Optimization of the protocol for Agrobacterium Mediated Gene Transformation of the Basmati Rice Cultivar by using Gus Expression

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FINAL APPROVAL

It is certified that we have read and evaluated the thesis "Optimization of the protocol for Agrobacterium mediated gene transformation of the basmati rice cultivar by using Gus expression" submitted by Ms. Shazia Burki and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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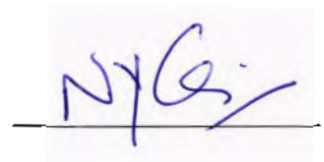

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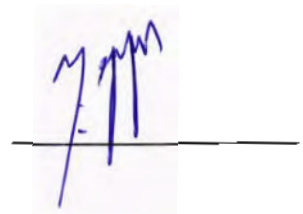

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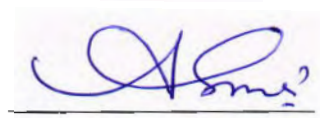

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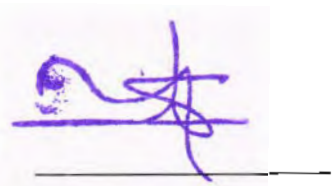

A thesis submitted to Department of Bioinformatics and Biotechnology of International Islamic University, Islamabad as a partial fulfillment of requirement for the award of degree Master of Science in Biotechnology.

Dedication

This meek exertion is dedicated to the being who is above and above of all, who is the most Exalted, the all preserver, the Sustainer of all and the one who cure the infirmities of human being, and has not left a single disease without a treatment.

Also I would like to dedicate this honor to my parents for their unconditional support, love and prayers which enabled me to reach this position.

Declaration

I hereby declare that the work presented in the thesis is our own effort and composition.

Shazia Burki

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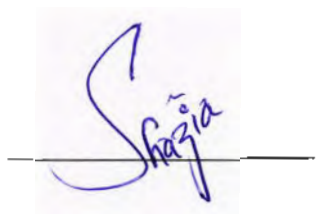

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TABLE OF CONTENTS

Acknowledgements

Thanks to Almighty Allah (SWT), the most merciful, the most beneficent. Praise is only for Allah. All the Knowing, who provided me with this opportunity, courage and ability to complete this humble contribution towards knowledge and His Holy Prophet MUHAMMAD (PBUH) the Most perfect and Exalted among the people and ever born on the surface of earth, who is forever torch of guidance and knowledge for humanity as a whole.

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Shazia Burki.

LIST OF ABBREVIATION

2,4-D	2,4- Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
Kn	Kinetin
NAA	Naphthanlene Acetic Acid
IBA	Indole-3-butyric Acid
OD	Optical density
MS	Murashige and Skoog
ddH_2O	Double distilled water
MES	2-(N-Morpholino) ethane Sulfonic Acid
HCl	Hydrocloric Acid
NaOH	Sodium hydroxide
ANOVA	Analysis of variance

LIST OF FIGURES

LIST OF TABLES

Abstract

Rice is the most important crop of Pakistan and second chief commodity after wheat. Productivity of rice is limited by number of biotic and abiotic stresses. These stresses can be controlled by introduction of novel genes transformation technology. Once the efficient and vigorous protocol for in vitro gene transformation by using reporter genes of rice would be established the desired transgenic plant production. The binary vector carrying NPTI gene under constitutive expression of CaMV35S promoter was successfully transformed into *Agrobacterium* strain. For this study local rice varieties are used, Kashmir basmati, Pusa basmati, Punjab basmati and basmati 385, on the basis of popularity due to aroma in basmati rice and availability in the local market. Tissue culture optimization is the pre-requisite for an efficient transformation establishment. Maximum callus induction frequency (Kashmir basmati=86.78% and Pusa basmati=81.48%) (Punjab basmati 84.77% and basmati 385 83.58%). A standardized tissue culture protocol for gene transformation and GUS expression was developed, which is considered as the first step for successful transformation. One step one reagent process for sterilization of seeds was used with commercially available bleach (Chlorox) and 50% was found an optimum dose. MS-media having three levels of growth regulator 2,4-D were examined for the four cultivars in terms of their callus induction potential. The concentration of 3 mg/l 2,4-D was observed suitable for efficient callus induction in Kashmir basmati while 4 mg/l was found best for genotype Pusa basmati and Basmati 385. Light and dark condition was evaluated and significant difference was observed in callus quality between the two conditions tested. Dark condition was found to have positive effect on callus quality. Some important factors effecting transformation have been optimized including acetosyringone level (100 µM), infection time and co-cultivation period (10 minutes and 3 days), use of filter paper in co-cultivation, Hygromycin concentration (50 mg/l) during selection and cefotaxime (300 mg/l) concentration for elimination of *Agrobacterium*. All these optimized condition lead to transformation efficiency in Kashmir basmati 45.33% and in Pusa basmati 26.66%. These study enhance gene transformation by using reporter gene of rice would be established the desired transgenic plant production.

CHAPTER 1

INTRODUCTION

Introduction

In a quite short time in the agrifood manufacturing, genetically modified plants with direct genes of interest and also of direct value are fashioned. It is known lately that markedly marker free transgenic can be more conventional (Yoder and Goldsborough, 1994; Komati *et al.*, 1996).Worldwide economy mostly depends on agricultural crops, in cash crops rice has a major role in advancement of economy. Some Asian parts have its more farming than the rest of the world, nearly 90% of the world's rice farming depending in Asian countries. Rice becomes the favorite dish of the world people by the rate of 1.8% per year (Islam*et al.*, 2015). It has predicted by the year 2025 the cultivation of rice can be raised by 50 %(Khush and Virk, 2000).

Pakistan is an agricultural country and it also depends in rice crop. One of the most note worthy cereal crop is rice belong to Poaceae family and for more than 10,000 years it has been cultivated (Sasaki, 2005). In regeneration of whole plant from tissue culture rice is the first main cereal crop (Vasil, 1983). Deferent verities of rice crop are facing many diseases in Pakistan and other countries of the world. It has been estimated that this crop is damaging by more than 40 diseases. Small harvesting is a side effect of this. One of the most critical and alarming disease of rice crop is rice blast whicb causes by *Pyriculariaoryzae*(Jia*et al.*, 2000).

Through Biotechnological process in modern era some chief species of rice are modified (Hao*et al.*, 2009; Skamnioti and Gurr, 2009; Cheng *et al.*, 1998). Plants breeding methods are improved in order to progress of inherently resistance against disease. This is possible only in sexually well-matched plants types and in a long period of 15 to 20 years (Rommens and Kishore, 2000). Some integrating genes that have been developed by genetic engineering can provide resistance proteins whose work is to improve immunity of plant species against disease (van der Biezen, 2001).

In retort to pathogen contagions that are recognized several intricate tools that have advanced in plants. Subsequently yeast invasions, the part of multiple genes that are involved in resistant reactions have been revealed (Islam 2006). Fungal resistance genetically modified plants can be produce by these immune boosting genes (Grover and Gowthaman, 2003). In the case of rice genetic modifications considered an important achievement (Xu*et al.*, 2012).

An infectious bacterium that cause crown gall disease in plants named *Agrobacterium tumefaciens* uses to transform plants. Now a day this bacterium is widely used to transfer foreign genes in targeted plants (Smith and Townsend, 1907).A unique facility which is gifted to *A. tumefaciens*is tumor-inducing (Ti) plasmid which is responsible to transfer a specific DNA slice (T-DNA) to the wounded cell nucleus. Then it starts integration and transcription into the host genetic material and causes crown gall tumor (Nester *et al.*, 1984; Binns and Thomashaw, 1988). Some applications can be successfully introduced by Agrobacterium into plants such as high co-expression of introduced genes, transfer of smaller quantity copies of genes into the plant hereditary material, alteration of comparatively large parts of DNA and high potency of genetically modified plants (Cheng *et al.*, 1997; Hiei*et al.*, 1994; Datta*et al.*, 1992). Transformation through *Agrobacterium tumefaciens*are widely used due to its some advantages over other techniques such as its low cost, simplicity, less copy number of foreign gene integration (Nishimura *et al.*, 2007; Larkin and Scrowcroft, 1981).

Rice (*Oryza sativa* L) is the most essential staple crop of the world and approximately half of the world's popnlation consumes it (White, 1994). In Pakistan rice is the second leading crop after wheat and is cultivated on an area of 2.57 million hectares (Government of Pakistan, 2012). A significant export commodity of Pakistan is Basmati rice (Naqvi*et al.*, 2005).New agronomic traits can be introduced into unlike crops as important advances are present that help in the gene delivery and

tissue culture. By the introdnction of useful genes through genetic engineering into basmati rice enable breeders to establish new varieties. In the history significant progress has been made in few years in optimizing and sanitization genetic transformation techniques in rice (orayza sativa L.) the japonica and afewindica varieties as an explant was reported (Toriyama *et al.*, 1998), the Direct DNA uptake into different rice (orayza sativa L.) (Rao*et al.*, 1995). To prevent this issue, Rice (Orayza sativa L.) transformation has been possiblethrough*Agrobacterium*-mediated techniques (AMT) (Finnegan *et al.*, 1994).

Basmati rice is also host to many insect, pest and diseases (Jain and Jain, 2000). Different environmental pressures limit its production. To improve resistance against the biotic and abiotic stresses; Necessary to established an efficient tissue culture system and genetic transformation procedure (Sujatha and Sonti., 2005).

The present study will focus on important rice cultivars Basmati-515, Basmati-385 and JP-5. In this regard concentration of different phytohormones and various chemicals snch as MES, cysteine and proline which have been recognized to play a significant role in callus induction and regeneration frequency will be recognized (Shahsavari*et al.*, 2010).

1.1. Plant habit

The Rice (Oryza sativa) Plant is called auto-game which can grow in the tropic to over 1M tall to 5m long in standing water. Rice is annual plant through cylindrical long leaves 50-100cm long and 2-2.5cm board. Rice was probably cultivated without submersion, although due to mutation it becomes a semi aquatics plant and grows in diverse and warm conductions. Rice plant contains spike and many tillers, panicles, nodes and influerences, and seeds, (5 -12mm) long and 2-3mm thick.

1.2. Classification of Rice

Rice belongs to the grass family (*Poaceae*) and a close genomic relationship is found between rice and many important cereal crops like maize, wheat and barley (Mooreet *al.*, 1995).

The genome size is small (430 Mbp) which provides an advantage in gene isolation and genomic sequencing compared to other cereal crops. These features (Synteny with other cereals and undersized genome) serve in the establishment of rice as a model plant of monocots (Ian and Curtis, 2004).

Table 1.1 Scientific classification of Rice

Kingdom	Plantae
Division	Magnoliophyta
Class	Liliopsida
Order	Poales
Family	*Poaceae*
Genus	*Oryza*

(http://en.wikipedia.org/wiki/scientific_classification)

Kato *et al.* (1928) classified rice into indica and japonica cultivars. Typical varieties of these cultivars differ in many characters but overlapping variations are observed in them (Singh *et al.*, 2000).

A significant export commodity of Pakistan is basmati rice. Different environmental pressures limit its production. So improving the existing germplasm of basmati cultivars is the need of the hour (Naqvi*et al.*, 2005).

1.3. Distribution in sub-contents

The cultivation of rice began above 6500 years ago. The first crop of rice was reported from different region of china more or less 5000 before Christ (B.C) as well as neighbor country Thailand approximately 4500B.C. Although the two species Japonica and indicia were later cultivated in Asian, Country that is, South Korea, Japans, Pakistan Indonesia and India. *Oryza sativa* was introduced in Middle-East and Europe in 800 B.C.in 15^{th} centuries the rice farming spread in the Italy and France. Later the Arabia coming from the famous East Coast from 7^{th}-11^{th} centuries. Rice as a staple food, The Mohenjo-Daro also introduced the rice in the age of 3millennium B.C, and almost in Punjab (2000 -1500) B.C (Oka, 1988).

