OVER EXPRESSION OF *AmA1*, A SEED ALBUMIN FROM *Amaranthus hypochondriacus* IN RICE TO IMPROVE NUTRITIONAL QUALITY

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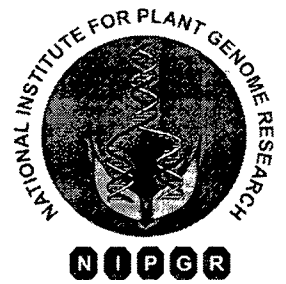

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CERTIFICATE

The research work embodied in this thesis entitled "**Over expression of AmA1, a seed albumin from *Amaranthus hypochondriacus* in rice to improve nutritional quality**" has been carried out at the National Institute for Plant Genome Research, New Delhi. The work is original and has not been submitted so far in part or in full for any degree or diploma of any university.

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Dedicated to my Parents, Wife & Son

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Abbreviations

BCIP	5-bormo-4-chloro-3-indolyl phosphate
Bp	Base pairs
BSA	Bovine serum albumin
DIG	Digoxigenin
2,4-D	2,4-dichlorophenoxyacetic acid
BAP	6-benzylaminopurine
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Gfp	Green fluorescent protein gene
Gus	β-glucuronidase
MS	Murashige and Skoog (1962) medium
Nos	Nopaline synthase promoter
NPT II	Neomycin phosphotransferase enzyme
*npt*II	Neomycin phosphotransferase gene
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	Phenolmethylsulphonyl fluoride
SD	Standard deviation
SDS	Sodium dodecyl sulphate
35S	Cauliflower mosaic virus 35S transcript promoter
Amp.	Ampicillin
dNTPs	Deoxy-nucleotide triphosphate
Kb	Kilo base
KDa	Kilo Dalton
LB	Luria broth
Min	Minutes

Hr	Hour
NAA	Alpha naphthalene acetic acid
Ng	Nano gram
O.D.	Optical density
Rnase	Ribonuclease
NBT	Nitro blue tetrazolium
TAE	Tris acetic acid
TE	Tris-EDTA
UV	Ultraviolate
PCR	Polymerase chain reaction
EDTA	Ethylenediaminotetra acetic acid
µg	Microgram
SSC	Saline sodium citrate
mM	Milli molar
gm	Gram
YEP	Yeast extract peptone
Ml	Milli litre
WHO	World Health Organization
DAP	Days after pollination
RT-PCR	Real time PCR
EtBr	Ethidium bromide
mm	Millimeter
var	Variety
β-ME	β-mercaptoethnol

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Rice (*oryza sativa*, 2n=24, family *Gramineae*) is the most important staple food crop for half of the world's population. The genus *oryza* has 24 species, out of which, 22 species are wild and two are cultivated viz. *sativa* and *glaberrima*. The *sativa* rice is cultivated in Asia, Europe, and America while *glaberrima* is cultivated in West Africa. The *sativa* rice species of the world are commonly grouped into three sub-species viz. *indica* (long grain), *japonica* (round grain) and *javanica*. Rice grown in India mainly belongs to the *indica* sub species. The varieties developed in Japan belong to *japonica* and *javanica, w*hich are cultivated mainly in Indonesia and are differentiated on the basis of grain starch content. Grain starch consists of amylose (linear polymer of glucose) and amylopectin (branched polymer of glucose) which have a major effect on physical properties of the grain. *Indica* rice grain contains 20-30% amylose, while *japonica* contains 17-22% (Shimada *et al.*, 1993). Rice has become a model system for cereal genomic research because of its simple diploid character, relatively small genome (4.2 X 10^8 bp), the availability of whole genome sequence (Yu *et al.*, 2002; Goff *et al.*, 2002; Sasaki *et al.*, 2002; Feng *et al.*, 2002) and several key genome mapping resources (Chen *et al.*, 2002; Wu *et al.*, 2002; Mc Couch *et al.*, 2002).

Rice is cultivated in India since ancient times. In fact, rice was known in India before the present era as per the reports based on the study of Sanskrit and various other languages in South-Eastern Asia. The origin and history of rice probably dates back to the antiquity. As per archaeological evidences and many references, Burma and India are regarded as the centre of origin of cultivated rice. It is predicted that rice has been the first cultivated staple food crop of Asia.

Rice is grown in at least 114 countries and more than 50 countries have an annual production of 100,000 tons or more. Asian countries account for 90% and 92% of world's area and production for rice, respectively. One third of Asia's rice production is consumed in China and one fifth in India. Rice is predominant staple food for 17 countries in Asia and pacific, nine countries in South and North America and eight countries of Africa. The rice producing countries, where annual production exceeds 1 million ton, rice has been the staple food. In Bangladesh, Cambodia, Indonesia, Myanmar, Thailand, and Vietnam, rice provides 50-80% of the total

calories consumed. Few exceptions are Egypt, Nigeria, and Pakistan, where rice contributes only 5-10% of per capita daily caloric intake. Rice ranks third after wheat and maize in terms of worldwide production. Rice provides 20% of the world dietary energy supply, while wheat supplies 19% and maize 5% (FAO, International year of rice 2004, Rice is life).

Rice is the most important crop of India (Fig 1.1) and it occupies 23.3% of gross cultivated area of the country. Rice contributes 43% of total food grain production and 46% of total cereal production. It continues to play vital role in the national food grain supply. Among the rice growing countries in the world, India has the largest area under rice crop (about 44 million ha.) and ranks second in production next to China in year 2003. India and China together accounts for 56% of the total production and about 50% of area of world's under rice during 1997-98, which is reduced to 51% and 46%, respectively in year of 2003. In terms of production, China ranks first in the world and accounts for 30.7% of total production of world during 1999-2003. While India accounts for 21.6% of total rice production of world during 1999-2003. Other important rice producing countries and their respective share to the world's production of rice during 1999-2003 were as follows: Indonesia 8.7%, Vietnam 5.6%, Thailand 4.4%, Japan 1.9%, Brazil 1.8%, United States of America 1.6%, Pakistan 1.2%, European Union 0.4%, Islamic republic of Iran 0.4% and rest of world 21.8%. The rice consumption in Asia is 80 kg/person/year which is much higher than other subtropics such as South America, Africa, and the Middle East, where per capita consumption averages between 30 and 60 kg/person/year.

1.2 Rice Nutritional Properties and Challenges

Rice, a carbohydrate rich food contains 85% carbohydrate, 7% fat, and 8% protein. In comparison to legumes which contain 30-40% protein, rice has much less protein. Moreover, the amino acids composition of rice storage proteins is not balanced with respect to all essential amino acids as recommended by WHO, having low levels of threonine, lysine, and methionine (Takaiwa *et al.,* 1999). In addition, significant parts of the rice storage proteins are indigestible and some albumins are known to cause allergenic reaction in hypersensitive patients (Limas *et al.*, 1990; Matsuda *et al.*, 1991).

A

B

Fig 1.1 (A) Rice crop growing in field
(B) Texture of seeds of different varieties of rice

The worldwide human consumption of protein is estimated as one hundred million tons per year. According to FAO about 70% of this comes from plant sources and rest 30% comes from animal sources, which again feed on seed meal (Report No. 24, FAO, United Nations, 1970). The cost of protein obtained from plant sources is much cheaper than animal. Storage organ, seed is the most important reservoirs among the plant protein sources. But unlike animal proteins (casein and egg albumin) which are nutritionally more balanced with respect to all essential amino acids, plant proteins are generally deficient in some of them. As essential amino acids cannot be synthesized in human beings, they must be supplied from other sources. Thus higher or better quality with reference to seed proteins can have many different meanings, depending upon the crop and its utilization. But by convention, the 'quality' of seed protein refers to how far its amino acid composition matches the balanced amino acid composition recommended for human diet by the World Health Organization (WHO) (Technical Report Series, No. 522, World Health Organization, 1973).

The world population is predicted to increase from 6.1 billion in 2001 to 7.2 billion in 2015 and 8.3 billion in 2030. Same time, rice consumers are increasing at the rate of 1.8% every year. But the rate of growth in rice production has slowed down. It is estimated that rice production has to increase by 50% by 2025 to meet the growing population (Khush and Virk, 2000). Facing pressure from this continuous population growth and shrinking agricultural lands, the challenges are not only to meet the future food needs of humans but also to deal with the need of nutritionally balanced crops/foods. Same time, the price of rice raised due to shortage of supply which limits the availability of it's to poor people. (BBC news, 7th may 2008). Major shortages of basic nutrients in the many parts of world restricted to a diet, based exclusively on staple food include vitamin A, iron, iodine, zinc, quality protein, and deficiencies in these nutrients cause micronutrient and protein-energy malnutrition throughout much of the developing world. Thus enhancing the nutritional quality of crops is an important undertaking for future food security and the nutritional well-being of world population (Biotechnology and Sustainable Agriculture 2006 and Beyond Proceedings of the 11th IAPTC&B Congress, August 31-18, 2006 Beijing, China).

Rice is a major staple crop for around half of the world. However, rice seed contains low amount of protein and its proteins are deficient in some of the essential

amino acids. If protein content and quality of rice is improved, it will be a source of balanced food. To expedite nutritional improvement in rice, genetic engineering can be used as a powerful tool. It helps to facilitate and accelerate the progress towards achieving these multi facted goals of nutritional and economic benefits of rice to farmer (Datta, 2000; 2001). Because, traditional breeding approach in this direction has not been very successful in overcoming the amino acids limitation of cereal and legume seeds (Mertzz *et al.*, 1964; Loesch *et al.*, 1976; Delancey and Bliss, 1991). An alternative approach using protein engineering and gene transfer for amino acid enhancement has been initiated earlier (Ohtani *et al.*, 1991; Dyer *et al.*, 1993). Success in protein quality improvement of rice will be the most significant achievement to elevate the nutritional standard in Asian countries. (Source: Seed Protein, Biochemistry, Genetics, Nutritive value edited by Werner Gottschalk, Kluwer Academic Publishers).

1.3 Seed Storage Protein

Seed protein can be broadly classified into two major categories, *viz.* housekeeping protein and seed storage protein. The housekeeping proteins are responsible for maintaining normal cell metabolism (Millerd, A., 1975). The seed storage proteins are non enzymatic and have the sole purpose of providing proteins to fulfill of nitrogen and sulfur source requirement during germination and establishment of a new seedling. Seed proteins were empirically classified many years ago by Osborne in 1924 on the basis of their solubility as follows:

Albumin: Soluble in water or dilute buffer at neutral pH; (1.6 S-2S).

Globulin: Soluble in dilute alkali or acid solution; (7S-13S).

Glutelin: Soluble in aqueous alcohols.

Prolamin: Soluble in 70-90% aqueous alcohols and is a major storage protein of the cereals.

Other protein storage bodies includes

Lectin: Capable of binding sugars and of agglutinating red blood cells; may have defensive role.

Late embryogenesis abundant (LEA): Highly hydrophilic proteins that accumulate late in seed development.

Albumins and globulins comprise the storage proteins of dicots (*e.g.* pulses), whereas prolamin and glutelin are major proteins in monocots (e.g. cereals). Table 1 depicted the amino acids composition of some representative seed storage protein with comparison to WHO recommended value for storage protein for crop improvement. The seed storage proteins can be distinguished from other proteins by some of their characteristics.

1. These accumulate in high amounts in seed during mid-maturation stage of seed development and are used up during germination.
2. These are synthesized only in the seed (in cotyledon or in endosperm) and not in other tissues.
3. They lack any other functional activity besides storage.
4. They are deposited mostly in special storage organelles called protein bodies.

Table 1.1: Amino acid composition of some seed proteins (mole %) and comparison with WHO recommendation for essential amino acids

Amino Acid	WHO	Maize 2S	B.Comp. (Yellow sarson) 2S	Chenopodium 2S Protein	Amaranthus AmA1	Pea Legumin
Ala (A)	-	13.7	4.2	4.3	5.3	6.0
Arg (R)	-	1.2	4.2	7.5	5.3	10.0
Asx(B)	-	4.7	1.6	5.0	16.6	13.1
Cys(C)	-	Trace	6.6	5.8	0.7	1.2
Gly(G)	-	2.1	6.6	5.8	12.0	6.9
His(H)	-	0.9	3.3	5.7	3.3	1.8
Ile(I)	4.0	4.1	4.2	3.8	5.0	4.0
Leu(L)	7.0	20.0	6.6	3.0	7.6	7.6
Lys(K)	5.5	0.2	6.6	4.9	6.6	4.2
Met(M)	3.5*	Trace	3.3	2.8	1.6	0.7
Phe(F)	6.0**	6.3	1.6	5.4	5.6	2.3
Pro(P)	-	9.7	8.2	6.1	3.6	5.5
Ser(S)	-	6.6	4.9	5.6	6.6	5.8
Thr(T)	4.0	2.4	3.3	5.1	5.3	3.1
Trp(W)	1.0	N.D.	0.82	N.D.	2.3	-
Tyr(Y)	**	3.1	0.82	3.0	4.8	3.6
Val(V)	5.0	3.6	4.9	3.4	5.6	5.0

†, Derived from DNA sequence; N.D., not determined; *, Met plus Cys; **, Phe plus Tyr.

Adapted: Surekha mandal and R.K. Mandal, CURRENT SCIENCE, VOL. 79, No. 5, 10 September 2000.

1.4 Transgenic Plants as a Factory for Human Useful Recombinant Protein

Plant system is potentially a cheap source of recombinant products (Whitelam *et al.*, 1995; Parmenter *et al.*, 1995). It is estimated that cost of recombinant protein production in plants could be 10 to 50 fold lower than producing the same protein by *E. coli* fermentation, depending upon the crops (Kusandi *et al., 1997*). The advantages of recombinant protein production in plants are described below.

1. Recombinant protein production in plants is economically cheaper than any other system.
2. Recombinant protein can be directed in seed endosperm, which can be easily collected and stored.
3. Post translation processing system of mammals is much closer to plant in comparison to microorganism.
4. Large scale production of recombinant protein is possible in plants.
5. Health risk due to contamination and other toxins are minimized.

There are two strategies used for recombinant protein production in agriculture system. **First strategy** includes; stable integration of recombinant protein gene in genome of crop plant using different method, *viz.* *Agrobacterium* mediated transformation, particle gun or other direct gene transfer methods. CaMV 35S promoter is frequently used to express recombinant proteins in constitutive manner. *Nicotiana tobaccum* is widely used as a model system to express a recombinant protein but same time other plants as well as staple crops have been used, including rice (Ganz *et al.*, 1996; Burkhardt *et al.*, 1997; Stoger *et al.*, 2000), potato (Chakraborty *et al.,* 2000; Ganz *et al.*, 1996; Tacket *et al.*, 1999; Tacket *et al.*,1997; Ma *et al.*,1997; Dixon *et al.*, 1997; Artsaenko *et al.*, 1998), wheat (Stoger *et al.*, 2000), Ethiopian mustard (Boothe *et al.*, 1999; Chaudhary *et al.*, 1998), oilseed rape (Boothe *et al.*, 1999; Vandekerckhove *et al.*, 1989; Goddijn *et al.*, 1995), turnip, banana (Dalsgaard *et al.*, 1997; Doran *et al.*, 2000) etc.

Second strategy is to infect non transgenic plant with viruses, which express a recombinant protein during their replication in host crop plant. The two host-virus systems which are frequently used are; Tobacco with tobacco mosaic virus (TMV) and cow pea with cowpea mosaic virus (CPMV). This strategy is particularly useful

for vaccine and biopharmaceutical production in crop plants. Vectors derived from TMV have been used for production of oral antihypertensive peptide (angiotensin-1-converting enzyme inhibitors) in tomato and potato and inhibitor of HIV replication (alpha-trichosanthin) in *N. bethemiana* (Kumagai *et al.,* 1993). Chimeric CPMV particles, expressing human rhinovirus -14 and HIV-1 have been purified from cow pea and found to be eliciting antibody production and to neutralize the infection of T cells by HIV-1 *in vivo*, respectively (Dalsgaard *et al.,* 1997).

1.5 Improvement of Protein Quality in Crop Plant

Protein is considered as a most important nutrient for human and animals, as manifested by the origin of its name from Greek word *proteios* for primary. The protein is made up of 20 amino acids among which 10 are essential because animals including humans are incapable of synthesizing them; consequently these 10 amino acids have to be supplemented from diet. However, for efficient protein synthesis, amino acids derived from ingested protein must be present in balanced amounts. Lysine is the most limiting amino acid in cereal grains, which are commonly used as a principal energy source in humans and livestock. Conversely, legume seeds, which are important sources of protein in human and animal's diet, generally contain an adequate supply of lysine, but are deficient in the sulfur containing amino acids, methionine and cysteine. Animals can convert methionine to cysteine, but not the converse, and therefore methionine can supply the complete requirement for S-amino acids but cysteine cannot. The S-amino acid manifested intake of animal must consequently contain a minimum amount of methionine. For example, for optimum performance of growing pigs, dietary protein must contain 3.5% by weight, of S-amino acids of which 1.6% must be methionine, the remaining 1.9% can be either methionine or cysteine. People who are dependent on vegetarian diet of limited diversity have detrimental consequences on developmental aspects. Amino acid imbalance can have detrimental effects on the physical and mental development of children and can irreversibly retard overall growth and development. *Kwashiorkor* is a disorder cause by protein deficiency in infants.

1.5.1 Strategies for Improvement of Protein Quality in Crop Plant

The protein of important staple crops must be enriched with essential amino acids, as deficiency of its limits the growth of humans and many livestock and limit the efficient utilization of more abundant amino acids in our foods and feeds. Ideally, the protein of the most important crops should be tailored to the specific dietary needs of the humans and livestock for which they are used (William R. Folk, 2003). Appropriate applications of biotechnology can help address problems by improving cereal and nutritive value.

"Nutritional genomics" is a term referring to a combination of biochemistry, genetics, molecular biology and genome-based technologies to investigate and manipulate plant compounds with nutritional value (Tian and Della Penna, 2001). This new technology has been applied to rice in two instances: golden rice and iron-enhanced rice (Tina *et al.*, 2001). Three basic strategies are being used to engineer improved content of storage proteins in co-operation with balanced essential amino acid content in plant storage organs like seeds or roots.

The **first strategy** involves engineering of the amino acid metabolism in order to increase the free amount of the respective essential amino acid. The transgenic plants were made by expressing the aspartate kinase (AK) isoenzymes and/or AK dihydrodipicolinate dehydrogenase (DHDPS), which are the key regulatory enzymes in the pathway of isoleucine, lysine, threonine, and methionine biosynthesis. In these transgenic plants the amount of free Thr and Lys were increased (Galili *et al.,* 1995; Karchi *et al.,* 1993). Seed specific expression of aspartate kinase and dihydrodipicolinate resulted in more than 100 fold increase in free lysine in transgenic soybean (*Glycine max*) and canola (*Brassica napus*) seeds, equating to nutritionally significant increase in total seed lysine 25% and 100% respectively (Falco *et al.*, 1995).

The **second strategy** involves engineering the endogenous storage proteins by *in vitro* mutagenesis to mutate appropriate amino acid codons into essential amino acid codons such as Lys and Met or to insert stretches of additional codons of these amino acids (De Clercq *et al.,* 1990; Randall *et al.,* 2000; Vandekerckhove *et al.,* 1989; Coleman *et al.,* 1997). For example, a gene for β-phaseolin from *Pheseolin vulgaris* was modified by the addition of a 45 bp nucleotide sequence encoding a

methionine rich region from maize and 15 KDa zein storage protein. The added peptide was predicted to form an alpha-helical structure and was inserted into the alpha-helical region of phaseolin. The modified phaseolin protein had 9 methionine residues instead of 3 in wild type. The modified phaseolin gene was expressed at the same level as wild type gene in seeds of transgenic tobacco as measured by the mRNA abundance. However, modified high methionine phaseolin accumulated to much lower concentration than the wild type protein. It was concluded that high methionine phaseolin was unstable in the developing seed (Hoffman *et al.*, 1988).

The **third strategy** involves transferring genes encoding storage proteins with a high content of essential amino acids. The gene *AmA1* from *Amaranthus hypochondriacus,* Met-rich 2S albumins from seeds of Brazil nut, sunflower and *Arabidopsis*, Met-rich zeins from maize and high-molecular-weight glutelin subunits from wheat, have been correctly integrated and expressed in transgenic plants (Chakraborty *et al.*, 2000; Altenbach *et al.,* 1992; Altpeter *et al.,* 1996; Rafiqul *et al.,* 1996; Coleman *et al.,* 1996; Blechl and Anderson 1996; Alvarez *et al.,* 1998, Sharma *et al.,* 1998). The allergenic properties of certain proteins, such as Brazil nut 2S albumin (Melo *et al.,* 1994), to humans limit their usefulness. Although high level expression of the methionine-rich 2S albumin of Brazil nut has been achieved in transgenic soybean, its allergenicity to humans only represents as a model to demonstrate the possibilities of this approach (Nordlee *et al.,* 1996). The development of sequence-based approaches to protein structure and function prediction and the increasing understanding of the rules that govern protein folding and topology have made it possible to artificially design storage proteins, which are rich in essential amino acids and do not cause allergic reaction in humans. Yang *et al.,* (1989) reported that a 292 bp synthetic storage protein gene (HEAAE-DNA) can be correctly integrated and expressed in transgenic potato plants. However, this gene could not be expressed and translated at desirable levels. A 284 bp synthetic gene *asp1* (modified from HEAAE-DNA) coding for a storage protein rich in several essential amino acids (80%) including 13% methionine residues has been transformed into tobacco (Kim *et al.*,1992) and sweet potato (*Ipomoea batatas*) (Prakash and Egnin 1997) and showed high protein and essential amino acid levels in leaves and root, respectively than control plants. The ASP1 (Artificial Storage Protein 1) were designed to have a stable storage protein like structure in plants based on the structurally well studied maize

storage zein proteins (Z19 and Z22) (Agros *et al.,* 1982). ASP1 is comprised of four helical repeating monomers, each 20 amino acids long. The helical region of ASP1 is amphipathic and stabilized by several Glu-Lys salt bridges. The four monomers of the ASP1 tetramer were connected with three β-turn (Gly-Pro-Gly- Arg) sequences which play an important role for structural stability of the ASP1 tetramer *in vivo* (Kim *et al.,* 1992). Table 1.2 depicts the transgenic approaches for improving nutritional quality of proteins in the crops.

Table1.2 Transgenic Approaches for Improving Nutritional Quality of Proteins.

Gene/Source	Promoter	Transformed Plants	Improvement	Ref.
2S (Brazil nut)	Phaeseolin Promoter	Rape Seeds	Met	Altenbach *et al.,* 1992
2S (Brazil Nut)	CaMV35S	*V. narborensis*	Methionine	Saalbach *et al.,* 1995
Beta-Phaseolin (common bean)	Rice glutelin Promoter	Rice	Lys	Zhenweiz *et al.,* 1995
HS-7Zein (maize)	27KDa Zein Promoter	Rice	Met	Falco *et al.,* 1995
Cruciferin (Rape Seeds)	Napin Promoter	Rape Seed Met,Lys,Cys		Kohno *et al.,* 1995
2S Protein (Brazil Nut)	CaMV35S	Tobacco	Met	Marcellino *et al.,* 1996
2SProtein (Brazil Nut)	*V.faba* legB4 Promoter	*V.narbornsis*	Met	Hefford *et al.,* 1996
S-rich Gamme Zein	CaMV35S	Alfalfa, Trefoil	S-content	Bellucci *et al.,* 1997
2S Protein (Sunflower)	CaMV35S,ER-retentional signal	Clover	S-content rumen Protected	Rafiqul *et al.,* 1996; Sharma *et al.,* 1998
2S Protein (Sunflower)	CaMV35S	Legumes	S-content	C. S. Prakash
Ferritin (soyabean)	Rice Glu-B-1	Rice	Fe-content	Goto *et al.,* 1999
AmA1 (*Amarnathus*)	GBSS	Potato	Essential Amino Acids	Chakraborty *et al.,* 2000

A. hypochondriacus is a highly nutritious crop, which contains 15-18% protein with respectable amount of lysine and methionine. Earlier the *AmA1* gene from *A. hypochondriacus* has been cloned and characterized in our laboratory (Raina and Datta 1992). This gene has been patented (Datta *et al.*, 1997, 1998). *AmA1* gene encodes a 35 KDa protein with 304 amino acids and 6.8 pI value. The AmA1 protein is hydrophilic in nature with small stretch of hydrophobic amino acids region at N terminus (Raina and Datta 1992). AmA1 Protein is nonallergenic in natural form (Chakraborty *et al.*, 2000) and value of its essential amino acids is close to WHO recommendations of essential amino acids in storage protein (Raina and Datta 1992). *AmA1* from *Amaranthus hypochondriacus* has been a candidate gene for nutritional improvement of crops. The seed albumin gene, *AmA1* from *Amaranthus hypochondriacus* has a great potential to increase protein quality of crop plants as it is proved in transgenic potato (Chakraborty *et al.*, 2000). The transgenic potato tuber expressing *AmA1* gene has shown increased amino acids content and more protein in comparison to control potato plant. The AmA1 protein has a great potential as a protein donor for the following salient features.

1. Unlike most seed protein, it is a well balanced protein in terms of amino acid composition and even better than the values recommended by the WHO for a nutritionally rich protein.
2. It is a non allergenic protein in its purified form.
3. Unlike many seed storage proteins, AmA1 is encoded by a single gene and thus would facilitate gene transfer into target plants with less difficulty.
4. An increased nutritive value of transgenic potato by expressing a seed albumin gene *AmA1* from *A. hypochondriacus* is proved earlier (Chakraborty *et al.*, 2000).
5. *AmA1* gene is obtained from a natural source of edible vegetable plant *A. hypochondriacus*.

1.6 Plant Regeneration and Transformation Approaches

Genetic transformation of plant is now a core research tool in plant biology and a practical tool for cultivar improvement. Over 3000 field trials of transgenic plants are in progress or completed in at least 30 countries (Birch 1997). These trials involve

over 40 plant species modified for various agronomic traits (Dale 1995; Whitman 2000). Genetic transformation has been used routinely for raising insect resistance and herbicide tolerant transgenic plants as well as for transfer of new agronomic trait in crop plants. Recently, the development of the golden rice, which contains high levels of β-carotene and other carotenoids (Ye *et al.,* 2000) and *AmA1* potato (Chakraborty *et al.*, 2000), which contains high level of protein and amino acids, paved the way for novel strategies to combat blindness and protein malnutrition, respectively. Three prerequisites for applying genetic transformation for any plant improvement are, a reliable regeneration system which is compatible with transformation methods allowing regeneration of transgenic plants, secondly, an efficient way to introduce DNA into the regenerable cells, and lastly a procedure to select and regenerate transformed plants at a satisfactory frequency (Birch 1997).

1.6.1 Plant Regeneration

Plant regeneration is the process of growing an entire plant from a single cell or group of cells. In plant regeneration system, the most important factor is a source of large number of totipotent cells which are feasible to the gene transfer treatment and which will retain their capacity of regeneration during target preparation, cell proliferation and selection treatments. Plant regeneration through tissue culture can be accomplished using one of three methods: meristem culture, somatic embryogenesis and organogenesis.

1.6.1.1 Meristem

Meristem is a part of plant with capability of growth and unlimited potential to divide. There are two types of meristem in plants eg; lateral meristem and apical meristem. Meristems isolated from e.g. zygotic embryos (apical) and axillary buds (lateral) are the natural growth centers of the plant and plant regeneration is initiated from multiple cells which can directly develop to shoots without dedifferentiation (Murashige *et al.,* 1974). Therefore, this system for micropropagation and transformation is easy, fast and relatively genotype independent. Applications of meristem culture include germplasm preservation, micropropagation, transformation and eliminating virus or disease from plant materials.

1.6.1.2 Somatic Embryogenesis

Somatic embryogenesis is the asexual production of embryo-like structures from somatic cells. The somatic embryo is an independent bipolar structure and is not vascularly attached to the tissue origin (Ammirato *et al.,* 1987). Such embryos can develop further and germinate into plantlets through developmental steps that correspond to those of the zygotic embryos. Production of somatic embryos from cell, tissue and organ cultures may occur either directly or indirectly. The direct mode of somatic embryogenesis involves the formation of an asexual embryo from a single cell or a group of cells on a part of the explant tissue without an intervening callus phase. The indirect mode of embryogenesis consists of establishing an explant in culture, subsequent proliferation of embryogenic callus or suspension, initiation of pro-embryos and inducing bipolar embryo formation from pro-embryo initials (Sharp *et al.,* 1980). Plant regeneration via somatic embryogenesis is widely applied in micropropagation, mutation breeding, cryopreservation and transformation (Bhojwani and Razadan 1996).

