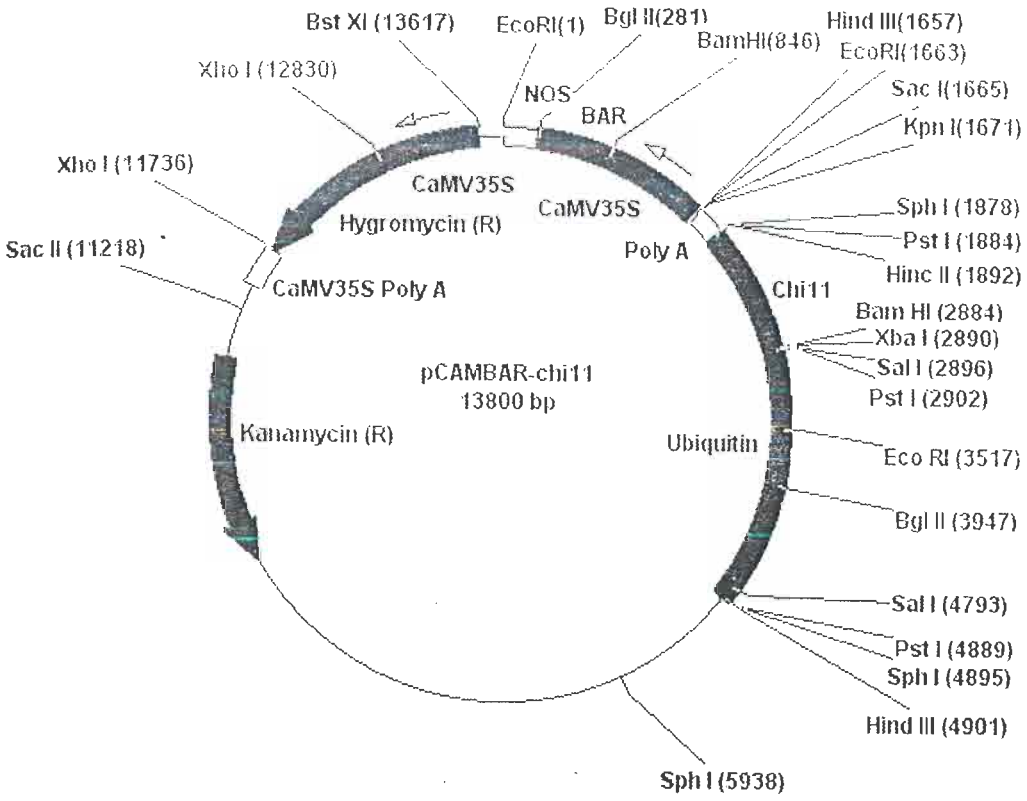

Fig.2. Physical map of pCAMBAR*chi11*

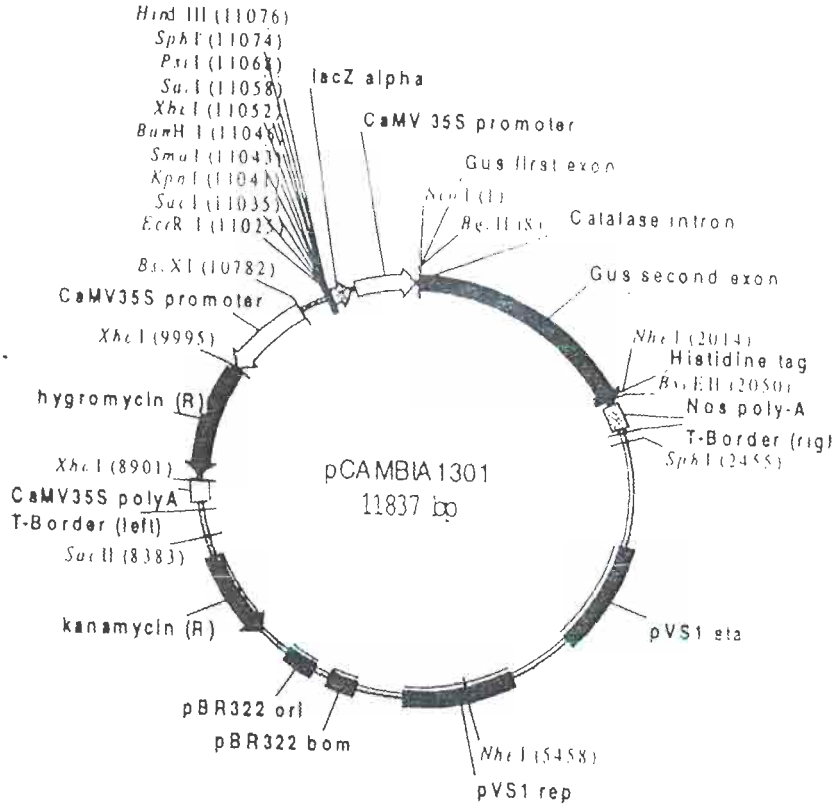

Fig.3. Physical map of pCAMBIA1301

containing 0.5XTBE (0.045 M Tris-borate and 0.001M EDTA) and the DNA fragment was collected in the dialysis tube by electrophoresis. The content of the dialysis tube was transferred to an Eppendorf tube and the DNA was purified using standard protocols (Sambrook *et al.*, 1989).

The plasmid pCAMBIA1301 was linearized with *Hin*dIII and the resultant product was purified following standard protocols. The linearized pCAMBIA1301 and the 3.1 kbp *chi11* cassette were ligated at 16 °C overnight. The ligated product was used to transform *E. coli* (DH5α) and the transformed cells were plated onto LB (Luria-Bertani medium; Miller, 1972) plate containing kanamycin (50 µg/ml) and X-gal (40 µg/l) for the selection of recombinants. Individual recombinant colonies (white colonies) were patched in LB agar plate containing kanamycin (50 µg/ml) and X-gal (40 µg/l). The plasmid DNA was isolated from transformants and restricted with *Hin*dIII to check the insertion of the desired fragment of 3.2 kbp. To confirm the orientation of the cassette, recombinant plasmid from two of the transformants were digested with *Bgl*II and *Bam*HI.

3.3.2. Mobilization of binary vector into *Agrobacterium*

3.3.2.1. Mobilization of pMKU-RF2 into *Agrobacterium* strain EHA105

The plasmid pMKU-RF2 (pCAMBIA1301 with *chi11* cassette) was mobilized into a supervirulent strain, EHA105 (Hood *et al.*, 1993) by triparental mating, as described by Ditta *et al.* (1980). The donor *E. coli* strain DH5α harbouring pMKU-RF2 and the *E. coli* conjugative helper strain, DH5α harbouring the plasmid pRK2013 were grown separately in LB agar. The *Agrobacterium* recipient strain, EHA105 was grown on a YEP (Chilton *et al.*, 1974) plate. Single colonies of *E. coli* (pMKU-RF2), *E. coli* (pRK2013) and a patch of *Agrobacterium* were mixed on a YEP plate and incubated at 28 °C for 12-16 h. The resultant bacterial cells were plated on an AB agar plate containing 100 µg/ml kanamycin and 10 µg/ml rifampicin to select transconjugants. The

plates were then incubated at 30 °C for 3-4 d and single colonies were patched on an AB agar plate with antibiotics to confirm the growth of transconjugants. The presence of intact plasmid in the *Agrobacterium* recipient strain was confirmed by Southern hybridization. Two micrograms of total DNA extracted from the transconjugants, total DNA from EHA105 (negative control), 50 ng of the plasmid DNA, pMKU-RF2 (positive control) were digested separately with *Bam*HI. The digested DNAs were resolved on a 0.9 % agarose gel and the DNA profile was transferred to a nylon membrane (Boehringer GmbH). The blot was probed with a DIG labelled 3.2 kbp *Hin*dIII fragment containing *chi11* cassette, following manufacturer's instructions (Boehringer GmbH). The blot was exposed to a x-ray film (Konica, India) for analysis.

3.3.2.2. Mobilization of pJRB3 into *A. tumefaciens* strain, LBA4404

The plasmid pJRB3 (Jayashree, 1998; Fig 4), based on pCAMBIA1301, containing a 1.58 kbp *chi11* cassette (1.1 kbp rice chitinase genomic DNA fragment flanked by 35S CaMV and nos terminator) was mobilized into the *A. tumefaciens* strain, LBA4404 as described in Section 3. 3. 2. 1. The presence of binary vector in LBA4404 was confirmed by amplifying GUS gene present in pJRB3. PCR reactions were performed as described in Section 3. 1. 6.

3.3.3. Transformation of rice genotypes

3.3.3.1. Transformation of local elite rice cultivars

Immature seeds of ASD16, IR50, ADT38 and White Ponni were collected 12 to 14 d after pollination, dehusked and surface sterilized with 0.1 % $HgCl_2$ for 5 min followed by three washings in sterile water. The immature embryos were isolated under a stereomicroscope and placed on the cocultivation medium directly (MS basal salts and vitamins, 2 mg/l 2,4-D, 10 g/l glucose 30 g/l sucrose 100 µM acetosyringone and 3 g/l phytagel).

A. tumefaciens LBA4404 harbouring pJRB3 or EHA105 (pMKU-RF2) was grown to A_{600} =1 in an AB broth containing hygromycin (50 mg/l) and

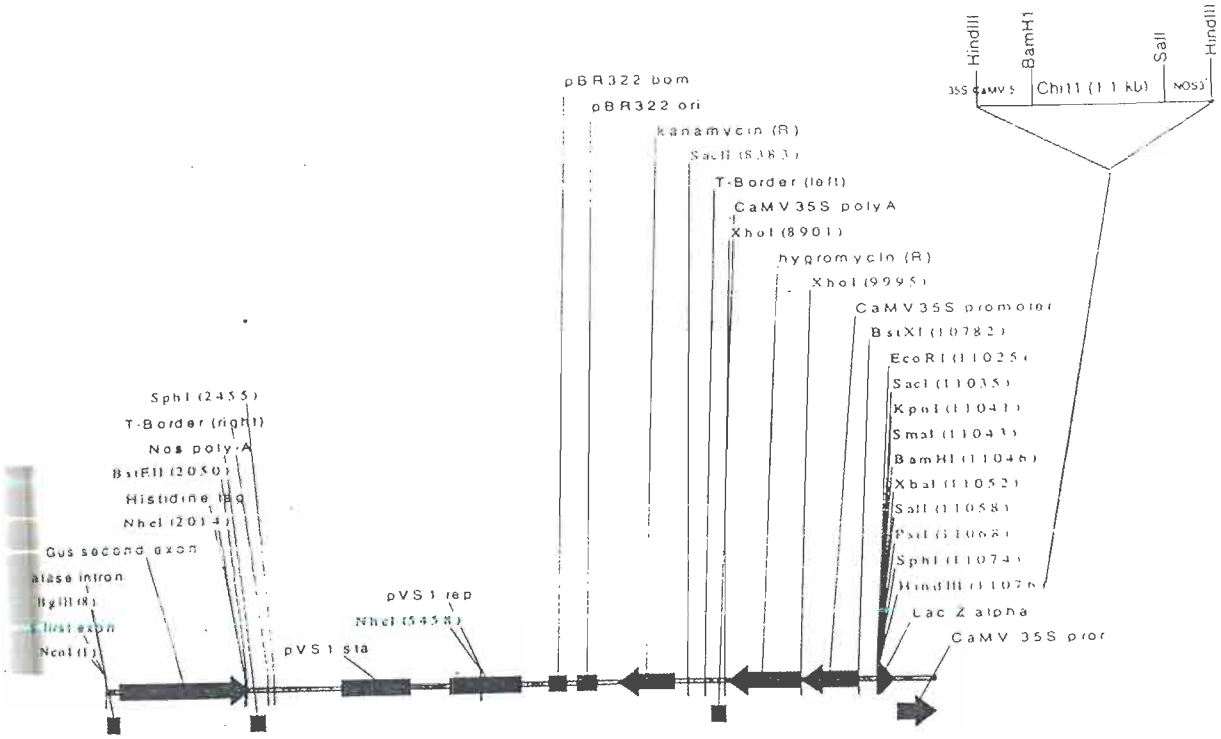

Fig. 4. Physical map of pJRB3

kanamycin (50 mg/l). The culture was centrifuged at 3000 rpm for 10 min and the pellet obtained was resuspended in half the volume of AAM medium containing 100 µM acetosyringone. Ten microlitres of the bacterial suspension was added onto each embryo and cocultivation was performed at 25 °C for 3 d. After the lapse of 3 d, the embryos were washed with cefotaxime solution (250 mg/l) for several times and placed on CC medium (Appendix 8) supplemented with 2 mg/l 2,4-D and 250 mg/l cefotaxime for 7 d. Then, the embryos were placed onto CC medium supplemented with 2 mg/l 2,4-D, 250 mg/l cefotaxime, 50 mg/l hygromycin. The actively growing calli were subcultured onto a fresh medium at 3 wk intervals. After 4 rounds of selection on hygromycin, a portion in each callus lines was tested for stable GUS expression as described elsewhere in this chapter. Callus lines, after 4 rounds of selection, were transferred onto CC medium containing cefotaxime (250mg/l) and hygromycin (30-40 mg/l) for regeneration. Regenerated shoots were transferred to MS rooting medium containing 30 mg/l hygromycin to recover whole plants.

3.3.3.2. Transformation of Pusa Basmati1 with the *Agrobacterium* strain EHA105 (pMKU-RF2)

Sterilised mature seeds of Pusa Basmati1 were cultured on MS callus induction medium (MS medium supplemented with 2.5 mg/l 2,4-D) for 21 d for callus induction. Embryogenic calli were subcultured onto a fresh callus induction medium for 4 d before cocultivation. Cocultivation and recovery of transgenic plants was performed as described in Section 3.2.1.

3.3.4. Biochemical analyses

3.3.4.1. GUS assay

GUS assays were performed on leaf tissues of *chi11* transformed plants following procedures described previously in this chapter.

3.3.4.2. Western blot analysis

Total protein was isolated from young leaves of putative transformants, fractionated by SDS polyacrylamide gel (12%) electrophoresis according to standard procedures (Laemmli, 1970). The fractionated protein was transferred to a nitrocellulose membrane (Protran BA85 Cellulosenitrat(e); Schleicher and Schuell, Germany) using the Trans-Blot semidry transfer apparatus (BioRad). The membrane was blocked in Tris-buffered saline (TBS; consisting of 10 mM Tris-HCl, 150mM NaCl, pH 8.0) with 0.05% Tween20 and 2.5% w/v gelatin for 3 h at room temp with constant shaking. The blot was then incubated for 3 h at room temp with gentle shaking in the antiserum containing antibodies raised against a barley chitinase (gifted by Dr. S. Muthukrishnan, Kansas State University, USA) diluted at 1:1500 in TBST (Tris-buffered saline-Tween 20). Unbound antibody was removed with four washes with TBS consisting of Tween 20 and the blot was incubated for 3 h at room temp with alkaline phosphatase-conjugated secondary antibody (at 1:1500 dilution) (goat-anti rabbit IgG, Sigma, USA). Unbound secondary antibody was removed with four washes of 30 ml TBS containing Tween20, followed by two washes with TBS. The protein bands of interest were detected using BCIP/NBT (Sigma). Molecular weights of the protein bands were determined using the pre-stained rainbow marker (BioRad).

Results

4. Results

4.1. Transformation of callus derived from embryogenic rice suspension culture using *A. tumefaciens* LBA4404 (pTOK233)

A significant increase in transient GUS expression was observed in calli pretreated with carborundum as compared to the untreated calli (Table 2 and Plates 1-6). Selective amplification of *gusA* and *hph* genes was observed in the DNA extracted from callus lines of Pusa Basmati1, IR50, Co46, TKM9, TKM10 and ASD16 (Plates 7 and 8). However, in this experiment, only two putative transgenic lines of Pusa Basmati1 could be recovered (Table 3). Those putative transformant lines proved positive to histochemical GUS assays. Further analysis could not be done on these plants, as both the lines died off during hardening process.