1.4. Ecology of Rice

The nature of Rice is water loving plants and cannot survival at higher latitude 53, N and longitude 35E and low humidity and temperature, 13c. The warm heated climate is so necessary because this plant is tropical plant. Due to unique nature of adaption the Rice grown in Himalayas and also grow in the sea of Coastal plain and Delta region through Asia and to highest of 2600m in slope of Himalayas in Nepal's (Salem *et al.*, 2003).

The quality and production of Rice depend in the availability of water (IRRI, 1997). In cereal crops the Rice is placed in 2nd position, the annual production at the start of the 1990s, was 350 million tons (Mt) and by the ending of the century it had reached 410Miillion tons (Mt). In 2015, approximately 450 million tons of rice is produced by Agriculture Organization of the United Nation Organization.

1.5. The status of Rice in Pakistan

Rice are cultivate in different warm region of Pakistan. The production of rice total 2.62 million hac area uses, Punjab province is main contributor in Pakistan due their warm climate conduction. Rice production in Punjab 3.17 million tons, Khyber Pakhtunkhwa 0.11 million tons, Sindh 1.7 million and Baluchistan 0.17 million tons. The 1.6 million hac areas use for the most vital production of Basmati Rice. The basmati rice is famous throughout globe and 1.6 million tones are produced in Pakistan (Anonymous, 2006). The 94% of Basmati rice produced by Punjab.

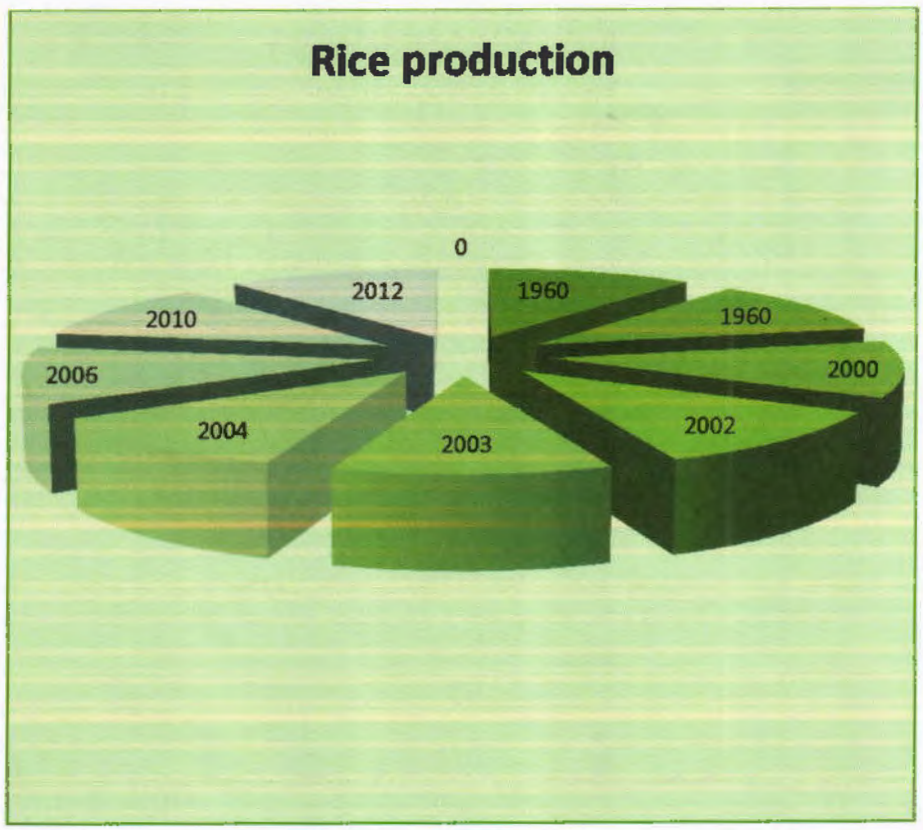

Figure.1 Rice Production in Pakistan

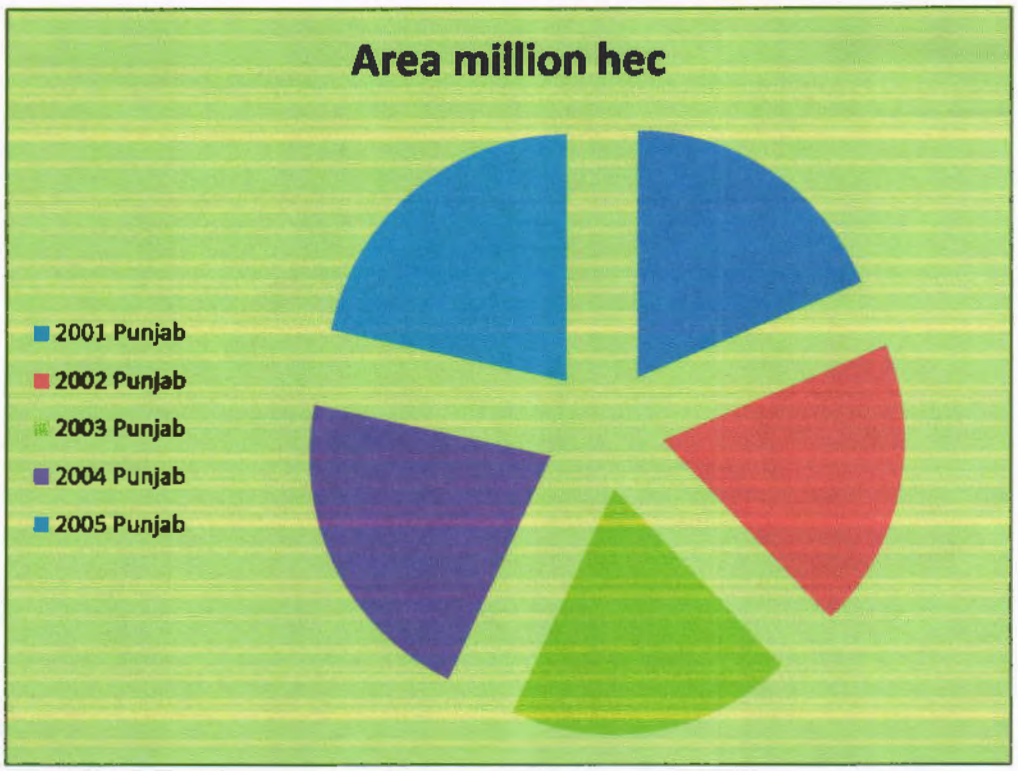

Figure.2 Sources: Pakistan agriculture survey

Table 1: Different Agro- climatic zones of Pakistan

Zone 1	Northern mountain areas
Zone 2	Area between Ravi and Chenab
Zone3	West bank of River Indus
Zone4	Indus delta

Climatic Zone

Table 2: Export of Basmati Rice from Pakistan

Years	Metric tones	Value US $
2001	502	255
2002	443	236
2003	410	359
2004	816	422

Rice export

1.6. Objective of study

The objectives are

- To extend the resourceful protocol for best callus induction and *in vitro* absolute plant GUS expression for different varieties of Pakistan rice (*Oryza sativa*) Kashmir basmati, Pusa basmati, Punjab basmati and basmati 385 as it is the pre-requisite for transformation.
- To select the best variety on the basis of GUS expression.
- To optimize a protocol for gene transformation of the best variety

CHAPTER 2

REVIEW OF LITERATURE

Review of literature

Several researchers have investigated successful callus induction and GUS expression improvement in rice.

Rao*et al.* (2012) initiated callus cultures on LS (Linsmaier and Skoog) medium containing 2mg/l from adult embryos of diverse cultivars. They establish that callus induction frequency was amplified in all the cultivars with increased concentration of 2,4-D and 2,4-5T (2,4,5-Trichlorophenoxyacetic acid). Maximum callus induction frequency was establishing in te cultivars Tellahamasa (65-75%) followed by DGWG, Yerragaluvadlu, sureckha, Basmati-370, Bala, cakkoambubi, Jaya and IR-8.

Hoque*et al.* (2013) developed a protocol for callus induction of diverse varieties of rice cultivated in Bangladesh. The varieties were pakhiBiroin, HatiBaromashi, KachaBiroin, BadalBoro, PoricokAmon, Khoiya, Boro, SoriaAman and BRRI Dhan 53. MS media along with varying concentration of 2,4-D (1.5, 2, 2.5 and 3mg/l) was used for callus induction. HatiBarmashi, BRRI Dhan 53 and PakhiBiroion showed 100% callus induction frequency. JoriaAman and KhoiyaBoro showed a failure in the callus induction. KachaBiroin sowed 75% efficiency.

Muhammad *et al.* (2014) established an efficient calli initiation and induction system cultivars retorted, to a particular concentration of 2, 4-D for calli initiation and induction. The cultivar IRRI-6 took minimum number of average days to calli initiation on 2mg/l concentration of 2, 4, -D, at 3mg/l show highest performance. KSK-282 showed maximum calli initiation at 4mg/l with 92.22% of calli induction.

Zaid*et al.* (2006) established a system for elevated frequency embryogenesis of indica rice cv. MDU5. For callus induction MS media was optimized by supplementing it with 2mg/l, 2,4-D (2,4-D Dichlorophenoxyacetic acid, 0.5mg/l kinetin, IAA (indole acetic acid), This optimized media was analyzed for response in 73 other indica genotypes out of which 64 produced 98.5% callus induction in eight days.

Rashid *et al*. (1996) a reproducible system has been developed for the production of transgenic plants in *indica rice* using *Agrobacterium*-mediated gene transfer. Three-week-old scutellacalli served as an excellent starting material. These were infected with an *Agrobacterium tumefaciens* strain EHA101 carrying a plasmid pIG121Hm containing genes for β-glucuronidase (GUS) and hygromycinresistnace (HygR). Hygromycin (50 mg/l) was used as a selectable agent. Inclusion of acetosyringone (50µM) in the *Agrobacterium* suspension and co-culture media proved to be indispensable for successful transformation. Transformation efficiency of Basmati370 was 22% which was as high as reported in *japonica* rice. Integration of foreign genes into the genome of transgenic plants was confirmed by Southern blot analysis. GUS and HygR genes were inherited and expressed in R1 progeny. Mendelian segregation was observed in some R1 progeny.

Revathi and pillai (2011) carried out callus induction of four rice varieties namely ASD 16, ADT 43, Basmati 37, Pusa Basmati and pokkali in six different medium having varing concentration of 2,4-D. The study was useful for selection suitable medium for callus induction. The variation in callus induction frequency was from 58.33% to 96.67%.

Kant *et al*. (2007) *Agrobacterium*-mediated transformation was done in rice (*Oryza sativa* L. var. *indica*). Co-cultivation with scutellar-calliderivative from mature seeds showed steady and extremely efficient transformation. In cvs. HKR126 and PB1, 35 % and 41 % of hygromycin resistant calli were obtained. The transformation efficiency in PB1 (22.0 %) was much higher than in HKR126 (12.5 %).

Tripathi*et al*. (2010) develop an efficient and reproducible protocol for Agrobacterium- mediated transformation of an ergonomically useful Oryza sativa variety, Pusa Basmati- 1 (IET- 10364) has been established. We produce an efficient and reproducible protocol. The callus derived from mature rice seeds were used as

explants for transformation studies. Callus were co-cultured with Agrobacterium tumefaciens EHA 105 harboring a binary vector p35SGUSINT that carried Betaglucuronidase (uid A) and It contains gene npt II, determining resistance to kanamycin, under the control of the constitutive (nos) promoter. Maximum Agrobacterium- mediated transformation frequency was observed at 350µM/l acetosyringone with 35 days old callus.

