# SEQUENCE MODIFICATION OF *Bacillus thuringiensis* INSECTICIDAL CRYSTAL PROTEIN GENE *cry1F*

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2003

# SEQUENCE MODIFICATION OF *Bacillus thuringiensis* INSECTICIDAL CRYSTAL PROTEIN GENE *cry1F*

By

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**A Thesis**
**submitted to the Faculty of Post Graduate School,**
**Indian Agricultural Research Institute, New Delhi,**
**in partial fulfilment of the requirements**
**for the award of the degree of**

**DOCTOR OF PHILOSOPHY**

**in**

**MOLECULAR BIOLOGY AND BIOTECHNOLOGY**

**2003**

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# CERTIFICATE

This is to certify that the thesis titled "**Sequence modification of *Bacillus thuringiensis* insecticidal crystal protein gene *cry1F***", submitted to the Faculty of the Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Molecular Biology and Biotechnology** embodies the results of *bonafide* research carried out by **Ms. Ratna Chandra** under my guidance and supervision and that no part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or source of information, has been duly acknowledged by her.

Place : New Delhi

Date : 4.9, 2003

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# ACKNOWLEDGEMENT

Chiselling through the cherished memories, I could not find words to convey the feeling that gushes from deep in my heart for all those who have helped me with their suggestions, views, appreciation, encouragement and support.

It is a great privelage for me to express my heartfelt gratitude and sincere thanks to Dr. P. Ananda Kumar, Principal Scientist, NRCPB and Chairman of my Advisory Committee for his guidance, unrelenting support and patience during the research work. He allowed me the complete autonomy of action while being supportive as well as providing right direction and supervision.

My sincere thanks are due especially to Dr. K.R. Koundal, Project Director, NRCPB also my Adivisory Committee Co-Chairman and Members, Dr. G.C. Srivastava, Head, Division of Plant Physiology, Dr. Anil Kumar Saxena, Division of Microbiology and Dr. G.T. Gujar, Division of Entomology.

I owe an immense debt of gratitude to my labmates Rajani, Dr. Ritu Mandaokar and Neeraj for their timely help and cooperation throughout my stay in lab.

I owe very much to Dr. Monica Vishwanathan for help in making the plasmid diagrams.

I would like to place on record my sincere thanks to Suman mam, Prashant, Shiv Kumar, Dipti, Vikrant, Shivsudha, Saraswati,

Jagdeesh,Priyanka, Pankaj and Samina for their help and cheerful and lively company.

It will be ungrateful if I fail to mention the help rendered by Chandeshwarji, Raiji and Arvind in the effective completion of research work.

Success at work is never possible without the family support. Their never ending sacrifices, blessings, constant encouragement are limitless. I owe it all to my parents, especially my mother. For timely submission of this thesis, I owe it to all to two people, the remarkably patient, extremely tolerant and always ready to help, my brother Dr. Ashish and my unstintingly supportive husband, Samuel.

A special thanks is also due to my sister-in-law, Dr. Poonam and to my parents-in-law for their blessing.

Last but not the least, I dedicate my thesis to my father, the only reason for my strength to continue the Ph.D. programme even though he himself could not live up to celebrate the day with me.

My sincere appreciation to Mr. P.K. Garg for neat and careful typing of this manuscript.

The wealth of information provided by the IARI Library and financial assistance by CSIR in the form of CSIR-JRF is duly acknowledged.

New Delhi
5th Sept , 2003

Ratna Chandra
(RATNA CHANDRA)

# ABBREVIATIONS

| | | |
|---|---|---|
| $A_{595}$ | : | Absorbance at wavelength 595 nm |
| α | : | Alpha |
| Amp | : | Ampicillin |
| β | : | Beta |
| BAP | : | 6-Benzyl amino purine |
| bp | : | Base pair |
| BSA | : | Bovine serum albumin |
| Bt | : | *Bacillus thuringiensis* |
| cm | : | Centimeter |
| Ci | : | Curie |
| Cry | : | Crystal |
| CTAB | : | Cetyltrimethyl ammonium bromide |
| cv | : | Cultivar |
| $^0C$ | : | Degree Celsius |
| δ | : | Delta |
| dATP | : | Deoxyadenosine 5'-triphosphate |
| dCTP | : | Deoxycystosine 5'-triphosphate |
| DEPC | : | Diethyl pyrocarbonate |
| dGTP | : | Deoxyguanosine 5'-triphosphate |
| DNA | : | Deoxyribonucleic acid |
| dNTP | : | Deoxynucleotide triphosphate |
| dTTP | : | Deoxythymidine 5'-triphosphate |
| DTT | : | Dithiothreitol |
| EDTA | : | Ehtylene diaminetetra acetic acid |
| ELISA | : | Enzyme-lined immunosorbant assay |

| | | |
|---|---|---|
| γ | : | Gamma |
| g | : | Gram |
| g | : | Acceleration due to gravity |
| h | : | Hour |
| ha | : | Hectare |
| kan | : | Kanamycin |
| kb | : | Kilobase |
| kDa | : | Kilodalton |
| LA | : | Luria agar |
| LB | : | Luria broth |
| M | : | Molar |
| m | : | Meter |
| MCS | : | Multiple cloning site |
| mg | : | Milligram |
| ml | : | Millilitre |
| mM | : | Millimolar |
| m mol | : | Millimole(s) |
| N | : | Normal |
| nM | : | Nanomolar |
| NAA | : | α-Naphthalene acetic acid |
| nt | : | Nucleotide |
| min | : | Minute |
| MS | : | Murashige and Skoog medium |
| µ | : | Micro |
| µg | : | Microgram |
| µ mole | : | Micromole(s) |
| µl | : | Microliter |

| | | |
|---|---|---|
| OD | : | Optical density |
| % | : | Per cent |
| PAGE | : | Polyacrylamide gel electrophoresis |
| $^{32}P$ | : | Phosphorus-32 (radionucleotide) |
| PCR | : | Polymerase chain reaction |
| *Pfu* | : | *Pyrococcus furiosus* |
| pH | : | Log $1/[H^+]$ |
| RNase A | : | Ribonuclease A |
| rpm | : | Revolutions per rninute |
| RT | : | Room temperature |
| SDS | : | Sodium dodecyl sulphate |
| SE | : | Standard error |
| s | : | Second |
| SSC | : | Sodium chloride-sodium citrate |
| TAE | : | Tris-acetate EDTA |
| TBE | : | Tris-borate EDTA |
| *Taq* | : | *Thermus aquaticus* |
| TE | : | Tris-EDTA buffer |
| TEMED | : | N,N,N',N'-tetramethylethylene diamine |
| Tris-Cl | : | Tris-(hydroxymethyl)-aminomethane hydrochloride |
| u | : | Unit |
| UV | : | Ultra vilolet |
| W | : | Watt |
| w/v | : | Weight/volume |
| X-Gal | : | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

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# INTRODUCTION

The desire for insect control in human society has increased significantly with two events : the realization that insects can spread human disease and the rise of agriculture. As the world's population increases, the need to keep insects from destroying food crops becomes even more imperitive. Currently insect pests destroy more than 13 per cent of crops worldwide (Miller, 1998).

All plants possess a certain degree of resistance to insects, and so only a limited range of herbivores are able to feed on each individual species. This inherent resistance is based on various defense mechanisms, including a wide range of noxious secondary metabolites produced by the plant. Individual plants within one genus or even one species, vary in their level of insect resistance, a fact long used by plant breeders to increase the insect resistance of crop cultivars.

Chemical treatments are highly efficient for controlling crop pests under a wide range of conditions. Howver, related environmental hazards and the possibility that insects rapidly develop resistance are major drawbacks of this strategy. Rational chemical control practices are combined with the use of resistant plant varieties developed through breeding programmes, biological control, rational crop techniques and biotechnology products, including transgenic plants.

Insect-resistant transgenes, whether of plant, bacterial or other origin, can be introduced in plants to increase the level of insect resistance, a technology that has dramatically extended the scope of resistance genes

available to plant breeder. The first reports of transgenic insect-resistant plant were published in 1987 (Hilder *et al.*, 1987) but technological development has been swift since then.

Over 90 species of naturally occurring, insect-specific (entomopathogenic) bacteria have been isolated from insects, plants, and the soil, but only a few have been studied intensively. Much attention has been given to *Bacillus thuringiensis*, a species that has been developed as a microbial insecticide. An estimated 95 per cent of the commercial biotechnology research for microbial insecticides focuses on the bacterium, *Bacillus thuringiensis.*

Bt insecticidal proteins have been used commercially for over 40 years, and now represent 98% of all biopesticides. Transgenic plants that produce Bt toxins provide a new way of dealing with pests that are difficult to control, i.e. those that develop inside plants. The main problem is mechanical, i.e. chemical contact pesticides or biological pesticides that are sprayed onto the plant primarily reach and protect external tissues. Plants that can produce Bt proteins are constantly protected from within. This technique atleast partly overcomes the need for chemical treatments. Moreover, the toxins produced only reach insects that feed on the plant, has faster biodegradability and greater resistance to weather conditions.

The advantages of Bt toxin producing transgenic plants over conventional Bt spray application are so obvious as to have made, in retrospect, development of transgenic Bt plants inevitable from a commercial perspective. Due to photosensitivity, conventional Bt sprays lack persistence and can only protect the plant surface. Root pests, stem and

fruit borers and sucking insects are not affected by sprays, these pests become controllable only with a transgenic plant expressing the toxin throughout the tissues.

Bt genes encoding toxin proteins have been introduced into crop plants such as maize, cotton, potato, tobacco, rice, walnut, apple, alfalfa, broccoli, soyabean etc. Genetically transformed crops with Bt genes have been deployed for cultivation in USA, China, India and Australia.

Transgenic plants with insecticidal genes are set to feature prominently in pest management in both developed and the developing world in future. Among the developing countries, China, India, Argentina, Mexico, Brazil, Pakistan and South Africa are pursuing the research on transgenic crops vigourously. Entomologists, breeders and the molecular biologists need to determine how to deploy this technology for pest management, and at the same time avoid or reduce possible environmental risks. To achieve these objectives, it is necessary to have an appropriate understanding of the insect biology, behaviour, its response to the insecticidal proteins, temporal and spatial expression of the insecticidal proteins in the plants, strategy for resistance management, impact of insecticidal proteins on natural enemies and non-target organisms. Equally important are the issues concerning the transfer of technology to the resource poor farmers.

Protease inhibitors, plant lectins, ribosome inactivating proteins, secondary plant metabolites, vegetative insecticidal proteins from Bt and related species and small RNA viruses can also be used alone or in combination with Bt genes to generate transgenic plants for pest control (Hilder and Boulter, 1999).

Bt till date is the most successful economical biological control agent of insect pests. It is an ubiquitous soil bacterium, isolated from soil, stored grains, insect cadavers and the phylloplane (plant surface). However, the ecology of Bt remains unclear. Bt is a Gram-positive, aerobic, endospore-forming bacterium belonging to morphological group I along with *B. cereus* and *B. anthracis*. All these bacteria have endospores. Bt, however, is recognized by its parasporal body (known as the crystal) that is proteinaceous in nature and possesses insecticidal properties. Considerable amount of information with respect to various aspects of Bt such as fermentation, biology and genetics (Aronson, 1986), molecular biology (Hofte and Whiteley, 1989; Kumar *et al.*, 1996), mechanism of action (Gill *et al.*, 1992; Knowles, 1994), application as biopesticide (Li *et al.*, 1975), and Bt transgenic plants (Peferoen, 1992; Schuler *et al.*, 1998; de Maagd *et al.*, 1999) is available. So far, more than 150 different genes encoding crystal proteins have been cloned from Bt.

A number of crystal protein genes (*cry*) ranging upto 150 have been sequenced and classified. Among these Cry1F is a 143 kDa molecular weight protein and is found to be toxic to order Lepidoptera. The nucleotide sequence study of *cry1F* gene showed its homology of only ~67 to 68 per cent to those of *cry1A* subgroup, *cry1B, cry1C, cry1D* and *cry1E* genes. *cry1F* has considerable homology to *cry1Aa* nucleotide sequence (77.6% homology) (Chambers *et al.*, 1991).

The insect control proteins are highly expressed in their natural host, Bt upto 50 per cent of the total protein in sporulated cultures of Bt are insect control proteins deposited as crystals within the cell. Native *cry* genes are, however, found to express poorly in the plants because of (1)

Instability of *cry* mRNA due to 5' to 3' degradation process which occurs in normal mRNA turnover, (2) Codon usage of *cry* genes is biased for expression in Gram-positive prokaryotes and this differs significantly from the codon usage of plants.

Truncating the gene, keeping essentially the N-terminal half of the protein intact (Fischhoff *et al.*, 1987), use of different promoters, fusion proteins and leader sequences have shown to increase the insect control protein gene expression significantly (Vaeck *et al.*, 1987). Efforts are being made to modify the coding sequence without affecting the amino acid sequence. This inhibition arose from the fact that Bt genes are rich in A/T content whereas plant genes have higher G/C content. Thus partial or complete resynthesis of these genes to contain a higher G/C content could resolve this problem to a greater extent (Perlak *et al.*, 1990). Also the DNA sequence predicted to inhibit efficient plant gene expression at both translational and mRNA level can be selectively removed throughout the coding sequence to partially modify the gene without changing the amino acid sequence. Modification can be designed in such a way so as to exclude from *cry* gene the numerous motifs seldom found in plant exons including (Perlak *et al.*, 1990) :

1. Stretches of AT rich sequences resembling plant mRNA processing signals.

2. Motifs which cause mRNA instability (these include potential poly A signal, potential intron 5' splice recognition sequence and ATTTA motifs).

3. Codons which are rarely used in plant genes can be altered to those more typical of dicot plant genes (Koziel *et al.*, 1993b).

Resulting modification can lead to partially modified gene or fully modified synthetic gene. The fully modified genes encode proteins nearly identical in amino acid sequence to the wild type gene. The utility of partially and fully modified genes to provide protection from insects has far-reaching implications for the future of insect-resistant plants and for the application of these gene modification principles to the design of heterologous gene for high level expression in plants.

The present research work is aimed to study and further improve the expression of *cry1F* gene in a plant system by sequence modification strategy.

# 2. REVIEW OF LITERATURE

## 2.1 Bt (*Bacillus thuringiensis*)

*Bacillus thuringiensis*, commonly known as Bt is a rod shaped (1.0-1.2 by 3-5 micron), Gram-positive, facultative, anaerobic, spore forming bacterium that occurs naturally in the soil. For years bacteriologists have known that some strains of Bt produce proteins that kill certain insects with alkaline digestive tracts. When these insects ingest the protein produced by Bt, the function of their digestive systems is disrupted producing slow growth and ultimately, death. Each strain produces its own unique Bt insecticidal crystal protein, or delta-endotoxin, which is encoded by a single gene on a plasmid in the bacterium (Whalon and McGaughey, 1998). The protein crystals of several strains of this bacterium have been used as microbial insecticides since the 1950s. Bt toxins have been transferred and expressed in at least 26 different plant species (Table 1). However, the level of resistance they confer will, in most cases, depend on whether native bacterial or truncated, codon-optimized genes have been used. There are 34 recognized subspecies of Bt. Some of the most commonly used include subspecies *kurstaki* (against Lepidoptera), subspecies *israelensis* (against Diptera, primarily mosquitoes and blackflies) and subspecies *tenebrionis* against *Leptinotarsa decemlineata* (Colorado potato beetle) (Whalon and McGaughey, 1998).

### 2.1.1 History of Bt

The first record on Bt goes back to 1901, when Ishiwata discovered a bacterium from diseased silkworm larvae that he named *Bacillus sotto*

**Table 1. List of transgenic plants carrying Bt genes**

| Plant | Genes | Target insects | References |
|---|---|---|---|
| Tobacco | *cry1Aa* | *Manduca sexta* | Barton *et al.*, 1987 |
| | *cry1Ab* | *Heliothis virescens* | Vaeck et al., 1987 |
| | *cry1Ac* | *Helicoverpa zea* | Warren *et al.*, 1992 |
| | *cry1C* | *Helicoverpa armigera* | Sutton *et al.*, 1992 |
| | *cry1Aa* | *Spodoptera littoralis* | *Vander Salm et al.*, 1994 |
| | *cry1Aa* | *Spodoptera exigua* | McBride *et al.*, 1995 |
| | *cry12Aa* | *Leptinotansa* | *Strizhov et al.*, 1996 |
| | *cry19Aa* | *decemileata* | *Selvapandiyan et al.*, 1998 |
| | *cry1IA5* | *Phthorimaea* | *Gleave et al.*, 1999 |
| | *cry1operon* | *operculella* | Cosa *et al.*, 2001 |
| Tomato | *cry1Ab* | *M. sexta* | Fischhoff *et al.*, 1998 |
| | *cry1Ac* | *H. zea* | *Delannay et al., 1995* |
| | *cry1C* | S. exigua | Mc Bride *et al.*, 1995 |
| | | H. virescens | USDS/APHIS |
| | | *Keiferia lycopersicella* | Mandaokar *et al.*, 2000 |
| | | *P. operculella* | |
| *Potato* | *cry1Ab* | *M. sexta* | *Perlak et al.,1993* |
| | *cry3A* | *P. operculella* | *Adang et al.,1993* |
| | *cry1Ab Bt884* | *Helicoverpa amigera* | *Jansens et al.,1995* |
| | *cryV-Bt* | *Symmaetrichimas* | *Li et al., 1999* |
| | | *tangolias* | *Chakrabarthi et al., 2000* |
| Eggplant | *cry1Ab* | *Leucinoides orbonalis* | USDA/APHIS |
| | *cry1Ac* | *L. decemlineata* | Arpaia *et al.*, 1997 |
| | *cry3Aa* | | *Innocone et al.*, 1997 |
| | *cry3B* | | Kumar *et al.*, 1998 |
| Cotton | *cry1Ab* | *H. virescens* | Perlak *et al.*, 1990 |
| | *crylAc* | *H. zea* | *Wibon et al.*, 1992 |
| | *cry2Aa* | *H. armigera* | *Perlak et al.*, 1993 |
| | *Cry1A* | *S. exigua* | *Benedict et al.*, 1996 |
| | | *Pectinophora* | |
| | | *gossypiella* | |
| | | *H. armigera* | |
| *Chickpea* | cry1Ac | *H. armigera* | Kar *et al.*, 1997 |
| Peanut | *cry1Ac* | *Elasmopalpus lignosellus* | Singsit *et al.*, 1997 |
| Alfalfa | *cry1C* | *S. littoralis* | Strizhov *et al.*, 1996 |
| | | | USDa/APHIS |
| Canola | *cry1Ac* | *Plutella xylostella* | Stewart *et al.*, 1996 |
| | | *Trichoplusia* | |
| Rutabaga | *cry1Ac* | *Pieris rapae* | *Li et al.*, 1995 |

| Plant | Genes | Target insects | References |
|---|---|---|---|
| Walnut | *cry1Ac* | *Cydia pomonella* | Dandekar *et al.*, 1992 |
| Apple | *cry1Ac* | *Cydia pormonella* | Dandekar *et al.*, 1992 |
| White spruce | *cry1Aa* | *Chorisoneura fumigenera* | Ellis *et al.*, 1993 |
| Polar | *cry1Aa*<br>*cry1Ab* | *Lymntaria dispara*<br>*Malacosama disstria* | McCroun *et al.*, 1991<br>*Kleiner et al.*, 1995 |
| Crauberry | *cry1Aa* | *Rghopodota* | |
| Coffee | *cry1Ac* | *Perileucoplera coffetella* | Leroy *et al.*, 2000 |
| Maize | *cry1ab*<br>*cry1B*<br>*cry1H*<br>*cry1Ac*<br>*cry9c*<br>*cry3Bb* | *Ostrinia nubilalis*<br>*Diatraea grandilosa*<br>*D. saccharalis*<br>*S. exigua*<br>*S. frugiperda*<br>*Diabrotica virgifera* | Koziet *et al.*, 1993<br>Estruch *et al.*, 1994<br>Duch and Evola *et al.*,1997<br>Janeseus *et al.*, 1997<br>Gray, 2000<br>Moellenbeck *et al.*, 2001 |
| Rice | *cry1Ab*<br>*Cry1Ab*<br>*Cry2A*<br>*cry1Ab*<br>*cry1Ac* | *Chilo suppressalis*<br>*Scripophago incertulus*<br>*Cnaphatocrosis medianalis*<br>*Marasmia patnalis* | Fujimoto *et al.*, 1993<br>Wunn *et al.*, 1996<br>*Ghareyazie et al.*, 1997<br>Maqbool *et al.*, 1998<br>Datta *et al.*, 1998<br>Cheng *et al.*, 1998<br>Alam *et al.*, 1999<br>Shu *et al.*, 2000<br>Tu *et al.*, 2000 |
| Sugarcane | *cry1Ab* | *Diatraea saccharalis* | Arencibia *et al.*, 1997 |

(Ishiwata, 1901). Then in 1911, Berliner (Baum *et al.*, 1999) working at a research station for grain processing in Berlin, investigated an infectious disease of the Mediterranean flour moth (*Ephestia kuehnielia*). The infected insects were originally obtained from a mill in the district of Thuringia. Berliner described a spore forming bacterium as the causative agent and designated it *B. thuringiensis.* In 1938, in France, *Bacillus thuringiensis* became available for the first time as a commercial insecticide, sporeine and much later in 1950s entered commercial use in the United States.

However, it was not until after the second world war, when problems with synthetic pesticides were realized, that serious attempts were made to establish Bt as a biological pesticide (Krieg, 1986). This led to the introduction of viable Bt biopesticides like Thuricide and Dipel.

In 1983, the World Health Organization used Bt in West-Africa to control disease-carrying blackflies. Commercial interest in Bt grew very rapidly in 1980s as many popular synthetic insecticides became ineffective due to insect resistance or became unusable due to environmental restrictions. In 1987 came the first reports of insertion of genes encoding Bt delta-endotoxins into plants. The first transgenic plants to express Bt toxins were tobacco and tomato plants (van Franken-Huyzen, 1993). The first Bt transgenic crop corn, was registered with the United States Environmental Protection Agency in 1995 (USEPA, 1999b). Today, Bt transgenics include a wide range of crop plants. In 2002, Bt cotton, corn and potatoes covered nearly 15.4 million acres of land in more than 10 countries. These crops have also been commercialized and are in wide use in Canada, Japan, Mexico, Argentina and Australia (Frutos *et al.*, 1999). While using Bt in the form of transgenic crops is now very common, the more traditional spray form of Bt is still widely used (Liu and Tabashnik, 1977).

### 2.1.2 Insecticidal toxins of Bt

#### (i) ICP (Insecticidal crystal proteins)

During sporulation phase strains of Bt produce protein inclusions called parasporal inclusions adjacent to the endospore. The parasporal inclusions consist of one or more insecticidal proteins in the form of a

crystal or crystal-complex. These insecticidal proteins are commonly known as Insecticidal Crystal Proteins (ICP) or delta-endotoxins. It is the active ingredient of 90% of the microbial insecticides produced in the world. Most susceptible species belong to Lepidoptera, Diptera and Coleoptera. By far the greatest number of Bt strains produce large bipyramidal crystals which are toxic only to lepidopteran larvae (Whitely *et al.*, 1986). These crystals consist of one or more related protoxin polypeptides having a molecular mass of 130-140 kDa. Some strains also contain small cubidal, flat rhomboid or composite of two or more crystal types (Sharpe and Baker, 1979). The crystal protein is highly insoluble in normal conditions, so it is entirely safe to humans, higher animals and most insects. However, it is solubilised in reducing conditions of high pH (above pH 9.5) the conditions commonly found in the mid-gut of lepidopteran larvae. Hence, Bt is a highly specific insecticidal agent. Besides ICP and endospore, some Bt subspecies produce Beta Exotoxins which are toxic to all forms of life including humans.

**(ii) Endospores**

Endospores are resistant to inactivation by heat and dessication and persist in the environment under adverse conditions and provide a mechanism for long term survival of Bt. Endospores are pathogenic to some insects, particularly when combined with ICP.

**(iii) Beta Exotoxin**

During vegetative growth, some Bt subspecies (*thuringiensis, galleriae* and *darmstadiensis*) produce Beta Exotoxin, an ATP analogue which is a water soluble and heat stable secondary metabolite. It is an

inhibitor of RNA polymerase and acts competitively with ATP in various biological processes. Beta Exotoxin is toxic to almost all forms of life including humans and has a broad-spectrum insecticidal activity.

## 2.2 Classification of Insecticidal Toxin Genes

Genes coding for ICPs are located on large plasmids (> 30 MDa). Their sequences are not fixed, and they naturally evolve by recombination, which can give them new specificities. These genes were initially classed into two main families - *cry* genes, for crystal, and *cyt* genes for cytolysin. A new family of VIP genes has recently been described (Estruch *et al.*, 1996).

Generally, the sequences of genes encoding proteins active on different orders of insects are not well conserved. Rather, the gene sequences encoding a given crystal phenotype and proteins active against the same insect order are significantly more related. The sequence relatedness of ICPs as well as their insecticidal activity spectrum have been used to define an ordered classification of genes encoding Bt ICPs (Hofte and Whiteley, 1989).

Six major classes of ICP genes *cryI, cryII, cryIII, cryIV, cryV* and *cryVI* and several subclasses characterised by both the structural similarities and the insecticidal spectra of encoded proteins have been identified (Hofte and Whiteley, 1989 and Feitelson *et al.*, 1992). Over 150 of the genes that encode Cry toxins have been sequenced and enable the toxins to assigned in more than 15 groups on the basis of sequences similarities.

The nomenclature of Hofte and Whitely (1989), based mainly an insecticidal activity, failed to accomodate genes that were highly

homologous to known genes but with different insecticidal spectrum. Based on amino acid identity of full length gene product, Crickmore *et al.* (1998) have introduced a systematic nomenclature for classifying the *cry* genes and their protein products. It is based on amino acid sequence homology, where each protoxin acquired a name consisting of Cry (or Cyt) and four hierarchical ranks consisting of numbers, capital letters, lower case letters and numbers (e.g. Cry25Aal), depending on the phylogenetic tree. Thus, the proteins with less than 45% sequence identity differ in primary rank (Cry1, Cry2 etc.) and 78% and 95% identity constitute the borders for secondary and tertiary rank, respectively.

