'Construction and use of a novel chloroplast transformation vector: Introduction of the *cry1Ac* gene of *Bacillus thuringiensis* in tobacco plastome'

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By

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CERTIFICATE

This is to certify that the thesis entitled, "**Construction and use of a novel chloroplast transformation vector: Introduction of the *cry1Ac* gene of *Bacillus thuringiensis* in tobacco plastome**'" submitted to Madurai Kamaraj University, Madurai for the award of Doctor of Philosophy in Biotechnology, is a record of research work done by **Mr. M.B. Mohamed Sathik** under my guidance and supervision during 1997-2003 at the Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai. I further certify that such help or source of information as has been availed of in this connection is duly acknowledged and that no part of this thesis whatsoever has been submitted anywhere else for any degree, diploma, associateship, fellowship or other similar titles.

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DECLARATION

I do hereby declare that this thesis submitted for the degree of Doctor of Philosophy in Biotechnology entitled "Construction and use of a novel chloroplast transformation vector: Introduction of the *cry1Ac* gene of *Bacillus thuringiensis* in tobacco plastome" is my original work and that it has not been previously submitted in part or whole for any degree, diploma, associateship, fellowship or other title.

Place: Madurai M.B.MOHAMED SATHIK

Date:

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ABBREVIATIONS

amp	ampicillin
BCIP	5 bromo –4 –chloro-3-indolyl phosphate
bp	base pair
βME	Beta Mercaptoethanol
BSA	bovine serum albumin
cm	Centimeter
CFU	Colony forming units
dATP	2' deoxyadenosine 5' triphosphate
dCTP	2' deoxycytidine 5' triphosphate
dGTP	2' deoxyguonosine 5' triphosphate
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNase	deoxyribonuclease
dNTP	2' deoxynucleotide 5' triphosphate.
DTT	dithiothreitol
dTTP	2' deoxythymidine 5' triphosphate
EDTA	ethylene diamine tetra acetic acid
hr	hour
IPTG	isopropyl β -D-thiogalactopyranoside
kg	Kilo gram
kb	kilo base
kDa	kilo Dalton
Mha	Million hectare
min	minutes
µl	Micro liter
mM	Milli molar
MT	Million tonnes
NBT	nitro blue tetrazolium
Nt	Nucleotide
nm	Nano meter
ORF	open reading frame
PCR	polymerase chain reaction
PMSF	phenyl-methyl-sulphonyl fluoride
RNase	Ribonuclease
Sp.	species
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS- PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
TEMED	N, N,N' N' – tetramethyl ethylene diamine
UV	Ultraviolet
X- gal	5 bromo–4 chloro-3-indolyl β-D-galactopyranoside

……..for a greener and hunger free India

Introduction……

INTRODUCTION

In India, cotton is one of the traditional cash crops of the peninsular region. Cotton cultivated here covered an area of 9.12 M ha in 1996-97 (the largest in the world) with an average yield of 266 kg ha^{-1} (Chowdhary and Laroia, 2001). This yield is well below the 943 kg ha^{-1} for China and also compares adversely to the current world average of 584 kg ha^{-1}. Cotton yield in India is mainly affected by the problems due to insects and pests and 45% of the all pesticides and 58 % of all insecticides used in Indian agriculture are applied to cotton alone. The major pests of cotton in India are *Heliothis* sp., *Helicoverpa armigera*, *Spodoptera* sp., *Pectinophora* sp., and *Earias* sp. which are collectively called as the 'boll worm complex' and they belong to the insect Order, *Lepidoptera*. Sixty percent of the insecticides are used to control these insects. White flies and aphids also affect cotton albeit to a lesser extent. Although insecticides are effective in controlling boll worm complex, the damage caused by bollworm infestation can be controlled only in the early stages after which it can cause extensive damage.

The economic and environmental costs of insect control in agriculture and the losses incurred have always been high ever since man started using the chemical pesticides. The worldwide annual expenditure on insecticides for cotton is 1870 million US $. Apart from cotton, the expenditure for the fruit and vegetables, rice, maize and other crops worldwide are (US $ million) 2465, 1190, 620 and 1965 respectively as per the 1994 data (Krattiger *et al.*, 1997). Despite the use of such sophisticated crop protection measures (chemical pesticides), the worldwide crop damage inflicted by phytophagous insects is staggering. The worldwide crop losses due to insect pests for Rice, maize, fruits and vegetables are (US $ million) 45 000,

8000, 20 000 and 25 000 respectively. The percentage of the total crop volumes lost are maize 12 %, fruit 6%, vegetables 9% and rice 27 % (de Maagd *et al.*, 1999). Though there are many insecticides and pesticides available in the market the problem of insects and pests continues. Individual farmers facing the possible devastation of their crops by insect infestations every year quit agriculture and look for job opportunities at cities. Some of the Indian farmers even resort to suicide to get rid of their debts due to pest influenced crop loss (Sharma, 1999). In this scenario, there is an urgent need to evolve and apply biotechnological improvements to reduce the crop loss and to develop insect resistant transgenic varieties in India. Especially insect resistant cotton varieties are the need of the hour to save the farmers from quitting agriculture.

In view of this, genetic engineering of insect resistance into crops represents an attractive opportunity to reduce insect damage with several clear advantages over traditional chemical pesticides. Over the past years success in producing insect resistant crops through gene transfer has been impressive, and the culmination of this process occurred in 1996 when the first generation of insecticidal plants, generically as *Bt* plants were introduced in the market. The *Bt* transgenic plants, which replaces insecticide is a better alternate to control insects and to reduce the use of highly toxic conventional insecticides. *Bacillus thuringiensis* (*B.t.*) has been one of the thoroughly studied organisms over the past few decades and much investigations made on its insecticidal crystal protein (Cry) have proven to be one of the best alternate for chemical pesticides. *B.t.* is a rod shaped, ubiquitous gram-positive spore forming bacterium that exists in many locations such as the soil, plant surfaces , grain storage dust, bird's feather, etc. It is known for its production of parasporal crystals during

sporulation which consist of one or more δ-endotoxins or Cry proteins of ~ 130 kDa. The crystal proteins upon ingestion, dissolve and release their constituent protoxins in the alkaline environment of the insect midgut. The protoxins are subsequently trimmed by insect gut proteases to an N-terminal 65-70 kDa truncated form to become the activated toxin. The toxin eventually binds to the receptors on the cell membranes of the midgut epithelial cells, and inserts itself into the membrane to form pores that kill the epithelial cells and subsequently the insect by colloid osmotic lysis (Knowles and Dow, 1993).

Spore and crystals of several strains of this bacterium have been used as microbial insecticidal sprays since the 1930's but the large scale production only started with the introduction of Thuricide TM in the late 1950's and this was followed by similar products from several companies. In spite of their environmental friendly reputation, *B.t.* sprays have never occupied a large share of the insecticide market. This is because of its lack of stability, and its narrow specificity, failure to penetrate tissues and therefore lack of ability to reach insects in all parts of the plant.. Crystal proteins also degrade rapidly in UV light and loose its activity. Since *B.t.* sprays are non-systemic insecticides, they are not effective against insects that do not come in direct contact with the crystals such as sap sucking and piercing insects, root dwellers, or larvae that bore and get burrowed into plant tissues.

To overcome this problem, transgenic plants that express crystal proteins were made and released in the market such as *Bt*-potato (New Leaf TM, Monsanto, St. Louis, MO, USA), *Bt*-cotton (BollgardRM, Monsanto) and *Bt*-Maize (YieldGardTM, KnockoutTM and BitegardTM). These plants produce toxin continuously and protect

themselves against the infesting larvae. The economic need for an effective insecticide, the availability of relevant *B.t.* genes that encode specific proteins against particular pests and proven safety of *B.t.* sprays made transgenic *B.t.* plants possible for commercial exploitation. Both full-length and truncated *cry* genes (exclusively encoding the activated toxin) were introduced into tobacco and tomato by *Agrobacterium tumefaciens* mediated transformation (Vaeck *et al.*, 1987, Fischoff, 1987, Barton *et al.*, 1987).

Plants transformed with full-length *cry* genes were not resistant enough to give protection against pests under field conditions. As *cry* genes are typical bacterial genes which have a high AT content compared with plant genes, the *cry* gene codon usage is inefficient in plants. The AT rich regions may contain transcription terminator (polyadenylation) sites (AATAA and its variations). Attempts were made to remove sequence motifs in the *cry1Ab* and *cry1Ac* genes (seven out of 18 polyadenylation sites and seven out of 13 ATTTA sequences) and thereby to get increased expression of *cry* genes. It indeed increased the production of crystal proteins upto 10 fold higher than the unmodified genes. Removal of the remaining polyadenylation sites and ATTTA sequences and changes in a total of 356 of the 615 codons, raised the levels still higher (up to 0.2 to 0.3 % of total soluble protein) which is 100 fold higher than the level for unmodified ones (Perlak, 1990, 1991).

Even higher levels of expression of *cry* genes were reported by using different promoters such as CaMV 35S promoter, chemically inducible promoters and tissue specific promoters (0.4 % of total protein in maize by Koziel *et al.*, 1993). A modified *cry1Ac*-coding region under a Rubisco small subunit promoter, which is

translationally fused to a chloroplast transit peptide produced up to 0.8 % *B.t.* protein in tobacco (Wong *et al.*, 1992). However, this level of toxin production occurred only in few transformed plants while most transformants produced between 0.001 to 0.6 % of their total soluble protein as toxin. This variation in the levels of protein production is reported to be due to variation in the positioning of transgene in the chromosomes (positioning effect) in the case of nuclear genome transformation.

The factors such as positioning effect, the low level of expression, need for codon modification of *cry* genes, spreading of transgenes to the weedy relatives of crops were the limiting factors in the nuclear genome transformed plants and the search for a transformation system without these limitation led researchers to the chloroplast transformation a decade ago. Chloroplast genome was successfully transformed first in *Chlamydomonas* by Boynton *et al.*, (1988) and later in tobacco (Svab and Maliga, 1993 and Zoubenko *et al.*, 1994). It is now being extended to other crops too. McBride *et al.* (1995) transformed tobacco chloroplast with unmodified full-length coding region of *cry1Ac* and reported expression of 3 to 5 % crystal protein in the total soluble protein. Such expression levels provided protection against even relatively *cry1Ac* tolerant plant pests too. Later, few other genes were also used such as EPSPS gene for glyphosate resistance (Daniel *et al.*, 1998), a combination of *aadA* gene for spectinomycin resistance and green fluorescent protein (GFP) (Khan and Maliga, 1999), hST gene for human somatotropin – a human therapeutic protein (Staub *et al.*, 2000) reporting higher level of expression by chloroplast transformation. The invention of gene gun as a transformation device also made the chloroplast transformation a big success in many systems.

The higher level of expression is attributed by the presence of high copy number of chloroplast genome per cell. In a typical leaf cell, there may be as many as 100 chloroplasts, each with around 100 copies of the plastome (chloroplast genome) giving a total of ~10 000 copies. Though there are species specific variations, this high copy number after transformation gives rise to high copy number of transformed gene per cell that leads to the higher level of expression of the introduced gene. Apart from higher level of expression, the chloroplast transformation has got more advantages. As it is well known that the transcriptional and translational machinery of the plastid is prokaryotic in origin and AT-rich, it is possible to transform plastome with any gene of prokaryotic origin without the need for codon modification.

Another advantage is its maternal inheritance. The plastids are present in the egg cell only and not present in the pollen. Hence, spreading of introduced foreign gene to other weedy relatives is impossible. This kind of maternal inheritance is common in most of the higher plants with few exceptions. Cotton is largely self pollinated, but 6 to 25 % (and up to 50 %) cross-pollination may occur (Purseglove, 1968). There is a strong possibility that the foreign in the pollen getting transferred to neighbouring fields and possibly to wild relatives of cotton in the case of nuclear genome transformed plants.

The facts such as the urgent need for the production of transgenic crops with insecticidal property to eliminate the application of chemical pesticides, feasibility of transforming chloroplast genome to get higher level of expression and the availability of *cry* genes which encodes for proteins that are insecticidal in nature made us to initiate this research. The objective is to develop a chloroplast transformation vector

and to transform crop plants especially cotton with *cry* gene to get insect resistant transgenic plants. Basically, our goal is to develop a vector which can transform most of the higher plants by introducing the gene of interest (*cry1Ac*) specifically between the *trnI* and *trnA* genes of 16S rRNA operon of the choloroplast genome. We have made an attempt to transform tobacco with this vector and the results are discussed in detail in the following chapters.

Review of literature.....

REVIEW OF LITERATURE

Cotton is a traditional crop in the peninsular regions of India. With the introduction of new varieties and pesticides, its cultivation has extended beyond these regions. The area under cotton has increased from 5.89 M ha in 1951-52 to 9.12 M ha in 1996-97 (the largest in the world), the production from 9.1 % to 35 % (Table 1 and 2) although the yield has increased from 88 kg ha^{-1} in 1951-52 to only 266 kg ha^{-1} in 1996-97. This figure is well below the 943 kg ha^{-1} for China and even the current world average of 584 kg ha^{-1} (Table 2 from Choudhary). However, in China, the yield increased from 225kg ha^{-1} in 1962 to 810 kg ha^{-1} in 1990 due to usage of improved cotton varieties (Choudhary and Laroia, 2001).

Cotton yield in India is mainly affected by insects and pests. The major insects that attack cotton are 'bollworm complex'. More than sixty percent of the insecticide spray is used to control a group of insects belonging to the class *Lepidoptera.* These include False American Bollworm - *Helicoverpa armigera;* Pink Bollworm – *Pectinophora gossypiella; Spotted Bollworm – Earias vittella* and Spiny Bollworm – *E. insulana* which are collectively referred to as the 'bollworm complex' (Manjunath and Mohan, 1999). To a lesser extent white fly and aphids also affect cotton crop. These insect pests currently reduce cotton yield by 50 % and more than 50 % of the production costs in cotton is in pesticides (Sundaramurthy and Gahukar, 1998). Pesticides such as Monocrotophos, Endosulphan, Chlorpyriphos, Quinalphos, Cypermethrin, Fnvalerate and Acephate are widely used to control these pests. The cost of management practices and chemical control of insects in global level approaches $ 10 billion annually, yet the loss

Table 1 Year-wise area under cotton cultivation, production, yield and % coverage under irrigation in India

Year	Area (M ha)	Production (MT)	Yield (Kg/ha)	% coverage under irrigation
1951-52	5.89	0.53	88	9.10
1961-62	7.98	0.78	103	13.0
1971-72	7.80	1.12	151	20.3
1981-82	8.06	1.43	166	27.7
1991-92	7.66	2.02	216	33.3
1996-97	9.12	3.00	266	35.0

Adopted from Choudhary and Laroia, 2001.

Table 2 Cotton production, yield and world market share (1997-98)

Country	Market share %	Area (M ha)	Production (MT)	Yield (kg/ha)
China	24.5	4.56	4.30	943
USA	16.5	5.37	4.13	769
India	15.2	8.9	2.86	321
Pakistan	7.5	2.89	1.59	552
Egypt	1.3	0.36	0.32	873
Turkey	4.6	0.71	0.75	1065
Uzbekistan	5.5	--	--	--
World	100	33.82	19.74	584

Adopted from Choudhary and Laroia, 2001.

due to insects still account for 20-30 % of the total production (Oerke, 1994). Every year the individual farmers face the possible devastation of their crops by insect infestations. Intense pest infestation along with the need for expensive pesticides led to high levels of debt in small farmers of Warangal district of Andhra Pradesh and as a result many resorted to suicide as a way out of insurmountable problems (Sharma, 1999). In view of this, it is imperative to develop genetically modified plants with insecticidal properties to reduce crop loss due to insects.

Over the past few years, the success in producing insect resistant crops through gene transfer has been impressive, and the culmination of this process occurred in 1996 with the first generation of insecticidal plants genetically known as *B.t.* plants were introduced into the market. Approximately, 40 different genes conferring insect resistance have been incorporated into crops since then (James, 1998). This new technology is seen as an additional tool for control of crop pests and could offer certain advantages over conventional insecticides such as more effective targeting of insects protected within plants, greater resilience to weather conditions, fast biodegradability, reduced operator exposure to toxins and financial savings (Gatehouse *et al.*, 1992) . The current status of transgenic crops expressing genes from *Bacillus thuringiensis* (*B.t.)* has been thoroughly reviewed (Schnepf *et al.*, 1998, deMaagd *et al.*, 1999, Estruch *et al.*, 1997 and Schuler *et al.*, 1998).

Biotechnology is being commercialized very fast. In 1996, 1.7 million hectares were planted with transgenic crops. This increased to 11 million hectares in 1997 and about 28 million hectares in 1998 (Table 3). In the first three years of commercialization

(between 1996 and 1998), eight countries excluding China – five industrial and three developing – have contributed to more than a fifteen fold increase in the area covered by transgenic crops globally. The number of countries growing commercialized transgenic crops increased from five in 1997 (USA, Argentina, Canada, Australia, and Mexico) to eight in 1998 when South Africa, Spain, and France grew transgenic crops for the first time.

Table 3.

Global Area[a] (million hectares) of transgenic crops in 1996, 1997 and 1998.

Year	Total area	Area in industrial countries	Area in developing countries
1996	1.7	1.7	-
1997	11.0	9.5	1.5
1998	27.8	23.4	4.4

[a] Excluding China
(James , 1998)

The main transgenic crops which are being commercialized include soybean (52%), corn (30%), cotton (9%), canola (9%), and potato (~1%). Transgenics have been produced for herbicide tolerance (71%), insect resistance (28%), and quality traits (1%). However, research is being done to genetically modify most plants with a high economic value such as cereals, fruits, vegetables, and floricultural and horticultural species.

Some of the current applications of plant biotechnology include

- Crop plants with improved agronomic properties – less need for agrochemicals, thrive on marginal land, resistant to pests or diseases, or give greater yields (even today, an estimated 30% of the food grown is lost to pests!)

- Crops with improved handling properties – delayed ripening and increased shelf life
- Crops with new qualities – improved nutritional status such as enhanced protein or starch content, added vitamins, and improved flavour
- Plants which yield entirely new products – pharmaceuticals, edible vaccines, oils, fuel, and other non-food products

In this world of fast moving biotechnology, developing countries have also taken a step ahead (Table 4). However, they still have a long way to go. Of the 159 field releases, Argentina leads with 43, followed by Chile, Mexico, Puerto Rico, and Republic of South Africa with 17–20 each. India is far behind with just two field releases (none of which are commercially released yet).

Table 4. Field releases of transgenic plants in developing countries (by species and introduced trait)

Species	Number of field releases[a]	Introduced trait				
		Herbicide resistance	Insect resistance	Virus resistance	Product quality	Others
Maize	46	29	16	1	3	5
Soybean	27	25	-	1	1	-
Cotton	24	16	15	-	-	-
Tomato	19	-	2	1	16	-
Potato	13	-	1	6	-	6
Subtotal	129	70	34	9	20	11
Other species	30					

[a] Total number of field releases is 159
(de Kathen. 1996)

Bacillus thuringiensis

Bacillus thuringiensis (*B.t.*) is a positive, spore forming bacterium that exists in many locations, such as the soil, plant surfaces and in grain storage dust. *B.t.* can be distinguished from related *Bacillus* sp. by the presence of parasporal crystals that are formed during sporulation. The parasporal crystals consist of one or more δ-endotoxins or crystal (Cry) protein of ~130 kDa (although truncated forms also occur) which makes *B.t.* an effective insect pathogen. Following ingestion, the alkaline environment of the insect mid gut causes the crystals to dissolve and release the constituent protoxins. The protoxins are subsequently trimmed by gut proteases to an N-terminal, 65-70 kDa truncated form-the activated toxin. The toxin binds to specific receptors on the cell membranes of the mid gut epithelial cells, inserts itself into the membrane and forms pores which causes the ion imbalance. This results in paralysing the gut cells which eventually kill the epithelial cells and later the insect by colloid osmotic lysis (Knowles and Dow, 1993).

This insecticidal property of *B.t.* led to the development of bioinsecticides for the control of certain insect species among the orders Lepidoptera, Diptera and Coleoptera (Hofte and Whiteley, 1989). Some *B.t.* strains have also been reported to be active against other insect families (Hymenoptera, Homoptera, Orthoptera and Mallophaga and also mites, nematodes, flatworms and protozoa (Feitelson *et al.*, 1992). *B.t.* δ-endotoxins are part of a large and still growing family of homologous proteins and more than 130 genes have been identified to date. These genes generate a rich source of diversity and contribute for their differing insect specificities. This specificity is an important aspect of

the *B.t.* Cry proteins. Each protein is active against one or few insect species. Specificity is to a large extent determined by the toxin-receptor interaction (VanRie *et al.*, 1990) although solubility of the crystal and protease activation also play a role. More information on *B.t.* is furnished in excellent reviews made by Schnepf *et al.*, (1998); Sekar, (2000) and Hofte and Whiteley (1989) about the different types of *B.t.* and crystal proteins. Crickmore *et al.*, (1998) revised the nomenclature for all the *B.t.* genes and their crystal proteins published then according to their degree of evolutionary divergence as estimated by their nucleotide sequence analysis. This allows closely related toxins to be ranked together and removes the necessity for researchers to bioassay each new protein against a growing series of organisms before assigning it a name.

Genetics and Molecular biology

B.t. strains have a genome size of 2.4 to 5.7 million bp. Most *B.t.* isolates have several extrachromosomal elements. Some of them circular and others linear (Carlson *et al.*, 1994). It has long been understood that the proteins comprising the parasporal crystal are generally encoded by large plasmids (Gonzalez *et al.*, 1981). However, it is not clear about the contribution of the chromosomal homologs which are also known to hybridize with the *cry* probes. The *B.t.* species harbour a large variety of transposable elements including insertion sequences and transposons. The transposons are postulated to be involved in the amplification of the *cry* genes in the bacterial cell and another possible role is one of mediating the transfer of plasmids by a conduction process involving the formation of cointegrate structure between self conjugative plasmids and chromosomal DNA or nonconjugative plasmids (Green *et al.*, 1989).

Regulation of insecticidal crystal protein production

The production of most Cry proteins in *B.t.* starts at the stationary phase and accumulates throughout the sporulation, resulting in the appearance of parasporal crystalline inclusions within the mother cell. The Cry proteins generally account for 20 to 30% of the dry weight of the sporulated cells. The high level of crystal protein synthesis in *B.t.* and its co-ordination with the stationary phase are controlled by a variety of mechanisms occurring at the transcriptional, post-transcriptional and post-translational levels.

Transcriptional mechanism

Only one type of RNA polymerase is present in prokaryotes. Temporal regulation of different genes is controlled by different sigma factors. In *B.t.* most of the *cry* genes are expressed during sporulation, except few genes that are expressed during vegetative phase.

Sporulation dependent *cry* gene expression

In *B. subtlis*, σ^{A} form of RNA polymerase transcribes the genes that should be transcribed during vegetative phase. The σ^{H} form of RNA polymerase transcribes genes to be expressed during stationary phase. σ^{E}, σ^{F}, σ^{G}, σ^{K} appear during sporulation. The σ^{A} and σ^{H} factors are active in the predivisional cell. σ^{E} and σ^{K} are active in the mother cell, and σ^{F} and σ^{G} are active in the forespore. The *cry1Aa* is expressed only in the mother cell compartment in *B. thuringiensis*. E and K homologs σ^{35} and σ^{28} of *B.t.* have been cloned and sequenced (Adams *et al.*, 1991). Two

transcription start sites (BtI and BtII) have been mapped in the *cry1Aa* transcripts, defining two overlapping, sequentially activated promoters (Wong *et al.*,1983). BtI is active between about T2 and T6 of sporulation and BtII is active from about T5 onwards. The σ^{35} and σ^{28} specifically direct transcription of *cry1Aa* from Bt.I and BtII, respectively. *In vitro* transcription experiments also indicated that at least two other *cry* genes *cry1Ba* and *cry2Aa* contain either BtI alone or BtI with BtII. In addition to *cry1Aa*, *cry1Ba* and *cry2Aa*, the transcription of many other *cry* genes, *cry4Aa*, *cry4Ba*, *cry11Aa*, *cry15Aa* is likely to be σ^{E} or σ^{K} dependent. The *cry18Aa* gene isolated from *Bacillus popilliae* is successively transcribed by σ^{E} and σ^{K} forms of RNA polymerase from a single promoter during sporulation (Zhang *et al.*, 1998). Even though the *cry4Aa, cry4Ba, cry11Aa* expression is sporulation dependent, low-level transcription has been detected during the transition phase (Yoshisue *et al.*,1995; Poncet *et al.*, 1997).

Sporulation independent *cry* gene expression

The *cry3Aa* gene, isolated from the coleopteran active *B. thuringiensis* var. *tenebrionis* was found to be expressed during vegetative growth, although at a lesser extent than during the stationary phase (Sekar, 1988, De souza *et al.*,1993; Malvar *et al.*,1994). It has been shown that the *cry3Aa* promoter is weakly but significantly expressed during vegetative growth, is activated from the end of exponential growth until stage 2 of sporulation (about T3) and remains active until stage 4 of sporulation (about T7) (Agaisse and Lereclus. 1994; Salamitou *et al.*,1996). The *cry3A* promoter, although located unusually far upstream of the start codon (position –558), resembles promoters recognised by the primary sigma factor (σ^{A}) of vegetative cells (Agaisse *et al.*,1994). A

similar promoter was found 542bp upstream of the start codon of the *cry3Bb* gene (Baum and Malvar, 1995). The results indicate that *cry3Aa* expression is activated by a nonsporulation dependent mechanism arising during the transition from exponential growth to the stationary phase.

Post transcriptional mechanisms

The half life of *cry* mRNA, about 10 min, is at least five fold greater than the half -life of an average bacterial mRNA (Glatron and Rapoport. 1972) which is an important contributer to the high level of toxin production in *B. thuringiensis.* Wong and Chang (1986) showed that the putative transcriptional terminator of the *cry1Aa* gene (a stem loop structure) acts as a positive regulator. It is likely that *cry1Aa* transcriptional terminator increases the *cry* mRNA stability by protecting it from exonucleolytic degradation from the 3′ end. Similar terminator sequences have been found downstream from various *cry* genes.

While the *cry3A* promoter is located in –560 to –600 from the translational start codon, after post transcriptional processing stable transcript appears from - 129 (Agaisse and Lereclus, 1994). In addition to normal Shine-Dalgarno sequence which is always found nearly 17 bases upstream of the start codon, another Shine-Dalgarno sequence is found between –125 and –117 in *cry3A* mRNA and this sequence has been designated as stability Shine-Dalgarno (STAB-SD) sequence (Agaisse and Lereclus, 1996). The stability of the *cry3A* mRNA result from an interaction between the 3′ end of 16SrRNA and STAB-SD. The binding of a 30S ribosomal subunit to this sequence may protect the mRNA against 5′-3′ ribonuclease activity, resulting in a stable transcript with a 5′ end at

nucleotide position –129. Potential STAB-SD sequences are also present in similar positions upstream of the *cry3Ba, cry3Bb* and *cry3Ca* (Schnepf *et al.*,1998).

Post translational mechanism

The ability of *B.t* to produce large quantities of insecticidal proteins after the onset of stationary phase and during sporulation phase is largely due to the ability of these proteins to form crystalline inclusions. The transition to an insoluble state presumably makes the Cry protein less susceptible to proteolytic degradation and allows them to accumulate within the mother cell. Proteolytic stability of the nascent protoxin is however a likely prerequisite for efficient crystal formation. Depending on their protoxin composition the crystals have various forms bipyramidal (Cryl), cuboidal (Cry2), flat rectangular (Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B) and rhomboidal (Cryl1A).

The 130-140 kDa Cryl protoxins can spontaneously form crystals. It is generally assumed that the cysteine rich C-terminal half of the Cryl protoxins contributes to the crystal structure through the formation of disulphide bonds (Bietlot *et al.*,1990). A similar mechanism of protein self-assembly may be responsible for the crystal formation of other 130 to 140 kDa protoxins (e.g., Cry4, Cry5 and Cry7). The cysteine rich C-terminal region is absent from the 73kDa Cry3A protoxins. It is postulated that the presence of four intermolecular salt bridges in the Cry3A toxin might participate in the formation of the crystal inclusion (Li *et al.*, 1991).

The smaller Cry proteins do not possess the conserved C-terminal domain characteristic of the 130-140 kDa proteins and indeed, several studies indicate that some

of these Cry proteins may require assistance in crystal formation. Two genes viz. orf1 and orf2 are present in the upstream of *cry11A* operon. The encoded product of *orf2* is essential for the crystal formation, but the *orf*1 does not have any effect on Cry11A crystal production (Crickmore and Ellar, 1992). Neither *orf*1 nor *orf2* in the *cry11C* operon appeared to be necessary for the formation of Cry11C inclusions in *B. thuringiensis* (Wu *et al.*,1991). *cyt1* and *cry11Aa1* genes, located on a 72 mDa plasmid are coordinately regulated during sporulation but are transcribed from divergent promoters. *cry11Aa1* operon contains three ORFs (*cry11Aa1*, P19 and P20). P19-encoding gene is localized in the upstream and P20 in the downstream of the *cry11Aa* (Adams *et al.*,1989; McLean and Whiteley, 1987; Visick and Whiteley, 1991). Wu and Federici (1995) reported that Cry11Aa1 production and crystal formation is dependent on the 20 kDa (P20gene product) protein and larger Cry11Aa1 crystals can be obtained by placing the P20 under the control of the sporulation (σ^E and σ^K) – specific Cry*1Ac* promoters. In contrast, Chang *et al.*, (1993) and Dervyn *et al.*, (1995) reported high-level production of Cry11Aa1 in the absence of the 20 kDa protein. Deletion of the p19 had also no apparent effect on Cry11Aa1 production (Dervyn *et al.*,1995).

The mechanisms by which the Cry2A orf2 protein and Cry11Aa1 20kDa protein exert their effect on crystal formation in *B. thuringiensis* are not well understood, although evidence provided by Visick and Whiteley (1991) suggests that the 20 kDa protein protects the nascent CytA peptide from proteolysis. Whether it protects the protoxin molecule from proteolysis or assists in proper folding of the nascent protein molecule to allow its deposition in the crystalline inclusion, or serves as scaffolding protein for crystal formation is not clear. An absence of these chaperonin-like function

would probably result in increased proteolytic degradation of the protoxin and reduced protoxin yield.

Toxin structure

To date, the structures of three crystal proteins-Cry3A (Li *et al.*, 1991), Cry1Aa (Grochulski *et al.*,1995) and Cyt2A (Li and Ellar, 1996) have been solved by X-ray crystallography. The Cry3A and Cry1Aa show about 36% amino acid identity and Cyt2A shows less than 20% amino acid identity. Cyt2A consists of a single domain in which two outer layers of alpha-helix wrap around a mixed beta sheet. CytA is believed to have a similar structure. Cry3A and Cry1Aa possess three domains. Domain I consists of a bundle of seven antiparallel α helices in which helix5 is encircled by the remaining helices. Domain II consists of three antiparallel β sheets joined in a typical "Greek key" topology, arranged in a so-called β-prism fold. Domain III consists of two twisted, antiparallel β- sheets forming a β-sandwich with a "jelly roll". Schnepf *et al.*, (1998) found three additional conserved blocks lying outside the active toxic core and 5 conserved blocks in N-terminal region as reported by Hofte *et al.*, (1989), when they compared the carboxy-terminal halves of the sequences with more than 1000 residues.

The group consisting of Cry1, Cry3, Cry7, Cry10, Cry16, Cry17, Cry19, Cry20 contains all the five of the core blocks. A second group consisting of Cry5, Cry12, Cry14, Cry21A, Cry13A contains recognizable homologs of blocks 1,2,4 and 5. Block 1 shows more variability within this second group of sequences than within the first. The proteins within this second subgroup also possess a block 2 variant; block 2 sequences show greater sequence similarity within the two groups than between them. Block3 is

completely absent from this second group of Cry proteins; an unrelated sequence, highly conserved within the second subgroup but absent from the first, lies between blocks2 and 4. For both groups, when a protein possess the C-terminal extension, blocks 6, 7 and 8 are invariably present. Members of a third sequence similarity group, composed of Cry2,Cry11,Cry18 possess block1 and a truncated variant of the block 2 core but lack convincing homologs of the other conserved blocks (Lereclus *et al.*,1989). An alternating arginine tract not otherwise homologs to block4 is found near the C-terminus of Cry11 and Cry18. A weak homolog of block5 may also be present among the proteins in this group, but its significance, if any, is uncertain. The other proteins Cyt1,Cyt2,Cry6,Cry15 and Cry22 have no recognizable homologs to the conserved blocks seen in three groups noted above.

Block1 encompasses helix5 of domainI. Block 2 includes helix 7 of domain I and the first β strand of domain II. These two structures comprise the region of contact between the two domains. Block 3, 4 and 5 each lie on one of the three buried strands within domain III. Block 3 contains the last β strand of domain II, a structure involved in interactions between domains I and III. In the current δ-endotoxin mode of action model, domain I has been thought to be responsible for the toxic activity in the membrane. It has been proposed that after the toxin binds to the receptor, there is a change in the conformation of this domain allowing the hydrophobic surfaces of the helices to face the exterior of the bundle, leading to insertion into the membrane and the formation of ion channels (Knowles. 1994; Li *et al.*,1991). The second domain of δ-endotoxin has been thought to be the receptor binding domain. Recently, it has been reported that the vitelline membrane outer layer protein (vmo-1) and domain II from Cry

proteins have similar three-dimensional structures. It has been proposed that the β prism-fold may be a structural domain associated with carbohydrate binding functionality (Shimizu and Morikawa, 1996). Both proteins may have a carbohydrate binding site, since binding of Cry*1Ac* toxin to its glycoprotein receptor is inhibited by N-acetylgalactosamine (Knowles *et al.*,1993). It has been proposed that domain III stabilizes the toxin by protecting against proteolysis (Li *et al.*,1991) and that this domain may be implicated in receptor binding (Aronson *et al.*,1995; Bosch *et al.*,1994; Lee *et al.*,1995).

Mechanism of action

The mechanism of action of *B. thuringiensis* Cry proteins involves solubilization of the crystal in the insect midgut, proteolytic processing of the protoxin by midgut proteases, binding of Cry toxin to midgut receptors, and insertion of the toxin into the apical membrane to create ion channels or pores.

Solubilization and proteolytic activation

The proteins are synthesized as insoluble crystalline protoxins in the insect gut before exerting their effects. The gut of most target insects has a very high pH (except coleopteran larvae whose gut pH is acidic) and is essential for dissolving most Cry proteins, which are usually soluble only above pH 9.5. After solubilization, many protoxins must be processed by insect midgut proteases to become activated toxins. The major proteases of the lepidopteran insect midgut are trypsin-like or chymotrypsin-like proteases. The degradation of Cry*1Ac* protein occurs in an ordered sequence of seven specific cleavages starting at the C-terminus of protoxin and proceeding towards the N-

terminal region. Fragments of approximately 10kDa are produced and rapidly proteolysed further into small peptides (Choma *et al.*,1990). An interesting and unexpected finding is that DNA is intimately associated with the crystal and appears to play a role in proteolytic processing (Bietlot *et al.*,1993; Clairmont *et al.*,1998).

In general, for Cryl protoxins, only limited proteolysis occurs at the N-terminus, while approximately 500 amino acids are removed from the C-terminus. The Cry1Ab toxic fragment resides between amino acid residues 29-35 and 599-607 (Hofte *et al.*, 1989) and *cry1Ac* toxin fragment lies between residues 29-695 (Toshida *et al.*,1989), while Cry4Bal toxic fragment residues between amino acids 39 and 677 (Pao-intara *et al.*,. 1988). In contrast, the Cry2, Cry3, Cry11Aa proteins do not undergo protease mediated C-terminal cleavage because these proteins appear to be naturally truncated. Even a small C-terminal truncation of 11 amino acids of the cry2A toxin results in loss of activity (Widner and Whiteley, 1989). Similarly, C-terminal protease cleavage of both the Cry3A and Cry11Aa toxins will likely result in loss of insecticidal activity, because one of three domains of Cry toxins is present in the C-terminal region of these proteins (Carroll *et al.*,. 1989). In the mosquito mid gut, the Cry11Aa protoxin is cleaved into 32-49 kDa that are found to be toxic to mosquito larvae (Cheung *et al.*, 1986). The 27-kDa CytA cytolytic toxin is also cleaved to an active product of 24 kDa resulting from both N- and C-terminal proteolysis (Gill *et al.*,1987).

The insect midgut structure and receptor binding

The midgut of lepidopteran larvae is a simple tube made of one layer of cells resting on a basal membrane. The two major cell types are columnar cells, with an

apical border of microvilli, and a unique goblet cell containing a large cavity (filled with a flocculent matrix of sulphated glycosaminoglycans) which is joined to the apical surface by a complex interdigitated valve (Flower and Filshie, 1976; Cioffi, 1979; Dow 1986; Moffett and Koch, 1992). The epithelial cells are joined primarily by septate junctions and gap junctions (Lane and Skaer, 1980; Lane *et al.*, 1989). The gap junctions provide electrical and chemical coupling between the goblet and columnar cells (Moffett and Koch, 1988b; Dow and Peacock, 1989). A peritrophic membrane, mainly composed of chitin and glycoproteins, lines the gut, separating the gut contents from the epithelium (Richards and Richards,1997; Adang and Spence, 1981).

Activated Cry toxins have two known functions, Receptor binding and ion-channel activity. The activated toxin binds readily to specific receptors on the apical brush border of the midgut microvilli of susceptible insects. In *Manduca sexta*, the Cry1Ab receptor is believed to be a cadherin like 210 kDa membrane protein (Vadlamudi *et al.*,1993; Francis and Bulla Jr.,1997; Keeton and Bulla. Jr., 1997), While the Cry*1Ac* and Cry1C receptors have been identified as aminopeptidase N proteins with molecular masses of 120 and 106 kDa respectively (Knight *et al.*, 1994; Luo *et al.*,1996; Sangadala *et al.*,1994). Binding is a two stage process involving reversible and irreversible steps. The latter step may involve a tight binding between the toxin and receptor, insertion of the toxin into the apical membrane or both. It has been generally assumed that irreversible binding is exclusively associated with membrane insertion. Recent reports indicates that truncated Cry1Ab molecules containing only domains II and III can still bind to midgut receptors but reversibly. This supports the notion that irreversible binding requires the insertion of domain I (Flores *et al.*,1997).