1.6.1.3 Organogenesis

Organogenesis is a process which lead to formation of outgrowth of monopolar organ e.g. shoots and roots from totipotent explants. These organs develop procambial strands which establish a connection with the pre-existing vascular tissue dispersed within the explant (Flick *et al.,* 1983). Plant regeneration via organogenesis can be achieved either through production of adventitious organs from a callus, internode, leaf or a suspension derived from the explant or through the emergence of adventitious organs directly from the explant without an intervening callus phase.

The regeneration systems based on organogenesis as well as somatic embryogenesis are an important tool in the application of plant biotechnology.

1.6.2 Gene Transfer Techniques

Gene transfer is a process of transferring DNA molecule, encoding a particular trait from one organism to another organism. Most commonly used technique to transfer foreign genes into plant cells are particle bombardment and *Agrobacterium*-mediated gene transfer. Other gene transfer methods, such as protoplast transformation (Shillito

1999; Paszkowski *et al.,* 1984), microinjecting DNA into cells or zygotes (Schnorf *et al.,* 1991; Leduc *et al.,* 1996), silicon carbide whisker-mediated DNA transfer (Thompson *et al.,* 1995) or electroporation (Fromm *et al.,* 1986; Lurquin 1997), have also been used in plant transformation. However, the transformation efficiency of these techniques is low as compared to *Agrobacterium* mediated gene transfer technique.

1.6.2.1 *Agrobacterium*-mediated Transformation

The plant pathogen, soil bacterium *Agrobacterium tumefaciens,* has the ability to transfer the T-DNA (24 kb in nopaline strain), a portion of Ti-plasmid (200-250 kb), delimited by the border flanking repeats of 25 bp, to the plant cells in nature (Van Larebecke *et al.,* 1974). Expression of the oncogenes located in the T-DNA cause the crown gall disease mainly on dicotyledonous plants and some gymnosperms (Smith and Townsend 1907; De Cleene and De Ley 1976). Only few monocotyledons plants in the family of *Liliaceae* and *Amaryllidaceae* have been reported to weakly susceptible to crown gall induction. Crown gall tissue synthesizes novel amino acids and sugar derivatives, collectively known as opines. The type of opine synthesized in the tumour is dependent on the strain of *Agrobacterium* that initiated tumour formation. Octopine and nopaline are two types of opines derived from arginine and easy to detect in crown gall tissues. The T-DNA transfer process initiates when *Agrobacterium* perceives certain signals from phenolic compounds, sugar compounds, lignin, and flavinoids secreted by wounded plant cells and activates the *Vir* gene expression (Stachel *et al.,* 1985). A single stranded form of T-DNA (ss-T-DNA) is transferred into the host plant cell nucleus and integrated into the genomic DNA. During the T-DNA transfer, the virulence proteins D2 (VirD2) and E2 (VirE2) containing short peptide sequences protect ss-T-DNA against nucleases and act as a nuclear localization signal (NLS) to target the incoming ss-T-DNA into the plant cell nucleus (Tinland *et al.,*1995; Mysore *et al.,*1998; Zupan and Zambryski 1997). It is believed that the T-DNA complex passes into the plant cell nucleus by active nuclear uptake, as the size of the T-DNA complex (12.6nm diameter) exceeds that of the diameter of the nuclear pores (9nm) (Forbes, 1992), although the size of the nuclear pore can increase to 23 nm during nuclear uptake (Citovsky *et al.,*1997; Forbes, 1992). Other virulence proteins on the bacterial surface form a pillus through which

the T-DNA and the transferred proteins may translocate. The *vir* genes can function in *cis* as well as in *trans*. Plant genes are also involved in T-DNA transfer and integration (Gelvin 2000). The oncogenic genes between the border repeats of the T-DNA, which define it for transfer (Wang *et al.,* 1984), cause the tumorigenic properties of the transformed cells. These oncogenes can be deleted or replaced by foreign genes without interfering with the capacity of T-DNA transfer. Two kinds of disarmed vectors were developed depending upon whether the DNA to be introduced is physically linked or separated from the *vir* genes of the Ti plasmid. The former type of vectors is known as co-integrative vectors or *cis* vectors (Fraley *et al.,* 1985; Zambryski *et al.,* 1983) and the latter type is referred to as *trans* vector or binary vectors (Hoekema *et al.,* 1983). Duplication of *VirG* region in transformation vectors has been shown to lead to increased virulence of *Agrobacterium* in rice, carrot, celery and cassava (Hiei *et al.,* 1994; Liu *et al.,* 1992; Arias-Garzón and Sarria 1995, Li *et al.,* 1996). Van der Fits *et al.,* (2000) reported that a constitutive *virG* mutant gene (*virG*N54D) on a compatible plasmid dramatically increased *Agrobacterium*-mediated plant transformation. Treatment of *Agrobacterium* with acetosyringone during inoculation and co-cultivation could increase the efficiency of transformation (Hiei *et al.,* 1994). Basic steps in transformation of plant cells using *Agrobacterium tumafacience* is depicted in fig 1.2. Today *Agrobacterium*-mediated gene delivery is routinely applied in important dicotyledonous crops such as cotton (Firoozabady *et al.,*1987), sunflower (Everett *et al.,*1987), tomato (Fillatti *et al.,*1987), potato (Sheerman and Bevan 1988), soybean (Hinchee *et al.,*1988), sugar beet (Lindsey and Gallois 1990), papaya (Fitch *et al.,*1993) and cassava (Li *et al.,*1996; Schöpke *et al.,*1996). However, monocotyledonous plants, especially graminaceous crops, were thought to be outside the host range of *Agrobacterium* (Bevan 1984, De Cleene 1985). After addition of a *vir* gene inducing compound acetosyringone to the co-cultivation medium and the use of appropriate target tissues, as well as selected *A. tumefaciens* strains harboring suitable binary vector, successful *Agrobacterium*-mediated transformation in monocots has been possible since the end of the 1980s, in many crops like asparagus (Byterbier *et al.,*1987), maize (Gould *et al.,*1991), banana (May *et al.,*1995), barley (Tingay *et al.,*1997), rice (Hiei *et al.*1994; Rashida *et al.,* 1996) and wheat (Cheng *et al.,*1997).

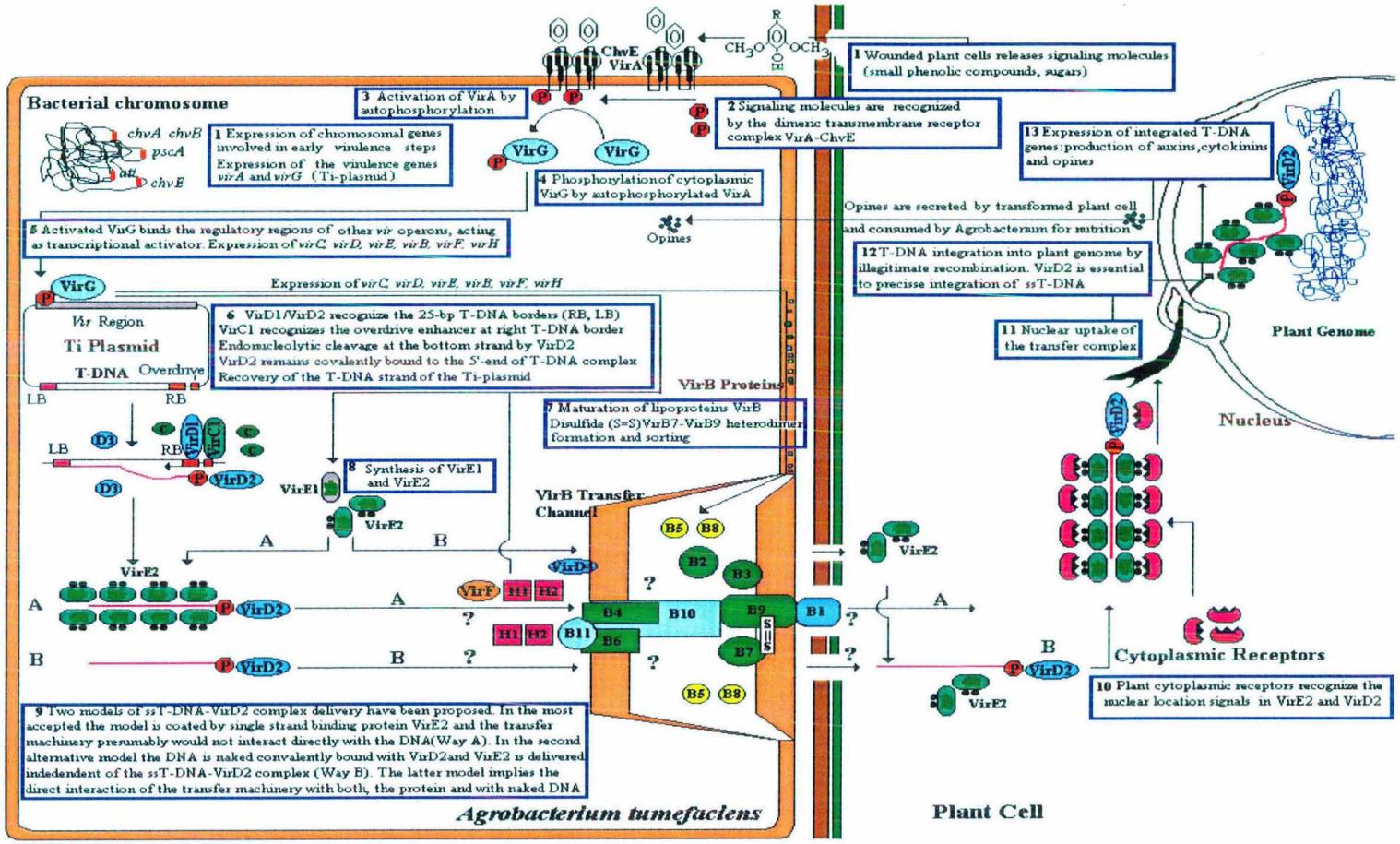


Fig 1.2 Basic steps in transformation of plant cells by *Agrobacterium tumefaciens*. The most important events are briefly mentioned in chronological order (boxes 1 through 13). Each step is described in the text.

Adaptad : Gustavo A. de la Riva, *Agrobacterium tumefaciens*: a natural tool for plant transformation, Electronic Journal of Biotechnology, Vol.1 No.3, Issue of December 15, 1998.

1.6.2.2 Biolistic Transformation

Methods of direct gene transfer avoiding the specific host range limitations of *Agrobacterium* have been developed as well. Sanford and co-workers established a method for gene delivery by microprojectile bombardment (Klein *et al.,* 1987). Microprojectile bombardment mediated transformation is one of the most promising gene transfer techniques even for those plants which have proved recalcitrant to transformation by any other procedure. DNA-coated microprojectiles are accelerated by means of an explosion or a burst of gas (helium) to a sufficient velocity to penetrate plant cell walls and release the DNA for expression and/or integration in the plant genome. Finer *et al.,* (1992) developed the system further and constructed the Particle Inflow Gun (PIG), which is inexpensive and simple to use and proved in by stable transformation in soybean and maize. Biolistic has now become the second most widely used technique for plant transformation after *Agrobacterium*-mediated transformation. This technique has been successfully used to transform meristems and tissues with high regeneration potential, to deliver foreign DNA into intact and regenerable cells, tissues and organs with no limitation of *Agrobacterium* related host specificity and tissue culture related regeneration difficulties, and to transform organelles such as chloroplasts (Luthra *et al.,* 1997; Klein and Jones 1999). Many important crop plants, for example, rice (Christou *et al.,* 1991), maize (Gordon-Kamm *et al.,* 1990), wheat (Vasil *et al.,* 1992, 1993), soybean (Mc Cabe *et al.,* 1988; Finer and McMullen 1991) and cassava (Shöpke *et al.,* 1996; Raemakers *et al.,* 1996) have been successfully transformed using biolistics.

1.6.3 Selectable and Screenable Markers

Transgenic plant cells have been traditionally selected by the introduction of genes conferring resistance to a selective chemical agent or genes conferring a phenotype allowing visual or physical screening or even by PCR screening to identify plants containing transgene (Birch 1997). Selective marker gene is introduced into plant genome to express into a protein with, generally, an enzymatic activity, allowing to distinguished transformed cells from non transformed cells (Brasilerieiro and dusi, 1999). The purpose of the use of a selective marker gene is to give to the transformed cells a selective advantage, allowing them to grow faster and better, and/or to kill untransformed cells. This expression should be easily distinguished from any

endogenous activity in the plant tissue, allowing differentiate the phenotype of transformed cells from untransformed cells. Therefore, the probabilities to recover transgenic plants in presence of a selective agent are greater than in its absence, especially because the transformation efficiency is always low.

Generally, selective marker gene is introduced into plant genome along with the gene of interest. These genes could be physically linked or separated in a same DNA vector. The frequency of co-transformation (*i.e.* cells with both genes integrated into genome) is 100% when they are linked and 50% when they are separated (Aragao *et al.*, 1996). In most cases, the expression of selection marker genes is under the control of constitutive promoter like the CaMV35S, nopaline and octopine synthase, and actin or ubiquitin promoter genes. A number of dominant genes encoding enzymes rendering the plants resistant to antibiotics or herbicides are widely used as selectable makers in plant transformation (Table 1.3).

Table 1.3 Selectable marker genes for plant transformation

Marker Gene	Enzyme encoded	Selective Agent	References
Antibiotic			
nptII	Neomycin phosphotransferase II	Kanamycin Neomycin Genticinin (G418) Paromomycin	Bevan *et al.*,. 1983 Fraley *et al.* 1983 Herrera-Estrella *et al.*, 1983 Present study
Dhfr	Dihydrofolate reductase	Methotrexate	Herrera-Estrella *et al.*, 1983
hph,hpt	Hygromycin phosphotransferase	Hygromycin B	Waldron *et al.*, 1985 Van den Elzen *et al.*, 1985
Ble	not known	Bleomycin Phleomycin	Hille *et al.*, 1986
aacC3, aacC	Gentamycin-3-*N*acetyltransferase	Gentamycin	Hayford *et al.*, 1988
SPT	Streptomycin phosphotransferase	Streptomycin	Jones *et al.*, 1987
aadA	16SrRNA aminoglycoside-3.-adenyltransferase	Spectinomycin Streptomycin	Svab *et al.*, 1990a Svab *et al.*, 1990b
Herbicides			
aroA	5-Enolpyruvylshikimate-3- Glyphosate	Phosphate synthase (Roundup)	Comai *et al.*, 1985 Shah *et al.*, 1986
bar	Phosphinothricin Acetyltransferase	Phosphinothricin	De Block *et al.*, 1987
Als	Mutant forms of acetolactate synthase	Chlorsulfuron Imidazolinones	Haughn *et al.*, 1988

The usefulness of a particular resistance marker depends on the characteristics of the selection agent, the resistance gene and the plant material. A selection agent that exerts inhibitory effect on the growth of untransformed cells but does not have adverse effects on the transformed cells is preferred in plant transformation. The sensitivity of the plant cells to the selection agent depends upon the genotype, the explant type, the developmental stage, and the tissue culture conditions.

*npt*II, the most commonly used selectable marker gene was isolated from the transposon Tn5 of *E.coli* and its codes for the enzyme neomycin phosphotransferase II (*NPT*II, E.C.2.7.1.95) also known as aminoglycoside 3'-phosphotransferase II (APH (3) II). The active aminoglycoside antibiotic inhibits the protein synthesis in prokaryotic calls, by the binding to the 30S subunit of the ribosome, blocking the formation of initiation complex and decreasing the fidelity of translation. In plants, these antibiotics exert its effect on mitochondria and chloroplast since these organs utilize 30S ribosome during protein synthesis. The enzyme nptII transfers the gamma-phosphate group of ATP to the 3'-hydroxyl group of the amino hexose portion of aminoglycoside antibiotic that is consequently detoxified. Due to this ATP dependent phosphorylation, the binding of antibiotic to the bacterial ribosome is prevented and allowing the protein synthesis. Amino glycoside antibiotic that contain the 3'-hydroxyl group like kanamycin A, B, and C, paromomycin sulfate, genticinin (G-418) are substrate for *nptII* (Norelli and aldwinckle, 1993). Endogenous *nptII* activity is very rare in plant tissue.

Another selectable marker system based on a new plant vector in which a chimeric *ipt* gene is inserted into the transposable element *Ac* has been developed (Ebinuma *et al.,* 1997). The *ipt* gene from the T-DNA of *A. tumefaciens* encodes the enzyme isopentenyl transferase involved in the biosynthesis of cytokinins (Barry *et al.,* 1984). Transgenic plants containing the *ipt* gene under control of the 35S promoter exhibited an "extreme shooty phenotype" and loss of apical dominance on hormone-free medium. The maize transposable element *Ac* has the ability to move to new locations within a genome since it codes on transposase (Fedoroff 1989). Therefore, phenotypically normal transgenic plants may be obtained when the chimeric *ipt* gene transposes or becomes lost along with *Ac* in transgenic cells.

Alternatively, visible scorable markers, e.g., β-glucuronidase (GUS) (Jefferson 1987), luciferase (Ow *et al.,* 1986) and green fluorescent protein (GFP) (Chalfie *et al.,*

1994), can be used to screen transgenic plants. Because conventional GUS assays are destructive to the tissue, a non-destructive assay has been developed which is based on secreted GUS in spent media of *in vitro* plant culture (Gould and Smith 1989). Assaying only parts of putatively transgenic plants, e.g. root or leaf pieces, may also allow selection of transgenic plants. The luciferase reporter gene (*luc*) from *Photinus pyralis* was used for selection of transgenic plants in *Dendrobium* (Chia *et al.*, 1994) and cassava (Raemakers *et al.*, 1996). Although the luciferase assay is expensive and labour intensive, its nondestructive nature has advantages over the GUS assay. GFP, another non-destructive scorable marker from the jellyfish *Aequorea victoria* can be directly visualized and was used to monitor the transformed cells as well as for selection (Haseloff *et al.*, 1997).

1.7 Genetic Engineering of Rice

Rice crop is adversely affected by various abiotic and biotic stresses. Same time nutritional properties of rice also have some limitations. Over the next 7 to 8 years, global rice plantings are predicted to remain static, which will result in a shortfall of about 130 million tons (Khush, 2001, Brookes and Barfoot, 2003) and need to increases 755 million tons to fulfill demand of growing population (Brookes and Barfoot, 2003).

Rice is considered as a model monocot (Goff, 1999; Shimamoto and Kyozuka, 2002) and biotechnological progress in this crop is moving fast. Some of milestone of progress in rice biotechnology is described in table 1.4. First fertile transgenic plant was produced about 18 years ago (Shimamoto *et al.*, 1989; Datta *et al.*, 1990), since then various scientific groups used biotechnology for improvement of rice with agronomically important traits as well as nutritional quality (Table 1.5).

Table 1.4 Milestone of rice biotechnology

Year	Development	References
1988	First transgenic rice obtained	Toriyama *et al.*, 1988; Zhang *et al.*, 1988; Zhang and Wu, 1988
1989	First fertile transgenic rice plants (*japonica*)	Shimamoto *et al.*, 1989
1990	First fertile transgenic rice plants (*indica*)	Datta *et al.*, 1990
1991	Use biolistics for generation of rice transgenics	Christou *et al.*, 1991
1993	Insect resistance rice by using δ-endotoxin (*Bt*)	Fujimoto *et al.*, 1993
	Transgenic rice with *bar* gene for sheath blight resistance	Uchimiya *et al.*, 1993
1994	First conclusive report of rice transformation by *Agrobacterium*	Hiei *et al.*, 1994
1998	Multigene transformation	Chen *et al.*, 1998
	First report of *Agrobacterium*-mediated rice transformation for agronomically important genes (*cryIAb and cryIAc*)	Cheng *et al.*, 1998
1999	Iron fortified transgenic rice with soybean ferritin gene	Goto *et al.*, 1999
	Resistance against rice yellow mottle virus(RYMV) derived by pathogen-derived resistance	Pinto *et al.*, 1999
2000	Field trial of hybrid rice containing *Bt* gene	Tu *et al.* 2000b
	Golden rice	Ye *et al.*, 2000
	Draft sequence of rice genome announced	Monsanto*
2002	Draft sequence of rice genome	Goff *et al.*, 2002; Yu *et al.*, 2002
	Gene targeting by homologous recombination	Terada *et al.*, 2002
	Complete sequence of rice chromosome 2	Sasaki *et al.*, 2002
	Complete sequence of rice chromosome 4	Feng *et al.*, 2002
2003	Complete sequence of rice chromosome 10	Rice chromosome 10 consortium, USA, 2003
2004	Completion of rice genome by IRGSP	Sasaki *et al.*, 2005

Table: 1.5 Genetic engineering of rice

Rice	Traits	Gene	Transferred Method Used	Ref.
Indica (IR72)	Herbicide Resistant	Bar gene	Protoplast (PEG)	Datta *et al.*, (1992)
Japonica	Stripe virus Resistant	CP-Stripe	Protoplast (Electroporation)	Hayakawa *et al.*, (1992)
Japonica	Insect resistance	Bt	Protoplast (Electroporation)	Fujimoto *et al.*, (1993)
Indica	Sheath Blight Resistant	chi11	Protoplast(PEG)	Lin *et al.*, (1995)
Japonica	Insect resistant	Cc	Protoplast	Irie *et al.*, (1996)
Japonica	Bacterial Blight Resistant	xa21	Biolistic	wang *et al.*, (1996)
Japonica	Insect resistant	Pin II	Biolistic and Protoplast	Duan *et al.*, (1996)
Indica	Stem Borer Resistant	Bt	Biolistic	Nayak *et al.*, (1997)
Indica	Stem Borer Resistant	Bt	Biolistic	Tu *et al.*, (1998a)
Japonica	SSB & YSB	Bt	*Agrobacterium*	Cheng *et al.*, (1998)
Indica	stem borer resistant	Bt (DWR)	Protoplast and Biolistic	Alam *et al.*, (1998)
Indica and *Japonica*	Biological N2 Fixation	Early Nodulin	Biolistic and protoplast	Reddy *et al.*, (1998)
Indica and *Japonica*	Stem borer Resistant	Bt	Biolistic and Protoplast	Datta *et al.*, (1998)
Indica (IR72)	Bacterial Blight Resistant	Xa-21	Biolistic	Tu *et al.*, (1998b)
Indica	Stem Borer Resistant	Bt ML for Hybrid rice	Biolistic	Alam *et al.*, (1999)
Indica and *Japonica*	Biological N2 fixation	Early Nodulin Early Nodulin		Dey *et al.*, (1999)
Japonica	Higher Photosynthesis	Pepc	*Agrobacterium*	Ku *et al.*,(1999)

Indica	Resistant RTSV and RTBV	Viral Replicate CP1,CP2,Cp 3	Biolistic	Ang *et al.*,(1999)
Japonica	High Iron Storage	Ferritin	*Agrobacterium*	Goto *et al.*, (1999)
Indica	Sheath Blight Resistant	PR genes	Biolistic	Datta *et al.*, (1999b) Balsakh *et al.*, (2000)
Indica	Stem Borer Resistant	Bt Resistant for hybrid rice	Biolistic	Tu *et al.*, (2000 b)
Japonica	β-Carotene Synthesis	psy,lyc,crtf	*Agrobacterium*	Ye *et al.*, (2000)
Japonica	Submergence	adh,pdc	Biolistic	Qiumio *et al.*, (2000)
Japonica	Amylose content	Waxy gene	Biolistic	Terada *et al.*, (2000)
Japonica	Insects resistant Tolerance	Bt	Biolistic	Ye *et al.*, (2001)
Indica	Sheath Blight Resistant	PR Genes	Biolistic	Datta *et al.*, 2000,2001
Rice	Abiotic and Biotic stress	OsBIHD1	*Agrobacterium* mediated	Luo H *et al.*, (2005)
Rice	Shoot Branching	OsNAC2	*Agrobacterium* mediated	Mao *et al.*, (2007)
Rice	Head Blight	trichothecene 3-O-acetyltr-ansferase		Ohsato *et. al.*, (2007)
Rice	Starch saccharification	Glucoamylase	*Agrobacterium*	Xu *et al.*, 2008
Oryza sativa L. cv. Donjin	Wide range of biological function	synthetic hGH gene (shGH)	Biolistic	Kim *et al.*, 2008
Rice	Edible cholera vaccine	synthetic cholera toxin B subunit (CTB)	Biolistic	Oszvald *et al.*, (2008)

DWR : Deep water Rice KL: Maintainer Rice

1.8 Aim and Scope

Rice is the major staple crop for half of the world population. It is a carbohydrate rich crop but with limited nutritive value due to deficiency in essential amino acids specially lysine, methionine and threonine (Takaiwa1999). The *AmA1* gene has been proved to be a candidate gene to improve nutritional value of potato for improvement of protein content and amino acid composition (Chakraborty *et al.*, 2000). Thus, *AmA1* gene can also be used to improve the nutritional value of rice in terms of protein quality and quantity.

A successful raising of genetically engineered plants require an efficient regeneration and transformation system as well as construction of controllable expression vector containing the gene of interest driven by specific promoter. Expression of the gene of interest in a tissue specific manner is better than constitutive expression of the foreign gene. In addition, the selection of the best transgenic event requires a detailed analysis of the transgenic lines at molecular level. Keeping these in mind, my objectives of the thesis work were as followed:

1. Construction of expression vectors using *AmA1* gene for plant transformation under seed specific promoters.
2. Establishing regeneration and transformation system for rice.
3. Raising transgenic rice expressing *AmA1* gene. Molecular analysis of transgenic plants and testing their agronomic performance.

CHAPTER 2

ESTABLISHMENT OF *IN-VITRO* REGENERATION AND TRANSFORMATION SYSTEM FOR *Indica* RICE

2.1 Introduction

Cereal crops serve as the nutritional food in many countries. Among the cereal crops, rice is the major staple crop for more than three billion people (FAO, 2004). Although it qualifies as the staple food crop but it is adversely affected by the abiotic and biotic stress (Baker *et al.*, 1997), as well as its nutritional properties are limited by the micronutrients and protein deficiency, which limits the quantity and quality of rice grain. To overcome these constraints, genetic improvement of rice is very much essential. One cannot deny that genetic improvement in staple crops can be achieved by conventional plant breeding approaches, but this method cannot cope up with the present situation as it has many limitations as discussed below:

1. There are limitations to use sexual crosses for introducing many useful aliegn genes from different source.
2. The crop improvement by plant breeding is very laborious and time consuming.
3. It is very difficult to break gene linkage between desirable and undesirable genes.
4. The sequence of gene is unknown because breeding transfer traits among the crops.
5. It can not transfer only the desirable gene/s of interest, thus might introduce many unknowns.

For successful introduction of a useful alien gene through genetic engineering, a reproducible regeneration and transformation system which is applicable to most of *indica* var. of rice is very much essential. There are two major subspecies of rice in the world viz. *indica* and *japonica.* Among the two subspecies, *indica* is widely grown in the world and occupies 80% of the cultivated rice area (Swaminathan 1982; Ayres and Park, 1994). But there are only few reports available on *indica* rice variety transformation (Zhang *et al.*, 1997; Mohanty *et al.*, 1999; Azhaknandam *et al.*, 2001; Kumria *et al.*, 2001; Kumria and Rajam., 2002; Khanna and Raina, 2002). True *Indica* rice varieties are more recalcitrant to tissue culture and transformation in comparison to *japonica* rice (Jain *et al.*, 1995; Wunn *et al.,* 1996; Ayres and park 1994). The main factor which influences the transformation efficiency in rice and

other crops includes donor seed genotype, seed quality and culture conditions. Rice has become first cereal crop to be regenerated from callus. Nishi *et al.,* (1968) reported differentiation and plant regeneration from rice callus and Tamura (1968) was able to induce shoot formation from embryo derived culture.

Regeneration in rice follows two classical Pathways *i.e.* **organogenesis** or **somatic embryogenesis**. The determining factor to follow either of the pathways was originally recognized to be the nature of the explants. Anther, embryo and root regenerate via either of two mechanisms. Whereas, immature panicle derived callus, scutellum derived callus regenerate via somatic embryogenesis. Few reports also confirmed that, rice regeneration could be direct somatic budding, organogenesis and combination of organogenesis and somatic embryogenesis.

