Towards improving protocols for *in vitro* plant **regeneration and *Agrobacterium-mediated*** transformation of *indica* rice genotypes

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

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CERTIFICATE

This is to certify that Mr. B. Chandra Shekhar has carried out the research work embodied in this thesis entitled "Towards improving protocols for *in vitro* plant regeneration and *Agrobacterium-mediated* transformation of *indica* rice genotypes" under the supervision and guidance of Prof. Arjula Ramachandra Reddy, for the full period prescribed under the Ph.D. ordinance of this University. We recommend submission of his thesis work for the award of the degree of Doctor of Philosophy from this University.

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DECLARATION

I hereby declare that the work embodied in this thesis entitled "Towards improving protocols for *in vitro* **plant** regeneration and *Agrobacterium*-mediated **transformation** of *indica* rice **genotypes"** has been carried out by me under the supervision of Prof. Arjula Ramachandra Reddy, and that this has not been submitted for any degree or diploma of any other University.

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ABBREVIATIONS

µg/L	Microgram per litre
µl	Microlitre
µM	Micro molar
2,4-D	2,4-Dichlorophenoxyacetic acid
BA	N_6-benzyladenine
B_5	B_5 medium (Gamborgs medium)
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
cm	Centimetre
cv	cultivar
d	Day(s)
DNA	Deoxyribo nucleic acid
dNTPs	Deoxyribo nucleotide triphosphates
EDTA	Ethylenediamine tetra acetic acid
GUS	â-glucuronidase
HCl	Hydrochloric acid
h	Hour(s)
kb	Kilo base pair
kinetin	kinetin (6-furfuryl aminopurine)
L	Litre
LB	Luria Broth
M	Molar
mg/L	Milligrams per litre
min	Minutes
ml	Millilitre
mm	Millimetre
mol wt	Molecular weight
mRNA	Messenger ribonucleic acid

MS	Murashige and Skoog medium
MSCI	MS callus induction medium
MSRE	MS regeneration medium
NAA	á-naphthaleneacetic acid
NaOH	Sodium hydroxide
N_6	N_6 medium
NB •	N6 (macro nutrients) + B5 (micro nutrients)
NBCI	NB callus induction medium
NBRE	NB regeneration medium
NBSR	NB selective regeneration medium
ng	Nanogram
°C	Degree centigrade
PCR	Polymerase chain reaction
pH	Negative logarithm of H+ ions
psi	Pounds per square inch
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
sec	Second(s)
SSC •	Sodium chloride and sodium citrate
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
T-DNA	Transfer DNA
TE	Tris-EDTA
Ti plasmid	Tumor inducing plasmid
uidA	aVglucuronidase gene
uv	Ultraviolet light
w/v	Weight per volume

LIST OF CONTENTS

INTRODUCTION

1. INTRODUCTION

Rice is the most cultivated major food crop in the world. It is the staple food for seventeen countries in Asia and the Pacific, eight countries in Africa, seven countries in Latin America and the Caribbean. Rice is cultivated in about 113 countries all over the world under wide eco-climatic conditions ranging from deserts to submerged conditions. The rice varieties are basically grouped under five major classes that are rain-fed lowland, deep water, tidal wetlands, upland and irrigated rice. Of the 23 reported rice sub-species *indica*, *japonica* and *javanica* are cultivated predominantly in the humid tropics of Asia. Certain rice cultivars exist that are tolerant to desert, hot, humid, flooded, dry and cool conditions, and are also grown in saline, alkaline and acidic soils (www.fao.org). Farmers have been domesticating the crop plants for long term sustainable agriculture. The profound evolutionary consequence of such conserved breeding was the creation of plant diversity. The natural selection pressures such as drought, submergence, flooding, soil nutrient deficiency, biotic and abiotic stresses and human selection have contributed to the vast genetic diversity in rice genotypes.

With limited natural resources such as water and arable land, rice production needs to be increased to meet the food demands of an ever increasing population. Drought floods, salinity and various plant diseases have always been the key constraints in rice production which cause tremendous yield losses. In the recent past, introduction of high yielding varieties produced by conventional plant breeding methods have boosted the rice production. Green revolution utilized the genetic

variability resulted from selection and breeding in producing new high yielding varieties that are used throughout the world. One such example was the introduction of "dwarf" genes into rice and wheat, which in conjunction with fertilizer applications. dramatically increased the yield of traditional food crops predominantly in China and India.

In India, since 1965 more than 600 improved *indica* rice varieties were developed and released for cultivation. However expected yields to meet the increasing demand could not be achieved because of the biotic and abiotic stress factors that limit the production and productivity of rice. Globally, an annual loss of 23% rice production is estimated, of which about 16% of the yield loss is due to the abiotic factors. The yield loss due to biotic factors was curtailed to a greater extent by use of pesticides and other chemicals and good management practices. Added to this in developing countries an additional rice loss of about 15 percent is recorded during critical operations such as drying, storage and milling. Most rice produced is milled to reduce cooking time and to increase storage life. However, this process removes a large percentage of nutrients. proteins. fiber. fat. iron and vitamin B there by further decreasing the quality of rice.

According to FAO analysis, there are about 800 million people who are deprived of sufficient food. In developing countries malnutrition is the cause for the death of more than 6 million children under age five. In addition to lack of food. deficiencies in micro-nutrients. especially vitamin A. iodine and iron are widespread. The modern methods of crop improvement have to be utilized for dramatic increase in the food production lo achieve the minimum necessary growth in total production of

global staple crops with the limited land under cultivation. Hence, it is important to increase the yield on land that is intensively cultivated by a way of generating new varieties using the recently developed methods.

Conventional and modern plant breeding methods have made significant contributions to the increase in food production. However, use of large amount of pesticides and fertilizers disturbed the ecological balance and most of these high yielding varieties are not tolerant to biotic and abiotic stresses. Despite past successes. the rate of increase of food production is either stagnant or declining as yield losses are significant due to various biotic and abiotic factors. Furthermore, the erratic or severe alterations in monsoon pattern and adverse climatic conditions resulting from global warming are exacerbating the problems of regional food production. The major challenge aliead is to increase world food production without further depleting the non-renewable natural resources and causing the environmental deterioration.-

The genetic diversity of some crop plants has also decreased and there are species without wild relatives with which to cross breed. There are fewer options available than previously to address current problems through traditional breeding techniques. although it is recognized that these techniques will continue to be important in the future.

. Transgenic crops research aims at modifying a biochemical pathway or adding or removing a character of choice in plants. It offers the possibility of bringing in desirable characteristics from diverse and unrelated species, genera! and beyond. Modification of qualitative and quantitative characteristics. such as the composition and

content of proteins, starch. fats or vitamins by modification of metabolic pathways. has already been achieved in some plant species. Such modifications enhance the nutritional status of the foods and help to improve human health by addressing malnutrition and under-nutrition problems. These nutritional improvements have rarely been achieved previously by traditional methods of plant breeding.

Since 1987, transgenic crops have been approved and grown in the United States since 1987. while at present china is one of the leading countries in the world with more than 2.1 million hectares of land under cultivation of transgenic crops. Besides these. the other major countries include Canada, Brazil and Argentina. Of the transgenic crops approved for commercial use in 2003, 34% were developed for insect resistance, 30% for herbicide tolerance, 16% for quality improvement in crop plants and the rest are for other qualities of improved agronomic traits.

Production and quality standards resulting from transgenic plants include increased flexibility in crop management. decreased dependency on chemical pesticides. to last longer and withstand commercial harvesting. to improve packing shipping and storage practices. less damage to environment and enhanced yields depending on the modifications introduced *in planta.*

Transgenic crop plants were developed to improve yields. taste. increased nutritional value, and to produce edible vaccines. Intense research is in progress on developing resistance to viral, bacterial. and fungal diseases; modification of plant architecture (e.g.. height) and development (e.g., early or late flowering or seed production); tolerance to abiotic stresses (e.g., salinity and drought); production of

industrial chemicals (plant-based renewable resources) and the use of transgenic plant biomass for novel and sustainable sources of fuel.

•... Production of golden rice is an example of pathway engineering in transgenic plants. The specific purpose behind the production of "Golden Rice" was to rescue millions of children around the world suffering from vitamin A deficiency. Vitamin A is essential for the functioning of the immune system and children who are vitamin A deficient are at much greater risk of dying from common childhood illnesses such as measles.

Transgenic plants carrying novel genes are most useful where no equivalent genetic source is available in that species. In particular, GM technology, coupled with important developments in other areas are demonstrated to increase the production of main food staples, improve the efficiency of production, and reduce the environmental deterioration.

Spectacular developments in whole genome sequencing of many organisms, including rice have generated huge data on gene structure, function and regulation. These zetabytes of data has huge potential in improving crop plants for various qualitative and quantitative traits.

One of the direct ways of mobilizing any gene into the target plant is genetic transformation. Parallel developments in genetic transformation protocols for a number of plants have made this route as almost an obligatory for genetic improvement of crop plants via novel gene transformation.

. Various physical, biological and biochemical methods of plant transformation

have been developed over the past decades or so. These methods have demonstrated the transformation of many plant species. Some of the cereals were also transformed with considerable success. However, literature reveals that not all methods of transformation were found efficient in transformation of these monocot species. In particular, rice transformation was found to be rather difficult and laborious since there are genetic variations among *Oryza* subspecies for their response to tissue culture.

Rice was transformed using most of the common methods described so far. However, transformation of rice using *Agrobacterium* is preferred over others because of several underlying advantages. Among the three widely cultivated rice subspecies. routine transformation of *indica* rice is far from complete. This is mainly due to lack of reproducible and well established methods that were reported in the other rice ecotypes and dicotyledonous species.

In the present study experiments were carried out to develop *Agrobacterium*-mediated transformation of *indica* rice that is applicable to different rice genotypes. This study resulted in the development of a simple and precise protocol by optimizing the critical parameters involved in transformation using *Agrobacterium*. The protocol is demonstrated to be effective for all tested rice genotypes. Further. the protocol is simple. requires relatively short time and reproducible.

Using the optimized protocol, transgenic rice plants carrying maize *apx1* gene, encoding cytosolic ascorbate peroxidase, in sense and anti-sense expression were developed. These *apxl* transgenic plants are potentially useful in understanding the role of ascorbate peroxidase in oxidative stress tolerance and in cross tolerance to other

physiological and biochemical stresses in plants. Such studies, particularly antisense *apx1* plants, will also help in elucidating the alternate pathways of oxidative stress tolerance in plants.

The specific objectives are as follows:

- To develop an efficient protocol for producing high frequency embryogenic calli from the scutellar region of mature embryos of *indica* rice genotypes.
- To develop an efficient protocol for *in vitro* plant regeneration from embryogenic callus.
- To optimize *in vitro* conditions *fox Agrobacterium* mediated transformation of *indica* rices using *Agrobacterium* strain EHA105/pCAMBIA1301.
- Apply the above protocol in transforming other *indica* rice genotypes.
- To transform the *indica* rice "Pusa Basmati" with maize cytosolic ascorbate peroxidase gene "*apx1*".
- Molecular analysis of the developed transgenic plants.

LITERATURE REVIEW

2. Literature Review

Plant transformation methods in conjunction with various molecular biology tools are vital for development of new varieties with desired agronomical traits within a very short time. Various physical, biochemical and biological methods of plant transformation were developed over a period of time. Particularly, transformation methods whose applications were restricted for specific explants have been widely reported. The wide applicability of these techniques to plants of different genera was found advantageous and supplement conventional breeding methods which are time consuming, laborious, cumbersome and has genotypic and biological limitations.

Among cereals and mono cots, rice has become the model plant for research because of its relatively small and diploid genome. However, success in rice transformation is largely achieved using *japonica* varieties. Genetic transformation of *indica* rice for improved agronomical values is needed as it accounts for nearly 80% *of* cultivated rice. Transformation of *indica* rice genotypes is still not a routine method as they exhibit high degree of recalcitrance. Recently, progress has been made towards transformation of *indica* rices in a few cases.

The present literature survey covers information on various plant transformation methods having the potential for genetic improvement in rice.

2.1 Progress in rice tissue culture

History of rice tissue culture dates back to mid twentieth century. Ameniya *et al*.,

(1956) were among the first to show *in vitro* culture of immature embryos of rice. The *in vitro* embryo culture methods were mainly developed to rescue interspecific F1 hybrid embryos. Embryos rescue methodologies had a history of wide applications in introgression breeding and gene transfer from wild relatives to cultivated species in many crop plants. Anther culture in rice was extensively investigated and this technique remained indispensable for the production of double haploid lines (Chu 1982). Various factors that affect *in vitro* anther culture were discussed in detail by Raina *et al,* (1989). A major problem associated with anther culture is the occurrence of albinos at a higher frequency during plant regeneration from anther derived calli. Plantlet regeneration directly from pollen embryoids in rice was also reported with lower efficiency (Chu *et al,* 1975). Ovary culture is also reported in rice (Zhou *et al,* 1983) though plant regeneration is at lower frequency as compared with anther culture. albino production was found at lesser frequency (Liu and Zhou 1984). Somatic cell culture in rice was developed primarily for large scale plant regeneration from single or a cluster of cells for propagation of specific genotypes and for studies involving mutation research and somaclonal variations (Raina *et al,* 1989).

Callus induction in rice using nodal segments was first reported by Furuhashi and Yatazawa (1964). Plant regeneration in rice was reported using callus derived from different explants such as embryos. roots. immature endosperm. shoot apex. immature panicles and leaf blades. Protoplast culture and regeneration technologies in rice have been most intensively investigated. Takebe *et al,* (1971) were the first to show successful regeneration of plants from protoplasts in tobacco. there after the technique

has been extended for other plant species owing to its flexibility. Studies using plant protoplasts opened up new avenues in plant research such as production of interspecific or intergeneric somatic cell hybrids, cybrids and asymmetric hybrids. Protoplasts have been the most amenable explants for plant transformation methods such as PEG mediated, heat shock, electroporation and microinjection. In rice isolation of plant protoplasts was reported from roots, callus, leaf blades and leaf sheaths. However, protoplast based methodologies are labor intensive and cumbersome.

2.2 Factors affecting rice tissue culture

Hiei et al., (1994) reported that scutellar callus derived from mature rice seeds are more amenable for *Agrobacterium*-mediated transformation when compared to other explants. Thereafter, all the rice transformation protocols reported have used these callus explants for further improvements in rice transformation. Since mature seed is the source for callus initiation, seed quality plays a pivotal role in rice tissue culture. Adkins *et al,* (1990) showed that an optimal gas exchange in the culture vessel results in better growth and reduced necrosis of rice callus. Rueb *et al.,* (1994) reported that regeneration in rice is dependent on the seed batch used and environmental factors such as time of harvest. drying procedures and storage conditions affect the quality of seed batch which inturn reflects on plant regeneration. Chun *et al,* (1997) showed that rice seeds that were surface sterilized using sodium hypochlorite showed greater seedling growth than those that were sterilized using mercury chloride. Miyoshi and Sato (1997) proved that the frequency of rice seed germination can be increased by treating the

dehusked seeds with ethanol.

2.3 Improving *in vitro* regeneration in rice

ABA and sorbitol were reported to be effective in somatic embryo formation from rice callus (Kobayashi and Okii 1990). Tsukahara and Hirosavva (1992) and Rance *et al,* (1994) reported that partial desiccation of rice callus leads to somatic embryo development and plant regeneration in *indica* and *japonica* rice varieties. Chowdhry *et al.,* (1993) showed that callus induction media when fortified with L-proline and L-tryptophan. the frequency of embryogenic callus formation was increased which inturn **reflected** on pl antl et regeneration. Rueb *et al.,* (1994) found that upon transferring the callus induced on N6 medium to N6 regeneration medium, the frequency of regeneration increased in *japonica* rice cultivar Taipei 309. Plant regeneration in rice is affected by several factors such as genotype, developmental stage of explant. hormonal composition of the medium, carbohydrate source. partial desiccation. and water stress **(Jain** *et al.,* 1996). By increasing the agar concentration and supplementing the media with mannitol, the frequency of plant regeneration can be increased (Jain *et al.,* 1996). Zhuo. *et al..* (1996) observed that replacing 2,4-D with Phenylacetic acid did not affect the frequency of callus induction. however it significantly improved the shoot differentiation from callus in *indica* rice. The pronounced recalcitrance can be minimized with changes in media composition for the culture of *indica* rice varieties (Sivamani *et al.,* 1996) which until recently, proved far less responsive than *japonica* rice (Faruque *et al..* 1998; Blackhall *et al,* 1999). Sahasrabudhe *et al,* (1999) showed

that increased concentration of boric acid promotes somatic embryogenesis in *indica* rice. Tsugava and suzuki (2000) reported that presence of aspartic acid, glutamine, and casamino acids or casein hydrolysate were effective in promoting regeneration. They also demonstrated that protoplasts isolated from cryopreserved callus preserved at 5°C for 5 months regenerated plants at a frequency higher than from non-preserved callus.

2.4 Genetics of rice tissue culture

Peng and Hodges (1989) and Abe and Futsuhara (1991). previously demonstrated that the response of rice cultivars to *in vitro* tissue culture is under a complex genetic control and that separate groups of genes are involved in the control of callus; induction, callus growth and plant regeneration. Using *FS* lines derived from two *japonica* rice cultivars. a Quantitative trait loci (QTL) controlling the regeneration ability of rice seed callus was detected using RFLP markers (Shiobara *et al.,* 1997). Takeuchi *et al,* (2000) in their study on F2 population derived from a cross between an *indica* and *japonica* rice, analyzed 103 RFLP markers and mapped a major QTL on rice chromosome 2 and a minor QTL on chromosome 4, for shoot regeneration from mature seed derived callus. Giraux and Pauls (1997) identified three alfalfa somatic embryogenesis-specific transcripts in the RNA samples from embryogenic tissues of the embryogenic genotype. Similarly, Dong and Dunstan (1999) characterized embryogenesis associated cDNAs from *Picea glauca.* However. such studies in rice are not reported.

2.5 Gene transfer methods

2.5.1 *Agrobacterium*-mediatedgene transfer

Agrobacterium, a gram negative soil bacterium causing crown gall tumors in dicotyledonous plants. was first discovered by Smith and Townsend (1907). To date. the *Agrobacterium*-mediated transformation is the most widely used plant transformation method. Its popularity can be attributed to the ease at which it can be used for transgene integration stably into plant chromosomes. Though monocots are not **the** natural hosts of *Agrobacterium* its host range is broadened with the use of v/r gene inducers such as acetosyringone, sinapinic acid, and potato suspension culture. Presently this method is the most preferred one as it results in stable integration of the transgene at a very low copy number where only a defined segment of plasmid DNA will be incorporated into the host genome. The major advantage is that the gene of interest will be integrated into the nucleus that results in stable transformation events. However, the only limitation in this technique appears to be the host range which is narrowed by using chemical inducer acetosyringone (Rashid *et al.,* 1996) and alkylsyringamides (Dye *et al,* 1997).

Raineri *et al,* (1990) showed stable GUS expression in transgenic calli. In their approach, mature seeds were cocultivated and the transformed seeds were kept on selection medium for callus induction. However, they could not show regeneration from the transformed calli. Chan *et al,* (1992) demonstrated *Agrobacterium*-mediated transformation of *indica* rice using different seedling explants such as stem. leaf and roots. They observed that only roots survived and produced callus. However. their

protocol did not show any regeneration of transgenic plants. The first successful *Agrobacterium*-mediated rice transformation was reported by Hiei *et al.*. (1994) using mature embryo derived callus. Vijayachandra *et al.*. (1995) analyzed different explains of rice for their ability to induce *Agrobacterium vir* genes and reported that rice scutellum from 4 d old seedlings carry high potential. Park *et al,,* (1996) transformed *japonica* rice cv Maybelle following *Agrobacterium* inoculation of isolated shoot apices. Among the key factors they analyzed, extra wounding treatment was found to increase the efficiency of transgenic rice production. They also reported that extra wounding treatment may favor the penetration of bacteria into rice shoot apex. However only 20 out of 721 transformed shoot apices survived on selection medium. Dong *et al,,* (1996) first reported *Agrobacterium*-mediated transformation of *javanica* rice. However, the time required to regenerate transgenic plants using this protocol was about 6 months. Rashid *et al,,* (1996) identified that, among the various factors that affect the transformation efficiency, establishment of suitable culture conditions for cocultivation and selection are vital. Aldemita and Hodges (1996) showed that immature embryos are the good source *for Agrobacterium*-mediated transformation of *indica* and *japonica* rice varieties. A routine system *for Agr obacterium*-mediated transformation *of japonica* rice cultivars was developed by Lee *et al.,* (2002). However, such a consistent protocol for transformation of many *indica* rice genotypes is lacking.

2.5.1.1 Factors affecting *Agrobacterium* mediated transformation

All the plant transformation methods developed till date require tissues with high regeneration potential as target explants (Binns 1990). hence choice of explant plays a vital role in plant transformation. Ou-Lee *et al,* (1986) used protoplast explants and demonstrated transient expression of CAT gene following electroporation. Raineri *et al,* (1990) used mature embryos for cocultivation and produced transgenic calli upon subsequent transfer to selection medium. Cao *et al..* (1992) transformed the rice suspension culture cells derived from a *japonica* cultivar following particle bombardment. Chan *et al.,* (1992) analyzed different explants suitable for rice transformation using *Agrobacterium.* They reported that among root. stem and leaf explants. only root explants showed proliferation of transformed calli. Chaudhury *et al.,* (1995) evaluated various explants such as leaves, coleoptiles and roofs for electroporation mediated gene transfer and shoot explants were reported to exhibit about 2-6 fold increase in GUS activity when compared with other explants. Vijaychandra *et al,* (1995) reported that scutellum from 4 d old rice seedlings have the ability to induce *Agrobacterium vir* genes. Aldemita and Hodges (1996) demonstrated that callus derived from immature embryos is more amenable for *Agrobacterium*-mediated transformation. Zhang *et al,* (1996) reported use of embryogenic suspension cell cultures for microprojectile transformation of *indica* rice varieties. Park *et al,* (1996) used shoot apices *for Agrobacterium*-mediatedtransformation *of ajaponica* rice cultivar. Hiei *et al,* (1994) and Rashid *et al,* (1996) showed that scutellar callus derived from mature rice seeds are the ideal explants for *Agrobacterium*-mediated rice

transformation and hence the subsequent protocols developed have used scutellar callus as the target explain for rice transformation.