## 2.3 Basic Structural Features of Endotoxins

Alignment of the Cry toxins reveals the presence of five conserved sequence blocks common to a large majority of the proteins. The C-terminal extention found in the longer protoxins is not part of the active toxin (it is digested by proteases in the insect gut) but it is believed to have a role in the formation of the crystal (Schnepf, 1998). The three-dimensional structures of three activated forms of Bt toxins, a Cry1 (Grochulski, 1995), a Cry2 and a Cry3 (Li *et al.*, 1991) have been solved by X-ray crystallography and are remarkably similar, each consisting of three domains (Figure 1). According to this, the first 285 residues are present as a bundle of seven amphipathic α-helices, wherein six are arranged in a circle, and helix 5 is in the center (domain I). Residues 286-500 are organised as β-prism, in which three anti-parallel β-sheets with similar topology are packed around a hydrophobic core (domain II). The remaining amino acids are also present as β-sheets and arranged like a sandwich (domain III). The core of the toxin molecule, encompassing the central

Fig. 1. **Three dimensional structure of an activated δ-endotoxin Cry3Aa** showing the three structural domains I, II and III. Domain I, corresponds to the N-terminus half of the activated toxin and is made of seven α-helices. This domain is involved in membrane insertion and pre-formation. Domain II has three exposed loops is involved in the recognition of a binding sites at the surface of the cell membrane and in the specificity of various proteins towards a given insect. Domain III, is thought to be involved in the stabilization of the toxin by protecting against proteases. Cry proteins produced by the transgenic plants usually correspond to the sum of domains I, II and III.

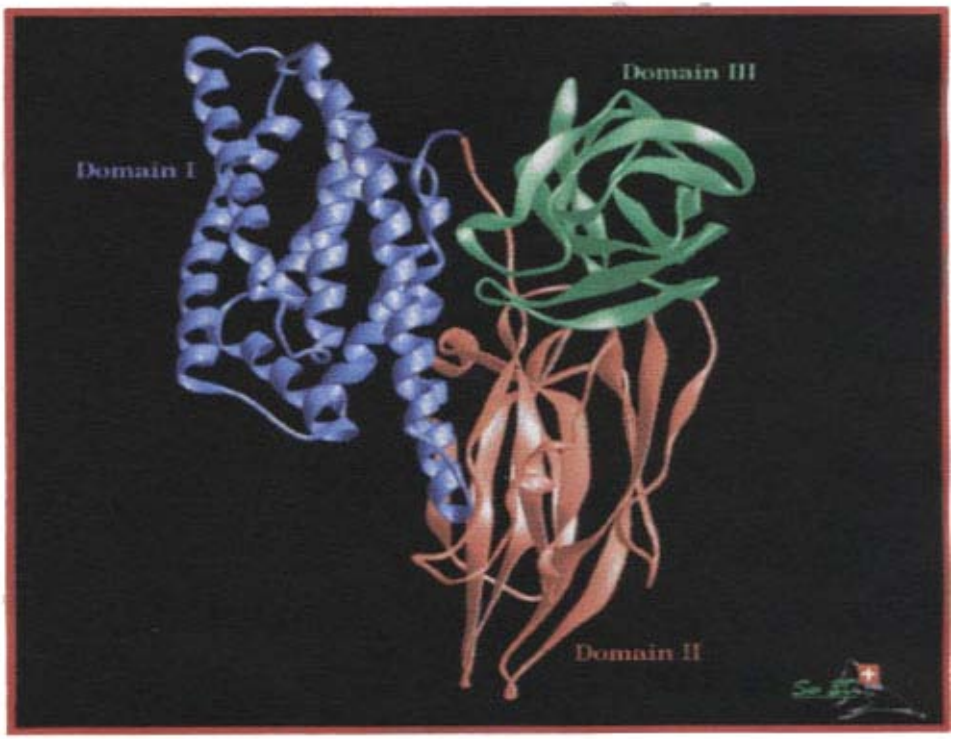



**Figure 1**

helix α 5 in the helical bundle and all the domain interfaces, are built up from five sequence blocks that are highly conserved.

The overall structure of the Bt toxin suggests a kind of "built-in" variability which gives Bt great flexibility in its actions in different target insects. The protein is made up of alternating conserved and variable regions. The N-terminal part contains the five conserved blocks and is responsible for toxicity and specificity. The C-terminal part is usually highly conserved and therefore responsible for crystal formation.

## 2.4 Mechanism of Action

The Cry proteins typically require both solubilization and activation steps before they became biologically active toxins. The main target for Bt toxins is insect midgut (Knowles, 1994). The crystalline protoxins are inactive until they are solubilized by gut proteases (Tojo and Aijawa, 1983; Gill *et al.*, 1992; Milne and Kaplan, 1993) which cleave rearly 500 amino acids from the C-terminus of 130 KDa protoxin and 28 amino acids from the N-terminus, leaving 55-65 KDa protease resistant active core comprising the N-terminal half of the protoxin (Hofte and Whitley, 1989). The Cry1A toxin is cleaved at the amino terminal R2 arginine residue (Nagamatsu *et al.*, 1984), and the carboxyl terminal K lysine residue (Bietlot *et al.*, 1989). A heterogenous DNA fragment of 20 Kb is involved in the proteolytic processing of the protoxin (Bietlot *et al.*, 1993). The 70 KDa Cry2, Cry3 and Cry4 proteins are naturally occurring truncated forms. The α-helices region of N terminal of toxin domain are considered important in penetrating the peritrophic membrane while the C terminal region and highly variable region (amino acid 280-460) are considered important in toxin specificity

by coding for open β-sheet that bind to glycoprotein receptors in the midgut.

Brush border membrane vesicles (BBMV) have been identified as the primary binding site for several insect species (Lee *et al.*, 1992). The active toxins bind to the specific receptors located on the apical brush border membrane of the columnar cells. There may be many toxin binding protein receptors, and some have been identified as 12 to 180 KDa glycoproteins (Garczynski *et al.*, 1991; Knowles *et al.*, 1991; Oddou *et al.*, 1991). A 210 KDa membrane protein is the receptor in *Manduca sexta* for Cry1Ab toxin (Vadlamudi *et al.*, 1995) and a 120 KDa aminopeptidase. N is the receptor for Cry1Ac toxin (Knight *et al.*, 1994). Cry1Ac binding amino peptidase in *Manduca sexta* has a glycosyl phosphatidylinositol anchor (Garczynski and Adang, 1995). After binding to the receptor, the toxin inserts irreversibly into the plasma membrane of the cell leading to lesion formation. There is a positive correlation between toxin activity and ability to bind BBMV (Gill *et al.*, 1992), and the toxicity is correlated with receptor number rather than receptor affinity (Van Rie *et al.*, 1989).

The toxicity of Bt lies in the organisation of α-helices derived from domain I. After binding to the midgut epithelial cells, the α-helices can penetrate the apical membrane to form an ion channel (Knowles and Dow, 1993). The formation of toxin induced pores in the columnar cell apical membrane allows rapid fluxes of ions. The pores are K+ selective (Sacchi *et al.*, 1986), permeable to cations (Wolfersberger, 1989), permeable to anions (Hendrickx *et al.*, 1989), permeable to solutes such as sucrose, irrespective of the charge (Schwartz *et al.*, 1991). Carroll and Ellar (1993) observed that midgut permeability in the presence of Cry1Ac was altered

for cations, anions, neutral solutes and water. Knowles and Dow (1993) suggested that Bt toxins lead to cessation of $K^+$ pump that leads to swelling of columnar cells and osmotic lysis. The disruption of gut integrity leads to death of the insect through starvation or septicemia. These pores possess both selective (only $K^+$ passes through) and non-selective ($Na^+$ and anions pass through) properties depending on the pH (Schwartz *et al.*, 1993). The lepidopteran insect midgut is alkaline and the pores probably permit $K^+$ leakage. Formation of this cation selective channel destroys the membrane potentials (English and Slatin, 1992) resulting in midgut necrosis, degeneration of peritrophic membrane and epithelium and ultimately bacterial septicemia, which occurs after larval death due to toxins (Sneh and Schuster, 1981; Salama and Sharaby, 1985).

Differences in the extent of solubilization of different toxins may explain the differences in the toxicity of various proteins (Meenakshisundaram and Gujar, 1998). Decreased solubility could be one potential mechanism of insect resistance to Bt proteins (McGaughey and Whalon, 1992). In cotton bollworm (*Helicoverpa zea*), Cry2A is less soluble than than CrylAc and fails to bind to a saturable binding component in the midgut brush border membrane (English *et al.*, 1995),. The unique mode of action of Cry2A may provide a useful tool for management of resistance to Bt toxins. Although binding of Cry toxins to the receptors determines the species sensitivity to various toxins, there are distinct exceptions, e.g.; Cry1Ac binds to the ligand bands of beet armyworm (*Spodoptera exigua*) brush border membrane proteins, but there is very little toxicity to the insect (Garczynski *et al.*, 1991). CrylAb is more toxic to the gypsy moth than CrylAc, but does not bind well with the receptors in the brush border membrane (Wolfersberger, 1990).

### Symptoms

Larvae affected by Bt become inactive, stop feeding, and may regurgitate or have watery excrement. The head capsule may appear to be overly large for the body size. The larvae become flaccid and die, usually within days or weeks. The body contents turns brownish-black as they decompose.

## 2.3 Delivery Systems of Bt Cry Proteins

Current research on Bt focuses not only on novel means of using more potent strains of Bt to kill a wider variety of insects, but also new ways to deliver the bioinsecticide to the field and insect-resistant plants. different techniques for using the genetically engineered Bt endotoxin gene to combat insect pest include sprays, transgenic microorganisms and transgenic crop plants.

### 2.3.1 Spray Formulations

*B. thuringiensis* has proven to be a valuable alternative to conventional insecticides. It is highly active and harmless to the environment owing to its specificity. Formulations of Bt spore-crystal mixtures are commercially available for use as biological insecticides in agriculture and forestry. Bt subspecies *israelensis*, active against larvae of mosquitoes and blackflies, is being used to control vectors of a variety of human and animal diseases. There were nearly 200 registered Bt products having more than 450 uses and formulations (Schnepf *et al.*, 1998). The earliest commercial production of Bt began in France in 1938 under the trade name sporeine (Luthy *et al.*, 1982). During the 1960s, several industrial formulations of Bt were manufactured in the United States,

France, Germany and Soviet Union. The isolation of the highly potent *kurstaki* variety by Kurstak in 1962 and by Dulmage in 1967 (Dulmage, 1970) provided a much needed boost to the commercialization of Bt. The HDI isolate of Dulmage is still the active ingredient in most Bt products used against caterpillar pests in agriculture, horticulture and forestry. The use of conventional Bt insecticides, however, was found to have limitations like narrow specificity, short shelf life, low potency, lack of systemic activity, and presence of viable spores (Lambert and Peferoen, 1992). These problems are now overcome by various approaches that utilize the tools of molecular biology and genetic engineering as well as conventional microbiological methods (Kumar *et al.*, 1996). Currently over 180 Bt products are registered in USA. It is compatible with many pesticides and can be used mixed with chemical insecticides. Pesticides made by using strain *B. thuringiensis* subspecies *kurstaki* include Biobit, Dipel, MVP, Steward, Thuricide which are effective on the larval stages of vegetable insects - cabbage looper, diamondback moth, tomato and tobacco hornworm, fruit worms; field and forage crop insects - European corn borer, alfalfa caterpillar, alfalfa webworm; tree and shrub insects - tent caterpillars, leaf rollers, red-humped caterpillar, pine budworm, jackpine budworm; fruit crop insects - leafrollers, Achemon, Sphinx; forest insects - leaf eating caterpillars, such as gypsy moth, spruce budworm, hemlock looper, tent caterpillar and others. Pesticides manufactured using subspecies *tenebrionis* include trident, M-One, M-Trak, Foil, Novodor etc. which are effective against the Colorado potato beetle, elm leaf beetle, cottonwood leaf beetle. Subspecies *israelensis* pesticides include Vectobac, Skectal, Gnatrol, Bactimos effective against larval stages of mosquitoes, blackflies, fungus gnats. Methods for application of Bt formulations include

aerial spraying (forest crops), water treatment by aerial or ground equipment (vector control), soil application by drip or overhead irrigation systems (field, fruit and vegetable crops), foliar application by spraying from a vehicle, backpack or hand held sprays (field, vegetables and fruit crops).

There can be 5 or 6 different plasmids in a single Bt strain, and these plasmids can encode different toxin genes. The plasmids can be exchanged between Bt strains by a conjugation-like process, so there is a potentially wide variety of chains with different combinations of Cry toxins. In addition to this, Bt contains transposons (transposable genetic elements that flank genes that can be excised from one part of the genome and inverted elsewhere). All these properties increase the variety of toxins produced naturally by Bt strains, and provide the basis for commercial companies to create genetically engineered strains with novel toxin combinations. One of the first such product was a Bt strain marketed as Raven® for enhanced control of Colorado potato beetle as well as for caterpillars that attack potato, tomato and aubergine plants (all Solanaceae family). This Bt strain contained two different beetle-active CryIII proteins (with different binding affinities for midgut cell membranes of Colorado beetle) as well as two caterpillar-active CryI proteins. Such an approach called gene pyramiding - is designed to delay development of resistance in target pests, because resistance would have to develop simultaneously to several different toxins.

The conjugational approach to create novel Bt strains has certain limitations. Not all the Bt toxin genes are located on transferable plasmids. Second, the toxin protein with useful insecticidal activity may be synthesized at low amounts. Plasmid incompatibility could also be a problem.

### 2.3.2 Transgenic microorganisms

Limited field stability is a problem related to commercial Bt preparation. To overcome this cloned Bt toxin genes were introduced into a number of microbial hosts to create more stable and compatible agents for the toxin activity.

The primary rationale for using live endophytic or epiphytic bacteria as hosts is to prolong the persistence of Cry proteins in the field by using a host that can propogate itself at the site of feeding and continue to produce crystal proteins.

Crystal genes were introduced into *E. coli, B. subtilis, B. megaterium* and *Pseudomonas fluorescens* long before there was an efficient transformation system available for *B. thuringiensis* (Gawron-Burke *et al.*, 1991).

Monsanto scientists were the first to report the expression of the *cry1Ab* gene in a root-colonizing bacteria (*Pseudomonas*) at levels sufficient to kill lepidopteran larvae. The gene was later cloned into Tn5 and transposed into the chromosome of six corn root-colonizing strains of *Pseudomonas fluorescens* and *Agrobacterium radiobacter.* Mycogen has pioneered a new biopesticide delivery system called Mcap, which encapsulates the Bt endotoxin inside a dead cell. the endotoxin gene is moved into a *Pseudomonas* bacteria. The bacteria is then treated so that the cells containing the bioinsecticide are killed, but the endotoxin is encapsulated and 'fixed' inside. Crop Genetics International has field tested a Bt derived insecticide which is designed to kill corn earworms in corn plants. The company has genetically engineered an endophyte

(*Clavibacter xyli*) to contain this *cry1Ac* gene. the recombinant bacteria can be inoculated into the stems to establish an endogenous supply of the toxin for protection against European corn borer (Lampel *et al.*, 1994).

Endophytic isolates of *B. cereus* have been used as hosts for the *cry2Aa* gene (Mahaffee *et al.*, 1994) and *B. megaterium* isolate that persists in the phyllosphere has been used as a host for *cry1A* genes. Similarly, *cry* genes have been transferred into other plants colonizers, including *Azospirillum* spp., *Rhizobium leguminosarum, Pseudomonas cepacia* and *P. fluorescens* (Obukowicz *et al.*, 1986). Alternative delivery system have also been sought for the dipteran-active toxins of Bt subsp. *israelensis* to increase their persistance in the aquatic feeding zone. Such host include *Bacillus sphaericus, Caulobacter crescentus* and the cyanobacteria *Agmenellum quadruplicatum* and *Synechococcus* spp.

### 2.3.3 Transgenic crop plants

An elegant and perhaps the most effective delivery system for Bt toxins is the transgenic plant. The major benefits of this system are economic, environmental, and qualitative. In addition to the reduced input cost to the farmers, the transgenic plants provide season long protection independent of weather conditions, effective control of burrowing insects difficult to reach with sprays, and control at all of the stages of insect development. The important feature of such a system is the only insects eating the crop are exposed to toxin. Genetic transformation of almost all the major crop species is now feasible. The first reports of transgenic insect-resistant plants were published in 1987 (Hilder *et al.*, 1987 and Vaeck *et al.*, 1987). Since then more than 40 different genes conferring insect resistance have been incorporated into crops, and the insect-resistant

crops have been commercialized in several countries since 1996 (Kumar, 2002) (Table 1).

The level of resistance Bt transgenics confer in most cases depends on whether native bacterial or truncated, codon optimized genes have been used (Peferoen, 1997). Codon optimized genes have been transferred in some crops, including cotton, maize, potato, broccoli, cabbage and alfalfa. It is not possible to use complete toxin genes in plants because these are not sufficiently soluble in plant cells (the protoxins are only soluble at pH greater than 9.5, whereas the pH in plant cells is around 7.6). This problem is circumvented by using truncated genes which produce almost fully activated toxin molecules and resides in the plant cells in solubilised form.

A number of factors have been developed for transferring the genes of interest into crop plants. The system involves a marker gene for resistance to antibiotics or herbicides, a replication site, and a multiple cloning site (MCS) with several restriction sites for DNA insertion. Foreign DNA (Bt gene) can be inserted in the vector using restriction enzymes that recognise a specific DNA sequence. Insertion of foreign DNA interscripts gene expression of an identifiable protein product to indicate DNA incorporation. For expression of the Bt gene in the higher plants, a recognisable promoter and a terminator sequence must border the Bt gene. Popular constitutive promoters include Cauliflower. Mosaic virus (CaMV35S) and ubiquitin. Tissue specific promoters include PEPC (Phosphenolpyruvate carboxylase) for green tissues and maize pollen specific promoter (Koziel *et al.*, 1993). The size of the vector ranges from 5000 to 10,000 bps depending on the Bt gene and the promoter

incorporated in the vector (Koziel *et al.*, 1993). Two commonly used methods for delivery of the vectors into the nucleus to obtain transgenic plants are bombarding plant tissues using a gene gun (biolistic method) or inoculating them with *Agrobacterium tumefaciens* bacterium. Transformed cells are identified selected and then cultured to regenerate plants.

The first transgenic tobacco plants with Bt were produced in 1987 (Barton *et al.*, 1987; Fischoff *et al.*, 1987 and Vaeck *et al.*, 1987). These plants expressed full length or truncated Bt toxin genes (*cry1A*) under the control of constitutive promoters. The expression was quite low in tobacco plants, resulting in only 20 % mortality of tobacco hornworm (*Manduca sexta*) larvae. Truncated *cry1A* genes encoding for the toxic N-terminal fragment provided better protection to tobacco and tomato plants. Plants transformed with truncated gene expressed about 0.2% of total leaf soluble protein. Gene truncation, use of different promoters, enhancer sequences and fusion proteins resulted in only a marginal improvement in gene expression (Barton *et al.*, 1987; Perlak *et al.*, 1990 and Carozzi *et al.*, 1992).

Tissue specific regulation of Bt *cry1Ab* gene has been utilised to achieve high and regulated expression in the leaves and pollen grains. The promoter derived from PEPC controls the expression of *cry1Ab* in green tissues (Hudspeth and Grula, 1989), while the promoter derived from calcium dependent protein kinase (CDPK) gene is pollen specific (Estruch *et al.*, 1994). Combination of green tissue specific PEPC and pollen specific CDPK tissue promoters provides high *cry1Ab* gene expression in leaves and pollen, where it is most effective in controlling

the Europaen corn borer (*Ostrinia nubilalis*). the intron 9 of maize PEPC is located between *cry1Ab* structural gene and the 35S terminator, and its presence increased the expression of Bt gene (Hudspeth and Grula, 1989). Three untranslated termination sequences from CaMV35S are present to the PEPC intron 9 and provide the polyadenylation site (Rothstein *et al.*, 1987). The catalytic activity of mature CDPK protein in maize is affected by calcium channels. Fusion of this sequence to *cry1Ab* does not manifest any changes in the calcium requirements of the maize plant.

Marker-free transformation system has also been used in creating Bt transgenics (Yoder and Goldsborough, 1994). Isopentyl transferase gene has also been used to develop marker free plants (Ebinuma *et al.*, 1997). The advances in genetic transformation over the past two decades are now being successfully employed to develop crop plants with durable resistance to insects.

Considerable progress has been made in developing transgenic crops with resistance to the target pests over the last decade (Milder and Boulter, 1999). Such transgenics have shown good promise in reducing insect damage, both in laboratory and field conditions. Successful control of pink bollworm (*Pectinophora gossypiella*) has been achieved through transgenic cotton (Wilson *et al.*, 1992). In transgenic cotton BTK, the mean per cent injury has been observed to be 2.3 in flowers and 1.1 in capsules compared to 23 and 12% in Coker 312, respectively (Benedict *et al.*, 1996). The cotton seed yield being 1050 kg per ha in Coker 312 compared to 1460 kg per ha in BTIC,. Significant variation in insecticidal activity has been observed in transgenic plants at different growth stages during the season and in different parts of the cotton plant (Zhao *et al.*,

1998a). The efficiency of Bt in leaf and squares were high during the second generation of the insect, but declined in the third and fourth generation in North China. The surviving third and fourth generation larvae, after feeding on flowers of Bt cotton, fed on the bolls until pupation, which could cause selection in the field population of *H. armigera*. The increase in resistance was 7.1 fold after 17 generations of selection in the laboratory, with an average mortality of 67.2% for each generation. The resistance level of Bt cotton declined from high resistance in insects of Bt cotton. Adamczyk *et al.* (1998) observed no differences in survival in fall armyworm (*Spodoptera frugiperda*) larvae between the normal and *cry1Ac* transformed cotton cultivars and in the number of larvae that pupated and emerged as adults.

Field trails of transgenic maize with Cry type toxins have shown that they are highly effective against the European corn borer and can withstand up to 50 larvae per plant at the whorl leaf stage and about 300 larvae at the anthesis stage (Armstrong *et al.*, 1995). Leaf feeding is restricted to pin holes and the stem tunnelling to 0.2 tunnels per transgenic plant compared to 9 tunnels per plant in the non-transgenic control plants. With 2400 larvae at the mid-whorl stage and 1200 larvae at the anthesis stage, the leaf damage rating in the transgenic plant was 1.6 compared to 7.2 in the controls (damage evaluated on a 1-10 scale) and stem tunnelling was 1.7 cm in the transgenic plants transformed with *cry1Ab* gene compared to 59 and in the control plants (Koziel *et al.*, 1993). Maize plants with *cry1Ab* gene was resistant to the sugarcane borers (*Diatraea grandiosella* and *Diatraea saccharalis*) (damage rating 2.4-2.6 compared to 10.0 in the susceptible control with 50 larvae per plant at the 6-leaf

stage). However, only a slight reduction in damage was recorded due to the fall (leaf damage rating 8.0-8.7 compared to 9.5-10.0 in the controls) (Vergvinson *et al.*, 1997). Bt transformed plants also showed better resistance to *D. grandiosella* than those derived from the conventional host plant resistance breeding programme. Larvae collected 25 days after infestation were fewer and smaller on the transgenic hybrid CML 139 x CML 167 than the larvae collected from the control hybrid. The effectiveness of the transgenic hybrid was less pronounced against the sugarcane borer (*Diatraea saccharalis*). The level of resistance was comparable to the conventional host plant resistance. Transgenic maize expressing Cry9C, an insecticidal crystal protein from *Bacillus thuringiensis* subsp. *tolworthi*, effectively controlled both generations of the European corn borer (Jansen *et al.*, 1997). CBH 351 tested in plots containing only Cry9C tested in plots containing only Cry9C transgenic plants had 0.14 and 0.09 cm tunnelling per stalk compared with more than 30 and 23 tunnelling per stalk for the negative controls in the field trials conducted in Belgium and Iowa, respectively. Williams *et al.* (1997), observed that transgenic hybrids sustained significantly less leaf feeding damage than the resistant check by the fall armyworm and southwestern corn borer (*Diabrotica undecimpuncta howardi*). The high levels of resistance to fall armyworm and near immunity to south-western maize borer of transgenic maize hybrids provided the highest levels of resistance documented for both pests.

Arencibia *et al.* (1997) used a truncated *cry1Ab* gene in transgenic sugarcane plants under the control of the CaMV35S promoter. Transgenic sugarcane plants showed significant larvicidal activity against neonate larvae of sugarcane borer (*D. saccharalis*) despite low expression of *cry1Ab*.

Transformation of high quality rice of group V is a feasible alternative to sexual hybridization (Ghareyazie *et al.*, 1997). Truncated *cry1Ab* gene has been introduced into several cultivars of rice (*indica* and *japonica*) by microprojectile bombardment and protoplast systems (Datta *et al.*, 1998). The expression was driven by two constitutive promoters (35S from CaMV and Actin-1 from rice) and two tissue specific promoters (pith tissue and PEPC for green tissue from maize). Eighty one transgenic plants caused 100% mortality of the yellow stem borer (*Scirpophaga incertulas*). The transgene, *cry1Ab*, driven by different promoters showed a wide range of expression (low to high) of Bt protein stably inherited in a number of cultivars with enhanced yellow stem borer resistance. Maqbool (1998) transformed the rice cultivars Basmati 370 and M7 by using *cry2A* insecticidal gene against the yellow rice stem borer and the rice leaf folder. Nayak *et al.* (1997) reported that two rice lines transformed with synthetic *cry1Ac* were highly toxic to yellow stem borer larvae and reduced the insect feeding. Rice plants expressing *cry1Ab* and *cry1Ac* genes were highly toxic to striped stem borer (*Chilo suppressalis*) and yellow stem borer (*Scirpophaga incertulas*), with mortalities of 97 to 100% within 5 days after infestation. Bt genes have also been inserted into an elite maintainer line, R68899B with *cry1Ab* gene (Alam *et al.*, 1999). Insect bioassay showed enhanced resistance to yellow stem borer.

