

**AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION OF
EGGPLANT (SOLANUM MELONGENA L.)**



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NOVEMBER 2011

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OF EGGPLANT (SOLANUM MELONGENA L.)**

A Thesis By

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Examination Roll No: MS-090701

Session: 2008-2009

Submitted To

**Biotechnology and Genetic Engineering Discipline, Life Science School,
Khulna University, Khulna-9208, Bangladesh,**

**In partial fulfillment of the requirement for the degree of Masters of
Science (MS) in Biotechnology and Genetic Engineering**

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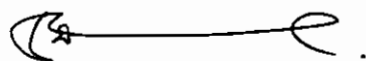
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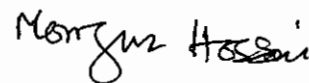


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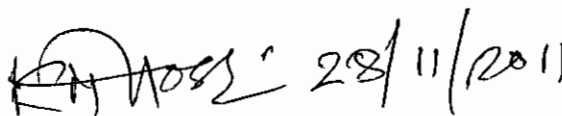


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CERTIFICATE

I do hereby certify that *ASIF AHAMED* has been working on his thesis entitled *AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION OF EGGPLANT (SOLANUM MELONGENA L.)* under my supervision, which is the record of bonafide research carried out at the Plant Breeding and Gene Engineering Lab., Dept. of Botany, University of Rajshahi, Rajshahi, Bangladesh. The results of the investigation, which are embodied are original and have not been submitted before in substance for any degree or diploma. He has fulfilled all the requirements of regulations relating to the nature and prescribed period of research for submission of thesis for the partial fulfillment of MS degree.

Date.....

(Professor Dr. M. Monzur Hossain)

DECLARATION

This thesis report is the original research work of the author, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/ will not be published anywhere without the concurrence of the concerned supervisor and co-supervisor.

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November 2011

DEDICATED
TO
MY BELOVED FAMILY
MEMBERS

Acknowledgement

At first, I would like to express my greatest and deepest gratitude to the Almighty Allah, to Whom all praises go for enabling me to complete this work. Guidance, help and co-operation have been received from several persons and authority during the tenure of the study, the author is immensely grateful to all of them. Although it is not possible to mention everyone by name, it will be an act of ungratefulness if some names are not mentioned here.

I would like to express my deep and sincere gratitude and profound indebtedness to my respected thesis supervisor **Prof. Dr. M. Monzur Hossain**, Department of Botany University of Rajshahi, Bangladesh for his continuous supervision, support and encouragement in every aspect from the very beginning to the completion of this research work.

I would like to express my sincere gratefulness to my teachers and supervisor **Md. Shamim Akhter**, Assistant Professor, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna-9208 for his kind co-operation, valuable suggestions and encouragement through out this research work. I am also grateful to **Mr. Rezaul Karim**, Lecturer Department of Botany, University of Rajshahi, for his valuable advice and technical support during the work.

Sincere appreciations and thanks to **Shaifur, Sayeem, Nasir and Mili** for their encouragement and help during this research work. I would also like to thank all the members of Plant Breeding and Gene Engineering Lab., Department of Botany, University of Rajshahi, Bangladesh.

Finally, I am very much grateful to my family members for their immeasurable blessing, continuous inspiration and uninterrupted support throughout this study.

The Author

ABSTRACT

Abstract: An efficient variety-independent method for producing transgenic eggplants (*Solanum melongena* L.) via *Agrobacterium tumefaciens*-mediated genetic transformation was developed. Two weeks old cotyledon explants were transformed by co-cultivation with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pVDH65 carrying the reporter gene β -glucuronidase intron (*GUS*-INT) and the marker gene neomycin phosphotransferase (*NPTII*). Putative transformed explants were cultured MS basal medium supplemented with different concentration of growth regulators and antibiotics. The best results were obtained when the explants were onto MS medium containing 0.5 mg/l NAA and 0.5 mg/l BA, 50 mg/l kanamycin, 100 mg/l cefotaxime and 25 mg/l acetosyringone. The putative transgenic shoot buds were allowed to elongate on the selection medium for subsequent histochemical *GUS* assay and PCR analysis. The presence of the *GUS* and *NPTII* genes were confirmed through *GUS* assay and PCR analyses.

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ABBREVIATIONS

The following abbreviations have been used throughout the text:

μ	: Micron
μ M	: Micro mole
cm	: Centimeter
0.1N	: 0.1 Normal
etc.	: Etcetera = And others
e.g.	: Exempli gratia, for example
<i>et al.</i>	: et alile = Other people
%	: Percentage
Fig.	: Figure
<i>GUS</i>	: β -glucuronidase
m	: Meter
mg	: Milligram
mg/l	: Milligram per liter
ml	: Milli-liter
min	: Minute
Kin	: 6-furfuryl amino purine
IAA	: Indol acetic acid
NaOH	: Sodium hydroxide
2,4-D	: 2,4-dichlorophenoxy acetic acid
BAP	: 6-benzyl amino purine
MS	: Murashige and Skoog (1962) medium
Na ₂ -EDTA	: Sodium salt of ferric ethylene diamine tetra acetate
NAA	: α -naphthalene acetic acid
No.	: Number
pH	: Negative logarithm of hydrogen ion (H ⁺) concentration
Sec.	: Second
gm	: Gram
<i>Nco</i> I	: <i>Nocardia corallina</i>
bp	: Base pair
<i>nos</i>	: Nopaline synthase
CaMv	: Cauliflower mosaic virus
<i>Eco</i> RI	: <i>Escherichia coli</i> RY13
<i>nptII</i>	: Neomycine phosphotransferase II
V/v	: Volume by volume
HgCl ₂	: Mercuric chloride
L.	: Linnaeus (Carolus Linnaeus)
T-DNA	: Transfer DNA
i.e.	: Id est = that is

mm	: Millimeter
nm	: Nanno-meter
<i>Viz.</i>	: Videlicet = namely
KOH	: Potassium hydroxide
rpm	: Rotation per minute
LB	: Luria-Borth
sp.	: Species
HCl	: Hydrochloric acid
⁰ C	: Degree Celsius

CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

For millennia, cultivators have selectively bred plants in an attempt to induce or enhance a desired trait. Early farmer who settled the banks of the Euphrates learned to better their crops by selecting specific seeds for their harvest for use in the next season planting. Although this was an effective practice, the process usually took hundreds of years and strictly limited to the alleles present in the same species, or closely related species populations. This need to control and predict the amount of plant food has been a fundamental motivator in developing new technologies in plant science. Since the advent of recombinant DNA technology in the 1970's, the recent years have shown remarkable advances in plant genetics and the rise of plant transformation. These new advances both biological and mechanical have given researchers the ability to express foreign genes, in desired plant hosts, essentially breaking the species barriers. This powerful technology of great importance to many aspects of human society. It has opened the door to molecular farming, a novel system of genetics research and new crops adapted to our civilization.

The ability of vastly increasing population may very well rest in the hand of plant geneticists. Currently, the practical success of plant genetic manipulation can be seen in agricultural products such as Bt cotton and Bt brinjal, resistant to insects, the NewleafTM potato which express a natural insecticide and the FlavrSavr tomato whose ripening is inhibited until exposure to ethylene. With many more products are on the way, it is clear that plant transformation will continue to have a profound impact on plant genetics and global agricultural practices. Plant transformation has been defined as the stable incorporation and expression of foreign genes into host plant. In 1983, Robert Fraley and colleagues first reported the successful incorporation of kanamycin resistance genes into the tobacco genome. Within a year other researchers reported similar accomplishments, making the beginning of plant transformation. Since then, successful transformations have been described for over 120 species in 35 different families (Birch 1997) and the number is increasing every year.

The process of plant transformation is currently a long complex multistage procedure which involving three general phases. Phase-I involves the selection and application of delivery system which incorporate the genes of interests into variable host cells. Phase-II involves integration, expression and selection of inserted DNA segment indicating proper integration and expression of the gene products. Finally, Phase-III involves the recovery of viable transgenic plants.

In true sense, the plant transformation celebrates the combination of several plant biochemicals, physiological and genetic technologies. Currently, plant transformation technologies come in many different forms and are a long and expensive process. However, continued research is devoted to improving these techniques and in time it will become more available and affordable. It is clear that the implications of this advancement are and will become an integral part of our society. With this new technology come new responsibilities to produce and release transgenic plants in safe and responsible manner. Finally as the production of transgenic is now a reality, scientist must extend their knowledge beyond the laboratory and learn to deal with the economic, social, religious and legal implication of plant transformation.

1.1. GENERAL ACCOUNT OF THE TARGET PLANT

Brinjal or eggplant (*Solanum melongena* L.) is an important solanaceous crop of subtropics and tropics. The name brinjal is popular in Indian subcontinents and is derived from Arabic and Sanskrit whereas the name eggplant has been derived from the shape of the fruit of some varieties, which are white and resemble in shape to chicken eggs. It is also called aubergine (French word) in Europe.

The brinjal is of much importance in the warm areas of Far East, being grown extensively in India, Bangladesh, Pakistan, China and the Philippines. It is also popular in Egypt, France, Italy and United States. In India, it is one of the most common, popular and principal vegetable crops grown throughout the country except higher altitudes. It is a versatile crop adapted to different agro-climatic regions and can be grown throughout the year. It is a perennial but grown commercially as an annual crop. A number of cultivars are grown in India, consumer preference being dependent upon fruit color, size and shape.

The varieties of *Solanum melongena* L. display a wide range of fruit shapes and colors, ranging from oval or egg-shaped to long club-shaped; and from white, yellow, green through degrees of purple pigmentation to almost black. Most of the commercially important varieties have been selected from the long established types of the tropical India and China. Brinjal fruit (unripe) is primarily consumed as cooked vegetable in various ways and dried shoots are used as fuel in rural areas. It is low in calories and fats, contains mostly water, some protein, fiber and carbohydrates. It is a good source of minerals and vitamins and is rich in total water soluble sugars, free reducing sugars, amide proteins among other nutrients.

1.1.1. Geographic Origin and Distribution

The plant is native to India. It has been cultivated in southern and eastern Asia since prehistory, but appears to have become known to the Western world no earlier than ca. 1500. Brinjal is considered a native to India where the major domestication of large fruited cultivars occurred. In "Origin of cultivated plants" published in 1886 De Candolle, stated that the species *S. melongena* has been known in India from ancient times and regarded it as a native of Asia. Vavilov (1928) was of the opinion that its centre of origin was in the Indo-Burma region.

Various forms, colours and shapes of brinjal are found throughout South-East Asia, suggesting that this area is an important centre of variation. A centre of diversity is believed to be in the region of Bangladesh and Myanmar (Former India-Burma border). Evidence to this was given by Isshiki et al (1994) based on the isoenzyme and morphological variation noticed in large germplasm collection from India.

