DEVELOPMENT OF *cry2Ac* TRANSFORMANTS OF TOBACCO AND THEIR ANALYSIS FOR TRANSGENE EXPRESSION AND LARVICIDAL ACTIVITY

Thesis submitted in part fulfillment of the requirements for the award of the
Degree of **Doctor of Philosophy** in **Biotechnology** to the
Tamil Nadu Agricultural University, Coimbatore

By

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CERTIFICATE

This is to certify that the thesis entitled "**DEVELOPMENT OF *cry2Ac* TRANSFORMANTS OF TOBACCO AND THEIR ANALYSIS FOR TRANSGENE EXPRESSION AND LARVICIDAL ACTIVITY"** submitted in part fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY** to the Tamil Nadu Agricultural University, Coimbatore is a bonafide record of research work carried out by **Ms. SONA S.DEV** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published earlier in part or full in scientific or popular journal or magazine.

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ABSTRACT

DEVELOPMENT OF *cry2Ac* TRANSFORMANTS OF TOBACCO AND THEIR ANALYSIS FOR TRANSGENE EXPRESSION AND LARVICIDAL ACTIVITY

By

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Genetic engineering of crop plants with Bt toxin encoding genes offers an environment friendly method of crop protection. The management of resistance development in insects to Cry proteins is one of the major challenges facing the prospects of Bt crops. The Cry2A proteins of Bt are promising candidates for management of resistance development in insects due to their difference in structure and mode of action from the widely used Cry1Ac protein. In the present study, codon optimized synthetic *cry2Ac* gene and its original version (native *cry2Ac* gene) were tested individually for their expression in transgenic tobacco plants.

A new plant transformation vector, p2AP1, harbouring synthetic *cry2Ac* gene under the control of CaMV 35S promoter was constructed and mobilized into *Agrobacterium tumefaciens* strain EHA105 by electroporation. The transformed *A. tumefaciens* strain was used for carrying out transformation of tobacco leaf discs, in two batches. Shoot regeneration in untransformed tobacco was suppressed at 50 mg/l concentration of kanamycin. Among the 15 putative transformants of tobacco transferred to pot culture condition, 12 plants established well. Nine out of the twelve putative transformants of tobacco tested were

positive for the amplification of synthetic *cry2Ac* gene by PCR. A comparison of the reaction of Cry2Ac polyclonal antibody to different Cry proteins by ELISA revealed that it reacted with Cry2Ac protein in the range of 0.5 to 4 ng per well, but failed to react efficiently with Cry2Ab and Cry1Ac proteins even upto 10 ng. Among the nine PCR positive transgenic tobacco plants that were screened by ELISA, NT2 showed a maximum of 0.11 per cent of Cry2Ac protein in total soluble protein. The level of Cry2Ac protein expression in the transgenic tobacco plants at vegetative (24 DAP) and flowering stages (33 DAP) were almost the same, as revealed by ELISA. *Helicoverpa armigera* (Hubner) bioassays showed maximum larval mortality (77 per cent) in the transgenic tobacco plant NT2 and 0 to 3.3 per cent mortality in control plants. The leaf area fed by *H. armigera* was nearly four fold more in control tobacco plants as compared to transgenic tobacco plants. The reduction in the leaf area fed by *H. armigera* in transgenic tobacco plants expressing synthetic *cry2Ac* gene over the control tobacco plants ranged from 47 to 71 per cent. Southern blot analysis with P^{32} labelled synthetic *cry2Ac* gene fragment showed a signal corresponding to that of positive control in the tobacco transformant NT2, thus confirming the integration of the gene. Northern blot analysis with the same probe also revealed the presence of *cry2Ac* transcript in the plant NT2.

In another experiment, transgenic tobacco plants containing native *cry2Ac* gene under the control of *rbc*S promoter and its transit peptide sequences were screened by ELISA and *H. armigera* bioassay. Results of the ELISA revealed a maximum of 0.33 per cent of Cry2Ac protein in total soluble protein of the transgenic tobacco plant D14-3. In *H. armigera* bioassay also, the same plant (D14-3) showed 87 per cent larval mortality. The leaf area fed by *H. armigera* in the plant D14-3 was 40.2 ± 4.9 mm^2 only, while in control tobacco plant, it was as high as 208.1 ± 19.7 mm^2. The reduction in the leaf area damage of the (native *cry2Ac*) transgenic tobacco plant, D14-3 over that of control tobacco plant was 81 per cent.

In order to get efficient expression of *cry2Ac* gene by *ubiquitin*I promoter in monocot plants, the synthetic *cry2Ac* gene was cloned in the *Bam*HI site of plant transformation vector, pUH.

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LIST OF ABBREVIATIONS

BAP	:	6-benzyl aminopurine
bp	:	Base pair
BSA	:	Bovine Serum Albumin
DAP	:	Days After Planting
EDTA	:	Ethylene Diamine Tetraacetic Acid
ELISA	:	Enzyme Linked Immunosorbant Assay
EtBr	:	Ethidium Bromide
F	:	Flowering stage
HEPES	:	N-2 hydroxy ethyl piperazine – N'2 ethane sulphonic acid
IBA	:	Indole-3-butyric acid
IPTG	:	Isopropyl-beta-thiogalactopyranoside
kb	:	kilo base
kDa	:	kilo Dalton
kV	:	kilo Volt
LB	:	Luria-Bertani broth
mA	:	milli Amperes
MS	:	Murashige and Skooge medium
NAA	:	α-naphthalene acetic acid
OD	:	Optical Density
PBST	:	Phosphate Buffer Saline Tween
PCR	:	Polymerase Chain Reaction
PMSF	:	Phenyl Methyl Sulfonyl Fluoride
RM	:	Rooting medium

rpm	:	revolutions per minute
SDS	:	Sodium Dodecyl Sulphate
SDS-PAGE	:	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SM	:	Shoot regeneration medium
TAE	:	Tris-Acetate Buffer
TBA	:	Tris Borate Ascorbic acid buffer
TE	:	Tris-EDTA buffer
TEMED	:	N,N,N',N'- Tetramethyl ethylene diamine
V	:	Vegetative stage
X-Gal	:	5-bromo-4 chloro – 3 indolyl - β-D- galactopyranoside
XIK	:	LB agar plates with Xgal, IPTG and kanamycin
YEB	:	Yeast Extract Beef extract broth
%	:	per cent
μF	:	micro Farad

CHAPTER I
INTRODUCTION

All organisms have a genetic blueprint made up of DNA that determines its characteristics, which makes it unique. Prior to genetic engineering, the exchange of DNA material was possible only between individual organisms of the same species. In the field of agriculture for instance, farmers have continuously modified naturally occurring species through seed selection and controlled breeding. With the advent of the recombinant DNA technology, it has however been possible to identify specific genes associated with desirable traits in one organism and transfer those genes within and beyond the boundaries of species into other organisms.

A large majority of the Indian farmers have been and are employing chemical pesticides as the only measure to combat the insect pest. The estimated annual spending on agricultural pesticides in India is 28 billion rupees, out of which 16 billion is spent on cotton. Approximately 45 per cent of the pesticide consumption in India is for the cotton crop and one third of the pesticides used for cotton are applied to control the boll-worm complex alone. The pest associated losses minimizing crop production has been estimated as 14 per cent of total agricultural production (Sharma and Ortiz, 2000). Applications of genetic engineering or recombinant DNA technology if properly integrated into crop production systems, offer new opportunities to increase production and productivity in a more sustainable and eco-friendly agricultural system.

Genetic engineering has lead to the development of novel varieties of crop plants with desirable characteristics like, increased nutritive value, resistance to insect pests, diseases, stress etc. Of these, insecticidal proteins of *Bacillus thuringiensis* (Bt) have emerged as the proteins of choice to be expressed in transgenic crops towards an environment friendly mode of insect pest management in agriculture (Kumar, 2004). Although *Bt* has been under extensive use as a biopesticide over the past five decades, its efficacy and potential have been realized only recently, because of its effective deployment in transgenic crops. Large scale planting of *Bt* crops began in 1996 and

increased quickly to 12.2 million hectares or 18 per cent of the global GM crop area world wide in 2003 (James, 2003). However, there has been many concerns about the efficiency and durability of Bt toxin expression in the transgenic plants. The major one relates to the development of resistance in insects to Cry proteins. The requirement of producers and farmers to develop and implement strategies to combat the development of insect resistance to Bt crops is a very significant step forward in resistance management. Resistance management strategies that include, gene pyramiding, refugia, optimum dosage etc., have to be appropriately modified to suit local conditions where the crop is being cultivated.

The development of resistance in insects against the Bt protein due to continous exposure to a single type of toxin can be overcome by several strategies, one of them being deployment of a different gene like the *cry2* genes. The Cry2A proteins of Bt are promising candidates for management of resistance development in insects due to its difference from the currently used Cry1A proteins, in structure (Morse *et al.,* 2001), and mode of action (English *et al.,* 1994). Moreover, Akhurst *et al.* (2003) have reported that Cry1Ac-resistant *Helicoverpa armigera* was not cross-resistant to Cry2Aa and Cry2Ab. Kumar *et al.* (2004) have cloned *cry2Aa* and *cry2Ab* genes from Indian isolates of Bt. The Cry2A proteins in combination with the most widely used Cry1Ac protein are expected to have positive implications for resistance management in insects (Perlak *et al.,* 2001).

High-level expression of proteins in specific plant organelles can be another efficient strategy for management of resistance in insects. The CaMV 35S promoter is used in more than 80 per cent of transgenic plants developed to express the gene of interest. Although it is a strong constitutive promoter, further research on finding novel promoters is imperative to improve the expressing levels of proteins in specific tissues and organs vulnerable to the insect attack. Jang *et al.* (1999) reported organellar localization of green fluorescent protein (GFP) in transgenic rice plants using synthetic GFP (*sgfp*). The transformed rice lines, generated by *rbc*S – Tp – *sgfp* exhibited 20 times higher fluorescence as compared to versions without plastid targeting.

Studies conducted at Tamil Nadu Agricultural University have shown that toxicity of protein encoded by an indigenous *cry2Ac* gene against rice leaf folder was comparable to Cry1Ac protein (Udayasuriyan *et al.*, 2001). The present study was carried out for testing the expressions of the native *cry2Ac* gene driven by *rbc*S promoter and synthetic *cry2Ac* gene driven by CaMV 35S promoter in tobacco as model plant.

Thus, the present study was undertaken with the following objectives:

1. Transformation of tobacco with synthetic *cry2Ac* gene by *Agrobacterium* mediated method.
2. Molecular analysis and screening of transformants of tobacco containing the synthetic *cry2Ac* gene.
3. Screening of transformants of tobacco containing native *cry2Ac* gene by ELISA and *H. armigera* bioassay.
4. Attempts were also made to clone the synthetic *cry2Ac* gene under the control of *ubiquitin*I promoter of plant transformation vector, pUH.

CHAPTER II
REVIEW OF LITERATURE

The insecticidal proteins produced by *Bacillus thuringiensis* (Bt) have provided a uniquely specific and effective tool for the control of a wide variety of insect pests. The use of Bt as a commercial insecticide is based on the remarkable ability to produce large quantities of δ-endotoxin proteins that form crystalline inclusion bodies during sporulation. The multitude of insecticidal crystal proteins of Bt sub-species has spurred their use as a natural control agent with application in agriculture, forestry and human health. Trends in agriculture suggest that biological control will become increasingly important, particularly as a part of the strategies for integrated pest management. Novel insecticidal Bt toxin with an extended target spectrum could enhance environmentally safe biocontrol practices and lead to increased food production and post-harvest protection (Federici, 1999).

2.1 Important events in the development of Bt as an insecticide

The insecticidal properties of *B. thuringiensis* have long been recognized. The bacterium was first isolated by Ishiwata Shigetene in 1901 from silkworm larvae suffering from the disease 'flacherie'. The organism was named *B. thuringiensis* by Berliner in 1911, who isolated it from diseased flour moth larvae. In 1938, the first commercial product that contain Bt, an insecticidal preparation of 'Sporeine' was marketed in France. In 1954, Angus demonstrated that the crystalline protein inclusions produced by *B. thuringiensis* in the course of sporulation were responsible for insecticidal action. The advancement in genetic engineering and molecular biology in the early eighties led to the cloning of Bt crystal protein (*cry*) gene for the first time in 1981 (Schnepf and Whitely,1981). Since then, more than 100 *cry* genes have been successfully cloned and added to the growing list. Today, the most efficient way to deliver the *cry* genes seems to be the development of transgenic plants expressing them. The first reported use of insecticidal crystal protein (ICP) genes from Bt could be introduced and expressed in plants for insect control occurred in 1987 (Barton *et al.,* 1987; Vaeck *et al.,* 1987).

Ever since the commercialization of Bt cotton in USA in 1996 the area under Bt crops has been steadily increasing. In 2003, of the total global GM crop area of 67.7 million hectares, Bt crops occupied 12.2 million hectares (James, 2003). India also has made its long-awaited entry into agricultural biotechnology, after the Government of India approved three Bt cotton varieties (MECH 12, MECH 162 and MECH 184) in 2002 for commercial cultivation. During 2004, the fourth Bt cotton variety (RCH 2) has been approved by the Indian government for commercial cultivation in the southern and central parts of the country. Thus Bt serves as a paradigm for transgenic plants that can augment productivity and bring about more sustainable agriculture.

2.2 Classification of Bt toxins

The first attempt to systematically classify Bt toxins was proposed by Hofte and Whiteley (1989). The classification was limited to the 42 then known Bt genes. The 42 *Bt* genes were classified into 14 distinct types based on the protein they encoded; 13 for crystal (Cry) proteins & 1 for the cytolytic (Cyt) proteins. Since, *Bt* genes encoding cytolytic proteins were totally unrelated to *cry* genes, they were designated as *cyt* genes. The Bt crystal protein encoding genes were categorized into 4 major classes designated by a Roman numeral based on their structural similarities and insect specificity (Table 1).

However, the nomenclature of Hofte & Whiteley (1989) based mainly on insecticidal activity failed to accommodate genes that were highly homologous to known genes but with a different insecticidal spectrum. Hence Crickmore *et al*. (1998) introduced a system of classification based on amino acid homology using the ClustalW software. In this system of classification, each protoxin acquired a name consisting of the mnemonic Cry or (Cyt) and four hierarchial ranks consisting of numbers, capital letters, lower case letters and numbers (eg. Cry25Aa1). Thus, proteins less than 45 per cent homology differ in primary rank (Cry1, Cry2) and 78 per cent and 95 per cent identity constitute the border for secondary (Cry1A, Cry1B) and tertiary ranks (Cry1Aa, Cry1Ba), respectively. Quaternary rank is given to those proteins, which are more than 95 per cent similar in amino acid sequence (Cry1Aa1, Cry1Aa2).

2.3 Structure of Bt toxin

Hofte and Whitely (1989) compared the sequences among a number of Bt toxins with varying specificities and found five well conserved regions and designated it as blocks. On the basis of the conservation of the defined blocks, it was postulated that the Bt crystal toxin consists of three major domains. Because of this typical structure, this class of proteins is sometimes referred to as three domain Cry proteins. Among these proteins, to date, the structure of five crystal proteins Cry3A (Li *et al*., 1991), Cry1Aa (Grochulski *et al*., 1995), Cyt2A (Li *et al*., 1996), Cry2Aa (Morse *et al*., 2001) and Cry3Bb (Galitsky *et al*., 2001) have been confirmed by X-ray crystallography. The characteristics of these domains along with their proposed functions are listed in Table 2.

The structure of the Cry2Aa from *B. thuringiensis* subsp *kurstaki* was determined by Morse *et al*. (2001). The structure of the three domains is surprisingly similar in overall topology to those of the activated toxin Cry3Aa and Cry1Aa. These toxins have little sequence homology to Cry2Aa (20% and 17%, respectively). In the mature toxin, the N terminal domain (residues 1-272) is a pore-forming seven α-helical bundle (wherein, six are arranged in a circle and helix 5 is in the center) resembling the pore-forming domain of colicin A . The long hydrophobic and amphipathic helices of domain I suggest that this domain might be responsible for the formation of lytic pores in the intestinal epithelium of the target organism (Schnepf *et al.,*1998).

The second domain (residues 273-473) is organized as a three β sheet structure. The domain II is involved in receptor binding and this is supported by site directed mutagenesis and segment swapping experiments (Jurat-Fuentes and Adang, 2001).

The third domain of Bt toxin (474-633) also comprises of three β sheets and arranged like a sandwich. The domain III could play a number of key roles in the biochemistry of the toxin molecule. Its main function consists of maintaining the structural integrity of the toxin molecule by shielding it from the proteases during proteolysis within the gut of the target pest (Li *et al.,*1991).

2.4 Mode of action of Bt toxins

The mode of action of Bt toxins has been studied quite extensively (Aronson *et al*., 1986; Hofte & Whiteley, 1989; Knowles, 1994; Schnept *et al*., 1998; de Maagd *et al*., 2001; de Maagd *et al.,* 2003). The Bt insecticide proteins are toxic only after ingestion by the susceptible insects.

The main steps involved when the Cry protein is ingested by the insect comprises of, solubilization of the protoxin, its enzymatic activation by terminal cleavage, receptor binding in brush border membrane of the midgut, pore formation, consequent disruption of ionic potential, destruction of epithelial membrane, leading to cell death.

The biochemical properties of Cry proteins & their mode of action are responsible for the insect specificity demonstrated by these proteins. At each step of their reactivity inside the insect, the outcome may vary depending on the target insect. Initial step of solubilization of the protoxin inside the gut is highly dependent on alkaline pH. This pH is found in lepidopterans and dipterans but in coleopterans the gut pH ranges from neutral to weakly acidic. Any toxins that is insoluble at this pH will not be able to manifest toxicity against coleopteran insect due to lack of its activation by gut proteases. Differences in gut physiology from one order to the other can play an important role at the second step of activation. eg. key digestion proteases of lepidoptera and diptera are serine proteases whereas that of coleoptera are cysteine & aspartic proteases. Thus the specific protease requirement for the cleavage of C-terminal of the protoxin decides whether a toxin is potent against a particular insect or not. The widely studied aspect of receptor binding and pore formation, is the central and decisive step of the specificity that finally leads to disruption of the brush border membrane of the midgut causing death of the insect.

Several Cry toxin receptors (120-170 kDa) were identified as aminopeptidase N (APN) in *Heliothis virescens* Fab. (Luo *et al.,* 1997; Karim *et al*., 2000 and Hua *et al*., 2001) and in addition 210 kDa cadherin like glycoprotein has been identified as a Cry1Ab binding protein in BBMV of *Manduca sexta* (L) larvae (Vadlamudi *et al.*, 1993 and 1995 and Karim *et al*., 2000). When the activated toxin binds to specific receptors on the brush

border membrane of a susceptible insect, it induces disruption of the activity of K^+ pump leading to osmotic imbalance. This leads to the swelling of microvilli and destruction of epithelial membrane and finally to cell death of the insect (Lorence *et al*., 1997).

The mode of action of Cry1, Cry2 and Cry3 insecticidal toxins have been studied in detail. Although Cry1 & Cry3 toxins have a similar mode of action, English *et al*. (1994) reported unique attributes to the mode of action of Cry2 toxin. Cry2A was less soluble than Cry1Ac, but like proteolytically activated Cry1Ac, Cry2A was stable in the digestive environment of the insect. Unlike Cry1 toxins on the lepidopteran brush border & Cry3A on brush border of coleoptera, Cry2A failed to bind to a saturable binding component on the midgut brush border of *H. zea*. In addition Cry2 toxin formed voltage dependent and not highly cationic selective channels in planar lipid bilayer unlike Cry1Ac & Cry3 toxin.

2.5 Vegetative insecticidal proteins (Vips) of *B. thuringiensis*

Vegetative insecticidal proteins (Vips) is a novel class of protein produced by *B. thuringiensis* during its vegetative phase of growth (non-sporulation phase). This toxic protein has been identified by Estruch *et al*. (1996) and Warren *et al*. (1996). Unlike the δ-endotoxins, Vip proteins are not parasporal and secreted from the cell. The symptoms produced by Vips are similar to those caused by Cry proteins, but it develops 48-72 hours after ingestion whereas in the latter it takes only 16-24 hours (Yu *et al*., 1997). An operon encoding a binary toxin comprising of Vip1A(a) (100 kDa) and Vip2A(a) (52 kDa) were identified by Warren (1997) in a *Bt* strain AB78. Based on sequence homology studies, it has been suggested that Vip2 is the cytotoxic subunit and Vip1 is the receptor binding subunit (de Maagd *et al*., 2003). Warren *et al*. (1996) reported that the 100 kDa protein encoded by *vip1A* gene yields 80 kDa active protein after processing, which is highly toxic to Western Corn Root worm (WCR) in conjunction with the Vip2A protein. Another 88 kDa protein, Vip3A from strain AB88, identified by Estruch *et al*. (1996) exhibits toxicity against some of the economically important lepidopteran pests e.g. *Agrotis ipsilon, Spodoptera frugiperda, Spodoptera exigua* and *Helicoverpa zea* (Estruch *et al*., 1996 and de Maagd *et al*., 2003). The Vip3A protein shows 260 times higher activity than some of the Cry1A proteins (Estruch *et al*., 1996). In context of the LD50 value also, the Vips are required at much less concentrations for corn root worm

and black cut-worm (Warren, 1997). The *vip3V* gene of *B. thuringiensis* was cloned and characterized by Doss *et al*. (2002) which showed high toxicity against *Bombyx mori* and *Culex quinquefasiciatus*. Recently, Chen *et al*. (2003) reported that the N-terminal sequences of the Vip3A protein plays an important role in their secretion and toxicity.

2.6 Screening of *B. thuringiensis* genes by PCR

The Polymerase Chain Reaction (PCR) is a molecular tool widely used to characterize the insecticidal bacterium *Bacillus thuringiensis* strain collections (Gleave *et al.*, 1993; Ceron *et al*., 1995; Ben-Dov *et al*., 1997; Bravo *et al*., 1998 and Ferrandis *et al*., 1999). This technique is a highly sensitive method of rapidly detecting and identifying target DNA sequences, requires minute amounts of DNA and allows screening of many *B. thuringiensis* samples to classify them and predict their insecticidal activities. The PCR based identification of *B. thuringiensis cry* genes was first developed by Carozzi *et al.* (1991) and they designed primers from *cry1Ab, cry3A* and *cry4A* genes, for identification of Lepidoptera, Coleoptera and Diptera active strains, respectively. The efficacy of PCR in identifying the large family of *cry* genes, with amino acid identities ranging from less than 45 per cent to more than 95 per cent, is based on the presence of conserved regions. For practical reasons, primer pairs designed from highly conserved regions and recognizing entire *cry* gene sub family are often used in a preliminary screening prior to performing a second PCR with specific primers. Another strategy for the screening is based on the multiplex PCR which uses more than two primers in a mixture of the same reaction (Juarez Perez *et al*., 1997). Usually, a single universal primer is combined with several specific oligonucleotide that recognize individual genes.

2.7 Cloning of new *Bt* genes by PCR

The use of PCR for amplification of nucleic acids has expanded vastly the power of gene cloning and construction (Rashtchian, 1995). Kalman *et al*. (1993) proposed a strategy to identify variants of the *cry1C* group through PCR making use of series of primer designed to anneal through *cry1Ca1* sequence. Ceron *et al.* (1995) have reported an amplified fragment that may correspond to a new *cry* gene, when using a multiplex PCR with specific primers. A combination of PCR and restriction fragment length polymorphism (RFLP) was the first method specifically designed by Kuo and Chak (1996)

for cloning new genes. However the PCR-RFLP analysis was unable to detect differences between *cry1Ca, cry1Cb, cry1Ea* and *cry1F* gene. Juarez-Perez *et al*. (1997) developed a novel approach to detect new *cry* genes based on the use of two sequential PCR reactions, using a multiplex PCR with specific and universal primers. This strategy is called E-PCR and starts with the amplification of already described *cry1* genes followed by a second conditional amplification that will occur only if a new putative *cry1* gene(s) is present in the strain (*cry1* subfamily was taken as a model to test this technique). Kuo *et al.* (2000) reported the first primer pair able to recognize all members of the *cry1* gene family known at that time, as well as some other genes coding for the actual *cry3, cry4* and *cry7* genes.

2.8 Bt and its current application in agriculture

2.8.1 Bt spray formulations

The Cry proteins studied till date have been verified to be non pathogenic to mammals, birds, reptiles and amphibians. These toxins are specifically lethal to insects and invertebrate pests. Individual toxins have narrow specificity but the vast array of toxins reported in literature can cover a very large spectrum of insect pests. The Cry proteins have thus been successfully applied against agricultural pests, forest pests, mosquitoes and black flies. The US Environment Protection Agency (EPA) registered 127 commercially available Bt-based products against lepidopterans in 1961. By 1995, such products had occupied two per cent of the global pesticide market, with an estimated sales value of 90 million dollars (Schnepf *et al.*, 1998).

