Development of Broad- Spectrum Insect Resistance in Crop Plants

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CERTIFICATE

This dissertation submitted by **Zaheer Abbas** is accepted in its present form by National Institute for Biotechnology and Genetic Engineering, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirement for the
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DEDICATED TO

MY FAMILY

&

ZAHID MUKHTAR CH.

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ABSTRACT

Insect pests are among one of the major factors limiting crop productivity destroying 20-30% of the world's food supply. Insects not only cause direct losses to agricultural produce but also act indirectly as vectors for various plant pathogens. Many different strategies are being employed for pest control including chemical pesticides, biological pesticides, integrated pest management etc. Arthropods pests have been controlled almost exclusively with chemical insecticides. However, the use of chemical insecticides has been under extensive pressure due to their adverse effects on the environment, food and beneficial organisms. This has necessitated the search for safer and more effective pest control strategies. *Bacillus thuringiensis* (*Bt*) has been successfully used as a bio-control agent against major insect pests for the last many decades. In addition to this, a number of *Bt* genes have been identified which are effective against different insect pests. The expression of wild type *Bt* genes in early 1990s showed that for optimum expression of bacterial genes in plants, they must be modified according to plant preferred codons in addition to many other factors, which enhance mRNA stability. To date, a large number of different crop plants have been developed expressing different *Bt* genes. Recently, however, reports regarding the development of resistance against *Bt* crops among insect pests have necessitated evolving strategies for delaying resistance against *Bt* crops. It has been suggested that deployment of two or more *Bt* genes can impart durable and long lasting resistance against insect pests.

The current study is an endeavor to develop different constructs of *Bacillus thuringiensis* synthetic cry1Ac gene under different plant expressible promoters (rbcS and 35S), with and without introns. Another objective of this study was to develop and characterize transgenic tobacco expressing the *Bt* cry1Ac fused with the funnel web spider's ω-atracotoxin-Hv1a (ω-ACTX-Hv1a) gene. This study shows that some of the transgenic tobacco plant lines expressing the cry1Ac-ω-ACTX-Hv1a resulted in 100% mortality in armyworm, which is otherwise reported to be resistant to cry1Ac.

INTRODUCTION

We are in a race between growing population and food production. This era was cast in Paul Ehrlich's *The Population Bomb* (Ehrlich, 1975) perhaps prematurely, at a time when population would outpace the earth's resources, including its capacity to produce food. The threat of the Malthusian crisis forecast by Ehrlich appears to have diminished as we have witnessed a slowdown in the rate of population growth. But the challenge of feeding a world population growing by up to 160 people every minute (>90% of them in developing countries) remains daunting. It is forecasted that, by 2050, world population will increase from the current level of 6 billion to >8 billion people (Anonymous, 1996). Feeding this population will require an astonishing increase in food production. In fact, it has been estimated that the world will need to produce as much food during the next 50 years as was produced since the beginning of agriculture 10,000 years ago (James, 1997).

According to a World Food Program estimate, hunger affects one out of seven people on the planet. In 1996, the World Bank estimated that more than one billion of the world's people do not have enough food to lead healthy and productive lives. Furthermore, another 1-2 billion are at risk of falling into the ranks of the hungry and, if trends continue, the number is expected to grow dramatically (World Bank, 1996; Foster, 1992).

According to the Food and Agriculture Organization (Anonymous, 1997), 840 million of the world's 1.1 billion poor live in rural areas, where 15 million die each year from starvation and related diseases. About 80 countries do not produce enough food to feed their populations. Sub-Saharan Africa produces less food per person today than it did 30 years ago (McLaughlin, 1984).

In the middle of the 20th century the world was predicting a starvation catastrophe. However, the Green Revolution of the 1960s and 1970s "provided such a remarkable short term solution to such a horribly devastating long term problem that some have come

to hail it as a miracle" (Everett, 2001). By using high-yielding varieties of wheat, rice, and maize, together with irrigation, chemical fertilizers and pesticides, world grain production doubled without using more farmland. But more importantly, it led to what is now been termed the "Green Revolution" (Borlaug, 1988). The price of rice and wheat is 40 % lower than it was in the 1950s. This lower price has helped the poorer sections of society, who spend 50-60% of their income on food. The proportion of the population in the developing world that is malnourished fell from 46.5% in the early 1960s to 31% in 1995. However, there is still 1.3 billion of the population who go to bed hungry every day (Khush, 2001).

The problem is that the first Green Revolution focused only on three crops and it did not work in poor, fragile, harsh, and risk-prone environments, omitting the poorest of the poor. Further, "population growth hasn't stopped, so the Green Revolution has to happen all over again (Mann, 1997; Anonymous, 1996; Anonymous, 2003).

Many hope for a second Green Revolution, also called a biotechnology revolution, grey to green revolution, evergreen revolution, and gene revolution, which could be "very relevant to the problems of food security, poverty reduction, and environmental conservation in the developing world" (Serageldin, 1999).

Supporters of a biotechnology revolution claim that it could accomplish "a safer, more stable and lower-cost food supply and responsible stewardship of the environment" (Parrott and Paterson, 2000). Some advantages of the new techniques, together with conventional plant breeding and better agricultural methods, include, for example, the possibility of crop adaptability to marginal areas, lower water needs, lower pesticide and chemical fertilizer use, higher and more stable productivity, higher quality and nutritional characteristics, and more environmentally-friendly agricultural techniques (Mann, 1997; Borlaug, 2002).

There are five basic categories to be considered, ranging from general contributions to economic growth to very specific contributions that reduce malnutrition (Maarten *et al.*, 2003).

A. Biotechnology can improve yields of basic crops through insect protection, drought resistance and modifications to basic crop biology that could not be achieved through traditional breeding techniques.

B. Productivity gains for agricultural systems in degraded and hostile environments can be achieved through biotechnology because genetic potential already exists in some plants that thrive in these environments. Problems include salinity, aluminum toxicity and chronic drought. Many of the world's poorest farmers live in these difficult agricultural environments and improved productivity could have a direct effect on poverty in such settings.

C. Productivity gains for non-grain crops and livestock are possible from biotechnology, and these products have better demand opportunities as incomes increase and a middle class emerges. These gains will help stimulate agricultural diversification and thus permit farmers to get out of the "trap" of growing staple grain commodities with low income potential. China, in its commitments upon joining the World Trade Organization (WTO), is betting heavily on this strategy to solve its problem of rural poverty.

D. Genetically modified crops offer the possibility of reduced input use, especially of pesticides, which have had very serious health consequences for farm workers in countries with inadequate safety regulations on the use of hazardous chemicals. The potential for biotechnology to contribute to sustainable agricultural systems, through much more efficient utilization of water, nutrients and agricultural chemicals, may be its most important promise in the long run.

E. Biofortification of key foodstuffs with better availability of micronutrients such as iron and vitamin A is possible through biotechnology (Steven *et al.*, 2002).

Using modern biotechnology, plants can be made more resistant to insects, bacteria, fungi, and viruses, all of which lead to global production losses of well over 35 percent. The cost of these enormous losses is estimated at over US$200 billion annually (Krattiger 1997). But modern biotechnology can do more than simply increase crop yields. Food quality enhancement also offers great benefits. Reducing certain enzymes in fruits and perishable vegetables, for example, reduces their perishability and significantly cuts post harvest losses (Neupane *et al.*, 1998). In addition, certain naturally occurring substances in plants can be increased such as anticancer compounds naturally found in soybeans, (Wang and Wixon 1999), vitamin A in rice (Burkhardt *et al.*, 1997), iron content in cereals (Theil *et al.*, 1997), or more non-saturated fatty acids in canola (Kramer and Sauer, 1993), and other oil crops. Plants can also be used to deliver edible vaccines, which would have a tremendous impact in developing countries (Arntzen 1996, 1998).

Several crop plants in current commercial production have been transformed with genes that alter the quality of the plant and products derived from these plants. For example, canola was transformed to increase the lauric acid content (Budziszewski *et al.*, 1996; Krimsky and Wrubel, 1996), thus improving the quality of the oil derived from those plants. Similarly, soybeans have been transformed to increase the oleic acid content (Budziszewski *et al.*, 1996; James, 2002; Mazurt *et al.*, 1999).

Scientists are developing crops that can tolerate extreme conditions, such as drought, flood and harsh soil. For instance, researchers are working on a rice that can survive for long periods under water (CGIAR) as well as rice and corn that can tolerate aluminum in soil. A tomato plant has been developed to grow in salty water that is 50 times higher in salt content than conventional plants can tolerate and nearly half as salty as seawater. About one third of the world's irrigated land has become useless to farmers because of high levels of accumulated salt (Owens and Susan, 2001).

Biotechnology has been rapidly and widely adapted by the agricultural research and development community and their products have rapidly gained acceptance among farmers. By the end of the 1998 growing season, 22% of the maize and 36% of the soybean grown in the USA contained one or more transgenes. Similarly, 50% of the

canola produced in Canada and 60% of soybean produced in Argentina was of transgenic origin (James, 2002).

It is now accepted that among different approaches one practical means of achieving greater yields is to minimize the pest associated losses, which are estimated at 14% of the total agricultural production: 52% in wheat, 83% in rice, 59% in maize, 74% in potato, 58% in soybean and 84% in cotton (Oerke *et al.*, 1994). In addition to destroying an estimated 20-30% of the world's food supply arthropod pests are responsible for the transmission of many new and reemerging human diseases (Brogdon *et al.*, 1998).

Insects not only cause direct loss to the agricultural produce, but also indirectly due to their role as vectors of various plant pathogens. In addition to direct losses caused by insects, there are additional costs in the form of pesticides applied for pest control, currently valued at US $10 billion annually. In crops such as pearl millet, sorghum, pigeonpea, chickpea and groundnut grown under subsistence farming conditions in the semi-arid tropics, the losses due to various biotic and abiotic factors have been estimated to be over US $ 2 billion annually (Anonymous, 1992).

Arthropods pests are one of the most diverse animal groups on the planet. Their ability to inhabit a vast array of ecological niches has inevitably brought them into conflict with humans. Although only a small minority is classified as pest species, they nevertheless destroy about a quarter of the world's annual crop production and transmit an impressive array of pathogens of human and veterinary public health importance.

Arthropod pests have been controlled almost exclusively with chemical insecticides since the introduction of DDT in the 1940s (Tedford *et al.*, 2004). Spraying broad-spectrum chemical insecticides has largely controlled arthropod pests. However, the long-term application of a small armament of insecticides that act on a restricted number of invertebrate nervous system targets has inevitably led to the development of resistance in most agrochemically and medically important arthropods (Brogdon *et al.*, 1998; Feyereisen, 1995). Furthermore, most chemical insecticides are relatively non-specific. It

has been estimated that they kill about 70 million birds and 10 million fish per year (Le Couteur *et al.*, 1999). Moreover, there is now strong epidemiological (Le Couteur *et al.*, 1999) and experimental (King *et al.*, 2002) evidence linking certain neurological disorders, such as Parkinson's disease, to pesticide exposure. Massive application of pesticides results in adverse effects on the beneficial organisms, leaves pesticide residues in the food and results in environmental pollution. As a result, the chemical control of pests is under increasing pressure. Pesticide use in the world is declining, largely due to major reduction in Europe as a result of regulatory mechanisms, environmental activism and public pressure. This has necessitated the use of target specific compounds with low persistence, and an increase in emphasis on integrated pest management based on host plant resistance to insect pests. Although the benefits to agriculture from the pesticide use to prevent insect associated losses, cannot be overlooked, yet there is a greater need to develop alternative or additional technologies, which would allow a rational use of pesticides, and provide adequate crop protection for sustainable food, feed and fiber production in the future.

Integrated pest management (IPM) has historically placed great hopes on host plant resistance (HPR). Host plant resistance (HPR) to insects is an effective, economical, and environment friendly method of pest control. The most attractive feature of HPR is that farmers virtually do not need any skill in application techniques, and there is no cash investment by the resource poor farmers. Considerable progress has been made in identification and development of crop cultivars with resistance to the major pests in different crops. There is a need to transfer resistance genes into high-yielding cultivars with adaptation to different agro-ecosystems. Resistance to insects should form one of the criteria to release varieties and hybrids for cultivation by the farmers. Genes from the wild relatives of crops, and novel genes, such as those from *Bacillus thuringiensis* can also be deployed in different crops to make HPR an effective weapon to minimize the losses due to insect pests. HPR will not only cause a major reduction in pesticide use and slowdown the rate of development of resistance to insecticides in insect populations, but also lead to increased activity of beneficial organisms and reduction in pesticide residues in food and food products (Sharma and Ortiz, 2002). However, conventional host-plant

resistance to insects involves quantitative traits at several loci, and as a result, the progress has been slow and difficult to achieve.

The evolution of resistance to many insecticides, coupled with increased awareness of the potential environmental, human and animal health impacts of these chemicals, has stimulated the search for new insecticidal compounds, novel molecular targets, and alternative control methods (Tedford *et al.*, 2004).

With the advent of genetic transformation techniques based on recombinant DNA technology, it is now possible to insert genes into the plant genome that confer resistance to insects (Bennett, 1994). One approach is to engineer plants to produce insect specific toxins, as exemplified by the engineering of genes encoding insecticidal δ-endotoxins from the soil bacterium *Bacillus thuringiensis* into a variety of agricultural cultivars (Shelton *et al.*, 2002). Genes from bacteria such as *Bacillus thuringiensis* (*Bt*) and *Bacillus sphaericus* have been the most successful group for use in genetic transformation of crops for pest control on a commercial scale (Gill *et al.*, 1992; Charles *et al.*, 1996). Protease inhibitors, plant lectins, ribosome inactivating proteins, secondary plant metabolites, vegetative insecticidal proteins from *Bt* and related species, and small RNA viruses can also be used alone or in combination with *Bt* genes to generate transgenic plants for pest control (Hilder and Boulter, 1999).

Plants modified to express insecticidal proteins from *Bacillus thuringiensis* (referred to as Bt-protected plants) provide a safe and highly effective method of insect control (Betz , *et al.*, 2001). Initially δ endotoxin encoding genes such as cry 1Aa, cryl Ab and cry1Ac from the Bt sub species kurstaki were introduced in tomato and in potato in their native and truncated forms using *Agrobacterium* mediated transformation. No significant insecticidal activity was observed in plants transformed with genes encoding the full length of crystal protein. Tobacco and Tomato transformants with truncated forms of the crystal protein genes that coded for the insecticidal fragment consisting the N-terminal half of the crystal protein were found to produce significant levels of the protein and were protected from feeding damage by some insect pests. In spite of these reports, different

research groups conducting field trials with the transgenic plants expressing the Bt proteins found that the level of insecticidal protein produced in transgenic plants were generally inadequate to provide field protection against agronomically important insect pests. These findings culminated in various attempts to enhance the level of Bt-gene expression (Fischoff *et al.*, 1987; Lambert and Peferoen, 1992).

The coding sequence was apparently found to be responsible for inhibiting the proper levels of expression. Therefore, efforts were made to modify the coding sequence without affecting the amino acid sequence. This inhibition arose from the fact that Bt genes are rich in A/T content whereas plant genes have a higher G/C content. Thus, partial or complete re-synthesis of these genes to contain a higher G/C content resolved this problem to a great extent. Sequence modification in areas of the gene with predicted mRNA secondary structures in combination with powerful promoters resulted in 100 fold increased level of insecticidal protein in transgenic cotton encoding the cry1Ab or cry1Ac genes thereby leading to effective control of cotton pests (Perlak *et al.*, 1991).

For expression of the Bt gene in the higher plants, a recognisable promoter and a terminator sequence must bracket the Bt gene. Popular constitutive promoters include cauliflower mosaic virus (CaMV35S) and ubiquitin. Tissue specific promoters include PEPC (phosphoenolpyruvate carboxylase) (for green tissue) and maize pollen specific promoter (Koziel *et al.*, 1993).

Bt-protected corn, cotton, and potato were introduced into the United States in 1995/1996 and grown on a total of approximately 10 million acres in 1997, 20 million acres in 1998, and 29 million acres globally in 1999. The extremely rapid adoption of these Bt-protected crops demonstrates the outstanding grower satisfaction of the performance and value of these products. These crops provide highly effective control of major insect pests such as the European corn borer, southwestern corn borer, tobacco budworm, cotton bollworm, pink bollworm, and Colorado potato beetle. B*t* crops have reduced reliance on conventional chemical pesticides. They have provided notably higher yields in cotton and corn. The estimated total net savings to the grower using Bt-protected cotton in the

United States was approximately $92 million in 1998. Other benefits of these crops include reduced levels of the fungal toxin fumonisin in corn and the opportunity for supplemental pest control by beneficial insects due to the reduced use of broad-spectrum insecticides. Insect resistance management plans are being implemented to ensure the prolonged effectiveness of these products. Extensive testing of Bt-protected crops has been conducted which establishes the safety of these products to humans, animals, and the environment. Acute, sub-chronic, and chronic toxicology studies conducted over the past 40 years establish the safety of the microbial Bt products, including their expressed insecticidal (Cry) proteins, which are fully approved for marketing. Mammalian toxicology and digestive fate studies, which have been conducted with the proteins produced in the currently approved Bt-protected plant products, have confirmed that these Cry proteins are nontoxic to humans and pose no significant concern for allergenicity. Food and feed derived from Bt-protected crops, which have been fully approved by regulatory agencies, have been shown to be substantially equivalent to the food and feed derived from conventional crops. Non-target organisms exposed to high levels of Cry protein are virtually unaffected, except for certain insects that are closely related to the target pests. Because the Cry protein is contained within the plant (in microgram quantities), the potential for exposure to farm workers and non-target organisms is extremely low. The Cry proteins produced in Bt-protected crops have been shown to rapidly degrade when crop residue is incorporated into the soil. Thus the environmental impact of these crops is negligible. The human and environmental safety of Bt-protected crops is further supported by the long history of safe use for Bt microbial pesticides around the world (Betz *et al.*, 2001).

Worldwide, the areas planted to transgenic crops jumped more than twenty-fold in the past six years, from 3 million hectares in 1996 to nearly 44.2 million hectares in 2000 (James, 2000).

Insects can and have developed resistance to nearly every type of insecticide. Insecticide resistance develops due to genetic variation in large insect populations. A few individuals in the original insect population are unaffected by a given insecticide. Generally,

unaffected (resistant) individuals differ from affected (susceptible) individuals either in the nature of the insecticide's target molecules in the insect, or in the method the insect uses to break down toxin molecules (Michaud, 1997). When the insecticide is applied, individuals who are unaffected by it are those who survive to pass their genes on to following generations. Over time, a greater and greater proportion of the insect population is unaffected by the insecticide. In addition to the damage done by the increasingly large number of surviving, resistant insects, attempts to control insecticide resistance can indirectly cause secondary pest outbreaks that do yet more crop damage (Hoy, 1998).

Transgenic insect- resistant crops that express single toxin from *bacillus thuringiensis* offered significant advantages but these advantages may be lost due to evolution of resistance in targeted insect pest. Multiple toxins, refugia and high toxin doses have been proposed as strategies for minimizing resistance development (McGaughey *et al.*, 1998).

The use of two or more toxins in the very same variety (pyramiding) can reduce the amount of refuge required to delay resistance for an extended period (Roush *et al.*, 1998). For such strategies to work, cross- resistance must not occur among the different toxin (Roush, 2000).

Relative to transgenic tobacco with the Bt protein the transgenic tobacco with Bt and CpTI proteins delays resistance development to BtICP in *Helicoverpa armigera*, and gene pyramiding could be a valuable strategy for resistance management and the sustainable use of Bt transgenic crops (Zhao *et al.*, 1997). Theoretical models suggest that pyramiding of two dissimilar toxin genes in the same plant has the potential to delay the onset of resistance much more effectively than single-toxin plants released spatially or temporally and may require smaller refuges (Roush, 1998). To minimize the potential for cross-resistance, it is highly advantageous for new insecticides to act at new or under exploited targets (Sparks *et al.*, 2001). Keeping in view the management of insect resistance, the present study is designed for the following purpose:

OBJECTIVES

The objectives of the present study are

1. Designing and optimization the truncated *Bacillus thuringiensis* cry1Ac gene with triplet codon specific to plant genome.
2. Translational Fusion of cry1Ac and neurotoxic gene.
3. Cloning of expression cassettes in binary vector.
4. Targeting of the expressed toxin protein to the plant organelle genome.
5. *Agrobacterium* mediated transformation.
6. Molecular analysis of transgenic plants.

REVIEW OF LITERATURE

The continuing growth of the human population will, for the foreseeable future, require that we find new ways to increase food production. Reducing losses caused by pests and diseases is one possible approach. Current estimates put global losses caused by pests (insects, nematodes, diseases, and weeds) at US$ 300 billion annually (Anonymous, 1992) which equals around 30-40% of potential global food, fiber, and feed production (Anonymous, 1992) with substantially higher proportions in particular developing countries. Moreover, these estimates generally concern losses to crop production only. If losses caused by post harvest pests and diseases are added, then figures approaching 60-70% may be more typical for the developing world (Kumar, 1984).

Over the past 50 years, application of chemical pesticides has come to be the dominant form of pest control in developed, and increasingly in developing, countries (Anonymous, 1992). Future approaches to reducing the damage caused by pests are, however, likely to be very different from those that predominate today. Indeed, while pests have been a chronic problem in agriculture since it's beginning, many of the serious pest problems in the developing world today are the direct consequence of actions taken to improve crop production (Waage, 1993). These pest problems associated with agricultural intensification particularly apply to insects. In recent decades, the dependence on chemical insecticides has led in some crop systems to a high frequency of insecticide resistance recorded in more than 500 insect species worldwide (Georghiou, 1990) pest resurgence, acute and chronic health problems, environmental pollution, and uneconomic crop production. All of these problems are particularly severe in developing countries, where pesticide use is poorly regulated and farmers often lack appropriate information or training. For many of these farmers, pesticide use is becoming a rising and unreliable component of the cost of crop production.