4.2. Transformation of *indica* genotypes with *A. tumefaciens* LBA4404 (pTOK233) using mature seed-derived calli as explants

4.2.1. Cocultivation and recovery of transgenic plants

Two different methods (Section 3.2.1) *viz.*, Rashid *et al.* (1996) (AgR) and a modification thereof (mAgR) were used for transforming different *indica* genotypes *viz.*, Pusa Basmati1, IR50, White Ponni, ASD16 and ADT38. A random sample of a few of the cocultivated explants was drawn and was assayed for histochemical GUS expression. Transient GUS expression in cocultivated calli of Pusa Basmati1 varied from 55 to 57% when AgR method was used, while it was from 26 to 46% when mAgR method was followed (Table 4 and Plate 9). After four rounds of selection on hygromycin (Plates 10-11), 86% of the calli

Table 2: Effect of carborundum treatment on transient GUS expression in the embryogenic suspension calli cocultivated with *A. tumefaciens* LBA4404 (pTOK233)

Genotypes	Percentage of GUS positive callus	
	Cocultivation without carborundum pre-treatment	Cocultivation with carborundum pre-treatment
Co46	16	0
TKM9	0	20
Pusa Basmati1	16	64
ASD16	0	16
IR50	Not done	64

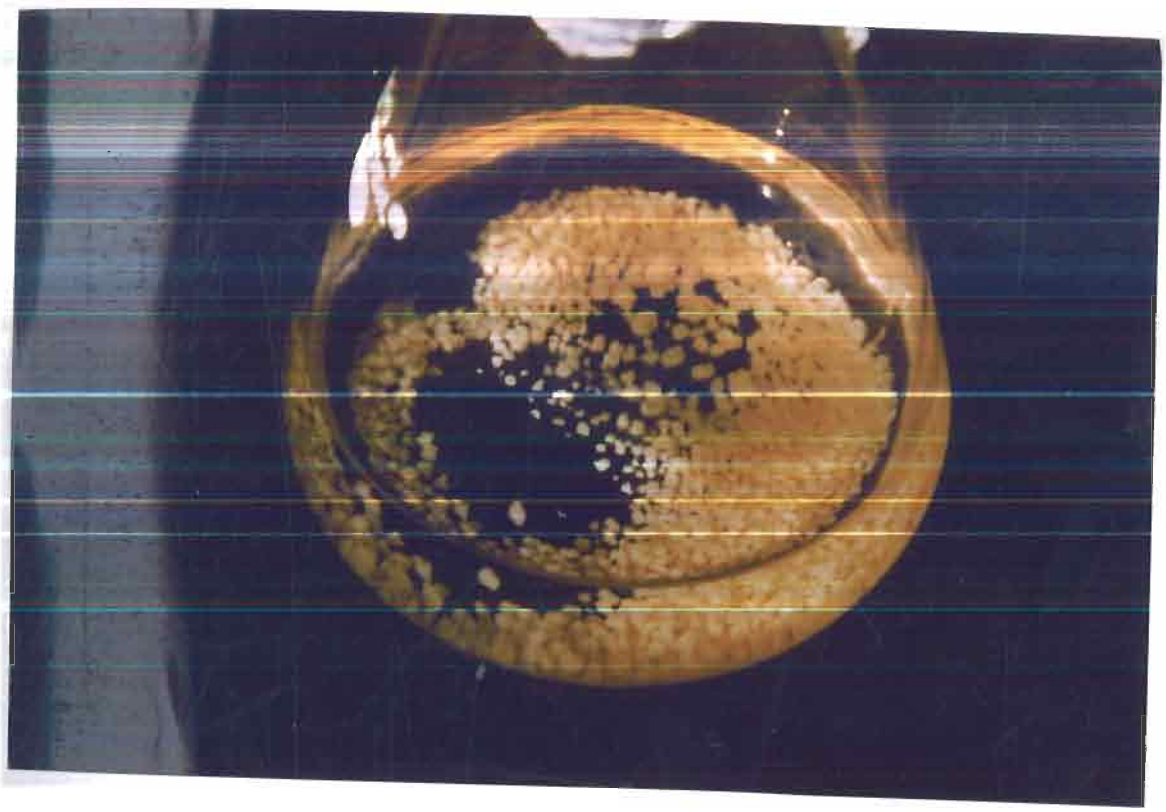

Plate 1. Established suspension culture of Pusa Bamati1 8 wk after initiation.

Plate 2. Established suspension culture of IR50 8 wk after initiation

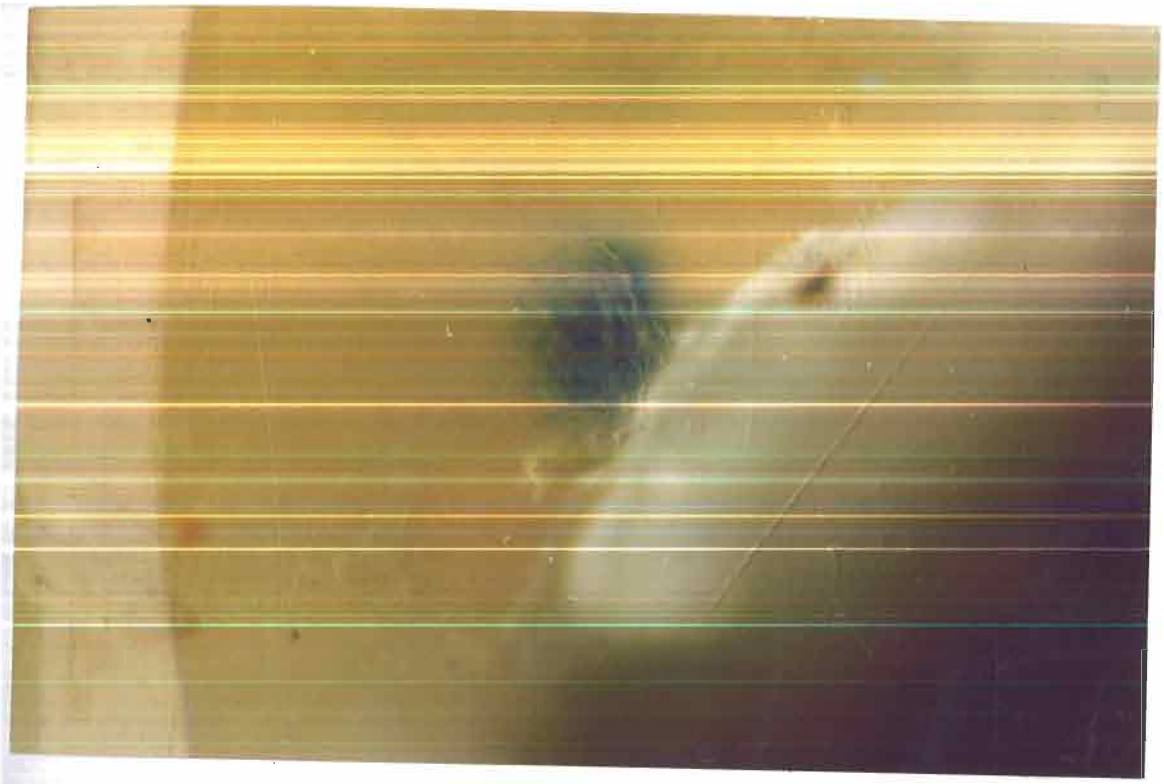

Plate 3. Transient GUS assay in IR50 suspension calli cocultivated with *A. tumefaciens* LBA4404 (pTOK233)

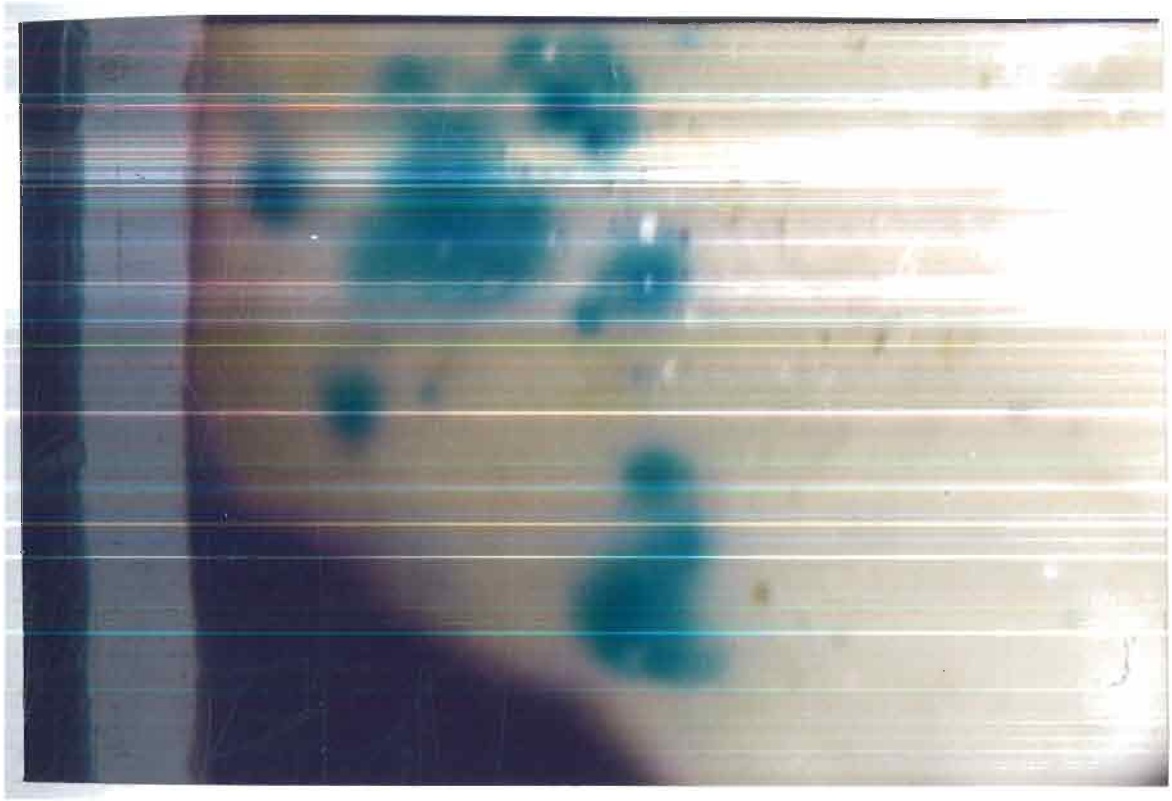

Plate 4. Transient GUS assay in TKM9 suspension calli cocultivated with *A. tumefaciens* LBA4404 (pTOK233)

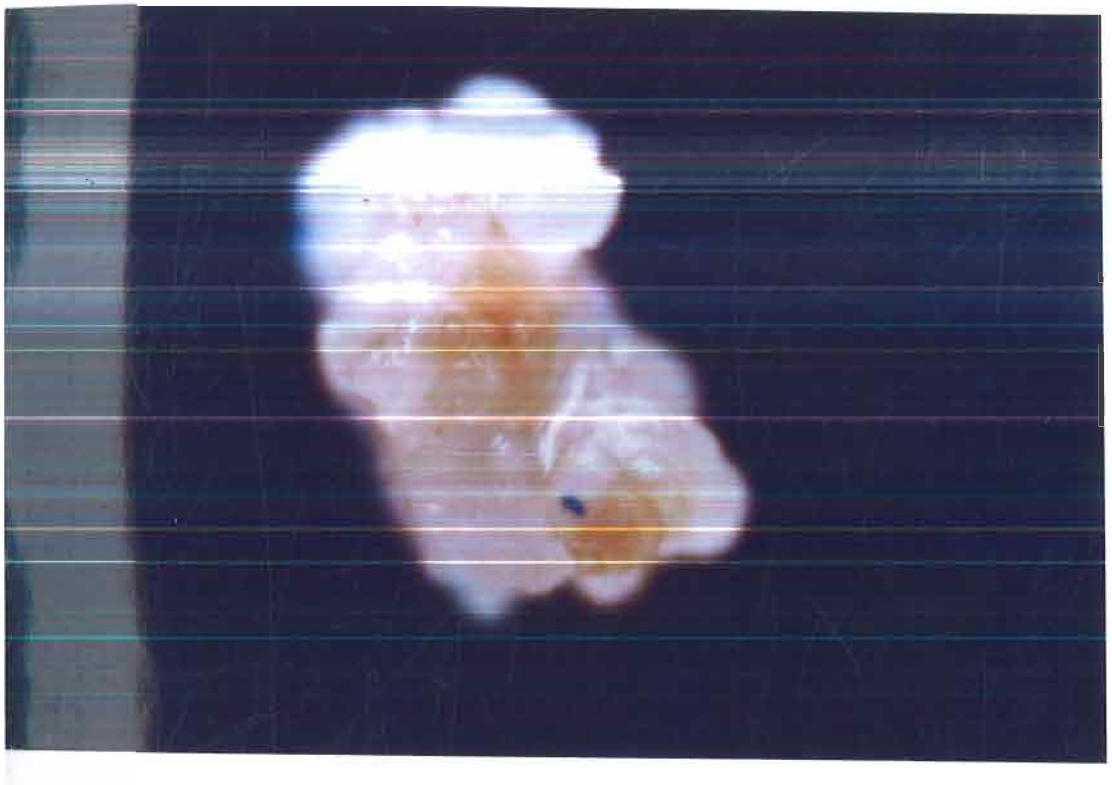

Plate 5. Transient GUS assay in Co46 suspension calli cocultivated with *A. tumefaciens* LBA4404 (pTOK233)

Plate 6. Transient GUS assay in ASD16 suspension calli cocultivated with *A. tumefaciens* LBA4404 (pTOK233)

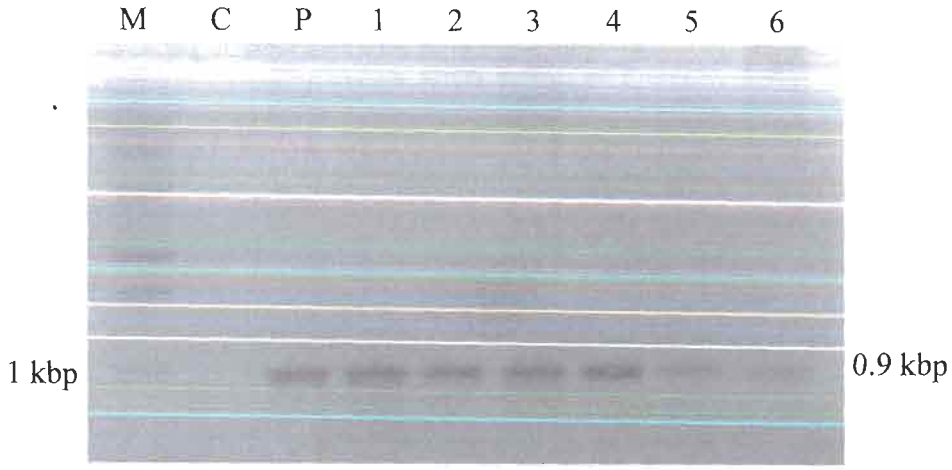

Plate 7. PCR amplification of *hph* gene in hygromycin resistant calli of different indica cultivars

M, 1kb ladder; C, negative control (non-transformed calli); P, positive control (pRQ6); Lane1, Pusa Basamati1; Lane 2, IR50; Lane 3, Co46; Lane 4, TKM9; Lane 5, TKM10; Lane 6, ASD16

