DEVELOPMENT OF EFFICIENT TRANSFORMATION SYSTEMS TO ENHANCE INSECT RESISTANCE IN RICE (*Oryza sativa* L.)

Ph. D. THESIS

by

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CERTIFICATE-I

This is to certify that the thesis entitled **"Development of efficient transformation systems to enhance insect resistance in rice (*Oryza sativa* L.)"** submitted in partial fulfillment of the requirement for the degree of "Doctor of Philosophy in Biotechnology" of the Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.), is a record of the bonafide research work carried out by **Shri. Jadhav Pravin Vishwanathrao** under our guidance and supervision. The subject of the thesis has been approved by Student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma (certificate awarded etc.) or has been published / published part has been fully acknowledged. All the assistance and help received during the course of the investigation have been duly acknowledged by him.

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CERTIFICATE-II

This is to certify that the thesis entitled **"Development of efficient transformation systems to enhance insect resistance in rice (*Oryza sativa* L.)"** submitted by **Shri. Jadhav Pravin Vishwanathrao** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur in partial fulfillment of the requirements for the degree of Ph. D. in the Department of Biotechnology has been approved by the external examiner and Student's Advisory Committee after oral examination.

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Date: **Jadhav Pravin Vishwanathrao**

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Abbreviations	Details
%	Percentage
µl	Microliter
2, 4-D	2, 4 – dichlorophenoxy acetic acid
AB	*Agrobacterium* medium
ABA	Abscisic acid
Agro-inoculum	*Agrobacterium* inoculum
BAP	Benzyleamino purine
bp	Base pairs
Bt	*Baccillus thuriengiensis*
CaMV	Cauliflower Moisac virus
cry	Crystal
CTAB	Cetryl trimethyl ammonium bromide
cv.	Cultivar
d	Day
DMSO	Dimethylsulfoxide
DNA	Deoxyribose Nucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene Diamine Trtra-acetic acid
g/L	Gram per liter
GUS	β-glucuronidase
INT	Intron
kb	Kilo base
KIN	Kinetin
L3	Lin and Zhang medium
LB	Luria broth
LF	Leaf folder
LS	Linsmaier and Skoog medium
mg	Milligram
min	Minute
mM	Millimolar
MS	Murashige and Skoog
MSM	Murashige and Skoog Modified medium
*m*VIP	Modified vegitative insecticidal protein
NAA	α-Napthalene acetic acid
ng	Nano gram
NT	Non transformed
PCR	Polymerase Chain Reaction
psi	Pressure per square inch
rpm	Revolution per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SSB	Stripped stem borer
T-DNA	Target –DNA
TE	Tris EDTA
U	Units
vs	Versus
X- gluc	5-bromo-4-chloro-3indolyl-β-D- glucuronide
Yr	Year
YSB	Yellow stem borer

CHAPTER I

INTRODUCTION

Rice is the most consumed cereal grain in the world, constituting the dietary staple food for more than half of the planet's human population. About 80% of the world's rice is grown by small- scale farmers in developing countries (FAO, 2004). Asia produces 90 % of the rice in the world (James, 2006). Rising demand, shrinking paddy fields and low productivity due to inclement weather, cope of biotic and abiotic stresses are likely to cause a supply shortage and drive rice prices up in the near future. More than 200 million tones of world's production of rice are lost every year due to biotic and abiotic factors (Wunn *et al.,* 1996). Rice is severely attacked by many insects and among them Yellow stem borer (*Scripophaga incertulas*), Striped stem borer (*Chilo suppressalis*) and leaf folder (*Cnaphalocrocis medinalis*) are the most destructive and economically important in India. Chemical control is ineffective against the insects which feed internally like YSB and SSB. At the same time increases the input cost and having well known for adverse effects on human and animal health.

Several efforts for breeding rice varieties with inherent resistance against devastating stem borers have not been successful due to non availability of sufficient level of resistance in rice lines screened for this insect (Teng and Revilla, 1996). With the development of transferring genes from the alien sources using genetic engineering methods for *indica* rice, several rice

transgenics have been developed with various *cry* genes (Tu *et al.,* 2000; Ye *et al.,* 2001; Datta *et al.,* 2004; Chandel, 2005).

Rice genetic transformation has taken rapid strides since the first transgenic rice plant was produced nearly 15 years ago. Transgenic insecticidal crops expressing the *Bacillus thuringiensis* (*Bt*) toxins provides an alternative method of remarkable levels of insect control unsurpassed by any other insecticidal treatment (Tyagi and Mohanti, 2000). There are several significant environmental benefits from the absence of pesticide drifts, residual pesticide in soil and effects of non-target insects with their unique insecticidal activity and efficacy. Therefore, this approach to pest control offers many promises in developing agricultural practices which are perfect harmony with environment.

Development of transgenic crops during the 1990's is an important landmark in the history of crop improvement. Since the first commercial release in 1994, transgenic crops have registered steady increase in area (102 m ha, 2006) and have slowly spread across nations. Between the years 1996-2005, a total of 24 countries, 12 developing and 12 developed countries contributed over 50- fold increase in the global area of transgenic crops from 1.7 m ha in 1996 to 90 m ha in 2005. Adoption rates for transgenic crops during this period are unprecedented and by recent agricultural industry standards, they are the highest adoption rates for improved crops (James, 2006).

Transgenic rice is developed by genetic engineering technique by directing genetic manipulation at the single cell and organ level, there by shortening the time required for conventional breeding procedures. *Japonica* and *indica* are two major subspecies growing in different regions of the world. *Indica*

rice (long grain tropical rice) alone accounts for ~80% of the cultivated rice. It is sensitive to several biotic and abiotic stresses and is a poor yielder. There are eminent genetic transformation systems which have been applied in several crops including rice. Among them, *Agrobacterium*- mediated, particle gene gun system, PEG- mediated and electroporation transformation systems are tissue culture based. These methods involved two major steps in transformation i.e. subculture and regeneration which accounts for transformation efficiency. With the development of first rice transgenic in 1990 by Datta *et al.,* using electroporation and PEG in *japonica* and *indica,* respectively. Efforts have been made to develop high efficiency transformation system for *indica* rice varieties. Most of the transformation techniques need single cell based plant regeneration system, which allows introduction of foreign DNA in to totipotent cell and later the ability of this cell to develop complete plant. Plant regeneration of *indica* rice is highly genotype dependent and most of the popular rice varieties have reported to be recalcitrant to *in-vitro* response (Chandel, 2005; Datta *et al,* 2004).

The establishment of high efficiency *Agrobacterium*- mediated transformation (Hiei *et al.,* 1994) has greatly facilitated the wide spread application of transformation in japonica rice (*Oryza sativa* spp. *Japonica*). Currently this technique is widely used not only to introduce genes of interest in the rice genome for the purpose of varietal improvement (Lin *et al.,* 2002; Sallaud *et al.,* 2003), but also as a common means for testing gene function by enhancing or inhibiting expression of target genes (Moori *et al.,* 2002). In contrast there have been very few successful cases reported of *Agrobacterium*-mediated transformation in *indica* rice (*O. sativa* spp. *Indica*). Even in reports in

which transformation succeeded, the results showed either low transformation efficiency (Garg *et al.,* 2002; Wang *et al.,* 2002) or success in only with very specific varieties (Zhang *et al.,* 1997), which has severely hindered the application of transformation in *indica* rice compared to *japonica* rice. This may be due largely to the fact that the technical maturity of tissue culture in *indica* rice lags behind that of japonica. The high efficiency tissue culture systems for *japonica* rice have long been established and are technically robust and mature (Chu *et al.,* 1975). In *indica* rice however, robust and widely applicable methods for *in vitro* regeneration have not been established despite large efforts investigated in the *in vitro* culture of *indica* rice. The success of all the published protocols for culturing *indica* rice was largely genotype dependent, which limits the reuse of such protocols (Lin and Zhang, 2005). Hence the genotype dependent nature of tissue culture based system has been a limiting factor for *indica* rice which has proven to be highly recalcitrant to *in vitro*.

Recently techniques have been evolved where in tissue culture step is eliminated, which are commonly termed as *in planta* transformation. Some recalcitrant crop species *viz.,* sunflower, safflower and peanut have been transformed till date by using *in planta* system of transformation. This method essentially involves infection of embryos, immature flowers (Feldmann and Marks, 1987; Feldmann, 1992; Betch-told *et al.,* 1993), germinating shoots or meristematic region (Chee and Slightom, 1995; Brich, 1997). The non- tissue culture based method of transformation provides minimum genomic changes and genetic distortion among the transformants compare to that of tissue culture based methods (Datta, 2004). Use of transformation tools for improving *indica*

rice is of great importance in modern agriculture. Genotype dependent *in vitro* response of *indica* rice and led to the need of developing independent non-tissue culture based method which would enhance the speed of using transformation technology for incorporation of desirable traits like insect resistance in *indica* rice.

Looking to the potential of rice transgenic in enhancing insect resistant varieties mainly against lepidopterous insects *viz.*, YSB, SSB and LF, this study was planed to assess the efficacy of two popular tissue culture based transformation systems, *Agrobacterium*- mediated and Particle gun and a non tissue culture based flower dip method for *indica* rice varieties *viz.*, Swarna and Mahsuri. We have examined the various factors affecting the transformation efficiency for tissue culture based method and physical, environmental and biological factors affecting the transfer of foreign gene in to developing flowers of rice plants with following objectives:

1. Development of efficient protocols for producing transgenic rice using different transformation systems:
   A. *Agrobacterium* mediated
   B. Biolistic gun based
   C. *In planta* Floral dip method
2. Development of high frequency *in vitro* regeneration protocol for efficient transformation in selected *indica* rice varieties.
3. Development of insect-resistant transgenic rice and its confirmation using molecular analysis.

CHAPTER II

REVIEW OF LITERATURE

Rice is the most consumed cereal grain in the world, consisting the dietary staple food for more than about half of the planet population (FAO, 2004). About 80% of the world's rice is grown by small farmers in developing countries. Rice consumers are increasing at the rate of 1.8 % every year. But the rate of growth in rice production has slowed down. It is estimated that rice production has to increase by 50% by 2025 (Khush, 2001; Saharan *et al.,* 2004). This will require acceleration in rice production. Solving this problem will entail development of rice varieties, which have higher yield, excellent grain quality and resistance to biotic and abiotic stresses. Losses due to pests and diseases have been estimated at 37% of agricultural production world wide, with 13% due to insect pests (Gatehouse *et al.,* 1992). Of the several insect pests, breeders rank the lepidopteron Yellow stem borer (*Scirpophaga incertulas* L.) as the most serious pest in low land rice for which 50% of all insecticidal sprays in Asian countries were targeted (Heong, 1994; Wang *et al.,* 2002). The average yearly yield losses caused by SSB, YSB and LF (*Cnaphalocrocis medinalis* and *Marasmia patnatis*) world wide are estimated at about 10 million tones (Herdt, 1991), or about 5% grain losses by stem borer in Asia (IRRI, 1996). In eastern India only the annual yield loss goes to 671,000 tonnes which comprise 235,000 tonnes in rainfed lowland and 86,000 tonnes in deepwater areas (Widawsky and O' Tool, 1995). The losses due to egregious pests like stem borers, gall midge, green leaf hopper, rice hispa, brown leaf hopper and thrips are quite high

(Kalode *et al.,* 1986) ranging from 4.8 to 14.5 % in Eastern India including Chhattisgarh. Stem borer is a serious pest that causes considerable yield loss in Asia. Figure 2.1 shows the features of Stem borer and symptoms of rice plants after infestations. At the vegetative stage, insect feeding inside the stem can result in death of the young leaf whorl causing "dead heart" and during reproductive stage it feeds inside the panicle stalk leading to unfilled grains-white heads (Rubia *et al.,* 1995).

2.1 Efforts to develop Stem borer resistance in rice

Wide application of chemical insecticides not only increases the rice production cost but also contributes to health concerns of rice farmers as well as deterioration of the rice field environment (Pingali, 1995). Hence, it is urgency to develop a more eco-friendly agriculture to overcome the limitations of agrochemicals; it has been aimed in view to develop insect pest resistance rice varieties.

Conventional efforts of two decades have made to the development of rice varieties with moderate level of resistance to stem borer (Kalode *et al.,* 1986). The results are not convincing and identification of donor varieties with high level of resistance is still a challenge (IRRI, 1995). Moreover, the efforts of screening more than 30,000 rice accessions for the stem borer resistance genes have not been successful in identifying sufficient degree of resistance in any of the accessions (Teng and Revilla, 1996).

The approach consisting transgenic *Bt* (*Bacillus thuringiensis*) rice can be cited as one of the exemplary success story of agricultural biotechnology to date and several rice varieties have been transformed with various *cry* genes and

shown to have high levels of resistance against Lepidopterous insects (YSB and SSB) under green house and field conditions (Ye *et al.*, 2001; Datta *et al.*, 2004).

2.2 Use of *Bacillus thuringiensis* δ-endotoxins*:*

The bacterium *B. thuringiensis* was first discovered by Ishiwata in Japan during 1902 in a silkworm rearing unit and named it *Bacillus scotto*. In 1995, it was renamed as *Bacillus thuringiensis.* In the early twentieth century, Berliner provided the first inkling that microbes could control insect pests. *Bacillus thuringiensis* is a gram positive, sporulating bacteria differing from *Bacillus cereus* only by the synthesis of several insecticidal crystal proteins (ICPs). *Bt* is a complex species spanning more than 30 varieties of flagellar serotypes based on serological and biochemical tests. The ICPs are synthesized during the late growth phase of bacteria and are accumulated in the cytoplasm as crystals or inclusion bodies. The crystal may account for up to 30% of the dry weight of sporulated culture. The ICPs produced by *Bt* are alpha-, beta- and gamma exotoxins and beta- endotoxins are used in agriculture. The δ- endotoxin is the most extensively studied toxin; its larvicidal specificity includes members of lepidopteran, dipterans and coleopteran insects. In the natural form *Bt* has been used by farmers practicing organic and other sustainable growing methods since 1950s as a spray to kill pests without damaging non-targeted insects or other wildlife. For more than 40 years *Bt* proteins have had a safe history as biopesticides preparations and are approved for organic farming. Rats fed with very high doses of *Bt* proteins showed no detectable toxic effects whereas synthetic pesticides, such as organophosphates and chlorinated biphenyles are toxic (http:www.akademienunion.de). The growing realization of deleterious

effects of inorganic insecticides to the environment and human health spurred a renewed interest in *Bt* in 1960 and viable biopesticide like Dipel, Thuricide etc. were introduced.

The inclusion produced by *Bacillus thuringiensis* consists of proteins exhibiting a highly specific insecticidal activity. These observations led to the development of biopesticide based on *Bt* for the control of certain insect species among the orders, Lepidoptera, Diptera and Coleoptera (Beegla and Yamamoto, 1992). Fietelson *et al.,* (1992) reported that *Bt* was also found active against Hymenoptera, Homoptera, Orthoptera, Mallophaga and against Nematodes, Mites and Protozoa.

2.2.1 Insecticidal crystal Proteins (ICPs)

In early 1980s, intensive research was directed towards the localization of the ICP genes among different *Bt* species. A number of early studies revealed direct correlation between the presence of plasmids and insecticidal crystal production. Plasmid curing, conjugation (mating) and gene cloning experiments provided further evidence that the ICP genes are very often located in larger (730 MDa), low copy number cryptic plasmids (Gonzalez *et al.,* 1986; Whiteley and Schnepf, 1986). However there are conflicting reports about the possible excistance of ICP genes in the chromosome of some subspecies such as *'kurustaki* HD1, *entomocidus, aizawai* 7.29, *dendrolimus* and *wahnensis* (Held *et al.,* 1982). Carlton and Gonzalez (1985) made a survey of the plasmid present in most of the *Bt* strains. Their studies on the plasmid present on 21 *Bt* subspecies revealed that the number of plasmid varies from two to twelve between strains. In the case of B. *thuringiensis* subspecies *israelenis,* four *dipteran* specific toxin

genes and one cytolysin coding gene are located on a single 72 MDa plasmid (Aronson *et al.,* 1986).

Following localization of toxin genes, many researchers groups began to clone ICP genes from several B. *thuringiensis* subspecies. Schnef and Whiteley (1981) reported the first cloning and expression of *cry* type genes from *Bt 'kurustaki* HD1 "Dipel" plasmid preparation. They expressed the ICP gene in *E. coli* and showed its activity to *Manduca sexta* larvae. Following this report, protoxin genes were cloned and sequenced from numerous other *Bt* species toxic to the members of the insect order lepidoptera, diptera and coleopteran. At the present time over 60 ICP sequences are given in gene databases or the journal and present literature.

2.2.2 Classification of ICP genes

Held *et al.,* (1982) first used the abbreviation "*cry*" (for crystal) to represent the insecticidal crystal producing genes of *Bt* strains. Based on the primary target insect specificity and sequence similarity, insecticidal toxins have been classified in to five major classes (Hofte and Whiteley, 1989). Table 2.1 shows a summary of *Bt* crystal protein genes and specificity.

Research efforts in the past few years have led to the discovery of novel ICP which are produced by certain isolates of *B. thuringiensis*. These proteins unlike well-characterized crystal proteins are produced during vegetative growth of cells and are secreted in to the growth medium. These proteins have been termed as Vegetative insecticidal protein (VIP). Sequences encoding for a VIP have been cloned, sequenced and the protein has been expressed in *E. coli* (Estruch *et al.,* 1996). The 88 kDa vegetative insecticidal protein has a putative

Table 2.1: Classification of *B. thuringiensis* δ-endotoxin genes and specificity

***cry* gene**	***Bt* subspecies/ Bacterial strain**	**Target Insect**		**Reference**
		Order	**Species**	
cryIAa	HD1, *Scotto, entomocidus*	L	*B. mori, M. sexta*	Schnepf *et al.*, 1985; Shibano *et al.*, 1985; Masson *et al.*, 1989
*cry*IAb	*Kurustaki*, HD1, berkiner, NRD 12, ICI, IPL 7	L/D	*M. sexta, Pieris brassicae, Aedes agypti*	Thorn *et al.*, 1986; Geiser *et al.*, 1986; Hoftte *et al.*, 1986
*cry*IAc	HD 73, *BT*S 89A	L	*H. virescens, T. ni*	Adang *et al.*, 1985; Dardenne *et al.*, 1990
cryIB	HD2	L	*P. brassicae*	Brizzard & Whieteley, 1988
cryIC	*entomocidus, aizawai*	L	*S. littoralis*	Sanchis *et al.*, 1989; Honee *et al.*, 1988
cryICb	*Galleriae*	L	*S. exigua*	Kalman *et al.*, 1993
cryID	*HD 68*	L	*S. exigua, M. sexta,*	Hofte *et al.*, 1990
cryIE	*Kenyae*	L	*S. littoralis*	Bosse *et al.*, 1990
cryIF	*EG 6346*	L	*Ostrinia nubilalis, S. exigua*	Chambers *et al.*, 1991
cryIG	*galleriae, DSIR 517*	L		Smulevitch *et al.*, 1991; Gleave *et al.*, 1992
cryIX	*Galleria*	L		Shevelev *et al.*, 1993
cryIIA	*HD1, HD 263*	L & D	*L. dispar, A. aegypti*	Widner & Whiteley, 1989; Donovan *et al.*, 1988
cryIIB	*HD 1*	L	*L. dispar, T. ni*	Widner & Whiteley, 1989
cryIIC	*Shanghai S1*	L	*M. sexta, L. dispar*	Wu *et al.*, 1991
cryIIIA	*Tenebrionis, San diego, EG2158*	C	*Leptinotarsa decemlineta, Phaedon cochleriea*	Sekar *et al.*, 1987; Donovan *et al.*, 1988
cryIIIB	*Tolworthi*	C	*L.decemlineta*	Sick *et al.*, 1990
cryIIIC		C	*Diabrotica undecimpunctata*	Lambert *et al.*, 1992
cryIIID	*Bt* 1109P	C		Lambert *et al.*, 1992
cryIVA	*Israelensis*	D	*A, aegypti, Culex pipiens*	Ward and Ellar, 1987
cryIVB	*Israelensis*	D	*A. aegypti*	Tungpradubkul *et al.*, 1988
cryIVC	*Israelensis*	D	*A. aegypti*	Thorn *et al.*, 1986
cryIVD	*Israelensis*	D	*A. aegypti, C. pipiens*	Donovan *et al.*, 1988
cryV	*JHCC 4835*	L/C	*O. nubilalis, Diabrotica spp.*	Tailor *et al.*, 1992
VIP3 *AaI*	*AB 88*	L/C	*A. ipsilon*	Estruch *et al.*, 1996
VIP3 *Aa2*	*AB 424*	L/C	*S. frugiperda*	Warren., 1997
VIP3 *Aa14*	*Bt. toworth*	L/C	*H. armigera S. litura E. vittela P. brassicae P. xylostella*	Bhalla *et al.*, 2005

Note: L. Lepidoptera; D. Diptera; C. Coleoptera

bacillar secretary signal at the N-terminal which is not processed during its secretion. It does not show any homology with the known crystalline insecticidal proteins.

This structural dissimilarity is indicative of a possible divergent insecticidal mechanism than the expressed receptor to *Bt*- toxin of polyphagous pest *S. litura* was titrated against VIP toxin no interaction between these legends was observed (Bhalla et al., 2005).

These results together with observed structural divergence of VIP with other toxins make them an ideal candidate for deployment in insect management programme together with the other category of *Bt*- toxins described earlier. Individually VIP has been successfully expressed in monocots and dicots plants and efforts to pyramid VIP in the *Bt* transgenic crops are underway in several laboratories (Ranjekar *et al.,* 2003).

2.2.3 Mode of action

Most studies of histopathological and mode of action of *Bt* have been carried out on lepidopteran larvae with toxin preparations derived from whole *Bt* crystals. These investigations showed that mechanism of action of *Bacillus thuringiensis cryI* proteins involve solubalization of the crystals in insect midgut receptors and insertion of the toxin in to the apical membrane to create ion channels or pores (Luhty and Ebersold, 1981). For most lepidopteron, protoxins are solubilized under the alkaline conditions of the insect midgut proteases (Tojo and Aizawa, 1983), to become activated toxins. Major proteases are trypsin like or chymotrypsin like (Novillo *et al.,* 1997). Activated *cryI* toxins have two known functions, receptor binding and ion channel activity. Hoffman *et al.,*

(1988) have shown that the activated toxin binds to specific receptors on apical brush border of the midgut microvillae of susceptible insect binding being a two stage process involves reversible and irreversible steps (Raja Mohan *et al.*, 1995). Irreversible step involves a tight binding between the toxin and the receptor, insertion into apical membrane of the columnar epithelial cells follows the initial receptor mediated binding rendering the toxin insensitive to proteases and monoclonal antibodies and induces ion channels or non-specific pores in the target membranes. The formation of toxin induced pores in the columnar cell apical membrane allows efflux of cellular content/ions. The disruption of gut integrity results in the death of insect from starvation or septicemia.

2.3 Methods of transformation system for cereals

Most of the cereal crops, including some of the most important food crops, such as wheat, rice, maize, sorghum and barley are now routinely being transformed using various methods. Although numerous methods have been employed to transform the monocots like rice, Particle gun mediated gene transfer system has been commonly used.

2.4.1 Tissue culture based techniques

2.4.1.1 *In vitro* culture of rice

In vitro tissue culture is an experimental approach for basic and applied research. It is an essential component of breeding, biotechnology and genetic improvement of plants. Every plant cell is considered to be totipotent. The potentiality to readily regenerate plants from cultured tissue has been considered as a powerful tool for improvement and the simplest form of genetic engineering (Larkin and Scowcroft, 1981). Tissue culture techniques are well established for

most monocotyledonous plants but difficult to culture gramineous species because of their extremely recalcitrant behavior in *in vitro* manipulation. The prerequisite of *in vitro* culture is the identification of tissues or cells which are competent for regenerating whole plant.

In vitro tissue culture of rice was started by Fujiwara and Ojima, (1955) with the culture of excised roots and immature embryos (Amemiya *et al.,* 1956). Tissue culture constraints were reduced by culturing of different explant in nutrient medium supplemented with varying concentrations of phytoharmones (Vasil and Vasil, 1982; Vasil, 1988; Pipatpanukul *et al.,* 2003). Rice was the first cereal to regenerate in to whole plant (Vasil, 1983). Abe and Futstuhara (1986) and they observed large difference for the tissue culture ability of *japonica* and *indica*. They reported that *japonica* varieties showed high callus yield and regeneration than the *indica in vitro* tissue culture.

2.4.1.2 Protoplast mediated DNA uptake

Rice Transformation has been achieved through uptake of DNA by isolated protoplast, facilitated by poly ethylene glycol (PEG) treatments and electroporation.

2.4.1.2.1 PEG mediated gene transfer in isolated protoplast

Poly ethylene glycol (PEG) has been identified as an efficient agent for protoplast fusion in the development of somatic hybrids and also in uptake of transgene in protoplast based transformation system. Several monocots including, wheat, sugarcane, maize, jawar and rice have been transformed using PEG mediated DNA uptake (Jahne *et al.,* 1995).

PEG mediated gene transfer method was first employed in *japonica* rice varieties by Uchimiya *et al.,* 1986. Later, successful transformation of rice protoplast was reported by Toriyama *et al,* (1988) ; Zhang and Wu (1998). Transgenics developed by this method is mostly restricted to *japonica* cultivars, due to their better amenability to *in vitro* techniques. Several transgenic lines were developed in *japonica* cultivars through PEG-mediated transformation into isolated protoplasts. Later, Datta *et al.,* (1990) reported recovery of fertile transgenic *indica* rice cultivars through PEG mediated transfer of DNA into isolated protoplast. Although PEG mediated transformation methods suffers from many limitations like source of explants for isolation of intact protoplast from suspension culture of rice. The maintenance and regeneration of plants from protoplast is also genotype dependent, laborious and most cases time consuming. Besides these regeneration of fertile plants is very low and somaclonal variation is unavoidable (Bao *et al.,* 1996). However, despite these difficulties, this method has been used to fertile transgenic rice lines with herbicide resistance (Datta *et al.,* 1992), fungal resistance (Lin *et al.,* 1995) and insect resistance (Irie *et al.,* 1996).

2.4.1.2.2 Electroporation of Protoplasts

In this method, short pulse of high field strength is applied so as to increase the permeability of cell membrane of protoplasts for efficient DNA uptake. Maize was the first monocot to be transformed through electroporation of protoplasts (Fromma *et al.,* 1986).

Shimamoto *et al.,* (1989) were the first to report recovery of fertile transgenic rice plants by electroporation of protoplasts. Thereafter, several

researchers have reported the development of transgenic plants in *japonica* and *indica* rice cultivars. An insect resistant *Bt* rice lines, carrying *Bt*-gene was developed through electroporation of insolated protoplasts by Fujimoto *et al.*, (1993).

Bhattacharya *et al.,* (1994) developed transgenic *indica* rice through this method. In 1998, Arencibia *et al.,* and Wu *et al.,* (1997) reported transgenic *japonica*, rice through electroporation of protoplasts. This method also has same limitations associated with the protoplast isolation, culture and regeneration.

2.4.1.3 Biolistic approach

The biolistic process (accelerated particle bombardment) was invented by John Sanford and his co-workers at Cornell University, USA. It involves high velocity acceleration of micro projectile (Gold and Tungsten) carrying foreign DNA to penetrate to the target tissue. The advantages of this approach are:

1. It is genotype independent
2. Intact tissues can be employed for transformation
3. In species where Agro-infection is not possible, it provides an alternative approach.
4. In comparison to protoplast approach, it requires much less involvement of *in vitro* cell manipulations.

Particle gun approach to transformation has been successful in several cases and therefore, it is the method of choice in such case where *Agrobacterium* system is not effective. Also there is no different in the transformation efficiency of monocots and dicots so far as DNA delivery is concerned (Sanford, 1990). Christou *et al.* (1991) were the first to obtain transgenic rice by this method.

Since then several transgenic rice lines, including different *indica* varieties have been developed (Khanna *et al.,* 2002; Nayak *et al.,* 1997; Datta *et al.,* 1998; Alam *et al.,* 1998, 1999; Reddy *et al.,* 1998; Bano *et al.,* 1999; Sharan *et al.,* 2004). More significantly, this method is said to be genotype independent with more than 40 rice varieties already transformed and transformation frequency as high as in dicots has been reported in some cases (Tyagi *et al.,* 1999).

Many agronomically important genes have been introduced in rice. Transgenic rice having herbicide resistance has successfully completed its field trial (Oard *et al.,* 1996). The main drawbacks of the biolistic process are as below:

1. In some cases, the copy number and the frequency of rearrangement of the introduced gene is high, thereby rendering transgene(s) prone to gene silencing and casing genomic changes (Tyagi *et al.,* 1999).
2. The cost of equipment is very high. Also, each bombardment process involves expensive consumable.
3. Bombardment may cause considerable tissue damage.

In spite of a few disadvantages, the Particle gun approach was considered to be the most versatile and successful approach, until *Agrobacterium*-mediate transformation in rice was successfully reported (Hiei *et al.,* 1994).

2.4.1.4 *Agrobacterium*-mediated gene transfer

At present *Agrobacterium*-mediated gene transfer is the most widely used system for genetic transformation of higher plants. *A. tumefaciens* is a gram negative soil bacterium which infects a wide range of dicot plant species. It produces crown gall tumors by introducing a piece of DNA (T-DNA) in to the

host genome (Smith and Hood, 1995). It is this T-DNA delivery system which has been exploited for developing transformation techniques through the *A. tumefaciens* approach. It is now known that for foreign gene transfer only the T-DNA borders (25 bp repeats) and some flanking sequences are needed in *cis* position. Thus by deleting T-DNA region and adding selectable markers, "disarmed" plasmid vectors can be prepared and effectively used for transferring foreign genes without disturbing the endogenous hormone balance of the host plant. *Agrobacterium*-mediated gene transfer method in dicots is the most popular technique to obtain transgenic plants. This method generally results in higher rate of transformation (5%), more efficient and predictable for integration of the foreign DNA (Smith and Hood, 1995). Complications generally associated with biolistic method of transformation such as multiple copy integration, DNA rearrangements, are less frequent in *Agrobacterium* system.

Agrobacterium-mediate gene transfer was not successful in cereals and most of the monocots. However, during the last few years significant progress has been achieved in this direction. Earlier success in this field came through use of germinating embryos of *japonica* variety of rice and by employing a wide host range super virulent strain (Raineri *et al.,* 1990).

The first convincing report of monocot transformation through *Agrobacterium*-mediated methods was by Hiei *et al.,* (1994). Transformation of a *japonic* rice cultivars was achieved by using some super-virulent vectors such as LBA 4404 (pTOK 233) and EHA 101 (pTOK 233). Following this report several laboratories have been successful in rice transformation in *indica*,

japonica, as well as, in *Javanica* varieties. (Rashid *et al*., 1996; Aldemita and Hodges, 1996; Datta, *et al.,* 1996; Toki, 1997; Cheng *et al.,* 1998; Khanna and Raina, 1999; Rao *et al.,* 1999; Opabode, 2006).

Now a days most of the transformation experiments are carried out using *Agrobacterium*-mediated gene transfer methods, followed by biolistic method, in dicots and monocots.

