

**“CLONING AND CHARACTERIZATION OF Ti PLASMID
BASED VECTORS CARRYING *Bt* GENES FOR
DEVELOPING HIGH EFFICIENCY PLANT
TRANSFORMATION SYSTEM”**

M.Sc. (Ag.) THESIS

by

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INDIRA GANDHI KRISHI VISHWAVIDYALAYA
RAIPUR (C.G.)**

2009

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Thesis

Submitted to the

Indira Gandhi Krishi Vishwavidyalaya, Raipur

by

GULFISHAN QURESHI

**IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE
DEGREE OF**

Master of Science

in

**Agriculture
(Biotechnology)**

Roll No. 9900

I D No. UG/Ag./RYP/2003

August, 2009

CERTIFICATE – I

This is to certify that the thesis entitled “**Cloning and characterization of Ti plasmid based vectors carrying *Bt* genes for developing high efficiency plant transformation system**” submitted in partial fulfilment of the requirements for the degree of “**MASTER OF SCIENCE IN AGRICULTURE**” of the Indira Gandhi Krishi Vishwavidyalaya, Raipur, is a record of the bonafide research work carried out by **Miss GULFISHAN QURESHI** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of Instructions.

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

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This is to certify that the thesis entitled “**Cloning and characterization of Ti plasmid based vectors carrying *Bt* genes for developing high efficiency plant transformation system**” submitted by **Miss GULFISHAN QURESHI** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur in partial fulfilment of the requirements for the degree of **M. Sc. (Ag.)** in the **DEPARTMENT OF BIOTECHNOLOGY** has been approved by the external examiner and Student's Advisory Committee after oral examination.

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Acknowledgement

First and foremost I would like to show my humble and whole hearted prostration before the Almighty "GOD" for showing his unprecedented blessing on me.

It is my profound privilege to acknowledge gratitude and indebtedness to my Major Advisor and Chairman Dr. G. Chandel, for his valuable guidance, inspiring suggestions and constant encouragement throughout the course of investigation. His everlasting support and guidance proved to be the flambeau showing me the right way towards my destination.

I have immense pleasure in expressing my whole hearted sense of appreciation for all members of my Advisory Committee Dr.D.K.Sharma, Dr.Tapas Choudhary, Dr.A.S.Kotasthane and Dr. R. R. Saxena, College of Agriculture, Raipur for their priceless guidance, useful suggestions, critical comments and kind help rendered as and when needed.

With profound respect, I extend my gratitude to Dr. M. P. Pandey, Hon'ble Vice Chancellor, Dr. A. S. R. A. S. Shastri, D. R. S. Dr. R. L. Pandey, Dean and Dr. S. S. Kohle, Director of Instruction, I.G.K.V., Raipur for providing their administrative and technical help which facilitated my research work,

I record my sincere gratitude to Head of the Department of Biotechnology Dr. D.K.Sharma, I.G.A.U. for all the co-operation extended towards me whenever required. I am grateful to entire staff for their encouragement and advice.

With great reverence, I express my sincere thanks to my teachers, Dr. A.S. Kotasthane, Dr. Zenu Jha, for their valuable support, scholarly advice and genuine guidance, whenever, I sought throughout my course work and research work, I extend my thanks to Shri C.M. Sharma, senior A.D.O., Department of Biotechnology, for his fatherly help and co-operation.

Words would not suffice for the appreciation I acknowledge for the exceptional staff of college and PhD for their constant encouragement and untiring help.

Many, many thanks to my wonderful research scholar Ms. Shubha Banerjee and Miss. Sudeshna See No way, this piece of work has been completed without their help.

My genuine thanks to my friend cum unsparing critic, Paritosh for his tuff comments accompanied with gentle support.

I am especially thankful to my seniors Dr. Pravin Jadhav, Ms. Shubha, Ms. Sweta, Ms. Anradha, Mr. Bhubaneswara, Mr. Piyush, Mr. Susheel, Mr. Umesh, and Mr. Jitendra for their gentle support.

I can't forget the moments shared with my friends, Priyanka, Taslima, Lovejot, Paritosh, Ashish, Pramod, Kaushalendra and Vikrant whose support and help always strengthened me.

I want to express my sincere thanks to my dear friend Priyanka whose pampered support, care and constant encouragement always gave showers to my ideas.

I will be failing in my duties if I don't convey my sincere thanks to Pusau Bhaiya, Vinod Bhaiya, Ramachandra Bhaiya, Manoj Bhaiya and Maan bai of the Department of Biotechnology.

Last but not the least, there aren't enough words to express my gratitude to my most beloved parents Mr. A. H. Qureshi and Mrs. Shabnam Qureshi, and my family members for their encouragement, sincere prayers, expectations and blessings which have always been the most vital source of inspiration and motivation in my life. I acknowledge their perseverance and endurance over the years. It is my turn now to take care of them. I must thank God for giving me place among them.

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LIST OF ABBREVIATIONS

AB	<i>Agrobacterium</i> medium
@	At the rate
AS	Acetosyringone (3,5-dimethoxy-4-hydroxy Acetophenone)
BAP	Benzyl amino purine
Bp	Base pairs
<i>Bt</i>	<i>Bacillus thuriangiensis</i>
CaMV	Cauliflower Mosaic Virus
<i>Cry</i>	Crystal
C-terminal	Carboxyl (COOH)-terminal
CTAB	Cetyl trimethyl ammonium bromide
DMSO	Dimethylsulfoxide
ER	Endoplasmic Reticulum
EtBr	Ethidium Bromide
F pilus	Flagella Pilus
GFP	Green Fluorescent Protein
GUS	1,4-Glucuronidase
H	Hour
HPT	Hygromycin Phosphotransferase
kb	Kilobase
kDa	Kilodalton
L	Liter
LB	Luria Bertini
<i>mcr1Ac</i>	Modified crystal protein
μg	Microgram
min	Minute
μl	Microliter
ml	Milliliters
MS	Murishage and Skoog
<i>mVIP</i>	Modified Vegetative Insecticidal Protein
NAA	α-Naphthalene acetic acid
NPT II	Neomycin Phosphotransferase II

Nos poly-(A)	Nopaline Synthase Poly (adenylation)
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PIV2	Potato Intervening Sequence 2
psi	Pressure per square inch
rpm	Revolution per minute
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
TAE	Tris Acetic acid EDTA bffer
Ti plasmid	Tumor-Inducing Plasmid
T-DNA	Transfer-DNA
T-Pilus	Transfer Pilus
Tra protein	T-DNA transfer protein
<i>vir</i> gene	<i>virulence</i> Gene

CHAPTER- I

INTRODUCTION

Bacillus thuringiensis (*Bt*) is a gram-positive sporulating soil inhabiting bacteria produces a proteinaceous parasporal crystal (δ -endotoxin), which is toxic against a number of lepidopteron and dipterans (Burgess 1982). The crystal protein genes are mostly located in large plasmids (15–120 Md) (Lereclus *et al* 1982). Transgenic insecticidal crops expressing the BT toxins provide an alternative method of remarkable level of insects control unsurpassed by any other insecticidal treatment.

Over past several years, more than 100 different polypeptides have been identified and several of them have been employed in insect pest management programs. The *cry* protein has been used as bio-pesticide spray for more than 30 years. The expression of *mcry1Ac* genes provides protection against *S. exigua*, *M. sexta*, and *H. virescens*. Hence, it is feasible to increase the level of resistance in transgenic plant and also employ different genes for managing development of resistance to *Bt* in insect population. The two *Bt* genes (*mcry1Ac* and *mVIP*) modified, characterized and shown highly effective against the lepidopteran pest especially *S. litura*, *Earias insulana*, *Chilo partellus*, *Spilosoma obliqua*, *Maruca testulalis*, *H. armigera* and *L. orbonalis* (Selvapandiyan 1998).

In addition to the crystal protein, some insecticidal polypeptides produced during the vegetative growth of the bacteria known as *VIP* (Vegetative Insecticidal Protein) are gaining importance in recent years as bio-pesticides. The *VIP* protein possess insecticidal activity against a wide spectrum of lepidopteron insects and display bioactivity towards the black cutworm, fall armyworm & beet army worms.

Development of vectors suitable for plant transformation is one of the prerequisites in transgenic research. Several cloning vectors were developed in the 1980s for higher plant transformation and their use has led to the development of genetically modified (GM) crops. It is a DNA molecule that carries foreign DNA into a host cell, replicates inside a bacterial (or yeast) cell and produces many copies of itself and the foreign DNA. Features of all cloning vectors: (1) Sequences that permit the propagation of itself in bacteria (or in yeast for YACs). (2) A cloning site to insert foreign DNA; the most versatile vectors contain a site that can be cut by many restriction enzymes. (3) A method of selecting for bacteria (or yeast for YACs) containing a vector with foreign DNA; usually accomplished by selectable markers for drug resistance. Two types of prokaryotic cloning vectors can be distinguished: extra-chromosomal plasmids, which cause antibiotic resistance of transformed cells, and bacteriophages like phage λ and filamentous phages, which are mostly selected by plaque formation. Among plasmids the most versatile vectors for *Escherichia coli* transformation are those which are derived from the ColE1 plasmid, such as pBR322, pBR325 and pBR328 (Bolivar *et al.*, 1977., Bolivar, 1978).

One of the binary vectors constructed in the early days was pBin19 (Bevan, 1984), and pBI121 was created shortly after by adding a marker gene to pBin19 (Jefferson, 1987). Soon these vectors and their derivatives were widely distributed among plant scientists. Another popular series of vectors are pPZP vectors (Hajdukiewicz *et al.*, 1994) and pCAMBIA vectors (www.cambia.org), which are modified from pPZP vectors. Recent modifications of binary vectors provide a number of user-friendly features, such as wide selection of cloning sites, high copy numbers in *E. coli*, high cloning capacity, improved compatibility with strains of choice, a wide pool of selectable markers for plants, and a high frequency of plant transformation. Using a binary tumor-inducing (Ti)

plasmid vector system, several plant species were transformed with a kanamycin resistance marker (*npt II* gene).

Two novel insecticidal protein encoding genes *mcry1Ac* and *mVIP* cloned into expression vector pET-29(a) and characterized by ICGEB (International Center for Genetic Engineering and Biotechnology), New Delhi were obtained through MTA (Material Transfer Agreement). Development of suitable vector (Ti plasmid) for high efficiency transformation system in major agronomically important crops like rice, wheat, maize and other crops, the *Agrobacterium* offers a DNA delivery system requires less sophisticated and low copy integration of foreign DNA. Considering these facts a study entitled **“Cloning and characterization of Ti plasmid based vectors carrying *Bt* genes for developing high efficiency plant transformation system.”** was carried out to clone two *Bt* genes into a binary vector pBI 121 and develop the LBA 4404 *Agro* clones for plant transformation with the following specific objectives:

1. Cloning of *Bt* genes into the Ti plasmid background (pBI121) for high efficiency transformation.
2. Screening and identification of positive clones in *E. coli* system.
3. Characterization of clones using restriction digestion analysis.

CHAPTER- II

REVIEW OF LITERATURE

Biotechnology has opened unprecedented avenues for exploring biological systems and genetic engineering tools have paved transfer of genes across species and genus. Plant genetic engineering or transformations involves transfer of recombinant DNA (rDNA) into plant cells to generate transgenic species. Genes and genomes from a wide range of organisms are being manipulated for the benefit of mankind.

Since the development of transgenic plants efforts has been made to improve and develop new plant transformation vectors and methodologies to increase the efficiency of plant transformation and to achieve stable expression of transgenes. Plant transformation is performed using a wide range of tools of which *Agrobacterium* and micro- projectile bombardment are currently the most extensively used methods Dai, S. *et al.*, (2001). Recent developments in these two technologies along with the phenomenon of ‘gene silencing’, which has come to centre stage after a large number of transgenic plants, have been carefully evaluated for transgene expression in successive generations. Due to the simplicity of the transformation system and precise integration of transgenes, the *Agrobacterium* Ti plasmid-based vector continues to offer the best system for plant transformation. The use of commercial crops expressing *Bacillus thuringiensis* (*Bt*) toxins has increased in recent years due to their advantages over crops that require traditional chemical insecticides.

In Alabama alone, between 300,000 and 400,000 acres of *Bt* cotton have been grown annually since 1996. Tobacco was the first transgenic plant developed in 1983 and today more than 50 other transgenic plant species viz., tomato, potato, sunflower, cotton,

carrot, grapes, etc. (Table 2.1), have been developed with foreign genes integrated in their genome. Initially, the production of transgenics was restricted to dicotyledonous plants but, now it has been extended to several monocots like wheat, maize, rice, oats, etc.

Table 2.1. Transgenic crop plants developed with insect and disease resistance genes

Crops	Genes Transferred	Purpose
Tobacco	<i>B. thuringiensis</i> CpTI Chitinase gene <i>aro A</i>	Insect resistance Insect resistance Disease resistance Herbicide resistance
Tomato	<i>Bt</i> <i>aro A</i> Sucrose phosphate synthase	Insect resistance Herbicide resistance Increased sucrose and reduced starch
Potato	<i>Bt</i> Bacteriophage T4-lysosome	Insect resistance Disease resistance
Cotton	<i>Bt</i>	Insect resistance
Rice	<i>Bt</i>	Insect resistance
Maize	<i>Bt</i>	Insect pest resistance
Sugarcane	<i>Bt</i>	Insect pest resistance

* CpTI - Cowpea trypsin inhibitor;

2.1 *Bacillus thuringiensis*: a natural insecticide

Ishiwata in Japan first discovered the bacterium *B. thuringiensis* during 1902 in silkworm rearing unit and named it *Bacillus scotto*. In 1995, it was renamed as *Bacillus thuringiensis*. In the early twentieth century, Berliner provided the first insight that microbes could control insect pests. *Bacillus thuringiensis* exists in many locations, such as the soil, plant surfaces and in grain storage dust. *B. thuringiensis* can be distinguished from related *Bacillus* species by the presence of parasporal crystals that are formed during sporulation. The parasporal crystals consist of one or more δ -endotoxins or crystal (Cry) proteins of ~130 kDa (although truncated forms also occur). It is these that makes

Bt an effective insect pathogen. *B. thuringiensis* was first discovered in 1911 as a pathogen of flour moths from the province of Thuringia, Germany. It was first used as a commercial insecticide in France in 1938, and then in the USA in the 1950s. However, these early products were replaced by more effective ones in the 1960s, when various highly pathogenic strains were discovered with particular activity against different types of insect.