To fully realize the potential of Bt δ-endotoxins as biopesticides, progress is required in several areas. First, we must increase the yield or efficiency of toxin protein production. Second, we must gain a sufficient understanding of the mechanism of toxicity to allow engineering of the toxins for maximum activity. Third, we must continue to isolate new strains with novel toxin structures and activities.

The Cry and Cyt ICPs are the primary cause of insect death. Viable bacterial spores are also present in Bt spray formulations. The other components in sprayed Bt pesticides can contribute especially in insects that are not very sensitive to Cry proteins. The Bt spore germinates after the gut is damaged and then begins the vegetative growth producing other insecticidal proteins which includes vegetative insecticidal proteins (Vips), βexotoxin, zwittermicin A, chitinases & phospholipases.

Bt products, when applied as sprays have certain limitations like difficulty in getting uniform coverage on the crops, can be easily washed away by rain or may get degraded by solar radiation, thus requiring repeated applications, need for labour and equipment etc. While the use of Bt as a spray formulation remains significantly behind chemical pesticides in agriculture, the long and environmentally benign use of Bt products has weighed in favour of Bt transgenic crops from a safety stand point. Private companies and public funded institutions interested in agricultural biotechnology quickly realized the potential of the existing rich gene pool of *Bt* insecticidal genes which can be exploited in the development of insect tolerant crops.

2.8.2 Why transgenic technology, why not pesticides?

Biotechnology has provided access to novel molecules, ability to change the level of gene expression, capability to change the expression pattern of genes and develop transgenics with different insecticidal genes.

Development and deployment of transgenic plants with insecticidal genes will lead to

- Reduced exposure of farmers, farm labour and non-target organisms to the pesticides.
- Increased activity of 'natural enemies' because of reduction in pesticide sprays
- Reduced amounts of pesticide residues in the food and food products.
- A safer environment to live because of reduction in pesticide use (Sharma and Ortiz, 2000).

There are various reports indicating that plants modified to express insecticidal proteins from *B. thuringiensis* provide a safe and highly effective method of insect control. Betz *et al*. (2000) summarized the benefits of using Bt-protected crops as follows:

a. Reduced chemical insecticide treatments for target pests

b. Highly effective pest control
c. Increased crop yields
d. Supplemental pest control by preserving or enhancing populations of beneficial organisms and
e. Less damaged seeds (due to reduced attack by corn borers) have less fungal infection. Hence less aflatoxin, fusarial toxins etc in feeds used for animals, poultry etc.,

2.8.3 Transgenic plants developed using *Bt* genes

2.8.3.1 Expression of *cry* genes in plant cells

Procedures are now available to introduce any isolated gene from any organism into crop plants or model plant species. However bacterial genes require significant modification before these can be expressed in plants. One major modification was the introduction of regulatory regions (promoters and polyadenylation signals) that can be used for expression in the plant cells. A number of promoters that provide high level of expression (CaMV 35S and *rbc*S) have been characterized. However identification and use of such regulatory sequences is not sufficient for the expression of *Bt* genes in plants. The process becomes complicated due to the differences in GC content (plants are relatively GC rich as compared to *Bacillus*) and in amino acid codon preferences of the two organisms. Hence, the level of expression of native *Bt* genes in plant cells was very poor (Perlak *et al*., 1991; Diehn *et al.* 1998). Another major limitation in using *Bt* genes is the presence of AT rich stretches that function as potential polyadenylation signals causing interrupted transcription of those genes. The role of putative signals in limiting the transcription of *cry1Ac* gene has been experimentally demonstrated by Diehn *et al*. (1998). Therefore, specific nucleotide changes are essential for optimal transcription and translation of *cry* genes in transgenic plants.

The principal criteria for these modifications are as follows:

a. Nucleotide changes made in *Bt* genes to shift codon bias towards plants without disturbing the amino acid sequence.

b. Modification of sequences that might cause premature termination of transcription. e.g. AT rich regions mimicking plant polyadenylation signals.
c. Removal of putative splice signals from bacterial genes to prevent disruption of the mRNA transcript in eukaryotic cells.

Use of full length *Bt* genes for plant expression is not essential as the long C-terminal of the translated full length protein (or protoxin) is not required for toxicity. The role of C-terminal stretch is in the formation of crystal body inside the bacterial cell. Therefore codon modification and use of truncated genes emerged as two key features of modifying *cry* gene sequences for their effective expression in plant cells.

In a pioneering study by Perlak *et al*. (1991), a drastic improvement in expression of modified *Bt* genes was demonstrated as opposed to wild type and partially modified nucleotide sequences. The fully modified *cry1Ab* gene showed 100 times higher expression in cotton plants (var.Coker312) than the wild type gene. This was achieved through a combination of powerful promoters (ie., CaMV 35S with duplicated enhancers) and sequence modification in areas of the gene with predicted mRNA secondary structures. Transgenic potatoes providing better protection against Colarado potato beetle was achieved by increasing overall GC content of *cry3A* gene from 36 to 49 per cent (Perlak *et al.*, 1993).

These initial accomplishments were restricted to dicotyledonous plants. Expression of *Bt* genes in monocotyledonous plants was advanced when Koziel *et al*. (1993) transformed elite cultivars of maize with a synthetic truncated *cry1Ab* gene with GC content of 65% and using tissue specific promoters. These transgenic maize plants provided excellent protection against European corn borer (*Ostrinia nubilalis*) even when challenged in the field with over 2000 larvae per plant, an insect pressure several hundred fold higher than natural infestations.

2.8.3.2 Bt crop plants

Using recombinant DNA techniques, the crystal protein (*cry*) gene was transferred and expressed in plants for the first time in 1987. Three pioneering reports were published on insect resistant transgenic plants in 1987. Barton *et al*. in tobacco; Fischhoff *et al*. in tomato and Vaeck *et al*. in tobacco demonstrating resistance to *Manduca sexta* larvae. But the extent of protection was low as in all these studies native *Bt* genes without codon alterations were used. The wild type bacterial gene sequences were not able to produce sufficient amounts of protein. Later reports used truncated *Bt* genes carrying codon modified sequences for their optimal expression in plants. Since the first report of the transgenic Bt plant, more than 30 plant species have been transformed by using a range of *Bt* genes. Currently, one or the other of the three transgenic Bt crops (cotton, corn and potato) are under cultivation in USA, China, South Africa, Australia, Argentina, Mexico, Indonesia and India (Bambawale *et al*., 2004). Approximately 12 mha of insect protected transgenic crops incorporating Bt Cry proteins are now planted annually world wide and the information on the insect resistant transgenic plants harbouring Bt *cry* genes is summarized in Table 3.

2.9 Insect resistance to Bt

Insects have demonstrated a high capacity to develop resistance to a wide array of chemical insecticides. More recently, field populations have been shown to be equally adapt at developing resistance to microbial sprays based on the Bt δ-endotoxins (Shelton *et al*., 2002). These cases are limited and have been associated with frequent and prolonged use of the microbial products on geographically isolated insect populations. However, there is an industry wide recognition of the need for research to address the potential for resistance to Bt δ-endotoxin.

2.9.1 How insects developed resistance against Bt toxins?

Mc Gaughey (1985) demonstrated that *Plodia interpunctella* became resistant to Bt when reared for several generations in the laboratory with a sublethal dose of a Bt commercial product. Van Rie *et al.* (1990) found that the receptor sites on BBMV for Cry1Ab were greatly reduced in Bt resistant *P. interpunctella*. Ferre *et al*. (1991) reported

that in Bt resistant *P. xylostella* binding sites for Cry1Ab was lost but retained sites for Cry1Ba and Cry1Ca. Tabashnik *et al*. (1994) found no binding of Cry1Ac to BBMV isolated from a resistant *P. xylostella* population. The resistance was lost when the Bt pressure was removed.

Tabashnik *et al*. (2000) observed that two strains of pink boll worms, *Pectinophora gossypiella* selected in the laboratory for resistance to Bt toxin Cry1Ac had substantial cross resistance to Cry1Aa and Cry1Ab but not to Cry1Da, Cry1Ea, Cry1Ja, Cry2Aa and Cry9Ca. The narrow spectrum of resistance and cross resistance to activated toxins suggested that reduced binding of the toxin to midgut target sites could be an important mechanism of resistance.

A second mechanism of resistance involves gut proteinases, that interact with Bt toxins. Two *B. thuringiensis* resistant strains of Indian meal-moth were found to lack a major gut proteinase that activates Bt protoxins (Oppert *et al*., 1997). In USA, the *Heliothis virescens* is a major pest of transgenic cotton expressing the Bt *cry1Ac* gene. Alteration of receptor, a 170 kDa aminopeptidaseN is implicated as a mechanism for *H. virescens* resistance to Cry1A toxins (Jurat-Fuentes and Adang, 2001).

2.9.2 Management of resistance in Bt crops

The first step in resistance management is to establish the target pest baseline susceptibility to the insecticidal protein. The base line should be determined by geographical location, insect species and selective agents like microbial spray or transgenic plants. Once a base line has been established, regular monitoring can be used to detect changes in susceptibility that may indicate early stages of resistance in local insect populations.

In an attempt to develop insect resistant transgenic crops, the following points may be considered (Kumar, 2004):

1. Critical evaluation of the target pests, its biology and susceptibility to a range of insecticidal proteins (Bt & non-Bt sources).

2. Selection of two or more effective toxins based on their efficacy, mechanism of action and receptor binding.
3. Evaluation of the biosafety of the insecticidal proteins.
4. Optimization of gene expression as evidenced by studies in model systems like tobacco.
5. Selection of suitable and effective promoters based on spatial and temporal aspects of insect infestation.
6. Expression of multiple insecticidal genes with different modes of action driven by different promoters in transgenic crop of interest (either by co-transformation or by plant breeding). This technique is called gene stacking strategy.
7. Selection of the transformed plants with a single copy transgene insertion and high levels of toxin expression. Transgenic plants producing very high levels of insecticidal proteins should be produced in an effort to eliminate heterozygotes carrying a resistance allele.
8. Adoption of refugia strategy to provide a source of susceptible insects for mating with the selected populations to prevent the fixation of resistance. Through random mating, rare recessive resistance genes are diluted out of the insect population and prevented from providing a selective advantage in off spring of the matings. This strategy will not be effective if cases develop where resistance is dominant. The effectiveness of a refuge depends on consistent, appropriate implementation and monitoring.

Tang *et al*. (2000) based on the green house tests using *B. thuringiensis* transgenic broccoli plants expressing *cry1Ac* reported that resistance of diamond backmoth, *P. xylostella* could be delayed by increasing the proportion of refuge plants and separate refuges delayed resistance better than mixed refuges.

A study released by entomologists at Indian Agricultural Research Institute in New Delhi has cast doubts on the long-term benefits of Bt cotton. The study indicated that the protection afforded by *Bt* gene is at its best for six years. The study by Chandrasekar and Gujar (2004) reported that the bollworm developed 31-fold resistance to the Cry1Ac toxin

within six generations. The bollworms also developed cross-resistance to Cry1Aa and Cry1Ab toxins. The Cotton farmers in India spend nearly 12 billion on pesticides to protect their crops from the cotton bollworm. What this study means is that cotton farmers may have to go back to spraying pesticides after six seasons unless scientists come out with Bt cotton hybrids that produce a high dose of the Cry1Ac toxin. Hence, the findings of the scientists mandate the necessity of Bt resistance management. One way of doing this is by ensuring that each farmer allots a part of his land for non-Bt crops -- a requirement unlikely to be followed by farmers with small land holdings.

2.10 *Agrobacterium* mediated plant transformation

Plant transformation is performed using a wide range of tools such as *Agrobacterium* Ti plasmid vectors, microprojectile bombardments, microinjection, chemical (PEG) treatment of protoplasts and electroporation of protoplasts. Among these the *Agrobacterium* mediated transformation is widely used and offers many unique advantages in plant transformation as reviewed by Veluthambi *et al*. (2003). It includes

a. The simplicity of *Agrobacterium* gene transfer makes it a poor man's vector.
b. A precise transfer and integration of DNA sequences with defined ends.
c. A linked transfer of genes of interest along with the transformation marker.
d. The higher frequency of stable transformation with many single copy insertions.
e. Reasonably low incidence of transgenic silencing.
f. The ability to transfer long stretches of T-DNA (> 150kb).

The current status of our understanding of *Agrobacterium* T-DNA transfer process has been reviewed by Gelvin (2000) and Zupan *et al*. (2000). Three genetic elements, *Agrobacterium* chromosomal virulence genes (*chv*), T-DNA delimited by a right border and a left border and Ti plasmid virulence genes (*vir*) constitute the T-DNA transfer machinery. The T-DNA transfer is mediated by the 30 kb virulence region, comprising of six operons *vir*A, B, D, G, C and E. *vir*C and *vir*E are required for increasing the transfer efficiency (de la Riva *et al*., 1998). Krishnamohan *et al*. (2001) reported that efficient *vir* gene induction in *A.tumefaciens* requires VirA, VirG and Vir box from the same Ti plasmid.

The *Agrobacterium* mediated genetic transformation is successfully being employed in crops like peanut (Rohini and Rao, 2000), blackgram (Karthikeyan *et al*., 1996), pigeonpea (Geetha *et al.*, 1999), chillies (Manoharan *et al.*, 1998), brinjal (Kumar *et al*., 1998), cabbage (Bhattacharya *et al*., 2002) and other fruit crops (De Bondt *et al*., 1996 and Peri *et al*., 1996). For long, the inability of *Agrobacterium* to transfer DNA to monocotyledonous plants was considered its major limitation. However with effective modifications in Ti plamid vectors and finer modifications of transformation conditions, a number of monocotyledonous plants including rice (Roy *et al*., 2000 and Mohanty *et al*., 1999), wheat (Singh and Chowla, 1999) etc. have been transformed.

2.10.1 Ti plasmid - based vector system

2.10.1 .1 *Agrobacterium vir* helper strains

The co-integrate or *cis* vectors carry the T-DNA on the same replicon as the *vir* gene and have a region of homology to small cloning vectors that replicate only in *E.coli*. The binary vectors or *trans* vectors are based on plasmids that can replicate both in *E.coli* and *Agrobacterium* and have T-DNA and *vir* genes on separate plasmids (Gelvin, 2000).

A typical binary vector system comprising an octopine – type *vir* helper strain such as LBA4404 (Hoekema *et al*., 1983) that harbours the disarmed Ach5 Ti plasmid and a binary vector such as pBin19 (Bevan, 1984) is very commonly used for plant transformation. The available range of *vir* helper strains has been expanded with the nopaline-type MP90 (Koncz and Schell, 1986) and L, L-succinaminopine-type EHA101 (Hood *et al*., 1986). The bacterial kanamycin resistance gene in EHA101 was deleted to develop the *vir* helper strain EHA 105 (Hood *et al*., 1993). EHA101 and EHA105, by virtue of harbouring the 'super virulent' *vir* genes exhibit broader host-range and higher transformation efficiency. Many recalcitrant plants such as rice (Rashid *et al*., 1996), wheat (Cheng *et al*., 1997) and barley (Tingay *et al*., 1997) have been transformed using EHA101 and EHA105.

2.10.1.2 Binary vectors

Beginning from the binary vector pBIN19, constructed by Bevan in 1984, many modifications have been made in these vectors to expand the range of their utility and to

improve their transformation efficiency. The pBIN19 has been completely sequenced (Frisch *et al*., 1995) and its improved version, pBIN20, with many additional single restriction sites in the MCS was reported by Hennegan (1998). A new series of pPZP vectors have been developed which are small in size and stable in *Agrobacterium* (Hajdukiewicz *et al*.,, 1994). The pPZP vector back bone was used to construct the pCAMBIA series of vectors with *nptII, hpt* or *bar* as selection markers and *gus* or *gfp* as reporters. Plant expression vectors of pRT100 series constructed by Topfer *et al*. (1993) permitted constructions of gene cassettes with CaMV 35S promoter and its poly A signal. These cassettes can be excised and placed in the MCS of binary vectors. Katiyar-Agarwal *et al*. (2002) reported the construction of a series of binary vectors having high strength constitutive promoters of either *ubiquitin*I (pUH, pUB) or *actinI* gene (pAH-1, pAH-2, pAB-1, pAB-2) and CaMV 35S promoter driven genes encoding either resistance to hygromycin or phosphinothricin for selection of transformants for efficient genetic transformation of rice. The foundation vectors used for the construction of these vectors belong to pCAMBIA series.

2.11 Kanamycin resistance gene as a plant selectable marker

The original and most widely used selectable marker is a bacterial gene for neomycin phosphotransferase (*npt*II). The enzyme *npt*II, inactivates by phosphorylating, a number of aminoglycoside antibiotics such as kanamycin, neomycin, geneticin (or G418) and paromomycin. Of these G418 is routinely used for selection of transformed mammalian cells while the other three are used in a diverse range of plant species (Bevan *et al*., 1983). It has been reported by various workers that in transformation experiments using cotyledonary leaves of tomato (Mandaokar *et al*., 2000) and brinjal (Kumar *et al*., 1998) as explants, 100 mg/l and 50 mg/l kanamycin was used in shoot regeneration medium and rooting medium, respectively. Most of the transformation experiments adopt the above concentrations of kanamycin in selection medium.

Considerable research on the *npt*II has shown that it is safe for both the environment and the consumer (Fuchs *et al*., 1993). It has been concluded from extensive experimentation that the potential for compromising the efficacy of kanamycin or

neomycin for therapeutic use in humans or animals by consuming the food and feed products are derived form genetically modified plants containing them is effectively zero because:

- The transfer of the *npt*II gene from plant material to gut microflora is extremely unlikely because there is no evidence that such transfer can occur. This conclusion is supported by studies, which demonstrated that horizontal gene transfer from plants to microbes did not occur under a variety of test conditions.

- In the unlikely event of a transfer occurring by an unknown mechanism from the genome of genetically modified plants or products derived from them to gut microflora and this event being maintained, this would not add significantly to the inherently large microbial population of kanamycin and neomycin resistant microbes in the gut of either humans (Kelch and Lee, 1978; Levy *et al*., 1988; Nap *et al*., 1992; Shaw *et al*., 1993) or animals (McAllan *et al*., 1973; Nap *et al*., 1992).

- The expression of the *npt*II gene in genetically modified plants is controlled by a plant specific promoter which is not expected to function in bacteria. In the unlikely event of transfer of the *npt*II gene and stable propagation of the intact gene fragment in bacteria, the gene is unlikely to be expressed and even less likely that a DNA rearrangement event occurs that places the functional *npt*II encoding open reading frame in front of a bacterial promoter.

- Even if expressed in intestinal bacteria, antibiotic therapy would not be compromised as the co-factors necessary for the enzyme to inactivate kanamycin and neomycin are not present at the required concentration range in the gut. Moreover, the nptII protein would be rapidly degraded in the gut.

Hence, the overall risk is considered to be effectively zero and the therapeutic use of antibiotics in humans or animals will not be impacted by the commercialization of transgenic crops containing antibiotic resistant selectable marker genes.

2.12 Transgenic Bt technology in India

The approval given by GEAC, Government of India, in March 2002 for the commercial release of Bt cotton sets the stage for the cultivation of genetically engineered crops in India. Indian groups have made impressive progress in the transformation of crops like potato (Chakrabarti *et al*., 2000), rice (Khanna and Raina, 2002), brinjal (Kumar *et al.*, 1998), tomato (Mandaokar *et al*., 2000, Kumar and Kumar, 2004). However, we still face hurdles in transforming other important crops such as legumes, cotton and sugarcane. The traditional strength of many Indian laboratories in plant tissue culture has facilitated a successful transition to plant genetic engineering. Any general improvement in transformation methods reported at the international level, whether in the form of vector development or in the form of methodology is immediately adopted and improved upon in Indian laboratories. This success is reflected by the fact that more than 10 Indian laboratories routinely perform rice transformation. An account of the Bt transgenic plants developed in Indian research laboratories that have reached the field trial is summarized in Table 4.

The scientific expertise that many Indian laboratories have been developing over the years in molecular biology must now be converted into 'technologies' so that most of the problems faced by the Indian farmers can be solved by plant genetic engineering approaches.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and Media

3.1.1 The chemicals used in this study are of analytical grade. The chemicals, media, antibiotics and plant growth hormones were purchased from Himedia Laboratory Pvt Ltd., Mumbai and E.Merck (India) Ltd. PCR chemicals while, DNA modifying enzymes, protein and DNA molecular weight markers were purchased from Bangalore Genei, Pvt.Ltd., India and MBI Fermentas Ltd. The chemical compositions of the media are given in Annexure I.

3.2 Plant material used

Tobacco (*Nicotiana tabaccum* cv Havana) obtained originally from the Department of Microbiology and Cell Biology, Indian Institute of Sciences, Bangalore was used for the transformation studies.

3.3 Bacterial strains and cloning vectors

For the construction and maintenance of the plant transformation vector expressing the synthetic *cry2Ac* gene, the *Escherichia coli* DH5α, a recombinant deficient strain was used. The recombinant pUC19 carrying the synthetic *cry2Ac* gene, the pRT103 (Fig.1), the pCAMBIA2300 (Fig.2) and the pUH (Fig.3) were used to construct the plant transformation vector. The *Agrobacterium tumefaciens* strain EHA105 was used for mobilizing the plant transformation vector carrying the synthetic *cry2Ac* gene. The source for different plasmids and bacterial strains are given in Table 5.

3.4 Cloning of the synthetic *cry2Ac* gene in the pRT103 vector

The recombinant pUC19 plasmid carrying synthetic *cry2Ac* gene and pRT103 were subjected to double digestion with the enzymes *Nco*I and *Sac*I using Buffer E (Bangalore Genei, India) in 20µl reaction volume. Restriction digestion was carried out at 37°C for 3 hours.

The digested products were run on 0.8 per cent agarose gel pre incorporated with ethidium bromide. The synthetic *cry2Ac* fragment and pRT103 fragment were eluted out from the gel and ligated. The ligated mixture was run on 0.8 per cent agarose gel to check the concentration and used for the *E.coli* transformation. The transformed *E.coli* cells harbouring the pS2CRT were selected on LB plates containing ampicillin (200 mg/l).

3.5 Cloning of synthetic *cry2Ac* expression cassette (from pS2CRT) in pCAMBIA2300 vector

The pS2CRT plasmids were isolated from the transformants of *E.coli* and digested with the *Pst*I enzyme. The pCAMBIA2300 plasmids were also digested with *Pst*I enzyme. The digested products were run on 0.8 per cent agarose gel and the desired fragments were eluted out. The two *Pst*I fragments from pS2CRT of equal size were ligated into pCAMBIA2300 at the *Pst*I site. Ligation of 50 to 100 ng of the vector DNA with 150 to 300 ng of insert DNA was performed in 20μl reaction mixture containing 40U of T4 DNA ligase at 16°C for 10 hours.

The ligated mixture was used for transformation of *E.coli*. The transformants of *E.coli* carrying recombinant plasmid (designated as p2AP1) were selected on medium containing 50 μg/ml kanamycin.

3.6 The α-complementation test

To select the transformed colonies of *E.coli* carrying the 2.6 kb fragment containing the CaMV 35S promoter, synthetic *cry2Ac* & poly A sequences and to eliminate the colonies with self ligated vector, the α complementation test was carried out as described by Sambrook *et al*. (1989). The XIK plates (X-gal, IPTG and kanamycin plates) were prepared by spreading 40 μl of 0.1 M IPTG and 40 μl of 10 per cent (w/v) X-Gal on LB agar medium containing kanamycin (50 mg/l) half an hour before streaking. The transformed *E. coli* colonies were selected randomly from the LB agar plates with kanamycin (50 mg/l) and streaked further onto the XIK plates with a sterile tooth pick. The plates were wrapped with aluminium foil and incubated at 37°C for 12-16 hours.

The plates were stored at 4°C for 6 hours to allow full colour development and the results were recorded.

3.7 Selection of ampicillin susceptible colonies

The white colonies of *E. coli* from XIK plates were inoculated into 3 ml LB broth containing kanamycin (50 mg/l). A loop full of broth culture was further streaked on to LB agar plates with 200 mg/l ampicillin. Simultaneously the broth culture was streaked on to LB agar plates with kanamycin (50 mg/l). The *E.coli* harbouring ampicillin resistance gene in pRT103 plasmid vector was used as positive control for checking growth in the ampicillin plate.

3.8 Confirming the presence of synthetic *cry2Ac* gene in p2AP1

The bacterial (*E. coli*) colonies grown on kanamycin plates but not in ampicillin plates were also screened by PCR with synthetic *cry2Ac* gene specific primers. These colonies were inoculated into 3ml LB broth containing kanamycin for plasmid isolation. The recombinant plasmids p2AP1 were isolated from the over night grown culture and digested with the *Pst*I enzyme.

3.9 Transformation of *Agrobacterium* with p2AP1

3.9.1 Preparation of electro-competent cells of *Agrobacterium*

Electrocompetent cells of *A. tumefaciens* strain EHA105 were prepared as described by Cangelosi *et al*. (1991) with certain modifications.