2.3.2 Non tissue culture methods *in planta* transformation systems

2.3.2.1 Floral dip based *in planta* transformation

Plant transformation is a key methodology that has fostered diverse form of scientific inquiry and technology development (Brich, 1997; Gelvin, 1998; Hansen and Wright, 1999). Plants have been engineered to resist insect pests, tolerate herbicide and grow under stress, among other traits. The production of such transgenic plants requires three important procedures. First, a DNA delivery system is needed to transfer the gene of interest in to plant cells. Secondly, a selection system is required for separating transformed from non transformed cells. Lastly, we needed a reproducible regeneration protocol. This last step is often the rate limiting step, requiring specialized facilities and often resulting in tissue culture- induced variation (somaclonal variation). Further, for many plant species, the generation of lines carrying stable heritable transformation events remains a technical challenge. Transformation can be labor intensive process that is plagued by low success rate, excessive mutagenesis, and/or the need for highly skilled practioners. Research with *Arabidopsis* has benefited from the development of high throughput transformation method that avoids tissue culture (Azpiroz-Leehan and Feldmann, 1997). In particular the development of the

Agrobacterium tumefaciens mediated vacuum infiltration method (Bechtold *et al.,* 1993) has had a major impact on *Arabidopsis* research. *Arabidopsis* can now be transformed routinely in laboratories that have little or no expertise in tissue culture and transformation, allowing *in planta* analysis of multiple DNA constructs (Azpiroz-Leehan and Feldmann, 1997; Clough and Bent, 1998). A number of academic and industrial laboratories have carried out large scale transformation projects, generating thousand of independent transformed *Arabidopsis* lines from which T-DNA tagged mutants can be identified (Mollier *et al.,* 1995; Azpiroz-Leehan and Feldmann, 1997; Hirsch *et al.,* 1998; Richardson *et al.,* 1998). Large scale tagged population can even be used for reverse genetic identification of plant lines that carry in a gene of known DNA sequence (Krysan *et al.,* 1999; http://www.biotech.wisc.edu/*Arabidopsis*).

In planta transformation is a method of producing a transgenic seed comprising infection of recombinant *Agrobacterium* in to floral tissue of a plant without using a needless-hypodermic injection (Bent, 2000). Germ line transformation is the common feature that allows avoidance tissue culture and regeneration in the vacuum infiltration, seed transformation, *in planta* transformation and floral dip methods (Feldmann and Marks, 1987; Feldmann, 1992; Bechtold *et al.,* 1993; Cheng *et al.,* 1994; Katavic *et al.,* 1994; Clough and Bent, 1998). With all of these methods, selection with antibiotic or herbicide is not applied to the *Agrobacterium* treated T_o plant. Instead, progeny seed are harvested and selection is applied to the resultant seedlings as they germinate. Transformation events occurring prior to or early in floral development of the To plant would be expected to give rise to identical transformed male and female

gametophyte, which upon self-fertilization could produce a significant number of T_1 plants that are homozygous for T-DNA insertion. The rarity or total absence of such lines indicates that the relevant transformation events usually occurs during germ line development after divergence of male and female gametophyte cell lineages or possibly soon after fertilization of the T_1 embryo. Separate transformants from a single plant carry independent-DNA insertions even in methods that apply *Agrobacterium* to T_o seed in the growth of plant, again suggesting that transformation occurs late floral development (Bechtold *et al.,* 1993).

Despite above, the cell type that is transformed and timing of the transformation have remained unknown. The success of the floral dip method, in which flowering plants are simply dipped into a solution of *Agrobacterium*, suggested that the cellular targets are present on the exterior of the plant. Transformants are often derived at high frequency (as high as of all T_1 seed; Clough and Bent, 1998), again suggesting that the transformed germ line plant cells are readily accessible to *Agrobacterium.* Although the benefits of vacuum filtration and or floral dip transformation methods are evident, efforts to apply these methods to other plant species have generally been successful. However, reports of success with *Brassica compestris* subsp. *Chivesis* and *Medicago trucatula* suggest that the method is not uniquely restricted to *Arabidopsis* (Liu *et al.,* 1998.). A mechanistic understanding of the successful *Arabidopsis* method should foster application of similar transformation methods to other plant species.

Presently there is considerable interest in developing plant transformation methods that exclude tissue culture steps and rely on simple protocols; these methods are *in planta* transformation. Because transgenes are delivered into intact plants in the form of naked DNA or via *Agrobacterium*. In many cases these methods have targeted meristem or other tissues that will ultimately give rise to gametes (Chee and Slightom, 1995; Brich, 1997). Injection of naked DNA into castor ovaries have also been reported to produce transformed progeny (Zhou *et al.,* 1983). A mixture of DNA and pollen was either applied to receptive stigmatic surfaces or DNA was injected directly into rice floral tillers (Brich, 1997) or soybean seeds were imbibed with DNA (Shou *et al.,* 2002). These procedures proved to be impractical because of their low reproducibility.

Direct access to the gamete is an option that can be achieved by the delivery of DNA into the pollen. Subsequent pollination with the bombarded pollen led to recovery of transgenic plants. This method could also be applied to other crops (Desfux *et al.,* 2000). However, this apparently clear strategy, might in fact be complicated by a variety of factors including the presence of nucleases and methylases in pollen, pollen survival and polyploidy (Bent, 2000).

Recently, there are several successful results reported in cereal crops. Supartana *et al.,* (2005) have been developed transgenic rice by using *in planta* system. In this, embryo containing an apical meristem was inoculated with *Agrobacterium* by piercing a site of the husk overlying the apical meristem with needle. They obtained 40-43% transformation efficiency by PCR and histochemical assay of GUS gene respectively. Chumakov *et al.,* (2006) performed *in planta* treatment of maize silk with *Agrobacterium* and then

pollinated with the pollen of the same cultivar. They recorded the amplification of *nptII* gene showing the presence of about 60.3% of PCR positive plants. In hexaploid wheat (*T. aestivum* L.), Zale and Steber (2006) attempted *in planta* transformation and suggested that timing of the *Agrobacterium* application to the developing inflorescence is critical to the success of this method.

2.3.2.2 Rice Floral Organs, Pollination and Fertilization Mechanisms

Rice is a self pollinated crop, although out crossing up to 6.8% has been observed in some varieties and under certain conditions (Sahadevan and Namboodiri, 1963).

A rice plant bears perfect flowers in single-flowered spikelets borne on a panicle. A flower consists of six stamens, each composed of two-lobed, four-loculed anther, borne on a slender filament and a pistle containing one ovule (Fig 2.2). The short style bears a feathery stigma with two branches. The flower is fully developed and the stigma fully receptive at the time when pollen sheds. At this stage lodicules become turgid and force the lemma and palea apart. Anther dehiscence and extrusion occurs more or less simultaneously so that the stigma of a flower receives pollen from the same flower, hence resulting in self-pollination. Lemma and palea close after about 50 to 90 min (Virmani and Athwal, 1973).

Rice pollen grains after shedding from the anther are comparatively short-lived and generally lose their viability within 5 min under ordinary conditions; in some exceptional cases a few pollen grains remain viable for 15 min (Koga *et al.,* 1971). On the other hand, wild rice pollen grains have longevity of up to 9 min (Oka and Morishima, 1967).

Soon after being deposited on the stigma, pollen grains start the germination process. The first step in the process is expansion of the pollen grains by the absorption of liquid from the moist surface of the stigma and the protrusion of the intine through a germ pore. Germination occurs 2 to 3 min after pollination. Two generative and vegetative nuclei move into the pollen tube together with the pollen cytoplasm 3 to 5 min after pollination. After all the contents of pollen grains move into the pollen tube, the tube makes it way between the stigmatic papillae in to the tissues of the style. Only the distal part of the tube has living cytoplasm. About 9 hrs after pollination the pollen tube reaches the micropyle of the nucleus (Namai, 1987). Double fertilization starts after 9 to 12 hrs of pollination and finishes completely 18 to 24 hrs after pollination. The tip of the pollen tubes penetrates in to the embryo sac through a synergid, and then it bursts and gives off the generative nuclei. One of them fertilizes the egg cell and the other fertilizes the polar nuclei. The fertilized polar nucleus is the primary nucleus of the endosperm, which begins cell division soon after fertilization. The events occur during pollination and fertilization is presented in Figure 2.3.

The fertilized egg undergoes embryo development takes about 10 days to complete. Within 5 to 6 days after fertilization, the ovary grows longer and becomes the same length as the mature grain. It is possible to determine whether florets are fertilized or not 2 days after pollination. After about 15-16 days following fertilization, the young seed reaches the maximum width. The grain thickness then increases slowly until about 25 days after fertilization. Then the ripening of rice seed starts.

2.4 Selection and Molecular Characteristics of Transgenic Rice plant

Selection of transformed calli or plants is an important factor in plant transgenic technology. A simple but efficient protocol is essential in selecting transformants from which transgenic plants can be regenerated. A number of reporter genes Beta-glucuronidase (GUS), luciferase (LUC), chloromphenical acetyl transferase (CAT) and anthocyanins are being used in cereal transformation in order to analyze gene expression. In rice, the most commonly used reporter gene is B-glucuronidase (Peng *et al.,* 1992). The GUS gene was originally isolated from *E. coli* (Jefferson *et al.,* 1987). The intrinsic GUS like activity was observed in rice tissue by Hu *et al.,* (1990). It was controlled by treating tissue at alkaline pH 8.0 or by inducing 20% methanol in the assay. Selectable markers such as antibiotic resistance had increased the transformation screening. The neomycin phosphotransferase (*NPTII)* confers resistance to kanamycin and hygromycin phosphor tranferase (*hpt* also called *hph)* to Hygromycine antibiotic (Uchimiya *et al.,* 1986).

The apparent leakiness of Hygromycine being employed to select transformed cells (Matsuki *et al.,* 1989; Shimamoto *et al.,* 1989; Datta *et al.,* 1990; Meijer *et al.,* 1991 and Husnain *et al.,* 1995). In addition to transformation with selectable marker, the feasibility of conformation with a non selectable gene and a selectable marker had been used for the production of transgenic plants by Shimamoto *et al.,* (1989). Co-transformation frequencies were found to be independent of the pair of marker genes used (Peng *et al.,* 1990). The problem of Sterility in transgenic rice plants was found by Li *et al.,* (1990) and Datta *et al.,* (1991, 1992). Insertion of one or a few foreign genes did not generally adversely

affect the agronomic characters of transgenic plants (Uchimiya *et al.,* 1986). Early selection and screening of putative transformants for stable transformation is essential. GUS expression can be used as rapid early screening method but due to intrinsic GUS activity detected in many plants including rice (Hu *et al.,* 1990) and being a destructive assay, made GUS gene difficult for screening of stable transformants. Putative transformants could easily and quickly be screened using polymerase chain reaction (Potrykus, 1990). A rapid method for isolating plant DNA suitable for analysis by PCR was necessary and recently an efficient protocol for isolation of plant DNA and preparation of intact plant material has developed for PCR (Edward *et al.,* 1991). The application of PCR technique readily revolutionized the rice biotechnology programmes. PCR helped in assessing the presence of foreign DNA sequence in limited amount of putative transformed tissue. After identification of putative transformants, rigorous proof of transformation is needed for the judgment of stable transformation. Potrykus (1990) discussed different criteria necessary for confirmation of stable transformation included proper positive and negative controls, correlation between physical and genotype data (e.g. southern analysis and gene expression study by western blot), comparison of predicted and actual results, and data allowing discrimination between false and true positives.

Different protocols have been developed to investigate the presence of introduced foreign gene in the rice genome. The most important protocols are Polymerase Chain reaction (PCR), DNA-DNA Hybridization (Dot blot and Southern blotting), Western blotting and insect bioassay. PCR is quick and sensitive method in screening transgenic plants. The polymerase chain reaction

(PCR) has greatly simplified *procedures* for cloning, modification of nucleic acid and efficient detection of specific DNA sequence in individuals. PCR is a simple and powerful method invented and patented by Cetus Corporation (Saiki *et al.,* 1995; Schafr *et al.,* 1986). This method allows amplification of DNA, *in vitro*, through a succession of incubation steps at different temperatures. Typically, the double stranded DNA is heat denatured primers are annealed at low temperature and extended at an intermediate temperature. One step of these three consecutive steps is referred to as cycle. PCR is based on the repetition of these cycles.

Now –a-days PCR is routinely used for confirming the presence of gene in transgenic plants. Very small amount of genomic DNA either in pure or crude form is required for PCR. PCR can also be used to detect gene of interest in intact tissue either callus or leaf without isolating DNA (Victor *et al.,* 1993). The stable integration of gene in rice genome could be confirmed by dot blot hybridization or southern blotting. Southern blotting confirms the right integration as well as the copy number of introduced gene in plants (Curtis *et al.,* 2000).

2.5 Transgenic *Bt* approaches for pest control and plant protection

Bacillus thuringiensis is the most widely used biologically produced pest control agent. As of early 1998, there were nearly 200 *Bt* products registered by U.S. Environmental Protection Agency (Torla, 1998). Thought the use of biological pesticide in agriculture remains behind that of synthetic chemical pesticides, several safety and environmental consideration favor the development

of *Bt* in future. *cryI* proteins that have been studied so far, are not pathogenic to mammals, birds, amphibians or reptiles, but are specific to the target insects.

The most effective *Bt*-mediated pest control system is the development of *Bt* transgenic plants. Other systems like *Bt* transgenic microbes, *Bt*-transgenic viruses and *Bt* formulations suffer many inherent disadvantages (Kumar *et al.*, 1996). Recent advances in plant transformation technology have facilitated stable introduction of foreign genes into many crop including monocots. So there is considerable potential for genetic engineering of plants to express introduced *Bt* genes.

The main advantages of *Bt* transgenic plants against different pests include:

1. Protection of crop for whole season independent of weather condition.
2. Effective control of burrowing insects which escape the reach of sprays.
3. Control at all stages of insect pest development.
4. Non-injurious to beneficial insects like pollinators, predators/natural enemies of pests and also non-injurious to mammals including human beings.

The first transgenic plants expressing δ-endotoxin of *cry* proteins, having native coding sequences of *cry* gene was developed against tobacco horn worm in tobacco by Vaeck *et al.*, (1987). Barton *et al.*, 1987, analyzed expression of the full length and truncated native *cry*IAa gene in tobacco and found that no transformant produced detectable levels of *Bt* protein or mRNA. Northern blot analysis of these plants showed truncated mRNA species. Existence of this incomplete mRNA was attributed to inefficient post transcriptional processing or rapid turnover of the full length transcript.

Fischhoff *et al.*, (1987) was successful in transforming tomato plants with a native *cry*IA(b) gene which showed insecticidal activity. Similarly a native c*ry*IA (c)- npt II fusion in potato showed little insecticidal activity (Ebora *et al.*, 1994). Carozzi *et al.,* (1992) characterized the expression of native *cry*IA(b) endotoxin gene in field grown tobacco and observed that the level of *Bt*, (-endotoxin increased through the course of plant development with a substantial increase at the time of flowering).

When unmodified crystal protein genes are fused with expression signals used in the plant nucleus, protein production is quite poor as compared to that of similar genes containing typical plant marker genes. However, truncation of the unmodified genes to synthesize only the toxin protein of the protein notably results in much improved expression. The relatively AT-rich Bacillus genome contains a number of sequences that could provide deleterious signals to gene expression in plants; such signals may be splice sites, poly 'A' addition sties, ATTTA sequences, transcription termination sites, as well as bias in codon usage. Plants show strong preference for coding sequence (Murray *et al.,* 1989). When the native gene sequences which is poor in GC content in coding sequence. When the native gene sequences are modified with synonymous codons to reduced the potential deleterious sequence and generate a codon usage more like that of a plant, expression improves dramatically. (Murray *et al.*, 1989). Instability of mRNA could be due to endonucleolytic / exonucleolytic degradation at specific sequences that destabilize the message during transcription or create pausing due to formation of secondary structures.

Perlak and others at Monsanto in 1990-91 made a pioneering study of the expression of partially modified and fully modified (synthetic) c*ry*IAb and c*ry*IAc genes in cotton. The level of insecticidal protein was increased by 100 fold, resulting in the effective control of cotton boll worm.

Adang *et al.,* (1993) modified *cry*3A gene in a similar way and transformed potato plants conferring resistance to Colorado potato beetle. This improved expression of c*ry*3A gene was due to increase of GC- content from 36 per cent to 49 per cent in modified gene.

2.6 Development of *Bt* rice

Tu *et al.,* (2000) developed transgenic elite rice lines expressing a *Bt* fusion gene derived from *cry*IA(b*)* and *cry*IA(c) under the control of rice *actinI* promoter. The level of *Bt* fusion protein *cry*IA(b) / *cry*IA(c*)* detected in Minghui 63 (T51 – 1) plants was 20 ng/mg soluble protein. The *Bt* Shanyou 63 was field-tested in natural and repeated heavy manual infestation of two lepidopteron insects, leaf folder and yellow stem borer.

Fearing *et al.,* (1997) reported that the range and stability of expression of the transgenic *cryIA(b)* protein was examined in Ciba seeds *Bt* maize plants derived from Event 176. *cryIA(b)* expression in maize plants derived from transformation Event 176 was stable over at least four successive generations. On a per acre basis, the highest amount of *cry*IA(b) protein (estimated to be 2- 4 g *cry*IA(b) protein/acre) was found to occur at anthesis, consistent with the stage at which maximum plant vegetative biomass is reached. *cry*IA(b) was not detected in silage prepared from *cry*IA(b) - expressing plants. The milestones in the development of transgenic rice are given in Table 2.2.

Table 2.2: Milestone in rice transgenics

Year	Development	Reference
1988	First transgenic rice obtained	Toriyama *et al.*, (1988); Zhang *et al.*, (1988); Zhang and Wu, (1988)
1989	First fertile transgenic rice plants (*japonica*)	Shimamoto *et al.*, (1989)
1990	First fertile transgenic rice plants (*indica*)	Datta *et al.*, (1990)
1991	Use biolistics for generation of rice transgenics	Christou *et al.*,(1991)
1993	Insect resistance rice by using δ-endotoxin(*Bt*) transgenic rice with bar gene for sheath blight resistance	Fujimoto *et al.*,(1993); Uchimiya *et al.*, (1993)
1994	First conducive report of rice transformation by *Agro bacterium*	Hiei *et al.*, (1994)
1995	Resistance against bacterial blight obtained by using cloned *Xa*21 gene	Song *et al.*, (1995)
1996	Insect resistance transgenic *indica* rice harboring *cry*IAb gene field trail of herbicide resistance transgenic rice harboring *pin*II gene	Wunn *et al., (*1996); Oard *et al.*,(1996); Duan *et al*., (1996)
1997	Use of reconstructed *cry*IAc gene for high level resistance against pests	Nayak *et al.*, (1997)
1998	Multigene transformation First report of *Agrobacterium*-mediated rice transformation for agronomically important gene (*cry*IAb & *cry*IAc	Chen *et al., (*1998); Cheng *et al.*, (1998)
1999	Iron fortified transgenic rice with soybean ferritin gene Resistance against rice yellow mottle virus (RYMV) derived by pathogen derived resistance	Goto *et al.*, (1999); Printo *et al.*, (1999)
2000	Field trial of hybrid containing *Bt* gene Golden rice	Tu *et al.*, (2000); Ye *et al.*, (2000)
2001	Field trials shown high protection against SSB, YSB Wound-inducible expression of *cry*Ab against SSB	Ye *et al.*, (2001); Breitler *et al.*, (2001); Maqbool *et al.*, (2001)
2002	Use of different promoters for tissue specific *Bt* gene expression Different varieties transformed for insect-resistance	Husnain *et al.*, (2002) ; Khanna & Raina (2002)
2003	Constitutive or endosperm- specific expression of barely trypsin inhibitor *BT*I-CMe for protection against rice weevil	Alfonso-Rubi *et al.*, (2003)
2004	Field-trial of *Bt* rice in Pakistan	Bashir *et al.*, (2004)
2005	Development of efficient *in planta* transformation method for rice using *Agrobacterium tumefaciens* Two *cry*I genes *(*single *cry*IAb and fused *cry*IAb */cry*IAc*)* were introduced in to *indica* rice varieties	Supartana *et al.*, (2005) ; Chandel, (2005)
2006	Commercialized *Bt* rice in Iran	James, (2006)

Wu *et al.,* (1997) developed *japonica* rice variety Taipei 309 was co transformed by particle bombardment of immature embryo derived embryogenic calli with a modified δ-endotoxin gene *cry*IA(b) of *Bacillus thuringiensis* (*Bt*) under the control of the rice Actin 1 promoter, and the hygromycin resistance gene, *hph* driven by the CaMV35S promoter.

Bohorova *et al.,* (2001) reported that the twenty five percent of the transformed maize plants with *cry*IB expressed a protein that is active against the southwestern corn borer and sugarcane borer.

Maqbool and Christou (1999) reported that the *Bacillus thuringiensis* (*Bt*) δ -endotoxin gene *cry*IAc and *cry*IIA were carried on separate vectors, while the *gna* (snowdrop lectin) and *hpt* (hygromycin phosphotransferase) genes were linked on the same, co-integrate vector. They evaluated the molecular and expression profiles of 29 independently derived transgenic lines over two generations. The *gna* and *hpt genes* co-integrated with a frequency of 100% as expected. Furthermore 60% of the transgenic plants carried all four genes. They observed wide variation in the expression levels of the non selected transgenes among independently derived lines.

Maqbool *et al.,* (2001) reported that the simultaneous introduction of three insecticidal genes (the *Bt* genes *cry*IAc and *cry*IIA and the snowdrop lectin gene *gna*) into commercially important *indica* rice varieties M7 and Basmati 370, by particle bombardment. Transgenic plants expressed *cry*IAc, *cry2A* and *GNA* at different levels, either singly or in combination at 0.03-1%, 0.01-0.5% and 0.01-2.5% of total soluble protein respectively.

Wu *et al.,* (2002) indicated that the *cry*IAb gene was stably transmitted in an intact manner via successive sexual generations, and the concentration of the *cry*IAb protein was kept quantitatively stable up to the R_6 generation. The *cry*IAb gene driven by the maize *ubiquitin* promoter, displayed certain kind of spatial and temporal expression patterns under field conditions. The content of the *cry*IAb protein varied in different tissues of the main stems, the primary tillers and the secondary tillers.

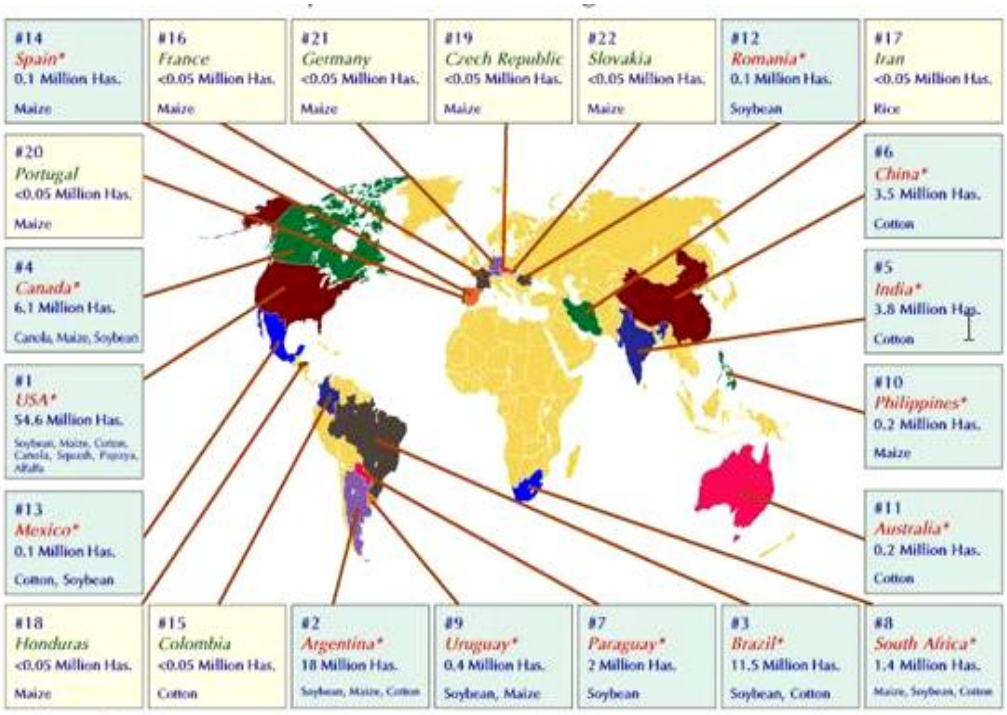
Maagd *et al.,* (1999) observed that the introduction of transgenic crops that are engineered to express a *Bacillus thuringiensis* toxin that confers resistance to insect predation.

2.7 Global status of transgenic crops

The promise of transgenic crops and their potential benefits to the farmers and the consumers, an environment, has led to the rapid increase in global under transgenic crops. This increase has been substantial in the developed world as compared to the developing countries. In the 10 years period since the commercial cultivation of transgenic crops stated, the global area under these crops increased by more then 60 fold, from 1.7 million hectares in 1996 to 102 million hectares in 2006. This rate of adaptation is the highest rate of crop technology adoption for any crop technology and reflects the growing acceptance of biotech crops by farmers in both large and small farmers in industrial and developing countries.

According to recent data, in 2006, 14 out of 22 countries reflect broadening, deepening and stabilizing in transgenic crop adoption by planting transgenic crops under more then 50,000 hectares. These mega countries

included the USA, Argentina, Brazil, Canada, India, China, Paraguay, South Africa, Uruguay, Philippines, Australia, Romania, Mexico and Spain (Fig. 2.4).



*14 biotech mega- countries growing 50,000 hectares, or more, of biotech crops (Source: James, 2006)

Figure 2.4: Transgenic crop countries and Mega countries during 2006

Globally, more than 15 transgenic crops have been approved for commercial use, mentioned in Table 2.3. The predominant transgenic crops are herbicide tolerant soybean, canola, maize, cotton; insect resistant maize and cotton and transgenic cotton and maize tolerant to both insect pests and herbicide (James, 2006). Area under these four dominant transgenic crops is as follows: 64% of Soybean, 19% of maize, 37.5% of cotton and 11.5% of canola of the total global area under cultivation for each crop (James, 2006).

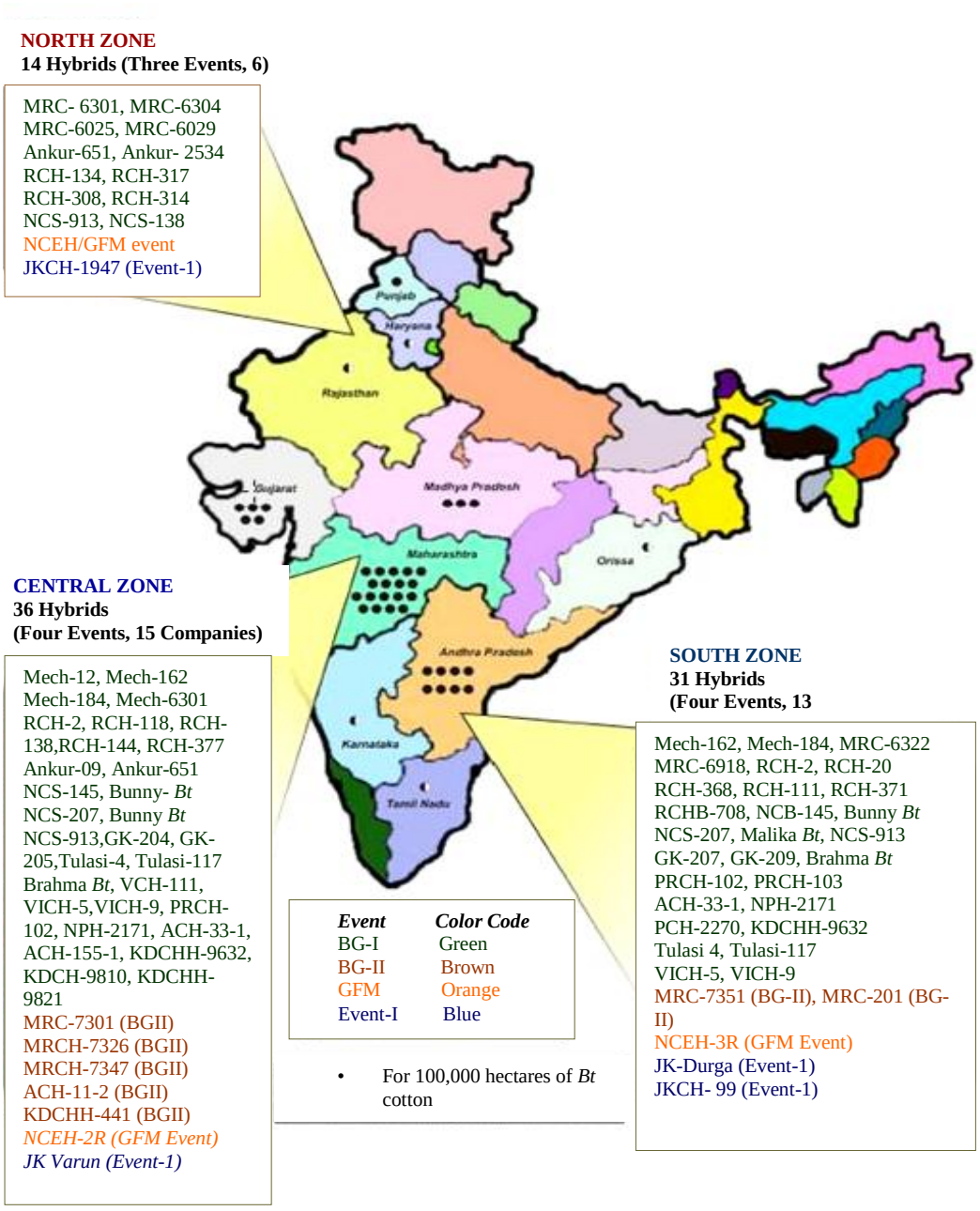
Table 2.3: Transgenic crops approved for commercial use

S. No.	Crop	Traits	Countries where approved
1.	Alfalfa	Herbicide tolerance	USA, Canada, Mexico
2.	Argentine	Herbicide tolerance and improved protection against weeds	Canada, USA, Japan, Australia
3.	Carnation	Increased shelf life by delayed ripening modified flower color and Herbicide tolerance	Australia, European Union
4.	Chicory	Herbicide tolerance, tolerance against weeds and higher Yields	European Union
5.	Cotton	Improved insect protection, herbicide tolerance and improved protection Against Weeds	Japan, Australia, USA, China, Mexico, South Africa, Argentina, India
6.	Flax, Linseed	Herbicide tolerance, antibiotic resistance improved	Canada, USA
7.	Green pepper	Virus resistance	China
8.	Maize	Herbicide tolerance, improved against weed Protection, resistance insects and restored fertility of seeds	Canada, Japan, USA, Argentina, European Union, South Africa, Philippines, Switzerland, Taiwan,China, U.K., Korea, Russia, Uruguay
9.	Melon	Delayed ripening	USA
10.	Papaya	Virus Resistance	USA, Canada
11.	Polish Canola	Herbicide tolerance and improved weed control	Canada
12.	Potato	Improved protection from insect and leaf roll virus Philippines	USA, Canada, Japan, Australia,
13.	Rice	Herbicide resistance	USA, Iraq
14.	Soybean	Improved weed control and herbicide tolerance, increased cooking quality	USA, Argentina, Japan, Canada, Uruguay, Mexico, Brazil and South Africa, Czech Republic, European Union, Korea, Russia, Switzerland, Taiwan, U.K., Philippines and Australia
15.	Squash	Resistance against watermelon mosaic virus and zucchini yellow mosaic virus	USA, Canada
16.	Sugar beet	Herbicide tolerance	USA, Canada, Japan, Philippines, Australia
17.	Sunflower	Herbicide tolerance	Canada
18.	Tobacco	Herbicide tolerance	USA
19.	Tomato	Improved shelf life, taste, color and texture, improved insect resistance, virus resistance	USA, Mexico, Japan, China, Canada

Source: Premier on GMOs. Biosafety information kit, India, 2006.