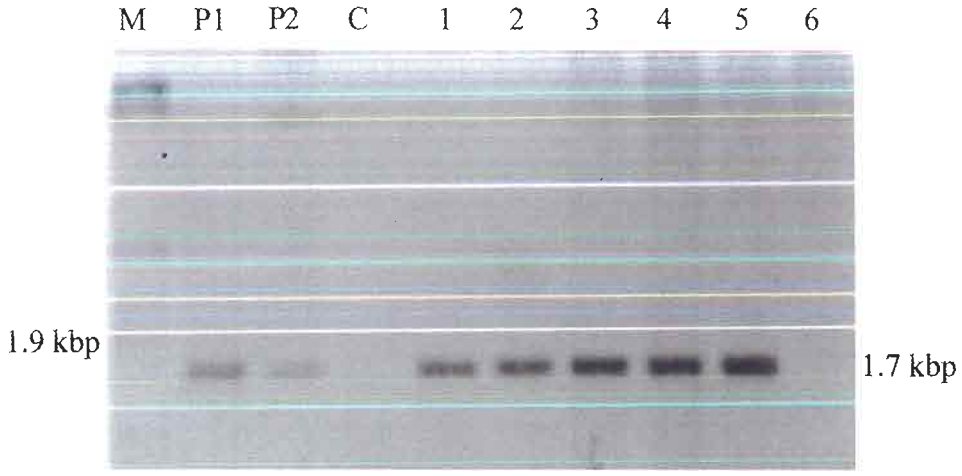

Plate 8. PCR amplification of *gusA* gene in hygromycin resistant calli of different indica cultivars

M, λ double digest; P1, positive control (pAHC27); P2, positive control (pUC19/RGN); C, negative control (non-transformed calli); Lane1, Pusa Basamati1; Lane 2, IR50; Lane 3, Co46; Lane 4, TKM9; Lane 5, TKM10; Lane 6, ASD16

Table 3: Transformation of calli derived from suspension cultures with *A. tumefaciens* LBA4404 (pTOK233)

Genotype	No. of callus cocultivated	No. of callus lines after two rounds of selection on hygromycin	No. of lines regenerated
Co46	90	20	-
TKM9	105	19	-
Pusa Basmati1	86	25	2
ASD16	110	21	-
TKM10	90	15	-
IR50	95	14	-

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Table 4: Mature seed-derived callus of Pusa Basmati1 cocultivated with *A. tumefaciens* LBA4404 (pTOK233)

Cocultivation method	Expt. No.	No. of explants cocultivated	Percentage calli showing transient GUS activity	No. of callus lines selected during first round of selection on hygromycin	No. of callus lines obtained after four rounds of selection on hygromycin	No. of callus lines exhibiting stable GUS activity	No. of lines regenerated	Frequency of transformation (%)
Rashid *et al.* (1996) (AgR) method	I	172	55	20	9	8	6	3.50
	II	189	57	29	13	11	9	4.80
Modification of Rashid *et al.* (1996) (mAgR) method	I	160	46	45	18	16	14	8.75
	II	195	42	56	24	20	19	9.70
	III	85	26	85	22	22	22	25.8

AGRICULTURAL

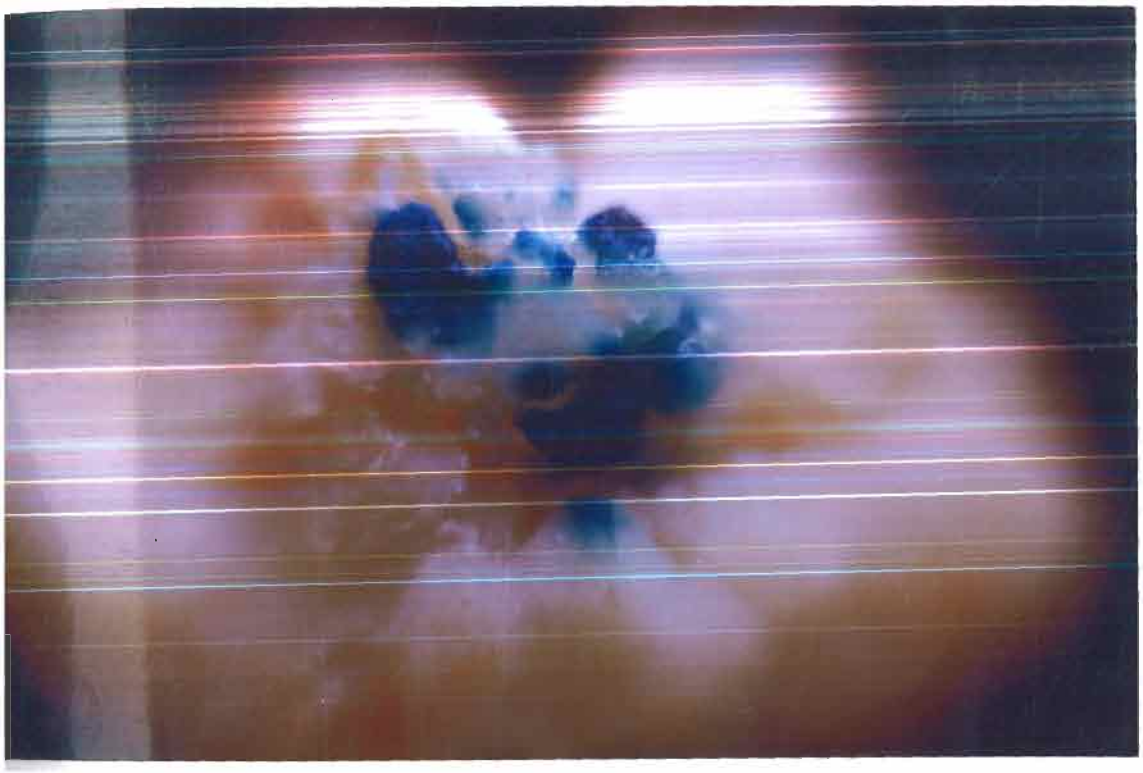
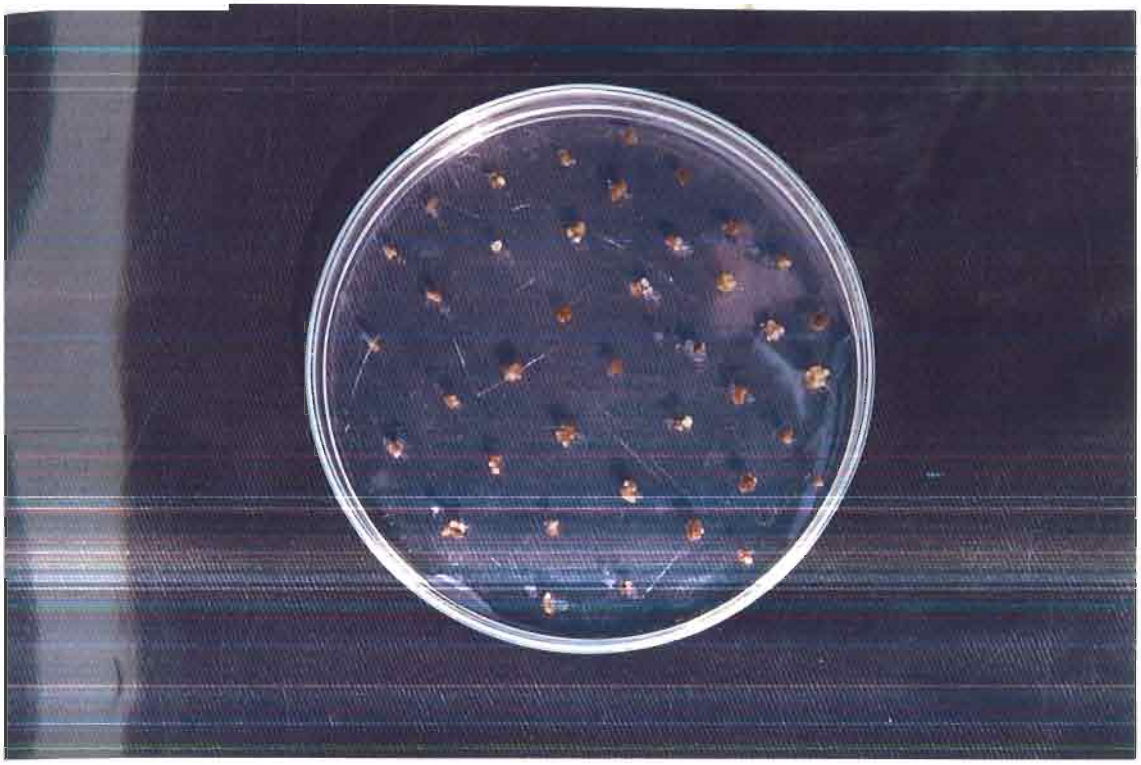
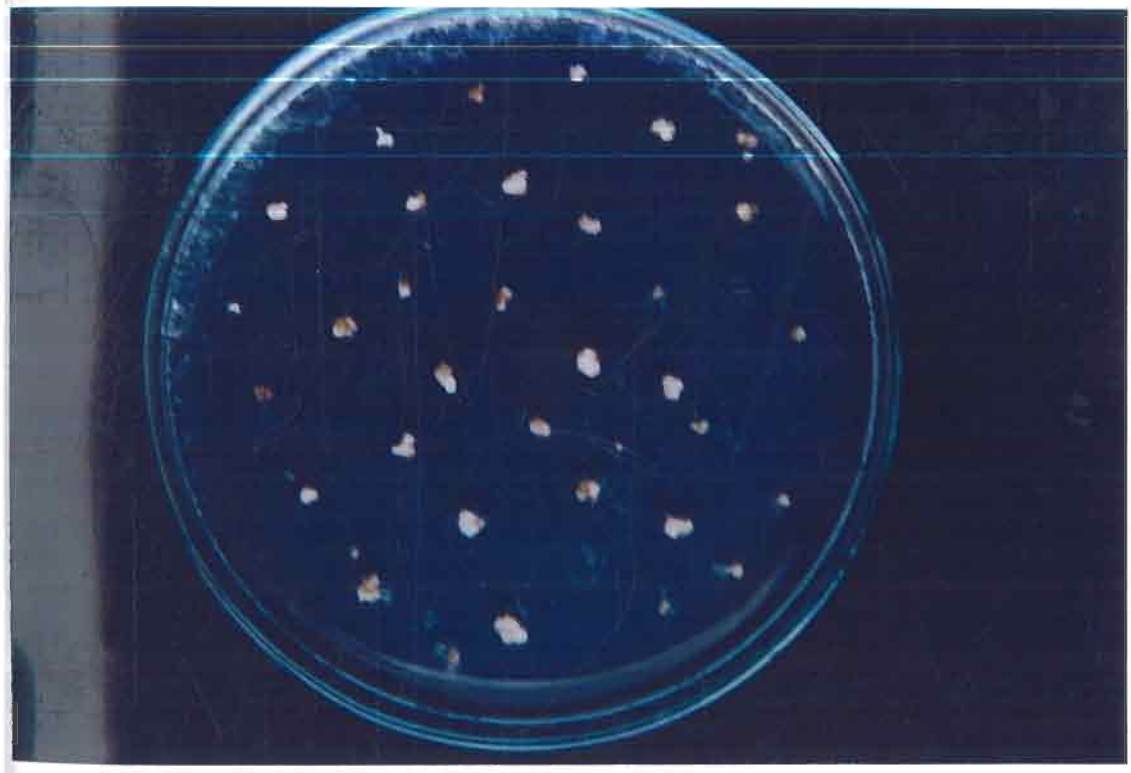


Plate 9.Transient GUS assay in Pusa Basmati1 callus 72 hr after cocultivation with LBA4404 (pTOK233)



a. Rashid method (AgR)



b. Modified Rashid method (mAgR)

Plate 10. Pusa Basmati1 calli during the first round of selection on hygromycin

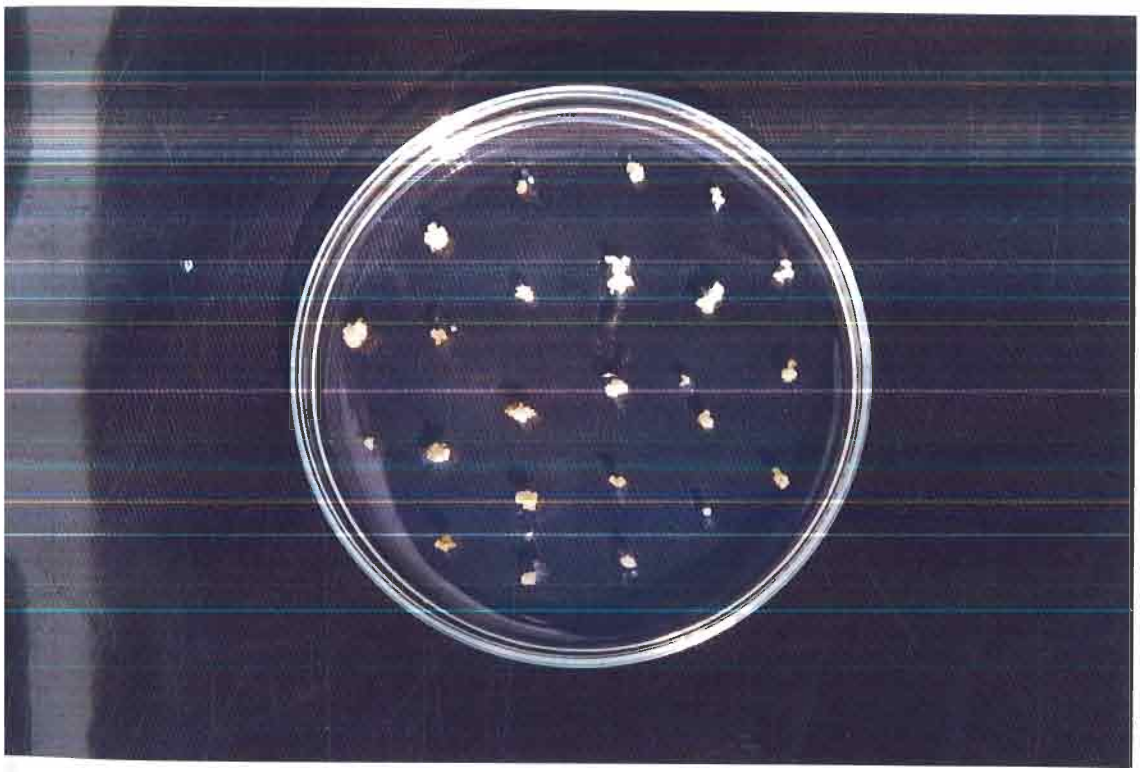


Plate 11. Proliferation Pusa Basmati1 calli during the third round of selection on hygromycin

lines proved GUS-positive in histochemical assays when AgR method was followed, while it was 91 % when mAgR method was used (Plate 12 and Table 4). Ninety five per cent of the GUS-positive lines were regenerated into whole plants using mAgR method, whereas it was only 75% when AgR method was followed (Plate 13). Transformation efficiency in Pusa Basmati1 ranged from 3.5 to 4.8 % in case of AgR method whereas it was 8.8 to 25.8% with mAgR method (Table 4). However, callus lines of IR50, White Ponni, ADT38 and ASD16 exhibited neither stable GUS expression nor did they regenerate into plantlets (Table 5).

4.2.2. Biochemical and Molecular analyses of transgenic plants

4.2.2.1. GUS assay

Histochemical GUS assays were performed in leaf and root tissues from 70 lines of putative T_0 Pusa Basmati1 transformants (Plate 14-15). All of them were GUS positive.

4.2.2.2. PCR assays

Total DNA isolated from leaf tissues of putative transformants (T_0) was subjected to polymerase chain reaction for selective amplification of *hph* and *gusA* (Plates 16-17). All 7 of them proved positive for the presence of *hph* gene, and all the five of them proved positive for the presence of *gusA* gene.

4.2.2.3. Southern hybridization analysis

Putative transformed plants (T_0) recovered from six callus lines were studied in this experiment.