According to Zeven and Zhukovsky (1975), it originated in India but spread eastward and by the 5th century B.C. was in China, which became a secondary centre of variation. Thus, it has been known for the last 1500 years in China. Arabic traders were responsible for subsequent movement to Africa and Spain. Brinjal cultivation in the Mediterranean region is relatively recent. Portuguese colonies took it to Brazil. It is now widely cultivated for its fruits in the tropical, subtropical and warm temperate zones, especially in Southern Europe and the Southern United States. Sampson (1936) suggested the African origin of this crop, but there is no evidence that *S. melongena* is native there though there are spiny African brinjal plants.

1.1.2. Botanical Aspect

Brinjal belongs to the family *Solanaceae* and is known under the botanical name *Solanum melongena* L. The family contains 75 genera and over 2000 species, out of which, about 150-200 are tuber bearing and belong to section *Tuberarium*. The majority of species (about 1800) are non tuber bearing. Cytological studies have indicated that basic chromosomal number $2n = 24$ is same in almost all the varieties and species.

There are 3 main botanical varieties under the species *melongena* (Choudhury, 1976a). The common brinjal, to which large, round or eggshaped fruited forms belong, are grouped under var. *esculentum*. The long, slender types are included under var. *serpentinum* and the dwarf brinjal plants are put under var. *depressum*.

Scientific classification

Kingdom: Plantae

Class: Magnoliopsida

Sub class: Asterids

Order: Solanales

Family: Solanaceae

Genus: *Solanum*

Species: *S. melongena* L

1.1.3. Agronomy of Brinjal

In tropical and subtropical climates, eggplant can be sown directly into the garden. Eggplant grown in temperate climates fares better when transplanted into the garden after all danger of frost is passed. Seeds are typically started eight to ten weeks prior to the anticipated frost-free date. Many pests and diseases which afflict other solanaceous plants, such as tomato, pepper (*capsicum*), and potato, are also troublesome to eggplants. For this reason, it should not be planted in areas previously occupied by its close relatives.

Four years should separate successive crops of eggplants. Common North American pests include the potato beetles, flea beetles, aphids, and spider mites. (Adults can be removed by hand, though flea beetles can be especially difficult to control.) Good sanitation and crop rotation practices are extremely important for controlling fungal disease, the most serious of which is *Verticillium*.

Spacing should be 45 cm (18 in.) to 60 cm (24 in.) between plants, depending on cultivar, and 60 cm to 90 cm (24 to 36 in.) between rows, depending on the type of cultivation equipment being used. Mulching will help conserve moisture and prevent weeds and fungal diseases. The flowers are relatively unattractive to bees and the first blossoms often do not set fruit. Hand pollination will improve the set of the first blossoms. Fruits are typically cut from the vine just above the calyx owing to the somewhat woody stems. Flowers are complete, containing both female and male structures, and may be self pollinated or cross pollinated.

1.1.4. Genetic Basis of Brinjal

Solanum is a very large genus. It includes both tuberous and non tuberous group of species. The chromosome number of many species under non tuberous group is fairly stable as $2x = n = 24$. There are 38 asian species which include 22 Indian species. There is a group of 5 related ones, namely *S. melongena* L., *S. incanum* L. (often considered synonymous to *S. coagulans* L.), *S. xanthocarpum*, *S. indicum* L. and *S. maccani*. (Choudhury, 1976b).

The species of *S. melongena*, *S. aethiopicum* and *S. macrocarpon* are interfertile with their respective wild ancestors (Lester and Niakan, 1986; Hasan, 1989; Daunay *et al.*, 1991). For each of these, the wild ancestor constitutes the primary gene pool according to Harlan and de Wet (1971). In nature, the morphological continuum observed between the primitive forms of each cultivated species, its semi-cultivated forms and the wild species indicates spontaneous gene exchanges. However, these exchanges, which are frequent between primitive forms of each cultivated species and wild species, are much less frequent between advanced forms and wild species.

As regards affinities and origin of *S. melongena* L., hybridization experiments between *S. melongena* and other species of *Solanum* have been performed only in few cases and definite conclusions are not available. Efforts have been initiated for studying evolutionary relationship and discerning varieties within crop germplasm (Doganlar Sami, 2002 and Singh *et al*, 2006)

1.1.5. Importance of Brinjal

It has been reported that on an average, the oblong-fruited eggplant cultivars are rich in total soluble sugars, whereas the long-fruited cultivars contain a higher content of free reducing sugars, anthocyanin, phenols, glycoalkaloids (such as solasodine), dry matter, and amide proteins (Bajaj *et al.*, 1979). A high anthocyanin content and a low glycoalkaloid content are considered essential, regardless of how the fruit is to be used. For processing purposes, the fruit should have a high dry matter content and a low level of phenolics. Bitterness in eggplant is due to the presence of glycoalkaloids which are of wide occurrence in plants of *Solanaceae* family. The glycoalkaloid contents in the Indian commercial cultivars vary from 0.37 mg/100 g fresh weight to 4.83 mg (Bajaj *et al.*, 1981). Generally, the high content of glycoalkaloids (20mg/100 g fresh weight) produce a bitter taste and off flavor. The discoloration in eggplant fruit is attributed to high polyphenol oxidase activity. The cultivars which are least susceptible to discoloration are considered suitable for processing purposes.

Brinjal is known to have ayurvedic medicinal properties and is good for diabetic patients. It has also been recommended as an excellent remedy for those suffering from liver complaints (Shukla and Naik 1993). Studies of the Institute of Biology of São Paulo State University, Brazil, have shown eggplant is effective in the treatment of high blood cholesterol. It helps to block the formation of free radicals and is also a source of folic acid and potassium. Eggplant is richer in nicotine than any other edible plant, with a concentration of 100 ng/g (or 0.01 mg/100g). However, the amount of nicotine from eggplant or any other food is negligible compared to passive smoking. On average, 20 lbs (9 kg) of eggplant contains about the same amount of nicotine as a cigarette.

Brinjal has been cultivated in India for the last 4,000 years, although it is often thought of as a Mediterranean or mid-Eastern vegetable. The global area under brinjal cultivation has been estimated at 1.85 million ha with total production of brinjal fruit of about 32 million MTs (FAO data, 2005, <http://faostat.fao.org/>). India accounts for about 8.7 million MTs with an area of 0.53 million hectares under cultivation. Brinjal is also exported in the fresh or frozen form. In 2007-08, 34 million kg worth of Rs. 19 million was exported mainly to UK, Netherland, Saudi Arabia and Middle East countries (DGCIS, 2008)

Table 1-The nutritional composition of edible portion of brinjal

Calories	24.0	Sodium (mg)	3.0
Moisture content (%)	92.7	Copper (mg)	0.12
Carbohydrates (%)	4.0	Potassium (mg)	2.0
Protein (g)	1.4	Sulphur (mg)	44.0
Fat (g)	0.3	Chlorine (mg)	52.0
Fiber (g)	1.3	Vitamin A (I.U.)	124.0
Oxalic acid (mg)	18.0	Folic Acid (µg)	34.0
Calcium (mg)	18.0	Thiamine (mg)	0.04
Magnesium (mg)	15.0	Riboflavin (mg)	0.11
Phosphorus (mg)	47.0	B-carotene (µg)	0.74
Iron (mg)	0.38	Vitamin C (mg)	12.0
Zinc (mg)	0.22	Amino Acids	0.22

Source: National Institute of Nutrition, 2007

1.2. RATIONALE AND OBJECTIVE OF THE PRESENT INVESTIGATION

Brinjal (*Solanum melongena* L.) one of the most ergonomically and economically important plants and it is the most productive, common, and multiuse horticultural vegetable crop. In the present investigation *Agrobacterium* mediated transformation of Brinjal was carried out to establish an efficient transformation protocol.

Bangladesh is an agricultural country where brinjal grows in a large scale and fulfill a substantial amount vegetable demand. For the importance of brinjal in our agriculture and national economy it was selected for the present study to standardize a protocol for *Agrobacterium* mediated genetic transformation. The use of recombinant DNA techniques holds the promise to increase the productivity and quality improvement of brinjal in a wide range. So the wide experimentation on brinjal transformation may give rise new opportunity for production of new varieties with desirable traits. Therefore, the transgenic brinjal may be used in human diet to treat diabetic and high blood cholesterol patients, as an excellent remedy for those suffering from liver complaints (Shukla and Naik 1993), to prevent free radicals formation and can also be used as a source of folic acid and potassium.

The aim of the present study is to evaluate the feasibility of the production of the transgenic brinjal plants through *Agrobacterium* mediated transformation process. The specific objective is to study the different parameters in the development of transgenic brinjal plants.

1.2.1. Objectives

In the present investigation *Agrobacterium* mediated transformation of Brinjal was carried out to establish an efficient transformation protocol.

- A. To establish a suitable protocol for *Agrobacterium* mediated genetic transformation of eggplants.
- B. Selection and characterization of putative transgenic eggplants.



CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Based on the available information on cross ability and different species and subtropics of brinjal, it could be concluded that as such there is no natural crossing among cultivated and wild species of brinjal. Further under forced crossing situations, even if crossing was possible, the viability and subsequent development stable crosses have not been successful.

There are no reports of any gene transfer from brinjal to unrelated plant species. Further it may be noted that such a transfer of any gene is highly improbable because of pre-and post-zygotic genetic incompatibility barriers that are well documented for distantly related plant groups. No evidence for transfer of genes from brinjal to other plant taxa has been identified.

Horizontal gene transfer from plants to animals (including humans) or microorganisms is extremely unlikely. No evidence has been identified for any mechanism by which brinjal genes could be transferred to humans or animals, nor any evidence that such gene transfer has occurred for any plant species during evolutionary history, despite animals and humans eating large quantities of plant DNA. The likelihood of brinjal genes transferring to humans and other animals is therefore effectively zero. Similarly gene transfer from brinjal, or any other plant, to microorganisms is extremely unlikely. Horizontal gene transfer from plants to bacteria has not been demonstrated experimentally under natural conditions (Nielsen *et al.*, 1997; Nielsen *et al.*, 1998; Syvanen 1999) and deliberate attempts to induce such transfers have so far failed (Schlüter *et al.*, 1995; Coghlan 2000).

Brinjal researchers use a variation on the *Agrobacterium* mediated transformation technique to induce mutation in the plant. Studying the effect of mutation in a particular gene often yields critical information about the normal function of that particular gene. Because *Agrobacterium* inserts its transforming DNA randomly in the genome in many cases the DNA get inserted directly within a gene sequence. This usually disrupts the function of the affected gene, resulting in a “knockout mutant” (Meinke *et al.* 1998).

S. melongena L can be transformed after co cultivation of explants with *Agrobacterium*. Transformation approaches on *Solanum melongena* with *Agrobacterium* were published by a large number of researcher groups, using various types of explants (root, shoot, cotyledon, internodes etc).