2.8 Current status of transgenic crops in India

In view of the importance and potential of transgenic crops, extensive efforts have been initiated in India for development of transgenic crops. As of now, *Bt* cotton containing the *cry*IAc gene from *Bacillus thuringiensis* is the only transgenic crop approved for commercial cultivation in India. The approval accorded to MAHYCO in 2002 and it was the first to receive approval for 3 *Bt* cotton hybrids i.e. MECH 12, MECH 162 and MECH 184 for commercial cultivation in the central and southern cotton growing zones in India. The deployment of the 62 *Bt* cotton hybrids in 2006 as well as their respective events in three regions is summarized and illustrated in the Figure 2.5. Subsequently, several other companies have taken sub-license from MAHYCO and in 2006, 62 hybrids of *Bt* cotton were approved for commercial cultivation in nine states in the country. The total area under *Bt* cotton has increased from 50,000 hectares to 3.8 million hectors in 2006. *Bt* hybrids available during 2006 are listed in the Table 2.4. Beside *Bt* cotton, ten food crops mentioned in Table 2.4 have been developed with traits such as resistance to insect pests, diseases, tolerance to drought/salinity and improving quality and extended shelf life.



(Source: James, 2006)

Figure 2.5: Approval of events and *Bt* cotton hybrids in India during 2006

Table 2.4: Transgenic crops approved for conducting contained limited field trials (including multi-location field trials)

S. No	Crop	Organization	Transgene
1.	Brinjal	Mahyco, Mumbai Sungro Seeds Ltd, New Delhi IARI, New Delhi	*cry*IAc *cry*IAc *cry*IF
2.	Cabbage	Sungro Seeds Ltd, New Delhi	*cry*IAc
3.	Cauliflower	Sungro Seeds Ltd, New Delhi	*cry*IAc
4.	Corn	Monsanto, Mumbai Metahelix Life Sciences, Bangalore	*cry*IAb Modified Mu-element (Turbo-Mu)
5.	Groundnut	ICRISAT, Hyderabad	Coat protein of IPCV Nucleo Capsid Protein of PBNV
6.	Mustard	UDSC, New Delhi	barnase & barstar
7.	Okra	Mahyco, Mumbai	*cry*IAc,
8.	Pigeonpea	ICRISAT, Hyderabad	*cry*IAc
9.	Rice	IARI, New Delhi Mahyco, Mumbai Metahelix Life Sciences, Bangalore	*cry*IAc, *cry*IAa + *cry*IB *cry*IAc NHX gene
10.	Tomato	IARI, New Delhi Mahyco, Mumbai	antisense replicase gene of tomato leaf curl virus, *cryI*Ac

Source: Premier on GMOs. Biosafety information kit, India, 2006.

Many of these transgenics are under green house testing and in some cases under field testing as per the biosafety regulations of Department of Biotechnology, GOI, New Delhi. Transgenic *Bt* rice cultivars are being developed in many ICAR laboratories and other labs. At NRC on Plant Biotechnology, *Bt* transgenic rice in agronomically superior cultivars such as IR 64 has been developed and tested for green house trials (Khanna and Raina, 2002). Elite *Bt* lines are being field tested at IARI (Bansal *et al.*, 2004). In addition to rice transgenic vegetable crops like brinjal, tomato, cabbage and cauliflower with *Bt* genes have been developed (Ranjekar *et al.*, 2003). The transgenic brinjal and tomato line has been field tested at IARI and multilocation trials with *Bt* brinjal are in progress (Bansal *et al.*, 2004).

CHAPTER-III

MATERIALS AND METHODS

The present study entitled **"Development of efficient transformation systems to enhance insect resistance in rice (*Oryza sativa* L.)**" was carried out at Plant Tissue Culture and Genetic Engineering Laboratory of the Department of Biotechnology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur, C.G.

3.1 Materials

3.1.1 Rice varieties

In the present study, two *indica* rice varieties namely Swarna and Mahsuri, popularly grown in Central and South East Asia were used. The details of rice varieties used are mentioned in Table 3.1.

Table 3.1: General characteristics of the two *indica* rice varieties used in the study

Genotype	Parentage	Released By/ Yr	Duration / Grain Type	Salient features
Swarna	Vasishtha X Mahsuri	Andhra Pradesh (1982)	145-150 days Medium slender	• Yields well at low (20Kg) of nitrogen level • Good grain quality, dark green leaf high, photosynthetic efficiency and high tillering ability. • Recommended for low lying areas of Krishna and Godavari zones • Semi dwarf stature and susceptible to biotic and abiotic stresses
Mahsuri	Taichung 65 X Mayong Abos 608012	Tamil Nadu (1971)	140-145 days Medium slender	• Yields high under average management • High tillering, BLB resistant • Recommended for *'thaladi'* • Tall stature (135-140 cm) • Developed by Malaysia

Two *Bt* genes, *cry*IAc and *m*VIP were used to develop pest resistant rice and GUS, as a reporter marker gene was also used to develop efficient transformation system for *indica* rice. These gene constructs were obtained from International Center of Genetic Engineering and Biotechnology (ICGEB), New Delhi through Material Transfer Agreement (MTA).

3.1.2 Gene Constructs

A strain of *Agrobacterium tumefaciens*, LBA 4404 strain harboring the plasmid vector pCAMBIA 1301 was used for transformation of rice calli in *Agrobacterium* - mediated and Particle gun mediated transformation systems as well as to infect emasculated rice florets during Floral dip based *in planta* transformation system. The plasmid vector pCAMBIA 1301 contained *Bt* gene *cry*IAc, *m*VIP and a reporter GUS gene with intron driven by a constitutive promoter CaMV35S respectively.

The partial construct map and gene sequence is shown in Figure 3.1, 3.2 and 3.3. The intron-containing GUS gene in T-DNA region was used to assay the efficiency of transformation. Secondly, the plasmid contains Hygromycine resistant (*hpt*) antibiotic gene as well as kanamycin resistant (*npt II*) gene driven by CaMV 35S promoter for the selection of transformed plant and bacterial cells respectively.

3.2 Rice transformation

3.2.1 Tissue culture protocol for *indica* rice transformation

Both rice varieties, Mahsuri and Swarna were used to transform using two tissue culture based methods, *Agrobacterium*-mediated and Particle gun mediated method and non- tissue culture based *in planta* system.

For tissue culture based methods, mature seeds of the rice varieties were inoculated on different callus induction medium. Succulent scutellar cells of mature embryos were used for Agro-infection and Particle gun mediated transformation system. The schematic procedure for tissue culture based transformation methods used is presented in Figure 3.4.

3.2.1.1 Seed inoculation

Seeds were carefully hand dehusked and selected grains were surface sterilized by using two sterilants namely, sodium hypochlorite and mercuric chloride with different concentrations and treatment time were used to find out suitable sterilizing agent with optimum concentration (Table 3.2).

Table 3.2: Treatments of sterilants used for effective seed sterilization

S. No.	Sterilizing agent			
	Sodium hypochlorite (NaOCl)		Mercuric chloride ($HgCl_2$)	
	Concentration (%)	Time (Min)	Concentration (%)	Time (Min)
1	0.2	45	0.1	10
2	0.5	30	0.5	06
3	0.6	20	0.6	05
4	0.7	10	0.7	03
5	0.8	05	0.8	02

The sterilization of selected embryos were done by using following protocol. The selected rice grains were washed using 3-5 ml of labolin with continuous shaking for 3-4 min and rinsed with sterile water for 2-3 times. Then in laminar air flow, the grains were rinsed with sterile water. These grains were washed in 70% ethanol with shaking for 45-50 seconds and then rinsed with sterile water to remove traces of ethanol. Surface sterilization of seeds were carried out on a shaker using a solution of mercuric chloride (0.1% $HgCl_2$ with

active chlorine) and Tween- 20. After 4-6 min, the solution was removed and seeds were thoroughly washed 5-6 times with sterile water. Sterilized seeds of the rice varieties were blot for few minutes on sterile blotting paper and transferred on to a Petri disc containing different callus induction medium.

3.2.1.2 Optimization of media composition for callus induction

Different compositions of MS basal medium (Table 3.3) were used to get high efficiency callus induction and plant regeneration for rice varieties, Swarna and Mahsuri. Those callus induction media were, MS media with 2.5 g/L 2,4-D, 30 g/L sucrose, 8 g/L agar agar; LS media supplemented with MS basal salts, 30 g/L sucrose as carbon source, 3 g/L gel rite, 4 g/L thiamine HCl, MSM medium containing MS basal salts, 0.500 g/L L-proline, casein hydrolysate and kinetin respectively. However, L3 medium supplemented with MS basal salts, 0.5 g/L glutamine, 3.00 g/L 2, 4-D were used with pH 5.7-5.8 before autoclaving. Sterilized mature embryos were cultured on MS, LS, MSM and L3 media and incubated at $28\pm1^{o}C$ in dark for callus induction. After 3 weeks of incubation under dark at $28\pm 1^{0}C$, calli initiated transformation after overnight of desiccation. The embryogenic callus inducing frequency was determined as following:

$$\text{Callus induction (\%)} = \frac{\text{Number of calli induced}}{\text{Number of seeds incubated}} \times 100$$

Table 3.3: Composition of different callus induction medium used in study

Source	Component	Concentration (g/L)			
		MS [a]	**LS [b]**	**MSM[c]**	**L3[d]**
MS I	NH_4NO_3	1.70	1.65	1.65	1.65
MS II	KNO_3	1.90	1.90	1.90	1.90
	$MgSO_4.7H_2O$	0.37	0.37	0.37	0.37
	$MnSO_4.4H_2O$	0.100	0.223	0.223	0.223
	$ZnSO_4$	0.20	0.106	0.86	0.86
	$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025
MS III	$CaCl_2.2H_2O$	0.400	0.440	0.440	0.440
	KI	0.75	0.80	0.83	0.83
	$CoCl_2.6H_2O$	0.025	0.025	0.025	0.025
MS IV	KH_2PO_4	0.170	0.170	0.170	0.170
	H_3BO_3	0.30	0.62	0.62	0.62
	$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25
MSV	$FeSO_4.7H_2O$	4.18	2.78	2.78	2.78
	Na_2EDTA	5.59	3.12	3.73	3.73
Vitamins	Nicotnoic acid	0.5	0.5	0.5	0.5
	Pyridoxine HCl	0.5	0.5	0.5	0.5
	Thiamine HCl	0.1	0.4	0.1	0.1
	Glycine	2.0	2.0	2.0	2.0
Meso inositol		0.100	0.100	0.100	0.100
L-Protein		-	-	0.500	0.500
Glutamin		-	-	-	0.500
Casein Hydrolysate		-	-	0.500	-
Kinetin		-	-	0.500	-
Hormones	2,4-D	2.5	2.5	2.5	3.0
Sucrose		30.0	-	-	30.0
Maltose		-	30.0	30.0	-
Agar agar		8.0	8.0	-	8.0
Gelrite		-	-	3.0	2.5
pH - 5.6-5.8		5.8	5.8	5.8	5.8

Note: **a**- Murashige and Skoog, 1962; **b**- Linsmaier and Skoog, 1965; **c**- Murashige and Skoog Modified; **d**- Lin and Zang, 2005

3.2.2 Transformation systems used

Transformation of the subjected *indica* rice varieties for the introduction of recombinant construct carrying target gene (GUS, *cry*IAc and *m*VIP) has been done by using Tissue culture based and Non-tissue culture based systems. The

schematic protocol for production of transgenic rice plants using tissue culture based method *viz.*, Particle gun mediated and *Agrobacterium* mediated system is shown in Figure 3.4.

3.2.2.1 Tissue culture based *Agrobacterium*-mediated transformation

3.2.2.1.2 Preparation of *Agrobacterium* culture for co-cultivation

The *Agrobacterium* inoculum of strain LBA 4404 containing desired gene construct from the stock stored at -20^{o}C was grown on the minimal media containing plates having 50mg/L kanamycin for 2 days at 28$\pm$1^{o}C in the dark. *Agrobacterium* was scrapped from freshly grown AB medium plates and inoculated the culture in 5 ml of liquid AB media (Table 3.4) with respective antibiotic, kanamycin. Next day the same overnight grown 5 ml media again inoculated in 50 ml freshly prepared AB medium supplemented with kanamycin @ 50 mg/L and 40 mg/L AS on rotary shaker @150 rpm at 28^{o}C for 2-3 hrs till the inoculum density OD_{600} reaches 0.6. Inoculum density for the bacterial suspension was measured at OD_{600} using Spectrophotometer.

Table 3.4: Composition of AB Media used for *Agrobacterium* culture

Stock	Components	Amount (g/ 500 ml)
Stock I	K_2HPO_4 NaH_2PO_4	30.0 10.0
Stock II	NH_4Cl $MgSO_4$. $7H_2O$ KCl $CaCl_2$, $2H_2O$ $FeSO_4$ $7H_2O$	10.0 3.0 1.5 0.1 0.025
Stock III	Glucose Agar	5.0 15.0

3.2.2.1.3 Infection and co-cultivation of rice embryogenic calli with *Agrobacterium tumefaciens*

Three-four weeks old embryogenic calli of size ~1-2 mm were used for Agro- infection after overnight desiccation. The desired extent of desiccation was obtained by transferring calli to sterile empty Petri dishes containing sterile Whatman- No.1 filter paper. The Petri dishes were sealed with Para film and kept at 26±1^{o}C in dark for 18-24 hrs to obtain the desiccation of calli. After desiccation treatment, the calli were immersed in *Agrobacterium* inoculum aided with acetosyringone before 2 hours for 20-30 min Agro-infected calli were blotted dry on sterile Whatmann No. 1 filter paper and transferred on to the co-cultivation medium under dark at 26±1^{0}C for 2-3 days. After co-cultivation for three days the calli were shifted on fresh media containing cefataxim 250 mg/L to inhibit the over growth of *Agrobacterium* and Hygromycin with appropriate selection for 7 days at 28±1^{o}C in dark.

3.2.2.2 Isolation of Ti plasmid from *Agrobacterium* strain

For Particle gun mediated transformation, the two gene constructs, *cry*IAc and *m*VIP were transformed in to *E. coli* cells, to get the high copy multiplication of Ti plasmids. The Ti plasmids carry *Ori* gene for high copy multiplication in *E. coli* cells. The Ti plasmid was isolated using Alkali lysis method from *Agrobacterium* strain and subsequently transformed into *E. coli* DH5α strain by Freez thaw method as described by Maniatis *et al.,* (1982).

3.2.2.2.1 Plasmid DNA isolation

Cloned plasmid vector pCAMBIA 1301 was isolated from the *Agrobacterium* strain LBA 4404 by using alkali lysis method given by Sambrook *et al.,* (1989). The stepwise protocol of alkali lysis method is described below:

- Bacterial cells, LBA 4404 containing the desired clone (pCAMBIA 1301) were grown overnight at 28^{o}C in LB medium containing suitable antibiotic (Kanamycin @50 mg/L).
- The cells were harvested by centrifuging 1.5 ml of culture at 12000 rpm for 7-8 min at room temperature (RT).
- The supernatant was discarded completely and left the tubes in an inverted position on a tissue paper for 3-4 minutes to drain off AB completely.
- 50 µl of Lysozyme was added and the pellet was resuspended in 200 µl of solution I by gentle vortexing or with the help of pipettman.
- 400 µl of freshly prepared solution II was added and mixed immediately by gently inverting the tubes few times till cell suspension became clear and incubated in ice for 5 min.
- 300 µl of chilled solution III was added (stored at -20^{o}C) and mixed thoroughly but gently by inverting the tubes several times till a white coarse precipitation was visible then incubated on ice for 15 min. The composition of Solution I, II and III are given in Table 3.5.
- Centrifuged at 12000 rpm at 4^{o}C for 20 min and transferred ~700µl of clear supernatant to a fresh micro centrifuge tube. Centrifuged again at 12,000 rpm at RT for 3 minutes and transferred 600 µl of clear supernatant to a fresh micro centrifuge tube.
- 450µl of isopropanol was added and mixed well and incubated at RT for 10 min then pellet down plasmid DNA by centrifuging at 12,000 rpm for 5 min at RT.

- The supernatant was discarded carefully by decanting and left the tubes in an inverted position on a tissue paper for few minutes to drain-off isopropanol completely.
- The pellet was washed by adding 800 µl of 70% ethanol to the micro centrifuge tubes and then centrifuged at 12,000 rpm for 5 min at RT. The supernatant was discarded carefully by decanting. Centrifuged once again at above conditions for 1 min only and discarded the remaining ethanol with the help of pipetteman.
- The pellet was air dried for 5 min and dissolved the DNA pellet in 25-50 µl of TE Buffer containing RNase A (50 µg/ml).
- The DNA was resolved on 1% agarose gel by electrophoresis as well as Nanodrop Spectrophotometer to check the quality and concentration of DNA.

Table 3.5: Solutions used in alkali lysis method of plasmid DNA isolation

Chemical	Components	Final Conc.
Solution I (pH 8.0)	Glucose	50 mM
	Tris (pH 8.0)	25 mM
	EDTA (pH 8.0)	10 mM
Solution II	SDS	1.0 %
	NaOH	0.2 N
Solution III	Ammonium acetate	7.5 M
TE Buffer	Tris (PH 8.0)	1.0 M
	EDTA (pH 8.0)	0.5 M

3.2.2.2.2 Preparation of Gold particles for bombardment

Proceeded with a freshly opened bottle of ethanol and sterile water only

- 50 mg of Gold powder was taken in a microfuge tube and added 1ml of freshly opened absolute ethanol.
- Centrifuged for 10 seconds at high speed (2000 rpm) in a microfuge and discarded the supernatant and re-suspended the gold particles in 1 ml of fresh ethanol and transferred into 15 ml round bottom falcon plastic tube.
- Vortexing was done thoroughly for five minutes each for three times with 1 min interval in cold room or low temperature and transferred the gold suspension to an eppendorf tube.
- Centrifuged 10 seconds at high speed and discarded the supernatant and washed gold particles with sterile double distilled water 3 times.
- The particles were re-suspended finally in 1 ml water and stored aliquots of 50 µl at -20^{o}C for further use till 1 month.

3.2.2.2.3 Coating of DNA to gold particles (Micro carrier)

The following components were mixed maintaining in the order with slow vortexing before adding the next component

1. 50µl tungsten solution
2. 10µl DNA in TE buffer (1µg/µl conc.)
3. 50µl of $CaCl_2$ (2.5M)
4. 20µl spermidine free base (0.1M. Sigma Cat No. S0266)

- Vortexing was done for 30 min in cold room and proceeded further with the sample preparation.

- The sample was kept on ice throughout the preparation period.
- 200µl ethanol was added and centrifuged for 10 sec @ 10,000 rpm and removed supernatant and added 200µl ethanol and re-suspended very well by pipetting or mild vortexing.
- Centrifuged for 10 seconds and replaced the ethanol and the same step of above washing were repeated for 3 times.
- The final DNA coated gold particles was re-suspended in 30µl of ethanol.
- 5µl-7µl sample was used for shooting each plate containing embryogenic calli.

3.2.2.2.4 Loading of DNA coated with gold particles

Aliquot of 5 µl-7µl of DNA coated gold suspension was loaded onto macro carrier disc at the centre which was previously inserted into the macro carrier holder and the alcohol was allowed to evaporate in a sterile environment (laminar air flow hood).

3.2.2.2.5 Bombardment of coated gold suspension on to the targeted calli

Eventually, Plasmid DNA was precipitated as a mixture of 3 mg gold particles (M 10 Bio Rad), 10µl mixture of plasmid DNA (8-10 µg), 20 µl spermidin free base (0.1 M) and 50 µl $CaCl_2$ (2.5 M). The mixture was centrifuged in a microfuge for 15 second at 1000 rpm and the supernatant was removed. The pellet mixture (Plasmid DNA and gold particles) was washed 2-3 times with ethanol three times and re-suspended in 50 µl absolute ethanol. For each bombardment 10-12 µl of particle DNA suspension was spreaded on to the surface of the micro carrier film of the PDS-1000/ He micro projectile delivery

system (Fig 3.4). Plates containing embryogenic calli (3-4 weeks old) were placed at the second stage of machine, 6 cm from the stopping screen. Each plate was bombarded with helium pressure of 900, 1100, 1350, 1800, 2000 psi under partial vacuum at 20 lbs. The plates were kept in dark for 3-4 days. Then the bombarded calli were transferred to selection medium containing optimum dose of Hygromycine.

3.3 Selection of transformed calli

The bombarded as well as Agro-infected calli were transferred on to the selection medium (MS modified) containing 30 g maltose, 0.3% gel rite, 2.5 mg/L 2,4-D, 500 mg/L casein hydrolysate, 500 mg/L L-proline and 35 and 45 mg/L Hygromycine B for Swarna and Mahsuri derived calli respectively.

In *Agrobacterium*-mediated transformation system, infected calli were transferred to selection medium after 3 days of co-cultivation whereas, in Particle gun mediated system, bombarded calli were shifted to selection medium after 48 hr of bombardment. The selection cycle was carried out for 6-8 weeks within the change of medium every 3 weeks. The brown colored dead calli / cells were separated and transferred onto fresh selection medium for each cycle of selection.

3.4 *In vitro* regeneration of *indica* rice

To get highly efficient *in vitro* regeneration of Swarna and Mahsuri varieties we have used three different compositions of growth hormones. The composition M 1 was MSM basal medium; composition of M2 medium contains MSM supplemented with kinetin 2.5 g/L, NAA 0.5 g/L and composition M3 having MSM supplemented with kinetin 2.0 g/L, NAA 1.0 g/L and BAP 0.5 g/L

(Table 3.6). Partially desiccated and non-desiccated four-five weeks old calli were transferred in culture tubes containing M1, M2 and M3 regeneration

Table 3.6: Composition of co-cultivation and regeneration medium

Source	Component	Culture Medium (g/L)			
		MSM	**Co-Cultivation**	**Shoot Induction**	**Root Induction**
MS I	NH_4NO_3	1.65	1.65	1.65	0.825
	KNO_3	1.9	1.9	1.9	0.95
	$MgSO_4.7H_2O$	0.370	0.370	0.370	0.185
	$MnSO_4.4H_2O$	0.223	0.223	0.223	0.1115
	$ZnSO_4$	0.86	0.86	0.86	0.43
	$CuSO_4.5H_2O$	0.025	0.025	0.025	0.0125
MS II	$CaCl_2.2H_2O$	0.440	0.440	0.440	0.22
	KI	0.83	0.83	0.83	0.415
	$CoCl_2.6H_2O$	0.025	0.025	0.025	0.0125
MS III	KH_2PO_4	0.170	0.170	0.170	0.085
	H_3BO_3	0.62	0.62	0.62	0.31
	$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.125
MS IV	$FeSO_4.7H_2O$	2.78	2.78	2.78	1.39
	Na_2EDTA	3.73	3.73	3.73	1.865
MSV	Nicotnoic acid	0.500	0.500	0.500	0.250
	Pyridoxine HCl	0.500	0.500	0.500	0.250
	Thiamine HCl	0.100	0.100	0.100	0.050
	Glycine	2.00	2.00	2.00	1.00
Vitamins	Meso inositol	0.100	0.100	0.100	0.050
	L-Protein	0.500	0.500	0.500	0.250
	Casein hydrolysate	0.500	0.500	0.500	0.250
	Kinetin	-	-	2.0^{A} 2.5^{B}	-
	NAA	-	-	1.0^{A} 0.5^{B}	-
	BAP	-		0.0^{A} 0.5^{B}	-
	D-Glucose		18.00	-	-
	Acetosyringone	-	200uM	-	-
	Maltose	30.0	22.00	30.00	15.00
	Gelrite	4.00	-	4.00	4.00
	pH	5.8	5.2	5.8	5.8

Note: A - The medium containing A^{th} concentration of the respective component for shoot induction.

B - The medium containing B^{th} concentration of its respective component for shoot induction.

respectively to know the effect of treatment of desiccation and auxin: cytokinin ratio on regeneration. For desiccation, before calli were transferred on to regeneration medium, the calli were kept for desiccation. To do so, calli were in light at 26^{o}C. After 25 days regenerants with 3-4 cm long shoots were transferred on to half MS medium, for root induction, half MS medium was used and plant regeneration frequency was calculated as follows:

$$\text{Plant regeneration (\%)} = \frac{\text{Number of regenerated calli}}{\text{Number of calli incubated}} \times 100$$

The well rooted plants were cultured hydroponically in Yoshida's culture solution (Yoshida *et al.*, 1976) for one or two weeks and then transferred to pots containing soil mixture and placed in the transgenic green house. The composition of Yoshida's culture is given in Table 3.7.

Table 3.7: Composition of Yoshida's culture solution (Yoshida *et al*., 1976)

Stock	Components	Stock solution (g/ 10 L)
A 1	NH_4NO_3	914.0
B2	$NaH_2PO_4.2H_2O$	403.0
C3	K_2SO_4	714.0
D4	$CaCl_2$	886.0
E5	$MgSO_4.7H_2O$	3240.0
F Dissolved separately and then combined with 500ml of concentrated sulphuric acid and made up to 10L with distilled water. **pH- 5.0**	$MnCl_2.4H_2O$ $(NH_4)_6Mo_7O_{24}.4H_2O$ H_3BO_3 $ZnSO_4.7H_2O$ $CuSO_4.5H_2O$ $FeCl_3.6H_2O$ Citric acid (Monohydrate)	15.0 0.74 9.34 0.35 0.31 77.0 119.0

3.2.2.3 Non-Tissue culture based method: Floral dip based *in planta*

3.2.2.3.1 Floral dip based *in planta* transformation

Of the many variables present in the germ line transformation protocol, one of the most important is the target cell of the flower and developmental stage of the plant at the time of inoculation with *Agrobacterium*. Although, it has previously been shown that transformation can occur when *Agrobacterium* is applied to rosettes at the site of bolt initiation or even to seed just prior to planting (Cheng *et al.,* 1994; Feldmann and Marks, 1987; Feldmann, 1992; Katavic *et al.,* 1994), reproducible high frequency transformation is reliably achieved with plants in which the inflorescence has developed and floral buds are evident (Betchold *et al.,* 1993). To identify the floral target cell that is most susceptible to transformation, we used floral dip based *in planta* transformation. The outline of floral dip based *in planta* transformation method is given in Fig. 3.5.

In this study, six to seven week old individual Swarna plants (before flowering) were uprooted and transferred in pots and brought to containment green house. The unopened emasculated rice flowers and un-emasculated flowers at 25, 50 and 100% anthesis stage were treated with *Agrobacterium* inoculum. The secondary shoots and panicles were clipped close to the base, leaving very few tillers carrying panicles were grown and used in this study. The pots were kept in green house under 12-14 hr day photoperiod at 27-30^{o}C temperature without irrigation for two day before to infect. When plants grown, attained to flowering stage, inflorescence of Swarna genotype at different floral stages were selected and plants used as female were hand emasculated. Emasculated flowers of Swarna were treated with *Agrobacterium tumefaciens*

harboring *cry*IAc, *m*VIP and GUS genes respectively, following different treatments of *Agrobacterium* inoculation density, dipping time of emasculated flowers in Agro-inoculum stored in the wide mouth beaker with Jack and platform system (Fig. 3.6). Treatment OD_{600} of *Agrobacterium* inoculum with 0.2 and 0.4 were used with the flower dipping time of 2.0 and 4.0 min. with gentle shaking and glass spray to mist onto rice flowers were used for *in planta* transformation. The treated inflorescences were hand pollinated using anthers from freshly opened flowers of Swarna genotype after 16-17 hrs of drying as per the conventional method of developing crosses between rice varieties. After pollination, covering of treated panicle was done to maintain humidity and allowed to grow naturally. After ~20 days seed setting took place. At proper ripening when all seeds became yellowish, they were harvested. Those T_1 seeds were again grown in containment green house and used leaf and root tissues of 40-45 day old T_1 plants for GUS assay. The integration of transgenes, GUS and *cry*IAc was confirmed using PCR analysis.

3.2.2.3.2 Preparation of *Agrobacterium* inoculum

The plates of *Agrobacterium* strain LBA 4404 carrying pCAMBIA 1301 plasmid vector harboring GUS and c*ry*IAc were prepared on AB medium containing kanamycin antibiotic @ 50 mg/ L. Single colony from the freshly grown plate was inoculated in 5 ml of AB suspension culture medium with respective antibiotic and grown at $28\pm1^{o}C$ for overnight. Next day, the inoculum was taken from 5 ml overnight grown inoculum and re- inoculated in 50 ml of AB media supplemented with *vir* gene inducer acetosyringone for 2-3 hrs at $28\pm1^{o}C$ with aeration in dark. Then OD_{600} was checked and dilutions were

prepared with AB medium of OD_{600} 0.2 and 0.4. The diluted inoculum of treatment OD_{600} (0.2 & 0.4) containing acetosyringone was used for floral dip treatment.

3.5 Quantitative GUS assay

The leaf and root tissues of 30 days old T_1 plants were used for histochemical GUS assay using X-gluc test as per the method described Jefferson, (1987). The leaf and roots sample were dipped into the X- gluc solution and incubated at 37^{o}C for 12-24 hrs. The expression of GUS gene expressing indigo blue color in plant sample were recorded after washing with 70% ethanol. The plants showing the GUS expression were promoted for PCR analysis and to calculate transformation efficiency.

3.6 Molecular screening of putative transgenic T_1 plants by PCR analysis

3.6.1 Genomic DNA isolation from T_1 plants

Genomic DNA was isolated by homogenizing 100-150 mg of leaf tissue and extracting essentially according to CTAB method given by Saghai Maroof *et al.,* (1984). The step wise protocol is given below:

- ~100 mg of tender shoot was grinded in 400 µl 2X CTAB exaction buffer with a glass rod on spot plate.
- 400 µl more of 2X CTAB extraction buffer was added and mixed thoroughly, in ~ 700 µl of solution and then transferred, into 1.5 ml eppendorf tube.
- Incubated at 65°C on water bath for 15 min and then cooled briefly and 700 µl of Chloroform Isoamyl Alcohol was added.

- The contents were shaken by hands intermittently and kept at room temperature for 15 min. Tubes were centrifuged at 13,000 rpm for 3 min.
- 600 µl of upper aqueous phase was transferred into a new 1.5 ml eppendorf tube. 900 µl of absolute ethanol was added and mixed gently and the tubes were kept for 2 hrs at –20°C.
- The sample was centrifuged for 3 min at 10,000 rpm, the supernatant was decanted. The pellet was washed with 70 % ethanol and air-dried.
- DNA pellet was air dried and then dissolved in 50 µl of TE buffer
- The extracted DNA was quantified by using Nanodrop spectrophotometer and 0.8% agarose gel electrophoresis. The DNA samples diluted to make the concentration as 50 ng/µl to use as template for PCR analysis.

3.6.2 Quantification of Genomic DNA

Genomic DNA extracted from T_1 plants was quantified with Nanodrop spectrophotometer as well as 0.8% agarose electrophoresis. 3µl of DNA samples isolated from each plant along with the standards of known quantity of DNA was loaded on 0.8% agarose gel. The DNA was stained with ethidium bromide and observed under UV trans-illuminator. The quantity of DNA was estimated by comparing with fluorescence of standards. After the quantification DNA was diluted with sterile water such that the final concentration of DNA was 50 ng/µl.

3.6.2.1 Genomic DNA isolation Reagents and solutions

- **1M Tris- HCl (PH-8.0)**

30.28g of Trizma base was dissolved in 200 ml of distilled water. The pH was set to 8.3 using concentrated HCl. The solution was allowed to cool at room

temperature before making a final adjustment of pH. The final volume was adjusted to 250 ml with distilled water and sterilized by autoclaving.

- **0.5M EDTA (pH-8.0)**

186.1 g EDTA was dissolved in 800 ml distilled water stirred vigorously on a magnetic stirrer and the pH adjusted to 8.0 with NaOH. The volume was made up to 1 L.

- **CTAB extraction Buffer**

5 g CTAB, 20.35 g NaCl dissolved in 200 ml double distilled water later 25 ml 1 M tris HCl 10 ml 0.5 M EDTA were added and stirred vigorously on a magnetic stirrer. Volume was made upto 250 ml and stored in room temperature and 20 µl /20 ml 2- Mercaptoethanol added into it prior to use.