2.1.1 Use of *Bacillus thuringiensis* δ -endotoxins

Bacillus thuringiensis, δ -endotoxin that is toxic against a number of lepidopterans and dipterans (Burgess 1982). The gene(s) coding for crystal protein in different species and subspecies of *Bt* are plasmid encoded (Carlton and Gonzalez 1985) although examples of chromosomal location of the gene(s) have also been reported (Aronson *et al* 1986). The crystal protein genes are mostly located in large plasmids (15–120 Md) (Lereclus *et al.*, 1982). Toxin activity appears to be mediated by binding of toxins to cell membrane phospholipids (Drobniewsky and Ellar 1987; Chow *et al* 1989). Several studies revealed that the expression of the crystal protein gene is functionally related to sporulation specific events (Monro 1961; Lecadet and Dedonder 1971) and it was also found that one of the subunits of spore coat protein shares some similarities with the crystal protein (Lecadet *et al* 1972; Meenakshi and Jayaraman 1979). However, desynchronization of sporulation and crystal formation could be achieved by short-term temperature shift-up (Arescaldins 1969) or addition of chloramphenicol (Meenakshi and Jayaraman 1979) or erythromycin (Arescaldins 1969; Somerville 1971).

B. thuringiensis δ -endotoxins are part of a large and still growing family of homologous proteins – more than 130 genes have been identified to date (see *Bt* toxin nomenclature at: http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html).

These genes generate a rich source of diversity on which to draw for differing insect specificities. This specificity is an important aspect of the *Bt* Cry proteins: each protein is active in only one or a few insect species.

2.1.2 Insecticidal crystal proteins (ICPs) of *Bacillus thuringiensis*

The abbreviation “*cry*” (for crystal) was first used by Held *et al.*, (1982) to represent the insecticidal crystal producing genes of *Bt* strains. These crystalline proteins are highly insecticidal at very low concentrations. The ICPs are synthesized during the late growth phase of bacteria and are accumulated in the cytoplasm as crystals or inclusion bodies. The crystal may account for up to 30% of the dry weight of the sporulated culture. The ICPs produced by *Bt* are alpha-, beta- and gamma exotoxin and δ -endotoxins are used in agriculture. The δ -endotoxins are the most extensively studied toxin; its larvicidal specificity includes member of lepidopterons, dipteran and coleopteran insects.

As these proteins are non-toxic to mammals and other organisms, *Bt* strains and their insecticidal crystal proteins (ICPs) have acquired acceptability as eco-friendly biopesticides all over the world and have been under extensive use in agriculture, horticulture, forestry, animal health and mosquito control for the past four decades. With the advent of molecular biology and genetic engineering, it has become possible to use *Bt* more effectively and rationally by introducing the ICPs of *Bt* in crop plants. The first transgenic plants using *cry* genes were developed in 1987. The tobacco plants engineered with truncated genes encoding *Cry1Aa* and *Cry1Ab* toxins were found to be resistant to the larvae of tobacco hornworm Jouanin *et al.*, (1998). However, the levels of *Cry* protein expression in the plant tissues were not very high. A significant breakthrough was made in 1990 by researchers at Monsanto Company (USA) who modified the *cry* genes

(*cry1Ab* and *cry1Ac*) for better expression in plant cells. An interesting example of native gene (*cry1IA5*) expression resulting in significant resistance to *H. armigera* in transgenic tobacco was provided by Selvapandiyan *et al.*, (1998). Another important landmark is the introduction of a native *cry1Ac* gene into the chloroplast genome of tobacco, which expressed the *Cry* protein to a very high level (3–5% of leaf soluble protein).

Most families of Lepidoptera include species susceptible to the *Cry1* and *Cry2* crystal proteins produced, in particular, by *Bt* serotypes *kurstaki* and *aizawai*. Currently, the crystal toxins are classified on the basis of amino acid sequence homology. The ICPs fall under 40 different classes with some toxins exhibiting specificity to multiple insect orders (www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt/).

2.1.3 Vegetative insecticidal proteins (VIPs) of *Bt*

Research efforts in the past five years have led to the discovery of novel insecticidal proteins that are produced by certain isolates of *B. thuringiensis*. These proteins unlike well-characterized crystal proteins are produced during vegetative growth of cells and are secreted into the growth medium. These proteins have been termed as vegetative insecticidal proteins (*VIP*). Sequences encoding for a *VIP* have been cloned, sequenced and the protein has been expressed in *E. coli* (Estruch *et al.*, 1996). The 88 kDa vegetative insecticidal proteins have a putative bacillar secretory signal at the N-terminal which is not processed during its secretion. It does not show any homology with the known crystalline insecticidal proteins. This structural dissimilarity is indicative of a possible divergent insecticidal mechanism than the other known *Bt*-toxins of polyphagous pest *S. litura* was titrated against *VIP* toxin no interaction between these legands was observed (Bhalla *et al.*, 2005). These results together with observed structural divergence

of *VIP* with other toxins make them an ideal candidate for development in insect management programmes together with the other category of *Bt*-toxins described earlier. Individually *vip* has been successfully expressed in monocots and dicot plants and efforts to pyramid *vip* in the *Bt*-transgenic crops are under way in several laboratories (Ranjekar *et al.*, 2003).

2.1.4 Classification of ICP genes

B. thuringiensis strains produce two types of toxin. The main types are the *Cry* (crystal) toxins, encoded by different *cry* genes, and this is how different types of *Bt* are classified. The second types are the *Cyt* (cytolytic) toxins, which can augment the *Cry* toxins, enhancing the effectiveness of insect control. Over 50 of the genes that encode the *Cry* toxins have now been sequenced and enable the toxins to be assigned to more than 15 groups on the basis of sequence similarities. Based on the primary target insect specificity and sequence similarity, insecticidal toxins have been classified into five major classes (Hofte and Whiteley, 1989). Table 2.2 shows the *cry* genes with their encoded protein sizes and specificity.

It has been known since the 1980s that the *Cry* toxins are encoded by genes on plasmids of *B. thuringiensis*. There can be 5 or 6 different plasmids in a single *Bt* strain, and these plasmids can encode different toxin genes. The plasmids can be exchanged between *Bt* strains by a conjugation-like process, so there is a potentially wide variety of a strain with different combinations of *Cry* toxins. In addition to this, *Bt* contains transposons (transposable genetic elements that flank genes and that can be excised from one part of the genome and inserted elsewhere). All these properties increase the variety of toxins produced naturally by *Bt* strains, and provide the basis for commercial companies to create genetically engineered strains with novel toxin combinations.

Table 2.2: Classification of *B. thuringiensis* delta-endotoxin genes, protein sizes and specificity

<i>cry</i> genes	Crystal shape	Protein size(kDa)	Insect activity
<i>cry</i> I (Several sub groups:A(a), A(b), A(c), B, C, D, E, F, G.	bipyramidal	130-138	Lepidoptera larvae
<i>cry</i> II (subgroups A, B, C)	cuboidal	69-71	Lepidoptera & diptera
<i>cry</i> III (subgroups A, B, C)	flat/irregular	73-74	Coleopteran
<i>cry</i> IV (subgroups A, B, C, D)	bipyramidal	73-134	Diptera
<i>cry</i> V-IX	various	35-129	Various

2.1.5 Mode of Action

Most studies conducted on histopathological reactions and mode of action of *Bt* have been carried out on lepidopteron larvae with toxin preparations derived from whole *Bt* crystals. These investigations showed that mechanism of action of *Bacillus thuringiensis cryI* proteins involve solubilization of the crystals in the insect midgut receptors and insertion of the toxin in to the apical membrane to create ion channels or pores (Luhty and Ebersold, 1981). For most lepidopteran, protoxins are solubilized under the alkaline conditions of the insect midgut proteases (Tojo and Aizawa, 1983), to become activated toxins. Major proteases are trypsin like or chymotrypsin like (Novillo *et al.*, 1997). Activated *cry1* toxins have two known function, receptor binding and ion channel activity. Hoffman *et al.*, (1988) have shown that the activated toxin binds to specific receptors on apical brush border of the midgut microvilli of susceptible insect binding being a two stage process involves reversible and irreversible steps (Rajamohan *et al.*, 1995). Irreversible steps involves a tight binding between the toxin and receptor, insertion into the apical membrane of the columnar epithelial cells follows the initial receptor mediated binding rendering the toxin insensitive to proteases and monoclonal

antibodies and induces ion channels or non-specific pores in the target membranes. The formation of toxin induced pores in the columnar cell apical membrane allows efflux of cellular content/ions. The disruption of gut integrity results in the death of insect from starvation or septicemia. The insecticidal properties of variety of *Bt* toxins produced naturally by *Bt* strains have provided basis for their commercial use to create genetically engineered strains with novel toxin combinations and their further use in insect resistance crop plants. A suitable cloning vector is prerequisite to develop any transgenic organisms and is especially developed to suit cytological parameters of both donor (generally microorganisms) and hosts (generally eukaryotes).

2.2 Cloning Vectors for Plant Transformation

Cloning vectors for higher plants were developed in the 1980s and their use has led to the genetically modified (GM) crops. It is a DNA molecule that carries foreign DNA into a host cell, replicates inside a bacterial (or yeast) cell and produces many copies of itself and the foreign DNA. Features of all cloning vectors: (1) Sequences that permit the propagation of itself in bacteria (or in yeast for YACs). (2) A cloning site to insert foreign DNA; the most versatile vectors contain a site that can be cut by many restriction enzymes. (3) A method of selecting for bacteria (or yeast for YACs) containing a vector with foreign DNA; usually accomplished by selectable markers for drug resistance. Two types of prokaryotic cloning vectors can be distinguished: extra-chromosomal plasmids, which cause antibiotic resistance of transformed cells, and bacteriophages like phage/1 and filamentous phages, which are mostly selected by plaque formation. Among plasmids the most versatile vectors for *Escherichia coli* transformation are those which are derived from the ColE1 plasmid, such as pBR322, pBR325 and pBR328 (Bolivar *et al.*, 1977; Bolivar, 1978).

Types of cloning vectors: (1) Plasmid: A small circular DNA molecule found in bacteria that replicates independently of the chromosome. Plasmids are used as cloning vectors. Example: PUC19, ColE1 origin of replication, high copy number, ampicillin resistance gene, 500-5000 kb inserts. Low copy number plasmid vectors such as pHOS (TIGR) and pSmart (Lucigen) series are thought to increase the randomness of small insert libraries. (2) Phage: A virus that infects bacterial hosts and may be utilized to introduce genes. Phages are/were widely used as cloning and expression vectors. Examples: M13, lambda phage. The M13 vectors are derivatives of the single-stranded, male-specific filamentous DNA bacteriophage M13. Double-stranded circular DNA (replicative form, or RF) can be isolated from cells by standard plasmid preparation techniques and used for cloning, while the single-stranded viral DNA (+ strand) can be isolated from phage particles collected from culture medium. Lambda phage has a linear 45 kb chromosome, in which 9-23 kb can be replaced with DNA to be cloned. P1 bacteriophage can hold more DNA than lambda and is the basis of PAC vectors. (3) Phagemid: A phage-plasmid vector able to replicate as single- or double-stranded DNA. Phagemids can be induced to produce phage particles containing single-stranded DNA. Example: pBluescript series (Stratagene), which contains a filamentous f1 phage intergenic region including the origin of replication. (4) Cosmid: Artificially constructed cloning vector containing the cos gene of phage lambda. Cosmids can be packaged in lambda phage particles for infection into *E. coli*; this permits cloning of larger DNA fragments (up to 45 kb) than can be introduced into bacterial hosts in plasmid vectors. Cosmids and cosmid recombinants replicate as plasmids. Likely to be less stable than plasmids because of large insert and high copy number. (5) Fosmid: vector containing the single copy *E. coli* F-factor replicon, developed as an improved method for constructing

libraries of cosmid-sized (approximately 40 Kb) clones. The stability of inserts cloned into fosmid vectors has been shown to be substantially greater than in high copy vectors. Copy control fosmids, e.g. pCC1fos, contain both the *E. coli* F-factor replicon and the oriV high copy origin of replication, thus providing the user the clone stability afforded by single-copy fosmid cloning and the high yields of DNA that can be realized from cosmid clones. (6) Artificial chromosomes: YACs: yeast artificial chromosomes (replicate in yeast) BACs: bacterial artificial chromosomes (replicate in *E. coli*); contain the *E. coli* F-factor single-copy origin of replication. PACs: DNA constructs that are derived from the DNA of P1 bacteriophage. They can carry large amounts (about 100-300 kb) of other sequence.

2.2.1 Binary Vectors of Ti Plasmid

One of the binary vectors constructed in the early days was pBin19 (Bevan, 1984), and pBI121 was created shortly after by adding a marker gene to pBin19 (Jefferson, 1987). Soon these vectors and their derivatives were widely distributed among plant scientists. Another popular series of vectors are pPZP vectors (Hajdukiewicz *et al.*, 1994) and pCAMBIA vectors (www.cambia.org), which are modified from pPZP vectors. Recent modifications of binary vectors provide a number of user-friendly features, such as wide selection of cloning sites, high copy numbers in *E. coli*, high cloning capacity, improved compatibility with strains of choice, a wide pool of selectable markers for plants, and a high frequency of plant transformation. Using a binary tumor-inducing (Ti) plasmid vector system, several plant species were transformed with a kanamycin resistance marker (*npt II* gene). Four *Nicotiana* species, seven tomato cultivars, two potato cultivars, and *Arabidopsis thaliana* were transformed by the binary vector transformation method.

In this method, various plant organ pieces were co-cultivated with *Agrobacterium tumefaciens* cells carrying the binary vector, pGA472, and a helper Ti plasmid.

The *Agrobacterium*-mediated transformation method was improved by developing modern binary Ti vectors after the removal of all the genes for tumor induction and opines synthesis. Ti plasmids without the tumor-inducing function are called disarmed plasmids (non-oncogenic Ti plasmid). Ti plasmids have been engineered to separate T-DNA and *vir* regions into two distinct plasmids, resulting in a binary vector and a *vir* helper plasmid, respectively (Hoekema *et al.*, 1983).