Genetic transformation of eggplant via *Agrobacterium* was first reported by Guri & Sink (1988b), using leaf explants and a co-integrate vector, although no success was achieved with a binary vector. Later, Fillipone & Lurquin (1989) reported the transformation of leaf and cotyledon explants using the wild supervirulent strain A281. Transgenic plants were obtained by Rotino & Gleddie (1990) and Fári *et al.* (1995), using organogenic regeneration systems. An optimization of factors that influence transformation efficiency, including length of pre and post-coculture periods, explant type, and genotype was performed using a TDZ-based organogenic system (Magioli *et al.* 2000). The efficiency of transformation protocols based organogenesis may be influenced by the antibiotic used to eliminate *A. tumefaciens*. For example, augmentin can cause enhanced shoot proliferation induced by TDZ (Billings *et al.* 1997). Recently, an organogenic system from root explants was applied in a protocol for transformation of variety MEBH 11. These explants demonstrated a high susceptibility to *Agrobacterium* and quick regeneration capacity on selection media, resulting in 82.5% of transgenic calli induction with a means of 24 transgenic shoots per callus (Franklin & Sita 2003).

The production of transgenic eggplant through somatic embryogenesis either fails to occur (Fillipone & Lurquin 1989) or is achieved with very low efficiency (Fári *et al.* 1995). It has been demonstrated that both co-cultivation with *Agrobacterium* and the presence of bactericidal antibiotics used in transformation protocols cause a reduction of 80-99% in the number of embryo/explants.

The inhibitory effect on somatic embryos development may result from the interaction between the physiological alterations caused by these treatments and the delicate processes of gene regulation that are induced in early culture stages (Magioli *et al.* 2001a). Following the establishment of basic protocols, successful introduction of agronomic traits into eggplant was achieved.

Resistance to Colorado Potato Beetle (*Leptinotarsa decemlineata* Say) (CPB), a pest that has developed resistance to synthetic insecticides and became a serious problem for agriculture in Europe and America (Arpaia *et al.* 1997), has been pursued by a number of groups. Chen *et al.* (1995) have produced transgenic eggplant lines with the introduction of *Bacillus thuringiensis* (*Bt*) genes, but resistance to CPB was not observed. Later, different groups obtained lines resistant to CPB by using mutagenized versions of *cryIIB* (Arpaia *et al.* 1997; Iannacone *et al.* 1997) and a synthetic version of *cryIIIA* *Bt* genes (Jelencovic *et al.* 1998). Field trials demonstrated high levels of resistance in transgenic plants produced after the introduction of a mutagenized *Bt cryIIIB* gene, without detrimental effects on non target arthropods (Acciarri *et al.* 2000). These resistant transgenic eggplants can potentially be used for the development of new varieties.

Besides CPB resistance, there are other examples of genetic improvement of eggplant via *A. tumefaciens*. Resistance to *Leucinodes orbanalis* was obtained in transgenic plants harboring the *Bt* (*CryIAb*) gene. Tolerance against osmotic stress induced by salt drought and chilling stress was achieved in transformants expressing the bacterial mannitol-1- phosphodehydrogenase (*mtlD*) gene (Prabhavathi *et al.* 2002) and transgenic hybrids harboring the parthenocarpic gene *DefH9-iaaM* presented a significant yield increment that resulted in a 10% reduction in cultivation costs (Donzella *et al.* 2000).

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Plant Materials

Cotyledons of 10-12 days old *in vitro* grown and filter paper grown plants were used as explants for *Agrobacterium* mediated genetic transformation.

3.1.2. Bacterial Strain and Plasmid

Agrobacterium tumefaciens strain LBA4404 (pVDH65, pTOK47) were used for the infection of explants. The binary vector pVDH65 **Figure 3.1** based on the pBIN19 derivative pMOG18 carried a T-DNA with the chimeric *nos.nptII nos* and intron-GUS genes. Here the selectable marker neomycin phosphotransferase gene (*nptII*) conferring kanamycin resistance to explants. The single colony and the liquid culture of that bacterium *A. tumefaciens* LBA4404 were used for infections which are shown in **PLATE 2.2**.

3.1.3. Chemicals

All chemical compounds including micro and macro nutrients, organic and inorganic acids, sugar, agar, KOH, HgCl₂, ethanol etc used in present study were the reagent grade products of either BDH, England, E-Merk, Germany or MERCH India. The vitamins, amino acids, different growth regulators and antibiotics were the product of Sigma Chemical Company, USA and Phytotech, USA. The following chemicals were used in investigation:

3.1.3.1. Sterilant & Surfactant

In the present investigation mercuric chloride (HgCl₂) was used as surface sterilizing agent and tween-80 and savlon (ACI Pharma, Bangladesh) were used as surfactant cum detergent.

PLATE 3.1



Figure – Different stages (A,B,C) of explants development for *Agrobacterium* mediated genetic transformation of *Solanum melongena* L

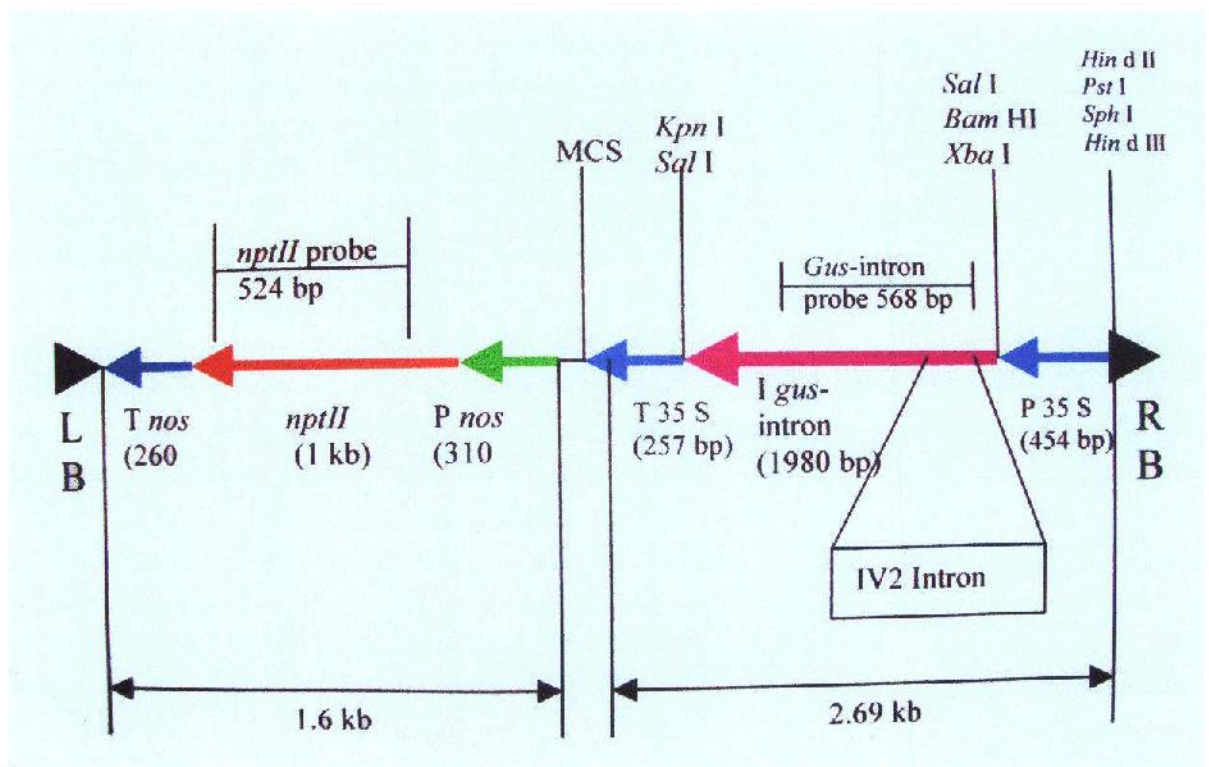
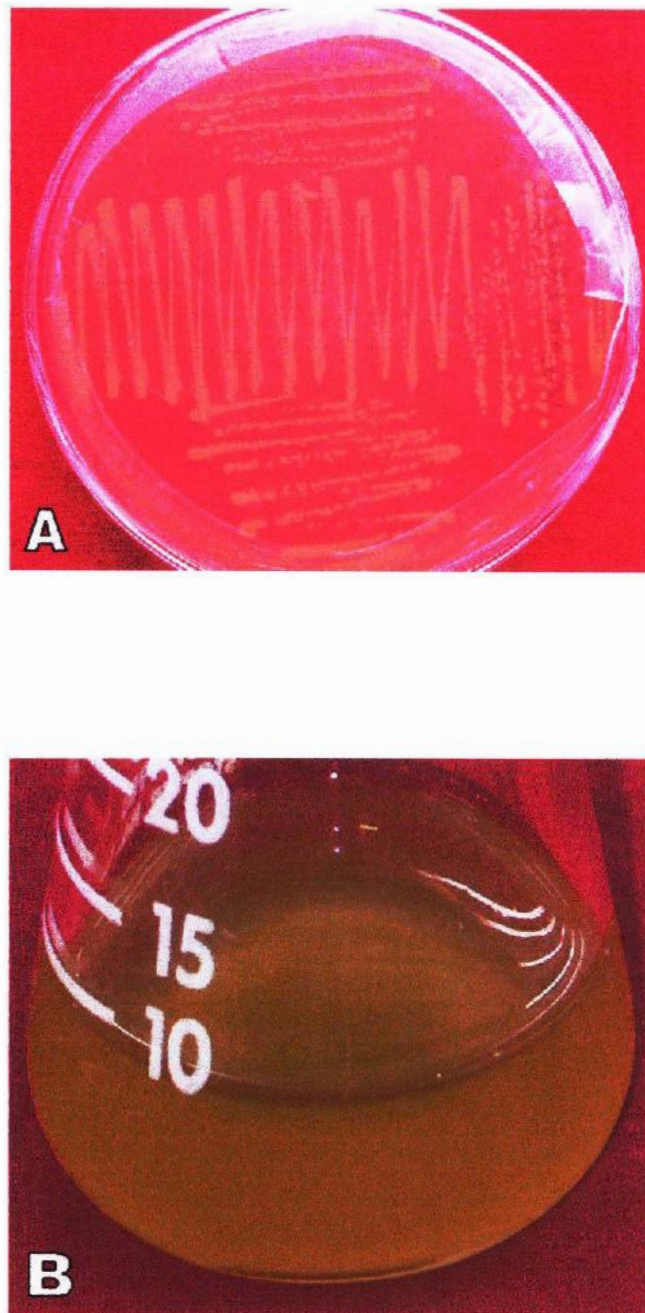


Figure 3.1 Plasmid pVDH65 carried in *Agrobacterium tumefaciens* Strain LBA4404

PLATE 3.2



Culture of *A. tumefaciens* strain LBA4404 containing pVDH65 recombinant vector.