- **Ethanol (70%)**

  70 ml of absolute ethanol was taken in measuring cylinder and volume was made up to 100 ml using distilled water.

- **TE buffer (pH-8.0)**

  10 ml 1 M Tris HCl mixed with 2 ml 0.5 M EDTA the volume was adjusted by using 988 ml of sterile double distill water.

- **PCR Reagents**

- **dNTPs: (dATP/dCTP/dGTP/dTTP)**

  100mM stock of each dNTP was diluted to 1mM of dNTP (i.e. 10 µl of each dNTP + 990 µl of sterile water).

- **2.5 M $CaCl_2$**

36.8 g of $CaCl_2$ was dissolved in 50 ml of Autoclaved double distilled water.

- **PCR Buffer (10X)**

Components	Concentration
1 M Tris (pH 8.3)	4.0 ml (100mM final Conc.)
1M KCl	10.0 ml (500 mM final Conc.)
1.5 mM $MgCl_2$	2.0 ml
Gelatin	2.0 ml (1 mg/ml final Conc.)
Sterile water	4.0 ml
Total	**20.0 ml**

- **LB Broth medium, Lennox (Difco, 240230)**

20 g of the LB powder was suspended into 1 L of autoclaved distilled water and autoclaved at 121^{o}C for 15 min.

- **50X TAE buffer**

Components	Concentration
Trizma base	121.00 g
Acetic acid (glacial)	28.55 ml
0.5M EDTA (pH 8.0)	100.00 ml
Water	50.45 ml
Total	**300 ml**

3.6.3 PCR analysis

The initial screening for the presence of transgene in regenerated plants was done using PCR technique as per the methods described by Datta *et al.*, (1999).

Plant DNA isolated from leaf tissue was used as template to obtain amplification product of GUS and *cry*IAc genes for screening of putative transgenic plants. For integration of transgenes, following gene specific primer pairs were used.

GUS primer

Forward-5’CGT GCT GCG TTT CGA TGC GG-3’

Reverse-5’ACC ATT GGC CAC CAC CTG CCC-3’

***cryIAc* primer**

Forward-5’ATG GAT AAC CCA AAC ATT AAC-3’

Reverse-5’GTA CTC AGC CTC AAG AGT GGC-3’

***m*VIP primer**

Forward-5’GTT GAC CAC TAG AGC TTT GC-3’

Reverse-5’CTT AAT AGA GAC ATC GTA G-3’

The 100 ng of genomic DNA isolated from putative transgenic rice plants, the negative control (non-transgenic/non-infected) plant and plasmid DNA which was used for transformation were used as template for PCR reaction. The PCR reaction mixture consisted of 10 ng of template DNA, 50ng/µl of each the primer, 0.5mM dNTPs, 10X PCR buffer and 1-2 Unit of *Taq* DNA polymerase in final volume of 20ul (Table 3.8).

Table 3.8: PCR components with their quantity used for screening the putative transformants of rice

Components	**Concentration**	**Quantity (µl)**
1. Genomic DNA	10 ng/ul	2.0 µl
2. Taq Buffer	10 X	2.0 µl
3. dNTPs	0.5mM	2.0 µl
4. Primer F	50ng	1.0 µl
5. Primer R	50ng	1.0 µl
6.*Taq* Polymerase	1-2 U/µl	1.0 µl
7. Sterile Water	-	11.0 µl
Total		**20.0** µl

The PCR condition was performed in Thermal Cycler (MJ Research, PTC 100, USA). The PCR conditions were set as per the transgene and primer combinations used (Table 3.9a, 3.9b & 3.9c). PCR products were separated on 1% agarose gel (in 1X TAE electrophoresis buffer) containing 0.5 µg/ml ethidium bromide. Separated products were visualized under UV light and photographed using gel documentation system (Bio Rad, USA) to examine the size of the amplification products. Based on the size of DNA band and its comparative position with amplified product of a plasmid DNA, transgenic plants were designated as positive.

Table 3.9a: Temperature profile used for the amplification of reporter GUS gene

Steps	Temperature (^{o} C)	Duration (min)	Cycles	Activity
1	94	2.0	1	Denaturation
2	94	0.30	↑	Denaturation
3	52	0.45	35 cycle	Annealing
4	72	1.30	↓	Extension
5	72	1.0	1	Final extension
6	4	720	1	Storage

Table 3.9b: Temperature profile used for the amplification of *Bt* gene, *cry*IAc

Steps	Temperature (^{o} C)	Duration (min)	Cycles	Activity
1	94	2.0	1	Denaturation
2	94	0.45	↑	Denaturation
3	50	0.45	35 cycle	Annealing
4	72	0.45	↓	Extension
5	72	4.0	1	Final extension
6	4	720	1	Storage

Table 3.9c: Temperature profile used for the amplification of *Bt* gene, *m*VIP

Steps	Temperature (^{o} C)	Duration (min)	Cycles	Activity
1	94	2.0	1	Denaturation
2	94	0.45		Denaturation
3	55	0.45	35 cycle	Annealing
4	72	0.45		Extension
5	72	4.0	1	Final extension
6	4	720	1	Storage

3.4 Southern Blot Analysis

Genomic DNA of putative rice transformants, the control non transgenic rice and 1.8 kb fragment of *Eco*RI- *Hind* III digest of *cry*IAc Plasmid were sent Bangalore Genie lab, Bangalore and ICGEB New Delhi for southern analysis

Ten μg genomic DNA of transgenic rice, control (non–transgenic) rice and plasmid DNA were double digested over night at 37^{0} C (Fermentas Life sciences) for *cry*IAc with *Eco*R1/ *Hind*III. One μl of each sample was checked for digestion in 1% (w/v) agarose gel in a 1X TAE buffer stained with ethidium bromide (0.5 μg/ml) and visualized under UV. The digested DNA was electrophorsed on 1% (w/v) agarose gel. Then gel was depurinated in 0.25 N HCl for 30 min followed by equilibration in 0.4 M NaOH for 20 min. The DNA fragments were transferred from the gel to Hybond-N+ nylon membrane under alkaline (0.4 M NaOH) denaturation condition (Amersham, Arlington Heights, IL) according to manufacturer's instructions. The membrane was prehybridized for 2-3 hours in hybridization buffer (5X SSPE, 5X Denhardt's solutions, 0.5 % SDS) 50 ml per membrane to which sheared single standard salmon sperm DNA (10 μg/ml) was added for blocking. Digested 1.84 Kb *cry*IAc sized DNA

fragment from coding region of *cry*IAc from pCAMBIA 1301 were radio-labeled with (α - 32P) dCTP using Rediprime Labeling Kit (Amersahm, Arlingtom heights, IL) were used as probes. After hybridization for over night, the membranes were washed thrice each in wash solution I (2X SSC, 0.1 % SDS) for 20 min followed by wash solution 2 (1X SSC, 0.1 % SDS) and wash solution 3 (0.5X SSC, 0.1 % SDS) for 30-40 min and 15-30 min, respectively. The filters were wrapped with saran wrap after removing the excess wash solution and exposed with X-ray film for autoradiography.

CHAPTER- IV

RESULTS

The present study was undertaken to assess the various rice transformation methods including recently developed *in planta* transformation method and to develop transgenic *Bt* (*cry*IAc and *m*VIP) rice using two i*ndica* rice cultivars, Swarna and Mahsuri. The study also aimed to evaluate the factors affecting rice tissue culture, its subsequent transformation efficiency and characterize transformants at molecular and gene expression level.

4.1 Refinement of tissue culture based transformation systems

4.1.1 Callus induction in rice varieties Swarna and Mahsuri

Callus formation invariably developed from the scutellar region of the seeds and was visible in 7-10 days. Both the rice varieties responded differently with respect to callus induction percentage and nature of callus, derived from mature seeds (Table 4.1). Figure 4.1 shows the difference in response of Swarna and Mahsuri rice varieties on callus induction i.e. hard, fragile and globular in shape.

Overall induction of embryogenic calli in case of Swarna and Mahsuri was the most efficient (89.0% and 92.0%, respectively) in the MS Modified (MSM) medium supplemented with 3% maltose, 0.5 g/L of L-proline, casein hydrolysate and kinetin gelled with 0.3% of gel rite.

Table 4.1: Effect of Different culture medium on callus induction % for Swarna and Mahsuri varieties

Culture medium	Composition	Callus induction %	
		Swarna	**Mahsuri**
L3	MS basal salts, vitamins, 3% sucrose, 0.5 g/L glutamin, 0.5 g/L L- proline, 3 g/L 2,4-D, 0.8% agar agar, pH 5.8	76.8 (8.8)	78.1 (8.9)
LS	MS basal salts, vitamins, 0.4 g/L thymine, 3% sucrose, 2.5 g/L 2,4-D, 0.8% agar agar, pH 5.8	69.4 (8.4)	61.4 (7.9)
MS Modified	MS basal salts, vitamins, 3% maltose, 0.5 g/L casein hydrolysate, 0.5 g/L L- proline, 0.5g/L kinetin, 2.5 g/L 2,4-D, 0.3% gel rite, pH 5.8	89.0 (9.5)	92.0 (9.6)
MS	MS basal salts, vitamins, 3% sucrose, 2.5 g/L 2,4-D, 0.8% agar agar, pH 5.8	61.0 (7.8)	53.8 (7.4)
Mean		**75.05 (8.6)**	**71.32 (8.47)**
SEm±		**0.0575**	**0.08555**
CD **(P= 0.05)**		**0.12189**	**0.18135**

Note : Transformed values using *Square root* transformation are mentioned in parenthesis

4.1.2 Effect of proline, maltose and casein hydrolysate on callus induction

Figure 4.1 shows the effect of proline, maltose and casein hydrolysate on callus induction for both rice varieties, Swarna and Mahsuri under study. The callus in MS modified medium induced the most prolifilic, compact, nodular, creamish and larger than the callus grown in L3 and LS medium which looks hardy, rooty and non prolifilic. This medium is supplemented with maltose as a carbon source, kinetin and amino acids like L- proline and casein hydrolysate. These results suggest that the application of supplementary of amino acids and suitable carbon source could promote callus induction. Figure 4.2 showed the increased callus induction of Swarna and Mahsuri in MS modified (MSM) medium over the MS basal medium was 49% and 71% respectively. It suggests that maltose promotes rice callus induction more effectively than the sucrose

added in MS basal medium because it regulate osmotic potential of cellular environment of callus when sucrose promotes the *in vitro* production of ethylene in excised tissues causing browning of the callus. Hence, the substitution of sucrose may help to protect the calli by reducing the production of ethylene. Another factor promoting callus induction as well as regeneration was the addition of amino acids. Casein hydrolysate provides a source of amino acids and addition of casein hydrolysate in MS modified medium shown to increase the production of embryogenic calli.

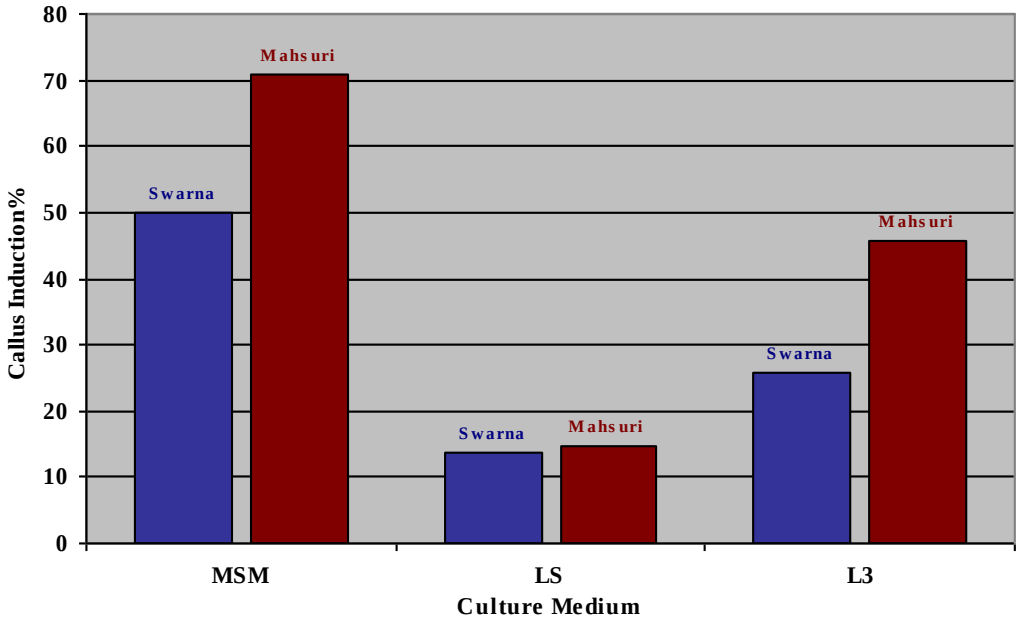


Figure 4.2: Per cent increase in callus induction of MSM, L3 and LS medium over MS medium in Swarna and Mahsuri varieties

These results suggested that MSM medium which is supplemented with 0.500 mg/L of L-proline, casein hydrolysate and kinetin, respectively with 30 g/L maltose as carbon source gelled with 0.3% gel rite was found comparatively superior to the rest of the three culture medium i.e. L3, LS and MS basal to get high callus induction % from both the subjected rice varieties.

4.1.3 Effect of gelling agent

The callus induction percentage and rate of the callus growth were recorded for both the rice varieties, Swarna and Mahsuri.

Figures 4.3 and 4.4 showed the effect of gelling agent i. e. gel rite and agar agar on callusing of rice varieties under study. Irrespective of the varieties, gel rite was found to show significant enhanced effect both on callus induction and nature of calli. The calli produced on gel rite containing medium were embryogenic, fragile, globular compare to that of succulent, watery calli produced on agar agar containing medium. Similarly, the callus induction in Swarna was 89.9% and Mahsuri 93.4% on gel rite medium (Figure 4.3). The calli formed on culture media solidified with 0.3% gel rite were characterized by having whitish in color, small size and compact and globular in shape (Figure 4.4).

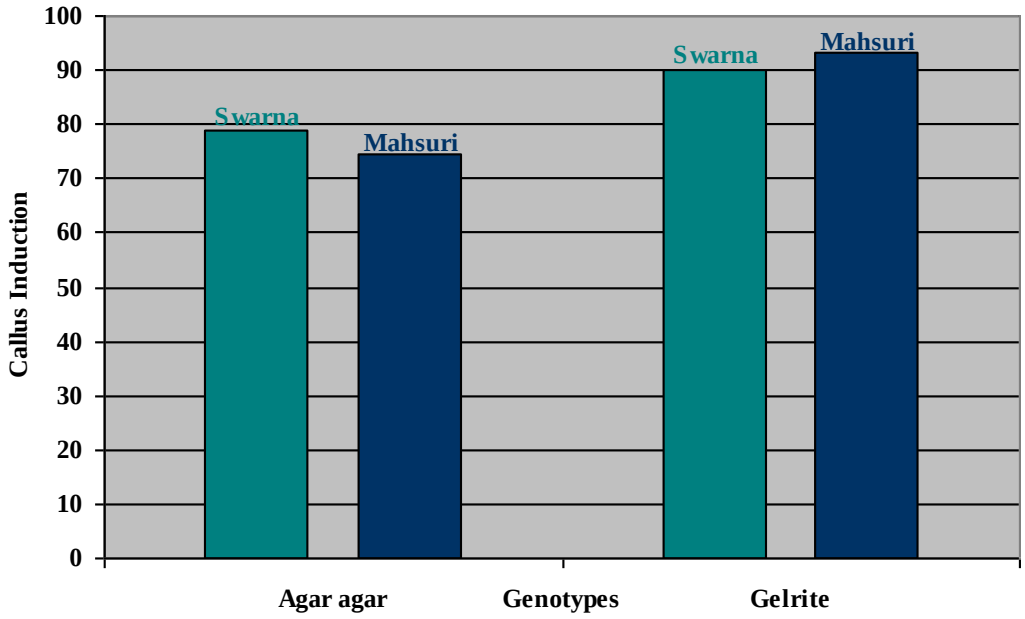


Figure 4.3: Effect of agar agar and gel rite on callus induction% in Swarna and Mahsuri varieties.

Thus by considering these agents as variables, it was observed that gel rite @3.0 g/L showed 14.23% and 25.03% increased callus induction over the medium containing agar agar in Swarna and Mahsuri varieties respectively.

4.1.4 *In vitro* regeneration system in rice

Shoot regeneration started within ten days transfer of seed derived embryogenic calli to M1, M2 and M3 mediums. Within four weeks, calli were observed with green shoot bulbs (Fig 4.5). Partial desiccation was showed the significant effect on plant regeneration irrespective of the varieties used in rice. Out of total 160 desiccated calli inoculated on M2 media, 145 Regenerants were obtained in rice varieties Swarna with the regeneration efficiency of 90.62% and 190 regenerants for Mahsuri with efficiency 95.0% (Table 4.2 and 4.3). This clearly indicated that stress in calli leads to the formation of stress inducing hormones like abscisic acid (ABA)**,** which ultimately provoked the cell physiological and biochemical changes which is necessary for efficient regeneration in rice.

For both the varieties, highest regeneration efficiency was observed in partial desiccated calli placed on to M2 medium supplemented with MSM salts, 2.5 g/L KIN, 0.5g/L NAA and 0.4% gel rite (Fig. 4.6). The results of this study clearly showed the enhanced effect of desiccation of calli on plant regeneration.

Table 4.2: Effect of desiccation on shoot regeneration from mature seeds derived calli of rice varieties Swarna cultured on different regeneration medium.

Medium	Desiccation			Non- Desiccation			Average
	No. of Calli kept	No. of regenerants	Regeneration %	No. of Calli kept	No. of regenerants	Regeneration %	
M1	150	0	00.00 (0.005)	175	00	00.00 (0.006)	00.00 (0.005)
M2	160	145	90.62 (0.962)	140	95	67.85 (0.771)	79.23 (0.792)
M3	185	65	35.10 (0.351)	175	21	12.00 (0.275)	23.55 (0.313)
Average	165.00	70.00	41.90 (0.422)	163.33	37.00	28.51 (0.285)	

Source	SEm ±	CD (p= 0.05)
Medium (M)	0.0430	0.1021
Treatment (D & ND)	0.0351	0.1766
Interaction (M x Treatment)	0.0607	NS

Note: M1- MS Modified medium (MSM); M 2- MSM+ KIN 2.5 g/L + NAA 0.5 g/L; M 3- MSM+ KIN 2.0 g/L + NAA 1.0g/L + BAP 0.5 g/L; D- Desiccated calli; ND- Non desiccated calli; Transformed values using *Arc sign* are given in parenthesis; NS- Non significant

Table 4.3: Effect of desiccation on shoot regeneration from mature seeds derived calli of rice varieties Mahsuri cultured on different regeneration medium.

Medium	Desiccation			Non- Desiccation			Average
	No. of Calli kept	No. of regenerants	Regeneration %	No. of Calli kept	No. of regenerants	Regeneration %	
M1	210	0	00.00 (0.005)	190	0	00.00 (0.006)	00.00 (0.005)
M2	200	190	95.00 (0.950)	175	135	77.14 (0.771)	86.07 (0.861)
M3	215	40	18.00 (0.186)	190	16	08.40 (0.464)	13.50 (0.325)
Average	208.33	76.66	37.86 (0.380)	185.00	50.33	28.51 (0.285)	

Source	SEm ±	CD (p= 0.05)
Medium (M)	0.0449	0.1306
Treatment (D & ND)	0.0366	NS
Interaction (M x Treatment)	0.0634	0.1844

Note: M1- MS Modified medium (MSM); M2- MSM+ KIN 2.5 g/L + NAA 0.5 g/L; M3- MSM+ KIN 2.0 g/L + NAA 1.0g/L + BAP 0.5 g/L; D- Desiccated calli; ND- Non desiccated calli; Transformed values using *Arc sign* are given in parenthesis; NS- Non significant

4.1.5 Identification of optimum concentration of Hygromycine B for selection of transformants

Seed derived embryogenic calli of both the rice varieties Swarna and Mahsuri were inoculated onto different medium supplemented with various levels of Hygromycine B (0, 25, 30, 35, 40, 45, 50 mg / L). The time and number of callus mortality was recorded to identify the concentration of Hygromycine to be used for selection of putative transformed calli (Fig.4.7 & 4.8).

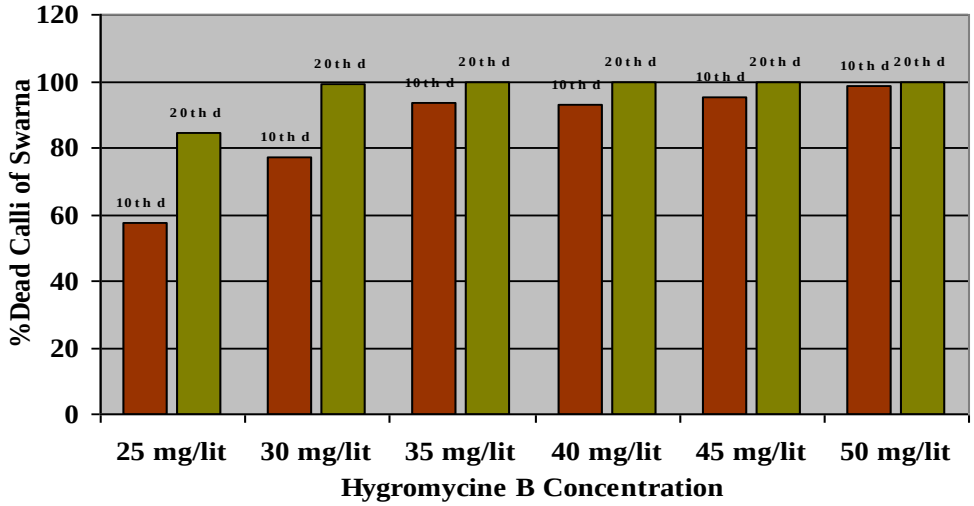


Figure 4.7: Effect of Hygromycine B concentrations on Mahsuri calli on 10th and 20th day of selection.

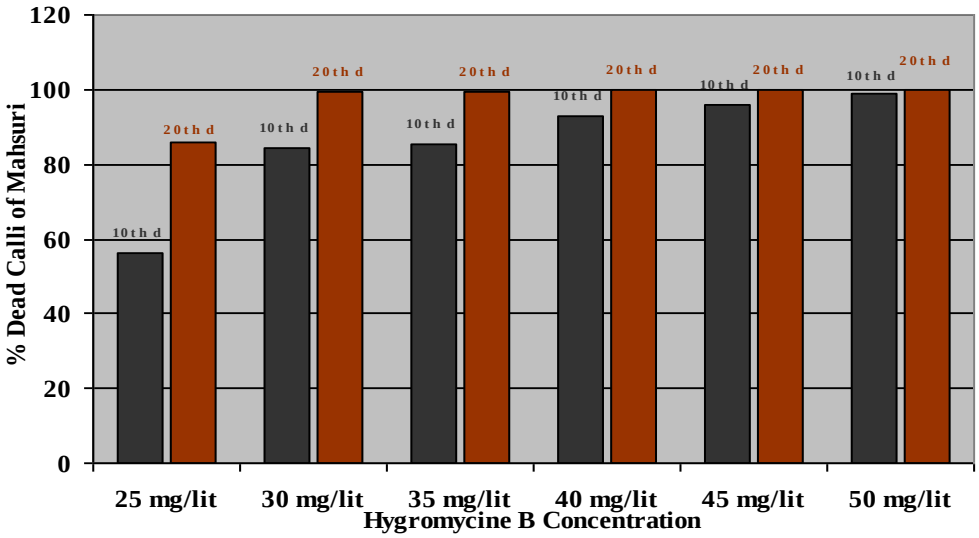


Figure 4.8: Effect of Hygromycine B concentrations on Swarna calli on 10th and 20th day of selection.

Genotypic difference for Hygromycine B concentration required to kill the embryogenic calli was observed. The Hygromycine concentrations of 35 mg/L for Swarna and 45 mg/L for Mahsuri varieties found optimum. These concentrations of Hygromycine B were used in further studies to select the transgenic calli and killing of non transformed calli, which is a prerequisite to develop transformants in tissue culture based transformation methods, *viz.*, Particle gun method and *Agrobacterium*- mediated systems. The calli were started browning after 6-7 days of inoculation in Swarna calli and 10-12 days in Mahsuri calli, subsequently the calli turned black in15 days for Swarna and 20 days for Mahsuri (Fig 4.9a & b).

4.1.6 Statistical Analysis

To assessment of different factors influencing *in vitro* regeneration as well as antibiotic concentration for the selection of transformed calli the data of rice varieties, Swarna and Mahsuri in four replicates were recorded separately during pilot experiments. All results were analyzed by analysis of variance (ANOVA) using Completely Randomized Design to show statistical significance of the difference among the means. The tables of ANOVA are presented in Appendix.

4.2 Molecular screening of putative transformants generated through Tissue Culture based methods

4.2.1 PCR analysis for *cry*IAc

All putative transformants generated from tissue culture based method i.e., *Agrobacterium* and Particle gun mediated transformation systems were selected on Hygromycine B containing medium and subjected to PCR analysis

for initial screening of presence or absence of gene of interest. A specific primer for *cry*IAc gene was designed to yield 780 bp amplification products. In order to select the positive transformants, each of putative transformants was tested for the gene specific primers.

Out of 19 putative transformants produced from the tissue culture based method for both the rice varieties, Mahsuri and Swarna analyzed by PCR, only two transformants # 7 and #18 derived from the Particle gun mediated transformation methods for Swarna rice varieties were found positive for *cry*IAc gene as shown in Fig 4.10a, indicating lower efficiency of transformation in rice by tissue culture based method.

All these plants were transferred into pots and kept in controlled transgenic green house of Department of Biotechnology, IGKV, Raipur. The plants were not reached to flowering and maturity due to heavy infestations of BPH insects and failure in maintaining the Green House conditions.

When 25 rice regenerants produced using *m*VIP gene construct were analyzed by PCR, no one was found positive for gene of interest indicating that regenerants selected on the Hygromycine plates were escape rather true transformants.

4.2.2 Southern analysis for *cry*IAc gene

In order to ensure the integration of *cry*IAc gene into the genome of Swarna transformants, the genomic DNA was isolated from the leaf samples as per the methods outline in section 3.4. The genomic DNA of putative transformants were digested with *Eco*RI- *Hind*III enzymes, which restricts the

coding region of *cry*IAc gene and releases 1.8 kb DNA fragment of coding region.

For hybridization 1.8 kb DNA fragment of *Eco*RI and *Hind*III digest of *cry*IAc constructs/ plasmid were used as probe. Autoradiography revealed some of the fragments showed hybridization signals, where as control non transformed plants DNA did not show any signals as expected. Fig. 4.10b showed the southern blot of set of transformants of rice *cv.* Swarna, only two transformants #7 and # 18 showed the presence of distinct band of 1.8 kb that got hybridized with probe suggesting the integration of *cry*IAc gene into genome of rice transformants #7 and # 18.

4.3 Floral-dip based *in planta* transformation in rice

4.3.1 Effect of various factors affecting *in planta* transformation in rice

Initially, to asses the various factors *viz.*, target cells, age of flowers, inoculum density, time of flower dipping and pollination. The plants were inoculated with *Agrobacterium* carrying a 35S-GUS/intron fusion reporter gene in the T-DNA, allowing the GUS expression only after insertion in to the rice genome.

The inoculum density of *Agrobacterium* to be used for inoculation and time of flower dipping was found to affect the seed setting and subsequent plant development and insertion of transgene into the rice genome. The *Agrobacterium* inoculum density with (OD_{600} 0.2 and 0.4) and dipping time (2.0 and 4.0 min) has found to affect high seed setting on crossing and transformation efficiency (Fig 4.11).

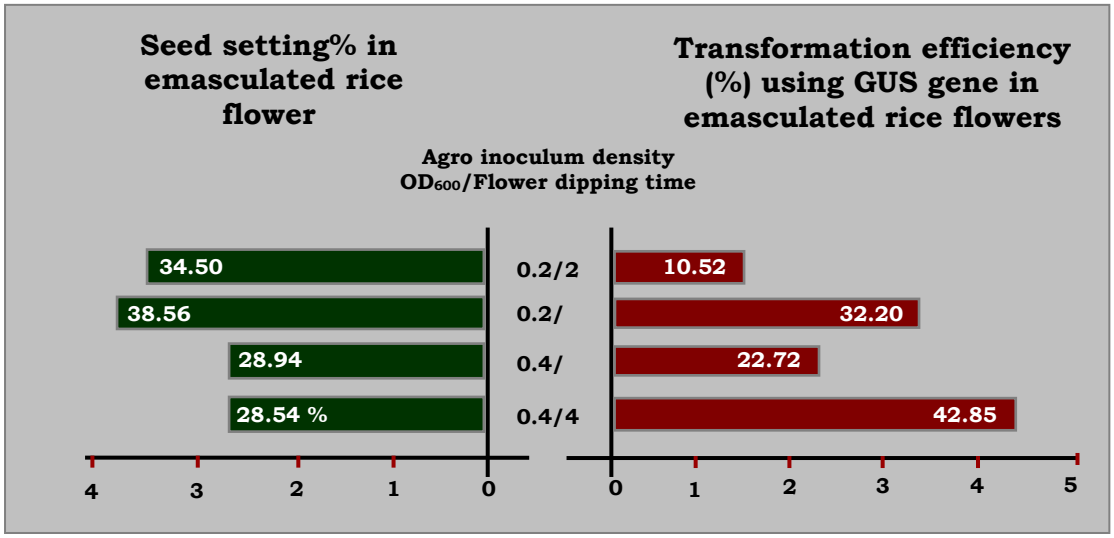


Figure 4.11: Seed setting and transformation efficiency for GUS gene in rice flowers inoculated by different *Agrobacterium* inoculum density for different dipping time and pollinated to emasculated rice flowers.

The left histogram in the Figure 4.11 shows the rate seed setting obtained according to OD_{600} and dipping time; right histogram shows the transformation efficiency obtained from the emasculated flowers; Y axis shows *Agrobacterium* inoculum density and inoculated flowers for different dipping time; X axis shows rate of transformation obtained in emasculated flowers. The highlighted values in % are the rate of transformation according to OD_{600} and flower dipping time.

4.3.2 Target Cell for T-DNA transfer

Standard floral dip transformation procedure as described by Bent, 2000 for *Arabidopsis* flowers, were used for assessment of plant growth stage and *Agrobacterium* inoculum density with modifications required for rice crop (Fig. 2.3) as per the developmental stages of rice flower. Emasculation of rice flowers by removing the anthers of unopened flowers before anthesis and subsequent pollination using pollen grains of male parent anthers is being practiced since the beginning of hybridization program for genetic modification of rice. With the

success of transforming *Arabidopsis* flowers by dipping it in *Agrobacterium* inoculum and allowing the T-DNA transfer in to female germ line cells, we attempted to develop rice transgenics using floral dip methods and examined the various factors affecting the transfer of T-DNA into germ line cells of developing rice flowers. The stepwise protocol of floral dip based *in planta* transformation followed is given in Fig. 4.12.

The target cell type for delivery of T-DNA was identified for rice *cv.* Swarna. The emasculated and un-emasculated at 25, 50 and 100% anther dehiscence stage, rice flowers were treated with *Agrobacterium* inoculum. Immature flower of rice plants were emasculated and pollinated using pollen grains of rice plants of Swarna cultivar only, as it is being practiced for developing hybridization between the two rice varieties in conventional breeding approach. By taking the clue from conventional; hybridization procedure, an attempt was made to inoculate the female organ/ flower with *Agrobacterium* and allowing T-DNA transfer in to target cells of embryo sac mainly egg cells and crossing by pollinating with un inoculated pollen grains of same rice cultivar i.e. Swarna. The emasculated and non-emasculated rice florets were inoculated by dipping (no vacuum infiltration) and infected by spraying *Agrobacterium* culture onto opened rice flowers. Candidate T_1 transformants seeds (cross seeds) were then generated by performing crosses in which the male parents (pollen donor) had not been inoculated. The pollen grains of non-inoculated Swarna plants were used for pollination of inoculated and emasculated rice flowers. The schematic procedure of transformation followed using floral dip in Swarna variety is depicted in Fig. 4.13.