Successful expression of Bt gene has also been obtained in tomato (Delannay *et al.*, 1989). Transgenic potato plants containaing *cry1Ab* gene Bt884 and a truncated gene *cry1A(b)6* against potato tuber moth (*Phthorimaea operculella*) resulted in less damage to the leaves. However, the size of the leaf tunnels increased over time in plants containing only

Bt884 gene, while there was no increase in those containing *cry1A(b)6* (Jansen *et al.*, 1995). The latter also resulted in 100% mortality of the insects in tubers stored upto six months. Transformed brinjal plants have shown a significant insecticidal activity of transgenic brinjal fruits against the larvae of fruit borer (*Leucinodes orbonalis*) (Kumar *et al.*, 1998). A modified gene of *Bacillus thuringiensis* var. *tolworthi* encoding a coleopteran insect-specific Cry3B toxin has been used to transform the female parent of the eggplant commercial hybrid Rimina (Arpaia *et al.*, 1997). Twenty-three out of fourty-four plants showed significant insecticidal activity towards neonate larvae of Colorado potato beetle. Brinjal cultivar Picentia and the wild species *Solanum integrifolium* have also been transformed with both a wild type (wt) and four mutagenized versions of Bt43 belonging to Cry3 class (Innacone *et al.*, 1997). Transgenic plants obtained with the more modified versions, BtH and BtI are fully resistant to Colorado potato beetle (*Leptinotarsa decemlineata*) first and third-instar larvae, while Bt43Wt, BtE and BtF genotypes did not cause mortality and did not effect larval development. Synthetic *cry1C* gene introduced in broccoli (*Brassica oleracea* ssp. *italica*) provided protection not only from susceptible diamond back moth (*Plutella xylostella*) larvae, but also from diamond back moth selected for moderate levels of resistance to *cry1C*. *cry1C* containing transgenic broccoli was also resistant to the cabbage looper (*Trichoplusia ni*) cabbage butterfly (*Pieris rapae*). Selvapandian *et al.* (1998) transformed tobacco plants using Cry1Ia5 insecticidal toxin from Bt strain from India, which provided complete protection against *H. armigera*. The effectiveness of this toxin was comparable to *Cry1Ab* or *Cry1Ac* genes.

Bt transgenic broccoli has been transformed with *cry1Ac* and *cry1C* genes (gene pyramiding) against diamond back moth. After 24 generations of selection resistance to pyramidded two-gene plants was significantly delayed as compared with resistance to single gene plants) (Jan-Zhou Zhoa, 2003).

Following an alternative strategy, in 2003, Maliga and Svab (Watsman Institute), expressed *crylAc* gene in tobacco chloroplasts using chloroplast transformation vectors and particle bombardment technique. The transplastomic tobacco expressed the Bt toxin at very high level and achieved control of lepidopteran larvae. The advantages of such a strategy are manyfold :

1. The Bt gene does not need as modification because the chloroplast transcriptional and translational apparatus are typically prokaryotic.
2. It is possible to have many copies of the Bt gene in each cell.
3. The expression of the gene will be high if driven by promoters like rbcl and cab.
4. Because chloroplast are naturally inherited, there is no risk of pollen transfer of the Bt gene to related plant species or weeds.

The disadvantage of this approach lies in its tissue specificity. The stem and fruit borers cannot be controlled following this method.

Most of the transgenic plants developed so far contained Bt toxin genes under the control of powerful, constitutively active 35S promoter. However, expression of the Bt toxin gene throughout the plant growth and development and in tissues in which it is not needed may encourage resistance development by the target insect (Harris, 1991). Considerable

research effort is now directed towards concentrating expression in these parts of plant attacked by insects. An example is the deployment of a phloem specific promoter for genes providing resistance to phloem sucking insect pests such as aphids (Shi *et al.*, 1994). There is also potential for the use of wound-induced promoters, which lead to gene expression only when the plant is actually attacked (Finch, 1994 and Lazzeri, 1996). Other specific promoters that have been used with insect-resistance genes include seed-specific and pollen-specific promoters and promoters especially suited for use in monocotyledonous plants.

## 2.4 Sequence Modifications of Bt genes

In early experiments, both full length and truncated *cry* genes were introduced into tobacco and tomatoes. However, when these genes were fused with expression signals used in plant nucleus, Cry toxic expression was comparatively low to confer sufficient protection against pests under field conditions (Fischoff *et al.,*1987; Vaeck *et al.,* 1987)

The AT rich *Bacillus* DNA contains a number of sequences that could provide signals deleterious to gene expression in plants, such as splice sites, poly (A) addition sites, ATTTA sequences, mRNA degradation signals, and transcription termination sites, as well as codon usage biased away from that used in plants.

The Bt gene should be first converted from AT-rich (typical of bacteria) to GC rich (typical of higher plants) to increase toxin expression. Most changes are made to the third codon thereby minimising changes in the amino acid sequence and increasing the expression of Bt toxin by 10 to100 fold (Perlak *et al.,* 1991). Perlak followed two approaches to modify

the *cry1ab* and *cry1Ac* genes. One approach included selective removal of DNA sequences predicted to inhibit efficient expression of Bt gene at both translational and mRNA levels by site directed mutagenesis. These genes were termed partially modified (PM) genes. The other approach was to generate a synthetic gene with a fully modified (FM) nucleotide sequence taking into account factors such as codon usage in higher plants, potential secondary structure of mRNA, and potential regulatory sequences. The PM-*cry1ab* gene is approximately 96% homologous to the native gene with a GC content of 41% with the number of potential plant polyadenylation signal sequences (PPSS) reduced from 18 to 7 and the number of ATTTA sequences reduced from 13 to 7. The FM-*cry1Ab* is approximately 79% homologous to the native gene, with a GC content of 49% and the number of PPSS reduced to one and all ATTTA sequences removed. The toxin protein levels in transgenic tobacco and tomato harbouring these modified genes increased upto 100 fold over levels seen with the wild type Bt gene in plants.

Perlak *et al.* (1990) made a gene construct in which the first 1359 nucleotides were derived from FM-*cry1Ab* gene and the remaining sequence from PM-*cry1Ac* gene. The variant gene was placed under the control of CaMV 35S promoter containing a duplicated enhancer region. Cotton variety Coker 312 was transformed and the transgenic plants were shown to have total protection from *Trichoplusia ni* (Cabbage looper), *S. exigua* and *H. zea* (cotton boll worm). The maximum level of toxin protein was 0.1% of total soluble protein. Mansanto group also placed the FM-*cry1Ac* gene under the control of *Arabi dopsis thaliana* Rubisco small subunit promoter with its associated chloroplast transit peptide sequence (Wong *et al.*, 1992). Transgenic tobacco plants expressing this gene provided a

10 to 20 fold increase in *cry1Ac* mRNA and protein compared to gene constructs in which CaMV 35S promoter with duplicated enhancer region was used to express the same gene. The toxin protein was localized in the chlorolast and in the tobacco plants that produce the Bt protein nearly 1% of the leaf protein had the highest levels of Bt toxin proteins yet reported. The enhancement of Bt toxin protein levels in tissues in which Rubisco expression is highest may lead to very effective control of certain insect pests that feed on leaves and other green tissues.

Based on the codon usage of known rice genes, 66.6% of the codons in the coding regionof *cry1Ab* gene were attended to enhance its expression in rice plants (Fujimoto *et al.,* 1993). The overall GC content of the modified gene was 59.2% whereas that of the original gene was 37.6%. The monocotyledons, including cereals have higher GC content than thsoe from dicots. Thelevel of expression of the modified gene in transgenic rice was 0.05% of total soluble leaf protein. The plants were signifcantly resistant to two lepidopteran rice pests, leaf folder (*Cnaphalocrosis medinalis*) and yellow stem borer (*Chilo suppressalis*) Synthetic *cry3A* genes have also been expressed in tobacco and potato plants for the control of Colorado potato beetle (*Leptinotarsa decemlineata*) (Perlak *et al.,* 1993). The Russet Burbank potatoes were protected from damage by all insect stages in the laboratory, and dramatic protection was discernible at multiple field location (Perlak *et al.,* 1993) Tobacco and tomato plants expression of *cry1Ab* and *cry1Ac* genes have also been developed (Van der Salm *et al.,* 194) to lepidopteran insects. The sequence motiffs that affect mRNA stability in plant cells were removed from the Bt

genes. The expression of *cry1Ab* - *cry1Ac* genes provided protection against *S. exigua, M. Sexta* and *H. virescens.*

A codon modified *cry1Ac* gene has been introduced into groundnut (Singsit *et al.,* 1997). Feeding bioassay indicated various levels of resistance to lesser corn *stalkborer* (*Elasmopalpus lignosellus*), from complete larval motality to 66% reduction in larval weight.

### *2.5* Bt *cry1F* gene

*cry1F* gene was first isolated from a novel grain dust isolate of *B. thuringiensis* subsp. *aizawai* by Chambers *et al.* in 1991. The Cry1F was found to be distinctly different in protein sequence and insecticidal specificity from the other Cryl proteins. It is a 133.6 KDa molecular weight protein and found to be toxic to insects of the order Lepidoptera.

The nucleotide sequence analysis of *cry1F* (Chambers *et al.*, 1991) shows sequence homology of *cry1F* and Cry1F to other ICP genes/proteins as summarised in Table 2. The nucleotide sequence of *cry1F* gene is only about 67 to 78% homologous (positionally identical) to those of the *crylA* subgroup, *crylB, crylC, crylD* and *crylE* genes. Among these crystal protein gene sequences, the DNA sequence of *cry1F* is most homologues to crylA(a) nucleotide sequence, with 77.6% of the nucleotides conserved between the two genes. Comparison with *cry2, cry3* and *cry4* genes revealed significantly less homology. The sequence of *cry2A* gene is most divergent with only 43.9% of the nucleotide conserved between the two genes. The insecticidal activity spectrum of the Cry1F protein is distinct from those of other Cryl crystal protein. Significant larvicidal activity is observed for a number of lepidopteran pests, including *H. virescens*

**Table 2. Sequence homology of *cry1F* and Cry1F to other ICP genes and proteins**

| Gene | Homology with *cry1F* | | |
|---|---|---|---|
| | | Amino acid | |
| | DNA | Total | *N-terminal region |
| *Cry1A(a)* | 77.6 | 71.7 | 51.0 (1-608) |
| *cry1A(b)* | 75.8 | 70.4 | 52.0 (1-609) |
| *cry1A(c)* | 75.8 | 69.9 | 49.0 (1-610) |
| *cry1B* | 66.6 | 58.3 | 40.1 (1-637) |
| *cry1C* | 75.3 | 70.0 | 48.8 (1-617) |
| *cry1D* | 75.6 | 71.5 | 52.0 (1-593) |
| *cry1E* | 77.2 | 69.8 | 48.1 (1-602) |
| *cry2A* | 43.9 | 24.6 | |
| *cry3A* | 53.0 | 35.6 | |
| *cry4D* | 44.5 | 20.8 | |

*Amino acid 1 to 602 of the Cry1F protein compared with the N-terminal regions of other Cryl proteins.

(tobacco budworm), *S. exigua* (beet armyworm) and *O. nubilalis* (Europaen corn borer). All the five consumed domains or homology boxes identified in Cry1 and Cry3 ICPs are also present in CrylF. The box I and 2 conserved domains are highly hydrophobic and comprise a toxicity domain capable of membrane insertion.

Synergistic effect of *Cry1Ac* and Cry1F δ-endotoxins observed on *Helicoverpa armigera* (cotton bollworm) suggests that the two toxins can be expressed together in transgenic crops for effective control of *H. armigera* (Chakrabarti *et al.*, 1998).

## 2.6 *Spodoptera litura* - Biology and pest status

The tobacco caterpillar, *Spodoptera litura* (Fabricus) (Lepidoptera :Noctuidae) is a polyphagous insect distributed throughout South East Asia and Eastern Australia. It has been reported to feed on 112 cultivated food plants belonging to 44 families all over the world (Moussa *et al.,* 1960) of which 60 are known from India (Lefrey 1908; Basu, 1943; Garad *et al.,* 1984). It is primarily a foliage feeder. It is the larval stages of this pest that cause severe damage to crops. It feeds on a wide range of food plants belonging to diverse botanical origin, several economically important crops such as cotton, peas, groundnut, castor, brinjal, banana and tobacco.

Tobacco caterpillar moths are medium sized and stout bodied, with front wings pale grey to dark brown in colour having wavy markings and whitish hind wing (Fig. 2). This pest can breed, develop at temperatures between $10^0$C to $30^0$C. Females lay numerous eggs in masses covered with brown hairs on tender leaves and they hatch in a period of four to five days. A single female can lay upto 2000 eggs. The caterpillars which are darker in colour, on hatching start feeding on the soft green layers of leaves during the night, both in seedbeds and planted fields. The caterpillars are pale greenish-brown and smooth, with dark marking and prothoracic plate and are about 37.5 mm long when full grown. The larval period lasts for 15-20 days. They pupate in rough earthen cocoons. The pupae are dark reddish brown in colour and the pupal period lasts from 7 to10 days and the total period of their lifecycle is 30 to 40 days.

High reproductive capacity and its ability to migrate over large distances in the adult stages are the main characteristics that makes *S. litura* a major pests throughout the world. In India, it is particulary notorious

**Fig. 2.** **Different stages of the life cycle of *Spodoptera litura***

(a) neonate larva
(b) Mature caterpillars
(c) and (d) Adult moth

(a)

(b)

(c )

(d)

Figure 2

for the damage it causes to tobacco (Nair, 1986). However, during the last 30 years it has become increasingly important on other crops -cotton, groundnut and mungbean in particular. On groundnut crop alone *S. litura* along with leaf viners cause an estimated loss of US$ 300 million world wide (Sharma and Ortiz, 2000a).

*S. litura* was one of the first pests of agricultural importance in India to develop resistance to insecticides (Armes *et al.*, 1997). By 1965 resistance to benzene hexachloride (BHC) was reported in field populations from Rajasthan and later to endosulfan and carboryl in Haryana and West Bengal, in Andhra Pradesh to lindane, endosulfan, carbaryl and malathion.

*Spodoptera litura* has become an increasingly important pest of groundnut, particularly in Andhra Pradesh, Karnataka, Tamil Nadu and Maharashtra which accounts for 15% of country's annual production (Amin and Mohammad, 1980).

Bt δ-endotoxins have also failed to show adequate insecticidal activity against this notorious pest. The genetic resistance induced by engineering novel insecticidal genes in crops, together with simple and appropriate management practices would be useful in alleviating the crop losses by this pest.

## 2.7 Other Insect Resistance Genes from Higher Plants

Currently, these are two major groups of plant derived genes used to confer insect resistance on crops - inhibitors of digestive enzymes and lectins. These have been transferred into crop plants without major alteration and expression has been at a similar level to codon optimised

Bt toxins. This approach, has however, so far, not resulted in the same high levels of insect control.

### 2.7.1 Protease inhibitors

Disruption of amino acid metabolism by inhibition of protein digestion has been a key target for use in insect control (Johnson *et al.,* 1989; Hilder *et al.,* 1992). Many insects, particularly lepidoptera, depend on serine proteases (trypsin, chymotrypsin and elastase endoproteases) as their primary protein digestive enzymes. Genes encoding number of various serine protease inhibitors (SPIs) have been cloned and introduced into transgenic plants. Other species of insects rely on thiol proteases (cysteine proteases) as their primary digestive protease. These can be targeted with thiol proteases inhibitors (TPIs). TPIs have been reported to be effective for controlling the maize rootworm (*Diabrotica* spp.) against which there are no effective Bt proteins (Edmonds *et al.,* 1996) transgenic tobacco plants expressing trypsin inhibitor gne at nearly 1% (derived from cowpea via CaMV35S constituve promoter) have resulted in increased mortality, reduced insect growth and reduced plant damage by *H. virescens* (Hilder *et al.,* 1987). Similar results have been shown against *H. zea, Spodoptera littoralis* and *M. sexta* (Hoffmann *et al.,* 1992; Thomas *et al.,* 194; Yeh *et al.,* 1997).

### 2.7.2 Alpha amylase inhibitors

Carbohydrate metabolism in insects has been targeted through the use of α-amylase inhibitors. Amylase inhibitors from wheat (WA AI) and common bean (BAA1) have been characterised. Transgenic tobacco expressing WAAI gene has been reported to increase mortality of the lepidopteran larvae between 30 to 40% (Carbmero *et al.,* 1993). Genes

encoding BAAI have been expressed in pea by the *Pha*1 gene promoter to direct high levels of expression in seeds to increase the level of resistance to *Collosobruchus* spp. (Shade *et al.,* 1994).

### 2.7.3 Plant lectins

Plant lectins are a heterogenous group of sugar binding proteins, which have a protective function against a range of organisms. Lectins from snowdrop, pea, wheat, rice, castor, soyabean, mungbean, garlic, sweet, potato, tobacco, chickpea and groundnut have been isolated and characterised. Lectins produce chronic effects on survival and development of insect pests belonging to different insect orders. Transgenic tobacco expressing pea lectin has shown adverse effects against *H. virescens* (Boulter *et al.,* 1990). Greater insecticidal activity has been observed in chitin binding lectins from wheatgerm and common bean. Sap sucking Hemiptera can be controlled by certain lectins. A gene encoding the mannose specific lectin from snowdrop expressed in tobacco showed enhanced resistance to peach potato aphid (*Myzus persicae*). Rice cystatin I in transgenic potato caused upto 53% mortality in larvae reared on transgenic leaves, compared with < 17% mortality in the control (Lecardonnel *et al.,* 1999). Concanavalin A inhibits development of tomato and peach-potato aphid when expressed in transgenic potato plants (Cao *et al.,* 1999). Transgenic sugarcane plants engineered to express either the potato inhibitor II or the snowdrop lectin gene showed increased antibiosis to larvae of sugarcane grubs (*Antitrogus consanguineus*) in glass house trials (Nutt *et al.,* 1999).

### 2.7.4 Vegetative insecticidal proteins

Supernatants of vegetative *Bacillus cereus* cultivar have two compounds : VIP1 and VIP2, which have been shown to possess toxic effects towards insects (Estruch *et al.,* 1997). VIP3 has been isolated from *B. thuringensis* supernatants, which is highly toxic to *Agrotis* and *Spodoptera* (Estruch *et al.,* 1996). The activity of these proteins is similar to δ-endotoxins. They induce gut paralysis, followed by complete lysis of the gut epithelium cells resulting in larval mortality.

### 2.7.5 Secondary plant metabolites

Many secondary plant metabolites such as alkaloids, steroids, foliar phenolic esters (rutin, chlorogenic acid etc.) terpenoids, cyanogenic glycosides, saponins, flavonoids, pyrethrin acts as potent protective chemicals. Some of the secondary plant metabolites are produced in response to insect feeding, infection by pathogens, and abiotic stress factors. These compounds are called phytolexins (Sharma and Norris, 1991) *Arabidopsis* mutants deficient in linoleic acid cannot synthesise jasmonate and are susceptible to the fungal gnat (*Bradasia impatiens*). Xu *et al.* (1993) observed enhanced resistance in rice by inducing methyl jasmonate and Abscissic acid in transgenic plants.

# 3. MATERIALS AND METHODS

## Materials

The bacterial strains and plasmids used in this study are listed in Table 3.

**Table 3. Bacterial strains and plasmids used in the study**

| | Strains/plasmids | Relevant characters |
|---|---|---|
| 1. | *Agrobacterium tumefaciens* (LBA 4404) | $Rif^R$ $Strep^R$ |
| 2. | *E. coli* (DH5α) | $Nal^R$ |
| 3. | *E. coli* (BL-21DE3) | Specific for expression vector pET-29a (+) carrying His.Tag sequence |
| 4. | Plasmid p39/66/6 | $Kan^R$, 44734 bp recombinant plasmid which contains modified *cry1F* gene domain I insert |
| 5. | Plasmid pET-29a (+) | $Kan^R$, 5371 bp carrying His-tag sequence |
| 6. | Plasmid pBluescriptII KS | $Amp^R$, 2961 bp, phagemid |
| 7. | Plasmid pBinAR | $Kan^R$, 11045 bp disarmed, plant transformation binary vector |

## Composition of culture media used

### Bacterial culture media

**Luria Bertani** (LB & LA) Complete medium for *E. coli*

| | | |
|---|---|---|
| Bacto Tryptone | - | 10 g |
| Yeast Extract | - | 5 g |
| Sodium chloride | - | 5 g |

Agar - 15 g (for plating medium LA)

Distilled water - 1000 ml

pH was adjusted to 7.2 with 0.1 N NaOH (Sambrook *et al.*, 1989).

**Yeast Extract Mannitol Broth/Agar** (YEM)

Yeast extract - 1 gm

Mannitol - 10 gms

NaCl - 0.1 gms

$MgSO_4.7H_2O$ - 0.2 gms

$K_2HPO_4$ - 0.5 gms

Agar - 1.5 gm (for plating medium)

Distilled water - 1000 ml

pH was adjusted to 7.2 with 0.1 N NaOH

**Plant tissue culture medium**

**Murashige and Skoog (1962) medium**

| **Constituents** | **Amount (mg/l)** |
|---|---|
| ***Macronutrients*** | |
| $NH_4NO_3$ | 1650.0 |
| $KNO_3$ | 1900.0 |
| $MgSO_4.7H_2O$ | 370.0 |
| $KH_2PO_4$ | 170.0 |
| $CaCl_2.2H_2O$ | |
| ***Micronutrients*** | |
| $H_3BO_4$ | 6.20 |
| $MnSO_4.H_2O$ | 16.90 |

| | |
|---|---|
| $ZnSO_4.7H_2O$ | 8.60 |
| $Na_2MoO_4.2H_2O$ | 0.25 |
| $CuSO_4.5H_2O$ | 0.025 |
| $CoCl_2.6H_2O$ | 0.025 |
| KI | 0.83 |
| ***Iron source*** | |
| $FeSO_4.7H_2O$ | 27.8 |
| $Na_2EDTA.2H_2O$ | 37.3 |
| ***Meso-inositol*** | |
| Myo-Inositol | 100.0 |
| ***Organics (with B5 vitamins)*** | |
| Glycine | 2.00 |
| Nicotinic acid | 1.00 |
| Pyridoxin HCl | 1.00 |
| Thiamine HCl | 10.00 |
| ***Carbon source*** | |
| Sucrose | 20,000.00 |

**Autoclaving and sterlization**

Sterilization of media, stock solutions, micropipette tips and glassware was done by autoclaving in a vertical autoclave at 15 lbs generating a temperature of 120$^0$C for 20 minutes.

**Antibiotics and hormones**

Stock solution of antibiotics and hormones were prepared in sterile distilled water and were stored in refrigerator at 4$^0$C. Antibiotics were added after media cooled to 50$^0$C.

**Enzymes and chemicals**

Various restriction endonucleases, calf intestinal alkaline phosphate enzyme and T4 DNA ligase enzyme were purchased from MBI Fermentas. All enzymes were used according to the instructions of the manufacturers.

Most of the chemicals were obtained from Sigma Chemical Company, St. Louis, MO, USA and were of Molecular Biology Reagent grade. Isopropyl alcohol and other chemicals were from Qualigens, Fine Chemicals, Bombay. Luria broth and agar were from Hi-Media Laboratories, Bombay. Chloroform and phenol were from BDH while ethanol was purchased from Bengal Chemicals, Calcutta.

$^{32}P$ dCTP used in the study was obtained from Bhabha Atomic Research Centre, Trombay, Mumbai.

**Other materials**

The two sets of primers used in the study for carrying PCR experiments were obtained on order from Genset Singapore Biotech. Ptc Ltd, Singapore.

Photographic and X-Ray Films (X - OMAT) was obtained from Kodak, USA along with developer and fixer. Polaroid film type 667 (3000 ASA) was obtained from Polaroid Corp., MA, USA.

Hybond N+ membranes were produced from Amersham.

## Methods

### 3.1 Truncation of the native full length *cry1F* gene

The wild type (WT) full length *cry1F* gene was truncated to ~1.9 Kb size through PCR to enable easy cloning and expression of the gene.

#### 3.1.1 Design of PCR primers

The set of gene specific primers (30 & 31 bp size) were designed from the published sequence for *crylF* (Gene bank Accession No. M63897) to truncate the gene to exactly 1.861 Kb size with additons of termination codon (TAA) and restriction site of endonuclease *Sal* I (GTCGAC) flanking the truncated gene to enable its cloning in vectors.

**Sequence of designed primers**

Forward primer : (nucleotide position 486 to 517)

5' CCGGTCGACATGGAGAATAATATATTCAAAATC - 3'
*Sal* I

Reverse primer : (nucleotide position 2330 to 2340)

5' GGCGTCGACTTAATTCACCGCCTTTTGTGC - 3'
*Sal* I

#### 3.1.2 PCR reaction set up

| | | |
|---|---|---|
| Primers 1 (concentration 300 nM) | - | 1 µl |
| Primers 2 (concentration 300 nM) | - | 1 µl |
| dNTPs (200 µM each) | - | 1 µl |
| *Pfu DNA polymerase enzyme* (5 unit/µl) | - | 0.5 µl |
| Template DNA (100 ng) | - | 2 µl |
| *Pfu* enzyme buffer (10 X) | - | 5 µl |
| Sterile distilled water | - | 39.5 µl |
| | | 50 µl |

**Temperature conditions**

Reaction was heated up at 95°C for 5 minutes followed by :-

| | | |
|---|---|---|
| Denaturation temperature | - | 94°C for 1 minute |
| Annealing temperature | - | 55°C for 1 minute |
| Primer Extention | - | 72°C for 2 minutes |

PCR programme was set for 30 cycles. At the end of 30 cycles additional incubation at 72°C for 5 minutes was performed to extend any premature synthesis of DNA.

The PCR products were purified through PCR purification kit (Qiagen) following the prescribed instructions.

### 3.1.3 PCR product digestion with Restriction Endonuclease *Sal* I and Agarose Gel Electrophoresis

Restriction was carried out in the following manner

| | | |
|---|---|---|
| PCR product | - | 10 µl |
| *Sal* I enzyme | - | 1 µl |
| *Sal* I buffer (10 X) | - | 2 µl |
| Sterile Distilled Water | - | 7 µl |
| | | 20 µl |

All the ingredients were mixed and the Eppendorf tube was incubated at 37°C in a water bath for 2 hours for complete digestion. The digestion was stopped by incubating the sample mixture at 65°C for 2 minutes.