Figure (A) Plate culture on LBA medium

(B) Suspension culture in LB liquid medium

3.1.3.2. Plant Growth Regulators (PGRs)

Growth regulators play significant role on *in vitro* culture the following PGRs were used either individually or in various combinations to the nutrient media. Auxins and Cytokinin were added to the nutrient media before autoclaving. However, GA₃ was used in filter-sterilized condition after autoclaving the nutrient medium.

- (i) **Auxins:** Auxin promote cell enlargement and root initiation (Kyte 1987); the following type was used to fulfill the experimental purpose.

Naphthalene Acetic Acid (NAA)

- (ii) **Cytokinins:** Cytokinins promote cell division and shoot initiation (Kyte 1987); the following two types were used to fulfill the experimental purpose.

6-Benzyl Amino purine (BAP).

- (iii) **Gibberellins:** Gibberellins influence cell enlargement and elongation (Kyte 1987).

Gibberellic acid (GA₃).

3.1.3.3. Nutrient basal salts

MS (Murashige and Skoog, 1962) formulation was used as nutrient basal salts in the present study. All chemical compounds including macro and micro nutrient, organic acid and inorganic acids, sugar, agar, KOH, HgCl₂, 70% ethyl alcohol etc. used in the present study were the reagent grade products of either BDH, England or E-Merk, Germany. The vitamins, amino acids and different growth regulators were the products of Sigma Chemical company U. S. A. The following basal nutrient salts were used in this study which contain macronutrients, micronutrients and vitamins for plants.

Macronutrients: MgSO₄·7H₂O, KNO₃, KH₂PO₄, CaCl₂·2H₂O, NaH₂PO₄·H₂O, NH₄NO₃, (NH₄)₂SO₄.

Micronutrients: H₃BO₃, NaMoO₄·2H₂O, MnSO₄·2H₂O, ZnSO₄·7H₂O, CuSO₄·5H₂O, KI, CoCl₂·6H₂O, FeSO₄·7H₂O, Na₂EDTA.

Vitamins: Thiamine HCL, Pyridoxine HCL, Nicotinic Acid, Myo-Inositol.

3.1.3.4. Chemicals used in Polymerase Chain Reaction

Mg⁺⁺ [uM]

DNA Template [10ng/ul]

10X PCR Buffer

MgCl₂ [25mM]

Primer [2.5uM]

dNTP (10mM, 2.5mM each)

ddH₂O

Taq Polymerase (0.5 u/ul)

3.1.3.5. Other Chemicals

- a) Kanamycin (50 mg/l) for the selection of transformants.
- b) NaOH and HCL for pH adjustment.
- c) Cefotaxime (50 mg/l) for the bacterium (*Agrobacterium*) cleansing.
- d) Acetosyringone (T-DNA activator).

3.1.3.6. Equipments

The plant materials and nutrient media were used for different tissue culture experiments contained within various types of culture vessels, the whole constituting a culture system. The culture vessels such as Petri dishes, test tubes (150×25 mm), conical flasks (250 ml, 500ml, 1000 ml), measuring cylinders glass rods, beakers, micro pipette, pipette pumps, PCR machine, centrifuge machine, gel electrophoresis machine, cotton plugs, rubber bands, filter paper, aluminum foils, marker pen, spirit lamp, forceps, needle, sharp blade, firebox, stereomicroscope, electronic balance, autoclave, pH meter, magnetic stirrer, Laminar airflow machine etc. were also used in the present investigation.

3.1.3.7. Gelling and Solidifying Agents

Gelling and solidifying agents are commonly used for preparing semisolid or solid tissue culture media. Agar, a polysaccharide obtained from seaweed can be used as a gelling agent in the most nutrient media. In the present investigation, 0.8% agar (BDH, England) was used as a gelling agent.

3.1.3.8. Carbon Sources

Plant cells and tissues in the culture medium lack of autotrophic ability and therefore, need external carbon for energy. The most preferred carbon source in the plant tissue culture is sucrose. In the present investigation, 3% sugar was used in the culture media for the sources of carbon.

3.1.4. Glassware and Instruments

Glassware and instruments are divided in to three parts according to use.

3.1.4.1. For media preparation

Different types of glass vessels including test tubes, culture bottles, conical flasks, measuring cylinders, separating funnels, pipettes, micro-pipette, magnetic stirrer, micro-wave oven, pH meter, electronic balance, squijar etc.

3.1.4.2. Instrument for aseptic transfer

Autoclave, laminar air flow machine, spirit lamp, fitter paper disc, forceps of various sizes, fire box, needles, scissors, surgical scalpel holder with disposable blades, Petri dishes, marker pen etc.

3.1.4.3. For incubation

The culture were maintained at in a culture room condition under a 16h photoperiod of 3000 lux light provided by cool white florescent tubes and controlled temperature $25 \pm 1^{\circ}\text{C}$ maintained with air cooler.

3.2. METHODS

The methods involved experiments in plant tissue culture in the present investigation described are under the following heads.

3.2.1. Nutrient Media

The medium on which plant cells or organs are cultured is known as nutrient medium, culture medium or simply medium. MS (Murashige and Skoog, 1962) medium was used for culture purpose. In all cases sucrose was added at the rate of 30 gm/l. Plant growth regulators were added separately to different media according to requirements. Agar was added at the rate of 6-8 gm/l for the preparation of semi-solid MS medium. However, liquid medium was used at the time of primary culture and blotting paper bridges were used in liquid to support the seeds in primary culture medium. Different constituents of the MS medium including plant growth regulators were prepared in to stock solutions for ready use during the preparation of culture medium. Different constituents of MS medium and the procedure preparation of stock solution MS medium and the procedure preparation of stock solution MS salts are given in APPENDIX.

3.2.1.1. Preparation of stock solution

In the first step of the preparation of culture medium stock solutions were made. Various constituents of the respective nutrient medium were prepared into stock solutions for ready use during the preparation of media for different experiments. As different constituents were required in different concentrations, stock solutions of macro-nutrients, micro-nutrients, organic compounds (vitamin and amino acids) and growth regulators were prepared separately.

Stock solution I

NH_4NO_3	33g
KNO_3	38g
KH_2PO_4	3.4g

The above listed nutrients were weighted accurately with the help of an electronic balance and dissolved in 200 ml of distilled water. The final volume was made up to 400 ml (for 20 liters). This stock solution was stored in a refrigerator at 4°C for several weeks.

Stock solution VI

KI	0.166g
CuSO ₄ .5H ₂ O	0.005g
NaMoO ₄ .2H ₂ O	0.050g
CoCl ₂ .6H ₂ O	0.050g

The above listed nutrients were weighted accurately with the help of an electronic balance and dissolved in 200 ml of distilled water. Then the final volume made up to 400 ml and stored in a refrigerator at 4°C.

Stock solution VII

Myoinositol	5.000g
Nicotinic acid (vitamin B ₃)	0.025g
Pyridoxine-HCl (vitamin B ₆)	0.025g
Thiamine-HCl (vitamin B ₁)	0.025g
Glycine (amino acid)	0.100g

The above listed vitamins and amino acids were weighted accurately with the help of an electronic balance and dissolved in distilled water. The final volume was made up to 100ml. This stock solution was poured into a bottle and stored in a refrigerator at 0°C for several weeks.

3.2.2. Stock solutions of plant growth regulators (PGRs)

In addition to the inorganic and organic nutrients, it is generally necessary to add different growth regulators to the media for supporting suitable growth and development of tissue and organs (Bhojwani and Razdan, 1983).

The following growth regulators were used in the present investigation. Stock solution of growth regulators was prepared separately. Details of the preparation method of stock solution are given in the following **Table 3.1**

Table 3.1. Preparation of stock solutions of growth regulators.

Growth regulators	Amount taken (mg)	Appropriate solvents	Final volume with distilled water (ml)	Strength of the stock solution (mg/ml)
Auxin	NAA 10	0.1N KOH	100	1
	IBA 10	70% ETOH	100	1
	IAA 10	0.1N KOH	100	1
	BA 10	0.1N KOH	100	1
Cylokinin	KIN 10	0.1N KOH	100	1
Gibberellins	GA ₃ 10	70% ETOH	100	1

Growth regulators were dissolved in appropriate solvent as shown against each of them (plant cell culture catalogue 1992). For the preparation of any are of the previously mentioned PGR stock solution 10mg of the powdered growth regulator was taken in a clean plastic weighing boat and dissolved in 1 or 2ml of respective solvent. The mixture was then washed of with distilled water and collect in 100 ml measuring cylinder. It was then filtered, poured in to a clean plastic container and stored in a refrigerator at 4°C for several weeks.

3.2.3. Preparation of Antibiotics

In case of antibiotic stock solution preparation(Kanamycin, Cefotaxime) desired mg of antibiotic was dissolved in 10 ml distilled water inside the laminar airflow cabinet and filter sterilized separately and stored in effendroff tubes at -4°C in dark as stock solution. For the preparation of Acetosyringone stock solution, 25mg of acetosyringone was dissolved in 2.5mg of dimethylsulphodioxide (DMSO). Then filter sterilized separately and stored in effendroff tubes at -19°C in refrigerator.

3.2.4. Preparation of Culture Media

Following steps were followed for the preparation of 1 litre MS medium:

i) Assembling of the medium components

For the preparation of 1 litre MS medium, 20 ml of stock solution MS-I, 20 ml of stock solution MS-II, 20 ml of stock solution MS-III, 20 ml stock solution MS IV, 20 ml stock solution MS V, 2 ml stock Solution MS-VI and 2 ml MS-VII were added in 1 liter flask containing distilled water for final volume and mixed well.

ii) Addition of sucrose

30g sugar was dissolved in 200ml distilled water and filter through filter paper. Filtered sucrose solution, salts, vitamins, amino acids, growth regulators were poured into measuring cylinder. The final volume was made 1000ml and shake well to mix up the components uniformly in the solution

ii) Addition of plant growth regulators

Stock solutions of plant growth regulators were added in appropriate concentrations and combinations in above solutions and were mixed well.

iv) pH of the medium

In all tests the pH of the medium was adjusted to 5.8 using a digital (TOA, Japan) pH meter with the help of 0.1N HCL or 0.1N KOH (where necessary) before addition of agar.

v) Solidifying agent

Solidifying agents are used for the preparation of semi solid tissue culture medium, which provide support for the tissues growing in static conditions. Usually 6.6gm/l agar was used in the medium to form semi solid medium. Then the medium was heated for 5 minutes in a microwave oven to melt the agar completely.

vi) Medium dispensing to culture vessels

The prepared melted medium was dispensed into culture vessels such as test tubes or Petri dishes, through separating funnel. The culture vessels were plugged with cotton plugs, wrapped with cheesecloth, which were inserted tightly at the mouth of culture vessels. The culture vessels were marked to designate specific hormonal supplements.

vii) Sterilization

Finally the culture vessels containing medium were autoclaved at 15lb/sq inch's pressure and at the temperature of 120°C to 121°C for 20 minutes to insure sterilization. Then the vessels with the medium were allowed to cool as vertically and then marked with a glass marker pen to indicate specific hormonal supplements and stored in the culture room for ready use.