After binding to a specific receptor, toxins insert rapidly and irreversibly into the plasma membrane of the gut cell. There is evidence from in vitro studies that (at least in the case of CytA) the proteins then form oligomers of up to 16 toxin monomers (Maddrell *et al.*, 1988; Chow *et al.*, 1989). The next step involves the formation of a pore or lesion in the plasma membrane. It is not yet known whether the toxin alone forms the pore *in vivo*, or whether the toxin and receptor together form a complex. Two theories were proposed for the mechanism of cell lysis. According to the "proton peril" hypothesis, the toxin induced or formed a K^+ selective channel in the columnar cell apical membrane, making the columnar cells leaky to K^+ but not affecting active K^+ pumping in the goblet cell (Harvey *et al.*, 1986; Crawford and Harvey, 1988). Such studies led to the hypothesis that formation of a cation leak in the normally K^+ impermeable columnar cell apical membrane would result in depolarization of this membrane and a consequent efflux of H^+ down the large pH gradient. The apical membrane separates compartments with a greater than 10,000 fold H^+ concentration gradient. The rise in columnar cell cytoplasmic pH would kill the cells, leading to gut breakdown and eventual death of the larvae (Harvey *et al.*, 1986; Wolfresberger, 1995).

Knowles and Dow (1993) produced the following model. The formation of nonselective pores in the columnar cell apical membrane results in entry of K^+ and efflux of H^+, which rapidly depolarizes this membrane. Small anions can probably also enter through the toxin pore. Both the rise in intracellular pH and membrane depolarization would probably lead to closure of gap junctions (Loewenstein, 1981), isolating the goblet cells from the damaged columnar cells. The columnar cells, containing macromolecules which can not leak out through a 0.6 nm pore, would absorb water

osmotically and thus swell and burst by the process of colloid-osmotic lysis (Knowles and Ellar, 1987). Elevated cytoplasmic pH probably accelerates the death of the columnar cells (Harvey *et al.*,1986).

B.t. formulations

B.t. is the most widely used biopesticide in the recent years and it represents about 2% of the total global insecticide market (Lambert and Peferoen, 1992). *B.t.* formulations (spore and crystal mixture) were used as insecticidal sprays in the 1930's but large scale production started with the introduction of Thuricide TM in the late 1950's and similar products from several countries followed it soon (Beegle and Yamamoto., 1992). In spite of their environment friendly reputation, *B.t.* sprays have never occupied a large share of the insecticide market, and are largely used by organic farmers and gardeners and in forestry. The main three factors responsible for this are the lack of stability, failure to penetrate tissues and therefore to reach insects in all parts of the plant and too narrow a specificity (De Maagd *et al.*, 1999). Crystal proteins degrade rapidly in UV light, loosing their activity and therefore multiple applications are needed throughout the growing season which becomes very expensive. This is the biggest drawback of *B.t.* sprays though some improvements have been made.

The fact that *B.t.* sprays are non systemic insecticides and are ineffective against insects that are not reachable with sprays such as sap sucking and piercing insects, root dwelling pests and larvae that burrow or bore into the plant tissues. These two problems were effectively addressed by developing transgenic plants that produce crystal proteins continuously and the toxins are protected against degradation. The third problem is of its narrow specificity. For *e.g.* the Cry1Ab protein is highly toxic against the European

corn borer and is used in current *B.t.* corn hybrids (Koziel *et al.*, 1993 and Armstrong *et al.*, 1995). The Cry1Ac protein is highly toxic to both tobacco budworm and cotton bollworm larvae and is expressed in the *B.t.* cotton varieties commercialized in USA and Australia (Perlak *et al.*, 1990). In *B.t.* potato varieties, the Cry3A protein is engineered to express and to give protection against Colarado potato beetles (Perlak *et al.*, 1993).

Vegetative Insecticidal Proteins

In the search for novel products with insecticidal activities it was discovered that clarified culture supernatant fluid collected during vegetative phase (*i.e.* log phase) growth of *Bacillus* species are a rich source of insecticidal activities (Warren *et al.*, 1996, Estruch, 1996). Supernatant of certain *Bacillus* cereus isolates possess acute bioactivity against Northern and Western corn root worms (*Diabrotica longicornis barnieri*, *D. virgifera virgifera* respectively). Similarly supernatant fluids of certain *B.t.* cultures had potent insecticidal properties against black cut worm and fall army worm (Estruch *et al.*, 1996). The cloning and characterization of the gene encoding this activity yielded a novel insecticidal protein, Vip3A that has no homology with any known protein. The N-terminal sequences of Vip proteins possess a number of positively charged residues followed by hydrophobic core region that are similar to other signal peptides of *Bacillus*. Potency studies performed on Vip proteins show that they afford acute bioactivity against susceptible insects in the range of nanograms per ml of diet (Estruch and Warner), a level comparable to that exerted by δ-endotoxins (Koziel *et al.*, 1993). In particular, Vip3A displays insecticidal activity against a wide spectrum of Lepidopterans insects particularly black cut worm and beat army worm (*S. exigua*) with LC50 between 30-100 ng per cm-2 (Estruch *et al.*, 1996). A complete degeneration of the epithelium columnar

cell was observed in second instar larvae of black cut worm fed on Vip3A-containing diet after 48 hours. *In vivo* immunological studies have also revealed specific binding of the Vip3A protein to gut epithelium cells of susceptible insects, particularly to columnar cells. (discuss about victor's work briefly here)

Insect resistance to *B.t.* toxins

Of the $ US 8.1 billion spent annually on insecticides worldwide, it is estimated that nearly $ 2.7 billion could be substituted with *B.t.* biotechnology applications (Krattiger *et al.*, 1997). At least 16 companies are presently developing transgenic crops with *B.t.* genes and about 18 *B.t.* transgenic crops have been approved by US Department of Agriculture (USDA) for field testing (Mellon *et al.*, 1998). In transgenic *B.t.* plants, the *B.t.* protein are made much more persistent and effective even against insects at sites difficult or impossible to reach with sprays (Roush, 1994). For example, the *B.t.* cotton needed fewer insecticide treatment compared with an average of 5.12 sprays per year in US. Despite the considerable advantages of *B.t.* transgenic crops, both to the farm environment and to farm worker safety, concern is widespread that these gains will be short lived because of evolution of resistance in the pests.

Over 500 species of insects are reported to have become resistant to one or multiple synthetic chemical insecticides (Georghiou, 1991). It had long been believed that insects would not develop resistance to *B.t.* toxins, since *B.t.* and insects have coevolved. The degree of resistance observed in an insect population is typically expressed as the resistance ratio (the number of LC50-resistant insects/number of LC50-sensitive insects). Examples of laboratory selected insects developing resistant to

individual Cry toxins include the Indian meal moth (*Plodia interpunctella*), the almond moth (*Cadra cautella*), the Colarado potato beetle (*Leptinotarsa decemlineata*), the cotton wood leaf beetle (*Cadra scripta*), the cabbage looper (*T. ni*), the cotton leaf worm (*Spodoptera littoralis*), the beat army worm (*S. exigua*), the tobacco budworm (*H. virscens*), the European corn borer (*O. nubilalis*) and the mosquito *Culex quinquefasciatus* (Schnepf *et al.*, 1998).

Resistance management

Resistance management strategies are necessary to prevent or diminish the selection of rare individuals carrying resistance genes and hence to keep the frequency of resistance genes sufficiently low for insect control. The strategies proposed include the use of multiple toxins (stacking or pyramiding). Crop rotation, high or ultrahigh dosages and spatial or temporal refugia (McGaughey and Whalon, 1992 and Tabashnik, 1994). Recent analysis of resistance development does support the use of refugia. The real value of the various tactics proposed have to be tested in larger scale field trials to implement a specific pest management strategy for each crops growing in different agroclimatic conditions. It is equally important to design a resistance management strategy acceptable to various people like technology suppliers, seed companies, extension workers, crop consultants, regulators and most all, the growers (Kennedy and Whalon, 1995). In transgenic crops, selection pressure could be reduced by restricting the expression of the crystal protein genes to certain tissues of the crop (most susceptible to pest damage) so that only certain parts of the plant are fully protected, the remainder providing a form of spatial refuge (Mallet and Porter,1992). It has been proposed that

cotton lines in which *cry* gene expression is limited to the young bolls may not suffer dramatic yield loss from *Heliothis* larvae feeding on other plant structures since cotton plants can compensate for a high degree of pest damage (Gould, 1988). The wound inducible promoters can be used that can trigger the resident *cry* gene expression only when the insect starts feeding on them (Peferoen, 1990). This might be a better mechanism to minimize toxin exposure to insects.

Alternately, chemically inducible promoters (Williams *et al.*, 1992) can be used to control the toxin expression and by this method the farmer has an option to have the *cry* gene in the crops to express when the insect population exceed an economic threshold. If transgenic plants can be engineered to express *cry* gene at doses high enough to kill even homozygous resistant insects, the crop can become a non-host. This is possible with the currently alterable levels of Cry expression in plants (Jansens, 1997). Higher levels of expression has been reported by transforming tobacco chloroplasts with an unmodified, full length *cry1Ac* coding region. The effects of amplification of the integrated gene in the plastid genome resulted in Cry protein expression to a level of 3-5% of total soluble protein. This level of expression even provided protection against relatively Cry*1Ac* tolerant plant pests (McBride *et al.*, 1995). Colorado potato beetle population which were 100 fold resistant to Cry3A protein spray could not survive on transgenic potato expressing the Cry toxin (Whalon *et al.*, 1993). It is yet to be established if a combination of toxins with ultrahigh expression can overcome all homozygous resistant alleles, changing the crops to non host plants.

Another attractive option is combination of high dose strategy with the use of refugia (toxin free areas). The principle is to express Cry toxins at such dose that nearly all heterozygotic carriers of resistance alleles will be killed. The survivors would most likely mate with the sensitive insects harbored in the nearby refuge. Consequently, a population of homozygous resistant insects would be unlikely to emerge. Metz *et al.*, (1995) demonstrated that F1 larvae from a cross between a susceptible laboratory *P. xylostella* colony and a field resistant colony did not survive on transgenic broccoli expressing Cry1Ac (Shelton *et al.*, 1993). A 10% refuge was reported to delay resistance over a nine generation test in diamond back moth population that had evolved resistance to Cry1Ab and Cry1Ac in the field (Liu and Tabashnik, 1997).

Depending on the plant, the refugia may be naturally present or created by planting nontransgenic plants. The refugia should be uncontaminated and allowing random mating between resistant and nonresistant insects (Gould, 1994, 1998). Prepacked refugia (mixture of toxin expressing and toxin free seeds) is also recommended even though it is still controversial as it is effective only to larvae that move very little between plants or to adults that can mate visually over a short distance.

The most attractive option for resistance management in connection with the use of refugia is to produce transgenic plants engineered to express multiple Cry proteins or by spraying multiple proteins, provided these toxins have different modes of action with respect to insects' mechanism of resistance (Roush, 1994). *B.t.* Cry toxins can be even combined with other insecticidal proteins. This kind of multiple attack strategy has a

benefit that even if insects homozygous for one resistance gene are rare, than insects homozygous for one or two resistance genes are yet heterozygous for another gene. A critical condition for the success of this strategy is that each of the insecticides on its own should have high mortality for susceptible homozygotes. The field collected *C. quinquefasciatus* readily developed resistance (in laboratory conditions) to a single toxin (Cryl1A) but they were highly sensitive when selected with more toxins (Georghiou and Wirth, 1997). These studies suggest that a thorough understanding of the biology of the crop-pest complex, the possible mechanisms of resistance and the frequency of resistant alleles in the insect population would be necessary to devise an optimum resistance management strategy.

Transgenic *B.t.* plants

The urgent need for an effective biopesticide, the discovery of different *B.t.* genes encoding relevant insecticidal activity and the established safety of *B.t.* commercial formulations have all made transgenic *B.t.* plants a promising candidate for early commercial exploitation of plant biotechnology. In early experiments, both full length and truncated *cry* genes (which almost encodes the activated toxin) were introduced into tobacco and tomato by *Agrobacterium tumefaciens* mediated transformation (Vaeck *et al.*, 1987; Fischoff *et al.*, 1987 and Barton *et al.*, 1987). Constructs containing the truncated genes were shown to have some measure of protection against insect predation better than those containing the full length genes. Later, it became evident that the expression level of unmodified *cry* genes was too low (to a level of few ng mg^{-1} total protein) to confer enough protection against pests under field conditions.

In 1995, *B.t.*-potato (NewLeafTM, Monsanto, St Louis, MO, USA) was released as the first commercial *B.t.*-crop. These potatoes were engineered to express the Cry3A protein for protection against Colorado potato beetles and reported to have reduced the insecticide use up to 40% for *B.t.* plants in 1997. Subsequently in 1996, *B.t.*-cotton (BollgardTM, Monsanto) was introduced in the market with a reported yield increase of 14%. It expresses Cry1Ac protein and was reported to be resistant to tobacco budworm and to lesser extent to cotton boll worm and pink bollworm.

Several companies have developed *B.t.*-Maize and commercialized its use since 1996 by licensing it to seed companies: Novartis, basel, Switzerland (YieldgardTM, KnockoutTM and BitegardTM), Mycogen, San Diego, CA, USA (NatureGardTM), Monsanto (YieldGardTM) and DEKALB Genetics, IL, USA (Nt-maize was reported to give 99% control of first generation European corn borer larvae.

Limiting factors of *cry* gene integration in higher plants

B.t. cry genes are typical bacterial genes which are AT rich (60-70%) compared to plant genes (40-50%). Because of this difference, the *cry* gene codon usage is inefficient in plants. The AT rich regions may contain transcription termination (polyadenylation sites CAATAAA and variations there of), cryptic mRNA splice sites and mRNA instability motifs (ATTTA). The truncated transcripts were often found and the sequence motifs that causes the truncations were removed with an intention to increase the expression levels. The effects of different degrees of gene modification were investigated in the *cry1Ab* and *cry1Ac* genes (Perlak *et al.*, 1991). Removal of seven out

of 18 polyadenylation sites and seven out of 13 mRNA instability motifs (ATTTA) from *cry1Ab* raised the *B.t.* protein concentrations to detectable levels (upto tenfold higher than unmodified genes). Removal of the remaining polyadenylation sites and ATTTA sequences and changes to a total of 356 of the 615 codons raised the expression levels further up to (0.2-0.3 % of total soluble protein) 100 fold higher than the level for unmodified genes. These studies were made in transgenic tobacco, tomato and cotton (Perlak *et al.*, 1990 and 1991).

Apart from rare codons, some other factors were also found to be involved in low level of expression. For example, some portion of the unmodified sequences are homolgous to plant introns resulting in mRNA slicing when expressed in plants (Van Aarssen *et al.*, 1995). When the *cry* gene sequences were extensively modified with synonymous codons to reduce or eliminate the potentially deleterious sequences and generated a codon bias more like that of a plant, expression improved dramatically (Adang *et al.*, 1993; Fujimoto *et al.*, 1993).

Cry3A gene that was used in potato to give resistance to Colarado potato beetle too has been codon modified to increase the level of its expression (Perlak *et al.*, 1993). The gene *cry1Ab* too was subjected to major changes to make it suitable to maize (maize specific codon usage) the first cereal to be transformed, leading to resistance against European corn borer (Koziel *et al.*, 1993). After effecting codon changes in *B.t.* genes to suit the specific plant system, both the full length and truncated versions of these genes were demonstrated successfully to express the transgenes in plants (Koziel *et al.*, 1996).

Attempts were made with a variety of plant promoters in combination with *cry* genes to increase the expression level. For example, CaMV 35S promoter is the most commonly used one and the other promoters such as wound inducible promoters (Vaeck *et al.*, 1987), chemically inducible promoters (Williams *et al.*, 1992) and tissue specific (Koziel *et al.*, 1993) promoters (in maize, the level increased up to 0.4% of total soluble protein) have also been used. In contrast to expressions from the nucleus, an unmodified *cry1Ac* gene was expressed at high levels in the chloroplasts of tobacco (McBride *et al.*, 1995).

Much importance and attention is given to the commercial development of crops such as cotton and corn these days. *B.t.* cotton is being licensed directly to the growers to an area over 2 million hectares in the USA and 4,00,000 hectares in Australia. The overall value in these markets is in the range of US $ 250 million. The first large scale (around 8,00,000 hectares) production has tempered the euphoria around *B.t.* cotton. An exceptionally high level of bollworms appeared to have caused severe feeding damage and some additional insecticide spraying was required to the *B.t.* crops. This situation warranty the immediate need to develop transgenic plants that can produce very high levels of insecticidal proteins. Such a high level of expression was made possible when McBride *et al.*, (1995) transformed chloroplast genome of tobacco with *cry1Ac* gene and reported a level of 3-5 % Cry protein to the total soluble protein. Such a high level of expression was possible due to the development of chloroplast transformation techniques then becoming popular after the particle bombardment delivery of DNA into plants became possible in late 1980's. The chloroplast transformation technique was identified to have plenty of advantages over nuclear genome transformation method.

_B.t._ cotton in India

In India, *B.t.* cotton had been released by Mahyco Monsanto Biotech in the yea 2002 and nearly 55,000 farmers haved planted these seeds in six states (viz Maharashtra, Gujarat, Andhra Pradesh, Karnataka, Madhya Pradesh and Tamil Nadu) The results of first year of harvest are available except in Tamil Nadu where the crop ha not attained harvestable stage. Mahyco says farmers have reported a nearly 70 per cen reduction in the use of pesticide and an increase in income of about Rs 7,000 an acre. I claims an increase of yield by 30 percent. In Karnataka, the irrigated *B.t.*-cotton fiel was more vigorous and healthier than the rain fed crop. Farmers applied two rounds o spray for sucking insects and one for bollworm whereas the farmers having non *B.t.*-cro were spraying more than 20 sprays. The loss of yield was around 20 to 40 percen (Times News Network, 2003; Kameswara Rao, 2002)

In Gujarat, though there was a severe monsoon failure the yield was not a expected. It is premature to give a verdict on the performance of *B.t.*-cotton in any par of India. This year's results cannot be taken to proclaim *B.t.*-cotton either as success or failure.

Status of genetically modified plants in India

The first experiment on transgenic plants in the field was started in 1995 whe *Brassica juncea* plants containing *Barnase*, *Barstar*, and *Bar* genes were planted a Gurgaon (Haryana) to assess the extent of pollen escape. Subsequently, severa

experiments have been started in the field in different locations using transgenic mustard, cotton, and tomato. Several Indian institutes and organizations claim that they have developed transgenic plants which are ready for greenhouse/screenhouse/polyhouse evaluation, and some for field evaluation as well. Table 5 (adopted from www.teriin.org/gmp/status.htm) gives a summary of the major developments in transgenic plants in India.

The Government of India through the Department of Biotechnology (DBT) has been mainly responsible for the propagation of research in transgenic plants and for the development of transgenic cultivars in the country. The DBT has spent about 6750 million rupees during 1986–97 March on all aspects of biotechnology development in the country. During 1989–97, nearly 270 million rupees were spent by the DBT alone on plant molecular biology research in which the projects primarily focused on developing transgenic plants of higher economic value. There had also been marginal support from the Indian Council of Agricultural Research, Department of Science and Technology, Council of Scientific and Industrial Research, and certain other agencies; the combined expenditure of all these agencies would not exceed 70 million rupees. Private sector units have started allocating some funds during the last three years and their investment could be estimated to be of the order of 100 million rupees. Thus, the total money allocated for research in transgenic plants in the country could be estimated at about 440 million rupees up to the present time. It is anticipated, however, that the private sector would make substantial investments in development and production in the future.

Table 5. Transgenic research in India

Institute	Plants/crops used for transformation	Transgenes inserted	Aim of the project and progress made
Central Tobacco Research Institute, Rajahmundry	Tobacco	*Bt* toxin gene – *Cry1A(b)* and *Cry1C*	To generate plants resistant to *H. armigera* and *S. litura*. One round contained field trial completed. Further evaluation under progress.
Bose Institute, Calcutta	Rice	*Bt* toxin genes	To generate plants resistant to *lepidopteran* pests. Ready to undertake greenhouse testing.
Tamil Nadu Agricultural University, Coimbatore	Rice	Reporter genes like *hph* or *gus*	To study extent of transformation frequency.
University of Delhi, South Campus, New Delhi	Mustard/rapeseed	*Bar*, *Barnase*, *Barstar*	Plant transformations completed and ready for greenhouse experiments
	Rice	Selectable marker genes, e.g., *hph* resistance and *gus*	Gene regulation studies. Transformations completed.
National Botanical Research Institute, Lucknow	Cotton	*Bt* toxin gene	To generate plants resistant to lepidopteran pests. Laboratory transformations in progress.
Indian Agricultural Research Institute, substation at Shillong	Rice	*Bt* toxin gene	To impart resistance to lepidopteran pests. Transformations in progress.
Central Potato Research Institute, Shimla	Potato	*Bt* toxin gene	To generate plants resistant to lepidopteran pests. Ready to undertake greenhouse trials.
ProAgro–PGS India Ltd, New Delhi	*Brassica* (Mustard)	*Barstar*, *Barnase*, *Bar*	To develop better hybrid cultivars suitable for local conditions. Contained field trails in over 15 locations completed. Further contained open-field research trials in progress at many locations.
	Tomato	*Cry1A(b)*	To develop plants resistant to lepidopteran pests. Glasshouse experiments and one-season contained field experiment completed. Further experiments in progress.
	Brinjal	*Cry1A(b)*	To develop plants resistant to

			lepidopteran pests. Glasshouse experiments in progress.
	Cauliflower	*Barnase, Barstar, Bar*	To develop hybrid cultivars for local use. Glasshouse experiments in progress.
	Cauliflower	*Cry1H/Cry9C*	To develop resistance to pests. Glasshouse experiments in progress.
	Cabbage	*Cry1H/Cry9C*	To develop resistance to pests. Glasshouse experiments in progress.
Mahyco, Mumbai	Cotton	*Cry1A(c)*	To develop resistance to lepidopteran pests. Multicentric field trials in over 40 locations completed and further contained field trails in progress.
Rallis India Ltd, Bangalore	Chilli	Snowdrop (*Galanthus nivalis*) Lectin gene	Resistance against lepidopteran, coleopteran, and homopteran pests. Transformation experiments in progress.
	Bell pepper	Snowdrop (*Galanthus nivalis*) Lectin gene	Resistance against lepidopteran, coleopteran, and homopteran pests. Transformation experiments in progress.
	Tomato	Snowdrop (*Galanthus nivalis*) Lectin gene	Resistance against lepidopteran, coleopteran, and homopteran pests. Transformation experiments in progress.
Indian Agricultural Research Institute, New Delhi	Brinjal, Tomato, Cauliflower, Mustard/rapeseed	*Bt* toxin gene	To impart resistance to lepidopteran pests. Transformation completed, greenhouse trials completed, and one-season field evaluation completed for brinjal and tomato.
Jawaharlal Nehru University, New Delhi	Potato	Gene expressing for protein containing lysine obtained from *Amaranthus* plants	Transformation completed and transgenic potato under evaluation.

hloroplast genome transformation

Chloroplast genome transformation was first reported in the unicellular algae *lamydomonas reinhardtii* by Boynton *et al.*, (1988). They could successfully roduce foreign gene through the double membrane of the plastid by using the biolistic stem developed by Sanford *et al.*, (1988) and refined by Johnston (1990). This nsformation studies were started with an objective to address plastid genetics, to aracterize promoter strength (Blowers *et al.*, 1990) trans-splicing (Goldschmidt-ermont *et al.*, 1991), photosynthetic functions, to achieve targeted disruption of plastid nes and to get the introduced foreign DNA expressed. A typical plastid genome in wering plants is 120-160 kb in size and includes a region of ~25 kb that is duplicated an inverted orientation (Palmer, 1991). In any particular species, all plastid types carry ntical multiple copies of the same genome. The meristematic cells contain around 10-plastids each containing around 50 copies of plastome, whereas the leaf cell may have many as 100 chloroplasts each with around 100 copies of plastome giving in total of und 10,000 copies of the plastid genome per cell. This number may vary from species species in the range of 1900-50,000 (Bendich, A.J. 1987).

Progress of *Chlamydomonas* system has had a major influence on the direction of earch in plastid transformants in flowering plants. However, the plastid nsformation in flowering plants is different from *Chlamydomonas*. Flowering plants ve many plastids whereas *Chlamydomonas* have only one. In addition, plastid nsformation in *Chlamydomonas* is carried out in photoautotrophic cells whereas in wering plants selection of transplastomic lines occurs in cells cultured on media ntaining sucrose – photoheterotrophic system. The two system respond differently to

the spectinomycin, an antibiotic used to select plastid transformants. Spectinomycin inhibits plastid protein synthesis, but not of protein synthesis by the eukaryotic type cytoplasmic ribosomes. Spectinomycin will prevent growth of sensitive *Chlamydomonas* cells since colony formation is dependent on photosynthesis (Goldschmidt-Clermont, 1991) whereas the sensitive tobacco cells continue to proliferate in culture. Since sucrose in the culture medium obviates the requirement for photosynthesis sensitive cells become white due to inhibition of plastid protein synthesis (Svab *et al.*, 1990, 1993, Staub and Maliga, 1992) whereas the resistant, transformed cells are green.

In chloroplast genome transformation, the desired gene is incorporated into the plastid genome by two homologous recombination events. In the case of gene replacement, a cloned plastid DNA vector is incorporated into the recipient chloroplast genome, resulting in complete or nearly complete replacement of the homologous region of resident genome by the donor DNA (Staub and Maliga, 1992). Insertion of foreign genes was later obtained by two homologous recombination events via flanking plastid DNA sequences. Insertion of DNA in both the repeat region (Staub and Maliga, 1993) and in the large single copy region (Svab and Maliga, 1993) had also been obtained.

Basic criteria in construction of various chloroplast transformation vector are that the vector should insert the heterologous gene at specific sites, the gene should be placed under the control of plastid specific regulatory elements to get constitutive expression, and it should have a selectable marker gene. In the previous reports of chloroplast transformation, a number of insertion sites have been used such as between *trnV* and *rps12/7* loci (McBride *et al.*, 1995, Sidkar *et al.*, 1998, Khan and Maliga, 1999, Staub *et*

al., 2000) between *rbcL* and *orf512* or *accD* gene (Daniell *et al.*, 1998 and Kota *et al.*, 1999); between *trnI* and *trnA* (De Cosa *et al.*, 2001), *etc.* which are non essential intergenic regions. Introduction of foreign DNA at these sites did not affect the function of the plastome. The vector DNA contains *trnI* and *trnA genes* which introduces the foreign DNA between *trnI* and *trnA* of plastome by homologous recombination as these genes of higher plants are highly conserved (Daniell *et al.* 1998).

The regulatory elements used are 16S rDNA promoter region (P*rrn*) and the *rps16* 3' region (T*rps16*) as terminator as they have been shown to be performing well in the previous reports. The P*rrn* had been the one that has been used throughout the chloroplast transformation experiments. Apart from T*rps16*, the terminator portion used is the 3' region of *psbA* gene. Details of the regulatory elements used in various transformation events reported are given in Table 6. The marker genes used were mainly *aadA* gene which encodes for the protein 5-aminoglycoside 3-adenyltransferase giving resistance to spectinomycin and streptomycin. In some reports, gene for kanamycin resistance (*kan*) had been used. Khan and Maliga (1999), used a transcriptionally and translationally fused *aadA* gene and *gfp* (which encodes the Green Fluorescent Protein). This helps to trace the transformed leaf portions early by green fluorescence. Various combination of chloroplast transformations vectors (*viz.* regulatory elements, DNA homology used to facilitate the insertion and the marker genes for selection) made by different groups are enlisted in the Table 6.

Table 6

List of chloroplast transformation events using different combination of foreign genes and regulatory elements

SL. NO.	PLANT	INTEGRATION SITE	GENE	PROMOTER	TERMINATOR	MARKER	REFERENCE	REMARKS
1.	Tobacco	*trnV* and *rps12/7 loci*	*cry1Ac*	16S rDNA promoter (P*rrn*)	*rps16* 3' region (T*rps16*)	*aadA*	McBride et al., 1995	Stable integration and successful
2.	Tobacco	*rbcL* and *orf512* (*accD*)	EPSPS	P*rrn*	*psbA* 3'region (T*psbA*)	*aadA*	Daniell et al., 1998	Stable integration and successful
3.	Tobacco	*trnI* and *trnA*	*cry2Aa2* operon	P*rrn*	T*psbA*	*aadA*	Cosa et al., 2001	Stable integration and successful
4.	Tobacco	*rbcL* and *accD*	*cry2Aa2*	P*rrn*	T*psbA*	*aadA*	Kota et al., 1999	Stable integration and successful
5.	Arabidopsis	*trnV* and *rps12/7 loci*	*aadA*	P*rrn*	*TpsbA*		Sidkar et al., 1998	Not fertile and low regeneration
6.	Tobacco	*trnV* and *rps12/7 loci*	*hST Human somato-tropin*	P*rrn*	T*rps16*	*aadA*	Staub et al., 2000	High yield
7.	Tobacco and rice	*trnV* and *rps12/7 loci*	*aadA*-gfp	*Prrn*	psbA	*aadA/gfp*	Khan and Maliga 1999	Higher level of expression

Methods of Transformation

Plastid transformation requires a method for DNA delivery through the double membrane of the plastid, an efficient selection for the transplastome and integration of the heterologous DNA without interfering with the normal function of the plastid genome (Maliga, 1993). There are several ways by which foreign DNA is introduced into plastome *viz.Agrobacterium* vectors (DeBlock *et al.*, 1985), Polyethylene Glycol (PEG) treatment (Sporlein *et al.*, 1991), biolistic DNA delivery (Daniell *et al.*, 1990, 1991, Guang-Ning *et al.*, 1990), by UV laser microbeam (Weber *et al.*, 1991) and by a novel galinstan expansion femtosyringe method (Knoblauch *et al.*, 1999).

In the biolistic method, plastome is hit by DNA coated tungsten particle carrying 20 to 50 copies of the donor plasmid. However, the transforming DNA probably interacts with only one or almost a few of the many plastid genomes in the organelles. Based on reconstruction experiments, (Moll *et al.*, 1990) achieving the homoplasmic state of transgenomes takes at least 16 to 17 cell divisions during which time between $5x10^4$ and 10^5 cells are obtained from the founder cell. Therefore, selection pressure should be maintained throughout cellular proliferation to obtain a homoplasmic organelle and finally a homoplasmic cell (Maliga, 1993).

A novel development of mode of DNA delivery is the injection of DNA material (vector) into chloroplasts using syringes with extremely narrow tips (Knowblauch, *et al.*, 1999). The microinjection of DNA in plant cells had not been successful because of the cell wall rigidity and also because of the damage to the turgor of the plant cell due to the rigorous electrode penetration. Electrodes with smaller tips would cause less damage but

require extremely high pressure for fluid injection. The novel microinjection principle combines a reduced tip size with the required injection force. Micropippettes with a diameter of 0.1 micron are drawn from capillaries of borosilicate or quartz glass, backfilled with a mixture of silicone oil and galinstan (an alloy of gallium, indium and tin) and sealed with a miniature glass cap. The fluid in the tip is expelled by the pressure exerted by warming the mixture in the capillary. This galinstan expansion femtosyringe (GEF) allows injection of volumes in the femtolitre range into nuclei. The GEF tip is also suitable for insertion into chloroplasts (Knoblauch, 1999). The injection of constructs containing the *bla* gene into the filamentous cyanobacterium *Phormidium laminosum* resulted in a stable transformation conferring ampicillin resistance. The injection of plasmid DNA containing the *gfp* gene under the control of a chloroplast ribosomal RNA promoter (Hibberd *et al.*, 1998) into chloroplasts of marginal mesophyll cells of intact tobacco plants led to visible expression of GFP in the chloroplasts. The advantages of this procedure are 1, the cells survive the injection 2, the transformed cell can be spotted easily and 3, the cellular contents remain intact.

Selection strategies

The incorporation of reporter genes in the DNA construct that is integrated into the plastome is mandatory for tracing the transformed cells. In previous reports, selection of plastid transformants was made by spectinomycin resistance encoded by mutant 16S rRNAgenes (Svab *et al.*, 1990; Staub and maliga, 1992, 1993) and a chimeric *aadA* gene encoding aminoglycoside-3"-adenyltransferase (Svab and Maliga, 1993). Goldschmidt Clermont (1991) demonstrated the use of *aadA* gene as selectable marker in transformation of *Chlamydomonas*. Selection for *aadA* yielded a 100 fold increase in

transformation frequency than with the mutant 16S rRNA genes which yielded at least one plastid transformant per sample on an average. This relatively high frequency was believed to be due to an improved recovery of the newly formed transplastomes by the dominant *aadA* gene and makes plastid transformation a practical experimental tool in higher plants. The lower transformation frequency obtained with the mutant 16S rDNA genes was explained to have occurred by elimination of 99 out of 100 copies of the integrated DNA before the recessive selectable marker can be expressed (Svab and Maliga, 1993). An additional marker gene encoding resistance to kanamycin (*kan*) was also tried and it was not more encouraging (Carrer *et al.*, 1993). In an another attempt, the bacterial gene *aphA-6* which encodes an aminoglycoside phosphotransferase, was added to the limited assortment of selectable markers for chloroplast transformation in *Chalamydomonas* (Bateman and Purton, 2000). Insertion of this gene conferred kanamycin and amikacin resistance to the transformants.

Several new reporter gene strategies for the identification of plastome transformation have been developed over the past few years. Khan and Maliga (1999), developed a vector with GFP-*aadA* fusion construct which produced a bifunctional marker gene product (FLARE) that combines antibiotic resistance with optical tracking of Green Fluorescent Protein (GFP) in the transplastomic plants. The *aadA* gene was translationally fused to the GFP. The drugs used in the selection procedure suppress chlorophyll production and inhibit shoot formation on plant regeneration media. The transformed lines can thus be distinguished by their ability to form green shoots on bleached wild type leaf sections. This fusion strategy gives visual support to the identification of the transplastomic leaf vectors and helps isolate a section of leaf that can

be used for a second cycle of plant regeneration. The inclusion of resistance marker genes has the disadvantage that transformed cells must be traced by straight methods. Plant regeneration from a cell embedded in the cellular environment killed by lethal agents is a risky undertaking and not fully understood. In the case of optical marker like GFP, difficulties arise with the regeneration of a plant from a single GFP expressing cell. Given the additional problems of genetic contamination, the reporter gene strategy may not be the most ideal solution for selecting transformants. Therefore alternative selection scenarios that eliminate the use of marker genes are encouraged.

Advantages of chloroplast genome transformation

Overexpression of the introduced foreign DNA in chloroplasts of plant cells is possible because of the very high copy number (5 to 80 for *Chlamydomonas* and between 1900-50,000 in higher plants) plastome per cell (Bendich, 1987, Maliga 1993, and Daniel *et al.*, 1998). Moreover the transcriptional and translational machinery of the chloroplast is prokaryotic in nature and relatively AT rich (Shimada and Sugiura, 1991), genes of prokaryotic origin can be introduced and expressed at extraordinarily high levels in chloroplasts (Daniell *et al.*, 1998 and McBride *et al.*, 1995). Recently, Staub *et al.*, (2000) could successfully transform and express chloroplast genome with a human somatotropin gene, a secretory protein in a soluble biologically active, disulfide bonded form to an extent of more than 7 % of the total soluble protein. Apart from overexpression of the foreign gene, the above report proves that chloroplast transformation can be used for the production of pharmaceutical proteins in plants. Similarly, unmodified full length *cry1Ac* gene was introduced into chloroplast genome of

tobacco to get an expression of up to 5 % of the total soluble protein in tobacco leaves as protoxin (McBride *et al.*, 1995).

Overexpression of *B.t.* gene (*cry2Aa2*) was also reported by Kota *et al.*, (1999) in which the transformed leaves expressed Cry*2Aa2* protoxin at levels between 2 % and 3 % of total soluble protein. Recently, it has been demonstrated that even the whole *cry2Aa2* operon (4.0 kb in size) can be introduced into the plastome to get higher level of expression and crystal formation by De Cosa *et al.*, (2001). This level of protein expression was reported to be the highest ever of 45.3 % of the total soluble protein in a transgenic plant to date. The attraction here is the possibility of transforming plastome with polycistronic operon and expression (that is the stacking of multiple genes in a single transformation event).

Another advantage is insertion of foreign genes at specific site. The foreign genes are delivered on leaves by particle bombardment and the DNA gets integrated into the plastome by homologous recombination. Stable transformation requires that all 10,000 copies be uniformly converted. The location of foreign gene integration by this method is certain. Stable transformation requires the insertion at specific sites (intergenic or nonessential regions) to avoid gene silencing or disturbing the function of other important genes.

It is known that *B.t.* toxin genes are AT rich compared to plant genes and have been speculated that the low level of expression in nuclear genome transformed plants may be due to number of factors such as premature transcription termination, aberrant mRNA splicing, mRNA instability or efficient codon usage (Perlak *et al.*, 1991 and

Murray *et al.*, 1991). To avoid such undesirable features, synthetic genes were constructed and the expression improved dramatically to certain extent (Perlak, 1991 and Adang *et al.*, 1993). However, such codon modification is not necessary if the prokaryotic genes are used to transform chloroplast genome (McBride *et al.*, 1995).

The escape of foreign genes through pollen from transgenic plants has been reported at varying distances and in different directions (Llewellyn and Fitt, 1996; Umbeck *et al.*, 1991). They have observed the dispersal of pollen from a central test plot containing transgenic cotton plants to surrounding nontransgenic plants which is a serious environmental concern. One common concern is the pollen from transgenic plants containing gene for herbicide resistance which is a potential threat to transform their weedy relatives in the neighbouring areas thus creating 'super weeds' (King, 1996). The frequencies of marker genes reported in wild sunflowers averaged about 28-38 %. In wild strawberries growing within 50 m of a strawberry field, more than 50 % of the wild plants contained marker genes from cultivated strawberries. Similarly, transgenic oil seed rape, genetically engineered for herbicide resistance outcrossed with *Brassica campestris*, field mustard- a weedy relative) and conferred herbicide resistance even in the first back-cross generation under field conditions (Mikkelsen *et al.*, 1996). High rates of gene flow from crops to wild relatives of 60 important crop plants and wild relatives has been summarized by Daniell (1999).

Production of transgenic plants through chloroplast transformation method is the best alternative to remedy the problems associated with transgene dispersal into the wild plant population that are weedy relatives of the transformed crops. As there are no

plastids in pollen, the introduced foreign gene are not available as well and any gene introduced into chloroplast engineered plants is unlikely to be transferred *via* pollen to next generation. But there are plants with exceptions too for *e.g.* alfalfa and possibly rice and pea (Stewart and Prakash, 1998).