**Agrose Gel Electrophoresis**

The *Sal* I digested amplified DNA was subjected to gel electrophoresis. For this purpose 0.8% (w/v) agarose was mixed in I X TAE buffer (40 mM Tris acetate, 1mM EDTA) and boiled for 2 minutes for complete melting. After cooling to about 50$^0$C, ethidium bromide (0.5 µg/ml) was added and poured into a gel casting tray fitted with comb. The solidified gel along with the tray was then placed in a running buffer tank. Running buffer ( I X TAE) was added in the buffer tank. comb was removed to make wells for loading DNA samples. The sample DNA was then mixed with 1/6$^{th}$ volume of DNA loading dye [0.25% (w/v) bromophenol blue, 40% (w/v) sucrose in water] and loaded into wells. Standard molecular marker (Gene Ruler$^{TM}$ 1 Kb DNA ladder, MBI fermentes) was also loaded. The DNA was electrophoresed at 10 V/cm$^2$ of gel using EC 105 power supply for 2 hours. The stained gel was kept directly on a UV transilluminator (UVP Inc., UK) and photographed.

## 3.2 Cloning of truncated *cryIF* gene in pBluescriptII KS vector

### 3.2.1 Elution of the PCR amplified DNA fragment

The required ~1.9 Kb PCR product (*t-cryIF* gene), to be used as the insert in cloning was eluted using QIAquick Gel Extraction Kit (Qiagen, Germany) standard protocol. Purity of the eluted DNA was checked by using UV spectrophotometer.

### 3.2.2 Isolation Restriction and Dephosphorylation of the plasmid pBluescriptII KS

**Isolation of the plasmid DNA**

Plasmid isolation by the Qiagen Midi prep plasmid DNA isolation Kit is based on optimised alkaline lysis method of Birnboim and Doly (1979).

*E. coli* carrying pBluescript IIKS were grown overnight in 100 ml LB at 37°C with ampillicin (100 µg/ml).

**Digestion of pBluescript-II KS DNA with restriction endonuclease *Sal* I**

Reaction was carried out following manner

| | | |
|---|---|---|
| plasmid DNA | - | 10 µl |
| (5 units/µl) *Sal* I enzyme | - | 1 µl |
| (10 X) optimum buffer | - | 2 µl |
| Sterile distilled water | - | 7 µl |
| Reaction volume | | 20 µl |

Contents were mixed by gentle tapping of the Eppendorf tube and incubated at 37°C for 2 hours for complete digestion. Reaction was stopped by incubating the tube again at 65°C for 2 minutes.

5 µl of the restricted vector DNA was observed on agarose gel against the standard molecular weight marker 1 Kb DNA ladder (from Gene Ruler, MBI Fermentas). The remaining sample DNA was immediately used for the dephosphorylation.

**Dephosphorylation of the restricted plasmid (pBluescriptII KS)**

Dephosphorylation was done using the enzyme calf intestinal alkaline phosphatase (CIAP). This enzyme removes 5' phosphate group of linear DNA thereby preventing self ligation of the vector.

**Reaction mixuture set up**

| | | |
|---|---|---|
| *Sal* I restricted pBluescriptII KS vector | - | 15 µl |
| (0.5 units/µl) CIAP | - | 0.5 µl |
| (10 X) CIAP buffer | - | 3 µl |
| Sterile distilled water | - | 11.5 µl |
| | | 30 µl |

The contents were mixed by gentle tapping and the Eppendorf tube incubated at 37°C in water bath for one hour. The reaction was terminated by incubating the tube at 75°C for 15 minutes.

### 3.2.3 Ligation and Transformation

Restricted and dephosphrylated vector was purified using QIA quick(R) PCR Purification kit as per manufacturer's instructions for further use in ligation experiment. Ligation of ~1.9 kb truncated *crylF* gene fragment to linearised pBluescriptII KS vector involved the formation of new bond between phosphate residues located at the 5' termini of double stranded DNA and adjacent 3' hydroxyl moities. Formation of this phosphodiester bond is mediated out by the enzyme T4 DNA ligase. For efficient ligation, vector and insert DNA were taken in the ratio of 1:1, 1:3 and 1:5 using the formulae :

$$\text{ng of vector} = \frac{\text{ng of insert x size of insert in kb x vector : insert ratio}}{\text{size of vector in kb}}$$

The following reactions was performed in 1.5 ml of Eppendorf tube:

| | | |
|---|---|---|
| (a) | Insert DNA (100 - 1000 ng) | - 7 µl |
| | Vector DNA (50 - 100 ng) | - 1 µl |
| | T4 DNA ligase enzyme (Weiss unit) | - 1 µl |
| | T4 DNA ligase enzyme buffer (10X) | - 1 µl |
| | | 10 µl |
| (b) | Control ligation set up : | |
| | Vector DNA (50 - 100 ng) | - 4 µl |
| | T4 DNA ligase (Weiss unit) | - 1 µl |
| | T4 DNA ligase buffer (10X) | - 1 µl |
| | Sterile distilled water | - 4 µl |
| | | 10 µl |

Both the reaction mixtures were incubated at 4°C for 16 hours overnight followed by heat inactivation of the enzyme at 65°C for 15 minutes and then transformed in *E. coli* strain DH5α.

**Preparation of competent cells and transformation**

**Preparation of competent cells of *E. coli* strain DH5α for transformation**

* Innoculated single colony of *E. coli* DH5α from LA plate in 5 ml of LB and kept for shaking at 220 rpm at 37°C overnight.

* Transferred 1 ml of overnight grown culture to 500 ml flask containing 100 ml of LB and 10 µl of nalidixic acid, incubated at

37°C with shaking at 220 rpm for 3 hours until the $A_{600}$ is 0.5 - 0.6.

* Transferred the overnight grown culture to chilled 50 ml polypropylene centrifuge tubes and kept on ice for 15 minutes.
* The cells were pelleted down by centrifugation at 7000 rpm for 5 minutes at 4°C.
* The supernatant was discarded and the pellet was resuspended in 30 ml of 0.1 M $CaCl_2$ (chilled).
* Incubated on ice for one hour.
* Cells were centrifuged at 3000 rpm for 15 minutes at 4°C.
* Pellet was resuspended in chilled 0.1M $CaCl_2$ and 30% ultrapure glycerol.
* Incubated the cells on ice for 15 minutes.
* Transferred 100 ml aliquots into 1.5 ml microfuge tubes, frozen in liquid nitrogen and stored at -70°C to be further used in transformation.

**Transformation of *E. coli* DH5α with truncated *cryIF* cloned in vector pBluescriptII KS**

Transformation was carried out following the method described by Mandel and Higa (1970). Competent cells of *E. coli* DH5α strain were thawed and placed on ice. Competent cells were mixed with 5 µl of the ligated mixture and kept on ice for 30 minutes. A heat shock at 47°C for 60 seconds was given and the cells were immediately incubated on ice for 2 minutes. The cells were kept at room temperature for 5

minutes. 900 µl of LB medium was added to the tube and incubated at 37°C with shaking (200 rpm) for one hour. The cells were pelleted down and resuspended in 100 µl of LB medium. LA medium containing Kanamycin (50 mg/ml) and spread plated with 20 µl of 0.2 M IPTG and 40 µl of 5% (w/v) X - Gal plates were prepared. The plates were kept in dark for 20-30 minutes. Transformed *E. coli* cells were spread on the plates and plates were incubated overnight at 37°C.

The recombinants were picked up by blue - white colour selection. White colonies are the *E. coli* that carry recombinant vectors which can be further checked by restriction analysis.

## 3.3 Expression Analysis of *crylF* gene in *E. coli* (BL-21DE3)

### 3.3.1 Cloning of truncated *crylF* in expression vector pET29a

pET29a is an expression vector which contains a powerful T4 polymerase promoter that generates large amount of mRNA complementary to cloned sequences of foreign DNA. It is an efficient system for cloning and expression of recombinant proteins in *E. coli.*

The ~1.9 kb truncated *crylF* gene was extracted from the pBluescript KS-IF vector by restriction with enzymes *Kpn* I and *Hin*d III and further cloned into pET29a (Novagen) vector previously digested with the same restriction enzymes (*Kpn* I and *Hin*d III).

**Transformation of pET-If vector in *E. coli* strain BL-21DE3**

BL-21DE3 *E. coli* strain is employed for high level expression of genes cloned in expression vectors containing bØT7 promoter. ØT7 RNA polymerase is carried on the ØλDE3 which is integrated into the chromosome BL21.

Transformation protocol followed was same as in the case of pBluescriptKS vector. Recombinat colonies were checked on plate containing LA medium with antibiotic Kanamycin (50 mg/l).

### 3.3.2 Isolation of CrylF crystal protein from over expressing *E. coli*

100 ml of LB medium was inoculated with *E. coli* BL-21DE3 cells harbouring truncated *cryl F* gene cloned in vector pET 29a and was grown overnight at 37°C at 220 rpm. IPTG (1 mM) was added to it and incubated again at 37°C for 24 hrs. The cells were harvested by centrifugation at 7000 rpm for 10 minutes. Pellets was resuspended in 50 ml lysis buffer with 0.5 mg/ml lysozyme. The reaction mixture was incubated at 37°C with slow shaking for 3-4 hours. The cells were pelleted and resuspended in chilled crystal wash I solution. The sample was subjected to sonication with large tip on heat system sonicator set on timed output-2 second pulse, 50% duty cycle, output control setting 8 and timer on 2 minutes. The cycle was repeated 3 times for one minute each. Sonicated cells were pelleted and resuspended in 50 ml crystal wash I solution and centrifuged. Pellet was washed twice with crystal wash I solution, centrifuged and washed thrice with crystal wash II solution and finally thrice with sterile distilled water.

**Solubilization of the crystal protein**

The sample was centrifuged and the supernatant was carefully decanted. The pellet was resuspended in 10 ml of solubilization buffer and incubated at 37°C for 4 - 6 hours with slow shaking. The suspension was centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a sterile Eppendorf tube and is referred to as protoxin.

The final yield of the solubilized protoxin was estimated by Bradford protein quantification assay. Analysis of the protoxin was done by SDS - PAGE.

### 3.3.3 Analysis of the t-CryIF (truncated) protein by SDS-PAGE

In order to analyse for the expression of the *t-crylF* gene cloned in expression vector pET29a, SDS - Polyacrylamide Gel Electrophoresis was carried out. The denatured polypeptides of the protein bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS - polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide.

**Preparation of SDS - polyacrylamide Gels :**

The glass plates in the SDS - PAGE apparatus were assembled according to manufacturer's instruction 10 ml of 10% resolving gel was prepared in the following manner

| | | |
|---|---|---|
| $H_2O$ | - | 4.0 ml |
| 30% acrylamide mix | - | 3.3 ml |
| 1.5 M Tris (pH 8.8) | - | 2.5 ml |
| 10% SDS | - | 0.1 ml |
| 10% ammonium persulphate | - | 0.1 ml |
| TEMED | - | 0.004 ml |

The resolving gel components were rapidly poured into the gap between the glass plates. Polymerization was allowed to complete. One ml of 5% stacking gel was prepared in the following manner

| | | |
|---|---|---|
| $H_2O$ | - | 1.4 ml |
| 30% acrylamide mix | - | 0.33 ml |
| 1.0 M Tris (pH 6.8) | - | 0.25 ml |
| 10% SDS | - | 0.02 ml |
| 10% ammonium persulphate | - | 0.02 ml |
| TEMED | - | 0.02 |

Clean Teflon comb was immediately inserted into the stacking gel solution, avoiding air bubbles and allowed to polymerise.

**Preparation of sample protein**

The protein samples were prepared by heating them to 100°C for 3 minutes in 1 X SDS gel loading buffer to denature the proteins. The marker proteins of known molecular weight were also denatured by boiling at 100°C for 2 minutes.

**Preparation of the Tris-glycine electrophoresis buffer**

5 X stock was made by dissolving 15.1 g of tris base and 94 g of glycine in 900 ml of deionized $H_2O$. 50 ml of a 10% (w/v) stock solution of electrophoresis - grade SDS is added, and the volume is adjusted to 1000 ml with $H_2O$.

15 µl of sample and marker protein were loaded in the wells and allowed to run at a voltage of 15 v/cm.

**Staining and Destaining of the Gel**

Polypeptides separated by SDS-polyacrylamide were fixed by placing the gel in staining solution for 3-4 hours with slow shaking. The excess dye was then allowed to diffuse out from the gel by destaining for 2-4 hours.

The destained gel was stored in water and later photographed.

## 3.4 Analysis of toxicity of truncated CryIF protein to *Spodoptera litura*

The toxicity of truncated CryIF protein was tested against the neonate larvae of *Spodoptera litura*. Different concentrations of truncated toxin were spread on leaf disks of the host plant (castor).

### Leaf disk assay

Leaf disk assays were performed in 6-well culture plates (Grenier Labrotechnick, Germany). Disks of 3 cm diameter were cut using a cork borer from castor leaves. Stock solutions of the truncated toxin were prepared in autoclaved distilled water and desired concentrations were spread on the leaf disks (ng/cm$^2$) and air dried. A single disk was placed on moist Whatman Number 1 filter paper (cut to well size) in each well. Ten first instar larvae were released on each leaf disk with a paint brush and the plates were tightly sealed with Saran wrap to prevent the larvae from escape. The plates were kept at 28 ± 2$^0$C, 60 ± 5% humidity and 14 hours photophase. Change in feeding habit, weight and mortality were recorded after 5 days. Each treatment was replicated five times.

## 3.5 PCR based substitution of domain I in truncated *cryIF* by modified *cryIF* domain I

The synthetic *cryIF* gene was designed by using Lasergene programme DNA STAR. The synthetic gene was subdivided into six fragments for convenient synthesis and assembly. Fragments carry restriction sites at 5' and 3' ends. p39/66/6 vector carries four such fragments which constitute the modified domain I.

### 3.5.1 Designing of PCR primers and PCR reaction

The set of gene specific primers (24 and 23 bp in length) were designed so as to incorporate *Xba* I restriction enzyme site in forward primer and *Pst* I restriction enzyme site in the reverse primer. The primer sequences are

Forward : 5' TCT AGA ATG GAGAAC AAC ATC CAG 3'
*Xba* I

Reverse : 5' GCT CTG CAG TTA CGA CGA AAG AG 3'
*Pst* I

**PCR reaction**

The PCR reaction was performed in 50 µl of reaction volume containing

| | | |
|---|---|---|
| Primer 1 (250 mM of each) | - | 1 µl |
| Primer 2 (250 mM of each) | - | 1 µl |
| dNTPs (200 µm each) | - | 1 µl |
| *Pfu DNA polymerase enzyme* (5 units/µl) | - | 0.5 µl |
| Template DNA (100 ng) | - | 2 µl |
| *Pfu* DNA polymerase enzyme buffer (10X) | - | 5 µl |
| Sterile distilled water | - | 39.5 µl |
| | | 50 µl |

**Temperature conditions**

Reaction was heated upto 95°C for 5 minutes followed by

| | | |
|---|---|---|
| Denaturation temperature | - | 95°C for 1 minute |
| Annealing temperature | - | 52°C for 1 minute |
| Primer extention temperature | - | 72°C for 2 minutes |

The programme was set for 32 cycles and at end of the last cycle additional incubation at 72°C was performed for 5 minutes to extend any premature synthesis of DNA.

**Agarose Gel Electrophoresis**

Amplified DNA was subjected to gel electrophoresis. The stained gel was checked for PCR amplified DNA (920 bp) under the UV transilluminator and photographed.

**Elution of DNA from the Agarose Gel**

Following electrophoresis, the desired 920 bp DNA fragment was eluted using QIA quick(R) Gel extraction kit (Qiagen, Germany) as per the manufacturer's instructions.

### 3.5.2 Ligation of domain I of modified *crylF* with domain II and III of native truncated *crylF*

10 µg of pBinIF vector (~13 kb) carring the truncated *crylF* gene (*t-crylF*) was partially digested with 0.1 u of *Pst* I restriction enzyme for 5 minutes at 37°C and completely digested with 10 units of restriction enzyme *Xba* I for 2 hours at 37°C. The restricted product was electrophoresed on 1% agarose gel and double digested pBinIF vector (~ 13 kb) was isolated using Qiagen gel extraction kit.

The DNA fragment (920 bp) from V(a) was subjected to complete digestion with 5 units of *Xba* I for 2 hours and 5 units of *Pst* I for 2 hours at 37°C. The restricted product was PCR purified using QIA quick(R) PCR purification kit (Qiagen, Germany).

100 ng of vector DNA (pBinIF excluding *crylF* domain 1 sequence) and 300 ng of insert (920 bp modified *crylF* domain sequence) were used for ligation using 10 units of T4 DNA ligase at 4°C for 16 hours. The reaction was inactivated by heating in a water bath at 65°C for 10 minutes. After ligation, transformation of the resultant plasmid DNA was carried out as described in section II(c).

The recombinant colonies were picked up from LA medium plates containing antibiotic kanamycin (50 mg/l) and further analysed for the presence of chimeric *crylF* gene by restriction analysis.

## 3.6 *Agrobacterium tumefaciens* - mediated transformation of tobacco with pBinAR carrying truncated *crylF* gene

### 3.6.1 Cloning of *t-crylF* (truncated *crylF*) gene in plant transformation vector pBinAR

BinAR is a Bin19 derivative containing expression cassette for constitutive expression of chimeric genes in plants. It has a multiple cloning site between CaMV 355 promoter and *nos* terminator (Hofgen and Willmitzer, 1990).

Cloning at restriction enzyme site *sel* I was carried out following the protocols used earlier. Recombinant colonies were checked on LA medium plates containing kanamycin (50 mg/l).

### 3.6.2 Transformation of *Agrobacterium tumefaciens* strain LBA4404 with *t-cry1F*

The binary vector pBinT-1F carrying truncated *cry1F* gene was transformed into *A. tumefaciens* strain LBA 4404 containing the Ti helper plasmid pAL4404 (which encodes virulence genes needed for T-DNA

transfer) by freeze-thaw transformation method (Hofgen and Willmitzer, 1988). The plasmid pBinT-1F was isolated from *E.coli* using Qiagen Midiprep isolation kit following the standard protocol. *A. tumefaciens* LBA 4404 stock from -70°C was streaked on YEM medium containing 10 µg/ml rifampicin and 15 µg/ml streptomycin, solidified with 1.5% (w/v) agar and incubated at 28°C for 2 days. Single colony was picked up using a sterile inoculating loop and inoculated in 5 ml of YEM medium containing 10 µg/ml rifampicin and 15 µg/ml streptomycin and allowed to grow overnight at 28°C with 150 rpm.

For preparation of competent cells, 1 ml of this culture was inoculated in 50 ml YEM medium containing 10 µg/ml rif and 15 µg/ml strep and grown at 28°C until OD reached 0.6. This procedure requires that cells be growing actively at eary or mid-log phase. At this stage the culture was prechilled and centrifuged at 1500 x g at 4°C to pellet down the cells. The pellet containing *Agrobacterium* cells was resuspended in 1 ml sterile ice-cold 20 mM $CaCl_2$ solution and an aliquot of 100 ml ws taken in a 2 ml microfuge tube. 1 µg of plasmid DNA (pBinT-1F) was added to the microfuge tube and frozen in liquid nitrogen for a minute. The tube was thawed at 37°C for 5 minutes. 1 ml of YEM was added to the microfuge tube and incubated at 28°C with gentle shaking (80 rpm) for 5 hours. The cells were centrifuged at 10000 x g for 1 minute at room temperature. The pelleted cells were mixed in 100 µl YEM and spread on YEM agar plates containing 50 µg/ml streptomycin, 10 µg/ml rifampicin and 50 µg/ml kanamycin.

### 3.6.3 Plasmid DNA isolation from transformed *Agrobacterium*

Single colony out of the few colonies that developed after 2-3 days was inoculated in 5 ml YEM broth containing antibiotics and grown at 28°C for 20 hours. The cells were pelleted by spinning at 13,000 rpm for 1 minute. After the supernatant was completely removed, the pellet was resuspended in 500 µl of STE solution [0.1 M NaCl, 10 mM, Tris Cl (pH 8.0)] and 1 mM EDTA (pH 8) and again pelleted the cells at 13,000 rpm for 2 minutes. This step removes the polysaccharides from the bacteria. The pellet was resuspended in 150 µl of GTE solution (50 mM Glucose, 25 mM TrisCl (pH-8) and 10 mM EDTA). 300 µl of freshly prepared solution of 0.2 M NaOH and 1% SDS were added to the tube. After incubation for 15 mins, the mixture was centrifuged at 13,000 rpm for 5 minutes. The supernatant was decanted in a sterile Eppendorf tube and 675 µl of absolute alcohol was added. The mixture was centrifuged at 13,000 rpm for 5 min. The supernatant was discarded carefully and the plasmid pellet was further washed with 1.5 ml of 70% ethanol by centrifugation at 13,000 rpms for 2 minutes. The pellet was dried and resuspended in 30 µl of sterile TE (pH 8.0).

The presence of the *t-cry1F* gene cloned pBinAR (pBinT-1F) vector in the transformed *Agrobacterium* was checked by performing the PCR, using the same primers which were used for turncating the full length native *cry1F* gene.

### 3.6.4 Transformation of tobacco with pBint1F (truncated *cry1F*)

**Plant material**

Tobacco (*Nicotiana tabacum* L.) var. Petit Havana SR-1 was used for the transformation.

**Growth of seedlings**

Tobacco seeds were sterilized with 0.01% (w/v) mercuric chloride for 10 minutes and washed four times with sterile distilled water. The sterilized seeds were germinated in Borosil™ culture tubes (150 mm x 25 mm) and plastic caps containing 10 ml of half-strength Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium solidified with agar-agar (0.8%). About five seeds were inoculated in each test tube and incubated at 25°C under 16 hours photoperiod in tissue culture room. After 15 days seedlings were transferred to MS basal medium in tissue culture bottles (135 mm x 60 mm) and maintained by transferring to fresh medium every four weeks.

**Cocultivation**

A leaf from tissue culture grown tobacco was excised and cut into small sections (~ 5 mm x 5 mm) in laminar air flow. About 100-200 such leaf explants were incubated for two days in preculturing medium (CCM) (Table 4) in petri dishes (50 mm x 90 mm). *Agrobacterium* strain LBA 4404 containing *t-cry1F* gene was grown overnight in 10 ml YEM containing 50 µg/ml kanamycin, 10 µl/ml rifampicin and 15 µg/ml streptomycin. One ml of this culture was centrifuged at 10000 x g for 30 seconds in an Eppendorf tube. The pellet containing the *Agrobacterium* cells was washed twice in half strength liquid MS medium and resuspended in 20 ml of liquid MS medium in a petri plate. Leaf explants from CCM medium were dipped in this *Agrobacterium* solution and incubated at room temperature for 15 minutes with gentle skahing. The infected explants were blotted free of excess *Agrobacterium* on Whatman # 1 filter paper and transferred back to the same CCM medium.

**Table 4. Media composition used for transformation of tobacco**

| Media | Modified as compared to MS medium |
|---|---|
| Seed germination medium (SGM) | Half strength MS medium + 0.8% Agar |
| Cocultivation medium (CCM) | Full MS + 2 mg/l BAP 0.1 mg/l NAA + 0.8% Agar |
| Selection medium (SM) | CCM + 500 mg/l cefotaxime + 300 mg/ml Kanamycin sulfate |
| Rooting medium (RM) | Full MS + 0.1 mg/l NAA + 0.8% Agar + 500 mg/l cefotaxime + 300 mg/l Kanamycin sulfate |

* Antibiotics were filter sterlized and added at 60°C into autoclaved medium

The plates were incubated for cocultivation for 2 days in tissue culture room.

**Selection and regeneration**

At the end of the cocultivation period, the explants were transferred to the selection medium (SM). The medium was changed at 2 weeks interval. After 5-6 weeks, small shoots resistant to kanamycin were obtained from the callus and these shoots were excised and transferred to the rooting medium (RM). Profuse rooting was obtained after 3-4 weeks of transfer. The rooted plantlets were transferred to sterile soil rite in small pots (10 cm 9cm) for hardening and later to normal soil in bigger pots.

## 3.7 *Agrobacterium tumefaciens* - mediated transformation of tobacco with pBinAR carrying chimeric *cry1F* gene

### 3.7.1 Construction of vector pBinM-1F (pBinAR vector carrying chimeric *cry1F* gene)

The transformation vector pBinM-1F in *E. coli* strain DH5α was constructed as described in section V and mobilised into *Agrobacterium tumefaciens* strain LBA 4404 for transformation of tobacco explants (variety Petit Havana SR-1).

### 3.7.2 Transformation of tobacco with pBinm1F vector

The transformation, cocultivation, selection and regeneration protocols followed for the experiment were similar to that described for the transformation of tobacco with pBinT-IF vector (section 3.6.4).

## 3.8 Molecular analysis of the putative transgenic tobacco plants

Putative transformed shoots of tobacco plants were analysed for the presence of *t-crylF* and *m-crylF* transgenes. The molecular analysis was done by PCR and Southern hybridiztion.

### 3.8.1 Plant DNA Isolation

Total genomic DNA was isolated according to Doyle and Doyle (1990). About 5 mg of leaf tissue (after removing midribs) was ground to a fine powder with pestle and mortar using liquid nitrogen. The powdered leaf material was transferred into Oakridge tube containing 20 ml of preheated CTAB buffer [0.1 M Tris-HCl, 0.02 M $Na_2$ EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol and 2% hexadecyltrimethyl ammonium bromide (CTAB) pH 8.0. The sample was incubated in 65$^0$C for 1 hour with occasional swriling. The sample was extracted once with equal

volume of chloroform : isoamyl alcohol (24:1) by mixing gently but thoroughly followed by centrifugation at 5000 x g (Sorvall RC 5C) for 15 minutes. The aqueous phase was transferred into a new tube and 0.6 volume of isopropanol was added to precipitate the DNA. The precipitate was recovered by centrifugation at 5000 x g for 15 minutes and pellet was washed with wash buffer (70% ethanol and 10 mM ammonium acetate), air dried and dissolved in 1 ml of TE buffer (pH 8.0). DNA was further purified by treating with 3 ml RNAase A (10 mg/ ml) at 37°C for 30 minutes followed by extraction twice with phenol : chloroform : isoamyl alcohol (25:24:1) and once with chloroform : isoamyl alcohol (24:1). DNA was precipitated by adding 1/10th volume of 3M sodium acetate (pH 4.8) and two volumes of chilled ethanol. Precipitated DNA was recovered by centrifugation at 10,000 x g (Microfuge E, Beckman) for 10 minutes. Pellet was washed with 70% ethanol, air dried and dissolved in a suitable volume of TE buffer. Spectrophotometer (Beckman, UK) measurements at $A_{260}$ and $A_{280}$ were taken to determine the purity and concentration of DNA samples. The following formula was used to calculate the concentration of DNA [($A_{260}$ x 50 X dilution factor)/1000] µg/ml.