Various scientific groups working on *indica* rice species have tried to overcome recalcitrance problem by transforming the mature embryo derived calli (Hiei *et al.* 1994), immature embryo (Aldemita & Hodges 1996) and scutellum derived calli of mature seeds (Rashid *et al.*, 1996). Regeneration from mature seeds of any plant offers various advantages. Some of the obvious advantages include the continuous availability of starting material, avoids the need to grow plants to maturity in order to get alternative explants such as immature embryos, young inflorescence, panicle, anthers etc. and ease to store them at room temperature.

A number of reports showed that various factors control regeneration from mature seeds in plants (Julio Alfonso *et al.*, 1999). Key factors identified in this context are genotype, health of seed material in terms of being free from pest and any other infection, chemical composition of basal media in terms of appropriate salts, carbon source, hormone, culture conditions, gelling agent and pH during culture.

A comprehensive study of rice verities showed that both callus induction and plant regeneration were highly genotype dependent (Kamia *et al.*, 1988; Peng and Hodges 1989). A study carried on nutritional requirement of rice cells in culture medium also *indica*ted the involvement of genotype factors in rice tissue culture (Schmitz and Lozh., 1990). Earlier study in our laboratory showed low frequency of transformation (1%) of rice via. particle bombardment technique (Biswas 1997). Thus, present study is an attempt to establish a protocol for rice regeneration and

transformation which is applicable to different *indica* rice cultivars, for e.g. *IR64, Budda, Pusa Basmati-1* by *Agrobacterium* mediated transformation.

2.2 Materials and Method

2.2.1 Rice Cultivar, Explant and Seed Sterilization

Rice (*Oryza sativa, sub sps. indica*) cultivars used for studying regeneration and transformation are *Budda, IR64* and *Pusa Basmati-1*. Rice *indica* varieties *Budda, IR64* and *Pusa Basmati-1* mature seeds were dehusked and washed with autoclaved sterile MQ water for 5-6 times to remove all dust and small particles. Mature seeds were then rinsed with 70% ethanol (Bengal Chemicals, Calcutta) for 2 min followed by several washes with sterile distilled water. These seeds were surface sterilized with continuous stirring in 2% sodium hypochlorite (Qualigens chemicals) solution for 20 min followed by several washes with sterile distilled water. Seeds were soaked on 3 mm sterile Whatmann filter paper before transferring them to callus induction media.

2.2.2 Callus Induction and Somatic Embryogenesis

Callus induction medium consisted of MS media (Murashige and Skoog, 1962) with basic salts, supplemented with 30g/l sucrose or 3% maltose, and 1-4 mg/l 2, 4-D (CIM1, CIM2, CIM3, CIM4, and CIM5). After adjusting the pH to 5.8 and adding gelling agent 0.7% Agarose Type-I, media were autoclaved at 121°C and 15 bars for 20 min. Autoclaved media were cooled to ~ 55°C temperature and poured in petri plates. Sterilized seeds of rice were transferred onto callus induction media by keeping horizontally in dark at 25°C for 7-9 days or till callus emerged from scutellum portion.

Scutellum derived calli from mature seeds of 7-9 days old were separated from seeds and subcultured on callus induction media. Further, subculturing was done for another 14 days to develop embryogenic calli. 21-23 days old scutellum derived callus were used for further regeneration and transformation.

Evaluation of Callus Induction Ability

Evaluation of callus induction efficiency was carried out by counting the number of seeds transferred onto callus induction media to the seed induced with callus after 21 days in callus induction media.

2.2.3 *Agrobacterium* Strain and Co-cultivation

Agrobacterium strain EHA105 harboring pBI121 plasmid was used to study transient expression of GUS in rice callus. Single colony of *Agrobacterium* strain *EHA105* was grown in YEP media containing 50mg/l rifampicin and 50 mg/l kanamycin. Secondary culture was initiated by adding 200 µl of primary culture and grown till it reached OD 0.8. *Agrobacterium* Strain EHA 105 cells were pelleted by centrifugation at 4°C, 5000 rpm for 10 minutes in (Sorvall kendro, SS 34). Pellet was then resuspended in liquid co-cultivation media. Liquid co-cultivation media consisted of MS basal media (Sigma Cat.No.5519), 3% maltose and pH 5.4. A virulence inducing agent acetosyringone was added in 100µM conc. to co-cultivation medium after filter sterilization.

Calli were submerged in *Agrobacterium* suspension for 20 min followed by soaking on 3 mm sterile Whatmann paper. Calli were kept for infection on a callus induction media supplemented with 100 µM of acetosyringone for 3 days in the dark at 25°C. These calli were washed with sterile water containing 250 mg/l cefotaxime sulfate and blotted on sterile Whatmann paper. Further, calli were transferred onto callus induction media containing 250 mg/l cefotaxime sulfate and 50 mg/l paromomycin sulfate in dark for one month. Fresh embryogenic calli were separated from brown calli and subcultured in the callus induction media added with antibiotic after every 10 days interval till one month.

2.2.4 Selection

To identify suitable selection concentration of antibiotic for *npt II* in three varieties of rice, paromomycin sulfate was used during callus induction, regeneration and seed germination. For preparation of selective callus induction and regeneration media, selective agent paromomycin sulfate was added in increasing concentration and

named as CP1, CP2, CP3, CP4 and CP5 in callus induction media (CIM3) and RP1, RP2, RP3, RP4 in regeneration media (Reg2 in case of *Budda* and *IR64*, Reg4 in case of *Pusa Basmati-1*) after filter sterilization to the lukewarm autoclaved media. An equal number of 21-23 day old embryogenic calli were transferred onto selective callus induction and regeneration media for callus proliferation and regeneration, respectively.

An evaluation of selective agent in regeneration for *Budda, IR64* and *Pusa Basmati-1* rice varieties was carried out after 18 days by counting the number of calli with induced shoot bud to that which did not induce shoot buds and turned brown. Similarly an evaluation of selective agent in callus induction media was carried out by counting the calli at three different stage of callus induction to the total callus in selective media. Three different stages of callus were identified; stage A *indica*ted the callus without browning, stage B *indica*ted the callus browning upto 50% and stage C *indica*ted the callus browning between 50 % to 100%.

In case of seed germination, paromomycin concentration of 0 mg/l, SP1 and SP2 was added to seed germination media consisted of MS Basal salts, 3% sucrose, 0.4% agarose Type-I and pH adjusted to 5.8. An equal number of seeds were transferred on germination media and evaluated by counting the germinated seeds to died seeds.

2.2.5 Regeneration

21-23 days old embryogenic calli induced from scutellum portion of rice seed on callus induction medium were used for regeneration study. Regeneration of rice calli were carried out in MS media supplemented with different combination of BAP, Kinetin and NAA with 3% sucrose (Reg1, Reg 2, Reg 3, Reg 4). After adjusting the pH of media to 5.8 with NaOH and HCl, 0.7% of gelling agent Agarose Type -1 was added. Fresh compact, dry, globular and embryogenic calli were transferred in regeneration media and kept under light at 25°C in 14/10 hours day/night photoperiod. After every 7 days calli were monitored for brown calli and separated from fresh calli during subculture till plants were regenerated. Rice regeneration media was evaluated after 4 weeks on the basis of number of green calli, shoot induction and elongation. In

case of transformation, paromomycin sulfate was added to lukewarm autoclave regeneration media.

2.2.6 Rooting

Shoot bud induced from embryogenic calli on regeneration media were used for root induction and shoot elongation. Shoot bud above 2 cm length was suitable for root induction as it is easy to handle in culture tubes. Root induction media consisted of MS plain and NAA supplemented with 3% sucrose. pH of media was adjusted to 5.8 and 0.4% gelling agent agarose Type-I was added. Once roots established in root induction media, plants were transferred to a liquid rooting media which is similar to root induction media but without solidifying agent agarose. An evaluation of root induction was carried out by counting rooted plants to the shoot bud transferred in rooting media. In case of transformation, paromomycin sulfate was added to lukewarm autoclaved rooting media.

2.2.7 Transient GUS Expression

Transient GUS expressions of rice callus were carried out after 7 days of transformation. Transformed calli with *Agrobacterium* strain EHA105 harboring pBI121 expression vector were washed with cefotaxime sulfate (250mg/l) containing water. Calli were assayed for transient GUS expression using 5 –Bromo -4- chloro-3-indolyl-D-Glucourinide(X-Gluc) as a substrate. Calli were fixed in 0.3% paraformaldehyde, 10mM MES (pH-5.6), 0.3M mannitol, 50mM NaH_2PO_4 (pH-7.0) for 45 min at room temperature. After fixation, calli were washed in 50mM NaH_2PO_4 (pH-7.0). Then histochemical assay was performed in X-Gluc staining solution (2mM X-Gluc, 0.01%chloremphenicol, 50 mM NaH_2PO_4, 20% Mannitol) by vacuum infiltration for 15 min followed by overnight incubation at 37°C. Further, calli were washed in 70% ethanol to bleach out chrophylly from leaf tissue. An evaluation of transient expression was carried on the basis of blue calli to the total calli examined for assay.

2.2.8 Molecular Analysis

PCR amplification was carried out using DNA sample isolated from leaf tissue of putative transgenic rice plants. Suitable pair of GUS gene specific oligonucleotides were synthesized for amplification of almost full length of the gene. Forward GUS Primer (5'-GTGGAATTGATCAGCGTTGG-3') and reverse primer (5'-GCA CCGAAGTTCATGCCAGT-3') were used for amplification of 1.6 kb region of GUS from genomic DNA of transgenic rice plants. PCR reaction was performed in 25 µl of reaction volume containing 1X PCR buffer, 2.0mM $MgCl_2$, 0.25mM dNTP, 10pm moles each of forward and reverse primers, 100 ng of genomic DNA and 2 units of *Taq* Polymerase. PCR reaction was carried out in MJ research PCR machine. The thermal condition of PCR is described below.

Step	Temperature	Time
Step-1	94 °C	5min
Step-2	94°C	30 Sec
Step-3	56°C	30 sec
Step-4	72°C	1 min 30 sec
Step-5	Go to step 2 and repeated for 30 cycles	
Step-6	72°C	10min
Step-7	4°C	5 min

2.3 Results and Discussion

2.3.1 Callus Induction

Induction of scutellar callus was observed in all cultured genotypes. Different genotypes showed minor differences in callus colour, texture and development (fig 2.1 B-E, 2.2 B-E, 2.3 B-E). Plumule and radical also emerged from seed after 2-3 days but further growth was suppressed. The results of callus induction efficiency in different cultivar on CIM1, CIM2, CIM3, CIM4 and CIM5 are depicted in the figure 2.4 A. The callus induction from scutellum portion of seeds was visible after 3-4 days. Small compact callus turned into globular, embryogenic calli. The callus induction

efficiency at CIM1, CIM2, CIM3, CIM4, and CIM5 after three weeks from culture initiation was 78% to 95%. One limiting factor in callus induction was browning of callus. In order to protect calli from browning, calli were transferred in callus induction medium supplemented with 3% maltose instead of 3% sucrose. Callus growth in 3% maltose was almost similar to 3% sucrose but browning of calli was decreased drastically. Callus induction from rice scutellum initiated between 2-4D concentrations of 1-4 mg/l of 2-4 D. But callus induction media CIM 3 was observed to be the best among the tested combinations with callus induction efficiency of 95%, 92% and 88% for *Pusa Basmati-1*, *Budda* and *IR64,* respectively. Similar results were also reported by several other workers (Kabir *et al.*, 2008; Islam *et al.*, 2000).

Callus induction efficiency in different cultivar of rice was calculated as given below.

$$\% \text{ callus induction} = \frac{\text{Number of Calli X 100}}{\text{Number of mature seeds inoculated callusing}}$$

Apart from callus induction, 2-4D has undesirable property of suppressing germination (Lu *et al.,* 1984). Rhizogenesis of calli is rare during callus induction but was found in *Budda* var. than *Pusa Basmati-1* and *IR64.* When seeds were placed densely in the callus induction media, rhizogenesis was observed more frequently in *Budda* variety compare to *IR64* and *Pusa Basmati-1.* This may be due to scarcity of rhizogenesis suppressing agent 2-4D in callus induction media. Once rhizogenesis started in callus, growth of callus stopped or retarted. *IR64* and *Pusa Bsmati-1* var. rarely showed rhizogenesis during callus induction.

2.3.2 Shoot Regeneration

Regeneration of plant from callus was observed in all three genotypes with significant difference among the cultivars (Fig 2.1 F-I, 2.2 F-I, 2.3 F and G). Regeneration started with greening of callus which leads to the development of shoot bud. Greening of callus was seen after 10-12 days in regeneration media. But all green calli clusters not necessarily regenerated into shoot bud as some of them remained dormant.

Proliferation of callus simultaneously with regeneration was observed for long time culture in *Pusa Basmati-1* cultivar during subculture. Root initiation from shoot bud was also observed after long time culturing on regeneration media. Comparative study of regeneration efficiency among three genotypes with different hormone combinations of BAP, NAA, and Kinetin is depicted in fig 2.4 B.

Regeneration rate in different cultivar of rice in regeneration media was calculated as given below.

$$\% \text{ Regeneration rate} = \frac{\text{Number of calli with shoot bud} \times 100}{\text{Number of calli inoculated on regeneration media}}$$

From the data obtained (Fig. 2.4 B), it is concluded that Reg 2 is most suitable among tested combination of hormones for Budda and *IR64* with regeneration efficiency 55% and 44%, respectively. For *PB-1*, Reg4 was most suitable with 44% regeneration efficiency.

2.3.3 Root Induction

Root induction in *Pusa Basmati -1*, *Budda* and IR64 was observed in MS basic medium with 3% sucrose, NAA and 0.5% of Agarose Type-I (Fig 2.1 J, 2.2 J, 2.3 I). Root induction was also observed in same media without any hormone but was delayed compared to NAA added root induction media. Root induction was initiated after 4-5 days and the density of roots increased after 10 days. For rapid growth of plants and to save time, rooted rice plants from the solidified media were transferred to liquid root induction media. Because liquid culture media is more easily accessible to plant for rooting and reduced chances of wounding due to subculturing compared to the solid media. The root induction efficiency for *Budda*, *IR64* and *Pusa Basmati-1* was 98%, 86% and 88%, respectively (Fig 2.4 C). Rooting of rice in root induction media was calculated as given below.

$$\% \text{ Rooting} = \frac{\text{Number of rooted plants} \times 100}{\text{Number of shoots transferred in root induction media}}$$

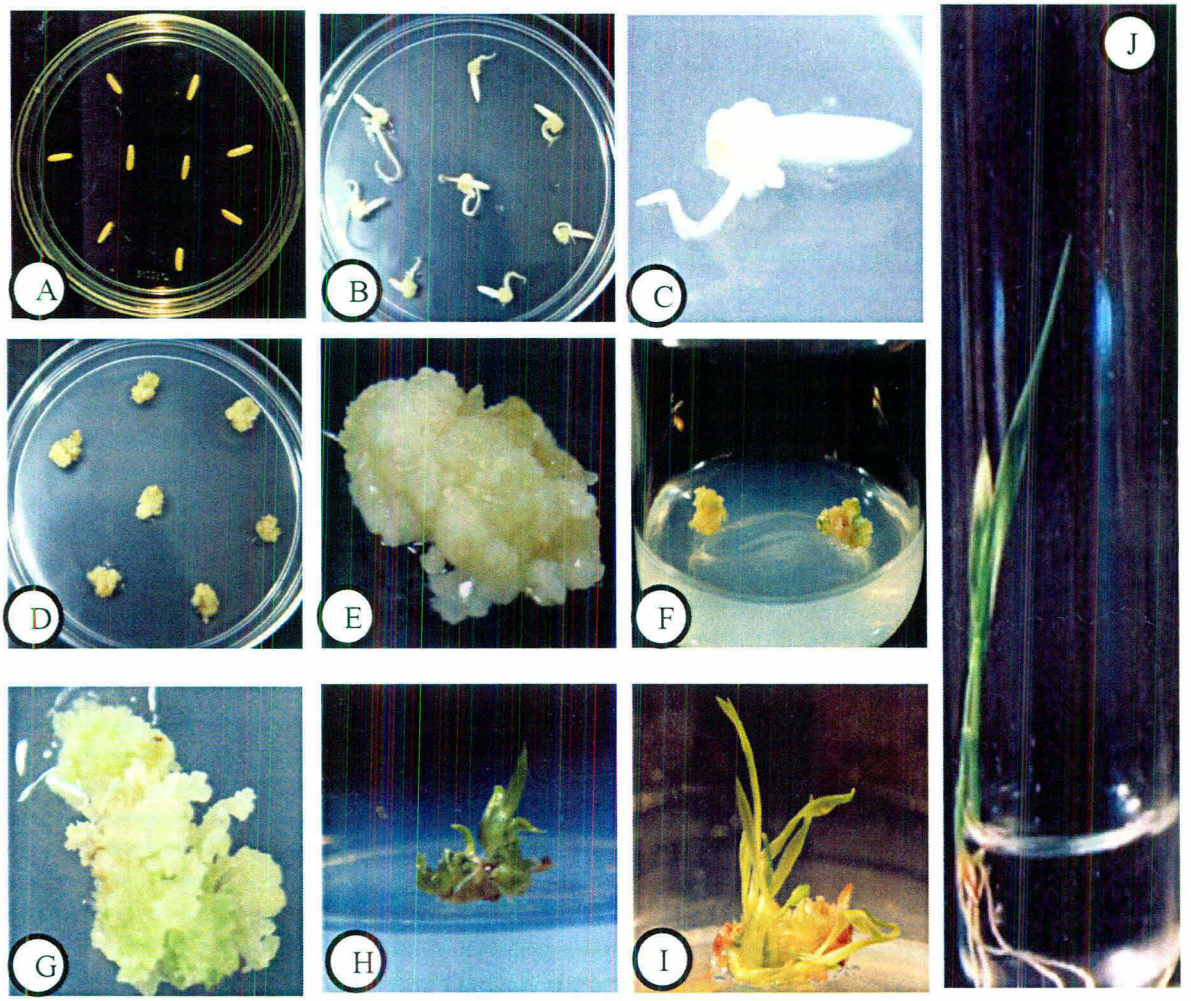


Fig 2.1 Regeneration from scutellum of IR64 rice *(oryza sativa, indica)* seeds.
(A) IR64 rice seeds on callus induction media. (B) Scutellum derived calli after 5 days.
(C) Scutellum derived callus after 5 days. (D) Scutellum derived callus after 14 days.
(E) 23 days old scutellum derived callus.
(F) Greening of callus after two weeks on regeneration media.
(G) Greening of callus after two months on regeneration media.
(H) Shoot bud induction from callus.
(I) Shoot bud elongation on regeneration media .
(J) Rooted plants in liquid rooting media after 7 days.

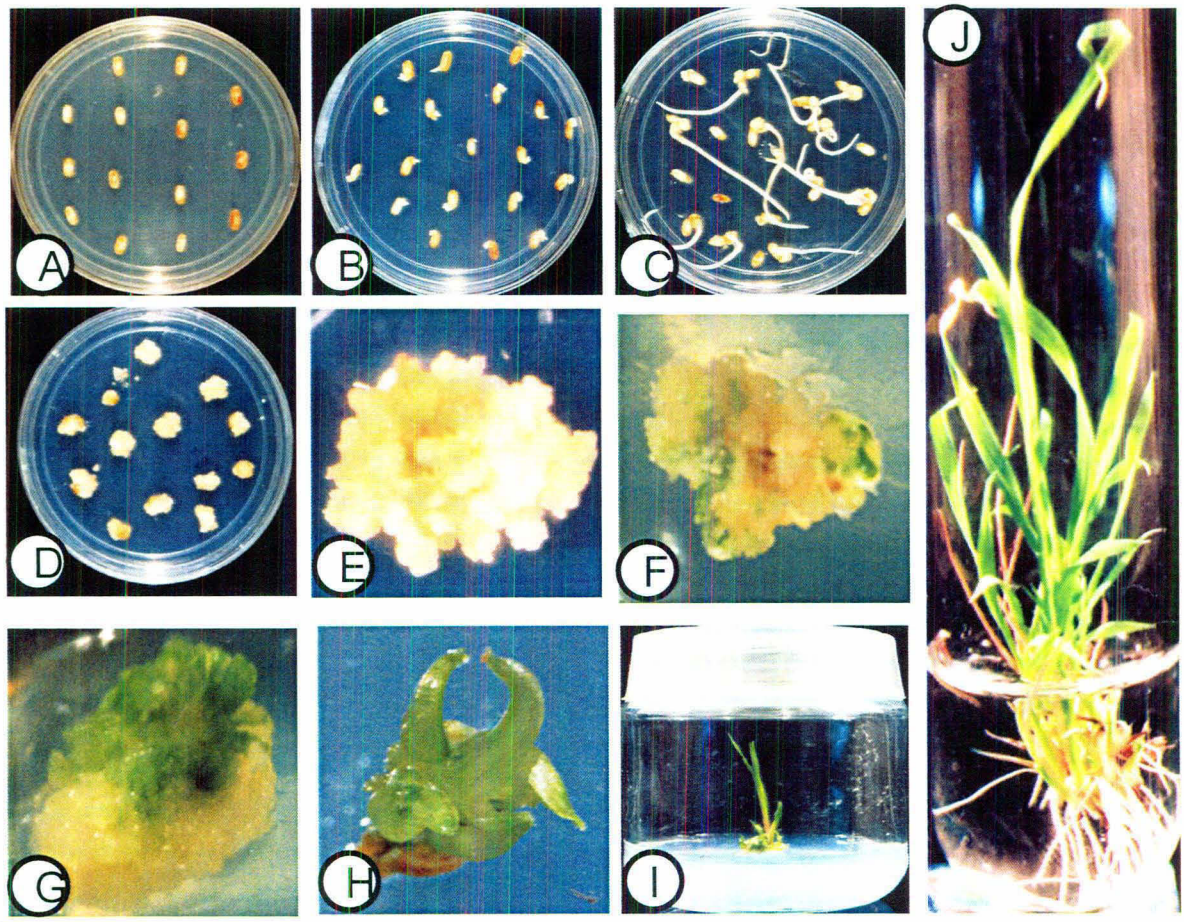


Fig 2.2 Regeneration from scutellum of *Budda* rice seeds (*Oryza sativa, indica*)
(A) *Budda* rice seeds on callus induction media.
(B) Scutellum derived calli after 2 days.
(C) Scutellum derived calli after 5 days.
(D) Scutellum derived calli after 14 days.
(E) 23 days old scutellum derived callus.
(F,G) Greening of callus after three weeks on regeneration media.
(H) Shoot bud induction from callus.
(I) Shoot bud elongation on regeneration media.
(J) Densely rooted plants after 15 days in liquid rooting media

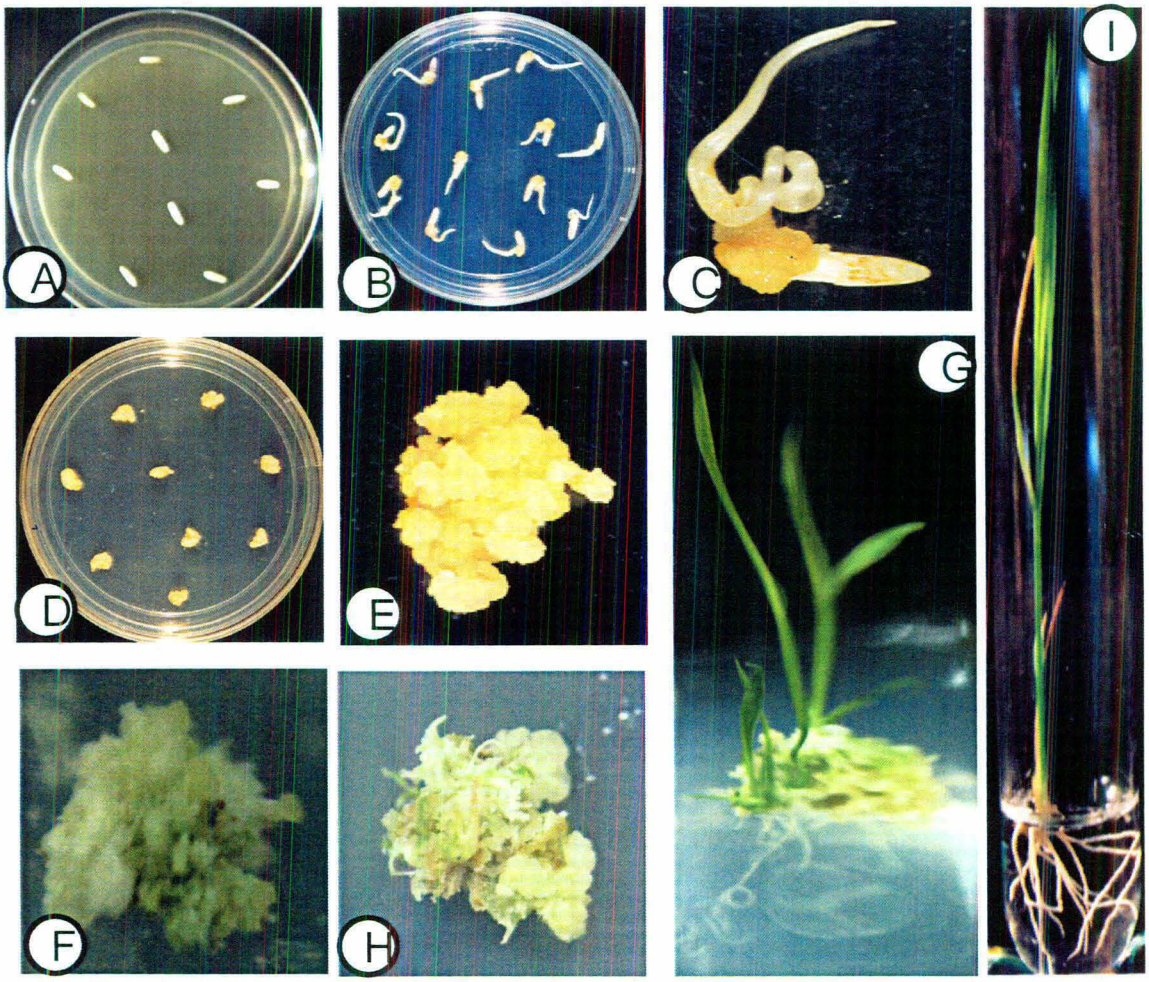


Fig 2.3 Regeneration from the scutellum of *Pusa Basmati-1* rice *(Oryza sativa, indica)* seeds.

(A) *Pusa Basmati-1* rice seeds on callus induction media.
(B) Scutellum derived calli after 3 days.
(C) Scutellum derived callus after 5 days.
(D) Scutellum derived calli after 14 days.
(E)23 days old scutellum derived callus.
(F) Greening and shoot bud induction of callus after two weeks on regeneration media.
(G) Shoot bud elongation on regeneration media.
(H) Dormant callus for shoot induction after 2 months on regeneration media.
(I) Rooted plants in liquid rooting media after 7 days.