Universality of the vector

The chloroplast transformation vectors employed in the previous reports were designed to target the foreign gene at specific insertion sites *viz.* between *trnI* and *trnA* (De Cosa *et al.*,2001), between *trnV* and *rps 12/7* loci (Khan and Maliga 1999 and Staub *et al.*, 2000, McBride *et al.*, 1995, Sikder *et al.*, 1998), between *rbcL* and *accD* (Kota *et al.*, 1999 and Daniell *et al.*, 1998). The vector which inserts the foreign gene between the *rbcL* and *accD* of tobacco chloroplast genome is known as tobacco vector (Daniell 1998). On the other hand, the vector which introduces foreign gene between the *trnI* and *trnA* genes of chloroplast genome is called Universal transformation vector.

The *trnI* and *trnA* (chloroplast transfer RNA genes coding for adenine and isoleucine) genes are highly conserved among the higher plants and thus the vector containing these sequences as flanking sequences can transform several plant species by homologous recombination. These genes are situated in the Inverted Repeat (IR) region between 16S and 23S rDNA genes of the 16S rDNA operon. The intron of these genes which belong to subgroup IIA show little variation among the genes analyzed (rice, tobacco and liverwort) with a homology of more than 80 % (Shimada and Sugiura, 1991). When a multiple alignment was performed (CLUSTLAW), the *trnI* and *trnA* genes of pea, tobacco, spinacia, *Helianthus*, and *Arabidopsis* showed a high percentage of

similarity except the deletion regions (Sathik *et al.*, 2003). This study confirm the highly conserved nature of the *trnA* and *trnI* gene sequences among different higher plants and thus ensures the possibility of using the intergenic region to be used as the insertion site in universal transformation vector.

Materials.....

MATERIALS

Chemicals and Enzymes

All commonly used media chemicals used in this study were purchased from Hi-Media, India. Other chemicals and stains of analytical grade were from Hi-Media, Qualigens, Glaxo and Fischer of India. Most of the fine chemicals were from Sigma Chemical Co., St. Louis, Mo. Prestained protein standard markers were from New England Biolabs Inc. USA. Protein molecular weight markers were purchased from Sigma Co., St.louis., USA as well as from Bangalore Genei Pvt. Ltd., India. Chemicals and nitrocellulose membrane used for immunoblotting were purchased from Bio-Rad laboratories, Richmond, Calif. Hybond N+ nylon membrane was used for all purposes. The [α^{32}-P]dCTP was from Bhabha Atomic Research Centre, India. All photographic solutions were prepared from chemicals purchased locally. The Random primer labeling kit was purchased from Bangalore Genei., India. The 1kb+ DNA ladder used was purchased from Life technologies, USA. The λ DNA markers were obtained from Bangalore Genei Pvt. Ltd., India. The pGEM-T and pGEM-T easy vectors were obtained from Promega, USA.

Instruments

The Table gives the list of instruments used during this study.

Analytical and top loading balances	...	Sartorius, Model L220S, West Germany; Shimadzu, AEL-40SM, Japan.
Automatic refrigerated high speed centrifuge	...	Himac-SCR20BA centrifuge, Hitachi, Japan.
Freezer (-20°C)	...	Blue Star, Freezefast, India.
Freezer, Ultra Low (-70°C)	...	Revco, Scientific Inc. ULT 790

Gel documentation system	...	Alpha Innotech Corporation
Hybridization oven	...	Boekel inst. Ltd. UK
Incubators, Oven and Water bath	...	Techno Instruments, India; Universal Bio-chem, India; Julabo, SW-20C
Laminar Flow System	...	Cleanair, Atlantis, India.
Liquid Scintillation Counter	...	1217 LKB Rackbeta, Wallac, Sweden.
Microfuge	...	Microspin 24, Sorvall Instrument, Dupont
Phase contrast microscope	...	Nikon, Microflex, PFX, Japan.
Microwave oven	...	National, Japan.
Minicycler	...	MJ. Research, USA.
Orbit environ and waterbath shakers	...	Labline Instruments Inc. USA.
pH meter	...	Biochem, Model M606, India; Ellico, LI-120 (Digital) India.
Powerpacks	...	LKB 2301, Sweden, Bangalore Genei, India.
Protein gel separating systems	...	Hoefer-HS1 (SE250), USA., Biorad, USA.
Reciprocal shaker	...	Recipro-shake-Luckham, England.
Refrigerated circulating waterbath	...	Haake DSV, West Germany; Hoeffer hsi, RCB-300, USA.
Spectrophotometer UV-vis	...	Model 100-60, Hitachi, Japan; Model U2000, Hitachi, Japan.
Semi-Phor-Semi-Dry Transfer Unit	...	T370, Hoefer, Scientific Instruments
Table top centrifuge	...	Remi Laboratory Equipment, India.
UV transilluminator	...	Fotodyne Inc., USA.

Vacuum Oven	...	Spinco, Model, IRI 021, India.
Vortex mixer, Magnetic stirrer and Hot plate	...	Techno Instruments, India; Remi Laboratories, India; Vibrofix VF-1 Electronic, Kinematica Switzerland.
Biolistic PDS1000/He Hepta Systems	...	BioRad, USA.

Bacterial strains

E. coli strains:

Strain Name	Genotype
JM109	McrA, recA1, edA1, gyrA96, thi-1, hsrR17 (rk^-, mk^+), supE44, relA1 (*lac*-proAB), [F^1 tra36, proAB, $LacI^qz$ MIS]
XL1-Blue MRA	Δ(*mcrA)* 183 Δ(*mcrCB-hsdSMR-mrr) 173endA1 supE44 thi-1 gyrA96 relA1 lac*
DH5α	F^- ϕ80d *lacZ* ΔM15 Δ(*lacZYA – argF*) U169 *end*A1 *rec*A1 *hsd*R17 (r_k^- m_k^+) *deo*R *thi*-1 *pho*A *sup*E44 λ^- *gyr*A96 *rel*A1

Construct containing the *aadA* gene used in this study was obtained from Dr. Daniel Zeigler, Bacillus Genetic Stock Centre (BGSC), Ohio State University, USA and transformed into *E.coli* for our use. The clone pTB9 and pTB23 containing the tobacco *trnI*, *trnA* and promoter region of 16S rDNA (used as positive control in PCR) were obtained from Dr. M. Sugiura, Nagoya University, Japan.

Plants used in this study

The tobacco plant Wisconsin 38 used in this study for transformation was kindly provided by Dr. K. Veluthambi, Department of Plant Molecular Biology, School of Biotechnology, Madurai Kamaraj University, Madurai, India.

Oligonucleotides and sequencing

All primers and probes used in this work was synthesized at Microsynth Co., Switzerland. Sequencing was carried out by Microsynth, Co., Switzerland and by the DBT National facility for sequencing, Department of Biochemistry, IISc., Bangalore

PLASMID VECTORS USED IN THIS STUDY:

Plasmid Name	Marker	Features
pGEM-T Easy (Promega)	Amp	T-tailed vector
pBS-KS (Stratagene)	Amp	Cloning vector

ANTIBIOTICS

Spectinomycin and ampicillin were purchased from Sigma, USA. The stock concentrations and working concentrations of the antibiotics are given below. The stock solutions were stored at 4°C.

Antibiotics	Stock solution (mg/ml)	Working concentration µg/ml
Ampicillin	50 (in water)	50 – 100
Spectinomycin	100 (in water)	500

Restriction enzymes and Modifying enzymes

The restriction enzymes were obtained from GIBCO BRL, USA and Amersham Pharmacia Biotech, U.S.A. All the modifying enzymes like T4 DNA ligase, Lysozyme, Dnase, Rnases, *Taq* DNA Polymerase, etc. were purchased from Sigma Co. USA, Gibco Brl, USA, Amersham Pharmacia Biotech, USA and Promega, USA.

Media used

Luria-Bertani Broth (LB) (Maniatis et al., 1981)

Tryptone	- 10 g
Yeast extract	- 5 g
NaCl	- 10 g
pH adjusted to 7.2 with NaOH	
Double distilled water to	- 1000 ml

1.5% agar was added to Luria broth before autoclaving to make solid agar medium (LA) and 25 ml was poured into each Petri plate.

Nutrient Broth (NB) (Maniatis et al., 1981)

Peptone type I	- 5.0g
Sodium chloride	- 5.0g
Yeast Extract	- 3.0g
Beef Extract	- 3.0g
pH was adjusted to 7.2 with NaOH	
Water added to	- 1000ml

1.5% agar was added to the medium to get Nutrient agar medium (NA) before autoclaving.

SCG Minimal media for sporulation (Spizizen, 1958)

10X salts (100ml)

K_2HPO_4	- 15.2g
KH_2PO_4	. 4.8g
Sodium citrate	- 0.5g
Ammonium sulphate	- 2.0g

1M $MgSO_4$

$MgSO_4$	- 2.465 in 10 ml of water

Casamino acids

Casamino acids	-	0.1g in 87.9ml of water

Add 1.5g Agar and autoclave

Glucose stock

Glucose	-	25g in 100ml of water

All stocks are autoclaved individually.

Casamino acid stock	-	87.9 ml
10 X salts	-	10 ml
Glucose stock	-	2.0 ml
MgSO4 stock	-	0.1 ml

Poured 25 ml of this in to each petri dishes and allowed to solidify.

BUFFERS and SOLUTIONS

Only double distilled water was used in the preparation of all buffers and solutions.

2% SDS

SDS	-	2 g
Water	-	100 ml

5 M NaCl

NaCl ...	-	29.220
Water	-	100 ml

Phenol

Phenol was distilled and hydroxyquinoline was added to the distilled phenol to a final concentration of 0.1%. Distilled phenol was equilibrated once with 1.0 M Tris.Cl [pH 8.0] and repeatedly with 0.1 m Tris.Cl [pH 8.0] till the pH of phenol reached 7.8. To the

equilibrated phenol 0.1 volume of Tris.Cl [pH 8.0] containing 0.2% β-mercaptoethanol was added. The phenol solution was stored in a dark bottle at 4 ^{0}C.

Chloroform : Isoamyl Alcohol Solution

Chloroform	-	24ml
Isoamyl alcohol	-	1ml

RNase

10 mg/ml in TEN Buffer. Working concentration was10μg to 50μg/ml. Incubation was for 30min at 37°C.

DNase I

Dissolved10mg DNase I (Sigma Co Ltd., USA) in 1 ml of water and stored at 4°C.

Reagents for Plasmid preparation

Solution I (TEG)

Tris.Cl (pH 8.0)	-	25mM
Glucose	-	50 mM
0.5 M EDTA pH 8.0	-	10mM

Solution II

NaOH	-	0.2 M
SDS	-	1%

Solution III (3M Potassium acetate, pH 4.8)

Glacial acetic acid	11.5 ml
Water	28.5 ml
5 M Pot. Acetate (29.44 g in 60 ml)	60.0 ml

All solutions were sterilized by autoclaving.

5 M Sodium Acetate pH 5.2

Sodium acetate	- 408.1g
Water	- 1000ml

pH adjusted to 5.2 with glacial acetic acid. Sterilized by autoclaving.

Total DNA extraction from bacteria

0.5M EDTA pH 8.0

EDTA	- 18.6g in 100ml of water

pH adjusted to 8.0 with HCl.

TEN buffer (100ml)

Trizma (10mM), pH 7.9	- 0.121 g
EDTA (10mM)	- 2.0 ml of 0.5M EDTA
NaCl (100mM)	- 0.5844 g

Water added to 100 ml

NaCl/EDTA buffer (100ml)

NaCl (150mM)	- 0.8766 g
EDTA (100mM)	- 20 ml of 0.5M EDTA

Water added to 100 ml

Tris/NaCl/SDS (100mL)

Trizma (100mM) pH 7.9	- 1.21 g
NaCl (100mM)	- 0.5844 g
SDS (2%)	- 2 g

Water added to 100 ml

Total DNA extraction from Plants

The total DNA from the leaf of tobacco plants were prepared by using the Plant DNA kit by Quiagen.

Preparation of chloroplast DNA

1. Buffer A

Components	Final concentration
Mannitol 54.65 g	0.2 M
50 ml of 1 M Tris-HCl, pH 8.0	0.05 M Tris, pH 8.0
6 ml of 0.5 M EDTA, pH 8.0	0.003 M
71 µl of B-Mercaptoethanol	0.001 M
Make up to 1000 ml with water	

2. Buffer B

Sucrose 102.69 g	0.3 M
100 ml of 1 M Tris-HCl, pH 8.0	0.04 M
40 ml of 0.5 M EDTA, pH 8.0	0.02 M
Make up to 1000 ml with water	

3. Sucrose Gradient Buffer 300 ml

18 ml of 1 M Tris-HCl, pH 8.0	60 mM
1.8 ml of 0.5 M EDTA	3 mM
21 µl of 1 mM B-mercaptoethanol	1 mM
0.3 g of BSA	0.1 % BSA
Make up to 300 ml with water	

4. SDS 10 g in 100 ml 5 %
5. Tris 1 M, pH 8.0
6. EDTA 0.5 M, pH 8.0
7. Neutral Phenol
8. TE Buffer.

DNA Agarose gel electrophoresis

10X TBE buffer

Tris (89 mM)	-	107.78 g
EDTA (2.5 mM)	-	8.41 g
Boric acid (89 mM)	-	55.0 g
pH adjusted to 8.3		
Double distilled water to	-	1000 ml

50X TAE buffer

Tris base	-	242 g
Glacial acetic acid	-	57.1 ml
EDTA (0.5M, pH 8.0)	-	100 ml
Distilled water	-	1000 ml

DNA loading dye 5X (1 ml)

Glycerol	-	0.7 ml
EDTA pH 8.0	-	0.3 ml
Bromophenol blue	-	0.05%

Gel elution

The DNA to be eluted from the agarose gel were cut and stored separately. The DNA gel elution kit from Roche, Bangalore Genei were used at different occasions. The electoelution method by using dialysis bag was performed.

Electroelution

Dialysis tube cut into convenient sizes

Boiling buffer

NaHCO3 - 2%

EDTA - 1mM

Ethanol/sterile water 50:50 (V/V)

TAE – 1X and Sodium acetate – 3M

POLYMERASE CHAIN REACTION (PCR)

Reaction Mix (2X)

10X buffer	...	20 (1X)
25 mM $MgCl_2$	...	16 (2 mM)
10 mM dNTP	...	4 (0.2 mM)
20 µM forward primer	...	2 (0.2 µM)
20 µM reverse primer	...	2 (0.2 µM)
Taq DNA polymer	...	1 (5 units)
Water	...	55
		100
2X reaction mix	...	20 µl
Template DNA	...	20 µl

Reaction Cycle

I step	...	94°C for 1 min
II step	...	92°C for 1 min
III step	...	55°C for 1 min
IV step	...	72°C for 1.30 min
V step	...	Go to step II for 20 times
VI step	...	72°C for 10 min
VII step	...	4°C

Ligation

10X Ligase buffer from Amersham	...	1 µl
vector DNA	...	1 µl
insert DNA	...	5 µl
T4 DNA ligase (1U/µl)	...	1 µl
Water	...	2 µl

Transformation Protocols

1. One step transformation of *E. coli*

TSS (Transformation and Storage buffer)

Tryptone	- 1.0g
Yeast Extract	- 0.5g
Sodium chloride	- 1.0 g
PEG	- 10g
$MgCl_2.6H_2O$	- 1 g
DMSO	- 5%
$D.H_2O$	- 90ml

The solution was made up and the pH was adjusted. Filter sterilized DMSO was added only after autoclaving. Stored at 4°C.

Reagents for Vertical Slot Lysis

10X TES

Tris Base (300mM)	- 36.3g /1 litre
EDTA (5mM)	- 18.6g
NaCl (5mM)	- 29.2g
pH 8.0	

Protoplast solutions

Solution A	- 40% Sucrose
Solution B	- 4% SDS
Solution C	- RNase (2mg/ml) in TES and boil for 20min)

Protoplast Buffer

Solution A	- 5 ml
Solution C	- 1 ml
10X TES	- 1 ml
Water	- 7.5ml to get 10 ml.

Add lysozyme (20mg/ml) in case of *Bacillus* spp.

Cell Lysis Solution (in 1X TES)

Solution A	- 1.25ml
Solution B	- 5.0 ml
10X TBE	- 1 ml
Bromo Phenol Blue	- 4 mg
Water	- 2.75 ml to get 10 ml.

Stored in the refrigerator. The addition of RNase is optional. All enzymes were added only after autoclaving.

Random primer labeling

Reagent mix (dATP, dGTP, dTTP and random hexadeoxyribonucleotides) Klenow fragment α^{32}-PdCTP.

Probe preparation:
Dye mix

Blue dextran	- 6 mg
Orange G	- 1 mg
EDTA (pH 8.0) (0.5 M)	- 1 ml

Column buffer

1 M NaCl	- 10 ml
2 M Tris HCl (pH 7.0)	- 0.5 ml
0.5 M EDTA (pH 7.0)	- 0.48 ml
Water to	- 100 ml

Scintillation Fluid

0.5% PPO in Toluene

Southern hybridization solutions
Depurination Solution

Concentration HCl (0.25 M)	- 21.0 ml
Water	- 979.0 ml

Denaturation Solution

NaOH (0.5 M)	- 20 g
NaCl(1.5 M)	- 87.66 g
Water to	- 1000 ml

Neutralization buffer

1 M Tris HCl (pH 8.0)	- 121.10 g
NaCl(1.5 M)	- 87.66 g
Water to	- 1000 ml

20X SSC

NaCl (3 M)	- 175.3 g
Sodium citrate (0.3 M)	- 88.2 g
Adjusted to pH 7.0	
Water to	- 1000 ml

Solution A

1 M Na_2HPO_4 pH 7.2

Solution B

20% SDS

Prehybridization and Hybridization solution (50mL)

Solution A	- 25mL
Solution B	- 18mL
EDTA	- 1 mM

Post hybridization Wash solutions (50mL)

<u>First Wash</u>

Solution A	- 2 ml
Solution B	- 12.5ml
EDTA	- 1 mM
Solution A	- 2 ml

Solution B	- 2.5 ml
EDTA	- 1 mM

Photography solutions

Developer

Mettol	- 1.75g
Hydroquinone (quinol)	- 4.5g
Pot. Bromide	- 2.5 g
Sod. sulphite anhydrous	- 37.5g
Sod. Carbonate	- 20.0g

Water to 500ml.

Add each component and dissolve before adding the next one. The preparation should be done in a brown glass bottle. Store at room temperature in a brown bottle. The solution may be reused several times till it turns yellow.

Fixer

Sod. thiosulphate	- 100g
Ammonium chloride	- 25g
Sod. metabisulphite	- 10g

Water to 500ml.

The fixer may be stored in a bottle. This can be used several times till the suspension becomes cloudy.

PROTEIN ESTIMATION

Bradford reagent

Coumassie Brilliant Blue G-250	- 20mg
88% W/V H_3PO_4	- 20mL

Crystal Solubilization Buffer

$NaHCO_3$	- 50 mM

DTT - 10 mM

pH adjusted to 10.2

SDS-polyacrylamide gel electrophoresis

30% Acrylamide stock

Acrylamide - 29.2 g

Methyl bisacrylamide - 0.8 g

Double distilled water to 100 ml

The acrylamide solution was deionized with Amberlite (1 g/100 ml), filtered through Whatman No.1 paper and stored in brown bottle at 4°C.

10% Separating gel (10 ml)

Water - 4 ml

Acrylamide : Bis stock - 3.1 ml

1.5 M Tris HCl, pH 8.8 - 2.5 ml

Degas the solution for 5 min and proceed.

20% SDS - 100 µl

10% Ammonium per sulfate - 100 µl

TEMED - 10 µl

4% Stacking gel (10ml)

Water - 6.8 ml

30% acrylamide stock - 1.7 ml

0.5 M Tris HCl, pH 6.8 - 1.25 ml

20% SDS - 100 µl

10% Ammonium persulfate - 100 µl

TEMED - 10 µl

1X Tank buffer

Tris (0.025 M, pH 8.3) - 12 g

Glycine (0.192 M) - 57.6 g

SDS (0.1%)	- 4 g
Distilled water to	- 4 litre

2X Sample dye

0.125 M Tris HCl, pH 6.8	- 2.5 ml
1% SDS	- 4 ml
Glycerol	- 2 ml
2 mercaptoethanol	- 1 ml
Bromophenol Blue	- 0.02 mg
Water	- 0.5 ml

Staining solution

Coomassie brilliant blue	- 100 mg
Methanol (50%)	- 50 ml
Acetic acid (10%)	- 10 ml
Water to	- 100 ml

Coomassie brilliant blue was first dissolved in methanol, before adding acetic acid and water.

Destaining solution I

Methanol (50%)	- 50 ml
Acetic acid (10%)	- 10 ml
Water to	- 100 ml

Destaining solution II

Methanol (5%)	- 5 ml
Acetic acid (7%)	- 7 ml
Water to	- 100 ml

IMMUNOBLOTTING

Towbin transfer buffer (200ml)

Glycine	- 2.882g
Tris pH 8.3	- 0.605g
SDS	- 0.075g.
Methanol	- 40ml (20%)
DDH_2O	- 160ml

Tris buffered saline (TBS) (200ml)

Tris (pH 7.5)	- 0.4844g (20mM)
NaCl	- 5.8445g (500 mM)

Wash solution (TTBS)

0.05% ofTween 20 in TBS (10µl of tween 20 + 20mL of TBS)..

2% Blocking solution

Milk powder	- 2 g
TBS	- 100 ml

Antibody buffer

Milk powder	- 0.1 g
TBS	- 100 ml

Alkaline phosphate (AP) colour development buffer.

Tris (pH 9.5)	- 0.1 M

Solution A

Nitro Blue tetrazolium	- 30mg in 70% Dimethyl Formamide.

Solution B

BCIP	- 15mg in 1 ml of DMF

Stored both stocks at -20°C. 1ml each of A and B to 100 ml of AP buffer were added and used immediately. .

K- Reagents for purification of Antibody by CNBr method:

1. 1mM HCl

2. **Coupling buffer:**

$NaHCO_3$	-	0.1M
NaCl	-	0.5M

The pH was adjusted to 8.3 and autoclaved.

3. **0.1 M Tris – HCl :**

0.1M .Tris – HCl, adjust pH to 8.0 with HCl and autoclaved.

4. **0.1M Acetate buffer:**

Sodium acetate	-	0.1M
NaCl	-	0.5 M

Adjusted the pH to 4.0

5. **Tris buffer:**

Tris	-	0.1 M
NaCl	-	0.5 M

Adjusted the pH to 8.0 and autoclaved.

Other Chemical stocks

X-Gal

40mg /ml of Dimethyl Formamide

Stored at -20°C away from light. Per plate 25 µl was added.

1M IPTG

240mg/ml of water

Stored at 4°C. Per plate 50µl was added.

Ethidium bromide

10mg in 1 ml of water.

Materials for tissue culture:

Reagents for Tissue culture:

1. Sulphates (10X) :1000 ml

$MgSO_4.7H_2O$	-	3700.0mg
$MnSO_4.H_2O$	-	338.0mg
$ZnSO_4.H_2O$	-	86.0mg
$CuSO_4.H_2O$	-	0.250mg

Storing temperature 4°C.

2. Non sulphates (10X) :1000ml

NH_4NO_3	-	16500mg
KNO_3	-	19000 mg
KH_2PO_4	-	1700 mg
$CaCl_2.2H_2O$	-	4400 mg
KI	-	8.20 mg
H_3BO_3	-	62.0 mg
$CoCl_2.6H_2O$	-	0.250 mg
Na_2MoO_4	-	0.250 mg

Storing temperature 4°C

3. Iron EDTA: (200Xstock)

$FeSO_4.7H_20$	-	55.7 mg/lit
Na_2EDTA	-	745 mg/lit

4. Vitamins:

Vitamin	mg/lit	Stock
1.Nicotinicacid	0.5	500
2.Pyridoxine HCl	0.5	500
3.Thiamine HCl	0.1	100
4.Glycine	2.0	2000

5. Myoinositol - 100 mg/lit as powder or solid

6. Sucrose - 30gm/lit

7. Agar - 0.8%

Adjust the pH to 5.8 with 0.1 N NaOH .

Growth regulators:

Auxins :

Auxins were dissolved in 0.5 ml of DMSO completely and distilled water was gradually added into it while stirring and made upto 100 ml.

NAA (Naphthalene Acetic Acid):

1mM stock - 18.62 mgs in 100 ml.

Cytokinins:

Dissolve in 0.5 ml of 5N Hcl and make upto 100 ml by adding double distilled water slowly.

BAP (Benzyl Amino Purine):

1mM - dissolve 22.5 mg in 100 ml.

Murashige skoog's Medium (1000 ml)

Sulphate (10x)	-	100ml
Non sulphate (10x)	-	100ml

Iron-EDTA (200x)	-	5ml
Sucrose	-	30 gm
Myoinosital	-	10 gm
pH	-	5.7
Agar	-	0.8 %

The working concentrations of Growth regulators for tobacco regeneration:

	NAA	BAP
1. Callus induction	10μM	1μM
2. Shoot induction	0.5μM	4μM
3. Root induction	10μM	0.1μM

RMOP medium for shoot regeneration

MS salts as in MS medium

Thiamine - 1 mg per liter

Inositol - 100 mg per liter

NAA –0.1 mg per liter

BAP – 1 mg per liter

pH – 5.8

Agar 0.6 %

Sugar 30 g per liter

Materials required for Particle bombardment:

- Device PDS 1000/He
- ExplantTobacco leaf discs of Wisconsin 38.
- Microcarrier Gold particles(Au 1μ diameter)
- Rupturedisc 1100psi
- Plasmid pMSVS7A vector DNA 1μg/μl
- Vaccum28 inches

Other materials:

- $CaCl_2$
- Spermidine.
- Alcohol.
- Double distilled water.
- Helium gas cylinder
- Vortex machine.

Methods.....

METHODS

Maintenance of *E.coli* strains:

E. coli strains were stored as single colonies on LB agar plates containing the appropriate antibiotics in a refrigerator for routine purpose. For long term storage cultures were preserved in 40% glycerol at –70 °C or as stabs at 4°C.

Maintenance of *Bacillus* strains:

Bacillus strain HD73 was maintained as sporulated cultures on nutrient agar plates with or without antibiotic in a refrigerator for routine use. For long-term storage, cultures were preserved in 40% glycerol at –70°C.

Sterilization:

All media buffers and reagents were sterilized at 15 lbs/inch^2 for 20 min unless otherwise stated. Glasswares like pipettes and Petri dishes were sterilized in a hot air oven at 120°C for 6 hrs and also were autoclaved alternatively. All antibiotics were filter sterilized.

Growth conditions for *E.coli* and *Bacillus* strains:

E.coli cultures were grown at 37°C and *Bacillus* cultures were grown at 30°C in an orbital shaker.

DNA PROTOCOLS

Alkaline lysis procedure

Plasmid extraction from *E.coli* was done according to the alkaline lysis procedure of Birnboim and Doly (1979).

1. An overnight 2 ml culture was inoculated into 100ml of Luria Bertani broth containing the respective antibiotics and kept under constant shaking for 12-14 hr at 37°C.
2. Cells were pelleted down by centrifugation at 8000 rpm for 10 min. They were resuspended in 100 µl of Solution I by vortexing. RNase A was added to a final concentration of 50µg/ml (if the cells were *Bacillus*).
3. Cells were lysed by addition of 200µl of solution II and the tubes were gently inverted to mix the solution. The lysate was kept on ice for 10 min.
4. Finally, 150µl of solution III was added to the lysate and the contents mixed gently. It was chilled on ice for 10 min and centrifuged at 10,000 rpm for 10 min at 4°C.
5. The supernatant was washed once with equal volumes of Phenol:chloroform: isoamylacohol (25:24:1) mixture and washed once with chloroform:isoamylalcohol (24:1). Centrifuging was done at maximum speed on a table top centrifuge for 10 min at 20°C.
6. The plasmid DNA from the supernatant was precipitated by addition of 0.6 volumes of isopropanol.
7. DNA was pelleted by centrifugation at 12,000 rpm for 10 min. Pellet was air dried and the DNA was dissolved in 20µl of TE.

Note: Alternatively, whenever high purity of DNA was required as in protoplast transformation, the Quiagen Plasmid kits were used to get RNA free high purity DNA. The procedure was essentially as described in the accompanying manual.

Chloroplast DNA Isolation from Cotton

1. Cotton leaves (about 100 g) were collected from cotton plants kept in dark for a day (destarched).

2. Washed with distilled water for 3 times and blotted dry on blotting paper.
3. The leaves were cut in to small pieces using a sterile blade and ground with liquid nitrogen with the aid of a mortar and pestle.
4. The homogenized powder was suspended in 500 ml of Buffer A with constant stirring. Later, the suspension was homogenized again using a Kinematica homogenizer at high speed for 3 – 4 min with intervals.
5. The suspension was filtered through 3 layers of cheese cloth and centrifuged at 1000 g for 3 minute to remove the debris.
6. The supernatant was carefully transferred to another centrifuge bottle and centrifuged at 5500 x g for 10 min (6000 rpm in rotor JA14 of Beckman centrifuge).
7. The supernatant was discarded and the pellet was suspended in a minimal volume (5 ml) of Buffer B.
8. The suspended pellet was laid over the top of a sucrose step gradient (25-34-51 %) in a corex tube and centrifuged in a Hitachi RPRS 10-457 swing out rotor with a speed of 3000 x g for 30 min.
9. The green band of intact chloroplast at the interphase of 34 and 51 % of sucrose layers was collected using a pasteur pipette without disturbing the broken chloroplasts settled at the interphase of 34 and 25 %.
10. The thick purified intact chloroplast suspension was diluted with 3 volumes of Buffer B and mixed gently.
11. The suspension was centrifuged at 10 000 x g for 10 min and the pellet was collected after discarding the supernatant.

12. The pellet was suspended in a minimal volume (2 ml) of Buffer B and the chloroplast was lysed by the addition of sodium sarcosinate to a final concentration of 5 % and Pronase E to a final concentration of 1mg ml^{-1} at 37° C for an hour.

13. The lysate was added with equal volume of neutral phenol was and vortexed thoroughly.

14. The mixture was spun at 10 000x g for 5 min and the aqueous phase was extracted with equal volume of phenol:chloroform 1:1 mixture to spin again.
The supernatant was extracted with chloroform:IAA (24:1 ratio) and spun to collect the upper phase.

15. The aqueous phase collected was added with $1/10^{th}$ volume of 3 M sodium acetate and 3 volumes of 100% ethanol (double distilled). The mixture was incubated at –20° C for overnight or at –70° C for 30 min.

16. The mixture was centrifuged at 10 000 x g for 15 min after incubation and the pellet was washed with 2 volumes of 80 % ethanol.

17. Centrifuged again and the supernatant was discarded to collect the pellet.

18. The pellet was air dried or vacuum dried and resuspended in minimal volume of TE buffer. The concentration of DNA was spectrophotometrically estimated.

Preparation of template DNA for PCR reactions

A 2 ml culture of overnight grown *E.coli* (in case of *Bacilus* spp. the cells should be grown for 6-7 hours or till an OD of 0.7) was pelleted down quickly by spinning down at maximum speed for 30 sec. The pellet was suspended in 50 µl of sterile ddH_2O and boiled for 10 min. The suspension was spun down and the supernatant was used as template.

PCR reaction

A loop of *E. coli* cells from a single colony was transferred to 100 µl of water, and the mixture was boiled for 10 min to lyse the cells. The resulting cell lysate was briefly spun and 15 µl was used in the PCR reactions. The chloroplast DNA was used in the range of 100 ng per microcentrifuge tube containing 0.2 µM of forward and reverse primers, 0.2 mM each of the four deoxynucleotides and 2.5 U of *Taq* DNA polymerase from GIBCO BRL in a total volume of 50 µl. PCR was performed for 30 cycles with denaturation of template DNA at 94°C for 1 min, annealing templates and oligonucleotide primers at 55°C for 2 min, and extension of PCR products at 72°C for 10 minutes. In some of the reaction conditions the annealing temperature was reduced to 45°C. The PCR products were separated and analysed on a 0.7% to 2% agarose gel.

Agarose gel electrophoresis of DNA:

Agarose gels were prepared by adding the required amount of agarose to 1X TAE and melting it to homogeneity. It was allowed to cool for some time and then poured into casting trays of required sizes with combs of fitting size to form slots. After the gel has set, the comb was gently removed and the gel was put in an electrophoretic tank containing 1x TAE. DNA samples were mixed with 5X DNA loading dye and was loaded into the wells with suitable marker. Electrophoretic separation was carried out at 100 volts till the dye front reached the end of the gel. DNA was visualized under a UV transilluminator after staining the gel in a solution of Ethidium Bromide (10µg/ml). For restriction fragments and checking eluted DNA fragments, a 1% agarose gel is usually used. For genomic and total DNA preparations 0.8% agarose gels were used.

The DNA gel was stained with 10μg/ml Ethidium bromide solution for 5 min and destained for 10 min before visualizing it under UV (254nm). The gels were photographed using an Alpha Imager 2000 gel documentation system.

Restriction digestions

The required amount of DNA (normally 1μg) was digested with respective endonuclease using ddH_2O. Buffer concentration, duration and temperature of incubation were according to manufacturers instructions.

Elution of DNA from agarose gels.

The DNA bands were cut out from the lane after viewing the gel over long wavelength UV radiation quickly so as to avoid nicks. The gel slice was macerated well in an Eppendorf tube using a 1 ml pipette tip and then frozen at -70°C for half an hour. The tube was spun down at maximum speed for 10 min and the supernatant was collected. The supernatant was diluted to 100μl and precipitated using 2.5 vol of ethanol and $1/10^{th}$ volume of 5M Sod. acetate. The DNA was resuspended in appropriate quantity of TE (10μl) and used. This protocol was used mainly for preparation of probes and not for ligation. Elution of DNA band from agarose gel had also been performed by using Agarose gel DNA extraction kit from Roche Diagnostics, GmBH, Germany according to the manufacturer's instructions.

Electroelution

Preparation of dialysis tube

The dialysis tube was cut into pieces of convenient length. Boiled for 20 minute in a large volume of 2% NaHCO3 and 1mM EDTA buffer. The tube was rinsed with boiled distilled water thoroughly. Later the tubes were boiled in 1mM EDTA for 10 min.

Allowed to cool and stored in 50% ethanol in sterile distilled water at 5° C . Before use, the tubes were washed with DD water. The tubes were always handled with gloves only.

Dialysis

The eluted gel was placed inside the dialysis tube which is sealed at one end. The tube was filled with autoclaved 1X TAE and the gel was allowed to sink to the bottom of the bag. The excess TAE was removed and only very little quantity of TAE was retained in the bag to keep the gel slice in constant contact with the buffer. Care was taken to avoid air bubbles. The other end was also sealed and kept in the gel electrophoresis tank and run at 5V per cm for one hour. At the end the polarity was reversed to run for 30 sec to facilitate the release of DNA sticking on to the wall of the tube. The bag was gently massaged to remove the DNA from the wall. Opened the bag and carefully transferred all the buffer surrounding the slice to a fresh tube. The bag was washed with a small quantity of 1X TAE and added into the other tube. The eluted DNA was precipitated using 0.1 volume of 3 M sodium acetate and 2.5 volume of ethanol by incubating at –20° C overnight or 30 min at –70° C.

Ligation

Ligation of digested DNA was carried out at 16°C for 16 hr or at 22°C for 3 hours. Prior to ligation, the restricted digested fragments were heated to 70°C to inactivate the resident restriction endonucleases and then the appropriate amounts were directly used for ligation. T_4 DNA Ligase was used along with the buffer supplied by the manufacturer. Usually a vector to insert ratio of 1:3 was used for ligation of cohesive ends and 1:10 for blunt ended fragments. After ligation, the appropriate amounts of the ligation mix was used

for cloning into the desired vector. The ratio of insert and vector was determined using following formula.

$$\text{Amount of the insert} = \frac{\text{ng of vector X kb size of insert X molar ratio of insert : vector}}{\text{Kb size of the vector}}$$

Cloning of vectors

Generally, due to the inherent problems in cloning using eluted DNA fragments, shot gun cloning was practiced for all purposes. The restricted fragments from the donor plasmid was quantified and mixed in appropriate ratio with the recipient vector and then subjected to ligation so that the desired insert fragment to vector ratio was 3:1. The clones were then transformed into the *E.coli* and the transformants checked for plasmid mobility shift on a 0.8% horizontal slot lysis gel as described below. The smaller the size of the insert, the faster will the bottom most supercoiled band move. According to the mobility of the bottom plasmid band, the clone having the right size insert is selected.

Transformation protocols for *E.coli*

The one step transformation protocol of Chung *et al* (1989) was used for all purposes.

1. The *E.coli* strain was cultured in 2 ml LB overnight at 37°C with shaking.
2. It was then subcultured into LB till the an OD of 0.3-0.5 is reached (3-4 hours).
3. The cells were harvested at 6000rpm for 10 min at 4°C.
4. The cells were resuspended in $1/100^{th}$ volume of TSS and quickly frozen as aliquots of 50µl at -80°C.

For transformation one of the aliquots were taken and the appropriate quantity of the ligated mix or DNA was added (normally 10-20ng) and incubated on ice for 30min.

Then 450 µl of LB or TSS or SOC was added and incubated at 37°C for 60 minutes at 225 rpm. It was then plated on suitable selection plates.

Slot-lysis gel Electrophoresis

The presence of plasmids in the transformants was checked by modifying the method of Gonzalez *et al.* (1981).

Bacillus transformants (Vertical slot lysis):

1. Cells were streaked on SCG minimal agar and incubated at 30°C for 6-7hr.
2. A vertical agarose gel (0.6%) was cast with a 2% agarose plug at the bottom of the gel (to prevent sliding of the gel), in TBE.
3. A loopful of young cells from the SCG plates were gently scooped out and resuspended in protoplasting buffer by vortexing at least for 1 min.
4. The suspension was incubated at 37°C for nearly 30 min to generate protoplasts. Wells were flushed clean and 12 µl of cell lysis buffer was loaded.
5. The protoplast suspension was gently loaded under the lysis buffer and electrophoresis was started immediately. Initially, electrophoresis was carried out at 3 mA, then at 7 mA for 30 min and finally at 28 mA till the end of the run. The gel was stained in ethidium bromide solution and DNA was visualized under UV.

E.coli transformants (Horizontal slot lysis):

The procedure is essentially the same except for the following changes

1. The transformant colonies from the selection plates were patched using sterile toothpicks on selection plates and incubated overnight at 37°C.
2. The patches were scooped out and resuspended in Protoplasting buffer (without lysozyme) till an OD of 1.0 or so.

3. The suspension was immediately loaded under the lysis buffer in the agarose wells. A horizontal agarose gel system was used for this purpose. The gel was casted as a 0.8% gel with 0.1% SDS incorporated. The gel was then run in 1X TAE supplemented with 0.05% SDS. The gel was run and viewed under the same conditions described as above.

SOUTHERN HYBRIDIZATION

Random primer labelling:

The procedure was as outlined in the manufacturer's manual (Random Primer labeling kit by Bangalore Genei Pvt. Ltd., India).