Li *et al,* (1992) studied various factors influencing transient GUS expression following *Agrobacterium* infection and reported that shoot explants derived from 4 d old rice seedlings produced higher levels of GUS expression than root and seed remnants. All the 21 rice lines they tested exhibited GUS expression when cocultivated for 4 - 6 d. Liu *et al.,* (1992) proved that preinduction of seedling explants with acetosyringone prior to cocultivation for 4 d improved the transformation efficiency in the tested *indica* rice cultivars IR64 and Lemont. Among the three strains of *Agrobacterium,* agropine and nopaline strains are supervirulent and were reported to be more efficient in plant transformation than octopine strains (Hood *et al,* 1993). Narasimhulu *et al,* (1996) showed that a minimum of 2 h of cocultivation is required to detect the transgene expression in tobacco. but for rice. longer cocultivation time of 3 d is needed for efficient transformation (Hiei *et al,* 1994). Chan *et al,* (1993) and Hiei *et al,* (1994) reported that a key factor for *Agrobacterium*-mediated rice transformation is the use of potato suspension culture rich in acetosyringone and sinapinic acid. However, Rashid *et al,* (1996) showed that acetosyringone is alone is sufficient for transformation of rice scutellar callus. New inducers of *vir* genes were also reported by Dye *et al.* (1997).

Apart from the above, callus cocultivation in acidic pH (Morbe *et al,* 1989; Turk *et al,* 1991) and at a temperature of below 28 °C (Morbe *et al,* 1998) was also reported to affect the efficiency of rice transformation.

2.5.1.2 Progress in *Agrobacterium*-mediated *indica* rice transformation

Chan *et al,* (1992) were among the first to show the feasibility of *indica* rice transformation using *Agrobacterium.* In their study, different seedling explains *of* an *indica* rice (cv Taichug Native 1) were evaluated. However they could not regenerate transgenic plants. Rashid *et al.,* (1996) were the first to report successful production of transgenic plants from an elite *indica* rice cultivar Pusa basmati. Since then this cultivar has become the model *indica* rice variety for the investigators working to improve *Agrobacterium*-mediated *indica* rice transformation. In the same year Aldemita and Hodges (1996) also reported the successful production of *indica* rice transgenics. Khanna and Raina (1999) showed transformation of two *indica* rice cultivars IR64 and Karnal local. They reported that preinduction of *Agrobacterium* is needed for efficient transformation of rice calli. Mohanty *et al,* (1999) reported high frequency transgenic plant production from *indica* rice cultivar Pusa basmati 1. They reported that preinduction of explants prior to cocultivation, is needed to increase the frequency *of* rice transformation. Kumria *et al,* (2001) developed an efficient protocol for regeneration of transgenic plants from transformed calli at high frequency in the *indica* rice cultivar Pusa basmati. Very few *indica* genotypes were reported to be transformed using *Agrobacterium* and a reproducible protocol for *Agrobacterium*-mediated transformation of *japonica* is reported (Lee *et al,* 2002). such efficient protocols for *Agrobacterium*-mediated *indica* rice transformation have to be developed and this can be achieved by establishing the ideal conditions needed for transformation.

2.5.1.3 Vectors for *Agrobacterium*-mediated gene transfer

Plant genetic transformation vectors are developed with essential features such as unique multiple cloning site to facilitate cloning of the gene of interest, a bacterial origin **of** replication for plasmid maintenance in bacteria. a selectable marker gene for selection and proliferation of transformed cells from a mixed population comprising both transformed and untransformed cells and a reporter gene whose expression will be an indirect proof for the presence of transgene. For transformation using *Agrobacterium,* two types of vectors, binary vectors (Hoekacma *et al,* 1983) and cointegrate vectors (Fraley *et al,* 1985) were used. Disarmed and modified forms of these vectors were developed for routine use in plant transformations. BIBAC vectors (Hamilton 1997), TAC vectors (Liu *et al.* 2000), pGREEN vectors (Hellens *et al.*, 2000). pCAMBIA vectors (www.cambia.org) are to mention a few and more recently super binary vectors using super promoters of *Agrobacterial* origin were reported, these vectors showed a 156fold increase in gene expression in transgenic plants when compared with the constitutive 35S promoter (Ni *et al.*, 1995).

2.5.2 Microprojectile-mediated gene **transfer**

Microprojectile bombardment is commonly referred to as the biolistic or the particle gene gun method. The technique involves plant tissues or cells being bombarded with gold or tungsten particles coated with plasmid DNA containing the genes to be transferred. The DNA coated on the particles is incorporated into the host genome through a recombination and repair process. The plant tissue treated is then

subjected to a selection process where, only the transformed tissues proliferate and plants are regenerated. This method was first described by Sanford *et al,* (1987).

Since the method involves physical bombardment of high velocity DNA coated gold or tungsten particle onto the target tissue. this method can be applied to all the regenerable cells or explants. The versatility of the technique is proven with its efficiency in transformation of different crop plants such as Soybean (Christou *et al,* 1990), Cotton (McCabe and Martinell 1991), Poplar (McCrown *et al,* 1991). Spruce (Ellis *et al,* 1991), Papaya (Fitch *et al,* 1990), Canberry (Serres *et al,* 1990). Maize (Fromm *et al,* 1990), Sugarcane (Bower and Birch 1992). Wheat (Vasil *et al,* 1991) and Sorghum (Hagio *et al,* 1991). Rice transformation using particle bombardment was first reported by Christou *et al,* (1991) using immature embryos of *indica* and *japonica* cultivars. The transformed immature embryos were subjected for callus induction in presence of hygromycin selection and the transgenic plants were regenerated from the proliferated embryogenic calli. Thereafter. numerous rice transformation protocols were reported with considerable modifications. Zhang *et al,* (1996) developed a simple, efficient, repeatable. genotype and environment independent transformation of *indica* rice through particle bombardment. The limiting factor for group 1 *indica* rice transformation is apparently related to genotype and culture dependent components. Rooting in the absence of hygromycin may be important to retain fertility of the T_0 plants. Sivamani *et al,* (1996) generated a homogenous population of embryogenic subcultured calli by selectively propagating a small number of regeneration proficient calli derived from mature seeds. Fauquet *et al,* (1995) reported a routine and efficient

protocol for biolistic transformation of *indica* and *japonica* varieties.

The major limitation with this method is the insertion of multiple copies of transgene. The transgenic plants may also exhibit maternal inheritance if the gene is directed to cell organelles such as mitochondria or chloroplasts.

2.5.3 Protoplast-mediated transformation

Polyethylene Glycol (PEG) mediate protoplast transformation involves the removal of the cell wall by using cell-wall-degrading enzymes and then treating the exposed cells with a mixture of polyethylene glycol (PEG) and transforming DNA. The exogenous DNA molecules are passively absorbed by plant protoplasts. Krens *et al,* (1982) and Draper *et al,* (1982) were the first to report PEG mediated DNA uptake into tobacco and petunia protoplasts. Fujimura *et al,* (1985) showed successful regeneration of plants from rice protoplasts. They used suspension cell cultures generated from immature and mature embryos of *japonica* cultivars. Yamada *et al.,* (1986) evaluated the regeneration potential of protoplasts derived from 26 *japonica* rice cultivars. Toriyama *et al.,* (1986) reported that both haploid and diploid plants can be regenerated from anther derived calli from *japonica* cultivars. The initial reports on regeneration from protoplasts in *indica* cultivars were from Kyozuka *et al.,* (1988) who showed regeneration in 4 out of 14 cultivars tested. Three different methods of protoplast transformation, PEG mediated transformation (Zhang and Wu 1988), Electroporation (Toriyama *et al,* 1988) and Heat shock (Thompson *et al,* 1987) were widely used. Datta *et al,* (1990) produced transgenic plants from the protoplasts of an *indica* rice cv

Chinsurah Boroll. however some of the plants regenerated were found to be sterile. Later. Datta *et al,* (1992) reported an efficient fertile plant regeneration method from protoplasts of the *indica* rice IR72. A major limitation is that the technique is very time consuming and the efficiency of the protoplasts regeneration is relatively low. However. this method appears to be an alternate choice for plant transformation besides callus transformation either by biolistics or by *Agrobacterium*-mediated.

2.5.4 Gene transfer by electroporation

Transformation by eleetroporation was designed as an alternative to PEG transformation method. After the cell wall of the tissue is removed, the cell is exposed to an electric field that polarizes the membrane components and causes it to develop a potential difference across it. When the voltage exceeds the threshold level. the membrane breaks down in localized areas and the cells become permeable to exogenous DNA molecules. The simplicity and reproducibility in this technique proved advantageous, however the limitations with this method was that the transformed cells were produced at lesser frequency than that of PEG.

2.5.5 Transformation using silicon carbide whiskers

Kaeppler *et al,* (1990) were the first to report transformation using silicon carbide fibres in maize suspension cells. Silicon carbide fibres play a key role in cell wall perforations through which DNA enters with the turbulence of vortexing. The method has successfully generated stable transgenic plants. However its applications are

limited to certain tissue types and plants.

2.5.6 Pollen-mediated gene transfer

Pollen-mediated transformation was first reported by Ohta (1986) in maize. The plasmid DNA was applied to flowers shortly after pollination and this DNA is thought to be transported in association with the germinating pollen tube to the newly fertilized egg cell. This technique showed very little progress and needs to be well established.

2.5.7 Gene transfer by imbibition

Hess (1969) was among the first to demonstrate gene transfer by imbibition in petunia. The uptake of exogenous DNA occurs during the imbibition of dehydrated plant tissue. Transformation of rice through imbibition was reported by Yoo and Jung (1995). These methods although are simple failed to produce stable heritable transgenic plants.

2.5.8 Gene transfer by macroinjection

The first successful report using this technique was reported by De la Pena *et al.*, (1987) for rye transformation, where DNA is directly injected into developing floral tillers. This method however failed to produce stable transgenic plants.

2.5.9 Ultrasound induced DNA uptake (Sonication)

Joersbo and Brunstedt (1990) were the first to demonstrate sonicated assisted

plant transformation into sugar beet and tobacco protoplasts. The method was also employed for transformation of rice tissues by Trick and Finer (1997). Plant tissues were subjected to ultrasound exposure that generates micro perforations on tissue **surfaces enabling** passive uptake of exogenous DNA. Though the method is simple and **used** considerable for different plant species, its application is limited by factors such **as** cell damage and high temperature.

2.5.10 Laser mediated gene transfer

Weber *et al,* (1988) were the first to **report** the use of laser micro beams for plant transformation in *Brassica napus*. The significant limitation of the technique is the high cost of equipment and **low** transformation efficiency.

2.5.11 Gene transfer by microinjection

The method was first described for transformation of animal cells by Jaenisch and Mintz (1974). Crossway *et al,* (1986) were the first to show tobacco protoplast transformation by microinjection.

2.6 Transgenic rice with improved agronomical traits

Following *Agrobacterium*-mediated rice transformation, Agarwal *et al,* (2003) produced transgenic *indica* rice Pusa basmati over expressing the *Arabidopsis* hsp101 gene. These transgenic plants showed better growth in the recovery phase following high temperature stress of 45 -50 °C. Transgenic *indica* rice with high tolerance to salt

stress were generated by Mohanty *et al,* (2002). They introduced *codA* gene from *Arthrobacter globiformis* into rice through *Agrobacterium.* The transgenic plants showed tolerance upto 0.15M NaCl stress. Garg *et al.* (2002) produced *Agrobacterium*-mediated *indica* rice transgenics carrying *otsA* and *otsB* genes isolated from *E.coli.* which mediate trehalose biosynthesis in rice. Expression of *otsA* gene under stress responsive ABA inducible promoter and *otsB* gene under rice rbcS promoter led to 3-10 fold accumulation of trehalose in transgenic rice. The transgenic plants developed showed increased tolerance to abiotic stresses such as salt tolerance and drought tolerance. These plants were also reported to increased photosynthetic activity under non-stress conditions. Rohila *et al,* (2002) produced *indica* rice transgenics following *Agrobacterium*-mediated approach. These transgenic plants expressing *Hval* gene under stress inducible promoters showed increased tolerance to salt and drought. Datta *et al,* (2001) produce several *indica* rice transgenics through biolistics and PEG mediated protoplast transformation. The constitutive expression of infection specific rice chitinase gene RC7 in *indica* rice transgenics were reported to show enhanced resistance to sheath blight caused by fungal pathogen. Pathway engineering in rice endosperm for a-carotene biosynthesis is a major advancement in plant biotechnology reported by Ye *et al,* (2000). The golden rice was produced using *Agrobacterium*-mediated transformation. where three genes "*psy*", "*crtI*" and "*lcy*" were introduced by cotransformation, two of these genes *psy* and *lcy* were isolated from Daffodil *(Narcissus pseudonarcissus)* and *crtI* was isolated from *Erwinia uredovora.* UNICEF estimated that this transgenic rice with increased levels of provitamin A (a-carotene) could

prevent about 2 million annual deaths among children. Goto *et al.* (1999) reported production of transgenic rice plants with increased iron content. These transgenic plants produced by *Agrobacterium*-mediated transformation showed upto three fold increase in iron content in rice endosperm when the ferritin gene from soybean was expressed under the rice seed specific glutelin promoter. Similar results were demonstrated by Vasconcelos *et al.* (2003) where increased iron and zinc accumulation is reported. Wang *et al,* (1996) produce transgenic rice plants expressing *Xa-21* gene. The transgenic plants produced showed resistance to bacterial blight resulting from 29 diverse isolates of the pathogen *Xanthomonas oryzae pv. oryzae.* Fujimolo *et al,* (1993) were the first to produce transgenic rice expressing *Bt* genes. The transgenic rice plants expressing the truncated *cryIIA(b)* gene showed resistance to lepidopteran rice pests such as leaf folder and stem borer where upto 50% mortality rate was observed in insect larvae. The transgenics produced by these methods have been very successfully used in molecular genetic analysis and appear to be promising agronomically.

2.7 Marker genes for plant transformation

2.7.1 Selectable marker genes

Genetic transformation of plants requires markers which play a vital role to ascertain and confirm the presence of the transgene introduced into the plant cells through various methods described above. Selection of few transformed cells from the untransformed cells constitutes the key step in gene transfer methods that allows proliferation and regeneration of transformed cells or tissues that are unequivocally

distinguished with untransformed tissues (Bowen 1993). These marker genes in plant transformation studies are often expressed under the control of a constitutive promoter (Birch 1997).

The most commonly used selectable marker genes for rice transformation include those that confer resistance to amino-glycoside antibiotic hygromycin B (van den Elzen *et al,* 1985; Waldron *et al,* 1985). The *hpt* (hygromycin phosphotransferase) gene was isolated from *E.coli* and was considerably modified for expression in plants.

Genes coding for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). a critical enzyme for aromatic amino acid biosynthesis. and phosphinothricin acetyl transferase (PAT) provide tolerance to glyphosate and glufosinate ammonium herbicides. respectively. The enolpyruvylshikimate-phosphate synthase (CP4) gene was isolated from *Agrobacterium* strain CP4. The enzyme glyphosate oxidoreductase (GOX) also provides tolerance to glyphosate by degrading glyphosate into aminomethyl phosphoric acid. Methotrexate has also been evaluated as a selective agent for rice transformation. It binds to the catalytic site of dihydrofolate reductase and causes cell death due to thiamine deficiency. Meijer *et al.,* (1991) concluded that Methotrexate is a poor selective agent when compared with hygromycin selection.

Mannose was recently reported as a novel selectable agent and the enzyme phosphomannose isomerase "*pmi*" as a suitable selectable marker gene (Joersbo and Okkels 1996). In contrast to herbicides or antibiotics. mannose has no direct adverse effects on plants. Genetically transformed cells carrying *pmi* gene were able to utilize mannose as carbon source and acquire a growth advantage on mannose-containing

media, it is therefore called as positive selection (Joersbo and Okkels 1996), Successful genetic transformation with *pmi* as selectable marker was reported in sugar beet (Joresbo *et al.* 1998) maize (Negrotto *et al.*. 2000) and rice (Lucca *et al.*, 2001).

2.7.2 Scorable marker genes

These genes are also called as reporter genes or screenable marker genes. The most commonly used reporter genes include *cat* gene encoding chloramphenicol acetyltransferase (Herrera-Estrella *et al.*. 1983). *uidA* gene encoding a glucuronidase enzyme (Jefferson *et al.*, 1987), *lux* gene coding for luciferase and *gfp* gene for the expression of a green fluorescent protein (Chalfie *et al.*. 1994). Most of the above genes are of bacterial origin except *gfp* which was isolated from jelly fish. Recently. Wenck *et al.*, (2003) identified five novel non-bioluminiscent fluorescent proteins as visual non destructive reporter genes from reef corals that can be used in plant transformation methods.

2.8 Ascorbate peroxidase and oxidative stress

- In plants stress leads to a multitude of biochemical changes and such major event is oxidative burst as a result of which reactive oxygen species are generated. These reactive oxygen species bring about the detrimental effects in plants. To circumvent this effect, the cellular internal antioxidant mechanism will be elevated and the notable antioxidant enzymes in higher plants include superoxide dismutase. catalase, glutathione transferase and ascorbate peroxidase (Smirnoff 2000). Ascorbate peroxidase

has high affinity to deloxify the H_2O_2 an intermediate produced under stress condilions which involve reduction of reactive oxygen species. in particular $OH^{\cdot}$ radicals. In plants APX is encoded by a multigene family with distinct isoforms localized in various cellular components. An increased cytosolic *apx* transcript was observed in virus infected tobacco plants without a concomitant increase in protein (Mittler *et al,* 1998). Over expression of peroxisomal APX of Arabidopsis in tobacco led to increased protection against oxidative stress resulted from Aminotriazole (Wang *et al.,* 1999). Similar studies in rice plants by over expressing or suppressing the apx gene may decipher the role of APX in oxidative stress tolerance.

MATERIALS & METHODS

3. MATERIALS AND METHODS.

3.1 MATERIALS

3.1.1 *Chemicals and reagents*

All the chemicals and reagents used in molecular biology experiments were obtained from US Biochemical's (distributors-Amersham Pharmacia). Restriction enzymes and T_4 DNA Ligase was obtained from New England BioLabs. Klenow fragment. *Taq* polymerase and RNaseA were obtained from MBI Fermentas. DNA labeling kit and radio labeled aP^{32} dCTP were obtained from BARC India. Nylon membrane Hybond N+ was purchased from Amersham Pharmacia. Oligo nucleotide primers of HPLC grade purity were synthesized from MWG. Germany.

All the inorganic salts and organic supplements used in preparation of culture media were obtained from Hi-Media, India. and are of tissue culture grade purity. Biochemical's used for microbiological studies were also obtained from Hi-Media. All phytohormones and antibiotics except hygromycin-B were obtained from Sigma Chemicals, USA. Antibiotic hygromycin-B was obtained from Calbiochem, La Jolla, USA. Filter purified "RO" grade water obtained from Fristream Multipure system was **used** for media preparation.

All the glassware used in routine tissue culture practice such as 50 ml test-tubes, 90 mm petri-plates. conical flasks and beakers were obtained from Borosil, India. Glass culture bottles 7 mm X 11 mm (diameter x length) with autoclavable screw caps, used **for** growth and hardening of tissue culture raised plants were obtained from local manufacturer. Glass petri plates (10cm diameter x 2.5 cm height) were specifically used

for *in vitro* regeneration experiments.

3.1.2 Plant genotypes

Mature seeds of *indica* genotypes "Manasarovar", "Pusa basmathi" and "Nagina22W" were obtained from Directorate of Rice Research, Rajendra Nagar, Hyderabad, India. Seeds of genotype "Nootripattu" were obtained from Tamil Nadu Agricultural University. Coimbatore, Tamil Nadu. India. These cultivars were grown in 18" clay pots containing black soil enriched with organic compost. The plants were grown till maturity in green house.

3.1.3 Bacterial strains

E. coli strain DH5a obtained from "Bangalore Genie" was used for general cloning and clone maintenance. *Agrobacterium tumefaciens* strain EHA 105 used for rice transformation was obtained from Dr. Gelvin. Purdue University, USA.

3.1.4 Plasmid vectors

Binary vector pCAMIA1305.1 obtained from GAMBIA, Australia. was used in optimizing rice transformation protocol.

Plasmid "pMAPX1" harboring the "*apx1*" cDNA encoding maize cytosolic ascorbate peroxidase. was obtained from Dr. Van Montagu (Belgium).

Super promoter driven plant expression vector "pE1805" was obtained from Dr. Gelvin (Purdue University).

3.2 METHODS

3.2.1. Tissue culture and plant transformation methods

3.2.1.1. Seed pretreatment

After complete seed filling, mature seeds were harvested and sun dried for three days in the mid day sunlight from 11a.m. to 3 p.m. and then incubated at 42°C for 16 h to break their dormancy.

3.2.1.2. Seed surface sterilization

Mature seeds were manually dehusked and healthy seeds were selected. For each experiment involving callus induction, 100 seeds were dehusked and thoroughly washed in a 100 ml glass beaker with ample quantities of sterile double distilled water before rinsing them for two min in 25 ml of 70% ethanol. The seeds were washed three times with sufficient quantities of sterile distilled water to remove traces of ethanol. Finally, the seeds were rinsed in 25 ml of 0.1% $HgCl_2$ for 10 min with occasional swirling, this was followed by five times washing with ample quantities of sterile double distilled water. These surface sterilized seeds were blotted dry on sterile whatman # 1 filter papers before placing them on callus induction medium.

3.2.1.3. Callus Induction

Callus induction medium (NBC1) was prepared by adding all the constituents stated in Table 1. The NBCJ medium containing 2.25 mg/L of 2,4-D was autoclaved in conical flasks for 15 min at 15 lbs pressure on liquid cycle. The autoclaved media was

poured into 9 mm sterile glass petri-plates under laminar hood and allowed to solidify for 30 min. About 25 ml of medium was added to each petri-plate.

Scutellar callus was initiated from the surface sterilized mature rice seeds by placing them on the callus induction medium. Ten seeds were placed in each plate containing about 25 ml of NBCI medium and the plates were sealed with parafilm. The cultures were maintained in dark at 27 ± 1°C.

3.2.1.4, In vitro plant regeneration

Regeneration medium (NBRE) was prepared by adding 3 mg/L of BA to NB (N_6 macro nutrients + B_5 micro nutrients) basal medium (Table 2). All the plant hormones, except 2.4-D. were dissolved in DMSO and added to media after autoclaving. The 18 d old embryogenic callus was cut to about 3 mm size and placed over NBRE medium for *in vitro* regeneration. For regeneration from callus cultures, about 20 calli were transferred to each 10 cm diameter glass petri-plate containing about 40 ml of NBRE regeneration medium. Calli on regeneration medium were initially given a dark treatment for one week and later transferred to continuous light at 27 ± 1°C. The illumination was from four fluorescent tubes each of 40 W capacity.