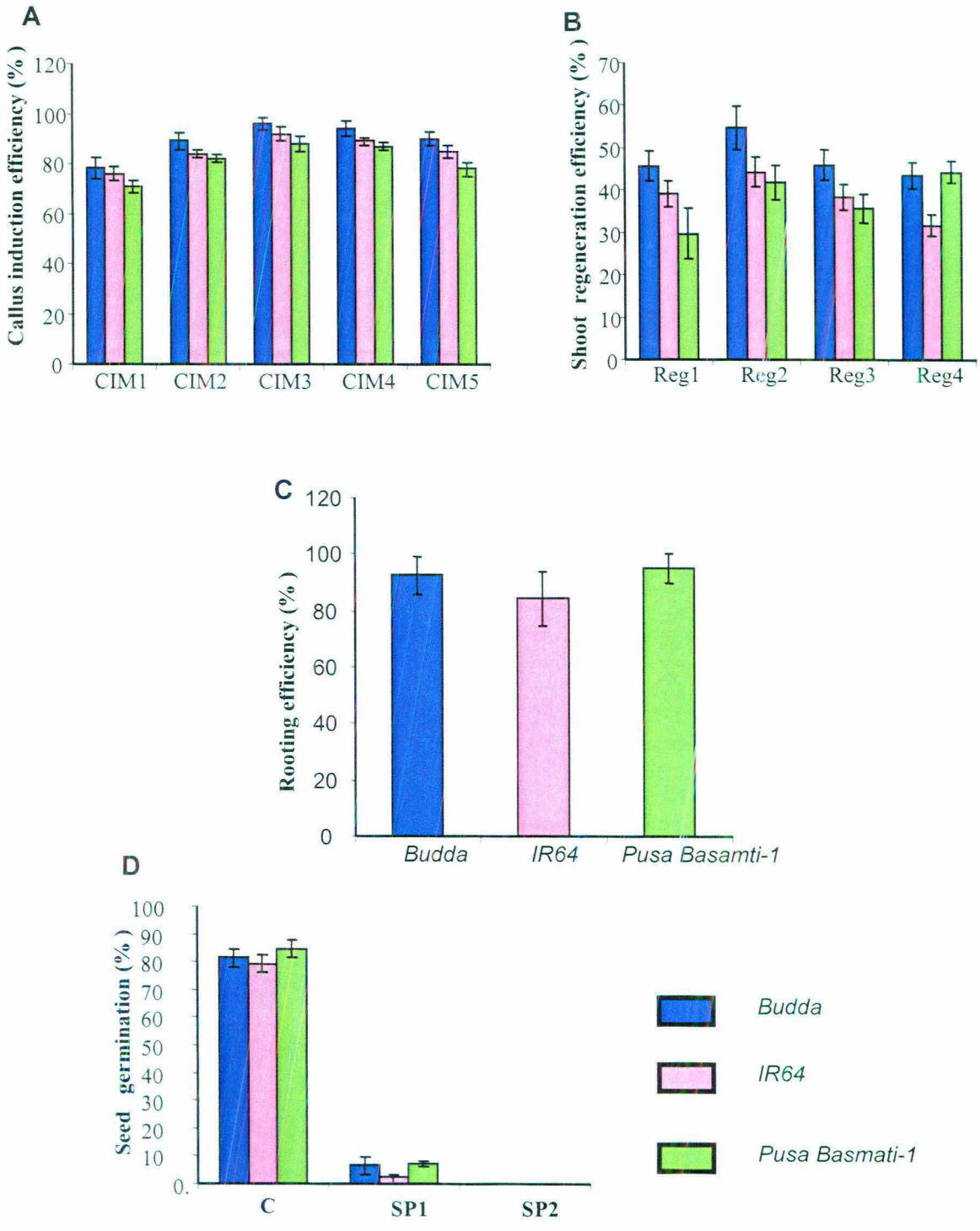


Fig 2.4 Genotypic variation among *Budda* , *IR64* and *Pusa Basmati-1* rice varieties. Three variety were checked for genotypic variation during (A) callus induction in different media composition (CIM1, CIM2, CIM3, CIM4 and CIM5), (B) shoot induction in different media composition (Reg1, Reg2, Reg3 and Reg4) (C) rooting in rooting media (D) seed germination in different antibiotic concentration (C, SP1 and SP2).

2.3.4 Selection

Selection of transformed calli or explants is a key factor in screening of transgenic plants. The regenerated transgenic plants could be screened for gene of interest by molecular analysis but early screening on the basis of antibiotic selection made it easier to handle large number of putative transgenic lines. In transformation of dicotyledonous plant based on *npt*II selectable marker gene, kanamycin sulfate is commonly used. However, it has been shown that this compound has no clear selection effect in cereals (Dekeyser *et al.*, 1989). Kanamycin sulfate not proved to be a good selectable marker for rice transformants (Vasil, 1994). The selectable marker gene *npt*II also confers resist to paromomycin sulfate and genticinin (G418). Various attempts have been made to use G418 against selection of *npt*II gene in rice (Chan *et al.*, 1993; Peng *et al.*, 1992; Toriyama *et al.*, 1988). The performance of G418 as a selective agent for monocots was better than kanamycin, but there was poor transformation efficiency since G418 also interferes in greening of calli. Paromomycin sulfate was the superior selective agent for transformation of oat (Torbert *et al.*, 1995) and turfgrass (*Lolium pereene* L. and *Festuca rubra* L.) (Altpeter *et al.*, 2000) and paromomycin sulfate could be a better antibiotic agent for *npt*II based selection in rice (Japan Tobacco, 1998).

The effect of paromomycin sulfate was tested on *IR64*, *Budda*, and *Pusa Basmati-1* during callus subculture, regeneration and seed germination. The influence of antibiotic during seed germination, regeneration and subculture of callus among three different cultivars is summarized in fig 2.4D, 2.5, 2.7.

The data for seed germination on different concentration of paromomycin sulfate showed that seed germination was inhibited with increasing concentration of paromomycin sulfate. Seed germination media added with SP2 concentration of Paromomycin sulfate completely suppressed the plant growth and all seeds showed dying of radical and plumule. The paromomycin sulfate at SP1 concentration in germination media resulted in albino, leaf chlorosis, stunted plants which did not further elongate and resulted in dying of plumule and radical.

The effect of paromomycin sulfate on callus during callus proliferation and regeneration of three varieties is depicted in Table 2.1, 2.2, 2.3 and 2.4. The regeneration of scutellum derived calli was inhibited by paromomycin sulfate at a

concentration of RP1 and completely suppressed at RP2 for IR64 and *Pusa Basmati-1* and RP3 in *Budda*, when subcultured for more than one month. In case of callus proliferation CP1 and CP2 concentration of paromomycin sulfate inhibited the callus proliferation with browning and CP3 concentration almost stopped callus proliferation in all three varieties when subcultured for 30 days.

Table 2.1 Effect of Antibiotic Paromomycin Sulfate on Proliferation of Callus in three Varieties of Rice (*Budda, IR64, Pusa Bsmati-1*)

Conc. of Paromomycin sulfate	*Budda*				*IR64*				*Pusa Basmati-1*			
	Callus	A	B	C	Callus	A	B	C	Callus	A	B	C
0 mg/l	50	38	9	3	50	37	10	3	50	42	7	1
CP1	50	33	12	5	50	29	14	7	50	38	9	3
CP2	50	22	14	14	50	19	18	13	50	33	15	12
CP3	50	3	23	24	50	1	15	34	50	5	21	24
CP4	50	0	17	33	50	0	11	39	50	0	13	37
CP5	50	0	6	44	50	0	2	48	50	0	4	46

Table 2.2 Effect of Different Concentration of Paromomycin Sulfate on Rice Regeneration of Pusa Basmati-1 Callus

Paromomycin sulfate	Browning after 8 Days					Browning after18 Days					Browning after 30 Days				
	Callus	A	B	C	plants	Callus	A	B	C	plants	Callus	A	B	C	plants
0 mg/l	86	83	2	1	0	86	80	4	2	2	86	70	6	10	25
RP1	69	60	7	2	0	69	52	14	3	0	69	40	20	9	7
RP2	88	38	47	3	0	88	30	49	9	0	88	10	60	18	0
RP3	72	21	40	11	0	72	13	49	10	0	72	2	18	52	0
RP4	43	3	30	10	0	43	0	6	37	0	43	0	3	40	0

Table 2.3 Effect of Different Concentration of Paromomycin Sulfate on Rice Regeneration of IR64 Callus

Paromomycin sulfate	Browning after 8 Days					Browning after18 Days					Browning after 30 Days				
	Callus	A	B	C	plants	Callus	A	B	C	plants	Callus	A	B	C	plants
0 mg/l	64	36	20	8	0	64	20	32	12	4	64	14	26	24	16
RP1	27	7	9	11	0	27	5	8	14	2	27	4	3	20	4
RP2	53	2	16	35	0	53	0	8	45	0	53	0	2	51	0
RP3	55	0	11	44	0	55	0	2	53	0	55	0	0	55	0
RP4	31	0	5	26	0	31	0	0	31	0	31	0	0	31	0

Table 2.4 Effect of Different Concentration of Paromomycin Sulfate on rice regeneration of *Budda*

Paromomycin sulfate	Browning after 8 Days					Browning after18 Days					Browning after 30 Days				
	Callus	A	B	C	plants	Callus	A	B	C	plants	Callus	A	B	C	plants
0 mg/l	48	32	12	4	0	48	29	9	7	8	48	24	14	10	21
RP1	37	9	14	14	0	37	6	16	15	6	37	5	14	19	17
RP2	40	8	17	15	0	40	4	20	16	5	40	3	15	22	8
RP3	42	5	24	11	0	42	3	18	21	0	42	0	12	30	0
RP4	40	2	17	21	0	40	1	14	25	0	40	0	8	32	0

A- No browning, B- Browning upto 50%., C- Browning 50 to 100%.

2.3.5 Transient Expression and Molecular Analysis

An expected 1.6 kb fragment of GUS region was amplified during PCR of putative transgenic line for *Gus* gene (Fig 2.6 A). Transient expression of GUS gene in rice callus was also confirmed by GUS staining (Fig 2.6B). Transient expression of GUS in transformed calli of *IR64*, *Budda*, and *PB-1* turned blue after GUS staining. The transient Gus expression efficiency was 44%, 51% and 31% for *Budda*, *IR64* and *Pusa Basmati-1*, respectively. Result of GUS assay of callus was showed an unequal distribution of blue clusters because a different expression level of *GUS* gene due to

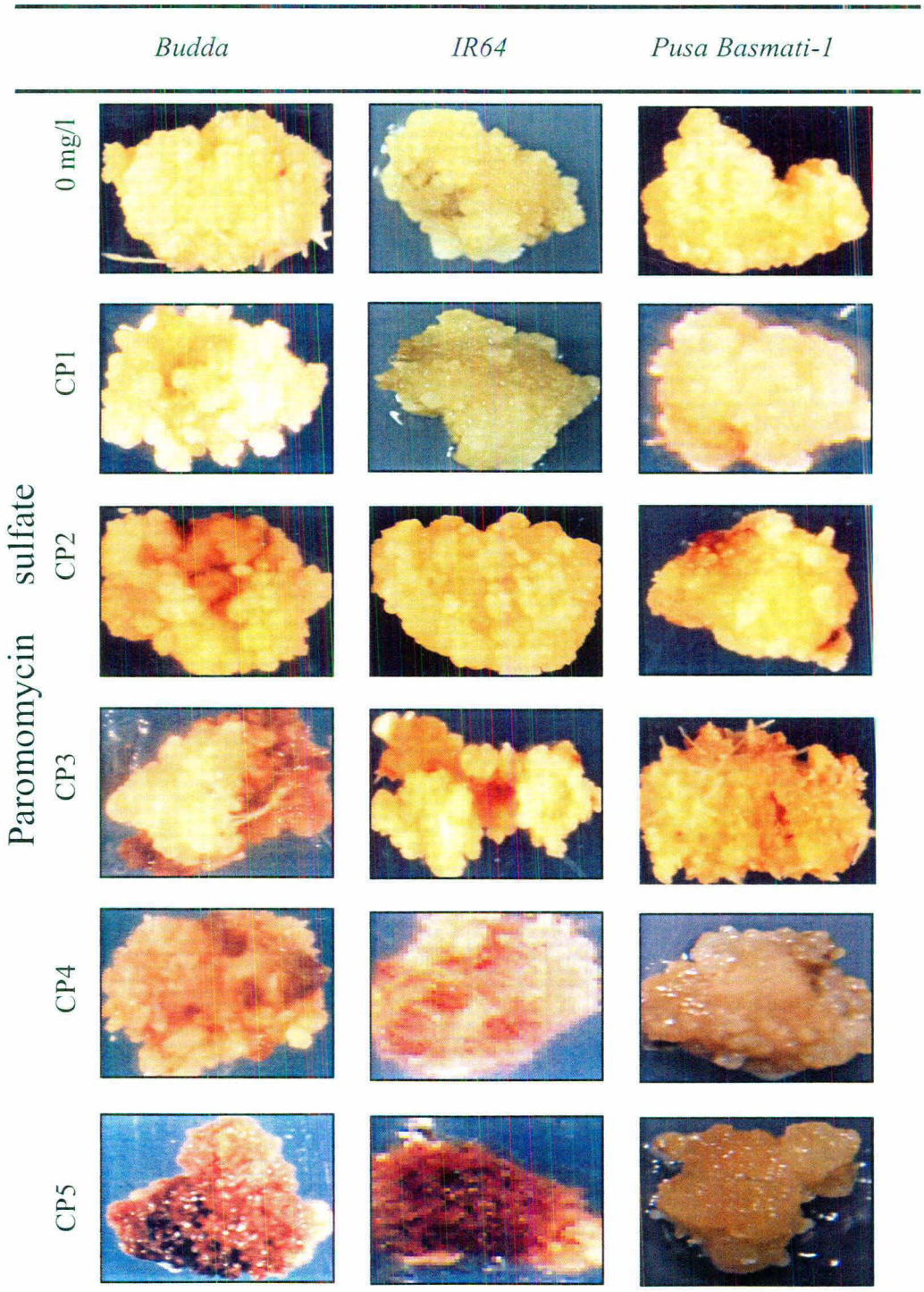


Fig 2.5 Effect of the different concentration of antibiotic paromomycin sulfate (CP1, CP2, CP3, CP4, CP5)on growth and proliferation of callus from *Budda*, *IR64* and *Pusa Basmati-1* rice varieties.

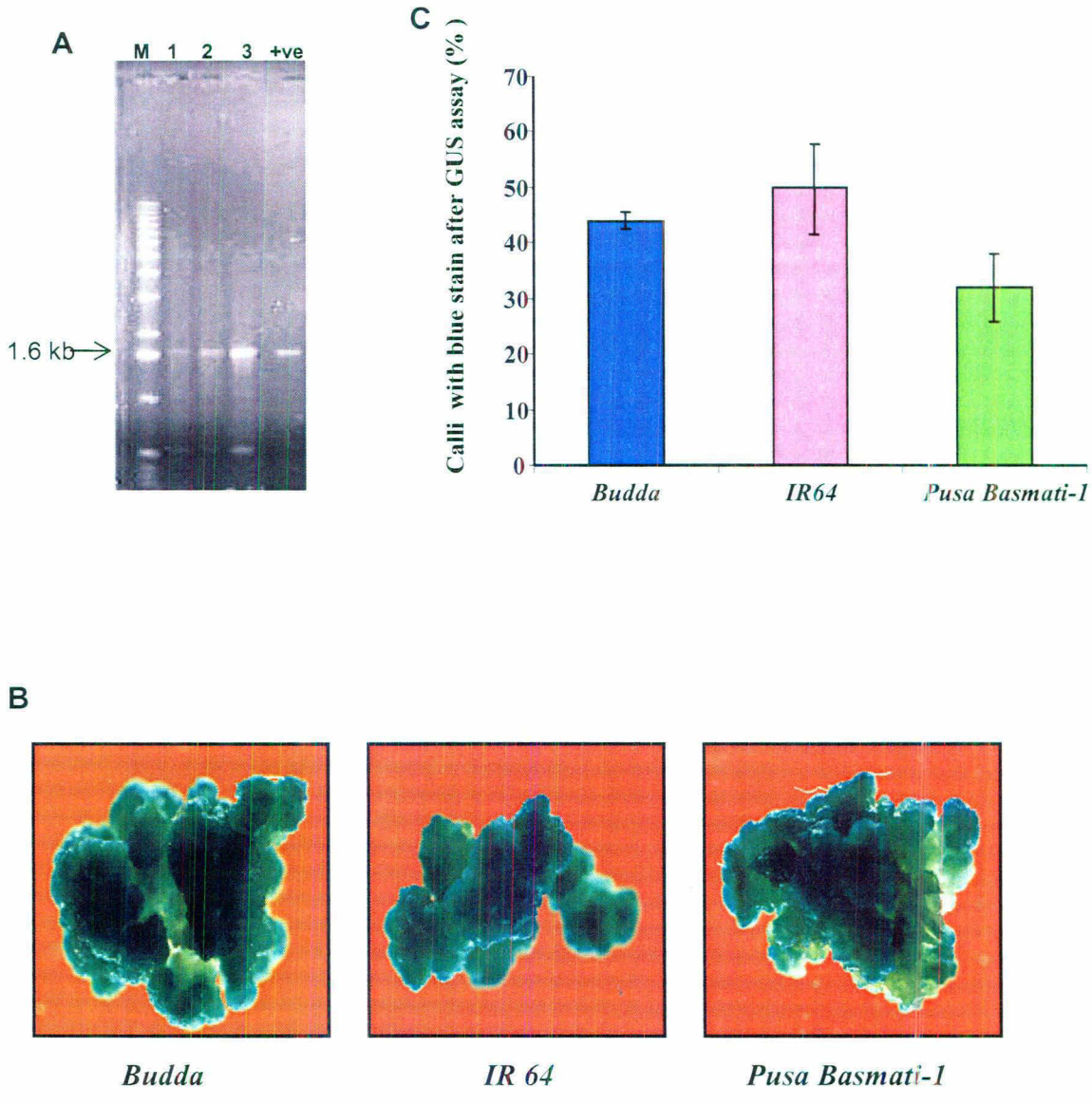


Fig 2.6 Analysis of *indica* rice transformed with pBI121 construct. (A) PCR amplification of GUS gene, an expected 1.6 kb fragment was amplified (B) Transient Gus staining after 7 days in *Budda, IR64, Pusa Basmati-1* callus. (C) Transient GUS efficiency after 7 days in *Budda, IR64, Pusa Basmati-1* callus.

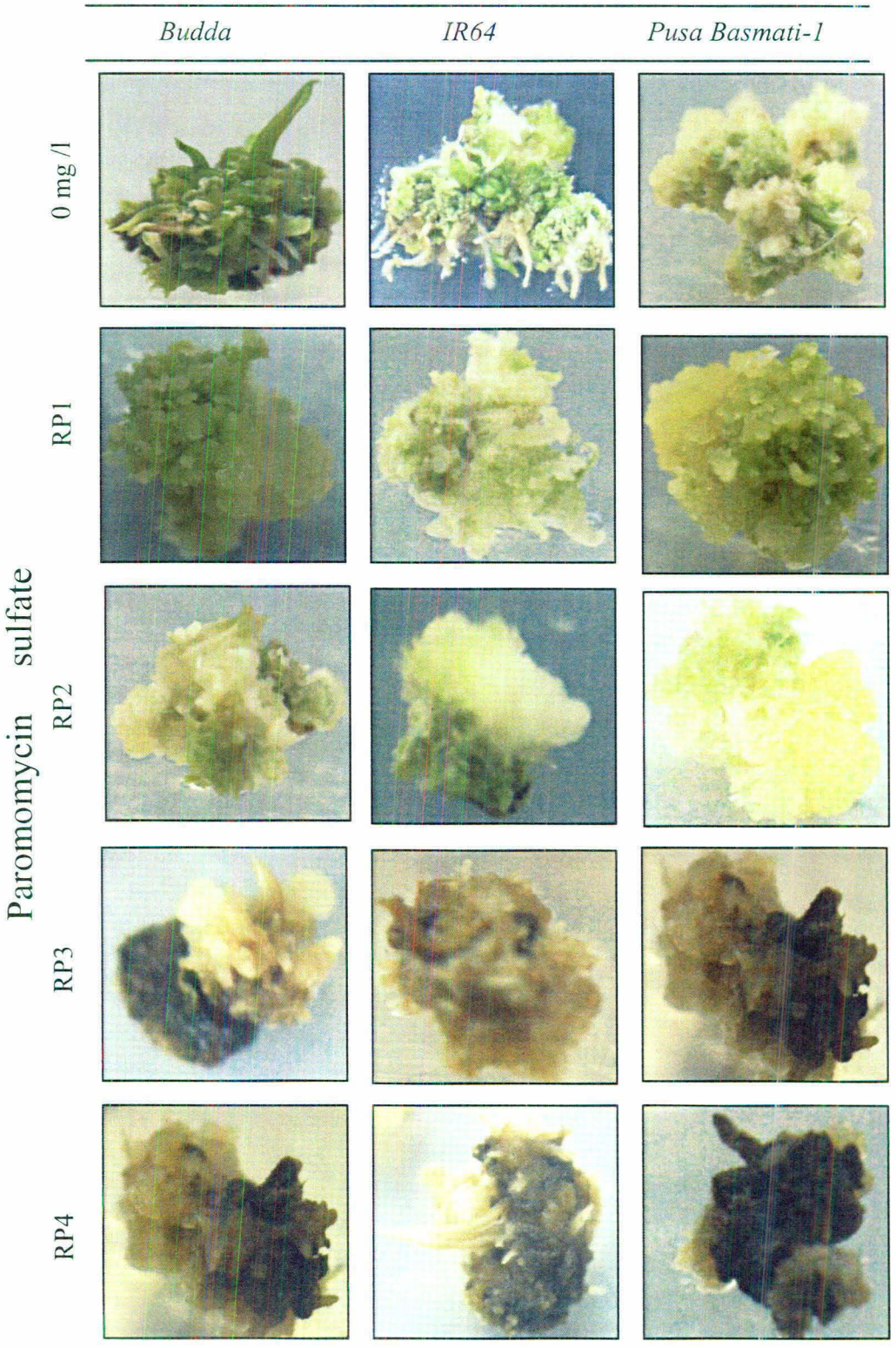


Fig 2.7 Effect of the antibiotic paromomycin sulfate on regeneration of callus from *Budda*, *IR64* and *Pusa Basmati-1* rice var. Different antibiotic concentration (RP1, RP2, RP3, RP4) were used in regeneration media.

position effect of transgene in host plant genome. Untransformed clusters of callus can escape on a selective media added with paromomycin sulfate. An efficiency of Transient Gus expression after 7 days is depicted in fig 2.6 C.

2.4 Conclusion

We have developed a regeneration and transformation system for different cultivar of *indica* rice. A range of protocols are available for rice regeneration and transformation using different explants like mature seed (Kyozuka *et al.*, 1988, Nasuda *et al.*, 1989, Svamani*et al.*, 1996) mature and immature embryo (Koetje *et al.*,1989; li *et al.*,1993; lee *et al.*,2002), anther and microspore (Gupta and Borthakur1987; Lentini *et al.*, 1995; Lee *et al.*, 2004), leaf blade (Yan and Zhao 1982), Coleoptile (Oinam and Kothari 1993; Saharawat and Chand 2001), root (Kawata and Ishihara.,1968; Abe and Futsuhara., 1985; Sticklen 1991; Mandal *et al.*,2003). In the present study, we used mature seeds as explant which is a better than other explants because of its availability of this throughout the year and was easy to store. A most popular and conventional Murashige and Skoog (1962) were used throughout the regeneration. We have made an effort to standardize a regeneration and transformation protocol which is simple and applicable to more than one genotypes of *indica* rice.

CHAPTER 3

AmA1 GENE TRANSFER IN RICE AND MOLECULAR ANALYSIS OF TRANSGENIC PLANTS

3.1 Introduction

Eight hundred million people worldwide live below absolute poverty levels, and the majority of these are malnourished as they do not have access to the essential food basket. Availability of nutritious rice will benefit poor people (Datta, 2001; Datta, S.K. 2001). Seed storage proteins, intended as a source of nitrogen for germinating seedlings, form an important source of dietary protein for human beings. Humans require a diet with a balanced amino acid composition, but often seeds are deficient in some of these essential amino acids. For years, plant breeders have tried to improve the balance of essential amino acids in important crop plants (Larkins, 1983) but have met with limited success.

Rice being major staple crop for more than half of world's population and is a primary source of protein for humans, especially in Asia. Rice is rich in carbohydrate but low in protein, vitamin A and minerals. Compared to other major cereals, rice grain has the less protein content. The amino acid composition of rice storage proteins is not well balanced due to the low content of lysine and threonine. Moreover, a considerable percent of rice storage protein is not digested by monogastric animals (Resurreccion *et al.*, 1982; Tanaka *et al.*, 1975). A typical daily diet of 300gm rice provides only 10% of the requirement of essential amino acids resulting in deficiency (FAO 2002). Protein deficiency is serious cause of ill health and death as it causes disease like *kwashiorkor* which is more frequent in many part of world.

To overcome the protein deficiency, we need genetically engineered crops that are tailored to nutrient dense crops which can reduce malnutrition. Golden rice (Ye *et al.*, 2000) represents a good genetic engineering concept to improve nutritional value of rice seed. One of the major and efficient methods for improvement of seed protein quality is over expression of gene encoding seed storage protein in crop plants. Rice endosperm tissue has advantage over other tissues as a production platform for foreign recombinant proteins. Takaiwa *et al.*, (2007) expressed hybrid peptide of seven major T-cell epitope in rice, which were mainly localized in the endoplasmic reticulum-derived protein bodies, designated as protein body I (PB-I). Although RNA transcript was also detected in leaf, embryo, stem but expression was mainly in seed and accumulated in protein bodies. This proved that seed has advantage over the other tissue in crop plants for recombinant protein expression. The Promoter is generally regarded as the most important element in regulating gene expression in quantitative

and qualitative ways (T.J. Guilfoyle, 1997; Meisel *et al.,* 1997). A seed specific expression of storage protein in rice is preferred in comparison to a constitutive expression in whole plants. Tissue specific expression is often a desirable characteristic because constitutive expression of foreign gene may interfere with normal plant development, growth and reproduction in transgenic plants (Kusnadi *et al.*, 1997). In rice, constitutive expression of gene for nutritional improvement is futile because seed is considered as a main edible part. Many seed specific promoters have been isolated from seed storage protein. Seed storage proteins are synthesized during seed development. In cereal crops, seed storage protein accumulate in short span and reach up to 15% of dry weight of seed. During endosperm development, the transcription of genes encoding storage protein is regulated in specific temporal and spatial pattern to provide these protein reserves (Chrispeels and Shannon, 1996). Such a time and tissue specific patterns of gene expression, along with high expression levels make the promoters of the seed storage protein ideally suited for controlling the expression of target protein in cereals grains. There are various report of *Agrobacterium* as well as biolistic mediated transformation in rice. It has been frequently stated that frequency of single insert events in biolistic DNA delivery system is low in comparison to *Agrobacterium* mediated gene delivery (Cheng *et al.,* 1997).

Cloning and characterization of *AmA1* gene encoding a seed storage albumin protein from *Amaranthus hypochondriacus* was reported from our laboratory. The study revealed that *AmA1* gene is the best donor candidate gene for protein improvement in crop plant as has been discussed in earlier section.

Present study is directed towards increasing the nutritional quality of rice with respect to protein by over expressing the said *AmA1* gene. For fulfillment of this, we constructed *AmA1* expression vectors driven by endosperm specific promoters to be transformed to rice var. *Budda, IR64, Pusa Basmati-1.* Putative transgenic lines were characterized at molecular and biochemical level.

3.2 Construction of Rice Seed Specific Expression Vector for *AmA1* Gene

3.2.1. Material and Methods

3.2.1.1 Cloning of NRP33 and Bx-7 Promoters in pGEM-T Easy Vector

3.2.1.1.1 PCR Amplification of Seed Specific Promoter Region

The promoter region of rice prolamin (NRP33, NCBI Accession Number A 63901) and wheat glutelin (Bx-7, NCBI Accession Number X13927) were amplified by PCR from Rice *Budda var.* (*oryza sativa, indica*) and wheat *UP2338* genomic DNA, respectively. All the PCR amplifications were carried out in MJ Research INC PCR machine. The PCR reaction was performed in 25 µl reaction volume containing 100 ng intact genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTP mix, 10 pM of each forward and reverse primers and 0.5 units of *Taq* polymerase. PCR thermal conditions and promoter specific primer sequences are as given below.

Promoter	Primers	Primer sequence	Length of primer	Amplified product	Tm
NRP33	NRP33 F	5′ TTC CTT CTA CAT Cgg CTT Agg 3′	21bp	647 bp	58°C
	NRP33 R	5′ATT CTA gAA TTg Tgg TgA Agg ATg 3′	24 bp		
Bx-7	Bx7 F	5′ CgT CgT CTC TgC Agg CCA ggg AAA gAC AAT g 3′	31bp	993 bp	62°C
	Bx7 R	5′ CgC TTA TCT AgA TCA gTg AAC TgT CAg Tg 3′	26bp		

Step	Temperature	Time	Specification
Step-1	94 °C	5 min	
Step-2	94°C	30 Sec	
Step-3	58°C	30 sec	In Case of NRP33
	62°C	30 sec	In case of Bx-7 promoter
Step-4	72°C	1 min	
Step -5	Go to step-2 and repeated for 30 cycle		
Step-6	72°C	10 min	
Step-7	4°C	5 min	

3.2.1.1.2 Elution of DNA from Agarose Gel

The PCR amplified product was run on 0.8% agarose containing with 0.5μg/ml EtBR. The band representing NRP33 (674 bp) and Bx-7(993 bp) promoters were gel excised and gel eluted. Gel elution was performed by using Mini Elute gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions with few modifications.