Maximum number of insects showing resistance to insecticides has been recorded in cotton, vegetables and tobacco (Rajmohan, 1998). *Helicoverpa armigera* (which is the most serious pest on cotton, legumes, vegetables and cereals) has shown resistance to

several groups of insecticides in cotton, tomato, chillies, sunflower, groundnut, pigeonpea and chickpea. This has resulted in widespread failure of insect control causing extreme debts, at times even forcing the farmers to commit suicide. The cotton whitefly (*Bemisia tabaci*) has shown resistance to insecticides in cotton, brinjal and okra; while tobacco caterpillar (*Spodoptera litura*) has been found to be resistant to insecticides on cotton, cauliflower, groundnut and tobacco. Green peach and potato aphid (*Myzus persicae*), cotton aphid (*Aphis gossypii*), mustard aphid (*Lipaphis erysimi*) and diamond back moth (*Plutella xylostella)* have also been found to exhibit resistance to insecticides in several crops. Development of resistance to insecticides has necessitated the application of higher doses of the same pesticide or increased number of pesticide applications. The farmers often resort to insecticide mixtures to minimize the insect damage to crops. This not only increases the cost of pest control, but also results in insecticidal hazards and pollution of the environment. It is in this context that the use of biotechnological techniques to contain the pest damage, both in the developed and the developing countries, becomes all the more important.

The insecticidal toxins from *Bacillus thuringiensis* (Bt) represent a class of biopesticides that are attractive alternatives to broad-spectrum "hard" chemistries organophosphate insecticides. The advantage of using a biopesticide like Bt is that it is fairly specific and quite lethal. Bt particularly suits integrated pest management (IPM) and ICM (Integrated Crop Management) strategies because it is only effective directly against the pest. Unlike organophosphates, which are quite general in their effect, Bt's toxins are very specific to certain harmful insects and are therefore safe to most beneficial insects and other animals. Additionally, Bt toxins are biodegradable and do not persist in the environment (Van Frankenhuyzen, 1993). This encourages farmers to intervene only when there is a pest problem rather than to spray their fields with poisons as a standard precaution. There are also several disadvantages to Bt: wind and rain can sharply reduce its effective biological life; the pest has to attack the crop before Bt can be used, so some damage is always sustained; grubs that eat the underside of the plant foliage can escape a Bt spray or dusting; and insects that bore into the plant can escape the effects altogether. The traditional use of Bt by organic farmers and horticulturists has thus been limited to the

control of pests that eat the outer leaves of plants. Pests that burrow into the plant such as the European corn borer or the rice borer can withstand traditional Bt sprays or dustings (Whalon and Wingerd, 2003).

After the invention of recombinant DNA technology in late 70's and later on rapid advances in this modern tool make possible, to successfully modify the already existing genotype of an organism like plants and etc. Genetic engineering allows the use of several desirable genes, which was not possible to use in conventional tools because of sexual barrier.

Research on transgenic crops, as is the case with conventional plant breeding and selection by the farmers, aims to selectively alter, add or remove a character of choice in a plant, bearing in mind the regional need and opportunities. It not only offers the possibility of bringing in a desirable character from closely related plants, but also of adding desirable characteristics from the unrelated species. After the transformation event, the transformed plant becomes a parent for use in conventional breeding programmes. Development and deployment of transgenic plants in an effective manner will be an important prerequisite for sustainable use of biotechnology for crop improvement. As a result of advances in genetic transformation and gene expression during the last decade (Hilder and Boulter, 1999; Sharma *et al.*, 2000). There has been a rapid progress in using genetic engineering for crop improvement, of which protection of crops against the insects is a major goal. The potential of this technology has now been recognized widely. Once efficient protocols for tissue culture and transformation are developed, the production of transgenic plants with different genes is fairly routine (Sharma and Ortiz, 2002).

One approach is to engineer plants to produce insect specific toxins, as exemplified by the engineering of genes encoding insecticidal δ-endotoxins from the soil bacterium *Bacillus thuringiensis* into a variety of agricultural cultivars (Shelton *et al.*, 2002). Genes from bacteria such as *Bacillus thuringiensis* (*Bt*) and *Bacillus sphaericus* have been the most successful group of organisms identified for use in genetic transformation of crops

for pest control on a commercial scale (Gill *et al.*, 1992; Charles *et al.*, 1996). Protease inhibitors, plant lectins, ribosome inactivating proteins, secondary plant metabolites, vegetative insecticidal proteins from *Bt* and related species, and small RNA viruses can also be used alone or in combination with *Bt* genes to generate transgenic plants for pest control (Hilder and Boulter, 1999).

Bacillus thuringiensis was discovered first in Japan in 1901 by Ishawata and then in 1911 in Germany by Berliner (Baum *et al.*, 1999). It was subsequently found that thousands of strains of *B. thuringiensis* exist (Lereclus, 1993). Each strain produces its own unique insecticidal crystal protein, or delta-endotoxin, which is encoded by a single gene on a plasmid in the bacterium (Whalon and McGaughey, 1998). The insecticidal activity of the toxins from each Bt strain differs. Nevertheless, the set of Bt delta-endotoxins affects a variety of species from the orders Coleoptera (beetles), Lepidoptera (moths and butterflies), and Diptera (flies and mosquitoes) (Gould and Keeton, 1996).

There are 34 recognized sub species of *B. thuringiensis* some of the most commonly used include subspecies *kurstaki* (against Lepidoptera), subspecies *israeliensis* (against Diptera, primarily mosquitoes and black flies), and subspecies *tenebrionis* (against *Leptinotarsa decemlineata*, the Colorado potato beetle) (Whalon and McGaughey, 1998). Two general groups of insecticidal crystal proteins made by this wide variety of subspecies are cytolysins (Cyt) and crystal delta-endotoxins (Cry). Hofte and Whiteley (1989) defined four classes of Cry genes and two classes of Cyt genes. CryI and CryII toxins are active against lepidopterans, CryII and CryIV against dipterans, and CryIII against coleopterans. While CryIII toxins are produced by subspecies *tenebrionis* and *tolworthi* and CryIV by *israelensis*, generally very little correlation between certain toxins and certain subspecies exists. Cry toxins bind to specific receptors on cells in the insect midgut. Cyt genes are active against dipteran and coleopteran pests, and additionally have shown action against hemipterans (true bugs) and dictyopterans (roaches and termites) (Frutos *et al.*, 1999; Gould and Keeton, 1996). Cyt toxins, unlike Cry toxins, do not recognize specific binding sites (Lereclus *et al.*, 1993).

The mode of action of *B. thuringiensis* toxins involves ingestion followed by crystal solubilization and proteolytic activation of protoxin in the insect midgut. Activated toxin binds to receptors in the midgut epithelial membrane and inserts into the membrane, leading to cell lysis and death of the insect (Schnepf *et al*, 1998). Binding of *B. thuringiensis* toxins to specific sites in the epithelial membrane is a key step in toxin specificity (Hofmann *et al.*, 1998; Van Rie *et al.*, 1989).

Initially δ-endotoxin encoding genes such as cry1Aa, cry1Ab and cry1Ac from the Bt sub species kurstaki were introduced in tomato and potato in their native and truncated forms using *Agrobacterium* mediated transformation. No significant insecticidal activity was observed in plants transformed with genes encoding the full length of crystal protein. Tobacco and tomato transformants with truncated forms of the crystal protein genes that coded for the insecticidal fragment consisting the N-terminal half of the crystal protein were found to produce significant levels of the protein and were protected from feeding damage by some insect pests. Inspite of these reports, different research groups conducting field trials with the transgenic plants expressing the Bt proteins found that the level of insecticidal protein produced in transgenic plants were generally inadequate to provide field protection against agronomically important insect pests (Fischhoff *et al.*, 1987; Lambert and Peferoen, 1992).

Later on it was discovered that gene should be first converted from AT-rich (typical of bacteria) to GC-rich (typical of higher plants) to increase toxin expression. Most changes are made to the third codon thereby minimizing changes in the amino acid sequence and increasing the expression of Bt toxin by 10 to 100 fold (Perlak *et al.*, 1991). For expression of the Bt gene in the higher plants, a recognisable promoter and a terminator sequence must bracket the Bt gene. Popular constitutive promoters include cauliflower mosaic virus (CaMV35S) and ubiquitin. Tissue specific promoters include PEPC (phosphoenolpyruvate carboxylase) (for green tissue) and maize pollen specific promoter (Koziel *et al.*, 1993).

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

Truncated cryIAb gene has been introduced into several cultivars of rice (*indica* and *japonica*) by microprojectile bombardment and protoplast systems (Datta *et al.*, 1998). The expression was driven by two constitutive promoters (35S from CaMV and Actin-1 from rice) and two tissue-specific promoters (pith tissue and PEPC for green tissue from maize). Eighty-one transgenic plants caused 100% mortality of the yellow stem borer *(Scirpophaga incertulas)*. The transgene, cryIAb, driven by different promoters showed a wide range of expression (low to high) of Bt proteins stably inherited in a number of rice cultivars with enhanced yellow stem borer resistance. Maqbool *et al.*, (1998) transformed the rice cultivars Basmati 370 and M7 by using Cry 2A insecticidal gene against the yellow stem borer and the rice leaf folder. Nayak *et al.*, (1997) reported that two rice lines transformed with synthetic cry IAc were highly toxic to yellow stem borer.

Cotton cultivar Coker 312 was first transformed by using modified partially modified (PM) cry1Ac gene under the control of CaMV35S promoter containing a duplicated enhancer region. The transformed plants showed total protection against *Trichplusia ni*, *Spodoptera exigua* and *Heliothis zea*. The maximum level of toxin protein was 0.1% of the total soluble protein. By placing the fully modified (FM) cry1Ac gene under the control of *Arabidopsis thaliana* small subunit promoter with its chloroplast transit peptide sequence, produced tobacco plants with 10 to 20 fold increase in cry1Ac mRNA and protein compared to gene constructs with CaMV35S promoter with duplicated enhancer region. This increased the Bt toxin production to nearly 1% of the total leaf protein (Wong *et al.*, 1992).

Tissue specific regulation of Bt cry1Ab gene has been utilised to achieve high and regulated expression in the leaves and pollen grains. The promoter derived from PEPC controls the expression of cry1Ab in green tissue gene (Hudspeth and Grula, 1989), while

the promoter derived from calcium dependent protein kinase (CDPK) gene is pollen specific (Estruch *et al.*, 1994). Combination of green tissue specific PEPC and pollen specific CDPK tissue promoters provides high cry1Ab gene expression in leaves and pollen, where it is most effective in controlling the European corn borer (*Ostrinia nubilalis)*. The intron 9 of maize PEPC is located between cry1Ab structural gene and the 35S terminator, and its presence increased the expression of Bt gene (Hudspeth and Grula, 1989).

NuCOTN 33B, a Bt transgenic variety of upland cotton *Gossypium hirsutum* expressing the insecticidal protein cry1Ac from *Bacillus thuringiensis Berliner* sp. *kurstaki*, was evaluated for resistance to *Helicoverpa armigera* (Hubner) during 1998-2000 in northern China. The results indicated that there was no significant difference in egg densities between NuCOTN 33B and three non-transgenic varieties (DP5415, Zhongmian12, and Shiyuan321) during the season, although the survival of larvae on NuCOTN 33B seemed significantly reduced. High larval densities observed on non-Bt cotton appeared in great contrast to the low larval populations observed on NuCOTN 33B plants during the seasons. In an environment without insecticide sprays, the annual ginned cotton yields in NuCOTN 33B plots, ranging from 1391.17 to 1511.35 kg/ha, were significantly higher than those in non-Bt cotton (340.34-359.58 kg/ha). These high levels of field efficacy for NuCOTN 33B against *H. armigera* in northern China may pave the way for reduced pesticide applications and an expansion of alternative pest-control strategies (Wu et *al.*, 2003).

Van Rensburg (1999) reported that transformed maize was effective against the spotted stem borer and the maize stalk borer (*Busseola fusca*), which are the two most important pests of sorghum in Asia and Africa. Spotted stem borer was more susceptible than the maize stalk borer to the same events. Effectiveness of Bt toxins has also been demonstrated in maize against the stem borers infesting sorghum in Latin America (Bergvinson *et al.*, 1997).

Today, major Bt transgenic crops include corn, cotton, potatoes, and rice. The engineering of plants to express Bt delta-endotoxins has been especially helpful against pests that attack parts of the plant that are usually not well protected by conventional insecticide application. A prime example of this is protection against *Ostrinia nubilalis*, the European corn borer. Larvae of this lepidopteran bore into the stalk of a corn plant and destroy its structural integrity. In the stalk, the pest is relatively safe from pesticide application. With toxins engineered into the plant, *O. nubilalis* is exposed and its damage becomes easier to control (Ely, 1993). Overall, because of benefits such as these, Bt has become a major presence in agriculture. In 1997, Bt cotton, corn, and potatoes covered nearly 10 million acres of land in the United States alone. These crops have also been commercialized and are in wide use in Canada, Japan, Mexico, Argentina, and Australia (Frutos *et al.*, 1999).

Insects have great natural potential to develop resistance. Many field populations of diamondback moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae), have evolved resistance to *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) (Tabashnik, 1994). One gene in diamondback moth confers resistance to four *Bacillus thuringiensis* toxins (Tabashnik *et al.*, 1997).

Insecticide resistance develops due to genetic variation in large insect populations. A few individuals in the original insect population are unaffected by a given insecticide. Generally, unaffected (resistant) individuals differ from affected (susceptible) individuals either in the nature of the insecticide's target molecules in the insect, or in the method the insect uses to break down toxin molecules (Michaud, 1997). When the insecticide is applied, individuals who are unaffected by it are those who survive to pass their genes on to following generations. Over time, a greater and greater proportion of the insect population is unaffected by the insecticide. In addition to the damage done by the increasingly large number of surviving, resistant insects, attempts to control insecticide resistance can indirectly cause secondary pest outbreaks that do yet more crop damage (Hoy, 1998).

There are several factors that increase the rate at which insecticide resistance is generally developed. Some factors are related to the insect population itself. Species with higher reproductive rates, shorter generation times, greater numbers of progeny, and larger, more genetically varied local populations develop a large resistance population more quickly (Pimentel and Burgess, 1985). Whether the genetic basis of insect resistance is dominant or recessive is also of importance (Wearing and Hokkanen, 1995). Other factors are dependent upon the insecticide. Resistance develops more rapidly to more persistent insecticides; their staying power in the environment increases the chance that susceptible individuals are exposed to the toxin and die, thus not passing on their insecticide-susceptible traits to the next generation. This selects more strongly on resistant insects because only the resistant insects thrive. By similar logic, frequent application of non-persistent insecticides has the same effect (Wood, 1981). Insect populations with little immigration into the gene pool of new, non-exposed susceptible individuals also develop resistance more readily (Comins, 1977). Populations that have in the past been exposed to an insecticide with a mode of action similar to that of a new insecticide are quick to develop resistance to the new toxin. This phenomenon is known as cross-resistance (Pimentel and Burgess, 1985).

Several resistance strategies (Perlak *et al.*, 1993) interwoven with integrated pest management (IPM) have been proposed, which can be categorized into short and long term strategies. Short-term strategies involve the use of high dose expression of Bt genes, use of host plants for sensitive insects as refugia, the use of agronomic practices that minimizes insect exposure to Bt and IPM insect management systems. Long-term strategies involve the use of multiple genes encoding insect control proteins with unique mode of action in the same crop (Shah *et al.*, 1995).

Different *B. thuringiensis* toxins to work, cross-resistance must not occur among them. So far, the best method of predicting cross-resistance among *B. thuringiensis* toxins is determining which toxins share a common binding site in a given insect. Sequences or

combinations of toxins that share a common binding site are not likely to be useful for managing resistance.

Reduced binding of toxin to midgut membrane target sites is the best-known mechanism of resistance to *B. thuringiensis* toxins (Ballester *et al.*, 1999, Ferré *et al.*, 1991, Herrero *et al.*, 2001). Furthermore, alteration of a common binding site has been found in cases where insects evolved high levels of resistance simultaneously to more than one *B. thuringiensis* toxin (Lee *et al.*, 1995; Sayyed *et al.*, 2000; Tabashnik *et al.*, 1997).

Most lepidopteran insects tested for binding of cry1Aa, cry1Ab, and cry1Ac toxins to midgut brush border membrane vesicles (BBMV) share a common binding site for these toxins (Ballester *et al.*, 1999; Escriche *et al.*, 1997; Karim *et al.*, 2000). This is not surprising, because these three cry1A toxins have 73 to 88% amino acid sequence identity for the activated toxin (as determined by using the BLAST program (Altschul *et al.*, 1997).

cry1Ja is a lepidopteran active toxin (Donovan *et al.*, 1994) with low sequence identity with cry1A toxins (e.g., 47% amino acid sequence identity with cry1Ac). Nonetheless, two strains of *Plutella xylostella* selected with *B. thuringiensis* products containing cry1A toxins but not cry1Ja evolved resistance to cry1A toxins and strong cross-resistance to cry1Ja (Donovan *et al.*, 1994; Tabashnik *et al.*, 1997). In this pest, one gene confers resistance to cry1Aa, cry1Ab, cry1Ac, cry1Fa, and cry1Ja toxins (Tabashnik *et al.*, 2000; Tabashnik *et al.*, 1997). It was shown elsewhere that binding of cry1Aa, cry1Ab, and cry1Ac toxins was strongly reduced in some resistant populations (Ballester *et al.*, 1999; Tabashnik *et al.*, 1997). Because cry1Fa competes for cry1A's binding site (Granero *et al.*, 1996), it has been proposed that binding of this toxin might also be affected in the resistant populations.

P. xylostella cross-resistance to cry1Ja suggests that this toxin might also share a binding site with the cry1A toxins and cry1Fa. Recently, a common binding site for cry1Aa, cry1Ab, cry1Ac, cry1Fa, and cry1Ja was reported for *Heliothis virescens* (Noctuidae)

(Jurat-Fuentes, and Adang, 2001). Reduced toxin binding/binding sites and decreased activation of the toxin, are thought to occur together in *H. virescens* (Michaud, 1997).

Keeping in view the phenomenon of cross resistance, unrelated genes were transformed in same variety for broad spectrum and long lasting resistance to insect pests. One such type of study was conducted from 1999 to 2001 to evaluate the efficacy of the transgenic cotton, *Gossypium hirsutum* genotype, Bollgard II (Monsanto 15985), which expresses two *Bacillus thuringiensis* Berliner (Bt) proteins (cry1Ac + cry2Ab) that are active against lepidoptera pests. Bollgard II was compared with Bollgard (DP50B), which expresses only one Bt protein (cry1Ac), and, in all tests, the conventional variety, DP50, was used as a non-Bt control. Larval populations of the bollworm, *Helicoverpa zea* (Boddie), and the soybean looper, *Pseudoplusia includens* (Walker), were significantly lower in Bollgard II than in Bollgard and conventional cotton, and the proportion of fruit damaged by *H. zea* was also lower. Fall armyworm, (*Spodoptera frugiperda)* populations were lower in Bollgard II than in Bollgard, although not significantly. Field tests were supplemented with laboratory bioassays in 2001 to compare mortality of *S. frugiperda*, and beet armyworms, *Spodoptera exigua* (Hubner), feeding on these genotypes. Mortality of both species was significantly greater on Bollgard II plant material than on either Bollgard or conventional cotton. This study demonstrated that the dual-toxin Bollgard II genotype is highly effective against lepidopterous pests that are not adequately controlled by the current single-toxin Bollgard varieties (Chitkowski *et al.*, 2003).

Transgenic tobacco with *Bacillus thuringiensis* (Bt) and cowpea trypsin inhibitor (*CpTI*) genes was evaluated for insecticidal activity on the cotton bollworm *(Helicoverpa armigera)*, and *H. armigera* ability to adapt to these transgenic tobaccos studied. A total of 200 adults were collected from 6 counties in 3 northern Chinese provinces to initiate a laboratory strain. Instars (48 first, 24 second, 20 third, 20 fourth and 20 fifth) were exposed to either a transgenic tobacco (Kongchong 931) expressing Bt and *CpTI*; a transgenic tobacco expressing Bt only; a non-transgenic tobacco (NC89); or an artificial control diet. *H. armigera* survivorship was recorded after 3 days and after pupation. Mortality of 1st-, 2nd- and 3rd-instars after 3 days was significantly lower on transgenic

tobacco expressing Bt alone than that expressing both *Bt* and *CpTI*, 88.2, 55.6 and 50.0% compared to 99.3, 83.3 and 80.5%, respectively. Fourth- and 5th-instars were able to pupate on Bt tobacco, but only 5th-instars were able to pupate on Bt and *CpTI* expressing tobacco. After 11 generations, 61.5% of 2nd-instar larvae survived on tobacco expressing *Bt* and *CpTI*, and 59.2% on tobacco expressing Bt genes only. After 11 generations of selection, *H. armigera* reared on transgenic tobacco expressing *Bt* and *CpTI* genes had resistance ratios (RR) to *Bt* insecticidal crystal protein of 2.4 (LD50) and 1.9 (LD90), *H. armigera* reared on tobacco expressing only the Bt gene had RR of 5.5 (LD50) and 13.6(LD90). It is concluded that relative to transgenic tobacco with the Bt protein the transgenic tobacco with *Bt* and *CpTI* proteins delays resistance development to BtICP in *H. armigera,* and gene pyramiding could be a valuable strategy for resistance management and the sustainable use of Bt transgenic crops (Zhao *et al.*, 1997).

To minimize the potential for cross-resistance, it is highly advantageous for new insecticides to act at new or under exploited targets (Sparks *et al.*, 2001). Theoretical models suggest that pyramiding two dissimilar toxin genes in the same plant has the potential to delay the onset of resistance much more effectively than single-toxin plants released spatially or temporally and may require smaller refuges (Roush, 1998).

Many of the candidate genes, that have been used in genetic transformation of crops, are either too specific or are only mildly effective against the target insect pests. Some insect species are also insensitive to some of these genes. Therefore, to convert transgenics into an effective weapon in pest control, e.g., by delaying the evolution of insect populations resistant to the target genes, it is important to deploy genes with different modes of action in the same plant. Thus far, scientists have been successful in finding several new Bt proteins for these purposes. Cotton containing both cry1Ac and the new insecticidal protein, cry2Ab, is marketed in the United States as Bollgard II (registered in 2002). cry2Ab is relatively non-homologous to cry1Ac. It displays different modes of action and midgut binding properties, and one insect species resistant to cry1Ac has been successfully controlled with Bollgard II (Tabashnik *et al.*, 2002). And expression of these two proteins has resulted in an increase in the number of economically important

lepidopteran pests controlled. However, Bollgard II is still active only against lepidoptera and is engineered on the basis of the expression of two genes cryIAc and cry2Ab. These two genes cry2Ab and cry1Ac were combined, based on the idea that cryIAc and cry2Ab have limited homology (Charles *et al.*, 2000), strong cross-resistance between plants producing these toxins is not expected. Cry2Ab is toxic to some lepidopteran larvae, including the major cotton pests *Heliothis virescens* and *Helicoverpa zea* (Dankocsik *et al.*, 1990). Although the pyramiding of these two genes had shown satisfactory results in the first cotton generation but still there is chances of developing resistance in insects population because these two proteins have homology in their amino acids sequence and these two genes are derived from same bacterial origin. Several other Bt proteins, such as cry1F and the first Bt non-Cry protein, Vip3A, are currently under review for registration. The resistance level of Bt cotton declined from high resistance against a non-selected population to medium levels of .esistance against the selected population, indicating a potential problem of development of resistance in insects to Bt cotton. It is therefore need of the time to look for alternates to Bt genes so that new genes can either be used alone or in combination with Bt to develop transgenic plants with durable and long lasting resistance to insect pests.