From the glycerol stock of *A. tumefaciens* strain EHA105, one per cent of the culture was inoculated into 5ml YEB broth containing 10 mg/l of rifampicin and incubated at 28°C in a rotary shaker set at 200 rpm. From the seed culture, one per cent culture was inoculated into 50 ml YEB broth containing 10 mg/l rifampicin and grown to an OD of 0.6 at 600 nm in a shaker at 28°C and 200 rpm. The cells were pelleted down at 5000 rpm for 15 minutes at 4°C. The supernatant was discarded and equal volume of 1mM ice cold HEPES buffer was used to re-suspend the cells. The suspension was incubated in ice for 30 minutes. The contents were centrifuged at 5000 rpm for 15 minutes at 4°C and resuspended the pellet in half volume of 1mM HEPES buffer.

The cell suspension was centrifuged at 5000 rpm for 15 minutes and the pellet was resuspended in 40 per cent glycerol (300 µl for every 100 ml culture). Aliquots of 40µl were made and stored immediately at -70°C.

3.9.2 Electroporation of *Agrobacterium*

To the 40 µl of electro-competent *Agrobacterium* cells retrieved from -70°C and thawed in ice, about 1 µg of recombinant plasmid DNA (p2AP1) was added and mixed well. The mixture was immediately transferred to sterile ice cold cuvette ((Bio Rad, USA). Electroporation was performed at 25F capacitance, 200 ohm resistance and 2.5 kV using a Biorad Gene Pulser$^{(R)}$ with Pulse Controller Plus$^{(R)}$. The electroporated cells were immediately resuspended in 960 µl of YEB broth and incubated at 28°C in a rotary shaker at 150 rpm for 6 to 7 hours. From the culture, 50 µl and 100 µl were spread on YEB plates with 10 mg/l rifampicin and 50 mg/l kanamycin and incubated at 28°C for 2 days.

3.10 Sensitivity of tobacco to kanamycin

To identify the killer concentration of kanamycin for effective selection of transgenic tobacco plants, sensitivity test using different concentrations of kanamycin was carried out.

In the first set of experiment, control plant leaf discs were tested for sensitivity to kanamycin. The MS media containing 1mg/l BAP and 0.1 mg/l NAA was used as shoot regeneration media (SM). Different concentrations of kanamycin were used in shoot regeneration media for different treatments. The treatments carried out were as follows:

Control leaf discs on SM without kanamycin,

Control leaf discs on SM with kanamycin 50 mg/l,

Control leaf discs on SM with kanamycin 100 mg/l,

Control leaf discs on SM with kanamycin 200 mg/l.

The second set of experiment was conducted for both cocultivated leaf discs as well as for control plant leaf discs, with 3 replications for each treatment. A control plate without adding both cefotaxime as well as kanamycin was also maintained. The various treatments tried were as follows:

Control leaf disc on SM with kanamycin 100 mg/l+cefotaxime 300 mg/l,

Control leaf disc on SM with kanamycin 200 mg/l+cefotaxime 500 mg/l ,

Cocultivated leaf disc on SM with kanamycin 100 mg/l+cefotaxime 20 mg/l

Cocultivated leaf disc on SM with kanamycin 150 ppm+cefotaxime 300 mg/l

Cocultivated leaf disc on SM with kanamycin 150 ppm+cefotaxime 500 mg/l

3.11 *Agrobacterium* mediated transformation of tobacco

The *A. tumefaciens* strain EHA105 harbouring p2AP1 (Fig.4) was used for the transformation of tobacco *N. tabacum* cv Havana.

3.11.1 Preparation of explants

From the leaves of *in vitro* raised tobacco plants, leaf discs of about $5mm^2$ were cut out using a cork borer and inoculated into shoot regeneration medium in petriplates. Fifteen to twenty leaf discs were inoculated per plate, with their dorsal surface touching the medium.

3.11.2 Pre incubation

Pre incubation or preculturing of tobacco leaf bits was done for two days in regeneration medium at 25±2°C, 16 hours photoperiod and 2000 lux light intensity, provided by the cool white fluorescent lamps.

3.11.3 Co-cultivation and transformation

The co-cultivation experiment was performed twice (November, 2001 and March, 2002) as mentioned below. The *A. tumefaciens* strain EHA105, harbouring the p2AP1 plasmid was grown in YEB broth containing 10 mg/l rifampicin and 50 mg/l kanamycin at 28°C on a shaker at 175 rpm. The *Agrobacterium* culture was used in the late log phase (O.D. of 1 to 1.5 at 600 nm) for infection of explants. Pre cultured explants were treated with the *Agrobacterium* suspension. The leaf discs were immersed in *Agrobacterium* suspension under gentle agitation, 50 rpm for 15 minutes at 28°C, then dried on sterile filter paper and placed on shoot regeneration medium under the culture conditions

already described. In control experiment, explants were placed on regeneration medium for two days without *Agrobacterium* infection.

3.11.4 Selection and plant regeneration

The explants after co-cultivation were washed 4 to 5 times with sterile water, blot dried and transferred to selection medium containing 300 mg/l cefotaxime and 100 ppm kanamycin. The control plants were subcultured at 3 weeks interval in shoot regeneration medium. The putative transgenic plants were subcultured twice at 4 to 5 weeks interval in selection medium (shoot regeneration medium with 300 mg/l cefotaxime and 100 ppm kanamycin). The shoots of putative transformants that are longer than 2 cm were transferred to rooting medium containing 1mg/l IBA, 250 mg/l cefotaxime and 50 ppm kanamycin.

3.11.5 Establishment of transgenic plants

The shoot tip of the plantlets that were established in rooting medium was cut and the leaves were removed. Approximately 1.5 to 2 cm long shoot tip with two nodes were transferred to rooting medium containing 1 mg/l IBA, 250 mg/l cefotaxime and 50 ppm kanamycin. The remaining basal portion of the transgenic plants were then transferred to potting mixture in mud pots after thoroughly washing off the media from the roots. It was then grown under glass house conditions. The transformation and regeneration protocol for tobacco leaf explants is depicted as flow chart in Fig.5.

3.12 Screening of putative transgenic plants of tobacco containing synthetic *cry2Ac* gene by PCR

The plants that were successfully rooted in the potting mixture were screened for the presence of synthetic *cry2Ac* gene by polymerase chain reaction.

3.12.1 Extraction of DNA for PCR analysis

A leaf bit ($1cm^2$) was ground in 200 µl of extraction buffer (200 mM Tris HCl; pH 7.5, 200mM NaCl, 25 mM EDTA & 0.5% SDS) in Eppendorf tube with fine sterilized sand using pellet pestle (Genei). The homogenate was centrifuged at 10000 rpm for 10 minutes. To the supernatant that was retrieved, equal volume of isopropanol was added for chromosomal DNA precipitation and kept on ice for 30 minutes. The DNA was

pelleted down at 10000 rpm for 10 minutes. The pellet was suspended in 50 µl of 1X TE and stored at -20°C.

3.12.2 Polymerase Chain Reaction

A 20 µl reaction mixture containing 100 ng of genomic DNA, 2.0 µl of 10X Taq DNA polymerase assay buffer (10 mM Tris HCl; pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$), 6 mM each of dNTPs (Bangalore Genie, India) 50 ng each of forward and reverse primers and 1.5U of Taq DNA polymerase (Bangalore Genei, India) was used to amplify the regions of synthetic *cry2Ac* gene at temperature profile described in Table 6.

Two sets of primers S2F1 and S2R1, S2F3 and S2R3 were used to amplify 800 bp and 600 bp, respectively from the synthetic *cry2Ac* gene (Primer sequence mentioned in Table 7). The S2F1 and S2R1 primers were used to initially screen all the putative transformants of tobacco. The results were further confirmed using S2F3 and S2R3 primers for selected tobacco transformants, NT2 and NT6.

The PCR program for the amplification of fragments of synthetic *cry2Ac* gene is depicted in Fig 6.

3.13 Screening of transgenic tobacco plants containing synthetic *cry2Ac* gene by ELISA

Some of the plants that were tested positive in PCR screening were subjected to ELISA test. From the first batch of putative tobacco transformants, NT2, NT3, NT4 and NT6 were screened by ELISA. The putative tobacco transformants that were screened by ELISA from the second batch included MT8a, MT8b, MT11a, MT11b and MT14b.

3.13.1 Protein extraction

Protein extraction from leaf bits of putative transgenic tobacco plants containing the synthetic *cry2Ac* gene was carried out using two different buffers TBA and PBST as

mentioned below. All the steps for protein extraction from tobacco leaf samples were carried out at 4°C or on ice.

3.13.1.1 TBA buffer method

Materials required (Annexure 2.1.1)

TBA buffer.

Procedure

Leaf sample weighing 0.5 g was homogenised with 1000 µl of 1X TBA buffer using pestle and mortar after placing on ice. The contents were then centrifuged at 10000 rpm for 10-15 minutes. The supernatant was then transferred to new Eppendorf tubes. If the supernatant was not clear, the centrifugation was repeated. Finally the supernatant was dispensed in aliquots and stored at -20°C.

3.13.1.2 PBST method

Materials required *(Annexure 2.1.2)*

10X PBST, β mercaptoethanol, PMSF

Procedure

Tobacco leaf sample weighing 0.5 – 1.0 g was homogenized in 500 µl – 1 ml of 1X PBST buffer containing 1µl / ml of β mercapto-ethanol and 2mM PMSF using pestle and mortar. The contents were centrifuged at 10000 rpm for 10 minutes. The supernatant obtained was stored at -20°C.

The plant protein samples extracted using the TBA buffer and PBST buffer were analyzed using SDS-PAGE for checking the concentration.

3.13.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970)

Materials required (Annexure 2.3)

Solution A, Solution B, Solution C, Ammonium persulphate (10%), TEMED, sample loading buffer (4X), electrode buffer (10X), staining solution and destaining solution.

Procedure

Electrophoresis was carried out in a vertical mini – gel unit in a discontinuous buffer system using 8 per cent acrylamide gel. The glass plates were cleaned thoroughly with water followed by alcohol and acetone. The plates were sealed at the bottom and sides. The gels were casted as per the recipe given in Annexure - 3. The separating gel was overlaid with a film of alcohol to accelerate polymerization. After polymerization, the alcohol layer was removed, rinsed with water and blotted with filter paper. The stacking gel was poured and the comb was placed on top of the sandwich. The comb was carefully removed from the slots after polymerization of the stacking gel and slots were rinsed with electrode buffer before loading the samples. The protein samples (5 - 20 µg concentration) were mixed with 1X loading dye, boiled for 2 minutes and then loaded. The gel was initially run at constant current of 15 mA till the dye front reached the separating gel. The current supply was increased to a constant supply of 30 mA till the dye front reached the bottom. After the electrophoretic run, the gel unit was dismantled and gel was stained for 30 minutes. The gel was destained until the background became colourless and the gel was documented using Alphaimager (AlphaInnotech, USA).

3.13.3 Protein quantification

3.13.3.1 Lowry's Method

The concentration of all the plant protein samples tested for ELISA was determined using Lowry's Method (Lowry *et al.,* 1951).

Materials required (Annexure 2.2)

Reagent A, Reagent B, Reagent C, Reagent D, Standard BSA

Procedure

A measured quantity of the solubilized protein was taken in a clean test tube and the volume was made upto 1.0 ml with distilled water. Five ml of reagent C was added to the above and mixed well. After 10 minutes, 0.5 ml of reagent D was added and mixed well. The contents were incubated in dark for 30 minutes and absorbance was taken at 660 nm in Systronics spectrophotometer -106. The standard graph was prepared in the

range of 0-100 µg (0, 10, 20 ……. 100 µg) of BSA and the amount of solubilized protein present per ml of sample was calculated from this graph.

3.13.3.2 Spot Densitometric Analysis for Protein Quantification

Procedure

The Alpha Imager ™ 2200 Imaging System is a complete package including all the hardware and software needed for image capture, enhancement and analysis. Alpha Ease FC software which includes the 2D spot densitometric analysis tool was used to quantify the Cry protein.

In the tool box, Analysis Tools, a tab labeled 'SPOT DENSO' opens a set of tools with which the density of bands, spots or other objects on the image of a SDS-PAGE gel can be measured. A two dimensional area of interest is created and the density is obtained through the pixel intensity values designated as IDV or Integrated Density Value.
The steps involved in this process are as follows.

- Autospot is a tool that opens in the tab labeled SPOT DENSO. It is designed to find multiple spots within a region of interest.
- After selecting Autospot the area of interest was drawn on the image of the gel by clicking and dragging the mouse. The smaller and more defined the area of interest is, the better the resulting data.
- Once the correct area of interest was drawn the Find Spot button was selected. A green outline will be drawn around the detected spots.
- A satisfactory spot outline when achieved, the Get Data button was selected. This will convert all drawn objects into standard spot densitometry objects with associated density numbers.
- Once the objects were defined the Standard Curve button was clicked which opened a set of tools that created a calibration curve. The calibration curve function allows quantitation of the bands on a gel based on a set of standards. A minimum of two standard bands must be input.
- When the standard curve tool box appeared, the units in which the results should be reported was entered.
- The <Enter> key on the keyboard was then pressed after typing the units.

- To designate the standard band, one of the bands whose value is known was clicked
- In the dialog box displayed, the value for the band was entered, using either the keyboard or the numeric keypad in the dialog box.
- The entry if correct, the OK button was clicked. The band number changes from white to green, indicating that it was now a standard.
- To enter the next value, another band was clicked. The dialog box opened again and showed an estimated value (based on the object's IDV value and on the standard value entered).
- If the value was correct, the OK button was clicked, else entered a new value.
- As the values for the standard bands have been entered the quantitation values of the unknown bands were automatically calculated.
- The calculated values of the unknowns could be seen by clicking on the Exit button in the standard curve tool box.

The third column in the data box contains values for the standards (entered by user) and the values for the unknown bands (calculated based on the curve).

3.13.4 Rapid ELISA using direct adsorption of antigen to solid phase (Clark *et al*., 1986)

Materials required (Annexure 2.4)

10X PBST, coating buffer, substrate, substrate buffer, primary antibody, secondary antibody.

3.13.4 .1 ELISA of Cry1Ac, Cry2Ab and Cry2Ac proteins with Cry2Ac polyclonal antibody

Procedure

The Cry1Ac, Cry2Ab and Cry2Ac protein samples obtained from Bt lab were analysed by SDS-PAGE and the concentrations of these proteins were determined by Spot Densitometric Analysis as mentioned in 3.13.3.2. The above mentioned Cry proteins were diluted to appropriate concentration using 1X PBST buffer and 100 μl was dispensed in each well of the ELISA plate. One hundred μl of 1X PBST buffer alone was also dispensed in duplicate as blank. The plate was placed in a humid container and incubated at 4°C for 12-15 hours. The samples were then poured off and the plate rinsed with 100 μl 1X PBST per well, allowing 3 minutes for the wash. Rinsing with 1XPBST was again repeated twice. This was followed by dispensing 100 μl per well of the primary antibody

diluted (1:10000) using coating buffer. The plate was then incubated at 4°C for 12 hours. Rinsing the plate with 1X PBST was carried out 3 times as mentioned above. The secondary antibody diluted (1:2000) using the coating buffer was then dispensed, 100 µl per well and the plate was incubated at 4°C for 5-6 hours. This was followed by washing the plate 3 times with 1X PBST as mentioned above. Substrate (pNPP) was then finally added, 100 µl per well and incubated at room temperature, until colour developed. The absorbance of yellow colour was read at 405 nm wave length using ELISA reader and recorded as OD value (A). The OD value obtained for the blank was then subtracted from the OD value obtained for each sample and the data was recorded

3.13.4. 2 ELISA of protein extracts of synthetic cry2Ac transformants of tobacco by Cry2Ac polyconal antibody

Procedure

Protein extracts of tobacco plants (from both batches) prepared using TBA buffer were diluted to appropriate concentration using 1X PBST buffer. It was then dispensed at the rate of 100 µl per well of the ELISA plate. The positive control (Cry2Ac protein) was also diluted to required concentration using 1X PBST 100 µl was dispensed in each well. One hundred µl 1X PBST buffer alone was also dispensed as blank. The steps mentioned in 3.13.4 starting from incubation of the plate after addition of protein sample till the recording of the OD value (A) are followed in this experiment also. The OD value obtained for the blank was then subtracted from the OD value (A) obtained for each sample. Let the OD value obtained for each control plant sample and transgenic plant sample after deducting blank OD value be denoted as 'B' and 'C', respectively. When there was more than one control plant in an experiment, the average of control plant OD values was calculated as 'D'. The final OD value for each of the transgenic tobacco plant was then determined as 'C – B' or 'C – D'. The equivalent concentration of Cry protein in ng for each of the transgenic tobacco plant final OD was determined from the Cry2Ac standard graph. Finally, the per cent of Cry2Ac protein in total soluble protein of tobacco plant was estimated as follows:

$$\text{Per cent of Cry2Ac protein in total soluble protein of tobacco plant} = \frac{\text{Equivalent concentration of Cry2Ac} * 100}{\text{Amount of total soluble protein of tobacco for ELISA}}$$

3.14 Bioassay of tobacco plants for toxicity against *H. armigera* larvae

The adult moths of *H. armigera* were collected and transferred to jars containing cotton swabs dipped in sugar solution. Eggs were allowed to be laid on muslin cloth and larvae emerged after three days. Third leaf from the top was selected from each of the transgenic plant and the corresponding control plants for carrying out the bioassay. The third leaf was cut into ten leaf bits of size 1.5 x 3.0 cm^2 (approximately). The leaf bit was placed on the moist filter paper taken in a plastic cup. In each of the plastic cup, a single third instar larvae of *H. armigera* was released. Proper care was taken to maintain the moisture content in the plastic cup. The experiment was carried out at 28°C and 70 per cent relative humidity. The mortality per cent, area of leaf fed and the per cent reduction in the leaf area damaged over control was recorded periodically. The area of leaf fed was calculated by placing the leaf bit on a graph paper, drawing the outline (before feeding) and then recording the area fed. The experiment was repeated two more times at an interval of one week, with 10 replications for each treatment. Each experiment lasted for 5-6 days until the leaf bit started drying.

3.15 Screening of transgenic tobacco plants containing synthetic *cry2Ac* gene by Southern hybridization

3.15.1 Plant DNA extraction (CTAB method)

Materials required (Annexure 2.5)

CTAB extraction buffer, liquid nitrogen, RNaseA solution, chloroform: isoamyl alcohol (24:1), ethanol (70 %), TE buffer, absolute alcohol, isopropanol, NaCl (3M), $NaOCH_3$ (3M).

Procedure

Isolation of total genomic DNA from the leaves of NT2 transgenic plant which exhibited high levels of insect protection in bioassays as well as NC1 control plant was performed by CTAB method (Gawel and Jarret, 1991).

Leaf tissue weighing 2 g was homogenised in liquid Nitrogen using a pestle and mortar and transferred to 50 ml centrifuge tubes. The homogenized leaf tissue was suspended thoroughly in 15 ml of CTAB extraction buffer preheated to 65°C. The suspension was incubated at 65°C for 30 minutes with occasional mixing. The mixture was cooled to room temperature. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the suspension and mixed well to get an emulsion by inverting the tube several times for 15 minutes. The mixture was centrifuged at 4000 g for 15 minutes at 4 ^{o}C and the aqueous layer was carefully transferred to a new 50 ml centrifuge tube. The chromosomal DNA was precipitated by adding 0.8 volume of cold isopropanol and mixed carefully. It was kept at room temperature for 15 minutes. The DNA pellet was recovered with a blunt ended Pasteur pipette (or centrifugation at 10000 g for 10 minutes at 4 ^{o}C can pellet the DNA). The DNA pellet was washed in 70 per cent ethanol and allowed to dry. The DNA was dissolved in minimum volume of sterile H_2O or TE buffer. To accelerate the dissolution of DNA and to inactivate residual DNaseI, the dissolved DNA was incubated at 60°C for 10 minutes. This was followed by addition of 1/100 vol. of 10 mg/ml RNaseA solution. It was mixed by inversion and incubated at 37°C for 30 minutes. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added and mixed well, followed by centrifugation at 12000 rpm for 10 minutes. To the aqueous layer two volumes of absolute alcohol and 1/10 volume of 3 M sodium acetate was added. Centrifugation was again repeated for 5 minutes at 10000 rpm (If no proper precipitation was obtained, the product was kept in freezer overnight at –20°C). The supernatant was discarded and the precipitate was washed with ethanol. The pellet was air dried and dissolved in TE buffer.

3.15.2 Southern blot of transgenic tobacco plant

The transgenic tobacco plant NT2 was analyzed by Southern hybridization to confirm the presence of synthetic *cry2Ac* gene.

Materials required (Annexure 2.6)

Denaturation solution, neutralization solution, 20X SSC, pre-hybridization solution, washing solutions (3X SSC and 0.1% SDS, 2X SSC and 0.1% SDS, 0.5X SSC and 0.1% SDS, 0.3 X SSC and 0.1% SDS), developer solution, fixer solution.

Procedure

Southern blotting involves digestion, agarose gel electrophoresis, membrane transfer, pre-hybridization, hybridization and autoradiography.

3.15.2.1 Digestion of DNA

Ten µg of the plant DNA extracted from transgenic tobacco plant NT2 and control tobacco plant NC1 was digested with ten µl of *Bam*HI enzyme. The positive control p2AP1 was also digested with *Bam*HI enzyme. Overnight digestion of these samples with *Bam*HI was carried out and loaded in 0.8 per cent agarose gel.

3.15.2.2 Agarose gel electrophoresis of DNA

The digested product was run slowly at 40V in 1 per cent agarose gel stained with EtBr for 6-7 hours. The gel was observed in the UV illuminator.

3.15.2.3 Gel treatment after electrophoresis

3.15.2.3.1 Depurination

The gel was kept in 0.2 N HCl for 15-20 minutes and gently shaken in a shaker (40-50 rpm), until the blue colour changes to yellow. The gel was then rinsed with sterile distilled water.

3.15.2.3.2 Denaturation

The gel was transferred to denaturation solution and gently shaken for 1 – 1½ hours. This was followed by rinsing with sterile distilled water.

3.15.2.3.3 Neutralization

The gel was placed in neutralization solution and gently shaken for 1 – 1½ hour. Before transferring to membrane, it was rinsed 2-3 times with sterile distilled water.

3.15.2.4 Membrane transfer

Nitrocellulose membrane was wetted in sterile water first and then in 20X SSC before keeping it on the gel. The membrane transfer set up was assembled as described here under.

A tray with 20X SSC or 10X SSC and Whatman No.3 sheet (1 layer) of gel tray size and touching 20X SSC on both sides was taken. First, three layers of Whatman No.3 sheet of gel size wetted with 20X SSC was placed on the glass plate. The gel was placed above it in an inverted position. Above the gel, the wet nylon membrane marked 'T' on the marker side was placed. Three Whatman No.3 sheets of gel size, wetted with 20X SSC were placed above the nylon membrane. One layer of dry Whatman No.3 sheet also of gel size was placed on top of the three Whatman No.3 sheets. Dry country filter papers were stacked to 2 – 2.5 inch height above. A weight was placed on top of the stacked papers. The entire set up was kept as such for 15 – 16 hours for the DNA to transfer onto membrane. After 15-16 hours, the set up was dislodged and the membrane was rinsed in 2X SSC for 50-60 seconds. The membrane was dried and covered with polythene sheet and stored at 4 ^oC.

3.15.2.5 Preparation of radio active probe

The radioactive probe used for carrying out the Southern hybridization was ^{32}P-dATP labeled synthetic *cry2Ac* insert (1.9 kb) separated from pS2Ac plasmid by *Bam*HI digestion. Template DNA (40-50 ng) and sterile distilled H_2O (made upto 20µl) was denatured in boiling water (100^oC) for 5 minutes. It was cooled immediately on ice. The radioactive probe preparation mixture was added to the template DNA (Annexure 2.6).

The mixture was then centrifuged at 10000 rpm for few seconds, followed by incubation at 37^oC for 20 minutes (can be upto 1 – 2 hours). To stop the reaction, 3 µl of 3 M NaOH and 7 µl of 0.1X TE was added and a brief spin was given. The reaction can also be stopped by keeping the mixture at 100^oC (water bath) for 5 minutes. The denatured probe was then used for hybridization.

3.15.2.6 Pre-hybridization

All the steps were done at 65^oC. The hybridization chamber was set at 65^oC (10-20 minutes earlier). Pre-hybridization solution was prepared freshly and kept at 65^oC. The membrane was taken from 4^oC, and kept inside the hybridization tube, in such a way

that 'T' side was facing up. Into the hybridization tube, 25 – 30 ml of prehybridization solution prewarmed to 65°C was added and allowed to rotate for 1 – 1½ hours.

3.15.2.7 Hybridization

After 1 – 1½ hours, the pre-hybridization solution was removed and 30 ml of fresh pre-hybridization solution prewarmed to 65°C was added. Then the radioactive labelled probe (35 µl reaction mixture) was carefully added into the tube. The hybridization was carried out at 65°C, overnight (15-18 hours).