1. Three volumes (one volume of gel, 100 mg ~ 100 μl) of buffer QG was added to the eppendrof containing the gel slice and incubated at 50°C for 10 min to dissolve the agarose.
2. After the gel slice was dissolved completely, one gel volume of isopropanol was added and mixed by inverting the tubes 4-5 times.
3. Then sample was loaded into the Mini Elute column which was kept on a 2 ml collection tube and centrifuged at 13,000 rpm for 1 min.
4. After discarding the flow-through 500 μl of QG buffer was loaded onto the column and centrifuged at 13,000 rpm for 1min. The flow through was discarded and column was again placed in the same collection tube.
5. To wash the column, 750 μl buffer PE was loaded onto the column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and column was washed again with 250 μl of buffer PE.
6. Further the column was centrifuged for additional 1 min to remove the residual ethanol.
7. The Mini Elute column was then placed in clean 1.5 ml microcentrifuge tube. To elute DNA from column, 10 μl of elution buffer (100 mM Tris-Cl, pH 8.0) or sterile nuclease free water was loaded directly on the matrix. The column was allowed to stand for 2 min and then centrifuged at 13,000 rpm for 2 min. DNA was obtained as flow through.

3.2.1.1.3 Ligation of the Eluted DNA Fragment to pGEM T –Easy Vector

The eluted product of NRP33 and Bx-7 promoter was ligated separately with pGEM-T Easy vector (Promega) following protocol described below.

1. In a 0.2 ml tube, 50 ng of the vector was taken and 150 ng of the gel eluted fragment was added.
2. To this, 2X ligation buffer was added to make its final concentration to 1X.
3. Contents were mixed with the pipette and volume was made up to 9 µl with deionized autoclaved water.
4. Finally, 1 µl of T4 DNA ligase was added gently and mixed with the pipette, spun briefly and incubated at 4°C for 24 hr.

3.2.1.1.4 Preparation of Competent Bacterial Cells

For cloning, *E. coli* DH5α and GM2163 strain were made competent by calcium chloride method, as described (Sambrook *et al.*, 2001) with few modifications.

1. From overnight grown preculture of bacterial cells, 1 ml of inoculum was used to inoculate 100 ml LB medium in a culture flask.
2. This culture was grown at 37°C with constant shaking (200-250 rpm) till A_{600} OD reached 0.3-0.4.
3. The culture was chilled on ice for 15-20 min and centrifuged at 5000 rpm for 5 min at 4°C in Sorvall® RC5C plus centrifuge (Kendro, USA) with SA-600 rotor.
4. The pellet in each tube was gently suspended in 0.5 volumes (of original culture) of ice-cold 100 mM $CaCl_2$ by gently swirling the tubes and incubated on ice for 30 min.
5. The cells were collected by centrifugation as above and resuspended in 0.1 volumes ice-cold 100 mM $CaCl_2$ by gently swirling the tube.
6. Then the cells were aliquoted and snap frozen in liquid nitrogen and stored at -80°C.

3.2.1.1.5 Transformation *of E.coli* DH5-alpha and GM2163 Competent Cells

For each transformation, 100 µl aliquot of the competent cells were used.

1. Competent cells were thawed on ice.

2. 5 µl of the ligation mix and 45 µl of TE was added to 100 µl aliquots of competent cells and incubated for 30 min on ice with gentle tapping at regular intervals.
3. The cells were subjected to heat shock at 42°C in a water bath for 90 sec and then chilled on ice for 5 min.
4. 900 µl of 2XL broth was added to the cells after it attained room temperature. The cells were allowed to grow for 90 min at 37°C at 200 rpm.
5. Transformation efficiency was determined by counting the number of colonies /µg of DNA on selection plate.

3.2.1.1.6 Screening of the Transformants

1. After the transformation, 200 µl of the transformation mix was spreaded on LB agar added with 50 µg/ml ampicillin and 40 µl of X-Gal and 4 µl of IPTG then incubated at 37°C for overnight.
2. Next day the plates were kept at 4°C for 2 h for colour development.
3. Then the white colonies were randomly picked up and checked for the presence of the insert by isolating the plasmid.
4. The transformants were confirmed based on their retarded migration in comparison to the control (control 3 kb plasmid).

3.2.1.1.7 Small Scale Plasmid DNA Isolation (Mini Preparations)

Small scale plasmid DNA isolation from *E.*coli *DH5-alpha* and GM2163 strain cells was carried out using Promega Wizard plus SV Miniprep DNA Purification system. The protocol was followed as according to manufacturer's instruction with few modifications.

1. A single colony containing the plasmid from LB agar plate supplemented with antibiotic was inoculated in 3-5 ml LB medium supplemented with the same antibiotic. The cells were grown overnight (16-18 hours) at 200 rpm in 37°C shaker.
2. Cells were pelleted by centrifugation at 8000 rpm at 4°C for 5 minutes.

3. Pellet was resuspended in 250 µl of cell suspension buffer.
4. Then 250 µl of cell lysis solution was added to the sample and inverted 4 times to mix.
5. 350 µl of neutralization solution added to sample and again inverted 4 times to mix.
6. Then centrifuged at 12000 rpm for 10 min at room temperature.
7. The supernatant was decanted carefully into DNA binding spin column inserted in collection tube.
8. The column was centrifuged at 12000 rpm for 1 min at room temperature and the flow through was discarded.
9. 500 µl wash solution was added to the spin column and centrifuged at 12000 rpm for 1 min and the flow through was discarded from collection tube.
10. Step 9 was repeated with 500 µl wash solution and centrifuged at 12000 rpm for 2 min.
11. The DNA binding spin column was transferred to a new 1.5 ml microcentrifuge tube and centrifuged for 2 min at 12000 rpm.
12. Then DNA binding spin column was transferred to a new 1.5 ml microcentrifuge tube and 50 µl nuclease free water added and centrifuged for 1min.
13. Step 12 was repeated again with 50 µl Nuclease free water.
14. Plasmid DNA was visually quantified on 0.8% agarose gel added with EtBr.

3.2.1.1.8 Site Directed Mutagenesis of Promoter Region

The site directed mutagenesis of pG-N and pG-B clones was carried out using QuikChange® XL Site-Directed Mutagenesis Kit. The sequence of the primers used in site directed mutagenesis are mentioned below. The protocol followed as according to manufacturer's instruction with few modifications.

1. Site directed mutagenesis reaction was performed in a 50 µl reaction mix containing 1X reaction buffer, 10 ng of Plasmid, 125ng of forward and reverse primer, 1 µl of dNTP mix, 3µl of quick solution and the volume was made to 50 µl with autoclave MQ water. The thermal condition and primer sequences are given below.

Clone	Required Mutation	Primer Name	Length	Sequence of primer
pG-N	CT to TC	NRP33FM	31bp	5'CAT AAT ATT ATT TTC TTT GCT ACC CAT CAT G3'
		NRP33R	30 bp	5'CAT GAT GGG TAG CAA AGA AAA TAA TAT TAT G3'
pG-B	G to A	Bx-7 FM1	28bp	5'CCA ACT AAA TGA CAA GCA ACA AAA CCT G3'
		Bx-7RM2	28bp	5' CAG GTT TTG TTG CTT GTC ATT TAG TTG G 3'
pG-B	Insertion of T	Bx-7 FM2	26bp	5' CTT ATC CAG CTT TCT TTT GTG TTG GC 3'
		Bx-7 RM2	26bp	5' GCC AAC ACA AAA GAA AGC TGG ATA AG 3'

Step	Temperature	Time
Step-1	94°C	1 min
Step-2	94°C	40sec
Step-3	60°C	40 sec
Step-4	68°C	4 min
Step-5	Go to step 2 for 18 more cycle	
Step-6	68°C	10 min

2. The reaction tubes were placed on ice for 2 minutes to cool the reactions mix.
3. Then 1µl of the *Dpn*I restriction enzyme (10 U/µl) was added directly to each of the amplification reaction.
4. The reaction mix was mixed gently and thoroughly by pipetting the solution up and down several times. The reaction mix was spun down in a microcentrifuge for 1 min and immediately incubated at 37°C for 1 hour to digest the parental super coiled dsDNA.

5. The XL10-Gold ultra competent cells were gently thawed on ice. For each reaction mixture to be transformed, 45 µl of the ultra competent cells were aliquoted to a prechilled eppendrof tube and to it 2 µl of β-ME was added.
6. The contents of the tubes were mixed gently and incubated on ice for 10 minutes. The cells were swirled every 2 minutes.
7. The 2 µl of the *Dpn*I-treated DNA from each sample was transferred to separate aliquots of the ultra competent cells.
8. Then heat pulse was given at 42°C in water bath for 30 seconds and incubated for 2 min on ice. Then 0.5 ml of LB media was added to each tube and incubated at 37°C for 1 hour with continuous shaking at 250 rpm.
9. 200 µl of each of the transformation mix was spreaded on LB ampicillin (50µg/ml) plate and kept at 37°C for overnight.
10. Then plasmid DNA was isolated from single colony and sequenced.
11. The clone which showed original sequence was named pG- N and pG-B for NRP33 and Bx-7, respectively.

3.2.1.2 Sub Cloning of NRP33 and Bx-7 Promoter in pBS KSII(+/-)

NRP33 and Bx-7 promoter were cloned in pBS KSII (+/-) plasmid to get *Hind*III - *Xba*I fragment of promoter to replace CaMV35S promoter in pSB8 vector.

3.2.1.2.1 Vector Preparation

1. 10 µg of pBS KS II (+/-) plasmid was digested in 20 µl reaction volume consisting of 1X NEB Buffer 2, 1X BSA and 10 units each of *Pst*I and *Xba*I enzyme and incubated at 37°C for 2 hours.
2. The reaction was stopped by heating the reaction mix at 80°C for 20 min.
3. The digested plasmid was loaded on a 0.8% agarose preparative gel and run at 70 V.
4. The 3 kb linear fragment of pBS KS II (+/-) was gel excised, followed by gel elution using mini elute gel extraction kit (Qiagen, Germany) according to protocol mentioned in section 3.1.1.1.2.

3.2.1.2.2 Insert Preparation

1. 20 µg plasmid DNA of each plasmid pG-N and pG-B was separately digested with *Pst*I and *Xba*I enzyme as described above in section 3.1.1.2.1.
2. The digested product of both the reactions were loaded separately on 0.8% preparative agarose gel and run at 70 V till 0.67 kb of NRP33 and 1 kb of Bx-7 fragment were well separated from 3 kb linear fragment of pGEM-T Easy vector back bone.
3. *Pst*I-*Xba*I fragment of NRP33 and Bx-7 were gel excised and eluted as described previously and visually estimated on 0.8% agarose gel.

3.2.1.2.3 Ligation

Ligation reaction was set up using 50 ng of *Pst*I-XbaI digested 3kb linear fragment of pBS KSII (+/-) and *Pst*I–*Xba*I digested 0.67 kb of NRP33 fragment in 10 µl reaction volume containing 1X ligation buffer and 1 µl T4DNA ligase at 4°C for 24 hr. Similar protocol was used to ligate *Pst*I-*Xba*I fragment of Bx-7 with 3kb linear fragment of pBS KSII (+/-).

3.2.1.2.4 Transformation and Screening of Recombinants

2 µl of each ligation mixture was used to transform *E.coli* strain GM2163 competent cells as described in section 3.1.1.1.5. 100 µl of transformation mix was spreaded on LB ampicillin agar plate and incubated at 37°C for 14 hours. Plasmid DNA from colonies grown on LB ampicillin plate were screened for the presence of insert using promoter specific primer in a 25 µl reaction volume containing 100 ng intact plasmid DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTP mix, 10 pM of each forward and reverse primers and 0.5 units of *Taq* polymerase. The thermal condition is described below. Resultant positive clones were named as pBS-N and pBS-B for NRP33 and Bx-7 promoter region, respectively.

Step	Temperature	Time	Specification
Step-1	94 °C	5min	
Step-2	94°C	30 Sec	
Step-3	58°C	30 sec	In Case of NRP33
	62°C	30 sec	In case of Bx-7 promoter
Step-4	72°C	1 min	
Step -5	Go to step-2 and repeated for 30 cycle		
Step-6	72°C	10min	
Step-7	4°C	5 min.	

3.2.1.3 Cloning of NRP33 and Bx-7 Promoter in pSB8 Construct

NRP33 and Bx-7 gene promoters were used for seed specific expression of *AmA1* gene in rice. Replacement cloning strategy was used to replace 0.8 kb *Hind*III-*Xba*I fragment of pSB8 representing CaMV35S promoter with *Hind*III-*Xba*I fragment of NRP33 and Bx-7 gene promoter.

3.2.1.3.1 Vector Preparation

1. 10 µg of pSB8 vector plasmid was double digested in a reaction volume of 20 µl consisting of 1X NEB buffer 2, 1X BSA, 5 µg plasmid DNA, 10 units of each *Hind*III and *Xba*I enzyme and incubated at 37°C for 6 hour.
2. Digested product was resolved on 0.8% agarose gel to separate 11.47 kb linear vector back bone from 0.8 kb fragment of CaMV 35S.
3. Then the 11.47 kb linear fragment was gel excised and eluted as described in section 3.1.1.1.2.
4. The eluted product was visually estimated on 0.8% EtBr agarose gel.

3.2.1.3.2 Insert Preparation

1. Plasmid DNA of pBS-B isolated from GM2163 strain was digested completely in a reaction volume of 20 µl containing 1X NEB buffer 2, 1X

BSA, 5 µg plasmid DNA, 10 units of each *Hind*III and *Xba*I enzyme and incubated at 37°C for 6 hour. But in case of pBS-N, 5 µg plasmid DNA was digested completely with *Xba*I and partially with *Hind*III. For fulfillment of this, reaction was set up in 20 µl volume containing 1X NEB buffer 2, 10 unit of *Xba*I, 1X BSA, 10 µg of plasmid DNA for 6 hours at 37°C. This is followed by addition of 2.5 unit *Hind*III enzyme in reaction mixture and incubated at 37°C, after every 5 min, digestion product was analyzed by resolving 2 µl reaction mixture on 0.8% agarose gel added with EtBr. The time point noted at which 0.67 kb fragment was visualized with partial fragment of 0.35 kb and 0.32 kb in agarose gel. This is followed by setting a reaction in replicate following same reaction condition upto a time point to get sufficient amount of insert DNA.

2. The digested product of both the reactions were loaded separately on 0.8% preparative agarose gel and run at 70 V till 0.67 kb of NRP33 and 1 kb of Bx-7 fragment were well separated from 11.47 kb linear fragment of pSB8 backbone.
3. *Hind*III-*Xba*I fragment of NRP33 (0.67kb) and Bx-7(1.0kb) were gel eluted as described previously and estimated on 0.8% agarose gel.

3.2.1.3.3 Ligation and Transformation

Ligation reaction was set up separately using 11.47 kb linear fragment of pSB8 with *Hind*III-*Xba*I fragment of NRP33 (0.67kb) and Bx-7(1.0kb) in 10 µl reaction volume as described previously. 2 µl of ligation mixture was used to transform *E.coli DH5–alpha* competent cells using protocol described previously and spreaded on LB-agar plate supplemented with 50 µg/ml kanamycin.

3.2.1.3.4 Screening and Selection of Recombinants Clones

Plasmid DNA was isolated from transformed colony using Promega wizard plasmid isolation kit and confirmed by PCR using promoter specific primers. Further, the PCR positive clones were confirmed by restriction digestion and southern blotting.

3.2.1.3.4.1 PCR Confirmation of Recombinant Clone

PCR amplification of NRP33 and Bx-7 promoter from both recombinant clones was carried out using promoter specific primers as described in section 3.1.1.2.4. PCR product was resolved on 0.8% agarose gel with EtBr.

3.2.1.3.4.2 Restriction Digestion

1. 5 µg plasmid DNA of each pSBN and pSBB was double digested separately in 20 µl volume consisting of 1X NEB buffer 2, 1X BSA, 10 unit of each *Hind*III and *SnaB*I at 37°C for 6 hours. The reaction was stopped by incubating the reaction mix at 80°C for 20 min.

3.2.1.3.4.3 Southern Blotting of Recombinant Clone

Restriction Digestion

Plasmid DNA of both the recombinant clones was digested with *Hind*III and *Xba*I at 37°C for 4 hour to excise out NRP33 and Bx-7 promoter from pSB8 vector backbone according to protocol described earlier.

Agarose Gel Electrophoresis

1. Digested product was mixed with 1X Endo R dye and loaded on 0.8% agarose gel containing 0.5µg/ml EtBr with 1kb DNA ladder as a marker.
2. The gel was run at constant 70 V with 1X TAE buffer until dye reached 2/3 of gel. After electrophoresis, the gel was photographed to mark the position of bands. Digested product was transferred from gel to nylon membrane (GE biosynthesis) membrane as per manufacturer's instruction.

Southern Transfer

1. The gel was rinsed with MQ-water and depurination of the DNA was done by immersing the gel in 300 ml of 0.25 N HCl for 10 minutes in a baking dish with mild shaking (80 rpm) on gyratory platform shaker.

2. The solution was discarded and the gel was rinsed with MQ-water to wash off excess HCl and treated with 150 ml denaturing solution (0.6 M NaCl and 0.4 N NaOH) for 30 minutes, under similar conditions.
3. The solution was then exchanged with 150 ml neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl (pH 7.5) for 30 minutes.
4. Gene Screen Plus® membrane and 3 mm whatmann® paper were pre-wet for few seconds in MQ-water and equilibrated in 10X SSC for 15 minutes.
5. Whatmann paper and the Gene Screen Plus® membrane were aligned and the wells were marked on the membrane with a needle.
6. DNA was transferred onto the positively charged nylon membrane by capillary transfer method using 20X SSC as transfer buffer, for 16-18 hrs.
7. After transfer, the membrane was agitated in 0.4 N NaOH for 1 minute. This step was performed to denature the DNA. The membrane was then neutralized in 0.2 M Tris-HCl (pH 7.5)/1X SSC for 1 min.
8. UV-cross linking of nucleic acids to the membrane was done in Strata linker (Stratagene) for one minute at 1200 joules/sec of energy.

Probe Preparation

1. Plasmid DNA from pSBN and pSBB recombinant clones were PCR amplified using NRP33 and Bx-7 specific primers, respectively as described previously.
2. PCR Product was run on 0.8% agarose gel with EtBr and both 0.67 kb and 1.0 kb fragment of NRP33 and Bx-7 were gel eluted as described previously.
3. Gel eluted fragment were visually estimated by running on 0.8% agarose gel.
4. 50 ng of insert DNA was denatured in 17.5 µl MQ in a boiling water bath for 5 min followed by quick chilling on ice.
5. 2.5 µl of 10X labeling buffer, 1.0 µl of each dNTPs, and 1.0 µl klenow fragment was added to denature DNA and volume made up to 25 µl.
6. Then reaction mixture was incubated at 37°C for 1 hour followed by deactivation at 65°C for 20 min.

7. The probe was purified on 1 ml Sephadex G-50 column saturated in TE (pH-8.0), as per Sambrook *et al.*, (1989)

Prehybridization

1. The membrane was pre-wet in 2X SSC for 1 minute.
2. The membrane was then placed in a hybridization bottle and pre hybridized overnight, with 50 µl prehybridization buffer per cm^2 membrane (~9 ml for 12 cm x 14 cm membrane), at 42°C in hybridization incubator (Robbins Scientific).

Hybridization

The membrane was hybridized by adding the probe to prehybridization solution and hybridization was done at 42°C, for 16 hours in a hybridization incubator (Robbins Scientific).

Post-hybridization Washing

1. The membrane was washed with 2X SSC at room temperature for 10 minutes.
2. The second washing was done with 2X SSC/1% SDS at 42°C for 20 minutes. The washing was repeated depending on the counts.
3. The membrane was then washed with 0.2X SSC/1% SDS at 42°C for 20 minutes.
4. After each washes the background count was monitored with a handheld monitor (Grieger Muller counter) to avoid washing off of specifically bound signal.

Exposing and Developing of Film

1. The membrane was kept securely in a poly bag in wet condition.
2. The blot was exposed to Kodak X-Omat™ film in a film cassette, and kept at -80°C.
3. The film was developed and aligned with the marker to identify the signals.

3.2.1.4 Mobilization of Expression Plasmid in *Agrobacterium* and Confirmation

Successes in transformation of plants widely vary depending on the cultivar, *Agrobacterium* strain and antibiotic marker. The *Agrobacterium* strain EHA105 has been used to transform rice in present work. The strain contains L, L-succinamopine Ti plasmid which makes it hyper virulent. Constructs were mobilized into the strain by triparental mating. Transformed *Agrobacterium* strain EHA 105 was confirmed by colony hybridization for presence of binary plasmid.

3.2.1.4.1 Triparental Mating

1. *E.coli* DH5-alpha containing the *AmA*1 constructs and *E.coli* HB101 harboring the helper plasmid pRK2013 were streaked on LB kanamycin plate and incubated at 37°C for 10 -16 h.
2. *Agrobacterium tumefaciens* EHA105 was streaked on YEP plate supplemented with rifampicin (50 mg/ml) and incubated at 28°C for 48 hr.
3. Single colonies of *E coli* and *A. tumefaciens* were inoculated in 2 ml LB and YEP, respectively supplemented with appropriate antibiotics. The cultures were grown overnight.
4. 100 µl of each culture was mixed in tube and incubated for 5 min. The mix was spotted onto YEP agar plate and incubated at 28°C for 48 h.
5. The spotted cell were then streaked on to selection YEP agar plate containing 50 µg/ml rifampicin and 50 µg/ml kanamycin and incubated at 28°C for 48 h.
6. The presence of intact plasmids in *A. tumefaciens* EHA105 was checked by colony hybridization.

3.2.1.4.2 Colony Hybridization

1. The membrane (NEN DuPont) was placed facing the colony side of the agar plate without trapping air bubbles for 2 min. Three asymmetric orientation marks were made by piercing the membrane and agar with a sterile needle. The needle holes were marked on to the membrane as well as on the plate.

2. The membranes were treated with denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 2 min at room temperature with colony side up such that the solution does not run over the colony side.
3. The membrane was lifted from the solution and excess solution was allowed to drain on 3 mm Whatmann paper. The membrane was treated with neutralization solution (1.5 M NaCl and 0.5 Tris-HCl, pH 8.0) for 5 min at room temperature in the similar manner as in step 3.
4. The membrane was washed with 2X SSC and 0.2 M Tris-HCl, pH 7.5 for 5 min at room temperature.
5. The membrane discs were blotted on filter paper and allowed to air dry and the DNA on the membrane was cross linked using UV cross-linker (Strata-linker) for 30 sec at 1200 joules/cm.
6. Hybridization and washing of the membrane was carried out as described previously till the counts reduced to 10 cpm. Then membrane was exposed to KODAK film for 24 hours.

3.2.1.4.4 Picking up the Positive Colonies

1. The asymmetric marks on the membrane were aligned with the film orientation marks and then marked.
2. Positive signals were marked on the plate and an area of 2 mm was picked up containing the positive colony with sterilized toothpick.

3.2.1.4.5 Storage of Bacterial Strains

For long term storage, overnight grown bacterial cultures were stored as glycerol stock. Bacterial cultures were grown overnight and sterile glycerol was added to a final concentration of 15% and stored at -80°C.

3.2.2 Results and Discussion

3.2.2.1 Construction of *AmA1* Gene Expression Vector for Rice

Seed specific expression of *AmA1* gene in rice was driven by rice prolamin NRP33 gene promoter (0.674 kb) and wheat Glutelin Bx-7 (0.993kb) gene promoters. These two promoters have been proved in rice for endosperm specific expression of *GUS*

gene. A comparative study of GUS expression in rice driven by seed specific promoters Bx-7 (wheat glutelin), Glb (rice globulin), GluB2 (rice glutelin), Gt3 (rice glutelin) after 7-9 DAP in immature endosperm concluded that Bx-7 promoter has highest expression (Hwang, Y. *et al.*, 2001). Chaun Y *et al.*, (1998) reported that 13 KDa rice prolamin NRP33 promoter activity throughout endosperm of rice with stronger expression in aleurone and sub aleurone. Therefore, considering these previous reports, we used NRP33 and Bx-7 promoter for seed specific expression of *AmA1* gene in rice. The Bx-7 promoter region (Nucleotide No. 15055 to 16033 of Glu-B1-1b gene, Acc. No. X13927) and NRP 33 (Nucleotide No. 3 to 673 of NRP33 gene for 13 KDa proteins, Acc. No. A 63901) was amplified from wheat and rice genomic DNA, respectively (Fig 3.1 A, B). The primers were designed to amplify promoter region upto translation initiation site. Primers for NRP33 and Bx-7 promoter were appended with *Pst*I and *Xba*I site to forward primer and reverse primer, respectively to get compatible ends to sub clone in pBS KSII (+/-). PCR amplified product of both the promoters were cloned in pGEM-T Easy vector (Fig 3.1) and the sequences of both the promoters were confirmed by sequencing to identify any mismatch and mutation. The results of sequencing showed, two adjacent nucleotide mismatches in NRP33 promoter at 416 and 417 nucleotide position (CT to TC). In case of Bx-7 promoter, two single nucleotide mismatches were identified at position 15452(A to G) and a deletion at position 15826 deleted in comparison to original sequence deposited in NCBI. Since, spatial expression and various regulation of gene are controlled by DNA motif of promoter region. Thus mutation in promoter may affect expression of gene at various levels. Therefore to correct the mutation, we performed site directed mutagenesis of the mutated or mismatched nucleotide to revert it back to the original sequence (As deposited in NCBI). For fulfillment of this, two complimentary PAGE purified oligonucleotides, containing the desired mutation in middle flanked by unmodified sequence were used for site directed mutagenesis in each reaction. The rectified clones were further sequenced to rule out any mismatch in comparison with original sequence of NRP33 and Bx-7 gene promoter and named as pG-N and pG-B, respectively. A schematic representation of cloning of promoter region in pGEM-T Easy is depicted in fig 3.1.

*Pst*I-*Xba*I fragments of pG-N and pG-B were ligated separately with *Pst*I-*Xba*I fragment of pBS KS II (+/-). Resultant recombinant clone were confirmed by PCR

amplification using promoter specific primers (Fig 3.2A) and named as pBS-N and pBS-B for NRP33 and Bx-7 insert, respectively. The pBS-N and pBS-B recombinant clones were transformed to dam^{-ve} strain GM2163 to get demethylated *Xba*I site since it was blocked by methylation due to overlapping sequences. A schematic representation of subcloning of promoter region in pBS KSII (+/-) is depicted in fig 3.2.

To excise out, *Hind*III-*Xba*I promoter fragment, partial and complete digestion strategy were used, for pBS-N and pBS-B, respectively because pBS-N was carrying to two *Hind*III sites within the NRP33 promoter region (Fig 3.3 A, B). Similarly 0.8 kb CaMV35S promoter region in pSB8 was also excised out by restriction digestion using *Hind*III and *Xba*I digestion (Fig 3.3 C).Further, 0.8 Kb *Hind*III-*Xba*I fragment of CaMV35S promoter in pSB8 (Chakraborty *et al.*, 2000) was replaced separately with *Hind*III- *Xba*I fragment of NRP33 (0.67 kb) and Bx-7(1.0 kb). Resultant recombinant clones were confirmed by PCR amplification and restriction digestion. PCR result showed an amplification product of 674 bp and 993 bp of NRP33 and Bx7 (Fig 3.4 C, D). PCR positive clone were further confirmed by restriction digestion and southern blotting (Fig 3.5 C, D). Restriction digestion of pSB8N recombinant clone with *Hind*III-*Xba*I excised out NRP33 promoter in two fragments of each 0.35 kb and 0.32 kb since it had two restriction site for *Hind*III (Fig 3.4 A). But in case of pSB8B recombinant clone, *Hind*III- *Xba*I digestion excised out 1.0 kb fragment of Bx-7 promoter (Fig 3.4B). Further, pSB8N and pSB8B vector were digested with *Pst*I-*SnaB* I to excise out full length promoter (674 bp of NRP33, 1 kb of Bx-7) with partial fragment of *AmA1* gene (306bp) to confirm directional cloning. An expected 1.0 kb and 1.3 kb fragments were obtained from pSB8N and pSB8B recombinant clone, respectively (Fig3.5 A).