Individual seeds from these crosses were harvested separately and seeds were stored in separate place. Further seeds were germinated and planted onto trays and kept in the transgenic green house of IGKV, Raipur for growth and development. More than 82 individual rice plants were inoculated and crossed with Swarna pollen, has generated four hundred and forty seven seeds, of which one hundred and one seeds germinated and screened for presence of transgene. Out of 183 seeds screened from 82 successful crosses in which the pollen donor plants were not inoculated with *Agrobacterium* but female plants were inoculated with *Agrobacterium*, fifty six transformants were recovered (Fig 4.14a & b).

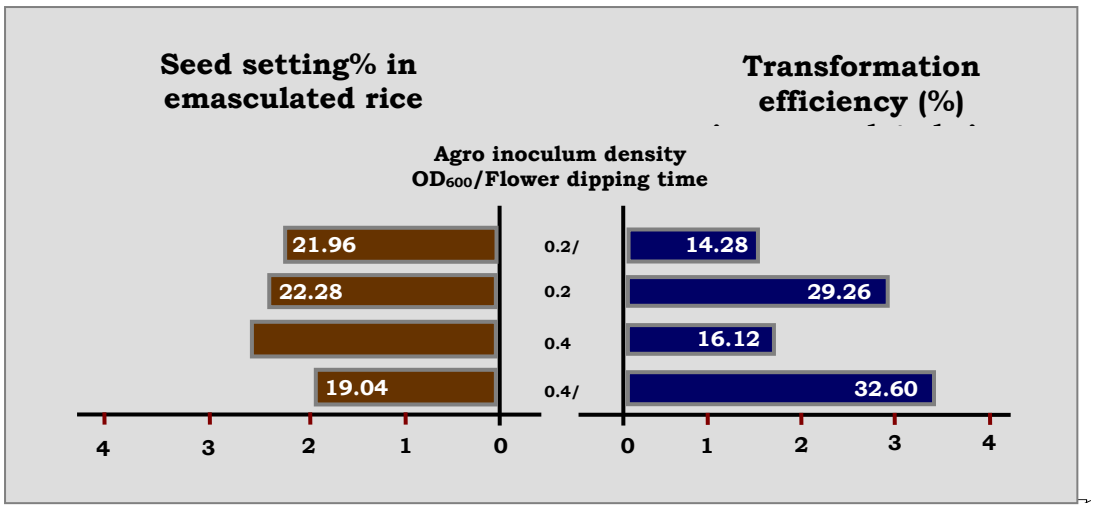


Figure 4.14a: Seed setting and transformation efficiency in rice flowers inoculated by different *Agrobacterium* inoculum density for different dipping time and pollinated to emasculated rice flowers.

In the Figure 4.14a, left histogram shows the rate of seed setting obtained according to OD_{600} and dipping time; right histogram shows the transformation efficiency obtained from the emasculated flowers; Y axis shows *Agrobacterium* inoculum density and inoculated flowers for different dipping time; X axis shows

rate of transformation obtained in emasculated flowers. The highlighted values in % are the rate of transformation according to OD_{600} and flower dipping time.

In contrast, no transformants were recovered from 20 separate attempts in which rice flowers at different level of anther dehiscence stage (25%, 50% & 100% anthesis) i.e. un-emasculated were inoculated with *Agrobacterium* (Fig. 4.12). These results suggests that T-DNA transfer occurs on female floral structures particularly in embryo sac cells *viz*., egg cells prior to fertilization and that they do not occur during early stage of anther/or pollen tube development prior to fertilization in rice.

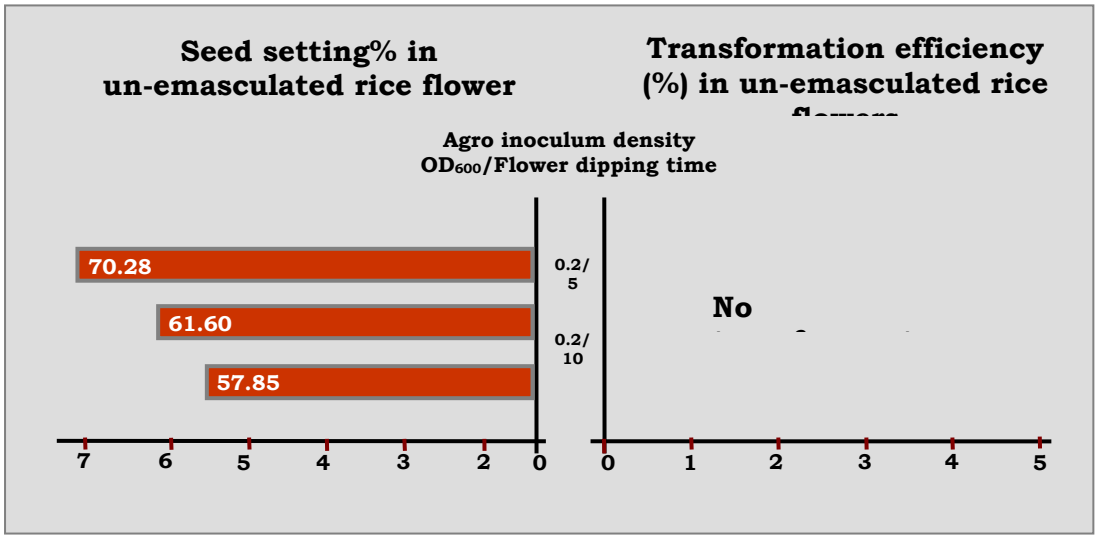


Figure 4.14b: Seed setting and transformation efficiency in rice flowers inoculated by different *Agrobacterium* inoculum density for different dipping time to un-emasculated rice flowers.

In the Figure 4.14b, left histogram shows the rate seed setting obtained according to OD_{600} and dipping time; right histogram shows no transformants obtained from the non-emasculated flowers; Y axis shows *Agrobacterium* inoculum density and inoculated flowers for different dipping time; X axis shows

transformation efficiency obtained. The values highlighted are the rate of seed setting according to OD_{600} and flower dipping time.

Double fertilization in rice embryo starts after 9-12 hrs of pollination and finishes completely in 18 to 24 hours after pollination. The tip of the pollen tube penetrates in to the embryo sac through synergid, and then it burst and gives off the generative nuclei. One of them fertilizes the egg cell and the other fertilizes the polar nuclei. The fertilized polar nucleus is the primary nucleus of the endosperm, which begins cell division soon after fertilization (Fig. 4.15).

Rice plant bears perfect flower in single flowered spikelet bloom on panicle that typically carries flowers of wide range of developmental stage. In an attempt, as shown in Fig. 4.12 crosses were made by dusting un- inoculated pollen grains of the stigma of donor plants (Swarna) on to the stigma of emasculated female flower after 12-14 hours of the inoculation. This provided information about the developmental stage at the time of *Agrobacterium* inoculation, stigma receptivity, pollen tube growth, double fertilization in rice flowers. Productive transformation events occurred in the emasculated flowers shows that target cells for T-DNA transfer could be egg cells and or cells of embryo sac prior to fertilization. Flowers at different levels of anthesis produced no transformants indicated that T-DNA transfer did not occur to the pollen nuclei.

All T_1 putative transformants recovered from the above attempts were analyzed using histochemical X-gluc analysis and by PCR and transformation efficiency was recorded.

4.3.3 *Agrobacterium* inoculum density and dipping time

To obtain higher efficiency of T-DNA transfer using floral dip method, two *Agrobacterium* concentrations with OD_{600} 0.2 and 0.4 with dipping time 2.0 and 4.0 min were examined. The *Agrobacterium* inoculum density was found to affect stigmal receptivity during floral dip, and reduced the seed setting on crossing with pollen grains of same plants. Whereas, the 4.0 min of flower dipping time produced a transformation efficiency that was more than 10.0% higher than that of 2.0 min dipping time irrespective of the *Agrobacterium* inoculum density. Table 4.4 & 4.5 showed the percentage of seed setting and transformation efficiency according to the treatment of OD_{600} and time of flower dipping.

Table 4.4: Effect of *Agrobacterium* inoculum density and flower dipping time on rice transformants efficiency with GUS gene using floral dip method

Inoculation Density OD_{600}	Flower dipping time (Min)	Seed setting percentage	Percentage of GUS positive plants
0.2	2.0	34.50	10.52
0.2	4.0	38.56	32.00
0.4	4.0	28.94	22.72
4.0	4.0	28.57	42.85

Table 4.5: Effect of the density of *Agrobacterium* inoculum in the transformation efficiency with *cry*IAc of rice *cv.* Swarna performed using floral dip method

Inoculation Density OD_{600}	Flower dipping time (Min)	Seed setting percentage	Percentage of *cry*IAc positive plants
0.2	2.0	32.26	16.66
0.2	4.0	31.94	21.73
0.4	4.0	20.41	12.5

4.0	4.0	16.07	16.66

4.3.4 Floral dip with gentle shaking vs Floral spray

The emasculated rice flowers were inoculated with *Agrobacterium* inoculum by dipping with gentle shaking (Fig. 4.12) and by spraying of *Agrobacterium* culture using glass sprayer releasing mist of Agro culture on to emasculated rice flowers. Seed setting percentage on crossing with pollen grains of the same cultivar rice plants were significantly affected (Table 4.6).

Table 4.6: Seed setting performance found using spray of inoculum and gentle shaking of emasculated Swarna flowers

Treatment	No. of plants treated	No. of flowers/ spikelets	No. of seed set	Seed setting %
Gentle shaking	68	2038	447	21.93
Spraying	24	521	46	8.82

The treatment spraying with Agro culture produced lower seed setting compare to that of dipping with gentle shaking indicated that spraying causes physical injury to the stigma cells of the emasculated rice flowers leads to the lowering of stigma receptivity, pollen tube growth and double fertilization when hand pollinated.

4.3.5 Molecular analysis of T_1 plants produced by floral dip method

4.3.5.1 Histochemical GUS assay using X-gluc

Two tissue samples, leaf and root of 35-40 days old T_1 plants of rice *cv.* Swarna produced from the *Agrobacterium* inoculated rice flowers pollinated by crossing with un-inoculated donor pollen were analyzed using X-gluc test. Fig. 4.16a & b showed the appearance of indigo blue color after 24 hours of incubation in X-gluc solution. The leaf and root samples of control non-

transformed Swarna plants did not show any blue culture. Out of 128 T_1 plants 38 produced the GUS positive reaction indicating the integration and expression of reporter gene GUS in to transformants of Swarna.

4.3.5.2 Floral dip transformation using *Agrobacterium* carrying GUS

The GUS positive transformants were analyzed by PCR using gene specific primers amplifying amplicon of size 750 bp from the coding region of gene of interest (GUS). All X-gluc positive transformants showed the amplification of 750 bp indicating the presence of GUS gene with higher transformation efficiency of 31.66% in to the genome (Fig. 4.16c).

4.3.5.3 Floral dip transformation using *Agrobacterium* carrying *cry*IAc

With the standardization of various factors for floral dip method in rice, an attempt was made to inoculate 473 emasculated flowers of Swarna rice plants were inoculated with *Agrobacterium* carrying 35S-*c*ryIAc constructs in T-DNA (OD_{600} value of 0.2 and 0.4 for 2.0 and 4.0 min dipping time) and pollinated. Out of total one hundred and eleven T_1 transformants seeds produced from eighteen were found positive by PCR analysis (Fig. 4.17).

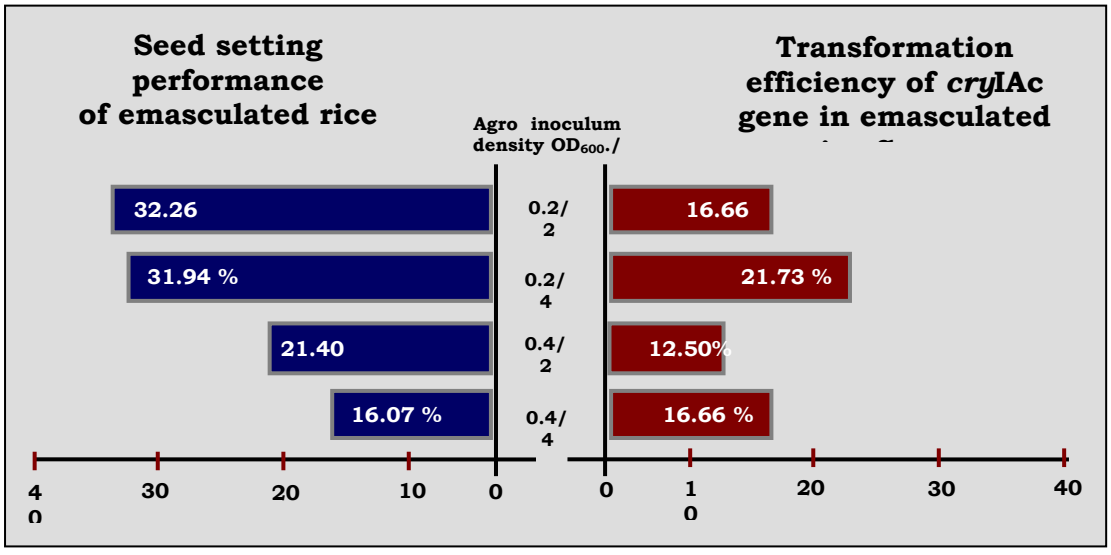


Figure 4.17: Rate of seed setting and transformation efficiency using *cry*IAc gene in *Agrobacterium* inoculated rice flowers pollinated by crossing with un-inoculated donor pollen of Swarna plants.

In the figure 4.17, left histograms shows number of T_1 seeds subjected to analysis; right histograms shows the rate of transformation from corresponding treatments of *Agrobacterium* concentration and dipping time; X axis shows the number of plants analyzed; Y axis shows the different treatments used for flower dip method of transformation.

Fig. 4.18 showed the amplification of 780 bp DNA fragment specifying the coding region of the *cry*IAc gene with high transformation efficiency (16.20%).

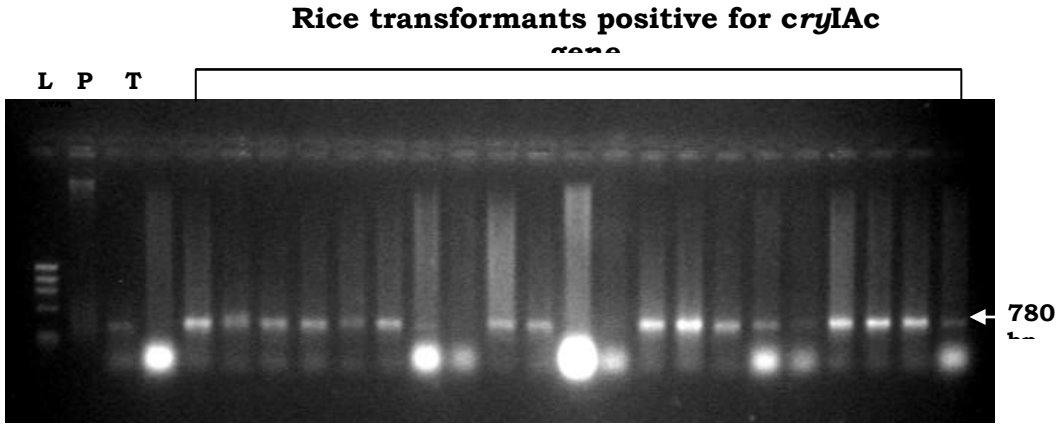


Figure 4.18: PCR detection of *cry*IAc transgene in transformants developed by Floral dip based *in planta* system.

CHAPTER V

DISCUSSION

In Asia stem borer bears to cause about 5% grain losses (IRRI, 1996) and 50% insecticides employed in rice field are targeted at lepidopterous insect species (Heong *et al.,* 1994). All over the world the average yield losses are estimated to be about 10 million tonnes caused by SSB, YSB and LF (Herdt, 1991). Use of toxic chemicals not only increases the rice production cost but also causes health harms to rice farmers as well as deteriorated the rice field environment (Pingali and Roger, 1995). Since Fujimoto *et al.*,(1993) reported insect resistant rice generated by introduction of a modified endotoxin gene *cry*IAb of *Bacillus thuringiensis* driven by different promoters, several many genes have been transferred into various types of rice (Ghareyazie *et al.*, 1997; Nayak *et al.,* 1997; Wu *et al.,* 1997; Cheng *et al.,* 1998; Xiang *et al.,* 1999, Datta *et al*., 2004) mainly using tissue culture based DNA delivery systems like *Agrobacterium*- mediated and Particle gun mediated transformation methods. However, some technological limitations like complicated method of transformation, varieties dependent, recalcitrant nature of rice to *in vitro* regeneration, low rate of transformation efficiency, somaclonal variation and epigenetic changes (Christou, 1995; Hooykaas and Schilperoorts, 1992; Clough and Bent, 1998; Bent, 2000) has limited the routine use of this technology .

It is therefore, imperative to look for the novel methods not only to increase the transformation efficiency of existing tissue culture based transformation method but also to look for an alternative method of DNA

transfer for rice varieties. In recent years, a novel method of transformation i.e. *in planta* transformation system has been demonstrated in a model crop *Arabidopsis* with high efficiency of about 60% with the success of high rate of transformation using floral dip, embryo infiltration, floral spray, vacuum filtration, efforts has been initiated to repeat the same for major staple crops like rice.

During the present investigation entitled **"Development of efficient transformation systems to enhance insect resistance in rice (*Oryza sativa* L.)**", an effort was made to assess various factors affecting tissue culture response and subsequent transformation efficiency in rice, transformed two rice varieties namely Swarna and Mahsuri with *Bt* genes (*cry*IAc and *m*VIP) and critically examine the biological and physical factors affecting transfer of T-DNA using non tissue culture based Floral dip (*in planta*) method in rice. This chapter discusses the various aspects of results obtained in different studies conducted towards fulfilling the objectives of the study.

5.1 Tissue Culture based transformation methods

During the past few years, the success in designing insect-resistant crop plants through gene transfer has been impressive. Development of transgenic rice has become novel technique to combat the attack of major pests of rice *viz.*, YSB and SSB, causing 10-30% yield losses (Ramaswamy and Jatileksono, 1996). This technique offers much promise in developing agricultural practices that are in perfect harmony with the environment. There are several transgenic rice has been developed showing 100% resistance to stem borer and leaf folders under

green house and natural field conditions (Wu *et al.,* 1997; Alam *et al.,* 1998; Datta *et al.,* 1998; Tu *et al.,* 1998; Ye *et al.,* 2001; Chandel, 2005).

Transgenic rice is typically produced by complex methods that require sophisticated *in vitro* regeneration protocols that imply technical abilities, which are complicated with somaclonal variation and epigenetic changes (Christou, 1996; Hooykaas and Schilperoorts, 1992; Clough and Bent, 1998; Bent, 2000).There have been very few successful cases reported for *Agrobacterium* mediated transformation of *indica* rice. Even in reports in which transformation succeeded, the results showed either low transformation efficiency (Aldemita and Hodges, 1996; Garg *et al.,* 2002; Khanna and Raina, 1999, 2002; Mohanty *et al.,* 2002; Nayak *et al.,* 1997; Wang *et al.,* 2002) or success only with very specific varieties (Zhang *et al.,* 1997), which is severely hindered the applications of transformation in *indica* rice.

The high efficiency tissue culture system for *japonica* rice has long been established, and it technically robust and mature (Chu *et al.,* 1975). In *indica* rice however, robust and widely applicable method for subculture and regeneration have not been established, despite the large efforts invested in the *in vitro* culture of *indica* rice (Opabode, 2006; Datta *et al.,* 2001).

5.1.1 Callus induction and Plant regeneration

In this study, seed derived embryogenic calli developed from the mature embryos were used as explant for tissue culture based transformation systems as they are easily available throughout the year and can be preserved for a long time, hence ideal for many experiments (Zaidi *et al.,* 2006; Lentini *et al.,* 1995). But for rice transformation experiments immature embryos have been suggested

as better source for producing embryogenic calli with high regeneration potential. Meda and Radi (1988) pointed out that not seed but the immature embryo is better material from which plantlets easily regenerated and callus tissue are mainly induced from scutellar epithelium of rice embryos (Christou, 1991).

A highly efficient system for production of good quantity embryogenic calli with high regeneration potential is a pre-requisite for development of rice transgenic.

Despite many years of research, tissue culture of *indica* rice varieties is still not easy and none of the nutritional composition (medium) published so far can produces a satisfactory outcome. During the long course of tissue culture in the commonly used transformation processes of approximately 100 days, subculture (on medium with and without antibiotics) takes a total of approximately 60 days, resulted deterioration in quality of callus and reduced the regeneration potential in rice (Meneses *et al*., 2005).

Most of the reported studies used MS as the callus induction medium in transforming *indica* rice which indicated MS might still have utility for this purpose. Therefore, using MS as our starting point we changed the relative proportions of the nutrients and modified the ratios of macronutrients and micronutrients, taking into account the bioavailability of the nutrients under the given pH range. Our study identified MSM as the most suitable basic medium for callus induction of both subjected rice varieties. This medium not only significantly increased the rate of growth of the calli, but also greatly improved the vigor and hence the quality of the calli, compared with other mediums tested.

This medium supplemented with the carbohydrate maltose, KIN and amino acids like L- proline and casein hydrolysate in addition with 2,4-D. The quality of the callus is very important for the regeneration and improvement of the quality of callus in induction medium is also a crucial factor affecting regeneration frequency. Earlier, large number of old, empty cells with thinner cytoplasm, bigger vacuoles and smaller nucleus was found in the calli induced on 2, 4-D alone medium. However, the cells with thicker cytoplasm, bigger nucleus and vigorous rate of mitosis in the calli induced on medium containing 2, 4-D and kinetin (Wenzhong *et al*, 1994). When mostly 2, 4-D had been used as the only growth regulator in callus induction media by Katiyar *et al.*, 1999; Zhenyu *et al.,* 1999). Use of casein hydrolysate was found to be beneficial for generation of embryogenic calli in *japonica* (Hiei *et al.,* 1994; Toki, 1992) as well as *indica* rice varieties (Zhang *et al.*, 1997; Saharan *et al.*, 2004). In the present investigations it is observed that the callus induction and regeneration was greatly affected by the carbon source, amino acids and gelling agent used in the background of MS medium for both rice varieties, Swarna and Mahsuri. The frequency of callus induction with the presence of maltose, L-proline, casein hydrolysate and KIN was found increased by 49% and 71% in Swarna and Mahsuri over the MS basal medium. Similarly several other have reported that maltose is a better carbon source compared to that of sucrose for callus induction as well *in vitro* regeneration (Kumaria *et al.*, 2001; Kumaria and Rajam, 2002; Kumar *et al.*, 2005). Maltose not only works as good carbon source but also as an agent that regulate osmotic potential of cellular environment of calli which ultimately enhanced the regeneration potential (Lentini *et al.*, 1995; Zaidi *et al.*,

2006). Moreover, because sucrose promotes the *in vitro* production of ethylene in excised tissue causing the browning of the callus, the substitution of maltose for sucrose may help to protect the calli by reducing the production of ethylene. As observed in our study, the enhanced effect of maltose on callusing and differentiation in rice has been reported by Lin and Zhang, 2005. They found the difference in bioavailability of two sugars, sucrose and maltose. Similarly, the use of proline in the medium has also been reported to be effective for the initiation and maintenance of embryogenic calli (Datta *et al.,* 1992; Kishor *et al.,* 1999).

In present investigation, the MSM medium supplemented with 2, 4-D, Casein hydrolysate, Proline and Maltose has shown higher induction of embryogenic calli from mature seed scutella of rice. Here proline provides an additional amino acid and has previously been shown to increase the production of embryogenic calli in *indica* rices (Moura *et al.,* 1997; Zaidi *et al.,* 2006). The addition of cytokinin (KIN) to callus induction medium supplemented with 2,4-D had shown enhanced callus induction in both the rice cultivars under study. The auxin 2, 4-D is used routinely as a callus inducing agent in rice while very few reports indicates the use of cytokinin as well.

5.1.2 Gelling agent in culture medium

Role for the solidification of gelling agent in tissue culture medium have profound influence in retention of water and regulation of moisture regimes of the medium, which influence *in vitro* regeneration response immensely (Suprasanna *et al.,* 2000; Khaleda and Al- Forkan, 2006).

In this study, the effect of two gelling agents agar agar and gel rite has been assessed. Gel rite was found to enhance callus induction and *in vitro* regeneration of Swarna (89.9%) and Mahsuri (93.4%) varieties. Although use of agar agar as a gelling agent to develop *in vitro* regeneration of rice has also been reported (Wenzhong *et al*, 1994; Patpanukul *et al.,* 2004; Katiyar *et al.,* 1999; Datta *et al.,* 1992; Khaleda and Al- Forkan, 2006; Suprasanna *et al.,* 2000; Lee *et al.,* (2002) but recently, Saharan *et al.,* 2004 and Menses *et al.,* 2005 have reported higher regeneration frequency in *indica* rice varieties when media was solidified with gel rite. Our findings with two rice varieties, Swarna and Mahsuri have also shown the enhanced effect on callusing and regeneration.

5.1.3 *In vitro* regeneration of *indica* rice

Availability of an efficient regeneration system is a prerequisite for undertaking any transformation study. Till date most of the regeneration studies in rice have been done in *japonica* rice. The *indica* rice including Swarna and Mahsuri are considered recalcitrant to tissue culture manipulation (Pandey *et al.,* 1994; Seraj *et al.,* 1997) and number of groups are still investigating the optimum media requirement and other culture conditions for efficient plant regeneration (Khanna and Raina, 1997; Datta *et al.,* 1992; Kishor *et al.,* 1999; Lin and Zhang, 2005).

The results presented here suggest that regeneration of the two rice varieties were influenced by the type of calli and medium with the concentration of auxin and cytokinin (Jain, 1997; Saharan *et al.,* 2004). Strategies to improve plant regeneration frequency in rice have been steadily evolved during last decade (Kyozuka *et al.,* 1988; Datta *et al.,* 1992). Partial desiccation treatment of calli

has been reported to be beneficial for embryogenesis and plant regeneration in several plant species including rice. Jain *et al.,* (1996) and Chand and Sharwat (2001) have also reported three fold increases in shoot regeneration frequency following partial desiccation for 24 hours in *indica* rice.

In the present investigation, the partial desiccation has found promotive to plant regeneration than the non desiccated calli. These results are in agreement with Diah and Bhalla, 2000; Urshibara *et al.,* 2001). The partial desiccation treatment (18-24 hrs) gave maximum shoot regeneration 90.62% and 95.00% in Swarna and Mahsuri rice varieties respectively.

The present results demonstrated that partial desiccation of embryogenic calli prior to inoculation in to regeneration medium, have significant effect on shoot bud initiation and ultimately enhance the regeneration efficiency in rice, irrespective of the genotype. We found 33.55% and 23.15% increased regeneration efficiency over the non desiccated calli in Swarna and Mahsuri varieties. It suggests that partial desiccation of calli leads to the reduction of water content of callus but also starvation of calli by desiccation provoked the cell physiological and biochemical change which is necessary for efficient regeneration (Wenzhong *et al,* 1994). Desiccation had shown not only enhanced callus growth but also green bud initiation on MS medium supplemented with 2.5 mg/L KIN and 0.5 g/L NAA in both the rice varieties (Fig. 4.5 & 4.6). Our results are in confirmatory with the findings of Saharan *et al.,* 2004; Diah and Bhalla, 2000; Chand and Sahrawat, 2001). Saharan *et al.,* (2004) have reported that partial desiccation of *indica* rice calli produced 1.2 to 5.6 fold increased more shoots compared to that of non desiccated calli.

5.1.4 Hygromycine based selection of rice transformants

Among the various selectable marker genes, Hygromycine phosphotransferase is one of the widely used antibiotic resistant marker gene transformation of *japonica* and *indica* rice varieties (Zaidi *et al.,* 2006; Pipatanukul *et al.,* 2004; Chen *et al.,* 1992; Cheng *et al.,* 1998). The concentration of Hygromycine is a crucial factor for the selection of transformed cells because it is amino glycoside antibiotic which cause harmful death to plant cells by inhibiting transcription and translation. At the higher concentration levels of Hygromycine the transformed cells could not grow normally and leads to death. In present investigation, we have identified the sufficient levels, 35 mg /L for Swaran and 45 mg /L for Mahsuri calli of Hygromycine required to kill the non transformed seed derived calli of two rice cultivars, Swarna and Mahsuri. The Hygromycine levels was found to be genotype specific and it is first pre requisite for using tissue culture based transformation system (Datta *et al*., 2004). While working with bold seeded rice genotype BR-29, Baisakh *et al*., (2002) and Alam *et al*., (1998) have reported Hygromycine levels up to 80 mg /L for high rate of transformants from bombarded rice calli. Other workers have reported a range 30 to 100 mg /L Hygromycine for selection of rice transformants.

5.2 Development of Transgenic rice

In this study total of 58 transformants were produced using three methods of transformation, which includes 20 *Bt* (*cry*IAc) and 38 GUS positive transformants. Out of twenty *Bt* rice transformants, two were produced from tissue culture based Particle Gun mediated transformation system and 18 were

from *Agrobacterium*- mediated floral dip transformation method as outlined in Fig 3.5. All thirty eight GUS positive transformants were produced by floral dip method only. The potential of *cry* IAc gene in controlling the yellow stem borer in rice has previously been reported by several workers (Wu *et. al.,* 1997; Datta *et.al.,* 1998; Tu *et.al.,* 1998, 2000; Baisakh, 2000). In this study, several putative transformants were developed from Hg^{+} resistant calli of two rice cultivars (Swarna and Mahsuri) with *cry*IAc gene through Particle gun mediated method and total 20 regenerants were found positive for *cry*IAc gene by PCR. Similarly, Chandel (2005) has also reported the development of transgenic *Bt* rice *cv.* Swarna using Particle gun mediated transformation method with the efficiency raging from 0.5 to 3.0 %. As reported by Kohli *et al.,* 1999; Datta *et al.,* 2002, 2004, we also find integration of high rearranged transgene fragments on southern blots of Swarna transformants developed through Particle Gun mediated transformation. This suggests that the high amount of plasmid DNA bombarded into the primary rice calli and introduction of DNA by physical forces have led to the integration of rearranged DNA fragments among the transformants generated by Particle gun mediated method (Register *et.al.,* 1994). Several *cry* genes have been introduced in many rice cultivars using Particle gun mediated method and were found to show 100% YSB mortality in lab and field conditions (Table 5.1). Ye *et al., (*2001) has evaluated the performance of two independent events of transgenic rice carrying a hybrid *cry*IAb/*cry*IAc gene at Hangzhou, China and reported the control of three insects, YSB, PSB and LF.