4.2.2.3.1. Experiment I

This experiment was performed with a view to check stable integration of *gusA* in the putative transgenic (T_0) lines. DNA extracted from those lines, untransformed control plant and *A. tumefaciens* [LBA4404 (pToK233)] were digested with *Hin*dIII and the products transferred onto a nylon membrane. When a 3.1 kbp *gusA* gene fragment was used to probe the blot, a band of size 3.1 kbp

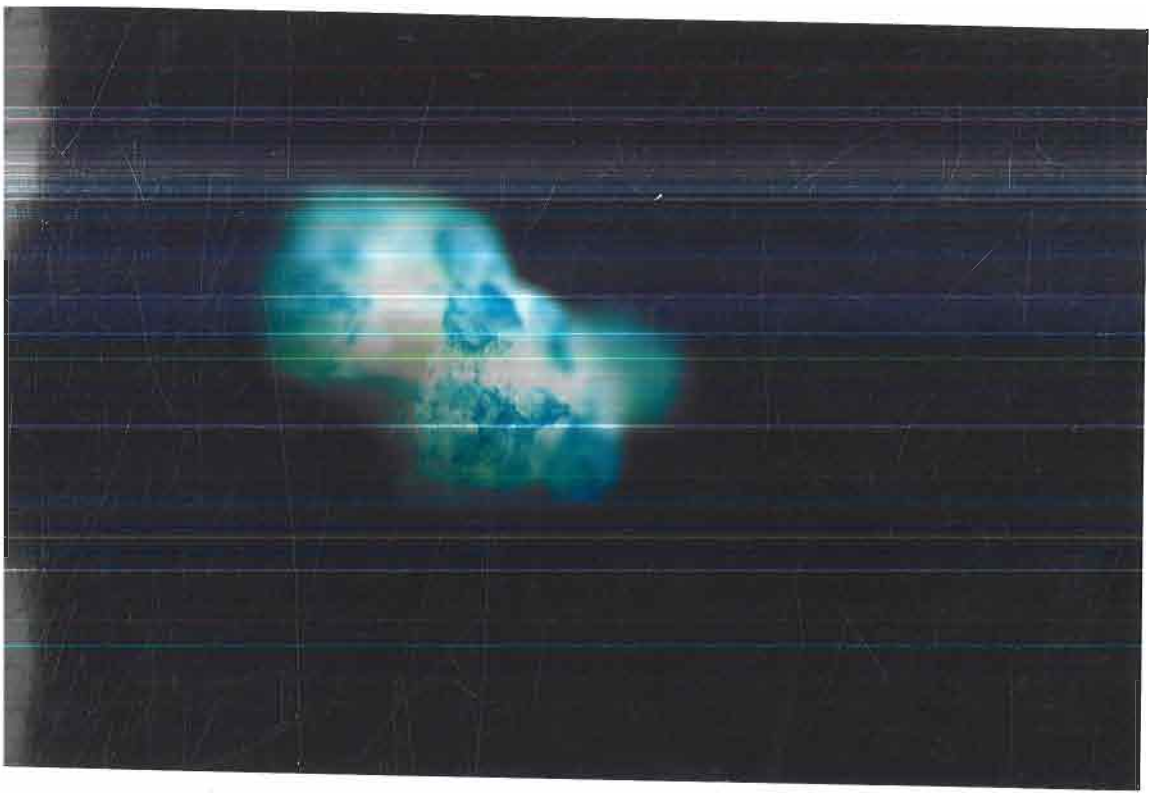


Plate 12. Stable GUS assay in Pusa Basmati1 calli after four rounds of selection on hygromycin

Plate 13. Pusa Basmati1 plantlets regenerating on hygromycin

Table 5: Transformation of local elite rice cultivars with LBA4404 (pTOK233)

Genotype	No. of calli used in cocultivation.	Percentage calli showing transient GUS activity	No. of callus lines selected on hygromycin during the fourth round	No. of callus lines regenerated
White Ponni	140	5.2	20	-
	126	4.2	24	-
IR50	105	4.1	19	-
	119	5.6	18	-
ASD16	129	3.3	14	-
	144	3.7	17	-
ADT38	120	4.9	12	-
	136	5.9	19	-

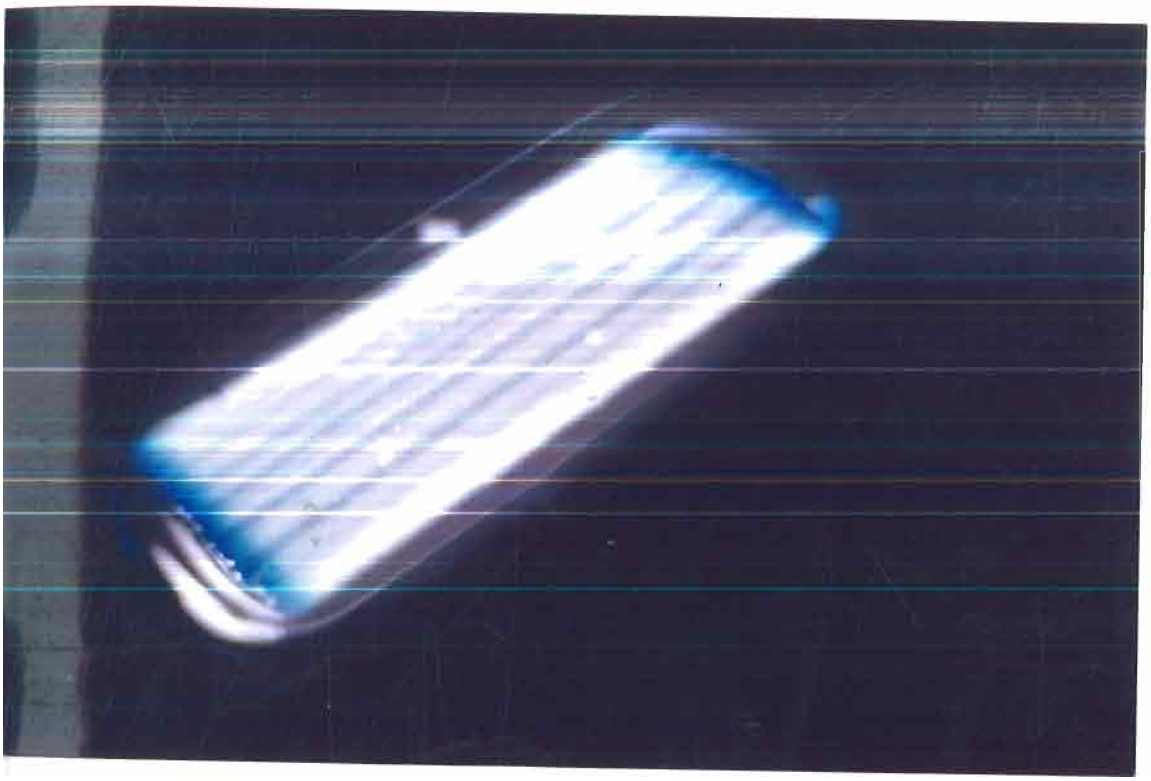


Plate 14. Stable GUS assay in the leaf from T_0 Pusa Basmati1 plants

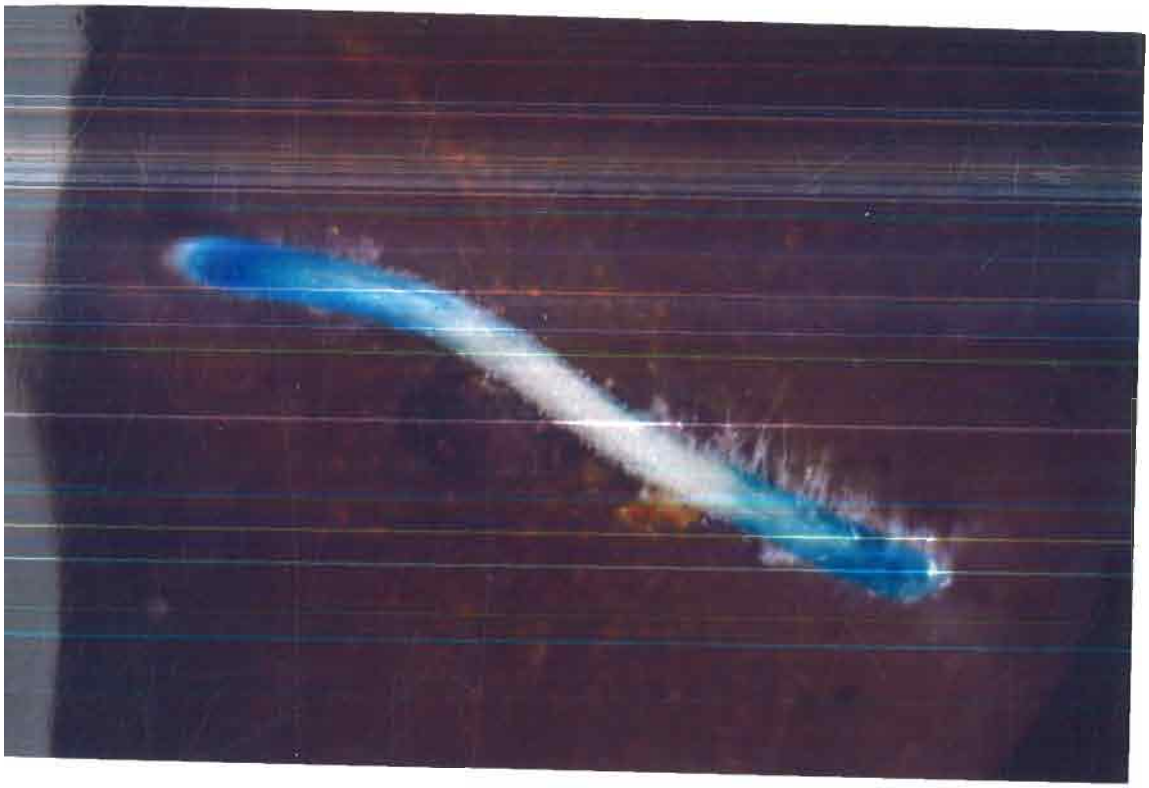


Plate 15. Stable GUS assay in a root tissue of T_0 Pusa Basmati1 plant.

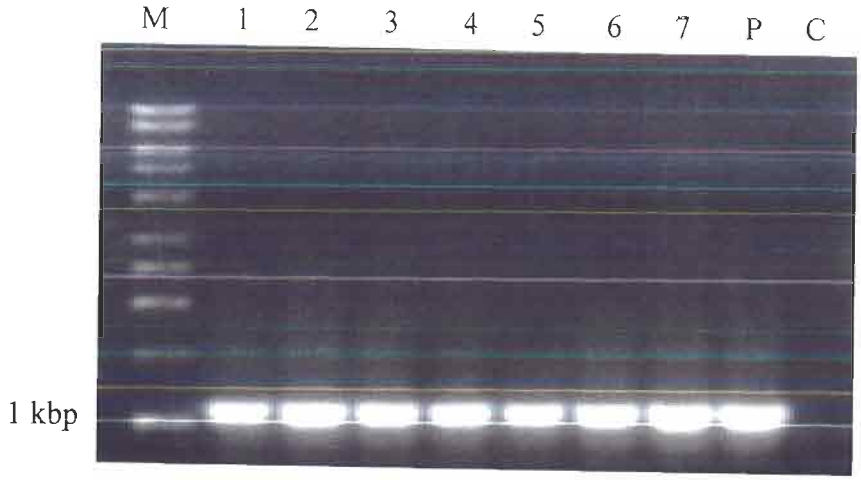


Plate 16. PCR analysis of *hph* gene in putative transgenic T_0 Pusa Basmati1 lines

M, 1 kbp ladder; Lane 1-7, putative transformed lines; P, positive control (pRQ6); C, negative control (non-transformed Pusa Basmati1)

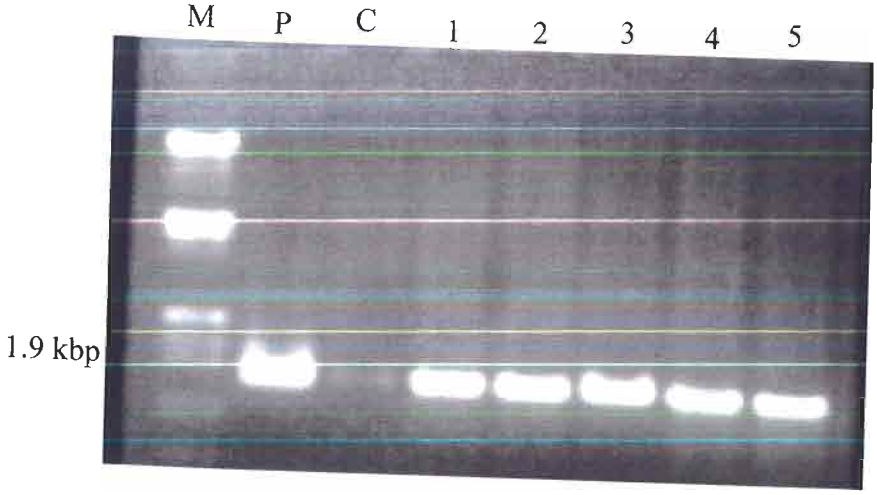


Plate 17. PCR analysis of *gusA* gene in putative transgenic T_0 Pusa Basmati1 lines

M, λ double digest; P, positive control (pRQ6); C, negative control (non-transformed Pusa Basmati1); Lane 1-5, putative transformed lines

was associated with all putative transformants and *A. tumefaciens* (Plate 18), While such a 3.1 kbp band was not associated with the untransformed plants.

4.2.2.3.2. Experiment II

A Southern blot of genomic DNA extracted from six putative transformants, subsequently digested with *Hin*dIII and probed with a 2 kbp fragment (representing the *nptII gene, nos* promoter and *nos* terminator) is presented in Plate 19. The analysis resulted in a hybridisation band of size 5.1 kbp in *A. tumefaciens*. Hybridisation bands of size larger than 5.1 kbp were associated with all putative transformants confirming that all the bands seen in the blot were due to true T-DNA integration. No such a band was observed in the untransformed control plant. A single hybridisation band was observed in the plant # 2 indicating the phenomenon of single copy integration. However, a faint band of size 11 kbp was also observed above the dark intense band. Plant # 6 exhibited two hybridisation bands indicating the presence of two-copies of the transgenes. Plant #1, 3 and 5 were associated with three copies of the transgenes. However, in these three plants the pattern of hybridisation bands was similar. A four-copy integration of the transgene was evident in the plant # 4. A faint band of size 3.8 kbp was also associated with all the plants analysed.

4.2.3. Progeny analysis

All T_0 lines were fertile and there was no discernible difference in seed production or maturation as compared to the control plants (data not shown). Histochemical GUS assays on T_0 seed and T_1 progeny plants were positive (Plates 20–21). GUS assay was performed on individual T_1 progenies of some of the transformed lines to study the segregation pattern of the *gusA* marker gene (Table 6). Segregation pattern of GUS ranged from 13:1 to 20:1 in T_1 plants. Similar results were obtained in PCR assays for *hph* transgene in progeny plants.