Most spider venoms are likely to be rich sources of insecticidal compounds since their primary role is to kill or paralyze arthropod prey. Thus, it seems surprising that spider venoms, which as a whole are likely to contain more than a million different pharmacologically active peptides (King *et al.*, 2002) have not been explored as thoroughly as those of other venomous creatures such as scorpions and cone snails. While numerous peptide neurotoxins have been isolated from spider venoms (Escoubas *et al.*, 2000; Rash *et al.*, 2002).

The ω-atracotoxin-Hv1a (ω-ACTX-Hv1a) gene from funnel web spider (*Hadronyche versuta*) was designed and optimized at the National institute for biotechnology and genetic engineering (NIBGE) and commercially synthesized (Patent pending). This gene was characterized in tobacco (*Nicotiana tabaccum* L. cv. Samsun). Bioassay of transgenic plants revealed its strong effectiveness against lepidopteron pests including

armyworm, which is resistant to *Bacillus thurengiensis* toxin proteins. Similarly for enhanced expression of *Bacillus thuringiensis* cry1Ac, it was designed and optimized with triplet codon specific to plant genome (Patent pending).

Australian funnel-web spiders (Araneae: Mygalomorphae: Hexathelidae: Atracinae) are currently divided into two genera, *Atrax* and *Hadronyche* (Gray *et al.*, 1988). There are at least 40 different species, although many remain undescribed. The spiders are nocturnal, and most are terrestrial burrowers. They spend the day huddled in a small chamber at the bottom of a long burrow in the ground (Brunet *et al.*, 1998). The burrow is lined with a silk tube, hence the name "funnel-web spider". At night the spiders ascend the silken tube and position themselves at the burrow entrance waiting for insect prey to stumble into striking range. As a group these are probably the world's deadliest spiders (Miller *et al.*, 2000), with envenomation by the Sydney funnel-web spider, *Atrax robustus*, having caused at least 14 deaths prior to the introduction of antivenom in 1980 (Sutherland, 1980).

Atkinson and coworkers were the first to recognize the potent insecticidal properties of Australian funnel-web spider venom (Atkinson *et al.*, 1996). By testing the venoms of a taxonomically diverse group of Australian spiders (Atkinson *et al.*, 1996), they showed that funnel-web venom was the most lethal against larvae of the moth *Helicoverpa armigera* (cotton bollworm), a refractory agricultural pest. This led to the identification of a potent insect-specific neurotoxin (Atkinson *et al.*, 1996) that is now referred to as ω-atracotoxin-1 (Fletcher *et al.*, 1997). All toxins from Australian funnel-web spiders are referred to by the generic name atracotoxin as all of these spiders, regardless of genus, belong to the Atracinae subfamily (Fletcher *et al.*, 1997).

A detailed study of venom from the Blue Mountains funnel-web spider, *Hadronyche versuta* was conducted which led to the discovery of several families of novel insect-specific neurotoxins (Wang *et al.*, 2001; Wang *et al.*, 2000). It should be stressed that these toxins are highly insect-specific; they have no activity in any vertebrate system that has been tested thus far.

The first family of insect-specific neurotoxins isolated from Australian funnel-web spiders was the ω-ACTX-1 family (Atkinson *et al.*, 1998). These peptides each contain 36-37 residues with six strictly conserved cysteine residues that form three disulfide bonds. A single species of spider may contain six or more variants of the toxin, with some variants differing by only a single conservative residue substitution (Wang *et al.*, 1999). The ω-ACTX-1 families of toxins are lethal to a wide range of insects, including members from the orders coleoptera, orthoptera, lepidoptera, and diptera, but they are harmless when injected at high doses into newborn mice (Atkinson *et al.*, 1998; Fletcher *et al.*, 1997; Tedford *et al.*, 2001). Injection of toxin into American cockroaches (*Periplaneta americana*) causes a loss of locomotion, high frequency twitching of limbs with loss of righting reflexes, followed by paralysis and death (Fletcher *et al.*, 1997). Direct application of toxin to the cockroach metathoracic ganglion abolishes hind-limb reflexes, whereas the forelimbs, which are not directly innervated by motor neurons of the metathoracic ganglion, are unaffected (Fletcher *et al.*, 1997). These peptides can therefore be classified as depressant neurotoxins. Electrophysiological studies (Fletcher *et al.*, 1997) revealed that the phylogenetic specificity of the toxins derives from their ability to block insect, but not vertebrate, voltage-gated calcium channels (VGCCs).

These toxins display exceptional phylogenetic specificity, with at least a 10,000-fold preference for insect *versus* vertebrate calcium channels. These toxins are the most potent blockers of insect voltage-gated calcium channels reported to date, but they are virtually inactive on vertebrate ion channels, making them ideal biopesticide candidates. The structure of one of the toxins reveals a compact, disulfide-rich core and a structurally disordered lipophilic extension that is essential for channel blocking activity. (Wang *et al.*, 2001).

Recently isolated a second family of ω-atracotoxin that specifically block insect VGCCs (Wang *et al.*, 2001). These toxins contain 41-45 residues with six conserved cysteines that form three disulfide bonds. The sequences are unrelated to the ω-ACTX-1 toxins and there is no homology in the protein/DNA sequence databases. As for the ω-ACTX-1

family, a single spider may contain more than one variant of the toxin. These toxins cause a prolonged but reversible paralysis in a range of insects including dipterans. At moderate to high doses the paralysis is sustained for ~6 h in house crickets (*Acheta domestica*) and ~10 h in flies (*Musca domestica*).

There are several lines of evidence suggesting that the ω-ACTX-1 and ω-ACTX-2 toxins target different subtypes of insect VGCCs: (I) the ω-ACTX-1 toxins are lethal to orthopterans and dipterans, whereas ω-ACTX-Hv2a causes sustained but reversible paralysis in these insects; (ii) at saturating concentrations of toxin, ω-ACTX-Hv1a and ω-ACTX-Hv2a differ in the extent of calcium current blockage in various insect neurons (Wang *et al.*, 2001; Fletcher *et al.*, 1997); (iii) the 3D structures of the toxins are vastly different. These two families of VGCC blockers presumably play different but synergistic roles in funnel-web spider venom; it is likely that the ω-ACTX-2 toxins are important for the initial rapid incapacitation of prey whereas the ω-ACTX-1 toxins contribute to slower-onset, but irreversible, paralysis.

In order to help understand ω -Atracotoxin-Hv1a mechanism of action and to enhance its utility as a lead compound for insecticide development, the combination of protein engineering and site-directed mutagenesis is used to probe the toxin for key functional regions. First, hairpinless mutant is constructed in which the C-terminal beta-hairpin, which is highly conserved in this family of neurotoxins, was excised without affecting the fold of the residual disulfide-rich core of the toxin. The hairpinless mutant was devoid of insecticidal activity, indicating the functional importance of the hairpin. Subsequently developed a highly efficient system for production of recombinant toxin and then probed the hairpin for key functional residues using alanine-scanning mutagenesis followed by a second round of mutagenesis based on initial "hits" from the alanine scan. This revealed that two spatially proximal residues, Asn (27) and Arg (35), form a contiguous molecular surface that is essential for toxin activity. It is proposed that this surface of the beta-hairpin is a key site for interaction of the toxin with insect calcium channels (Tedford *et al.*, 2001).

From these studies, it can be concluded that the atracotoxins described above are useful lead compounds to develop transgenic plants for the following reasons:

1. They are active against a broad range of arthropod pests of agricultural and medical importance
2. They act on non-conventional neuronal targets, making them invaluable tools for the characterization of new insecticide targets
3. They are efficiently expressed in heterologous systems, making them suitable for biopesticide applications.

Once the gene has been identified, isolated and designed, it is now ready for transformation, which involves the delivery of a transgene into the nucleus of a recipient plant cell and incorporation into a chromosome so it can be passed onto offspring. Transformation is the most important step in every genetic engineering experiment.

During the last decades, a wide range of methods and approaches for gene transfer into plant cells has been explored, as microinjection, incubation of dry seeds with DNA, protoplast electroporation and transformation with PEG, electrophoresis etc. However, most of them had a limited success. Protoplast transformation by electroporation or PEG are mainly used to analyze expression of new chimeric gene constructions. Although the high efficiency of these methods, the tissue culture techniques necessary to regenerate plants from protoplasts are long and laborious (Carneiro *et al.*, 1999). In fact, the majority of transgenic plants obtained to date has been accomplished using only two approaches for gene transfer: the *Agrobacterium*-based vectors and the biolistic strategy (Brasileiro and Carneiro, 1998). The biolistic strategy is based on the acceleration of metal microparticles coated with DNA that carries the genes of interest (Sanford *et al.*, 1987). These microparticles are able to reach several layers of cells and penetrate, in a non-lethal way, into virtually every type of cell or tissue when accelerated to high velocities. Once delivered in the cell, the transferred DNA becomes stably incorporated into the genome of the bombarded cell of the explant. In the biolistic system, there is no limit to the range of species into which DNA can be introduced and it shows to be effective for both dicotyledonous and monocotyledonous plant species (Luthra *et al.*, 1997).

Virulent strains of *A. tumefaciens* and *A. rhizogenes*, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crow gall and hairy roots, respectively. These strains contain a large megaplasmid (more than 200 kb), which play a key role in tumor induction and for this reason it was named Ti plasmid, or Ri in the case of *A. rhizogenes*. Ti plasmids are classified according to the opines which are produced and excreted by the tumors they induce. During infection the T-DNA, a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated into the plant chromosome. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. The 30 KB virulence (*vir*) region is a regulon organized in six operons that are essential for the T-DNA transfer (*virA, virB, virD,* and *virG*) or for the increasing of transfer efficiency (*virC* and *virE*) (Hooykaas and Schilperoort, 1992; Zupan and Zambryski, 1995; Jeon *et al.*, 1998).

The initial results of the studies on T-DNA transfer process to plant cells demonstrate three important facts for the practical use of this process in plant transformation. Firstly, the tumor formation is a transformation process of plant cells resulting from transfer and integration of T-DNA and the subsequent expression of T-DNA genes. Secondly, the T-DNA genes are transcribed only in plant cells and do not play any role during the transfer process. Thirdly, any foreign DNA placed between the T-DNA borders can be transferred to plant cells, no matter where it comes from. These well-established facts, allowed the construction of the first vector and bacterial strain systems for plant transformation (Hookyaas and Schilperoort, 1992; Deblaere *et al.*, 1985; Hamilton, 1997; Torisky *et al.*, 1997).

Gene transfer systems based on *Agrobacterium* take advantage of the natural DNA transfer ability of this pathogen (Tinland, 1996; Zupan and Zambryski, 1995). Plant

transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most widely used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants (Smith and Townsend, 1907). However, *Agrobacterium tumefaciens* naturally infects only dicotyledonous plants and many economically important plants, including the cereals, remained inaccessible for genetic manipulation during long time due to lack of wound response. For these cases, alternative direct transformation methods have been developed (Shillito *et al.*, 1985; Potrykus, 1991), such as polyethyleneglycol-mediated transfer (Uchimiya *et al.*, 1986), microinjection (de la Pena *et al.*, 1987), protoplast and intact cell electroporation (Fromm *et al.*, 1985, 1986; Lörz *et al.*, 1985; Arencibia, 1995) and gene gun technology (Sanford, 1988). However, *Agrobacterium*-mediated transformation has remarkable advantages over direct transformation methods. It reduces the copy number of the transgene, potentially leading to fewer problems with transgene cosuppresion and instability (Koncz *et al.*, 1994, Hansen *et al.*, 1997). In addition, it is a single-cell transformation system and not forming mosaic plants, which are more frequent when direct transformation is used (Enriquez-Obregón *et al.*, 1997, 1998).

Agrobacterium-mediated gene transfer into monocotyledonous plants was not possible until recently, when reproducible and efficient methodologies were established on rice (Hiei *et al.*, 1994; Cheng *et al.*, 1998; Rashid *et al.*, 1996), banana (May *et al.*, 1995), corn (Ishida *et al.*, 1996), wheat (Cheng *et al.*, 1997) and sugarcane (Enriquez-Obregón 1997, 1998, Arencibia *et al.*, 1998).

The rapid development of rice transformation technology not only provides a valuable method for introducing useful genes into rice to improve important agronomic traits, but also helps in studying gene function and regulation (Hiei, *et al.*, 1997; Tyagi, *et al.*, 1999).

Endosperm, the edible part of rice grains, lacks several essential nutrients, such as provitamin A. Vitamin A deficiency causes symptoms ranging from night blindness to those of xerophthalmia and keratomalacia, leading to total blindness. *Agrobacterium*

transformation methods have been used to transfer genes encoding several *b*-carotene biosynthetic pathway enzymes into rice endosperm (carotenoid-free) in a single transformation effort (Ye *et al.*, 2000).

Phosphoenolpyruvate decarboxylase (PEPC) is the key enzyme of C4 plants. It catalyses the initial fixation of atmospheric CO_2, even in the presence of high concentrations of O_2. The photosynthetic pathway of C3 rice plants was modulated by transferring the C4 *PEPC* gene. The maize *PEPC* gene of the C4 photosynthetic pathway, including all the exons, introns, promoter and terminator sequence, was transferred into C3 rice through *Agrobacterium*-mediated transformation. The transgenic rice plants showed high levels of expression of the maize *PEPC* gene and accumulated high levels of PEPC protein (12% of the total leaf soluble protein) (Ku *et al.*, 1999).

It is concluded that there are many potential benefits of biotechnology for the rural poor in developing countries. Biotechnology may help achieve the productivity gains needed to feed the growing global population. It can help impart resistance to insect pests, diseases and abiotic stress factors, improve the nutritional value and enhance the durability of products during harvesting and storage. New crop varieties and biocontrol agents will reduce the reliance on synthetic pesticides and thus reduce farmers' crop protection costs. Research on genetic modification to achieve appropriate weed control will increase farm incomes and reduce the time farmers need to spend on weeding. Biotechnology would also offer cost-effective solutions to micronutrient malnutrition, such as vitamin A and iron. Research in biotechnology, on increasing the efficiency of utilizing the farm input could lead to the development of crops that use water more efficiently and extract phosphate from the soil more effectively. The development of cereal plants capable of capturing nitrogen from the air could contribute greatly to plant nutrition, helping the poor farmers, who often cannot afford fertilizers. By increasing the crop productivity, agricultural biotechnology could help reduce the need to cultivate new lands and conserve biodiversity. So there is an urgent need to use biotechnological techniques to increase the productivity potential of crops that play a major role in the life of the rural poor in the developing countries. In addition to the reduction in losses due to

insect pests, the development and deployment of transgenic plants with insecticidal genes will also lead to: (i) A major reduction in insecticide sprays; (ii) Reduced exposure of farm labour and non-target organisms to pesticides; (iii) Increased activity of natural enemies; (iv) Reduced amounts of pesticide residues in the food and food products; and (v) A safer environment to live. So biotechnology holds great hopes to provide food security to the rapidly growing population

MATERIALS AND METHODS

The materials used and the methods applied for this research project are discussed as under.

Gene Designing and Plasmid constructs

The sequence of the cry1Ac gene was deduced from the known amino acids sequence of the protein (Perlak *et al.*, 1991) and modified according to the plant prefered triplet codons as described in Appendix-15.The gene was commercially synthesized from Medigenomics, Germany. To facilitate the cloning of this gene under desirable expression cassette, few restriction sites were added to the flanking regions of the gene at the 5' as well as 3' ends of the gene. The plant expression vector was developed at the Plant Molecular Biology and Transformation Lab at NIBGE which contained the 2477 bp synthetic fragment containing rbcS promoter, transit peptide, intron, cry1Ac fused with ω-ACTX and ocs terminator. This vector was named as pSAK-IV (Fig.1a).

a) Development of cry1Ac construct with intron under rbcS promoter

In order to make different constructs, pSAK-IV was digested with *Kpn*1-*Pst*1 to isolate the cry1Ac from the ω-ACTX. The desirable fragment which consisted of rbcS promoter, transit peptide, intron and cry1Ac was eluted from the gel following protocols as described in Appendix-9. pJIT60 was also restricted with *Kpn*1-*Pst*1 to remove the double 35S promoter. The eluted fragment from pSAK-IV was ligated with pJIT60 (without double 35S) to produce pZS-60 following ligation procedure as described in Appendix-33. The purpose of cloning in pJIT60 was to take terminator from the pJIT60 to complete the expression cassette. The resultant pZS-60 was digested with the *Kpn*1 and *Xho*1 and expression cassette was released from the pZS-60 and was eluted from the gel and cloned in pGreen0029 binary vector to produce pZS-23.

b) Development of cry1Ac construct without intron under rbcS promoter

Another construct of cry1Ac was made in which the intron sequence was absent. For this purpose the pZS-60 was digested with *Nhe*1, as the intron is flanked by *Nhe*1. Self-ligation reaction was performed following ligation procedure as described in Appendix-34. The resultant vector was named as pZS-59. The pZS-59 was manipulated with *Kpn*1 and *Xho*1 to remove the cry1Ac expression cassette without intron and to clone in pGreen0029 binary vector. The resultant binary vector is called pZS-22.

c) Development of cry1Ac construct with intron under 35S promoter

To develop cry1Ac expression construct under 35S promoter with intron the pJIT60 was restricted with *Eco*R1 and used as a vector in cloning procedure. To prevent self-ligation, pJIT60 was treated with alkaline phosphatase after elution from agarose gel. Alkaline phosphatase treatment was given following the procedures as described in Appendix-35. Alkaline phosphatase removes 5' phosphatase group from DNA and RNA and in subsequent ligation reactions, this treatment prevents self-ligation of the vector and thereby facilitates ligation of other DNA fragments into the vector. Calf intestinal alkaline phosphatase (CIAP) was used in the reaction because it is effectively destroyed by protease digestion or heat. After alkaline phosphatase treatment phenol choloroform treatment is given following the procedure given in Appendix-36 to destroy the proteins and to precipitate the pure DNA. The transit peptide, intron and cry1Ac was lifted with *Eco*R1 from pZS-60 and ligated with alkaline phosphatase treated pJIT60. The resultant vector was named as pZS-62 in which the cry1Ac is under 35S.

Upon digestion with *Kpn*1 and *Xho*1 pZS-62 releases the cry1Ac cassette *with* intron under 35S promoter. This fragment was cloned in pGreen0029 binary vector. The resultant construct was given the name as pZS-35.

d) Development of cry1Ac construct without intron under 35S promoter

Intron is flanked by *Nhe*1 sites. To remove the intron the pZS-62 was treated with *Nhe*1 enzyme and self ligated to generate the pZS-61. cry1Ac expression cassette without intron under 35S promoter was lifted from pZS-61 while using *Kpn*1 and *Xho*1 enzymes and cloned at the same sites in the pGreen0029 binary vector. The resultant vector is named as pZS-34.

e) Development of bacterial expression construct

Synthetic cry1Ac fused with the ω-ACTX-Hv1a was amplified (Fig. 1b) from plasmid pSAK-IV using full-length primers (Appendix-2). The following PCR profile was optimized for the amplification of synthetic cry1Ac fused with the ω-ACTX-Hv1a.

PCR profile optimization

Denaturation temperature	94 °C	1 cycle
Time	5 min	
Denaturation temperature	94 °C	30 cycles
Time	1 min	
Annealing temperature	55 °C	
Time	1 min	
Extension temperature	72 °C	
Time	2.5 min	
Final extension temperature	72 °C	1 cycle
Time	10 min	
Hold	4 °C	

Eppendorf thermal cycler was used for PCR and amplified products were analyzed by electrophoresis on 1% agarose gels (Appendix-1) along with DNA marker (Appendix-19).

These specific primers were designed following the procedure as given in Appendix-24. The forward primer was based on cry1Ac 5' region while the reverse primer was designed based on the 3' region of the ω-ACTX-Hv1a gene. The amplified product was cloned with correct orientation in T/A cloning vector pTZ57R. The resultant vector was given name as pZSTA (Fig. 1c). *Eco*R1-*Hin*dIII enzymes were used to lift the cry1Ac fused with the ω-ACTX-Hv1a gene from the pZSTA. PET32a(+) was digested with *Eco*R1-*Hin*dIII and eluted from the gel and used as a vector in cloning strategy. The *Eco*R1-*Hin*dIII lifted fragment of pZSTA was ligated with the pET32a(+). The resultant bacterial expression construct was named as pSAK-V.

Expression of pSAK-V in *E. coli* strain BL21

The pET system is considered the most powerful system developed for the cloning and expression of recombinant proteins and prokaryotic systems. Target genes are cloned in pET plasmids under the control of strong bacteriophage T7 transcriptional and translational signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. The T7 RNA polymerase is so selective and active that, when fully induced, almost all of the cell's resources are converted to target gene expression; the designed product can comprise more than 50% of total cell protein in few hours after induction. It is also possible to attenuate expression levels simply by lowering the concentration of inducer. Decreasing the expression level may enhance the soluble yield of some target proteins. Another important benefit of this system is its ability to maintain target genes transcriptionally silent in the uninduced state. The electrocompetent cells of BL21 were prepared following the procedure as described in Appendix-25. The BL21 host containing a chromosomal copy of T7RNA polymerase gene under *lacUV5* promotor is transformed with pSAK-V following the procedure as described in Appendix-25. The *lacUV5* promoter is responsible for driving the expression of T7RNA polymerase. It is inducible by (IPTG) isopropyl beta D thiogalactopyranoside. pET32a (+) carries the target gene under T7 promoter. When IPTG is added it activate the *lacUV5* promoter and turn on synthesis of T7 RNA polymerse. This in turn transcribes the gene in pET vector.