Karthikeyan*et al.* (2012) a greatly efficient protocol for *Agrobacterium tumefactions*-mediated transformation of *indica* rice (*Oryza sativa* L. subsp. *indica* cv. ADT 43) was reputable. earlier to transformation, embryogenic callus were induced from mature seeds incubated on Linsmaier and Skoog (LS) medium supplemented with 2.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg l^{-1} thiamine-HCl. Callus, intact mature seeds, and other in vitro derived explants (leaf bases, leaf blades, coleoptiles, and root-tips) were immersed in a bacterial suspension culture of *A. tumefaciens* strain EHA 105, OD600 of 0.8, and co-cultivated on LS medium for 2 days in the dark at 25 ± 2°C. Based on GUS expression analysis, 10 min incubation time of explants on a co-cultivation medium containing 100 µMacetosyringone was optimum. Following *β*-glucuronidase (GUS) assay and polymerase chain reaction (PCR) analysis, transformants were identified.

Ignacimuthu*et al.* (2011) the rice leaffolder (RLF), *Cnaphalocrocismedinalis* is a vital pest of rice that causes cruel damage in numerous areas of the world. The plants were transformed with abundant modified (plant codon optimized) synthetic *Cry1C* coding sequences as well as with the *hpt* and *gus*genes, coding for hygromycinphosphotransferase and β-glucuronidase, respectively. *Cry1C*sequences placed under the power of doubled 35S promoter plus the AMV leader sequence, and *hpt* and *gus* genes driven by cauliflower mosaic virus 35S promoter, were used in this study.

Amitabh *et al*., (1992) rice, indica varieties are measuredrefractory to tissue culture and genetic manipulation. We described here *Agrobacterium*-mediated transformation of an economically important. In this study, we report *Agrobacterium*-mediated transformation of abestindica variety Pusa Basmati 1. A large number of fertile transgenic plants, mostly from free transformation events, have been obtained. Stable integration and transmission of the transgenes in the R0, R1 as well as in the R2 progeny, as established by molecular analysis, are also reported.

CHAPTER 3

MATERIALS AND METHODOLOGY

Materials and Methods

Tissue culture is a pre-requisite for gene transformation. Therefore, first of all tissue culture protocol will be optimized. Seeds would be used as explants source for tissue culture and gene transformation. The research work was carried out partly at Applied Biotechnology and genetic engineering lab (ABGE), IIUI, Quaid-e-Azam University and National institute of genomics and Advance Biotechnology (NIGAB), National Agriculture Research center (NARC) Islamabad.

3.1. Material

3.1.1. Plant Material

Experiment was performed on four rice (*Oryza sativa)* cultivars namely Basmati 385, Kashmir basmati, Pusa basmati, and Punjab Basmati. Collected from NARC, Islamabad, and Crop institute (CSI) was used as preparatory material in the present study.

Scutellum callus Obtained from mature seeds was used as explants for transformation process.

3.1.2. Cloning vector

The cloning vector was provided by Plant Biotechnology Programmed, National Institute for Genomics and Advance Biotechnology (NIGAB), NARC Islamabad. The structure of the T-DNA region of the binary vector p35SGU is given in fig.3.1

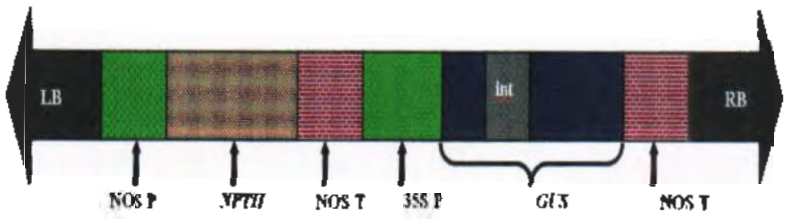

Fig 3.1 Schematic representation of T-DNA region of p35SGUSint

LB: Left border; **NOS P**: Nopalinesysthase promoter; **NOS T**: Nopline synthase terminator, NPTII: Neomycin phosphotransferase gene; **35S P**: CaMV35S promoter;GUS: ß-glucuronidase gene; **int**: intron; **RB**: Right border

3.1.3. Bacterial strain

The recombinant vector was replicated in *E. coli* LBA4404 strain and then transferred to *Agrobacterium tumefaciens* EH101 strains.

3.1.4. Equipment.

Laminar flow cabinet (Forma scientific), Balance (Denver Instrument), pH meter, Microwave oven (Dawlance), Glassware, Tubes (pyrex,Germany), Micropipettes (Gilson France), Falcon tubes, Centrifuge machine, Incubator, shaking incubator, Eppendorf, Refrigerator (04°C, -20°C,and 80°C) and Electroporator (Eppendorf 2510).

Table 3.1 Media used for rice tissue culture and transformation

Medium	Culture time	Media Composition
Callus Induction Medium	**12 days**	MS Salts (Murashige and Skoog*et al.*, 1962), 2,4-D (2 mg/l,3 mg/l and 4 mg/l), Casein hydrolysate 0.3 g/l, Myoinositol 0.1 g/l, Sucrose 3%, Gellan Gum Agar 0.3%, pH 5.8
Callus Maintenance Medium	**04 days**	N6 Salts, 2,4-D 2 mg/l, Vits., Casein hydrolysate 0.3 mg/l,Myoinositol 0.1 mg/l, Sucrose 3%, Gellan Gum Agar 0.3%, pH 5.8
Bacterial suspension medium	**2 days**	LB medium, sucrose 3%, Acetosyringone 100 µM, pH 7
Pre-induction medium	**1 day**	MS Salts (Murashige and Skoog, 1964), Sucrose 3%, Acetosyringone100 µM, pH 7
Infection Medium	**5-10 minutes**	Pre-induction medium ($O.D_{600}$ of 0.4)
Co-cultivation Medium	**1-3 days**	N6 salts, 2,4-D 2 mg/l, Casein hydrolysate 0.3 g/l, Myoinositol 0.1 g/l, Sucrose 3%, Acetosyringone (100µM, 200µM, 300µM), Gellan Gum Agar 0.3%, pH 5.8
Washing Medium	**10 minutes**	MS basal medium, Sucrose 30 g/l, Cefotaxime 500 mg/l, pH 5.8
Selection Medium	**3-4 weeks**	MS salts, 2,4-D 2 mg/l, Hygromycin (25 mg/l, 50 mg/l & 75mg/l), Sucrose 30%, Cefotaxime (250 mg/l, 300mg/l, 500mg/l), Gellan Gum Agar 0.3%, pH 5.8

Table 3.2 List of Antibiotics and concentrations

Antibiotics	Group	Source	Use	Concentration
Kenamycinsulphate	Broad spectrum aminoglycosides antibiotic	Streptomyces kanamyceticus	Selective agent (*npt II* gene)	50 mg/l
2.2 Tetracycline	Broad spectrum polyketideantibiotic	Streptomycesgenus of Actinobacteria	Selective agent (*tet*gene)	250 mg/l
Hygromycin B	Broad spectrum aminoglycosides antibiotic	*Streptomyces hygroscopicus*	Selective agent (*hpt* gene)	25 to 75 mg/l
Cefotaxime sodium	cephalosporinantibiotic	Synthetic	Bacterial elimination	250 to 500 mg/l

3.2. Methodology

3.2.1. Tissue culture

The culture conditions were optimized by using the following methodology.

3.2.2. Surface sterilization of seeds

Mature rice seeds were dehusked by hand manually. Then these seeds washed through detergent by using distilled water for 2 mints. These seeds were first rinsed in ethanol (70%) for 60 seconds then washing with commercially available (50%) chlorox ($NaOCl_2$) for 15-20 minutes. After that the seeds washed with autoclaved distilled water for five times to eradicate the remnants of chlorox. Autoclaved filter papers were used to dry up the seeds. Sterilizing agents are very important and can affect the efficiency of generation.

3.2.3. Inoculation of seeds

MS- media (Murashige and Skoog 1962) and N6 (Chu, 1978) media consisting of vary concentration of growth hormones with different combination will be used for callus induction and regeneration. The tissue culture media was autoclaved before use.

3.2.4. Callus Induction

For callus initiation, the surface sterilized desiccated seeds were reserved on callus induction media (N6- media, as reported by (Chu *et al*, 1976). Three different concentrations of 2, 4-D (2.5mg/l, 3mg/l, and 3.5mg/l) were tested for their callus induction capability. N6-basal salt containing B5 vitamins, sucrose (3%) casein hydrolysate (0.3 g/l), gelatin gum agar (0.3%) and fortified with different levels of 2,4-D (2.5mg/l,4mg/,5mg/l). 2,4-D. Callus induction media with supplements (glycine, proline, and MES) were also used to study its affect. After addition of all the

components, pH was adjusted to 5.7 -5.8 using M HCl or 0.5 M NaOH and autoclaved at 121°C for 15 minutes at 15 psi. The media was transferred into clean and dried test tubes and then it was autoclaved at 121°C and 15 psi for 20 minutes. The surface sterilized seeds were inoculated into the test tubes under sterilized condition in laminar air flow hood. The culture tubes were kept in both light (16/8 hrs photoperiod) as well as dark conditions provided by energy saver (Philips) for 12 days. After the specific incubation period of 10-13 days, embryogeniccalli induced induced from seed scutellum were obtained and the data was recorded. The callus induction frequency was calculated as follows:

$$\text{Callus Induction frequency (\%)} = \frac{\text{No. of Calli of calli obtained}}{\text{Total number of seeds culture.}} \times 100$$

3.2.5. Callus Maintenance

The scutellum derived embryogeniccalli of all rice genotypes were shifted to subculture media and kept under comparable condition for four days under light (16/8 hrs photoperiod) conditions. The composition of maintenance media was similar to that of callus initiation medium with 2mg/L 2, 4-D.

3.2. Gene transformation

Calli from each variety will be cut into small pieces. These calli will be transformed using Agrobacterium *tumefaciens* has boring the binary vectors containing selectable marker and reporter gene.

3.2.1.Agrobacterium mediated genetic transformation

Agrobacterium approach is the most popular method to deliver genes to plant cells because of its clean insertion, low-copy number of the inserted genes, easy to handle, higher efficiency, more predictable pattern of foreign DNA integration. Studies on *A.*

tumefaciens provided the basis that has made this soil bacterium as dominant customer for plant transformation.

3.2.2. Agrobacterium culture preparation

A single colony of cloned Agrobacterium strain EHA105 from selection plates was inoculated into liquid LB- medium supplemented with kenamycin antibiotic (50mg/l) and kept 28°C at 170 rpm for 48 hours in shaking incubator. Subsequently the Agrobacterium culture prepared was equally divided in several Eppendorf tubes and centrifugation was carried out at 8000 rpm for five minutes. The pellets thus formed were re-suspended in MS liquid containing 100 µM acetosyringone and was kept in the incubator at 28°C, 170 rpm for 24 hours.

3.2.3. Co-cultivation

The bacterial cell suspension was adjusted to three different density levels (0.2, 0.4 and 0.6) and three different pH levels (5, 5.5, and 6.5) to test its efficacy. The embryogeniccalli were infected with suspension of *Agrobacterium* having three different OD's for 10 minutes. After infection calli were dried on filter paper for 10 minutes. These calli were shifted then to the N6 Co-cultivation plates containing N6-media containing 2, 4-D level of 2mg/l and 100 µM acetosyringone for 2 days.

3.2.4. Selection

The calli were washed 4-5 times with ddH2O to remove excess bacteria. The Agrobacterium culture was eradicated from the infected calli by washing 5 times with liquid MS-media containing 500mg/l cefotaxime and then dried on sterilized filter papers. Later these calli were shifted to the selection plates consisting of N6-media with three different levels of hygromycin (35mg/l, 50mg/l, and 75mg/l) and three different cefotaxime levels (25mg/l, 500mg/l and 750mg/l). These selected calli were kept in incubator at 25 ± 2°C in dark conditions for a period of 3-4 weeks.