1. About 50 ng of DNA was made upto 10 µl in a milli Q water and boiled for 5 min to denature.
2. Then it was immediately chilled on ice for 5 to10 min and centrifuged at 8000 rpm for 30 sec.
3. Added 2.5 µl of 10X labeling buffer.
4. Added 1µ l of 100 ng/µl of random primer (hexamer).
5. Then 2.5µl of 20mM dNTP (without dCTP) solution was added.
6. To this 3µl of $\alpha[P^{32}]$dCTP (Sp. activity >3000Ci/mMole or 10µCi/µl) was added.
7. Then 3µl of nuclease free autoclaved water was added.
8. To this 1 µl of 3u/µl of klenow (Large fragment of DNA polymerase I) was added and mixed gently. The mix was incubated at room temperature for atleast 2 hours.

Purification of probe through Sephadex G-50 column :

1. 1 gm of Sephadex G-50 was weighed into 40 ml of TE of ph 8.0 and autoclaved. It was stored in fridge.
2. Blue dextran dye was prepared by dissolving 10 mg in 0.5M EDTA.
3. A 10 ml disposable pipette was taken and cut at 2 ml level from the top (around 15 cm). Then it was plugged with siliconised glass wool.

4. The G- 50 matrix was poured upto 4 ml level (or 2 inches below the top). It was allowed to settle and slowly TE was added. The dye and the probe are added in the 1:1 ratio so totally 100 μ l was added on the column when the TE level was just above the matrix. TE was added only after the probe moved to few mm into the matrix and the washing was continued till the blue region reaches near the nozzle of the column. Then the blue fraction was collected in a Eppendorf tube upto 0.5 ml and stored in the freezer till use.

Such purification helps to remove all the small nucleotides and other unincorporated nucleotides to avoid background signals in the blots.

Blotting of Membrane

The method is based on the procedure developed by Southern (1975). The gel was properly documented before proceeding with blotting. Measure the distances on the gel and also its size. The gel was processed for transfer as follows:

1. DNA was depurinated by soaking in a solution of 0.25 N HCl for 15 min. Then the gel was rinsed with distilled water twice briefly.
2. Denaturation of the DNA was carried out by treating the gel in denaturation solution for 15 min with gentle shaking. Then it was rinsed with distilled water.
3. It was neutralized by soaking in neutralization buffer for 15 min.

   Care was taken to see that gel is completely immersed in all solutions while treatment.

Transfer procedure

For all purposes, the capillary transfer procedure was adopted for DNA transfer (Sambrook et al., 2001)

1. The gel after neutralization was briefly washed in 20X SSC and kept ready.

2. A trough was filled to a height of 5 cm with 20X SSC. A suitable platform with dimensions bigger than the gel was placed in the trough.
3. The surface of the platform was covered with three layers of Whatman 1 filter paper presoaked in 20X SSC, so that the ends of the papers is immersed in the 20X SSC.
4. Then 3 square sheets of Whatman 1 filter paper was placed after being cut to the same dimensions of the gel and presoaked in 20X SSC on top of the platform. Any airbubbles was removed by rolling the surface with a tube.
5. The gel was placed carefully on top of this and then place a Hybond N+ nylon membrane presoaked in 20X SSC on top of the gel. The nylon membrane should be cut slightly smaller than the gel dimensions. Any airbubbles were removed by gently rolling a falcon tube on the surface.
6. Two sheets of the presoaked filter paper was placed on top of this. Three more sheets of clean dry filter paper was then stacked on it over which crude filter papers cut to the gel dimensions were stacked to a height of 15 cm. Over this, a suitable weight of around 200-500 grams was placed. The weight should not crush the gel but should be able to compress the papers tightly.
7. The transfer was allowed to proceed overnight for a period of 12-16 hours.
8. After the transfer the assembly was disassembled and the nylon membrane was briefly washed in 5X SSC and air dried.
9. The membrane was fixed using a UV cross linker at 12000 J/cm^2. The membrane was stored between crude filter paper in the fridge till use.

Preparation of the Probe

After labeling and purification, the probe was briefly boiled for 5 min and then cooled immediately in ice for 10min. The probe was then added to the required quantity of

hybridization buffer before proceeding with the hybridization. The probe preparation may be stored in -20°C and used again after thawing. About 100ng of labeled probe was mixed into 20ml of hybridization buffer for hybridization.

Pre-hybridization and hybridization

The protocol used for this was as outlined in the Biorad's zetaprobe membrane manual.

1. The blotted membrane was placed in a hybridization tube containing 50ml of prehybridization solution. Prehybridisation was carried out at 65°C for 10 min in an hybridization oven.
2. The prehybridization solution was poured out and 20ml of hybridisation solution (prehybridisation buffer containing denatured probe DNA labelled with $\alpha^{32}P$) was poured into the tube and then again incubated with slow rotation for 12-24 hr at 65°C.
3. After hybridisation, the membrane was washed twice at 65°C for 30 min each with first-wash solution followed by 2 washes with the second-wash solution at 65°C for 30 min each. The membrane was briefly washed with 5X SSC and airdried and subjected to autoradiography. If stripping of the membrane was required, then the membrane was not allowed to get dry. It was immediately wrapped in cling film and then used for exposure.

Stripping of blot

To strip the probe from the blot, the following protocol was used.

1. Prior to re-probing, membranes may be stored, wrapped in Saran, in a refrigerator (2-8°C).
2. When ready to commence re-probing, rinse membrane in 5x SSC for 1-2 minutes.

3. Add the membrane to a boiling solution of 0.1% (w/v) SDS using approximately 5 ml of SDS solution per cm2 membrane. Place on a bench-top shaker for 10 minutes. Repeat the operation twice more, using freshly boiling SDS each time.
4. Rinse the membrane in 5X SSC
5. Prehybridize, probe and detect as in standard protocol.

Exposure to X-ray plates

The membrane was fixed on top of a aluminium foil covered sheet cut to the same dimensions as the X-ray cassette. It was again covered with plastic wrap and placed inside the X-ray cassette. An X-ray sheet was placed over it after marking the orientation. A amplifying screen was placed over this assembly and the cassette was closed tightly and placed in -70°C for 1-2 days.

After this, the X-ray sheet was removed and developed in the developer solution under safe red light. As soon as the spots develop, the X-ray sheet was cleaned with water and quickly placed in the fixer for a few minutes. The sheet was extensively washed in water and air dried.

Black and White Photography

Keep three photographic trays ready. The first tray must contain the developer, the next some clean tap water and the third one the fixer.

1. Developing and fixing

The Developer was made as detailed in the Materials section. The developer has to be kept in the dark always. The Photographic plate to be developed should be developed only under safe light conditions or under red light.

The film was quickly immersed in the developer and the tray was moved to and fro till the image starts developing which can be visualized under the safe light. After the required intensity and contrast of the image was achieved, the film was quickly washed in water thoroughly and transferred into the fixer for a few minutes. The developed and fixed film was then extensively washed and hung out to dry.

PROTEIN PROTOCOLS

Preparation of Crystal Protein

Cells were grown on SCG minimal plates until sporulation. The spore crystal mixture was scooped out of the plate and washed twice with 0.5 M NaCl followed by two washes with distilled water. It was resuspended in distilled water containing 1 mM PMSF and stored at -20°C.

Solubilization of protein

Fifty ml of three days old *B.t.* culture was spun at 8 K for 5 min and the pellet was washed with 0.5 M NaCl thrice. The pellet was later washed with distilled water twice and resuspended in 5 ml of solubilization buffer. The suspension was kept at 37°C overnight. Later, it was spun at 8 K for 5 min. The supernatant was saved and the protein concentration was estimated by Bradford method.

Preparation of protein samples for SDS-PAGE

In case of total protein from cells, the 2 ml culture was pelleted down by spinning and then suspended in protein sample dye (~50µl) containing mercaptoethanol. The suspension was boiled for 5 min and spun again. The supernatant was then loaded on the gel. The protein quantification was done by Bradford assay

Sonication:

E.Coli cells were grown over night in L broth. The cells were pelleted by centrifugation and concentrated to 100 folds by suspending in sonication buffer. The cell suspension was immersed on ice and was sonicated with 30s bursts at maximum power. (Brausonic 1510, B.Braun). The lysates were stored at -20^0 C. (Adang et al, 1985).

Bradford assay

The procedure is adopted from Bradford, (1976).

1. Standards were prepared from a 1mg/ml of BSA stock as 2, 4,6,8,8,10,15 and 20 μg samples in 1ml Eppendorf tubes. The volume was made upto 100μl with water. Then 1ml of Bradford reagent was added to each tube and shaken well.
2. The samples were prepared by taking 5μl of the protein preparation and making it up to 100 μl with water after which 1 ml of Bradford reagent was added and shaken well. Blanks were also prepared with 100μl of water.
3. The samples were incubated at room temperature for 10-30 minutes.
4. The absorbance of each sample at 595 nm was measured using a UV-visible spectrophotometer. The instrument has to warm up for at least 15 minutes prior to use.
5. The absorbance values of each BSA standard was plotted as a function of its theoretical concentration. The plot was linear.
6. The concentration of the sample proteins was interpreted by interpolating the sample absorbance value on the graph.

SDS-PAGE

Electrophoretic separation of protein was done according to Laemmli (1970) using a Hoefer Mighty Small vertical slab unit SE250.

- The resolving gel consisted of 10% acrylamide and was poured in between the glass plates after assembling the apparatus. The top was layered with water saturated butanol and allowed to polymerize at room temperature for half an hour. Then the 0.4% stacking gel was poured after removing the butanol and rinsing the surface. The appropriate combs were fixed and the gel allowed to polymerize. 1X SDS-PAGE buffer was prepared and poured into the apparatus till the top of the well.
- The protein sample was prepared by adding equal volume of 2X treatment buffer and boiling in a waterbath for 5 min.
- Samples were loaded into wells and electrophoresis was carried out initially at 80V until the due front reached the separating gel. Subsequently, electrphoresis was continued at 100V till completion.
- The gel was stained in coomassie blue R-250 stain and destained first in solution I for 2-3 hr with gentle shaking and then with destaining solution II for another 4 to 5 hr.
- The bands were visualized as blue bands and documented.

Immunoblotting

Protein transfer onto nitrocellulose membrane was done using a TE-70 Semiphor semi-dry transfer unit (Hoefer Scientific Instruments, San Francisco) according to the manufacturer's instructions.

1. The stacking gel was removed and the size of the resolving gel was measured.
2. The nitrocellulose membrane and blotter sheets were cut to the same size as the gel. They were pre-wet in transfer buffer. A mylar mask with an opening slightly bigger than the gel was centered on the electrode.

3. Three sheets of presoaked blotter paper were placed first, then the nitrocellulose membrane, then the gel and finally few more papers of buffer saturated blotter paper. At each step care was taken to remove air bubbles.
4. The transfer was carried out at 100 mA for 60 min.
5. The membrane was immersed in the blocking solution and incubated at room temperature for 3 hours to overnight with gentle shaking. It was then washed once with TTBS for 10 min.
6. After decanting the TTBS, the primary antibody in antibody buffer was added. It was incubated for 2 hr at room temperature. Unbound antibody was removed by washing twice for 5 min each with TTBS.
7. The secondary antibody in antibody buffer was added and incubated for 1 hr. The conjugate solution was decanted and the membrane was washed twice for 5 min each with TTBS at room temperature.
8. It was given a final wash with TBS and then ddH_2O and processed for colour development. Equal amounts (100µl) of colour reagent A (containing NBT) and colour reagent B (containing BCIP) were added to the colour development buffer and the membrane was immersed in this buffer and kept in the dark for 10 to 15 minutes till the colour developed. The membrane was then washed extensively in distilled water and stored in polythene bags after air-drying.

CNBr-Sepharose purification of antiserum

1. 0.5 gms of CNBr-Sepharose was weighed and suspended in 1mM HCl. Swirl to mix occasionally and leave for 30 min. The beads were pelleted by centrifuging at 1000 rpm for 10 min. The pellet was washed for 3 times with 1mM HCl , each time with 10 ml of HCl (0.5 gms of beads swell up to 1.6 ml volume).

2. The ligand was added to the coupling buffer and subsequently mixed with the gel. The mixture was rotated gently for one hour at room temperature or at 10^0C overnight.
3. The excess ligand was washed away with at least 5 gel volumes of coupling buffer. The remaining active groups were blocked by transferring the gel to 0.1 M Tris HCl, pH 8.0 and allowed to stand for 2 hours.
4. The gel was washed for at least 3 cycles of alternating pH (at least 5 gel volumes of each buffer). Each cycle contained a wash with 0.1M acetate buffer, pH 4.0 and 0.5M NaCl followed by a wash with 0.1M Tris HCl, pH 8.0 containing 0.5M NaCl.
5. The pellet was washed and mixed with antiserum for Cry1Ac and left at 6°C for 10 hours and on a slow rotating shaker at room temperature for 1 hour. It was centrifuged and the supernatant was aliquoted in eppendorf tubes to store at – 20° C.

Tissue culture Protocols:

Maintenance of Explants of Tobacco:

MS medium supplemented with1μM BAP and 10μM NAA were prepared. The tobacco leaf discs of Wisconsin 38 were placed on the callusing medium.

Multiple shoot generation:

MS media supplemented with 4μM BAP and 0.5μM NAA were prepared and the small portion of friable callus was transferred to the media.

Rooting: MS media supplemented with 0.1μM BAP and1μM NAA were prepared and a shoot with a small portion of callus was transferred to the medium.

Regeneration of a plant:

MS media without hormones were made and transferred the shoot with leaves from the rooting media into the regeneration media.

Spectinomycin standardization:

MS medium with 10μM NAA and 0.1μM BAP was made. Different concentrations of spectinomycin was added to the media at the temperature of 53 °C so that the antibiotic doesn't loose its activity. Then the tobacco leaf discs were kept on the media supplemented with spectinomycin

Transformation of tobacco plant by biolistic method:

24 hours before bombardment:

1. MS medium prepared with hormones was autoclaved and poured in petriplates.
2. Tobacco leaf discs were kept in the center of the plate in a circle of 2 cm diameter and keep over night in the dark.
3. Next day morning the hood was wiped with alcohol and the vortex machine was kept in the hood.
4. Double distilled alcohol was poured in petriplates in which following the following things are placed.
    - Rupture discs.
    - Macrocarrier disc.(to which stop screen is attached later.)
    - Metal mesh.
    - Socket for macro carrier.
    - Socket for the disc (from the equipment)

Coating the DNA to the Gold particles :

1. For 20 Petri plates, 6 gms of gold was taken in a 1.5 ml siliconised Eppendorf tube.

2. 100µl of double distilled alcohol was added and vortexed for 3-5 min..

3. Incubated in ice for 15 min.

4. Centrifuged at 10k for 1 min.

5. The supernatant was discarded. This step was repeated for 3 – 5 times (optional)

6. The pellet of gold particles was resuspended in 100µl of double distilled water and vortexed thoroughly.

7. The suspension was allowed to settle in ice for 2-3 min or room temperature for 10 min.

8. The suspension was divided into two 50µl of aliquots while vortexing continuously.

9. To each add the following in the order while vortexing,

   a. 5µg of DNA,

   b. 50 µl of $CaCl_2$ (2.5 M)

   c. 20 µl of 1M spermidine.

10. Vortexed at high speed for 5-10 min.

11. Allowed to settle in ice.

15. The solution was removed without disturbing the gold particles.

16. Resuspended the pellet with 80 µl of double distilled alcohol.

(Spermidine is poly cation and it stabilizes the DNA by binding to DNA as it is negatively charged. It can also precipitate the DNA at 4°C).

Bombardment:

1. The whole system was rinsed with alcohol.

2. The macro carrier socket was wiped with alcohol, dried in air flow and fixed to its socket.

3. Similarly, the macro carrier discs dipped in alcohol were air dried.

4. The macro carrier discs were coated with 10µl of DNA- gold as a smear in the center.

5. Allowed to dry for some time.

6. The rupture disc sockets were removed from the alcohol, air dried and were placed into its groove after which it was fixed to the top of the chamber with the help of a tighter.

7. In the hole of the macro carrier socket, dried wire mesh was placed over which DNA-gold coated disc was placed. This assembly was fixed to the chamber tightly in its groove.

8. The petri plate containing the leaf discs to be bombarded was kept below in a groove at 12 cm distance.

9. The chamber was closed tightly.

10. Vaccum was created after which the helium gas was allowed to enter. .

11. The rupture disc breaks at the appropriate pressure forcing the DNA-gold particles into the leaf disc.

12. The vaccum was released after the bombardment.

13. This was repeated once again.

16. The Petri plates were kept in dark for another 24 hours after which the leaf discs were cut into small pieces of about 2 mm square size and were kept in MS media with 500mg/lit of spectinomycin.

Maintainence and selection of transformants:

After bombardment, the leaf discs were cultured on MS callusing media supplemented with a spectinomycin concentration of 500 μg / ml. The bombarded leaf discs were repeatedly subcultured for every fortnight to get homoplasmicity. The putative transgenic calli (Spectinomycin resistant) which were obtained after repeated subculturing on spectinomycin were transferred to MS shooting medium to get multiple shoots.

Bioassay

Neonate larvae of *Helicoverpa armigera* were used for the bioassay studies. The eggs were purchased from Mass production lab, Project directorate of Biological control, Bangalore and the insects were reared in artificial diet. The neonate larvae were lifted using a paint brush and left to feed on the leaf materials to be tested. The survival/mortality rate was monitored.

Chapter 1.....

Construction of chloroplast transformation vector

INTRODUCTION

Basic criteria in construction of various chloroplast transformation vectors are that the vector should insert the heterologous gene at specific sites, the gene should be placed under the control of plastid specific regulatory elements to get constitutive expression, and it should have a selectable marker gene. In previous reports of chloroplast transformation, a number of insertion sites have been used such as the region between *trnV* and *rps12/7* loci, between *rbcL* and *orf512* or *accD* gene, between *trnI* and *trnA*, *etc.* which are nonessential and intergenic regions. Introduction of foreign DNA at these sites did not affect the function of the plastome.

In this study, we designed a vector to insert the foreign DNA between *trnI* and *trnA* as these genes are highly conserved among the higher plants and the vector having these genes as the plastid DNA homology can transform a variety of higher plants. The regulatory elements used are 16S rDNA promoter region (P*rrn*) and the *rps16* 3' region (T*rps16*) as terminator as they had been shown to work well by several authors (Table 6). The 16S rDNA promoter region had been the one that has been used in all published chloroplast transformation experiments. Apart from T*rps16*, the 3' region of *psbA* gene has also been used.

The marker genes used were mainly *aadA* gene which encodes for the protein 5-aminoglycoside 3-adenyltransferase giving resistance to spectinomycin and streptomycin. In some reports, gene for kanamycin resistance (*kan*) had been used. Recently, Khan and Maliga (1999) used a transcriptionally and translationally fused *aadA* gene and *gfp* (which encodes the green fluorescent protein). This helps to trace

the transformed leaf portions by green fluorescence. The various combination of chloroplast transformations vectors (*viz.* regulatory elements, DNA homology used to facilitate the insertion and the marker genes for selection) made by different groups are enlisted in Table 6 (page 43).

The flanking genes *trnI* and *trnA*, regulatory elements *Prrn* and *Trps16*, and the marker gene *aadA* were PCR amplified with the addition of appropriate restriction sites to facilitate the subsequent cloning exercises. The coding region of *cry1Ac* of *Bacillus thuringiensis* (*B.t.*) that encodes the Cry1Ac protein (bioinsecticide) has been used as the foreign DNA. All the fragments were placed in proper order to construct the final transformation vector (Fig. 1.15). Preparation of various fragments and the details of vector construction are explained in this chapter.

MATERIALS AND METHODS

I PCR amplification of various fragments

Polymerase Chain Reaction (PCR)

About 100 ng of chloroplast DNA was used per reaction mix containing 0.2 μM of forward and reverse primers, 0.2 mM each of the four deoxynucleotides and 2.5 units of *Taq* DNA Polymerase (from GibcoBRL) in a total volume of 50μl. PCR was performed for 30 cycles with denaturation of template DNA at 94°C for 1 min, annealing templates and oligonucleotide primers at 55°C for 2 min, primer extension at 72°C and finally extension of PCR products at 74°C for 10 minutes. For some samples, the annealing temperature was reduced to 45°C. The PCR products were separated and analyzed on a 0.7% to 2% agarose gel based on their expected size.

PCR amplification

1. 16S rDNA promoter: (P*rrn*)

The P*rrn* was PCR amplified from cotton plastome. The forward primer adds a *Hind* III and *Sfi* I sites at the 5' end and the reverse primer introduces a RBS (Ribosome Binding Site), *Bam*H I, *Sma* I and *Spe* I restriction sites at the 3' end of the amplicon.

Forward primer:

5' ACACCCAAGCTTGGCCNNNNNGGCCGCCCAATGTGAGTTTTTG 3'

Reverse primer:

5'CTAGACTAGTCCCGGGGGATCCCATTCCAAGGCCTCCTTGTATCCGTGC GCTTCGTATTCG 3'

2. Primers for *trnI*

Forward primer: 5' ACACCGCTCGAGGGGTTTCTCTCGCTTTTG 3'

Reverse primer:

5' ACACCCAAGCTTGGCCNNNNNGGCCGCTTCTTCTATTCTTTTCCCTG 3'

The resultant amplicon is mutagenized to have a *Xho* I restriction site at the 5' region and a *Hind* III site at the 3' region by the forward and reverse oligonucleotides.

3. Primers for *trnA*:

Forward primer:

5' AAATATGCGGCCGCGGGAAAAGAATAGAAGAAGC 3'

Reverse primer:

5' GCGAGCGAGCTCTTCACGAGTTGGAGATAAG 3'

The forward and reverse primers inserts *Not* I and *Sac* I restriction sites at the 5' and 3' ends respectively for future cloning purposes. The *trnA, trnI* genes and P*rrn* were PCR amplified using the primers designed based on the gene sequences of tobacco plastome.

4. Primers for *aadA*

The spectinomycin resistance gene (*aadA*) used in this vector had been PCR amplified from clone ECE 101 (containing the full length *aadA* gene) obtained from BGSC, Ohio. The primers used were given below. The forward primer introduces a *Bam*H I site while the reverse primer a *Spe* I restriction site at the 5' and the 3' end of the amplicon. Primer annealing was done at 55° C temperature.

Forward Primer:

5' CGCGGATCCGTGAGGAGGATATATTTG 3'

Reverse Primer:

5' CGGACTAGTAATCTGATTACCAATTAG 3'

5. Primers for T*rps16*

The T*rps16* fragment was PCR amplified from tobacco plastome and the primers used are as follows. The forward and reverse primers were designed to introduce *Spe* I and *Xba* I at the 5' and 3'ends respectively.

Forward primer:

5' CGGACTAGTCGTCCTAATCAATCCTAATCAACCG 3'

Reverse primer:

5' CTAGTCTAGAGTCGTTATAGAACACGGAATTC 3'

II Cloning into pGEM-T vector

The PCR amplified *trnI* and *trnA* amplicons were cloned into pGEM-T vector (following the manufacturer's protocol) by incubating the ligation mixture at 16° C for 16 hours. The ligated DNA had been used to transform *Escherichia coli* (*E. coli*) cells (JM109). The transformed colonies were picked up from LB agar plates containing 50 μg per ml after incubating at 37°C overnight. The transformed colonies were selected by slot lysis method and the plasmid DNA samples prepared from these colonies were isolated by culturing them in LB medium overnight. The subsequent DNA isolation was done by alkaline lysis method. The vector details are given in Fig. 1.16.

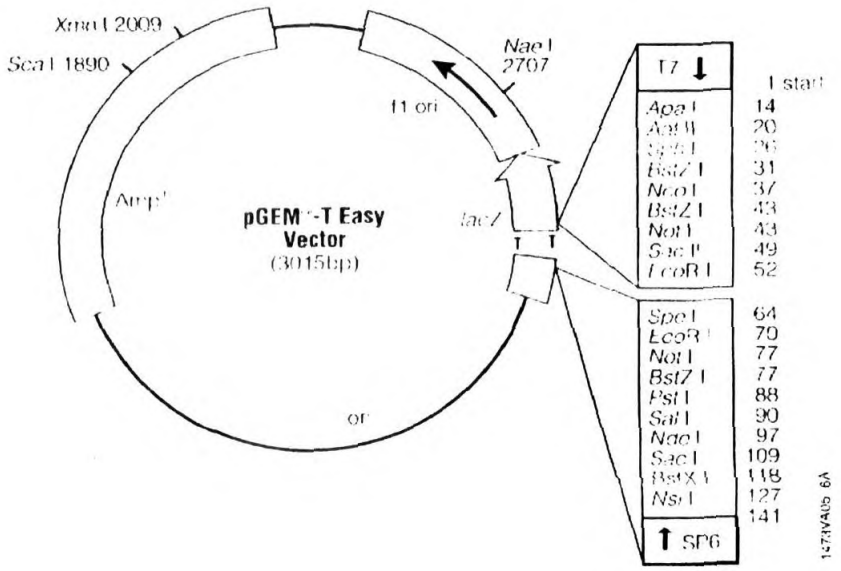
Sequencing of the pGEM-T cloned fragments

The DNA of transformed clones were sequenced by Microsynth, Switzerland and at Indian Institute of Science, Bangalore.

The basic vector

The pBluescript II (KS+) had been used as the basic backbone of the chloroplast transformation vector. The details of the vector and the restriction sites are given in Fig. 1.17.

Fig. 1.16 Schematic representation of pGEM-T Easy vector



Partial digestion of DNA

The DNA was digested with 0.1, 0.5 U of enzymes for 5 min, 10 min, 15 min and the reaction was stopped by adding 0.1 M EDTA. The samples were run on agarose gel by electrophoresis and the intact fragments were gel eluted to use in further experiments.

Southern

The pGEM-T cloned cotton *trnI* and *trnA* genes were run on agarose gels, and transferred on to nylon membrane. The *trnI* and *trnA* genes amplified from the positive control from tobacco (pTB9 -obtained from Dr. Sugiura, Japan) were radiolabelled using ^{32}P and were used as probes. They were allowed to hybridize with the *trnI* and *trnA* genes of cotton transferred on to the membrane at higher stringency conditions (60°C).

Fig. 1.17 Schematic representation of vector pBluescript being used in cloning of chloroplast transformation vector

pBluescript® II KS (+/-) phagemid

The pBluescript® II KS (+/-) phagemid is a 2961-bp phagemid derived from pUC19. The KS designation indicates the polylinker is oriented such that *lacZ* transcription proceeds from *Kpn* I to *Sac* I.

f1 (+) origin: (3-459 bp) f1 filamentous phage origin of replication allowing recovery of the sense strand of the *lacZ* gene when a host strain containing the pBluescript II phagemid is co-infected with helper phage.

f1 (-) origin: (3-459 bp) f1 filamentous phage origin of replication allowing recovery of the antisense strand of the *lacZ* gene when a host strain containing the pBluescript II phagemid is co-infected with helper phage.

ColE1 origin: (1032-1972 bp) Plasmid origin of replication used in the absence of helper phage.

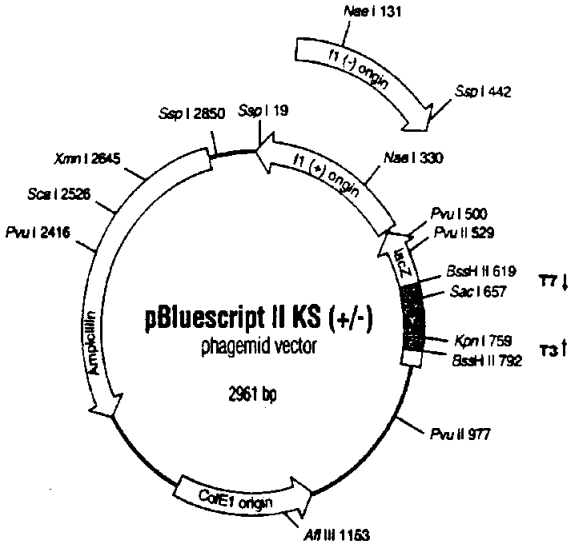
***lacZ* gene:** (*lac* promoter: 816-938 bp) This portion of the *lacZ* gene provides α-complementation for blue/white color selection of recombinant phagemids.
An inducible lac promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.

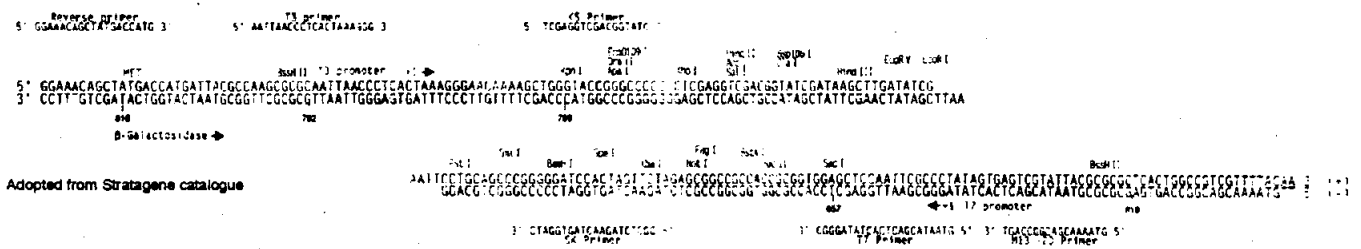
MCS: (657-759 bp) Multiple cloning site flanked by T3 and T7 RNA promoters (please see the polylinker sequence below).

Ampicillin: (1975-2832 bp) Ampicillin-resistance gene (Amp^r) for antibiotic selection of the phagemid vector.

Please Note: The upper strand is designated the (+) strand and the lower strand is designated the (-) strand

GenBank® # X52327 (KS(+)) & # X52329 (KS(-))





Adopted from Stratagene catalogue

Ligation

Ligation of digested DNA was carried out at 16°C for 16 hr. T_4 DNA Ligase was used along with the buffer supplied by the manufacturer. Usually a vector to insert ratio of 1:5 was used for ligation of cohesive ends. High concentration T4 DNA Ligase (10 U per μl) was used in the case of blunt end ligation. Blunt end ligation was carried out at 16° C.

RESULTS AND DISCUSSION

PCR amplification of *trnI*, *trnA* genes and the 16SrDNA 5' region

PCR amplicons of *trnI* and *trnA* genes and the 16SrDNA 5' region were run on agarose gel along with the amplicons of tobacco as positive control (Fig. 1.1). Later, they were cloned into pGEM-T vector and the transformed clones were selected based on slot-lysis method. The transformed clones containing these amplicons were confirmed by restriction digestion analysis with *Not* I enzyme (Fig. 1.2). To confirm the authenticity of the amplicons of 16S rDNA, *trnI* and *trnA* from cotton, a Southern experiment was performed with the radiolabelled 16S rDNA, *trnI* and *trnA* genes of tobacco as probes (Fig. 1.3). The heterologous probes hybridized with the cotton amplicons thus confirming the authenticity of the amplicons. The clones were sequenced and the sequences were deposited in the GenBank database. Their accession numbers are as follows: *trnA* - **AF 175761**, *trnI* - **AF189149**, and 16S rDNA 5' region -**AF189150**. The nucleotide sequences of the above and *rps16* 3' region are given in Fig. 1.4. The exon sequences of *trnI* and *trnA* of both cotton and tobacco were similar (discussed more in chapter III).

PCR amplification of *aadA* and T*rps16*

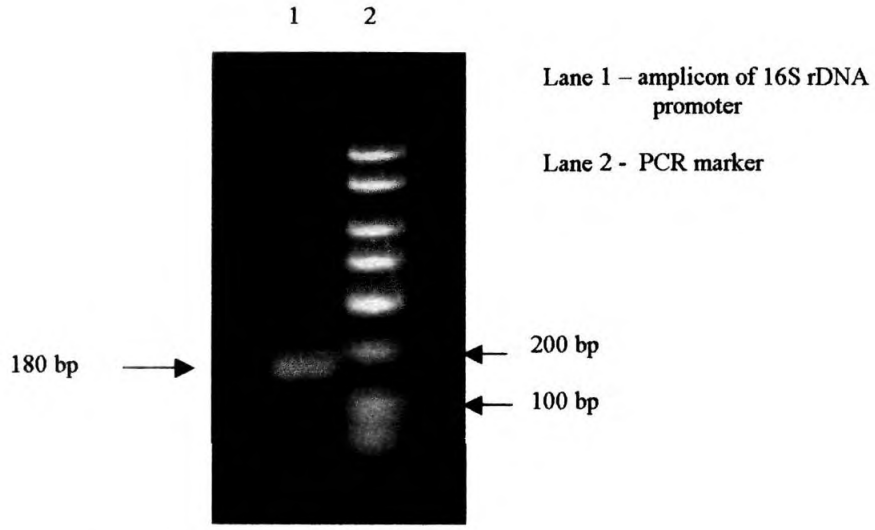
The *aadA* gene and the T*rps16* region were amplified and amplicons are shown in the Fig. 1.5. The sizes of the *aadA* gene and the T*rps16* used in this construct are 867 bp and 196 bp respectively. Nucleotide sequence of *aadA* is given in Fig. 1.5.