3.2.2.2 Conjugation of Recombinant Plasmid into *Agrobacterium* and Confirmation

The expression vectors for plant transformation can be mobilized in *Agrobacterium* strain either by direct DNA transformation (known as electroporation) or conjugation (known as triparental mating). Triparental mating method involves the *E.coli* harboring the helper plasmid *pRK2013*, the *E.coli* strain containing the binary vector

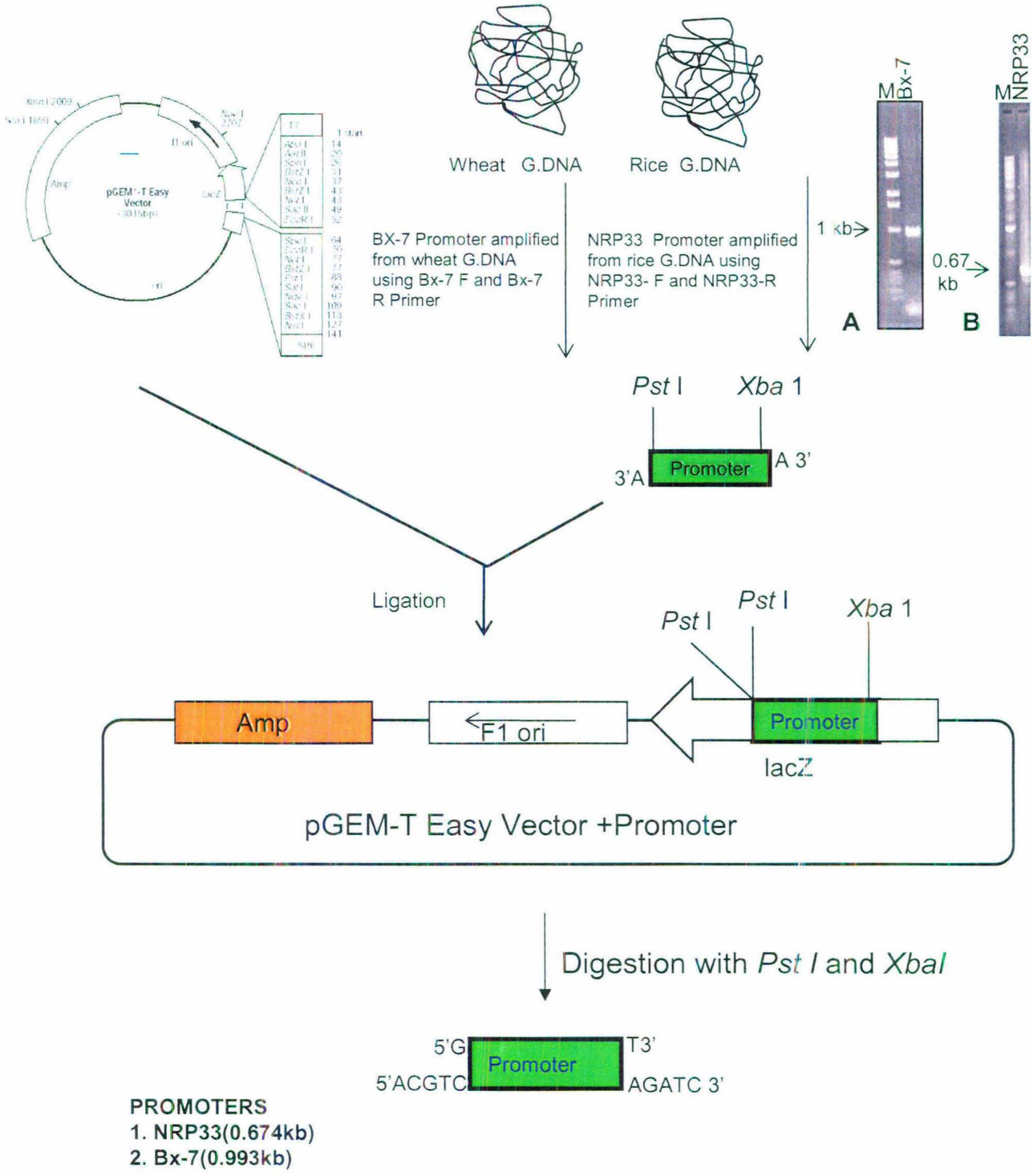
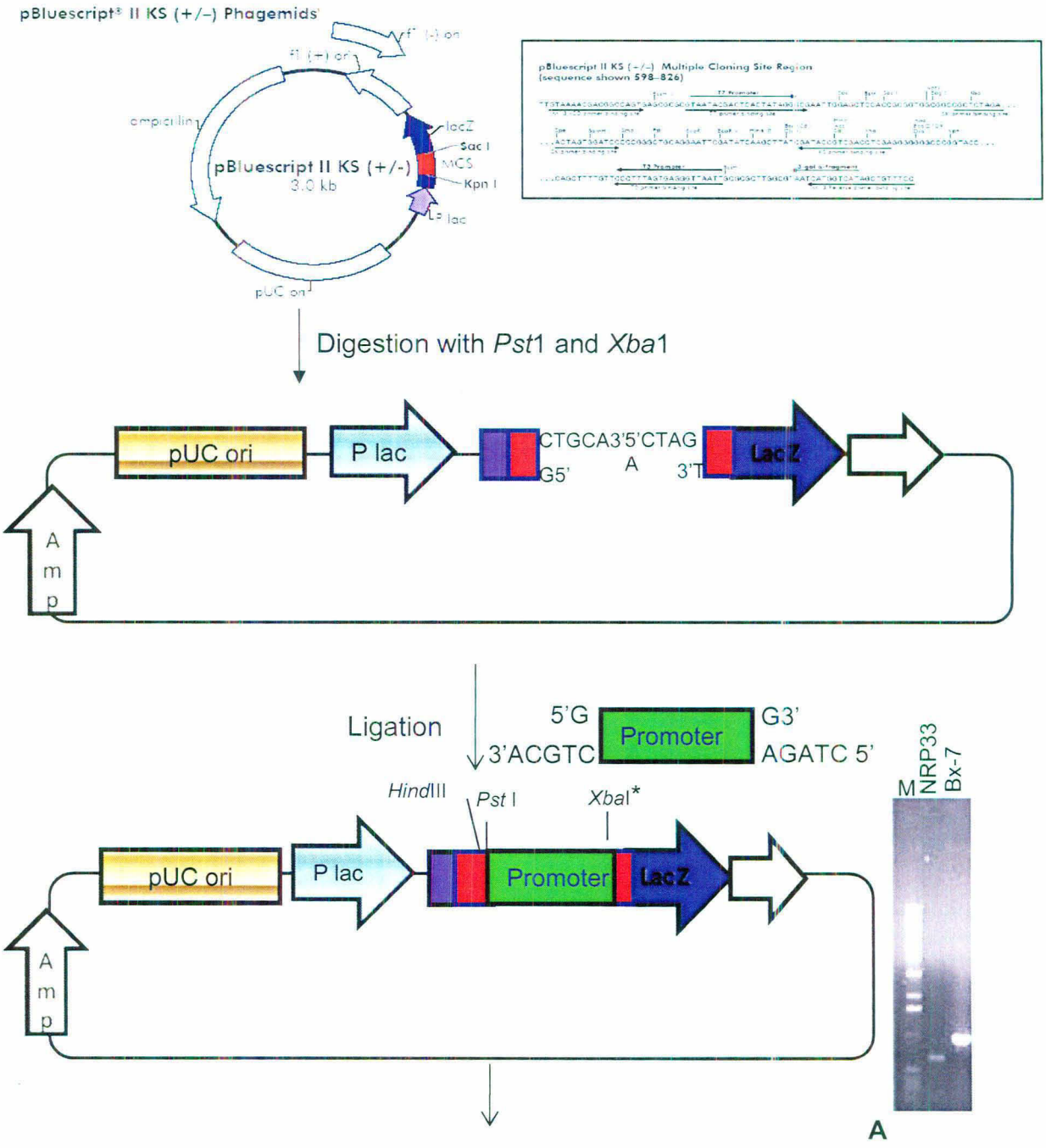


Fig 3.1 Cloning of NRP33 and Bx-7 promoter in pGEM-T Easy vector
Endosperm specific promoter Bx-7 (A) and NRP-33 (B) were amplified with a primer set appended with *Pst*I and *Xba*I site and ligated in pGEM-T Easy vector.



*: Restriction site is dam methylated

Fig3.2 Subcloning of NRP 33 and Bx-7 Promoter in pBS KS II(+/-). *Pst*I -*Xba* I fragment of promoter NRP33 and Bx-7 were ligated to *Pst*I-*Xba*I 3kb linear fragment of pBS KS II(+/-) and transformed in dam–ve strain GM2163 followed by PCR conformation using promoter specific primers (A).

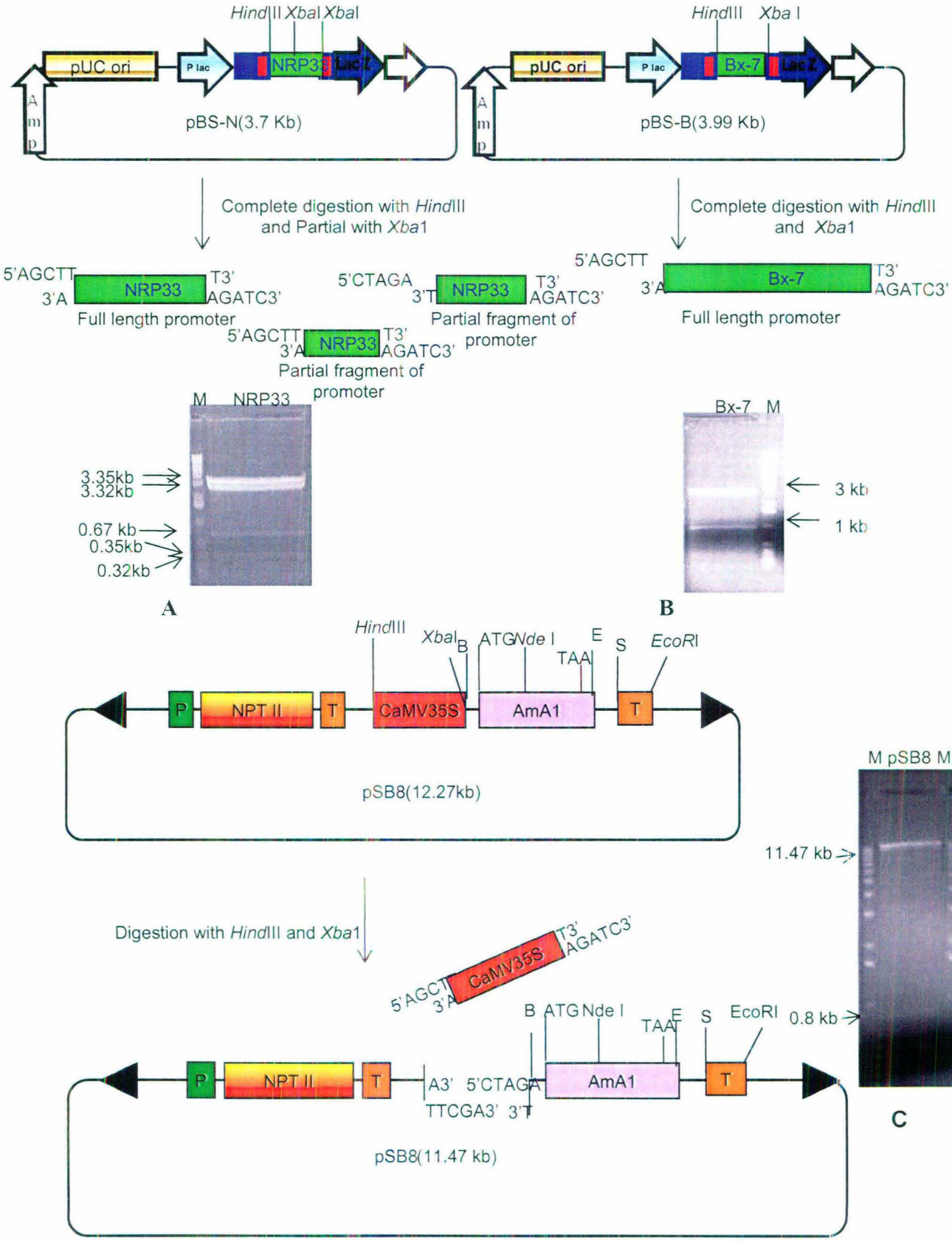


Fig 3.3 Schematic Diagram showing digestion of pBS-N, pBS-B and pSB-8 with *Hind*III and *Xba*1. To replace CaMV 35S promoter with NRP33 and Bx-7 promoter in pSB8, *Hind* III and *Xba* I digestion were performed in pBS-N(A), pBS-B(B), and pSB8 (C) constructs.

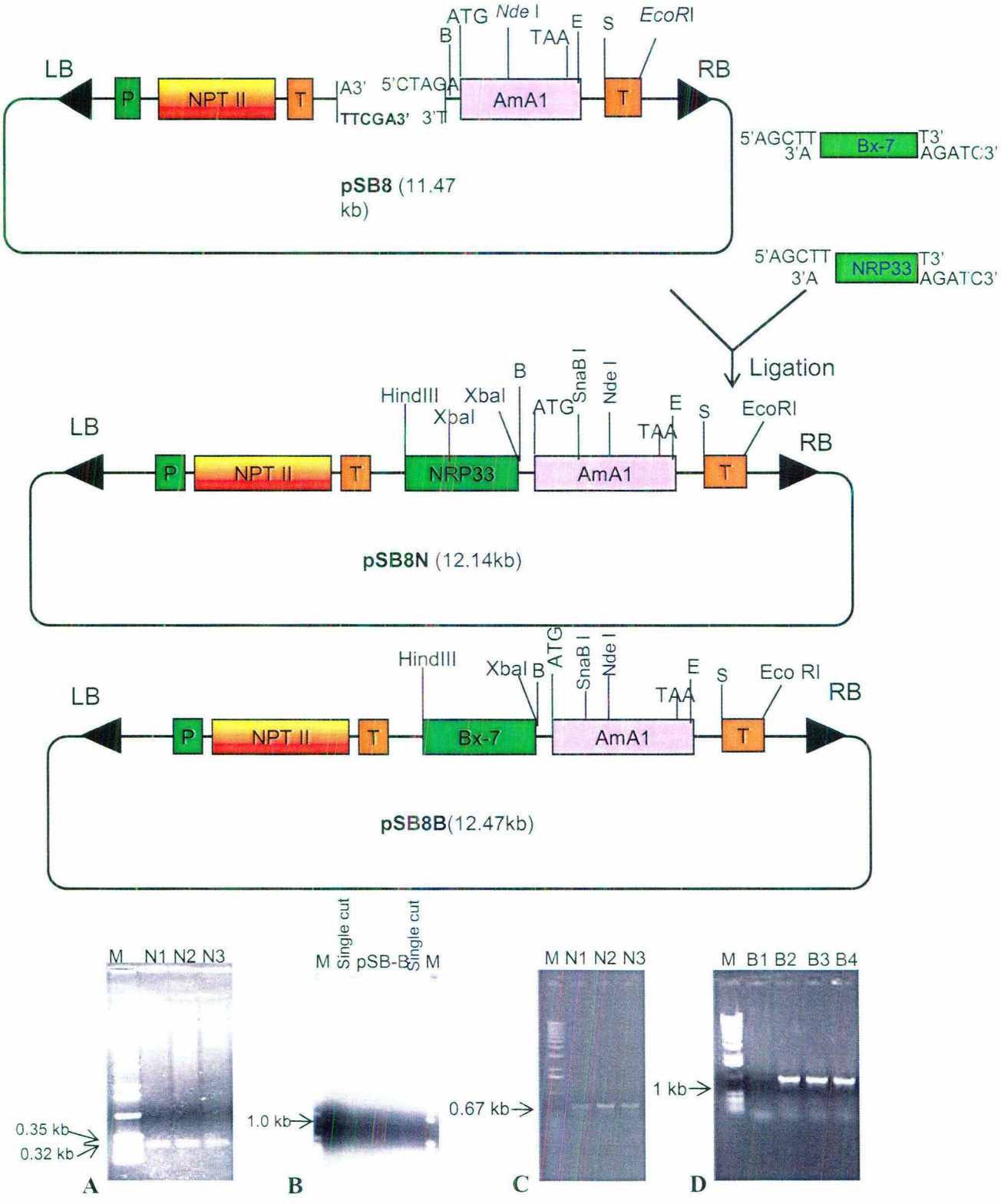


Fig 3.4 Subcloning NRP33 and Bx-7 promoter in pSB8 Construct . 0.67 kb of NRP33 promoter and 1kb of Bx-7 promoter was cloned into pSB8 using *Hind*III and *Xba*I site for endosperm specific expression of *AmA1*. Resultant construct pSB8N and pSB8B is confirmed by restriction digestion(A,B) and PCR amplification (C,D) with promoter specific primers and restriction enzymes (*Hind*III-*Xba*I), respectively.

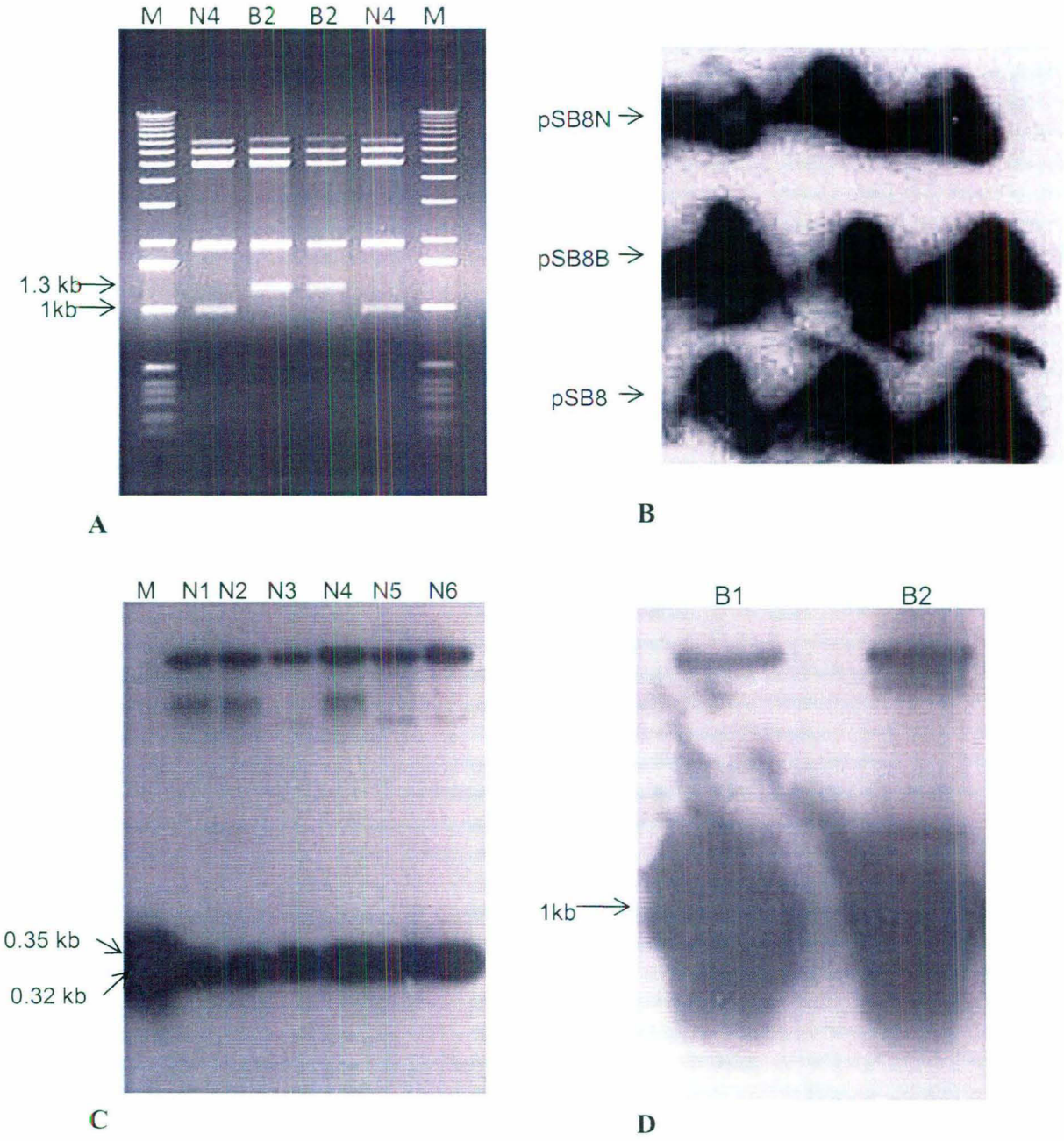


Fig 3.5 Confirmation of *AmA1* expression construct for rice

(A) Restriction digestion of pSB8N (N4 plasmid) and pSB8B (B2 plasmid) using *Hind*III and *SnaB*1 enzyme to excise full length promoter of NRP33(0.674 kb) and Bx-7 (1kb), respectively with partial fragment of *AmA1* (0.302 kb).

(B) Colony hybridization of different colony of pSB8N, pSB8B and pSB8 using AmA1 probe.

(C) Southern blotting of different colonies of pSB8N construct using NRP33 Promoter probe .

(D) Southern blotting of different colonies of pSB8B construct using Bx-7 Promoter probe.

or expression vector and the *Agrobacterium* host strain. A hyper virulent strain *EHA105* of *Agrobacterium* was used to transform rice in the present study. The strain contains L. L-Succinamopine Ti plasmid which makes it hyper virulent. *AmA1* expression vector were mobilized into *Agrobacterium* EHA 105 strain by triparental mating using helper plasmid *pRK2013*. After mobilization of expression vector, conjugates were confirmed by colony hybridization using *AmA1* specific probe (Fig 3.5 B).

3.3 *Agrobacterium*-mediated Transformation of Rice

Mature seeds of rice (*oryza sativa* Sub sps. *Indica*) varieties *IR64*, *Budda* and *Pusa Basmati-1* were used for *AmA1* gene transformation. Surface sterilization of mature seeds is described previously.

3.3.1 Materials and Methods

3.3.1.1 Callus Induction

1. Mature seeds were transferred to callus induction media consisting of MS basal media (sigma M5519), 3% sucrose, 2-4D, 0.7% agarose Type-1, pH5.8 (CIM 3) in dark at 25°C.
2. After 7-8 Days, scutellum derived calli were separated out from seeds and subcultured onto fresh callus induction medium.
3. 21-23 days old embryogenic calli were used for *Agrobacterium* mediated transformation.

3.3.1.2 Co-cultivation, Infection and Selection

1. *Agrobacterium* strain EHA105 harboring *AmA1* expression vector were grown till OD reached to 0.8 in YEP medium and pelleted by centrifugation at 5000 rpm for 10 min at 4°C. The pellet was dissolved in MS basal medium with 3% sucrose, 100 µM acetosyringone and pH was adjusted to 5.4. Embryogenic calli of 21-23 days were suspended and co-cultivated in *Agrobacterium* suspension for 30 min.
2. The calli were blotted onto sterile 3 mm whatmann paper. Co-cultivated calli were transferred on callus induction media supplemented with filter sterilized 100µM acetosyringone for three days.
3. *Agrobacterium* infected calli were washed in sterile MQ water with 250 mg/l cefotaxime sulfate followed by blotting on 3mm Whatmann paper.
4. Then infected calli were subcultured onto callus induction media supplemented with 250mg/l cefotaxime sulfate and 50 mg/l paromomycin sulfate.

5. After every 10 days, infected calli were subculture onto callus induction media supplemented with 250mg/l cefotaxime sulfate and 75mg/l paromomycin sulfate. In each subculture, brown calli were separated out from compact, yellowish, globular, dry calli. Selections of *transformed* rice calli were continued for one month.

3.3.1.3 Regeneration

1. In case of *Budda* and *IR64*, Compact, globular, dry, yellowish, embryogenic calli transferred on selective regeneration media consisting of MS basal media, 3% Sucrose, BAP, NAA, pH 5.8, paromomycin sulfate, 0.7% agarose type-1(Reg2). In case of *Pusa Basmati-1*, regeneration media consisting of BAP, NAA, Kinetin (Reg4).
2. A green, fresh, embryogenic, compact calli were separated out from brown calli and subcultured after every 10 days on selective regeneration media. Shoot bud induction was started after 2-3 weeks. This was followed by shoot elongation in same media composition. Elongated shoot buds were transferred on selective rooting media.

3.3.1.4 Rooting

1. 2-3 cm long shoot buds were transferred on rooting media consisting of MS basal media, 3% Sucrose, NAA and 0.4% agarose type-1, pH 5.8 and antibiotic paromomycin sulfate.
2. Rooted rice plants were further subcultured on liquid rooting media for vigorous growth.

3.2.1.5 Hardening

1. Well rooted plants of rice were transplanted in soil, vermiculate, agro pit in 3:1:1 in green house maintained at 28°C temperature and relative humidity at 65% with 14 /10 hours photo period for day and night.

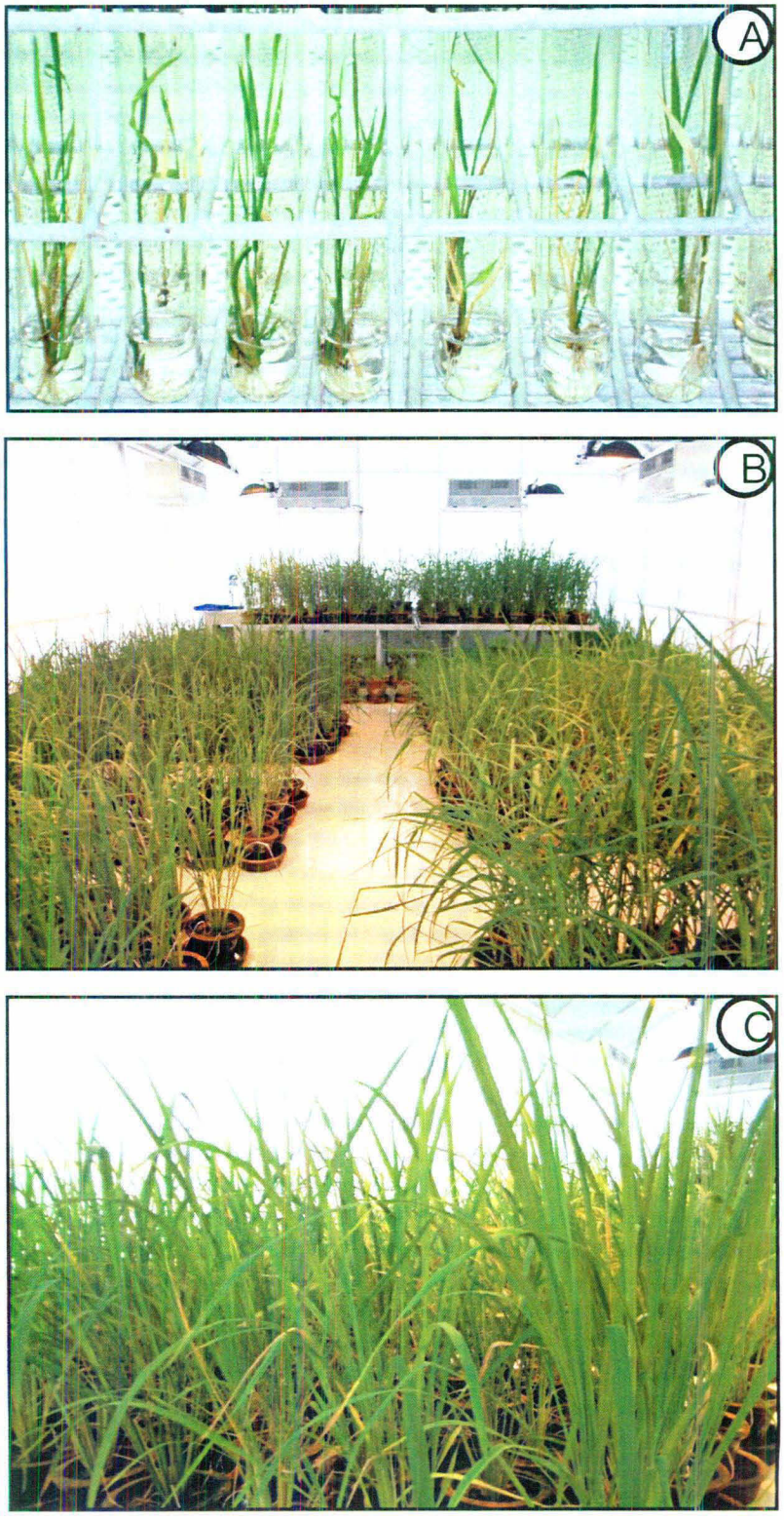


Fig 3.6 Putative transgenic lines transformed with *AmA1* gene.
(A) Rooted Transgenic lines,
(B,C) Putative transgenic lines growing in green house.