3.3. Gus expression

The GUS reporter system (*GUS*: β-glucuronidase) is a reporter gene system, particularly useful in plant molecularbiologyand microbiology. GUS reporter gene provided useful desirable characteristics in transformation. Several kinds of GUS reporter gene assay are available, depending on the substrate used. The term GUS staining refers to the most common of these, a histochemical technique.

The purpose of this technique is to analyze the activity of a promoter (in terms of expression of a gene under that promoter) either in a quantitative way or through visualization of its activity in different tissues. The technique is based on β-glucuronidase, an enzyme from the bacterium Escherichiacoli this enzyme, when incubated with some specific colorless or non-fluorescent substrates, can transform them into coloured or fluorescent products.

3.3.1. Assay of ß-glucuronidase (Gus) activity

Histochemical Gus assay was approved out basically as described by Jefferson (1987). Calli were incubated in X-Gluc solution containing 1 mg/l 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5% triton X-100, 20% Methanol and 50 mM Sodium phosphate buffer (pH 7.0). The reaction mixture was incubated at 37°C for 2-3 days and calli were examined under microscope.

3.4. Experimental design

Factorial design was used for all the experiments. Each experiment was composed of 3 replications. For callus induction each replication comprised of 18 seeds per treatment while each replication consisted of 10 calli. And each variety consist transformed calluses.

The treatment parameters were based on the following factors.

- Co-cultivated calli showing Gus expression at different level of acetosyringone concentration.
- Infection period and co-cultivation time with *Agrobacteriumtumefaciens* strain EHA101 (pTCL5) Gus expression in rice (*Oryzasativa* L.).
- Co-cultivation media with filter paper on browning of calli and over growth.
- Transformed calli selection by using hygromycin.
- Effect of Cefotaxime on *Agrobacterium* transformation efficiency.

3.5. Data Analysis

Data analysis was performed to analyze which variety is best and which media or combination at which concentration of growth regulators, provided the higher callus induction frequency and regeneration efficiency of the transgenic plants.

CHAPTER 4

RESULTS

Results

4.1. Tissue culture

Tissue culture conditions were optimized for the four rice cultivars Kashmir basmati, Pusa basmati, Punjab basmati and basmati 385 by evaluating the following factors.

4.1.1. Callus induction

4.1.1.1. Effect of chlorox Concentration on Seed Sterilization

The efficacy of different percentage of antiseptic chlorox was evaluated for controlling the contamination incidence of the explants (Seeds). The results clearly showed that 50% chlorox significantly reduce the contamination frequency of the four cultivars with maximum number of calli induced (Table 4.1). The ANOVA results revealed significant differences between the three concentrations of chlorox and among the four genotypes for contamination and callus induction frequency (Table 4.1). The effect of genotype and chlorox interaction also shows significant differences. The results of LSD further clarify that at 50% chlorox, contamination frequency was lower while the callus induction frequency was higher and moving above this will hamper the growth of the seeds.

Table 4.1 Effect of chlorox on contamination and callus induction frequency

Chlorox (%)	Genotype	Contamination (%)	Range (5%)	Callus induction (%)	Range (5%)
30	Kashmir Basmati	13.17±1.63 b	A	77.09±2.16	C
	Pusa Basmati	23.61±1.62 a		66.82±3.50	
	Punjab Basmati	13.71±52 b		52.65±2.10	
	Basmati 385	21.52±43 a		42.51±1.08	
50	Kashmir Basmati	06.36±1.27 c	B	73.21±6.25	A
	Pusa Basmati	12.77±3.31 b		71.65±2.52	
	Punjab Basmati	05.25±1.37 c		51.43±1.30	
	Basmati 385	13.76±3.21 b		41.52±1.02	
70	Kashmir Basmati	00.00±00.00 d	C	00.00±00.00	B
	Pusa Basmati	00.00±00.00 d		00.00±00.00	
	Punjab Basmati	00.00±00.00 d		00.00±00.00	
	Basmati 385	00.00±00.00 d		00.00±00.00	

Mean values followed by the same letter with a column shows no significant differences (α= 0.05).

4.1.1.2. Effect of growth regulator on Number of Days to callus induction

Callus induction is one of the most extensive step for genetic transformation. Callus induction largely depends on genotype of the donor plant, type of culture media, growth regulators and various other factors. Mature rice seeds of four different rice genotype were inoculated on MS media with three different levels of 2,4-D to determine the optimum level for high frequency callus induction.

The results revealed that growth regulator 2,4-D level have significant effect on callus induction. Maximum callus induction (85.12%) was observed at 2 mg/l concentration of 2,4-D for the genotype Kashmir basmati while increasing the 2,4-D level result a decline in callus induction frequency in this genotype. Genotype Pusa basmati shows maximum callus induction (75.34%) at 4mg/l 2,4-D level (Table 4.2). Both the genotypes show difference in the callus induction frequency and their response to different level of 2,4-D was different (Fig 4A and 4B). These results are clarified by LSD. The ANOVA result further stated that 2,4-D and 2,4-D×Genotype interaction have significant effect on callus induction in four different rice genotypes at 5% probability level.

Table 4.2: Effect of 2,4-D on callus induction frequency

2,4-D (mg/l)	Callus induction (%)			
	Kashmir basmati	Range (5%)	Pusa Basmati	Range (5%)
2	42.22±3.41 (76.22)	A	31.01±1.24 (55.55)	B
3	37.23±1.85 (86.78)	AB	34.23±0.77 (63.51)	Ab
4	30.00±1.15 (62.50)	B	29.58±1.67 (52.58)	A

Mean values followed by the same letter within a column shows no significant differences (α= 0.05)

Table 4.2 Effect of 2,4-D on callus induction frequency

2,4-D (mg/l)	Callus induction (%)			
	Punjab basmati	Range (5%)	Basmati 385	Range (5%)
2	31.12±3.31 (65.21)	A	24.01±1.23 (45.34)	B
3	45.13±1.75 (74.67)	AB	38.21±0.63 (54.38)	Ab
4	25.00±1.13 (42.30)	B	20.54±1.68 (30.28)	A

Mean values followed by the same letter within a column shows no significant differences (α= 0.05)

Fig 4.2 Callus induction in Basmati 385, Kashmir basmati, Pusa basmati, and Punjab basmati

Fig 4.2 Callus Induction in Kashmir and Pusa basmati

Fig 4.2 Callus induction in Punjab basmati and basmati 385

4.1.1.4. Effect of light and dark condition on callus induction and quality

Seeds of the rice genotypes were cultured on MS media with 2,4-D under both light and dark conditions. Light conditions of 16/8 hrs photoperiod and dark conditions at 25 °C were applied to cultured seeds of both rice genotypes.

It was observed that under both light and dark conditions, the callus induction frequency remained the same. But light and dark conditions induction significant effect on callus quality (Table 4.4). The calli induced under light conditions were yellowish, smaller in size, globular, compact and was termed as type I calli while those produced under dark conditions were whitish, larger in size, friable and was termed as type II calli (fig.1 and 2). These results allocate that under dark conditions, nutrient supplement enhance callus quality. The effect of treatment, genotype and their interaction has no significant effect on callus induction (ANOVA $p < 0.05$)

Table 4.4: Effect of light and dark on callus induction and its quality

Treatment	Genotype	Callus induction (%)	Callus quality
Light	Kashmir basmati Pusa basmati	36.32±0.77 (86.67) 35.57±2.10 (79.36)	Type I Type I
Dark	Kashmir basmati Pusa basmati	42.24±2.30 (88.22) 40.58±1.39(83.62)	Type II Type II
Light	Punjab basmati Basmati 385	34.23±0.76 (74.66) 32.13±0.73 (72.63)	Type I Type I
Dark	Punjab basmati Basmati 385	44.33±0.86 (72.56) 41.23±0.73 (62.72)	Type II Type II

Mean values followed by the same letter within a column shows no significant differences (α= 0.05)

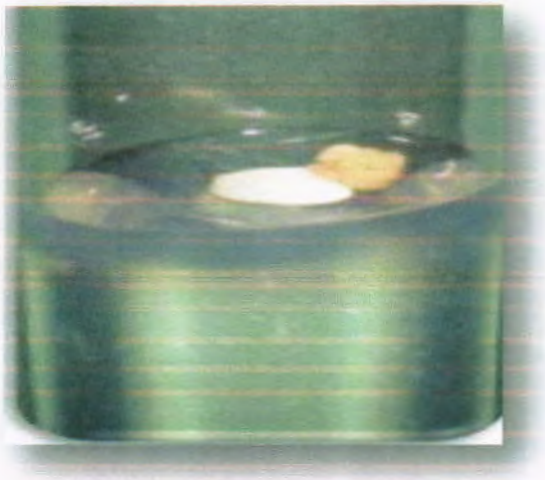

Fig 1 Type I callus: Yellowish, Smaller in size, globular

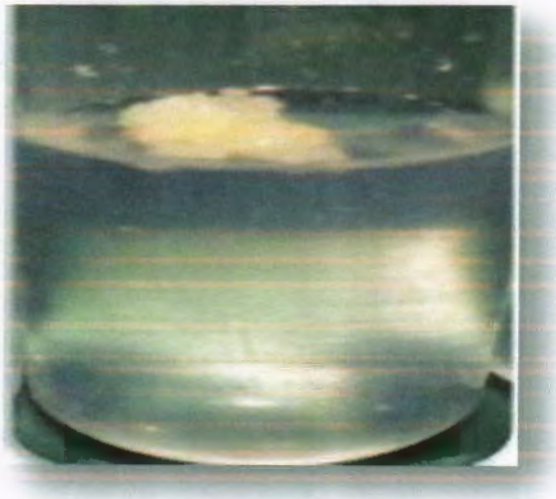

Fig 2 Type II callus: Whitish, larger, friable

4.2. GUS analysis

The GUS reporter system (*GUS*: β-glucuronides) is a reporter gene system, useful in plant molecular biology and microbiology. GUS reporter gene provided useful desirable characteristics in transformation. Several kinds of GUS reporter gene assay are available, depending on the substrate used. The term GUS staining refers to the most common of these, a histochemical technique.

The purpose of this technique is to analyze the activity of a promoter (in terms of expression of a gene under that promoter) either in a quantitative way or through visualization of its activity in different tissues. The technique is based on β-glucuronidase, an enzyme from the bacterium *Escherichiacoli* this enzyme, when incubated with some specific colorless or non-fluorescent substrates, can transform them into colored or fluorescent products.

To evaluate transgenic and constant T-DNA transformation, GUS expression analysis was receiving out direct calli 4 weeks after co-cultivation as well as in transient GUS expression was observed in calli of all varieties. For stable GUS expression, for weeks old transformed and untransformed calli were assayed. Calli that developed from untransformed explants (seeds) showed no detectable GUS expression, while calli of transformed explants strained blue.

Compared to the 90% of the explants showing GUS expression after two days of co-cultivation, only 40% explants developed hygromycin resistance calli and showed GUS expression after four weeks. Each variety showed stable GUS expression in calli in basmati varieties. More stable GUS expression showed the calli deep blue strain.

4.3. *Agrobacterium* mediated transformation

Agrobacterium Mediated transformation has long been used for successful transformation of monocots. Limited research has been undertaken for the improvement of our local rice cultivars through genetic engineering. Various factors that affect *Agrobacterium* mediated transformation in four different rice cultivars have been optimized in the present study.

4.3.1. Co-cultivated calli showing GUS expression at different level of acetosyringone concentration

The allowance and transmit of T-DNA from *Agrobacterium tumefaciens*to plant cells is mediated by virulence genes and these genes are induced byphenolics compound acetosyringone provided in the co-cultivation medium. Co-cultivation of calli with *Agrobacterium* in the presence of acetosyringone has become a usual exercise in the transformation of monocots. *Agrobacterium* show unlike sensitivity level to varying concentrations of acetosyringone. Therefore, we have tested three unlike levels (100 µM, 200 µM and 300 µM of acetosyringone to see its effect on virulence induction of the *Agrobacterium*EH101strain binary vectors and its transformation efficiency (Table 4.1).