Construction of the vector

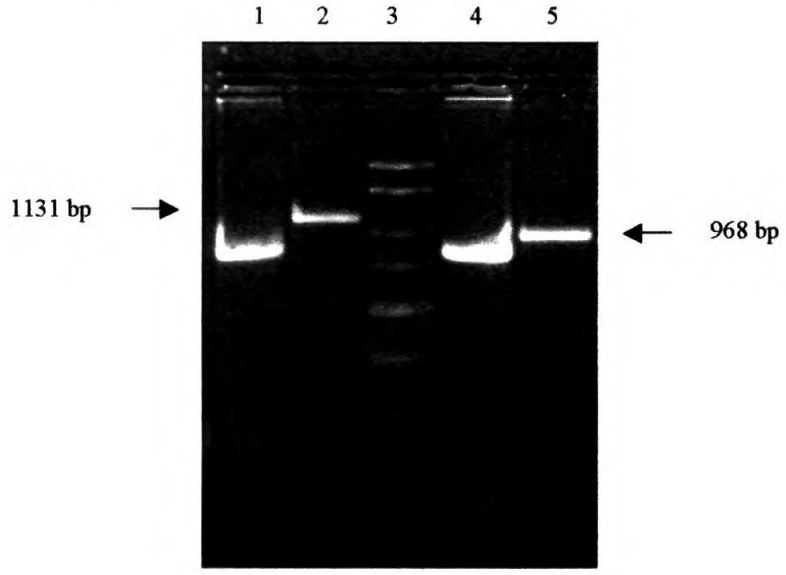
Construction of the transformation vector was done in 4 steps. 1. Introduction of *trnI* and *trnA* genes in pBluescript II KS+ (pBS); 2. Cloning in the P*rrn*, *aadA* and T*rps16* in pBS vector; 3. Introduction of cassette containing P*rrn*, *aadA* and T*rps16*

Figure 1.1

PCR amplicon of 16S rDNA promoter from cotton



PCR amplicon of *trnI* and *trnA* from cotton



Lane 1 – PCR amplicon of *trnI* (tobacco)
Lane 2 – PCR amplicon of *trnI* (cotton)
Lane 3 - PCR marker
Lane 4 - PCR amplicon of *trnA* (tobacco)
Lane 5 – PCR amplicon of *trnA* (cotton)

Figure 1.2

***Not*I Restriction digestion pattern of pGEM-T vector containing various amplicons**

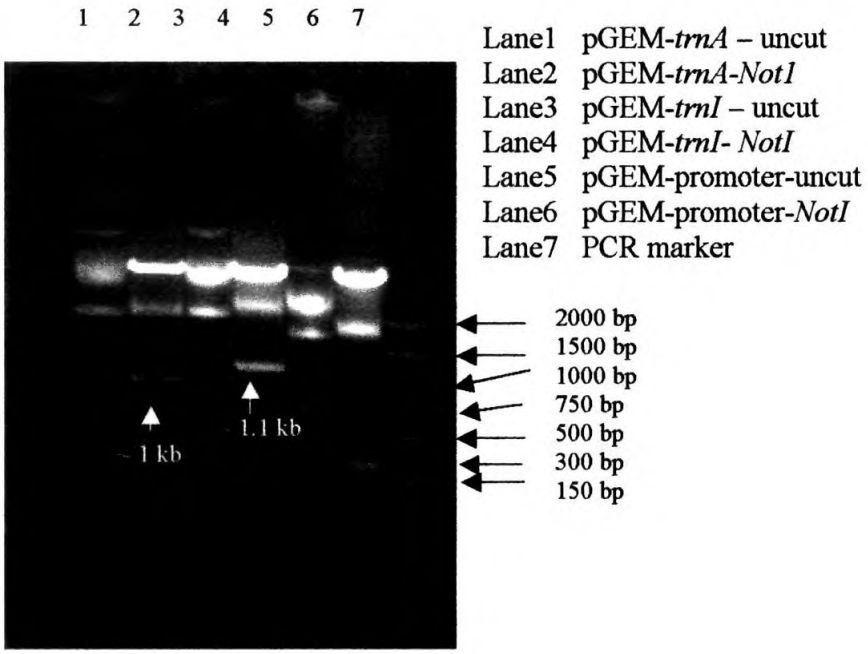


Figure 1.3

Southern hybridization result of 16S rDNA, *trnI* and *trnA* amplified from cotton

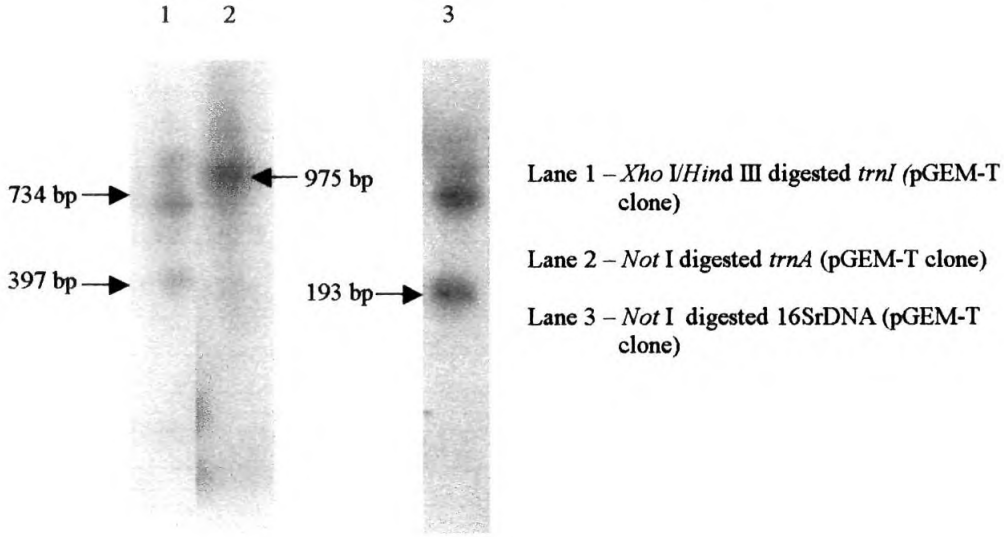


Figure 1.4

DNA sequence of *trnI* from cotton plastome

```
   1  GGGCTATTAG CTCAGTGGTA GAGCGCGCCC CTCATAATTG CGTCGTTGTG
  51  CCTGGGCTGT GAGGGCTCTC AGCCACATGG ATAGTTCAAT GTGCTCATCA
 101  GCGCCTGACC CTGAGATGTG GATCATCCAA GGCACATTAG CATGGCGTAC
 151  TTCTCCTGTT CGAACCGGGG TTTGAAACCA AACTTCTCCT CAGGAGGATA
 201  GATGGGGCGA TTCAGGTGAG ATCCAATGTA GATCCAACTT TCTATTCACT
 251  CGTGGGATCT GGGCGGTCCG GGGGGGACAC CACGGCTCCT CTCTTCTCGA
 301  TAATCCATAC ATCCCTTATC AGTGTATGGA CAGCTATCTC TCGAGCACAA
 351  GTTTAGGTTC GGCCTCAATG GGAAAATAAA ATGGAGCACC TAACAACGTA
 401  TCTTCACAGA TCAAGAACTA CGAGATCGCC CCTTTCATCC TGGGGTGACG
 451  GAGGGATCGT ACCATTCGAG CCTTTTTTTT TCATGCTTTT CCCGGAGGTC
 501  CGGAGAAAGC TGCAATCAAT AGGATTTTCC TAATcCTCCC TTCCCGAAAA
 551  GGAAGAACGT GAAATTCTTT TTCCTTTCCG CAGGGACCAG GAGATTGGAT
 601  CTAGCCGTAA GAAGAATGCT TGGCTGATAA ATAATTCACT TCTTGGTCTT
 651  CGACCCCCTC AGTCACTACG AACGCCCCCG ATCAGTGCAA TGGGATGTGT
 701  CTATTTATCT ATCTCTTGAC TCGAAATGGG AGCAGGTTTG AAAAAGGATC
 751  TTAGAGTGTT TAGGGTTGGG CCAAGAGGGT CTCTTAACGC CCTCTTTTTT
 801  TTTCTTCCCA TCGGAGTTAT TTCTTATTTC ACAAATACTT GCCATGGTAA
 851  GGAAGAAGGG GGGAACAAGC ACACTCGGAG AGCGCAGTAC AACGGAGAGT
 901  TGTATGCTGC GTTCGGGAAG GATGAATCGC TCCCGAAAAG GAATCTATTG
 951  ATTCTCTCCC AATTGGTTGG ACCGTAGGTG CGATGATTTA CTTCACGGGC
1001  GAGGTCTCTG GTTCAAGTCC AGGATGGCCC A
```

DNA sequence of *trnA* from cotton plastome

```
   1  GGGGATATAG CTCAGTTGGT AGAGCTCCGC TCTTGCAATT GGGTCGTTGC
  51  GATTACGGGT TGGGTGTCTA ATTGTCCAGG CGGTAATGAT AGTATCTTGT
 101  ACCTGAACCG GTGGCTCACT TTTTCTAAGT AATGGGGAAG AGGACCGAAA
 151  CATGCCACTG AAAGACTCTA CTGAGACAAA GATGGGCTGT CAAGAACGTA
 201  GAAGAGGTAG GATGGGCGGT TGGTCAGATC TAGTATGGAT CGTACATGGA
 251  CGGTAGTTGG AGTCGGCGGC TCCCCTAGGT TCCCCCATCT GGGATCCCTG
 301  GGGAAGAGGA TCAAGTTGGC CCTTGCGAAC AGCTTGATGC ACTATCTCCC
 351  TTCAACCCTT TGAGCGAAAT GCGGCAAAAG GAAGGAAAAT CCATGGACCA
 401  ACCCCATCGT CTCCACCCCG TAGGAACTAC GAGATCACCC CAAGGACGCC
 451  TTCGGTATCC AGGGGTCGCG GACCGACCAT AGAACCCTGT TCAATAAGTG
 501  GAATGCATTA GCTGTCCGCT CTCAGGTTGG GCAGTAAGGG TCGGAGAAGG
 551  GCAATCACTC ATTCTTAAAA CCAGCATTCT TAAGACCAAA GAGTCGGAGG
 601  GGGGAAAGCT CTCCGCCGTT CCTGGTTTTC CTGTAGCAGG ATCCTCCGGA
 651  ACCACAAGAA TCCTTAGTTA GAATGGGATT CCAACTCAGC ACCTTTTGAG
 701  ATTTTGAGAA GAGTTGCTCT TTGGAGAGCA CAGTACGATG AAAGTTGTAA
 751  GCTGTGTTCG GGGGGGAGTT ATTGTCTATC GTTGGCCTCT ATGGTAGAAT
 801  CAGTCGGGGG CCTGAGAGGC GGTGGTTTAC CCTGTGGCGG ATGTCAGCGG
 851  TTCGAGTCCG CTTATCTCCA
```

DNA sequence of 16S rDNA promoter

```
   1  GCCCAATGTG AGTTTTTGAT TTTGATTTGC TACCCCGCCG TGATTGAATG
  51  AGAATGGATA AGAGGCTCGT GGGATTGACG TGAGGGGGCA GGGATGGCTA
 101  TATTTCTGGG AGCGAACTCC GGGCGAATAT GAAGCGCATG GATACAAGTT
 151  AGGCCTTGGA ATGAAAGACA ATTCCGAATC CGCTTTGTCT ACGAACAAGG
 201  AAGCTATAAG
```

Nucleotide sequence of *rps16* 3' region

Spe I

TA[illegible]CGT CCTAATCAAT CCTAATCAAC CGAAATTCAA TTAAGGAAAT AAATTAAGGA

AATACAAAAA GGGGGGTAGT CATTTGTATA TAACTTTGTA TGACTTTTCT CTTCTATTTT

TTTGTATTTC CTCCCTTTCC TTTTCTATTT GTATTTTTTT ATCATTGCTT CCATTGAATT

CCGTGTTCTA TAACGAC[illegible] *Xba* I

Figure 1.5

Nucleotide sequence of *aadA* gene

(arrows and letters in green shade indicate the ORF, RBS is underlined, Restriction sites are denoted with purple shade)

*Bam*H I

TAGGGGATCC GTGAGGAGGA TATATTTGAA TACATACGAA CAAATTAATA AAGTGAAAAA

AATACTTCGG AAACATTTAA AAAATAACCT TATTGGTACT TACATGTTTG GATCAGGAGT

TGAGAGTGGA CTAAAACCAA ATAGTGATCT TGACTTTTTA GTCGTCGTAT CTGAACCATT

GACAGATCAA AGTAAAGAAA TACTTATACA AAAAATTAGA CCTATTTCAA AAAAAATAGG

AGATAAAAGC AACTTACGAT ATATTGAATT AACAATTATT ATTCAGCAAG AAATGGTACC

GTGGAATCAT CCTCCCAAAC AAGAATTTAT TTATGGAGAA TGGTTACAAG AGCTTTATGA

ACAAGGATAC ATTCCTCAGA AGGAATTAAA TTCAGATTTA ACCATAATGC TTTACCAAGC

AAAACGAAAA AATAAAAGAA TATACGGAAA TTATGACTTA GAGGAATTAC TACCTGATAT

TCCATTTTCT GATGTGAGAA GAGCCATTAT GGATTCGTCA GAGGAATTAA TAGATAATTA

TCAGGATGAT GAAACCAACT CTATATTAAC TTTATGCCGT ATGATTTTAA CTATGGACAC

GGGTAAAATC ATACCAAAAG ATATTGCGGG AAATGCAGTG GCTGAATCTT CTCCATTAGA

ACATAGGGAG AGAATTTTGT TAGCAGTTCG TAGTTATCTT GGAGAGAATA TTGAATGGAC

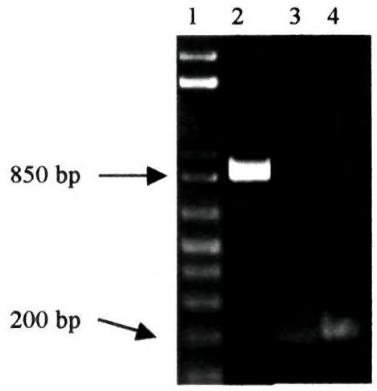
TAATGAAAAT GTAAATTTAA CTATAAACTA TTTAAATAAC AGATTAAAAA AATTATAAAA

AAATTGAAAA AATGGTGGAA ACACTTTTTT CAATTTTTTT GTTTTATTAT TTAATATTTG

GGAAATATTC ATTCTAATTG GTAATCAGAT TACTAGTCGT

Spe I

Agarose gel analysis of *aadA* and *rps16*



Lane 1 – 1 kb plus ladder
Lane 2 – *aadA* (867 bp)
Lane 3 – *rps16* -3' (196 bp)
Lane 4 – PCR amplicon (204 bp)

(from the first step) between the *trnI* and *trnA* (of second step); 4. Placing *cry1Ac* between the P*rrn* and *aadA* gene to get the final transformation vector.

Introduction of *trnI* and *trnA* into pBS.

In vitro mutagenesis to abolish *Bam*H I site

The pGEM-T clone containing *trnA* was digested with *Bam*H I and the sticky ends were end filled using T4 DNA polymerase from GibcoBRL. The end filled DNA was religated using T4 DNA Ligase (Amersham Pharmacia). The ligation mix was transformed into *E. coli* (JM 109) and the transformed colonies containing the *Bam*H I mutated clones were selected by restriction digestion analysis. The clone that do not get digested (linearized) with *Bam*H I were selected for further use.

Cloning *trnI* into pBS

The *trnI* gene cloned in pGEM-T vector had been excised and introduced in to the *Xho* I and *Hin*d III sites of pBS (2961 bp). But the *trnI* gene after sequencing was identified to have an internal *Xho* I site which had a possibility of getting cut while restriction digesting the pGEM-T clone to excise the intact *trnI*. In order to get the full length *Xho* I/*Hin*d III fragment of *trnI* (1131 bp) without getting the internal *Xho* I site cut, a partial restriction digestion with *Xho* I restriction enzymes was performed. To achieve this, the pGEM-T clone containing the *trnI* was first linearized with *Hin*d III enzyme and partially digested with 0.1 and 0.5 U of *Xho* I restriction enzyme for about 10 min. The Digested sample was run on agarose gel and the band of full-length intact *trnI* was excised out to electroelute. After electroelution, it (1131 bp)was cloned into the *Xho* I/*Hin*d III sites of pBS (size of *Xho* I/*Hin*d III digested pBS is 2940 bp) to get the clone pMSVS1 (Fig.1.6). The size of the vector is 4071 bp.

Figure 1.6a

Schematic representation of clone pMSVS1

(pGEM-T clone containing *trnI* (1131 bp)

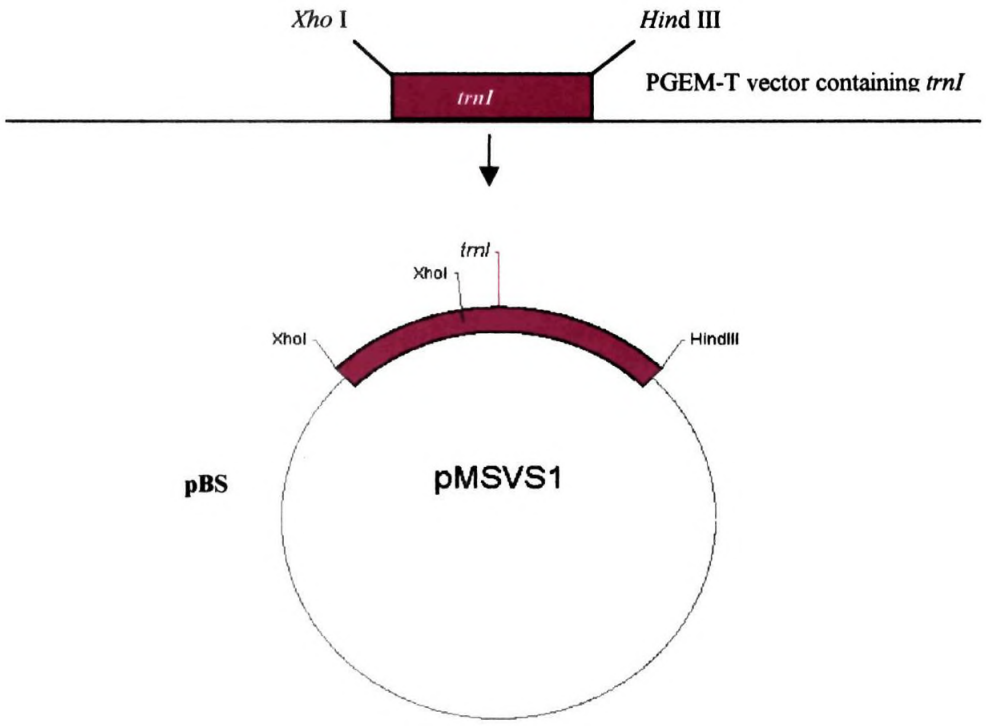
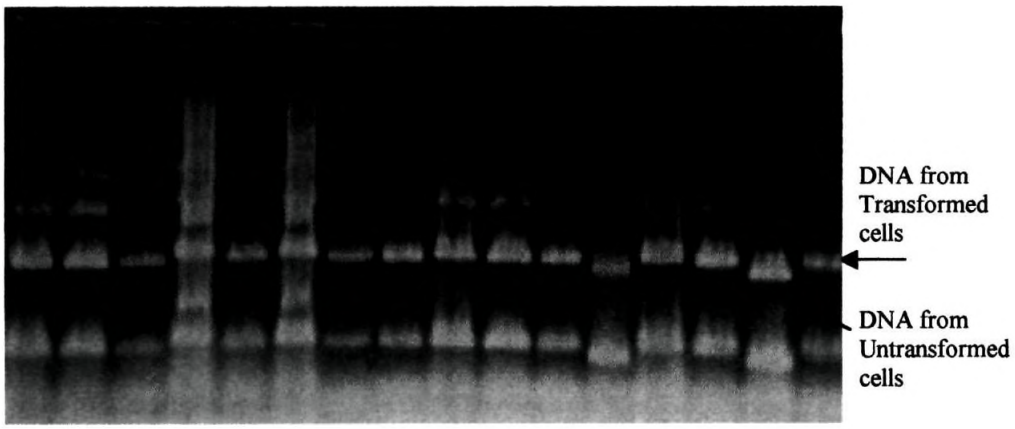


Fig. 1.6b

Identification of *E. coli* colonies transformed with pMSVS1 by slot lysis method



Introduction of *trnA* into pMSVS1

The *trnA* gene cloned in pGEM-T vector had been found to have two internal *Bam*H I restriction sites which had to be abolished to avoid inconvenience while introducing the *cry1Ac* as *Bam*H I fragment at the last step. For this purpose, a partial digestion of pGEM-T/*trnA* clone with *Bam*H I enzyme was performed. The linearized full length DNA band from the agarose gel after electrophoresis was cut and electroeluted. The sticky ends of *Bam*H I restriction site were end filled to get blunt ends with T4 DNA polymerase and religated with T4 DNA Ligase to transform into *E.coli* cells later. The selected clone with only one internal *Bam*H I restriction site was again linearized with *Bam*H I enzyme and the end filling process was performed again as mentioned earlier. The positive clone does not get digested when restriction digested with *Bam*H I. The selected clone was restriction digested with *Not* I restriction enzyme to release 975 bp *Not* I fragment and cloned into the *Not* I site of pMSVS1 and the resultant clone was named as pMSVS2 (Fig.1.7). The size of the clone is 5046 bp.

Orientation confirmation

To check whether the *trnA* had been cloned in the right orientation, the clones were restriction digested with *Kpn* I and *Ava* I. The clone with right orientation releases around 3.5, 0.8, 0.3 and 0.2 kb fragments. Among the clones tested, clone number 13 was found to be releasing the expected fragments and that was chosen for the further cloning steps (Fig.1.8 a and b).

Figure 1.7 Schematic representation of the construction of pMSVS2

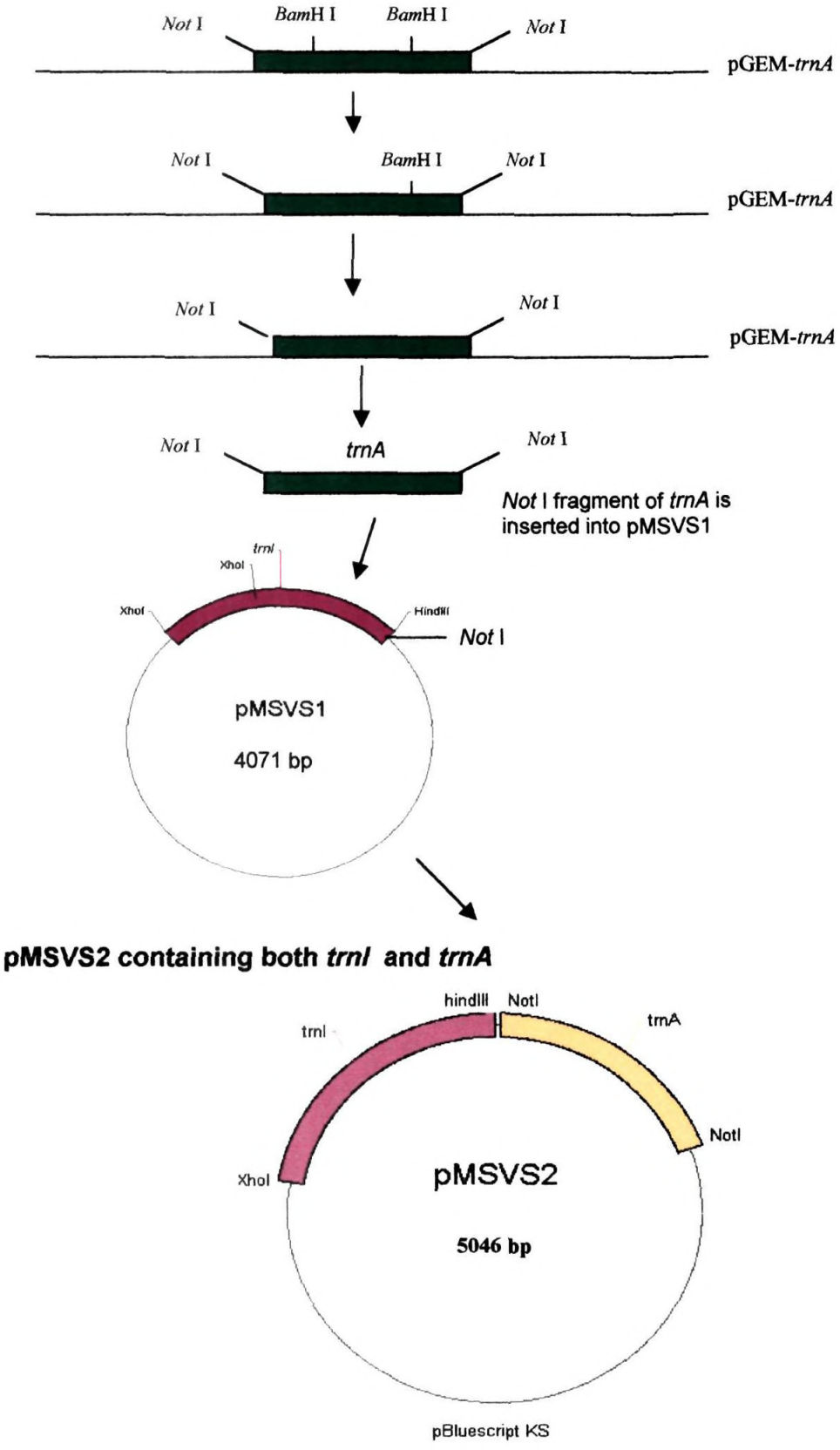
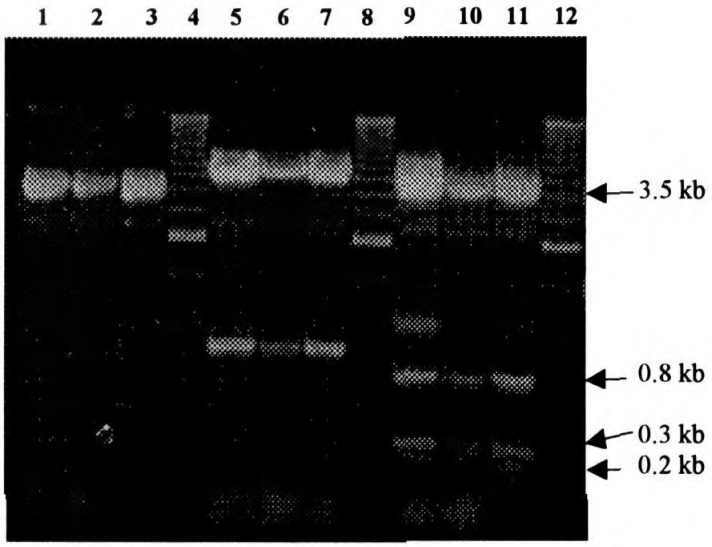


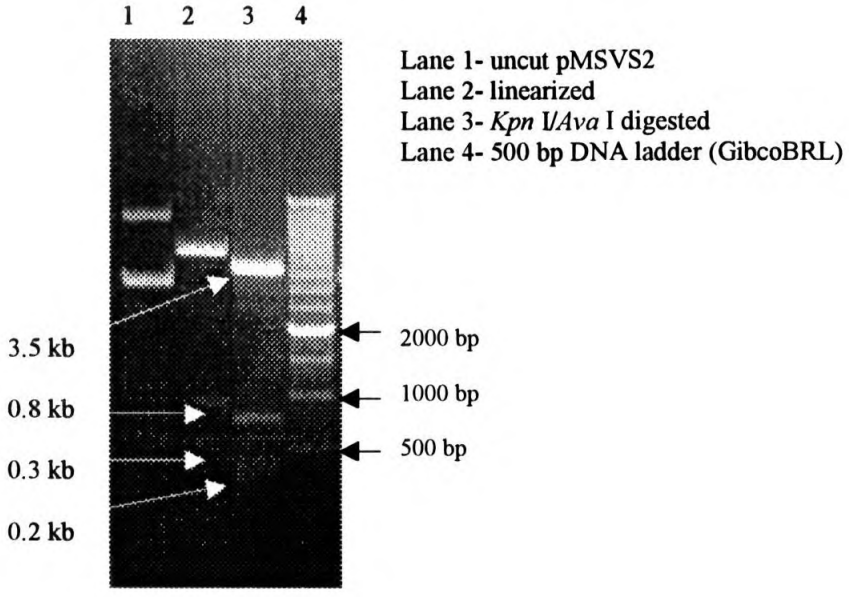
Figure 1.8

a. Restriction digestion analysis of three pMSVS2 clones (orientation analysis)



Lane 1 to 3 – uncut DNA of 3 putative clones
Lane 4, 8 and 12 – DNA ladder
Lane 5 to 7 – *Not* I digested plasmid DNA of 3 clones
Lane 9 to 11 – *Kpn* I/*Ava* I digested DNA of 3 clones

b. Restriction digestion analysis of the pMSVS2 clone with the right orientation



Lane 1- uncut pMSVS2
Lane 2- linearized
Lane 3- *Kpn* I/*Ava* I digested
Lane 4- 500 bp DNA ladder (GibcoBRL)

B. Introduction of P*rrn* and T*rps16* in pBS

Introduction of P*rrn* into pBS to get pMSVS3

The P*rrn* (16S rDNA promoter) was PCR amplified from cotton chloroplast DNA using the primers mentioned in the methodology. The Forward primer introduces a *Hin*d III restriction site and the reverse primer introduces a ribosome binding site (RBS) – AGGAGG, *Bam*H I, *Sma* I and *Spe* I restriction sites at the 3' end of the amplicon. The *Bam*H I site will be used to introduce the *cry1Ac* at the later stage. *Sma* I site introduced here is to have some more genes introduced at the later stage if necessary. The PCR amplicon was cloned into pGEM-T vector and the transformants were selected based on blue/white selection. Plasmid DNA was isolated from the transformed colonies and were confirmed by performing restriction digestion with *Not* I enzyme which releases the amplicons from the pGEM-T vector (Fig.1.9c). The clone was sequenced and submitted in the database. (Fig.1.9a)

The P*rrn* from pGEM-T was restriction digested with *Hin*d III and *Spe* I enzymes to release the 193 bp fragment. This fragment was subsequently cloned into the corresponding sites of the pBS (after *Hin*d III and *Spe* I digestion, the size is 2925 bp) vector to get the clone pMSVS3 (Size 3118 bp; Fig.1.9b). Restriction digestion analysis was performed to confirm the clone.

Introduction of T*rps16* into pMSVS3 to get pMSVS4

The T*rps16* (terminator) used in the chloroplast transformation vector was PCR amplified as 196 bp fragment from tobacco chloroplast genome using the primers mentioned in the methodology. The forward primer introduces a *Spe* I restriction site at the 5' end and the reverse primer introduces an *Xba* I site in the amplicon to facilitate cloning it as *Spe* I/*Xba* I fragment in pMSVS2. The amplicon

Figure 1.9

a. Nucleotide sequence of 16S rDNA promoter

(This fragment starts with *Hind* III restriction site and ends with *BamH* I, *Sma* I and *Spe* I sites. RBS, -35 and –10 regions are underlined.)

Hind III

AAGCTTGGCC ACTATGGCCG CCCAATGTGA GTTTTTGATT TTGATTTGCT ACCCCGCCGT

-35

GATTGAATGA GAATGGATAA GAGGCTCGTG GGATTGACGT GAGGGGGCAG GGATGGCTAT

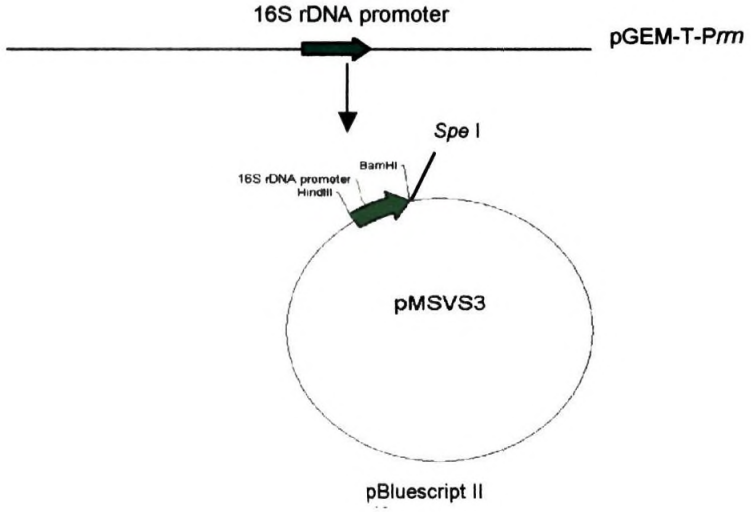
-10 RBS

ATTTCTGGGA GCGAACTCCG GGCGAATACG AAGCGCACGG ATACAAGGAG GCCCTGGAAT

BamH I *Sma* I *Spe*

GGGATCCCCC GGGACTAGTC

b. Schematic representation of the clone pMSVS3



c. PCR amplicon of 16S rDNA promoter (P*rrn*)

was restriction digested with *Spe* I and *Xba* I enzymes and cloned into the appropriate sites in pMSVS3 (3118 bp) to obtain pMSVS4 (3314 bp; Fig.1.10a). *E. coli* cells were transformed with the cloned DNA and the transformed cells were selected based on blue/white colour. *E. coli* colonies with the right clone were confirmed by performing a restriction digestion analysis after isolating the plasmid DNA (Fig. 1.10b).

Introduction of *aadA* gene into pMSVS4 to get pMSVS5

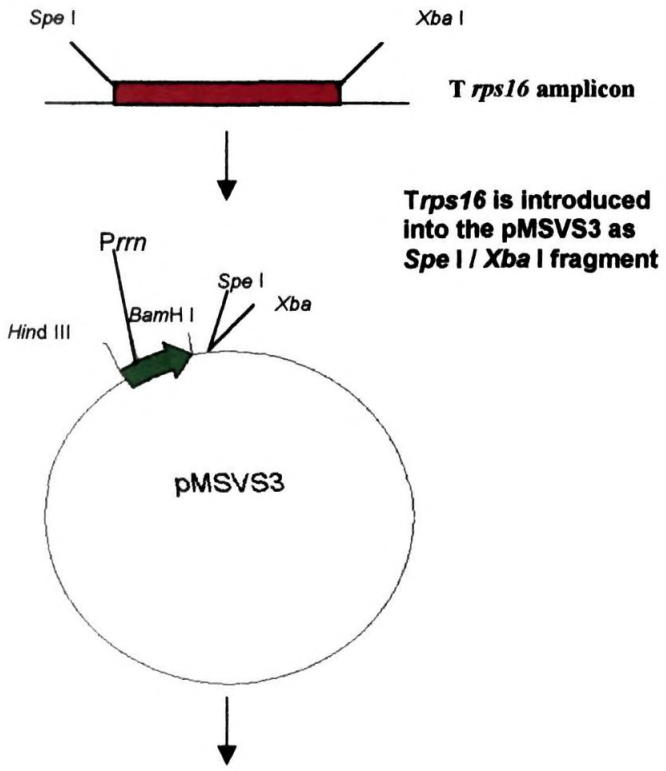
The *aadA* gene encoding aminoglycoside 3"-adenyltransferase which gives resistance to spectinomycin and streptomycin has been used as marker gene in this vector. The coding region of *aadA* gene was PCR amplified using the primers mentioned in the methodology. The forward primer inserts a *Bam*H I and the reverse primer inserts a *Spe* I restriction sites at the 5' and 3'end respectively. The construct ECE101 (obtained from BGSC) containing the full-length *aadA* gene was used as template. The PCR amplified amplicon was cloned into the pGEM-T Easy vector and then it was later restriction digested as *Bam*H I/*Spe* I fragment (867 bp) to clone into the corresponding sites in pMSVS4 to obtain the clone pMSVS5 (4169 bp; Fig. 1.11). After cloning and transformation in to *E. coli,* the colonies harbouring the resultant clone were selected by slot lysis method. Further, they were confirmed by restriction digestion analysis (Fig.1.12).

C. Introduction of the expression cassette from pMSVS5 to pMSVS2.

In this step, the expression cassette of pMSVS5 containing the promoter (P*rrn*), *aadA* gene and the T*rps16* was digested as *Hind* III/*Xba* I fragment (1244 bp) and introduced into the appropriate sites of the clone pMSVS2. The size of the clone pMSVS2 is 5004 bp after *Hind* III/*Xba* restriction digestion. The resultant clone

Figure 1.10

a. Schematic representation of the construction of pMSVS4



pMSVS4 containing both P*rrn* and T*rps16*

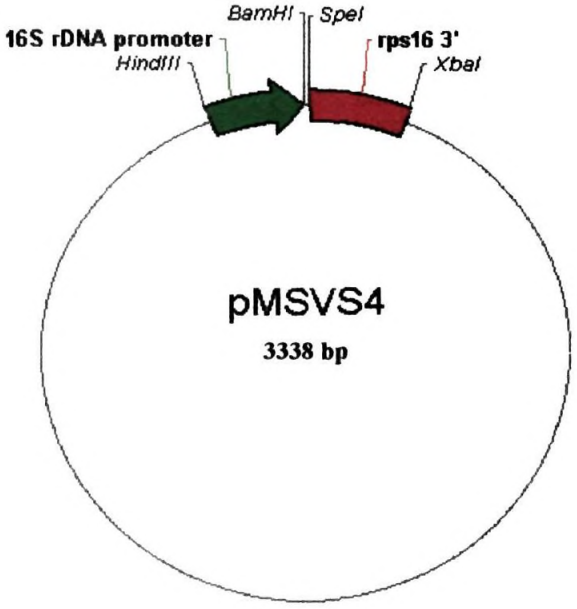
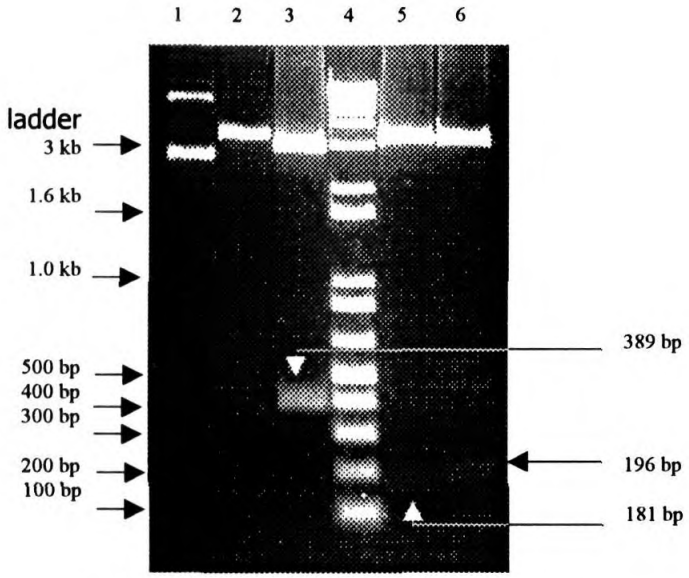
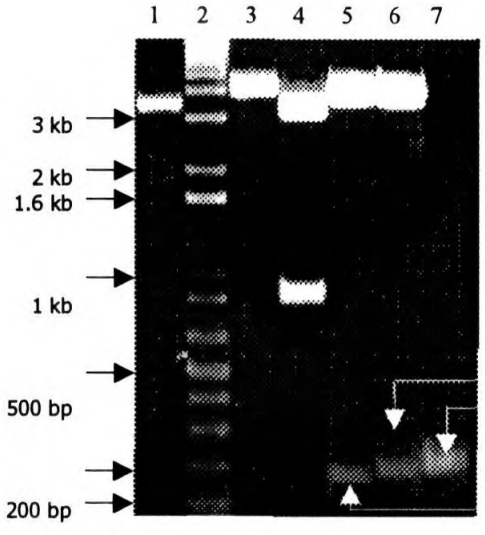


Figure 1.10b
Restriction digestion pattern of pMSVS4



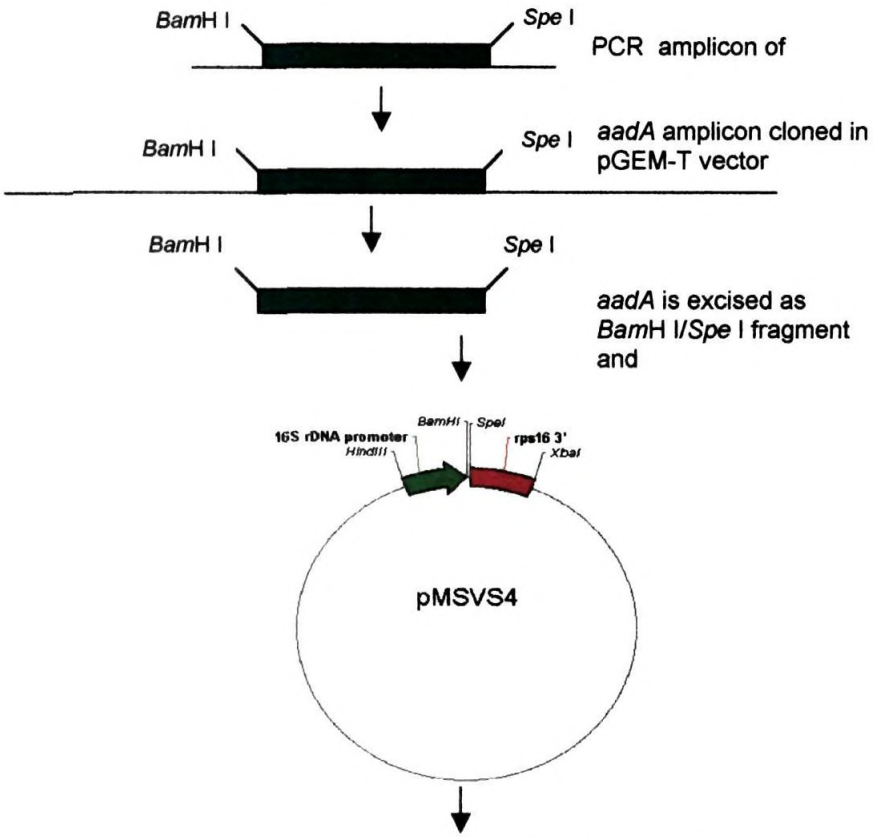
Lane 1 – pMSVS4 – uncut
Lane 2 - pMSVS4- *Bam*H I linearized
Lane 3 - pMSVS4 – *Hin*d III/*Xba* I digested
Lane 4 – 1 kb plus DNA ladder
Lane 5 - pMSVS4 – *Hin*d III/*Bam*H I digested
Lane 6- pMSVS4 – *Spe* I/*Xba* I digested

Figure 1.12
Restriction digestion analysis of pMSVS5

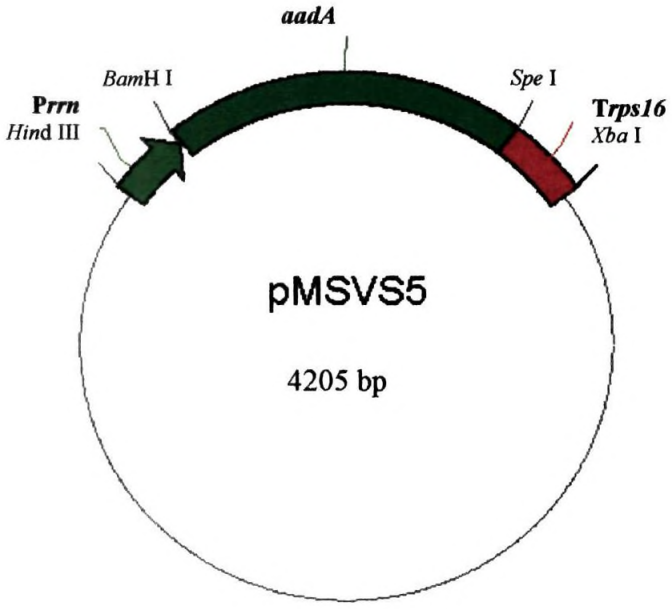


Lane 1 – uncut pMSVS5 DNA
Lane 2 – 1 kb plus DNA ladder
Lane 3 – *Bam*H I linearized
Lane 4 – *Bam*H I/*Spe* I digested
Lane 5 – *Hin*d III/*Bam*H I digested
Lane 6 – *Spe* I/*Xba* I digested
Lane 7 – T *rps16* PCR amplicon

Figure 1.11
Schematic representation of the construction of pMSVS5.



pMSVS5 containing marker gene *aadA* in between P*rrn* and T*rps16*



pMSVS6 is 6248 bp in size (Fig.1.13a). The cloned DNA samples were transformed into *E. coli* cells and the transformed colonies with the pMSVS6 plasmid were screened by slot lysis method. Plasmid DNA was isolated from the transformed *E. coli* cells and the clones were confirmed by restriction digestion analysis (Fig. 1.13b).