After transformation and confirmation of pSAK-V in BL21. Expression of the target gene is induced by the addition of IPTG (1 mM IPTG). Colony was picked from a freshly streaked plate and inoculated 3 ml LB containing the ampicilin and chloramphenicol for the plasmid and BL21 host strain respectively in a 15 ml falcon tube. This 3 ml culture was Incubated with shaking at 37°C for over night. 50 ml of LB containing ampicilin and chloramphenecol was inoculated with overnight culture. This 50 ml of medium was incubated at 37°C with shaking until OD at 600 nm reaches 0.4–1 (0.6 recommended; about 3 h). Uninduced control sample was removed and to the remainder, IPTG was added from a 100 mM stock to a final concentration of 1 mM. Samples were taken at regular intervals after induction i.e 0.5h, 1h, 1.5h, 2.0h, 2.5h. The cells were harvested by centrifugation at 13400 rpm for 1.5 min. The cells were frozen at -20°C for 30 minutes.

The harvested cells were thawed, sonicated and then BugBuster Protein Extraction Reagent method was used to gently disrupt the cell wall of *E. coli* resulting in the liberation of soluble protein. 10% SDS PAGE was prepared following the procedure as described in Appendix-27. The expression of target genes was assessed quickly by analysis of total cell protein on an SDS-polyacrylamide gel followed by Coomassie blue staining and destaining procedures as given in Appendix-31.

Plasmid isolation from *E. coli*

For the isolation of pSAK-IV plasmid from *E. coli*, protocol was followed as described in Appendix-6.

Transformation of pSAK-IV in *Agrobacterium* strain LBA4404

Electrocompetent cells of *Agrobacterium* strain LBA4404 already harbouring pSoup plasmid were prepared using the procedures as described in the Appendix-12. pSAK-IV plasmid was transformed into *Agrobacterium* strain LBA4404 through electroporation method as described in the Appendix-13.

Plant Transformation

Two types of plant transformations were carried out.

Transient

Stable

For transient transformation the Biolistic gun was used. Gold particles (1 μm in diameter) (BIO-RAD) were used as DNA carriers. DNA was precipitated onto the particles according to the $CaCl_2$-spermidine method as described in the Appendix-23. PDS-1000/He Biolistic gun bombardment chamber was sprayed with ethanol and allowed to air dry in sterile conditions, in laminar flow cabinet. Rupture discs, stopping screens, macro-carriers and macro-carrier holders were dipped in 70% ethanol in sterilized petri plates in laminar airflow cabinet. After 10 minute ethanol was completely removed and all these supplies were allowed to dry under the aseptic conditions.

Bombardment by PDS-1000/He Biolistic Gun

a. Loading the rupture disc: Unscrewed the rupture disc retaining cap from the gas acceleration tube and placed the ruptured disc of 1100 psi burst pressure in the recess of the rupture disc cap. The rupture disc-retaining cap was screwed on to the gas acceleration tube.

b. Loading the Microcarrier Launch assembly: Micro carrier launch assembly was removed from the sample chamber and unscrewed the macro carrier cover lid from the assembly. Placed the sterile stopping screen on the stopping screen support. Macro carrier holder with DNA coated micro carriers was placed on the top rim of the fixed nest and placed the lid on the assembly and screwed. Then the macro carrier launch assembly was placed in the sample chamber in the second slot from the top.

c. Positioning the sample:

Petri dish containing the sample was put on the petri dish holder and placed the petri plate holder on stage III approximately at a distance of 9 cm from the micro carrier launch assembly. Closed and latched the sample chamber door.

Firing the PDS-1000/He Biolistic Gun:

PDS-1000/He power was turned on and pressure was set at 1400 psi from the gas regulator fixed at the He cylinder. The vaccum pump was turned on and set the vaccum switch on PDS 1000/He to VACUUM position tom evacuate the chamber up to 27 inches of mercury. Then the vaccum switch was put in the HOLD position. The FIRE switch was pressed to allow gas pressure to build in the acceleration tube. Released the FIRE switch immediately after the disc ruptured. The vaccum was released, opened the sample chamber door and removed the petri plate out. Also removed the macro carrier launch assembly. Discarded the macro carrier and stopping screen from assembly. The rupture disc-retaining cap was unscrewed from gas acceleration tube and removed the ruptured disc. Repeated the whole procedure for other bombardements. After bombardment the biolistic gun was turned off. After bombardment the explants were spread on the medium with the cut ends touching the surface of the medium. The plates were sealed with parafilm and incubated at 25±1°C in dark for 48 hours.

The leaf discs were ground, and then BugBuster buffer was added followed by strip analysis for cry1Ac.

Stable transformation of pSAK-1V through *Agrobacterium* mediated transformation in *Nicotiana tabacum cv spade.*

Materials required

Sterilized nano pure water for rinsing seeds, whatman No.1 filter papers (9cm) and some filter papers to fit the base of 9 cm, petri dishes, scalpel holder, forceps and spatula.

Transformation

1. Single colony of *A. tumefaciens* having pSAK-1V and pSOUP was picked and inoculated in 25 ml of liquid LB medium (Appendix-5) having 10 µg/ml

tetracycline and 50 µg/ml Kanamycin in 50/100 ml flask and shaken at the rate of 150-250 rpm for 48 hrs in dark.

2. Cut leaf discs from top 2-3 leaves under aseptic conditions and placed on MS0 medium (Appendix-20), having 15 discs per plate. The leaf discs were placed upside down. Plates were sealed with parafilm and incubated at 16/8 light and dark cycle at 25± 1°C. Explants were left to pre incubate for 48 hours.

3. Poured *Agrobacterium* suspension into sterile petriplate and place all leaf discs in the bacterial suspension for 25-30 minutes with gentle shaking after regular intervals.

4. The leaf discs were removed and blotted dry on sterile filter paper and transfered to co-culture medium (Appendix-17). Plates were sealed with parafilm.

5. Co-culture was performed for 48 - 72 hours at 25 °C (depending on the growth of bacteria) under light conditions.

6. Leaf discs were removed from co-culture medium and placed on selection medium (Appendix-18) after washing with cefotaxime to remove the excess of *Agrobacterium* (250mg/L).

7. Leaf discs were placed with cut edges in contact with the medium.10 -15 leaf discs were placed per petri plate, plates were sealed with para film and incubated under 16 hours photoperiod at 25° C for two weeks.

Regenerated leaf disc were transferred to fresh selection medium (MS0 selection medium; Appendix-18). When shoots became large for the petri dishes, they were shifted into magenta containers.

Rooting

Shoots were cut from callus when at least 1 internode was formed. Shoots were transferred into rooting media with reduced antibiotic concentration.

Transfer to soil

Plants were transferred to sterile soil pots and kept at 25± 2°C under 16 hours photoperiod for hardening. Media was removed by washing under running tap water. Plants were covered with polyethylene bags to retain humidity. After 7-10 days, envelopes were backed off to reduce humidity gradually until plants were acclimatized to ambient humidity and temperature conditions.

Control experiment was also performed. Non-transformed leaf discs were placed on two different media, one having antibiotic and other without antibiotic, to observe the effect of selection agent (Kanamycin).

PCR Analysis of Transgenic plants

Putative transgenic plants were screened through PCR analysis. The forward primer (5'-TGC CAA CTT GCA TCT CTC TG-3') and the reverse primer (5'-TCG GTG AAT CCA TGA GAA CA-3') were based on the internal sequence of the cry1Ac gene.

The PCR profile is given below

Denaturation temperature	94 °C	1 cycle
Time	5 min	
Denaturation temperature	94 °C	30 cycles
Time	1 min	
Annealing temperature	55 °C	
Time	1 min	
Extension temperature	72 °C	
Time	1 min	
Final extension temperature	72 °C	1 cycle
Time	10 min	
Hold	4 °C	

Eppendorf thermal cycler was used for PCR and amplified products were analyzed by electrophoresis on 1% agarose gels (Appendix-1) along with DNA marker (Appendix-19).

Total Genomic DNA extraction was done using the procedure as given in Appendix-8 from 12 transformed tobacco plants.

Western blot analysis

Transgenic tobacco plants were analyzed for protein expression by SDS PAGE analysis. Leaf tissue was frozen in liquid nitrogen, ground to a fine powder and then the total soluble protein was extracted using the Trizole reagent. The protein concentration of the supernatant was determined by the Bradford assay. Fifty µg of protein from both transformed and non-transformed plants was run per lane on 10% SDS-PAGE gels. The protein was run quickly on SDS-polyacrylamide gel. Western blot analysis was done using polyclonal antibodies raised against fused ω-ACTX-Hv1a protein. The different buffers used in western blots are given in Appendix-32.

Nitrocellulose membrane was wetted in water and then equilibrated for 15 minutes in transfer buffer. Gel was also equilibrated in transfer buffer for 15 minutes, especially if transfer buffer contains MeOH which will cause the polyacrylamide gel to shrink slightly.Transfer "sandwich" was assembled. During sandwich assemblance the following points were kept in mind.

1. Avoid bubbles which will block transfer of proteins
2. Re-wet layers in transfer buffer
3. Assemble by laying layers down beginning near the center and lowering the edges
4. Roll pipette or test tube across sandwich to expel trapped air bubbles
5. Trim layers to size of gel to prevent "short-circuiting" which will reduce transfer efficiency

6. Use enough Whatmann 3MM filter paper to make sandwich tight in cassette
7. Lower cassette into electrophoresis tank (wet electroblotting system) or between electrodes

Protein were transferred at lower voltage i.e 50 volts or in the cold room because swelling may occur during transfer due to excessive increase in temperature. lower acrylamide concentrations was used to better transfer of higher molecular weight proteins. The protein transformation on membrane was confirmed by using the pre-stain marker.

Different Steps in western blot

1. Transfer proteins to nitrocellulose
2. Orient and mark blot
3. Block nonspecific binding sites using the BSA
4. Bind primary antibody over night at cold room.
5 Three time washing each for 15 minutes
6. Bind secondary antibody over night
7.Three time washing each for 15 minutes
8. Detection using the AP substrate

Insect Bioassays:

For insect bioassays, potted plants were exposed to 2^{nd} instar larvae of spodoptera littaralis (army worm). Four larvae were placed on each plant. Data was collected every 24h until 96h after exposure to the armyworm larvae.

RESULTS

Bacillus thuringiensis truncated Cry1Ac gene (1839 bp) was designed and optimized for enhanced expression in plants at the Plant Biotechnology Division of the National Institute for Biotechnology and Genetic Engineering (NIBGE) (Patent pending). Similarly the ω-atracotoxin-Hv1a (ω-ACTX-Hv1a) gene from funnel web spider (*Hadronyche versuta*) was designed and optimized at NIBGE and commercially synthesized (Mukhtar *et al.*, 2004). The present study describes the cloning, development of different constructs based on these two genes and subsequent transformation of plants (tobacco) for characterization of these genes to determine their ability and efficacy for control of major insect pests of cotton.

a) Cloning of Cry1Ac and ω-ACTX-Hv1a genes in bacterial expression vector

The 1968 bp DNA fragment DNA containing the full length synthetic Cry1Ac fused with the ω-ACTX-Hv1a gene was amplified from plasmid pSAK-IV (Fig. 1a) using full-length primers (Appendix-2). The forward primer was based on the cry1Ac 5' region while the reverse primer was designed based on the 3' region of the ω-ACTX-Hv1a gene. These primers amplified 1968 bp fragment as shown in Fig. 1b. This 1968 bp fragment was cloned in T/A cloning vector pTZ57R and the orientation of the insert was confirmed using *Sac*1. Since there is a unique *Sac*1 site in pTZ57R in addition to one *Sac*1 site in the insert, therefore, the digestion with *Sac*1 should produce two fragments of either 742 bp and 4112 bp (orientation I) or 1300 and 3554 (Orientation II). Fig 1d shows that upon digestion with *Sac*1, the resultant vector produced two fragments of 742 and 4112 bp indicating that the insert has been cloned in correct orientation I. The resultant vector was named as pZSTA. (Fig 1c).

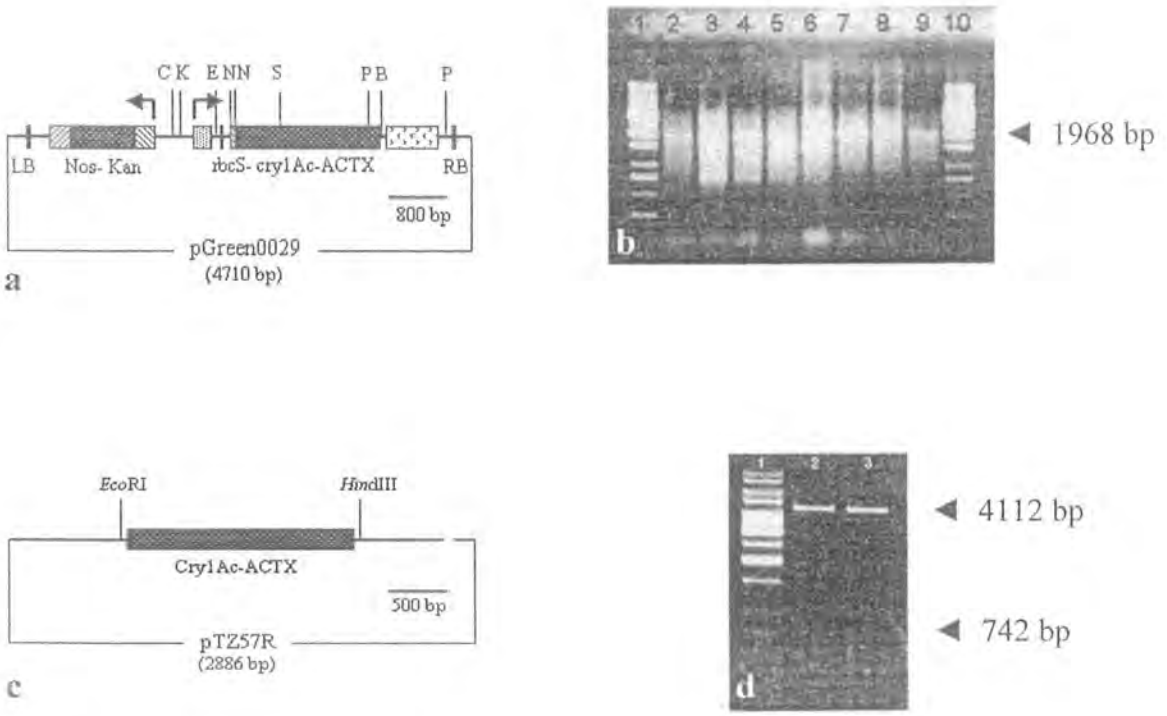


Fig. 1: Different steps in cloning of Cry1Ac-ω-ACTX genes. **a)** Physical map of plasmid pSAK-IV, (C, *Cla*1; K, *Kpn*1; E, *Eco*R1; N, *Nhe*1; S, *Sac*1; P, *Pst*1; B, *Bam*H1; RB, right border; LB, left border); **b)** PCR amplification of the full length synthetic Cry1Ac-ω-ACTX-Hv1a gene (1968 bp) from pSAK-IV; lane 1 and 10, 1 kb DNA ladder ; lanes 2-9 1968 bp fragment amplified from pSAK-1V; **c)** cloning of PCR amplified 1968 bp fragment in pTZ57R to from pZSTA; **d)** confirmation of the cloning through digestion of pZSTA with *Sac*1; lane 1, 1 kb DNA ladder ; lanes 2 and 3, restriction with *Sac*1.

pZSTA was digested with *Eco*R1-*Hin*dIII to clone the Cry-ACTX genes in frame with the coding TAG sequence of the pET32a(+) as shown in Fig. 2a. The resultant vector was named as pSAK-V (Fig. 2b). The cloning was confirmed through digestion of pSAK-V with *Eco*R1 and *Hin*dIII, which should remove the insert (2048 bp fragment) from the parent vector. Upon digestion with these enzymes, pSAK-V released the 2048 bp fragment along with the vector backbone (5900 bp) as shown in Fig. 2d. This confirmed the cloning of cry-ACTX genes in pET32a(+). To further confirm the cloning in pET32a(+), pSAK-V was digested with *Sac*1-*Hin*dIII, which should produce three fragments (i.e. vector backbone 5900 bp, 1318 bp and 730 bp). Fig. 2e shows that upon digestion with *Sac*1-*Hin*dIII, pSAK-V produced three fragments of the expected sizes thus confirming the cloning of Cry-ACTX genes in pET32a(+). Further confirmation of the clone was made by PCR using Cry1Ac specific primers which produced 883 bp internal fragment of the cry1Ac gene as shown in Fig. 2c indicating that the insert has successfully been cloned into pET32a(+).

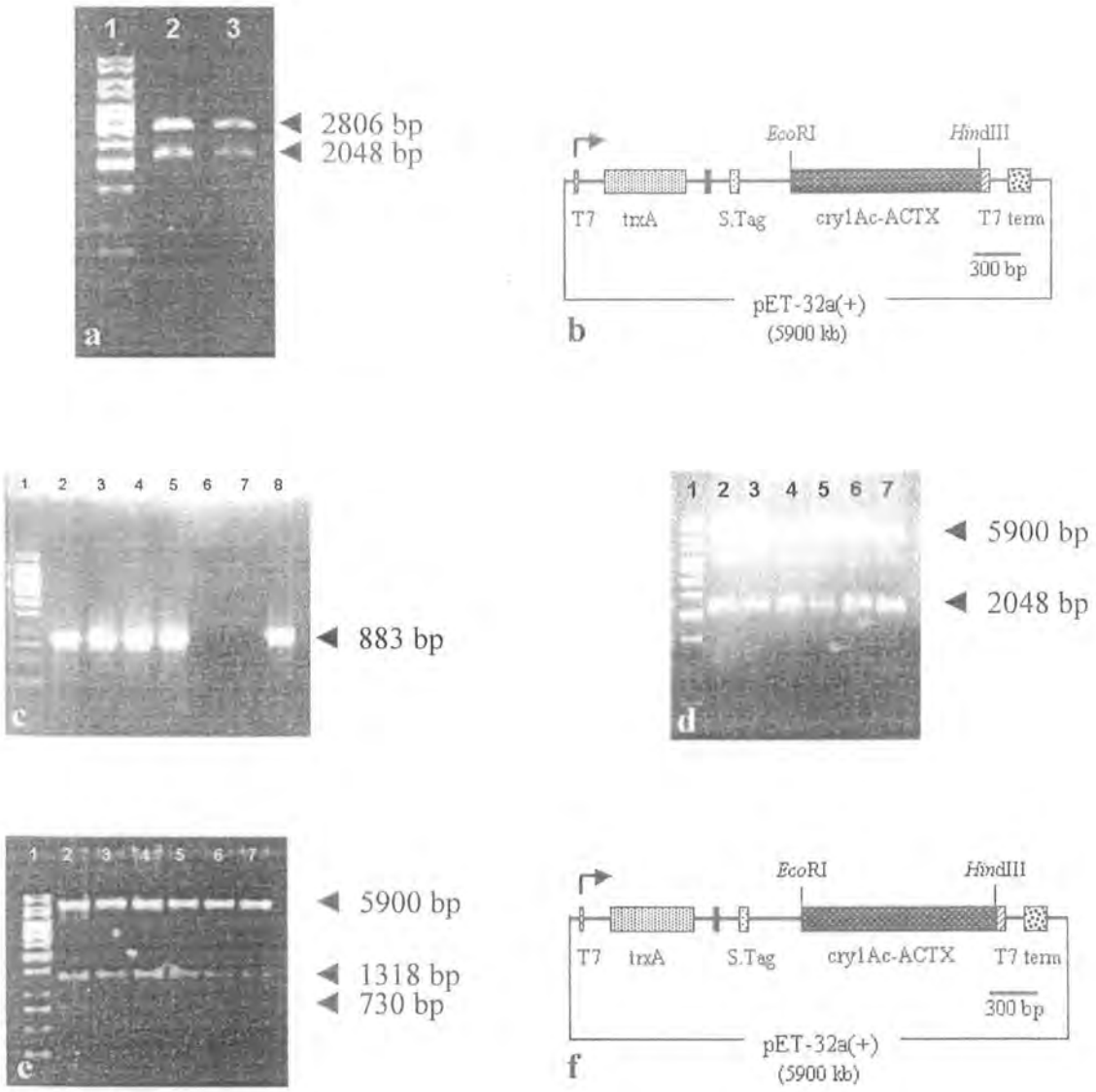


Fig. 2: a) Digestion of pZSTA with *Eco*R1 and *Hin*dIII lanes 2 and 3; lane 1, 1 kb DNA ladder;**b)** cloning of EcoR1-HindIII fragment (2048 bp) in pET32a(+) to from pSAK-V; **c)** PCR amplification for confirmation of pSAK-V, lane 1, 1 kb DNA ladder (Appendix-19); lanes 2-5, 883 bp fragment amplified from pSAK-V; lane 6, Negative control pET32a(+); lane 7, water control; lane 8, plasmid (pSAK-1V) control; **d)** confirmation of cloning of Cry1Ac –ACTX genes in pET32a(+) through restriction analysis, lane 1, 1 kb DNA ladder; lanes 2-7, pSAK-V digested with *Eco*R1-*Hin*dIII; **e)** confirmation of pSAK-V through restriction analysis with *Sac*1-*Hin*dIII, lane 1, 1 kb DNA ladder; lanes 2-7, pSAK-V digested with *Sac*1-*Hin*dIII; **f)** physical map of the resultant vector pSAK-V.

pSAK-V was introduced into *E. coli* strain BL21 through electroporation. The selected bacterial colonies were analysed for the presence of pSAK-V through PCR analysis using cry1Ac specific primers (Appendix-2). The amplification of 883 bp fragment (Fig.3) confirmed the transformation of plasmid pSAK-V in *E. coli* strain BL21.

After transformation and confirmation of pSAK-V in BL21, expression of the target gene is induced by the addition of IPTG (1 mM IPTG). IPTG was added from a 100 mM stock to a final concentration of 1 mM. Samples were taken at regular intervals after induction i.e 0.5h, 1h, 1.5h, 2.0h, 2.5h. The samples were processed and analysed on SDS PAGE. The intensity of desired protein (74 kDa) was increased with increase in time after induction i.e. 30, 60, 90, 120 and 150 minutes as shown in Fig 4.

In another experiment after processing of samples immunoblot was done for cry1Ac detection. In control sample, the total soluble protein was extracted from IPTG induced BL21 strain transformed with pET 32a(+) and was used for immunoblot. The cry1Ac was not detected in control sample, while a strong signal appeared when total soluble protein from BL21 strain transformed with pSAK-V was used in immunoblot. (Fig. 5).

Fig. 3: Agarose gel electrophoresis of PCR amplified product of *E. coli* strain BL21 transformed with pSAK-V using cry1Ac specific primers. Lane 1-5, selected BL21 colonies; lane 6, non-transformed BL21 culture; lanes 7-10, selected BL21 colonies; lane 11, 1 kb DNA ladder.