Acetosyringone concentration of 200µM in co-cultivation media resulted in advanced transformation efficiency and Gus expression in four rice cultivars Kashmir basmati, (44%), pusa basmati (26.6%), Punjab basmati (22.20%) and basmati 385 (18.32%).Increase of the AS concentration to 300µM caused the percentage of hygromycin resistance calli to refuse in four varieties tested. The percentage of hygromycin resistant calli was decreased by 6% in Kashmir basmati while the same tendency was observed for pusa basmati 5%, Punjab basmati 4% and basmati 385 3%. For rice genotypes have responded differently to the three levels of acetosyringone with Kashmir basmati having highest transformation efficiency

compared to other three varieties (Table 4.1). The ANOVA result illustrate that both acetosyringone and variety have significant effect on transformation efficiency and Gus expression while their interaction shows non-significant effect. The transformation efficiency using AS at a concentration of 200μM was significantly higher than the two other concentrations used (LSD, $P< 0.05$).

Table4.1:Co-cultivated calli showing GUS expression at different concentration of Acetosyringone

Genotype	Average no of calli showing GUS expression at acetosyringone concentration			Mean of each variety
	100µM	200 µM	300 µM	
Basmati 385	0.0±0.00	2.0±1.20	1.0±1.25	4.06 ab
Punjab basmati	0.0±0.00	5.0±2.00	2.0±1.73	3.400 ab
Pusa basmati	1.0±0.58	7.0±0.58	3.0±1.00	1.40 bc
Kashmir basmati	2.0±0.90	10.0±2.08	4.0±1.70	3.40 bc
Mean of treatment	2.11^{d} **	3.66^{c} **	4.76^{ab} **	

SD 0.01 for variety = 1.891

LSD 0.01 for treatment = 1.325

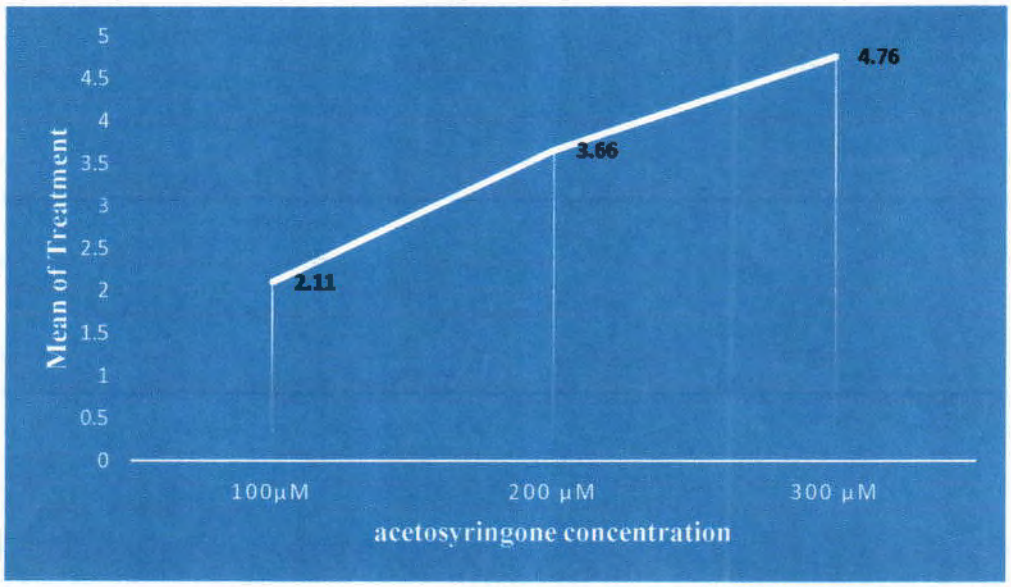

Fig 4.1: A graph representing GUS expression in different varieties at different concentration of Acetosyringone

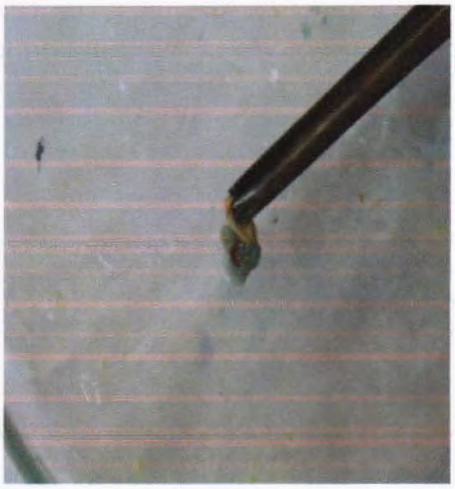

Fig 4.1 GUS expression in Kashmir basmati

Fig 4.1 GUS expression in Punjab basmati

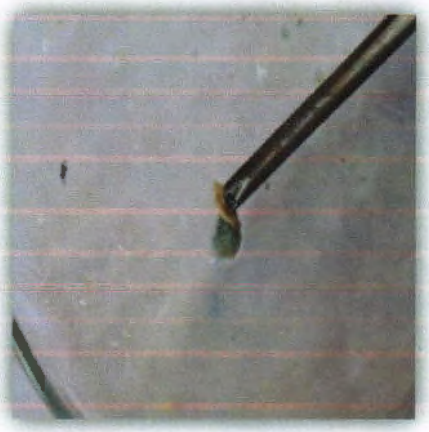

Fig 4.1GUS expression in Pusa basmati

Fig 4.1GUS expression in Basmati 385

4.3.2. Infection period and co-cultivation time with *Agrobacteriumtumefaciens* strain EHA101 (pTCL5) GUS expression in rice (*Oryzasativa* L.)

The most significant and serious factors that affect *Agrobacterium* mediated transformation are the infection time and co-cultivation period. Four different co-cultivation period of 1, 2, 3 and 4 days on transient Gus expression of 21-day old rice calli were tested for the four rice cultivars in this experiment, but the difference among genotypes was significant. The average number of Gus expression (7.2%) was shown when co-cultivation time period where kept for 2 days and normal bacterial growth was observed. The lowest standard number of calli showing Gus expression (1.3%) was obtained when co-cultivation time period was 1 day and bacterial growth was below normal. No Gus expression was observed in culture co-cultivated for 4 days. The tissues were adversely affected during prolonged co-cultivation period. It is clear from the result that the gain efficient expression of Gus in rice, 2 days' cultivation with Agrobacterium is significantly best time.

Table 4.2: Effect of infection time and co-cultivation period with *Agrobacterium tumefaciens* train EA101 (pTCL5) GUS expression in rice (*Oryza sativa* L.)

Genotype	Average No of GUS calli co-cultivated of different varieties				Mean of each variety
	1 day	2 day	3 day	4 day	
Basmati 385	0.00±0.01	5.00±0.678	2.00±0.476	0.00±0.00	2.40
Punjab basmati	1.00±0.155	6.21±1.427	3.00±0.782	0.00±0.00	3.00
Pusa basmati	1.00±0.10	7.33±1.156	5.00±0.55	0.00±0.00	5.50
Kashmir Basmati	2.00±1.55	9.66±1.125	7.00±1538	0.00±0.00	6.24
Mean of each treatment	1.23	7.12	2.32	0.00	

LSD 0.01 for variety = 1.03

Fig 4.2 Co-cultivation in Basmati 385

Fig 4.2 Co-cultivation in Punjab basmati

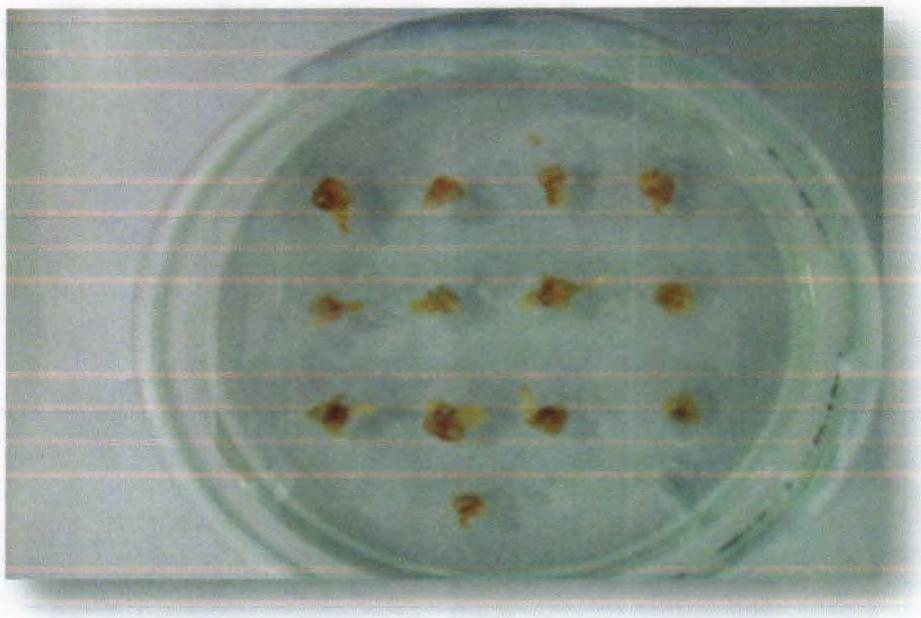

Fig 4.2 Co-cultivation in Kashmir basmati

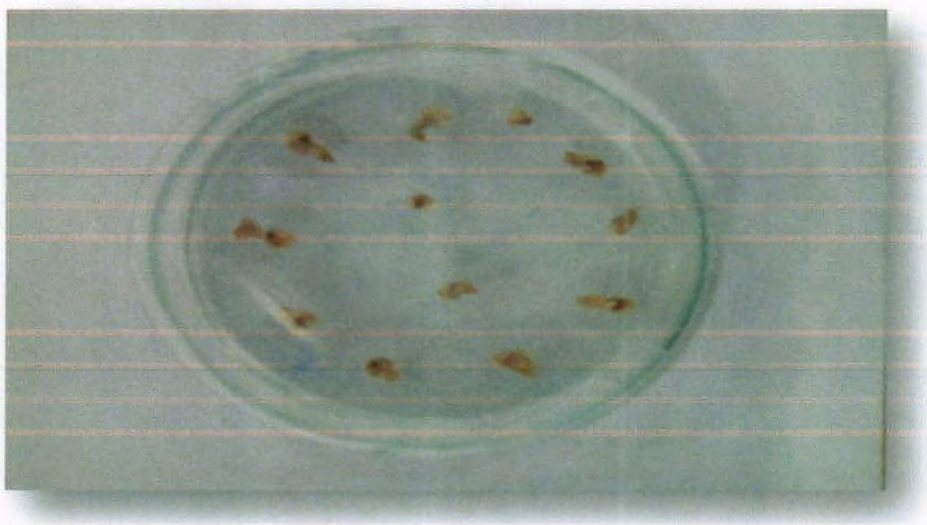

Fig 4.2 Co-cultivation in Pusa basmati

4.3.3. Effect of Co-cultivation media with filter paper on browning of calli and over growth

When the infected calli were subjected to long term cultivation period of four days, the transformation efficiency was increased but the calli shows' browning due to quick proliferation of *Agrobacterium* and overgrowth of *Agrobacterium* was hard to control after washing with cefotaxime. Therefore, to overcome these conditions to some extent, the solid co-cultivation medium in plates was roofed with filter paper.

The result point out that when the media were roofed with filter paper, the *Agrobacterium*EH101 proliferate quickly because of less access to the media and the browning (6.00% calli in Kashmir basmati, 11.31% in pusa basmati, 18.21% Punjab basmati, and 19% in basmati 385) and overgrowth (0.00) were controlled. The ANOVA table reveals that solid media with filter paper have significant effect on calli browning and overgrowth in both rice genotypes at $p < 0.005$ (Table 4.3).