D. Introduction of *cry1Ac* into pMSVS6

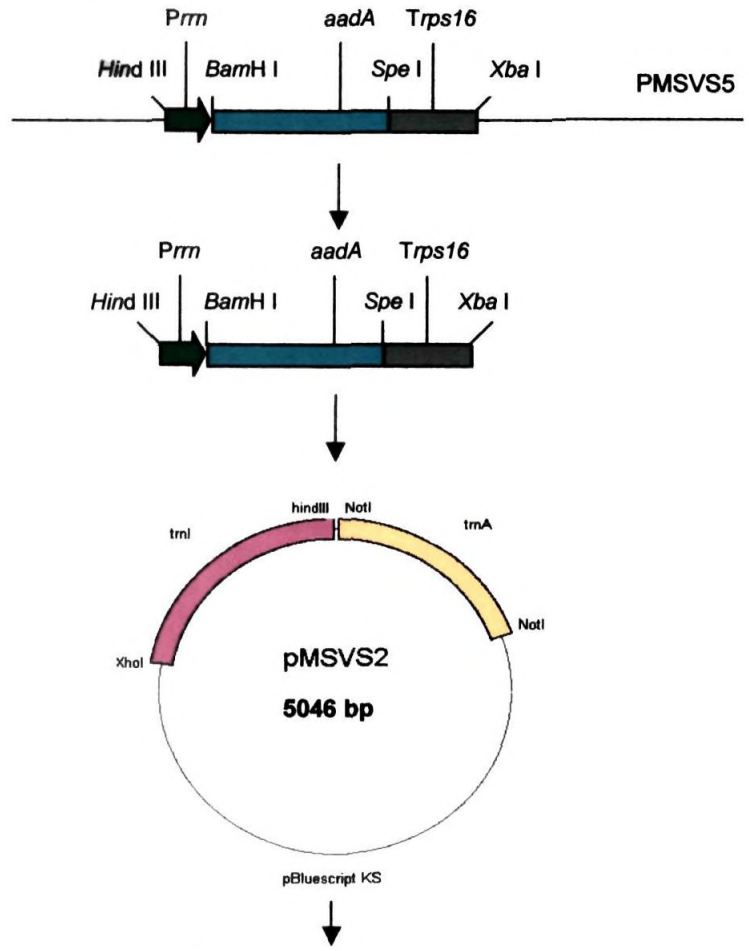
In this final step, the *cry1Ac* gene for insect resistance was introduced into the *Bam*H I site between the promoter and *aadA* gene of the clone pMSVS6 to get pMSVS7 (9952 bp). The coding region of unmodified *cry1Ac* (3704 bp) was obtained from the clone pVS6.6 (Fig.1.14) as *Bam*H I fragment and cloned into pMSVS6. The transformants were selected by restriction digestion analysis (Fig. 1.14a). The clone with right orientation was further selected based on *Hin*d III digestion. The clone with *cry1Ac* gene placed in the right orientation releases ~ 3 kb and the clone with the negative orientation releases ~1 kb. The selected right clone was restriction digested with *Bam*H I to release the 3.7 kb *cry1Ac* fragment. The *Bam*H I digested pVS6.6 had been used as the positive control (Fig.1.14b). Schematic representation of construction of this clone is shown in Fig.1.15.

Expression of pMSVS7A in *E.coli*

The *E. coli* cells were transformed with the final chloroplast transformation vector (pMSVS7A) and the expression of the Cry1Ac protein was analyzed. The protein extract of *E.coli* cells was dot blotted on nitrocellulose membrane and allowed to cross react with the Cry1Ac antiserum. Results of this experiment confirmed the expression of Cry1Ac protein in *E. coli* (Fig. 14c). The antiserum cross reacted with only the *E.coli* with pMSVS7A and not with the *E. coli* having the vector pBS.

Figure 1.13

a. Schematic representation of the construction of pMSVS6.



Clone pMSVS6A containing the expression cassette in between the *trnI* and *trnA* flanking genes

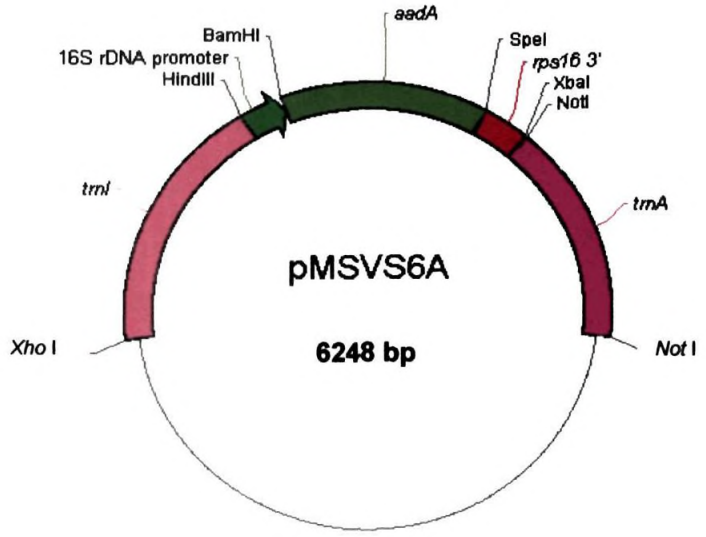
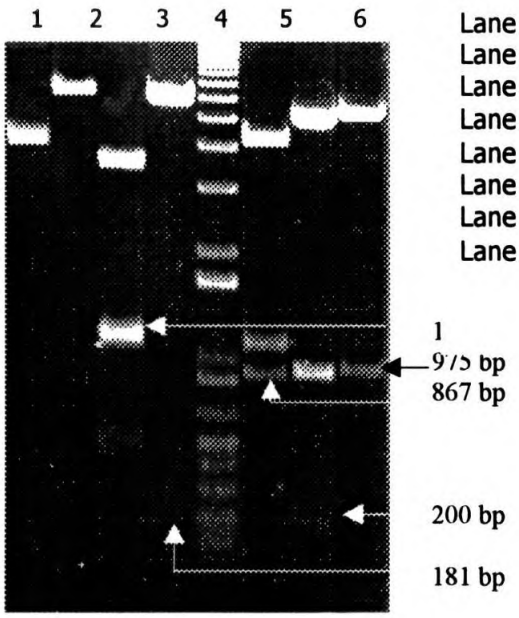
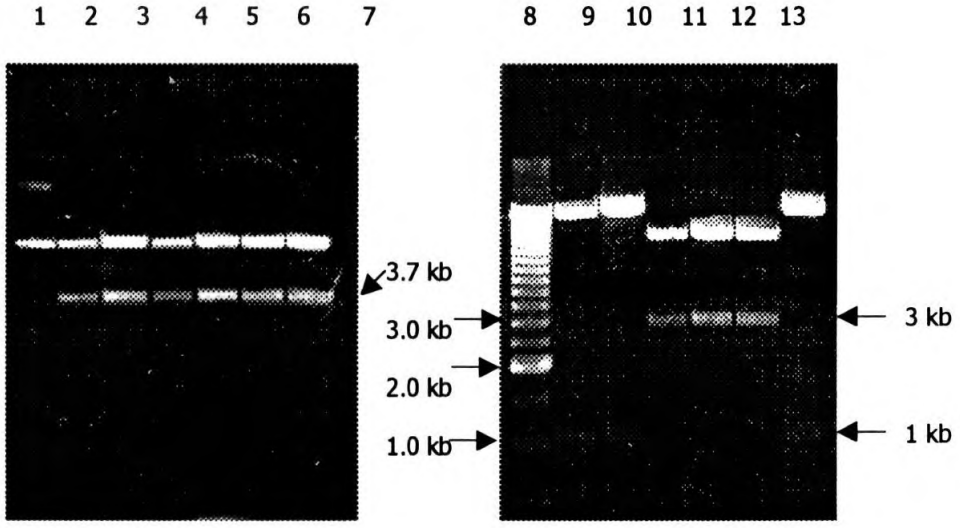


Figure 1.13b
Restriction digestion pattern of pMSVS6A



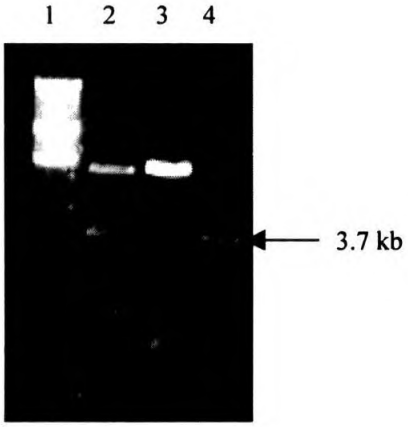
Lane 1 – uncut pMSVS6A DNA
Lane 2 – *Bam*H I linearized
Lane 3 – *Kpn* I/*Hin*d III digested
Lane 4 – *Hin*d III/*Bam*H I digested
Lane 5 - 1 kb plus ladder marker
Lane 6 – *Bam*H I/*Spe* I digested
Lane 7 – *Spe* I/*Xba* I digested
Lane 8 – *Not* I digested

Figure 1.14a
Restriction digestion analysis of *E. coli* [pMSVS7A] clones



Lane 1 – uncut trnasformant DNA
Lane 2 to 7 - clones digested with *Bam*H I (all releasing 3.7 kb)
Lane 8 – 1 kb ladder
Lane 9 to lane 14 - clones digested with *Hin*d III
Lane 9, 10 and 14 are clones releasing ~1 kb fragment (wrong orientation)
Lane 11, 12 and 13 are clones releasing ~ 3.7 kb fragment (right orientation)

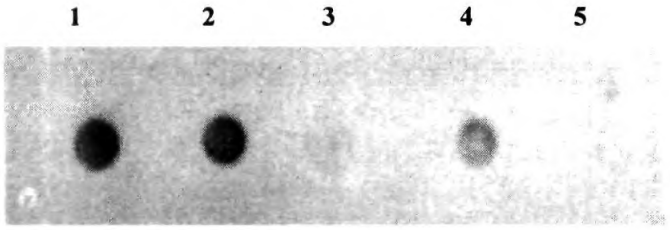
Figure 1.14b
Restriction digestion analysis of the selected MSVS6A & 7A clones



Lane 1. Lambda *Hind* III marker
Lane 2. pMSVS7A digested with *Bam*H I
Lane 3. pMSVS6A digested with *Bam*H I
Lane 4. pVS6.6 digested with *Bam*H I (+ control)

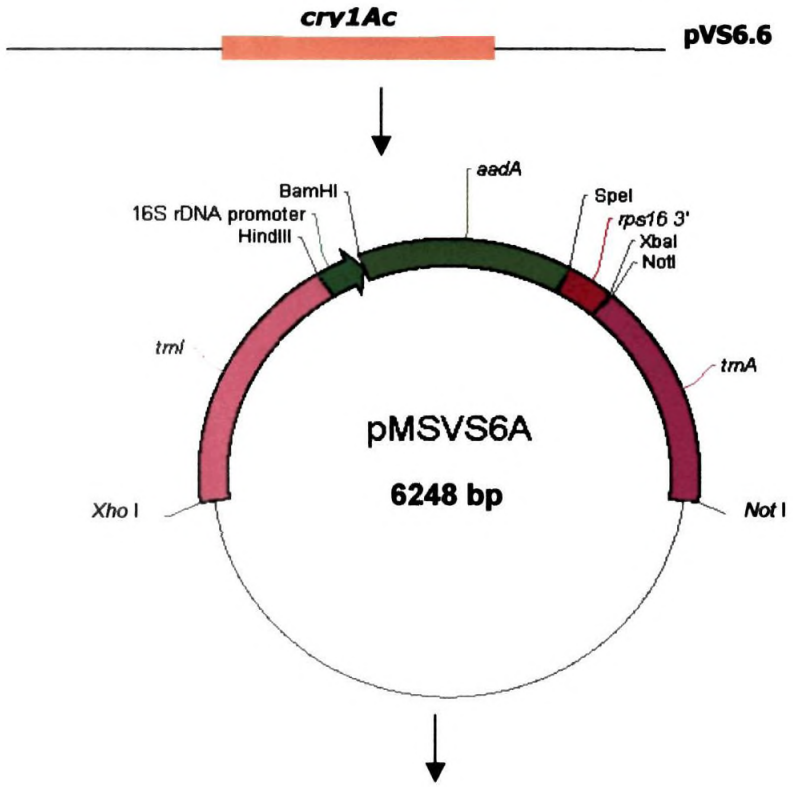
Figure 1.14c

Expression of Cry1Ac in *E. coli* (pMSVS7A)

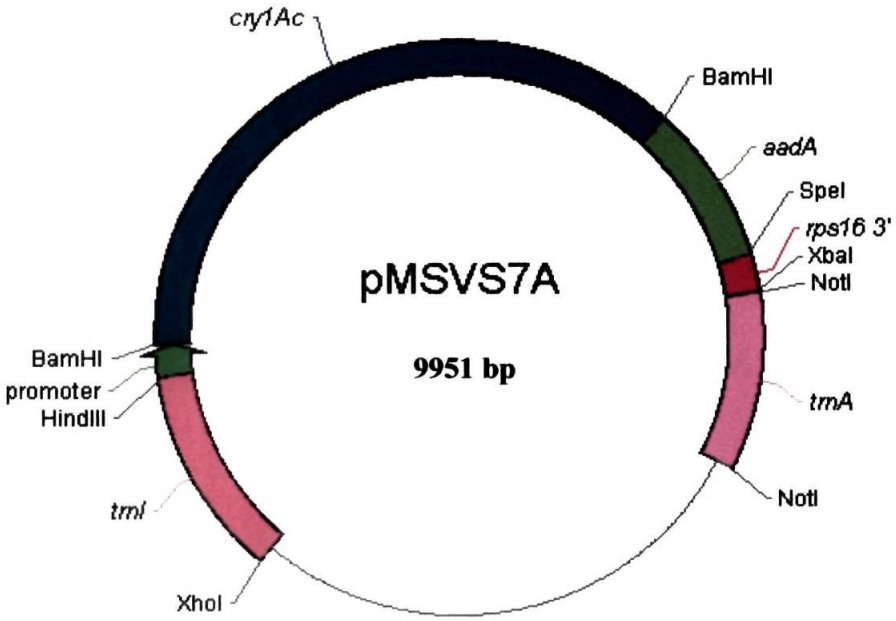


Dot 1 - *Bacillus thuringiensis* HD 73 cell extract.
Dot 2 - Purified Cry1Ac toxin
Dot 3 - pMSVS6A in *E.coli*
Dot 4 - pMSVS7A in *E.coli*
Dot 5 - pBluescript in *E.coli*

Figure 1.15
Schematic representation of the the construction of pMSVS7



The final chloroplast transformation vector containing *cry1Ac*



Findings from various research groups show that transforming chloroplast genome with heterologous genes is highly advantageous over the nuclear genome transformation technique. Even though chloroplast genome has been initially demonstrated to transform with prokaryotic genes, transforming plastome with gene for human somatotropin (hST) and with the eukaryotic gene for a biopolymer have clearly indicated that plastome can be transformed successfully with both eukaryotic and prokaryotic genes.

Over expression is another factor that makes it attractive. A recent report (DeCosa *et al.*, 2001) indicating a very high level of expression (~ 45 %) of *cry2Aa2* in tobacco, makes the yesteryear's wonder possible now. Apart from that, the maternal inheritance of chloroplast genome makes it a safe system of transformation these days. The escape of foreign genes to nonspecific plants or weedy relatives is the main concern of plant breeders to prevent creating super weeds while transforming crops with weedicide resistance gene. Even the *cry* genes were reported to be inherited by crops growing in the neighbouring plots of *B.t.* transformed crops. Such threat of ecological concern from genetically modified crops is on the rise and the need to develop techniques with a safer system of transformation encourages us to proceed with the chloroplast transformation which is reported to be highly advantageous while comparing the nuclear genome transformed plants.

Majority of the chloroplast transformation vectors (Table 6) target the heterologous gene between *trnV* and *rps12/7* loci. But only few of the vectors were designed to target the foreign gene in between the spacer region of *trnI* and *trnA* genes. This region is reported to be highly conserved and hence, vectors based on this target site are called 'universal chloroplast transformation vectors' (Daniell *et al.*,

1998). Therefore, we decided to design our vector to target in between these two genes so that the vector can be utilized to transform most of the higher plants. Computational analysis made by us on the *trnI* and *trnA* genes of the species available in the database also confirmed that these two genes are highly conserved and can be utilized in the vector to transform plants across the spectrum.

Chapter 2 .....

Transforming plastome of tobacco with the transformation vector

INTRODUCTION

Tobacco has been a very good model system for studies on transformation, regeneration and tissue culture. It has also been a suitable model system for chloroplast transformation since these attempts were initiated little more than a decade ago. Plastome of tobacco had been transformed with a variety of genes such as *aadA* gene, *cry1Ac*, hST, *gfp*, EPSPS gene, EPSPS-*gfp* chimeric gene, *cry2Aa2*, gene for biodegradable protein based polymer, *cry2Aa2* operon, *etc.* (Tab. 6).

As there was a need to develop insect resistant cotton plants of Indian varieties, we initiated an attempt to construct a chloroplast transformation vector which can transform cotton. To widen the use of this vector, it had been designed to transform a variety of higher plants apart from cotton. In order to achieve this, the expression cassette in the vector is flanked with *trnI* and *trnA* genes which will insert the foreign DNA (of interest) at specific sites (between *trnI* and *trnA* genes) of the plants being transformed. As the *trnI* and *trnA* genes are highly conserved among higher plants, this vector is supposed to transform a variety of plants. Though this vector can specifically transform cotton because of the presence of cotton *trnI* and *trnA* gene fragments as flanking sequences, we carried out the transformation attempts only in tobacco to confirm the transforming ability of this particular vector. The details of the transformation and the results are discussed in this chapter.

METHODOLOGY

1. Determination of antibiotic concentration

The leaf bits from tobacco plants raised aseptically was placed on RMOP medium containing various levels spectinomycin such as 0, 100 250, 500, 750, and 1000 µg per ml. The regeneration was assessed after two weeks.

2. Tissue culture techniques of tobacco

Regeneration: Tobacco explants of Wisconsin 35 (obtained from Dr. K. Veluthambi, Department of Plant Molecular Biology, School of Biotechnology, Madurai Kamaraj University, Madurai, India) were used for this study. The leaves were cut into small pieces (1 cm^2) and were subcultured on RMOP medium (for more details please refer Materials and Methods) containing MS salts, NAA (0.1 mg/L), BAP (1mg/L) along with thiamin (1mg/L), inositol (100 mg/L) and sucrose (30g/L) with a pH of 5.8.

Rooting: The regenerated shoots were shifted to rooting medium containing MS salts, thiamin HCl, Pyridoxin HCl, Myoinositol, sucrose, NAA (10 µM) and BAP (0.1 µM). Plants were kept for at least 4 weeks in rooting medium before transferring to individual flasks for single plant culture.

Particle bombardment

The leaves were cut into small pieces (0.5 cm^2) aseptically and arranged closely in a circular fashion in the petri plates containing MS medium for two days before bombardment. After two days they were particle bombarded with the vector DNA containing the foreign gene at a pressure of 1100 *psi*. The bombarded leaves were maintained in the same medium for two days and were later transferred to RMOP medium containing the selection pressure. The calli were transferred to fresh RMOP medium with spectinomycin every fortnight. Shoots regenerated from

transformed calli were transferred to rooting medium with spectinomycin every fortnight. Shoots regenerated from transformed calli were transferred to rooting medium with spectinomycin. The rooted plantlets were transferred to MS medium for further growth.

Confirmation of foreign DNA integration by PCR

Total DNA containing the plastome DNA also was isolated from the leaves of putative transformants as well as control plants using the 'Qiagen - Plant DNA Isolation Kit'. This DNA was used as template for performing PCR with various set of oligonucleotides. The primers used were as follows:

PrimerA: 5' GAACCCTGAACAGACTGCCG 3'

Primer B: 5' GGAATAGCGGTTGTAAGGG 3'

Primer C: 5' CGCGGATCCGTGAGGAGGATATATTTG 3'

Primer D: 5' GTTGTCTCTTGCCTGCCCATG 3'

The transformants were selected based on the amplification of products in the expected range.

Southern hybridization

The total DNA prepared from the leaves of control and putative transformants were restriction digested overnight with *Bam*H I restriction enzyme at 37°C. About 10 µg DNA per sample was used for digestion and 5 units enzyme per µg DNA was used for digestion. A small aliquot of restriction digested DNA was checked by agarose gel electrophoresis. Restriction digestion was repeated in samples, which were not digested properly after overnight incubation. Restriction digestion was repeated in DNA samples that were not fully digested by precipitating the DNA again. The properly digested DNA of control as well as the putative clones were loaded on

0.7 % agarose gel and run at slow speed (2 V/cm^2). The construct pVS6.6 containing the *cry1Ac* gene was restriction digested with *Bam*H I to release the 3.7 kb fragment and run along as positive control. The gel was visualized in UV transilluminator after staining with ethidium bromide and documented. The gel was later destained in distilled water and subsequently processed with subsequent washes such as depurination, denaturation and neutralization. The DNA from the gel was transferred on to nylon membrane by using a semidry transfer apparatus. After transfer, the DNA on membrane was cross-linked by baking at 80° C for an hour under vacuum. The membrane was later incubated with prehybridization buffer at 60° C.

The construct pVS6.6 containing the coding region of *cry1Ac* (3.7 kb) was digested with *Spe* I and *Xho* I enzymes to be released as ~1.5 kb fragment and was radiolabelled using ^{32}P to be used as probe in the Southern hybridization reaction. The probe was purified using Sephadex column and at least 10^9 cpm/μg was added for each hybridization experiment. The reaction was allowed for 16 hours at 65°C. Later, the membrane was washed with post hybridization buffer and dried between blotting papers. The membrane was immediately kept on a board and wrapped with cling film. Autoradiography was performed by exposing an X ray film to the membrane at –80° C overnight and the film was developed further as per the manufacturer's instructions.

Immunoassay

The leaf samples from control and the putative transformants were ground with extraction buffer (50mM Tris, pH 7.9, 1 mM PMSF, and 10 mM βME) in ice-cold conditions. The samples were centrifuged at 3000 rpm for 3 minutes at 4°C. The supernatant was removed carefully and precipitated with TCA (10% final

concentration) by incubating in ice for 30 min. After incubation the samples were centrifuged at 10000 g for 5 min. The pellet was resuspended in a minimal volume of 0.1 N NaOH. About 100 µg of total protein was loaded per lane of SDS-polyacrylamide gel. After running the gel, the protein was transferred to nitrocellulose membrane by semi-dry transfer apparatus followed by immunoblotting prescribed by Towbin *et al* (1979). The membrane was incubated in blocking buffer, primary antibody (raised against Cry1Ac protein and purified by CNBR-sepharose conjugated total tobacco protein) with a 1:10000 dilution, and anti IgG conjugated with HRP (Horse Radish Peroxidase). This HRP develops blue black colour in the presence DAB (3,3'-diaminobenzidine) as substrate and cobalt and nickel salts as transition elements.

Bioassay

The leaves from control and putative transformants were fed to at least five neonate larvae of *Helicoverpa armigera* in each petri plate and their mortality /survival rate was monitored every day. The percentage of mortality rate was worked out. *Helicoverpa armigera* eggs were collected from 'Project Directorate of Biological Control' Bangalore, India.

RESULTS AND DISCUSSION

Determination of antibiotic concentration

The leaf bits on antibiotic free RMOP medium (control) started regeneration after two weeks whereas the leaf bits cultured at and above the concentration of 100 µg /ml spectinomycin started yellowing and got completely bleached later (Fig. 2.1). Though this experiment confirmed that a minimum level of 100 µg/ml spectinomycin is enough for selection, a safe level of 500 µg/ml had been selected to be used as selection pressure to induce homoplasmicity in the transformants. In the previously published reports also, the spectinomycin concentration had been in the range of 500 µg/ml for inducing homoplasmicity after repeated subcultures (Svab and Maliga, 1993; Zoubenko *et al.*, 1994).

Transformation

The gold particle coated with chloroplast transformation vector (pMSVS7A) was particle bombarded on the leaves of tobacco at a pressure of 1100 *psi*. After bombardment, the leaf bits were maintained on RMOP medium for inducing regeneration in the transformed cells. The untransformed calli started losing colour and turned pale in the beginning. Later, they turned into dark brown colour during the subsequent subculture steps (Fig. 2.2). The transformed region of the calli maintained the green colour throughout and gave rise to the shoot formation after a period of around 20 weeks. The shoots were allowed to grow well and the leaves collected from the shoots were again cut into smaller pieces and cultured on RMOP medium under selection pressure for inducing regeneration of shoots further (Fig. 2.3). The shoots regenerated from these leaf bits were collected to proceed with confirmation

Fig. 2.1

Tobacco leaf bits cultured on different levels of Spectinomycin (from 100 µg per ml to 1000 µg per ml) for two weeks

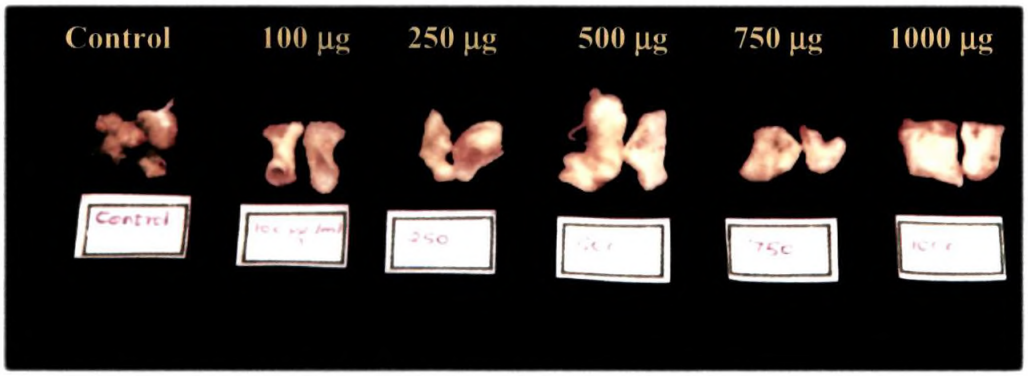


Fig. 2.2

Different steps involved in tissue culture of tobacco before and after bombardment

A – Tobacco leaf bits arranged in circular fashion for bombardment.
B - The leaf bits were cut into smaller pieces after bombardment.
C - The calli are grown in the subsequent subculture steps.
D – Shoot regeneration from the transformed calli.

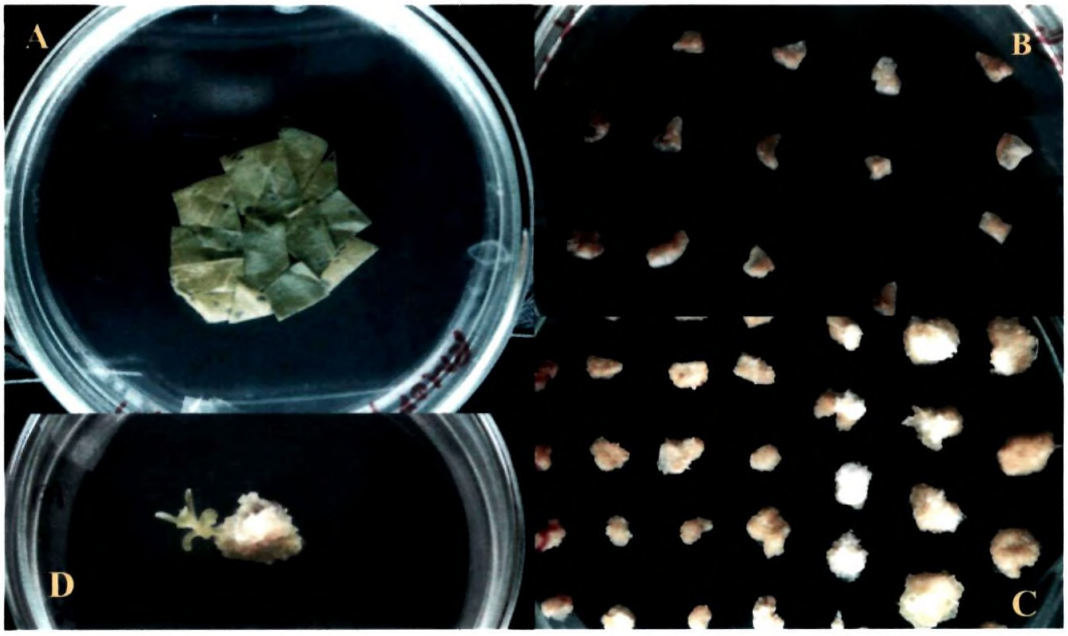
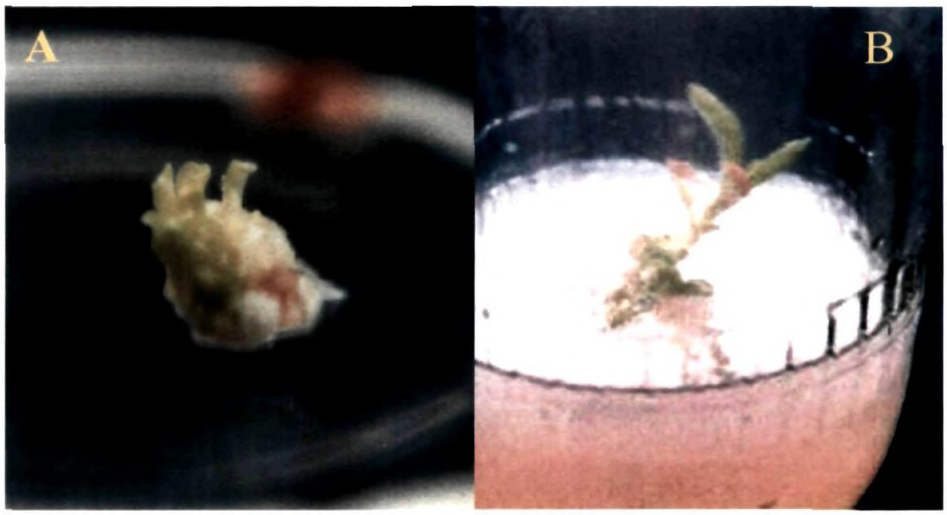


Fig. 2.3 Shoot regeneration and rooting to develop individual plants

A – Shoot regeneration from the transformed calli

B – Shoots transferred on to rooting medium



experiments such as Southern hybridization, PCR, immunoassay (western) and for insect bioassay using *Helicoverpa armigera.*

Southern hybridization

The result of the Southern hybridization is shown in Fig. 2.4. The radiolabelled *cry1Ac* (*Spe* I/*Xho* I fragment) hybridized at a position in lane four exactly matching with the 3.7 kb fragment of *cry1Ac* in lane six (*Bam*H I digested *cry1Ac* from pVS6.6) being run as positive control. The lanes having control (lane 1) and other putative transformants (lane 2 and 3) did not hybridize with the probe, confirming the non integration of the *cry1Ac* gene in them. The faint hybridization in lane 6 above the 3.7 kb band may be the undigested pVS6.6 plasmid. From this result, it is evident that the clone three (lane 4) is the real transformant having the *cry1Ac* gene incorporated. To confirm the integration of foreign gene in plastome further, PCR was performed with various primers.

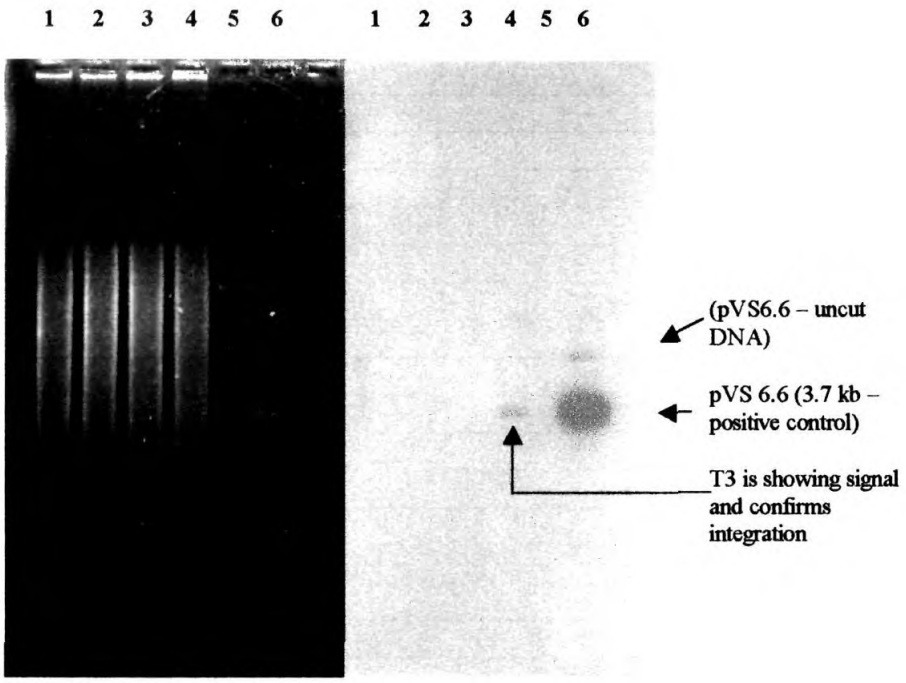
Confirmation of transformants by Polymerase Chain Reaction (PCR)

The results of the PCR carried out as one of the parameters of confirmation is given in Fig. 2.5. The primers are designed in such a way that they confirm the integration of the expression cassette at the specific sites. The primer A and primer B anneal in 16S rDNA region and *cry1Ac* region respectively amplifying a product with a size of ~2.5 kb in the transformed plants. The untransformed plants do not have a site for the primer B to anneal with and hence there would not be amplification. In a second PCR, primers C and D that can anneal at *aadA* gene and 23S rDNA, respectively amplifying a product of ~2.3 kb were used to identify transformed clones. As the untransformed plants do not have an *aadA* gene in it, there will not be any amplification. The third PCR set up was designed to get amplification in control

Figure 2.4

Southern Hybridization

*Bam*H I digested total DNA from control and the putative transformants were run on agarose gel and transferred on nylon membrane. They were allowed to hybridize with radiolabelled *cry1Ac* probe. The autoradiography result shows the presence of *cry1Ac* gene in clone 3 (Lane 4).

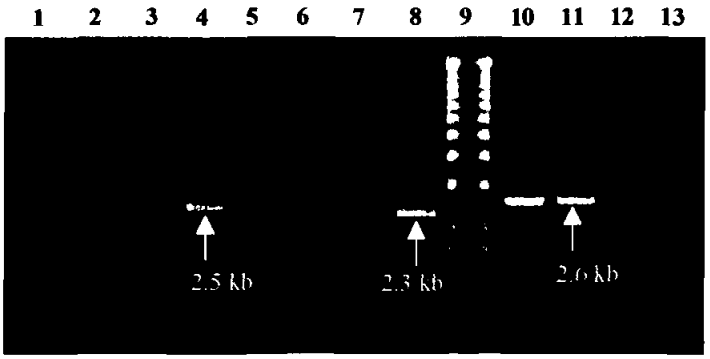


Lane 1 - Control
Lane 2 – Putative clone 1
Lane 3 – Putative clone 2
Lane 4 – Putative clone 3
Lane 5 – 1 kb DNA ladder
Lane 6 – pVS6.6 – *Bam*H I digested

Figure 2.5

Confirmation of transformation by PCR

PCR was performed using the DNA from control and putative plants as template. The results shows that only clone 3 is the transformant. Primer A and B amplifies around 2.5 kb fragment in the transformant Primer C and D amplifies around 2.3 kb fragment in the transformant Primer A and D amplifies around 2.6 kb fragment in the control plant and untransformed plant



Lane – 1, 5 and 10 – control
Lane – 2, 6 and 11 – clone 1
Lane – 3, 7 and 12 – clone 2
Lane – 4, 8 and 13 – clone 3
Lane – 9 - 1 kb DNA ladder

Lane 1 to 4 – Primer Aand B
Lane 5 to 8 – Primer C and D
Lane 10 to 13 – Primer A and D

plants and untransformed plants only in which primer A and primer D anneal with 16S rDNA and 23S rDNA respectively to amplify a product of ~2.6 kb size.

The results indicate that the expected size of amplicon was found only in clone 3. The first set of primers (A and B) show an amplification of ~2.5 kb product in lane 4 confirming the insertion of *cry* gene next to the 16S rDNA. The control in lane 1 and other putative transformants in lane 2 and 3 did not show amplification proving the non-availability of the *cry1Ac* gene. In the second set of primers used (C and D), the expected size of ~2.3 kb amplicon was obtained only in clone 3 (lane 8) whereas the other clones did not amplify as missing in control. The third set of primers (A and D) amplified only in control and untransformed plants (lane 10, 11 and 12 respectively) in the expected range of ~2.6 kb. The lane 13 (clone 3) did not show amplification confirming the presence of foreign DNA.

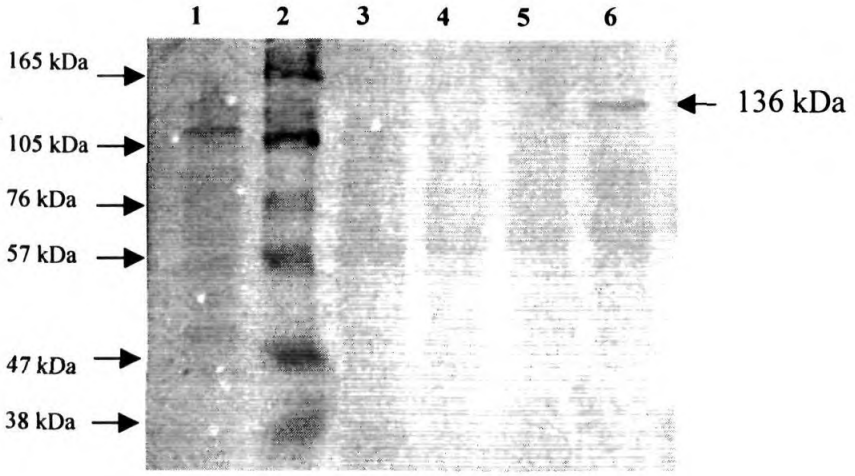
Immunoassay

Protein samples prepared from the leaves of control as well as the putative transformants were run on SDS polyacrylamide gels and later transferred on nitrocellulose membrane by semi-dry blotting method. The membrane containing the protein was processed as mentioned in the methodology and allowed to hybridize with antibodies which were raised against the Cry1Ac protein and immunoprecipitated against total protein by (CNBR activated sepharose) affinity purification. The lane 1 in Fig.2.6 has been loaded with around 750 ng of solubilized Cry1Ac protein, which is the positive control. Lane 2 is run with pre-stained protein marker (from New England Biolabs Inc.). Lane 3 is loaded with protein samples from control and lanes 4 to 7 contain protein samples from the putative transformants. The results indicate

Figure 2.6

Immunoasssay

Protein samples from control and putative transformants were run on SDS-polyacrylamide gel and transferred to nitrocellulose membrane. When they were allowed to cross react with the purified Cry1Ac antiserum only the clone 3 (Lane 6) got lighted up confirming the expression of Cry1Ac protein.