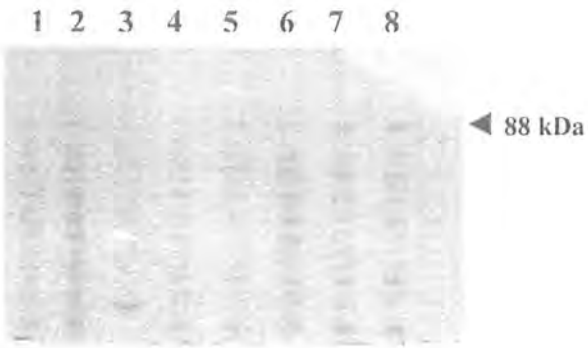


Fig.4: SDS-PAGE analysis of total soluble protein isolated from bacterial strain BL21 transformed with pSAK-V. From left to right Lane 1-2, un-iduced protein; lane 3, protein marker (MBI Fermentas #SM 0441; lane 4, protein induced after 30 minutes; lane 5, protein induced after 60 minutes; lane 6, protein induced after 90 minutes; lane 7, protein induced after 120 minutes; lane 8, protein induced after 150 minutes.

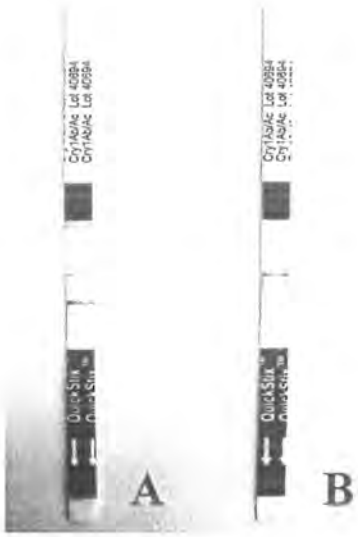


Fig.5: Immuno blots showing the detection of expressed *Bacillus thuringiensis* Cry1Ac protein in bacterial strain (BL21). A, BL21 having pET32-a with cloned Cry1Ac gene; B, BL21 having wild type vector (pET32-a)

Transient transformation was done through Biolistic gene gun. Gold particles (1 μm in diameter) (BIO-RAD) were used as DNA carriers. Initially plasmid DNA was precipitated onto the gold particles according to the $CaCl_2$-spermidine method. The whole process was carried out in complete sterilized conditions. The leaves were detached from the in-vitro plants and cuttings of leaves were used as target material. The pieces of leaves were placed on MS0 medium. The gold particles coated with the pSAK-IV plasmid were fired on the leaf discs. After bombardment the explants were spread on the medium with the cut ends touching the surface of the medium. The plates were sealed with parafilm and incubated at 25±1°C in dark for 48 hours.

The leaf discs were ground, and then BugBuster buffer was added followed by strip analysis for cry1Ac. The results were found negative for transient assay while the same immunoblot has given positive results in case of bacterial expression system as shown in Fig. 5. The reason of negative results of transient assay may be some sort of post-translational modification of protein in eukaryotic system. The reason of positive result in prokaryotic system may be the absence of post-translational modification. And later on processing of samples with disruption which contain SDS which will convert all protein to rod shape and mercaptoethanol which will break disulphide linkages in protein.

b) Cloning of Cry-ACTX genes in plant expression vector:

The plant expression vector pSAK-IV having Cry1Ac fused to the ω-atracotoxin-Hv1a gene used in this study was already made in the Plant Molecular Biology and Transformation Lab of the Plant Biotechnology Division, NIBGE. The strategy used to construct this plant expression vector is as follows. The synthesis of truncated cry1Ac fused to the ω-ACTX 2477 bp fragment was made commercially. Several restriction sites as shown in Fig. 6a were introduced to facilitate cloning and manipulation. *Cla*I and *Bam*H1 sites were introduced upstream and downstream of the fragment, respectively. The 107 bp intron had flanking *Nhe*1 sites on either side. ω-ACTX can be easily removed from the cry1Ac by *Pst*1 and *Bam*H1.

The first step in the construction of pSAK-IV involved the removal of *ocs* terminator from pN6 (Appendix-37) by *Xba*1-*Not*1 and its cloning at the same sites in pGreen0029. The resultant vector was named pZS-19. In the second step, the 2477 bp fragment containing the rbcS promoter, transit peptide, intron, truncated Cry1Ac gene fused to the ACTX-Hv1a gene was cloned at *Cla*1-*Bam*HI sites in pZS-19. The resultant vector was named as pSAK-IV (Fig. 6a).

c) Cloning of Cry1Ac synthetic gene in plant expression vector:

i) Cloning of cry1Ac gene with intron under rbcS promoter:

pSAK-IV was digested with *Kpn*1-*Pst*1 to remove 2349 bp fragment (Fig.6b), which contained the rbcS promoter, transit peptide, intron and the cry1Ac coding sequence. Plasmid pJIT60 was also digested with *Kpn*1-*Pst*1 and run on 0.8% agarose gel. It produced two fragments of 700 bp and 3020 bp (Fig. 6c) The 700 bp fragment contains the double 35S promoter while the 3020 bp fragment contained the CaMV terminator and the vector backbone. 2349 bp *Kpn*1-*Pst*1 fragment of the pSAK-IV was eluted from the gel and ligated with 3020 bp fragment of pJIT60. The resultant vector was named as pZS-60 (Fig. 6d). The cloning of Cry1Ac in pJIT60 was confirmed by restriction digestion. pZS-60 was digested with *Kpn*1-*Pst*1 to remove the insert (2349 bp) from the vector backbone (3000 bp) as shown in Fig. 6e. Out of the twelve colonies analysed by restriction digestion with *Kpn*1-*Pst*1, only three were non-recombinant indicating the absence of the insert, while nine colonies (represented by Fig. 6e lanes 3-11) showed cloning of cry1Ac gene in pJIT60. Further confirmation was made by digestion of pZS-60 with *Sac*1. It produced two fragments each of 4137 and 1212 bp, which further confirmed the cloning of cry1Ac in pJIT60 (Fig. 6e). The expression cassette (3049 bp) from pZS-60 containing the rbcS promoter, transit peptide, intron, cry1Ac coding sequence and the CaMV terminator was lifted with *Kpn*1-*Xho*1 (Fig. 7b) and cloned at the same restriction sites in the binary vector pGreen0029. The resultant vector was named as pZS-23 (Fig. 7e). The cloning of cry1Ac expression cassette was confirmed first by restriction digestion followed by PCR. PZS-23 was digested with *Kpn*1-*Xho*1. The release of 3049 bp expression cassette along with 4710 bp vector backbone

confirmed the cloning as shown in Fig. 7c. The cloning of Cry1Ac was then confirmed by PCR amplification of the cry1Ac internal fragment using specific primers (Appendix-2) The amplification of 883 bp fragment shown in Fig. 7d confirmed the cloning of cry1Ac expression cassette in the binary vector.

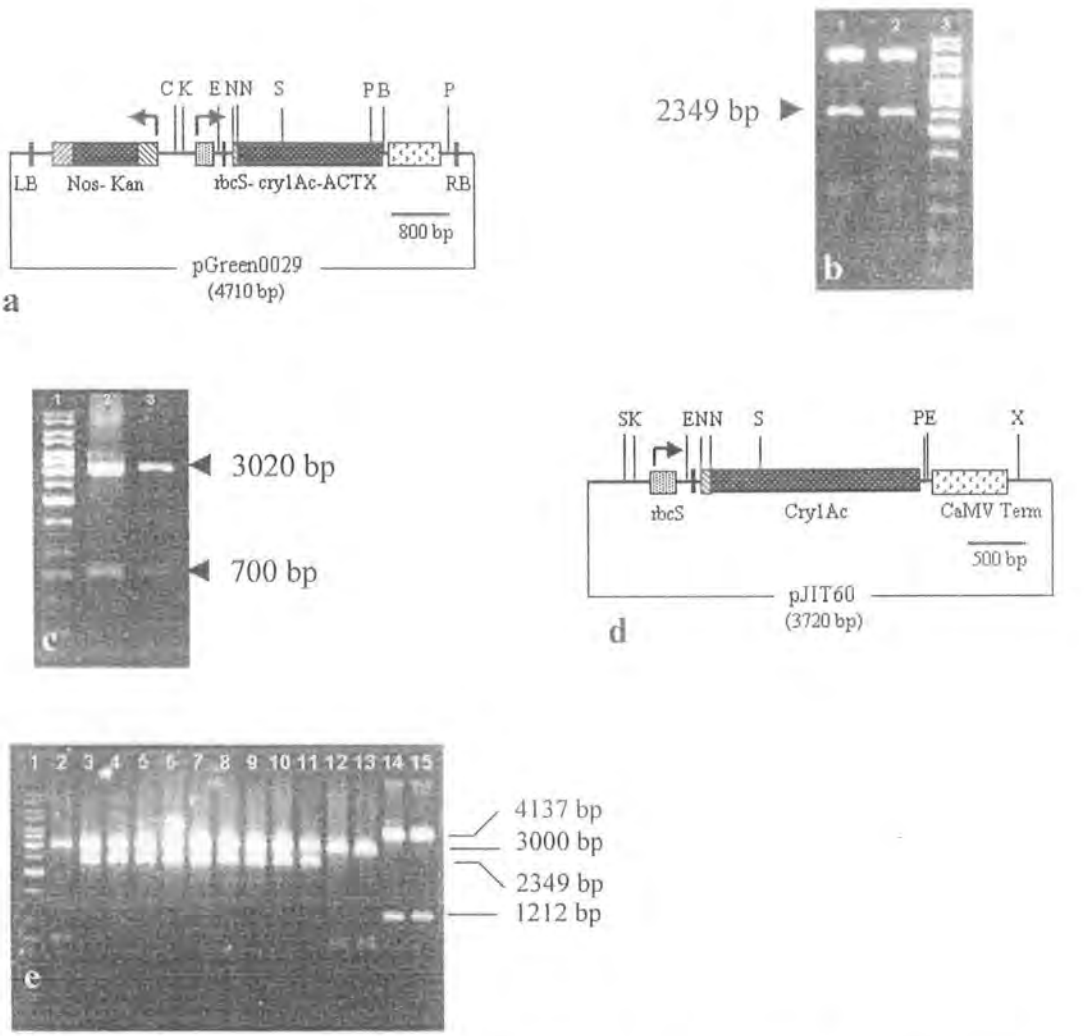


Fig. 6: a) Physical map of plant expression vector pSAK-IV; **b)** Agarose gel electrophoresis of pSAK-IV digested with *Kpn*1-*Pst*1 (lanes 1 and 2); lane 3, 1 kb DNA ladder ; **c)** agarose gel electrophoresis of plasmid pJIT60 digested with *Kpn*1-*Pst*1 (lanes 2 and 3); lane 1, 1 kb DNA ladder **d)** Physical map of resultant vector pZS-60; **e)** Confirmation of cloning of cry1Ac under rbcS promoter with intron in pJIT60, lane 1, 1 kb DNA ladder; lanes 2-13 represent pZS-60 digested with *Kpn*1-*Pst*1; lanes 14-15 represent pZS-60 digested with *Sac*1. Restriction sites are indicated by following abbreviations: C, *Cla*1; K, *Kpn*1; E, *Eco*R1; N, *Nhe*1; S, *Sac*1; P, *Pst*1; B, *Bam*H1; X, *Xho*1.

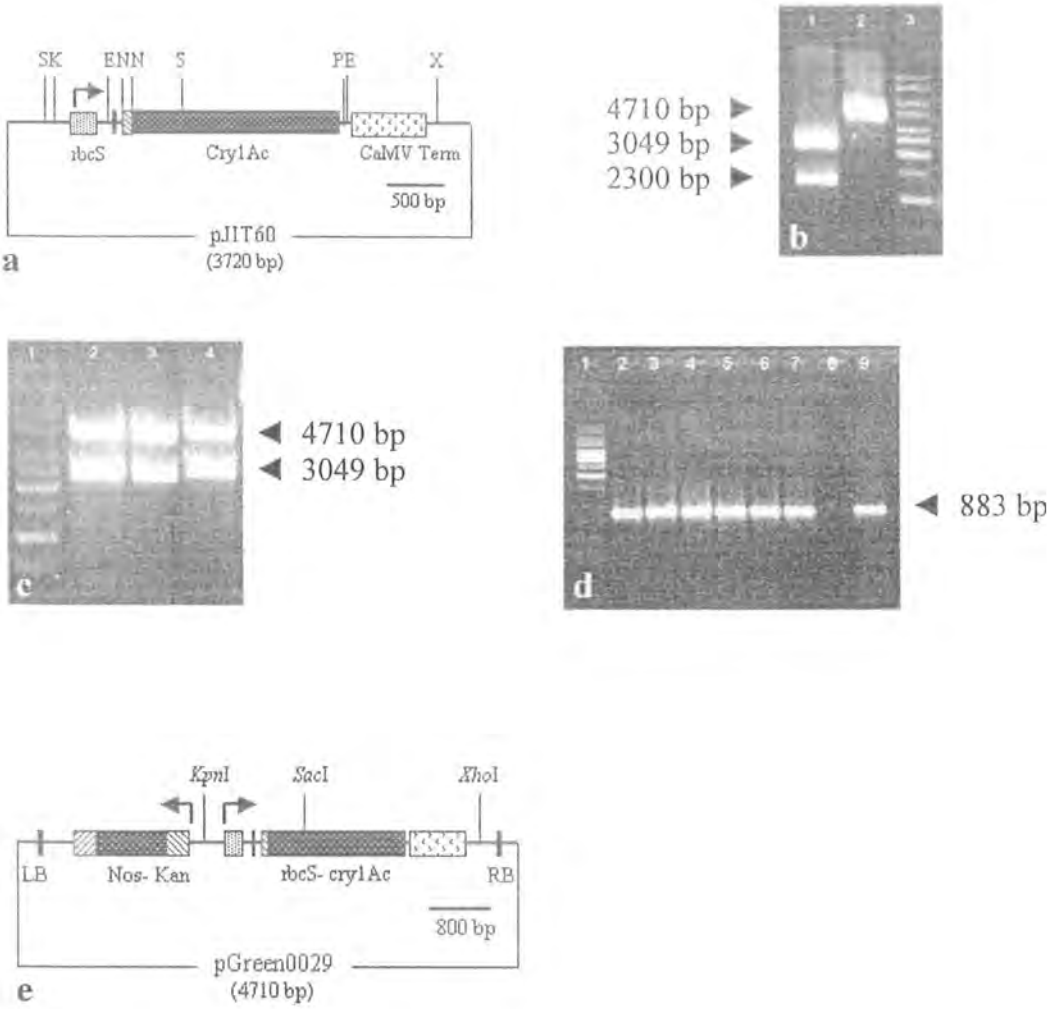


Fig. 7: a) Physical map of plasmid pZS-60; **b)** Agarose gel electrophoresis of pZS-60 and pGreen0029 digested with *Kpn*1-*Xho*1 (lane 1 and lane 2, respectively; lane 3, 1 kb DNA ladder ; **c)** confirmation of pZS-23 by agarose gel electrophoresis through restriction digestion with *Kpn*1-*Xho*1 (lanes 2-4), lane 1 represents 1 kb DNA ladder; **d)** confirmation of pZS-23 by agarose gel electrophoresis of PCR amplified product using cry1Ac specific primers (Appendix- 2); **e)** Physical map of the resultant vector pZS-23. Restriction sites are indicated by following abbreviations: S, *Sac*1; K, *Kpn*1; E, *Eco*R1; N, *Nhe*1; P, *Pst*1; X, *Xho*1.

ii) Cloning of cry1Ac gene without intron under rbcS promoter:

Another plant expression vector was designed which was almost similar to pZS-23 but without intron. For this purpose pZS-60 (5349 bp) was digested with *Nhe*1 to remove the 107 bp intron (flanked by *Nhe*1 sites on either side). The digest plasmid was run on 0.8% agarose gel and the 5242 bp fragment was eluted and self-ligated. The resultant vector was named as pZS-59 (Fig. 8c). To confirm whether the intron has been removed from the cry1Ac expression cassette of pZS-59, the plasmid was digested with *Kpn*1-*Nhe*1 to produce two fragments of 4849 and 400 bp. The digestion was run on 2% agarose gel (Fig. 8b). This confirmed that the intron has been removed from the cry1Ac expression cassette otherwise another fragment of 107 bp should also have been produced because the digestion was run on 2% agarose gel. Since the 107 bp intron fragment is small enough which may not been seen even on 2% gel, therefore it was required to further confirm the removal of intron from pZS-59. For this purpose, the cry1Ac expression cassette (2942) from pZS-59 first removed with *Kpn*1-*Xho*1 as shown in Fig. 8d and cloned at the same restriction sites in pGreen0029. The resultant vector was named as pZS-22 (Fig. 8e). In order to confirm the removal of intron from the cry1Ac expression cassette of pZS-22, it was compared with pZS-23 (having cry1Ac expression cassette with intron). The plasmid DNAs of pZS-22 and pZS-23 were digested with *Eco*R1-*Sac*1. Both the vectors should produce four fragments as shown in Fig. 9c. The three fragments (4877, 1137 and 750 bp) were of equal size in both pZS-22 and pZS-23 while the fourth fragment varied in size. pZS-23 having the intron produced the fourth fragment of a larger size (995 bp) compared to pZS-22 (without intron), which produced the fourth fragment of 888 bp as shown in Fig. 9c.

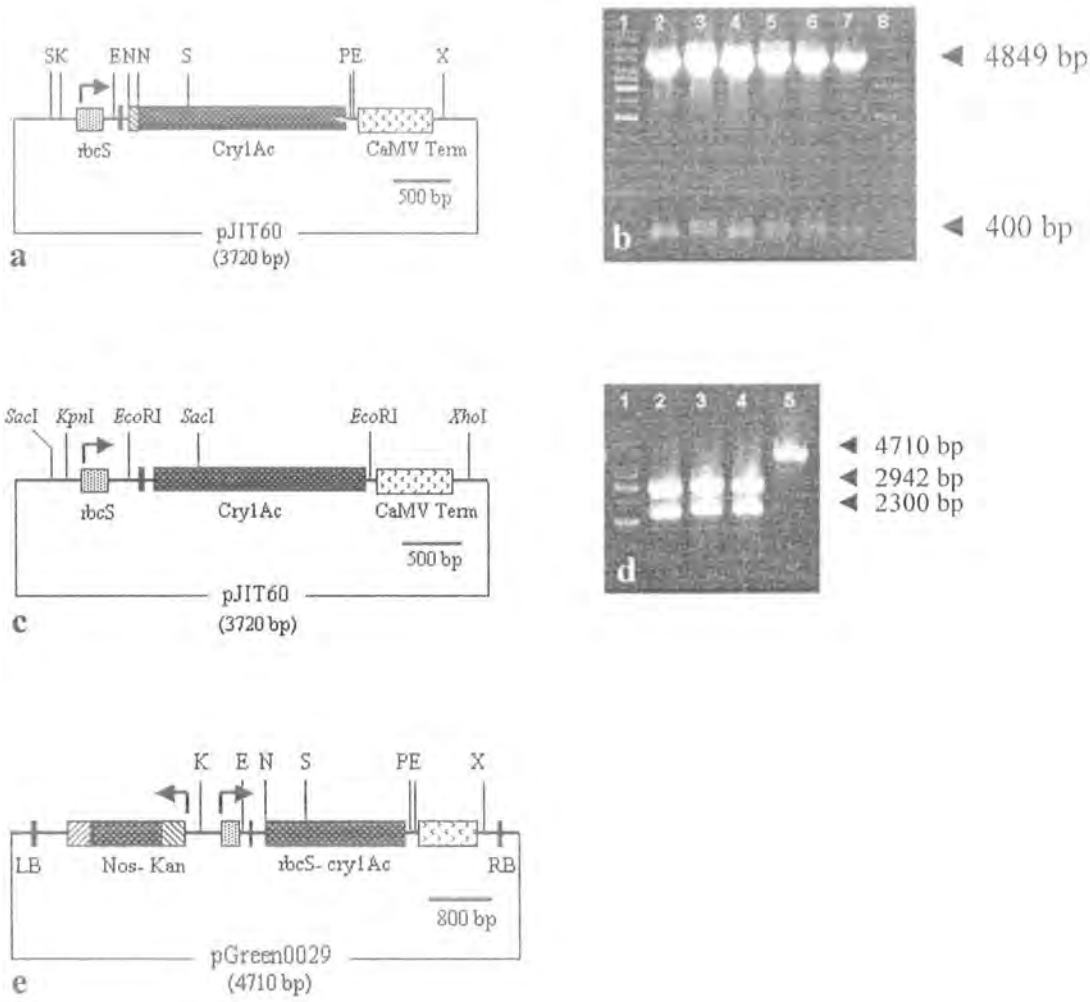


Fig. 8: a) Physical map of pZS-60; **b)** Agarose gel electrophoresis of pZS-60 digested with *Kpn*1-*Nhe*1 (lanes 2-7); lanes 1 and 8 represent 1 kb DNA ladder ; **c)** physical map of the resultant vector pZS-59 **d)** Agarose gel electrophoresis of pZS-59 and pGreen0029 digested with *Kpn*1-*Xho*1 (lanes 2-4 and lane 5) respectively; **e)** physical map of resultant binary vector pZS-22. Restriction sites are indicated by following abbreviations: S, *Sac*1; K, *Kpn*1; E, *Eco*R1; N, *Nhe*1; P, *Pst*1; X, *Xho*1.