Table 4.3: Effect of co-cultivation solid media, media with filter paper on browning of calli and growth control

Genotype	Solid media		Media with Filter paper	
	Browning	Over growth	Browning	Over growth
Kashmir basmati	16.32±0.87	5.76±0.72	6.00±0.67	00±
Pusa basmati	14.23±0.67	4.32±0.22	11.31±0.56	00±
Punjab basmati	24±0.56	6.72±0.62	18.21±0.34	00±
Basmati 385	28±0.45	7.82±0.74	19.00±0.35	00±

Media with filter paper showing significant effect on calli browning and over growth at ($p < 0.05$)

4.3.4. Transformed calliSelection by using Hygromycin concentration

Hygromycinphosphotransferase gene is widely employed marker gene for selection of transgenic cells in rice calli. We introduced this marker gene in our construct and tested three different concentrations of hygromycin B antibiotics to determine the best dose for selection of transgenic cells. In the recent experiment, the hygromycin used was maintained at different levels to evaluate its effectiveness in selection of transformed cells. Data was recorded for surviving calli. Transformed calli were observed within 2-3 weeks of culture calli on selection media. All the three levels of Hygromycin (25mg/l, 50mg/l and 75mg/l) tested reveal significance difference in terms of Hygromycin resistance calli. It was observed that elevated dose of hygromycin (75mg/l) result showed completely died calli. This might suggest that calli were more responsive to hygromycin and increase in its concentration have negative effect on transformation efficiency. Lower dose of hygromycin (25 mg/l) may have showed fake positive result with the escape of untransformed cells. So, it was concluded that 50 mg/l hygromycin was the optimized conc. with less escape.

Table 4.4 Selection of transformed calli on hygromycin concentration

Concentration of hygromycin mg/l	Calli showing browning (%)	Percentage of transformed calli showing necrosis (%)	Percentage of transformed calli showing growth (%)	Response
25	23	3	62	**Well proliferation**
50	2	0	82	**Average proliferation**
75	0	95	0	**Calli completely died**

Total transformed calli cultured = 50

Fig 4.4 Calli showing necrosis

Fig 4.4 Calli showing browning

Fig 4.4 Calli showing growth

4.3.5. Effect of Cefotaxime on the growth of calli in rice (*Oryza sativa L.*)

Cefotaxime is a broad spectrum antibiotic effectively used to stop the growth of Agrobacterium in selection media during transformation of rice calli. Three different concentrations (250mg/l, 300mg/l and 500mg/l) of Cefotaxime have been used in this experiment to test its efficacy for controlling the bacterial explosion.

It was found that when calluses were subjected to concentration (300 mg/l) of bacteriostatic agent led to a significant increase in the transformation efficiency. At 300 mg/l concentration the *Agrobacterium* is fully controlled and no overgrowth is recorded throughout the experiment. When calluses were subjected to the lower concentration (250mg/l) of bacteriostatic agent led the growth of calli become normal and the concentration (500mg/l) the growth of calli become decrease. It is clear that increasing the concentration may lead to negative effect on calli growth result a refuse in transformation efficiency as well as Gus expression (Table. 4.5). Significant difference was observed among the three levels of Cefotaxime tested.

Fig 4.5 Effect of cefotaxime on the growth of calli in rice

Genotype	Growth of calli showed normal proliferation on cefotaxime	Average growth of calli proliferation on cefotaxime	Growth of calli decrease on cefotaxime	Mean of each variety
	250 mg/l	300mg/l	500mg/l	
Kashmir basmati	5.00±1.53	9.00±3.14	2.00±1.00	11.66
Pusa basmati	4.00±0.24	7.00±2.32	1.00±1.01	10.33
Punjab basmati	3.00±0.12	6.00±1.22	1.00±0.00	7.23
Basmati 385	2.00±0.10	5.00±0.11	1.00±1.00	4.65
Mean of each treatment	4.44 **	7.77 **	2.88**	

LSD 0.01 for variety = 3.223

LSD O. O1 for variety = 1.856

Fig 4.5 Normal growth of calli on cefotaxime conc.

Fig 4.5 Average growth of calli on cefotaxime conc.

Fig 4.5 Decreased growth of calli on cefotaxime conc.

CHAPTER 5

DISCUSSION

Discussion

The protocol Optimization for tissue culture is the pre-requisite for establisbing an efficient genetic transformation system. The present study was carried out on four basmati rice cultivars (Kashmir basmati, Pusa basmati, Punjab basmati and basmati 385) to develop an efficient tissue culture system. Basmati rice genotypes are mostly obstinate toward callus induction, gene transformation, GUS expression, and subsequent transformation.

5.1.1. Surface sterilization

Surface sterilization is a major factor limiting the contamination frequency in rice seeds cultured for callus induction. One step one reagent process of surface sterilization using commercially accessible chlorox containing 5% sodium hypochlorite bas been used for the rice seeds and the process is in close commitment with that of (Oyebanji*et al.*, 2009). They stated that two step processes were difficult and costly. Three different concentrations of chlorox have been used, among which 50% have been found to reduce contamination frequency with maximum callus induction potential in both rice genotypes. This result is in agreement with that of (Rashid *et al.*, 2005). Whereas increasing concentration of chlorox to 70% totally eliminated the contamination but it retards the growth of the seeds showing no callus induction.

5.1.2. Callus induction

Mature rice seed has long been used as an initial material for embryogeniccalli formation. Immature rice seeds are highly responsive to tissue culture system (Li *et al*, 1993) but are arduous work to execute and have seasonal limitations (Ge*et al.*, 2006). Mature rice seeds produce embryogeniccalli with fewer efficiency as compared to immature embryo but have some advantages of its eagerly availability throughout the year, easy storage and it's simple to handle procedure (Lee *et al*,2002;

Shahsavari*et al.*, 2010; Yan *et al.*, 2010). Embryogeniccalli have been produced by culturing mature rice seeds of the four local rice genotypes (Kashmir basmati, Punjab basmati,basmati 385 and pusa basmati). MS media supplemented with three different levels of growth regulators 2,4-D have been tested to induce calli. The growth regulator 2,4-D alone have been tested to be more successful for embryogeniccalli formation (Rashid *et al.*, 2001; Shahsavari*et al.*, 2010).

The two rice genotypes responded differently; 2 mg/l 2,4-D level in the media shows better frequency of callus induction for Kashmir basmati while 4mg/l have been found effective for pusabasmati as well as for Punjab basmati and basmati 385. Our result for callus induction are close harmony with the observations that 2,4-D level of 2mg/l better for callus initiation and growth (Tyagi*et al.*,2007; Karthikeyan*et al.*, 2009) but there are some reports suggesting that rice genotypes also shows better response to elevated level of 2,4-D (Niroula*et al.*, 2005; Islam *et al.*, 2005). There are genotypic differences toward callus induction response (En-hui*et al.*, 2004; Lin and Zhang 2005; Ge*et al.*, 2006)

5.1.3. Effect of growth regulator on callus induction

We bave used mature rice seeds as a starting material in our experiment. Using mature seed embryos as a starting material for *in vitro* callus formation have several benefits over others (khatun*et al.*, 2003 and Wijesekera *et al.*, 2007). Callus formation is largely dependent on genotype (Rashid *et al.*, 2003 and Wagiran*et al.*, 2008, Khatun*et al.*, 2010), form of media and growth hormones (Ge*et al.*, 2006; Lee *et al*, 2002 and Rueb*et al*, 1994). The difference practical for callus formation were significant among the four genotypes tested at 5% significant level. Our results are favoured by other researchers (khalequzzaman*et al.*, 2005, Abe *et al.*, 1985 and Khatun*et al.*, 2010) whose findings suggest that differences in genotype play a key role in callus induction response.

The growth regulator 2,4-D alone are vital for callus formation (Shahsavari*et al.*, 2010) and was reputable as being sufficient for development of embryogeniccalli and subsequent transformation (Geng*et al.*,2008). Three different 2,4-D levels has been checked for calli formation. Calli were induced at 2.5 mg/l in all the four varieties tested but showed better response at different 2,4-D level. Our resultsfavoured have demonstrated that Kashmir basmati,pusa basmati, Punjab basmati and basmati 385 have better response on 3.5 and 2.5 mg/l respectively. (Tyagi*et al.*, 2007, Ge*et al.*, 2006 and Khaleda and M.Al-Forkan, 2006) suggested that 2 mg/l have better response in callus induction which supports our results. (Sikder*et al.*, 2006) concluded from them conclude that 2,4-D (2.0 to 3.0 mg/l) was suitable for the formation of calli. The genotype Kashmir basmati showed better callus induction response (86.78%) at 3 mg/l 2,4-D which are opposing to other workers revealing genotypic differences.

5.1.4. Effect of light and dark conditions on callus induction and quality

Light and dark conditions were found to have no significant effect on callus induction frequency. These results are opposing with the research findings of other workers (Maneewan*et al.*, 2005;Thadavong*et al.*, 2002), whose findings exposed differences in callus induction frequency between the two conditions. These opposing results might arise because of the differences in genotype and culture media. Light and dark conditions showed significant effeet on callus quality which are in agreement with other workers (Maneewan*et al.*, 2005; Summart*et al.*, 2008). (Wanichananan*et al.*, 2010) finding suggested that under light condition, the calli were smaller in size, brown and compact, when compared with those in the dark conditions.

5.2. *Agrobacterium* mediated transformation

5.2.1. Acetosyringone level

Acetosyringone is a *vir* gene inducer normally employed in the transformation of monocots (Ozawa *et al*, 2009; Veluthambi*et al.*, 2003) because efficiency of tansformation is very low in the absence of this phenolic compound in rice transformation (Hiei*et al*, 1997; Ozawa *et al*, 2009). Various studies (Rashid *et al.*, 1996; Khanna and Raina, 1999; Gould, 1997; Shoukat*et al.*, 2007) have implicated the role of acetosyringone to *Agrobacterium* measures of co-cultivation. The main complexity was the fixed level of acetosyringone to utilize because transformation efficiency varies with different level of acetosyringone and their interaction with different genotypes. So we have used three different level of acetosyringone in the co-cultivation media (Table 4.1). Maximum transformation efficiency was observed by utilizing 200 µM acetosyringone for the genotypes Kashmir basmati (44%), pusa basmati (26.6%), (Punjab 22.20%), and (basmati 385 18.32%). Our results are in commitment with the research outcomes of Shoukat*et al.*, (2007), Bernal*et al.*, (2009) and Wagiran*et al.*, (2010) whereas opposing with that of Ozawa *et al*, (2009) and Terada *et al.*, (2004) who recommended that 200 µM acetosyringone is the best concentration for rice transformation but these variances might be due to the different co-cultivation conditions. Increasing the concentration to 300 µM results a decrease in transformation efficiency which are supported by other workers (Amoah*et al.*, 2001, Wagiran*et al.*, 2010, Shoukat*et al.*, 2007; Clercq*et al.*, 2002).

5.2.2. Infection time and co-cultivation period

Determining the optimum infection time and co-cultivation period is very important for maximum transformation efficiency. Increasing these time period may lead to colonization of Agrobacterium resulting in cell necrosis and death (Kumaria*et al.*, 2001; Kuta and Tripathi, 2005; Bernal*et al.*, 2009). Significant difference was found

between all treatment (infection time and co-cultivation period) tested at 5% probability level. Less infection time and co-cultivation period leads to low number of transformed calli because it does not ensure integration and working of transformed DNA. The Infection time of 10 minutes and 2 days co-cultivation period have maximum transformation efficiency (Kashmir basmati=45.33%,pusa basmati=24%, Punjab=12 %, and basmati 385=10 %) and Gus expression (Kashmir basmati=9.66, pusa basmati=7.33, Punjab=6.21, and basmati 385=5.00). Our results are uniform with Samiphak*et al.* (2006) who suggested that 15 minutes and 3 days' co-cultivation period have high transformation efficiency. The optimum co-cultivation period communicates closely with that of other workers (Yang *et al.*, 2000; Nishimura *et al.*, 2007; Wanicbananan*et al.*, 2010) who obtain most efficient expression of the introduced gene and yield the maximum transformation efficiency.