### 3.8.2 Polymerase chain reaction

Genomic DNA isolated from putative transformed and untransformed lines was used as templates to amplify the transgenes. Primers specific to *t-cryIF*, *m-cryIF* and antibiotic resistant marker *npt* II gene were used. Sequences of these primers for *t-cryIF* and *m-cryIF* have been mentioned earlier and for *npt* II is as follows:

Forward primer : 5' - CAATCGCTGCTCTGATGCC - 3'

Reverse primer : 5' - GGCGATAGAACGCGATGCG - 3'

The reaction components were combined in the order as described earlier. However, the folowing modifications were incorporated. One unit of *Taq* DNA polymerase with 1 X *Taq* buffer was used for amplification. The annealing temperatures (°C) for *t-cryIF, m-cryIF* and *npt* II were 55, 52 and 59 respectively. The polymerization time was 2 minutes at 72°C for all primers. The melting temperature (Tm) was obtained from manufacturer (Genset Oligos, Singapore) on the basis of base composition of the primer. The following simple formula was followed to calculate the Tm (Rychlik and Rhoads, 1989) : 4 x (G + C) + 2x (A+T). However, the final annealing temperature was experimentally determined for each primer template combination. Positive and negative control reactions for *npt*II and *cry* genes were also included. After the completion of PCR, the products were resolved on 0.7% agarose gel and photographed.

### 3.8.3 Southern Hybridization

**Restriction of plant genomic DNA and Agarose Gel Electrophoresis**

Total genomic DNA from untransformed (control) and putative transformed plants were digested with *Bam* HI restriction enzyme as follows :

| | | |
|---|---|---|
| Total genomic DNA | - | 10 µg |
| 10 X reaction buffer | - | 10 µl |
| Restriction enzyme | - | 100 units |
| Sterile distilled wate (Final volume) | - | 100 µl |

Restriction was done at 37°C for overnight. Reaction was stopped by adding 1 µl of 0.5 M EDTA. DNA was precipitated with 2/3 volume of 5M ammonium acetate and 2 volumes of ice cold absolute ethanol followed by incubation at -20°C for 30 min. DNA pellet was then recovered by centrifugation at 10,000 x g (Microfuge E, Beckman) at 4°C for 10 minutes and washed with 70% ethanol, dried at room temperature and dissolved in 30 µl of sterile water. To this DNA, 3 µl of 10 X gel loading dye was added and mixed before loading on 0.8% agarose gel (without ethidium bromide) in 1 X TAE. Molecular weight marker (1 kb ladder) was also loaded on gel for determining the size of DNA band that appeared after autoradiography. The DNA was run at 2 volts/cm$^2$ for 12-15 hours in 1 X TAE buffer.

**Southern blotting**

After electrophoresis, the gel was rinsed in sterile water and treated with 5 volumes of 0.25 N HCl for 10 minutes with gentle shaking. The acid treated gel was rinsed in sterile water and placed in 5 volumes of 0.4 M of NaOH for 30 minutes. DNA transfer from gel to membrane was done as follows. In a shallow tray, gel tray was put upside down and about 200 ml of 0.4 M NaOH was added. Gel tray was covered with a Whatman filter paper strip of same width with upper and lower sides dipped in buffer and allowed it to saturate with NaOH. Over this, gel was placed upside down and air bubbles between filter paper and gel were removed by rollling a pipette. Hybrid N$^+$ membrane (Amersham Inc.) was cut exactly to the size of gel, soaked in 0.4 M NaOH for a minute and placed carefully on gel without trapping air bubbles. Two layers of wet 3 mm Whatman filters were placed over the membrane

followed by one inch stack of dry blotting papers. A glass plate was kept on the blotting paper stack and about 500 gms of weight was placed over the glass plate. Blotting was done overnight at room temperature. Next day, membrane was removed by sequencially removing the weight, blotting papers and position of wells was marked with the pencil. Membrane was then rinsed in 2 X SSC (0.3 M NaCl, 0.03 M Trisodium citrate $2H_2O$, pH 7.0) and placed on Whatman 3 mm paper and dried at room temperature and stored in a vaccum until used for hybridization.

**Prehybridization and Hybridization**

Membrane with DNA side up was carefully placed in a hybridisation tube (Amersham Inc.) and 50 ml (100 µl/sq. µm of membrane) of prehybridization solution [0.5 M phosphate buffer pH 7.2, 7% (w/v) SDS and 10 µM $Na_2$ EDTA] (Church and Gilbert, 1984) was added. Prehybridisation was carried out at 65°C in a hybridisation oven (Amersham Inc.) with gentle isolation for at least 2 hours.

For hybridisation of DNA, probes were prepared using 920 bp *m-cryIF* gene carrying 920 bp insert and ~1.9 kb *t-cryIF* gene for separate southern hybridisation experiments. Decalabel™ DNA labeling kit (MBI Fermentas) was used. The method of probe preparation is as follows :

| | | |
|---|---|---|
| Transgene insert | - | 10 µl (~50 ng) |
| 5X reaction buffer | - | 10.0 µl |
| Deionised water | - | 30 µl |

Mixture was vortexed and spinned down for 3-5 seconds and boiled for 10 minutes in water bath and chilled in ice for at least one minute.

The entire content was collected by centrifugation at the bottom of the tube. To this 3.0 µl of Mix C (0.33 mM each of dGTP, dATP, dTTP aqueous solution 5.0 ml of [$\alpha$-$^{32}$P] dCTP (50 µCi) and 1.0 µl of Klenow fragment exo$^-$ was added and the tube centrifuged for 3-5 seconds and was incubated at 37$^0$C for 5 minutes. Again 4.0 ml of dNTP mix (0.25 mM each dATP, dCTP, dGTP, dTTP) was added in a reaction and incubated for additional 5 minutes at 37$^0$C. The reaction was stopped by adding 1 µl of 0.5 M EDTA. Labelled probe was denatured in boiling water for 5 minutes, snapped cool in ice for 2-3 minutes and added into a hybridization bottle containing membrane and hybridisation solution (reduced to 30 ml). Hybridization was carried out 14-16 hours at 65$^0$C.

**Washing and Autoradiography**

The hybridization solution was decanted in a radioactive waste bottle and membrane filter was rinsed with 2 X SSC/ 0.1% SDS at room temperature. Subsequently washing was done at 65$^0$C with 2 X SSC/ 0.1% SDS for 30 minutes; 1 X SSC / 0.1% SDS by monitoring the counts on the membrane until radioactivity counts per minute (cpm) declined to 40-50 min. Washed membrane was wrapped in a saran wrap, placed in a exposure cassette with intensifying screens (Amersham Inc.) and exposed to a X-Ray film (Hyper film MP, Amersham, UK) in a dark room. The lead cassette was locked tightly and kept at -70$^0$C for 3-4 days (depending on the counts on membrane). The film was developed in a developer (Kodak) for 5 minutes, rinsed in water and again kept in a fixer (Kodak) solution for about 5 minutes. the film was then washed in running tap water and air died at room temperature.

**Determination of DNA fragment size**

The migration of unknown DNA was compared to the migration of the standard DNAs estimate the sizes of unknownDNA fragments using the following equation :size of unknown DNA = B (1-Z)* $S^Z$ (Ausubel *et al.,* 1992) where B and S are sizes of bigger and smaller flanking standards (either in bp or kb) and Z is the unit less fraction of the distance between and S that the unknown has migrated. The fraction Z is smaller for the bands closer to B and approaches the unknown band gets closer to S. For instance if an unknown migrants 1/4 way down from the 5020 bp standard to 3180 standard then the size of the unknownis $5020^{0.75}$ + $3180^{0.25}$ = 4545 bp.

## 3.9 Insect Bioassay

Insect bioassays were performed to study the efficacy of the *cryIF* expressed in transgenic tobaccos against first instar larvae of *S. litura*. For this purpose leaf disks bioassays were carried out in the following way.

Ten transgenic plants (5 each transformed with pBin*M-IF* and pBin*T-IF*) each representing an independent transformation event were selected for the bioassay. Two leaf disks of 3 cm diameter each were cut using a cork borer from one mature leaf taken from each test plant and placed on moist Whatman filter paper in a 60 welled culture plate (Cellstar, Grenier Labortecnik, Germany). The cork borer was washed each time before cutting the leaf of different plants to prevent transfer of any fluids. Six larvae of first instar stage were released on each disk and the plates were tightly covered with saran wrap to prevent the

larvae from escaping the wells. At one time two leaf disks from a test plant harbouring six larvae each were tested. The number of larvae surviving on each disk was counted after 24 hrs interval for six consecutive days after release. The experiment was replicated 3-4 times on different days.

# 4. RESULTS

## 4.1 Truncation of native full length *cry1F* gene

The primer sequences for truncation of the native full length *cry1F* gene were derived on the basis of the published nucleotide sequence of the gene (Chambers *et al.*, 1991) so as to amplify the required sequence (1.9 kb) and also to incorporate a restriction endonuclease (*Sal* I) site for convenient cloning.

PCR analysis showed specific amplification of 1.9 kb fragment of *cry1F* (Fig. 3). No non-specific band at any other position was observed in any lane. Multiple PCR reactions using *Pfu* DNA polymerase were set to generate enough DNA for further manipulation and cloning. This enzyme retains the proofreading activity (3'→5' exonuclease) as a result of which the chances of incorporating an error/mismatch in the amplified sequence are minimized.

## 4.2 Cloning of truncated *cry1F* gene in pBluescript KS vector

In order to facilitate further cloning of *t-cry1F* in *E. coli* expression vector pET29a and plant expression vector pBinAR, the PCR product was cloned in pBluescript-II KS cloning vector and the resultant vector named as pBluescript KS T-*cry1F* (Figure 4). Since *Sal* I restriction site had been introduced in the truncated gene through forward and reverse primers, the PCR product was cloned in *Sal* I site in the vector. This being a single site cloning, dephosphorylation of the plasmid vector and keeping a control ligation reaction become essential.

**Fig. 3.** **PCR based truncation of native full length *cry1F* gene**

M : 1 kb ladder

Lane 1 - 3 : Truncated *cry1F* gene fragments electrophoresed on 1% agarose gel

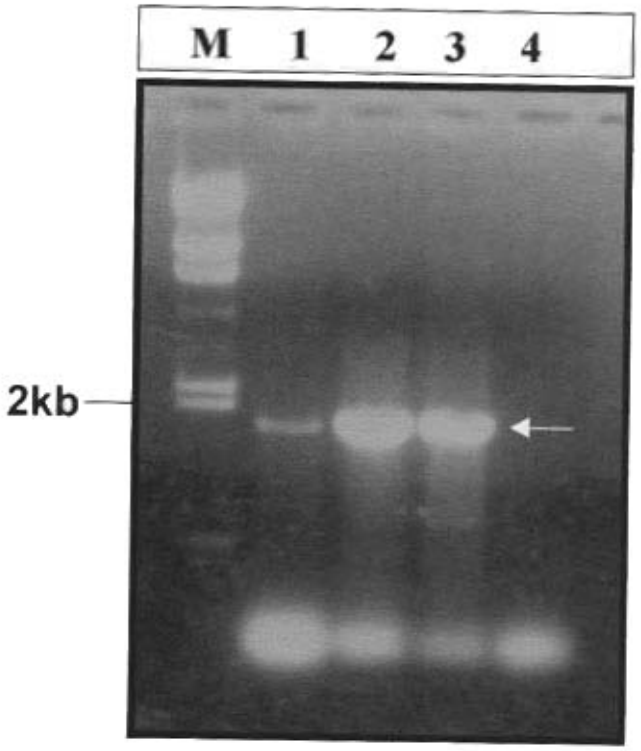



**Figure 3**

**Fig. 4.** **Vector map of pBST-*cry1F***

Restriction map of pBST-*cry1F* containing *t-cry1F* gene

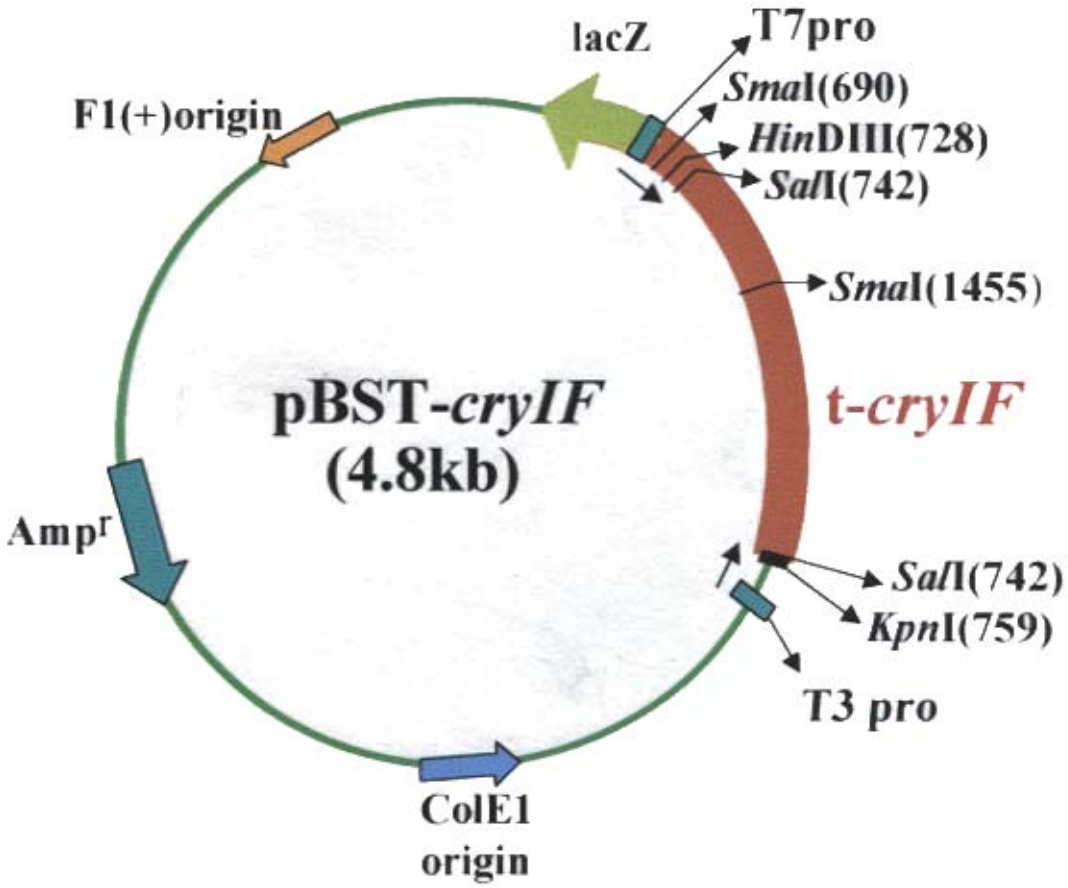

lacZ
T7pro
SmaI(690)
HinDIII(728)
SalI(742)
SmaI(1455)
F1(+)origin
pBST-cryIF
(4.8kb)
t-cryIF
Ampr
SalI(742)
KpnI(759)
T3 pro
ColE1
origin

Figure 4

**Fig. 5. Restriction analysis of pBS-T*cry1F***

M : 1 kb ladder

Lane 1 : Unrestricted pBS-T*cry1F*

Lane 2 : pBS-T*cry1F* restricted with *Sal* I showing 1.9 kb fragment of *cry1F* gene and 3.0 kb fragment of pBluescript vector

Lane 3 : pBS-T*cry1F* restricted with *Sma* I for checking correct orientation of the *cry1F* gene

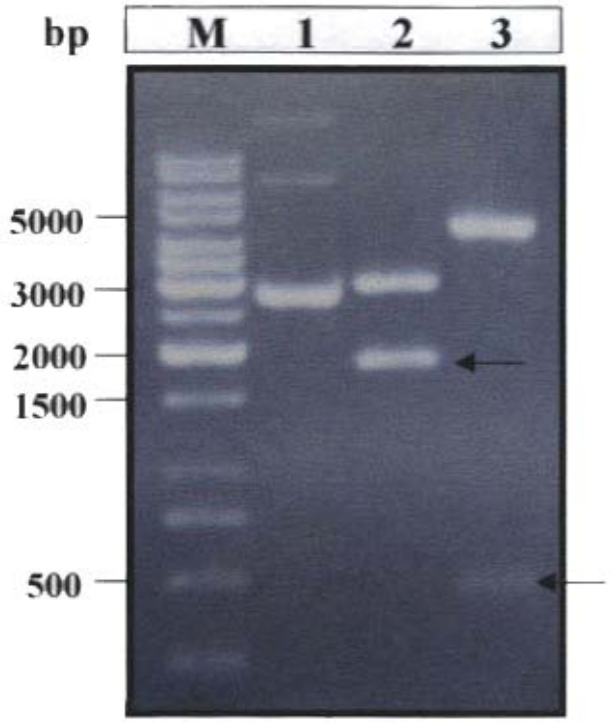



**Figure 5**

The recombinant colonies were selected using blue/white colony selection. The presence of insert in the plasmid was checked by restriction analysis with *Sal* I enzyme (Figure 5, Lane 2). Correct orientation of the *t-cry1F* gene was checked by restriction analysis with *Sma* I enzyme (Figure 5, Lane 3). Colonies with plasmid showing the insert of expected size were saved and stored as glycerol stocks in -70$^0$C for further use.

### 4.3 Expression analysis of *t-cry1F* gene in *E. coli* (BL-21DE3)

The bacterium *Escherichia coli* is the most favourite organism for studying molecular and functional aspects of the introduced genes. In order to conduct the functional analysis of Cry1F toxin, *cry1F* was introduced in *E. coli*. The ~1.9 kb truncated fragment already cloned in pBluescript KS T-*cry1F* was isolated by restriction with *Kpn* I and *Hin* DIII enzymes and further cloned into high expression vector pET29a (the cloned vector was named pETT-*cry1F*, Figure 6). The recombinant plasmid with *t-cry1F* gene was then transformed into *E. coli* strain BL-21DE3 for protein expression. The recombinant colonies were selected on LA medium plates containing kanamycin @ 50 µg/ml. The cloning was confirmed by restriction analysis of the plasmid isolated from transformed single colony obtained after overnight incubation. The colony that showed correct size (Figure 7a) of the insert (~1.9 kb) after restriction with *Kpn* I and *Hin* DIII was preserved as glycerol stock and used for further studies. Even though in directional cloning two enzymes were involved, orientation check was done by restriction with *Sma* I enzyme (Figure 7b).

Extraction of Cry1F protein from *E. coli* clone pETT-*cry1F* was done according to the method described earlier. IPTG induction at final concentration of 1 mM at 15$^0$C for overnight was found to be ideal for

**Fig. 6.** ***E. coli*** **expression vector pETT-*cry1F***

Restriction map of pETT-*cry1F* containing truncated *cry1F* gene under the control of T7 promoter; *npt* I, kanamycin resistance gene

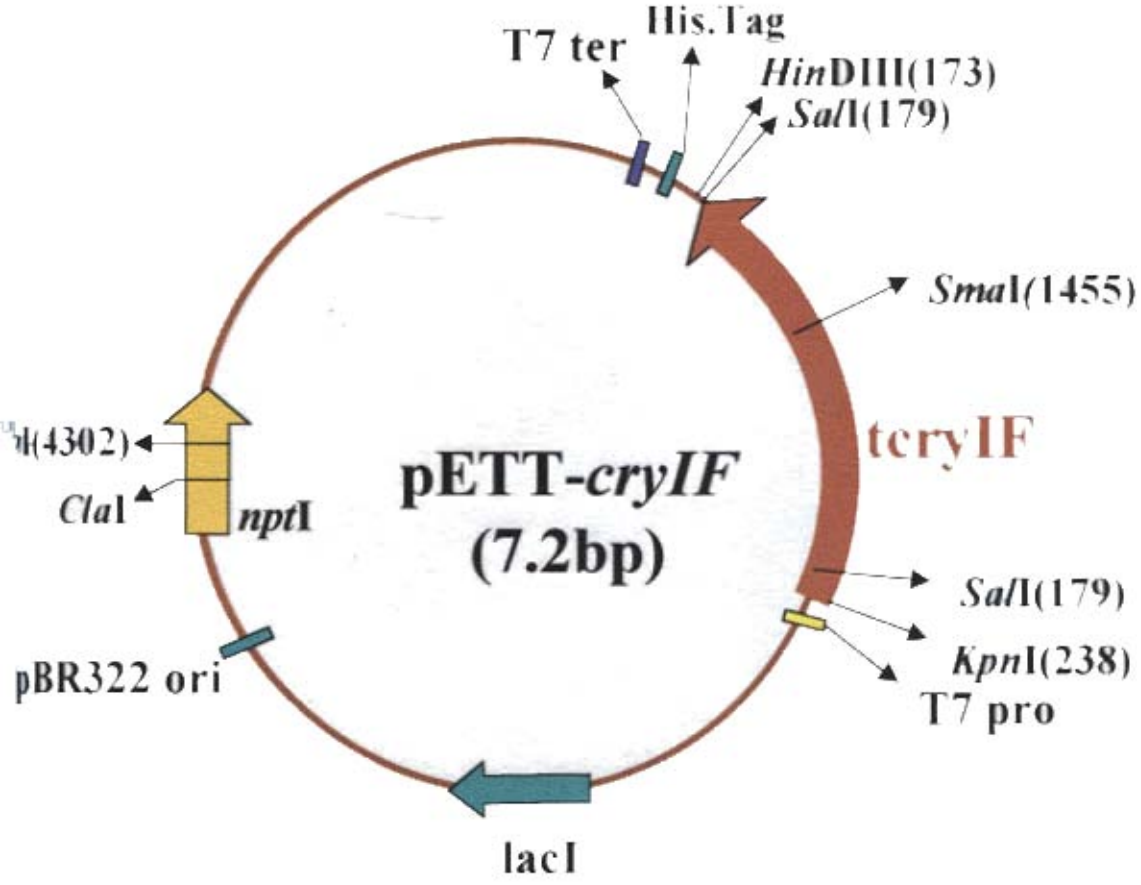

T7 ter
His.Tag
HinDIII(173)
SalI(179)
SmaI(1455)
teryIF
SalI(179)
KpnI(238)
T7 pro
lacI
pBR322 ori
ClaI
nptI
pETT-cryIF
(7.2bp)

Figure 6

**Fig. 7.** **Restriction analysis of pETT-*cry1F* carrying truncated *cry1F* gene**

**(a)**

M : 1 kb ladder

Lane 1 : pET29a vector (unrestricted)

Lane 2 : pET29a vector cut with *Sal* I showing 5.4 kb vector size

Lane 3 : pETT-*cry1F* (unrestricted)

Lane 4 : pETT-*cry1F* restricted with *Kpn* I and *Hin* DIII showing the 1.9 kb *t-cry1F* gene and 5.4 kb pET29a vector

**(b)**

M : 1 kb ladder

Lane 1 : peTT-*cry1F* restricted with *Sma* I

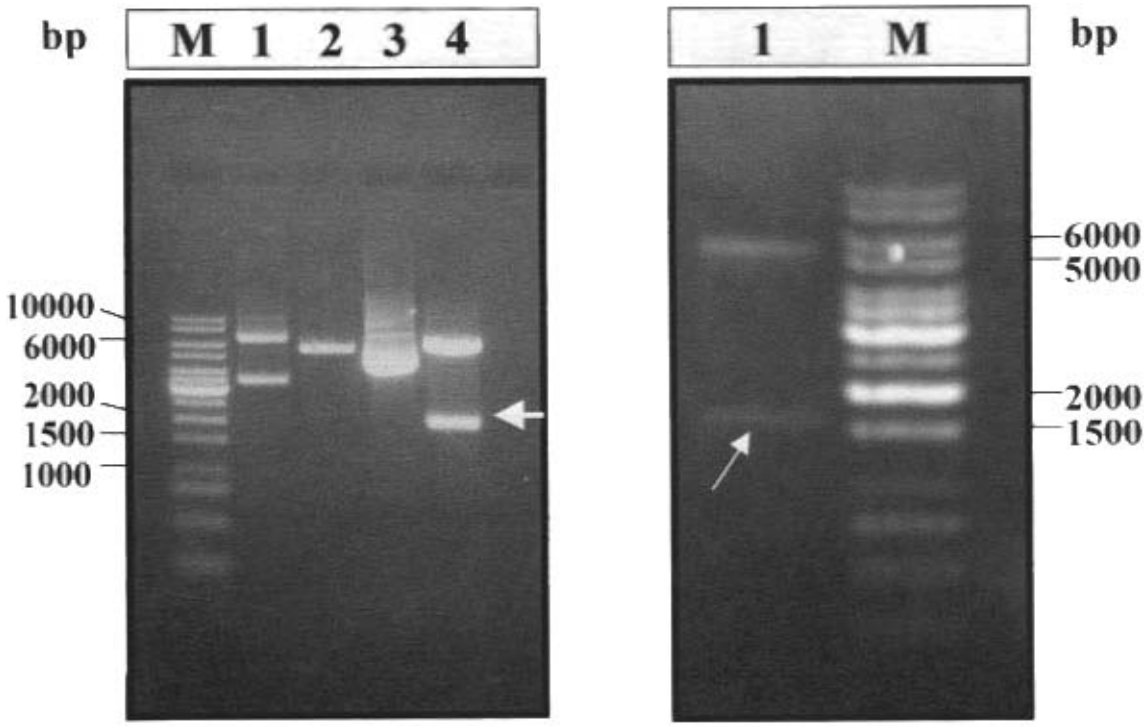

bp
M 1 2 3 4
10000
6000
2000
1500
1000
(a)
1 M
bp
6000
5000
2000
1500
(b)

**Figure 7**

**Fig. 8. Expression of Cry1F in *E. coli* on 10% SDS-PAGE**

S1 and S2 : Truncated Cry1F toxin in the total protein extracted from *E. coli* clone pETT-*cry1F*

M : Protein standard

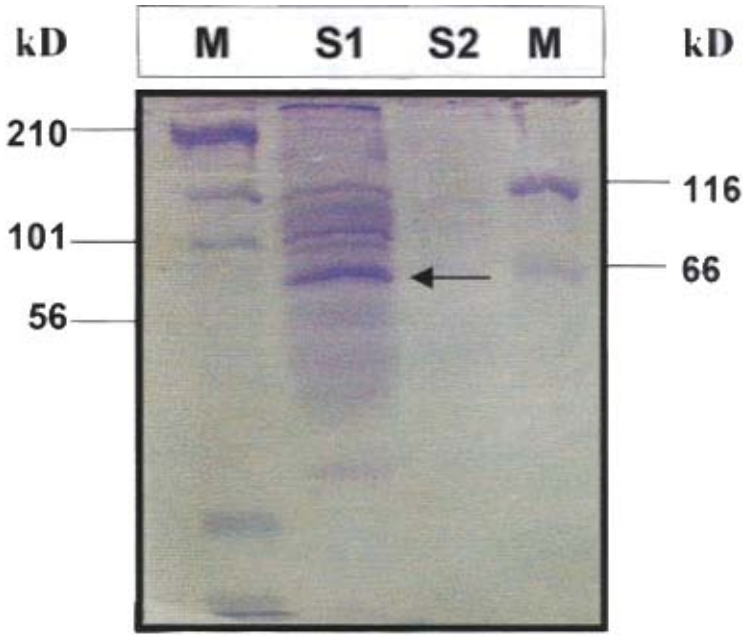



Figure 8

obtaining the desired expression level of Cry1F toxin in total *E. coli* protein without causing any protein degradation. Total protein extracted from the *E. coli* culture of pETT-*cry1F* was subjected to 10% SDS-PAGE. Approximately 5 µg of total protein was resolved by SDS and compared with standard molecular weight markers (Figure 8). In addition to this faint bands of other host proteins were also observed in both the lanes.