Since disarmed binary plasmids, containing the T-DNA region, do not have the ability to move a T-DNA into the plant, they need the help of another separate plasmid containing the *vir* genes. Many *Agrobacterium* strains containing non-oncogenic *vir* helper plasmids (LBA 4404, GV3101 MP90, AGL0, EHA101, and its derivative strain EHA 105) have been developed (Gelvin, 2003).

Binary vector plasmids are small and easy to handle in *E. coli* and *Agrobacterium* when the wild type Ti plasmid is around 200 kb. The sizes of the processed binary vectors from wild type Ti plasmids have been reduced to less than 10 kb. The binary vector has a replication origin for both *E. coli* and *Agrobacterium*, an antibiotic selectable marker for bacteria and plants, a reporter gene and a T-DNA region containing a multi-cloning site for insertion of genes of interest. The binary vector is transformed to *Agrobacterium* harboring a disarmed Ti-plasmid, called the helper plasmid, providing *vir* gene functions. The T-DNA region from the binary vector is transferred to the plant by expression of the *vir* gene in the helper plasmid (Klee *et al.*, 1983). A binary vector consists of T-DNA and the vector backbone. T-DNA is the segment delimited by the border sequences, the right border (RB) and the left border (LB), and may contain

multiple cloning sites, a selectable marker gene for plants, a reporter gene, and other genes of interest.

A binary vector was invented soon after it had been elucidated that crown gall tumorigenesis was caused by genetic transformation of plant cells with a piece of DNA, T-DNA for transferred DNA, from a Ti plasmid (tumor-inducing plasmid) harbored by the soil bacterium *Agrobacterium tumefaciens* (Fraley *et al.*, 1986). A key finding was that the virulence genes, which are involved in the transfer of T-DNA, could be placed on a replicon separate from the one with T-DNA (Hoekema *et al.*, 1983). Thus, combination of a “disarmed” strain, which carries a Ti plasmid without the wild-type T-DNA, and an artificial T-DNA within a plasmid that can be replicated both in *Escherichia coli* and *A. tumefaciens* turned out to be fully functional in plant transformation. The term “binary vector” literally refers to the entire combination, but the plasmid that carries the artificial T-DNA is usually called a binary vector.

The binary vector pBI121 has been widely used in plant transformation. The total size of pBI121 is 12.8 kb according to its construction map (Jefferson *et al.*, 1987); From construction experiments involving replacement of beta- glucuronidase (*gus*) gene in pBI121 by plant genes, it was found that a *SmaI-SacI* fragment (1.8 kb) carrying GUS coding region was the same size as predicted; however, the remaining *SmaI-SacI* fragment was approximately 13 kb, 2 kb larger than expected. In order to confirm the size of pBI121, the plasmid was double digested with *HindIII* and *EcoRI* (each restriction enzyme has only one recognition site within the plasmid), resulting in two fragments of 3 kb and 12-13 kb. The 3 kb *HindIII-EcoRI* fragment carrying the cauliflower mosaic virus (CaMV) 35S promoter (835bp), GUS coding region (1812 bp) and nopaline synthase

(NOS) terminator (253 bp); however, the 12-13 kb *EcoRI-HindIII* fragment would have been expected to be 9.8 kb.

Standard methods using pBI121 as the transforming vector for plant transformation have been successfully documented in Tobacco and *Arabidopsis* plants (Newell, 2000). More recently, *Agrobacterium*-mediated transformation using pBI121 as a donor for the Kanamycin resistance was developed for several plants including Peanut (Rohini and Sankara Rao 2001; Sharma and Anjaiah 2000), shallot (Zheng *et al.* 2001), spruce (Le *et al.* 2001), buckwheat (Kojima *et al.* 2000), poppy (Park and Facchini 2000) and pine (Tang 2001). Thus, the binary vector pBI121 is a valuable vector for plant transformation.

Great progress has been made in recent years in studies on the mechanism of *Agrobacterium*-mediated transformation and its application. Many details of the key molecular events within the bacterial cells involved in T-DNA transfer have been elucidated, and it is notable that some plant factors which were elusive before are purified and characterized. Vast kinds of species, which were either recalcitrant to or not included in the host range of *Agrobacterium*, can now, be transformed by this bacterium.

Agrobacterium tumefaciens has played a major role in the development of plant genetic engineering and the basic research in molecular biology. It accounts for about 80% transgenic plants produced so far. Initially, it was believed that only dicots, gymnosperms and a few monocot species could be transformed by this bacterium; but recent achievements totally changed this view by showing that many “recalcitrant” species not included in its natural host-range such as monocots and fungi can now be transformed. In addition, the transformed cells usually carry single or low copy number T

DNA integrated in their genome with less rearrangement, and very large DNA segments can be transformed into the plants.

2.2.2 Expression vectors

Expression systems are designed to produce many copies of desired protein within a host cell. In order to accomplish this, an expression vector is inserted into a host cell. This vector contains all of the genetic coding necessary to produce the protein, including a promoter appropriate to the host cell, a sequence which terminates transcription, and the sequence which codes for ribosome binding (Purves *et al.*, 2001) that yielded the protein products of the cloned genes. The main function of an expression vector is to yield the product of a gene, therefore a strong promoter is necessary. The more mRNA is produced, the more protein product is made.

A pET vector is a bacterial plasmid designed to enable the quick production of a large quantity of any desired protein when activated. This plasmid (pictured below) contains several important elements - a *lacI* gene which codes for the *lac* repressor protein, a T7 promoter which is specific to only T7 RNA polymerase (not bacterial RNA polymerase) and also does not occur anywhere in the prokaryotic genome, a *lac* operator which can block transcription, a polylinker, an f1 origin of replication (so that a single-stranded plasmid can be produced when co-infected with M13 helper phage), an ampicillin resistance gene, and a ColE1 origin of replication (Blaber, 1998).

2.3 Important *Bt* transgenic crops

As of now, more than 30 plant species have been transformed with *Bt cry* genes Schuler, *et al.*, 1998. (Table 2.3). The commercialization of *Bt*-crops started in 1996 with the introduction of bollworm-resistant cotton ('Bollgard') in USA. Subsequently, potato and maize were also commercialized Krattiger, A. F., (1997). In India, intensive efforts

are underway to introduce *cry* genes in crop plants such as rice, potato, cotton, sorghum and vegetables. The first transgenic plants of tobacco (*cv.* Hema and Jayasri) developed at the Tata Energy Research Institute by using modified *cry1Ab* and *cry1C* (obtained from Dr Bert Visser, CPRO-DLO, and the Netherlands) showed considerable protection against tobacco caterpillar (*Spodoptera litura*) in limited field trials conducted at the Central Tobacco Research Institute (CTRI).

Table 2.3: Transgenic *Bt* Crops

Crop	Gene	Target pests	Reference
Cotton	<i>cry1Ab/cry1Ac</i>	Bollworms	Perlak <i>et al.</i> , (1990).
Corn	<i>cry1Ab</i>	European corn borer	Koziel M. G. <i>et al.</i> , (1993).
Potato	<i>cry3a</i>	Colorado potato beetle	Perlak F. J. <i>et al.</i> , (1993).
Rice	<i>cry1Ab/cry1Ac</i>	Stem borers & leaf folders	Nayak P. <i>et al.</i> , (1997).
Tomato	<i>cry1Ac</i>	Fruit borers	Mandaokar, <i>et al.</i> , (2000).
Potato	<i>cry1Ab</i>	Tuber moth	Jansens <i>et al.</i> , (1995).
Eggplant	<i>cry1Ab/cry1B</i>	Shoot and fruit borer	Kumar <i>et al.</i> , (1998).
Canola	<i>cry1Ac</i>	Diamondback moth	Stewart <i>et al.</i> , (1996).
Soybean	<i>cry1Ac</i>	Soybean looper	Stewart <i>et al.</i> , (1996).
Corn	<i>cry1H/cry9C</i>	European corn borer	Jansens, S. <i>et al.</i> , (1997).

2.3.1. Transgenic *Bt* approaches for pest control and plant protection

Bacillus thuringiensis is the most widely used biologically produced pest control agent. As of early 1998, there were nearly 200 *Bt* products registered by U.S. Environmental Protection Agency. Though the use of biological pesticide in agriculture remains behind that of synthetic chemical pesticides, several safety and environmental considerations favor the development of *Bt* in future. *cryI* proteins that have been studied

so far, are not pathogenic to mammals, birds, amphibians or reptiles, but are specific to the target insects.

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

The main advantages of *Bt* transgenic plants against different pests include: (1) Protection of crop for whole season independent of weather condition. (2) Effective control of burrowing insects which escape the reach of sprays. (3) Control at all stages of insect pest development. (4) Non-injurious to beneficial insects like pollinators, predators/natural enemies of pests and also non-injurious to mammals including human beings.

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

Fischhoff *et al.*, (1987) was successful in transforming tomato plants with a native *cryIA(b)* gene which showed insecticidal activity. Similarly a native *cryIA(c)-npt II* fusion in potato showed little insecticidal activity (Ebora *et al.*, 1994). Carozzi *et al.*,

(1992) characterized the expression of native *cryIA(b)* endotoxin gene in field grown tobacco and observed that the level of *Bt*, (-endotoxin increased through the course of plant development with a substantial increase at the time of flowering). When unmodified crystal protein genes are fused with expression signals used in the plant nucleus, protein production is quite poor as compared to that of similar genes containing typical plant marker genes. However, truncation of the unmodified genes to synthesize only the toxin protein of the protein notably results in much improved expression. The relatively AT-rich *Bacillus* genome contains a number of sequences that could provide deleterious signals to gene expression in plants; such signals may be splice sites, poly 'A' addition sites, ATTTA sequences, transcription termination sites, as well as bias in codon usage. Plants show strong preference for coding sequence (Murray *et al.*, 1989). When the native gene sequences which is poor in GC content in coding sequence. When the native gene sequences are modified with synonymous codons to reduced the potential deleterious sequence and generate a codon usage more like that of a plant, expression improves dramatically. (Murray *et al.*, 1989). Instability of mRNA could be due to endonucleolytic / exonucleolytic degradation at specific sequences that destabilize the message during transcription or create pausing due to formation of secondary structures.

Perlak and others at Monsanto in 1990-91 made a pioneering study of the expression of partially modified and fully modified (synthetic) *cryIAb* and *cryIAc* genes in cotton. The level of insecticidal protein was increased by 100 fold, resulting in the effective control of cotton boll worm. Adang *et al.*, (1993) modified *cry3A* gene in a similar way and transformed potato plants conferring resistance to Colorado potato beetle. This improved expression of *cry3A* gene was due to increase of GC- content from 36 per cent to 49 per cent in modified gene.

Table 2.4. Milestones in transgenic crop research involving *Bt*

Year	Milestone	Reference
1998	20% of US cotton crop was <i>Bt</i> cotton & 9% of corn was <i>Bt</i> corn	Wadman 1999
1996 onwards	<i>Bt</i> transgenic cultivar of maize and cotton released for commercial use	Estruch <i>et al.</i> , 1997
1995	Used rational protein design to engineer PI with greater inhibitory activity	Urwin <i>et al.</i> 1995
1991	Modified the GC content of <i>Bt</i> gene for higher expression in plants	Perlak <i>et al.</i> , 1991
1987	Expressed cowpea TI gene in tobacco and showed the transgenic plant to be insect-resistant	Hilder <i>et al.</i> , 1987
1987	Expressed <i>Bt</i> gene in tobacco and tomato and showed the transgenic plant to be insect-resistant	Vaeck <i>et al.</i> , Barton <i>et al.</i> , Fishchoff <i>et al.</i> , 1987
1981	Gene for <i>Bt</i> crystal protein cloned in <i>E.coli</i>	Schnepf and Whitely 1981

2.4 Methods of transformation of tobacco

2.4.1 Tissue culture based techniques for tobacco

In the past, plant tissue culture techniques have been used in academic investigations of totipotency and the roles of hormones in cytodifferentiation and organogenesis. Currently, tissue-cultured plants that have been genetically engineered provide insight into plant molecular biology and gene regulation. Plant tissue culture techniques are also central to innovative areas of applied plant science, including plant biotechnology and agriculture. In addition, the management of genetically engineered cells to form transgenic whole plants requires tissue culture procedures.

A tissue culture cycle involves the establishment of a more or less differentiated cell or tissue culture under defined conditions, proliferation for a number of cell generations and subsequent regeneration of plants (Larkin and Scowcroft, 1981). The concept of cell culture was first originated by Haberlandt (1902) who attempted to cultivate isolated plant cells *in vitro* on an artificial medium. The first plant tissue culture,

in the sense of long-term culture of callus, involved explants of cambial tissues isolated from carrot (Gautheret, 1939; Nobecourt, 1939) and tobacco tumor tissue from hybrid tobacco (White, 1939). *In vitro* organogenesis could be chemically controlled by a balance between cytokinin and auxin was first indicated by Skoog (1944). According to White (1951) the selection of right plant material and the formulation of satisfactory nutrient medium are the two main factors for successful culturing of excised plant material. At present plant tissue culture technology has been reached in its much advance stage and making significant contributions to agriculture and industry.

2.4.1.1 Plant tissue culture of tobacco- a model plant

In vitro tissue culture is an experimental approach for basic and applied research. It is an essential component of breeding, biotechnology and genetic improvement of plants. Every plant cell is considered to be totipotent. The potentiality to readily regenerate plants from cultured tissue has been considered as a powerful tool for improvement and the simplest form of genetic engineering (Larkin and Scowcroft, 1981). The prerequisite of *in vitro* culture is the identification of tissues or cells which are competent for regenerating whole plant. Sub-culturing of tobacco twigs (with nodal segments) in the MS basal medium produce sufficient leaves for the transformation. Plant tissue culture is typically performed using a defined basal media, which is supplemented with hormones when optimum growth and differentiation are desired. A defined media usually consists of macro- and micro- salts, an exogenous energy source like sucrose. For the culturing of tobacco (*Nicotiana*) we will use a modification of the well-known media formulation of Murishige and Skoog (1962). Leaf discs were obtained from four week-old tobacco plantlets grown *in vitro*. Sterile tobacco leaves were cut from plantlets and soaked in MS liquid media in a sterile Petri dish to avoid de-hydration. Fifteen leaf disks

were placed per Petri dish on the co-cultivation media containing per liter: 4.4 g MS basal salts, 1.0 mg nicotinic acid, 1.0 mg pyridoxine HCl, 0.1 mg thiamine HCl, 100 mg *myo*-inositol, 1.0 µg 6-benzylaminopurine (BA), 0.1 µg indole-3-acetic acid (IAA), 200 µM AS, 30.0 g sucrose, 10.0 g agar at pH 5.6 without antibiotic (Burow *et al.*, 1990). Petri dishes were covered and sealed well with a strip of parafilm.