3.2.1.5. Callus cocultivation

Cocultivation media (NBCC; Table 1) was prepared by adding 100 µM of filter sterilized acetosyringone to NBRE medium (Table 1). Eighteen day old scutellar embryogenic callus was cut to about 2-3 mm in size and were infected with 1.0 O.D.

Agrobacterial culture, strain EHA105. A 200 ml *Agrobacterial* culture was grown to an $O.D._{600}$ ~ 0.6 and centrifuged at 5000 rpm for 5 min at room temperature. The obtained bacterial pellet was resuspended in 100 ml of NBCC medium so as to get a final $O.D._{600}$ ~ 1.0, and used for infecting the rice calli. For cocultivation, the calli after infecting for 10 min in *Agrobacterium* culture were blotted dry on whatman # 1 filter papers and transferred onto filter paper discs (5 mm diameter. whatman # 1) wetted with 700 µl of liquid cocultivation medium. Cocultivation was performed in petri-plates containing only filter papers discs wetted with 700 µl of liquid NBCC medium (without any semi-solid medium) and the calli were incubated in dark for 2.5 d at 25 °C. Cocuitivation was also performed as reported by Rashid *et al.*, (1996) where the explants were placed on filter papers overlayed on semi-solid medium containing 100 µM acetosyringone,

After cocuitivation, the calli were washed thoroughly in sterile distilled water till the bacteria was removed. Final wash was with cefotaxim solution (500 mg/L) and dried on whatman # 1 filter paper for 5 min. before transferring to the selective regeneration medium (NBSR; Table 1).

3.2.1.6. Selective regeneration of transgenic plants

NBSR medium consists of NB basal medium supplemented with 3 mg/L BA, 300 mg/L cefotaxim and 50 mg/L hygromycin-B. The above cocultivated calli were transferred onto NBSR medium and incubated in dark for the initial one week at 25°C. later the cocultivated calli were transferred to continuous light. After two weeks. the transformed calli that survived the selection pressure and proliferating on NBSR

medium were sub-cultured onto NBRE medium containing 350 mg/L cefotaxim and maintained under the same growth conditions for next 30 d (Kumaria *et al.*. 2001). The number of plants regenerated after 30 d on NBRE medium was scored and the frequency of plant regeneration was calculated as stated by Rubulo *et al.*, (1984) and Khanna and Raina (1998)..

3.2.1.7. Rooting and acclimatization

The *in vitro* regenerated shoots after attaining a height of about 2 cm were transferred to NB basal medium for rooting. The cultivars "Nootripattu" and "Pusa basmati-1" were rooted successfully when transferred to NB basal medium. Cultivar "Manasarovar" failed to root on NB basal medium. Rooting was induced by transferring the shoots to "M" shaped filter paper boats placed in 5 ml of liquid NB medium fortified with 1 mg/L NAA, in 50 ml glass tubes capped with paper plugs. The shoots were given a 16/8 light/dark regime to enhance rooting.

3.2.2. Vector construction: Gene cloning and analysis

3.2.2.1 Plasmid DNA isolation

Plasmid DNA was isolated following the standard protocols (Sambrook *et al.*, 1989). The *E. coli* culture (DH5a) harboring the plasmid pMAPXl was grown overnight at 37°C in 5 ml liquid LB medium containing 50 µg/ml of ampicillin, on an orbital shaker maintained at 220 rpm. The culture was raised in 50 ml Borosil tubes capped with paper plugs. A 1.5 ml of the overnight grown culture was transferred into

1.8 ml microfuge tubes and centrifuged at 5000 rpm for 5 min at $4^{O}C$. The supernatant was discarded and the bacterial pellet was suspended by vortexing in 100 µl of ice cold Solution I and briefly incubated on ice for 5 min. The cell suspension was lysed by adding 200 µl of freshly prepared Solution II followed by a brief incubation at room temperature for 5 min. Finally. 150 u1 of ice cold Solution III was added and mixed by slow inversion. The lysate was centrifuged at 5000 rpm for 5 min at 4°C and the supernatant was purified by extracting once with Phenol-Chloroform (1:1) mix. After centrifugation at 5000 rpm for 5 min at room temperature, the top aqueous phase was collected and the plasmid DNA was precipitated by adding two volumes of ice-cold absolute ethanol with a brief incubation on ice for 10 min. The DNA pellet was obtained by centrifuging at 5000 rpm for 5 min at 4°C and the plasmid DNA pellet was washed once with 70% ethanol. The DNA pellet was air dried and dissolved in 50 µl of TE buffer or in sterile double distilled water and stored at 4°C. RNA that was co-precipitated with plasmid DNA was removed by treating the sample with 1 µl of 20 mg/ml RNaseA and incubating it at 37°C for 2 h. The plasmid DNA was purified again using Phenol-Chloroform mix and the DNA was precipitated by adding 1/10 volume of Ammonium acetate (pH-5.2) and 2.5 volumes of absolute ethanol. The DNA was pelleted by centrifugation at 5000 rpm for 5 min at $4^{O}C$, washed with 100 µl of 70% ethanol, air dried and dissolved in 50 µl TE buffer or in sterile double distilled water. Plasmid DNA was quantified using spectrophotometer by taking the O.D values corresponding to 260 nm and 280 nm absorbance.

Reagents:

Solution I: 50 mM Glucose, 25 mM Tris (pH -8.0). 10 mM EDTA (pH -8.0)

Solution II: 0.2 N NaOH. 1% SDS.

Solution III: 10 ml solution contains 6 ml of 5 M Potassium acetate. 1.15 ml of Glacial acetic acid and 2.85 ml H_2O.

TE Buffer: 10 mM Tris (pH -8.0) and 1 mM EDTA (pH -8.0)

RNase A: 10 mg/ml RNase A in 10 mM Tris-HCl (pH -7.5) and 15 mM NaCl

3.2.2.2 Restriction digestion

In a 30 ul reaction volume. 1 µg of the plasmid DNA (pMAPX1) was double digested with Xho I and *XbaI* restriction enzymes (10U each and NEB buffer 2) to release a 1.1 kb fragment corresponding to the full length cDNA coding for maize cytosolic ascorbate peroxidase. The sample was incubated at 37°C overnight and the digested sample was fractionated on 0.8% agarose gel.

Reaction setup:

Plasmid DNA pMAPX1 (1µg)	x µl
NEB2 buffer (10X)	3 µl
Xho I enzyme (10U)	1 µl
XbaI enzyme (10U)	1 µl
Sterile water	y µl
Total volume	30 µl

(Note: x + y = 25µl)

Similarly 400 ng of the binary pEI805 was digested with *SmaI* restriction enzyme by incubating the sample at 25°C for 4 h. After digestion the sample was purified using Phenol-Chloroform mix and then precipitated using ammonium acetate and ethanol.

Reaction setup:

Plasmid pE1805 (400 ng)	a µl
NEB4 buffer (10X)	2 µl
SmaI enzyme (10U)	1 µl
Sterile water	b µl
Total volume	20 µl

(Note: a + b = 17 µl)

3.2.2.3 Fragment elution

The digested plasmid DNA was run on 1% agarose gel casted in 0.5X TBE and the gel was cut to about 5 mm x 10 mm dimensions having the band of interest. The cut gel slice was placed inside pretreated dialysis tubing (Sigma D9777, MW 12,400. cellulose membrane dialysis tubing with a 16 mm average diameter) sealed at one end using clips. The dialysis bag was then filled with nearly 400 ul of 0.5 X TAE buffer and sealed the other end with clips, ensuring no air bubbles inside.. The sealed dialysis tube was placed in an electrophoresis tank in such a way that the DNA band was parallel to the electrodes. The electrophoresis was carried out in 0.5 X TBE buffer by powering the apparatus with a 5 V/cm electric field for 30 min. The electric field was reversed for 30 seconds and the buffer in the dialysis tubing was transferred to a 1.5 ml microfuge tube.

The sample was purified by extracting once with Phenol-Chloroform and the DNA was precipitated with ethanol. The DNA pellet was washed once with 70% ethanol and then dissolved in 10 µl of sterile water. Finally the amount of DNA present was quantified using spectrophotometer.

3.2.2.4 End filling

The 1.1 kb *Xho I-Xba I* fragment corresponding to the cDNA sequence encoding **maize** cytosolic ascorbate peroxidase was gel eluted using dialysis tubing method and **then** end filled using Klenow fragment (MBI Fermentas). About 500 ng of insert DNA was **end** filled using Klenow fragment *(E.coli* DNA polymerase 1, large fragment). The reaction mix containing 20 µl of DNA solution. 5 µl of 10 X reaction buffer. 10 µl of 0.25 mM dNTP mix. 1 ul of Klenow fragment (10U) and 14 ul of sterile water with a total reaction volume of 50 µl was incubated at 37°C for 10 min and then heat inactivated at 75°C for 10 min. The end filled DNA was purified by Phenol-Chloroform extraction and the DNA was precipitated using ethanol. The DNA pellet was washed in 70% ethanol and dissolved in 10 u1 of sterile water.

3.2.2.5. Ligation

Plasmid DNA ligations were carried out in a 20 µl reaction volume containing 200 ng of combined DNA concentrations of vector and insert, in a molar ratio of 1:10. The ligation mix containing, 2 µl of 10 X reaction buffer. 1 µl (400U) of T_4 DNA ligase (NEB), 85 ng of end filled insert DNA and 115 ng of vector DNA digested with *SmaI*

was incubated overnight at room temperature.

Calculating DNA concentrations based on the molar ratios of vector to insert Formula: $I = (S_I * T) / [(S_V/R_{IV})+S_I]$ & $V = T / [(1+(S_I*R_{IV}/S_V)]$; (Gruenwald and Heitz 1993).

I = Amount of insert DNA in ng needed for ligation reaction.

V = Amount of vector DNA in ng needed for ligation reaction.

T = Amount of total DNA required for ligation reaction.

S_I = Size of insert DNA

Sv = Size of vector DNA

R_{IV} = Insert to vector molar ratio.

3.2.2.6 Preparation of Ca Ch Competent cells

One ml of overnight grown *E. coli* culture (DH5a) was added to 99 ml of fresh LB medium and the culture was allowed to grow to an $O.D_{600}$ of 0.4-0.6 on an orbital shaker maintained at 37°C and 220 rpm. The log phase culture obtained was chilled on ice for 30 min and centrifuged at 5000 rpm for 5 min at 4°C. The pellet was suspended in equal volume of ice cold sterile water and centrifuged at 5000 rpm for 5 min at 4°C. The bacterial pellet was resuspended in 100 ml of ice cold 0.1 M $CaCl_2$ and incubated on ice for 1 h prior to centrifugation at 5000 rpm for 5 min al 4°C. The bacterial pellet was resuspended in half volume of 0.1 M $CaCl_2$ and centrifuged at 5000 rpm for 5 min at 4°'C. Finally the pellet was resuspended in 2 ml of ice cold 0.1 M $CaCl_2$ and 200 µl

aliquots of the competent cells were made and stored at -70°C till further use.

3.2.2.7 Bacterial transformation

The 20 u1 ligated sample was added to a 200 µl aliquot of $CaCl_2$ competent cells and incubated on ice for 30 min. The competent cells were given a heat shock for 90 sec. at 42°C and incubated on ice for 5 min. To the heat shock treated competent cells. 800 u1 of fresh LB medium was added and the culture was incubated at 37°C for 1 h on an orbital shaker maintained at 220 rpm. A 200 u1 of the transformed bacterial culture was plated on LB semi-solid medium containing kanamycin at 50 µg/ml and plates were incubated overnight al 37°C.

3.2.2.8 Colony hybridization

About 133 transformed colonies were picked and plated in two replicates on the selection medium and the colonies were allowed to grow for exactly 12 h. A rectangular nylon membrane, cut to the size of the plate was placed over colonies and pressed gently and evenly. The membrane was transferred onto the whatman # 1 filter disc soaked in 10 ml of Solution A for exactly one minute and then subsequently transferred to whatman # 1 filter discs containing Solutions B, C, D' and E in sequential order. At each step the nylon membrane was placed on filter paper for exactly 1 min. Finally the membrane was air-dried and baked at 80°C for 1 h.

Reagents:

Solution A: 25% Sucrose solution and 50 mM Tris (pH-8.0). Lysozyme at 1.5 mg/ml was added after autoclaving.

Solution B: 0.5 M NaOH and 0.2% SDS.

Solution C: 0.5 M NaOH.

Solution D: 1 M Tris (pH-7.5).

Solution E: 0.15 M NaCl in 0.1 M Tris (pH-7.5).

3.2.2.9 Preparation of electrocompetent cells

A starter culture of *Agrobacterium tumefaciens* strain EHA105 was raised in 5 ml liquid LB medium having tetracycline at 15 mg/L. *Agrobacterium* was cultured for 48 h at 28°C on an orbital shaker maintained at 220 rpm. Starter culture of 1 ml was added to 100 ml of fresh LB medium containing tetracycline 15 mg/L and the culture was allowed to grow to log phase $O.D_{600}$ 0.4 - 0.6. The log phase culture was chilled on ice for 30 min and centrifuged at 5000 rpm for 15 min at 4°C. The pellet was suspended in equal volumes of ice cold sterile double distilled water and centrifuged at 5000 rpm for 5 min at 4°C. The bacterial pellet was resuspended in equal volume of 0.1 mM HEPES (pH-7.0) and incubated on ice for 1 h before centrifuging at 5000 rpm for 5 min at $4^{O}C$. The obtained bacterial pellet was suspended in equal volume of 10% ice-cold glycerol and centrifuged at 7000 rpm for 10 min at 4°C. Finally the pellet was suspended in 300 µl of 10% glycerol and aliquots of 40 µl were made and stored at -$70^{O}C$ till further use.

3.2.2.10 Agrobacterium transformation by electroporation

To a 40 µl of electrocompetent cells about 10 ng of plasmid DNA was added and an electric pulse of Resistance-201 Ω, Capacitance-25 µF and Voltage-2500 V was given in an electroporator. For all the successful electroporations. pulse time of 5.25 milli sec was recorded. Immediately after the pulse treatment. 1 ml of fresh LB medium was added to the electro-transformed competent cells and incubated for 1 h in an orbital shaker maintained at $28^{O}C$ and 220 rpm. After 1 h of expression. 50 µl of the transformed cells were plated on LB semi-solid plates with corresponding antibiotics for selection of transformed clones, and the plates were incubated at 28°C for 2 d.

3.2.3. Analysis of transgenic plants

3.2.3.1 GUS assay

Transient expression of the *uidA* gene in calli immediately after cocultivation and slable expression in calli that were proliferating on selective regeneration medium were analyzed. Also expression in transgenic leaf tissue was examined following the protocols stated in IRR1 reports (Datta *et al.*, 1997) with slight modifications. Explants were immersed in 5 ml of GUS staining solution containing 10 mg X-Gluc (5-bromo-4-chloro-3-indolyl-â-D-glucuronide), 100 mM Tris (P^{H}-7.0), 50 mM NaCl, 2 mM Potassium ferricyanide. 0.1% Triton X-100. 0.2% Sodium azide and sterile water. Explants were incubated for 16 h in dark at 37°C and decolorized with 2-3 rinses of absolute ethanol 30 min each before detecting the blue spots under a low magnification laboratory compound microscope. Photographs were taken using a high sensitive

photographic film (Kodak 400 ASA) using the attached camera facility.

3.2.3.2 In vitro HPT assay

In vitro HPT assay using rice leaf tissues was performed as described by Wang *et al.,* (1997). A fully juvenile leaf grown to about 10-12 cms was cut into pieces of about 1 cm in length. The leaf pieces were placed over HPT assay medium (Table VII) consisting of MS basal medium fortified with 1 mg/L BA and 100 mg/L hygromycin. The leaf tissues were incubated at 25°C for one week with a 16/8 light/dark regime.

3.2.3.3 Isolation of genomic DNA "Mini-prep"

Genomic DNA was isolated following a genomic DNA mini prep protocol stated at website http://biologi.uio.no/molbiol/protocol/miniprep2.htm. About 50 mg of leaf tissue was ground to a fine powder in a 1.5 ml micro centrifuge tube using liquid nitrogen and micro pestle. To the powdered tissue 700 µl of prewarmed extraction buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0. 100 mM NaCl. 1% SDS and 10 mM â-mercaptoethanol) was added and incubated at 65°C for 15 min. To this. 220 µl of Acetate buffer (3 M Potassium + 5 M Acetate) was added and incubated on ice for 30 min, The cell lysate was centrifuged at 10000 rpm for 5 min at 4°C and to the supernatant 550 µl of isopropanol was added, mixed well by inversion and incubated at room temperature for 1 h. The precipitated DNA pellet was obtained by centrifuging at 11000 rpm for 30 min at 4°C. The DNA pellet was washed in 70% ethanol and dissolved in 100 µl of TE after air drying.

3.2.3.4 Genomic DNA isolation from rice leaves using modified CTAB method

Genomic DNA was isolated from young leaves following modified CTAB stated in CIMMYT protocols (Saghai-Maroof *et al.,* 1984). Leaf tissue of 300 mg was **frozen in liquid** nitrogen and ground to a fine powder using mortar and pestle. The powdered tissue was transferred to 50 ml conical flasks and 9 ml of pre warmed CTAB extraction buffer (500 mM NaCl. 50 mM Tris-HCl (pH 8.0). 50 mM EDTA. 1% (v/v) ß-mercaptoethanol) was added, followed by addition of 6% polyvinylpyrrolidone (PVP. 25kDa) and 2% SDS before incubating the mixture for 1 h at 65°C. The sample was centrifuged at 12000 rpm for 10 min at room temperature. The top aqueous phase was collected and to it 30 ul *of* 10 mg/ml RNaseA was added and incubated at room temperature for 1 h. To the aqueous phase 5 M Potassium acetate was added to a final concentration of 0.5 M and the DNA was precipitated with the addition of 0.6 volume isopropanol. The DNA pellet was dissolved in TE and purified by Phenol-Chloroform-Isoamyl alcohol extraction. The genomic DNA was precipitated by adding 50 al of 5 M NaCl and 2.5 ml of ethanol and washed once with 76% ethanol containing 10 mM ammonium acetate, air dried and dissolved in 250 ul of TE. The DNA concentration was measured by spectrophotometer, and its quality was checked on 0.8% agarose gel.

3.2.3.5 PCR analysis of putative transgenic rice plants of cv "Manasarovar"

The gene specific primer sequences were designed using online Primer3 software at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi and the following primer sequences were synthesized from MWG. Germany.

Primer sequences for *uidA* gene

gusF 5' - GCCATTTGAAGCCGATGTCACGCC - 3' (Tm 62°C)

gusR 5' - GTTCTGCGACGCTCACACCGATAC - 3' (Tm 60°C)

Primer sequences for *hpt* gene

hptF 5' - GAAGATCTTACCATGAAAAAGCCTGAACTCACCG - 3' (Tm 65°C)

hptR 5' - ATTCGAGCTCCTATTTCTTTGCCCTCGGACGA - 3' (Tm 68°C)

Plants regenerated from the transformed calli on selective regeneration medium were initially analyzed by PCR. Gene specific primers that are homologous to the *uidA* gene and *hpt* gene were used. PCR amplifications using 40 ng of genomic DNA were performed in 20 ul volumes containing 100 mM Tris-HCl (pH 9.0). 500 mM KCl, 25 mM MgCl2, 1% Triton X-100, 2.5 mM of each dNTP. 5 uM of primers. 0.8U of *Taq* DNA polymerase and a primer concentration of 0.1 mM each.

Amplifications were performed in a DNA engine (PTC 200. M J Research, Inc.). The amplification temperature cycle for the *uidA* gene was as follows. Preheating at 94°C for 5 min and 1 cycle of 1 min each at 94°C. 59°C and 72°C and 34 cycles of 1min each at 94^{O}C. 55^{O}C and 72° C. The amplification temperature cycle for the *hpt* gene was as follows - pre-heating at 94°C for 5 min and 1 cycle of 1 min each at 94°C. 55°C and 72°C and 34 cycles of 1min each al 94°C. 61^{O}C and 72° C. The programs ended at 4°C for cooling. Products were electrophoresed on 1.0% agarose gel and visualized under UV light by ethidium bromide staining.

3.2.3.6 PCR analysis of apxl rice transgenics cv Pusa basmati

Genomic DNA was isolated from 8 *apxl* sense expressing and 7 *apxl* anti-sense expressing independent putative transgenic rice lines. following modified CTAB method. About 40 ng of genomic DNA isolated from rice transgenics was used to amplify a 512 bp region corresponding to maize *apxl* coding. using gene specific primer sequences at an annealing temperature of 59°C.

apx1F 5' - CGGTCGACAAGGCCAAGCGTAAG - 3' (Tm 60°C)

apx1R 5' - TGGGCGGAAGGATGGATCAGAGA - 3' (Tm 60°C)

Similarly. a 1.029 kb region corresponding to *hpt* gene was amplified using the same reaction conditions as staled in section 3.2.3.5. The amplified samples were fractionated on 1% agarose gel stained with ethidium bromide.

3.2.3. 7 RT-PCR amplification of maize apxl gene in rice transgenics

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen) following manufacturer's protocols. RNA was isolated from leaf tissues of *apxl* sense and anti-sense expressing transgenic rice lines that have shown *hpt* and *apxl* gene amplifications using genomic DNA.

By using the above RNA samples. RT-PCR was performed using "SUPERSCRIPT™ One-Step RT-PCR" kit obtained from Invitrogen. RT-PCR reaction was carried out in a 50 ul reaction volume containing 1 µg of total RNA. 1.2 mM $MgSO_4$. 0.2 mM dNTPs. 0.5 µM forward and reverse primers corresponding to maize *apxl* cDNA. 1 ul of RT/Platinum *Taq* mix and 2 X reaction buffer. The reaction mix

was initially incubated at 50°C for 30 min and the PCR amplification was performed at an annealing temperature of 49°C for 40 cycles, as recommended in the manufacturers guide lines. The amplified samples were resolved on 1% agarose gel stained in ethidium bromide.

3.2.3.8 Restriction digestion and agarose gel electrophoresis

About 30 µg of genomic DNA isolated from *apx1* sense and anti-sense expressing independent transgenic lines was digested with 30U of *Eco RI* restriction enzyme (NEB) in a 50 µl reaction volume, incubated at 37°C for 16 h. The genomic DNA isolated from untransformed plant (negative control) was also digested with *Eco RI*. The digested samples were fractionated on 1% agarose gel (13 cm gel length) casted in TAE buffer. Electrophoresis was carried out for 12 h in a 25 cm X 13 cm gel tank (Broviga) with an applied voltage of 0.5 V/cm.