3.2.5. Preparation of Transformation Medium

3.2.5.1. Preparation of *Agrobacterium* culture mediums

For one liter *Agrobacterium* culture mediums following concentration of components were used.

Table-3.2. Composition of *Agrobacterium* culture medium

Items	g/l
Bacto Peptone	10
Bacto Yeast Extract	05
NaCl	10

Ingredients were taken in a conical flask containing appropriate amount of distilled water. These ingredients were dissolved properly in distilled water using a magnetic stirrer. pH was adjusted at 7.0 with 1N NaOH and 1N HCL. Then the medium was sterilized by autoclaving 120 C for 20 minutes. To solidify the medium 20mg/l agar was added except the liquid medium. When the temperature came down antibiotic solution was added and poured into Petri dishes according to the necessity and wait until the solidification of media. All the activities were carried out in laminar airflow cabinet.

3.2.5.2. Preparation of inoculation medium

In order to facilitate the infection, liquid MS₀ media were prepared and mixed with bacterial suspension culture into a ratio of 1:1 combination.

3.2.5.3. Preparation of co cultivation medium

For the purpose of co-cultivation (culture of leaf with *Agrobacterium*) MS₀ (semi solid) media were used and the pH was adjusted to 5.8 prior to use.

3.2.5.4. Preparation of selection regeneration medium

Agrobacterium strain used in the investigation contains the *nptII* gene encoding neomycin phosphotransferase, so the antibiotic kanamycin was used for the selection of transformed tissue. The selection medium composed of MS + 0.1mg/l NAA + 50mg/l kanamycin + 50mg/l cefotaxime. The pH was adjusted at 5.8. The media was autoclaved and cooled at 40 C in subsequent steps and antibiotics were added into the medium inside the laminar airflow cabinet. The media were then separated and poured into Petri dishes, test tubes or bottles as per requirement.

3.2.5.5. Preparation of antibiotics

For the preparation of antibiotics stock solution (Kanamycin, Cefotaxime), desired mg of antibiotics was dissolved in 10ml of distilled water. Then all the solutions were filter sterilized separately and stored in eppendorf tubes at -4°C in dark. For the preparation of acetosyringone stock solution, 25 mg of acetosyringone was dissolved 2.5 ml of dimethyl sulphoxide (DMSO). Then the solutions were filter sterilized and stored in Eppendorf tubes at -20°C in refrigerator.

3.2.6. Preparation of Plant Material

For this experiment, 10-12 days old cotyledon of *in vitro* and *ex vitro* grown Jessor variety of brinjal were used as explants. Seeds were sterilized with 0.1% HgCl_2 for 3 minutes inside the laminar airflow cabinet. In order to remove disinfectant the seeds were rinsed with double distilled water for 4 to 5 times. Sterilized seeds were cultured on MS_0 basal medium fortified with 3% sucrose. Cultures were kept at the temperature of $25\pm 2^{\circ}\text{C}$ and a photo period of 14 hours maintained by cooled white fluorescent light. Cotyledons excised from 10-12 day old seedlings were used for *Agrobacterium* co-cultivation. *In vitro* explants were collected from plant breeding and gene engineering lab. In order to the effects of pre-culture on transformation efficiency, explants were either used directly or pre-cultured before transformation.

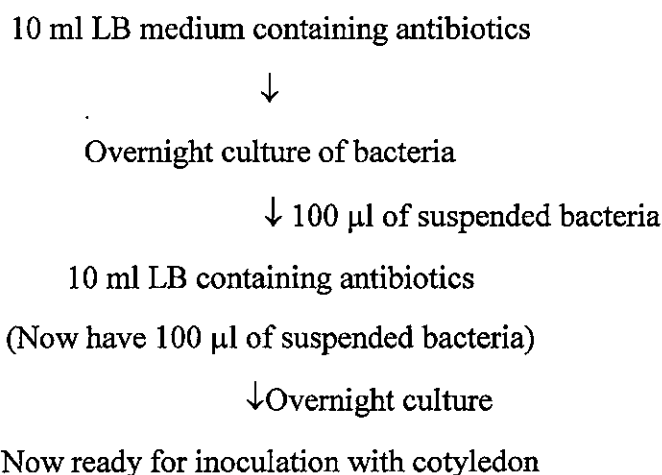
3.2.7. Transformation Technique

3.2.7.1 Maintenance and culture of bacteria

For growth and maintenance of *A. tumefaciens* strain LBA4404, one single colony from previously maintained *Agrobacterium* stock was streaked into freshly prepared petridishes containing LB medium having Kanamycin and Cefotaxime. The Petri dishes were sealed with parafilm and kept in dark at 28°C in the incubator for at least 48 hours. These were then kept 4°C to check the growth. Such culture of *Agrobacterium* strain was thus ready to use for liquid culture. The culture was sub cultured regularly in freshly prepared media to maintain the stocks.

3.2.7.2. Preparation of bacterial cultures for transformation

Liquid bacterial cultures were initiated by transferring a loopful of bacteria collected from 3 days old bacterial colonies on semi-solid LB to 20 ml aliquots of liquid LB, containing the appropriate antibiotics in 100 ml conical flasks. Cultures were incubated in dark on a horizontal shaker (180 rpm, $28 \pm 1^\circ\text{C}$) for 24 hours. Overnight cultures were grown to an OD (600 nm) of 0.5-1.5, before dilution [1:1 (v/v)] with liquid MS₀ medium for infection of potato explants. It can be shown as follows:



3.2.7.3. Infection of explants with *A. tumefaciens*

Cotyledons were excised from 10-12 days old *in vitro* grown brinjal plants and were notched by sharp scalpel. Notched leaves were submerged in bacterial suspension for different periods. The explants were then taken out of the suspension and soaked with sterile filter paper to remove excess of bacterial suspension.

3.2.7.4. Co-cultivation of infected explants with *A. tumefaciens*

After the infection phase, the explants were co-cultivated with *A. tumefaciens* on co-cultivation medium. The petridishes were sealed with parafilm and incubated under dark condition at 25°C in an incubator. In order to evaluate the effect of duration of cultivation, the explants were co-cultivated for different periods of time (12, 24, 36, 48, and 72h).

3.2.7.5. Selection and regeneration of putative transformants

For selection and regeneration after 24-48 hours of co-cultivation, the explants were transferred to selection medium containing 50-mg/l kanamycin and 50-mg/l cefotaxime for selection of transgenic tissue. Explants were cultured in fresh selection and regeneration medium with appropriate antibiotic. Plantlets appeared to be regenerated within 15-22 days at the cut ends of the explants. Then these regenerated plantlets attached to the explants were fully regenerate into shoots and then they were sub cultured every 14 days to the same fresh medium with appropriate antibiotics. Non-infected explants were also cultured onto selection media as control environment.

3.2.7.6. Rooting of regenerated shoots

For rooting Individual shoots (each 2-4 cm in length) were excised from the calli and transferred to MS₀ medium containing the antibiotics. Putative transformed rooted plants were regenerated and maintained properly. Putative transformed rooted plants were used for *GUS* histochemical assay and were also analyzed by PCR for the presence of *nptII* gene.

3.2.8. Analysis of Putative Transgenic Plants

3.2.8.1 *GUS* histochemical Assay of the transformed plants

The β -glucuronidase (*gus*) gene, encoded by the *Uid A* locus of *E. coli*, is most popular reporter gene used in plant transformation. The product of this gene i.e. enzyme β -glucuronidase break down glucuroides giving a colored reaction. A blue color develops in the cell due to the reaction. *GUS* activity was assayed using X-glue (*5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid*; Gold Biotechnology, St. Louis, USA). This substrate gives a blue precipitate which is insoluble and highly colored indigo-dye 5, 5'-dibromo-4' dichloro-indigo (CIBr-indigo) at the site of *gus* enzyme activity. This dye is formed by oxidative dimerization of the colorless indolyl derivative that results for *gus* hydrolysis of X-glue.

Table 3.3. Histochemical reagents for *gus* assay

Components	Amounts per 10ml
X-glue	8.89mg
NaH ₂ PO ₄ (1.0M)	0.5mg
TritonX (10%)	0.1mg
Chloramphenicol	1.0mg
Methanol	20%
pH	7-8

The *GUS* histochemical assay solution modified from Jefferson et al., (1987) and was prepared by dissolving above chemicals one by one in 300ml of distill water while stirring in beaker. X-glue solution was prepared by 250mg in 5ml dimethyl sulfoxide (DMSO). Thee final volume was made to 500ml by adding distill water and then filter sterilized.

GUS histochemical assay was performed using 10-15 explants per treatment, 5 days after inoculation to monitor *GUS* activity and 28 days after inoculation with *Agrobacterium* to estimate stable transformation. Leaf tissues were assayed for *GUS* activity in regenerated putative transgenic plants. Assay was performed in microtitre plates. Preparation were incubated overnight at 37° C after which the histochemical assay solution was removed and placed with 99% (v/v) ethanol. For leaf explants, ethanol was replaced every 5 hours to remove chlorophyll. The localization and intensity of blue staining were quantified by eyes. If no blue stained plant tissue were detected, plant samples were scored as *GUS* negative and if any blue spots or streaks were present in the tissues, it was scored as *GUS* positive.

3.2.8.2. PCR analysis for the detection of transgene

Putative transformants and control (non-transformants) plants were used for PCR to detect the presence of *gus* gene. Plant DNA used for PCR analysis was extracted from young leaves using the protocol of Edwards *et al.* (1991). The process of DNA extraction is summarized below:

- (i) Placed about 20 mg of plant tissue in Eppendorf tube.
- (ii) Break up tissue with pellet pestle (Kontes: Vineland, NJ),
- (iii) Added a little sterile sand and 400 μ l of extraction buffer (200mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS),
- (iv) Grind on ice with pellet pestle until completely ground up.
- (iv) Centrifuged in table-top microfuge at 4°C for 2 min.,
- (v) Transferred supernatant to new Eppendorf tube (about 250 μ l),
- (vi) Added 1 volume of ice cold isopropanol and incubate on ice for 5-30 min.,
- (vii) Centrifuged in microfuge at room temperature for 5 min.,
- (ix) Discard supernatant and dry pellet,
- (x) Re-suspended in 100 μ l of TE, store at -20°C,
- (xi) A 1:100 dilution of this extract was used for PCR reaction.

PCR analysis for the presence of the *gus* gene was carried out using the following pair of primers.