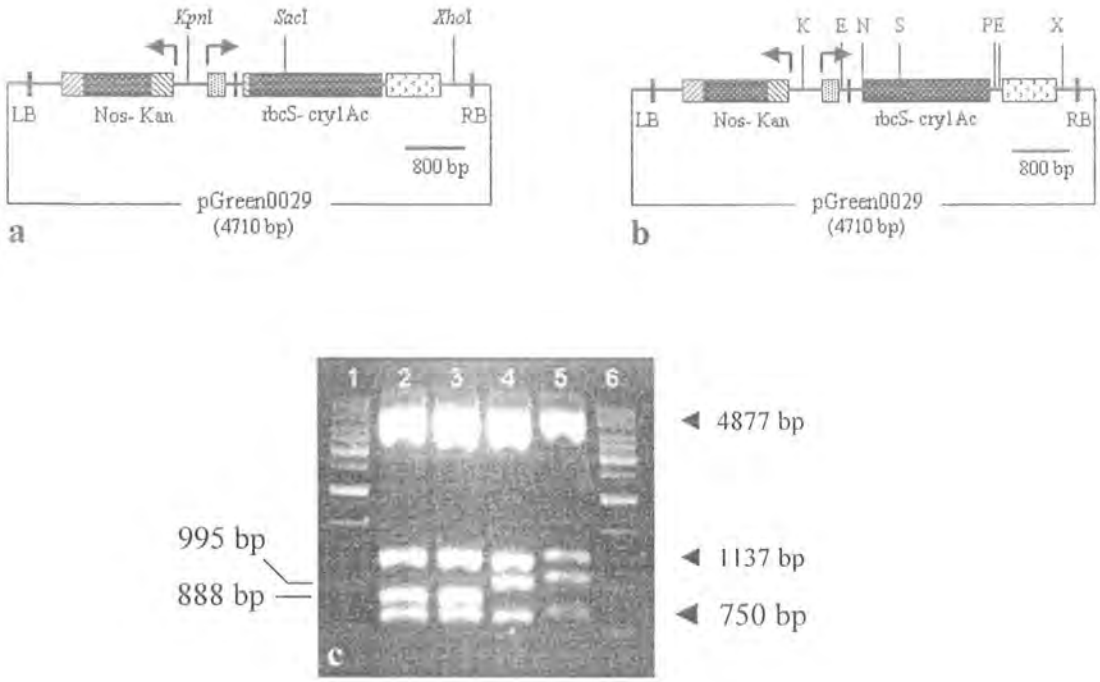


Fig. 9: a-b) Physical maps of pZS-23 and pZS-22; **c)** Agarose gel electrophoresis of pZS-22 (lanes 2-3) and pZS-23 (lanes 4-5) digested with *Eco*R1-*Sac*1, respectively; Restriction sites are indicated by following abbreviations: K, *Kpn*1; E, *Eco*R1; N, *Nhe*1; S, *Sac*1; P, *Pst*1; X, *Xho*1

iii) Cloning of cry1Ac gene with intron under 35S promoter:

The plasmid pZS-60 (Fig. 10a) was digested with *Eco*R1 to produce two fragments, a 3149 bp fragment which contained the vector backbone and the rbcS promoter and a 2200 bp fragment containing the transit peptide, intron and Cry1Ac gene (Fig. 10b). This 2200 bp was gel purified and ligated to the *Eco*R1 digested pJIT60. The resultant vector was named as pZS-62 (Fig. 10c). To find out the orientation of the insert and to confirm the cloning of the insert, pZS-62 was analysed through PCR. The forward and reverse primers from 35S internal sequence and cry1Ac (Appendix-2) were used to amplify 2013 bp fragment. A total of 17 colonies were used for PCR analysis out of which only four produced the 2013 bp fragment, as shown in Fig. 10d, indicating the cloning and correct orientation of the cry1Ac cassette. Plasmid DNA from these four colonies was further analysed by restriction digestion. The plasmid DNA was digested with *Eco*R1, which released the 2200 bp insert from the vector backbone (3720 bp) (Fig. 10e) indicating the successful cloning of cry1Ac expression cassette under 35S promoter. Further confirmation of pZS-62 was made with *Sac*1. Since there are two sites of *Sac*1, one in the vector backbone and one in the insert, therefore, pZS-62 produced two fragments of expected sized of 4125 and 1750 bp as shown in Fig. 10e. Therefore, the cloning of cry1Ac gene having the transit peptide and intron under double 35S promoter and CaMV terminator was successfully accomplished.

In order to clone the cry1Ac gene under 35S promoter from pZS-62 (Fig. 11a) in a binary vector, the *Kpn*1-*Xho*1 lifted (3600 bp) fragment (Fig. 11c) having double 35S promoter, transit peptide, intron, cry1Ac gene and CaMV terminator was ligated with *Kpn*1-*Xho*1 digested pGreen0029. The resultant vector was named as pZS-35 (Fig. 11b). To confirm the cloning of cry1Ac expression cassette in pGreen0029, the plasmid DNA isolated from bacterial colonies was isolated and digested with *Kpn*1-*Xho*1. The digestion resulted in production of two fragments of 3600 bp (insert) and 4710 (vector) as shown in Fig. 11d.

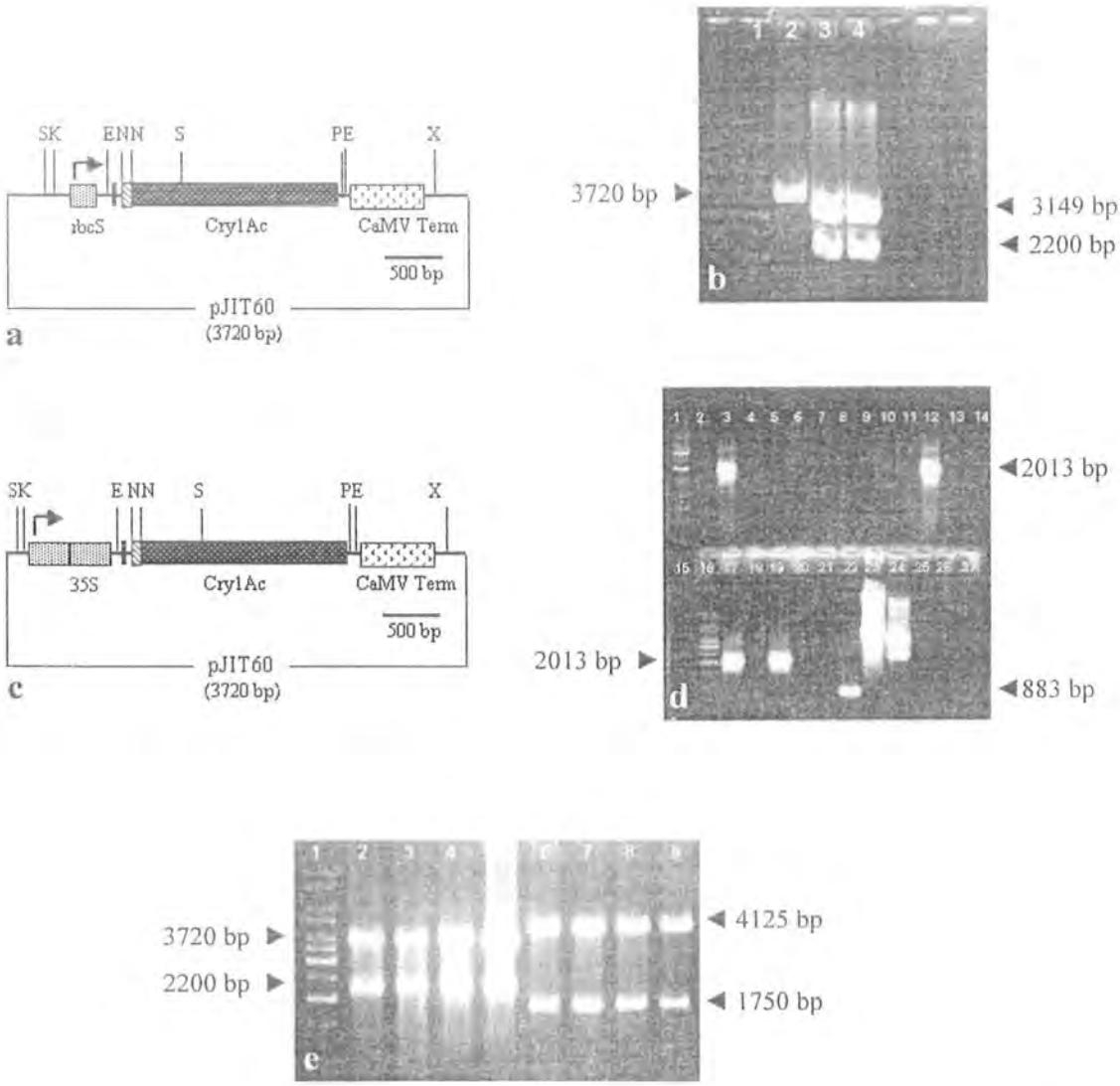


Fig. 10: a) Physical map of pZS-60; **b)** Agarose gel electrophoresis of pJIT60 (lane 2) and pZS-60 digested with *Eco*R1 (lanes 3-4); lane 1, 1 kb DNA ladder ; **c)** physical map of resultant vector pZS-62; **d)** agarose gel electrophoresis of amplified PCR products for the confirmation of pZS-62 using 35S forward and cry1Ac specific reverse primers; **e)** agarose gel electrophoresis of pZS-62 digested with *Eco*R1 (lanes 2-5) and *Sac*1 (lanes 6-9), lane 1, 1 kb DNA ladder ; Restriction sites are indicated by following abbreviations: S, *Sac*1; K, *Kpn*1; E, *Eco*R1; N, *Nhe*1; P, *Pst*1; X, *Xho*1.

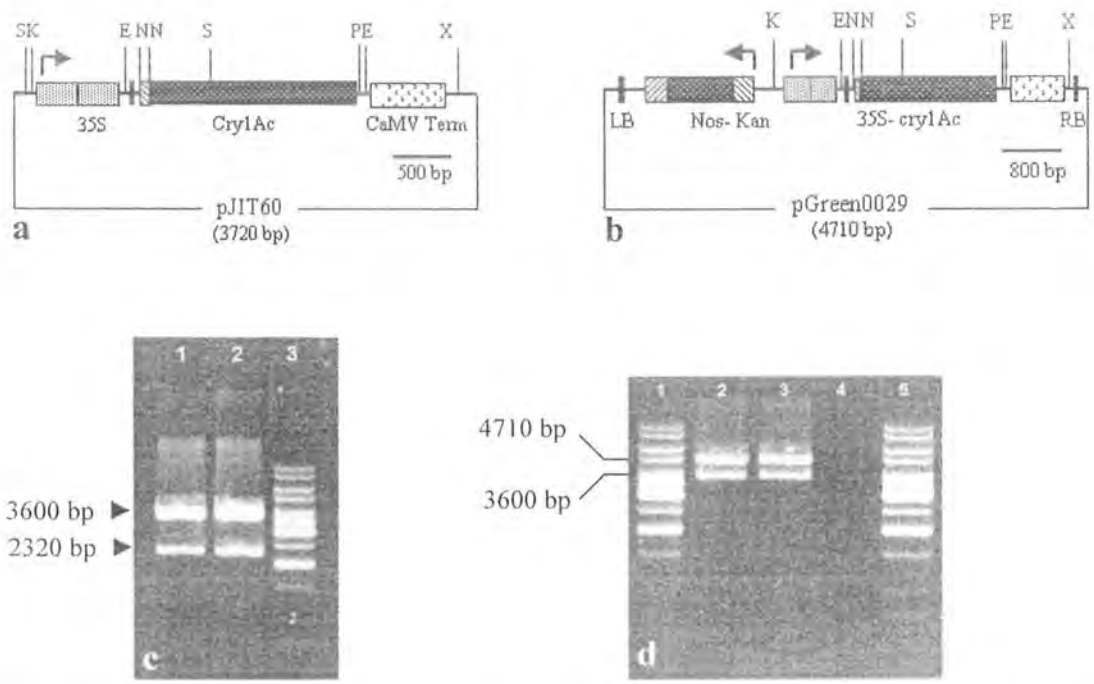


Fig. 11: a-b) Physical maps of plasmids pZS-62 and pZS-35; **c)** agarose gel electrophoresis of pZS-62 digested with *Kpn*1-*Xho*1 (lanes 1-2); lane 3 represent 1 kb DNA ladder **d)** agarose gel electrophoresis of pZS-35 digested with *Kpn*1-*Xho*1 (lanes 2-3), lanes 1& 5 represent 1 kb DNA ladder ; Restriction sites are indicated by following abbreviations: S, *Sac*1; K, *Kpn*1; E, *Eco*R1; N, *Nhe*1; P, *Pst*1; X, *Xho*1.

iv) Cloning of cry1Ac gene without intron under 35S promoter:

For cloning of cry1Ac gene without intron under the double 35S promoter and CaMV terminator, pZS-62 (Fig. 12a) was digested with *Nhe*1 to remove the intron (107 bp) and was then self ligated. The resultant vector was named as pZS-61 (Fig. 12b). In order to confirm the removal of intron from the cry1Ac expression cassette of pZS-61, it was compared with pZS-62 (having cry1Ac expression cassette with intron). The plasmid DNAs of pZS-61 and pZS-62 were digested with *Eco*R1-*Sac*1. Both the vectors should produce four fragments as shown in Fig. 12c. The three fragments (3000, 1137 and 750 bp) were of equal size in both pZS-61 and pZS-62 while the fourth fragment varied in size. pZS-62 having the intron produced the fourth fragment of a larger size (995 bp) compared to pZS-61 (without intron), which produced the fourth fragment of 888 bp as shown in Fig. 12c.

The cry1Ac expression cassette (35S promoter, transit peptide, cry1Ac and CaMV terminator) was removed from pZS-61 with *Kpn*1-*Xho*1 (Fig. 12e) and cloned at the same sites in pGreen0029. The resultant vector was named as pZS-34 (Fig. 12d). To confirm the cloning of cry1Ac expression cassette, the plasmid pZS-34 was digested with *Kpn*1-*Xho*1. The digestion produced two fragments of 3500 and 4710 bp representing the insert and the vector, respectively as shown in Fig. 12f This confirmed the cloning of cry1Ac gene without intron under double 35S promoter.

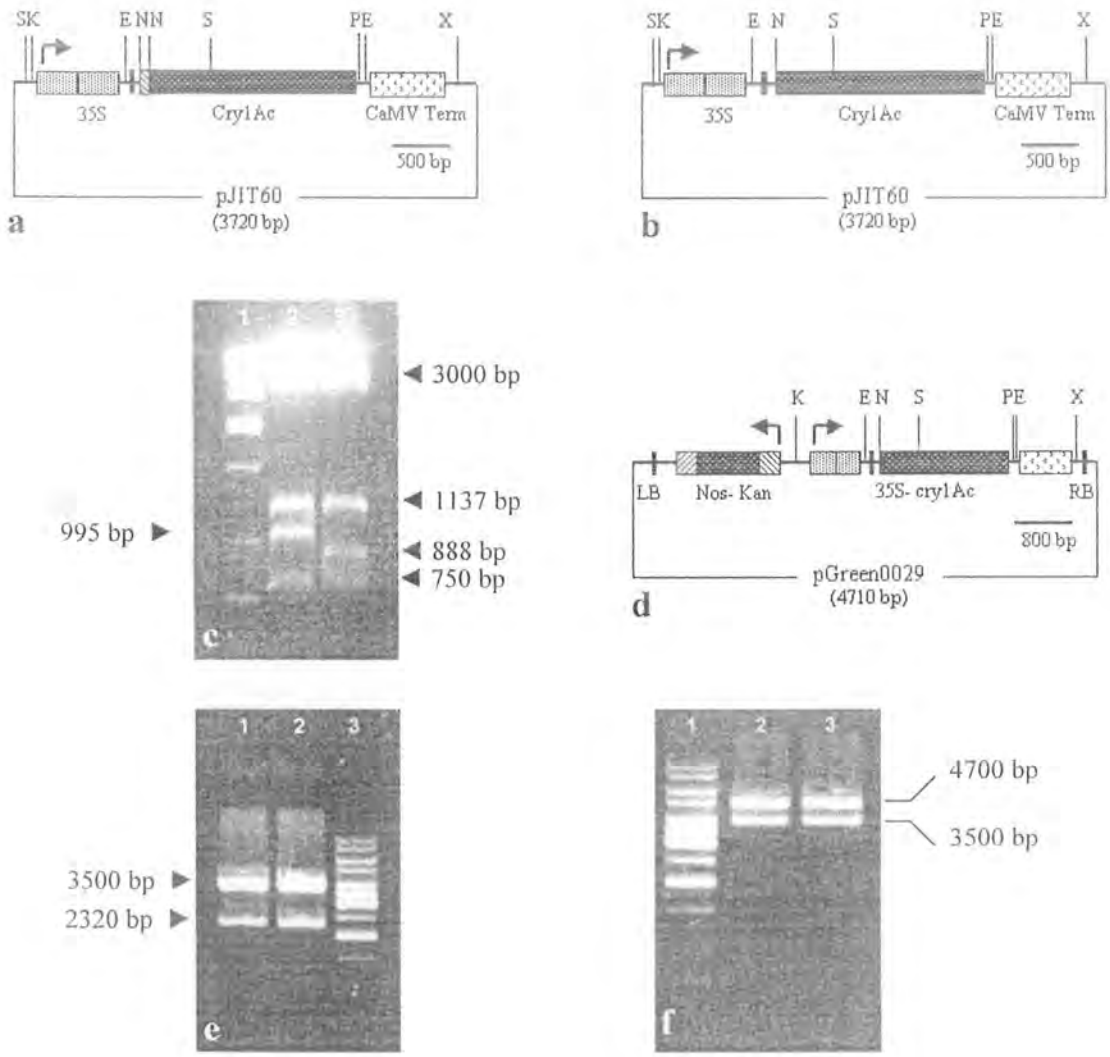


Fig. 12: a-b) Physical maps of plasmids pZS-62 and pZS-61; **c)** agarose gel electrophoresis of plasmids pZS-62 (lane 2) and pZS61 (lane 3) digested with *Eco*R1-*Sac*1, respectively; lane 1, 1 kb DNA ladder ; **d)** physical map of the resultant vector pZS-34; **e)** agarose gel electrophoresis of plasmid pZS-61 digested with *Kpn*1-*Xho*1 (lanes 1-2); lane 3, 1 kb DNA ladder ; **f)** agarose gel electrophoresis of plasmid pZS-34 digested with *Kpn*1-*Xho*1 (lanes 2-3); lane 1, 1 kb DNA ladder ; Restriction sites are indicated by following abbreviations: S, *Sac*1; K, *Kpn*1; E, *Eco*R1; N, *Nhe*1; P, *Pst*1; X, *Xho*1.

d) Transformation of pSAK-IV in *Agrobacterium* and tobacco:

The plant expression vector pSAK-IV, having the Cry1Ac and ω-ACTX-Hv1a genes under rbcS promoter, was transformed into *Agrobacterium tumefaciens* strain LBA4404 through electroporation. The selected bacterial colonies were analysed by PCR for the presence of transformed plasmid using cry1Ac specific primers (Appendix-2). The amplification of 883 bp fragment (Fig. 13) from the bacterial culture indicated that pSAK-IV has been successfully transformed into *A. tumefaciens* strain LBA4404.

Exp. No	Total explants used	No. of kanamycin resistant explants	Transformation efficiency (%)	Total plants produced	No. of plants transferred to soil
1	95	16	16.8	28	6
2	109	49	44.6	95	18
3	96	45	46.9	137	28
Total	300	110	36.1	260	52

About 300 explants (leaf discs) of *Nicotiana tabaccum* L. cv. Spade 28 were transformed with *A. tumefaciens* having pSAK-IV in three different experiments. Out of 300 explants, only 110 explants could survive on selection media and regenerate plants with a average transformation efficiency of 36.1% as shown in above table. The rest of the explants bleached out indicating that these were not transformed. The first signs of regeneration of plantlets were evident after 35 days from the date of transformation with the bacteria. A large number of kanamycin resistant plants were recovered as shown in table and successfully established in soil. The different steps involved in transformation of plants through *Agrobacterium* are shown in Fig. 14.

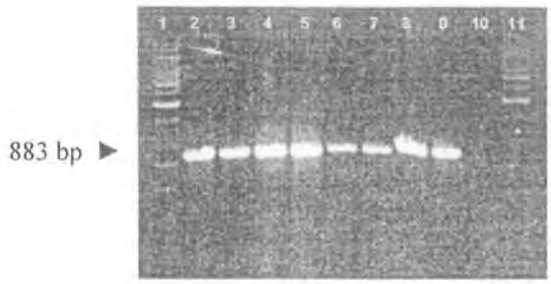


Fig. 13: Agarose gel electrophoresis of PCR amplified products from *Agrobacterium tumefaciens* strain LBA4404 colonies transformed with pSAK-IV using cry1Ac specific primers.