2.4.1.2 *Agrobacterium*-mediated gene transfer approach

Plants have been genetically engineered for the purpose of developing resistance to herbicides, insects or viruses; tolerance to drought, salt or cold; and increasing yield (Birch, 1997). At present *Agrobacterium*-mediated gene transfer is the most widely used system for genetic transformation of higher plants. *A. tumefaciens* is a gram negative soil bacterium which infects a wide range of dicot plant species. Transgenic tomatoes do not express the gene for polygalacturonase, an enzyme that degrades pectin, leading to softening of fruit tissues. As a result, the tomatoes can accumulate flavor components for a longer period of time. Cotton, potato and maize were genetically engineered for insect resistance, and soybean, canola and cotton were genetically developed for herbicide resistance. The *Agrobacterium*-mediated transformation method has not only been used for commercial purposes, but also for basic biology research to study gene regulation or protein function in transgenic plants (McIntosh *et al.*, 2004). Although it has been possible to make a genetically modified plant in some plant species, it is still not easy to make transgenic plants of all plant species. This may be due to the poor transformation event caused by improper environmental conditions during bacterial infection of the plant, poor plant regeneration frequency, gene silencing due to position effects or transgene copy number after stable integration into the plant chromosome. The

Agrobacterium-mediated transformation method still requires improvement in these aspects.

2.5 Molecular Mechanism of *Agrobacterium*-mediated DNA Transfer.

2.5.1 Bacteriology, Host Range and Opines

The *Agrobacterium tumefaciens*-mediated transformation method is commonly used to create transgenic plants because it has several merits compared with direct gene transfer methods such as particle bombardment, electroporation, and silicon carbide fibers. The advantages are: (1) Stable gene expression because of the insertion of the foreign gene into the host plant chromosome; (2) Low copy number of the transgene; and (3) Large size DNA segments can be transferred. Agronomically and horticulturally important crops, flowers and trees have been genetically modified using this method (Ko and Korban, 2004; Lopez *et al.*, 2004).

Agrobacterium tumefaciens is a gram-negative bacterium and soil phytopathogen that genetically transforms host plants and causes crown gall tumors at wound sites (Smith and Townsend, 1907). The interaction of *Agrobacterium* and eukaryotic cells is the only known mechanism for DNA transport between the different kingdoms in nature. *Agrobacterium* is usually classified by the disease symptomology (type of opine) and host range. *Agrobacterium* can transfer DNA to a broad group of organisms: angiosperms (dicots and monocots), gymnosperms and fungi, such as yeasts, ascomycetes, and basidiomycetes. Recently, it was found that *Agrobacterium* can transfer DNA to human cells (Gelvin, 2003). The genetic mechanism of host range determination is still obscure, but it was reported that several virulence (*vir*) genes on the Ti plasmid, *virC* (Yanofsky and Nester, 1986), *virF* (Regensburg-Tuink and Hooykaas, 1993), and *virH* (Jarchow *et al.*, 1991) were involved in determination of the range of plant species. Hood *et al.*

(1987) reported that the Ti plasmid in a natural host strain *A. tumefaciens* Bo542 cannot transform leguminous plant species, but when it is placed in the C58 chromosomal background, pTi Bo542 causes tumors in soybeans and other legumes. Interaction of the Ti plasmid with a particular chromosomal background in bacteria may also control the host range. However, it appears that the plant host also plays a role in transformation, because many monocot plant species such as maize, rice, barley and wheat can not be genetically transformed by *Agrobacterium* without addition of chemicals, or these plants do not develop a crown gall tumor after transformation (Gelvin, 2003). Complex genetic mechanisms of the host plants are also necessary for transformation by *Agrobacterium*.

Agrobacterium is an effective tool for plant genetic engineering, since a portion of the plasmid DNA from *Agrobacterium* is incorporated into higher plant cells and results in crown gall in the host plant (Chilton *et al.*, 1977). Tumor induction is initiated by bacterial recognition of monosaccharides and phenolic compounds secreted by the plant wound site. “Activated” *Agrobacterium* transfers a particular gene segment, called transfer DNA (T-DNA), from the Ti plasmid, and T-DNA is stably integrated into the chromosomal DNA in nucleus of the host plant; the genes for opine synthesis and tumor inducing factors in T-DNA are transcribed in the infected cells. This expression of the foreign gene in the host plant results in neoplastic growth of the tumors, providing increased synthesis and secretion of opines for bacterial consumption (Nester *et al.*, 1984). Opine is the condensation product of an amino acid with a keto acid or sugar, and is a major carbon and nitrogen source for *Agrobacterium* growth. *Agrobacterium* is classified based on the type of opine. Different *Agrobacterium tumefaciens* strains produce different opine phenotypes of crown gall tumors, because a particular opine expressed in the tumor is used for particular bacterial growth. Most common

Agrobacterium strains produce an octopine or nopaline form of opines (Hooykaas and Beijersbergen, 1994). Octopine and nopaline are derivatives of arginine. Agropine was found in octopine-type tumors, and it is derived from glutamate (Guyon *et al.*, 1980).

2.5.2 Tumor-inducing (Ti) Plasmid

Several components of *Agrobacterium* are necessary for transferring the piece of bacterial DNA into the plant cell. One component is the chromosomal virulence A (*chvA*) gene, which is on the *Agrobacterium* chromosome and activated by sugars. ChvA protein triggers bacteria to bind to the wounded plant tissue and to respond to a specific chemical (chemotaxis). The Ti plasmid in bacteria contains the other main components, which are generated or activated efficiently for causing crown gall in host plants after bacteria attach to the plant wound site. The first is T-DNA, which is actually integrated into the plant cell chromosome. The second is the 35-kb virulence (*vir*) region, which is composed of seven loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virG*, and *virH*). Expression of *vir* genes is triggered by a phenolic compound, which is secreted from the wound site of the host plant. The main functions of Vir proteins are to mediate the T-DNA excision from the Ti plasmid, export of the T-DNA piece from the bacteria, and insert it into the host plant chromosome (Gelvin, 2003). These two components are essential for a successful gene transfer. The Ti plasmid also has other components, an opine catabolism region, a conjugal transfer region, and a vegetative origin of replication of the Ti plasmid (*oriV*). Engler *et al.* (1981) found that these *vir* regions have sequence conservation between the octopine and nopaline Ti plasmids.

2.5.3 Transfer DNA (T-DNA) of Ti Plasmid

T-DNA is the DNA segment transferred into the plant cell. The T-DNA is present on the Ti-plasmid of the wild type *Agrobacterium* and its size is an average of 25 kb,

ranging from 10 to 30 kb. The T-DNA region is flanked and delineated by two 25bp direct repeats, known as the right border and left border (Sheng and Citovsky, 1996). These border sequences are highly homologous and are targets of the border-specific endonuclease (VirD1/VirD2). The excised single strand of T-DNA from the Ti plasmid is exported from the bacterial cell to the plant cell by the activity of the other *Agrobacterium* Vir proteins. Howard *et al.* (1992) showed that deletion of the right border leads to a reduction of virulence, whereas the left border does not. These data suggested that the right border is essential for *Agrobacterium* pathogenicity. They concluded that the transfer of T-DNA is directed from the right to left border by the polarity. Additional evidence, that the right border is more important than left border, is that the VirD2 protein can alone bind to the single stranded right border sequence and cleaves a single-stranded T-DNA. The VirD2 protein remained on the 5'-end (right border) of the resulting single stranded T-DNA molecule, termed the T-strand (Jasper *et al.*, 1994). T-DNA of octopine-type Ti plasmid has an overdrive sequence near the right border, but not left border, which may enhance the functional polarity of right border and left border (Gelvin, 2003).

Wild type T-DNA also has genes that are involved in plant hormone synthesis in the host plant. They are the *tml*, *tms*, and *tmr* regions for leafy tumor, shooty tumor, and rooty tumor, respectively, in the plant wound site (Ream *et al.*, 1983). The opine synthase region is also located within the T-DNA. *Agrobacterium* strains are classified based on opine type, and components of the T-DNA are different in different opine types. If the Ti plasmid is a nopaline type, all components on T-DNA are a contiguous stretch, whereas the octopine type T-DNA consists of three individual parts, left (13kb), central (1.5kb), and right (7.8kb) Each segment has T-DNA border repeat sequences (Sheng and

Citovsky, 1996); therefore, these segments are transferred separately to the other organism due to the sequence. After T-DNA is integrated into the host plant, opine is synthesized, and then secreted out of the cell and imported into *Agrobacterium*. The absorbed opine molecule is catabolized by a specific enzyme in *Agrobacterium*. Opine is degraded into amino acid and the sugar moieties, which can be used as carbon and energy sources for bacterial growth.

2.5.4 Factors affecting transformation Efficiency

Transformation efficiency can be increased by the manipulation of either the plant or bacteria for enhancing competency of plant tissue and *vir* gene expression, respectively (Henzi *et al.*, 2000; Mondal *et al.*, 2001; Lopez *et al.*, 2004; Chakrabarty *et al.*, 2002). Seedling age and pre-culturing of explants have been tested to increase the transformation efficiency. These trials were conducted to determine the best conditions for plant cell infection or increasing the number of dividing plant cells before bacterial infection (Amoah *et al.*, 2001; Chakrabarty *et al.*, 2002; Mets *et al.*, 1995). To increase the virulence of bacteria by inducing the *vir* gene expression, temperature (Dillen *et al.*, 1997; Chakrabarty *et al.*, 2002; De Clercq *et al.*, 2002), medium pH (Mondal *et al.*, 2001; Holford *et al.*, 1992; Godwin *et al.*, 1991; De Clercq *et al.*, 2002) and chemical inducers, such as acetosyringone (SomLeva *et al.*, 2002; Lopez *et al.*, 2004; Le *et al.*, 2001; Chakrabarty *et al.*, 2002; Henzi *et al.*, 2000; Cervera *et al.*, 1998; De Clercq *et al.*, 2002; Stachel *et al.*, 1985), have been tested. These factors likely enhance bacterial pili formation required for gene transfer between bacteria, as well as between the bacteria and plants. Manipulation of other factors, such as bacterial density, co-cultivation duration, surfactant, and vacuum infiltration, have also increased transformation efficiency in many experiments (Mondal *et al.*, 2001; Lopez *et al.*, 2004; Cheng *et al.*, 1997; Amoah *et al.*,

2001). According to previous experiments, inducing *vir* gene expression seems most important and effective for increasing plant transformation efficiency, regardless of the type of plant being studied. Although low temperature was very important to induce bacterial pili formation by inducing the *vir* gene, optimization experiments were conducted at room temperature or over 25°C for co-cultivation.

2.5.4.1 Effect of *Agrobacterium* Concentration

It has been considered in several experiments that transformation efficiency might be affected by bacterial growth phase and bacterial cell density. Different concentrations of the *Agrobacterium* have been used by different research groups and for different plant materials. In the standard protocol, cells are grown to the stationary phase ($A_{600nm} \approx 2-2.4$), pelleted and resuspended in inoculation medium to stationary or log or mid-log phase ($A_{600nm} \approx 0.1-1.15$). High concentrations of bacteria at the stationary phase have normally been used for rice, legume and tobacco transformation (Hood *et al.*, 1987; Kapila *et al.*, 1997; Dillen *et al.*, 1997), and low concentrations of bacteria at the log or mid-log phase have been used for broccoli (Mets *et al.*, 1995), cabbage (Henzi *et al.*, 2000), wheat (Cheng *et al.*, 1997), cottonwood (Han *et al.*, 2000), and tobacco (Krugel *et al.*, 2002).

2.5.4.2 Effect of Co-cultivation Duration

Co-cultivation for 2 to 7 days has been normally used in *Agrobacterium*-mediated transformation under various co-cultivation temperatures (Han *et al.*, 2000; Mondal *et al.*, 2001; Cervera *et al.*, 1998; SomLeva *et al.*, 2002). Co-cultivation for 3 days resulted in high transformation efficiency. Many transformation experiments in different plant species, such as tea (*Camellia sinensis* L.), cauliflower, white spruce (*Picea gluca*) and citrange, showed that 2 to 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; Cervera *et al.*, 1998). The co-cultivation duration recommended from published protocols for tobacco leaf disk transformation varies from one research group to another. They are 2, 3 and 4 days at 26°C, 24°C, and room temperature cocultivation conditions (Svab *et al.*, 1995). Most previous experiments indicated that 2 to 3 days were optimal at 25°C co-cultivation conditions, regardless of plant species. No experiments have been conducted yet to find an optimal co-cultivation duration at lower than 25°C.

2.6. Current status of transgenic crops in India

India recently made its long-awaited entry into commercial agricultural biotechnology when the Genetic Engineering Approval Committee (GEAC), Ministry of Environment and Forests, Govt of India, at its 32nd meeting held in New Delhi on 26th March 2002 approved three *Bt*-cotton hybrids for commercial cultivation. This is a historic decision as *Bt*-cotton became the first transgenic crop to receive such an approval in India. These transgenic hybrids were developed by MAHYCO (Maharashtra Hybrid Seed Company Limited) in collaboration with Monsanto. These contain Monsanto's lepidopteron specific Bollgard® *Bt* gene, *cry1Ac*, which offers protection against all the major species of Indian bollworms - *Helicoverpa armigera*, *Pectinophora gossypiella*, *Earias vittella* and *E. insulana*. These bollworms, especially *H. armigera*, have been responsible for heavy yield losses. Annual losses caused by bollworms alone are estimated at about US\$ 300 million despite repeated spraying of insecticides (6 to 16 times or more for each crop). It is estimated that insecticides valued at \$ 700 million are used on all crops annually in India, of which about 50% are used on the cotton crop alone. Since dependable alternative methods were not available, farmers had no option

except to 'spray' or 'pray.' *Bt*-cotton has brought in a ray of hope (Barwale *et al.*, 2004; Mohan and Manjunath, 2002; Manjunath, 2004).