5.2.3. Co-cultivation with filter paper

Co-cultivation of rice calli on solid media results in browning because the calli were damaged by the rapid proliferation of *Agrobacterium* that might lead to decrease in transformation efficiency. So we have covered co-cultivation media with two filter papers and significance difference was observed in the browning and overgrowth frequency. These results are supported by other workers (Toki *et al.*, 1997; Kumar *et al.*, 2005; Ozawa *et al.*, 2009).

5.2.4. Selection of transformed calli(Hygromycin)

Hygromycinephosphotransferase (*hpt*) gene is used as selectable marker in plant genetic transformation experiment (Rashid *et al.*, 1996) because rice has no native resistance to this antibiotic (Christou and Ford, 1995). The 3 different levels of hygromycin B antibiotic have been employed in this experiment to select the transformed cells and the resultant Gus expression with significant differences observed among results. Hygromycin level of 50 mg/l was found suitable for the

selection process, providing more tough selection in which minimum calli were runaway whereas it has less negative effect on Gus expression. Increasing hygromycin concentration to 75 mg/l strongly reduced Gus expression capacities of rice calli. Our result is in close agreement with (Cheng *et al.*, 1998; Rafiqueet *al.*, 2010;Rachmawati*et al.*, 2006; En-hui*et al.*, 2004; Yookongkaew*et al*, 2006), who reported the presence of 50 mg/l hygromycin as suitable dose in their transformation experiment. Hygromycine level of 30-50 mg/L has been widely adopted for most of the transformation experiment using *hpt*as a selectable marker gene (Hiei and Komari, 2006, Toki *et al.*, 2006; Rachmawati*et al.*, 2004). The optimum level of antibiotic hygromycin B to select transgenic cells varied with plant genotypes and experimental assessment were made to determine this dose (Wagiran*et al.*, 2010). Hygromycin at 50 mg/l dose was used to select transformed cells of both *Indica* and *Javanica* rice cultivars in the first successful *Agrobacterium* transformation (Dong *et al.*, 1996; Rashid *et al.*, 1996).

5.2.5. Elimination of *Agrobacterium* (Cefotaxime)

Cefotaxime is an antibiotics used to destroy*Agrobacterium* after co-cultivation process. Three different level of cefotaxime (250 mg/l, 300 mg/l and 500 mg/l) have been used in the present experiment to avoid the proliferation of *Agrobacterium* in the selection medium. We have been successful in eliminating *Agrobacterium* at minimum level of 300 mg/l in selection media while increasing the concentration lead to reduced transformation and Gus expression efficiency. Qiu*et al.* (2001) also used 300 mg/l concentration for bacterial elimination.

Our results are contradictory with that of other workers (Asghar*et al.*, 2007;Rafiqueet *al.*, 2010) who used 1000 mg/l cefotaxime to inhibit bacterial proliferation whereas Rashid *et al.*, (1996) used 500 mg/l carbencillin to control bacterial growth. They have found that below this concentration have less negative effect on calli growth but control of *Agrobacterium* is difficult. Barrett *et al.* (1997) reported that levels of

antibiotics (carbenicillin and cefotaxime) higher than 500 mg/l would be necessary to get rid of *Agrobacterium*, but the problem of phytotoxicity arose. Therefore, they could not be used at higher concentrations.

5.3. Conclusion and Recommendations

The current research was conducted to established transformation system by evaluating different factors such as callus induction and Gus expression and few other transformation parameters for optimizing tissue culture in four varieties. Kashmir basmati, Punjab basmati, Pusa basmati and basmati 385 were checked and optimized for their callus induction frequency, organic supplement casein. Data analysis by using ANOVA (5%) showed difference in response toward all the four treatment tested. Highest callus induction formation frequency observed for Kashmir basmati 93.06%, Pusa basmati 93.2%Punjab87% and basmati 385 84%. The calli of basmati 385 were prone to browning, therefore casein hydrolysate was used at different concentration (0.3mg/l, 0.4mg/l, and 0.5mg/l) to reduce browning of calli. It was observed that 0.5mg/l concentration was best for reducing browning without damaging calli. Kashmir basmati (93.06%) and Pusa basmati (93.2%) showed better GUS expression frequency. Optimization of *Agrobacterium* mediated transformation protocol was performed for Kashmir basmati, Pusa basmati, Punjab basmati and basmati 385 only. Different parameters like optical Density, pH, Hygromycin and cefotaxime was optimized. Furthermore, the finding of this study can easily be applied for the improvement of other rice cultivars.

5.4. Future prospects

Productivity of rice is limited by number of biotic and abiotic stresses. These stresses can be controlled by introduction of novel gene through gene transformation technology. Once efficient and robust protocol for *in vitro* gene transformation by using reporter genes of rice would be established the transgenic plant production can be enhanced.

CHAPTER 6

REFERENCES

References

Anonymous (2006). Agricultural statistics of Pakistan (govr of Pakistan). Ministry of food, Agriculture and livestock (Economic using) Islamabad. P. 13-17

Ahmad M. Akhtar M. and Anwar M., (2005). Basmati rice, progress issues and prospects for Pakistan. In proceeding of the international seminar in rice crop. Oct 2-3 Rice Research Institute, Kala Shah kaku. P. 1-7

Amoah B.K. H. Wu C. Sparks and D.H. Jones. (2001). Factors influencing Agrobacterium-mediated transient expression of μidA in wheat inflorescence tissue. J. Exp. Bot. 52, p. 1135-1142

Amitabh Mohanty N.P. SarmaAkhilesh K. Tyagi. (1992) indica varieties are measured refractory to tissue culture and genetic manipulation. p. 127–137

Asghar A. H. Rashid M. Ashraf M.H. Khan and Z. Chaudhary. (2007). Improvement of basmati rice against fungal infection through gene transfer technology. Pak. J. Bot. 39(4), p. 1277-1283

Barrett C. E. Cobb R. McNicol and G. Lyon. (1997). A risk assessment study of plant genetic transformation using Agrobacterium and implications for analysis of transgenic plants. Plant Cell Tiss. Org. Cult. 47, p. 135-144

Bernal M.P. C. Hernandez M.T. Barcelo M. Delgado R. Armas. (2009). Quantitative transient GUS expression in J-104 rice calli through manipulation of in vitro culture conditions. Rev. Columb. Biotechnol. 11(2), p. 75-84

Binns A.N. and Thomashow M.F. (1988). Cell biology of Agrobacterium infection and transformation of plants. Annual Review of Microbiology 42, P. 575-606

Christou P. and T. Ford. (1995). Parameters influencing stable transformation of rice embryogenic tissue and recovery of transgenic plants using electric discharge particle acceleration. Ann. Bot. p. 407-413

Cheng X.Y. R. Sardana H. Kaplan and I. Altosaar. (1998). Agrobacterium-transformed rice plants expressing synthetic cryIA(c) gene are highly toxic to striped stem borer and yellow stem borer. Proc. Nat. Acad. Sci. USA. 95, p. 2767-2772

Cheng X.Y. Sardana R. Kaplan H. Altosaar I. (1998). Agrobacterium-trasformed rice plants expressing synthetic cryIA (c) gene are highly toxic to stripped stem borer and yellow stem borer. Proc Nat AcadSci USA 95, P. 2767-2772

Cheng M. Fry J.E. Pang S. Zhou H. Hironaka C.M. Duncan D.R. Conner T.W. Wan Y. (1997). Genetic transformation of wheat mediated by Agrobacterium tumefaciens. Plant Physiol. 115(3), P. 971-980

Datta K. Potrycus I. Dutta S.K. (1992). Efficient fertile plant regeneration from protoplasts of the indica rice breeding line IR-72 (Oryza sativa L.) variety. Aust J Agric Res. 51, P. 305-312

Dong J., Teng W., Buchholz W.G and Hall T.C (1996). Agrobacterium mediated transformation of Javanica rice. Mol breeding 2, p. 267-276

En-hui C. Z. Ping Z. Shi-min L. Ai-hong, Z. Ya-fang C. Zong-xiang and P. Xue-biao. (2004). System of rice (Oryza sativa L.).Plant Sci. 176(4), P. 522-527

Ge X. Z. Chu, Y. Lin and S. Wang. (2006). A tissue culture system for different germplasms of indica rice. Plant Cell Rep. (Cell Biol. and Morphogenesis). 25, p. 392-402

Grover A. Gowthaman R. (2003). Strategies for development of fungus-resistant transgenic plants. CurrSci 84, P. 330–340

Ge X. Z. Chu Y. Lin and S. Wang. (2006). A tissue culture system for different germplasms of indica rice. Plant Cell Rep. (Cell Biol. and Morphogenesis). 25, p. 392-402

Hiei Y. and Komari T., (2006). Improved protocol for transformation of rice mediated by Agrobacterium tumefaciens. Plant cell-tissue and organ-culture. 85, p. 271-283

Hoque N. M. A. Azdi Z.A. and prodhan S. H. (2013). Development of callus initiation and regeneration system of different indigenous indica rice varieties. Journal of botany, 01(2), p. 46-51

Hao Z.N. Wang J. Wang L.P. Tao R.X. (2009). Influences of the disease resistance conferred by the individual transgenes, Pi-d2, Pi-d3 and Xa21, on the transgenic rice plants in yield and grain quality. Afr. J. Biotechnol. 8, P. 4845–4848

Hiei Y. Ohta S. Komari T. Kumashiro T. (1994). Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J. 6(2), P. 271-282

Ignacimuthu S. &Raveendar S. Euphytica. (2011) studied the rice leaffolder (RLF), Cnaphalocrocismedinalis is a vital pest of rice that causes cruel damage in numerous areas of the world. p. 277–286

Ian S. Curtis (Ed) (2004). Transgenic crop of the world. Essential protocol. Springer science and Business media

IRRI (International Rice Research Institute). (1997). Rice almanac. 2, p. 3-5

Islam A. (2006). Fungus resistant transgenic plants: strategies, progress and lessons learnt. Plant Tissue Cult Biotechnol 16, P. 117–138

Islam M.M. Khalekuzzaman M. (2015). Development of Transgenic Rice (Oryza sativa L.). Plant Using Cadmium Tolerance Gene (YCFI) through Agrobacterium Mediated Transformation for Phytoremediation. Asian J. Agric. Res. 9(4), P. 139-154

Islam M.M. M. Ahmed and D. Mahaldar. (2005). In vitro callus induction and plant regeneration in seed explants of rice (Oryza sativa L.). Res. J. Agric. Biol. Sci. 1(1), p. 72-75

Jefferson R.A., (1987). Assaying chimeric genes in plants. The GUS gene fusion system, plant Mol. Biol. Rep. 5, p. 387-405

Jia Y.S.A. McAdams G.T. Bryan H.P. Hershey and B. Valent., (2000). Direct interaction of resistance genes and avirulence gene products confers rice beat resistance. Embo J., 19, P. 4004-4014

Jefferson R.A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant MolBiol Rep. 5, p. 387-405

Karthikeyan A. Shilpha. J. KaruthaPandian S. (2012) a greatly efficient protocol for Agrobacterium tumefaciens-mediated transformation of indica rice (Oryza sativa L. Plant Cell Tiss Organ Cult. p. 153–165

Komari T. Hiei Y. Saito Y. Murai N. and Kumashiro T. Plant J. 1996, 10, 165–174

Karthikeyan A. S.T.K. Pandian and M. Ramesh. (2009). High frequency plant regeneration from embryogenic callus of a popular indica rice (Oryza sativa L.). Physiol. Mol. Biol. Plants. 15(4), p. 391-399