## 4.4 Analysis of toxicity of truncated Cry1F protein to *Spodoptera litura*

The efficiency of *E. coli* expressed truncated Cry1F toxin was evaluated against the neonate larvae of *Spodoptera litura* (Tobacco caterpillar). Table 5 shows the mortality (± SE) of larvae at various concentrations of truncated toxin. Each treatment was replicated five times. In each replicate one control, treated with water, was included to determine insect mortality under control conditions. The maximum mortality was observed at a concentration of 400 ng and onwards showing almost 100% mortality after 48 hours. At a low (100 ng) concentration, little infestation was observed on the first day which reduced to minimum on the second day with much reduced activity of the larvae. Within four days the larvae showed stunted growth, developmental delays and changes in feeding habit.

## 4.5 PCR based substitution of domain I in truncated *cry1F* by modified *cry1F* (domain I)

In order to substitute domains of truncated and modified *cry1F*, presence and absence of restriction endonuclease recognition sites was utilized. *Pst* I restriction site was already present in truncated *cry1F* gene at 920 bp position which covered the entire domain I. Since *Pst* I site was

**Table 5. Analysis of toxicity of truncated Cry1F protein to *Spodoptera* larvae[1]**

| Dosage (T-Cry1F) ($mg/cm^2$) | No. of larvae released | Infes-tation | Time taken to cause mortality | % Insect mortality[2] |
|---|---|---|---|---|
| 100 | 10 | Minimum | 3-4 days | 78 ± 2.6 |
| 200 | 10 | Nil | 3-4 days | 94 ± 2.8 |
| 400 | 10 | Nil | 48 hours | 100 |
| 800 | 10 | Nil | 48 hours | 100 |
| 1000 | 10 | Nil | 48 hours | 100 |
| Control | 10 | Complete | None | None |

1 Neonate larvae were used for bioassay

2 Percentage was calculated on the basis of 10 larvae per replicate and the data represents average of at least five replicates.

absent at the same position in the modified/ synthetic *cry1F* gene, it was added through PCR. The strategy for domain substitution is summarised in Figure 9.

The PCR result shows very sharp and clear band at 920 bp position and a few faint bands at further lower molecular weight as a result of non-specific primer binding (Figure 10). Due to the presence of non-specific binding the entire PCR product was electrophoresed and then required band was eluted from the gel to be further used in ligation.

### 4.6 *A. tumefaciens* mediated transformation of tobacco with pBinAR carrying truncated *cry1F* gene

Truncated *cry1F* gene was cloned into plant expression vector, pBinAR containing CaMV 35S promoter and Octopine synthase poly (A)

Fig. 9. **Schematic representation of construction of plant transformation vector pBinM-*cry1F***

Step A : Partial digestion of pBinM-*cry1F* with *Pst* I and complete digestion with *Xba* I

Step B : PCR amplification of p39/66/6 carrying the synthetic *cry1F* gene to obtain domain I fragment using *Pfu* DNA polymerase. PCR product was further restricted with *Xba* I and *Pst* I

Step C : Ligation of restricted pBinT-*cry1F* and Step B PCR product to obtain pBinM-*cry1F*

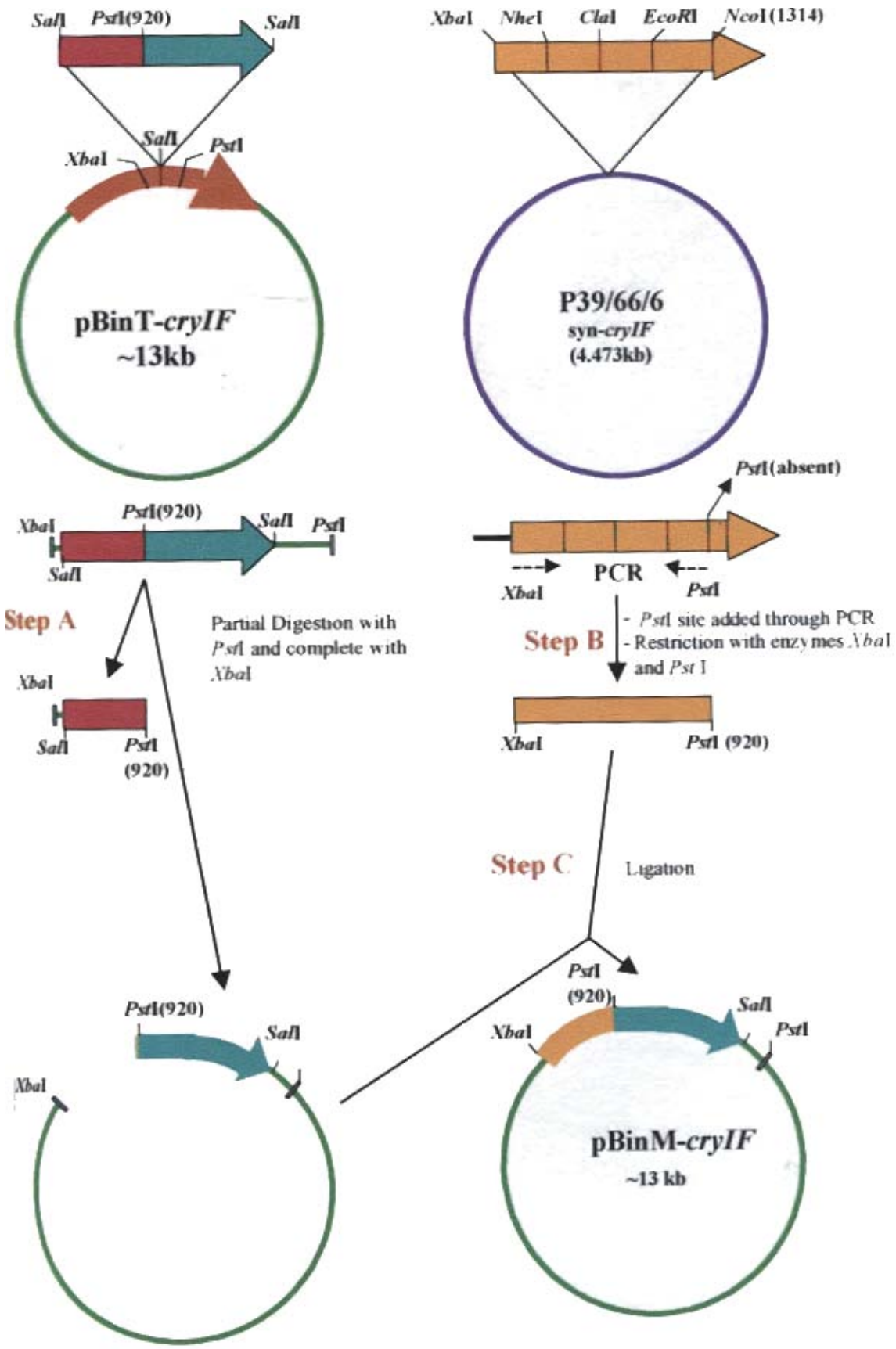

SalI
PstI(920)
SalI
XbaI
SalI
PstI
pBinT-cryIF
~13kb
XbaI
NheI
ClaI
EcoRI
NcoI(1314)
P39/66/6
syn-cryIF
(4.473kb)
PstI(absent)
XbaI
PstI(920)
SalI
PstI
SalI
PCR
XbaI
PstI
Step A
Partial Digestion with PstI and complete with XbaI
Step B
- PstI site added through PCR
- Restriction with enzymes XbaI and Pst I
XbaI
SalI
PstI (920)
XbaI
PstI (920)
Step C
Ligation
PstI(920)
SalI
XbaI
PstI
(920)
SalI
XbaI
PstI
pBinM-cryIF
~13 kb

**Figure 9**

Fig 10. **PCR based amplification of synthetic *cry1F* gene fragment comprising domain I nucleotide sequence**

M : 1 kb ladder

Lane 1-4 : 920 bp amplified synthetic *cry1F* gene fragment

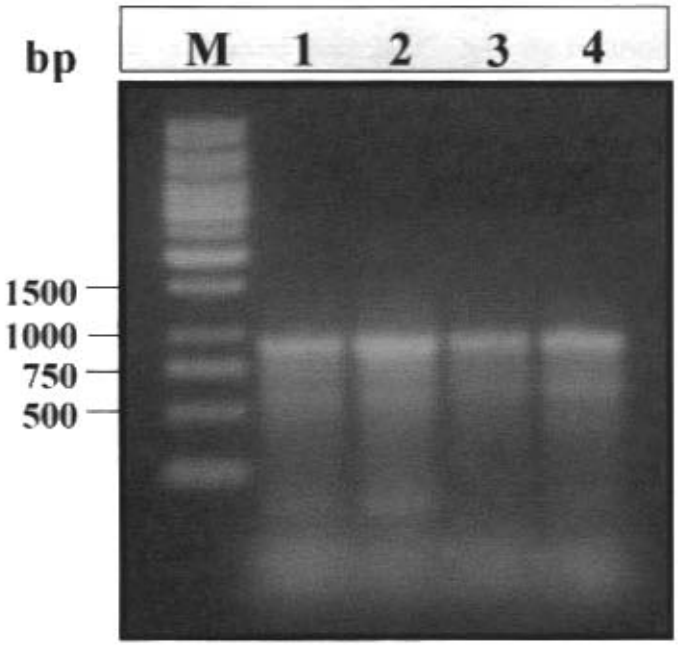



**Figure 10**

sequence (Hofgen and Willmitzer, 1990). *Sal* I site present in MCS region of BinAR was used to insert the gene. The resultant plasmid pBint-*cry1F* (Figure 11) was mobilised into *Agrobacterium tumefaciens* strain LBA4404. The transformed cells were selected on YEM medium containing rifampicin (10 µg/ml), streptomycin (15 µg/ml) and Kanamycin (50 µg/ml). Presence of the vector construct was confirmed by restriction analysis of the plasmids isolated from single colonies (Figure 12) of transformed *A. tumefaciens.* The clone was stored as glycerol stock and used for present study.

*Agrobacterium tumefaciens* strain LBA 4404 containaing the binary vector plasmid pBinT-*cry1F* (carrying truncated *cry1F* gene), for insect resistance and *npt* II gene for selection of putative transformants on the medium containing kanamycin were used for the transformation of the tobacco.

The leaf explants (precultured on MS medium supplemented with BAP (2 mg/l) and NAA (0.1 mg/l) and solidified with agar (0.8%) were infected with *A. tumefaciens* carrying truncated *cry1F* gene construct and cocultivated for 2 days. Subsequently, they were transferred to selection medium (Table 4) containing kanamycin (300 ng/l) (for selection of putative transformants) and cefotaxime (500 mg/l). Within six weeks several kanamycin resistant shoots (Figure 13b) were obtained which were excised and transferred to rooting medium. The majority of the excised shoots readily formed roots within 4 weeks of transfer to the rooting medium (Figure 14a). At this time PCR analysis (described later) was done and following which PCR positive young plants were transferred to small pots containing Soilrite (Figure 14b) for hardening and subsequently into big

**Fig. 11. Plant transformation vector pBinT-*cry1F***

Restriction map of pBinT-*cry1F* containing *t-cry1F* gene; *npt* II, neomycin phosphotransferase II; LB, left border; RB, right border; DCST, terminator of octopine synthase gene

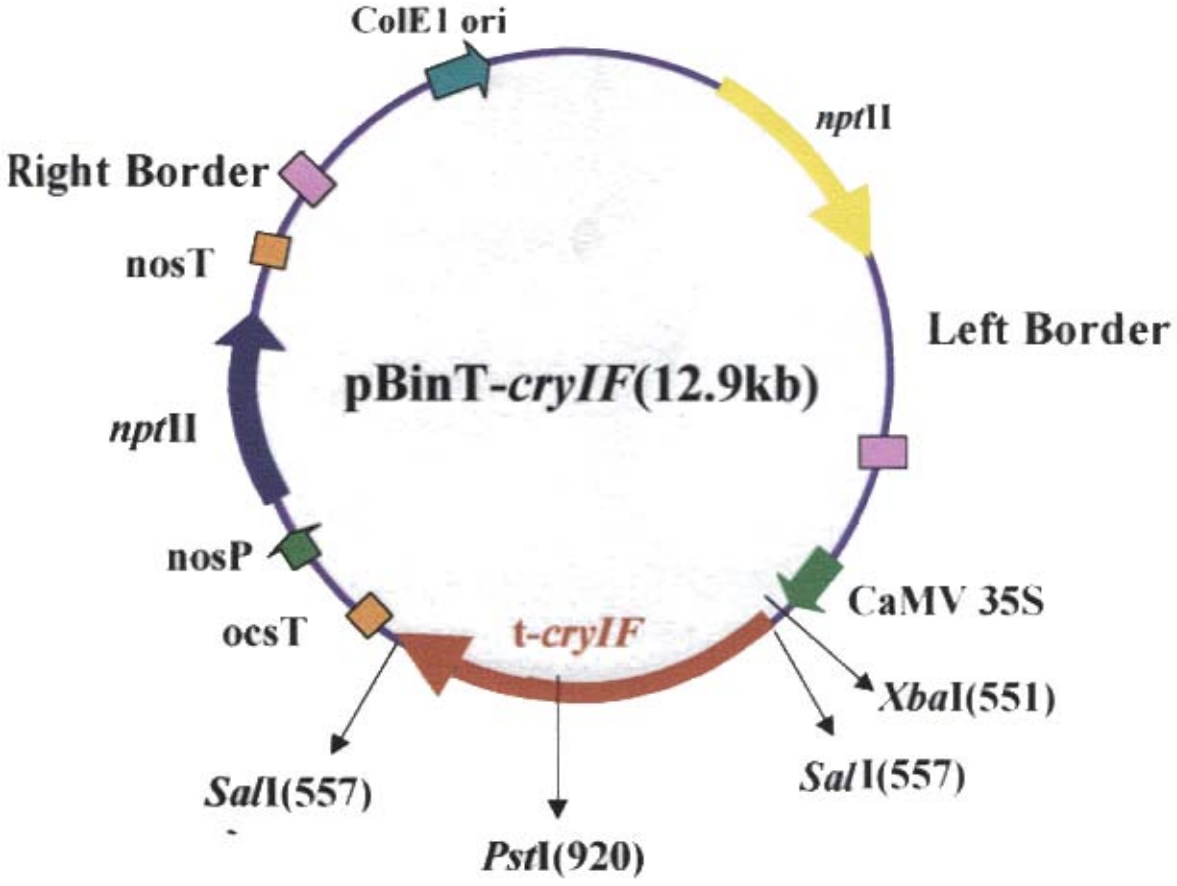

ColE1 ori
nptII
Right Border
nosT
Left Border
pBinT-cryIF(12.9kb)
nptII
nosP
CaMV 35S
ocsT
t-cryIF
XbaI(551)
SalI(557)
SalI(557)
PstI(920)

Figure 11

**Fig 12. Restriction analysis of pBinT-*cry1F***

M : 1 kb ladder

Lane 1 : pBinT-*cry1F* (unrestricted)

Lane 2 : pBinT-*cry1F* restricted with *Sal* I showing 1.9 kb fragment of *cryt-1F* gene and ~11.0 kb fragment of pBinAR vector

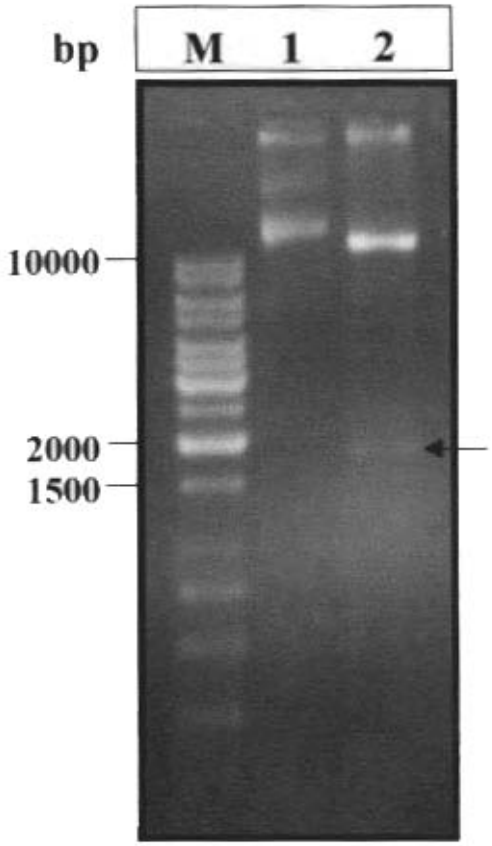
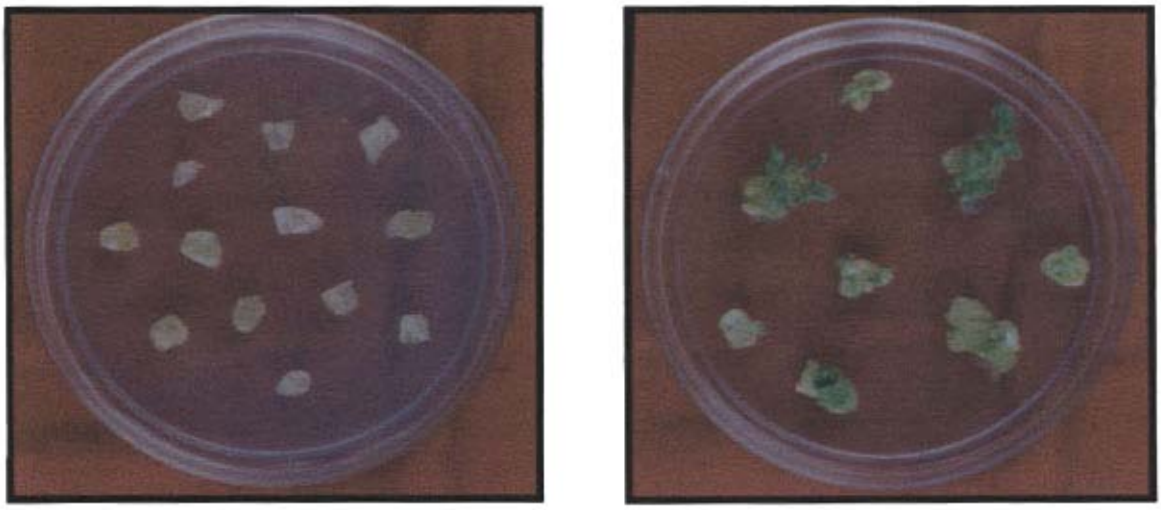



**Figure 12**

**Fig. 13.** (a) Control leaf explants on selection medium showing complete bleaching after 4 weeks

(b) Leaf explants after 4 weeks of incubation on selection medium showing green shoots emerging from the callus



(a) (b)

Figure 13

**Fig. 14.** (a) Kanamycin resistant shoots in rooting medium. Rooting was obtained after 2-3 weeks

(b) Putative transformant in its hardening stage

(a) (b)

Figure 14

pots. The plants in big pots were transferred to net house and grown until the completion of studies.

Leaf explants (control) that were infected with *Agrobacterium* without the binary vector construct carrying *t-cry1F* gene construct, turned chlorotic when subsequently selected on kanamycin containing medium (Figure 13a).

## 4.7 *Agrobacterium tumefaciens*-mediated transformation of tobacco with pBinAR carrying chimeric *cry1F* gene

Construction of the vector pBinAR carrying chimeric *cry1F* gene (pBin*M-cry1F*) involved PCR based domain substitution and partial and complete digestion of the vector by *Pst* I and *Xba* I enzyme, respectively. The resultant construct was mobilised into *Agrobacterium tumefaciens* strain LBA4404 for transformation of tobacco explants in the similar pattern as for transformation with pBinT-*cry1F* (section 3.6.4).

The restriction map of the construct, pBinAR carrying chimeric *cry1F* gene can be seen in Figure 15. Cloning of the construct was confirmed by restriction analysis of the plasmid DNA isolated from single colonies.

## 4.8 Molecular analysis of the putative transgenic tobacco plants

Putative transformant regenerated on the selection media need to be analyzed to confirm the presence of the foreign gene. Molecular analysis provides a direct evidence for the presence of the foreign gene sequences.

**Fig. 15. Restriction analysis of pBinM-*cry1F***

M : 1 kb ladder

Lane 1 : pBinM-*cry1F* (unrestricted)

Lane 2 : pBinM-*cry1F* restricted with *Xba* I and *Pst* I

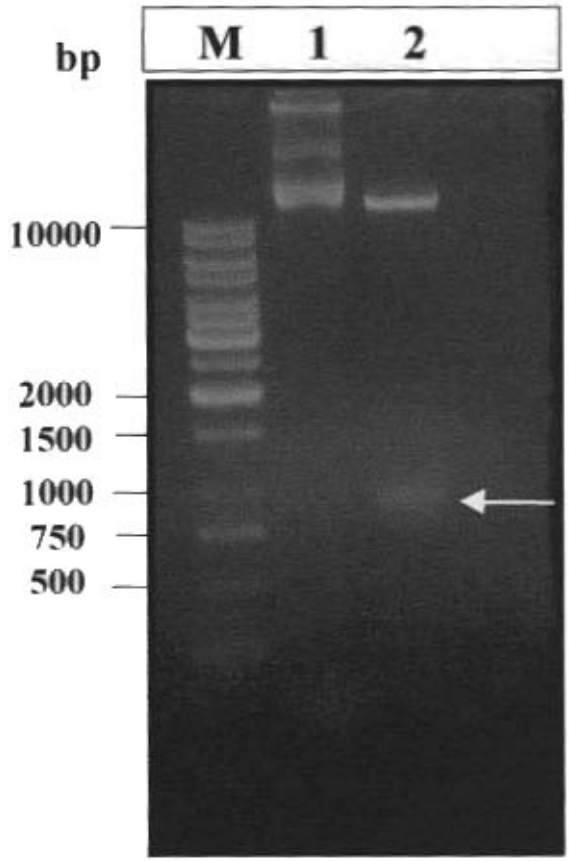



**Figure 15**

### 4.8.1 PCR (Polymerase chain reaction) analysis of putative transformants

Using total genomic DNA from each putative transformant as template and primers that specifically amplify a portion of the foreign gene construct, the amplification of a DNA of the expected size as determined by gel electrophoresis, provides good evidence for the presence of the foreign gene.

Thirty transformants (15 each of modified *cry1F* and truncated *cry1F* gene) selected on the kanamycin-containing medium were subjected to PCR analysis to confirm the presence of *npt II* and *m-cry1F* and *t-cry1F* genes. Approximately, 100 mg of the leaf segment without the midrib portion was taken in each case and genomic DNA was isolated. PCR screening was carried out at 6-7 leaf stage for all the putative transformants with well developed roots.

Specific primers for *npt* II, *m-cry1F* and *t-cry1F* genes were designed to yield 700 bp, 920 bp and 1.9 kb amplification products, respectively. In order to confirm the presence of the full length T-DNA in the transformants, both the primers were used. Interestingly, all the 30 transformants tested with *npt* II, *m-cry1F* and *t-cry1F* gave positive amplification, Figure 16 for *m-cry1F* and Figure 17 for *t-cry-1F* (result not shown for *npt* II). However, no amplification was observed in the DNA isolated from the leaves of wild type plants. The PCR was repeated to confirm the results. The putative transformants which exhibited PCR positive signals were transferred to pots for further growth and subsequent analysis by Southern hybridization.