3.15.2.8 Post-hybridization washing

The temperature of washing solutions was maintained at 65°C. After overnight hybridization, the hybridization solution was carefully removed. Then 30 ml of 3X SSC and 0.1X SDS solutions was added and allowed to run for 30 minutes at 65°C. Before washing with hot 3X SSC and 0.1X SDS, cold washing with the same solutions was carried out. The solution was removed and 30 ml of 0.5X SSC and 0.1 per cent SDS solution (65°C) was added and run for 15-30 minutes at 65°C. Here also, the membrane was rinsed with cold solution of 0.5X SSC and 0.1 per cent SDS before adding the hot solution. The radioactivity was read using the radioactive counter. Based on the counts per second, the washing and stringency of washing solution was increased or decreased. After washing, the membrane was taken out and dried to remove the moisture and kept inside a polythene sheet. The membrane in the polythene sheet was kept inside the cassette and stored at -70°C.

3.15.2.9 Detection of signal

The membrane was first exposed by Phosphoimager to get immediate result. Inorder to confirm the above result, the membrane was stored in the cassette and later exposed by autoradiography (X-ray film).

3.15.2.9.1 Phospho imaging

Phosphoimager is highly sensitive (100 times) when compared to X-ray film. (For example, 30 minutes exposure is enough if counts per second are 200-250). The imaging plate was kept in such a way that black side faced down inside the eraser in order to

remove (or) erase previous records. The imaging sheet (plate) was then kept over the membrane in such a way that black side faced up inside the cassette and kept as such for exposing. The imaging plate was then kept with the black side facing down inside the scanner to read the signals. The image was scanned using BAS-1800 software and processed using L-process software. After saving the picture, the portion required was selected, copied and pasted in photoshop file for further processing.

3.15.2.9.2 Autoradiography

The membrane (covered with polythene bag) was kept inside the cassette in such a way that 'T' side faced up. The X-ray film was kept over the membrane and the cassette closed and kept as such for exposing. The duration of exposure time varied according to the radioactive counts. The cassette was kept at –70°C for exposure. (eg.) If 250 counts per second, 1 hour exposure was enough. After exposing on X-ray film, the X-ray sheet was taken out and kept in developer solution for 1-2 minutes. It was then rinsed with sterile water for 1 minute and kept in fixer solution for 1-2 minutes. The X-ray sheet was washed in sterile distilled water and air dried. The X-ray sheet was developed in dark.

3.16 Screening of transgenic tobacco plant by Northern blot

Materials required (Annexure 2.7)

10X MOPS, 5X loading buffer, Northern prehybridization solution, Northern hybridization solution.

Procedure

3.16.1 RNA extraction

RNA was extracted from the putative tobacco transgenic plant, NT2 and the control plant, NC1 using the RNA extraction kit (Genei) as per the manufacturer's instruction.

3.16.2 Electrophoresis of RNA (Denaturing gel)

Denaturing 1 per cent agarose gel was casted as follows. To 1.2 g agarose, 86.6 ml of sterile water was added and boiled. The mixture was cooled to 60°C and 12 ml of 10X MOPS and 21.1 ml of formaldehyde (37%) and 4 µl of EtBr (10 mg/ ml) was added. It was mixed well and the gel was casted. The RNA sample was resolved using the denaturing 1 per cent agarose gel. The sample preparation was carried out as follows:

RNA - 4 µl

10X MOPS -1.56 µl

Formaldehyde - 3.12 µl

Deionized formamide - 8.88 µl

The above ingredients were mixed and heated to 60°C for 7.5 minutes. After cooling on ice for 5 minutes, 4 µl of 5X loading buffer was added. The gel was pre-run for 10 minutes in 1X MOPS prior to loading the samples. Three and fifteen µl of samples, namely transgenic tobacco plant NT2 RNA and control tobacco plant NC1 RNA was loaded on the gel and run at 5V /cm for 3 hours till the dye front reached half way down the gel. The gel was then observed under UV transilluminator.

3.16.3 Northern hybridization of transgenic tobacco

All the steps are same as that of Southern blotting. The only difference is in the composition of the pre hybridization solution. The transgenic plant (NT2) and control plant (NC1) RNA was resolved in the denaturing agarose gel and blotted on to the nitrocellulose membrane. This was followed by hybridization with the ^{32}P labeled *Bam*HI fragment of synthetic *cry2Ac* insert (1.9 kb) released from the pS2Ac plasmid.

3.17 Screening of transgenic tobacco plants containing native *cry2Ac* gene

Two transformants of tobacco plants harbouring p2AcP1 (containing native *cry2Ac* gene under the control of *rbcS* promoter and its transit peptide sequences, Fig.7) were obtained from earlier studies and tested for expression of the Cry2Ac protein by ELISA and leaf bioassay with *H. armigera* larvae. As per the earlier studies these transformants were selected in shoot regeneration and rooting media containing 3 mg/l Basta. Also they were proven positive for presence of native *cry2Ac* gene by PCR test. The plants were also tested for Basta® sensitivity by patch test and found resistant to herbicide application. Hence, the transgenic status of the tobacco plants and expression of the *bar* gene was already confirmed. These two transformants of native gene construct were selected as the best ones among several other transgenics of the native *cry2Ac* gene construct based on preliminary bioassay with *H. armigera* (Udayasuriyan, Unpublished)

3.17.1 Screening of native *cry2Ac* gene containing tobacco transformants by ELISA

The protein concentration of the tobacco transformants containing native *cry2Ac* gene namely, D14-2 and D14-3 along with control tobacco plants DC1 and DC2 was determined by Lowry's method as mentioned in 3.13.3.1. The per cent expression of Cry2Ac protein in the total soluble plant protein was determined for all the above mentioned plants by ELISA as described in 3.13.4.2. The average OD value for the control plants, DC1 and DC2 was calculated and denoted as DC in table 13. The OD value corresponding to DC was subtracted from transgenic plant OD value to determine the per cent expression of Cry2Ac to total soluble plant protein.

3.17.2 Bioassay of native *cry2Ac* gene containing tobacco transformants for toxicity to *H. armigera* larvae

The leaf bits of transgenic tobacco plants D14-2 and D14-3 as well as the control tobacco plants DC1 and DC2 were tested for toxicity to *H. armigera* larvae by bioassay conducted as mentioned in 3.14.

3.18 Construction of recombinant pUH harbouring synthetic *cry2Ac* gene

3.18.1 Cloning vectors

The vectors used for the study were pS2Ac harbouring the synthetic *cry2Ac* gene and pUH vector which was kindly provided by Dr.Anil Grover, University of Delhi, South Campus, New Delhi.

3.18.2 Analysis of plasmid DNA samples by agarose gel electrophoresis (Sambrook *et al*., 1989)

Materials required (Annexure 2.9)

TAE buffer (10X), loading dye (6X), Ethidium bromide (EtBr).

Procedure

Agarose gel (0.8%) was prepared to analyze the DNA samples. Agarose was added to 1X TAE buffer and melted in a microwave oven. The mixture was cooled to 60°C and EtBr was added, mixed gently to avoid bubble formation. The mixture was

poured into the gel mould containing comb and was allowed to solidify. The gel buffer reservoir in the electrophoretic apparatus was filled with 1X TAE buffer to about 2mm above gel surface. DNA samples were mixed with loading dye at 5:1 ratio. Contents were loaded in respective gel slots and electrophoresis was carried out at 50 V. After the run, the gel was examined on the trans-illuminator (Bio Rad, USA) using UV light and documented using Alpha imager (Alpha Innotech.).

3.18.3 Restriction digestion of pUH and pS2Ac plasmids

Restriction digestion was carried out as per the manufacturer's instruction. The pUH plasmid as well as pS2Ac plasmid were digested with *Bam*HI enzyme.

Restriction digestion was set up for 40 µl as follows.

DNA : 50 ng

Buffer (10X) : 4 µl

Restriction enzyme : 10 U

Sterile distilled H_2O : to make up to 40 µl

The restriction digestion was carried out at 37°C for 1-3 hours.

3.18.4 Elution of synthetic *cry2Ac* gene and pUH digested by *Bam*HI

After restriction digestion, DNA fragments were resolved in agarose gel. The 1.9 kb synthetic *cry2Ac* gene fragment released by digestion of pS2Ac plasmid with *Bam*HI and 11.3 kb pUH vector also digested with the same enzyme were eluted using the 'Gen Elute' Kit (M/s. Sigma Genosys, UK).

3.18.5 Ligation of DNA fragments

Ligation was carried out as per the manufacturer's instruction. Vector (pUH) and insert (synthetic *cry2Ac*) were taken in 1:3 ratio for ligation reaction. Ligation mixture was incubated at 16°C over night.

Vector : 50 ng

Insert : 150 ng

Ligase buffer (10X) : 2.0 µl

T_4 DNA ligase : 1.0 µl (40U)

Sterile distilled H_2O : made upto 20.0 µl

3.18.6 Transformation of *E. coli* (Hanahan, 1983)

3.18.6.1 Competent cell preparation

Materials required (Annexure 2.10)

TfbI, TfbII

Procedure

The *E. coli* strain DH5α to be transformed was inoculated into 4 ml LB broth and grown over night. This was used as mother culture for competent cell preparation. To 25 ml of LB broth, 225 µl of the mother culture was inoculated and grown with vigorous shaking at 37°C for about 2 hours until OD_{600} = 0.6. The culture was transferred to polypropylene tube and chilled on ice for 10 minutes. The cell suspension was pelleted by centrifugation at 5000X g for 10 minutes at 4°C. The supernatant was discarded completely and the pellet was resuspended in sterile ice cold solution of Tfb1 (10 ml). The cell suspension was placed on ice for 20 minutes and centrifuged at 5000X g for 10 minutes at 4 °C. The supernatant was discarded completely and the pellet was resuspended in sterile ice cold solution of Tfb2 (1.0 ml), followed by incubation in ice for 15 minutes and used for transformation.

3.18.6.2 Transformation of *E. coli* with ligated mixture containing pUH and synthetic *cry2Ac* gene

Materials required (Annexure 1.2)

SOC broth

Procedure

To transform a fixed aliquot of 100 µl of competent cells, 5-10 µl of ligated mixture was added and kept on ice for 30 minutes. The cells were subjected to heat shock at 42°C for 1 minute followed by chilling on ice for 5 minutes. Nine hundred µl of preheated (kept at 37°C) SOC broth was added to the cells and incubated at 37°C with shaking for 2-3 hours. Appropriate quantity (75-150 µl) of cells were plated on selective plates (LB agar containing kanamycin) and incubated at 37°C. The results were recorded after overnight incubation.

3.18.7 Screening of the transformed colonies

Some of the randomly picked colonies were screened using synthetic *cry2Ac* gene specific primers (S2F1 and S2R1) for checking the presence of insert as described in 3.12.2.

3.18.8 Isolation of plasmid DNA from positive clones by alkali lysis (Sambrook *et al*., 1989)

Materials required (Annexure 2.8)

Solution I, Solution II, Solution III, Buffered phenol, Sodium acetate.

Procedure

A single colony or loop full cells of *E.coli* was inoculated into 4 ml LB broth supplemented with suitable antibiotics. The cells were grown over night at 37°C with vigorous shaking. Two ml of the culture was centrifuged at 6000X g for 5 minutes. The supernatant was discarded and the pellet was suspended in 100µl of ice cold solution I. Cells were kept in ice for 5 minutes. A freshly prepared, 200 µl of solution II was added and the suspension was gently inverted 2-3 times and kept in ice for 5 minutes. Ice cold solution III (150 µl) was added and the suspension was mixed well and kept in ice for 10 minutes. Tubes were centrifuged at 10000X g for 10 minutes at room temperature. The supernatant was carefully transferred to a fresh tube and mixed with 400 µl of phenol : chloroform mixture (1:1). It was centrifuged at 10000X g for 10 minutes at 4°C. Aqueous layer was separated and 2.5 volume of ice cold ethanol and $1/10^{th}$ volume of 3M sodium acetate (pH 5.2) was added and incubated at -70°C for 30 minutes. The mixture was then centrifuged at 10000 g for 10 minutes at 4°C. Precipitated plasmid DNA was washed with 70 per cent ethanol, air dried and resuspended in TE buffer (pH 8.0). Small aliquots of the plasmid DNA was analyzed on 0.8 per cent agarose gel.

3.18.9 Restriction digestion of recombinant pUH

The recombinant pUH plasmid DNA was subjected to restriction digestion by *Bam*HI as well as *Xho*I as mentioned in 3.18.3 to confirm the presence of the insert (synthetic *cry2Ac*) in pUH vector.

CHAPTER IV
RESULTS

The main objective of the study was to construct a new plant transformation vector p2AP1 carrying the synthetic *cry2Ac* gene and mobilize it into *Agrobacterium tumefaciens* strain EHA105. *Nicotiana tabacum* cv Havana was transformed using the recombinant plasmid *A. tumefaciens* strain harbouring the p2AP1. Various molecular techniques, such as PCR, ELISA, Southern and Northern hybridization were used to analyze the putative transformants of tobacco. Bioassays were also carried out with *Helicoverpa armigera* larvae to screen the transgenic plants.

In addition, transgenic tobacco plants containing native *cry2Ac* gene under the control of *rbc*S promoter and its transit peptide sequences (obtained from earlier studies) were also screened by ELISA and bioassay with *H. armigera* larvae.

Another part of the study was the construction of a recombinant pUH vector harbouring the synthetic *cry2Ac* gene under the control of ubiquitin promoter, which could be more efficient in the transformation of monocots.

4.1 Construction of a new plant transformation vector with synthetic *cry2Ac* gene

4.1.1 Cloning of synthetic *cry2Ac* gene in pRT103 vector

Recombinant pUC19 plasmid (pS2Ac) when digested with *Nco*I and *Sac*I enzymes yielded a 1.9 kb fragment of synthetic *cry2Ac* gene and a 2.6 kb fragment of pUC19 plasmid vector. A linear fragment of 3.3 kb size was obtained when the pRT103 vector was digested using *Nco*I & *Sac*I enzymes.

The synthetic *cry2Ac* fragment of 1.9 kb was ligated with the 3.3 kb linearized pRT103 vector at the *Nco*I and *Sac*I sites to obtain a recombinant plasmid pS2CRT of size 5.2 kb. Transformation of the *E. coli*, DH5α strain with the ligated mixture gave about 100 putative transformants on LB plates containing ampicillin (200 mg/l). Double digestion of the plasmids isolated from the putative transformants of *E.coli* with enzymes *Nco*I and *Sac*I released the 1.9 kb synthetic *cry2Ac* fragment.

4.1.2 Subcloning of *cry2Ac* expression cassette from pS2CRT into pCAMBIA2300 vector

A single fragment of about 2.6 kb, which resolved into two adjacent fragments after long run of the gel was obtained when pS2CRT plasmid was digested with *Pst*I enzyme. Restriction digestion of the plasmid pCAMBIA2300 with *Pst*I enzyme, gave a linear fragment of size 8.7 kb.

A recombinant vector p2AP1 was made by ligating the 2.6 kb fragment (*cry2Ac* expression cassette: CaMV35S promoter + synthetic *cry2Ac* + poly A sequences) with pCAMBIA2300 at the *Pst*I site. The ligated mixture was used to transform *E.coli* (DH5α strain) cells. The transformation resulted in the development of numerous colonies in the LB agar plates containing kanamycin (50 mg/l). Few of these colonies were then screened on XIK plate for blue or white colonies. The white colonies failed to grow in LB agar plates containing ampicillin (100 mg/l).

4.1.3 Confirming the presence of *cry2Ac* cassette by restriction digestion

From one of the ampicillin susceptible white colony, the recombinant plasmid p2AP1 was isolated. This plasmid on digestion with *Pst*I enzyme gave fragments of size 8.7 and 2.6 kb as was expected (Plate 1).

4.2 Transformation of *A. tumefaciens* with plant transformation vector p2AP1

The *A. tumefaciens* (EHA105 strain) was transformed with plasmid p2AP1 by electroporation and grown for short period without selection pressure. The culture was then spread on YEB plates containing rifampicin (50 mg/l) and kanamycin (50 mg/l). Numerous colonies of *A. tumfaciens* developed within 2 days. Few of the single colonies were sub cultured in YEB agar plate and PCR was carried out using a portion of the colony and primers specific to the synthetic *cry2Ac* gene .As expected agarose gel electrophoresis gave amplified fragments of about 800 bp, only in the case of transformed colonies (Plate 2). Glycerol stock was made for the PCR positive transformants of *A. tumefaciens*.

4.3 Establishment of tobacco plants *in vitro*

Tobacco leaf discs placed on shoot regeneration medium produced well-grown callus within 3 weeks. Shoot tips emerging from the callus were further subcultured thrice at 3 to 4 weeks interval in the same shoot regeneration medium. After the third subculture, the shoots of 1.5 to 2 cm length were transferred to rooting medium containing IBA (1 mg/l). Rooting of the plantlets was observed within 15 to 20 days. These rooted plants maintained *in vitro,* served as source of explants for transformation.

4.4 Kanamycin sensitivity test of shoot regeneration

Explants placed on shoot regeneration medium without kanamycin showed good shoot regeneration within 2 to 3 weeks. Out of the three concentrations of kanamycin (50, 100 and 150 mg/l respectively) tested, the callus formation and shoot regeneration were severely inhibited by kanamycin at 50 mg/l concentration. The shoot regeneration of tobacco was totally absent and browning / yellowing of explants observed at 100 mg/l concentration of kanamycin. At 150 mg/l of kanamycin, complete scorching of the leaf discs were observed within 4 weeks after the start of the experiment (Plate 3).

4.5 *Agrobacterium* mediated transformation of tobacco

Leaf discs of size $5mm^2$ from *in vitro* grown tobacco plants were pre incubated for two days in shoot regeneration medium. After co-cultivation, these leaf discs were transferred to the same medium. The transfer of the leaf discs to the selection medium was done after two days.

4.5.1 Regeneration of putative transformants of tobacco

In the control explants (uninfected with *A. tumefaciens*) placed on regeneration medium without kanamycin and cefotaxime, shoot bud initiation was noticed by 8 to 10 days. Shoot bud initiation occurred in infected leaf discs placed on selection medium containing kanamycin (100 mg/l), cefotaxime (300 mg/l) by 4 to 5 weeks (Plate 4). The green and healthy regenerated shoots from the infected leaf discs of size 1 to 1.5 cm were then subjected to three more subculture in shoot regeneration medium containing kanamycin (100 mg/l) and cefotaxime (300 mg/l). The regenerated shoots from the control explants were also subjected to two more subculture in shoot regeneration medium without kanamycin and cefotaxime (Plate 5).

4.5.2 Rooting of putative transformants of tobacco

The regenerated shoot tips of the putative transformants were transferred to rooting medium containing kanamycin (50 mg/l) and cefotaxime (250 mg/l). These shoot tips induced roots after 25 to 30 days of transfer (Plate 6).

The co-cultivation was carried out in two batches and the putative transformants obtained were designated as I batch (2001 November co-cultivation) and II batch (2002 March co-cultivation) transgenics.

From the two co-cultivation experiments 24 shoot tips were transferred to the rooting medium. Nine shoots were stunted and did not produce roots. The remaining 15 shoots produced roots and established as plantlets in rooting medium. Out of the 15 putative transformants of tobacco transferred to pots, only 12 established successfully in green house condition (Plate 7).

4.6 Screening of putative transformants of tobacco by PCR

The forward and reverse primers, namely S2F1 and S2R1 amplified the 800 bp long internal region of synthetic *cry2Ac* gene in nine out of the twelve putative tobacco transformants screened (Table 8). This was also in confirmation with the size of the amplified product produced by p2AP1 plasmid harbouring the synthetic c*ry2Ac* gene, which was used as the positive control. Control plant DNA, when screened with S2F1 and S2R1 primers did not produce any amplification (Plate 8). The presence of the synthetic *cry2Ac* gene was confirmed by another set of primers, S2F3 and S2R3, also specific for synthetic *cry2Ac* gene. The putative transformants of tobacco, NT2 and NT6 showed amplification of 600 bp long internal region of synthetic *cry2Ac* gene (Plate 9). Summary of the two co-cultivation experiments of tobacco performed with p2AP1 plasmid containing the synthetic *cry2Ac* gene is recorded in Table 9.

4.7 Reaction of Cry2Ac polyclonal antibodies to different Cry proteins

SDS-PAGE of alkali solubilized Cry2Ab, Cry2Ac, Cry1Ac proteins showed prominent bands of about 65 kDa in Cry2A samples (Plate 10a) and ~ 135 kDa in Cry1Ac sample (Plate 10b). The protein concentration of Cry2Ab, Cry2Ac and Cry1Ac

samples were determined by densitometric analysis. The final output obtained as per the software was 930, 390, and 650 ng/µl for Cry2Ab, Cry2Ac and Cry1Ac, respectively.

An experiment was carried out by Rapid ELISA using direct adsorption of antigen to solid phase to compare the reactivity of Cry2Ac polyclonal antibody with Cry2Ab Cry2Ac and Cry1Ac protein samples. The results indicated that Cry2Ac polyclonal antibody showed good reaction with Cry2Ac protein in the range of 0.5 to 4.0 ng per well. However the Cry2Ac polyclonal antibody failed to react efficiently with Cry2Ab and Cry1Ac proteins upto a concentration of 10.0 ng per well (Table 10).

4.8 Screening of transgenic tobacco plants expressing synthetic *cry2Ac* gene by ELISA

Plant leaf extraction was made using PBST buffer (Plate 11) and TBA buffer (Plate 12). The concentration of protein in the extract obtained by the first method was less than that obtained by the second method in SDS-PAGE analysis.

Protein concentration of the leaf extracts of tobacco plants made during vegetative stage (24 DAP) and flowering stage (33 DAP) are presented in Table 11 & 12.

4.8.1 Screening of I batch of transgenic tobacco plants

Four putative transformants from the I batch were screened for expression of the Cry2Ac protein. Different concentration of the above plant protein samples (extracts made after 33 DAP) were analysed for Cry2Ac protein. The per cent of Cry2Ac protein in total soluble plant protein was maximum in transgenic tobacco plant, NT2 (0.11) followed by NT6 (0.067). The Cry protein expression level for the other transgenic tobacco plants NT3 and NT4 were 0.031 and 0.019 per cent, respectively (Table 13).

Another ELISA experiment was carried out with higher concentrations of plant protein for transgenic plants that showed low concentration of Cry protein expression in the previous ELISA experiment. The results indicated low concentration of Cry2Ac protein, inspite of using more amount of total soluble protein for the plant samples NT3 and NT4 (Table 14).

4.8.2 Screening of II batch of transgenic tobacco plants

Among the five protein samples extracted during the vegetative stage (24 DAP) of the second batch of transgenic plants, MT14b showed the maximum expression of Cry2Ac, 0.0305 per cent. The transgenic plants, MT8a and MT8b recorded moderate level of Cry2Ac expression 0.0145 and 0.0194 per cent respectively. On the other hand, the transgenic plants, MT11a and MT11b did not show detectable level of expression of the Cry2Ac protein (Table 15).

In the protein samples of flowering stage (33 DAP) as in the case of vegetative stage, the transgenic plant MT14b showed maximum Cry2Ac protein expression of 0.034 per cent, whereas MT8a and MT8b showed a moderate level of expression of 0.0164 and 0.0205 per cent, respectively. MT11a and MT11b showed very low level of expression, 0.0033 and 0.0028 per cent, respectively (Table 16). The per cent of Cry2Ac protein in total soluble protein of the transgenic plants screened by ELISA is summarized in Table 17.

4.9 Screening of transgenic tobacco plants expressing synthetic *cry2Ac* gene for toxicity against *H. armigera*

Bioassays conducted with leaf bits showed that the larvae on the transgenic tobacco plants NT2 and NT6 expressing the synthetic *cry2Ac* gene recorded larval mortality of 76.7 and 66.7 per cent, respectively. Lower mortality of 27 and 37 per cent was observed in NT3 and NT4, respectively. The mortality on the control plant ranged from 0 to 3.3 per cent (Table 18).

The leaf area fed in the control plants by *H. armigera* ranged from 437± 25.2 to 476.3± 21.8 mm^2 (Plate 13). Among the transgenic plants, the area fed was minimum in NT2 (137.3 ± 11.3 mm^2) and maximum in NT4 (232.2 ±16.4 mm^2) as given in Table 19. The per cent reduction in the leaf area fed by *H. armigera* in transgenic tobacco plants expressing synthetic *cry2Ac* gene over control tobacco plants ranged from 46.9 to 71.0 (Table 20).

4.10 Southern Blot analysis

The concentration and intactness of the tobacco plant DNA extracted by CTAB method was checked by loading in 0.8 per cent agarose gel (Plate 14). Five and ten µg of the plant DNA was taken for digestion with *Bam*HI enzyme. In order to check the extent of digestion, an aliquot of the digested sample was loaded in 0.8 per cent agarose gel (Plate 15).

The *Bam*HI digested samples of NT2, NC3 and p2AP1 were loaded in 0.8 per cent agarose gel. The digested fragments resolved on the gel were transferred to a nylon membrane. The membrane was hybridized with P^{32} labelled synthetic *cry2Ac* gene fragment obtained by digestion of p2AP1 with *Bam*HI. The control tobacco plant (NC3) did not show any hybridization signal whereas the transgenic tobacco plant (NT2) showed a signal corresponding to that of synthetic *cry2Ac* gene fragment obtained by restriction digestion of p2AP1 plasmid (Plate 16).