Table 5.1: Transgenic rice generated using insect- resistant genes

Gene	Target insects	Highlights	References
*cry*IAb	SSB, YSB	Field trials show high protection against these insects	Shu *et al*., (2000); Ye *et al*., (2001)
*cry*IAb, *cry*IAc	YSB, Leaf folder	Field-tested insect-resistant *Bt indica* hybrid rice with high insect protection and no reduction in yield	Tu *et al*., (2000b)
*cry*IAb or *cry*IAc	SSB, YSB	Transfer of insect-resistant trait into commercial varieties and/or stable expression over several generations	Shu *et al*., (2002); Wang *et al*., (2002); Wu *et al*., (2002)
*cry*IAa or *cry*IAb	SSB	*Bt* rice for Mediterranean conditions; already field-tested	Breitler *et al.,* (2000); Marfa *et al.,* (2002); Breitler *et al.,* (2004)
*cry*IAb and/or *cry*IAc	YSB	Different varieties transformed for insect-resistance	Khanna and Raina (2002); Ramesh *et al.,* (2004)
*cry*IAb	SSB	Marker-free insect resistant transgenic rice plants	Cotsaftis *et al*., (2002)
*cry*IAb	SSB	Wound-inducible expression of *cry*IAb	Breitler *et al*., (2001)
*cry*IAb	YSB and leaf folder	Use of different promoters for tissue-specific *Bt* gene expression	Husnain *et al*., (2002)
*cry*IAb, *cry*IAc and/ or *gna*	Lepitopteran and sap-sucking insects	Gene-pyramiding for protection against broad-range of insects	Maqbool *et al*., (2001); Ramesh *et al*., (2004)
cry, *Xa21* and *RC7*	YSB, bacterial blight and sheath blight	Gene-stacking for multiple disease tolerance	Datta *et al.,* (2002)
gna	Several homopteran, coleopteran and lepitopteran insects	Different varieties for multiple-range of insect resistance and/or no expression in seeds	Foissac *et al*., (2000); Tinjuangjun *et al*., (2000); Tang *et al*., (2001); Wu *et al*., (2002); Nagadhara *et al*., (2003)
gna and *cry*IAc	BPH and SSB	Use of linear constructs to avoid any integration of back-bone sequences in transgenic plants	Loc *et al.,* (2002)
Itr1	Rice weevil	Constitutive or endosperm-specific expression of barley trypsin inhibitor BTI-CMe for protection against rice weevil	Alfonso-Rubi *et al*., (2003)
cryIAb/cryIAc	YSB and leaf folder	Insect bioassay	Chandel, 2005
*cry*IAc; *cry*IIA	YSB and leaf folder	Field-trial of *Bt* rice in Pakistan	Bashir *et al*., (2004)

Note: BPH, brown plant hopper; *Itr1*, *Hordeum vulgare* gene encoding for trypsin inhibitor BTI-CMe; SSB, striped stem borer; YSB, yellow stem borer.

5.3 Non Tissue Culture based floral dip (*in planta*) method of transformation

Developing tissue culture independent genetic transformation system is of great interest because such system would avoid constraints imposed by genotype specificity in transformation, regeneration and eliminate tissue culture induced genetic variation. In addition, transgenic plants could be produced inexpensively and rapidly. The *Agrobacterium*- mediated floral dipping method developed for the model plant *Arabidopsis thaliana* has greatly impacted biological research and is being routinely used for transferring transgenes in gene validation studies (Bent, 2000).

Research with *Arabidopsis* has benefited from the development of high throughput transformation methods that avoid plant tissue culture (Leehan and Feldmann, 1997). In particular, the development of the *Agrobacterium*- mediated vacuum infiltration method (Bechtold *et al*., 1993) has had a major impact on *Arabidopsis* research. The *Arabidopsis* transformation can not be performed routinely in laboratories that have little or no expertise in plant tissue culture and transformation, allowing *in planta* analysis of multiple DNA construct (Leehan and Feldmann, 1997; Clough and Bent, 1998)

5.3.1 Target cell for T-DNA transfer

Germ line transformation is the common feature that allows avoidance of tissue culture and regeneration in the vacuum infiltration, seed transformation, *in planta* transformation and floral dip methods (Feldmann and Marks, 1987; Feldmann, 1992; Bechtold *et al.*, 1993; Chang *et al.*, 1994; Katavic *et al.*, 1994; Clough and Bent, 1998). With all of these methods selection with antibiotics or herbicide is not applied to the *Agrobacterium*- treated plants. Instead, progeny

seeds are harvested and selection is applied to the resultant seedlings as they germinate. Previous studies have shown that T_1 transformants are typically hemizygous carrying T-DNA at only one of the two alleles of a given locus (Feldmann, 1991; Bechtold *et al.,* 1993). Transformation events occurring prior to or early in floral development of the T_o plant would be expected to give rise to identically transformed male and female gametophytes, which upon self-fertilization could produce a significant number of T_1 plants that are homozygous for the T-DNA insertion.

Here, we investigated the target site and timing of transformation in the *Agrobacterium* floral dip method for *indica* rice. The transformants were obtained from the crosses (Swarna pollen grains) between *Agrobacterium* inoculated female (emasculated flower - pollen recipient) and un-inoculated male (pollen donor) plants of Swarna rice. Whereas, when the un-emasculated rice flowers at 25-100% anthesis stage were inoculated with *Agrobacterium* inoculums, no transformants obtained. This suggested that the productive transformation events occur at female flower structures and that they do not occur during early stages of anther or pollen/ microspores development to pollen release. Our results indicated that cells of ovary, mainly egg cells are the primary sites for T-DNA insertion.

Rice plants bear an intermediate inflorescence that typically carries flower of a wide range of developmental stage. The rice flowers are cleistogamous in nature and opened only after bursting of anthers in closed flower and pollination for self fertilization. In our experiment, the opened rice flowers with different level of anthesis (Fig. 4.12) when inoculated with

Agrobacterium did not produced any positive transformants. Whereas, when flowers were cut opened and emasculated by removing un-bursted anthers manually, then exposed to the *Agrobacterium* inoculums, which allows exposure of *Agrobacterium* to the target cells, which may be cells of ovules in developing flowers. Similarly, Bechtold *et al*., (2000) have performed experiments to identify the genome (megagametophyte or microgametophyte) that receives the T-DNA insert in floral transformation of *Arabidopsis*. Their findings are consistent with our work, indicating that the predominant target is the female genome. However they did observe apparent T-DNA integration into the male genome in one of the 26 cases examined.

Ye *et al*., (1999) also examined *Agrobacterium*- mediated floral transformation of *Arabidopsis*. In a smaller crossing study that did not monitor timing of floral development, they observed 15 transformants when *Agrobacterium* was applied to the pollen recipient flower and zero transformants when *Agrobacterium* was applied to the pollen- donor.

The identification of ovules and/ or megagametophytes as the primary target for transformation brings up the question of how *Agrobacterium* gains access to the interior of the ovary in *Arabidopsis* flowers. The success of transformation after gentle dipping of inflorescences into *Agrobacterium* solution had suggested that the transformed tissue were exposed at or near the surface of the plant. Developing anthers present one such target, as do germinating pollen grains present on the stigmatic surface (Desfux *et al*., 2000). However, in extensive studies they did not obtain evidence for pollen transformation in *Arabidopsis*. The possibility remains that germinated pollen

tubes occasionally carry *Agrobacterium* from the stigma down the style to mature ovules, but the crossing experiments did not produce transformants in flowers pollinated during the 1st four day after inoculation.

Pollen transformation or pollen tube mediated delivery of *Agrobacterium* to the embryo sac also do not account for the uniformly GUS stained ovules that were frequently observed in non- pollinated flowers (Desfeux *et al*., 2000; Ye *et al*., 1999). These delivery methods are also not consistent with the recent findings of Bechtold *et al.,* (2000) implicating the female genome as the primary target of transformation for *Arabidopsis* transformation.

5.3.2 *Agrobacterium* inoculum density and dipping time

Clough and Bent (1998) reported that *Agrobacterium* inoculum OD_{600} of 0.15 to 1.70 did not modify the transformation efficiency in floral dip method of transformation in *Arabidopsis*. They found that when *Agrobacterium* plants were inoculated drop by drop using *Agrobacterium* having more than 2.0 OD_{600} density, the transformation efficacy increased by two fold probably a matter of probability, as more bacteria are available to enter the opened flower. Here, in our study we did not find higher transformation efficiency with the increased *Agrobacterium* inoculum density. When cut opened, emasculated rice flowers were inoculated with > 0.8 OD_{600} inoculum, the seed setting percentage reduced significantly upon crossing. This indicated that higher concentration of *Agrobacterium* may have affected the stigma receptivity and pollen tube growth, which ultimately leads to reduced seed setting. Similarly the dipping time more than 4.0 min with *Agrobacterium* inoculum has low seed setting.

5.3.3 Flower dipping *vs* spraying with *Agrobacterium*

The flowering stage and application of *Agrobacterium* inoculum on to the target cell surface have very important role in the success of *in planta* transformation (Bent 2000). We examined two methods of applying *Agrobacterium* inoculum on to emasculated rice flowers *viz*., dipping with gentle shaking and spraying with *Agrobacterium* culture. Low seed setting recorded in the plants treated with spraying method compare to that of dipping method, subsequently no T_1 plants generated from spraying method were found positive for gene of interest, GUS and *cry* IAc. This clearly indicated that spraying of *Agrobacterium* cultures on to emasculated rice flowers caused injuries to stigma/style and ultimately affects the double fertilization of seed development. In contrast to this finding, Chung *et al*., (2000) reported the high transformation efficiency in *Arabidopsis* of floral spray method compared to that of vacuum infiltration and floral dip method using *Agrobacterium*. This opens the possibilities of *in planta* transformation of plant species which are too large for dipping or vacuum infiltration.

5.4 Comparison of transformation efficiency among different methods of transformation systems

Low rate of transformation efficiency in tissue culture based transformation methods Particle gun and *Agrobacterium*- mediated transformation systems have limited the routine use of genetic engineering techniques for crop improvement (Azhakanandam *et al.,* 2000; Jiang *et al.,* 2000; Upadhyaya *et al.,* 2000; Lin and Zhang, 2005). The transformation efficiency ranging form 6.40 to 7.30% for *Agrobacterium*- mediated (Breilter *et al.,* 2001) and by Particle gun mediated system from16.0 to 18.0 (Kumar *et al.,* 2005).

Supartana *et al.,* (2005) has reported maximum transformation efficiency of 43.0% using meristem infiltration method in rice. In the recent development in gene validation research, a high through put transformation method i.e. *in planta* has been developed in *Arabidopsis* by dipping the flowers into *Agrobacterium* inoculum, embryo infiltration, with the highest transformation efficiency of 40.0% in *Arabidopsis* (Ye *et al.,* 1999). In our study, we found 22.95% high throughput transformation technique for tissue culture recalcitrant *indica* rice. Similarly, the attempts of *in planta* transformation method for various crops, *viz.,* transformation efficiency and developed for soybean, maize, *Medicago trunctulata* have been transformed with the transformation efficiency of 2.0%, 60.0%, 7.0% respectively (Trieu *et al.,* 2000; Shou *et al.,* 2002; Chung *et al.,* 2000; Chumakov *et al.,* 2006). The high efficiency transformation system developed for *indica* rice varieties has great potential in gene validation studies in post genomic era and also transform non tissue culture responsive varieties of rice with agronomically important genes.

CHAPTER VI

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH WORK

6.1 Summary

Transgenic *Bt* rice is a proven technology having shown a great potential for environment friendly sustainable and cost effective solutions for control of lepidopterous pests *viz.,* YSB, SSB and LF. Most of the transgenic rice developed till date has used tissue culture based methods mainly, Particle gun mediated and *Agrobacterium*- mediated DNA delivery systems. Although the rate of developing transgenic crops using these technologies are with very low transformation efficiency, genotype dependent and leads to genomic changes and genetic distortion. The present study undertaken to assess various factors affecting tissue culture response, to develop transgenic *Bt* rice using popular rice varieties Swarna and Mahsuri. This study also aimed to develop a high efficiency transformation method using floral dip based *Agrobacterium*- mediated *in planta* transformation system.

Four factors, carbon source, gelling agent, L- proline and partial desiccation of calli on callus induction and regeneration of recalcitrant *indica* rice varieties Swarna and Mahsuri were studied. We have successfully examined the effect of these factors and standardized a high efficiency plant regeneration protocol for two rice varieties, Swarna, one of the popular rice variety ranking top five among the rice varieties grown in Eastern India including Bangladesh and Mahsuri which is popular medium slender aromatic rice variety.

We found maltose, as better source of carbohydrate, additional supplementations of proline, casein hydrolysate, and a cytokine, kinetin along with 2,4-D favoring high callusing and regeneration in rice varieties, Swarna and Mahsuri. The rate of callusing increased by 49% in Swarna and 71% in Mahsuri. Similarly, the partial desiccation of embryogenic calli (18-24 hours) prior to regeneration had significantly enhanced the regeneration efficiency by 23.15% in Swarna and 33.55% in Mahsuri indicating that stress in calli induces the cell physiological and biochemical changes , which is necessary for efficient regeneration.

In a pilot experiment, the concentrations of Hygromycine required to kill the non transformed cell of targeted rice cultivars, Swarna and Mahsuri were examined. Variations for the concentration of Hygromycine in selection medium were observed. Swarna calli required low dose of Hygromycine (35 mg/L) compared to that of Mahsuri (45 mg/L) to be used for the selection of transformed and killing of non transformed control calli. These doses were used for the selection of transgenic calli on tissue culture based Particle gun and *Agrobacterium*- mediated transformation methods.

From our tissue culture based transformation experiments, nineteen putative transformants were produced for both the rice varieties, Swarna and Mahsuri. Two Swarna transformants #7 and #18 derived from the Particle gun mediated transformation were found positive for *cry*IAc gene. Whereas, no transformants had shown amplification of DNA when analyzed using gene specific primers of *m*VIP gene by PCR analysis, indicating lower efficiency of transformation in *indica* rice by tissue culture based method. In order to ensure

the integration of *cry*IAc gene into the genome of Swarna transformants, the genomic DNA was isolated from the leaf samples and subjected to southern analysis. Two PCR positive Swarna transformants, #7 and #18 had shown the presence of distinct band of 1.8 kb band that got hybridized with transcriptional cassette of *cry*IAc gene construct used as probe in a southern blot analysis suggesting the integration of *cry*IAc gene into genome of rice transformants #7 and # 18. We also found integration of high rearranged transgene fragments on southern blots of Swarna transformants.

Looking to success of *Agrobacterium* based in-planta transformation methods; embryo & vacuum infiltration, floral spray and meristem infiltration as developed in a model crops *Arabidopsis* (Ye *et al.,* 1999, Desfux *et al.,* 2000, Clough and Bent, 1998) and limitations of tissue culture based transformation systems, we attempted to examine the various factors affecting the T- DNA transfer in *Agrobacterium* mediated floral dip method of transformation for *indica* rice using a reporter gene GUS and a *Bt* gene *cry*IAc driven by constitutive promoter CaMV35S.

Here, we investigated the target site and timing of transformation in the *Agrobacterium* floral dip method for *indica* rice. The transformants were obtained from the crosses (Swarna pollen grains) between *Agrobacterium* inoculated female (emasculated flower - pollen recipient) and un-inoculated male (pollen donor) plants of Swarna rice. Whereas, when the un-emasculated rice flowers at 25-100% anthesis stage were inoculated with *Agrobacterium* inoculums, no transformants obtained. This suggests that the productive transformation events occur at female flower structures and that they do not

occur during early stages of anther or pollen/ microspores development to pollen release. Our results indicated that cells of ovary mainly egg cells are the primary sites for T-DNA insertion.

Rice plants bear an intermediate inflorescence that typically carries flower of a wide range of developmental stage. The rice flowers are cleistogamous in nature and opened only after bursting of anthers in closed flower and pollination for self fertilization. In our experiment, the opened rice flowers with different level of anthesis, when inoculated with *Agrobacterium* did not produced any positive transformants. Whereas, when flowers were cut opened and emasculated by removing un-bursted anthers manually, then exposed to the *Agrobacterium* inoculums, allows the exposure of *Agrobacterium* to the target cells and T-DNA insertion occurs. Second important factor affected the transformation efficiency is *Agrobacterium* inoculum density. The *Agrobacterium* inoculum density > 0.8 OD_{600} has shown significant effect on seed setting of emasculated rice flowers after inoculation and subsequent pollination. Similarly, the time of flower dipping onto *Agrobacterium* inoculum had also reduced the rate of seed setting on crossing. This indicated that higher concentration of *Agrobacterium* may have affected the stigma receptivity and pollen tube growth, which ultimately leads to reduced seed setting. Similarly the dipping time more than 4.0 min. with *Agrobacterium* inoculum has lowered the seed setting.

We also examined two methods of applying *Agrobacterium* inoculum on to emasculated rice flowers *viz.*, dipping with gentle shaking and spraying with *Agrobacterium* culture. Low seed setting recorded in the plants treated with

spraying method compare to that of dipping method had shown that with the spraying of *Agrobacterium* culture on emasculated flowers caused injuries to stigma/style and ultimately affects the double fertilization of seed development.

6.2 Conclusions

- The *in vitro* study revealed that maltose, as better source of carbohydrate, additional supplementations of proline, casein hydrolysate, and a cytokine, kinetin along with 2, 4 –D favoring increase callusing by 49% in Swarna and 71% in Mahsuri rice.
- Similarly, the partial desiccation of embryogenic calli (18-24 hours) prior to regeneration had significantly enhanced the regeneration efficiency in both the rice varieties under study.
- The medium containing 0.3% gel rite has significantly enhanced both callus induction and nature of calli by 89.9% and 93.4% in Swarna and Mahsuri respectively. It accounts 14.23% and 25.03% increased callus induction over the medium containing agar agar in both the varieties.
- The concentration levels of Hygromycine-B were found to be genotype dependent for selection of transgenic rice calli. Swarna calli required low dose of Hygromycine (35 mg/L) compare to that of Mahsuri (45 mg/L) to be used for the selection of transformed and killing of non transformed control calli.
- Total of 58 transformants were obtained with the transformation efficiency of 0.4% and 22.95% for tissue culture based Particle gun- mediated and non tissue culture based floral dip (*in planta*) transformation method respectively.

- Total of 56 transformants were produced using non tissue culture based floral dip including 18 were transgenic *Bt* (*cry*IAc) and 38 were of GUS positive with transformation efficiency of 16.66% and 29.68% for *cry*IAc and GUS gene .
- The PCR positive Swarna transformants, #7 and #18 had shown the presence of distinct band of 1.8 kb that got hybridized with transcriptional cassette of cryIAc gene construct used as probe suggesting the integration of transgenes by southern analysis.
- The PCR positive plants with reporter gene GUS had shown the expression of β-glucuronidase enzyme by X-gluc test.
- The target cell type for DNA transfer in the *Agrobacterium* mediated floral dip method of transformation for *indica* rice was identified. The transformants were obtained only from the attempts in floral dip method where, emasculated flowers were exposed with *Agrobacterium* inoculum and then pollinated with un-inoculated pollen grains (Swarna pollen grains). Whereas, when the un-emasculated rice flowers at 25-100% anthesis stage were inoculated with *Agrobacterium* inoculums, no transformants obtained. This suggests that the T-DNA transfer occur into egg cell of developing rice flowers. When flowers were cut opened and emasculated by removing un-bursted anthers manually, the *Agrobacterium* culture comes in contact with the surface of the target cells and allows transfer of T-DNA.
- Lowe seed setting and reduced transformation efficiency from the high density *Agrobacterium* inoculum infection (> 08 OD_{600}) and dipping time more than 4.0 min indicated reduced stigma receptivity and pollen tube

growth on crossing. Similarly the dipping time more than 4.0 min with *Agrobacterium* inoculum has low seed setting.

- The development of high efficiency floral dip based *in planta* transformation method in *indica* rices provide a less sophisticated, cheap and easy non tissue culture based transformation, which will speed up the development of useful rice transgenic for various agronomically important genes.

6.2 Suggestions for future work

- The tissue culture medium developed in this investigation for two *indica* rice varieties, Swarna and Mahsuri should be tested for more popular rice varieties to be used for transformation of genes.
- The gene integration in to T_o plants form tissue culture based and T_1 plants from *in planta* based methods should be analyzed and characterized for the transgene integration and its expression in subsequent generations.
- The *Bt* transgenic rice developed should be bioassayed against target insects *viz.*, YSB, SSB and LF.
- Further experiments to assess the factors affecting the *in planta* transformation system in *indica* rice and also to elucidate the prices of role *Agrobacterium* inoculum density and target cell for T-DNA transfer should be carried out.

Development of efficient transformation systems to enhance insect resistance in rice (*Oryza sativa* L.)

by

Jadhav Pravin Vishwanathrao

ABSTRACT

Rice is the world's important food crop, serving as a major staple food for about 3 billion people. The pests particularly stem borers causes million dollars worth of loss annually. Development of resistant varieties plays a pivotal role in management of insect pests such as yellow stem borer (*Scripophaga incertulus*), stripped stem borer (*Chilo supperessalis*) and leaf folder (*Cnaphalocrocis medinalis*). Despite of the fact that more than 30,000 rice accessions have been screened for resistance till date, the sufficient level of resistance was not found in rice collection (Wu *et al.,* 1999). The only viable option to control borers and leaf folder insects of rice are either through the use of chemical insecticides or use of genetic engineering to introduce genes of alien origin conferring resistance to these pests. Genetic engineering using *Bt* gene(s) is an attractive alternative of developing rice genotypes resistant to stem borers. Most of the rice genotypes have been transformed using tissue culture based techniques, *Agrobacterium*- mediated and Biolistic approaches. However, some technological limitations like these methods of transformation are genotype dependent, time consuming and recalcitrant nature of *indica* rice to *in-vitro* regeneration with very low transformation efficiency has limited the routine use of this technology.

The present study was therefore conceived with the objectives to assess the various factors affecting tissue culture response and subsequently the tissue culture based transformation systems *viz.*, *Agrobacterium*- mediated and Particle gun mediated DNA delivery and to develop insect resistant *indica* rice plants using *cry*IAc and *m*VIP genes. For developing high efficiency callusing and plant regeneration systems for two rice varieties, Swarna and Mahsuri, suitable concentrations of carbohydrate maltose, a gelling agent gel rite and plant growth

regulators were identified and a high efficiency *in vitro* regeneration protocol from seed derived calli were developed for *indica* rice varieties under study. Partial desiccation of embryogenic calli (18-24 hours) prior to regeneration has significantly increased regeneration efficiency by 23.15% in Swarna and 33.55% in Mahsuri.

The *cry*IAc gene was successfully introduced into rice variety Swarna using Particle gun mediated transformation system for developing insect resistant rice plants. Twenty of 58 putative transformants of rice variety Swarna were found positive for *cry*IAc gene, whereas, no transformants were found positive for *m*VIP gene. Later, the integration of *cry*IAc gene was confirmed initially by PCR and then by southern analysis.

To develop a high efficiency non- tissue culture based *in planta* transformation system for *indica* rice, various factors for *Agrobacterium* based floral dip method were examined using a reporter gene, GUS/INT driven by CaMV35S promoter. Our study suggested that female floral part mainly egg cells of emasculated rice flowers are the target of T- DNA transfer in floral dip method. The parameters *viz.*, density of *Agrobacterium* inoculum, dipping time with gentle shaking, removing of excess *Agrobacterium* culture adhered on the surface and pollen density to allow double fertilization and seed development were standardized for *indica* rice. The high transformation efficiency of 22.95% was recorded using the procedure developed in this study. This opens up avenues for developing rice transgenic rapidly using less sophisticated, easy and by less technically skilled personals. Development of genotype independent non-tissue culture based high efficiency transformation procedure for *indica* rice will also enhance the gene validation research in rice.

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Table 1 : Differences in callus induction% of Swarna genotype on different medium

SUMMARY

Groups	Count	Sum	Average	Variance
S-L3	5	43.94909	8.789817	0.044557
S-LS	5	41.81134	8.362268	0.010946
S-MSM	5	47.29215	9.458429	0.000329
S-MS	5	39.20413	7.840827	0.010454

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	7.025667	3	2.341889	141.32**	1E-11	3.238872
Error	0.265143	16	0.016571			
Total	7.29081	19				

Table 2: Differences in callus induction% of Mahsuri genotype

SUMMARY

Groups	Count	Sum	Average	Variance
M-L3	5	44.31132	8.862263	0.055174
M-LS	5	39.32716	7.865432	0.051335
M-MSM	5	48.08258	9.616517	0.020966
M-MS	5	36.82457	7.364915	0.018921

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	15.23894	3	5.079647	138.79**	1.15E-11	3.238872
Error	0.585583	16	0.036599			
Total	15.82452	19				

Table 3: Standardization of Hygromycine concentration for selection of Transformed calli of Swarna

10TH DAY

SUMMARY

Groups	Count	Sum	Average	Variance
S-25mg/l	5	2.883	0.5766	0.0003573
S-30mg/l	5	3.866	0.7732	0.0004967
S-35mg/l	5	4.65	0.93	0.0004695
S-40mg/l	5	4.65	0.93	0.000189
S-45mg/l	5	4.767	0.9534	0.0002023
S-50mg/l	5	4.931	0.9862	0.0001792
		Mean	**0.86**	

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	0.611405	5	0.1222	387.374**	4.03E-22	2.620654147
Error	0.007576	24	0.0003			
Total	0.618981	29				

20TH DAY

SUMMARY

Groups	Count	Sum	Average	Variance
S-25mg/l	5	4.217	0.8434	0.0003623
S-30mg/l	5	4.963	0.9926	7.68E-05
S-35mg/l	5	2.01	0.402	0.2970075
S-40mg/l	5	1.994	0.3988	0.2922827
S-45mg/l	5	2.01	0.402	0.2970075
S-50mg/l	5	0.02	0.004	0
		Mean	0.51	

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	3.1786922	5	0.635738	4.301**	0.006183	2.62065
Error	3.5469472	24	0.147789			
Total	6.7256394	29				

Table 4: Standardization of Hygromycine concentration for selection of transformed calli

10th Day

SUMMARY

Groups	Count	Sum	Average	Variance
M-25mg/l	5	2.8	0.56	0.0004
M-30mg/l	5	4.22	0.844	0.00088
M-35mg/l	5	4.28	0.856	0.00048
M-40mg/l	5	4.64	0.928	0.00032
M-45mg/l	5	4.8	0.96	0.0002
M-50mg/l	5	4.937	0.9874	0.0003023
		Mean	**0.86**	

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	0.60512	5	0.12102	281.2048	1.77E-20	2.62065
Error	0.01032	24	0.00043			
Total	0.61545	29				

20th Day

SUMMARY

Groups	Count	Sum	Average	Variance
M-25mg/l	5	4.3	0.86	0.0002
M-30mg/l	5	4.957	0.9914	0.0001083
M-35mg/l	5	4.957	0.9914	0.0001083
M-40mg/l	5	4.957	0.9914	0.0001083
M-45mg/l	5	2.013	0.4026	0.2964108
M-50mg/l	5	0.025	0.005	0
		Mean	**0.707**	

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	4.2576	5	0.85152	17.20619851	3.00E-07	2.6206
Error	1.1877	24	0.04948			
Total	5.4453	29				

Table 5: Callus induction response of Swarna genotype on medium containing different gelling agent

	SWARNA-MSM+ Agar agar	SWARNA-MSM+Gelrite
Mean	8.896847129	9.488043622
Variance	0.057638958	0.021285275
Observations	5	5
Pooled Variance	0.039462117	
Hypothesized Mean Difference	1	
df	8	
t Stat	-12.66495435	
P(T<=t) one-tail	7.09958E-07	
t Critical one-tail	1.859548033	
P(T<=t) two-tail	1.41992E-06	
t Critical two-tail	2.306*	

Table 6: Callus induction response of Mahsuri genotype on medium containing different gelling agent

	Mahsuri -MSM+ Agar agar	Mahsuri -MSM+Gelrite
Variance	0.060749438	0.007116748
Observations	5	5
Pooled Variance	0.033933093	
Hypothesized Mean Difference	0	
df	8	
t Stat	-8.755098856	
P(T<=t) one-tail	1.13438E-05	
t Critical one-tail	1.859548033	
P(T<=t) two-tail	2.26877E-05	
t Critical two-tail	2.306*	

- **Antibiotic Stocks**
  - **Kanamycine:50mg/lit**

    500 mg Kanamycine was added in 10 ml of autoclaved distilled water and aliquots were prepared.

- **Ampicilin: 100mg/lit**

  1 gm Ampicilin was added in 10 ml of autoclaved distilled water and aliquots were prepared.

- **Acetosyringone: 40 mg/lit (Freshly Prepared)**

  0.039 mg Acetosyringone was added in 300 µl DMSO and made up to 1 ml with sterile water.

- **Cefateximine: 250mg/lit**

  250 mg Cefateximine was added in 1 ml of distilled water.

- **Hygromycine: 50 mg/ml**

  500 mg Hygromycine was added in 10 ml of distilled water and then filter sterilized and after that distributed in aliquots.