**Fig. 16. PCR analysis of putative transformants (with *m-cry1F* gene) selected on kanamycin**

M : 1 kb ladder

Lane 1 : Amplification of *m-cry1F* gene in pBinM-*cry1F*

Lane 2-10 : Putative transformants analysed for *m-cry1F* gene

Lane 11 : Wild type tobacco negative for *m-cry1F* gene

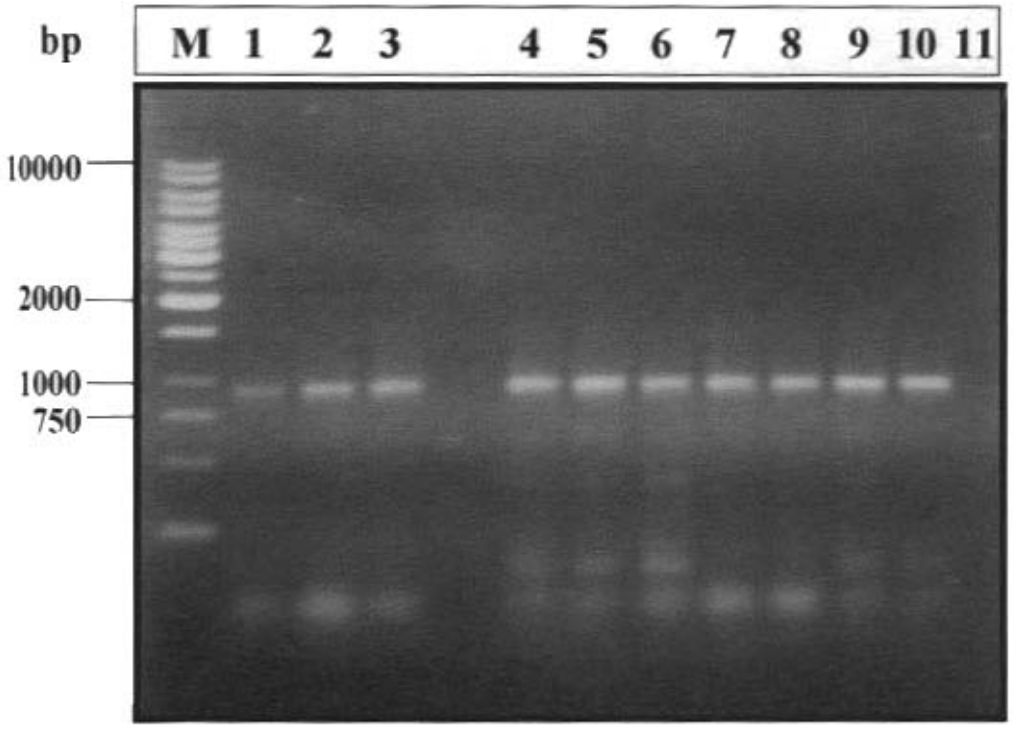



**Figure 16**

**Fig. 17. PCR analysis of putative transformants (with *t-cry1F* gene) selected on kanamycin**

M : 1 kb ladder

Lane 1 : Amplification of *t-cry1F* gene in pBinT-*cry1F*

Lane 2-7 : Putative transformants analysed for *t-cry1F* gene

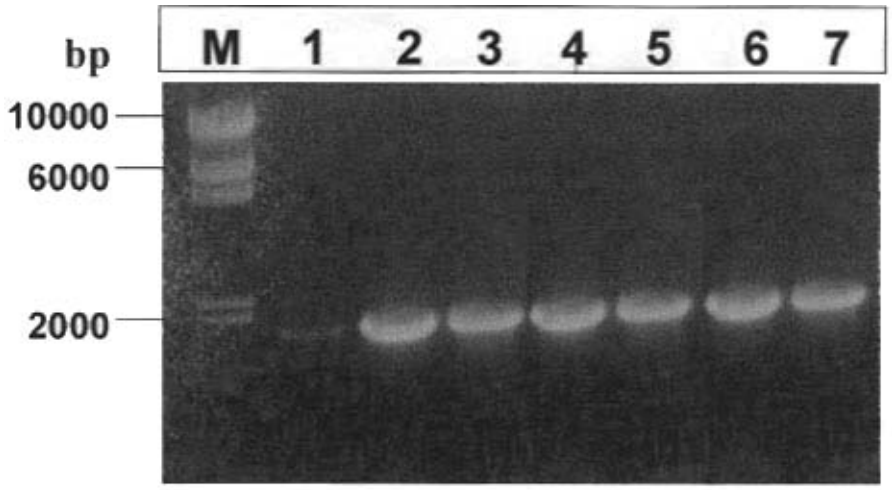



Figure 17

### 4.8.2 Southern hybridization analysis

Southern blot analysis of putative transformants can be carried out using the gene of interest or the marker gene sequence as a specific radiolabelled probe to hybridize with the restriction fragments of total DNA blotted on nitrocellulose/ nylon membrane. The presence of complimentary sequences, as detected by autoradiography, provides a good evidence for the integration of foreign gene.

In order to ensure the insertion of the truncated and modified *cry1F* gene in *Nicotiana tabacum* (Petit Havana SR1) genome and to determine the number of loci where the gene got integrated in each of the transformants that were found to be PCR positive, the genomic DNA was isolated from wild type and putative transformants. The DNA was digested with *Bam* HI enzyme. *Bam* HI restriction enzyme was chosen as it restricts the T-DNA of pBinT-*cry1F* and pBinM-*cry1F* only at one site that is at the 5' end of the genes. Since the cloned *cry1F* genes do not have any internal site for *Bam* HI and also *Bam* HI has only one site in MCS of binary vector BinAR, where the genes are cloned, when the DNA from PCR positive plants were restricted with *Bam* HI, one restriction was expected to be at the 5' end of the gene (within the T-DNA region) and other site could be within plant genome. Hence every independent event of integration was expected to show up as a seperate band in Southern hybridization. The number of bands obtained in the autoradiogram would give the number of loci where the fusion gene got integrated in the plant genome. For Southern hybridization *t-cry1F* gene and *m-cry1F* gene (carrying 920 bp insert) were used as the probes.

**Fig. 18. Southern hybridization of *m-cry1F* gene in transgenic tobacco plants**

The hybridization was performed with [$\alpha^{32}$P] radio-labelled m-cry1F gene carrying 920 bp insert

M : 1 kb ladder

Lane 1 : *m-cry1F* gene

Lane 2 : Non-transgenic tobacco plants

Lane 3-11 : Transgenic plants with *m-cry1F* gene

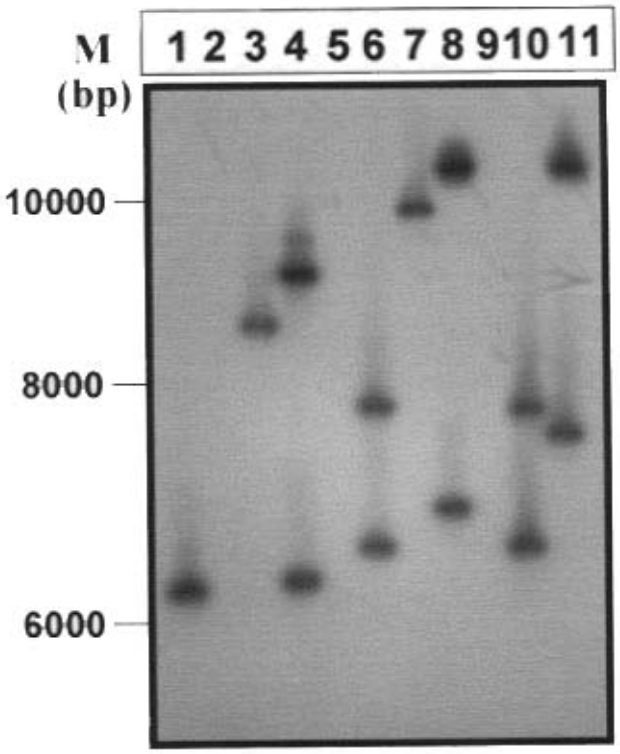



Figure 18

**Fig. 19. Southern hybridization of *t-cry1F* gene in transgenic tobacco plants**

Genomic DNA (5 µg) isolated from leaves of transgenic and non-transgenic tobacco plants was fully digested with *Bam* HI. The hybridization was performed with [$\alpha^{32}$P] radio-labelled t-cry1F gene (~ 1.9 kb as probe)

M : 1 kb ladder

Lane 1 : *t-cry1F* carrying pET vector

Lane 2 : Non-transgenic tobacco plants

Lane 3-9 : Transgenic plants with *t-cry1F* gene

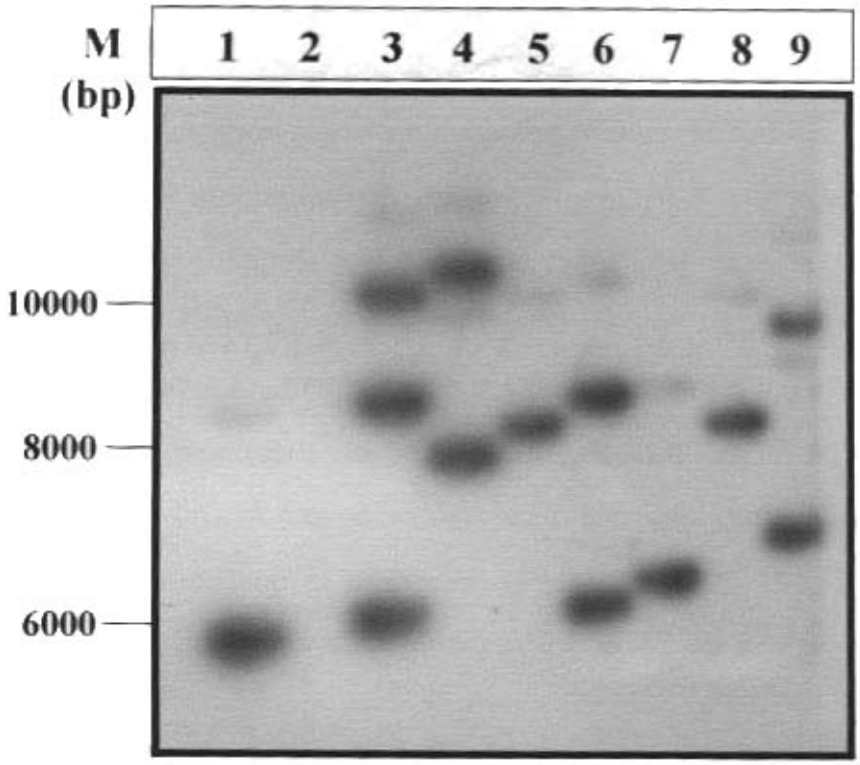



**Figure 19**

The results of the Southern blot analysis of the sixteen plants (nine of BinM-*cry1F* and seven of BinT-*cry1F*) are shown in Figure 18 and Figure 19, respectively. Autoradiogram of BinM-*cry1F* revealed that out of the nine PCR positives only seven showed hybridization signals. Lane 5 and lane 9 of Figure 18 show negative results. Wild type plant DNA did not show any hybridization signal as expected (Figure 18, Lane 2). While for the BinT-*cry1F* autoradiogram, all the seven PCR positives tested showed hybridization signals and wild type plant DNA showed negative results (Figure 19, Lane 2). Presence of 1-3 distinct bands in both the autoradiograms that got hybridized with the probe suggests the integration of the genes at independent positions in the genome. In order to know whether full length T-DNA has got integrated in the plant genome, the size of the band was calculated as described in section 4.8.3. The estimated size of the different bands varied between 6.7 to 12 kb.

### 4.9 Insect Bioassay

After molecular analysis, the plants showing *m-cry1F* and *t-cry1F* gene integration were evaluated for their effect on survival of the first instar larvae of *S. litura*. For this purpose leaf disk assays were performed. When evaluating the survival and changes in feeding habits of the first instar larvae of *S. litura* on the leaf disks cut from transgenic (*cry1F*) and non- transgenic tobacco plants, it was observed that the transgenic plants had significant impact on infestation and survival of the larvae (Figure 20 and Figure 21).

Survival of first instar *S. litura* larvae on leaf disks of non-transgenic tobacco tended to remain between 90-100% with maximum infestation and activity of the neonate larvae. However, a significant difference was

**Fig. 20.** **Leaf disk bioassay of putative transformants with first instar *S. litura* larvae (Dead larvae indicated by arrows)**

**(a)**

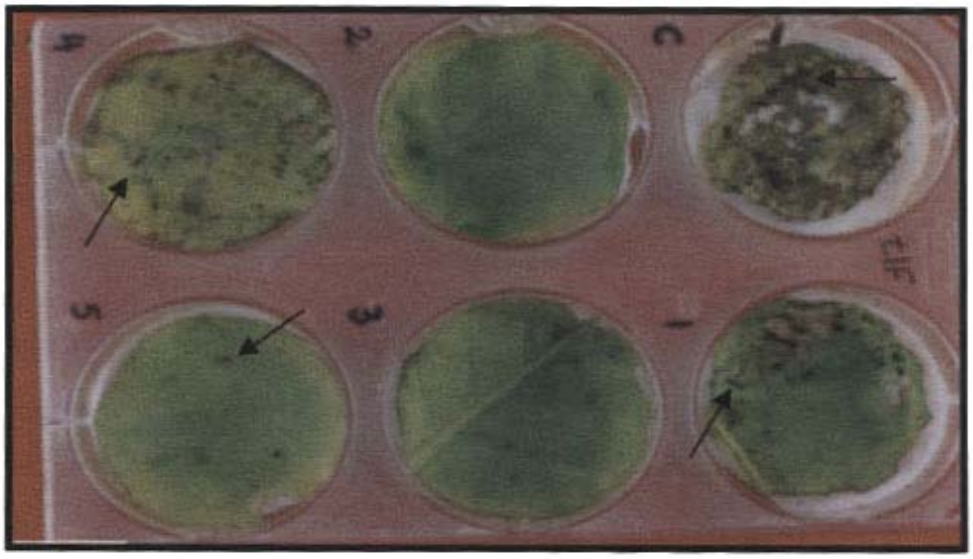
Control (c) : Non-transgenic tobacco plant

1-5 : Putative tobacco transformants with *t-cry1F* gene

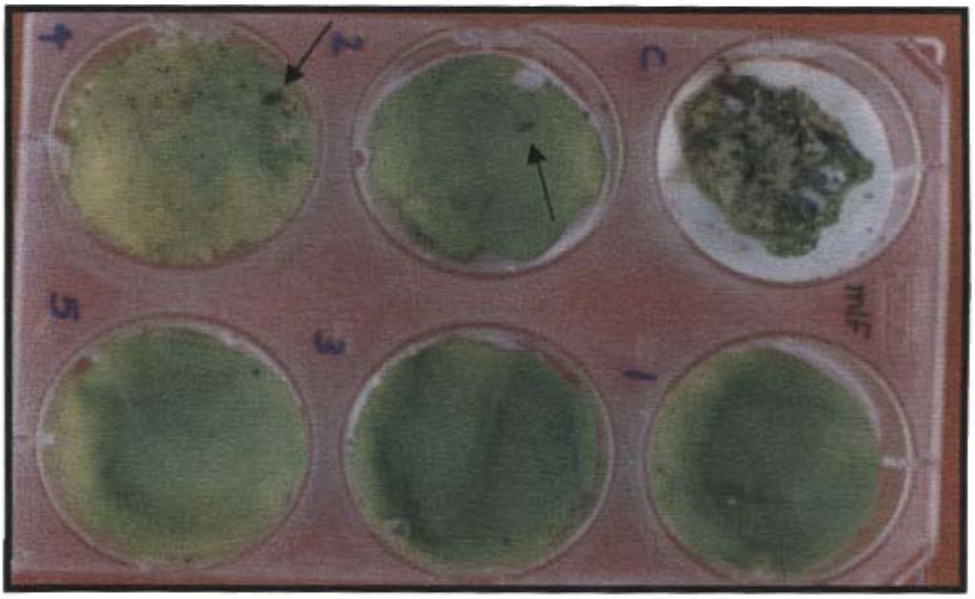
**(b)**

Control (c) : Non-transgenic tobacco plants

1-5 : Putative transformants with *m-cry1F* gene




(a)




(b)

Figure 20

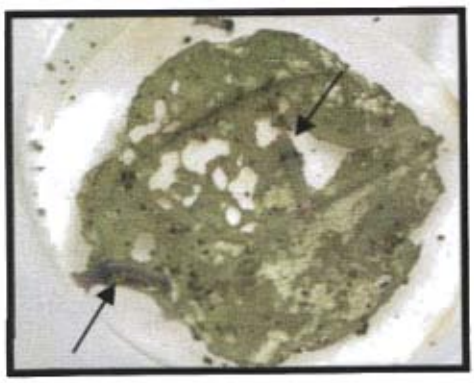
Fig. 21. **Enlarged view of the individual wells of the culture plates showing the infestation and growth retardation of the first instar larvae of *S. litura***

**(a)**

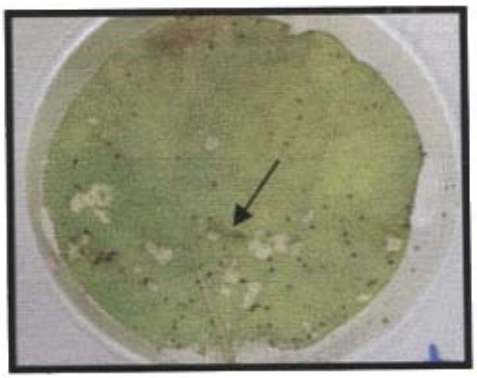
Control : Non-transgenic tobacco plants

**(b) - (d)**

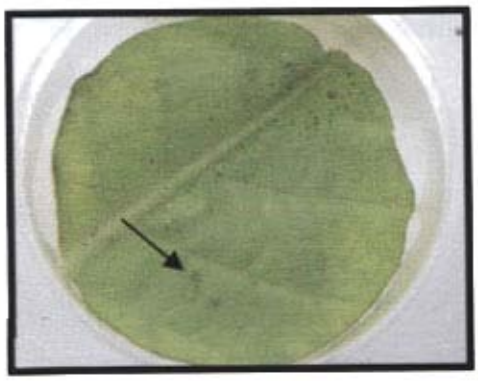
Putative tobacco transgenics



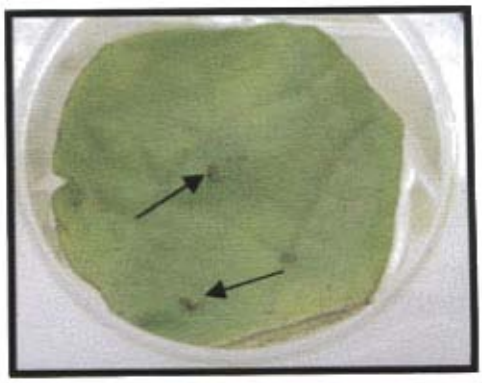
(a)



(b)



( c)



(d)

Figure 21

At 48 hr survival on *t-cry1F*-transgenic leaves dramatically dropped to average of 82.0% and for *m-cry1F*-transgenic leaves it dropped to an average of 80.9% as compared to 95.2% on non-transgenic tobacco. At 72 h, survival on *t-cry1F*-transgenic showed a precipitatious drop to average of 47.4% and a notable drop in case of *m-cry1F*-transgenic to an average of 30.6%, while on non-transgenic tobacco the survival percentage recorded was 94.5%. At 96 h, the survival of the larvae on leaf disks of *t-cry1F*-transgenic plants declined to an average of 37.8% and 15.8% in case of *m-cry1F*-transgenic plants when compared to average of 93.9% of non-transgenic tobacco. At 120 h, the percentage survival was further lowered in case of *t-cry1F*-transgenic to 22.8% and 7.5% in *m-cry1F*-transgenic tobacco plants when compared to 92.% in non-transgenic tobacco.

# 5. DISCUSSION

The major form of biotic stress faced by nearly all agricultural crops is insect infestation. It is estimated that about 13 per cent of 37 per cent of the crop product lost due to pest and diseases is accounted to insect pests (Gatehouse, 1992). Massive applications of pesticides not only leave behind harmful residues in the food, but also cause adverse effects on non-target organisms and the environment. Insect resistant cultivars provide a substantial return on economic investment. The current world economic value of this resistance is several hundred million dollars per year (Smith, 2000). These cultivars interact synergistically with biological, chemical and cultural control methods. The ecological value of insect resistant cultivars has greatly decreased world pesticide usage.

Having realized the necessity of developing insect resistant genotypes of various crop species, the breeders have been trying to explore natural gene pool/ genetic diversity and in identifying the wild genotypes of conserved crop plant species that have potential to show resistance to infestation by insect pests and subsequently transfer the conserved traits through breeding into the susceptible cultivar of crop plant species. Secondly, in isolating plant against infestation by insect pests from plants/ organisms and in introducing these genes into genotypes/ cultivars of susceptible crops through genetic engineering technology.

Impressive results have been obtained with the expression of *Bt* δ-endotoxins and plant derived genes in several crops. The first generation of insecticidal plants expressed δ-endotoxin (*cry*) genes from

*B. thuringiensis.* These genes have demonstrated the feasibility of using genetically modified plants for insect control. However, the native *cry* genes are poorly expressed in plants due to instability of mRNA and biased codon usage. Methods like truncating the gene, use of different promoters and fusion proteins have shown to increase the insect control protein gene expression significantly (Vaeck *et al.*, 1987). Increased expression of the insect control protein genes of *Bt* in plants has been critical in the development of genetically improved plants with agronomically acceptable levels of insect resistance.

The partially and fully modified genes have dramatically increased insect control protein levels in plants (Perlak *et al.*, 1991). This appears to be one of the largest increases in gene expression in any system obtained solely through modification of the coding sequence. The rationale for these designs is based on the elimination of sequences such as potential polyadenylation signal sequences, ATTTA sequences, and AT rich regions.

In the present study, it was observed that deletion of nucleotide sequence of *cry1F* gene from full length native 3525 bp to truncated 1861 bp corresponding to 550 amino acids (Figure 22a) at N-terminal end of Cry1F toxin did not have any negative effect on the toxicity of the protein. PCR was utilised to truncate and amplify the truncated *cry1F* gene. *Pfu* DNA polymerase was used for amplification as the proof-reading activity (3'→5' exenuclease) of this enzyme minimizes the chances of incorporating errors in the amplified sequence. The error rate of *Pfu* DNA polymerase is $2.6 \times 10^{-6}$ errors per nucleolide per cycle or conversely the accuracy, the inverse of error rate, an average number of nucleotides the polymerase incorporates before making an error is $3.8 \times 10^{5}$ (Lundberg *et al.*, 1991).

Fig. 22. **Restriction maps of the *cry1F* gene in various modified forms as used in the study**

(a) : Native full length *cry1F* gene

(b) : Truncated *cry1F gene*

(c) : Partially modified *cry1F* gene

(d) : Truncated *cry1F* gene showing the three domains I, II and III

(e) : Synthetic *cry1F* (fully modified) gene

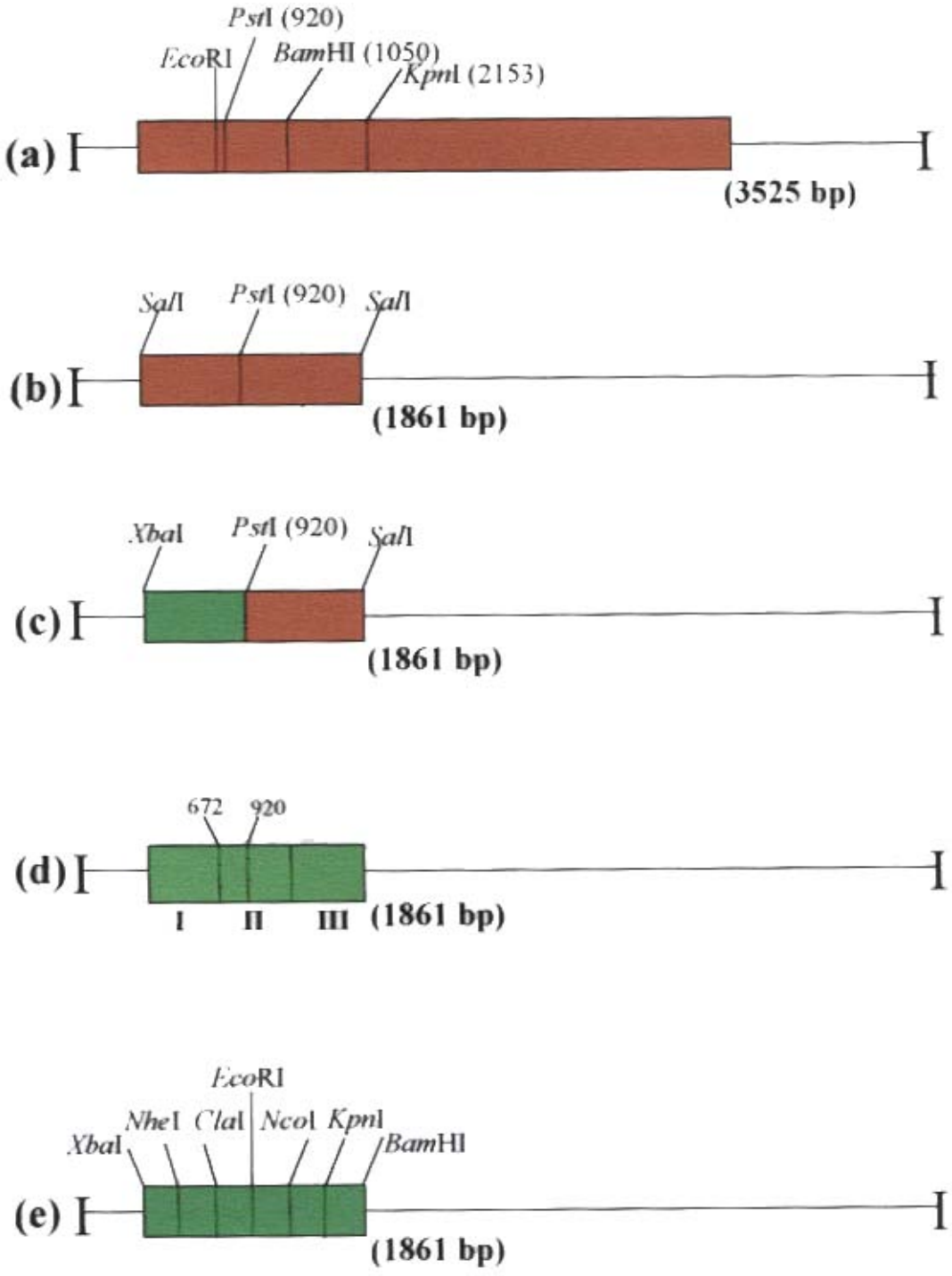

PstI (920)
EcoRI
BamHI (1050)
KpnI (2153)
(a)
(3525 bp)
SalI
PstI (920)
SalI
(b)
(1861 bp)
XbaI
PstI (920)
SalI
(c)
(1861 bp)
672
920
(d)
I
II
III
(1861 bp)
EcoRI
NheI
ClaI
NcoI
KpnI
XbaI
BamHI
(e)
(1861 bp)

Figure 22

An elementary step in determining the merit of a gene encoding an insecticidal protein for transgenic expression will be to define its toxicity spectrum. Expression of toxin genes in *E. coli* is a convenient way to produce adequate quantities of proteins to facilitate insect bioassays. The advantage of using this system include simplicity of growth medium and rapid growth of the *E. coli* (the division time ranges from 20 min to 1 hr), which means that a very large mass of cells can be obtained quickly.

pET 29a vector originally developed by Studier and colleagues (Moffatt and Studier, 1986) was the vector of choice as it is a powerful vector for the cloning and expression of heterologous genes in *E. coli.* Expression of target genes is under the control of T7 lac promoter, which allows the expression of recombinant protein only in the *E. coli* hosts containing a chromosomal copy of T7 RNA polymerase gene. BL21DE3 is the most widely recommended *E. coli* host for pET vector expression. In *E. coli,* a greater fraction of *cry1F* was isolated from the bacterial culture.