4.11 Northern Blot analysis

RNA isolated from the transgenic plant NT2 and control plant NC3, was resolved in denaturing agarose gel for checking the quality and concentration (Plate 17). Ten µg of RNA from each of the plants was used for carrying out Northern analysis. The Northern blot showed a signal in NT2 transgenic plant while no signal was detected in the control plant, NC3 (Plate 18).

4.12 Screening of putative transgenic tobacco plants containing native *cry2Ac* gene by ELISA

The protein concentration of the tobacco transformants harbouring the native *cry2Ac* gene as well as that of the control tobacco plants are presented in Table 21. The ELISA result showed Cry2Ac expression level as 0.12 and 0.33 per cent of total soluble protein in transgenic plants, D14-2 and D14-3, respectively (Table 22).

4.13 Screening of transgenic tobacco plants expressing native *cry2Ac* gene for toxicity against *H. armigera*

Bioassays carried out with leaf bits of tobacco plants, (D14-2 and D14-3) expressing native *cry2Ac* gene, showed mortality of *H. armigera* ranging from 76.7 to

86.7 per cent after six days. On the other hand, the control tobacco plants (DC1 and DC2) showed mortality of 0 to 3.3 per cent only (Table 23). The leaf area fed by *H. armigera* larvae upto six days in the transgenic tobacco plants, D14-2 and D14-3 were 56.3 ± 6.3 and 40.2 ± 4.9 mm^2, respectively (Table 24). The per cent reduction in leaf area damaged on the transgenic tobacco plants expressing native *cry2Ac* gene over that of the control tobacco plants after six days were 71.5 and 80.8 per cent in D14-2 and D14-3, respectively (Table 25).

4.14 Construction of recombinant pUH vector containing synthetic *cry2Ac* gene

The pUH vector was linearised by digestion with *Bam*HI and the 11.3 kb fragment was eluted from gel (Plate 19). The pS2Ac plasmid harbouring the synthetic *cry2Ac* gene was also digested with *Bam*HI and the gene fragment corresponding to 1.9 kb was eluted (Plate 20). The eluted fragments were ligated and used to transform the *E.coli* (DH5α strain) cells.

4.14.1 Screening of the putative recombinant pUH clones by PCR for the presence of *cry2Ac* gene

Transformed colonies of *E.coli* were screened by PCR using the S2F1 and S2R1 primers, specific for the synthetic *cry2Ac* gene. Amplification of expected size of 800 bp was observed in the positive control (pS2Ac) and two of the *E. coli* transformants (Plate 21). The two clones of recombinant pUH showing presence of synthetic *cry2Ac* gene by PCR were named as p2P3A and p2P3B.

4.14.2 Restriction digestion of p2P3A and p2P3B

Restriction digestion of the plasmid isolated from the positive clones, p2P3A and p2P3B with *Bam*HI enzyme released the 1.9 kb insert (synthetic *cry2Ac* gene fragment) from the vector backbone of size 11.3 kb (Plate 22).

Restriction digestion of the recombinant pUH clones with *Xho*I enzyme showed four fragments of size while the pUH vector showed only three fragments of size 7.3, 2.8 and 1.1 kb, respectively (Plate 23).

CHAPTER V
DISCUSSION

5.1 Construction of a new plant transformation vector with synthetic *cry2Ac* gene

A new plant transformation vector, p2AP1 carrying the synthetic *cry2Ac* gene was constructed and mobilized into *Agrobacterium tumefaciens* strain EHA105. The Cry2A proteins have unique characteristics and behaviour as compared to other Cry proteins. Hence the *cry2A* gene can be used in combination with other *Bt* genes for pyramiding resistance in transgenic plants (Maqbool *et al*., 1998).

The DNA fragment containing the synthetic *cry2Ac* gene (flanked by *Nco*I and *Sac*I sites at 5' and 3' ends, respectively) was released from the recombinant pUC19 plasmid by *Nco*I and *Sac*I digestion. The *cry2Ac* gene was ligated into pRT103 at *Nco*I and *Sac*I sites. The pRT103 vector was used as the source for the 35S promoter and poly A sequences. The recombinant pRT103 is designated as pS2CRT. Since the pRT103 vector has ampicillin resistance gene, the *E. coli* cells transformed with recombinant plasmid pS2CRT were selected on LB agar plates containing ampicillin.

The 35S promoter and poly A sequences are flanked by *Pst*I site in pRT103 so that digestion using *Pst*I enzyme released two fragments: a 2.6 kb expression cassette consisting of CaMV 35S promoter, *cry2Ac* gene and poly A signal and another 2.6 kb fragment consisting of the back bone of pRT103 vector carrying the ampicillin resistance gene. The two fragments of equal size were indistinguishable when resolved on agarose gel. So both the fragments were eluted together and used for ligation with pCAMBIA2300 at the *Pst*I site.

The multiple cloning site of pCAMBIA2300 is the *lac*Z site. Hence the insertion of a foreign gene at this site inactivates the *lac*Z gene. To avoid the self ligated clones, the transformed colonies were subjected to the α complementation test by streaking on LB agar plates containing X-Gal, IPTG and kanamycin. The recombinant clones were white while, the self-ligated ones were blue in colour. Only 6-12 per cent of the colonies screened were found to be recombinants.

The recombinant pCAMBIA2300 clones carrying the expression cassette of *cry2Ac* (2.6 kb) were distinguished from those having the backbone of pRT103 (containing the ampicillin resistance gene) by checking the growth of white colonies on ampicillin plates. Therefore the ampicillin susceptible white colonies were expected to harbour the p2AP1 (recombinant pCAMBIA2300 carrying the expression cassette of the synthetic *cry2Ac* gene). Moreover, the release of the expression cassette (2.6 kb) by digestion with *Pst*I enzyme and amplification of expected size (800 bp) fragment by PCR with synthetic *cry2Ac* gene specific primers confirmed the presence of *cry2Ac* expression cassette in the newly constructed plant transformation vector. The plant transformation vector p2AP1 carrying the expression cassette of the synthetic gene *cry2Ac* gene was mobilized into *A. tumefaciens* strain EHA105 by electroporation and selected on YEB agar plates containing kanamycin.

5.2 Establishment of tobacco plants *in vitro*

Availability of an efficient regeneration system is usually a pre-requisite to genetic modification of a particular plant species. Tobacco has good organogenic potential and amenability to genetic transformation. Most techniques for genetic transformation and regeneration depend on the use of plant growth regulators in complex. Also, maintaining plants under aseptic conditions obviates the need for sterilization of explants. The tobacco plants established under *in vitro* conditions were used for transformation studies.

5.3 Sensitivity of tobacco to kanamycin

The bacterial gene for *neomycin phosphotransferse* (*npt*II), which inactivates the antibiotic kanamycin is most widely used as a selectable marker in transformation experiments. After introduction of constructs containing the *npt*II gene into plant cells, kanamycin is applied to kill untransformed tissue. Transformed cells expressing *npt*II are protected from the effects of antibiotics and using appropriate cell culture media can regenerate into whole transgenic plants. The concentration of kanamycin used for selection purposes in transformation experiments varies depending on the plant species. It has been reported that in transformation experiments using cotyledonary leaves of

tomato (Mandaokar *et al*., 2000) and brinjal (Kumar *et al*., 1998) as explants, 100 mg/l and 50 mg/l kanamycin was used in shoot regeneration medium and rooting medium, respectively. Selvapandiyan *et al*., (1998) reported regeneration of transgenic tobacco (*N. tabaccum* var. Xanthi) in the presence of 100 µg/ml kanamycin containing shooting medium. In the present study, the killer concentration of kanamycin for *N. tabaccum* cv. Havana was found to be 50 mg/l. For stringent selection of transformants, kanamycin at 100 mg/l was used in selection media.

5.4 *Agrobacterium* mediated transformation of tobacco

The *A. tumefaciens* strain EHA105 carrying the synthetic *cry2Ac* gene was grown to an OD of 1 to 1.5 to obtain late log phase culture. The pre-incubated explants were infected with the late log phase culture of *Agrobacterium* as reported by De Block *et al.* (1987).

Untransformed shoots of tobacco proliferated in a comparable manner in shoot regeneration medium containing cefotaxime 300 and 500 mg/l. At a low concentration of 200 mg/l, cefotaxime has been previously found to have stimulatory effect on regeneration from leaf explants of apple and pear (De Bondt *et al.,* 1996). Moreover, Cheng *et al*., (1998) reported that growth of *A. tumefaciens* could be suppressed by cefotaxime at a lower concentration of 250 mg/l. Hence, for further selection, cefotaxime was used at 300 mg/l concentration.

Shoots of putative transformants were normal and green when cultured in selection medium containing kanamycin (100 mg/l) while the untransformed shoots died out indicating the effective selection of transformed plants using kanamycin. For more stringent selection of transformed plants, kanamycin was included in RM at 50 mg/l. Majority of plants transferred to RM produced roots except nine plants that may be non-transformants.

5.5 Screening of putative transgenic tobacco plants by PCR

PCR provides a quick and convenient mechanism for confirming the presence of the gene of interest using specific primers. Majority (nearly 75 per cent) of the putative transgenic tobacco plants screened by PCR for the presence of synthetic *cry2Ac* gene

gave positive results. The genomic DNA from control plants gave no amplification, while bands of expected size (of about 800 bp) were seen only in transformed plants when screened using synthetic *cry2Ac* gene specific primers S2F1 and S2R1. The result was further confirmed by another set of synthetic *cry2Ac* specific primers, S2F3 and S2R3 which produced bands of expected size (of about 600 bp) in the putative tobacco transformants.

5.6 Reaction of Cry2Ac polyclonal antibody to different Cry proteins

The Cry2Ac polyclonal antibody was tested for its reaction with different Cry proteins namely Cry2Ab, Cry2Ac and Cry1Ac. The Cry2Ac polyclonal antibody reacted with Cry2Ac protein even at low concentration of 0.5 to 4.0 ng per well. On the other hand, the Cry2Ac polyclonal antibody did not react efficiently with Cry2Ab and Cry1Ac proteins, even at higher concentrations of upto 10 ng. The result indicated that the Cry2Ac polyclonal antibody is reasonably specific to detect the Cry2Ac protein.

5.7 Screening of c*ry2Ac* transgenic tobacco plants by ELISA

Leaf extracts from the transgenic as well as the control plants were made using PBST buffer and TBA buffer. The concentration of the samples was analyzed in SDS-PAGE. The results indicated a lower concentration of the protein in similar amount of samples extracted by PBST buffer as compared to TBA buffer (also same quantity of buffers used for extraction). Hence, for the further screening of the transgenic plants by ELISA, the leaf extracts made using TBA buffer was used. Protein concentration of the tobacco leaf extracts was determined by Lowry's method and the amount of Cry2Ac protein detected by ELISA in the transgenic tobacco plants were expressed as percentage of the total plant soluble protein.

In the I batch of putative transformants of tobacco screened for Cry2Ac protein by ELISA, the transformant NT2 showed a maximum expression of 0.11 per cent followed by 0.067 per cent expression in NT6. Another ELISA was carried out with the I batch of plants by taking higher concentrations of plant protein for transgenic plants NT3 and NT4 which recorded 0.03 and 0.019 per cent expression only in the earlier ELISA experiment.

However the results indicated that, the Cry2Ac protein expression was not increased inspite of taking more amount of the plant protein sample (Table 7). This clearly indicated that the synthetic *cry2Ac* gene expression level is very low in these plants.

Among the II batch of putative tobacco transformants that were screened for the level of Cry2Ac protein, the transformant MT14b recorded a maximum of 0.030 per cent. The putative transgenic plants, MT8a and MT8b showed Cry2Ac expression of 0.015 and 0.019 per cent, respectively. MT11a and MT11b did not show any detectable amount of Cry2Ac protein (Table 13). This ELISA was carried out with leaf extracts made at vegetative stage (24 DAP).

To test whether there occurs any variation in the Cry2Ac expression at flowering stage (33 DAP), another ELISA was carried out with samples made at this stage from the same plants of the II batch. The putative transgenic plant MT14b recorded Cry2Ac protein expression level of 0.034 per cent followed by 0.0164 and 0.021 per cent in MT8a and MT8b respectively. A very low level of expression of 0.0033 and 0.0028 per cent was recorded in the case of MT11a and MT11b, respectively (Table 14). The results of both the ELISA were comparable and there was only a slight increase in the Cry2Ac expression level during flowering stage.

It has been reported by various authors that different plant species transformed with *cry* genes under the control of constitutive promoter like CaMV 35S exhibited a wide range of levels of foreign protein expression. For example, out of total soluble protein, 0.1 per cent of Cry3A in potato (Adang *et al.,* 1993), 0.03 per cent of Cry1Ab in tobacco (Perlak *et al.,*1991), 0.05 per cent of Cry1Ab in rice (Fujimoto *et al.,*1993), 0.4 per cent of Cry1Ab in maize (Koziel *et al.,*1993), 0.4 per cent of Cry1Ac in canola (Stewart *et al.,*1996) were observed. The Cry2Ac protein expression levels recorded in the transgenic tobacco plants that were screened in the present study is comparable to previous studies.

5.8 Screening of transgenic tobacco plants expressing synthetic *cry2Ac* gene for toxicity against *Helicoverpa armigera*

Insect bioassays have been previously demonstrated as the most sensitive method to quantitate Bt protein levels in transgenic plants (Fuchs *et al*., 1990). It has been reported by earlier workers (Fischhoff *et al*., 1987; Vaech *et al*., 1987) that expression of *cry* genes of *Bt* can confer insect tolerance on transgenic plants. In the present study, *H. armigera* bioassays were carried out to test whether the expression of synthetic *cry2Ac* gene confers insect suppression on transgenic tobacco plants.

The results of the *H. armigera* bioassay showed larval mortality of 76.7 and 66.7 per cent, in the tobacco transformants NT2 and NT6, respectively which recorded the maximum level of Cry protein expression (0.138 and 0.069 per cent, respectively) in the ELISA. Transgenic tobacco plants NT3 and NT4 that recorded lower Cry protein expression (0.027 and 0.018 per cent, respectively) with a larval mortality of 26.7 and 36.7 per cent, respectively. The effect of cry protein concentration in transgenic tobacco plant on mortality of *H. armigera* larvae is graphically represented in Fig.8. The fact that, insect control activity was seen even under conditions of low level of gene expression is a reflection of the high potency of the Cry2Ac protein. The growth of the larvae was severely inhibited in the case of *H. armigera* larvae that survived upto two to three days on some of the transgenic plants tested. The leaf area fed by *H. armigera* larvae in the control plants was about two–to-four fold more as compared to the damage caused on the transgenic plants. A comparison of the leaf area damaged by *H. armigera* larvae in transgenic and control tobacco plants is shown in Fig.9. The leaf bits of transgenic tobacco plants sustained little damage, because larval feeding was significantly reduced. These effects *viz.,* feeding inhibition and stunting of growth of larvae at sublethal doses are known properties of Bt proteins (Luthy and Ebersold, 1981).

5.9 Southern blot analysis of *cry2Ac* transgenic tobacco tobacco plant.

Southern blot analysis was carried out in one of the transgenic tobacco plants that showed maximum level of expression of Cry2Ac protein in ELISA. In p2AP1 the synthetic *cry2Ac* gene is flanked by the *Bam*HI site. So hybridization of P^{32} labelled synthetic *cry2Ac* gene fragment as probe showed a single signal in the case of the positive control. Absence of any signal in the control plant and the presence of a signal

corresponding to that of the positive control confirm the presence of *cry2Ac* gene integration in the transgenic plant NT2.

5.10 Northern blot analysis of *cry2Ac* transgenic tobacco plants

For Northern blot analysis, RNA was used from NT2, the transgenic plant expressing the maximum Cry protein as per the ELISA results and the control plant, NC3. The result of the Northern blot analysis with P^{32} labelled synthetic *cry2Ac* gene fragment showed the presence of the transcript in the transgenic plant NT2.

5.11 Screening of transgenic plant expressing native *cry2Ac* gene by ELISA

Leaf extracts made from transgenic tobacco plants harbouring native *cry2Ac* gene under the control of *rbc*S promoter and transit peptide sequences were screened for the expression level of the Cry2Ac protein by ELISA. The transgenic tobacco plants, D14-2 and D14-3 recorded Cry2Ac protein expression of 0.12 and 0.33 per cent, respectively. The Cry2Ac expression level of 0.33 per cent recorded by the tobacco plant harbouring native *cry2Ac* gene construct is higher than that of the transgenic plants harbouring synthetic *cry2Ac* gene construct. The two transgenic plants harbouring the native *cry2Ac* gene construct (D14-2 and D14-3) were selected as the best ones among several others by preliminary bioassay with *H. armigera* larvae. So it does not mean that all transgenic plants of native gene construct are superior to transgenic plants of synthetic gene construct. Moreover, this may be due to the *rbc*S promoter and its transit peptide sequences used to express the native gene construct. Organellar targeting of proteins alleviates cytoplasmic toxicity of gene products and often results in an increase in expression levels. Wong *et al.* (1992) could get an enhanced expression (10 to 20 fold increase in expression) of truncated *cry1A(c)* gene in tobacco using *Arabidopsis thaliana* *rbc*S promoter and its transit peptide. Hence, it may be possible to get transgenic plants of synthetic gene construct having higher level of expression than these two transgenic plants of the native gene construct by screening more number of transgenic plants of synthetic gene construct.

5.12 Screening of transgenic tobacco plants expressing native *cry2Ac* gene for toxicity against *H. armigera*

Bioassays carried out with leaf bits of tobacco plants (D14-2 and D14-3) expressing the native *cry2Ac* gene, showed mortality of *H. armigera* ranging from 77 to 87 per cent after six days. On the other hand, the control tobacco plants (DC1 and DC2) showed mortality of 0 to 3.3 per cent only. Significant amount of insecticidal activity of tobacco plants transformed with native *Bt* gene construct is also documented by earlier workers (Vaeck *et al.,* 1987; Selvapandiyan *et al.,* 1998). The leaf area fed by *H. armigera* larvae upto six days in the transgenic tobacco plants, D14-2, D14-3 and control plants DC1, DC2 was 56.3 ± 6.3, 40.2 ± 4.9, 197.2 ± 16.7 and 208.1 ± 19.7 mm^2, respectively. One early sign of Bt intoxication is inhibition of feeding by larvae. The per cent reduction in leaf area damage of transgenic tobacco plants expressing native *cry2Ac* gene over that of control tobacco plants was 71.5 and 80.8 per cent in D14-2 and D14-3, respectively.

5.13 Construction of plasmids to express synthetic *cry2Ac* gene by *ubi*I promoter

The availability of effective vector system is a pre-requisite for genetic manipulation of plants through recombinant DNA technology. The pUH vector constructed by Katiyar-Agarwal *et al*., (2002) possesses *ubiquitin*I promoter to express foreign gene and hygromycin phosphotransferase (*hph* / hyg^r) as selectable marker gene. The maize *ubiquitin*I promoter has a high strength of expression and hence often used for rice transformation. Besides the promoter and selectable marker, the other useful feature of the pUH vector is the availability of unique restriction sites *Bam*HI*, Sma*I*, Kpn*I and *Sac*I in the MCS. As the foundation vector used for the construction of pUH belongs to pCAMBIA series, the pUH vector inherit several useful features like kanamycin based selection in bacteria, high copy number plasmid replication, Kozak sequences for improved expression, minimal extraneous DNA sequences and stability of the plasmid under non-selection condition.

In the present study, the synthetic *cry2Ac* gene (1.9 kb) flanked by *Bam*HI sites was ligated at the *Bam*HI site (in MCS) of the pUH. The ligated mixture was used to transform *E.coli* cells (DH5α strain). Since the pUH vector has kanamycin based selection in bacteria, the transformed *E.coli* cells were selected on LB agar plates containing kanamycin. These transformed colonies were screened for the presence of the

synthetic *cry2Ac* gene by specific primers namely, S2F1 and S2R1. The two recombinant plasmids that were positive in the screening by PCR were named as p2P3A and p2P3B.

The presence of the synthetic *cry2Ac* gene was further confirmed by restriction digestion. Restriction digestion of the plasmids isolated from the positive clones, p2P3A and p2P3B with *Bam*HI released the 1.9 kb synthetic *cry2Ac* gene fragment from the vector backbone of size 11.3 kb. The pUH vector has three restriction sites for *Xho*I. The synthetic *cry2Ac* gene fragment (insert) cloned in the *Bam*HI site of the pUH vector also has a *Xho*I site at 770 bp, from the 5' end (Fig.10). Hence, the presence of the insert could be further confirmed by restriction digestion with *Xho*I. Restriction digestion of untransformed pUH and the recombinant plasmid p2P3A with *Xho*I showed three fragments and four fragments respectively. Sizes of the *Xho*I fragments of pUH were 7.3, 2.8, 1.2 and that of p2P3A were 7.3, 2.5, 2.2 and 1.2 kb. Further studies need to be carried out to know the orientation of the synthetic *cry2Ac* gene to the *ubiquitin*I promoter so that, this vector finds applicability in genetic transformation of monocots, especially rice.

CHAPTER VI
SUMMARY

The present study was undertaken to test the expression of synthetic and native *cry2Ac* genes driven by CaMV 35S and *rbc*S promoters, respectively, in transgenic tobacco plants. Another plasmid p2P3A harbouring synthetic *cry2Ac* gene under the control of *ubiquitin*I promoter for efficient expression of the *cry* gene in monocots was also constructed as a part of the study.

The results of the study are summarized hereunder:

1. A synthetic *cry2Ac* gene from recombinant pUC19 plasmid was cloned into *Nco*I and *Sac*I sites of pRT103 vector. The recombinant plasmid was designated as pS2CRT.

2. The expression cassette containing the CaMV 35S promoter, *cry2Ac* gene and poly A sequences from pS2CRT was subcloned into pCAMBIA 2300 vector at the *PstI* site and the recombinant plasmid was designated as p2AP1.

3. The new plant transformation vector p2AP1 harbouring the synthetic *cry2Ac* gene was mobilized into *Agrobacterium tumefaciens* strain EHA105 by electroporation.

4. Shoot regeneration in untransformed tobacco was suppressed at 50 mg/l concentration of kanamycin.

5. *Nicotiana tabacum* cv Havana was successfully transformed with the synthetic *cry2Ac* gene by *Agrobacterium* mediated method of transformation carried out in two batches (Nov. 2001 and March, 2002).

6. Fifteen putative transgenic lines of tobacco were recovered after selection in shoot regeneration and rooting medium containing kanamycin at 100 and 50 mg/l concentration, respectively. However, only twelve plants established successfully in pot culture.

7. Nine out of twelve putative transgenic lines of tobacco were found to be positive for the amplification of the synthetic *cry2Ac* gene by PCR.
8. Results of ELISA (using direct adsorption of antigen to solid phase) revealed the reaction of Cry2Ac polyclonal antibody with Cry2Ac protein in the range of 0.5 to 4.0 ng per well. But the Cry2Ab and Cry1Ac proteins failed to react efficiently with the Cry2Ac polyclonal antibody, even upto a concentration of 10.0 ng per well.

9. Screening of the first batch of transgenic tobacco plants by ELISA showed that the per cent of Cry2Ac protein in total soluble plant protein was maximum in transgenic tobacco plant NT2 (0.11) followed by NT6 (0.067).

10. Inspite of using twelve fold more of the total soluble protein in the next ELISA experiment, the tobacco transformants NT3 and NT4 showed low level of Cry protein expression.

11. Screening of the second batch of transgenic tobacco plants by ELISA, using protein samples extracted during vegetative stage (24 DAP), revealed 0.031, 0.015 and 0.019 per cent expression of Cry2Ac in the transformants MT14b, MT8a and MT8b, respectively.

12. Screening of the second batch of transgenic tobacco plants by ELISA, using protein samples extracted during flowering stage also showed similar levels of Cry2Ac protein, as in the case of vegetative stage for the transformants MT14b, MT8a and MT8b (0.034, 0.016 and 0.024 per cent, respectively).

13. *Helicoverpa armigera* bioassays with tobacco leaf bits showed that the tobacco transformants NT2 and NT6 recorded larval mortality of 76.7 and 66.7 per cent, respectively, while mortality in control plant ranged from 0 to 3.3 per cent.

14. The bioassay experiments also revealed that the leaf area fed in control plants by *H. armigera* ranged from 437 ± 25.2 to 476.3 ± 21.8 mm^2, while the tobacco transformant NT2 recorded 37.3 ± 11.3 mm^2 only.

15. The per cent reduction in the leaf area fed by *H. armigera* in transgenic tobacco plants expressing synthetic *cry2Ac* gene over control plants ranged from 46.9 to 71.0.

16. Southern blot analysis carried out with P^{32} labelled synthetic *cry2Ac* gene fragment showed absence of any signal in control plant and presence of a signal in transgenic tobacco plant corresponding to that of the positive control confirming the presence of *cry2Ac* gene integration in the transgenic plant NT2.