The approval of *Bt*-cotton or Bollgard® (Bollgard® is the registered brand name for Monsanto's *Bt*-cotton) in India was preceded by about 500 field trials carried out in different agro-climatic regions between 1998 and 2001 to assess its efficacy against bollworms and the concomitant agronomic benefits. Experimental data on the bio-safety of *Bt*-cotton were generated by several public funded research institutions as per the direction of Department of Biotechnology (DBT), Ministry of Science & Technology, Government of India.

In addition to many trials conducted by MAHYCO as per DBT's guidelines and supervision, several *Bt*-cotton hybrids were also evaluated by ICAR (India Council of Agricultural Research) in multi-location field trials in 2001.

The *Bt*-cotton hybrids approved were MECH 12, MECH 162 and MECH 184. Following the approval, Mahyco-Monsanto Biotech Limited (MMB), a joint venture of Mahyco and Monsanto, which had only a limited stock of the *Bt* cotton seeds, sold these in six states of south and central India (Andhra Pradesh, Karnataka, Tamil Nadu, Maharashtra, Madhya Pradesh and Gujarat) to cover about 29,415 hectares. Thus, the first commercial planting of *Bt*-cotton occurred in the second half of 2002. Following its success, the area of *Bt*-cotton increased to 86,240 ha in 2003 and to 530,800 ha in 2004.

Table 2.5. Status of field trials of GM/Biotech Crops in India 2008

Institute	Crops	Transgene(s)	Purpose
Central Institute for Cotton Research, Nagpur	Cotton	<i>Bt. cry</i> gene(s)	resistant to lepidopteran pests
Central Potato Research Institute, Simla	Potato	<i>Bt. cryIA(b)</i>	resistant to lepidopteran pests
Central Tobacco Research Institute, Rajahmundry	Tobacco	<i>Bt.cryIA(b)</i> and <i>cryIC</i>	resistant to <i>Helicoverpa armigera</i> and <i>Spodotera litura</i>
Narendra Dev University of Agriculture, Faizabad	Rice	<i>CryIA(b) gene</i>	resistant to lepidopteran pests, bacterial and fungal diseases
National Botanical Research Institute, Lucknow	Cotton	<i>CryIE</i> and <i>CryIC</i> with terminal altered at C-end	resistant to <i>Spodopotera litura</i> and <i>Heliiothisis armigera</i>
Directorate of Rice Research, Hyderabad	Rice	<i>Xa-21, cryIA(b)</i>	resistant to lepidopteran pests and bacterial and fungal diseases
Indian Agricultural Research Institute, New Delhi	Brinjal Tomato Cauliflower Cabbage	<i>Bt. cryIA(b)</i> <i>Bt. cryIA(b)</i> <i>Bt. cryIA(b)</i> <i>Bt. cryIA(b)</i>	resistant to lepidopteran pests resistant to lepidopteran pests resistant to <i>Plutella scylostella</i> resistant to <i>Plutella scylostella</i>
International Centre for Genetic Engineering and Biotechnology, New Delhi	Tobacco	<i>Bt. cryIIa5</i>	resistant to <i>Spodoptera litura</i>
Punjab Agricultural University, Ludhiana	Rice	<i>CryIAb,</i> <i>CryIAc</i>	resistance against yellow stem borer

CHAPTER- III

MATERIALS AND METHODS

The present investigation entitled “**Cloning and characterization of Ti plasmid based vectors carrying *Bt* genes for developing high efficiency plant transformation system.**” was carried out at Department of Biotechnology, Indira Gandhi Krishi Vishwavidyalaya, Raipur. The detail of the various experiments performed is explained below.

3.1 Materials

3.1.1 Plant material

Tissue cultured tobacco (*Nicotiana tabacum* cv. petit Havana) plants available in the Plant Tissue Culture lab of Department of Biotechnology, college of Agriculture, I.G.K.V., Raipur was used for the study. Fresh leaves, about 20-25 days old, were taken as explants and cut into square sections of about 0.5 cm², 0.75 cm² and 1.0 cm² by using a scalpel.

3.1.2 Bacterial strains

Escherichia coli - DH5- α

Agrabacterium strain- LBA4404

Binary vector- pBI121 (plasmid backbone)

3.2 Construction of recombinant vectors

Two *Bt* genes (*mVIP* and *mcry1Ac*) were cloned into the expression vector pET-29(a) (Fig.3.1) having T7 promoter and T7 termination sequences (Appendix I), obtained from ICGEB, New Delhi through material transfer agreement (MTA). The two genes have been tested for insecticidal activity against major lepidopteran pests viz., Pink boll

worm, borers or brinjal, cabbage etc. The modified genes *mcry1Ac* and *mVIP* have shown highly effective against the lepidopteran pest especially *Scodoptera litura*, *Earias insulana*, *Chilo partellus*, *Spilosoma obliqua*, *Maruca testulalis*, *H. armigera* and *L. orbonalis* (selvapandiyan 1998).

In the present investigation *mcry1Ac* and *mVIP* genes were cloned into Ti plasmid pBI121 (Fig. 3.2) which is a binary vector containing NPT II marker gene for developing *Agrobacterium* based plant transformation. Cloning strategies adopted for cloning *Bt* genes from pET-29(a) vector carrying *mVIP* and *mcry1Ac* genes into binary vector (pBI121). The pET-29(a) vector carrying *mVIP* and *mcry1Ac* genes was digested with *BamH* I and *SacI* restriction endonucleases to obtain 1.2 kb *mVIP* and 1.8 kb of *mcry1Ac* genes (inserts). The binary vector pBI121 was also digested with *BamH* I and *SacI* to obtain the vector backbone DNA having similar cohesive ends as that of pET-29(a). A suitable vector for high efficiency transformation must possess following characteristics (1) beta-glucorinidase (GUS) cassette with CaMV 35S promoter in the binary vector pBIN 19 (2) high levels of GUS activity upon Ti-mediated transformation of tobacco cells (3) large DNA molecule transfer capacity (10^6 - $10^7/\mu\text{g}$) (4) Kanamycin resistance gene for selection in bacterial and plant cells:.

The one step ligation reaction was performed to clone the 800 bp insert have *BamH* I and *Sac* I fragment into the pBI121 vector backbone having the sticky ends for the insert used for cloning. The *mVIP* gene was cloned into pBI121 backbone in between 35S promoter and Nos terminator. Many putative bacterial colonies grown on the selective medium supplemented with kanamycin 50mg/l LB plates. The plasmid DNA was isolated from the putative positive clones and checked by restriction digestion method to identify the right clone to be used for transformation.

3.2.1 Isolation of plasmid DNA

Methods

- Bacterial cells, *E. coli* containing the desired clone pET-29(a) and pBI121 were grown overnight at 37° C in LB (Luria-Bertani) medium containing suitable antibiotic (Kanamycin) @ 50mg/L.
- The cells were harvested by centrifuging 1.5 ml of *E. coli* culture at 12000 rpm for 7-8 min at room temperature (RT).
- The supernatant was discarded completely and left the eppendorf tubes in an inverted position on a tissue paper for 4-5 minutes to drain off LB completely.
- 50 µl of Lysozyme was added and the pellet was resuspended in 200 µl of solution I by gentle vortexing or with the help of pipetteman.
- 400µl of freshly prepared solution II was added and mixed immediately by gently inverting the tubes few times till cell suspension became clear and incubated on ice for 5 minutes.
- Then 300 µl of chilled (stored at -20°C) solution III was added and mixed thoroughly but gently by inverting the tubes several times till a white coarse precipitation was visible and then incubated on ice for 15 min.
- Centrifuged at 12,000 rpm at 4°C for 20 min and ~700 µl of clear supernatant was transferred to a fresh micro centrifuge tube. Centrifuged again at 12,000 rpm at RT for 3 min and transferred 600 µl of clear supernatant to a fresh micro centrifuge tube.
- 450 µl of isopropanol was added and mixed well and incubate at RT for 10 minutes then pellet down plasmid DNA by centrifuging at 12,000 rpm for 5 minutes at RT.

- Then supernatant was discarded carefully by decanting and left the tubes in an inverted position on a tissue paper for few minutes to drain off isopropanol completely.
- The pellet was washed with 80 μl of 70% ethanol to the micro centrifuge tube and then centrifuged at 12,000 rpm for 5 min at RT. The supernatant was discarded carefully by decanting. Centrifuged once again at above conditions for 1 min only and discarded the remaining ethanol with the help of pipette man.
- The DNA pellet was air dried for 5 min and dissolved in 25-50 μl of TE buffer containing RNase A (50 μg /ml).
- The DNA was resolved on 1% agarose gel by electrophoresis as well as Nanodrop Spectrophotometer to check the quality and concentration of DNA.

3.2.2 Restriction Digestion of plasmid DNA

Restriction digestion of plasmid DNA was set-up in the following way. It is desirable to make the cocktail and dispense into eppendorf tubes containing plasmid DNA.

Method: Setting-up digestion reaction

Table 3.1. Reaction mixture for Single digestion

Components	Volume (μl)
Plasmid DNA	4.0
Restriction buffer (10 X)	2.0
Restriction enzyme (<i>EcoR</i> I)	1.0
Water (Millipore)	13.0
Make up the final volume	20.0 μl

Table 3.2. Reaction mixture component for Double Digestion

Components	Volume (μl)
Plasmid DNA	4.0
Restriction buffer (10 X)	2.0
Restriction enzyme (<i>Bam</i> H I)	1.0
Restriction enzyme (<i>Sac</i> I)	1.0
Water (Millipore)	12.0
Make up the final volume	20.0 μl

The micro centrifuge tubes containing reaction mixture was incubated at 37⁰C for 1.5-2 hours. The digested sample was resolved on 1% Agarose gel to check proper digestion.

3.2.3 Spectrophotometer based quantification of Nucleic Acid

The quality and quantity of the nucleic acid was determined by measuring the absorbance at 260 nm and 280 nm. The amount was calculated taking 1.0 μ l (A_{260nm}) = 50 μ g/ml for DNA. The purity of the nucleic acid was determined by calculating the ratio A_{260}/A_{280} for each sample.

3.2.4 Elution of Plasmid DNA from the Agarose gel using silica column:

- The gel slice was cut from the agarose gel containing plasmid DNA (pBI121 as vector backbone and inserts *mVIP* and *mcry1Ac* from the separate gels).
- The vector backbone and inserts were collected along with gel slice in separate eppendorf tubes.
- 700 μ l of binding buffer was added and incubated at 60⁰C for 10-15 minutes.
- Kept at room temperature for 5 min.
- The gel mixed buffer was passed through the silica column twice by spinning at 12,000 rpm for 1 min.

- The column was washed with 750µl of wash buffer by spinning at 12,000 rpm for 1 min.
- Then air dried the column for 10 min.
- 50µl of 10mM Tris-HCl (pH-8) was added for elution of DNA.

3.2.5 Ligation of insert with vector backbone

The ligation of digested plasmid DNA with the gene of interest (*mVIP* and *mcry1Ac* separately) was set-up in the following way: The reaction mixture of 20 µl was kept for ligation of *mVIP* and *mcry1Ac* (inserts) into pBI121 vector backbone with the ligase enzyme. In general, vector: insert ratio of 1:3 was recommended. In most cases either 1:1 or 1:3 molar ratio of vector: insert works well. It works great, if the insert size was small or may be the same size as the vector. This has been suggested by Iruela-Arispe Lab University of California, Los Angeles.

Table 3.3 Components for ligation reaction

Components	Volume (µl)
Digested plasmid DNA (vector DNA)	1.0
Gene of interest (insert)	1.0
Ligase buffer	2.0
Ligase enzyme	2.0
Water (Millipore)	14.0
Make up the final volume	20.0 µl

The stepwise protocol followed for ligating the two genes, one at each turn, to the vector backbone is as under

- Millipore water (14.0 µl) was taken in sterile 1.5 ml tube.
- 2 µl of ligation buffer was added to the tube.

- Buffer was gently vortexed before use and due care was taken because buffer contained ATP, so repeated freeze, thaw cycles can degraded the ATP thereby efficiency of ligation may decreased.
- 1.0 μl of insert was then added to the tube containing buffer along with 1.0 μl of vector DNA.
- 2.0 μl of ligase was then added and whole ligation reaction mixture was incubated at 22.5°C for 30 minutes.
- The ligase enzyme was denatured at 65°C for 10min and dialyzed for 20 minutes and finally stored at -20°C.

3.3 Recombinant DNA techniques for cloning and DNA analysis

3.3.1 Preparation of competent *E. coli* cells for transformation

- Single bacterial (*E coli*- DH5- α) colony was picked and suspended into 5 ml of LB medium. Grown at 37⁰C, 200 rpm for 16-20 hours.
- Then 1 ml of the growing culture was transferred into 50 ml LB broth and was grown under similar conditions for 2.5 to 3 hours, till the optical density of the culture reaches 0.4 at 600 nm.
- The culture was transferred into sterile polypropylene tubes and was stored in ice for 10 min.
- Then it was centrifuged at 3000 rpm for 10 min at 4⁰C, to recover the cells.
- The cells were resuspended in 20 ml of 100mM CaCl₂ by gentle swirling. The cells were again recovered by centrifugation at 3000 rpm for 10 min at 4⁰C.
- The pellet was resuspended in 3 ml of cold 100 mM CaCl₂ and 1.5 ml of 50% sterile glycerol was added into it.
- Aliquot of cells was taken in sterile eppendorfs (150 μl) and store at -80⁰C.

Table 3.4 Composition of the LB medium (Luria-Bertani)

Constituents	Concentration (g/L)
Nacl	10g
Tryptone	10g
Yeast extract	5g
Agar	15g

3.3.2 Bacterial transformation using heat-shock method

- The competent *E. coli* (DH5- α) cells were removed from -70°C on ice and add the plasmid DNA (pBI121 backbone + insert) to be transformed into it. The DNA should not be more than 50 ng in volume less than $10\mu\text{l}$. Incubate on ice for 20-25 min.
- The tubes carrying vector backbone (pBI121) and inserts (mVIP and/or mcry1Ac) were transferred to a water bath at 42°C for 90 seconds. Immediately chilled the cells on ice for 1-2 min.
- $850\mu\text{l}$ of LB medium was added to the cells and was grown for half an hour at 37°C , 200 rpm.
- The appropriate amount of cells on LB medium was plated and supplemented with the suitable antibiotic (in this study, kanamycin) to select the transformed colonies.
- The plates were incubated at 37°C for 12-16 hrs for transformed colonies to appear.