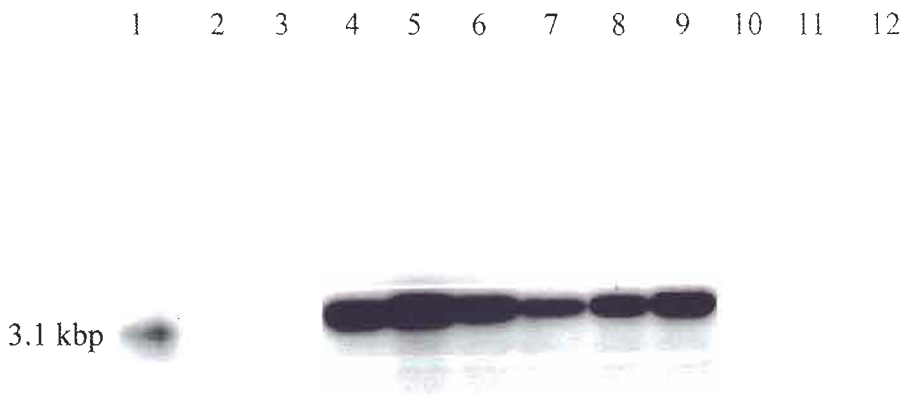


Plate 18. Southern analysis of genomic DNA of Pusa Basmati1 plants transformed with *A. tumefaciens* LBA4404 (pTOK233)

Lane 1, unlabelled probe DNA; Lane 2, lambda *Hin*dIII DNA marker; Lane 3, negative control (non-transformed Pusa Basmati1); Lane 4-9, putative transformed plants 1# to 6#; Lane 10-12, Positive control (Total DNA of LBA4404 (pTOK233))

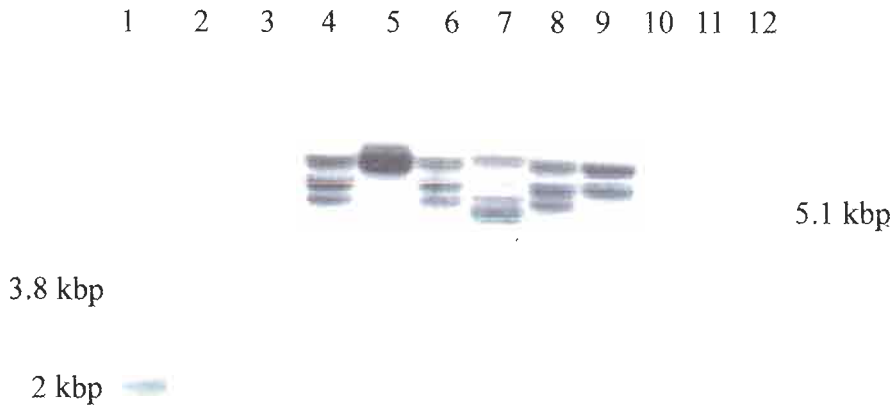


Plate 19. Southern analysis of genomic DNA of Pusa Basmati1 plants transformed with *A. tumefaciens* LBA4404 (pTOK233)

Lane 1, unlabelled probe DNA; Lane 2, λ *Hin*dIII DNA marker; Lane 3, negative control (non-transformed Pusa Basmati1); Lane 4-9, putative transformed plants 1# to 6#; Lane 10-12, positive control (Total DNA of LBA4404 (pTOK233))

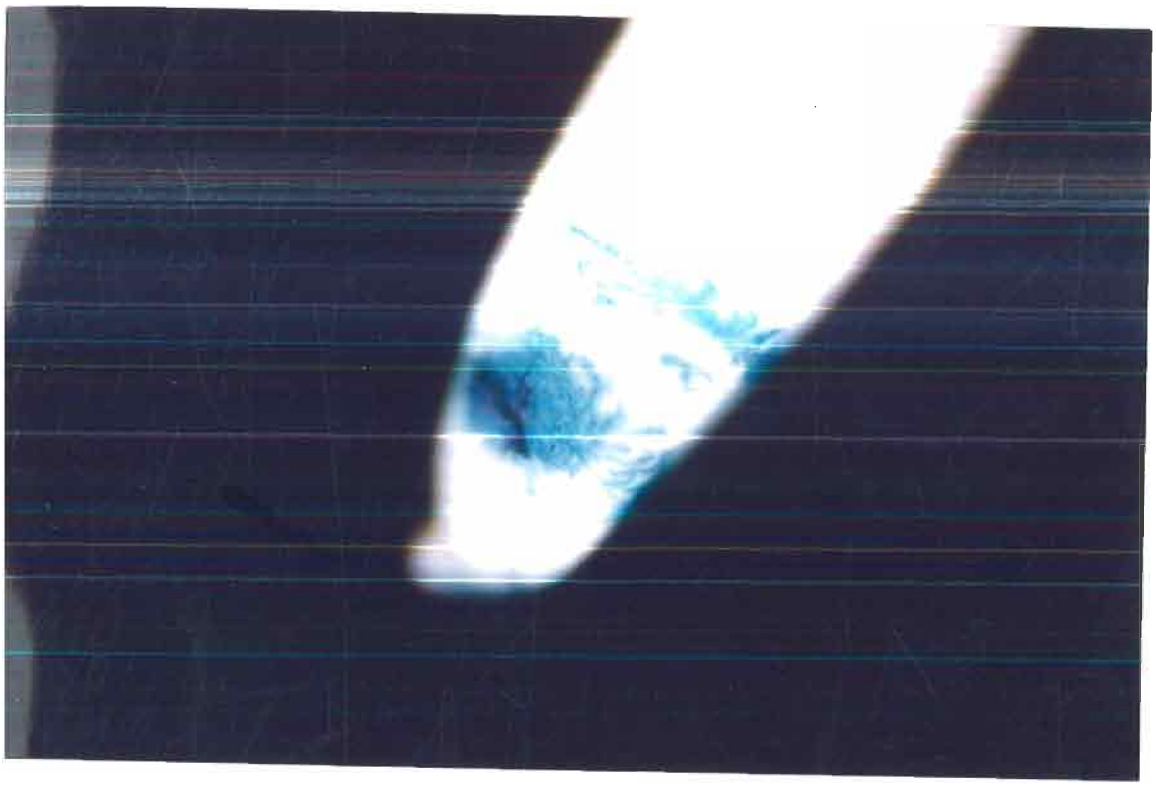


Plate 20. Stable GUS assay in Pusa Basmati1 T_0 seed

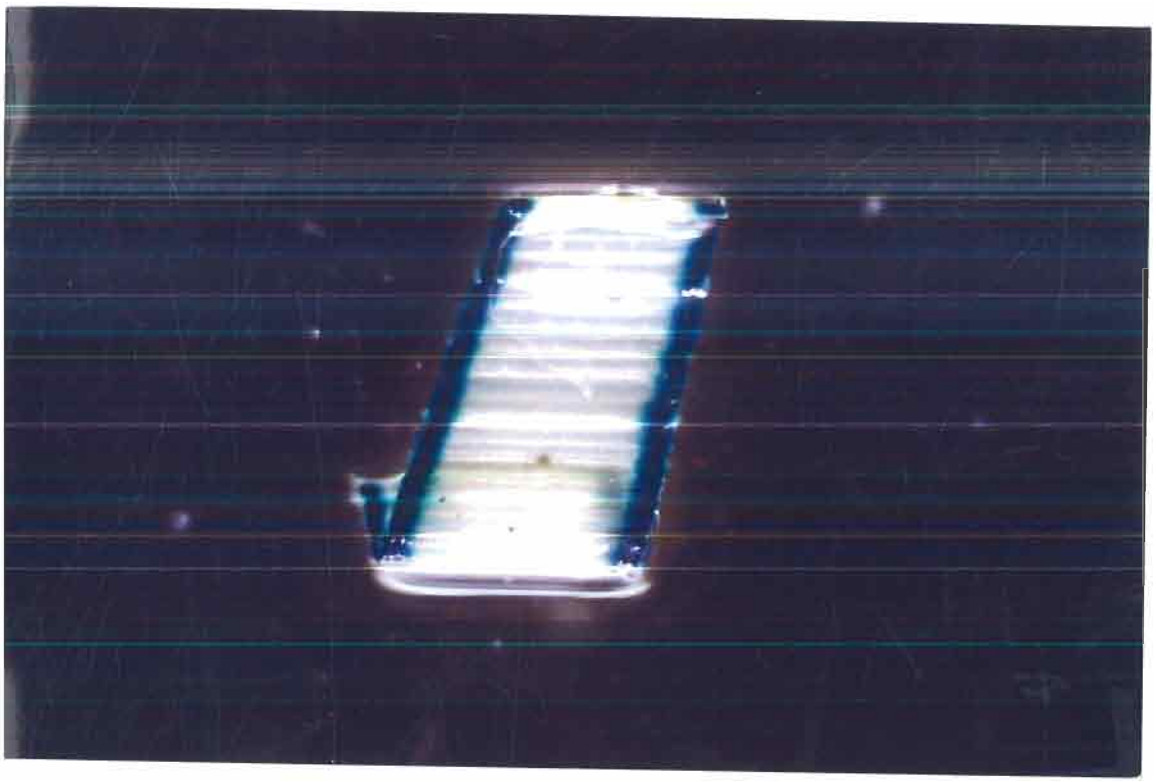


Plate 21. Stable GUS assay in a leaf from a T_1 Pusa Basmati1 plant

Table 6: GUS assay of T_1 progeny plants

T_1 Lines	GUS-positive	GUS-negative	Segregation ratio
PB1.3	25	2	12.5:1
PB1.5	27	2	13.5:1
PB1.6	20	1	20:1

4.3. Traqnsformation of rice genotypes with rice chitinase gene, *chi11*

4.3.1. Construction of *A. tumefaciens* binary vector containing *chi11*

The plasmid pCAMBAR*chi11* when digested with *Hin*dIII released a 3.2 kbp fragment consisting *chi11* cDNA clone flanked by ubiquitin promoter and polyA terminator on the upstream and downstream sides respectively (Fig.2). The fragment was cloned into the *Hin*dIII site of multiple cloning site of pCAMBIA1301. The ligated product was amplified in *E. coli* (DH5α strain) cells, which were plated on LB medium containing kanamycin (50 µg/ml) and X-gal (40 µg/ml). Seven individual recombinant colonies (white colonies) were obtained. Plasmid DNA isolated from four of such clones, when restricted with *Hin*dIII, released a 3.2 kbp fragment (Plate 22). To confirm the orientation, two of them were selected and digested with *Bgl*II and *Bam*HI. This digestion released 3.0 kbp and 2.0 kbp fragment in both clones. The correct orientation is shown in Fig. 5. One among these four clones was selected and the plasmid isolated was named pMKU-RF2.

4.3.2. Mobilization of binary vector into *A. tumefaciens*

4.3.2.1. Mobilization of pMKU-RF2 into *A. tumefaciens* strain EHA105

Presence of plasmid pMKU-RF2 in EHA105, after triparental mating, was confirmed by a non-radioactive (DIG) Southern hybridisation. Total DNA isolated from six individual transconjugants, positive and negative controls were digested with *Bam*HI, transferred to a nylon membrane and probed with a DIG-labelled 3.2 kbp *Hin*dIII fragment (*chi11* cassette released from pCAMBAR-*chi11*). The probe was specifically hybridized to 2 kbp and 13 kbp fragments of the transconjugants and positive control (EHA105-pMKU-RF2) as shown in the autoradiogram (Plate 23). No band was observed in untransformed control.

4.3.2.2. Mobilization of pJRB3 into *Agrobacterium* strain LBA4404

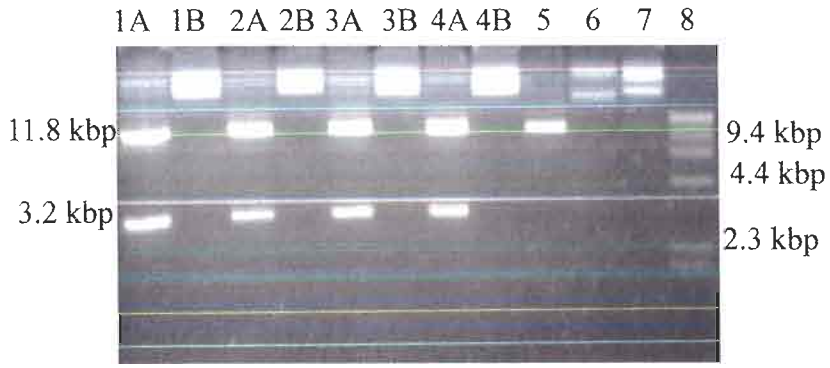


Plate 22. Restriction analysis of recombinant clones containing *chi*11 cassette.

Lane 1A to 4A, *Hin*dIII digest of clones 1-4; Lane 1B to 4B, undigested clones 1to 4; Lane 5, *Hin*dIII digest of pCAMBIA 1301, Lane 6, pCAMBIA 1301 undigested; Lane 7, pCAMBAR.*chi*11 undigested; Lane 8, λ *Hin*dIII DNA marker

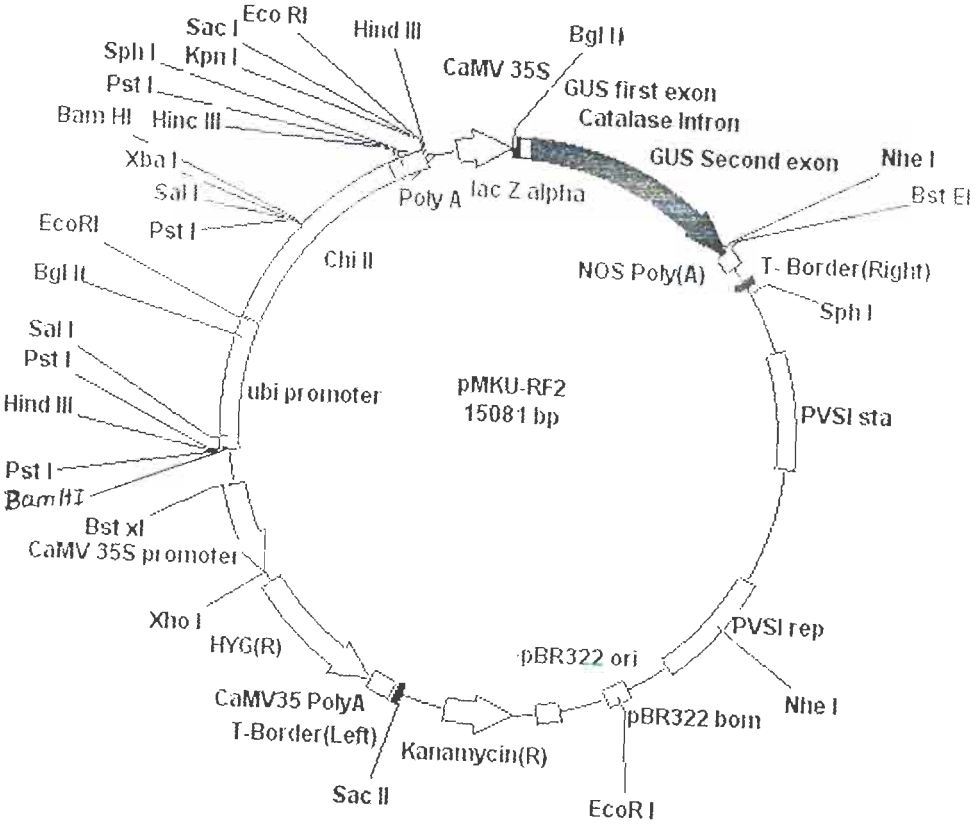
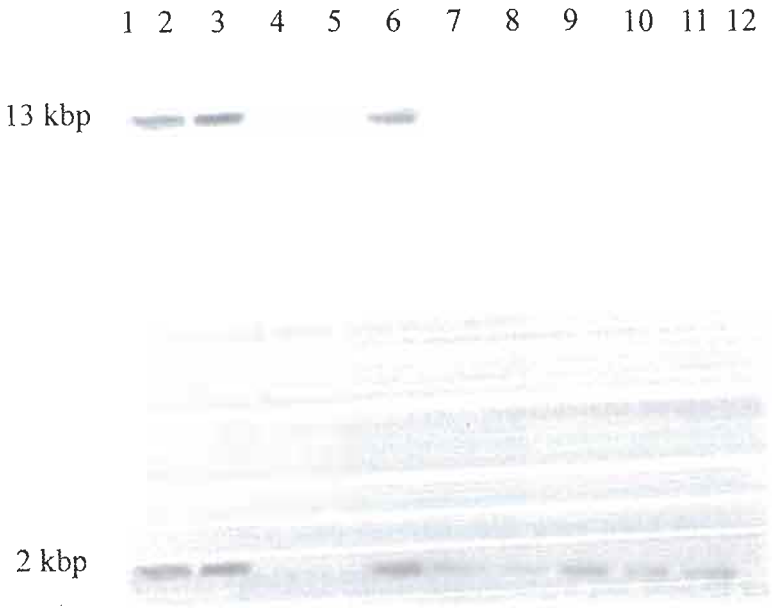


Fig.5. Physical map of pMKU-RF2



Plaste 23. Southern blot (non-radioactive) analysis of the transconjugants, EHA105 (pMKU-RF2).