17. Northern blot analysis with P^{32} labelled synthetic *cry2Ac* gene fragment showed the presence of the transcript in the transgenic plant NT2.

18. In another ELISA the transgenic tobacco plants D14-2 and D14-3 containing native *cry2Ac* gene, showed Cry2Ac expression level of 0.12 and 0.33 per cent of total soluble plant protein, respectively.

19. In the *H. armigera* bioassay the transgenic tobacco plants D14-2 and D14-3 expressing native *cry2Ac* gene, showed larval mortality ranging from 77 to 87 per cent, where as the control tobacco plants showed 0 to 3.3 per cent mortality only.

20. The leaf area fed by *H. armigera* upto six days in the transgenic tobacco plants, D14-2 and D14-3 was 56.3 ± 6.3 and 40.2 ± 4.9mm^2 respectively, while in control plants DC1 and DC2 it was 197.2 ± 16.7 and 208.1 ± 19.7, respectively.

21. The reduction in leaf area damage of transgenic tobacco plants expressing native *cry2Ac* gene over that of control tobacco plants was 71.5 and 80.8 per cent in D14-2 and D14-3, respectively.

22. In another experiment, the synthetic *cry2Ac* gene was cloned under the control of *ubiquitin*I promoter in the *Bam*HI site of pUH vector. Two recombinant pUH plasmids obtained were designated as p2P3A and p2P3B.

23. The presence of the synthetic *cry2Ac* gene in the recombinant plasmids, p2P3A and p2P3B was confirmed by PCR with primers specific for synthetic *cry2Ac*

gene as well as by restriction digestion with *Bam*HI and *Xho*I. Further experiments are needed to confirm the orientation of the synthetic *cry2Ac* gene to the *ubiquitin*I promoter.

ANNEXURE – I

MEDIA COMPOSITION

1.1. LB Broth

Yeast extract	: 5.0 g
Sodium chloride	: 10.0 g
Tryptone	: 10.0 g
Distilled water	: 1000.0 ml
pH	: 6.8 to 7.0

LB agar: 1.8 g of bacteriological agar was added to 100 ml of LB broth.

1.2. SOC broth

Tryptone	: 20.0 g
Yeast extract	: 5.0 g
Sodium chloride	: 0.58 g
Potassium chloride	: 0.186 g
Magnesium chloride	: 2.033 g
Magnesium sulphate	: 1.20 g
Glucose	: 3.60 g
Distilled water	: 1000 ml
pH	: 6.8 – 7.0

1.3. YEB medium

Beef Extract	: 5.0 g
Yeast Extract	: 1.0 g
Peptone	: 5.0 g
Sucrose	: 5.0 g
$MgSO_4$	: 2.0 ml
Distilled water	: 1000 ml
pH	: 7.2

Agar: 1.5 g was added to 100 ml of YEB broth.

1.4. Shoot regeneration medium (SM)

MS medium powder (Hi Media)	: 4.2 g
Calcium chloride	: 440 mg
BAP (1mg/mlstock)	: 1.0 ml
NAA (1mg/mlstock)	: 0.1 ml
Sucrose	: 30.0 g
Agar	: 8.0 g
Distilled water	: 1000 ml
pH	: 5.8 (adjusted using KOH)

1.5. Rooting medium (RM)

MS medium powder (Hi Media)	: 4.2 g
Calcium chloride	: 440 mg
IBA (1mg/mlstock)	: 1.0 ml
Sucrose	: 30.0 g
Agar	: 8.0 g
Distilled water	: 1000 ml
pH	: 5.8 (adjusted using KOH)

ANNEXURE – II

BUFFERS AND SOLUTIONS

2.1. Plant protein extraction

2.1.1. TBA buffer method

TB buffer (10X)

Tris (100 mM)	: 12.114 g
Sodium borate (100 mM)	: 38.137 g
$MgCl_2$ (5 mM)	: 1.02 g
Tween 20 (0.05% v/v)	: 0.5 ml

pH adjusted to 7.8 with HCl and made upto 1 litre.

TBA buffer

L-Ascorbic acid 0.2% (w/v) was added to 1X TB buffer on the day of use.

2.1.2. PBST method

10X PBST buffer

KH_2 PO_4 (0.015M)	: 2.0 g
Na_2 $HPO_4.7H_20$ (0.08M)	: 11.5 g
KCl (0.03M)	: 2.0 g
NaCl (1.4M)	: 80.0 g
Tween 20	:7 ml

Made up the volume to 1 litre.

2.2 Estimation of protein

Reagent A : Sodium carbonate (2%) dissolved in 0.1 N sodium hydroxide

Reagent B : Copper sulphate (0.5%) dissolved in sodium potassium tartarate(1%)

Reagent C : 50 ml reagent A was mixed with 1.0 ml of reagent B before use

Reagent D : Folin – Ciocalteau's phenol dye stored at 4ºC

Standard BSA: Bovine serum albumin (BSA) stock 1 mg/ml stored at 4ºC

2.3. SDS-PAGE

Reagents

Solution A	: Acrylamide, 29 g and 1 g bis-acrylamid were dissolved in 100 ml water, filtered and stored at 4°C in a brown bottle
Solution B	: Tris – HCl 1.5 M (pH 8.8) (stored at 4°C)
Solution C	: Tris – HCl 0.5 M (pH 6.8) (stored at 4°C)

Ammonium persulphate (10%)

10 mg of ammonium per sulphate was dissolved in 100µl distilled water prepared fresh each time.

TEMED

It was used as such and stored at 4°C.

Sample loading buffer (4X)

Tris-HCl, 0.25 M (pH 6.8) was added to 8 per cent SDS, 40 per cent glycerol, 20 per cent β-mercaptoethanol, 0.5 per cent bromophenol blue and dispensed into aliquots for use; stored at room temperature.

Electrode buffer (10X)

Tris base (0.25M) was added to glycine (1.92 M) and SDS (10%) and stored at room temperature; 1X electrode buffer was used for running gels.

Staining solution

Two hundred and fifty milligram of Coomassie brilliant blue R 250 was dissolved in 40 ml methanol followed by addition of 10 ml of acetic acid and then the volume was made upto 100 ml with distilled water.

Destaining solution

Prepared from acetic acid, methanol and water in the ratio of 10:40:50 v/v.

Composition of separating and stacking gels

Composition	Separating gel (8%)	Stacking gel (4.5%)
Water	4.55 ml	1.8 ml
Solution A	2.10 ml	0.45 ml
Solution B	2.25 ml	-
Solution C	-	0.75 ml
APS (10%)	0.080 ml	.030 ml
TEMED	0.005 ml	0.003 ml

2.4. ELISA

10X PBST buffer

NaCl (1.4 M) : 80.0g

Na_2 HPO_4 $7H_2O$ (0.08 M) : 21.7g

KH_2 PO_4 (0.015 M) : 2.0 g

KCl (0.03 M) : 2.0 g

Tween 20 : 7.0 ml

Made up the volume to 1 litre

Coating buffer

1X PBST : 20.0 ml

Polyvinyl pyrollidone (PVP) : 0.4g

BSA : 0.04 g

Substrate buffer

Diethanol amine : 10.0 ml

Distilled H_2O : 60.0 ml

Adjusted pH to 9.8 with HCl and made up the volume to 100ml.

Substrate

0.5 mg of PNPP / ml of the substrate buffer.

2.5. Plant DNA extraction (CTAB method)

CTAB extraction buffer

Tris HCl (pH 8.0) 100 mM	:24.22 g
NaCl 1.4 M	: 16.36 g
EDTA (pH 8.0) 20 mM	:37.22 g
CTAB 2% w/v	:4.0 g
2-Mercapto ethanol 0.1% w/v	:0.2 g

Make up the volume to 200 ml.

RNaseA solution : 10 mg/ml

Chloroform : Isoamyl alcohol (24:1) : Chloroform – 480.0 ml
Isoamyl alcohol – 20.0 ml

Ethanol : 70% w/v

TE buffer

Tris HCl (pH 8.0) 10 mM	: 1.21 g
EDTA (pH 8.0) 1 mM	:0.37 g

Make up the volume to 1000 ml.

NaCl (3M)	: 87.66g / 500 ml
$NaOCH_3$ pH 5.2 (3M)	: 204.1g / 500 ml

2.6. Southern Hybridization

Denaturation solution

NaCl (1M)	: 29.22 g
NaOH (0.5 M)	: 10.0 g

The volume was made to 500 ml, autoclaved and stored at room temperature.

Neutralization solution

NaCl (1.5 M)	: 43.83 g
Tris (0.5 M)	: 30.28 g

Dissolved in 350 ml distilled water and pH adjusted to 7.0 with conc. HCl. The volume was made up to 500 ml with distilled water, autoclave and stored at room temperature.

20X SSC

NaCl :175.3 g

Sodium citrate : 88.2 g

Dissolved in 800 ml water, pH adjusted to 7.0 with conc. HCl and the volume made upto 1 litre.

Radioactive probe preparation mixture

10X Klenow buffer : 3.5 µl

Random primers : 3.0 µl

dGTC : 3.0

Klenow enzyme : 1.5 µl

Dithiothreitol : 2.5 µl

dATP (^{32}P) : 1.5 µl

Total : 15.0 µl

Pre-hybridization solution

Stock	Final conc. required	Stock required for				
		25 ml	30 ml	50 ml	100 ml	150 ml
2 M Na_2HPO_4	0.5 M	6.25 ml	7.5 ml	13.5 ml	25.0 ml	37.5 ml
10% SDS	7%	17.5 ml	21.0 ml	35.0 ml	70.0 ml	105.0 ml
0.1 M EDTA (pH 7.0)	1 mM	250.0 µl	300 µl	500 µl	1.0 µl	1.5 µl
Sterile H_2O	-	1.0 ml	1.2 ml	2.0 ml	4.0 ml	8.0 ml

Washing solution : 3X SSC & 0.1% SDS

20X SSC	: 150 ml
10% SDS	: 10 ml

The volume was made upto 1000 ml using sterile distilled water.

Washing solution : 2X SSC & 0.1% SDS

20X SSC	: 100 ml
10% SDS	: 10 ml

The volume was made upto 1000 ml using sterile distilled water.

Washing solution : 0.5X SSC & 0.1% SDS

20X SSC	: 25 ml
10% SDS	: 10 ml

The volume was made upto 1000 ml using sterile distilled water.

Washing solution : 0.3X SSC & 0.1% SDS

20X SSC	: 15 ml
10% SDS	: 10 ml

The volume was made upto 1000 ml using sterile distilled water.

Developer solution

Metol	: 0.5 g
Hydroxy quinine	: 1.25 g
Sodium sulphite (anhydrous)	: 25.0 g
Borax	: 0.5 g

The volume was made upto 250 ml using sterile distilled water.

Fixer solution

Sodium thiosulphate	: 62.5 g
Sodium / Potassium metabisulphite	: 6.25 g

The volume was made upto 250 ml using sterile distilled water.

2.7. Northern Blotting

10X MOPS buffer

MOPS 0.2M : 41.86 h

NaOAC 50 mM : 6.8 g

EDTA 10 mM : 3.362 g

Adjusted pH to 7 using NaOH and the volume made upto 1 litre.

5X Loading buffer

EDTA (0.5M) :1.9 g

Bromophenol blue (0.25%) :0.03

Xylene cyanol (0.25%) :0.03

Glycerol (50%) :5.0 ml

Make up the volume to 10.0 ml with sterile distilled water.

Northern prehydridization solution

Potassium phosphate 25mM : 12.5 ml

5X SSC : 125 ml

5X Denhardt's solution : 25 ml

Salmon sperm DNA (50μg/ml) : 5ml

50% formamide : 250 ml

The final volume was made upto 500 ml and stored at -20°C.

Northern hybridization solution

To the prehybridization solution, 10% dextran sulphate (50g for 500 ml of pre hybridization solution) was added.

2.8. Isolation of Plasmid DNA

Solution I : Tris – HCl (pH 8.0) 25mM, EDTA (pH 8.0) 10 mM, Glucose 50 mM

Tris HCl 1M : 2.5 ml

Glucose 1M : 5.0 ml

EDTA 0.1 M : 10.0 ml

Distilled water : 72.5 ml

Autoclaved and stored at 4°C.

Solution II : NaOH 0.2 M, SDS (1%)

NaOH (2M)	: 100 µl
SDS (10%)	: 100 µl
Distilled water	: 800 µl

Freshly mixed before use.

Solution III : 3M Potassium, 5M Acetate

Potassium acetate (5M)	: 60.0 ml
Glacial acetic acid	: 11.5 ml
Distilled water	: 28.5 ml
Total	: 100.0 ml

Autoclaved and stored at 4°C.

Buffered phenol

Distilled phenol was equilibrated first with equal volume of 1M Tris-HCl (pH 8.0) and then with equal volume of 0.1M Tris – HCl (pH 8.0). Equilibrated phenol was stored overlaid with solution of 0.1M Tris – HCl at 4°C.

Sodium acetate, 3M (pH 5.2)

40.8 g of sodium acetate was dissolved in 80 ml of water and the pH was adjusted to 5.2 with glacial acetic acid and made upto 100 ml.

2.9. Analysis of plasmid DNA samples by agarose gel electrophoresis

TAE buffer (10X)

Tris	: 121 g
Glacial Acetic Acid	: 5.71 ml
EDTA	: 50 ml from 0.5M stock

Made upto 500 ml with distilled water.

Loading dye (6X)

Bromophenol blue	: 0.25 per cent

Glycerol : 30 per cent in water

Ethidium bromide (EtBr)

5 mg of sodium salt of EtBr was dissolved in 1ml of sterile water and stored at room temperature in amber vials.

2.10. Preparation of competent cells of *E.coli*

Composition of TfbI

Chemical	Amount (per 200 ml)	Final conc.
Potassium acetate	0.588 g	30 mM
Rubidium chloride	2.42 g	100 mM
Calcium chloride	0.294 g	10 mM
Manganese chloride	2.0 g	50 mM
Glycerol	30 ml	15% v/v

pH 5.8, adjusted with dilute acetic acid

Composition of TfbII

Chemical	Amount (per 100 ml)	Final conc.
MOPs	0.21 g	10 mM
Calcium chloride	1.1 g	75 mM
Rubidium chloride	0.121 g	10 mM
Glycerol	15 ml	15% v/v

pH 6.5, adjusted with dilute NaOH

Table 1. Classification of Bt toxins[a]

Class	MW (kDa)	Specificity	Crystal shape	Exception
CryI	130-140	Lepidoptera	Bipyramidal	CryIB[b]
CryII	70	Lepidoptera / Diptera	Cuboidal	CryIIB[c]
CryIII	70	Coleopteran	Rhomboidal	CryIIIC[d]
CryIVA, B	130	Diptera	Bipyramidal	
CryIVC, D	70	Diptera	Bar shaped	
Cyt	27	Diptera / Cytolytic	Amorphous	

[a] based on the classification scheme of Hofte and Whiteley (1989)

[b] CryIB family has a member (originally designated CryV) that kills both Lepidoptera and Coleoptera

[c] CryIIB is non-toxic to Diptera

[d] CryIIIC is 130 kDa and has cryptic coleopteran activity (Adapted from Knowles, 1994)

Table 2. Structure function relationship of the three Bt crystal protein domains and their characteristics (Schnepf *et al*., 1998; de Maagd *et al*., 2001)

Domain	Length[a]	Structure	Function
Domain I	~250 N-terminal amino acids	Six hydrophobic and amphipathic α-helices surrounding a central core helix (α5)	Pore formation
Domain II	~central 200 amino acids	Three anti-parallel β-sheets arranged in three dimensional symmetry (β-prism) forming a greek key conformation. Consists of hypervariable regions	Proposed role in receptor binding
Domain III	C-terminal 150 amino acids	β-sheet sandwich or 'jelly-roll'	Proposed roles in maintenance of structural integrity of the toxin, defining specificity, ion channel formation, binding etc.,

[a] The length is only a generalized prediction based on structural studies done on Cry3A (Li *et al*., 1991) and Cry1Aa (Grochulski *et al*., 1995) .

Table 3. Summary of reports on insect resistant transgenic plants harbouring *Bt cry* genes

Crop	Insect	Gene	Protein analysis (expression levels)	Bioassays	Protection levels	Reference
Tobacco	*Manduca sexta* (tobacco hornworm), *Heliothis virescens* (cotton bollworm), *Heliothis zea* (corn earworm), and *Spodoptera exigua* (beet armyworm)	*Bt* gene (native)	Western	Whole plant assays using eggs	Lethal to all larvae in 4 days	Barton *et al.,* 1987
Tomato	*Manduca sexta*	*Btk* (native)	Not done	Whole plant using larvae	50-80% mortality	Fischhoff *et al.,* 1987
Tobacco	*Manduca sexta*	*Bt2* (native)	ELISA (inefficient protein synthesis)	Larvae on whole plant and leaves	100% mortality after 6 days	Vaeck *et al.,* 1987
Cotton	Cabbage looper and beet armyworm	*cry1A(c)*	Western (upto 0.1% of total protein)	Leaf tissue assays on larvae	100% protection	Perlak *et al.,* 1990
Tobacco	-	*cry1A(b)*	ELISA (0.01% of the total soluble protein)	-	-	Carozzi *et al.,* 1992
Potato	Colorado potato beetle	*cryIIIA*	Western (0.025% of the total soluble protein or 25 ng in 100 µg plant protein)	First and third instar larvae	Protection varying from low to high	Adang *et al.,* 1993

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Rice	Striped stem borer (*Chilo suppressalis*) and leaf folder (*Cnaphalocrocis medinalis*)	*cry1A(b)*	0.05% of the total soluble protein	One week old seedling assay using larvae	10-40% mortality	Fujimoto *et al.,* 1993
Potato	Colorado potato beetle (*Leptinotarsa decemlineata*)	*cryIIIA*	Western (0.002-0.3% of total leaf protein)	Whole plant assays using larvae at different life stages	100% mortality	Perlak *et al.,* 1993
Tobacco and Tomato	*Spodoptera exigua, Heliothis virescens* and *Manduca sexta*	*cry1A(b)* and *cry1C* (independently and translationally fused)	Western (data not shown)	Whole plant assay using larvae	100% protection	van der Salm *et al.,* 1994
Potato	Potato tuberworm (*Phthorimaea operculella*) and *Manduca sexta*	*cry1Ab6*	ELISA	Leaf and tuber damage assays using larvae	100% mortality	Jansens *et al.,* 1995
Canola	Cabbage looper (*Trichoplusia ni),* diamond back moth (*Plutella xyostella*), corn earworm (*Helicoverpa zea*) and beet armyworm (*Spodoptera exigua*)	*cry1Ac*	Western	Detached leaf assay using neonates	Complete protection	Stewart *et al.,* 1996a
Soybean	Corn earworm (*Helicoverpa zea*), soybean looper (*Pseudoplusia includens*), tobacco budworm (*Heliothis virescens*) and velvetbean caterpillar (*Anticarsia gemmatalis*)	*cry1Ac*	ELISA (0-46 ng/mg)	Detached leaf insect bioassay	Complete protection	Stewart *et al.,* 1996b

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Alfalfa and Tobacco	Egyptian cotton leafworm (*Spodoptera littoralis*) and beet armyworm (*Spodoptera exigua*)	*cry1C*	Western (alfalfa 0.01-0.1% and tobacco 0.02 – 0.1% of total soluble protein)	Leaf bioassays using larvae at different stages of life cycle	100% mortality of larvae	Strizhov *et al.,* 1996
Rice	Stripped stem borer (*Chilo suppressalis*) and yellow stem borer (*Scirpophaga incertulas*)	*cry1A(b)*	ELISA	Detached leaf and whole plant assays using larvae	Average mortality of 80%	Wunn *et al.,* 1996
Sugarcane	Sugarcane borer (*Diatraea saccharalis*)	*cry1A(b)* (native)	Western and immunoradiometry (higher expression of 27.23 ng/g of foliar tissue)	Whole plant assay using neonates	Low to high resistance to the insects	Arencibia *et al.,* 1997
Eggplant	Colorado potato beetle (*Leptinotarsa decemlineata*)	*cryIIIB*	Western and ELISA (320 ng/ml of the plant extracted protein)	Leaf disc assays using larvae	100% protection	Arpaia *et al.,* 1997
Rice	Striped stem borer (*Chilo suppressalis*) and yellow stem borer (*Scirpophaga insertulas*)	*cry1A(b)*	Not done	Cut stem and whole plant assays using neonate larvae	70-100% mortality	Ghareyazie *et al.,* 1997
Eggplant	Colorado potato beetle (*Leptinotarsa decemlineata*)	*cry3B*	Western, ELISA	Leaf assays using larvae	100% mortality	Iannacone *et al.,* 1997
Chickpea	*Heliothis armigera*	*cry1Ac* (native)	Western, ELISA	Young shoots using larvae	Highly reduced growth of larvae and most parts of the plant were protected	Kar *et al.,* 1997

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Tobacco	Egyptian cotton leafworm (*Spodoptera littoralis*)	*cry1C* (native)	Western	Detached leaf assays using larvae	91-100% mortality	Mazier *et al.*, 1997
Rice	Yellow stem borer (*Scirpophaga insertulas*)	*cry1Ac*	Western blot and ELISA, (0.02-0.025% of the total soluble protein)	Cut stem assay with first instar larvae	75.9 – 92.4% mortality	Nayak *et al.*, 1997
Rice	Yellow stem borer	*cry1A(b)*	Western	Cut stem assays using neonates	Complete protection	Alam *et al.*, 1998
Rice	Striped stem borer (*Chilo suppressalis*) and yellow stem borer (*Scirpophaga incertulas)*	*cry1A cry1Ac* and *cry1A(b)*	Dot blot Cry1Ac (levels between 0.2 and 2% of the soluble protein)	Stem cutting assays using eggs	97-100% mortality after 4-5 days	Cheng *et al.*, 1998
Rice	YSB (*Scirpophaga incertulas*)	*cry1A(b)*	Western (0.01 – 0.2% of the total soluble protein)	Cut stem assays using neonates	100% mortality	Datta *et al.*, 1998
Eggplant	Fruit borer (*Leucinodes orbonalis*)	*cry1A(b)* (codons modified according to monocot bias)	Cry1Ac (0.02% of the total soluble protein)	Fruit infestation on whole plant using larvae	Completely damage free	Kumar *et al.*, 1998
Rice	Yellow stem borer (*Tryporyza incertulas*) and the rice leaf folder (*Cnaphalocrocis medinalis*)	*cry2A*	Western (0.01 – 1% of the total soluble protein)	Detached leaf assays using second instar larvae	60-100% mortality	Maqbool *et al.*, 1998

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Sweet potato	Sweet potato weevil (*C.formicarius*)	*cryIIIA*	Western (no higher than 0.005% of the total soluble protein)	Whole plant assays	Two to five times lower damage than the control	Moran *et al.,* 1998
Tobacco	*Heliothis armigera*	*cryIIa5* (native)	Western (0.06% of the total soluble protein)	Leaf feeding assay using neonates	Complete protection	Selvapandiyan *et al.,* 1998
Rice	YSB (*Scirpophaga incertulas*)	*cry1A(b)*	Protein study not done	Cut stem feeding assays using neonates	100% mortality	Alam *et al.,* 1999
Mediterrranean rice	Striped stem borer	*cry1Ba*	Western blotting (0.01-0.04% of the total soluble protein)	Whole plant assay using larvae	100% mortality of insects after 7 days	Breitler *et al.,* 2000
Potato	*Helicoverpa armigera*	*cry1Ab*	ELISA (0.005 – 0.04% of the total protein)	Detached leaf assays using neonates	Total protection	Chakrabarti *et al.,* 2000
Coffee	Coffee leaf miner (*Perileucoptera coffeella* and other Leucoptera spp.)	*cry1Ac*	Western blotting	Scored on basis of two week larval exposure and pupae formation 1 week later	Highly resistant	Leroy *et al.,* 2000
Brassica campestris ssp. *parachinensis*	Diamond back moth (*Plutella xylostella*)	*cry1A(b)* and *cry1Ac*	Western blotting	Whole plant assay using larvae	100% mortality of insects after 7 days	Xiang *et al.,* 2000

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Tropical maize	Southern western cornborer, sugarcane borer and fall armyworm	*cry1B* and translational fusion *cry1B – cry1A(b)*	Not done	Neonates	High levels of toxicity against the insects	Bohorova *et al.,* 2001
Chinese cabbage	Diamond back moth (*Plutella xylostella*) cabbage worms (*Pieris rapae*) and cabbage loopers (*Trichoplusia ni*)	*cry1C*	Not done	Eggs or freshly hatched larvae on whole plant or leaves	100% protection	Cho *et al.,* 2001
Tobacco, potato, cauliflower and turnip rape	Diamond back moth (*Plutella xylostella*)	*cry9Aa*	Western (0.3% of the total soluble protein)	Leaf feeding assays using second or third instar larvae	40-100% mortality	Kuvshinov *et al.,* 2001
Rice	Rice leaf folder (*Cnaphalocrocis medinalis*), yellow stem borer (*Scirpophaga incertulas*) and brown plant hopper (*Nilapavata lugens*)	*cry1A(c), cry2A* and GNA (co-transformed via particle bombardment)	Western Cry1Ac (0.03-1%) Cry2A (0.01-0.5%) and GNA (0.01-2.5%) of the total soluble protein	Detached leaf assays using 2nd instar larvae	100% mortality	Maqbool *et al.,* 2001
Rice	Yellow stem borer (*Scirpophaga incertulas*)	*cry1Ac*	ELISA, Western (0.5 – 0.2% of total soluble protein)	Cut stem and whole plant assays using neonates	80-100% mortality	Khanna and Raina, 2002
Tomato	Tomato fruit borer (*Helicoverpa armigera*)	*cry1Ab*	Western not done	Whole plant assays using eggs	Damage absent	Kumar and Kumar, 2004

Table 4. Bt crops field trials in India

Organism common name: Gene: Organization: Purpose of release:	Cotton *cry1A(c)* M/s. MAHYCO, Mumbai-joint venture with Monsanto To develop resistance against lepidopteran pests Has been granted approval
Organism common name: Gene: Organization: Purpose of release:	Brinjal *cry1A(b)* M/s. Proagro PGS (India) Ltd., New Delhi To develop plants resistant to lepidopteran pests
Organism common name: Gene: Organization: Purpose of release:	Brinjal Bt gene Indian Agricultural Research Institute, New Delhi To impart lepidopteran pest resistance.
Organism common name: Gene: Organization: Purpose of release:	Cabbage **cry1H / cry9C** M/s. Proagro PGS (India) Ltd., New Delhi To develop resistance to pests.
Organism common name: Gene: Organization: Purpose of release:	Cauliflower *cry1H / cry9C* M/s. Proagro PGS (India) Ltd., New Delhi To develop resistance to pests
Organism common name: Gene: Organization: Purpose of release:	Cauliflower Bt gene Indian Agricultural Research Institute, New Delhi To impart lepidopteran pest resistance.