Gene sequence of *cry* IAc

GGCGAATTGGGTACCGGATCCATGGATAACAACCCAAACATTAACGAGTG
CATTCCATACAACTGCTTGAGCAACCCAGAGGTTGAGGTTCTTGGAGGAG
AGCGCATTGAGACCGGATACACTCCCATCGACATCTCCTTGTCCTTGACTC
AGTTCCTCCTCAGCGAGTTCGTGCCAGGAGCTGGGTTCGTTCTCGGACTTG
TTGACATCATCTGGGGAATCTTCGGACCATCTCAATGGGACGCCTTCCTCG
TGCAAATTGAGCAGTTGATCAACCAGAGGATCGAGGAGTTCGCCAGGAA
CCAGGCCATCTCTAGGTTGGAGGGATTGAGCAACCTCTACCAAATCTACG
CTGAGAGCTTCAGAGAGTGGGAGGCCGATCCAACTAACCCAGCTCTCCGC
GAGGAAATGCGTATTCAATTCAACGACATGAACAGCGCCTTGACCACTGC
TATCCCATTGTTCGCCGTGCAGAACTACCAAGTTCCACTCTTGTCCGTGTA
CGTTCAAGCTGCTAACCTTCACCTCAGCGTGCTTCGTGACGTTAGCGTGTT
CGGCCAAAGGTGGGGATTCGATGCTGCAACCATCAACAGCCGTTACAACG
ACCTTACTAGGCTCATTGGAAACTACACCGACCACGCTGTTCGTTGGTAC
AACACTGGCTTGGAGCGTGTCTGGGGACCCGATTCTAGAGATTGGATCAG
GTACAACCAGTTCAGGAGAGAGTTGACCCTCACTGTTTTGGACATTGTGT
CTCTCTTCCCCAACTACGACTCCAGAACCTACCCTATCCGTACTGTGTCCC
AACTTACCAGAGAGATCTACACTAACCCAGTTCTTGAGAACTTCGACGGT
AGCTTCCGCGGATCTGCTCAGGGCATCGAGGGCTCCATCAGGAGCCCACA
CTTGATGGACATCTTGAACAGCATAACTATCTACACCGATGCTCACAGAG
GAGAGTACTACTGGTCTGGACACCAGATCATGGCCTCTCCAGTTGGATTC
AGCGGGCCCGAGTTCACCTTCCCACTCTACGGAACTATGGGAAACGCCGC
TCCACAACAACGTATCGTTGCTCAACTTGGACAGGGAGTCTACAGGACCT
TGTCTTCCACCTTGTACAGAAGGCCCTTCAACATCGGAATCAACAACCAG
CAACTTTCCGTTCTTGACGGAACTGAGTTCGCCTACGGAACCTCTTCCAAC
TTGCCATCCGCTGTTTACAGAAAGAGCGGAACCGTTGATTCCTTGGACGA
GATCCCACCACAGAACAACAATGTGCCACCCAGGCAAGGATTCTCCCACA

GGTTGAGCCACGTGTCCATGTTCCGTTCCGGATTCAGCAACAGTTCCGTGA
GCATCATCAGAGCCCCTATGTTCTCTTGGATTCACCGTTCTGCCGAGTTCA
ACAACATCATCGCTTCTGATAGCATTACTCAGATCCCAGCCGTGAAGGGA
AACTTCCTTTTCAACGGAAGCGTTATCAGCGGACCAGGATTCACTGGCGG
AGACCTTGTGAGACTTAATAGCTCTGGCAACAACATTCAGAATAGAGGCT
ACATCGAGGTTCCTATCCACTTCCCATCCACATCTACTAGATATAGAGTTA
GGGTTAGATACGCCTCTGTGACCCCAATCCACCTTAACGTGAACTGGGGC
AACTCATCTATCTTCTCCAACACCGTTCCAGCTACTGCTACCTCTCTTGAT
AACCTTCAATCCAGCGATTTCGGATACTTCGAGAGCGCCAACGCTTTCAC
TTCTTCCTTGGGCAACATCGTGGGAGTTAGGAACTTCAGCGGTACTGCAG
GAGTGATCATTGACAGATTCGAGTTCATTCCAGTTACTGCCACTCTTGAGG
CTGAGTACAACCTTTAAGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTA
ATT

Gene sequence of VIP (unmodified)

CTGTCGTAATACCACTCACTATAGGGCGAATTGGGTACCGGATCCATGAC
AAGAACAACACTAAGTTGAGCACTAGAGCTTTGCCAAGCTTCATTGATTA
TTTTAACGGAATCTACGGATTCGCAACTGGAATCAAGGATATTATGAACA
TGATTTTCAAGACTGATACTGGAGGTGATCTTACCCTTGATGAAATCTTGA
AGAACCAGCAATTGCTTAACGATATTTCTGGTAAGTTGGATGGAGTTAAC
GGAAGCTTGAACGATCTTATTGCACAAGGAAACTTGAACACTGAGTTGTC
TAAGGAGATTTTGAAGATTGCAAACGAGCAAAATCAGGTTTTGAACGATG
TTAACAACAAGCTTGATGCTATAAACACTATGCTTCGTGTTTACCTTCCAA
AAATTACCTCTATGTTGAGCGATGTAATGAAGCAAAACTACGCTCTTAGC
CTGCAAATAGAATACTTGAGCAAGCAATTGCAAGAGATTTCTGATAAGTT
GGATATTATTAACGTAAACGTTCTTATTAACTCTACACTTACTGAAATTAC
ACCAGCTTACCAAAGGATTAAGTACGTGAACGAGAAATTCGAGGAATTG
ACTTTCGCTACAGAAACTAGCTCTAAGGTTAAGAAGGATGGCTCTCCTGC
AGACATTCTTGATGAGTTGACTGAGTTGACTGAACTTGCGAAGAGCGTTA
CTAAGAACGATGTGGACGGTTTCGAATTCTACCTTAACACCTTCCACGAT
GTGATGGTGGGAAACAATTTGTTCGGCCGTTCTGCTTTGAAGACTGCTTCC
GAATTGATCACCAAGGAGAACGTGAAGACTAGCGGCAGCGAGGTCGGAA
ACGTTTACAACTTCTTGATTGTTTTGACTGCTCTTCAGGCCAAGGCTTTCCT
TACTTTGACAACTTGCCGTAAGTTGTTAGGCTTGGCTGACATTGATTACAC
TTCTATTATGAACGAGCATTTGAACAAGGAAAAGGAGGAATTCAGAGTTA
ACATCCTCCCAACTCTTTCTAATACTTTCTCTAACCCCAACTACGCTAAAG
TTAAGGGAAGCGACGAGGACGCTAAGATGATTGTGGAGGCTAAGCCAGG
ACATGCCTTGATTGGGTTCGAGATTAGCAACGATTCTATTACTGTTTTGAA
GGTCTACGAGGCTAAGCTTAAGCAGAATTACCAAGTGGATAAGGATTCCT
TGTCTGAGGTTATATACGGAGACATGGATAAGTTGTTGTGCCCAGATCAG
TCTGAACAAATCTACTACACAAACAACATAGTTTTCCCAAATGAATACGT
GATTACTAAAATTGATTTCACTAAGAAGATGAAGACTTTGAGATACGAGG
TGACCGCCAACTTCTACGATTCTTCTACTGGAGAAATTGACTTGAACAAG

AAAAAGGTTGAGTCTAGCGAGGCTGAGTACAGAACTTTGAGCGCTAACG
ACGATGGCGTGTACATGCCATTGGGAGTCATCAGCGAGACTTTCTTGACT
CCAATTAACGGATTCGGCCTTCAAGCTGACGAGAACTCTAGATTGATTAC
TTTAACCTGCAAATCTTACTTGAGAGAGCTTCTCCTTGCTACTGACTTGAG
CAACAAGGAGACTAAATTGATCGTCCCCCCAAGCGGATTCATTAGCAACA
TTGTTGAGAACGGGTCCATTGAAGAGGACAATTTGGAGCCCTGGAAGGCT
AACAACAAGAATGCTTACGTTGACCATACTGGCGGAGTGAACGGAACTA
AGGCTTTGTACGTTCATAAGGACGGAGGAATTTCTCAATTCATTGGAGAT
AAGTTGAAACCTAAGACTGAGTACGTTATCCAATACACTGTTAAGGGAAA
GCCATCTATTCATTTGAAAGATGAAAACACTGGATACATTCATTACGAGG
ATACTAACAACAACTTGGAGGATTACCAAACTATTAACAAACGTTTCACT
ACAGGAACTGATTTGAAGGGAGTGTACTTAATTTTGAAGAGCCAAAACGG
AGATGAGGCTTGGGGAGATAACTTCATTATCTTGGAGATTAGCCCATCTG
AAAAGTTGTTAAGCCCAGAGTTGATTAACACTAACAACTGGACGAGCACG
GGATCTACTAACATTAGCGGTAATACTCTCACTCTTTACCAAGGAGGACG
TGGGATTCTTAAGCAAAACCTTCAATTAGATAGCTTCTCTACTTACAGAGT
TTACTTCTCTGTGTCCGGAGATGCTAACGTTAGGATTAGAAACTCTAGAG
AGGTGTTGTTCGGAAAAAGATACATGAGCGGTGCTAAGGATGTTTCTGAG
ATGTTCACTACAAAGTTCGAGAAAGATAACTTCTACATTGAGCTTTCTCAG
GGGAACAACTTGTACGGAGGTCCAATTGTTCATTTCTACGATGTCTCTATT
AAGTAAGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATT

Table 1 : Differences in callus induction% of Swarna genotype on different medium

SUMMARY

Groups	Count	Sum	Average	Variance
S-L3	5	43.94909	8.789817	0.044557
S-LS	5	41.81134	8.362268	0.010946
S-MSM	5	47.29215	9.458429	0.000329
S-MS	5	39.20413	7.840827	0.010454

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	7.025667	3	2.341889	141.32**	1E-11	3.238872
Error	0.265143	16	0.016571			
Total	7.29081	19				

Table 2: Differences in callus induction% of Mahsuri genotype

SUMMARY

Groups	Count	Sum	Average	Variance
M-L3	5	44.31132	8.862263	0.055174
M-LS	5	39.32716	7.865432	0.051335
M-MSM	5	48.08258	9.616517	0.020966
M-MS	5	36.82457	7.364915	0.018921

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Treatment	15.23894	3	5.079647	138.79**	1.15E-11	3.238872
Error	0.585583	16	0.036599			
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Groups	Count	Sum	Average	Variance
S-25mg/l	5	2.883	0.5766	0.0003573
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S-40mg/l	5	4.65	0.93	0.000189
S-45mg/l	5	4.767	0.9534	0.0002023
S-50mg/l	5	4.931	0.9862	0.0001792
		Mean	**0.86**	

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	0.611405	5	0.1222	387.374**	4.03E-22	2.620654147
Error	0.007576	24	0.0003			
Total	0.618981	29				

20TH DAY

SUMMARY

Groups	Count	Sum	Average	Variance
S-25mg/l	5	4.217	0.8434	0.0003623
S-30mg/l	5	4.963	0.9926	7.68E-05
S-35mg/l	5	2.01	0.402	0.2970075
S-40mg/l	5	1.994	0.3988	0.2922827
S-45mg/l	5	2.01	0.402	0.2970075
S-50mg/l	5	0.02	0.004	0
		Mean	0.51	

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	3.1786922	5	0.635738	4.301**	0.006183	2.62065
Error	3.5469472	24	0.147789			
Total	6.7256394	29				

Table 4: Standardization of Hygromycine concentration for selection of transformed calli

10th Day

SUMMARY

Groups	Count	Sum	Average	Variance
M-25mg/l	5	2.8	0.56	0.0004
M-30mg/l	5	4.22	0.844	0.00088
M-35mg/l	5	4.28	0.856	0.00048
M-40mg/l	5	4.64	0.928	0.00032
M-45mg/l	5	4.8	0.96	0.0002
M-50mg/l	5	4.937	0.9874	0.0003023
		Mean	**0.86**	

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	0.60512	5	0.12102	281.2048	1.77E-20	2.62065
Error	0.01032	24	0.00043			
Total	0.61545	29				

20th Day

SUMMARY

Groups	Count	Sum	Average	Variance
M-25mg/l	5	4.3	0.86	0.0002
M-30mg/l	5	4.957	0.9914	0.0001083
M-35mg/l	5	4.957	0.9914	0.0001083
M-40mg/l	5	4.957	0.9914	0.0001083
M-45mg/l	5	2.013	0.4026	0.2964108
M-50mg/l	5	0.025	0.005	0
		Mean	**0.707**	

ANOVA

SV	SS	df	MS	F	P-value	F crit
Treatment	4.2576	5	0.85152	17.20619851	3.00E-07	2.6206
Error	1.1877	24	0.04948			
Total	5.4453	29				

Table 5: Callus induction response of Swarna genotype on medium containing different gelling agent

	SWARNA-MSM+ Agar agar	SWARNA-MSM+Gelrite
Mean	8.896847129	9.488043622
Variance	0.057638958	0.021285275
Observations	5	5
Pooled Variance	0.039462117	
Hypothesized Mean Difference	1	
df	8	
t Stat	-12.66495435	
P(T<=t) one-tail	7.09958E-07	
t Critical one-tail	1.859548033	
P(T<=t) two-tail	1.41992E-06	
t Critical two-tail	2.306*	

Table 6: Callus induction response of Mahsuri genotype on medium containing different gelling agent

	Mahsuri -MSM+ Agar agar	Mahsuri -MSM+Gelrite
Variance	0.060749438	0.007116748
Observations	5	5
Pooled Variance	0.033933093	
Hypothesized Mean Difference	0	
df	8	
t Stat	-8.755098856	
P(T<=t) one-tail	1.13438E-05	
t Critical one-tail	1.859548033	
P(T<=t) two-tail	2.26877E-05	
t Critical two-tail	2.306*	

- **Antibiotic Stocks**
  - **Kanamycine:50mg/lit**

    500 mg Kanamycine was added in 10 ml of autoclaved distilled water and aliquots were prepared.

- **Ampicilin: 100mg/lit**

  1 gm Ampicilin was added in 10 ml of autoclaved distilled water and aliquots were prepared.

- **Acetosyringone: 40 mg/lit (Freshly Prepared)**

  0.039 mg Acetosyringone was added in 300 µl DMSO and made up to 1 ml with sterile water.

- **Cefateximine: 250mg/lit**

  250 mg Cefateximine was added in 1 ml of distilled water.

- **Hygromycine: 50 mg/ml**

  500 mg Hygromycine was added in 10 ml of distilled water and then filter sterilized and after that distributed in aliquots.

Gene sequence of *cry* IAc

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Gene sequence of VIP (unmodified)

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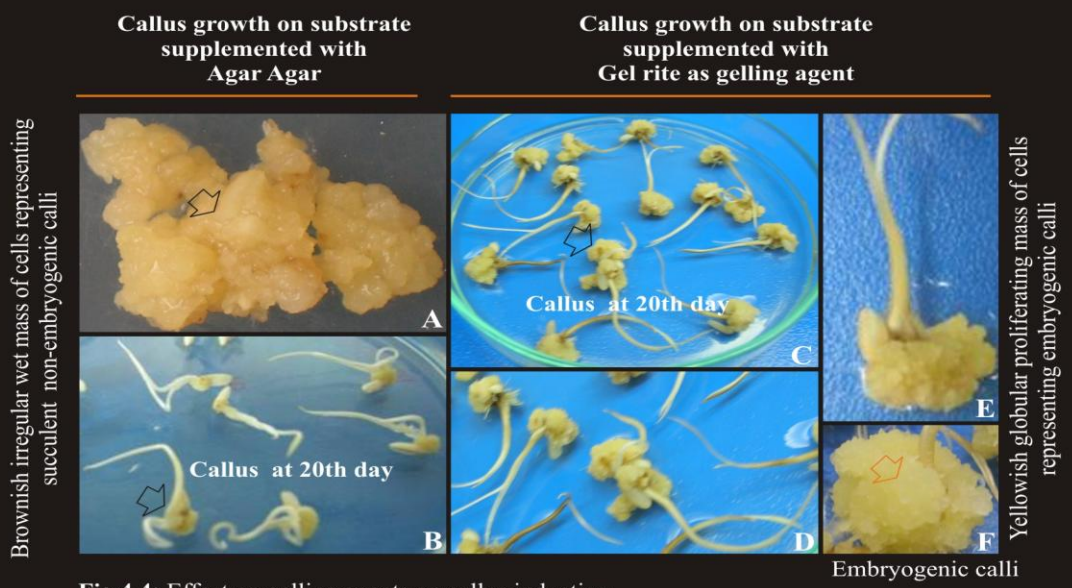
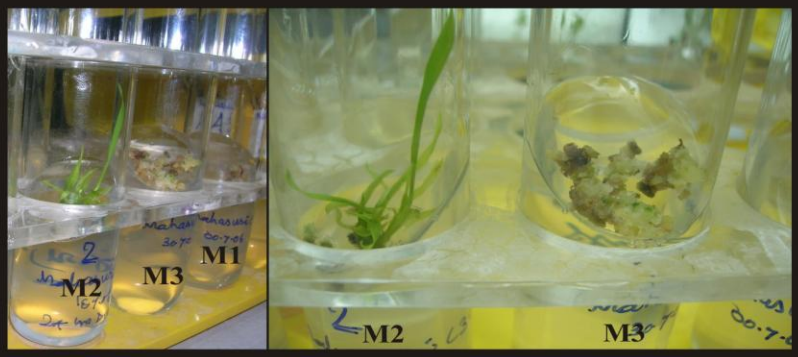


Fig 4.4: Effect on gelling agents on callus induction

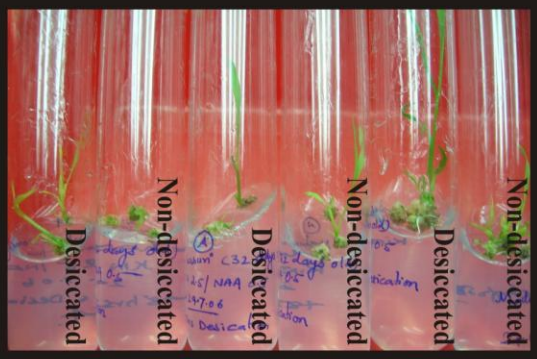
A= Callus on substrate containing agar agar as gelling agent
B= Callus (20 days after inoculation) growth on substrate containing agar agar as gelling agent
C & D= Callus growth (20 days after inoculation) on substrate containing gel rite as gelling agent
E & F= A magnified view of proliferating embryogenic calli (20 days after inoculation) on substrate containing gel rite as gelling agent



Effect of different hormonal (Kinetin, BAP, NAA (in various combinations) supplements on shoot induction of *indica* rice *cv*. Mahsuri

M1=MS
M2=MS + Kinetin (2.5 g/l) + NAA (0.5 g/l)
M3=MS+ Kinetin (2.0 g/l) + BAP (0.5 g/l)+ NAA (1.0 g/l)

Effect of partial desiccation (18-24 hrs.) of calli on regeneration of *indica* rice *cv*. Mahsuri on **M1**=MS and **M2**=MS + Kinetin (2.5 g/l) + NAA (0.5 g/l)

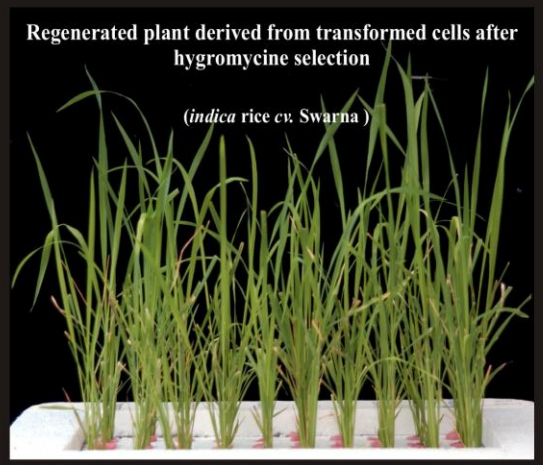


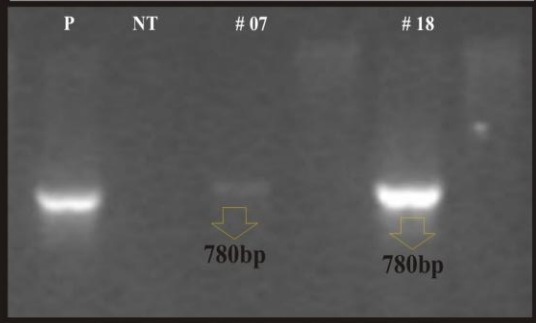
Effect of partial desiccation (18-24 hrs.) of calli on regeneration of *indica* rice *cv*. Mahsuri

Fig 4.6: Response of calli (desiccated / non desiccated) on MS media supplemented with different hormone(s) for plant regeneration

Screening of embryogenic calli after transformation(Particle gun) on hygromycine supplemented MS(M) medium
T= Transformed cell; **NT=**Non-transformed cells

Putative viable transformants (after hygromycine selection) showing shoot induction





P= Plasmid DNA with *cry* 1AC; **NT=**Non-transformed; **# 07 and # 18**= PCR amplicon (780 bp) confirming Transformation (Particle gun)

Fig 4.10a: Screening of putative rice transformants generated through tissue culture based particle gun mediated transformationsystem

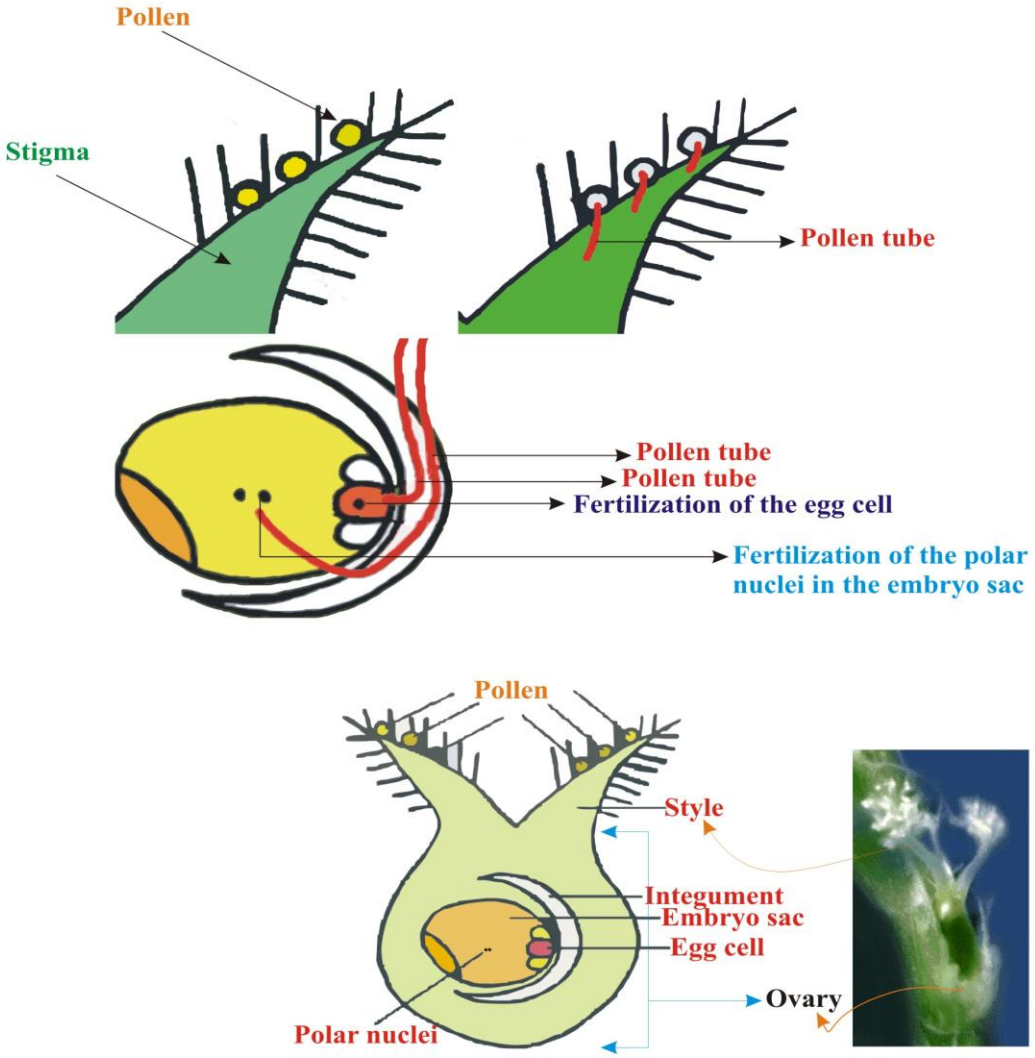


Fig 4.15: Target cell of rice flower for floral dip based *in planta* transformation

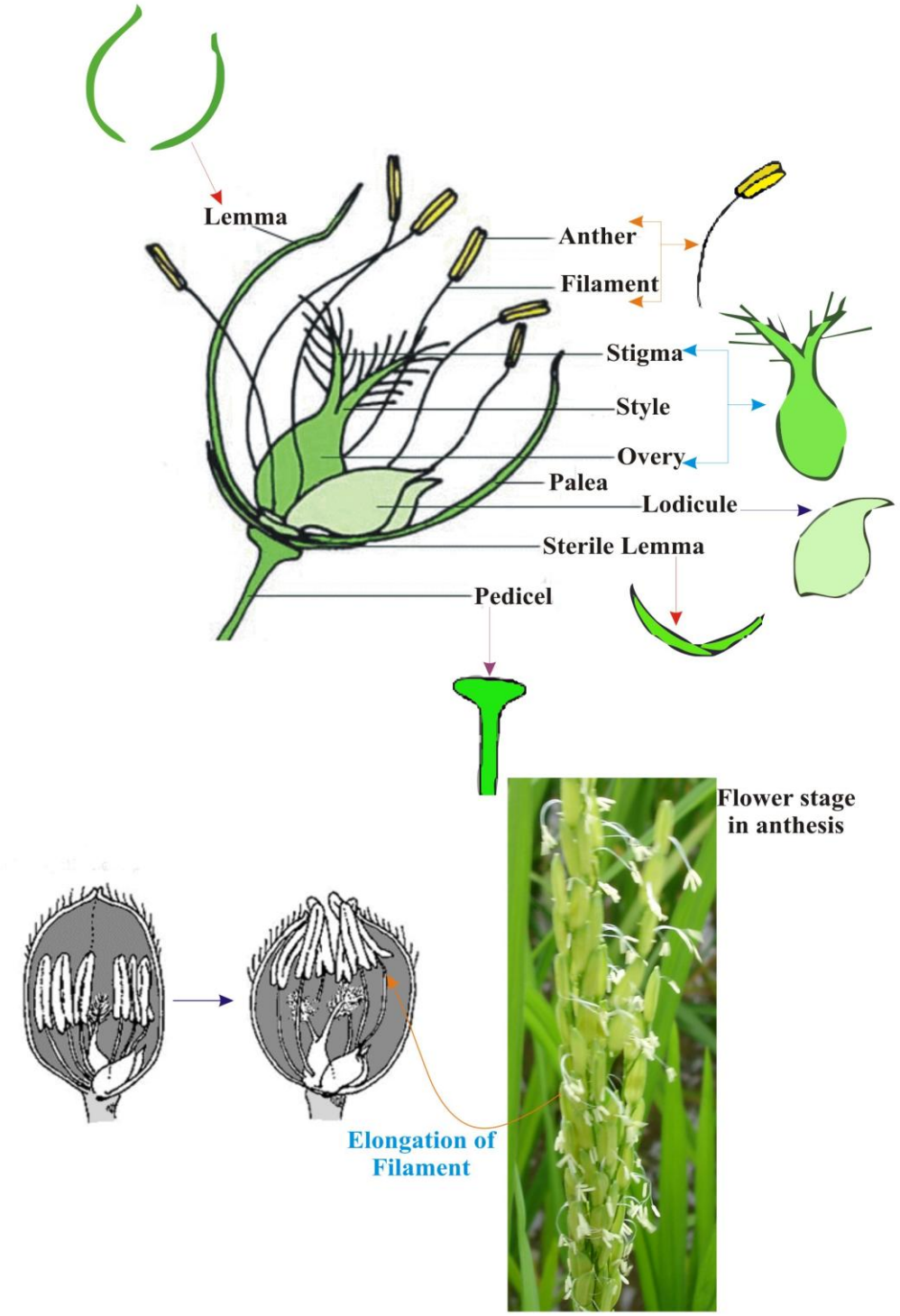


Fig 2.2: The structure of rice flower

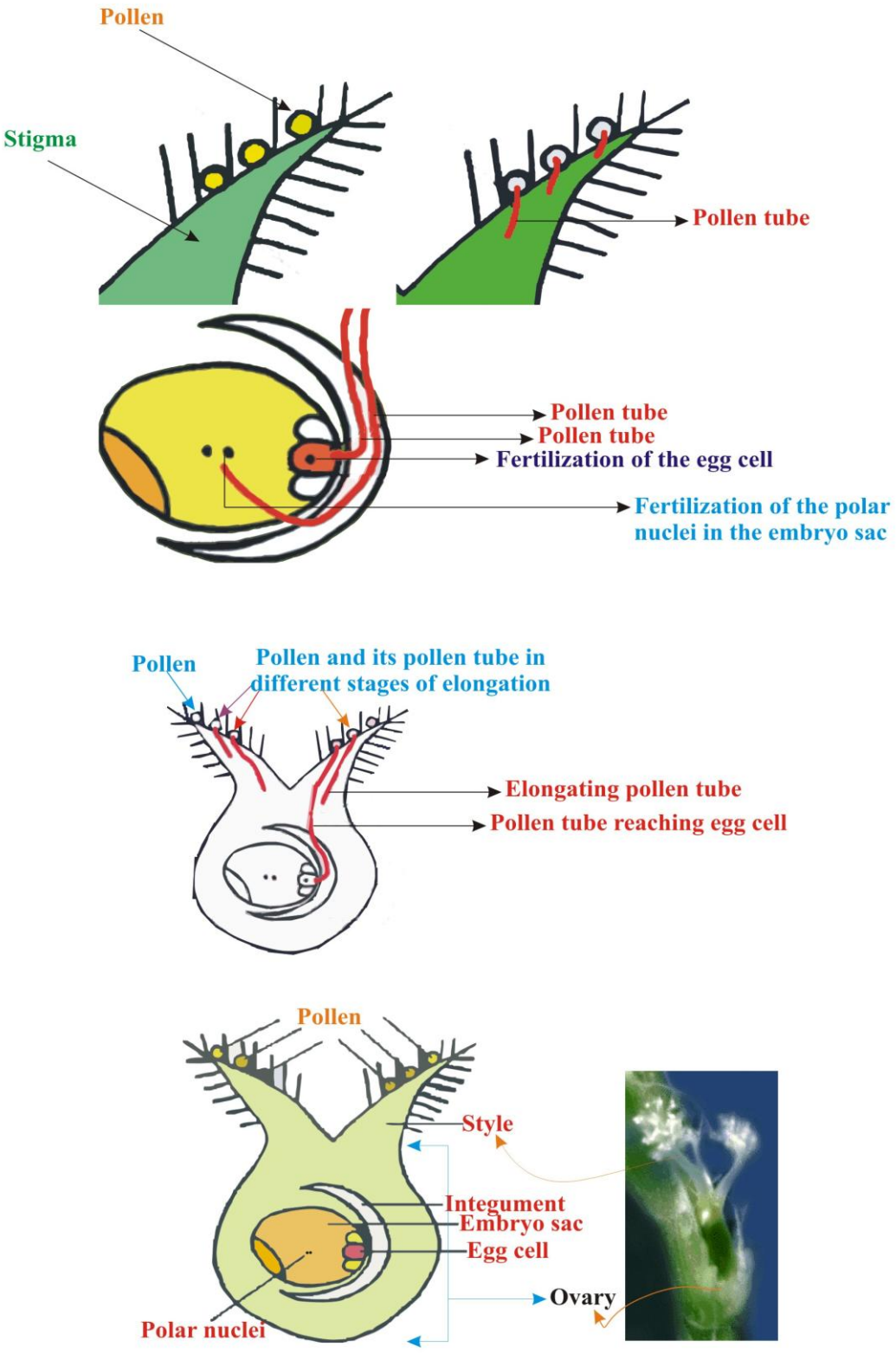


Fig 2.3: Pollination and fertilization in rice

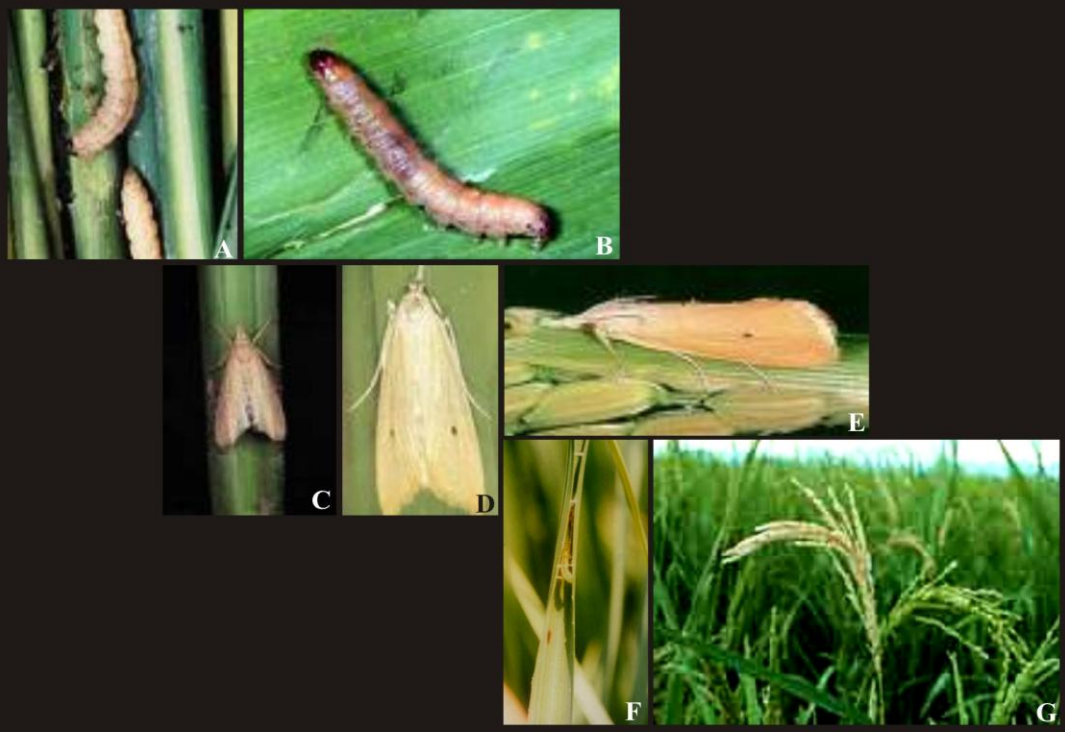


Fig 2.1: Stem borer infestation in rice

A. Striped rice stem borer larva
B. Rice stem borer
C. Adult striped rice stem borer
D. Adult yellow stem borer
E. Adult stem borer
F. Leaf folder (emerging larvae)
G. Dead Heart