3.2.3.9 Southern blotting

After electrophoresis, the agarose gel resolving the restricted DNA was depunnated in 250 ml of 250 mM HCl solution for 30 min followed by washing with sterile double distilled water. The depurinated gel was denatured for 15 min in 250 ml of denaturation solution (1 M NaCl and 0.5 M NaOH) on a rocker maintained at 15 cycles per minute. The depurinated gel was thoroughly washed using double distilled water and neutralized for 15 min in 250 ml of neutralization solution [1.5 M NaCl and 0.5 M Tris (pH-7.0)] and the gel was kept overnight for capillary transfer.

Capillary blotting

Restricted DNA from the above pretreated gel was transferred onto N+ Nylon membrane (Amersham Pharmacia) by capillary blotting. In a 25 cm x 25 cm glass trough. the gel casting tray (13 cm x 13 cm) was kept in an inverted position with the bottom side up and whatman # 1 filter paper wick was placed on top with its free ends dipped in 500 ml of 20X SSC (175.3 gms/L NaCl and 88.2 gms/L Sodium citrate. pH-7.0) solution present in the glass trough. Three whatman # I sheets. cut to the size of the gel. were placed and above this the gel was placed in an inverted position so that the bottom even surface faces up. A nylon membrane cut to the size of the gel was placed over the gel ensuring no air bubbles trapped in between nylon membrane and the agarose gel. Above the nylon membrane, three whatman # 1 papers. cut to the size of the gel. were placed and a pile of rough filter papers cut to the size of the gel were placed over the whatman # 1 filter papers. On top of all, a weight of about 200 gms was placed and the setup was left undisturbed overnight. After capillary transfer the blot was air dried and baked at 80°C for 1 h in vacuum.

Random labeling of DNA fragments

To 200 ng of purified DNA fragment corresponding to maize *apx1* coding sequence. 5 µl of random primer was added and the DNA was denatured by placing the tube in boiling water for 5 min and immediately transferred to ice. To this denatured DNA. 4 ul of each dATP. dGTP, dTTP. 5 µl of $\propto P^{32}$ dCTP. 5 ul of 10 X reaction buffer and 1 µl of klenow (10U) was added and the total volume was made up to 50 µl using sterile water. The reaction mix was incubated at 37 C for 1 h and the synthesized probe

was purified using Sephadex G-50 spin column.

Southern Hybridization:

a) Pre-hybridization.

The nylon membrane after baking was placed inside a hybridization bottle containing 150 ul/cm^2 pre-hybridization solution (0.5 M Na_2HPO_4, 7% SDS and 1 mM EDTA pH-7.0) and incubated for 30 min in a hybridization oven maintained at 65°C.

b) Hybridization

The synthesized probe was denatured in boiling water for 5 min and immediately transferred to ice for 10 min. The Prehybridization solution was removed and equal volume of fresh Prehybridization solution was added, to this the denatured probe was added and kept for overnight hybridization at 65°C in the hybridization oven.

The hybridization solution was decanted and the membrane was washed in solution containing 2X SSC and 1% SDS, for 30 min at 65°C. The blot was then washed twice with solution containing 1% SDS and 0.5X SSC for every 10 min. Finally, the membrane was rinsed in 2 X SSC solution at room temperature, air-dried and covered with Saran wrap for autoradiography.

3.2.3.10 Autoradiography and X-ray film development

An X-ray (Kodak) film was placed over the dried Southern blot wrapped in saran wrap, in the dark room under red light. The film was allowed to expose for sufficient length of time in the X-ray cassette depending on the intensity of counts recorded using a GM counter. The X-ray cassette was stored at -70°C by wrapping it in a black cloth. The autoradiogram was developed in the dark room first by rinsing it in

the developer solution for 2 min followed by a brief wash using distilled water for 1 min. Finally the film was immersed in fixer solution for another 2 min and washed thoroughly using tap water and later air-dried.

RESULTS

4. RESULTS

As regeneration in rice is through somatic embryogenesis, production *of* good quality callus is a pre-requisite for any laboratory experiment involving regeneration and transformation. Callus cultures are also needed to produce cell suspension cultures for isolation of protoplasts. Thus the present study on protocol development was emphasized on identifying and optimizing the critical parameters involved in rice tissue culture and transformation. This was achieved by establishing optimal conditions for regeneration and transformation that were found conducive for transformation of the four *indica* rice genotypes tested, namely "Manasarovar", "Nootripattu", "Nagina22W" and "Pusa basmati-1". All tissue culture and transformation protocols were optimized using mature seed derived scutellar callus from an elite *indica* rice genotype "Manasarovar".

4.1 Culture conditions and plant regeneration

4.1.1 Effect of basal medium on callus induction

Callus was initiated from mature, dehusked, surface sterilized seeds on three callus induction media i.e., MSCI (Table 3), B5CI (Table 4) and NBCI (Table 1), each having the 2.4-D at a concentration of 2 mg/L. The experiments were replicated thrice with a sample size of 100 seeds per experiment, and on an average 10 seeds were kept in each petri-plate having 25 ml of semi-solid medium. The frequency of callus induction was scored after 21 d of culture in dark at 27°C. Callus induction from the

scutellum was observed in all the media tested. However, a high frequency of 96% total callus induction was observed from the seeds cultured on NBCI medium (Fig. 1).

4.1.2 Effect of2,4-D in inducing embryogenic callus

Initially, callus cultures were raised using various concentrations of 2.4-D to identify the threshold concentration required to produce embryogenic callus cultures. It is well known that the frequency of embryogenic calli in a given experiment and genotype varies considerably. Thus callus cultures were initiated on NBC1 medium having 1.75. 2.0. 2.25 and 2.5 mg/L of 2.4-D. Maximum percentage of embryogenic callus (about 61%) was observed in NBCI medium containing 2.25 mg/L 2.4-D (Fig. 2). Further NBCI medium containing 2.25 mg/L 2.4-D was effective in inducing callus at high frequencies in the three *indica* rice genotypes "Nagina22W". "Pusa basmati" and "Nootripattu" when tested (Fig. 3).

4.1.3 Effect of age of callus on plant regeneration

The age of the explant was found to be critical in regeneration experiments with all genotypes. using callus cultures. Hence. the regeneration efficiencies of 15. 18. 21. 25 and 28 d old callus cultures was tested on NBRE medium fortified with 3 mg/L BA. These callus cultures were initially given a dark treatment of 1 week at 25°C and later transferred to continuous light. Frequencies of regeneration were scored after 30 d of treatment on NBRE medium. It is observed that 18 d old callus cultures that were raised

on NBCI medium (with 2.25 mg/L 2.4-D), produced a high frequency of 60.86% *in vitro* plant regeneration on NBRE medium (Fig. 4).

4.1.4 Effect of basal medium on plant regeneration

To identify a suitable basal medium for plant regeneration. 18 d old callus cultures raised on NBCI medium containing 2.25 mg/L 2.4-D were regenerated on MS. NB and B5 media. all supplemented with 3 mg/L BA. Calli on regeneration medium were initially given a dark treatment for 1 week at 25°C and then transferred to continuous light. Green plants regenerated with the formation of multiple shoot on NB and MS regeneration media. The frequency of regeneration in terms *of* relative shoot yield index was calculated based on the total number of plants regenerated after 30 d of treatment. A high relative shoot yield index value 106.024 (Fig. 5) was obtained from calli that were regenerated on NBRE medium.

4.1.5 Effect of cytokinins on plant regeneration

To determine the effective combination of cytokinins required for high frequency *in vitro* regeneration, 18 d old embryogenic callus was transferred to NB medium supplemented with three different combinations of cytokinins (NB medium + 3 mg/L BA. NB medium + 3 mg/L kinetin. and NB medium + 1.5 mg/L BA + 1.5 mg/L kinetin). The calli on regeneration media were given continuous light treatment at 25°C and the results of the frequencies of regeneration were scored after 30 d. Calli regenerated on NBRE medium fortified with 3 mg/L BA produced a high frequency of 60.86% regeneration as compared with the other combinations (Fig. 6). As regeneration

frequency was higher in presence of BA at 3 mg/L. the *in vitro* plant regeneration frequencies of the three *indica* rice genotypes "Pusa basmati", "Nootripattu" and "Nagina22W" was also tested on NB medium supplemented with 3 mg/L BA. and the results were shown in Figure 7.

4.2 Callus transformation with *Agrobacterium*

4.2.1 Effect of duration of cocultivation on callus transformation

Eighteen day old embryogenic calli (Fig. 8). cut to 2-3 mm in size, were cocultivated with 1.0 $O.D_{600A}$ culture of *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pCAMBIA1305.1 (Fig. 9). Cocultivation was performed in the presence of 100uM acetosyringone for 3 different time periods such as 2. 2.5 and 3 d, in dark at 25°C. on filter papers placed over semi-solid medium and wetted with 1 ml of NBCC liquid cocultivation medium (Table 1). The efficiency of transformation based on transient GUS expression, was recorded by counting the number of GUS positive calli obtained after staining with X-Gluc solution. Transformation efficiency was significantly higher in calli that were cocultivated for 2.5 and 3 d (Fig. 10).

4.2.2 Effect of basal semi-solid medium on cocultivation

To determine the effect of filter papers on callus transformation by cocultivation. 18 d old embryogenic calli were cocultivated on whatman # 1 filter papers with and without semi solid NBCC medium. The filter papers were wetted with 700ul of liquid NBCC medium and the cocultivation was carried out for 2.5 d in dark at 25°C. The

experiment was replicated thrice with a sample size of about 75 calli in each experiment and the transient GUS expression was recorded after staining in X-Gluc solution (Table 5). Transient GUS expression was higher (Fig. 11) in calli that were cocultivated on filter papers without semi-solid medium. The transformation efficiencies recorded based on percentage of transient GUS expression observed in the four *indica* rice genotypes tested was shown in Figure 12.

4.3 Regeneration of transgenic plants

4.3. J Selective regeneration of transgenic plants

Cocultivated calli were thoroughly washed in water and cefotaxim solutions and later the cocullivated calli were transferred to NBSR medium (Table 1) containing cefotaxim and hygromycin. About 350 cocultivated calli were transferred to petri-plates (10 cm diameter x 2.5 cm height) each containing about 40 ml of semi-solid NBSR medium. Calli on NBSR medium were initially kept in dark for one week at 25°C before they were transferred to continuous light. After two weeks on NBSR medium. the proliferating calli were transferred to NBRE medium with 300 mg/L cefotaxim and kept under continuous light at 25°C. Figure 13, shows selective regeneration of transgenic plants. A frequency of about 23% transgenic plant regeneration was recorded from transformed calli cv. "Manasarovar". after 30 d on NBRE medium containing cefotaxim (Fig. 14). Figure 14. also shows the frequency of transgenic plant production from the *indica* rice genotypes "Pusa basmati" and "Nootripattu". The schematic representation of the various steps involved in *Agrobacterium*-mediated *indica*

transformation was shown in Figure 15. The figure also highlights the changes made in the transformation protocol.

4.3.2 Rooting of transgenic plantlets

Of the genotypes tested, genotype "Manasarovar" failed to root on NB basal medium even in presence of NAA at a concentration *of* 1 mg/L. However, the regenerated shoots rooted when transferred to "M" shaped filter paper boats (2 cm breadth and 10 cm length) placed in 5 ml of liquid NB basal medium (Table 2) containing 1 mg/L NAA in 50 ml glass tubes capped with paper plugs. Rooting of transgenic shoots was observed after 8 d under 18 h light and 6 h dark treatment at 28°C.

4.4 Analysis of transgenic plants

4.4.1 Histochemical analysis

To visualize the transformation events, the explants were incubated in X-Gluc solution (Table 5) for overnight that resulted in deep blue color GUS staining (Datta *et al,* 1997). The explants were photographed using simple or compound microscopes with the attached camera. Transient GUS expression in cocultivated calli (Fig. 16). stable GUS expression in calli that proliferated on NBSR medium (Fig. 17) and GUS expression in the vascular regions of juvenile leaf tissue of a transgenic plant were observed (Fig. 18).

4.4.2 Molecular analysis using PCR

Total genomic DNA was isolated following genomic DNA miniprep protocol. About 40 ng of the genomic DNA from an independent GUS positive, transgenic plant **was** used for PCR amplification of *hpt* and *uidA* genes using gene specific primers. A 1083 bp amplicon corresponding to *hpt* (Fig. 19) and 1029 bp amplicon corresponding to *uidA* gene (Fig. 20) were observed when the PCR amplified samples were resolved on a 1% agarose gel stained with ethidium bromide.

4.5 Production and analysis of rice transgenics carrying maize *apxl* gene

4.5.1. Construction of apxl sense and anti-sense expression vectors

The schematic representation of the various steps involved in cloning the maize *apxl* gene downstream to a constitutive superpromoter in plasmid pE1805 was detailed in Figure 21. A 1.1 kb fragment from the plasmid pMAPXl (Fig. 22) corresponding to "*apxl*" coding sequence was isolated following restriction digestion using the two restriction enzymes *Xba* I and *Xho* I. The fragment was gel eluted using dialysis tubing method and the eluted fragment was end filled using Kelnow. The filled in fragment. corresponding to *apxl* coding sequence, was ligated using T4 DNA ligase at the *Sma* 1 site in the binary vector pE 1805 (Fig. 23a). downstream to the super promoter for constitutive over expression. The recombinant clones were screened by colony hybridization (Fig. 24) and the sense and anti-sense expressing clones were identified following a restriction digestion with *Pst* I enzyme (Fig. 25). The clones C1 from blot 1 and C1 from blot 2. released a 700 bp fragment upon digestion with *Pst* I indicating that

the: gene was cloned in sense orientation. The clones Cl and F2 from blot1 were identified as sense and antisense expressing clones and were designated as pE1805-apx1.s and pE1805-apx1.as respectively. These clones were further confirmed by sequencing using MegaBase 500 (Amersham Pharmacia). with gene specific primers.

The sense and antisense clones. pE1805-apx1.s and pE1805-apx1.as (Fig.23b and 23c). were independently transferred into *Agrobacterium tumefaciens* strain EHA105 by electroporation (Mozo and Hooykaas. 1991) and were used to generate *apxl* sense and antisense transgenic plants.

4.5.2 Agrobacterium-mediated transformation of "Pusa basmati-1" with "apx1" gene

By using the above optimized protocol for *Agrobacterium*-mediated transformation of rice. 47 putative *apxl* anti-sense expressing and 36 *apxl* sense expressing independent transgenic lines were recovered (Fig. 26). These putative transgenics were acclimatized in small plastic pots (12cm length 6cm diameter) having sand and soil in 1:1 ratio. bagged with plastic bags. The fully acclimatized plants were transferred to big pots (40 cm length and 30 cm diameter) in glass house where they were grown to maturity and fully viable seeds were harvested.

4.5.3 In vitro HPT assay

The transgenic leaf tissues were evaluated based on the *in vitro* HPT assay as described by Wang *et al.*, (1997). Small section of juvenile leaf (10 cm) from a healthy transgenic plant was cut to about 1.5 cm long and assayed for HPT in glass petri-plates containing HPT assay medium (MS basal medium with 100 mg/L hygromycin). The

leaf tissues were incubated for 7 d. the leaf pieces from the non transgenic plant showed complete bleaching by 5 d. whereas the leaf tissues from the transgenic lines remained green even after one week of treatment (Fig. 27),

4.5.4 PCR amplification of hpt gene in apxl rice transgenic

Total genomic DNA was isolated following the modified CTAB method (Saghai-Maroof. 1984) from 8 independent *apxl* sense expressing and from 7 independent *apxl* anti-sense expressing putative transgenic rice lines. By using the gene specific primers. the *hpt* gene in transgenic plants was PCR amplified in a 20 \\\ reaction volume containing 40 ng of genomic DNA and 5 pM of each forward and reverse primer. DNA amplification was performed in the Thermal cycler (MJ Research PTC 200) at corresponding Tm values of primers. The amplified fragments were resolved on 1% agarose gel (Fig. 28). Based on the PCR analysis for *hpt* gene. 7 out of 8 sense expressing transgenic lines (S1. S2. S3. S5. S6. S7 and S8) and 6 out of 7 anti-sense expressing transgenic lines (ASK AS3. AS4. AS5. AS6 and AS7) were found positive.

4.5.5 PCR amplification of maize apxl gene in rice transgenics

The transgenic rice lines that were confirmed positive to *hpt* gene amplification were further subjected to amplification of *apxl* gene with corresponding primers. The results of the amplifications were shown in (Fig. 29). Based on the results of *apxl* amplifications. it was evident that 4 out of 7 sense expressing *hpt* positive transgenic rice lines (S1. S5. S6 and S8) were positive for *apxl* amplifications, and 4 out of 6 anti-

sense expressing *hpt* positive transgenic lines (AS1. AS3. ASS and AS7) were found positive for *apxl* gene amplification,

4.5.6 RT-PCR analysis of maize apxl gene in rice transgenics

The transgenic plants were further subjected to RT-PCR analysis. PCR conditions were set according to the manufacturers protocols and the amplified products were resolved on 1% agarose gel (Fig. 30). The *apxl* sense expressing rice lines (S1. S3 and S8) have shown amplification of *apxl* gene by RT-PCR. The *apxl* anti-sense expressing transgenic lines (S5 and S7) have also shown the similar amplifications by RT-PCR.

4.5.7 Southern blot analysis of transgenic plants

The genomic DNA was isolated from the *apxl* sense expressing transgenic rice lines (S1, S5 and S8) and from the anti-sense expressing transgenic lines (AS5 and AS7) that were earlier confirmed positive based on PCR and RT-PCR results. The genomic DNA was isolated and digested with *EcoR]* restriction enzyme and were fractionated on 1% agarose gel before transferring them onto nylon membrane either by capillary blotting or by vacuum transfer. The DNA on the nylon membrane was made to bind irreversibly by baking at 80°C for 1h and then used for hybridization with radio labeled probe corresponding to maize *apxl* gene. Southern blot analysis revealed the transgene integration and copy number in the five independent transgenic lines tested (Fig. 31).

The 3 *apxl* sense (S1, S5 and S8) and 2 anti-sense (AS5 and AS7) expressing

independent transgenic lines were analyzed by Southern blotting for the presence of transgene. All the plants analyzed showed the presence of transgene. Out of the 5 independent transgenic lines tested, 4 plants (S1, S5. S8 and AS7) showed single copy insertions where as only 1 plant (AS5) showed 3 copies of transgene.

FIGURES

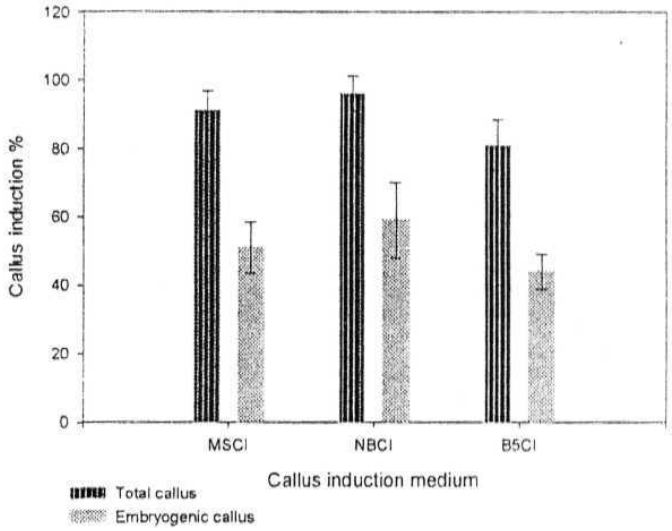

Figure 1. Frequencies of callus induction from mature seeds of cv "Manasarovar" *on* three different callus induction media containing 2 mg/L 2,4-D.

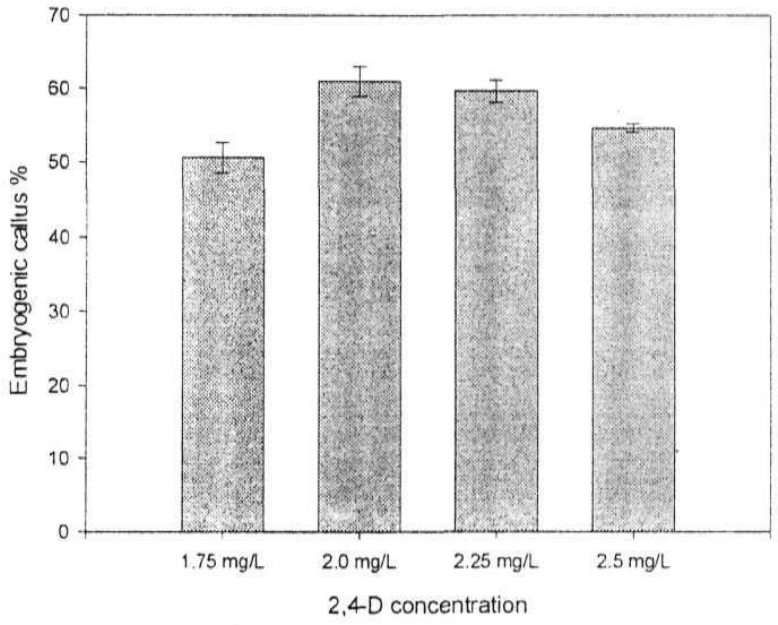

Figure 2. The effect of 2,4-D concentration in inducing embryogenic callus from mature seeds *oi' indica* rice cultivar "Manasarovar".

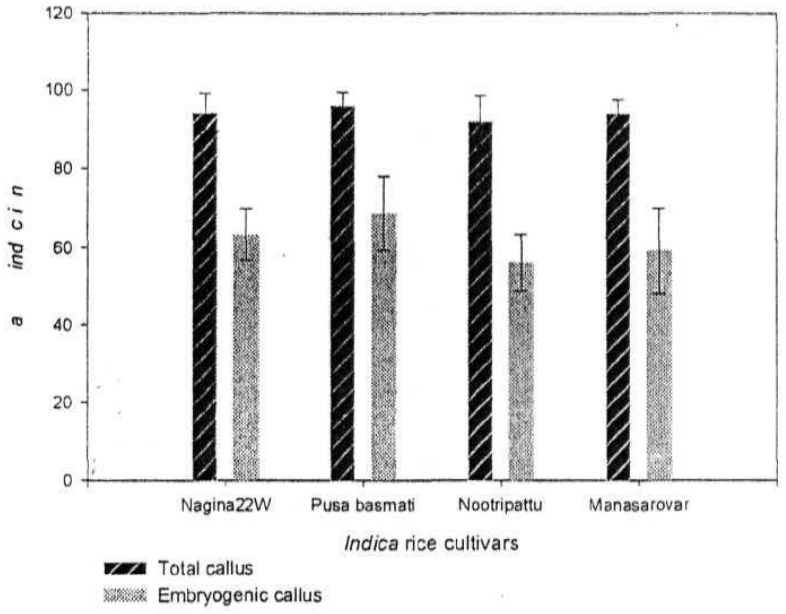

Figure.3. Frequencies of callus induction in four *indica* cultivars tested on NBCI medium containing 2.25 mg/L 2.4-D.