3.3.2 Results

Agrobacterium mediated transformation technique was used for raising transgenic lines for *AmA1* gene. *Indica* rice varieties *Budda, IR64 and Pusa Basmati-1* were used for *Agrobacterium* transformation. Endosperm specific expression of *AmA1* was driven by rice prolamin NRP33 gene promoter and wheat glutelin Bx-7 gene promoter using pSB8N and pSB8B constructs, respectively however constitutive expression of *AmA1* gene was driven by CaMV35S promoter using pSB8 construct (Chakraborty *et al.,* 2000). A scutellum portion of mature seed was induced for callusing. Callus were further transformed and followed by prolonged selection on the selective media before regeneration. This reduced the chances of escapants for the transgene. Selection was also continued during regeneration and rooting. The rooted transgenic plants as well as grown in green house is depicted in fig 3.6. The transformation efficiency was found between 3 to 4% for *Budda, IR64, Pusa Basmati-1, respectively.*

3.4 Molecular Analysis of Putative Transgenic Rice Plants

Transgenic plants need to be evaluated at four basic levels; (1) transgene integration (2) transgene expression (3) transgene expression stability and (4) trait fulfillment by transgene.

Transgene Integration: DNA analysis for transgene integration is basic requirement to evaluate transgenic plants. Polymerase chain reaction is widely used for this purpose. The polymerase chain reaction (PCR, Mullis and Faloona, 1987) has become a standard procedure to screen putative transgenic plants at an early developmental stage, when plant material is a limiting factor for other confirmation assays. In this method a defined fragment, unique for transgene is amplified *in-vitro* by elongation of transgene specific oligonucleotides sequence and visualized in agarose gel after electrophoresis on the basis of size. Amplified product can be further confirmed by sequencing or southern blotting. High sensitivity, specificity, less time consuming and low costs are some advantages of PCR.

However, in most cases further information is required concerning the number of copies of the inserted transgene in the transgenic. Transgene copy number is traditionally estimated by southern hybridization, fluorescence in situ hybridization (Kallioniemi *et al.*, 1996). In southern blotting method genomic DNA is digested, separated by gel electrophoresis, transferred and linked to a membrane and hybridized with labeled DNA strands complementary to the transgene that can visualized on radioactive sensitive film. DNA analysis by southern blotting is more time-consuming than other method like Real Time PCR. Some other techniques to detect copy number involve comparative genomic hybridization (Larramendy *et al.*, 1998), multiplex amplifiable probe hybridization (Armour *et al.*, 2000) and microarray (Lucito *et al.*, 2000). But all of these methods are laborious, time consuming and requires large quantity of genomic DNA and use of hazardous radioisotope. Moreover copy number detection using all these techniques does not reflect the rearranged transgene copies lacking with relevant restriction site (Mason *et al.*, 2002). Real time PCR for copy number detection is an effective, sensitive technique and consumes less time than the southern blotting.

Transgene expression: Transgene expression can be evaluated at the RNA, protein, and bio-analytical levels. The transgene which is finally expressed as protein can be

confirmed by western blot analysis or bio analytical assay. Western blot is performed with a protein specific antibody which is visualized by biochemical reaction.

Transgene expression stability: The stability of the transgene in consecutive meiotic generations is the final requirement and has central importance if the transgene is going to be introduced into a breeding programme. A molecular and genetic screening is necessary to identify individual transformants with desired characteristics, such as single inserts, desired expression level and single locus integration.

Trait fulfillment by transgene: This is desired to be fulfilled by any transgene in transgenic line. This has to be evaluated on the basis of physiological and biological assay related to trait of the gene for example if our aim to rise a transgenic for protein improvement than it has to show increases in protein profile in comparison to wild type.

Here, we described the molecular analysis of putative transgenic plants using conceptual tools of applied technical procedures. *AmA1* transgenic rice plants were analyzed for integration, expression, trait fulfillment and stability. *AmA1* gene integration in putative transgenic rice plants was confirmed by polymerase chain reaction and DNA sequencing. However, copy number of transgene was identified by Real Time PCR analysis. The expression of transgene was confirmed by western blot analysis and trait fulfillment by Kjeldahl method for increasing protein content. Stability of transgene in subsequent generation was confirmed by segregation ratio.

3.4.1 Materials and Methods

3.4.1.1 Transgene Insertion

3.4.1.1.1 Plant Genomic DNA Isolation

Genomic DNA isolation of rice leaf sample was performed by using Dneasy Plant Mini Kit (Qiagen, Germany) according to manufacturer's instructions with few modifications.

1. Leaf tissue of rice was collected and shock frozen in liquid nitrogen.
2. 100mg of leaf sample was ground to a fine powder with liquid nitrogen and transferred to 2 ml eppendrof.

3. 400µl of buffer AP1 and 4 µl of RNaseA stock solution (100mg/ml) was added to the leaf powder and vortexed vigorously.
4. The mixture was incubated for 10 min at 65°C and mixed 2-3 times during incubation by inverting tube.
5. 130 µl of AP2 buffer was added to lysate mix and incubated for 5 min on ice.
6. Lysate from step 5 was applied to the QIA shredder spin column (lilac) sitting on a 2ml collection tube and centrifuged for 2 min at maximum speed.
7. Flow through fraction from step 6 was transferred to a new tube without disturbing the debris.

1.5 volume of buffer AP3/E was added to the clear lysate and mixed by pipetting.

8. 650 µl of the mixture from step 8 was transferred to the Dneasy mini spin column, sitting on a 2 ml collection tube. Dneasy mini spin column was centrifuged for 1 min at 8000rpm and the flow through was discarded.
9. Dneasy mini spin column was transferred in a new 2 ml collection tube and 500 µl of buffer AW was added and centrifuged for 1 min at 8000 rpm. Flow through was discarded and to it 500µl AW buffer was added and centrifuged at maximum speed for 2min.
10. Dneasy spin Column was transferred in a new 1.5 ml eppendrof tube and 50µl of buffer AE was added directly onto Dneasy membrane. Dneasy spin column was kept at room temperature for 5 min followed by centrifugation at 8000 rpm for 1 min.
11. Step 11 was repeated once with 30µl volume of Buffer AE.
12. Genomic DNA from step 11 & 12 was pooled and run in 0.8% agarose gel. Genomic DNA of rice was visually quantified in UV light after EtBr staining.

3.4.1.1.2 PCR Analysis

PCR analysis of rice genomic DNA of putative transgenic lines transformed with pSB8, pSB8N and pSB8B for *AmA1* gene was carried out using F51 forward primer (5'-CACCATGGCGGGATTACCAGTG-3') and R1044 reverse primer (5'-CAAGGAAGAACCCTCTTGT- TTCC-3') (Chakraborty *et.al.,* 2000). PCR reaction was performed in 25µl volume consisting of 1X PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTP mix, 10 pM of each primer, 1.25 units of *Taq* polymerase and 100ng

genomic DNA of rice using thermal cycle condition described below. All PCR amplification was carried out using MJ Research INC. PCR machine and the product was analyzed on 0.8% agarose gel added with 0.5μg /ml EtBr along with 1 kb ladder (Invitrogen) as DNA marker.

Step	Temperature	Time
Step-1	94°C	3 min
Step-2	94°C	30 Sec
Step-3	56°C	30 Sec
Step-4	72°C	1 min
Step-5	Go to step-2 and repeated for 30 cycle	
Step-6	72°C	5 min
Step-7	4°C	5 min

3.4.1.1.3 DNA Sequencing

A 1.03 kb PCR amplified product from randomly selected transgenic rice lines, transformed with *AmA1* expression construct was gel eluted using Mini Elute Gel Extraction Kit (Qiagen, Germany) according to protocol described previously. Eluted product was sequenced by Applied Biosystem DNA Analyzer Model 3700, using *AmA1* specific primer. Resultant sequence was aligned with *AmA1* gene sequence using NCBI Blast 2 Sequences tool.

3.4.1.1.4 Copy Number Detection by Real Time PCR

Copy number detection of *AmA1* in putative rice transgenic lines was carried out using *AmA1* specific primers and *Taqman* probe which were labeled with the fluorescent reporter dye 6-carboxy-fluoroscein (FAM) on the 5' end and the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) on the 3' end. *Taqman* RT-PCR carried out in 20 μl volume consisted of 20X ABD mix, 2 X *Taqman* master mix, quantified genomic DNA of *AmA1* transgenic lines and the final volume made up to 20μl with autoclave MQ water. The thermal cycle condition is

described below. Each sample was run in triplicate. The RT PCR data were analyzed with 7500 Real Time Applied Biosystem software. To generate standard curve for *AmA1* containing pSB6-Hyg (6.72 kb) (Biswas, S. Ph.D thesis 1998) were serially diluted and subjected to quantitative RT-PCR assay in triplicate.

Stage	Cycle	Temperature	Time
Stage 1	1	50°C	2min
Stage 2	1	95°C	10min
Stage 3	1	95°C	15sec
Stage 4	Go to stage 3 and repeated for 40 cycle		
Stage 5	1	60°C	1min

3.4.1.2 Transgene Expression

3.4.1.2.1 Protein Extraction from Seed Materials

Total protein extraction from mature rice seed was carried out as described below.

1. 100mg of mature dry seeds were ground in liquid nitrogen with pestle and mortar.
2. Resultant rice flour of mature seed was transferred to 1.5 ml tube and vortexed vigorously after adding 500µl of protein extraction buffer consisting of 62.5mM Tris-HCl (pH-6.8), 30% Glycerol, 2mM PMSF, 1mM EDTA and 0.5% Triton-x.
3. Protein extracts were transferred to ice bucket and incubated for 1 hour on rotary shaker in 150 rpm.
4. Protein extracts were centrifuged at 13000 rpm for 10 min at 4°C and the supernatant was transferred in a new tube.
5. Step 4 was repeated 2-3 times till clear supernatant was obtained.
6. Protein quantity was estimated using Bradford assay reagent.

3.4.1.2.2 SDS-polyacrylamide Gel Electrophoresis (PAGE) of Proteins

1. 100 μg of total protein was dissolved in 35μl of loading dye or Lammeli Dye (Lammeli,1970) consisting of 2% strongly anionic detergent SDS, 5% reducing agent β-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol and 0.002% bromophenol blue.
2. Crude protein sample together with lammeli dye was boiled for 5 min to denatured protein. Since the amount of bound SDS is proportional to the molecular weight of the polypeptide, therefore SDS-polypeptide complexes migrate in accordance with their size.
3. 100μg of soluble protein was loaded on 12.5% SDS-PAGE. Electrophoresis was carried out using electrophoresis buffer containing 25mM Tris, 250mM glycine (pH 8.3) and 0.1% SDS in mighty small apparatus (Hoefer) at 70 volt constant till all the proteins were stacked and then 100 V constant for resolving .
4. Stacking had 4% acrylamide, 0.125 M Tris-Cl (pH 6.8), 0.04% TEMED and 0.5% APS. Reservoir buffer consisted of 0.025 M Tris base, 0.192 M Glycine and 0.1% SDS (pH 8.3).

3.4.1.2.3 Western Blot Analysis

Protein from SDS–PAGE gels were transferred onto nitrocellulose Hybond membrane (Amersham-GE) and immunodetection was carried out to visualize interested protein as described below.

3.4.1.2.3.1 Transfer

1. The protein gel was briefly equilibrated in transfer buffer; the transfer was carried out in an electrotransfer apparatus (Amersham Pharmacia) at 150 mA constant current for 3 h at room temperature.
2. After the transfer, blots were stained with 0.5% Ponceau S (Salinovich and Montelaro, 1986) in 1% acetic acid for 5 min. The position of the molecular weight markers was marked and the blots were destained completely in water.

3.4.1.2.3.2 Immunodetection

1. The blots were rinsed in TBS and blocking was done overnight in 5% non fat dry milk in TBST at 4°C.
2. The blots were washed three times at RT with TBST (15 min each) in orbitary shaker at 50 rpm.
3. The TBST solution was replaced by primary antibody diluted to 1:5000 in TBST and incubated for an hour at RT.
4. The blots were washed twice with TBST for 5 min and then three times, each of 15 min duration in orbitary shaker.
5. Secondary antibody rabbit IgG alkaline phosphatase conjugate (Sigma, USA) was added to the blot at a dilution of 1:5000 in TBST and incubated for 1 hr at RT.
6. The blots were washed similarly as described in step 4, and developed using NBT/BCIP (Bio-Rad) in AP buffer as a substrate for alkaline phosphatase. Once the colour developed, the reaction was stopped by rinsing the blots in MQ H_2O.

3.4.1.3 Trait Fulfillment (Kjeldahl Method)

The Kjeldahl method consists of three steps (1) Digestion of sample (2) Distillation (3) Titration. A detailed method for determining protein in transgenic rice seeds is described below.

3.4.1.3.1 Digestion of the Sample

The sample was first digested in strong sulfuric acid in the presence of a catalyst, which converts the amine nitrogen to ammonium ions.

1. Mature rice seeds were kept at 70°C for overnight to release moisture.
2. Rice seeds were powdered in pastel and mortar and the sample was transferred into a digestion flask, along with 25 ml of concentrated sulfuric acid (H_2SO_4).

3. 5 gm of potassium sulfate and 0.25 gm catalyst copper sulfate was added in the sample.
4. Digestion flasks were fitted to the heating block and temperature increased to 100°C followed by increment of 100°C after every 30 min till 380-400 ° C.
5. Heating the mixture in the digestion flask was done until white fumes could be seen, and then continued for about 60-90 min until the flask became transparent.
6. Digestion sample was equally distributed in two tubes and added with 50 ml of water and 100 µl of phenopthalene.

3.4.1.3.2 Distillation

The purpose of the distillation, is to separate the ammonia (that is, the nitrogen) from the digestion mixture.

1. The pH of the mixture was increased by adding 50-90ml of sodium hydroxide (40% NaOH solution) till chocolaty colour appeared in the sample. This is due to conversion of ammonium (NH_4^+) ions (which are dissolved in the liquid) to ammonia (NH_3), which is a gas.
2. Separating the nitrogen away from the digestion mixture by distilling the ammonia (converting it to a volatile gas, by raising the temperature to boiling point) and then trapping the distilled vapors was done in a special trapping solution consisting of 25ml of 0.1N H_2S0_4, 50µl of 1% methylene red and volume made up to 50 ml with distilled water.
3. Further the trapping flask and condenser was rinsed with water to completely dissolve ammonia.

3.4.1.3.3 Titration

As the ammonia dissolves in the acid trapping solution, it neutralizes some of the H_2S0_4. The amount of acid left in the trapping solution after neutralization with ammonia was again neutralized by "back titration" with a standard solution of NaOH and the 'end point' was detected when color changed from red to pink. In this way the

amount of ammonia distilled off from the digestive solution was calculated, and hence the amount of nitrogen in the protein determined.

1. Known Normality solution of NaOH (close to 0.1) was titrated with trapping solution. NaOH solution was slowly added to the acid solution and change in colour of trapping solution was noted.
2. The point, at which the methylene red dye turns pale yellow, *indica*ted the "endpoint". The volume of neutrilizing base (sodium hydroxide solution) that was required to reach the endpoint was recorded properly.

To determine the exact normality of NaOH solution (in burette) following procedure was followed.

Normality of NaOH: 0.3gm of potassium pthalate and 100µl 1%phenopthalene of was dissolved in 100ml of water and titrated with NaOH solution and endpoint was noted.

To calculate percent nitrogen in sample, percent protein in sample and normality of NaOH, following formula has been used.

$$\text{Normality of NaOH:} \quad \frac{\text{Pot. Phthalate in gm} \times 1000}{\text{Consumed Vol. of NaOH in ml}} \times 204.22$$

$$\text{Nitrogen calculation:} \quad \frac{(\text{Blank} - \text{sample}) \times 100 \times 14 \times \text{Normality of NaOH}}{1000 \times \text{weight}}$$

$$\text{Protein calculation:} \quad 6.25 \times \% \text{ Nitrogen}$$

3.4.1.4 Transgene Stability

The T1 seeds of rice transgenic lines for *AmA1* gene as well as selective marker *nptII* gene were tested on selective seed germination media consisting of MS basal media, 3% sucrose, pH 5.8, 0.4% agarose Type-I. The selective agent paromomycin sulfate was added in the concentration of 50mg/l to lukewarm autoclaved seed germination

media. An equal number of seeds from different transgenic lines were transferred to selective seed germination media for germination. Seed germination ratio was evaluated by counting the antibiotic resistant seeds to susceptible seeds after 15 days.

3.4.2 Results and Discussion

3.4.2.1 Transgene Insertion

3.4.2.1.1 PCR Analysis

Integration of *AmA1* gene in putative transgenic lines of rice varieties *Budda*, *IR64* and *Pusa Basmati-1* was confirmed by PCR using F51 and R1044 forward and reverse primers, respectively. An amplified product of 1.03 kb was detected in agarose gel but intensity of band varied amongst transgenics. No amplification was observed in wild type plants (Fig3.7 B, 3.8 B, 3.9 B, and 3.10 B). The intensity of the band varied with the copy number with few exceptions where it could be due to unequal amount of template DNA used for amplification as it was difficult to correctly quantify the DNA due to presence of polysaccharides and small amount of RNase resistant RNA. Large difference was observed between the visual estimation of the DNA against known amount of DNA standard and spectrophotometric estimation.

3.4.2.1.2 DNA Sequencing

DNA sequencing of amplified product of expected size from putative transgenic lines can give reliable confirmation of the presence of transgene in transgenic plant. Because, nonspecific amplification of DNA during PCR is always possible depending upon, reaction conditions, homology of primer to close sequence and annealing temperature. Therefore, we did sequencing of expected 1.03 kb amplicon from rice plants transformed with *AmA1* gene construct. The produced sequencing data was aligned with *AmA1* gene sequence and verified for 100% homology.

3.4.2.1.3 Real Time PCR for Copy Number Detection

Randomly selected transgenic lines of rice transformed with *AmA1* gene were detected for copy number of *AmA1* gene in rice genome. The result of Real Time PCR

using Taqman probe for *AmA1* gene in rice transgenic lines showed the presence 1-6 copies of the transgene with majority of lines having single copy.

3.4.2.2 Transgene Expression

Western Blotting

The objective of raising transgenic plants is not confined to the incorporation of gene of interest in the genome of target plant. The ultimate objective of raising a transgenic is to rightly encoding of transgene into specific desired protein. Expressed foreign protein could be identified by the western blot analysis. In this method, total protein from putative transgenic was isolated and electrophoresed on SDS-PAGE gel and transferred on to nitrocellulose membrane. After treating membrane with *AmA1* specific antibodies, detection was carried out by AP conjugated secondary antibody using chromogenic substrate. The result of western blot analysis of rice transgenic line transformed with *AmA1* gene is depicted in figure 3.7(C), 3.8(C), 3.9(C), 3.10(C). The analysis showed expression of 35 KDa of AmA1 in transgenic plants and no signal was detected in wild type plants. Few non specific bands were also detected which may be because of polyclonal nature of AmA1 antibody. The level of expression of any heterologus protein in transgenic environment could vary due to several reasons. First, copy number of introduced gene varies among transformants though it has often been shown that there is no positive correlation between increase in copies number and expression of the genes (Jones *et al.*, 1085, Shirsat *et al.*, 1989). On the other hand there are even reports that single copies result in higher expression (Odel *et al.*, 1987; Rogers *et al.*, 1985). The insertion of foreign gene is random within the plant genome and the activity of the introduced gene may be affected by the position effect; however this has been found not to be the simple, exclusive cause of variable expression in many instances (Jones *et al.*, 1987; Dean *et al.*, 1988). Second, truncation, rearrangement or repetition of the introduced gene may also affect gene expression as there are direct correlations shown between low expression, deletions, rearrangements (Delores and Gardner, 1988) and/or inverted repeat (Jones *et al.*, 1985). Third, plants with single insertion had high expression whereas those plants with multiple insertions, at one or more loci, had low expression which was

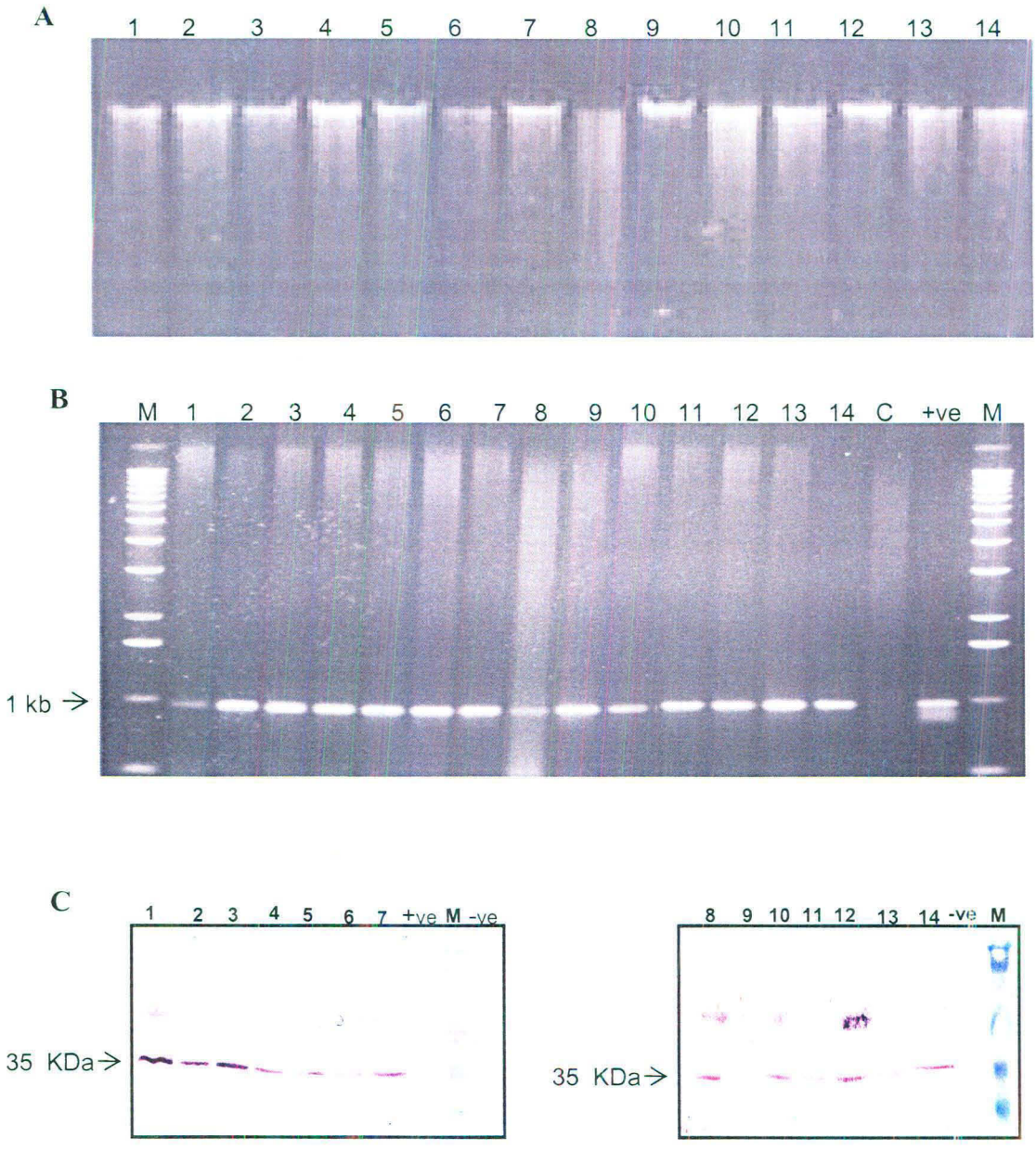


Fig 3.7 Molecular analysis of *Pusa Basmati-1* plants transformed with pSB8B.

(A) Genomic DNA of transgenic lines were resolved on 0.8% agarose gel.

(B) PCR analysis of transgenic lines using *AmA1* gene specific primers (F51 and R1044). An expected 1.03 kb fragment of *AmA1* gene was amplified .

(C) Western blot analysis of seed protein of different transformants using AmA1 antibody Showing a 35 KDa band, 10 ng of purified protein was loaded as a positive control and seed protein from untransformed rice plants were used as a negative control.

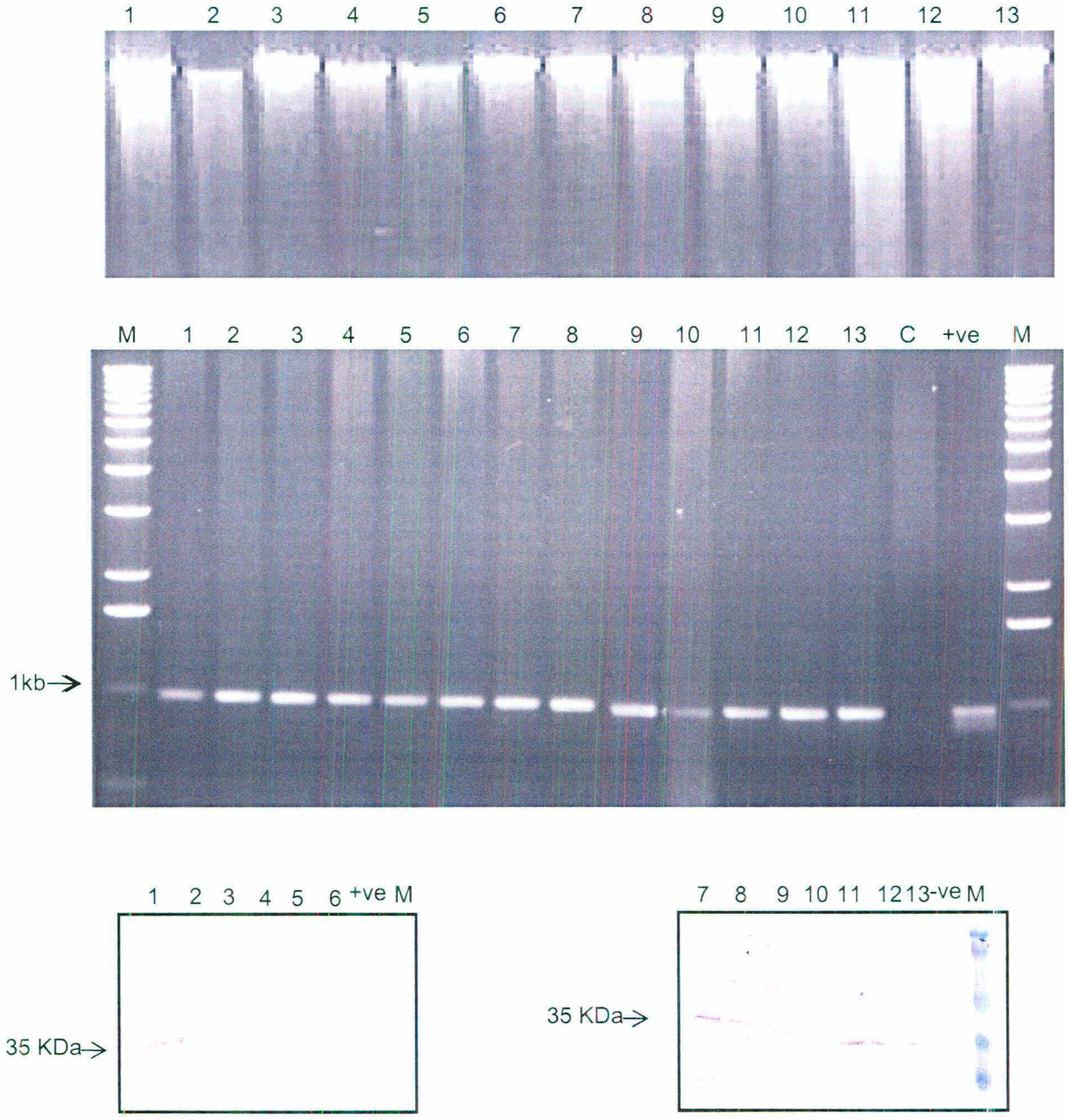


Fig 3.8 Molecular analysis of *Pusa Basmati-1* plants transformed with pSB8N.

(A) Genomic DNA of transgenic lines were resolved on 0.8% agarose gel.