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Organism common name: Gene: Organization: Purpose of release:	Potato Bt toxin gene Central Potato Research Institute, Simla To generate plant resistant to lepidopteran pests
Organism common name: Gene: Organization: Purpose of release:	Rice Bt toxin gene Bose Institute, Calcutta To generate plants resistant to lepidopteran pests
Organism common name: Gene: Organization: Purpose of release:	Rice Bt toxin gene Indian Agricultural Research Institute Sub-station at Shillong To impart lepidopteran resistance
Organism common name: Gene: Organisation: Purpose of release:	Tobacco Bt toxin gene *cry1A(b)* and *cry1C* Central Tobacco Research Institute, Rajahmindri To generate plants resistant to *H.armigera* and *S.litura*
Organism common name: Gene: Organization: Purpose of release:	Tomato *cry1A(b)* M/s. Proagro PGS (India) Ltd., New Delhi. To develop plants resistant to lepidopteran pests.
Organism common name: Gene: Organization: Purpose of release:	Tomato Bt gene Indian Agricultural Research Institute, New Delhi To impart lepidopteran pest resistance.

Source : Adapted from the Biosafety Information Network and Advisory Service (BINAS), United Nations Industrial Development Organization (UNIDO), and Biotech Consortium India Ltd., Biosafety Issues Related to Genetically Modified Organisms (Sept. 2002).

Table 5. Source of plasmids and bacterial strains

Sl No	Plasmid/Strain	Antibiotic markers	Application	Source
1.	pS2Ac	Ampicillin	For synthetic *cry2Ac* gene	Dr. V. Udayasuriyan, TNAU, Coimbatore
2.	pRT103	Ampicillin	For CaMV 35S promoter and poly A sequence	Dr. Savithri, IISC, Bangalore.
3.	pCAMBIA2300	Kanamycin	Binary vector	Dr. Anil Grover, University of Delhi, South Campus, Delhi.
4.	pUH	Kanamycin	For *ubiquitin*I promoter	Dr. Anil Grover, University of Delhi, South Campus, Delhi.
5.	*A. tumefaciens* EHA105	Rifampicin	For plant transformation	Dr. Anil Grover, University of Delhi, South Campus, Delhi.
6.	*E. coli* strain DH5α	-	For maintaining plasmid constructs	Bangalore Genei, Bangalore.

Table 6. Temperature profile for PCR amplification of synthetic *cry2Ac* gene fragment

Sl. No.	Step	Temperature	Time
1.	Initial denaturation	94°C	2 minutes
2.	Denaturation	94°C	40 seconds
3.	Annealing	55°C	40 seconds
4.	Primer extension	72°C	1 minute
5.	Step 2 to 4	30 cycles	
6.	Final extension	72°C	5 minutes

Table 7. Primer sequences for amplification of fragments of synthetic *cry2Ac* gene

Sl. No.	Primer name	Sequence (5'→3')	Size of the product (bp)
1.	S2F1	ATGAACACCGTGCTCAACAAC	800
2.	S2R1	TGGTACTTGAAGAGGGACCAG	
3.	S2F3	CTCCGGCGTGGTGGGCAC	600
4.	S2R3	AGAGTGGCGGGAGGTTGGTC	

Table 8. Screening of putative transgenic tobacco plants for presence of synthetic *cry2Ac* gene by PCR

Sl.No	*Putative transformants*	Amplification for *cry2Ac* gene
1	NT1	-
2	NT2	+
3	NT3	+
4	NT4	+
5	NT6	+
6	MT4a	-
7	MT8a	+
8	MT8b	+
9	MT11a	+
10	MT11b	+
11	MT12a	-
12	MT14b	+

Table 9. Summary of two co-cultivation experiments of tobacco with *A. tumefaciens* harbouring p2AP1 plasmid containing synthetic *cry2Ac* gene

Batch No.	Explants used	Individual shoots obtained from SM	Plantlets established in RM	Putative transgenic tobacco established in pot culture	Transgenic tobacco plants showing presence of *cry2Ac* by PCR
I.	14	13	7	5	4
II.	14	11	8	7	5

Table 10. Comparative study of Cry2Ac, Cry2Ab and Cry1Ac proteins with Cry2Ac polyclonal antibody

Protein conc (ng)	Cry2Ac		Cry2Ab		Cry1Ac	
	OD value	OD value-blank	OD value	OD value-blank	OD value	OD value-blank
Blank	0.160	-	0.160	-	0.160	-
0.5	0 .270	0.110	NT	NT	NT	NT
1.0	0.472	0.312	0.110	-	0.112	-
1.5	0.674	0.514	NT	NT	NT	NT
2.0	0.889	0.729	0.129	-	0.187	0.027
2.5	1.128	0.968	NT	NT	NT	NT
3.0	1.314	1.154	0.134	-	0.193	0.033
3.5	1.438	1.278	NT	NT	NT	NT
4.0	1.663	1.503	0.164	0.004	0.203	0.043
5.0	NT	NT	0.200	0.040	0.212	0.052
6.0	NT	NT	0.203	0.043	0.283	0.123
7.0	NT	NT	0.210	0.050	0.294	0.134
10.0	NT	NT	0.218	0.058	0.295	0.135

* NT : Not tested

Table 11. Total protein concentration of transgenic and control tobacco plant leaf extracts (I Batch)

Sl.No	Plant Sample	Vegetative stage	Flowering stage
		Protein conc (µg/µl)	Protein conc (µg/µl)
1	Transgenic NT2	10.0	8.0
2	Transgenic NT3	9.5	9.2
3	Transgenic NT4	9.3	8.6
4	Transgenic NT6	8.0	9.4
5	Control NC3	8.8	9.0
6	Control NC4	8.6	9.3

Table 12. Total protein concentration of transgenic and control tobacco plant leaf extracts (II Batch)

Sl.No	Plant Sample	Vegetative stage	Flowering stage
		Protein conc (µg/µl)	Protein conc (µg/µl)
1	Transgenic MT8a	10.7	9.8
2	Transgenic MT8b	9.7	8.6
3	Transgenic MT11a	8.9	8.8
4	Transgenic MT11b	8.5	8.1
5	Transgenic MT14b	8.0	7.9
6	Control MC2	9.8	9.4
7	Control MC3	9.4	9.2

Table 13. Screening of I batch of transgenic tobacco plants by ELISA for expression of synthetic *cry2Ac* gene

Sl No	Sample	Conc of protein /well (ng or µg)*	OD value	OD value - Blank	Transgenic plant OD value – Control plant OD value	Equivalent conc. of Cry protein in ng (from graph)	Per cent of Cry2Ac in total soluble protein of plant
1	Cry2Ac	0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0	0.252 0.463 0.661 0.883 1.124 1.319 1.433 1.650	0.112 0.323 0.521 0.743 0.984 1.179 1.293 1.510			
2	Blank		0.140				
3	Transgenic NT2 (F)	0.5 1.5 4.5	0.300 0.791 1.621	0.160 0.651 1.481	0.160 0.629 1.416	0.5 1.95 4.35	0.1 0.13 0.097 **Mean 0.109 ± 0.018**
4	Transgenic NT3 (F)	0.5 1.5 4.5	0.183 0.311 0.617	0.043 0.171 0.477	0.043 0.149 0.412	0.175 0.450 1.260	0.035 0.030 0.028 **Mean 0.031 ± 0.004**
5	Transgenic NT4 (F)	0.5 1.5 4.5	0.113 0.229 0.477	- 0.089 0.337	- 0.067 0.272	- 0.33 0.81	- 0.022 0.018 **Mean 0.020 ± 0.003**
6	Transgenic NT6 (F)	0.5 1.5 4.5	0.252 0.470 1.280	0.112 0.330 1.140	0.112 0.308 1.075	0.340 0.900 3.330	0.068 0.060 0.074 **Mean 0.067 ± 0.007**
7	Control NC3 (F)	0.5 1.5 4.5	0.105 0.156 0.196	- 0.016 0.056	- - -	- - -	- - -
8	Control NC4 (F)	0.5 1.5 4.5	0.123 0.167 0.214	- 0.027 0.074	- - -	- - -	- - -
9	Control NC (F)[1]	0.5 1.5 4.5	0.114 0.162 0.205	- 0.022 0.065	- - -	- - -	- - -

* ng for Cry2Ac samples and µg for plant protein samples

Table 14. Screening of higher concentrations of leaf protein samples of I batch of transgenic tobacco plants by ELISA for expression of synthetic *cry2Ac* gene

Sl No	Sample	Conc of protein /well (ng or µg)*	OD value	OD value - Blank	Transgenic plant OD value - Control plant OD value	Equivalent conc. of Cry protein in ng (from graph)	Per cent of Cry2Ac in total soluble protein of plant
1	Cry2Ac	0.5	0.253	0.08			
		1.0	0.494	0.321			
		1.5	0.656	0.483			
		2.0	0.794	0.621			
		2.5	0.915	0.742			
		3.0	1.053	0.880			
		3.5	1.283	1.110			
		4.0	1.413	1.240			
2	Blank		0.173				
3	Transgenic NT2 (F)	0.5	0.383	0.210	0.210	0.70	0.140
		1.5	0.819	0.646	0.646	2.05	0.135
							Mean 0.138 ± 0.004
4	Transgenic NT3 (F)	6.0	0.495	0.322	0.280	0.90	0.015
		12.0	1.515	1.342	1.256	4.20	0.035
		18.0	1.929	1.756	1.683	5.40	0.030
							Mean 0.027 ± 0.010
5	Transgenic NT4 (F)	6.0	0.425	0.252	0.210	0.72	0.012
		12.0	0.929	0.756	0.670	2.16	0.018
		18.0	1.539	1.366	1.293	4.14	0.023
							Mean 0.018 ± 0.006
6	Transgenic NT6 (F)	1.5	0.529	0.356	0.356	1.08	0.072
		3.0	0.809	0.636	0.670	1.98	0.066
							Mean 0.069 ± 0.004
7	Control NC4 (F)	0.5	0.152	-0.021	-	-	-
		1.5	0.189	-0.006	-	-	-
		3.0	0.189	0.016	-	-	-
		6.0	0.215	0.042	-	-	-
		12.0	0.259	0.086	-	-	-
		18.0	0.246	0.073	-	-	-

* ng for Cry2Ac samples and µg for plant protein samples

Table 15. Screening of II batch of transgenic tobacco plants by ELISA for expression of synthetic *cry2Ac* gene at vegetative stage

Sl No	Sample	Conc of protein /well (ng or µg)*	OD value	OD value - Blank	Transgenic plant OD value - Control plant OD value	Equivalent conc. of Cry protein in ng (from graph)	Per cent of Cry2Ac in total soluble protein of plant
1	Cry2Ac	0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0	0.250 0.490 0.637 0.785 0.918 1.042 1.250 1.440	0.066 0.306 0.453 0.601 0.734 0.858 1.066 1.256			
2	Blank		0.184				
3	Transgenic MT8a (V)	3 9	0.314 0.768	0.130 0.584	0.105 0.430	0.4 1.4	0.013 0.016 *Mean 0.015 ± 0.002*
4	Transgenic MT8b (V)	3 9	0.393 0.787	0.209 0.603	0.184 0.449	0.65 1.50	0.021 0.017 *Mean 0.019 ± 0.003*
5	Transgenic MT11a (V)	3 9	0.134 0.386	- 0.202	- 0.048	- 0.005	- -
6	Transgenic MT11b (V)	3 9	0.156 0.364	- 0.180	- 0.026	- -	- -
7	Transgenic MT14b (V)	3 9	0.514 1.113	0.33 0.929	0.305 0.775	1.0 2.5	0.033 0.028 *Mean 0.030 ± 0.004*
8	Control MC2 (V)	3 9	0.198 0.324	0.014 0.140	- -	- -	- -
9	Control MC3 (V)	3 9	0.219 0.352	0.035 0.168	- -	- -	- -
10	Control MC (V)[1]	3 9	0.209 0.338	0.025 0.154	- -	- -	- -

* ng for Cry2Ac samples and µg for plant protein samples

[1] MC(V) – Average of MC2(V) and MC3(V)

Table 16. Screening of II batch of transgenic tobacco plants by ELISA for expression of synthetic *cry2Ac* gene at flowering stage

Sl No	Sample	Conc of protein /well (ng or µg)*	OD value	OD value - Blank	Transgenic plant OD value - Control plant OD value	Equivalent conc. of Cry protein in ng (from graph)	Per cent of Cry2Ac in total soluble protein of plant
1	Cry2Ac	0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0	0.250 0.490 0.637 0.785 0.918 1.042 1.250 1.440	0.066 0.306 0.453 0.601 0.734 0.858 1.066 1.256			
2	Blank	-	0.184				
3	Transgenic MT8a (F)	3 9	0.362 0.829	0.178 0.645	0.139 0.478	0.450 1.60	0.015 0.018 *Mean 0.016 ± 0.002*
4	Transgenic MT8b (F)	3 9	0.423 0.842	0.239 0.658	0.200 0.491	0.70 1.65	0.023 0.018 *Mean 0.020 ± 0.004*
5	Transgenic MT11a (F)	3 9	0.145 0.471	- 0.287	- 0.120	- 0.30	- 0.003 *Mean 0.0030*
6	Transgenic MT11b (F)	3 9	0.167 0.450	- 0.266	- 0.099	- 0.25	- 0.0028 *Mean 0.0028*
7	Transgenic MT14b (F)	3 9	0.596 1.296	0.412 1.112	0.360 0.945	1.20 3.00	0.040 0.028 *Mean 0.034 ± 0.008*
8	Control MC2 (F)	3 9	0.209 0.335	0.025 0.151	- -	- -	- -
9	Control MC3 (F)	3 9	0.236 0.366	0.052 0.182	- -	- -	- -
10	Control MC (F)[1]	3 9	0.223 0.351	0.039 0.167	- -	- -	- -

* ng for Cry2Ac samples and µg for plant protein samples

[1] MC(F) – Average of MC2(F) and MC3(F)

Table 17. Summary of ELISA results for transgenic tobacco plants expressing synthetic *cry2Ac* gene

Sl. No	*Transgenic plant*	Per cent of Cry2Ac in total soluble protein of leaf	
		Vegetative stage (24 DAP)	**Flowering stage (33 DAP)**
1	NT2	NT	0.138
2	NT3	NT	0.027
3	NT4	NT	0.018
4	NT6	NT	0.069
5	MT8a	0.015	0.016
6	MT8b	0.019	0.020
7	MT11a	ND	0.003
8	MT11b	ND	0.0028
9	MT14b	0.030	0.034

NT : Not Tested

ND : Not Detected

Table 18. Mortality of *Helicoverpa armigera* larvae fed with transgenic tobacco plant leaves expressing synthetic *cry2Ac* gene

S. No	Plants tested	Larval mortality (%)		
		Day 3	Day 4	Day 5
1	Transgenic NT2	20 20 20	40 30 40	80 80 70
	Mean	**20.0 ± 0**	**36.7 ± 5.8**	**76.7 ± 5.8**
2	Transgenic NT3	20 10 10	20 20 20	30 20 30
	Mean	**13.3 ± 5.8**	**20.0 ± 0**	**26.7 ± 5.8**
3	Transgenic NT4	0 10 10	10 20 30	30 40 40
	Mean	**6.7 ± 5.8**	**20.0 ± 10**	**36.7 ± 5.8**
4	Transgenic NT6	10 10 10	60 50 60	70 70 60
	Mean	**10.0 ± 0**	**56.7 ± 5.8**	**66.7 ± 5.8**
5	Control NC3	0 0 0	0 0 0	0 0 0
	Mean	**0 ± 0**	**0 ± 0**	**0 ± 0**
6	Control NC4	0 0 0	0 0 0	10 0 0
	Mean	**0 ± 0**	**0 ± 0**	**3.3 ± 5.8**

Table 19. Leaf area damaged by *H. armigera* larvae in the control and the transgenic tobacco plants expressing synthetic *cry2Ac* gene

S. No	Plants tested	Leaf area damaged (mm^2)		
		Day 3	**Day 4**	**Day 5**
1	Transgenic NT2	74.4 80.3 65.2	97.8 116.4 90.0	133.5 152.6 125.8
	Mean	**73.3 ± 6.2**	**101.4 ± 11.1**	**137.3 ± 11.3**
2	Transgenic NT3	99.5 100.4 81.2	141.8 158.2 129.3	218.2 229.6 192.4
	Mean	**93.7 ± 9.7**	**143.1 ± 11.8**	**213.4± 5.6**
3	Transgenic NT4	95.4 118.6 112.4	140.2 169.5 155.6	210.3 249.9 236.9
	Mean	**108.8 ± 9.8**	**155.1± 12**	**232.2 ± 16.4**
4	Transgenic NT6	60.4 74.0 71.4	86.8 112.0 109.6	140.4 169.2 160.8
	Mean	**68.6 ± 5.9**	**102.8 ± 11.4**	**156.8 ± 12.1**
5	Control NC3	162.8 199.2 187.0	250.2 305.7 285.0	406.7 468.3 438.1
	Mean	**183 ± 15.1**	**280.3 ± 23**	**437.7± 25.2**
6	Control NC4	196.8 223.6 181.4	310.4 333.2 287.9	484.8 497.7 446.4
	Mean	**200.6 ± 17.4**	**310.5 ±18.5**	**476.3 ± 21.8**

Table 20. Reduction in leaf area damaged by *H. armigera* larvae in transgenic tobacco plants expressing synthetic *cry2Ac* gene

S. No	Transgenics tested	Per cent reduction in leaf area damaged over control		
		Day 3	Day 4	Day 5
1	NT2	62.2 64.1 64.1	68.5 65.1 68.7	72.5 69.3 71.3
	Mean	**63.5 ± 1.1**	**67.4 ± 2.03**	**71.0 ± 1.62**
2	NT3	49.4 55.1 55.2	54.3 52.5 55.1	55.0 53.9 56.8
	Mean	**53.2 ± 3.32**	**54.0 ± 1.33**	**55.3 ± 1.52**
3	NT4	41.4 40.5 39.9	44.0 44.6 45.4	48.3 46.6 45.9
	Mean	**40.6 ± 0.75**	**44.7 ± 0.57**	**46.9 ± 1.23**
4	NT6	62.9 62.9 61.8	65.3 63.4 61.5	65.5 63.9 63.1
	Mean	**62.5 ± 0.64**	**63.4 ± 1.90**	**64.2 ± 1.22**

Table 21. Total protein concentration of control and transgenic tobacco plants expressing native *cry2Ac* gene

Sl.No	Plant Sample	Protein conc (µg/µl)
1	Transgenic D14-2	8.5
2	Transgenic D14-3	8.2
3	Control DC1	7.0
4	Control DC2	7.5

Table 22. Screening of transgenic tobacco plants by ELISA for expression of native *cry2Ac* gene

Sl No	Sample	Conc of protein /well (ng or µg)*	OD value	OD value - Blank	Transgenic plant OD value - Control plant OD value	Equivalent conc. of Cry protein in ng (from graph)	Per cent of Cry2Ac protein in total soluble protein of plant
1	Cry2Ac	0.5	0.250	0.066			
		1.0	0.490	0.306			
		1.5	0.637	0.453			
		2.0	0.785	0.601			
		2.5	0.918	0.734			
		3.0	1.042	0.858			
		3.5	1.250	1.066			
		4.0	1.440	1.256			
		4.5	1.816	1.632			
		5.0	1.866	1.682			
2	Blank		0.184				
3	Transgenic D14-2 (F)	0.5	0.368	0.184	0.153	0.48	0.096
		1.0	0.731	0.547	0.400	1.35	0.135
							Mean 0.120 ± 0.028
4	Transgenic D14-3 (F)	0.5	0.570	0.386	0.355	1.62	0.320
		1.0	1.321	1.137	0.990	3.3	0.33
							Mean 0.33 ± 0.007
5	Control DC1 (F)	0.5	0.206	0.022	-	-	-
		1.0	0.317	0.133	-	-	-
6	Control DC2 (F)	0.5	0.224	0.04	-	-	-
		1.0	0.345	0.161	-	-	-
7	Control DC (F)[1]	0.5	0.215	0.031	-	-	-
		1.0	0.331	0.147	-	-	-

* ng for Cry2Ac samples and µg for plant protein samples

[1] DC(F) – Average of DC1(F) and DC2(F)

Table 23. Mortality of *H. armigera* larvae fed with transgenic tobacco plant leaves expressing native *cry2Ac* gene

S. No	Plants tested	Larval mortality (%)			
		Day 3	**Day 4**	**Day 5**	**Day 6**
1	Transgenic D14-2	0 10 10	60.0 50.0 50.0	70.0 60.0 60.0	80.0 80.0 70.0
	Mean	**6.70 ± 5.80**	**53.3 ± 5.8**	**63.3 ± 5.8**	**76.7 ± 5.8**
2	Transgenic D14-3	0 10 20	50.0 60.0 50.0	60.0 70.0 70.0	90.0 80.0 90.0
	Mean	**10.0 ± 10**	**53.3 ± 5.8**	**66.7 ± 5.8**	**86.7 ± 5.8**
3	Control DC1	0 0 0	0 0 0	0 0 0	10.0 0 0
	Mean	**0.0 ± 0**	**0.0 ± 0**	**0.0 ± 0**	**3.30 ± 5.8**
4	Control DC2	0 0 0	0 0 0	0 0 0	0 10.0 0
	Mean	**0.0 ± 0**	**0.0 ± 0**	**0.0 ± 0**	**3.30 ± 5.8**

Table 24. Leaf area damaged by *H. armigera* larvae in the control and the transgenic tobacco plants expressing native *cry2Ac* gene

S. No	Plants tested	Leaf area damaged (mm^2)			
		Day 3	**Day 4**	**Day 5**	**Day 6**
1	Transgenic D14-2	32.8 33.5 27.1	46.8 50.1 37.8	53.8 58.2 44.6	59.1 62.2 47.6
	Mean	**31.8 ± 2.9**	**44.9 ± 5.2**	**52.2 ± 5.7**	**56. 3 ± 6.3**
2	Transgenic D14-3	31.4 27.2 23.6	39.1 33.2 30.6	40.2 35.1 30.9	45.5 41.4 33.7
	Mean	**27.4 ± 3.2**	**34.3 ± 3.6**	**35.4 ± 3.8**	**40.2 ± 4.9**
3	Control DC1	60.0 68.6 55.3	90.3 104.0 82.9	139.4 148.8 124.0	192.9 219.4 179.3
	Mean	**61.3 ± 5.5**	**92.4 ± 8.7**	**137.4 ± 10.2**	**197.2 ± 16.7**
4	Control DC2	71.1 60.7 58.4	108.4 93.5 89.1	159.6 142.0 133.4	226.2 217.4 180.0
	Mean	**63.4 ± 5.5**	**97.0 ± 8.3**	**145 ± 10.9**	**208.1 ± 19.7**

Table 25. Reduction in the leaf area damaged by *H. armigera* larvae in the transgenic tobacco plants expressing native *cry2Ac* gene