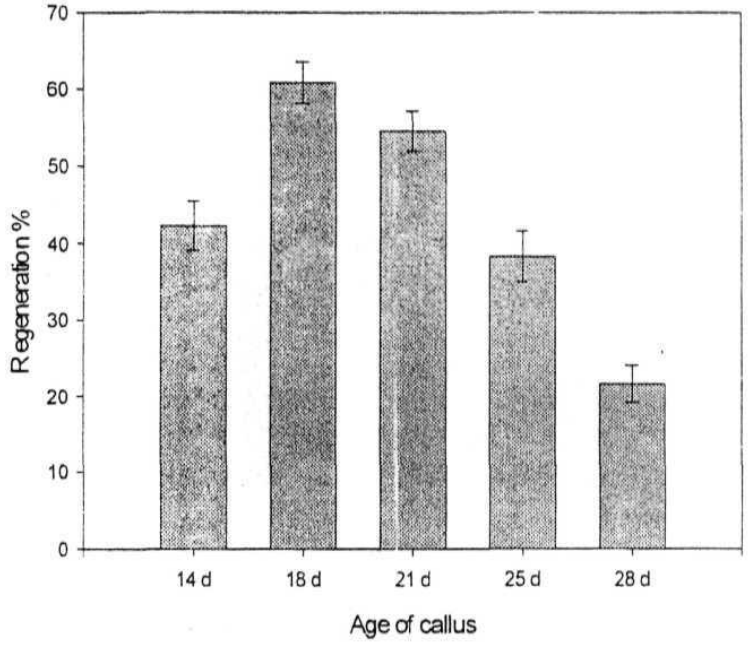

Figure 4. Effect of age of explant on plant regeneration. Calli of different age groups were cultured on NBRE medium containing 3 mg/L BA.

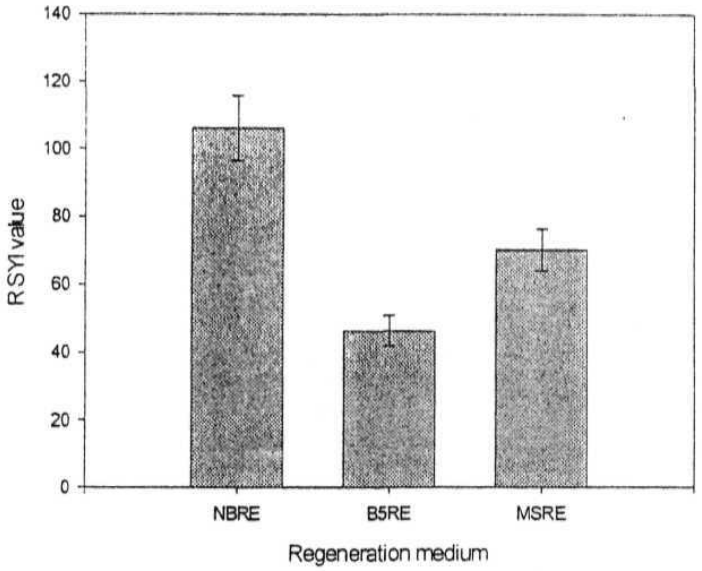

Figure 5. The effect of culture medium on frequency of plant regeneration (in terms of Relative Shoot Yield Index). The 18 d old embryogenic calli (cv Manasarovar) were cultured on three different regeneration media NBRE, B5RE and MSRE containing 3 mg/L BA.

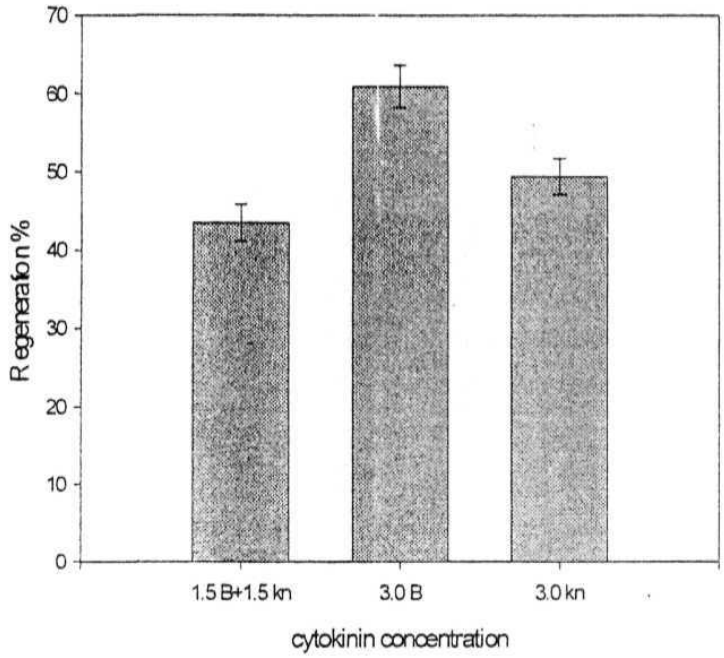

Figure 6. The effect of cytokinin(s) on plant regeneration. Eighteen day old calli regenerated on NBRE medium containing two different cytokinins.

1.5B + 1.5K: NBRE medium containing 1.5 mg/L BA and 1.5 mg/L kinetin.
3.0B: NBRE medium containing 3.0 mg/L BA
3.0K: NBRE medium containing 3.0 mg/L Kinetin

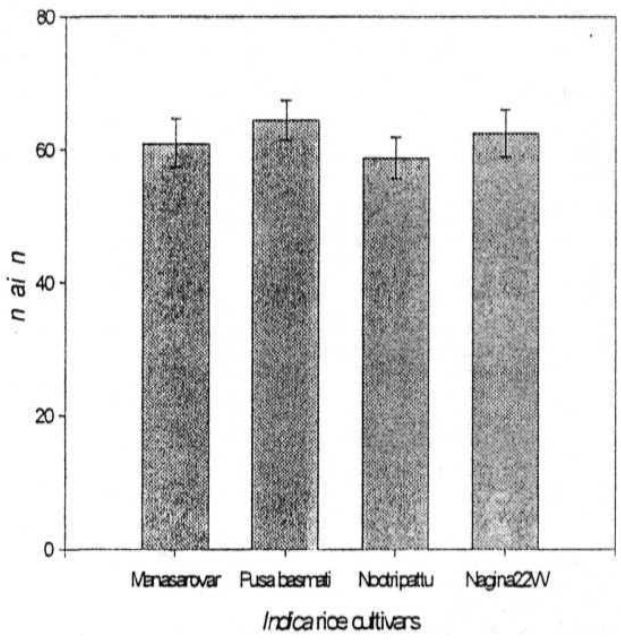

Figure 7. Plant regeneration frequencies of four different *indica* rice genotypes on NBRE medium containing 3 mg/L BA.

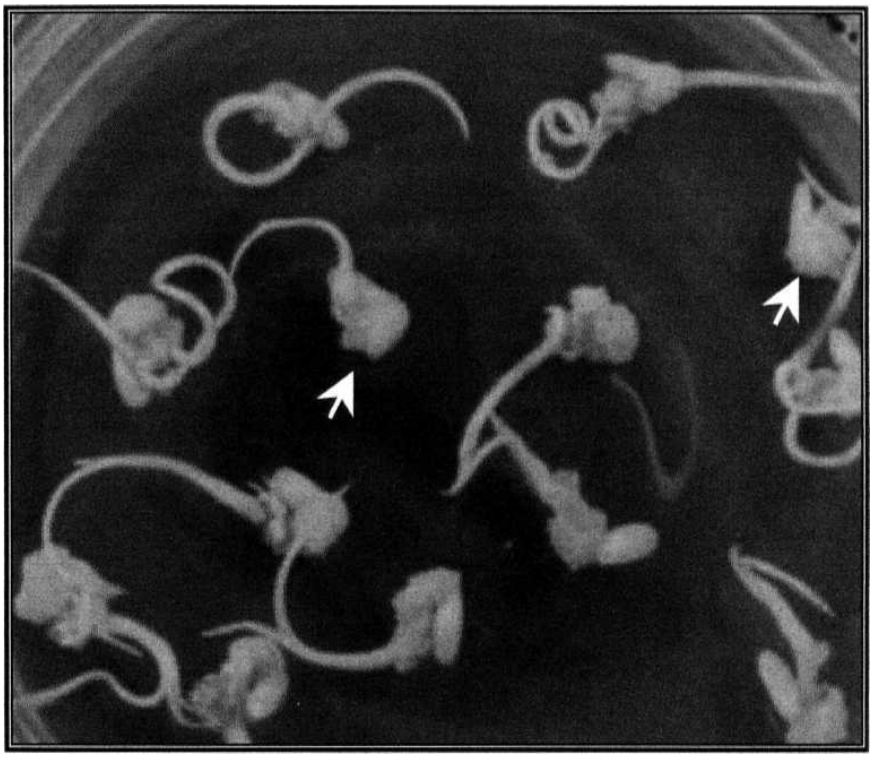

Figure 8. Eighteen day old embryogenic calli proliferating on NBCI medium containing 2.25 mg/l 2,4-D. Arrows point at embryogenic calli that were distinguished from the non-embryogenic calli based on creamy, nodular appearance.

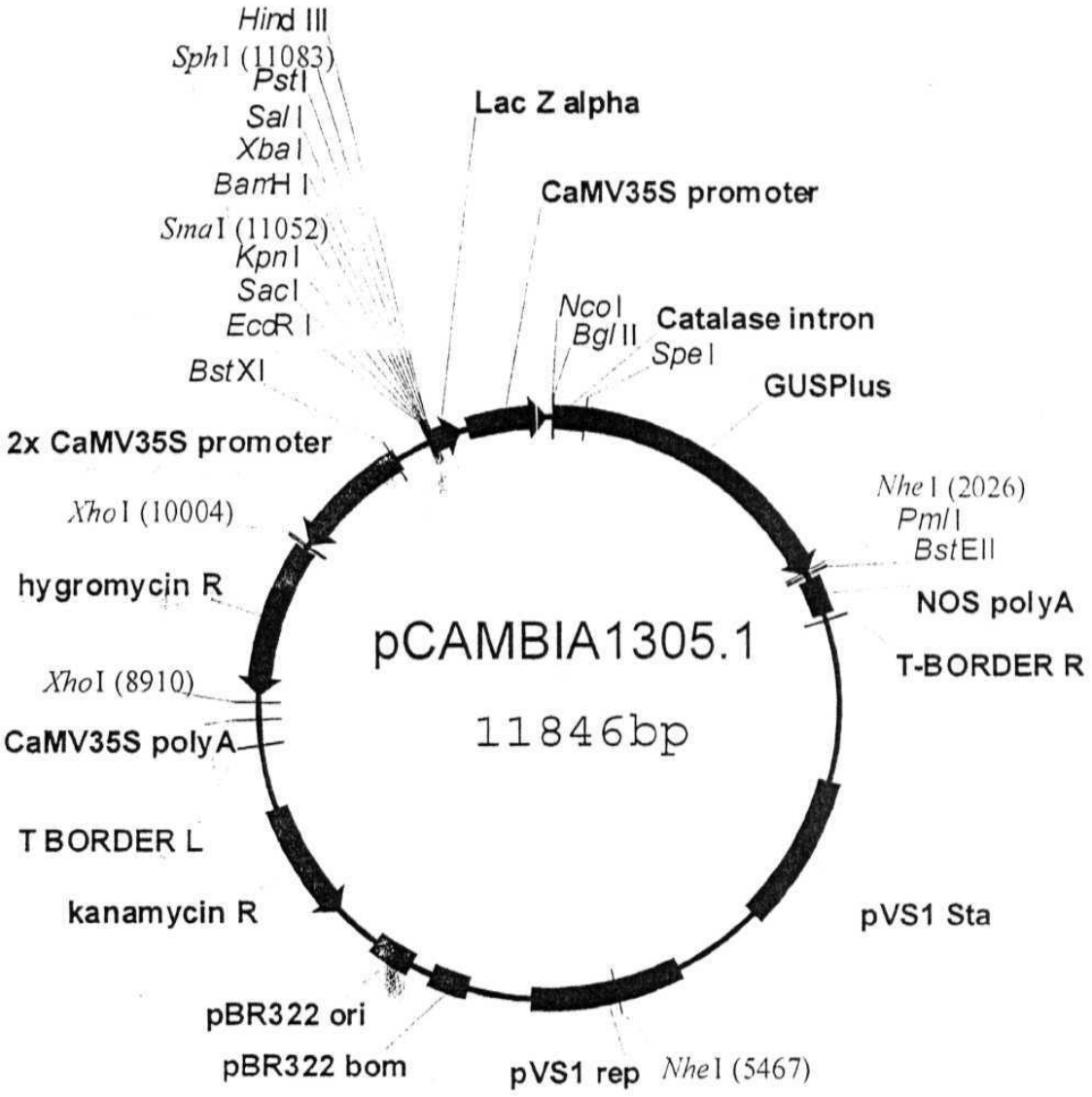

Figure 9. Plasmid map of the binary vector pCAMBIA1305.1, used in *Agrobacterium*-mediated rice transformation.

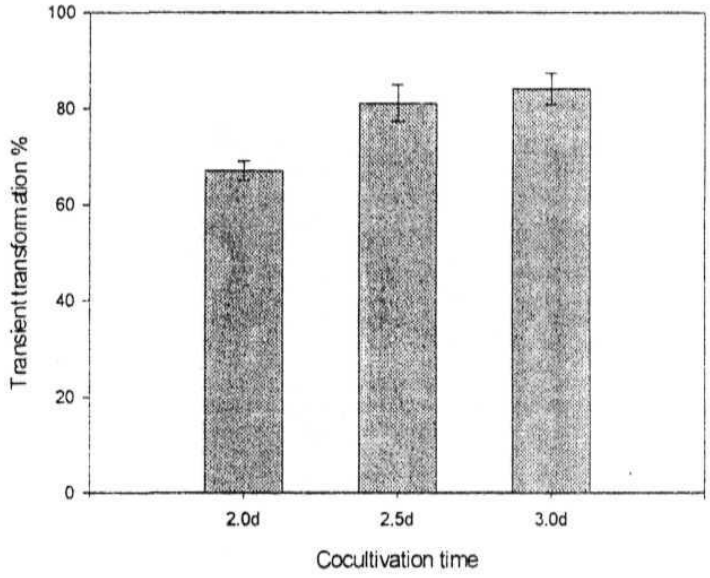

Figure 10. Effect of cocultivation time on transient GUS expression

2.0d: cocultivation carried out for 2 d.
2.5d: cocultivation carried out for 2.5 d.
3.0d: cocultivation carried out for 3 d.

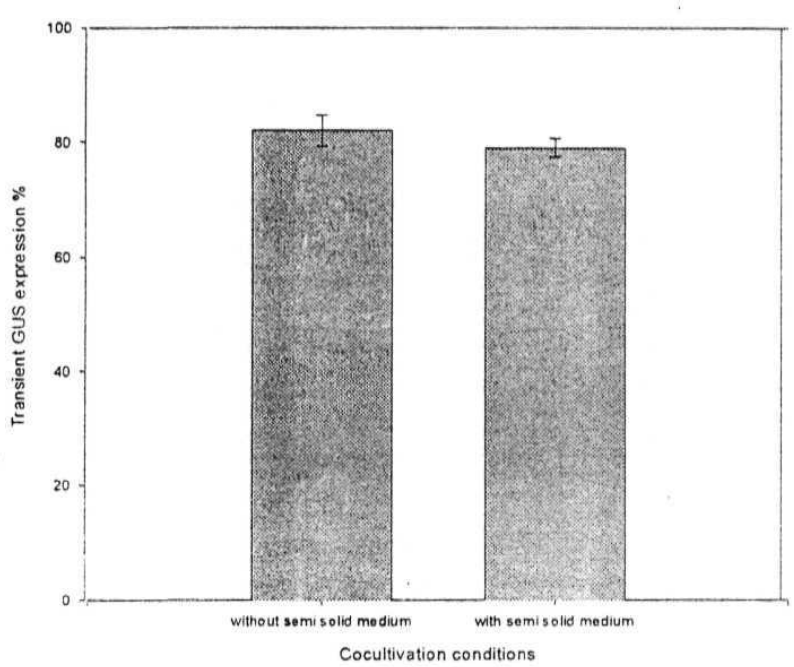

Figure 1.1. Significance of NBCC semi-solid medium on callus transformation during cocultivation. Eighteen day embryogenic calli were cocultivated with and without semi-solid medium.

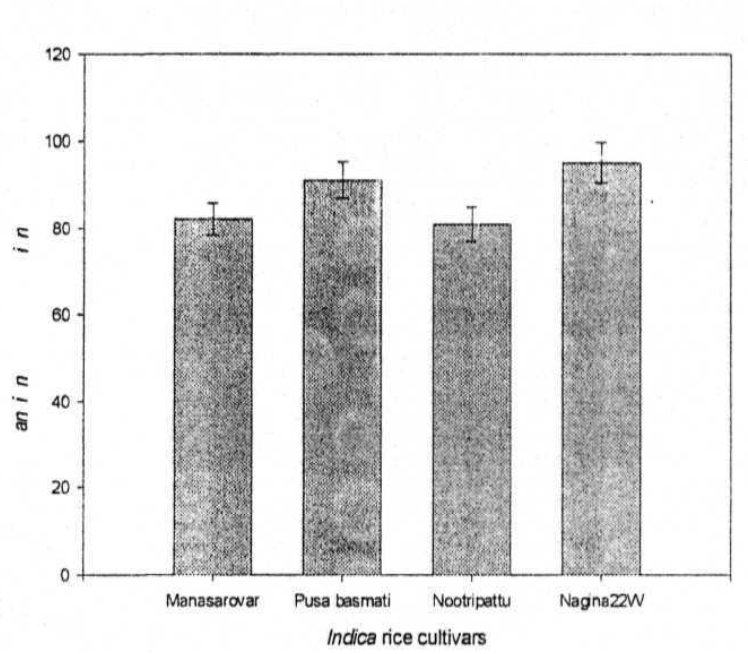

Figure 12. Transformation efficiencies of four indica rice genotypes obtained using NBCC liquid cocultivation medium.

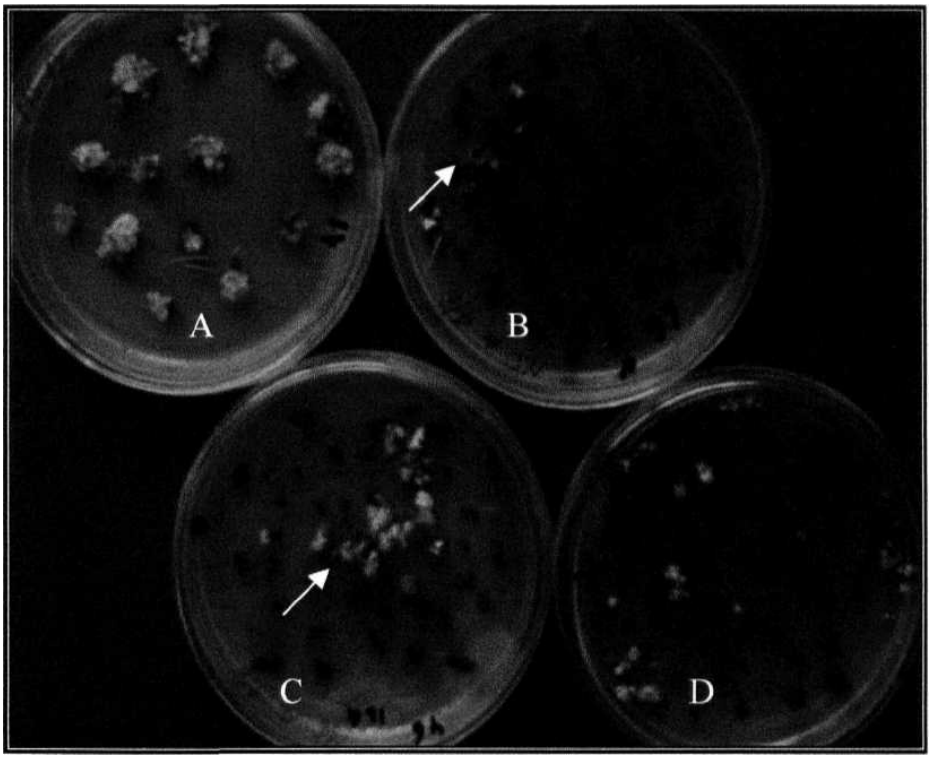

Figure 13. Selective regeneration of transgenic plantlets.

Plate A: Untransformed calli regenerating on NBRE medium (without hygromycin).
Plates B, C and D: Transformed calli regenerating on NBSR medium containing 50 mg/L hygromycin. Arrow shows putative transgenic plants regenerating in presence of selection.

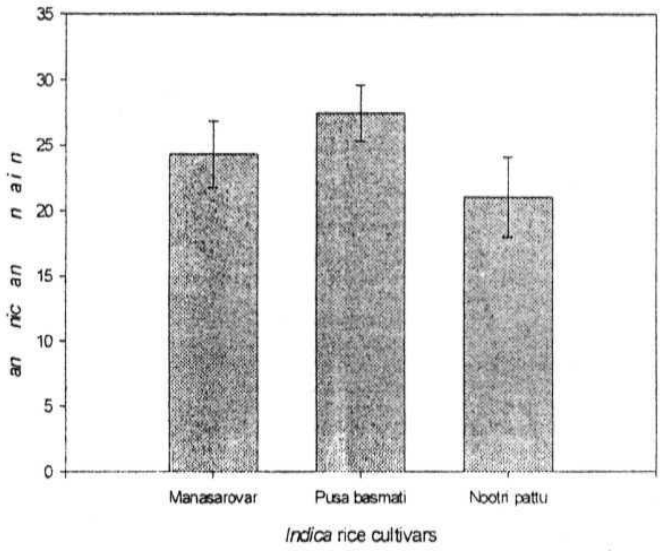

Figure 14. The frequencies of transgenic plant production from the three *indica* rice cultivars.