Lane1, λ *Hin*dIII marker DNA; Lane 2-3, positive control Plasmid pMKU-RF2 digested with *Bam*HI; Lane 4, negative control total DNA of *Agrobacterium* helper strain EHA105 digested with *Bam*HI; Lane 5, uncut transconjugant1; Lane 6-11, total DNA of transconjugant TC1 to TC6 digested with *Bam*HI. Lane 12, 1 kbp ladder. The blot was probed with 3.2 kb DIG- labelled *Hin*dIII fragement containing *chi11* gene cassette.

The binary vector pJRB3 containing *chi11* cDNA flanked by 35S CaMV promoter and *nos* terminator was mobilized into *Agrobacterium* helper strain LBA4404 by triparental mating. The presence of the binary vector pJRB3 in *Agrobacterium* stain LBA4404 was confirmed by PCR assay of *gusA* gene (Plate 24).

4.3.3. Transformation of rice genotypes

4.3.3.1. Transformation of local elite rice cultivars.

Immature embryos isolated from immature seeds (collected 12-14 d after pollination) of ASD16, White Ponni, ADT38 and IR50 were cocultivated with LBA4404 (pJRB3) and EHA105 (pMKU-RF2). Selection of regenerants was made on hygromycin (30 mg/l) (Table 7 and 8). Molecular analysis of the recovered plants showed that they were non-transformants (data not shown).

4. 3. 3. 2. Transformation of Pusa Basmati1 with *A. tumefaciens* strain EHA105 (pMKU-RF2)

In this experiment, mature seed derived calli were used for cocultivation with *A. tumefaciens* EHA105 (pMKU-RF2). Eighty eight per cent of callus lines that survived four rounds of selection (Plate 25) were positive for stable GUS assay (Plate 26). Fifty seven per cent of the GUS-positive lines regenerated into whole plants (Table 9 and Plate 29-30).

4.3.4. Biochemical analyses

4.3.4.1. GUS assay

Histochemical GUS assay was carried out in putative T_0 lines of Pusa Basmati1 and all recovered plants were found to be positive.(Plate 27).

4.3.4.2. Western blot analysis

To demonstrate stable integration and expression of rice chitinase transgene in putative transformed plants, Western blot analysis was performed with five randomly selected independent T_0 lines (Plate 28). All the five

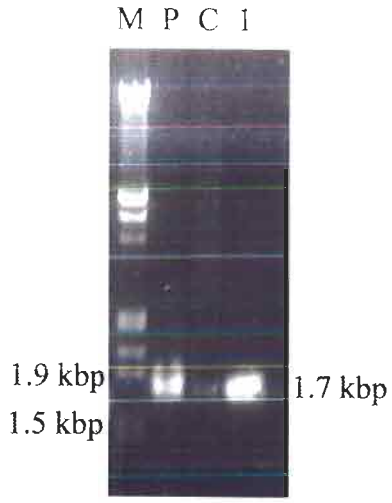


Plate 24: PCR amplification of the *gusA* gene in transconjugant, LBA4404(pJRB3)

M, λ double digest (*Eco*RI and *Hin*dIII); P, positive control (pJRB3); C,negative control (LBA4404 total DNA); Lane 1, transconjugant [LBA4404 (pJRB3)]

Table 7: Transformation of IR50, White Ponni, ASD16, and ADT38 with LBA4404 (pJRB3)

Genotype	No. of Immature embrysed for cocultivation	No. of callus selected during first selection on hygromycin	No. of callus lines selected after four round of selection on hygromycin	No. of lines regenerated	Percentage efficiency
White Ponni	75	15	5	-	-
	90	20	6	2	2.22
	89	14	7	1	1.12
	105	16	9	-	-
IR50	120	20	7	-	-
	78	18	12	2	2.56
	86	16	9	3	3.50
	95	25	10	-	-
ASD16	80	20	10	1	1.25
	89	17	7	2	2.24
ADT38	65	15	5	2	3.07
	72	12	6	1	1.38

Table 8: Transformation of IR50, White Ponni, ASD16, and ADT38 with *A. tumefaciens* EHA105 (pMKU-RF2) containing rice chitinase gene

Genotype	No. of immature embryo used for cocultivation	No. of callus selected during first selection on hygromycin	No. of callus lines selected after the fourth round of selection on hygromycin	No. of lines regenerated	Percentage efficiency
White Ponni	87	17	7	2	2.3
	93	16	7	3	3.2
IR50	74	12	8	3	4.1
	69	9	6	4	5.8
ADT38	83	9	6	2	2.4
	87	11	8	5	5.7
ASD16	63	8	4	2	3.2
	76	10	7	2	2.6

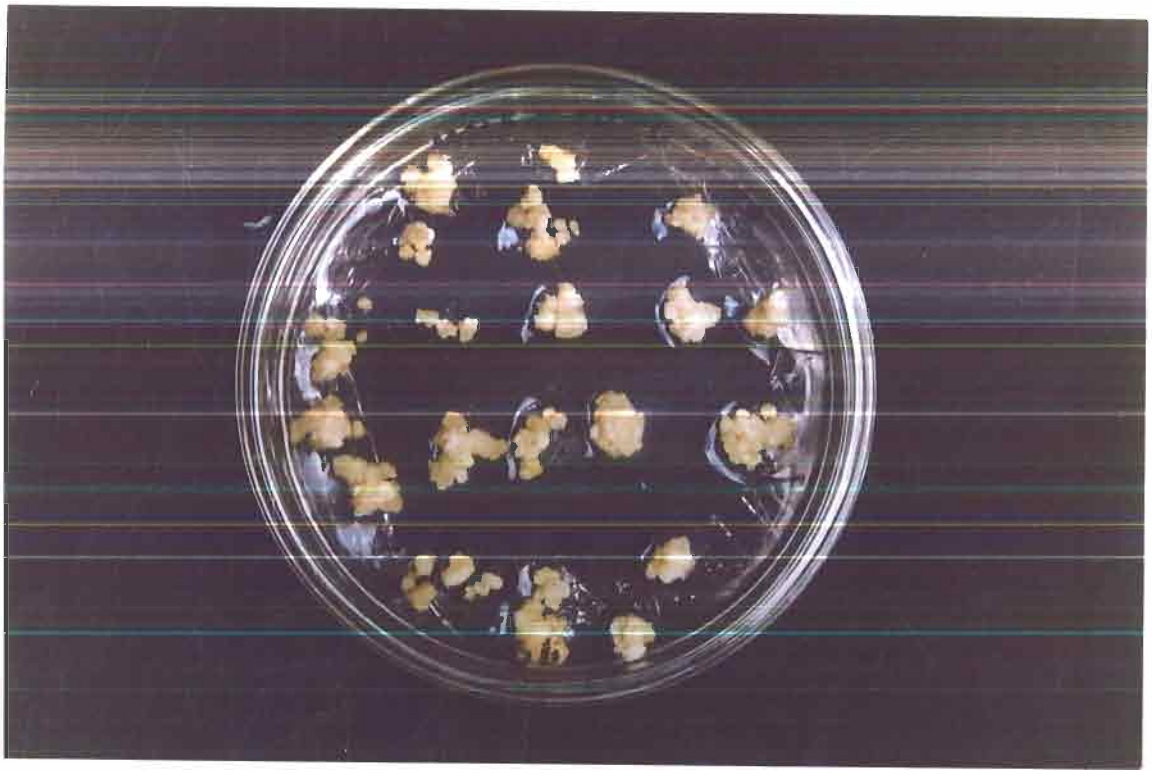


Plate 25. Proliferating Pusa Basmati1 calli during the fourth round of selection on hygromycin

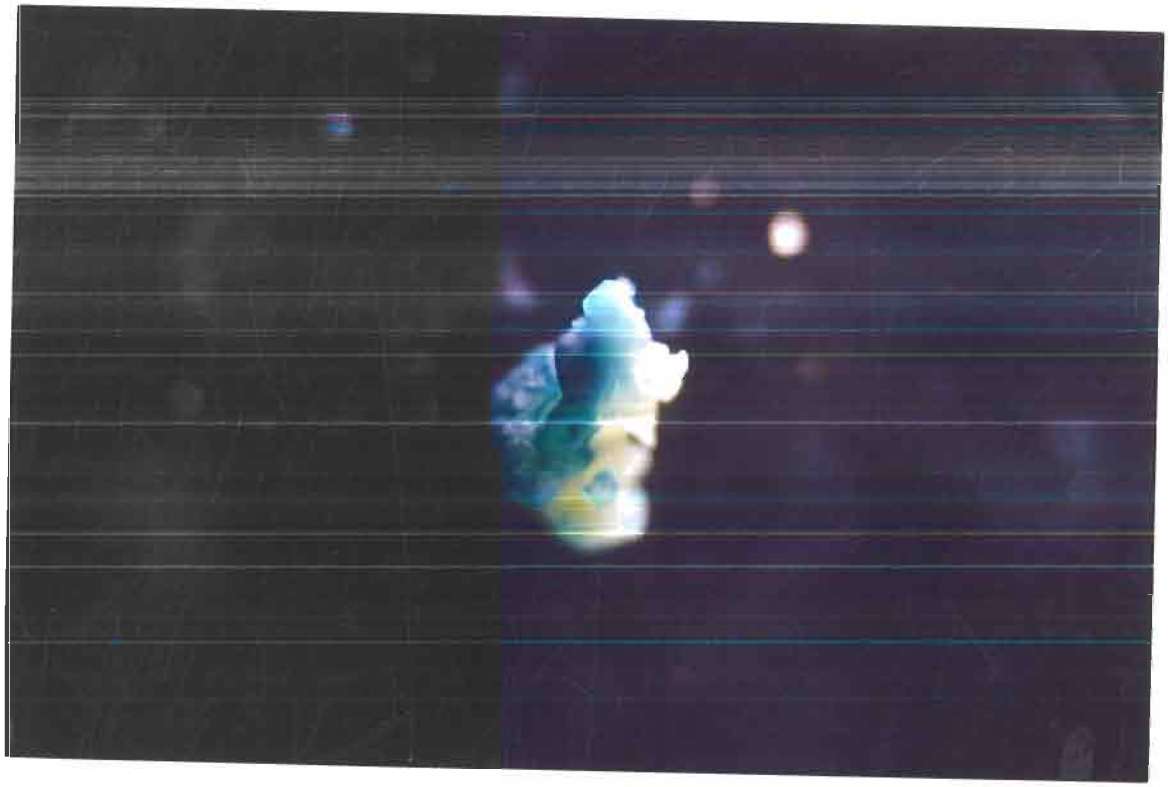


Plate 26. Stable GUS assay in putative transformed calli of Pusa Basmati1

Table 9: Transformation of Pusa Basmati1 with *A. tumefaciens* EHA105 (pMKU-RF2) containing the rice chitinase gene*

No. of calli used in cocultivation	No. of callus line selected after the fourth round of selection on hygromycin	No. of callus lines showing stable GUS activity	No. of lines regenerated	Transformation efficiency (%)
94	24	21	12	13

* using mAgR method

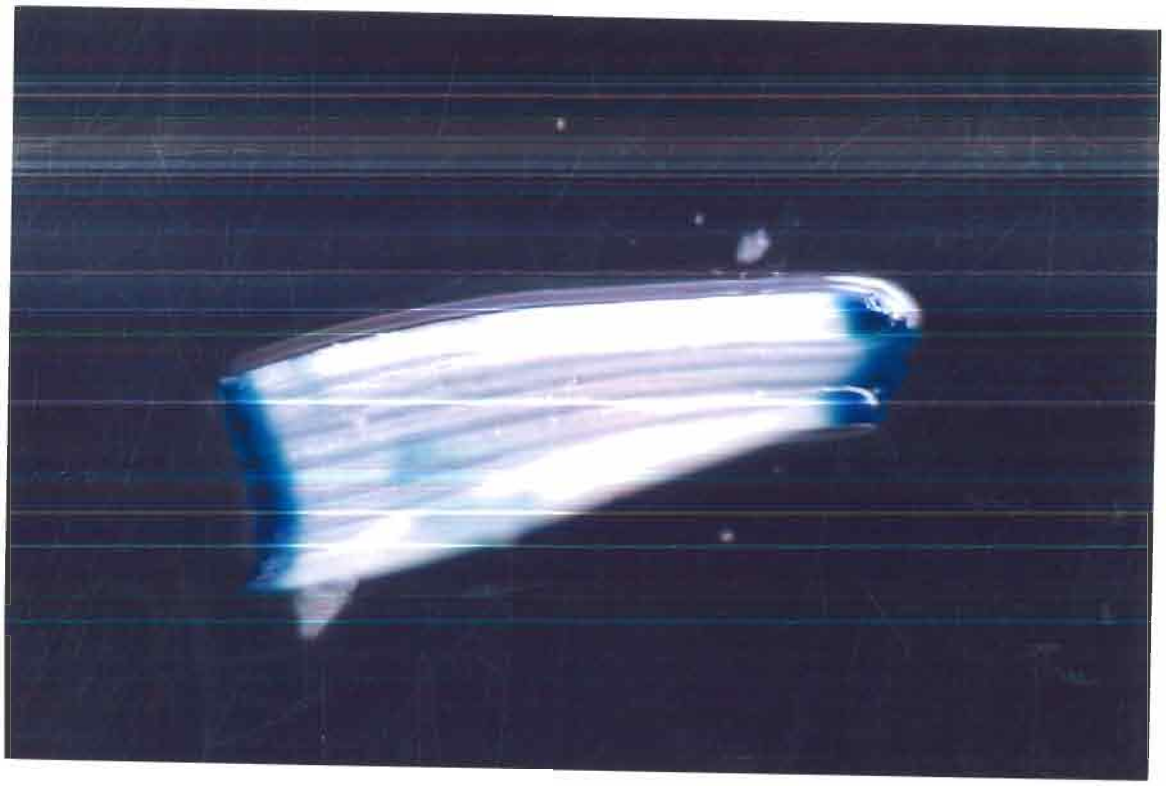


Plate 27. GUS assay on a leaf from putative transgenic Pusa Basmati1 transformed with EHA105 (pMKU-RF2)

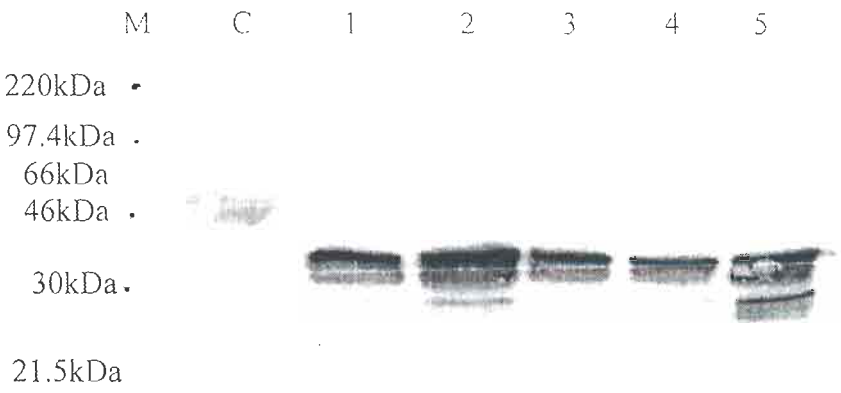


Plate 28. Western blot analysis of chitinase in T_0 Pusa Basmati 1 rice plants

M, Rainbow protein marker (BioRad); C, negative control (non-transformed Pusa Basmati 1); Lane 1-5, putative transformants of Pusa Basmati 1

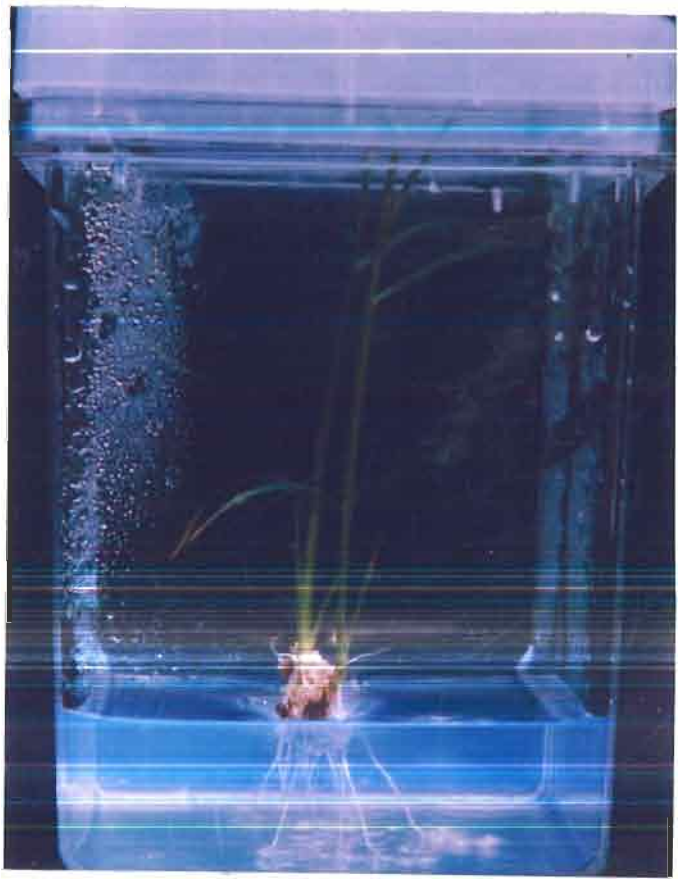


Plate 29: Pusa Basmati1 putative transgenic plant in the rooting medium.