S. No	Transgenics tested	Per cent reduction in leaf area damaged over control			
		Day 3	**Day 4**	**Day 5**	**Day 6**
1	D14-2	45.3 51.2 51.0	48.2 51.8 54.4	61.4 60.9 64.0	69.4 71.6 73.5
	Mean	**49.2 ± 3.35**	**52.7 ± 3.11**	**62.1 ± 1.66**	**71.5 ± 2.05**
2	D14-3	55.8 55.2 59.6	63.9 64.5 65.7	74.8 75.3 76.8	79.9 81.0 81.4
	Mean	**56.9 ± 2.39**	**64.7 ± 0.92**	**75.6 ± 1.04**	**80.8 ± 0.78**

Plate 1	**Agarose gel electrophoresis of p2AP1**
Lane 1.	Marker (λDNA / *Hin*dIII)
Lane 2 & 3.	p2AP1 / *Pst*I
Lane 4.	p2AP1 undigested

Plate 2.	**Screening of transformants of *A.tumefaciens* by PCR with primer specific for *cry2Ac***
Lane 1 to 3.	Transformants of *A. tumefaciens*
Lane 4.	100 bp marker
Lane 5.	Positive control (p2AP1 plasmid)

Plate 3	**Sensitivity of tobacco shoot regeneration to kanamycin**
1.	Control
2 to 4.	Kanamycin at 50, 100 &150 mg/l

Plate 4	**Sensitivity of control and *Agrobacterium* infected tobacco leaf discs to kanamycin & cefotaxime**
1.	Control : without kanamycin & cefotaxime
2.	Control : with kanamycin 100 mg/l and cefotaxime 300 mg/l
3.	Control : with kanamycin 50 mg/l and cefotaxime 500 mg/l
4.	Infected : with kanamycin 100 mg/l and cefotaxime 300 mg/l
5.	Infected : with kanamycin 150 mg/l and cefotaxime 300 mg/l
6.	Infected : with kanamycin 150 mg/l and cefotaxime 500 mg/l

Plate 5 Regeneration of tobacco in shooting medium

A. Second subculture of tobacco transformants in shooting medium

B. Third subculture of tobacco transformants in shooting medium

Plate 6 Establishment of transgenic tobacco in rooting medium

Plate 7 Control and transgenic tobacco plants in green house

Plate 8	**Screening of tobacco transformants by PCR using S2F1 and S2R1 primers**
Lane 1.	Untransformed tobacco plant
Lane 2 to 7.	Putative transformants of tobacco
Lane 8.	100 bp marker
Lane 9 & 10.	Positive control
Lane 12	Negative control

Plate 9	**Screening of putative tobacco transformants by PCR with two sets of primers specific for synthetic *cry2Ac* gene**
Lane 1 & 2.	Amplicon of 800 bp from transgenic plants NT2, NT6 by S2F1 and S2R1 primers
Lane 3.	Control plant screened with S2F1 and S2R1 primers
Lane 4.	100 bp marker
Lane 5.	Negative control
Lane 6.	Control plant screened with S2F3 and S2R3 primers
Lane 7 & 8.	Amplicon of 600 bp from transgenic plants NT2, NT6 by S2F3 and S2R3 primers

Plate 10a.	**SDS-PAGE of Cry2Ab and Cry2Ac proteins**
Lane 1.	Protein molecular weight marker
Lane 2 & 3.	Solubilized Cry2Ab protein
Lane 4 & 5.	Solubilized Cry2Ac protein

Plate 10b.	**SDS-PAGE of Cry1Ac protein**
Lane 1.	Protein molecular weight marker
Lane 2.	BSA
Lane 3 & 4.	Solubilized Cry1Ac protein

Plate 11.	**SDS-PAGE of tobacco plant leaf protein extracted by TBA buffer**
Lane 1.	Protein molecular weight marker
Lane 2 & 3.	Control plants' (NC3, NC4) extracts - 15µl per well
Lane 4 to 7.	Transgenic plants' (NT2, NT3, NT4, NT5, NT6) extracts - 15µl per well

Plate 12.	**SDS-PAGE of tobacco plant leaf protein extract by PBST buffer**
Lane 1.	Protein molecular weight marker
Lane 2 & 3.	Control plants' (NC3, NC4) extracts - 15µl per well
Lane 4 to 7.	Transgenic plants' (NT2, NT3, NT4, NT5, NT6) extracts - 15µl per well

Plate 13.	***Helicoverpa armigera* bioassay of tobacco leaf bits**
A.	Transgenic tobacco plant leaf bit
B.	Control tobacco plant leaf bit

Plate 14.	**Agarose gel electrophoresis of tobacco plant DNA**
Lane 1.	Control plant, NC3 (5μg)
Lane 2.	Transgenic plant, NT2 (5μg)
Lane 3.	Control plant, NC3 (20μg)
Lane 4.	Transgenic plant, NT2 (20μg)
Lane 5 & 6.	Marker (λDNA / *Hin*dIII)

Plate 15.	**Agarose gel electrophoresis of tobacco plant DNA digested by *Bam*HI**
Lane 1.	Aliquot from 10μg of NC3 DNA digested by *Bam*HI
Lane 2.	Aliquot from 5μg of NC3 DNA digested by *Bam*HI
Lane 3.	Aliquot from 10μg of NT2 DNA digested by *Bam*HI
Lane 4.	Aliquot from 5μg of NT2 DNA digested by *Bam*HI
Lane 5.	Marker (λDNA / *Hin*dIII)

Plate 16.	**Southern blot analysis of transgenic tobacco plant DNA digested with *Bam*HI**
Lane 1.	NT2 DNA sample digested with *Bam*HI
Lane 2.	NC3 DNA sample digested with *Bam*HI
Lane 3.	p2AP1 digested with *Bam*HI

Plate 17.	**Tobacco plant RNA resolved in denaturing agarose gel**
Lane 1.	Transgenic plant, NT2 RNA (3μl)
Lane 2.	Control plant, NC3 RNA (3μl)
Lane 3.	Transgenic plant, NT2 RNA (15μl)
Lane 4.	Control plant, NC3 RNA (15μl)

Plate 18.	**Northern blot analysis of transgenic tobacco plant**
Lane 1.	Control Plant (NC3) RNA
Lane 2.	Transgenic plant (NT2) RNA

Plate 19. **Agarose gel electrophoresis of pUH vector**

Lane 1. Undigested pUH vector

Lane 2. Marker (λ DNA / *Hin*dIII)

Lane 3. pUH vector digested by *Bam*HI

Plate 20. **Agarose gel electrophoresis pS2Ac**

Lane 1. Undigested pS2Ac

Lane 2. Marker (λ DNA / *Hin*dIII)

Lane 3. pS2Ac digested by *Bam*HI

Plate 21.	**Screening of putative recombinant pUH clones by PCR with S2F1 and S2R1 primers**
Lane 1.	100 bp marker
Lane 2.	pUH
Lane 3.	Positive control (pS2Ac)
Lane 4.	p2P3A
Lane 5.	p2P3B
Lane 6.	Negative control

Plate 22.	**Restriction digestion of p2P3A and p2P3B by *Bam*HI**
Lane 1.	Marker (λ DNA / *Hin*dIII)
Lane 2.	p2P3A / *Bam*HI
Lane 3.	p2P3B / *Bam*HI

Plate 23.	**Restriction digestion of p2P3A by *Xho*I**
Lane 1.	Digestion of pUH with *Xho*I
Lane 2.	Marker (λ DNA / *Hin*dIII)
Lane 3.	Digestion of p2P3A with *Xho*I

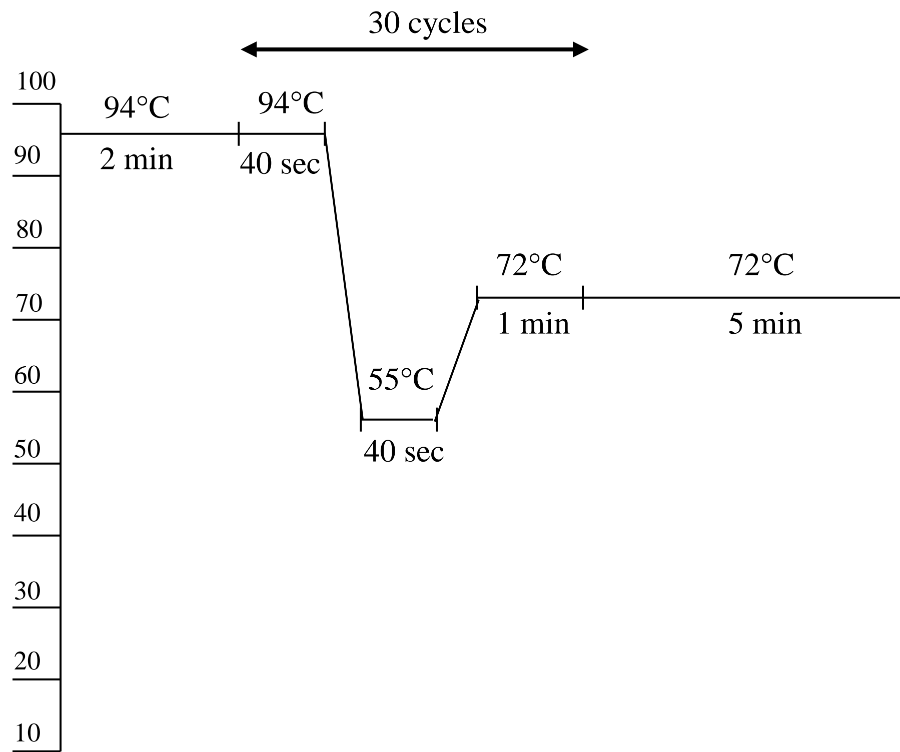


Fig.6. PCR cycle program for amplification of fragments of synthetic *cry2Ac* gene

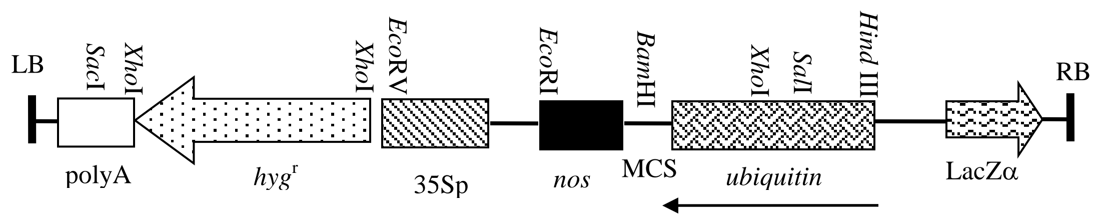


Fig.3. Diagrammatic representation of the T-DNA region of pUH vector

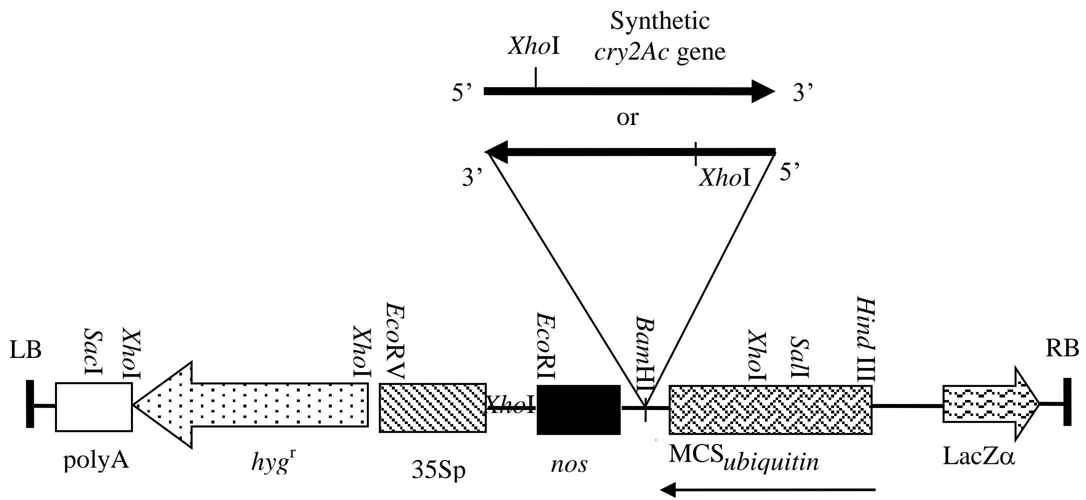


Fig.10. Diagrammatic representation of the T-DNA region of recombinant pUH vector, P2P3

In vitro grown tobacco plants

15 to 20 leaf discs each of 5mm^2 pre incubated in non-selective shoot regeneration medium

2 days

The above leaf discs were infected with *A.tumefaciens* (strain EHA105 harbouring p2AP1)

15 min at 28°C

Blot dried leaf discs were transferred to same non-selective shoot regeneration media

2 days

Transferred to selection media containing kanamycin 100 mg/l and cefotaxime 300 mg/l

30 days

First stage callus from the leaf discs were transferred to bottles containing SM with kanamycin 100 mg/l, cefotaxime 300 mg/l

20 days

Regenerated shoot tips transferred to bottles (3 per bottle) containing SM with kanamycin 100 mg/l, cefotaxime 300 mg/l

15-20 days

Regenerated shoot tips transferred to bottles (2 per bottle) containing SM with kanamycin100 mg/l, cefotaxime 300 mg/l

20 days

Individual shoot tips > 1.5 to 2 cm transferred one per bottle in RM containing kanamycin 50 mg/l, cefotaxime 250 mg/l

1.5 to 2 cm long shoot tips from 2 nodes of established plantlets transferred to RM

The basal portion of the plantlets transferred to pots in green house.

Fig.5. Flow chart depicting transformation and regeneration protocol for tobacco leaf explants

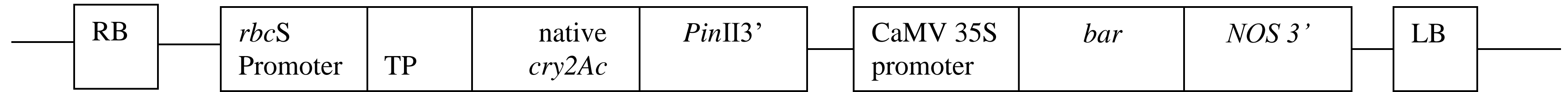


Fig.7. Physical map of the plasmid (p2AcP1) carrying the native *cry2Ac* gene

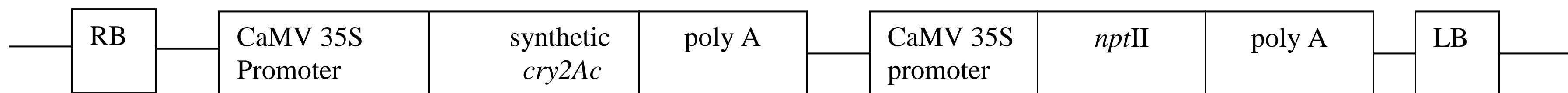
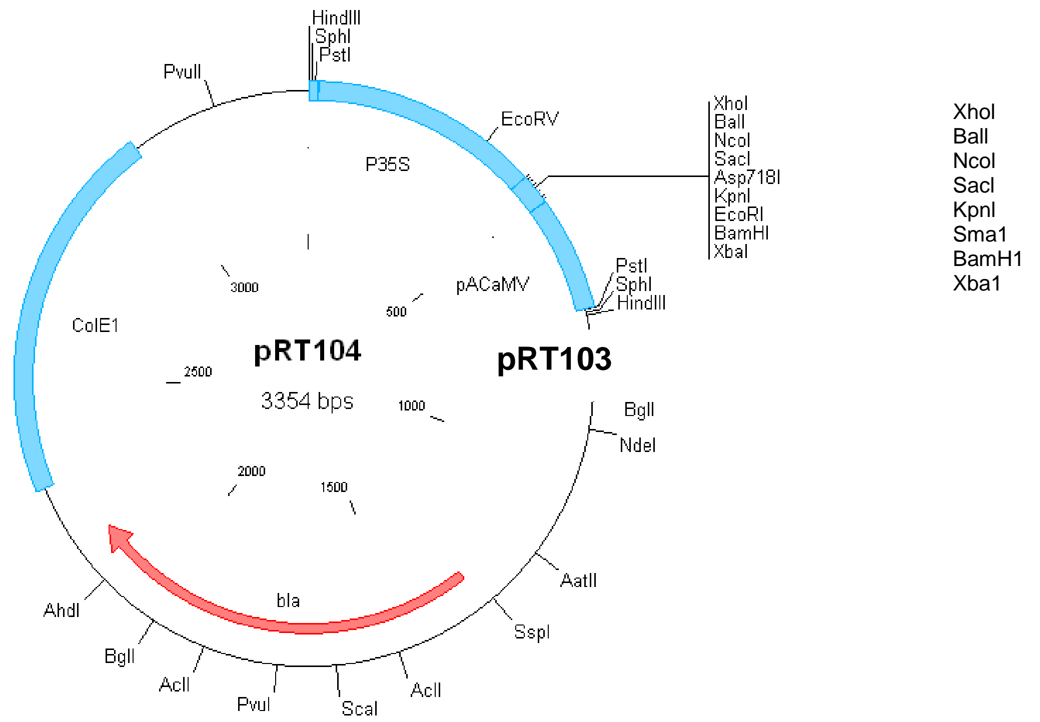


Fig.4. Physical map of the plasmid (p2AP1) carrying the synthetic *cry2Ac* gene



(Source: Topfer *et al*., 1987)

Fig. 1. Vector map of pRT103

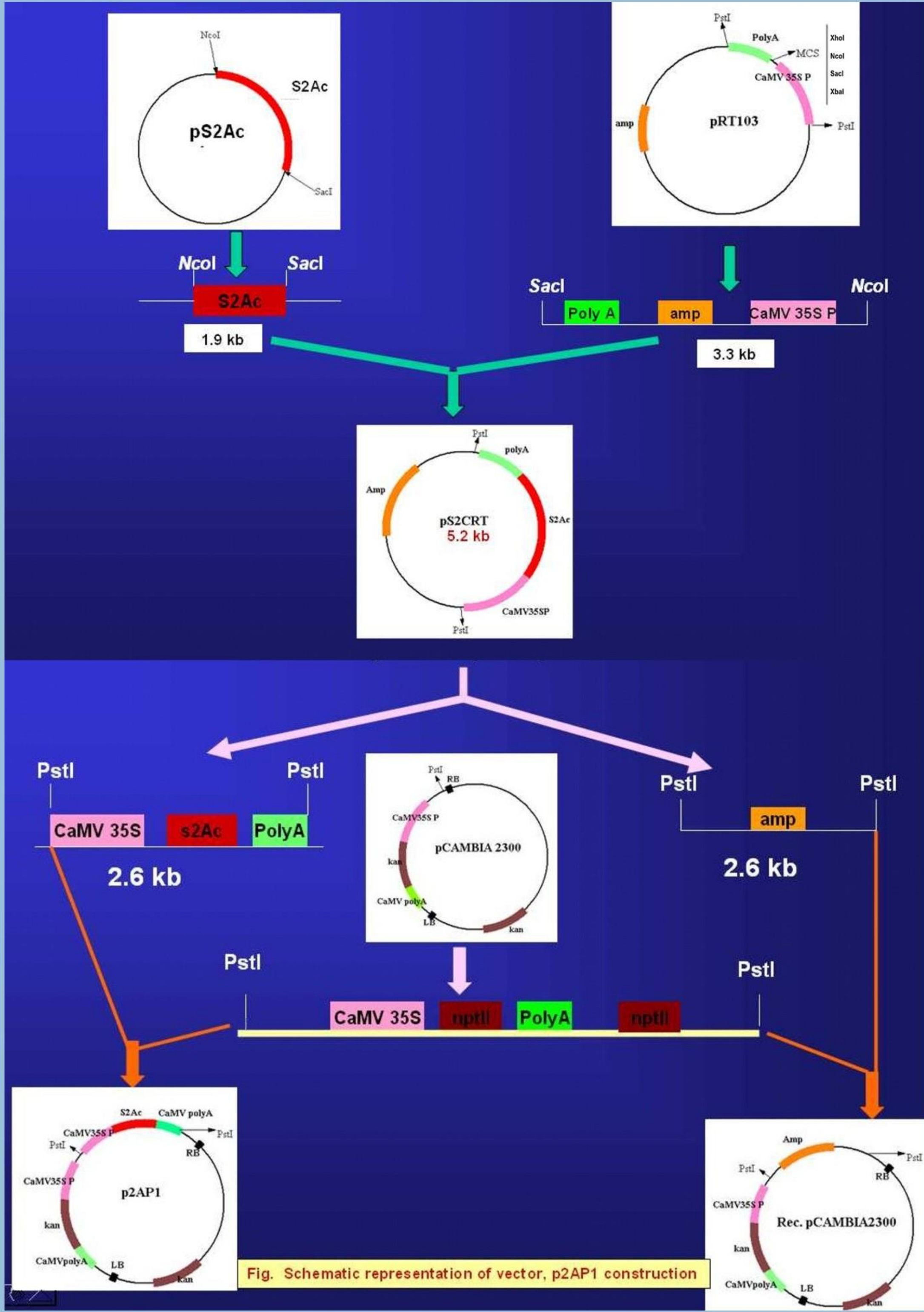


Fig. Schematic representation of vector, p2AP1 construction

Fig.8. Effect of Cry protein concentration in transgenic tobacco plants on mortality of *H. armigera*

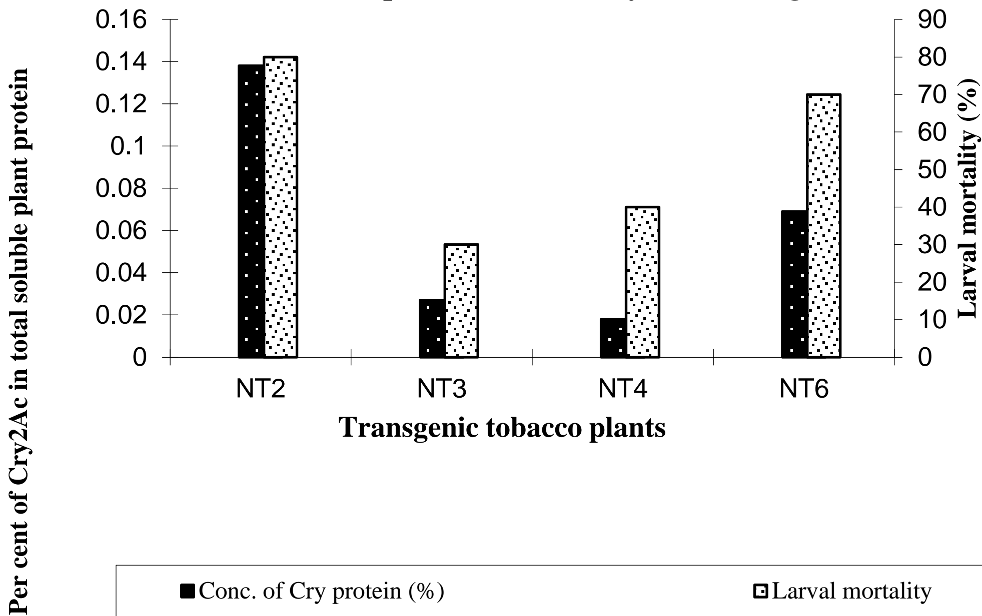
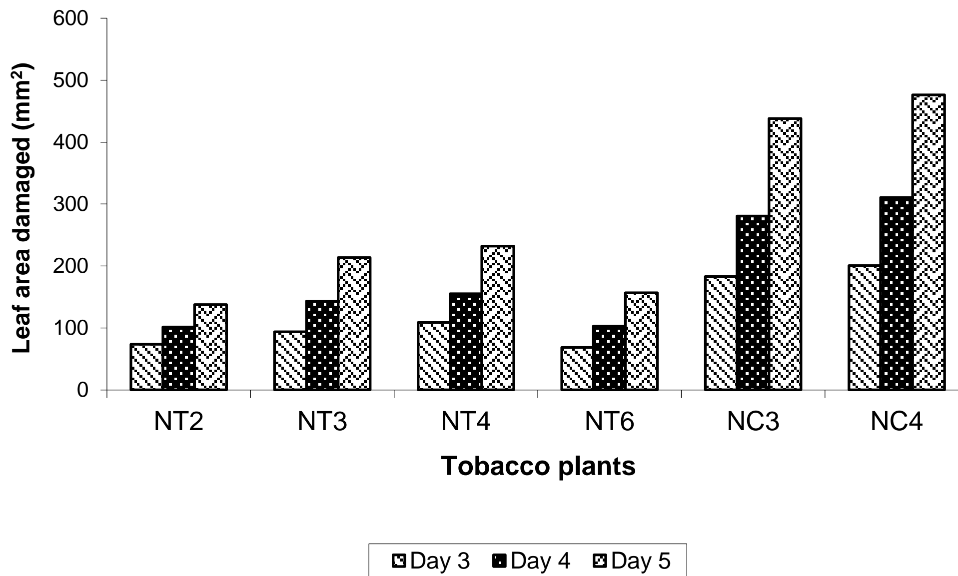


Fig.9 Comparison of leaf area damaged by *H. armigera* in control and transgenic tobacco plants



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