Table 3.4. List of primer name and sequences for PCR analysis

Primer name	Primer sequences 5' 3'
Forward primer	5'- GTCAT CTCAC CTTGC TCCTG CC -3'
Reverse primer	5' - GTCGC TTGGT CGGTC ATTTC GG - 3'

Table 3.5. List of components in the PCR reaction mix

<i>Components</i>	Volume (μl)
Mg ⁺⁺ [μM]	1.5
DNA Template [10 ng/μl]	2.0
10X PCR buffer	2.5
MgCl ₂ [25 mM]	1.5
Primer F [2.5 uM]	2.0
Primer R [2.5 uM]	2.0
dNTP (10 mM, 2.5 mM each)	2.0
dd H ₂ O	12.9
Taq Polymerase (0.5u/μl)	0.1
Total Volume (μl)	25.0

Table 3.6. PCR Reaction Condition

Parameters	Temperature	Duration	Number of cycles
Denaturation	94 °C	45 sec	1
Annealing	58 °C	40 min	
Polymerization	72 °C	2 min	35

Here, reaction was performed in a tag DNA thermal cycler (Progene, Cambridge, UK). PCR products were separated in 1% agarose gel containing 0.5ml/ml ethidium bromide (EtBr) by electrophoresis at 50 volts for 1.5 hours. After separation, the products were visualized under UV light and photograph was taken through red filter. The size of PCR product was measured using 100 bp size molecular marker (promega).

3.2.9. Data collection and calculation

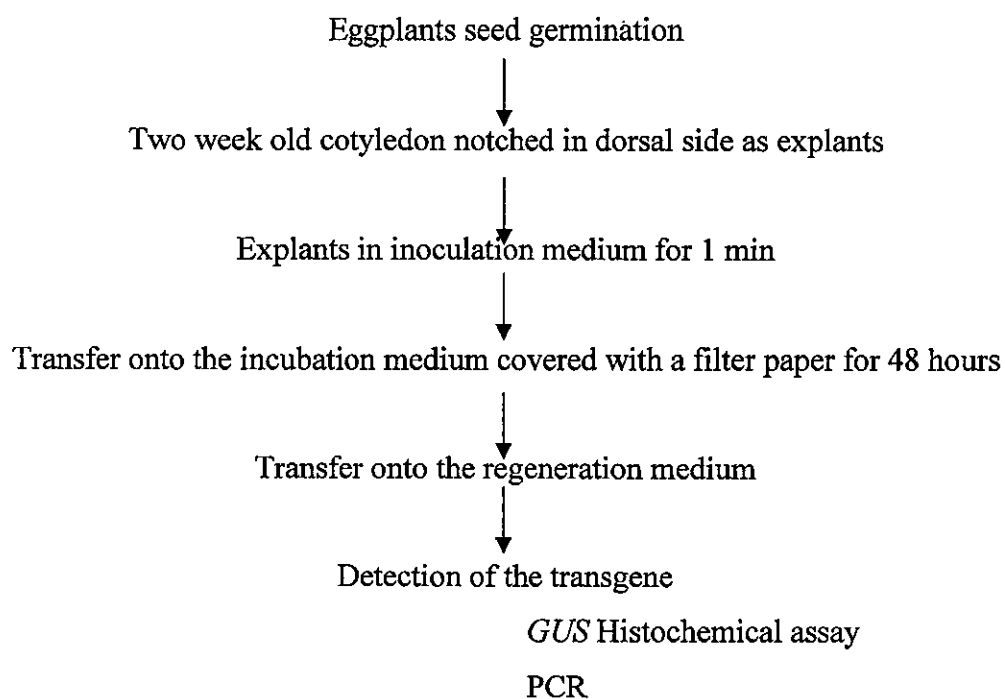
For the suitable protocol of Agrobacterium mediated genetic transformation, different parameters were used in different amount. Each experiment was replicated at least thrice and data were recorded properly.

3.2.10. Statistical analysis of data

Collected data were analyzed by using Microsoft Excel software. Each experiment was replicated at least thrice. Transformation efficiencies was calculated as follows:

$$\text{Transformation efficiencies} = (\text{Number of kanamycin resistant plant} / \text{Total number of plant tested}) \times 100$$

3.2.11. Flow chart of the present investigation



CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

4.1. Standardization of plant regeneration from *Solanum melongena* L. Explants

Though the main objective of this study is to transform eggplants with *gus* gene, plant regeneration media were standardized. To do his seeds of Jessori variety were collected from market, surface sterilized with 0.1 HgCl₂ for 5 minutes followed by several subsequent wash with distill water. The sterilized seeds were placed on agar gelled MS₀ media in 9cm petridishes (25 seeds/dish) for germination. The cotyledon of 10-12 days old seedlings were excised and cultured on MS basal medium supplemented with different combination of NAA and BA and incubated in growth chamber. The effects of different media formation on shoot regeneration are shown in table 3.1. the experiment reveals that MS medium supplemented with 0.5mg/l NAA and 0.5mg/l BA was the most effective formulation where the highest 68 percentage of explants were induced to regeneration of shoots.

Table 4.1. Effects of different media formulation on plant regeneration from aseptically grown seedlings of eggplants.

Growth Regulators	No. of Inoculated Explants	No of Explants Induced Shoot Regeneration	% of Explants regenerated shoots
NAA + BA			
0.5 mg/l + 0.5 mg/l	25	17	68
0.5 mg/l + 1.0 mg/l	25	13	52
2.5 mg/l + 1.0 mg/l	25	10	40
1.0 mg/l + 2.5 mg/l	25	9	36

4.2. OPTIMIZATION OF TRANSFORMATION PARAMETERS

Effective transformation of plant is affected by number of factors such as kanamycin sensitivity of the explants, inoculation density, co-cultivation period, inoculation time etc. A number of experiments were conducted in order to standardize these parameters of

Agrobacterium mediated genetic transformation of eggplants.

4.2.1. Determination of Kanamycin Concentration for the Selection of Kanamycin Resistant Tissue

The cotyledon of eggplants was co cultured with *Agrobacterium* and cultured on plant regeneration media. The sensitivity of explants to kanamycin was assayed by culturing the cotyledon without the o-cultivation with *Agrobacterium* on selection medium supplemented with different concentrations of kanamycin (0, 25, 50, 75, 100mg/l) is shown in the table 3.2. The growth and survival rate were decreased as compare to control (explants without kanamycin). Observed results are shown in the table 3.2. The explants growth was completely inhibited at the kanamycin concentration 50 mg/l. Under continuous pressure of kanamycin the explants become necrotic and ultimately died. Therefore 50mg/l kanamycin was considered to be effective for the inhibition of normal growth and developments. So, 50mg/l kanamycin was chosen for the selection of kanamycin resistant tissue in subsequent transformation experiment.

Table 4.2. Effect of Kanamycin concentration in the selection of kanamycin resistant tissues.

Kanamycin Concentration	Control Explants			Experimental Explants		
	No. of Inoculated Explants	No. of survival Explants	Percentage of survived Explants	No. of Inoculated Explants	No. of survival Explants	Percentage of survived Explants
0 mg/l	25	0	0	25	0	0
25 mg/l	25	0	0	25	7	28
50 mg/l	25	0	0	25	10	40
75 mg/l	25	0	0	25	5	20
100 mg/l	25	0	0	25	2	8

4.2.2. Optimization of Acetosyringone Concentration for Stable Transformation

Acetosyringone is a phenolic substance. The use of Acetosyringone compounds has been considered to enhance the transformation events mediated by *A. tumefaciens*. Different concentrations of acetosyringone were added into the co-cultivation medium to test the influence of acetosyringone on transformation mediated by *A. tumefaciens* strain LBA4404. The acetosyringone at 0, 10, 15, 20, 25 and 30 mg/l was added to co-cultivation media and the explants were incubated for 24-48 h and then transferred to the selection medium at 50 mg/l kanamycin, 50 mg/l cefotaxime, with different combination of NAA and BA.

The experimental results showed that none of the explants survived on the selection medium when the explants were co-cultivated in co-cultivation medium without acetosyringone, after the several days of transfer. On the other hand, explants continued to grow on the selection medium when acetosyringone was added to the co-cultivation medium. The highest percentage of kanamycin resistant explants growth was obtained at the 25 mg/l concentration of acetosyringone.

4.2.3. Effects of infection time on transformation efficiency

To determine the effect of incubation time on transformation efficiency of explants were infected with different period of time (36, 48, and 90 seconds) with *Agrobacterium tumefaciens* Strain LBA4404. The infected explants were cultured on selection medium containing 50mg/l kanamycin and 50mg/l cefotaxime. Initial transformation efficiency was measured after three weeks of inoculation by counting the number of explants forming kanamycin resistance tissues. The results are shown in the table 3.3. It was found that the highest numbers of survived explants were 11 out of 25 and the highest percentage was 55 and these results were obtained after infection at 1 minute. On the other hand, the lowest percentage was observed at 30 seconds of infection. However, the higher infection period caused the subsequent death of all explants. Therefore, 60 seconds period of infection found standard for *Agrobacterium* genetic transformation of eggplants.

Table 4.3. Effects of infection period on survival percentage of in vitro grown explants of eggplants.

Explants type	Incubation period (sec)	No. of explants inoculated	No. of explants survived	Surviving percentage of explants
<i>In vitro</i>	30		7	28
	60	25	11	44
	90		5	20

4.3.4. Effects of co-cultivation period on transformation efficiency

Notched leafs were treated with *Agrobacterium* and incubated in the dark for 24, 48 and 72 h in co-cultivation medium. After co-cultivation the explants transferred to the selection medium containing 50mg/l kanamycin and 50 mg/l cefotaxime. The explants were sub cultured at regular intervals, usually 15-20 days onto same fresh selection medium for better growth. In every case, kanamycin resistant tissues were selected and dead explants were discarded the results of co-cultivation period on transformation rate are presented in the Table3 .5 and Graph 3.5. Among different co-cultivation period, 48h was found to be optimal for explants transformation (**Plate 4.1, Figure A**).