Plate 30: Pusa Basmati1 putative transgenic plants in pots.

transformants tested in this analysis were originally positive for histochemical GUS assay. Results of this experiment showed the presence of one immunologically related polypeptide with an apparent molecular weight of 35 kDa in all the transgenic plants. Another immunologically related polypeptide with the molecular weight one 30 kDa was also seen in all the transgenic plants with varying intensities. However, both bands were missing in the non-transformed Pusa Basmati1 (negative control).

Discussion

5. Discussion

Plant genetic engineering has now become one of the important tools for the genetic improvement of crop plants. *Agrobacterium*-mediated transformation was extensively used mostly for transforming dicotyledonous plants alone during the recent past. Since monocotyledons are not a natural host of *A. tumefaciens*, various other direct DNA delivery methods such as protoplast-based transformation (Toriyama *et al.*, 1988; Zhang and Wu, 1988), electroporation (Shimoda *et al.*, 1990; Toriyama *et al.*, 1988) and particle bombardment method (Christou *et al.*, 1991, 1992; Zhang *et al.*, 1996) are used for transforming rice. All the above direct DNA delivery methods suffer from integration of multiple copies of foreign gene, which leads to sterility (Finnegan and McElroy, 1994). With a view to overcoming these limitations, *Agrobacterium*-mediated transformation of rice is attempted in many laboratories the world over.

Use of *Agrobacterium*-mediated gene transfer in rice appears to have several desirable features such as a high efficiency of transformation, transfer of pieces of DNA with defined ends, and the ease of transferring of relatively larger segments of target DNA efficiently, besides introducing fewer copies of the gene of interest, the number being critical to avoid gene silencing (Hiei *et al.*, 1994). Susceptibility of rice to agrobacterial infection was first demonstrated by Raineri *et al.* (1990), while Chan *et al.* (1993) obtained the first *Agrobacterium*-transformed transgenic rice plant. Hiei *et al.* (1994) used a super-binary vector system to transform *japonica* rices and demonstrated inheritance of transgenes in subsequent generations. Later, Rashid *et al.* (1996) and Aldemita and Hodges (1996) transformed *indica* rice varieties using similar methods based on Hiei *et al.* (1994). Although a large number of *japonica* rice varieties were efficiently transformed by *Agrobacterium* (Hiei *et al.*, 1994), only a few *indica* rices like IR72, Basmati370 and Basmati385 were successfully transformed (Aldemita and

Hodges, 1996; and Rashid *et al.*, 1996). Such successes in *Agrobacterium*-mediated transformation of rice could be attributed to use of super-virulent strains or super-binary vectors, use of acetosyringone in the cocultivation medium, use of actively proliferating mature seed-derived callus or immature embryo as explants and use of hygromycin in the place of kanamycin as the selective agent (Hiei *et al.*, 1994; Rashid *et al.*, 1996; Aldemita and Hodges, 1996). In the present study, an attempt was made to standardize *Agrobacterium*-mediated transformation of local *indica* genotypes popular among farmers of the state using standard marker genes such as, *gusA* and *hph*. Such an optimized protocol developed in the present study was subsequently used to transform Pusa Basmati1, another *indica* rice, with an agronomically important gene namely, *chi11*, a rice chitinase gene which has been implicated in rice sheath blight resistance (Lin *et al.*, 1995).

5.1. Transformation of callus derived from rice suspension culture with LBA4404 (pTOK233)

Embryogenic suspension cells were successfully used to transform both *japonica* and *indica* genotypes using particle bombardment or *Agrobacterium*-mediated transformation methods (Zhang *et al.*, 1996; Hiei *et al.*, 1994). In the present study, Pusa Basmati1 was successfully transformed through *Agrobacterium*-mediated transformation using mature seed-derived suspension cultures (Table 3). Two to three month-old embryogenic suspension cell clumps of ASD16, IR50, TKM9, TKM10, Co46 and Pusa Basamti1 were cocultivated with LBA4404 harbouring pTOK233, following a modification of the protocol developed by Rashid *et al.* (1996). The vector system was developed by Hiei *et al.* (1994) to transform *japonica* varieties using mature seed-derived suspension cultures and a transformation efficiency as high as 9% in the genotype Nipponbare. In the present study, an improved protocol based on Hiei *et al.* (1994) or Rashid *et al.* (1996) could successfully be used to transform an *indica* genotype namely, Pusa Basmati1 (Table 3), though the transformation efficiency was found to be lower than that of Hiei *et al.* (1994).

In order to enhance agrobacterial infection, at least two pre-treatment methods namely, enzymatic digestion of rice cell walls (Chen *et al.*, 1992) or mechanical wounding (Raineri *et al.*, 1990) could be used. In the present study, pre-treatment of embryogenic suspension cells with carborundum (an abrasive known to make fine wounds on plant surfaces) enhanced the transient GUS activity of most of the *indica* rice varieties tested (Table 2). However, Mooney and Godwin (1991) demonstrated, in their by electron micrograph studies, that agrobacterial attachment to wheat embryos *in vitro* was not wound-dependent. Though the agrobacterial cells attached themselves to both wounded and unwounded cell surfaces of embryos, a preferential adherence of the bacteria at the wound site was also observed (Mooney and Godwin, 1991).

PCR analyses of transformants evolved in this experiment showed the presence of *gusA* or *hph* gene in some of the callus lines after two rounds of selection on hygromycin. Most of these callus lines did not regenerate into whole plants excepting for two lines of Pusa Basmati1. Possibly a hypersensitive response due to wounding, a higher concentration of hygromycin in the regeneration medium or an unpredictable tissue culture response of the genotypes used could be attributed as reasons for such a failure.

5.2. Transformation of Pusa Basmati1 with LBA4404 (pTOK233) using mature seed-derived callus as explants

With a view to optimizing an *Agrobacterium*-mediated transformation protocol suitable for *indica* rices, diverse *indica* genotypes *viz.*, Pusa Basmati1, IR50, White Ponni, ASD16 and ADT 38 were tried to be transformed with the supervirulent strain, LBA4404 harbouring pTOK233. Excepting for Pusa Basmati1, none of the above genotypes were amenable for such a transformation. The transformation protocols tried in this experiment were basically the one suggested by Rashid *et al.* (1996) (AgR) and a modified one (mAgR) (Section 3.2.1). The basic differences between mAgR and AgR methods were: i.)

Subculturing explants on the callus induction medium 4 d before cocultivation in mAgR method as against an immediate transfer to cocultivation medium in AgR method, ii.) addition of 100 μM acetosyringone into the cocultivation medium in mAgR method as against 50 μM of the chemical used in AgR method. These two factors appeared to have significantly enhanced transformation efficiency of putative transgenic lines of Pusa Basmati1. A transformation efficiency as high as 25.8% was obtained in the present experiment. Though Hiei *et al.* (1994) and Rashid *et al.* (1996) could obtain transformation frequencies of 23% and 22% respectively in Tsukinohikari and Basmati370, the protocol followed appeared to be genotype-dependent. Results obtained by Hiei *et al.* (1994) on Tsukinohikari indicated that the step involving the subculture of callus 4 d prior to cocultivation was indispensable to achieving such a high frequency of transformation. However, Basmati370 could be transformed with a comparable efficiency by Rashid *et al.* (1996) without this step. In this present study, these two modifications in the basic protocol of Rashid *et al.* (1996) appeared to have significantly increased the transformation efficiency of Pusa Basmati1 from 5.3% to 12.3% (as indicated in the frequency of stable GUS-positive plants obtained; Table 4) even though the transient GUS expression frequency in Pusa Basmati1 was considerably lower in mAgR method (38.2% as compared to 56% when AgR method was tried) on the test-variety of the present study. Subculturing for 4 d appeared to have helped the explants switch to an active phase of growth and cocultivation at this phase was found to be more efficient. However, use of mature seed callus explants in cocultivation without subculturing them for 4 d led to commencement of tissue browning with eventual reduction in regeneration on selection medium with hygromycin (Plate 10). Other *indica* genotypes tried in the present study though failed to exhibit stable GUS expression during their callus phase or regenerate into plantlets. Only a very few genotypes like Basmati370 (Rashid *et al.*, 1996), Pusa Basmati1 (Mohanty *et al.*, 1999) and IR72 (Aldemita and Hodges, 1996) have been transformed so far by *Agrobacterium*.

Stable integration of *hph* and *gusA* gene was demonstrated by selective amplification of the transgenes in the recovered plants through PCR (Table 4).

All the recovered plants were PCR-positive for both of these genes. All these plants also proved positive for histochemical GUS expression (Table 4). Stable integration of *gusA* and *npt*II genes was further demonstrated by Southern hybridisation analysis. All putative transformants were positive when the blot was probed with a radioactively labelled *gusA* sequence (Plate 18).

In order to eliminate the possibility of false positive results due to contamination of *Agrobacterium* in putative transformants and to estimate the number of copies of transgenes, the genomic DNA from select putative transgenic plants were digested with *Hin*dIII and probed with an *npt*II sequence. The enzyme, *Hin*dIII has a site in *nptII* gene and another site outside the right border. All the hybridisation bands in the blot was larger than 5.1 kbp indicating that the positive results are due to true T-DNA integration and not due to the contamination of *Agrobacterium.* In majority of the plants tested in this experiment the copy number was more than one. Earlier workers obtained a large proportion of plants with a single copy of transgenes (Hiei *et al.*, 1994; Rashid *et al.*, 1996). In the present study, only one plant possessed a single copy of the transgene. However, all the other plants had copy numbers ranging from 2-4. Aldemita and Hodges (1996) observed that the majority of plants recovered by them possessed 2-3 copies of transgene. In the present study, hybridisation bands of plant # 1, 3 and 5 were found to be similar and these plants could have recovered from the same clone (Plate 19).

With the exception of a few, all the Pusa Basmati1 transgenic plants were fertile and yielded normally as the wild type Pusa Basmati1 plants. Similar observations were made by several other workers too (Hiei *et al.*, 1994; Dong *et al.*, 1996; Aldemita and Hodges, 1996 and Rashid *et al.*, 1996). However, Rashid *et al.* (1996) reported that 11% of the Basmati370 plants were albinos.

Histochemical staining of mature seeds from T_0 plants revealed GUS activity in the embryo and testa but there was little activity in the endosperm. Dong *et al.* (1996) observed the similar pattern of staining in *javanica* rice

transformed with *gusA* gene. Inheritance pattern of progenies for GUS expression in the present study (Table 6) showed a segregation ratio close to 15:1 indicating a two-point integration of *gusA* transgenes at two different chromosomes. Since the number of seeds tested were not sufficiently large to determine the segregation ratio, it was difficult to conclude on the integration pattern. PCR analysis on T_1 generation of Pusa Basmati1 further provided evidence of stable inheritance of the transgene. Stable inheritance of GUS gene in transgenic rice plants was also reported by several workers (Hiei *et al.*, 1994; Rashid *et al.*, 1996; Dong *et al.*, 1996; Aldemita and Hodges, 1996 and Wang *et al.*, 1997).

5.3. Transformation of rice genotype with rice chitinase gene, *chi11*

Chitin, a major structural component of the cell walls of many fungi, is unique in that it is not found in plants (Vidhyasekaran, 1997). Although, physiological role of chitinases in plants is unknown, there is strong correlative evidence that these are defense proteins exhibiting antifungal activity. Low constitutive activities of chitinase found in many plants can be dramatically increased by wounding or fungal or bacterial infection (Majeau *et al.*, 1990; Roby *et al.*, 1990).

Chitinases are PR-3 group of pathogenesis-related (PR) proteins induced in plants. Purified chitinases lyse fungal hyphae *in vitro* (Boller *et al.*, 1983; Dunsmuir and Suslow, 1989). Genes encoding chitinases from heterologous sources were successfully used to evolve transgenic lines expressing chitinases antagonistic to certain fungal pathogens of plants. To date, transgenic lines of tobacco (Brogli *et al.*, 1991; Oppenheim and Chet, 1992; Wang *et al.*, 1996), canola (Benhamou *et al.*, 1993), *Arabidopsis* (Samac and Shah, 1994), potato (Wyke *et al.*, 1994), rice (Lin *et al.*, 1995; Oliva *et al.*, 1996), tomato (Jongedijk *et al.*, 1995; Harikrishna *et al.*, 1996), cucumber (Punja and Raharjo,1996) carrot (Punja and Raharjo, 1996), and *Brassica* (Grison *et al.*, 1996) expressing chitinases have been evolved.

So far, a very few agronomically important genes have been transformed in rice following *Agrobacterium*-mediated method. They include transfer of soybean ferritin gene into rice (Goto *et al.*, 1999), introduction of intact phosphoenol pyruvate carboxylase (PEPC) gene of maize into *japonica* rice (Ku *et al.*, 1999) and *cry1A(b)* and *cry1A(c)* genes into *japonica* rice (Cheng *et al.*, 1998). This report appears to be the first report of *Agrobacterium*-mediated transformation of a disease resistance gene (or agronomically important gene) in *indica* rice. Previously, rice chitinase gene was transformed into *indica* rice variety (Chinsurah Boro II) by using the PEG mediated transformation of rice protoplast (Lin *et al.*, 1995).

Transformation of local elite *indica* rice cultivars

The *indica* genotypes ASD16, ADT38, IR50 and White Ponni were used in this study. After cocultivation, were kept on a medium without selective agent (hygromycin) for 7 d. Then, they were transferred onto selection medium containing 30 or 50 mg/l of hygromycin. The plants regenerated on 30 mg/l of hygromycin were found to be non-transgenic. This is in contrast to the report by Aldemita and Hodges (1996) that less than 50% of the plants were transgenic when regenerated without hygromycin. Aldemita and Hodges (1996) found that the use of LBA4404 harbouring super-binary vector pTOK233 was critical for *indica* rice transformation.

Transformation of Pusa Basmati1 with the *Agrobacterium* strain EHA105 (pMKU-RF2)

Mature seed-derived callus of Pusa Basmati1 was used as target tissues for cocultivation. After 4 rounds of selection on hygromycin, when the calli were tested for stable GUS expression, about 88% of the calli lines proved positive for

GUS assays. Over 50% of these positive lines resulted in recovery of whole plants.

Western blot analysis confirmed the stable integration and expression of rice chitinase gene, *chi11* in all the five putatively transformed transgenic plants. One prominent band of 35 kDa and another band of 30 kDa were seen in all the five transgenic plants. This is in agreement with the report of Lin *et al.* (1995) and Zhu *et al.* (1998).