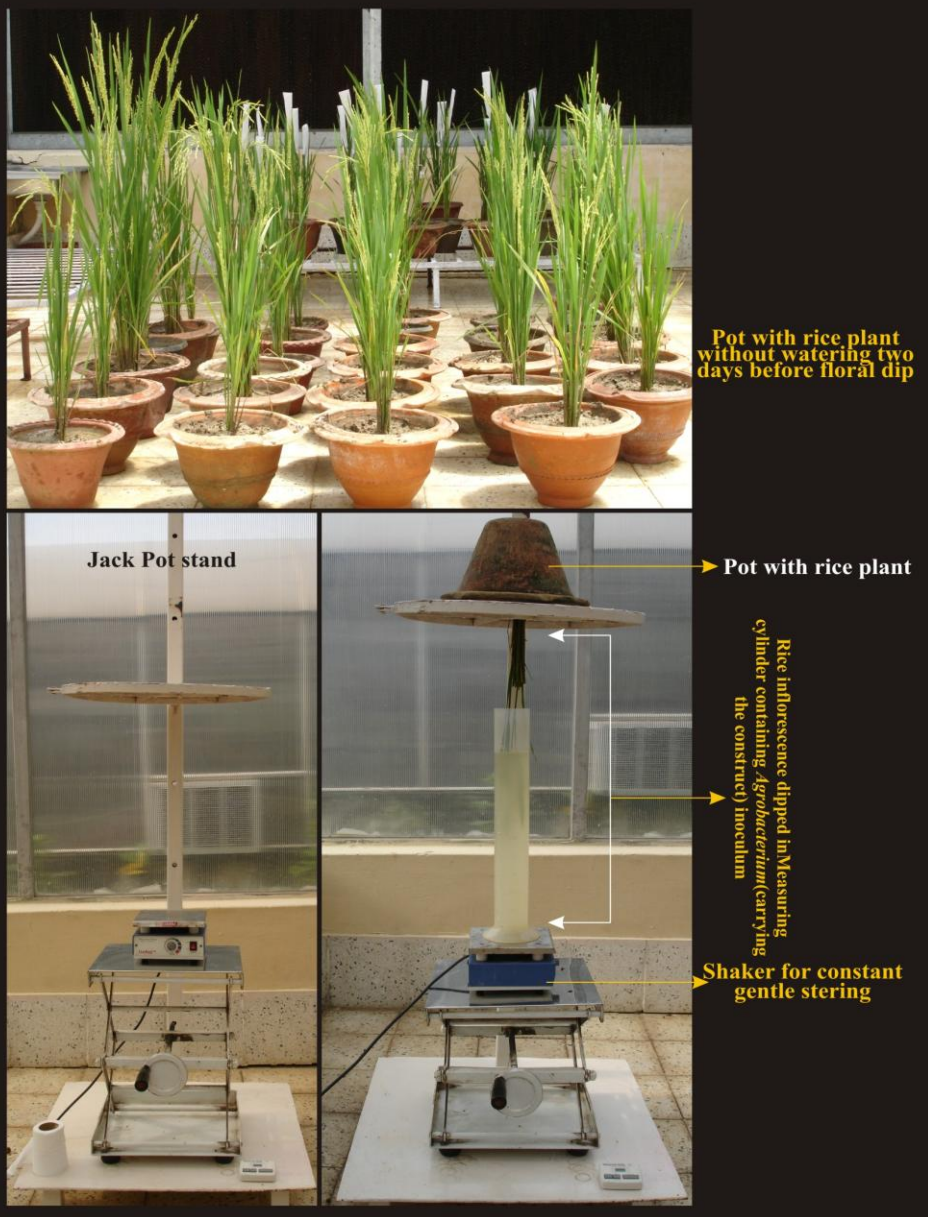


Fig 3.6: Jack pot stand developed for efficient floral dip based *In Planta* transformation system in *indica* rice

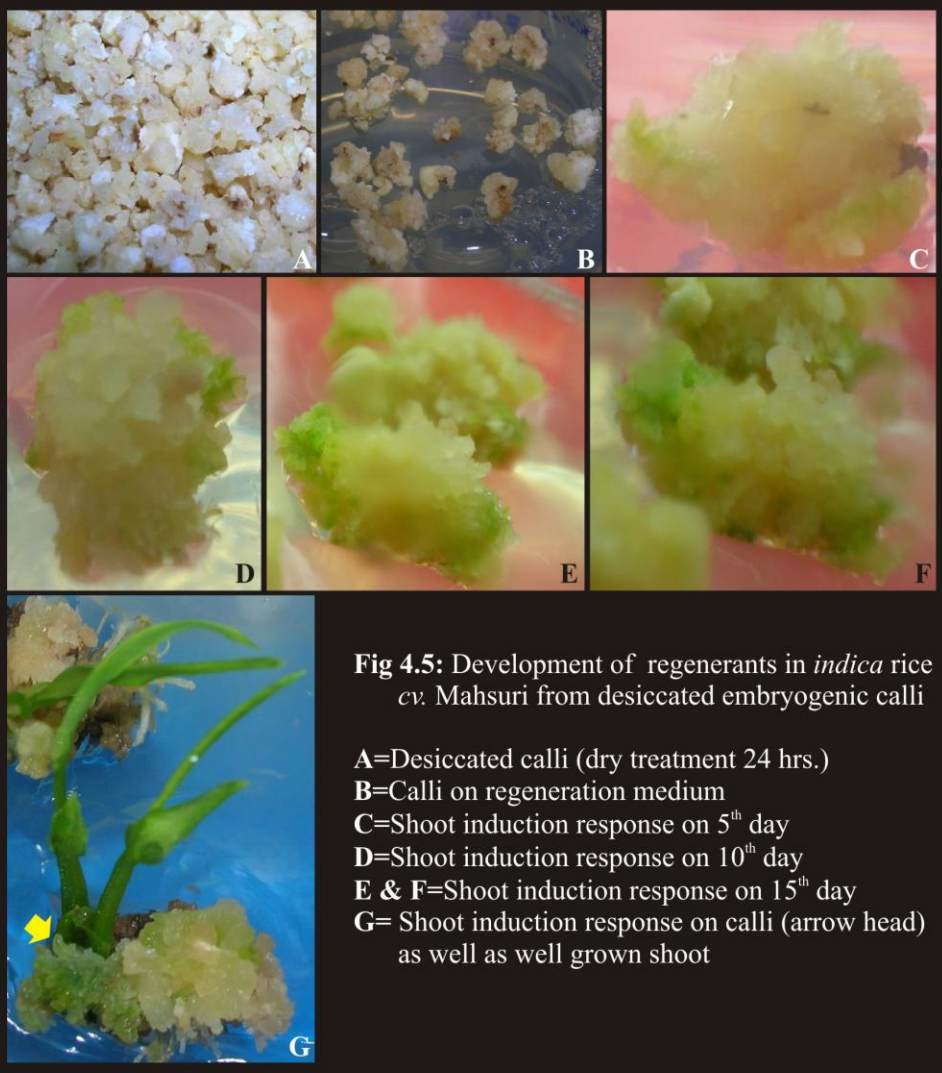


Fig 4.5: Development of regenerants in *indica* rice *cv.* Mahsuri from desiccated embryogenic calli

A=Desiccated calli (dry treatment 24 hrs.)
B=Calli on regeneration medium
C=Shoot induction response on 5th day
D=Shoot induction response on 10th day
E & F=Shoot induction response on 15th day
G= Shoot induction response on calli (arrow head) as well as well grown shoot

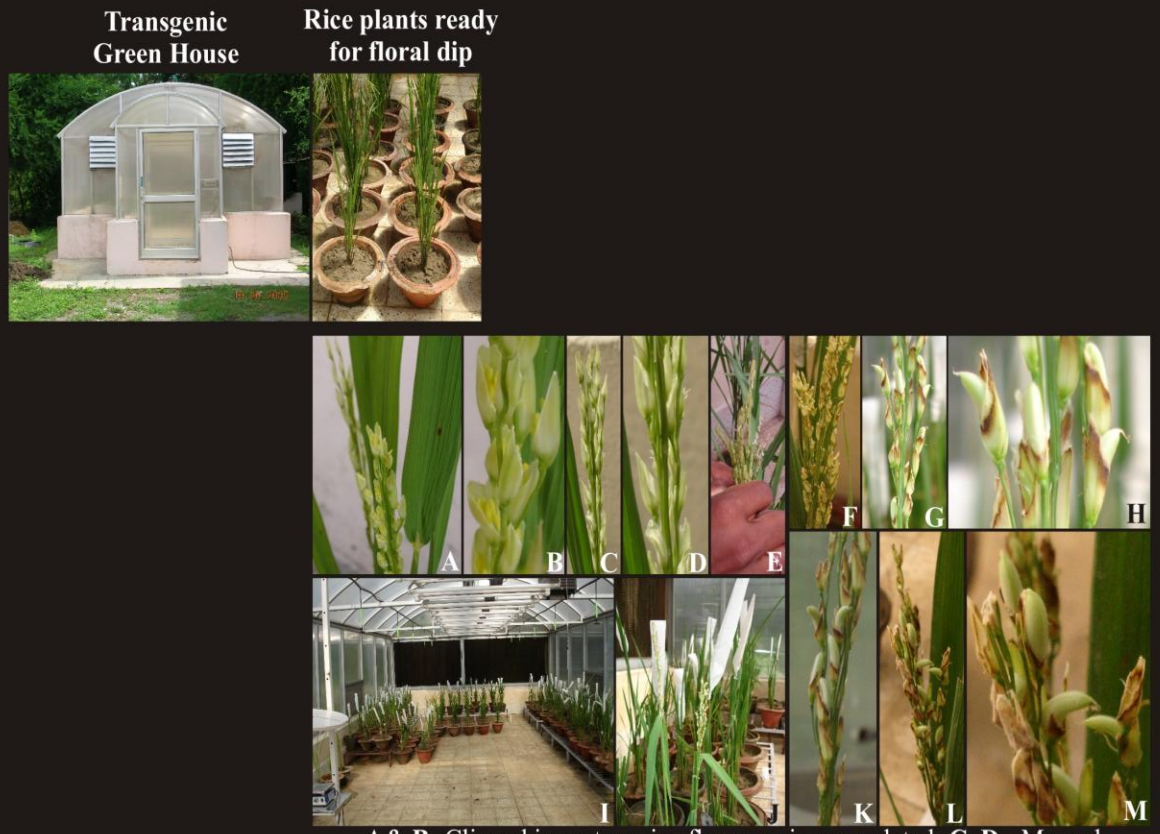


Fig 4.13: Schematic procedure followed for floral dip based i*n planta* transformation in rice *cv*. Swarna

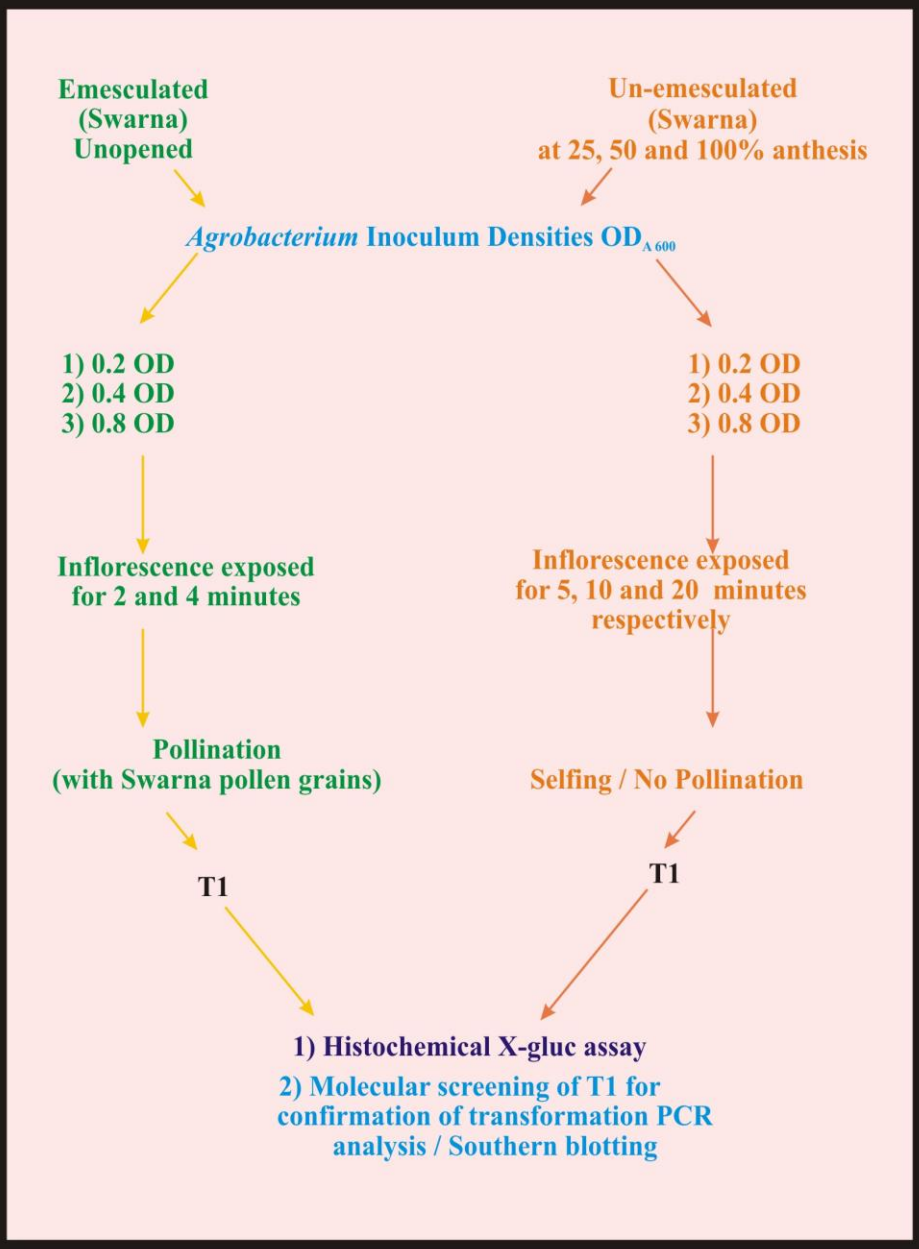


Fig 3.5: Step wise procedure for *Agrobacterium* mediated floral dip transformation in rice

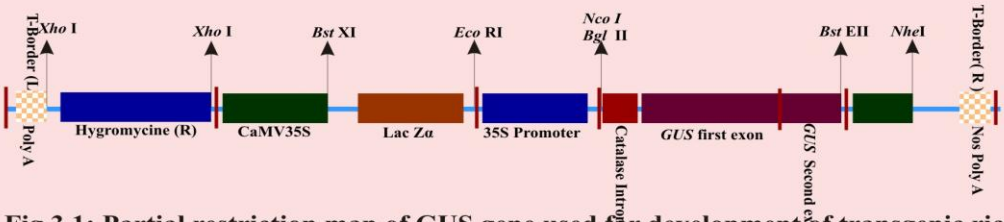


Fig 3.1: Partial restriction map of GUS gene used for development of transgenic rice

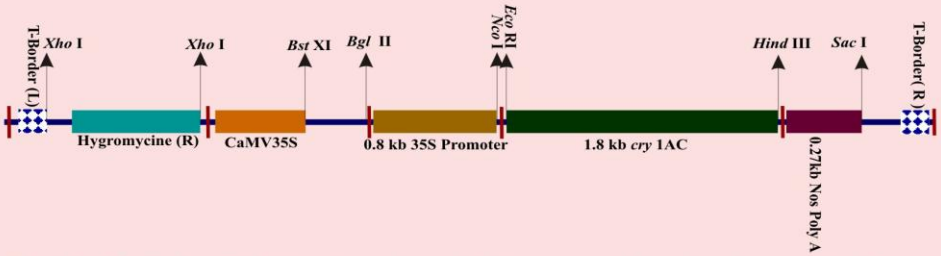


Fig 3.2: Partial restriction map of *cry* IAC gene used for development of transgenic *Bt* rice

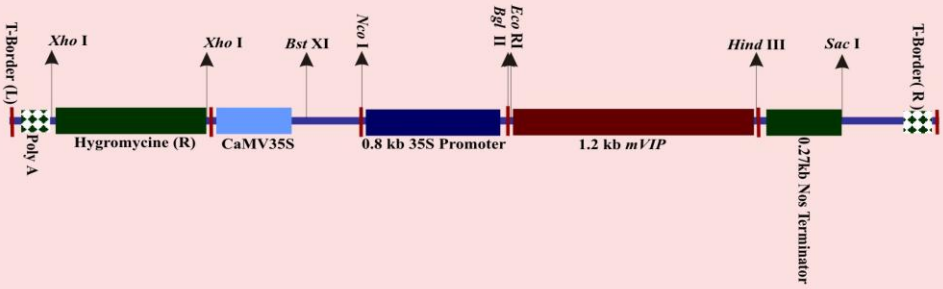


Fig 3.3: Partial restriction map of *mVIP* gene used for development of transgenic *Bt* rice

Fig 4.1: Callus growth response on different culture medium after 15 days of inoculation in rice *cv* Mahsuri and Swarna.

A & E:- Lin and Zang (2005)
B & F:- Murashige and Skoog Modified (MSM)
C & G:- Linsmaier and Skoog Modified (1965)
D & H:- Murashige and Skoog (1962)

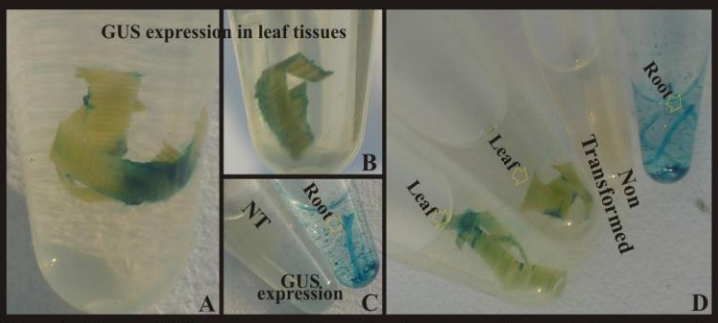


Fig 4.16a: GUS expression in leaf and root tissues in transformants (A to D)

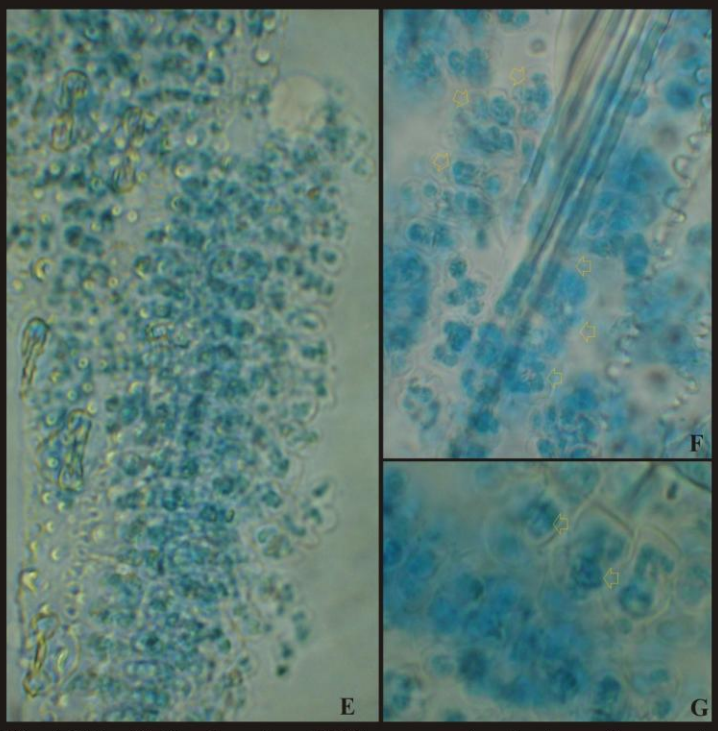


Fig 4.16b: Cells showing GUS expression in transformants (E to G arrow head)

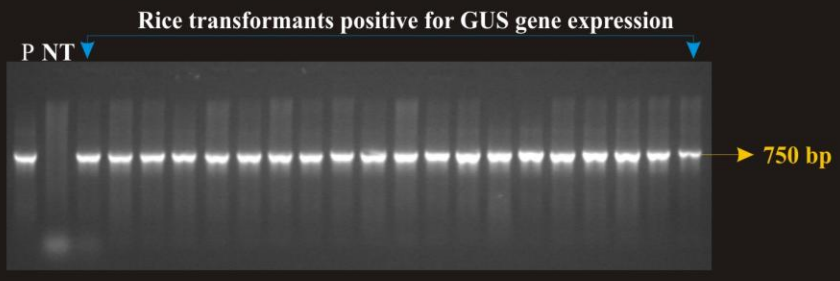


Fig 4.16c: PCR amplicon indicating confirmation of putative transformants (GUS +ve) generated following floral dip based *in planta* system

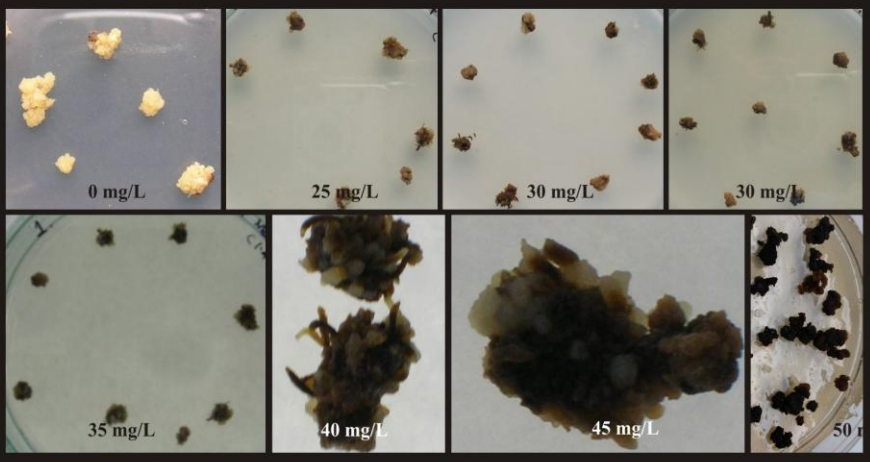


Fig 4.9a: Optimization of Hygromycine concentration for selection of transformed calli derived from rice *cv.* Swarna

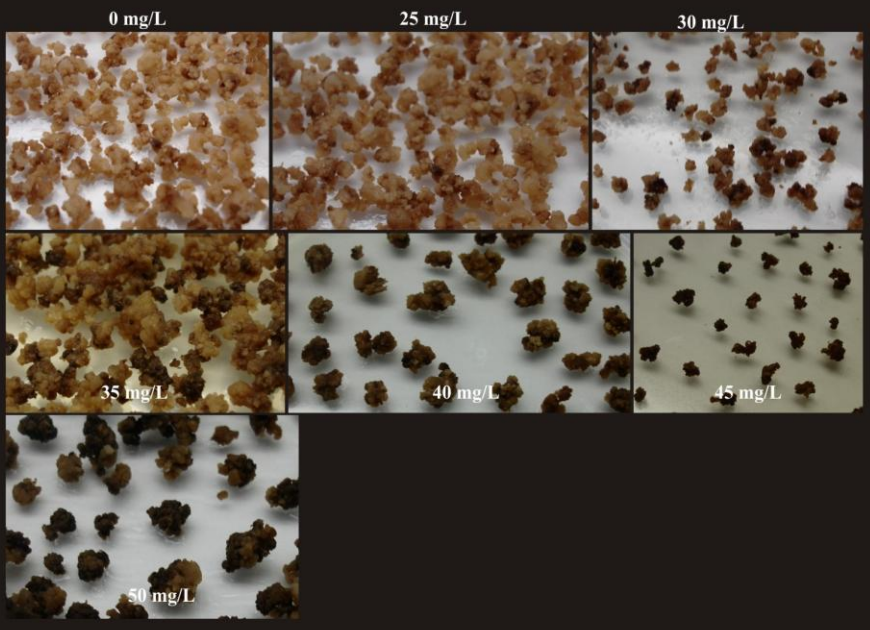


Fig 4.9b: Optimization of Hygromycine concentration for selection of transformed calli derived from rice *cv.*Mahsuri

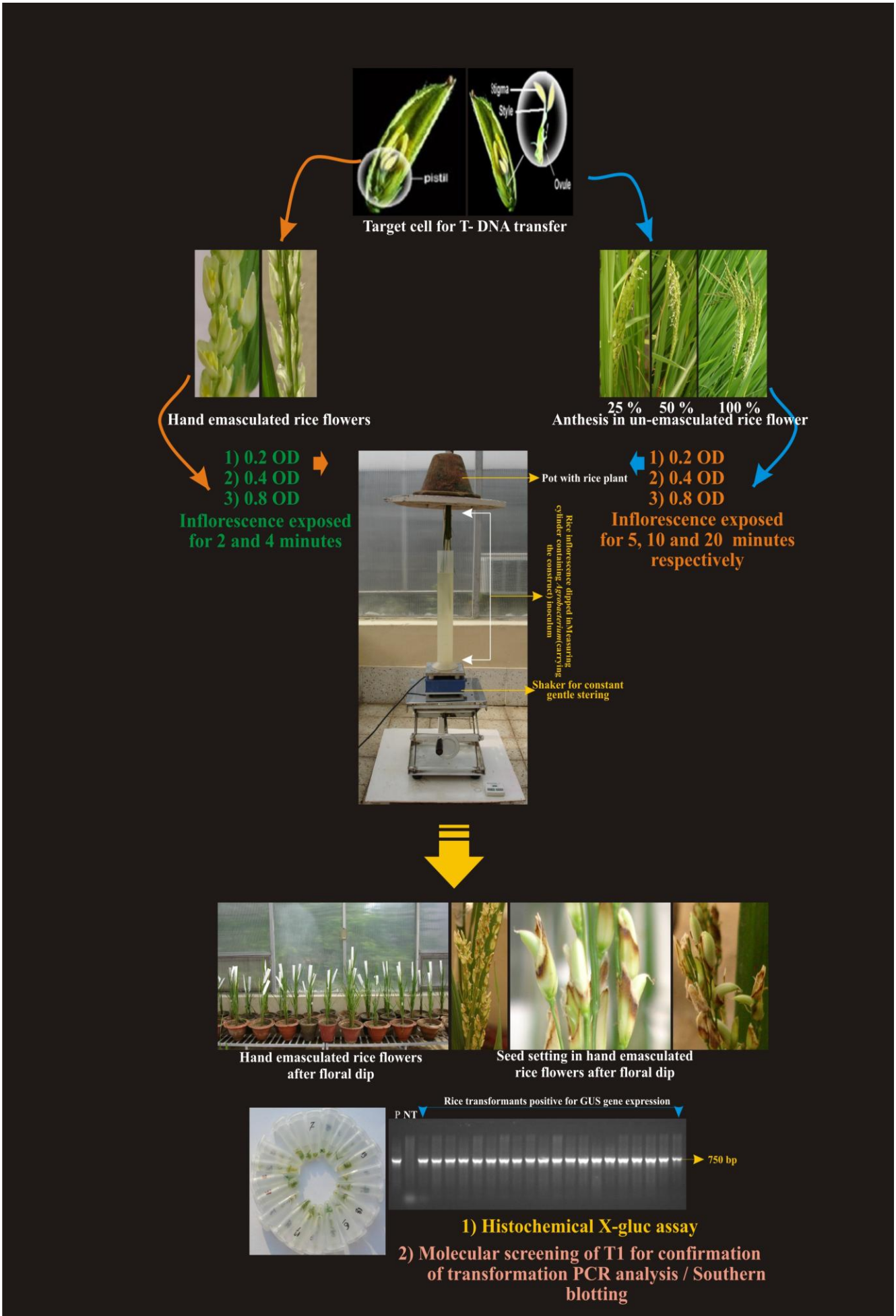


Fig 4.12: Step wise protocol of floral dip based in planta transformation in rice

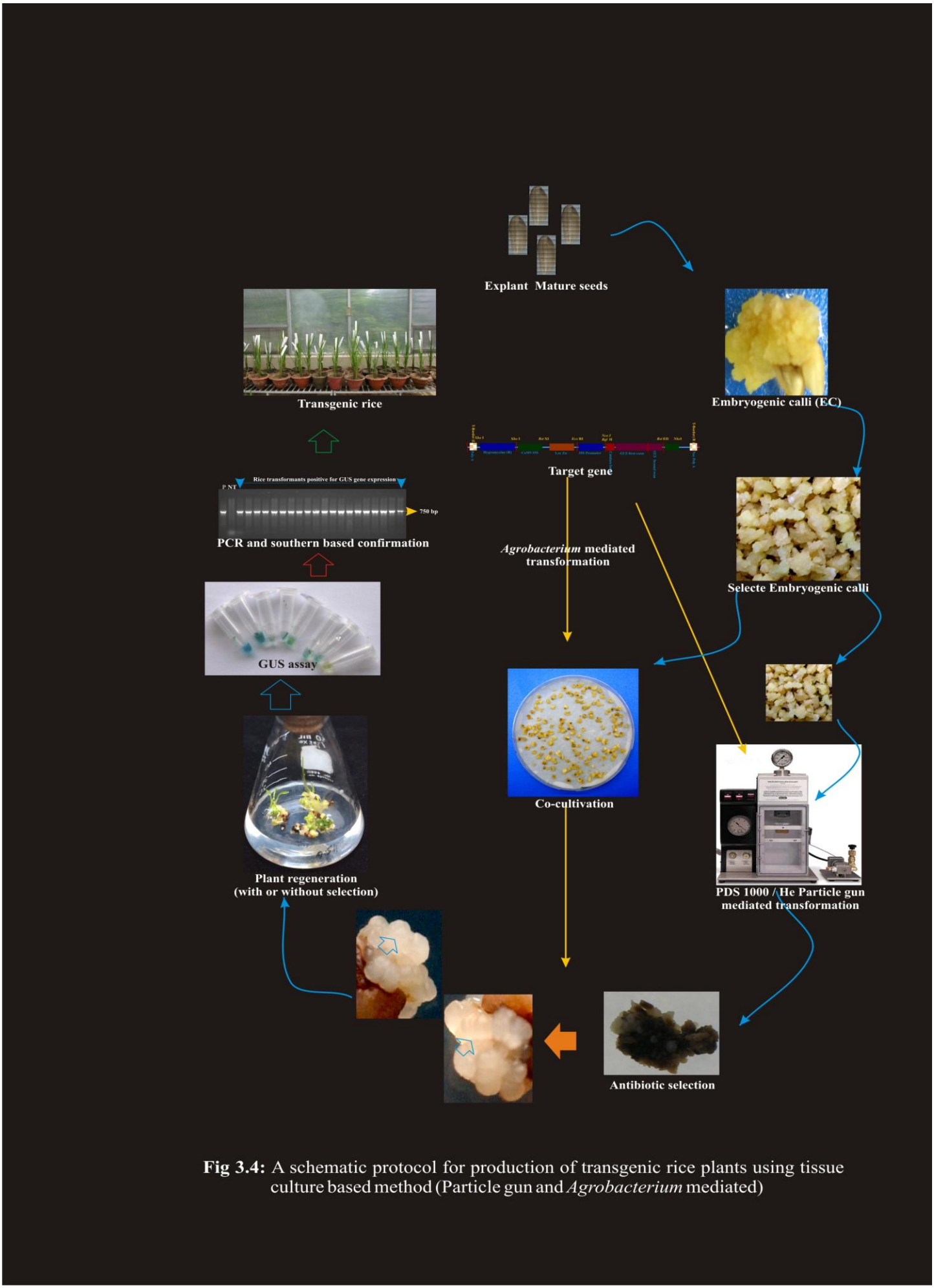


Fig 3.4: A schematic protocol for production of transgenic rice plants using tissue culture based method (Particle gun and *Agrobacterium* mediated)