(B) PCR analysis of transgenic lines using *AmA1* gene specific primers (F51 and R1044). An expected 1.03 kb fragment of *AmA1* gene was amplified .

(C) Western blot analysis of seed protein of different transformants using AmA1 antibody Showing a 35 KDa band, 10 ng of purified protein was loaded as a positive control and seed protein from untransformed rice plants were used as a negative control.

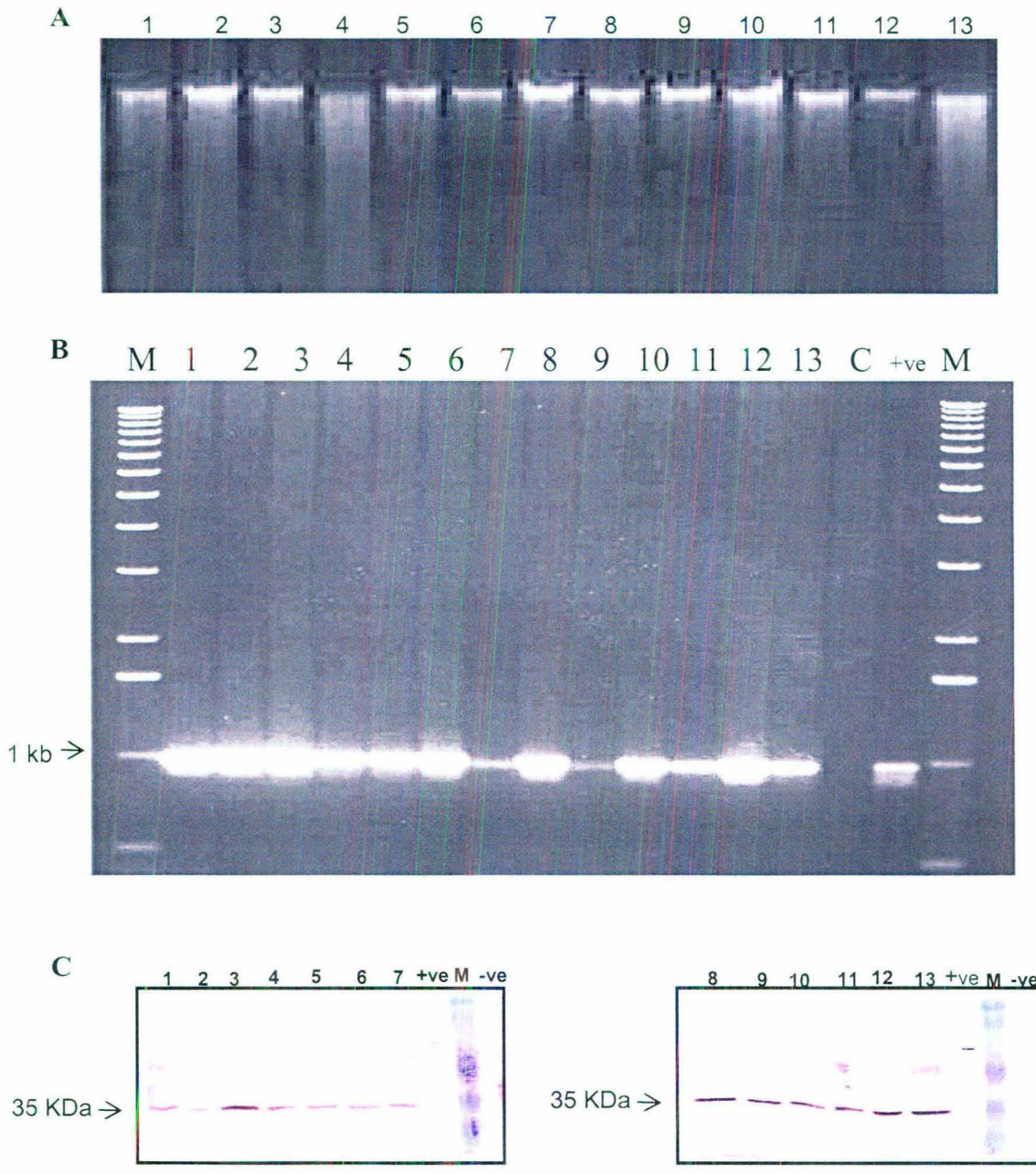


Fig 3.9 Molecular analysis of *Pusa Basmati-1* plants transformed with pSB8.

(A) Genomic DNA of transgenic lines were resolved on 0.8% agarose gel.

(B) PCR analysis of transgenic lines using *AmA1* gene specific primers (F51 and R1044). An expected 1.03 kb fragment of *AmA1* gene was amplified .

(C) Western blot analysis of seed protein of different transformants using AmA1 antibody Showing a 35 KDa band, 10 ng of purified protein was loaded as a positive control and seed protein from untransformed rice plants were used as a negative control.

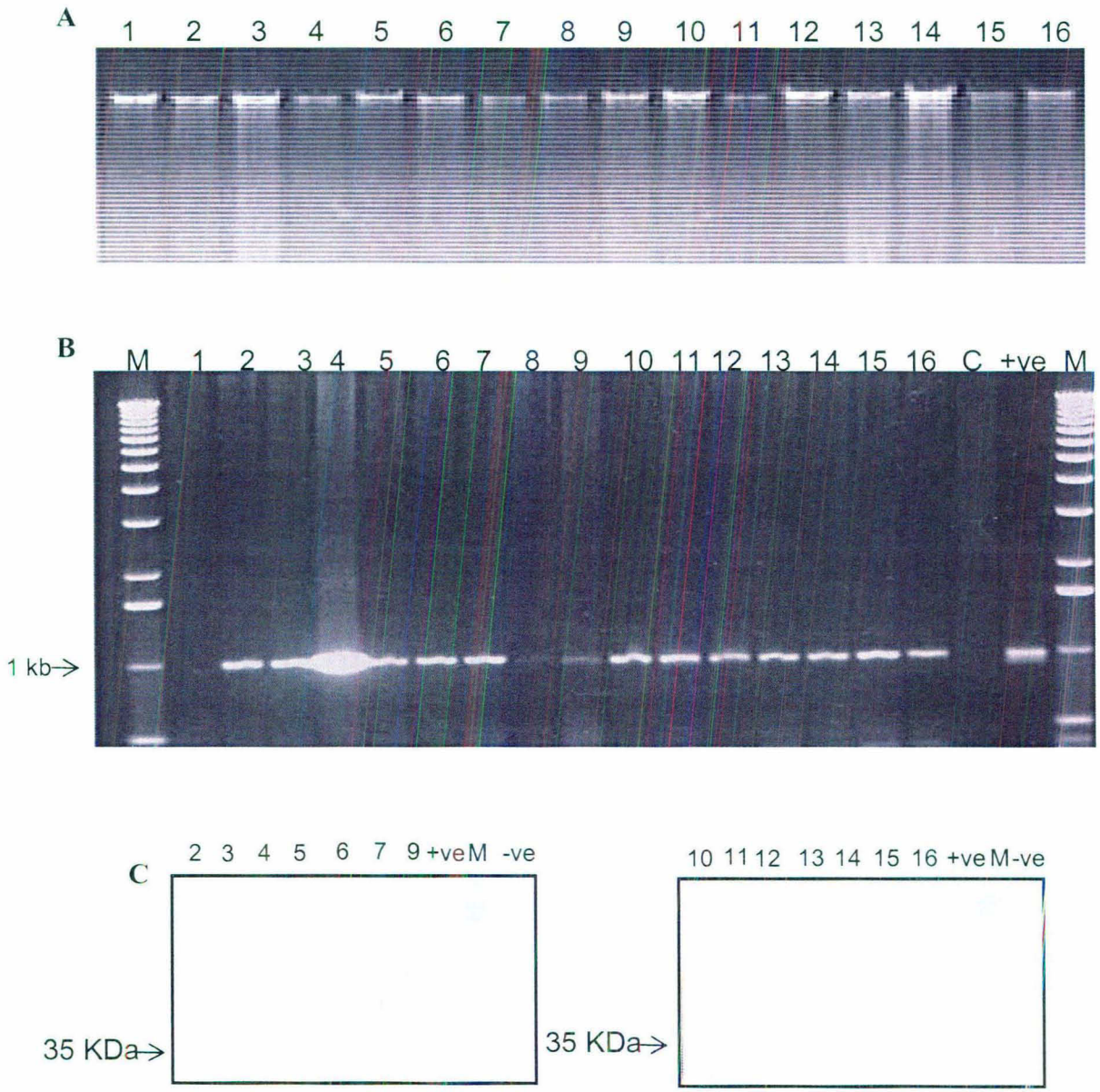


Fig 3.10 Molecular analysis of IR64 plants transformed with pSB8N.

(A) Genomic DNA of transgenic lines were resolved on 0.8% agarose gel.

(B) PCR analysis of transgenic lines using *AmA1* gene specific primers (F51 and R1044). An expected 1.03 kb fragment of *AmA1* gene was amplified .

(C) Western blot analysis of seed protein of different transformants using AmA1 antibody Showing a 35 KDa band, 10 ng of purified protein was loaded as a positive control and seed protein from untransformed rice plants were used as a negative control.

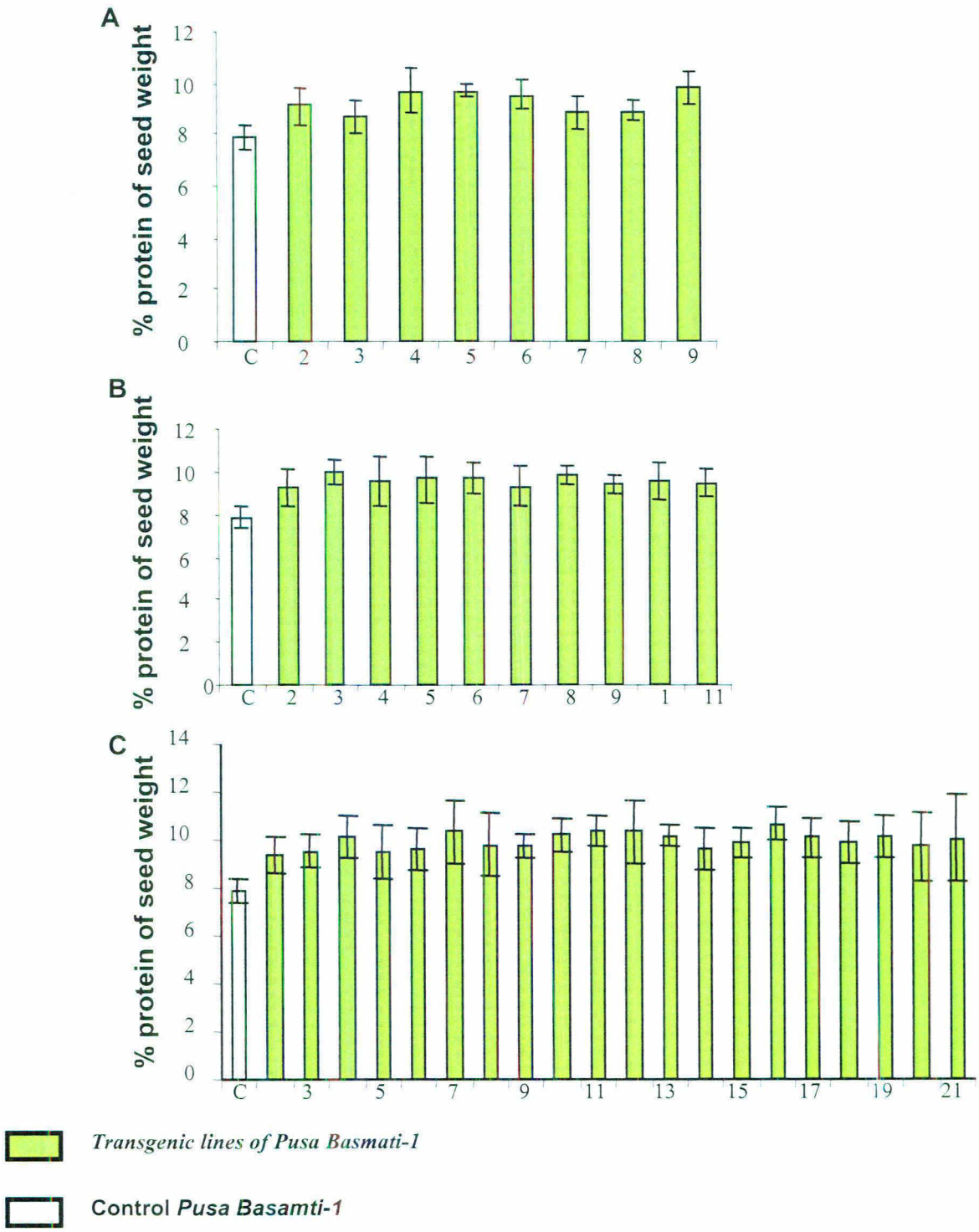


Fig 3.11 Analysis of protein content of *AmA1* transformed rice seeds in comparison to the wild type

(A) *Pusa Basmati-1* transgenic lines transformed with pSB8.
(B) *Pusa Basmati-1* transgenic lines transformed with pSB8B.
(C) *Pusa Basmati-1* transgenic lines transformed with pSB8N.

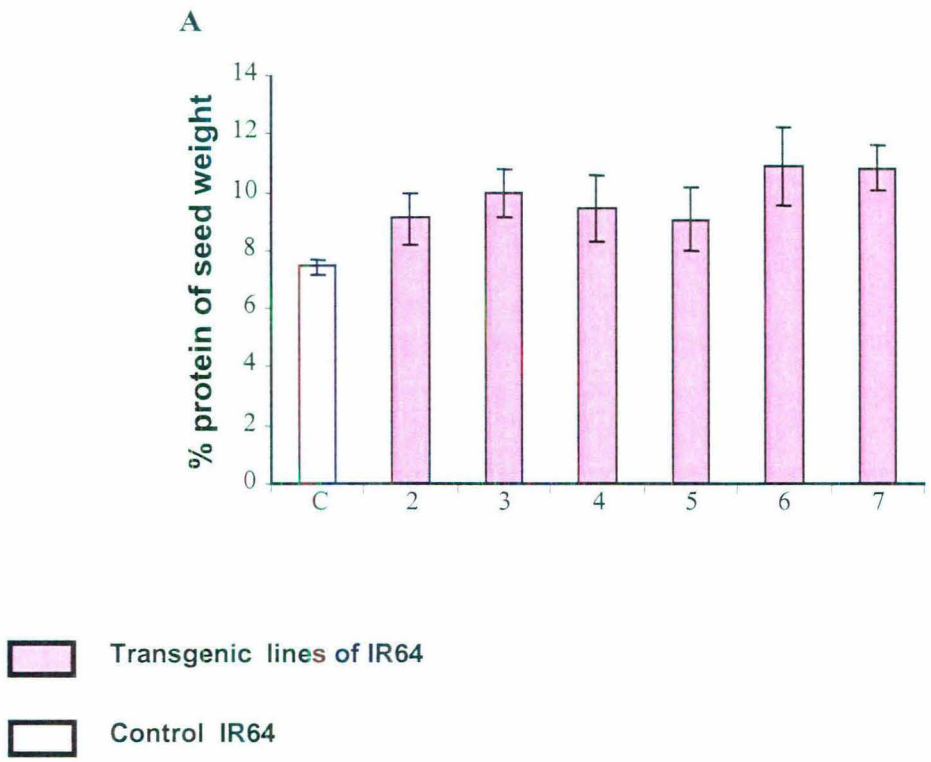


Fig 3.12 Analysis of protein content of IR64 rice seeds transformed with pSB8N.

associated with increased methylation of integrated gene per se. There was no *indica*tion of position effect within high and low groups (Hobbes *et al.*, 1990).

3.4.2.3 Trait Fulfillment

Kjeldahl Analysis

Nitrogen is one of the five major elements found in organic materials such as protein. The most popular method for determining nitrogen is the Kjeldahl method, developed in 1883. It is based on the conversion of the bound nitrogen to ammonia (NH_3), which is then separated by distillation and determined by titration. The Kjeldahl method of nitrogen analysis is the worldwide standard for calculating the protein content in a wide variety of materials ranging from human and animal food, fertilizer, waste water and fossil fuels. Nitrogen amount can be further converted in the protein by multiplying a factor which is usually 6.25 in most of cases.

The Kjeldahl analysis result of AmA1 expressing rice seeds is depicted in fig 3.11 and 3.12. The Kjeldahl analysis revealed that there was significant increase in seed protein quantity of *AmA1* expressing lines. Constitutive expression of *AmA1* driven by CaMV35S promoter in *Pusa Basmati-1* showed upto 24% increase in seed protein. Endosperm specific expression of AmA1 in *Pusa Basmati-1* driven by NRP33 promoter showed upto 35% increase with 10.67% protein in comparison to wild type which is 7.86% protein of seed weight. *AmA1* expression in *IR64* driven by NRP33 promoter showed an increase of protein quantity upto 45% with 10.87% protein content in comparison to 7.63% in wild type. *AmA1* expression driven by Bx-7 promoter in rice seed showed an increase upto 26% with 9.97% of seed weight in comparison to 7.86% in wild type.

3.4.2.4 Transgene Stability

The selfed T1 progeny of transgenic lines transformed with *Agrobacterium* strain harboring *AmA1*and *nptII* expression constructs were evaluated on NPTII selection by seed germination. The result showed that most of the lines having single insertion followed 3:1 Mendelian segregation ratio which confers stability of transgene in next generation.

CHAPTER 4

SUMMARY

4. Summary

Rice (*oryza sativa*, 2n=24, family *Gramineae*) is a major staple food for more than half of the world's population with production of 645 million tons in the year 2007. Rice provides 20% of the world dietary energy supply and ranks third after wheat and maize in worldwide production. Asia accounts for 90 and 92% of world's rice area and production respectively. Among the rice growing countries in the world, India has the largest area under rice crop (about 44 million ha.) and ranked second in production next to China in the year 2003.

The world population is predicted to increase from 6.1 billion in 2001 to 7.2 billion in 2015 and 8.3 billion in 2030. Same time, rice consumers are increasing at the rate of 1.8% every year, but the rate of growth in rice production has slowed down. It is estimated that rice production has to increase by 50% by 2025 to meet the growing population (Khush and Virk, 2000). Facing pressure from this continuous population growth and shrinking agricultural lands, the challenges are not only to meet the future food needs of humans but also to deal with the need of nutritionally balanced crops/foods. Major shortages of basic nutrients in the many parts of world is restricted to a diet, based exclusively on staple food including vitamin A, iron, iodine, zinc, quality protein, and deficiencies in these nutrients cause micronutrient and protein-energy malnutrition throughout much of the developing world. Thus enhancing the nutritional quality of crops is an important undertaking for future food security and the nutritional well-being of world population (Biotechnology and Sustainable Agriculture 2006 and Beyond Proceedings of the 11th IAPTC&B Congress, August 31-18, 2006 Beijing, China).

Rice is a high-carbohydrate food with 85% of carbohydrate, 7% of fat, and 7-8% of protein. The protein content of rice is much less than the legumes (30-40% protein). Moreover, the amino acid composition of rice storage protein is not well balanced, having low level of threonine, lysine, and methionine (Takaiwa, 1999). Therefore, if protein content of rice is increased, it will be a very important protein source. To expedite genetic improvement in rice, genetic engineering can be a powerful tool. It helps to facilitate and accelerate the progress towards achieving these multi-faceted goals of nutritional and economic benefits of rice to farmer (Datta, 2000; 2001). The traditional breeding approaches in this direction have not been very successful in overcoming the amino acid limitation of cereal and legume Bseeds

(Mertzz *et al.*, 1964; Loesch *et al.*, 1976; Delancey and Bliss, 1991). An alternative approach using protein engineering and gene transfer for amino acid enhancement has been initiated earlier (Ohtani *et al.*, 1991; Dyer *et al.*, 1993).

The *AmA1* gene from *Amaranthus hypochondriacus* was cloned and characterized in our laboratory (Raina and Datta 1992). This gene has been patented (Datta *et al.*, 1997, 1998). AmA1 protein is nonallergenic in purified form and has essential amino acids close to WHO recommendations of essential amino acids for an ideal storage protein. The seed albumin *AmA1* gene has a great potential to increase protein quality of crop plants as it is reported in potato (Chakraborty *et al.*, 2000).

This work describes the overexpression of *AmA1* gene from *A. hypochondriacus* in rice to improve protein quality. To develop *AmA1* expressing transgenic lines, a reproducible regeneration and transformation system was a prerequisite. *AmA1* expression constructs for rice with constitutive as well as endosperm specific promoter was made to drive tissue specific expression. After development of putative transgenic lines, it was needed to analyze transgene integration and expression followed by the trait fulfillment and transgene stability.

In order to establish a regeneration and transformation system, we used three varieties (*Budda, IR64, and Pusa Basmati-1*) *of indica* rice. A regeneration protocol was developed using a scutellum derived calli from mature seeds for three varieties. It was revealed from the study that MS basal media supplemented with 2-4D, 3% sucrose, gelling agent 0.7% Agarose Type-I and pH 5.8 (CIM3) was most suitable among tested combinations for callus induction. The efficiency of callus induction was 95%, 92% and 88% for *Pusa Basmati-1*, *Budda* and *IR64,* respectively. For transient expression, we used 21 days old calli and co-cultivated with *Agrobacterium* strain EHA105 harboring pBI121. The GUS assay of these calli after 7 days revealed transformation efficiency of 44%, 51% and 31% for *Budda, IR64* and *Pusa Basmati-1*, respectively. The regeneration media consisting of MS basal salts, 3% sucrose, BAP, NAA, and 0.7% agarose type-I (Reg2) was best for *Budda* and *IR64* among tested combination with regeneration efficiency of 55% and 44% respectively. In case of *Pusa Basmati-1* MS basal salts with 3% sucrose, BAP, NAA and kinetin (Reg4) was best among tested combinations with 44% regeneration efficiency. Rooting of shoot bud was initiated on MS basal media, NAA, 0.4% Agarose Type-I with pH 5.8. The root induction efficiency for *Budda, IR64 and Pusa Basmati-1* were 98%, 86%

and 88%, respectively. In the present study, paromomycin sulfate was used as selective antibiotic to confer resistance against *NPT*II gene.

In order to construct, *endosperm* specific *AmA1* expression vector for rice, replacement cloning strategy was used. The endosperm specific expression of *AmA1* gene was driven by NRP33 (Rice prolamin), Bx-7(Wheat Glutelin) and a constitutive expression by CaMV35S promoter. In the replacement cloning strategy, CaMV35S promoter fragment of pSB8 (Chakraborty *et al.,* 2000) was replaced with 0.674 kb of NRP33 and 1.0 kb of Bx-7 promoter fragment. For fulfillment of this, NRP33 promoter and Bx-7 promoter was amplified from rice and wheat genomic DNA, respectively and cloned in pGEM-T Easy vector. This was followed by sub- cloning of promoter in pBS KSII (+/-) vector to get compatible sites to replace CaMV35S promoter in pSB8 construct (Chakraborty *et al.,* 2000). The *Hind*III-*Xba*I fragment containing CaMV35S promoter was replaced with NRP33 and Bx-7 promoter, separately. Resultant constructs were confirmed by restriction digestion, PCR amplification and southern blotting for presence of promoter region in right orientation and named as pSB8N and pSB8B for NRP33 and Bx-7 promoter constructs, respectively. Both pSB8N and pSB8B *AmA1* expression vectors were mobilized to *Agrobacterium* strain EHA105 using triparental mating method and confirmed by colony hybridization for presence of AmA1 gene.

Budda, IR64 and Pusa Basmati-1 rice were transformed with *Agrobacterium* strain harboring *AmA1* expression construct pSB8N and pSB8B for endosperm specific expression and pSB8 for constitutive expression. The putative rice transgenic lines for *AmA1* gene were analyzed for transgene insertion by PCR amplification of *AmA1* gene and sequencing of PCR product. Copy number was estimated by Real Time PCR and the transgene expression was confirmed by western blotting using AmA1 antibody. The protein content was estimated by Kjeldahl method and transgene stability by segregation of the gene in next generation. The result of molecular analysis of transgenic lines confirmed the insertion of *AmA1* and expression of 35 KDa AmA1 protein. The Real Time PCR using Taqman probe showed presence 1 to 6 copies of *AmA1* with majority of transgenics having single insertion. The *AmA1* gene in transgenic lines showed an increment of total protein. Constitutive expression of *AmA1* driven by CaMV35S promoter in *Pusa Basmati-1* showed upto 24% increase in seed protein. Endosperm specific expression of *AmA1* in

Pusa Basmati-1 driven by NRP33 promoter showed upto 35% increase with 10.67% protein in comparison to wild type which is 7.86% protein of seed weight. *AmA1* expression in *IR64* driven by NRP33 promoter showed an increase of protein quantity upto 45% with 10.87% protein content in comparison to 7.63% in wild type. *AmA1* expression driven by Bx-7 promoter in rice seed showed an increase upto 26% with 9.97% of seed weight in comparison to 7.86 % in wild type. *AmA1* gene showed a segregation pattern during germination in presence of selection agent paromomycin sulfate which revealed the stability of transgene in subsequent generation.

Future Objective and Perspective

In order to improve nutritional quality of rice, *AmA1* expressing transgenic lines have been generated. Once seeds of homozygous line expressing *AmA1* gene is harvested, analysis of their amino acid composition will be carried out. Future studies will focus on sub cellular localization of AmA1 storage protein. Efforts will also be made to identify transformation events, and to know the site of T-DNA sequence of insertion in the rice genome by genome walking or/and TAIL PCR technique. Agronomic and food safety evaluation needs to be carried out towards the development of the high protein variety of rice expressing *AmA1* gene.

The technology and protocols established in this thesis paves the way for introducing value-added traits in rice to overcome various constraints like nutritional improvement, abiotic stress, and biotic stress. In the future transgenic rice could play very important role in food security and elevating nutritional requirement in developing countries.

CHAPTER 5
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Appendix

Bacterial strains and Plasmids

Escherichia coli: DH5α, Helper strain -HB101:: pRK 2013 (Clontech), GM2163

Agrobacterium tumefaciens EHA105

Plasmid: pBI121 (Clontech)

Media and Solutions

YEP:	1% Yeast Extract 1% Bacto Peptone 0.5% NaCl
Luria broth (LB):	25 g/l
LB agar:	32 g/l
TE (pH 8.0):	10 mM Tris-HCl (pH 8.0) 1 mM EDTA (pH 8.0)
20X SSC:	175.3 g NaCl 88.2 g Trisodium citrate pH 7.0
6X Endo-R:	30% Ficoll 400 60 mM EDTA (pH 8.0) 0.6% SDS
Antibiotics:	Kanamycin 100 mg/ml of water Rifampicin 50 mg/ml of methanol Cefotaxime 250 mg/ml of water Paromomycin sulfate 50mg/l of water

High Salt TE:	10 mM Tris (pH 8.0) 1 mM EDTA
Fixative:	0.3% paraformaldehyde 10 mM MES pH5.6 0.3 M Mannitol 50 mM NaH_2PO_4 (pH 7.0)
2X Extraction Buffer:	50 mM Tris (pH 6.8) 1% 2-mercaptoethanol 1 mM PMSF 1 mM EDTA
4X Stacking Buffer:	0.5 M Tris HCl (pH 6.8)
8X Resolving Buffer:	3 M Tris HCl (pH 8.8)
12.5% polyacrylamide gel: (40 ml)	Acrylamide (30:0.8) - 16.68 ml 4X buffer (pH 8.8) - 10 ml MilliQ - 12.92 ml SDS (20%) - 200 µl APS (10%) - 200 µl TEMED - 15 µl
Reservior Buffer (10X):	0.25 M Tris (pH 8.3) 1.92 M Glycine 1% SDS
Towbins Buffer:	25 mM Tris 190 mM Glycine 20% Methanol
TBS:	10 mM Tris-HCl, pH 8.0

	100 mM Tris, pH 8.0 150 mM NaCl
TBST:	TBS, 0.05% Tween 20
Blocking Solution:	TBS + 5% Fat free dry milk
AP Buffer:	100 mM Tris-Cl, pH 9.5 100 mM NaCl 50 mM $MgCl_2$
AP Colour Development Solution:	10 ml AP Buffer 66 µl NBT (50 mg/ml, 70% DMF) 33 µl BCIP (50 mg/ml DMF)
X-gal:	20 mg/ml (in DMF)
IPTG:	200 mg/ml in H_2O