Summary

Summary

1. *Agrobacterium*-mediated transformation of elite *indica* rice using plant transformation vectors, pTOK233, pJRB3 or pMKU-RF2 was attempted.
2. Transient GUS expression studies on elite *indica* rices of local importance, ASD16, ADT38, IR50, White Ponni and an aromatic rice genotype, Pusa Basmati1 showed the transfer of T-DNA from *Agrobacterium*.
3. Putative transgenic lines of Pusa Basmati1 expressing *hph* or *gusA* gene were evolved and the presence of those transgenes was confirmed by GUS assays, PCR or Southern analysis.
4. Copy number of *nptII* in putative transgenic lines of Pusa Basmati1 ranged from 1 to 4. Southern blot analysis confirmed the T-DNA integration.
5. Such attempts on transforming the *indica* rices of local importance failed to produce any stable transformants.
6. Segregation analysis for transmission of *gusA* using histochemical GUS assays indicated multiple integration of the transgene.
7. A rice chitinase gene cassette under a ubiquitin promoter (pCAMBAR-*chi11)* was cloned into *Agrobacterium* binary vector, pCAMBIA1301.
8. A rice chitinase gene driven by CaMV 35S promoter (pJRB3) was mobilized into an *Agrobacterium* strain, LBA4404. The same chitinase gene placed under the control of a ubiquitin promoter (pMKU-RF2) was mobilized into a supervirulent *Agrobacterium* strain EHA105 and the presence of those binary vectors into *Agrobacterium* was confirmed by PCR and Southern blotting.
9. Putative transgenic lines of Pusa Basmati1 expressing the rice chitinase gene were evolved and the presence of the chitinase gene was confirmed by Western blotting.

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Appendices

Appendices

Appendix 1

MS medium

Chemical	Final conc. per litre of the medium	Stock/250 ml	Quantity to be dispensed for 1 litre of MS medium
NH_4NO_3	1.65 g	16.5 g	25 ml
KNO_3	1.90 g	19.0 g	
$MgSO_4.7H_2O$	0.37 g	3.70 g	
KH_2PO_4	0.170 g	1.70 g	
$CaCl_2$	0.440 g	4.4 g	25 ml
$MnSO_4$	22.3 mg	223 mg	25 ml
$ZnSO_4$	8.60 mg	86 mg	
H_3BO_3	6.2 mg	62 mg	
KI	0.83 mg	8.3 mg	
Na_2MoO_4	0.25 mg	2.5 mg	
$CuSO_4$	0.025 mg	0.25 mg	
$CoCl_2.6H_2O$	0.025 mg	0.25 mg	
FeEDTA	40 mg	1 g	10 ml
Nicotinic acid	0.5 mg	12.5 mg	10 ml
Pyridoxine	0.5 mg	12.5mg	
Thiamin HCl	0.1 mg	2.5 mg	
Glycine	2.0 mg	50 mg	
Myo-inositol	100 mg	2.5 g	
Sucrose	30 g		
pH	5.8		

Appendix 2

Modified R_2 medium

Chemical	Final conc. per litre of the medium	Stock/250 ml	Quantity to be dispensed for 1 litre of MS medium
NaH_2PO_4	240 mg	2.40 g	25 ml
$(NH_4)_2SO_4$	330 mg	3.30 g	
$MgSO_4$	120 mg	1.20 g	
KNO_3	4.04 g	40.40 g	
$CaCl_2.\ 2H_2O$	147 mg	1.47 g	25 ml
$ZnSO_4.7H_2O$	2.20 mg	55.0 mg	10ml
$MnSO_4.H_2O$	1.535 mg	38.38 mg	
H_3BO_3	2.860 mg	71.5 mg	
$CuSO_4.5H_2O$	0.1285 mg	3.21 mg	
$Na_2MoO_4.\ 2H_2O$	0.126 mg	3.15 mg	
FeEDTA	40 mg	1 g	10 ml
Thiamin HCl	1 mg	25mg	10 ml
Pyridoxine	0.5 mg	12.5 mg	
Nicotinic acid	0.5 mg	12.5 mg	
Myo-inositol	100 mg	2.5 g	
Maltose	30 g		
pH	5.8		

Appendix 3

AB medium for growing *Agrobacterium*

Label AB buffer	gm/L	10 X stock/250 ml (in gm)
K_2HPO_4	3.0	30
NaH_2PO_4	1.0	10

Label AB salt I	gm/L	10 X stock/250 ml (in gm)
NH_4Cl	1.0	10
$MgSO_4.7H_2O$	0.3	3.0
KCl	.015	1.5
$FeSO_4.7H_2O$	0.0025	0.025

Label AB salt II	gm/L	10 X stock/250 ml (in gm)
$CaCl_2(CaCl_2.2H_2O)$	0.01(0.013)	0.1(0.13)

Glucose	5.0 gm

Prepare and autoclave AB buffer and AB salt stock separately. To prepare one litre of AB medium, take 5 g glucose, 15 g agar, and distilled water 925 ml. Adjust the pH to 5.5 using 1M KOH/NaOH, and autoclave. Add 25 ml from the autoclaved stocks, under aseptic conditions, when agar solution was still hot. Finally dispense to Petri plates. Add hygromycin 50 mg/l and kanamycin 50 mg/l at bearable warmth before plating.

Appendix 4

AAM medium

chemical	Final conc. per litre of the medium	Stock/250 ml	Quantity to be dispensed for 1 litre of MS medium
KCl	2.950g	29.50g	25 ml
KNO_3	1.90 g	19.0 g	
$MgSO_4.7H_2O$	0.250 g	2.50 g	
$NaH_2PO_4.H_2O$	0.150 g	1.50 g	
$CaCl_2.2H_20$	0.150 g	1.50g	
KI	0.75 mg	75 mg	2.5 ml
H_3BO_3	3.0 mg	300 mg	
$MnSO_4.H_2O$	10.0 mg	1000 mg	
$ZnSO_4.H_2O$	2.0 mg	200mg	
$Na_2MoO_4.2H_2O$	0.25 mg	25 mg	
$CuSO_4.5H_2O$	0.025 mg	2.5 mg	
$CoCl_2.6H_2O$	0.025 mg	2.5 mg	
FeEDTA	40 mg	1 g	10 ml
Nicotinic acid	0.5 mg	12.5 mg	10 ml
Pyridoxine	0.5 mg	12.5mg	
Thiamin HCl	0.1 mg	2.5 mg	
Glycine	2.0 mg	50 mg	
Myo-inositol	100 mg	2.5 g	

L-glutamine	876 mg
Aspartic acid	266 mg
Arginine	174 mg
Casamino acid	500 mg
sucrose	30 g
Glucose	10 g

Mix components before adjusting pH. Filter sterilize

Appendix 5

GUS assay

X-Gluc stock solution

A stock containing 2mM X-Gluc stock was prepared by dissolving 5.2 mg of X-Gluc in 50 µl of DMSO and made up to 5 ml using the histochemical reagent (50 mM sodium phosphate buffer pH 7.0, 1mM EDTA pH 7.0, 0.1 mM potassium ferricyanide and 0.1 mM potassium ferrocyanide).

X-Gluc staining solution

X-Gluc staining solution prepared by mixing 2.5 ml of X-Gluc stock solution (2mM), 1 ml of methanol and 1.5 ml of sodium phosphate buffer (50mM).

Appendix 6

Modified CTAB method

1. Freeze one gram of plant material or/ callus in liquid nitrogen and grind to a fine powder using mortar and pestle.
2. Weigh approximately 350 mg of powder in each of three microfuge tubes and add equal volume (w/v) of hot (65° C) 2X CTAB buffer (2% CTAB (w/v), 100mM Tris-Cl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 1% PVP).
3. Mix well by inversion and add equal volume (700µl v/v) of ice-cold chloroform: iso-amyl alcohol (24:1) mixture.
4. Mix thoroughly by inversion to from an emulsion and centrifuged at 15,000 rpm for 5 minutes at room temperature.
5. Transfer the aqueous phase to a new microfuge tube by using a cut yellow tip and add $1/5^{th}$ volume of 5% CTAB solution (5% CTAB (w/v), 0.35 M NaCl) and mix well by gentle inversion.
6. Add equal volume of chloroform: iso-amyl alcohol (24:1) mixture, mix thoroughly to form an emulsion and centrifuge for 5 minutes at 15,000 rpm.
7. The top aqueous phase was transferred to a fresh microfuge tube using a cut yellow tip and add equal volume of CTAB precipitation buffer (1% CTAB, 50mM Tris-Cl pH8.0, 10mM EDTA pH 8.0) and mix gently by inversion
8. Centrifuged for a minute at 15,000 rpm and discard supernatant.
9. Dissolve the DNA pellet in 200 µl of high salt TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH8, 0.1M NaCl) by gently tapping the tube.
10. Add 2.5 volume of cold 95% ethanol and mix gently by inversion.
11. Centrifuged for 10 minutes at 15,000 rpm, DNA pellet washed in 70% ethanol, dried in a speed Vac and dissolved in 50 µl in 0.1 X TE.

Appendix 7

Southern analysis

Southern blotting

1. *Plant DNA isolation*

Plant DNA is isolated from the leaves of rice plants following the modified CTAB method (Porebski *et al.*, 1997; Appendix 6).

2. Isolation of total DNA form Agrobacterium tumefaciens.

Total DNA is isolated from cultures of *A. tumefaciens* strain LBA4404 (pTOK233) using the method described by Chen and Kuo, (1993). Bacterial cells are harvested by centrifugation at 12,000 rpm for 5 minutes and the pellet dissolved in minimal volume of the remaining medium by vortexing. Cells are lysed by adding 200 µl of lysis buffer (10 mM Tris-acetate, pH 7.8, 20 mM sodium acetate, 1mM EDTA and 1% SDS) with mild vortexing. To this 66 µl of 5 M NaCl is added, mixed well and left at –20° C for 10 minutes. The viscous mixture is centrifuged at 12,000 rpm for 10 minutes at room temperature. The supernatant is extracted with equal volume of phenol/chloroform followed by twice extracting with water saturated diethyl ether and DNA is precipitated with 2.5 volumes of ethanol, followed by washing the DNA pellet once with 70% ethanol. The pellet is air dried and dissolved in 50µl of 0.1X TE buffer.

3. Southern transfer

Ten microgram of DNA is digested with appropriate restriction enzymes and electrophoresed in 1% agarose gels at 60Volts in 1XTBE buffer. The gel is soaked in 250 ml on denaturation solution (1M NaCl, 0.5 M NaOH) in a glass tray with gentle shacking for 90 minutes on a platform shaker and the gel washed with sterile distilled water for four times (250 ml each time). The gel is soaked in 250ml of neutralization solution (1.5 M NaCl, 0.5M Tris-HCl pH 7.0) with shacking for 90 minutes on a shaker. Three Whatman No.3 sheets, cut to the size of the gel are wetted with 20X SSC and arranged over a glass plate. Over this the gel is placed with the bottom side facing up. Nylon

membrane (BM membrane) cut to the size of the gel, is wetted with water, soaked in 20X SSC and placed over the gel. Three Whatman No. 3 sheets wetted with 20 X SSC are placed over the membrane and a dry Whatman no.3 sheet is kept on the top. Stacked further to about one inch with paper towels. The blot is removed after overnight transfer and given a rinse in 2X SSC for 30 seconds, air-dried and the DNA is cross-linked to membrane with a UV cross linker.

Southern hybridization

1. Probe DNA preparation

Plasmid DNA was isolated by alkali lysis method (Brimboim and Doly, 1979). The plasmid DNA restricted digest with suitable enzyme and electrophoresed in an agarose gel. The desired fragment is separated from the gel by electroelution method (Sambrook *et al.,* 1989).

Radiolabelling of Probe DNA

Radiolabelling of probe DNA was performed by the random primer oligo labelling method (Feinberg and Vogelstein, 1983) using a kit from Amersham International Plc, England. Thirty nanograms of electro-eluted DNA and 5µl of random primer are taken in microfuge tube. The final volume is made up to 33 µl with sterile distilled water. DNA is denatured by heating for 5 minutes over a boiling water bath and cool down to room temperature gradually. The content is centrifuged for 5 sec and the volume made up to 33µl with sterile distilled water. To the denatured DNA, 10 µl of labelling mix, 5 µl of (α -^{32}P) dCTP and 2 µl of Klenow fragment are added and mixed gently by tapping and incubated at 37°C for 20 minutes. After 20 minutes, 50 µl of nick translation dye (6mg of Blue dextran and 1 mg of Orange-G added to 1ml of 0.5 M EDTA; store at room temperature) is mixed to stop the reaction.

Purification of labelled probe DNA

The radiolabelled probe DNA is purified by passing through a Sephadex G-50 column. A 2ml Sephadex G-50 [0.4 gm of Sephadex G-50 in 6ml of column buffer (0.1 M NaCl; 12mM Tris-HCl pH7.0; 2.5mM EDTA pH7.0)] column is prepared using a

Pasteur pipette plugged with glass wool and washed with 10 ml of column buffer. The labelled DNA mixture is loaded onto the top of the column. The blue fraction is collected separately in a 1.5 ml microfuge tube.

2. Pre-hybridization

The Membrane is kept inside the hybridization bottle by using a sterile flat forceps and prehybridization solution (0.5 M Na_2HPO_4, SDS 7%, 1mM EDTA (pH7.0)) is added at the rate of 150 µl/cm^2 membrane size. Air bubbles are removed using a thin spatula. The bottle is incubated at 65°C for 30 minutes in a hybridization oven.

3. Hybridization

The labelled probe is denatured in a boiling water bath for 5 minutes and cooled rapidly on ice for 10 minutes. The prehybridization solution is removed and fresh prehybridization solution (same volume) is added to the bottle. The denatured probe is added to the bottle, mixed by gentle shaking and incubated at 65°C for 12-20 hrs in the hybridization oven.

4. Post-hybridation washes (High stringency wash)

The hybridization solution is discarded and the membrane is rinsed briefly with 20 ml of 2X SSC/0.1% SDS. The blot is washed for 30 minutes at 65° C with 40 ml of 3X SSC/0.1% SDS, followed by 0.5X SSC/0.1% SDS, and finally with 0.3X SSC/0.1% SDS. Finally, the blot is rinsed with 2X SSC at room temperature. The bottle is filled up with 2X SSC and the blot is removed carefully. The membrane is air dried and expose it to X-ray film. The film is kept at –70° C till the blot is developed.

5. *Developing the x-ray film*:

X-ray cassette along with the film is brought to room temperature before developing. The x-ray film is removed in a dark room from the cassette and transferred to developer solution for 2 minutes, then to water for 1 minute and then to fixer solution for 2 minutes. After developing, the film is washed thoroughly and air dried.

Appendix 8

CC medium

chemical	Final conc. per litre of the medium	Stock/250 ml	Quantity to be dispensed for 1 litre of MS medium
KNO_3	1212 mg	12.12 g	25 ml
NH_4NO_3	640 mg	6.4 g	
$MgSO_4.7H_2O$	247 mg	2.47 g	
KH_2PO_4	136 mg	1.36 g	
$CaCl_2$	588 mg	5.88 g	25 ml
$MnSO_4.7H_2O$	11.5mg	115 mg	25 ml
$ZnSO_4.7H_2O$	5.76mg	57.6 mg	
H_3BO_3	3.1mg	31 mg	
KI	0.83 mg	8.3 mg	
Na_2MoO_4	0.24 mg	2.4 mg	
$CuSO_4$	0.025 mg	0.25 mg	
$CoCl_2.6H_2O$	0.025 mg	0.25 mg	
FeEDTA	40 mg	1 g	10 ml
Nicotinic acid	6 mg	60 mg	25 ml
Pyridoxine	1.0 mg	10 mg	
Thiamin HCl	8.5 mg	85 mg	
Glycine	2.0 mg	20 mg	
Myo-inositol	90 mg	0.9 g	

Casein hydrolysate	300 mg
Mannitol	15 g
Sorbitol	15 g
Sucrose	20 g
pH	5.8

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