Table 4.4. Effects of co-cultivation period on development of kanamycin resistant tissue with *Agrobacterium tumefaciens* LBA4404

Explants type	Co-cultivation period (h)	No. of explants inoculated	Number of explants survived	Surviving percentage of explants
<i>In vitro</i> grown cotyledon	24	25	7	28
	48		13	52
	72		3	12

4.4. Transformation of explants using optimum parameters

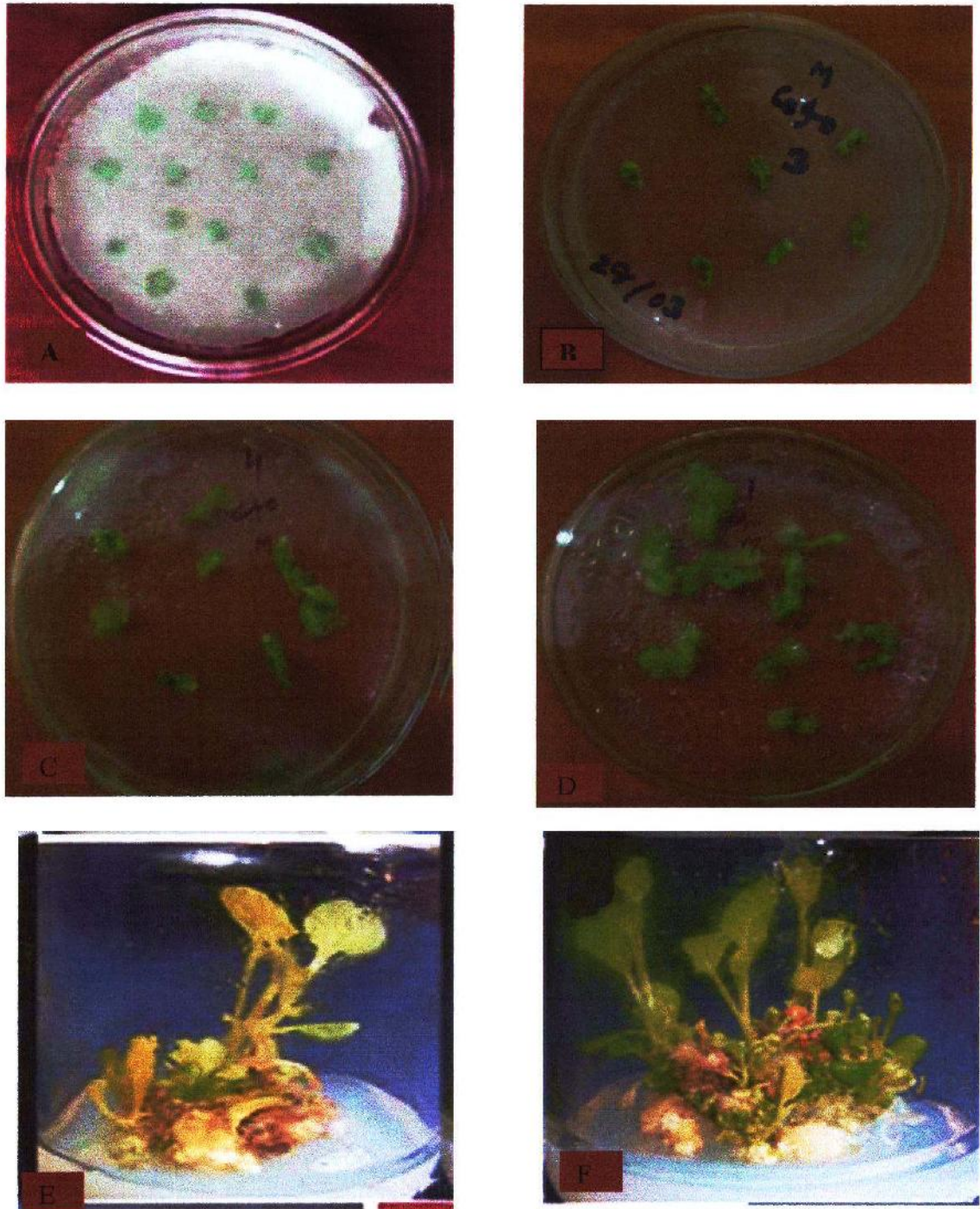
For the successful transformation via *Agrobacterium*, optimum values of kanamycin concentration, inoculums density, incubation period, co-cultivation period are the key factors, which are described under the previous heading of this section. After incubation with *Agrobacterium*, notched leaf were co-cultured to secure better infection and the infected discs were transferred to the selection medium composed of MS basal medium + 50mg/l kanamycin +50 mg/l cefotaxime + different combination of growth regulator(NAA, BA). In case of positive control uninfected explants were also placed on the media with same combination. Infected explants responded to the new growth. Whereas, uninfected explants when cultured on selection medium died within 8-12 dys. After 4-5 weeks shoot began to regenerate in the selection medium from the cut surface of leaf discs. Four experiments were carried out using above mentioned optimal parameters and data were recorded. The results are shown in Table 3.6 and Plate 3.1 and the Fig B and C.

Among four experiments, the highest no of transient plants were recovered from the experiment 1 which showed the highest transformation efficiency (40). On the other hand, experiment 3 and 4 showed better transformation efficiency (17.33 and 20.33) than experiment no 2 (13.33). The putative transformants and subsequent regeneration from different experiments shown in the table 3.6. The in vitro grown healthy plantlets were sub cultured at least 3-4 times in MS Medium with appropriate concentration of antibiotic to ensure that the transgenic plants free from *Agrobacterium* and resistant to kanamycin. (Plate 4.1 Fig D, E and F)

Table 4.5. Transformation efficiency of explants after co cultivation with *Agrobacterium tumefaciens* LBA4404 carrying *gus* gene.

Explants type	Number of experiment	No. of explants inoculated	Number of kanamycin resistant plantlets recovered	Transformation efficiency (%)
In vitro cotyledon	1. 0.5 mg/l + 0.5 mg/l	25	8	40.00
		25	10	
		25	12	
	B. 0.5 mg/l + 1.0 mg/l	25	6	17.33
		25	4	
		25	3	
	C. 2.5 mg/l + 1.0 mg/l	25	2	13.33
		25	5	
		25	3	
	D. 1.0 mg/l + 2.5 mg/l	25	4	20.33
		25	9	
		25	3	

PLATE 4.1



Steps involved in *Agrobacterium* mediated transformation of eggplants

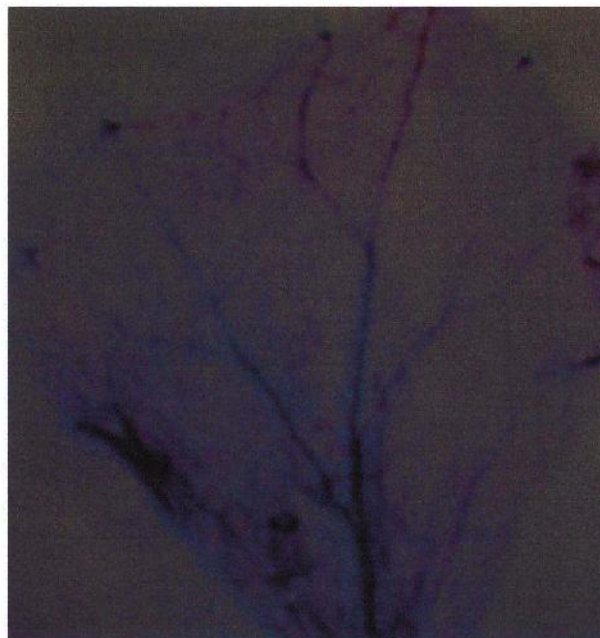
Figure A) Co-cultivation of cotyledon B) Infected explants in regeneration medium C) Regenerated plantlets after 25 days D) Regenerated putative transformed plantlets in shoot induction medium after 4 weeks E) Plantlets in multiplication medium F) Multiplication of transformed plantlets after 6 weeks.

4.5. *GUS* Gene Expressions in Kanamycin Resistant Plants

The plantlets regenerated in the presence of kanamycin were tested for *gus* expression. All kanamycin resistant had the characteristics blue color produced by *gus* activity was detected in untransformed control plants (Plate 3.2, Fig A and B). A positive reaction of the *gus* gene was found in their leaves. The intensity of blue *gus* staining varied between different plants. *GUS* activity was present in all kanamycin resistant plants tested, confirming that they were transgenic.

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PLATE- 4.2



Histochemical *gus* assay of the leaf from putative transformed and non transformed plant.

Figures A) A *gus* stained leaf from kanamycin resistant plant.

B) A leaf of non transformed plant

4.6 Molecular Analysis of Putative Transformed Plant through PCR

Polymerase Chain Reaction (PCR) has the ability to amplify specific segments of DNA from very small amount of substrate DNA. The PCR analysis for *nptII* gene of putative plants of *Solanum melongena* regenerated from kanamycin resistant explants presented in the **Figure 4.1**

Here,

Lane M	: 1000 bp size marker
Lane 1 -11	: Transformed plants
Lane 12	: Positive control
Lane 13	: Plasmid control
Lane 14	: Non Transformed Plants
Lane 15	: water (control).

It was found that putative transformed plants (lane 2-11) expressed the expected 0.3 kb band of *nptII* gene. The same band also expressed in positive control (lane 12) that confirmed the integration of *nptII* gene in the genome of *Solanum melongena*.

PLATE 4.3

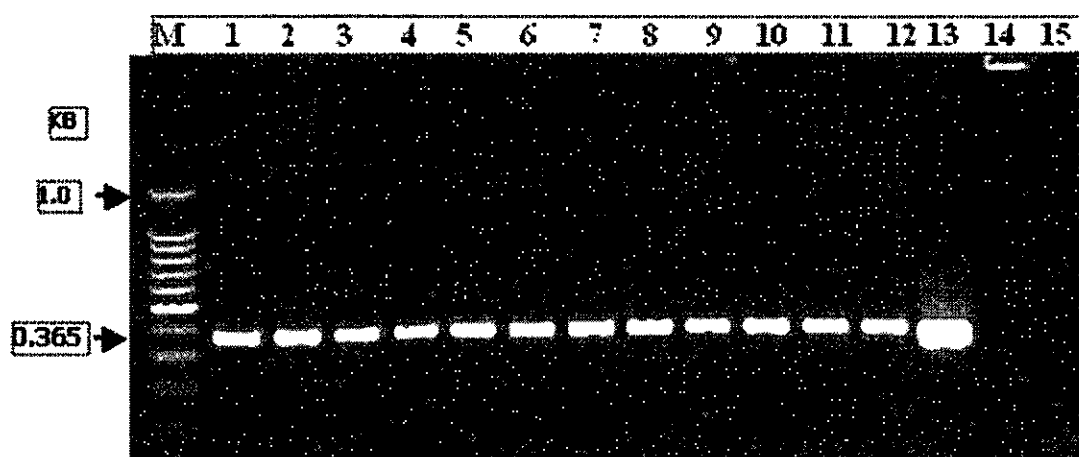


Figure 4.1- PCR analysis of genomic DNA of putative transformed plants of *Solanum melongena* for *nptII* gene

Lane M	: 1000 bp size marker
Lane 1 -11	: Transformed plants
Lane 12	: Positive control
Lane 13	: Plasmid control
Lane 14	: Non Transformed Plants
Lane 15	: water (control).

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

Science and technology education have enjoyed a meaningful partnership across most of the century. The work of scientists embraces an array of technologies, and major accomplishments in science are often accompanied by sophisticated applications of technology. As a result, a complete science education has, in principle, involved a commitment to the inclusion of technology, both as a tool for learning science content and processes and as a topic of instruction in itself.