Lane 1 – *B.t. Kurstaki*, HD73 – positive control
Lane 2 – prestained marker (New England Biolabs)
Lane 3 – control plant
Lane 4 – putative clone 1
Lane 5 – putative clone 2
Lane 6 – putative clone 3

that only the lane 7 loaded with protein sample from clone three got lighted up at the position exactly equivalent to the band in the positive control (lane 1).

This result confirms the previous results of Southern and PCR. So, among the three clones tested, only the clone 3 has displayed the integration of *cry1Ac* and its expression. The level of expression in this clone when compared to the positive control in lane 1 appears to be around 500 ng which works out to be around 0.5% of the total protein. The expression level seems to be low when compared to the previous reports (3 to 5 % McBride *et al.*, 1995 and Daniell *et al.*, 1998; 7% by Staub *et al.*, 2000; 2 to 3% by Kota *et al.*,1999; and a highest of 45.3% by De Cosa *et al.*, 2001). Details are given in Tab. 5. The reason for low level of expression in our case is not known as on now. It is assumed that the copy number of plastome may be less compared to the previous reports because of the high stringency condition maintained throughout the subculture steps. Probably by obtaining more number of transformants, we may be able to select clones with higher level of expression. Alternately, it would give us some information on the levels of expression in various transformants. If it varies between clones, then we may be able to select clone with higher level of expression for our future use.

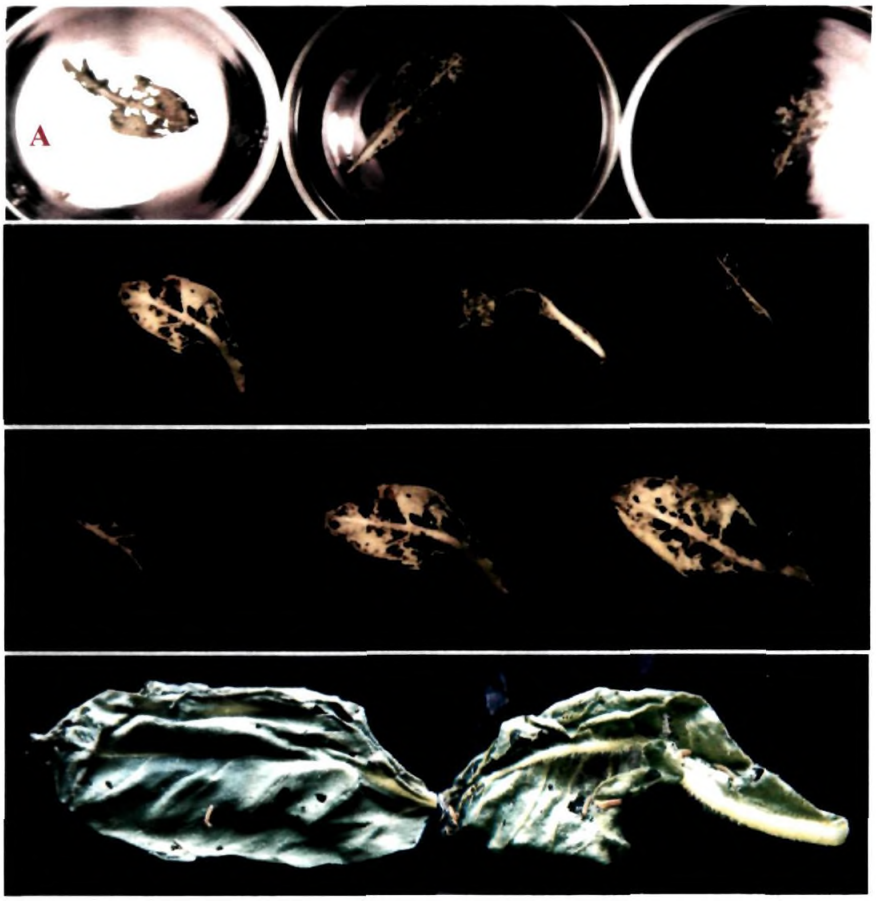
Bioassay

The neonate larvae of *Helicoverpa armigera* were allowed to feed on the excised leaf from the putative transformants and control plants to estimate the rate of mortality. At least five larvae were allowed to feed on the leaf kept in a petri plate and were monitored every day. The larvae feeding on leaves from control as well as putative transformants except clone T3 did not show any mortality. The neonate larvae feeding the leaf from clone 3 showed a kind of slow down in its activity after 3

days and at the end of 5 days all were dead. A minimum of 3 replications were maintained for each treatment. The leaf of control and the untransformed putative clones were mostly eaten by the larvae (Fig. 2.7). The larvae were growing healthy. This result further confirms the insecticidal property of the clone 3 due to the integration and expression of foreign gene. Similarly, Kota *et al.*, (1999) and McBride *et al.*, (1995) also reported 100% mortality in all neonates feeding the transformed leaves. The results of McBride *et al.*, (1995) demonstrated that the leaves were toxic to both *H. virescens* and *H. zea* causing 100% mortality where as the rate was closer to 90% in *S. exigua*. In this study, though the expression level is not found in the expected range i.e. above 2 to 3%, the obtained level of expression of Cry1Ac protein had been lethal enough to cause 100% mortality in the neonates.

It has been found that expression level vary in different transformants and it is important to screen the various transformants and select one with highest level of expression for further multiplication. In order to obtain quite a good number of transformants, more number of bombarding can be attempted. Again, the rate of transformation per bombardment is very low in this attempt. Out of ten bombardment events, we could obtain only seven putative transformants among which four got contaminated and could not be saved. Among the three putative transformants we were left with, only one had the gene incorporated. The rate of plastid transformation in land plants is found to be very difficult and less frequent (Svab and Maliga, 1993). The low rate of transformation in this attempt is quite acceptable because there appears to be a similarity with the previous reports. One bombardment in nuclear genome transformation experiments yield 2 to 20 transformants where as one plastid transformant is recovered in about 100 bombarded samples (Svab *et al.*, 1990; Staub and Maliga, 1992). Svab and Maliga (1993) could get 84 spectinomycin resistant

Figure 2.7
Bioassay of leaf from putative transformants with *Helicoverpa armigera* larvae



A – Leaves from control plants
B - Leaves from putative transformant (T1)
C – Leaves from putative transformant (T2)
D – Leaves from putative transformant (T3)

clones from 79 bombarded leaf cultures among which 40 out of 50 clones had the *aadA* gene integrated. The remaining 10 were spontaneous mutants. The transformation rate was comparable in the report by Daniell *et al.,* (1990) in which out of 16 bombarded leaves, 10 independently transformed shoots were identified. Out of ten shoots, six plants were transformants and four were mutants. Whereas all the transgenic lines transformed with the universal transformation vector examined by them showed integration of the *aadA* gene. The rate of transformation was still lesser in attempts made by McBride *et al.,* (1995) in which they got 8 independently transformed lines from 28 bombarded leaves.

In this attempt, though we could get seven transformants from 10 bombardments, we could save only three clones resistant to the selective pressure. Out of these only one happened to be the real transformant. Although the rate of transformation is quite low, we could confirm from these results that the chloroplast transformation vector developed by us can transform tobacco also. This also proves that the vector could be used in a system other than cotton. The cotton genes used as flanking sequences in this vector have transformed plastome of tobacco confirming the ability of the vector to transform plants other than cotton as expected. More number of bombardments are needed to get considerably good number of transformants which can give us a choice of plants with varying levels of expression. Transformants with higher level of expression can be selected for further use. The vector can also be used to transform a variety of species to check its ability to transform various plant species.

Chapter 3 ……

Comparative analysis of the plastomic *trnI* and *trnA* gene sequences

INTRODUCTION

The chloroplast genome ('plastome') of land plants is highly conserved with respect to size, structure gene content and linear order of genes among major lineages of land plants (Palmer 1985b, 1991). In spite of this feature, variations in both gene content and sequence organization, including the loss of one copy of Inverted Repeat (IR) region by various additions or deletions in non-coding DNA have also been found and their contribution to the phylogenetic difference among various plants have been studied to some extent. The *trnI* and *trnA* genes situated in the IR region between 16S and 23S rDNA genes of the 16S rDNA operon are known to be highly conserved among higher plants. The introns of these genes which belong to subgroup II A show little variation among the genes analyzed (rice, tobacco and liverwort) with a homology of more than 80 % (Shimada and Sugiura 1991).

The conserved nature of the chloroplast genes has been the subject of phylogenetic analysis with an aim to find out the variations during the course of evolution (Downie and Palmer 1992). The availability of complete chloroplast genome sequences of several species (*Nicotiana tabacum*, Shinozaki *et al.* 1986; *Marchantia polymorpha*, Ohyama *et al.* 1995; *Oryza sativa*, Hiratsuka *et al.* 1989; *Zea mays*, Maier *et al.* 1995; *Oenothera*, Hupfer *et al.* 2000; *Spinach*, Schmitz-Linneweber *et al.* 2001) as well as the reported partial sequences of various species have facilitated the genetic analysis. Johnson and Hattori (1996) have reported the presence of hot spot(s) that led to deletions and additions within the intron of *trnI* and *trnA* genes from 36 plants representing a wide range of plant families. The *trn* genes are used as the site of integration of foreign genes because of their highly conserved nature. The chloroplast transformation vectors (Daniell *et al.* 1998) which

contain these genes as flanking regions are called universal chloroplast transformation vectors as they can transform most land plants by inserting the foreign gene between the *trnI* and *trnA* genes by homologous recombination.

As the chloroplast transformation of cotton is a long-term objective of our team, we had PCR amplified and sequenced the *trnI* and *trnA* genes of cotton . The sequence similarity of the *trnI* and *trnA* genes among land plants (including cotton) and the major changes and gene conservations that have occurred during the course of evolution is discussed in this chapter.

MATERIALS AND METHODS

I PCR amplification of *trnI* and *trnA*

Polymerase Chain Reaction (PCR)

About 100 ng of chloroplast DNA was used per reaction mix containing 0.2 μM of forward and reverse primers, 0.2 mM each of the four deoxynucleotides and 2.5 units of *Taq* DNA Polymerase (from GibcoBRL) in a total volume of 50μl. PCR was performed for 30 cycles with denaturation of template DNA at 94°C for 1 min, annealing templates and oligonucleotide primers at 55°C for 2 min, primer extension at 72°C and finally extension of PCR products at 74°C for 10 minutes. The PCR products were separated and analyzed on a 0.7% agarose gel.

Primers for *trnI*

Forward primer: 5' ACACCGCTCGAGGGGTTTCTCTCGCTTTTG 3'

Reverse primer:

5' ACACCCAAGCTTGGCCNNNNNGGCCGCTTCTTCTATTCTTTTCCCTG 3'

The resultant amplicon is mutagenized to have a *Xho* I restriction site at the 5' region and a *Hind* III site at the 3' region by the forward and reverse oligonucleotides.

Primers for *trnA*:

Forward primer:

5' AAATATGCGGCCGCGGGAAAAGAATAGAAGAAGC 3'

Reverse primer:

5' GCGAGCGAGCTCTTCACGAGTTGGAGATAAG 3'

The forward and reverse primers inserts *Not* I and *Sac* I restriction sites at the 5' and 3' ends respectively for future cloning purposes. The *trnA*, *trnI* genes and P*rrn* were PCR amplified using the primers designed based on the gene sequences of tobacco plastome.

Ligation

Ligation of digested DNA was carried out at 16°C for 16 hr. T_4 DNA Ligase was used along with the buffer supplied by the manufacturer. Usually a vector to insert ratio of 1:5 was used for ligation of cohesive ends. High concentration T4 DNA Ligase (10 U per μl) was used in the case of blunt end ligation. Blunt end ligation was carried out at 16° C.

Cloning into pGEM-T vector

The PCR amplified *trnI* and *trnA* amplicons were cloned into pGEM-T vector (following the manufacturer's protocol) by incubating the ligation mixture at 16° C for 16 hours. The ligated DNA had been used to transform *Escherichia coli* (*E. coli*) cells (JM109). The transformed colonies were picked up from LB agar plates containing 50 μg per ml after incubating at 37°C overnight. The transformed colonies were selected by slot lysis method and the plasmid DNA samples prepared from these colonies were isolated by culturing them in LB medium overnight. The subsequent DNA isolation was done by alkaline lysis method. The vector details are given in Fig. 1.16.

Sequencing of the pGEM-T cloned fragments

The DNA of transformed clones were sequenced by Microsynth, Switzerland and at Indian Institute of Science, Bangalore.

Southern

The pGEM-T cloned cotton *trnI* and *trnA* genes were run on agarose gels, and transferred on to nylon membrane. The *trnI* and *trnA* genes amplified from the positive control from tobacco (pTB9 -obtained from Dr. Sugiura, Japan) were radiolabelled using ^{32}P and were used as probes. They were allowed to hybridize with the *trnI* and *trnA* genes of cotton transferred on to the membrane at higher stringency conditions (60°C).

Sequence analysis

Multiple alignment of gene sequences were performed by using the programme CLUSTLAW of GCG software.

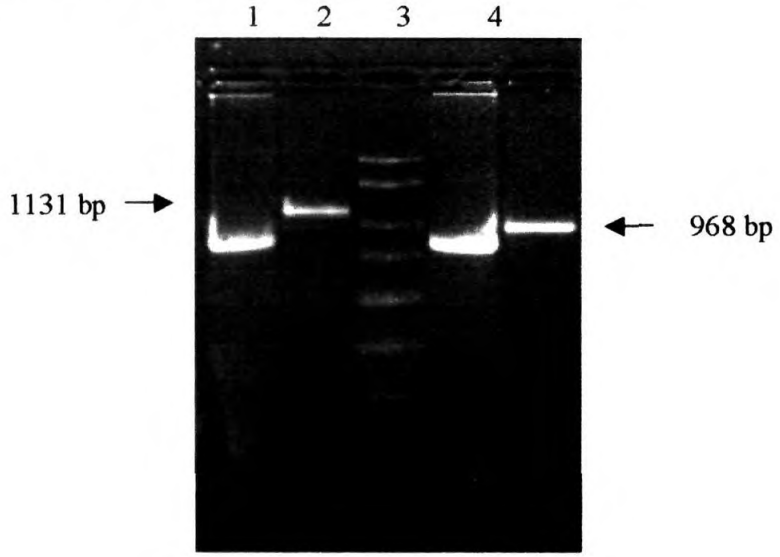
RESULTS AND DISCUSSION

PCR amplification of *trnI*, *trnA* genes

PCR amplicons of *trnI* and *trnA* genes were run on agarose gel along with the amplicons of tobacco as positive control (Fig. 3.1). Later, they were cloned into pGEM-T vector and the transformed clones were selected based on slot-lysis method. The transformed clones containing these amplicons were confirmed by restriction digestion analysis with *Not* I enzyme (Fig. 1.2). To confirm the authenticity of the amplicons of *trnI* and *trnA* from cotton, a Southern experiment was performed with the radiolabelled *trnI* and *trnA* genes of tobacco as probes (Fig. 3.2). The heterologous probes hybridized with the cotton amplicons thus confirming the authenticity of the amplicons. The clones were sequenced and the sequences were deposited in the GenBank database. Their accession numbers are as follows: *trnA* - AF 175761 and *trnI* - AF189149, The nucleotide sequences of these genes are given in Fig. 3.3.

The cotton *trnI and trnA* sequences were subjected to sequence comparison with the similar DNA sequences of tobacco (Figure 3.4 and 3.5). A major deletion of about 234 bp in *trnI* and about 98 bp in *trnA* of tobacco was found. These deletions in the *trnI* and *trnA* introns map to the loop region of domain III (ii) of the standard group II (subgroup IIA1) intron structure (Michel *et al.* 1989). These deletions which occur principally in non-coding intergenic spacer regions and introns lead to minor rearrangements that can result in a different phylogeny. The analysis was extended to the rest of the sequences available in the database to find out the presence of conserved sequences flanking the deletion areas. The deletions are known to be occurring in these hotspot regions, which are more vulnerable.

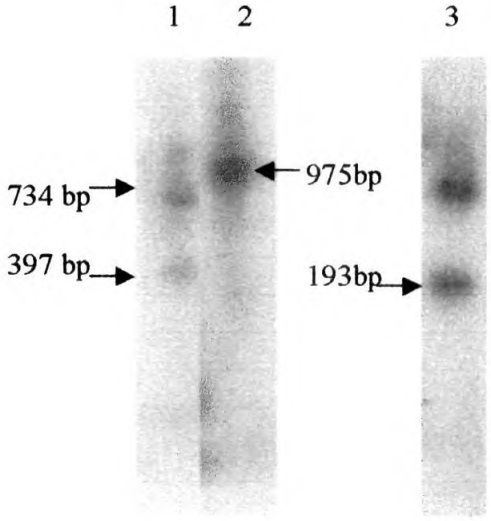
Fig. 3.1 PCR amplicon of *trnI* and *trnA* from cotton



Lane 1 – PCR amplicon of *trnI* (tobacco)
Lane 2 – PCR amplicon of *trnI* (cotton)
Lane 3 - PCR marker
Lane 4 - PCR amplicon of *trnA* (tobacco)
Lane 5 – PCR amplicon of *trnA* (cotton)

Figure 3.2

Southern hybridization result of 16S rDNA, *trnI* and *trnA* amplified from cotton



Lane 1 – *Xho* I/*Hin*d III digested *trnI* (pGEM-T clone)

Lane 2 – *Not* I digested *trnA* (pGEM-T clone)

Lane 3 – *Not* I digested 16SrDNA (pGEM-T clone)

Figure 3.3

DNA sequence of *trnI* from cotton plastome

```
   1  GGGCTATTAG CTCAGTGGTA GAGCGCGCCC CTCATAATTG CGTCGTTGTG
  51  CCTGGGCTGT GAGGGCTCTC AGCCACATGG ATAGTTCAAT GTGCTCATCA
 101  GCGCCTGACC CTGAGATGTG GATCATCCAA GGCACATTAG CATGGCGTAC
 151  TTCTCCTGTT CGAACCGGGG TTTGAAACCA AACTTCTCCT CAGGAGGATA
 201  GATGGGGCGA TTCAGGTGAG ATCCAATGTA GATCCAACTT TCTATTCACT
 251  CGTGGGATCT GGGCGGTCCG GGGGGGACAC CACGGCTCCT CTCTTCTCGA
 301  TAATCCATAC ATCCCTTATC AGTGTATGGA CAGCTATCTC TCGAGCACAA
 351  GTTTAGGTTC GGCCTCAATG GGAAAATAAA ATGGAGCACC TAACAACGTA
 401  TCTTCACAGA TCAAGAACTA CGAGATCGCC CCTTTCATCC TGGGGTGACG
 451  GAGGGATCGT ACCATTCGAG CCTTTTTTTT TCATGCTTTT CCCGGAGGTC
 501  CGGAGAAAGC TGCAATCAAT AGGATTTTCC TAATcCTCCC TTCCCGAAAA
 551  GGAAGAACGT GAAATTCTTT TTCCTTTCCG CAGGGACCAG GAGATTGGAT
 601  CTAGCCGTAA GAAGAATGCT TGGCTGATAA ATAATTCACT TCTTGGTCTT
 651  CGACCCCCTC AGTCACTACG AACGCCCCCG ATCAGTGCAA TGGGATGTGT
 701  CTATTTATCT ATCTCTTGAC TCGAAATGGG AGCAGGTTTG AAAAAGGATC
 751  TTAGAGTGTT TAGGGTTGGG CCAAGAGGGT CTCTTAACGC CCTCTTTTTT
 801  TTTCTTCCCA TCGGAGTTAT TTCTTATTTC ACAAATACTT GCCATGGTAA
 851  GGAAGAAGGG GGGAACAAGC ACACTCGGAG AGCGCAGTAC AACGGAGAGT
 901  TGTATGCTGC GTTCGGGAAG GATGAATCGC TCCCGAAAAG GAATCTATTG
 951  ATTCTCTCCC AATTGGTTGG ACCGTAGGTG CGATGATTTA CTTCACGGGC
1001  GAGGTCTCTG GTTCAAGTCC AGGATGGCCC A
```

DNA sequence of *trnA* from cotton plastome

```
   1  GGGGATATAG CTCAGTTGGT AGAGCTCCGC TCTTGCAATT GGGTCGTTGC
  51  GATTACGGGT TGGGTGTCTA ATTGTCCAGG CGGTAATGAT AGTATCTTGT
 101  ACCTGAACCG GTGGCTCACT TTTTCTAAGT AATGGGGAAG AGGACCGAAA
 151  CATGCCACTG AAAGACTCTA CTGAGACAAA GATGGGCTGT CAAGAACGTA
 201  GAAGAGGTAG GATGGGCGGT TGGTCAGATC TAGTATGGAT CGTACATGGA
 251  CGGTAGTTGG AGTCGGCGGC TCCCCTAGGT TCCCCCATCT GGGATCCCTG
 301  GGGAAGAGGA TCAAGTTGGC CCTTGCGAAC AGCTTGATGC ACTATCTCCC
 351  TTCAACCCTT TGAGCGAAAT GCGGCAAAAG GAAGGAAAAT CCATGGACCA
 401  ACCCCATCGT CTCCACCCCG TAGGAACTAC GAGATCACCC CAAGGACGCC
 451  TTCGGTATCC AGGGGTCGCG GACCGACCAT AGAACCCTGT TCAATAAGTG
 501  GAATGCATTA GCTGTCCGCT CTCAGGTTGG GCAGTAAGGG TCGGAGAAGG
 551  GCAATCACTC ATTCTTAAAA CCAGCATTCT TAAGACCAAA GAGTCGGAGG
 601  GGGGAAAGCT CTCCGCCGTT CCTGGTTTTC CTGTAGCAGG ATCCTCCGGA
 651  ACCACAAGAA TCCTTAGTTA GAATGGGATT CCAACTCAGC ACCTTTTGAG
 701  ATTTTGAGAA GAGTTGCTCT TTGGAGAGCA CAGTACGATG AAAGTTGTAA
 751  GCTGTGTTCG GGGGGGAGTT ATTGTCTATC GTTGGCCTCT ATGGTAGAAT
 801  CAGTCGGGGG CCTGAGAGGC GGTGGTTTAC CCTGTGGCGG ATGTCAGCGG
 851  TTCGAGTCCG CTTATCTCCA
```

Figure 3.4.

Comparison of *trnI* intron sequences of cotton and tobacco.

The intron sequences of *trnI* of cotton and tobacco were aligned by using gel assembly program of GCG. The tobacco sequence was obtained from Genbank (Z00044). A similarity (and identity) of 97.3% was observed between the plant species. Identical bases are shown by the symbol | and · indicates gaps. A major deletion of about 234 bp in tobacco corresponding to the 444-676 bp position of cotton was found.

```
                .         .         .         .         .

Cotton  1 TTGCGTCGTTGTGCCTGGGCTGTGAGGGCTCTCAGCCACATGGATAGTTC 50
          ||||||||||||||||||||||||||||||||||||||||||||||||||
Tobac   1 TTGCGTCGTTGTGCCTGGGCTGTGAGGGCTCTCAGCCACATGGATAGTTC 50
                .         .         .         .         .
       51 AATGTGCTCATCAGCGCCTGACCCTGAGATGTGGATCATCCAAGGCACAT 100
          |||||||||||| ||||||||||||||||||||||||||||||||||||
       51 AATGTGCTCATCGGCGCCTGACCCTGAGATGTGGATCATCCAAGGCACAT 100
                .         .         .         .         .
      101 TAGCATGGCGTACTTCTCCTGTTCGAACCGGGGTTTGAAACCAAACTTCT 150
          |||||||||||||| |||||||||||||||||||||||||||||| ||
      101 TAGCATGGCGTACTCCTCCTGTTCGAACCGGGGTTTGAAACCAAACTCCT 150
                .         .         .         .         .
      151 CCTCAGGAGGATAGATGGGGCGATTCAGGTGAGATCCAATGTAGATCCAA 200
          |||||||||||||||||||||||| ||||||||||||||||||||||||
      151 CCTCAGGAGGATAGATGGGGCGATTCGGGTGAGATCCAATGTAGATCCAA 200
                .         .         .         .         .
      201 CTTTCTATTCACTCGTGGGATCTGGGCGGTCCGGGGGGGA.CACCACGGC 249
          ||||| ||||||||||||||| ||||||||||||||| ||||||||
      201 CTTTCGATTCACTCGTGGGATCCGGGCGGTCCGGGGGGGGACCACCACGGC 250
                .         .         .         .         .
      250 TCCTCTCTTCTCGATAATCCATACATCCCTTATCAGTGTATGGACAGCTA 299
          |||||||||||||| ||||||||||||||||||||||||||||||||||
      251 TCCTCTCTTCTCGAGAATCCATACATCCCTTATCAGTGTATGGACAGCTA 300
                .         .         .         .         .
      300 TCTCTCGAGCACAAGTTTAGGTTCGGCCTCAATGGGAAAATAAAATGGAG 349
          |||||||||||| ||||||         ||||||||||||||||||||
      301 TCTCTCGAGCACAGGTTTAG.........CAATGGGAAAATAAAATGGAG 341
                .         .         .         .         .
      350 CACCTAACAACGTATCTTCACAGATCAAGAACTACGAGATCGCCCCTTTC 399
          |||||||||||| |||||||||| ||||||||||||||||||||||||
      342 CACCTAACAACGCATCTTCACAGACCAAGAACTACGAGATCGCCCCTTTC 391
                .         .         .         .         .
      400 ATCCTGGGGTGACGGAGGGATCGTACCATTCGAGCCTTTTTTTTTCATGC 449
          || ||||||||||||||||||||||||||||||||| ||||||
      392 ATTCTGGGGTGACGGAGGGATCGTACCATTCGAGCCGTTTTTT..... 434
                                  .
                                  .
                                  .
                .         .         .         .         .


      650 GCAATGGGATGTGTCTATTTATCTATCTCTTGACTCGAAATGGGAGCAGG 699
                                  ||||||||||||||||||||||||
      435 ........................TCTTGACTCGAAATGGGAGCAGG 457
                .         .         .         .         .
```

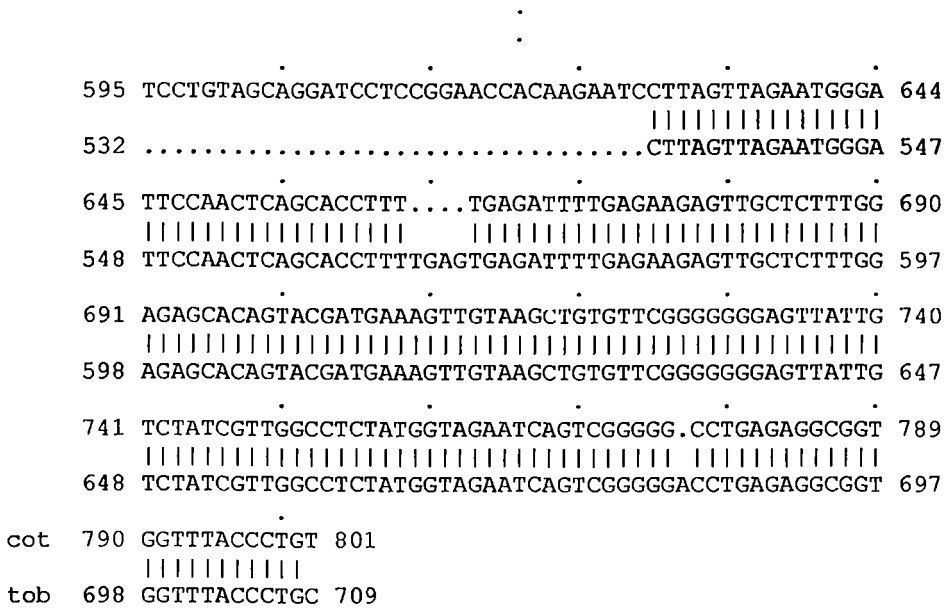
```
      700 TTTGAAAAAGGATCTTAGAGTGTTTAGGGTTGGGCCAAGAGGGTCTCTTA 749
          |||||||||||||||||||||||| |||||||||||| ||||||||||||
      458 TTTGAAAAAGGATCTTAGAGTGTCTAGGGTTGGGCCAGGAGGGTCTCTTA 507
                    .         .         .         .         .
      750 ACGCCCTCTTTTTTTTTCTTCCCATCGGAGTTATTTCTTATTTCACAAAT 799
          ||||   ||| ||||||||||| ||||||||       ||||||||||||
      508 ACGC...CTTCTTTTTTCTTCTCATCGGAG.......TTATTTCACAAAG 547
                    .         .         .         .         .
      800 ACTTGCCATGGTAAGGAAGAAGGGGGGAACAAGCACACTCGGAGAGCGCA 849
          |||||||| |||||||||||||||||||||||||||| ||||||||||
      548 ACTTGCCAGGGTAAGGAAGAAGGGGGGAACAAGCACACTTGGAGAGCGCA 597
                    .         .         .         .         .

      850 GTACAACGGAGAGTTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGA 899
          |||||||||||||||||||||||||||||||||||||||||||||||||||
      598 GTACAACGGAGAGTTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGA 647
                    .         .         .         .         .

      900 AAAGGAATCTATTGATTCTCTCCCAATTGGTTGGACCGTAGGTGCGATGA 949
          |||||||||||||||||||||||||||||||||||||||||||||||||||
      648 AAAGGAATCTATTGATTCTCTCCCAATTGGTTGGACCGTAGGTGCGATGA 697
                    .

Cot   950 TTTACTTCAC 959
          ||||||||||
Tob   698 TTTACTTCAC 707
```

Figure 3.5.

Comparison of *trnA* introns sequences of cotton and tobacco

Alignment of *trnA* intron sequences of cotton and tobacco by using gel assembly programme. The sequence of tobacco was obtained from Genbank (Z000444). It shows 96.99 % similarity and 96.4 % identity. Identical bases are shown by the symbol | and the · indicates gaps. A major deletion of about 98 bp in tobacco corresponding to the 531-629 bp position of cotton was found.

```
Cotton   1 TTGGGTCGTTGCGATTACGGGTTGGGTGTCTAATTGTCCAGGCGGTAATG  50
           ||||||||||||||||||||||||| |||||||||||||||||||||||
tobac    1 TTGGGTCGTTGCGATTACGGGTTGGATGTCTAATTGTCCAGGCGGTAATG  50

          51 ATAGTATCTTGTACCTGAACCGGTGGCTCACTTTTTCTAAGTGATGGGGA 100
             |||||||||||||||||||||||||||||||||||||||| |||||||
          51 ATAGTATCTTGTACCTGAACCGGTGGCTCACTTTTTCTAAGTAATGGGGA 100

         101 AGAGGACCGAAACATGCCACTGAAAGACTCTACTGAGACAAAGATGGGCT 150
             ||||||||||||| ||||||||||||||||||||||||||||||||||||
         101 AGAGGACCGAAACGTGCCACTGAAAGACTCTACTGAGACAAAGATGGGCT 150

         151 GTCAAGAACGTAGAAAAAGTAGGATGGGCGGTTGGTCACGATCTAGTATG 200
             |||||||||||||| | .| |||||||||| |||||||| ||||||||||
         151 GTCAAGAACGTAGAGGAGGTAGGATGGGCAGTTGGTCA.GATCTAGTATG 199

         201 GATCGTACATGGACGGTAGTTGGAGTCGGCGGCTC.CCCTAGGTTCCCCC 249
             |||||||||||||||||||||||||||||||||| |||  ||||||| |
         200 GATCGTACATGGACGGTAGTTGGAGTCGGCGGCTCTCCCAGGGTTCCCTC 249

         250 ATCTGGGATCCCTGGGGAAGAGGATCAAAGTTGGCCCTTGCGAACAAGCT 299
             |||||  ||||  ||||||||||||||| |||||||||||||||||| ||||
         250 ATCTGAGATCTCTGGGGAAGAGGATC.AAGTTGGCCCTTGCGAAC.AGCT 297

         300 TGATGCACTATCTCCCTTCAACCCCTTTGAGCGAAATGCGGC.....AAA 344
             |||||||||||||||||||| | ||||||||||||||||||     |||
         298 TGATGCACTATCTCCCTTCAA.CCCTTTGAGCGAAATGCGGCAAAAGAAA 346

         345 AGGAAGGAAAATCCATGGACCAACCCCATCGTCTCCACCCCGTAGGAACT 394
             |||||||| ||||||||||||| |||||||| ||||||||||||||||||
         347 AGGAAGGAAAATCCATGGACCGACCCCATCATCTCCACCCCGTAGGAACT 396

         395 ACGAGATCACCCCAAGGACGCCTTCGGTATCCAGGGGTCGCGGACCGACC 444
             |||||||||||||||||||||||||| | |||||||||| ||||||||||
         397 ACGAGATCACCCCAAGGACGCCTTCGGCATCCAGGGGTCACGGACCGACC 446

         445 ATAGAACCCTGTTCAATAAGTGGAATGCATTAGCTGTCCGCTCTCAGGTT 494
             |||||||||||||||||||||||| ||||| ||||||||||||||||||
         447 ATAGAACCCTGTTCAATAAGTGGAACGCATTAGCTGTCCGCTCTCAGGTT 496

         495 GGGCAGTAAGGGTCGGAGAAGGGCAATCACTCATTCTTAAAACCAGCATT 544
             |||||||  |||||||||||||||||||  |||||||
         497 GGGCAGTCAGGGTCGGAGAAGGGCAATGACTCATT.............. 531
```


595 TCCTGTAGCAGGATCCTCCGGAACCACAAGAATCCTTAGTTAGAATGGGA 644
532 .....CTTAGTTAGAATGGGA 547
645 TTCCAACTCAGCACCTTT....TGAGATTTTGAGAAGAGTTGCTCTTTGG 690
548 TTCCAACTCAGCACCTTTTGAGTGAGATTTTGAGAAGAGTTGCTCTTTGG 597
691 AGAGCACAGTACGATGAAAGTTGTAAGCTGTGTTCGGGGGGGAGTTATTG 740
598 AGAGCACAGTACGATGAAAGTTGTAAGCTGTGTTCGGGGGGGAGTTATTG 647
741 TCTATCGTTGGCCTCTATGGTAGAATCAGTCGGGGG.CCTGAGAGGCGGT 789
648 TCTATCGTTGGCCTCTATGGTAGAATCAGTCGGGGGACCTGAGAGGCGGT 697
cot 790 GGTTTACCCTGT 801
tob 698 GGTTTACCCTGC 709

The multiple alignment (CLUSTLAW) performed on the available *trnI* and *trnA* sequences (representing dicots, monocots, gymnosperm and bryophtes) including cotton chloroplast DNA (Table 3.1) indicated a high degree of sequence conservation among the species compared except *Triticum* (Fig. 3.6 and 3.7). The major deletions that have occurred in *trnI* show two uniform patterns (Fig. 3.6). For both patterns, the 3' end point of deletion looks almost similar. But the 5' start point of deletion shows variation and suggests the existence of two regions that are vulnerable for deletion. For the four species, pea, tobacco, *Spinacia* and *Arabidopsis*, the 5' deletion start point falls on or adjacent to a T- rich region (from 473 to 490) 'TTTTTTTTTCATGCTTTT' (15/17 pyrimidines) and the 3' end which maps to a 25 bp region that falls adjacent or on another T rich region (from 699 to 725) TGGGATGTGTCTATTTATCTATCTCTT (18/26 pyrimidines). The second pattern of deletion starts from an AT rich region (from 567 to 579) occurs adjacent to 'ATTCTTTTTCCTTT' and ending at the T rich regions of the first pattern (699 to 725) in *Cucumis* and *Hordeum* whereas no deletion was found in plants such as cotton, soya, alnus, *Oenothera*, maize, *Marchantia*, *Pinus* and rice. From this analysis, it is evident that deletion that has occurred in the sequences do not fall in any order across the lower and higher plants studied. For example, the *Marchantia* (Bryophyte) and *Pinus* (Gymnosperm) show similarity to most of the higher plants where no deletion event has occurred.