3.3.3 Selection of positive clones

- The competent *E. coli* cells were transformed with ligation mixture (carrying rDNA plasmids) using freez thaw method as given by Sambrook *et al.*, (1989).

- After 24 hours at 37°C, the single transformed cells were identified on the minimal medium containing respective antibiotic (Kanamycin) were isolated and reviewed in LB suspension culture. Selection of positive clones has been done on the selective medium containing Kanamycin @ 50 mg/L (Fig.3.3).

3.3.4 Development of *Agrobacterium* with positive clones

Recombinant plasmid construct (modified pBI121) was transformed into *Agrobacterium tumefaciens* strain- LBA 4404 by freeze-thaw method. Transformation of *Agrobacterium* (LBA 4404) with recombinant pBI121 (having vector backbone and insert) plasmid construct was carried out by mixing 10 µg of DNA with competent *E. coli* cells (DH5- α) followed by immediate freezing in liquid nitrogen. Subsequently cells were thawed by incubating the eppendorf tubes at 37°C for 5 minutes. One ml of LB medium was added to the tube and incubated at 28°C for 3 hours. Cells were spread on LB agar plates supplemented with 50 µg/ml Kanamycin and incubated at 28°C transformed colonies that appeared after 1-2 days were analyzed either by PCR or the positive colonies were confirmed by restriction digestion of the purified recombinant plasmids.

3.4 Tobacco Transformation

3.4.1 Tissue culture protocol for tobacco (*Nicotiana tabacum* cv. petit Havana) Transformation

Tobacco variety (*Nicotiana tabacum* cv. petit Havana) was used for *Agrobacterium*-mediated transformation (Fig.3.4). Propagation of sterile tobacco plants as a source of leaf disks. It is preferable to maintain continuously sterile tobacco plants than to surface-sterilize plants before each transformation. A tissue culture technique provides healthy, viruses, insect-pest and disease free plants.

3.4.1.1 Plantlet propagation

- Plantlets containing two leaves with the connecting stem were cut with a sharp knife and transferred to Magenta boxes containing fresh MS medium.
- Plantlets were then allowed to grow for 4 to 5 weeks. At this stage, they may be used as a source of sterile leaf disks and can be cut and transferred again.

3.4.1.2 Selection of explants

Tobacco (*Nicotiana tabacum*) cultivar petit Havana was used as the source of leaf disks in the study. Havana is regenerated easily from leaf disks. The leaves of tobacco plant used for transformation were selected as of age not more than 30 days. Two tobacco plantlets were sufficient to provide 50 leaf disks.

3.4.1.3 Co-cultivation Method of tobacco leaf disks with *Agrobacterium*

- Tobacco plants were grown in sterile condition (in sterile magenta jar containing MS 30 Solid Medium.) for 1-2 months, or until fully expanded green leaves obtained.
- Fully expanded, young leaves (2nd-6th leaves) in sterile condition were collected.
- The leaves were cut into MS 15 Liquid Medium in Petri plates and then resuspended in overnight grown *Agrobacterium* culture in MS15 Liquid Medium (0.4-0.6 OD at A_{595nm}).
- The leaves were transferred into Petri dish containing the MS 15 Liquid Medium and overnight grown *Agrobacterium* culture.
- The leaves were cut with forceps and blade and left for 10 minutes with occasional agitation.
- The cut leaves were blotted between two sheets of sterile Wattman filter paper.

- The leaf segments were then placed on MS 30 Solid Medium for co-cultivation for 2-3 days at 28°C in growth chamber.
- After incubation the leaves were transferred to regeneration media (MSBN Medium).
- The infected leaves were kept in growth chamber under light for 3-4 weeks with intermittent transferred into regeneration medium in every 7 days.
- When regenerated shoots were about 1.5-3.0 cm long, they were cut with sharp scalpel.
- The shoot lets were transferred to selective medium and checked for rooting. If transformed, roots will appear within 2-4 weeks depending on gene constructs.
- Selected transformed plants having roots were cut to multiply and transferred into magenta jars containing selective medium.
- Plantlets were then transferred to primary hardening in sterile substrate under controlled growth conditions.
- PCR screening of putative transformants was performed.

3.4.1.4 Preparation of *Agrobacterium* culture for co-cultivation

The *Agrobacterium* inoculums of strain LBA 4404 containing desired gene construct were grown on the minimal media plates containing 50mg/L Kanamycin for 2 days at 28±1°C in the dark. *Agrobacterium* was scrapped from AB medium plates and inoculated the culture in 5 ml of liquid AB media (Table 3.5) with respective antibiotic, Kanamycin. The overnight grown 5 ml media was again inoculated in 50 ml AB medium supplemented with Kanamycin @ 50 mg/L on rotary shaker @ 200 rpm at 28°C for 2-3 hours till the inoculums density OD₆₀₀ reaches 0.4- 0.6. Inoculums density for the bacterial suspension was measured at OD₆₀₀ using Spectrophotometer.

Table 3.5 Composition of AB Media used for *Agrobacterium* culture

Stock	Components	Amounts (g/500 ml)
Stock -I	KH ₂ PO ₄	30.0
	NaH ₂ PO ₄	10.0
Stock-II	NH ₄ Cl	10.0
	MgSO ₄ .7 H ₂ O	3.0
	KCl	1.5
	CaCl ₂	0.1
	FeSO ₄ .7H ₂ O	0.025
Stock-III	Glucose	5.0
	Agar	15.0

3.4.1.5 Infection and co-cultivation of tobacco Leaf Discs with *Agrobacterium tumefaciens*

Agrobacterium tumefaciens was grown at 28°C in AB minimal medium in the presence of 50 mg L⁻¹ Kanamycin. The cells were harvested in log phase, washed, and resuspended in liquid MS media at an OD₆₀₀ of 0.6–0.8. Leaf sections were incubated with resuspended cells for 5-15 min and blotted dry on sterile Wattman No.1 filter paper and transferred on to the co-cultivation medium under dark at 28°C for 2-3 days Leaf sections were co-cultivated with *Agrobacterium* for different periods of time ranging from 5 to 15 minutes before transfer to shoot induction media containing Cefatoxime for control of *Agrobacterium* and Kanamycin for selection of transformed tissue (Fig.3.4). Shoots were regenerated in the presence of 50 mg L⁻¹ Kanamycin and 250 mg L⁻¹ Cefatoxime using the method previously described and rooted with antibiotics of the same concentrations in MS media without PGR (Table 3.6 and 3.7).

3.4.1.6 Plant Regeneration Protocol

- After 4-6 days of co-cultivation leaf discs were washed with liquid MS having antibiotic Cefatoxime 250mg/L to prevent *Agrobacterium* overgrowth followed by drying on sterile filter paper.
- Leaf pieces were transferred to Petri plates containing selective medium having antibiotic Kanamycin 50 mg/L and wrapped with Para film.
- Incubated in a 28°C growth chamber under 16/8-day/night photoperiod for 2 weeks.
- Leaf pieces were transferred to new MS Magenta jars with fresh selection antibiotic (Kanamycin), and incubated for an additional 10-14 days during which the 1st or additional shoots would appear.
- When shoots attached to the explants reached 2-3 cm height they were transferred to the MSO medium (without any hormone) with Kanamycin which prevents rooting of the escapes but facilitate the rooting of the transformants.

Plant regeneration frequency was calculated as follows:

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

Table 3.6 Composition of MS Media

(a) Macro-nutrients	Amounts (mg/lit.)
KNO ₃	1900
CaCl ₂ .2H ₂ O	440
MgSO ₄ .7H ₂ O	370
NH ₄ NO ₃	1650
KH ₂ PO ₄	170
(b) Micro-nutrients	Amounts (mg/lit.)
KI	0.83
H ₃ BO ₃	6.2
MnSO ₄ .H ₂ O	22.3
ZnSO ₄ .7H ₂ O	8.6
Na ₂ MoO ₄ .2H ₂ O	0.025
CuSO ₄ .5 H ₂ O	0.025
AlCl ₃	0.025
FeSO ₄ .7H ₂ O	27.85
Na ₂ .EDTA. 2H ₂ O	37.25
(c) Organic constituents	Amounts (mg/lit.)
Inositol	100.0
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Nicotinic Acid	0.5
Glycine	2.0

Agar-Agar- 8 gm/lit. **Sucrose-** 30 gm/lit, pH-5.7

Note: FeSO₄.7H₂O & Na₂.EDTA.2H₂O dissolved separately in 450 ml distil water. Boil EDTA solution and add it to FeSO₄ gently & pH -5.5 was adjusted. Then added distilled water to make the final volume to 1 liter.

Table 3.7 Shoot and root induction media (MSBN) for tobacco

Components	Callus/shoots (mg/l)	Rooting (mg/l)
MS salts	4,400.0	4,400.0
Thiamine	0.6	10.0
Nicotinic acid	0.5	1.0
Pyridoxine	0.5	1.0
Inositol	100.0	100.0
Sucrose	30,000.0	30,000.0
BAP	2.0	-
NAA	0.1	-
pH	5.8	5.8
Agar agar	8,000.0	8,000.0
Kanamycin	50.0	50
Cefotaxime	250.0	-

Note: Neomycin phosphotransferase (NPT) gene fusion confers resistance to Kanamycin (selectable marker). All hormones were added prior to autoclaving and all antibiotics are added post autoclaving. Antibiotics for bacteria control. The antibiotics should be sterilized and stored at 20°C.

Table 3.8 Concentrations of various antibiotics used

Antibiotic	Abbreviation	Concentration (µg/ml)
Kanamycin	Kan	50
Cefatoxime	Cefa	25

Antibiotic Solutions:

- Kanamycin monosulfate (Sigma K 1377) was prepared as a 50 mg/ml stock solution in distilled water and filter sterilized. Stock solution was prepared fresh for each experiment or stored at -20°C for no longer than 2 weeks.
- Cefotaxime (Sigma C 7039) was prepared as a 50 mg/ml stock solution in distilled water and filter sterilized.

Note: All other antibiotic solutions were prepared in appropriate solvents (water or ethanol) and added to media which had been cooled to 55°C.

Table 3.9 Different media used for tobacco transformation

Medium	Components	Amount (g/L)
MS 30 Liquid Medium (1 liter)	MS salts	4.32
	Sucrose (3%)	30.0
MS 30 Solid Medium (1 liter)	MS salts	4.32
	Sucrose (3%)	30.0
	Agar (0.8%)	8.0
MS 15 Liquid Medium (1 liter)	MS salts	4.32
	Sucrose (1.5%)	15.0
Shoot Regeneration Medium (1liter)	MS30 Solid Medium	1Liter
	BA (Benzyl Amino purine)	2.0 mg
	NAA	1.0 mg
	Kanamycin	100 mg
	Cefotaxime	250 mg
Root Regeneration Medium (1 liter)	MS30 Solid Medium	1Liter
	NAA	1.0 mg
	Kanamycin	100 mg
	Cefotaxime	250 mg
Selective Medium (1 liter)	MS30 Solid Medium	
	Kanamycin	100 mg
	Cefotaxime	250 mg

3.5 Molecular screening of putative transgenic plants by PCR analysis

3.5.1 Genomic DNA isolation from T₁ plants

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

The stepwise protocol is given below:

- About 100 mg of tender shoot was grinded in 400 μ l 2X CTAB exaction buffer with a glass rod on spot plate.
- 400 μ l more of 2X CTAB extraction buffer was added and mixed thoroughly, in ~ 700 μ l of solution and then transferred, into 1.5 ml eppendorf tube.
- Incubated at 65°C on water bath for 15 min and then cooled briefly and 700 μ l of Chloroform: Isoamyl Alcohol (24:1) was added.
- The contents were shaken by hands intermittently and kept at room temperature for 15 min. Tubes were centrifuged at 13,000 rpm for 3 min.
- 600 μ l of upper aqueous phase was transferred into a new 1.5 ml eppendorf tube. 900 μ l of absolute ethanol was added and mixed gently and the tubes were kept for 2 hrs at -20°C.
- The sample was centrifuged for 3 min at 10,000 rpm, the supernatant was decanted. The pellet was washed with 70 % ethanol and air-dried.
- DNA pellet was air dried and then dissolved in 50 μ l of TE buffer.

3.5.2 Quantification of Genomic DNA

Genomic DNA extracted from T₀ plants was quantified with Nanodrop spectrophotometer as well as 0.8% agarose electrophoresis. About 3 μ l of DNA samples isolated from each plant along with the standards of known quantity of DNA was loaded on 0.8% agarose gel. The DNA was stained with Ethidium Bromide and observed under UV trans-illuminator. The quantity of DNA was estimated by comparing with fluorescence of standards. After the quantification DNA was diluted with sterile water such that the final concentration of DNA was 50 ng/ μ l.

3.6. Reagents and solutions used for Genomic DNA isolation

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

30.28g of Trizma base was dissolved in 200 ml of distilled water. The pH was set to 8.3 using concentrated HCl. The solution was allowed to cool at room temperature before making a final adjustment of pH. The final volume was adjusted to 250 ml with distilled water and sterilized by autoclaving.

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

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

- **CTAB extraction Buffer**

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

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

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

- **PCR Reagents**

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

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

- **2.5 M CaCl₂**

36.8 g of CaCl₂ was dissolved in 50 ml of Autoclaved double distilled water.

➤ **PCR Buffer (10X)**

Components	Concentration
1 M Tris (pH 8.3)	4.0 ml (100mM final Conc.)
1M KCl	10.0 ml (500 mM final Conc.)
1.5 mM MgCl ₂	2.0 ml
Gelatin	2.0 ml (1 mg/ml final Conc.)
Sterile water	4.0 ml
Total	20.0 ml

• **50X TAE buffer**

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

3.6.1 PCR analysis

The initial screening for the presence of transgene in regenerated plants was done using PCR (Polymerase Chain Reaction) technique as per the methods described by Datta *et al.*, (1999). Plant DNA isolated from leaf tissue was used as template to obtain amplification product of *mcry1Ac* and *mVIP* genes for screening of putative transgenic plants. For integration of transgenes, following gene specific primer pairs were used.