The advancement of science and technology has done the dawn of modern civilization enhanced the overall standard of human in the planet. Science directs technological innovation and technology accelerates the progress of science and with their help people of the world has standardized their lives. The unprecedented development of science in the 20th century particularly in the broad field of agriculture, if, has ensured these fundamental needs in the world, which is still a mirage to the over whelming majority of population in the so called developing world. The knowledge of molecular biology and biotechnology were broadly used in agriculture food science and medicine.

Transgenic plants are genetically modified. Biotechnology or genetic engineering technology creates transgenic plants where inserting genes directly in to a single plant cell. Transgenic plants are now generally produced in a laboratory by adding one or more genes to a plant's genome and the techniques frequently called genetic transformation. Genetic transformation is a powerful tool for crop improvement of agronomic traits it is a source of agriculture innovation, providing new solution to the age-old problems. Transgenic plants have been deliberately developed for a variety of reasons: longer shelf life, disease resistance, herbicide resistance, and pest resistance, non-biological stress resistance, such as to drought or nitrogen starvation and nutritional improvement. The first modern transgenic crop approved for sale in the US, in 1994, was the Flavravr tomato, which was intended to have a longer shelf life, the first conventional transgenic cereal created by plant breeders was actually a hybrid between wheat and rye in 1876.

Unlike traditional plant breeding, which involves the crossing of hundreds or thousands of genes, plant biotechnology allows for the transfer of only one or a few desirable genes. This more precise science allows plant breeders more precise science allows plant breeders to develop crops with specific beneficial traits and without undesirable traits. Transgenic methods for higher plants have great impact not only on basic molecular biology of plants but also on the strategy for crop improvement (Confalonieri *et al.* 2003) and genetic transformation opens an avenue for incorporating novel sources of resistance, or other added value traits, in to the genome, which can subsequently be utilized in breeding program along with endogenous traits (Hansen and Wright, 1999; Parsley, 2000).

Transformation is usually achieved using gold particle bombardment or a soil born bacterium (*Agrobacterium tumefaciens*) carrying an engineered plasmid vector, or a carrier of selected extra genes. *Agrobacterium*-mediated genetic transformation is the dominant technology used for the production of genetically modified transgenic plants (Tzfira and Citovsky, 2006). The capacity to introduce and express diverse foreign genes in pants was first described in tobacco by *Agrobacterium* mediated (Horsch *et al.* 1984; De Block *et al.* 1984) and vector less approach (Paszowski *et al.* 1984.) Potatoes were one of the first crop plants in which transgenic plants were successfully regenerated (An *et al.* 1986; Shanin and Simpson. 1986). Genetic transformation of eggplant via *Agrobacterium* was first reported by Guri & Sink (1988b), using leaf explants and a co-integrate vector, although no success was achieved with a binary vector. Later, Fillipone & Lurquin (1989) reported the transformation of leaf and cotyledon explants using the wild super virulent strain A281.

The present study was undertaken to establish an improve protocol for efficient *Agrobacterium* mediated transformation of *Solanum melongena* L. The choice and selection of suitable explants source was also an important factor in the present investigation. In this study 10 -12 days old cotyledon were used as a starting material for transformation with *Agrobacterium tumefaciens* strain LBA4404 harboring binary plasmid pVDH65 containing nptII gene as selectable marker and *gus* as reporter gene.

In the present investigation, different concentration of acetosyringone were added into the co-cultivation medium but it was found that 25 mg/l concentration of acetosyringone showed the highest percentage of kanamycin resistant explants. Results from a wheat inflorescence transformation experiment showed that T-DNA could not be transformed to the plant tissue without acetosyringone (Amoah *et al.* 2001). Similar results subjected to acetosyringone and co-cultivation period have been reported in Maize (Gould *et al.* 1991), Rice (Hiei *et al.* 1994, Rashid *et al.* 1996) and banana (May *et al.* 1995). The co-cultivation explants did not survive and the transformation was not successful at different co-cultivation period with acetosyringone in co-cultivation medium.

Incubation period is the time elapsed between exposure to a pathogenic organism or chemical or radiation, and symptoms and sign are first apparent. The period may be short as minutes to as long as thirty years in the case of variant. Transformation efficiency depends on the incubation period of explants. In this experiment, incubation period of 60 second was found to be optimum for inoculation of explants. The performance of Bangladeshi ecotype of *Arabidopsis* was best at incubation period of 25 min for survival rates and GUS positive activity in leaf and nodal segment explants (Biswas, 2006). Found the highest surviving percentage of lettuce explants at the incubation period of 3 minutes (Ahmed *et al.* 2007).

Bacterial growth phase and bacterial cell density play an important role on genetic transformation efficiency. Concentrations of the *Agrobacterium* vary to different research groups and plant materials. In the study bacterial cell concentration OD=1.50 was accepted as optimum.

Transformation efficiency was found to be affected by the co-cultivation period. Co-cultivation for 2-7 days has been normally used in *Agro bacterium*-mediated transformation under various co-cultivation temperatures (Han *et al.* 200; Mondal *et al.* 2001; Cervera *et al.* 1998). Many transformation experiments in different plant species, such as tea (*Camellia sinensis* L.), Cauliflower, white spruce (*Picea gluca*) and citrange, showed that 2-3 days of co-cultivation resulted in high transformation efficiency under room temperature co-cultivation conditions (Lopez *et al.* 2004; Chakrabarty *et al.* 2002; Le *et al.* 2001; Covera *et al.* 1998).

Too short co-cultivation period is not favorable for transformation. On the other hand, too long co-cultivation period results in over growing *Agrobacterium* and therefore is harmful to plant cells (Wang and Fang, 1998). Normally 1-3 days of Co-Cultivation are standard for most transformation protocols (Men *et al.* 2003; Weber *et al.*, 2003) presence of Kanamycin in the culture medium at the very initial stage greatly hampers integration of *Agrobacterium* to the explants tissue as well as organogenesis of explants and as a result many putative transformants may be lost. For this reason, immediately after incubation with *Agrobacterium* (inoculation), The infected explants were not allowed to grow directly in selection medium containing Kanamycin rather they were rather they were allowed to grow for 24-48 hours on co-cultivation medium devoid of antibiotic (Ahmed *et al.* 2007). In the present study, among different co-cultivation period with co-cultivation medium in the presence of 25 mg/l acetosyringone, 48 hours was found to be optimal for highest percentage of surviving of explants.

Transformation efficiency of eggplants explants was carried out in three repeated experiments and 40% of transformation efficiency was observed. The transformed explants were then placed on regeneration medium containing proper selective agent (50 mg/l Kanamycin).

Then the *GUS* histochemical assay was conducted in order to select the putative transformed plants. Histochemical analysis of vegetative and floral parts from transgenic plants of all the varieties tested showed a characteristic deep blue color throughout the tissue, indicating constitutive *GUS* gene expression. There were, however, variations in the intensity of the blue coloration in the different organs and also variations as the plant matured. An intense blue color was observed in the leaf of the transformed eggplants. Control leaves taken from plants regenerated normally did not show any blue coloration. Since the *GUS*-INT gene was used, we can safely conclude that the blue color is due to the actual integration of this gene after splicing and not due to *Agrobacterium* contamination. Fari *et al.* (1995) found a strong cell-type preference for *GUS* expression in the phloem tissues and in the veins, whereas the mesophyll and epidermis cells showed very little activity. They also found that in the vegetative tissues. Finally the integration of *nptII* gene into the genome of eggplants was confirmed through Polymerase Chain Reaction.

CHAPTER VI

SUMMARY

CHAPTER VI

SUMMARY

Genetic transformation is an important and powerful tool for the improvement of crops, and in understanding gene structure and expression of integrated gene. In the present study *Agrobacterium* mediated genetic transformation was used as a transformation method to deliver and integrate the foreign gene in the genome of eggplants. Notched leaves obtained from *in vitro* grown seedlings were infected with *Agrobacterium tumefaciens* strain LBA4404 harboring binary plasmid pVDH65 containing nptII gene as selectable marker and *gus* as reporter gene

Different parameters (kanamycin concentration, *Agrobacterium* inoculum density, incubation and co-cultivation period) were optimized to achieve the results. In the current study Kanamycin concentrations of 0, 25, 50, 75, 100 mg/l were used as a transformed tissue selective agent and acetosyringone (0, 10, 15, 20, 25 and 30 mg/l) was used as a signal molecule. Various densities (0.50, 1.0, 1.5 and 2.00,) at 600 nm of bacterial inoculum, different incubation period (30, 60 and 90 sec) and co-cultivation period of 24 and 48 hour were also evaluated for finding out optimization of the parameters of transformation of potato explants. Among different parameters it was observed that 50 mg/l kanamycin was suitable for the selection of kanamycin resistant tissues in subsequent transformation experiment. Also, transformation efficiency was found best when explants were incubated for 60 seconds in bacterial suspension having OD₆₀₀ 1.50 and 48 h co-cultivation period in the presence of acetosyringone (25 mg/l). Three experiments were done by using above mentioned optimum parameters. The present investigation showed that without acetosyringone, none of the explants survived on the selection medium when the explants were co-cultivated in co-cultivation medium.

In this experiment *in vitro* cotyledon showed the maximum kanamycin resistant and positive explants with the highest transformation frequency. Successfully transformed and regenerated plantlets were further analyzed at molecular level to be confirmed about the transformation process.

Putative transformed plants were harvested from selection medium and identified on the basis of GUS histochemical assay. Polymerase chain reaction (PCR) analysis confirmed that the putative transformed plants expressed the presence of expected 0.365 band of nptII gene in the genome of eggplants

CHAPTER VII

REFERENCES

CHAPTER VII

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APPENDIX

Composition of MS (Murashige and Skoog, 1962) basal medium.

Components	Concentrations (mg/l)
Macro-nutrients:	
KNO ₃	1900
KH ₂ PO ₄	170
NH ₄ NO ₃	1650
CaCl ₂ .2H ₂ O	440
MgSO ₄ .7H ₂ O	370
Micro-Nutrients:	
KI	0.83
H ₃ BO ₄	6.20
CoCl ₂ .6H ₂ O	0.025
ZnSO ₄ .7H ₂ O	8.60
FeSO ₄ . 7H ₂ O	27.8
CuSO ₄ .5H ₂ O	0.025
MnSO ₄ .4H ₂ O	22.30
Na ₂ MoO ₄ .2H ₂ O	0.25
Na ₂ EDTA. 2H ₂ O	37.3
Vitamins and organics:	
Glycine	2.0
Sucrose	30g
<i>myo</i> -Inositol	100.0
Nicotinic acid	0.50
Thiamine-HCl	0.10
Pyridoxine-HCl	0.50