Khush G.S. Virk P.S. (2000). Rice breeding: Achievements and future strategies. Crop Improv. 27, P. 115-144

Khanna H.K. and S.K. Raina. (1999). Agrobacterium-mediated transformation of indica rice cultivars using hinary and superbinary vectors. Aust. J. of Plant Physiol. 26, p. 311-324

Kant P. Kant S. Jain R.K. and Chaudhury V.K. (2007). Agrobacterium mediated transformation was done in rice (Oryza sativa L. varindica). Biol Plant. 51 (1), p. 61-68

Kumaria R. B. Waie and M.V. Rajam. (2001). Plant regeneration from transformed embryogenic callus of elite indica rice via Agrobacterium. Plant Cell Tiss. Org. Cult. 67, p. 63-71

Kuta D.D. and L. Tripathi. (2005). Agrobacterium-induced hypersensitive necrotic reaction in plant cells: a resistance response against Agrobacterium-mediated DNA transfer. Afr. J. Biotechnol. 4(8), p. 752-757

Khaleda L. and M. Al-Forkan. (2006). Genotypic variability in callus induction and plant regeneration through somatic embryogenesis of five deepwater rice (Oryza sativa L.) cultivars of Bangladesh. Afr. J. Biotechnol. 5(16), p.1435-1440

Lin Y.J. and Q. Jhang. (2005). Optimising tissue culture conditions for bigh efficiency transformation of indica rice. Plant Cell Rep. 23, p. 540-547

Larkin P.J. Scrowcroft W.R. (1981). Somaclonal variation a novel source of variability from cell culture for plant improvement.The or Appl Genet. 60, P. 197-214

Li L. R. Qu A. Dekochko C. Fauquet and R.N. Beachy. (1993). An improved rice transformation system using the biolistic method. Plant Cell Rep. 12, p. 250-255.

Lee K. H. Jeon and M.Y. Kim. (2002). Optimization of mature embryo-based in vitro culture system for high frequency somatic embryogenic callus induction and plant regeneration from Japonica rice cultivars. Plant Cell Tiss. Org. Cult. 71, p. 237-244

Muhammad I. Zia M. A. Ali S. Roomi S. Iqbal A. Abbas Z. and Ali M.G. (2014). Development of efficient regeneration system in different recalcitrant rice cultivars and expression analysis of putative transgenic plants. International journal of agriculture and Biology, 16(4), p. 701-706

Niroula R.K. B.P. Sah, H.P. Bimb and S. Nayak. (2005). Effect of genotype and culture media on callus induction and plant regeneration from matured rice grain culture. J. Inst. Agric. Anim. Sci. 26, p. 21-26

Naqvi S. M. S., Sultana R. and Rasheed H., (2005). Tissue Culture Studies in Oryza Sativa L. Cvs. Basmati 385 and Super Basmati. Pakistan Journal of Botany, 37(4), p. 823-828

Naqi S.M.S. Sultana R. and Rasheed H., (2005). Tissue culture studies in Oryza sativa L. CVS. Basmati 385 and super Basmati. Pakistan general of botany. 37, p. 823-828

Nester E.W. Gordon M.P. Amasino R.M. and Yanofsky M.F. (1984). Crown gall: a molecular and physiological analysis. Annual Review of Plant Physiology 35, P. 387-413

Nishimura A. Aichi I. Matsuoka M. (2007). A protocol for Agrobacterium mediated transformation in rice. Nat. Prot. 1, P. 2796-2802

Ozawa K. (2009). Establishment of a high efficiency Agrobacterium-mediated transformation system of rice (Oryza sativa L.). Plant Sci. 176, p. 522–527

Oka (1988). Origin of cultivated rice developments in crop science 4. Japan scientific societies press, Tokyo and Elsevier, Amsterdum. P. 254

Rao S.S., Lippincott B.B. and Lippincott J.A., (1882). Agrobacterium adherence involves the pectin protein of the cell wall and its sensitivity to the degree of pectin methylation. physiolSplant 56, p. 374-380

Rshid H., Yokoi S., Toriyama K. and Hinata K., (1996). Transgenic plant production mediated by Agrobacterium in indica rice plant cell Rep. 15, p. 727-730

Rommens C.M Kishore G.M. (2000). Exploiting the full potential of disease-resistance genes for agricultural use CurrOpinBiotechnol. 11, p. 120–125

Rashid H. Yokoi S. Toriyama K. and Hinata K. (1996). Transgenic plant production Mediated by Agrobacterium in indica rice. Plant Cell Reports, 15, P. 727-730

Revathi S. and pillai M.A. (2004). The rice leaf blast pathogen undergoes developmental processes typiOyebanji O. B. O. Nweke O. Odebunmi N.B. Galadima M.S. Idris U.N. Nnodi A.S. Afolabi and G.H. Ogbadu. 2009. Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds. Afr. J. Biotechnol. 8(20): 5395-5399. Cal of root-infection fungi. Nature, 431, p. 582-586

Rao A.M. Kumar I.S. and Kishdor P. B. K. (2012). Effect of growth regulators and physiological Gradients on the High Frequency plant regeneration from the long-term callus cultivars of different germplasms of rice (Oryza sativa L.) Journal of phytology 4(2), p. 06-15

Rachmawati D. H. Anzai. (2006). Studies on callus induction, plant regeneration and transformation of Javanica rice cultivars. Plant Biotechnol. 23, p. 521-524

Rafique M.Z. M. Zia H. Rashid M.F. Chaudhary and Z. Chaudhry. (2010). Comparison of transgenic plant production for bacterial blight resistance in Pakistani local rice (Oryza sativa L.) cultivars. Afr. J. of Biotechnol. 9(13), p. 1892-1904

Rashid H. S.Y. A. Bokhari and A. Quraishi. (2001). Callus induction, regeneration and hygromycin selection of rice (Super Basmati). OnLine J. Biol. Sci. 1, p. 1145-1146

Samiphak K. and N. Siwarungson. (2006). Embryogenichva 1 Gene in Indica Rice KDML 105 by Agrobacterium tumefaciens EHA 105. J. Sci. Res. 31(1), p. 331-339

Shahsavari E., Maheran A.A., Sitinorakmar A. and Hanafi M.M., (2010). The effect of plant growth regulators on optimization of tissue culture system in Malaysian upland rice. African Journal of Biotechnology, 9, p. 2089-2094

Sujatha S. and Sonti R.V., (2005). Growth deficiency of a XanthomonasOryzaepv. Oryzae fur mutant in rice leaves is rescued by ascorbic acid supplementation. Molecular Plant-Microbe Interactions, 18, p. 644-651

Sanford J.C., (1988). The Biolistic process, trends Biolchemica. 6, p. 299-302

Sivamani E., shen P., Opalka N., Beachy R.N. and Fauquet C.M., (1996) selection of large quantities of embryonic calli from indica rice seeds for production of fertile transgenic plants using the biolistic method. Plant cell report. 15, p. 322-327

Singh R.K., Singh U.S. and Khush G.S., (2006). Aromatic Rice. International Rice Research institute

Smith E.F. and Towsend C.O. (1907). A plant tumor of bacterial origin. Science 25 P. 671-673

Sasaki T. (2005). The maped base sequence of the rice genome. Nature 436, P. 793-800

Skamnioti P. Gurr S.J. (2009). Against the grain: safeguarding rice from rice blast disease. Trends Biotechnol. 27(3), P. 141-150

Shahsavari E. A.A. Maheran A.S.N. Akmar and M.M. Hanafi. (2010). The effect of plant growth regulators on optimization of tissue culture system in Malaysian upland rice. Afr. J. Biotechnol. 9(14), p. 2089-2094

Toki S. (1997). Rapid and efficient Agrobacterium-mediated transformation in rice. Plant Mol. Biol. Rep. 15, p. 16–21

Toriyama k. and Hinata k., (1985). Cell suspension and protoplast culture in rice plant sci, 41, p. 179-183

Tripathi R.M. Bisht H.S and Singh R.P. (2010) an efficient and reproducible protocol for Agrobacterium- mediated transformation of an agronomically useful Oryza sativa variety, Pusa Basmati- 1 (IET- 10364) has been established. International Journal of Pharma and BioSciences. p. 0975-6299

Tyagi H. S. Rajasubramaniam and I. Dasgupta. (2007). Regeneration and Agrobacterium-mediated transformation of a popular indica rice variety ADT 39. Curr. Sci. 93(10), p. 678-683

Tyagi H. S. Rajasubramaniam and I. Dasgupta. (2007). Regeneration and Agrobacterium-mediated transformation of a popular indica rice variety ADT 39. Curr. Sci. 93(10), p. 678-683

Vasil I.K. (1983). Isolation and culture of protoplasts of grasses. Int. Rev. Cytol. Suppl. 16, P. 79-88

Veluthambi K. A. K. Gupta and A. Sharma. (2003). The current status of plant transformation Technologies. Curr. Sci. 84(3), p. 368–380

Van der Biezen E.A. (2001). Quest for antimicrobial genes to engineer disease-resistant crops. Trends Plant Sci 6, P. 89–91

Wagiran A. I. Ismail C.R.C.M. Zain and R. Abdullah. (2010). Agrobacterium tumefaciens-mediated transformation of the isopentenyltransferase gene in japonica rice suspension cell culture. Aust. J. crop Sci. 4(6), p. 421-429

Wanichananan P. T. Teerakathiti, S. Roytrakul C. Kirdmanee and S. Peyachoknagul. (2010). A highly efficient method for Agrobacterium mediated transformation in elite rice varieties (Oryza sativa L. spp. indica). Afr. J. of Biotechnol. 9 (34), p. 5488-5495

Wagiran A. I. Ismail C.R.C.M. Zain and R. Abdullah. (2010). Agrobacterium tumefaciens-mediated transformation of the isopentenyltransferase gene in japonica rice suspension cell culture. Aust. J. crop Sci. 4(6), p. 421-429

Xu X. Liu X. Ge S. Jensen J.D. Hu F. Li X. Dong Y Gutenkunst R.N. Fang L. Huang L. Li J. He W Zhang G. Zheng X. Zhang F. Li Y. Yu C. Kristiansen K. Zhang X. Wang J. Wright M. McCouch S. Nielsen R. Wang J. Wang W. (2012). Resequencing 50 accessions of cultivated and wild rice yields markers for identifying agronomically important genes. Nat. Biotechnol. 30, P. 105-111

Yookongkaew N. M. Srivatanaku and J. Narangajavana. (2006). Development of genotype-independent regeneration system for transformation of rice (Oryza sativa ssp. indica). J. Plant Res. 120(2), p. 237-245

Yoder J. I. and Goldsbrough A. P. Bio/Technology, (1994), 12, P. 263–267

Yan L. X. Li and D. Wu. (2010). The comparison in tissue culture ability of mature embryo in different cultivars of rice. Agri. Sci. inChina. 9(6), p. 840-846

Yang G. A. Morita Y. Matsubayashi K. Nakamura and Y. Sakagami. (2000). A rapid and efficient system of Agrobacterium infection mediated transient gene expression in rice Oc cells and its application for analysis of the expression and antisense suppression of preprophytosulfokine, a precursor of phytosulfokine, encoded by OsPSK gene heping Plant cell physiol. 41(6), p. 811-816

Zahid M.A. and Akhtar M., Sabir., M., Anwar M. and Jamal A., (2005). Genotype and phenotypic correlation and path analysis in coarse grain rice. Proceeding of the international seminar in rice crop. Oct 2-3, 2005. Rice research institute, Kala shah kaku. P 29

Zaid M.A. Narayanan M. Sardana R. Taga I. Postel S. Johans R. McNulty M. Mottiar Y. Mao J. Loit E. and Altorsaar I. (2006). Optimized tissue culture media for efficient transformation of different indica rice genotypes.Agronomy Research 4(2), p. 563-567