***cryIAc* primer**

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

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

***mVIP* primer**

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

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

The 100 ng of genomic DNA isolated from putative transgenic tobacco plants, the negative control (non-transgenic/non-infected) plant and plasmid DNA which was used for transformation were used as template for PCR reaction. The PCR reaction mixture consisted of 10 ng of template DNA, 50ng/ μ l of each the primer, 0.5mM dNTPs, 10X PCR buffer and 1-2 Unit of Taq DNA polymerase in final volume of 20ul (Table 3.10).

Table 3.10 PCR components with their quantity used for screening the putative transformants of tobacco

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

The PCR was performed in Thermal Cycler (MJ Research, PTC 100, USA). The PCR conditions were set as per the transgene and primer combinations used (Table 3.10(a) and 3.10(b)). PCR products were separated on 1% agarose gel (in 1X TAE electrophoresis buffer) containing 0.5 μ g/ml ethidium bromide. Separated products were visualized under UV light and photographed using gel documentation system (Bio Rad, USA) to examine the size of the amplification products. Based on the size of DNA band

and its comparative position with amplified product of a plasmid DNA, transgenic plants were designated as positive.

Table 3.10(a) Temperature profile used for the amplification of *Bt* gene, *mcryIAc*

Steps	Temperature (° C)	Duration (min)	Cycles	Activity
1	94	2.0	1	Denaturation
2	94	0.45	↑	Denaturation
3	50	0.45	35 cycle	Annealing
4	72	0.45	↓	Extension
5	72	4.0	1	Final extension
6	4	7.20	1	Storage

Table 3.10(b) Temperature profile used for the amplification of *Bt* gene, *mVIP*

Steps	Temperature (° C)	Duration (min)	Cycles	Activity
1	94	2.0	1	Denaturation
2	94	0.45	↑	Denaturation
3	55	0.45	35 cycle	Annealing
4	72	0.45	↓	Extension
5	72	4.0	1	Final extension
6	4	7.20	1	Storage

3.7 Standard deviation (SD)

Standard deviation is the root of the sum of squares of deviation divided by their number. Calculated by the formula

$$\text{Standard deviation} = \sqrt{\frac{d^2}{n}}$$

CHAPTER- IV

RESULTS AND DISCUSSION

The present study entitled “**Cloning and characterization of Ti plasmid based vectors carrying *Bt* genes for developing high efficiency plant transformation system**” was undertaken to develop the Ti plasmid based suitable vectors for *Agrobacterium* mediated transformation of crops. Two *Bt* genes (*mcry1Ac* and *mVIP*) cloned into pET-29(a) expression vector were obtained from ICGEB (International Center for Genetic Engineering and Biotechnology), New Delhi and cloned into the binary vector pBI121 by replacing a reporter gene GUS with coding region of both genes. Further, the efficiency of cloned plasmids were assessed using transformation of tobacco (*Nicotiana tabacum* cv. petit Havana) - a model plant system for plant genetic engineering research. The study also aimed to evaluate the factors affecting tobacco tissue culture, its subsequent transformation efficiency and molecular screening of transformants.

4.1 Construction of Vector

The two modified *Bt* genes (*mcry1Ac* and *mVIP*) have been characterized and found highly effective against the lepidopteron pests especially *Scodoptera litura*, *Earias insulana*, *Chilo partellus*, *Spilosoma obliqua*, *Maruca testulalis*, *H. armigera* and *L. orbonalis* (Selvapandiyar 1998). These two modified *Bt* genes were generously provided by ICGEB, New Delhi in the pET-29(a) plasmid under the control of T7 promoter. The coding regions of 1.2 Kb of modified *VIP* (Vegetative Insecticidal Protein) and 1.8 Kb *mcry1Ac* (Crystal protein) fragments were cloned into binary vector pBI121, which is one of the popular vectors used in plant transformation (Jefferson *et al.*, 1987). The

binary vector pBI121 is very popular vector used for the *Agrobacterium* mediated transformation of several crop plants. The uses of pBI121 as the high efficiency transforming vector for plant transformation have been reported by several workers (Hofgen and Willmitzer, 1990, Chen *et al.*, 2003, Newell 2000). *Agrobacterium* mediated transformation using pBI121 as donor for the kanamycin resistance has been developed for many crops viz; peanut (Rohini and Rao, 2001, Sharma and Anjaiah, 2000), shallot (Zheng *et al.*, 2001), spruce (Lee *et al.*, 2001), buckwheat (Kojima *et al.*, 2000) and rice (Jadhav *et al.*, 2008). The two clones were developed in the vector backbone of pBI121 by replacing the reporter gene GUS with the *mVIP* and *mcry1Ac* coding regions in the present study. The cloning strategy used for the construction of two recombinant vectors is illustrated in Fig 4.1 and 4.2.

The pET-29(a) vector carrying *mVIP* and *mcry1Ac* was digested with two restriction enzymes *BamH* I and *Sac* I to obtain the 1.2 kb and 1.8 kb fragments coding regions of *mVIP* and *mcry1Ac* genes respectively and fragments were gel eluted. Similarly, the binary vector pBI121 was also digested with *BamH* I and *Sac* I enzymes to obtain the vector backbone (Fig 4.1 for *mVIP* and 4.2 for *mcry1Ac*). The sticky end ligation for two different restriction endonucleases assured correct orientation of the genes into binary vector pBI121 (Fig. 4.3). The orientation of the genes was then confirmed by restriction digestion analysis using complete digestion of newly constructed vectors with *BamHI* and *SacI* enzymes (Fig 4.4 (a) and (b)). The resultant vectors pBI121 *mVIP* and pBI121 *mcry1Ac* were 1.2 kb and 1.8 kb in length, respectively. The reconstructed binary vectors obtained as pBI121 containing the *mVIP* and *mcry1Ac* gene each driven by CaMV35S promoter and Nos terminator.

4.2 Refinement of tissue culture based *in vitro* systems of Tobacco

The tissue culture response for all the crops including tobacco is influenced by several factors viz; genotypes, age of the plants, target tissues, PGR concentrations etc. leaf area factors has been reported (Gu and Zhang, 2005; Raghu *et al.*, 2006). To validate the cloned vectors on plant transformation, tobacco (*Nicotiana tabacum* cv. petit Havana) has been transformed using leaf disc method mediated by *Agrobacterium tumefaciens*. Sub-culturing of tobacco twigs (with inter-nodes) in the MS basal medium produced sufficient leaves to be used as explants for the transformation experiments.

4.2.1 Effect of leaf age on shoot regeneration

To analyze and understand effect of age of leaf on shoot regeneration, explants obtained from 20-30 days, 30-40 days and 40-50 days old leaves (Fig 4.5) were used. Shoot regeneration started within 25 days after the transfer of leaf explants to MSBN medium (regeneration media). Within four weeks, calli were observed with green shoot bulbs. Marked difference in regeneration was observed between leaf explants of different age. The results indicated that explants taken from 20-30 days old leaves had 85.33 % higher shoot regeneration on an average as compared to those explants obtained from 30-40 and 40-50 days old leaves. As the age of the leaf increased, the shoot induction capacity from the cut ends of explants was found to reduced (Table 4.1). A variety of related work done on the other plant species has demonstrated that age of explants donors affected regeneration of shoot lets, although the specific factor influencing regeneration capacity remains a matter of debate and varies among plant species (Grant and Hammatt, 2000). Our results suggested that the young leaves of 20-30 days old *in vitro* cultured plant showed better regeneration capacity than the fully developed leaves taken from 30-40 and 40-50-days old plants which is in agreement with the findings of Perez-Tornero *et*

al., (2000) and Gu and Zhang (2005) in Apricot (*Prunus armeniaca* L.) and Zhanhua winter jujube (*Zizyphus jujube* Mill) plants. Whereas in contradiction to results reported by Lane *et al.* (1998), Rout (2002) and Mahalakshmi *et al.*, (2006), who suggested that primary leaf explants (cut at the node) taken from two cultivars of mungbean from four-day-old and ten-day-old seedlings showed better regeneration than mature leaves. The varying response reported for regeneration capacity in relation to age of source explants in different species indicated that age of leaf in turn influences other metabolic or growth factors that affect shooting efficiency. Similarly Rodriguez *et al.*, 2008 reported the high tissue culture response in young /juvenile internodal young leaf segment of sweet orange (*Citrus sinensis* L.) compare to that of mature leaf and adult/mature tissues of the same crop.

Table.4.1. Effect of age of explant on shoot induction

S. No	Age of leaf	No of explants	No: of Callus induced	% of Callus induced	No: of Shoot per explant
1	20-30	75	64	85.33	8 ± 5
2	30-40	75	56	74.47	6 ± 3
3	40-50	75	44	58.67	4 ± 3

Data was collected on 25 day of incubation. Data presented here shows the effect of age of tobacco plant on callus induction in three treatments. 20-30 days old sub-cultured plant is giving good number of shoots.

4.2.2 Effect of sizes of explants on *in vitro* callus induction of *N. tabacum* cv. petit Havana

Earlier studies have revealed that the leaf disc method is very simple and convenient, since the selection and plant regeneration process is rapid. It has become the standard method for a number of dicots such as tobacco, petunia and tomato, etc (Li Wei

et al., 2000). In order to study the effect of size of the leaf disc on in vitro callus induction, leaf disc of different sizes viz. 0.5, 0.75 and 1.0 square cm. area (Table 4.2 and Fig.4.6) were placed on callus induction medium with the adaxial surface toward the medium.

It was observed that the leaf discs of 0.5 cm.sq. was most suitable for callus induction as maximum number of calli were obtained from these leaf discs. Similar size of leaf discs has been used for *Agrobacterium*-mediated transformation of tobacco lines in a routine laboratory procedure by Horsch *et al.*, 1985; Rogers *et al.*, 1986; Burow *et al.*, 1990. Therefore in our investigation also leaf discs of about 0.5 square cm were used for further transformation experiments.

Table 4.2: Effect of size of explants of tobacco on callus induction

S. No	Size of leaf disk in cm ²	No: of leaf disc inoculated	No: of discs callusing	% of calli induced*
1	0.50	50	40	80 ± 12.5
2	0.75	50	38	76 ± 11.25
3	1.00	50	29	58 ± 8.87

Data collected after 25 days of incubation. The table showed the effect of size of leaf disc taken from the lower portion of sub cultured 20-30 days old plant. Out of three treatments, 0.5 cm.sq. showed the maximum percentage of callus induction. * Data is presented as ± SE

4.2.3 Optimization of concentration of Kanamycin for selection of transformants

Sensitivity of leaf discs to Kanamycin was established prior to actual transformation experiments in order to determine the effective concentration for selection. Higher Kanamycin concentration has been found to be too toxic to leaf disc and caused immediate browning (Husaini *et al.*, 2008) while a lower concentration will allow regeneration of non-transformants and hence produce misleading results. To optimize the concentration of kanamycin for selection the maximum callus regeneration showing leaf discs of 0.5 cm² area obtained from 25 to 30 days old tissue cultured plants

developed through subculture of nodal explants, were used. These leaf discs were inoculated onto different medium supplemented with four levels of Kanamycin as 0, 50, 80 and 100 mg/L. The time and number of leaf disc mortality was recorded to identify the optimum concentration of Kanamycin to be used for selection of *Agrobacterium* infected leaf discs of tobacco. The leaf discs were infected with *Agrobacterium tumefaciens*, as tobacco control plants (wild type) carry no selectable markers genes they will become necrotic when grown in regeneration medium containing Kanamycin at 50 mg/L concentration while, explants carrying *Agrobacterium* plasmid which confers resistance to Kanamycin will grow easily. Table 4.3 shows the appropriate concentration of Kanamycin for the selection, a 50 mg/l Kanamycin was used for selection of transformed cell in leaf discs (Fig.4.7).

Table 4.3 Effect of various concentration of Kanamycin on tobacco explants.

S. No	kanamycin Concentration (mg/l)	No of leaf discs co-cultured	Mortality of leaf discs			
			3 rd of inoculation		7 th of inoculation	
			No of Dead leaves	%	No of Dead leaves	%
1	00	25	All survived			
2	50	25	10	40	25	100
3	80	25	20	80	00	00
4	100	25	25	100	00	00

Data was collected on 3rd and 7th day of incubation of explants on selection media containing different concentration of kanamycin. Same size (0.5 cm.sq.) leaf disc and age of leaf was kept constant to find the effect of kanamycin.

In the absence of Kanamycin, the all leaf discs regenerated normally and produced multiple shoots on the periphery. The leaf discs showed total mortality on 3rd day at 100mg/L Kanamycin, similarly at 80mg/L Kanamycin the leaf discs showed total

mortality within 7th days. However, the growth of leaf discs was considerably restricted and bleaching was complete at 50mg/L Kanamycin on 7th day (Fig. 4.8). Hence, 50mg/L Kanamycin concentration was identified as optimum for selection of transformed cells. Xu, *et al.*, 2008 have also suggested the use of 50 mg/L Kanamycin as a selective growth system for genetically transformed *M. zumi*.

4.3 *Agrobacterium* mediated transformation of model plants Tobacco using leaf disc method

4.3.1 Transformation of Tobacco leafs with pBI121mVIP and mcry1Ac

Burow *et al.*, (1990) has standardized the tobacco co-cultivation protocol which indicated that optimum effective *Agrobacterium* density from 0.4-0.6 at A₆₀₀ and co-cultivation period. Sterile tobacco young leaves of size 0.5 cm.sq. were cut from 20-30 days old plant. Forty-five leaf discs were placed into one Petri dish for bacterial inoculation. Leaf disks were soaked in 10 ml of *Agrobacterium tumefaciens* harboring pBI121 with mVIP and mcry1Ac. 15-20 leaf discs were placed in one Petri dish and kept in co-cultivation media.