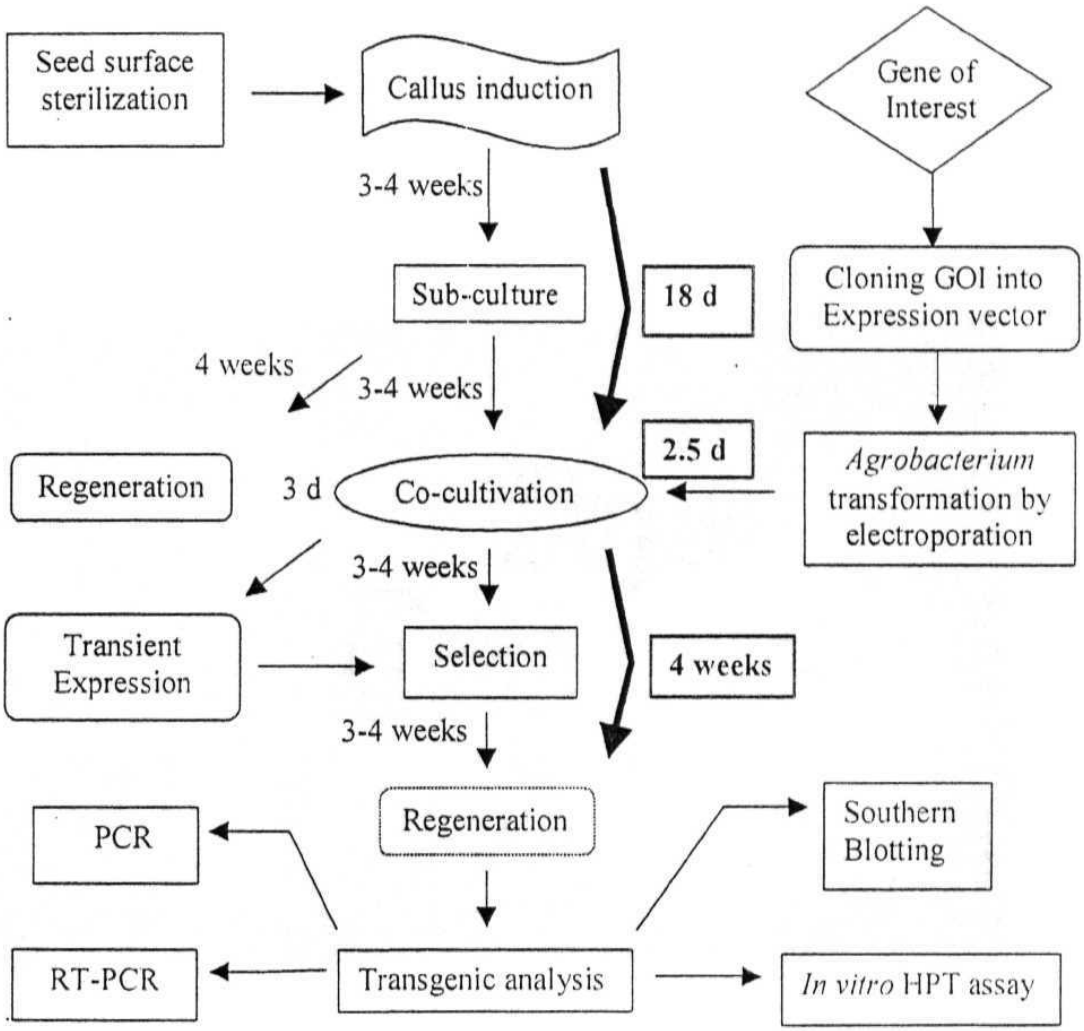

Figure 15. Schematic representation of the various steps involved in *Agrobacterium*-mediated rice transformation. Dark lines indicated the modifications made in the protocol.

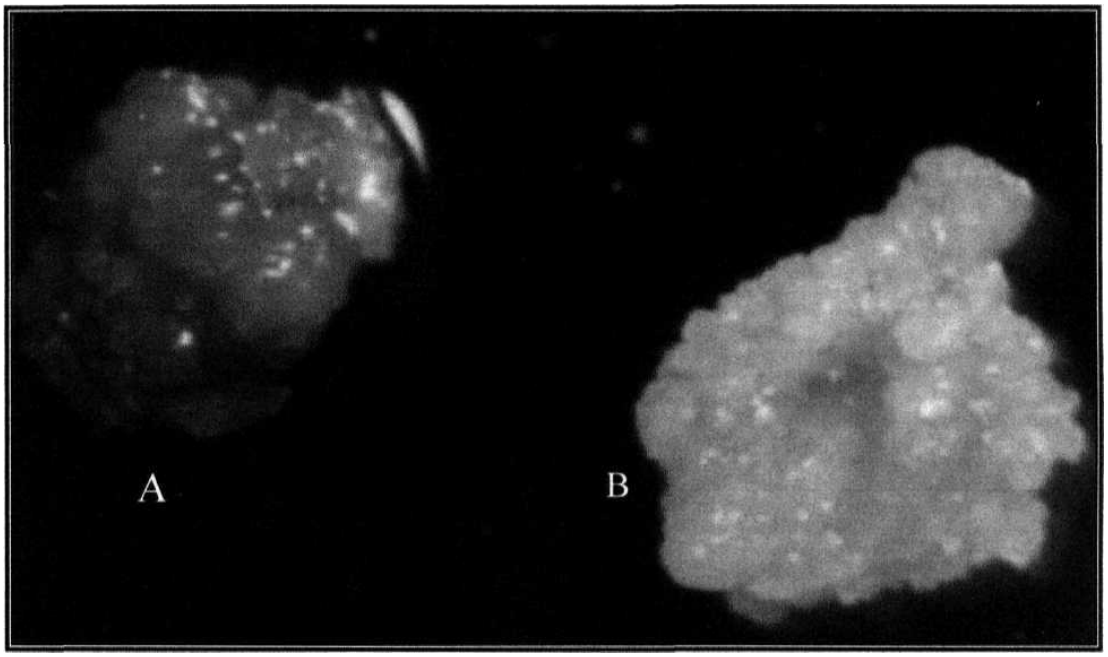

Figure 16. Transient GUS expression in callus observed after 2.5 d of cocultivation.

Callus A: Transformed callus showing GUS expression
Callus B: Untransformed callus showing no GUS expression

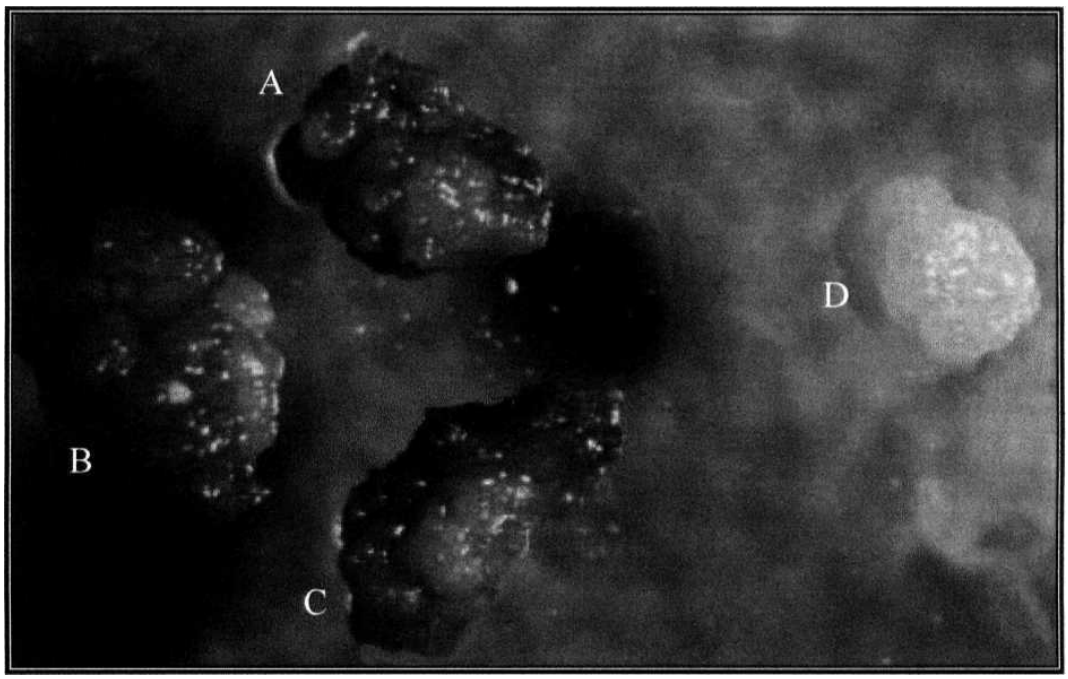

Figure 17. Stable GUS expression in transformed calli

Callus A, B and C: GUS staining in transformed calli that proliferated on NBSR medium Callus D: Untransformed callus showing no GUS expression

Figure 18a

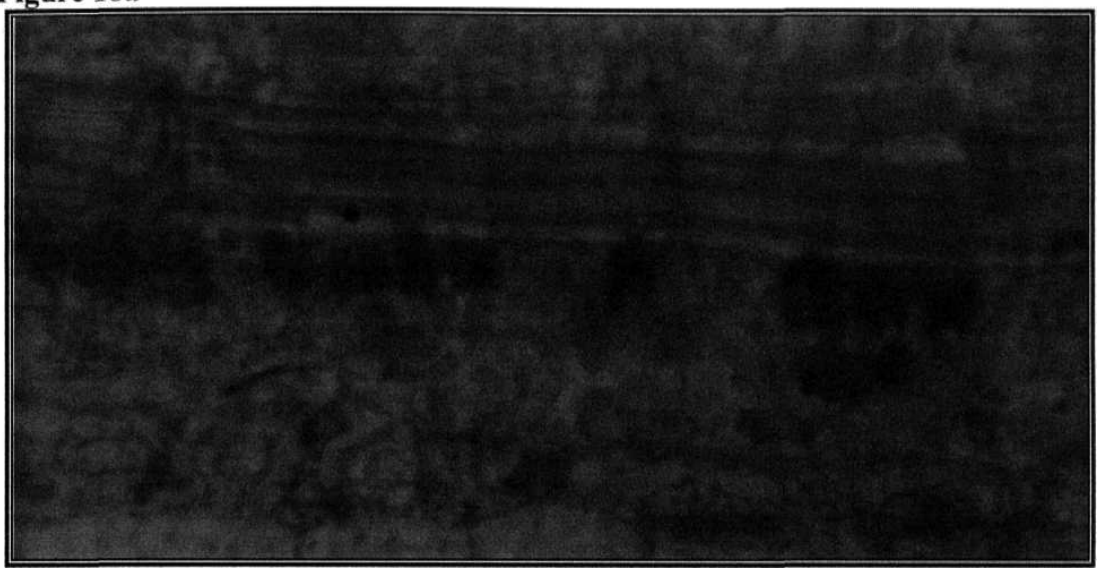

Figure 18b

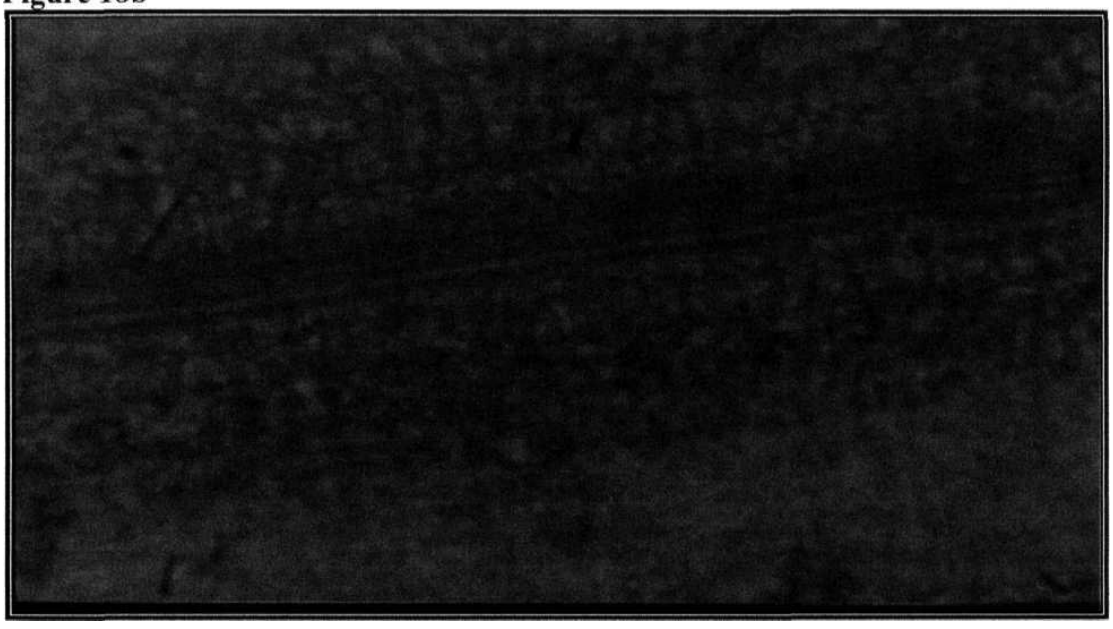

Figure 18a. GUS staining in the vascular region of a juvenile transgenic rice leaf

Figure 18b. GUS staining in the vascular region of a juvenile untransformed rice leaf

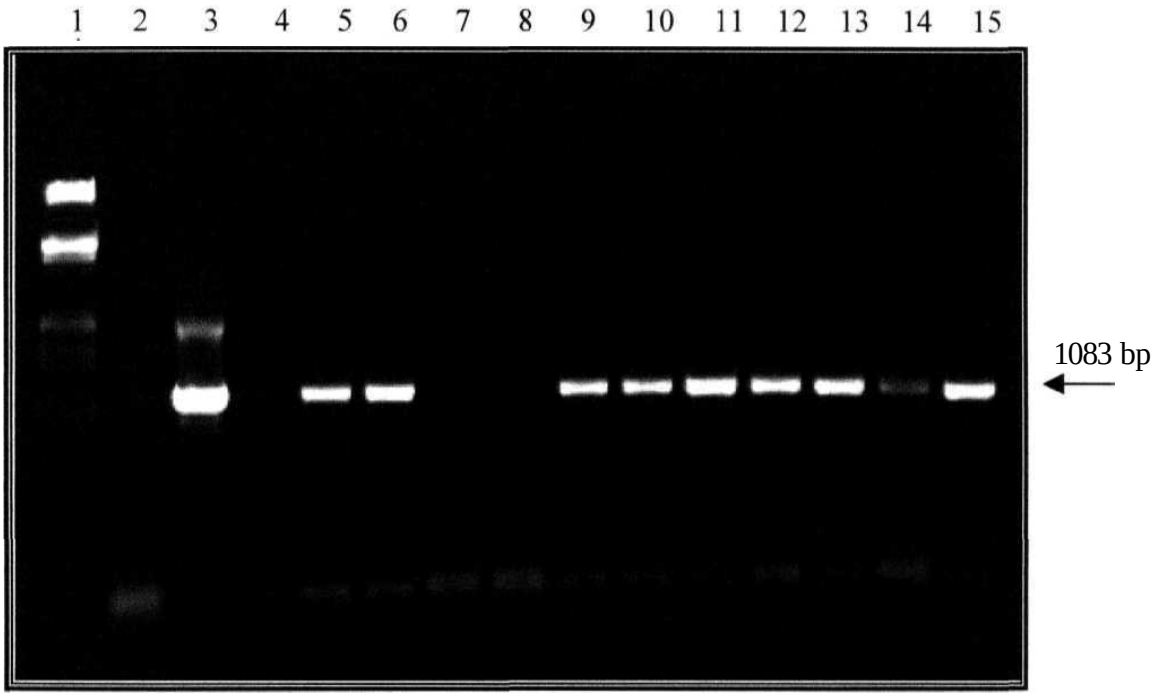

Figure 19. PCR analysis of *hpt* gene in putative transgenic rice plants cv "Manasarovar".

Lane1: *X* DNA, *Eco RJ - Hind HI* double digest. Lane2: Amplification using genomic DNA from untransformed plant (Negative control). Lane3: Amplification using 1 ng of plasmid DNA pCAMBIA1305.1 (Positive control). Lane 4-15. Amplification of *hpt* gene using the genomic DNA isolated from 12 independent putative transgenic plants.

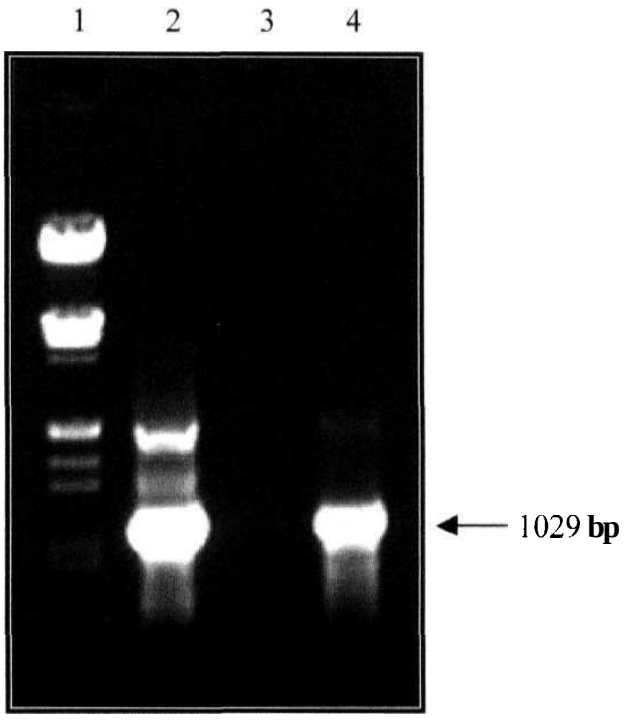

Figure 20. PCR analysis of *uidA* gene in putative transgenic rice plants (cv Manasarovar).

Lane 1: λ DNA *Eco RI - Hind III* double digest. Lane 2. Amplification of *uidA* gene using 1 ng of plasmid DNA, pCAMBIA 1305.1 (positive control). Lane 3: Amplification using genomic DNA isolated from untransformed leaf tissue (negative control). Lane 4: Amplification using genomic DNA isolated from the transgenic plant that showed GUS staining in juvenile leaf tissue.

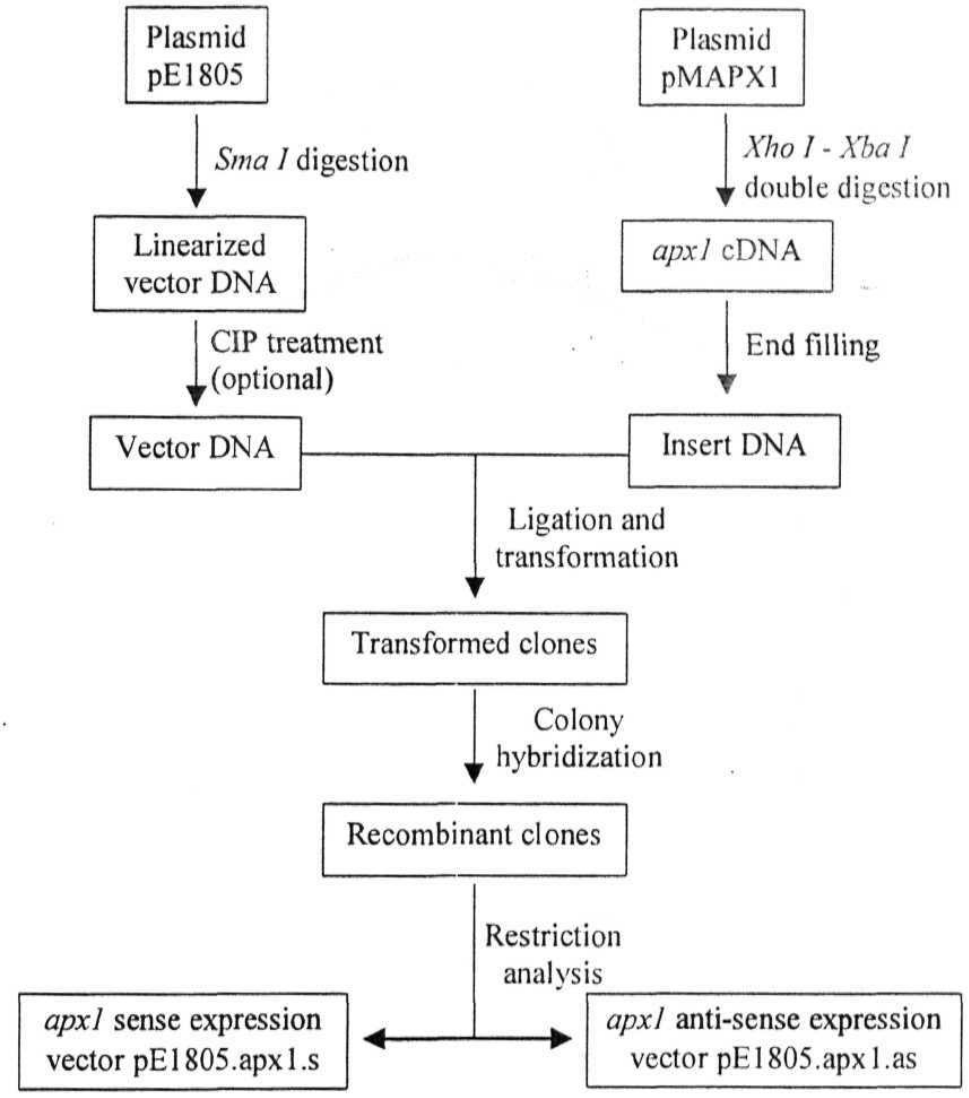

Figure 21. Step wise methods followed in cloning maize *apxl* gene downstream to the super promoter.