The *trnA* gene analyzed among 12 other species shows a high percentage of similarity except in the case of tobacco (Figure 3.6) which shows a major deletion from 557 to 681 bp. The 5' and 3' deletions in *trnA* of tobacco too fall on or adjacent to a T rich block 'TTCTTAA' (550 to 556) and TTAGTTAGAAT (678-688), respectively. *Marchantia* and *Pinus* also exhibit a deletion of a small fragment (26 bp length) at this

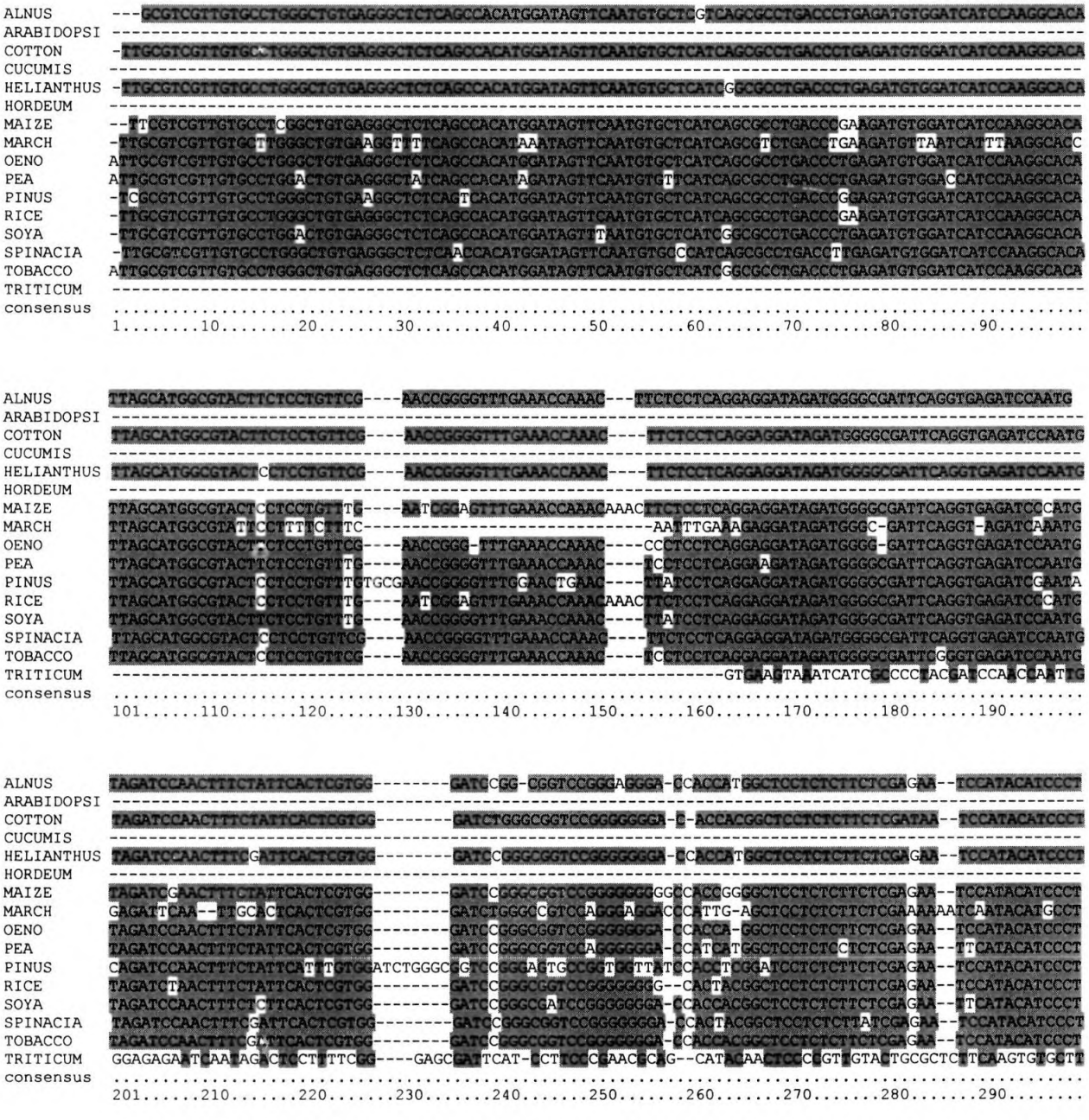
Table 7 List of Plants used for the analysis and their accession number.

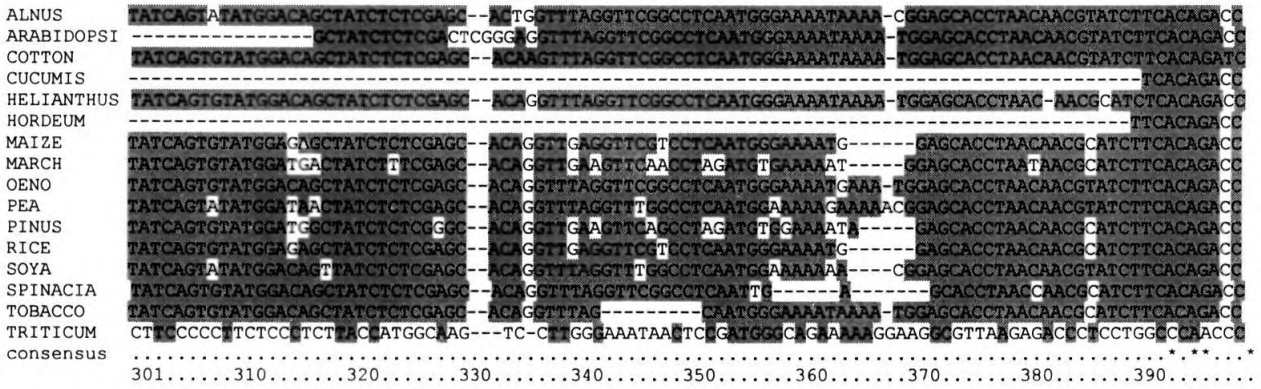
No.	Plants	GenBank accession No.
1.	*Alnus incana*	M76450
2.	*Arabidopsis thaliana*	L43905 (*trnI* only)
3.	*Gossypium hirsutum*	AF189149(*trnI*) and AF175761(*trnA*)
4.	*Cucumis sativus*	L43907
5.	*Glycine max*	M37149
6.	*Helianthus tuberosus*	L43908
7.	*Hordeum vulgare*	L43909
8.	*Marchantia polymorpha*	X04465
9.	*Nicotiana tabacum*	Z00044
10.	*Oenothera grandiflora*	X97294
11.	*Oryza sativa*	NC_001320
12.	*Pisum sativum*	X55033
13.	*Pinus thunbergii*	D17510
14.	*Spinacia oleracea*	M21453
15.	*Triticum aestivum*	AB042240
16.	*Zea mays*	X86563

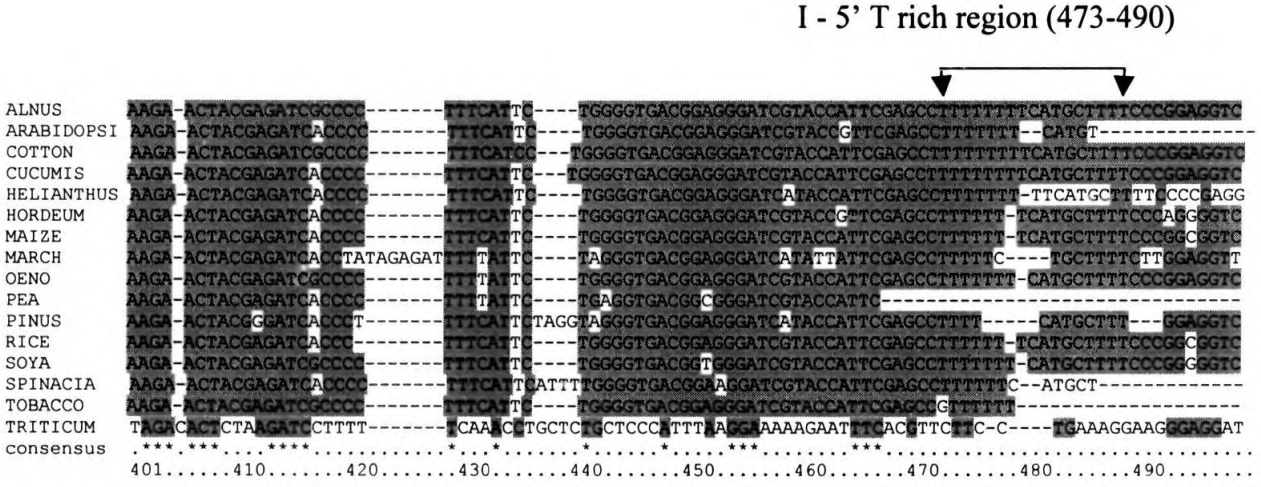
Figure 3.6

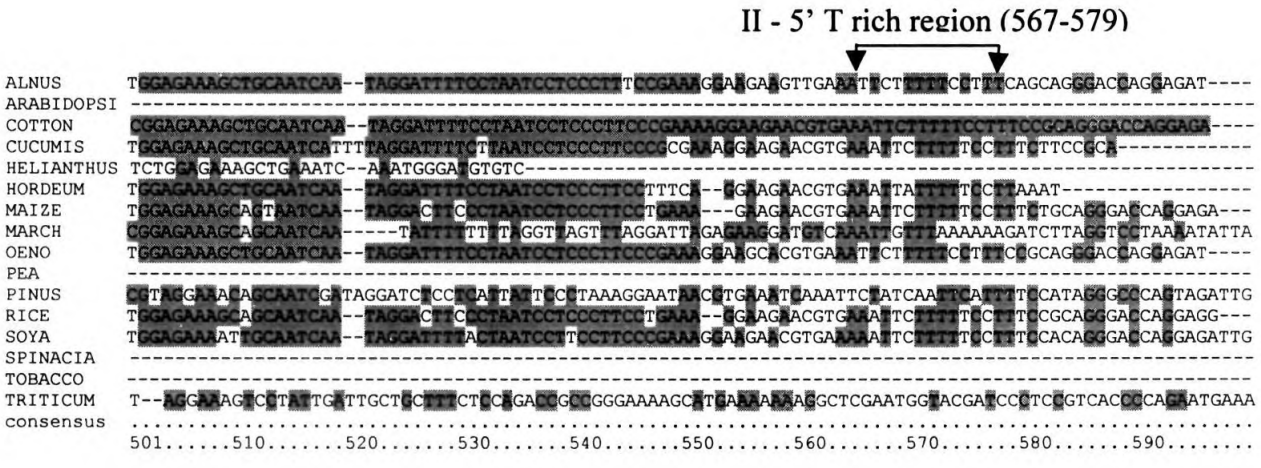
Multiple alignment (CLUSTLAW) of *trnI* intron sequences

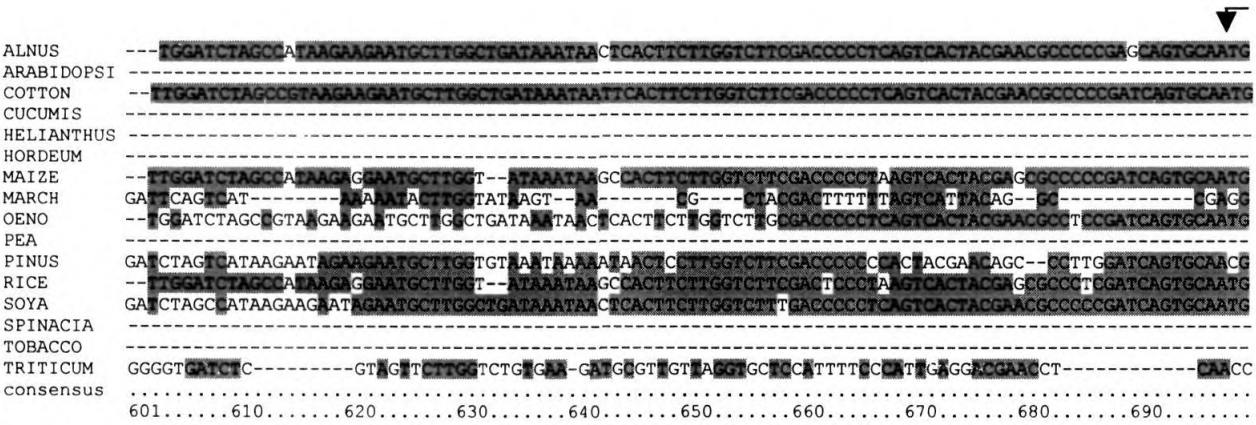
All intron sequences analyzed (except *Gossypium*) were obtained from the database and their accession number are given in the Table 3.1. The deletion gaps are indicated by – and the identical bases are shaded with BOXSHADE program. The highly conserved bases are indicated with * symbol. The two 5' T rich regions and the 3' T rich region where the deletion has occurred are indicated.



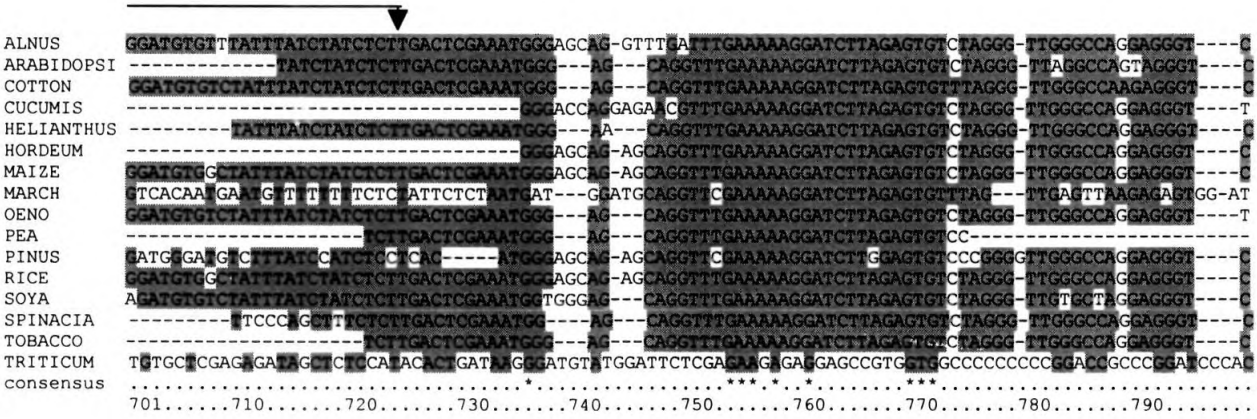

ALNUS
ARABIDOPSI
COTTON
CUCUMIS
HELIANTHUS
HORDEUM
MAIZE
MARCH
OENO
PEA
PINUS
RICE
SOYA
SPINACIA
TOBACCO
TRITICUM
consensus
301.....310.....320.....330.....340.....350.....360.....370.....380.....390.....

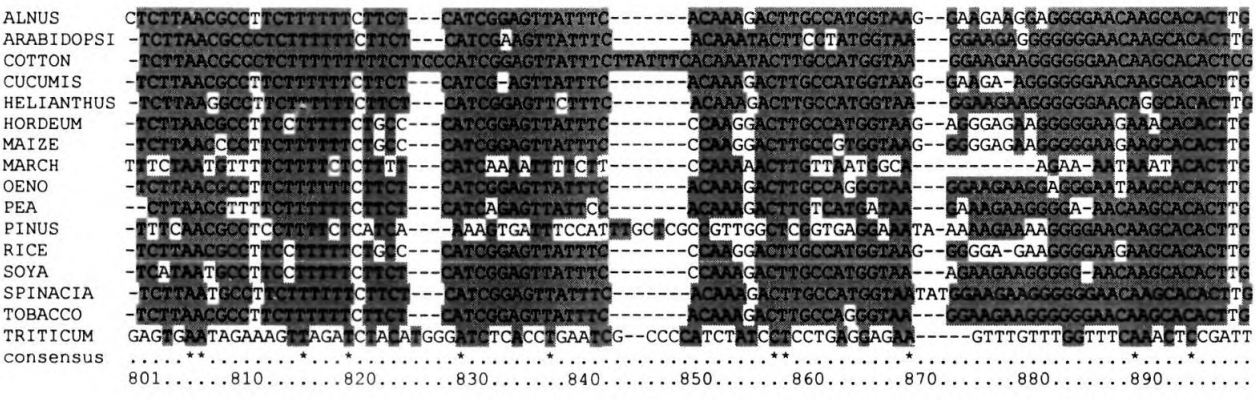

I - 5' T rich region (473-490)
ALNUS
ARABIDOPSI
COTTON
CUCUMIS
HELIANTHUS
HORDEUM
MAIZE
MARCH
OENO
PEA
PINUS
RICE
SOYA
SPINACIA
TOBACCO
TRITICUM
consensus
401.....410.....420.....430.....440.....450.....460.....470.....480.....490.....


II - 5' T rich region (567-579)
ALNUS
ARABIDOPSI
COTTON
CUCUMIS
HELIANTHUS
HORDEUM
MAIZE
MARCH
OENO
PEA
PINUS
RICE
SOYA
SPINACIA
TOBACCO
TRITICUM
consensus
501.....510.....520.....530.....540.....550.....560.....570.....580.....590.....



3' T - rich region (699-725)





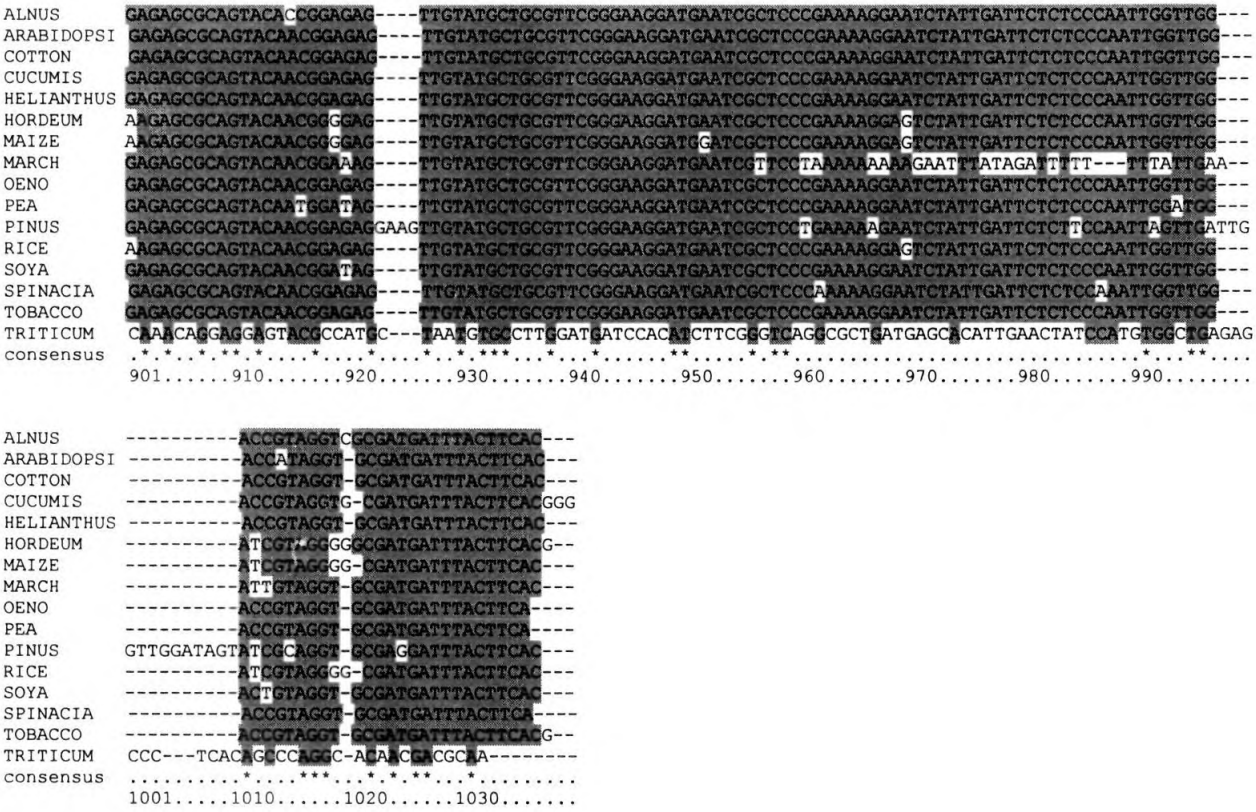
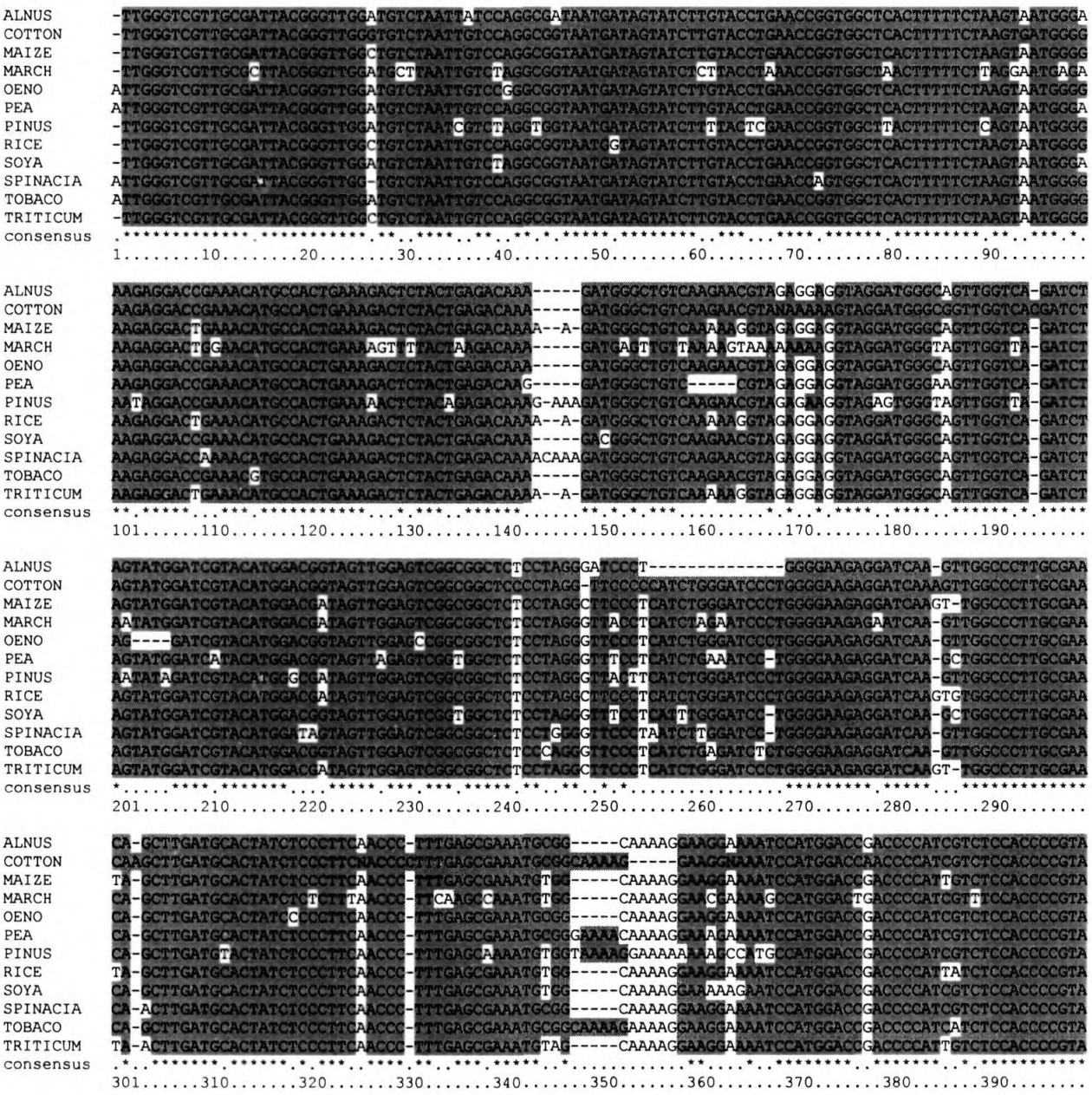
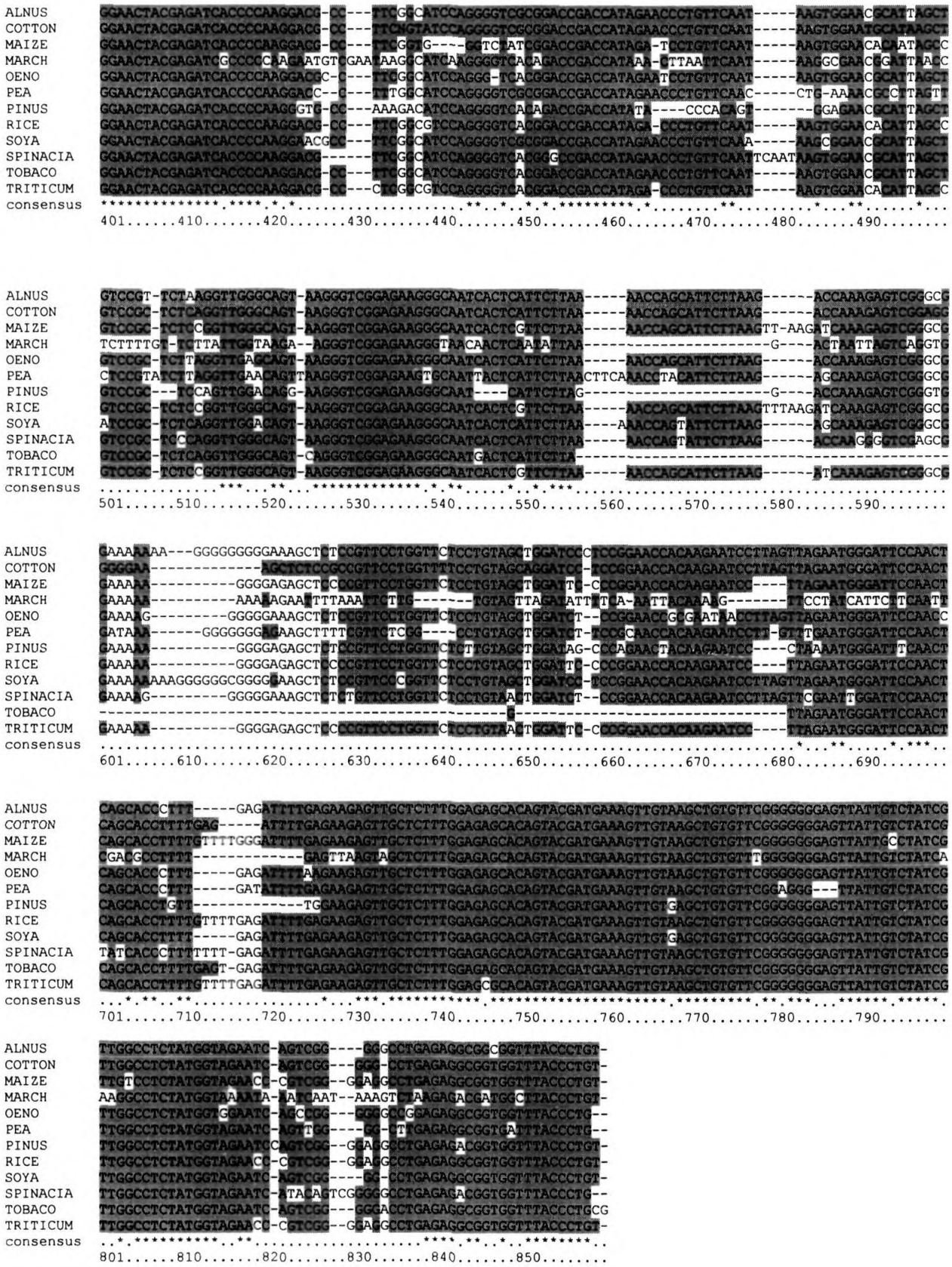

ALNUS GAGAGCGCAGTACACCGGAGAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAATCTATTGATTCTCTCCCAATTGGTTGG---
ARABIDOPSI GAGAGCGCAGTACAACGGAGAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAATCTATTGATTCTCTCCCAATTGGTTGG---
COTTON GAGAGCGCAGTACAACGGAGAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAATCTATTGATTCTCTCCCAATTGGTTGG---
CUCUMIS GAGAGCGCAGTACAACGGAGAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAATCTATTGATTCTCTCCCAATTGGTTGG---
HELIANTHUS GAGAGCGCAGTACAACGGAGAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAATCTATTGATTCTCTCCCAATTGGTTGG---
HORDEUM AAGAGCGCAGTACAACGGGGAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAGTCTATTGATTCTCTCCCAATTGGTTGG---
MAIZE AAGAGCGCAGTACAACGGGGAG----TTGTATGCTGCGTTCGGGAAGGATGGATCGCTCCCGAAAAGGAGTCTATTGATTCTCTCCCAATTGGTTGG---
MARCH GAGAGCGCAGTACAACGGAAAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGTTCCTAAAAAAAAAGAATTTATAGATTTTT---TTTATTGAA--
OENO GAGAGCGCAGTACAACGGAGAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAATCTATTGATTCTCTCCCAATTGGTTGG---
PEA GAGAGCGCAGTACAATGGATAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAATCTATTGATTCTCTCCCAATTGGATGG---
PINUS GAGAGCGCAGTACAACGGAGAGGAAGTTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCTGAAAAAGAATCTATTGATTCTCTTCCAATTAGTTGATTG
RICE AAGAGCGCAGTACAACGGAGAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAGTCTATTGATTCTCTCCCAATTGGTTGG---
SOYA GAGAGCGCAGTACAACGGATAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAATCTATTGATTCTCTCCCAATTGGTTGG---
SPINACIA GAGAGCGCAGTACAACGGAGAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCAAAAAGGAATCTATTGATTCTCTCCAAATTGGTTGG---
TOBACCO GAGAGCGCAGTACAACGGAGAG----TTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAATCTATTGATTCTCTCCCAATTGGTTGG---
TRITICUM CAAACAGGAGGAGTACGCCATGC---TAATGTGCCTTGGATGATCCACATCTTCGGGTCAGGCGCTGATGAGCACATTGAACTATCCATGTGGCTGAGAG
consensus .*.*..*.**.*....*....*....*..*.***...*...*.....**.....*.**.....*...**....
901.....910.....920.....930.....940.....950.....960.....970.....980.....990.....
ALNUS ----------ACCGTAGGTCGCGATGATTTACTTCAC---
ARABIDOPSI ----------ACCATAGGT-GCGATGATTTACTTCAC---
COTTON ----------ACCGTAGGT-GCGATGATTTACTTCAC---
CUCUMIS ----------ACCGTAGGTG-CGATGATTTACTTCACGGG
HELIANTHUS ----------ACCGTAGGT-GCGATGATTTACTTCAC---
HORDEUM ----------ATCGTAGGGGCGATGATTTACTTCACG--
MAIZE ----------ATCGTAGGGG-CGATGATTTACTTCAC---
MARCH ----------ATTGTAGGT-GCGATGATTTACTTCAC---
OENO ----------ACCGTAGGT-GCGATGATTTACTTCA----
PEA ----------ACCGTAGGT-GCGATGATTTACTTCA----
PINUS GTTGGATAGTATCGCAGGT-GCGAGGATTTACTTCAC---
RICE ----------ATCGTAGGGG-CGATGATTTACTTCAC---
SOYA ----------ACTGTAGGT-GCGATGATTTACTTCAC---
SPINACIA ----------ACCGTAGGT-GCGATGATTTACTTCA----
TOBACCO ----------ACCGTAGGT-GCGATGATTTACTTCACG--
TRITICUM CCC---TCACAGCCCAGGC-ACAACGACGCAA--------
consensus .....*....***...*.*.**...*.....
1001.....1010.....1020.....1030.....

Figure 3.7

Multiple alignment (CLUSTLAW) of *trnA* intron sequences

All intron sequences analyzed (except *Gossypium*) were obtained from database and their accession number are given in Table 1. There are no major deletions in any introns except in *Nicotiana* where a deletion (from the nt 557 to nt 681) was seen.




ALNUS
COTTON
MAIZE
MARCH
OENO
PEA
PINUS
RICE
SOYA
SPINACIA
TOBACO
TRITICUM
consensus

point. Another interesting point is an uniform deletion pattern of GA rich 15 bp region (610-625) in most of the plants except soya. This region confirms the idea that some spots are more vulnerable for the deletion to occur. Apart from these deletions, there are no major changes in the *trnA* genes of the plants analyzed. The sequence alignment analysis carried out in this study confirms the high degree of sequence similarity among the various species studied except for the presence of two different deletion patterns in *trnI* of six species. Pea, tobacco, *Spinacia* and *Arabidopsis* showed one pattern of deletion and *Cucumis* and *Hordeum* showed another pattern. It may be possible that the presence of deletion in some closely related species is due to inheritance for example in wheat and barley (Johnson and Hattori, 1996). These deletion regions are useful in certain cases for identifying closely allied species but the general use of deletion for phylogenetic analysis may be limited (Morton and Clegg, 1993) These deletions in the *trnI* and *trnA* introns are reported to be mapping to the loop region of domain III (ii) of the standard group II (subgroup IIA1) intron structure (Michel *et al.*, 1989). These deletions occur principally in non-coding intergenic spacer regions and introns lead to minor rearrangements that can result in a different phylogeny. Observations of these studies indicate the existence of independent deletion events rather than deletion through the course of evolution and more investigations are needed to see whether these deletion patterns can be used as phylogenetic markers. These studies also suggested various possible events that could have led to the deletion/addition across the spectrum of land plants by multiple independent insertion of transposons and subsequent deletion of the same element in the later course.

Johnson and Hattori (1996) have proposed that the T rich block functions as a part of novel imprecise site specific recombination system or alternately possesses a unique structure or property that renders it more recombinagenic and that this function is conserved in many plant genera. They have also discussed about the possibility of deletion by

homologous recombination in the direct repeats. Our study also confirms the view that there are preferred vulnerable sequences that function as hotspots for deletion, which results in loss of particular fragment of DNA. The present study suggests that the T- rich regions may be responsible for site- specific recombination (Figure 3.6).

Apart from the deletions that exist in few species, the *trnI* and *trnA* genes of pea, tobacco, *Spinacia, Helianthus*, and *Arabidopsis*, showed a high percentage of similarity. This high degree of homology makes this region suitable for genetic transformation experiments. The chloroplast transformation vectors using these genes as flanking sequences to insert the foreign gene specifically in between *trnI* and *trnA* of chloroplast genome by homologous recombination can be used as universal transformation vectors. Generally, the flanking genes having above 30% similarity with the insertion site of plants to be transformed has more chance of inserting the foreign DNA by homologous recombination. As these exon esquence of *trnI* and *trnA* are generally similar, any addition or deletion in the intron region does not affect the function of the gene. This study confirms that the *trnI* and *trnA* gene sequences analyzed are highly conserved except the deletion regions and can be used as specific insertion sites for transformation experiments. More studies on *trnI* and *trnA* of various land plants would further strengthen this view in due course of time.

Summary .....

SUMMARY

Chloroplast genome transformation is emerging as an attractive alternate method of transforming plants with foreign gene. This method has many advantages over the nuclear genome transformation method such as over-expression of introduced foreign gene due to the high copy number of chloroplasts, maternal inheritance of plastids which prevents spreading of foreign gene to non-target plants, prokaryotic origin of the chloroplasts makes it amenable for transformation with genes from prokaryotes. In this work we have attempted to transform tobacco plastome with *cry1Ac*, which encodes for an insecticidal crystal (Cry1Ac) protein, and makes the plant insect resistant.

The first chapter deals with construction of the chloroplast transformation vector. This involves preparation of the various components and assembling them in proper order in a basic vector back bone. The second chapter deals with transforming tobacco with the constructed chloroplast transformation vector. This involves transformation as well as evaluation of the putative transformants to confirm the occurrence of transformation event. The third chapter discusses the highly conserved nature of *trnI* and *trnA* genes that are used as flanking sequences in this vector. This property of flanking sequences facilitates the insertion of foreign DNA in to the plastome of most higher plants by homologous recombination.

Construction of chloroplast transformation vector

P*rrn* (16S rDNA, the promoter region) and the T*rps16* (*rps16* 3' region, the terminator region) were used as the regulatory elements. *aadA* gene has been used as marker gene (for spectinomycin resistance which encodes for the protein 5-aminoglycoside 3-adenyltransferase). The *trnI* and *trnA* genes were used as flanking

region to facilitate the insertion of foreign DNA by homologous recombination. The regulatory elements P*rrn* and T*rps16*, *trnI*, *trnA* and the marker gene *aadA* were PCR amplified with the addition of appropriate restriction sites to facilitate the subsequent cloning exercises. The coding region of *cry1Ac* that encodes the Cry1Ac protein (bioinsecticide) has been used as the foreign DNA. All these fragments were placed in proper order to construct the final transformation vector.

The construction process was carried out in four steps.

- First, the PCR amplified *trnI* and *trnA* gene fragments were placed in pBS vector in appropriate restriction sites to obtain the clone pMSVS2.
- Second, the PCR amplified 16S rDNA promoter, *aadA* gene and *rps16* 3' region were placed in appropriate restriction sites of another pBS vector to generate clone pMSVS5.
- Third, the expression cassette containing the promoter, marker gene and the terminator from pMSVS5, was excised and later introduced into the appropriate sites of pMSVS2 to generate clone pMSVS6A.
- And at last, the ~3.7 kb *cry1Ac* coding region was introduced into the *Bam*H I site of pMSVS6A (in between the promoter and the marker gene) to obtain the final chloroplast transformation vector pMSVS7A..
- Restriction digestion analysis and Southern analysis were performed to confirm the proper integration and the orientation of specific fragments at various steps.

Transformation of tobacco plastome

The vector pMSVS7A was transformed into *Escherichia coli* and the immunoassay was performed to confirm the production of Cry protein. Subsequently,

the vector was used to transform tobacco leaves by particle bombardment. Antibiotic resistant calli were repeatedly subcultured on medium with selection pressure. Regenerated shoots were allowed to root and then later to develop as independent plants. Experiments such as Southern, immunoassay, PCR and bioassay were performed to confirm the integration and expression of foreign DNA in these plants.

- Assay of gene integration: Integration of *cry1Ac* gene in the putative clones was analyzed by performing a Southern analysis. The results showed the presence of *cry1Ac* in only one clone of the three putative clones obatined.
- Assay of site specific integration: Specific PCR primers were synthesized to check the specific integration of *cry1Ac* gene between the *trnI* and *trnA* of plastome of tobacco. The results confirmed the specific integration in one clone of the three transformants.
- Immunoassay: The immunoassay performed with protein samples of various transformants confirmed the production of Cry1Ac protein in one clone. The level of expression in this clone appears to be around 0.5% of the total protein.
- Bioassay: Bioassay performed on neonatal larvae of *Helicoverpa armigera* by feeding with the leaves from the transformants confirmed the occurrence of insecticidal property in one clone..

The results of Southern, PCR, immunoassay and bioassay were satisfactory to prove the integration of the expression cassette between the *trnI* and *trnA* of tobacco and its expression. The protein produced was sufficient enough to cause mortality in the neonate larvae. The results established the transforming ability of the vector by homologous recombination in plants other than cotton. The level of protein expression in the transformant was sufficient enough for the plant to be insecticidal.

Comparative analysis of *trnI, trnA* and their intron sequences

The chloroplast genome ('plastome') of most land plants is highly conserved with respect to size, structure, gene content and linear order of genes. In spite of this feature, variations in both gene content and sequence organization, including the loss of one copy of Inverted Repeat (IR) region by various additions or deletions in non-coding region have also been found and their contribution to the phylogenetic difference among various plants have been studied to some extent. The availability of complete chloroplast genome sequences of several species viz. *Nicotiana tabacum*; *Marchantia polymorpha*, *Oryza sativa*, *Zea mays*, *Oenothera*, *Spinach* as well as the reported partial sequences of various species have facilitated this genetic analysis.

- The chloroplast DNA from cotton leaves were isolated and was used to PCR amplify the *trnI* and *trnA* genes using heterologous primers. Multiple alignment of gene sequences were performed by using the programme CLUSTLAW of GCG software.
- The *trnI* genes of *Cucumis, Hordeum, Triticum* and *Arabidopsis* showed major deletions towards the 5' end while those of pea, tobacco, spinach and *Arabidopsis* showed different patterns. No significant differences were noticed in the *trnI* sequences of rest of the eight species.
- The *trnA* gene, on the other hand, was highly homologous in most plants except tobacco.
- This study confirms that the *trnI* and *trnA* gene sequences analyzed are highly conserved (except in the deletion regions) and can be used as specific insertion sites for transformation experiments.

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