4.3.2. Factors affecting the transformation efficiency

4.3.2.1 Co-cultivation Duration Effect on transformation efficiency

The known protocols for tobacco leaf disc transformation have revealed different duration of co-cultivation varying from 2, 3 and 4 days at 26°C, 24°C at room temperature (Svab *et al.*, 1995). The reports on tobacco transformation have indicated that *Agrobacterium* concentration as well as co-cultivation time affects the transformation efficiency (Lopez *et al.*, 2004 and Chakrabarty *et al.*, 2002). Here in the study we found that both mVIP and mcry1Ac inserts showed best result at 0.4 O.D concentration of the *Agrobacterium* inoculum for 5 min (Table 4.4). A total of 100 explants were infected for each experiment and were kept in selection media after infection. The rate of callus

formation was determined after 4 weeks when the leaf discs were in 3rd selection. It was observed that most of the explants co-cultivated with *Agrobacterium* strain-LBA4404 turned brown with more co-cultivation time, on transfer to the selection media (Table 4.4). Figure 4.9(e) shows the optimum growth of shootlets in regeneration medium (MSBN medium). In our investigation we found that the extension of incubation of *Agrobacterium*-infected leaf disks from two days to seven days was the modification that contributed the most to the success of the transformation experiment.

Table 4.4 Effect of *Agrobacterium* inoculum density and its exposure time on tobacco leaf disc transformation.

S.No	<i>Agrobacterium</i> density/Incubation time	Number of explant infected	Number of explants survived on selection medium after 30 days of inoculation
1	0.4 / 5 min	100	28
2	0.4 / 10 min	100	18
3	0.6 / 5 min	100	23
4	0.6 / 10 min	100	14

Table shows the effect of culture density out of two treatments of optical density and time in minutes, tested for high efficiency of regenerants for two clones. Maximum callus induced after infection for 5 min at 0.4 O.D was recorded the highest than other treatment.

4.3.3 Selection of transformants

Keeping the selection pressure on callusing and shoot induction media increases the chances of producing more number of positives among the regenerants and enhances the transformation efficiency. Similarly efficient transformation systems for leaf discs of *Nicotiana tabacum* were achieved with *Agrobacterium* was, 55.6 % by using the *uidA* and the *luc* reporter genes that encode beta-glucuronidase (GUS) and luciferase, respectively with neomycin phosphotransferase (*nptII*) selectable marker gene for kanamycin (E. Arican, N. Gozukirmir, K. Bajrovic, N. Gozukirmir 1997). *Agrobacterium*

strain LBA4404 harboring a binary vector pBI121 containing neomycin phosphotransferase (*nptII*) gene as selectable marker was used for transformation (Fig.4.9). Kanamycin at a concentration of 50mg/l was used to select transformed cells. Inclusion of selection agent in the regeneration and rooting media was responsible for the production of putative transformed shoots that stably expressed transgene. The explants inoculated in MSBN media containing 50mg/L Kanamycin produced transformed shootlets in 3rd selection. The calli survived in 3rd selection were transferred into Magenta box containing 50 ml of rooting medium (as recommended by Burow *et al.*, 1990; Horsch *et al.*, (1985) and Rogers *et al.*, (1986).

Table 4.5 Transformation efficiency of *Agrobacterium* LBA 4404 strain with Kanamycin (50 mg/l).

S.No.	No of leaf discs co-cultivated	<i>mVIP</i>		<i>mcry1Ac</i>	
		No: of leaf discs survived on selection media	% of putative transformants	No: of leaf discs survived on selection media	% of putative transformants
1	90	5	5.56	5	5.56
2	90	20	22.22	2	2.22
3	90	10	11.20	19	21.11
4	90	7	21.11	11	28.89
Total	360	42	11.66	37	10.27

Total leaf disc infected were 360 for both *mVIP* and *mcry1Ac*. Putative transformants are the callus with shoots. Data collected from all the experiment and compiled to get the average transformants in *mVIP* (11.66) and *mcry1Ac* (10.27). Each attempts are compiled data of 14 days.

Out of total 360 leaf discs co-cultivated, 42 (11.66 %) and 37 (10.27 %) putative transformants were recovered for *mVIP* and *mcry1Ac* infected agro cultures respectively after 3rd selection (Table 4.5). Fig 4.9(c) showed the putative transformants on selection medium. Leaf disc based transformation of tobacco for the gene expression and vector validation has also been reported (Jahdav, 2008). Optimal transformation efficiency of

22.2% was obtained using high concentrations of augmenting as a bacteriostatic agent for a short period to inhibit the growth/ persistence of *Agrobacterium*, without compromising the regeneration potential of the tissue. Using a viral promoter-driven GUS reporter gene, morphologically normal fertile plants were obtained

4.4 Molecular screening of putative transformants by PCR

Gene specific primers designed from the coding region of both the genes were used for molecular screening of putative transformants. Four completely grown plants from the *mVIP* and *mcry1Ac* clones were analyzed by PCR.

The putative transformants generated from tissue culture based method i.e., *Agrobacterium*-mediated transformation systems were selected on Kanamycin containing medium and were subjected to PCR analysis for initial screening of presence or absence of gene of interest. A specific primer for *cryIAc* and *mVIP* gene was used to yield 780 bp and 900 bp amplification products respectively. Out of 42 (11.66%) putative transformants produced for *mVIP* and 37 (10.27%) for *mcryIAc* infected explants, four each of fully developed regenerants were tested for the presence of the gene of interest by PCR analysis. All four plants produced after *mVIP Agro*-infection were found positive (Fig 4.10a) whereas, the regenerants infected with *mcryIAc Agro* culture were found to be negative by PCR analysis (Fig 4.10b). Use of gene specific primers for PCR based screening of large number of transformants have been the routine practice in the development of transgenic crops (Kim and Li., 2001) .The identification of *mVIP* positive plants are in rooting medium and will be tranfered into pots.

CHAPTER - V

SUMMARY, CONCLUSION AND SUGGESTION FOR FUTURE RESEARCH WORK

Genetic Engineering can be used to introduce specific traits into plants from any known organism, theoretically. Plant genetic engineering will not replace conventional breeding but can add to its efficiency as it widens the gene pool for crop improvement. The totipotency of plants i.e the ability to regenerate into a new plant from a single cell is the most remarkable feature that makes them feasible for genetic manipulation. Plant transformation vectors and methodologies have been improved to increase the efficiency of plant transformation and to achieve stable expression of transgenes in plants. Due to the simplicity of the transformation system and precise integration of transgenes, *Agrobacterium* Ti plasmid-based vectors continue to offer the best system for plant transformation. Being a natural parasite of dicots, *Agrobacterium tumefaciens* based transformations technique was more used in the dicots crops species. Genes are cloned into plant expression vectors that carry the right and left border sequences. They are introduced into plants with the aid of a disarmed Ti plasmid whose virulence gene products allow the genes to be transferred to the plant nucleus where they are integrated into the genome. In this case, callus tissue is regenerated on media containing an antibiotic to select transformants. It is often advantageous for plants to express the introduced transgenes in specific tissues or under specific conditions. As a result many genes are cloned downstream of tissue-specific or inducible promoters. The 35S promoter of the cauliflower mosaic virus is commonly used in dicots.

A binary vector was invented soon after it had been elucidated that crown gall tumorigenesis was caused by genetic transformation of plant cell with a piece of DNA, T-DNA for transferred DNA, from a Ti plasmid (tumor-inducing plasmid) harbored by soil bacterium *Agrobacterium tumefaciens*. A key finding was that the virulence genes, which are involved in the transfer of T-DNA, could be placed on a replicon separate from the one with T-DNA. Thus, combination of a “disarmed” strain, which carries a Ti plasmid without the wild-type T-DNA and an artificial T-DNA within a plasmid that can be replicated both in *Escherichia coli* and *A. tumefaciens* turned out to be fully functional in plant transformation. The term “binary vector” literally refers to the entire combination, but the plasmid that carries the artificial T-DNA is usually called a binary vector. Tobacco is referred as most feasible plant system for transformation and expression of transgenes from a variety of organisms. Among the many genes from different organisms expressed in tobacco plants, genes of bacterial origin are most commonly used to genetically engineer tobacco and then the same system is followed in other crop plants with some modifications. Increasing transformation efficiency in tobacco for insecticidal genes will significantly contribute to development of high efficiency transformation system in commercially important crops. The present study **“Cloning and characterization of Ti plasmid based vectors carrying *Bt* genes for developing high efficiency plant transformation system.** “ was thus undertaken using bacterial genes (*mVIP* and *mcry1Ac*) to genetically engineer tobacco for genes conferring resistance against lepidopteron insects and also to assess various factors affecting its *in-vitro* regeneration efficiency in order to develop high efficiency *Agrobacterium*-mediated transformation system using *Nicotiana tabacum* cv. petit Havana.

The *mVIP* and *mcry1Ac* gene earlier cloned in pET-29(a) vector provided by ICGEB, New Delhi were excised using *Bam*H I and *Sac*I enzymes and were subcloned in the plant expression vector pBI121 having CaMV 35S promoter and NOS terminator sequences. The *gus* reporter gene encoding Beta-glucuronidase (GUS) was replaced with *mVIP* or *mcry1Ac* genes. The resultant binary vector pBI121 was then mobilized into *A. tumefaciens* strain-LBA 4404 by freeze and thaw method and was grown on LB media with Kanamycin selection. This was analyzed for *mVIP* and *mcry1Ac* genes by restriction digestion of cloned pBI121 and positive clones carrying both *Bt* genes were used for tobacco transformation.

The optimum leaf age showing maximum calli induction and age of explants from which leaf discs were taken, were analyzed in *in-vitro* regeneration study for their further use in tissue culture based transformation of tobacco and development of high efficiency plant transformation system. As tissue culture provides sterile, young, and disease free plants the tissue cultured tobacco plants of cultivar petit Havana were used for developing high efficiency plant transformation system. The tobacco leaf discs of 0.5 cm² size taken from 20-30 days old plant was found suitable for *Agrobacterium tumefaciens* mediated transformation. It was observed that MS medium supplemented with 2.0 mg/L and 0.1 mg/L was efficient in regenerating transformed cells into shoots from leaf disc explants of 20-30 days old leaf.

The optimum concentration of Kanamycin required to prevent growth of non-transformed cells of infected tobacco and not toxic for growth of positive transformants was also determined. Variations for the concentration of Kanamycin in selection medium were used for optimization of selection media. It was observed that tobacco calli required low dose of kanamycin (50 mg/L) for selection of transformed plants and killing of non

transformed control calli. The 50 mg/L dose was used for the selection of transgenic calli on tissue culture based Particle gun as well as *Agrobacterium*- mediated transformation methods.

PCR analysis with gene specific primers for *mVIP* and *mcry1Ac* gene was used for screening of putative transformants selected in the selection medium. It was noted that four positive tobacco transformants exhibited PCR positive signal for *mVIP* gene, it had shown the presence of distinct band of ~900 bp of *mVIP* gene, while no amplification have been observed in PCR screening of the *mcry1Ac* infected putative transformants. Four putative transformants (carrying *mVIP* gene) were produced from *Agrobacterium*-based transformation experiments, for *N. tabacum cv. petit Havana*. Whereas, no transformants were obtained for *mcry1Ac* gene as no amplification with *mcry1Ac* gene specific primers was produced in PCR analysis, indicating lower efficiency of transformation by tissue culture based method.

Conclusions:

- The binary vector pBI121 carrying *Bt* genes (*mVIP* and *mcry1Ac*) under CaMV 35S promoter and NOS terminator sequences was developed and mobilized into *Agrobacterium tumefaciens* strain- LBA4404.
- Two high efficiency *Agrobacterium* clones of LBA 4404 carrying the novel *Bt* genes *mVIP* and *mcry1Ac* were developed.
- About 20 to 30 days old explants leaves and the leaf discs corresponding to 0.5 cm.sq area were found to be most responsive for callusing and subsequent shooting/ plant regeneration.
- Lethal dose 50 mg/l Kanamycine has been identified for selection of transformed calli.

- The effects of optical densities (O.D) and duration (minutes) of *Agro*-infection, showed that tobacco leaf disc exposed to bacterial culture with density 0.4 O.D for 5 minutes gives 28% of calli regenerated for mVIP and 23% of calli regenerated for *mcry1Ac* as shown in Table 4.4.
- PCR screening was performed to trace the positive plants. Out of 42 putative transformants of mVIP and 37 putative transformants of *mcry1Ac* were transferred to rooting and shooting media, only four of them from each clone showed significantly higher shoot development and were selected for PCR analysis.
- Four mVIP positive transformants were obtained after PCR screening and no transformants for *mcry1Ac* were developed.

Suggestions for future work:

- The regenerants developed on selection medium should be analyzed by PCR using gene specific primers.
- PCR positive plants should be analyzed by southern analysis to confirm the integration of transgene.
- A study should be planned to develop *Bt* transgenic in commercial crops using novel *Agro* strains LBA 4404 carrying pBI121 mVIP and pBI121 *mcry1Ac* binary vector.

“Cloning and characterization of Ti plasmid based vectors carrying *Bt* genes for developing high efficiency plant transformation system.”

by

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ABSTRACT

Two *Bt* genes *mcryIAc* and *mVIP* were cloned in to the widely used vector pBI121 for plant transformation. The T-DNA region contains the right border, expression cassettes for neomycin phosphotransferease (*nptII*) selectable marker and β -glucuronidase reporter gene and the left border. The pET-29(a) containing two *Bt* genes (*mVIP* and *mcryIAc*) were obtained from ICGEB, New Delhi (Selvapandiyan 1998). Two constructs, pBI121 *mVIP* and pBI121*mcryIAc* were made by replacing the reporter gene GUS from the pBI121 at *Bam*HI and *Sac*I sites. The resultant vectors were transferred into *Agrobacterium* strain LBA 4404 and used in *Agrobacterium*-mediated transformation of Tobacco (*Nicotiana tabacum* cv. petit Havana) a model plant transformation system. Various factors for the leaf disc method of *Agrobacterium* transformation have been standardized. With 0.4 OD densities and 5 min *agro* incubation 42 *mVIP* and 37 *mcryIAc* putative transformants were regenerated. Out of total 79 putataive transformants, only four completely developed plants analyzed were found positive for *mVIP* gene by PCR analysis. The novel LBA 4404 *Agro* clones carrying *mVIP* and *mcryIAc* in the binary vector pBI121 background are useful in transformation of important food crops like rice, wheat and corn. The *Agrobactruim*-medtaied transformation is most widely accepted transformation method having added advantage of low copy and stable integration of genes with minimal distortion of target genome.

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