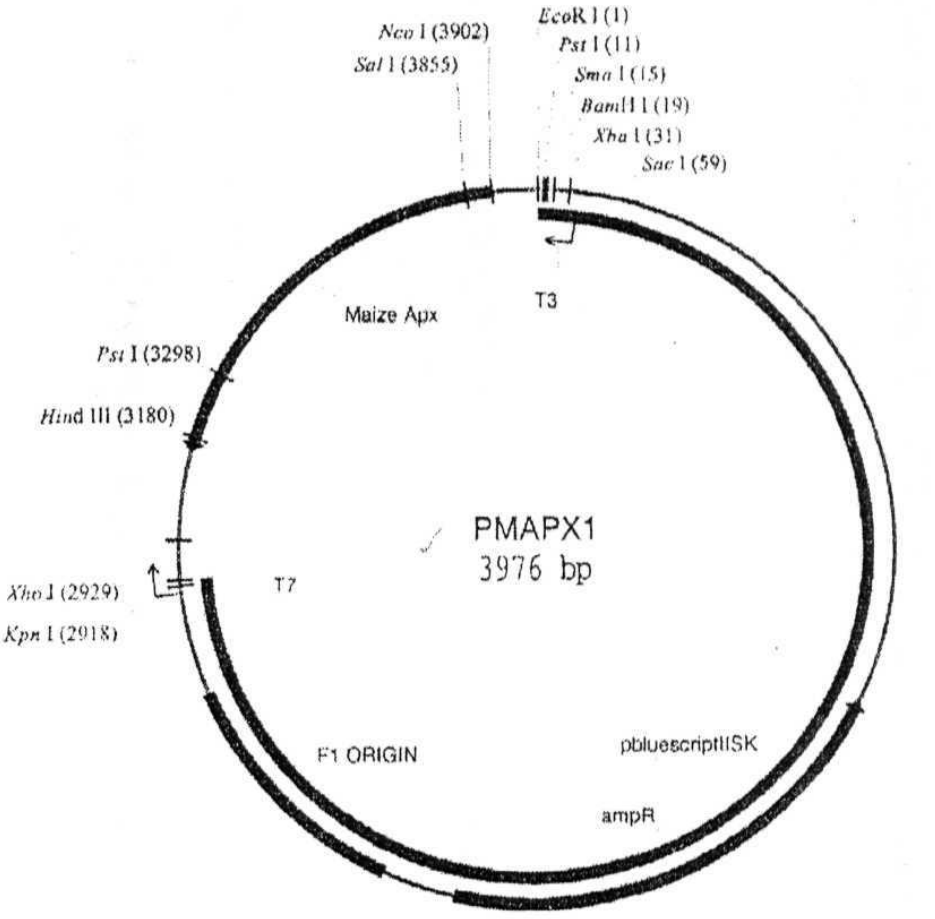

Figure 22. Restriction map of plasmid pMAPXl harboring maize cDNA encoding cytosolic ascorbate peroxidase, *apx1*.

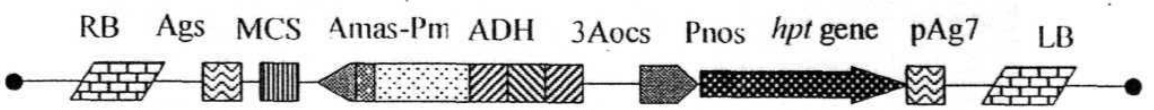

Figure 23a. Diagramatic representation of the T-DNA region of the plasmid pE1805, containing the superpromoter driven plant expression vector.

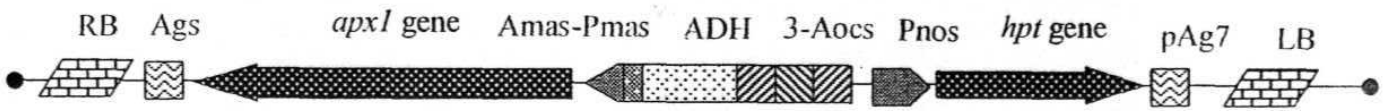

Figure 23b. Diagramatic representation of T-DNA region of the plasmid pE1805.apx1.s, showing the cloning of maize *apx1* gene in sense orientation.

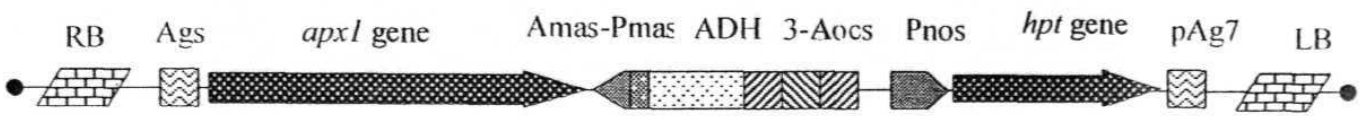

Figure 23c. Diagramatic representation of T-DNA region of the plasmid pE1805.apx1.as, showing the cloning of maize *apx1* gene in anti-sense orientation.

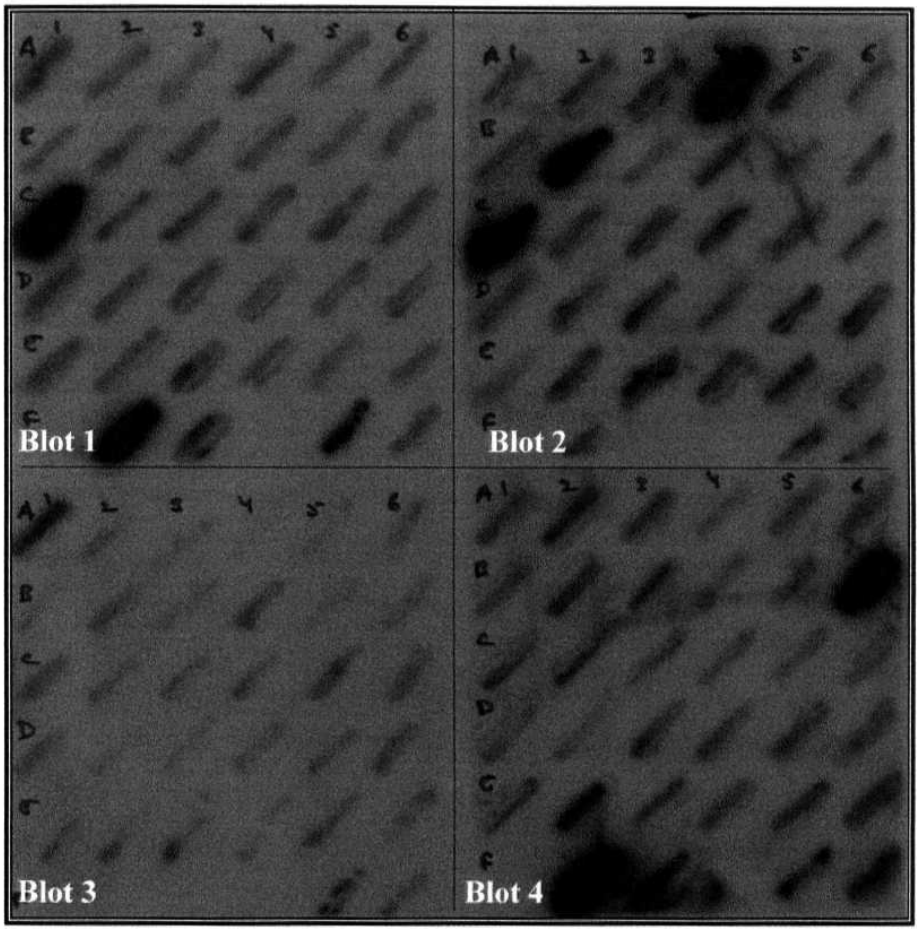

Figure 24. Colony hybridization blots highlighting the recombinant clones having maize *apx1* gene.

The clones Cl and F2 in Blot 1, A4, B2 and Cl in Blot 2, and B6 in Blot 4 are identified as recombinant clones in which the maize *apxl* gene was cloned downstream to a super promoter in plasmid pE1805. The clone F2 in Blot 4 is a positive control having plasmid pMAPX1.

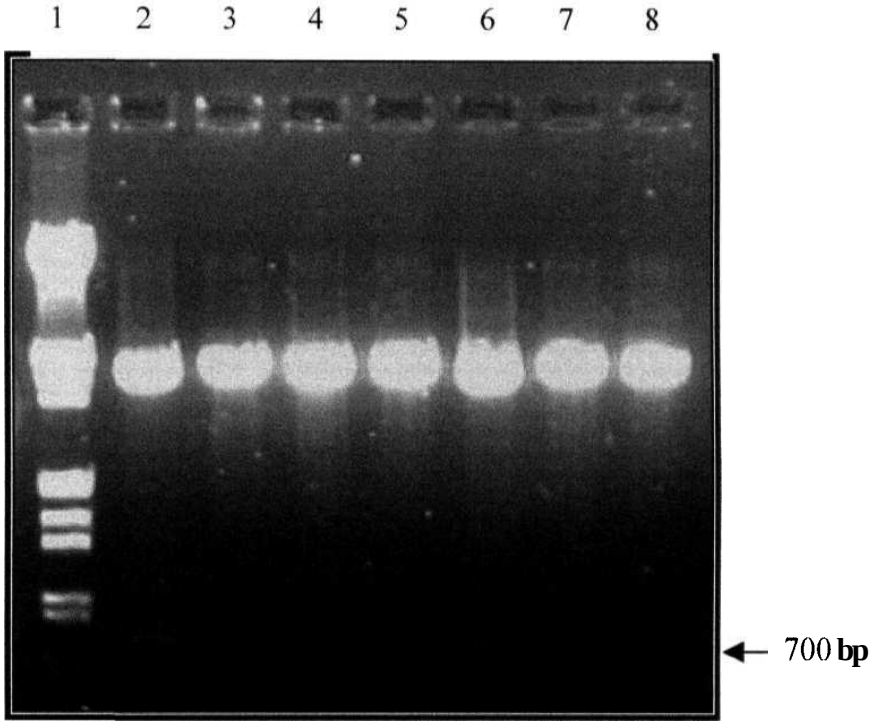

Figure 25. Restriction analysis of recombinant clones. About 500 ng plasmid DNA of each clone was digested with *Pst* 1.

Lane1: λ DNA *Eco RI-Hind III* double digest, Lane 2: clone Cl from blot1, Lane 3: clone F2 from blotl, Lane 4: clone A4 from blot2, Lane 5: clone B2 from blot2, Lane 6: clone Cl from blot2, Lane 7: clone B6 from blot4, and Lane 8: plasmid pE1805.

Figure 26. Fully acclimatized transgenic *indica* rice plants (cv Pusa basmati) carrying maize *apx1* gene.

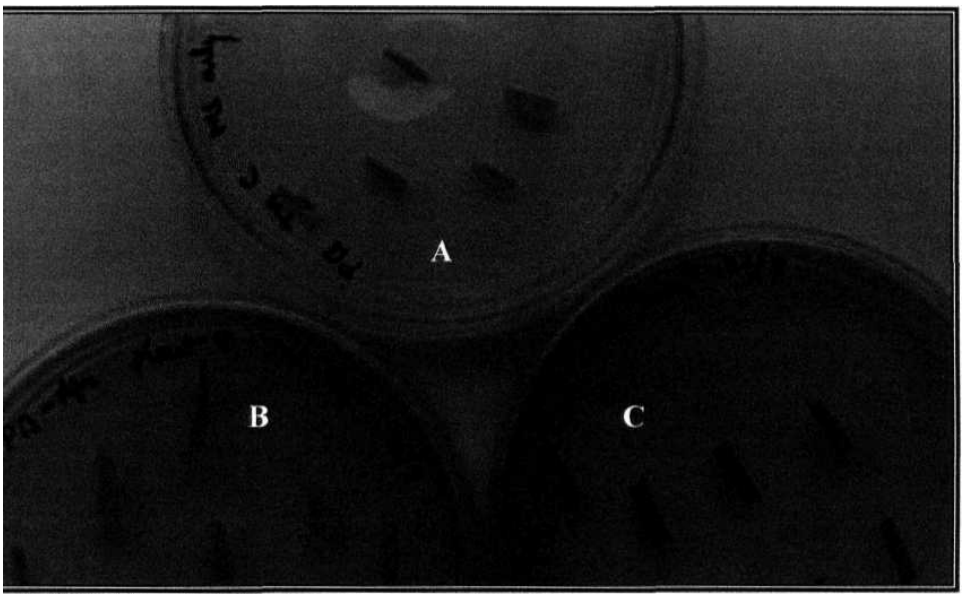

Figure 27. *In vitro* HPT assay using transgenic leaf tissues.

Plate A: Untransformed leaf tissue placed over MS basal medium without hygromycin.
Plate B: Transgenic leaf tissue placed over HPT assay medium containing hygromycin at 100mg/L.
Plate C: Untransformed leaf tissue placed over HPT assay medium having hygromycin at 100mg/L.

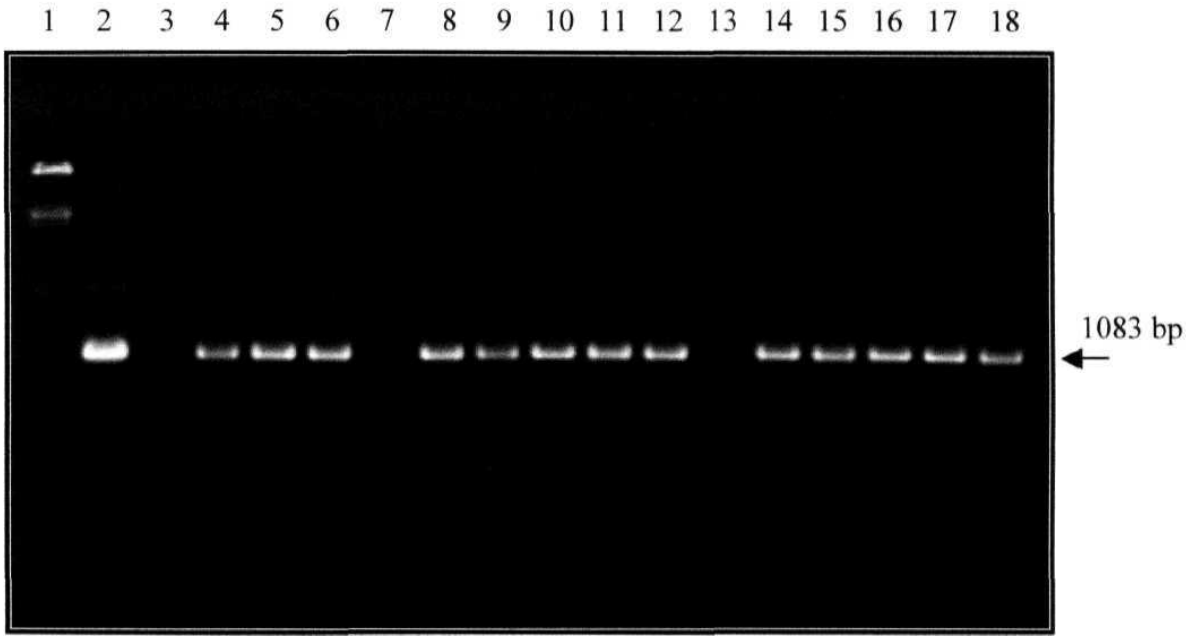

Figure 28. PCR amplification of *hpt* gene in putative rice transgenic plants (cv Pusa basmati) carrying maize *apxl* gene.

Lane 1: λ DNA *Eco RI-HindIII* double digest. Lane 2: Amplification using 1 ng of plasmid DNA pE1805. Lane 3: Amplification using genomic DNA from untransformed plant. Lanes 4 -11. Amplification using genomic DNA isolated from 8 independent *apxl* sense expressing transgenic plants (S1, S2, S3, S4, S5, S6, S7 and S8) and Lanes 12-18: Amplification using genomic DNA isolated from 7 independent *apxl* antisense expressing transgenic plants (AS1, AS2, AS3, AS4, AS5, AS6 and AS7).

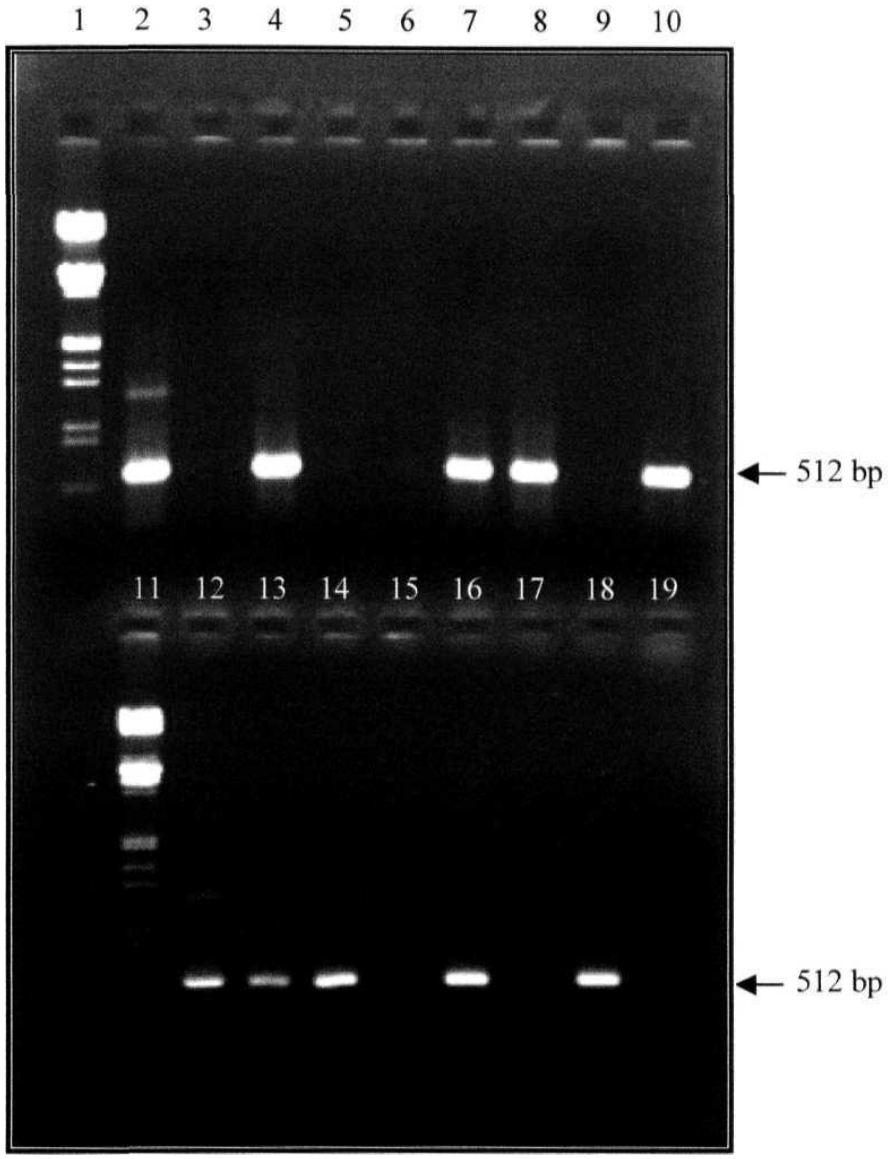

Figure 29. PCR amplification of maize *apxl* gene in transgenic plants.

Lane 1 & 11: *X* DNA *Eco RI-HindIII* double digest, Lane 2: Amplification using 1 ng of plasmid DNA pE1805-apx.s, Lane 3: Amplification using genomic DNA from an untransformed plant Lanes 4-10: Amplification using genomic DNA isolated from 7 *apxl* sense expressing transgenic rice plants (S1, S2, S3, S5, S6, S7 and S8) Lane 12: Amplification using 1 ng of plasmid DNA pE1805-apx.as. Lane 13-18: Amplification using genomic DNA isolated from 6 *apxl* antisense expressing transgenic plants (AS1, AS3, AS4, AS5, AS6 and AS7), Lane 19: Empty.

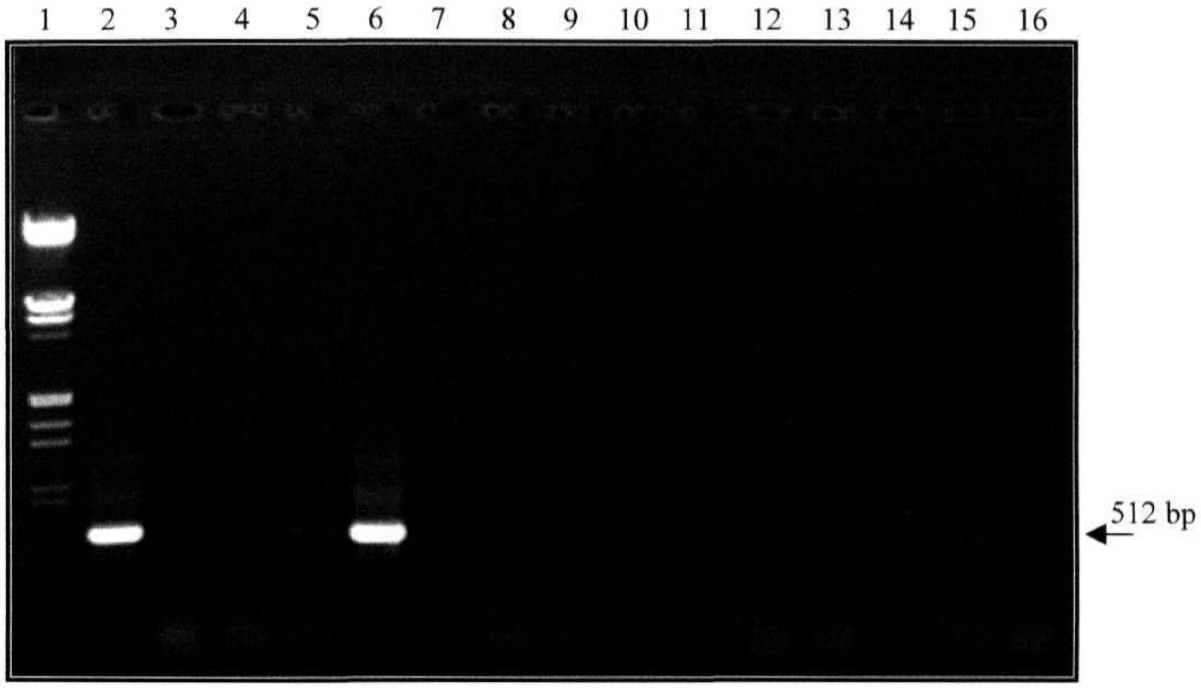

Figure 30. RT-PCR analysis of *apx1* sense and antisense transgenic plants

Lane1: *X* DNA *Eco RI-HindIII* double digest, Lane 2: Amplification using 1 ng of plasmid DNA pE1805-apx.s. Lanes 3-5: Amplification at 3 different Tm values 62°C, 60°C and 58°C, using total RNA isolated from plant S1. Lane 6: Amplification using 1 ng of plasmid DNA pE1805-apx.as. Lane 7: Damaged lane. Lanes 8-11: Amplification using 500 ng of total RNA isolated from 4 independent *axp1* sense expressing transgenic plants (S1, S5, S6 and S8). Lanes 12-15: Amplification using 500 ng of total RNA isolated from 4 independent *apx1* anti-sense expressing transgenic rice plants (AS1, AS3, AS5 and AS7). Lanel6: Amplification using 500 ng of total RNA isolated from an untransformed plant.