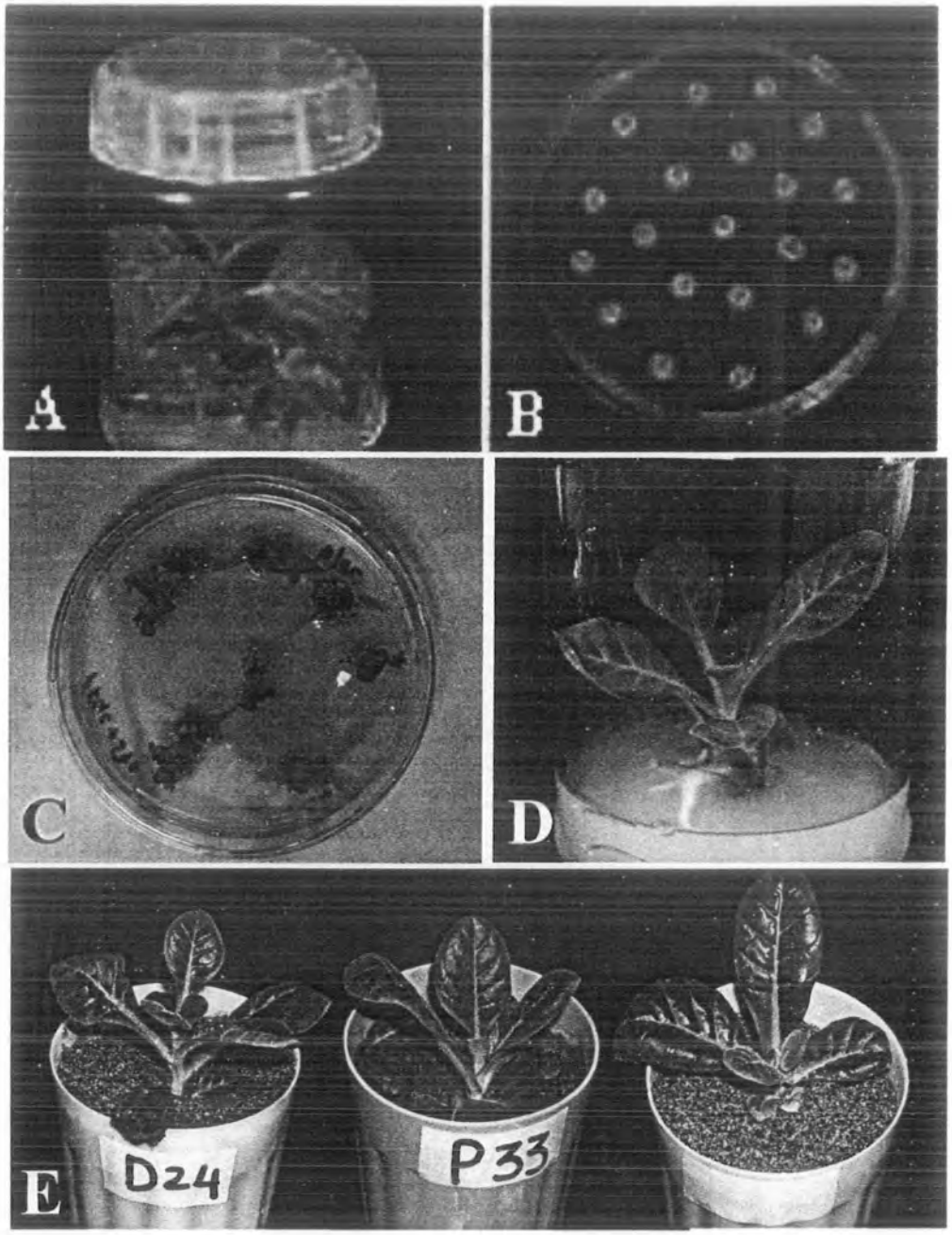


Fig. 14: Different steps in transformation of plants through Agrobacterium. A) In-vitro plants used for cuttings leaf discs, B) freshly cut leaf discs on MS0 medium; C) plant regeneration from leaf discs on selection media; D) In-vitro kanamycin resistant plant ready for transfer to soil; E) putative transgenic plants established in soil.

e) Molecular analysis of putative transgenic plants:

i) PCR analysis:

A large number of putative transgenic plants were recovered but it was not possible to test or screen all the regenerated plants. Therefore, only few plants representing each experiment, from individual transformation events were randomly selected and analysed by PCR. The DNA isolated from 12 putative transgenic plants and a non-transformed control was subjected to PCR analysis using cry1Ac specific forward and reverse primers (Appendix-2). The results of PCR analysis of the putative transgenic plants are shown in Fig. 15. The figure shows that all the 12 putative transgenic plants were positive for the transgene. No amplification from the non-transformed control authenticates our results.

ii) Western blot analysis:

Transgenic tobacco plants were analyzed for protein expression by SDS PAGE analysis. Leaf tissue was frozen in liquid nitrogen, ground to a fine powder and then the total soluble protein was extracted using the Trizole reagent. The protein concentration of the supernatant was determined by the Bradford assay. Fifty µg of protein from both transformed and non-transformed plants was run per lane on 10% SDS-PAGE gels. Each sample was run in duplicat on the same gel. One part of the gel was stained with coomassive blue dye and the other part was developed as western blot (as in material methods). Western blot analysis was carried out using already raised polyclonal antibodies against ω-ACTX-Hv1a protein as a pimary antibodies and used the anti-rabit goat antibodies conjugated with alkaline phosphotase as a secondry antibodies. Developed the blot using the NBT and BCIP as substrate for the alkaline phosphatase, which gave the purple color for the positive samples as shown in the Fig. 16.

f) Insect bioassays

Transgenic plants were randomly selected from different experiments and subjected to bioassay. Each plant was exposed to second instar larvae of armyworm, which is resistant to cry1Ac. Individual plants were given a treatment with four larvae of armyworm and

data were collected after 24, 48, 72 and 96 hours. The different lines showed variable resistance to second instar larvae of armyworm as shown in table below.

Transgenic line No 01 showing strong resistance to second instar larvae of armyworm. The table also indicating that line No. 30 and 14 also given 100% results after 96 hours. But the difference between line No. 30 and 14 is that after 72 hours, three larvae of army worm were set to death by line No.30 while in line No. 14 the mortality of two larva were observed. The difference among the line No. 30, 14 and 1 is that 100% mortality was observed in line No. 1 after 72 hours. The reason for different levels of resistance among different lines may be the site of integration of the gene and the number of the copies that have been integrated in plant genome. Detached leaf bioassay of transgenic tobacco line No. 1 is shown in Fig. 17.

Plant lines	Number of Insect larvae	Mortality Time after treatment (h)			
		24	48	72	96
Control	4	0	0	0	0
01	4	1	2	4	-
14	4	0	1	2	4
16	4	0	0	1	2
24	4	0	1	2	2
30	4	1	2	3	4
33	4	0	1	1	2

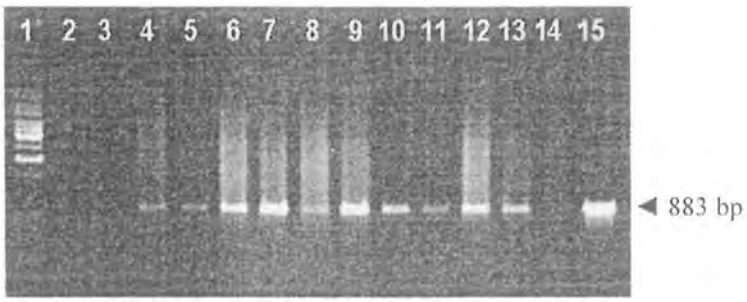


Fig. 15: Agarose gel electrophoresis of PCR amplified products, from DNA extracted from putative transgenic and non-transformed (control) plants, using cry1Ac specific primers. Lane 1, 1 Kb DNA ladder , lanes 2-13, DNA from putative transgenic plants, lane 14, DNA from a non-transformed plant (negative control), lane 15, plasmid control (pSAK-IV).

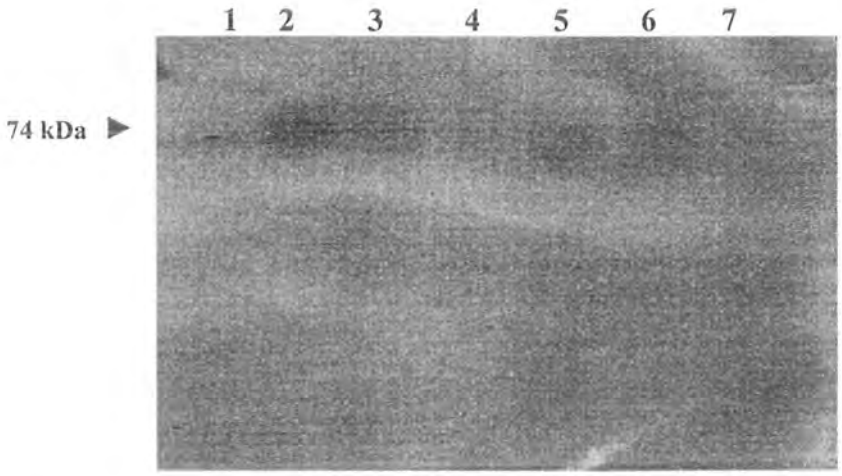
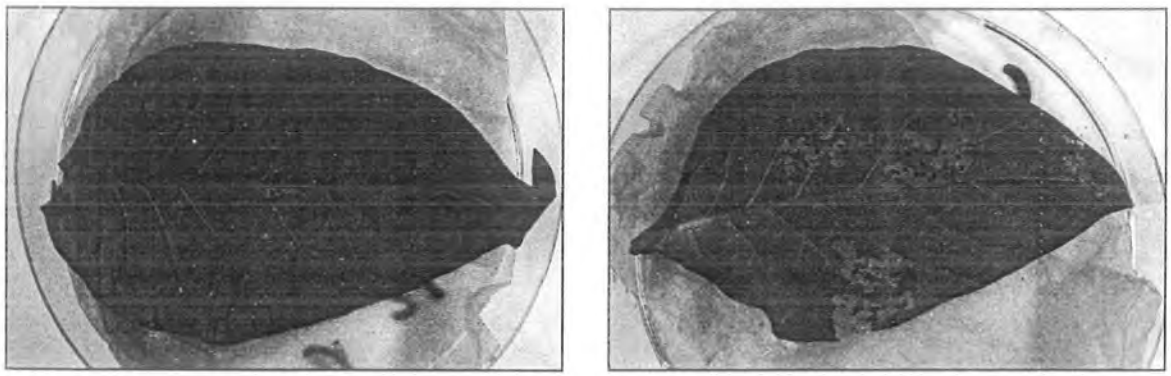


Fig. 16: Western blot analysis of total soluble protein isolated from transgenic tobacco transformed with pSAK-IV expressing cry1Ac-ω-ACTX-Hv1a fusion protein. . Lane 1, Prestained marker #SM0441 , lanes 2-6, protein from putative transgenic plants, lane 7, protein from a non-transformed plant (negative control).



a b

Fig. 17: Detached leaf bio-assays of transgenic tobacco line 01(a) expressing synthetic cry1Ac-ω-ACTX-Hv1a genes along with control (b) exposed to 2nd instar larvae of armyworm. Photograph was taken 48 h after exposure.

DISCUSSION

The objective of the present study was to develop different constructs of *Bacillus thuringiensis* synthetic cry1Ac gene under different plant expressible promoters (rbcS and 35S), with and without introns, and to develop and characterize transgenic tobacco expressing the *Bt* cry1Ac fused with the funnel web spider's ω-atracotoxin-Hv1a (ω-ACTX-Hv1a) gene.

Genes from bacteria such as *Bacillus thuringiensis* (*Bt*) and *Bacillus sphaericus* (Gill *et al.*, 1992; Charles *et al.*, 1996) have been the most successful group of organisms identified for use in genetic transformation of crops for pest control on a commercial scale. The scientific challenge in utilizing the *B. thuringiensis* protoxin is to create a transgenic plant that expresses and synthesizes a functional form of this prokaryotic insecticidal protein at levels sufficient high to prevent damage by insect predation. In initial experiments, the unmodified *B. thuringiensis* subsp. *kurstaki* insecticidal protein genes, cry1Aa, cry1Ab, and cry1Ac, were not particularly well expressed in plants (Vaeck *et al.*, 1987; Barton *et al.*, 1987). Since high levels of expression of these insect control proteins are needed in order to produce commercially viable insect-resistant plants, therefore, to raise the level of the expressed protein, truncated *Bt* gene was used having only the N-terminal portion, the part which contained the toxin, of the insecticidal protoxin and expressed under a strong plant promoter. This study showed a significant increase in the level of insecticidal toxin produced, affording transgenic plants some protection against damage from insect predation (Perlak *et al.*, 1991). This study also showed that all of the insecticidal toxin activity resides within the first 646 amino acids from the N terminus of the 1,156 amino acid protoxin.

Fischhoff *et al.*, (1996) demonstrated that the level of expression can be increased by changing any DNA sequences that cause destabilization, inappropriate polyadenylation, degradation and termination of RNA thus inhibiting efficient transcription or translation in a plant host. They demonstrated that "partially" modified gene having a nucleotide sequence 96.5% unchanged from the wild type gene encoded the identical insecticidal toxin protein. Transgenic plants that expressed this partially modified sequence produced

a 10 fold higher level of insecticidal toxin protein than did plants that were transformed with the wild type gene.

In another attempt, a "fully" modified version of the insecticidal toxin gene was designed and chemically synthesized in order to contain codons preferred by plants to attain an A+T content in nucleotide base composition substantially that found in plants, as opposed to those favored by gram positive bacteria such as *B. thuringiensis*. This gene was also modified to eliminate any potential mRNA secondary structure or chance polyadenylation sequences, which might decrease gene expression (Fischhoff *et al.*, 1996). Transgenic plants that were transformed with this highly modified synthetic protoxin gene have an approximately 100 fold higher level of toxin protein that did plants transformed with the wild-type gene. Moreover, higher level of insecticidal toxin synthesis directly correlated with increased insecticidal activity.

It is now well established that the wild-type *Bt* insecticidal protein genes cannot be well expressed in plants unless they are modified. High level expression of non-plant genes in plants requires the use of plant preferred codon usage. In designing synthetic *Bt* cry1Ac and Spider ω-ACTX genes used in this study, the DNA sequence of the wild-type genes was modified in order to contain codons preferred by highly expressed plant genes, to attain an A+T content in nucleotide base composition substantially that found in plants, and also preferably to form a plant initiation sequence, and to eliminate sequences that cause destabilization, inappropriate polyadenylation, degradation and termination of RNA and to avoid sequences that constitute secondary structure hairpins and RNA splice sites, for high level of expression in plants

Atkinson and co-workers (Atkinson *et al.*, 1996) were the first to recognize the potent insecticidal properties of Australian funnel web spider venom. By testing the venoms of a taxonomically diverse group of Australian spiders, they showed that funnel-web venom was the most lethal against larvae of the moth *Helicoverpa armigera*, a refractory agricultural pest. This led to the identification of a potent insect specific neurotoxin that is now referred to as ω-atracotoxin-1 (Tedford *et al.*, 2004).

This animal toxin, acting on novel ion channel of excitable cells, is principally highly potent short peptide that is present in limited amounts in the venom of *Hadronyche versuta* species (Mouhat *et al.*, 2004). ω-ACTX-Hv1a is lethal to a wide range of insects, including coleopterans, lepidopterans, and dipterans (Fletcher *et al.*, 1997; Atkinson *et al.*, 1998), but it has no adverse effects when high doses are injected subcutaneously into newborn mice and rabbit (Atkinson *et al.*, 1998; Mukhtar *et al.*, 2004). Mukhtar *et al.* (2004) showed that this synthetic ω-ACTX-Hv1a gene when expressed in transgenic plants produced ACTX-Hv1a protein at about 0.25% of total soluble protein. They showed that leaves from the transformed tobacco plants were totally protected and 100% mortality of neonate cotton bollworm larvae was observed. Similarly, a much less sensitive insect to *Bt* crop or *Bt*-pesticide, armyworm (*Spodoptera littoralis*), was also used in detached leaf bioassay and absolute insect mortality was achieved in all the lines tested.

One of the major objectives of the present study was to develop different constructs of *Bacillus thuringiensis* synthetic cry1Ac gene under different strong plant expressible promoters, like CaMV35S and rbcS, with and without introns, for high level expression of cry1Ac genes in plants. Fischhoff *et al.*, (1996) used a number of different promoters like 35S, nopaline synthase (NOS), mannopine synthase (MAS) and ribulosebisphosphatecarboxylase small subunit (RUBISCO) for expression of synthetic *Bt* cry1Ac genes in a number of plant species. 35S provided maximum expression of fully modified cry1Ac gene in the transgenic tobacco. This present study resulted in the development of different constructs of cry1Ac gene under CaMV35S and rbcS promoters for high level expression in plants. The transformation of these vectors will provide an insight into the role of these promoters and intron in expressing the cry1Ac protein at levels sufficient to resist major insect pests.

This study also demonstrated that expression of cry1Ac-ω-ACTX-Hv1a fusion protein in tobacco confers resistance to armyworm, which is otherwise resistant to cry1Ac. This new protein will help solve the problems emerging rapidly with the so far successful *Bt* protein e.g. the specificity of the *Bt* protein to specific insect and the development of

resistance in insect population to *Bt* crops. The specificity of the Bt toxin to specific insect reduces its significance in agriculture, i.e. cry1Ac is effective against the *Helicoverpa* and not against the *Spodoptera* insects, although these two are the major cotton pests. Early studies (Perlak *et al.*, 1990) indicated some control of beet armyworm (*Spodoptera exigua*) with *Bt* cotton. However, some of these plants produced Cry1Ab protein, a protein more effective on beet armyworm and less effective on cotton bollworm when compared to Cry1Ac. Cotton containing both Cry1Ac and the new insecticidal protein, Cry2Ab, is marketed in the United States as Bollgard II (registered in 2002). Cry2Ab is relatively non-homologous to Cry1Ac and cry2Ab has different receptor site than cry1Ac for pink bollworm. That is why one insect species (i.e. pink bollworm) resistant to Cry1Ac has been successfully controlled with Bollgard II (Tabashnik *et al.*, 2002) but the scenario is different for different insect species. Expression of these two proteins resulted in an increase in the number of economically important lepidopteron pests controlled. However, Bollgard II is still active only against lepidopteron and is engineered on the basis of the expression of two genes cryIAc and Cry2Ab. These two genes were combined, based on the idea that CryIAc and Cry2Ab have limited homology (Charles *et al.*, 2000) and strong cross-resistance between plants producing these toxins is not expected. Cry2Ab is toxic to some lepidopteron larvae, including the major cotton pests *Heliothis virescens* and *Helicoverpa zea* (Dankocsik *et al.*, 1990). Although the pyramiding of these two genes had shown satisfactory results in the first cotton generation but still there are chances of developing resistance in insects population because these two proteins have at least some homology in their amino acids sequence and derived from same bacterial origin. Several other Bt proteins, such as Cry1F and the first Bt non-Cry protein, Vip3A, are currently under review for registration.

Thus it seems that effective control of major insect pests and delaying the development of resistance in insect population is only achievable with pyramiding of different Cry genes, having different receptors in the same insect species or other genes with a different mode of action like spider toxins as shown in this study. It is therefore need of the time to look for alternates to *Bt* genes so that new genes can either be used alone or in combination

with *Bt* genes to develop transgenic plants with durable and long lasting resistance to insect pests. The present work demonstrated that combination of cry1Ac and ω-ACTX results in 100% mortality in armyworm within 48-72 h after exposure to the 2nd instar larvae. This suggests that this combination of two genes has immense potential for controlling major lepidopteron insect pests. Since the two genes have different mode of action, therefore, it is also expected that development of resistance in the insect population will also be minimized or delayed.

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APPENDICES

Appendix-1

Agarose Gel Electrophoresis

DNA fragments were separated by electrophoresis on 1% (w/v) agarose gels in 0.5 X TAE buffer containing ethidium bromide. Fragment sizes were estimated by comparison with Fermentas 1kb DNA ladder. Fermentas 6 X DNA loading dye was used.

Appendix - 2

Sequence of primers used

Specific Primers

Cry1Ac Forward primer1

5'-TGCCAACTTGCATCTCTCTG-3'

Cry1AcReverse primer

5'-TCGGTGAATCCATGAGAACA-3'

35S Forward primer

5'-GGTGGCTCCTACAAATGCC-3'

Full Length Primers

Cry1Ac Forward primer11

5-' GCATGGATAATAACCCTGGA-3'

ACTX Reverse primer

5-' ATCACACGTATATCAGACTC-3'

Appendix - 3

50X Tris-acetate EDTA buffer (TAE):

Tris base	242 gm
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Make up the final volume with distilled water to 1000 ml.

Appendix - 4

6X Gel Loading Buffer

1. Bromophenol blue	0.25% (w/v)
2. Xylene cyanol FF	0.25% (w/v)
3. Glycerol	30.0% (v/v)

Dissolve in distilled water.

Appendix - 5

LB (Luria-Bertini) Medium:

Tryptone	1.0 %
Yeast extract	0.5 %
NaCl	0.5 %
Bacto agar	1.5 %

Adjust pH to 7.5 and autoclave.

Appendix - 6

Mini Prep Solutions

Solution I (Suspension buffer)

Tris (pH 7.4-7.6)	50 mM
EDTA	1 mM
RNase	100 µg/ml

Solution II (Denaturation soln.)

NaOH	0.2 N
SDS	1 %

Solution III (Neutralization soln.)

Potassium acetate	3 M
Glacial acetic acid (pH 4.8-5.0)	11.5ml/100ml

Appendix - 7

PLASMID ISOLATION FROM *E. coli*: ALKALINE LYSIS METHOD (MINIPREP)

Following protocol was used for the isolation of plasmid DNA from *E. coli*.

1. A single *E. coli* colony was cultured in 3 ml liquid LB medium containing ampicillin or kanamycin and grown overnight at 37 °C.
2. The *E. coli* culture was centrifuged in 1.5 ml Eppendorf tube at 14000 rpm for 1 minute.
3. The supernatant was discarded and the pellet was allowed to dry on tissue paper.
4. 200 ml of solution I (Appendix 6) was added to Eppendorf tube and the pellet was suspended in the solution with the help of vigorous vortex.
5. 200 ml of solution II (Appendix 6) was added to eppendorf tube and mixed well by inverting gently and incubate for 5 minutes at room temperature.

6. 200 ml of solution III (Appendix 6) was added to eppendorf tube mixed well and incubate for 5 minutes on ice then centrifuged at 14000 rpm for 5 minutes.
7. The supernatant was taken in fresh Eppendorf tube and two volume of absolute ethanol was added.
8. Eppendorf tube was kept at -20 °C for 20 minutes and then centrifuged at 14000 rpm for 10 minutes.
9. The supernatant was discarded and the pellet was washed with 70% ethanol and pellet was air-dried.
10. 20 µl of sterile distilled water was added to the pellet to dissolve DNA and was stored at -20 °C.

The plasmid concentration and quality were detected by 1% agarose gel using stranded DNA markers.

Appendix - 8

CTAB method for isolating total genomic DNA

1. Take 15 ml 2X cetyltriethylmethylammonium bromide (CTAB) and 100µl 2-mecraptoethanol in 50ml falcon tube. Keep the tube in water-bath at 65 °C for 30 minutes.
2. Collect fresh leaves in liquid nitrogen. Grind the sample (1 gm) to a fine powder.
3. Pour the hot 2X CTAB in ground powder suspend and incubate at 65 °C for 30 minutes with occasional swirling.
4. Add equal volume of chloroform: isoamyl alcohol (24:1) and mix gently.
5. Centrifuge at 9000 rpm for 10 min at room temp. Take the upper phase in a 50 ml tube.
6. Again add equal volume of chloroform: isoamyl alcohol (24:1) and mix gently.
7. Centrifuge at 9000 rpm for 10 min at room temp. Take the upper phase in a 50 ml tube and add 0.6 volume of isopropanol.
8. Centrifuge at 9000 rpm for 5 min at room temperature.
9. Discard the supernatant and wash the pellet with 70% ethanol.

7. Air dry the palette. Dissolve the pellet in 0.4-0.6 ml double distilled autoclaved water or TE buffer.
8. Run 2 µl on 1% agarose gel to check the concentration of genomic DNA.

Reagents:

2x CTAB:

2% CTAB (w/v)

100 mM Tris (pH8.0)

20 mM EDTA (pH8.0)

1.4 M NaCl

1% PVP (polyvinylpyrrolidone)

Appendix - 9

RAPID GEL EXTRACTION PROTOCOL

NOTE:

Perform all centrifugation at room temperature.

Before beginning preheats an aliquot of TE to 65 to 70 °C. Equilibrate a water bath or heat block to 50 °C. Verify that ethanol has been added to wash Buffer (L2).

1. GEL SLICE EXCISION:

Cut the area of gel containing the DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment.