In the present study, the *E. coli* expressed Cry1F toxin displayed very high toxicity against *Spodoptera litura* neonate larvae. Toxicity effects were seen at low concentrations (100 ng) on the second day and onwards. At concentrations of 400 ng and onwards almost 100% mortality was observed after 48 h. Growth and developmental delays were observed in the first instar larvae with respect to control from the first day of the experiment. These observations indicate the effectiveness of truncated Cry1F protein against the tobacco caterpillar (*Spodoptera litura*) larvae even at low concentrations. This promising result facilitated the further modification of *cry1F* gene towards its much more effective expression.

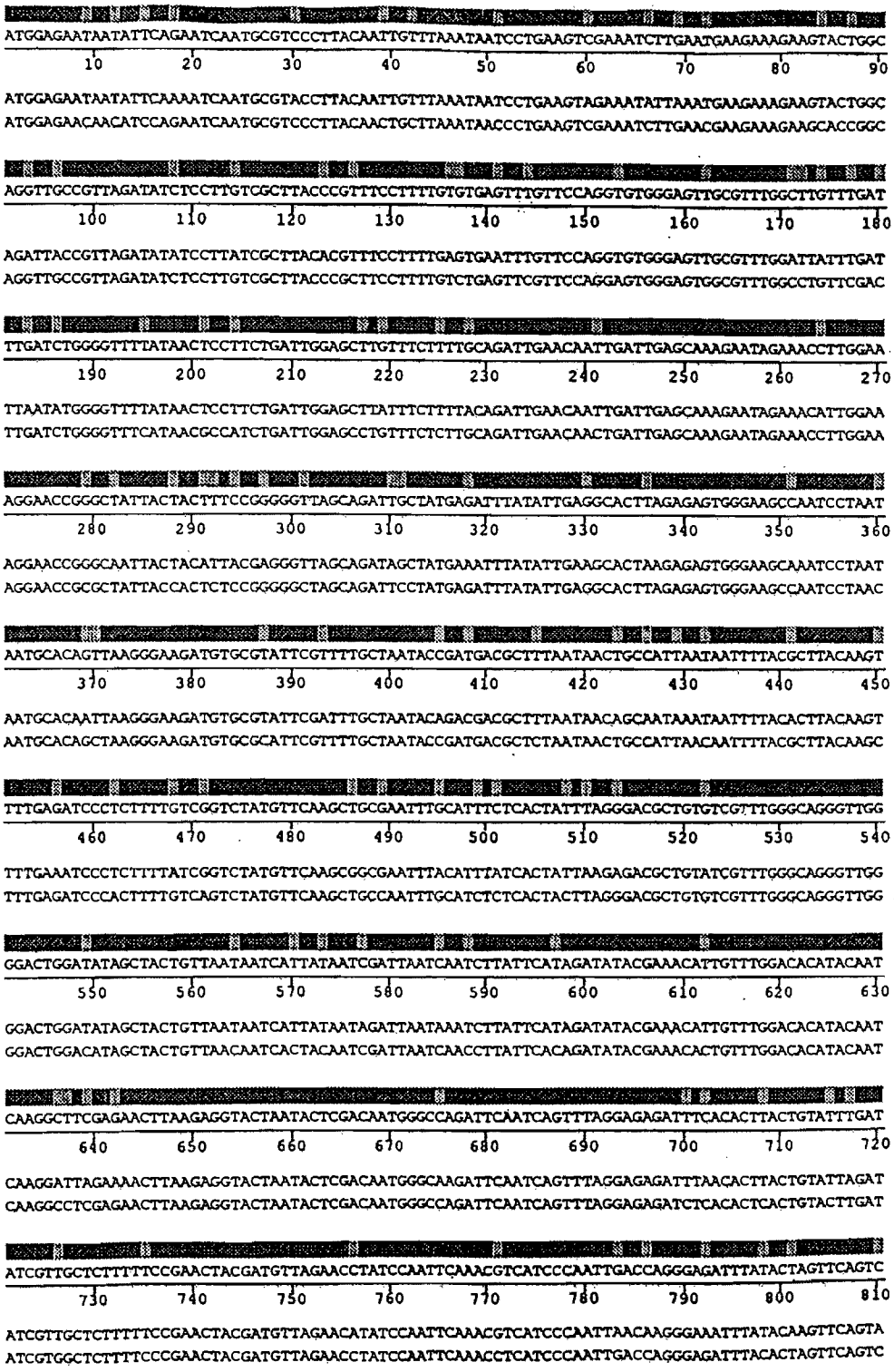
*Bt* genes coding for δ-endotoxins differ in their mode of action, receptor binding and sequence homology. However, alignment of Cry toxins reveal the presence of five conserved sequence blocks common to majority of the proteins. These homology boxes are present at the junction of the three domains I, II and III. Utilising this information the domain I boundary was marked for *cry1F* gene in the present study. In order to substitute domain I of truncated *cry1F* with that of synthetic *cry1F* gene the *Pst* I restriction enzyme recognition site present at 920 bp in *t-cry1F* gene was marked (This enzyme recognition site was absent in synthetic *cry1F* at the same position). The choice of *Pst* I enzyme however added an extra 83 amino acids that belong to domain II from synthetic *cry1F* gene (Figure 22d).

Domain I is the most essential of the three domains in imparting toxicity to the δ-endotoxin. The α helices of domain I bind to the midgut epithelial cells of the larvae, the α helices can penetrate the membranes to form an ion channel which ultimately leads to ceastion of $K^+$ pump and death of the larvae due to septicemia.

High level of expression of Bt toxin genes has been achieved by modifying the sequences of *cry* genes (Perlak *et al.*, 1991). The construction of partial or fully modified *cry1F* gene involved introduction of several changes along the entire stretch of the gene (Table 7). Synthetic *cry1F* (FM) gene was designed after analysis of *cry1F* gene nucleotide sequence was made using lasergene DNA STAR computer programme (Figure 23). Synthetic gene was subdivided into six fragments carrying convenient restriction sites at 5' and 3' ends. These six fragments were later assembled together and cloned in pET29a (Figure 24).

Fig. 23. **Published sequence of *cry1F* gene**

(Chambers *et al.*, 1991, NCBI Accession number M638978) **compared with the fully modified (synthetic) *cry1F* gene sequence**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Synth-cry1F.SEQ ATGGAGAACAACATCCAGAATCAATGCGTCCCTTACAACTGCTTAAATAACCCTGAAGTCGAAATCTTGAACGAAGAAAGAAGCACCGGC 90
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100 110 120 130 140 150 160 170 180
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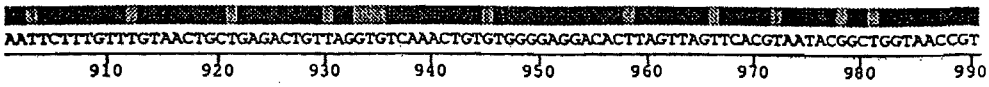


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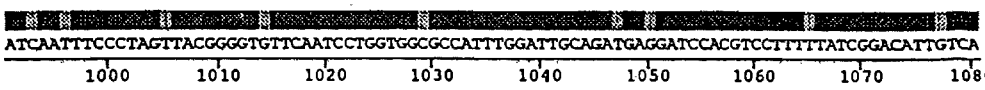


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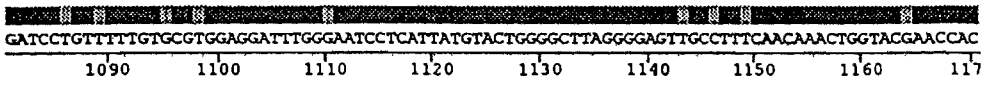


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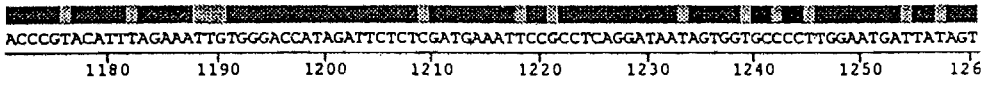


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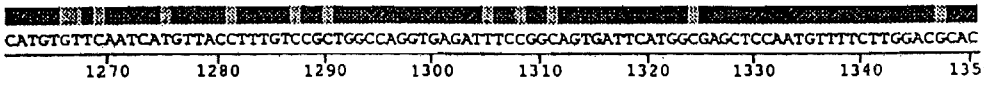


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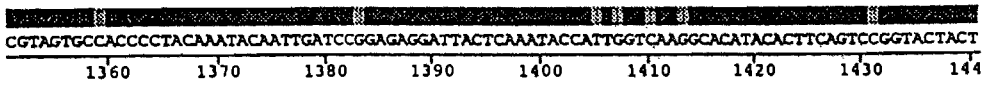


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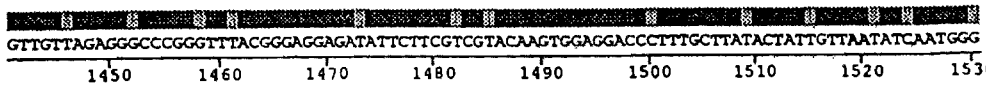


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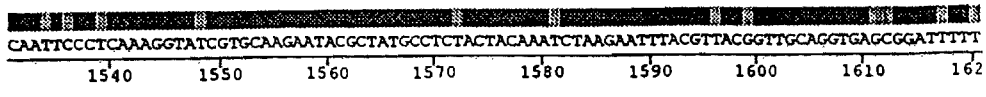


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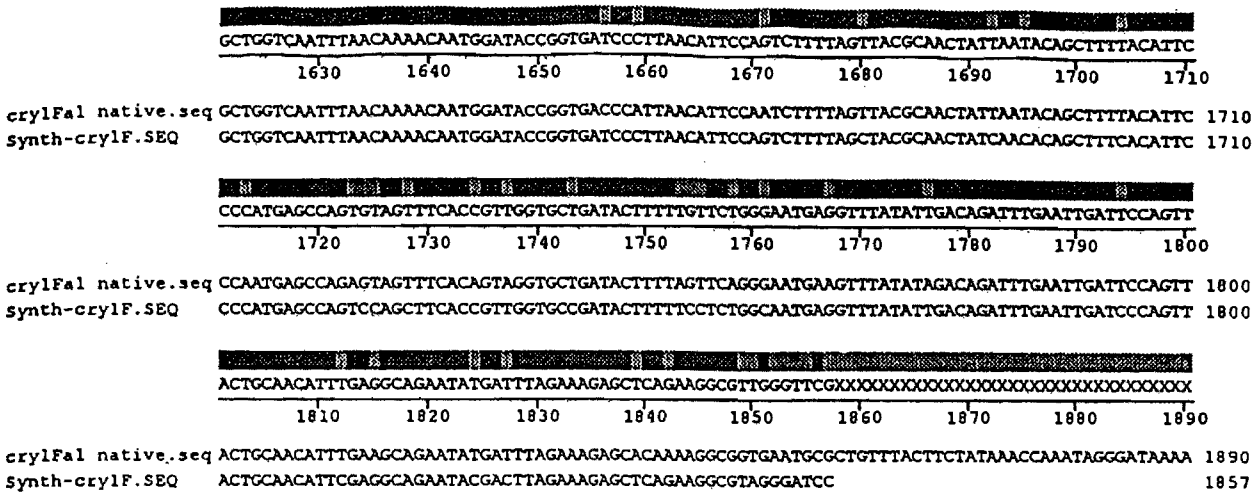
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1810 1820 1830 1840 1850 1860 1870 1880 1890
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**Fig. 24. Vector map of pET-syn*cry1F***

Restriction map of pET-syn*cry1F* containing synthetic *cry1F* gene

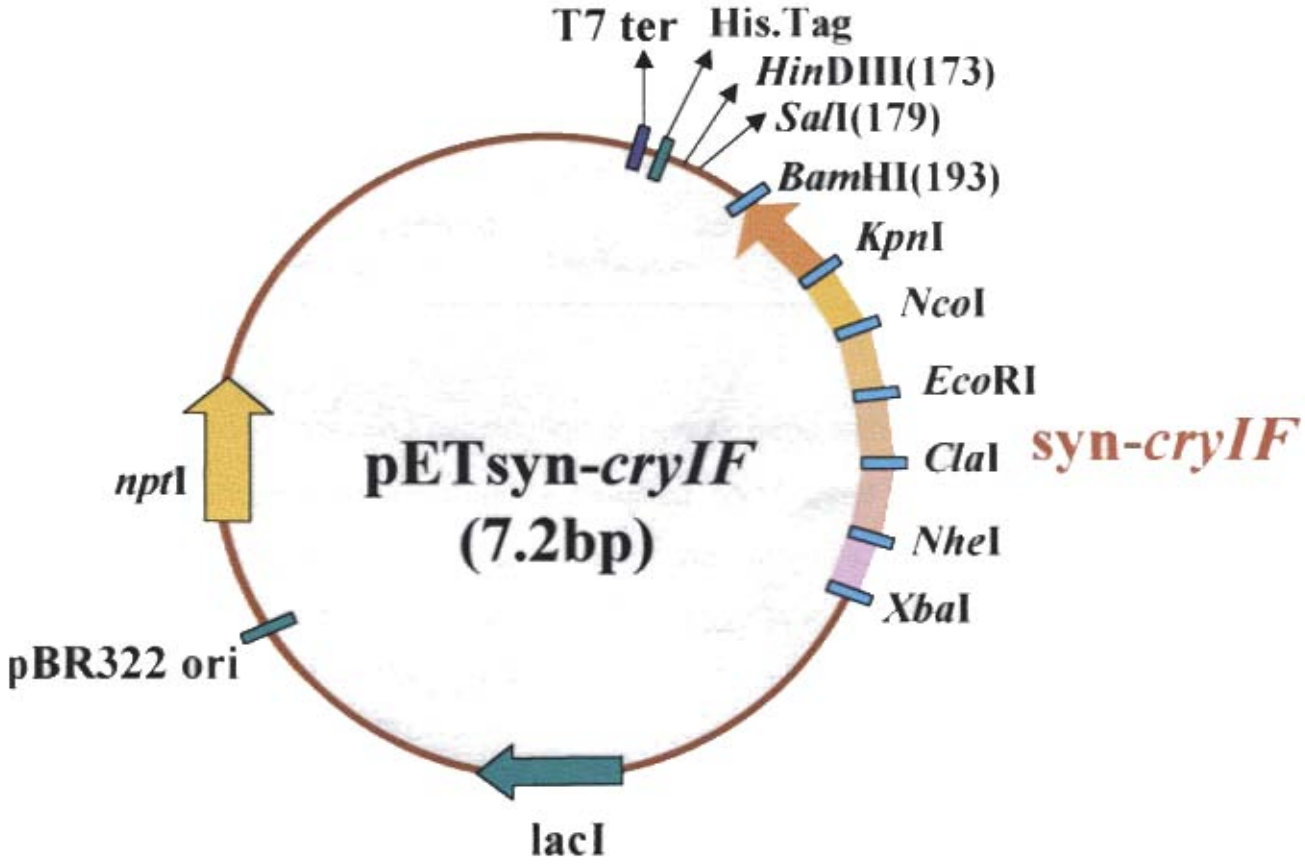
T7 ter
His.Tag
HinDIII(173)
SalI(179)
BamHI(193)
KpnI
NcoI
EcoRI
ClaI
NheI
XbaI
syn-cryIF
pETsyn-cryIF
(7.2bp)
nptI
pBR322 ori
lacI

Figure 24

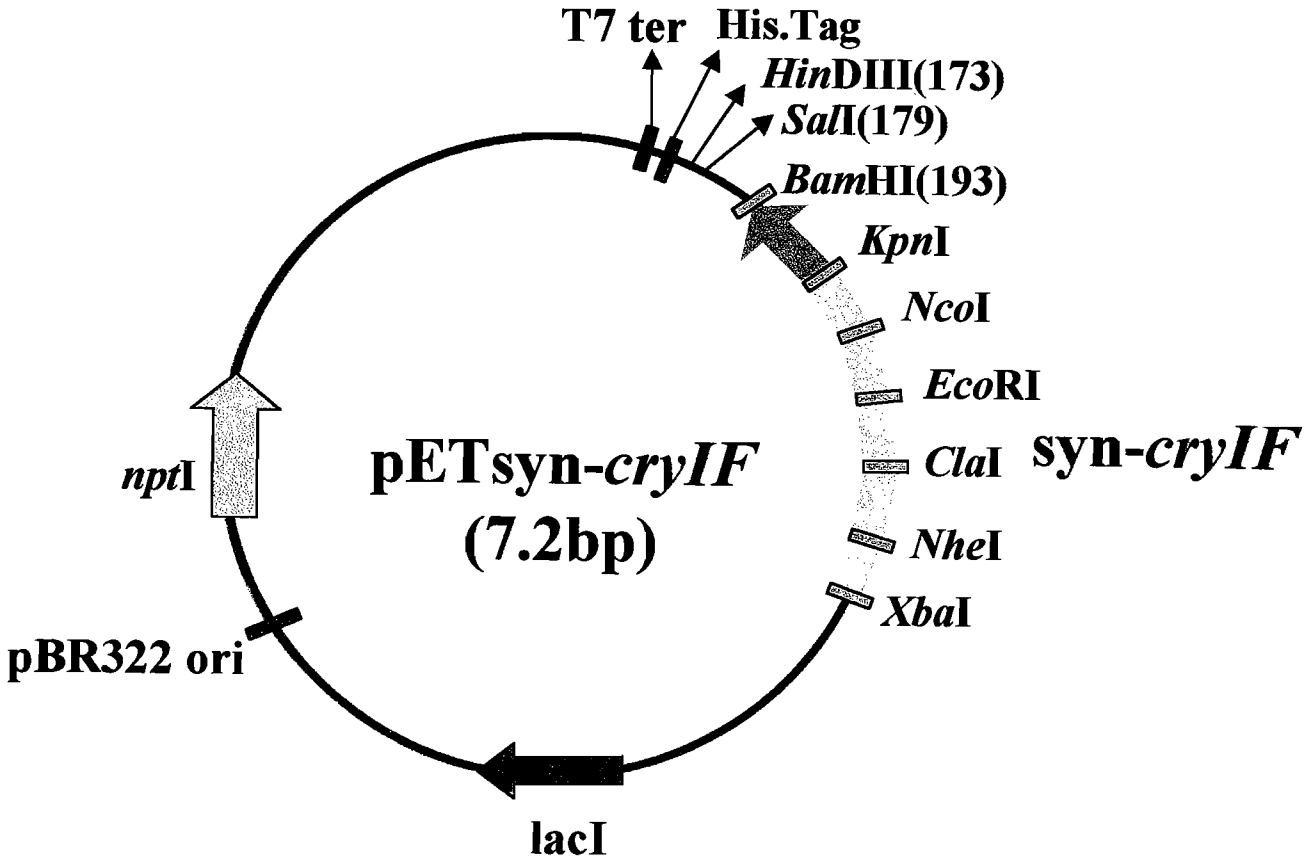
T7 ter
His.Tag
HinDIII(173)
SalI(179)
BamHI(193)
KpnI
NcoI
EcoRI
ClaI
NheI
XbaI
syn-cryIF
pETsyn-cryIF
(7.2bp)
nptI
pBR322 ori
lacI

**Figure 24**

**Table 7. Comparative analysis of native and modified (FM) *cry1F***

| | Native | Synthetic |
|---|---|---|
| AT content | 61.10 % | 53.90 % |
| GC content | 38.90 % | 45.50 % |
| Poly A signals | 7 | Nil |
| mRNA detabilising sequences | 26 | Nil |

The domain I substitution of *t-cry1F* gene with synthetic (FM) *cry1F* gene represents the partially modified *cry1F* gene (*m-cry1F*) containing the subset of the changes found in the FM gene. The PM or *m-cry1F* gene shows 11.3% increase in GC content in domain I than in the native *cry1F* gene.

In the present study, tobacco plants were transformed with truncated and modified *cry1F* genes. *Agrobacterium tumefaciens* mediated transformation was the method of choice as tobacco is very amenable to genetic transformation by this method. Tobacco has been used as a model system to study the expression of various heterologous genes including many insecticidal genes (Vaeck *et al.*, 1987; Sutton *et al.*, 1992; De Cosa *et al.*, 2001). Leaf disk infection method for tobacco transformation is a very simple and reliable method (Horsh *et al.*, 1985) which gives regenerants with a high transformation frequency. The other methods of tobacco transformation include particle bombardment, which can randomly integrate foreign genes in nuclear as well as chloroplast genome.

Incorporation of selecting agent (kanamycin) in the rooting medium minimised the number of escapes. Bleaching of control leaf explants

(which were infected with *Agrobacterium* LBA4404 without the binary plasmids constructs) shows the efficiency of selection of transformants with kanamycin (300 mg/l). PCR analysis resulted in the identification of transformants that were selected on medium containing kanamycin. The presence of full T-DNA was ensured by using gene specific primers for *npt* II as well as *t-cry1F* and *m-cry1F* genes. Interestingly all 30 transformants analysed, showed positive signals for *npt* II as well as the *m-cry1F* and *t-cry1F* genes.

Even though PCR is a quick and easy method for identification of transformants it can give false positive results. Southern hybridization is a more reliable method. In this analysis, sixteen (nine for *m-cry1F* and seven for *t-cry1F*) showed presence of one or three distinct bands that got hybridised with the *cry1F* gene specific probes. It is known that *Agrobacterium* mediated transformation often results in insertion of multiple copies of the transgenes (Gheysen *et al.*, 1991). There are reports on frequent occurrence of abberrant T-DNA insertions where one or both the ends of T-DNA were missing (Castle *et al.*, 1993).

Most of the *cry*-transgenic tobacco plants were highly lethal to first instars of the tobacco caterpillar larvae. Survival of the first instar larvae in case of both *t-cry1F*-transgenic and *m-cry1F*-transgenic plants was dramatically lowered after 48 h of infestation. This can be explained by the fact that symptoms provoked by δ-endotoxin ingestion develop over a period of 48 h to 72 h. However, in case of *m-cry1F*-transgenic plants after 72 h of infestation a sharp decline in survival percentage was observed when compared to *t-cry1F*-transgenic plants which show a steady decline pattern. This difference in percentage survival pattern can be attributed

to increase in gene expression due to sequence modification in *cry1F* gene. The larvae found alive on transgenic leaf disks were stunted and lethargic whereas on non-transgenic tobacco leaf disks the larvae had molted to the next instar.

At present, however, it is not clear if these structural differences are solely responsible for observed high level of expression and protection against insect predation. Nevertheless, it is apparent that sequence divergence of different insecticidal toxin proteins and less extensive changes in the partially modified gene, can be utilized for obtaining transgenic plants without resorting to drastic modifications in the sequence composition.

# SUMMARY

Engineering transgenic plants to express alien insecticidal proteins is a means of producing crops with enhanced levels of insect resistance. This technology has the potential to move farming closer to economically sustainable practices, both in developed and developing countries. The ecological value of insect resistant cultivars has greatly decreased world pesticide usage, contributing to healthier environment for humans, livestock and wild life. Agricultural producers have benefitted from crops with insect resistance through decreased production costs. Consumer benefit derived from these crops include safer and more economically produced crops.

In the present study, the gene of interest is a *cryI* gene, *cry1F*, designated so by Chambers (1991). The Cry1F is distinctly different in protein sequence and insecticidal specificity from the other CryI proteins. The *cry1F* gene was truncated and cloned in expression vector pET29a and its toxicity towards *Spodoptera litura* neonate larvae was observed. The results indicated the high toxicity of T-Cry1F endotoxin towards the larvae.

The presence of number of sequences in the prokaryotic *Bacillus* DNA that could provide signals deleterious to gene expression in plants, such as splice sites, poly(A) addition sites, ATTTA sequences, mRNA degradation signals and transcription termination sites, as well as codon usage biased away from that used in plants. The Bt gene hence should be first converted from AT-richness to GC-richness (typical of higher plants) to increase toxin expression. Most changes are made to the third codon

thereby minimising changes in the amino acid sequence and increasing the expression of Bt toxin by 10 to 100 fold (Perlak *et al.*, 1991). Modification of Bt gene has now become a common practice to increased toxicity expression of δ-endotoxins. In the present study, the *t-cry1F* gene sequence was fully modified with the help of Lasergene DNA STAR computer programme to obtain a custom made synthetic *t-cry1F* gene.

Changes as less extensive as 3% - 11% of the total nuicleotides have shown to produce large increases in ICP levels (Perlak *et al.*, 1999). Such partially modified genes therefore have a greater advantage over the fully modified genes. In the present study, domain I which is the functionally most essential domain in the δ-endotoxin protein was substituted between the native truncated *cry1F* gene and the custom made synthetic/fully modified *cry1F* gene. The resultant *m-cry1F* gene was cloned in pBinAR and mobilised in *Agrobacterium tumefaciens.* Tobacco transgenics transformed by *m-cry1F* and *t-cry1F* gene were produced and insect bioassays (neonate larvae of *Spodoptera litura*) carried out against these transgenic plants. The results were promising with percentage survival decreasing drastically in *m-cry1F*-transgenic after 72 h in comparison with *t-cry1F*-transgenics. This also indicated the successful use of *Agrobacterium*-mediated transformation method for introduction of *m-cry1F* and *t-cry1F* gene in the cells of leaf explants of tobacco.

PCR amplification with specific primers for *npt* II and *cry1F* genes (modified and truncated) genes was useful in initial screening of putative transformants. Southern hybridization with specific gene probes confirmed the integration of *m-cry1F* and *t-cry1F* in the genome of the tobacco genotype (Petit Havana SRI).

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