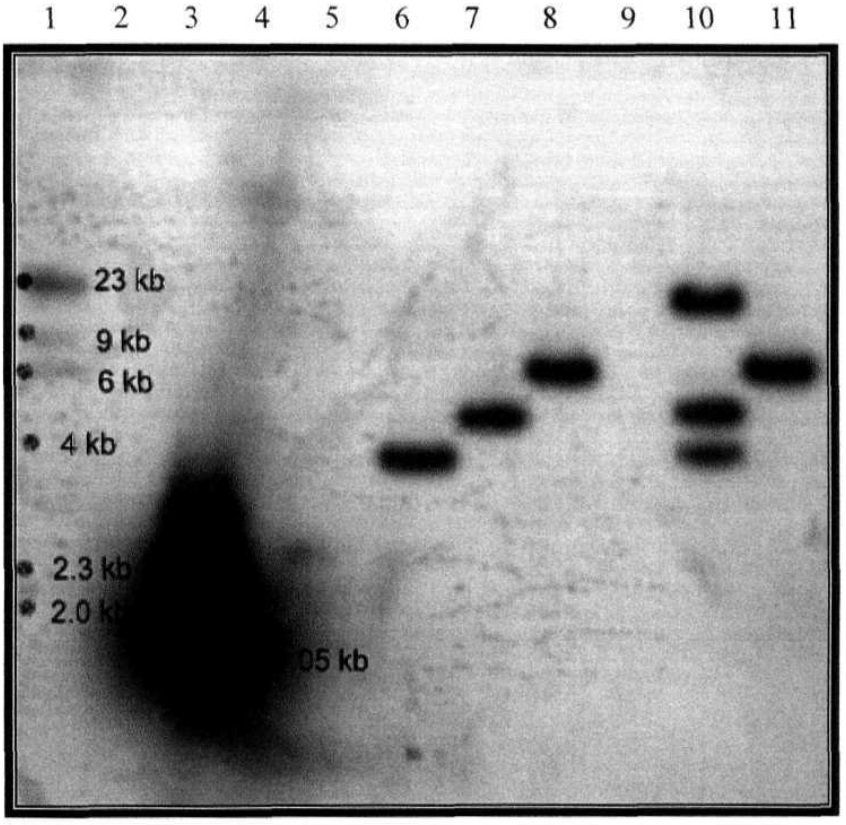

Figure 31. Southern blot analysis of genomic DNA from T_0 transgenic plants of Pusa basmati. About 35 µg of DNA digested with *Eco RI,* was loaded in each lane.

Lane 1: *X* DNA *Hind III* digest. Lane 2: Empty. Lane 3: 5 ng of plasmid pMAPX1 doubly digested with *Xho I - Xha I* to release *apxl* cDNA (1047 bp). Lane 4: Empty. Lane 5: Genomic DNA from an untransformed plant digested with *Eco RI.* Lane 6, 7 and 8: Genomic DNA from 3 independent *apxl* sense expressing transgenic plants S1, S5 and S8, digested with *Eco RI.* Lane 9: Empty lane. Lanes 10 & 11: Genomic DNA from *apx1* anti-sense expressing transgenic plants AS5 and AS7, digested with *Eco RI.*

TABLES

Table 1. List of culture media and their compositions, used in the present study

	NBCI	NBRE	NBCC	NBSR
NB Basal medium	+	+	+	+
2,4-D	2.25 mg/L	-	-	-
BA	-	3 mg/L	3 mg/L	3 mg/L
Acetosyringone	-	-	100 µM	-
Agar	0.8%	1.0%	-	1.0%
Hygromycin	-	-	-	50 mg/L
Cefotaxim	-	-	-	300 mg/L
pH	5.8	5.8	5.2	5.8
Temperature	27±1	27±1	25	25±1

Table 2. NB basal medium - composition.

SL#	Compound	Concentration
	Macro nutrients	
1.	Potassium Nitrate (KNO_3)	2.83 grams / liter
2.	Ammonium Sulphate {$(NH_4)_2SO_4$}	463 mg / liter
3.	Potassium Dihydrogen ortho Phosphate (KH_2PO_4)	400 mg / liter
4.	Magnesium Sulphate ($MgSO_4.7H_2O$)	185 mg / liter
5.	Calcium Chloride ($CaCl_2.2H_2O$)	166 mg / liter
	Micro nutrients	
6.	Manganese Sulphate ($MnSO_4.H_2O$)	10 mg / liter
7.	Boric acid (H_3BO_3)	3 mg / liter
8.	Zinc Sulphate ($ZnSO_4.7H_2O$)	2 mg/ liter
9.	Potassium Iodide (KI)	0.75 mg / liter
10.	Sodium Molybdate ($Na_2MoO_4.2H_2O$)	0.25 mg / liter
11.	Cobalt Chloride ($CoCl_2.6H_2O$)	0.025 mg / liter
12.	Copper Sulphate ($CuSO_4.5H_2O$)	0.025 mg / liter
	Vitamins	
13.	Thiamine Hydrochloride	10 mg / liter
14.	Pyridoxine Hydrochloride	1 mg / liter
15.	Nicotinic acid	1 mg / liter
16.	Ferrous Sulphate ($FeSO_4.7H_2O$)	27.8 mg / liter
17.	Disodium EDTA (Na_2EDTA)	37.35 mg / liter
18.	Proline	500 mg / liter
19.	Glutamine	500 mg / liter
20.	Casein Enzymatic Hydrolysate	360 mg / liter
21.	Myo-inositol	100 mg / liter
22.	Sucrose	30 grams / liter
23.	pH	5.8

Table 3. MSCI medium - composition.

SL#	Compound	Concentration
	MS sulphates	
1.	Copper Sulphate ($CuSO_4.5H_2O$)	0.025 mg / liter
2.	Manganese Sulphate ($MnSO_4.H_2O$)	16.9 mg / liter
3.	Zinc Sulphate ($ZnSO_4.7H_2O$)	8.6 mg/ liter
4.	Magnesium Sulphate ($MgSO_4.7H_2O$)	370 mg / liter
	MS non-sulphates	
5.	Ammonium Nitrate (NH4NO3)	1.65 gms / liter
6.	Potassium Nitrate (KNO_3)	1.9 gms / liter
7.	Potassium Dihydrogen Phosphate (KH_2PO_4)	170 mg / liter
8.	Potassium Iodide (KI)	0.83 mg / liter
9.	Calcium Chloride ($CaCl_2.2H_2O$)	440 mg / liter
10.	Cobalt Chloride ($CoCl_2.6H_2O$)	0.025 mg / liter
11.	Boric acid (H3BO3)	6.2 mg/ liter
12.	Sodium Molybdate ($Na_2MoO_4.2H_2O$)	0.25 mg / liter
	MS organic supplements	
13.	Thiamine Hydrochloride	0.5 mg /liter
14.	Pyridoxine Hydrochloride	0.5 mg / liter
15.	Nicotinic acid	0.5 mg / liter
16.	Myo-inositol	100 mg / liter
17.	Glycine	2 mg/ liter
18.	Ferrous Sulphate ($FeSO_4.7H_2O$)	27.8 mg / liter
19.	Disodium EDTA (Na_2EDTA)	37.35 mg / liter
20.	2,4 Dichlorophenoxyacetic acid	2.0 mg/L
21.	Casein Hydrolysate	500 mg / liter
22.	Proline	500 mg/L
23.	Glutamine	500 mg/L
24.	Sucrose	30 grams / liter
25.	pH	5.8

Table 4. B5Cl medium – composition.

SL#	Compound	Concentration
	Macro nutrients	
1.	Potassium Nitrate (KNO_3)	2.5 grams / liter
2.	Ammonium Sulphate $\{(NH_4)_2SO_4\}$	134.00 mg /liter
3.	Sodium Dihydrogen ortho Phosphate (NaH_2PO_4)	130.44 mg / liter
4.	Magnesium Sulphate ($MgSO_4.7H_2O$)	121.56 mg / liter
5.	Calcium Chloride ($CaCl_2.2H_2O$)	113.23 mg /liter
	Micro nutrients	
6.	Manganese Sulphate ($MnSO_4.H_2O$)	10 mg/ liter
7.	Boric acid (H_3BO_3)	3 mg / liter
8.	Zinc Sulphate ($ZnSO_4.7H_2O$)	2 mg/ liter
9.	Potassium Iodide (KI)	0.75 mg / liter
10.	Sodium Molybdate ($Na_2MoO_4.2H_2O$)	0.25 mg / liter
11.	Cobalt Chloride ($CoCl_2.6H_2O$)	0.025 mg / liter
12.	Copper Sulphate ($CuSO_4.5H_2O$)	0.025 mg / liter
	Vitamins	
13.	Thiamine Hydrochloride	10 mg/ liter
14.	Pyridoxine Hydrochloride	1 mg / liter
15.	Nicotinic acid	1 mg / liter
16.	Ferrous Sulphate ($FeSO_4.7H_2O$)	27.8 mg / liter
17.	Disodium EDTA (Na_2EDTA)	37.35 mg / liter
18.	2,4 Dichlorophenoxyacetic acid	2.0 mg / L
18. •	Proline	500 mg / liter
19.	Glutamine	500 mg / liter
20.	Casein Enzymatic Hydrolysate	360 mg / liter
21.	Myo-inositol	100 mg / liter
22.	Sucrose	30 grams / liter
		—
23.	pH	5.8

Table 5. Composition of GUS staining solution

SL#.	Compound.	Volume added.
1.	X-Gluc 16mg dissolved in one ml of DMSO.	1000 µl
2.	0.5M Phosphate Buffer (pH-7.0).	1000 µl
3.	Potassium Ferrocyanide (32mg/ml stock)	5µl
4.	Potassium Ferricyanide (42mg/ml stock).	5µl
5.	0.5M Sodium EDTA (pH-8.0).	200µl
6.	Sterile double distilled water.	7.790 ml

DISCUSSION

5. DISCUSSION

Transformation or rice has become the most important route for introgressing target genes from diverse sources into rice, these include both input traits and output traits. While the former include the genes conferring resistance to diseases. pests. high/low temperature, drought and salt, the latter include enriched vitamin production. iron, proteins of therapeutic importance and vaccines. It has become clear from the experiments carried out in the past decade or so that the rice transformation protocols are rather cumbersome and usually take more than 12 weeks from seed explant to plantlet. Among the rice genotypes, *indica* varieties are particularly known to exhibit high recalcitrance towards tissue culture methods and therefore, relatively few successful transformation cases were reported. Simple and rapid transformation protocols will save time and cost and greatly facilitate gene transfer into *indica* rices. Our objective is to improve the efficiency of transformation through the development of protocols that require less time and complex operations and can be applied lo diverse genotypes.

In the present study an efficient and reproducible protocol that requires about 6 weeks to produce transgenic rice plants by *Agrobacterium*-mediated transformation was developed. All the experiments in establishing the *indica* rice transformation protocol were carried out using mature seed derived scutellar callus from an elite *indica* rice cultivar Manasarovar, unless otherwise stated. The efficiency of the developed protocol was tested on three randomly selected *indica* rice genotypes

Nagina22W, Nootripattu and Pusa basmati.

5.1 Improved protocols for callus induction and regeneration

Among the three basal media tested for callus induction in cv "Manasarovar". NB medium with 2 mg/L 2,4-D (NBCI) was found to be marginally better with 96% callus induction (Fig.1) compared to MS with 91% and B5 with 81% respectively. Based on the phenotypic observations, the number of embryogenic calli produced and the frequency of embryogenic callus induction, NBCI medium was found to be an effective medium for the tested rice cultivars. The embryogenic and non-embryogenic calli were visually discriminated based on the criteria reported by Rueb *et al.*, (1994).

The production of embryogenic callus with high regeneration potential appears to be largely dependent on 2,4-D concentration. It is well known that all calli will not be embryogenic. The frequency of embryogenic callus formation on NBCI medium was enhanced with 2.25 mg/L 2.4-D (Fig 2). It is clear from figure 2 that with the increase in 2,4-D concentration from 1.75 mg/L to 2.25 mg/L, the percentage of embryogenic callus also increased. However, a marginal decrease was observed using 2.5 mg/L 2.4-D. indicating that a concentration of 2.25 mg/L was ideal for the cultivar "Manasarovar". Also the three other *indica* rice genotypes (Nagina22W. Nootripattu and Pusa basmati) produced similar increased callus induction frequencies when tested on NBCI medium containing 2.25 mg/L 2,4-D (Fig.3). These results suggest that a combination of NBCI medium and 2.25 mg/L 2,4-D is ideal for embryogenic callus induction from the *indica* rice genotypes tested. This is some what different from the

earlier reports of Kumria *et al,* (2001) and Mohanty *et al,,* (1999) which used less concentration of 2,4-D.

As the age of explant is a critical factor that significantly affects the embryogenicity in callus cultures, callus of 5 different age groups, that is. 14. 18, 21. 25 and 28 d old were tested for their regeneration efficiency upon transfer to NBRE medium (Table 1) having 3 mg/L BA. Among the calli of the tested age groups. 18 d old callus cultures showed a high regeneration efficiency of 60.86% (Fig. 4). However, the frequency of regeneration declined with increase in the age of the callus explant, indicating that 18 d old embryogenic cultures carry a high regeneration potential. This stage of the explant was found critical for all the *indica* rice cultivars tested. This result also signifies that 18 d old callus explants needs to be cultured on NBC1 medium containing 2.25 mg/L 2.4-D. These results are different from the earlier reports of (Kumria *et al,* 2001) and (Hiei *et al.,* 1994; Khanna and Raina 1998) which suggested the use of either 21 d or 28 d old callus cultures for *in vitro* regeneration in rice.

The regeneration protocol described here is simple, efficient and reproducible, and the time needed to regenerate transgenic plants is relatively short. Plant regeneration is observable by the end of fifth week. Earlier reports on production of transgenic plants reveal that atleast 12 weeks time is needed to initiate plant regeneration from transformed callus (Hiei *et al,* 1994; Rashid *et al,* 1996: Mohanty *et al,* 1999; Kumria *et al,* 2001; Lee *et al,* 2002). Most of the regeneration protocols mentioned in the above reports either have a pre-regeneration step or a subculture step

before transferring callus to regeneration under light. However, in the present work, such in-between steps were eliminated since the 18 d old primary calli was directly used for regeneration or cocultivation. Use of primary callus directly for regeneration was recently reported by Kumria *et al.,* (2001), however the time needed to regenerate transgenic plants using their protocol was longer than the optimized protocol described in the present work.

,. *In vitro* regeneration from callus cultures was carried out on three different media NBRE, MSRE and B5RE. Plant regeneration was relatively better on NBRE medium containing 3 mg/L BA (Fig. 5). This increased regeneration frequency was accompanied by a high RSYI (relative shoot yield index) value. A high RSYI value signifies higher regeneration with the formation *of* multiple shoots (Rubluo *et al,* 1984). Observations from the preliminary experiments on the effect of cytokinin on plant regeneration using BA and kinetin both independently and in combination (Fig 6) suggest that BA is more suitable for *in vitro* regeneration in the tested rice genotypes.

5.2 Improved protocols for *Agrobacterium*-mediated rice transformation

Agrobacterium-mediated transformation usually requires 3 d of cocultivation. cocultivation was performed for 2.5 d instead of 3.0 d. This decrease in cocultivation time did not significantly affect the transformation efficiency (Fig 10). Instead, it turned out to be beneficial to transformed calli as this facilitated easy removal of *Agrobacterium* using less stringent washes.

Transformation frequency was significantly higher when calli were

cocultivated with *Agrobacterium* on NBCC liquid cocultivation medium (Fig 11). The results indicate that the semi-solid medium used in most of the transformation protocols reported till date was not obligatory for callus transformation. Also the need for a separate cocultivation medium, such as AA medium, was eliminated as callus transformation was achieved using liquid NBCC medium containing 100 uM acetosyringone. Eliminating such optional steps accelerated transgenic production, reduced cost and complexity of operation.

One of the major changes that reduced the time needed for production of transgenic plants is the use of selective regeneration medium. The transformed calli when transferred to selective regeneration medium for two weeks resulted in proliferation hygromycin resistant calli that showed stable GUS expression (Fig 17). Regeneration of transgenic shoots was observed after two weeks when the above hygromycin resistant calli were transferred to NBRE medium containing cefotaxim. Based on the frequency of transgenic plants produced, it is concluded that the developed rice transformation protocol was efficient in transformation of the three *indica* rice genotypes (Fig. 14) tested. Also, this protocol being efficient and relatively short, this protocol for the production of transgenic plants is suited for *indica* rice genotypes.

5.3 Ascorbate peroxidase (Apx1) rice transgenics

In order to assess the developed transformation protocol, we transformed rice

genotypes with maize *apxl* gene that encodes cytosolic ascorbate peroxidase. Accordingly, the maize *apxl* cDNA was cloned in sense and anti-sense orientation downstream to. a super promoter that was reported to enhance gene expression by 156 fold (Ni *et al,* 1995) in tobacco and *Arabidopsis.* The present study using such a promoter is the first report in rice.

Transgenic plants were initially tested for *in vitro hpt* assay using leaf sections (Fig.27). Transgenic leaves remained healthy and green after one week on hygromycin selection medium that was supplemented with 100 mg/L hygromycin-B, while untransformed leaves showed severe necrosis and turned brown, indicating the constitutive expression of *hpt* gene in transgenic plants. These observations are in agreement with the reports of Wang and Waterhouse (1997).

The presence or *hpt* gene was confirmed in 7 independent To transgenic plants (S1, S2, S3, S5. S6, S7 and S8) out of 8 by PCR analysis using primers specific to *hpt* gene (Fig. 28). Similarly, in 6 independent *apxl* antisense expressing transgenic lines (AS1, AS3. AS4. AS5. AS6 and AS7) out of 7 tested revealed hpt specific amplicon. The transgenic lines S4 and AS2 failed to show the corresponding amplification of *hpt* gene and hence were assumed as escapes.

As the *apxl* gene of maize and rice share 80.26% sequence homology, care was taken in designing primers where a majority of the sequence was unique to maize *apxl and* not to rice. A primer sequence of 25 nucleotides was chosen which shared only 60% of its sequence with rice *apxl* gene. These primers specifically amplified a 512 bp amplicon from the maize *apxl* gene in transgenic rice plants (Fig. 29). Since

the primers designed were more specific for maize *apxl* gene. no amplification was observed in untransformed control plants. Further. the rice *apxl* homologue is a large gene with introns and therefore did not lead to amplification under the given conditions. Eight putative transgenic plants (S1, S5, S6. S8. AS1. AS3. AS5 and AS7) showed the expected amplicons. Five of these transgenic plants (S1, S5. S8 ASS and AS7) showed maize *apxl* specific transcript as revealed by RT-PCR experiments (Fig 30). Notably, both sense and anti-sense plants revealed the transgene. However. we have not detected any dramatic increase in transcript in any transgenic plant though all carry super promoter that was expected to enhance expression by more than 156 fold (Ni *et al.*, 1995).

Transgene integration and copy number was confirmed by southern blot hybridization using the maize *apxl* cDNA sequence as a probe. The T_0 transgenic plants S1. S5, S8. and AS7 showed single copy insertions, while the plant AS5 showed multiple insertions (3 copies) of transgene (Fig. 31). Production of transgenic plants carrying multiple copy insertions of the transgene is not uncommon (Hiei *et al.*, 1994). Since no hybridization signals were observed in untransformed DNA. it is inferred that the synthesized radio labeled probe corresponding to the cDNA of maize *apxl* did not cross hybridize to rice *apxl* gene sequence under the stringent hybridization conditions used in the experiments.

The above study demonstrates a well defined rice transformation protocol that results in production of *indica* rice transgenics in the shortest possible time of 6 weeks. All the steps involved in rice transformation were critically evaluated and the protocol

was simplified, **and** made less cumbersome to a considerable extent to minimize the costs involved. The rapid rate with which the *apx1* transgenics were produced is a direct proof for the efficiency of the developed protocol. Further, the general applicability of this protocol was proven by transformation of 4 different rice cultivars. This protocol, however, needs further evaluation by testing it on more *indica* genotypes and analyzing transgenics beyond T_2 generation. We expect this procedure will accelerate the process of *indica* rice transformation in a cost effective manner.

SUMMARY

SUMMARY

The following is the summary of results from the present study.

- Four selected *indica* rice genotypes were tested in the present study for their response to callus induction on different media. The results highlight that NB medium is the most suited medium for *in vitro* culture of all *indica* rice genotypes tested.
- A 2,4-D concentration of 2.25 mg/L was found optimum in inducing embryogenic callus at higher frequencies from the scutellar region of mature rice seeds.
- Among various age groups of the callus tested. 18d old callus cultures showed higher regeneration frequency on NBRE medium containing BA.
- Primary callus produced from the scutellar region of mature rice seeds was directly used for regeneration without any subculture.
- NBRE medium produced high frequency *in vitro* regeneration with the formation of multiple shoots from the embryogenic callus of *indica* rice genotypes. Plant regeneration was observed with a high Relative Shoot Yield Index value.
- Cytokinin. BA at 3.0 mg/L was found effective for *in vitro* shoot regeneration from the scutellar embryogenic callus.
- A cocultivation period of 2.5 d was found optimal for *indica* rice transformation. The decrease in cocultivation time from 3 d to 2.5 d was found

beneficial to calli as more number of calli survived on the NBSR selection medium subsequently.

- Better transient GUS expression was observed in calli that were cocultivated on filter papers using NBCC liquid cocultivation medium.
- Rooting of transgenic plants was accomplished using "M" shaped paper boats in the presence of liquid NB basal medium.
- The optimized rice transformation protocol developed in the present study showed shoot regeneration from transformed calli from fifth week onwards and transformed plants with well developed roots were produced within 2 months.
- All four randomly selected *indica* rice genotypes were transformed using the optimized rice transformation protocol, indicating that this protocol may be applicable for *indica* rice transformation in general.
- Enhanced plant regeneration (27%) was observed in *indica* rice genotype "Pusa basmati".
- Sense and anti-sense *indica* rice transgenics cv "Pusa basmati" carrying maize *apxl* gene were developed using the optimized transformation protocol.
- Rice transgenics carrying maize *apxl* have been demonstrated to be positive by PCR analysis.
- The *apxl* transgene expression was detected in transgenic plants by RT-PCR analysis.

Southern blot hybridization of T_0 transgenic plants revealed both single and multiple copy insertions.

The regeneration and transformation protocol reported here for *indica* rice is efficient for the production of high frequency of transformants and the system has been successfully utilized to produce *indica* rice transgenic plants. Therefore, this system could be generally applicable for the biotechnological improvement of *indica* rice genotypes. More variations in the methodologies are to be tested for a routine application. Further, an in-depth analysis of later generation transgenic plants would finally establish the potential of this protocol in rice transformation.

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