2. GEL SLICE WEIGHING:

Weigh the gel slice.

a. For $\leq$ 2% agarose gel, place up to 400 mg of gel into a 1.5 ml polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 30 µl of Gel solubilization Buffer (L1) for every 10 mg of gel.

b. For $\geq$ 2% agarose gel, place up to 400 mg of gel into a 5 ml polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 60 µl of Gel solubilization Buffer (L1) for every 10 mg of gel.

3. **GEL SOLUBILIZATION:**

Incubate at 50 °C for ≥ 15 min. Mix every 3 min to ensure gel dissolution. After gel slice appear dissolved, incubate for 5 min longer.

4. **CARTRIDGE LOADING:**

Place a spin cartridge into a 2 ml wash tube. Pipette the mixture from step 3 into the spin cartridge. Centrifuge the mixture in a microcentrifuge at > 12,000 rpm for 1 min. Discard the flow through.

Note: Load no more than 400 mg agarose per cartridge.

5. **(OPTIONAL CARTRIDGE WASH):**

Place the spin cartridge back into the 2 ml wash tube. Add 500 µl gel solublization buffer (L1) to the spin cartridge. Incubate at room temperature for 1 min, then centrifuge at > 12,000 rpm for 1 min. Discard the flow through.

6. **CARTRIDGE WASH:**

Place the spin cartridge back into the 2 ml wash tube. Add 700 µl of Wash Buffer (L2) (containing ethanol) to the spin cartridge and incubate for 5 min at room temperature. Centrifuge at > 12,000 rpm for 1 min. Discard the flow through. Centrifuge again for 1 min to remove residual wash buffer.

7. **DNA ELUTION:**

Place the spin cartridge into a 1.5 ml recovery tube (supplied). Add 50 µl of warm TE Buffer (TE) directly to the center of the spin cartridge. Incubate for 1 min at room temperature, then centrifuge at > 12,000 rpm for 2 min.

Appendix- 10

Stock solutions

Vitamin B5 (100X)

Sr/No.	Name of Vitamin	Quantity in mg/L of the medium	Quantity in g/L of the 100X Stock
1	Nicotinic acid	0.100	00.10
2	Thiamine HCL	10.00	01.00
3	Pyridoxine HCL	01.00	00.10
4	Myo-Inositol	100.0	10.00

Appendix – 11

Antibiotics

Antibiotics	Stock Conc.	Working Conc.	Solvent
Kanamycin	50mg/ml	50µg/ml	Water
Ampicillin	100mg/ml	100µg/ml	Water
Rifampicin	50mg/ml	50µg/ml	Methanol
Tetracycline	10mg/ml	10µg/ml	90% Ethanol

Appendix - 12

Preparation of electro competent cells of *Agrobacterium tumefaciens* (LBA 4404)

1. A single colony from a freshly grown plate of LBA 4404 was picked and inoculated into 100 ml LB liquid medium in 250 ml autoclaved flask using sterile toothpick and incubated at 28 ^{0}C for 48 hours with vigorous shaking.
2. 5 ml of the 48 hours grown culture was re-inoculated into 1 liter flask containing 250 ml of the same LB medium and incubated at 28 ^{0}C until OD_{600} of cells were become 0.5-1.0 (10^{10} cells/ml).

3. The cells were transferred aseptically to ice cold 50 ml propylene tube and kept cool on ice for 30 minutes.
4. The cells were then centrifuged at 4000 rpm for 10 minutes at 4 ^{0}C. The supernatant was discarded and the cells were paletted and then resuspended in 50 ml of sterile cold $dddH_2O$.
5. The cells were again centrifuged at 4000 rpm in the same centrifuge at 4 ^{0}C for 10 minutes. The supernatant was discarded and the cells were paletted, then resuspended in 25 ml of sterile cold $dddH_2O$.
6. After another wash cells were resuspended in 10 ml sterile cold $dddH_2O$ containing filter sterilized cold 10% glycerol. This wash was repeated.
7. Finally the cells were resuspended in 1-1.5 ml filter sterilized cold 10% glycerol, aliquoted in 100 µl and stored at -70 ^{0}C.

Appendix - 13

Transformation of pSAK-1V in *Agrobacterium tumefaciens* strain LBA 4404 by electroporation

Only 2 µl of each clone was used for electro-transformation by electro cell manipulator 600 (BTX San Diego, California).

For electroporation the following protocol was used.

1. Electroporation cuvettes 1 mm gap were placed on ice. Vials of frozen electro-competent cells of *Agrobacterium* were allowed to thaw on ice.
2. 200 ng DNA of the recombinant plasmid was mixed with 100 µl of electro-competent cells in the electroporation cuvettes on ice.
3. The condition for electroporation were set as recommended by the manufacturer:

Choose mode T	2.5 KV
Set resistance R	R5 (129ohm)
Set charging voltage	1.44 KV

4. The electro-competent cells containing the DNA mixture were transferred to electroporation cuvette.

5. Pulse was given and 1 ml of liquid LB medium was added immediately, mixed gently and transferred to a 1.5 ml eppendorf tube and incubated at 28°C for 3 hour with vigorous shaking.
6. 100 µl and 200 µl of transformed culture were spread on petriplates containing solid LB medium supplemented with rifampicin, tetracycline and kanamycin so that only transformed cells should multiply.
7. When the liquid was absorbed completely the plates were sealed with parafilm and kept at 28 ^{0}C for 2-3 days.
8. At the end of incubation colonies were picked with sterile toothpicks and cultured in 5ml liquid LB medium in 50 ml tube containing required antibiotics.
9. Culture tubes were kept at 28 ^{0}C on shaker in *Agrobacterium* growth room for 48 hours with vigorous shaking.
10. Transformants were confirmed through PCR

Appendix –14

Components of MS Salt

Macro Elements	**mg/l**	**20x(g/l)**
KNO_3	1900	3.8
KH_2PO_4	170	3.4
$MgSO_4$	370	7.4
$CaCl_2$	440	8.8
NH_4NO_3	1650	3.3
Micro Elements	**mg/l**	**50xg/500ml**
H_3BO_3	6.2	0.31
$MnSO_4$	16.9	1.115
$ZnSO_4.7H_2O$	8.6	0.43
KI	0.83	0.0415
$NaMoO_4\ 2H_2O$	0.25	0.0125
$CuSO_4\ 2H_2O$	0.025	0.00125
$CoCl_2\ 6H_2O$	0.025	0.00125

Appendix - 15

Table Preffered Codon Usage in *Gossypium hirsutum* [gbpln]

325 CDS's (116006 codons)

fields: [triplet] [frequency: **per thousand**] ([number])

UUU 19.9(2305)	UCU 17.6(2043)	UAU 18.1(2097)	UGU 9.0(1049)
UUC 21.6(2509)	UCC 13.3(1543)	UAC 14.8(1720)	UGC 8.7(1004)
UUA 9.9(1154)	UCA 15.8(1828)	UAA 1.2(137)	UGA 1.1(128)
UUG 20.5(2377)	UCG 6.9(797)	UAG 0.5(59)	UGG 14.3(1660)
CUU 23.6(2733)	CCU 20.5(2376)	CAU 14.3(1659)	CGU 8.6(993)
CUC 14.0(1623)	CCC 8.8(1017)	CAC 9.2(1071)	CGC 4.3(496)
CUA 6.9(795)	CCA 17.7(2051)	CAA 21.3(2472)	CGA 6.2(717)
CUG 10.4(1212)	CCG 5.7(661)	CAG 15.5(1801)	CGG 4.6(535)
AUU 24.0(2783)	ACU 18.6(2163)	AAU 22.7(2639)	AGU 12.5(1450)
AUC 18.2(2111)	ACC 13.9(1615)	AAC 22.2(2576)	AGC 11.8(1374)
AUA 10.7(1237)	ACA 15.4(1782)	AAA 25.6(2975)	AGA 14.8(1712)
AUG 26.3(3046)	ACG 5.4(626)	AAG 33.0(3830)	AGG 13.5(1571)
GUU 27.4(3183)	GCU 31.6(3671)	GAU 34.9(4044)	GGU 25.2(2921)
GUC 12.7(1479)	GCC 16.3(1890)	GAC 17.0(1977)	GGC 12.2(1417)
GUA 8.0(933)	GCA 19.0(2209)	GAA 33.4(3870)	GGA 22.5(2610)
GUG 17.4(2020)	GCG 5.7(656)	GAG 29.8(3457)	GGG 13.4(1557)

Coding GC 45.84% 1st letter GC 51.81% 2nd letter GC 41.48% 3rd letter GC 44.21%

Appendix-16

MS0 Medium (For seed germination)

pH 5.7-5.8 in dist. Water and autoclaved

1	MS Salt	4.3g/l
2	Vitamin B5	5ml/l
3	Sucrose	30g/l

Appendix -17

Co-culture medium

1) MS Salts	4.4 g/L
2) Sucrose	30 g/L
3) Phytagar	15 g/L
4) B5 vitamin (100X)	5 ml/L
5) 1-naphthylacetic acid (NAA) (1mg/ml)	0.1 mg/L
6) 6-benzylaminopurine (BAP) (1mg/ml)	1 mg/L

Adjust pH: 5.8 with 0.1 N KOH.

After autoclaving add filter sterilized:

Appendix – 18

Selection medium

1) MS Salts	4.4 g/L
2) Sucrose	30 g/L
3) Phytagar	15 g/L
4) B5 vitamin (100X)	5 ml/L
5) 1-naphthylacetic acid (NAA) (1mg/ml)	100 µl/l
6) 6-benzylaminopurine (BAP) (1mg/ml)	200 µl/L

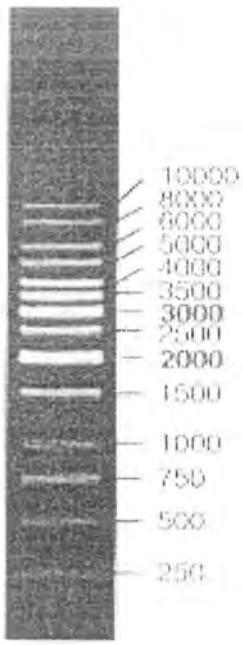
Adjust pH: 5.8 with 0.1 N KOH.

After autoclaving add filter sterilized:

7) Cefotaxime (100mg/ml)	250 mg/L
8) Kanamycin (50 mg/ml)	50 mg/L

Appendix- 19

1Kb DNA ladder from Fermentas



Appendix -20

MS0 Medium (for tobacco)

pH 5.7-5.8 in dist. water

1	MS Salt	4.3g/l
2	Vitamin B5	5ml/l
3	Sucrose	30g/l

Appendix - 21

Rooting medium

1) MS Salts	4.4 g/L
2) Sucrose	30 g/L
3) Phytagar	15 g/L

Adjust pH: 5.8 with 0.1 N KOH.

After autoclaving add filter sterilized kanamycin

Appendix- 22

Benzyl amino purine

Dissolve 50.0 mg benzyl amino purine in 1 ml 1 M NaOH, then add distilled water drop by drop while shaking and make volume upto 10 ml. Use 200 µl of this stock for 1L of medium to make concentration of 1 mg/l.

Naphthalene Acetic Acid

Dissolve 10 mg naphthalene acetic acid in 1 ml 1 M NaOH, then add distilled water drop by drop while shaking and make volume up to 10 ml. Use 100 µl of this stock for 1L of medium to make concentration of 1 mg/l.

Appendix - 23

Preparation of gold particles

1. Weighed 3 mg, 1-µm diameter gold particles (BioRad, USA) in a sterile Eppendorf tube.
2. Added 500 µl absolute ethanol and vortexed at high speed for 10 min.
3. The gold particles were paletted by spinning for 5 seconds at 14,000 rpm.
4. Ethanol was discarded without disturbing the gold pellet.

5. Gold particles were washed with sterile water by adding 500 µl deionized double distilled autoclaved water. Vortexed for 5 minutes, spun for 30 seconds and finally the water was discarded without disturbing the gold pellet. Repeated this washing step three times.
6. Finally gold particles were resuspended in 50 µl sterile double distilled deionized water. Vortexed and briefly immersed the tube in sonicating waterbath to break any gold aggregates before proceeding to DNA precipitation.

DNA precipitation

1. To the 50 µl gold suspension in water, 5 µl plasmid DNA (DNA concentration 1.0 µg/µl) was added, followed by 20 µl spermidine free base (0.1 molar) and 50 µl calcium chloride (2.5 molar). The contents were mixed by vortexing at low speed. Vortexing was continued for three minutes.
2. Allowed the particles to settle down by keeping tube in ice for 1 minute.
3. The contents were spun at low speed for 2 seconds and the supernatant was discarded. Pellet was washed with 250 µl cold absolute ethanol. After washing, ethanol was removed and finally the DNA coated particles were resuspended in 50 µl ethanol.
4. Pipetted 8 µl suspension and dispensed in the center of the macro carriers fixed in steel holders. Allowed to air dry for two to three minutes in low humidity and vibration free environment.

Appendix- 24

Primer designing parameters

Following criteria were considered for primer designing.

- Target sites selected for primer designing should not contain restriction sites used for cloning.
- Cheap and easily available restriction enzymes should be selected.
- Primers should be at least 20 bases long.
- GC contents should be around sixty percent.

- Primer pairs should not be complimentary to avoid primer dimmer formation.
- Primer pairs should have similar melting temperature.
- Four to five bases should be left at five prime end for better enzyme activity.

Appendix- 25

The procedure for Preparation of Electro competent cells of BL21 is same as described in appendix- 12 Instead of rifampicin and tetracycline chloramphenicol was used.

Appendix- 26

Electroporation of BL21 strain of *E.coli*

The procedure for electroporation of BL21 is same as described in appendix 13, except

1. 2mm cuvette was used.
2. Instead of 1.44 voltage 2.34 was used.
3. Instead of rifampicin and tetracycline chloramphenicol was be used.

Appendix - 27

10% SDS PAGE

Running gel 10%

Solution I	6.67 (Appendix-28)
Solution II	5 ml (Appendix -29)
d_3H_2O	8.33 ml
APS 10%	200 µl
TMED	20 µl

Stacking gel 4.5%

Solution I	1.5 ml (Appendix-28)
Solution III	2.5 ml (Appendix-30)
d_3H_2O	6 ml
APS10%	100 µl
TMED	10 µl

Appendix-28

Solution I 30% stock
Acrylamide 30%
Bis acrylamide 0.8%
Amberlite 1%

Appendix- 29

Solution II 4X 250 ml stock

Tris base	45.5 g
SDS	1 g
pH	8.8

Appendix- 30

Solution III 4X 250 ml stock

Tris base	15.125 g
SDS	1 g
pH	6.8

Appendix- 31

Stainer for Protein Gel

Coomassie Brilliant Blue	0.5g
Glacial acetic acid	50ml
Methanol	225ml
Water	225ml

The gel was stained for 3-4 hours while shaking

Destainer for Protein Gel

Glacial acetic acid	50ml
Methanol	225ml
Water	225ml

The gel was destained for 2-3 hours while shaking

Appendix – 32

Transfer buffer	1000 ml
Tris base	50 mM
Glycine	380 mM
SDS	0.1%
Methanol	20%

Tris buffer saline

Tris-HCl	10 mM
NaCl	150 mM
PH	8.0

Alkaline phosphatase buffer

NaCl	10 mM
$MgCl_2$	5mM
Tris HCl	100mM
PH	8.0

Appendix- 33

Vector	4 µl
Insert	2 µl
T4 DNA ligase	1 µl
10X ligation buffer	2 µl
PEG	2 µl
d_3H_2O	9 µl

Appendix- 34

Vector	4 µl
T4 DNA ligase	1 µl
10X ligation buffer	2 µl
d_3H_2O	13 µl

Appendix- 35

Vector	15 µl
CIAP	1 µl
Buffer CIAP	5 µl
d_3H_2O	29 µl

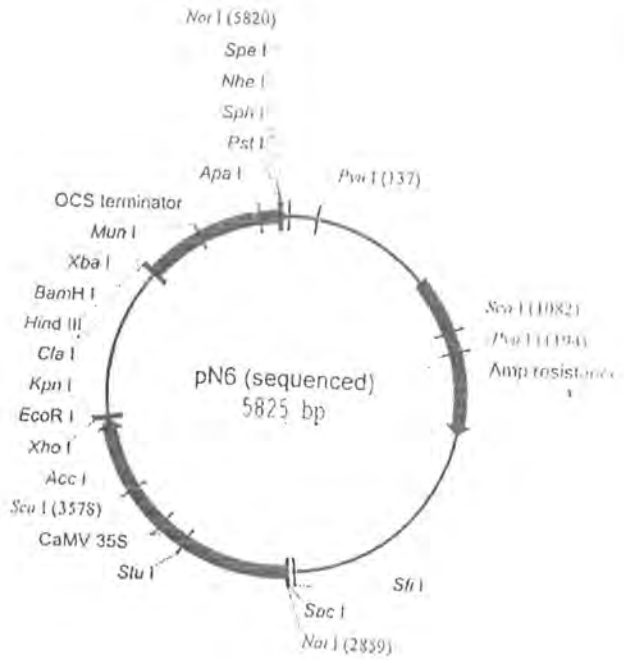
Appendix- 36

Phenol-chloroform precipitation

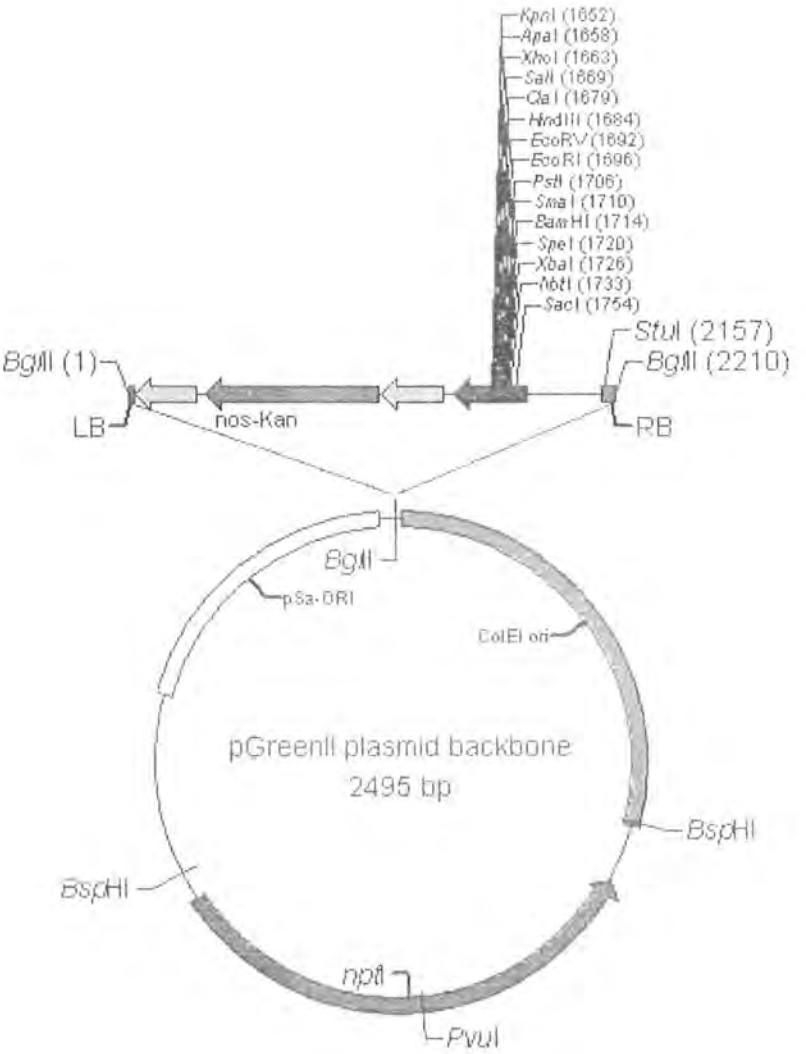
1. Add dH_2O (50 µl) in reaction mixture (50 µl) to make up volume to 100 µl.
2. Add equal volume Phenol: chloroform (100 µl) and mix it.
3. Centrifuge for 6 min.
4. Take supernatant in fresh tube.
5. Add 3M sodium acetate1/10th (8 µl pH 5.2) and 2.5 volume absolute ethanol (300 µl).
6. Freeze at –20°C for 30 min.
7. Centrifuge for 10 min.
8. Dispose ethanol.
9. Wash the pellet with 70% ethanol (100 µl), spin for 2 min

Dry the pellet and dissolve in 15 µl dH_2O.

Appendix - 37



Appendix- 38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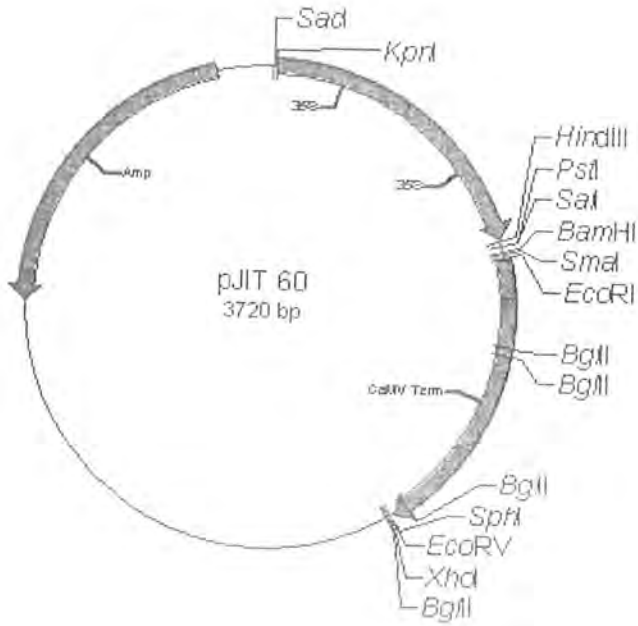
XhoI (1663)
SalI (1669)
ClaI (1679)
HindIII (1684)
EcoRV (1692)
EcoRI (1696)
PstI (1706)
SmaI (1710)
BamHI (1714)
SpeI (1720)
XbaI (1726)
NotI (1733)
SacI (1754)
StuI (2157)
BglII (2210)
BglII (1)
LB
nos-Kan
RB
BglII
pSa-ORI
ColEI ori
pGreenII plasmid backbone
2495 bp
BspHI
BspHI
npt
PvuI

Appendix- 39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35S
HindIII
PstI
SalI
BamHI
SmaI
EcoRI
Amp
35S
pJIT 60
3720 bp
BglII
BglII
CaMV Term
BglII
SphI
EcoRV
XhoI
BglII